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Exon-primed, intron-crossing (EPIC) loci for five nuclear genes in deep-sea protobranch bivalves: primer design, PCR protocols and locus utility

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Abstract

We describe PCR primers and amplification protocols developed to obtain introns from conserved nuclear genes in deep-sea protobranch bivalves. Because almost no sequence data for protobranchs are publically available, mollusk and other protostome sequences from GenBank were used to design degenerate primers, making these loci potentially useful in other invertebrate taxa. Amplification and sequencing success varied across the test group of 30 species, and we present five loci spanning this range of outcomes. Intron presence in the targeted regions also varied across genes and species, often within single genera; for instance, the calmodulin and β-tubulin loci contained introns with high frequency, whereas the triose phosphate isomerase locus never contained an intron. In introns for which we were able to obtain preliminary estimates of polymorphism levels in single species, polymorphism was greater than traditional mitochondrial loci. These markers will greatly increase the ability to assess population structure in the ecologically important protobranchs, and may prove useful in other taxa as well.

Keywords: bivalve, deep-sea, intron, nuclear markers, population genetics

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Introduction

Protobranchs are a diverse group of approximately 600 species of bivalve mollusks found in many marine environments, including coastal zones, hydrothermal vents and submarine caves (Zardus 2002). Several lines of morphological and molecular data suggest that they are the oldest extant lineage of bivalves (Giribet & Wheeler 2002; Zardus 2002), representing the original diversification of bivalves throughout the world oceans. Their high species diversity in the Atlantic (Allen & Sanders 1996) and their abundance in the deep-sea make them an intriguing group in which to study evolution and the invasion of species into deep-sea environments. Ecologically, protobranchs are intriguing because of their unusual success in the deep sea, which covers more than two-thirds of Earth's surface. Although the deep-sea floor was originally thought to be devoid of life, we now know that it supports a rich and highly endemic fauna. Significant progress has been made in understanding the modern ecological processes that maintain this high species richness (Etter & Mullineaux 2001; Levin *et al.* 2001; Snelgrove & Smith 2002; Carney 2005; Rex *et al.* 2005; Smith *et al.* 2008; Levin & Dayton 2009). In stark contrast, we know virtually nothing about how this diverse fauna originated and evolved (Etter *et al.* 2005; Zardus *et al.* 2006). Because protobranchs are typically the third most abundant taxon in deep-sea communities, they provide a model system for unravelling how evolution unfolded in this vast and remote ecosystem. The deep-water fauna also play key roles in global biogeochemical cycling and other essential ecosystem processes (Danovaro *et al.* 2008, 2009; Smith *et al.* 2008, 2009). As the deep sea is impacted by anthropogenic activities and the accelerating pace of climate change, it is vital to understand how this fauna might respond on evolutionary timescales.

The rapid development of molecular methods in the 'genome era' has facilitated production of the multilocus genetic data sets required to investigate evolution in understudied taxa, such as protobranchs. These data sets are finally allowing researchers to test complex hypotheses concerning population admixture, selective pressures and adaptation (e.g. Burke *et al.* 2010; Reagon *et al.* 2010). Although multilocus data sets are relatively easy to acquire in model organisms, researchers working on non-model organisms are increasingly at a disadvantage. Single loci are inadequate for many population genetic

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and phylogenetic analyses because of gene tree heterogeneity and coalescent stochasticity (e.g. Knowles & Maddison 2002; Brito & Edwards 2009; Degnan & Rosenberg 2009), as well as the effects of selection, linkage to selected genes and recombination (Maddison 1997), indicating that multiple independent loci are required to accurately infer evolutionary relationships (Edwards & Beerli 2000; Knowles 2009a,b). With no protobranch genome or EST database from which to obtain sequences for genes better suited to population-level analysis, one must proceed gene by gene, designing degenerate primers from alignments of related taxa and screening for variation. Until it becomes feasible for genome sequencing to reach every twig of the tree of life, this slow process is the only tool available to gain a better foothold into the genetics of nonmodel organisms. These organisms are frequently of great interest precisely because of their remote habitats, unusual life histories and the complex forces that have shaped their evolution.

