Application for Ph.D. student position in Marine Ecology

Ref. no. U 2011/171

May 3, 2011

Anna Godhe, Associate Professor
Department of Marine Ecology, University of Gothenburg

Application Lovisa Hansén

To Dr. Anna Godhe

I am writing in response to the open position for Ph.D. student in Marine Ecology at the University of Gothenburg. I am currently a Master of Science in Biology, focusing on population dynamics and genetics of plankton communities. I believe my experience and commitment to this field of marine research make me well qualified to contribute to your project.

Marine biology has been my passion since gymnasium. At the age of 17 I moved away from home to pursue my scientific interest and study marine biology at Gullmarsgymnasiet, Lysekil. During this time my interest in marine research increased proportionally with the knowledge I gained. During my third year I was one of only a few students invited to attend one week of practical work aboard R/V Argos, Institute of Marine Research, Swedish Board of Fisheries.

During the course of my undergraduate career, I pursued coursework in marine biology and oceanography. I gained invaluable experience in experimental design and field sampling of coastal and open water regions. My knowledge in oceanography along with experiences in handling of large data sets, mathematical modeling and statistical analysis may prove very useful when investigating correlation between genetic structure of populations and different environmental conditions.

In 2004, under the guidance of Dr. Anna Godhe and Dr. Melissa McQuoid, I examined the genetic diversity within and between spatially distinguished populations of *Skeletonema marinoi* by sequencing of the ITS region ("Genetic variability of Skeletonema costatum (Bacillariophyceae) based on ribosomal DNA ITS sequences"). Parts of the data collected were later published in Godhe et al. (2006) *J.Phycol.* 42:2.

My second project, "Molecular studies of mating behaviour in Indian *Skeletonema"*, was funded by a Minor Field Study scholarship (SIDA) and was carried out at College of Fisheries, Mangalore, India in 2005. The method we used in this study was new to all of us which resulted in me taking on a greater responsibility. Working abroad also provided me with experience in more independent work and adapting to new environments.

Lovisa Hansén

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Application for Ph.D. student position in Marine Ecology

Ref. no. U 2011/171

May 1, 2011

Anna Godhe, Associate Professor
Department of Marine Ecology, University of Gothenburg

On two occasions I worked as a project assistant to Dr. Anna Godhe. In 2008 I was asked to sample the spring bloom while Dr. Godhe was in India. Water samples and sediment core were collected from Gullmarsfjorden. During the course of both projects and as a project assistant, I worked extensively in isolation, culturing and extraction of DNA from clonal cultures of *S. marinoi*. I believe my experience in independent laboratory work and different molecular methods, makes me an ideal candidate for the current position and I requiring little introduction. This would give the project a head start.

I am also interested in the development of improved analytical models and methods for further study of population dynamics within plankton communities. This last year during my free time, I have been working towards starting a new project. At this moment the project is pending funding, which I am now in the process of securing. By using a statistical method devised from Social Network Analysis one should be able to analyze and better visualize population structures and dynamics using genetic data and calculate the correlation with environmental factors.

With this PhD student position I would like to contribute to the increased understanding of population dynamics and the impact of environmental conditions on population structure. I would be gracious for an opportunity to further discuss my thoughts and ideas regarding your project. Enclosed are my curriculum vitae, letters of recommendation, a copy of my examination certificate, my master's theses and a transcript of my grades. If you require any additional information or supporting documents, I am happy to provide it. I look forward to hearing from you.

Sincerely

Lovisa Hansén

Lovisa Hansén

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Curriculum Vitae

Education

Degree of Master of Science in Biology, Marine Biology

Thesis title: Molecular studies of mating behaviour in Indian *Skeletonema* (2005)
Funded by SIDA, Minor Field Study scholarship
Supervisor: Dr. Anna Godhe

Brief summary of the research project:
A working protocol for AFLP analysis was established and the hypothesis that heterothallic recombination occurs in *Skeletonema sp.* was examined by an intercrossing experiment mixing two parental single cell clonal cultures. From that mix an F1-generation was isolated and genetic relationship was assessed. Results showed that both hetero- and homothallic recombination occurred.
Full thesis enclosed.

Thesis title: Genetic variability of *Skeletonema costatum* (Bacillariophyceae) based on ribosomal DNA ITS sequences (2004)
Supervisors: Dr. Anna Godhe and Dr. Melissa McQuoid

Brief summary of the research project:
Single cell clonal cultures was established from sediment samples from Canada, Portugal and Sweden. Canadian clones were found to be genetically distinct whereas the two populations from Atlantic waters were mixed within the trees. Determining intraspecific genetic diversity by sequencing the ITS region rendered a good result on a large oceanic scale.
Full thesis enclosed.

1997 - 2000 Natural Science Programme Marine Biology, Gullmarsgymnasiet, Lysekil, Sweden

Employment (a selection)

December 2010 -
Shop Assistant, Media Markt Bäckebol, Gothenburg, Sweden.

May 2010 - September 2010
Guide, Kosterhavet National Park, Strömstad, Sweden.
Duties mainly consisted of informing visitors about the park and the reasons for such a high biodiversity in the area. It was a great pleasure to get the chance to share my knowledge and passion for the sea.

February 2008 - May 2008
Project Assistant, Inst. Marine Ecology, University of Gothenburg, Sweden.
I worked as part of Dr. Anna Godhe's research project investigating phytoplankton bloom dynamics. Field work included collection of water samples and sediment core, establishment of clonal cultures and extraction of DNA for future analysis.

September 2005 - November 2005
Project Assistant, Inst. Marine Ecology, University of Gothenburg, Sweden.
As an assistant within Dr. Anna Godhe's project, investigating phytoplankton bloom dynamics, my main responsibilities was to process clonal cultures of *Skeletonema.* The work included microscopic work followed by extraction and purification of DNA, PCR, sequencing and subsequent analysis primarily using Sequencher.

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Curriculum Vitae

Participation in expeditions and fieldwork:

February 2008 Fieldwork Sven Lovén Center for Marine Sciences (formerly Kristineberg Marine Research Station), Gullmarsfjord, Sweden

April-May 2001 R/V Skagerak, Kristineberg Marine Research Station. A three week undergraduate oceanography field course. Expedition in Kattegat, Skagerrak and eastern North Sea and following fieldwork at Bornö Hydrographic Field Station, Gullmarsfjord.

December 2000 R/V Arne Tiselius, Kristineberg Marine Research Station. A week long Undergraduate oceanography field course. Expeditions in the Skagerrak Koljöfjord and Gullmarfjord and following fieldwork at Bornö Hydrographic Field Station, Gullmarsfjord.

September 1999 R/V Argos, Institute of Marine Reasearch, Swedish Board of Fisheries

Skills

General skills in laboratory work and experimental design.
Experience and proficiency in:

Computer software:

- Applications: Sequencher, GelComparII, PAUP, MatLab, GIS, UCINET, Microsoft Office, Apple iWork.
- Operative systems: Mac OS X, Windows XP, Windows Vista/7, Unix/Linux

Molecular methods:

- Establishing clonal cultures by using micropipette and microscope
- DNA extraction using CTAB or Phenol-Chloroform-Isoamylalcohol methods
- Real-Time PCR, sequencing, AFLP, microsatellite

Language:

- Swedish - native
- English - advanced

Other:

- Drivers license
- PADI Advanced Open Water Certificate

Scholarships and grants

2004 Minor Field Study scholarship, SIDA

References

Supervisor
Dr. Anna Godhe
Inst. of Marine Ecology, University of Gothenburg
Telephone: +46 (0)31 7862709 E-mail: anna.godhe@marecol.gu.se

Anita Tullrot
National Park Vice Manager, Kosterhavet National Park
Telephone: +46 (0) 31 607198 E-mail: anita.tullrot@lansstyrelsen.se

Lovisa Hansén
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GÖTEBORGS UNIVERSITET

EXAMENSBEVIS
OFFICIAL TRANSCRIPT OF RECORD

2011-02-01

Name

Lovisa Hansén

Date of birth
year month day pers code

1981 05 25 - 4661

has passed the following courses and been awarded the following degree at University of Gothenburg in accordance with the Higher Education Ordinance (SFS 1993:100).

Course	*Credit Points*	*Grade *)*	*Date*
Biology: Basic Course	40	Pass with Distinction	2002-01-17
Oceanography: Intermediate Level	20	Pass	2002-06-06
Marine Organisms and their Habitat	10	Pass	2002-10-31
Marine Botany 1	10	Pass	2003-01-16
Comparative and Marine Zoophysiology	10	Pass	2003-03-25
Marine Population Ecology	10	Pass with Distinction	2003-06-06
Degree Project in Marine Botany	20	Pass with Distinction	2004-02-19
Marine Ecology: Biology Project	10	Pass	2005-06-30
Oceanography: Marine System Analysis	10	Pass with Distinction	2005-10-25
Marine Ecology: Degree project	20	Pass	2006-02-10

Degree awarded: **Filosofie magisterexamen**
(Degree of Master of Science in Biology (Marine Biology))

Date of degree: 1 February 2011

Maria Knutsson
Degree Officer

Vidimeras 27/3-2011
Åsa Norén Klingberg
070-7766375

Robert Schaffelt 076-6373371

**) Grades: Pass or Pass with Distinction, if nothing else is stated*

20 credit points correspond to full-time study during one semester. 20 credit points correspond to 30 ECTS credits.
For further information, see Diploma Supplement.

