GENETIC AND MORPHOLOGICAL VARIATION IN *MACROCYSTIS* C. AGARDH (LAMINARIALES, PHAEOPHYCEAE) OFF THE NORTH AMERICAN COAST

by

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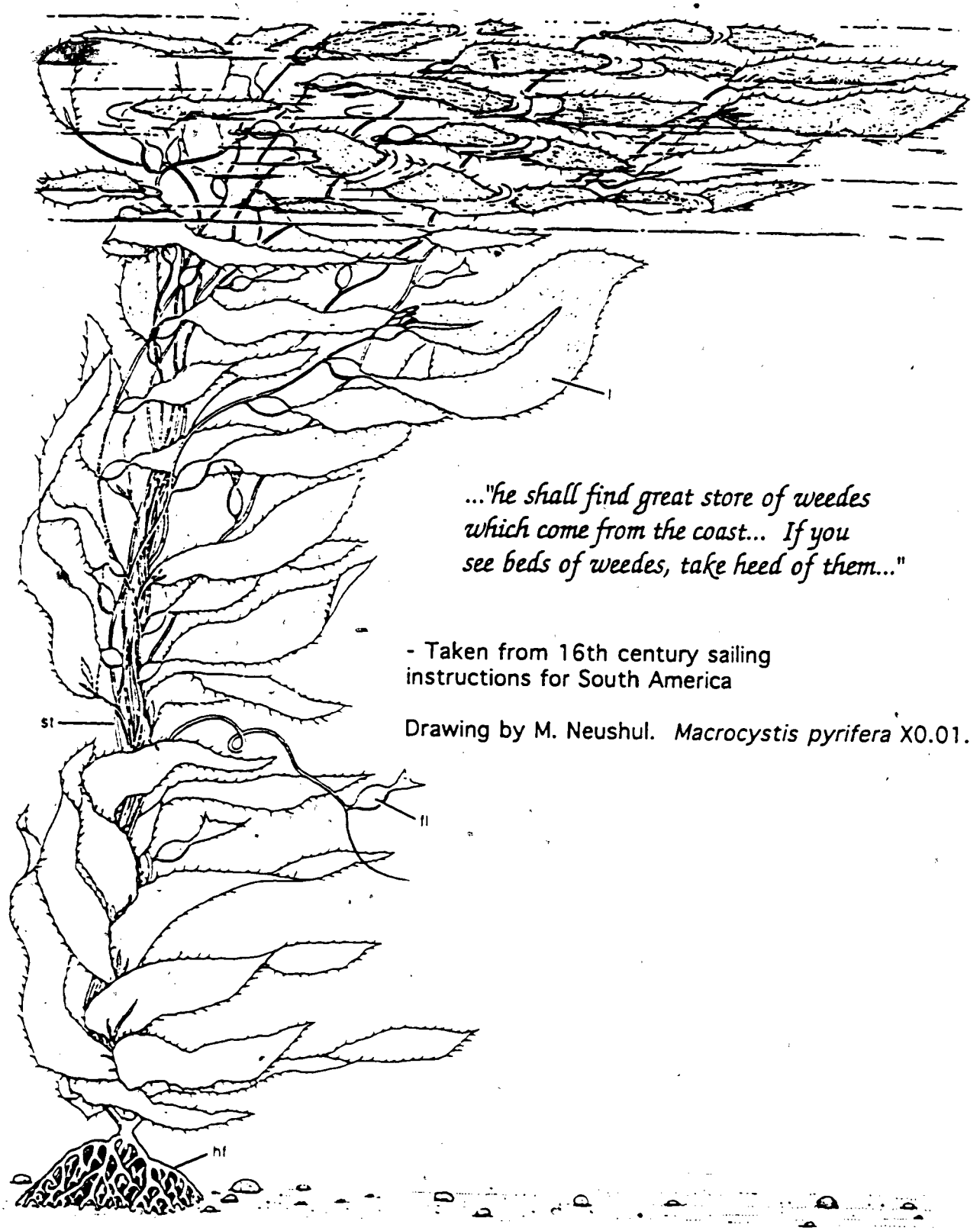

..."he shall find great store of weedes which come from the coast... If you see beds of weedes, take heed of them..."

- Taken from 16th century sailing instructions for South America

Drawing by M. Neushul. *Macrocystis pyrifera* X0.01.

APPROVAL

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Degree: MASTER OF SCIENCE

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GENETIC AND MORPHOLOGICAL VARIATION IN *MACROCYSTIS C. AGARDH* (LAMINARIALES, PHAEOPHYCEAE) OFF THE NORTH AMERICAN COAST.

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ABSTRACT

Concurrent analyses of morphological and genetic diversity within the kelp genus *Macrocystis* C. Agardh (Laminariales: Phaeophyceae) revealed a pattern of nuclear ribosomal Internal Transcribed Spacer (ITS1) divergence not in accordance with morphological differentiation or taxonomy. Five individuals were sampled from each of eleven populations of *M. integrifolia* Bory and *M. pyrifera* (L.) C. Agardh from the eastern Pacific and southern Atlantic oceans. Variation was found between rDNA repeats within individuals and between populations. Levels of population divergence were relatively low ($\leq$1%), and the distribution of ribotypes at a polymorphic putative stem-loop region suggest the possibility of gene flow between the Southern and Northern Hemispheric plants. The pattern of the population divergence indicates that the primary taxonomic character, the holdfast, has undergone convergent evolution, and does not adequately reflect phylogenetic relationships within the genus.

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This research was done at the Bamfield Marine Station, and I owe a great deal to the staff and researchers there, particularly John Boom, who knew it then, too.

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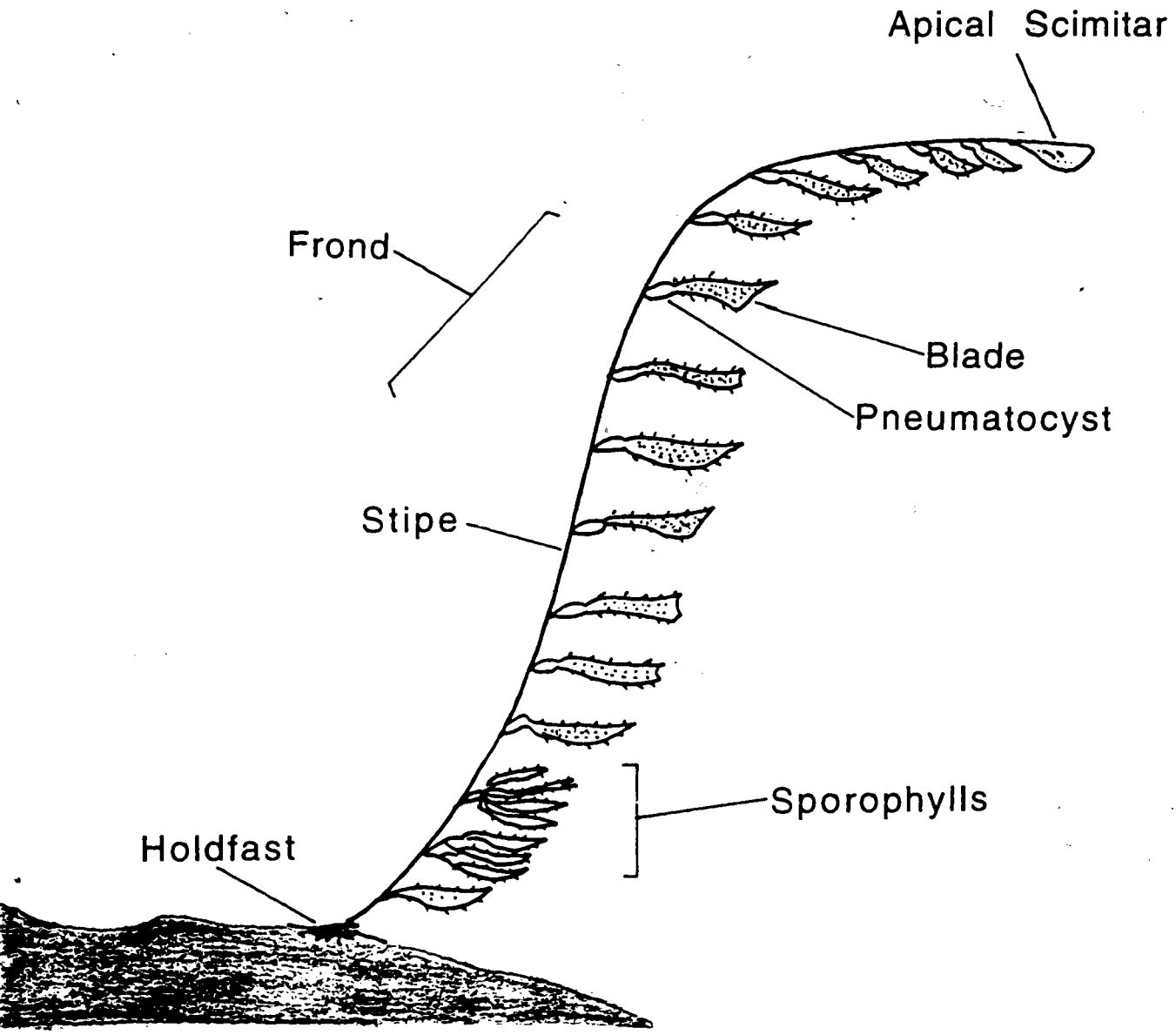
INTRODUCTION

Macrocystis C. Agardh (Laminariales, Phaeophyceae) is a distinctive genus of algae of the upper sublittoral zone of many temperate oceans (Womersly 1954). The large, dense canopies it forms on the fringes of the Southern Ocean and eastern Pacific (Womersly 1954, Druehl 1969, North 1971, Neushul 1971) have inspired many an imagination and phycologist alike. Although the genus is both morphologically distinct and easily recognizable, species delineation within the genus has troubled phycologists for over a century (Hooker 1847, Skottsberg 1907, Womersly 1954, North 1971, Neushul 1971, Hay 1986, Fain 1986, Brostoff 1988, Van Tussenbroek 1989a). The genus has not been adequately resolved on morphological characters (Fain 1986, Druehl and Saunders 1992), so this study hopes to further elucidate phylogenetic relationships within *Macrocystis*, incorporating modern methods of molecular biology.

Macrocystis is the largest of all algae (Druehl et al, in prep), anchored to the substrate by a holdfast, which can be either conical and hapteral, or flattened, creeping and rhizomatous (Womersly 1954). From this holdfast sprouts numerous fronds (Fig. 1), whose terete stipes can attain lengths over 30 meters and possess more

Figure 1: A typical frond of *Macrocystis*.

Figure 1


Apical Scimitar
Frond
Blade
Pneumatocyst
Stipe
Sporophylls
Holdfast

than a hundred flattened, rugose laminae, or blades. Each blade is subtended by a gas filled pneumatocyst which buoys the frond to the ocean surface (Lobban 1978).

An intercalary meristem is found in the apical scimitar, at the apex of each frond (Fig. 1). Growth proceeds as the stipe elongates and successive blades are split off the proximal side of the apical scimitar.

Macrocystis has a heteromorphic, biphasic life history. The conspicuous diploid sporophyte produces biflagellated haploid meiospores in patches of unilocular sporangia, or sori, often found on the blades proximal to the holdfast. These proximal blades, when lacking pneumatocysts, are referred to as sporophylls, but sori can be found on any part of the plant. After release from the sori, the meiospores settle and germinate into inconspicuous filamentous gametophytes. Male and female gametophytes produce sperm and egg, respectively, and the biflagellated sperm is released to swim to the egg, attracted by the pheromone Lamouxirene released by the egg (Müller et al 1985). The range of attraction of the Lamouxirene must be quite limited, since successful fertilization is dependent on close proximity of the male and female gametophytes (Anderson and North 1966). Fertilization initiates growth of the new sporophyte generation, which overgrows the female gametophyte. The sporophyte can persist for many seasons, though estimates of its typical longevity range from 2 years in British Columbia and Chilé

(Druehl and Wheeler 1986, Pérez et al 1972) to 8 years in California (Lobban 1978).

Taxonomic Studies

Taxonomic studies of *Macrocystis* C. Agardh constitute a progressive search for phylogenetically informative characters (Womersly 1954, North 1971, Neushul 1971, Hay 1986, Brostoff 1988) amongst considerable phenotypic plasticity (Womersly 1954, North 1971, Brostoff 1988, Druehl 1978, Druehl and Kemp 1982, Van Tussenbroek 1989b). The taxonomy has undergone numerous revisions and continues to be questioned (North 1971, Fain 1986, Druehl and Saunders 1992). It is not through lack of attention that the problematic taxonomy has persisted; *Macrocystis* is one of the most studied algal genera. Instead, the morphological species within *Macrocystis* seem to resist traditional taxonomic approaches with the plasticity of their morphological characters, and the interfertility they display, both between species (Lewis et al 1986, Lewis and Neushul 1994) and with other genera (Sanbonsuga and Neushul 1978, Coyer et al 1992, Lewis and Neushul 1995).

