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Expanding dispersal studies at hydrothermal vents through species identification of cryptic larval forms

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Abstract The rapid identification of hydrothermal vent-endemic larvae to the species level is a key limitation to understanding the dynamic processes that control the abundance and distribution of fauna in such a patchy and ephemeral environment. Many larval forms collected near vents, even those in groups such as gastropods that often form a morphologically distinct larval shell, have not been identified to species. We present a staged approach that combines morphological and molecular identification to optimize the capability, efficiency, and economy of identifying vent gastropod larvae from the northern East Pacific Rise (NEPR). With this approach, 15 new larval forms can be identified to species. A total of 33 of the 41 gastropod species inhabiting the NEPR, and 26 of the 27 gastropod species known to occur specifically in the 9° 50′ N region, can be identified to species. Morphological identification efforts are improved by new protoconch descriptions for *Gorgoleptis spiralis*, *Lepetodrilus pustulosus*, *Nodopelta subnoda*, and *Echinopelta fistulosa*. Even with these new morphological descriptions, the majority of lepetodrilids and peltospirids require molecular identification. Restriction fragment length polymorphism digests are presented as an economical method for identification of five species of *Lepetodrilus* and six species of peltospirids. The remaining unidentifiable specimens can be assigned to species by comparison to an expanded database of 18S ribosomal DNA. The broad utility of the staged approach was exemplified by the revelation of species-level variation in daily planktonic samples and the identification and characterization of egg capsules belonging to a conid gastropod *Gymnobela* sp. A. The improved molecular and morphological capabilities nearly double the number of species amenable to field studies of dispersal and population connectivity.

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Introduction

Larval dispersal in patchy and disturbed ecosystems such as hydrothermal vents is essential for population maintenance and colonization of nascent or disturbed habitat. Gastropods are emerging as a model group on which to focus studies about dispersal, colonization, and population dynamics at vents (e.g., Mullineaux et al. 2003; Mullineaux et al. 2005; Adams and Mullineaux 2008; Matabos et al. 2008a). Gastropod abundances and ecological influence across the range of vent habitats make them key players in structuring macrofaunal communities (e.g., Micheli et al. 2002; Mullineaux et al. 2003; Govenar et al. 2004; Mills et al. 2007). High abundances of gastropod larvae in the plankton (Metaxas 2004; Mullineaux et al. 2005), multiple modes of development (Lutz et al. 1984, 1986), and relative ease of larval identification (Mullineaux et al. 1996) allow researchers to address questions such as the following: how do larval development and behavior, and hydrodynamics combine to disperse and/or retain individuals (Lutz et al. 1980; Marsh et al. 2001; Adams and Mullineaux 2008); and what is the impact of dispersal and recruitment on community structure and dynamics?

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Difficulty in identifying larval stages to the species level can limit studies of larval dispersal (Metaxas 2004; Mullineaux et al. 2005). Larval identifications have traditionally relied on the culturing of larvae and metamorphosis of collected larvae to an identifiable juvenile stage. To date, very few larval stages of vent-endemic species have been cultured, e.g., *Alvinella pompejana* (Pradillon et al. 2004; Pradillon et al. 2005), *Riftia pachyptila* (Marsh et al. 2001), and *Bythograea thermydron* (Epifanio et al. 1999); no vent organisms have been successfully cultured through the entire lifecycle. Thus, identifications of vent larvae have instead relied on similarities between larval and adult morphology, larval structures preserved in adult morphology (Gustafson et al. 1991; Mullineaux et al. 1996) and, more recently, molecular identification (Epifanio et al. 1999; Comtet et al. 2000; Pradillon et al. 2007).

Although gastropod larvae are more readily identifiable than most other taxa due to the preservation of morphologically distinct protoconchs (larval shells) on adults and juveniles, less than half of the gastropod species (17 of 41 species) inhabiting the northern East Pacific Rise (NEPR), from 21° N to 9° N and the Galápagos Rift, can be unequivocally identified to species using the morphological characteristics of the protoconch (e.g., Mullineaux et al. 1996; Warén and Bouchet 2001). Most embryos and trochophores do not have morphological characteristics that allow for species-level identification. Species-level identification of protoconchs has been hampered by poor preservation of larval shells (especially for Caenogastropoda), lack of descriptions of sister species, and strong similarities within genera and families. Regardless, comparisons of preserved protoconch morphology in adult and juvenile gastropods to field-collected larvae has enabled the morphological identification of selected larval vent gastropods to species (Mullineaux et al. 1996). All species of the Sutilizonidae and Neomphalidae known to occur on the NEPR can be identified to the species level morphologically (Turner et al. 1985; McLean 1989a; Mullineaux et al. 1996; Warén and Bouchet 2001). In contrast, representatives of the most abundant taxa, the Lepetodrilidae (7 out of 8 species) and Peltospiridae (8 out of 12 species), and all of the Caenogastropoda (4 species) cannot be distinguished morphologically to species. The caenogastropods, seven peltospirids, and six other species lack *any* information on protoconch morphology.

A main goal of the present study is to improve the capability, efficiency, and economy of identifying vent gastropod larvae. Since we cannot identify all species with morphology alone, we employ a staged approach that involves visual examination of larval shell morphology, followed when necessary, by molecular genetic analysis (Fig. 1). Gastropod specimens can be divided into three categories based on morphology alone: (1) those with larval shell morphology that is distinct at the species level, (2) those with larval shell morphology distinct only at the family or genus level, and (3) those with uninformative larval shell morphology (hereafter referred to as 'unknowns'). From this morphological categorization, the appropriate molecular techniques are selected for each grouping to obtain species-level identification. This approach takes advantage of easily obtained morphological information and optimizes the efficiency of molecular genetic identifications.

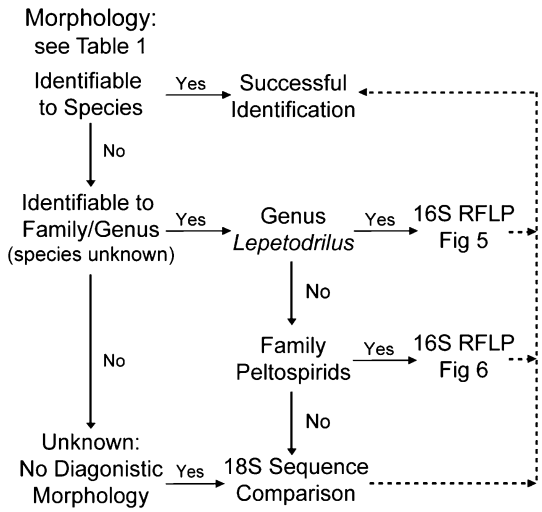

Fig. 1 Flow chart of staged identification procedure

We have three objectives to increase the capability, efficiency, and economy of the staged approach. The first is to expand the number of species that can be identified solely by larval shell morphology. The second is to develop a fast and inexpensive molecular genetic method that is useful for identifying species whose larval shell morphology is informative, but not distinct at the species level. The third is to expand a sequence database of morphologically identified gastropod species ('barcode') that can be compared to sequences of unknowns—embryos, trochophore larvae, and shelled larvae whose morphologies do not allow for classification. To demonstrate the effectiveness of this three-step approach, it is used to identify field-collected larval and benthic samples.

