Exploring *Chara* as a model system to study the function of homeobox genes in macroalgae

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By

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CERTIFICATE

This is to certify that this dissertation entitled ''**Exploring *Chara* as a model system to study the function of homeobox genes in macroalgae"** towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Nava Krishna Chowdary D at IISER Pune under the supervision of Dr. Anjan K Banerjee, Assistant Professor, Biology Division, IISER Pune during the academic year 2013-2014.

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DECLARATION

I hereby declare that the matter embodied in “**Exploring *Chara* as a model system to study the function of homeobox genes in macroalgae**„ are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Anjan K Banerjee, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

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Abbreviations

NTA	Nitrilo acetic acid
RACE	Rapid amplification of cDNA ends
IAA	Indole acetic acid
GA3	Gibberellic acid
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
kb	kilo base
bp	base pair
min	minutes
sec	seconds
hr	hours
SAM	Shoot apical meristem
rcf	Relative centrifugal force

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ABSTRACT

Chara is a multicellular green algae belonging to the family *Characeae*. Our literature survey showed that land plants might have evolved from the Charophyceans algae. In the present investigation, we aimed to establish *Chara* as a model system to study the evolutionary origin of land plants. Because *Chara* exhibits apical cell mediated growth, and also shows the presence of plasmodesmatal connections between nodal and internodal cells, a characteristic of intracellular communication in higher plants. Homeobox gene *KNOX* is shown to have multipurpose role in the maintenance of shoot apical meristem in higher plants. Hence, an attempt has also been made to identify and isolate the homeobox gene in *Chara,* which may provide insights into the apical cell mediated growth of *Chara* development. In this study, sterile cultures of *Chara zeylanica have been established in our lab.* Germination protocol of *Chara* oospores has also been standardized followed by the optimization of growth of germinated sporelings aseptically. Based on the phylogenetic analysis of *rbcL* and *18S* sequences, the species has been successfully identified and routinely cultured in the lab. Both morphological and histological observations are made and documented for our study. Since, our overall interest was to understand the role of *KNOX* genes in *Chara*, in this attempt, conserved homeodomain sequence from *Chara* zeylanica has been isolated and sequence verified. Isolation of full length sequence of *KNOX* is presently in progress.

1. INTRODUCTION

Chara belongs to the family *Characeae* which means it contain green pigments. It is a green multicellular algae that is closely related to ancestory of land plants (KAROL *et al*. 2001; DELWICHE *et al*. 2002). They resemble land plants because of stem like, leaf like structures.

1.1 Morphology of *Chara*

Chara thallus is haploid and represents the gametophyte stage, while oospore is the only diploid part of the life. The plant body of *Chara* is differentiated into three parts namely main axis, lateral branches, and rhizoids. The main axis is composed of two types of cells; long single internodal cells alternating with much shorter nodal cells from which whorls of laterals arise. Internodal cells can extend up to 10 cm and internodal cells of many species of *Chara* are surrounded by cellulose cell wall having deposits of calcium carbonate. Because of these calcium carbonate deposits these plants are rough to touch. Internodal cells have special characteristics like large central vacuole, and many discoid chloroplasts. Internodes are made of multinucleated single cells (Shen et al., 1967).The cytoplasmic fluid inside the internodal cells" (Williamson,exhibit*et*p *al*., 1975) .Movement of cytoplasmic fluid around the cell with the help of molecular motors is called (Cyclosis).In many species of *Chara*, internodal cells of main axis remain covered by cortex. Interestingly first half of the internodal cell is covered by corticating filaments developed from lower node and second half of the internodal is covered by corticating filaments developed from upper node. Nodal cells have special characteristics like much number of small vacuoles and central vacuole is ill-developed, dense and granular cytoplasm, and having many discoid chloroplasts with no pyrenoids.

There are reports that cell walls between internodal cells and nodal cells are porous of pore size ranging from 0.4 to 0.6um.These pores allows continuous cytoplasmic connections between the two cells (Franceschi *et al*., 1980).The ability to establish and control cell-to-cell communication played an important role in the evolution of an organism. *Chara* is the ancestral form of land plants that is forming plasmodesmatal connections between nodal and intermodal cells. But the formation of plasmodesmata in Chara is fundamentally different from the processes that give rise to the formation of the higher-plant primary plasmodesmata.

Nodes bears lateral branches which are of two types namely branches of unlimited growth (Long laterals) and branches of limited growth (Branchlets). Branchlets are short branches which are arranged in the form of whorl containing nodes and internodes. Sex organs are present on the nodes of these branchlets. Stipulodes are the unicellular outgrowths that are located at the first lowermost node of the branchlet. Long laterals appear to be main axis of *Chara* plant having nodes and internodes. Nodes of these long laterals develop branchlets. It means that if we cut long laterals and put them in a medium, it will eventually grow into a *Chara* plant.

Rhizoids function similar to roots of the land plant which helps in the uptake of nutrients and water from the surrounding environment and also helps in anchorage to the limestone that are present in lakes(Wang-cahil *et al*., 1995).

1.2 Apical cell mediated growth of *Chara*

Growth of *Chara* takes place with the help of a single dome shaped cell situated at the tip. Basically apical cell mediated growth is the characteristic of land plants and the first plant that is showing apical cell mediated growth in the phylogenetic tree is *Chara*, it means that it can be treated as closest extant relative of land plants (Richard M. McCourt *et al*., 2004).

1.3 Reproduction in *Chara*

Chara reproduces either by vegetative reproduction or by sexual reproduction. Mode of reproduction varies from species to species.

Chara reproduces vegetatively by the following methods:

a) In some *Chara* species, amylum starch containing cells aggregate on the lower nodes of the cells and develop amylum into stars". Stars once they are detached from the main axis they develop into new *Chara* plants.

b) In some *Chara* species, round shaped bodies that are located at the nodes of the rhizoids or nodes of the main axis will germinate to give a new plant once they are detached from the nodes.

c) Amorphous bulbils are small cells at root nodes or stem nodes which will form lateral outgrowths which lead to a new *Chara* plant.

Chara reproduce sexually by sex organs that are present on the branchlets. Oogonium (Female) and antheridium (Male) are the sex organs that are present on *Chara*. Although microscopic, sex organs are readily identified by their shape and colour. The sperm bearing antheridia are bright orange, while the egg bearing oogonium is green with a distinctive crown of cells.

Oospores are the product of fertilization of egg cell by sperm cell. Oospore is a hard nut like body of various colours. The colour of oospore changes from species to species. Oospore is surrounded by four layered wall, of which two outer layers are coloured and other two inner layers are colourless. At the time of germination the diploid nucleus moves towards the upper side of the oospores and divide by meiotic division to give four haploid nuclei. At this stage oospore cell wall appears and small distal uninucleate will cut off from the other three nucleate. Soon the lower part containing three nuclei degenerates. The oospore wall now bursts open at the apex and the distal cell is exposed.

1.4 Chara oospore in vitro germination

Many people have put their constant efforts in *in vitro* germination of *Chara* oospores. To grow *Chara* in the lab aseptically, one should start from the germination of oospores. Germination of oospores is governed by both environmental and physiological factors.

For an oospore to germinate, water is essential requirement especially for algal spores as the plant is grown fully in water. Spores imbibe water so that enzymes will become hydrated and these hydrated enzymes become active and the spores increase its metabolic activities to produce energy for the growth of sporelings. Temperature is one of the key factor that regulates germination of *Chara* oospores. Temperature at which oospore showing highest germination vary from species to species. For example oospores of *Chara zeylanica* shows maximum germination at 28°C than at 24°C.Similarly *Chara* contraria shows maximum germination at 18°C than at 24°C or 28°C(Proctor et al.,1967). Light also have an important role in the germination of *Chara* oospores through phytochrome mediation. There have been reports that white light promotes oospore germination but not dark light. And also a flash of red light irradiation promotes oospore germination, but blue and far red light has little effect (Seiji Taka tori *et al*., 1971). The type of substratum that it is adhered will influence the germination rate and germination efficiency of oospores. For example some oospores

required 2 days to germinate on glass, whereas same oospores took less than 12hr for germination on cotton threads (Boney *et al.*, 1978).

1.5 Culturing *Chara*

Chara is a plant that grows under water in lakes. An attempt to culture them under controlled conditions in lab deals with many difficulties like sterile germination of *Chara* oospores, and then growing those germinated sporelings in certain media so that they grow into a fully developed plant with reproductive structures. Germination of spores aseptically involves surface sterilization to prevent contamination. There are various media for growing *Chara* plant like Forsberg media and Broyer-Barr medium. Growth substances like hormones, temperature, and media for providing micro and macro nutrients are the essential factors for the growth of any plant.

There are reports that IAA (Jacobs *et al.*, 1987), gibberellins like substances are present in the thallus of *Chara* plant (Murakami *et al.*, 1966).Gibberellins has a role in accelerating the development of sex organs like oogonium and antheridium. Gibberellins will increase the number of spermatozoids that were produced in antheridium. (Godlewski *et al.*, 1967). It has also been shown that decreasing the levels of endogenous gibberellins by inhibitor AMAO-1618 will cause an elongation of internodes. IAA has a role in elongation of internodes in the main axis (Kwiatkowski *et al.*, 1967).It means both IAA and gibberellins
acting antagonistically.

Growth of *Chara* takes place well at 24°C, whereas the formation of reproductive structures takes place at almost all temperatures between 16°C and 32°C except at 20°C

(Robirt *et al.*, 1956).

1.6 Role of plant home domain box genes in shoot apical maintenance

Homeobox genes are known to encode for transcriptional factors, usually involved in plant

developmental programs and response to stress. For the first time, isolation of *Kn1* gene in maize by transposon tagging revealed the presence of homeobox genes in plants (Vollbrecht *et al*., 1991). After *kn1* discovery in maize, related homeobox have been isolated in all plant species. Based on the conserved sequences within the homeobox region as well as presence of additional sequences outside the domain they were classified mainly into four classes namely *KNOX*, *BELL*, PHD-finger and HD-Zip genes.

The crucial part of plant development is maintenance of apical meristem as it provides a reservoir of undifferentiated cells from which the aerial parts of the plant are subsequently arise. In land plants *KNOX* Class I genes are known to have a role in maintenance of shoot apical meristem (Hake *et al*., 2004; Jackson *et* al., 1994).

