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Differentiation of *Chara gymnopitys* A. Br. and *Chara hydropitys* Reich. by morphological characters, isozyme analysis and oospore wall ornamentation

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Abstract — Morphological, electrophoretic and SEM analyses are used to assess the differences between *C. gymnopitys* A. Braun and *C. hydropitys* Reichenbach. A comparative study of the twelve isozymes through polyacrylamide gel electrophoresis supports the findings of the morphological studies which had demonstrated that the two taxa investigated are distinct. The SEM study of the oospore wall ornamentation of the two species also shows distinct variations in the ornamentation pattern. The present study has provided evidence for the establishment of *C. gymnopitys* and *C. hydropitys* as two distinct species.

***Chara gymnopitys* / *Chara hydropitys* / isozyme / oospore wall ornamentation**

Résumé — Différentiation entre *Chara gymnopitys* A. Braun et *Chara hydropitys* Reich. à l'aide des caractères morphologiques, de l'analyse des isozymes, et de l'ornementation de la paroi de l'oospore. En vue d'évaluer les différences entre *C. gymnopitys* A. Braun et *C. hydropitys* Reichenbach, leurs morphologies, comprenant les données du microscope électronique à balayage (SEM), ont été confrontées aux données de l'électrophorèse. L'étude électrophorétique comparée de douze isozymes, à partir de gel polyacrylamide, est en accord avec les données morphologiques, lesquelles vont dans le sens de la distinction des deux taxa considérés. De plus, l'ornementation de la paroi de l'oospore, observée au SEM, montre aussi de nettes différences entre les deux espèces. La présente étude rétablit la distinction de *C. gymnopitys* et de *C. hydropitys*.

***Chara gymnopitys* / *Chara hydropitys* / isozyme / ornementation de la paroi de l'oospore**

INTRODUCTION

The genus *Chara* L. is cosmopolitan in distribution and comprises about 116 species (Wood, 1952), occurring in a wide range of habitats. Morphological characters such as cortication of the axes and branchlets, rows of stipulodes and spine cells, amongst others, have been commonly employed in the delineation of species, though it is known they are variable. Wood & Imahori (1965) merged

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many species and reduced the number from 116 to 19. Many charophytologists strongly disapproved of these taxonomic realignments based on developmental morphology (Sundaralingam, 1963, 1966; Bharathan, 1987; Bharathan & Sundaralingam, 1984a), cytology (Bharati & Chennaveeriah, 1980; Ray & Chatterjee, 1988; Khan & Sarma, 1967; Sarma & Ramjee, 1971; Tindall & Sawa, 1964), reproductive isolation-hybridization (Proctor *et al.,* 1967; Proctor, 1971; Bharathan & Sundaralingam, 1984b) and numerical studies (McGuire, 1971).

Wood & Imahori (1965) included *C. gymnopitys* A.Br. and *C. hydropitys* Reich as synonym or variety of the species *Chara fibrosa* Agardh, respectively (*C. gymnopitys* = *C. fibrosa* var. *fibrosa* Ag. ex Bruz. em. R.D.Wood, and *C. hydropitys* = *C. fibrosa* var. *hydropitys* (Reich.) R.D.Wood em. R.D.Wood). However, a lack of reference on Indian material of *C. gymnopitys* and *C. hydropitys* makes unclear whether the two mentioned species should be considered within *C. fibrosa.* Zaneveld (1940) retained the name *C. hydropitys* in his "Malaysian Charophytes", and Sundaralingam (1959) also described the material collected from the same Indian localities visited for this study as *C. gymnopitys* and *C. hydropitys.* Proctor (1971); Grant & Proctor (1980) and John *et al.* (1990) also referred to these two species as *C. gymnopitys* and *C. hydropitys.* Taking into account the above considerations the original names *C. gymnopitys*, as used by Braun, and *C. hydropitys,* as referred to by Reichenbach are retained.

In most water bodies in and around Chennai (Madras), Tamil Nadu, India, these two species, *C. gymnopitys* and *C. hydropitys* occur together in association with *C. zeylanica* Klein ex Willd. Though both *C. gymnopitys* and *C. hydropitys* appear similar in stature and have black oospores, a careful examination reveals differences in the morphological features. *Chara hydropitys* is particularly interesting as it may form a "bridge" between subsections Agardhia and Willdenowia of Wood & Imahori (1965), as postulated by Proctor (1980).

In the present investigation, the variability of morphological features, differences in proteins and isozymes (analysed using electrophoresis), and characteristics of the oospore wall ornamentation studied with the Scanning Electron Microscopy (SEM), are used to determine the validity of *C. hydropitys* and *C. gymnopitys* as separate entities.

MATERIALS AND METHODS

The materials required for the present study were collected from six rain-fed fresh water lakes (seasonal) around Chennai (Madras), India (Tab. 1). Entire plants were collected, washed thoroughly and each collection was assigned a number. The two species *C. gymnopitys* and *C. hydropitys* were grown in the biphasic medium (Imahori & Iwasa, 1965; Forsberg, 1965, Proctor *et al.*, 1967; Bharathan & Sundaralingam, 1984 and Krishnan & Bharathan, 2001, 2004). The cultures were maintained in an aseptic environment. Oospores for SEM studies were collected from 30-40 days old culture, maintained in the laboratory and prepared for SEM, adopting the method of John & Moore (1987).

Samples from 28-30 days old culture were removed before the sex organs appeared and were subjected to biochemical analyses (1 g fresh weight sample was used for each enzyme and protein). After the estimation of protein by the

Table 1. Morphological features of field and culture specimens of *C. gymnopitys* and *C. hydropitys*

Morphological features / *Collection Sites*		*Chara gymnopitys*				*Chara hydropitys*			
		Mudichur	*Manimangalam*	*Potheri*	*Padappai*	*Mudichur*	*Manimangalam*	*Potheri*	*Padappai*
Plant Height in cm	F	8 – 10	5.5 – 6.5	8 – 9	8.5 – 9	7 – 8.5	4.5 – 6	8 – 9	7 – 9
	C	19 – 20.5	15 – 16	26 – 28	26.5 – 28	9.5 – 12	8.5 – 11	10 – 12.5	10 – 12
Axis Thickness in µm	F	434 – 476 (M:458±1.8)	364 – 392 (M:378±0.8)	420 – 434 (M:424±0.2)	504 – 532 (M:520±1.1)	350 – 364 (M:354±1.2)	224 – 238 (M:227±0.1)	392 – 406 (M:396±0.2)	350 – 364 (M:356±1.3)
	C	448 – 518 (M:490± 2.9)	476 – 532 (M:504±1.9)	476 – 532 (M:504±1.6)	434 – 490 (M:462±2.7)	350 – 392 (M:378±2.9)	336 – 378 (M:352±2.6)	392 – 434 (M:406±2.2)	336 – 378 (M:355±2.8)
Internode length compared to branchlet	F	1 1/4 -1 1/2 times	Same	1 1/4	2	1 1/2	Same or 1/4	Same	Same
	C	2 times	1 1/2 times	2 1/2 - 3	2 1/2 - 3	1 1/2 - 2	1 1/4	1 1/2	2
Axis Cortex	F	Diplo	Diplo	Diplo	Diplo	Diplo/triplo	Diplo/triplo	Diplo/triplo	Diplo
	C	Diplo	Diplo	Diplo	Diplo	Diplo/triplo	Diplo/triplo	Diplo/triplo	Diplo
Spine Cell	F	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary
	C	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary
Spine Cell Length in µm	F	84 – 112 (M:96±1.1)	112 – 126 (M:120±0.4)	154 – 168 (M:161±0.2)	70 – 98 (M:78±0.3)	Rudimentary	Very rudimentary	Rudimentary	Rudimentary
	C	336 – 392 (M:378±2.9)	392 – 462 (M:424±3.1)	238 – 308 (M:282±1.8)	252 – 308 (M:270±2.3)	Very small	Rudimentary	Rudimentary	Very small
Stipulode Type	F	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo
	C	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo	Haplo
No. of stipulodes compared to branchlets	F	Twice	Twice	Twice	Twice	Same	Same	Same	Same
	C	Twice	Twice	Twice	Twice	Same	Same	Same	Same
No. of branchlets	F	9 – 10	10	9 – 10	10	9 – 10	8 – 9	10 – 12	10 – 12
	C	10 – 12	10	11	9 – 10	10	10	10 – 12	10