Early Interests in *Macrocystis*

The first known classifications of *Macrocystis* were based on its traditional uses; Nitinaht and Hesquiat indians on the west coast of Vancouver Island included *Macrocystis* in ʔoʕaqʷapt, their word for those algae, particularly the kelp, on which the herring would attach their spawn (Turner et al 1983). Europeans first came in contact with *Macrocystis* on the sailing voyages of exploration in the 16th century. The usefulness of these "weedes" (reviewed in North 1971) was noted, in that the band of buoyed kelp, presumably *Macrocystis*, provided an isobath (a contour line connecting areas of equal depth) when approaching shallow water.

These early voyages returned to Europe with specimens for scientific study. One such specimen reached Bauhine, an early botanist, whose illustration in 1651 is clearly recognizable as *Macrocystis*. His label "Bulbus Marinus Crinitus" is the earliest known western scientific name for this alga (North 1971).

Linnaeus, and on to *Macrocystis*

Specimens of drifting *Macrocystis* were collected by Emmanuel Koenig while sailing to India via the Cape of Good Hope, and were brought to Linnaeus. These were classified as *Fucus pyriferus* and described as having a dichotomous stem and blades that were

"terminal, stalked, and provided with bladders" (Linnaeus 1771, *in* North 1971). Subsequent authors described a number of other species, often based on dried fragments or incomplete plants. These species were described largely on the basis of varying morphology and arrangement of the blades and stipes, since most were obtained as drift.

Lamouroux (1812, *in* North 1971) subdivided the genus *Fucus*, creating the genus *Laminaria*, which included *Macrocystis* until C. A. Agardh (1821, *in* North 1971) erected the current genus of *Macrocystis* nine years later. By mid century, as many as 22 species had been described, including numerous varieties (Skottsberg 1907, *in* Womersly 1954). A variety of morphological characters were utilized in describing these species, making consistency difficult (North 1971). Hooker, in a more thorough treatment of the genus, recognized the weaknesses in the taxonomy of this period:

"Out of some 30 specimens brought home by 10 different collectors, ... not one conveys any notion of the variations which even a solitary individual can assume... In thus bringing together under one, the 10 species which have been described by 5 authors, of whom hardly one has ever seen the genus in a living state, we are only taking advantage of opportunities which a long residence in the southern hemisphere has afforded. Without studying these plants on the coasts they inhabit, it is impossible to judge of the influence of

local causes on their plastic forms," (Hooker 1847 *in* North 1971, p. 11).

Skottsberg (1907) recommended the genus be collapsed back to the single species *Macrocystis pyrifera* (L.) C. Agardh (Womersly 1954).

Here began the current taxonomic trend, with Howe (1914) separating South American species on the basis of holdfast morphology. The current species *M. integrifolia* Bory possesses a flattened, rhizomatous holdfast, while *M. pyrifera* has a conical holdfast with pyriform haptera and an upright, terete primary dichotomy (Fig 2). Setchell (1932) applied these names to similar North American forms. Womersly (1954) concurred with this approach, and further delineated *M. angustifolia* Bory on the basis of its distinctive holdfast, which is intermediate in morphology between that of *M. integrifolia* and *M. pyrifera* (Fig. 2).

With agreement on the importance of the holdfast, the type specimens of all three species became a frustration to taxonomists, for they all lack the holdfast. These species were uprooted from the typical taxonomic base, for now the type specimens were not useful in species clarification.

The fundamental importance of the holdfast morphology in taxonomic studies of *Macrocystis* has endured until the present, resulting in these three species having disjunct populations in many temperate oceans (Fig. 3).

Figure 2: Types of *Macrocystis* holdfasts seen in North and South America. **A.** *M. pyrifera* (Peru, Baja California and California Channel Islands). **B.** *M. pyrifera* (La Jolla, California, Tierra del Fuego and Patagonia). **C.** *M. angustifolia* (southern Califonia, southern Australia). **D.** *M. integrifolia* (central Chile, Monterey, California and Barkley Sound, B. C.) (after Neushul 1971, Womersly 1954). CS= location of cross section taken for illustration of hapteral organization around basal dichotomy of the stipe.

Figure 2

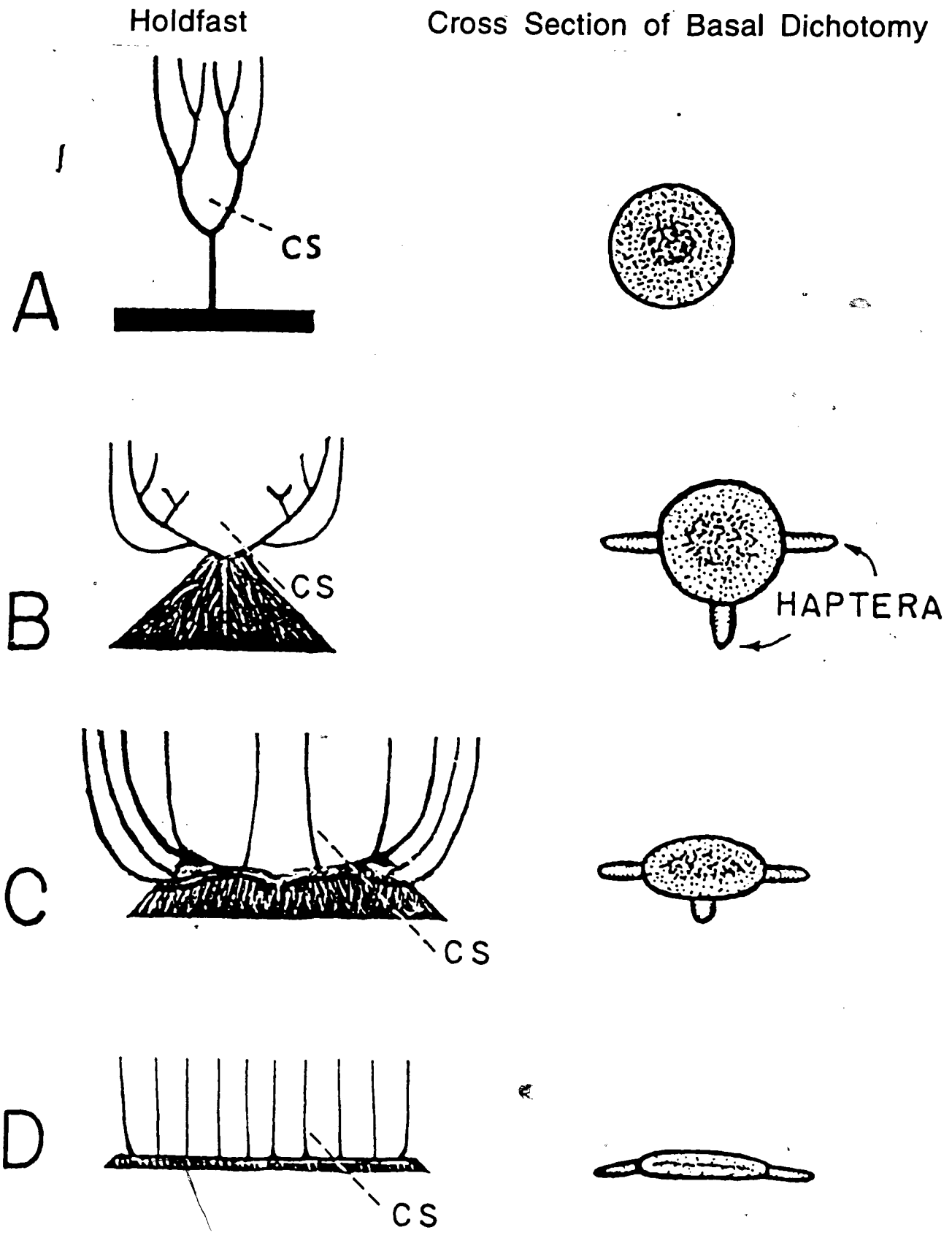
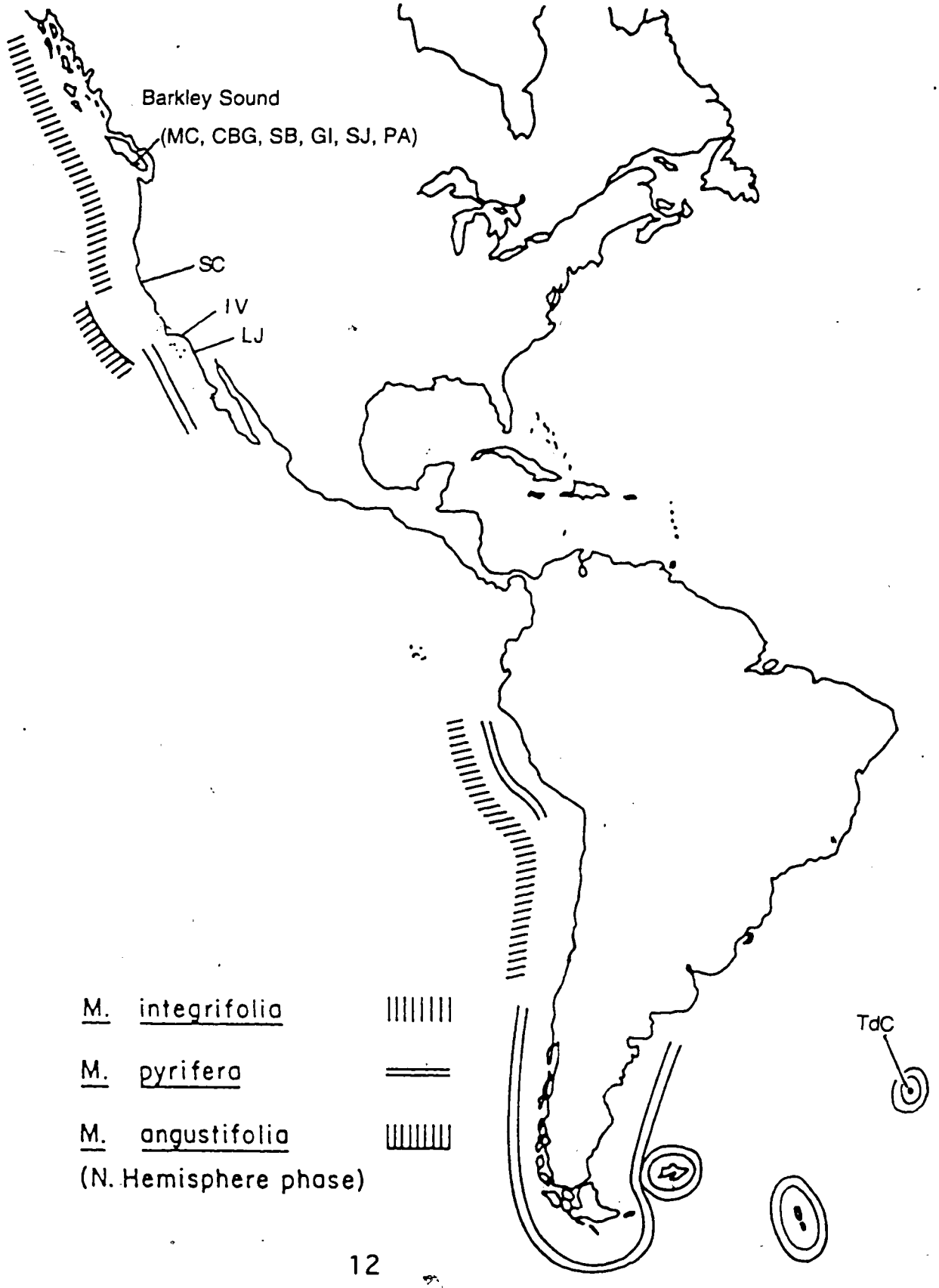

Holdfast
Cross Section of Basal Dichotomy
CS
A
CS
B
HAPTERA
CS
C
CS
D

Figure 3: Distribution of *Macrocystis* in North and South America, showing sites sampled for genetic analysis with respect to the morphological species (Neushul 1971). See Figure 4 for details of Barkley Sound. Stillwater cove (SC), Monterey, California, is the southern extreme of the distribution of *M. integrifolia*. Samples were taken from a population of *M. angustifolia* sensu Neushul at Isla Vista (IV), near Santa Barbara, California, and *M. pyrifera* was taken from La Jolla, California (LJ) and Tristan da Cunha (TdC) in the southern Atlantic (Neushul 1971, van Tussenbroek 1989a).

Figure 3


Barkley Sound
(MC, CBG, SB, GI, SJ, PA)
SC
IV
LJ
TdC
M. integrifolia
M. pyrifera
M. angustifolia
(N. Hemisphere phase)

Taxonomic Difficulties

The use of the holdfast as a taxonomic character has not been without its difficulties. Neushul's (1971) proposal that *M. angustifolia* was present on the central Californian coast was based on the presence of holdfasts matching Womersly's (1954) key for *M. angustifolia*. However, Brostoff (1988) later showed that the holdfasts in the region intergraded. Although transplant and reciprocal crossing experiments had suggested that there was stable genetic differentiation, *M. pyrifera* and *M. angustifolia* sensu Neushul constituted extremes of a continuum of characters, and as such were not discrete taxonomic entities, even though those plants proposed to be *M. angustifolia* in California were indistinguishable from *M. angustifolia* (Neushul 1971) in Australia using the key of the time (Womersly 1954).

