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ORIGINAL ARTICLE

Toward a mechanistic understanding of larval dispersal: insights from genomic fingerprinting of the deep-sea hydrothermal vent tubeworm *Riftia pachyptila*

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Abstract

Larval dispersal is critical for the maintenance of species populations in patchy and ephemeral hydrothermal vent habitats. On fast-spreading ridges, such as the East Pacific Rise, rates of habitat turnover are comparable to estimated life-spans of many of the inhabiting species. Traditionally, dispersal questions have been addressed with two very different approaches, larval studies and population genetics. Population genetic studies of vent-endemic species have been informative for determining whether patterns of dispersal are suggestive of stepping stone or island models and estimating rates of gene flow (effective migrants per generation) over broad geographic ranges. However, these studies leave fundamental questions unanswered about the specific mechanisms by which larvae disperse and species maintain their populations and biogeographic ranges. With the goal of examining genetic structure and elucidating alternative larval dispersal mechanisms, we employed a genomic DNA fingerprinting technique, amplified fragment length polymorphisms (AFLPs). To assess the potential utility of AFLPs, and genetic structure of the hydrothermal vent tubeworm *Riftia pachyptila*, genomic 'fingerprints' were recovered from 29 individuals from five vent fields spanning a distance of up to *c.* 5000 km along the East Pacific Rise. In contrast to previous population genetic studies that found little to no genetic structure using allozymes and mitochondrial DNA, genetic analyses of 630 polymorphic AFLP loci identified distinct subclades within *R. pachyptila* populations. Significant levels of differentiation were observed among populations from all vent regions as well as within each region. Discrete assemblages of tubeworms separated by as little as *c.* 400 m within a given vent region were genetically distinguishable and cohorts (based on size-frequency distribution) within an aggregation were found to be most closely related. These results suggest that mechanisms of larval dispersal act to retain cohort fidelity in *R. pachyptila*.

Problem

Since the discovery of deep-sea hydrothermal vents, questions have persisted as to how populations along the mid-ocean ridge system are maintained through larval dispersal and successful colonization. The highly ephemeral nature and the vast distances separating these disjunct habitat 'islands' were expected to affect the genetic structure of individual species (Grassle 1985; Jollivet *et al.* 1995a; Vrijenhoek 1997; Jollivet *et al.* 1999). Early hypotheses suggested that the fragmented nature of vent habitats would produce high levels of genetic variation among populations (Grassle 1985; Bucklin 1988) and that such fragmentation would lead to isolation by distance in non-planktotrophic

vent species (Lutz *et al.* 1986; Vrijenhoek 1997; Jollivet *et al.* 1999). Most population genetic investigations of vent-endemic species have been focused on allozyme markers, which revealed low levels of genetic variation (Vrijenhoek 1997 for review; Black *et al.* 1998; Shank *et al.* 1998; Maas *et al.* 1999) and concluded that sufficient migrants are exchanged per generation ($Nm > 1.0$) to prevent genetic isolation between widely separated populations (sensu Nei 1973). However, allozymes could be limited in sensitivity for several reasons, including the small number of loci available for analyses (David *et al.* 1997), variation not expressed at the enzyme level (Murphy *et al.* 1996), and environmental influences on protein expression (Karl & Avise 1992; Jollivet *et al.* 1995a,b; Eanes 1999), all of which would promote inferences of genetic homogeneity. Conservative mitochondrial gene sequence data (*e.g.* CO1) obtained for several vent-endemic species (*e.g.* vestimentiferans, Black *et al.* 1997 and Hurtado *et al.* 2004; shrimp, Shank *et al.* 1999; clams, Peek *et al.* 1997; mussels, Maas *et al.* 1999, and see also gastropods, Kojima *et al.* 2000) have repeatedly shown limited intraspecific variation, raising the concern that both allozyme and mitochondrial surveys have markedly under-represented genetic diversity in hydrothermal vent systems. More importantly, fundamental questions about the specific mechanisms by which vent species maintain their populations and geographic ranges still remained unanswered. Alternative approaches are needed to investigate temporal change in genetic structure of propagules, to resolve contemporary from historical influences, and to evaluate the role of spatial variation in larval genetic structure on the genetic structure of adult populations.

The first genetic study of the vestimentiferan tubeworm *Riftia pachyptila* reported moderate levels of genetic divergence based on allozyme gene frequencies between populations inhabiting the Galápagos Rift and 21°N on the East Pacific Rise (EPR) and suggested limited larval dispersal ability and the potential existence of barriers to larval dispersal (Bucklin 1988). Subsequent allozyme data based on additional polymorphic loci suggested that barriers to gene flow do not exist over 4000 km along the northern EPR and the Galápagos Rift (Black *et al.* 1994). This weak isolation by distance trend supports dispersal occurring predominantly in a stepping-stone fashion along the ridge axis. Similarly, weak patterns have been observed from allozyme gene frequencies among populations of the tubeworms *Tevnia jerichonana* and *Oasisia alvinae* (Black *et al.* 1998). Single-locus mitochondrial DNA sequences (Cytochrome *c* Oxidase subunit I, Black *et al.* 1997; Hurtado *et al.* 2004) have also revealed little (one polymorphic site out of *c.* 650 bp among a total of 32 individuals from 27°N, 21°N, and 31°S) to no genetic variability in *R. pachyptila* populations along the northern and southern EPR to 17°S. A marked mtCOI divergence among *R. pachyptila* populations was identified just south of the Easter Microplate (31–32°S), which is consistent with a historical dispersal barrier (Hurtado *et al.* 2004). Processes limiting effective population size, and ultimately genetic diversity, have been proposed to explain genetic homogeneity among vent populations and in the deep-sea (*e.g.* Black *et al.* 1994; Creasey & Rogers 1999), while genetic divergence has been attributed to physical barriers (*e.g.* France *et al.* 1992), shifting of available habitat (Jollivet *et al.* 1999), frequent extinction and recolonization events (Black *et al.* 1994; Vrijenhoek 1997), and species succession (Vrijenhoek *et al.* 1998). To understand the role these processes play in defining the genetic structure of populations and the evolution of species in these deep-sea ephemeral habitats, new integrated fine-scale (metapopulation) genetic and time-series ecological approaches are needed.