UNIVERSITY OF GOTHENBURG

University of Gothenburg
Department of Zoology

Official transcript of records for

LOVISA JANSSON
(Civic registration number 810525-4661)

Admitted to study programme:
Study Program in Marine Science

240 Credits

Courses	Credits	Grade	Date	Note
OC0200 Oceanography: Basic Level Course	30.0	VG	2001-01-19	1,k
1040 Marine Environment	(7.5)	G	2000-10-27	k
1030 Introduction to Oceanography	(7.5)	VG	2000-11-01	k
1080 Coastal Oceanography	(7.5)	VG	2000-11-28	k
1060 Case Studies	(7.5)	G	2001-01-19	k
BIN580 Biology: Basic Course	60.0	VG	2002-01-17	1,k
1590 Cell Biology I	(7.5)	VG	2001-02-15	k
1591 Cell Biology II	(7.5)	VG	2001-03-21	k
1584 Plant Physiology	(7.5)	G	2001-04-27	k
1583 Plant Morphology	(7.5)	G	2001-06-07	k
1592 General Ecology and Evolution	(6.0)	VG	2001-09-27	k
1593 Ecology & Nature Conservation	(9.0)	VG	2001-11-01	k
1585 Animal Morphology	(7.5)	VG	2001-12-04	k
1586 Animal Physiology	(7.5)	G	2002-01-17	k
OC2200 Oceanography: Intermediate Level	30.0	G	2002-06-06	1,k
2090 Geosphere Dynamics	(15.0)	G	2002-03-22	k
2060 Ocean Circulation	(7.5)	VG	2002-04-29	k
2080 Field Course	(7.5)	G	2002-06-06	k
BIN440 Basic Course in Floristics and Faunistics	7.5	G	2002-08-30	1,k
1440 Floristics	(3.8)	G	2002-06-19	k
1441 Faunistics	(3.7)	VG	2002-08-30	k
BIN180 Marine Organisms and their Habitat	15.0	G	2002-10-31	1,k
1180	(15.0)	G	2002-10-31	k
BIN160 Marine Botany 1	15.0	G	2003-01-16	1,k
1160	(15.0)	G	2003-01-16	k
BIN251 Comparative and Marine Zoophysiology	15.0	G	2003-03-25	1,k
2510	(15.0)	G	2003-03-25	k
BIN380 Marine Population Ecology	15.0	VG	2003-06-06	1,k
1380	(15.0)	VG	2003-06-06	k
ES0180 Marine Pollution	7.5	G	2003-06-06	1,k
1010 Marine Pollution	(7.5)	G	2003-06-06	k
BIN170 Degree Project in Marine Botany	30.0	VG	2004-02-19	1,k
1170	(30.0)	VG	2004-02-19	k

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BOX 463
405 30 GÖTEBORG

Camilla Pettersson

2010-05-10

Continued on next page ---->

Official transcript of records for

LOVISA JANSSON
(Civic registration number 810525-4661)

BIN563 Marine Ecology: Biology Project	15.0	G	2005-06-30 1,k
1563	(15.0)	G	2005-06-30 k
OC2600 Oceanography: Marine System Analysis	15.0	VG	2005-10-25 1,k
1011 Theory	(4.5)	VG	2005-09-27 k
1010 Practical Model Work	(10.5)	G	2005-10-25 2,k
NGN120 Geographical Information Systems	15.0	G	2006-02-01 1,k
1020 Theory in GIS	(7.5)	G	2006-01-28 k
1010 Practice Course in GIS	(7.5)	G	2006-02-01 k
BIN531 Marine Ecology: Degree project	30.0	G	2006-02-10 1,k
1531 Marine Ecology	(30.0)	G	2006-02-10 k

Total sum: 300.0 credits

The above is an excerpt from the register of student records.

Göteborg, May 10, 2010

Camilla Pettersson

GÖTEBORGS UNIVERSITET
EXAMENSBEVIS OCH UTBILDNINGSINFORMATION
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405 30 GÖTEBORG

Notes:
k Credits converted 1 July 2007

Grading systems:
1 VG=Pass with Distinction, G=Pass
2 Pass course only, G=Pass

60 credits represent a full academic year.

Vidimeras 27/3-2011
Åsa Norén Klingberg 070-7766375
Pernilla Sikefjäll 0706-637331

Minor Field Studies No 350

Molecular studies of mating behaviour in Indian *Skeletonema* using AFLP

Lovisa Jansson

Marine Ecology, Göteborg University

February 2006

Supervisor: Anna Godhe

Swedish University of Agricultural Sciences
SLU External Relations
Uppsala, February, 2006
ISSN 1402-3237

Minor Field Studies are carried out within the framework of the Minor Field Studies (MFS) Scholarship Programme, which is funded by the Swedish International Development Cooperation Agency (Sida).

The MFS Scholarship Programme offers Swedish university students an opportunity to undertake two months´ field work in a developing country to be analysed, compiled and published as an in-depth study or graduation thesis work. The studies are primarily made on subjects of importance from a development perspective and in a country supported by Swedish development assistance.

The main purposes of the MFS programme are to increase interest in developing countries and to enhance Swedish university students´ knowledge and understanding of these countries and their problems and opportunities. An MFS should provide the student with initial experience of conditions in such a country. A further purpose is to widen the Swedish human resource base for international development cooperation.

The SLU External Relations administers the MFS programme for the rural development and natural resources management sectors.

The responsibility for the accuracy of information presented rests entirely with the respective author. The views expressed are those of the authors and not necessarily those of the SLU External Relations.

Abstract

Previously observations of auxospore formation have been made in single clone cultures, hence it is known that sexual recombination occurs. But it is not known if *Skeletonema* sp. is homothallic or heterothallic. Amplified Fragment Length Polymorphism (AFLP) has previously been used for taxonomic and population studies with the widest application in analyses of genetic variation on the sub-species level. An intercrossing experiment was set up with two parental clones of *Skeletonema* sp. isolated from plankton samples collected of the coast off Mangalore, Karnataka, India, one year apart. The parental clones were mixed, and from the mix F_1-generation was isolated. There was a high reproducibility of the method and results of the experiment showed that similarity values of 93% or higher were strong indications of identical clones, while similarity ranging from 73% to 90% indicated homothallic recombination. Heterothallic reproduction seemed to give similarity values of 60% to 70% and different clones of *Skeletonema* were less than 60% similar.

Introduction

Background

Skeletonema is a diatom genus, which is widespread all over the world's oceans. It is one of the dominating phytoplankton in temperate regions, sometimes called "the Dandelion of the sea" referring to it's rapid expand in numbers. In many parts of the world *Skeletonema* is commonly found throughout the year, e.g. in India (Subrahmanyan, 1959), but in the temperate regions the highest density occurs during spring blooms.

Recent studies have examined *Skeletonema costatum*-like species using electron microscopy and the small and large subunit (SSU and LSU) rRNA genes (Sarno et al., 2005). Genetic differences and morphological divergence detected in electron microscope, which was observed in the collected material, have led to the description of several new species within the genus. Most individuals formerly classified as *S. costatum* have new species designations. We have not been able to examine the clones isolated from the Arabian Sea in electron microscope, neither sequencing species specific fragments of the genome, and thus we can not classify the organism to species level. Henceforth we will therefore refer to the organism as *Skeletonema* sp.

Little is known about the reproduction of *Skeletonema* sp. They primarily use asexual reproduction by vegetative division, but it is also known that they produces gametes and form auxospores as a part of their sexual reproduction (Drebes, 1977; Hargraves et al., 1983).

Figueroa et al. (2005) showed that homothallic reproduction or self-fertilisation, i.e. fusion of genetically identical gametes were present in some strains of the dinoflagellate *Gymnodinium catenatum,* while other strains could only have sex by outcrossing (fusion with gametes from two different strains) and this way of reproduction is called heterothallic.

Amplified Fragment Length Polymorphism (AFLP) has previously been used for taxonomic and population studies with the widest application in analyses of genetic variation on the sub-species level (Balasaravanan et al., 2003; González et al., 2003; Baydar et al., 2004; Liu et al., 2005; Zhou et al., 2005). The method combines the advantages of Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) meaning that no prior sequence information is needed (John et al., 2004) and there is a high reproducibility (Jones et al., 1997). The key feature of AFLP is the simultaneous screening of many different regions distributed randomly across the entire genome. AFLP provides a powerful tool to fingerprint individual clones and establish genetic distances and relationships between clones based on genetic similarities (Beismann et al., 1997).

AFLP includes several steps. Extracted DNA is digested with one rare cutting and one frequent cutting restriction enzyme. The digested DNA is then ligated with double-stranded restriction-site specific adapters. These adapters have a specific adaptor sequence that serve as attachment sites for PCR primers. Subsequent PCR is used to amplify the digested DNA fragments. This method uses two consecutive PCR amplifications. The first called pre-amplification uses the site-specific primer-sequences with an additional extra base at the 3' end. Selective amplification use the same sequence but with three additional bases. The additional bases at the 3' end reduces the number of fragments amplified by a factor of 4 (one base) and 64 (three bases) to give a clearer separation of bands.

The aim of this study was to establish a working protocol for AFLP analysis on *Skeletonema* sp., and to investigate the genetic diversity within a small group of closely related clones to examine the means of reproduction. Previous observations of auxospore formation have been made in monoclonal cultures (McQuoid, pers. com.), hence it is known that sexual recombination occurs and that it is homothallic. But is *Skeletonema* sp. also heterothallic? Can gametes only fuse with genetically identical gametes or also with gametes from other strains?