F – Specimens from the field
C – Specimens from the culture
M – Mean
Diplo – Diplostichous
e – Ecorticate
c – Corticate
Haplo – Haplostichous
Triplo – Triplostichous
lo – Lower segment
u – Upper segment

Table 1. Morphological features of field and culture specimens of *C. gymnopitys* and *C. hydropitys (continued)*

Morphological features	*Collection Sites*	*Chara gymnopitys*				*Chara hydropitys*			
		Mudichur	*Manimangalam*	*Potheri*	*Padappai*	*Mudichur*	*Manimangalam*	*Potheri*	*Padappai*
No. of segments per branchlet	F	4 – 5	4 – 5	6 – 7	4 – 5	5 – 6	4 – 5	5 – 6	5 – 6
	C	4 – 5	4 – 5	6 – 7	4 – 5	5 – 6	5 – 6	5 – 6	5 – 6
Branchlet cortication	F	Ecorticate	Ecorticate	Ecorticate	Ecorticate	e (lo – 1, u – 2,3) c–2,3	e (lo – 1, u – 1,2) c–2,3	e (lo – 1, u – 1,2) c–3,4	e (lo – 1, u – 2) c–2,3
	C	Ecorticate	Ecorticate	Ecorticate	Ecorticate	e (lo – 1, u – 2,3) c–2,3	e (lo – 1, u – 2,3) c–2	e (lo – 1, u – 2,3) c–2	e (lo – 1, u – 1,2) c–3
Bract cell length in µm	F	1162 – 1204 (M:1182±1.8)	826 – 840 (M:831±0.8)	826 – 854 (M:841±1.1)	1316 – 1344 (M:1328±1.8)	910 – 952 (M:926±2.1)	966 – 994 (M:978±2.2)	1386 – 1400 (M:1392±0.9)	686 – 700 (M:690±0.7)
	C	1330 – 1722 (M:1698±5.2)	1232 – 1316 (M:1275±2.9)	1232 – 1358 (M:1292±3)	1638 – 1820 (M:1711±3.1)	868 – 896 (M:882±1.9)	840 – 938 (M:872±2.6)	1092 – 1162 (M:1120±2.1)	840 – 896 (M:864±2.3)
No. of fertile nodes per branch	F	All nodes	All nodes	Lower 4 nodes	Lower 2 nodes	All	All	Lower 3	All
	C	All nodes	Lower 2 nodes	3 or 4 nodes	Lower 2 nodes	Lower 4	All	Lower 4	Lower 3 or 4
Diameter of antheridia in µm	F	336 – 364 (M:348±1.4)	196 – 224 (M:208±2.1)	182 – 210 (M:192±1.1)	266 – 294 (M:274±1.3)	224 – 252 (M:232±1.2)	182 – 210 (M:192±1.4)	266 – 280 (M:271±0.5)	224 – 252 (M:238±2.5)
	C	336 – 378 (M:364±1.9)	434 – 462 (M:448±1.8)	364 – 392 (M:381±0.9)	448 – 476 (M:460±1.2)	210 – 238 (M:224±0.8)	210 – 238 (M:218±0.9)	266 – 294 (M:278±1.3)	224 – 252 (M:242±2.9)
Oogonia length including coronula in µm	F	728 – 770 (M:749±2.4)	546 – 574 (M:558±2.6)	336 – 364 (M:345±2.1)	616 – 649 (M:632±1.8)	546 – 574 (M:554±1.1)	406 – 434 (M:418±1.3)	490 – 504 (M:494±0.4)	504 – 518 (M:512±0.8)
	C	770 – 826 (M:798±2.6)	812 – 868 (M:830±1.4)	756 – 812 (M:775±3.1)	770 – 826 (M:789±2.7)	532 – 588 (M:560±2.9)	546 – 588 (M:566±1.8)	546 – 588 (M:564±2.4)	504 – 532 (M:514±1.5)

F – Specimens from the field
C – Specimens from the culture
M – Mean
Diplo – Diplostichous

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Haplo – Haplostichous
Triplo – Triplostichous

lo – Lower segment
u – Upper segment

Table 1. Morphological features of field and culture specimens of *C. gymnopitys* and *C. hydropitys (continued)*

Morphological features / *Collection Sites*		*Chara gymnopitys*				*Chara hydropitys*			
		Mudichur	*Manimangalam*	*Potheri*	*Padappai*	*Mudichur*	*Manimangalam*	*Potheri*	*Padappai*
Oogonia width in µm	F	434 – 462 (M:445±1.8)	322 – 350 (M:340±2.2)	210 – 238 (M:216±0.8)	476 – 564 (M:512±5.2)	308 – 322 (M:316±1.4)	238 – 252 (M:242±0.8)	238 – 266 (M:244±2.2)	266 – 280 (M:270±0.2)
	C	448 – 476 (M:462±1.7)	532 – 560 (M:542±2.3)	448 – 476 (M:458±1.2)	490 – 518 (M:504±2.1)	350 – 392 (M:308±2.4)	280 – 294 (M:284±0.5)	336 – 350 (M:340±0.8)	280 – 322 (M:302±2.9)
Coronula height in µm	F	70 – 98 (M:81±1.6)	70 – 84 (M:78±0.6)	56 – 70 (M:61±0.4)	56 – 70 (M:62±0.8)	56 – 70 (M:62±1.2)	56 – 70 (M:63±0.7)	56 – 70 (M:61±1.6)	56 – 70 (M:63±0.6)
	C	112 – 140 (M:120±1.2)	70 – 84 (M:76±0.8)	56 – 70 (M:62±0.8)	98 – 112 (M:102±1.8)	42 – 84 (M:67±1.8)	56 – 70 (M:64±0.2)	56 – 84 (M:74±1.4)	56 – 70 (M:62±0.4)
Coronula width in µm	F	168 – 210 (M:178±1.3)	140 – 154 (M:146±0.9)	140 – 168 (M:155±3.2)	126 – 140 (M:132±1.1)	140 – 168 (146±0.7)	140 – 168 (M:152±0.9)	140 – 168 (M:150±0.8)	140 – 168 (M:152±0.8)
	C	140 – 182 (M:150±3.2)	140 – 168 (M:151±1.1)	154 – 168 (M:159±1.6)	154 – 168 (M:161±0.8)	98 – 154 (M:130±2.9)	126 – 140 (M:132±0.7)	126 – 168 (M:142±1.5)	126 – 140 (M:133±0.5)
Convolutions	F	8 – 9	8	9	10	8 – 9	8 – 9	9 – 10	9 – 10
	C	9	8	8	10	8 – 10	8 – 9	9 – 10	9 – 10
Colour of oospore	F	Black	Black	Black	Black	Black	Black	Black	Black
	C	Black	Black	Black	Black	Black	Black	Black	Black
Oospore length in µm	F	350 – 378 (M:360±2.5)	406 – 434 (M:420±2.1)	308 – 336 (M:318±2.3)	308 – 322 (M:314±0.9)	294 – 336 (M:308±1.2)	336 – 350 (M:342±0.4)	294 – 322 (M:312±0.8)	280 – 294 (M:287±0.7)
	C	350 – 405 (M:378±4.1)	476 – 504 (M:486±2.4)	392 – 462 (M:428±4.6)	392 – 434 (M:412±2.1)	350 – 392 (M:364±2.9)	294 – 330 (M:315±1.8)	336 – 350 (M:343±1.3)	336 – 350 (M:342±1.7)
Oospore width in µm	F	238 – 266 (M:244±1.6)	238 – 266 (M:245±2.4)	196 – 224 (M:204±1.6)	280 – 308 (M:292±2.5)	196 – 210 (M:201±0.3)	168 – 196 (M:178±1.1)	196 – 238 (M:218±1.1)	196 – 210 (M:202±0.2)
	C	266 – 308 (M:280±4.2)	364 – 420 (M:392±2.3)	266 – 308 (M:280±3.1)	322 – 364 (M:340±2.7)	182 – 196 (M:184±0.2)	126 – 154 (M:132±2.3)	182 – 190 (M:184±0.15)	224 – 266 (M:243±2.1)

