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Molecular and morphological identification of settlement-stage vent mussel larvae, *Bathymodiolus azoricus* (Bivalvia: Mytilidae), preserved in situ at active vent fields on the Mid-Atlantic Ridge

Abstract—This paper describes the first successful attempt to trap and identify the larvae of a deep-sea vent organism using a combination of sediment traps and molecular analysis. During the European Union-funded MARVEL cruise in August and September 1997, sediment traps containing a high-salt DNA preservative were deployed around active black smoker chimneys on the newly discovered Rainbow vent field in an attempt to collect larval stages of the dominant vent bivalve *Bathymodiolus azoricus*. A total of 2,055 shelled mussel larvae was collected within a 2-week period, all of which were of identical size and shell morphology that indicated that they were at the settlement, prodissoconch II stage. Scanning electron microscopy of the shell hinge indicated that they belonged to the family Mytilidae, but it required molecular analysis to confirm their species identity. Polymerase chain reaction (PCR) amplification of the ITS2 region was performed on a subset of the larvae, and the resulting PCR products were cut with diagnostic restriction endonucleases to allow comparison with a DNA database based on adult specimens. The DNA restriction patterns typifying the Rainbow larvae were identical to those of adult *B. azoricus* from the Menez Gwen, Lucky Strike, and Rainbow vent sites and were significantly different from those that typified *Bathymodiolus thermophilus* (Pacific) and *Bathymodiolus puteoserpentis* (Snake Pit vent field on the Mid-Atlantic Ridge), which clearly points to the Rainbow larvae having their origin on that part of the ridge close to the Azores. These findings point to the value of sediment traps as a way to study the temporal and spatial aspects of larval settlement in deep-sea hydrothermal vent environments.

Larval dispersal and colonization are key stages in the life history of planktonic spawning marine invertebrates. The direct study of these processes requires the accurate identification of often microscopic larvae, which in many cases lack diagnostic morphological characters, particularly when dealing with the earliest development stages. Previously, several biochemically based techniques, including immunological probes (Miller et al. 1991) and allozyme electrophoresis (Hu et al. 1992), were developed to assist in larval identification. More recently, new more powerful molecular methods have been brought to bear on this subject. In particular, randomly amplified polymorphic DNA (RAPD) (Coffroth and Mulawka 1995; André et al. 1999), restriction fragment length polymorphism (RFLP) (Toro 1998), and species-specific DNA probes (Olson et al. 1991; Medeiros-Bergen et al. 1995) have been used successfully to identify larvae of marine invertebrates, including those of bivalve molluscs.

In the highly fragmented deep-sea hydrothermal environment, larval dispersal and colonization are the two most important biological factors determining the distribution and composition of vent communities on a range of spatial scales. In the case of hydrothermal vent organisms, larval identification is made more difficult because few descriptions exist for the early developmental stages. Numerous larval stages of archaeogastropods, polychaetes, and bivalves have been collected from various hydrothermal vent areas in the Pacific (Mullineaux et al. 1995, 1996). However, except in the case of five archaeogastropod species, it was not possible to identify these larvae to the species level based on morphological characters alone. Molecular identification utilizing diagnostic DNA sequences derived from adult specimens has already been used successfully for vent shrimps (Dixon et al. 1998; Shank et al. 1998) and has the potential to be applied to a wide range of organisms (Dixon et al. 1995a). In an earlier study, we proposed the use of sediment traps as a way to study the long-term fluctuations in larval abundance of at least some dominant vent species (Khrifpounoff et al. 2000), which could be combined with molecular identification. However, a prerequisite for such time-series studies is the in situ preservation of samples in a condition suitable for subsequent DNA analysis. Here we present the results of the first successful attempt to preserve vent mussel larvae in the field using sediment traps containing a salt-based preservative buffer that is compatible with DNA analysis.

Description of the preservation method—To preserve larval DNA, we used a solution composed of 5 M NaCl, 10% dimethylsulfoxide (DMSO), and 0.001% bromophenol blue. Buffers of this type have already been shown to work effectively on the tissues of a range of organisms, including marine invertebrates (see Dawson et al. 1998 for a review). This high-salt buffer was previously tested on 12-h-old larvae of the coastal blue mussel *Mytilus edulis* and the serpulid tubeworm *Pomatoceros lamarckii*, because vent larvae were unavailable at the time and would have been too valuable for this type of preliminary work. The larval DNA was extracted following the method of Corte-Real et al. (1994), and to test the efficiency of the preservation method, a short segment of the 28S rRNA gene was amplified using universal PCR primers following 1, 15, 182 and 365 d preservation at 4°C. No difference in the PCR products or amplification efficiency was observed between any of these samples.