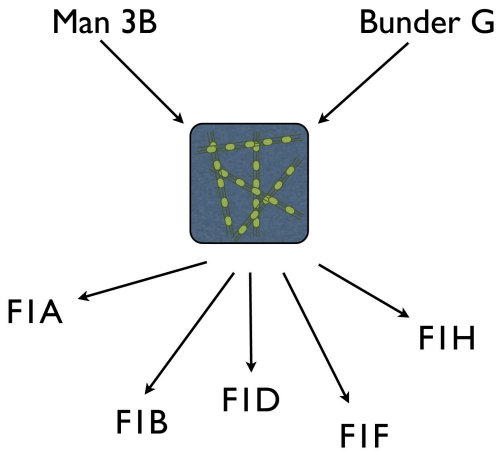

Figure 1. Schematic drawing of experiment set up.

Materials and Method

Experiment set up

Water samples where collected at the same location, off the coast of Mangalore, Karnataka, India, but separated in time by one year. From these water samples single chains of *Skeletonema* sp. were isolated and cultured in f/2+Si media (Guillard 1975) in 12/12 light/dark at 27°C.

The experiment was set up with two clones of *Skeletonema* sp. isolated from plankton samples collected one year apart, Man 3B (P1) and Bunder G (P2). A small volume from each of these clonal isolates where mixed. Within a time-period of 1 to 3 weeks single chains of cells where isolated from the parental-mix, denoted F1A-F1F (Figure 1).

For comparison a clonal isolate (Pond 6A) sampled from Pondicherry, Tamil Nadu, on the east coast of India was also cultured under the same conditions.

Extraction of DNA

Prior to extraction, all cultures where scanned in microscope to confirm that they where not contaminated. The cultures were then transferred to 50 ml centrifuge tubes and centrifuged at 5000 rpm for 7 minutes. The supernatant was discarded and cell pellets were transferred to labeled Eppendorf tubes and processed immediately.

DNA was extracted using the CTAB protocol (Rynearson and Armbrust 2000) and Qiagen DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) following the instructions of the manufacturer. Concentration of the extracted DNA was measured using an UV-Visible spectrophotometer (Shimadzu Corporation, Japan).

AFLP

AFLP analysis was performed following a modification of a protocol described by Vos et al. (1995). Approximately 2000 ng of extracted DNA from each sample was digested with 5 units of EcoRI and MseI restriction enzymes (Van der Lee et al., 1997; John et al., 2004) (Table 1) in a total reaction volume of 50 μl for 3 hours in 37°C. Every hour the tubes were tapped, and after 3 hours the enzyme was inactivated at 65°C for 10 minutes.

Adaptors were prepared by mixing EcoRI linker 1 and EcoRI linker 2, MseI linker 1 and MseI linker 2 (Table 1) in separate tubes, kept at 65°C for 10 min, followed by 37°C for 10 min and 25°C

Restriction enzyme	Recognition Sequence
EcoRI	5'-G AATTC-3' 3'-CTTAA G-5'
MseI	5'-T TAA-3' 3'-AAT T-5'
Adaptors	**Sequence**
EcoRI linker 1 linker 2	5'-CTC GTA GAC TGC GTA CC-3' 3'-CAT CTG ACG CAT GGT TAA-5'
MseI linker 1 linker 2	5'-GAC GAT GAG TCC TGA G-3' 3'-TA CTC AGG ACT CAT-5'
Primers	**-AS- -RS- -SB-**
EcoRI+A	5'-GACTGCGTACC AATTC A-3'
EcoRI+AAG	5'-GACTGCGTACC AATTC AAG-3'
EcoRI+ACC	5'-GACTGCGTACC AATTC ACC-3'
MseI+C	5'-GATGAGTCCTGAG TAA C-3'
MseI+CTA	5'-GATGAGTCCTGAG TAA CTA-3'
MseI+CTT	5'-GATGAGTCCTGAG TAA CTT-3'

Table 1. Oligonucleotide sequences used in AFLP analysis. Restriction enzyme, adapter and primers designed according to Vos *et al.* 1995. AS: adapter sequence,

for 10 min. Ligation to the ends of the DNA fragments was made by adding 10 μl ligation-mix consisting of T4-ligase (1U, Bangalore Genei), 2.5 pmol of EcoRI adaptor, 25 pmol of MseI adaptor and 1x T4 DNA ligase buffer to the restriction digested DNA and incubate at room temperature for 3 hours (tapped every hour). The enzymes was inactivated at 65°C for 10 min.

Pre-amplification reactions were set up in a total volume of 20 μ1,l containing 1xPCR buffer, 4 nmol dNTP, 25 ng of primer EcoRI+A and MseI+C (Table 1), 1.2 U Taq polymerase (Bangalore Genei) and 1-2 μl of restricted ligated DNA (2 μg). The pre-amplification reactions were performed using a PTC-100, (Peltier-Effect Cycling, MJ Research inc., Waltham, MA, USA) and carried out using 92°C for 2 min, 26 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 1 min), 72°C for 5 min and hold at 4°C. Duplicates of the pre-amplification samples and negative controls were run out on a 2% agarose gel to check quality and contamination.

The PCR products from the pre-amplifications were diluted with 100 μl sterile double-distilled H_2O and used as template for a consecutive PCR reaction. Selective amplifications were set up using 1xPCR buffer, 4 nmol dNTP, 1.2 U Taq polymerase, 25 ng of EcoRI+AAG and EcoRI+ACC, 30 ng of MseI+CTA and MseI+CTT (Table 1) and 10 μl of template DNA from the diluted pre-amplification PCR product. Amplification by touchdown PCR with initial denaturation at 94°C for 2 min and a first cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. In the following 12 cycles, the annealing temperature was reduced by 0.7°C each cycle. Touchdown was then followed by 23 cycles (94°C for 30 s, 56°C for 30 s, 72°C for 1 min), 72°C for 5 min and hold at 4°C.Selective amplification products were loaded on a 12% Novex® TBE Gel (Invitrogen) using the XCell SureLock™ Mini-Cell and the fragments were separated at 200V for approximately 40 minutes. For visualization of the bands the gel was stained using SilverExpress® Silver Staining Kit (Invitrogen), following the protocol supplied by the manufacturer. The results were documented using a digital camera and stored as TIFF-files for further processing.

Analyses

A consensus for each clone was manually created by measuring fragment sizes from different photographs. This was done separately by two persons and then put together to a consensus. It was assumed that bands of the same size where homologous.

Data analyses were performed using the software GelcomparII, Applied-Maths BVBA, Belgium. The percentage of similarity was calculated using the Dice coefficient (Van der Lee et al., 1997; Figueroa et al., 2005). For cluster analysis the neighbor joining algorithm (Hagen et al., 2002; Furini et al., 2003) was used to create a dendrogram.

To assess the reproducibility of the method the clonal isolates F1A and F1D where extracted on two occasions 10 days apart. The second extraction was denoted F1A2 and F1D2 respectively and underwent the same protocol as the other DNA extracts.

Results

Reproducibility

The samples denoted F1A, F1A2 and F1D, F1D2 originated from the same cultured strain but were extracted on two occasions 10 days apart to asses the reproducibility of the AFLP protocol. Figure 2 reveals the banding patterns of the replicates of strain F1A and F1D. A dendrogram created using neighbor joining algorithm (Figure 3) shows clustering of the replicates. A matrix (Figure 3) was obtained from analysis using the Dice coefficient that calculated the similarity within the replicates of F1A and F1A2 and within F1D and F1D2 to 93.3% in both cases. The similarities between the replicates F1A, F1A2 and F1D, F1D2 were less than the similarity within F1A and F1A2 and within F1D and F1D2 (Figure 3). The calculated similarity between F1A, F1A2, F1D, F1D2 and the parent Bunder G ranged from 66.8% to 75%.

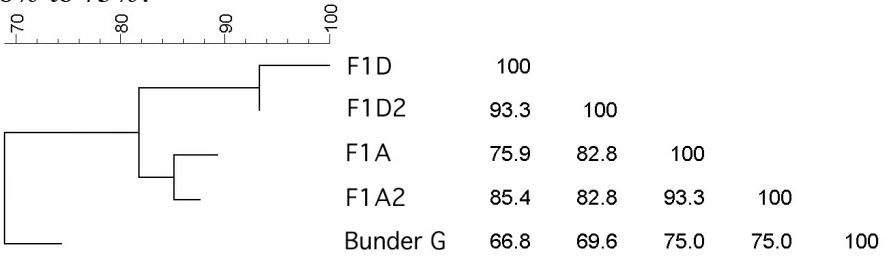

Figure 3. Dendrogram using Neighbor Joining algorithm of two clonal replicates extracted on two different occasions 10 days apart. To the right is a similarity matrix calculated using the Dice coefficient.

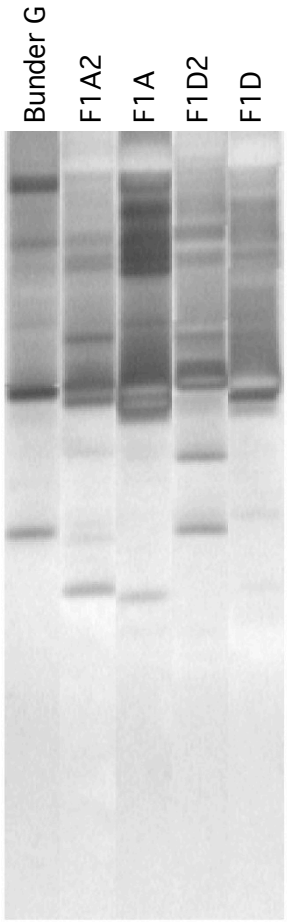

Figure 2. AFLP banding pattern for replicates of F1A and F1D and parental strain Bunder G.

