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Molecular evolution in natural populations of *Skeletonema costatum*: Restriction mapping and analysis of the chloroplast genome

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City University of New York, 1994

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Molecular Evolution in Natural Populations of *Skeletonema costatum*: Restriction Mapping and Analysis of the Chloroplast Genome

by

JOSEPH E. STABILE

A dissertation submitted to the Graduate Faculty in
Biology in partial fulfillment of the requirements
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Abstract

Molecular Evolution in Natural Populations of Skeletonema costatum: Restriction Mapping and Analysis of the Chloroplast Genome

by

Joseph E. Stabile

Advisors: Professor Jane Gallagher
Professor Eleanore Wurtzel

A wealth of background information on the ecology, physiology and population genetics of the marine diatom Skeletonema costatum makes this a unique model system in phytoplankton because new molecular genetic data can be compared with prior results. The genetic divergence among 29 isolates representative of Narragansett Bay populations of S. costatum was quantified using restriction analysis of chloroplast DNA (cpDNA). CpDNA data were compared to earlier results obtained using allozymes in order to study the process of speciation in this genetically diverse and widely distributed species. Data from terrestrial plants indicates that cpDNA evolves more slowly than allozymes and provides markers useful for the detection of genetic relationships over higher phylogenetic levels. The pattern of cpDNA variation in Skeletonema spp. was investigated to determine if this general relationship is also true of chromophytes.

The chloroplast genome of S. costatum is 131 kilobases (Kb). Its organization is typical of most plants in that an inverted repeat separates a large and small single copy region. Chloroplast DNA restriction site maps of two strains representative of the most diverse allozyme groups, reveal that their are extensive restriction site polymorphisms, but no major rearrangements among their cpDNAs. Most observed differences appear to be the result of restriction site mutations. The colinearity of these chloroplast genomes indicates that restriction analysis of chloroplast DNA is appropriate for studying lower phylogenetic relationships in diatoms.

Five homologous cpDNA probes were used to observe restriction fragment length polymorphisms (rflps) among the 29 Narragansett Bay isolates, 3 strains of S. costatum isolated from other areas and 2 strains of a sister taxon S. tropicum. The pattern of divergence observed among all Skeletonema spp. suggests that the distribution of chloroplast lineages follows a relationship based on a light and thermal gradient. Among the Narragansett Bay isolates, there is a strong concordance of pattern between the cpDNA and allozyme data. However, the overall genetic diversity observed with the allozyme data is greater than that of the cpDNA rflps, confirming the hypothesis that cpDNA evolves more slowly in diatoms as well as in terrestrial plants.

Both cpDNA and allozyme data suggest that there is limited gene flow between the most diverse allozyme groups in Narragansett Bay. Analysis of the chloroplast and allozyme data sets within the theoretical framework of the phenetic, biological and phylogenetic species concepts suggests that all strains from Narragansett Bay are one species. The observed pattern of divergence among the Narragansett Bay populations suggests that they are probably in the incipient stages of speciation. However, more data on the extent and pattern of gene flow are needed to confirm this conclusion.

Acknowledgements

I thank the members of my advisory committee for their critical comments and suggestions. I am especially grateful to Dr. John Lee for his help in revising this thesis, but most importantly for giving me an appreciation of the diversity of marine organisms. The training I received in Dr. Eleanore Wurtzel's molecular biology laboratory was outstanding, and for this I can never thank her enough. I am also grateful to Dr. Jane Gallagher for all her help and perseverance throughout my doctoral training. I thank Dr. Kate Goddard for her participation and earlier reviews of this thesis. I am grateful to Dr. Thomas Jensen for agreeing to serve on my committee at the last minute.

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Preface

What is a species? All biologists work on species or parts of them, yet the question of what constitutes a species has never really been completely resolved or applicable to all organisms. Perhaps it never will be. The species problem is due to two reasons: One, specialists have diverse opinions on how to interpret characters. For example, one may study the process of speciation by examining key characters, while other specialists will prefer to analyze all characters on an equal basis. In practice, these different interpretations can lead to different conclusions on whether or not two populations have separated into new species. Therefore, decisions on what constitutes a new species can be arbitrary. The second aspect of the species problem is that speciation is a complex process which has two paradoxical aspects, diversity and discontinuity (Dobzhansky, 1951). Descent with modification does not often lead to clear cut phenotypic or genetic discontinuities that biologists can use to unambiguously identify species. In addition, discontinuities in pattern of phenotypic variation do not always allow us to pinpoint the mode of speciation. Different species concepts have attempted to provide a theoretical framework within which to address the above problem.

A specialist's concept of species influences his/her interpretation of the speciation process. There are three species concepts that have a major role in the characterization of species. The typological species concept is the oldest and perhaps most commonly used in the characterization of multicellular organisms and many protists. This is a type subjective phenetic concept in that overall morphological similarity is the most important criterion used for defining a species (Sokal, 1974). Each species has a characteristic form in reference to some phenotypically described key characters. The botanical and zoological codes of nomenclature require that a "type" specimen be deposited in a museum collection. This serves as a reference specimen for future workers. Newly collected individuals or strains must be within the range of variation written in the original description of the species. If the new specimen is differentiated beyond that description, then a decision must be made as to whether it is the same, in which case the original description is amended, or a new species.

Adansonian or numerical taxonomy is a quantative phenetic approach. Here, all characters, are analyzed and treated equally without weighting them *a priori*. Species boundaries are determined by the analysis of these characters using multivariate statistics at the population level (Sokal, 1974). Distinct species should be represented as discrete clusters of populations in a branch of a phenogram or dendrogram (Sneath, 1984). Since both phenetic species concepts base species designations on degrees of differentiation, they lead to arbitrary decisions. The advantage of these two phenetic species concepts is that they can be applied to all organisms living or extinct, without regard to their mode of reproduction. Secondly, these phenetic species concepts may be the easiest to use in the sense that extensive knowledge of the evolutionary history of the characters in other members of the group is not required.

The most widely accepted species concept in practice, particularly when it is applied to multicellular organisms, has been that of the biological species. Here, species are composed of populations genetically isolated from other species, by reproductive isolation. Species have distinct gene pools and genetic systems whose integrity is maintained either by internal cohesion (recognition via premating mechanisms) or post mating mechanisms. In this concept, speciation is driven by selection. Populations are thought to adapt to local environmental conditions and reproductive isolation serves to maintain coadapted gene complexes (Mayr, 1957; Dobzhansky, 1951).

The advantage of using the biological species concept is that it has a clear criterion, reproductive isolation, for establishing a discontinuity between populations. It is also this criterion that sometimes makes interpretations difficult. For instance, if 2 species hybridize and their offspring have a reduced fecundity, a decision to maintain 1 or 2 species may be arbitrary. Secondly, this concept excludes organisms that only reproduce asexually, and is difficult to apply to organisms that reproduce sexually on an intermittent basis. This limits the application of the biological species concept in many terrestrial plants and protists. Representatives of these two groups sometimes form hybridizing "species" complexes called syngameons (Grant, 1957).

Recently, the phylogenetic species concept has been proposed (Cracraft, 1983). Phylogenetic species are clusters of populations that should be diagnostically distinct from other populations and in which there is a pattern of descent from a common ancestral population

(Cracraft, 1983). There are two versions of this theory. In one version, autapomorphic, uniquely derived characters are used as evidence for monophyly. In the second, unique combinations of characters, not necessarily derived, are used to establish monophyletic groups. The phylogenetic species concept is applicable to all organisms regardless of their mode(s) of reproduction. One weakness of the phylogenetic species concept is that the number of species recognized depends on the resolving power of the characters analyzed (Avise and Ball, 1990). Characters can be chosen arbitrarily and different numbers of species can be recognized depending upon which characters one uses in the phylogenetic analysis (Avise and Ball, 1990).

All three species concepts attempt to do the same thing: characterize species. This characterization would be easy if divergent populations always became completely distinct. This does not happen however, in every case. Sometimes there is clinal variation in which a particular character changes gradually over a geographic transect. In other instances, morphological variation is slight, yet speciation has occurred due to cytogenetic processes such as polyploidy or karyotypic change. Natural hybridization (introgression) between two or more "species" may also obliterate the original discontinuities that were used to distinguish them (Grant, 1957).

Traditionally most protists are characterized as species on the basis of their light micrographic or fine structural characteristics. Sonneborn (1957) found genetically isolated, but morphologically similar populations of _Paramecium_ _aurelia_. He proposed a new term, syngen be used to denote an evolutionary unit, while species, based on morphological criteria, be used as a taxonomic unit of identification. A syngen is a group of populations that has attained an irreversible evolutionary divergence from other such groups. Unlike a species, syngens need not show recognizable morphological differences. In sexually reproducing protists, the test for a syngen is gene flow (Sonneborn, 1957). As for asexually reproducing protists, the test for a syngen is to compare different strains of a species for all types of characters that are genetically determined (not environmentally induced) and then arrive at a judgement on discontinuities between groups (Sonneborn, 1957). This is a practical approach, in that groups of populations, can be called syngens, without changing the taxonomic designation.

Other terms are commonly used to describe variation at the intraspecific level. Although variability has always been recognized in original taxonomic descriptions, in some cases an expert may want to formally recognize variation without describing a new species. Variety has been commonly used to describe plant and algal populations that are distinct in some characters, yet maintain most characters typical of the species (Cronquist, 1988). Diatom taxonomists typically recognize variation at the subspecific level by describing distinct populations within the formal taxonomic rank of variety. The common practice has been to describe diatom varieties on the basis of morphological characteristics. For example, _Chaetocerus compressus var. hirtisetus_ differs from _C. compressus_ by generally having a smaller apical axis, differences in chain torsion, and differences in the common intercalary and terminal setae (Rines and Hargraves, 1990).

The botanical code of nomenclature places the taxonomic rank of subspecies between that of species and variety. Subspecies designations are usually reserved for extraordinarily distinguished populations. The subspecies rank is not as commonly used as variety in diatom taxonomy. Unlike the different concepts of species (e.g. biological species concept), there is no underlying theoretical framework with which to judge the application of these subspecific ranks. Thus, the application of both variety and subspecies is subjective and based on the past experience of the expert and the common practice in that particular field.

No single concept is adequate in describing species because the process is complex. Diversity and discontinuity are products of mutation and microevolutionary forces, such as gene flow, genetic drift and natural selection acting alone or simultaneously. Information regarding the extent and pattern of variation within and among populations is necessary to understand how these microevolutionary forces operate and how they affect the speciation process in a particular group of organisms.

Higher Level Systematics and Diatoms

At the present time there is a great deal of debate regarding the conceptual framework which should be erected to organize the highest levels of taxonomy of protists and higher eukaryotes. The arguments center around the significance of the new and still incomplete data obtained from fine structural and comparative molecular studies. One conceptual scheme based on molecular, ultrastructural and palaeontological data, developed by Cavalier-Smith (1993), divides the eukaryotes into five kingdoms: Plantae, Animalia, Fungi, Chromista and Protozoa. This classification scheme shows one approach in which the protozoans are placed within their own kingdom. The Kingdom Protozoa is defined as a paraphyletic group on the basis of both positive and negative characters. Cavalier-Smith justifies this classification of the protozoa by its greater utility to biologists (Cavalier-Smith, 1993). The chromophyte algae are placed in their own kingdom; Chromista (Cavalier-Smith, 1993). Diatoms are one of the most diverse groups of Chromophytes. Approximately 100,000 living and fossil diatom species have been described, although one taxonomist believes that the number of valid species should be closer to 12,000 (Tappan, 1980). Only six species of diatoms were examined in the phylogenetic tree of Cavalier-Smith (1993). There also appears to be a problem in this part of the tree, because the centric diatom S. costatum is shown to share a more recent common ancestor with 3 pennate diatoms rather than other centric diatoms. This error is probably due to a high number of nucleotide base pair changes in S. costatum as is indicated by the long branch length. This part of the tree will probably be corrected in future phylogentic analyses that examine more species of diatoms.

Wainright et al., (1993) show an alternative approach to higher level classification, although their phylogentic tree based solely on 16s-like rRNA sequence data, is not presented as a classification scheme. Here the protozoans are separated into several kingdoms along phylogenetic lines. Also the Chromista have been renamed, straminophiles (Wainright et. al., 1993). For the purposes of this thesis, I will follow the classification scheme of Cavalier-Smith (1993).

The Genus Skeletonema

One of the most ecologically important genera of centric diatoms is Skeletonema. Six species have been described on the basis of morphology and distribution. The six species are S. costatum, S. tropicum, S. potamus, S. subsalum, S. menzelii and S. cylindraceum (Hulbert and Guillard, 1968; Hasle, 1973; Hasle and Evensen, 1976). Recently, Medlin et. al.(1991), described a seventh species, S. pseudocostatum, on the basis of morphology and DNA sequence data. The evolutionary relationships among these species are not well understood. The results of a recent cladistic analysis of morphological characters such as, position of labiate and strutted processes, extent of silicification, markings of the valve, geographic distribution, and number of chloroplasts per cell suggested that S. costatum and S. menzelii are sister taxa (Gallagher unpublished). A phenetic analysis of the same morphological characters suggested that S. costatum and S. tropicum are more closely related (Gallagher, unpublished).

Skeletonema costatum - Population Background

Skeletonema costatum has a worldwide distribution and is the most abundant species in the genus (Hasle, 1973). It is a dominant species in virtually every habitat where it occurs for at least part of the year. Previous studies have suggested that this ecologically successful species is genetically diverse and that this diversity is responsible for the existence of a wide array of physiological phenotypes (Gallagher, 1982). Allozyme electrophoresis suggests that 3 seasonal populations of S. costatum coexist in Narragansett Bay, Rhode Island (Gallagher, 1980). Two allozyme groups, "WH" and "Whet" were dominant in the winter. The "SH" allozyme group was dominant in the summer. Individuals observed to be dominant in the interbloom population had some alleles common to both the "WH" and "SH" allozyme groups and were thus designated as "Mixed" (Gallagher, 1980).

Each seasonal population was dominated by a particular allozyme type and the cycling in abundance of the three allozyme types according to season was a stable feature of the Narragansett Bay ecosystem for two years (Gallagher, 1980). The pattern of variation observed with allozyme electrophoresis was also correlated with physiological traits. Strains of S. costatum representative of the seasonal populations were shown to differ in the size and number of photosynthetic units, pigment ratios, photosynthesis - irradiance characteristics, and growth rates when they were grown in light saturated and light limited environments (Gallagher et al., 1984; Gallagher and Alberte, 1985). On the basis of these data, seasonal types would be expected to grow best under the climatic conditions observed during their time of maximum abundance. The correlation between allozyme and photosynthetic traits is interpreted as an adaptation to light and temperature within these populations. This is of particular importance because photosynthetic traits may determine survival in coastal habitats during different seasons (Hitchcock and Smayda, 1977).

Populations can diverge on an ecological and/or geographic basis. Protist species distributions to different environmental gradients has shown extensive variation in their ecophysiologies (Gallagher, 1986;

Haynes, 1992). The correlation of divergent populations with ecogeographic distribution has been used to illustrate the process of speciation. Ecophenotypes, populations exhibiting environmentally induced variation, and ecotypes, genetically adapted populations, have both been used to describe populations that have differentiated in a particular environment. It is common for researchers to state that certain populations of a species are ecotypes without providing strong evidence on whether the observed pattern of variation is induced environmentally or has some genetic basis (Haynes, 1992).

In order to experimentally demonstrate ecotypic differentiation, it is important that a researcher is working with clones. Clones here are defined as a lineage of individuals produced asexually from one cell (Futuyma, 1979). The establishment of clonal cultures allows one to determine whether the differences observed between experimental and control groups have a genetic base. If clonal cultures are not used, then one can not be certain that the results obtained from such experiments are due to differential effects on combinations of genotypes within one culture. The protist, Ammonia beccarii, is a widely distributed common temperate zone foraminifer. Tank experiments extensively quoted as demonstrating ecotypic differentiation never established what was genetically or environmentally induced variation because the investigator was not working with clones (Haynes, 1992). In one set of experiments, strains isolated from Montsweag Bay, Me. to Beufort, N.C. were observed to have different temperature regimes for optimum growth and reproduction (Schnitker, 1974). These experiments failed to demonstrate ecotypic differentiation because it is not certain that there is a genetic base for the observed differences among these populations of A. beccarii (Haynes, 1992).

Characters used in the study of evolution should be discrete, independent and heritable (Farris, 1990). Genetic evidence for ecotypic differentiation in S. costatum was originally provided by allozyme banding patterns (Gallagher, 1980). Only four stains yielding bands representative of 5 loci function well or repeatedly. Therefore, there is a limited amount of genetic data for each strain using this method. I speculated that a stronger demonstration of ecotypic differentiation could be achieved through the examination of characters at the molecular level. I also felt that S. costatum could be an excellent model system for studying microevolutionary and speciation processes.

Which Molecular Tool?

Photosynthetic eukaryotes have three genomes available for a molecular genetic analysis: the chloroplast, nucleus and mitochondrion. Mitochondrial (mt) genomes have not been extensively used in comparative studies of terrestrial plants because this genome (200 Kb unlike vertebrates - 16 Kb) rearranges frequently among closely related species (Palmer, 1992).

Little is known about the mtDNA of chromophyte algae. It is extremely difficult to extract and thus did not seem to be a good place to start a molecular study of S. costatum (Goff and Coleman, 1991). Only recently, have researchers been able to successfully extract it from the chromophytes (Goff and Coleman, 1991). The mitochondrial genome of most chromophytes is a linear molecule ranging in size from 25 -50 Kb (Goff and Coleman, 1991). Its utility for evolutionary studies is unknown.

Chromophytes are evolutionarily distant from terrestrial plants, and this severely limits the number of higher plant nuclear probes which are useful in Southern hybridization experiments. Goff and Coleman (1988) demonstrated that only conserved nuclear ribosomal DNA probes from terrestrial plants hybridized to red algal nuclear DNA. Initially it was thought that these genes evolve too slowly to be useful in analyzing population level divergence. Recently, the polymerase chain reaction (PCR) and direct sequencing have facillitated the analysis of nuclear ribosomal DNA in systematic and population level studies. Several workers have successfully used these techniques in examining inter and intraspecific diversity in protists (Medlin et. al., 1991; Rowan and Powers, 1992). However, these techniques were not readily available in our laboratory, nor was this considered a good approach for intraspecific studies at the time (1988) I started this thesis research project. One can also argue that the allozyme data collected by Gallagher (1980) should be representative of the nuclear genome (Hillis and Moritz, 1989).

I speculated that the analysis of chloroplast DNA (cpDNA) would be a good approach in a molecular level examination of the S. costatum Narragansett Bay populations. It seemed reasonable to suspect that the large differences in the photoadaptive strategy of the three seasonal populations could be reflected by molecular level changes within the chloroplast. Technically it should be easier to extract and analyze chloroplast DNA since it is found in multiple copies and is more abundant than mitochondrial DNA (Palmer, 1992).

Finally, since a wealth of information exists on the molecular systematics of terrestrial plants using cpDNA, general comparisons could be drawn between higher plants and chromophytes; and in particular, whether higher plant models of evolution could be applied to chromophyte algae.

Chloroplast DNA Background

Terrestrial Plants

The DNA in terrestrial plant chloroplasts ranges in size from 120 - 270 kb. The DNA of most chloroplasts is approximately 150 kb (Palmer, 1991) and has an inverted repeat (IR) section that divides the genome into small (SSC) and large single copy regions (LSC) (Palmer, 1983; Kolodner and Tewari, 1979). Genes within the inverted repeat section usually encode ribosomal RNA's, several tRNA's and ribosomal proteins (Palmer, 1991). The LSC region of the chloroplast genome contains many of the genes needed for photosynthesis. Order and arrangement of these genes, across higher plant taxa are highly conserved (Palmer, 1991). Most lineages are characterized few inversions within their chloroplast genomes. For example, even though 400 million years have passed since the divergence of Marcantia polymorpha, Oryza sativa, and Nicotiana tobaccum (Ohyama et al., 1986; Hiratsuka et al., 1989; Shinozaki et al., 1986), only three inversions distinguish their cpDNA maps (Palmer, 1991). DNA sequence and restriction fragment length polymorphisms from various chloroplast genomes have also confirmed that the rate of divergence is very slow (Zurawski and Clegg, 1987; Sytsma and Schaal, 1985, Wolfe et. al., 1987).

Due to its conservative nature, cpDNA has been used successfully in quantifying genetic variation at or above the species level in higher plants. There are polymorphic "hotspots" in the large coding region near the inverted repeat and many small insertion/deletion type mutations in non-coding regions which facilitate chloroplast genome research (Zurawski and Clegg, 1987; Coates and Cullis, 1987, Jansen and Palmer, 1988; Shinozaki et al., 1986). Major rearrangements of the chloroplast genome have been used to establish relationships in the major plant family, Asteraceae (Jansen and Palmer, 1987; Jansen and Palmer, 1988). As stated above, major rearrangements of cpDNA within plant lineages are infrequent and typically occur only when one half of the inverted repeat section is absent (Lavin et. al., 1990).

Is chloroplast DNA a good tool for examining population level differences?. Several initial studies suggested it is not, although this conclusion appears to be contradicted by the actual data. The goal of early investigations (Palmer and Zamir, 1982; Clegg et al., 1984a+b; Palmer et al., 1985) was to interpret the evolutionary relationships at the higher taxonomic levels. These studies examined between 6-11 intraspecific isolates, just to obtain a baseline knowledge of the variability. Yet even with small sample sizes, intraspecific variation was always observed. The pattern of divergence among individuals was useful in interpreting the evolutionary relationships between these taxa and in determining the loss of genetic variation in the domestication of certain agricultural crops (Palmer and Zamir, 1982; Clegg et al., 1984a; Palmer et al., 1985).

An early study of one Lupinus texensis population revealed only three variant chloroplast restriction fragment patterns among 100 individuals (Banks and Birky, 1985). Two of the three cpDNA types were observed only in single individuals. This initial finding suggested that there was little, or no, intraspecific variation in chloroplast genomes.

Several recent studies using hundreds of individuals isolated from different populations demonstrate more intraspecific variation. One study examined cpDNA rflps among 400 individuals of Pinus contorta and P. banksiana. Intraspecific variation was observed throughout their distributional ranges. Also, interspecific cpDNA polymorphisms distinguish these two species unambiguously (Wagner et. al., 1987). Recombinant cpDNA genomes were observed in areas of sympatry between P. contorta and P. banksiana. These unusual sympatric cpDNA's are evidence that variant genotypes of hybrid zones are not limited to the nuclear genome (Govindaraju et. al., 1990).

Intraspecific variation was observed in Helianthus bolanderi (Rieseberg et al. 1988). The cpDNA sequence difference between the weedy and serpentine races of H. bolanderi suggested (if the calibration of the molecular clock is correct) that these races diverged some 3 million years ago. This refuted a hypothesis that the weedy race had originated in the recent past, through introgressive hybridization from H. annus (Heiser, 1949).

In another study, 245 accessions of wild barley, Hordeum vulgare, were shown to have restriction fragment length polymorphisms. Three chloroplast genotypes, within this species, were observed to have disjunct distributions. The authors concluded that this non-random segregation of cpDNA genotypes suggested that there was adaptation to local environments (Neale et al., 1988).

Extensive population level differences were observed in Trifolium pratense (Milligan, 1991). A total of ninety-six individuals isolated from three different populations, were observed to have 22 different chloroplast DNA restriction fragment patterns. One of the 22 was the dominant genotype in all three populations, but each population had unique genotypes which occurred at low frequencies. This study used only one restriction enzyme, and therefore underestimates the true level of intraspecific variation.

Chloroplast DNA Background

Chromophyte Algae

In comparison to higher plants, there is little data on the chloroplast genomes of Chromophytes. Presently, there are nine chloroplast DNA maps for chromophyte algae. The chloroplast genome of these nine chromophytes are organized into LSC, SSC and IR regions (Cattolico and Loiseaux-de Goër, 1989; Kowallik, 1989; Douglas, 1988). Several features distinguish Chromophyte cpDNA's from those of terrestrial plants. One, is the presence of the small subunit of the ribulose 1-5, bisphosphate carboxylase gene, rbcS, and the protein elongation factor protein tufA. These genes are nuclear - encoded in higher plants (Reith and Cattolico, 1986; Baldauf et al., 1991). Second, the variation in size of chromophyte chloroplast genomes ranges between 118 to 130 Kb (Kowallik, 1989; Stabile, Chapter 3). Much of this variation in size is due to differences in the inverted repeat, which ranges in size from 4.7 to 22 Kb among several genera of chromophytes (Kushel and Kowallik, 1987; Reith and Cattolico, 1986). Some of the first chromophyte IR's examined were small in comparison to those of higher plants; the former are approximately 6 Kb while the latter are typically 20 Kb. It has been suggested that the smaller chromophyte inverted repeat section may not have the same stabilizing effect on the chloroplast genome as it does in higher plants leading to the extensive rearrangement of genes observed between distantly related chromophytes (Kowallik, 1989; Cattolico and Loiseaux-de Goër, 1989).

The degree of variation present in the chloroplast genomes of Chromophyte algae is not known because of the small number of species examined thus far. As mentioned earlier, current work on these organisms has focused almost exclusively on the description of size and location of genes within the chloroplast (Palmer, 1991). Presently it is not clear that cpDNA analysis will have the same utility as a systematic tool as it does in higher plants (Bourne et. al., 1992). For these reasons, I initiated a study of ecotypic differentiation in natural populations of the marine diatom S. costatum using chloroplast DNA as a molecular marker.

The development of a diatom-specific DNA extraction technique is outlined in the first chapter which was published in Biochem. Syst. Ecol. (18, 5-9, 1990) entitled "Molecular Analysis of Intraspecific Variation in the Marine Diatom Skeletonema costatum." This was necessary because extraction methods useful in terrestrial plants did not work in S. costatum. After high molecular weight genomic DNA was extracted, heterologous probes (tobacco chloroplast genome) were used as a first approach in analyzing the extent of variation among the different strains in Southern hybridizations. The goal of these experiments was to screen representative strains of the seasonal populations in order to determine whether it was practical to use cpDNA as a molecular tool.

The second chapter of this thesis reports the analysis of chloroplast DNA restriction fragment length polymorphism (rflp) analysis of the Narragansett Bay "winter type" strains. This chapter was published in the Journal of Phycology (28, 90-94, 1992) and was entitled, "Comparison of chloroplast DNA and allozyme variation in winter strains of the marine diatom *Skeletonema costatum* (Bacillariophyta)." Winter strains were analyzed first in order to determine whether cpDNA rflp's were conservative within one allozyme group.

The physical chloroplast DNA map of *S. costatum* is described in chapter three. This chapter will be submitted to the journal, *Plant Molecular Biology* for publication. The map is compared to the one of the centric freshwater diatom *Cyclotella meneghiniana* (Bourne et al., 1992).

The full extent of cpDNA divergence among the winter, summer and mixed strains is presented in chapter 4. This chapter will be submitted to the *Proceedings of the National Academy of Sciences, USA*.

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Chapter 1

MOLECULAR ANALYSIS OF INTRASPECIFIC VARIATION IN THE MARINE DIATOM *SKELETONEMA COSTATUM*

Biochem. Sys. Ecol. 18: 5-9 (1990)
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ABSTRACT

Restriction fragment length polymorphisms (rflps) were examined in 3 strains of the marine diatom Skeletonema costatum using higher plant chloroplast and nuclear gene probes. Two of the strains, isolated from Narragansett Bay, R.I., are representative of different allochronic seasonal populations. The third is a standard strain of this species obtained from the Culture Collection of Marine Phytoplankton. DNA hybridization probes revealed rflps among these strains, a finding which is consistent with an earlier hypothesis that local population diversity has a genetic basis. The large number of DNA polymorphisms observed indicate that a systematic revision of this genus using molecular approaches should begin with population-level studies. A method for the extraction of high molecular weight DNA from diatoms and other microalgae is reported.

INTRODUCTION

Populations of the cosmopolitan marine diatom Skeletonema costatum (Grev.) Cleve have high levels of intraspecific diversity [1,2]. In Narragansett Bay, R.I., the most common components of the local populations cycle in their relative abundance during different seasons [1]. Strains isolated from populations of S. costatum, when grown under identical environmental conditions [3,4], have been found to be morphologically identical but differ in allozyme banding patterns and physiological traits, such as growth rates, size and number of photosynthetic units, and photosynthesis-irradiance characteristics. Breeding experiments cannot be used to confirm the genetic basis of these phenotypic differences because sexual reproduction is rare and cannot be controlled [5]. The genetic basis of phenotypic variability has been inferred from the observed stability of expression when strains are grown under identical conditions for over ten years in culture. Genetic differences among populations can be established directly by examination of the DNA of the strains. The goal of this preliminary investigation was to determine if the DNA differed among three strains of S. costatum whose allozyme banding patterns and physiological traits also varied [2].

Analysis of the chloroplast genome is of particular interest due to its extensive use in higher plant systematics [6,7]. However, with only a few exceptions

[8,9,10], higher plant chloroplast DNA evolves too slowly to be useful in population studies [7] and is used mainly for intergeneric comparisons. The higher levels of intraspecific diversity in microalgae [11] imply that there may be a greater probability of detecting cpDNA differences within diatom populations. Determination of DNA variation within populations requires the screening of large numbers of individuals and a culture collection with global scope is available for *S. costatum* and other species in this genus. Therefore, a second goal of this investigation was to determine a strategy for the systematic examination of this extensive collection. In the process of conducting this study we developed a method to extract high molecular weight DNA from *S. costatum* that appears to have utility for genetic studies in a wide variety of microalgae.

RESULTS AND DISCUSSION

Isolation of high molecular weight DNA from diatoms has been difficult due to the high concentration of intracellular proteins and polysaccharides. Therefore, we developed a method for the extraction of total genomic DNA suitable for rflp analysis and cloning. Using this method, total genomic DNA was extracted from the three strains of S. costatum listed in Table 1. DNA digested with <u>Hind</u> III was electrophoresed on an agarose gel as shown in Fig. 1. This method was used successfully in extracting DNA from other microalgae that have been intractable for DNA analysis using other techniques [Stabile et al., unpublished].

In separate experiments, total genomic DNA was digested with <u>Hind</u> III and hybridized to chloroplast or nuclear DNA probes. The autoradiogram obtained using chloroplast probe, Ba4, is shown in Fig. 2. Standard strain Skel and NY17 (a winter strain) had 5 major bands of approximately 5.5, 3.8, 2.4 Kb, 1.9 and 0.7 Kb, whereas UP45 (a summer strain) had 2 major bands of approximately 11.7 and 4.4 Kb. The autoradiogram obtained using a second chloroplast probe, Ba5, also revealed rflps among the three strains (Fig. 3). All strains shared a fragment of approximately 540 bp. In addition, Skel had a 3.4 Kb fragment, UP45 had fragments of approximately 8.3 and 6.5 Kb and NY17 had fragments of 16.0, 12.5 and 3.4 Kb. Fig. 4 shows restriction fragments which hybridize to nuclear probe <u>atp</u>2-1. Skel and NY17 shared a fragment of approximately 5.7 Kb, while UP45 had a 2.9 Kb fragment.

These data indicate that rflps exist among strains of a single species of diatom isolated from a single location. In general, the hybridization patterns shown here parallel the degree of allozyme and physiological divergence of these strains [2]. Although Skel and NY17 were not identical, they were more similar to each other than to UP45. In previous experiments, NY17 and UP45 were found to be at opposite ends of the physiological-ecological continuum shown by the Narragansett Bay populations of S. costatum [2]. The existence of strains intermediate between the summer (UP45) and winter (NY17) ecotypes preclude dividing these strains, and the populations they represent, into different species. However, further strains need to be examined. These data, including the DNA hybridization patterns reported here, support the hypothesis that the differences among populations of this species are genetically determined and that results obtained from a "standard" strain cannot be extrapolated to the species as a whole. Furthermore, rflps detected using other restriction enzymes and chloroplast probes support this interpretation [Stabile et al., unpublished].

The existence of rflps detected by hybridization to the chloroplast probes indicate that cpDNA restriction fragment analysis might be useful for determining population-level differences in S. costatum. This would contrast with many higher plants where cpDNA polymorphisms are observed only at or above the genus level [7]. However, further research is required using homologous probes and the construction of a cpDNA map for S. costatum. Heterologous probes obtained from phylogenetically distant organisms are limited in use beyond initial surveys. The restriction fragment patterns obtained can not be compared with the DNA restriction maps of the heterologous probes due to rearrangements and mutations that may occur during evolution. For example, DNA sequences commonly found in the nucleus of higher plants may be in the chloroplasts of algae [12] or may be repeated in other cellular organelles [13]. Although there are some reports that the location of genes in the chloroplast and nuclear genomes of diatoms is similar to that found in higher plants [14,15], more research needs to be undertaken. I am currently in the process of cloning cpDNA from one strain of S. costatum. This is a necessary step in the development of probes needed to screen the large number of strains in our collection. The preliminary investigation reported here indicates that this effort is warranted and that the Narragansett Bay strains should be examined in more detail before global studies of this species and other members of the genus can be undertaken with confidence.

EXPERIMENTAL

Strains and Culture Conditions. *Skeletonema costatum* strains are listed in Table 1. The standard strain Skel was obtained from the Culture Collection of Marine Phytoplankton (C.C.M.P.), Bigelow Laboratory, West Boothbay Harbor, Maine. Cultures were axenic or were treated with antibiotics before being grown to a maximum density (approximately 10^6 cells/ml) in 8 liter cultures of f/2 medium [18], as described in [2] with bubbling air.

Rapid Total Genomic DNA Preparation from Diatoms. Cells were harvested by allowing cultures to settle overnight prior to centrifugation at 3000 g for 10 minutes. The extraction methods were modified from Wurtzel et al. [17]. Except where indicated, the following steps were carried out at room temperature. The cells were resuspended in a total volume of 15 ml of 1x lysis buffer (10x lysis buffer: 3.5 M NaCl; 0.1 M Tris-HCl pH 7.6; 10 mM EDTA pH 8.0) with 0.2% dithiothreitol (DTT) and ruptured by a French press at 6000 p.s.i. The exudate was added directly into 60 ml of extraction buffer (12 ml 10x lysis buffer, 25 g urea, 4.0 ml buffered phenol, 12 ml 0.5 M EDTA, 6 ml 20% sodium sarcosinate (Sigma) and 0.2% DTT. One ml of Triton X-100 (Sigma) was added and this solution was inverted gently for 15 minutes. An equal volume of buffered phenol:chloroform:isoamyl alcohol (300:100:1) was added and inverted for an additional 10 minutes. The extracted aqueous layer was recovered by centrifugation at 3000 g for 10 minutes and then re-extracted. Equal volumes of chloroform:isoamyl alcohol (24:1) were used to extract the aqueous layer until it lost its pink color. Nucleic acids were precipitated by mixing in one-tenth volume of 3 M sodium acetate (pH 5.2), adding one volume of ethanol, and incubating overnight at -20°. Total genomic DNA was pelleted by centrifugation at 12,000 g for 10 minutes. The nucleic acid pellets were then rinsed with 70% ethanol and centrifuged at 12,000 g for 10 minutes. The pellets were lyophilized briefly to remove traces of ethanol and then resuspended in 1 ml of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). The following were added sequentially: 20 ul of proteinase K (stock at 20 mg/ml), 15 ul 5M NaCl, and 10 ul 20% SDS. After incubation for 10 min at 65° followed by 2 hours at 37°, the suspension was extracted with buffered phenol-chloroform-isoamyl alcohol followed by chloroform-isoamyl alcohol. The nucleic acids were then precipitated as above, resuspended in 500 ul TE buffer and stored at -20°. Approximately two-thirds of the total nucleic acid present was RNA. This procedure typically yielded 400-950 ug of DNA.