Unlike animals, plants undergo alternations of generations with both haploid and diploid generations in their life cycle. *KNOX* homeobox gene have a role in transition from an ancestral haploid (n) dominant life cycle to a diploid (2n) dominant life cycle, with diploid specific functions of *KNOX* gene in almost all plant lineages. In unicellular algae like Chylamydomonas algae, the molecular regulation of haploid to diploid transition is contributed by Gsm1 and Gsp1, orthologs of *KNOX* and *BELL* in land plants respectively (Lee *et al*., 2008). In bryophytes like moss *P patens*, there are reports that *KNOX* genes are not involved in shoot development in the moss *Physcomitrella patens* but do function in sporophyte development (Sakakibara et al., 2008). The role of *KNOX* in lower algae like Chylamydomonas algae is different from that it plays in higher plants like angiosperms. Our knowledge about the development of higher plants is still in primitive phase. There are no reports about the identification of *KNOX* genes in *Chara*. Chara is a good model system to study the origin and evolution of apical cell, as it is showing apical mediated modular growth.

2. Objectives

The main objective of this study is to establish *Chara* as model system and to know the function of homeobox genes in higher algae. Following approaches have been considered for the present study.

1) Standardization of aseptic germination of *Chara* oospores.

2) Optimization of nutrient medium and phytohormones to achieve faster growth in vitro.

3) Phylogenetic analysis and species identification

4) Morphological observation of *Chara zeylanica*

5) Histological study of *Chara zeylanica* vegetative body and reproductive organs

6) Isolation of conserved *KNOX* homeobox domain from *Chara* by using RT-PCR.

3 Material and methods

3.1 Plant Material

Soil was collected from a pond in University of Pune and then separated around 4000 *Chara* oospores using brush and a needle. Oospores were aseptically germinated and germinated sporelings were cultured in a Forsberg medium along with some plant hormones of definite concentration.

3.2 *Chara* growth medium

Chemical	Concentration (mg/l)
$CaNO_3$	80
$Mgso_4 7H_2O$	100
Na_2CO_3	20
Na_2SiO_3	10
KCl	30
K_2HPO_4	0.056
$FeCL_3$	0.090
TRIS	500
$ZnCL_2$	0.010
Co_2CL_6	0.002
$CuCL_3$	0.004
H_3BO_3	0.040
Mo(Na salt)	0.010
NTA	10

Table 1: Composition of Forsberg medium

Chemical	Concentration(mg/l)
$NaHCO_3$	0.5mM
NaCl	1.0mM
$NaNO_3$	0.2mM
KH_2PO_4	0.017mM
K_2SO_4	0.05mM
$MgSO_4$	0.1mM
$CaCl_2$	0.1mM
$FeSO_4$	3.6µM
$MnSO_4$	0.91µM
$ZnSO_4$	0.76µM
$(NH_4)_6Mo_7O_{24}$	0.014µM

Table 2: Composition of Broyer-Barr medium

3.3 Primers

Primer Name	Sequence Name(5'->3')	Purpose
KNOXD4F	GCATTACAAGTGGCCTTAYCCNWSNGA	Homeodomain isolation
KNOXD8R	CTCTTCCTCTGGTTGATGAACCARTTRTT	Homeodomain isolation
KNOXD10R	TGCCTCTTCCTCTGGTTDATRAACCA	Homeodomain isolation
CZNW KNOXF	CAGATATACAATTGGTTCATCAACC	3' RACE or 5' RACE
CZNW KNOXR	GGTTGATGAACCAATTGTATATCTG	
CZPY KNOXF	TGCATTCCATTNGGCCTTACCTG	
CZPY KNOX R	CAGGTAAGGCCNAATGGAATGCA	
rbcL RH1F	ATGTCACCACAAACAGAAACTAAAGC	Phylogenetic analysis
r*bcL* 972R	ATCACCACCAGAAAGACGAAG	Phylogenetic analysis
18GF	AGGGCAAGTCTGGTGCCA	Phylogenetic analysis
18HR	CCCTTCCGTCAATTCCTTTAAGTTTCAGC	Phylogenetic analysis

Table 3: Primers sequences used in this study.

3.4 Bacterial culture media

All bacterial culture media were sterilized by autoclaving at 121 ^{o}C for 20 minutes.

LB: 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl.

LB Agar: prepared by adding 10 g bacto-agar to one litre of LB prior to autoclaving.

3.5 Chemicals

Chemicals used in this study are Triton X -100, 0.1% $HgCl_2$, 0.8% NaClO, IAA, GA3, Kinetin, TRIzol, Chloroform, Isopropanol, Xylene, Ethanol, Acetic acid, LB, LB Agar.

3.6 RNA isolation

100mg of tissue was homogenized with the help of liquid nitrogen, without allowing the tissue to thaw. TRIzol reagent (1 ml) was added to the tissue and vortexed thoroughly for 2 to 5 min, and then incubated at room temperature for 5 min. 200µl of chloroform was added to each sample and inverted the tube 5-6 times. Incubated at room temperature for 10 min. For phase separation, samples were centrifuged at 12000 rcf at 4 degree for 15 min. Top most aqueous phase was collected in a new tube. RNA was precipitated by adding Isopropanol (500µl) and the tubes were inverted to mix. The samples were incubated at room temperature for 10 min. The tubes were centrifuged at 12,000 rcf for 10min and then discarded the supernatant. Pellet was observed after centrifugation step.

1ml of 75% ethanol was added to each tube and mixed it by inverting. Centrifuged at 7500 rcf at 4^{0}C for 5 min. Discarded supernatant. Removed excess ethanol by air drying for 15 min. Suspended RNA pellet in DEPC water (10µl).Incubate RNA for 15min at 55 degrees. RNA concentration was determined using Nanodrop 2000 Spectrophotometer (Thermo Scientific,Wilmington,USA).The extracted total RNA was then stored at -80^{0}C.

3.7 RT-PCR for amplification of *18S*, *rbcL* and home domain TF loci

3.7.1 Amplication of 18S gene from *Chara* RNA using one step RT-PCR

18S rRNA gene (~550 bp) was successfully isolated using single step RT-PCR. The primers that are used for this single step PCR are 18GF and 18HR.The reaction conditions of this reaction are as follows:

No of cycles from third step to fifth step = 35

Purpose	Temp	Time
Reverse transcription	45 ^{o}C	30 min
Initialization step	94 ^{o}C	2 min
Denaturation step	94 ^{o}C	15 sec
Annealing step	52 ^{o}C	30 sec
Elongation step	68 ^{o}C	60 sec
Final extension	68 ^{o}C	10 min

The PCR product was loaded in 1.2% agarose gel and observed using UV spectrophotometer and the image is shown in Fig. 7.

3.7.2 Amplication of *rbcL* gene from *Chara* RNA using one step RT-PCR

rbcL gene (~1000 bp) was successfully isolated using single step RT-PCR. The primers that are used for this single step PCR are *rbcL*RH1F and *rbcL*972R.The reaction conditions of this reaction are as follows:

No of cycles from third step to fifth step = 35

Purpose	Temp	Time
Reverse transcription	45 ^{o}C	30 min
Initialization step	94 ^{o}C	2 min
Denaturation step	94 ^{o}C	15 sec
Annealing step	52 ^{o}C	30 sec
Elongation step	68 ^{o}C	90 sec
Final extension	68 ^{o}C	10 min

The PCR product was loaded in 1.2% agarose gel and observed using UV spectrophotometer and the image is shown in Fig. 6.

3.7.3 Amplication of conserved homeobox gene from *Chara* RNA using one step RT-PCR

To amplify homeobox gene (~109 bp) from isolated RNA using single step RT-PCR. The degenerate primers that are used for this single step PCR are D4F and D8R.The reaction conditions of this reaction are as follows:

No of cycles from third step to fifth step = 35

Purpose	Temp	Time
Reverse transcription	45 ^{o}C	30 min
Initialization step	94 ^{o}C	2 min
Denaturation step	94 ^{o}C	15 sec
Annealing step	52 ^{o}C	30 sec
Elongation step	68 ^{o}C	90 sec
Final extension	68 ^{o}C	10 min

The PCR product was loaded in 1.2% agarose gel and observed using UV spectrophotometer and the image is shown in Fig. 11.

3.8 Phylogenetic analysis of various *Chara*

Species name	*18S* accession number	*rbcL* accession number
Chara longifolia	**AF032741**	**-**
Chara globularis	**Y16465**	**AF097165**
Chara connivens	**CCU18493**	**AF097162**
Chara australis	**CAU18104**	**AB440260**
Chara braunii	**AF032728**	**AB440259**
Chara tomentosa	**AF032745**	**AB440255**
Chara drouetii	**CDU18495**	**-**
Chara hornemannii	**CHU18497**	**-**
Chara hispida	**CHU18496**	**-**
Chara curtissii	**CCU18494**	**-**
Chara aspera	**AF032725**	**-**
Chara andina	**AF032724**	**-**
Chara baltica	***AF032727***	**-**
Chara vandalurensis	**KJ094039**	**-**
Chara stuartiana	**AF032744**	**-**
Chara polyacantha	**AF032742**	**-**
Chara imperfecta	**AF032739**	**-**
Chara hydropitys	**AF032738**	**HQ380464**
Chara haitensis	**AF032737**	**-**
Chara corallina	**AF032730**	**-**
Chara rusbyana	**-**	**AF097168**
Chara vulgaris	**AF032747**	**AF097166**

Chara gymnopitys	**-**	**AB440261**
Chara zeylanica	**AF032748**	**AB440257**

Table 4: NCBI accession numbers of 18S and rbcL of various species

Finally, the *18S* and *rbcL* sequences were imported in MEGA 4 software (Tamura K.*et.al.*, 2011) and aligned using multiple sequence alignment (ClustalW). Phylogeny was built using UPGMA with bootstrap method and 500 replications.

3.9 Cloning and Sequencing

The PCR products were analyzed using agarose gel electrophoresis. And the purified PCR products using PCR purification were then cloned in the TA cloning vector pGE-MT vector and transformed into *Escherichia coli* DH5 αcells. For screening, the recombinant colony (white colony) was picked with a sterile tip and inoculated in a Luria-Bertani medium containing ampicillin. The same tip was then used to streak the colony onto LB agar plate containing ampicillin, x-gal, and IPTG. Plasmids were isolated from the bacterial culture using alkaline lysis method.

