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ECOLOGICAL GENETICS OF THE HYDROTHERMAL VENT TUBEWORM, *RIDGEIA PISCESAE* AND THE HYDROCARBON SEEP MUSSEL, *BATHYMODIOLUS CHILDRESSI*

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by

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ABSTRACT

Chemosynthetic communities far below the surface of the ocean present new opportunities for studies of the interaction of organisms with their environments. The hydrothermal vents of the northeast Pacific Ocean and the cold seeps of the Gulf of Mexico serve as the setting for the work in this dissertation. Populations of the vent tubeworm *Ridgeia piscesae* at the Juan de Fuca Ridge exhibit a number of diverse phenotypes. Like all deep-sea tubeworms, *R. piscesae* relies on a symbiosis with sulfur-oxidizing bacteria within an organ in its trunk region and requires a constant supply of sulfide and oxygen from its surroundings. Different *R. piscesae* phenotypes each inhabit distinct microhabitats ranging from low temperature, low sulfide, diffuse flow, basalt substrate to vigorous flow, high temperature, sulfide chimneys. This study first uses a genome-wide DNA fingerprinting survey to test whether genetic variation undetected by previous studies underlies the two most extreme *R. piscesae* phenotypes from chimney and basalt environments. Our results confirm that this is a genetically undifferentiated population, supporting that this tubeworm species displays a high degree of phenotypic plasticity. Next, focusing on hemoglobin as a candidate gene, this study examines whether chimney and basalt *R. piscesae* differentially express the chains used to assemble their extracellular hemoglobin molecules, the direct link between the animal and its local environment. The gene expression analysis shows that *R. piscesae* on high-flow chimneys express higher levels of globin chains than animals from low-flow basalt environments, and within a phenotype, gene expression varies according to collection sites and different chemical environments. The genomic DNA sequences of the six globin chains examined in the expression analysis revealed no phenotype-specific

nucleotide variation within or upstream of the genes, thus implicating *trans*-regulation of this tubeworm hemoglobin system.

In the Gulf of Mexico, two mitochondrial and six nuclear markers were used to test whether gene flow between populations of the mussel *Bathymodiolus childressi* is limited over its range of depth, geographic distance, and environmental conditions. The results from this study yield no evidence for differentiation between *B. childressi* populations in the Gulf of Mexico, however they do suggest that the combination of geographic distance and depth may limit dispersal over the extremes of this species' range.

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PREFACE

Contributions to the research and writing of multi-authored chapters

Chapter 2: John Peoples assisted with some of the data collection (AFLP amplifications) in the final stages of the project.

Chapter 3: David Butterfield contributed chemical data from collection sites as well as pertinent text regarding the chemistry of the sampling environments.

Chapter 4: Marisa Formica assisted with the cloning and sequencing of some of the globin gene regions in multiple individuals.

Chapter 5: Kimberlyn Nelson was involved in the collection and preparation of mussel samples, and she developed the variable microsatellite marker as well as fine-tuned some of the methods involved. Himani Divatia assisted with the scoring of one of the nuclear loci, and Marisa Formica assisted with the development and scoring of three others.

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CHAPTER 1

Introduction and Motivation

The discovery of thriving communities of tubeworms and mussels at hydrothermal vents along the Galapagos Rift (Lonsdale, 1977a) and at cold seep communities off the Gulf Coast of Florida (Paull *et al.*, 1984) spurred investigations to determine how these organisms survive in what appeared to be a formidable environment. Our understanding of these systems, ultimately fueled by the energy of the earth rather than the sun, has entailed collaborative efforts of scientists from a range of disciplines. The geology of vents and seeps drives the chemistry of the environment, which in turn fuels the chemosynthetic- and methanotrophic-based communities that inhabit these settings. The work in this dissertation takes a molecular biological approach to address two main ecological questions about how deep-sea organisms reproduce and survive in vent and seep environments. First, I examine the role that the hydrothermal vent environments of the Juan de Fuca Ridge play in the differentiation of a morphologically-diverse tubeworm, *Ridgeia piscesae*. Second, I examine how physical factors such as depth, distance, or environmental site conditions may play a role in limiting gene flow between populations of a cold seep mussel, *Bathymodiolus childressi*, among hydrocarbon seeps in the Gulf of Mexico.

Hydrothermal vents

Mid-ocean ridges illustrate the dynamic nature of the earth's surface, a system of constantly shifting plates. To life on land, the most dramatic manifestation of plate

tectonics occurs in the form of earthquakes or volcanoes. At mid-ocean ridges, hydrothermal fluid emitted through the opening of a vent after relatively minimal movement of plates is a comparably dramatic occurrence that can fuel the existence of life. Movement of plates 10 to >90 mm per year sustains and creates both vigorous and diffuse flow regimes (Lonsdale, 1977b). Ocean bottom water, entrained in cracks in the seafloor along ridges, returns to the seafloor surface as hot, mineral-laden, chemically-rich fluid. Some of the chemicals and minerals precipitate out of solution into often spectacular sulfide edifices or chimney structures, and some components of the fluid are used by chemosynthetic bacteria that are the primary producers in vent systems. Invertebrates such as tubeworms, mussels, and clams have developed symbioses with chemosynthetic bacteria at vents and form the bulk of the biomass found at most sites along mid-ocean ridges worldwide.

The Endeavour segment of the northeast Pacific Juan de Fuca Ridge is the setting for most of the work in this dissertation. The Juan de Fuca Ridge is characterized by a broad heterogeneity of chemical environments and vent flow regimes. The mosaic of habitats ranging from vigorous flow, high temperature sulfide chimneys to diffuse flow, near ambient temperature (~2 °C) basalt substrate support a range of biological communities (Sarrazin *et al*., 1997; Urcuyo *et al*., 2005).

Vestimentiferan tubeworms

While a number of different taxa are found in deep-sea hydrothermal vent environments, the work in Chapters 2 through 4 of this thesis focuses on a single tubeworm species. Vestimentiferan tubeworms, currently classified as annelids within

the family Siboglinidae (Rouse, 2001), depend on a symbiotic relationship with sulfur-oxidizing bacteria inside an organ in their trunk region for their nutrition (Jones, 1985). The branchial plume is a gas exchange organ where extracellular hemoglobin molecules bind sulfide and oxygen from the environment for transport through the tubeworm's vascular system to the symbionts (Arp and Childress, 1981). The widespread distribution of tubeworms at vents and seeps worldwide (reviewed in McMullin *et al.*, 2003), is likely due in part to adaptations of the various species for different sulfide acquisition strategies. In environments where sulfide levels around the plume are low to undetectable, at least one species takes up sulfide through posterior extensions of its tube (Julian *et al.*, 1999; Freytag *et al.*, 2001). Population genetic analysis of allozymes and nucleotide sequences of vent tubeworms have supported the hypothesis that tubeworms are capable of long-distance dispersal via long-lived, planktotrophic larvae (Bucklin, 1988; Black *et al.*, 1994; Southward *et al.*, 1996; Black *et al.*, 1998). The radiation of deep-sea tubeworms is hypothesized to have occurred from seep to vent environments around 100 million years ago (Suzuki *et al*., 1993; Black *et al*. 1997; Halanych *et al*., 2001). Adaptation to life on exposed hard substrate and increasing levels of sulfide, from low levels at seeps and sedimented vent sites to higher levels at bare rock vents, has been proposed as a driving force behind the evolution of siboglinids (Schultze and Halanych, 2003).

Ecological genetics of Ridgeia piscesae

Populations of the vent tubeworm *Ridgeia piscesae* at the Juan de Fuca Ridge show a degree of phenotypic variability that spans the range of growth forms seen among different species of tubeworms from vents and seeps worldwide (Southward *et al.*, 1995).

Whether this species is composed of distinct genotypes that are selected for in an appropriate microhabitat or whether *R. piscesae* is a single species that exhibits a classic case of phenotypic plasticity was unclear when these studies were initiated. Chapter 2 of this thesis describes the use of a highly reproducible DNA fingerprinting approach, amplified fragment length polymorphism (AFLP), to test whether *R. piscesae* is one or multiple species. AFLP allows a survey of variable markers throughout the genome to determine if there is significant genetic differentiation between the two most extreme *R. piscesae* phenotypes, from chimney and basalt environments. This whole genome approach provides a more comprehensive view of the underlying variation in *R. piscesae* than previous genetic studies of this species that only examined single gene regions. The AFLP results provide strong evidence that morphologically distinct forms of *R. piscesae* are drawn from the same metapopulation. If the morphotypes of *R. piscesae* from different microhabitats are members of the same species, then what is the molecular basis of this morphological differentiation?

In Chapter 3, the focus shifts to a candidate gene, hemoglobin, which provides a tangible link between the environment and the endosymbionts within the tubeworm host. This chapter examines differences between chimney and basalt *R. piscesae* phenotypes at the level of gene expression of the hemoglobin subunits. Quantitative-real time PCR (QRT-PCR) was used to examine if the basalt- and chimney dwelling phenotypes show differential expression of the chains that are used to assemble the hemoglobin molecules. The QRT-PCR results demonstrate that *R. piscesae* on sulfide chimneys in areas of high vent flow and temperature consistently express higher levels of six globin chains than tubeworms that live in low-flow, ambient temperature, basalt environments.

Additionally, gene expression of tubeworms of the same phenotype, but from different sulfide chimneys, also varies in relation to more fine scale differences in vent fluid chemistry at similar environments. The expression level differences could result from nucleotide sequence variation either at *cis*-acting sites upstream of the structural genes for the hemoglobin subunits or from *trans*-acting factors that regulate hemoglobin expression.

Chapter 4 presents a molecular population genetics analysis designed to test whether *cis*-acting factors are responsible for differential hemoglobin chain expression in *R. piscesae* phenotypes. The genomic sequences for the six globin chains examined in the gene expression study show no phenotype- or collection site-specific nucleotide differences. As *cis*-variation was not detected, this implies that the globin genes are controlled by *trans*-regulatory elements. The presence of hypoxia response element motifs in the upstream and intronic regions of the globin chains yields a preliminary indication that oxygen may be a controlling factor in this system.

Cold seeps

Hydrocarbon and brine seeps, often found in subduction zones and at continental margins, represent areas away from mid-ocean ridges that, while geologically distinct from vents, are driven by similar chemosynthetic processes (Sibuet and Olu, 1998). This dissertation will also examine population structure of a mussel species from the seep environments of the Gulf of Mexico. The Gulf of Mexico cold seep environments, much like those at vents, are driven by the geologic setting, which in this case is the movement of a bed of buoyant, incompressible salt beneath the Gulf of Mexico seafloor (Humphris,

1979; Kennicutt *et al*., 1988). Centuries of uneven sedimentation on top of this salt layer have caused it to deform, and in some areas rise in the form of salt domes and pillars (Kennicutt *et al*., 1988). This has led to a dynamic system of conduits through the overlying sediments, through which hydrocarbons and gases, including methane, seep to the seafloor. The primary producers at seeps, methanotrophic and chemoautotrophic bacteria, derive their energy from fluid seepage, and in turn they support macrofauna through symbiotic associations. Mussels with sulfur-oxidizing, methanotrophic, and/or chemoautotrophic symbionts, are found at areas of active seepage of reduced chemicals along with other organisms similar to those found at Pacific hydrothermal vents (Kennicutt *et al*., 1985; reviewed in Sibuet and Olu, 1998).

Bathymodioline mussels

Deep-sea bathymodioline mussels, within the family Mytilidae, have a broad distribution at vents and seeps worldwide. Their success is likely due in part to their ability to supplement their nutritional input from symbionts with filter-feeding (Fisher *et al*., 1989; Page *et al.*, 1991). Vent and seep mussels can harbor methanotrophic and/or chemoautotrophic symbionts within their gills (Childress *et al.*, 1986; Fisher, 1990; Cavanaugh *et al.*, 1992; Fisher *et al.*, 1993; Distel *et al*., 1995; Le Pennec and Benninger, 2000). Recently, a new species was found in the one biogeographic province thought not to include bathymodioline mussels (McKiness *et al.*, in press). Phylogenetic analysis of mussels from seeps, vents, and from whale fall and sunken wood environments on the sea floor suggests that deep-sea mussels, much like tubeworms, radiated from shallow water habitats to seeps, and they subsequently invaded vent environments (Craddock *et al.*,

1995a; Distel *et al.*, 2000). Population genetic studies of vent bivalves have demonstrated that mussels at vents also have the capability of long-range dispersal (Craddock *et al.*, 1995b; Jollivet *et al.*, 1998; O'Mullan *et al.*, 2001; Won *et al.*, 2003a).

Population structure analysis of Bathymodiolus childressi

Population genetic characterizations of seep fauna are not as prevalent as those of their vent relatives. A recent analysis of microsatellite DNA from two seep tubeworms has shown little or no evidence for differentiation of populations within the 1000 km depth range of the upper Louisiana Slope of the Gulf of Mexico (McMullin *et al*., in prep.). The genetic structure of populations of the mussel *Bathymodiolus childressi* from the same sites and from additional sites over its broader range of depth and geographic distance has not been characterized. A previous study identified limited evidence for differentiation between two Gulf of Mexico *B. childressi* populations that differ in depth by almost 1700 m (Craddock *et al.*, 1995a). Substantiation of these results would reveal that the dispersal limitations imposed on *B. childressi*, whether they are as a result of depth, distance or alternate factors, are greater than those faced by mussels from vent sites which have greater depth and geographic ranges (Craddock *et al.*, 1995b; O'Mullan *et al.*, 2001). An estimate of gene flow in this species will also give us an indication of the stability of the population and its potential ability to recover from habitat disturbance. Hydrocarbon seeps, unlike vent environments, are currently of significant economic importance because of the oil and gas industry. The impact of oil drilling on biological communities in deep regions of the Gulf of Mexico is not known. Chapter 5 presents an analysis of molecular markers in the deep-sea mussel *Bathymodiolus childressi,* from

hydrocarbon and brine seeps of the Gulf of Mexico to determine if gene flow is limited or extensive between populations of this mussel throughout its depth, geographic and environmental range. Nucleotide sequence and restriction fragment length polymorphism (RFLP) analysis of eight molecular markers was used to genotype 183 mussels from nine localities. Analysis of these markers provides no evidence that even the most disparate populations are genetically differentiated. There was, however, a slight trend for isolation with geographic distance suggesting that gene flow in *B. childressi* is panmictic throughout the Gulf of Mexico.

CHAPTER 2

AFLP analyses of genomic DNA reveal no differentiation between two phenotypes of the vestimentiferan tubeworm, *Ridgeia piscesae*

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Introduction

Ridgeia piscesae Jones (1985) is the only vestimentiferan tubeworm species at the Juan de Fuca Ridge of the northeast Pacific, yet the range of microhabitats it inhabits and phenotypes it expresses encompasses that of all known vestimentiferan tubeworms known at seeps and vents throughout the world. Initially, two species of *Ridgeia* were formally described, and up to five were proposed based on distinct morphological phenotypes (Jones, 1985; Tunnicliffe and Fontaine, 1987; deBurgh *et al*., 1989; Tunnicliffe, 1991). Studies of variation in allozymes, in the mitochondrial cytochrome oxidase I gene, and in nuclear ribosomal genes, however, have detected no significant differentiation between different *Ridgeia* morphs (Southward *et al*., 1995; Southward *et al*., 1996; Black *et al*., 1997). The vent habitats on the Juan de Fuca Ridge are diverse, varying in vent fluid flow rate, chemical composition, temperature, and substrate types. The morphological forms of *R. piscesae* that inhabit these microhabitats differ considerably in length, diameter, and details of their body morphology, as well as in characteristics of their tube (Jones, 1985; Southward *et al*., 1995).

The present study focuses on two of the most extreme phenotypes, which we refer to as long-skinny and short-fat. Long-skinny *Ridgeia* are found on basalt substrate in weak, diffuse flow environments of low sulfide levels, where temperatures generally range from ambient (2 ˚C) to only a few degrees above (Robigou *et al*., 1993; Urcuyo *et al*., 1998). Tubes of mature long-skinny individuals are relatively sturdy, rigid, and average one meter in length, tapering to approximately 1 mm in diameter posteriorly. Long-skinny *Ridgeia* resemble the seep vestimentiferan *Lamellibrachia* cf. *lumeysi* van der Land and Nørrevang (1975) from the Gulf of Mexico which has been shown to

acquire sulfide through its roots at levels sufficient to sustain net chemoautotrophy (Freytag *et al*., 2001). Based on similar posterior tube permeability characteristics, it has been hypothesized that the long-skinny morph of *Ridgeia* may also supplement its sulfide uptake in this manner, a useful adaptation for tubeworms that live in environments where sulfide is often undetectable around the animals' plumes (Urcuyo *et al*., submitted 2001)[1]. Short-fat individuals are found on sulfide edifices, in areas with visibly active venting fluid that is up to 30˚C and with sulfide concentrations around 200 µM (Robigou *et al*., 1993; Sarrazin *et al*., 1997). Short-fat tubes are very thin and relatively flimsy, averaging 15 to 20 cm in length, 1 cm in diameter, and displaying pronounced flanges along their length.

Several pieces of evidence suggest that induction of phenotype in *R. piscesae* is due to environmental cues. In a single heterogeneous flow aggregation of *Ridgeia* at the Endeavor Segment of the Juan de Fuca Ridge, Urcuyo *et al*. (submitted 2001) found two clusters of newly settled individuals. The tubeworms that had settled in an area of lower flow developed roots, while those in a higher vent flow area did not. Studies currently being conducted on the blood physiology of short-fat and long-skinny *Ridgeia* have detected morph-specific differences in the structure and function of hemoglobin (J. Flores, PSU, pers. comm.). Initial studies have detected higher concentrations of hemoglobin in the coelomic fluid of the short-fat morph as compared to the long-skinny morph. This suggests that short-fat *Ridgeia* can bind considerably greater concentrations of sulfide from their high-flow, high sulfide environments. These studies have also found that the hemoglobin in the long-skinny morph has a very high affinity for sulfide. This again might be a valuable adaptation to low-sulfide environments. Ongoing studies by

[1] This work was subsequently published as Urcuyo *et al*. 2003.

Flores *et al*. will attempt to ascertain the physiological differences between the hemoglobins of short-fat and long-skinny *R. piscesae* at the level of protein structure.

The basis of the differentiation of distinct *R. piscesae* phenotypes is still unknown. Although the previous genetic studies of *Ridgeia* have detected no significant differentiation between morphs, these studies only surveyed limited gene regions and allozymes. Allozymes have been criticized for their potential to be subject to selection and for their frequent underestimation of population differentiation due to an inability to detect silent nucleotide substitutions and amino acid changes that do not result in detectable changes in proteins (Hartl and Clark, 1997). Undetected genetic differentiation could still leave open the possibility of selection of genotypes by microhabitats as a driving force for phenotypic differentiation. If indeed no fixed genetic differences exist between the two morphs, then the mechanism for differentiation might be due to differences in gene expression. This study takes a more comprehensive approach, comparing amplified fragment length polymorphisms (AFLPs) between sample populations of short-fat and long-skinny *R. piscesae*.

The AFLP technique generates highly reproducible fingerprints of genomic DNA (Vos *et al*., 1995). Genomic DNA is digested with two restriction enzymes, and the ligation of double-stranded adapters onto the resulting fragments provides specific binding sites for primers in PCR amplifications. Selectivity is introduced by the addition of different three-nucleotide 3' extensions onto the primers. This, along with high annealing temperature PCR cycles, amplifies only a select subset of the genomic fragments, generating a unique genomic fingerprint with each different primer combination. By surveying the whole genome, this method is more likely to detect subtle

genetic differentiation that might otherwise go undetected. Our results with this technique confirm the findings of previous genetic studies, suggesting that the very different phenotypes of *R. piscesae* represent one species. This more complete determination of the genetics of these two distinct morphotypes provides a foundation for understanding how a single organism can tolerate and exploit such a broad range of environmental conditions.

Materials and Methods

Ridgeia piscesae were collected with the manipulator arm of ROPOS at the Endeavour Segment of the Juan de Fuca Ridge in May 2001. Long-skinny tubeworms were retrieved from a basalt environment at Clam Bed (47º 57'N, 129º 05'W) while short-fat animals were retrieved from a portion of the Smoke and Mirrors sulfide edifice named Strawberry Fields (47º 56'N, 128º 05'W). The animals were brought to the surface in a temperature-insulated box and were kept in chilled seawater for up to eight hours before dissection. Pieces of the vestimentum and trophosome were immediately frozen in liquid nitrogen and then transferred back to the lab on dry ice where genomic DNA extraction was performed via a standard phenol/chloroform technique. DNA from each vestimentum sample was subjected to AFLP analysis using the AFLP System I kit (Life Technologies, Inc.). One of the two primers used in each selective amplification of the AFLP process was fluorescently-labeled (Resgen, Inc). Fragment sizes were then detected by use of the Fragment Analysis System of the Beckman CEQ 2000 automated sequencer. Samples were scored for the presence or absence of fragments after visual confirmation of peaks (detection threshold = 5%), and the method of Zhivotovsky (1999)

was used to estimate allele frequencies between and within the two sample populations based on the frequency of null alleles.

Results

No fixed differences were found in either the short-fat or the long-skinny populations of *R. piscesae*; there were no fragments found in all twelve individuals of one morphotype and absent in the other. Of the 677 fragments generated through the selective amplifications with ten primer combinations, 68.4% were found in both populations (Table 2-1). 10.1% of the fragments generated were found in all of the 24 individuals sampled. Of the fragments that were found only in some individuals of one population and not in the other, 22.7% were singletons, found in only one of the 24 individuals sampled.

Table 2-1. Summary of AFLP data for 12 long-skinny and 12 short-fat individuals of *R. piscesae*

Primer combination	N	Polymorphic SF/ Absent LS	Shared	Polymorphic LS/ Absent SF
E-AGG/M-CTA	36	3	25	8
E-AGG/M-CTC	44	8	29	7
E-AGG/M-CTG	73	13	48	12
E-AGG/M-CTT	54	9	35	10
E-AGG/M-CAG	48	12	29	7
E-AGG/M-CAC	54	4	43	7
E-AGG/M-CAT	140	23	90	27
E-ACT/M-CTC	67	9	48	10
E-ACT/M-CTG	72	8	49	15
E-ACT/M-CAT	89	6	67	16
Total	677	95	463	119

N = total number of fragments; SF = short-fat morph; LS = long-skinny

Data from all of the fragment analysis runs were compiled into a presence/absence matrix. AFLPs are dominant markers, i.e., heterozygotes cannot be distinguished from homozygotes because both genotypes show the presence of a fragment. Thus, the frequency of heterozygotes was estimated from the frequency of null alleles with Bayesian methods (Zhivotovsky, 1999). The levels of heterozygosity averaged over AFLP fragments were used to estimate F_{ST} and *Nm*. F_{ST} is the inbreeding coefficient due to nonrandom mating in subpopulations relative to the total population. F_{ST} values can vary from 0 to 1, where 0 equals no differentiation and 1 equals complete differentiation. F_{ST} values around 0.25 indicate great differentiation (Hartl and Clark, 1997). The neutral migration parameter, *Nm*, estimates the number of migrants per generation that are exchanged between the two populations (Hartl and Clark, 1997). A value of *Nm* greater than or equal to 1 is sufficient to prevent differentiation between populations. We used 10,000 random permutations that shuffled the 24 individuals among the populations to derive a 95% confidence interval for our observed estimates of F_{ST} and *Nm*. The AFLP data for the two *R. piscesae* morphs yield an overall F_{ST} estimate of 0.0386 (95% confidence interval = 0.0317 to 0.0458) and an *Nm* estimate of 6.2 migrants per generation (95% confidence interval = 5.2 – 7.6).

Discussion

The results of this genome-wide survey of DNA fragments between short-fat and long-skinny *R. piscesae* populations suggest that no significant differentiation exists between these two extreme phenotypes based on the individuals sampled. The estimated level of migration of 6.2 migrants per generation implies that a free exchange of gametes

is occurring between these two populations. Thus, the dramatic phenotypic variation seen in this vestimentiferan species does not result from fixed genetic differences that accumulated through limited gene flow. Given that the short-fat and long-skinny morphotypes exist in very different microhabitats, it is likely that environmentally induced differences in gene expression are responsible for the development of such extreme phenotypes. One of the key environmental differences between the habitats of long-skinny and short-fat *Ridgeia* is the concentration of sulfide in the water around their plumes. A possible scenario for the differentiation of phenotypes could be that a single, genetically identical larval population exists in the water column after reproduction. The development of habitat-specific growth forms is then induced by the microenvironment where the larvae settle. The sulfide levels, temperature and other factors may trigger the development of larvae into short-fat *Ridgeia* where vent flow is high and into long-skinny *Ridgeia* where vent flow is low. The results of this AFLP study provide more conclusive evidence that the morphs of *R. piscesae* represent a single, highly variable species, and thus lay the groundwork for future studies to detect morph and tissue-specific differences in gene expression.