Endonuclease Digestion and Agarose Gel Electrophoresis. Total genomic DNA (5ug) was digested with 20 units of Hind III (Bethesda Research Lab.) for 3-4 hr at 37° in the presence of 4 mM spermidine in the vendor's buffer. Electrophoresis in 0.6% agarose gels was carried out in TBE buffer (89 mM Tris pH 8.0; 89 mM borate; 2 mM EDTA) at 1.4 V/cm. The gels were stained with ethidium bromide and photographed on a transilluminator. To serve as a reference between this study and studies by other investigators, DNA from strain Skel was run on each gel as a control. Lambda DNA digested with Hind III was electrophoresed on all gels to provide size markers.

Southern Blot Hybridization. DNA from the agarose gels was transferred to nitrocellulose [18] by blotting for a minimum of 20 hr. Filters were prehybridized in 25% formamide, 75 mM sodium citrate, 0.75 M sodium chloride, 5x Denhardts, 0.5 mg/ml salmon sperm DNA, and 0.4% SDS at 37° for 3-4 hr [12]. DNA hybridization probes were labelled with ^{32}P by the random prime method [19]. Hybridizations were carried out in 1 ml of the above solution with 10^6 dpm/ml/filter at 37° for 36-48 hr. The filters were washed twice at room temperature with 2x SSC and several times with 1x SSC at 42-45°. Filters were exposed to X-ray film for 1-3 d at -70°.

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Table 1. Isolation Data for the Strains of Skeletonema costatum. The designation follows the terminology of [2].

Strain Designation	Isolation Date	Location	Source
Skel Standard	1956	Millford Conn.	C.C.M.P.
UP45 Summer Homozygote	8/17/76	Narragansett Bay, R.I.	J.C.G.
NY17 Winter Homozygote	1/6/76	Narragansett Bay, R.I.	J.C.G.

Figure 1. Stained agarose gel of total genomic DNA digested with *Hind* III. Source of DNA is noted above each lane. Shown at the left are lengths (in Kb) of DNA size markers generated by digestion of Lambda DNA with *Hind* III.

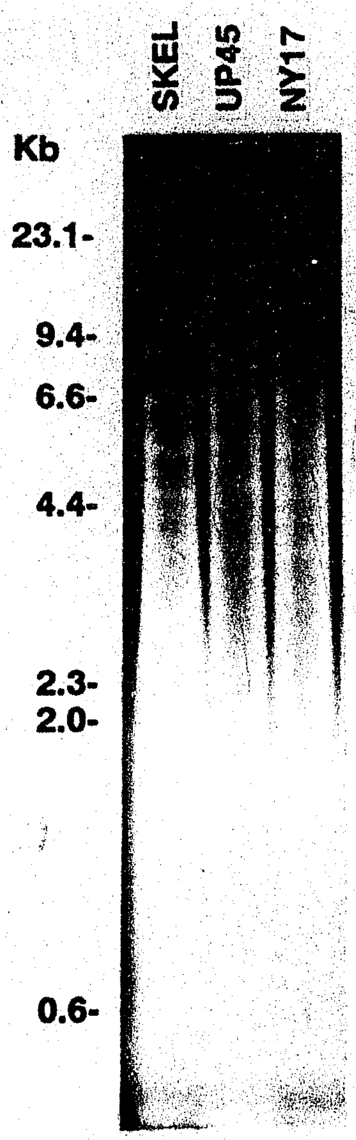

SKEL
UP45
NY17
Kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
0.6-

Figure 2. Hybridization of *Hin*d III digested total genomic DNA with the 9.0 kb tobacco chloroplast probe, Ba4. This probe is from the large single coding region and contains the following genes: *trnR*, *atpA*, *atpF*, *atpH*, *atpl*, *rpoC* [20]. Sizes, in Kb, of the hybridized restriction fragments are shown on the right.

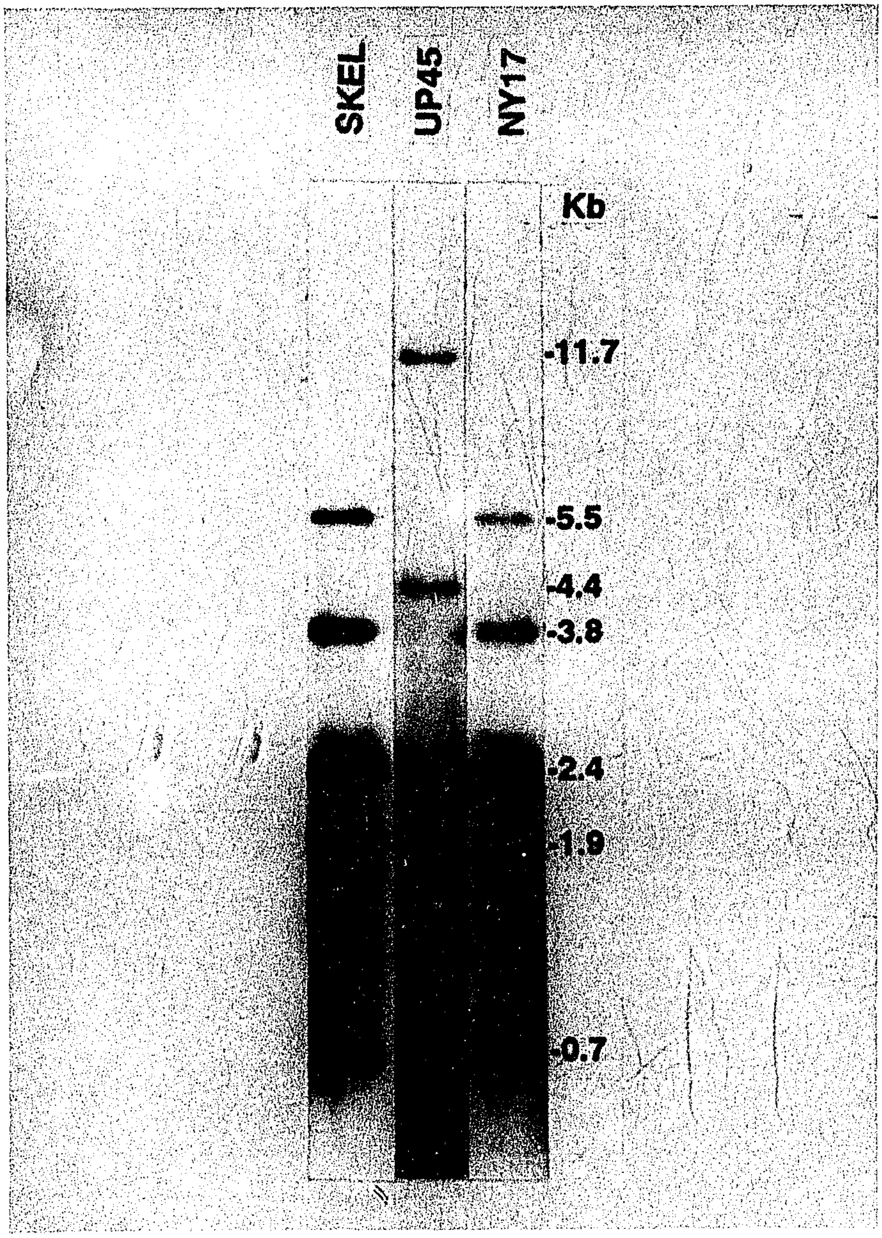
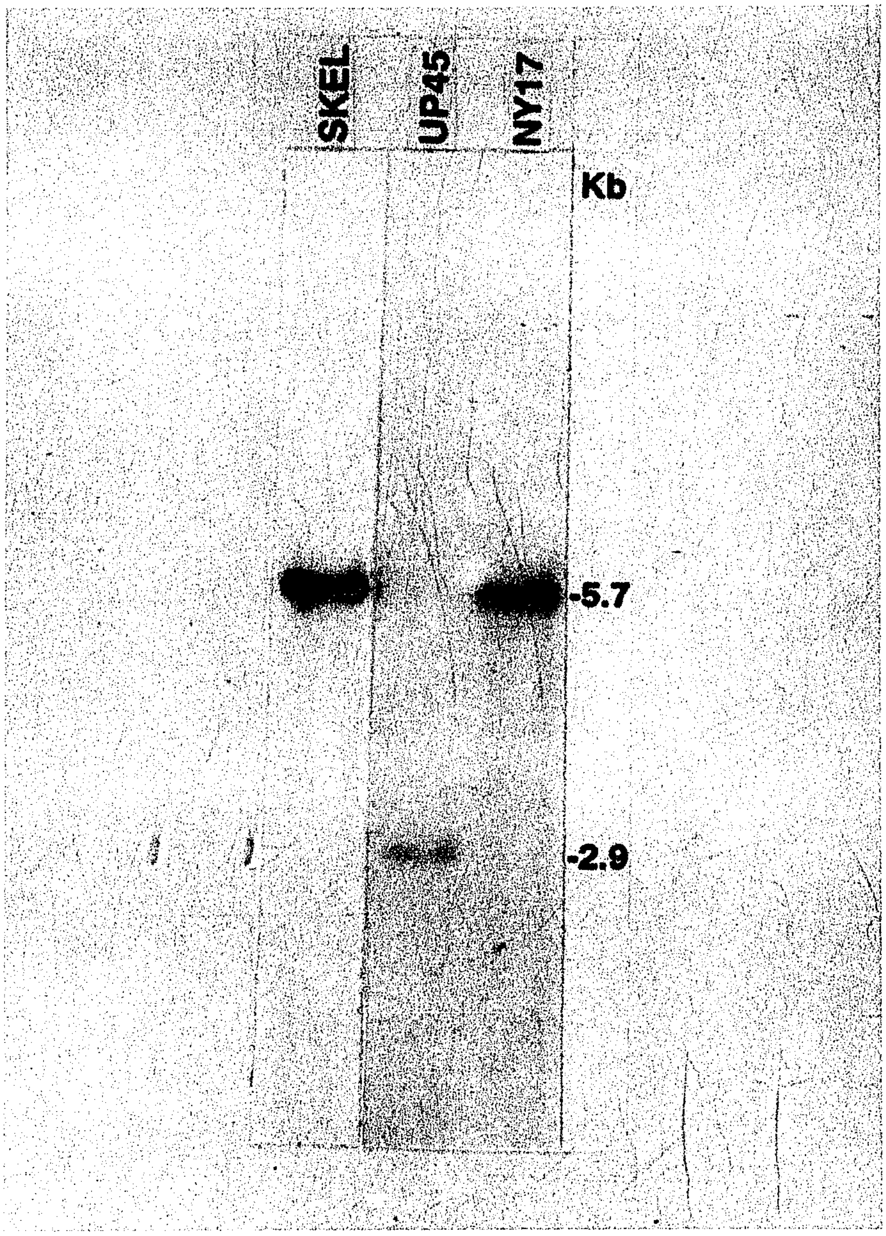

SKEL
UP45
NY17
Kb
11.7
5.5
4.4
3.8
2.4
1.9
0.7

Figure 3. Hybridization of Hind III digested total genomic DNA with the 800 bp Xho I fragment of probe Ba5. This probe is from the inverted repeat section of the tobacco chloroplast genome and contains the gene for the 23S rDNA [20]. Sizes, in Kb, of the hybridized restriction fragments are shown on the right.


SKEL
UP45
NY17
Kb
-16.0
-12.5
-8.3
-6.5
-3.4
-0.54

Figure 4. Hybridization of Hind III digested total genomic DNA with the 1.7 Kb probe atp2-1. This is a nuclear cDNA probe from maize and contains the gene for the beta subunit of the mitochondrial ATP synthase [21]. Sizes, in Kb, of the hybridized restriction fragments are shown on the right.


SKEL
UP45
NY17
Kb
-5.7
-2.9

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Chapter 2

A COMPARISON OF CHLOROPLAST DNA AND ALLOZYME VARIATION IN THE WINTER STRAINS OF THE MARINE DIATOM <u>SKELETONEMA</u> <u>COSTATUM</u>

<u>J. Phycol.</u> 28: 90-94 (1992)
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ABSTRACT

Restriction fragment length polymorphisms (rflps) were examined in twelve strains of the marine diatom *Skeletonema costatum* (Grev.) Cleve using homologous chloroplast gene probes. The winter strains examined included eight different allozyme genotypes exhibiting physiological differences. These twelve strains are representative of the least diverse genetic group present in Narragansett Bay populations. Five chloroplast DNA probes and four different restriction enzymes were used to analyze the 12 Narragansett Bay strains and a reference strain "Skel". A total of 46 restriction fragments were identified. All twelve of the winter strains had identical patterns. Strain Skel exhibited 2 rflps in comparison to the Narragansett Bay strains. Calculated diversity within the winter strain group was 0.0 and 0.85 for the chloroplast DNA and allozyme data, respectively. The chloroplast DNA polymorphisms revealed by this study are expected to represent a minimum level of the chloroplast DNA diversity present in Narragansett Bay seasonal populations.

INTRODUCTION

Early studies of higher plant chloroplast DNA (cpDNA) revealed that rflps are rare at the intraspecific and population level (Palmer and Zamir, 1982; Banks and Birky, 1985, Sytsma and Schaal, 1985). Recent findings have indicated that the chloroplast genome has a conservative structure and a low rate of sequence divergence (Palmer, 1987, Zurawaski and Clegg, 1987). This lack of diversity has limited rflp analysis of cpDNA to studies of taxonomic relationships above the species level in higher plants. Recently, some exceptions to the higher plant archetype have been reported. These studies demonstrate that intraspecific variation does exist for a few species of higher plants (Neale et al., 1988; Soltis et al., 1989).

In contrast, two studies have implied that cpDNA rflps may be commonplace at lower taxonomic levels in chromophytic algae. One study demonstrated that intraspecific variation exists in the xanthophyte Vaucheria sessilis. Eighteen strains isolated from different geographic locations were shown to exhibit rflps. Even two strains isolated from one location exhibited differences (Linne von Berg and Kowallik, 1987). We have recently shown that three strains of the marine diatom Skeletonema costatum (Grev.) Cleve also exhibited numerous rflps when using heterologous cpDNA probes (Stabile et al., 1990). However, the small number of strains examined from each location in these two studies did not allow for the quantification of the population divergence.

One of the most completely characterized populations of chromophytic algae are those of S. costatum isolated from Narragansett Bay, R.I. These strains are morphologically identical, yet differ in physiological traits such as growth rates, size and number of photosynthetic units, photosynthesis-irradiance characteristics, and allozyme banding patterns (Gallagher, 1980; Gallagher et al., 1984; Gallagher and Alberte, 1985). These characteristics have remained stable during their maintenance in culture since 1975-1977. Summer and winter populations of S. costatum are assemblages of strains that vary in the prevalence of different genotypes. Individual strains isolated from these populations can be designated as "winter", "summer", or "mixed" on the basis of their allozyme pattern and corresponding physiologies (Gallagher, 1982). Winter populations have the lowest genetic diversity based on allozyme data (Gallagher, 1980; Gallagher, 1991). Eight of the strains examined in this study were isolated during the winter and are representative of the winter bloom populations. The other four strains have similar allozyme banding patterns to those prevalent in the winter blooms but were isolated during the summer. These strains differ slightly in physiology from those isolated in the winter (Gallagher, 1982).

Since the winter strains can be separated into groups on the basis of allozyme and physiological data, and preliminary rflp data indicated that very few cpDNA fragments were shared among the winter, summer, and mixed strains (Stabile et al., 1990), it was determined that a more comprehensive analysis of DNA variation was warranted. The primary goal of the present study was to determine if cpDNA polymorphisms exist among the 12 winter strains which have similar, but not identical, allozyme patterns and physiological characteristics.

MATERIALS AND METHODS

Strains and culture conditions. The winter strains of Skeletonema costatum are listed in Table 1 along with their allozyme banding patterns and isolation date. Notation follows that of Gallagher (1982). Strains isolated from winter populations can be divided into two groups with respect to allozyme patterns. Designation of strains as homozygous, "WH(w)", or heterozygous, "Whet", is made on the basis of the allozyme genotype of the superoxide dismutase (TO-2) locus. Strains with banding patterns similar to the winter bloom populations are a minor component of the summer blooms. Four of these strains designated, WH(s), were also examined (Table 1). Strain "Skel" was obtained from the Culture Collection of Marine Phytoplankton, Bigelow laboratory, West Boothbay Harbor, Maine. Culture conditions were as previously described (Stabile et al. 1990).

Total genomic DNA preparations. Total genomic DNA was extracted and purified as previously described with the exception that 3M sodium acetate was not added at the first precipitation step (Stabile et al., 1990).

Cloning of cpDNA. Axenic cultures of Skel were used as a source for cloning plastid DNA. Total genomic DNA preparations from Skel were centrifuged in cesium chloride (CsCl)/bisbenzamide density gradients (Aldrich and Cattolico 1981) overnight in a VTi65.2 rotor (Beckman) at 45,000 rpm. After centrifugation, two bands were visible. An upper band, corresponding to chloroplast DNA, was isolated and centrifuged several more times to remove any contaminating nuclear DNA present in the lower band. The DNA was extracted several times with isopropanol saturated with CsCl and water to remove bisbenzamide, and dialyzed against T.E. (10mM Tris-HCl, pH 8.0; 1mM ethylene diaminetetraacetic acid, pH 8.0), to remove CsCl. DNA from the upper band was digested with Hind III, ligated to plasmid vector pT7/T3 alpha-19 (Bethesda Research Laboratories) previously digested with Hind III and treated with calf intestinal alkaline phosphatase, and transformed into E. coli. Recombinants were identified by a white colony phenotype after plating on media containing 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) and isopropylthiogalactoside (IPTG) (Sambrook et al., 1990). Colony hybridizations were performed on recombinant DNA clones using individual photosynthetic genes and cloned fragments of the chloroplast genome of tobacco as hybridization probes (Shinozaki et al., 1986). Positive clones were confirmed by using cloned diatom Hind III fragments as probes to nylon filters containing the cloned fragments of the tobacco chloroplast genome. For each identified recombinant colony, the cloned DNA was purified and shown to hybridize to the tobacco cpDNA used in screening. In addition, both diatom and the corresponding tobacco cpDNA probes were hybridized to total genomic DNA to insure that both hybridize to a common DNA fragment generated by either Hind III or Pst I digestion. The five diatom probes used in this study are described below.

Probe 4. Tobacco probe Ba1 (notation of all tobacco cpDNA fragments follows Shinozaki et al. [1986]) was used to recover recombinant probe 4, a 2.3 Kb Hind III fragment. Probe 4 hybridizes to Ba1 DNA. Both of these probes hybridize to a 2.3 Kb Hind III fragment and a 16 Kb Pst I fragment of genomic Skel DNA.

Probe 19. Tobacco probe, B13, was used to select a recombinant colony containing diatom probe 19, a 1.4 Kb Hind III fragment. Probe 19 hybridizes to B13 DNA. Also, 19 and B13 hybridize to a 1.4 Kb Hind III fragment and a 16 Kb Pst I fragment.

Probe 20. Tobacco probe, Ba1, was used to select a recombinant colony containing diatom probe 20, a 3.6 Kb Hind III fragment. Probe 20 hybridizes to Ba1 DNA. In addition, both 20 and Ba1 hybridized to a 3.6 Kb Hind III and to an 8 Kb Pst I fragment of a total genomic DNA digest.

Probe 27. Tobacco probe Ba8 hybridized to a recombinant containing diatom probe 27, an 8.5 Kb Hind III fragment. Ba8, contains the photosystem II gene psbA. Probe 27 hybridizes to Ba8, and also to Bam HI fragment Ba2, and Xho I fragment X6. Ba2 and X6 correspond to overlapping regions in the small single coding region of tobacco and are distant from Ba8 on the tobacco cpDNA map. When used as hybridization probes to genomic diatom DNA, probe 27, Ba2, and X6 hybridize to an 8.5 Kb Hind III fragment and to both a 22 Kb and an 18 Kb Pst I fragment.

Probe 64. Tobacco probe Ba16 was used to select a recombinant containing probe 64, a 6.3 Kb Hind III fragment. Probe 64 hybridizes to Ba5, B13, and Ba16 of the tobacco chloroplast genome. Ba5 and B13 map to the inverted repeat of the tobacco chloroplast genome. Ba16 is a Bam HI fragment distantly located in the large single coding region and encodes the photosystem I gene psaA as well as a small piece of psaB. When used as hybridization probes, 64, Ba5, B13, and Ba16 all hybridize to a 6.3 Kb Hind III fragment and a 22 Kb Pst I fragment of Skel genomic DNA. In addition, probes 64, Ba5, and B13 hybridize to an 18 Kb Pst I fragment, although Ba16 does not.

Restriction enzyme digestion, agarose gel electrophoresis and Southern hybridizations. Genomic DNA, 0.2 - 0.4 ug, from the 12 winter strains and Skel was digested with the following restriction enzymes: BstN I, Hind III, Eco RI, Eco RV, (Bethesda Research Lab.) and subjected to gel electrophoresis as previously described (Stabile et al., 1990) and then transferred to nylon filters (Zetaprobe, Biorad) according to the method of Southern (Southern, 1975). Hybridization probes were labelled using the random prime method (Feinberg and Vogelstein, 1978) and hybridization conditions followed the method of Wurtzel et al. (1987)

Data Analysis. Genetic diversity was calculated using the method of McArthur et al., 1988. Following the equation, $D = 1 - \sum (x_i)^2$ where D is diversity and (X_i) is the proportion of the same genotype within the sample. This diversity calculation has the advantage of being independent of all models of evolution and can be used with both cpDNA rflp and allozyme data. It has the disadvantage that it will probably increase at some unknown rate as both the sample size and the proportion of the genome examined increases, and therefore can only be used to analyze approximately equivalent number of characters and taxa. Other methods cannot be used to analyze these data because they either cannot be applied to both allozyme and rflp data or because they are dependent on models of evolution whose applicability to these data is unknown.

RESULTS

Twelve strains of S. costatum examined in this study are representative of the winter strains isolated from the seasonal populations of Narragansett Bay. The eight different allozyme genotypes of the winter strains are shown in Table 1. Skel has identical allozyme patterns as NY17, NY27, and ST37. As shown in Table 2, a total of 46 cpDNA fragments were visualized using the five diatom probes. No differences were exhibited among any of the Narragansett Bay winter strains. The Narragansett Bay strains shared a total of 44 fragments. Strain Skel shared 44 restriction fragments with the winter strains but had unique restriction fragments when digested with <u>Eco</u> RI and hybridized to probe 20 (Figure 1a) and probe 64 (Figure 1b). To rule out the possibility of partial DNA digestion in Figure 1b, the filter was reprobed with a subfragment of probe 20. Probing with this subfragment resulted in only one band in each lane (data not shown). The 12 winter strains exhibited 8 different allozyme genotypes when variation at all 5 loci is considered, but only 1 chloroplast genotype was identified. This yields genetic diversity values of 0.85 and 0 for the allozyme data and cpDNA data respectively. If Skel is included in the calculation, the values for diversity are 0.83 for the allozyme and 0.14 for the cpDNA data.

DISCUSSION

One cpDNA pattern and eight allozyme patterns were observed among the 12 winter strains. The low level of cpDNA diversity is not surprising since studies of higher plants have indicated that cpDNA evolves at a slower rate than does nuclear DNA (Wolfe et al., 1987). The lack of correspondence between allozyme banding patterns and cpDNA rflp data might be due to different modes of inheritance of the chloroplast and nuclear genomes. The chloroplast genome is generally inherited uniparentally in higher plants and is not subject to recombination (Palmer, 1987). Skeletonema costatum is oogamous (Migita, 1967) suggesting that the chloroplast genome may be maternally inherited when sexual reproduction occurs. In contrast, allozyme loci are representative of nuclear genes that are biparentally inherited and subject to recombination.

CpDNA polymorphisms were exhibited between strain Skel and the Narragansett Bay strains even though Skel shared identical allozyme banding patterns with three of the latter strains. This may reflect either spatial or temporal differences between the Narragansett Bay and Long Island Sound populations of S. costatum that were not detected previously. Both the allozyme and cpDNA data support the cohesiveness of the winter type strains as compared to the summer and mixed groups isolated from Narragansett Bay (Stabile et al., in prep.). The winter populations from Narragansett Bay have the least allozyme diversity of any seasonal population. Therefore, the cpDNA diversity reported here is expected to represent a minimum level of diversity for all the Narragansett Bay populations.

Our earlier study using heterologous cpDNA probes suggested that winter and summer type strains had divergent chloroplast genomes (Stabile et al., 1990). Few restriction fragments were shared by both seasonal strains in that preliminary work. However, under the conditions of low stringency necessary for hybridization, heterologous cpDNA probes have the potential to hybridize to nonchloroplast DNA (Li and Cattolico, 1987). It was also evident from that preliminary study and from characterization of cpDNA recombinant clones reported here, that the chloroplast genome of S. costatum is rearranged as compared to that of tobacco. The use of heterologous probes made it difficult to interpret whether the polymorphisms observed between the winter and summer type strains were due to length polymorphisms, rearrangements, base pair substitutions or hybridization to other organelle genomes. This necessitated the isolation and use of homologous cpDNA probes. Furthermore, homologous chloroplast probes can now be used to generate cpDNA maps which would allow for the determination of the underlying causes of the observed variation among strains. This determination is critical for the purpose of data analysis. Most methods, such as the site difference method or fragment length difference method (Nei and Li, 1979; Nei, 1988) calculate genetic divergence by comparing the proportion of shared sites or fragments between two individuals. Both of these methods are based on a model of evolution that assumes changes are due to base pair substitutions at a particular restriction site and are not due to rearrangements and length polymorphisms. A comparison of chloroplast maps indicates that rearrangements are common during the evolution of this organelle within the chromophytic algae (Cattolico and Goër, 1989; Douglas, 1988; Kowallik, 1989). At present it is not clear whether length polymorphisms or rearrangements play a significant role in the evolution of cpDNA at the population level. Examination of the summer and mixed strains should reveal the full extent of cpDNA variation within the Narragansett Bay populations. If the preliminary data obtained with heterologous probes is correct, then construction of a cpDNA map will be instrumental in developing a cohesive understanding of the modes of chloroplast DNA evolution at the population level.

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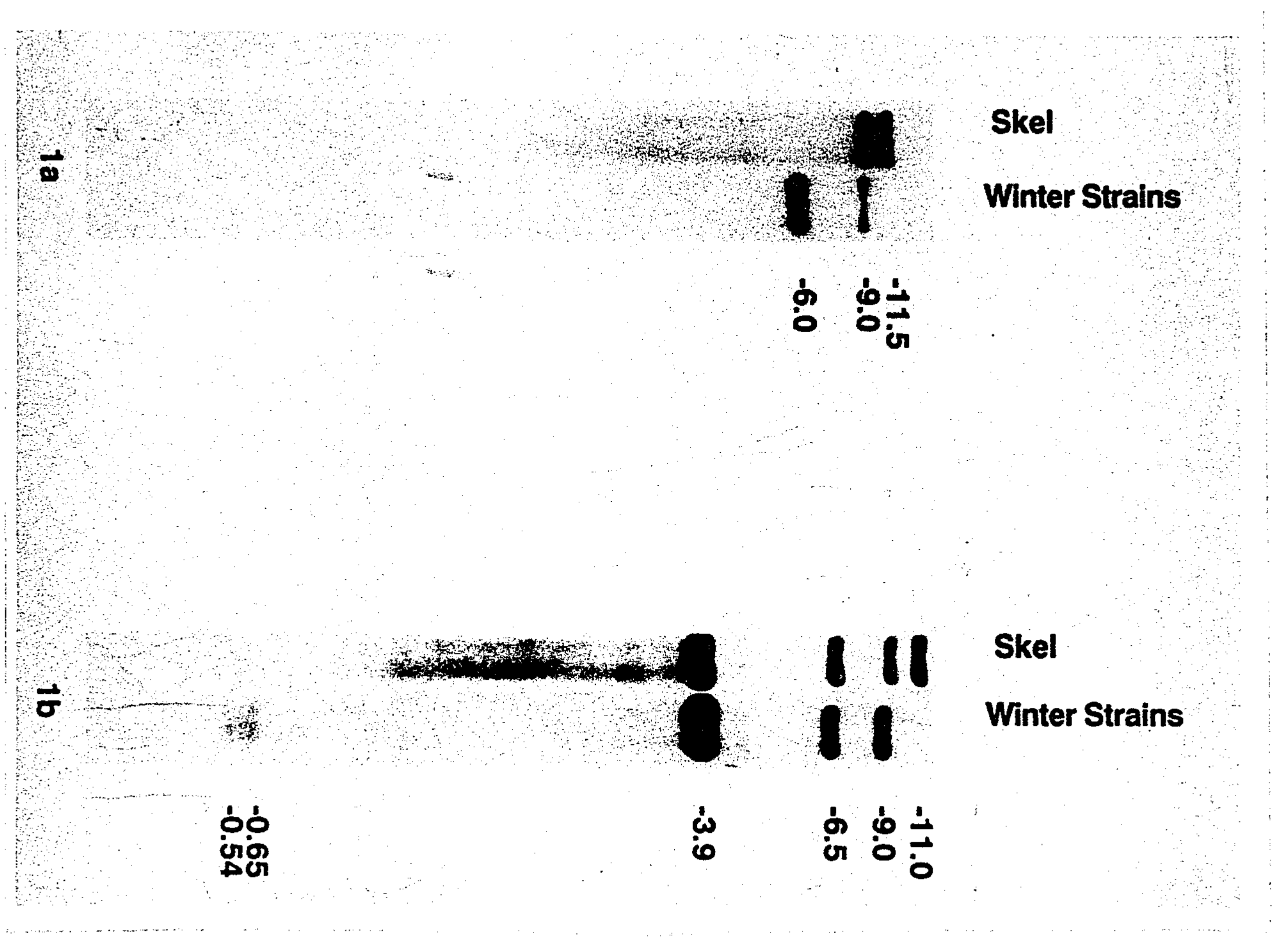
Table 1. Genotypes based on allozyme banding patterns of S. costatum strains. Notation as in Gallagher (1980, 1982) where PGI, MDH, GDH, TO1, TO2 are loci and each letter represents an allele.

Strain		Isolation	Genotype				
		Date	PGI	MDH	GDH	TO1	TO2
NY17	WH(w)	Jan. 6, 1976	BB	KK	GG	UU	RR
ED2	WH(w)	Jan. 11, 1977	BB	KK	EE	UU	RR
NY27	WH(w)	Jan. 6, 1976	BB	KK	GG	UU	RR
MF10	WH(w)	Feb. 1, 1977	BB	KK	EE	UU	RR
HB17	Whet	Jan. 27, 1976	BB	KK	HH	UU	RQ
ED1	Whet	Jan. 11, 1976	BB	KK	GG	UU	RQ
MF15	Whet	Feb. 1, 1977	BB	KK	GG	VV	RQ
MF55	Whet	Feb. 1, 1977	BB	KK	FF	UU	RQ
ST10	Whet	Aug. 17, 1976	BB	KK	GG	UU	RQ
ST37	WH(s)	Aug. 17, 1976	BB	KK	GG	UU	RR
SF73	WH(s)	Aug. 3, 1976	BB	KK	HH	WW	QQ
UP3	WH(s)	Aug. 17, 1976	BB	KK	HH	UU	RR
Skel	Standard	Season unknown 1956	BB	KK	GG	UU	RR

Table 2. Sizes of restriction endonuclease fragments (kb).

Probe	Strains	*Hind* III	*Eco* RI	*Bst*N I	*Eco* RV
4	All Strains	2.3	8.9, 0.7	1.6, 1.5	11.5
19	All Strains	1.4	5.2, 4.7	2.1, 0.7	6.4
20	Skel	3.6	11.5, 9.0	4.8, 1.6	6.1, 2.4
	Winter Strains	3.6	9.0, 6.0	4.8, 1.6	6.1, 2.4
27	All Strains	8.5	11.5, 10.5, 6.6, 2.5	4.7, 4.3 3.1, 2.6	7.2, 4.3, 4.1 3.8, 0.7
64	Skel	6.3	11.0, 9.0, 6.5, 3.9, 0.65, 0.54	2.6, 1.2, 0.9	8.2, 7.5
	Winter Strains	6.3	9.0, 6.5, 3.9, 0.65, 0.54	2.6, 1.2, 0.9	8.2, 7.5

Figure 1. Restriction fragment length polymorphisms detected in cpDNA. Genomic DNA digested with _Eco_ RI and probed with diatom probe 20 (a) and diatom probe 64 (b). Fragment sizes are in kb.


1a
Skel
Winter Strains
-11.5
-9.0
-6.0
1b
Skel
Winter Strains
-11.0
-9.0
-6.5
-3.9
-0.65
-0.54

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Chapter 3

THE CHLOROPLAST GENOME OF THE MARINE DIATOM SKELETONEMA COSTATUM (BACILLARIOPHYTA)

ABSTRACT

The chloroplast genomes of three isolates of the marine diatom *Skeletonema costatum* were mapped. These data are compared to chloroplast DNA maps of three other diatom species in order to determine the appropriateness of restriction site analysis in the evolutionary studies of this group. Chloroplast DNA of *S. costatum* is a circular molecule of 131 ± 2 Kb. It contains both large and small single copy regions separated by an inverted repeat. This repeat contains rRNA and *psbA* genes in orientation similar to that of *Coscinodiscus* and *Cyclotella*, two other diatom genera. I map the *rbcS* gene to the same chloroplast DNA fragments as the *rbcL* gene using an oligonucleotide probe, derived from the alignment of published sequences. The large single copy region features an apparent inversion relative to the freshwater diatom *Cyclotella meneghiniana*. Low resolution maps of two other strains of *S. costatum* reveal no rearrangements and differences appear to be the result of site mutations. These maps also demonstrate that extensive restriction site variation exists among these three strains. I conclude that simple restriction fragment analysis of the chloroplast genome will be

useful in comparative studies at lower taxonomic levels of diatoms and that results obtained with heterologous chloroplast DNA probes may be misleading.

INTRODUCTION

Chloroplast DNA (cpDNA) has been widely used as a molecular marker to determine the phylogenetic relationships among groups of higher plants (Clegg and Zurawski, 1992). Extant information obtained from over 1000 maps of terrestrial plant cpDNA's shows that rearrangements occur infrequently (Palmer, 1991). Commonly held rearrangements therefore, provide strong evidence for monophyly and have been important in studying relationships above the intergeneric level (Jansen et. al., 1992; Downie and Palmer, 1992). Below the genus level, restriction analysis of the chloroplast genome has been used extensively in elucidating evolutionary relationships (Doebly, 1989; Soltis et al., 1991; Milligan, 1991).