A continuum in holdfast morphology was also noted by Scagel (1947) when looking at *M. integrifolia*, but in this case the continuum was expressed ontogenetically. The holdfast of juvenile *M. integrifolia* was seen to be upright and terete, as Womersly (1954) described *M. pyrifera*, but as the plant matured, the primary stipe progressively flattened, as Womersly (1954) described *M. angustifolia*, and eventually acquired the prostrate, creeping form typical of *M. integrifolia*.

In situ studies of Northern Hemispheric plants indicate that holdfast morphology may be affected by environmental factors and by

plant vigour (Neushul 1971). In Baja California, holdfast morphology ranged from upright and hapteral (Fig. 2A) in cool upwelling water, to noticeably peaked (Fig. 2B) in an adjacent area of warmer water (Dawson 1952). North (unpublished, in Neushul 1971) has also noticed peculiar holdfast morphology in areas exposed to higher water temperature.

Populations of *Macrocystis* have been found in the Falkland Islands which lack holdfasts altogether, and instead perpetually drift, reproducing through fragmentation (van Tussenbroek 1989a). Species status for these populations was not proposed, nor was it suggested how this example could be accounted for in the taxonomy.

Another species, apparently distinct both genetically and morphologically, has been described based on characters apart from the holdfast. *Macrocystis laevis* in the southern Atlantic was described primarily on the morphology of its smooth blades (Hay 1986). The typical range of blade rugosity was not found; the blades were found to be smooth regardless of the exposure regime (Hay 1986). However, in the past, blade morphology has been shown to be plastic, being highly susceptible to environmental inducement (North 1971, Druehl 1978, Druehl and Kemp 1982, Van Tussenbroek 1989b). As such, blade morphology had been abandoned as a taxonomic character. Hay's (1986) description of *M. laevis* reintroduces the problem of different authors utilizing different characters to delineate species. The morphological characters of the blade could

possibly fall within the range of plasticity of other populations, confounding comparisons of individual specimens.

Several morphological characters have been explored extensively, including blade dimensions, pneumatocyst dimensions, stipe dimensions and number, frond growth rates and more (Lobban 1978, Fain 1986) and yet the findings with respect to taxonomic utility, have been less than conclusive (North 1971, Druehl 1978, Druehl and Saunders 1992). Currently, one cannot confidently assign an individual specimen to a species without prior knowledge of the geographic origin of the sample. Coupled with the highly variable extent of morphological plasticity, this uncertainty could reflect the inadequacy of the characters in describing the taxa, or could reflect the inadequacy of the current taxonomy.

A further shortcoming of the current taxonomy has been noted by Fain (1986), in his examination of divergence of chloroplast DNA sequence within the genus, as inferred from RFLP (Restriction Fragment Length Polymorphism) analysis. *Macrocystis integrifolia* and *M. pyrifera* from North America were found to display less sequence divergence (0.08%) than populations of *M. integrifolia* from North and South America (0.3%). The inference is that Northern populations of *M. integrifolia* and *M. pyrifera* have shared a more recent common ancestor than have the two conspecific populations of *M. integrifolia.* Fain (1986) concludes that the species *M. integrifolia* and *M. pyrifera* are polyphyletic. The holdfast morphology could thus be a symplesiomorphy, reflecting relict variation in the ancestral

population, or it could have evolved twice convergently. Evidence suggesting convergent evolution of the primary taxonomic character necessitates further attention.

If there has been convergent evolution of the holdfast, this would abrogate its reliability as a taxonomic character. The apparent inconsistency between morphological and genetic characters could be the result of several factors: (1) drastically varying rates of base substitution between the populations, such that the conclusion of polyphyly (Fain 1986) is incorrect; (2) failure to identify the appropriate morphological traits; (3) insufficient genetic variation for the populations to be consistently differentiated.

(1) **Varying rates of base substitution:** The possibility that the data from the chloroplast DNA study have produced an inaccurate phylogeny was recognized by Fain (1986). The study was expanded to access a greater portion of the chloroplast genome, however the results were not published (Druehl pers. com.), even though they reinforced the conclusion of polyphyly within the species of *Macrocystis*. A substitution rate in accordance with that of angiosperms was determined using the sequence divergence values from Fain's 1986 study along with the oceanic warming event which initiated the Pliocene, 4.8 mya, as the onset of reproductive isolation. Fain's (1986) result suggests it is unlikely the *Macrocystis* chloroplast genome has been evolving at an aberrant rate compared to other plant groups. If we accept the apparent divergence pattern to

be accurate, then we must reconcile its discordance with the taxonomy as it stands today.

(2) **Inappropriate taxonomic characters:** The difficulty in identifying unambiguous taxonomic characters could simply indicate that taxonomists have not yet recognized the appropriate traits, though they may be present amidst the considerable morphological diversity within the genus. However, most of the conspicuous external morphological characters of *Macrocystis* have been investigated (Lobban 1978, Druehl and Kemp 1982). A recognition of the remarkably broad morphological plasticity (Druehl 1978, Druehl and Kemp 1982) has been the prevalent outcome of these studies. Vast size, conspicuous habit and economic importance have predisposed *Macrocystis* to extensive study, and may have had the effect of exaggerating the differences within the genus. Further studies will need to access a set of characters independent of phenotypic plasticity to attain a taxonomy more reflective of the phylogeny of the genus. Molecular genetics could provide such a character set.

(3) **Insufficient genetic differentiation:** A third possible cause for the lack of distinct morphological differentiation could be an underlying genetic similarity between the species. A lack of genetic divergence could produce an intergrading of morphological characters, low observed sequence divergence and the demonstrated interfertility of the species.

Genetic differentiation has been demonstrated with transplant experiments (Brostoff 1988) and with interspecific hybridization experiments (Lewis et al 1986). The interfertility between species of *Macrocystis* was demonstrated with reciprocal crossing experiments (Lewis et al 1986, Lewis and Neushul 1994). Reciprocal crosses have even been successful between *Macrocystis pyrifera* and other genera (Sanbonsuga and Neushul 1978, Lewis and Neushul 1995), suggesting that gene flow between species is certainly possible. A high degree of genetic similarity is necessary for hybridization to proceed and produce fertile offspring, and the wide success of experiments crossing species of *Macrocystis* infers that such genetic similarity exists. Conversely, any hybridization that does occur will work to homogenize the genetic structure of the populations.

Interspecific hybrids of *Macrocystis* (Lewis et al 1986) could be discerned from either parental species on the basis of growth rates, suggesting that the distinct species can be genetically differentiated, but does not quantify the differentiation. In the case of the Californian *Macrocystis angustifolia* sensu Neushul, this differentiation was later characterized as clinal morphological variation on a geographic scale, with very little accompanying genetic differentiation, since the populations were not reproductively isolated. Thus the basis for taxonomic segregation of *M. angustifolia* North Hemisphere phase *sensu* Neushul was removed (Brostoff 1988).

The intergradation of supposedly diagnostic holdfast morphologies, whether geographically or ontogenetically influenced, is another type of plasticity that is not accounted for in the taxonomy.

The inference of genetic similarity is reiterated by the high degree of similarity seen in the restriction maps of the chloroplast genome of *Macrocystis integrifolia* (North America) and *M. pyrifera* (North America), and corroborated by direct nuclear genomic sequence comparisons (Saunders 1991, Druehl and Saunders 1992, Liptack unpublished).

The level of chloroplast sequence divergence inferred between species of *Macrocystis* in the previous studies is an order of magnitude less than that seen in other plant groups, such as angiosperms (Clegg et al 1984, Palmer 1987, Ritland et al 1993), rhodophytes (Bird et al 1990) or chlorophytes (Bakker et al 1995).

Currently, four morphological species of *Macrocystis* are recognized. M. pyrifera, M. integrifolia and M. angustifolia are separated on the basis of holdfast morphology, and M. laevis is distinguished by its smooth blades. The distinctiveness of the holdfast types has been questioned (Dawson 1952, Brostoff 1988), and initial genetic studies (Fain 1986) have suggested that the species in North and South America are polyphyletic. It would appear that the morphological species of *Macrocystis* are in need of the clarification and augmentation available through direct genetic analysis.

The purpose of this study was to clarify the phylogeny of the distinct morphological species of *Macrocystis* in North America, using concurrent analyses of morphological and genetic variation.

Molecular Approach

Modern cladistics, widely accepted as the most rigorous approach for phylogenetic inference (Davis and Nixon 1992), is typically a numerical method (parsimony) of inferring descent relationships through the inference of synapomorphies, or shared derived characters. Cladistics can accept any data, regardless of the biological significance, and construct a tree. For such a tree to reflect phylogeny (Hennig 1966, *in* Davis and Nixon 1992), certain conditions must be satisfied. First, descent relationships among the populations examined must be hierarchic, which necessitates the careful choice of groups to be used as terminals. For instance, if a phenotypically distinct population of *Macrocystis* is simply an ecotype within a larger panmictic group, then the assumption of hierarchical descent would be violated if it were used as a terminal. Second, an attribute must be transferred from the ancestor to all its descendants. Therefore, the characters used in constructing the phylogeny must be fixed in the population, for if the population were polymorphic for that character, not all descendants of an heterozygotic individual would necessarily receive this character. Individuals possessing a specific allele would appear more related to

each other than to more closely related individuals not possessing that allele.

Hennig's (1966) conditions were central to the derivation of the phylogenetic species concept (PSC) (Cracraft 1983, Nixon and Wheeler 1990, Davis and Nixon 1992), thus, phylogenetic species are the appropriate phylogenetic terminals for cladistic analysis (Cracraft 1989). These terminals are properly delimited by population aggregation (Nixon and Wheeler 1990), where local panmictic populations are compared to neighbouring populations until fixed character differences indicate mutual reproductive isolation.

The reconciliation of the PSC with the biological species concept (Dobzhansky 1937) has been problematic. Dobzhansky contended that species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism or perhaps by a combination of several such mechanisms (Dobzhansky 1937). There has been no agreement as to whether or not a level of DNA divergence can be assumed to indicate sufficient reproductive isolation, and therefore, to indicate a separate species. The degree of interfertility between populations will determine, in part, the probability of gene flow between the populations. The kelp (Laminariales) are notorious for not adhering to this tenet of the BSC; hybridization has been observed in the wild (Coyer et al 1986, Lewis and Neushul 1994), and demonstrated in the laboratory up to the inter-familial level (Lewis and Neushul 1995). Interfertility between the three major species of *Macrocystis* (*M.*

integrifolia, M. pyrifera and *M. angustifolia*) has been demonstrated (Lewis et al 1986, Lewis and Neushul 1994), so it is likely that the species of *Macrocystis* do not comprise satisfactory biological species. The species concept used in this study is an addition of the PSC to the morphological species concept currently used for *Macrocystis*. A satisfactory species will be comprised of individuals that are both morphologically and demonstrably genetically distinct from individuals of other species.

Choice of Method

Hennig's (1966) conditions affect the choice of molecular method, since the method must be able to diagnose the unique combinations of character states (Nixon and Wheeler 1990) which delimit the minimal branch tips for cladistic analysis, or phylogenetic species. Direct sequence analysis was chosen, since it provides character data, can diagnose heteroplasmic individuals (when sequencing is performed on populations of amplified DNA fragments), and the homology (ancestral origin) of characters is reasonably clear (Avise 1984, Gyllenstein 1989, Nichols and Raben 1994).

Ribosomal Cistron

To some cladists, the nuclear ribosomal cistron is an ideal system from which suitable data can be obtained for phylogenetic reconstruction (Hillis and Dixon 1991). The cistron contains ribosomal RNA (rRNA) genes and associated spacers in a tandomly repeated array. The rRNA coded therein form the ribonucleic acid backbone of the ribosome, the cellular structure responsible for protein synthesis (Mathews and van Holde 1990).