We present the results of our effort to develop a suite of highly polymorphic markers to allow multilocus analysis of the phylogenetics and population genetics of protobranchs. Our approach was to target evolutionarily conserved introns (i.e. consistently found in the same gene region) in single-copy nuclear genes, for use as exon-primed, intron-crossing loci (EPIC; Palumbi 1996; Jarman *et al.* 2002). To the extent that introns are absent or inconsistently found, the resulting exon-only sequences could potentially serve as phylogenetic markers if they show appropriate levels of polymorphism. Because these primers were developed from alignments of a wide-range of metazoans, their utility probably extends beyond protobranchs. Although this gene-by-gene process was slow and tedious, we show its potential in developing multiple loci suited to analysis at a variety of evolutionary scales.

Materials and methods

Gene selection and motivation

Five conserved housekeeping genes were selected because they appeared to be single- or low-copy, frequently possessed introns in stable positions, and/or they had proven useful as markers in related organisms: calmodulin (CAL), actin (MAC), β-tubulin (BTUB), triose phosphate isomerase (TPI) and the adenosine nucleotide transporter (ANT). Several other loci were also explored, but were not pursued after very low initial success (aminopeptidase N, lysyl-tRNA synthetase, phosphoglucomutase, phosphoglycerate kinase and pyruvate kinase).

Calmodulin, an important calcium-binding protein (Chin & Means 2000), appears to exhibit variable copy number in metazoans (Simpson *et al.* 2005), but possesses at least one highly conserved intron (Friedberg & Rhoads 2001). This CAL intron has been used as a population genetic marker in marine snails of the genus *Conus* (Duda & Kohn 2005) and the marine bivalves *Spisula solidissima* (Hare & Weinberg 2005) and *Mytilus edulis* (Côrte-Real *et al.* 1994).

Two conserved structural genes were also selected. Actin is a ubiquitous component of eukaryotic cells, important in functions from cell scaffolding to muscle contraction (Pollard 1990). The gene sequence is also highly conserved, and gene structure appears to be relatively stable. MAC introns have been used to determine hybridization zones in *M. edulis* (Daguin *et al.* 2001; Bierne *et al.* 2003), and in phylogeographic studies of freshwater *Schistosoma*-transmitting snails in the genus *Biomphalaria* (DeJong *et al.* 2003). BTUB is also a ubiquitous structural component of eukaryotic cells; its heterodimerization with α-tubulin forms the basic building block of microtubules (Oakley 2000; McKean *et al.* 2001). A survey of gene structure in diverse eukaryotes revealed some conserved intron positions, and several strongly conserved, widely spaced amino acid motifs in BTUB were convenient for primer design.

The final two genes are important in metabolism and energy production. The enzyme TPI catalyses the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, an important step in glycolysis (Lehninger *et al.* 2008). There is some indication that introns in TPI separate successive αβ-barrels of the protein (Elder 2000), implying conservational constraints. Finally, the ANT (also called ADP/ATP translocase) carries ADP to the inner mitochondrial space and ATP to the outer mitochondrial space (Alberts *et al.* 1994). Jarman *et al.* (2002) designed degenerate primers (employed here initially but with low success) to flank an intron found in *Homo sapiens*, *Drosophila melanogaster* and *Caenorhabditis elegans*, although four other metazoans appear to lack this intron (*Anopheles gambiae*, *Rattus norvegicus*, *Rana rugosa* and *Halocynthia roretzi*).

Primer design

For the selected genes, sequences were obtained from GenBank and available EST/genome projects. We first obtained all possible mollusk sequences available for a gene, then added the phylogenetically closest metazoans until an alignment of sufficient size resulted, with preference given to entries containing annotated introns. Table 1 tallies the mollusk species by class, and other species by phylum, used for each gene; Table S1 (Supporting information) lists the species and accession number for each downloaded sequence.