3.10 Confirmation of recombinant Plasmid

Plasmids were digested with *EcoRI*-HF for about 2hr 30min.

Cut smart Buffer	2 µl
Plasmid	2 µl
EcoRI-HF	0.4 µl
Water	15.6 µl

Table 5: Restriction digestion of plasmid containing insert with EcoRI-HF

3.11 Histology

Chara tissues like main axis containing internodes and nodes, primary branchlets with sex organs oogonium and antheridium, and apical part of the plant, were collected and fixed in 3:1 ratio of ethanol and acetic acid for overnight at 4°C according to (Cai *et al.*,2006) with the following modifications. The material was dehydrated with 75% ethanol for 30 min with two changes at 4°C. 95% ethanol for 30 mins and 100% ethanol with 3 changes, each with 45 mins intervals. The material was then treated with 50-50% ethanol-xylene for 45 mins, followed by clearing with 100% xylene for 45 min with 3 changes. The xylene was replaced with paraplast wax for several times at 45°C.Then the tissue was embedded in the paraplast blocks. The tissues were sectioned with 11 microns thickness and the paraplast sections were then mounted on the slides with water at 50°C.

4. Results

4.1 Effect of Surface sterilizing agents on *Chara* oospores

Collected soil from a pond in University of Pune and then separated spores using a brush and a needle. *Chara* oospores were then placed in eppendorf tubes containing distilled water. We performed three protocols for surface sterilizing the spores so as to remove dust on it. Surface sterilization is an important process before placing them in six well plates for the sterile germination. Various surface sterilizing agents were used at different concentrations and duration to determine the most efficient procedure for initiation of tissue culture of *Chara* using oospores as explant. Germination of *Chara* oospores started within 7 to 10 days of incubation. To achieve surface sterilization we tried chemicals like1% Triton X -100 (detergent), 0.1%mercuricchloride ($HgCl_2$), and 0.8%sodiumhypochlorite (NaClO). Among these three, 0.1%mercuric chloride gave me the best results. But 0.1%sodium hypochlorite also gave us the results which are promising. As 0.1% mercuric chloride is hazardous chemical, we are using 0.8% sodium hypochlorite as surface sterilizing agent. Germinations of *Chara* oospores were carried out at 24°C under long day (L: D=16:8) conditions.

Surface Sterilizing agent used	Treatment duration	Set of Germinat-ions	Number of spores inoculated	Number of spores germinated after 12 days	Germination percentage after 12 days	Contamination Percentage (Contaminated wells) / Total no of wells
$HgCl_2$ (0.1%)	3 min	2	360	27	7.5 %	Yes (6/12) = 50%
$HgCl_2$ (0.1%)	4 min	4	720	137	19 %	Yes (5/24) = 21%
$HgCl_2$ (0.1%)	5 min	4	720	130	18 %	Yes (2/24) = 8.3%
NaClO (0.8%)	5 min	2	360	40	11 %	Yes (3/12) = 25%
Without surface sterilization	0 min	3	540	260	36 %	Yes (12/12)= 100%

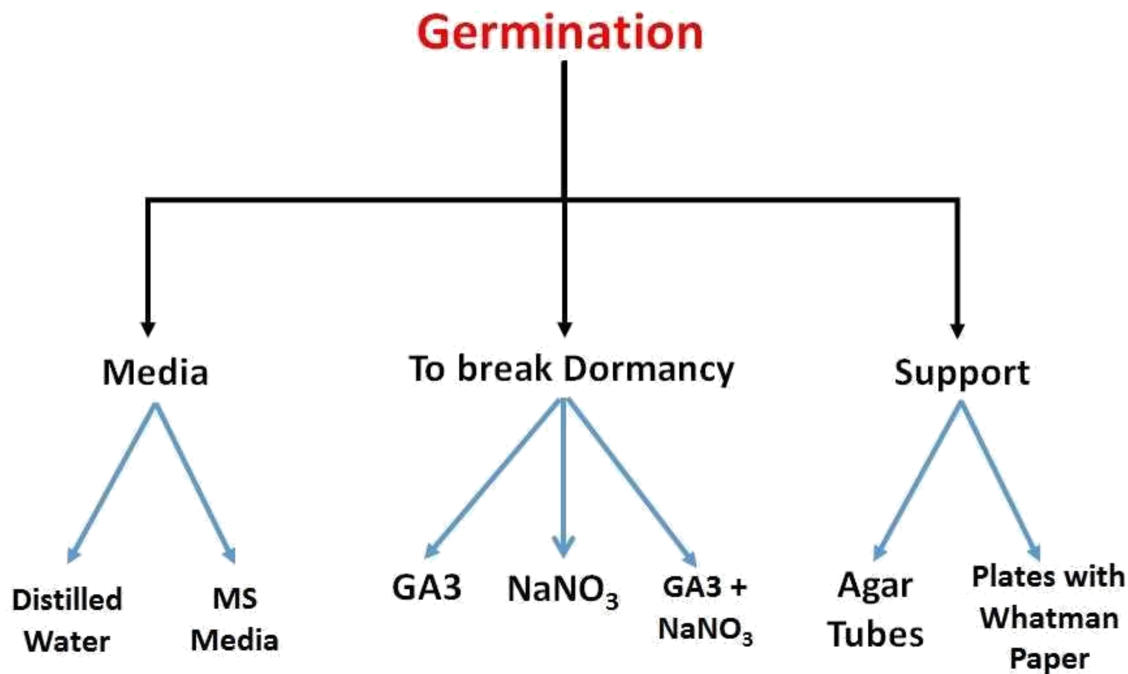
Table 6: Effect of surface sterilizing agents on germination percentage and contamination

Figure 1: Aseptic cultures of Chara from germinated oospores

(A) Germinated sporelings from *Chara* oospores treated with0.1%$HgCl_2$ (B) Germinated sporelings from *Chara* oospores treated with 0.8% Sodium hypochlorite (C) Germinated sporelings from *Chara* oospores without any chemical treatment.

4.2 Sterile germination of *Chara* oospores

For a spore to germinate, it requires certain media. For that we tried distilled water and MS media. Among these two distilled water gave best results. There are reports that Gibberellins and $NaNO_3$ have a role in breaking the dormancy of other algal oospores, there by favoring germination. For that we tried both GA3 and $NaNO_3$ and also the combination of both at various concentrations. None of them gave me improvement in germination percentage**.** And for a spore to germinate, it requires proper support so that newly coming rhizoids could anchor it. We tried agar tubes and plates with whatman paper soaked in water. Among these two supports whatman paper soaked in water gave the best results.



4.3 Protocol for aseptic germination of *Chara* oospores

Collected spores from soil and then transferred 30 spores to each 1.5ml eppendorf tube. 1 ml of distilled water was added to the eppendorf and inverted the tube multiple times for 5 min. Repeated for 3 times and kept it on rotor for overnight washing. 1ml of 1% Triton X -100 was added to eppendorf tubes, to remove the dust on spores. Eppendorf tubes were inverted several times for 5 minutes, to ensure all the spores are exposed to the detergent. Spores were not kept in detergent for longer than 3 minutes. Pipetted off the detergent solution and 1ml of sterile deionised water was added, to remove residual detergent from the spores. Tubes were inverted with sterile deionised water multiple times to ensure thorough washing of the spores. 1ml of 70% ethanol was added to eppendorf and inverted the tube multiple times for 3min.1ml of 0.8% sodium hypochlorite was added to the eppendorf tubes and inverted each eppendorf tube several times for 4 minutes. Do not leave spores in sodium hypochlorite for longer than 4 minutes. Pipetted off the sodium hypochlorite solution and 1 ml of sterile deionised water was added for washing. After each surface sterilization event, spores were thoroughly rinsed 5 times with sterilized distilled water. The majority of the water was removed, leaving a small volume in the base of the tube to facilitate plating of spores into six well plates. Using pipette distributed the spores individually at regular intervals over the well of the six well plates. A maximum of 30 spores were plated per well.

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4.4 Establishment of aseptic cultures of *Chara*

After establishing protocol for sterile germination of *Chara* oospores, it is very important to establish protocol for growing *Chara* aseptically. For that we tried two media namely Forsberg medium (Forsberg *et al*., 1965) and Broyer-Barr medium. There is precipitate that is forming after preparing original composition of Forsberg medium. To avoid that precipitate formation we tried many combinations like removing chemicals that are causing precipitate immediately after adding that chemical. We observed that $MnCL_2$ and NTA are the two chemicals that are responsible for precipitate formation. Adding less amount of NTA is not causing any precipitate, so that we included NTA (Fewer amounts) in Forsberg medium. But we excluded $MnCL_2$ from the Forsberg medium. There is one more reason to exclude $MnCL_2$ from the Forsberg medium because it is causing tissue to turn it to brown. As there is no Mn salt in media, we tried adding another Mn salt ($MnSO_4$) and still it is giving precipitate. After excluding $MnCL_2$ from Forsberg medium also *Chara* is growing fine. We tried Broyer-Barr medium but it is also giving precipitate as well as the *Chara* plant is not growing in this medium. So that we have decided to use Forsberg medium as a main media for growing *Chara*. To speed up the growth of *Chara*, it is very much essential to add plant hormones along with Forsberg medium. There are reports that certain combinations of plant hormones enhance the growth of Chara (Maria Kwiatkowski *et al*., 1991).For that we tried different combinations of hormones with water and Forsberg medium as control. With water we didn"t observe growth at all.

4.4.1 Effect of auxin on *Chara* growth

IAA (10^{-7} M) along with modified Forsberg medium caused an increase in the number of nodes from 4 to 5 and 5 to 6 observed after 10 and 20 days respectively.

Medium	IAA(M)	Growth after 20 days of incubation
Forsberg	10^{-7}	Good Growth
Forsberg	10^{-6}	Moderate Growth
Forsberg	10^{-5}	No Growth
Forsberg	0	No Growth
Water	0	No Growth

Table 7: Growth of *Chara* plant in a Forsberg medium containing different concentrations of auxin hormone with water as control.

