RIBOSOMAL DNA ASSESSMENT OF GENETIC RELATIONSHIPS AMONG *ALARIA* GREVILLE (PHAEOPHYTA) STANDS FROM VANCOUVER ISLAND AND VICINITY.

by

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Ribosomal DNA Assessment of Genetic Relationships among *Alaria* Greville (Phaeophyta) Stands from Vancouver Island and Vicinity

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August 11 1989
(date)

ABSTRACT

Alaria taxonomy is based on quantitative morphological variation. Usually stands of A. marginata Postels et Ruprecht, A. tenuifolia Setchell and A. nana Schrader, are morphologically distinct within their respective ranges around Vancouver Island. However, in some locations taxonomy can be confusing, possibly as a result of environmental influences on morphology. Some stands contain individuals which exhibit intermediate A. marginata crossed with A. tenuifolia, and A. marginata crossed with A. nana hybrid-like morphologies, which suggests inter-breeding or phenotypic plasticity. Molecular biological methods were used to investigate genetic relationships between Alaria stands. Individuals from various stands were collected, identified using a standard morphological key, and examined for differences in their ribosomal (rDNA) repeat units using a ribosomal RNA gene (small subunit) as a probe. Restriction fragment length polymorphism (RFLP) analysis revealed that Alaria rDNA repeat units have differentiated into five major variants. Differences between repeat units were found to be restricted to the intergenic spacer regions and consisted of two length polymorphisms and three restriction site polymorphisms. Distribution of these variants among individuals indicated that genetic

differentiation into three distinct breeding groups had occurred. Most individuals contained variants characteristic of their traditionally defined species. However, breeding group membership did not always correspond with traditional species identification based on morphology. In addition, individuals with more than one variant indicated that hybridization between breeding groups had occurred. The evidence suggested that phenotypic plasticity had a greater influence on morphology than breeding group membership or hybridization, and had created the areas of apparent phenotypic intergression observed between these species.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Kelp, a large and diverse group of brown algae, are important, common members of subtidal and intertidal temperate ocean ecosystems. Impressive sizes, growth rates and interesting life histories within this group, have inspired numerous studies on many aspects of their biology. Although, little is known about the inconspicuous, filamentous gametophyte stage of kelp, considerable interest has centered on the extreme range of sporophyte morphology of these heteromorphic plants.

Intra-specific phenotypic variation in kelp has been studied using various methods, such as morphometrics and numerical taxonomy, quantitative genetics and transplant experiments. Some studies suggest that kelp are phenotypically plastic and vary in morphology as a response to environmental variation (Sundene 1964, Svendsen and Kain 1971, Druehl and Kemp 1982). This implies that environmental factors impinge on genetically controlled developmental processes early in sporophyte development, thereby altering the adult plant morphology (Russel and Fielding 1981). Physiological and morphological plasticity, when defined in this sense, therefore excludes any phenotypic variation that has purely genetic origins (Bradshaw 1965).

In many kelp genera it is unclear to what extent environment influences morphology relative to genetic

influence. It has been demonstrated that many seaweeds can be greatly modified or influenced by their environments. Kelp sporophytes especially exhibit intraspecific variaton in response to a variety of stimuli. Seasonality can effect the thickness and texture as well as the relative growth and decay rates of some kelp (Kain 1976, Norton and Burrows 1969). Tidal height determines both degree of desiccation and wave influence and may cause some kelp species to display deep and shallow water forms (Chapman 1961, Edelstein et al 1969, Norton et al 1981). Although uncommon in most areas, unattached seaweeds can develop distinct floating forms (Norton and Milburn 1972). Changes in light intensity (Burrows 1964, Widdowson 1965), chemical factors (Chapman and Craigie 1977, Druehl 1984) and physical damage (Norton 1971) all have been reputed to influence kelp morphology. It is difficult to determine how much of this induced morphological variation as observed in the field is due to phenotypic plasticity, opposed to longer term genetic adaptation.

The genus Alaria Greville provides an excellent example of varying morphology in closely related species. Alaria is a mid-sized intertidal kelp found along both the Atlantic and Pacific north temperate shores. Moderately complex in morphology, members of this genus have a branched hapteral holdfast connected to a thin blade by a flexible trunk (Fig. 1). The trunk is divided into a lower stipe and an upper rachis on which reproductive lateral

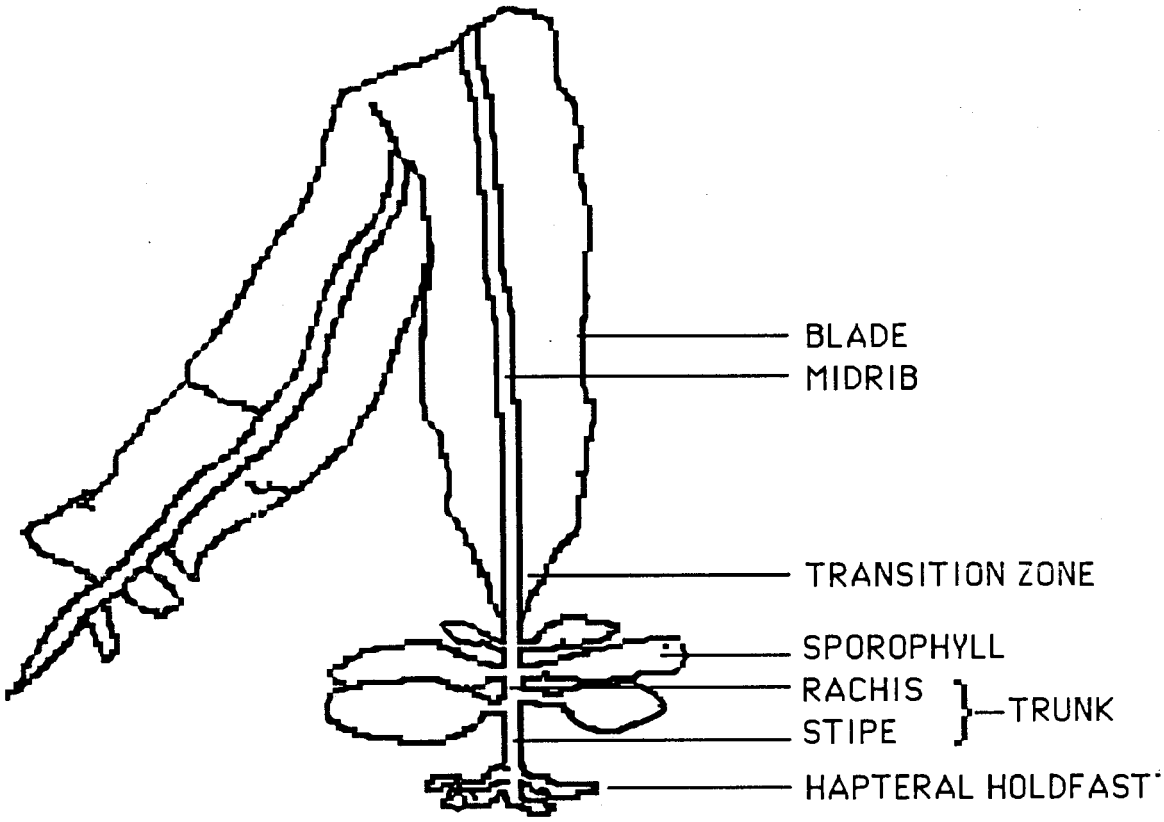


Figure 1. Diagram of a typical Alaria showing general morphological features common to local species.

sporophylls are borne. The transition zone immediately above the rachis, marks the origin of the midribbed blade and meristematic activity. In this genus, these common morphological features exhibit quantitative variation, forming the basis of taxonomic delineation for most species. However, morphological variation between stands may be large enough to cause confusion when classifying particular groups of plants. Widdowson (1971a, 1971b) used morphometric analyses to determine which features of Alaria were more useful in discriminating species and then used these features to construct a dichotomous key to the genus. With these numerical taxonomic techniques, many Alaria populations could be unambiguously identified to a particular species.

Some stands of plants, however, displayed morphologies that Widdowson (1964) found suggestive of hybridization between species, and he proposed that continuums of hybrid populations contributed to the morphological variation in those stands. Interbreeding may be common in other kelp species, and can be accomplished between different species in the laboratory (Cosson and Oliveri 1982, Bolton et al 1983). Chances of interbreeding are further increased for Alaria due to the similar organization and number of chromosomes in northeast Pacific Alaria species (Robinson and Cole 1971).

Stands of such proposed natural hybridization occur around Vancouver Island in limited areas (Widdowson

1971a). Generally A. tenuifolia Setchell and A. marginata Postels et Ruprecht are easily recognizable throughout most of their respective ranges around Vancouver Island. However, near the southern tip of Vancouver Island, Alaria stands are less morphologically distinct and the two species tend to blend together in appearance, suggesting various degrees of hybridization. A similar relationship appears to exist between A. marginata and A. nana Schrader on the west coast of Vancouver Island. Here the two species are more easily separated along their northern ranges, but become more similar in appearance towards the south, again suggesting hybridization.

The genetic relationships of these three common species, comprises the main focus of this research. Are these functional species which are able to interbreed in certain areas, or are the hybrid-like morphologies examples of the normal phenotypic plasticity of the genus, when found growing in many different environments?

The purpose of this study was to examine the underlying genetic relationships between local stands of Alaria, and determine limits of population interbreeding, thereby defining breeding groups. Comparing home environment and morphology of individual plants with their genetic affinity was thereby used to separate genetic and environmental influences on their morphology.

Quantitative genetic techniques have been used for this purpose in other kelp species (Chapman 1974), although they

are usually applied in agriculture where pure, inbred lines are used (Russel and Fielding 1971). These methods were designed to determine heritability of traits regardless of environmental influence through crossing experiments. By comparing trait measurements of individuals of known genetic relatedness, Chapman (1974) discovered that differences in stipes between two Laminaria species were due mainly to additive genetic effects, and only slightly to environmental influence. However, data obtained from such techniques may be biased due to complex life history traits (Mitchell-Olds and Rutledge 1986) and limited when applied to wild populations of unknown genetic variabilty (Russel and Fielding 1981). In the latter situation, individuals of each different genotype should be compared. Consequently, the large number of crosses required, may make this method very difficult to apply to natural kelp populations.

Many researchers have attempted to relate environment, especially water movement, to phenotype by performing reciprocal transplant experiments between sheltered and wave exposed areas. Such experiments have yielded much information on the relative phenotypic plasticity of various species of seaweed (Sundene 1958, Norton 1969, Svendson and Kain 1971, Gerard and Mann 1979, Druehl and Kemp 1982, Gerard 1987). However, transplant experiments have serious limitations which must be considered before meaningful conclusions may be drawn (Norton 1981).

To surmount some of these problems, alternate methods involving biochemical techniques have been used, providing some useful information on genetic divergence between groups of organisms. Enzyme electrophoresis (Shaw et al 1987) and amino acid sequence analysis (Wyss et al 1987) have limited use in uncovering evidence of genetic differentiation and species subdivision (Nei 1987). Since these features are ultimately dependent on the genome, an alternate approach would involve direct analysis of the DNA.

Recent advances in molecular biology have made this possible providing more informative techniques to allow researchers to readily identify genetic variation in organisms. An individual's genome is organized into two separate forms, organelle and nuclear DNA. Occurring as simple easily studied molecules, organelle DNA has been used extensively. For example, in seaweeds, differences in chloroplast DNA have been used to delineate red algal species and populations (Goff and Coleman 1988) and have been used to construct a kelp phylogeny (Fain et al 1988). Such studies provide much information on the evolution of organelle DNA molecules, but may not provide accurate phylogenetic information. Unlike the nuclear genome, plastid DNA is maternally inherited. Consequently plastid derived phylogenies may not be representative of the organismal phylogeny.