Materials and methods

Sample collection

Adult, juvenile, and larval gastropods were collected by submersible (*DSV Alvin*) or autonomous underwater pump. Adult and juvenile gastropods used in morphological studies were collected on basalt blocks (10 cm each side) or from washings of mussel, tubeworm and sulfide collections

during multiple cruises to the EPR, 9° 50′ N area between 1995 and 2004 (Table S1). Larvae were collected in the same region, near active vent sites, via Mclane WTS-LV plankton pumps between 1998 and 2000 (Table S1). All specimens used for morphology were preserved in 80% ethanol. For molecular investigation, adult gastropods were collected from washings of mussel, tubeworm and sulfide collections from the EPR, 9° 30′–9° 51′ N and 21° N between 2000 and 2006 (Table S1). Adult specimens were sorted and morphologically identified to species onboard the *RV Atlantis* before freezing at −70°C.

Morphological identification

To expand the suite of species that can be identified by larval shell morphology, we compiled morphological descriptions from the published literature to identify gaps in our knowledge, and we imaged protoconchs retained on juveniles from species lacking larval descriptions. If juveniles can be accurately identified to species, and the retained protoconchs on those juveniles are morphologically distinct at the species level and have little to no within-species variation, then new species-specific morphological descriptions can be generated (Mullineaux et al. 1996). Standard diagnostic features used for morphological characterization and identification of the protoconchs included shell size (maximum diameter), sculpture and shape, and aperture flare and shape (e.g., sinuous or straight margin). We focused on obtaining descriptions for the genus *Lepetodrilus* and for the family Peltospiridae, whose species are abundant and ecologically important (e.g., Mullineaux et al. 2003; Van Dover 2003; Govenar et al. 2004; Mills et al. 2007). Individuals with smaller than average shell length and sufficient adult morphology for species-level identification (herein referred to as juveniles) were screened under a dissecting scope for the preservation of an attached protoconch. Juveniles of *Clypeosectus delectus*, *Echinopelta fistulosa*, *Gorgoleptis spiralis*, *Lepetodrilus cristatus*, *L. elevatus*, *L. ovalis*, *L. pustulosus*, *Nodopelta rigneae*, *N. subnoda*, and *Peltospira operculata* were found with attached protoconchs and subsequently imaged using scanning electron microscopy (SEM). Select and common larval morphotypes from pump collections were also imaged using SEM. Micrographs of these unknown larval morphotypes were compared to SEM images of protoconchs retained on juveniles that yielded taxonomically informative descriptions. These larval micrographs sometimes revealed or clarified protoconch characteristics that were not apparent on the juveniles due to juvenile growth or partially corroded protoconch sculpture.

For SEM, juvenile gastropods with attached larval protoconchs and larvae were cleaned in a diluted 3:1 (Clorox) bleach solution at 50° C for 5 min, air-dried, and then mounted on circular glass slides using a small amount of white glue. Slides were glued to SEM stubs with silver polish, then silver-coated in a SAMSPUTTER 2a automatic sputter-coating machine and imaged on a JEOL JSM-840 Scanning Electron Microscope. For each species, juveniles were imaged until an informative SEM image was obtained or all available specimens of that species with an intact protoconch were used. In all, 16 juveniles and 45 larvae were imaged with SEM.

Identification of a defined group of species: RFLP design

Restriction fragment length polymorphism assays (RFLPs) were developed as a cost effective molecular method for identifying *Lepetodrilus* spp. and peltospirids, which represent twelve of the morphologically unidentifiable species (taking into consideration the new morphological descriptions described herein). RFLPs use restriction enzymes to cut PCR products into unique banding patterns based on species-specific differences in nucleotide sequence. This method can be cost efficient for identification of a finite number of candidate species for which species-specific banding patterns could be characterized. Since many of the reagents are one time purchases rather than per sample, cost efficiency increases with increased sample number. Thus, *Lepetodrilus* spp. and peltospirids are well suited for this assay, rather than sequencing, due to high abundance in the benthos (Van Dover 2003; Dreyer et al. 2005) and as larvae in the plankton (Mullineaux et al. 2005), and the ability for morphological assignment to a defined species group (genus or family, respectively).

We developed RFLP assays for the genus *Lepetodrilus* and unidentifiable species of the family Peltospiridae (*Echinopelta fistulosa*, *Hirtopelta hirta*, *Nodopelta heminoda*, *N. rigneae*, *N. subnoda*, *Peltospira delicata*, and *P. operculata*) using part of the mitochondrial 16S rDNA gene. The mitochondrial 16S rDNA gene has established use for species-level lineage determination in gastropod phylogenetics (e.g., Reid et al. 1996; Douris et al. 1998). While mitochondrial genes can be subject to hybridization and introgression, the use of mitochondrial markers for species identification has been broadly accepted by the community, as evidenced by large sequencing initiatives such as the Barcode of Life (Savolainen et al. 2005). Additionally, we saw no evidence for either hybridization or introgression in this study. Nuclear 18S rDNA was also attempted, but abandoned due to insufficient nucleotide variability at potential restriction sites among sister species (see "Results"). Part of the 16S gene was amplified and sequenced for at least two adult individuals each of *Lepetodrilus cristatus*, *L. elevatus*, *L. ovalis*, *L. pustulosus*, *L. tevnianus, P. operculata*, *P. delicata*, *E. fistulosa* and *N. subnoda* (see Table S2). Only one individual of each *N. rigneae* and *N. heminoda* were sequenced due to

availability. No *H. hirta* specimens were available, but the partial 16S sequence from GenBank (AY163397) was included in the alignment and RFLP design. *Echinopelta fistulosa* was included in the RFLP because it was not morphologically identifiable at the time of initial RFLP development. *Peltospira lamellifera* was the only morphologically unidentifiable species in the NEPR region from these two groups not included, due to availability. The absence of this species in this study is not likely to compromise identifications since only three specimens of *P. lamellifera* (all from the 13° N area) have ever been recorded.

All PCRs were performed in an Eppendorf Master Gradient thermocycler in 25 µl reaction containing 0.75–1.00 µl genomic DNA extracted using a DNAeasy Kit (Qiagen), 1× buffer (Promega), 1 mM $MgCl_2$, 1 mM each dNTP, 500 nM each primer, and 1 unit of Taq DNA polymerase (Promega). *Lepetodrilus* spp. were amplified and sequenced using the "universal" primers, 16sar-L (forward) and 16sbr-H (reverse) (Palumbi 1996). The peltospirids were amplified and sequenced using the 16sar-L forward primer and a new reverse primer, Pelto16sR: 5′ GCTTCTRCACCMACTGGAAATC. Failure to amplify *Nodopelta rigneae* using 16sar-L and 16sbr-H necessitated the design of the new primer for the peltospirids using Primer3 (http://frodo.wi.mit.edu/primer3/). Amplifications were performed using the following cycling parameters: 2 min initial denaturation at 96°C followed by 30 cycles of 30 s at 94°C, 30 s at 48°C, and 1 min at 72°C. PCR products were visualized on a 1.5% agarose gel with ethidium bromide using the ChemImager or AlphaImager system (Alpha Innotech Corporation). PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen) before sequencing on an ABI 377 or 3730xl sequencer (Applied Biosystems). Sequences were edited in EditView (Applied Biosystems) and aligned using Sequencher v. 4.2.2 (Gene Codes Corp.) and MacClade (Maddison and Maddison 2000). Restriction enzymes were chosen by viewing cut sites using Sequencher v. 4.2.2.

All restriction enzyme digestions were performed in 15 µl reactions containing 500–1000 ng of purified PCR product, 5 units of each restriction enzyme, 1× buffer (enzyme specific, provided by Promega or New England Biolabs), and 100 µM BSA. Digestions were visualized on a 2% agarose gel containing ethidium bromide using the ChemImager or AlphaImager system (Alpha Innotech Corporation).

