

DNA BARCODING OF *LEPETODRILUS* LIMPETS REVEALS CRYPTIC SPECIES

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ABSTRACT Lepetodrilid limpets are common inhabitants of deep-sea hydrothermal vents worldwide, but the frequent occurrence of morphologically cryptic species makes their identification very difficult. To facilitate these identifications, we provide DNA barcodes based on ~1,000 bp of cytochrome-*c*-oxidase subunit I (*COI*), for 20 taxa within the genus *Lepetodrilus*. The method was also used to identify lepetodrilids that were found living on vent decapods. A preliminary phylogenetic analysis resolved relationships among members of several cryptic species complexes; however, *COI* sequences alone were unable to resolve higher-level systematic relationships caused by saturation of synonymous nucleotide substitutions.

KEY WORDS: vetigastropoda, *Lepetodrilus*, mitochondria, cytochrome-*c*-oxidase subunit I, phylogeny, DNA barcoding

INTRODUCTION

More than 600 new species of animals have been described since the discovery of chemosynthetic environments at deep-sea hydrothermal vents and hydrocarbon seeps (Sibuet & Olu 1998, Tunnicliffe et al. 1998), yet biogeographical studies are hampered by a lack of information on species' distributions. A considerable part of the vent and seep faunas is known from a single site and major portions of the world's oceans remain unsampled, not because they lack chemosynthetic environments, but because they lie at latitudes that preclude exploration with human occupied or robotic submersibles. Deep-sea exploration is difficult, risky and expensive even at midlatitudes, so geographic samples rarely provide comprehensive size-series or the variety of life-stages needed to adequately assess species' identities and distributions. Furthermore, traditional systematic methods that only rely on morphological species descriptions (morphospecies) may be prone to errors for several reasons. First, species ranges may be overestimated, because putatively widespread marine species actually consist of morphologically cryptic species, a problem commonly encountered with deep-sea molluscs described from shell morphology alone (Vrijenhoek et al. 1994, Peek et al. 1997, Goffredi et al. 2003, Kojima et al. 2004, Johnson et al. 2006, Matabos et al. 2007). On the other hand, geographical ranges may be underestimated in other taxa, because some researchers are prone to describing each morphological variant as a distinct species, ignoring the possibility that morphotypes might represent different ontogenetic stages, sexes, or ecotypes of a phenotypically plastic species. For example, juvenile and adult stages of the mid-Atlantic vent shrimp, *Rimicaris exoculata* Williams & Rona, 1986, were variously described as distinct genera and species, until molecular studies clearly linked the life stages (Shank et al. 1998). Discrete morphospecies of siboglinid tubeworms from eastern Pacific vents were found on examination with molecular markers to be developmentally plastic ecophenotypes (Black et al. 1994, Southward et al. 1995, Carney et al. 2002). Given the problems with morphospecies identifications it is difficult to assess what we really know about biodiversity and biogeography of chemosynthetic environments.

DNA-barcoding (Hebert et al. 2003) offers considerable promise for solving some of the problems associated with traditional biological identifications, because it can unmask cryptic species, link distinct life stages or dimorphic sexes, identify partial specimens, and provide a cost-effective means for species identifications by nonspecialists. An approximate 700 base pair (bp) region at the 5' end of mitochondrial cytochrome-*c*-oxidase subunit I gene (*COI-5*) has proved to be a useful barcoding marker for invertebrate animals (Hebert et al. 2003, Neigel et al. 2007) because "universal" invertebrate primers are available (Folmer et al. 1994). *COI-5* is highly conserved at the amino acid level, allowing for easy sequence alignments among distantly related taxa, whereas synonymous nucleotide substitutions at degenerate codon positions provide variation within and among closely related species. Currently, several hundred thousand *COI-5* sequences from diverse animal taxa have been deposited in international DNA databases such as GenBank, and the Barcode of Life Data Systems (BOLD; <http://www.boldsystems.org>) lists more than 30,000 described species that have been barcoded (Ratnasingham & Hebert 2007), including many molluscs.

We applied *COI* DNA barcoding to deep-sea lepetodrilid limpets (Gastropoda: Lepetodrilioidea), which are the most abundant and diverse gastropod group at hydrothermal vents worldwide. Experts have recognized 14 nominal species (Warén & Bouchet 2001, Desbruyères et al. 2006), but genetic studies have begun to reveal geographically widespread complexes involving cryptic species that have syntopic or allopatric distributions (Beck 1993, Craddock et al. 1997, Johnson et al. 2006, Matabos et al. 2007). The present analysis involves a subsampling of *COI* sequences, mostly from collections that we (RCV) have made during the past 20 y of deep-sea exploration. We have now obtained *COI* barcodes from several hundred specimens representing 12 of the 14 nominal species. Unfortunately, two species were unavailable for our analysis because descriptions were based on single specimens that are now held as holotypes (probably fixed in formalin). Here we report *COI* barcodes for 20 "molecular operational taxonomic units" (MOTUs, Blaxter et al. 2005) that are currently nested within these nominal species. We do not attempt at this time to resolve the species status of MOTUs. Alpha-level taxonomic studies are currently underway in our laboratories, but they require characterization of MOTUs with more comprehensive geographic samples, additional genetic markers, and detailed morphological analyses.