In contrast, nuclear DNA provides a better record of

phylogeny for an organism. Comparisons of nuclear DNA have been used for widely different purposes, such as characterizing closely related plant species (Bhave et al 1986, Hallden et al 1987) and 'finger-printing' individual birds (Wetton et al 1987).

One way of assessing DNA similarity between organisms, is by DNA-DNA hybridization (Olsen et al). This can provide some useful information about the amount of divergence between species. Another common way of examining DNA differences between genomes is by digesting the DNA with enzymes of known recognition sequences. The resulting DNA fragments may be size fractionated by gel-electrophoresis (Southern 1975). Banding patterns produced with this method are used to uncover restriction fragment length polymorphisms (RFLPs) and may be characteritic of species, populations or individuals, depending on the enzyme used and the level of relatedness sought.

DNA RFLPs are not necessarily directly related to gene function in an organism, and therefore may not be subject to selection. However, changes occurring within restriction site recognition sequences on DNA do provide easily detectable measures of genetic divergence between populations of organisms. When the entire nuclear genome of a kelp is digested with a restriction enzyme, the number of fragments produced is too high for examining all restriction site polymorphisms revealed. Visualizing one

small region of DNA and then comparing it with homologous regions in other individuals can be accomplished usingknown or random probes.

Random sequences of DNA can uncover useful species markers and have been used to distinguish between closely related species such as African and European honey bees (Hall 1988), and have useful applications in such fields as fisheries science, where identification of breeding groups is required (Hallerman and Beckman 1988).

Repeated DNA sequences have the added advantage of being physically easier to manipulate in the laboratory, and have been used to characterize species of the angiosperm families Brassicaceae (Hallden et al 1987) and Cucurbitaceae (Bhave et al 1986). The high copy numbers within individuals are thought to be kept homogenized (concerted evolution) by the various mechanisms of molecular drive (Dover 1986, Gerbi 1986). This includes such processes as gene conversion, which results in one copy of a repeated sequence being used to convert the remaining copies within the genome to identical sequences. As well, unequal crossing over during meiosis could result in the high copy numbers observed.

The nature of tandemly repeated gene families makes them highly suitable for uncovering sequence divergence. The gene family coding for the small and large subunit ribosomal RNA, has the further advantage of being well studied and characterized in many organisms ranging from

mice (Cory and Adams 1977) and frogs (Gourse and Gerbi 1980) to dozens of plant species (Appels and Dvorak 1982, Learn and Schaal 1987, Rogers and Bendich 1987).

As well the nature of rDNA evolution results in a highly conserved, functionally constrained, rRNA gene coding region with small internal spacers. This coding region alternates with a less conserved intergenic spacer region (IGS) (Fig. 2). The IGS has little sequence constraint and therefore has a much faster rate of evolution than the adjacent coding regions (Federoff 1979). The combined coding and spacer regions are collectively termed the rDNA repeat unit. Repeat units are often similar within an individual's genome and within breeding groups, but may diverge between breeding groups.

A common difference in rDNA between individuals is repeat unit copy number, but this may be too highly variable in some organisms to be of any use in distinguishing populations or species (Rogers and Bendich 1987). Cullis (1979) found that the percentage of rDNA in genomes of flax genotrophs varied greatly and unpredictably between individuals, and even between parents and offspring.

Other changes may also occur within the sequences of repeat units resulting in rDNA polymorphisms within or between individual organisms. Most of these mutations occur within spacer regions leaving the coding regions conserved (Gerbi 1986). For example, length differences,

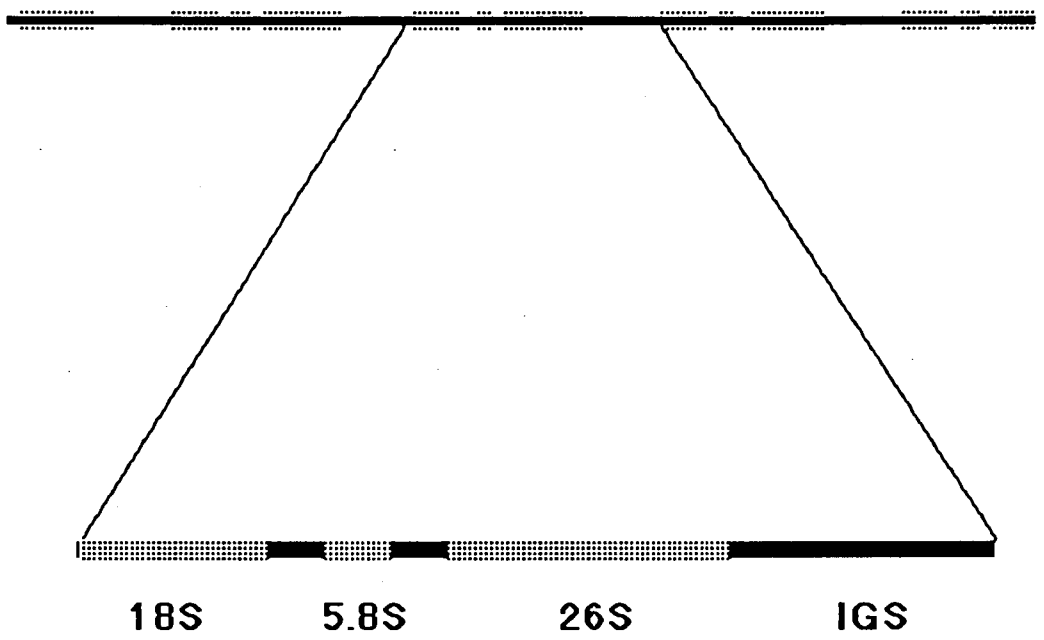


Figure 2. Schematic representation of general eukaryotic ribosomal DNA organization showing tandem repeat nature. Horizontal lines represent DNA strands, with coding regions and intergenic spacer identified as stippled and solid lines respectively. A single repeat unit is expanded for closer examination.

common in repeat units, may be due to insertions or deletions of IGS subrepeats as in maize (McMullen et al 1986), wheat (Barker et al 1988) and broadbeans (Yakura et al 1984).

Alternatively, restriction site recognition sequences may be altered by point mutations or base methylation, the latter of which may be very common in higher plants (Siegel and Kolacz 1983, Ganal and Hemlebon 1986).

Both site and length mutations are heritable in a predictable Mendelian fashion (Reeder et al 1976, Polans et al 1986, Russel et al 1988). They may range in variability from invariant within a species (Varsanyi-Brenner et al 1979) to highly varible within single populations (Black et al 1989). In between this range, some species exhibit more moderate amounts of repeat length variation (Appels et al 1980, Oono and Sugura 1980) which may be useful in providing a measure of genetic relatedness between organisms. Such moderate rDNA length and site variation allowed Bhattacharya and Druehl (1988) to uncover several breeding groups of the kelp Costaria costata (Turn.) Saunders. In this study the authors discovered that certain morphologically distinct populations of C. costata were genetically homogeneous and consequently interbreeding. Since these populations grew in different environments, the authors therefore concluded that environmental influences played a larger role in phenotypic differentiation than did genetic variation of morphological

traits alone.

In the Costaria study, the rDNA system was used to distinguish between populations of plants recognized as belonging to a single species. In the present study, the analogous rDNA system of three recognized Alaria species is characterized in a similar manner, using the 18S rDNA gene, cloned from C. costata (Bhattacharya 1988) as ahybridization probe (see Materials and Methods for description). Comparing rDNA between Alaria plants defines breeding groups, which can be examined for evidence of hybridization. Comparisons of morphology, species identification, environment and breeding group membership can then be made among stands. This aids in the separation of purely genetic influences such as genetic differentiation and hybridization, from environmental influences on morphology, and gauges the extent of phenotypic plasticity in these species.

Plant Collection and Identification

Alaria plants were collected from 20 sites along the circumference of Vancouver Island and Vancouver, Canada, and in Puget Sound, Washington, USA (Fig. 3, Table 1.). Two outlying sites, in Oregon and Alaska, USA, were also included. Collections were made from the lower intertidal zone during low tides between October 1986 and August 1988. Usually only one species was found at any one collection site; however, *A. nana* and *A. marginata* were collected together at two sites, Frank Island and Botany Beach, from different levels of the intertidal zone. Attempts were made to select individuals randomly, however this was not always possible for some sites. Generally, four or five large (>100 g, wet weight) plants were collected from separate clumps located more than 1 m apart. In sites with small populations this separation was smaller, while in others, collections were further spaced.

Initial identification of individual plants was based on known species range, collection site location and overall species description (Widdowson 1971a). Positive identification was based further on the dichotomous key developed by Widdowson (1971a). Possible confusion with other species due to subjective wording of the key was not considered a problem when the ranges of these other species were considered.

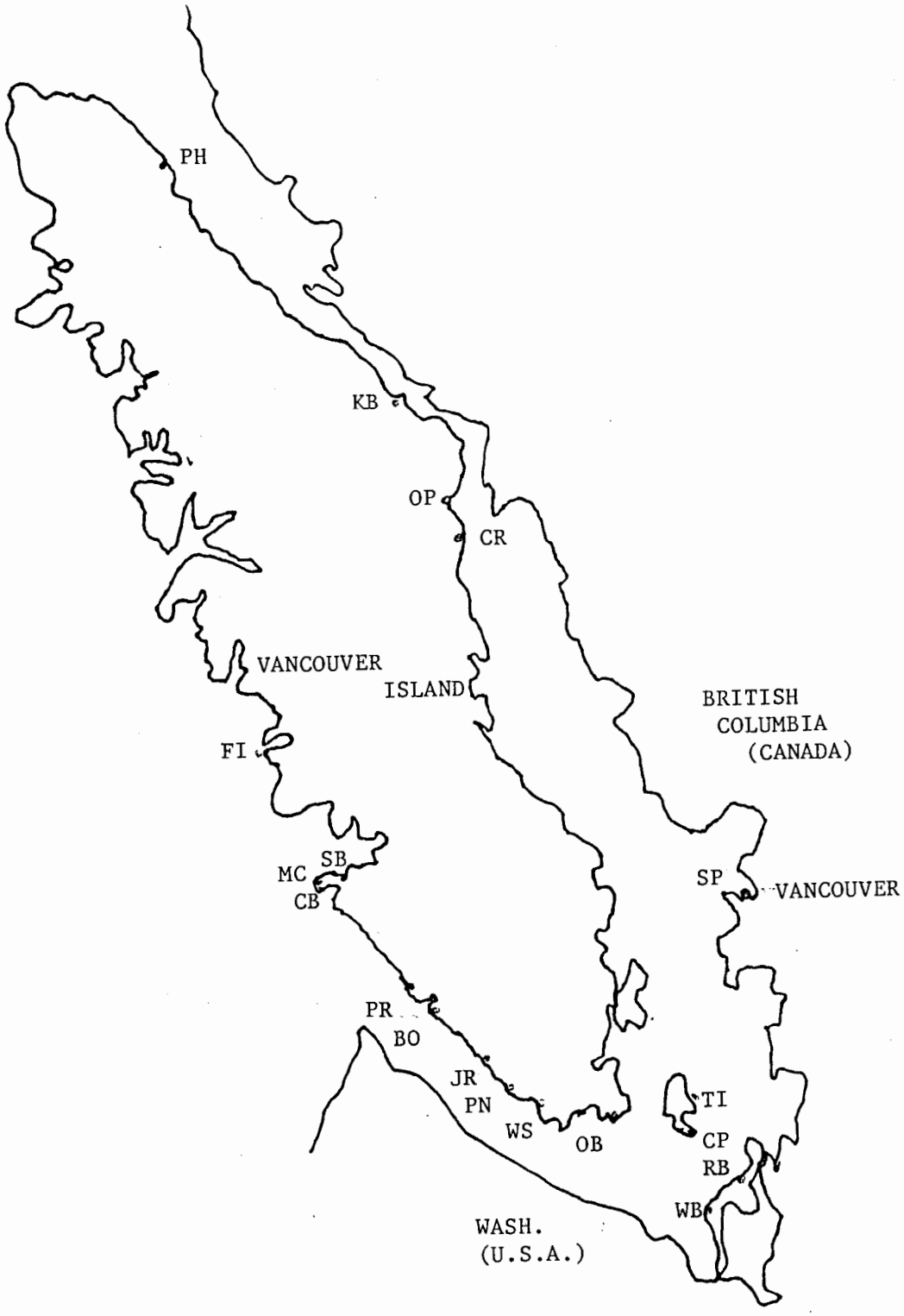


Figure 3. Map showing Alaria collection sites from Vancouver Island and Puget sound (Outlying sites not shown). See Table 1 for location names.