Fifteen individuals of each species, except *Hirtopelta hirta*, *Nodopelta rigneae*, and *N. heminoda*, from at least two ridge segments (e.g., 9° 50′ N and 21° N) were digested as described earlier to test for false negatives and false positives. Initial morphological screening into the two taxonomic groups, the genus *Lepetodrilus* and unknown peltospirids, eliminated false-positive identification of species not included in the RFLP design.

Identification with no morphological information

In order to expand the database for comparison with sequences from unidentified larvae, partial nuclear 18S rDNA sequences were obtained from all available adult gastropods species from the NEPR (20 out of 41, Table S2). The nuclear 18S rDNA gene was chosen to take advantage of existing sequences in GenBank and because of the established use of the 18S region in gastropod phylogeny (Harasewych and McArthur 2000). If necessary, adult identifications were compared to the reference collection at the Los Angeles County Natural History Museum or identified by Anders Warén (Swedish Museum of Natural History). Genomic DNA was purified using the DNAeasy Kit (Qiagen). Part of the 18S rDNA gene was amplified and sequenced using polymerase chain reaction with the primers AGM-18F (forward): 5′ GCCAGTAGTCATATGCTTGTCTC and AGM-18R (reverse): 5′ AGACTTGCCCTCCAATRGATCC (Harasewych and McArthur 2000) using the procedure and PCR conditions described earlier. Sequences were aligned for comparison using Sequencher v. 4.2.2 (Gene Codes Corp.) and MacClade (Maddison and Maddison 2000). Parsimony trees and neighbor-joining trees were made in PAUP 4.0 (Swofford 2003). To determine the confidence level of the monophyletic groups, bootstrap analyses were performed using five hundred replicates.

Application to field samples

The staged procedure developed in this study was applied to identify larvae from a sub-set of time-series sediment trap collections near 9° 50′ N EPR. Larvae were collected daily in a 21 sample Mclane PARFLUX time-series sediment trap moored 4 m above bottom at a location 10 m south of the Choo Choo vent site (9° 49.60′ N, 104° 17.37′ W, 2512 m) during the November 2004 AT11-20 cruise. The trap opening was 0.5 m^2 and is covered by baffle with a cell diameter of 2.5 cm. Samples were preserved in a saturated salt-20% DMSO solution (Khripounoff et al. 2000) to preserve morphology and DNA. Larvae from four of the samples were sorted using a Zeiss Stemi 2000-C dissecting scope and then identified morphologically to species using a Zeiss Axiostar Plus compound scope.

Those larvae not identifiable to species were sorted into three groups for molecular identification: *Lepetodrilus* spp., peltospirids and unknowns. *Lepetodrilus* spp. were identified based on small size, 170–190 µm, punctate sculpture, and a straight aperture margin that was even with the axis of coiling. Unfortunately, *L. pustulosus* has not been successfully imaged and juveniles are difficult to identify (Warén and Bouchet 2001); thus, the morphology assessment was based on the consistency of size, shape, and sculpture characteristics within *Lepetodrilus* species on the EPR, Galápagos, Juan de Fuca, and Mid Atlantic Ridges

and within the family in general (Mullineaux et al. 1996; Warén and Bouchet 2001). Peltospirids were identified based on ridged ornamentation and shape. Genomic DNA was extracted from each sorted larva not identified to species, using the QiaAmp DNA Micro Kit (Qiagen), a Chelex extraction (Walsh et al. 1991), or by dropping larvae directly into the PCR solution. Successful extractions were then sequenced or processed for RFLP as described earlier.

The identification procedure was also applied to unidentified egg capsules to demonstrate the utility of the technique on other early-stage specimens without morphological descriptions. Egg capsules were collected from caged (6 mm mesh) and uncaged basalt colonization blocks placed on the seafloor as part of a larger colonization study (Micheli et al. 2002; Mullineaux et al. 2003). Nine blocks collected in beds of vestimentiferan tubeworms or mussels, during the May 1998 cruise, contained egg capsules with embryos and developing veligers. Larvae in the egg capsules had not yet formed identifiable shells preventing morphological identification; therefore, they were identified by direct 18S sequence comparisons, following DNA extraction using the DNAeasy kit (Qiagen) and PCR amplification of part of the 18S gene as described earlier. Sequences obtained from the egg capsules were compared directly to the gastropod 18S sequences from known adults using Sequencher v. 4.2.2 (Gene Codes Corp.) and MacClade (Maddison and Maddison 2000). The shape, size, and number of embryos per capsule were characterized for 20 egg capsules under a Zeiss Stemi 2000-C dissecting scope.

Results

Morphological identification and descriptions

Morphological characteristics of twenty-seven vent gastropod protoconchs from the NEPR were compiled from the literature and from our new descriptions of SEM images presented in this study (Table 1). With these new morphological descriptions, 20 descriptions are diagnostic to the species level, five descriptions are diagnostic to the genus level, and two descriptions are diagnostic to the family level. All descriptions from the literature, except for one, were from protoconchs preserved on identified or identifiable juveniles. The exception is a larval description of *Phymorhynchus* sp., based upon veligers found within egg capsules collected on the Galápagos Rift morphologically identified as belonging to the genus *Phymorhynchus* (Gustafson et al. 1991). Unnamed archaeogastropods in Lutz et al. (1984, 1986) are now identifiable as *L. cristatus* and *L. ovalis*, respectively (McLean 1988). Unnamed *Rimula?* in Turner et al. (1985), Fig. 11a–c, has since been identified as *Temnozaga parilis* (McLean 1989a). The specimen in Mullineaux et al. (1996), Fig. 1f, i, was mistakenly identified as *Lepetodrilus ovalis* instead of *L. elevatus*.

SEM images yielded new protoconch descriptions for three species, *Gorgoleptis spiralis*, *Echinopelta fistulosa* and *Nodopelta subnoda*. The protoconch of *G. spiralis* is characterized by a small size (~150 μm) and an overall coarse punctuate sculpture which forms close parallel rows away from the axis (Fig. 2a, b). This description of the *G. spiralis* protoconch allows it to be differentiated from the *G. emarginatus* protoconch (Fig. 2c) which is similar in shape, sculpture, and aperture (Mullineaux et al. 1996), but is larger in size (~180 μm). *G. spiralis* is distinguished from another close relative, *Clypeosectus delectus* (Fig. 2d), by the scalloped aperture. Additional images of *C. delectus* protoconchs on two juveniles (not shown) were consistent with the previous protoconch description and larval identification.

In the Peltospiridae, the protoconch of *Echinopelta fistulosa* (Fig. 3) is distinct at the species level, but the protoconch of *Nodopelta subnoda* (Fig. 4) is not. Both protoconchs were similar to protoconchs of previously described peltospirids based on the presence of ridges. *E. fistulosa* protoconchs can be easily distinguished from other members of the peltospirid family by the restriction of ridges to the apex and indentations or "shelves" at the axis of coiling. The protoconch of *N. subnoda* (Fig. 4a, b) is not distinguishable to species due to a high degree of similarity to *P. operculata* (Mullineaux et al. 1996, Fig. 3e). Both species are characterized by smooth parallel ridges and moderate size (215–220 μm). However, if all peltospirid protoconchs were imaged, the number, spacing, or pattern of ridges may be determined to be species-specific.

