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Genetic differences between two growth-forms of *Lithophyllum margaritae*

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GENETIC DIFFERENCES BETWEEN TWO GROWTH-FORMS OF
LITHOPHYLLUM MARGARITAE.

A Thesis

Presented To

The Faculty of Moss Landing Marine Laboratories

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

in

Marine Sciences

By:

Timothy Nicholas Schaeffer

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ABSTRACT

GENETIC DIFFERENCES BETWEEN TWO MORPHOLOGIES OF LITHOPHYLLUM MARGARITAE IN BAJA CALIFORNIA SUR.

By

Timothy Nicholas Schaeffer

Unattached, non-geniculate coralline algae or rhodoliths exhibit a range of morphological variability seemingly dependent on environmental factors. Rhodoliths have an extensive fossil record and may have environmentally dependent characteristics, making them potentially reliable paleoindicators. The rhodolith forming genus Lithophyllum spp. found in Baja California Sur, Mexico was recently consolidated. Under the new classification, L. margaritae consists of several growth forms presumably reflecting local environmental conditions. In this study the genetic structure of several populations of this species from Baja California Sur, Mexico were examined using Amplified Fragment Length Polymorphisms (AFLP) with the goal of characterizing the extent of genetic variation associated with the foliose and fruticose morphologies described for this species. The data indicate these morphologies are genetically distinct. These results suggest exchange of genetic information between foliose and fruticose morphologies of L. margaritae is limited and that environmental conditions are not the only influence on growth forms of L. margaritae.

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INTRODUCTION

Recent attention has been given to reconstructing past environmental conditions to better understand global change. The fossil record has been utilized as a source of indicators providing a record of ancient environments (Markwick, 1998; Pirrie, Marshall, and Crame, 1998; Ketcher and Allmon, 1993; Walker and Yamada, 1993). Critical to the reconstruction process for some organisms is assessing environmental dependence of morphological characteristics of extant representatives and fossil species. Uncertainty in this approach stems from a lack of knowledge regarding the genetic basis of morphological variation within a species compared to environmental influences. At one time, it had been proposed that in any organism "the demands of the environment may call forth any type of vegetative growth" (Wood-Jones, 1910, pg. 520). Later research, however, suggests the environment may not be the primary cause of morphological variability within some species (Chapman, 1974; Stearn, 1982; Espinoza and Chapman, 1983; Willis and Ayre, 1985). Stearn (1982) noted that most research testing environmental effects on the coral Acropora could not be understood exclusively on the basis of mechanical restraints. Willis and Ayre (1985) grant that environmental gradients modify coral morphology but found a high correlation between genotype and growth-form, and suggested that major divisions in colony morphology are genetically determined. Such observations require that environmentally induced morphological variability be partitioned from heritable morphology in order to estimate ancient environmental conditions from living organisms. To test if morphology is

environmentally induced, genetic analyses can be used to detect differences or similarities among morphologies of a species.

Organisms with calcium carbonate skeletons may be exceptional reef builders and can be well preserved in sediments. In some reef-building organisms, environmental factors are thought to promote the expression of a particular phenotype making them ideal paleoindicators. An example of such organisms are rhodoliths, free-living nongeniculate coralline algae (Corallinales, Rhodophyta), found in tropical, temperate, and arctic waters (Adey and MacIntyre, 1973; Johansen, 1981) at depths over 200 m to the intertidal zone (Littler, Littler, and Hanisak, 1991). New rhodoliths can arise in non-muddy environments when crustose or branching corallines settle on unattached substrates or are detached from existing hard substrates (Bosence, 1983), and can be maintained by sexual and asexual reproduction. Rhodoliths are thought to grow slowly: deep water laminar forms, 20 to 30 cm diameter, could be 500-800 years old (Adey and MacIntyre, 1973). Currently, rhodoliths are placed in the Phylum Rhodophyta, Order Corallinales. Common names include Lithothamnion balls, algal balls, algally coated-grains, rhodolites, algal nodules, maerl and chicharrones (Foster et al., 1997).

The effects of environmental variables, such as water motion, light availability, and temperature on rhodolith morphology have been discussed extensively (Foslie, 1894; Lemoine 1910; Bosellini and Ginsburg, 1971; Adey and MacIntyre, 1973; Bosence, 1976; Bosence, 1983; Steneck, 1986; Braga & Martin, 1988; Steller and Foster, 1995; Foster et al., 1997). Rhodolith morphologies in Bermuda Bay contained a consistent record of wave-induced rhodolith movement (Bosellini and Ginsburg, 1971). Bosence

(1983) also noted that hydraulic energy influences crustose and branching growth-forms and is actually required for rhodolith survival. Braga and Martin (1988) state that for fossil material "the type of algae and their growth-forms seem to be controlled by the characteristics of the environment in which they developed" (pg. 300). Foster et al. (1997) reported two types of rhodolith beds in Baja California, Mexico, one type is characterized by gentle slopes with moderate wave action, called wave beds, and the other type is found relatively deeper and influenced by tidal currents, called current beds. They found that particular branching characteristics (e.g. sphericity, branch shape, branch density) of rhodoliths varied according to water motion within wave beds. Additionally, in rhodolith beds in Baja California Sur, Mexico, fruticose growth-forms are relatively high in abundance in both types of beds while foliose growth forms are restricted to deeper water (Yabur-Pacheco, 1998).