Relatively little information exists on the chloroplast genomes of the other two major photosynthetic eukaryotic lineages, Chromophytes and Rhodophytes. The nine cpDNA maps that exist for chromophyte algae reveal that this genome is extensively rearranged among representatives of the distantly related Cryptophyta (Douglas, 1988), Chrysophyta (Cattolico and Louiseaux-de Goër, 1989), Phaeophyta (Kushel and Kowallik, 1987), Xanthopyta (Linne von Berg and Kowallik, 1988) and Bacillariophyta (Kowallik, 1989; Bourne et. al., 1992). Even within the Bacillariophyta, cpDNA maps of the centric diatoms *Odontella sinensis*, *Cyclotella menghiniana* and *Coscinodiscus granii* are highly rearranged (Kowallik, 1989; Bourne et. al., 1992) and indicate that there are many differences between the generalized terrestrial plant and diatom chloroplast genomes. First, the diatom genome is smaller, typically between 120 - 130 Kb. Second, diatom cpDNA's possess several unique genes, such as the *rbcS* and *tufA* genes, that are nuclear-encoded in higher plants (Hwang and Tabita, 1991; Bourne et al., 1992). Also within the diatom genera, *Coscinodiscus* and *Cyclotella*, the photosystem II gene *psbA* has been mapped to the inverted repeat (Kowallik, 1989; Bourne et. al., 1992).

At this juncture, the utility of cpDNA restriction analyses in diatoms and other chromophytes has not been determined (Bourne et. al., 1992). If rearrangements are frequent at all taxonomic levels, then restriction analyses used so successfully in terrestrial plants are not appropriate in this group of algae. As more information accumulates on the size and structure of diatom chloroplast genomes, it will be possible to determine the utility of cpDNA analyses in these organisms. I present here the cpDNA maps of three strains of the marine diatom species, Skeletonema costatum. This species is perhaps the most popular laboratory test organism in phytoplankton ecology (Gallagher, 1982). Prior research has indicated that these strains are extremely divergent for a variety of genetic and physiological characters (Gallagher, 1982; Gallagher et al., 1984). Also, their cpDNA restriction fragment profiles are divergent as determined by heterologous cpDNA probes (Stabile et al., 1990).

MATERIALS AND METHODS

Culture Conditions. Strain "Skel" was obtained from the Culture Collection of Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, Maine. This strain was mapped in greater detail because of its general availability and its common use as a test strain in phytoplankton ecology. The strains "ED1" and "UP45" were isolated from Narragansett Bay, Rhode Island by Gallagher (1980) and are representative of the prevalent winter and summer bloom populations respectively. Culture conditions for all strains were as previously described (Stabile et al., 1990).

Total Genomic DNA Extractions and Isolation of cpDNA. Chloroplast DNA was purified from total genomic DNA as previously described (Stabile et al., 1992). One to three ug aliquots of cpDNA were digested with *Pst* I, *Sst* I or *Bgl* II according to vendor's instructions (Bethesda Research Laboratories). Fragments were resolved on agarose gels of 0.4 - 0.8% and then isolated by electroelution. Because fragments P2, P3, P6, P7, and P8 were similar in size, they were additionally gel purified by electrophoresis in 0.6% low melting point agarose (Sea Plaque, FMC Corp.) at 0.5 V/cm for approximately 60 hours. All isolated fragments, as listed in Figure 1, were used directly as homologous hybridization probes to generate the cpDNA maps.

Restriction Enzyme Digestion, Agarose Gel Electrophoresis, Southern Blotting and Homologous Hybridizations. One hundred ng of purified cpDNA was digested with *Bgl* II, *Pst* I or *Sst* I. Double digestions were also performed for each possible combination. Digested DNA was subjected to electrophoresis on a 0.4% gel (20 X 24 cm) and transferred to nitrocellulose by a

Possiblot Pressure Blotter (Stratagene Inc.) according to the manufacturer's instructions. DNA was crosslinked to the nitrocellulose using ultraviolet radiation (UV Stratalinker, Stratagene, Inc.). Radioactive labeling of probes and homologous hybridizations were performed as previously described (Stabile et al., 1992).

Localization of Individual Chloroplast Genes. The heterologous chloroplast genes used in this study were obtained from Nicotiana tabacum or Chlamydamonas rienhardtii. In most instances, individual genes were obtained by single or double restriction enzyme digestion. Heterologous hybridizations were done according to Stabile et. al. (1990). An oligonucleotide probe, identical to a conserved area in the 5' region of the rbcS gene was synthesized. This conservative area was discovered by aligning the rbcS sequence of Olisthodiscus luteus (genbank accession M24288) and Cryptomonas **Φ** (Boszcar et al., 1989; Douglas and Durnford, 1989). The 18 base pair sequence we used is as follows: TGAGACTTACACAAGGAG and is complimentary to sequence position 2-19 of Cryptomonas **Φ**. The oligonucleotide was radiolabeled by phosphorylation using T4 Kinase (Sambrook et al., 1989). Hybridizations were at 25°C in 6XSSPE (0.9 M Nacl, 60 mM NaH_2PO_4, 6 mM EDTA), 10X Denhardt's (1% w/v of each Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.5% sodium pyrophosphate, 0.1% sodium dodecyl sulfate (SDS) and 100 ug/ml salmon sperm DNA. The filters were washed two times each in 6XSSPE at 37° C for 20 minutes and then exposed to x-ray film for 7 days at - 85° C.

Mapping of Strains "ED1" and "UP45". Approximately 100 ng of genomic DNA from "ED1" and "UP45" was digested with *Bgl* II, *Pst* I or *Sst* I, or double digested with a combination of these three enzymes. Digested DNA's were subjected to electrophoresis and transferred to nitrocellulose as described above. Fragments S1, S3, S5, S6, S7, B1, B2, B3, B4, B5, B8, B10, P3, P6, P7, P8, and P10, all from strain "Skel", were used as hybridization probes.

RESULTS

Physical mapping. The chloroplast DNA restriction site map of "Skel" was generated using the cloned and gel purified fragments covering 100% of the chloroplast genome (Table 1). Hybridizations were repeated to the same regions using fragments obtained with all three enzymes to confirm their proper alignment.

The sizes of the large _Bgl_ II fragments (B1, B2, B3), _Sst_ I fragments (S1, S2, S3, S4, S5) and _Pst_ I fragments (P1, P2, P3, P4, P5) listed in Figure I, were calculated by probing with each of these fragments and then adding the lengths of internal fragments generated by double digestion. The sum of the fragments for each, _Bgl_ II, _Sst_ I, and _Pst_ I, yields a total genome size of approximately 131 ± 2 Kb. In order to estimate the size of the inverted repeat section, cpDNA was digested with enzymes that cut it frequently (_Eco_ RI and _Eco_ RV). Then the digested DNA was hybridized to probes B2 and B3, which cover both sides of the IR. The sum of the lengths of identical size fragments, distinguished with these two probes yields a value of slightly greater than 20 Kb. Additional data are necessary in order to make a more precise size estimate of this region.

Gene Mapping. Individual gene probes, their sources and the fragments they hybridize to are listed in Table 2. A linear representation of the cpDNA of "Skel" is shown in Figure 3. Genes are mapped to the smallest hybridizing fragment. The inverted repeat contains the _psbA_ gene as well as the 16S and 23S ribosomal RNA genes. Located just outside the inverted repeat (IR) in the large single copy region (LSC) is the _psaA_ gene, followed by the rbcL, atpA, atpB, and psbC genes in sequential order. Hybridization patterns of the rbcS, rbcL, 23S and atpA genes are shown in Figure 2. The rbcS gene mapped to the same fragments as the rbcL gene.

Mapping of S. costatum Seasonal Strains "ED1" and "UP45". The mapped Pst I and Sst I sites for these two strains are illustrated in Figure 3. We only report the Bgl II map for these strains in the IR and small single copy region (SSC) to illustrate the only differences between "Skel" and "ED1". Also, UP45 had three extremely large Bgl II fragments in the LSC that were difficult to distinguish. Apparent differences among the three strains of S. costatum appear to be simple site mutations. Three of the Pst I fragments observed in UP45 were slightly smaller that those from ED1 and Skel. I could not determine whether these differences are due restriction site changes or small insertions/deletions since we did not observe hybridization signals from fragments under 560 bp. The cpDNA's of these three genetically divergent strains of S. costatum appear to be colinear with no major rearrangements present.

DISCUSSION

As in most plant taxa, the chloroplast genome of *S. costatum* is arranged into a LSC and SSC region separated by an IR (Palmer, 1991). The size of the cpDNA of *S. costatum* is 131 ± 2 Kb, which is similar to the 128 of *Cyclotella meneghiniana* (Bourne et. al., 1992). *Coscinodiscus granii* and *Odontella sinensis* have smaller chloroplast genomes of approximately 118 Kb (Kowallik, 1989). This variation in genome size seems to be mainly due to differences in the size of the inverted repeat. The IR of both *C. granii* and *O. sinensis* are only 13 and 8.7 Kb respectively, while those of *C. meneghiniana* and *S. costatum* are 17 and 20 Kb respectively.

The maps of *C. granii*, *C. meneghiniana* and *S. costatum* demonstrate that the location of several chloroplast genes are conserved among these centric diatoms (Fig. 4). In all three, the IR contains the *psbA* gene, and flanking the IR in the LSC is *psaA*. Also, the *atpB* gene appears to be in a similar position on all three maps. In contrast, the *rbcL* and *atpA* gene are in different locations among all three maps. When one includes *O. sinensis* in this comparison, only the location of the *psaA* gene is conserved, and it appears that rearrangements have played an important role in the evolution of cpDNA at higher taxonomic levels within diatoms (Kowallik, 1989; Bourne et. al., 1992).

The rbcS gene of chromophyte algae is chloroplast encoded. DNA sequence data suggests that the chromophyte rbcS gene may have arisen through lateral gene transfer from purple sulfur bacteria (Reith and Cattolico, 1986; Douglas and Durnford, 1989). DNA sequence alignment revealed that a complimentarity of only 66% exists between the rbcS genes of the two chromophytes Heterosigma akashiwo and Cryptomonas Φ (Stabile et al., unpublished). A probe for the rbcS gene from Cryptomonas Φ failed to hybridize to C. meneghiniana cpDNA and further reflects the extreme sequence divergence between distantly related chromophytes (Bourne et. al., 1992). One region in the 5'end of the rbcS gene appears to be conserved across distantly related chromophytes. I synthesized an oligonucleotide probe complimentary to this region and used it to localize the rbcS gene on the cpDNA map of S. costatum. Presently, there has been no determination of the usefulness of cpDNA analyses for the phylogenetic studies of diatoms, since the chloroplast genomes of the distantly related species C. meneghiniana, C. granii and O. sinensis are highly rearranged (Kowallik, 1989; Bourne et. al., 1992). I demonstrate that two more closely related centric diatoms from the

Thalassiosiraceae, S. costatum and C. meneghiniana, are similar in size and in location of the majority of mapped genes. The only exception is an apparent inversion of the atpA and rbcL genes in the LSC. Although, higher resolution mapping data may reveal other differences between C. meneghiniana and S. costatum, the overall similarity between their chloroplast maps implies that restriction analysis will be useful at taxonomic levels, lower than genus. In order to make a precise judgement, however, additional mapping data are needed from closely related species.

Microalgae have much greater levels of intraspecific variation than most other organisms (Gallagher, 1986). Previous allozyme data, indicate that the seasonal strains of S. costatum isolated from Narragansett Bay, Rhode Island have the second highest genetic diversity indices recorded to date (Stabile et. al., submitted; Gallagher in prep). My preliminary study indicated that these seasonal strains have divergent chloroplast genomes (Stabile et al., 1990). Yet I could not infer the extent of the divergence or the underlying mutational processes, because it was difficult to interpret the hybridization patterns we observed using heterologous cpDNA probes (Stabile et. al., 1990). Consequently, it was important to establish the colinearity of the chloroplast genomes of the different seasonal strains of S. costatum in order to confirm the appropriateness of restriction data for the evolutionary study of this group. It appears that the genetically and physiologically distinct strains of S. costatum have colinear chloroplast genomes and the observed differences are predominantly the result of site mutations. There are 16 observed site mutations between the two seasonal strains, "ED1" and "UP45", and a one site difference between "Skel" and "ED1". The extensive site differences observed with these three enzymes is probably an underestimate of the extent of the polymorphisms that would be revealed if more restriction enzymes were used. These findings are in agreement with observations of extensive site differences, but no major cpDNA rearrangements, among isolates of another chromophyte Vaucheria sessilis (Linne von Berg and Kowallik, 1988). From these data I conclude that restriction analysis of cpDNA in diatoms is most appropriate at the population and intraspecific levels.

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Table 1. Fragments hybridizing to radioactive cpDNA probes. Isolated cpDNA was digested with Bgl II, Sst I and Pst I. Hybrization probes are listed in order of decreasing size. A. Hybrization with Bgl II. B. Hybridization with Sst I. C. Hybridization with Pst I.

A. Radioactive Fragments	Hybridizing cpDNA Fragments		
	Bgl II	Sst I	Pst I
B1	B1	S3, S5, S6	P1, P2, P4, P9, P10, P11, P13, P15 (a+b)
B2	B2, B3	S2, S4	P1, P2, P8
B3	B2, B3	S1, S2, S4	P1, P2, P6, P14
B4	B4	S1	P5, P7, P16, P18
B5	B5	S1, S7	P3
B6	B6	S1	P5, P12
B7	B7	S1	P3, P12
B8 (a+b)	B8 (a+b)	S2, S4	P1, P2
B10	B10	S1	P17

B. Radioactive Fragments	Hybridizing cpDNA Fragments		
	Bgl II	*Sst* I	*Pst* I
S1	B3, B4, B6, B7, B9, B10	S1	P3, P5, P6, P12, P14, P16, P17
S2	B2, B3, B8 (a+b)	S2, S4	P1, P2
S3	B1	S3	P4, P9, P10, P11, P13
S4	B2, B3, B8 (a+b)	S2, S4	P1, P2, P6
S5	B1	S5, S6	P1, P2, P4, P15 (a+b)
S6	B1	S5, S6	P1, P2, P15 (a+b)
S7	B5	S7	P3

C. Radioactive Fragments	Hybridizing cpDNA Fragments		
	Bgl II	Sst I	Pst I
P1	B2, B3, B8 (a+b), B11 (a+b)	S2, S4	P1, P2
P2	B2, B3, B8 (a+b), B11 (a+b)	S2, S4	P1, P2
P3	B2, B5, B7	S1, S2, S7	P3
P4	B1	S3, S5	P4
P5	B4, B6, B9	S1	P5
P6	B3	S1, S4	P6
P7	B4	S1	P7
P8	B2	S2	P8
P9	B1	S3	P9
P10	B1	S3	P10
P15 (a + b)	B1	S5, S6	P15 (a + b)
P16	B4	S1	P16
P17	B3, B10	S1	P17

Table 2. Hybridizations with heterologous individual gene probes. Tobacco probes were obtained from Shinozaki et. al., 1986. The *rbcL* and *atpB* genes from *C. reinhardtii* are from Dron et al., 1982 and Woessner et al., 1986, respectively. Fragments that were used as hybridization probes are listed according to their size and the enzyme(s) that were used to isolate them. Their clonal designations are listed in parenthesis.

Probe	Source	Fragment	*Hybridized Fragments*		
			Bgl I	*Sst* I	*Pst* I
rRNA 16 S	*N. tabacum*	3.1 Kb *Bam* HI (Ba11)	B2, B3	S2, S4	P1, P2
rRNA 23 S	*N. tabacum*	0.8 Kb *Xho* I (Ba5)	B2, B3	S2, S4	P1, P2
psaA	*N. tabacum*	2.5 Kb *Bam* HI (Ba16)	B2	S2	P1
atpA	*N. tabacum*	1.2 Kb *Sal* I/*Bgl* II (Ba4)	B5	S7	P3
psbC	*N. tabacum*	1.1 Kb *Pst* I (Ba9)	B3	S1	P14, P17
psbA	*N. tabacum*	1.7 Kb *Bam* HI/*Pvu* II (Ba8)	B1	S5, S6	P15 (a + b)
atpB	*C. reinhardtii*	3.0 Kb *Eco* RI/*Kpn* I (Bam 10)	B4	S1	P16
rbcL	*C. reinhardtii*	2.1 Kb *Pst* I/*Xho* I (Eco 14)	B2	S2	P8
rbcS	Oligonucleotide		B2	S2	P8

Figure 1. 0.5% ethidium bromide stained agarose gel of cpDNA digested with *Bgl* II, *Sst* I, and *Pst* I. Fragments are listed to the right of each lane in order of decreasing size. Fragment sizes (in kb) are listed to the left. Some stained bands are not labelled, because they do not map to the chloroplast genome and probably are mitochondrial DNA.

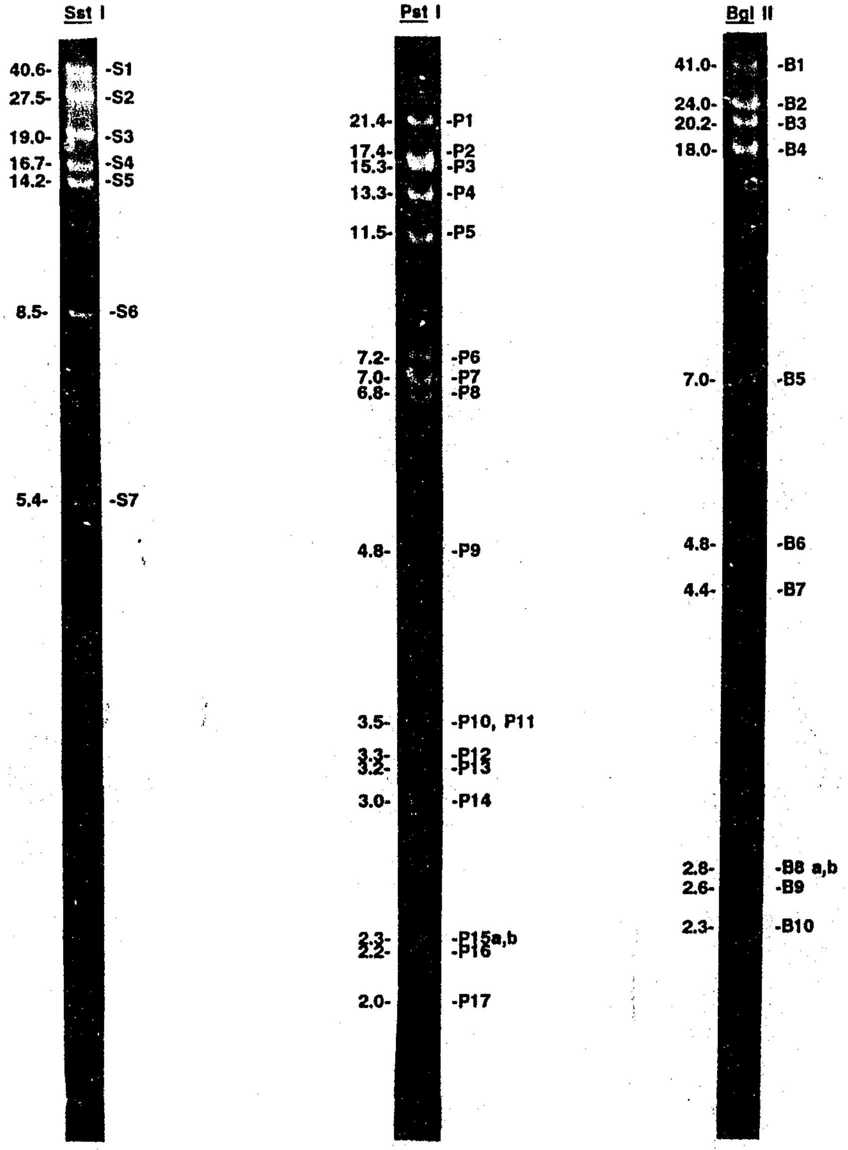
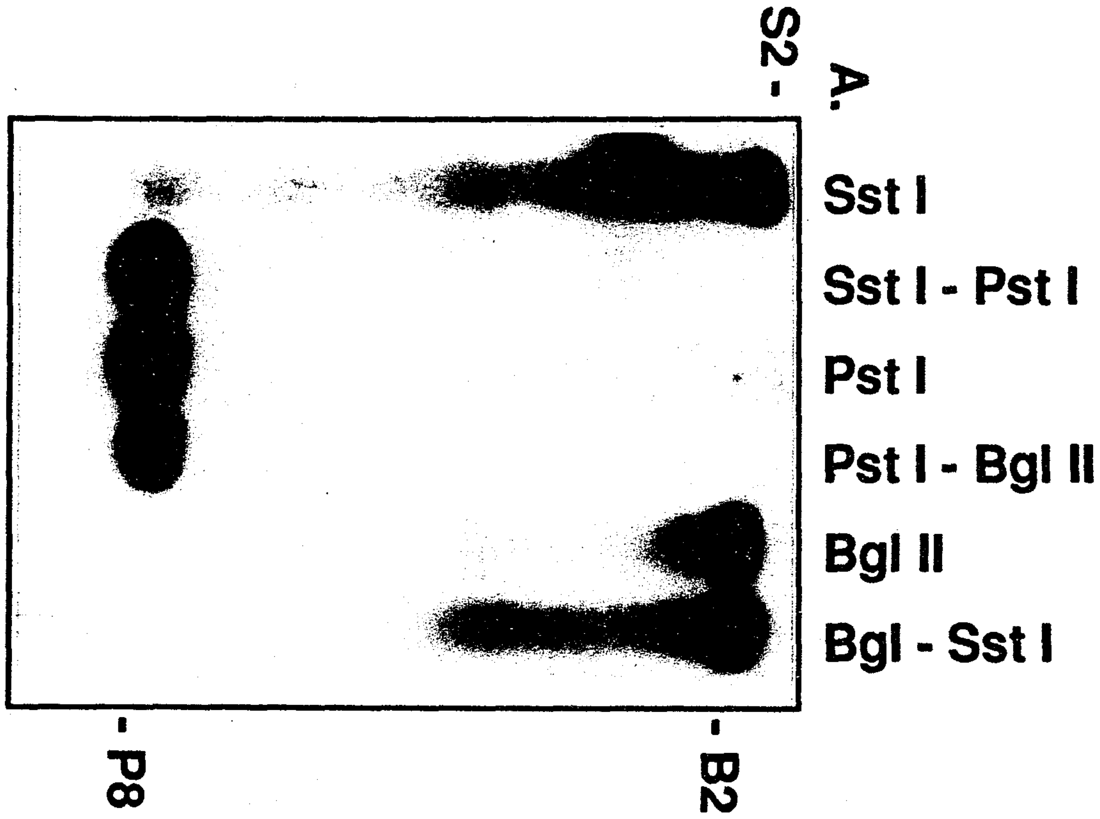
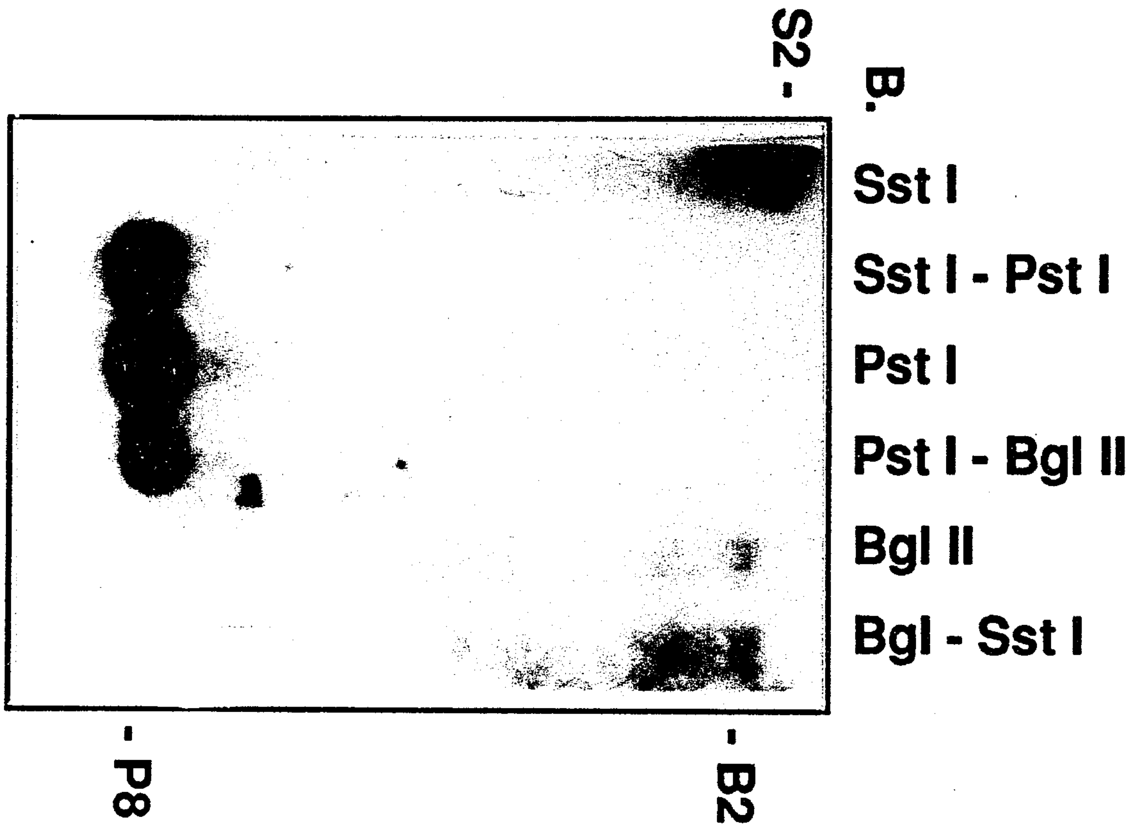
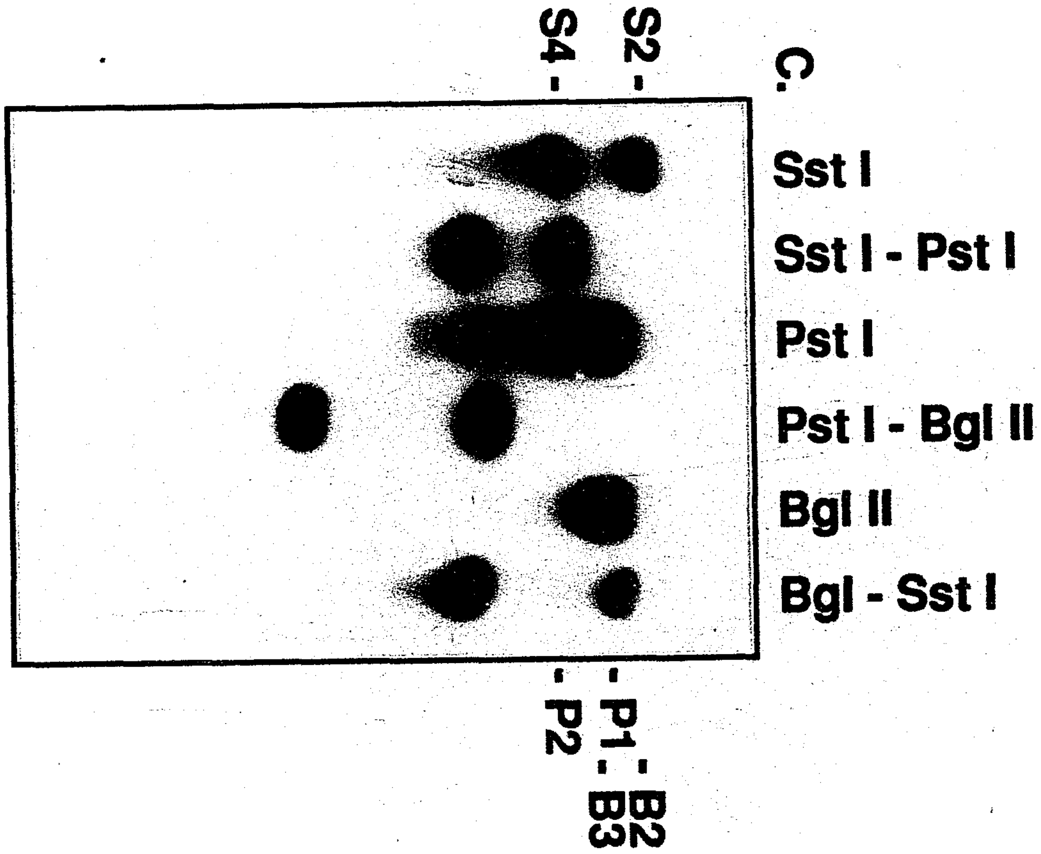

Sst I
40.6- -S1
27.5- -S2
19.0- -S3
16.7- -S4
14.2- -S5
8.5- -S6
5.4- -S7
Pst I
21.4- -P1
17.4- -P2
15.3- -P3
13.3- -P4
11.5- -P5
7.2- -P6
7.0- -P7
6.8- -P8
4.8- -P9
3.5- -P10, P11
3.3- -P12
3.2- -P13
3.0- -P14
2.3- -P15a,b
2.2- -P16
2.0- -P17
Bgl II
41.0- -B1
24.0- -B2
20.2- -B3
18.0- -B4
7.0- -B5
4.8- -B6
4.4- -B7
2.8- -B8 a,b
2.6- -B9
2.3- -B10

Figure 2. Hybridization of cpDNA with individual gene probes. The enzyme(s) used to digest the cpDNA are listed on top of each lane. Probes are: A. rbcS. B. rbcL. C. 23S. D. atpA. Hybridized restriction fragments are shown on the right and left.


A.
S2 -
Sst I
Sst I - Pst I
Pst I
Pst I - Bgl II
Bgl II
Bgl - Sst I
- P8
- B2


B.
S2 -
Sst I
Sst I - Pst I
Pst I
Pst I - Bgl II
Bgl II
Bgl - Sst I
- P8
- B2


C.
S4
S2
Sst I
Sst I - Pst I
Pst I
Pst I - Bgl II
Bgl II
Bgl - Sst I
P2
P1
B3
B2

D.

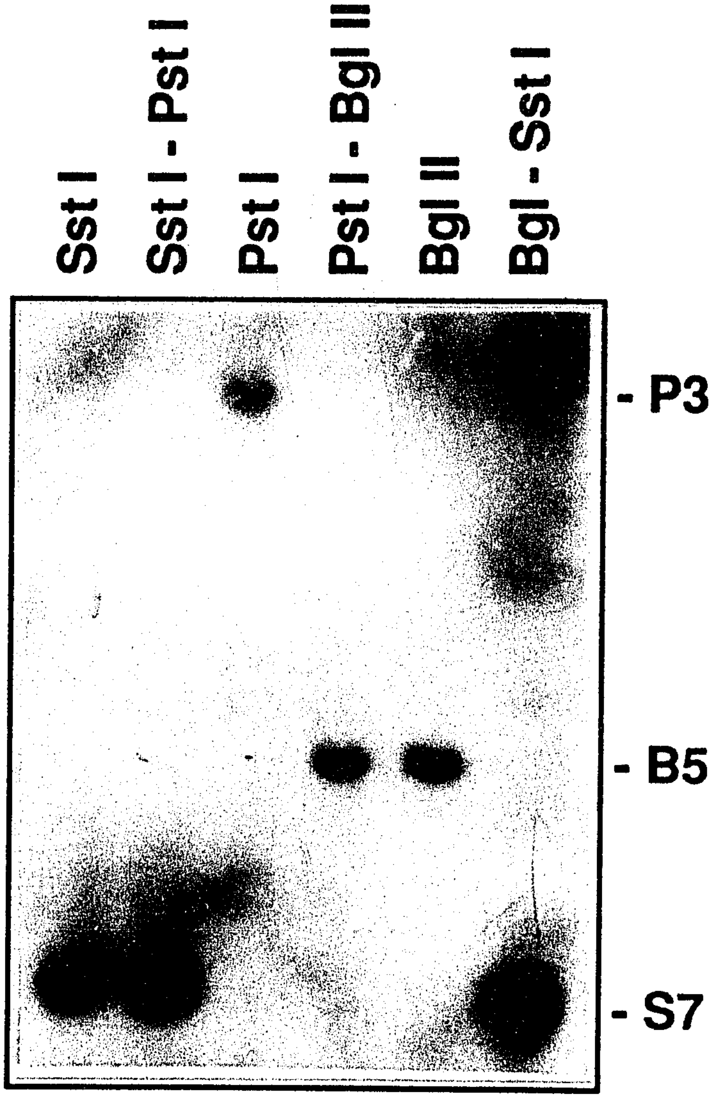


Figure 3. Linear representation of the chloroplast genomes of *S. costatum.* A. *Bgl* II, *Sst* I and *Pst* I restriction site map of strain "Skel". B. *Sst* I and *Pst* I restriction site map of strain "ED1". C. *Sst* I and *Pst* I restriction site map of strain "UP45". The approximate map positions of the *Hin*d III cloned fragments used as hybridization probes in Chapters 2 and 4 are shown under the map of strain "Skel". (.) identifies cloned fragments, (//) indicates undetermined order between two fragments, and the thickened black line represents the approximate area of the IR.