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CHAPTER 3

Phenotypic- and site-specific differences in hemoglobin expression in the hydrothermal vent tubeworm, *Ridgeia piscesae*

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Abstract

Ridgeia piscesae, the only vestimentiferan tubeworm species at the hydrothermal vents of the Juan de Fuca Ridge in the northeast Pacific, displays a wide range of phenotypes that lack significant levels of genetic differentiation. Each phenotype settles into a distinct microhabitat, ranging from low-temperature diffuse flows issuing from cracks in basalt to higher-temperature more vigorous flows on sulfide chimneys. It has been hypothesized that local microhabitats play a role in the differentiation of *Ridgeia* growth forms. Vestimentiferans rely on a symbiosis with intracellular, sulfide-oxidizing bacterial symbionts as their sole means of nutrition. The symbionts exist within a well-vascularized organ, the trophosome. Extracellular hemoglobins bind and transport sulfide and oxygen from the environment to the symbionts that use the chemical energy to fix carbon. Because of the essential role of hemoglobin in sustaining this symbiosis, we examined gene expression of hemoglobin chains of the two most extreme *Ridgeia* phenotypes from two very different habitats. Quantitative real-time PCR analyses of six different globin subunits were performed on "long-skinny" and "short-fat" *R. piscesae* to determine if globin gene expression differs in animals that are genetically indistinguishable. Long-skinny tubeworms from a diffuse flow basalt habitat show lower globin expression levels compared to short-fat individuals collected from three different sulfide chimneys. Within the short-fat population, gene expression also varied by collection site. These data suggest that local environmental factors influence gene expression within this phenotypically diverse species.

Introduction

Chemically and geologically diverse vent fields along the Juan de Fuca Ridge in the northeast Pacific Ocean provide habitat for a variety of vent fauna, including a single species of vestimentiferan tubeworm, *Ridgeia piscesae*. The vestimentiferan tubeworms act as foundation species of many Juan de Fuca Ridge hydrothermal vent communities because they form dense aggregations that provide habitats for numerous other vent organisms and they supply much of the biomass to the community through their association with chemosynthetic endosymbionts (Tunnicliffe, 1991; Bruno *et al.*, 2003; Urcuyo *et al.*, 2005).

Early investigators suggested as many as five different species within the genus *Ridgeia* were present in the northeast Pacific because of the wide variety of different morphological growth forms, and two species were initially described from vents along the Juan de Fuca Ridge (Jones, 1985; Tunnicliffe, 1988; Tunnicliffe and Juniper, 1990). Despite the considerable phenotypic variation of these tubeworms, the different growth forms are not genetically differentiated, and they are all currently considered to be a single species, *R. piscesae* (Southward *et al.*, 1995; Southward *et al.*, 1996; Black *et al.*, 1998; Carney *et al.*, 2002). Such a range of distinct phenotypes and the lack of detectable genetic variation suggest that *R. piscesae* may display a classic case of phenotypic plasticity, where a given genotype can produce multiple phenotypes depending upon how the animal responds to different environmental conditions (Zhivotovsky *et al.*, 1996). It has been suggested that phenotypic plasticity may be a common feature of vestimentiferan tubeworms because *Riftia pachyptila*, a close relative found at the hydrothermal vents at the East Pacific Rise (EPR), shows modest morphological variation

among vent sites in the absence of significant genetic differentiation (Black *et al.*, 1994). Black *et al*. (1994) proposed that variable details of body morphology in *R. pachyptila* might be a result of different microhabitat conditions. It has also been hypothesized that this might be the case in *R. piscesae*, which exhibits a much greater degree of plasticity. To date, the underlying mechanism that causes morphological differentiation is unknown in either species (Tunnicliffe, 1988; Southward *et al.*, 1995; Southward *et al.*, 1996).

This study focuses on two of the most extreme *Ridgeia* phenotypes which we designate as "short-fat" and "long-skinny" (previously referred to as "short and thick" and "long and thin" (Southward *et al.*, 1995; Sarrazin *et al.*, 1997). The general anatomy of all *R. piscesae* phenotypes is similar to that of their seep and vent vestimentiferan relatives (Jones, 1985; Southward *et al.*, 1995). A chitinous tube that can vary in color and rigidity encases and supports the body of the tubeworm (Gaill and Hunt, 1986; Southward *et al.*, 1995). The body of an adult individual consists of four main regions (Figure 3-1). Located anteriorly, the obturacular region includes the branchial plume, a gill-like respiratory organ and primary site of gas exchange with the environment (Jones, 1981; Arp *et al.*, 1985). Below the obturaculum is a muscular region, the vestimentum. Found within this region are the excretory ducts, heart, a simplified brain, and the gonopores (Jones and Gardiner, 1989; Schulze, 2001). The trophosome, which makes up the bulk of the trunk region, is a mass of tissue composed primarily of symbiont-containing bacteriocytes (Hand, 1987). The posterior-most region is the segmented opisthosome (Jones, 1981).

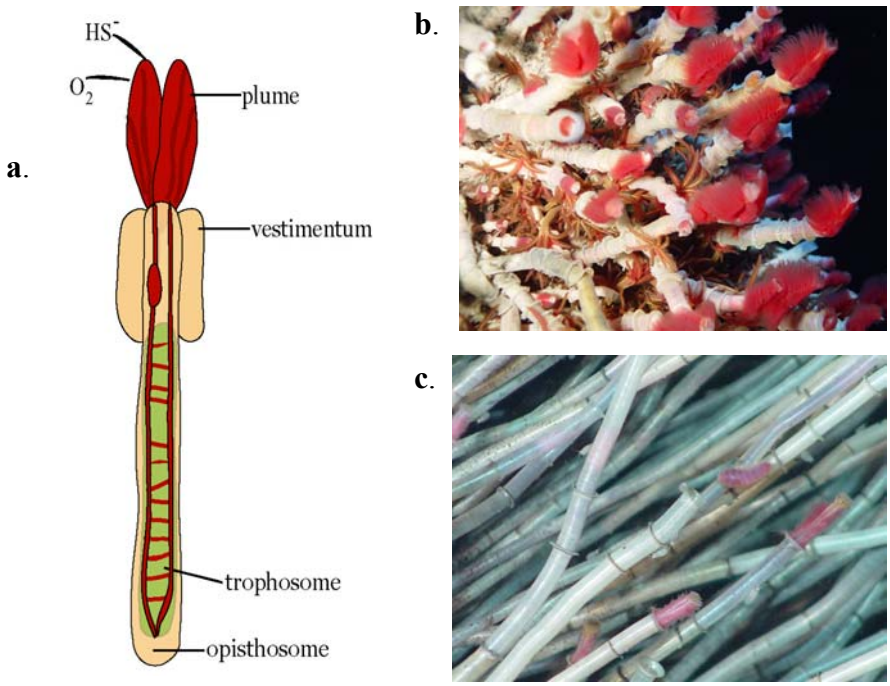

Figure 3-1. The vestimentiferan body plan. The cartoon on the left (a) shows the four main regions of all *R. piscesae* phenotypes. The plume is the only visible part of the animal outside of the tube. The photo on the top (b) shows short-fat *R. piscesae* on a chimney at S&M, while the photo on the bottom (c) shows the long-skinny phenotype at Clam Bed. Photos from ROPOS.

Some of the most distinct variations that are seen between different tubeworm species in deep-sea environments throughout the world are also present within *R. piscesae*. Long-skinny *R. piscesae* are supported by rigid tubes that can support the animal often as high as a meter above the surface (Southward *et al*., 1995). Often, these tubes display posterior extensions that are thin and permeable to sulfide and that extend posteriorly into basalt cracks (Urcuyo *et al*., 2003). This growth form of *R. piscesae* resembles that of seep vestimentiferans within the genus *Lamellibrachia*, while short-fat *R. piscesae* are similar to miniature versions of the giant tubeworm *R. pachyptila* from the EPR.

In most vent tubeworms, hydrogen sulfide, in the form HS^-, and oxygen are taken into the body through the plume and carried to the bacterial symbionts in the trophosome

bound to extracellular hemoglobins with high affinities for oxygen and sulfide (Arp and Childress, 1981; Arp *et al.*, 1985; Arp *et al.*, 1987; Fisher *et al.*, 1988; Goffredi *et al.*, 1997). Using substrates (HS^-, CO_2, O_2) provided by the tubeworm, the symbionts use the energy captured from the oxidation of sulfide to fix carbon into organic compounds, which in turn are supplied to the host (Cavanaugh, 1983; Childress and Fisher, 1992; Felbeck and Jarchow, 1998; Bright *et al.*, 2000). The internal location of the symbionts requires the circulatory system to deliver the substrates required for carbon fixation. The vestimentiferan body plan, with the vascularized plume, circulating hemoglobins, and musculature allowing the worm to position its body optimally within its tube, is crucial to the success of this symbiosis (Felbeck and Childress, 1988).

Short-fat and long-skinny *R. piscesae* are found in distinctly different environments along the Juan de Fuca Ridge (Tunnicliffe and Fontaine, 1987; Tunnicliffe *et al.*, 1990; Southward *et al.*, 1995; Scott *et al.*, 1999; Urcuyo *et al.*, 2003, 2005). Short-fat *R. piscesae* are found attached to sulfide chimneys by the posterior ends of their tubes in areas of relatively high flow rate vent fluid that bathes the entire length of the worm (Sarrazin *et al.*, 1997). The tubes of this phenotype are flexible, and individuals are arrayed in dense aggregates which, in combination with the vent flow, offer physical support. The long-skinny morph is found in low-temperature, low-flow basalt habitats such as Clam Bed, often with a sizeable portion of its posterior end anchored in the substrate. Tubes of long-skinny *R. piscesae* are more rigid and can support the animal upright with its plume often more than 0.5 m above the seafloor, where temperatures have been recorded to be less than or equal to 1 °C above ambient temperatures 99% of the time (Urcuyo *et al.*, 2003, 2005). Corresponding with differences in flow and

temperature regimes, the chemistry of the vent water surrounding the distinct phenotypes also differs. Average concentrations of sulfide around the plume of short-fat *R. piscesae* have been measured at 38.32 ± 47.2 μM at the Smoke and Mirrors (S&M) vent structure (Sarrazin *et al.*, 1999), while those at plume level for the long-skinny phenotype have been calculated to be less than 0.1 μM on average (Urcuyo *et al.*, 2003, 2005).

Chemical heterogeneity among the hydrothermal vents of Endeavour may be an important factor that influences the biology of *R. piscesae*. The hydrothermal system on the Endeavour segment is extremely vigorous, with many high-temperature vents, gradients in composition across the Main Endeavour Field (MEF), and significant areas of diffuse flow (Schultz *et al.*, 1992; Delaney *et al.*, 1992; Lilley *et al.*, 1993; Butterfield *et al.*, 1994; Tivey *et al.*, 1999). All tubeworms require a supply of sulfide and oxygen in a tolerable temperature range, so fluid flow and mixing are critical physical parameters. Two of the most crucial aspects of the fluid chemistry that are relevant to microbial production in diffuse flow vents are the ratio of H_2S to heat and H_2S to iron. The high-temperature fluids at Endeavour generally have relatively high total H_2S/Fe ratios (typically 3 to 10; Butterfield *et al.*, 1994; Seyfried *et al.*, 2003). If flow rates are fast and residence time along the flow path from the hot source fluid to the diffuse vent habitat are short, which would be typical of diffuse flow on sulfide edifices in the immediate vicinity of a hot smoker vent, then the major impact on chemistry is caused by relatively fast metal sulfide precipitation reactions. This generates diffuse fluids that, after sub-seafloor precipitation of metal sulfides, have even higher H_2S/Fe ratios, leaving sulfide in solution largely uncomplexed and available for utilization by *R. piscesae* and other sulfide-oxidizing organisms. In basalt-hosted diffuse flow, the flow path and residence time are

probably longer, the flow rates and temperatures are certainly lower (Urcuyo *et al*., 2003, 2005) and oxidation of sulfide can occur (e.g. Butterfield *et al*., 2004). This can lead to significantly lower H_2S/heat ratios and highly variable H_2S/Fe ratios (Butterfield *et al.*, 2004).

Gradients in chemistry and temperature in the MEF were quite stable until a July 1999 earthquake swarm that involved sub-seafloor magmatic activity (Lilley *et al.*, 2003; Seyfried *et al.*, 2003). The impact of this event on temperature in long-skinny tubeworm habitat at Endeavour is described by Johnson *et al.* (2000) and seen in Figure 4D and E in Urcuyo *et al*. (2005). The most notable effects on the hot source fluid were significant, temporary (< 1 year) increases in gas content (particularly CO_2, but also H_2S) and decreases in Cl content (Lilley *et al.*, 2003; Seyfried *et al.*, 2003). Vent fluid compositions in the MEF have been relatively stable since 2001 (Butterfield, unpublished data). Urcuyo *et al*. (2005) present sulfide-temperature data that are consistent with data from vent fluid samples taken at Clam Bed in 1995 and 2003. Clam Bed consistently has a higher H_2S/deltaT and higher H_2S/Fe ratio than either S&M or Milli-Q, which are quite similar to each other (Table 3-1).

Short-fat and long-skinny *R. piscesae* each have unique physical characteristics in their respective chemical environments (Figure 3-1). The phenotypes differ considerably in size, overall appearance, and in a number of fine-scale morphological details (Jones, 1987). One of the most striking differences between these two phenotypes is the presence of a posterior "root", which is a thin-walled, tapered extension of the tube that is sometimes present in the long-skinny form. Uptake of sulfide through the roots of vestimentiferans at levels sufficient to sustain chemoautotrophy has been

Table 3-1. Vent fluid compositions at short-fat *R. piscesae* collection sites in 2003. Concentrations are calculated to zero Mg endmember values. H_2S/deltaT calculated as endmember H_2S concentration in µmol/kg divided by [maximum (endmember) vent temperature – background T], where background T is 2.0 °C.

	Dive Number	H_2S (mmol/kg)	Fe (µmol/kg)	Cl (mmol/kg)	Fe/H_2S	Sample temperature (°C)	H_2S/delT (µmol/kg-degC)
S&M	R719	5.6	1860	472	0.33	352	16
Milli-Q	R719	5.0	1600	489	0.32	318	15.5
Clam Bed – smoker	R729	5.3	897	408	0.17	41.6*	36

* Note - Clam Bed temp in 2003 is not the maximum present, but is the maximum measured at the sampling site. Measurements in 2004 were 305 °C at Clam Bed (Butterfield, unpublished data).

documented in *Lamellibrachia luymesi* from Gulf of Mexico hydrocarbon seeps, where sulfide levels are often very low to undetectable around the plumes (Julian *et al.*, 1999; Freytag *et al.*, 2001). The roots of long-skinny *R. piscesae* display sulfide permeability coefficients similar to those measured in *L. luymesi*, suggesting that this morphotype of *R. piscesae* may also use "roots" for sulfide acquisition in low-flow environments where plume-level sulfide concentrations are not consistently sufficient to sustain the animals' autotrophic lifestyle (Urcuyo *et al.*, 2003).

Hemoglobin provides a direct link between the external environment and *R. piscesae*'s symbionts whether or not sulfide is obtained across the posterior "root" or from vent water surrounding the plume. Hemoglobin concentration and function have been observed to be sensitive to exogenous factors, particularly oxygen tensions, in invertebrates, amphibians, and mammals (Hochachka *et al.*, 1998; Weber *et al.*, 2002; Paul *et al.*, 2004). The site of hemoglobin synthesis in tubeworms is not known, but preliminary studies have suggested that it is synthesized and secreted in afferent blood

vessels leading into the trophosome (Andersen *et al.*, 2001). Tubeworms contain three extracellular hemoglobin molecules: a giant, ~3.5 MDa hexagonal bilayer hemoglobin (V1) and a ~400 kDa hemoglobin (V2) in the vascular fluid, as well as a ~400 kDa hemoglobin (C1) in the coelomic fluid (Terwilliger *et al.*, 1980; Suzuki *et al.*, 1988; Zal *et al.*, 1996). The ~400 kDa hemoglobins are composed of 24 heme-bearing globin chains of four to six different types, depending on the species (Suzuki *et al.*, 1988; Zal *et al.*, 1998). Phylogenetic analysis of annelid globin chains has revealed two distinct clades, which have been designated as A and B (Gotoh *et al.*, 1987). Within vestimentiferans, these chains can be further subdivided into four chain types, A1, A2, B1, and B2, based on amino acid sequence data (Bailly *et al.*, 2002). Oxygen is bound to the heme group on each of the globin chains (Terwilliger, 1998). Sulfide-binding is associated with free cysteine and/or zinc residues in the globin chains (Suzuki *et al*., 1990a; Zal *et al.*, 1998; Flores *et al.*, 2005). The giant hemoglobin, V1, is composed of heme-bearing globin chains held together by non-heme-containing linker chains that play a key role in the assembly of these molecules into hexagonal bilayer structures and in sulfide binding (Suzuki *et al.*, 1988; Suzuki *et al.*, 1990b; Zal *et al.*, 1998).

Given the essential link of hemoglobin between the tubeworm, its endosymbionts, and the environment, we tested whether short-fat and long-skinny *R. piscesae* differentially express the six genes that encode the globin chains of the ~400 kDa and ~3.5 MDa hemoglobin molecules in response to differences in environmental chemistry. Using quantitative real-time PCR (QRT-PCR), we compared the relative levels of expression of each chain between the phenotypes to test the hypothesis that expression of hemoglobin genes in *Ridgeia* is phenotype-specific. Elevated expression of all globin

chains in one phenotype compared to the other would suggest that each phenotype responds to environmental differences by an overall increase or decrease in the synthesis of hemoglobin molecules. Elevated or decreased expression of some chains and not others would indicate differential usage of globin chains and perhaps the different hemoglobin molecules between long-skinny and short-fat *R. piscesae*.

Materials and Methods

Collection site descriptions

Long-skinny *R. piscesae* were collected from Clam Bed, a basalt site located approximately 1.5 km north of the Main Endeavour vent field of the Endeavour Segment on the Juan de Fuca Ridge. Previous studies have characterized environmental conditions around long-skinny tubeworms at this site (Urcuyo *et al.*, 2003, 2005). Short-fat animals were collected from three different chimneys during 2002 and 2003. At the S&M sulfide edifice, animals were taken from a portion of Strawberry Fields in 2002. The environment around tubeworms at S&M has also been characterized (Sarrazin *et al.*, 1997; Sarrazin and Juniper, 1999; Sarrazin *et al.*, 1999). Short-fat *R. piscesae* were collected at Milli-Q, a sulfide chimney located approximately 75 meters south of S&M on the southern end of the Main Endeavour Field and from a smoker at Clam Bed in 2003. Environmental measurements were not taken at the precise time of collections, but vent fluid measurements from these sites taken using ROPOS near the times and locations of collections have been compiled in Table 3-1. These data and historical data are used to characterize and compare the sample collection sites.

Sample collections

Samples of *Ridgeia piscesae* were collected during cruises to the Endeavour segment of the Juan de Fuca Ridge in 2002 and 2003 (Table 3-2). Tubeworms were collected with the manipulator arm of the remotely operated vehicle (ROV) ROPOS. Animals were transported to the surface inside a temperature-insulated box and upon recovery were immediately transferred to a cooler of chilled seawater at 4 °C. Live individuals were dissected immediately after each was removed from the cooler.

Table 3-2. Information regarding *R. piscesae* samples collected for gene expression analyses

Dive Number	Date	Site	Phenotype	Coordinates	Depth (meters)	Number of Individuals
R682	08/19/02	Clam Bed	Long-skinny	47°57.78 N, 129°04.58 W	2194	8
R724	08/14/03				2198	12
R683	08/20/02	Strawberry Fields	Short-fat	47°56.90 N, 129°05.88 W	2184	10
R720	08/11/03	Clam Bed	Short-fat	47°57.78 N, 129°04.58 W	2194	8
R730	08/21/03	Milli-Q	Short-fat	47°55.43 N, 129°06.51 W	2187	8

RNA extraction and cDNA synthesis

RNA was extracted from fresh tissue using RNAwiz (Ambion, Inc.) following the product protocol. RNA pellets were stored at -80 °C during transport back to the laboratory on dry ice and maintained at -80 °C until used. RNA pellets were resuspended in 50 µl nuclease-free water and quantified spectrophotometrically. Total RNA (2 µg) was reverse-transcribed in a total volume of 25 ul using the First Strand cDNA synthesis kit according to the manufacturer's protocol (Novagen, Inc.).

Globin chain amplification and sequencing

Each of the four globin chain types, A1, A2, B1 and B2, was amplified from the cDNA pool with PCR reactions using degenerate primers. The degenerate primers for the B1 globin chains were designed based on primers used in Bailly *et al.* (2002). For the A2, B2 and A1 chains, primers were designed based on the alignment of globin proteins from *Oligobrachia mashikoi*, *Sabella spallanzanii*, *Lamellibrachia* sp., *Lumbricus terrestris, and Tylorrhynchus heterochaetus* as well as the complete *Riftia pachyptila* A2 amino acid sequence (accession numbers as in Bailly *et al*. (2002) and P80592 from ExPASy (SIB)). Amino acid alignments were done using Megalign 3.1.7 from the Lasergene sequence analysis package (DNAStar). PCR reactions consisted of a 25 µl volume containing 0.5 µl of cDNA template, 2.5 µl 10X PCR buffer (with 15 mM $MgCl_2$, supplied by the manufacturer), 2.5 µl 0.5 mM dNTPs, 200 ng of each of the forward and reverse primer, and 0.625 U of Taq DNA polymerase (GeneChoice, Inc.). The forward and reverse primers for each globin subunit and the respective annealing temperature are shown in Table 3-3. The PCR amplification cycles were performed in a Perkin-Elmer Cetus thermal cycler as follows: one cycle of denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature for 1 min, and extension at 72 °C for 1 min, with an additional 10 minutes of extension at 72 °C at the end.

For each globin subunit, PCR products of the desired sizes were extracted from 2% agarose gels with a QIAquick Gel Extraction Kit™ (QIAGEN Inc.) and concentrated to 100 ng/µl. The PCR products were subcloned into chemically competent TOP10 *E. coli* cells using the TOPO TA cloning® method (Invitrogen). The subcloned inserts were

Table 3-3. Primers used in PCR and QRT-PCR reactions of globin chains A2, A1, B2, B1a, B1c and B1new in *R. piscesae*. ND4 primer sequences can be found in Maas *et al.* (1999), and B1 globin Degenerate primer sequences, as well as A1 F, are from Bailly *et al.* (2002).
F = forward primer; R = reverse primer; * = labeled at 5' end with 6-FAM and at 3' end with BHQ-1

Primer	Sequence (5' – 3')	Annealing temperature (°C)	Accession No.
	cDNA amplifications		
A1 F	TGG GCN RAR GCN TAY GG	60	———
A1 R	RTC RAA RCA NAC NCC RAA		
A2 F	TTA TGT GGG CCC TTG TCT TC	50	———
A2 R	TGC CAN GCR TCY TKR TC		
B1 F	GTN AYH AAR CAG TGG VA	56	———
B1 R	CCA NGC RTC NGG NTT RAA		
B2 F	ACG TCG TAT CGC CAT TGC C	50	AY250083
B2 R	TGG CGA CCT GGG GCA TGA		
Oligo dT	TTT TTT TTT TTT TTT TTT	50 – 60	——
	Quantitative real-time PCR		
A1 F	ACG ACC CAC GTA GCA TCG A		
A1 R	GCT ACC GTC CCA ATC AGT GC	60	
A1 probe*	CCG CCA ATT TCG TCG TGT TCA GAG A		
A2 F	GAC AAC ATC CAC ACC CCA GAA		
A2 R	TGT CGA GTC CAC CAA GCA CA	60	
A2 probe*	TCA GGG CCC ACG CCA CCC		
B2 F	AGG CTG TCT TCA AGG ACC TCT TC		
B2 R	TCA CAC CGC CGA ACA GG	60	
B2 probe*	CCA ACG TGC CCG CTG CCG TAT		
B1a F	CGA GAG TCA ACG TCG ACA ACA		
B1a R	CCA GAC CGC CCA TCA CAC	60	
B1a probe*	AGC CCC GAA TTC AAC GGT CAC GT		
B1new F	AAC TTC GAA TCA CCC GAA TTC A		
B1new R	TGA CCA GCA CGT CCA GTC C	60	
B1new probe*	CGG CCA CAT CAT GCG CGT G		
B1c F	AAT GTC GGC GAC ATG AGG A		
B1c R	CCT GTC ATC ACA CGC ACC A	60	
B1c probe*	CCC GCA GTT CAG CGC CCA G		

amplified from recombinant colonies using universal M13F and M13R primers, and PCR fragments of the appropriate size were treated with ExoSAP-IT (USB Corporation) to remove excess primers and dNTPs. These PCR products were sequenced directly in both directions with the M13F and M13R primers using dye terminator cycle sequencing, and the sequencing products were run on a Beckman CEQ2000 automated sequencer (Beckman Coulter). At least four clones were sequenced for each chain type to ensure an

unambiguous consensus sequence for each coding region. Sequence reads from both strands of each clone were assembled using the program Seqman II from the Lasergene sequence analysis package (DNAStar). Conflicts among the overlapping sequence reads were resolved by eye. Assembled, edited sequences were aligned to identify clone variants, and verification of the identity of different clones was determined using BLASTN and BLASTX (Altschul *et al.*, 1997).

After the identification of clones homologous to the A2, B2, A1, B1a, B1c and another variant of the B1 globin chains, designated here as "B1new", nucleotide sequences of each chain type were aligned to find unique regions to design chain-specific primer/probe sets for QRT-PCR. All globin sequences were deposited in GenBank (accession numbers: XXXXXX-XXXXXX).