Demonstrating statistically insignificant genetic variability between rhodolith morphologies of the same species would support the use of rhodolith morphology as a reliable paleoenvironmental indicator. Additionally, the extent of genetic variation within and among populations could reflect the predominant mode of reproduction and potentially the magnitude of recruitment into and out of populations. Available evidence suggests rhodoliths may have relatively low gene flow within and between populations. Clonal propagation, through mechanisms such as fragmentation, minimizes genetic variability in populations because genetic information among individuals is not subject to the randomizing effect of independent assortment. Toomey (1975) found rhodoliths from the western Gulf of Mexico commonly had no "alien" nucleus, rather a nucleus initiated

by a skeletal fragment suggesting individuals may grow from fragments. Proliferation through vegetative fragmentation has been noted by other researchers as well (Adey and MacIntyre, 1973; Bosence, 1976; Steller and Foster, 1995). Steller and Foster (1995) observed abundant pigmented fragments within beds and the absence of non-calcareous cores in larger rhodoliths. If propagation in rhodolith beds is primarily by fragmentation, then it is likely these populations should exhibit low genetic diversity. Depending on migration rates and clonal longevity, evidence for such clonal structure would highlight the importance of environmental control on phenotype in genetically similar individuals.

Genetic comparisons between morphologies both within and among populations could reveal associations between genetic and morphological characteristics, the degree of asexual reproduction, and the magnitude of genetic exchange between rhodolith populations. Alberte et al. (1994) pursued similar questions in their study of genetic similarity among and within populations of the seagrass Zostera marina. They found less genetic similarity between populations than within populations, suggesting interpopulation dispersal or recruitment from outside sources is limited relative to within population dispersal and recruitment. In other instances disjunct populations may not be synonymous with limited dispersal; Porphyra cuneiformes and P. nereocystis were found to have no within species variation even over a span of 1000 km (Lindstrom, 1993). Sosa and Garcia-Reina (1992) also found no correlation between genetic differences and geographical distance in Gelidium arbuscula. They did, however, find significant genetic variability between gametophytes and sporophytes indicative of asexual

reproduction. The magnitude of genetic diversity within and among populations of rhodoliths is unknown but could be investigated with similar methods.

In this study, genetic variability was examined using Amplified Fragment Length Polymorphism (AFLP) techniques. AFLP is a relatively new method of PCR-based DNA fingerprinting, developed by Vos et al. (1995). AFLP is based on selective PCR amplifications of restriction fragments with the ability to detect single base pair differences between individuals. The technique produces individual as well population specific markers providing a spectrum of resolution. Genetic variability in the rhodolith forming alga Lithophyllum margaritae was examined relative to morphology and geographic distance. Lithophyllum margaritae is an ideal choice for several reasons. First, L. margaritae consists of several morphologies, all believed to be environmentally influenced. Second, the species has been reclassified by Riosmena-Rodriguez (1997) based on several microscopic features (esp. conceptacle morphology) as developed by Woelkerling (1988). As a result, three of the five Lithophyllum species found by Dawson (1960) in the Gulf of California were lumped into one species, Lithophyllum margaritae. Genetic fingerprinting techniques described in the work presented here can provide independent molecular evidence for this reclassification. Lastly, L. margaritae is found along a wide geographical gradient (>500 km) and well suited for among population comparison.

The null hypothesis that there is no significant difference in AFLP fingerprints between foliose and fruticose individuals of Lithophyllum margaritae within and among populations was tested. The objective of these analyses is to clarify the extent to which

some rhodoliths can provide us with information from the past, and to what extent these organisms are exchanging genetic information. Bosence (1983) states "The realization that living rhodoliths may be hundreds to tens of thousands years old means that a relatively long term record of the sea floor conditions is preserved...they have been shown to preserve information on depth, geography, temperature and prevailing hydraulic energy" (pg. 218). The question is, does genetic variation affect the interpretation of this information?

MATERIALS & METHODS

Collection

Individual rhodoliths of the species Lithophyllum margaritae were collected from four populations in Baja California Sur, Mexico during 1998 (Fig. 1). A population of rhodoliths was defined as any contiguous bed of rhodoliths separated from another bed by at least 1 km. Rhodoliths were collected in the La Paz region on January 23, 1998 from two populations, Manto de Diana (24° 50' 47" N, 110°36' 26" W), referred to as LP-1, at 20 m depth and Manto de Pepe (24°52' 01" N, 110°33' 54" W), referred to as LP-2, at 12 m depth. Two populations in the Mulege region were sampled on March 20, 1998, Manto de James (27°02'42.54" N, 111°55'.59.28" W), referred to as PC-1, at a depth of 8 m and Manto de Zachary (27°03'6.6" N, 111°57'56.94" W), referred to as PC-2, at 10 m. Rhodoliths of the fruticose (Fr) and foliose (Fo) growth-forms (Fig. 2) defined by Woelkerling et al. (1993) were collected haphazardly along the length of each bed and sealed in ZipLoc bags underwater. Immediately after collection, the bags containing rhodoliths were punctured several times, marked with place and time collected and transferred to a cooler with seawater. The cooler was equipped with a battery powered air pump to aerate and mix the water in an effort to facilitate rhodolith survival during transportation back to the laboratory. That samples collected were L. margaritae was

confirmed by R. Riosmena-Rodriguez using taxonomic characteristics described by R. Riosmena-Rodriguez (1997).