High estimated rates of gene flow over wide geographic distance are mostly at odds with expectations from inferred and measured larval dispersal potentials (Mullineaux & France 1995; Tyler & Young 1999). The sessile vent tubeworm *R. pachyptila* is believed to produce neutrally buoyant, small (*c.* 100 μm) lecithotrophic eggs and free-swimming larvae capable of long-distance dispersal (Southward 1988; Jones & Gardiner 1989; Young *et al.* 1996). The metabolic capacity of cultured *R. pachyptila* larvae suggests larval lifespans of *c.* 38 days, a duration sufficient for passive dispersal between vents within the 9°50′N segment of the EPR (Marsh *et al.* 2001), and possibly between ridge segments separated by hundreds of kilometers elsewhere along the EPR (Chevaldonné *et al.* 1997). However, the frequent reversals documented in along-ridge currents near 9°50′N (Marsh *et al.* 2001) and the relatively high density of demersal larvae (Kim & Mullineaux 1998; Mullineaux *et al.* 2005) suggest a strong potential for hydrodynamic retention of larvae at smaller spatial scales (<100 km), as do laboratory (Helfrich & Battisti 1991) and field (Speer & Marshall 1995; Helfrich *et al.* 1998; Joyce *et al.* 1998) studies of secondary circulation in buoyant hydrothermal plumes.

We hypothesize that previously employed genetic markers have under-sampled genetic diversity, limiting the potential for identifying larval dispersal mechanisms and metapopulation genetic structure. Therefore, we assessed genetic diversity using a genomic fingerprinting approach, amplified fragment length polymorphisms (AFLPs) (Vos *et al.* 1995), and a vent-endemic species for which genetic and larval studies have been conducted. Genomic AFLP surveys typically recover hundreds of polymorphic loci (*versus* 5 to 20 for allozymes) for simultaneous comparison via the visualization of restriction fragments representative of the non-coding portion of the genome (Wong *et al.* 2001). Although frequently considered a dominant marker due to the inability to distinguish homozygous and heterozygous individuals (but see

also Bensch *et al.* 2002 and Parsons and Shaw 2001), AFLP fingerprinting has been shown to be less prone to problems of hidden heterogeneity and selection than allozymes (*e.g.* Escaravage *et al.* 1998), to yield greater levels of genetic diversity than allozymes, RFLPs and RAPDs (*e.g.* Lopez *et al.* 1999; Mueller & Wolfenbarger 1999; Loh *et al.* 2000), and through AFLP methodological variants (Bensch & Akesson 2005), to be utilized as a highly effective gateway for more detailed investigation of (co-dominant) variation in microsatellites (*e.g.* Yang *et al.* 2002), cDNA (*e.g.* Bachem *et al*. 1996), and DNA methylation (*e.g.* Sherman and Talbert 2002).

This paper represents a pilot study to address the above hypothesis, determine the suitability of AFLP markers for the recovery of fine-scale intraspecific genetic diversity and develop insights into the subsequent utility of developing additional AFLP-derived markers (*e.g.* microsatellites and population genomic markers) for ecology-based studies of vent species. Specifically, we examined the genetic diversity and structure of one of the foundation species of vent communities along the EPR, the vestimentiferan tubeworm *R. pachyptila* (Annelida: Polychaeta: Siboglinidae). From recovered genetic structure and levels of differentiation among individuals and populations, we infer likely mechanisms of larval dispersal and the potential utility of genomic fingerprinting for future metapopulation genetic studies of mid-ocean ridge populations.

Material and methods

Specimens and DNA preparation

Specimens of *Riftia pachyptila* were collected from three hydrothermal vent regions on the EPR (Guaymas Basin, 9°50′N, and 17°S) *via* the submersible DSV *Alvin* (Table 1). Individuals from two localities (*i.e.* discrete tubeworm assemblages) within each region were sampled. All specimens available to the authors from the 17°S region were utilized in this study. Following surface recovery of the submersible, individuals of *R. pachyptila* were removed from their tubes and frozen at −80 °C for subsequent DNA extraction. Genomic DNA was extracted immediately aboard the research support vessel R/V *Atlantis* from approximately 0.05 g of vestimentum (to avoid potential contamination from bacterial symbionts), using the DNeasy^{TM} Tissue extraction system (Qiagen, Inc.). Purified DNA extracts were subsequently stored at −20 °C.

AFLP data collection

Amplified fragment length polymorphism fingerprinting involves three main steps (1) restriction digest of the genomic DNA and ligation of oligonucleotide adaptors; 2) pre-selective and selective amplification of the produced fragments; and 3) visualization of the amplified fragments *via* gel electrophoresis. The AFLP Plant (large genome) Mapping Kit (PE Biosystems Corp.) was used for restriction/ligation reactions and subsequent amplification of genomic fragments in accordance with the manufacturer's protocols. Restriction enzyme digests were performed by combining 0.5 μg of genomic DNA from each sample with 1 μl 10X T4 DNA ligase buffer (with ATP), 1 μl of 0.5 M NaCl, 0.5 μl of 1 mg·ml^{-1} bovine serum albumin, 1 μl of the Enzyme/Digestion Mix, 1 μl of *Mse*I and *Eco*RI restriction adaptors (Applied Biosystems, Inc.). The Enzyme Digestion Mix consisted of 1 μl 10X T4 DNA ligase buffer (with ATP), 1 μl of 0.5 M NaCl, 0.5 μl of 1 mg·ml^{-1} bovine serum albumin, 10 units of *Mse*I (frequent cutter) enzyme, 50 units of *Eco*RI (rare cutter) enzyme, and 10 Weiss units of T4 DNA ligase for each sample. The restriction/ligation cocktail (10 μl total volume) was incubated for at least 12 h at room temperature. Following denaturation at 95 °C for 5 min and re-annealing at room temperature, double-stranded oligonucleotide adaptors (denatured) were ligated to the

Table 1. Collection information of *Riftia pachyptila* specimens.