Protoconchs on juveniles of the six additional species (*Lepetodrilus cristatus*, *L. elevatus*, *L. ovalis*, *L. pustulosus*, *Nodopelta rigneae*, and *Peltospira operculata*) were not informative to species level, and are not shown. Images of *N. rigneae* and *P. operculata* were uninformative due to corrosion or other damage. All imaged *Lepetodrilus* spp. protoconchs exhibited the previously described punctuate sculpture, but lacked visible species-specific characteristics.

Identification of a defined group of species: RFLP design

For the *Lepetodrilus* spp. and peltospirid groups, 16S rDNA sequences from morphologically identifiable adults and juveniles contained suitable variation among species to design species-specific RFLP assays (Fig. S1, GenBank accession numbers listed in Table S2). Species-specific banding patterns were obtained for *L. cristatus*, *L. elevatus*, *L. ovalis*, *L. pustulosus*, and *L. tevnianus* by digesting the initial PCR product with the restriction enzymes *Sty*I, *Stu*I, and *Dra*I (Promega) together, using Buffer B, for 3–4 h at 37°C (Fig. 5). Due to decreased efficiency (75–100%) of

Table 1 Summary of known protoconch and egg capsule characteristics for vent gastropods on the northern East Pacific Rise

Species	Range	Level of Morph ID	General protoconch description			Source	Figure
			Size µm	Sculpture/shape	Aperture		
Subclass Patellogastropoda							
Family Neolepetopsidae							
Eulepetopsis vitrea	21°N–17°S, Gal	Species	250	Deep side indentations, flattened, smooth surface looks grainy in light microscopy	str. flared	McLean (1990) and Mills (unpublished data)	
Neolepetopsis densata	12°–13°N, Gal	Genus	230	Deep side indentations, knobbed, and pnt. apex	str.	Warén and Bouchet (2001)	
Neolepetopsis occulta	21°N	–	–	–	–		
Neolepetopsis verruca	21°N	–	–	–	–		
Family Trochidae							
Bathymargarites symplector	13°N–17°S	Species	240+	Smooth apex, outer axial striations	sin. flared	Warén and Bouchet (1993)	
Moelleriopsis sp.	13°N	–	–	–	–		
Family Lepetodrilidae							
Clypeosectus delectus	21°N–17°S, Gal	Species	175	Coarse pnt., forms close rows at curve	sl. sin.	McLean (1989b)	2 d
Gorgoleptis emarginatus	21–9°N	**Species**	180	Coarse pnt., forms close rows at curve	scalloped	Mullineaux et al. (1996)	2 c
Gorgoleptis spiralis	13–9°N	**Species**	150	Coarse pnt., forms close rows at curve	scalloped	This study	2 a,b
Lepetodrilus cristatus	21–9°N, Gal	Genus	–	Pnt.	str.	Lutz et al. (1986)[a]	
Lepetodrilus elevatus	Gal, 21°N–17°S	Genus	170–180	Pnt.	str.	Mullineaux et al. (1996)	
Lepetodrilus ovalis	21°N–17°S, Gal	Genus	170–180	Pnt.	str.	Mullineaux et al. (1996)	
Lepetodrilus pustulosus	21°N–17°S, Gal	**Genus**	170–180	Pnt.	str.	This study	
Lepetodrilus tevnianus	11°–9°N	–	–	–	–		
Family Sutilizonidae							
Sutilizona theca	13°N	Species	250	Deep pnt. in lineations following shell curve	–	McLean (1989b)	
Temnozaga parilis	21°N	Species	170	Smooth	–	Turner et al. (1985)[b]	
Family Fissurellidae							
Cornisepta levinae	13°N	–	–	–	–		
Subclass Uncertain Superfamily Neomphaloidea							
Family Neomphalidae							
Cyathermia naticoides	21–9°N	Species	240	Initial bold reticulate web, distal smooth	sl sin.	Warén and Bouchet (1989)	
Lacunoides exquisitus	Gal	Species	160	Initial irreg. net, distal smooth, bulbous shape	str.	Warén and Bouchet (1989)	
Melanodrymia aurantiaca	21°N–17°S, Gal	Species	250	Fine irreg. reticulate, full	sin. flared, ridge above	Mullineaux et al. (1996)	
Melanodrymia galeronae	13°N	Species	250	Very fine reticulate net, full	extended	Warén and Bouchet (2001)	
Neomphalus fretterae	21–9°N, Gal	Species	260	Initial fine irreg. reticulate, distal smooth	sin. flared	Turner et al. (1985)	
Pachydermia laevis	21°N–17°S	Species	250	Reticulate web fading at aperture	str. flared	Warén and Bouchet (1989)	
Planorbidella planispira	21–9°N	Species	215	Initial coarse irreg. net, distal smooth, broad curvature	str.	Warén and Bouchet (1989)	
Solutigyra reticulata	21–13°N	Species	210	Initial irreg net, distal smooth, rounded curve	str.	Warén and Bouchet (1989)	

Table 1 continued

Species	Range	Level of Morph ID	General protoconch description			Source	Figure
			Size µm	Sculpture/shape	Aperture		
Family Peltospiridae							
Ctenopelta porifera	13–9°N	Species	325	Ridged parallel then become irreg. near apex, Ridges end abruptly at ½	scalloped	Warén and Bouchet (1993)	
Echinopelta fistulosa	21–9°N	**Species**	210	Ridges only at apex, deep side indentations	str.	This study	3
Hirtopelta hirta	21–13°N	–	–	–	–		
Lirapex granularis	21–9°N	Species	220	Ridges fade toward axis, pnt. apex	str.	Mullineaux et al. (1996) and	
Lirapex humata	21°N	Species	180	Strong ridges irreg. spaced at apex	str.	Warén and Bouchet (1989)	
Nodopelta heminoda	21–9°N	–	–	–	–		
Nodopelta rigneae	13–9°N	–	–	–	–		
Nodopelta subnoda	9°N–17°S	**Family**	215	Smooth parallel ridges	str.	This study	4 a,b
Peltospira delicata	13–9°N	–	–	–	–		
Peltospira lamellifera	13°N	–	–	–	–		
Peltospira operculata	21–9°N	Family	220	Smooth parallel ridges	str.	Mullineaux et al. (1996)	
Rhynchopelta concentrica	21°N–17°S	Species	290	Irreg. ridges, shelf at axis	str.	Mullineaux et al. (1996) and McLean (1989a)	
Order Neogastropoda							
Family Conidae							
Gymnobela sp. A	13–9°N	**EC Species**	EC 2-3	Egg capsules lenticular, white, yellow or pink, elliptical escape aperture	N/A	This study	7
Phymorhynchus sp.	21°–9°N, Gal	Genus	EC 14–16	Egg capsules lenticular, white to transparent, elongated escape aperture (*s*-shaped)	N/A	Gustafson et al. (1991)	
(*P. major*)	(13–9°N)	–	235	Protoconch PII: spiral raised ridges in direction of growth, crossed by perpendicular riblets	–	Warén and Bouchet (2001) Lutz et al. (1986)	
Order Mesogastropoda							
Family Provannidae							
Provanna ios	21°N–17°S, Gal	–	–	–	–		
Provanna muricata	21°N, Gal	–	–	–	–		

Taxonomic placement and range as in Warén and Bouchet (2001) with modifications to the range based on authors' unpublished collections. The third column indicates the taxonomic level to which larvae of the given species can be identified. Bold type represents a new description or a more refined level of taxonomic identification contributed by this study. Dashed lines indicate that the morphology is unknown. The size is the maximum length of the shell in micrometers or the maximum diameter of the egg capsule in millimeters, if preceded by EC. Figure numbers reference the appropriate figure showing morphology for the given species

[a] Unnamed archaeogastropod limpet in Fig. 2a–c, partial loss of sculpture

[b] Unnamed *Rimula*(?) Fig. 11a–c

N/A not applicable, *Gal* Galápagos, *irreg* irregular, *pnt* punctuate, *sin* sinuous, *str* straight, *sl* slightly

*Sty*I in Buffer B (Promega), digestion of PCR products from *L. ovalis* often resulted in the expected bands representative of the cut positions as well as a remaining uncut band. Inclusion of *Stu*I is optional but makes an additional cut which facilitates identification of *L. cristatus*.