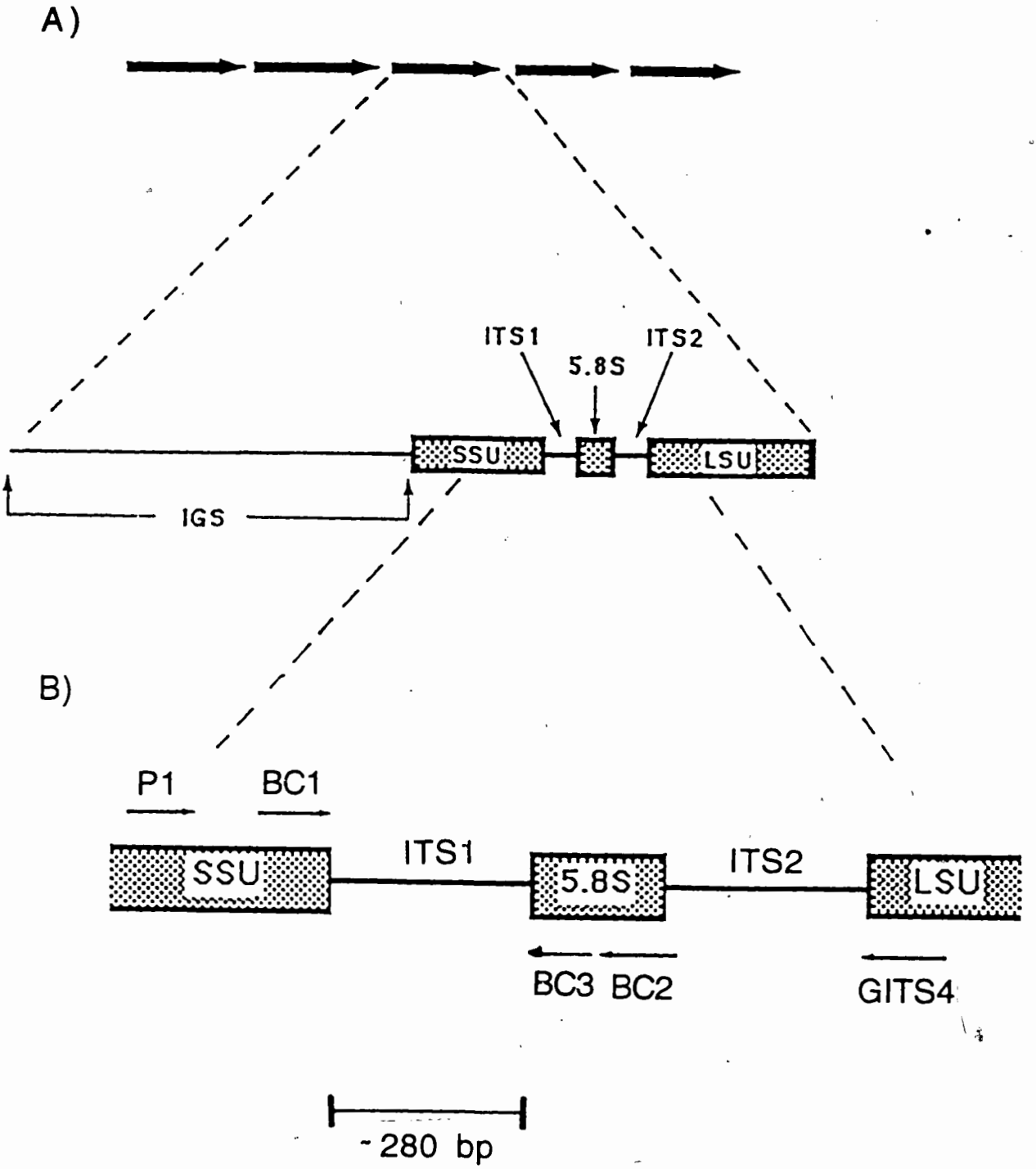
In higher eukaryotes such as the kelp, the tandem repeats often number in the thousands (Appels and Honeycutt 1986), and are separated by an intergenic spacer (IGS) (Fig. 4). The first gene is the 18S, or small subunit gene, which is separated from the 5.8S by the first internal transcribed spacer (ITS1). The 5.8S and the 28S, or large subunit gene, flank the second internal transcribed spacer (ITS2) (Hillis and Dixon 1991).

The popularity of the ribosomal cistron for phylogenetic studies can be attributed to its versatility, containing regions of diverse levels of functional constraint, resulting in some regions evolving at much different rates than others. Taxa can be resolved at almost any level by targeting the region with the appropriate variability (Hillis and Dixon 1991, Avise 1994).

Highly conserved regions can provide excellent locations for polymerase chain reaction primers, which, coupled with the typically

Figure 4: Diagram of the presumed structure of the nuclear ribosomal cistron from *Macrocystis*, showing **A**: organization of one tandem repeat unit. The intergenic spacer (IGS) separates repeat units. The first internal transcribed spacer (ITS1) separates the small subunit gene (SSU) from the 5.8S gene, and the second internal transcribed spacer falls between the 5.8S gene and the large subunit gene (LSU). **B**: Detail of the 3' SSU, ITS1, 5.8S, ITS2 and 5' LSU regions of a ribosomal cistron and the orientation of primers used in amplification reactions. (after Mayes 1993). P1: (5' TAATCT-GTTGAACGTGCATCG 3') and BC1 (5' GATTCCGGAC-TGTGGCTCCGGTG 3') are the forward primers, while GITS4 (5' CTTTTCCTCCGCTTATTGATATG 3'), BC2 (5' CGAGTGGTGTCAACAGACACTCC 3') and BC3 (5' GCGA-CGCAAGAAGTAGCAACACCC 3') are the reverse primers. The length of the target region (ITS1) averages 280 bp.

Figure 4


A)
ITS1
5.8S
ITS2
SSU
LSU
IGS
B)
P1
BC1
SSU
ITS1
5.8S
ITS2
LSU
BC3
BC2
GITS4
~280 bp

high copy number, make the system easy to amplify for sequencing (Hillis and Dixon 1991).

Multiple cistron copies do not evolve independently. Through concerted evolution, a process hypothesized to proceed through unequal crossing over, mismatch repair and gene conversion (Dover 1982), cistron copies tend to be homogenized both within individuals and within species (Hillis et al 1991). Concerted evolution allows the treatment of all copies found within an individual concurrently, as if only one copy existed (Sanderson and Doyle 1992). Variation has been found between individual repeats (Stewart et al 1983, Zimmer et al. 1988, Bobola et al. 1992) of the cistron, but this appears to be rare.

The utility of the ribosomal cistron for phylogenetic inference has led to its widespread use, which facilitates comparisons across broad groups of organisms (Hillis and Dixon 1991). The attention gained by the ribosomal cistron also promotes technical and theoretical development.

For resolution at the species level in the kelp, the genic regions have been found to be too highly conserved to yield sufficient phylogentically informative sites (Saunders 1991, Druehl and Saunders 1992, Saunders and Druehl 1993, Mayes 1993). The IGS spacers between rDNA repeats have been found to be highly variable, but perhaps they vary more quickly than concerted evolution can homogenize them, for intra-individual variation is seen (Appels and Dvorak 1982), which can complicate analysis. In contrast, ITS1 and

ITS2 are minimally conserved, not coding for ribosomal genes, yet containing signals for processing the primary transcript (Van Nues et al 1994) and have proven to be useful at the population - species - genus levels of resolution (Saunders 1991, Mayes 1993, Saunders and Druehl 1993, Bakker et al 1995). In *Drosophila*, ITS1 evolves at a rate approaching that expected for unconstrained regions, except for one region of secondary structure (Schlötterer et al 1994). A stem-loop structure has been found at the 3' end of the ITS1 in numerous groups of eukaryotes including insects, fungi (Schlötterer et al 1994), vascular plants (Torres et al 1990) and red, green and brown algae(Van Oppen 1995, Bakker et al 1994, Saunders 1991, Mayes 1993, Van Nues et al 1994). Schlötterer et al (1994) postulate that it is important for the assembly of the 18S ribosomal subunit (Schlötterer et al 1994).

MATERIALS AND METHODS

Site Selection

For concurrent morphological and genetic studies, individuals from six sites were chosen on the southern shore of Barkley Sound, British Columbia. A gradient of wave exposure (ranging from the most exposed in the southwest, to the most sheltered in the northeast) (Fig. 5) was chosen because this environmental factor has been reported to correlate with the most pronounced morphological variation (Druehl and Kemp 1982).

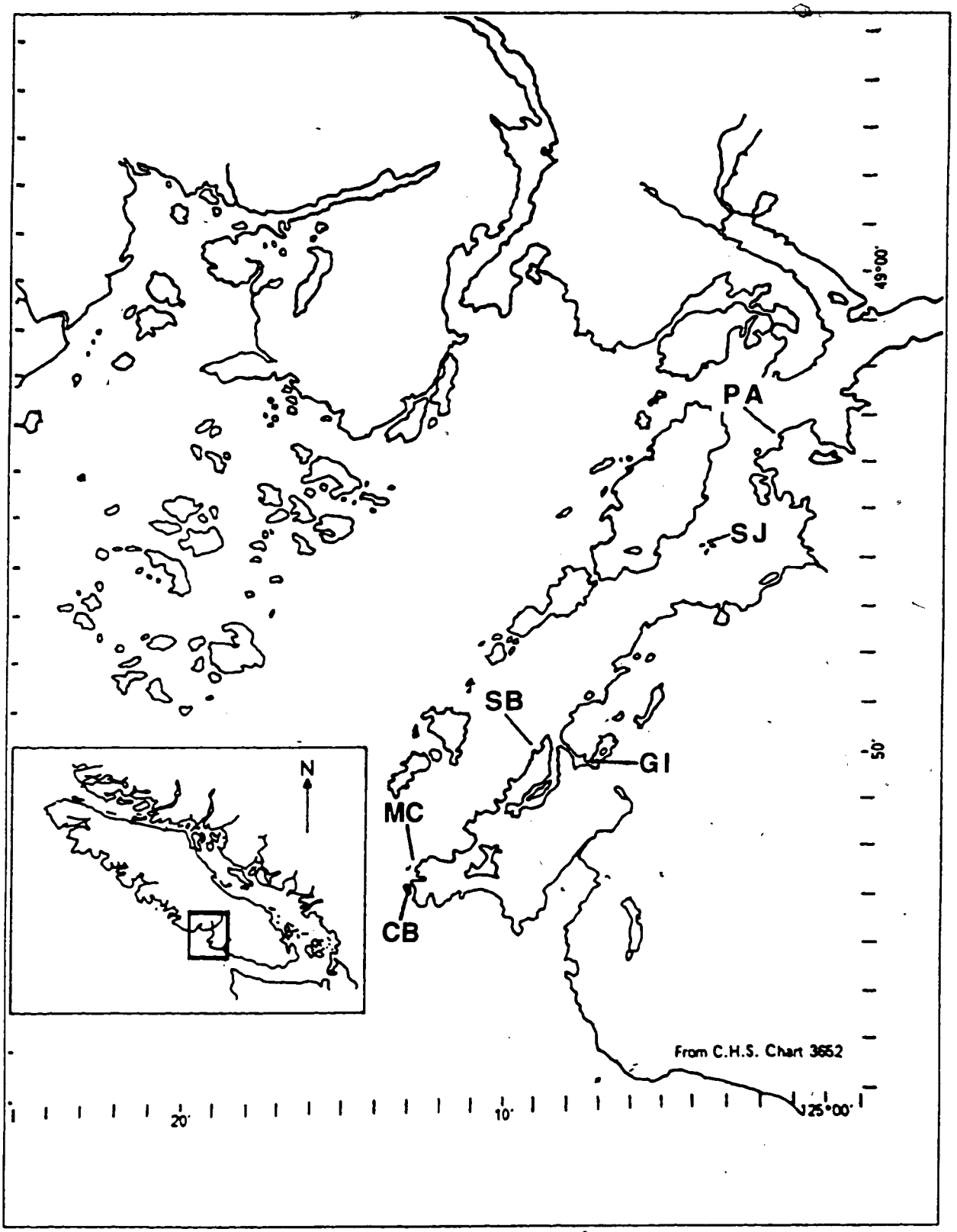
Genetic analysis alone was conducted on *Macrocystis integrifolia* from the Californian coast (Monterey) as well as *M. pyrifera* (including *M. angustifolia* sensu Neushul) from two sites in California and *M. pyrifera* from Tristan da Cunha (37.08 S, 12.28 W), in the southern Atlantic Ocean midway between Cape Horn and the Cape of Good Hope (Fig. 3).

Collection

In Barkley Sound, blades from *Macrocystis integrifolia* were collected from each of the six sites. Blades chosen were

Figure 5: Map of Barkley Sound, B. C., showing sites sampled for concurrent analyses of morphological and genetic differentiation. Diverse wave exposure regimes are represented: The Mud Cove (MC) site has the most exposed population of *Macrocystis* seen in Barkley Sound, subject to violent waves, sometimes reaching 10 m. The Gap at Cape Beale (CB) is subject to more moderate wave exposure. Scott's Bay (SB) is relatively sheltered site, but not nearly as calm as the San Jose Islets (SJ) and Port Alberni Inlet (PA), where wave action is rare. Grappler Inlet (GI) is peculiar, in that the *Macrocystis* population there is totally sheltered from any wave action, but is subject to considerable tidal current.

Figure 5



the youngest mature blade on the frond, as gauged by blade size, often the fifth or sixth blade below the apical scimitar. At each site, blades were haphazardly sampled from a broad area, taking care to avoid resampling individuals. The number of blades collected depended on the size of the kelp bed, and ranged from 15 (within the small bed in the Gap at Cape Beale) to 87 (from the large bed in front of Mud Cove). Blades were transported in seawater to the Bamfield Marine Station, where they were subjected to both morphological and genetic analyses.

Holdfasts were also collected from these sites by snorkeling throughout the bed with the purpose of obtaining several typical examples.