DNA Extraction

Two methods were employed to recover high molecular weight DNA from living rhodoliths; one using guanidine thiocyanate extraction buffer and the second a protease (Qiagen Cat#19155) based extraction. Both extraction methods require the rhodolith fragments to be ground in liquid N₂. For guanidine thiocyanate extraction, 1-3 grams of rhodolith fragments were added to 15 mL of buffer (4M guanidine thiocyanate, 25mM sodium citrate, pH 7, 0.5% (v/v) sarcosyl and 0.7% of 2-mercaptoethanol (added fresh)). This solution was heated at 65°C for 45-60 minutes. The buffer solution was then centrifuged at 3000 .xg for 10 minutes and transferred to a new 50 mL tube, leaving ground plant debris in the initial tube. DNA was then extracted by adding an equal volume of phenol and chloroform (1:1 ratio). The solution was mixed by inverting the tubes gently several times and their organic and aqueous phase separated by centrifuging at 3000 .xg for 10 minutes. The lower organic phase was removed and an equal volume of chloroform and isoamyl (24:1 ratio) was added to the remaining aqueous phase. Again, samples were mixed by gentle inversion and spun at 3000 .xg for 10 minutes. These steps were repeated until particulate matter was no longer visible at the interface separating the aqueous and organic phase. The upper aqueous phase was then removed and transferred to a new 50 mL tube. Total nucleic acids were precipitated by the

addition of 2/3 volume of 100% isopropanol and stored at -20°C for up to 2 weeks until DNA isolation.

For protease extractions, ground tissue was placed in a 50 mL tube with 10 mL of extraction buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 2.0% Sarcosyl, 0.5% PVPP, freshly added 2.0% 2-Mercaptoethanol and 0.4 mg/mL Qiagen Protease). Samples were incubated at 65°C for 30 minutes occasionally inverting, followed by a 37°C incubation and agitation at ca. 30 RPM for 2 hours. The solution was then transferred to a new 50 mL tube and subjected to phenol and chloroform extractions, as described above, in order to remove proteins and other lipophilic components.

Preliminary results demonstrated guanidine thiocyanate extraction methods resulted in moderate yields of intact, high molecular weight DNA (≥ 20 kb) while protease extraction methods resulted in high yields but relatively degraded DNA (≥ 10 kb) and RNA. Although guanidine thiocyanate consistently resulted in high molecular weight DNA (≥ 20 kb), this method required relatively large amounts of tissue (>2 g). The protease based method, on the other hand, provided ample quantities of DNA with less tissue. Choice of extraction method for a given rhodolith depended on individual wet weight.

DNA Isolation

After precipitation with isopropanol, all samples were centrifuged at 3000 $\times g$ for 20 minutes, pellets were washed with 70% ethanol and air dried. Once dry, pellets were

dissolved in 2 mL of TE (10 mM Tris, pH 8, 10mM EDTA) and incubated overnight at room temperature. Pellets were completely dissolved in TE and 2 g of CsCl was added to each sample. Samples were then transferred to Beckman 3.9 mL polyallomer quick seal tubes containing 45 μ L ethidium bromide (1 mg/ml) and 1 drop (ca. 10 μ L) of Triton-X-100 (Sigma #T9284). The tube was filled to the crown with CsCl/TE (50 g CsCl + 50 mL TE) and heat sealed. Samples were then spun at 100,000 $\times g$ for 4 hours at 20°C. After centrifugation, tubes were viewed in front of a UV light source in order to localize the high molecular weight DNA band. To isolate the DNA, bands were removed using a 1 mL syringe with an 18 gauge needle. The Cs/Cl solution containing rhodolith DNA was transferred to 1.5 mL centrifuge tubes and washed with salt saturated isopropanol (50% (v/v) 5M NaCl, 50% (v/v) isopropanol) in order to remove ethidium from the DNA. Samples were gently inverted and centrifuged at 15,000 $\times g$ for 3 minutes. This was repeated until samples no longer appeared pink (ca. 3 washes). The extracted DNA solutions were transferred to 15 mL snap cap tubes (Falcon #2059), then diluted with 2 volumes of TE (10 mM Tris, pH 8.0, 10mM EDTA). NaCl was added to a final concentration of 0.2 M from a 5 M stock and 2 volumes of -20°C 100% ethanol were added to each sample to precipitate the DNA. Samples were then incubated overnight at room temperature.

After overnight incubation, samples were centrifuged at 9000 $\times g$ for 30 minutes and decanted. The remaining pellets were washed once with 70% ethanol, air dried and resuspended in 200 μ L TE for 1-2 hours. Next, samples were transferred to 1.5 mL tubes and mixed with 100 μ L 7.5 M NH_4OAc and 600 μ L ethanol, incubated for 1 hour at room

temperature, centrifuged at 15,000 xg for 15 minutes, and washed with 70% ethanol.

Remaining pellets were then air dried and resuspended in 50 µL of TE overnight at 4°C.

Yield of DNA was quantified from each sample by using UV spectrophotometry. DNA samples were diluted in dH₂O and absorbances determined at 260 and 350 nm for

$$\text{Eq.1} \quad [(\text{OD}_{260} - \text{OD}_{350})50]/10 = X \text{ } \mu\text{g} \cdot \mu\text{L}^{-1}$$

use in Eq.1 yielding DNA concentration in µg·µL⁻¹. Once quantified, DNA samples were checked for integrity by adding 5 µL of sample to 1 µL of sample buffer (.025% bromophenol blue (v/v), 0.25% xylene cyanol FF (v/v), 1.5% Ficoll (w/v), 10% 10X TE, 80% H₂O) and run on a 0.7% agarose gel (30 mL 1X TAE (0.04 M Tris, 0.1% glacial acetic acid, .001 M EDTA, pH 8.0), 210 mg agarose (BioRad Molecular Biology Grade), 1.5 µL 1 mg/mL ethidium bromide) for 40 minutes at 50V.