Diagnostic banding patterns were obtained for the peltospirids (Fig. 6 and S2) by digesting the initial PCR product with *Dra*I (New England Biolabs) for 3–4 h at 37°C and, if necessary, with *Ssp*I and *Eco*RV (New England Biolabs) in buffer 3 for 3–4 h at 37°C in parallel. The first *Dra*I digestion identifies *Peltospira operculata*, *P. delicata*, and *Echinopelta fistulosa*, to species, and is predicted to identify *H. hirta* to species. The *Dra*I digestion identifies the genus *Nodopelta*, but does not distinguish among *Nodopelta* species. The second *Ssp*I and *Eco*RV digestion of the initial PCR product was only necessary to distinguish among *Nodopelta* species.

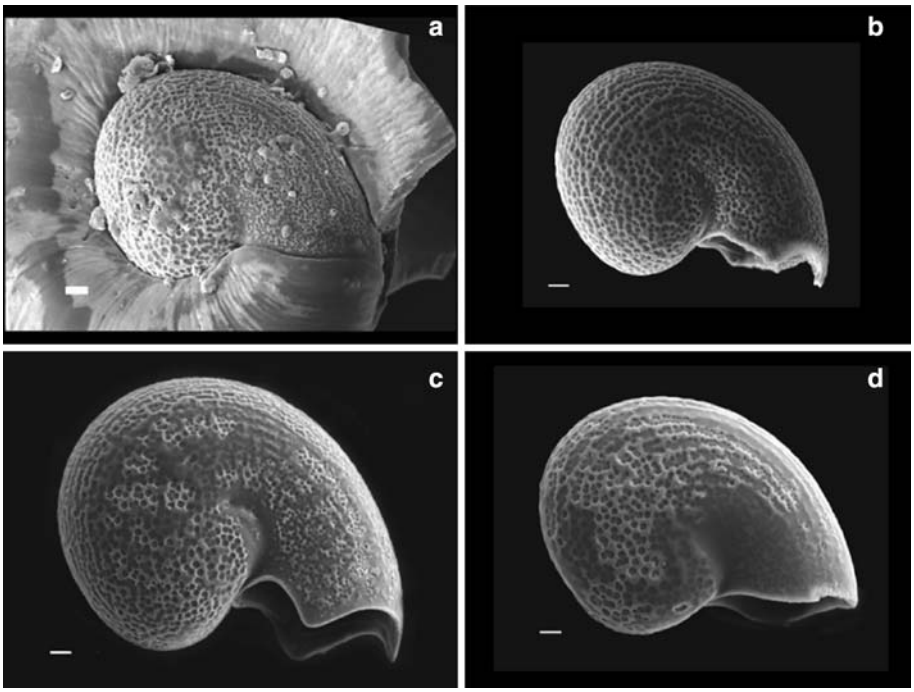

Fig. 2 SEM images of juvenile and larval *Gorgoleptis spiralis* and closely related species. **a** *G. spiralis* protoconch on juvenile. A broader view of the juvenile shell is not shown due to breakage during sample preparation. **b** *G. spiralis* larva. **c** *G. emarginatus* larva. **d** *Clypeosectus delectus* larva. *Scale bars* are 10 μm for all shells

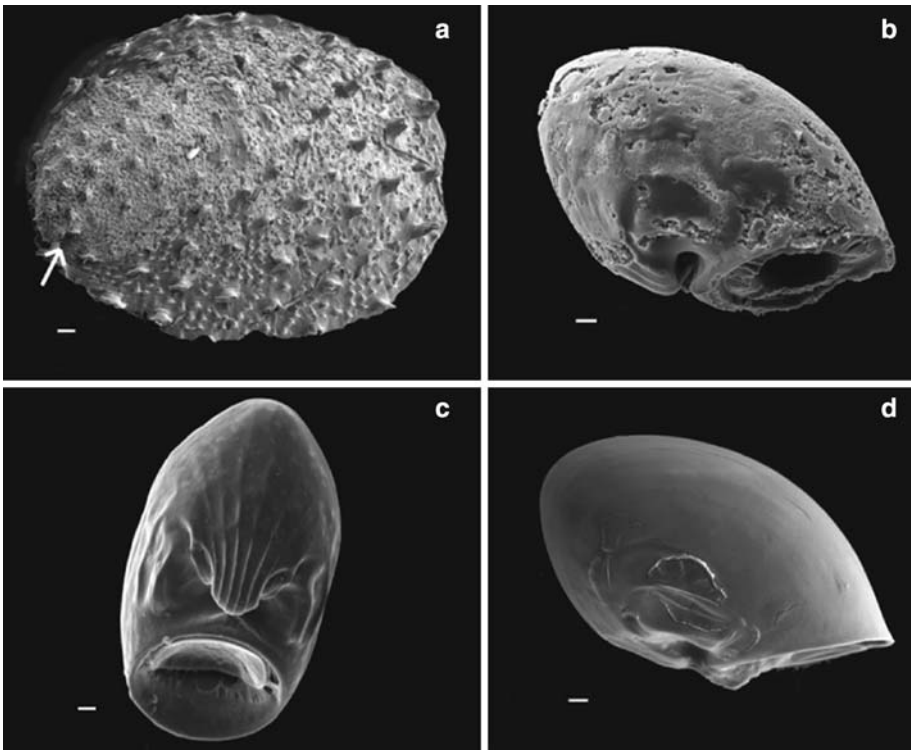

Fig. 3 SEMs of juvenile and larval *Echinopelta fistulosa*. **a** *E. fistulosa* juvenile. The *white arrow* denotes the location where the protoconch was previously attached. **b** Protoconch detached during manipulations of *E. fistulosa* juvenile pictured in **a**. Two *E. fistulosa* larvae are pictured to show the ridged sculpture restricted to the axis (**c**) and the indentations on the sides in the same orientation as the protoconch from the juvenile (**d**). *Scale bars* are 10 μm for all shells except **a** (100 μm)

Digestions to test for false positives and negatives produced the expected banding patterns for all adult individuals from each species with the exception of *Peltospira delicata* and a single specimen of *Lepetodrilus cristatus* (data not shown). *Ssp*I and *Eco*RV digestion of three individuals of *P. delicata* produced the banding patterns expected for *P. operculata*. However, the banding patterns in the initial Dra I digestion produced the expected banding patterns for both *P. delicata* and *P. operculata*. All *L. elevatus* specimens produced the same banding pattern, independent of vent field (9°N or 21°N) or vent site (tubeworm or mussel dominated), suggesting that this assay does not distinguish between the cryptic species or subspecies of *L. elevatus* (Johnson et al. 2008; Matabos et al. 2008b).