COLLECTION SITE	ABBREVIATION	APPROXIMATE POSITION	
VANCOUVER ISLAND			
FRANK ISLAND	FI	49° 08'N	125° 54'W
CAPE BEALE	CB	48° 47'N	125° 13'W
SCOTTS BAY	SB	48° 50'N	125° 08'W
BOTANY BEACH	BO	48° 32'N	124° 27'W
PORT RENFREW	PR	48° 32'N	124° 27'W
JORDAN RIVER	JR	48° 25'N	124° 03'W
POINT-NO-POINT	PN	48° 24'N	123° 59'W
WHIFFEN-SPIT	WS	48° 22'N	123° 47'W
OGDEN BREAKWATER	OB	48° 25'N	123° 20'W
ORANGE POINT	OP	50° 04'N	125° 17'W
CAMPBELL RIVER	CR	50° 05'N	125° 19'W
KELSEY BAY	KB	50° 24'N	125° 58'W
PORT HARDY	PH	50° 44'N	127° 30'W
VANCOUVER, MAINLAND B.C.			
STANLEY PARK	SP	49° 18'N	123° 08'W
PUGET SOUND			
WEST BEACH	WB	48° 20'N	122° 40'W
ROSARIO BEACH	RB	48° 25'N	122° 40'W
CATTLE POINT	CP	48° 26'N	122° 56'W
TURN ISLAND	TI	48° 33'N	123° 01'W
ALASKA			
SITKA	SK	57° 05'N	135° 15'W
OREGON			
SEAL ROCK	SR	44° 30'N	124° 05'W

Table 1. List of Alaria collection sites, with abbreviations and approximate positions.

The following criteria were used as a guide in local species identification. Locally, A. nana is restricted to highly wave exposed areas of the west coast of Vancouver Island and is very small with narrow sporophylls with thin petioles and short stout terete stipes. Sporophylls of similar thickness as the blade, are crowded on the rachis near the transition zone, becoming less so towards the stipe. Both rachis and midrib taper distally.

A. marginata is found locally in moderately wave exposed areas in the west coast of Vancouver Island and Puget Sound. Robust large plants have short thin stipes and long sporophylls which are considerably wider than those of A. nana. The stipe is clearly distinct from the rachis, the latter being flattened and lighter in colour. Sporophylls are often thicker than the blade, and have asymmetrical bases.

A. tenuifolia is restricted to wave sheltered regions, often with high tidal currents, from Puget Sound extending along the east coast of Vancouver Island up to Queen Charlotte Strait. It has a very long flexible trunk (greater than 15 - 20 cm) and large, wide, often irregularly shaped, sporophylls which can be irregularly spaced on the rachis.

Collection site locations were purposely chosen which had previously supported Alaria stands of unusual morphology, and had consequently been reported as possible hybrids (Widdowson 1964).

Morphometric data was not recorded, but individual plants were photographed or preserved in a herbarium for comparative purposes.

DNA Extraction and Analysis

Once harvested, entire plants were wrapped in moist paper and/or plastic, and stored on ice in insulated chests until removed to the laboratory. They were then transferred to refrigerated (8 - 10°C) seawater tanks until they could be photographed and then frozen in preparation for DNA extraction as in Fain et al (1988). Prior to freezing, individual blades of approximately 100 g wet weight were vigorously wiped clean with paper towel or cheesecloth and soaked in distilled water for two minutes to assist in removal of epiphytes. Blades were then blotted dry and cut into small pieces which were quick-frozen in liquid nitrogen, and then ground in a chilled mortar or Waring blender. Alternatively some plants were air dried for several hours in sunlight and ground at room temperature. Subsequent DNA extraction procedures were identical for both frozen and dried plants.

At least two plants from each collection site were further processed. Nuclear DNA was prepared by extracting organelles from the powdered tissue. Warmed powder (-10°C) was suspended in extraction buffer A (Table 2) and filtered through cheesecloth and miracloth (CalBiochem). The expressed solution was centrifuged in

SOLUTION	COMPOSITION
BUFFER A (pH 6.1)	1.65 M SORBITOL, 50 mM 2-[N-MORPHOLINO]-ETHANESULFONIC ACID (MES), 10 mM DISODIUM EHTHYLENEDIAMINETETRAACETIC ACID (EDTA), 2.0% POLYVINYLPYRROLIDONE-10 (PVP-10), 0.1% BOVINE SERUM ALBUMIN (BSA), 5 mM BETA MERCAPTOETHANOL (BME)
BUFFER B (pH 6.1)	1.65 M SORBITOL, 50 mM MES, 10 mM EDTA, 0.1% BSA, 5 mM BME
BUFFER C (pH 7.4)	1.65 M SORBITOL, 50 mM Tris(HYDROXY-METHYL)AMINOMETHAN (TRIS), 20 mM EDTA
BUFFER D (pH 8.0)	50 mM TRIS, 20 mM EDTA, 5% LAURYL SULFATE (SDS)
TRIS BUFFER (TE, pH 8.0)	10 mM TRIS-HCL, 1 mM EDTA

Table 2. List of DNA extraction and purification buffers and their chemical composition.

250 mL Sorval GSA bottles, at 5000 rpm (4000 G, in GSA rotor) for 10-20 minutes. The resulting pellet was resuspended in buffer B (Table 2) and recentrifuged as before. The pellet was again resuspended in buffer B and centrifuged in 50 mL Nalgene tubes at 5000 rpm (3000 G, in SS34 rotor) for 5 - 10 minutes. A similar wash was done with buffer C (Table 2) and the final pellet, consisting of chloroplasts, nuclei and mitochondria, was suspended in buffer D (Table 2) which lysed the organelles. When lysis was complete, CsCl was added to the solution to produce a final density of 0.939 g CsCl/mL. Flocculent debris was removed from the mixture by filtering through four layers of cheesecloth. The cleared solution was then loaded into 33 mL heat sealable ultracentrifuge tubes (Beckman polyallomer) with 400 ug bisbenzimide (Hoescht 33258, Sigma), and centrifuged at 45 000 rpm for 24 to 48 hours in a VTi50 rotor at 25°C. Resulting bands of nuclear DNA were visualized under UV light (375 nm) and withdrawn using 10 cc sterile syringes (Becton-Dickenson, 10cc21g1). Minor chloroplast and mitochondrial DNA bands were discarded. The genomic DNA was further cleaned by either a second CsCl spin, similar to the first except using ethidium bromide as a DNA specific stain (density 0.758 gCsCl/mL), or by passing through Sepharose (Sigma) spin columns (G. Kalmar and K. Eastwell, personal communication). For the latter process the DNA was first cleaned of dye by rinsing with NaCl saturated 2-propanol, precipitated in four volumes

of 95% ethanol, and then dissolved in Tris buffer (TE, Table 2). Nucleic acid concentration was determined by measuring absorbance at 260 nm and aliquots containing 40 ug DNA were diluted to 200 uL of TE. These samples were then loaded into spin columns which consisted of 1 mL syringes (BD Tuberculin, 1cc26g3/8) packed with Sepharose (Sigma) and plugged with porous polyurethane (Bolab Inc.). Dissolved DNA passed through these columns when centrifuged at 2000 rpm for 2 minutes, and rinsed with 50 mL aliquots of TE. Collected samples were precipitated in 95% ethanol and again dissolved in 40 uL TE. Either second CsCl spins or cleaning through spin columns resulted in clean restrictable nucleic acid for most plants. Yields varied from approximately 200 to 800 ug DNA per 100 g fresh tissue. DNA from Individual plants was extracted when possible, however, in some cases, especially for A. nana, tissue from many small plants were pooled to give 100 g total fresh tissue weights. Samples obtained from more than one plant are indicated in tables three and four. Restriction of purified DNA was conducted to the specifications of enzyme manufacturers (Pharmacia) and Maniatis et al (1982). Generally 20-30 units of enzyme were required to digest 3-5 ug of DNA to completion at 37°C for 3 - 6 hours. Aliquots of each DNA sample were digested with Pst I, Dra I and Bgl I, size fractioned on agarose gels, fixed onto nitrocellulose filters and probed with ^{32}P radiolabelled (nick translated, Maniatis et al

1982) complete plasmid pCc18 (plasmid plus insert). Plasmid pCc18 was kindly supplied by D. Bhattacharya and had been cloned in a shotgun genomic library of Costaria costata DNA using the restriction enzyme Eco RI and the plasmid vector pUC19. It contains a 1.8 kb (kilobase pairs) insert of small subunit (18S) rDNA and 200 bp (base pairs) of upstream spacer sequence (see Bhattacharya et al, 1988 for details).

Hybridizations were conducted between 58°C and 65°C in moderately stringent hybridization solutions (5X SSPE, .2% SDS, 5X BFP: Maniatis et al 1982). Stringency levels were chosen which resulted in strongly visible bands with little or no background and unspecific or weak binding. Minor or low copy number bands would consequently not yield strong images in subsequent autoradiographs. Resulting hybridized filters were washed in hybridization solution diluted with an equal volume of water, dried and used to expose X-ray film (Kodak Omat-K) overnight. Selected individuals of the three Alaria species were further digested with a number of other enzymes, to further restriction map their rDNA repeat units.

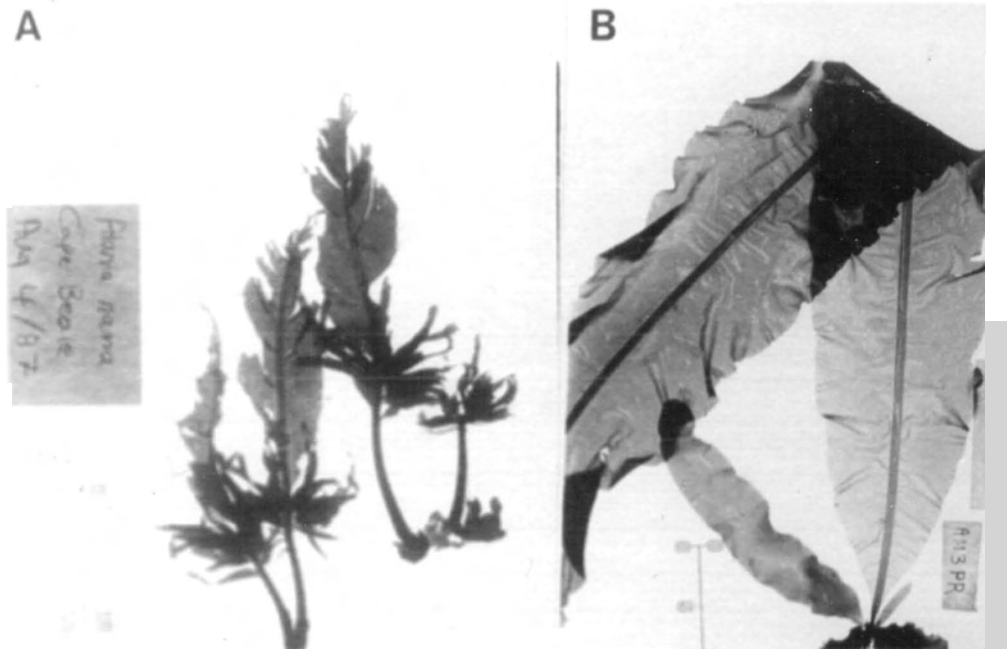
Plant Descriptions

After collection, photographs were taken of most of the plants analysed in this study. Where possible, portions of these plants, or similar ones from the same stand, were preserved by drying on herbarium paper. Identification of individual plants from most stands was unambiguous when Widdowson's (1971a) descriptions and key were followed. Members of each species were chosen as typical species representatives (Fig. 4, a,b and d), however, some plants were difficult to identify due to their similarity with other species (Fig. 5a, 5c, 6a, and 6b,).