Region	Alvin dive number	Date	Community locality	Number of Individuals	Latitude; Longitude	Depth (m)
Guaymas Basin	3518	01/15/00	Robin's Roost	5	27° 0.875N; 111° 24.622W	2012
	3521	01/18/00	Diffuse Vent	7	27° 0.638N; 111° 24.425W	1977
9°50′N	3408	05/24/99	Q vent	7	9° 50.767N; 104° 17.579W	2515
	3547	04/21/00	East Wall	4	9° 50.553N; 104° 17.514W	2530
17°S	3294	10/25/98	North vent	2	17° 24.943S; 113° 12.190W	2578
	3299	10/30/98	Miss Wormwood	4	17° 34.905S; 113° 14.668W	2595

Two localities were sampled within each region: Guaymas Basin (Robin's Roost: GBR35183, GBR35185, GBR35186, GBR35187, GBR35189; diffuse vent: GBR35211, GBR35213, GBR35214, GBR35215, GBR35216, GBR35217, GBR35219); 9°50′N (Q vent: 9N34081, 9N34082, 9N34083, 9N34084R; East Wall: 9N35474, 9N35478, 9N35477, 9N354711, 9N354713, and 9N354714); and the 17°S 'Spike' (North vent: S32941, S32942; Miss Wormwood: S32991, S32992 S32993, and S32994) hydrothermal area, a total linear distance of over 5235 km. Specific distances between localities within and between sites are shown in Fig. 1.

Table 2. Selective primer combinations and recovered loci.

Selective Primer Pair (*Mse*I/*Eco*RI)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci
CAA/AAG	85	85	100
CAC/ACT	67	62	93
CAT/AGC	37	25	68
CAG/ACC	56	39	71
CTA/AGG	63	63	100
CTC/ACA	80	75	94
CAA/AAC	162	161	99
CTC/AAG	118	118	100
Total	668	630	94

restriction fragments. Addition of the adaptors modifies the recognition sequence and prevents a second restriction.

The resulting genomic DNA fragments were amplified (pre-selective amplification) with one selective base on each primer (*Eco*RI) and (*Mse*I) reducing the number of fragments to be displayed. The pre-selective amplification thermocycler profile was 2 min at 72 °C followed by 20 repetitions of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2 min. This was followed by 30 min at 60 °C and a 4 °C final hold. Successful amplification was verified by visualization of 10 μl of each reaction on a 1.5% agarose gel. The remaining pre-amplification product (10 μl) was diluted with 190 μl of 0.1X TE buffer and used as a template for the selective amplification. Pre-selective amplification product (3.0 μl) was combined with 1.0 μl (10 μM) of each selective primer and 10 μl of AFLP Core mix (Applied Biosystems, Inc.). The selective amplification profile consisted of denaturation at 94 °C for 2 min, 94 °C for 20 s, annealing at 66 °C for 30 s, and extension at 72 °C for 2 min, followed by 10 cycles in which the annealing temperature was lowered 1 °C for each subsequent cycle. Following the 10th cycle, 20 additional cycles annealing at 56 °C were performed followed by a final extension (30 min at 60 °C) and a 4 °C final hold. Each selective primer possessed three distinct nucleotides at the 3′ end (*e.g.* *Eco*RI primer + AAC and *Mse*I primer + CAG; *Eco*RI primer + AGC and *Mse*I primer + CTG). Eight different primer pairs were used to amplify selectively different sets of genomic loci (Table 2).

Fragment visualization and analysis

For each sample, 0.4 μl of the selective amplification product was electrophoresed on a 5.0% denaturing acrylamide gel using an Applied Biosystems 377 automated DNA sequencer. An internal size standard, Rox 500, was included in the loading dye mixture of all samples. Collection and analyses of fragment banding patterns were performed using GeneScan Analysis Software (ver 3.1; Applied Biosystems). Discrete fragments with a threshold intensity below 50 florescent units were not included in the analyses. Moreover, fragments <50 nucleotides long were not considered in the analysis to avoid biases due to unincorporated fluorescent signal (*i.e.* noise). The local Southern size calling option and a split peak correction of 2500 option were chosen for GeneScan data collection. To assess the reproducibility of the banding patterns, we performed the following on randomly selected samples: (1) repeating the entire AFLP procedure from separate DNA extractions; (2) amplifying selective PCR product from the same pre-selective PCR product; and (3) visualizing the same selective PCR product multiple times over a 6-month period. In all cases, each specimen/sample reproduced identical fragment patterns.

Fragment sizes for each set of selective amplification primers were imported into a spreadsheet and sorted by size. Given the standard deviation of fragment sizing on the automated sequencer <0.25 (Applied Biosystems), fragments separated by ±0.5 nucleotides were considered different loci (*i.e.* not homologous). We conservatively group or 'bin' fragments that are not separated by ±0.5 nucleotide and consider them a putative locus (*i.e.* homologous). Putative AFLP loci are considered polymorphic if any fraction of the individuals contains a given size DNA fragment (*i.e.* gel band) produced by a given selective amplification primer pair. The resulting fragment table of polymorphic loci was recoded as presence/absence data.

Many previous AFLP studies have assumed AFLP markers to be exclusively dominant (whereby it is not possible to determine which fragments in different individuals represent alleles of the same AFLP locus) for practical considerations. Polymorphic AFLPs can be codominant, and as a result, conventional analytical parameters (heterozygosity and population differentiation, as measured using F_{ST}) may tend to underestimate the variability at each locus (Yan *et al.* 1999), particularly when the analyses include high levels of polymorphic AFLPs (discussed in Wong *et al.* 2001). We recognize this potential bias toward underestimating genetic variability and deemed it as acceptable for the present analyses given any underestimate would not compromise our ability to address the specific goals of this study. The assumption of dominant AFLP markers in this preliminary study was also made in light of the growing results from a number of recent studies comparing F_{ST} values (and their analogues) obtained from dominant AFLP markers that revealed overall similarity to codominant markers (*e.g.* RAPDs, allozymes, and microsatellites, depending on the number of loci) for the inference of population structure

(*e.g.* Mariette *et al.* 2002; Whitehead *et al.* 2003; Nybom 2004; Bensch & Akesson 2005).

Therefore, to assess the potential of AFLP data for revealing genetic subdivision, we estimated the distribution of genetic diversity, relative divergence, and an analog of Wright's F_{ST}, φ_{ST}, *via* an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) with the ARLEQUIN 2.0 software package (Schneider *et al.* 2000). *A priori* group definitions in AMOVA were constructed to examine the partitioning of genetic variation. Sample locations were grouped in two ways (1) all six sampled localities grouped separately; and (2) all three sampled regions grouped separately. This allowed investigation of hierarchical components of genetic variation including (1) variance due to differences between individuals within a sampled locality; (2) variance due to differences between populations within a region; and (3) variance due to differences among the three sampled regions. The significance of φ_{ST} estimates was determined *via* a Markov chain analysis as described by Raymond & Rousset (1995) using 5000 permutations (Schneider *et al.* 2000).