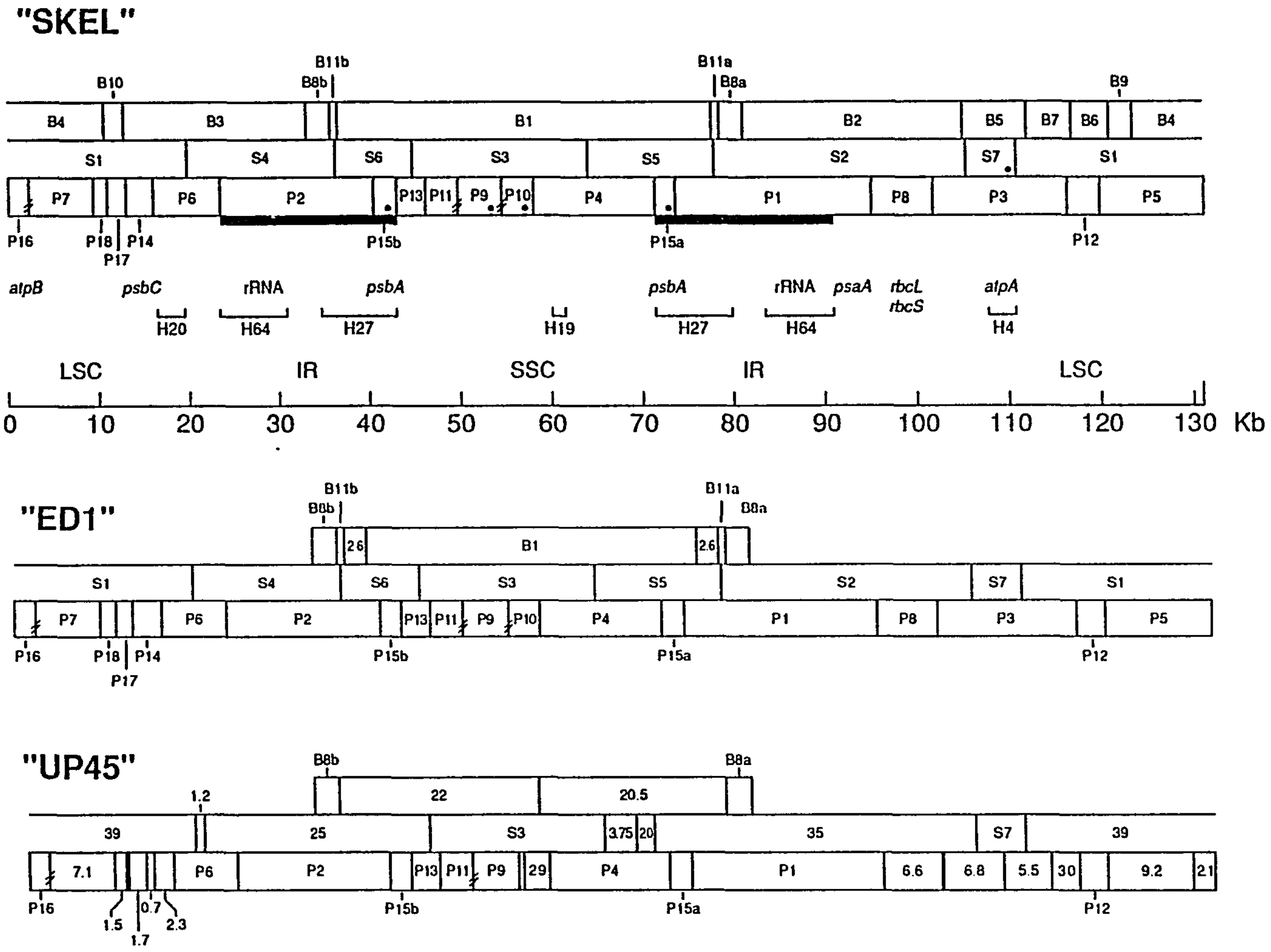
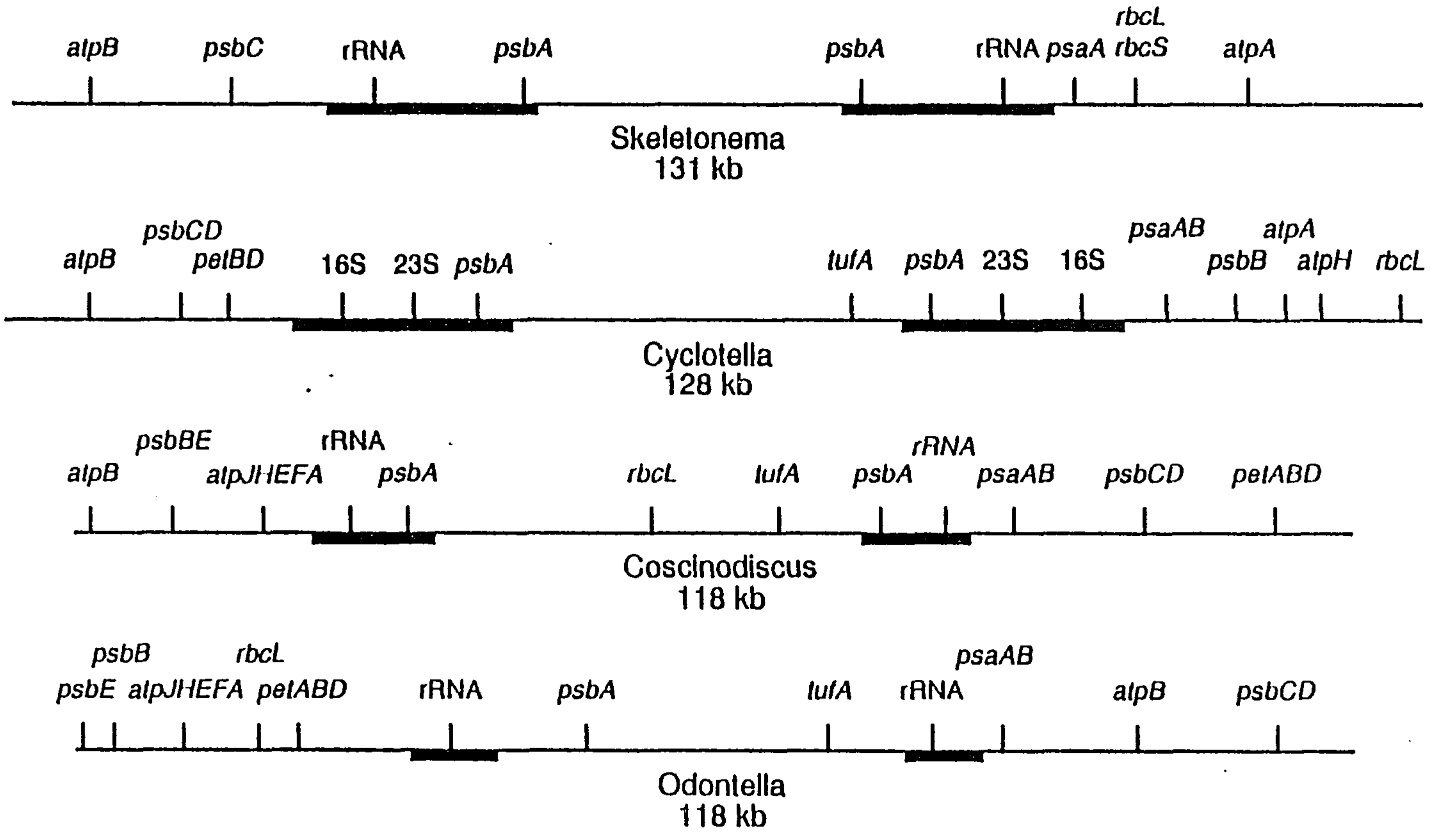

"SKEL"
B10
B8b
B11b
B11a
B8a
B9
B4
B3
B1
B2
B5
B7
B6
B4
S1
S4
S6
S3
S5
S2
S7
S1
P7
P6
P2
P13
P11
P9
P10
P4
P1
P8
P3
P5
P16
P18
P17
P14
P15b
P15a
P12
atpB
psbC
rRNA
psbA
psbA
rRNA
psaA
rbcL
rbcS
atpA
H20
H64
H27
H19
H27
H64
H4
LSC
IR
SSC
IR
LSC
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Kb
"ED1"
B11b
B8b
B11a
B8a
2.6
B1
2.6
S1
S4
S6
S3
S5
S2
S7
S1
P7
P6
P2
P13
P11
P9
P10
P4
P1
P8
P3
P5
P16
P18
P17
P14
P15b
P15a
P12
"UP45"
B8b
B8a
22
20.5
1.2
39
25
S3
3.75
20
35
S7
39
7.1
P6
P2
P13
P11
P9
29
P4
P1
6.6
6.8
5.5
30
9.2
21
P16
1.5
1.7
0.7
2.3
P15b
P15a
P12

Figure 4. The chloroplast genomes of the centric diatoms *Cyclotella meneghiniana*, *Skeletonema costatum*, *Coscinodiscus granii*, and *Odontella sinensis* (Kowallik, 1989; Bourne et. al., 1992).


atpB
psbC
rRNA
psbA
psbA
rRNA
psaA
rbcL
rbcS
atpA
Skeletonema
131 kb
atpB
psbCD
petBD
16S
23S
psbA
tufA
psbA
23S
16S
psaAB
psbB
atpA
atpH
rbcL
Cyclotella
128 kb
atpB
psbBE
atpJHEFA
rRNA
psbA
rbcL
tufA
psbA
rRNA
psaAB
psbCD
petABD
Coscinodiscus
118 kb
psbB
psbE
atpJHEFA
rbcL
petABD
rRNA
psbA
tufA
rRNA
psaAB
atpB
psbCD
Odontella
118 kb

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Chapter 4

EXTREME CHLOROPLAST DNA DIVERGENCE AMONG NATURAL POPULATIONS OF THE MARINE DIATOM *SKELETONEMA COSTATUM* (BACILLARIOPHYTA)

ABSTRACT

The genetic divergence of chloroplast DNA (cpDNA) within populations of the marine diatom, *Skeletonema costatum* was quantified. Natural populations of *S. costatum* have the second highest level of cpDNA nucleotide divergence of any photosynthetic species. The distribution of two distinct chloroplast lineages was closely correlated with the divergent patterns of seasonal variation illustrated by allozyme data collected from the same isolates. Representatives of an allozyme group intermediate between the seasonal extremes contained either one of the two chloroplast lineages found in the winter and summer groups or they had a unique chloroplast lineage. The observed pattern of concordance between the allozyme and chloroplast data sets, indicate that there is gene flow between genetically distinct diatoms. The chloroplast DNAs of different geographic isolates of *S. costatum* and a sister taxon *S. tropicum* suggest that the distribution of chloroplast lineages is closely correlated with the light and temperature environments rather than those predicted by the traditional species designations based on morphology alone. No single isolate was representative

of the local temporal population from which it was isolated. This is the first investigation of population level diversity in chloroplast DNA (cpDNA) of a photosynthetic protist.

INTRODUCTION

In theory, the large population sizes and fast growth rates of photosynthetic protists, coupled with their ability to reproduce both sexually and asexually should lead to fast rates of evolution and the formation of highly structured populations in time and space (1). However, very little data exist on the quantitative population genetics of protists. The few existing studies mainly have employed allozymes or measures of fitness in common garden experiments to estimate genetic divergence (2,3). These results indicate that the level of diversity in protist populations approaches the high levels of prokaryotes, rather than those of higher organisms (4,5). We report here the first molecular examination of variation in populations of the marine diatom Skeletonema costatum (Greve) Cleve using chloroplast DNA (cpDNA).

S. costatum is a dominant member of the phytoplankton community in coastal marine environments. The ecological importance of this species and its ease of cultivation has led to its use as a popular model system in plankton physiology and ecology. This organism was selected for this study because new molecular data can be analyzed in conjunction with the wealth of available background data. An earlier study of the population genetics of S. costatum in Narragansett Bay, R.I. of 457 isolates analyzing five allozyme loci, showed that all populations are genetically diverse and that the frequencies of the most prevalent genotypes varied between seasonal blooms (6). The genetic distance between summer and winter blooms was comparable to those found in different genera of higher organisms, although no morphological differences were detected among strains (6). Genetic intermediates that shared allozyme types at different loci between the prevalent seasonal blooms genotypes were also common and were the most abundant class during the interbloom periods (6). Strains representative of the major groups of multilocus genotypes (a genotype based on the five allozyme loci) (6) also showed patterns of physiological differences consistent with their time of maximal abundance in the field (7). The winter and summer bloom populations represent opposite extremes of a genetic continuum that cycles in frequency with time. The entire system is analogous to ecotypes observed in higher plants except that the cline varies in time on a scale of weeks rather than space and that the genetic divergence between extremes is large.

Cladistic analysis of the allozyme data from the Narragansett Bay strains, reveals that there is no resolution of these strains into different phylogenetic species. Therefore, all strains examined here are one species, S. costatum.

The conservative nature of gene order in the cpDNA of higher plants (8) has facilitated the use of restriction fragment length polymorphism (rflps) analysis of cpDNA as a phylogenetic tool. Recent investigations have demonstrated that rearrangements of gene order are common among cpDNA's of different classes and orders of chromophyte algae (9, 10). This finding has led some investigators to question the utility of cpDNA restriction analyses for phylogenetic reconstruction in diatoms and other chromophytes because the mutational processes responsible for different restriction patterns among taxa may be unclear (10). I have demonstrated that the maps of the most divergent intraspecific cpDNA genomes are colinear (11), and that they differ from the closely related diatom genus, Cyclotella meneghiniana by only an inversion (11). Conservation of gene order was also found at the species level in a xanthophyte, *Vaucheria sessilis* (9,12). These studies support the contention that cpDNA restriction fragment length polymorphisms can be used to explore the evolutionary relationships of chromophyte algae at the intraspecific level at least.

Restriction analysis was performed in order to determine whether the temporal cline in ecotypic frequency inferred by allozymes was reflected by changes at the molecular level in cpDNA. Narragansett Bay strains representative of all major groups were examined, as well as two strains of *S. costatum* isolated from other locations, two strains of a closely related species, *Skeletonema tropicum* and one strain of an undescribed species closely related to the genus *Skeletonema*.

MATERIALS AND METHODS

Strains and Culture Conditions.

The strains isolated from Narragansett Bay, R.I. were selected because they are representative of the major groups of allozyme types present in winter, summer and interbloom populations. Wherever possible, the strains selected for cpDNA analysis had been previously examined in common garden experiments for physiological differences (7). Allozyme group designations follow Gallagher (6) and are listed in Table I. For the allozyme data, four out of five loci varied in frequency with season. The strains in the "WH" group had the most common alleles in the winter bloom at all four loci. Members of the "WH" group were also a minor component of the summer blooms. Members of the "Whet" allozyme group comprised 8-27% of the winter blooms, but were also a minor component of the summer population. The strains in the "SH" allozyme group had the most common alleles in summer blooms at all four loci and constituted 65-75% of this population. However, this allozyme group was not detected in the winter. The "Mixed" allozyme group was intermediate in that it had some common summer bloom alleles at some loci and the most common alleles present in winter blooms at others. "Mixed" strains were present at all times of the year and were the prevalent group during the interbloom period. The low frequency of heterozygous loci observed within the "Mixed" strains suggests that they are not simple F1 hybrids of the strains prevalent in the winter and summer blooms. The observed pattern of homozygous loci within the "Mixed" strains is probably due to outbreeding and recombination that occasionly interupts the predominanat pattern of self-fertilization of the diatom life cycle (13).

All strains have been maintained together since their initial isolation and subsequent reexamination of their physiology and allozyme banding patterns has not revealed any changes (7, Stabile et al., unpublished). All strains of S. costatum and S. tropicum analyzed in this study are listed in Table I. Strains "Skel" (CCMP1332), "Fry-3" (CCMP777), "ABSkel" (CCMP782), "21-1" (CCMP789), and "Fry-1" (CCMP788) were obtained from the Culture Collection of Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, Maine. Culture conditions were as previously described (14) with the exception that clones of S. tropicum were grown at 25° C.

Molecular Techniques.

Genomic DNA extraction procedures and the five probes used in this study were as previously described (15). The location of these probes is shown in Fig. 1, a map of the cpDNA of strain "Skel" (9). DNA (0.1-0.2 ug) from 35 strains was digested with each of the restriction enzymes: BstN I, Hind III, Eco RI, Eco RV, Pst I and Bcl I, subjected to gel electrophoresis, according to ref. 12, and transferred to nitrocellulose membranes using the Posiblot Pressure Blotter (Stratagene Inc.) according to the manufacturer's instructions. DNA was crosslinked to the nitrocellulose by exposure to ultraviolet light (UV Stratalinker Stratagene Inc.). Radiolabeling of probes and hybridization conditions were as previously described (11), with the exception that a minimum of 6×10^6 d.p.m./ml /filter was added. The size of the filters was 11 cm x 14 cm.

Data Analysis.

Characters were scored on the basis of presence (or absence) of identical sized restriction fragments. Unrooted cladograms were calculated by Hennig86 (Farris, 1986) using the "ie option" which is guaranteed to find the most parsimonious tree and Paup version 3.0 for bootstrap analysis (D. Swofford, Illinois Natural History Survey, Champaign, Il. 61820).

Fragment data were also analyzed phenetically using the Fragment Length Difference method (16) as follows: (eq. 5.53) $F = 2m_{xy}/(M_x + M_y)$, (eq. 5.54) $G = \{F(3-2G_1)\}^{1\backslash 4}$, (eq. 5.55) $d = -(2/r)\log_e G$. Here m_x and m_y are the numbers of restriction fragments in DNA sequences X and Y, respectively, and m_{xy} is the number of fragments shared by the two sequences (16). For the purpose of this analysis, all strains having identical cpDNA restriction fragment profiles were pooled.

In order to back calculate to natural populations, a cpDNA type was assigned to each of the 457 Narragansett Bay isolates based on allozyme banding patterns and time of isolation. This assumed that strains with a particular ratio of seasonally variable allozymes had an increased probability of containing a particular cpDNA lineage. This assumption may underestimate the frequency of some cpDNA lineages. Nucleon (Haplotypic) diversity, h, was calculated using eq. 8.5, $h = n(1 - \sum x^2/(n-1)$ (16). Where, n is the number of individuals sampled and x is population frequency of the cpDNA genotype. This diversity calculation indicates the probability of randomly sampling two distinct genotypes in natural populations. Haplotype frequencies were then used to determine the genetic relationships among the winter, summer and mixed populations. Temporal heterogeneity in population frequency distribution of cpDNA was estimated by Chi-square analysis, using the Restriction Enzyme Analysis Package (REAP) software.

Fixation indices were also calculated to further examine deviations of cpDNA genotypic frequencies within and among populations. A comparison of $\mathbf{D_{st}}$ and $\mathbf{H_s}$ allows for the determination of among and within population diversity respectively. $\mathbf{F_{st}}$ is a further estimate of genetic differentiation among populations and is a correlation between cpDNA's sampled within populations to cpDNA's sampled from the whole population. These F statistics were calculated as follows (16). $\mathbf{H_s}$ = n/n-1 [1 - $\sum x^2$ - Ho/2n] (eq. 7.39) (16), $\mathbf{H_t} = 1 - \sum x^2 +$ $\mathbf{H_s}$/n - Ho/2ns (eq. 7.40) (16), $\mathbf{D_{st}} = \mathbf{H_t} - \mathbf{H_s}$, $\mathbf{F_{st}} = 1 -$ $\mathbf{H_s}/\mathbf{H_t}$ (eq. 7.43) (16). Where n is the harmonic mean of the sample size among the three populations, s is the number of populations. $\mathbf{F_{st}}$ estimate was tested for significance by the equation $x^2 = N_t$ (k-1) $\mathbf{F_{st}}$ and df = (k-1)(1-1), where k is the number of different cpDNA's sampled, N_t equals the total number of cpDNA's observed and 1 is the number of fragments (17).

RESULTS

Nine chloroplast types were observed within 3 main cpDNA lineages (Fig. 2). Thirty-eight fragments were unique to single strains. Twenty-eight of these unique fragments were observed in "Fry-3" and its hybridization pattern was different from all other strains for 4 out of the 5 probes used. Only one of the smaller probes (H20) did not reveal "unique" differences. These data suggest that length polymorphisms and/or rearrangements exist in "Fry-3" relative to the other isolates. Strains of S. costatum and S. tropicum shared a total of 14 fragments.

Their rflp patterns were all consistent with an interpretation due to simple point mutations (18).

The unrooted gene tree in Figure 2 shows that the primary divergence is between strains that are either predominant in the summer or winter bloom populations of Narragansett Bay. CpDNA restriction fragment profiles corresponded to the allozyme groups as shown in Table I. All strains of the allozyme groups, "WH" and "Whet" had a cpDNA type of B (Table I), regardless of season of isolation. "SH" strains had cpDNA type A, with the exception of ST7 which had one different fragment and is designated as cpDNA type A^1. In general, "Mixed" strains that share more allozyme characters with the "SH" group had cpDNA type A, while those that share more with "WH-Whet" groups had cpDNA type B. "Mixed" strain "IIA62" had a one fragment difference with the typical "SH" group and was designated cpDNA type A^2. Two "Mixed" strains however, "IIA51" and "IIA55", both had a 3:1 ratio of "SH" to "WH-Whet" alleles, but had a unique cpDNA lineage. These two strains contained cpDNA type E which formed an intermediate branch in the gene tree, that was not highly supported by the fragment data. This cpDNA type shared fragments with each cpDNA type A and B, but also had unique fragments. There may be two subgroups of cpDNA type E, because *Eco* RI did not digest genomic DNA from strain "IIA51", despite numerous attempts.

S. costatum strains "ABSkel" and "Skel", isolated from Alaska and Long Island Sound respectively, were most similar to the "WH-Whet" allozyme group but had distinct cpDNA types. Strain "Fry-3" from California was also unique in its cpDNA restriction fragment profile. The S. tropicum strains, "21L" and "Fry-1", were found in the same clade of the gene tree as the "SH" strains, but were not identical to them and designated as cpDNA type G.

Table 2 illustrates the estimated nucleotide sequence divergence values calculated from the fragment data in a pairwise comparison of the cpDNA types (16). This estimate is based on the assumption that differences in restriction fragment profiles are due to base pair substitutions because I have demonstrated that cpDNA types A, B, and C are colinear and that differences between cpDNA's are due to simple point mutations (11). This analysis is therefore appropriate. CpDNA type F ("Fry-3") was excluded from this last analysis because its restriction fragment pattern indicated that numerous length mutations and/or rearrangements exist between its cpDNA and those of all the other strains. Length mutations violate the assumptions upon which this calculation is based. The most divergent pair of cpDNA's are cpDNA A and cpDNA E, which had a sequence divergence of 0.059. CpDNA A and B were also divergent at 0.052. The least divergent pair are cpDNA C and B, 0.0013, and cpDNA C and D, 0.007. Also less divergence exists between cpDNA type G from S. tropicum and type A from S. costatum "SH" isolates, 0.014, than between cpDNA type A ("SH") and B ("WH-Whet").

In order to calculate nucleon diversity, "Mixed" strains with a 3:1 ratio of "WH-Whet" to "SH" alleles were assigned cpDNA type B and "Mixed" strains with a 3:1 ratio of "SH" to "WH-Whet" alleles were assigned cpDNA type A. Mixed strains "IIA51" and "IIA55" were assigned cpDNA type E. A nucleon diversity, h, of 0.50 was calculated for all Narragansett Bay isolates. The Winter bloom was least diverse, having an h of 0.0. The summer isolates had an estimated diversity of 0.23, while the interbloom was highest at 0.51. Chi-square analysis was also based on the frequency distribution of cpDNA types among the three populations. When all three populations were analyzed simultaneously, a chi-square of 231.73 was calculated. This value indicates extremely high levels of temporal heterogeneity exist among these three groups. Pairwise comparisons of winter, summer and interbloom populations furthermore reveal that significant differences of genotypic frequencies exist between each population in relation to the other two.

The fixation indices indicated that the within population diversity, $\mathbf{H_s}$ = 0.57, was greater than that observed among populations, $\mathbf{D_{st}}$ = 0.12, while the total diversity $\mathbf{H_t}$ was 0.69. The $\mathbf{F_{st}}$ was estimated to be 0.18 and was found to be significantly different than zero according to the chi-square analysis (17).

The nucleon and nucleotide sequence diversity calculated for cpDNA among Narragansett Bay strains of *S. costatum* is greater than that of terrestrial plant populations, with the one exception of *Trifolium pratense* (19). Most of the variation observed for *S. costatum* was within seasonal populations but significant variation also exists among populations as is exhibited by an $\mathbf{F_{st}}$ of 0.18, which is an order of magnitude higher than that shown in *T. pratense* (19). Temporal heterogeneity between the winter, summer, and interbloom populations was further demonstrated by a significantly high chi-square analysis based on the cpDNA haplotypes. This indicates that the three seasonal populations each have distinct cpDNA genotypic frequencies. These results are in agreement with similar analyses of allozyme genotypic frequencies (6).

DISCUSSION

There is a general pattern of concordance between the allozyme and cpDNA data, except that allozyme methods detect a higher diversity of genotypes than cpDNA. Both data sets indicate that the divergence among seasonal populations of the marine diatom, Skeletonema costatum, is greater than that typically found among genera of higher plants (6, 19). In contrast to the stable spatial differences in distributions of cpDNA's in higher plants (20), the cycling of the allozyme and cpDNA genotypes in the plankton occurs on a scale of weeks in a single location. These data support the hypothesis that fast growing protists can form highly structured populations that undergo rapid genetic change.

Allozyme banding patterns are commonly assumed to represent nuclear genes (21). A greater diversity of allozyme genotypes is similar to the results obtained in higher organisms and probably reflects faster rates of evolution in the nuclear genome (22). Although the general patterns of seasonal cycling are similar in both data sets, there is no 1:1 correspondence between a particular allozyme allele at a locus with any cpDNA lineage. The "Mixed" strains can have any cpDNA type. Therefore, the cpDNA's appear to be inserted in different nuclear backgrounds. This finding is consistent with the interpretation of the "Mixed" strains as the product of genetic recombination between the seasonal extremes (6). The combination of allozyme and cpDNA data suggest that genetically distant diatoms can exchange genes. Mann (23) also observed sexual reproduction between morphologically divergent isolates of the pennate diatom, Navicula protacta. In total, these findings suggest that there is gene flow among divergent diatom populations and that the summer and winter bloom isolates belong to a single biological species. The strains examined here are known to differ in their photophysiological characteristics (7) and "Mixed" strains in general have responses that are intermediate between the "Wh-Whet" and "SH" groups regardless of which chloroplast lineage they contain. Therefore, many of the observed phenotypic differences in physiology are probably due to nuclear control and regulation of chloroplast function.

The mechanism of chloroplast inheritance and the relative frequency of outbreeding in diatoms is unknown at present. It is difficult to interpret how intermediate cpDNA lineage E evolved within these seasonal populations. In most terrestrial plants, the chloroplast is inherited uniparentally (24). If uniparental inheritance of the chloroplast also occurs in diatoms, then the only way to account for cpDNA type E is to invoke that a series of parallel site gains and losses were retained in this chloroplast lineage while they were lost in others. An alternative hypothesis is that at least part of the time, cpDNA may be biparentally inherited, and that cpDNA type E is the result of recombination. Recombination of chloroplast types has been observed in _Chlamydomonas_ and in the terrestrial plant genus _Pinus_ (24,25). These issues can only be resolved by breeding experiments. The chloroplast rflp patterns reported here could be used as markers in controlled breeding experiments to resolve the issue of chloroplast inheritance.

Patterns of morphological divergence within the genus are not congruent with the molecular data. Isolates of the morphologically distinct species, _S. tropicum_ from Surinam and the Gulf of Mexico, have cpDNA's more closely related to the summer bloom strains of _S. costatum_ than the cpDNA's of the summer bloom _S. costatum_ strains are to those of the winter blooms in Narragansett Bay. Gene trees and species trees are not congruent when there is lineage sorting of ancestral polymorphisms and/or molecular introgression (26, 27). The data reported here are a clear example of the hypothesis that the examination of variation at single genetic loci will not necessarily yield an accurate portrayal of the evolution of a group of species and populations.

The presence of members of the winter bloom cpDNA clade in Alaska indicates that the geographic distribution of some of the cpDNA lineages extends beyond Narragansett Bay. The overall pattern of the three cpDNA clades is more consistent with a distribution according to temperature and light rather than geographical proximity or morphology. Since the biogeographical history of each cpDNA is not known, more data is needed from other members of the genus in order confirm the relationship of cpDNA type and ecological parameters.

Sampling methods for protistology usually involve the analysis of single strains from one or more geographic locations (28,29). Often, new species are described based on single isolates (e.g. 28). Recently, some investigators have incorporated data from single genes from these small sample sizes to describe new taxa. The data reported here clearly indicate that protist populations are extremely variable and highly structured. Thus, any taxonomic designations based on small samples is highly questionable.

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Table 1. Isolate information. Genotypes are based on allozyme banding patterns and cpDNA type of *S. costatum* strains. Allozyme designations, AD, follow (5,6) where PGI, MDH, GDH, TO1, TO2 are loci and each letter represents an allele.

Strain	AD	Time of Maximum Abundance	Isolation Date	Location	Genotype					cpDNA Type
					PGI	MDH	GDH	TO1	TO2	
ST2	SH	Summer	17.8.76	R.I.	AA	MM	GG	WW	PP	A
ST7	SH	Summer	17.8.76	R.I.	AA	MM	GG	WW	PP	A[1]
2B3	SH	Summer	15.8.75	R.I.	AA	MM	GG	XX	PP	A
2B11	SH	Summer	15.8.75	R.I.	AA	MM	GG	WW	PP	A
2B13	SH	Summer	15.8.75	R.I.	AA	MM	HH	WW	PP	A
UP45	SH	Summer	17.8.76	R.I.	AA	MM	HH	WW	PP	A
UP9	SH	Summer	17.8.76	R.I.	AA	MM	II	WW	PP	A
IIA35	SH	Summer	26.7.76	R.I.	AA	MM	GG	XX	PP	A
IIA49	M	Interbloom	26.7.76	R.I.	AA	MM	II	UU	PP	A
IIA62	M	Interbloom	26.7.76	R.I.	BB	MM	HH	WW	PP	A[2]
IB7	M	Interbloom	15.8.75	R.I.	BB	MM	GG	WW	PP	A
IIA51	M	Interbloom	26.7.76	R.I.	BB	MM	GG	XX	PP	E
IIA55	M	Interbloom	26.7.76	R.I.	AA	KK	GG	WW	PP	E

UP49	M	Interbloom	17.8.76	R.I.	AA	KK	FF	VV	QQ	B
SF73	M	Interbloom	3.8.76	R.I.	BB	KK	HH	WW	QQ	B
IIA54	M	Interbloom	26.7.76	R.I.	BB	KK	HH	UU	RP	B
NY10	M	Interbloom	6.1.76	R.I.	BB	MM	HH	UU	RR	B
NY17	WH	Winter	6.1.76	R.I.	BB	KK	GG	UU	RR	B
ED1	Whet	Winter	11.1.77	R.I.	BB	KK	GG	UU	RQ	B
ED2	WH	Winter	11.1.77	R.I.	BB	KK	EE	UU	RR	B
NY27	WH	Winter	6.1.76	R.I.	BB	KK	GG	UU	RR	B
MF10	WH	Winter	1.2.77	R.I.	BB	KK	EE	UU	RR	B
HB17	Whet	Winter	27.1.76	R.I.	BB	KK	HH	UU	RQ	B
MF15	Whet	Winter	1.2.77	R.I.	BB	KK	GG	VV	RQ	B
MF55	Whet	Winter	1.2.77	R.I.	BB	KK	GG	UU	RQ	B
ST10	Whet	Winter	17.8.76	R.I.	BB	KK	GG	UU	RQ	B
ST37	WH	Winter	17.8.76	R.I.	BB	KK	GG	UU	RR	B
UP3	WH	Winter	17.8.76	R.I.	BB	KK	HH	UU	RR	B
IIA23	WH	Winter	26.7.75	R.I.	BB	KK	FF	UU	RR	B
"Skel"		Standard	?.?.56	Conn.	BB	KK	GG	UU	RR	C
"Fry-3"		?	?.?.?	California	N/A	N/A	N/A	N/A	N/A	F
"ABSkel"		*S. costatum*	9.4.79	Alaska	N/A	N/A	N/A	N/A	N/A	D
"21- 1"		*S. tropicum*	5.6.65	Suriman	N/A	N/A	N/A	N/A	N/A	G
"Fry-1"		*S. tropicum*	?.?.73	TX.	N/A	N/A	N/A	N/A	N/A	G

Table 2. Estimates of Nucleotide diversity based on equations 5.53-5.55 in Nei (8). All identical strains from the winter, summer and mixed strains are represented as one group. Numbers above the diagonal indicate the number of shared fragments, while numbers below are the nucleotide diversity estimates.

	Type C	Type B	Type A	Type A^1	Type A^2	Type E	Type D	Type G
Type C	-	60	27	28	28	34	54	28
Type B	0.0013	-	25	26	26	34	53	26
Type A	0.047	0.052	-	57	55	22	23	45
Type A^1	0.044	0.049	0.002	-	54	23	24	44
Type A^2	0.044	0.049	0.003	0.004	-	23	24	44
Type E	0.034	0.035	0.059	0.057	0.057	-	35	22
Type D	0.007	0.008	0.057	0.054	0.054	0.033	-	27
Type G	0.045	0.049	0.014	0.015	0.015	0.058	0.046	-

Figure 1. A linear representation of the 131 Kb chloroplast genome of *S. costatum* based on strain "Skel" (Stabile et. al., submitted; Chapter 3). The location of the *Hind* III cloned fragments used as hybridization probes are shown underneath the map. Blackened areas illustrate the approximate position of the inverted repeat.

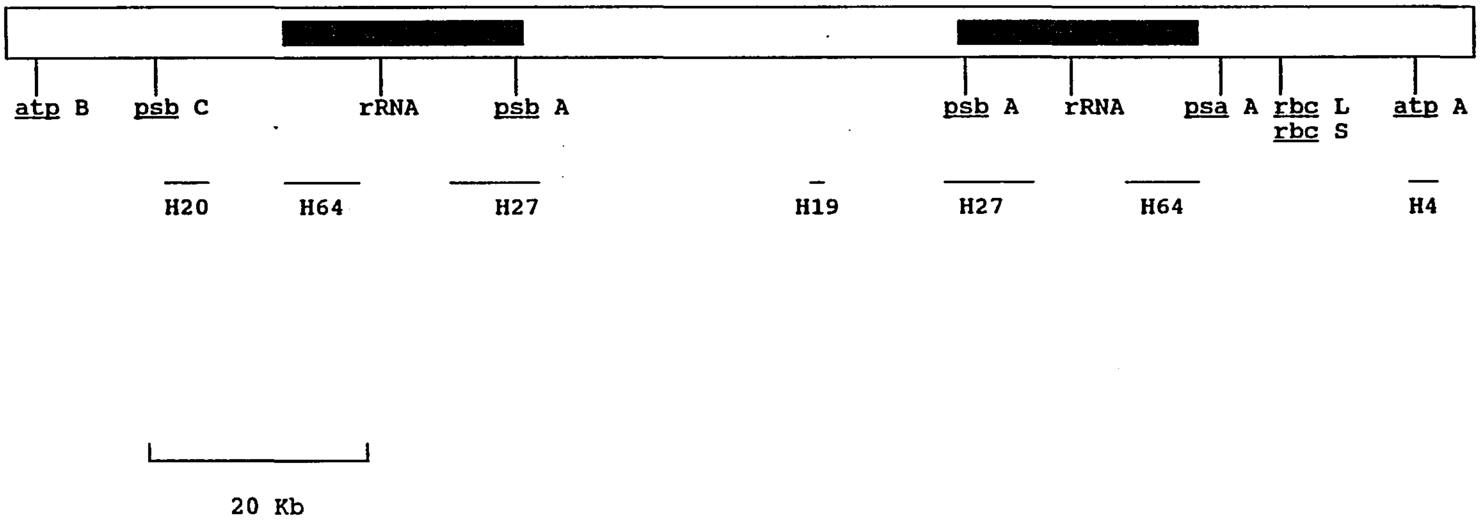
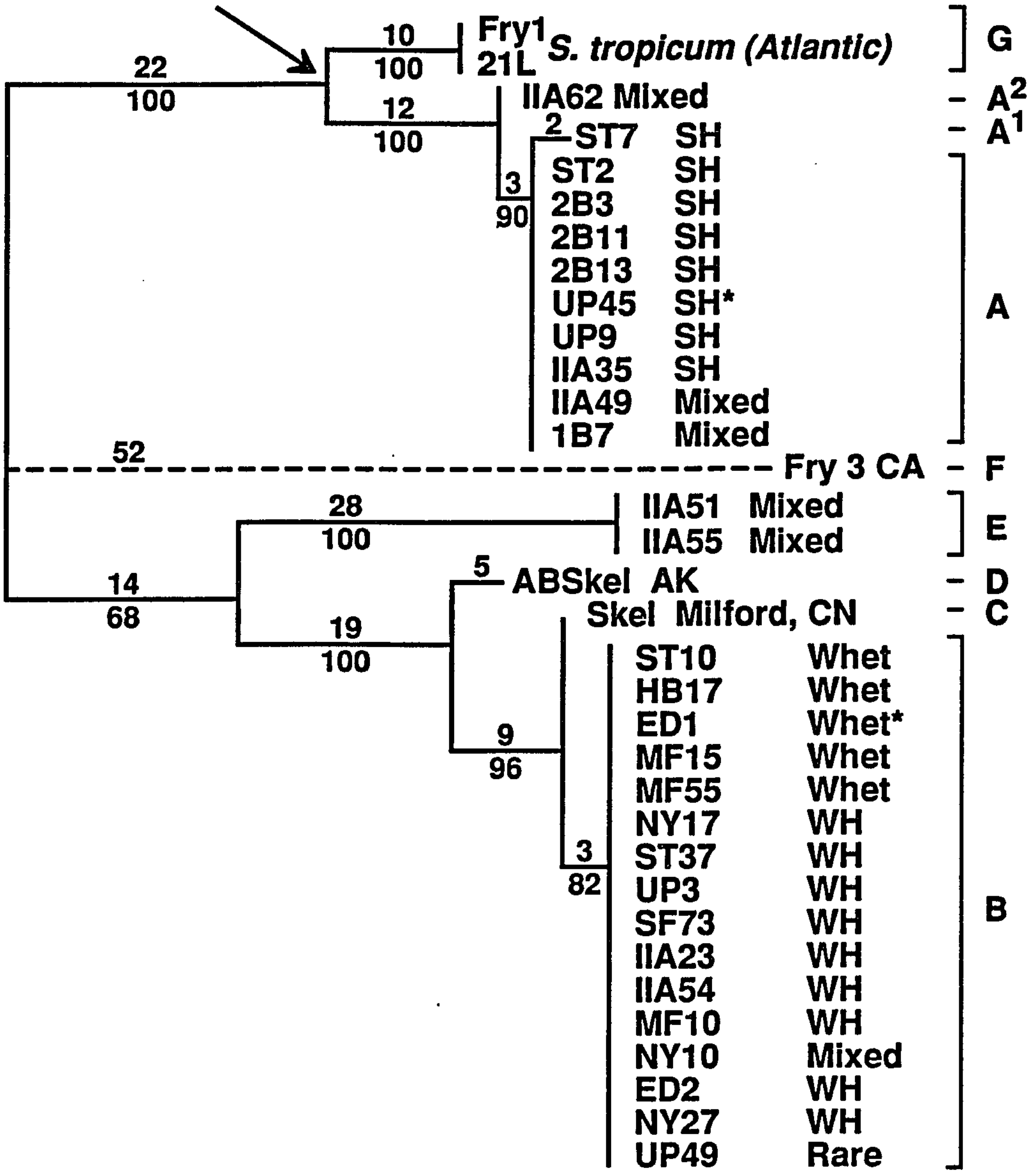

atp B
psb C
rRNA
psb A
psb A
rRNA
psa A
rbc L
rbc S
atp A
H20
H64
H27
H19
H27
H64
H4
20 Kb

Figure 2. Unrooted cladogram illustrating the relationships of chloroplast types A-G. The numbers above each branch indicate the number of apomorphies, while the number below each branch represents the number of times a clade was monophyletic in 100 bootstrap replicates. The arrow illustrates the theoretical root of the cladogram based on morphological data. The location of several strains are indicated in parentheses. 356 steps were needed to calculate the one parsimonious phylogram. The consistency index and retention index were 0.84 and 0.97, respectively.