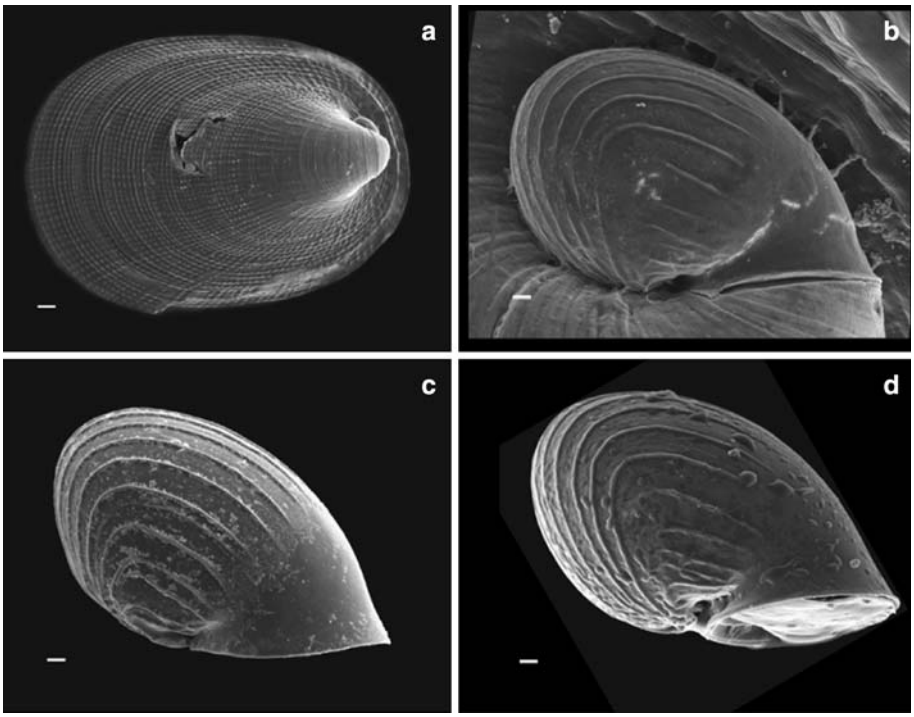

Fig. 4 SEMs of juveniles and larvae in the family Peltospiridae. **a** *Nodopelta subnoda* juvenile. **b** *N. subnoda* protoconch attached to juvenile pictured in **a**. **c**, **d** Peltospirid larvae that closely resembled both *N. subnoda* and *P. operculata* in shape and sculpture. *Scale bars* are 10 μm for all shells except **a** (100 μm)

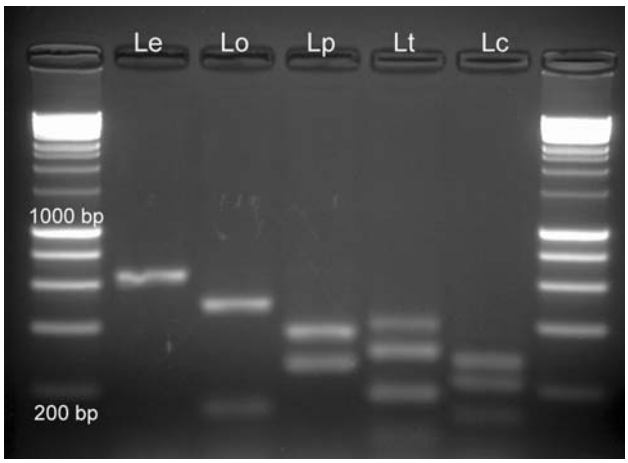

Fig. 5 Restriction fragment length polymorphism assays showing species-specific banding patterns using *Dra*I, *Stu*I, and *Sty*I. Le, *Lepetodrilus elevatus*; Lo, *L. ovalis*; Lp, *L. pustulosus*; Lt, *L. tevnianus*; Lc, *L. cristatus*. 100 bp ladder is included as size standard

Identification with no morphological information: application of 'barcodes'

Diagnostic 18S rDNA sequences were obtained from 39 adult gastropods representing 19 species (Table S2). GenBank contained two different sequences of the 18S rDNA region for each of *Eulepetopsis vitrea* and *Peltospira operculata*. To resolve possible sequence errors in these and other species, all of the existing GenBank sequences, except for *Melanodrymia aurantiaca* (specimens were not available), were verified with additional sequences in the present study. No other inconsistencies were uncovered. GenBank sequences and their accession numbers that were identical to sequences obtained during the present study are included in Table S2. Sequences representing 'barcodes' for thirteen new species were added to the public database, bringing the total number of NEPR vent-endemic gastropod species with 18S rDNA sequences to 20.

Genetic variation of the partial 18S sequence (~550 bp) was sufficient to resolve higher level systematic relationships and differentiate among the vent gastropod species, except among *Lepetodrilus* species (Fig. S3). Neomphalids showed the highest divergence among species with greater than 2.7% (15 bp), with a maximum of 6% (33 bp) divergence between species pairs. Genera within Peltospiridae differed by at least 1.3% (7 bp) and up to 3.5% (19 bp), but differences among species within genera were lower, 0.4–1.2% (2–9 bp) divergence. The pairwise difference between *Peltospira delicata* and *P. operculata* was 0.7% (4 bp) and between *Nodopelta heminoda* and *N. subnoda* was only 0.4% (2 bp). Lepetodrilids differ from all other families by greater than 8% (45 bp) sequence divergence; however, differentiation within the family was very low. *Lepetodrilus elevatus*, *L. ovalis* and *L. pustulosus* were identical over 540 bp and differed from *Gorgoleptis spiralis* and from *L. cristatus* by only 1 bp. In the Caenogastropoda, *Gymnobela* sp. A and *Phymorhynchus major* varied by only 1 bp (Fig. S4). No intraspecies variation was detected.

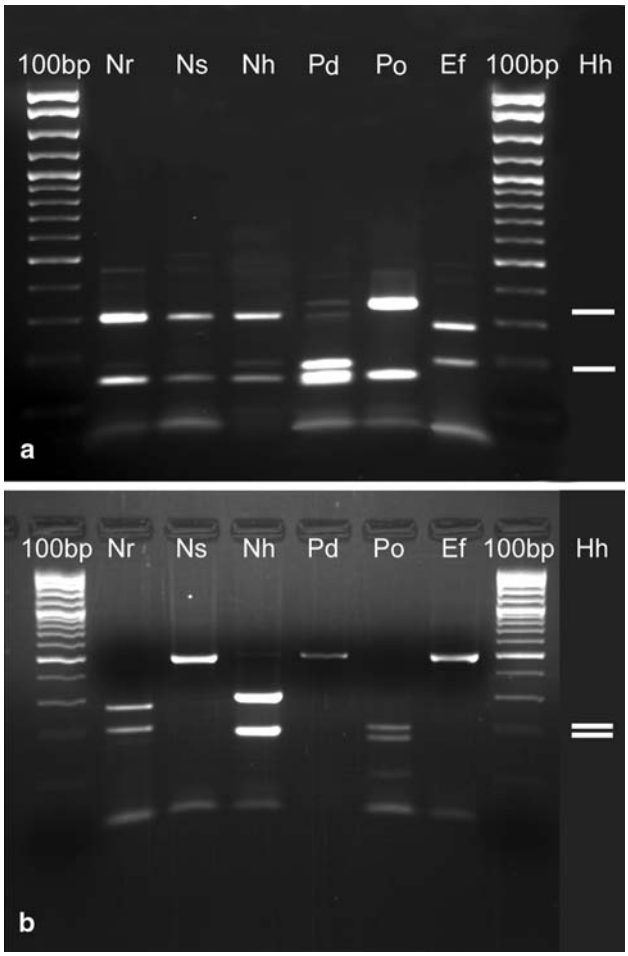

Fig. 6 Restriction fragment length polymorphism assays showing species-specific banding patterns for *Dra*I (**a**) and *Ssp*I with *Eco*RV (**b**). Nr, *Nodopelta rigneae*; Ns, *N. subnoda*; Nh, *N. heminoda*; Pd, *Peltospira delicata*; Po, *P. operculata*; Hh, *Hirtopelta hirta. H. hirta* digestions are predicted patterns inferred from sequence data, since no specimens were available. 100 bp ladder is included as size standard