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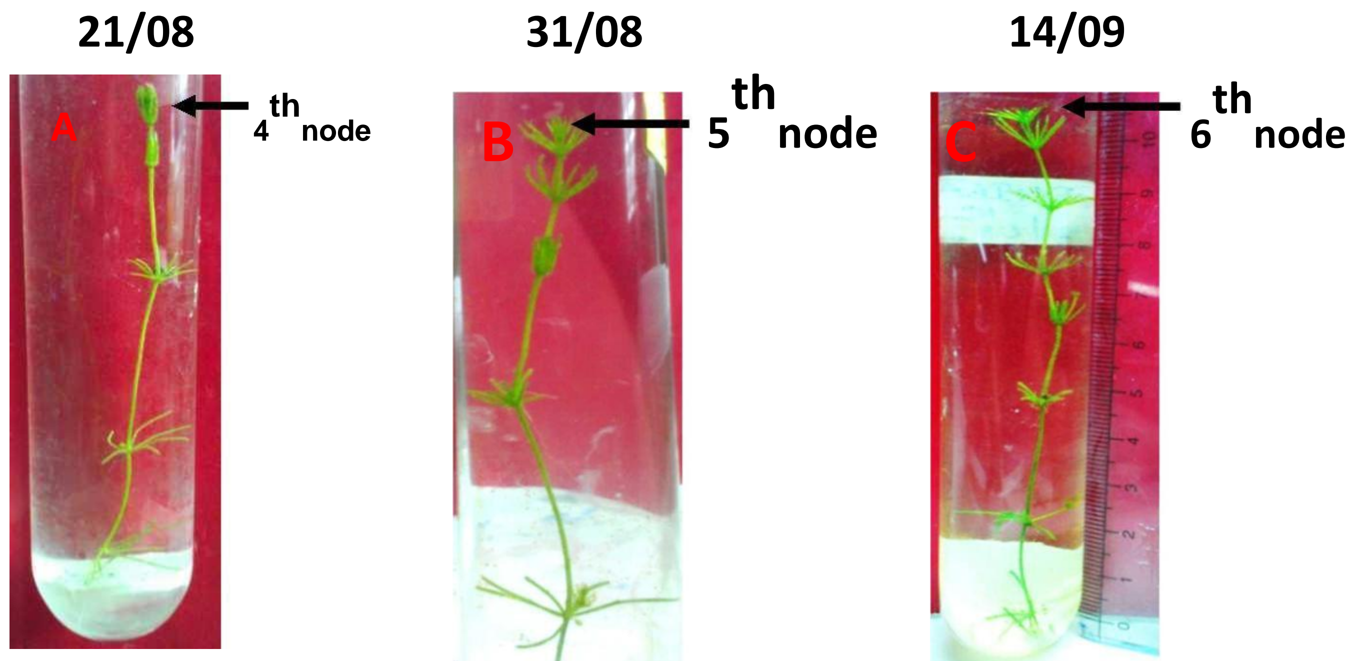

Figure 2: Growth of *Chara* after 20 days of inoculation in Forsberg medium with IAA (10-7 M).

(A) Four nodes on the day of inoculation. (B) Fifth node that is developed after 10 days of inoculation. (C) Sixth node that is developed after 20 days of inoculation.

4.4.2 Effect of auxin and GA3 on *Chara* growth

GA_3 **(**10^{-5} M) and IAA (10^{-7} M) along with modified Forsberg medium caused an increase in number of nodes as well as side branching after 20 days.

Medium	IAA(M)	GA3(M)	Growth after 20 days of incubation
Forsberg	10^{-7}	10^{-5}	Good Growth
Forsberg	10^{-6}	10^{-6}	Good Growth
Forsberg	10^{-5}	10^{-7}	No Growth
Forsberg	0	0	No Growth
Water	0	0	No Growth

Table 8: Growth of Chara plant in a Forsberg medium containing different concentrations of auxin hormone and gibberellin with water as control.

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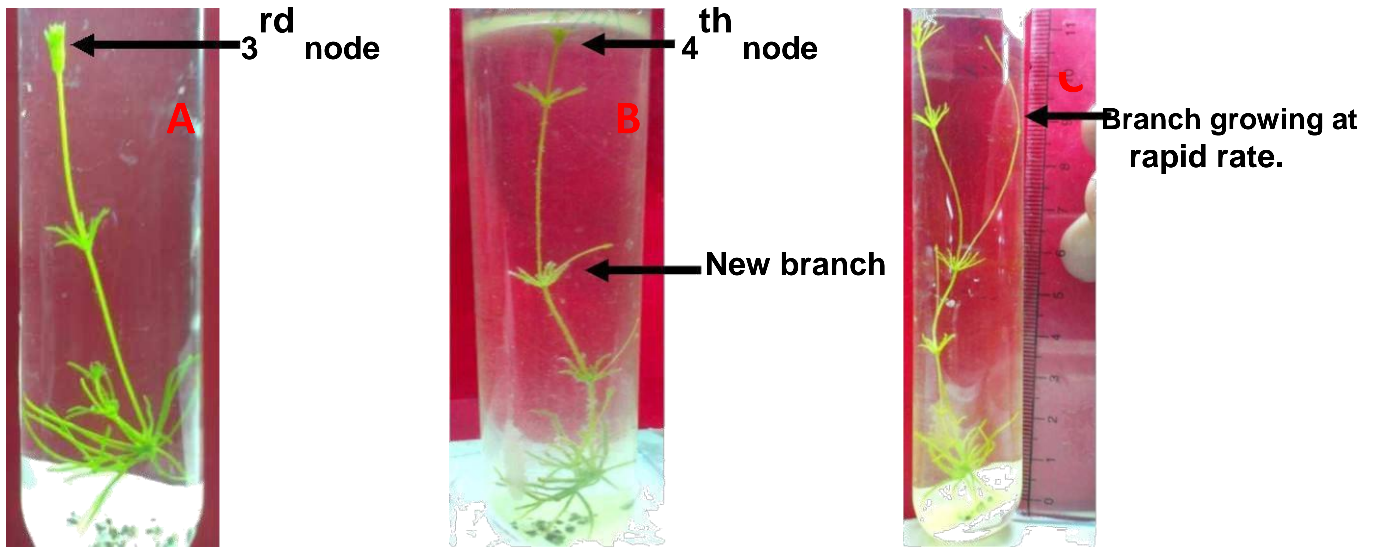


Figure 3: Growth of Chara after 20 days of inoculation in Forsberg medium with IAA (10^{-7}) and GA_3 (10^{-5}).

(A) Three nodes on the day of inoculation. (B) Fourth node that is developed as well as branching at second node after 10 days of inoculation. (C) Branch growing at a rapid rate after 20 days of inoculation.

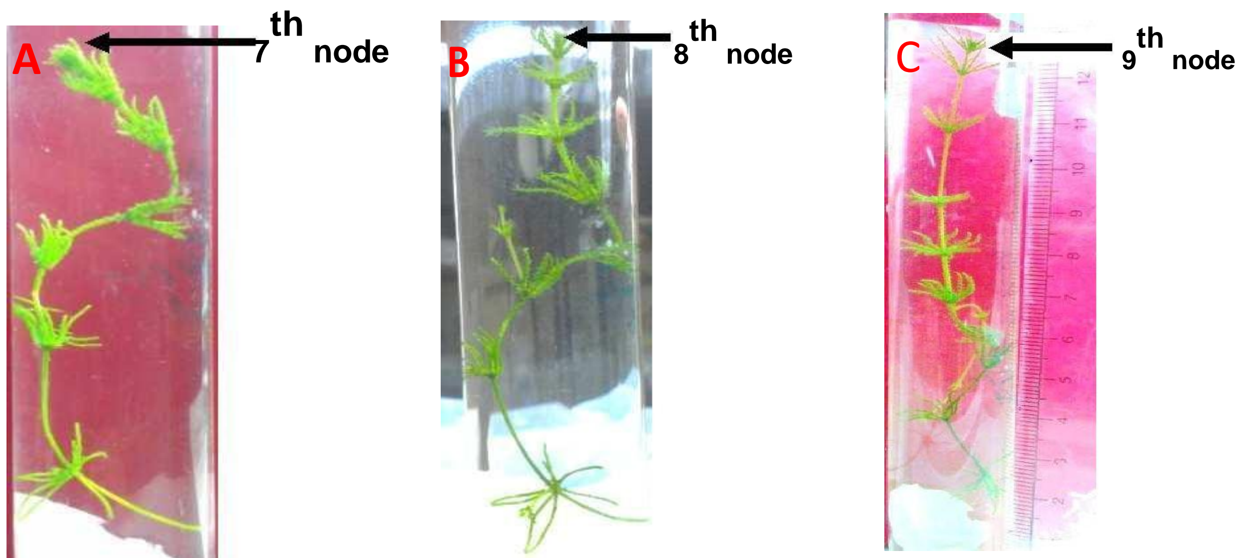


Figure 4: Growth of Chara after 20 days of inoculation in Forsberg medium with IAA (10^{-6}M) and GA_3 (10^{-6}M).

(A) Seven nodes on the day of inoculation. (B) Eighth node that is developed as well as branching at third node after 10 days of inoculation. (C) Fifth node that is developed after 20 days of inoculation.

4.4.3 Effect of kinetin on *Chara* growth

Kinetin (10^{-5} M) along with modified Forsberg medium caused an increase in the number of nodes observed after 10 and 20 days.

Medium	**Kinetin(M)**	**Growth after 20 days of incubation**
Forsberg	10^{-7}	No Growth
Forsberg	10^{-6}	Moderate Growth
Forsberg	10^{-5}	Good Growth
Forsberg	0	No Growth
Water	0	No Growth

Table 9: Growth of *Chara* plant in a Forsberg medium containing different concentrations of kinetin hormone with water as control.