On Botany Beach stands of A. nana were found high in the intertidal region on wave exposed points while stands of A. marginata (Fig. 4c) occupied more sheltered, lower shore positions. In areas of intermediate intertidal height and wave exposure, grew stands of plants which appeared intermediate to both species (Fig. 5c). Such intermediate plants had larger blades and wider sporophylls than other A. nana plants found at this site (similar to Fig. 4a), but were not as large as the A. marginata plants found there. These anomalous plants were identified (Widdowson 1971a) as 'marginata-like' A. nana.

Plants collected at Cattle Point, San Juan Island, were identified as A. marginata although reminiscent of A. tenuifolia (Fig. 5a). They had long stipes similar to the

Figure 4. Photographs of some Alaria individuals used in this study. See Tables 4 and 5 for banding pattern data, and Table 1 for collection site locations.
A. A. nana Schrader (AN00CB),
B. A. marginata Postels et Ruprecht (AM03PR),
C. A. marginata (AM04BO),
D. A. tenuifolia Setchell (AT02TI).


A
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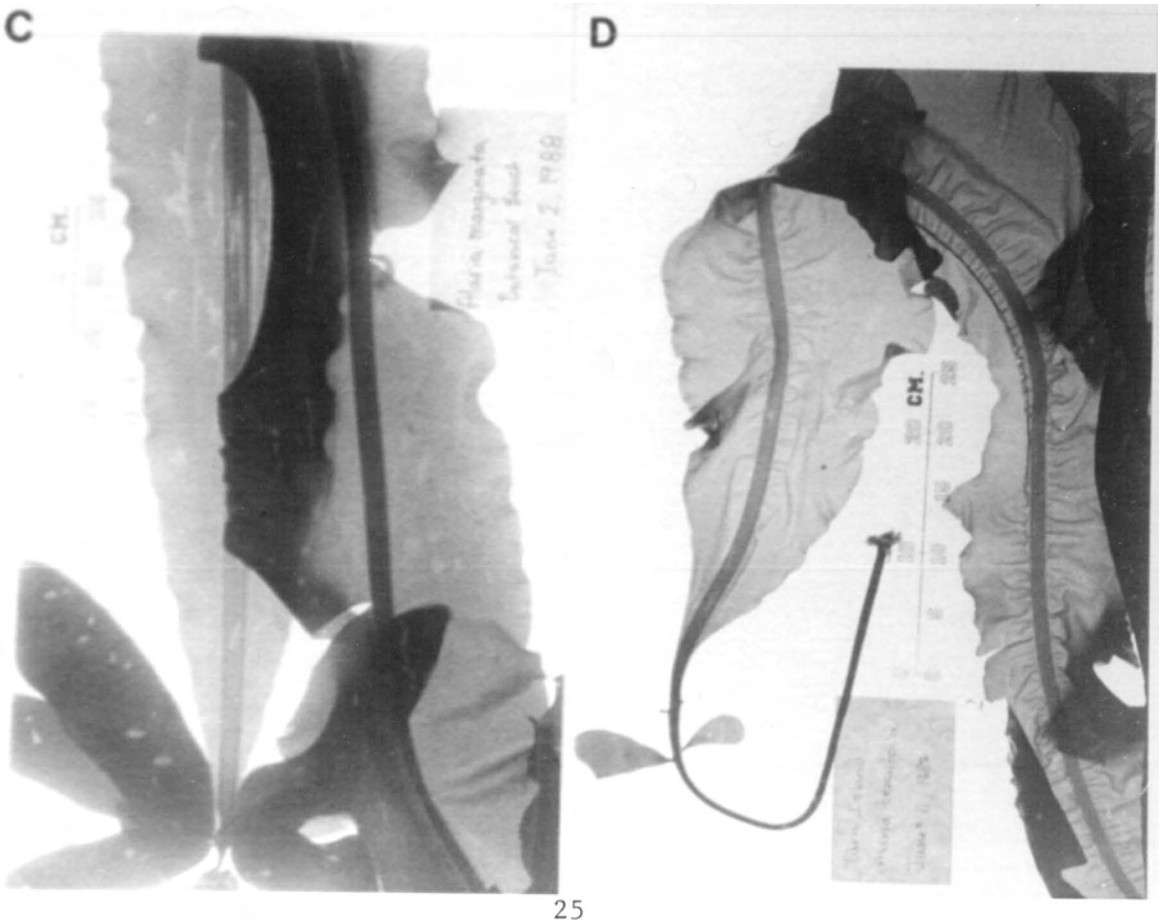
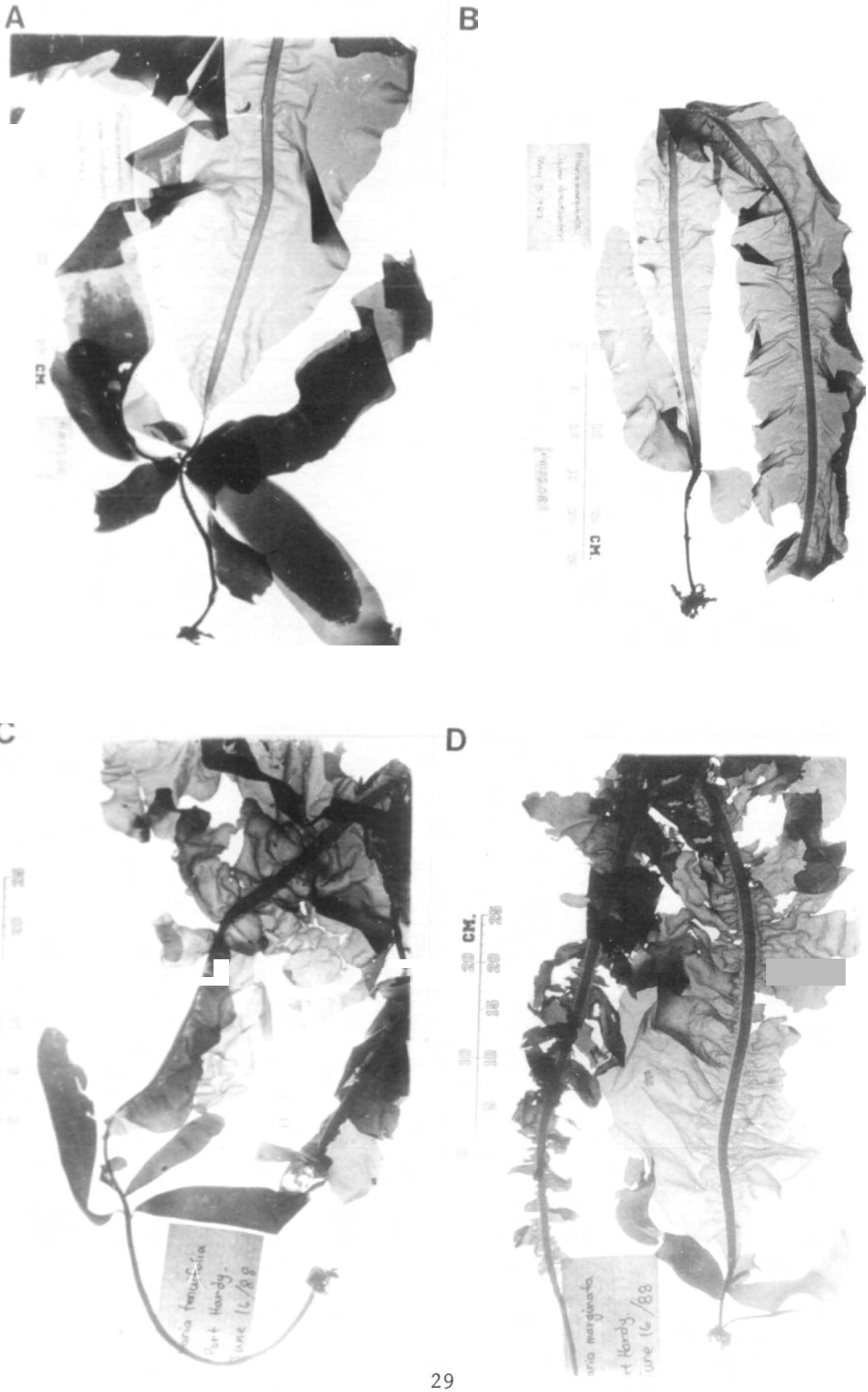

C
D

Figure 5. Photographs of some Alaria individuals used in this study. See Tables 4 and 5 for banding pattern data, and Table 1 for collection site locations.
A. A. marginata Postels et Ruprecht (AM02CP),
B. A. tenuifolia Setchell (AT03TI),
C. A. nana Schrader (AN10BO),
D. A. marginata (AM00SK).

A
B
C
D
Alaria nana
Botanical Beach
June 2 1988
CM
2s

Figure 6. Photographs of some Alaria individuals used in this study. See Tables 4 and 5 for banding pattern data, and Table 1 for collection site locations.
A. A. tenuifolia Setchell (AT03OB),
B. A. tenuifolia (AT02OB),
C. A. tenuifolia (AT01PH),
D. A. marginata (AM03PH).


A
B
C
D

latter, and sporophylls similar to the former species.

Other A. tenuifolia (Fig. 6a,b) plants from Ogden Breakwater (Strait of Juan de Fuca) and Kelsey Bay (Johnstone Strait), also had shorter stipes than typically found for this species, but were not shorter than allowed by Widdowson's (1971a) description.

Restriction Fragment Analysis

To begin the genetic analysis, purified DNA from a few individuals from different stands, was digested with a variety of enzymes, in search of a genetic marker that could be used to distinguish populations and species. Three enzymes, Pst I, Bgl II and Dra I, revealed polymorphisms among individuals and were considered suitable for this purpose. These enzymes were subsequently used to analyse each remaining DNA sample. Some of the supporting data in the form of autoradiographs resulting from digests of Alaria DNA samples probed with pCc18 (the 18S rDNA gene) are shown in the Appendix.