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was used to test whether levels of globin gene expression in the two *R. piscesae* phenotypes were different. This is a sensitive and accurate method of quantifying a target mRNA to assess differences in gene expression between samples (Wang *et al.*, 1989; Gibson *et al.*, 1996; Heid *et al.*, 1996). QRT-PCR uses two primers and a fluorogenic probe to allow levels of target transcript to be monitored at each cycle in a PCR reaction. Increasing levels of fluorescent signal emitted by the displacement of the transcript-specific fluorogenic probe over the course of a PCR reaction are measured to determine the PCR cycle number at which fluorescence first reaches a significant level above background, the C_t value. The C_t values, determined for each target transcript as well as for a concurrently amplified

internal control, are inversely proportional to the relative amount of target mRNA within a sample. A one cycle difference in C_t value between individuals represents roughly a two-fold difference in transcript expression, because PCR copy number increases exponentially over the course of a reaction. Therefore, relative differences in gene expression levels between individuals and populations can be approximately estimated by the relationship 2^N, where N equals the difference in C_t value between the two individuals or populations in question. The 18S rRNA transcript was used as an internal standard within each sample to control for potential variations in sample handling and reaction efficiency.

RNA samples from individuals were analyzed using the Perkin-Elmer ABI Prism 7700 sequence detection system for the A2, A1, and B2 chains and the Applied Biosystems 7300 Real Time PCR System was used for the B1a, B1c, and B1new chains. Duplicate samples run simultaneously on both machines have yielded similar C_t values (Deb Grove, PSU, personal communication). Primers and fluorogenic probes for QRT-PCR were designed from each of the different globin chain sequences using Primer Express® version 2.0.0 (Applied Biosystems). Sequences for all primers and probes can be found in Table 3-2. Globin probes were labeled at the 5' end with the fluorescent dye carboxyfluorescein (FAM) and a quencher dye (BHQ) at the 3' end (Biosearch Technologies, Inc.). When possible, at least one primer or the probe was designed to span an exon junction to avoid genomic DNA amplification. DNAse-treated RNA samples were heated to 65 °C for 5 minutes and then 50 ng were reverse transcribed in a total volume of 20 ul for one hour at 42 °C using the following reaction conditions: 20 U RNAse inhibitor, 2.0 ul RT buffer (Ambion), 4.5 mM $MgCl_2$, 0.5 uM globin-specific

reverse primer, 125 nM 18S reverse primer, 0.5 mM each dNTP, and 22 U MuLV reverse transcriptase (Applied Biosystems). A total of 20 ng of cDNA served as the template in the QRT-PCR reactions, which were run in triplicate for the A2, B2, and A1 chains using the following reaction conditions in a 50 ul total volume: 5.0 ul 10X TaqMan® Universal Master Mix Buffer, 4 mM $MgCl_2$, 0.2 uM each globin-specific primer, 50 nM fluorogenic globin-specific probe, 5 nM each 18S primer, 5 nM 18S probe, 200uM each dNTP, and 1.25 U TAQ Gold. QRT-PCR reactions were run at 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40-45 cycles of 15 sec at 95 °C, 1 min at 60 °C, with a 2 minute, 25 °C extension at the end. Because of the high degree of reproducibility of the reactions, only duplicate samples were run for B1a, B1c and B1new. The reverse transcription reactions for the B1 chains were done using the High Capacity RT mix according to the manufacturer's protocol (Applied Biosystems). All B1 QRT-PCR reactions were done using the same conditions as those just described, except the $MgCl_2$, probe and primer concentrations were increased in B1a and B1c to 5 mM, 0.4 uM, and 200 nM, respectively. Control reactions without template were also run to ensure that all C_t values represented only increases in fluorescence due to amplifications of the targeted globin chain. Increase in fluorescence of both globin and 18S over the course of each RT-PCR reaction was measured to determine the C_t values.

Statistical analysis

We performed an analysis of covariance (ANCOVA) among the log-transformed globin C_t values of the average of each individual's QRT-PCR replicates, using each corresponding log-transformed 18S C_t value as the covariate. This method of analysis

normalizes each globin C_t value based on the C_t value of the 18S internal standard and avoids the potentially false assumption that reaction efficiencies are identical. An analysis of variance (ANOVA) of the normalized C_t values was subsequently performed using site and phenotype as factors. Differences in expression levels were assessed using a Tukey-Kramer post-hoc test. Statistical significance for all tests was assigned at a level of $p < 0.05$. Values for each of the six different globin chains were analyzed separately. Fitted log values representing the normalized C_t value for each animal were averaged together by phenotype, by phenotype according to collection site, as well as by phenotype according to tissue type for three of the chains, and these values were back-transformed to yield mean C_t values for each phenotype by site and by tissue. Accurate comparisons between the relative levels of gene expression between the six different genes cannot be made at this time because each gene set was standardized and optimized independently. A paired *t*-test, comparing C_t values from samples taken immediately after recovery of the animals to those taken after holding animals for up to 12 hours in chilled seawater, was used to test whether time of processing may have had an effect on the measured levels of gene expression in samples. Results are reported as the mean ± standard deviation. All statistical analyses were performed using Minitab release 12.21.

Results

Globin sequences

PCR products of the A2, A1, and B2 globin chains, after cloning, yielded only one sequence for each chain type with high similarity to published sequences of the respective chains from *R. pachyptila*. No nucleotide differences were observed between

clones from short-fat and long-skinny *R. piscesae*. Phylogenetic analysis of 120 amino acid long partial sequences of the globin chains from *Ridgeia* and *Riftia* show that the corresponding globin chain types cluster together with 100% bootstrap support (Figure 3-2). Neighbor-joining and maximum parsimony methods yielded similar phylogenetic trees. The percent amino acid identity ranges from 67.5% for the B1c chain to 85.8% for the B2 chain (A2 – 80%; A1 – 76.7%; B1c – 67.5%; B1a – 78.3%; B2 - 85.8%). The degenerate B1 primers (Table 3-2) amplified three different PCR products with similarity to the B1a and B1c globin subunits of *R. pachyptila* and a new B1 subunit distinct from B1a and B1c. None of the isolated variants closely resembled the B1b chain that has been described from *R. pachyptila*. The B1c chain of *R. piscesae* has a glutamine residue located at position E7 within the globin helices of the protein's quaternary structure, as well as a tyrosine at the B10 position. These residues are also seen in *R. pachyptila.*

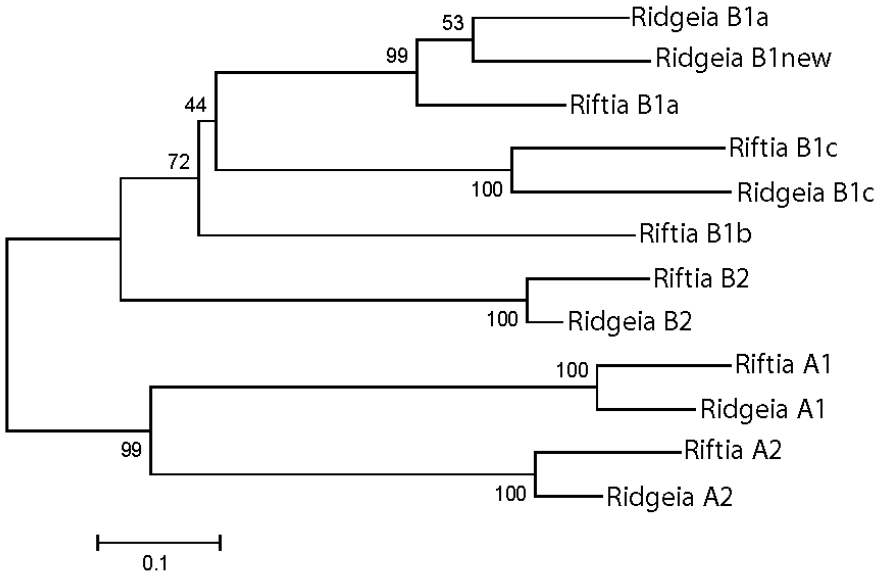

Figure 3-2. Neighbor-joining tree of amino acid chain sequences from *Ridgeia piscesae* and *Riftia pachyptila* globin chains. Bootstrap percentage values based on 1000 replicates are indicated at the nodes. Sequences for *R. piscesae* represent the consensus sequence between those from the short-fat and long-skinny phenotypes, which were identical.

Reproducibility of QRT-PCR reactions

The A2, B2, and A1 QRT-PCR reactions were run in triplicate to check reproducibility of the results. All three of the B1 chains' reactions were run in duplicate to maximize the number of individuals that could be analyzed. The raw C_t values for each of these globin chains as well as each corresponding 18S C_t value were consistent.

Statistical comparison of C_t values of animals from different processing time points

The datasets from two of the dives include animals that were processed immediately after recovery as well as those that were processed 10-12 hours later. The normalized C_t values of animals collected early were not significantly different from those of animals collected later on dives R683 (N = 5 of each phenotype) and R724 (N = 6 of each phenotype) ($p > 0.10$ for all six genes tested).

Comparison of gene expression between vestimentum and plume

The expression levels of the A2, B2 and A1 globin chains were compared between the plume and the vestimentum of both short-fat and long-skinny *R. piscesae*. Globin gene expression was significantly higher in vestimentum tissues than in the plume tissues ($p < 0.001$; Figure 3-3), but no significant interaction was observed between globin type, tissue, or phenotypes. Therefore, analyses for all of the B1 globin chains and comparisons between phenotypes and sites within each globin chain's data set focused only on levels of expression in the vestimentum.

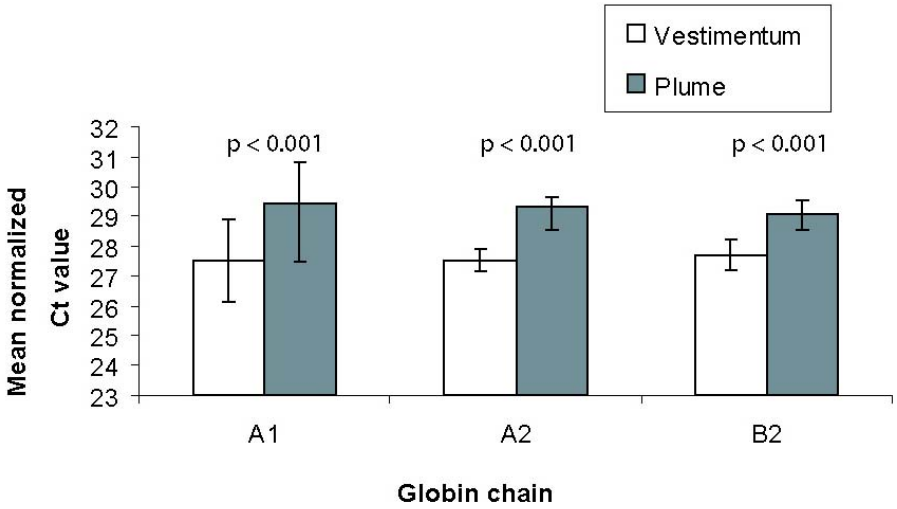

Figure 3-3. Mean globin chain expression compared between the vestimentum and plume of short-fat and long-skinny *R. piscesae* at Clam Bed. Error bars represent the standard deviation of the mean. Paired *t*-test performed on log-transformed data that were normalized to the 18S internal control.

Vent-fluid compositions at three chimney sites

The immediate environments around short-fat tubeworms at Clam Bed and Milli-Q have not been characterized, but measurements from high flow points at these sites along with measurements from S&M are used here to determine if significant chemical differences exist between fluids venting from these chimneys. Total sulfide concentrations in high temperature fluids are comparable between the three sites (Table 3-1), but Clam Bed has consistently had a ratio of H_2S/deltaT that is approximately a factor of 2 higher than Milli-Q and S&M. In 2003, maximum temperature and Fe and Cl^- concentrations increased from Clam Bed, to Milli-Q, to S&M (Table 3-1). In previous years, Fe/H_2S ratios at Clam Bed were lower (~0.02) than measured for the one fluid sample collected in August 2003, so the difference between Clam Bed and the two MEF vents may be larger than indicated in Table 3-1.

Site-specific differences in gene expression

Because long-skinny *R. piscesae* were only collected from Clam Bed, comparisons of the mean levels of expression of each globin chain between sites were done only within the short-fat *R. piscesae* population. The A1, A2, and B1new globin chains generally display the lowest C_t values in short-fat tubeworms at S&M, intermediate levels at Milli-Q, and the highest C_t values at Clam Bed, showing that gene expression levels increase from S&M to Milli-Q to Clam Bed (Figure 3-4). The B2 and B1a chains are also expressed most highly at S&M, but levels between Milli-Q and Clam Bed are not significantly different. The B1c chain shows a different trend, with short-fat animals at Milli-Q having the highest average C_t values, while S&M and Clam Bed show B1c C_t values that are lower and nearly identical to each other (Figure 3-4). Thus, Milli-Q animals demonstrate the lowest level of B1c expression. The average C_t value of this chain differed the greatest amount of any of the other chain-by-site comparisons, with the short-fat animals at Milli-Q showing C_t values that were 4.6 and 4.8 cycles greater than those values at Clam Bed and S&M, respectively, representing approximately a 21- to 23-fold increase in expression (Figure 3-4).

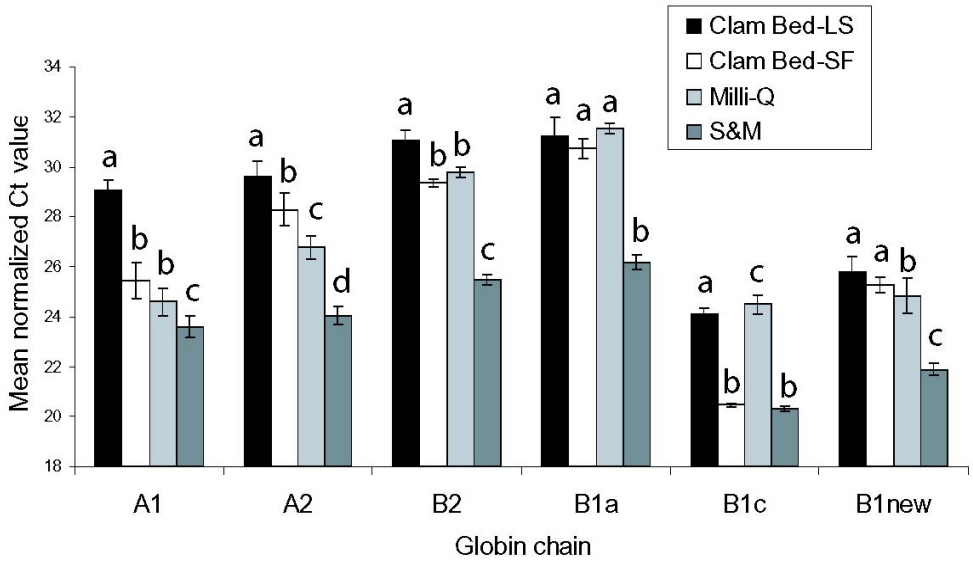

Figure 3-4. Comparison of site- and phenotype-specific globin chain expression of *R. piscesae*. Differences between phenotypes at Clam Bed and between sites were assessed using a Tukey-Kramer test. Different letters indicate values that are significantly different ($p < 0.05$) in intrachain comparisons between sites and phenotypes.

Phenotype-specific gene expression

Given the existence of a collection site effect on globin chain expression, phenotype comparisons were made only between short-fat and long-skinny *R. piscesae* from the same site, Clam Bed. Expression levels of the A1, A2, B2, and B1c globin chains are higher in short-fat individuals ($p < 0.05$). There is no significant difference in expression of the B1a or B1new chains between the phenotypes. The difference in expression of the B1c chain is the most dramatic, where short-fat animals average 6.6 cycles lower than long-skinny animals at the same site, representing nearly a 97-fold increase in expression level (Figure 3-4).

Discussion

Our analysis of the expression of six globin chains in short-fat and long-skinny *R. piscesae* at different sites along the Juan de Fuca Ridge tested whether gene expression in this tubeworm displays any phenotype- or site-specific differences. While no nucleotide differences were present between the cDNAs of the two phenotypes, expression levels between short-fat and long-skinny *Ridgeia* revealed that short fat individuals expressed four of the six chains at higher levels than long-skinny individuals (Figure 3-4). The B1a and the B1new chains are also expressed at higher average levels in the short-fat phenotype, however the differences are not significant, possibly due to small sample size. An overall increase in globin chain expression implies that short-fat *R. piscesae* may have higher concentrations of hemoglobin within their vascular and coelomic fluids. In addition, globin expression levels vary within a phenotype among collection sites, suggesting that even differences between similar microhabitats have an effect on gene expression.

The difference in mRNA levels observed among animals could be explained by differences in cDNA preparation time onboard ship or differences induced by the natural environment. The half-life of mRNA in eukaryotic cells can range from minutes to months (Ross, 1988). Thus, RNA isolation methods attempt to minimize the time between the death of an animal and processing. All of the tubeworms that were used during this study were alive until processing, however, RNA preparation varied from 1 hour to 12 hours after collection. This could lead to varying degrees of mRNA degradation. We can rule out differences in RNA preparation time as a factor influencing expression level differences because C_t estimates for animals processed up to 12 hours apart showed no significant differences in gene expression levels.

While the multitude of *Ridgeia* phenotypes that exist along the Juan de Fuca Ridge have been noted for years, no genetic basis for phenotypic differentiation has been implicated (Southward *et al.*, 1995; Southward *et al.*, 1996; Black *et al.*, 1998; Carney *et al.*, 2002). Findings here represent the first detectable molecular difference between extreme *Ridgeia* growth forms. Globin chains of short-fat tubeworms are generally expressed at higher levels than in their long-skinny counterparts. In light of previous characterizations around the environments of Clam Bed long-skinny animals and short-fat S&M animals, several possible explanations can be offered for the adaptive reasons as to why these extreme phenotypes display differences in globin expression levels. The demands of the internal environment imposed by the symbionts, as well as from the external environments imposed by vent fluid chemistry, likely play a role in influencing expression by the regulation of the factors that control this hemoglobin system.

The data from this work are in accordance with recent studies of the relative abundance of hemoglobin in the two systems of each phenotype showing that short-fat *R. piscesae* have roughly equal amounts of the 400 kDa and 3.5 MDa hemoglobins in their vascular and coelomic fluids (J. Flores, Ph.D. thesis). The long-skinny phenotype shows a different pattern of ~30% 400 kDa and ~70% 3.5 MDa hemoglobins in their vascular blood but the opposite (~75% 400kDa, ~25% 3.5 MDa) in the coelomic fluid (J. Flores, Ph.D. thesis). Estimates of the concentrations of hemoglobin in the two body fluids of these phenotypes show that while the concentration of the 3.5 mDa hemoglobin in the vascular fluid is roughly equal between the phenotypes, the concentration of the 400 kDa hemoglobin in the coelomic fluid is higher in short-fat animals (J. Flores, Ph.D. thesis).

Higher hemoglobin concentrations in short-fat *R. piscesae* could be advantageous for this phenotype in many ways. It has been noted that short-fat *R. piscesae* have more symbionts within their trophosome, and higher levels of dissolved inorganic carbon (DIC) in their coelomic fluid suggest that these symbionts fix carbon at higher rates than symbionts from long-skinny *R. piscesae* (Scott *et al.*, 1999). This phenotype lives in environments that generally are much higher in temperature and sulfide concentrations than the long-skinny phenotype, particularly around their plume. While sulfide is essential to sustain the tubeworms' symbiosis, over-exposure could potentially inhibit cellular respiration by poisoning cytochrome c oxidase (Smith *et al.*, 1977; Arp *et al.*, 1985; Fisher *et al.*, 1988). Increased amounts of hemoglobin in short-fat animals could serve to protect tissues from potential sulfide toxicity while transporting sulfide from the environment to their more numerous, more metabolically active symbionts (Weber, 1980; Powell and Somero, 1983; Bailly *et al.*, 2002; Bailly *et al.*, 2003; Bailly and Vinogradov, 2005). The possibility also exists that higher amounts of hemoglobin would allow short-fat individuals to be able to store more sulfide during times when levels around the plume may be diminished by tidal changes in vent flow (Williams, 2001). Long-skinny *R. piscesae* presumably receive a more stable and constant, albeit lower supply of sulfide through their "roots" in basalt cracks while short-fat animals experience exposure to transient vent flow around their plumes.

The detection of expression-level differences within the short-fat phenotype depending upon collection site further emphasizes the critical role of the environment in altering an organism's gene expression and ultimately physiological response. Vent fluid chemistry and temperatures from the openings and bases of the animals' tubes were not

measured for this study. However, a look at the chemistry of fluids from some of the highest flow points at the three short-fat collection sites shows that they are not identical in chemical composition (Table 3-1). While tubeworms on chimneys do not settle in the fastest flowing, hottest vent fluid, the differences in chemistry measured at these points suggest that some overall differences likely exist in the tubeworm-inhabited microhabitats at S&M, Milli-Q, and Clam Bed. Clam Bed has maintained a higher than average ratio of H_2S/heat, so that worms at an equivalent temperature would see higher levels of H_2S at Clam Bed than on the other sulfide-hosted sites. The variation in globin chain expression between these sites reflects that the animals, which outwardly appeared quite similar in form, respond differently to their microhabitats which presumably differ in chemical, thermal and/or other environmental aspects.

Alternatively, the presence of site-specific expression differences that were consistent among individuals from each collection site could reflect expression levels that are inherent within the different subpopulations. Especially for the short-fat phenotype, which lives on chimneys and experiences vent flow that can be highly variable in temperature and chemistry, consistent levels of gene expression within a subpopulation may suggest that individuals in an aggregation are genetically related as a result of a common settling event of closely related larvae. Some fine-scale genetic difference such as variation in the expression or repression of a *trans*-acting regulatory factor or in its binding capability at a *cis*-acting site might regulate globin genes in a population of animals that otherwise display no detectable genetic differentiation.

The lack of nucleotide differences between short-fat and long-skinny *R. piscesae* at the cDNA level is not surprising, given the conserved nature of hemoglobin molecules.

The expression differences seen here suggest that further investigation into the genomic sequences of each of these genes to identify potential *cis*- or *trans*-acting factors is warranted. Examples from other organisms provide leads for understanding how the expression of globin genes is regulated within the unique annelids studied here. In *Daphnia magna*, the expression of hemoglobin genes is regulated at the level of transcription (Tokishita *et al.*, 1997). Promoter regions in this crustacean contain several functional hypoxia response elements (Paul *et al.*, 2004). In the teleost fish, *Fundulus heteroclitus*, a single nucleotide difference in the promoter region of the lactate dehydrogenase-B gene between populations has shown that small variations in regulatory sequences can lead to adaptive expression level differences (Schulte *et al.*, 2000). Examination of hemoglobin genes of the mollusk *Scapharca inaequivalvis* has shown that functionally active regulatory sequences are located in the 3' end of the first intron (Gambacurta *et al.*, 2000).

Hypoxic conditions in animals can increase the expression of globin genes to order to increase the amount of hemoglobin available to bind oxygen from the environment (Heip *et al.*, 1978; Zeis, 2003; Zeis *et al.*, 2003). Temperature has also been shown to be a key regulatory factor of levels of globin expression in some systems, including vestimentiferans (Arp *et al.*, 1990; Paul *et al.*, 2004). Oxygen levels, sulfide levels and temperature are all confounding variables, and the identification of which of these, or perhaps other factors, might be involved in globin gene regulation in this multi-hemoglobin system will require additional work. However, more in-depth molecular population genetic analysis of the entire genomic sequence of these genes to identify potential morph-specific nucleotide

differences in non-coding intron and/or promoter regions should yield insight into the relative importance of *cis*- versus *trans*-acting factors in this system.

The presence of two particular amino acid residues in the B1c chain coupled with the QRT-PCR data raises intriguing possibilities. The B1c chain of both *Ridgeia* and *Riftia* has a glutamine residue located at position E7 in place of the E7His that is commonly found in most hemoglobins. Additionally, both *Ridgeia* and *Riftia* have a tyrosine located at B10 which, when coordinated with the E7 glutamine has been shown to increase oxygen affinity within the heme cavity (Weber and Vinogradov, 2001). The ability of B1c to potentially bind oxygen more strongly may help to explain why this is the only chain of the six analyzed which shows a different pattern of expression between the collection sites. Although zinc sites in the A2 globin chain have been recently implicated as playing a key role in sulfide binding (Flores *et al.*, 2005), nothing unique was revealed about this subunit from a standpoint of differential gene expression. Differential regulation of B1c and the other chains could allow independent responses to oxygen and sulfide.

The presence of phenotype-specific as well as site-specific globin chain expression differences within the short-fat phenotype of *R. piscesae* lends support for our hypothesis that environmental conditions play a key role in altering the physiology of different, and even similar, growth forms of this vestimentiferan species. Studies to determine if site-specific gene expression differences are also seen within the long-skinny *R. piscesae* phenotype, as well as studies looking at the differential expression of other genes, will provide better insight into how this unique species of tubeworm has evolved to exploit a broad diversity of habitats along the Juan de Fuca Ridge.