10
100
Fry1
21L
S. tropicum (Atlantic)
G
22
100
12
100
IIA62 Mixed
A2
2
ST7 SH
A1
3
90
ST2 SH
2B3 SH
2B11 SH
2B13 SH
UP45 SH*
UP9 SH
IIA35 SH
IIA49 Mixed
1B7 Mixed
A
52
Fry 3 CA
F
28
100
IIA51 Mixed
IIA55 Mixed
E
14
68
5
ABSkel AK
D
Skel Milford, CN
C
19
100
9
96
3
82
ST10 Whet
HB17 Whet
ED1 Whet*
MF15 Whet
MF55 Whet
NY17 WH
ST37 WH
UP3 WH
SF73 WH
IIA23 WH
IIA54 WH
MF10 WH
NY10 Mixed
ED2 WH
NY27 WH
UP49 Rare
B

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Summary Discussion

Observed Patterns of Divergence and Variation: Significant Findings

The most striking feature of the chloroplast DNA restriction site and fragment data (presented in chapters 3 and 4) is the extreme divergence observed at the intraspecific level. Five different cpDNA genotypes were found among the 29 Narragansett Bay isolates of *S. costatum*. Nine strains had cpDNA type A, while 16 had type B. Two subtypes of cpDNA genotype A were also observed. Genotypes A^1 and A^2, respectively had either a one or two fragment difference from cpDNA type A. A fifth cpDNA genotype was observed within two isolates and was designated as type E. While, chloroplast genotype E had some unique fragments, it also shared some restriction fragments with cpDNA types A and B. The calculated (Nei, 1987) sequence divergence between cpDNA genotypes A and E is 5.9%. This is the second highest sequence divergence between any two cpDNA's from a single species. The highest divergence known, 6.6%, is between two strains of the terrestrial plant, *Trifolium pratense* (Milligan, 1991). The extreme cpDNA divergence observed among strains of *S. costatum* is consistent with previously published data suggesting that protists have greater levels of variation than higher organisms (Gallagher, 1986).

Another significant finding reported in this thesis is the observed concordance between the cpDNA and allozyme data sets. Strains designated as winter homozygotes or winter heterozygotes, "WH-Whet", (Gallagher, 1980) on the basis of allozyme banding patterns, all had cpDNA type B. The summer homozygous strains, "SH", characterized by their allozyme banding patterns had cpDNA type A or A^1. The "Mixed" strains which have alleles at different loci common to both "Wh-Whet" and "SH", exhibited a variety of cpDNA types, however, some combinations were more common than others. Strains that had the alleles prevalent in winter blooms at three out of four enzyme loci had cpDNA type B. "Mixed" strains with a 3:1 ratio of "SH" to "WH-Whet" alleles had cpDNA type A, A^2, or E. There was no clear association between any particular electrophoretic allele at a locus and the cpDNA rflp pattern of the "Mixed" strains.

In addition, chloroplast DNA restriction fragment data was collected from three isolates of <u>S. costatum</u> from field stations distant from Narragansett Bay and two strains of a closely related species, <u>S. tropicum</u>. Strain "Skel" and "ABSkel" isolated from Milford, CN. and

Alaska have cpDNA genotypes closely related to cpDNA type B of the Narragansett Bay winter strains. *S. tropicum* strains, "21-L" and "Fry-1", have a cpDNA genotype related to the chloroplast genotype A of the Narragansett Bay summer strains. Thus, the genetic diversity of cpDNA's within populations of *S. costatum* exceeds the difference among the "summer" isolates of *S. costatum* and those of a morphologically different species, *S. tropicum*. "Fry-3", an isolate of *S. costatum* from an upwelling area off California has a unique cpDNA restriction fragment profile. In total, the rflp data suggest that chloroplast types within *Skeletonema* appear to be distributed according to light and thermal environmental gradients. However, more data are needed to confirm correlations of cpDNA type and the particular ecological distributions of different strains.

Possible Mechanisms Responsible for the Observed Patterns of Divergence and Variation

The observed pattern of genetic variation among isolates of *S. costatum*, is determined by the mechanism of chloroplast inheritance and the extent of outbreeding between the summer and winter ecotypes. In addition, the pattern of genetic variation can be explained by neutral and/or selective evolutionary processes acting upon the nucleus and chloroplast.

A. Mechanisms of chloroplast inheritance: What is the importance of uniparental or biparental chloroplast inheritance? How could intermediate chloroplast types evolve under both of these mechanisms?

The interpretation of cpDNA data is dependent upon whether the chloroplast is inherited uniparentally or biparentally. If it is inherited uniparentally, chloroplast DNA acts genetically as if it were a single gene whose patterns of divergence might not reflect the real patterns of evolution among the seasonal populations of S. costatum (Pamilo and Nei, 1988; Doyle 1992). Little is known about chloroplast inheritance in chromophyte algae in general or diatoms in particular. Mature cells of Skeletonema costatum have one or two chloroplasts (Hulbert and Guillard, 1968). In S. costatum, sperm cells may contain chloroplasts, because the cytoplasm of the spermatocyte is divided equally among four sperm cells after meiosis (Migita, 1967; Drebes, 1977; Jane Gallagher, pers. comm.). The presence of plastids in the sperm cells of Chaetoceros, another centric diatom, has recently been confirmed with fluorescence microscopy (Jan Rines, pers. comm.). Thus, the sperm cells of S. costatum may contain cpDNA, although this does not guarantee biparental inheritance. For example, Chlamydamonas with isogamous sexual reproduction, exhibits uniparental inheritance because copies of cpDNA from one parent are selectively destroyed after fertilization (Boynton, et. al. 1991). The only way to resolve this question in S. costatum is to conduct breeding experiments. These critical studies have not yet been attempted because we do not know the specific environmental conditions necessary to make S. costatum reproduce sexually in the laboratory. Consequently, the data presented in this thesis will be interpreted using both uniparental and biparental models.

Under a model of uniparental inheritance, the differences observed between cpDNA types A and B must have arisen through a series of parallel site gains and losses. CpDNA type E must have retained some combination of site changes observed in both the A and B lineages. CpDNA type E may have been maintained in the Narragansett Bay strains by (lingeage sorting) randomly being sorted among descendant lineages, while other possible cpDNA types were lost due to selection and/or genetic drift.

Under a model of biparental inheritance, both a maternal and paternal chloroplast type are inherited by the zygote. Although both parental chloroplasts are initially inherited, only one type may be maintained within the mature cells, due to selection or random loss of one chloroplast type. This situation has been observed in the terrestrial plant Oenothera, in which chloroplast inheritance is biparental. Here, the one descendent chloroplast is directly correlated with the nuclear type (Chiu and Sears, 1993). In rare instances, intermediate types of cpDNAs (cpDNA type E) could arise by recombination of the two parental types. This has been observed in Chlamydomonas (Boynton et. al., 1991) and is a possible mechanism for the evolution of cpDNA type E. Another possibility is that both the maternal and paternal chloroplast could be maintained. However, if there were heteroplasmy I would expect that some individual "Mixed" strains would contain both cpDNA types A and B. Since this was never observed, heteroplasmy will not be considered.

B. The role of outbreeding and inbreeding.

The frequency with which outbreeding or inbreeding occurs is important because these processes alter the associations between the different nuclear and chloroplast genomes. S. costatum, like other centric diatoms, is probably diploid and capable of self-fertilization (autogamy) (Migita, 1967; Drebes, 1977). In autogamy, two haploid nuclei from the egg cell unite restoring the diploid number of chromosomes.

The true extent of outbreeding is not known but the allozyme data suggests that it occurs on an occasional basis (Gallagher, 1980). If outbreeding never occurred, then one would not have observed the presence of the "mixed" allozyme types. Secondly, if outbreeding were common, then one would expect to see a higher frequency of heterozygotes among all three allozyme types. The frequency of observed heterozygotes is low, which suggests that outbreeding occurs on an occasional basis and has been followed by successive rounds of autogamy. This model of reproduction was invoked to account for the presence of "mixed" strains and to explain why the frequency of heterozygotes was low (Gallagher, 1980).

C. The relative importance of selection pressure.

Selection pressure may be important in determining the association between different nuclear and chloroplast types. Although, the five allozyme loci do not have any adaptive value per se, they can serve as molecular markers in analyzing the association of the different nuclear and chloroplast groups. The "mixed" allozyme group has cpDNA types identical with both the "WH-Whet" and "SH" strains, yet the "mixed" strains have intermediate physiologies when examined for chlorophyll content and mean carbon uptake (Gallagher, 1982). This strongly suggests that the nucleus is responsible for the phenotypic differences observed among strains. Nuclear control and regulation of photosynthesis may be the key factor in understanding the evolution of the winter and summer ecotypes. Consequently, the extreme divergence among chloroplast types is a secondary issue relative to the relationship between the chloroplast and the nucleus. Are all combinations of chloroplast and allozyme types viable? Is the observed association of some chloroplast types within some nuclear backgrounds due to genetic drift and the mating behavior of the Narragansett Bay strains? Are some combinations of chloroplast and nuclear types so strongly selected for by light and temperature, that only one cpDNA type is observed within one particular nuclear background? The pattern of association between cpDNA and allozyme types can lead to some conclusions regarding the relative strength of selection pressure or adaptive forces acting on these populations.

Table 1 presents a summary of the theoretical associations of cpDNA and allozyme types expected under neutral, moderate and strong selective pressure if inheritance of the chloroplast is uniparental or biparental. Also, it is assumed from the previous section, that outbreeding occurs with an indeterminate frequency followed by autogamy. If chloroplast inheritance occurs in a uniparental fashion, one would expect all three cpDNA lineages to be associated with "Mixed" strains. If there is no selective value to any cpDNA/allozyme association, one should observe a low percentage of strains that have cpDNA and allozyme types from different seasons, eg. cpDNA lineage B with "SH" allozymes. However, we did not observe this combination of cpDNA and allozyme types. Under moderate levels of selection, one would expect a strong correlation between cpDNA and "Mixed" allozyme types that have alleles predominantly of the same season, eg. cpDNA lineage A with a 3:1 ratio of "SH" to "WH-Whet" alleles. If the selection pressure was extremely strong, then one would expect that a cpDNA type would only be associated with the allozyme type of the same season and there would be no intermediate cpDNA types. Under uniparental inheritance therefore, the chloroplast and allozyme data suggest that moderate levels of selection can maintain the observed patterns of divergence among strains.

If we use a model of biparental inheritance with occasional recombination, we would expect all three cpDNA types to be associated with the "mixed" strains. If there is no selective value to any cpDNA/allozyme association, then a "Mixed" strain would be just as likely to have cpDNA A or B. Under moderate levels of selection, one would expect that cpDNA lineages A and B would be associated with "Mixed" allozyme types that have alleles predominantly of the same season. Also, intermediate cpDNA types (cpDNA type E) could be found within some "Mixed" strains. If the selection pressure was extremely strong, then one would expect that a cpDNA type would only be associated with the allozyme type of the same season and there would be no intermediate cpDNA types. The chloroplast and allozyme data suggest that modest levels of selection can maintain the observed patterns of divergence among strains.

Breeding experiments and the examination of more mixed strains may lead to a better understanding of the mechanisms responsible for the different chloroplast-nuclear associations and the relative roles of neutral and selective processes. The chloroplast DNA maps and RFLPs developed in this thesis, provide new markers that can be used in future breeding experiments. A winter and summer strain can be placed together in series of test tubes and exposed to conditions that may induce sexual reproduction. Some factors that induce sexual reproduction in diatoms are small cell size, light quality and intensity, temperature, salinity and nutrient concentrations (Geitler, 1935; Drebes, 1977). Sexual reproduction has been observed to occur within 24 hours of such treatments (Geitler, 1935). After sexual reproduction, isolates can be examined for their cpDNA and allozyme genotypes. These results should give a clearer picture of the mechanism of chloroplast inheritance. If the experiment is properly designed, the interpretation of the relative selection pressure of light and temperature in the natural environment on different cpDNA/allozyme associations will be on much firmer ground.

Additional, data from the "Mixed" group may be helpful in interpreting the relative role of selection on different cpDNA/allozyme associations. Although, all of the "Mixed" strains that have survived in culture were examined, the sample size of this group is small. The examination of more "Mixed" strains would provide better evidence for the patterns of cpDNA/allozyme associations outlined in Table 1. At present, there is no clear way to eliminate any of the possibilities outlined in Table 1, with the exception of extreme selection.

D. The distribution of chloroplast DNA lineages in *Skeletonema.*

There are several explanations for the global distribution of cpDNA lineages within the genus *Skeletonema*. Many natural and artificial (man made) processes can give a planktonic organism a global distribution. Consequently, a large array of genotypes can be introduced into new environments. The array of genotypes that survive in any environment may be due to selection. The correlation of cpDNA/allozyme genotypes with the physiological differences observed among strains (Gallagher, 1982) may be important in regulating niche breadth. This suggests that selection is important in determing the distribution of certain cpDNA lineages and can be used to explain the presence of cpDNA lineage B in Alaska (eg. ABSkel) and cpDNA lineage A in the tropics (eg. S. tropicum).

It is generally recognized that *S. tropicum* is the sister taxon to *S. costatum*. It was included in the experimental design in order to attempt to root the cladogram in Chapter 4. The close relationship between the cpDNA's of *S. tropicum* and *S. costatum* was unexpected because they have extremely different allozyme profiles

(Gallagher, per com.). Some authors have questioned whether they should be considered separate species (Hasle, 1973). The two species differ in chloroplast number, robustness of silicification, maximum cell size and distribution in the field (Hulbert and Guillard, 1968). They are easily distinguished by standard light microscopy. In any case, the data support the notion that the cpDNA of *S. tropicum* shares a more recent common ancestor with the cpDNA of the summer strains of *S. costatum* than do some of the winter strains or "Fry-3". Therefore, it can not serve as an appropriate outgroup in the chloroplast gene tree in chapter 4. One possible explanation for the close relationship of the cpDNA restriction fragment profiles of *S. tropicum* and *S. costatum* is that the ancestor of the *Skeletonema* genus was polymorphic for chloroplast DNA genotypes and these types were randomly sorted into descendent lineages.

Ecotypic Differentiation and Speciation

The concept of ecotypes has been used to explain the process of speciation. Populations evolve distinct characters which are a result of selective pressures of the local environment (Haynes, in press). The winter, summer, and interbloom populations of *S. costatum*, from

Narragansett Bay can be described as ecotypes. Each of the populations has a distinct genotypic frequency in regard to allozyme types and cpDNA genotypes. Many studies such as the one conducted on H. vulgare, assume that a population is adapted to local conditions, but present no specific data about the selective pressure of the physical environment (Saghai-Maroff et. al., 1992). It is common for investigators, to assume adaptation and selection are the major evolutionary forces responsible for the observed divergence among populations. Selection is difficult, if not impossible to prove. If it has occurred, then one should be able to at least illustrate the selective pressure with physical environmental data. The seasonal cycling in abundance of the "winter", "summer" and "mixed" strains can indeed be correlated to the light and temperature regimes observed during different times of the year (Gallagher, 1984). For example, under the light and temperature conditions found in the summer, the "SH" strains have significantly higher growth rates than the "WH" strains, while the "WH" strains have higher growth rates under "winter-like" conditions (Gallagher, 1982). The observed allochronic cycling of the different seasonal genotypes appears to follow the temporally changing physical environment.

Are these ecotypes different species? In order to address this question, we must analyze the data within the theoretical framework of the species concepts introduced in the preface.

Application of the typological species concept is dependent on the past experience of the expert and which key characters he/she chooses to analyze. Although there is no past experience in interpreting cpDNA data, we could describe two or three species of *Skeletonema* from Narragansett Bay based on the cpDNA rflps. One species to at least four species could be described based on the analysis of the allozyme data. These results are similar to those of Schoenberg and Trench (1970) in which 17 strains of *Symbiodinium microadriaticum* could be divided into 3 alloenzyme groups. I am not sure how many species of *S. costatum* could be designated using the typological species concept with both the allozyme and chloroplast DNA data. However, it seems reasonable that anywhere between 1 to 4 species could be designated using the typological approach.

Numerical taxonomy is a phenetic approach and has been useful in describing bacterial species (Sneath, 1984). A phenogram of the cpDNA data did not resolve any groups within the Narragansett Bay populations. A similar result was obtained with the allozyme data. Consequently, all strains isolated from Narragansett Bay are considered one species under this species concept.

The biological species concept has limited application to this data set. Diatoms are predominantly asexual and Mayr, 1982, categorically excludes predominantly asexual organisms from this concept. He suggests that the term "agamospecies" be applied to these forms. However, Round et. al., 1990 take an opposing view and advocate its use in diatoms. They note several studies where even morphologically different species of diatoms can hybridize and should thus really be considered varieties of a single species. A recent study of dinoflagellate sexual reproduction has also demonstrated that even morphologically different "species" form viable zygotes and should be considered varieties of a single species (Anderson et. al. in review). Presumably, the "Mixed" strains of S. costatum are the results of outcrossings between winter and summer strains. This suggests that they belong to a single species. Until formal breeding experiments are conducted, the applicability of the biological species concept to this organism is questionable.

The phylogenetic species concept is probably best to use in the analysis of both the allozyme and cpDNA data. Numerical programs allow one to analyze different types of data (eg. morphological and molecular) simultaneously.

In the autapomorphic phylogenetic species concept, species are the smallest monophyletic cluster, diagnosed with uniquely derived characters (Theriot, 1992). Autapomorphic characters were included in the cladistic analysis illustrated in chapter 4. The overall topology of this gene tree reveals that there is a dichotomy between the "winter" and "summer" cpDNA types. However, bootstrap analysis demonstrated that the node joining "IIA51" and "IIA55" with the "winter" strains is not well supported by the restriction fragment data. This node is observed in only 68 out of 100 bootstrap replicates. A recent study has demonstrated that a minimum of 70 out of 1000 bootstrap replicates are needed in order to demonstrate strong support for a clade. Moreover, this is true only if the rates of change and internodal change of characters are low (Hillis and Bull, 1993). When the rates of change are high, then confidence levels of over 50% are over estimates of accuracy. If the node uniting "IIA51" and "IIA55" with the predominantly winter strains is not strongly supported by the cpDNA data, then cladogram breaks down to an unresolved polytomy in which there is no resolution of the Narragansett Bay strains into different species. Again, this is similar to results obtained using the allozyme data in that there is only one species.

What is clear from the cpDNA gene tree, in concert with the allozyme data, is that complete differentiation or discontinuity is not present. The phenetic, biological and phylogenetic species concepts suggest that the populations of *S. costatum* are one species. Perhaps I have observed the incipient stages of speciation. Traditionally, species and varieties of diatoms have been established on the basis of morphology. Recently molecular techniques have been useful in examining the "species" problem in the symbiotic dinoflagellate, *Symbiodinium sp.* At one time, *Symbiodinium microadriaticum* was believed to be a single pandemic species. Subsequent, ribosomal RNA sequencing data has confirmed morphological, biochemical, physiological and behavioral data that there is more than one species of *Symbiodinium* (Rowan and Powers, 1992). Although analysis of the *Symbiodinium* species complex is incomplete, discontinuities among these "species" are observable because sexual reproduction is rare (Rowan and Powers, 1992). Complete differentiation among populations of *Symbiodinium sp.* can occur over time and be analyzed using molecular genetic techniques. In contrast, the cpDNA and allozyme data of *S. costatum* suggest that there is moderate outbreeding between representatives of the divergent winter and summer populations. This outbreeding will inhibit complete differentiation. Therefore, the "species" problem in S. costatum is not due to the techniques used to analyze the populations, but due to their ability to outbreed. Future research on these populations should focus on the extent and pattern of outbreeding in order to determine whether the Narragansett Bay populations of S. costatum are truly in the incipient stages of speciation.

Table 1. Theoretical associations of cpDNA and allozyme types with regard to neutral, moderate and strong selective pressure if inheritance of the chloroplast is uniparental or biparental. It is assumed that outbreeding occurs on an ocaasional basis. Observed patterns of diversity are underlined.

Uniparental Chloroplast Inheritance.

Neutral	Moderate	Strong
Good correlation between cpDNA type A with "SH" strains and cpDNA type "B" with "WH-Whet" strains. Observation of seasonal cpDNA types associated with "mixed" strains that have alleles generally of the same season.	Strong correlation between cpDNA type A with "SH" strains and cpDNA type "B" with "WH-Whet" strains. Observation of seasonal cpDNA types associated with "mixed" strains that have alleles predominantly of the same season.	Only observe cpDNA type "A" with "SH" strains, cpDNA type "B" with "WH-Whet". Observe no intermediate cpDNA types.

Biparental Chloroplast Inheritance.

Neutral	Moderate	Strong
Good correlation between cpDNA type A with "SH" strains and cpDNA type "B" with "WH-Whet" strains. Observation of cpDNA type A, B, or E associated with any "mixed" allozyme type.	Strong correlation between cpDNA type A with "SH" strains and cpDNA type "B" with "WH-Whet" strains. Observation of a few intermediate cpDNA types associated with some allozyme types. Observation of seasonal cpDNA types associated with "mixed" strains that have alleles predominantly of the same season.	Only observe cpDNA type "A" with "SH" strains, cpDNA type "B" with "WH-Whet". Observe no intermediate cpDNA types.

Appendix A

The following are detailed results of the hybridizations used in mapping the chloroplast genome of Skeletonema costatum. The complete map is shown in Chapter 3.

Results from Mapping Hybridizations
Sizes in Kilobases (Kb)

Bgl II Fragment 1 - 41 Kb

1. *Bgl* II/*Sst* I - 19, 14, 8.2
2. *Bgl* II - 41 (B1)
3. *Bgl* II/*Pst* I - 13.3, 4.8, 3.8, 3.5, 3.2, 2.8, 2.3
4. *Pst* I - 21.4 (P1), 17.4 (P2), 13.3 (P4), 4.8 (P9), 3.5 (P10), 3.5 (P11), 3.2 (P13), 2.3 (P15 a, b)
5. *Pst* I/ *Sst* I - 7.5, 5.8, 4.8, 4.4, 3.5, 2.3, 1.8
6. *Sst* I - 19 (S3), 14.2 (S5), 8.5 (S6)

Bgl II Fragment 2 - 24 Kb

1. *Bgl* II/*Sst* I - 24, 13.5
2. *Bgl* II - 24 (B2), 20.2 (B3)
3. *Bgl* II/*Pst* I - 14, 9, 7.2, 6.8
4. *Pst* I - 21.4 (P1), 17.4 (P2), 6.8 (P8)
5. *Pst* I/ *Sst* I - 17, 13, 6.8, 3.7
6. *Sst* I - 27.5 (S2), 16.7 (S4)

Bgl II Fragment 3 - 20.2 Kb

1. *Bgl* II/*Sst* I - 24, 13.5, 7.2
2. *Bgl* II - 24 (B2), 20.2 (B3)
3. *Bgl* II/*Pst* I - 14, 9, 7.2, 3.0

4. *Pst* I - 21.4 (P1), 17.4 (P2), 7.2 (P6), 3.0 (P14), 2.0 (P17)
5. *Pst* I/ *Sst* I - 17, 13, 3.7, 3.5, 3.0, 2.0
6. *Sst* I - 40.6 (S1), 27.5 (S2), 16.7 (S4)

Bgl II Fragment 4 - 18 Kb

1. *Bgl* II/*Sst* I - 18
2. *Bgl* II - 18 (B4)
3. *Bgl* II/*Pst* I - 7.0, 6.8, 2.2
4. *Pst* I - 11.5 (P5), 7.0 (P7), 2.2 (P16)
5. *Pst* I/ *Sst* I - 11.5, 7.0, 2.2
6. *Sst* I - 40.6 (S1)

Bgl II Fragment 5 - 7.0 Kb

1. *Bgl* II/*Sst* I - 5.4, 1.5, 0.58
2. *Bgl* II - 7.0 (B5)
3. *Bgl* II/*Pst* I - 7.0
4. *Pst* I - 15.3 (P3)
5. *Pst* I/ *Sst* I - 5.4
6. *Sst* I - 40.6 (S1), 5.4 (S7)

Bgl II Fragment 6 - 4.8 Kb

1. *Bgl* II/*Sst* I - 4.8
2. *Bgl* II - 4.8 (B6)

3. *Bgl* II/*Pst* I - 3.0, 1.8
4. *Pst* I - 11.5 (P5), 3.3 (P12)
5. *Pst* I/ *Sst* I - 11.5, 3.3
6. *Sst* I - 40.6 (S1)

Bgl II Fragment 7 - 4.4 Kb

1. *Bgl* II/*Sst* I - 4.4
2. *Bgl* II - 4.4 (B7)
3. *Bgl* II/*Pst* I - 4.2
4. *Pst* I - 15.3 (P3), 3.3 (P12)
5. *Pst* I/ *Sst* I - 6.2, 3.3
6. *Sst* I - 40.6 (S1)

Bgl II Fragment 8 - 2.6 Kb

1. *Bgl* II/*Sst* I - 2.6
2. *Bgl* II - 2.6 (B8)
3. *Bgl* II/*Pst* I - 2.6
4. *Pst* I - 21.4 (P1), 17.4 (P2)
5. *Pst* I/ *Sst* I - 17, 13
6. *Sst* I - 27.5 (S2), 16.7 (S4)

Bgl II Fragment 10 - 2.3 Kb

1. *Bgl* II/*Sst* I - 2.3
2. *Bgl* II - 2.3 (B10)
3. *Bgl* II/*Pst* I - 1.5

4. Pst I - 2.0 (P17)
5. Pst I/ Sst I - 2.0
6. Sst I - 40.6 (S1)

Sst I Fragment 1 - 40.6 Kb

1. Bgl II/Sst I - 18, 7.2, 4.8, 4.4, 2.5, 2.3
2. Bgl II - 20.2 (B3), 18 (B4), 4.8 (B6), 4.4 (B7), 2.5 (B9), 2.3 (B10)
3. Bgl II/Pst I - 7.0, 4.0, 3.3, 3.0, 2.5, 2.2, 1.8, 1.5
4. Pst I - 15.3 (P3), 11.5 (P5), 7.0 (P7), 3.3 (P12), 3.0 (P14), 2.2 (P16), 2.0 (P17)
5. Pst I/ Sst I - 11.5, 7.0, 6.3, 3.3, 3.0, 2.2, 2.0
6. Sst I - 40.6 Kb (S1)

Sst I Fragment 2 - 27.5 Kb

1. Bgl II/Sst I - 24, 13.5, 2.6
2. Bgl II - 24 (B2), 20.2 (B3), 2.6 (B8)
3. Bgl II/Pst I - 14, 9.0, 6.8, 2.6
4. Pst I - 21.4 (P1), 17.4 (P2), 6.8 (P8)
5. Pst I/ Sst I - 17, 13, 6.8
6. Sst I - 27.5 (S2), 16.7 (S4)

Sst I Fragment 3 - 19 Kb

1. Bgl II/Sst I - 19

2. Bgl II - 41 (B1)
3. Bgl II/Pst I - 13.3, 4.8, 3.5
4. Pst I - 13.3 (P4), 4.8 (P9), 3.5 (P10), 3.5 (P11), 3.2 (P13)
5. Pst I/ Sst I - 5.8, 4.8, 3.5, 1.4
6. Sst I - 19 (S3)

Sst I Fragment 4 - 16.7 Kb

1. Bgl II/Sst I - 24, 13.5, 2.6
2. Bgl II - 24 (B2), 20.2(B3), 2.6 (B8)
3. Bgl II/Pst I - 14, 9.0, 2.6
4. Pst I - 21.4 (P1), 17.4 (P2), 7.2 (P6)
5. Pst I/ Sst I - 17, 13
6. Sst I - 27.5 (S2), 16.7 (S4)

Sst I Fragment 5 - 14.2 Kb

1. Bgl II/Sst I - 14, 8.2, 0.52
2. Bgl II - 41 (B1), 0.78 (B11)
3. Bgl II/Pst I - 13.3, 3.8, 2.3, 0.78
4. Pst I - 21.4 (P1), 17.4 (P2), 13.3 (P4), 2.3 (P15 a,b)
5. Pst I/ Sst I - 7.5, 4.4, 2.3
6. Sst I - 14.2 (S5), 8.5 (S6)

Sst I Fragment 6 - 8.5 Kb

1. Bgl II/Sst I - 14, 8.2, 0.52
2. Bgl II - 41 (B1), 0.78 (B11)
3. Bgl II/Pst I - 14, 3.8, 3.3, 2.3, 0.78
4. Pst I - 21.4 (P1), 17.4 (P2), 3.2 (P13), 2.3 (P15 a,b)
5. Pst I/ Sst I - 4.4, 2.3, 1.8
6. Sst I - 14.2 (S5), 8.5 (S6)

Sst I Fragment 7 - 5.4 Kb

1. Bgl II/Sst I - 5.4
2. Bgl II - 7.0 (B5)
3. Bgl II/Pst I - 7.0
4. Pst I - 15.3 (P3)
5. Pst I/ Sst I - 5.4
6. Sst I - 5.4 (S7)

Pst I Fragment 1 - 21.4 Kb

1. Bgl II/Sst I - 24, 13.5, 2.6
2. Bgl II - 24 (B2), 20.2 (B3), 2.6 (B8)
3. Bgl II/Pst I - 14, 3.8, 2.65
4. Pst I - 21.4 (P1), 17.4 (P2)
5. Pst I/ Sst I - 17, 13, 4.4
6. Sst I - 27.5 (S2), 16.7 (S4)

Pst I - Fragment 2 - 17.4 Kb

1. *Bgl* II/*Sst* I - 24, 13.5, 8.2, 5.8, 2.6
2. *Bgl* II - 27.5 (B2), 20.2 (B3), 2.6 (B8)
3. *Bgl* II/*Pst* I - 14, 9.0, 3.8, 2.6
4. *Pst* I - 21.4 (P1), 17.4 (P2)
5. *Pst* I/ *Sst* I - 17 , 13, 4.4,
6. *Sst* I - 27.5 (S2), 16.7 (S4), 8.5 (S6)

Pst I - Fragment 3 - 15.4 Kb

1. *Bgl* II/*Sst* I - 5.4, 4.4, 1.3
2. *Bgl* II - 24 (B2), 7.5 (B5), 4.4 (B7),
3. *Bgl* II/*Pst* I - 7.5, 4.8, 3.3
4. *Pst* I - 15.3 (P3)
5. *Pst* I/ *Sst* I - 6.3, 5.4, 3.7
6. *Sst* I - 40.6 (S1), 27.5 (S2), 5.4 (S7)

Pst I - Fragment 4 - 13.3 Kb

1. *Bgl* II/*Sst* I - 19, 14
2. *Bgl* II - 41 (B1)
3. *Bgl* II/*Pst* I - 13.3
4. *Pst* I - 13.3 (P4)
5. *Pst* I/ *Sst* I - 7.5, 5.8
6. *Sst* I - 19 (S3), 14.2 (S5)

Pst I - Fragment 5 - 11.5 Kb

1. Bgl II/Sst I - 18, 4.8, 2.5
2. Bgl II - 18 (B4), 4.8 (B6), 2.5 (B9)
3. Bgl II/Pst I - 6.8, 2.5, 1.8
4. Pst I - 11.5 (P5)
5. Pst I/ Sst I - 11.5
6. Sst I - 40.6 (S1)

Pst I - Fragment 6 - 7.2 Kb

1. Bgl II/Sst I - 13.5, 7.2
2. Bgl II - 20.2 (B3)
3. Bgl II/Pst I - 7.2
4. Pst I - 7.2 (P6)
5. Pst I/ Sst I - 3.7, 3.5
6. Sst I - 40.6 (S1), 16.7 (S4)

Pst I - Fragment 7 - 7.0 Kb

1. Bgl II/Sst I - 18
2. Bgl II - 18 (B4)
3. Bgl II/Pst I - 7.0
4. Pst I - 7.0 (P7)
5. Pst I/ Sst I - 7.0
6. Sst I - 40.6 (S1)

Pst I - Fragment 8 - 6.8 Kb

1. *Bgl* II/*Sst* I - 24
2. *Bgl* II - 24 (B2)
3. *Bgl* II/*Pst* I - 6.8
4. *Pst* I - 6.8 (P8)
5. *Pst* I/ *Sst* I - 6.8
6. *Sst* I - 27.5 (S2)

Pst I - Fragment 9 - 4.8 Kb

1. *Bgl* II/*Sst* I - 19
2. *Bgl* II - 41 (B1)
3. *Bgl* II/*Pst* I - 4.8
4. *Pst* I - 4.8 (P9)
5. *Pst* I/ *Sst* I - 4.8
6. *Sst* I - 19 (S3)

Pst I - Fragment 10 - 3.5 Kb

1. *Bgl* II/*Sst* I - 19
2. *Bgl* II - 41 (B1)
3. *Bgl* II/*Pst* I - 3.5
4. *Pst* I - 3.5 (P10)
5. *Pst* I/ *Sst* I - 3.5
6. *Sst* I - 19 (S3)

Pst I - Fragment 15 a + b - 2.3 Kb

1. *Bgl* II/*Sst* I - 14, 8.2
2. *Bgl* II - 41 (B1)
3. *Bgl* II/*Pst* I - 2.3
4. *Pst* I - 2.3 (P15 a+b)
5. *Pst* I/ *Sst* I - 2.3
6. *Sst* I - 14.2 (S5), 8.5 (S6)

Pst I - Fragment 16 - 2.25 Kb

1. *Bgl* II/*Sst* I - 18
2. *Bgl* II - 18 (B4)
3. *Bgl* II/*Pst* I - 2.25
4. *Pst* I - 2.25 (P16)
5. *Pst* I/ *Sst* I - 2.25
6. *Sst* I - 40.6 (S1)

Pst I - Fragment 17 - 2.0 Kb

1. *Bgl* II/*Sst* I - 7.2, 2.3
2. *Bgl* II - 18 (B4), 2.3 (B10)
3. *Bgl* II/*Pst* I - 1.5, 0.5
4. *Pst* I - 2.0 (P17)
5. *Pst* I/ *Sst* I - 2.0
6. *Sst* I - 40.6 (S1)

Appendix B

Restriction Fragment Polymorphism Data: Tables and Figures

All of the restriction fragment data is contained within the following tables. They have been arranged by probe number. Hybridizing fragments are listed according to their size under the restriction endonuclease. Each column of data has a number(s) which refers to a particular Southern hybridization filter listed on pages 176-180. The corresponding figure can be found in the back of this appendix. Some hybridizations were repeated, because signals from strains 3, 5, 24 and 25 were consistently weak. If the hybridized fragments do not appear on the first set of autoradiograms of a particular set, then their fragments should appear on a subsequent autoradiogram.