To determine if localities represented discrete genetic entities, parsimony and neighbor-joining analyses were conducted with the PAUP ver 4.0*db2 software package; (Swofford 2000). For parsimony, heuristic searches employed TBR branch swapping and 50 random sequence-addition replication. Neighbor-joining employed a matrix of absolute character difference. Topological robustness (*i.e.* support of tree nodes) was assessed using non-parametric bootstrap with 500 iterations (Felsenstein 1985).

Results

Patterns of variation

A total of 668 loci were detected among 29 individuals of *Riftia pachyptila* using eight combinations of selective primers (Table 2). A total of 630 (94%) of these loci were considered polymorphic. Selective primer combinations amplified between 37 and 162 fragments ranging from 50 to 496 base pairs in length. The number of fragments differed greatly with different pairs of primers, and the numbers of bands decreased as fragment size increased. One selective primer combination (*Mse*I-CTC/*Eco*RI-ACA) did not successfully amplify fragments from individual ID S32994 and similarly three combinations (CTC/ACA, CAA/AAC and CTC/AAG) from individual ID 9N34084R (see Fig. 1). Inclusion (with absent data scored as missing) or exclusion of these two individuals did not alter the analytical results.

Each examined individual of *R. pachyptila* individual yielded a unique genetic fingerprint. Total character differences between individuals were the least ($d = 15/668$ loci or 2.2%) between individuals S32993 and S32994 from the 17°34′S locality (Miss Wormwood) and were the greatest ($d = 279$ or 42%) between Guaymas Basin individual GBR35185 (Robin's Roost) and 9° 50′N individual 9N34083 (Q Vent). Table 3 shows the average genetic differences between localities. Average genetic differences within regions (Table 4) were the highest at 9°50′N (132.02) followed by Guaymas Basin (122.33) and 17°S (47.53). Genetic differentiation between regions was the least between Guaymas Basin and 9°50′N populations ($\varphi st = 0.032$) and the greatest between 9°50′N and the 17°S populations ($\varphi st = 0.663$–0.684). Although 5000 permutations were conducted with the AMOVA analyses, caution is needed when interpreting these results due to differences in the available number of individuals who were sampled.

Genetic structure

Parsimony and neighbor-joining reconstructions grouped the 29 individuals into six discrete clades (defined as a group of branch tips sharing a common node corresponding to locality) (Fig. 1). High bootstrap values support the clustering of individuals according to locality. The longest branch length (indicated by an * in Fig. 1) occurred between 17°S and the two northern regions, indicating the two northern regions are more genetically similar to each other than to the southern EPR. This branch also represents the greatest geographical distance between regions. Two clades did not strictly correspond to geographic locality. Individual GB3518-6 from Robin's Roost consistently clustered within the Diffuse Vent clade (Guaymas Basin). Moreover, a subset of individuals from East Wall (9°50′N; 3547–4, 8, 7, 11) clustered together with individuals from Robin's Roost in Guaymas Basin. Individuals from Guaymas Basin (GB3521-3, 4, 6, and 3518-6) and individuals from 9°50′N (3547–4, 8, 7, 11) formed polytomies in their respective clades (Fig. 1).

To assess whether an adequate number of selective primer combinations was used and to identify the point at which a maximal, or near-maximal, amount of phylogenetic information (*i.e.* genetic structure as judged by tree topology) was obtained, the number of resolved tree nodes was plotted *versus* the number of primer pairs scored (Fig. 2). Twelve resolved bifurcating nodes were recovered after only three primer pairs were included (sequential addition from the smallest number of polymorphic loci). When four to eight primer pairs were included, the total of 16 nodes was recovered (Fig. 2). Random addition of primer pairs produced similar results, suggesting that an adequate number of primer pairs were used to recover the available information on genetic structure.

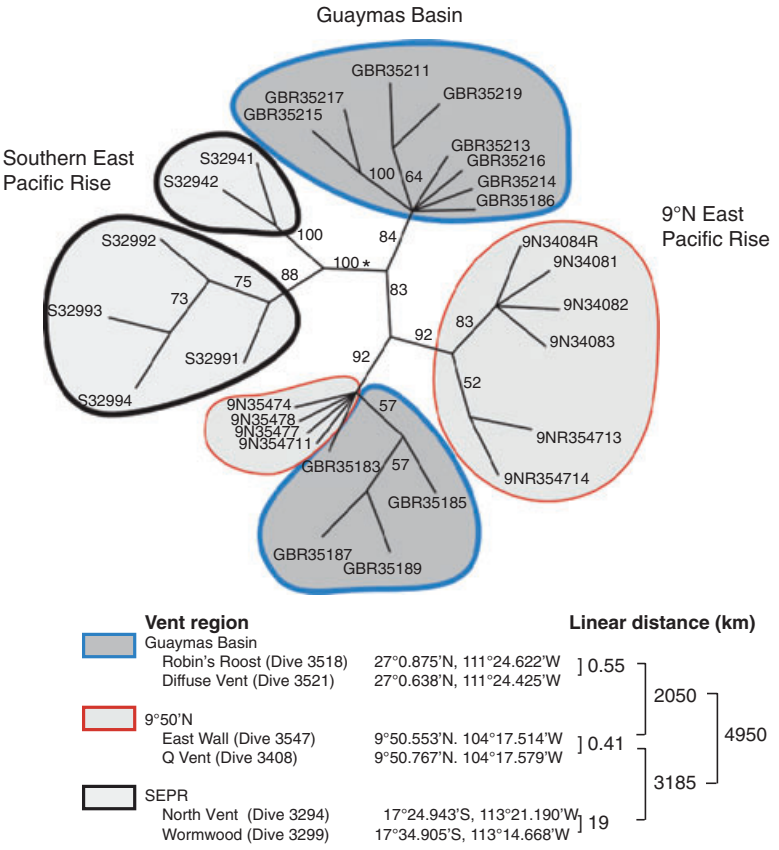

Fig. 1. Parsimony amplified fragment length polymorphism (AFLP) fingerprint relationships among individuals sampled from two localities within each of three regions (Guaymas Basin, 9°50′N, and 17°S EPR). The topology was constructed using 505 parsimony-informative sites from 630 polymorphic loci generated from eight primer pairs. The 29 individuals cluster into six clades, branching patterns that principally correspond to sampling localities (shaded regions) or discrete assemblages. Parsimony bootstrap values (500 iterations) are given next to the relevant node. Parsimony and neighbor joining produced congruent topological and bootstrap results. Branch lengths are to scale. Asterisks indicate that longest branch length resulting from neighbor-joining analyses. Distance between regions and localities is presented. Sample identification key: vent region: *S*, Southern EPR; 9N, 9°50′N; GBR, Guaymas Basin; *Alvin* Dive no. 3408, 3299, *etc.*; Specimen ID no. 1, 2, 3, *etc.*