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Triplo – Triplostichous
lo – Lower segment
u – Upper segment

Table 2. List of enzymes studied and staining schedule followed

Enzyme	*Abbreviation & Enzyme Number (IUBNC 1984)*	*Staining Schedule*
Acid phosphatase	(ACP - 3.1.3.2)	Soltis *et al*. (1983)
Alkaline phosphatase	(AKP - 3.1.3.1)	Reddy & Stahmann (1972)
Catalase	(CAT - 1.11.1.6)	Woodbury *et al.* (1971)
Esterase	(EST - 3.1.1-)	Reddy & Stahmann (1972)
Glucose-6-phosphate dehydrogenase	(G-6-PDH-1.1.1.49)	Cheney (1985)
Glutamate dehydrogenase	(GDH - 1.4.1.2)	Cheney (1985)
Hexokinase	(HEX - 2.7.1.1)	Soltis *et al.* (1983)
Lactate dehydrogenase	(LDH - 1.1.1.27)	Rice & Crowden (1987)
Malate dehydrogenase	(MDH - 1.1.1.37)	Cheney (1985)
Peroxidase	(PER - 1.11.1.7)	Reddy & Stahmann (1972)
Phosphoglucoisomerase	(PGI - 5.3.1.9)	Soltis *et al*. (1983)
Phosphoglucomutase	(PGM - 2.7.5.1)	Soltis *et al.* (1983)

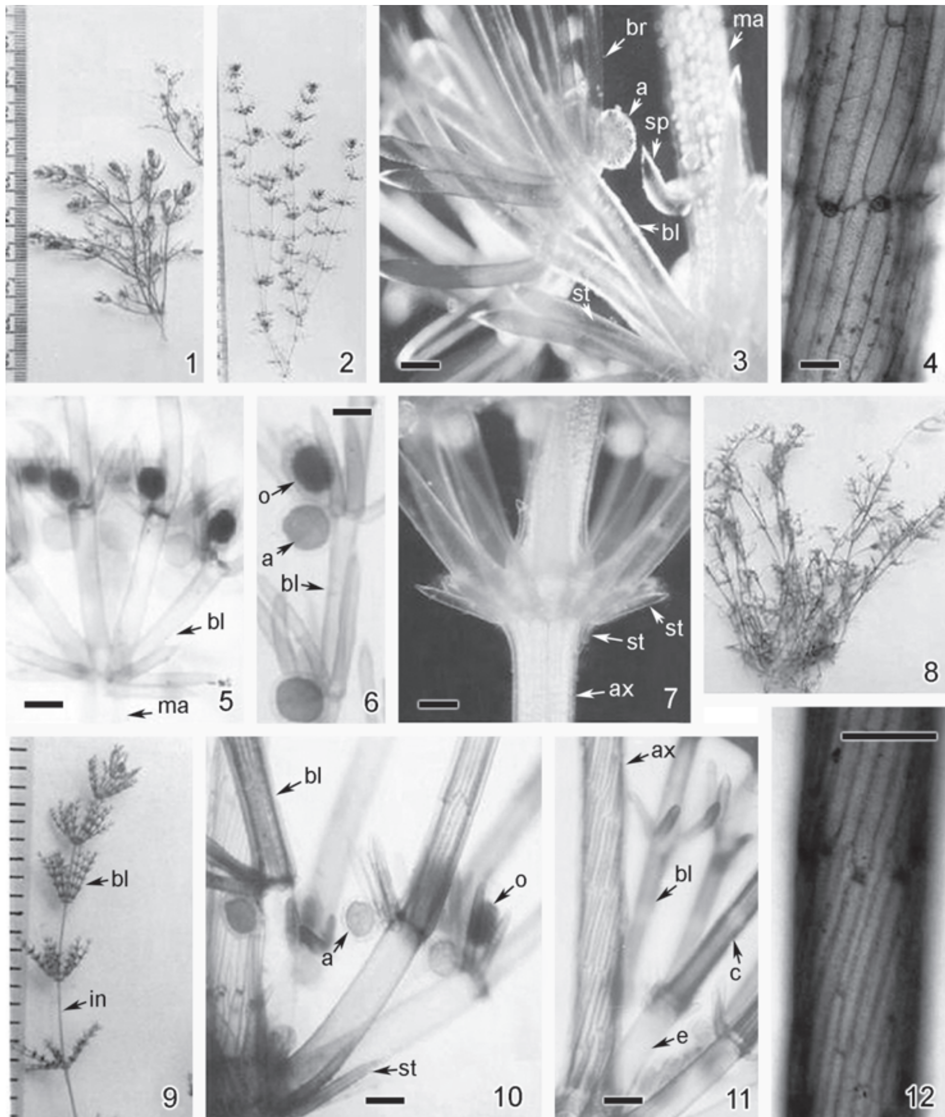
Lowry *et al.* (1951) method, Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out to separate and calculate the molecular weight of each protein subunit. The gel was then stained using modified Blum's silver staining method, described by Nesterenko *et al.* (1994). Replicate tests were run to ensure reproducibility.

Polyacrylamide gel electrophoresis (Davis, 1964) was performed for the study of isozyme banding pattern of twelve enzymes (Tab. 2). Protein supernatants (75 µl) were applied to 10% polyacrylamide gel. Electrophoresis was performed at 4 °C until reproducible banding patterns were obtained for all the enzyme systems examined. Relative mobility (Rm.) values were calculated as the ratio of distance migrated by the band in relation to the marker bromophenol blue dye front. The intensities of the bands on the gels were visually graded and zymograms were drawn. Each stained enzyme band was considered for comparison.