Size Selection of Genomic DNA

A compromise between DNA yield and quantity was encountered with the different extraction protocols. CsCl banded DNA samples were subjected to agarose gel purification to standardize the size range of DNA fragments subjected to AFLP analysis. DNA samples were separated on 0.7% agarose, 1X TAE gels as previously described. Samples of genomic DNA of 20-25 kb in size, based on migration relative to λ DNA digested with Hind III, were excised from the gel and placed in 1.5 mL centrifuge tubes. To each tube, 900 µL of lysis buffer (10 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 6.4, 0.2 M EDTA, pH 8.0 and Triton X-100) and 40 µL of glass milk (Bio 101 #1001-

604) was added. Samples were then vortexed and incubated at room temperature for 10 minutes. To keep the glass milk in suspension, samples were vortexed for approximately 5 seconds at 2 minute intervals during incubation. Samples were then centrifuged at 12,000 $\times g$ for 7 sec. and the supernatant was removed. Pellets were then washed once with wash buffer (10 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 6.4), vortexed, centrifuged again at 12,000 $\times g$ for 7 seconds and the supernatant discarded. Samples were then washed twice with 70% ethanol, once with 100% acetone, vortexed and centrifuged with the supernatant removed between every wash. After the supernatant was removed following the last wash, samples were kept at 56°C with the lids open to evaporate residual acetone. Thirty-five μL of water was then added to each sample, and samples were vortexed and placed at 56°C for ten minutes. After incubation samples were centrifuged at 12,000 $\times g$ for 2 minutes and the supernatant was pipetted to new 0.6 mL centrifuge tubes. To dissolve any residual DNA, another 35 μL of water was added again to the original pellet, and the sample was incubated at 56° C for ten minutes. After incubation, samples were spun at 12,000 $\times g$ for 2 minutes and the supernatant was added to the original supernatant corresponding to each sample. The 0.6 mL centrifuge tubes were then spun for 5 min. at 12,000 $\times g$ to concentrate any leftover glass milk. Samples were then transferred to a new 0.6 mL centrifuge tube.

Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphism (AFLP) methods, introduced by Vos et al. (1995), followed modifications by De Tomaso et al. (1998). The general protocol is depicted in figure 3. DNA (200 ng) was reduced in size by restriction enzyme digestion with 5 units EcoR I and 5 units MseI (New England BioLabs) for 2 hours at 37°C in a 30 µL reaction volume using the recommended buffer (New England Biolabs Buffer 2). Oligonucleotide adaptors (5 pMol - EcoR I, 50 pMol - Mse I) compatible with EcoR I and Mse I (Table 1) cohesive ends were ligated onto the rhodolith DNA fragments using 15 units of T4 ligase (Promega) in a total volume of 40 µL. Ligation reactions were incubated for 3-12 hours. After the ligation reaction, 60 µL of TE (10 mM Tris, pH 8.0, 10 mM EDTA) was added to each sample. The diluted adaptor ligated DNA (4 µL) was subjected to PCR amplification (preamplification) with 30 ng of pre-selection oligonucleotide primers (Table 1) encompassing the restriction site sequence plus one base at the 3' end, Buffer 2 (New England Biolabs) and 0.5 units Taq polymerase. The PCR reaction was carried out in a Model TC1 Thermocycler (Perkin Elmer) in the following manner: 1 cycle of 72°C for 2 min, followed by 20 cycles of 94°C (30 seconds), 56°C (30 seconds), and 72°C (1 minute). The preamplification reactions were diluted 1:20 in TE and 3 µL was used for AFLP fingerprinting with each primer containing 3 additional selective nucleotides. The EcoR I selective primer was labeled with ³³P-γATP using a polynucleotide kinase under standard reaction conditions (DeTomaso et al., 1998). Reaction conditions for the selective amplification consisted of

the following: initial 2 min. hold at 95°C for template denaturation followed by 1 cycle of 94°C for 30 sec., 65°C for 30 sec. and 72°C for 1 min. AFLP products specific for the selective primer combination were subsequently amplified during 24 cycles comprised of 94°C for 30 sec., 56°C for 30 sec. and 72°C for 1 min. Following PCR reactions, samples were incubated in an equal volume of stop solution (98% formamide, 10 mM EDTA pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol) for 5 min. at 95°C. Samples were loaded onto 38 x 50 cm denaturing acrylamide gels (42% Urea w/v, 12% 10X TBE v/v, 10 % Long Ranger (50%) v/v, .05% TEMED and .05% Ammonium Persulfate v/v) and run at 80 watts for approximately 2.5 hours using a SequiGen (BioRad) apparatus. Reference DNA was loaded the span of each gel to provide a check for gel induced curvature. Gels were transferred to paper, vacuum dried and placed under Kodak X-Omat film for approximately 36 hours.

Data Analyses

AFLP fingerprints for each sample were coded by the distance in centimeters traveled by each band. Data were entered in a spreadsheet and analyzed using SIM 1.01 (Zimmerman, 1993). SIM 1.01 calculates Similarity values (S), using Eq. 2 (Lynch,

$$\text{Eq. 2 } S_{xy} = 2n_{xy}/(n_x+n_y)$$

1990), where the S of two individuals, x and y, is twice the number of common AFLP bands in their fingerprint profiles (n_{xy}) divided by the total number of bands exhibited in both individuals or as the fraction of shared bands. Average genetic similarity within and

among populations is calculated by making all possible pairwise comparisons of S_{xy} among individuals. SIM 1.01 calculates the covariance caused by recursive comparisons (Lynch, 1990; Danforth and Freeman-Gallant, 1996) with the same individuals and adds this component to the total variance of each statistical population. From the pairwise comparisons, SIM 1.01 uses the F' (Eq. 3) statistic (Wright, 1951) to test the null

$$\text{Eq. 3 } F' = D_b / (D_w + D_b)$$

hypothesis that $F' = 0$. In Eq. 3, D_b is the average dissimilarity ($D = 1 - S$) between populations i and j and D_w is the average dissimilarity within population i . Foliose and fruticose samples from populations within geographic regions were treated as separate populations in statistical analyses to test the null hypothesis that similarity values are equal among morphologies within a population. Rejection of this null hypothesis would demonstrate a genetic difference between morphologies.