Intercrossing experiment

The resulting banding pattern from the intercrossing experiment is seen in Figure 4. Figure 6 shows a separation of the parents Man 3B and Bunder G with a similarity value of 59.3%. With 73.3% similarity F1A clustered together with Man 3B (Figure 5, Figure 6). The calculated similarity between F1D and F1H was 96.3%, between F1D and F1F it was 88.9% and 92.3% between F1H and F1F (Figure 6). Similarity values calculated between F1D, F1H, F1F and the parental strain Man 3B varied from 64.5% to 66.7% and the similarity to Bunder G from 50 to 60.9%. F1B was more similar to the out-group Pond 6A (66.7%) than to the two parental strains Man 3B (33.3%) and Bunder G (47.1%). The similarities between F1B and the other F1-generation (F1A, F1D, F1F and F1H) ranged from 40% to 50% (Figure 6). Similarity values between Pond 6A and Man 3B, Bunder G, F1A, F1D, F1F and F1H ranged from 31.6% to 44.4%.

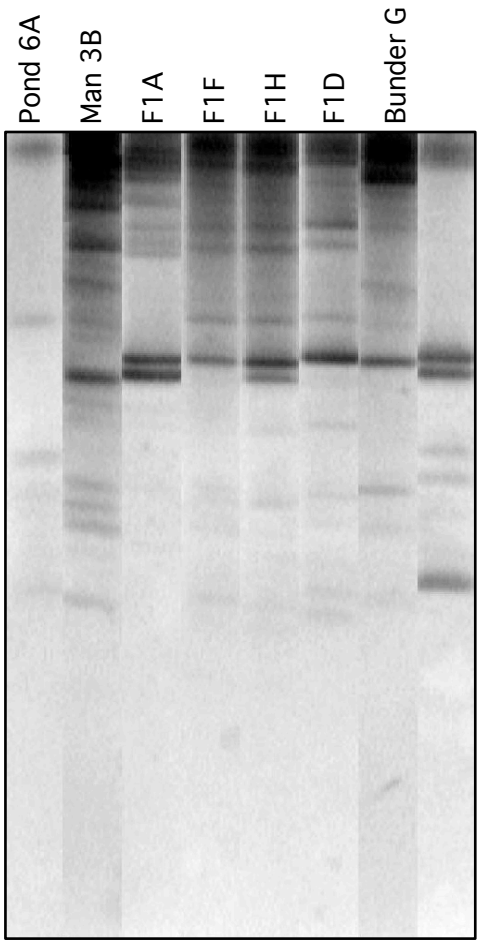

Figure 4. AFLP banding pattern produced by primer combination EcoRI+AAG, EcoRI+ACC, MseI+CTA and MseI+CTT.
Parental strains Man 3B and Bunder G along with F1 generation and out-group Pond 6A.

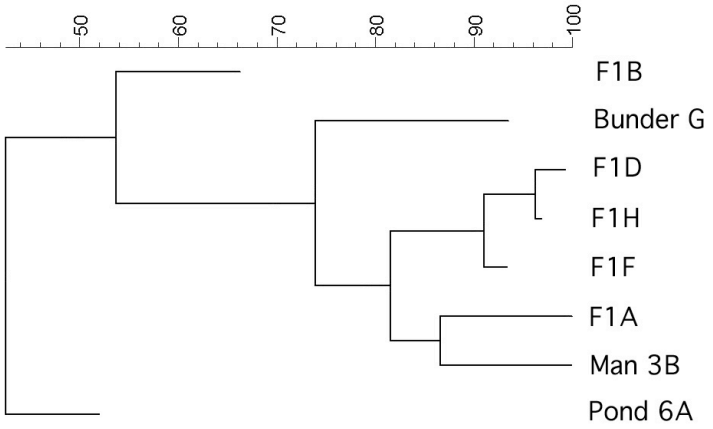

Figure 5. Dendrogram using Neighbor Joining algorithm of parental strains Man 3B and Bunder G and F1 generation F1A, F1B, F1D, F1F, F1H. and the out-group Pond 6A.

F1B	100							
Bunder G	47.1	100						
F1D	47.6	50.0	100					
F1H	50.0	52.2	96.3	100				
F1F	50.0	60.9	88.9	92.3	100			
F1A	40.0	52.2	66.7	69.2	69.2	100		
Man 3B	33.3	59.3	64.5	66.7	66.7	73.3	100	
Pond 6A	66.7	40.0	31.6	33.3	44.4	33.3	36.4	100

Figure 6. Similarity matrix calculated using the Dice coefficient for parental strains Man 3b and Bunder G and F1 generation F1A, F1B, F1D, F1F, F1H. and tha out-group Pond 6A.

Discussion

The reproducibility of the method seemed to be good, since the similarity within a single clone extracted more than one week apart is greater than that between different clones (Figure 3). The calculated similarity for F1A compared with F1A2 and F1D with F1D2 was 93.3% in both cases. That gives an error margin of 6.7% for within clonal strains.

The parental strains of Man 3B and Bunder G were separated from each other with a similarity value of 59.3%, in other words 40.7% difference. When comparing the similarity value of Man 3B and Bunder G, to the value calculated for replicates of the same strains, it is quite safe to make the assumption that Man 3B and Bunder G do not originate from the same strain. Also, since they are sampled one year apart the likelihood of such close relation is even smaller. F1D, F1H and F1F make up a cluster located in between the parental stains of Man 3B and Bunder G (Figure 5). Similarity values obtained when comparing F1D, F1H and F1F to Man 3B ranged from 64.5% to 66.7%. Comparisons of F1D, F1H and F1F to the parental strain of Bunder G varied from 50% to 60.9%. These similarities indicate that F1D, F1H and F1F were heterothallic recombination of the two parental strains.

The similarity between F1D and F1H was 96.3%, hence an error margin of 5.7%. A 5.7% difference between F1D and F1H is less than the difference calculated for the replicates of clones and indicate that F1D and F1H are identical clones, vegetative cells from a single strain. The similarity values calculated for F1F compared with F1D and F1H was high, 88.9% and 92.3%. An error margin of 7.7% to 11.1 % is to high to directly state that F1F also is a vegetative propagation along with F1D and F1H, but at the same time so low that the possibility of sexual variation between them is very small. One explanation to the similarity between F1F and F1D/F1H is vegetative propagation of F1D/F1H and a consecutive new sexual recombination that led to F1F. Another explanation could be bacteria. Since the cultures of *Skeletonema* sp. were not axenic, there is always the possibility that higher concentrations of bacteria in a culture can affect the banding pattern to some minor extent.

F1A was 73.3% similar to the parental strain of Man 3B (Figure 6). The error margin between F1A and Man 3B was 26.7%, which is 20% higher than what would be expected from identical clones, hence it can be assumed that F1A was not a vegetative propagation of Man3B. There is a possibility that F1A is a homothallic recombination of Man 3B. A homothallic progeny does not necessarily have to be identical to the parent. Gametes from the same clone fuse together and genetic recombination can occur, but in such cases the deviation is much smaller than in the case of heterothallic recombination, i.e. fusion of gametes from different clones. It seems that a similarity value of 93% or higher are strong indications of identical clones, while similarities ranging from 73% to 90% indicates homothallic recombination. Heterothallic reproduction seems to give similarity value of 60% to 70% and non-related clones of *Skeletonema* are less than 60% similar. To confirm any interbreeding between clones of *Skeletonema* sp. it is necessary to make backcross experiments and observe whether genetic distance will decrease.

The genetic pattern of F1B had the highest similarity to the strain Pond 6A. In this study Pond 6A was not part of the intercrossing experiment and was only used as a reference. Speculations about genetic resemblance between F1B and Pond 6A are futile as well as the divergence between F1B and the other strains involved in the experiment. A possible explanation to the outcome of F1B

is the method. According to John et al. (2004), the quality and integrity of the extracted DNA is of great importance when using AFLP for analysis since the method is based on restriction digestion and ligation of genomic DNA, and this can affect the resulting fingerprinting to a great extent. For a few clones the resulting banding pattern was just a faint smear with two or three bands and this pattern was seen again after a second DNA extraction. Marine diatoms are known to produce many secondary metabolites such as polysaccharides that can have a negative effect on restriction digestion and ligation. Isolation and cultivation of microalgae is a selection as such. Only clones well adjusted to culture conditions will survive, most probably this is displayed as a continuum in which clones well suited for culturing will yield satisfying DNA extracts, and other clones dying immediately after isolation. However, previous results suggest that compared to some other microalgal species (e.g. Evans et al., 2005), it is relatively easy to obtain a large numbers of clones and subsequently good quality genetic material from *Skeletonema* sp. (Godhe et al,. 2006).

One of the drawbacks using AFLP is that this technique does not distinguish between heterozygotes and dominant homozygotes. Another source of error is the assumption that bands of equal size are homologous, which might not always be correct. Also, Figueroa et al., (2005) found that there is a genetic variation within an asexual lineage within both homothallic and heterothallic clonal isolates of *G. catenatum*. Such variation could not be detected in this analysis due to the short time span.

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Genetic variability of *Skeletonema costatum* (Bacillariophyceae) based on ribosomal DNA ITS sequences

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Honors thesis in Marine Botany, 20 p
Göteborg University, Feb 2004

Supervisor: Melissa McQuoid and Anna Godhe

Abstract

Coastal sediments contain a rich assortment of viable diatoms and these benthic cells may be an important source of planktonic blooms. *Skeletonema costatum* is a cosmopolitan diatom species that is commonly found in coastal sediments. Sediment samples from Canada, Portugal and Sweden were cultivated and chains of *S. costatum* were isolated and cultured as clones. DNA was extracted and the ribosomal DNA ITS regions were amplified by Polymerase Chain Reaction (PCR). Sequences of the ITS 1, 5.8S, and ITS 2 were used to investigate the genetic diversity between and within different populations of *S. costatum*. When comparing the ITS sequences to that of *S. pseudocostatum* we found that a Swedish clone, KF M2, was an almost identical match and this species provided a useful outgroup for the analyses. Canadian clones were found to be genetically distinct whereas the two populations from Atlantic waters were somewhat mixed within the trees. This method was evaluated for future use in tracking the origin of phytoplankton blooms. The method of using sequences of the ITS regions to determine intraspecific genetic diversity renders a good result on a large oceanic scale. For use on a smaller scale, some refinements and improvements should be evaluated further.