RESULTS

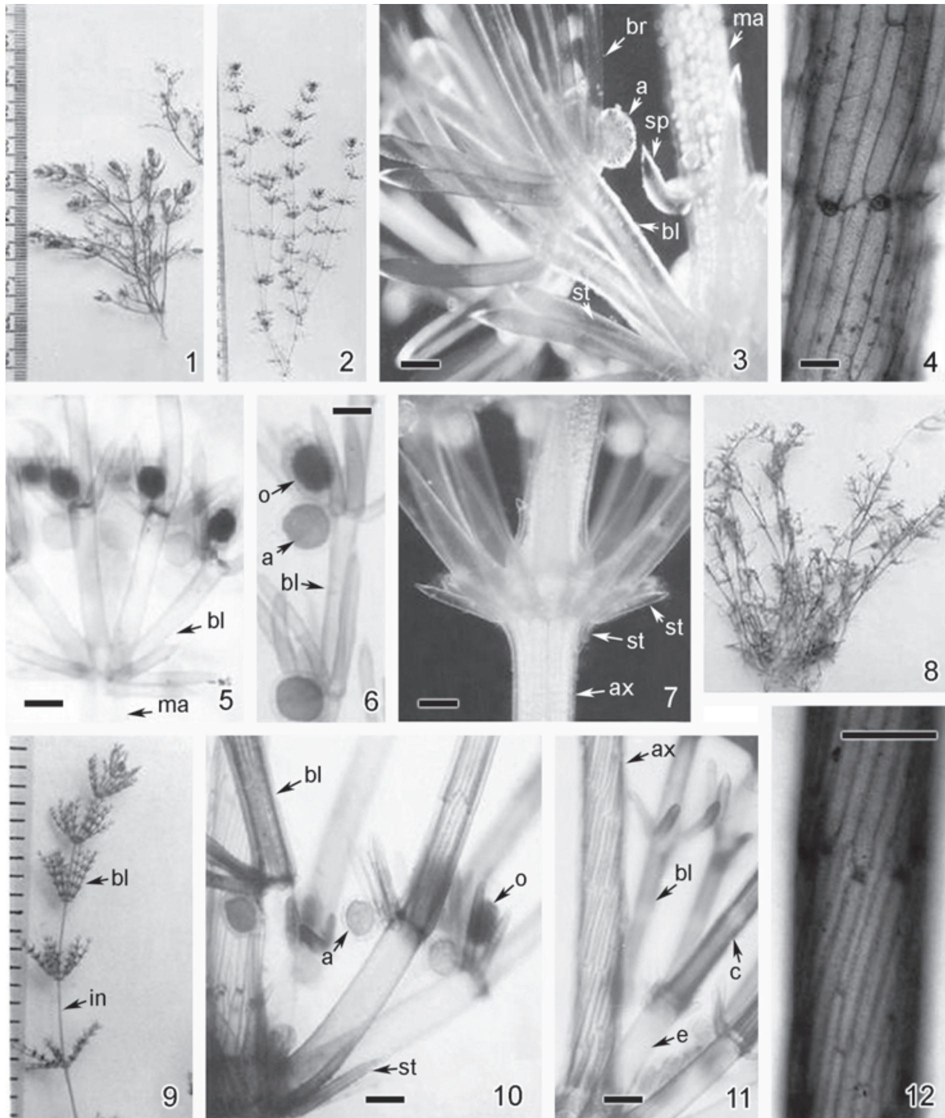
Morphological observations

The morphological features of field and culture specimens of *C. gymnopitys* are presented in Table 1. Materials collected from different locations show minor variations in the height of the plant, the thickness of the axis, the length of the spine cells and branchlets, the diameter of the antheridia and the length and width of the oogonia. These minor variations may have been influenced by environmental conditions. However, the distinguishing characters such as diplostichous cortex, ecorticate branchlets, haplostephanous nature of stipulodes, number of stipulodes per branchlet, well-developed solitary spine cells, remained the same for the isolates (Figs 1-6).



Figs 1-12. **1-6.** *Chara gymnopitys*. **1.** Field specimen. **2.** Culture specimen. **3.** A portion of the plant enlarged to show main axis, stipulodes, ecorticate branchlets, large bract cells and gametangia. **4.** Mature axial internode with diplostichous cortex. **5.** Main axis enlarged to show well-developed haplostephanous stipulodes and ecorticate fertile branchlets. **6.** Fertile branchlet node with conjoined gametangia. **7-12** – *Chara hydropitys*. **7.** Axial node showing irregular diplostephanous stipulodes. (polarisation). **8.** Field specimen. **9.** An enlarged portion of the cultured plant showing long internodes and branchlets with gametangia. **10.** Axial node with stipulodes and branchlets with gametangia. **11.** A portion of the plant enlarged to show cortication of axis and branchlets. **12.** Cortication (triplostichous) of the mature axis. All scales = 100 μm.

Abbreviations: a- antheridia; ax-axis; bl-branchlet; br-bract; c-corticate; e-ecorticate; g-gametangium; in-internode; ma-main axis; o-oogonium; sp-spine cell; st-stipulode.



Figs 1-12. **1-6.** *Chara gymnopitys*. **1.** Field specimen. **2.** Culture specimen. **3.** A portion of the plant enlarged to show main axis, stipulodes, ecorticate branchlets, large bract cells and gametangia. **4.** Mature axial internode with diplostichous cortex. **5.** Main axis enlarged to show well-developed haplostephanous stipulodes and ecorticate fertile branchlets. **6.** Fertile branchlet node with conjoined gametangia. **7-12.** – *Chara hydropitys*. **7.** Axial node showing irregular diplostephanous stipulodes. (polarisation). **8.** Field specimen. **9.** An enlarged portion of the cultured plant showing long internodes and branchlets with gametangia. **10.** Axial node with stipulodes and branchlets with gametangia. **11.** A portion of the plant enlarged to show cortication of axis and branchlets. **12.** Cortication (triplostichous) of the mature axis. All scales = 100 µm.

Abbreviations: a- antheridia; ax-axis; bl-branchlet; br-bract; c-corticate; e-ecorticate; g-gametangium; in-internode; ma-main axis; o-oogonium; sp-spine cell; st-stipulode.

Table 1 depicts the morphological features of isolates of *C. hydropitys* collected from the different locations and of the culture material. The diplostichous/triplostichous cortex with primary series prominent, partially corticate branchlets, number of stipulodes per branchlet, and rudimentary spine cells are the stable characteristic features in all the isolates (Figs 8-12). Variations are seen only in the length and the width of the vegetative and reproductive parts. These variations may, again, be due to environmental influence. The present observation on the variation in axis cortication — diplo/triplostephanous condition — is in conformity with observations made by Sundaralingam (1966) and Proctor (1980).

It is interesting to note, that few plants from the isolates ch12 and ch14 from Mudichur, have one or two incompletely formed lower stipulodes in addition to the regular upper whorl of stipulodes (Fig. 7). In all the plants, the lowermost segment and upper one or two segments are ecorticate, as reported by Zaneveld (1940); Sundaralingam (1959); Pal *et al.* (1962) and Wood & Imahori (1965). The specimens of *C. gymnopitys* and *C. hydropitys* from cultures did not show differences when compared with the field materials (Tab. 1), though, an increase in the height, the axis thickness and the size of the sex organs was observed.

