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Full Length Research Paper

Biodiversity of few Indian charophyte taxa based on molecular characterization and construction of phylogenetic tree

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The world charophyte flora suffered reshuffling in the taxonomic status of many taxa on the basis of certain morphological characters. Large numbers of species were reduced to the status of subspecies, variety or forma while some distant species were merged together. In this study, molecular characteristics such as band frequency, RAPD polymorphism, genetic identity index or similarity index, band sharing frequency and genetic distance within and in between *Chara* and *Nitella* were evaluated. With the help of scorable bands, range of molecular sizes was recorded in amplified products of 12 charophyte taxa by using five random primers. This investigation requires further elaboration to reach a definite conclusion.

Key words: *Chara, Nitella,* Charophyta, molecular characterization.

INTRODUCTION

RAPD based molecular characterization is an important tool to explore genetic biodiversity between morphologically identical species and genetic relatedness between distant species. Various forms of algae growing in varied environmental conditions show diversified morphology. RAPD was used for molecular identification and genetic variations in *Sargassum* (Ho et al., 1995), *Porphyra* (Dutcher and Kapraun, 1994), *Alexandrium tamarensis* (Haley et al., 1999) and seaweeds (Hong et al., 1996) . Cho et al. (1997) characterized seven isolates of green seaweed Ulvales. Alberto et al. (1997) gave DNA isolation method for RAPD analysis in *Gelidium sesquipedale*. Genetic polymorphism and genetic diversity in *Dunaliella salina* (Gomez and Mareila, 2001) and *Furcillaria lumbricalis* (Valatka et al., 2000) were studied by using RAPD-PCR method. Valatka et al. (2000) used 7 RAPD–PCR primers for studying genetic diversity. Besides these, molecular characterization and isolation of Fe-hydrogenase was attempted in *Chlorella fusca* by Winkler et al. (2002) while Meneses (1996) used it to assess the population of *Gracilaria*.

In Charophyta, Wood and Imahori (1965) either merged various independent species together or created new species on the basis of morphological characters. While providing this scheme of charophycean taxonomy, they either reduced many forms in their status i.e. from 'variety' to 'forma' or from 'species' to 'subspecies' or vice versa. It created unjustified speciation of various charophycean forms due to which molecular characterization of this important and highly divergent group became inevitable. Sundaralingam (2002), while reviewing over all investigations on Charophyta emphasized that biodiversity of this group can only be explored through molecular characterization. The available literature reveals no work from India on molecular characterization of Charophyta for genetic biodiversity, genetic relatedness and genetic distance between various taxonomic forms. In order to provide

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Table 1. Taxonomic status of Charophyte taxa investigated for molecular characterization.

Sr. No.	After Pal et al. (1962)	After Wood and Imahori (1965)
	Genus : *Chara*	
D1	*C. fibrosa*	*C. fibrosa* f. *tylacantha* (nordst) *em* RDW
D2	*C. wallichii*	*C. corallina* var. *wallichii* (A. Br) RDW **Male**
D3	*C. zeylanica*	*C. zeylanica* f. *elegans* (A. Br. Ex T.F.A) H. & J. Gr. RDW
D4	*C. erythrogyna*	*C. fibrosa* f. *erythrogyna* Griff. (RDW)
D5	*C. corallina*	*C. corallina* f. *corallina* Klein. ex Willd. RDW **Dioecious**
D6	*C. delicatula*	*C. globularis var. virgata* (Kutz.)RDW
D7	*C. braunii*	*C. braunii* Gm. RDW
D8	*C. wallichii*	*C. corallina* var. *wallichii (A. Br.)* RDW **Female**
D9	*C. socotrensis*	*C. socotrensis* Nordst. In Kuhn em RDW
	Genus : *Nitella*	
D10	*N. furcata*	*N. furcata* subsp. *mucronata* f. *wrightii* (A. Br.) RDW
D11	*N. furcata*	*N. furcata subsp. & var. mucronata f. oligospira* (A. Br.) RDW
D12	*N. furcata*	*N. furcata subsp. flagellifera (J. Gr & GOA)* RDW

Table 2. List of oligonucleotide random primers and their G-C ratio.