Blades from the Californian *Macrocystis integrifolia* and *M. pyrifera* as well as the *M. pyrifera* from Tristan da Cunha were collected and pressed dry, and then mailed to the author for genetic study.

Genetic analysis was performed on five individuals from each site, five being the minimum sample size for which there is a 95% probability that heterozygotes will be detected in a region 300 base pairs (bp) long, assuming a spontaneous mutation rate of .005/ nucleotide site (Clark 1990). An exception was the first site, Port Alberni Inlet, from which 10 individuals were sampled for genetic analysis.

Morphometrics

Fresh blades were measured for width, blade angle (where they attached to the pneumatocyst), pneumatocyst diameter, and substantiality. Blade width was measured at the widest point of the blade. Blade angle was measured as the angle made by the two sides of the blade where the blade departs from the pneumatocyst (Fig. 6). Substantiality is a measure of weight per projected area. A tissue disc 6 cm in diameter was cut from the blade at a distance 10 cm from the pneumatocyst, where the blade was no longer actively growing, yet still close enough to the pneumatocyst to avoid erosion (Fig. 6). Discs were then weighed. Dry weight of these discs was also assessed, but this did not affect the results, so wet weights are used here.

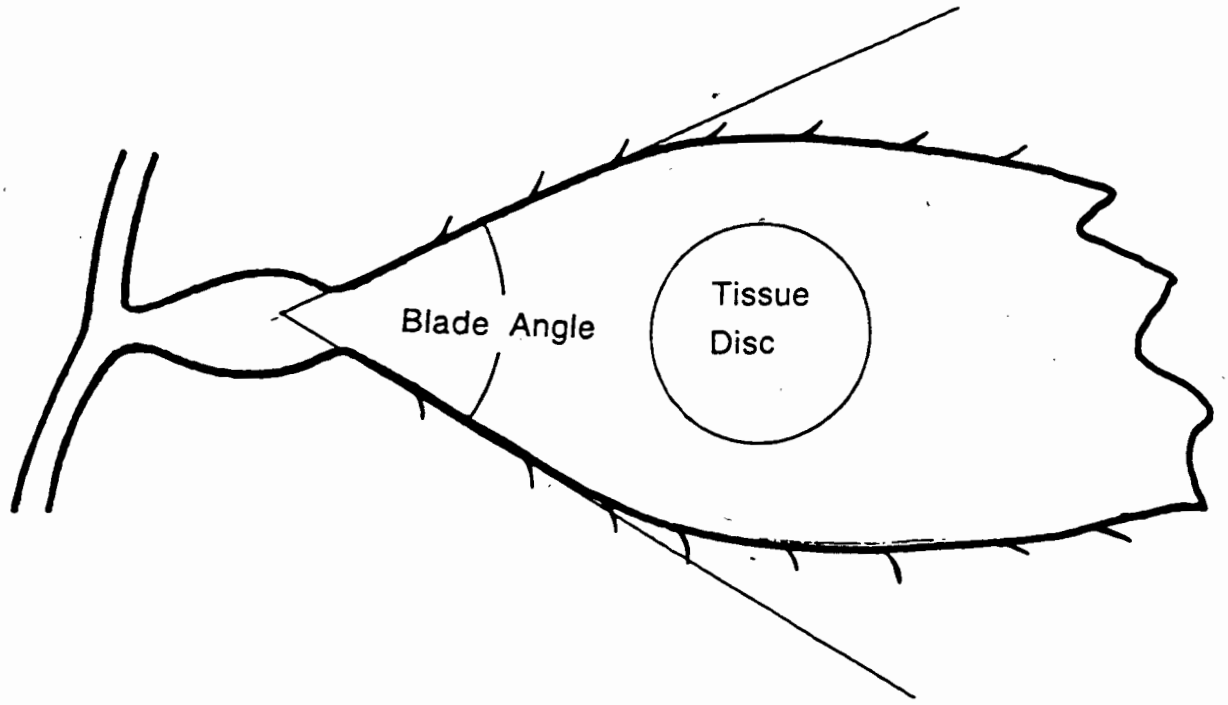
Statistical analyses were performed with Statview™SE + Graphics v. 1.03, ©Abacus Concepts Inc. 1988, Berkeley, California.

Fresh samples were pressed dry on labelled herbarium sheets and kept for molecular work.

Holdfasts were examined and compared to the key of Womersly (1954).

Figure 6: Illustration of morphometric measurements. Blades chosen were the youngest mature lamina on the frond, usually the 5th or 6th blade from the apical scimitar. Blade angle was measured where the blade departed the pneumatocyst using a modified protractor, and weighing the tissue disc (6 cm diameter) yielded substantiality. Substantiality is a measure of tissue density and blade rugosity.

Figure 6



DNA Extraction

Current kelp DNA extraction methods require approximately one day to process a set of samples, so to decrease sample processing time and enable the processing of large numbers of samples, an adaptation of the Promega Wizard™ DNA purification kit was utilized. A drastic reduction in handling time for DNA extraction, to approximately thirty minutes per set of samples was achieved.

Dry tissue was removed from the herbarium specimens and ground to a fine powder using a mortar and pestle. To prevent contamination, the mortar and pestle were scrubbed with soap and water, rinsed, then scrubbed with alcohol, and rinsed again between samples. Approximately 1 mg of tissue powder was suspended in 400 µl BS1 (50 mM Tris-Cl pH 7.5, 150 mM EDTA, 100 µg/ml RNAse A) and boiled for 5 minutes to lyse the cells. 700 µl PS4 (66.84 g/100 ml guanidine hydrochloride, 50 mM Tris, 20 mM EDTA, 4 g/ 100 ml diatomaceous earth) and 5 µl 1M dithiothreitol were added and mixed occasionally for five minutes, to allow the DNA to bind to the diatomaceous earth. The diatomaceous earth suspension was spun in a microcentrifuge at 5000 G for 15 seconds and the supernatant discarded. The pellet was resuspended in PS5 (200 mM NaCl, 20 mM Tris, 5 mM EDTA, 70% ethanol) and loaded onto

a Promega Wizard™ column and pushed through with a 2 cc syringe, trapping the diatomaceous earth with the bound DNA on the column. The column was rinsed with 1 ml PS5, and then with 2 ml 95% ethanol. Residual ethanol was removed by centrifugation at 15000 rpm for 2 minutes. DNA was resuspended by adding 50µl TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0) at 80°C to the column. DNA was removed from the column by spinning the column at 15000 rpm for 1 minute. The DNA solution was then frozen at -20°C for later use.

DNA Sequence Analysis

Sequence data were acquired by the dideoxy sequencing of single stranded amplification product (Gyllenstein 1989), amplified in two steps. A first amplification reaction produced double stranded product, which was then used as template for the second reaction, which amplified the single stranded product (Nichols and Raben 1994). A nested set of 4 primers was chosen to bracket the ITS1 region of the ribosomal cistron (Fig. 4B). With the nested primers, the external set could be used for the double stranded amplification, and the internal set for the single stranded amplification and sequencing, ensuring that degraded termini of products would not compromise sequence data (Gyllenstein 1989).

First Amplification

Double stranded amplification reactions were performed with the outer primer set (P1 and GITS4 initially; later P1 and BC2). Reactions consisted of 1X Promega DNA polymerase buffer, 2.5 mM MgCl, 1 µl 20-fold diluted DNA extract, 1 unit Promega Taq DNA polymerase, 200 µM each of dATP, dGTP, dCTP and dTTP and 0.5 µM of each primer. Reactions were overlaid with two drops of mineral oil. 25 µl reactions were run in a MJ Research Mini-cycler over a temperature profile of: 95°C for 3', (94°C, 1'; 52°C, 1'; 72°C, 1'40") for 27 cycles, then a final extension at 72°C for 10'. Once BC3 was designed and GITS4 was no longer used, reactions were performed with a combined annealing/extension step (72°C for 1'40") alternating with a denaturation (94°C for 1') for 27 cycles and a final extension at 72°C for 10'.

Second Amplification

Single stranded amplification reactions were 100 µl, consisting of 1X Promega DNA polymerase buffer, 2.5 mM MgCl, 4 µl of the unpurified double stranded amplified product, 4 units Promega Taq DNA polymerase, 200 µM each of dATP, dGTP, dCTP and dTTP, 0.25 µM internal primer (either BC1 or BC2 [or later BC3], depending on the target strand). Reactions were

overlaid with three drops of mineral oil. The reactions were run over a temperature profile of: 95°C, 3'; (94°C, 1'; 72°C, 1') for 29 cycles, then a final 10' extension at 72°C. The reactions were removed from the mineral oil and transferred to a Millipore® Ultrafree-MC filter. These filters allow the reaction product to be retained, while removing unincorporated nucleotides, salts and other small reaction components. The filters were placed in a microcentrifuge, and spun at 5000 G until approximately 10 µl remained. 200 µl water was added, and again spun down to 10 µl. A final rinse of 200 µl water was added, and this time spun down to 7 µl for use in the sequencing reactions.

Sequencing

Sequencing reactions were performed using Sequenase® 2.0 (United States Biochemicals) kits. The primer used for sequencing was the internal primer (BC1, BC2 or later, BC3) which was not used for the single stranded amplification. The Sequenase® 2.0 protocol was followed, except that there was no denaturation necessary for single stranded template, and annealing was performed with an initial temperature of 75°C, then slowly cooled to 40°C over 25 minutes (Nichols and Raben 1994). Sequencing reactions were electrophoresed on 6% acrylamide taurine gels for 2 - 6 hours (Sambrook et al 1989).

Gels were rinsed in a 10% methanol, 10% acetic acid bath, dried in a gel dryer, and exposed to Kodak PDB-1 X-ray film for 24 hours to 3 weeks.

Sequence Analysis

Sequence alignment was accomplished by eye using a manual sequence editing program (Eyeball Sequence Editor, version 1.09a, E. Cabot, Simon Fraser University).

Putative secondary structure was inferred using oligonucleotide analysis software (Oligo® 4.04, ©1992 Wojciech Rychlik, National Biosciences Inc, Plymouth, MN), and by aligning regions of conserved sequence between *Macrocystis* and other related kelp, for which analyses of secondary structure have been performed. Melting temperatures for the secondary structures were estimated using the %GC method in Oligo®.

RESULTS

Morphological Variation

Comparisons of blade angle and substantiality among samples collected in Barkley Sound are shown in Table 1. In general, wave exposed samples were much more streamlined, having a narrower blade and smaller blade angle than samples from more sheltered sites (Fig. 7). Wave exposed samples also had more substantial blades. Exceptions were the samples taken from Grappler Inlet, a site totally sheltered from all wave exposure, but subject to considerable tidal current. Samples taken here were very streamlined, possessing a narrow, acutely angled blade which had very low substantiality.

Differentiation among populations was clear for both blade angle (ANOVA $p<0.001$) and substantiality (ANOVA $p<0.001$).

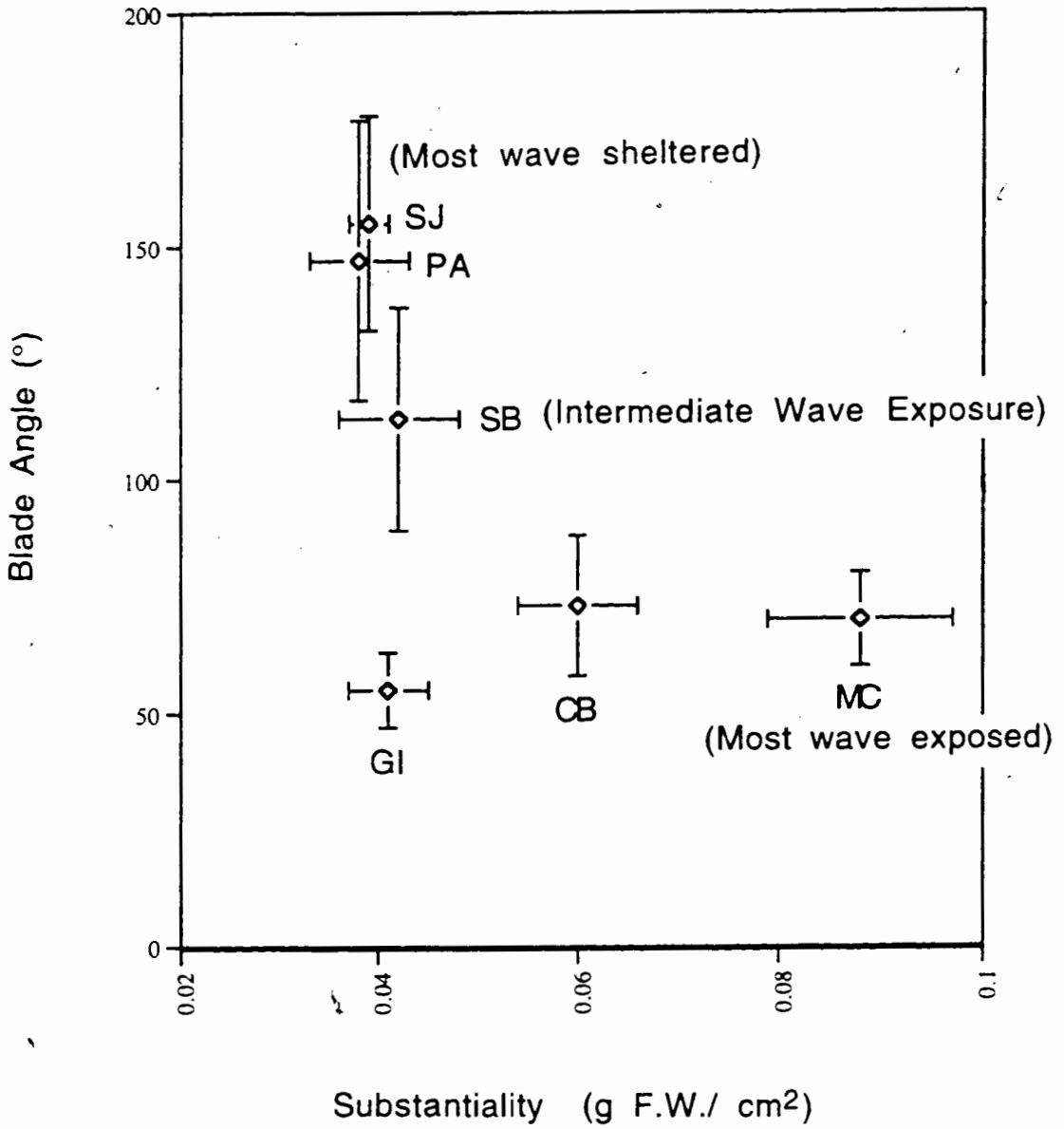
At all sites in Barkley Sound, holdfasts of mature plants were prostrate, flattened and creeping, as per *Macrocystis integrifolia* in the key of Womersly (1954). Occasionally, holdfasts of immature plants were seen to be upright and terete, as noted by Scagel (1947). Two such immature terete holdfasts were transplanted to a rope culture, where they