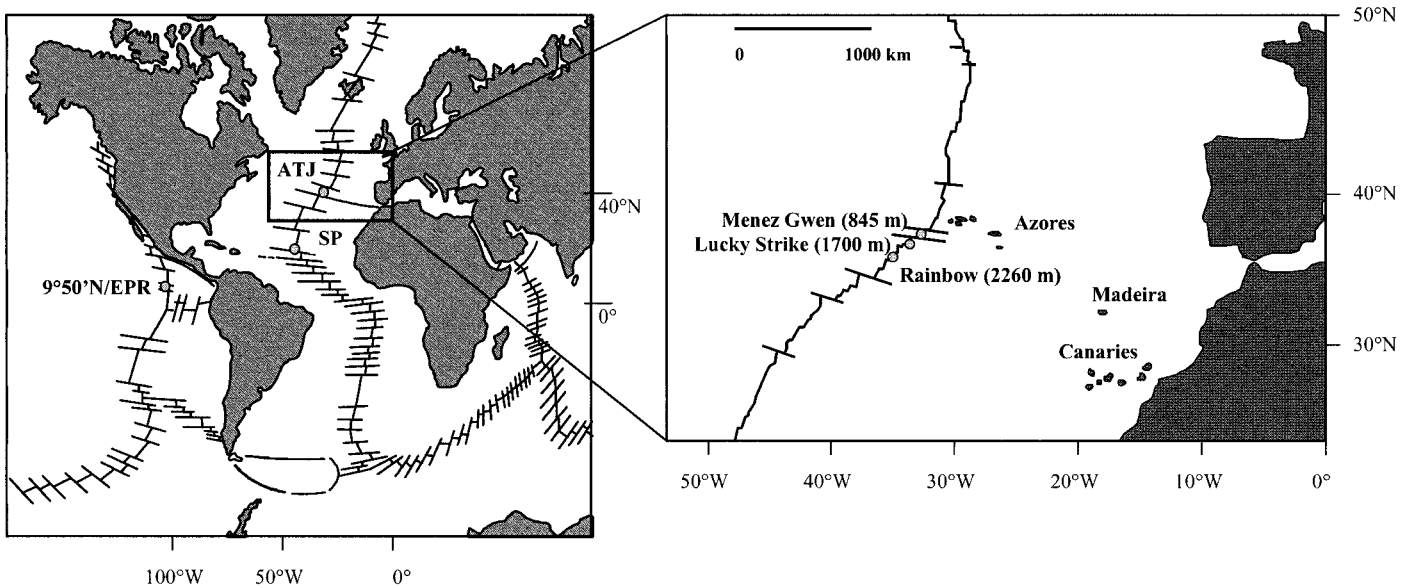
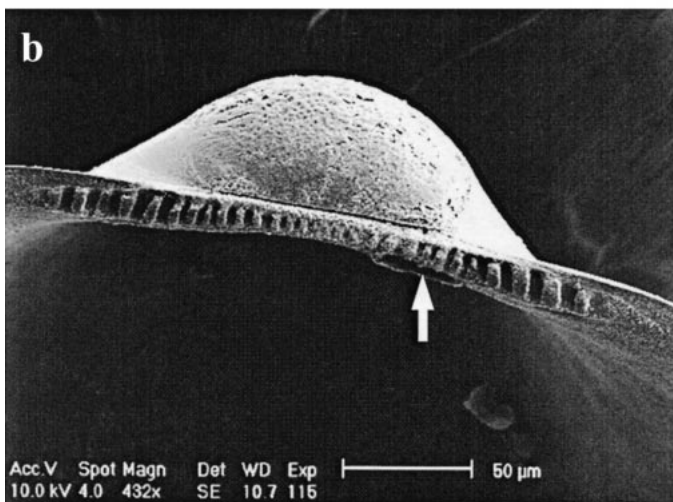
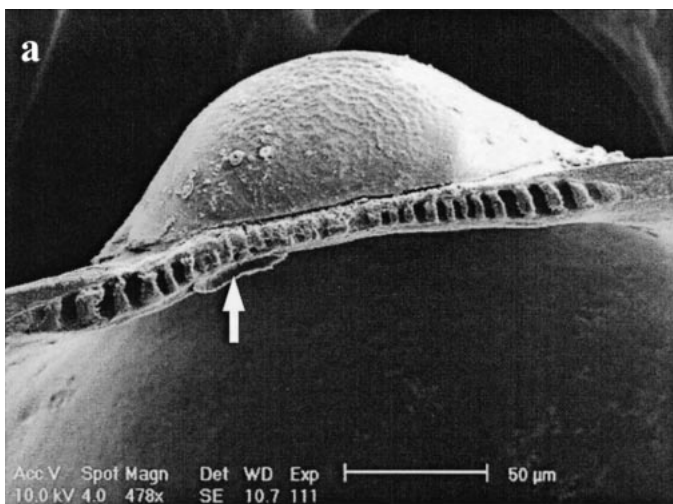