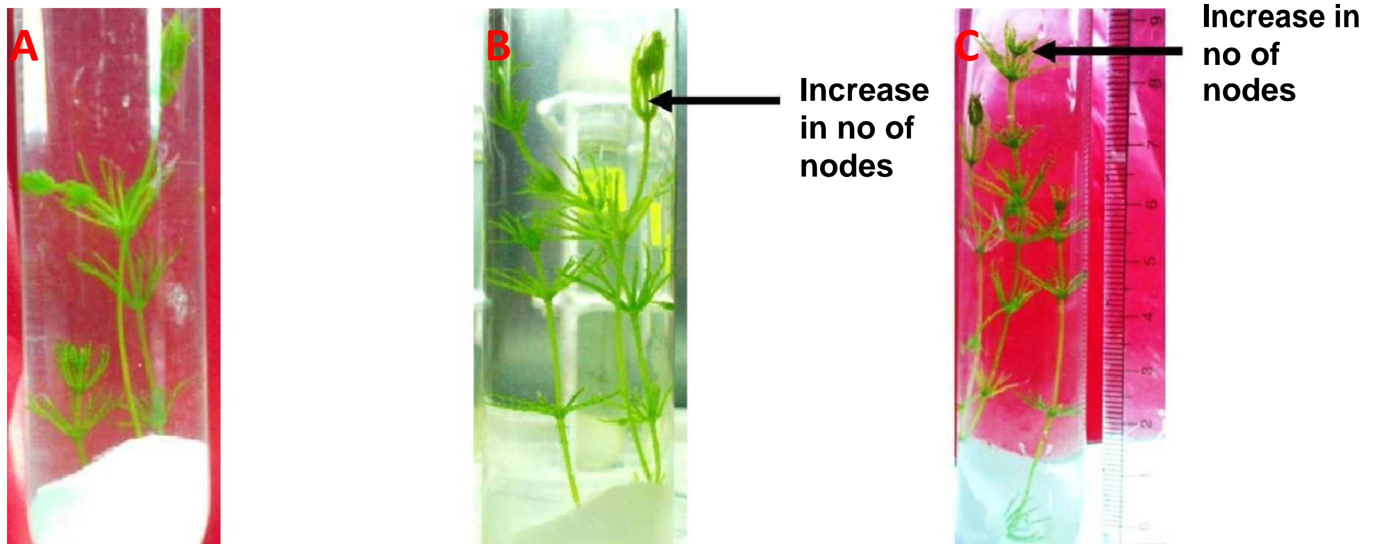


Figure 5: Growth of Chara after 20 days of inoculation in Forsberg medium with kinetin (10^{-5} M).

(A) Plants on the day of inoculation. (B) Increase in no of nodes after 10 days of inoculation. (C) More no of nodes that is developed after 20 days of inoculation.

4.5 Growth pattern of *Chara* plant from germinated sporelings

Germinated sporelings were transferred to the medium containing IAA (10^{-7} M) after 20 days of germination because IAA promotes rhizoid formation, which allow germinated sporelings to use the nutrients and absorb water very efficiently. To enhance the growth of *Chara, the* plants are being maintained in Forsberg medium along with IAA (10^{-7} M) and GA3 **(**10^{-5} M).

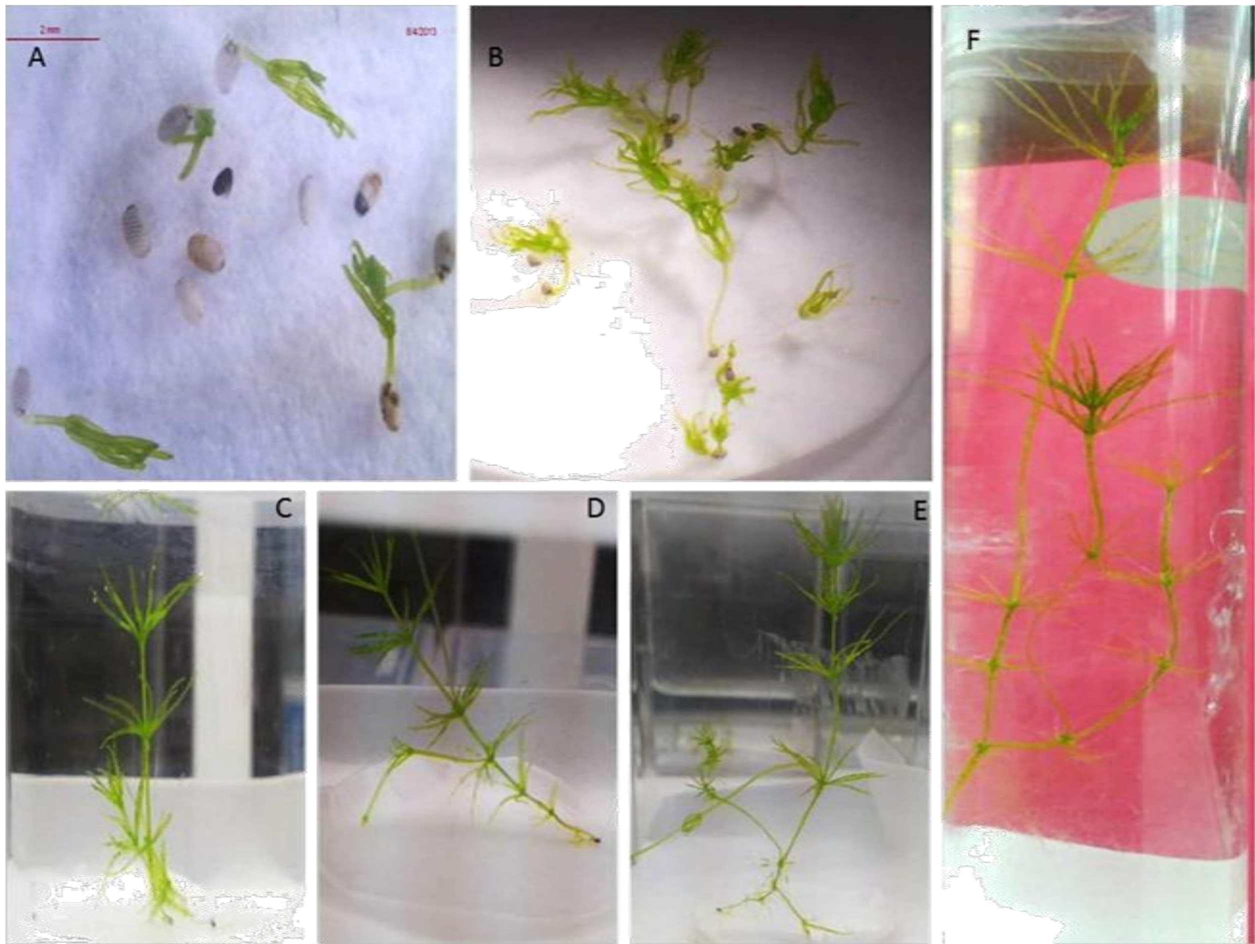


Figure 6: Growth of Chara from 18th day of germination

A) After 18 days (in water) B) After 40 days (in F+IAA 10^{-7}M) C) After 65 days (in F+ IAA 10^{-7}M + GA3 10^{-5}M) D) After 80 days (in F+ IAA 10^{-7}M + GA3 10^{-5}M) E) After 98 days (in F+ IAA 10^{-7}M + GA3 10^{-5}M) F) After 5 months (in F+ IAA 10^{-7}M + GA3 10^{-5}M).

4.6 DNA-based identification of Species of *Chara*

Once the culture is established and then to carry out the experiments it is very important to know about species that the growing *Chara* belongs to. We went ahead for the identification of species in six plants. There are reports that based on oospore morphology that one can identify what species it belongs to Since morphology based identification of species is not always reliable, so we used DNA sequences to establish the DNA-based identification of *Chara* species.

Plants in addition to nuclear genome, consists of chloroplast genome and mitochondrial genome. Most often it is the chloroplast genome, which is used for DNA based identification of species because it is present in abundance in plants, easy to extract and contain single copy genes (H. LI. *et al*., 1997). For DNA based identification of species we obtained sequences of chloroplast gene large subunit of the ribulose–biphosphate carboxylase gene (*rbcL*).We did DNA barcoding studies with one more gene that is *18S* (part of ribosomal RNA) a widely used gene in phylogenetic analysis of many organisms (Meiers ST, et al., 1999). Therefore, in our study, we used *rbcL* and *18S* sequences for DNA based identification of *Chara* species by phylogenetic analysis.

Using *rbcL* and *18S* primers, we were successful in amplifying ~1000bp of *rbcL gene* and ~550bp of *18S* gene respectively using single step RT-PCR by isolating RNA from six different *Chara* plants. Amplification results of *rbcL* and *18S* are shown in Figure 7 and Figure 8 respectively

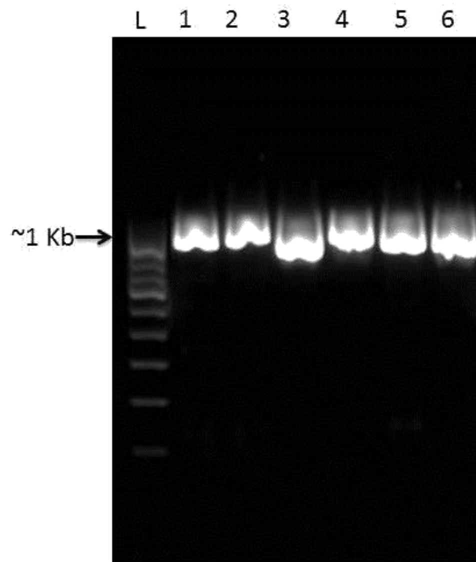


Figure 7: PCR amplification results of *rbcL* in *Chara* plant

L is the 100 bp ladder. 1-6 are the PCR amplified products of rbcL using single step RT-PCR by isolating RNA from *Chara* plantlets 2, 3, 9,10,11,12 respectively.

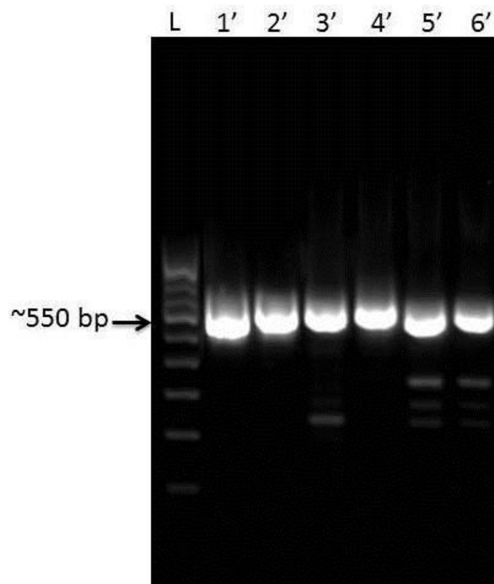


Figure 8: PCR amplification results of 18S in Chara plant

L is the 100 bp ladder.1‟-6‟ are the PCR amplified *18S* using single step products RT-PCR by isolating. RNA from *Chara* plantlets 2, 3, 9,10,11,12 respectively.