Table 1: Blade angle and substantiality (fresh weight/ projected surface area, measured by weighing a 6 cm tissue disc cut 10 cm from the pneumatocyst). Wave exposure is deemed high when the site is subject to violent wave action throughout the year. Moderate wave exposure refers to a site which is subject to occasional strong wave action. Low wave exposure sites receive little or no wave action. * - Grappler Inlet, while totally sheltered from wave action, is subject to considerable tidal current. Standard deviation is in brackets. MC - Mud Cove, CB - the Gap at Cape Beale, GI - Grappler Inlet, SB - Scott's Bay, SJ - San Jose Islets, PA - Port Alberni Inlet.

Site	n	Wave Exposure	Blade Angle (Degrees)	Substantiality (g F.W./cm^2)
MC	87	High	70 (10)	.088 (.009)
CB	15	High	73 (15)	.060 (.006)
GI	29	Low*	55 (8)	.041 (.004)
SB	40	Mod.	113 (24)	.042 (.006)
SJ	67	Low	155 (23)	.039 (.002)
PA	21	Low	147 (30)	.038 (.005)

Figure 7: The relationship between average blade angle and average substantiality for six populations of *Macrocystis* in Barkley Sound, B. C., representing a range of wave exposures. MC - Mud Cove. CBG - Cape Beale, the Gap. SB - Scott's Bay. GI - Grappler Inlet. SJ - San Jose Islets. PA - Port Alberni Inlet. MC is the most wave exposed site, while SJ, PA, and GI are the most sheltered. Grappler Inlet, while totally sheltered from wave action, is subject to considerable tidal current. Bars represent standard deviation.

Figure 7: Blade angle and substantiality among populations of *Macrocystis* in Barkley Sound, B. C.



subsequently developed flattened, creeping holdfasts, as would be expected of *M. integrifolia* (Scagel 1947).

DNA Sequence Variation

Complete ITS1 sequences were obtained for all samples from all sites in Barkley Sound, California, and Tristan da Cunha (Fig. 8). ITS1 boundaries were defined through comparisons of flanking 18S and 5.8S genic sequences with those of other kelp (Saunders 1991, Saunders and Druehl 1993). ITS1 length varied from 280 base pairs (bp) to 284 bp.

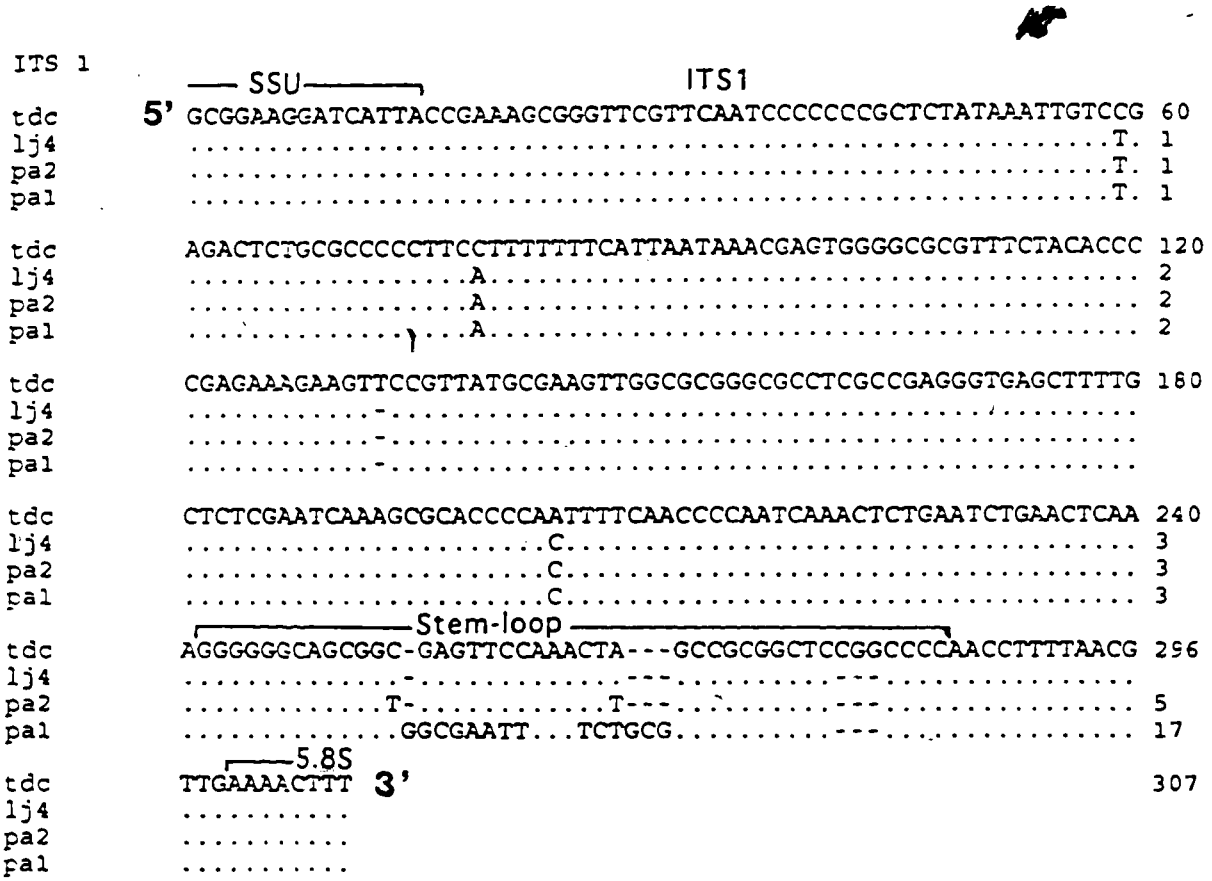
Four variants of the ITS1 sequence were found in total. Sequence variation fell into two broad categories: (1) variation within individuals and the populations in which they were found, and (2) fixed differences between populations.

(1) **Intra-individual Variation** One sequence variant, referred to by the sample to have first displayed it (ribotype PA1, Port Alberni Inlet individual #1), was ubiquitous in the Northern Hemispheric individuals, occurring in all 50 plants sampled. Variation within individuals was noted when the examination of sequencing autoradiographs showed that some individuals from the Northern Hemisphere (Port Alberni #2, Isla Vista #3, Isla Vista #5, La Jolla #4) had what appeared to be a second sequence superimposed upon the first in a 18 nucleotide (nt) region near the 3' end of the ITS1, adjacent to the 5.8S gene

Figure 8: Aligned *Macrocystis* ITS1 sequences for the four ribotypes seen. Ribotype TdC was seen in all individuals from Tristan da Cunha (n=5), ribotype PA1 was seen in all individuals from the Northern Hemisphere (n=50). One individual in Barkley Sound (Port Alberni #2) possessed both ribotype PA1 and ribotype PA2. Ribotype LJ4 was seen along with ribotype PA1 in one individual from La Jolla (La Jolla #4) and again in two individuals from Isla Vista, California (Isla Vista #1, Isla Vista #3).

Figure 8

tdc = ribotype TdC (n=5) - All samples from Tristan da Cunha
lj4 = ribotype LJ4 (n=3) - from heteroplasmic individuals of *M. pyrifera*
pa2 = ribotype PA2 (n=1) - from heteroplasmic individual of *M. integrifolia*
pa1 = ribotype PA1 (n=50) - All samples from North America

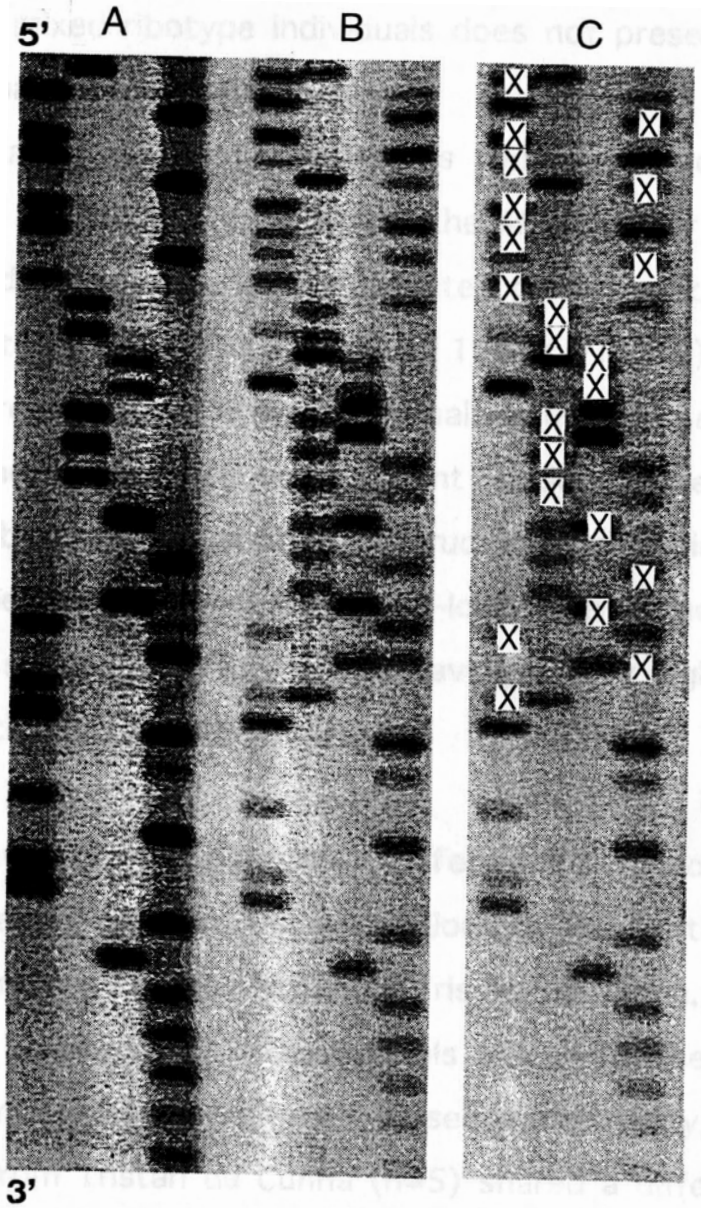


(Fig. 9B). The finding was initially regarded as a possible amplification artifact, and the entire DNA extraction and sequencing procedures were repeated for that individual (Port Alberni #2), to control for spurious amplification artifacts and contamination. In total, DNA extraction and sequencing was repeated four times for Port Alberni #2 over a period of 13 months, at first to resolve a presumed error, and then to confirm the finding. Since one ribotype was known from previous sequence (ribotype PA1), inferring the second sequence was possible by "subtracting off" (Clark 1990) the first (known) sequence by marking the bands on the autoradiograph which were present in the known sequence (Fig 9A), and then reading off the remaining unmarked sequence (Fig 9C). From this I hypothesized that two different copies of the ITS1 were present in this individual. Similar analyses of other individuals revealed a third variant (ribotype LJ4) in southern California, differing from the ribotype PA2 sequence by only two substitutions in this 18 nt region near the 3' end of the ITS1. All samples with dual sequences were sequenced at least twice, and both sequences appeared each time. The existence of ribotype LJ4 was also corroborated by its identity to sequence obtained from a single cloned copy of an amplified *Macrocystis* ITS1 from Catalina Island, California (M. Liptack unpublished).