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METHODS

Specimen Collections

Limpets were collected with manned and unmanned submersibles during oceanographic expeditions that spanned 1988 to 2007 and visited numerous hydrothermal vent, seep, wood-fall, and whale-fall localities worldwide (Fig. 1, Table 1). These tiny gastropods were typically sampled as by-catch with the larger vent taxa, primarily bivalve molluscs and vestimentiferan polychaetes, on which the limpets reside. Others were collected with geological specimens. At two localities, 38°S on the Pacific-Antarctic Ridge (PAR) and the North Fiji Basin, we found the limpets attached to bythograeid crab telsons that we had collected (Fig. 2). Once aboard the surface vessel, limpets were immediately frozen whole at -80°C or preserved in 95% ethanol. Frozen samples were transported on dry ice to the land-based laboratory and subsequently stored at -80°C .

Molecular Methods

For most specimens, genomic DNA was isolated with the Qiagen DNeasy DNA extraction kit (Qiagen Inc., Valencia, CA) from frozen or ethanol-preserved soft tissues. An approximate 1,200-bp portion of mitochondrial cytochrome-*c*-oxidase subunit I (*COI*) was amplified with invertebrate primers (Nelson & Fisher 2000). PCR was conducted in a 25- μl solution that included 30–100 ng of template DNA, 2.5 μl of $\times 1$ of PCR buffer (supplied by manufacturer), 2.5 μl of 2.5 μM MgCl_2 , 1 μl of each primer (10 μM final conc.), 2.5 units *Taq* polymerase (AmpliQ Gold, Applied Biosystems Inc., Foster, CA), 2.5 μl of 2 mM stock solution of dNTPs, and sterile water to final volume and occurred with a Cetus 9600 DNA Thermal Cycler (Perkin-Elmer Corp. CT), used an initial denaturation of $95^{\circ}\text{C}/10$ min, followed by $35 \times (94^{\circ}\text{C}/1$ min, $55^{\circ}\text{C}/1$ min, and $72^{\circ}\text{C}/1$ min), and a final extension at $72^{\circ}\text{C}/7$ min. PCR products were diluted in 40- μl sterile water and purified with Multiscreen HTS PCR 96 vacuum manifold system (Millipore Corp. Billerica, MA). An ABI 3,100 capillary sequencer and BigDye terminator v. 3.1 chemistry (Applied Biosystems Inc., Foster, CA) were used to sequence amplicons bidirectionally with the same primers used for PCR. DNA sequences were proofread and aligned using Sequencher v. 4.7 (Gene Codes Corp. Inc., Ann Arbor, MI) and edited by eye using MacClade v. 4.08 (Maddison & Maddison 2005). Unique sequences were deposited in GenBank and BOLD.

Statistical Methods

Statistical analyses of DNA diversity were conducted using Arlequin (v. 3.1, Excoffier et al. 2005) and DnaSP (Rozas et al. 2003). The program DAMBE (Xia & Xie 2001) was used to test for saturation among the Lepetodrilidae (Fig. 3). Parsimony networks were constructed using the program TCS v. 1.21 (Clement et al. 2000) with a connection limit of 95% and redrawn in Adobe Illustrator CS v. 11.0.0.

Phylogenetic analyses were conducted with the Mr. Bayes v. 3.1.3 program (Huelsenbeck & Ronquist 2001). Appropriate substitution models for *COI* were determined with standard procedures in PAUP (Swofford 1993) using Mr. Model Test (www.ebc.uu.se/systzoo/staff/nylander) and the Akaike information criterion (AIC) (Akaike 1974). Bayesian analyses

involving six chains were partitioned for each codon position, run for at least 50 million generations with a printing, sampling frequency, and burn-in period of 1,000. Analyses were run five times each and data were visualized using Tracer v. 1.3 (Rambaut & Drummond 2003) to determine the appropriate burn-in period and ensure data had reached convergence. Trees were visualized using FigTree V.1.0 (<http://tree.bio.ed.ac.uk/software/figtree/>). All nodes with less than 0.95 posterior probability support were collapsed to basal polytomys.

Gauging MOTUs

To assess the potential evolutionary significance of *COI* divergence, we refer to a prior study of the *L. fucensis* species complex from northeastern Pacific ridge systems (Johnson et al. 2006). DNA sequences including the *COI-5* region clearly revealed that *Lepetodrilus fucensis* sensu lato comprises a pair of sister-species separated by the 450-km-long Blanco Fracture Zone—*L. fucensis* sensu stricto from the Explorer and Juan de Fuca Ridges and *Lepetodrilus gordensis* from Gorda Ridge. *COI* divergence of 7.3% between the sister-species is reflected in divergent DNA sequences for a nuclear gene, in different allozyme frequencies, and in external soft part morphology. The maximum *COI* divergence within either species was less than 1.0%. These values are typical for recognized sister-species of other deep-sea invertebrates including bivalves, siboglinid tubeworms, and decapod crustaceans. Interspecific *COI* divergence in general exceeds 4%, whereas intraspecific divergence typically is less than 2% (Peek et al. 1997, Shank et al. 1999, Guinot et al. 2002, Goffredi et al. 2003, Guinot & Hurtado 2003, Kojima et al. 2003, Rouse et al. 2004, Jones et al. 2006). Though we do not recognize a particular degree of *COI* divergence alone as a reliable index of inter vs. intraspecific divergence, these values provide a guide for the present recognition of MOTUs and consequently indicate the need for additional alpha-level systematic studies.