4.6.1 Phylogeny of *Chara* based on *18S* DNA sequences

Nucleic acid sequences from *18S* rRNA are very much useful for analysing phylogenetic relationships between the species which lack any informative morphological and developmental differences (Sogin *et al*., 1977; Woese *et al*., 1987; Field *et al*., 1988).The *18S* rRNA is a major part of small ribosomal subunit.

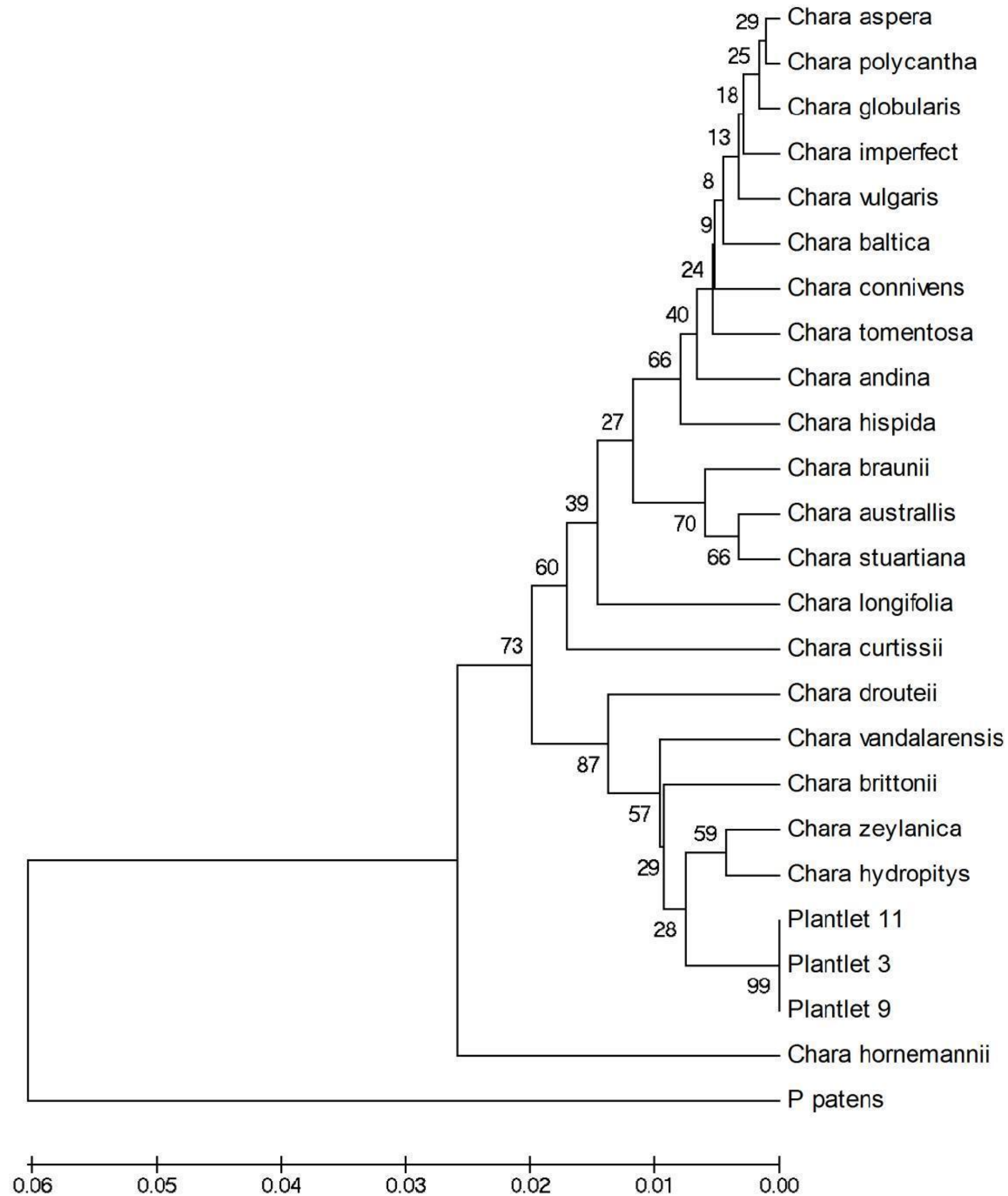


Figure 9: Phylogenetic tree showing the relationships among various *Chara* species based on *18S* gene sequence data and was constructed using the unweighted pair gap method

4.6.2 Phylogeny of *Chara* based on *rbcL* DNA sequences

Rubisco is a key enzyme in photosynthesis reaction and catalyzes first step carbon assimilation in the Calvin cycle. It is an oligomer composed of eight large subunits and eight small subunits. Large subunit of rubisco (*rbcL*) is encoded by chloroplast genome and whereas small unit of rubisco (*rbcS*) is encoded by nuclear genome. The *rbcL* is a single copy gene with no introns where as *rbcS* is a multi copy gene with introns. A single alteration in nucleotide sequence of *rbcL* gene is used to characterize which species and which family it is.

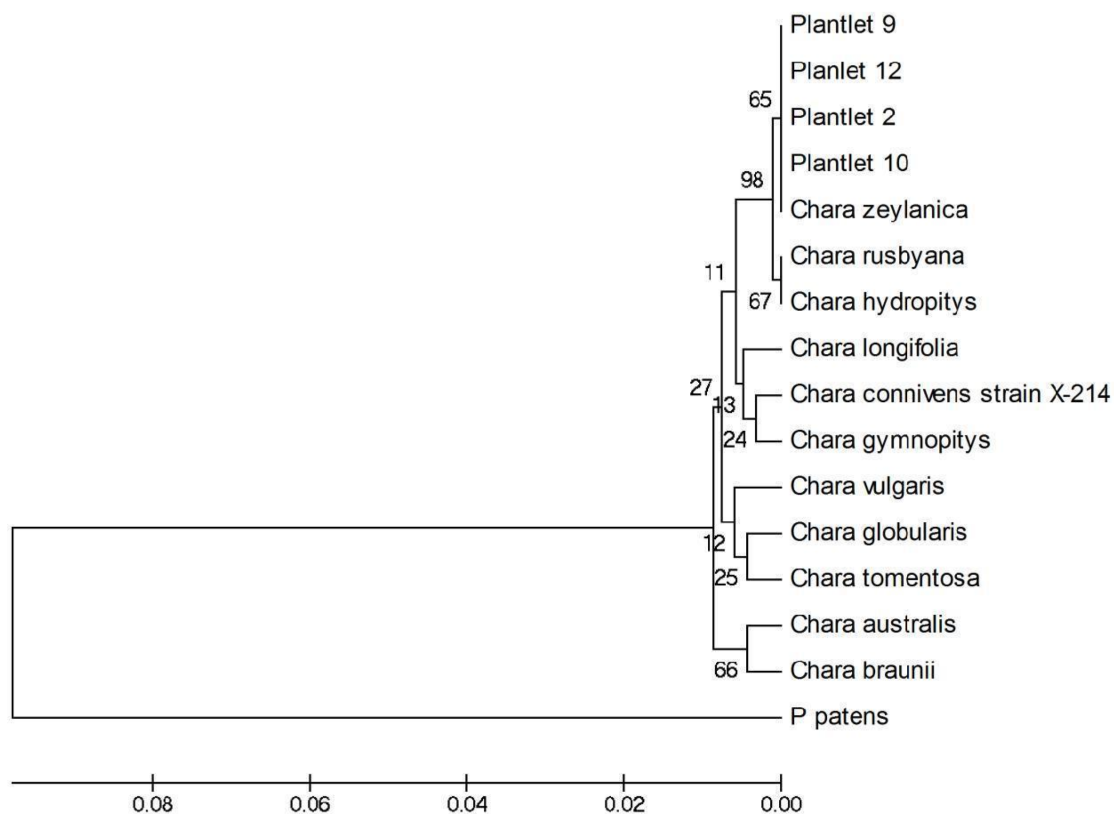


Figure 10: Phylogenetic tree showing the relationships among various *Chara* species. The tree is based on *rbcL* gene sequence data and was constructed using the unweighted pair gap method.

From the phylogenetic tree built on the DNA sequences of *rbcL* and *18S* from various *Chara* species, there are some conclusions that can be drawn from the above tree

(a) Plantelets 2, 9, 10, 12 are more related to *Chara zeylanica* with a bootstrap value of 66.

(b) *Chara zeylanica* is sharing some gene homology with *Chara* rusbyana and *Chara* hydropitys.

(c) *Chara* australis and *Chara* braunii are more related to each other with a bootstrap value of 66.

(d) *Chara* zeylanica and *Chara* hydropitys sharing gene homology when compared to plantlet 3,9,12 with a boot strap value of 27.A boot strap value of less than 50 indicating that both clades data are not sufficient to form a tree.

(e) DNA barcoding based on the *rbcL* sequences is sufficient to resolve the phylogenetic relationship at the species level in the above case.

From the phylogenetic tree (Figure 9 and Figure10) built on the *rbcL* and *18S* sequences of the various species of *Chara* suggest that both are very much useful for DNA barcoding within the species level. We were able to confirm that plantlets 3, 9,10,11,12 are *Chara* zeylanica species.

4.7 Isolation of high quality RNA from *Chara*

Extracting high quality RNA is very important to do reactions like RACE especially in the amplification of gene that is expressing in fewer amounts. We were successful in extracting high quality RNA after many isolations.