S.No.	Operons	Sequences (5' to 3' activity)	G-C ratio
1.	OPF 07	CCGATATCCC	60%
2.	OPG 02	GGCACTGAGG	70%
3.	OPG 03	GAGCCCTCCA	70%
4.	OBB 04	GGACTGGAGT	60%
5.	OPA 09	GGGTAACGCC	70%

justified taxonomic status to charophycean forms, RAPD-PCR based studies were carried out in Indian Charophyta for the first time.

MATERIALS AND METHODS

During present investigations, twelve charophyte taxa were studied for molecular characterization through RAPD-PCR method (Table-1). Genomic DNA from decolorized and epiphyte free charophyte taxa was isolated using the protocol of Bhatnagar et al. (2005). Genomic DNA samples were tested electrophoretically and spectrophotometrically for quality and quantity to ensure compatibility for RAPD-PCR amplification. The modified protocol of Plotsky et al. (1995) for RAPD-PCR was adopted. Five random oligonucleotide primers listed in Table-2 were procurred from Bangalore Genei (Pvt.) Ltd. The amplicons were analysed by agarose gel electrophoresis using 100 bp ladder and -*Hind* III/*Eco* RI double digest molecular markers and the results in few taxa have been presented in Figure 1.

RESULTS AND DISCUSSION

During present study, emphasis has been laid on the relatedness of various species of two charophyte genera *Chara* and *Nitella* on the basis of molecular characteristics like band frequency, RAPD polymorphism, genetic identity index or similarity index, band sharing frequency and genetic distance within and in between *Chara* and *Nitella.* Results obtained under various heads and their respective discussions have been presented below.

Band frequency

All amplified products of twelve species belonging to two charophyte genera *Chara* and *Nitella* were estimated for the frequencies of all scorable bands in all the five primers. Band frequencies of RAPD-PCR products in five different primers have been presented in Table 3.

RAPD polymorphism

Charophyte taxa of the genus *Chara* and genus *Nitella* were studied for scorable band pattern against five primers. The primer wise number of polymorphic bands and their per cent proportion in each primer has been presented in Table 4. A total of 66 bands were scored from 5 primers in both charophyte genera. In the primers OPF-07, OPG-02, OPG-03 and OBB-04 100% polymorphism was observed while in primer OPA-09, percentage polymorphism was 93.33 and exhibited monomorphic band. Thus it seems evident that various charophyte species show highest degree of polymorphism in four primers and monomorphic band was observed only in the primer OPA-09. Being the group of highly divergent morphology, Charophyta show high degree of polymorphism at molecular level which may be helpful in exploring its phylogenetic relationships. The monomorphic band observed in all charophyte spe-