The enzyme Pst I recognized at least two restriction sites in the Alaria rDNA repeating unit, yielding two bands for most individuals when probed with the 18S gene. The lower 2.3 kb band was found to be single and constant in size for all individuals examined, but the upper band varied in size and was doubled in some individuals (Tables 3 and 4; Appendix Figs. 1, 3b, 6a, 6b, 7a and 7b). This suggested that either Pst I restriction site polymorphisms

PLANT NUMBER	FRAGMENT SIZES FROM DIGESTS WITH: Pst I		Dra I		Bgl II	VARIANT
AM01FI	8.0	2.3	7.4	3.2	10.4	A
AM02FI*	8.0/6.8f	2.3	7.4	3.2	10.4	A/B
AM01CB	8.0/6.8f	2.3	7.4	3.2	10.4	A/B
AM01SB	8.0	2.3	7.4	3.2	10.4	A
AM01PR	8.0/5.7	2.3	7.4/7.1	3.2	10.4	A/A'
AM02PR	8.0	2.3	7.4	3.2	10.4	A
AM03PR	8.0	2.3	7.4	3.2	10.4	A
AM04PR	8.0/7.7	2.3	8.3/7.4	3.2	11.3/10.4	A/D
AM02BO	8.0	2.3	7.4	3.2	10.4	A
AM03BO	8.0	2.3	7.4	3.2	10.4	A
AM04BO	8.0	2.3	7.4	3.2	10.4	A
AM05BO	8.0	2.3	7.4	3.2	10.4	A
AM02JR	8.0	2.3	7.4	3.2	10.4	A
AM03JR	8.0	2.3	7.4	3.2	10.4	A
AM03PN	8.0	2.3	7.4	3.2	10.4	A
AM05PN	8.0	2.3	7.4	3.2	10.4	A
AM01WS	8.0	2.3	7.4	3.2	10.4	A
AM03WS	8.0	2.3	7.4	3.2	10.4	A
AM01WB	8.0	2.3	7.4	3.2	10.4	A
AM04WB	8.0/7.4	2.3	8.3/7.4	3.2	11.3/10.4	A/E
AM02RB	8.0	2.3	7.4	3.2	10.4	A
AM03RB	8.0	2.3	7.4	3.2	10.4	A
AM01CP	8.0/6.8	2.3	7.4	3.2	10.4	A/B
AM02CP	8.0/6.8	2.3	7.4	3.2	10.4	A/B
AM04CP	8.0/6.8	2.3	7.4	3.2	-	A/B
AM00PH*	6.8	2.3	7.4	3.2	10.4	B
AM01PH	6.8	2.3	7.4	3.2	10.4	B
AM02PH	6.8	2.3	7.4	3.2	10.4	B
AM03PH	7.4/6.8	2.3	8.3/7.4	3.2	11.3/10.4	B/E
AM02SK	8.0	2.3	7.4f	3.2/2.9	10.4	A/C
AM06SR	8.0	2.3	7.4	3.2	10.4	A

Table 3. Fragment sizes in kilobase pairs resulting from restriction digests of Alaria genomic DNA with the enzymes Pst I, Dra I and Bgl II and probed with the 18S rDNA gene (pCc18). Initial letters in plant identification code describe species name as follows: AM = A. marginata Postels et Ruprecht. Following numbers indicate individual plant sampled. Final letters are abbreviations of collection sites as in Table 2. '-' indicates absent data. '*' indicates more than one plant used in the sample preparation. 'f' indicates faint bands on autoradiographs. A' is a unique variant found only in this individual.

PLANT NUMBER	FRAGMENT SIZES FROM DIGESTS WITH: Pst I		Dra I		Bgl II		VARIANT
AT01SP	6.8	2.3	7.4	3.2	10.4		B
AT02SP	6.8	2.3	-	-	-		-
AT03SP	6.8	2.3	-	-	-		-
AT04SP	6.8	2.3	-	-	-		-
AT07SP	6.8	2.3	7.4	3.2	10.4		B
AT09SP	6.8	2.3	7.4	3.2	10.4		B
AT01TI	6.8	2.3	7.4	3.2	10.4		B
AT02TI	6.8	2.3	7.4	3.2	10.4		B
AT03TI	8.0/6.8	2.3	7.4	3.2	10.4		A/B
AT04TI	6.8	2.3	7.4	3.2	10.4		B
AT01OB	6.8	2.3	7.4	3.2	10.4		B
AT02OB	6.8	2.3	7.4	3.2	10.4		B
AT03OB	7.4/6.8	2.3	8.3/7.4	3.2	11.3/10.4		B/E
AT01CR	6.8	2.3	7.4	3.2	10.4		B
AT01OP	6.8	2.3	7.4	3.2	10.4		B
AT02OP	7.7/6.8	2.3	8.3/7.4	3.2	11.3/10.4		B/D
AT01KB	7.7/6.8	2.3	8.3/7.4	3.2	11.3/10.4		B/D
AT02KB	7.7/6.8	2.3	8.3/7.4	3.2	11.3/10.4		B/D
AT03KB*	7.7/6.8	2.3	8.3/7.4	3.2	11.3/10.4		B/D
AT00PH*	6.8	2.3	7.4	3.2	10.4		B
AT03PH	6.8	2.3	7.4	3.2	10.4		B
AT02PH	6.8	2.3	7.4	3.2	10.4		B
AN03FI*	6.8	2.3		3.2/2.9	10.4		C
AN02FI*	6.8	2.3		3.2/2.9	10.4		C
AN02MC	6.8	2.3		3.2/2.9	10.4		C
AN00CB*	6.8	2.3		3.2/2.9	10.4		C
AN01CB	6.8	2.3		3.2/2.9	10.4		C
AN00BO*	8.0	2.3	7.4	3.2/2.9f	10.4		A/C
AN01BO	8.0	2.3	7.4	3.2/2.9f	10.4		A/C
AN03BO	8.0	2.3	7.4	3.2	10.4		A
AN10BO	8.0	2.3	7.4	3.2/2.9f	10.4		A/C

Table 4. Fragment sizes in kilobase pairs resulting from restriction digests of Alaria genomic DNA with the enzymes Pst I, Dra I and Bgl II and probed with the 18S rDNA gene (pCc18). Initial letters in plant identification code describe species names as follows: AT = A. tenuifolia Setchell, AN = A. nana Schrader. Following numbers indicate individual plant sampled. Final letters are abbreviations of collection sites as in Table 2. '-' indicates absent data. '*' indicates more than one plant used in the sample preparation. 'f' indicates faint bands on autoradiographs.

or spacer length polymorphisms existed in Alaria rDNA.

The enzyme Bgl II was used to test for spacer length differences since it had only one recognition site in Alaria rDNA, and therefore yielded only one band representing the cistron and spacer size (some data in Appendix, Fig. 3a). All individuals were found to have a rDNA repeat unit size of 10.4 kb, but a number of plants contained an additional 11.3 kb size class. When compared, however, these larger cistron sizes did not correspond with the variation in band size from the Pst I digests. This indicated that both a Pst I polymorphism and a spacer size polymorphism were responsible for the observed banding pattern.

Further digests with a third enzyme, Dra I, revealed another restriction site polymorphism in some plants. This enzyme was found to cut at least twice within the rDNA repeat unit, yielding an invariant 3.2 kb band, and a second variable band (Appendix, Figs. 2a, 2b, 4a, 4b and 5b).

The combined information from banding patterns produced by each enzyme, suggested that at least five variants (Fig. 7) of the rDNA repeat unit existed in the Alaria stands studied, differing by restriction site and spacer size polymorphisms. Variants A, B and C were each 10.4 kb long and were primarily found in A. marginata, A. tenuifolia and A. nana respectively (Tables 3 and 4). Notable exceptions to this general distribution of these variants occurred in

Figure 7. Restriction maps of five variants of the ribosomal DNA repeat units found in the Alaria plants included in this study. Only variant enzymes are included, as follows: P = Pst I and D = Dra I, and are positioned with vertical lines. Horizontal lines represent linear DNA strands with stippled regions corresponding to the 18S gene. One complete repeat unit extends from the begining of one 18S gene, to the beginning of an adjacent one. Repeat unit sizes are 10.4 kilobase pairs for A, B and C, and 11.3 kb for D and E.

VARIANT A

VARIANT B

VARIANT C

VARIANT D

VARIANT E

1 kb

A. marginata plants from Port Hardy which contained variant B (Fig. 6d, Table 4). Other individuals contained copies of two of these variants. Some A. marginata plants from Cattle Point, Frank Island and Botany Beach, and a single A. tenuifolia plant from Turn Island contained both variants A and B. Some A. nana plants from Botany Beach and one A. marginata from Sitka, Alaska contained both variants A and C. The two larger size class variants of 11.3 kb, D and E, were usually found in A. tenuifolia and A. marginata respectively, and always co-occurred with another variant in any single plant. These two variants were rare, found in only eight plants, and differed from one another in either position or loss of a Pst I restriction site (Tables 3 and 4). Variant E was found only three times, and co-occurred with variants B and A in A. marginata plants from Port Hardy and West Beach respectively, and again co-occurred with variant B in a single A. tenuifolia plant from Ogden Breakwater. Variant D co-occurred with variant B in A. tenuifolia plants from Kelsey Bay and Orange Point, and co-occurred with variant A in a single A. marginata plant from Port Renfrew. A single plant from Port Renfrew contained unique bands in both Pst I and Dra I digests that do not correspond to any of these variants, and was indicated by A' in Table 3..

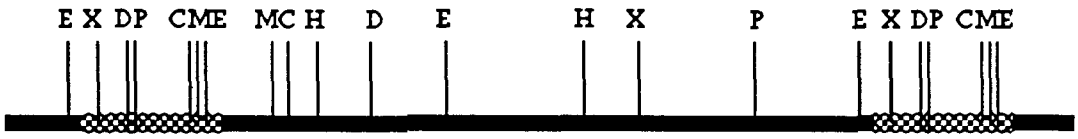
When any two variants appeared in single plants, the band intensities on the autoradiographs were usually the same for each variant band. Notable exceptions to this

occurred for some of the A. nana plants from Botany Beach, including both typical and large forms, which contained very faint bands representing variant C (Table 4). This implied that these plants contained only trace amounts of this variant in their genomes. In a similar fashion, band intensity differences for the plant from Alaska (Fig. 5d), implied that this plant contained proportionally more variant C than variant A in its genome.

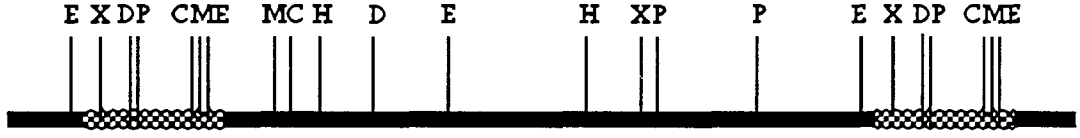
In addition to the three enzymes mentioned, five others, Hind III, Eco RI, Sma I, Sac I and Xba I, were used to further restriction map the rDNA repeat unit of three individual plants representing each species (Figure 8; Appendix Fig. 5b, autoradiograph for A. nana mapping digests only). Positions of restriction sites for these enzymes were found to be invariant among each of the three plants examined.

Figure 8. Restriction maps of ribosomal DNA repeat units found in three Alaria plants included in this study (AN01CB, AM03PN and AT03SP). Enzymes included, as follows: P = Pst I, D = Dra I, E = Eco RI, M = Sma I, C = Sac 1, X = Xba I and H = Hind III; and are positioned with vertical lines. Horizontal lines represent linear DNA strands with stippled regions corresponding to the 18S gene. One complete repeat unit extends from the beginning of one 18S gene, to the begining of an adjacent one. Repeat unit size is 10.4 kilobase pairs.

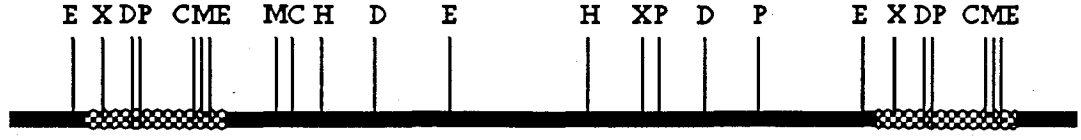
Alaria marginata



Alaria tenuifolia



Alaria nana



1 kb

DISCUSSION

Using the 18S gene as a probe, I uncovered both restriction site and length variation in the rDNA spacer regions of the three species of *Alaria* studied. Since both restriction site and length rDNA mutations exhibit simple Mendelian inheritance (Reeder *et al* 1976, Polans *et al* 1986, Russel *et al* 1988), they are often used in combination for distinguishing between species or populations. This, combined with the fact that all members of a breeding group are expected to share the same rDNA variants, is used in many fields to distinguish between genetically isolated strains of morphologically indistinguishable organisms (Simpson *et al* 1984, Finnerty *et al* 1988).

Alaria plants exhibited relatively little repeat unit length variation in the 50 individuals examined in this study, but at least one restriction site polymorphism was present in each of the three species. Such limited variation appears more commonly in domestic terrestrial plant populations than wild ones. For example, soybeans (*Glycine max*) were found to be homogeneous, with no rDNA variants discovered (Varsanyi- Brenner *et al* 1979). Both barley (Appels *et al* 1980) and rice (Oono and Sugura 1980) contained only two repeat length variants each. An exception to this was wheat, which contained a large amount of variation within single populations (Barker *et al* 1988).