Concentration of RNA –846 ng/µl. A260/A280 = 1.97

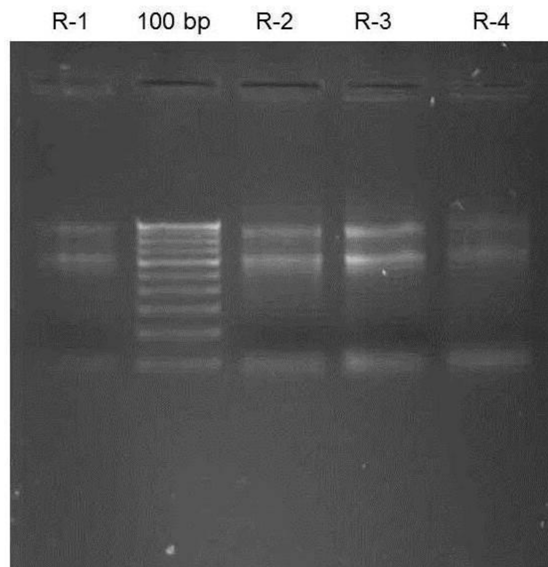


Figure 11: Isolation of RNA from whole Chara tissue

R-1 to R-4 are RNA isolated from same plant

4.8 Isolation of *KNOX* home domain gene from *Chara*

In order to isolate *KNOX* home domain genes that may have a role in apical cell mediated growth of *Chara*, we performed RT-PCR analysis with degenerate primers that are designed based on already known *KNOX* conserved homeodomain from various higher plants using the *Chara* tissues especially SAM and reproductive organs.

We were successful in pulling out homeodomain region of length 109 bp.

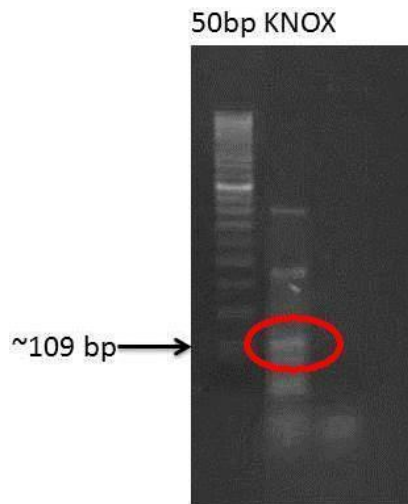
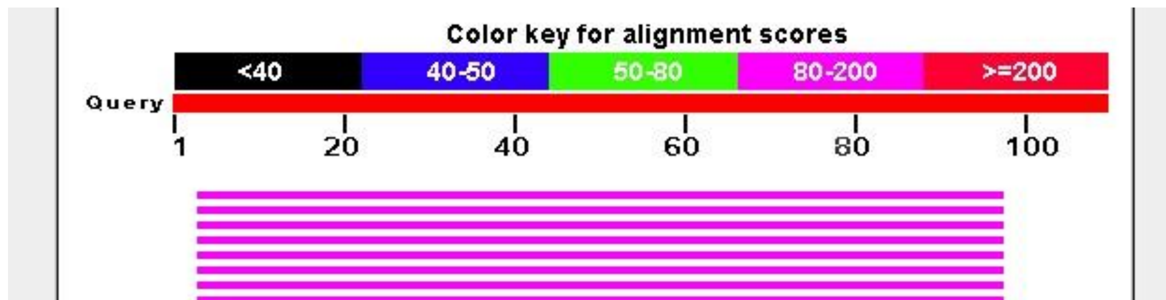


Figure 12: PCR amplification result of conserved home domain sequence of *Chara* zeylanica

Here we used degenerate primer that's why we got multiple bands on the gel. But we know the expected size so that we are able to clone that particular band of size ~109bp.