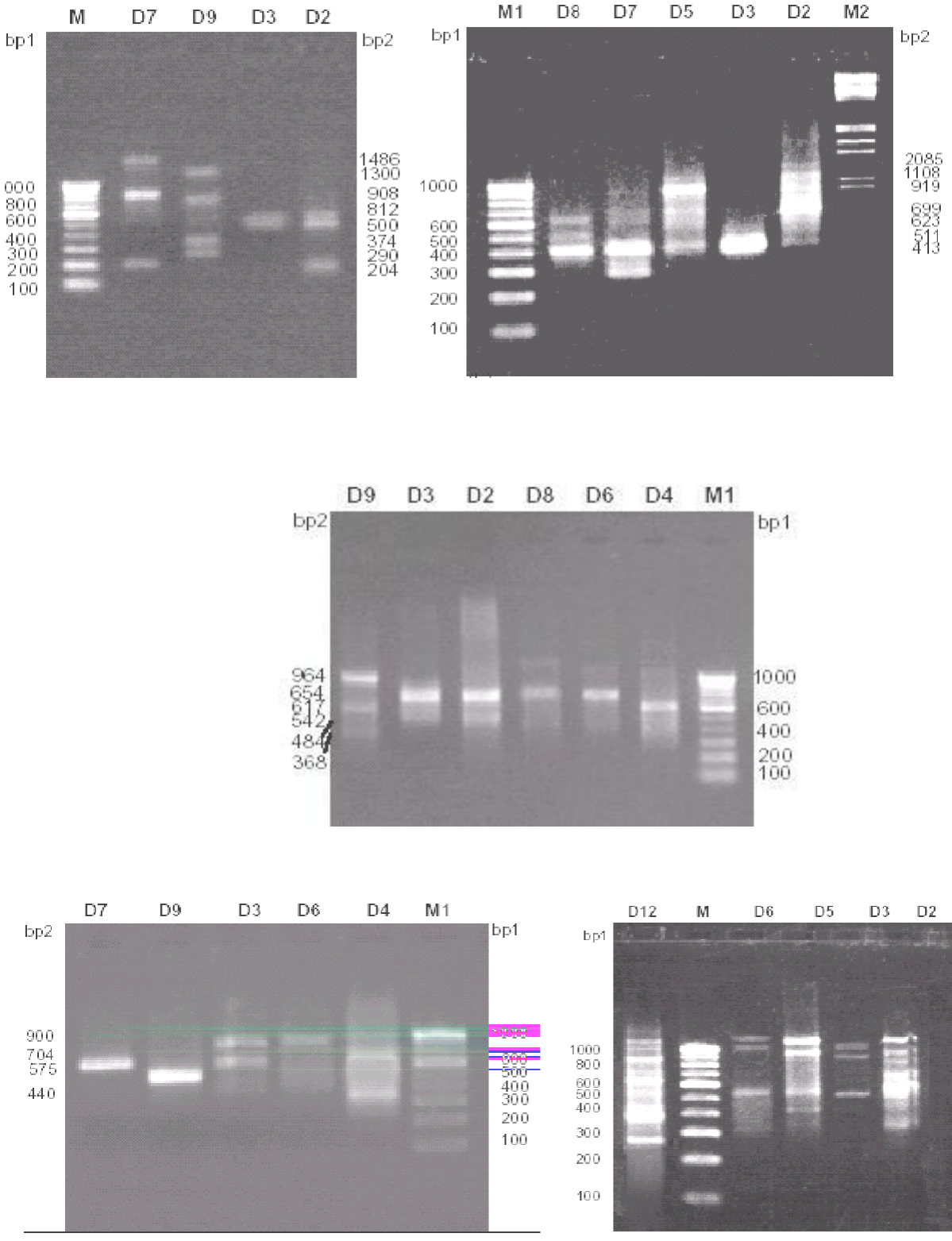

Figure 1. RAPD PCR amplification pattern of individual DNA samples of few Indian charophyte taxa with primers (a) OPF 07, (b) OPG 02, (c) OPG 03, (d) OBB 04 and (e) OPA 09. M1 and M2 are molecular markers, b1 and b2 are molecular sizes of markers and amplicons, respectively. See Table 1 for identities of the charophyte taxa.

Table 3. Band frequencies of various amplified products with different primers in the genus *Chara* and genus *Nitella*.

Primers	Molecular size of band (bp)	Band frequency	
OPF-07		***Chara***	***Nitella***
	1783	0.11	0.00
	1486	0.11	0.33
	1300	0.11	0.00
	1180	0.22	0.67
	908	0.22	0.33
	812	0.11	0.00
	742	0.22	0.33
	635	0.00	0.33
	562	0.33	0.67
	500	0.22	0.00
	443	0.22	0.00
	412	0.00	0.33
	374	0.22	0.67
	290	0.11	0.00
	204	0.22	0.00
OPG-02	2085	0.13	0.33
	1510	0.00	0.33
	1304	0.13	0.00
	1108	0.50	0.67
	919	0.25	0.00
	699	0.50	0.00
	623	0.25	0.33
	511	0.13	0.00
	413	0.63	1.00
	280	0.00	0.67
OPG-03	1087	0.11	0.33
	964	0.11	0.33
	847	0.00	0.67
	801	0.11	0.33
	753	0.11	0.00
	694	0.44	0.00
	654	0.11	0.67
	617	0.11	0.00
	542	0.33	0.67
	522	0.00	0.33
	484	0.22	0.00
	421	0.33	0.67
	368	0.11	0.00
OBB-04	1740	0.11	0.00
	1251	0.11	0.00
	1071	0.00	0.33
	993	0.00	0.33
	940	0.11	0.00

Cont.Table 3.