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CHAPTER 4

Molecular population genetics of six globin subunit genes in the Juan de Fuca Ridge hydrothermal vent tubeworm, *Ridgeia piscesae*

Susan L. Carney, Marisa I. Formica, and Stephen W. Schaeffer

Abstract

The variable phenotypes of the northeast Pacific hydrothermal vent tubeworm *Ridgeia piscesae* each inhabit microhabitats that differ in environmental conditions including substrate, vent flow rate, sulfide and oxygen levels, and temperature. Extracellular hemoglobin molecules, which bind and deliver sulfide and oxygen from the environment to chemosynthetic endosymbionts in the tubeworms' trunk region, provide a link between the animal and its environment. The populations of *R. piscesae* along the Endeavour segment of the Juan de Fuca Ridge have shown no evidence for genetic differentiation, including in an investigation of the coding sequences of six of the globin genes encoding the chains that assemble the hemoglobin molecules. However, two of the most extreme phenotypes, one from low-flow basalt substrate and the other from high-flow sulfide chimneys, express different levels of the globin subunits. *R. piscesae* living in chimney environments display consistently higher levels of globin chains than animals from basalt environments, and globin expression also differs between different chimney sites. In this study, we sequenced the entire genomic region of each of the six globin genes within five individuals each of the basalt and sulfide *R. piscesae* phenotypes. This molecular population genetic analysis showed that overall low levels of variation exist within this species, and no phenotype-specific nucleotide differences are present. The data presented here provide evidence that the phenotype- and environment-specific variation seen in *R. piscesae* globin gene expression is likely regulated by *trans*-acting factors.

Introduction

Phenotypic plasticity has been noted in the hydrothermal vent vestimentiferan tubeworm *Riftia pachyptila* at the East Pacific Rise (Black *et al.*, 1994), however the degree of microhabitat-specific morphological variability in the northeast Pacific species *Ridgeia piscesae* far exceeds the variation seen in *R. pachyptila.* In fact, the range of genetically indistinguishable phenotypes exhibited by *R. piscesae* spans the range of the tubeworm growth forms known from vent and seep habitats worldwide (Jones, 1985; Southward *et al*., 1995). The description of the genus *Ridgeia* with at least two proposed species (Jones, 1985) was subsequently revised based on evidence of no genetic differentiation between the growth forms (Southward *et al.*, 1995). Further support for a single species of *R. piscesae* along the Juan de Fuca Ridge has been noted in additional studies (Southward *et al.*, 1996; Black *et al.*, 1998; Carney *et al.*, 2002). The basis for the phenotypic differentiation of this species, particularly between the two most extreme phenotypes found either on sulfide chimneys or basalt substrate, remains a mystery.

Like all vestimentiferans, the growth forms of *R. piscesae* exhibit a typical tubeworm body plan with an anteriorly-located respiratory organ, the plume, supported by a muscular structure, the vestimentum, which helps to optimally position the animal within its chitinous tube (Jones, 1985). The trunk region comprises the bulk of the animal, housing a sac-like structure called the trophosome that contains the sulfide-oxidizing chemoautotrophic bacteria that live in an obligate symbiosis with the host tubeworm and are acquired each generation from the environment (Cavanaugh, 1983; Hand, 1987; Cary *et al*., 1993; Laue and Nelson, 1997). The tubeworm host delivers oxygen and sulfide, in the form of HS^-, from the plume where both are bound to

hemoglobin and transported through the vascular system to the bacteria in the trophosome (Arp *et al.*, 1985; Arp *et al.*, 1987; Fisher *et al.*, 1988; Childress and Fisher, 1992; Goffredi *et al.*, 1997). At least one species of hydrocarbon seep tubeworm, *Lamellibrachia luymesi*, that lives where plume-level sulfide concentrations are low to undetectable, acquires sulfide through the substrate via its "root", a posterior extension of its tube and body (Julian *et al.*, 1999). The long-skinny phenotype of *R. piscesae*, which lives in low-flow, basalt environments, displays sulfide-permeable posterior tubes that are hypothesized to function in the same role as those of *L. luymesi* (Urcuyo *et al.*, 2003). This suggests that the *Ridgeia* growth forms span not only the range of phenotypes known for vestimentiferan tubeworms, but also the sulfide acquisition strategies.

The discovery of giant hemoglobins in deep-sea tubeworms and the elucidation of their function have provided information necessary to understanding how the animals sustain their symbiosis. Sulfide and oxygen are transported from the external environment, through the vascular system, to the symbionts in the trophosome. Tubeworms have separate vascular and coelomic systems, in which two different hemoglobin molecules are found. In *R. pachyptila*, a giant ~3.5 MDa molecule circulates through only the vascular system, while both the vascular and coelomic systems contain a ~400 kDa molecule. Both the ~3.5 MDa and ~400 kDa hemoglobins are composed of the same six different types of globin chains. Additionally, the assembly of the vascular hemoglobin relies upon non-globin linker chains (Suzuki *et al.*, 1988; Suzuki *et al.*, 1990b). Phylogenetic analysis has determined that annelid globin chain types fall into two distinct clades, designated as A and B (Gotoh *et al.*, 1987; Negrisolo *et al.*, 2001; Bailly *et al.*, 2002). The A and B chains of tubeworms have been subdivided into six

subclades, A2, A1, B2, B1a, B1b, and B1c; there is evidence that at least one additional B1 variant exists (S. Carney, Ph.D. thesis). Heme groups associated with each globin chain are the site of oxygen binding, while sulfide is bound reversibly at separate sites. Both free cysteine residues (those not involved in interchain disulfide bonds) in the A2 and B2 chains and zinc residues associated with histidines within the A2 globin chain have been implicated in sulfide binding (Suzuki *et al.*, 1989; Suzuki *et al*., 1990a; Zal *et al.*, 1997; Zal *et al.*, 1998; Flores *et al.*, 2005). A1 and B1 globin chains do not have free cysteine residues or zinc residues and are not likely to be involved in sulfide binding (Suzuki *et al.*, 1989; Flores *et al.*, 2005).

The link provided by hemoglobin between tubeworms and their environment has been the basis for genetic and physiological studies of this protein in two of the most extreme *Ridgeia* phenotypes (S. Carney, Ph.D. thesis; J. Flores, Ph.D. thesis). From a physiological standpoint, short-fat *R. piscesae* from high-flow, high temperature sulfide chimney environments maintain different relative abundances of the ~3.5 MDa and the ~400 kDa hemoglobins in their vascular and coelomic fluids compared to the long-skinny phenotype that lives in low-flow, low temperature, basalt environments (J. Flores, Ph.D. thesis). From a genetic standpoint, chimney and basalt *R. piscesae* display no synonymous or non-synonymous nucleotide changes in the partial coding sequences of six globin chains (S. Carney, Ph.D. thesis). The chimney phenotype shows consistently higher levels of expression of the six globin genes than basalt *R. piscesae*, and even within a phenotype, levels of globin gene expression differ according to collection site (S. Carney, Ph.D. thesis).

There are two hypotheses for why globin chain expression differs between the basalt- and chimney-dwelling *R. piscesae*. The first hypothesis is that *cis*-regulatory sequences may be differentiated between the two morphotypes. In this case, *R. piscesae* populations would be polymorphic for globin chain alleles that differ in the *cis*-regulatory sequences that are upstream of each structural gene. One globin chain allele would be specific for animals that settle in basalt environments and the other, for animals settling on chimneys. This hypothesis predicts that sequences in the introns or 5' regions will be different between the two morphotypes. The second hypothesis is that the *cis*-acting regulatory sequences are similar between the two *R. piscesae* morphotypes, but that the regulation of the hemoglobin chains is controlled by *trans*-acting regulatory factors that differ depending on the environment to which the tubeworm is exposed. The prediction of this model is that the *cis*-acting regulatory sequences of the globin chains will not be differentiated between the basalt- and chimney-dwelling morphotypes.

A wide variety of mechanisms, both direct and indirect, have been implicated in the regulation of hemoglobin expression (reviewed in Hardison, 1998). Regulation of globin genes in annelids is not well-characterized, however, models of globin gene regulation from other invertebrates with extracellular hemoglobins are consistent with *trans*-acting factors binding to regulatory regions proximal to, or even within, the globin gene (Bunn and Poyton, 1996). In some systems, hemoglobin molecules are directly regulated by low oxygen levels, in particular by the binding of hypoxia response elements (HREs) and/or hypoxia inducible factors (HIFs) in promoter regions (Gorr *et al.*, 2004). The crustacean *Daphnia* has at least six globin genes that encode an extracellular, multidomain hemoglobin; four of the genes have been identified, and all are tandemly

arrayed on a single chromosome (reviewed in Paul *et al.*, 2004). *Daphnia* globin genes are transcriptionally-regulated (Tokishita *et al.*, 1997). The number and types of functional HREs in promoter sequences within 250 bp upstream of the genes determines the intensity of *D. magna*'s response to hypoxic conditions (Gorr *et al.*, 2004; Paul *et al.*, 2004). In the bivalve mollusk *Scapharca inaequivalvis,* regulatory sequences within globin introns and functionally active mini-genes within globin genes have been characterized (Gambacurta *et al.*, 2000). The detection of conserved regulatory motifs in *R. piscesae* globin genes and in the sequences of the other tubeworms examined here could serve as a preliminary indicator of how environmental variation plays a role in globin regulation in this tubeworm species. Identifying nucleotide differences in regulatory regions that might differentially affect the binding of a transcription factor(s) between the phenotypes could aid in explaining some of the differences in chain expression that have been observed between *R. piscesae* phenotypes and collection sites.

Hemoglobins also serve as a useful candidate molecule for a molecular population genetic analysis of the *R. piscesae* population from the Endeavour segment of the Juan de Fuca Ridge. To date, no significant genetic differentiation has been observed between the two most extreme *R. piscesae* phenotypes. Hemoglobin is an essential molecule, so one might predict that it might be unlikely to detect nucleotide differences within globin genes in *R. piscesae*. However, it has been demonstrated that the rate of evolution in the A1 and B1 chains is higher than that of the A2 and B2 chains; Bailly *et al.* (2002) propose that the A1 and B1 chains, which do not have free cysteine residues, are under relaxed constraint since they are not involved in sulfide binding. If this is the case,

perhaps the genomic sequences of the A1 and B1 chains may reveal slight population-specific variation.

The work presented here is a molecular population genetic analysis of the sequences of six of the genes encoding the globin chains that assemble the ~3.5 MDa and ~400 kDa hemoglobins in the basalt and chimney *R. piscesae* phenotypes. This study will determine if the coding sequence of the six globin chain genes is different at the amino acid level or at *cis*-acting sites between the basalt and chimney-dwelling phenotypes of *R. piscesae*. Non-coding sequences from *R. piscesae* globins were also compared to non-coding globin regions from tubeworms from other deep-sea environments to locate *cis*-acting sites that may be conserved between these deep-sea annelids.

Materials and Methods

Sample collections

Samples of *R. piscesae* were collected from three different sites along the Endeavour segment of the Juan de Fuca Ridge with the manipulator arm of the remotely-operated vehicle ROPOS in 2001, 2002, and 2003. Animals were transported to the surface in a temperature-insulated box, at which point they were transferred to a cooler of chilled seawater. Individuals of each of the short-fat and long-skinny phenotypes were dissected to obtain vestimentum samples that were stored at -70 ºC until further use.

DNA extractions and globin gene sequence determination

Standard phenol-chloroform extractions followed by ethanol precipitations were used to extract genomic DNA from each individual's tissue sample (Ausubel *et al.*, 1989). Sequences from the cDNA of each of the six globin chains, previously described in Carney (Ph. D. thesis), served as the basis for genomic amplifications and sequence determination of non-coding regions. Intron sequences were determined by identifying the boundaries of exons through amino acid sequence alignment with other annelid sequences and then primers were designed to amplify the region spanned between each of the three exons within each gene. Additional primers were designed to obtain upstream and downstream sequences via genome-walking by the method of Mishra *et al.* (2002). Finally, primers were designed to amplify and sequence as much of each gene as possible in five individuals of each of the two *R. piscesae* phenotypes. Primer sequences can be found in Table A-1. All PCR reactions were carried out in a 25 µl volume with 20 ng of template DNA, 2.5 µl 10X reaction buffer with 15 mM $MgCl_2$ (supplied by the manufacturer), 2.5 µl of 0.5 mM dNTPs, 200 ng of each primer, and 0.5 U of *Taq* polymerase (Genechoice, Inc.). Single PCR products were either sequenced directly or cloned into chemically-competent TOP10 *E. coli* cells using TOPO TA cloning® (Invitrogen) to obtain unknown terminal sequences. Cloned inserts as well as PCR products were sequenced using dye-terminator cycle sequencing according to the manufacturer's protocol (Beckman Coulter), and products were run on a CEQ2000 automated sequencer. Forward and reverse strands of each sequence were assembled with Seqman II from the Lasergene sequence analysis package (DNAStar) and sequence conflicts were resolved. At least four clones from every cloning reaction were

sequenced. In only three cases (portions of B1a intron 1, B1c intron 1, and A1 intron 1) were there single nucleotide conflicts between one of the clone sequences versus the others. Three additional clones for these reactions were sequenced to resolve these conflicts by majority consensus.

During cloning of PCR products from genome-walking in the B1a chain, a 507 bp portion of another B1 variant, referred to here as B1d, was identified. Attempts to amplify cDNA using primers designed to this sequence were unsuccessful, as were attempts to genome-walk upstream and downstream. The partial sequence for this additional B1 variant has been deposited in GenBank (accession numbers XXXXXX-XXXXXX).

Determination of globin sequences in other vestimentiferan species

Primers designed for *R. piscesae* exons were used to amplify coding and non-coding DNA globin chain genes from tubeworm species from two Gulf of Mexico hydrocarbon seep sites as well as from the East Pacific Rise hydrothermal vent sites. Samples of *Escarpia laminata* from Alaminos Canyon in the Gulf of Mexico were collected with the DSV Alvin in October 2003. *Lamellibrachia luymesi* and *Seepiophila jonesi* from the upper Louisiana Slope of the Gulf of Mexico were collected using the Johnson Sea-Link II in June 2002, and *Riftia pachyptila* samples from the East Pacific Rise were collected in December 2001 with the submersible Alvin. Genomic DNA extractions and PCR reactions were performed as described previously for *R. piscesae*. The first intron of the B2 globin chain in *S. jonesi* was sequenced in eight individuals,

while portions of globin sequences from *L. luymesi*, *R. pachyptila*, and *E. laminata* were sequenced in only one or two animals.

Anonymous marker sequence in R. piscesae

To determine if the levels of intraspecific nucleotide variation seen in the globin genes are unique to this gene type or are representative of the *R. piscesae* genome as a whole, we sequenced approximately 800 bp of an anonymous gene region in five individuals of the basalt- and chimney-dwelling phenotypes. This anonymous marker was identified by sequencing of a PCR product intended to be the upstream region of B1a; a BLAST search showed no similarity between this sequence and that of B1a or any known in GenBank. Primers used to amplify this region, AnonF/R, can be found in Table A-1. The sequence for this marker has been submitted to GenBank (accession numbers XXXXXX-XXXXXX).

Sequence analysis

Complete globin sequences for each of the six chains and sequences from the anonymous marker from five *R. piscesae* individuals of each phenotype were aligned using Megalign 3.1.7 (DNAStar) and intraspecific nucleotide differences were identified. Nucleotide diversity estimates (π) and their standard error values were calculated using MEGA version 3.0 (Kumar *et al*., 1993). To insure that our samples had the maximum genetic diversity, each set of five individuals of each morphotype consisted of animals collected from different dives, during different years, and from different collection sites (in the case of the short-fat phenotype; long-skinny animals were only collected at one

site). Sequences from introns, promoters, and 3' UTRs in *R. piscesae*, *L. luymesi*, *S. jonesi*, and *E. laminata*, where applicable, were searched for possible transcription factor binding sites using MatInspector (Quandt *et al.*, 1995; Cartharius *et al.*, 2005) and Match™ public version 1.0 (Goessling *et al.*, 2001), as well as by eye searching for HIF and HRE motifs as described in Rees *et al.* (2001). The partial sequence obtained from A1 intron 2 is not included in these analyses because this sequence's identity could not be confirmed. A2 intron 1 was only partially examined in one individual due to problems in sequencing through a microsatellite region, $(CATTA)_{24\text{-}27}$, that prohibited the completion of the 5' region of this intron. Genome walking from the first A2 exon upstream revealed at least three different alleles. The region almost immediately upstream of the first A2 start codon displayed a microsatellite $(GT)_n$ which varied in repeat copy number between two of the three upstream variants.

Results

R. piscesae globin gene sequences

All of the six globin genes in *R. piscesae* exhibit the three exon-two intron structure that is typical of vertebrate and other annelid globin genes (Jhiang *et al.*, 1988). The beginning and end of each intron have the established eukaryotic consensus sequences at the 5' and 3' boundary, GT..AG. Sequence comparisons within each globin chain type revealed overall low levels of variation, no amino acid variation, and no phenotype-specific nucleotide variation. A representation of the sequenced portions of all of the six genes examined in this study can be seen in Figure 4-1. Each of the six globin chains was sequenced in 10 individuals for a total of 14 kb of aligned nucleotides.

The same 10 animals were not sequenced for each globin gene; the aggregate set of sequences in this study is from 28 different tubeworms.

Of the six globin genes analyzed in this study, three (B1a, B1new, and B2) were sequenced in their entirety (Figure 4-1). Two of the globin chains, A2 and B1c, are approximately 95% complete, and A1 is roughly 60% complete. Completion of the first A2 exon and upstream region was successful by genome-walking from a small known portion of the first exon. However, the region from the 3' end of the first A2 exon to the microsatellite in the first intron remains unsequenced (Figure 4-1). The 5' end of B1c and the 3' end of A1 were not able to be sequenced despite multiple attempts to genome walk upstream and downstream respectively with different primer combinations (Figure 4-1). The second intron of the A1 gene was amplified by PCR using primers designed to the flanking exon sequences. Repeated attempts to directly sequence this PCR product as well as to clone and sequence it failed. The intron region was estimated to be ~1000 bp in length from an agarose gel using a 100 bp size standard, with only 800 bp of sequence being obtained.

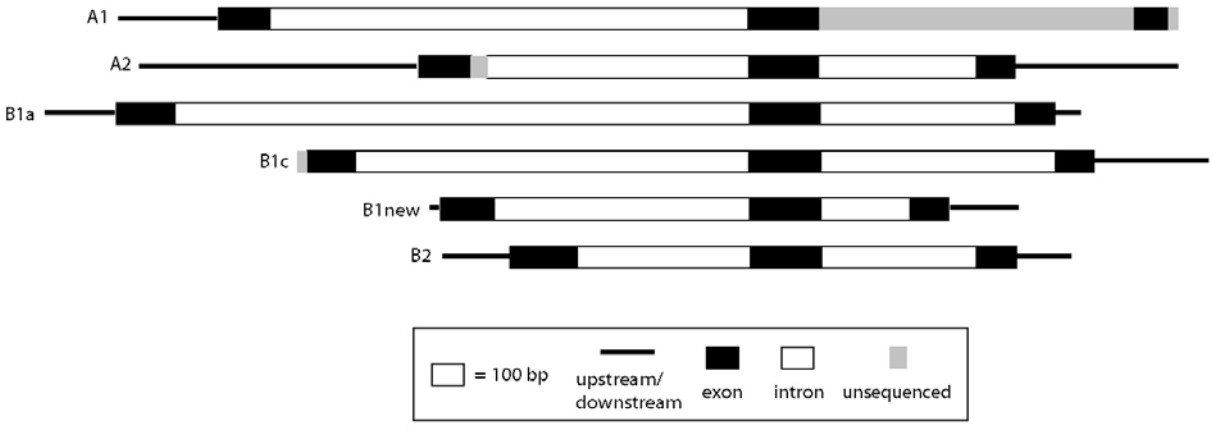

Figure 4-1. Representation of genomic regions of six globin genes sequenced in *R. piscesae*. The lengths of each gene/gene region are to scale.

Molecular population genetics of R. piscesae globin chains

A representation of the regions that were sequenced in the six *R. piscesae* globin genes in this work can be seen in Figure 4-1. The introns and/or downstream regions of all of the genes showed the greatest number of segregating sites. Fifteen of the 18 globin chain gene exons have no polymorphic sites. The three variable exons each had one segregating site; all were in synonymous positions. Available sequences for 5' flanking region revealed no segregating sites. Eight of the ten introns surveyed had segregating sites. Polymorphic sites were found in the first intron of all of the genes, and the B1c chain showed the overall highest number of segregating sites; 17 (Tables 4-1, 4-2, 4-3). Nucleotide diversity estimates for the six globin genes ranged from 0 to 0.0020 ± 0.0005 (Table 4-2). Indels were located within the non-coding regions of B1c (Table 4-3). None of the polymorphic sites or indels seen in the six genes sequenced here were specific to either of the two phenotypes. The standard errors of the nucleotide diversity estimates from chimney and basalt animals overlap in each case, suggesting no phenotype-specific differences (Tables 4-1, 4-2).

The portion of the B1d chain that was sequenced corresponds to 38 amino acids that most closely match, but are distinct from, the beginning of B1a and B1new exon 2 in *R. piscesae* (Figure 4-2). The remaining 393 nucleotides presumably comprise the 3' end of intron 1.

Globin genes from other vestimentiferan species

A few regions of the globin chain genes could be amplified from genomic DNA from *L. luymesi*, *S. jonesi*, *E. laminata* and *R. pachyptila* using *R. piscesae* primers.

Table 4-1. Nucleotide diversity (π) of each globin gene region in *R. piscesae*. * indicates partial intron or exon sequence included in analysis; N = number of animals sequenced; L = length of region sequenced; S = number of segregating sites.

Gene	Phenotype	Exons				Introns				Upstream				Downstream			
		N	L	S	$\pi \pm$ SE	N	L	S	$\pi \pm$ SE	N	L	S	π	N	L	S	$\pi \pm$ SE
A1	Both	10	546*	1	0.0005 ± 0.0004	10	1513*	1	0.0003 ± 0.0003	10	317	0	0	---	---	--	---
	Basalt	5		0	0	5		1	0.0003 ± 0.0003	5		0	0			--	---
	Chimney	5		1	0.0009 ± 0.0009	5		1	0.0004 ± 0.0004	5		0	0			--	---
A2	Both	10	496	0	0	10	499*	0	0	3	329	0	0	10	357	0	0
	Basalt	5		0	0	5		0	0	1		0	0	5		0	0
	Chimney	5		0	0	5		0	0	2		0	0	5		0	0
B1a	Both	10	516	1	0.0014 ± 0.0013	10	2439	5	0.0008 ± 0.0004	10	206	0	0		75	0	0
	Basalt	5		1	0.0016 ± 0.0015	5		4	0.0008 ± 0.0004	5		0	0			0	0
	Chimney	5		1	0.0013 ± 0.0013	5		5	0.0010 ± 0.0004	5		0	0			0	0
B1c	Both	10	480*	1	0.0008 ± 0.0008	10	1992	11	0.0019 ± 0.0006	---	---	--	--	10	350	5	0.0043 ± 0.0021
	Basalt	5		0	0	5		6	0.0016 ± 0.0007				-	5		2	0.0034 ± 0.0024
	Chimney	5		1	0.0013 ± 0.0013	5		10	0.0023 ± 0.0007					5		5	0.0054 ± 0.0025
B1new	Both	11	501	0	0	11	1106	8	0.0020 ± 0.0008	11	42	0	0	11	217	0	0
	Basalt	5		0	0	5		7	0.0028 ± 0.0010	5		0	0	5		0	0
	Chimney	6		0	0	6		3	0.0013 ± 0.0008	6		0	0	6		0	0
B2	Both	10	498	0	0	10	1040	2	0.0007 ± 0.0005	10	217	0	0	10	174	0	0
	Basalt	5		0	0	5		1	0.0008 ± 0.0005	5		0	0	5		0	0
	Chimney	5		0	0	5		1	0.0008 ± 0.0005	5		0	0	5		0	0

Table 4-2. Nucleotide diversity (π) of each globin gene in *R. piscesae*. * indicates partial intron or exon sequence included in analysis; N = number of animals sequenced; L = length of region sequenced; S = number of segregating sites.

Gene	Phenotype	Total			
		N	L	S	$\pi \pm$ SE
A1	Both	10	2310*	2	0.0003 ± 0.0002
	Basalt	5		1	0.0002 ± 0.0002
	Chimney	5		2	0.0005 ± 0.0003
A2	Both	10	1811*	0	0
	Basalt	5		0	0
	Chimney	5		0	0
B1a	Both	10	3257	6	0.0009 ± 0.0004
	Basalt	5		5	0.0009 ± 0.0004
	Chimney	5		6	0.0010 ± 0.0004
B1c	Both	10	2823*	17	0.0020 ± 0.0005
	Basalt	5		8	0.0016 ± 0.0005
	Chimney	5		16	0.0025 ± 0.0006
B1new	Both	11	1855	8	0.0013 ± 0.0005
	Basalt	5		7	0.0017 ± 0.0007
	Chimney	6		3	0.0008 ± 0.0005
B2	Both	10	1973	2	0.0005 ± 0.0003
	Basalt	5		1	0.0004 ± 0.0003
	Chimney	5		1	0.0006 ± 0.0004

Table 4-3. *R. piscesae* DNA sequence polymorphism in globin gene regions. Only genes and regions with polymorphic sites or indels are shown here. * indicates that sequence for region is incomplete.