1. Introduction

Harmful microalgal blooms are becoming an increasing problem around the world. These blooms can cause damage to both economies, and health of humans and ecosystems. What can we do to minimize these blooms and their affect on the ecosystem? To answer this question we first need to know about the diversity within a microalgal population and how these blooms are initiated.

The purpose of this study was to examine the genetic diversity within and between different populations of a diatom species and to test a possible method for identifying the origin of microalgal blooms.

Some species of microalgae are known to produce a resting spore or cyst. These are a very important contribution to the dynamics of microalgal blooms, since spore beds in the sediment are a potential seed bank for future blooms. It has been demonstrated that resting spores of different species can survive for many years in the sediment and then rapidly start to grow again when the conditions are favorable (McQuoid et al. 2002). If resting stages in the sediments are the source of a bloom, then the genetic signature of the benthic cells should match that of the planktonic cells. By comparing the genetic signature of a bloom to various populations of benthic resting stages, we should be able to determine the source of a local bloom.

I have chosen to work with *Skeletonema costatum* as a model organism because it can form resting cells, is easy to grow in culture, is cosmopolitan, grows to large quantities, and is commonly found in blooms along the west coast of Sweden. *Skeletonema costatum* forms resting cells. These cells are morphologically very similar to vegetative cells but have a highly reduced metabolic activity.

I chose to look at the Internal Transcribable Spacer (ITS) sequences (Figure 1), which are non-coding regions of the Ribosomal DNA. This region has been successfully used in genetic studies of other algal taxa (Coleman and Mai 1997, Kooistra et al. 2001, Lundholm et al. 2003). The spacers are grouped together with the18S small subunit (SSU), the 5.8S unit, and the 28S large subunit (LSU), in a cistron that is repeated hundreds of times in each genome (Kooistra et al. 2001).

The ITS regions are the most rapidly evolving parts of the rDNA because of their relative lack of functional constraints (Hillis and Davis 1988). Because of the repeating nature of the rDNA, it is a good base for PCR amplification because there are hundreds of exact copies in each genome. Mutations in these parts do occur frequently, but new variants are rapidly homogenized, a process referred to as concerted evolution (Hillis and Davis 1988, Kooistra et al. 2001). In rare cases, mistakes are made and the new variant or mutation is substituted throughout the chain of cistrons.

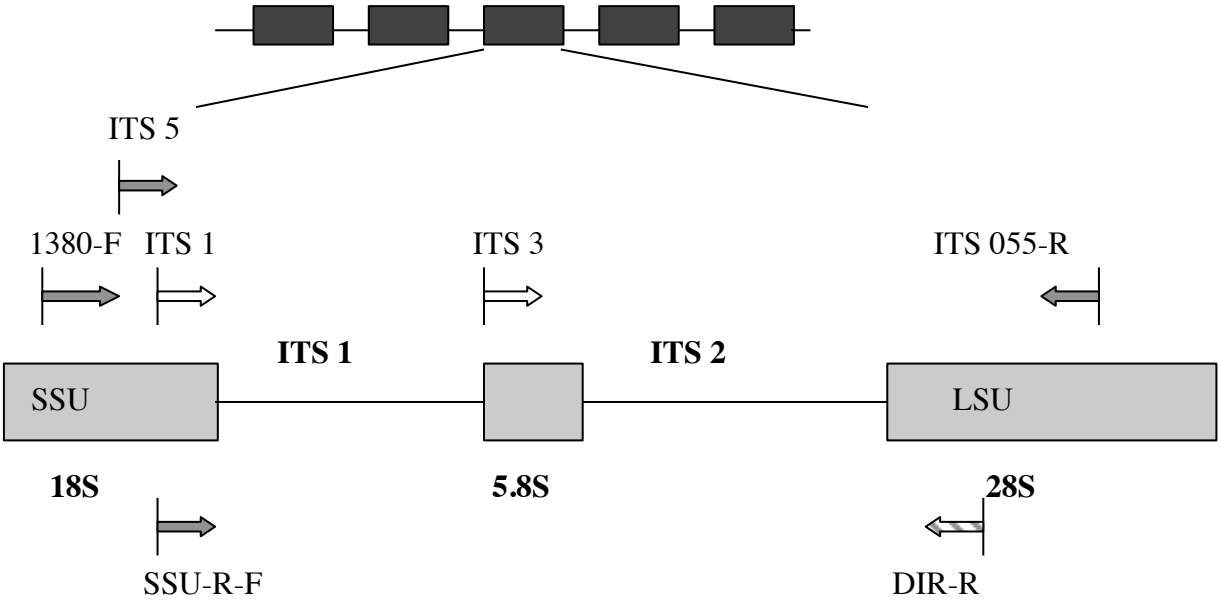


Figure 1. Schematic presentation of the ribosomal DNA repeat structure. SSU, ITS 1, 5.8S, ITS 2 and LSU. ⇒ primers used in PCR, ⇨ primers used in sequencing, ⇒ primer used in both PCR and sequencing.

Earlier, most intra- and interspecific population genetic studies on diatoms and other microalgae have used allozymes, electrophoretic bands, or sequencing of the SSU or LSU (Gallagher 1980, Gallager 1982, Medlin et al. 1991, Rehnstam-Holm et al. 2002, Lundholm et al. 2003). I chose to sequence the ITS 1, 5.8S and ITS 2 regions, because there does not seem to be enough genetic diversity within the SSU and LSU to find differences within a single species (Van der Auwera and De Wachter 1998, A. Godhe, personal communication). SSU and LSU sequencing has primarily been used for phylogenetic studies of one or more genera.

In this study, surface sediment from the west coasts of Portugal, Canada, and Sweden were cultivated and viable resting cells were isolated and cultured. These clones were used to examine the genetic diversity within and between different populations of *S. costatum*.

2. Materials and methods

2.1 Sediment collection and isolation of clones

2.1.1 Sediment cultures

Two replicate sediment cores (C2 and C3) were taken from Koljö Fjord (58°15´N, 11°35´E) on the Swedish west coast, using a Geminicorer (80 mm diameter) in November 2000 (McQuoid et al. 2002). The sediment cores were x-rayed and visually aligned to a previous core that was dated, using ^{210}Pb (McQuoid et al. 2002). Cores were sampled at 10 intervals: 0-2, 4-6, 8-10, 12-14,16-18, 20-22, 27-29, 34-36, 41-43 and 48-50 cm.

In 2002, surface (top 1-2 cm) sediment samples were taken from the west coast of Portugal (38°39´N, 9°24´W) by A. Amorin, close to Lisboa, and from the Strait of Georgia on the Canadian west coast (49°9´N, 123°23´W) by the Geological Survey of Canada. The samples were kept in darkness at 4°C until use.

Approximately 0.5 to 1 g of wet sediment was added to 45 ml of f/2 medium (Guillard and Ryther 1962) in 50 ml Nunc flasks. When settled, this amount was enough to cover the bottom of the flask with a 1 to 2 mm layer of sediment. The flasks were incubated at a temperature of 10°C with a 12:12 h light:dark cycle and an irradiance of 8×10^{14} to 4.5×10^{15} quanta $cm^{-2}s^{-1}$ from daylight fluorescent lamps (Osram L 36W Lumilux Plus, Osram, Germany). After about one week, *S. costatum* started to grow in the sediment cultures from 2 cm. Other diatoms and dinoflagellates were also observed. In the cultures from deeper sediment samples, only dinoflagellates were found and no diatom growth was observed.

2.1.2 Isolation and culturing of clones

To isolate *S. costatum* clones from the sediment cultures, a small aliquot of the incubated sediment was withdrawn from the flask using a Pasteur pipette. This included the top layer of sediment where *S. costatum* was most concentrated. Isolation of *S. costatum* clones was done under an inverted microscope using a fine micropipette. Micropipettes were made from glass capillary tubes that were heated and then drawn out to a very thin tip. With the micropipette, I picked a single chain of *S. costatum* and transferred it to a new, clean drop of f/2 medium. From the new drop I once again picked this single chain (clone) and move it to a new clean drop of medium. This procedure was repeated several times to clean the clone. The clone was then transferred to a small petri dish with f/2 medium and left to grow under the previously mentioned conditions.

After a few days, the clones had grown enough (minimum 20 to 30 chains) to be transferred to Nunc flasks with approximately 5 ml of f/2 medium. The content of the petri dish was transferred in equal volume to 2 or 3 50-ml Nunc flasks. In the beginning, the clones were kept in a smaller volume of medium. Once the culture became dense, I added an additional 40 ml of medium. The clones KF C33 and KF C23 were directly transferred from the petri dishes into separate Nunc flasks. Thereafter when growth was confirmed these clones were divided in two or more replicates.

2.2 DNA extractions

DNA extractions were made using two different methods, hexadecyltrimethylammonium bromide (CTAB) plus a commercial DNA purification kit from QIAGEN and a phenol-chloroform-isoamylalcohol mixture (Godhe et al. 2001) plus, when required, a commercial DNA purification kit from Amersham Pharmacia.

2.2.1 CTAB method

For DNA extractions, dense cultures were selected and scanned under the microscope to confirm that they were not contaminated. The culture was then transferred to a 50ml centrifuge tube and centrifuged for 5 min at 5000 rpm. The supernatant was discarded and the pellets transferred to labeled Eppendorf tubes. Pellets were processed immediately or frozen at –80 °C.