Fig. 1. Location of the sampled vent fields with detail of the Azores Triple Junction (ATJ) area (Menez Gwen, Lucky Strike, and Rainbow vent fields). SP, Snake Pit; 9°50'N/EPR, 9°50'N vent field on the East Pacific Rise.



Construction of a DNA sequence database—Adult specimens of *Bathymodiolus thermophilus* (9°50'N vent field on the East Pacific Rise [EPR], 2,500 m deep), *Bathymodiolus azoricus* from the Menez Gwen (37°50'N, 845 m), Lucky Strike (37°17'N, 1,700 m), and Rainbow (36°13'N, 2,260 m) vent fields on the Mid-Atlantic Ridge (MAR), and *Bathymodiolus puteoserpentis* from the Snake Pit vent field (23°22'N, MAR, 3,500 m) were collected using the deep-sea submersible *Nautilie* (Table 1; Fig. 1). Immediately following collection, the posterior adductor muscle was removed and deep-frozen at -80°C or preserved in BLB buffer (5% SDS, 250 mM EDTA (ethylenediaminetetraacetic acid), 50 mM Tris-HCl, pH 8), before storage at 4°C . In the laboratory, tissue samples were digested overnight with 10 μl of 20 mg ml^{-1} proteinase K in 0.5 ml PK-SDS lysis buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM EDTA, 1% SDS) at 55°C . Genomic DNA was purified using a standard phenol/chloroform extraction procedure (Sambrook et al. 1989). The internal transcribed spacer 2 (ITS2) region of the ribosomal DNA (rDNA) was amplified using PCR with the mytilid-specific primers BAF (5'-GCTTAAATTCAGCGGGTACT-3') and BAR (5'-ACATTGCGGCTTTGGGTCAC-3') using a Perkin Elmer Thermal Cycler 480. The PCR mixture contained 1 \times *Taq* DNA polymerase reaction buffer (Promega), 1 μM of each primer, 160 μM dNTPs, 1.5 mM MgCl_2 , and 0.625 units of *Taq* DNA polymerase (Promega). The PCR cycling parameters were as follows: initial denaturation 4 min at 96°C , followed by 35 cycles of 45 s at 96°C , 1.5 min

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Fig. 2. (a) Shell hinge structure of a mytilid larva collected in the sediment trap located at the Rainbow vent field (shell length 515 μm). (b) Shell hinge structure of a *B. azoricus* postlarva collected at Lucky Strike (MARVEL cruise, dive 1205). Arrow, subumbonal pit.

Table 1. *Bathymodiolus* species used in the comparison with larvae collected at Rainbow. *N*, number of individuals used in the RFLP analysis; numbers in parentheses are the number of individuals used for sequencing.

Vent field	Latitude (N)	Longitude (W)	Species	Cruise (year)	<i>N</i>
East Pacific Rise					
9°50'N	9°50'	104°17'	<i>Bathymodiolus thermophilus</i>	HOT'96 (1996)	3 (2)*
Mid-Atlantic Ridge					
Snake Pit	23°22'	44°57'	<i>Bathymodiolus puteoserpentis</i>	MICROSMOKE (1995)	3 (1)
Menez Gwen	37°50'	31°31'	<i>Bathymodiolus azoricus</i>	DIVA 2 (1994)	11 (2)
Lucky Strike	37°17'	32°16'	<i>Bathymodiolus azoricus</i>	DIVA 2 (1994)	14 (3)
Rainbow	36°13'	33°54'	<i>Bathymodiolus azoricus</i>	MARVEL (1997)	6 (1)

* Two additional individuals of *B. thermophilus* from the 13°N vent field (EPR) and one from the 18°S vent field (EPR) were also included in the sequence analysis.

at 55°C, 1.5 min at 72°C, and a final elongation step of 10 min at 72°C. The PCR products were visualized under ultraviolet (UV) light on a 2% agarose gel containing ethidium bromide.