Individuals with mixed ribotypes were relatively rare, comprising approximately 7% of the samples assessed.

Figure 9: A laser scanned (Macintosh Color OneScanner) image of a segment of a sequencing autoradiograph. **A**: Typical sequence ladder, showing a single set of bands (ribotype PA1, from individual La Jolla #5). **B**: Sequence ladder seen for individual with dual ribotypes. The arrow indicates the beginning of the two sets of bands. **C**: The same sequence ladder seen in B, with the ribotype PA1 bands marked with an X. The second sequence (ribotype LJ4) from individual La Jolla #4 can now be read as the unmarked bands. In positions where only one band is present, the two sequences share that base. Sequence shown is reverse sequence, 15 bp upstream of the 5.8S gene in individuals La Jolla #5 (**A**) and La Jolla #4 (**B**, **C**).

Figure 9



Ribotype PA2 occurred in one of 50 Northern Hemispheric plants sampled, and ribotype LJ4 occurred in three of those 50. While ribotype PA2 was seen only in *M. integrifolia*, and LJ4 was only seen in *M. pyrifera*, the frequency was so low that distribution of these mixed ribotype individuals does not present any obvious pattern (Fig. 10).

Putative stem-loop structures were constructed for this region of sequence obtained from the *Macrocystis* species, using conserved stem sequence from related genera of kelp to orient the structures (Saunders and Druehl 1993) (Fig. 11). The 18 nt variable region seen in the individuals with dual sequences corresponds to the loop and adjacent section of the stem of the most stable putative stem-loop structures, as indicated by melting temperature. Similar stem-loops are formed by the different sequences (Fig. 11), all having a very high melting point, estimated at 90° C.

(2) **Fixed Inter-population Differences** Fixed differences were apparent between the populations of the Northern Hemisphere and the population of Tristan da Cunha, in the Southern Hemisphere. All individuals of the Northern Hemisphere (n=50) shared an ITS1 sequence (ribotype PA1). All samples from Tristan da Cunha (n=5) shared a different ribotype (ribotype TdC). These two ribotypes differed by three substitutions, two small insertion/deletions, and a stretch of

Figure 10: Map showing the distribution of individuals with dual ribotypes.

Individual	Species	Ribotypes
A: Port Alberni #2	*M. integrifolia*	PA1 + PA2
B: Isla Vista #1	*M. pyrifera*	PA1 + LJ4
C: Isla Vista #3	*M. pyrifera*	PA1 + LJ4
D: La Jolla #4	*M. pyrifera*	PA1 + LJ4

Figure 10

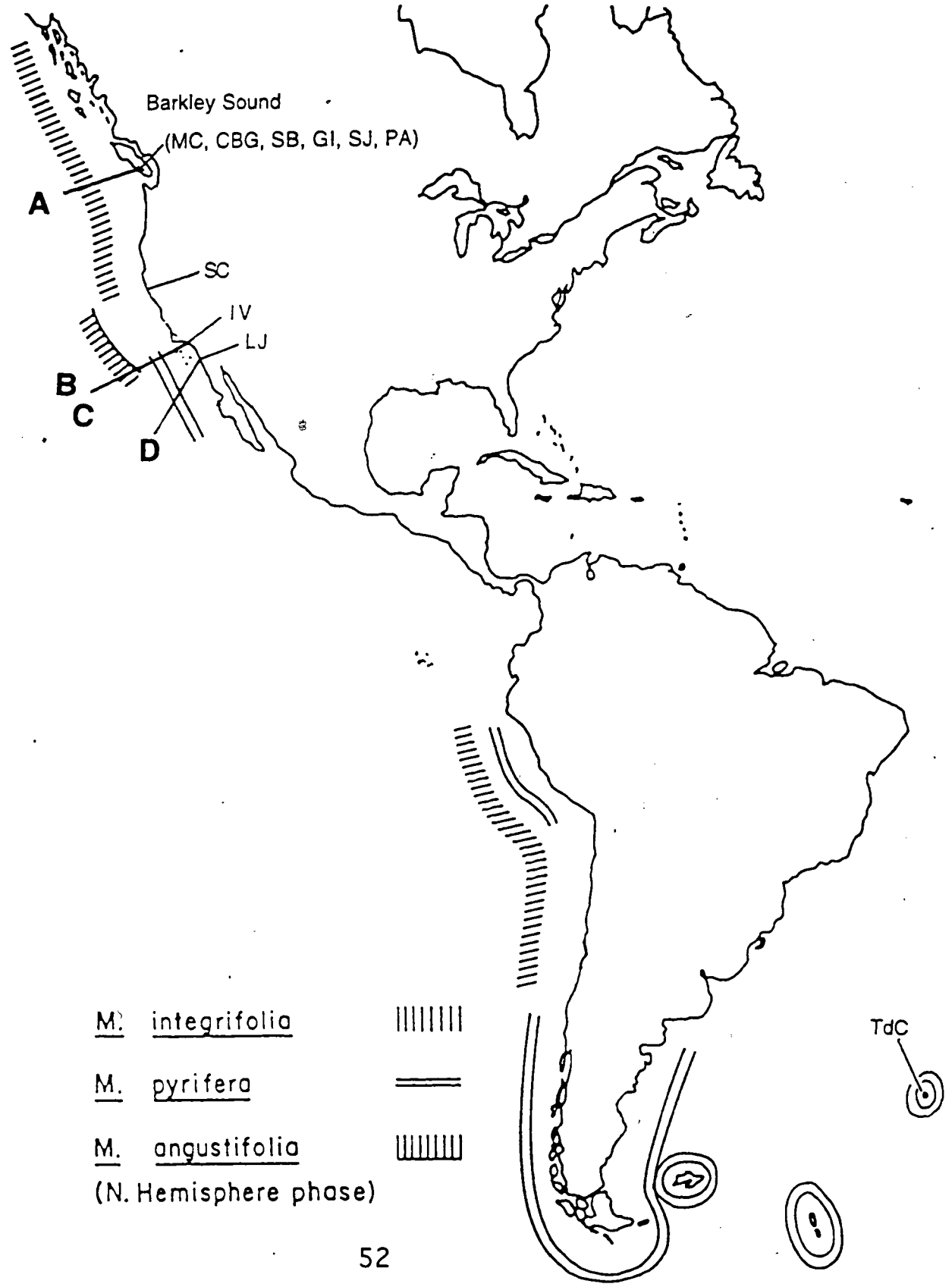
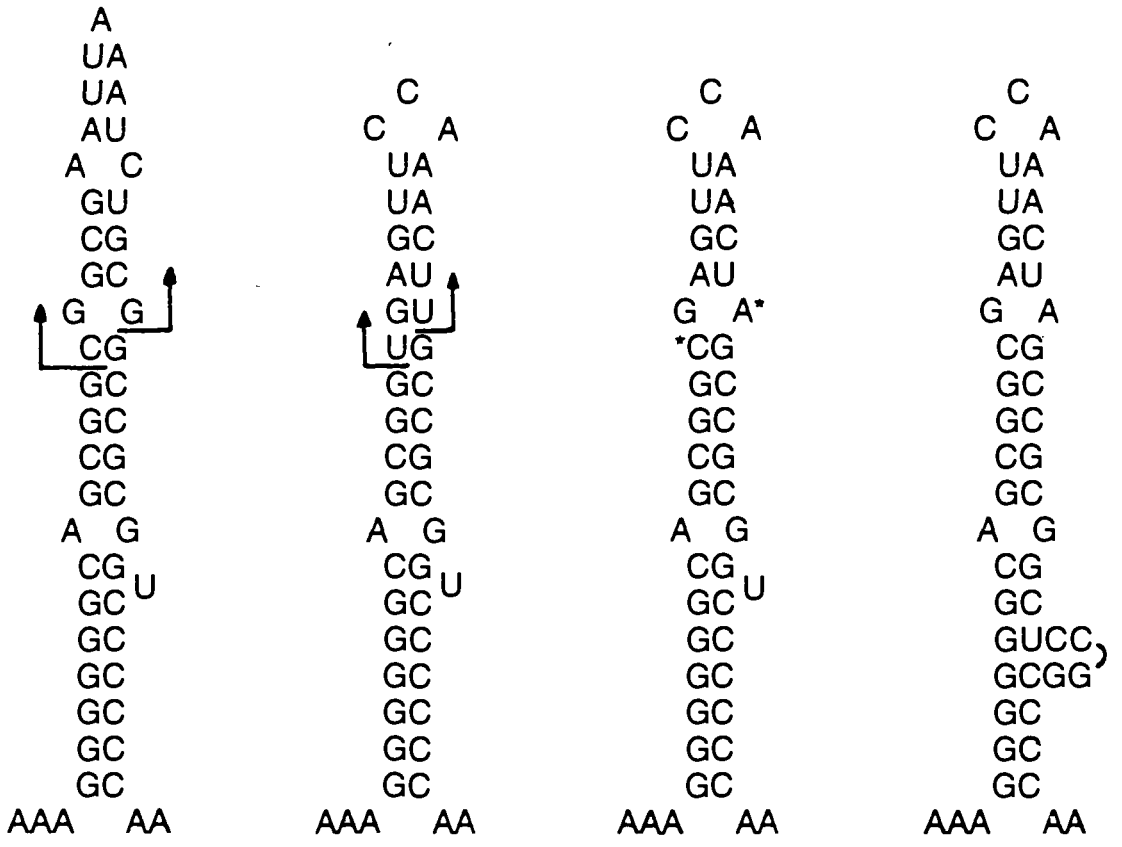

Barkley Sound
(MC, CBG, SB, GI, SJ, PA)
A
SC
IV
LJ
B
C
D
M. integrifolia
M. pyrifera
M. angustifolia
(N. Hemisphere phase)
TdC

Figure 11: Putative secondary structure models for the ITS1 stem-loop region of the four ribotypes observed in *Macrocystis* (after Saunders 1991, van Nues 1994). **A**: Putative stem-loop for individuals of *Macrocystis* with ribotype PA1 (found in all individuals from the Northern Hemisphere). **B**: Putative stem-loop for individual of *M. integrifolia* with ribotype PA2 (found in individual Port Alberni #2). Differs from A by the 18 nt of the loop and adjacent stem, as denoted by the arrow. **C**: Putative stem-loop for individuals of *M. pyrifera* with ribotype LJ4 (found in individuals Isla Vista #1,3 and La Jolla #4). Differs from A by the 18 nucleotides of the loop and adjacent stem, and differs from B by the two nucleotides marked with a "•". **D**: Putative stem-loop for individuals of *M. pyrifera* with ribotype TdC (found in all individuals from Tristan da Cunha). Same as C, except for a unique 5 nt insertion with respect to the other ribotypes.

Figure 11



A. PA1 **B. PA2** **C. LJ4** **D. TdC**

18 bases in a region hypothesized to form a stem-loop secondary structure in genera of the same order (Saunders and Druehl 1993) (Fig. 8).

Comparing the three stem-loop sequences from the Northern Hemisphere to the 18 nt region seen to vary between the Northern Hemisphere and Tristan da Cunha, ribotype LJ4 is found to be identical to ribotype TdC, and ribotype PA2 is found to differ by only two substitutions (Fig 11). Thus, the stem-loop region is polymorphic within the genus.

While a ribotype (ribotype LJ4) from California and that of Tristan da Cunha (ribotype TdC) share this stem-loop sequence, they do not share the three substitutions and two insertion/deletions seen to vary between the Northern and Southern Hemispheres (Fig. 8). These substitutions are fixed for different character states in the samples from the Northern Hemisphere (n=50) and the samples from Tristan da Cunha (n=5), delimiting two discrete populations on the basis of ITS1 sequence.

The three base substitutions constitute approximately 1% of the ITS1.

DISCUSSION

Morphological Variation

Within the species *Macrocystis integrifolia*, a broad range of morphological variation was seen over the gradient of wave exposures found in Barkley Sound, Vancouver Island, Canada (Fig. 7). As broad a range of morphological variation, in fact, as has been reported across the entire genus (Druehl and Kemp 1982, Fain 1986, Van Tussenbroek 1989b). Populations from wave exposed sites were discernible from wave sheltered sites on the basis of morphological differentiation. The cause of this morphological differentiation could be either random assortment of morphological variants, genetic response to selection, or phenotypic plasticity. Previous studies (Druehl and Kemp 1982, Van Tussenbroek 1989b) have demonstrated a phenotypic plasticity in the species that allows a wide range of morphologies to be elicited by varying environmental conditions.

Pilot studies revealed ITS1 variation in the first site examined (Port Alberni Inlet), which lead to the speculation that genetic variation may occur on a scale local to Barkley Sound, and so morphometric studies were initiated with the

intention of correlating the morphological variation to the supposed genetic variation. Subsequent expanded molecular sampling revealed no further genetic variation in Barkley Sound, and this left the relatedness between populations within Barkley Sound unresolved. Without knowledge of the underlying phylogeny of the populations, it is difficult to discern between the random assortment of morphological variants and genetic differentiation in response to selection. Without the resolution of genetic differentiation, phenotypic plasticity remains a potential source of morphological differentiation. It would, nonetheless, be a mistake to undertake studies of molecular differentiation without sufficient familiarity with the field biology of the subject.