Application to field samples

Forty-one gastropod larvae, collected in the sediment trap over the course of 4 days, were analyzed to determine what the staged approach could reveal about temporal variation of gastropod larvae in the field (Table 2). Twenty-one of the specimens could be identified under a light microscope by morphology alone. The remaining 20 specimens were divided into three morpho-groups, *Lepetodrilus* spp., peltospirids, and unknown for further identification. The *Lepetodrilus* spp. and peltospirids were suitable for RFLP analyses (Fig. 6); however, genomic extractions of *Lepetodrilus* spp. ($n = 3$) and the peltospirids ($n = 2$) failed to yield sufficient DNA for PCR and RFLP for all but one peltospirid. The unknown peltospirid was successfully identified as *P. operculata*.

Table 2 Abundances of gastropod larvae at Choo Choo vent site, 9° 49′ N East Pacific Rise, each day collected over a 0.5 m^2 area

Species	Date				Total
	13-Nov	14-Nov	15-Nov	16-Nov	
B. symplector		1			1
C. delectus		1			1
C. naticoides	5	10	2	1	18
G. spiralis	1				1
P. operculata			1		1
Unknown peltospirid		1			1
Lepetodrilus spp.		1		2	3
Unknown benthic sp. A	1	2	1	1	5
?Laeviphitus sp.	2	4	2		8
Unknown	1	1			2
Daily Total	10	21	6	4	41

The first four species were identified to species morphologically. *Peltospira operculata* was identified using RFLP assays. The morpho-types Unknown Benthic sp. A and ?*Laeviphitus* sp. were sequenced but were not successfully assigned to species

Two distinct morpho-types in the unknown group, *?Laeviphitus* sp. (EF549683) and Unknown Benthic sp. A (sensu Mullineaux et al. 2005) (EF549681), were sequenced for identification by direct comparison of 18S rDNA (100% success, $n = 2$ of each species). These morpho-types were chosen due to their relatively high abundances in this and other collections. Neither *?Laeviphitus* sp. nor Unknown Benthic sp. A matched any gastropod species within the current 18S database for gastropods along the northern EPR. Morphological identifications of larval *Cyathermia naticoides* and *Bathymargarites symplector* were verified through successful direct 18S rDNA sequence comparison of one individual each.

The sequence database was used to identify lenticular egg capsules (Fig. 7) collected on colonization blocks. Comparison of partial 18S rDNA sequences from the lenticular egg capsules revealed that the capsules were deposited by the conid gastropod *Gymnobela* sp. A. Sequences from six egg capsules, including yellow, pink and transparent capsules, had a 100% match over 540 bp with each other and adult *Gymnobela* sp. A, but differed from *Phymorhynchus major* by a single base pair (Fig. S4). The lenticular egg capsules occurred in abundances ranging from 1 to 390 egg capsules per block with densities up to 1.6 capsules per cm^2. Egg capsules are 2.0–3.0 mm (average 2.6 mm) in diameter, harbor ~90–200 embryos, and have a pink, yellow or transparent coloration.

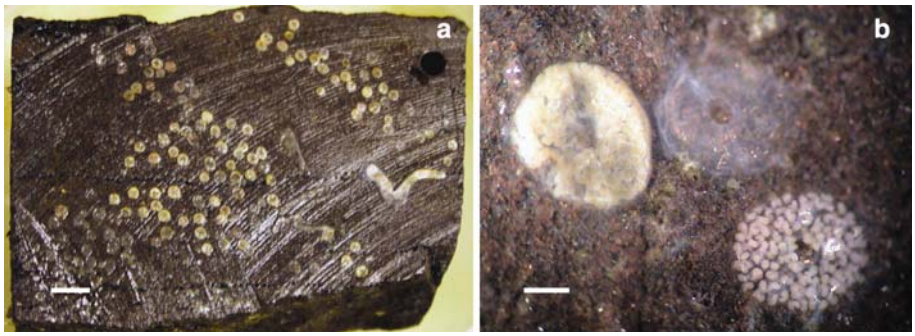

Fig. 7 Light micrographs of the lenticular egg capsules. **a** Egg capsules density deposited on a basalt block. The *arched striations* on the block are from cutting the blocks. *Scale bar* is 1 cm. **b** Close up of three egg capsules at different stages. The right case is *yellow* with yolky globular embryos inside. The empty middle capsule clearly shows the oval escape aperture from which the larvae escaped. The bottom right capsule is pinkish and contains developing larvae with bilobed vela but without fully developed protoconchs. *Scale bar* is 1 mm (color figure online)

Discussion

The staged approach to larval identification

Our results indicate that 33 of the 41 gastropod species inhabiting the northern EPR (NEPR) can now be identified to species at the larval stage using a combination of morphological and molecular techniques. This is nearly double the number of previously identifiable gastropod species at the larval stage. Twenty-six of the twenty-seven gastropod species known to occur specifically in the 9° 50′ N region can be identified to species, an increase of fifteen species. Only *Provanna ios* has no morphological or molecular information, due to scarce collection and poor preservation of the larval shell on juveniles and adults. New SEM protoconch descriptions of *Gorgoleptis spiralis*, *Echinopelta fistulosa* and *Nodopelta subnoda* increase the total of morphological protoconch descriptions for NEPR gastropods to 20 diagnostic to the species level, five diagnostic to the genus level, and two diagnostic to the family level. The RFLP assays allow for identification of five species within the genus *Lepetodrilus* and six species of peltospirids. 18S rDNA sequences for 20 species are available in GenBank, providing a 'barcode' with which to identify NEPR gastropod species at any stage.

Morphological and molecular techniques have advantages and disadvantages such that the combination of the two is better than either alone. The level of morphological identification in Table 1 is based on identification under a dissection and/or compound light microscope. Morphological identification under a light microscope requires little equipment and thus has a low direct cost. On average, more than 25 specimens can be identified in an hour. Specimens are not destroyed in the identification process.

Molecular identification techniques, though currently more costly and time consuming, contribute to new morphological descriptions and complement morphological identification techniques when morphology alone is insufficient. Molecular techniques require more specialized and expensive equipment and reagents. The procedure requires more steps, with each step ranging in time commitment from 15 min to 4 h. The longer steps do not require continuous labor and attention but make the entire process from sample to sequence or RFLP assay take 1–3+ days. Multiple samples can be processed during this time period. The use of RFLPs eliminates sequencing, which incurs a per sample cost, thus reducing the overall cost for identification of many samples. The restriction enzymes *Ssp*I and *Eco*RV are more expensive than *Dra*I; therefore, we suggest performing the *Dra*I digest for the peltospirids first and then performing an *Ssp*I and *Eco*RV digest only if necessary to distinguish among *Nodopelta* species. This will also prevent the potential for false identification of *Peltospira delicata* as *P. operculata*. *Peltospira* spp. are generally more common in adult collections than *Nodopelta* spp. at the 9° 50′ N area (TS and DA personal observation) and *Hirtopelta hirta* are not known from the 9° 50′ N area (Warén and Bouchet 2001); therefore, it is reasonable to predict that *Peltospira* spp. larvae, identifiable with the *Dra*I digestion alone, will be more common than other unknown peltospirids in the plankton.