RESULTS

During the course of our studies, we have sequenced 405 lepetodrilid specimens from 32 localities worldwide. Here we report *COI* sequences that represent 20 MOTUs (GenBank accession #'s EU306388-484). We have restricted the present analysis to a maximum of six individuals (when available) from each MOTU. The six sequences were chosen to represent the maximum diversity within each MOTU, as reflected across its sampled geographical range and in the depth of its internal nodes.

We identified 20 MOTUs within the genus *Lepetodrilus*. Pairwise distances within MOTUs ranged from 0.10% to 1.34%. Pairwise distances among MOTUs ranged from 3.01% to 31.25%. The majority of *Lepetodrilus* MOTUs differed by about 20% (Fig. 4). The most divergent taxon (average $d = 30.01\%$) was “*Lepetodrilus*” sp. 2 CIR from the Central Indian Ocean. However, its distance value was greater than the average distances to the outgroup, *Pseudorimula* aff. *marianensis* McLean, 1993, from the Fiji Basin (average $d = 26.03\%$).

Phylogenetic analyses revealed several distinct cryptic species complexes within the genus *Lepetodrilus*. High support (Bayesian Posterior Probabilities = 1.0) existed for many internal nodes, yet the deeper nodes were poorly resolved.

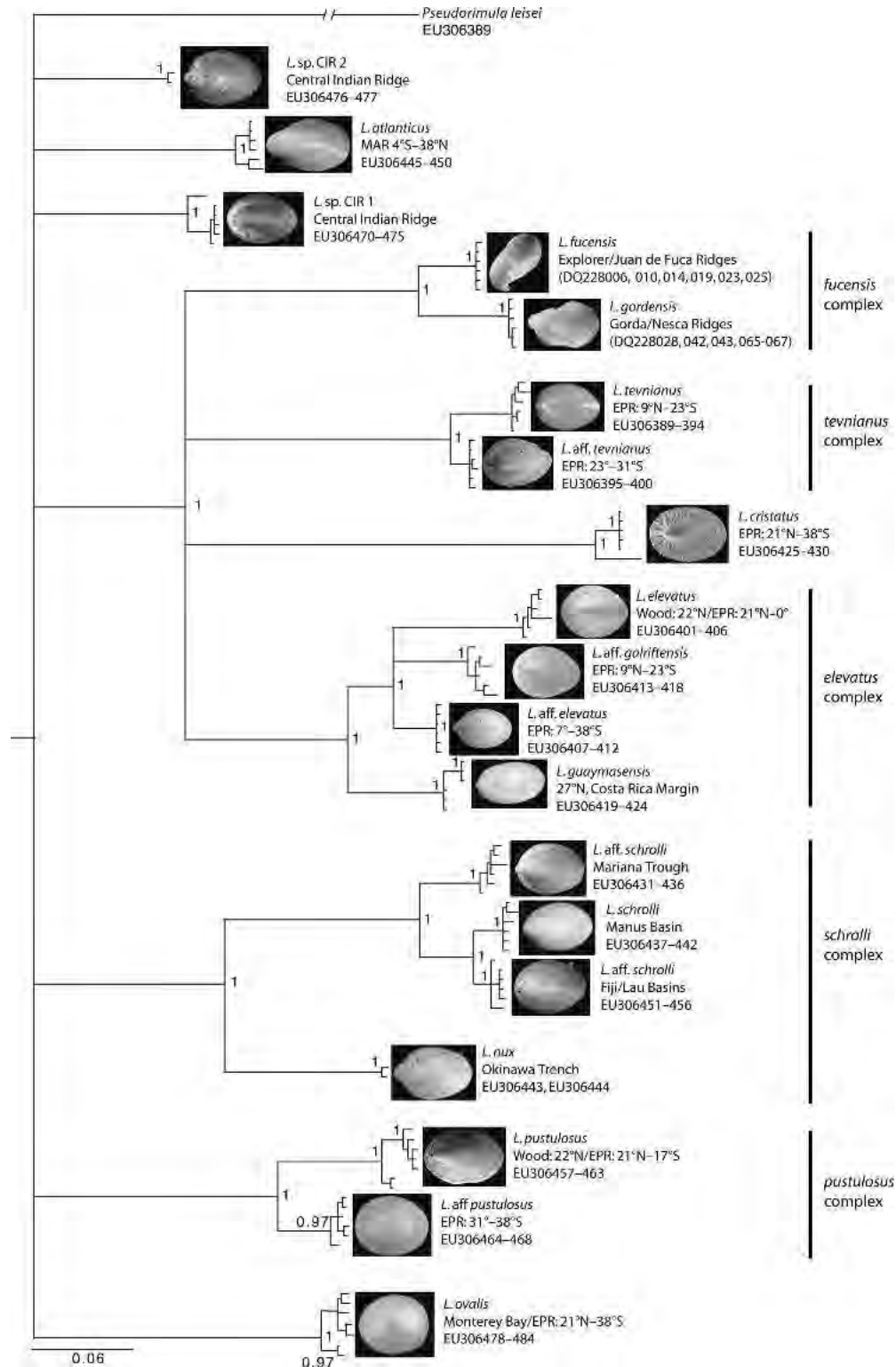


Figure 1. General-time-reversible (GTR + SS) Bayesian tree of lepetodrilid *COI* sequences with corresponding localities and GenBank accession numbers. Nodes with a posterior probability less than 0.95 were collapsed as polytomies. A vertical black bar highlights species complexes. Scale bar indicates % sequence divergence.