To each pellet I added 300 µl of lysis buffer (10 mM Tris, 1mM EDTA, 0.5% SDS, 100 µg ml^{-1} Proteinase K, pH 7) and incubated the sample at 60 °C for one hour. Thereafter, I added 50 µl of 5M NaCl, 40 µl of 10% CTAB (diluted in 0.7% NaCl) and 4 µl of RNase, and incubated the sample in 65 °C for 10 min. The extraction of DNA from the lysed cells and DNA purification was done with a DNeasy Plant Mini Kit (QIAGEN Gmbh, Germany) starting at step 5 in the manufacturer's instructions.

2.2.2 Phenol-chloroform-isoamylalcohol method

Cultures were selected and pelleted as above. In an Eppendorf tube, I resuspended the pellet in 420 µl of sterile milli-Q ultra-pure H_2O and thereafter added: 20 µl of 250 mM EDTA (pH 8.0), 50 µl of 10% SDS, 5 µl of 1M Tris-HCl (pH 7.5), and 5 µl of 1M NaCl. This mixture was incubated for 1 hour at 50 °C (Godhe et al. 2001).

DNA was extracted using buffered phenol:chloroform:isoamylalcohol (24:24:1). From our stocks this equaled 240 µl of phenol plus 250 µl of chloroform-isoamylalcohol mixture. Samples were vortexed and centrifuged at 13000 rpm for 3 minutes. The top layer, which contained DNA suspended in water, was moved to a new Eppendorf tube. The bottom layer, which was discarded, contained the phenol and chloroform solution, proteins, and cell debris, and was separated from the top layer by at thin layer of lipids and proteins. This procedure was repeated a second time with the same mixture and a third time with 490 µl of chloroform-isoamylalcohol solution and no phenol. This last step was done in order to minimize phenol contamination which will inhibit the PCR. For samples with a "milky" appearance the procedure was repeated until the top layer was transparent.

Thereafter, DNA was precipitated with 1/10 volume (45 µl) of Na-acetate (3M, pH 4.5) and 2 volumes (900 µl) of 96% EtOH at –20°C over night. Samples were centrifuged at maximum speed (13000 rpm) for 30 minutes to pellet the precipitated DNA. The supernatant was discarded and the pellet was washed with 250 µl of 70% EtOH. Samples were centrifuged again at maximum speed for 10 minutes, the supernatant discarded and the pellet dried for 5 minutes at 49°C. It is important that all the EtOH evaporates from the pellet otherwise it will inhibit the PCR. The washed DNA was diluted with AE-buffer (from the QIAGEN kit). Either 50 or 75 µl of buffer were added depending on the size of the pellet with larger pellets getting the larger volume. Extracted DNA was frozen (-20°C) until further analysis. Quantity and quality of the DNA was examined by agarose gel electrophoresis against known standards and by spectrophotometric measurement of the concentration and purity from proteins and RNA using a Pharmacia Biotech GeneQuant II.

In cases when the phenol-chlorophorm-isoamylalcohol method did not yield enough purity, the extract was further purified using Flexi Prep Kit (Amershan-Pharmacia) following the instructions of the producers.

2.3 PCR and Sequencing

2.3.1 PCR

PCR was used to amplify the ITS region (ITS-1, 5.8S and ITS-2) using the QIAGEN Taq DNA Polymerase (QIAGEN GmbH, Germany). The reaction was run in a total volume of 50 µl and contained: 1 µl of DNA extract, 5 µl of 10 x buffer 15 mM $MgCl_2$, 0.5 μg of forward primer and 0.5 μg of reverse primer (Table 1), 200 μM of each deoxynucleotide triphosphate (dNTP), 2.5 U of Taq DNA polymerase and 40.75 µl of milli-Q ultra-pure H_2O. Amplifications were run in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400, version 2.11, Norwalk, CT, USA) with an initial 5-minute heating step at 94 °C followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and an extension step at 72°C for 1 min. After the cycles, a final extension step was completed at 72°C for 5 min.

PCR products were examined by agarose gel electrophoresis against known standards. Products were purified using the QIAquick PCR Purification Kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. Final concentration for the PCR product was measured using a spectrophotometer (Pharmacia Biotech GeneQuant II).

Table 1. Primers used for PCR amplification and sequencing of the rDNA, ITS region.

Primer	Primer sequence	
1380-F.mod	5' GCARTGATTACGTCCCTGCC (forward)	PCR
ITS 5	5' GGAAGTAAAAGTCGTAACAAGG (forward)	PCR
SSU-R-F	5' GTAGGTGAACCTGCAGAAGGATCA (forward)	PCR
ITS 055-R	5' CTCCTTGGTCCGTGTTTCAAGACGGG (reverse)	PCR
DIR-R	5' TATGCTTAAATTCAGCGGGT (reverse)	PCR,Sequencing
ITS 1	5' TCCGTAGGTGAACCTGCGG (forward)	Sequencing
ITS 3	5' GCATCGATGAAGAACGCAGC (forward)	Sequencing

2.3.2 Sequencing

Approximately 50 ng of DNA template from the PCR product was used in each 20-μl sequencing reaction using the primers listed in Table 1. Nucleotide sequences were determined using the CEQ Dye Terminator Cycle Sequencing Kit following the manufacturer's instructions. Sequencing was done using a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA).

2.4 Alignment and statistical analyses

2.4.1 Alignment

A blast search (www.ncbi.nlm.nih.gov/BLAST) was run on all obtained sequences to ensure that the ITS sequences were related to those of other *Skeletonema* species. No ITS sequences for *S. costatum* have previously been reported. A minimum of five sequences were obtained for each clone and these were aligned using Sequencher 4.1.2 (Gene Codes Corporation, Michigan, USA). A consensus sequence for each clone was obtained from these five or more sequences, and two data sets, Data 1 and Data 2, were created for genetic diversity analyses of the ITS region. Data 1 was made by aligning 20 clones with an ITS sequence of *S. pseudocostatum* (Van der Auwera and De Wachter 1998, GenBank acc. no.Y11511). The *S. pseudocostatum* sequence was only 344 base pairs (bp), and all sequences in Data 1 were cut to this length. Data 2 contained the same 20 sequences used in Data 1 but *S. pseudocostatum* was not included and the sequences were cut to the length of the shortest fragment, 631 bp.

2.4.2 Phylogenetic analyses

Statistical analyses and creation of dendrograms were done using the program, Phylogeny Analysis Using Parsimony (PAUP) 4.0 beta-version (Swofford 2001). Separate analyses for Data 1 and Data 2 were performed following the method of Lundholm et al. (2003).

Data 1 and 2 were analyzed using distance, maximum likelihood (ML) and parsimony techniques. Distance and ML analyses were made by heuristic searches with 100 random addition replicates, the tree-bisection reconnection (TBR) branch-swapping algorithm, and the HKY substitution model (Hasegawa et al. 1985). Parsimony analyses were done using heuristic searches with random addition of sequences (1000 replicates) and the TBR algorithm. Characters were treated as multistate and unordered. In the case of multiple trees, a consensus tree was computed using a strict, 50% majority rule. Bootstrap analyses determined the robustness of the acquired tree topologies (Felsenstein 1985). A full heuristic search with 100 replicates was run for ML and parsimony. Due to lack of computer capacity, bootstrap analyses were not performed for the distance analyses.

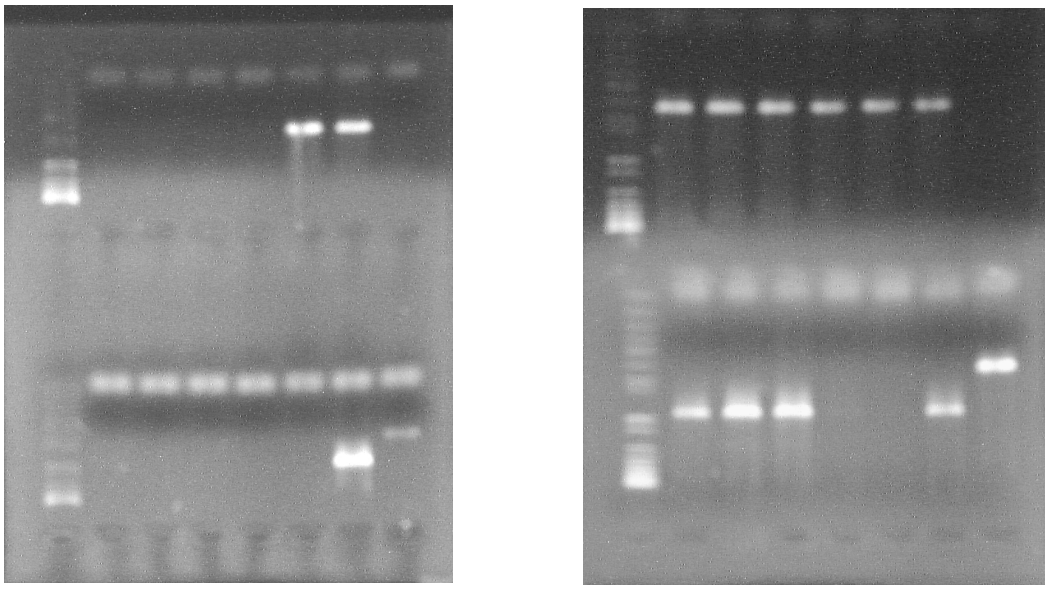
Ideally, the outgroup in this type of phylogenetic analysis should be a species that is not closely related to the taxa in the analysis. Attempts were made to find a different diatom species that would align with the samples, but there was no match. The only published ITS sequence that would align with my data was *S. pseudocostatum*. In the

analyses of Data 1, the *S. pseudocostatum* sequence was the defined outgroup, but the short length of the sequence limited the amount that the clones could be compared. For Data 2, the sequence from my own clone KF M2 was used as the defined outgroup. KF M2 was chosen because no other appropriate sequence was found. When looking at the results from the first analyses, I noticed that the clone KF M2 was different from all the other clones. After further examination, it was clear that the 344 bp ITS sequences of KF M2 and *S. pseudocostatum* were identical with the exception of one base pair. Because the sequence of KF M2 was much longer than that of *S. pseudocostatum*, more information could be used in the analyses when KF M2 was the defined outgroup.