Statistical assumptions of an F-test were tested using $F_{\max}$ and Kolmogorov-Smirnoff tests to verify the homoscedasticity of the error terms and that the data are distributed normally. To gain insight into the stratification of all populations, multiple comparisons were performed using SIM 1.01 to determine if a given morphology had a significantly different banding pattern between adjacent populations and between regions. If significant differences were not observed between adjacent populations then they were pooled. An alpha of 0.05 was determined *a priori* as the experimentwise error rate. To avoid a Type I error during the comparisons, the Dunn-Sidak method for multiple significance tests was used (Sokal and Rohlf, 1995). This method adjusts alpha for each comparison ($\alpha' = 1 - (1 - \alpha)^{1/k}$ where k is the k^{th} comparison).

To visualize genetic structure within and among the L. margaritae populations sampled, an unweighted pair group method with arithmetic averages (UPGMA) analysis was performed using the software package TFPGA v1.3 (Miller, 1997). This analysis is adapted to AFLPs by assuming the presence of a particular band represents one allele and the absence of the same band in another individual represents another allele. The analysis results in a graphical tree consisting of branches representing genetic distances (D) between statistical populations and bootstrap values indicating the degree of confidence for each node. Genetic distances are calculated using the equation provided by Nei (1978).

RESULTS

The two DNA extraction techniques employed resulted in different yields and integrity of DNA. DNA from foliose samples produced a mean of 5.8 μg DNA/g wet tissue ($\pm$ 5.1) when extracted with the protease and 1.5 μg DNA/g wet tissue ($\pm$ 1.4) with guanidine thiocyanate. Fruticose samples yielded an average of 6.9 μg DNA/g wet tissue ($\pm$ 4.8) with the protease and 2.3 μg DNA/g wet tissue ($\pm$ 1.7) with guanidine thiocyanate. Samples extracted with the protease yielded significantly more μg DNA per gram of wet tissue than guanidine thiocyanate extractions for both fruticose ($p < 0.05$, $t = 4.4$, $df = 17$) and foliose ($p < 0.05$, $t = 3.8$, $df = 20$) rhodoliths. In addition to differences in yield, the two extraction protocols differed in the molecular size range of DNA fragments produced (Fig. 4A & 4B). Guanidine thiocyanate extractions were characterized by the presence of a single band (23 kb) indicative that DNA remained intact while protease extractions were distinguished by higher yields but partially degraded (23 kb-1 kb range) DNA suggesting degradation.

Twenty-five samples of each morphology were collected from each population. Different primer combinations were attempted with the goal of obtaining more AFLP products for each sample. Bands from the F4 primer were not included in analyses because too many samples were unscorable, however, of the samples which were "clean", the patterns were similar to the E4 primer. After DNA extraction, AFLP processing, and using the E4 primer the number of unambiguous and usable samples within a morphology of a particular population ranged from 4-10 (Table 2). AFLP products

ranged in size from 100-400 bp. Among all samples, ninety-nine different loci were scored, 32 of which were common to both morphologies. The remaining 67 loci were exclusive to a particular morphology. Two bands, approximately 150 and 290 bp, were found in nearly every sample and there were 3 bands (175, 195, and 200 bp) present in every foliose sample while there were no consistent bands in all fruticose samples. Fruticose samples on average yielded 14 ($\pm$ 4.0 SD) bands while foliose samples generally had 22.8 ($\pm$ 3.0 SD) bands (Fig. 5).

Results from comparisons of like growth-forms between adjacent populations within a region suggest samples were not collected from genetically distinct populations. Similarity values of foliose populations ranged from 0.75-0.85 and fruticose populations from 0.55-0.67 (Table 3). Fruticose samples collected from the 2 populations in La Paz were not significantly different ($p > 0.05$) nor were foliose samples ($p > 0.05$). The same set of comparisons were performed on foliose and fruticose samples in Pta. Chivato. There was no significant difference ($p < 0.05$) between fruticose samples from the two Pta. Chivato populations but there was significant difference between foliose samples ($p < 0.01$). $F_{\max}$ tests performed on the data revealed heteroscedastic variances ($F_{\max, 0.01[8,9]} = 49$, $F_{\text{crit}} = 13.9$). Kolmogorov-Smirnoff tests concluded that data within all populations were normally distributed. Because of the low sample size from each population, the heteroscedastic data and the results of the multiple comparisons (despite not meeting the assumptions of the F test), populations of morphologies within each region were pooled. For example, LP1A and LP2A, both in the La Paz region, were pooled into one La Paz population (LP). Pooling the data increased the sample size of each group and produced

homoscedastic data ($F_{\max,01[4, 27]} = 2.77$, $F_{\text{crit}} = 3.3$). Similarity values for within pooled populations ranged from 0.83 (+/- 0.1 SD) to 0.76 (+/-0.2 SD) for foliose and 0.61 (+/-0.1 SD) to 0.57 (+/-0.2 SD) for fruticose. Similarity values between populations, including between morphology comparisons, ranged from 0.76 (+/- 0.1) to 0.38 (+/- 0.1). All S values from pooled populations can be found in Table 4. Analyses performed in the pooled populations resulted in a significant differences in genetic similarity between all populations (Table 5).