Lepetodrilus spp. and the peltospirids are two groups of species that exemplify the need to combine molecular and morphological techniques. SEM imaging of unknown peltospirid and *Lepetodrilus* sp. larvae and additional *Lepetodrilus* spp. juveniles yielded no additional information about species-specific protoconch characters. The similarity between *Peltospira operculata* and *Nodopelta subnoda* protoconchs and among the *Lepetodrilus* spp. protoconchs in SEM images indicates that morphology is not, at present, a useful tool for identifying these species in the larval stage. Additional imaging of juvenile specimens of the unknown peltospirids could yield species-specific descriptions such as that for *Echinopelta fistulosa*; however, peltospirids were rare in the collections from multiple cruises screened in this study and, like other gastropods, have a high

occurrence of protoconch loss and damage. The available morphological information does, however, allow for designation into defined groups to facilitate effective RFLP assays.

Such genetic approaches may also be needed for identifications of early stages of the Caenogastropoda. In the present study, the egg capsules of one species of caenogastropod in the NEPR, *Gymnobela* sp. A, were identified to species and described morphologically following molecular identification. Other egg capsules and veligers have been described morphologically by Gustafson and colleagues (1991) but have not been definitively assigned to a species. The protoconch and teloconch of caenogastropods quickly corrode such that additional morphological descriptions from retained protoconchs are unlikely. *Gymnobela* sp. A has not yet been described as a species due to high levels of corrosion of examined specimens (Warén and Bouchet 2001). Even juveniles with intact protoconchs may not yield species-specific protoconch descriptions because descriptions of juvenile shells are also rare. Direct sequence comparison can help guide morphological descriptions of caenogastropods' and other gastropods' protoconchs by identifying juveniles, by identifying egg capsules containing developed veligers, and by directly identifying planktonic larvae.

Similarity between species and lack of descriptions are just some of the problems that prevent morphological identification. Specimens in an embryo, egg case, or trochophore stage, or with a damaged shell, may have no taxonomically informative morphology. These specimens can still be identified using genetics, as demonstrated in the present study by the identification of the under-developed *Gymnobela* sp. A veligers within egg cases.

Daily larval collections

Identification of larvae from sediment trap collections demonstrated the utility of the combined morphological and molecular approach, but also illustrated some remaining challenges. Larval collections varied daily in abundance and species composition (Table 2). The high abundance of Unknown Benthic sp. A and *?Laeviphitus* sp. is intriguing because the corresponding adults have not been found in the nearby benthos, or in the sequence database. Species of *Laeviphitus* have not been found on the EPR as adults, but the genus was originally described from larvae, and the PI and PII on larval specimens from this study and Mullineaux et al. (2005) closely resemble other *Laeviphitus* spp. larvae. *?Laeviphitus* larvae may exhibit high abundances near vents due to the increased food supply in the plankton but not reside at vents as adults. Unknown Benthic sp. A does not have PII growth suggesting a non-feeding larval form, so increased food supply does not explain the high abundances for this morpho-species. Alternatively, adults of *?Laeviphitus* and Unknown Benthic sp. A may be present in the vent periphery which is not well sampled or be from the surrounding non-vent habitat.

Difficulties in DNA extraction prevented the identification of one unknown peltospirid (1 of 2) and three *Lepetodrilus* spp. (3 of 3). The identified *Peltospira operculata* and a *Lepetodrilus* were extracted within 3 months of collection, whereas attempts to extract DNA from the other larvae occurred >6 months after collection. DNA could have been too degraded after 6 months to successfully amplify by PCR. Extractions of *Lepetodrilus* spp. may not have been successful, even within 3 months, due to their relative small size. DNA was successfully extracted from larger larvae (>240 µm; see Table 1), such as *Cyathermia naticoides* (1 of 1) and *?Laeviphitus* sp. (1 of 1) up to 6 months after collection. The 20% DMSO-saturated salt solution was chosen for this experiment due to its successful application in a hydrothermal vent setting (Comtet et al. 2000) and its success in a study comparing preservation methods for other marine invertebrates (Dawson et al. 1998). The use of sediment traps limited the preservatives available to us, as the preservative needed to be heavier than seawater. Alternative preservatives, such as ethanol (Sawada et al. 2008), sampling techniques, such as plankton pumps, and minimizing the time between preservation and analysis could yield sufficient amounts of high-quality DNA for identification of unknown larvae using RFLP and direct sequence comparisons.

Egg capsules

The lenticular egg capsules (Fig. 7) were identified molecularly to belong to *Gymnobela* sp. A. Sequences from the egg capsules and *Gymnobela* sp. A differed from *Phymorhynchus major* by 1 bp (Fig. S4). The habitat in which the egg capsules were collected is consistent with the typical adult distribution of *Gymnobela* sp. A. *Gymnobela* sp. A have been collected in mussel aggregations near active venting where the egg capsules were found (DA and TS unpublished data). Blocks placed in the periphery, where *Phymorhynchus major* has been predominantly observed, did not contain any lenticular egg capsules. Additionally, the 6-mm-mesh cages would have prevented larger gastropods, like *Phymorhynchus major* (up to 72 mm) (Warén and Bouchet 2001), from entering and depositing eggs. The smaller size of *Gymnobela* sp. A, 12 mm maximum length (Warén and Bouchet 2001), would allow the gastropod to enter the cages and is consistent with the size of the egg capsules. *Phymorhynchus* sp. is believed to deposit large, 14–16 mm diameter, lenticular egg capsules found on the Galápagos Rift (Gustafson et al. 1991). The egg capsules have similar shapes which supports the close

phylogenetic relationship between the two species, but the different sizes and adult distributions suggest that the egg capsules collected on the basalt blocks belonged to *Gymnobela* sp. A.

Identification of the *Gymnobela* sp. A egg capsules serves as an example of how molecular identification contributes to our understanding of life histories and the ecology of vent gastropods. *Gymnobela* sp. A is a species for which little life-history data were previously known due to poor preservation of larval and juvenile shells on adult specimens. This early life-history information allows us to compare *Gymnobela* sp. A to other gastropod species with different larval dispersal potential, i.e., planktotrophic larvae and non-planktotrophic, lecithotrophic larvae. Comparisons of the population genetics, benthic ecology and larval supply at the species level for species with different life histories may provide additional insights into the role of larval dispersal in structuring benthic communities.

Application of molecular techniques is likely to be especially important for identifying larvae of species for which culturing is difficult, such as other hydrothermal vent species (not just gastropods), deep-sea species, and some polar species. However, coastal species may also require a combined molecular and morphological approach to yield species-specific identifications for closely related species (Pardo et al. 2009). Ideally, initial sequence comparisons would yield species-level identifications and new species-specific taxonomical descriptions, as exemplified here with the identification of the *Gymnobela* sp. A egg capsules. However, even after initial identification there may not be sufficient differences in morphological characteristics between closely related species to morphologically identify all larvae to the species level. We would then recommend application of our staged approach to identify a maximum number of species in an efficient and economical manner.

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