For some wild plants such as *Clematis*, extensive rDNA

variation was found to be too great along population transects to be useful in defining breeding groups (Learn and Schaal 1987). Alternatively, species of the plant genus Trillium each contained a characteristic set of the five possible rDNA repeat unit variants found in that genus. A single variant was common to all Trillium species, plus a closely related outgroup, and was thought to be a common progenitor repeat unit (Yakura et al 1983). Characteristic length and restriction site variations in the IGS of maize and related species, were used to organize them into phylogenetic groupings, which agreed with biochemical, karyotypic and morphologically based groupings (Zimmer et al 1988). Evolutionary relationships were elucidated in a similar manner for four closely related species of Cucurbitaceae (Ganal and Hemlebon 1986).

Superficially, it appeared that single rDNA polymorphisms could be used as Alaria species markers, since most plants contained what appeared to be species characteristic variants. Most A. marginata and A. nana plants had single characteristic variants, A and C, respectively, and A. tenuifolia plants usually contained either variants B or B plus D. Variants D and E never occurred alone, and were shared by A. tenuifolia and A. marginata plants from widely separated sites around Vancouver Island. Although dispersability in kelp is generally poorly understood (van den Hoek 1987), the fact that Alaria breeding groups extended over large geographic areas was not surprising

since this was also discovered in the kelp Costaria costata (Bhattacharya 1988). The distribution of variants A, B and C generally separated the stands into three main breeding groups sharing common variants, however, this did not always correspond with species identification based on morphology.

Three important examples illustrate this point. Two plants (Fig. 6a) sampled from a stand on Cattle Point (San Juan Island), one plant (Fig. 6b) from Turn Island (adjacent to San Juan Island) and single plants from both Frank Island and Cape Beale contained both A. marginata and A. tenuifolia variants (A plus B), suggesting that the plants were hybrids of the two species. This had been previously suggested based on morphological evidence from the Cattle Point plants. However, all Turn Island plants, regardless of variant content, were identified as A. tenuifolia, and showed no interspecific morphological intergression with A. marginata. As well, the Frank Island and Cape Beale plants were all identified as A. marginata.

A second anomalous situation occurred in Port Hardy. At this site, plants of two species were collected from two adjacent locations, A. marginata (Fig. 7d) from a moderately wave exposed stand and A. tenuifolia (Fig. 7c) from a moderately wave sheltered stand. All plants analysed were found to contain only A. tenuifolia rDNA variants B or B plus D, in spite of their distinctive

morphologies.

A third case occurred at Botany Beach, where A. marginata, A. nana and morphologically intermediate stands were found on the same stretch of shore. Typical, relatively large, A. marginata plants (Fig. 5c) were found lower on the intertidal region and in less wave exposed areas than typical, small, A. nana plants which grew in the most wave exposed areas in the higher intertidal region. Stands of large 'marginata-like' A. nana (Fig. 6c), were found growing between the two on areas of shore with intermediate wave exposure. All of the Alaria collected from this beach contained variant A regardless of which species they were identified as. Some A. nana plants from both typical and 'marginata-like' stands also contained variant C in their genomes. Variant C is found elsewhere only in A. nana plants further north (Fig. 5a) where no morphologically anomalous stands were found.

Each of these three cases share a common feature. Individuals with similar variant contents, indicating common breeding group membership, sometimes displayed distinct morphologies when collected from environments differing in levels of wave exposure or tidal currents. The Turn Island stand was in a less wave exposed area than Cattle Point, and was also in a tidal channel. Similarly, the location from which A. tenuifolia was collected in Port Hardy, is less wave exposed than the area where A. marginata was collected. In both cases, the different

locations were fairly close to one another (within 5 kms.), and plants from the more wave exposed locations displayed A. marginata morphologies while plants from the less wave exposed areas displayed A. tenuifolia morphologies. On Botany Beach, Alaria plants were collected within 20 m from one another, along short sections of beach varying in wave exposure and intertidal height.

The evidence suggests that environmental influences related to wave exposure levels, have a greater effect on morphology than breeding isolation or genetic differentiation alone.

Although not widespread, the presence of plants with both A. marginata and A. tenuifolia rDNA variants as well as plants with both A. marginata and A. nana variants indicates that hybridization is possible between these species. If true, then intermediate hybrid-like morphologies might have been expected as demonstrated when closely related kelp species of Undaria were crossed (Saito 1971). This was not the case for the putative A. marginata - A. tenuifolia hybrid from Turn Island, which resembled other A. tenuifolia plants collected from the same stand. In the same way, individuals collected from Frank Island and Cape Beale which contained variants A and B, were identified as A. marginata. The same situation exists for putative A. nana - A. marginata hybrids. In such individuals, the combination of variants A and C, cannot solely account for the 'marginata'-like A. nana

morphologies observed on Botany Beach. Not all plants identified as A. nana contained both variants, and the typical A. nana plants collected at Botany Beach were similar in morphology to the others found further north (Frank Island) which had variant C only. In spite of apparently conflicting morphological information, it appears that hybridization of Alaria breeding groups has occurred.

Interbreeding may be common in kelp and has been demonstrated between different species and genera in the laboratory (Saito 1971, Sanbonsuga and Neushal 1978, Cosson and Oliveri 1982, Bolton et al 1983). However, the artificial production of hybrids does not necessarily imply that hybridization is a common occurrance in wild kelp populations (Chapman 1978). For the three Alaria species in question the possibilty of hybridization is further enhanced by the fact that they share equal chromosome numbers (Robinson and Cole 1971). Increasing sample size may uncover more evidence of natural Alaria hybrid populations. There is presently insufficient data to characterize Alaria hybrid zones as found in both animals and plants (Barton and Hewitt 1985). However, it appears as though hybridization is not a major contributing factor to Alaria morphological variation.

The confirmation of phenotypic plasticity in Alaria is not surprising, and has been demonstrated in other kelp species with the use of transplant experiments. These

experiments have suggested that the use of morphology as taxonomic indicators may not always give correct phylogenetic relationships.

Laminaria digitata (Huds) Lamour. was found through transplant experiments to produce exposed and sheltered water forms (Sundene 1958), the latter of which had been previously given specific status. This form, called L. cuculata sensu Jorde and Klavestad, was reduced to ecad status of L. digitata when it was shown to be an inducible phenotype (Svendsen and Kain 1971). The kelps Macrocystis integrefolia Bory (Druehl and Kemp 1982) and Saccorhiza polyschides (Lightf.) Batt. (Norton 1969) were also shown to have phenotypically distinct wave exposed and sheltered water forms.

Other non-morphological, yet taxonomically important anatomical traits may also vary with environment. The presence of mucilage ducts is used to separate some morphologically similar Laminaria species but may be misleading due to possible environmental influences, such as water temperature (Burrows 1964). Such changes in morphology are considered to be adaptative. In Laminaria saccharina (L.) Lamour., Gerard (1987) found that mechanical stress produced narrow blades, and suggested that wave action has a similar effect which would enhance lamina stream-lining.

Contrasting results indicate that phenotypic plasticity is limited in some seaweed. Characteristics separating

varieties of the kelp L. japonica Aresch. having different economic value, varied only slightly in response to environment (Sanbonsuga 1978). From experiments on the non-kelp phaeophyte Fucus using correlation analysis, Rice et al (1971) showed that environmental variables such as longitude, latitude and wave exposure, accounted only for minor amounts of intraspecific or interstand morphological variation.

Results from transplant experiments must be examined carefully due to inherent errors with the techniques (Norton et al 1981). Survival of transplanted adult plants may be low due to their previous adaptation to their home environments. In addition, young sporophytes and gametophytes should be included in these studies, but rarely are due to the difficulties in handling them. Subjectivity on the researchers part when determining wave exposure levels, as well as the possibility of influence from other unstudied selective forces, such as competition are also potential problems. For seaweed, there is evidence that selective pressures may be primarily physical in wave exposed locations yet biotic in sheltered ones (Russel and Fielding 1981).

In addition, phenotypic plasticity itself is an heritable characteristic under genetic and selective control (Bradshaw 1965, Russel and Fielding 1981). It can therefore vary between and within organisms. Within an individual different traits may have different levels of

plasticity, as found in species of Undaria (Saito 1971). Alternatively, levels of stability of a single trait may change between genotypes. This could cause some common features, such as stipe length for example, to vary in heritability (Bradshaw 1965). Chapman (1974) found that variabilty and heritability levels for some traits in Laminaria saccharina were greater in sheltered populations relative to wave exposed locations.

For these reasons individual traits must be examined carefully within as well as between kelp species. For example, bullations were found to be under genetic control in L. saccharina and may therefore be useful taxonomically (Luning et al 1978). In Hedophyllum sessile Setchell populations, however, bullations were found to be environmentally induced by levels of light intensity (Widdowson 1965) and therefore would not be useful for classification of H. sessile varieties. In a similar fashion, the development of mucilage ducts in L. saccharina appears to be under differing levels of environmental and genetic control (Chapman 1975, Luning et al 1978), in populations from differing environments.

For Alaria, the implications of phenotypic plasticity are that morphology may not always be suitable for determining breeding group membership. This does not necessarily mean that genetic differentiation among these breeding groups has not occurred. The ranges of phenotypic variation which are typically used to separate traditional

Alaria species are relatively small and quantitative in nature. There are no presence or absence type features used to distinguish most Alaria species. Therefore phenotypes displayed by different breeding groups may overlap depending upon local variations in environment, and the ability of local genotypes to respond to it. As a result, morphology may not be the best indicator of which breeding group any particular individual Alaria may belong to. Considering the strong influences of phenotypic plasticity, it would be suitable to include local environment with criteria used in Alaria species identification.

Figure 1. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows (A is unknown):
B. AN00BO, C. AT02PH, D. AM01PH, E. AT03OB, F. AT02OB
G. AM02PR, H. AM01PR.