SEM study of oospores

In the present investigation, in addition to the measurements of the oospore length, the width and the number of striae, ultrastructural study of the oospore wall ornamentation by SEM has clearly helped in the identification of the two taxa. The terminology used by John & Moore (1987) and John *et al.* (1990) is adopted to describe the oospore wall ornamentation.

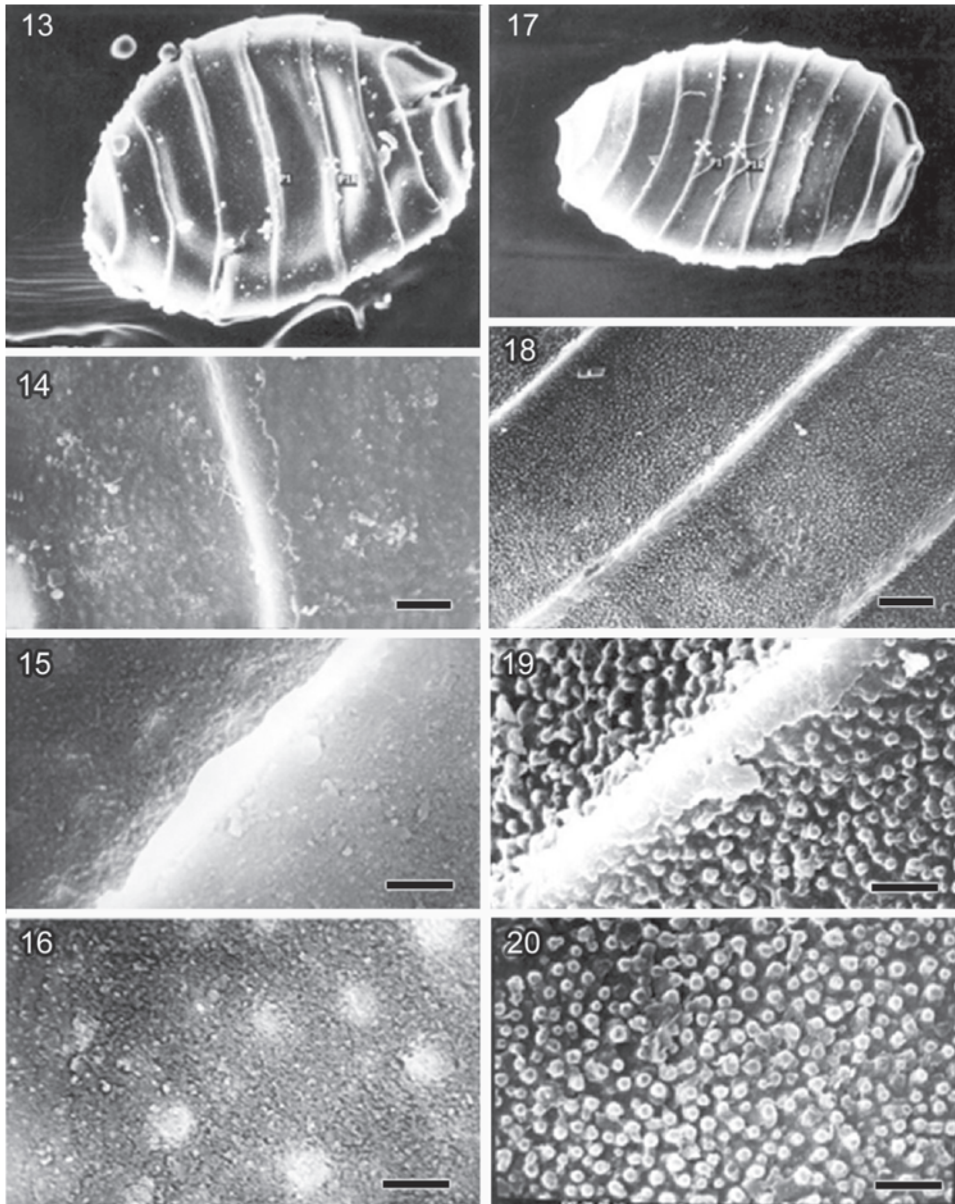
In *C. gymnopitys,* the fossa presents a finely granulated surface at a 3,000 magnification (Fig. 14). When viewed under a 15,000 magnification, these small granules appear to be fused to give a scabrous surface. Small elevations are seen on the surface at regular intervals and some of them have minute pores in the centre (Fig. 16). The fossa width is about 48 µm (Fig. 13). There are 7-8 striae (Fig. 13) with delicate ribbon-like projections with a smooth surface (Fig. 15).

The ornamentation on the fossa surface of *C. hydropitys* when viewed under a 3,000 magnification is densely verrucate (Fig. 18). These verrucae have irregular walls and many of them show central pores (Fig. 20). This ornamentation extends to the ridges for a short distance. The fossa width is about 28 µm (Fig. 17). Oospores have 8-14 striae (Fig. 17). The surface of the ridges forming the striae is irregular (Fig. 19).

Protein profile

The distinct protein bands obtained in an electrophoretic support media are classified based on the extent of migration. A combination of electrophoretic separation and staining of proteins enabled to compare and analyze the variations in proteins of the different species. As it is generally accepted that the protein variation detected by electrophoresis reflects genetic variation, the two species of *Chara* have been subjected to protein analysis by SDS-PAGE method (Fig. 21).

The scanning of the gel by the densitometer revealed polypeptides of low and high molecular weights in both species. In *C. gymnopitys,* 22 bands with approximate molecular mass of 10727 to 189611 daltons are observed and *C. hydropitys* reveals 21 polypeptides with molecular mass of 10789–189240 daltons.



Figs. 13-20. **13.** Scanning electron micrograph of oospore of *C. gymnopitys*. Figs 14-16. Oospore wall of *C. gymnopitys* under SEM. **14.** Portion of an oospore with finely granulate surface with one of the striae. **15.** Smooth, delicate ribbon-like projection from one of the striae. **16.** Fossa wall consisting of fused granules presenting scabrous surface. Small elevations with minute pores in the centre seen at regular intervals. **17.** Scanning electron micrograph of oospore of *C. hydropitys*. Figs. 18-20. Oospore wall of *C. hydropitys* under SEM. **18.** Portion of an oospore showing densely verrucate surface and a few striae. **19.** The irregular surface of the ridge forming the striae. Note the wall ornamentation extending onto the ridge. **20.** Fossa wall showing verrucae with irregular wall and central pores. Scales: 14, 18 = 2 µm; 15, 16, 19, 20 = 0.5 µm.