Globin gene	Position	Base variation or insertion	Frequency of polymorphism or insertion
A1	Intron 1	G/A	0.2
	Exon 3*	A/T	0.5
B1a	Intron 1	A/G	0.1
		A/G	0.2
		C/T	0.5
		G/A	0.1
		A/G	0.1
	Exon 2	G/C	0.5
B1c	Intron 1	TA	0.4
		T/A	0.1
		C/A	0.1
		AGGGATC	0.8
		A/G	0.2
		G/T	0.2
		C/T	0.1
		T/C	0.1
	Intron 2	T/A	0.2
		T/A	0.1
		T/C	0.1
		C/A	0.5
		AACGT	0.8
		T/G	0.4
	Exon 3	A/G	0.2
	Downstream	G/A	0.1
		T/G	0.3
		CT	0.7
		T/A	0.1
		TA/CG	0.1
B1new	Intron 1	G/A	0.1
		T/A	0.1
		C/G	0.1
		T/A	0.5
		T/A	0.1
		T/A	0.1
	Intron 2	C/T	0.1
		C/T	0.4
B2	Intron 1	T/A	0.2
	Intron 2	C/G	0.3

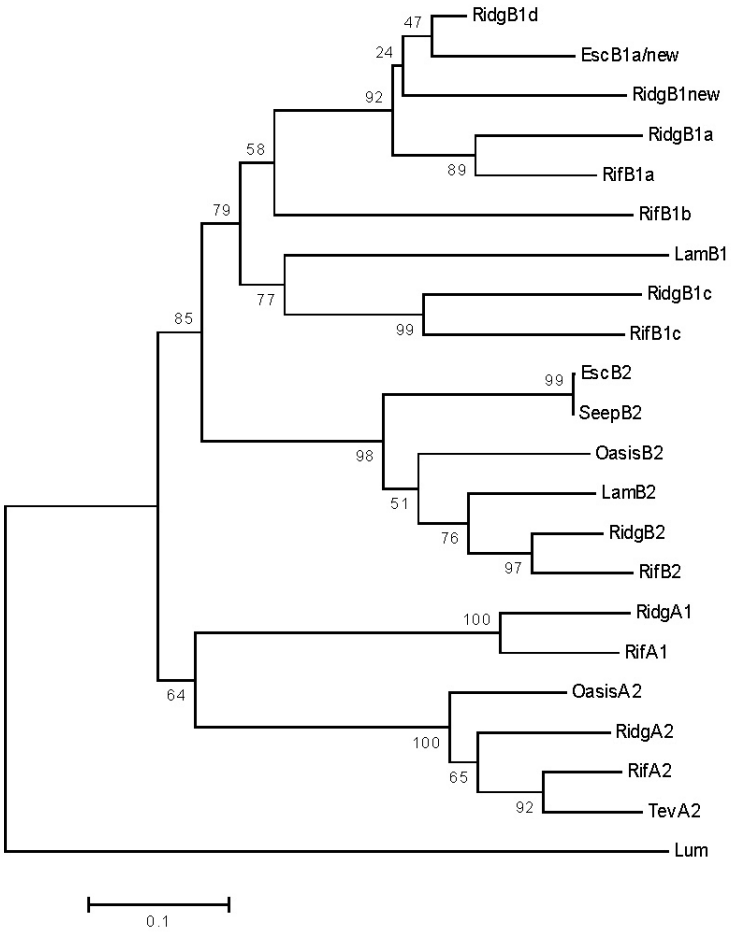

Figure 4-2. Neighbor-joining tree of vestimentiferan globin amino acid sequences using Kimura 2-parameter model of substitution. Sequences from the annelid *Lumbricus terrestris* are also included. Bootstrap values at nodes are based on 2000 replicates. Species abbreviations, with GenBank accession numbers in parentheses where applicable, are as follows: Ridg = *Ridgeia piscesae*; Esc – *Escarpia laminata*; Rif = *Riftia pachyptila* (AJ439735, AJ439734; AJ439732, AJ439736); Lam = *Lamellibrachia luymesi* (AAP40328); Lum = *Lumbricus terrestris* (AAC14536); Seep = *Seepiophila jonesi*; Oasis = *Oasisia alvinae* (AY250087, AY273264); Tev = *Tevnia jerichonana* (AY250086).

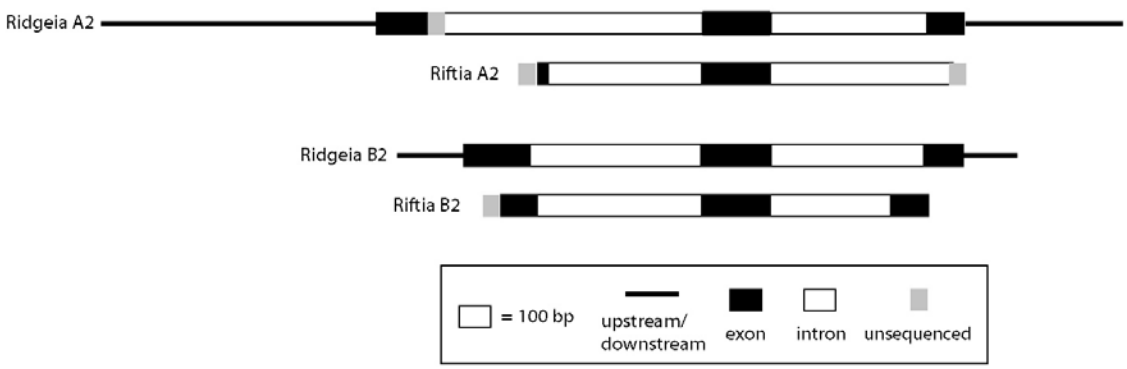

Figure 4-3. Representation of sequenced genomic regions in *R. pachyptila* A2 and B2 globins compared to *R. piscesae*.

A representation of the sequenced portions of A2 and B2 in *R. pachyptila*, in comparison to these genes in *R. piscesae*, can be seen in Figure 4-3. The coding sequences of B2 globin in *R. pachyptila* and *R. piscesae* show 14.0% nucleotide divergence and 11.5% amino acid divergence. None of the amino acid changes were in the residues highly conserved among nearly all invertebrate globins as described in Vinogradov *et al*. (1993). Comparison of B2 intron 1 in *R. piscesae* and *R. pachyptila* shows high sequence conservation for approximately 30 bp surrounding the beginning and ending splice sites, but the remaining intron sequence shows approximately 40% nucleotide divergence between the two species (Figure 4-4). Sequencing of the second A2 intron in *R. pachyptila* failed beyond a microsatellite region, $(TACA)_9$, followed by a number of (CA) repeats. The first intron of B2 globin in *S. jonesi*, amplified in its entirety and sequenced in eight individuals, revealed no nucleotide differences in 768 bp of sequence. Additionally, partial sequences of B2 intron 1 were obtained in *E. laminata* and *L. luymesi*, and a 547 bp portion of the second exon and intron from a B1 chain in *E. laminata* was also sequenced. Comparison of the nucleotide sequences from B2 intron 1 from five different species shows that *L. luymesi* is basal to the other two seep species, *S. jonesi* and *E. laminata*, while the sequences from the two vent species, *R. pachyptila* and *R. piscesae*, represent the most derived (Figure 4-5).

Possible transcription factors in R. piscesae and other vent and seep tubeworms

Searches of non-coding globin sequence regions with two different software programs designed to identify potential regulatory sequences yielded a number of motifs with matches to known and unknown transcription factor binding sites. There were not any motifs that were consistently found within or across given gene regions within *R.*

piscesae or compared to *R. pachyptila*, *S. jonesi*, *E. laminata*, or *L. luymesi*. Potential HIF and HRE binding sites, motifs of 5'-(A/G)CGTG-3', were identified within the upstream regions of A2, B2, A1, and B1a in *R. piscesae*, as well as within intron 1 of B1a and B1c, intron 2 of B1a, and the 3' UTR of B1c. Sufficient upstream sequence in B1new and B1c was not obtained in order to be searched. Possible HIF/HRE binding sites were also identified in B2 intron 1 of *S. jonesi*, both *R. pachyptila* B2 introns 1 and 2, and *L. luymesi* B2 intron 1. The sequences from B2 intron 1 of *S. jonesi* were not variable among the eight individuals sequenced, and the sequences from two *R. pachyptila* individuals were also identical. A sequence from only one *L. luymesi* individual was determined.

Non-globin sequence in R. piscesae

The 800 bp region of anonymous marker sequence in *R. piscesae* also revealed low levels of nucleotide variation. Only three variable nucleotides in this region (0.38%) were identified in a survey of eight animals, four of each phenotype.

```
                    ← exon 1↓ intron 1 →

RiftiaB2        TTCAAGGAGTGAGTACTTCGTTTCTAGTTCCGCACATATTTCTTTTTCCAAACTTCTTGT
RidgeiaB2       TTCAAGGAGTGAGTAGTTGGTTTCT---TCTTCAAATATTTTTTT---CAAAATTAATTA
                *************** ** ******   **  ** ****** ***   **** **  *

RiftiaB2        TTTGCTATTTGTTTTTTCTAAGGGCAATATTATAATACAACGTCTGCAGAGATGTTCTTT
RidgeiaB2       AATGCTACC-ACTTTGAATACAGTAAATATTTTG------CGCTAGCAAATATGTCTGCT
                  *****     ***   **  *  ****** *       **   *** * ****    *

RiftiaB2        TCGTGTTCCGGAGAAGAAATTTTTGTTT-------TGATATGATACATTTATTAGATATA
RidgeiaB2       TTGCATTCTGTAGTATAAATGTTTGTATATTTAAGTGATTTTAAACGGTTGTAATCCTTG
                * *  *** * ** * **** ***** *       **** * * **  ** * *    *

RiftiaB2        ACATATATAAAAGTTCGAATTTTCACAGAGATCTGTTTGTTTGGCTTTTT--AGTTA-TT
RidgeiaB2       TTAGGTCGAGTACTTTTGTTTTTCATACATAACTGTTTGGTTATTTCTGTTGAGTTAGTT
                  *  *  *  * **    ****** * * * ******* **   * * *  ***** **

RiftiaB2        TGTGTAGTGTTAGGTTGCATTT-----------------AAAATAT-GTAATATTTTCCA
RidgeiaB2       TATTTAGCCAAGGAGTGTAATTTTTTTACCAAACTGTCAAAAGTATTACGACATTTTGTA
                * * ***     *  ** * **                 *** ***    * *****  *

RiftiaB2        AAGCCA------AATATT-CGAAATTCTGTACT--GGTCTCCCAATATGCTGTTGG-GTT
RidgeiaB2       ATATTGTTTTATAATATTTCCAAATTCTAATTTTAAGTGGCTACATATGATATTTATATT
                *           ****** * *******    *   **  *   ***** * **    **

RiftiaB2        TGGT--TTTAATTACTAGCAACATGTTTTATTTGTGTTATACTTCTTTTATATAATGTGA
RidgeiaB2       TAGTGGTTAGTTTACTAATTA-ATGTTTTATCGATGTTATACTCCTTTCGTTTAAT----
                * **  **   ******   * *********   ********* ****  * ****

RiftiaB2        ACATATCTCTGTTATAGTAAATCTTTTTTAAATTGACTACATT-GTTTACTTCGTTCACA
RidgeiaB2       -CCAGTCCTTCGTGTAACAATAATTTTCAAATATAATTACGTTTATCTGCATACTTCGTC
                 *   **  *  * **  **   ****  **  * * *** **  * * * *  ***

RiftiaB2        CTTGCAACGTCTCAACAAAACAAATCCTTCACATTGACTAT-GTCTTAAGCTGTGCCACT
RidgeiaB2       CAT-TAAC-TCCCATTCCCGCCACCCCCGGCGATCATTTACCGAACTAAGCCGC-CTGCA
                * *  *** ** **      * *  **     **    **  *   ***** *  *  *

                                              ←intron 1 ↓ exon 2 →
RiftiaB2        CCTAGCGACTATCCGCTGAACGACTGATGCGTTCTTGCAGCCTCTTCGCCAACGTACCAG
RidgeiaB2       CCTGGCGATTATCCGCTGAACGACCGATGTCCTCTTGCAGCCTCTTCGCCAACGTGCCCG
                *** **** *************** ****   ********************** ** *

RiftiaB2        ATGCCGTTGGTCTCTTCGGTGCAGTCAAAGGTGACGAAGTCAACAGCAACGAATTCAAGG
RidgeiaB2       CTGCCGTATCCCTGTTCGGCGGTGTGAACGGTGAAAACATCAACAGCAACGAATTCAAGG
                 ******    ** ***** *  ** ** *****  *  ********************

RiftiaB2        CTCACTGCATCCGTGTCGTGAACGGT
RidgeiaB2       CTCATTGCATCCGTGTCGTGAACGGT
                **** ********************
```

Figure 4-4. Alignment of *Riftia pachyptila* and *Ridgeia piscesae* B2 intron 1. Arrows indicate location of splice sites at the beginning and the end of the intron.

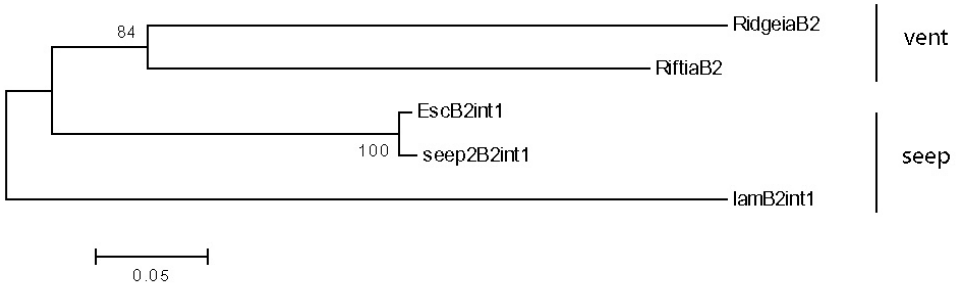

Figure 4-5. Neighbor joining tree of B2 intron 1 globin nucleotide sequences from *Lamellibrachia luymesi* (lam), *Ridgeia piscesae*, *Riftia pachyptila, Escarpia laminata* (Esc) and *Seepiophila jonesi* (seep), using Kimura 2-parameter model of substitution. Bootstrap values at nodes are based on 1000 replicates.

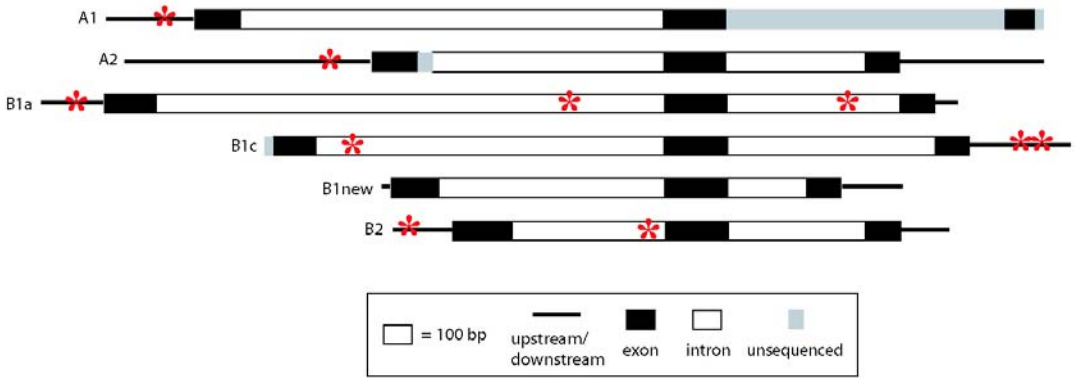

Figure 4-6. Location of motifs in *R. piscesae* globin genes with similarity to known hypoxia inducible factor (HIF) binding sites. Motif locations are indicated by asterisks.

Discussion

Despite the fact that globin chains in *R. piscesae* are differentially expressed between distinct phenotypes and within a phenotype across different environments, sequences of the nearly complete genomic regions of six of the globin chains revealed no significant differentiation between phenotypes. There were no amino acid differences in the coding regions of the six globin genes detected between the basalt- and chimney-

dwelling phenotypes of *R. piscesae*. Thus, expression-level differences in the hemoglobins of the different morphotypes cannot be attributed to nucleotide differences within or around the globin genes themselves.

The 14 kb of nucleotide sequence determined here show low levels of nucleotide diversity within *R. piscesae* at the Endeavour segment (Tables 4-1, 4-2). The lack of variation in *cis*-acting sites suggests that these regions do not play a role in differential gene expression of the six globin genes. One potential caveat is that there is *cis*-acting variation for the globin chains, but these sequences are located further upstream and were not detected in our study. The lack of phenotype- and site-specific nucleotide variation in the introns and the promoter regions sequenced in this study suggests that globin genes in this system are regulated by *trans*-acting factors.

The northeast Pacific, where *R. piscesae* lives, and the East Pacific Rise (EPR), home to *R. pachyptila*, were separated from each other ~28.5 million years ago as the North American continent migrated southwest to split the Farallon Plate (Tunnicliffe 1988; Atwater, 1989). Comparison of mtCOI sequences from another EPR tubeworm species, *Oasisia alvinae*, and *R. piscesae* has shown 13.1% nucleotide divergence and no nonsynonymous nucleotide changes between these species since the vicariance event (Chevaldonné *et al*., 2002). The coding sequences of the B2 globin chains from *R. pachyptila* and *R. piscesae* show approximately the same level of nucleotide divergence, 14.0%, but a higher frequency of nonsynonymous changes, 11.5%. Non-coding sequence from B2 intron 1 shows approximately 40% nucleotide divergence between the *R. pachyptila* and *R. piscesae* (Figure 4-4). The higher levels of amino acid and nucleotide divergence in the portion of the B2 globin chain examined from these two species suggest

that this chain is not under strong selective constraint. This may be consistent with the fact that the extracellular hemoglobin molecules of tubeworms are comprised of at least six different globin chain types, and some amount of functional redundancy may exist. While certain regions of the globin chains are conserved, interspecific evolution in non-conserved parts of the molecule may reflect that some specialization of the different chains may exist, perhaps due in part to pressures imposed by variable vent environments. Specialization of chains could accommodate needs of increased sulfide and/or oxygen binding in different microhabitats. The relationships depicted through the comparison of nucleotide sequences from B2 intron 1 from all five tubeworm species in this study (Figure 4-5) support those determined in previous studies showing that seep tubeworms are ancestral to vent tubeworms (Williams *et al*., 1993; Black *et al*., 1997; Halanych *et al*., 1998, 2001).

The *R. piscesae* population sampled in this study reveals a low level of nucleotide variation that is consistent with results from previous population genetic studies of this species. Two introns and some upstream and downstream sequence remain undetermined, but it seems likely that this pattern of low variation that is not phenotype-specific will apply in these regions as well. While only one non-globin marker was sequenced in this study, the results from that locus suggest that low levels of polymorphism in globins may not necessarily be a result of strong selection on these loci but are representative of the low nucleotide diversity levels in the population as a whole. The *R. piscesae* sequences determined from each phenotype were a haphazard sample of individuals collected on different dives over a three year period from a 1.5 km locale. The genetic similarity of individuals within our sample could reflect a common settling

event or a sample of offspring from highly related individuals. Studies of the water currents along the ~90-km-long Endeavour segment of the Juan de Fuca Ridge suggest that circulation is highly constrained and that its tendency would be to retain larval pools that are released into the water column (Thomson *et al.*, 2003). This would contribute to the low levels of genetic diversity that have been noted in the Endeavour *R. piscesae* population.

Regions with DNA microsatellites were found in at least two of the introns (*R. pachyptila* A2 intron 1 and *R. piscesae* A2 intron 2) as well as upstream in A2, and repetitive regions were found in most of the introns sequenced here. Microsatellites within introns and promoter regions have been hypothesized to play a role in gene expression, although their exact role is not clear (Li *et al.*, 2004). As sequences for these particular regions were not examined in multiple individuals, these represent ideal loci for future population genetic studies of the Juan de Fuca Ridge population of *R. piscesae*. In addition, differences in the number of repeats within some of these intronic and upstream microsatellites could play a role in phenotype- and site- specific globin expression variation, although no evidence of such was revealed here.

In a study of the expression of *R. piscesae* globin chains, a B1 globin variant in addition to the three that have been described from *R. pachyptila* was identified (Bailly *et al.* 2002; S. Carney, Ph.D. thesis). In this current study, a new B1 variant was identified, and this one is also most similar to the B1a chain (Figure 4-2). Evidence for expression of the B1d chain could not be found through amplifications of cDNA, suggesting that this may be a pseudogene. The diversification of this chain type raises questions about the function of the B1 chains within the hemoglobin molecules. The A1 and B1 globin

chains in deep-sea tubeworms have not been implicated in sulfide binding. All of the globin chains do, however, bind oxygen. Beyond their role in oxygen binding, the potential for functional specialization of different B1 globin chains within the hemoglobin molecule remains to be determined.

Considering that oxygen levels regulate hemoglobin expression in many other systems, it is not surprising that the results here suggest that oxygen may play a role in regulating the molecule that transports itself, along with sulfide, though the body of the animal. The detection of motifs indicative of HIFs and HREs within upstream regions as well as within several introns suggests that these areas merit further experimentation to determine if indeed they are functionally activated by *trans*-acting factors in response to environmental oxygen levels. Determining upstream sequence of B1c and B1new and the subsequent detection of similar motifs within would add strength to the hypothesis that oxygen may play a key role in the regulation of globin genes within tubeworms in this hydrothermal vent system. In the vent environment of constantly mixing fluid, levels of both sulfide and oxygen are difficult to discern and are inversely related. Low oxygen due to high sulfide or vice versa could differentially control *trans*-acting factors in animals in one environment compared to another. However, other, often confounding, environmental factors such as temperature could also play a role in affecting gene expression.

Molecular control of hypoxic response is well-characterized in mammals, however the characterization of the control of hypoxic response in marine invertebrates is less understood (Wu, 2002). HIF-1 is a common regulator of hypoxic-dependent gene expression (reviewed in Zagorska and Dulak, 2004). Regulation of the HIF-1

heterodimer occurs at multiple levels including transcription, posttranslational modifications, and protein stabilization (Zagorska and Dulak, 2004). If indeed globins in *R. piscesae* are controlled at least in part by hypoxic response involving HIF-1 or a related factor, any number of fine-scale nucleotide and/or gene expression level or timing differences could be responsible for regulating the factors that in turn regulate the *trans*-acting factors affecting the globin genes. Should experimentation reveal that the regions identified here are not functionally active HIF/HRE binding sites, the *trans*-regulation of *R. piscesae* globins may be directed by parameters alternative to, or in addition to, environmental oxygen/sulfide levels.

The dynamic and heterogeneous *R. piscesae* microhabitats at the Juan de Fuca Ridge together with the complex interactions of genes and their regulatory factors within the animal complicate our understanding of how this species responds to its environment. Much further analysis of genome sequence and experimentation is necessary to understand how this multihemoglobin system in *R. piscesae* is controlled. The results shown here suggest that all of the globin chains, whether they bind sulfide or not, could be regulated in part by the same *trans*-acting elements. It is of interest to find the penultimate gene(s) that regulates the hemoglobin polymorphism to better understand how environmental cues are translated into hemoglobin gene regulation. Certainly other regulatory elements remain to be determined as factors that could mediate the process of the expression of the appropriate combinations and stoichiometry of globin chains into their ultimate molecular assemblies.

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CHAPTER 5

Population structure analysis of the mussel *Bathymodiolus childressi*

from Gulf of Mexico hydrocarbon seeps

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Abstract

Hydrocarbon and brine seeps at the deep regions of the northern and western Gulf of Mexico support populations of the bathymodioline mussel, *Bathymodiolus childressi*. In this study, we use two mitochondrial and six nuclear DNA markers to investigate relationships within the metapopulation of *B. childressi* in the Gulf of Mexico from Mississippi Canyon to Alaminos Canyon over a range of 527 to 2222 m in depth. Restriction fragment length polymorphism (RFLP) analysis of these markers suggests that populations are not genetically differentiated and that overall levels of nucleotide variation are low. F_{ST} values display no clear trend according to collection site depth. F_{ST} values do increase according to the geographic distance between collection sites, a factor which is confounded by water currents whose effects on larval dispersal are unclear. The presence of a panmictic population of *B. childressi* over such a broad range of depth suggests that this species may be unique among members of Gulf of Mexico seep chemosynthetic communities, whose distributions are generally limited according to gradients in depth.

Introduction

While they are geologically quite distinct from hydrothermal vents, hydrocarbon and brine seep communities in the Gulf of Mexico support taxonomically similar, high biomass communities dominated by tubeworms, mussels, and clams (Paull *et al.*, 1984; Kennicutt II *et al.*, 1985; Brooks *et al*., 1987). Relative to vent habitats along mid-oceanic ridges, there are very small temperature anomalies at most seep sites, however, concentrations of sulfide and methane can be quite high, particularly in the sediments (Sibuet and Olu, 1998). As at vent communities, chemoautotrophic and methanotrophic symbioses are the basis for much of the biomass that is found in seep communities.