Following the EPIC approach, primers were designed to target known or suspected conserved introns, but were

Table 1 Genes selected for degenerate primer design, and taxonomic breakdown of species whose sequences were obtained from GenBank for alignment

Gene	Group	No. species (No. sequences)	Total sequences
Calmodulin (CAL)	Bivalvia	13 (17)	33
	Gastropoda	6 (7)	
	Cephalopoda	1 (5)	
	Annelida	1 (1)	
	Arthropoda	8 (8)	
Actin (MAC)	Bivalvia	22 (25)	34
	Gastropoda	5 (7)	
	Cephalopoda	1 (2)	
β-Tubulin (BTUB)	Bivalvia	3 (3)	13
	Gastropoda	1 (1)	
	Cephalopoda	2 (2)	
	Arthropoda	4 (5)	
	Nematoda	4 (4)	
Triose phosphate isomerase (TPI)	Bivalvia	7 (7)	31
	Arthropoda	24 (24)	
Adenosine nucleotide transporter (ANT)	Bivalvia	13 (13)	29
	Arthropoda	15 (16)	

Individual species names with accession nos are given in Table S1 (Supporting information).

placed in exons such that some exonic sequence was also amplified; these exon portions were useful in distinguishing pseudogenes and paralogs. Universally- or highly conserved amino acid sequences were chosen for primer sites, and all codon degeneracies were incorporated into the primer, except where selection pressure across many species for the same specific codon was evident. Primaclade (Gadberry *et al.* 2005) and Oligocalc (Kibbe 2007) web servers were used to ensure that primers had optimal qualities. The primers designed for each gene are listed in Table 2, along with primers designed by others and used here. The locations of these primers relative to the available gene structures from the alignment are given in Fig. 1.

PCR and sequencing strategies

Our goal was to robustly amplify the target gene regions across as many protobranch species as possible, without sacrificing specificity. Multiple primers were designed in most genes to facilitate nested PCR. Although initial PCR protocols employed nested PCR and/or cloning, we tried to develop optimized protocols that avoided these strategies. Because no data exist on gene structure for any protobranch, we had only rough expectations of amplicon size, and initially pursued as many amplified

Table 2 Primers taken from other work or designed here

Gene	Primer	Orientation	Sequence (5′–3′)	References
CAL	CAL1	F	GCCGAGCTGCARGAYATGATCAA	Duda & Palumbi (1999)
	CAL2	R	GTGTCCTTCATTTTNCKTGCCATCAT	Duda & Palumbi (1999)
	CAL5	F	TTYGACAAGGAYGGHGATGG	
	CAL6	R	TCGGCGGCACTGATGAANCCGTTNCCGTC	
	CAL7	R	TCDGCYTCNCKRATCATYTCRTC	
MAC	mac1F	F	GACAATGGMTCTGGTATGTGTAA	
	mac1R	R	TGCCAGATTTTCTCCATRTCRTCCCA	
	macR3	R	TCGAACATGATCTGDGTCATCTT	
	macR4	R	GTCATCTTYTCNCTGTTGGC	
BTUB	BtubF1	F	CAGGCYGGNCAGTGYGGHAACCAGATTGG	Mod. Einax & Voigt (2003)
	BtubR1	R	GCTTCATTGTAGTANACGTTGAT	
	BtubR1b	R	GCTTCATTGTAGTANACGTTGATYCTCTCCA	
	BtubR1c	R	GCCGTGRTARGTTCCGGTGGG	
	BtubF2	F	ATGTTYGAYGCHAAGAAYATGATGGC	
	BtubR2	R	TCCATGCCYTCNCCVGTGTACCAGTG	Mod. Einax & Voigt (2003)
	ABtub2r	R	GTTGTTNGGGATCCAYTCSACGAA	Mod. Einax & Voigt (2003)
	ABtub4r	R	GCYTCNGTGAARTCCATYTCGTCCAT	Mod. Einax & Voigt (2003)
TPI	TPIf1	F	GGMGGMAACTGGAAGATGAA	
	TPIr1	R	GTYTTNCCGGTNCCGATGGCCCA	
ANT	ANTf1	F	GTYAAYGTYATCAGGTACTTCCC	
	ANTr1	R	CGCCAGAACTGNGTNTTCTTGTC	
	ANTf3	F	CCRGTSCGRTTCGCIAAGGAYTTC	
	ANTf4	F	TAYTTYCCWACICAYGC	
	ANTr4	R	GCYTCACCAGATGTCATCATCAT	

Mod., modified from primers designed in this reference.