Table 3. Data on the molecular weights of polypeptides (Daltons) in *C.gymnopitys* and *C. hydropitys*

Cg	*Ch*	*Cg*	*Ch*
189611	189240	–	32911
135078	–	–	30889
126780	–	–	29668
118650	–	–	28495
108323	107886	26976	–
–	99523	25910	–
93409	–	–	24671
–	86168	23155	–
80179	81577	22433	–
71655	72278	21360	–
62040	62040	19090	–
56412	–	–	18335
52491	–	16768	–
–	48422	–	16012
–	442824	–	14899
39920	–	–	14435
36299	37145	11696	–
–	34563	10727	10789

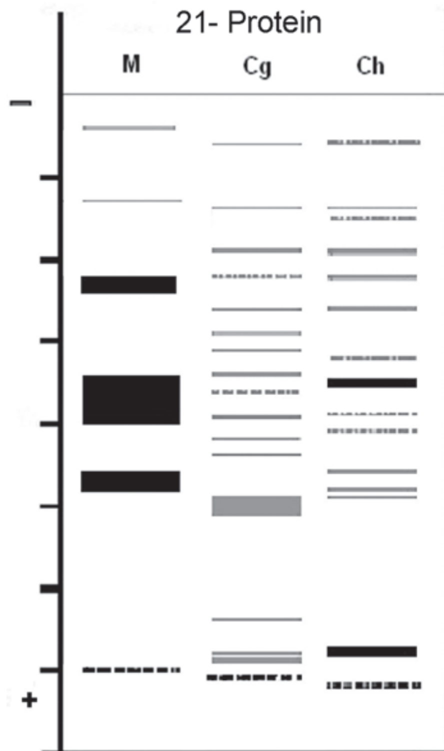


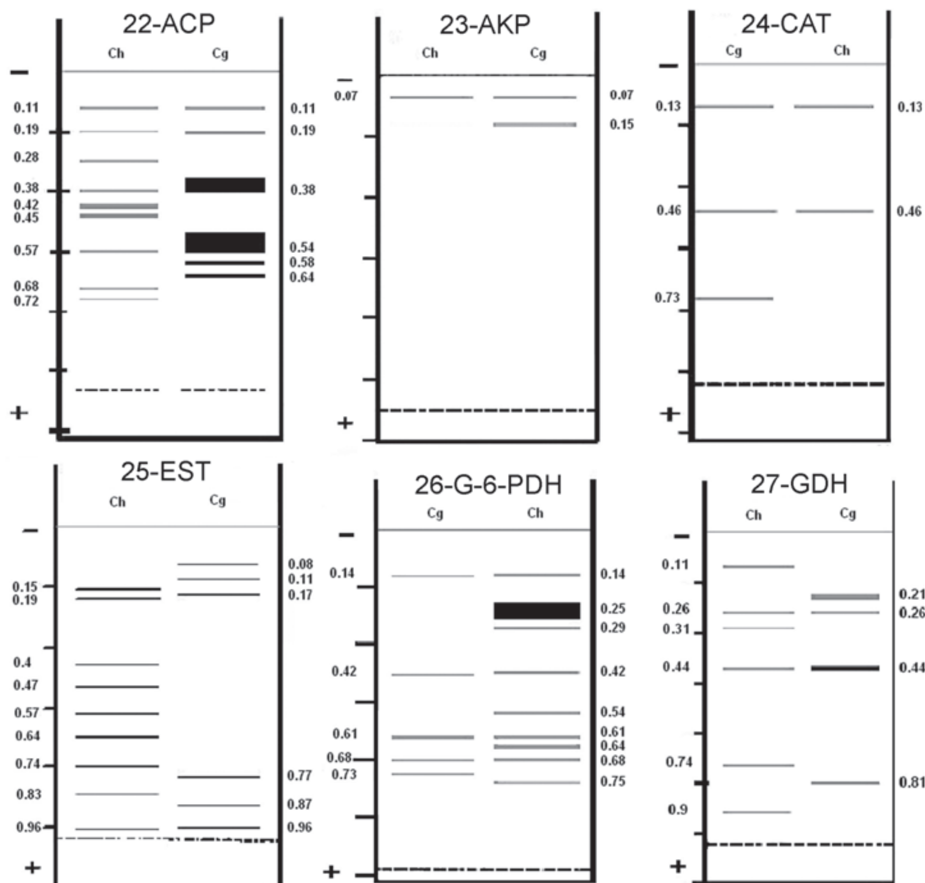
Fig. 21. Diagrammatic representation of gel stained for protein found in *C. gymnopitys* and *C. hydropitys*.

Though there are common polypeptide bands (of approximate molecular mass of 189, 108, 80, 72, 62, 36, 10 KDa) shared by the two species, the protein profile also reveals distinct differences between the two species (Tab. 3). It can be noticed that the bands seen in *C. gymnopitys* are different from those seen in *C. hydropitys*.

Isozyme analysis

The two species investigated reveal positive results for the twelve enzymes, viz. Acid phosphatase (ACP), Alkaline phosphatase (AKP), Catalase (CAT), Esterase (EST), Glucose-6-phosphate dehydrogenase (G-6-PDH), Glutamate dehydrogenase (GDH), Hexokinase (HEX), Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Peroxidase (PER), Phosphoglucoisomerase (PGI) and Phosphoglucomutase (PGM).

Acid phosphatase banding pattern is very complicated as they show many bands of varying intensities (Fig. 22). *Chara gymnopitys* and



Figs 22-27. Diagrammatic representation of gels stained for enzymes found in *C. gymnopitys* (Cg) and *C. hydropitys* (Ch). **22.** Acid phosphatase. **23.** Alkaline phosphatase. **24.** Catalase. **25.** Esterase. **26.** Glucose-6-phosphate dehydrogenase. **27.** Glutamate dehydrogenase.

C. hydropitys show similarity by recording bands at Rm. 0.11, 0.19 and 0.38, but distinct differences are seen between the two. The bands at Rm. 0.28, 0.42, 0.45, 0.68 and 0.72 are shown only by *C. hydropitys* and not by *C. gymnopitys.*

The enzyme alkaline phosphatase has low electrophoretic mobility, thereby revealing high molecular weight and/or low net ionic charges (Fig. 23). At Rm. 0.07 both the species reveal a band. *C. gymnopitys* records another band at Rm. 0.15 and both the bands recorded in *C. gymnopitys* are more intense when compared with the single band recorded in *C. hydropitys.*

Electrophoretic profile of catalase show restricted number of bands — 2 to 3. In C. *gymnopitys,* there are 3 bands recorded at Rm. 0.13, 0.46 and 0.73, while *C. hydropitys* shows only two bands at Rm. 0.13 and 0.46 (Fig. 24).