Saturation plots generated for *COI* revealed that transitions became saturated after 15% to 20% sequence divergence; however, transversions were not saturated (Fig. 3). For graphical purposes, we collapsed poorly resolved nodes (BPP < 0.95)

into polytomies. We are currently developing a multilocus phylogeny that includes *COI* and several nuclear genes to resolve higher relationships among these MOTUs (Johnson, Warén, and Vrijenhoek, in prep).

TABLE 1.
Lepetodrilid samples.

Region/location	Dive Numbers*	Latitude	Longitude	Depth (m)
Northeast Pacific Ridges (NEP)				
Explorer Ridge	R669	49.761	-130.257	1,798
Endeavor Ridge	J059	47.933	-129.100	2,265
Explorer Ridge	R590	47.952	-129.080	2,195
Axial Volcano	R623	45.916	-129.986	1,524
Cleft Segment	T180	44.658	-130.363	2,211
Cleft Segment	T184	44.990	-130.201	2,238
Gorda Ridge	T186, T188	42.755	-126.708	2,716
NESCA	T452	41.001	-127.495	3,222
Monterey Bay, CA	T609	36.614	-121.436	2,897
East Pacific Rise (EPR)				
27°N	T548	27.577	-111.450	1,778
22°N	T558	22.837	-108.118	3,139
21°N	T556	20.784	-109.149	2,549
13°N	A2228-A2227	12.802	-103.935	2,636
11°N	A2226-A2225	11.403	-103.783	2,516
9°N	A3540	9.828	-104.283	2,516
9°N	A2502	9.786	-104.269	2,518
9°N	A2358	9.503	-104.235	2,578
0°N	A2023-A2024	0.800	-86.217	2,460
7°S	A3322	-7.367	-104.460	2,737
11°S	A3323	-11.306	-110.540	2,791
17°S	A3330	-17.569	-113.250	2,599
23°S	A4097	-23.828	-115.459	2,649
31°S	A4094	-31.151	-111.919	2,237
32°S	A4092	-31.852	-112.048	2,334
Pacific-Antarctic Ridge (PAR)				
38°S	A4088-A4091	-37.799	-110.903	2,236
Western Pacific Back Arc Basins				
Kilo Moana (Lau)	J140	-20.055	-173.903	2,626
Tui Malila (Lau)	J144	-21.991	-176.568	1,900
Hine Hina (Lau)	J145	-22.519	-176.719	1,820
White Lady (N. Fiji)	J150	-16.996	-173.903	1,990
Manus Basin	PC7	-3.809	152.105	1,530
Okinawa Trough	S1371	24.850	123.833	1,473
Mariana Trough	S140-144	18.202	144.701	3,589
Central Indian Ridge (CIR)				
Edmund vent field	JL296-JL297	-23.883	69.597	2,432
Mid-Atlantic Ridge (MAR)				
Golden Valley	M68/1	-4.480	-12.220	2,994
Menez Gwen	A3117	37.833	-31.517	869
Costa Rica margin	FS M/22	8.983	-84.717	1,917

* Dive numbers are labeled: R = *Ropus*; A = *Alvin*; J = *Jason II*, T = *Tiburion S* = *Shinkai*, JL = *Jason I*, M = *Meteor*.

Cryptic Species Complexes

The present COI sequences revealed several cryptic complexes involving two or more MOTUs (Fig. 1). We treat them individually here.

The *Lepetodrilus tevnianus* Complex

Lepetodrilids identified initially as *L. tevnianus* McLean, 1993 occurred at East Pacific Rise (EPR) vents from 9°N to 31°S (Table 2). The complex contained two MOTUs ($d = 4.32\%$) subdivided by the Easter Microplate. Because the type



Figure 2. *Lepetodrilus* aff. *pustulosus* on the telson of a bythograeid crab collected at 38°S on the Pacific Antarctic Ridge.

locality for *L. tevnianus* is 11°N, we attribute the northern lineage to *L. tevnianus* sensu stricto (s.s.) (range: 11°N to 23°S EPR) and temporarily refer to the southern lineage as *L. aff. tevnianus* (range: 23° to 31°S EPR). Though females of the two MOTUs are similar, the penis of *L. tevnianus* s.s. males is short and blunt whereas the penis of *L. aff. tevnianus* males is longer and more pointed. In addition, *L. aff. tevnianus* has a well-developed sensory papillus on its neck and stronger dorsal ridge on its shell than *L. tevnianus* s.s.

The *Lepetodrilus pustulosus* Complex

Lepetodrilids identified initially as *L. pustulosus* McLean, 1988, occurred on wood found at 22°N on the EPR and at vents from 21°N to 23°S, the Galapagos Rift (GAR), and Pacific

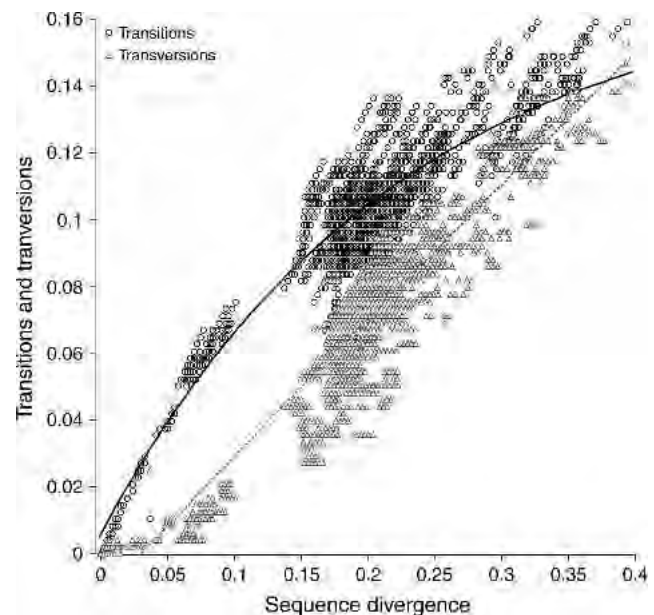


Figure 3. Transitions and transversions (Y-axis) versus sequence divergence (Fitch 84) for limpets included in COI phylogeny.