Table 3. Genetic differences within and between localities. The averages of pairwise differences within populations are on the diagonal.

Region-locality	GB-RR	GB-DV	9°N-EW	9°N-QV	17°S-NV	17°S-MW
Guaymas Basin-Robin's Roost	137.40	173.14	153.97	205.70	226.00	224.75
Guaymas Basin-Diffuse Vent	0.396 0.006*	78.47	188.59	182.71	167.42	165.75
9°50′N-East Wall	0.045 0.208	0.379 0.000*	155.52	164.78	226.57	212.46
9°50′N-Q vent	0.459 0.127*	0.568 0.002*	0.247 0.061	79.50	221.57	185.56
17°S-North Vent	0.515 0.610	0.591 0.000*	0.440 0.035*	0.704 0.048*	30.00	71.00
17°S-Miss Wormwood	0.601 0.012*	0.635 0.004*	0.496 0.005*	0.703 0.027*	0.576 0.068	30.16

Pairwise φ_{st} (upper number) and P-values (lower number) below the diagonal, and averages of pairwise differences between *R. pachyptila* populations above the diagonal.
*Values that are significant at $P < 0.05$.

Discussion

Amplified fragment length polymorphism fingerprinting of 29 individuals recovered 37-fold more polymorphic loci than previous genetic studies on *Riftia pachyptila* (Bucklin 1988; Black *et al.* 1994, 1998). Presumably, these loci were more representative of the entire genome than a limited number of expressed loci or a single mitochondrial locus. The amount of genetic resolution was sufficient to provide a unique AFLP fingerprint for each individual

Table 4. Genetic differences within and between regions. The averages of pairwise differences within populations are on the diagonal.

Region	Guaymas Basin	9°50′N	17°S
Guaymas Basin	122.33	156.66	175.97
9°50′N	0.188 0.000*	132.02	189.04
17°S	0.469 0.000*	0.479 0.000*	47.53

Pairwise φ_{st} (upper number) and P-values (lower number) below the diagonal, and averages of pairwise differences between vent regions above the diagonal.
*Values that are significant at $P < 0.05$.

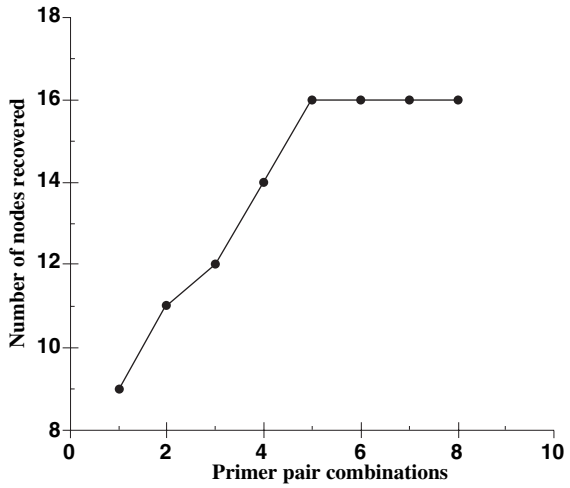

Fig. 2. Number of resolved nodes *versus* number of primer pairs scored. The total number of resolved nodes (12) using all eight primer pairs was reached after only three primer pairs were scored.

of *R. pachyptila*. Furthermore, the amount of genetic information produced was sufficient to recover historical information (Fig. 2). Our findings suggest that this increased genetic resolution may be sufficient to reveal previously undetected genetic structure along the northern EPR. Unlike the one other AFLP study involving vent fauna (the vestimentiferan tubeworm, *Ridgeia piscesae*; Carney *et al.* 2002) in which *R. piscesae* populations separated by *c.* 2 km showed little genetic structure and high levels of genetic connectivity (overall F_{st} estimate of 0.0386 and an *Nm* estimate of 6.2 migrants per generation), considerable diversity and structure were present in *R. pachyptila* populations along the EPR. The mechanisms controlling the apparent lack of genetic structure in *R. piscesae* are unknown, but probably reflect the variability of dispersal and colonization dynamics associated with different species and ridge axes. While each individual *R. pachyptila* yielded a unique haplotype, distinct populations of haplotypes over the species range were distinguishable. Genetic subdivision in this vent species may be extensive. φ_{st} values (0.188–0.479) based on AFLP loci suggest significant ($P < 0.05$) isolation between regions.

Phylogenetic relationships (Fig. 1) in general, support conclusions based on AMOVA analyses, yet some instances of migration can be inferred. For example, the placement of individual GBR3518-6 from Robin's Roost in the Diffuse Vent clade may be the result of a migration event. Similarly, the arrangement of the Guaymas Basin clades and the 9°50′N clades may reflect a historical migration event followed by subsequent diversification. Upon close examination, each of the *R. pachyptila* individuals comprising the polytomies from both 9°50′N and Guaymas Basin was >10 cm in length. If these individuals, composing a distinct size class, were in fact siblings, one would expect a polytomy rather than a resolved branching pattern. This observation raises the possibility of using polytomies to identify recruitment events or cohorts of related individuals. Interpretation of these and other alternative hypotheses will require analysis of more individuals and more localities. The reconstructed AFLP topology was a distinctively non-random pattern suggesting that *R. pachyptila* consists of more than a single large panmictic population with a weak isolation by distance trend (Black *et al.* 1994), and that unknown processes are structuring these genetically diverse populations. With such genetic diversity and structure to examine dispersal mechanisms and the genetic consequences of metapopulation dynamics, the traditional island and stepping-stone models will probably be of little utility (see Hellberg 2006).