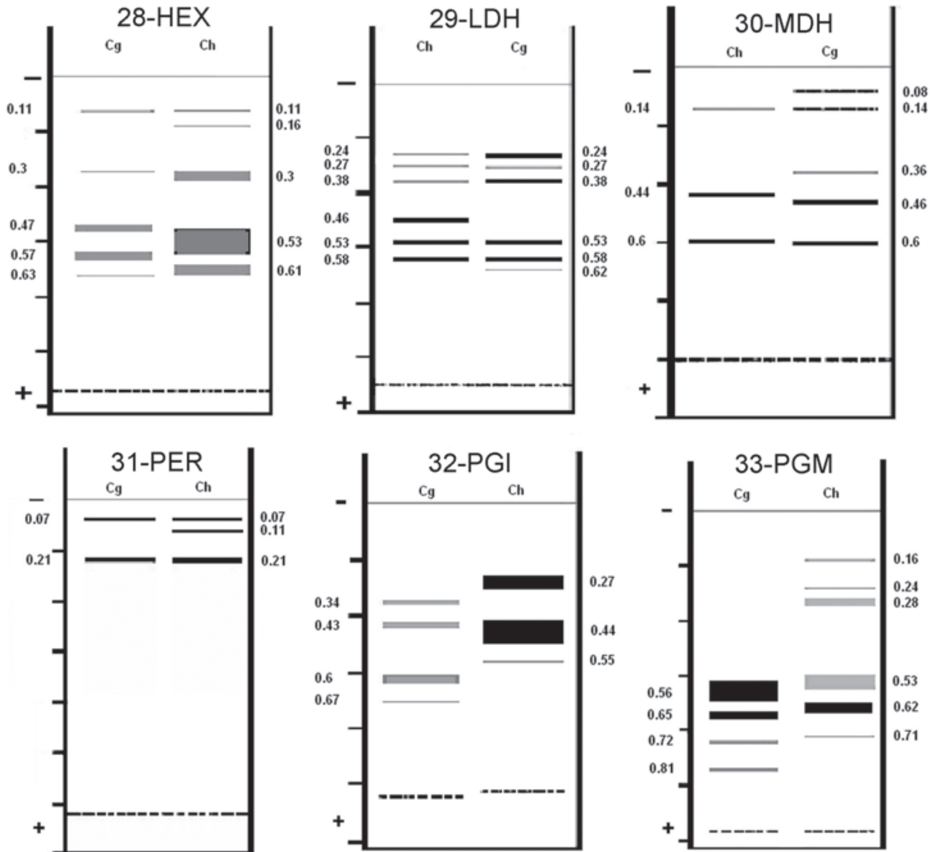
The esterase banding pattern is very complex. Both *C. gymnopitys* and *C. hydropitys* show slow as well as fast migrating bands. More number of bands are seen in *C. hydropitys* — at Rm. 0.15, 0.19, 0.4, 0.47, 0.57, 0.64, 0.74 and 0.83 — when compared with *C. gymnopitys. C. gymnopitys* shows bands at Rm. 0.08,

0.11, 0.17, 0.77 and 0.87, which are not seen in *C. hydropitys.* Both have a common band at Rm. 0.96 (Fig. 25).

The glucose-6-phosphate dehydrogenase banding pattern shows homogeneity between the two species by displaying common bands at Rm. 0.14, 0.42, 0.61, and 0.68. *C. hydropitys* differs from *C. gymnopitys* because of the presence of additional bands at Rm. 0.25, 0.29, 0.54, 0.64 and 0.75 (Fig. 26).

In *C. gymnopitys,* four bands of Rm. 0.21, 0.26, 0.44 and 0.81 of glutamate dehydrogenase are observed, whereas *C. hydropitys* reveals six bands at Rm. 0.11, 0.26, 0.31, 0.44, 0.74 & 0.90. These differences in the banding pattern and Rm. values contribute to the delineation of the two species (Fig. 27).

Hexokinase banding pattern shows slow and fast migrating bands in both the species and common bands at Rm. 0.11 & 0.3. The banding pattern (Fig. 28) exhibits a distinct difference between *C. gymnopitys* and *C. hydropitys* of *C. fibrosa* complex of Wood (1962). *C. hydropitys*, by having bands at Rm. 0.16, 0.53 and 0.61, differs from *C. gymnopitys*, which displays bands at Rm. 0.47, 0.57 and 0.63 (not recorded by the former).



Figs 28-33. Diagrammatic representation of gels stained for enzymes found in *C. gymnopitys* (Cg) and *C. hydropitys* (Ch). **28.** Hexokinase. **29**. Lactate dehydrogenase. **30**. Malate dehydrogenase. **31**. Peroxidase. **32**. Phosphoglucoisomerase. **33**. Phosphoglucomutase.

In lactate dehydrogenase, in addition to the five major bands, lightly stained minor bands are recorded. Occurrence of minor bands are common among animals and some plants, as mentioned by Wilkinson (1970). *C. gymnopitys* and *C. hydropitys* are identical in having five bands at Rm. 0.24, 0.27, 0.38, 0.53 and 0.58. *C. hydropitys* records a major band at Rm. 0.46, not seen in *C. gymnopitys*, which shows a minor band at Rm. 0.62 — lacking in *C. hydropitys* (Fig. 29).

While five bands of malate dehydrogenase are seen in *C. gymnopitys, C. hydropitys* records only three bands. *C. gymnopitys* varies from *C. hydropitys* in having bands at Rm. 0.08, 0.36 & 0.46, while *C. hydropitys* has a band at Rm. 0.44, which is not seen in *C. gymnopitys*. But both have a common band at Rm. 0.14 and 0.6 (Fig. 30).

Intense peroxidase activity is noticed in both the specimens. The enzyme shows low electrophoretic mobility, indicating high molecular weight and/or low net ionic charge. Of the total number of 3 slow migrating bands, two bands at Rm. 0.07 and 0.21 are common to both. The band at Rm. 0.11 is unique only to *C. hydropitys* (Fig. 31).

Diverse phosphoglucoisomerase enzyme system is observed in the present study (Fig. 32). *C. gymnopitys* and *C. hydropitys* display bands of varying mobility, thereby proving that each one is distinct. The bands at Rm. 0.34, 0.43, 0.6 and 0.67 shown by *C. gymnopitys* are not seen in *C. hydropitys*, which displays bands at Rm. 0.27, 0.44 and 0.55.

The differences in the number of phosphoglucomutase bands and the corresponding Rm. values recorded in *C. gymnopitys* and *C. hydropitys* contribute to the delineation of the two species (Fig. 33). In *C. gymnopitys,* no band is seen near the cathode end, whereas in *C. hydropitys*, three bands at Rm. 0.16, 0.24 and 0.28 are recorded. The most anodal band at Rm. 0.81 shown by *C. gymnopitys* is not recorded in *C. hydropitys*.

DISCUSSION

Morphological features

C. gymnopitys is found throughout the post North East Monsoon season (October-March), as long as water is available, while *C. hydropitys*, which has been considered as a variety of *C. fibrosa* by Wood & Imahori (1965) and as a distinct species by Zaneveld (1940); Sundaralingam (1959) and Pal *et al.* (1962), is found in the early period of the season. As has been pointed out by Zaneveld (1940), this species grows in the shallow regions of the lakes and disappears when water recedes during the middle of the season.

According to Wood & Imahori (1965), plasticity in ecological expression demands careful scrutiny while making a taxonomic decision, as it is difficult to know which of the "phenotypes" are environmentally induced and which are genetically controlled. In the present investigation, the field materials of both the species when in culture showed luxuriant growth, but retained the distinguishing features like axis and branchlet cortication, nature of spine cells, etc. In *C. hydropitys,* variation in the axis cortication is noticed from triplostichous to diplostichous, the diplostichous condition being more common, whereas in *C. gymnopitys*, only the diplostichous cortex is present. The spine cells are well

developed in *C. gymnopitys*, whereas they are rudimentary in *C. hydropitys*. *C. gymnopitys* exhibits completely ecorticate branchlets, but in *C. hydropitys,* 2 to 3 corticate internodal segments are seen, and the lowermost and terminal one or two segments are ecorticate. Wood & Imahori (1965) did not give much importance to cortication of the branchlets in the delineation of *C. gymnopitys* and *C. hydropitys.* But in the present study, the partial cortication of the branchlets is a stable feature of *C. hydropitys*, in contrast to the complete ecortication of the branchlets of *C. gymnopitys*, as also indicated by Zaneveld (1940) and Sundaralingam (1959).