Mussels within the family Mytilidae are represented in deep-sea reducing habitats by members of the subfamily Bathymodiolinae (Gustafson *et al.*, 1998; Distel, 2000). The genus *Bathymodiolus*, one of at least five genera within this subfamily, consists of members that live at hydrothermal vent and hydrocarbon seep environments (Distel, 2000). Bathymodioline mussels harbor methanotrophic and/or sulfide-oxidizing symbionts in their gills (Childress *et al.*, 1986; Fisher, 1990; Cavanaugh *et al.*, 1992; Fisher *et al.*, 1993; Distel *et al*., 1995; Le Pennec and Benninger, 2000). Evidence suggests that bathymodioline hosts acquire their symbionts through horizontal transmission, postspawning, rather than transgonadal, from parent to offspring (Le Pennec and Beninger, 2000; Won *et al.*, 2003b). It is believed that bacteria are obtained from the water soon after settlement by the pediveliger larvae via endocytosis directly into gill cells (Won *et al.*, 2003b).

Five bathymodiline species, including *B. childressi*, have been described from hydrocarbon and brine seep environments in the Gulf of Mexico (Gustafson *et al.*, 1998).

Populations of *B. childressi* have been described throughout the northern and western Gulf of Mexico at methane-rich sites over a minimum depth range of approximately 1660 meters (~540 m – 2200 m; Figure 5-1) (Gustafson *et al.*, 1998). Individuals of *B. childressi* harbor methanotrophic bacteria in enlarged gills (Childress *et al.*, 1986). The intracellular symbionts provide fixed carbon to the host as the basis for their nutrition either directly by translocation (Cary *et al.*, 1988) or indirectly by host digestion of symbionts (Streams *et al*., 1997). Additionally, filter feeding serves as a supplement for their dietary needs (Page *et al.*, 1991). The mixotrophic lifestyle typified by *B. childressi* likely plays a large role in the broad geographic distribution of bathymodiolines at vents and seeps worldwide (Fisher *et al.*, 1989; Le Pennec and Aloui Bejaoui, 2001).

B. childressi is a dioecious species that reproduces by broadcast spawning to produce numerous planktotrophic larvae (Eckelbarger and Young, 1999; Tyler and Young, 1999). Dispersal of individual larvae depends upon ocean currents as well as larval biology. Estimates from other *Bathymodiolus* species suggest that larvae can remain in the water column on the order of weeks to months (Lutz *et al*., 1980; Turner and Lutz, 1985). This dispersal time gives larvae the potential to travel long distances, although actual dispersal distances may be considerably reduced by the constraints of topography and water currents. Currents in the Gulf of Mexico over the depth range inhabited by *B. childressi* are quite variable but can often be intense within 400 meters of the seafloor, reaching speeds up to 73 – 86 km/day (Hamilton, 1990; Hamilton and Lugo-Fernandez, 2001). The potential water current speeds and ability of larvae to remain in the water column for several weeks suggest that *B. childressi* has the potential for long

distance dispersal throughout the Gulf of Mexico, although realized dispersal may be limited by the directions of different water currents.

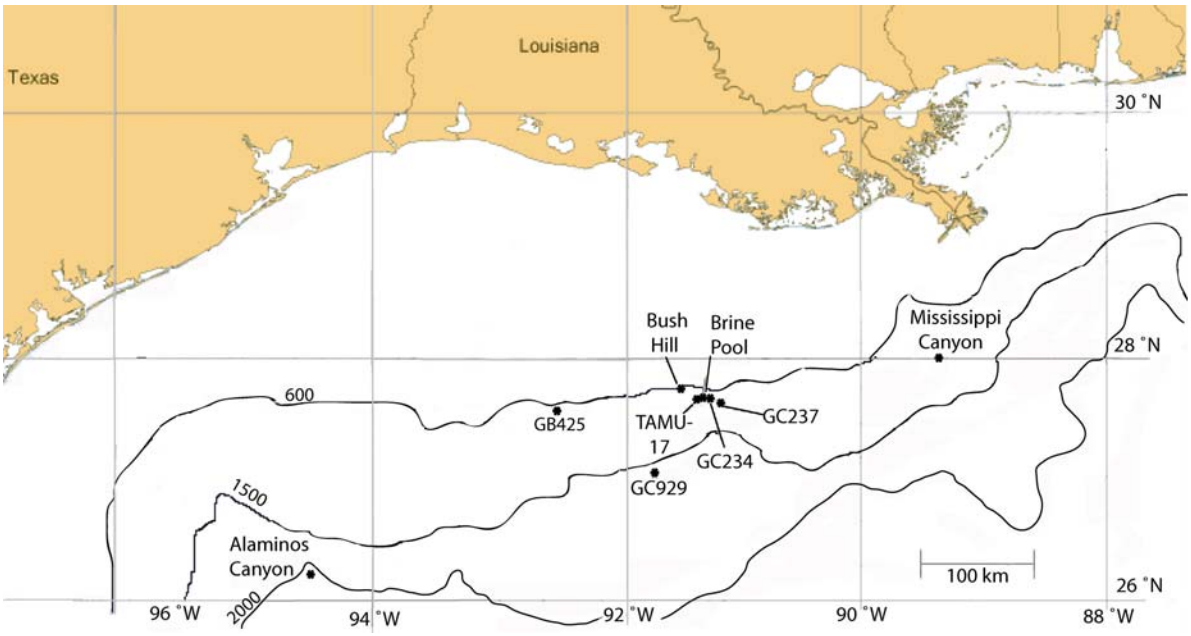


Figure 5-1. Map of collection sites for *B. childressi* samples in the Gulf of Mexico. Modified from Gulf of Mexico Lease Interface (MAFcom Services LLC) using Autodesk© DWF Composer ™ version 1.0.0.2161. Depth contour lines represent depth in meters.

At the East Pacific Rise, one single bathymodioline species, *B. thermophilus*, is present over a geographic range of 2370 km from the Galapagos Rift (GAR) to 13 ºN to 11 ºS on the East Pacific Rise (Craddock *et al.*, 1995b; Won *et al*., 2003a). In the Atlantic Ocean, two bathymodioline species exist over a nearly 3000 km span (von Cosel *et al.*, 1994; von Cosel *et al.*, 1999; O'Mullan *et al*., 2001). *B. azoricus* is found in the northern region of the Mid-Atlantic Ridge (MAR) over a depth range of 850-2250 m, while *B. puteoserpentis* is found at southern MAR vent fields at a deeper range, 3080-3650 m (O'Mullan *et al.*, 2001). The difference in depth between vent fields has been hypothesized as a barrier to the dispersal of organisms along the MAR (Van Dover *et al.*,

1996; Desbruyeres *et al.*, 2000). Genetic studies have also noted bathymetric patterns in the population structure of other deep-sea organisms. Populations of the protobranch bivalve *Deminucula atacellana* from the western North Atlantic were found to be genetically distinct above and below 2500 m depth on the basis of 16S rRNA sequence comparisons (Chase *et al.*, 1998). The amphipod *Eurythenes gryllus* from the eastern and western North Atlantic, the North Pacific, and the Arctic Ocean displays differentiation between populations shallower than 3200 m and deeper than 3500 m, although a rather homogeneous distribution exists up to 2000 m in depth (France and Kocher, 1996).

In a recent study, *B. childressi* was the only described species out of 162 examined from 40 different communities in the Gulf of Mexico that was found at both shallow (800 m) and deep (1800 m) sites (Cordes *et al*., in prep.). Depth is commonly attributed as a major factor to impose barriers to dispersal in the deep-sea, and that can lead to gradients in the diversity of biological communities (Rex, 1981). Within the Gulf of Mexico at several of the sites included in this study, ecological studies have shown a trend of decreasing species diversity with depth (Pequegnat *et al.*, 1990; MacDonald *et al.*, 2003; Cordes *et al.*, in prep.). The presence of *B. childressi* over such a broad depth range suggests that it may be unique among fauna in Gulf of Mexico chemosynthetic communities. An earlier allozyme study suggested that *B. childressi* from the upper Louisiana Slope (500 – 600 m) and Alaminos Canyon (2200 m) (seep mytilids Ia and Ib) are distinct populations of the same species (Craddock *et al.*, 1995a). These data, based on 18 allozyme loci, revealed one fixed difference between the populations, and Craddock *et al.* (1995a) suggested that this may be an indication of limited gene flow between these two sites, possibly as a result of their difference in depth.

In addition to depth, environmental conditions at hydrothermal vents and hydrocarbon seeps may play a role in creating distinctions between populations based on differences in physiological tolerance. At the Mid-Atlantic Ridge, variation in particulate matter and metal concentrations in vent fluids have been suggested to act in conjunction with depth differences to promote diversification of mussel populations whose physiological abilities might be compromised in more toxic conditions (Desbruyères *et al.*, 2000). More recently, the results from reciprocal transplants of populations of *B. childressi* between brine and petroleum-dominated sites (more and less favorable conditions respectively) found differences in the physiological performance of transplanted individuals of this species, and one suggested explanation for the data could be genetic differences between the populations (Bergquist *et al.*, 2004).

To date, it is not known whether populations of *B. childressi* throughout its range of depth and habitat conditions in the Gulf of Mexico show evidence of population subdivision. Empirical studies that track the movements of individual larvae through the water column are difficult if not impossible to perform. Molecular markers provide an indirect method to infer levels of gene flow among populations that determines if alleles are transferred among populations. Several population genetic studies of vent bivalves have been conducted (Craddock *et al.*, 1995b; Karl *et al.*, 1996; Jollivet *et al.*, 1998; O'Mullan *et al.*, 2001; Won *et al.*, 2003a), but currently the genetic structure of organisms at seeps is less well understood.

The primary goal of this study is to test whether gene flow in *B. childressi* is limited or extensive throughout its known range among Gulf of Mexico seeps using a molecular population genetic analysis of genetic markers. We use two mitochondrial and

six anonymous nuclear markers to determine whether the populations of this seep species are genetically subdivided due to habitat condition, over depth range, or over geographic range. Our results suggest that *B. childressi* dispersal is not limited over the depth and geographic range sampled nor between favorable and unfavorable site conditions.

Materials and Methods

Sample collection and DNA extraction

Research cruises between 1997 and 2004 were used to collect *B. childressi* samples from nine different sites on the Louisiana Slope within the Gulf of Mexico (Figure 5-1; Table 5-1). These sites span 527 to 2222 meters in water depth and a geographic range of approximately 560 km. Animals were collected with the submersibles Alvin and Johnson Sea-Link I and II and were brought to the surface in a temperature-insulated collection box. Symbiont-free mantle tissue was dissected from each mussel aboard ship and was frozen at -70 °C. DNA was extracted from the frozen mussel tissue with the method of Winnepenninckx *et al.* (1993) or with a modified CTAB + PVP method (Doyle and Doyle, 1987), followed by a standard ethanol precipitation (Ausubel *et al.*, 1989). DNA samples were stored at –80°C until use. A total of 183 mussels from nine sites were examined in this study.

Development of molecular markers

Microsatellite marker. We developed microsatellite markers from an enriched genomic library based on a protocol by Armour *et al.* (1994) as described in McMullin *et al.* (2003). Details of the methods for microsatellite development can be found in

Appendix A. The screening of approximately 3000 clones from these libraries yielded only 45 positive clones, 26 of which contained inserts greater than 200 base pairs, suitable for PCR primer design. Sequencing of these clones revealed only four microsatellites to which primers could be designed, and only one of these microsatellite loci was polymorphic. This variable locus was a dinucleotide repeat $(GA)_n$ (locus gA3c; Table 5-2), which produced a range of PCR product sizes that were scored by size using the Fragment Analysis System of the Beckman CEQ2000 automated sequencer. The forward primer in each PCR reaction was labeled with the fluorescent WellRED dye D4-PA (Proligo). Each fragment analysis run included 1 µl of PCR product and 0.25 µl of an internal reference size standard (CEQ DNA Size Standard-600; Beckman Coulter) in deionized formamide to a total volume of 40 µl. Analysis of the PCR product sizes against the size standard was done with the fragment analysis option of the CEQ 2000 software (Beckman Coulter). This locus showed evidence of stuttering (staggered, rather than distinct peaks) when scored by fragment analysis. This could have lead to an overestimate of the number of allelic classes. To overcome this problem, gA3c alleles were binned into size classes of *n* and *n*+1, which yielded a total of 27 allele bin classes.

RFLP markers. Whole genome fingerprinting methods like AFLP were not appropriate for this study because *B. childressi* have been documented to have high levels

Table 5-1. Collection site information for *B. childressi* samples

Site	Coordinates	Dive	Date of collection	Depth (m)	Sample size
GC234	27-44.07N, 91-13.30W	JSL2877	24-Jul-1997	538	28
	27-44.07N, 91-13.29W	JSL2889	29-Jul-1997	534	
	27-44.07N, 91-13.46W	JSL4717	12-Jul-2004	527	
Bush Hill (GC185)	27-47.03N, 91-30.52W	JSL2852	10-Jul-1997	553	50
	27-46.99N, 91-30.46W	JSL2857	12-Jul-1997	547	
	27-46.93N, 91-30.49W	JSL2865	15-Jul-1997	540	
	27-47.04N, 91-30.46W	JSL2868	16-Jul-1997	540	
	27-46.94N, 91-30.48W	JSL2873	21-Jul-1997	540	
	27-46.93N, 91-30.49W	JSL4042	12-Jul-1998	540	
TAMU-17 (GC233)	27-43.08N, 91-18.02W	JSL4049	15-Jul-1998	559	7
GC237	27-46.08N; 91-07.14W	JSL2908	11-Aug-1997	561	7
	27-46.08N; 91-07.14W	JSL2911	13-Aug-1997	561	
GB425	27-33.24N, 92-32.36W	JSL2882	26-Jul-1997	567	18
	27-33.24N, 92-32.36W	JSL2883	26-Jul-1997	567	
MS Canyon (MC929)	28-01.05N; 89-43.06W	JSL3340	08-Oct-2002	636	14
Brine Pool (GC233)	27-43.42N, 91-16.07W	JSL2850	09-Jul-1997	649	26
	27-43.42N, 91-16.07W	JSL2860	13-Jul-1997	649	
	27-43.42N, 91-16.07W	JSL2870	20-Jul-1997	649	
	27-43.42N, 91-16.77W	JSL4384	09-Mar-2002	649	
	27-43.42N, 91-16.77W	JSL4711	09-Jul-2004	651	
GC929	27-04.40N; 91-41.38W	JSL2029		1459	11
Alaminos Canyon	26-21.26N, 94-30.09W	A3924	19-Oct-2003	2222	22

of parasites (Powell *et al.*, 1999; Ward *et al.*, 2004). Individuals of *B. childressi* harbor body tissue parasites much like chlamydia and rickettsia greater than 67% of the time, and they also show infection by a *Bucephalus*-like trematode in 18 – 100% of the individuals, depending on the collection site (Powell *et al.*, 1999). Another Gulf of Mexico seep mussel from the Florida Escarpment was found to have as many as nine different types of parasites, including a rickettsia-like inclusion that exhibits no obvious tissue pathology but is found in the cytoplasm of mantle epithelial cells (Ward *et al.*,

2004). As the mantle tissue samples for this study were collected before this was known and without consideration of this factor, the extracted DNA samples from most of the mussels could contain contaminating parasitic DNA as well. For this reason, host-specific markers were essential.

Five of the anonymous nuclear molecular markers were developed based on published literature or sequences from *Bathymodiolus* spp. and other mytilids. Two of the primer sets used (Bchild EFBis F/R and PGM 1F/R) were designed initially to amplify introns based on intron/exon boundaries from alignments of gene sequences from several mytilid species, including an elongation factor (EFbis) (Bierne *et al.*, 2003a). Anonymous marker 1 (anonM1) and anonymous marker 7 (anonM7), were developed from primers to microsatellites in *Mytilus* spp. (Presa *et al.*, 2002). B84 was a locus developed by designing primers to a clone containing a non-microsatellite insert that was isolated from a genomic library used to develop gA3c. Marker PGM was developed from unpublished primers to an intron in phosphoglucomutase provided by Didier Jollivet (Station Marine de Roscoff, personal communication).

Primers were initially designed to amplify *B. childressi* genomic DNA for each locus. PCR amplifications for all loci were carried out in a 25 µl reaction volume containing approximately 20 ng of template DNA, 2.5 µl 10X reaction buffer with 15 mM $MgCl_2$ (supplied by the manufacturer), 2.5 µl of 0.5 mM dNTPs, 200 ng of each primer, and 0.5 U of *Taq* polymerase (Genechoice, Inc.). Any single PCR band that resulted from the amplification of several mussels from different sites was cloned into chemically-competent TOP10 *E. coli* cells using TOPO TA cloning® (Invitrogen). Both strands of each appropriately-sized clone insert were sequenced with universal M13

Table 5-2. Primer sequences used to amplify anonymous genomic markers in *B. childressi*. Mitochondrial ND4 primer sequences can be found in Maas *et al*. (1999). Restriction enzymes used to discriminate polymorphic sites within each marker are indicated in parentheses. Variation in mtND4 PCR products was detected with the enzyme *Bsa*J I[1]. 1 = New England Biolabs; 2 = Promega; 3 – Fermentas

Locus	Primer Name	Sequence (5'-3')	Annealing Temperature (°C)	Product size (bp)	Marker	GenBank Accession number
gA3c	gA3newF*	(dye 4)GTC GTA AAC ACT TCG CGG AC	55	177-235	Microsatellite	
	gA3new R	CAC CTG CCT CAT TTC GCG AC				
B84	B84F	CCG ATT CAG ACA ATG GAA GA	60	775	RFLP (*Hha* I[1])	
	B84R	GGG TAG ACG GAA TTG TTT A				
EFbis	Bchild EFbisF	CCG AAC CGA ACC ACC TAT CAG	55	643/687	RFLP (*Taq* I[2])	
	Bchild EFbisR	GGA TTG TAA CCA ATC TTT TTC AGG				
Pgm	PGM 1F	CTA CGT CAC ACG CAA GGA TAG T	60	592	RFLP (*Hpy*8 I[3])	
	PGM 1R	CCG TGT GGG CCA AAC AAA TA				
anonM1	Marker 1F	CTG ACT GCC TCA TGT TTT GT	55	557	RFLP (*Taq* I[2])	
	Marker 1R	GGT TCG TTC AGA TGG GTT TC				
anonM7	Marker 7F	GGA AGA AGT CGG CGA GAA GA	63	433	RFLP (*Eco*RI[1])	
	Marker 7R	CGC AAT TTA CAG TAA TCC AAA TG				
anonM2	M2F	GGG AAA GAC TGC CTA ACA AT	55	557	-----	
	M2R	CTC TTA CAT AGA AAA TGG TT				
anonM3	A5-8F	CTT CCT TGC AAT GAG TCG TC	53	575	-----	
	A5-8R	GGT AGC TCG GAT TTT GTT GT				
anonM4	M030304F	GCA TAC TAA GTG TGA CGA AC'	60	305	-----	
	M030304R	GAC ATT TAC CAA ATA ATG AA				
anonM5	MetF	GCC TGG ACC TTG TAA CTG TTG	62	439	-----	
	MetR	CAC ACC AGT GAA GAA CGG GAA				
anonM6	PGM2F	CAT GCT ATC TCA ACC GTG GA	58	383	-----	
	PGM2R	GTA GGA CCC ATT TCA ATT CG				

*Fluorescently-labeled with WellRED dye (Proligo LLC (Boulder, CO, USA))

primers using dye terminator cycle sequencing according to the manufacturer's protocol (Beckman Coulter). *B. childressi*-specific PCR primers were subsequently redesigned to each locus based on the clone sequences. PCR products from amplifications with the specific primers were sequenced in three or four mussels each from Alaminos Canyon, Mississippi Canyon, Bush Hill, Brine Pool, and GC929 to identify polymorphisms. Five non-variable loci were amplified by this method in addition to the variable ones reported here (Table 5-3). Polymorphic sites were examined using NEBcutter (Vincze *et al.* 2003) to determine if they could be discriminated by commercially available restriction enzymes (Table 5-3). Additionally, each sequence was searched for further restriction sites which, although not identified as variable through our sequence analysis, could potentially be variable in an RFLP screen of a larger sample size using available restriction enzymes. Fifteen additional restriction sites over five loci were assayed in 25 individuals from a mix of collection sites, including deep sites (Alaminos Canyon and GC929) as well as shallow and brine-versus petroleum-dominated sites (Brine Pool, Bush Hill, and GC234) (Table 5-3). Sequences from all markers have been deposited in GenBank (accession numbers XXXXXX-XXXXXX).

Table 5-3. List of non-discriminating restriction enzymes tested on PCR products from five nuclear loci

Locus	Restriction enzyme
EFBis	*Acc* I[1], *Bsa* I[1], *Bsa*J I[1]
anonM1	*Alu* I[4], *Hind* III[4]
PGM	*Alu* I[4], *Bsa* I[1], *Eco*R I[1],
B84	*Alu* I[4], *Hind* III[2], *Hpy*CH4 III[1]
anonM7	*Alu* I[4], *Hpy*CH4 III[1], *Nla* III[1], *Sau*3A I[1]

1 = New England Biolabs; 4 = Invitrogen

Mitochondrial markers. Mussels within the family Mytilidae, have separate male and female mitochondrial genomes that are each transmitted independently to offspring, a form of transmission termed sex-limited or doubly uniparental mitochondrial inheritance (Fisher and Skibinski, 1990; Skibinski *et al.*, 1994; Zouros *et al.*, 1994). This would suggest that data from mitochondrial markers should be viewed with caution unless information about the sex of each animal was also taken into account. Recent work, however, suggests that mussels within the genus *Bathymodiolus* do not have gender-specific mitochondrial types (R. Vrijenhoek, MBARI, personal communication). For this reason, data from locus mtND4 was included in this study.

Mitochondrial ND4 (mtND4) has been used to look at interspecific variation in two Mid-Atlantic Ridge bathymodiolid species (Maas *et al.*, 1999). Given the reported existence of some intraspecific variation, we tested previously published primers using the described PCR conditions (Maas *et al.*, 1999). The ND4 primers yielded a PCR product of approximately 800 bp in length. Direct sequencing of 510 bp of this product from 12 individuals from different collection sites showed a variable nucleotide that could be assayed by RFLP analysis, using the restriction enzyme *Bsa*J I according to the manufacturer's protocol (New England Biolabs, Inc.).

Additionally, approximately 465 base pairs of mitochondrial cytochrome c oxidase (mtCOI) were amplified and sequenced in 14 mussels from five different collection sites (Alaminos Canyon, Mississippi Canyon, Bush Hill, GC929, and Brine Pool) using primers of Folmer *et al.* (1994), and 457 base pairs of the 16S rDNA (16S) gene were sequenced as well in 10 mussels from the same five sites using primers of Palumbi (1996). Variable restriction sites were identified within the mtCOI sequences,

and the restriction enzymes *Bse*R I, *Dra* I, and *Hae* III (New England Biolabs) were used to discriminate mtCOI haplotypes in individuals from Alaminos Canyon, Bush Hill and Brine Pool.

Analysis of genetic variation

All genetic analyses were done using the program FSTAT version 2.9.3.2 (Goudet 2002), and confirmation of output values and analysis of the ND4 marker data were also done with ARLEQUIN (Schneider *et al.*, 2000). Allele frequencies and observed and expected levels of heterozygosity were calculated for each population. A chi square test was used to test for Hardy-Weinberg equilibrium. The methods of Weir and Cockerham (1984) were used to estimate Wright's fixation indices of reduced heterozygosity of individuals within subpopulation (F_{IS}), of subpopulations to the total population (F_{ST}), and individuals to the total population (F_{IT}). Each collection site was considered to be a subpopulation, each mussel was considered to be an individual within a subpopulation, and the 183 mussels over the nine collection sites constitute the total population. FSTAT computes the standard error of the *F*-statistics by a jackknifing approach over collection sites and uses random permutation tests to assess whether each *F*-statistic differs significantly from zero. Tests for Hardy-Weinberg equilibrium were conducted for each locus within and between sample populations, as well as over all loci. For F_{IT}, a test of overall Hardy-Weinberg equilibrium in the total population, alleles were permuted among collection sites. For F_{IS}, a test of Hardy-Weinberg equilibrium within collection sites, alleles were permuted among mussels within collection sites. Erring on the side of caution that alleles within each individual may not be independent, genotypes were

permuted among populations for F_{ST} to derive an estimate of any significant population differentiation. We used the sequential Bonferroni method to correct for multiple tests (Rice, 1989). Tests for linkage disequilibrium between all loci were conducted by means of the log-likelihood ratio *G*-statistic, where genotypes at each pair of loci were associated randomly via 420 permutations. The P-value was determined from the proportion of pseudo-random datasets that were greater than or equal to the observed *G* value. Regression analysis was used to determine whether a significant relationship exists between genetic distance (F_{ST}) and depth or geographic distance. The Mantel test, using depth and geographic distances as explanatory variables against pairwise F_{ST} values as the dependent variable, was used to test the significance of these correlations.