	900	0.22	0.33
	850	0.11	0.00
	775	0.11	0.67
	704	0.33	0.00
	660	0.11	0.33
	575	0.44	1.00
	481	0.11	0.00
	440	0.22	0.00
OPA-09	1700	0.00	0.50
	1330	0.43	0.50
	1170	0.29	0.50
	1035	0.57	0.00
	950	0.43	0.50
	870	0.14	0.00
	760	0.14	1.00
	680	0.57	0.50
	636	0.00	0.50
	550	1.00	1.00
	455	0.29	0.50
	410	0.14	1.00
	386	0.14	0.00
	340	0.29	0.00
	280	0.00	0.50

Table 4. Proportion of polymorphic bands pooled over varieties with different primers in Charophyte taxa.

Primer	No. of bands	No. of polymorphic bands	Proportion of polymorphic bands (%)
OPF-07	15	15	100.00
OPG-02	10	10	100.00
OPG-03	13	13	100.00
OBB-04	13	13	100.00
OPA-09	15	14	93.33

cies may represent the highly conserved region of primer binding sites in the genome. Similarly, the presence of polymorphic bands might be due to nucleotide changes in DNA sequence at primer binding sites (Williams et al., 1990).

Genetic identity index or similarity index

The genetic identity index (GII) or similarity index (SI) in two charophyte genera *Chara* and *Nitella* was calculated. Similarity index represents genetic relatedness or similarities between the species of genus *Chara* and the genus *Nitella* with respect to the amplified base sequences in RAPD-PCR. The estimation of SI was based on the frequencies of various bands generated by different primers (Zhu et al., 1996).

The primer wise genetic identity indices between *Chara* and *Nitella* have been presented in Table 5 along with their average values in all the five primers. The similarity indices in primer OPG-02 (0.351) and OPA-09 (0.417) were moderate while the lowest similarity index of 0.198 was observed in the primer OBB-04. Among other primers, OPF-07 and OPG-03 showed similarity indices of 0.295 and 0.85, respectively. The average SI between *Chara* and *Nitella* was 0.309 (SE 0.041) which indicates that both the genera have almost one third similarity with each other and constitute different phylogenetic lines.

Table 5. Similarity index and genetic distance estimated from RAPD-PCR analysis with different primers between the genus *Chara* and genus *Nitella*.

Primers	Similarity Index	Genetic distance
OPF-07	0.295	0.584
OPG-02	0.351	0.053
OPG-03	0.285	0.798
OBB-04	0.198	0.084
OPA-09	0.417	0.003
Average	0.309	0.304
SE	0.041	0.181

Table 6. Band sharing frequencies with different primers in the genus *Chara* and genus *Nitella.*

Primers	Band sharing frequency (BSF)		
	Within genus *Chara*	Within genus *Nitella*	Between the genera *Chara* and *Nitella*
OPF-07	0.148	0.185	0.297
OPG-02	0.291	0.5	0.362
OPG-03	0.189	0.232	0.094
OBB-04	0.177	0.444	0.258
OPA-09	0.449	0.429	0.44
Average	0.251	0.358	0.290
SE	0.062	0.070	0.065

Band sharing frequency

The band sharing frequency was calculated for both charophyte genera *Chara* and *Nitella.* (Table- 6). Band sharing frequency is an indicator of relatedness between different lines and is a simple expression of similarity measured in terms of sharing bands between the two genera *Chara* and *Nitella*. The band sharing frequency within the genus *Chara* and the genus *Nitella* were calculated individually along with the band sharing frequencies in between *Chara* and *Nitella* by using the statistical methods adopted by Lynch (1990) and Smith et al. (1996) with the help of scorable amplified products.

With all the five primers, genus *Chara* showed a frequency of shared bands between 0.449 and 0.148. The highest band sharing frequency was observed for the primer OPA-09 and the lowest for primer OPF-07. The other primers showed optimum band sharing frequency indicating that within this genus, like morphological diversity (Wood and Imahori, 1965), a high degree of diversification persists at molecular level also.

In *Nitella*, the band sharing frequency for all the five primers ranged between 0.5 and 0.185. Highest band sharing frequency of 0.5 was observed for primer OPG-02 while the lowest of o.185 was in primer OPF-07. As revealed by Table 3, over all band sharing frequency with in the genus *Nitella* was more (0.358 with SE 0.070) as compared to the genus *Chara* (0.251 with SE 0.062). However more data with more primers and Charophyte taxa is required to reach to a definite conclusion.