The PCR products from 12 adult mussels (Table 1) were sequenced according to Jollivet et al. (1998a). Sequences were analyzed with the automated Vistra[™] DNA Sequencer 725. Consensus sequences of the ITS2 region were then gen-

	1				50
<i>B. thermophilus</i>	GCTTAAATTC	AGCGGGTACT	CTCGTCTGAT	CTGAGGTCGG	AGAGGTGA.T
<i>B. azoricus</i>	-----	-----	-----	-----	---T---T-
<i>B. puteoserpentis</i>	-----	-----	-----	-----	---T---T-
	51				100
<i>B. thermophilus</i>	TGGGTTTGGC	GATGCCAAGC	CAACATTGTT	GCGAGGTCTC	CAGAGGAGAG
<i>B. azoricus</i>	-----	-----	-----	T-----	-----C----
<i>B. puteoserpentis</i>	-----	-----	---T----	T-----	-----
	101				150
<i>B. thermophilus</i>	GCCGTCGGTC	GTCTTTCTCT	CAAAAACAGA	GCATTTTGGC	ACCGAGGGAG
<i>B. azoricus</i>	-----	------G----	-----	G-AA-----
<i>B. puteoserpentis</i>	-----	-----A---G----	---G----	GGAA-----
	151				200
<i>B. thermophilus</i>	CAAGATAT..	TACACGTGTG	GGTTTTAAAA	CACACGTCAA	TTTCCGAGCA
<i>B. azoricus</i>	--,-----AA	--A-----	---,....C-	-----	-----
<i>B. puteoserpentis</i>	--,-----	..-A-----	---,....	-----	-----
	201				250
<i>B. thermophilus</i>	TACGTCCCTC	CGCACACGAG	AAAGATAAAC	CGTCGACCGG	AGACACCGGT
<i>B. azoricus</i>	-----	-----	-----	-----	-----
<i>B. puteoserpentis</i>	-----	-----	-----	-----	-----T---
	251				300
<i>B. thermophilus</i>	CTACATTTAA	GGTGACGAAG	CAAGACAAAA	AAGTACAGAG	CATATTTTGC
<i>B. azoricus</i>	-----	-----	-----G----	-----	---G-.------
<i>B. puteoserpentis</i>	-----	-----	-----	..-A-----	---G-.------
	301				350
<i>B. thermophilus</i>	TTGCTGCGAC	GACCCAAGGC	GCAAAAAA..	...TTAAT..	...TTTGCGA
<i>B. azoricus</i>	-----	-----	-----AA	AGA-----CT	TTT-----
<i>B. puteoserpentis</i>	-----	-----	-----AA	AGA-----T	TTT-----
	351				400
<i>B. thermophilus</i>	TGAATGTTTC	ACCGACCCTC	AGACAGGCGT	GGCTTCGGGA	GTGACCCAAA
<i>B. azoricus</i>	-----	-----	-----	-----	---T---G--
<i>B. puteoserpentis</i>	-----	---A-----	-----	-----	-----
	401				
<i>B. thermophilus</i>	GCCGCAATGT				
<i>B. azoricus</i>	-----				
<i>B. puteoserpentis</i>	-----				

Fig. 3. Consensus ITS2 sequences of *B. thermophilus*, *B. azoricus*, and *B. puteoserpentis* aligned using the GCG and GDE software packages. *B. thermophilus* sequence considered as the reference; dash, base similar to reference; dot, insertion/deletion.

Table 2. Numbers of bivalve larvae collected at Rainbow. Abundances are given for each trap of the triplicate device (traps 1 to 3). Means and standard deviations (SD) for each sampling period are also given. Sampling dates correspond to the beginning of the sampling period.

Sampling dates	Trap 1	Trap 2	Trap 3	Mean	SD
24 Aug 1997	355	266	249	290.00	56.93
28 Aug 1997	302	59	223	194.67	123.95
1 Sep 1997	235	237	80	184.00	90.07
5 Sep 1997	32	13	4	16.33	14.29

erated for each species following the majority rule and aligned using the GCG and GDE software packages. The sequence information was used to identify diagnostic restriction endonucleases that could be used in larval identification. Restriction digests were carried out following the manufacturer's instructions (Promega). Restriction products were visualized on a 2% agarose gel containing ethidium bromide under UV-light.