The development of the cell of the basal node of the branchlet shows a different mode in the ecorticate branchlets of *C. gymnopitys* from that of the partially corticate branchlets of *C. hydropitys* (Sundaralingam, 1966) which, again, supports the separation of the two taxa.

Though haplostephanous condition of stipulodes is noted, occasionally in a few specimens of *C. hydropitys*, an incompletely formed lower row — one or two lower stipulodes — has been observed. Proctor (1980), noting a similar condition in *C. hydropitys*, drew a comparison between *C. zeylanica* and *C. hydropitys* and suggested that the two species might form an intermediate link between subsections Agardhia and Willdenowia of Wood.

SEM study of oospores

In recent times, the application of SEM in studying the oospore wall ornamentation has helped in resolving charophyte taxonomy. Cáceres (1975); John & Moore (1987), Cáceres & García (1989); John *et al.* (1990), Haas (1994); Casanova (1991, 1997); Mandal *et al.* (1995); Mandal & Ray (1999) and Ray (2004) have shown that the characteristic features of the oospore can be used for identification, preparation of key and differentiation of the various species of charophytes.

In field conditions and under light microscope, there are not many visible differences in oospore colour and number of convolutions, though the oospore size is seen to be smaller in *C. hydropitys*. However, when viewed under SEM, the acid-cleaned oospores provided us with different surface characteristics of taxonomic importance. The two species, *C. gymnopitys* and *C. hydropitys,* which had been reduced to infraspecific levels by Wood & Imahori (1965), show distinct differences in wall ornamentation features, which help demarcate the two as different species. While in *C. gymnopitys,* the smaller granules on the oospore wall fuse to form a scabrous surface, in *C. hydropitys,* the ornamentation is significantly different, showing verrucae with irregular wall and central pores. Besides, the surface of the ridge in *C. gymnopitys* is smooth with a ribbon-like projection, whereas in *C. hydropitys,* the ridge is irregular. The differences in the ornamental pattern of the oospore of each species remained consistent across materials collected from the same and different water bodies. Cáceres (1975) has commented that the oospores provided surface features for distinguishing taxa and these features are more stable than those associated with the vegetative thallus. As pointed out by Casanova (1997) and Ray (2004), the variation in oospore wall structure can be taken as an indication of genetic differences between taxa. Apart from vegetative features, oospore wall characters can also be accorded an important place in the delineation of species, especially when there is a doubt regarding the validity of closely assigned species.

Based on the differences in the oospore wall ornamentation that have been analyzed in the present study, it is suggested that *C. gymnopitys* and *C. hydropitys* should be treated as two different species. Differences in oospore wall ornamentation have already been used to delineate species in the past (e.g. John & Moore (1990)).

Protein and isozyme analysis

The delineation of species of freshwater algae is mainly based on morphological variations. As Soudek Jr. & Robinson (1983) pointed out, morphological characters by themselves may be insufficient in distinguishing species. Biochemical analyses are often resorted to whenever controversies arise regarding the validity of any taxon in order to clarify and provide additional evidence for correct delineation of the taxa in question. As morphological characters are the end products of chemical pathways that are under the control of genes, it is necessary to analyze proteins and enzymes, which are the primary products of the genes. The technique of electrophoresis of proteins has become a powerful tool in studying genetic variation directly at the molecular level. Any change in the genetic constitution, i.e., any modification in the DNA nucleotide, will result in a change in the amino acid composition, which in turn will alter the net charge of the proteins and enzymes. This will affect the mobility of the enzymes in the charge field and can thus be detected. Proteins and isozyme patterns have served as an adjunct to morphological studies to resolve systematic controversies at the generic and specific levels.

As Shannon (1968) pointed out, more than hundred enzymes exist in multiple forms and each organism possesses a distinct enzyme composition. An analysis of these multiple forms has proved to be useful and is extensively applied in algal taxonomy.

Isozyme analysis had already been utilized to resolve taxonomic controversy. Gemayel (1988) had shown that the readings of the zymograms for PGM, PGI, IDH, MDH, EST, GOT and PAC reveal a clear interspecific differentiation between *C. globularis* Thuill., em. R.D. Wood and *C. aspera* Deth. ex Willd., which were classified by Wood & Imahori (1965) as a single species. Working on the green alga *Caulerpa* Lamour. Ballment *et al.* (1996) emphasized on the utility of consistent phenotypic pattern differences in discriminating the taxa. They are able to separate a cryptic taxon among the population of *C. serrulata* (Forssk.) J. Ag. based on the occurrence of fixed differences at many loci for seven of ten enzymes studied. Sosa & Lindstrom (1999), while reviewing the isozymes in macroalgae, had commented that isozymes are particularly useful as taxonomic characters because they are relatively easily observed, provide reliable data and have the power to discriminate among taxa.

Grant & Proctor (1980) made a detailed study of Phophoglucoisomerase (PGI) isozyme in *Chara* species (including *C. hydropitys*), and found the enzyme to be polymorphic. Each species was found to have more than five locations of enzyme activities. Apart from the number, the bands differed in their mobility also. Diverse PGI enzyme system is observed in the present study too. *C. gymnopitys* and *C. hydropitys* display bands of varying mobility, which contributes to differentiating them into two different species. Grant & Proctor (1980) had recorded five bands in *C. hydropitys*, whereas in the present investigation, only three bands are seen. The differences in the number of bands may be attributed to the different separating media and buffers used. Thomas &

Delcarpio (1971) have shown that the number of sites of reactions and the position of these sites for each enzyme were different for starch and polyacrylamide. Grant & Proctor (1980) had attributed the PGI enzyme diversity to polyploidization, resulting in functional gene duplication. They suggested Phosphoglucomutase (PGM) to be monomorphic in the species of *Chara*, except in one group of individuals of the *Chara globularis* complex. The present study shows PGM to be polymorphic in contrast to monomorphic, as suggested by Grant & Proctor (1980). Khan & Sarma (1984) had commented that enzymatic versatility due to functional gene duplication via polyploidy allowed greater ecological tolerance to develop, contributing to evolutionary significance.

In the present investigation, the protein profile of *C. gymnopitys* and *C. hydropitys*, show several variable bands (Tab. 3) and reveal only seven common bands. Although they show similarity by recording some common bands for Acid phosphatase, Alkaline phosphatase, Catalase, Esterase, Glucose-6-phosphate dehydrogenase, Glutamate dehydrogenase, Hexokinase, Lactate dehydrogenase, Malate dehydrogenase and Peroxidase, the number of sites of reaction and the positions of these sites for each enzyme are different. The differences found in the electrophoretic analyses indicate that *C. gymnopitys* and *C. hydropity* are separate species.

CONCLUSION

The present study reports for the first time SEM analyses of the oospore wall ornamentation of *C. gymnopitys* and *C. hydropitys* from India, and the first detailed report on the isozymes of the twelve enzyme systems present within the two species.

This investigation shows, as was discussed by other authors, that morphological differences of plants, oospore wall ornamentation, and isozyme analysis are important to discriminate species of charophytes. The differences found between *C. gymnopitys* and *C. hydropitys* using different techniques, justify therefore their separation as two different species.

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