The observed polytomies in Fig. 1 are consistent with the delivery of discrete larval cohorts whereby closely related individuals remain in close proximity during transport. For example, if larvae from multiple sources undergo extensive mixing once entrained in the neutrally buoyant vent plume, then population genetic structure would be expected to be effectively randomized (*i.e.* an island model). Alternatively, larvae may be vertically entrained into buoyant hydrothermal plumes (Kim *et al.* 1994; Kim & Mullineaux 1998), and subsequently transported into the neutrally buoyant plume, which can remain as distinct entities during transport away from the vent (Lupton *et al.* 1999). This mechanism raises the possibility that larvae entrained into such an eddy could disperse as a distinct cohort (Mullineaux & France 1995). As AFLPs produce unique patterns for individual organisms, mechanisms of larval transport and delivery may be elucidated for particular species. Our results are consistent with such a mechanism, referred to as the 'plume-eddy' or propagule pool model of dispersal. A similar result might be expected if post-settlement

selection plays a major role in structuring genetic diversity, as has been shown in intertidal mussel species (Gilig & Hilbish 2000); however, the non-coding nature and distribution of AFLPs throughout the genome suggest that this is not likely. Additional temporally and spatially constrained sampling of populations from key localities may allow the relative importance of discrete mechanisms (*e.g.* larval transport, delivery, and post-settlement selection processes, Butman 1987), which structure genetic diversity, to be assessed over a variety of spatial scales. Integrating temporal sampling with methods that address dispersal on short (inter- and intra-generational) timescales, such as elemental fingerprinting (Levin 2006), will advance our understanding of the currently unknown timescales over which integrated genomic fingerprinting approaches can resolve dispersal events as well as our mechanistic understanding of population dynamics in marine systems.

The extensive amount of genetic diversity recovered through genomic fingerprinting approaches (including specific variants of AFLP approaches; Bensch & Akesson 2005) may also permit detailed comparisons of genetic diversity from a variety of species to elucidate the role metapopulation processes (*e.g.* recurrent extinction and colonization) play in the genetic structure of vent species (Hellberg 2006). For example, high rates of vent habitat disturbance and the order of species colonization in ecological succession may affect levels of genetic diversity (Vrijenhoek 1997; Shank *et al.* 1998; Vrijenhoek *et al.* 1998). With extremely high levels of disturbance, some habitats may be arrested at permanent 'pre-climax' conditions, and never present opportunities for the establishment of later colonizers. Consequently, these later colonizing species would maintain a smaller total population size and lower genetic diversity than early colonizers (Hilbish 1996). Future studies utilizing genomic AFLPs fingerprinting of vent community assemblages will permit these hypotheses to be addressed at relevant ecological and genetic scales.

Current investigations are aimed at AFLP fingerprinting of selected vent species from temporally and spatially well-constrained collections of larvae, newly settled colonists, and adults from species with various larval life-histories acquired during time-series studies along the EPR ridge crest. This approach will permit hypotheses related to the mechanisms of dispersal, viability selection, cohort migration, and bottleneck effects, to be quantitatively examined within the context of genetic relatedness. Genomic surveys hold promise for detecting levels of previously undetected genetic structure as well as to provide insights into the inter-relationships and fine-scale patterns of larval dispersal, benthic community structure, and physical oceanographic processes.

Summary

Although the previous genetic investigations of *Riftia pachyptila* populations found sufficient numbers of effective migrants indicative of gene flow over large spatial scales, our AFLP results suggest greater population subdivision consistent with cohorts maintaining site fidelity and consistent with hydrodynamic retention models (Marsh *et al.* 2001). Significant levels of differentiation were observed among discrete, genetically distinguishable, putative cohorts (individuals forming polytomies and <10 cm in length) within an aggregation. These results are consistent with mechanisms of vent larval dispersal and transport that act to retain cohort fidelity. On the basis of our results, we assert that AFLPs and other new multilocus genotyping approaches, when combined with new likelihood and Baysian analysis of the coalescent (Beerli & Felsenstein 2001) and temporal population sampling (*e.g.* Wang & Whitlock 2003), hold great promise for providing insights into the mechanisms (*i.e.* larval behavior, metapopulation dynamics, and physical oceanographic processes) controlling larval dispersal and fine-scale pattern in marine systems (Hellberg 2006). Future studies aimed at the detailed examination of the temporal variation in (multilocus) genetic structure of vent larval assemblages and vent colonists over time and space may be capable of distinguishing local or regional sources, the effects of larval or post-settlement processes, and the impact of disturbance and habitat turnover on the genetic composition and evolution of vent metapopulations and the species they maintain.

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References

Bachem C.W.B., van der Hoeven R.S., de Bruijn S.M., Vreugdenhil D., Zabeau M., Visser R.G.F. (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *Plant Journal*, **9**, 745–753.

Beerli P., Felsenstein J. (2001) Maximum likelihood estimation of a migration matrix and effective population sizes using a coalescent approach. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 4563–4568.

Bensch S., Akesson S. (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, **14**, 2899–2914.

Bensch S., Akesson S., Irwin D. (2002) The use of AFLP to find an informative SNP: genetic differences across a migratory divide in willow warblers. *Molecular Ecology*, **11**, 2359–2366.

Black M.B., Lutz R.A., Vrijenhoek R.C. (1994) Gene flow among vestimentiferan tube worm (*Riftia pachyptila*) populations from hydrothermal vents of the Eastern Pacific. *Marine Biology*, **120**, 33–39.

Black M.B., Halanych K.M., Maas P.A.Y., Hoeh W.R., Hashimoto J., Desbruyères D., Lutz R.A., Vrijenhoek R.C. (1997) Molecular systematics of vestimentiferan tubeworms from hydrothermal vents and cold-water seeps. *Marine Biology*, **130**, 141–149.

Black M.B., Trivedi A., Maas P.A.Y., Lutz R.A., Vrijenhoek R.C. (1998) Population genetics and biogeography of vestimentiferan tube worms. *Deep-Sea Research II*, **45**, 365–382.

Bucklin A. (1988) Allozymic variability of *Riftia pachyptila* populations from the Galápagos Rift and 21°N hydrothermal vents. *Deep-Sea Research A*, **35**, 1759–1768.