The UPGMA analysis produced a dendrogram that grouped populations primarily by growth-form and secondly by geography (Fig. 6). After 1000 permutations the analysis resulted in 7 nodes and the associated proportion of similar replicates relative to the number of permutations. Node 1 included the 2 La Paz fruticose populations with a distance (D) of 0.016 (83% proportion of similar replicates). Node 2 included the 2 La Paz fruticose populations and a Pta. Chivato fruticose population (D=0.03, 83% proportion of similar replicates). Node 3 contained of all fruticose populations (D=0.055, 74% proportion of similar replicates). Node 4 consisted of the 2 La Paz Foliose populations (D=0.019, 78% proportion of similar replicates), and Node 5 the two Pta. Chivato foliose populations (D=0.037, 53% proportion of similar replicates). Node 6 included all foliose populations (D=0.058, 62% proportion of similar replicates) . Lastly, node 7 included all populations (D=0.087, 100% proportion of similar replicates).

DISCUSSION

Demonstrating a genetic trend correlated with morphological variability has long been "a central theme in phycology" (Bhattacharya and Druehl, 1989). This study represents the first application of AFLP analyses in marine algae and may be the only paper to examine morpho-genetic relationships in the corallines. While there is limited genetic data for corallines, studies utilizing different genetic techniques in other species of algae are available for comparisons. Sideman and Mathieson (1985) were able to correlate genetic trends among morphotypes of Fucus distichus associated with protected and exposed sites. John et al. (1993) used 18S rRNA genes to reveal morphotypes of the genus Microthamnion, previously believed to be genetically different, were indistinguishable. Using a combination of allozyme and random amplified polymorphic DNA (RAPD) analyses, Van Oppen, Oslen, and Stam (1995) were able to demonstrate a clear distinction between western and eastern North Atlantic species of Phycodrys rubens. Bhattacharya and Druehl (1989) attempted to delineate morphologically distinct populations of Costaria costata using DNA sequencing and RFLP analysis. One polymorphism was detected employing 42 RFLP probes. Based on the one polymorphism, they suggested morphological variability in Costaria costata is probably genetic. Relative to the results presented by Bhattacharya and Druehl (1989), the differences found in this study are relatively large. The present results were obtained using 1 primer set yielding ninety-nine polymorphisms each capable of detecting single base pair differences.

Analyses using AFLPs are reproducible and detect a relatively high number of polymorphisms (Vos et al., 1995) in addition to being reliable and robust (Becker et al., 1995). Due to the number of loci sampled (potentially screening the entire genome) in a single assay, polymorphisms revealed per reaction are much higher than by RFLPs or RAPDs (Paul et al., 1997). AFLPs can provide a range of resolution— some bands are invariably retained among samples while some are individual specific. AFLPs have been used to assess genetic variability between and within populations and to estimate inter- and intraspecific genetic variation of species in both plants and animals (Janssen et al., 1996; Majer et al., 1996; Paul et al., 1997; DeTomaso et al., 1998).

The data from this study illustrate a significant difference in AFLP band profiles between foliose and fruticose growth-forms of L. margaritae. Foliose individuals from one population are more closely related to foliose individuals from populations >250 km away than to fruticose individuals in the same population. These results indicate that there is severely limited or non-existent gene flow between the two growth-forms. The genetic distance based UPGMA results indicate that growth-form reflects a larger genetic component effect than geographic distribution when comparing between growth-form and among populations (Fig. 6). In this study, the genetic differences between growth-forms are used to examine L. margaritae as a species and as a paleoindicator.

A potential source of the observed genetic contrast between the foliose and fruticose growth-forms of L. margaritae are differences in reproductive events. Mechanisms of reproduction in the coralline algae are poorly understood, even relative to the other red algae. Nearly nothing is known about spore viability, dispersal, or habitat

selection in corallines (Steneck, 1986) except that coralline spores are negatively buoyant upon their release (Bold and Wynne, 1985). Edyvean and Ford (1986) estimated one species of Lithophyllum sp. had an estimated spore survival rate of .000003119%. Spore and rhodolith survival can also be affected by environmental conditions (Woelkerling, 1988). Because coralline spores, like other red algae, are not flagellated and their low survival rate, it seems gene flow could be affected by the ability of currents to quickly disperse spores.

Fragmentation in rhodolith beds is thought to be common (Adey and MacIntyre, 1973; Toomey, 1975; Bosence, 1976; Steller and Foster, 1995). Woelkerling (1988) reported that among some rhodolith populations examined, the "proportion of gametangial plants never exceeded 20.4% in any population" (pg. 187). furthermore, reproductive individuals in some populations of L. margaritae in the Gulf are approximately 98% tetrasporophytic and spore availability can be low (Riosmena, pers. comm.). The absence or low proportion of gametangial individuals in rhodolith populations suggests there is some degree of asexual reproduction (i.e. fragmentation or bispores). In this study, however, no identical individuals were found among all samples of both growth-forms indicating that fragmentation is either unsuccessful, an uncommon event, or only uncommon in current beds. Lower survivorship of fragments could explain genetic diversity above what is expected in a population seemingly dependent on fragmentation for propagation. Although rates of successful asexual reproduction may be low overall, it seems to occur more frequently in foliose samples than fruticose based on their higher within population similarity values.