Footnotes

a. Digestion with Eco RI was never achieved for strain IIA51.
b. The 15.0 Kb band is faint.
c. Data for Bcl I were removed because the hybridization signals were ambiguous.
d. Only one thick band is visible but appears to be a degraded doublet.
e. Only one dark band appears in lanes 9, 10, 12 and 13, but on a shorter exposure the 22 and 18 Kb fragments are clearly present in these lanes.
f. The 450 bp band appears on a longer exposure.
g. The 1.2 Kb fragment is faint.

Probe 4 (sizes in Kb)

	Hind III[1]			*Eco* RI[3,5]						*Eco* RV[8]				
	2.65	2.45	1.6	20.5	15.5	12	4.8	1.4	0.7	22	16	9.4	7.2	5.4
1. Skel	0	1	0	0	0	1	0	0	1	0	0	1	0	0
2. ST2 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
3. ST7 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
4. ST10 (Whet)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
5. 2B3 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
6. 2B11 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
7. 2B13 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
8. UP45 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
9. HB17 (Whet)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
10. ED1 (Whet)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
11. MF15 (Whet)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
12. MF55 (Whet)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
13. NY17 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
14. ST37 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
15. UP3 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
16. UP9 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
17. SF73 (R)	0	1	0	0	0	1	0	0	1	0	0	1	0	0

Probe 4 (Sizes in Kb)

	Hind III^{2}			Eco RI^{4,6,7}						Eco RV^{9,10}				
	2.65	2.45	1.6	20.5	15.5	12	4.8	1.4	0.7	22	16	9.4	7.2	5.4
1. Skel	0	1	0	0	0	1	0	0	1	0	0	1	0	0
18. IIA23 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
19. IIA35 (SH)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
20. IIA49 (M)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
21. IIA51 (M)a	0	1	0	?	?	?	?	?	?	0	1	0	0	0
22. IIA54 (M)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
23. IIA55 (M)	0	1	0	0	0	0	1	0	0	0	1	0	0	0
24. IIA62 (M)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
25. IB7 (M)	0	0	1	0	1	0	0	0	0	0	0	0	1	0
26. MF10 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
27. NY10 (M)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
28. ED2 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
29. NY27 (WH)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
30. UP49 (R)	0	1	0	0	0	1	0	0	1	0	0	1	0	0
31. Fry-1	0	0	1	0	1	0	0	0	0	1	0	0	0	0
32. 21-1	0	0	1	0	1	0	0	0	0	1	0	0	0	0
33. AB Skel	0	1	0	1	0	0	0	0	1	0	0	1	0	0
34. Fry-3	1	0	0	0	0	0	0	1	1	0	0	0	0	1

Probe 4 (sizes in Kb)

	BstN I[11,13]				Pst I[14,16]			Bcl I[17]	
	1.6	1.5	1.45	1.3	16.0	5.3	2.0	14.5	5.2
1. Skel	0	1	0	1	1	0	0	0	1
2. ST2 (SH)	0	1	0	1	0	1	0	0	1
3. ST7 (SH)	0	1	0	1	0	1	0	0	1
4. ST10 (Whet)	0	1	0	1	1	0	0	0	1
5. 2B3 (SH)	0	1	0	1	0	1	0	0	1
6. 2B11 (SH)	0	1	0	1	0	1	0	0	1
7. 2B13 (SH)	0	1	0	1	0	1	0	0	1
8. UP45 (SH)	0	1	0	1	0	1	0	0	1
9. HB17 (Whet)	0	1	0	1	1	0	0	0	1
10. ED1 (Whet)	0	1	0	1	1	0	0	0	1
11. MF15 (Whet)	0	1	0	1	1	0	0	0	1
12. MF55 (Whet)	0	1	0	1	1	0	0	0	1
13. NY17 (WH)	0	1	0	1	1	0	0	0	1
14. ST37 (WH)	0	1	0	1	1	0	0	0	1
15. UP3 (WH)	0	1	0	1	1	0	0	0	1
16. UP9 (SH)	0	1	0	1	0	1	0	0	1
17. SF73 (R)	0	1	0	1	1	0	0	0	1

Probe 4 (Sizes in Kb)

	BstN I[12]				Pst I[15]			Bcl I[18,19]	
	1.6	1.5	1.45	1.3	16.0	5.3	2.0	14.5	5.2
1. Skel	0	1	0	1	1	0	0	0	1
18. IIA23 (WH)	0	1	0	1	1	0	0	0	1
19. IIA35 (SH)	0	1	0	1	0	1	0	0	1
20. IIA49 (M)	0	1	0	1	0	1	0	0	1
21. IIA51 (M)	0	1	0	1	0	0	1	0	1
22. IIA54 (M)	0	1	0	1	1	0	0	0	1
23. IIA55 (M)	0	1	0	1	0	0	1	0	1
24. IIA62 (M)	0	1	0	1	0	1	0	0	1
25. IB7 (M)	0	1	0	1	0	1	0	0	1
26. MF10 (WH)	0	1	0	1	1	0	0	0	1
27. NY10 (M)	0	1	0	1	1	0	0	0	1
28. ED2 (WH)	0	1	0	1	1	0	0	0	1
29. NY27 (WH)	0	1	0	1	1	0	0	0	1
30. UP49 (R)	0	1	0	1	1	0	0	0	1
31. Fry-1	0	1	0	1	0	1	0	0	1
32. 21-1	0	1	0	1	0	1	0	0	1
33. AB Skel	0	1	0	1	1	0	0	0	1
34. Fry-3	1	0	1	0	0	1	0	1	0

Probe 19 (sizes in Kb)

		Hind III[20,22]		Eco RI[23,25]					Eco RV[26,28]				
		4.2	1.4	17	5.5	5.0	4.3	2.6	31	23	15	10.5	6.6
1.	Skel	0	1	0	0	1	1	0	0	0	0	0	1
2.	ST2 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
3.	ST7 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
4.	ST10 (Whet)	0	1	0	0	1	1	0	0	0	0	0	1
5.	2B3 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
6.	2B11 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
7.	2B13 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
8.	UP45 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
9.	HB17 (Whet)	0	1	0	0	1	1	0	0	0	0	0	1
10.	ED1 (Whet)	0	1	0	0	1	1	0	0	0	0	0	1
11.	MF15 (Whet)	0	1	0	0	1	1	0	0	0	0	0	1
12.	MF55 (Whet)	0	1	0	0	1	1	0	0	0	0	0	1
13.	NY17 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
14.	ST37 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
15.	UP3 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
16.	UP9 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
17.	SF73 (R)	0	1	0	0	1	1	0	0	0	0	0	1

Probe 19 (Sizes in Kb)

	Hind III[21]		Eco RI[24]					Eco RV[27,29]				
	4.2	1.4	17	5.5	5.0	4.3	2.6	31	23	15	10.5	6.6
1. Skel	0	1	0	0	1	1	0	0	0	0	0	1
18. IIA23 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
19. IIA35 (SH)	0	1	0	1	0	1	0	1	0	0	0	0
20. IIA49 (M)	0	1	0	1	0	1	0	1	0	0	0	0
21. IIA51 (M)	0	1	?	?	?	?	?	0	0	0	0	1
22. IIA54 (M)	0	1	0	0	1	1	0	0	0	0	0	1
23. IIA55 (M)	0	1	0	0	0	0	1	0	0	0	0	1
24. IIA62 (M)	0	1	0	1	0	1	0	1	0	0	0	0
25. IB7 (M)	0	1	0	1	0	1	0	1	0	0	0	0
26. MF10 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
27. NY10 (M)	0	1	0	0	1	1	0	0	0	0	0	1
28. ED2 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
29. NY27 (WH)	0	1	0	0	1	1	0	0	0	0	0	1
30. UP49 (R)	0	1	0	0	1	1	0	0	0	0	0	1
31. Fry-1	0	1	0	1	0	1	0	0	1	0	0	0
32. 21-1	0	1	0	1	0	1	0	0	1	0	0	0
33. AB Skel	0	1	0	0	1	1	0	0	0	0	1	0
34. Fry-[b]	1	0	1	0	0	0	0	0	0	1	0	0

Probe 19 (sizes in Kb)

	BstN $I^{30,32}$				Pst $I^{33,35}$			Bcl $I^{36,38}$		
	2.1	1.8	0.7	0.6	16	8	7	2.4	1.2	0.9
1. Skel	1	0	1	0	1	0	0	0	1	1
2. ST2 (SH)	0	1	1	0	1	0	0	1	0	0
3. ST7 (SH)	0	1	1	0	1	0	0	1	0	0
4. ST10 (Whet)	1	0	1	0	1	0	0	0	1	1
5. 2B3 (SH)	0	1	1	0	1	0	0	1	0	0
6. 2B11 (SH)	0	1	1	0	1	0	0	1	0	0
7. 2B13 (SH)	0	1	1	0	1	0	0	1	0	0
8. UP45 (SH)	0	1	1	0	1	0	0	1	0	0
9. HB17 (Whet)	1	0	1	0	1	0	0	0	1	1
10. ED1 (Whet)	1	0	1	0	1	0	0	0	1	1
11. MF15 (Whet)	1	0	1	0	1	0	0	0	1	1
12. MF55 (Whet)	1	0	1	0	1	0	0	0	1	1
13. NY17 (WH)	1	0	1	0	1	0	0	0	1	1
14. ST37 (WH)	1	0	1	0	1	0	0	0	1	1
15. UP3 (WH)	1	0	1	0	1	0	0	0	1	1
16. UP9 (SH)	0	1	1	0	1	0	0	1	0	0
17. SF73 (R)	1	0	1	0	1	0	0	0	1	1

Probe 19 (Sizes in Kb)

	BstN I[31]				Pst I[34]			Bcl I[37]		
	2.1	1.8	0.7	0.6	16	8	7	2.4	1.2	0.9
1. Skel	1	0	1	0	1	0	0	0	1	1
18. IIA23 (WH)	1	0	1	0	1	0	0	0	1	1
19. IIA35 (SH)	0	1	1	0	1	0	0	1	0	0
20. IIA49 (M)	0	1	1	0	1	0	0	1	0	0
21. IIA51 (M)	0	1	1	0	0	1	0	0	1	1
22. IIA54 (M)	1	0	1	0	1	0	0	0	1	1
23. IIA55 (M)	0	1	1	0	0	1	0	0	1	1
24. IIA62 (M)	0	1	1	0	1	0	0	1	0	0
25. IB7 (M)	0	1	1	0	1	0	0	1	0	0
26. MF10 (WH)	1	0	1	0	1	0	0	0	1	1
27. NY10 (M)	1	0	1	0	1	0	0	0	1	1
28. ED2 (WH)	1	0	1	0	1	0	0	0	1	1
29. NY27 (WH)	1	0	1	0	1	0	0	0	1	1
30. UP49 (R)	1	0	1	0	1	0	0	0	1	1
31. Fry-1	0	1	1	0	1	0	0	1	0	0
32. 21-1	0	1	1	0	1	0	0	1	0	0
33. AB Skel	1	0	1	0	1	0	0	0	1	1
34. Fry-3	0	0	0	1	0	0	1	0	0	1

Probe 20 (sizes in Kb)

	Hind III^{39,41}		Eco RI^{42}				Eco RV^{44,46}			
	3.6	2.6	15	11.5	7.0	6.0	21	15	6.1	2.4
1. Skel	1	0	0	1	0	0	0	0	1	1
2. ST2 (SH)	0	1	0	1	0	0	1	0	0	0
3. ST7 (SH)	0	1	0	1	0	0	1	0	0	0
4. ST10 (Whet)	1	0	0	0	0	1	0	0	1	1
5. 2B3 (SH)	0	1	0	1	0	0	1	0	0	0
6. 2B11 (SH)	0	1	0	1	0	0	1	0	0	0
7. 2B13 (SH)	0	1	0	1	0	0	1	0	0	0
8. UP45 (SH)	0	1	0	1	0	0	1	0	0	0
9. HB17 (Whet)	1	0	0	0	0	1	0	0	1	1
10. ED1 (Whet)	1	0	0	0	0	1	0	0	1	1
11. MF15 (Whet)	1	0	0	0	0	1	0	0	1	1
12. MF55 (Whet)	1	0	0	0	0	1	0	0	1	1
13. NY17 (WH)	1	0	0	0	0	1	0	0	1	1
14. ST37 (WH)	1	0	0	0	0	1	0	0	1	1
15. UP3 (WH)	1	0	0	0	0	1	0	0	1	1
16. UP9 (SH)	0	1	0	1	0	0	1	0	0	0
17. SF73 (R)	1	0	0	0	0	1	0	0	1	1

Probe 20 (Sizes in Kb)

	Hind III[40]		Eco RI[43]				Eco RV[45]			
	3.6	2.6	15	11.5	7.0	6.0	21	15	6.1	2.4
1. Skel	1	0	0	1	0	0	0	0	1	1
18. IIA23 (WH)	1	0	0	0	0	1	0	0	1	1
19. IIA35 (SH)	0	1	0	1	0	0	1	0	0	0
20. IIA49 (M)	0	1	0	1	0	0	1	0	0	0
21. IIA51 (M)	1	0	?	?	?	?	0	1	0	0
22. IIA54 (M)	1	0	0	0	0	1	0	0	1	1
23. IIA55 (M)	1	0	1	0	0	0	0	1	0	0
24. IIA62 (M)	0	1	0	1	0	0	1	0	0	0
25. IB7 (M)	0	1	0	1	0	0	1	0	0	0
26. MF10 (WH)	1	0	0	0	0	1	0	0	1	1
27. NY10 (M)	1	0	0	0	0	1	0	0	1	1
28. ED2 (WH)	1	0	0	0	0	1	0	0	1	1
29. NY27 (WH)	1	0	0	0	0	1	0	0	1	1
30. UP49 (R)	1	0	0	0	0	1	0	0	1	1
31. Fry-1	0	1	0	1	0	0	1	0	0	0
32. 21-1	0	1	0	1	0	0	1	0	0	0
33. AB Skel	1	0	0	1	0	0	0	0	1	1
34. Fry-3	?	?	0	0	1	0	1	0	0	0

	Probe 20 (sizes in Kb)[c]					
	BstN I[47]			Pst I[49]		
	4.8	2.3	1.6	12	10.5	8
1. Skel	1	0	1	1	0	1
2. ST2 (SH)	1	0	1	0	1	1
3. ST7 (SH)	1	0	1	1	0	1
4. ST10 (Whet)	1	0	1	1	0	1
5. 2B3 (SH)	1	0	1	0	1	1
6. 2B11 (SH)	1	0	1	0	1	1
7. 2B13 (SH)	1	0	1	0	1	1
8. UP45 (SH)	1	0	1	0	1	1
9. HB17 (Whet)	1	0	1	1	0	1
10. ED1 (Whet)	1	0	1	1	0	1
11. MF15 (Whet)	1	0	1	1	0	1
12. MF55 (Whet)	1	0	1	1	0	1
13. NY17 (WH)	1	0	1	1	0	1
14. ST37 (WH)	1	0	1	1	0	1
15. UP3 (WH)	1	0	1	1	0	1
16. UP9 (SH)	1	0	1	0	1	1
17. SF73 (R)	1	0	1	1	0	1

Probe 20 (Sizes in Kb)[c]

	BstN I[48]			Pst I[50]		
	4.8	2.3	1.6	12	10.5	8
1. Skel	1	0	1	1	0	1
18. IIA23 (WH)	1	0	1	1	0	1
19. IIA35 (SH)	1	0	1	0	1	1
20. IIA49 (M)	1	0	1	0	1	1
21. IIA51 (M)	1	0	1	1	0	1
22. IIA54 (M)	1	0	1	1	0	1
23. IIA55 (M)	1	0	1	1	0	1
24. IIA62 (M)	1	0	1	0	1	1
25. IB7 (M)	1	0	1	0	1	1
26. MF10 (WH)	1	0	1	1	0	1
27. NY10 (M)	1	0	1	1	0	1
28. ED2 (WH)	1	0	1	1	0	1
29. NY27 (WH)	1	0	1	1	0	1
30. UP49 (R)	1	0	1	1	0	1
31. Fry-1	0	1	1	0	1	1
32. 21-1	0	1	1	0	1	1
33. AB Skel	1	0	1	1	0	1
34. Fry-3	1	0	0	0	0	1

Probe 27 (sizes in Kb)

	Hind III[51]					*Eco* RI[53,55]							
	8.5	5.0	3.0	2.9	1.3	13	12	7.9	7.6	7.2	6.2	5.7	2.5
1. Skel	1	0	0	0	0	1	1	0	0	1	0	0	1
2. ST2 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
3. ST7 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
4. ST10 (Whet)	1	0	0	0	0	1	1	0	0	1	0	0	1
5. 2B3 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
6. 2B11 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
7. 2B13 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
8. UP45 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
9. HB17 (Whet)	1	0	0	0	0	1	1	0	0	1	0	0	1
10. ED1 (Whet)	1	0	0	0	0	1	1	0	0	1	0	0	1
11. MF15 (Whet)	1	0	0	0	0	1	1	0	0	1	0	0	1
12. MF55 (Whet)	1	0	0	0	0	1	1	0	0	1	0	0	1
13. NY17 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
14. ST37 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
15. UP3 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
16. UP9 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
17. SF73 (R)	1	0	0	0	0	1	1	0	0	1	0	0	1

Probe 27 (Sizes in Kb)

	Hind III[52]					Eco RI[54]							
	8.5	5.0	3.0	2.9	1.3	13	12	7.9	7.6	7.2	6.2	5.7	2.5
1. Skel	1	0	0	0	0	1	1	0	0	1	0	0	1
18. IIA23 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
19. IIA35 (SH)	1	0	0	0	0	0	1	0	1	0	0	0	1
20. IIA49 (M)	1	0	0	0	0	0	1	0	1	0	0	0	1
21. IIA51 (M)	0	1	1	0	0	?	?	?	?	?	?	?	?
22. IIA54 (M)	1	0	0	0	0	1	1	0	0	1	0	0	1
23. IIA55 (M)	0	1	1	0	0	0	0	1	1	0	0	1	0
24. IIA62 (M)	1	0	0	0	0	0	1	0	1	0	0	0	1
25. IB7 (M)	1	0	0	0	0	0	1	0	1	0	0	0	1
26. MF10 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
27. NY10 (M)	1	0	0	0	0	1	1	0	0	1	0	0	1
28. ED2 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
29. NY27 (WH)	1	0	0	0	0	1	1	0	0	1	0	0	1
30. UP49 (R)	1	0	0	0	0	1	1	0	0	1	0	0	1
31. Fry-1	1	0	0	0	0	0	1	0	1	0	0	0	1
32. 21-1	1	0	0	0	0	0	1	0	1	0	0	0	1
33. AB Skel	1	0	0	0	0	1	1	0	0	1	0	0	1
34. Fry-3	0	0	0	1	1	0	0	0	0	0	1	0	0

Probe 27 (sizes in Kb)

Eco RV^{56}

		31	18.5	8.1	6.2	5.6	4.8	4.4	4.3	3.8	3.3	2.75	2.7	2.6	2.5	1.2	0.64
1.	Skel	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
2.	ST2 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
3.	ST7 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
4.	ST10 (Whet)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
5.	2B3 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
6.	2B11 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
7.	2B13 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
8.	UP45 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
9.	HB17 (Whet)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
10.	ED1 (Whet)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
11.	MF15 (Whet)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
12.	MF55 (Whet)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
13.	NY17 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
14.	ST37 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
15.	UP3 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
16.	UP9 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
17.	SF73 (R)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1

Probe 27 (Sizes in Kb)

Eco RV[57,58]

	31	18.5	8.1	6.2	5.6	4.8	4.4	4.3	3.8	3.3	2.75	2.7	2.6	2.5	1.2	0.64
1. Skel	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
18. IIA23 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
19. IIA35 (SH)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
20. IIA49 (M)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
21. IIA51 (M)	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1
22. IIA54 (M)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
23. IIA55 (M)	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1
24. IIA62 (M)	?	0	0	1	0	0	0	0	0	0	1	0	1	0	1	1
25. IB7 (M)	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0
26. MF10 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
27. NY10 (M)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
28. ED2 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
29. NY27 (WH)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
30. UP49 (R)	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
31. Fry-1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1
32. 21-1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1
33. AB Skel	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1
34. Fry-3	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1

Probe 27 (sizes in Kb)

	BstN I[59]										Pst I[61e]					
	8.5	8.2	7.7	7.4	4.7	4.3	3.1	2.6	2.3	1.4	22	18	6.3	5.8	5.2	2.2
1. Skel	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
2. ST2 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
3. ST7 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
4. ST10 (Whet)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
5. 2B3 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
6. 2B11 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
7. 2B13 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
8. UP45 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
9. HB17 (Whet)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
10. ED1 (Whet)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
11. MF15 (Whet)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
12. MF55 (Whet)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
13. NY17 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
14. ST37 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
15. UP3 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
16. UP9 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
17. SF73 (R)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1

Probe 27 (Sizes in Kb)

	BstN I[60]										Pst I[62,63]					
	8.5	8.2	7.7	7.4	4.7	4.3	3.1	2.6	2.3	1.4	22	18	6.3	5.8	5.2	2.2
1. Skel	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
18. IIA23 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
19. IIA35 (SH)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
20. IIA49 (M)	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
21. IIA51 (M)	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1
22. IIA54 (M)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
23. IIA55 (M)	0	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1
24. IIA62 (M)[d]	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
25. IB7 (M)[d]	1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
26. MF10 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
27. NY10 (M)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
28. ED2 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
29. NY27 (WH)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
30. UP49 (R)	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1
31. Fry-1	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	1
32. 21-1	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	1
33. AB Skel	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1
34. Fry-3	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0

Probe 27 (sizes in Kb)

Bcl I[64,66]

	25	23	19.5	16	13	8.4	7.2	6.6	3.2	2.6	2.2	1.8	1.25	1.0
1. Skel	0	0	0	0	0	1	0	0	0	1	0	0	1	0
2. ST2 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
3. ST7 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
4. ST10 (Whet)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
5. 2B3 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
6. 2B11 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
7. 2B13 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
8. UP45 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
9. HB17 (Whet)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
10. ED1 (Whet)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
11. MF15 (Whet)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
12. MF55 (Whet)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
13. NY17 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
14. ST37 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
15. UP3 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
16. UP9 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
17. SF73 (R)	0	0	0	0	0	1	0	0	0	1	0	0	1	0

Probe 27 (Sizes in Kb)

Bcl I[65,67]

	25	23	19.5	16	13	8.4	7.2	6.6	3.2	2.6	2.2	1.8	1.25	1.0
1. Skel	0	0	0	0	0	1	0	0	0	1	0	0	1	0
18. IIA23 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
19. IIA35 (SH)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
20. IIA49 (M)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
21. IIA51 (M)	0	1	0	1	0	0	0	1	0	1	0	1	0	0
22. IIA54 (M)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
23. IIA55 (M)	0	1	0	1	0	0	0	1	0	1	0	1	0	0
24. IIA62 (M)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
25. IB7 (M)	0	0	0	0	1	0	1	1	1	0	1	0	0	0
26. MF10 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
27. NY10 (M)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
28. ED2 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
29. NY27 (WH)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
30. UP49 (R)	0	0	0	0	0	1	0	0	0	1	0	0	1	0
31. Fry-1	0	0	0	0	1	0	1	1	0	0	0	0	0	1
32. 21-1	0	0	0	0	1	0	1	1	0	0	0	0	0	1
33. AB Skel	0	0	0	0	0	1	0	0	0	1	0	0	1	0
34. Fry-3	0	0	1	0	1	0	0	0	0	0	0	0	0	0

Probe 64 (sizes in Kb)

	Hind III^{68}		Eco RI^{70}											Eco RV^{72,74}						
	6.3	6.0	19.5	12	11	6.5	3.9	2.6	2.5	1.9	0.65	0.56	0.45	30	23	16	13	11	8.2	7.5
1. Skel	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
2. ST2 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
3. ST7 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
4. ST10 (Whet)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
5. 2B3 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
6. 2B11 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
7. 2B13 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
8. UP45 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
9. HB17 (Whet)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
10. ED1 (Whet)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
11. MF15 (Whet)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
12. MF55 (Whet)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
13. NY17 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
14. ST37 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
15. UP3 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
16. UP9 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
17. SF73 (R)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1

Probe 64 (Sizes in Kb)

	Hind III^{69}		Eco RI^{71f}											Eco RV^{73}						
	6.3	6.0	19.5	12	11	6.5	3.9	2.6	2.5	1.9	0.65	0.56	0.45	30	23	16	13	11	8.2	7.5
1. Skel	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
18. IIA23 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
19. IIA35 (SH)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
20. IIA49 (M)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
21. IIA51 (M)	1	0	?	?	?	?	?	?	?	?	?	?	?	0	0	1	0	1	0	0
22. IIA54 (M)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
23. IIA55 (M)	1	0	1	0	0	1	0	0	1	1	1	1	0	0	0	1	0	1	0	0
24. IIA62 (M)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
25. IB7 (M)	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
26. MF10 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
27. NY10 (M)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
28. ED2 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
29. NY27 (WH)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
30. UP49 (R)	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1
31. Fry-1	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
32. 21-1	1	0	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0
33. AB Skel	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	1	0	0	1
34. Fry-3	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	0	0	0

Probe 64 (sizes in Kb)

	BstN I[75g]				Pst I[77]							Bcl I[80,82,83]			
	2.6	1.4	1.2	0.9	22	18	17	16	15	12	10	22	19	12	5.8
1. Skel	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
2. ST2 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
3. ST7 (SH	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
4. ST10 (Whet)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
5. 2B3 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
6. 2B11 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
7. 2B13 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
8. UP45 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
9. HB17 (Whet)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
10. ED1 (Whet)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
11. MF15 (Whet)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
12. MF55 (Whet)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
13. NY17 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
14. ST37 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
15. UP3 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
16. UP9 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
17. SF73 (R)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0

Probe 64 (Sizes in Kb)

	BstN I[76f]				Pst I[78,79]							Bcl I[81]			
	2.6	1.4	1.2	0.9	22	18	17	16	5	12	10	22	19	12	5.8
1. Skel	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
18. IIA23 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
19. IIA35 (SH)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
20. IIA49 (M)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
21. IIA51 (M)	1	0	1	1	0	0	0	1	0	1	0	1	1	0	0
22. IIA54 (M)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
23. IIA55 (M)	1	0	1	1	0	0	0	1	0	1	0	1	1	0	0
24. IIA62 (M)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
25. IB7 (M)	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
26. MF10 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
27. NY10 (M)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
28. ED2 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
29. NY27 (WH)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
30. UP49 (R)	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0
31. Fry-1	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
32. 21-1	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
33. AB Skel	1	0	1	1	0	0	1	0	0	1	0	1	1	0	0
34. Fry-3	0	1	0	1	0	0	0	0	1	0	1	1	1	0	0

Southern Hybridization and Filter Numbers

Probe 4

1. Hind III 22592 A
2. Hind III 22592 B
3. Eco RI 392 A
4. Eco RI 392 B
5. Eco RI 692 A
6. Eco RI 692 B
7. Eco RI 10991
8. Eco RV 22291
9. Eco RV 2491
10. Eco RV 1292
11. BstN I 22592 A
12. BstN I 22592 B
13. BstN I 72391
14. Pst I 392 A
15. Pst I 392 B
16. Pst I 22291
17. Bcl I 13191
18. Bcl I 1491
19. Bcl I 11692

Southern Hybridization and Filter Numbers

Probe 19

20. *Hind* III 22592 A

21. *Hind* III 22592 B

22. *Hind* III 6690

23. *Eco* RI 392 A

24. *Eco* RI 392 B

25. *Eco* RI 6690

26. *Eco* RV 3992 A

27. *Eco* RV 3992 B

28. *Eco* RV 6690

29. *Eco* RV 11692

30. *Bst*N I 22592 A

31. *Bst*N I 22592 B

32. *Bst*N I 72391

33. *Pst* I 392 A

34. *Pst* I 392 B

35. *Pst* I 6690

36. *Bcl* I 12392 A

37. *Bcl* I 12392 B

38. *Bcl* I 31792 A

Southern Hybridization and Filter Numbers

Probe 20

39. *Hin*d III 31392 A
40. *Hin*d III 31392 B
41. *Hin*d III 31590
42. *Eco* RI 41592 A
43. *Eco* RI 41592 B
44. *Eco* RV 3992 A
45. *Eco* RV 3992 B
46. *Eco* RV 31590
47. *Bst*N I 32092 A
48. *Bst*N I 32092 B
49. *Pst* I 31992 A
50. *Pst* I 31992 B

Southern Hybridization and Filter Numbers

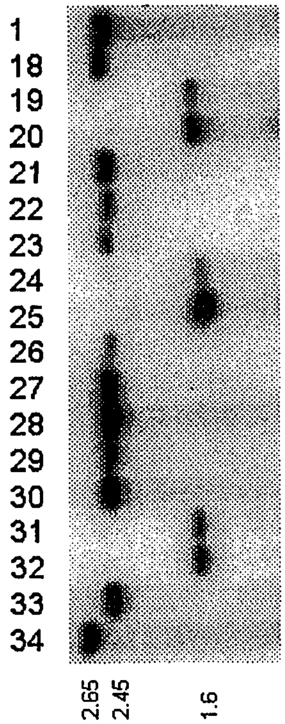
Probe 27

51. *Hin*d III 22592 A
52. *Hin*d III 22592 B
53. *Eco* RI 4192 A
54. *Eco* RI 4192 B
55. *Eco* RI 191
56. *Eco* RV 32092 A
57. *Eco* RV 32092 B
58. *Eco* RV 2491
59. *Bst*N I 22592 A
60. *Bst*N I 22592 B
61. *Pst* I 6690
62. *Pst* I 392 B
63. *Pst* I 2491
64. *Bcl* I 12392 A
65. *Bcl* I 12392 B
66. *Bcl* I 13191
67. *Bcl* I 2491

Southern Hybridization and Filter Numbers

Probe 64

68. Hind III 31392 A
69. Hind III 31392 B
70. Eco RI 41592 A
71. Eco RI 41592 B
72. Eco RV 3992 A
73. Eco RV 3992 B
74. Eco RV 32190
75. BstN I 22592 A
76. BstN I 22592 B
77. Pst I 31992 A
78. Pst I 31992 B
79. Pst I 13192
80. Bcl I 31792 A
81. Bcl I 31792 B
82. Bcl I 13191
83. Bcl I 692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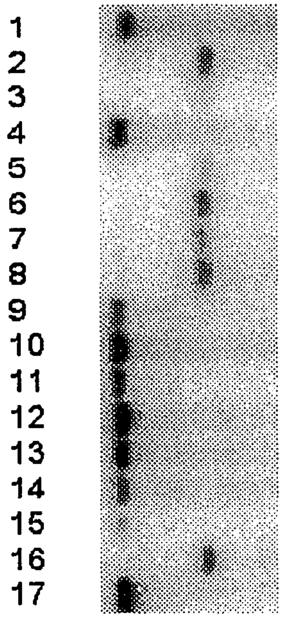
2.45
1.6