Butman C.A. (1987) Larval settlement of soft-sediment invertebrates: the spatial scales of pattern explained by active habitat selection and the emerging role of hydrodynamical processes. *Oceanography and Marine Biology: Annual Review*, **25**, 133–165.

Carney S.L., Peoples J.R., Fisher C.R., Schaeffer S.W. (2002) AFLP analyses of genomic DNA reveal no differentiation between two phenotypes of the vestimentiferan tubeworm *Ridgea piscesae*. *Cahiers de Biologie Marine*, **43**, 363–366.

Chevaldonné P., Jollivet D., Vangriesheim A., Desbruyères D. (1997) Hydrothermal-vent alvinellid polychaete dispersal in the eastern Pacific. 1. Influence of vent site distribution, bottom currents, and biological patterns. *Limnology and Oceanography*, **42**, 67–80.

Creasey S., Rogers A.D. (1999) Population genetics of bathyal and abyssal organisms. *Advances in Marine Biology*, **35**, 1–151.

David P., Perdieu M.A., Pernot A.F., Jarne P. (1997) Fine-grained spatial and temporal population genetic structure in the marine bivalve *Spisula ovalis*. *Evolution*, **51**, 1318–1322.

Eanes W.F. (1999) Analysis of selection on enzyme polymorphisms. *Annual Review of Ecology and Systematics*, **30**, 301–326.

Escaravage N., Questiau S., Pornon A., Doche B., Taberlet P. (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology*, **7**, 975–982.

Excoffier L., Smouse P.E., Quattro J.M. (1992) Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

France S.C., Hessler R.R., Vrijenhoek R.C. (1992) Genetic differentiation between spatially-disjunct populations of the deep-sea, hydrothermal vent-endemic amphipod *Ventiella sulfuris*. *Marine Biology*, **114**, 551–559.

Gilig M.R., Hilbish T.J. (2000) The relationship between allele frequency and tidal height in a mussel hybrid zone: a test of the differential settlement hypothesis. *Marine Biology*, **137**, 371–378.

Grassle J.P. (1985) Genetic differentiation in populations of hydrothermal vent mussels (*Bathymodiolus thermophilus*) from the Galápagos Rift and 13°N on the East Pacific Rise. *Bulletin of the Biological Society of Washington*, **6**, 429–442.

Helfrich K.R., Battisti T.M. (1991) Experiments on baroclinic vortex shedding from hydrothermal plumes. *Journal of Geophysical Research-Oceans*, **96**, 12511–12518.

Helfrich K.R., Joyce T.M., Cannon G.A., Harrington S.A., Pashinski D.J. (1998) Mean hydrographic and velocity sections near Pipe Organ vent at Juan de Fuca Ridge. *Geophysical Research Letters*, **25**, 1737–1740.

Hellberg M. (2006) Genetic approaches to understanding marine metapopulation genetics. In: Kritzer J.P., Sale P.F. (Eds), *Marine Metapopulations*. Elsevier Academic Press, Burlington, MA: pp. 431–455.

Hilbish T.J. (1996) Population genetics of marine species: the interaction of natural selection and historically differentiated populations. *Journal of Experimental Marine Biology and Ecology*, **200**, 67–83.

Hurtado L.A., Lutz R.A., Vrijenhoek R.C. (2004) Distinct patterns of genetic differentiation among annelids of eastern Pacific hydrothermal vents. *Molecular Ecology*, **13**, 2603–2615.

Jollivet D., Desbruyères D., Bonhomme F., Moraga D. (1995a) Genetic differentiation of deep-sea hydrothermal vent alvinellid populations (Annelida: Polychaeta) along the East Pacific Rise. *Heredity*, **74**, 376–391.

Jollivet D., Desbruyères D., Ladrat C., Laubier L. (1995b) Evidence for differences in the allozyme thermostability of deep-sea hydrothermal vent polychaetes (Annelida): a

possible selection by habitat. *Marine Ecology Progress Series*, **123**, 125–136.

Jollivet D., Chevaldonné P., Planque B. (1999) Hydrothermal-vent alvinellid polychaete dispersal in the eastern Pacific. 2. A metapopulation model based on habitat shifts. *Evolution*, **53**, 1128–1142.

Jones M.L., Gardiner S.L. (1989) On the early development of the vestimentiferan tube worm *Ridgeia* sp. and observations on the nervous system and trophosome of *Ridgeia* sp. and *Riftia pachyptila*. *Biological Bulletin*, **177**, 254–276.

Joyce T.M., Cannon G.A., Pashinski D., Helfrich K.R., Harrington S.A. (1998) Vertical and temporal vorticity observations at Juan de Fuca Ridge: hydrothermal signatures. *Geophysical Research Letters*, **25**, 1741–1744.

Karl S.A., Avise J.C. (1992) Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science*, **256**, 100–102.

Kim S.L., Mullineaux L.S. (1998) Distribution and near-bottom transport of larvae and other plankton at hydrothermal vents. *Deep-Sea Research II*, **45**, 423–440.

Kim S.L., Mullineaux L.S., Helfrich K.R. (1994) Larval dispersal via entrainment into hydrothermal vent plumes. *Journal of Geophysical Research*, **99**, 12655–12665.

Kojima S., Segawa R., Fujiwara Y., Hashimoto J., Ohta S. (2000) Genetic differentiation of populations of a hydrothermal vent-endemic gastropod, *Ifremeria nautilei*, between the North Fiji Basin and the Manus Basin revealed by nucleotide sequences of mitochondrial DNA. *Zoological Science*, **17**, 1167–1174.

Levin L.A. (2006) Recent progress in understanding larval dispersal: new directions and digressions. *Integrative and Comparative Biology*, **46**, 282–297.

Loh J.P., Kiew R., Hay A., Kee A., Leong G., Huat Y., Gan Y. (2000) Intergeneric and interspecific relationships in Araceae tribe Caladieae and development of molecular markers using amplified fragment length polymorphism (AFLP). *Annals of Botany*, **85**, 371–378.

Lopez J.V., Kersanach R., Rehner S.A., Knowlton N. (1999) Molecular determination of species boundaries in corals: genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. *Biological Bulletin*, **196**, 80–93.