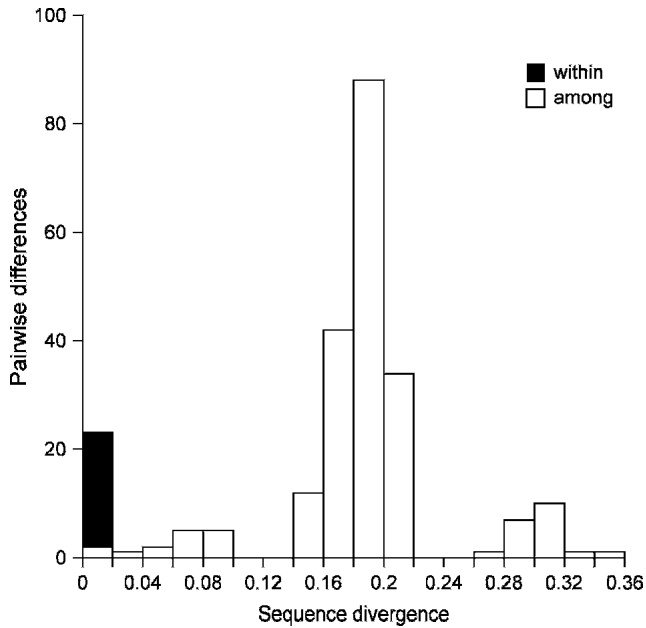


Figure 4. Within and among species sequence divergence (Kimura-2-parameter) for lepetodrilids in *COI* phylogeny.

Antarctic Ridge (PAR) from 31° to 38°S (Table 2). This complex is comprised of two distinct MOTUs ($d = 8.17\%$) subdivided by the Easter Microplate. Because the type locality for *L. pustulosus* is the Galapagos Rift, we attribute the northern lineage to *L. pustulosus* s.s. (range: 22°N to 23°S EPR) and the southern lineage to *L. aff. pustulosus* (range: 31°S EPR to 38°S PAR). *L. pustulosus* s.s. can be further subdivided into two MOTUs that are separated by the Equator ($d = 1.7\%$) and differ somewhat in shell morphology, with the northern form having larger and more pronounced pustules on its shell. In addition, all *L. pustulosus* s.s. found north of the Easter Microplate have an apomorphic penis, long and slender with a sperm gutter along its side, kept in a coil and ending with a small funnel-like point. Specimens of *L. aff. pustulosus* from south of the Easter Microplate have a penis that does not form a complete coil and is about 2.5 times as long as broad.

The *Lepetodrilus elevatus* Complex

The complex includes four MOTUs (Table 2): *Lepetodrilus elevatus* s.s. (EPR 22°N to 0°); *L. aff. elevatus* (EPR 7°S to 38°S PAR); *Lepetodrilus aff. galriftensis* (EPR 9°N to 23°S); and *Lepetodrilus guaymasensis* (Guaymas Basin 27°N and Costa Rica Margin Seeps). *COI* distances among MOTUs range from 5.9% to 9.3%. The type specimen for *L. elevatus* McLean, 1988, was from 21°N, so we attribute the northern lineage (EPR 22°N to 0° GAR) to *L. elevatus* s.s. and a distinct southern lineage (EPR 7°S to 38°S PAR) to *L. aff. elevatus*. The third MOTU was originally described as a morphologically distinct subspecies, *L. e. galriftensis*, from the Galapagos Rift, based on specimens that had a slightly lower apex (McLean 1988). Craddock et al. (1997) reported a genetically distinct *elevatus*-like taxon that occurred at 9°N to 13°N along the EPR, sympatrically with *L. elevatus* s.s. Because it was not found at 21°N, the type locality for *L. elevatus* s.s., they attributed the second taxon to *L. e. galriftensis* and recommended that it be

recognized as a distinct species, *L. galriftensis*, that did not hybridize with sympatric *L. elevatus* s.s. We have matched the allozyme patterns reported by Craddock et al. (1997) with corresponding *COI* sequences and concur with their recognition of *L. galriftensis* and *L. elevatus* s.s. (details will be reported in a separate phylogeographic study of this complex; Johnson et al. in progress). We have also found that the slightly flatter shells attributed by McLean (1988) to *L. e. galriftensis* are not diagnostic of the two species as did Matabos et al. (2007). Also the soft part morphology seems indistinguishable. The fourth MOTU, *L. guaymasensis* (McLean 1988), inhabits deeply sedimented hydrothermal vents at 27°N in the Gulf of California and cold seeps off Costa Rica (8°S). Shell structures of the Costa Rica limpets differ morphologically from Guaymas Basin limpets, but they occupy different habitats and lepetodrilids are known to be morphologically plastic.