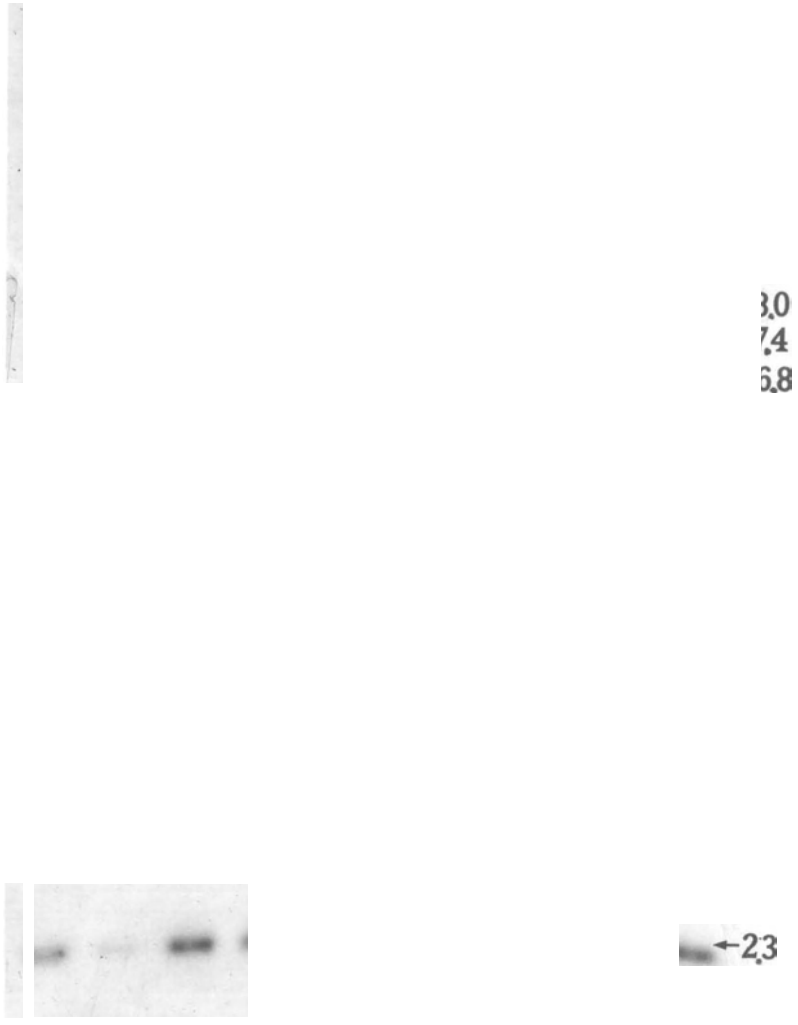
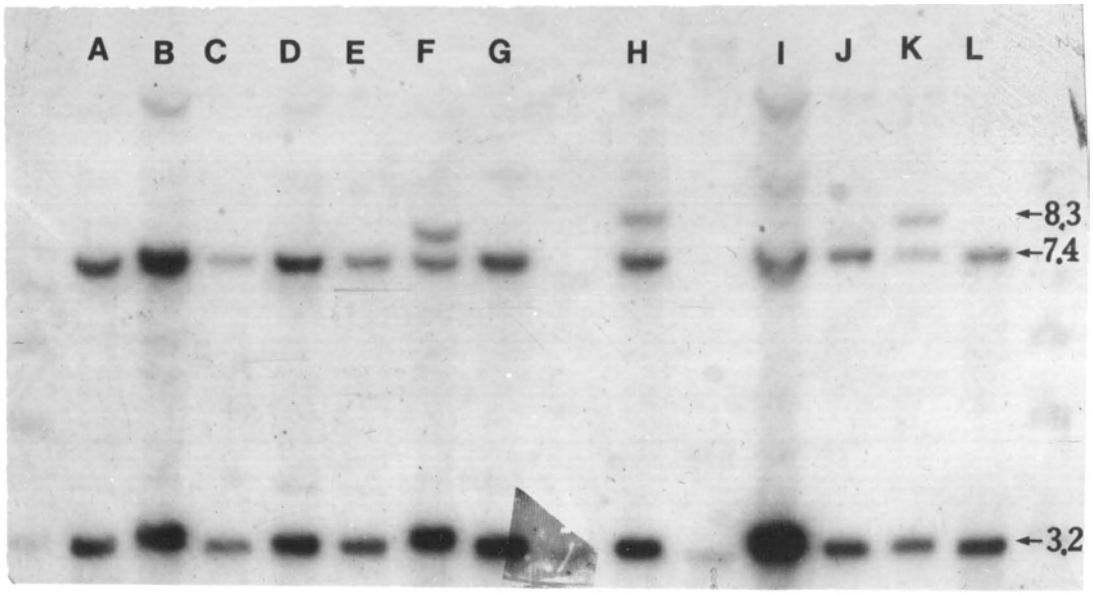

3.0
7.4
6.8
←2.3

Figure 2a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Dra I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AT03TI, B. AT02TI, C. AT01TI, D. AT09SP, E. AT07SP, F. AT02OP, G. AT01OP, H. AT03KB, I. AT02PH, J. AT00PH, K. AM03PH, L. AM01PH.

Figure 2b. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Dra I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AM03RB, B. AM02RB, C. AM01WB, D. AM01CP, E. AT03OB, F. AT02OB, G. AM03WS, H. AM05PN, I. AM03JR, J. AM02JR, K. AM03PR, L. AM01PR, M. AM02PR.


A B C D E F G H I J K L
8,3
7,4
3,2

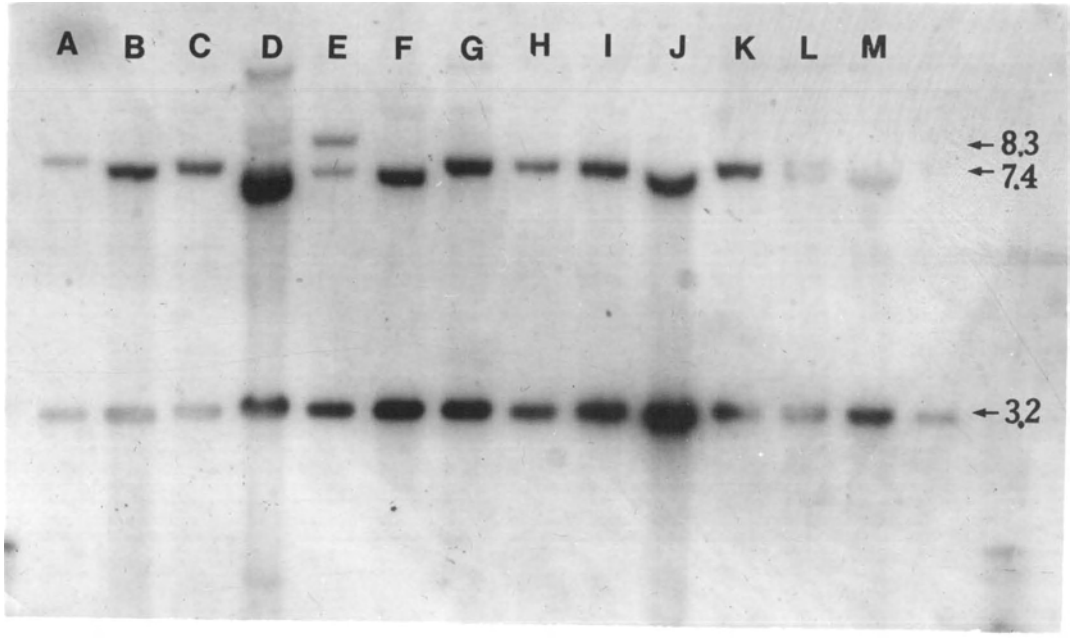

A B C D E F G H I J K L M
8,3
7,4
3,2

Figure 3a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Bgl II when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AT03TI, B. AT04TI, C. AT01TI, D. AT09SP, E. AT07SP, F. AT02OP, G. AT01OP, H. AT03KB, I. AT02KB, J. AT02PH, K. AT00PH, L. AM03PH, M. AM01PH.

Figure 3b. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows:
A. AM04WB, B. AN01CB, C. AM01CB, D. AM02FI, E. AN03FI, F. AN02FI.

a

b

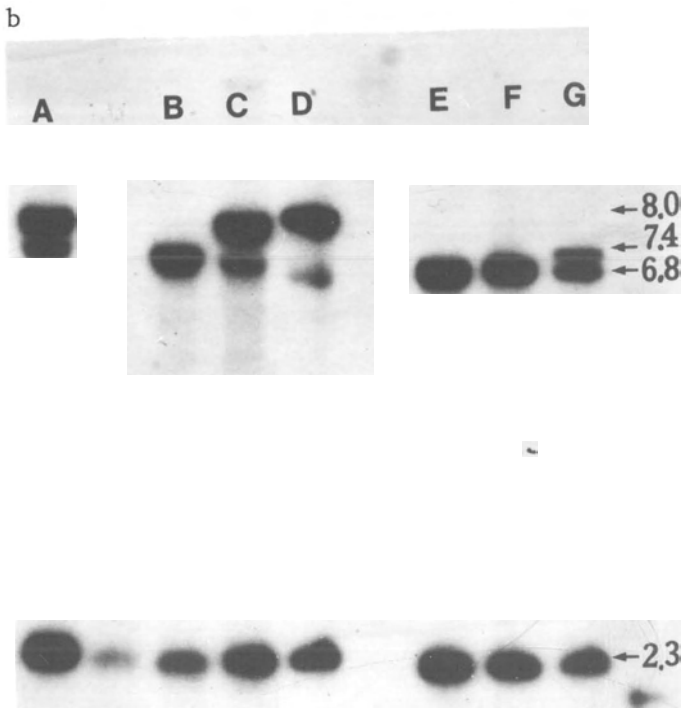
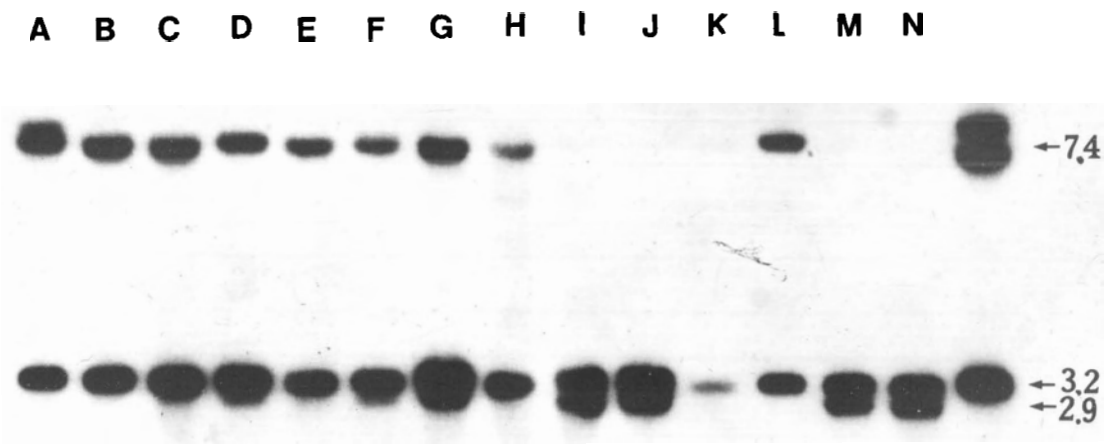


Figure 4a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Dra I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AM05BO, B. AM03BO, C. AM02BO, D. AN10BO, E. AN03BO, F. AN01BO, G. AN00BO, H. AM01CB, I. AN01CB, J. AN00CB, K. AM01FI, L. AM02FI, M. AN03FI, N. AN02FI.

Figure 4b. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Dra I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AT00PH, B. AT04TI, C. AT01SP, D. AT01KB, E. AM02SK, F. AM06SR, G. AM01PH, H. AM02CP, I. AM04WB, J. AT01OB, K. AM01WS, L. AM03PN, M. AM04BO, N. AM04PR, O. AM01FI.