Larval sampling and identification procedures—Larvae were collected during the European Union-funded MARVEL cruise (August–September 1997) on the Rainbow and Menez Gwen active hydrothermal vent fields (Fig. 1), by means of time series sediment traps deployed by the deep-sea submersible *Nautilie*. At each deployment site, three epoxy-fiberglass traps of cylindrical-conical shape were assembled on a single frame (later referred to as a triplicate trap) so that three replicate samples could be collected simultaneously. The tops of the traps were 1.5 m above the bottom (a.b.). Each trap had an aperture of 0.07 m² covered with a honeycomb baffle of 1-cm-diameter and 10-cm-deep cells (Khripounoff and Albéric 1991). At Rainbow, a triplicate trap was located 2 m to the southwest of the active and azoic Flores 5 vent site (36°13.81'N, 33°54.07'W, 2,275 m) and 125 m to the northeast of the nearest site colonized by mytilids, with sampling periods of four × 4 d (24 August–9 September). At Menez Gwen, a triplicate trap was located close to a vent (37°50.52'N, 31°31.23'W, 845 m), with sampling periods of two × 5 d (1–10 September). A reference triplicate trap was deployed 2 km to the east of Menez Gwen (37°50.80'N, 31°30.20'W, 913 m) over two × 5 d (1–10 September) in an area with no detectable hydrothermal activity. Prior to deployment, the sampling bottles were filled with either 3% neutral formalin (in the case of two of the three traps) or DNA preservative buffer. After recovery, the samples were stored in the dark at 4°C. The bivalve larvae were sorted and counted under a dissecting microscope.

For morphological identification purposes, four of the formalin-preserved individuals were used. The larval shell hinge structure was examined using a Philips XL30 scanning electron microscope (SEM) after the shell valves were separated in 5% sodium hypochlorite, rinsed in distilled water, and air-dried.

For molecular identification purposes, the DNA from 20 randomly selected larvae was extracted following the method described in Dixon et al. (1995b). Five additional larvae were digested overnight with 10 μl of 20 mg ml⁻¹ proteinase