Arabidopsis thaliana homeobox protein SHOOT MERISTEMLESS mRNA, complete cds

Sequence ID: ref|NM_104916.3| Length: 1363 Number of Matches: 1

Range 1: 926 to 1019 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
84.2 bits(92)	2e-13	77/95(81%)	2/95(2%)	Plus/Minus

```
Query  4     TTCCTCTGGTTGATGAACCAATTGTATATCTGTTTCTGGGCCTAACCCGGGGAATCC-CA  62
             ||||||||||| ||||||||||||| ||||||||||||| ||   ||||  || ||| ||
Sbjct  1019  TTCCTCTGGTTTATGAACCAATTGTTTATCTGTTTCTGGTCCAGCCCCGTTGATTCCGCA  960

Query  63    TGGGGGCAGCTTGTGTTGCTCGGCCAGGTAAGGCC  97
              ||| | ||||| |||||||| |   |||||||||
Sbjct  959   AGGGCG-AGCTTTTGTTGCTCCGAAGGGTAAGGCC  926
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Figure 13: Blast result of 109bp sequence of KNOX conserved homeodomain showing 81% identity with STM of Arabidopsis

4.9 Morphological and Histological Observations of *Chara* tissues

4.9.1 Morphological observations of *Chara*

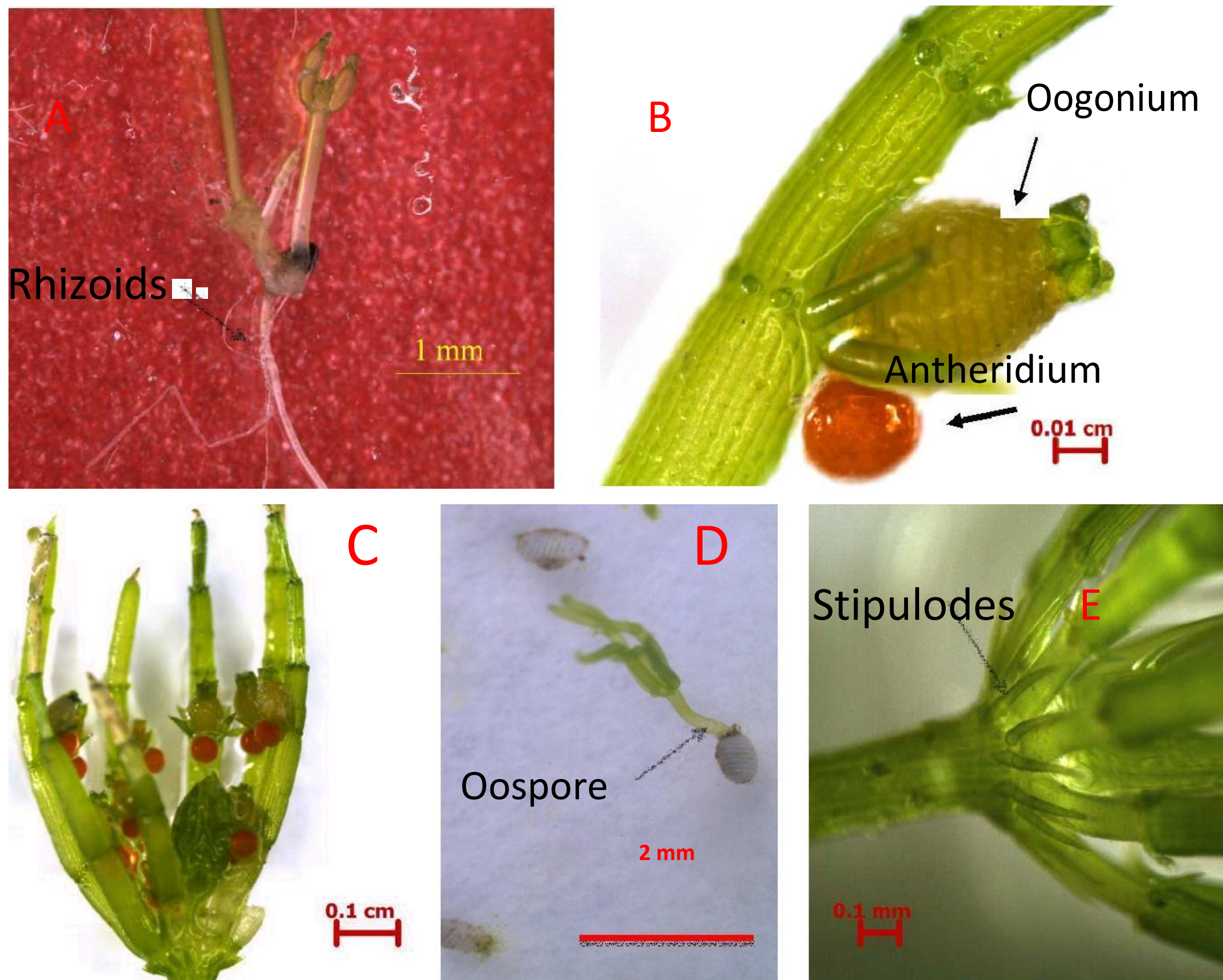

Figure 14: Thallus of *Chara* zeylanica

A) Rhizoids B) Oogonium and antheridium showing together C) Part of thallus consisting of a main axis and whorled branchlets with reproductive organs. D) Oospore E) Stipulodes those are present on the main axis of *Chara* plant.

4.9.2 Histological sections of *Chara*

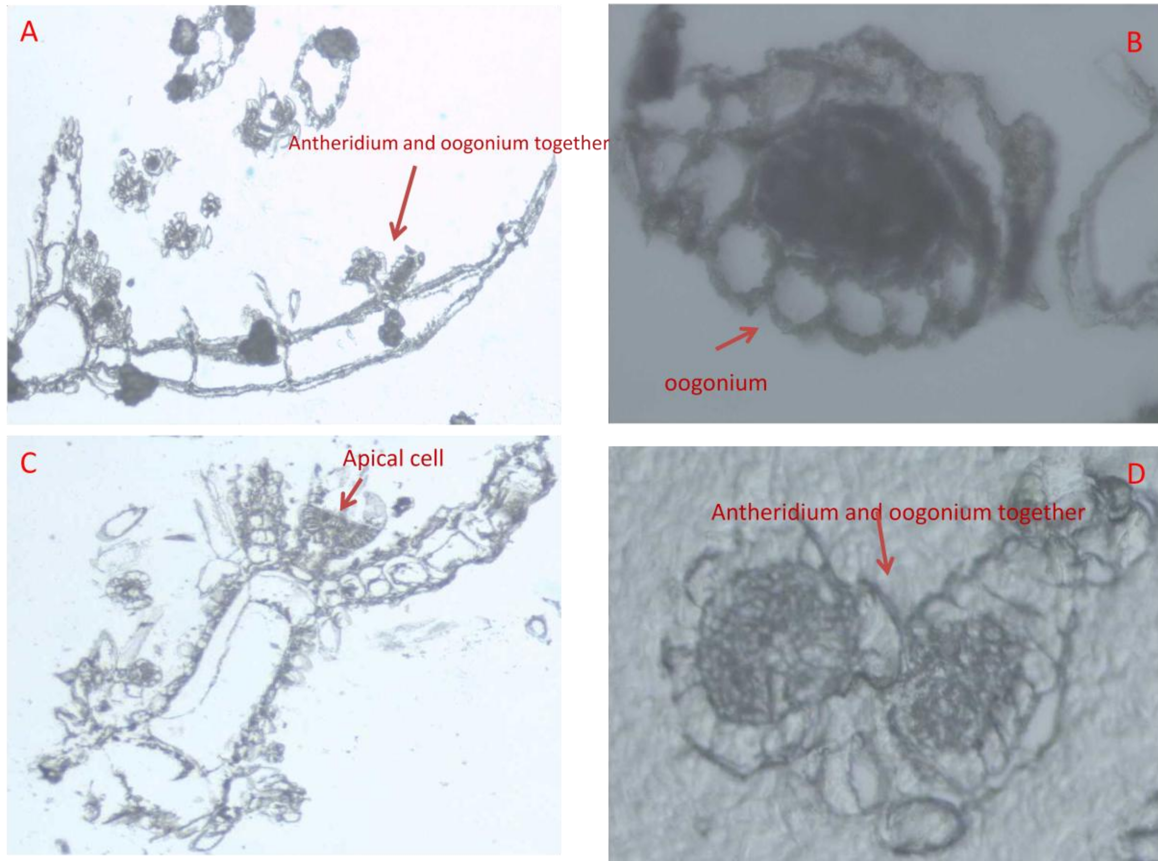

Figure 15: Histological sections of tissues of *Chara* zeylanica

A) Branches with oogonium and antheridium B) Antheridium C) Apical part of the plant. D) Oogonium and antheridium together.

5. DISCUSSION

Our Study mainly focus on the establishment of aseptic cultures of *Chara.* Achieving germination of oospores and growth of germinated sporelings aseptically is one of the milestone in an attempt to establish *Chara* as a model system in our lab. 0.1% HgCl2 as surface sterilizing agent gave us best results. As 0.1% HgCl2 is hazardous to the environment, we are using 0.8%sodiumhypochlorite (NaClO) as a surface sterilizing agent. We were successfully standardized the media that enhances the growth of *Chara* plant cutting. But there are some challenges that are yet to be faced regarding establishment of *Chara* as a model system. We don't have any strong evidence about the environmental and physiological factors that influence the formation of reproductive organs like oogonium and Antheridium on the *Chara* plant. There are reports that gibberellins have a role in the production of reproductive organs on the *Chara* plant. In higher plants photoperiod influences the induction of reproductive organs. As far as *Chara* is concerned, there are no reports about the influence of photoperiod on sex organ development.

During land plant evolution, there is a transition from a haploid (n) form to diploid (2n) form, *KNOX* genes are known to have a role in this transition. The vegetative part of the *Chara* thallus is a gametophytic body, where as reproductive organs are sporophytes. We are interested to know the expression of *KNOX* in the reproductive parts of *Chara* as it is a sporophytic body. Class I *KNOX* homeobox gene is shown to have a role in the maintenance of shoot apical meristem (Hake *et al*., 2004; Jackson *et* al., 1994). Growth of *Chara* takes place with the help of a single domed apical cell situated at the tip. *KNOX* might have a role in the apical cell mediated growth of *Chara.* The first step in an attempt to know the role of *KNOX* genes in *Chara* is to isolate the full length sequence of KNOX using RACE technique. To study the spatial expression of *KNOX* homeobox genes in *Chara*, we are planning to perform mRNA *in situ* hybridization by taking paraffin

embedded histological sections of various *Chara* tissues. Temporal expression of *KNOX* homeobox genes in *Chara* can be investigated by Real Time PCR. If KNOX has a role in both SAM maintenance and also transition from a haploid form to diploid in *Chara zeylanica,* then the expression of *KNOX* is restricted to SAM as well as reproductive organs.

6. Conclusions

1. $HgCl_2$ at concentration of (0.1%) and NaClO (0.8%) were found suitable for surface sterilization of *Chara* oospores in our study.
2. Germination percentage varied with different surface sterilizing agents and concentration of treatment tested.
3. Treatment of oospores with 0.1% $HgCl_2$ for a duration of 4 minutes resulted high germination percentage.
4. Forsberg medium with minor modifications has been standardized for the growth of the germinated sporelings. Removal of $MnCl_2$ from the original medium and adjustment of NTA concentration up to 10mM found effective in invitro condition.
5. Among all the phytohormone alone or in combinations tested to enhance the growth of Chara, IAA (10^{-7}M), kinetin (10^{-5}M), IAA(10^{-7}M)+ GA3(10^{-5}M) and IAA (10^{-7}M) + kinetin (10^{-5}M) promoted the growth of *Chara* plant cuttings along with basal medium.
6. Phylogenetic analysis based on *rbcL* and *18S* sequences from various *Chara* species, provided evidence that the cultured *Chara* sp belongs to *Chara* zeylanica.
7. Based on conserved *KNOX* domain, approximately 109bp amplicon has been isolated and sequence verified.
8. Isolation of full length sequence of *KNOX* gene is presently in progress.

7. Future goals

Once the full length sequence of KNOX is identified, we would proceed with expression profiles of KNOX in *Chara* vegetative body and reproductive organs. We also aim to continue with *in-situ* hybridization studies to understand the expression patterns of homeobox genes.

8. References

- Boney S.H.: Survival and growth of α-spores of Porphyra schizophylla HOLLENBERG (Rhodophyta:Bangiophyceae). J.Exp.Mar.Biol.Ecol. 35, 7–29 (1978).

- Boszke P., Pelechaty M. & Pukacz A. 2008. Oospore dimensions and wall ornamentation in *Charabraunii.* Biologica 63/4: 457-460.

- Delwiche C. F. K. G. Karol M. T. Cimino K. J. Sytsma 2002. Phylogeny of the genus Coleochaete (Coleochaetales, Charophyta) and related taxa inferred by analysis of the chloroplast gene *rbcL*. Journal of Phycology 38: 394-403.

- Forsberg, C., 1965b. Sterile germination of oospores of Chara and seeds of Najas marina. Physiologia Plantarum 18: 128-137.

- Forsberg, C., 1965c. Nutritional studies of Chara in axenic cultures. Physiologia Plantarum 18: 275-290.

- Franceschi, V.R., Lucas, W.J. (1980) Structure and possible functions of charasomes; complex plasmalemma-cell wall elaborations present in some characean species. Protoplasma 104, 253-271

- Godlewski, M. & KWIATKOWSKA, M., 1980. Effect of gibberellic acid on the formation and development of antheridia and oogonia in Chara vulgaris L. Acta Societatis Botanicorum Poloniae 49: 459-469.

- Godlewski, M., 1977. The role of plant growth substances in the regulation of the cell cycle in antheridial filaments of Chara vulgaris L. 1. Effect of gibberelic acid on some processes in the course of the cell cycle. Acta Societatis Botanicorum Poloniae 46: 317-329.

- H. LI. 1997. Molecular phylogeny of gymnosperms and seed plant evolution: analysis of 18S rRNA sequences. Mol.Biol. Evol. 14:56–68.

- Hake, S., Smith, H. M., Holtan, H., Magnani, E., Mele, G. and Ramirez, J. (2004). The role of Knox genes in plant development. Annu. Rev. Cell Dev. Biol.20, 125-151.

- Jackson, D., Veit, B. and Hake, S. (1994). Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120, 405-413.

- John, D. M., Moore, J. A. & Green, D. R. 1990. Preliminary observations on the structure and ornamentation of the oosporangial wall in Chara (Charales, Chlorophyta).Br. Phycol. J.25:1–24.

- Karol KG, McCourt RM, Cimino MT, Delwiche CF. The closest living relatives of land plants. Science 2001; 294:2351-3.

- Kwiatkowska , M., 1991b. Effect of the light factor on DNA and protein synthesis in non-generative cells of antheridium and regulation of spermatogenesis in Chara vulgaris L. Phycologia 30: 124-134.

- Kwiatkowska, M. (1991) Autoradiographic studies on the role of plasmodesmata in the transport of gibberellin. Planta 183, 294-299.

- Kwiatkowski, M., PALA, A. & MASZEWSKI, J., 1997. Size-variation in the antheridia and oogonia of Chara vulgaris under different experimental conditions. Acta Societatis Botanicorum Poloniae 66: 29-33.

- Lee, J. H., Lin, H., Joo, S. and Good enough, U. (2008). Early sexual origins of homeoprotein heterodimerization and evolution of the plant KNOX/BELL family. Cell 133, 829-840.

- Lewis L., McCourt R. M.2004 Green algae and the origin of land plants. Am. J. Bot. **91**, 1535–1556. McCourt, R.M. (1995) Green algal phylogeny. Trends Ecol. Evol. 10, 159–163

- Meiers ST, Proctor WV, Chapman RL (1999) Phylogeny and biogeography of Chara (Charophyta) inferred from 18S rDNA sequences. Austral J Bot 47:347–360

- MISHLER, B. D., AND S. P. CHURCHILL. 1985. Transition to a land flora: phylogenetic relationships of the green algae and bryophytes. Cladistics1: 305–328.

- Pal, R. & CHATTERJEE, P., 1986b. Feulgen cytophotometric determination of nuclear DNA in species of Chara (Charophyta) from India. Cryptogamie, Algologie 7: 317-322.

- Proctor, V.W., 1967. Storage and germination of Chara oospores. Journal of Phycology 3: 90-92.

- Sakakibara, K., Nishiyama, T., Deguchi, H. and Hasebe, M. (2008). Class 1 KNOX genes are not involved in shoot development in the moss Physcomitrella patens but do function in sporophyte development. Evol. Dev. 10, 555-566.

- Seiji Takatori and Kozo Imahori (1971) Light reactions in the control of oospore germination of Chara delicatula. Phycologia: September 1971, Vol. 10, No. 2 and 3, pp. 221-228.

- Shen, E.Y.F., 1967. Micro spectrophotometric analysis of nuclear DNA in Chara zeylanica. Journal of Cell Biology 35: 377-384.

- Vollbrecht, E., Veit, B., Sinha, N. and Hake, S. (1991). The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350, 241-243.

- Wang-cahil, F. & KISS, J.Z., 1995. The statolith compartment in Chara rhizoids contains carbohydrate and protein. American Journal of Botany 83: 220-229.

- Williamson, R. E. 1975. Cytoplasmic streaming in Chara: a cell model activated by ATP and inhibited by cytochalasin B. J. Cell Sci. 17:655-668.

- Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.