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

Susan L. CARNEY*, John R. PEOPLES, Charles R. FISHER, and Stephen W. SCHAEFFER
The Pennsylvania State University, Department of Biology
208 Mueller Laboratory, University Park, PA 16802, USA
*Fax: (1) 814-865-9131 - *E-mail: slc239@psu.edu*

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 sulphide microhabitats it populates and tube morphologies it expresses resemble those of other vestimentiferan tubeworm genera known at hydrothermal vent and deep-sea hydrocarbon seep sites 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 & Fontaine, 1987; 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., 1998). The vent habitats on the Juan de Fuca Ridge are diverse, varying in vent fluid flow rate, chemical composition, temperature, and substratum 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 substrata in weak, diffuse flow environments of low sulphide 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 metre in length, tapering to a thin-walled posterior that is approximately 1 mm in diameter. Long-skinny *Ridgeia* resemble the cold seep vestimentiferan *Lamellibrachia* cf. *lumeysi* van der Land & Nørrevang, 1975, from the Gulf of Mexico, which has been shown to acquire sulphide 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 sulphide uptake in this manner, a useful adaptation for tubeworms that live in environments where sulphide is often undetectable around the animals' plumes (Urcuyo et al., submitted). Short-fat individuals are found on sulphide edifices, in areas with visibly active venting fluid that is up to 30 °C and with sulphide 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. This phenotype displays no roots and uses its plume to uptake sulphide.

Two hypotheses can explain the morphological differences in *R. piscesae* in different microhabitats. The first hypothesis is that the various phenotypes are induced by environmental cues. In a single aggregation of *Ridgeia* in heterogeneous fluid flow at the Endeavour Segment of the Juan de Fuca Ridge, Urcuyo et al. (submitted) 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 haemoglobin molecules (J. Flores, PSU, pers. comm.). Initial studies have detected higher concentrations of the 400 kDa haemoglobin 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 sulphide

from their high-flow, high sulphide environments. These studies have also found that the 400 kDa coelomic haemoglobin has a higher affinity for sulphide in the long-skinny morph. This again might be a valuable adaptation to low-sulphide environments. Ongoing studies by Flores et al. will attempt to ascertain the physiological differences between the haemoglobins of short-fat and long-skinny *R. piscesae* at the level of protein structure.

Alternatively, *R. piscesae* morphotypes are selected by their local vent microhabitat. Previous genetic studies of *Ridgeia* have detected no significant differentiation between morphs at 10 variable allozyme loci, one mitochondrial gene, and one nuclear-encoded rDNA gene (Southward et al. 1995; Southward et al. 1996; Black et al., 1998). Allozymes provide a rapid method for assaying genetic differences, but these loci may not reflect true levels of differentiation among populations because they can be the targets of selection, and they are also unable to detect silent nucleotide substitutions and amino acid changes that do not result in detectable changes in proteins (Hartl & Clark, 1997). Undetected genetic differentiation leaves 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 is likely to be due to differences in gene expression. This study used the amplified fragment length polymorphism (AFLP) analysis to genetically fingerprint two populations of short-fat and long-skinny *R. piscesae* to determine if local selection discriminates between the two extreme populations.

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. The advantage of this method is that it allows more genetic variation within the genome to be surveyed. An important limitation of this approach is that one assumes that shared fragments among individuals are alleles of the same genetic locus. Our results with this technique confirm the findings of previous genetic studies, suggesting that the very different phenotypes of *R. piscesae* represent one species. The similarity of these phenotypes provides a foundation for future studies to understand how a single organism can tolerate and exploit such a broad range of environmental conditions.

Material and methods

R. 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 (47° 57'N, 129° 05'W) while short-fat animals were retrieved from a portion of the Smoke and Mirrors sulphide edifice named Strawberry Fields (47° 56'N, 128° 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 (Ausubel et al., 1989). DNA from each vestimentum sample was digested with EcoRI and *Mse* I and subjected to AFLP analysis according the manufacturer's protocol in 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%). Two methods were used to estimate allele frequencies at each AFLP locus. The method of Zhivotovsky (1999) assumes that populations are in Hardy-Weinberg equilibrium and the frequency of dominant homo- and heterozygotes can be estimated from the frequency of null alleles with Bayesian methods. The method of Holsinger et al. (2002), which also uses Bayesian methods to estimate allele frequencies, does not require the assumption of Hardy-Weinberg equilibrium. This allows one to incorporate uncertainty of an organism's breeding system into the estimates of genetic differentiation.

The AFLP loci are dominant genetic markers and the frequency of the null alleles at each locus was used to estimate heterozygosity. The average AFLP heterozygosity levels within morphotype and across morphotypes were used to estimate F_{st}, 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 & Clark, 1997). The neutral migration parameter, *Nm*, estimates the number of migrants per generation that are exchanged between the two populations (Hartl & Clark, 1997). An estimate of *Nm* can be determined from F_{st} assuming an island model of population structure (Hartl & 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 between the two *Ridgeia* populations to derive a 95% confidence interval for our observed estimates of F_{st} and *Nm* with the Zhivotovsky (1999) method. The Holsinger et al. (2002) method provides a 95% credibility method for F_{st}.

Results

Fragment analysis yielded nearly identical banding patterns between the short-fat and long-skinny populations of *R. piscesae*. No fixed differences were found in either population; there were no fragments found in all twelve individuals of one morphotype and absent in the other. A total of 463 fragments (68.4%) were found in multiple

individuals in both populations (Table 1). A total of 68 fragments (10.1%) generated were found in each of the 24 individuals sampled. Nearly one-fourth of the fragments (22.7%) were singletons, found in only one of the 24 individuals sampled.

Table 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; LS = long-skinny; SF = short-fat morph.

Data from all of the fragment analysis runs were compiled into a presence/absence matrix. The AFLP data for the two *R. piscesae* morphs yield an average 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) when one assumes that populations of *R. piscesae* are in Hardy-Weinberg equilibrium (Zhivotovsky, 1999). When Hardy-Weinberg equilibrium is not assumed, the average F_{st} value is 0.0004 (95% credible interval = 0.0002 - 0.0015) and the *Nm* estimate equals 624.7 (95% credible interval = 166.4 - 833.1) (Holsinger et al., 2002).

Discussion

The results of this genome-wide survey of DNA fragments between short-fat and long-skinny *R. piscesae* populations are consistent with the previous studies of genetic diversity, because no significant differentiation was observed between these two extreme phenotypes. The minimum 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 or through the selection of morphotypes in low or high sulphide environments. 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 physico-chemical differences between the habitats of long-skinny and short-fat *Ridgeia* is the concentration of hydrogen sulphide 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. Sulphide 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 strengthen existing evidence that the morphs of *R. piscesae* represent a single, phenotypically variable species and thus lay the groundwork for future studies to detect morph and tissue-specific differences in gene expression.

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