The *Lepetodrilus schrolli* Complex

Lepetodrilids from western Pacific back arc basins were initially attributed to *L. elevatus* (Hessler & Lonsdale 1991, McLean 1993). Subsequently, Manus Basin specimens were described as *L. schrolli* Beck, 1993, and Okinawa Trough specimens as *Lepetodrilus nux* (Okutani et al. 1993). We identified four MOTUs among the present specimens. We attribute the Manus Basin specimens to *L. schrolli* s.s. and Okinawa Trough specimens to *L. nux*, the type localities for each species (Table 2). Two additional MOTUs were identified; (1) *L. aff. schrolli* MT from the Mariana Trough and (2) *L. aff. schrolli* LF from the Lau and N. Fiji basins. Sequence divergence among the four MOTUs exhibits a broad range: from $d = 3.1\%$ between *L. schrolli* s.s. and *L. aff. schrolli* LF to $d = 15.3\%$ between *L. nux* and *L. aff. schrolli* MT. Shell morphology is uniform throughout the group; however, *L. nux* has no sensory papillus, whereas *L. aff. schrolli* from the Mariana Trough does have a sensory papillus.

Several examples were found of widely distributed *Lepetodrilus* that did not exhibit geographical subdivision or species crypticism, including *Lepetodrilus atlanticus* from the Mid-Atlantic Rise (MAR, 37°N to 04°S) and *Lepetodrilus ovalis* from 36°N in Monterey Bay and EPR 21°N to 38°S PAR, and *Lepetodrilus cristatus* (EPR 21°N to 38°S PAR). Whereas limited subdivision exists across the Easter Microplate for both *L. ovalis* ($d = 1.24\%$) and *L. cristatus* ($d = 1.34\%$), it is within species-levels of diversity for the *COI* gene (Fig. 3). In addition, all three species are morphologically uniform throughout their ranges.

Crab Limpets

To demonstrate the usefulness of *COI* barcodes, we identified lepetodrilids found attached to the telsons of bythograeid crabs from two distant localities (Fig. 2). *COI* sequences from the Lau Basin crab limpets (average length = 6.34 ± 2.4 mm) belonged to *L. aff. schrolli* LF (GenBank accession #'s EU306485-504). Those from 38°S PAR (average length = 10.88 ± 4.09 mm) belonged to *L. aff. pustulosus* (GenBank accession #'s EU306505-517). To determine whether these limpets might be hatchlings from a single maternal egg plaque, we compared their sequences with those of a comparable sample from the local vent population. In both cases, the crab limpets exhibited a diversity of *COI* haplotypes, indicating that

TABLE 2.
Lepetodrilus species and MOTUs discerned in this study.

<i>Lepetodrilus</i> spp.	Author	Year	Known Range	Type Locality	Hab.*	Known Depths (m)
<i>atlanticus</i>	Warén & Bouchet	2001	MAR 38°N–4°S	Menez Gwen	V	850–3,500
<i>corrugatus</i> **	McLean	1993	JdF	Middle Valley, JdF†	V	2,400
<i>cristatus</i>	McLean	1988	EPR 21°N–38°S PAR	EPR 21°N	V	2,200–2,800
<i>elevatus</i> s.s.	McLean	1988	EPR 22°N–17°S, GAR	EPR 21°N	VW	2,400–2,700
aff. <i>elevatus</i>	undescribed		EPR 7–38°S		V	2,400–2,700
<i>guaymasensis</i>	McLean	1988	Guaymas Basin, Costa Rica margin	Guaymas Basin	VS	2,000
<i>fucensis</i>	McLean	1988	JdF and Explorer ridges	Endeavor Segment, JdF	V	1,500–2,200
<i>gabriftensis</i>	McLean	1988	EPR, GAR	GAR	V	2,200–2,800
<i>gordensis</i>	Johnson et al.	2006	Gorda Ridge	Gorda Ridge	V	2,500–2,600
<i>japonicus</i> **	Okutani et al.	1993	Okinawa Back Arc Basin	Southern Okinawa Trough†	V	700
<i>nux</i>	Okutani et al.	1993	Okinawa Trough	Okinawa Trough Iheya Ridge	V	990–1,390
<i>ovalis</i>	McLean	1988	EPR 21°N–38°S PAR, Monterey Bay, CA	EPR 21°N	VC	2,200–3,100
<i>pustulosus</i> s.s.	McLean	1988	EPR 21°N–21°S	GAR	VW	2,200–2,800
aff. <i>pustulosus</i>	undescribed		EPR 31°S–38°S PAR		V	2,200
<i>schrolli</i> s.s.	Beck	1993	Manus Basin	Manus Basin	V	2,500–2,600
aff. <i>schrolli</i> MT	undescribed		Mariana Trough		V	3,600
aff. <i>schrolli</i> LF	undescribed		Lau and N. Fiji basins		V	1,820–2,650
sp. WA**	Warén & Bouchet	2008	West Africa	Congo River, Regab site	S	3,150
<i>tevnianus</i> s.s.	McLean	1993	9°N–7°S	EPR 11°N	V	2,200–2,800
aff. <i>tevnianus</i>	undescribed		23–31°S		V	2,200–2,700
sp. CIR-1	undescribed		Central Indian Ridge		V	2,400
sp. CIR-2	undescribed		Central Indian Ridge		V	2,400
sp. FL **	undescribed†		Florida Escarpment		S	3,300
sp. CR **	undescribed†		Costa Rica margin		S	1,900
sp. GOM **	undescribed†		Gulf of Mexico, Atwater Canyon		S	2,198

* Habitats: V = vent; S = seep; W = wood; C = whale carcass.