3. Results

3.1 Methods of extraction

Results suggest that the phenol-chloroform-isoamylalcohol method was the most effective for extracting DNA from *S. costatum*. Although this was not statistically tested, electrophoresis gels show bands of PCR products from each type of extraction (Figure 2). In both PCR reactions the same primers were used. The phenol-chloroform-isoamylalcohol method consistently resulted in more bands.



A B

Figure 2. Electrophoresis gels showing results after PCR amplification using different DNA extraction methods. A) CTAB extraction and B) Phenol-chloroform-isoamylalcohol extraction.

3.2 Genetic diversity

Analyses of the two data sets showed similar results. The tree topologies of the ML and parsimony analyses of the ITS data were similar, so only the parsimony trees are shown (Figures 3 and 4). Results support the existence of at least two groups.

In the results from Data 1, clone KF M2 differed from all the other clones but was almost identical to *S. pseudocostatum*. The overall pattern seemed to be that the clones isolated from the Canadian sediment samples (CAN A1, CAN B2, CAN D2, CAN J1 and CAN K2) clustered separately from the rest of the clones. In addition, the CAN B2 clone seemed to be somewhat different from all the other Canadian clones. Clones from Portugal and Koljö Fjord were not well separated from each other. For these clones, the only visible pattern was that clone KF Y2 seemed to differ from the other clones. The tree suggested that genetic variation in the ITS region within clones from Koljö Fjord and Portugal was larger than the variation between isolates from these sampling sites.

Trees from the analyses of Data 2 also showed that Canadian clones were clearly distinct from the "Atlantic" clones. The distinction between CAN B2 and the other Canadian clones was even stronger in this analysis. Clones from Portugal and Koljö Fjord were still not completely separated, but the resolution was better than in the previous analysis. Similar to the previous analysis, KF Y2 differed from all the other clones from Portugal and Koljö Fjord. In this analysis, clones from Portugal tended to cluster somewhat apart from the Koljö Fjord clones with the exception of PORT D2 and some intervening clones, such as KF C27 and KF C33. The clones PORT D1 and PORT D2 were originally the same isolate but did not group together in the analysis as would be expected. Although the overall results were the same from both data sets, the differences between the sampling locations were more clear when the longer sequences (Data 2) were used.

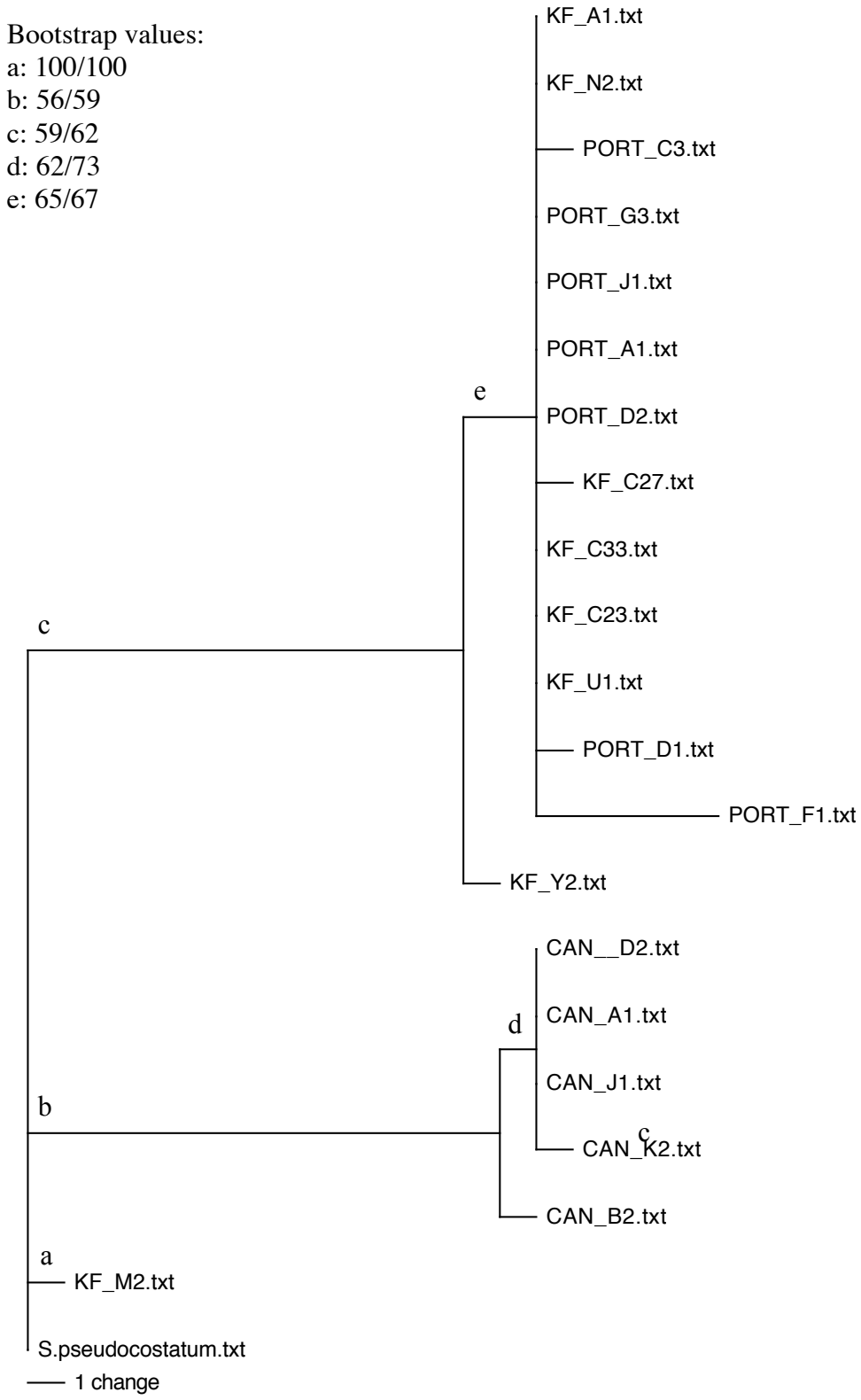


Figure 3. Phylogenetic analysis of Data 1 showing bootstrap values Likelihood/Parsimony. Bootstrap values below 5 % are not shown

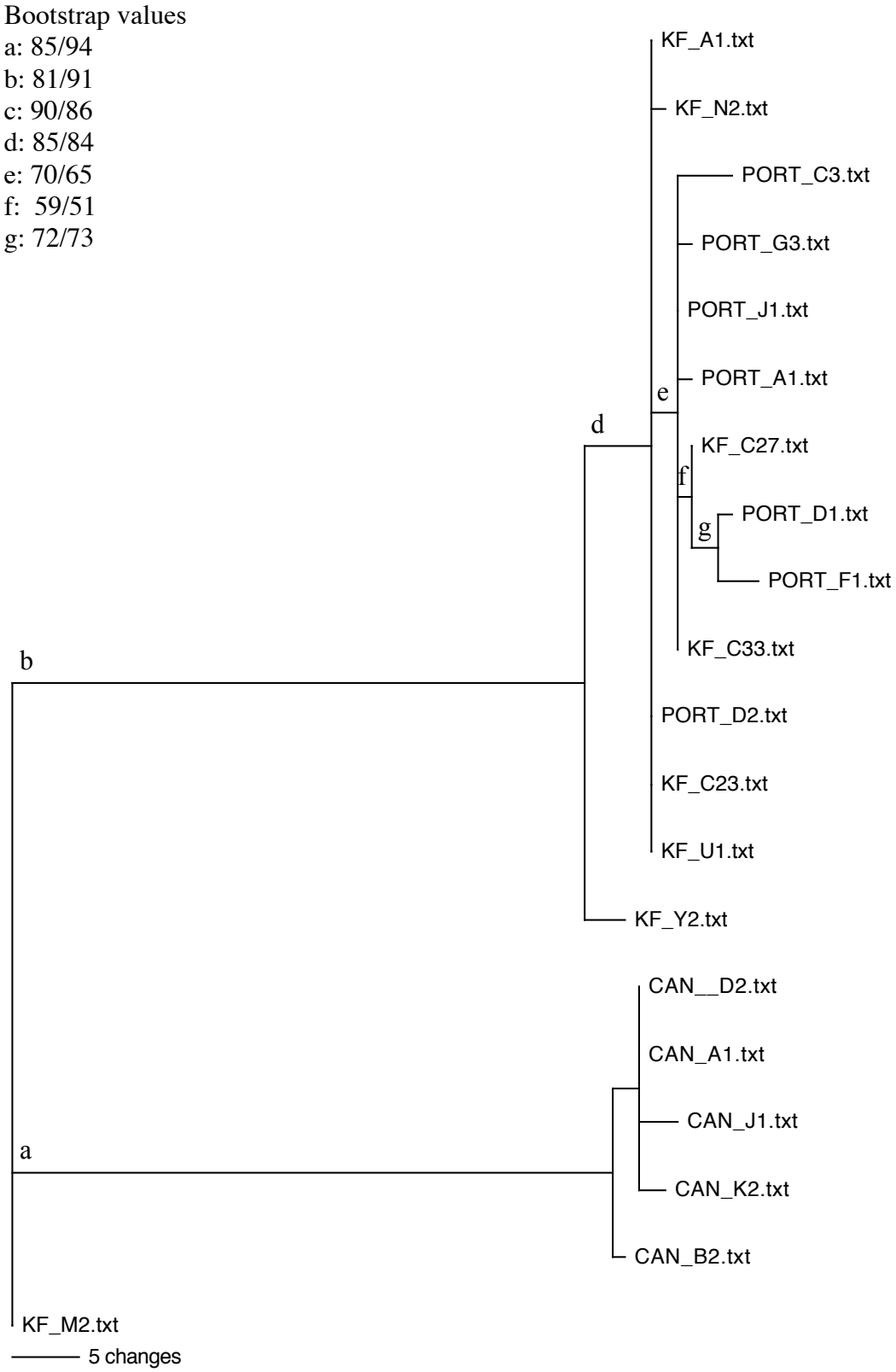


Figure 4. Phylogenetic analysis of Data 2 showing bootstrap values Likelihood/Parsimony. Bootstrap values below 5 % are not shown