Chara and *Nitella* are morphologically distinct lines in the division Charophyta which has also been established at molecular level (Table 6). The band sharing frequency between *Chara* and *Nitella* ranges from 0.44 to 0.094. Highest band sharing frequency was recorded for primer OPA-09 and lowest for primer OPG-03. The data in Table 3 also reveal that primer OPA-09 showed maximum BSF with in the genus *Chara* and in between *Chara* and *Nitella* perhaps because this primer got some common sites for amplification. It indicates that the genus *Chara* and genus *Nitella* possess some common nucleotide sites where a specific primer can bind.

Genetic distance

The genetic distance between the genus *Chara* and genus *Nitella* was estimated on the basis of band sharing frequencies within both genera and in between them as described by Smith et al. (1996). The genetic distance (Table 5) was highest with primer OPG-03 (0.798) and minimum with the primer OPA-09 (0.003). The average genetic distance calculated for the five primers was 0.304 (SE 0.181). It is evident that the primers showing maximum similarity index have lowest genetic distance and vice versa.

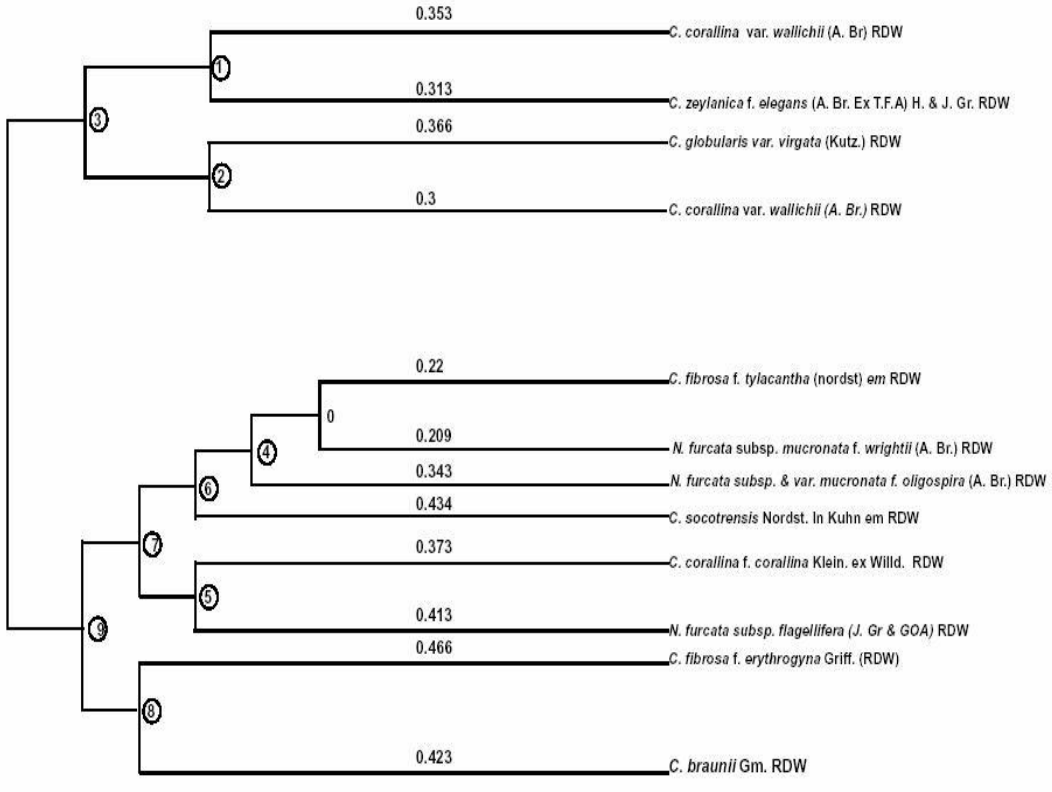

Figure 2. Dendrogram showing genetic relatedness between various species of Indian Charophyta belonging to the genus *Chara* and genus *Nitella*.

The genetic distance between *Chara* and *Nitella* was calculated using RAPDistance software version 1.04. Thus on the basis of genetic distance calculated for primer OPG-03, both the genera *Chara* and *Nitella* seem to form divergent phylogenetic lines after originating from the same ancestor as revealed by 0.003 genetic distance in primer OPA-09.