#2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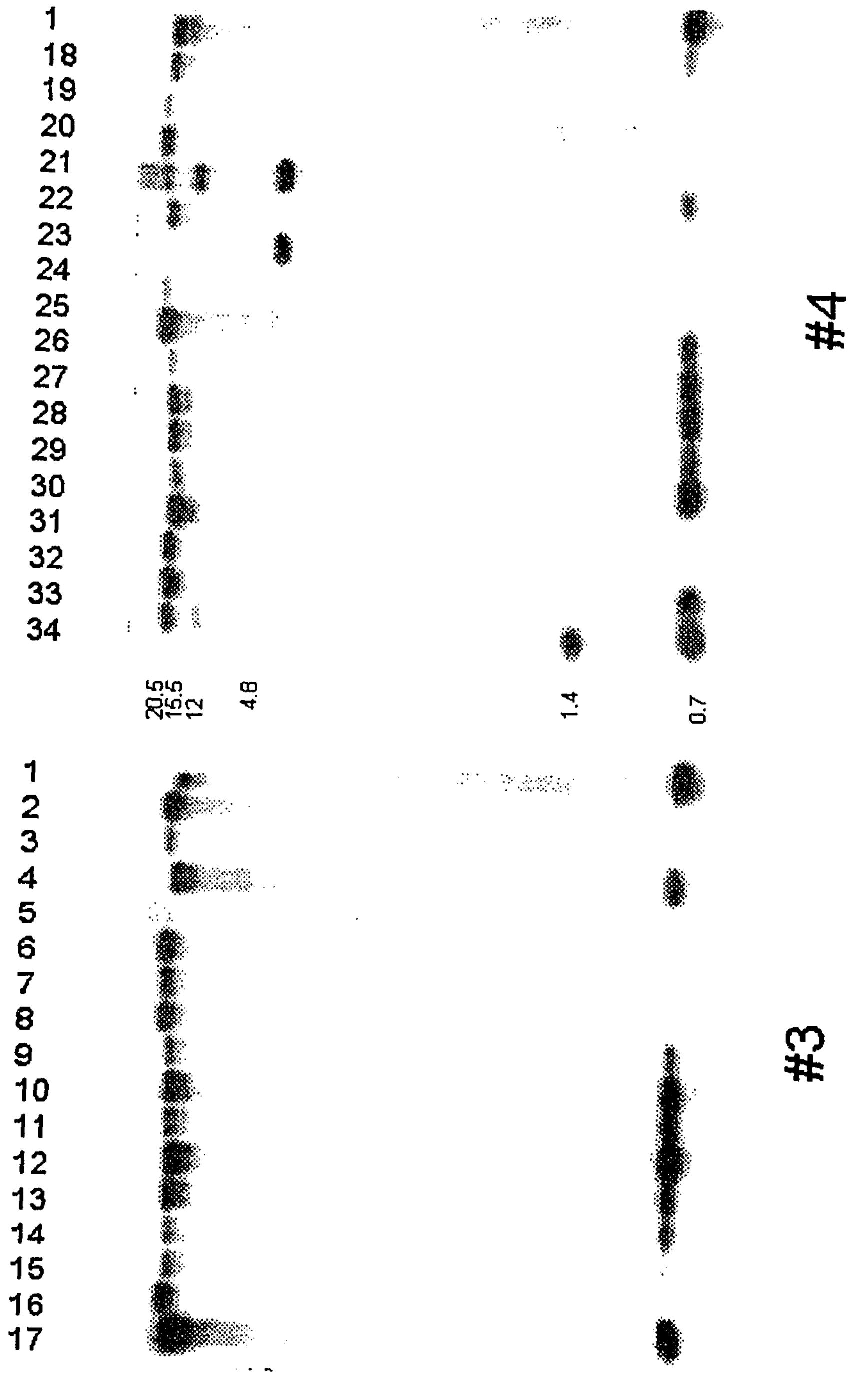
#1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20.5
15.5
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4.8
1.4
0.7
#4
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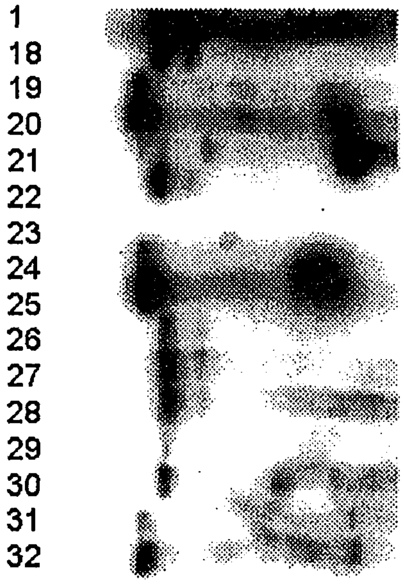
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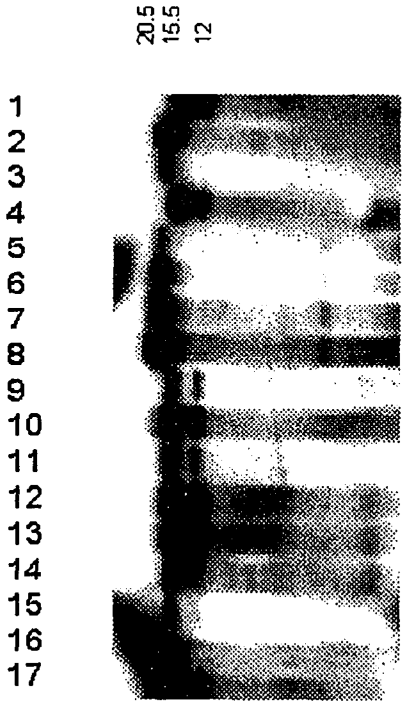
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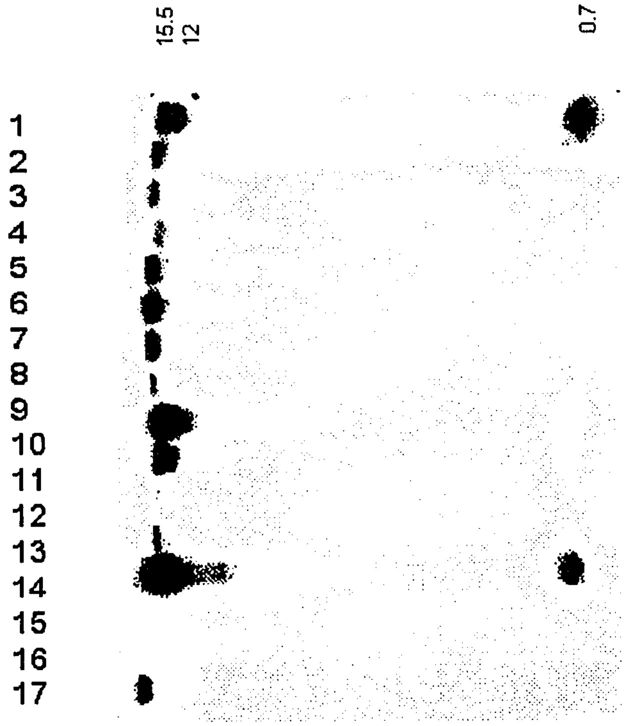
#5



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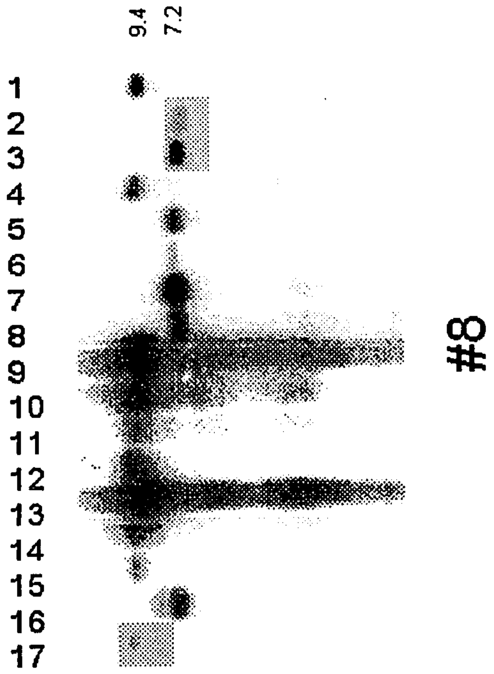
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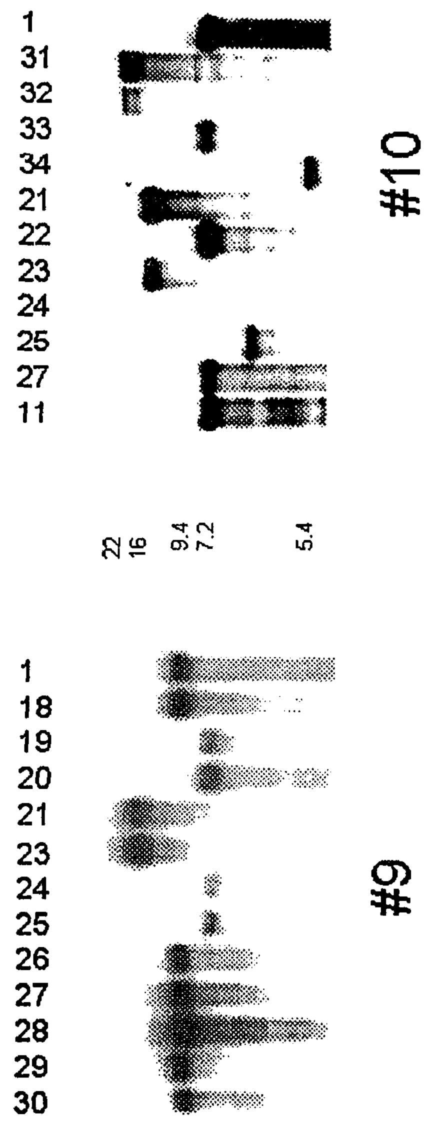
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#9
1
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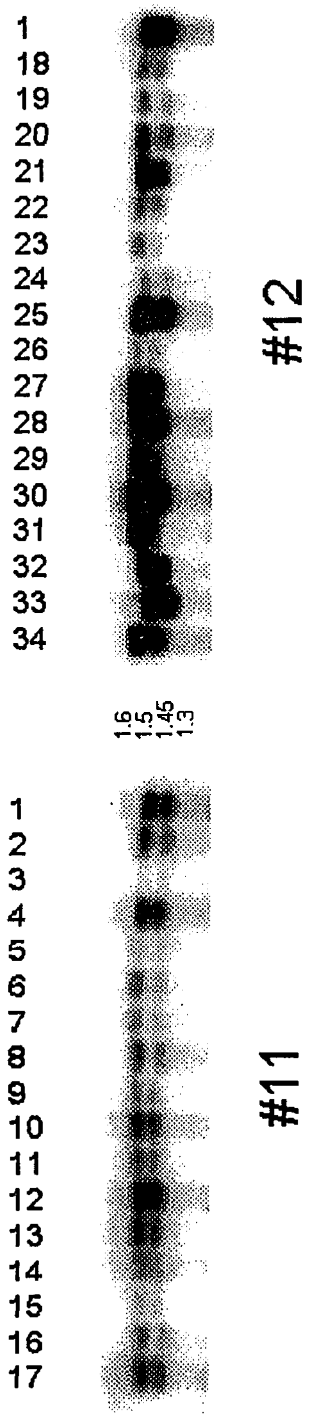
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33
34
#12
1.6
1.5
1.45
1.3
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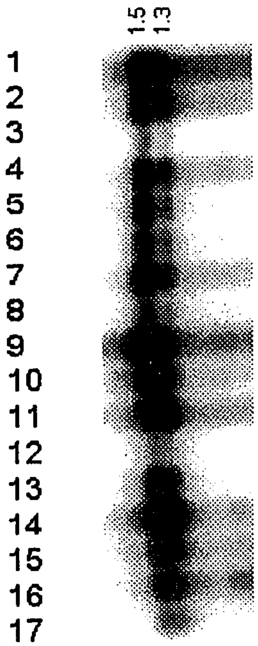
#11



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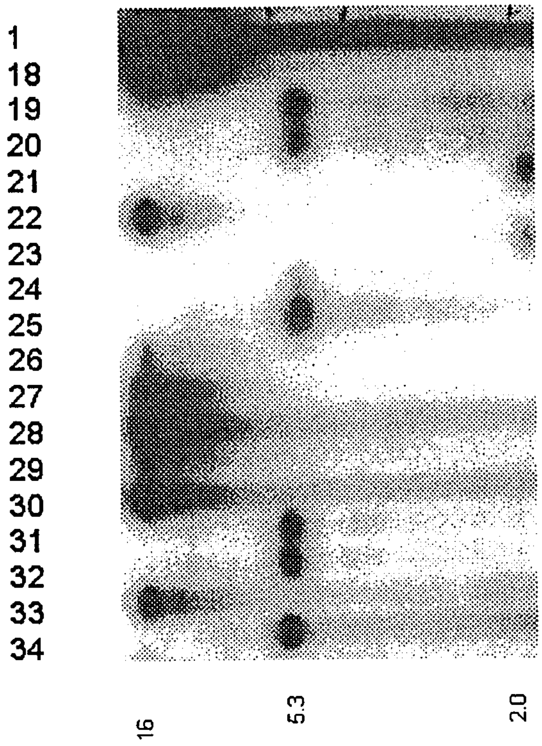
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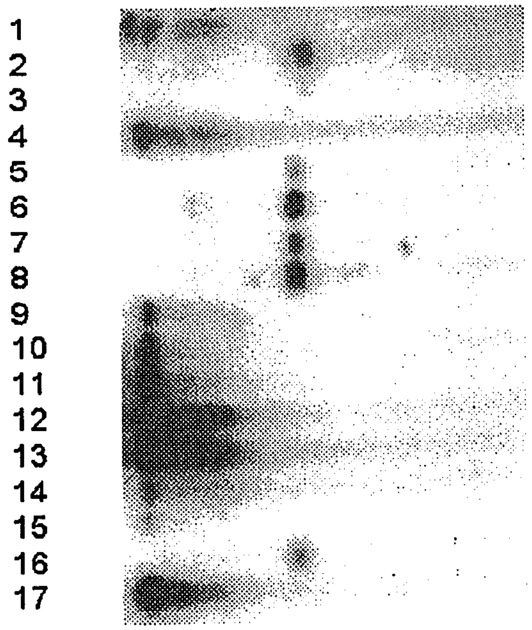
5.3
2.0

#15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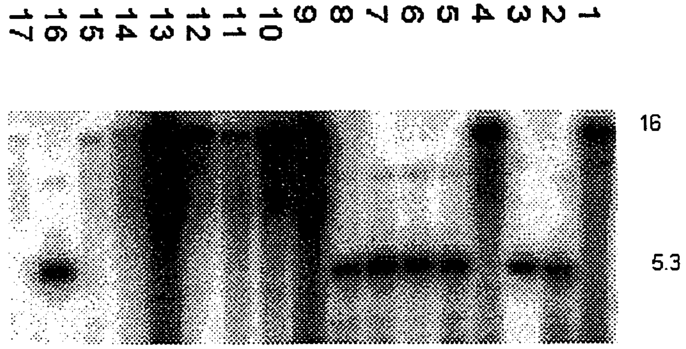

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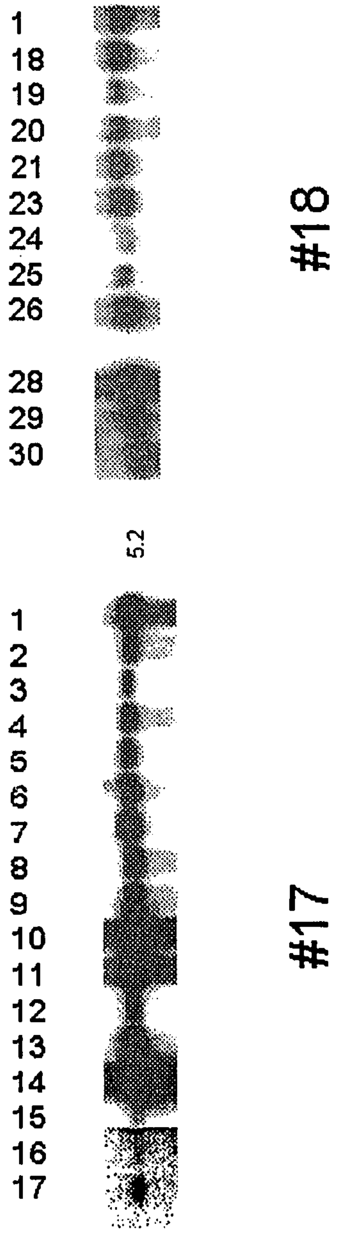
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#18
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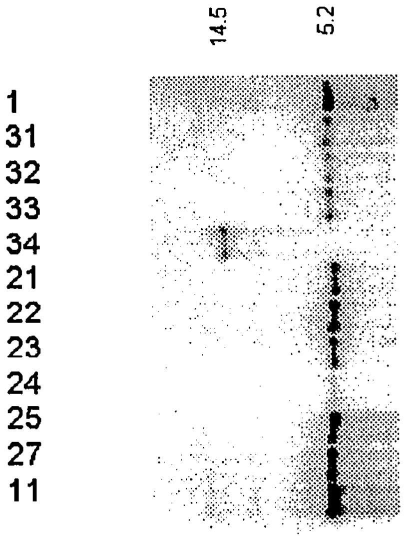
#17



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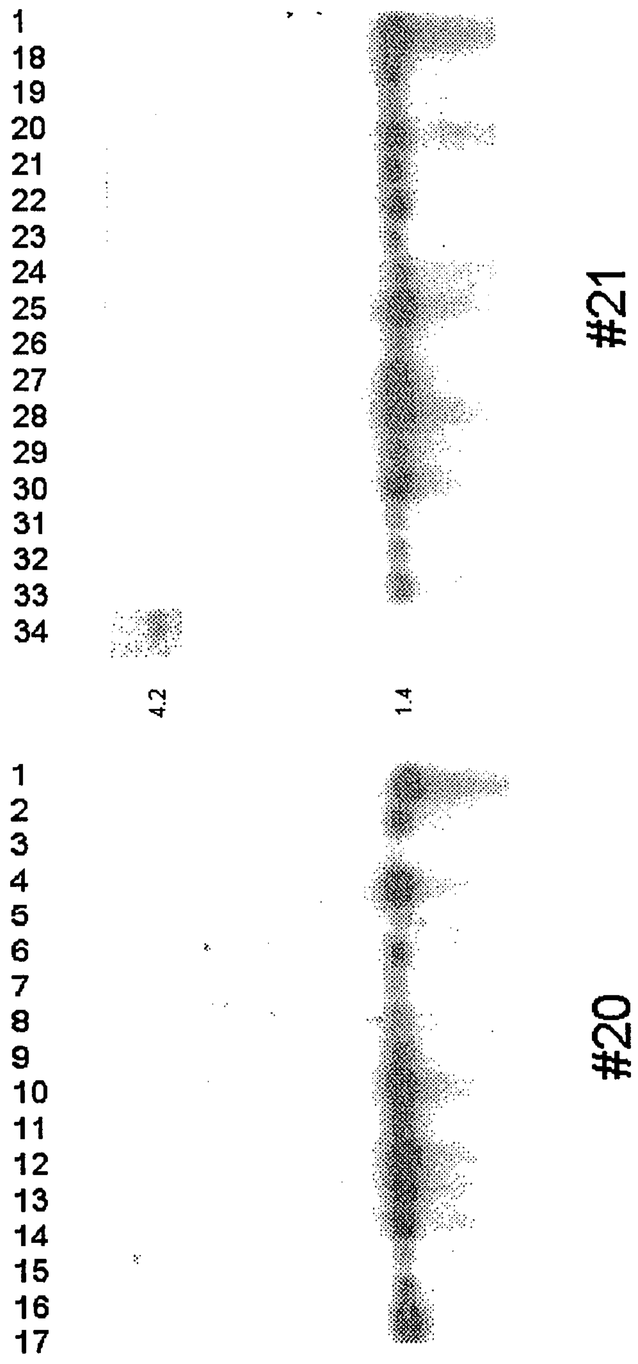
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#21
4.2
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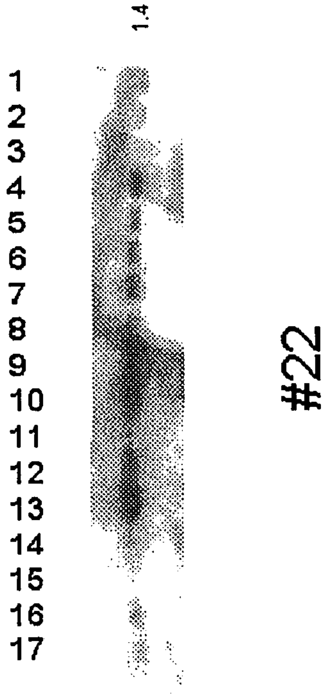
#20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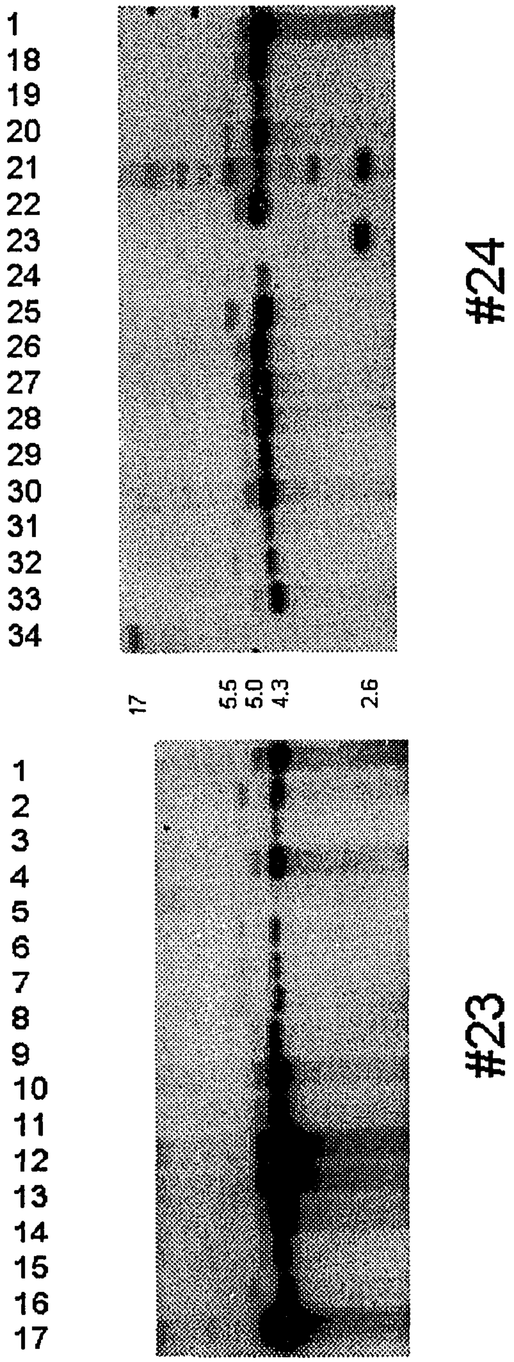
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4.3
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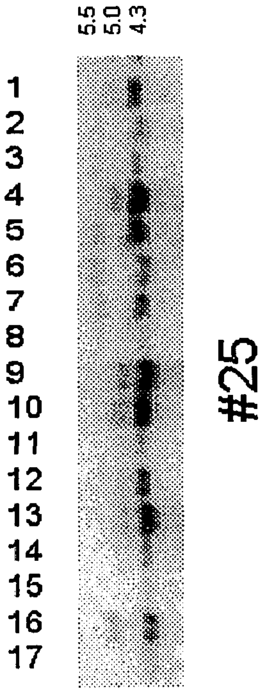
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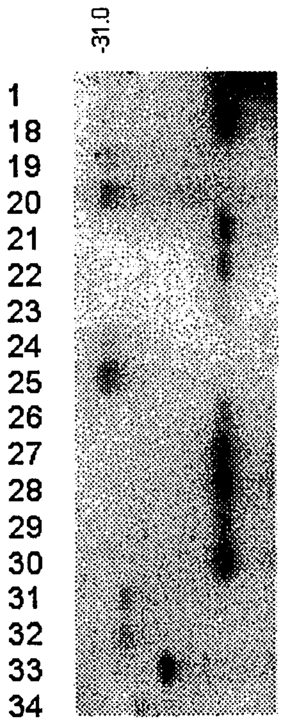
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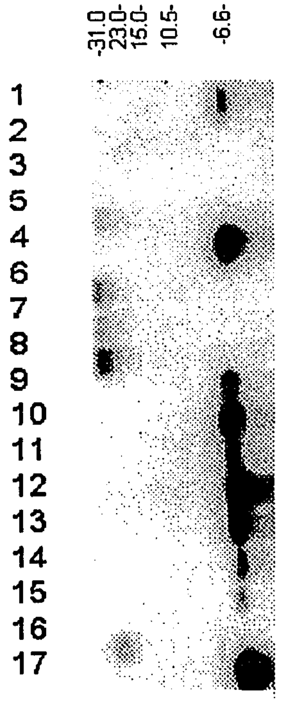
#27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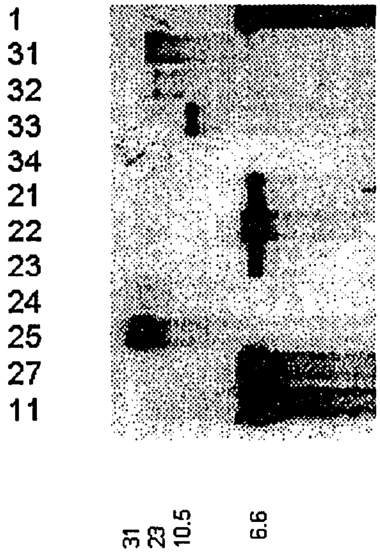
#26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6.6

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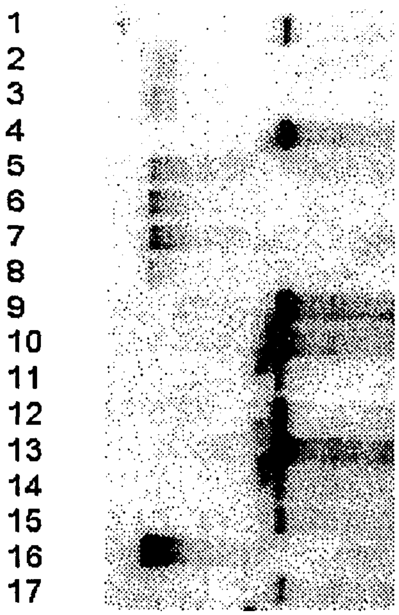
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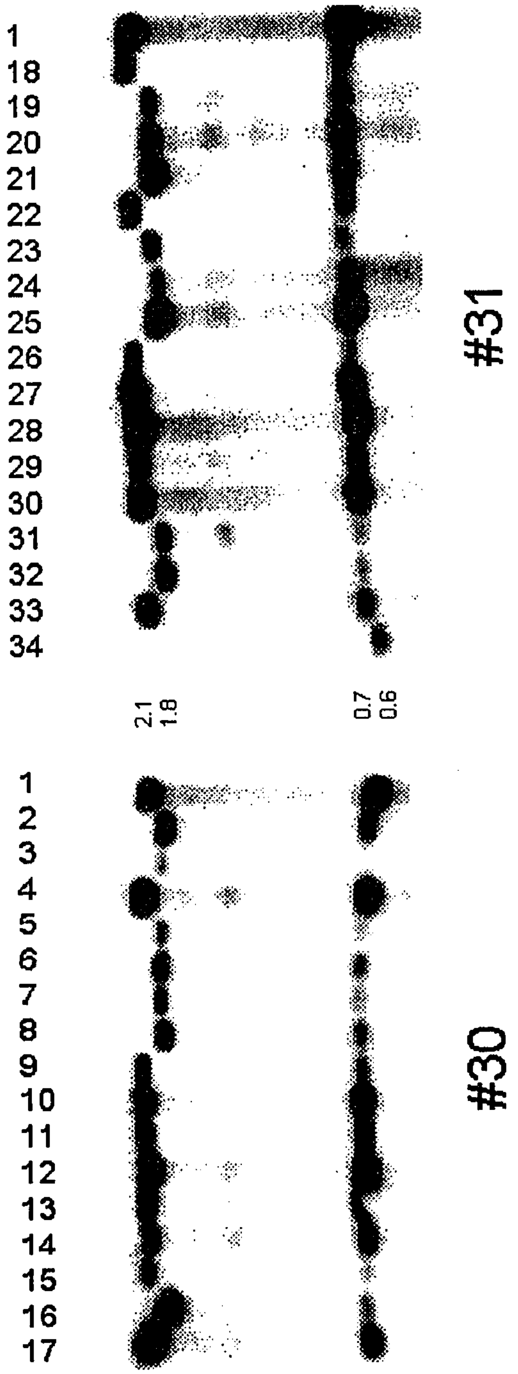
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34
#31
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1.8
0.7
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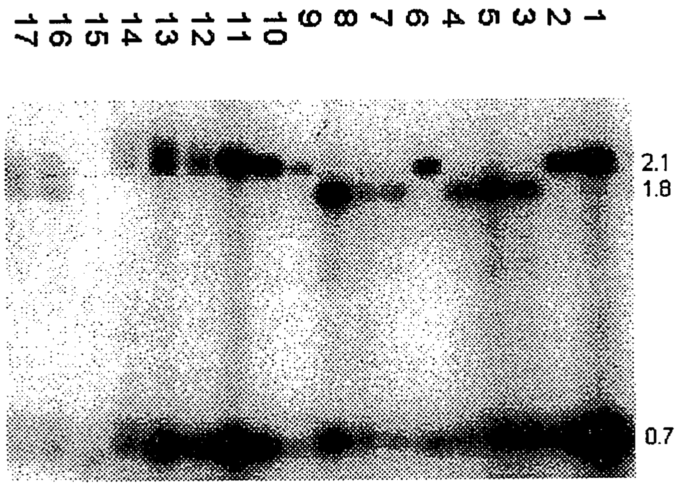

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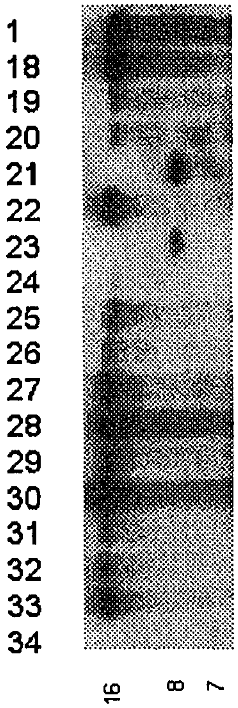
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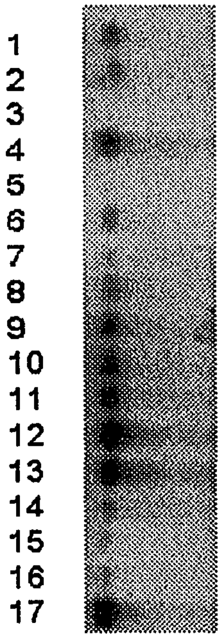
#34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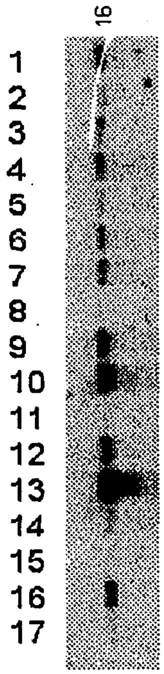

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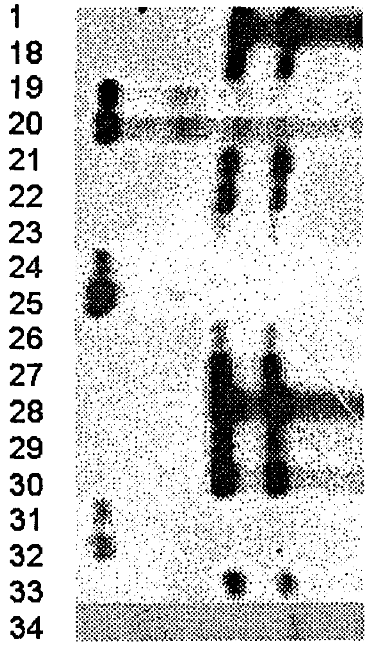
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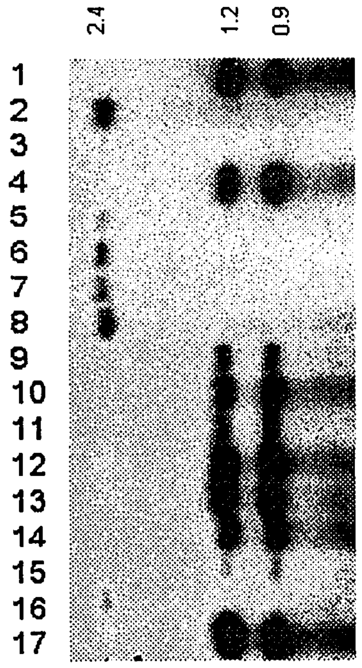
#37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17

#36


2.4
1.2
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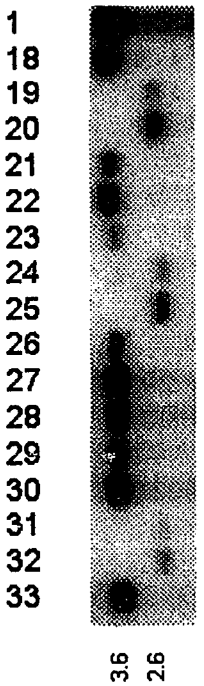
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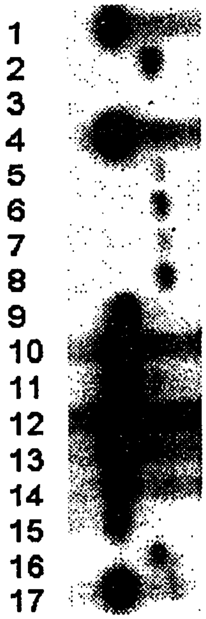
3.6
2.6

#40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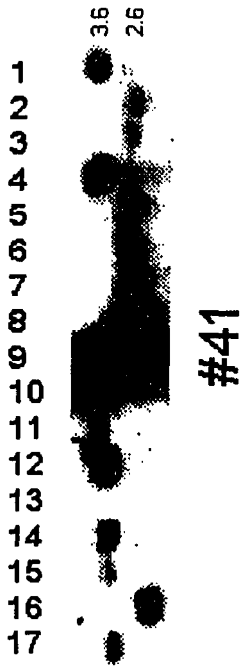
#39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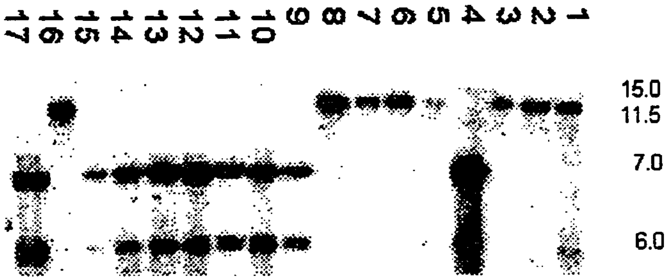
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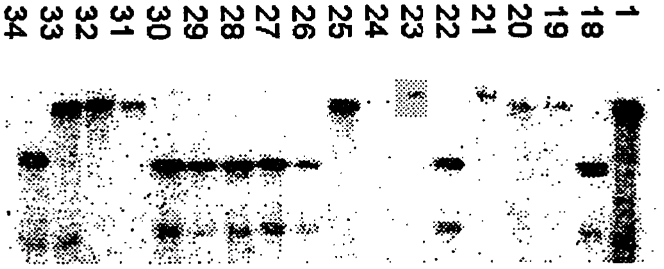
7.0
6.0

#42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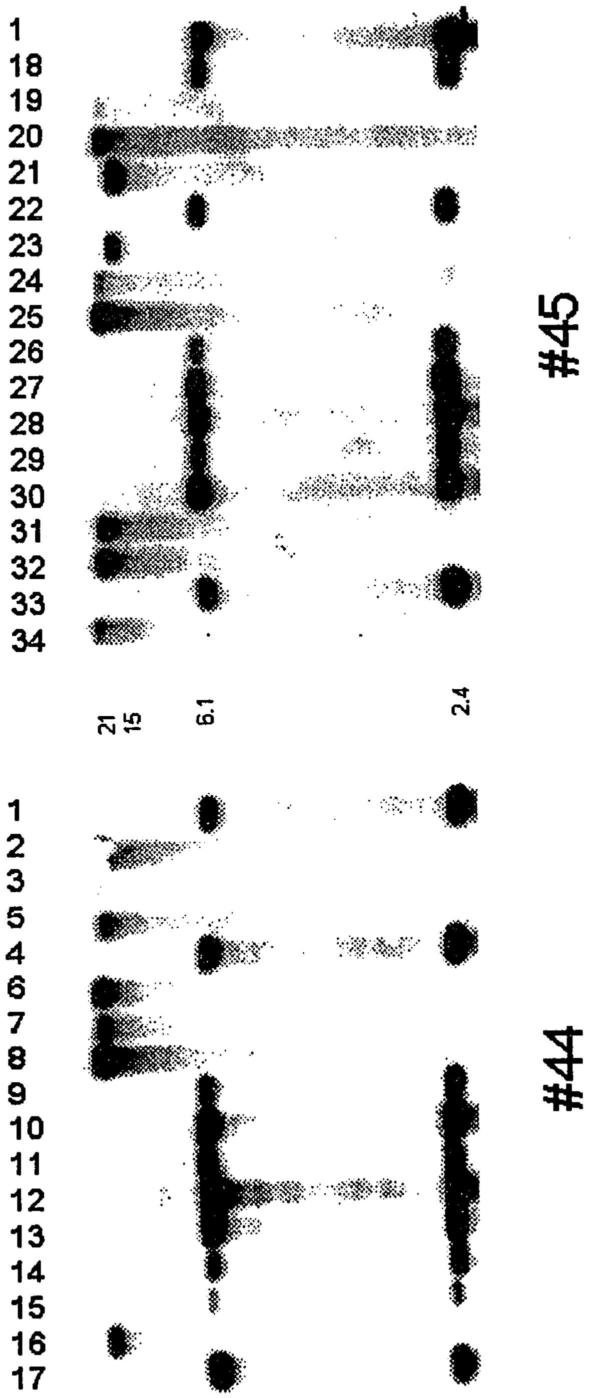
#43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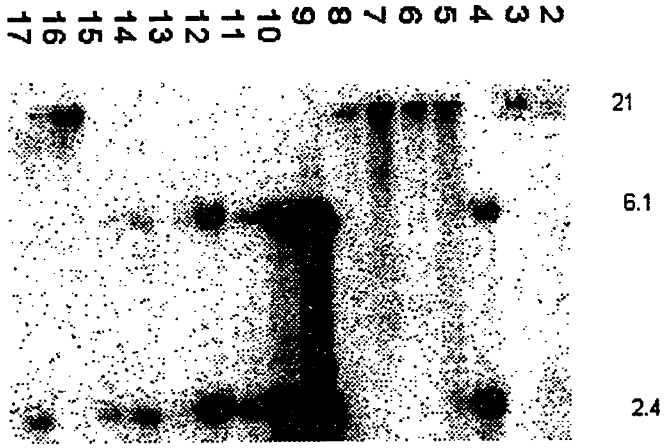
#44