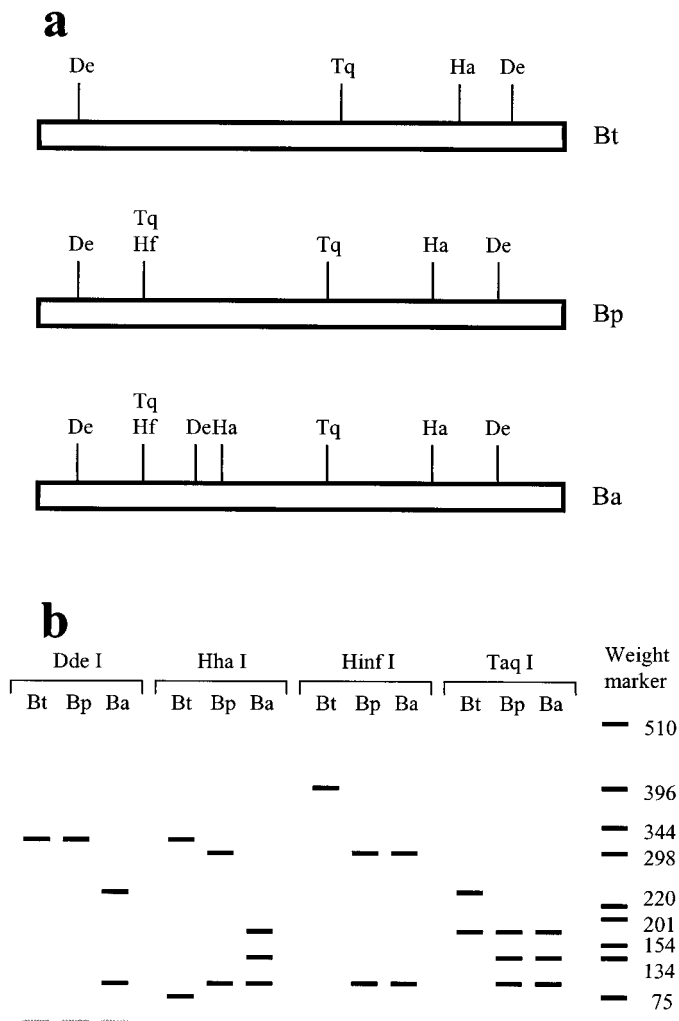


Fig. 4. *Bathymodiolus* spp. (a) Restriction maps of ITS2 as inferred from sequence data. *De*, *Dde*I; *Ha*, *Hha*I; *Hf*, *Hinf*I; *Tq*, *Taq*I. (b) Expected restriction patterns of ITS2 with four restriction enzymes (*Dde*I, *Hha*I, *Hinf*I, *Taq*I). *Bt*, *Bathymodiolus thermophilus*; *Bp*, *B. puteoserpentis*; *Ba*, *B. azoricus*. Weight marker fragment lengths in base pairs (bp). Grey bands represent fragments <75 bp, usually mixed with the primer heteroduplexes.

K in 100 μl PK-SDS lysis buffer at 55°C, then heated 10 min at 95°C. This larval DNA was then precipitated with 200 μl of cold absolute ethanol and 10 μl of 3 M sodium acetate, vacuum-dried and dissolved in distilled water. The ITS2 region was amplified and restriction-digested as described for the adults.

Larval identification—Prodissoconch II-stage larvae (2,055) were collected at Rainbow and Menez Gwen. At Rainbow, larval abundance ranged from 4 to 355 individuals per trap per 4-d sampling interval, whereas at Menez Gwen, only four larvae were collected, compared with no larvae at the nonhydrothermal reference site. The majority of these larvae were of a similar size (~500 μm shell length) and morphology. A single smaller specimen, which had a different shell morphology, was classified as a different species.