a



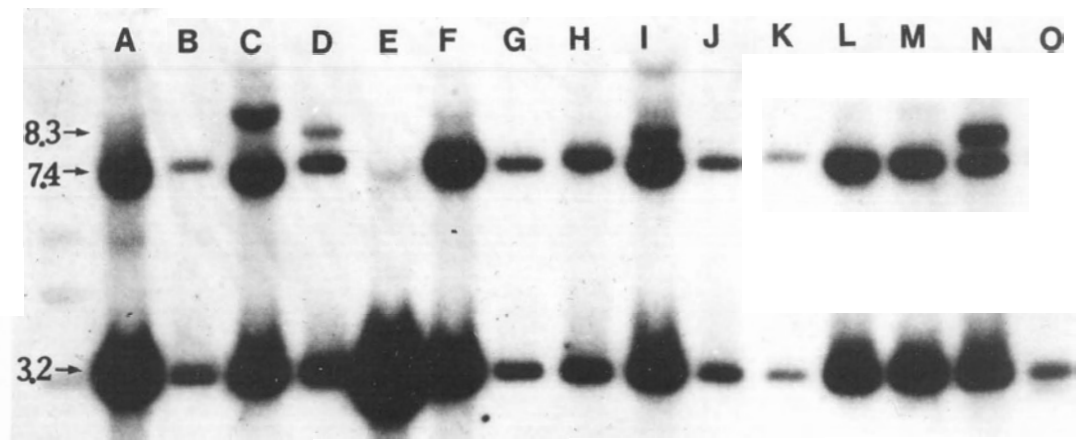
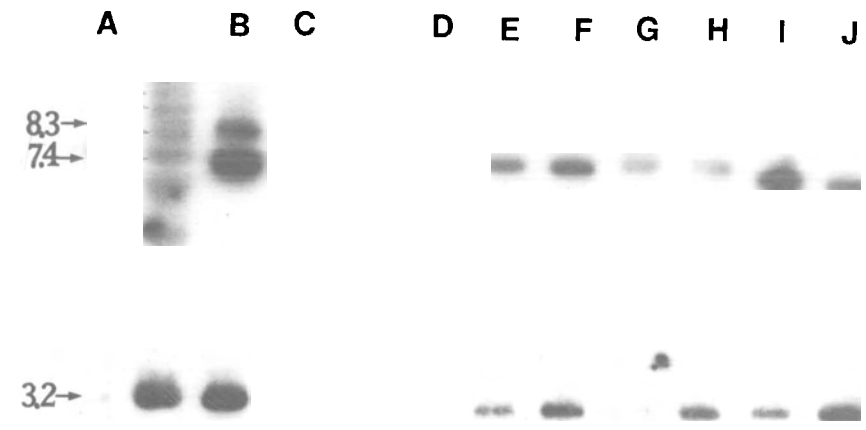
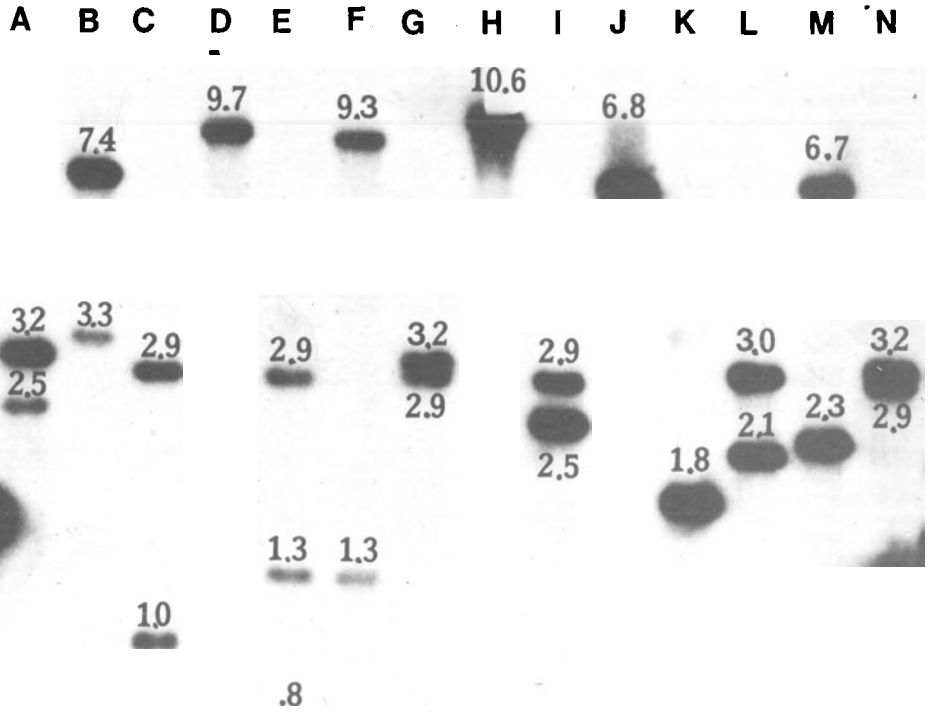


Figure 5a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Dra I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows:
A. AM01FI, B. AT01KB, C. AT03KB, D. AN10BO, E. AM03BO, F. AM02BO, G. AM05BO, H. AM04BO, I. AT01TI, J. AT04TI.

Figure 5b. Autoradiograph of A. nana Schrader (AN01CB) genomic DNA when digested with various restriction enzymes in single and double digests and probed with pCc18. Band sizes are indicated in kilobase pairs. See Figure 8 in Results section for resulting restriction map. Lane contents are as follows:
A. Dra I/Xba I, B. Xba I, C. Dra I/Sma I D. Sma I, E. Dra I/Sac I, F. Sac I, G. Dra I/Bgl II, H. Bgl II, I. Dra I/Hind III, J. Hind III, K. Eco RI, L. Dra I/Pst I, M. Pst I, N. Dra I.

a


A
B
C
D
E
F
G
H
I
J
8.3
7.4
3.2


A
B
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K
L
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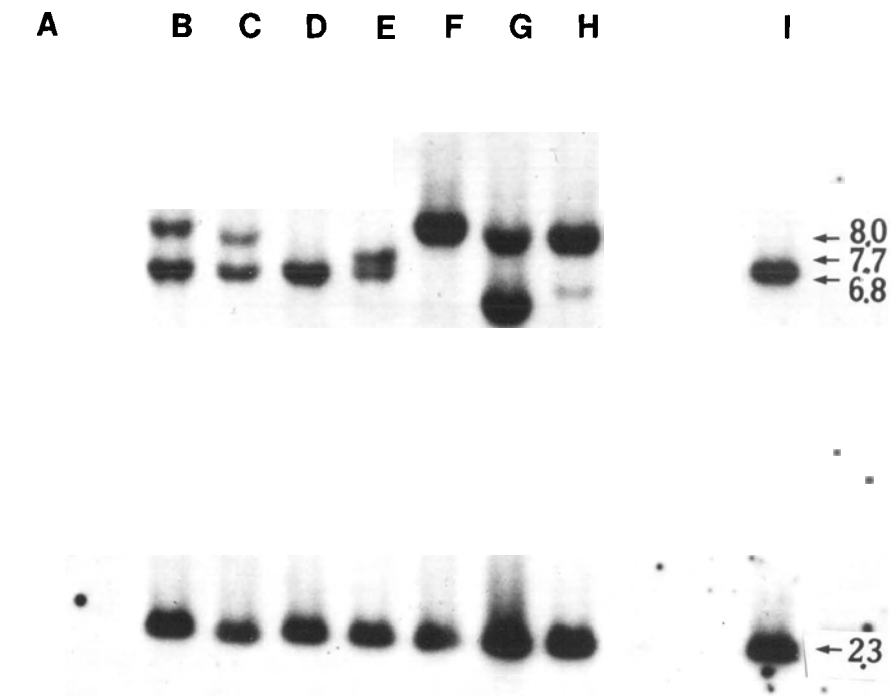
7.4
9.7
9.3
10.6
6.8
6.7
3.2
2.5
3.3
2.9
1.0
2.9
1.3
1.3
3.2
2.9
.8
2.9
2.5
1.8
3.0
2.1
2.3
3.2
2.9

Figure 6a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows:
A. AT02KB, B. AT01CP, C. AT03TI, D. AT09SP, E. AT03OB, F. AM03PN, G. AM01PR, H. AM02BO, I. AN02FI.

Figure 6b. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows:
A. AT01KB, B. AT03KB, C. AM01WS, D. AN10BO, E. AM03BO, F. AM02BO, G. AM05BO, H. AM04BO, I. AT01TI, J. AT04TI, K. AM01FI.

a



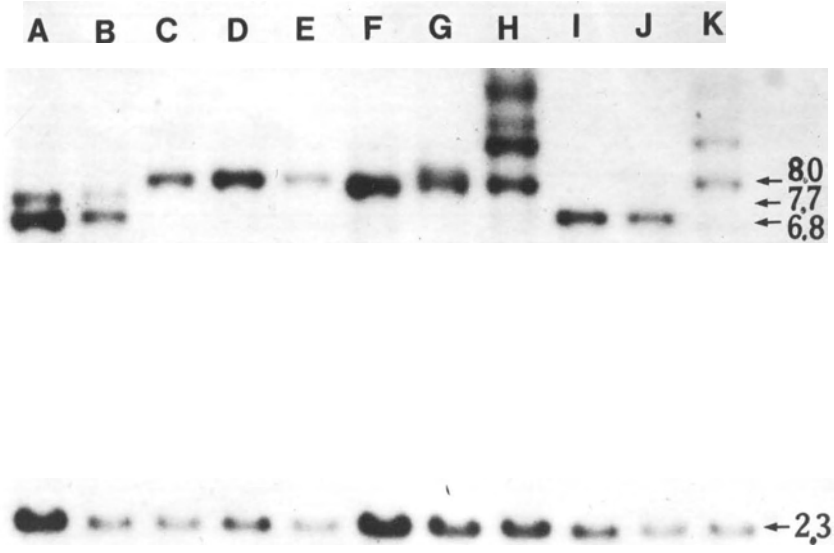
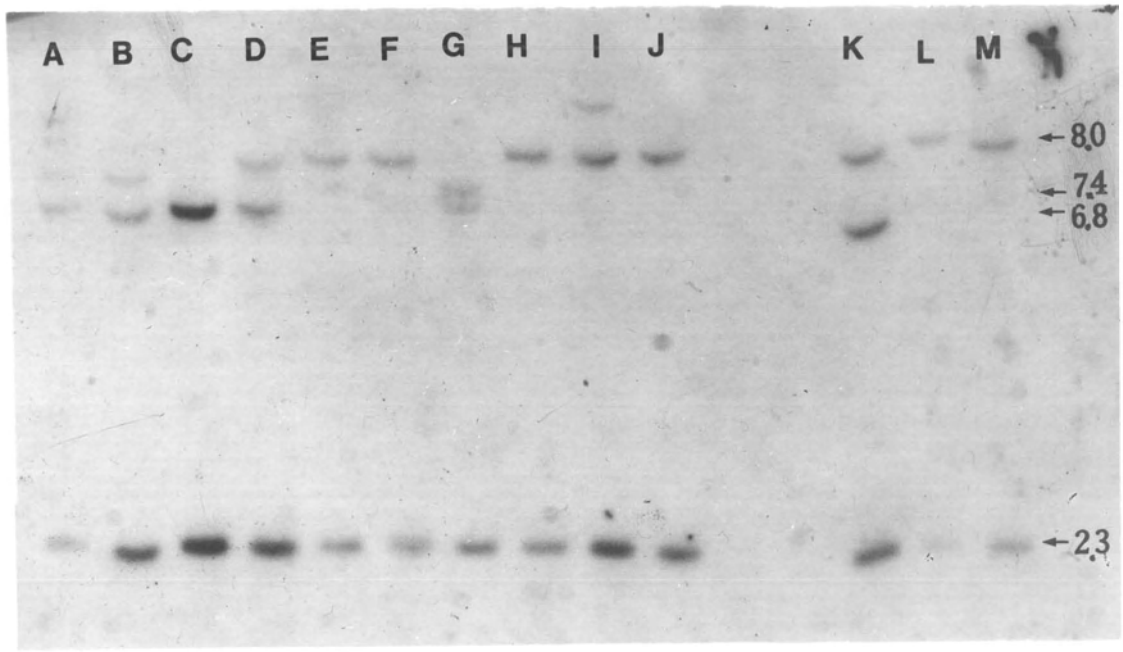


Figure 7a. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows: A. AM03RB, B. AM02RB, C. AM06SR, D. AM01PR, E. AT01OP, F. AT02OP, G. AM02JR, H. AM03JR, I. AM03PN, J. AM05PN.

Figure 7b. Autoradiograph of Alaria genomic DNA resulting from digests with the restriction enzyme Pst I when probed with pCc18 (18S ribosomal RNA gene). Initial letters in plant identification code describe species names as follows: AM = A. marginata Postels et Ruprecht, AT = A. tenuifolia Setchell, AN = A. nana Schrader. Band sizes are indicated in kilobase pairs. See Table 1 for collection site locations and Tables 4 and 5 for restriction banding pattern summary. Lane contents are as follows:
A. AT09SP, B. AT03TI, C. AT02TI, D. AT01CP, E. AM04WB, F. AM01WB, G. AT03OB, H. AM03WS, I. AM03PN, J. AM02JR, K. AM01PR, L. AN03B0, M. AN01B0.

A

b



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