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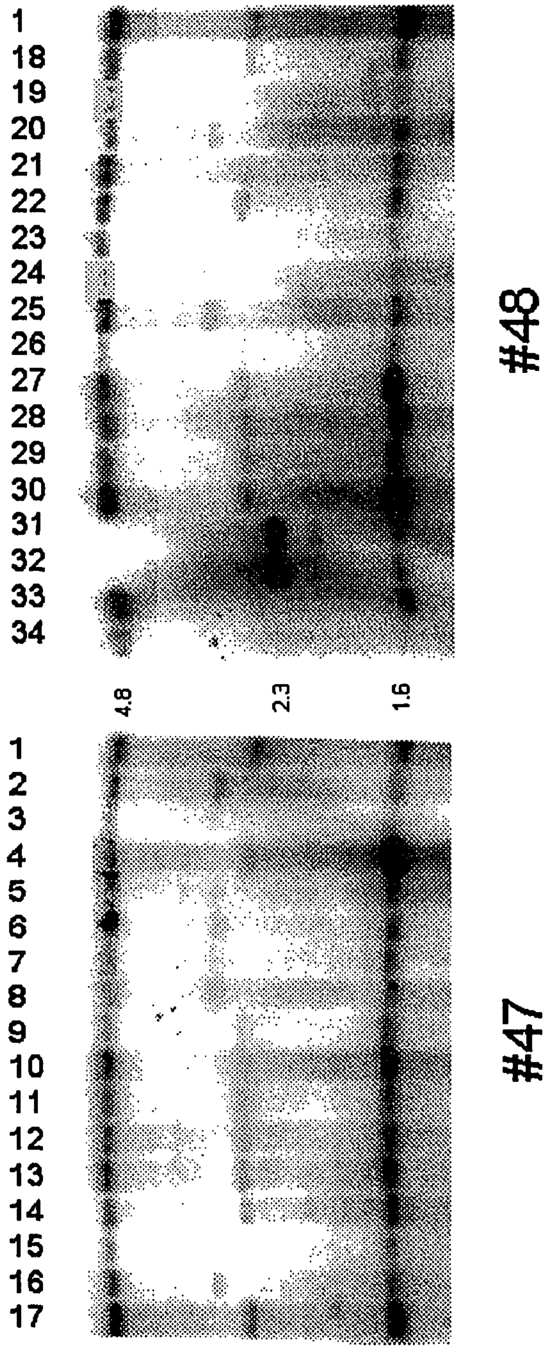
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2.3
1.6
1
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12
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14
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16
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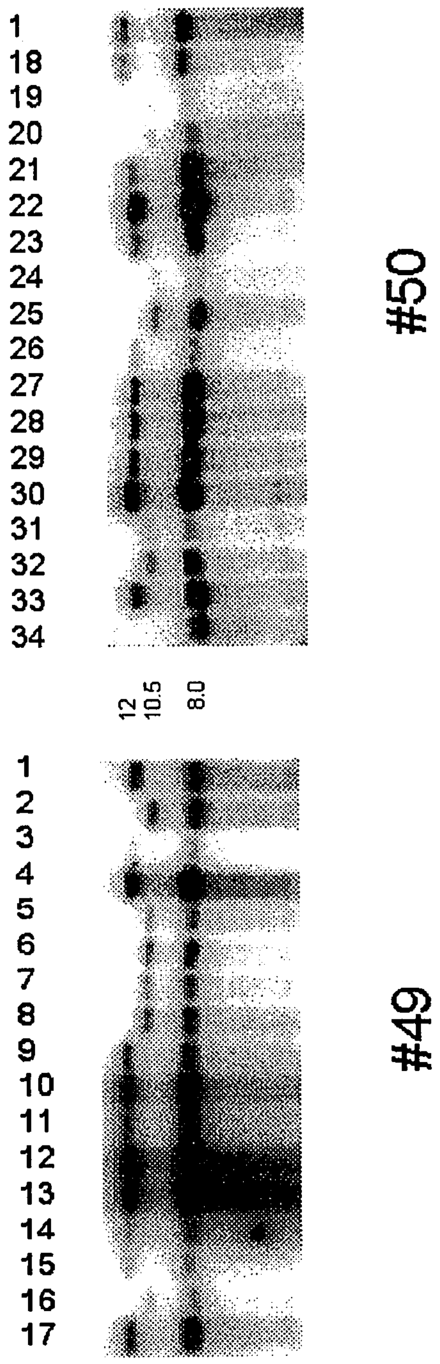
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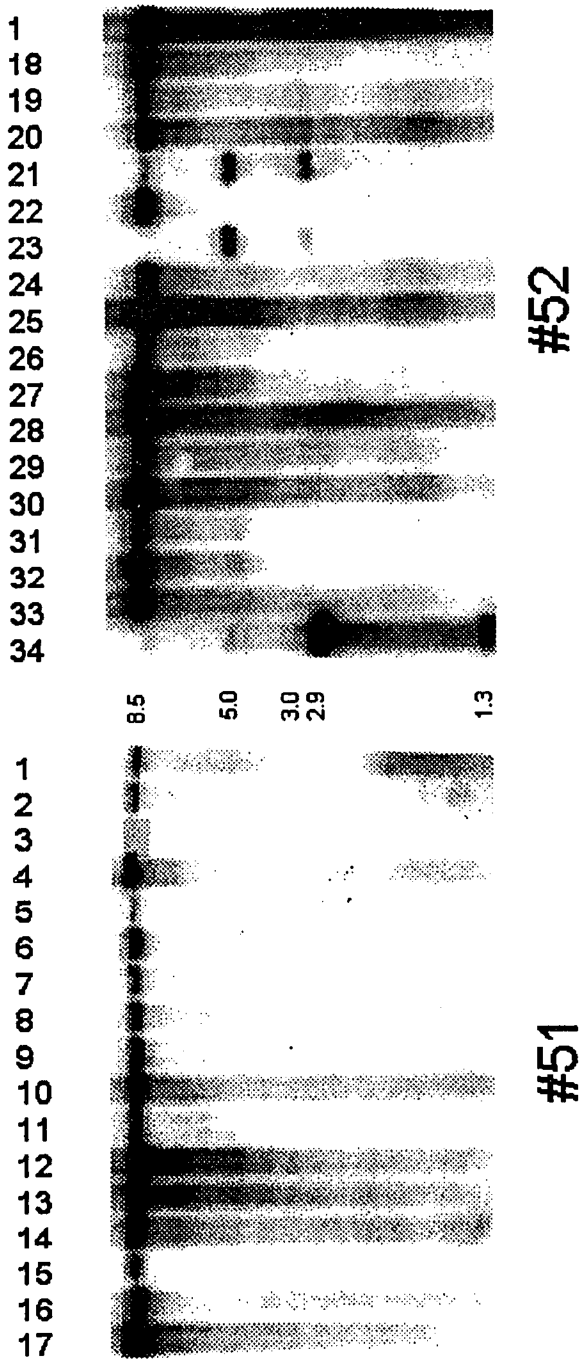
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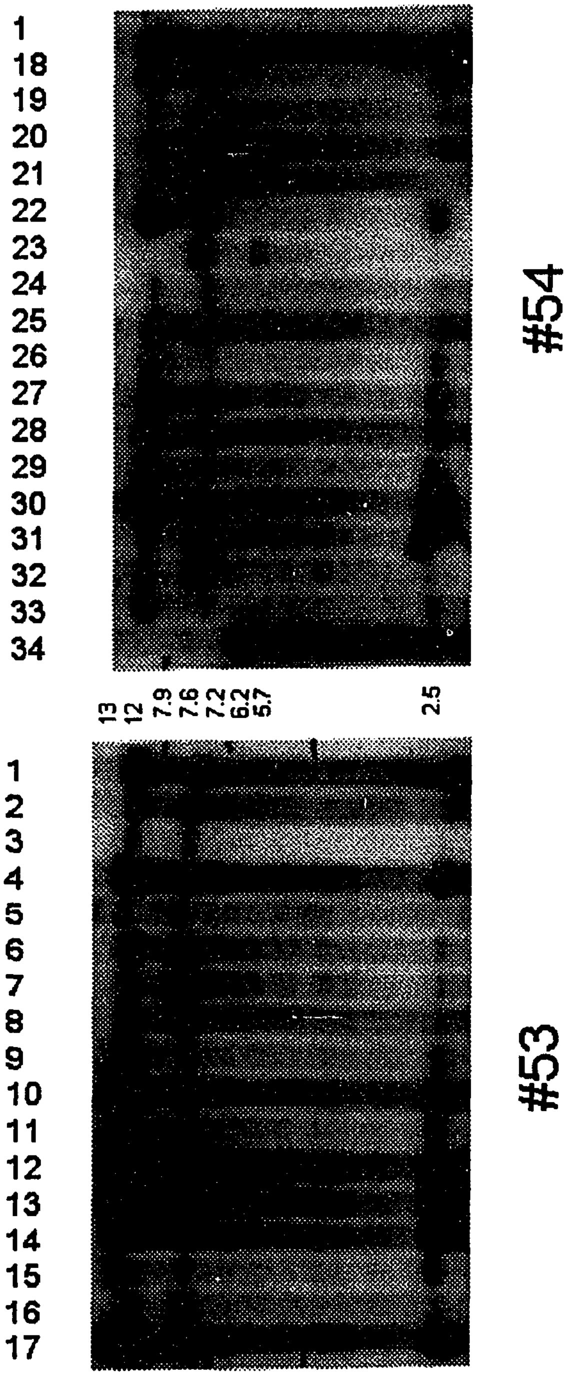
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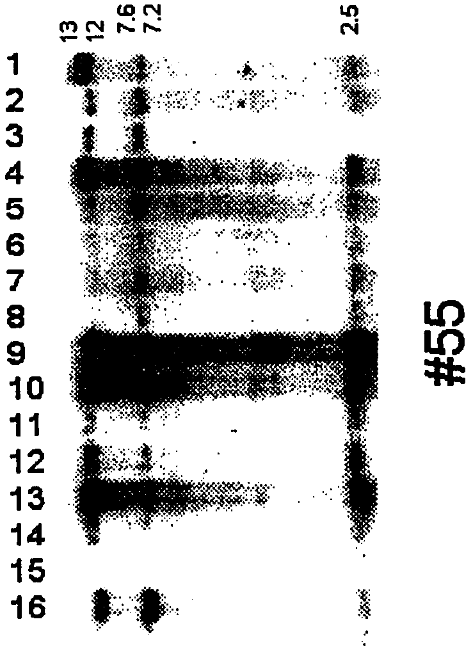
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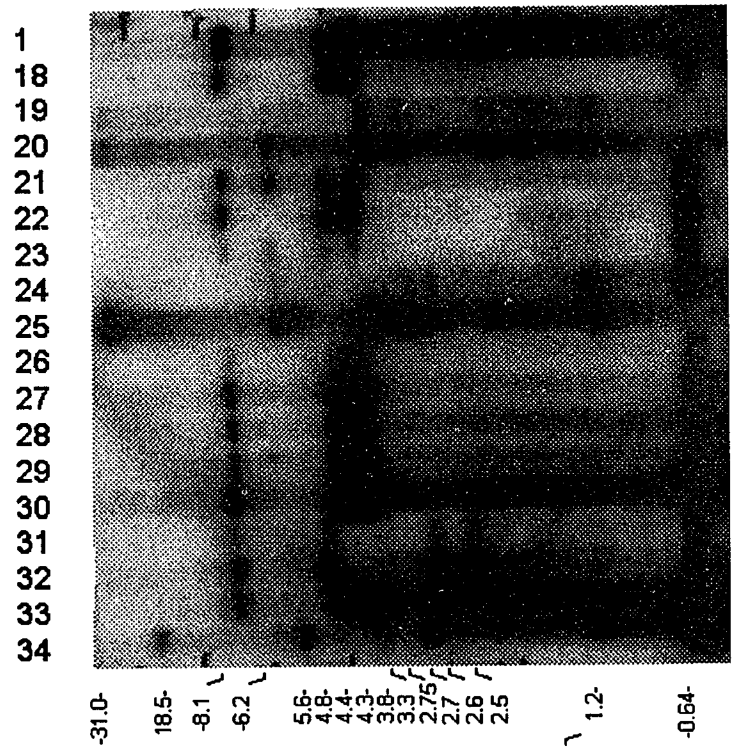
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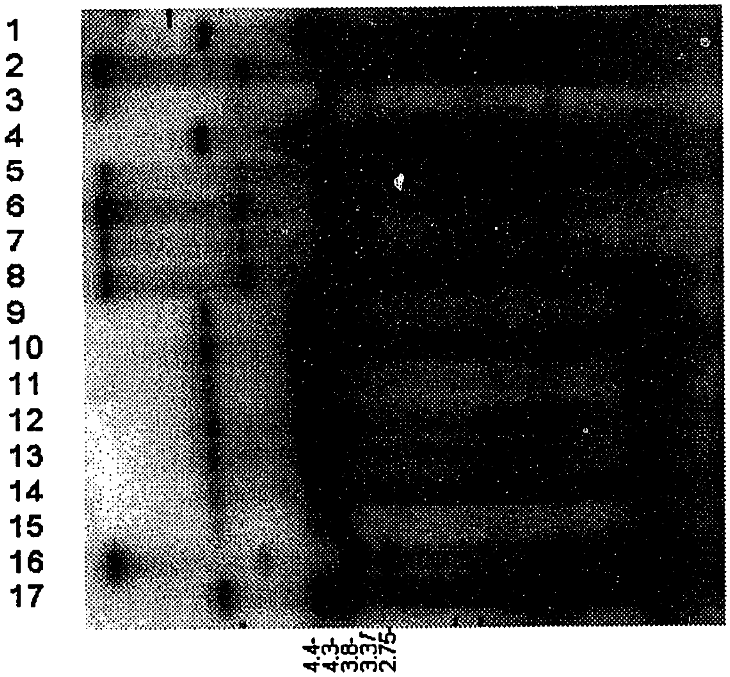
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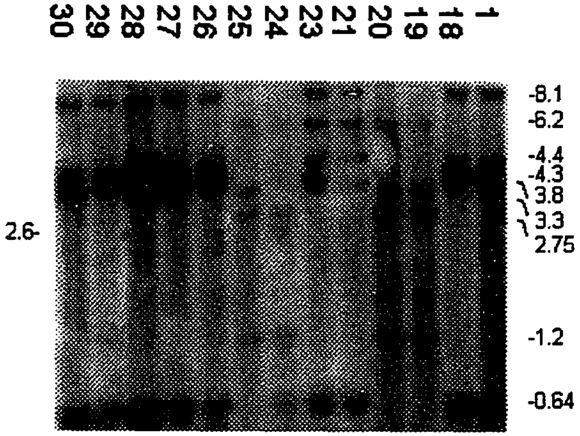

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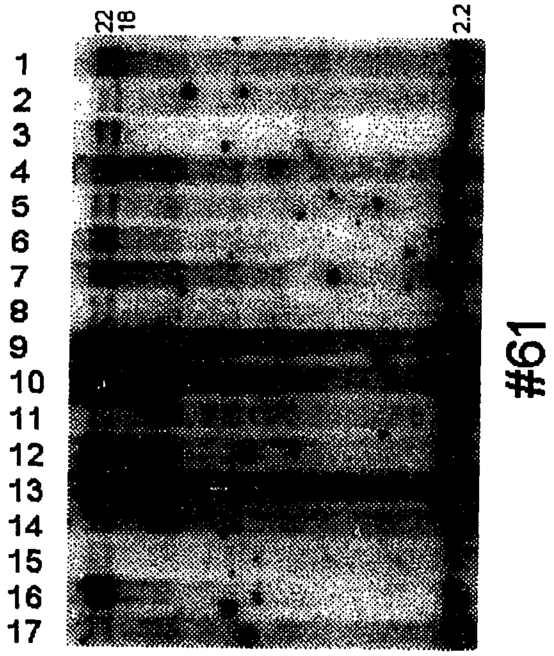
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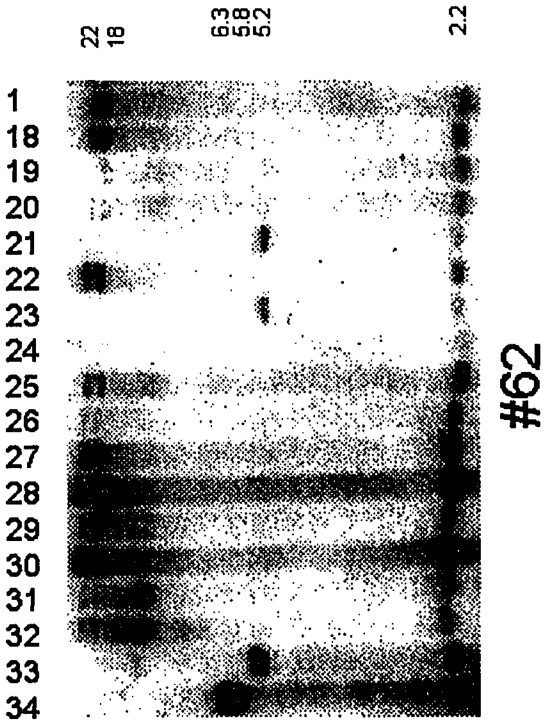
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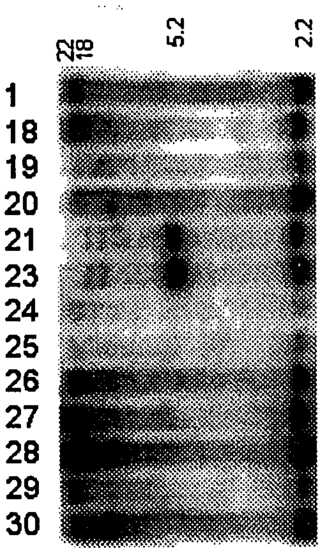

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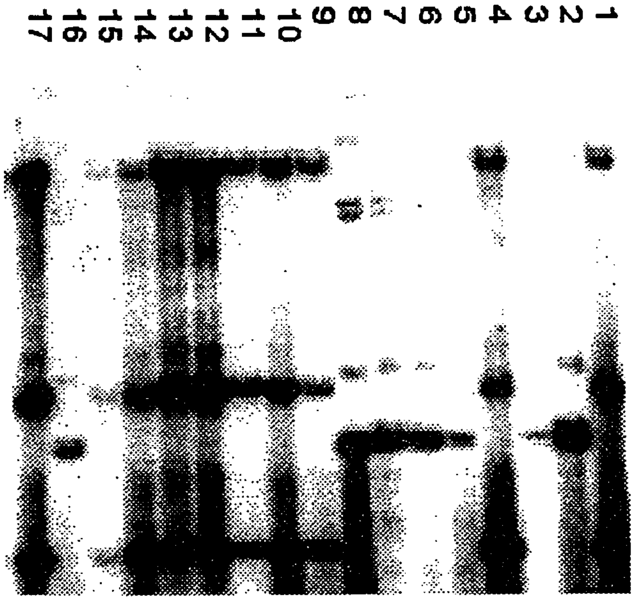
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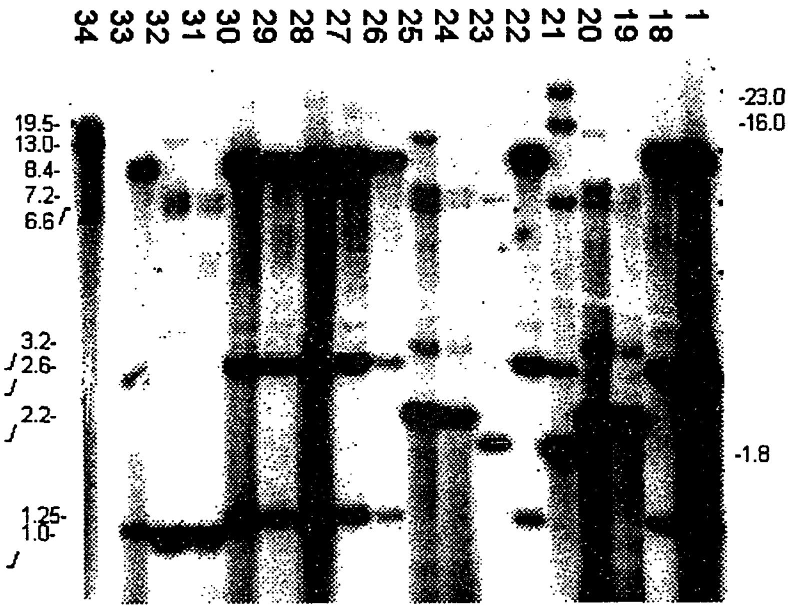
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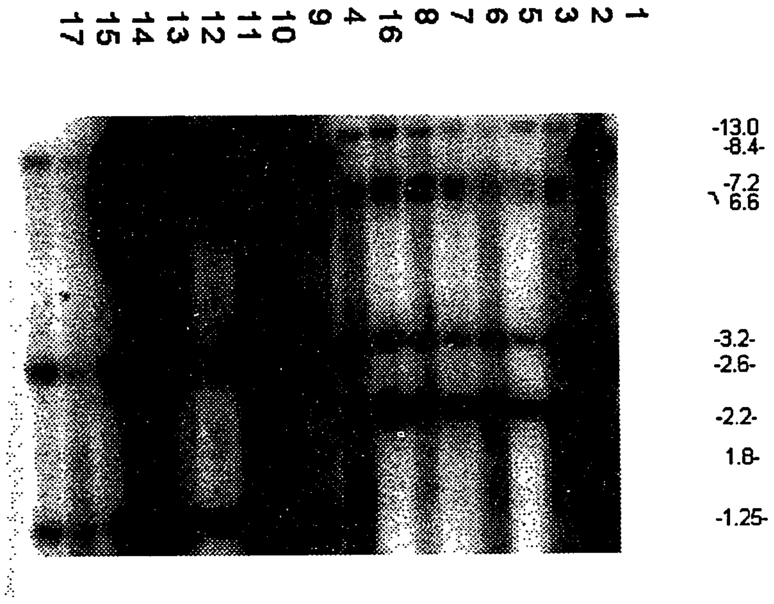
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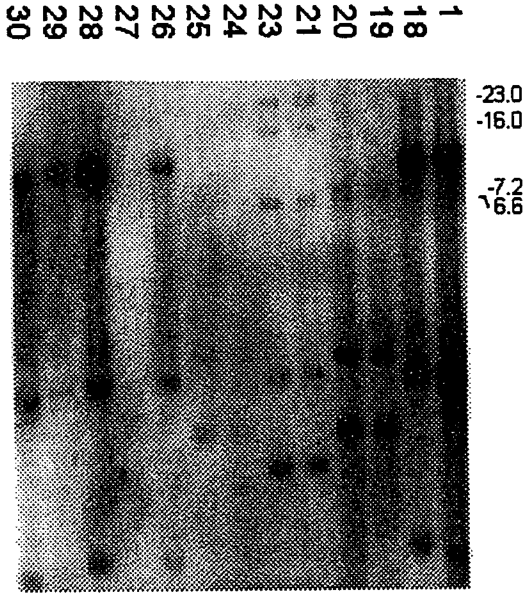
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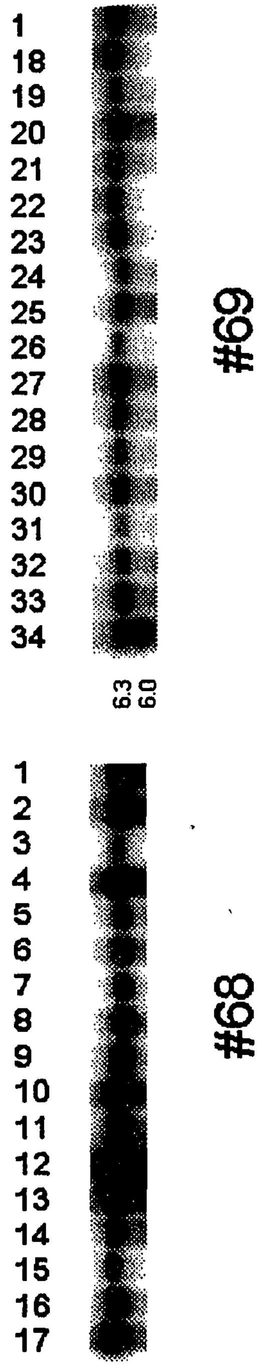
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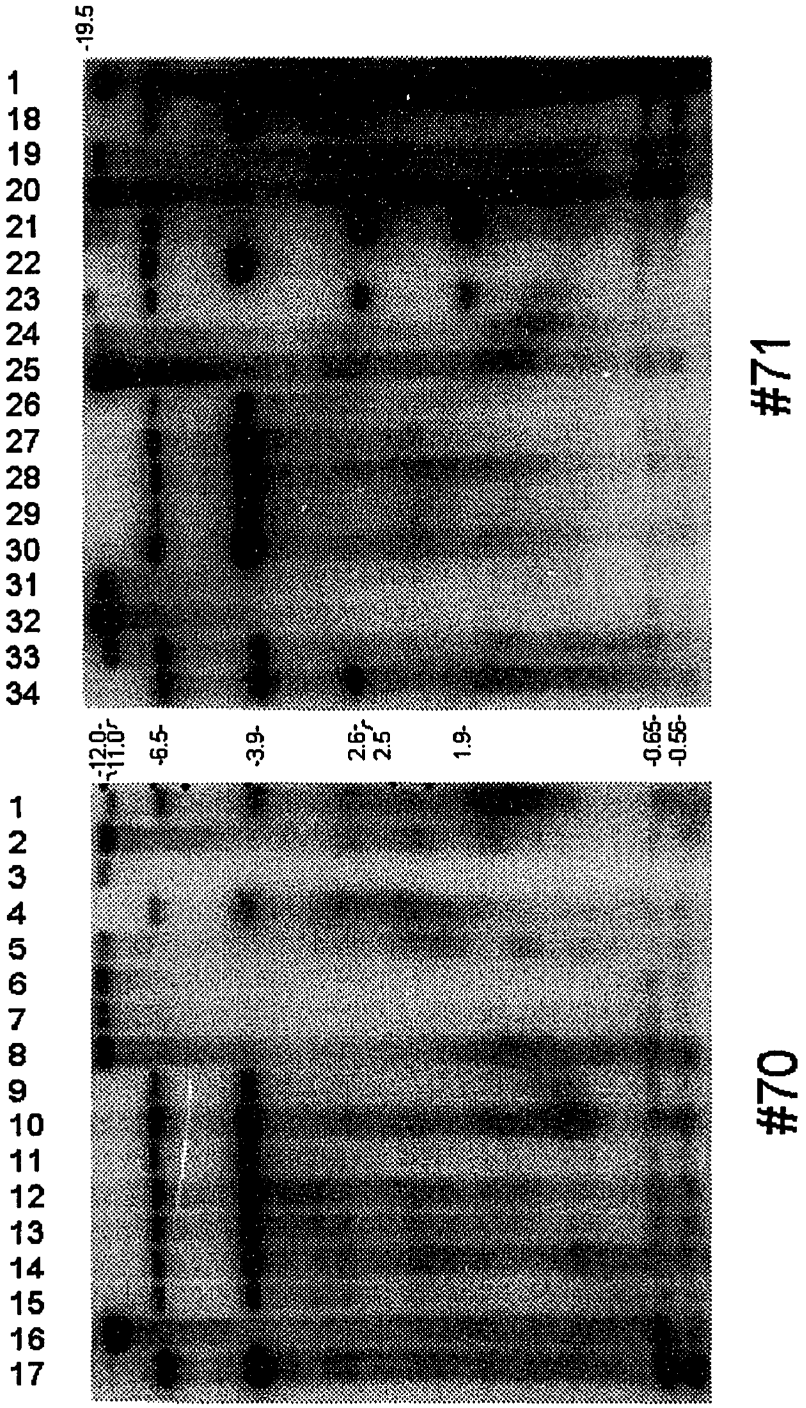
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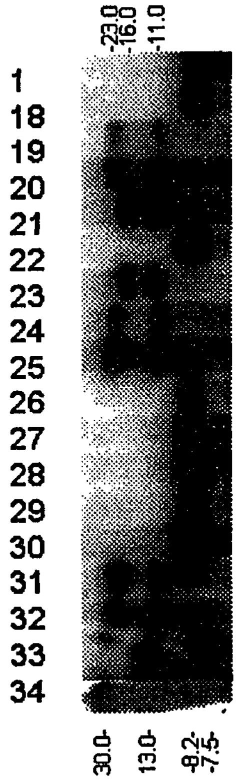

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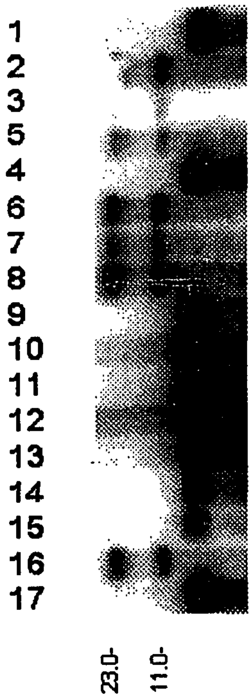

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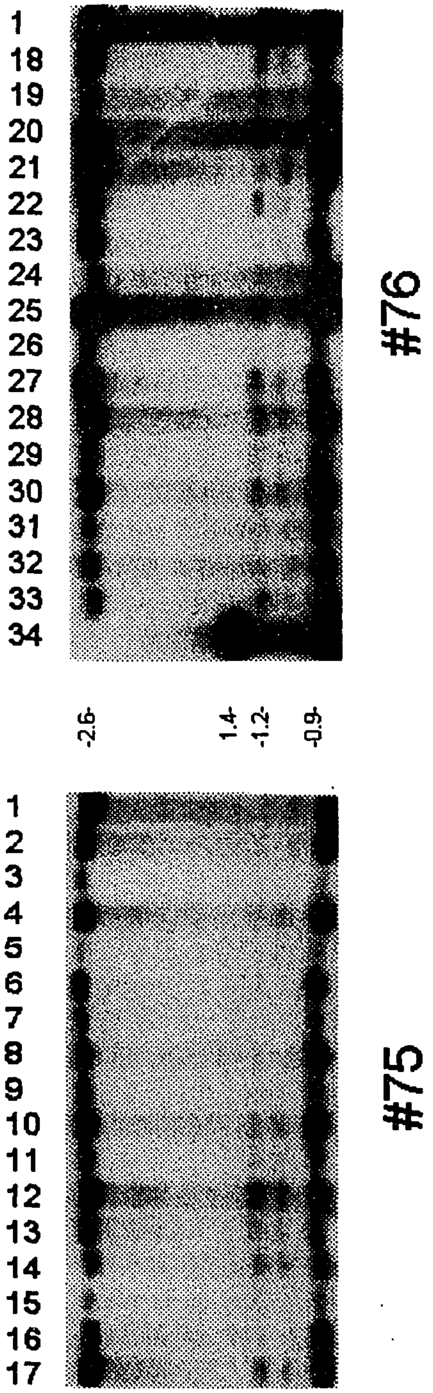
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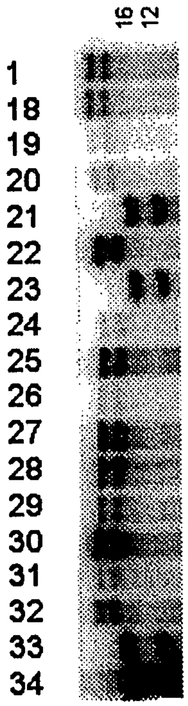
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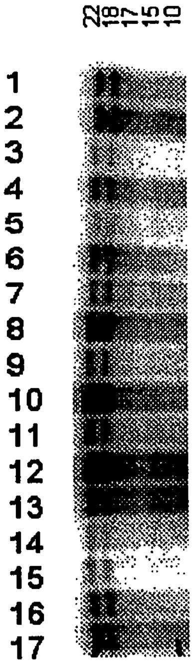
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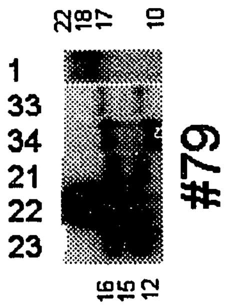
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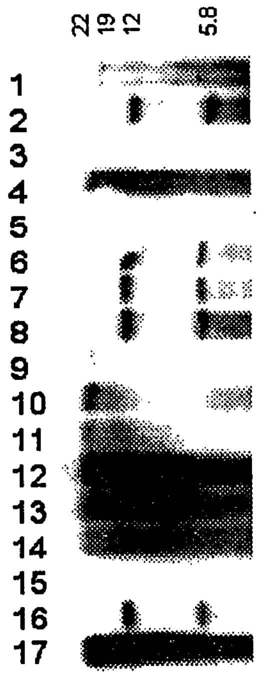

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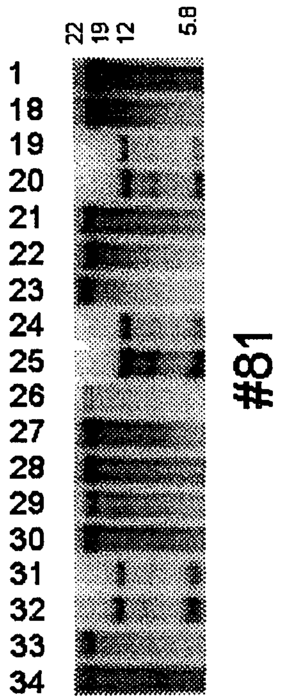
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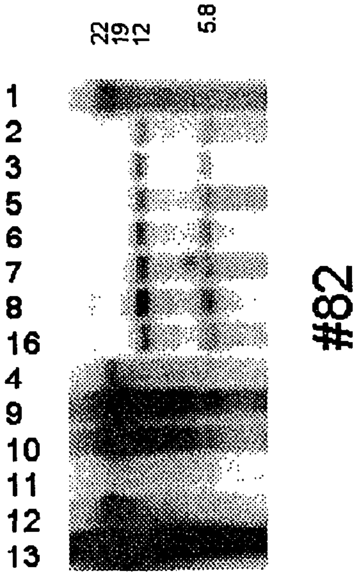
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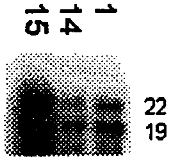

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