Genetic Variation

The presence of phenotypic plasticity does not preclude genetic differentiation among discrete populations. Anderson and North (1966) have demonstrated that dispersal of meiospores leading to successful fertilization of gametes can be limited to a few meters, which, if true, could restrict gene flow between populations, resulting in genetic differentiation. However, the presence of buoyant floats, along with the ability to survive and reproduce while drifting (North 1971) suggests that long range dispersal of mature plants is likely, which could

lead to genetic homogeneity between geographically distant populations.

Molecular methods have recently been developed (*in* Avise 1994 and Sambrook et al 1989) which allow the description of genetic variation within species (Bakker et al 1995, Van Oppen 1995, also see review of Manhart and McCourt 1992). One such method is the sequencing of the ITS1 region of the ribosomal cistron. Under minimal functional constraint (Schlötterer et al 1994), most of the ITS1 is thought to evolve very quickly (Hillis and Dixon 1991, Schlötterer et al 1994). Meanwhile, concerted evolution works to homogenize copies of the cistron both within individuals and within populations (Dover 1982, Sanderson and Doyle 1992), making ITS1 helpful for assessing differentiation among closely related taxa. In this study, adaptation of new methods of DNA extraction and direct sequencing allowed the sequencing of a large number of individuals quickly. Direct sequencing of amplified copies of the ITS1 allowed the description of variation within individuals, as well as between populations of *Macrocystis*.

Patterns of Variation

Concurrent analysis of morphological and genetic variation within *Macrocystis* has provided interesting results. In Barkley Sound, B.C., where populations were readily

recognized on the basis of morphological characters, no population divergence was seen in ITS1 sequence. This finding reinforced previous studies attributing morphological variation within *M. integrifolia* to phenotypic plasticity, and also provided an excellent starting point for a procedure of population aggregation (Davis and Nixon 1992), wherein sampling includes continually broader populations to discover the limits of genetic homogeneity.

On a greater geographic scale, no genetic divergence was seen between these morphologically distinct populations of *Macrocystis integrifolia* in Barkley Sound and a population at the southernmost extreme of the species' range, in Monterey, California (see Fig. 3). Genetic variation was observed within an individual (Port Alberni #2), which possessed two different ribotypes (ribotype PA1 and ribotype PA2, which differ by an 18 nt stretch of sequence), but no fixed differences between populations were seen.

To encompass a broader morphological range necessitated spanning the second species of *Macrocystis* found in North America, *M. pyrifera*, which is differentiated on the basis of holdfast morphology. However, no underlying genetic variation was observed. Similar intra-individual genetic variation was seen, with three individuals (Isla Vista #1,3 and La Jolla #4) possessing dual ribotypes (ribotype PA1 and ribotype LJ4), but still no genetic differentiation between populations was

apparent across the ITS1 region, on this broadest of morphological ranges. Included in the morphological range is a third putative species, *M. angustifolia* sensu Neushul.

To increase the geographical scale and aggregate further groups, a population of *Macrocystis pyrifera* from the South Atlantic, morphologically very similar to that of California, was compared and found to be divergent from all North American populations. Considering that the Northern and Southern hemisphere populations are thought to have been separated for the past 3.8 - .7 million years (Fain 1986, Druehl and Saunders 1992), genetic differentiation is not unexpected, considering the rapid speed at which the ITS regions have been reported to vary (Hillis and Dixon 1991, Hillis et al 1991, Schlötterer et al 1994). What is surprising is that one region, comprising 18 nt of the stem-loop, is seen to be polymorphic between the hemispheres. One ribotype was found in all northern samples (ribotype PA1), another ribotype in all southern samples (ribotype TdC), but several scattered individuals in the northern populations possessed both variants (Fig 10).

Origin of Intra-individual Variation

Variation between repeats of the ribosomal cistron within an individual occurs when the processes of concerted

evolution have not had sufficient time to homogenize copies in a population (Appels and Honeycutt 1986, Hillis and Dixon 1991). The variation itself can be introduced through mutation or gene flow from a diverged population. The similarity of the stem-loop of the odd ribotypes found in the heteroplasmic individuals in the North American populations to the stem-loop found in Tristan da Cunha leads to two hypotheses: either this variation was present in the ancestral population and has yet to become fixed in the northern populations, or there has been gene flow since the two populations diverged. Both are possible, but, the considerable time since divergence of the populations paired with the rapid rate of variation of the ITS regions make it seem less likely that the variation is relict (see Hillis et al 1991). If we turn our attention to gene flow, we must assess the likelihood of both natural dispersal and anthropogenic dispersal. While the most recent cooling event considered stable enough to allow dispersal through colonization advancing from one hemisphere to another is thought to have ended 3.8 - .7 million years ago (Fain 1986, Druehl and Saunders 1992), there have been more recent oceanic cooling events that could have allowed drifting plants to disperse great distances (Fain 1986). As recently as 10,000 - 15,000 years ago, there have been cool water corridors hypothesized, and *Macrocystis* is known to survive extensive periods while drifting (North 1971).

Increased global shipping traffic over the past few

centuries has been accompanied by an increase in anthropogenic dispersal of marine algae (Druehl 1969, Hay 1990). Before modern steam and diesel powered vessels, sailing ships often took on ballast in the form of stones from the seashore, often covered with intertidal organisms, making these sailing ships excellent vectors for the dispersal of marine life, including algae. When these ships arrived at their destinations, ballast stones were discharged and replaced with cargoes such as lumber. Druehl (1969) suggested this scenario to explain the disjunct populations of *Eisenia*, another kelp which is often found growing beside *Macrocystis* in Barkley Sound. *Eisenia*, more typical of warmer waters, is seen in Southern California, Barkley and Kyuquot Sounds on the west coast of Vancouver Island, and Japan, but nowhere in between. Records of shipping traffic lead Druehl (1969) to conclude, that ships, which proceeded from southern California to Barkley and Kyuquot Sounds, thence to Japan, and back to southern California, could have been responsible for transporting *Eisenia*. *Macrocystis* could conceivably have been similarly transported.

Regardless of the mode of dispersal, the species of *Macrocystis* are readily interfertile (Lewis et al 1986, Lewis and Neushul 1994), so any migrant individual would be able to interbreed with local populations, producing a chimerical hybrid genotype. Such chimeric genotypes could be seen as the odd individuals in the Northern Hemisphere which possess the

stem-loop typical of samples from Tristan da Cunha. It could be an interesting coincidence, but the single hetero-ribotype individual found in Barkley Sound was found at the mouth of the Port Alberni canal, a natural canal and a favourable location to discharge ballast, being both near Port Alberni, and at the onset of sheltered waters.

It is curious that the stem-loop typical of Tristan da Cunha may have introgressed into the Northern Hemisphere, but not the entirety of the ITS1. If concerted evolution is acting to homogenize copies within the ribosomal cistron of *Macrocystis*, perhaps this could be an example of an intermediate stage in that progression.

Population Delimitation

The existence of individuals with both variants of the stem-loop abrogates the usefulness of the stem-loop region for phylogenetic inference, since patterns of inheritance for a polymorphic trait are not hierarchic (Nixon and Wheeler 1990, Hennig 1966). In ITS1, there are still fixed character differences between two groups: the three substitutions and two insertion/deletions that distinguish the Tristan da Cunha population of *Macrocystis pyrifera* from the North American populations of *M. pyrifera* and *M. integrifolia* (Fig. 8). There are no less inclusive groups supported by variation within the ITS1;

ITS1 sequences from populations of *M. pyrifera* and *M. integrifolia* from the northern hemisphere are identical, suggesting that these North American populations have shared a more recent common ancestor than have populations of *M. pyrifera* on either side of the equator.

Comparative Levels of Variation

Approximately 1% of the 280-284 bases of the ITS1 was variable between populations. In the green alga *Cladophora vagabunda*, a comparably cosmopolitan species, intraspecific ITS1 variation was found that exceeded 15% (Bakker et al 1995), while intrageneric variation was much greater, making sequence alignments difficult. In the red alga *Phycodrys rubens*, geographic isolates in the Atlantic alone displayed intraspecific variation as high as 5% of the ITS1 (van Oppen 1994). At 1%, intrageneric ITS1 variation in *Macrocystis* is much less than even intraspecific variation in these other algae with comparably broad distributions.

In the kelp, to find a level of ITS1 variation similar to the levels of intraspecific variation seen in *Cladophora vagabunda* and *Phycodrys rubens*, one must look across the broad genus *Laminaria* (18%, Mayes 1993). The Laminariales have been observed to display considerable morphological differentiation, even in the absence of concomitant levels of genetic

differentiation (Bhattacharya and Druehl 1989, Bhattacharya et al 1991, Fain 1986, Fain et al 1988, Saunders 1991, Druehl and Saunders 1992). Druehl and Saunders (1992) have noted that the brown algal taxonomic hierarchy is inconsistent with genetic diversity as observed within other plant divisions .

Such a discrepancy between morphological and genetic differentiation could be the result of the recent radiation of the kelp, underestimations of the relatedness of taxa, or a combination of both.

The kelp are thought to be a relatively young group, having diverged from other brown algal orders approximately 8.5-40 million years ago (Estes and Steinberg 1988, Druehl and Saunders 1992). With such broad morphological differentiation arising from a relatively recent a common ancestor, it is not surprising that the level of genetic divergence between observed taxa is low.

Looks may have been deceiving; being conspicuous may have encouraged the exaggeration of taxonomic divisions, partly because morphological character differences are more easily measured and seem more important when the organisms are larger.

An over inflation of taxa within the kelp seems to have been the result of the recent radiation of the group and the conspicuous morphological diversity found within it (Druehl and Saunders 1992).

The taxonomy of the genus *Macrocystis* seems to fit this trend. In *M. integrifolia* in Barkley Sound, considerable morphological diversity is not reflected in the level of genetic differentiation as assessed with ITS1 sequence, apparently illustrating instead the phenotypic plasticity of morphological characters in response to a variable environment (Druehl and Kemp 1981). Holdfast morphology has been discovered to be independent of environmental inducement (Neushul 1971, Lewis et al 1986), and has seemed an appropriate taxonomic tool. The current study, however, finds genetic variation more related to geographic distribution than to holdfast morphology. Based on ITS1 sequence similarity, *M. integrifolia* and *M. pyrifera* appear to have shared a more recent common ancestor than have populations of *M. pyrifera* that are separated by the equator. Similarly, Fain (1986) concluded from chloroplast DNA variation that *M. integrifolia* and *M. pyrifera* from the Northern Hemisphere have shared a more recent common ancestor than have populations of *M. integrifolia* separated by the equator. Thus it appears that the distinct holdfast types have arisen more than once and a more critical revisitation of the holdfast as a taxonomic character is warranted.

Review of the ontogenetic development of *Macrocystis integrifolia* (Scagel 1947) illustrates how all three "distinct" holdfast types can be seen in the development of the flattened, creeping holdfast type characteristic of *M. integrifolia*

(Womersly 1954). Continuous clinal variation in holdfast morphology is seen on a geographic scale along the coast of central California (Brostoff 1988), where holdfasts vary from conical, upright and terete (*M. pyrifera*, Womersly 1954), to ovate and slightly flattened (*M. angustifolia*, Womersly 1954). Plant vigour or health also seems able to influence holdfast morphology (Dawson 1952).

Holdfast morphology, although not displaying as much phenotypic plasticity as some other morphological characters, appears to be sufficiently malleable to be misleading taxonomically. The holdfast types may constitute an ontogenetic progression that is subject to selection and differentiation even in the absence of significant genetic divergence.

I recommended that the holdfast morphology no longer be utilized in the delimitation of species within *Macrocystis*, for relationships inferred by its use do not reflect the phylogenetic relationships inferred from patterns of genetic variation. Without holdfast morphology, there is no reliable taxonomic character recognized to delimit species within *Macrocystis*.

The level of genetic similarity found within broadly distributed populations of *Macrocystis* is higher than found in other algal species examined to date. Considering the genetic similarity, in addition to the interspecific interfertility displayed within the genus and the lack of reliable

morphological characters, it does not seem unreasonable to conclude that the taxonomy of *Macrocystis* is currently overinflated.

I speculate that the lack of correlation between morphological variation, as reflected in the taxonomy, and genetic variation, as reported here and previously (Fain 1986) could be resolved by combining all currently recognized species into one. The low level of ITS1 sequence variation among the widely separated populations does not encourage taxonomic segregation.

Further work:

1) Inclusion of samples from all major geographically distinct populations of *Macrocystis* (Australia, New Zealand, South Africa, South America and the Falkland Islands) in the analysis would allow a thorough examination of variation within the genus.

2) Reassessment of morphological data with the purpose of differentiating populations that are currently conspecific, yet separated by the equator.

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