Rhodoliths in the Gulf of California can be found in both shallow and deep water habitats characterized by wave or current motion, respectively (Foster et al., 1997). Beds sampled in this study are in relatively deep water and are considered current driven beds. Generally, foliose individuals are restricted to relatively low hydrodynamic energy areas while fruticose individuals are found in a wide range of areas, including shallow habitats with relatively high hydrodynamic energy and deeper, relatively calmer habitats (Yabur-Pacheco, 1998). The AFLP analysis of L. margaritae indicate that similarity values within and among foliose populations are consistently higher than those observed for fruticose samples. It seems counter-intuitive that foliose individuals, generally restricted to deeper waters, are exchanging genetic information more effectively than a growth-form found in a range of wave-energy habitats. The differences in similarity values among populations of the growth-forms could be explained as varied responses of spores to the environment. It seems unlikely, however, that growth-form alone could account for the differences in population gene flow. It could be that potential allelic combinations in foliose individuals are fewer than in fruticose individuals or that vegetative propagation is more successful in one of the growth-forms. Another alternative is that populations should be tested with more AFLP primer sets. Different primer sequences could produce a range of among and within population similarity values; however, tests with the F4 primer revealed similar patterns between the two growth-forms.

Although the two growth-forms of L. margaritae can vary in relative abundance, samples were collected in areas where growth-forms were found living on top of one another. It is unlikely mechanisms of allopatric speciation are a source of genetic

differentiation. Another possible mechanism of divergence may be through reproductive isolation. For example, hybrids of the two growth-forms may be relatively inviable and consequently unable to propagate, failing to bridge the gap between growth-forms. Another potential source of reproductive isolation could be a difference in the timing of reproduction, which could limit interbreeding.

An alternative explanation of the genetic differences between foliose and fruticose growth-forms is that they represent different species although this is difficult to evaluate due to the experimental design of the present study. However, the validity of treating both growth-forms as L. margaritae is questionable because of their apparent reproductive isolation. Relatively recently, Riosmena-Rodriguez (1997) re-examined the taxonomic status of the rhodolith forming genus Lithophyllum in the Gulf of California. Prior to Riosmena-Rodriguez (1997), rhodoliths in this region had last been examined by Dawson (1960) who reported two species of Lithothamnion and five species of Lithophyllum based on 9 morphological characters. Riosmena-Rodriguez (1997) applied rhodoliths reported as Lithophyllum by Dawson (1960) to the generic concept of Lithophyllum set forth by Woelkerling (1983, 1988) and Irvine and Chamberlain (1994). Riosmena-Rodriguez (1997) found that “all species recognized by Dawson (1960), and their synonyms, indicated that most material fit the generic concept of Lithophyllum...” (pg. 5). Riosmena-Rodriguez (1997) further reviewed populations of the same rhodoliths using traits provided by Woelkerling and Campbell (1992), Irvine and Chamberlain (1994), Chamberlain (1996) and Woelkerling (1996), to evaluate species. Of 81 characteristics provided by Woelkerling and Campbell (1992) 13 were not applicable and

the remaining 68 could not be reliably used due to a wide range of variability or invariability. Consequently, 4 characteristics associated with conceptacle growth-form were used. The 4 characteristics used agreed invariably from population to population for Lithophyllum from the Gulf (Riosmena-Rodriguez, 1997). Despite these similarities, the genetic differences in this study are clear although the data are unclear as to whether they demarcate one species from another. A combination of genetic and taxonomic studies are necessary to determine if these differences are large enough to conclude that each growth-form represents a different species. Further molecular studies using markers more appropriate for higher phylogenetic resolution (eg. 18S rRNA) could test the relationships of rhodolith forming species by applying the same molecular test to related but validated species and estimating relatedness using standard phylogenetic methods.

Steller and Foster (1995) point out that more taxonomic studies are needed in order to make judgements regarding plant size and branch density when considering environmental effects on growth-form. They further point out that "variability in branch density and plant size among beds suggests that rhodolith morphology may not simply be a function of water motion" (pg. 210). Adey and MacIntyre (1973) claim that simplification of "the taxonomic structure of corallines has threatened to add considerable confusion to modern marine studies" (pg. 884). They further warn that "without some knowledge of the genera and their environmental limitations" (pg. 884) paleoenvironments cannot be interpreted simply from the occurrence of crustose corallines. While Adey and MacIntyre (1973) recognize that species of rhodoliths can be influenced by some physical factors they suggest "care should be taken on generalizing

this pattern until more information becomes available" (pg. 890). Reid and MacIntyre (1988) concluded that some morphological characteristics of rhodoliths in the eastern Caribbean have unreliable relationships with the environment. Thus using rhodoliths as paleoindicators may not be straightforward. The molecular data presented here suggest that more thought should be given to taxonomic uncertainties. It is clear that some morphological characteristics of rhodoliths such as shape and branch density are influenced by environmental factors (Bosellini and Ginsburg, 1971; Wray, 1971; Bosence, 1976; Steller and Foster, 1995; Foster et al., 1995). The results presented here, however, indicate a strong genetic component is also associated with the foliose vs. fruticose growth-forms of L. margaritae. While it is unknown what the loci sampled code for, the data suggest the loci sampled of the two growth-forms are significantly different from each other. Data on the full life history of L. margaritae, in particular, and rhodoliths in general would help clarify dynamics of these populations.

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Table 1. Sequences of primers and adaptors used for AFLP analyses.

Eco RI adaptor		5' -CTCGTAGACTGCGTACC-3'
		3' -CTGACGCATGGTTAA-5'
MseI adaptor		5'-AATTGGTACGCAGTC-3'
		3'-TACTCAGGACTCAT-5'
EcoRI +3 primers	E4	5'-AGACTGCGTACCAATTCAATG
EcoRI +3 primers	F4	5'-AGACTGCGTACCAATTCAAGT
Mse I +3 primers	E4	5'-GATGAGTCCTGAGTAATTTG
Mse I +3 primers	F4	5'-GATGAGTCCTGAGTAATTTG

Table 2. Quantity of samples collected, ran and used in statistical analyses. LP-1 samples are from Manto de Diana and LP-2 samples are from Manto de Pepe. PC-1 samples are from Manto de James and PC-2 samples are from Manto de Zachary.