** Specimens not available for barcoding analysis.

† Known or described from a single specimen.

single mothers had not produced them (Table 3, Fig. 5). From the haplotypic networks, the crab limpets appeared to represent random draws from the local population. No significant differentiation existed between the crab limpets and their local relatives (Lau $F_{ST} = -0.0055$, $P = 0.586$; 38°S PAR $F_{ST} = 0.0026$, $P = 0.423$).

DISCUSSION

DNA barcoding provided a valuable tool for distinguishing among *Lepetodrilus* lineages and defining molecular operational taxonomic units (MOTUs). Twelve described morpho-species, for which we had suitable samples, clearly represent distinct evolutionary lineages. Assuming a molecular clock previously calibrated for *COI* in lepetodrilids, the most closely related of the named species, *L. fucensis* s.s. and *L. gordensis*, differ by about 7% for *COI*, which translates into a most recent common ancestor that existed several million years ago (Johnson et al. 2006). Yet many of named morphospecies contained cryptic MOTUs with comparable or greater levels of evolutionary divergence. Consequently, our preliminary phylogenetic analysis of *COI* sequences identified 5 morphologically cryptic

“species” complexes: (1) the *pustulosus* complex includes two cryptic MOTUs that differ by 8%; (2) the *schrolli* complex includes three MOTUs that differ from 3% to 15%; (3) the *elevatus* complex includes four MOTUs that differ from 5% to 9%; (4) the *tevnianus* complex includes two MOTUs that differ by 4%; and (5) as previously mentioned, the *fucensis* complex is composed of two recently named species, *L. fucensis* s.s. and *L. gordensis* (Johnson et al. 2006). For the present purposes, we do not equate a particular level of *COI* divergence with “species” status, although *COI* divergence typically is $\geq 4\%$ between recognized sister-species of deep-sea bivalves (Vesicomidae and bathymodiolin mytilids) and decapod crustaceans (Bythogeraeidae); whereas intraspecific divergence is invariably $< 2\%$ (Peek et al. 1997, Shank et al. 1999, Guinot et al. 2002, Goffredi et al. 2003, Guinot & Hurtado 2003, Won et al. 2003). We leave the formal resolution of these cryptic species complexes to subsequent studies that survey multiple independent genes from more comprehensive geographical samples (Johnson, Warén and Vrijenhoek, in prep). The present *COI* sequences and images have been deposited in the Barcode of Life (BOLD) system, where we hope they facilitate future identifications of these relatively featureless deep-sea gastropods. Consequently,

TABLE 3.
Genetic variation in *Lepetodrilus* aff. *pustulosus* and *L.* aff. *schrolli* populations. Error estimates (one SD) in parentheses.

Parameter*	<i>L. aff. pustulosus</i>		<i>L. aff. schrolli</i>	
	38°S	Crab	Lau	Crab
<i>N</i>	12	31	10	24
<i>H</i>	8	11	8	20
<i>k</i>	19	20	14	48
<i>h</i>	0.9242 (0.0575)	0.8086 (0.0498)	0.9333 (0.0077)	0.9746 (0.0238)
π	0.00572 (0.00331)	0.00539 (0.00296)	0.00536 (0.00324)	0.00877 (0.00472)

* *N* = sample size per locus; *H* = number of haplotypes; *k* = number of polymorphic sites; *h* = haplotype diversity; π = nucleotide diversity.

we urge scientists who collect these animals to preserve representative specimens or subsamples in 95% ethanol to allow identifications *via* DNA barcoding.

More comprehensive phylogeographic studies of these *Lepetodrilus* species complexes are underway in our laboratories (see also Johnson et al. 2006, Matabos et al. 2007). Here we

briefly summarize our new findings regarding geographical subdivision within and among *Lepetodrilus* MOTUs and compare them with published information on other deep-sea vent taxa. The *L. pustulosus* and *L. tevnianus* complexes have highly divergent MOTUs that split across the Easter Microplate region of the southern East Pacific Rise. This region of inflated topography presents a known boundary for sister-species pairs of vent decapods and bathymodiolin mytilids (Guinot et al. 2002, Guinot & Hurtado 2003, Won et al. 2003), but not for some vent polychaetes (Hurtado et al. 2004). Likewise, two limpet species, *L. cristatus* and *L. ovalis*, traverse this boundary with limited impedance to gene flow. The Equator coincides with a significant dispersal barrier for the vent palm worm *Alvinella pompejana* (Desbruyères & Laubier 1980, Hurtado et al. 2004) and we observed similar separation of *L. elevatus* s.s. and *L.* aff. *elevatus*, whereas *L. galrifiensis* crosses the boundary with no apparent restriction to gene flow. All lepetodrilids share essentially similar protoconch morphologies and size (170–200- μ m diameter), which implies lecithrotrophic larvae and a free-swimming nonfeeding stage, and hence similar dispersal potentials (Lutz et al. 1984). Nevertheless, as previously recognized (Craddock et al. 1997), different species within and among these species complexes do not necessarily share identical patterns of population structure or dispersal capabilities.