Lupton J.E., Baker E.T., Massoth G.J. (1999) Helium, heat, and the generation of hydrothermal event plumes at mid-ocean ridges. *Earth and Planetary Science Letters*, **171**, 343–350.

Lutz R.A., Bouchet P., Jablonski D., Turner R.D., Waren A. (1986) Larval ecology of mollusks at deep-sea hydrothermal vents. *American Malacological Bulletin*, **4**, 49–54.

Maas P.A.Y., O'Mullen G.D., Lutz R.A., Vrijenhoek R.C. (1999) Genetic and morphometric characterization of mussels (Bivalvia: Mytilidae) from Mid-Atlantic Hydrothermal Vents. *Biological Bulletin*, **196**, 265–272.

Mariette S., Le Corre V., Austerlitz F., Kremer A. (2002) Sampling within the genome for measuring within-population diversity: trade-offs between markers. *Molecular Ecology*, **11**, 1145–1156.

Marsh A.G., Mullineaux L.S., Young C.M., Manahan D.T. (2001) Larval dispersal potential of the tubeworm *Riftia pachyptila* at deep-sea hydrothermal vents. *Nature*, **411**, 77–80.

Mueller U.G., Wolfenbarger L.L. (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, **14**, 389–394.

Mullineaux L.S., France S.C. (1995) Dispersal mechanisms of deep-sea hydrothermal vent fauna. In: Humphris S., Zierenberg R.A., Mullineaux L.S., Thomson R.E. (Eds), *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions*. American Geophysical Union, Washington, DC: pp. 408–424.

Mullineaux L.S., Mills S.W., Sweetman A.K., Beardreau A.H., Metaxas A., Hunt H.L. (2005) Vertical, lateral and temporal structure in larval distribution at hydrothermal vents. *Marine Ecology Progress Series*, **293**, 1–16.

Murphy R.W., Sites J.W. Jr, Buth D.G., Haufler C.H. (1996) Proteins: isozyme electrophoresis. In: Hillis D., Moritz C. (Eds), *Molecular Systematics*. Sinauer Associates, Inc., Sunderland, MA: pp. 45–126.

Nei M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the United States of America*, **70**, 3321–3323.

Nybom H. (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology*, **13**, 1143–1155.

Parsons Y.M., Shaw K.L. (2001) Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using amplified fragment length polymorphism. *Molecular Ecology*, **10**, 1765–1772.

Peek A.S., Gustafson R.G., Lutz R.A., Varijenhoek R.C. (1997) Evolutionary relationships of deep-sea hydrothermal vent and cold-water seep clams (Bivalvia: Vesicomyidae): results from mitochondrial cytochrome oxidase subunit I. *Marine Biology*, **130**, 151–161.

Raymond M., Rousset F. (1995) GENEPOP (version 1.2) Population genetic software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Schneider S., Roessli D., Excoffier L. (2000) *Arlequin ver. 2.000: a software for population data analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Shank T.M., Lutz R.A., Vrijenhoek R.C. (1998) Molecular Systematics of shrimp (Decapoda: Bresiliidae) from Deep-Sea Hydrothermal Vents: Enigmatic ''Small Orange'' Shrimp from the Mid-Atlantic Ridge are juvenile *Rimicaris exoculata*. *Molecular Marine Biology and Biotechnology*, **7**, 88–96.

Shank T.M., Black M.B., Halanych K., Lutz R.A. (1999) Miocene radiation of deep-sea hydrothermal vent shrimp (Caridea: Bresiliidae): Evidence from mitochondrial

cytochrome oxidase subunit I. *Molecular Phylogenetics and Evolution*, **13**, 363–366.

Sherman J.D., Talbert L.E. (2002) Vernalization-induced changes of the DNA methylation pattern in winter wheat. *Genome*, **45**, 253–260.

Southward E.C. (1988) Development of the gut and segmentation of newly settled stages of *Ridgeia* (Vestimentifera): implications for relationship between Vestimentifera and Pogonophora. *Journal of the Marine Biological Association of the United Kingdom*, **68**, 465–487.

Speer K.G., Marshall J. (1995) The growth of convective plumes at sea-floor hot-springs. *Journal of Marine Research*, **53**, 1025–1057.

Swofford D.L. (2000) *PAUP* 4.0 (Phylogenetic Analysis Using Parsimony)*. Sinauer Associates, Inc., Sunderland, MA.

Tyler P.A., Young C.M. (1999) Reproduction and dispersal at vents and cold seeps. *Journal of Marine Biological Association of the United Kingdom*, **79**, 193–208.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Research*, **2**, 4407–4414.

Vrijenhoek R.C. (1997) Gene flow and genetic diversity in naturally fragmented metapopulations of deep-sea hydrothermal vent animals. *Journal of Heredity*, **88**, 285–293.

Vrijenhoek R.C., Shank T.M., Lutz R.A. (1998) Gene flow and modes of dispersal in deep-sea hydrothermal vent animals. *Cahiers de Biologie Marine*, **39**, 363–366.

Wang J., Whitlock M.C. (2003) Estimating effective population size and migration rates from genetic samples over space and time. *Genetics*, **163**, 429–446.

Whitehead A., Anderson S.L., Kuivila K.M., Roach J.L., May B. (2003) Genetic variation among interconnected populations of *Catostomus occidentalis*: implications for distinguishing impacts of contaminants from biogeographical structuring. *Molecular Ecology*, **12**, 2817–2833.

Wong A., Forbes M.R., Smith M.L. (2001) Characterization of AFLP markers in damselflies: prevalence of codominant markers and implications for population genetic applications. *Genome*, **44**, 677–684.

Yan G., Romero-Severson J., Walton M., Chadee D.D., Severson D.W. (1999) Population genetics of the yellow fever mosquito in Trinidad: comparison of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. *Molecular Ecology*, **8**, 951–963.

Yang H., Shankar M., Buirchell B.J., Sweetingham M.W., Caminero C., Smith P.M.C. (2002) Development of molecular markers using MFLP linked to a gene conferring resistance to *Diaporthe toxica* in narrow-leafed lupin (*Lupinus angustifolius* L.). *Theoretical and Applied Genetics*, **105**, 265–270.

Young C.M., Vazquez E., Metaxas A., Tyler P.A. (1996) Embryology of vesimentiferan tube worms from deep-sea methane/sulphide seeps. *Nature*, **381**, 514–516.