	La Paz				Punta Chivato			
	Foliose		Fruticose		Foliose		Fruticose	
	LP-1	LP-2	LP-1	LP-2	PC-1	PC-2	PC-1	PC-2
Samples Collected	25	25	25	25	25	25	25	25
AFLP Samples Run	10	11	6	7	9	8	7	6
Samples Used in Analyses	10	5	4	4	6	8	5	4

Table 3. Similarity values (S) among individuals within populations prior to pooling data.

Population	S	SD	n
LP-Fo1	0.78	0.12	10
LP-Fr1	0.55	0.24	4
LP-Fo2	0.75	0.22	5
LP-Fr2	0.63	0.31	4
PC-Fo1	0.85	0.05	6
PC-Fr1	0.63	0.38	5
PC-Fo2	0.84	0.05	8
PC-Fr2	0.67	0.24	4

Table 4. Matrix of similarity values ($\pm$ SD) for within and among population comparisons. An asterisk indicates statistical significance, actual statistical values are in Table 5.

	La Paz Foliose	La Paz Fruticose	Pta. Chivato Foliose	Within Population
La Paz Foliose				0.76 ($\pm$ 0.2)
La Paz Fruticose	0.60 ($\pm$ 0.4) *			0.57 ($\pm$ 0.1)
Pta. Chivato Foliose	0.76 ($\pm$ 0.1) *	0.52 ($\pm$ 0.1) *		0.83 ($\pm$ 0.1)
Pta. Chivato Fruticose	0.38 ($\pm$ 0.1) *	0.51 ($\pm$ 0.3) *	0.62 ($\pm$ 0.6) *	0.61 ($\pm$ 0.1)

Table 5. F-Test testing for statistical significance between among and within population variation. α' is the alpha used for each successive comparison as outlined in the Dunn-Sidak method.

Source of variation	df	α'	F'	F' SE	P
LP-Fo v. LP-Fr	22	.05	.176	.061	<.01
PC-Fo v. PC-Fr	22	.03	.260	.099	<.01
LP-Fo v. PC-Fo	28	.02	.150	.024	<.01
LP-Fo v. PC-Fr	23	.01	.243	.117	<.01
LP-Fr v. PC-Fr	16	.01	.174	.062	<.01
LP-Fr v. PC-Fo	21	.01	.378	.064	<.01

Figure Captions

Figure 1. Map of Baja California Sur, Mexico with sampling site enlarged. An asterix represents a sampling site.

Figure 2. Photograph of range of growth-forms found in L. margaritae. Column A consists of fruticose individuals, column B consists of intermediate or hybrid morphologies and column C contains foliose individuals. Only fruticose and foliose rhodoliths were sampled.

Figure 3. AFLP flowchart.

Figure 4 A., B. Photograph of samples extracted with guanidine thiocyanate and protease. DNA in the 23-20 kb range was gel-purified from each sample.

Figure 5. Photograph of AFLP banding profile for samples from La Paz and Pta. Chivato. Lanes 1 and 2 are foliose from La Paz and lanes 3 and 4 are fruticose from La Paz. Lanes 5 and 6 are foliose from Pta. Chivato and lanes 7 and 8 are fruticose from Pta. Chivato. Arrows represent conserved bands.

Figure 6. UPGMA tree using genetic distance defined by Nei (1978). Each node represents genetic distance (D) between populations included in each "branch". Numbers in parenthesis indicate the proportion of similar replicates based on 1000 permutations.

Figure 1.

Figure 2.

Figure 3.

AFLPs

Amplified Fragment Length Polymorphisms

(Vos et al. 1996 NAR 23: 4407)

- (A) **Digest HMW DNA with *EcoRI* and *MseI***

AATTNNNN...NNNN
NNNN...NNNN

- (B) **Ligate *EcoRI* and *MseI* site-specific adaptors**

CTCTAGAGCTGGTACCAATTNNNN...NNNN TAATTCA...AATCAT
CATCTGAGGCAATGCTTAAGNNNN...NNNN ATTGATCTCTAGAGCTGGTAC

Selective Pre-amplification

- (C) **PCR with:
*EcoRI*_X / *MseI*_Y
Primers**

GACTGCGTACCAATTC
CTCTAGAGCTGGTACCAATTNNNN...NNNN TAATTCA...AATCAT
CATCTGAGGCAATGCTTAAGNNNN...NNNN ATTGATCTCTAGAGCTGGTAC
TAATTGAGTCCTGAGTAG

Selective Fingerprinting Amplification

- (D) **PCR with combinations of:
*EcoRI*_Xnnn / *MseI*_Ynnn
Primers**

GACTGCGTACCAATTC
CTCTAGAGCTGGTACCAATTNNNN...NNNN TAATTCA...AATCAT
CATCTGAGGCAATGCTTAAGNNNN...NNNN ATTGATCTCTAGAGCTGGTAC
TAATTGAGTCCTGAGTAG

- (E) **Gel Analysis**

CTCTAGAGCTGGTACCAATTNNNN...NNNN NGA
CATCTGAGGCAATGCTTAAGNNNN...NNNN NGTA

FN aflpmth.cdr

Figure 4a.

Figure 4b.

Figure 5.

Figure 6.