Lepetodrilids have previously been considered vent-endemic animals (McLean 1988, Warén & Bouchet 1993). Nevertheless, several of the taxa reported here were sampled from other deep-sea environments (Table 2). *Lepetodrilus guaymasensis*, a native of hydrothermal vents in the Gulf of California, was sampled from cold seeps off the Costa Rica margin, and *Lepetodrilus* n.sp. WA Warén & Bouchet, submitted, was sampled from cold seeps along the West African margin. Three additional unnamed species are known from seeps off Costa Rica, the Florida Escarpment and the Gulf of Mexico (Table 2).

Lepetodrilus elevatus s.s. and *L. pustulosus* s.s. were found on sunken wood sampled from 22°N on the East Pacific Rise. *Lepetodrilus ovalis*, a species previously known from vents between 21°N on the EPR and 38°S on the PAR, was found on whalebones collected in Monterey Bay, CA, at 36°N. Clearly, lepetodrilids are more versatile ecologically than previously believed.

Though lepetodrilids typically live on hard substrates (e.g., rocks, molluscan shells, and vestimentiferan tubes), on two occasions we found them attached in great numbers to the telsons of common bythograeid crabs. DNA barcoding identified these crab-limpets as conspecific with corresponding local populations of *L. aff. schrolli* LF at Lau Basin vents in the western Pacific and *L. aff. pustulosus* at 38°S PAR vents in the eastern Pacific. Additional analyses revealed that each crab-limpet sample was composed of genetically unrelated individuals, which as a group comprised a random draw of mitochondrial haplotypes from the local vent population. The highly mobile crabs might transport limpets among neighboring vent fields, but long-distance dispersal seems unlikely by this means. Alternatively, the crabs may provide little more than another hard surface on which the limpets can graze.

Despite the demonstrated utility of DNA barcoding for identifying cryptic species of *Lepetodrilus*, the *COI* gene was of limited use for inferring hierarchical relationships among

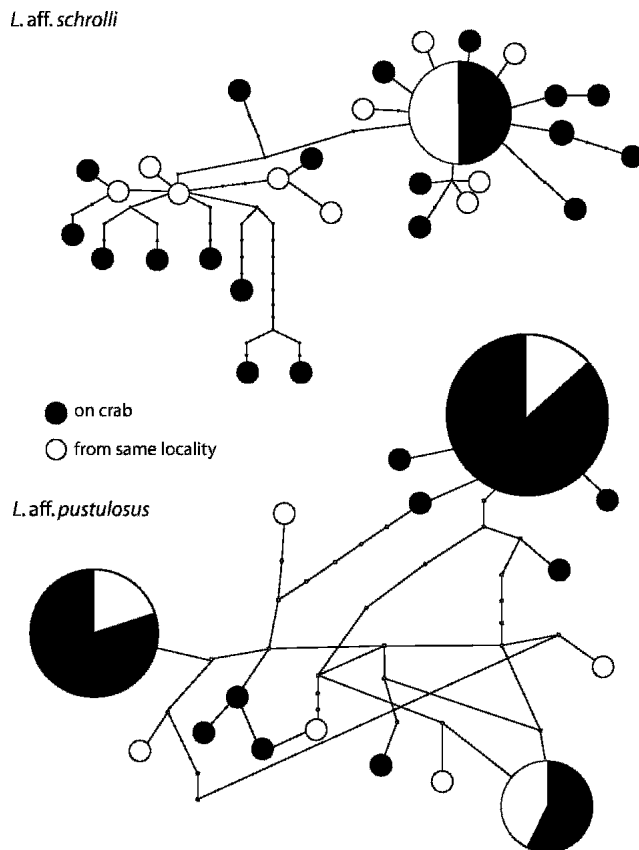


Figure 5. 95% parsimony network for *Lepetodrilus* aff. *schrolli* from the Lau basin and *Lepetodrilus* aff. *pustulosus* from PAR 38°S. Diameter of circle increases as number of haplotypes increase. Black and white areas indicates frequency of limpet haplotypes found on bythograeid crab versus surrounding localities, respectively. Small circles indicate inferred haplotypes. Ball sizes range from 1–13 haplotypes.

species complexes. Phylogenetic analyses of *COI* were compromised by saturation of synonymous nucleotide substitutions among more distantly related taxa. The same problem existed in an analysis of bathymodiolin phylogeny, but the inclusion of additional mitochondrial and nuclear genes provided a robust combined phylogeny that proved useful for tracing evolutionary patterns in the mussels (Jones et al. 2006). We are currently examining additional mitochondrial and nuclear genes in these *Lepetodrilus* MOTUs and hope in the near future to provide a comprehensive analysis of their phylogeny and relationships with other vetigastropods.

ACKNOWLEDGMENTS

The authors thank the submersible pilots of HOV *Alvin* and ROVs *Jason I* and *II* (Woods Hole Oceanographic Institute) and ROV *Tiburon* (Monterey Bay Aquarium Research Institute) for their patience and technical skills. Numerous former students and technicians helped to sort and preserve limpets. The authors also thank vent and seep explorers, who recognized unusual distribution patterns and contributed specimens, notably: P. Briand, P. Collins, E. Cordes, D. Desbruyères, C. Van Dover, O. Giere, J. Hashimoto, D. Jollivet, M. Matabos, T. Okutani, K. Olu, H. Sahling, T. Schleicher, M. Segonzac, M. Sibuet, and V. Tunnicliffe.

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