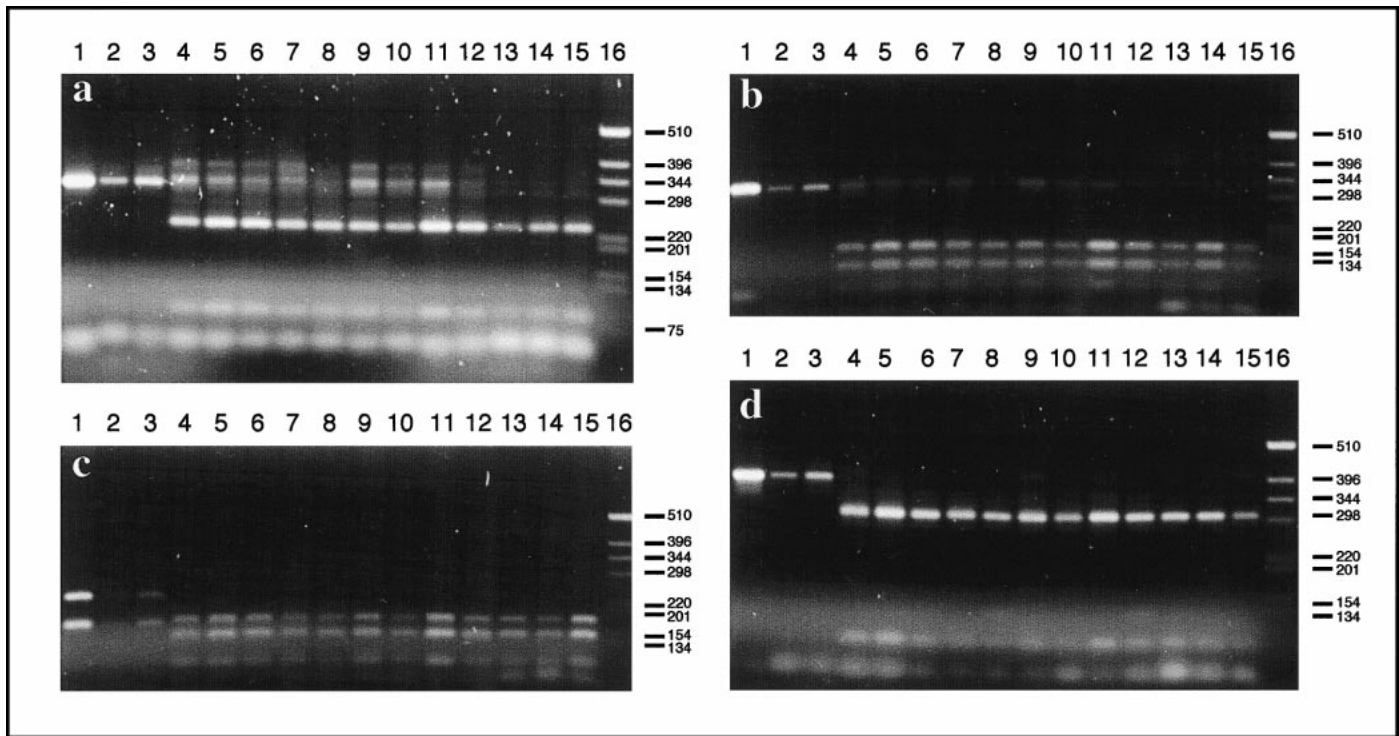


Fig. 5. Restriction patterns of ITS2 for *Bathymodiolus* spp. using *DdeI* (a), *HhaI* (b), *TaqI* (c), and *HinfI* (d). Lanes 1–3: adult *B. thermophilus* from the East Pacific Rise (9°50'N); lanes 4–6: adult *B. azoricus* from Lucky Strike; lanes 7–9: adult *B. azoricus* from Menez Gwen; lanes 10–12: adult *B. azoricus* from Rainbow; lanes 13–15: larvae collected at Rainbow; lane 16: weight marker 1 Kb (GibcoBRL). Weight marker fragment lengths in base pairs (bp).

At Rainbow, there was a decrease in larval numbers during the last sampling period (Table 2), which was observed in all three replicated samples. A Kruskal–Wallis nonparametric test showed that larval abundance differed significantly between the four sampling periods ($P < 0.05$), and it is likely that this was due to the decrease in number during the last sampling period. The results of a nonparametric multiple comparison test (Scherrer 1984) showed that larval abundance was significantly different ($P < 0.05$) between the first and the last period, with evidence of a gradient in between.

The larval bivalve hinge displayed a typical taxodont structure composed of numerous small teeth (between 25 and 30, $n = 4$), with evidence of a progressive increase in tooth size away from the umbone (Fig. 2a). The subumbonal pit, where the ligament originated, lay slightly in the rear of the umbone, which led to an asymmetry in hinge structure. These features have been described for several species within the Mytilidae (e.g., Fuller and Lutz 1989). Moreover, this structure is similar to that of postlarval individuals collected from mussel beds (*B. azoricus*) at both the Lucky Strike and Menez Gwen vent fields (Fig. 2b). The single Rainbow specimen, which showed a different morphology, had a hinge structure that was clearly not representative of the Mytilidae and has not so far been identified.

To confirm the larval species identity, the ITS2 rDNA region was amplified from 24 larvae. The PCR product had a length of ~400 base pairs (bp), which was identical to that of the adults of the three *Bathymodiolus* species used for

comparison. The sequence information (Fig. 3) provided evidence of four diagnostic restriction endonucleases for the three different *Bathymodiolus* species: *DdeI*, *HhaI*, *HinfI*, *TaqI* (Fig. 4). The observed restriction patterns for the adult specimens agreed well with the expected findings based on the sequence information (Fig. 5a–d), with the notable difference that there were also some additional, faint bands that were attributable to intraindividual polymorphism, a common feature of rDNA (e.g., Schlötterer and Hauser 1994; Jollivet et al. 1998b). The restriction patterns for the larvae were identical to those for the adults of *B. azoricus* from Menez Gwen, Lucky Strike, and Rainbow, but clearly differed in the case of *DdeI* and *HhaI* from those of *B. thermophilus* (EPR) and *B. puteoserpentis* (Snake Pit, MAR) (Figs. 4, 5a–d). In addition, they differed from the expected patterns of the *Bathymodiolus* species from the cold seeps of the Gulf of Mexico (D. Jollivet unpubl. data). The ITS2 restriction patterns of the vent larvae were also clearly different from those of adults of the shallow-water mytilid species *Mytilus edulis* and *Modiolus modiolus* (not shown), which adds weight to the specificity of the species “fingerprints” we obtained. These results confirmed unambiguously that the larvae we collected were *B. azoricus*.

In addition, they confirmed the temporal effectiveness of our preservation method based on larval RFLP patterns, which are identical to the typed adult specimens. It is important to note that the effect of poor preservation would lead to DNA degradation, thus resulting in a less efficient PCR reaction (quantitative effect), but this would not influ-

ence the quality of genetic fingerprints obtained after DNA digestion with restriction enzymes that are sequence-specific in their cutting sites. Consequently, the identity of one species could not be changed into another by poor preservation. Thus, the very fact that the PCR reactions on salt-preserved *Bathymodiolus* larvae worked effectively and yielded high-quality RFLP fingerprints shows the potential of this preservation method for long-term sampling in the future.