Results

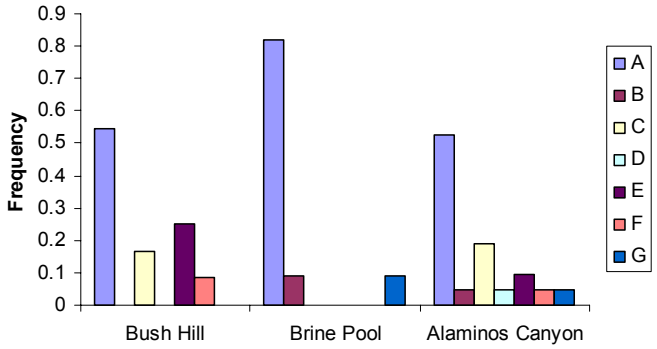
Marker identification and sequence analysis

The sequences for all loci other than the microsatellite (gA3c) were checked via BLASTN and BLASTX searches to see if each locus was closely related to any known protein or genomic DNA sequence, which could suggest that the marker could be under selective constraint if the marker sequence was in a protein coding gene (Altschul *et al.* 1997). None of the sequences from the nuclear loci showed a close match to a known protein in the NCBI database, and the sequences resulting from primers designed to PGM and EFBis introns could not be identified as the intended intron. Thus, we consider all nuclear markers in this work to be anonymous. The sequence for *B. childressi* mtND4 is currently in GenBank (accession number AY130248), and ~550 base pairs of sequences from this study were identical except for an insertion of eight Ts after nucleotide position

347 and variable nucleotides at positions 109 and 247. The sequence for 16S rDNA was deposited in GenBank (accession number XXXXXX).

The total frequency of polymorphic sites over all of the 14 loci sequenced in this study is approximately 0.36% (29/7946 nucleotides). The frequency of polymorphic sites in the six non-microsatellite loci from Mississippi Canyon, Alaminos Canyon, Bush Hill and GC929 was 0.41% (17 sites/4,186 bp). Screening of individuals with restriction enzymes in addition to those predicted to target variable sites revealed no additional polymorphism in a screen of at least 25 mussels from different sites (Alaminos Canyon, Mississippi Canyon, Bush Hill, GC929, GB425, GC234 and Brine Pool). In addition, approximately 2,260 bp of sequence from the five other markers (unpublished data) showed only two polymorphic sites that could not be discriminated with restriction enzymes. Comparison of the 457 bps of 16S rRNA sequence revealed no polymorphic nucleotide sites, and six variable sites were identified in 500 base pairs of mtCOI sequences. RFLP analysis targeting three of the polymorphic sites in mtCOI showed at least seven haplotypes exist. One haplotype is dominant in a sampling of individuals from the deepest site, Alaminos Canyon, as well as two shallow and environmentally-distinct sites, Bush Hill and Brine Pool (Figure 5-2a).

a. mtCOI



b. mtND4

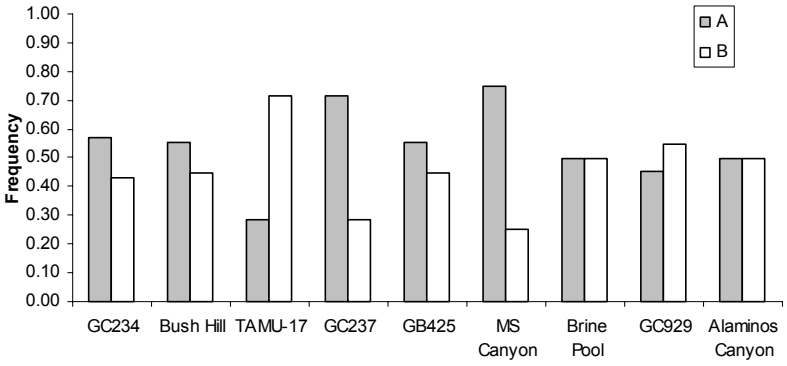


Figure 5-2. Mitochondrial haplotype distributions across populations

Genetic diversity

Five of the six nuclear loci were polymorphic at all localities; anonM7 was monomorphic at five of the nine localities. Two or three alleles were detected by RFLP analysis for five of the markers (Table 5-4). Levels of heterozygosity were low, with five of six markers displaying H_o values less than 0.5 (Table 5-4). Although most of the markers in this study showed low allelic diversity overall, the distribution of alleles for each marker was remarkably consistent between different collection sites, even those with small sample sizes like GC237 and TAMU-17 (Figure 5-3). The distribution of

mitochondrial ND4 haplotypes was similar among the populations, although a significant difference existed between the distribution at TAMU-17 and GC237 populations ($p = 0.0322$ (Bonferroni correction); Figure 5-2b).

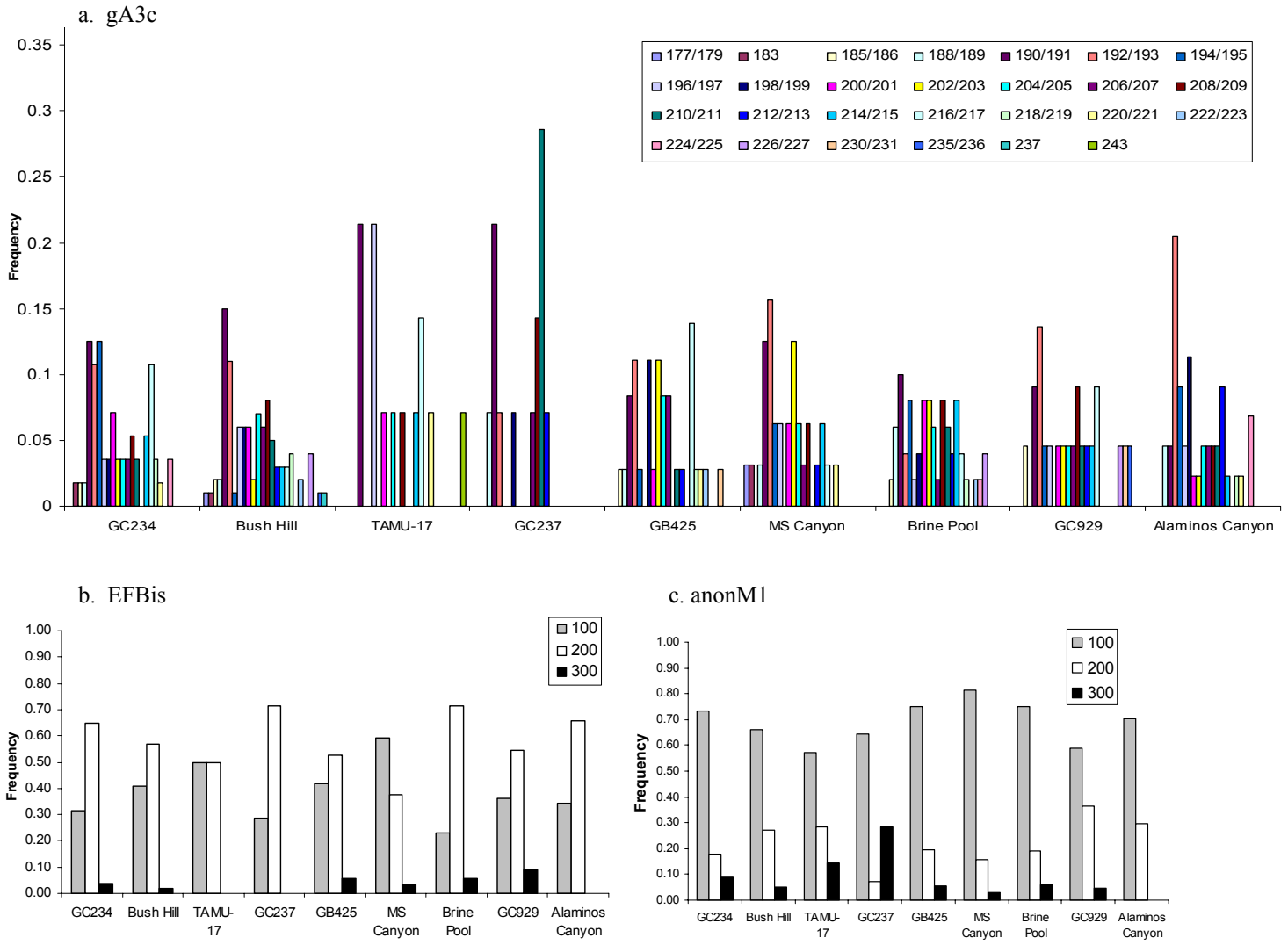
Genetic structure

FSTAT and ARLEQUIN yielded virtually identical *F*-statistics for the nuclear loci dataset. The overall value of F_{IS} was positive and significantly different from zero, as were the F_{IT} values for three of the individual loci (gA3c, anonM1, and anonM7) (Table 5-5). None of the single locus estimates or the overall estimate of F_{ST} differed significantly from zero (Table 5-5). Pairwise F_{ST} values ranged from -0.0181 to 0.0558; comparisons between each pair of collection sites yielded no significant differences ($p < 0.05$, Bonferroni corrected; Table 5-6). The overall value of F_{IT}, as well as F_{IT} for three of the loci (gA3c, anonM1, and anonM7), was also positive and significantly different from zero (Table 5-5). The overall value of F_{ST} based on haplotype frequencies using the mtND4 dataset was -0.01201.

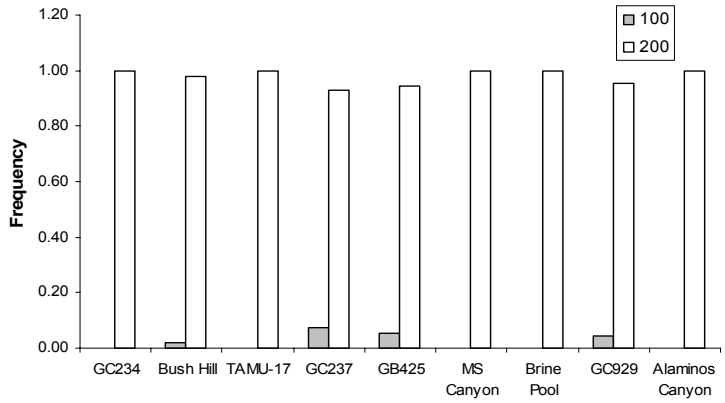
Table 5-4. Genetic diversity analysis at six nuclear loci from nine *B. childressi* collection sites. Values in bold indicate observed heterozygosity values that differ from those expected under Hardy-Weinberg equilibrium ($p < 0.05$; Chi square test). H_o = observed heterozygosity; H_e = expected heterozygosity

	Locus	gA3c	EFBis	M1	PGM	B84	M7
GC234	Sample size	28	28	27	28	28	28
	Number of alleles	18	3	3	2	2	1
	H_o	0.893	0.357	**0.296**	0.357	0.179	NA
	H_e	0.923	0.458	0.523	0.328	0.198	NA
Bush Hill	Sample size	50	50	50	50	50	50
	Number of alleles	22	3	3	2	2	2
	H_o	0.840	0.480	0.540	0.240	0.140	**0.000**
	H_e	0.910	0.480	0.512	0.288	0.165	0.059
TAMU17	Sample size	7	7	7	7	7	7
	Number of alleles	10	3	2	2	2	1
	H_o	0.857	0.571	0.429	0.142	0.287	NA
	H_e	0.967	0.703	0.538	0.275	0.385	NA
GC237	Sample size	7	7	7	7	7	7
	Number of alleles	7	3	2	2	1	2
	H_o	0.857	0.286	0.286	0.429	NA	0.143
	H_e	0.890	0.626	0.440	0.473	NA	0.275
GB425	Sample size	18	18	18	16	18	18
	Number of alleles	18	3	3	2	3	2
	H_o	**0.722**	0.500	0.500	0.313	0.389	0.111
	H_e	0.946	0.449	0.583	0.324	0.459	0.160
MS Canyon	Sample size	12	12	12	12	12	12
	Number of alleles	12	3	3	2	2	1
	H_o	0.938	0.375	0.438	**0.000**	0.125	NA
	H_e	0.935	0.375	0.569	0.181	0.179	NA
Brine Pool	Sample size	25	26	26	26	26	26
	Number of alleles	19	3	3	2	2	1
	H_o	0.920	0.385	0.423	**0.077**	0.154	NA
	H_e	0.949	0.437	0.473	0.242	0.180	NA
GC929	Sample size	11	11	11	11	11	11
	Number of alleles	16	3	3	2	2	2
	H_o	0.909	0.636	0.636	0.273	0.364	0.091
	H_e	0.961	0.571	0.589	0.437	0.385	0.091
Alaminos Canyon	Sample size	22	22	22	22	22	22
	Number of alleles	17	2	2	2	2	1
	H_o	0.818	0.409	0.318	0.318	0.136	NA
	H_e	0.947	0.470	0.504	0.312	0.130	NA
TOTAL	Sample size	182	183	182	181	183	183
	Number of alleles	27	3	3	2	3	3
	H_o	**0.862**	0.444	0.430	**0.239**	0.197	**0.038**
	H_e	0.936	0.508	0.526	0.318	0.231	0.065

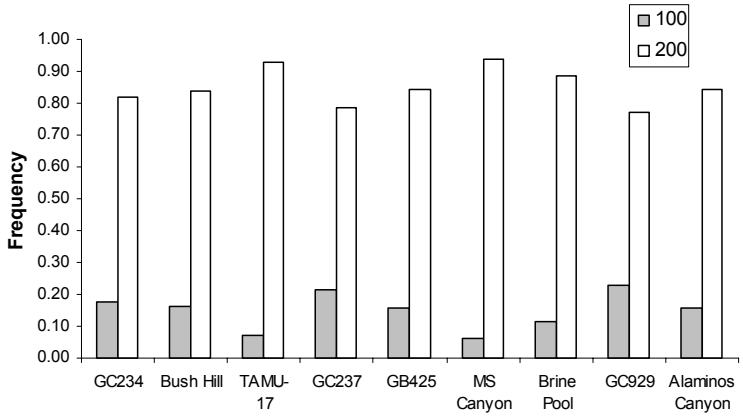
Figure 5-3. Distribution of alleles among different sample populations in *B. childressi*



d. PGM



e. B84



f. anonM7

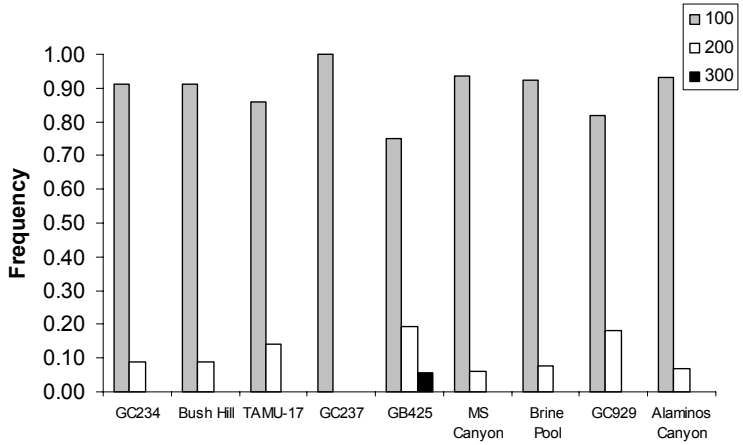


Table 5-5. *F*-statistics for each nuclear locus over all nine collection sites

Locus	F_{ST} ± SE	F_{IS} ± SE	F_{IT} ± SE
gA3c	0.004 ± 0.005	0.075 ± 0.022**	0.079 ± 0.024**
EFBis	0.002 ± 0.007	0.010 ± 0.048	0.012 ± 0.048
M1	0.009 ± 0.017	0.126 ± 0.085*	0.134 ± 0.085*
PGM1	-0.012 ± 0.005	0.092 ± 0.109	0.081 ± 0.111
B84	0.011 ± 0.020	0.002 ± 0.054	0.013 ± 0.057
M7	0.000 ± 0.022	0.325 ± 0.383*	0.325 ± 0.365*
ALL	0.004 ± 0.002	0.073 ± 0.020**	0.077 ± 0.021**

* = p < 0.05; ** = p < 0.001

Table 5-6. Pairwise F_{ST} values above the diagonal, with actual p-values in parentheses. No significant pairwise differences in F_{ST} values exist between any of the populations tested after Bonferroni correction (720 permutations).

	Bush Hill	TAMU-17	GC237	GB425	MS Canyon	Brine Pool	GC929	Alaminos Canyon
GC234	-0.0023 (0.578)	-0.0091 (0.835)	0.0019 (0.0019)	-0.0011 (0.471)	0.0184 (0.446)	-0.009 (0.890)	-0.0067 (0.682)	-0.0009 (0.069)
Bush Hill		-0.0082 (0.338)	0.0097 (0.256)	0.0014 (0.351)	0.0107 (0.272)	0.0064 (0.844)	-0.0133 (0.949)	0.0042 (0.051)
TAMU-17			0.0214 (0.089)	-0.0128 (0.696)	-0.0092 (0.908)	0.0085 (0.535)	-0.0082 (0.869)	0.0006 (0.135)
GC237				0.0203 (0.232)	0.0558 (0.042)	0.0083 (0.158)	0.0067 (0.447)	0.0212 (0.026)
GB425					0.0046 (0.615)	0.0104 (0.393)	-0.0181 (0.993)	0.0098 (0.065)
MS Canyon						0.0236 (0.536)	0.0227 (0.519)	0.0236 (0.318)
Brine Pool							0.0071 (0.678)	-0.001 (0.222)
GC929								-0.0067 (0.404)

Linkage disequilibrium

None of the pairs of loci used in this study showed a significant departure from linkage equilibrium with a Bonferroni correction ($p > 0.05$).

Tests for isolation by depth and distance

Plots of pairwise values of F_{ST} against the difference in depth between each collection site show no correlation between genetic distance and depth (Figure 5-4a; R^2 = 0.001; p = 0.851). A positive trend does exist between genetic and geographic distance (Figure 5-4b; R^2 = 0.105; p = 0.055). Differences in depth and geographic distance together account for 21.2% of the variation in F_{ST} values as assessed by a Mantel test (p = 0.019).

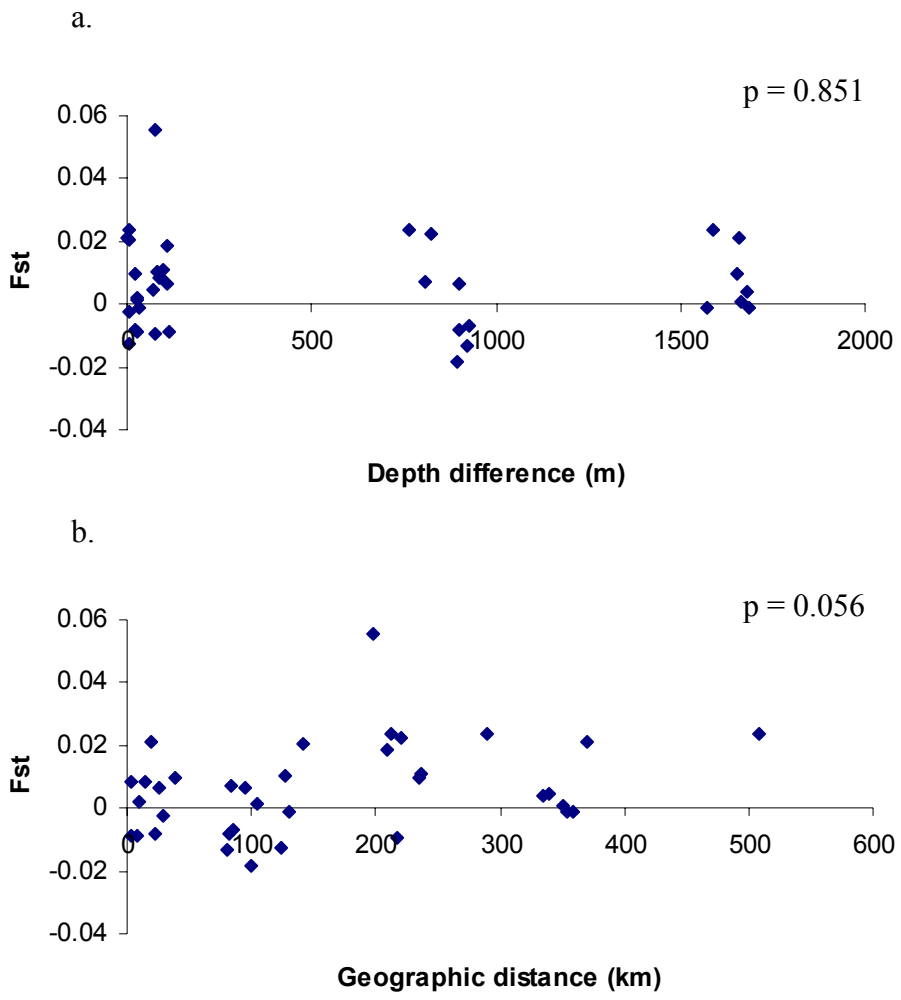


Figure 5-4. Pairwise genetic distance versus differences in depth (a) and geographic distance (b) between *B. childressi* collection sites. P values represent the significance of the correlation between F_{ST} and either depth difference or geographic distance as assessed by a Mantel test (2000 permutations).

Discussion

Craddock *et al.* (1995) suggested that depth may play a role in subdividing populations of *B. childressi* in the Gulf of Mexico. This would be consistent with general ecological patterns of differences in species diversity with depth seen in the Gulf of Mexico (Pequegnat *et al.*, 1990; MacDonald *et al.*, 2003; Cordes *et al.*, in prep) and in other deep-sea fauna (Rex, 1981; France and Kocher, 1996; Chase *et al.*, 1998) including the Mid-Atlantic Ridge bathymodiolines which occur in two different biogeographic provinces of differing depths (Van Dover *et al.*, 1996; Desbruyeres *et al.*, 2000). This would also be in accordance with a recent report showing physiological differences in the DNA repair capabilities of the MAR mussel *B. azoricus* from the extreme ends of its depth range (Dixon *et al.*, 2004). The data from this study show no evidence for differentiation between *B. childressi* populations at 527-636 m depth and those at 1459 and 2222 m (Figure 5a). The result that *B. childressi* does not appear to be depth-limited over a range from 527 to 2222 m may not be surprising. Bathymodiolines at the MAR do indeed show distinct species distributions according to depth, however, the depth range inhabited by *B. childressi* within the Gulf of Mexico is quite similar to that of *B. azoricus* alone (850 – 2250 m; O'Mullan *et al.*, 2001).

The work of Craddock *et al.* (1995a) found one fixed allozyme difference out of 18 loci examined between the Alaminos Canyon and Bush Hill populations of *B. childressi*, and the authors proposed that depth may limit dispersal between these sites. Hey (1991) has suggested that fixed differences are not likely to be found except when gene flow is limited. However, allozymes may not be appropriate markers in studies of gene flow because they may be subject to selection in different environments (Hoffmann

et al., 1995; Dhuyvetter *et al*., 2004; Piccino *et al*., 2004). In contrast, our data provide evidence for extensive gene flow over a 1700 m depth range. Thus, the fixed difference found by Craddock *et al*. (1995a) may potentially be due to differential selection pressures on the fixed locus (aconitase) imposed by the environments at Alaminos Canyon and Bush Hill.

An earlier study proposed that environmental conditions may play a role in selecting different subsets of the larval pool to promote population structure in *B. childressi* (Bergquist *et al.*, 2004). *B. childressi* requires methane in its environment to sustain its symbiosis, however populations are found in a variety of habitats ranging from those dominated by petroleum to those dominated by brine. Petroleum habitats, in addition to having higher levels of crude oil and sulfide that can be potentially toxic, support lower methane concentrations (Nix *et al.*, 1995; Bergquist *et al.*, 2004). Mussels in these environments also frequently harbor parasites and generally demonstrate poorer physiological condition (Nix *et al.*, 1995; Powell *et al.*, 1999; Smith *et al.*, 2000). Brine-dominated sites support higher methane concentrations, lower sulfide, and lower levels of crude oil (Smith *et al.*, 2000; Bergquist *et al.*, 2004). These conditions, combined with the low- to non-existent parasite loads in brine environments, support populations of *B. childressi* in generally better physiological condition (Smith *et al.*, 2000; Bergquist *et al.*, 2004). Reciprocal transplants of mussels between brine- and petroleum-dominated sites resulted in transplanted mussels only acquiring some of the physiological characteristics of mussels from their new environments (Bergquist *et al*., 2004). Bergquist *et al.* (2004) propose that post-settlement selection of *B. childressi* genotypes with different tolerances to different environments may explain the transplantation data. Studies in *Mytilus* spp.

have demonstrated that pre-settlement selection, possibly because different genotypes respond to distinct settlement cues, may account for small-scale ecological segregation of two species within a hybrid zone (Bierne *et al.*, 2003b). Whether potential selection upon different genotypes would occur pre- or post-settlement remains to be determined, however the results from this study of *B. childressi* also yield no evidence that different genotypes are favored at brine- and petroleum-dominated sites.

The lack of detectable genetic variation between brine- and petroleum-dominated populations could suggest that deep-sea mussels exhibit a high degree of physiological plasticity, which allows them to tolerate a wide range of hydrostatic and chemical environments. This result is consistent with findings from transplant studies in shallow water mytilids showing that physiological response to variation in environmental conditions is highly plastic (Dahlhoff and Menge, 1996). Rather than the local environment selecting for given *B. childressi* genotypes that are best suited for high versus low hydrostatic pressure or brine versus petroleum-dominated sediments, the response of the settled larvae may be that of different combinations and timings of gene expression that best suit the animal's physiological response to that environment. The results from this study coupled with the results of the transplant experiments of Bergquist *et al*. (2004) suggest that genetically similar mussels respond differently to the selection pressures imposed by their microhabitats in such a way that can lead to subpopulations with different physiological phenotypes.