The data recorded for scorable bands in 9 species of the genus *Chara* and 3 species of the genus *Nitella* was documented for Jaccard's coefficient (Jaccard, 1908) by using NJ Tree and TDRAW softwares. The analysis was made by using most popular Clustal software package (Jeanmougin et al., 1998). Clustal is usually used in conjunction with NJPlot, a simple program for tree reconstruction by the neighbour- joining method (Saitou and Nei, 1987). The relatedness of various Charophyte species on the basis of phylogenetic tree is presented in Figure 2.

As per the phylogenetic tree, two clusters have been observed which are marked as node 3 and node 9 respectively. Cluster starting from node 3 show furcation into two divergent lines having nodes 1 and 2. From the node 1, two charophyte taxa namely D-2 (*Chara wallichii*, male plant) and D-3 (*C. zeylanica* f. *elegans*) seem to originate thus showing greater relatedness. From node 2, the taxa D-6 (*C. delicatula*) and D-8 (*C. walliichii*, female plant) emerged in close association. In this cluster, *C. wallichii* show reasonable distance at molecular level on the basis of sexual dimorphism. It establishes that the sexual dimorphism in Charophyte taxa is a valid taxonomic criterion. *C. wallichii* has been ranked as a variety of *C. corallina* by Wood and Imahori (1965) but Pal et al. (1962) considered it as an independent species. Molecular characterization of *C. wallichii* justifies its status as an independent species rather then its merger in *C. corallina* complex.

On the other hand, *C. zeylanica* f. *elegans* originates from node 1 and *C. globularis* var. *virgata* originates from node 2. It indicates closer association between these two taxa. *C.zeylanica* and *C. globularis* var. *virgata* are corticated forms with ecorticated basal branch segment and upper branchlet segments respectively. On the basis of molecular characters, both these taxa seem to be close and originating from the same stock. Thus their independent status of species should be maintained however, they should be kept close to each other.

Cluster starting from node 9 bifurcates into two subclusters having node 7 and 8. Node 8 is a smaller furcation and bifurcates in to *Chara fibrosa* f. *erythrogyna* and *Chara braunii* indicating their close association. However, *C. braunii* is closer to the node then *C. fibrosa* f. *erythrogyna.* It is interesting that another forma of *C.*

fibrosa group i.e. *C. fibrosa* f. *tylacantha*, is forming the terminal line of the same node 9 and is far apart from *C. fibrosa* f. *erythrogyna. C. braunii* is known for complete ecortication whereas *C. fibrosa* f. *erythrogyna* show completely ecorticated branchlets. The ecortication and geminate gametangia seem to be the main characters for molecular relatedness between them. Since *C. fibrosa* group is far apart from *C. erythrogyna* on molecular basis, the merger of *C. erythrogyna* in *C. fibrosa* group seems unjustified.

Other nodes bifurcating from node 7 are nodes 5 and 6. Node 5 forms two lines leading to both ecorticated but genetically divergent forms. One belongs to the genus *Chara* (*C. corallina*, dioecious) while the other belongs to genus *Nitella* (*N. furcata* subsp. *flagellifera*). This data surprisingly indicate affinity between ecorticated species of *Chara* and ecorticated form of *Nitella* thus establishing ecortication as valid taxonomic criteria in charophytes.

These results are at preliminary stage and do not provide sufficient ground to interpret exact speciation pattern. Therefore exhaustive investigations in other charophyte taxa are desirable before reaching to a definite conclusion. Node 6 furcates into *C. socotrensis* line on one hand and node 4 on the other hand showing further bifurcation. *C. socotrensis* show very close alliance with *N. furcata* var. *mucronata* f. *oligospira* and complete ecortication is again a character in both charophytes despite being far away, genus wise. Besides this, node 4 show yet another furcation into node 0, which gives rise to *N. furcata* var. *mucronata*? Thus all the three ecortcated, monoecious species, two from genus *Nitella* and one from genus *Chara* seem to be closely aligned. However *C. fibrosa* f. *tylacantha* is also a part of this cluster but is far away from another member of *C. fibrosa* complex i.e *C. fibrosa* f. *erythrogyna*.

On the basis of phylogenetic tree, it seems evident that *C. wallichii*, *C. delicatula* and *C. erythrogyna* should be reverted back to the status of independent “species” as suggested by Pal et al. (1962) rather than their merger with other species.

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