Origin of the larvae—In addition to Rainbow, adult populations of *B. azoricus* are also known to occur at Menez Gwen, Lucky Strike, and Broken Spur (29°N, MAR) (Jollivet et al. 1998a; Cosel et al. 1999). Based on the present molecular information, it is not possible to identify the source population(s) of the larvae trapped at Rainbow. At the present time, we lack sufficient data on water currents, both within and among vent fields, as well as knowledge on larval dispersal (e.g., length of larval life and migration depth) to draw any firm conclusions. However, some data could help us understand our observations. At Rainbow, where the residual current is directed toward the NNW (close to the vents) and N(NE) (500 and 1,300 m to the NNE of the vents), semidiurnal tidal excursions are strong enough to transport larvae to the NE from a mytilid population located ~125 m to the SW of the trap (Khripounoff et al. 2000). This implies that the larvae may have stayed on the site over a long time interval because these developmental-stage larvae could be at least 3–6 weeks old (based on what is known for the blue mussel *Mytilus edulis*) and are probably older because of the low temperatures encountered at such depths (2–3°C). So if the larvae collected at Rainbow originated at this field, one must propose the existence of retention factors such as vent plume recirculation, as suggested by Mullineaux and France (1995). The surface hydrology in this area is characterized by the occurrence of the Subtropical (or Azores) Front (STF) at 35°–35°50'N and the eastward Azores Current (AC) (Fernandez and Pingree 1996; Pingree et al. 1999), which could prevent larval dispersal at the surface level between Rainbow and the southernmost vent fields (e.g., Broken Spur). In addition, given the significant depth difference between the two fields (Lucky Strike and Rainbow), depth in itself may play an important part in larval distribution patterns, but again we have too little data to go on at the present time.

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Chemically regulated feeding by a midwater medusa

Abstract—Planktonic cnidarians are principal predators in the vast habitats between the ocean’s surface and the deep-sea floor. Almost nothing is known, however, about the chemical ecology of these fragile midwater animals because of difficulties associated with collecting healthy specimens and conducting experiments in the field. With the use of a remotely operated vehicle, we found that the hydromedusa *Mitrocoma cellularia* is not a passive “drift-net” predator. This relatively simple gelatinous organism reacted to both the taste and smell of prey in the laboratory and in situ. Our results comprise the first definitive demonstration that a species of pelagic cnidarian responds behaviorally to chemical signals, and they lend new insight into the role of chemoreception in structuring midwater communities.

Gelatinous zooplankton are an integral part of oceanic midwater ecosystems. Predatory forms (including scyphomedusae, hydromedusae, cubomedusae, siphonophores, and ctenophores) often comprise up to 30% of the biomass in mesopelagic environments, which have no solid boundaries and little or no sunlight (Robison 1995; Pugh et al. 1997). As with all organisms, the success and distribution of gelatinous zooplankton is determined in large part by their ability to obtain food. Sensing molecules dissolved in fluids (distant

chemoreception or “smell”) and bound in solid materials (contact chemoreception or “taste”) have long been known to aid in the search for and selection of prey. Almost all organisms from bacteria (e.g., Adler 1969) to birds (e.g., Nevitt et al. 1995) have been found to acquire nutrition through the use of chemical signals.

Surprisingly, however, it has not been clearly demonstrated that any planktonic cnidarians or ctenophores utilize chemical cues. Most are thought to be relatively passive “drift-net” or random contact predators, relying either on active prey that swim into extended tentacles or on the predator moving through the water column for chance encounters with prey. Although it has been shown that mechanical stimuli are typically used by pelagic cnidarians and ctenophores when feeding, their ability to perceive chemical cues is largely a matter of speculation (see review by Purcell 1997).

A few studies have attempted to determine if various species of planktonic cnidarians or ctenophores respond behaviorally to compounds associated with prey (Loeb and Blanquet 1973; Swanberg 1974; Arai 1991; Falkehnaug and Stabell 1996). However, limitations in the methods used make the results of these studies difficult to interpret. Stronger support for the possibility that pelagic cnidarians may utilize chemical signals is found in the intracellular record-