The data from this study do show that a positive trend exists between F_{ST} and geographic distance (Figure 5-4). Given that *B. childressi* seemingly have the capability to remain in the water column for weeks, and knowing that other bathymodiolines have

the potential for dispersal over ranges much greater than 500 km, the direct relationship between F_{ST} and geographic distance emphasizes the important role of local water currents in the facilitation or limitation of larval transport. The Mantel test results suggest that the combined effects of depth difference and geographic distance may play a role in limiting dispersal between the most distant sites. However, even the F_{ST} values that are highest in this study (none greater than 0.0236 in comparisons between populations with sample sizes greater than 15) do not indicate that any of the populations are differentiated. The results here imply that further investigations into potential barriers to dispersal of populations of *B. childressi* are warranted. The identification of more variable genetic markers, if they exist, would certainly increase the power of any correlations between F_{ST} and these potential limiting factors. Additionally, factors not included in this study, such as more accurate measures of the speed and direction of water currents at and around the collection sites, need to be considered as other variables that could play a role, likely in addition to depth, in restricting the movement of larvae throughout the Gulf of Mexico.

Analysis of population structure using the eight molecular markers in this study revealed no significant differentiation between populations of *B. childressi* as evidenced by low values of Wright's F_{ST} and a relatively even distribution of mitochondrial haplotypes (Figure 5-2). The significant difference in the distribution of mtND4 haplotypes between GC237 and TAMU-17 is likely due to each of these sample populations containing only seven animals (Figure 5-2b). While the results seen here may be inconsistent with the proposals of Craddock *et al*. (1995a) and Bergquist *et al*. (2004), they are consistent with high levels of dispersal that have been seen in other

bathymodiolids at hydrothermal vent environments (Craddock *et al.*, 1995b). They are also consistent with genetic studies of the tubeworms with which these mussels are often associated in the Gulf of Mexico over sites shallower than 1000 m in depth (McMullin *et al.*, in prep.).

A recent population genetic study of two hydrocarbon seep tubeworms from sites along the upper Louisiana Slope of the Gulf of Mexico showed no evidence for differentiation between populations of *Seepiophila jonesi* and little evidence between populations of *Lamellibrachia luymesi* over a 540 km geographic range (McMullin *et al.*, in prep). These results, in combination with the results from the current study, suggest that water currents do not limit the dispersal of tubeworms and mussels over the Upper Louisiana slope at depths less than 1000 m. However, the occurrence of different species of escarpids as well as potentially different species of lamellibrachids at deep and shallow sites in the Gulf of Mexico suggests that dispersal of tubeworm larvae is limited, either by depth, water currents, or a combination of factors (Gardner *et al*., 2001; McMullin *et al*. 2003). The results from the current study suggest that mussel larvae are not limited over the same range. If higher resolution DNA markers would substantiate this result, this would suggest that currents may not be the primary limiting factor between deep and shallow Gulf of Mexico sites but rather that tubeworms and mussels may be differentially limited by depth.

The low frequency of polymorphic nucleotide sites within approximately 6,000 bp of anonymous DNA surveyed in this study suggests an overall low level of genetic diversity within this species. It must be taken into consideration that these markers likely underestimate the genetic diversity that could be present because restriction enzymes

only target a few nucleotide differences between individuals, and only a limited number of mussels were sequenced from different collection localities to determine the potential restriction sites. Additionally, this work would be strengthened with larger sample sizes for some of the collection sites.

The development of variable markers in *B. childressi* was not without challenges. The amplification of anonymous host genomic DNA seems at this point to be the best method for identifying variable molecular markers while avoiding the potential problems of low-microsatellite DNA frequency and parasite contamination. While it is not possible to know with complete certainty that any of the samples in this study were not contaminated, the results of this marker analysis suggest that parasitic contamination is likely not an issue here. There were only four cases in total over all of the animals and loci tested in which PCR amplification failed; these cases were restricted to three of the loci with a maximum of two failed reactions at any one locus (Table 5-4). A greater frequency of presence/absence variation would be more indicative that our markers were not amplifying only host DNA (McDonald *et al.*, 1996). Additionally, given that parasites have not been detected in mussels from Brine Pool (Powell *et al.*, 1999), the inclusion of samples from this collection site ensures that the markers in questions are host-specific.

This study represents our first steps towards understanding populations of *B. childressi* throughout the Gulf of Mexico from a genetic standpoint. While no differentiation between populations was detected, the data here are suggestive that depth and possibly water currents may play a role in limiting dispersal of larvae between some of the most distant collection sites. At this point, there is no evidence that hydrocarbon

and brine seep environments promote population subdivision by selecting for different genotypes. More variable genetic markers would increase the power of our ability to assess population subdivision, but for now the depth and geographic range of dispersal of *B. childressi* in the Gulf of Mexico is within that of other known bathymodiolids. This species' successful exploitation of a variety of Gulf of Mexico chemosynthetic environments mirrors the success of mytilids in their worldwide range, from coastal to bathyal and freshwater to marine.

Acknowledgments

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CHAPTER 6 – Summary and Implications

R. piscesae – a case of phenotypic plasticity

DNA fingerprinting and analysis of globin sequence differences show that genetic variation does not explain the microhabitat-specific phenotypic variability seen in *R. piscesae* along the Juan de Fuca Ridge. Analysis of globin gene expression shows that the different *R. piscesae* growth forms, as well as similar phenotypes in different microhabitats, respond differently to their local environments. Together, these results demonstrate the critical role of the environment in promoting a wide diversity of *R. piscesae* growth forms in the northeast Pacific vent system. This species presents a classic case of phenotypic plasticity and acclimation, where identical genotypes exhibit differential morphological and physiological responses to distinct environmental conditions (Kingsolver and Huey, 1998).

The mechanisms underlying phenotypic plasticity and acclimation in this species are due to a number of complex interactions of the animal with its environment from the time of the settling of its larval stage until it grows into an adult tubeworm. The results from this study suggest that oxygen may play a role at least in the differential response of chimney and basalt *R. piscesae* phenotypes at the level of globin gene regulation. However, hydrothermal vent environments are so dynamic that a number of confounding factors, including temperature, levels of sulfide and other chemicals that react with oxygen, or any combination of these and more, could ultimately drive the phenotypic and physiological differences seen in this species. An intriguing possibility may be that hemoglobin serves as a detector of oxygen and sulfide levels in recently settled larvae

and participates in the coordination of events that induce different phenotypes in distinct environments. The cues that induce tubeworm larvae to settle are unknown, although the use of biogenic cues has been proposed as means more reliable than the use of chemical signals, given that vent conditions can be so ephemeral (Mullineaux *et al*., 2000). Upon larval identification of cues and settlement, hemoglobin could act in coordination with developmental pathways to produce the most suitable growth form for the given environment.

Future directions for research in R. piscesae

Numerous questions remain unanswered regarding the Endeavour *R. piscesae* population and its multi-hemoglobin system. It would be interesting to see, knowing that collection site has an effect on gene expression, what is the effect of the age of the animal on its levels of gene expression? Are there ontogenetic shifts in the expression of globin subunits that might reflect the differential usage of one hemoglobin type over another in different life stages? Are the differences that we see truly due to differing environmental conditions alone, are they due to animals of different ages, or perhaps are they a result of a combination of factors? Does an environmental shift lead to changes in hemoglobin gene expression and the pattern of growth?

The work in this dissertation considers only questions surrounding the interactions between the tubeworm host and its local microhabitat. In addition to the external environment, interactions between the host and its symbionts seem likely to be an important component of the development of microhabitat-specific phenotypes. *R. piscesae* larvae acquire their symbionts from the environment each generation (Cary *et*

al., 1993). To date, no evidence exists for differences among the symbiont populations along the Juan de Fuca Ridge or between phenotypes. However, differential signaling of symbionts acquired from one environment versus another could potentially play a key role in phenotypic induction. The acquisition of symbionts differentially suited to microhabitats could serve as one of the biogenic cues proposed to be involved in larval settlement. Studies of the interactions between symbiont and tubeworm genomes, particularly looking at differences in gene expression of recently settled larvae in basalt and sulfide chimney environments, may yield insight into some of the forces behind phenotypic differentiation.

B. childressi population structure

In the Gulf of Mexico, the results of the genetic marker analysis suggest that while populations of *B. childressi* are not genetically differentiated over their range of habitats, geographic distance and depth constraints may play a role in limiting larval dispersal. If gene flow limitations do exist, this would suggest that the pressures faced by seep mussels are greater than those faced by vent mussels, which have demonstrated dispersal capabilities of greater geographic and depth ranges. This is particularly important in light of the fact that seep habitats are subject to anthropogenic disturbance as a result of their economic value.

Future directions for research in B. childressi

The genetic structure of the Gulf of Mexico *B. childressi* population would be better resolved with additional variable markers to conclusively establish whether factors

in this region indeed prevent unlimited mixing of populations from deep, shallow, favorable and less favorable environmental conditions. A few microsatellite markers are in the process of being developed in *B. thermophilus* (A. Baco, WHOI, personal communication), and testing of these primers on *B. childressi*, once they are published, would be worthwhile. Additionally, the questions surrounding the mitochondrial system in *B. childressi* still must be pursued further. Work by Maas *et al*. (in prep.) suggests that there is no correlation between gender and mitochondrial haplotypes in the vent mussel *B. thermophilus* (R. Vrijenhoek, MBARI, personal communication). Studies of multiple mitochondrial genes in *B. childressi* of known gender would help to resolve the question of whether the mytilid system of doubly uniparental inheritance exists in this system and whether mitochondrial markers are appropriate for population genetics studies.

Conclusion

Many questions remain unanswered, but the work in this dissertation marks a start to understanding some of the aspects of the genetic interactions of deep-sea tubeworms and mussels within their ecological settings. Molecular biological techniques give us insight into facets of deep-sea systems that would otherwise be inaccessible. Markers, quantitative real-time PCR, and DNA sequence analysis allow us to estimate dispersal, relatedness, and gene expression levels in organisms from 2200 m depth that do not easily lend themselves to experimentation. Advances in the sampling of organisms and environmental data, particularly in the northeast Pacific with the construction of an interactive undersea regional seafloor observatory (the NEPTUNE project), will hopefully increase our understanding of deep-sea biological systems. From what we do

know, however, even while vent and seep communities are unique as far as from where they draw their energy and carbon sources, the basic biological and ecological processes in these communities at the bottom of the ocean are fundamentally not much different from those in most other systems on earth.

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APPENDIX

Methods and results from *R. piscesae* chromosome preparation from Chapter 4

A protocol based on methods in Imsiecke *et al.* (1995), Cornet (2000), and Dixon *et al.* (2001) was used to attempt to determine the chromosome number in *R. piscesae* as well as to prepare chromosome spreads for use in fluorescent *in situ* hybridization with globin-specific probes to determine the location of globin genes among the chromosomes. All procedures were done on board the R/V Thomas G. Thompson during a research cruise in August 2003. Juvenile tubeworms (< 5 cm in length) among adult samples collected were removed from their tubes and incubated in a 0.01% solution of colchicine at 4 ºC for 4 - 24 hours to block cell division. Vestimentum tissue was removed from the animals. After removing the colchicine, the tissue was treated with a series of hypotonic seawater solutions for 45 min and then was fixed in a 3:1 methanol:acetic acid solution for three incubations of 10 minutes each. After fixation, the tissue was chopped up finely within a 50% acetic acid solution. Drops of the cell suspension were dropped from a Pasteur pipet from a height of approximately 30 cm onto glass microscope slides prewarmed to 42 ºC. After air drying, a few slides were stained with a 10% Giemsa solution and examined with an Olympus microscope to check the chromosome spreads. Most of the slides were stored unstained in ethanol at -70 ºC for later *in situ* hybridizations.

The Giemsa-stained slides, after examination, did not show any good chromosome spreads that would be useful for hybridizations with globin probes. Most of the slides showed several intact nuclei, but none that had been in the process of division. As a consequence, the number of chromosomes in *R. piscesae* was not able to be determined and the fluorescent *in situ* hybridization procedure was not done.

Table of genome walking and anonymous primers from Chapter 4

Table A-1. Primers used for amplification of introns, genome walking, and sequencing of globin genes and one anonymous marker in *R. piscesae*. * indicates primers used in genome-walking in conjuction with primers used for this method as described in Mishra *et al*. (2002).

Gene	Primer	Sequence (5' – 3')	Location	Accession No.
A1	A1F1	CGTTCTAAGTCTATGTGGCACG	5' UTR	
	A1F2	TGAGGCATACGGCATCGGCT	Exon 1	
	A1F3	GACGGTCATGACACGATGTT	Intron 1	
	A1F4	GAACGCAGAGGTCATCAGCA	Intron 1	
	A1F5	CCACCTGAAGTCTCAGCACGA	Exon 2	
	A1R1	GTCCGAACCGGTAATGCCCTG	Exon 3	
	A1R2	GCGACGAAAGGTCCTCCGAGTT	Exon 2	
	A1R3	GTATGCGGTGTGTTGACGTT	Intron 1	
	A1R4*	CGGAAGTCGGTTGTAACAACG	Intron 1	
	A1R5*	CGTATCGAGAAGTGTAACCGACTCAC	Intron 1	
A2	A2F1	CATGTCGAACAGTTTCATGCGT	5' UTR	
	A2F2	GCGGTATTAGAGCGTCTTACT	5' UTR	
	A2F3	GCCTGAAGGTTAAGCGCCAGTGGG	Exon 1	
	A2F4	CCTGCTGCTCGCGACTTGTT	Exon 2	
	A2F5	CATCGGCTCTGAGGTGTCG	Intron 2	
	A2R1	CGGTGGTCGGATACAGAATGTG	3' UTR	
	A2R2	CTAGTTCCCAGCGATGCCG	Exon 3	
	A2R3*	GATGTTGTCACCACGCACAC	Exon 2	
	A2R4*	GGAAGACACTGGAAACAGCGAAAG	Exon 2/Intron 1	
	A2R5*	CCCAGATGTAGTGTCCGAAG	Exon 1	
	A2R6*	CGTCCACTTCCGCTTCCGTAA	Exon 1	
B1a	B1aF1	CCATGTGCAGTTGATAAGAC	5' UTR	
	B1aF2	GCGATGTTTCGTGTCGGCAGAATCA	Exon 1	
	B1aF3	GCCGGCTACTCTGTATTGT	Intron 1	
	B1aF4	GGAATCCATGCTCGCTCATCT	Exon 2	
	B1aF5*	GATGCCATCTTCGTGAACATCTC	Intron 2	
	B1aF6*	GTTCTAGGTGATGGCGAAGGT	Intron 2/Exon 3	
	B1aR1	GTGTCGAAGTGGTGTGCATGCGT	3' UTR	
	B1aR2	CACGTCCAGACCGCCCATCACACGCATCA	Exon 2	
	B1aR3	CGAGGCGAGCAGAGTTAATTG	Intron 1	
	B1aR4*	CCTTAACATAGCGTATGCACAACAGG	Intron 1	
	B1aR5*	TCATACTCGTTATGACATGCCA	Intron 1	

B1c	B1cF1	CGTGTGCTGCAGTTATGGTGACTC	5’ UTR
	B1cF2	CTACGCCCTCTTCAGCAG	Exon 1
	B1cF3	CTGGACGTAGCCTGTGATTA	Intron 1
	B1cF4	TGCGTGTGATGACAGGACTTCG	Exon 2
	B1cF5*	CAGATGATGGAGCAGTCGAT	Exon 3
	B1cR1	GATCACGTGGTGGGTGCCTTGAGTG	3’ UTR
	B1cR2	CCACGCATCGGGGTTGAAGT	Exon 3
	B1cR3	CCTATCCAGGATGTGAACAG	Intron 2
	B1cR4	CTCCTCATGTCGCCGACATT	Exon 2
	B1cR5*	CAGATGGGTAACAAACGTGGGA	Intron 1
	B1cR6*	GAGGCAGTGAAGACGGCATC	Exon 1
B1new	B1newF1	GGATGCCGAGCCGATCATACAC	5’ UTR
	B1newF2	TCAGACGAAGCTCACCGGA	Exon 1
	B1newF3	CTTAACTCACCGGCGCATCT	Intron 1
	B1newF4	GACCCTCGACTCGCTACTGGC	Exon 2
	B1newF5*	CCACATTTGTGAATCTAGCC	Intron 2
	B1newF6*	GTTCTGACGCTCGATCTGAC	Intron 2
	B1newR1	GGTTTGTTATGATGAATGCC	3’ UTR
	B1newR2	CGACCACCTTGGGCAGACCG	Exon 3
	B1newR3	CACCAGGTGCCATTTCGAAG	Exon 2
	B1newR4*	GGTGATACTGAGGCCGCGAGAGA	Intron 1
	B1newR5*	GGACGACTCTGACTTACTTC	Intron 1
B2	B2F1	CTTGTTCGCACGTCTGCCTCT	5’ UTR
	B2F2	CAGTTCACTGGACGTCGTCT	Exon 1
	B2F3	GACCCAGCAACCCTCAACGCACAG	Exon 2
	B2F4	GTCGTTCCTGCGACTGATGCC	Exon 3
	B2R1	CGGTTTAAATGTCACTG	3’ UTR
	B2R2	CTATGCAGGCAGACCATCG	Exon 3
	B2R3	CTTGTGCTGAGTGGCCAAGT	Exon 2
	B2R4*	CAACTACTCACTCCTTGAAGACAGCCTGG	Intron 1/Exon 1
	B2R5*	CTGCATCTCCGTACGATCCTC	Exon 1
Anon	F	TGGGTAACAAACGTGGGACA	Anonymous
	R	CCCAGCGTATACCACACAAT	DNA

Methods for *B. childressi* microsatellite marker development from Chapter 5

Genomic DNA was digested with *Sau*3a I, and fragments between 250-850 bp in length were size-selected by extraction from a 1% agarose gel with a QUIquick Gel Extraction Kit (Qiagen, Inc.). Complimentary double-stranded linkers were constructed using the primers SAUL A: 5'-GCG GTA CCC GGG AAG GTT GG-3' and SAUL B: /5'Phos/- CGC CAT GGG CCC TTC GAA CCC TAG-3'. These linkers were ligated onto the digested fragments which were then hybridized with the repetitive probes $(GA)_{12}$, $(GAAA)_6$, $(GATA)_6$, and $(TACA)_6$. The MagnaSphere Magnetic Separation Kit (Promega) was used to capture repetitive DNA fragments that had annealed to the probes. Single-stranded DNA, removed from the kit's magnetic beads, was recovered and used as a template in a PCR reaction with SAUL A as a primer. These PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc.) and the linkers were removed before the insert DNA was ligated into the plasmid vector pBluescript (Stratagene). The vector was transformed into DH5α™ competent *E. coli* cells (Invitrogen) by a heat shock method. Transformation products were plated onto LB plates with 50µg/ml ampicillin and 100 µl of X-gal. After incubation overnight at 37 °C, positive colonies were transferred to 96-well microtiter plates containing LB broth and ampicillin. After growth for two hours at 37 °C, colonies were transferred from the microtiter plates to nylon filters on new LB/amp plates using a replica plater to be grown overnight again at 37 ºC. Cells were then lysed, and the DNA was denatured, neutralized, and UV-crosslinked to each nylon filter. Filters were hybridized to biotin-labeled repetitive probes (same four as before), and detection was done using the Biotin Luminescent Detection Kit (Boehringer Mannheim) followed by exposure to X-ray film to identify positive clones. Two genomic libraries were constructed with the enrichment step, and one was constructed without it.

Permission for Personal Communication

Chapter 3

Date: Wed, 20 Jul 2005 14:04:54 -0400
To: Susan Carney <slc239@psu.edu>
From: Deb Grove <dsg4@psu.edu>
Subject: Re: personal communication

You may use that statement.

Deb

Hi Deb,

In my thesis, I mention that QPCR runs on different machines yield similar Ct values, and I attribute that to you as a personal communication. I think that I need to have explicitly in writing that this is OK with you. If this is the case, could you reply to this email with your permission?

thanks!
Sue

At 04:29 PM 2/8/2005, you wrote:
I would hesitate to make any comparisons between genes without using a standard curve. If you have your gene sequence in a plasmid and we ran both in the assay and the same copy number came up with the same Ct, then I think you could cautiously make some comparisons.
This would probably be comparable between the two machines. I have run duplicates on both at the same time and see fairly similar Cts. Biggest difference is amount of fluorescence which doesn't really play a role in quantiation.

Deb

Chapter 5

Date: Wed, 20 Jul 2005 20:27:36 +0200
From: hourdez@sb-roscoff.fr
To: Susan Carney <slc239@psu.edu>
Subject: Primers for PGM

Dear Sue-

You can use the primer sequences Didier and I sent you for your thesis. These sequences will be published some time soon so I guess the best would be to cite

this as a personal communication from Didier Jollivet who will be the lead author on the publication.

Cheers,
Stephane

****
Dr. Stephane Hourdez
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Date: Tue, 07 Jun 2005 05:04:00 -0700
Subject: Re: question regarding Won et al. 2003
From: Bob Vrijenhoek <vrijen@mbari.org>
To: Susan Carney <slc239@psu.edu>
CC: "Paul A. Tyler" <pat8@soc.soton.ac.uk>

Hi Susan,

I attached a draft of the Maas note on heteroplasmy and DUI. As you can see, it was never finished, and I think it is a bit out of date now. It would be best to cite it as a pers. comm. from me, because Dr. Maas is now a dean and she doesn't have time to work on it. Please don't distribute it further, as someone may yet convince me to submit it somewhere. Let me know if it is helpful to your discussion.

Best wishes,
Bob Vrijenhoek

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Chapter 6

Date: Wed, 20 Jul 2005 20:13:09 -0400
To: Susan Carney <slc239@psu.edu>
From: Amy Baco-Taylor <abaco@whoi.edu>
Subject: Re: personal communication

Hi Sue,

Congratulations on being almost done! It is fine to use me a pers comm. We haven't gotten funding for developing the microsats yet, so they have been kind of stalled for a while, but that is still one of our goals.

-Amy

Hi Amy,

It's been a while since we've been in touch. I just defended my thesis last week and am getting ready to submit the final version to the graduate school. I am writing to ask you for permission to use you as a personal communication (an email reply to this would suffice, if you agree). In my conclusion chapter, under future directions, I have the following statement:

"Microsatellite markers are in the process of being developed in *B. thermophilus* (A. Baco, WHOI, personal communication), and testing of these primers in *B. childressi*, once they are published, would be worthwhile."

Is this statement appropriate for me to use you as a personal communication? Please let me know.

Best regards,
Sue

--
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Education

Ph.D. Biology - August 2005 **The Pennsylvania State University**, University Park, PA
B.S. Biology – May 1998 **Muhlenberg College**, Allentown, PA

Research experience

June 1999 – August 2005 **The Pennsylvania State University**, University Park, PA
Thesis: Ecological Genetics of the Hydrothermal Vent Tubeworm, *Ridgeia piscesae* and the cold seep mussel, *Bathymodiolus childressi* Ph.D. Thesis Advisor - Stephen W. Schaeffer
- Participated in 6 oceanographic research cruises and 3 deep submersible dives

May 1997 – August 1997 **The Wetlands Institute**, Stone Harbor, NJ
Research Intern, Horseshoe Crab Genetics

Publications

Carney, S. L., Peoples, J. R., Fisher, C. R. and S. W. Schaeffer. 2002. AFLP analyses of genomic DNA reveal no differentiation between two phenotypes of the vestimentiferan tubeworm, *Ridgeia piscesae. Cah Biol Mar* 43: 363-366.

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Grants

July 2003-2005 NSF Doctoral Dissertation Improvement Award
Molecular Evolution of Extracellular Hemoglobins in the Vestimentiferan Tubeworm *Ridgeia piscesae*

Oral presentations at meetings

Eastern Great Lakes Molecular Evolution Meeting – Cornell University - April 24, 2004
"Expression and molecular population genetic analysis of hemoglobin subunits in the deep-sea hydrothermal vent tubeworm, *Ridgeia piscesae*"

American Society for Limnology and Oceanography – Salt Lake City, UT – February 21, 2005
"Morph- and habitat-specific differences in the expression of hemoglobin subunits in two extreme phenotypes of the Juan de Fuca Ridge tubeworm, *Ridgeia piscesae*"

Northeast Ecology and Evolution Meeting – Penn State University – March 19, 2005
"Environment-specific levels of hemoglobin chain expression in variable phenotypes of the hydrothermal vent tubeworm*, Ridgeia piscesae*"

Poster presentations at meetings

Second International Symposium on Deep-sea Hydrothermal Vent Biology - Brest, France – October 2001
"AFLP analyses of genomic DNA reveal no differentiation between two phenotypes of the vestimentiferan tubeworm, *Ridgeia piscesae*"

10th Deep Sea Biology Meeting - Coos Bay, OR – August 25-29, 2003
"Sequencing, localization, and expression of globin genes in the Juan de Fuca Ridge vestimentiferan, *Ridgeia piscesae*"

SMBE National Meeting - Genomes and Evolution 2004 - Penn State University – June 17-20, 2004
"Expression and molecular population genetic analysis of hemoglobin subunits in the deep-sea hydrothermal vent tubeworm, *Ridgeia piscesae*"

Teaching experience

Teaching Assistant, Dept. of Biology, The Pennsylvania State University – 1999 – 2005
Biology 110 and 110H - Basic Concepts and Biodiversity – Fall 1999, 2000, 2002, 2004
Biology 220W and 220M - Populations and Communities – Spring 2000, 2001, 2005
Biology 427 – Evolution – Fall 2001