4. Discussion

4.1 Methods of DNA extraction

The extraction method using phenol-chloroform-isoamylalcohol seemed to give a better result than CTAB. Measurements done after extraction showed that the concentration of DNA was higher in the samples extracted with phenol-chloroform-isoamylalcohol. It is not clear why this method was better, but it may be that CTAB is not able to brake open the silica frustules of *S. costatum*. Phenol-chloroform-isoamylalcohol is a stronger and more hazardous mixture, which may make it more effective at opening the cells. One negative aspect of the phenol-chloroform-isoamylalcohol method is that the extracted DNA was not very clean. Despite this lack of purity, however, the extractions were still able templates for PCR amplification. When using CTAB for extraction, the DNA was cleaner, but the concentrations were low, sometimes less than 10 μg ml^{-1}. Even after extracting with CTAB for 17 hours, the concentrations were not much higher.

In the beginning of this work, it was difficult to find suitable primers that gave good PCR products. After some trial and error, I found that 1380-F together with ITS055R, and ITS5 together with DIR-R worked well for most samples. But the primer 1380-F did not work that well for clones originating from Portugal. Only 4 out of 16 PCR runs yielded a satisfying PCR product. This may indicate that there is something different in the SSU sequence of the clones from Portugal, and this may be worth further exploration.

4.2 Genetic diversity

Finding an appropriate outgroup turned out to be somewhat difficult. It would have been optimal to have an outgroup that was not a *Skeletonema* species but a close relative. Searching GenBank for ITS sequences of other diatoms provided some candidates (e.g. *Stephanodiscus* sp., *Cyclotella* sp., *Pseudo-nitzschia* sp., *Chaetoceros* sp.), but none would align with the *S. costatum* sequences, except *S. pseudocostatum*.

One interesting discovery during this project was that the KF M2 clone was significantly different from all the other *S. costatum* clones and almost identical to *S. pseudocostatum*. Further investigation of the isolate using electron microscopy is required to determine if this clone is really *S. pseudocostatum*.

Another interesting but puzzling result was the difference between the sequences of PORT D1 and PORT D2. These two cultures were the same clone originating from one isolated chain of cells. Because they originated from the same isolate, these two cultures should in fact have had the same or at least very similar sequences. One possibility is that the extracted DNA was contaminated before the PCR amplification and that the result was a mix of two clones from the same sediment sample. However, another ongoing genetic diversity study of *S. costatum* by RAPD (Random Amplified Polymorphic DNA) showed similar results (A. Godhe, personal communication) so the difference was not just a result of this analysis. The clones have been kept in

culture and reinoculated into new media for 6 months prior to DNA extraction. This short time of separation is not enough to generate such differences by mutations.

When comparing the results from the analyses of Data 1 and Data 2, I could see a difference in the resolution of the dendrograms. The overall pattern was the same, but the resolution and bootstrap values differed. There were lower values and resolution in the analyses of Data 1 and this may be a consequence of the smaller dataset. The outgroup sequence obtained from GenBank, *S. pseudocostatum*, was only 344 bp long, and therefore was a limiting factor for how long the dataset could be. If I were to use all of our obtained sequences, I would have a 176 bp dataset and analyses of that dataset showed no or a very small differentiation. Thus, I removed three clones with the shortest sequences and extended the fragment length to 344 bp (Data 1). Both the 176 and 344 bp datasets, especially the shorter one, were dominated by the 5.8S, coding region. Because these coding regions are more conserved there will not be large differences among the *S. costatum* clones. There was enough genetic information in Data 1 to show distinction on a large spatial scale (i.e. Pacific vs. Atlantic clones), but not within a smaller area.

By using Data 2 and selecting the KF M2 clone as an outgroup, there was a longer fragment to analyze. This longer sequence gave a higher resolution between different populations but the Portugal and Koljö Fjord clones were still not completely separated.

Earlier phylogenetic studies have shown that SSU and LSU sequencing is useful when comparing higher taxonomic levels (Bhattacharya and Medlin 1995, Van der Auwera and De Wachter 1998), but may not be suitable for differentiating clones of the same species. In contrast, the ITS region is the most rapidly evolving part of the rDNA sequence and should be more useful at species and population levels. The ITS region has been used successfully to define discrete biogeographic groups of *Cladophora albida* (Chlorophyta) (Bakker et al. 1992). In the present study, variations in the ITS sequence could reliably differentiate between *S. costatum* clones isolated from sediments in the Pacific vs. Atlantic Oceans, but not geographically closer populations. It is not clear why the clones from Portugal and Koljö Fjord could not be separated. The explanation may be a transport of genetic material between these locations by the prevailing ocean currents, or there may simply not be enough variation in the ITS region to show differences on an intra-ocean scale.

4.3 The use of ITS for population studies in microalgae

One objective of this project, was to evaluate whether or not ITS sequences could be used for further investigations of microalgal population dynamics, particularly with regard to the origin of planktonic blooms. The usefulness of the ITS region for determining the source of blooms requires that local populations from sediments contain distinctive ITS sequences. Thus, one could compare the ITS sequence of a bloom clone to the different "sediment signatures" and estimate the most likely source of the bloom population. Determining the source of a bloom would be a big step forward towards understanding and perhaps predicting potentially harmful blooms. So far, my results do not give enough resolution of spatial differences to be able to determine the source of a bloom. Because this method was not able to separate

between samples from Portugal and Koljö Fjord, it is unlikely that it would be able to detect differences among samples taken along the Swedish coast.

Earlier studies of allozyme banding patterns have shown large diversity within a population of *S. costatum* (Gallagher 1980). Those results were based on a survey of several hundred clones, so it may be that the number of clones used here was not enough to get a representative picture of the different populations. There may also be an effect of culture age. Cultures used in this study were sampled when cell densities were high, often post logarithmic growth. Perhaps old cultures are affected by mutation more than those that are actively growing. In addition, more information may have been gained by amplifying a longer sequence strand consisting of SSU, ITS 1, 5.8S, ITS 2 and LSU. Such a sequence would contain the entire ITS region, whereas the sequences used here were often incomplete. Further refinement of the techniques used here might increase the usefulness of the ITS region for population studies in microalgae.

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INTYG

Lovisa Hansén gjorde under hösten 2003 sitt examensarbete på Institutionen för Marin Ekologi med mig som handledare. Arbetes titel var ”Genetic variability of *Skeletonema costatum* (Bacillariophyceae) based on ribosomal DNA ITS sequences”. I korthet så isolerade Lovisa enskilda individer av *Skeletonema,* odlade dem till lämplig täthet och extraherade DNA från dem. Sen körde hon PCR, sekvenserade en del av en gen, analyserade sina fragment och konstruerade ett fylogenetiskt träd. Lovisa har också gjort en Minor Field Study (MFS) förlagd till ett universitet i Indien, då jag också fungerade som hennes handledare. Titeln på hennes studie utförd i Indien är ”Molecular studies of mating behaviour in Indian *Skeletonema* using AFLP”. I detta arbete undersökte Lovisa hur sexuell förökning sker hos *Skeletonema.* Tekniken vi använde i denna senare studie var ny för mig och för den indiska handledaren, vilket resulterade i att Lovisa hade ett stort ansvar som hon axlade galant. Trots att vi arbetade i en miljö som var helt ny för Lovisa–hon hade aldrig varit i Indien tidigare–så fann hon sig väl tillrätta och blev snabbt en uppskattad kollega och vän på Colleget i Mangalore.
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Göteborg som ovan

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Intyg

Härmed intygas att Lovisa Hansén har jobbat heltid som naturumguide i vårt naturum på Nordkoster mellan 14 juni och 22 augusti 2010. Naturumet hade nästan 3000 besökare under denna period.

Lovisa svarade kunnigt och villigt på alla frågor som besökarna ställde. Var det något hon inte kunde svara på omedelbart tog hon reda på svaret med hjälp av kollegor, böcker eller Internet. Dessutom höll hon på eget initiativ små guidningar med utgångspunkt i de foton vi hade i utställningen. Foton som var hämtade ur Stefan Edmans bok om Kosterhavet. Dessa guidningar var mycket uppskattade och vi hade besökare som kom tillbaka och ställde frågor om djur och växter de själva hittat och tackade än en gång för den fina guidningen de fått tidigare.

Lovisa kommer även att jobba hos oss som naturumguide under sommaren 2011. Hon kommer i år också att ha strandexkursioner för allmänheten två gånger per vecka.

den 25 mars 2011

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