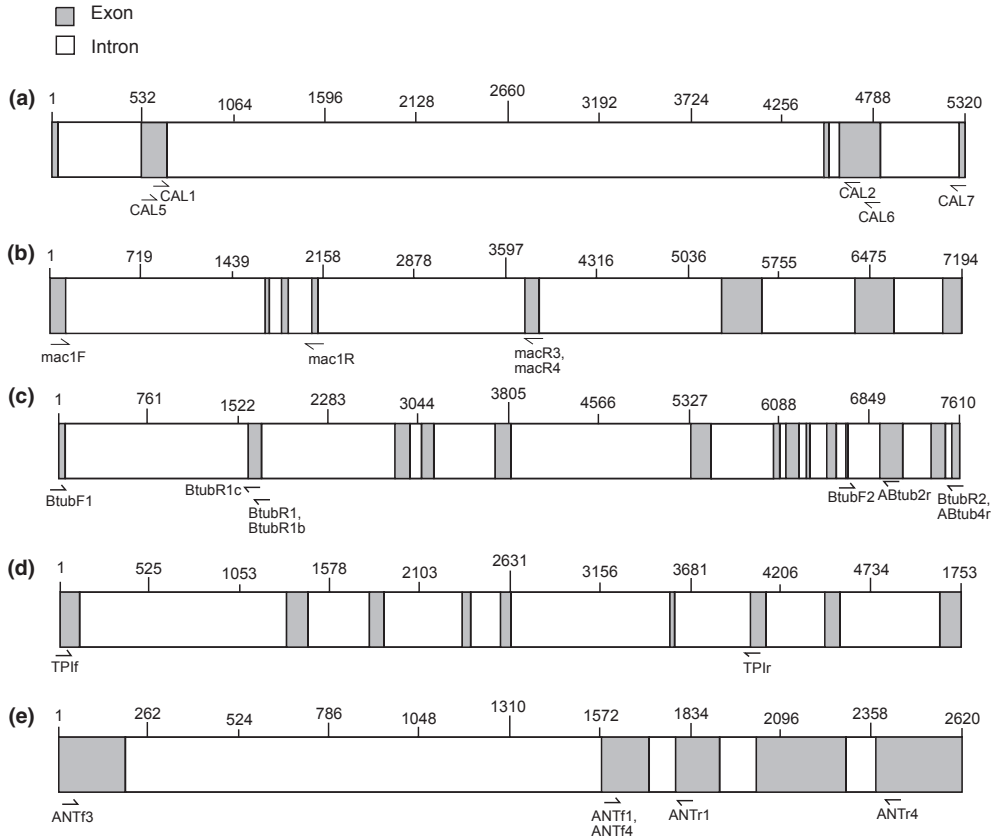

Fig. 1 Synthesis of gene structure based on all species present in multiple alignments. Introns are shown when present in any species in the alignment, with lengths corresponding to the longest intron observed. Primer locations and orientations are marked with arrows. Lengths are given in nucleotide basepairs. From (a) to (e) panels are CAL, MAC, BTUB, TPI and ANT.

bands as was feasible. Although it was possible that some degenerate primers might be suitable for direct use in single species at the population level, we anticipated that species-specific primers would be a more efficient alternative once sequences were in hand. PCRs employed the GoTaq-Flexi system (Promega, Madison, WI) in 50 μL reactions containing 1× buffer, 2.5 mM $MgCl_2$, 2 pmol dNTPs, 1.2 pmol each primer, 2 μL extracted genomic DNA and 1 U *Taq* polymerase. PCR cycling conditions for each gene are detailed next. As determined by gel electrophoresis, single-banded PCRs were sequenced directly; strong bands from multibanded PCRs were individually gel-purified, and either sequenced directly or cloned and sequenced, depending on yield. All PCR purification and capillary sequencing was performed by Beckman-Coulter/Agencourt, Inc. (Beverly, MA).

Species and specimens

Primers were tested on a suite of 30 protobranch species (mostly deep-sea), encompassing 14 genera and 7 families. Although protobranch phylogeny is poorly understood (Zardus 2002; Bouchet *et al.* 2010), these 30 species span 2 of the current major divisions of protobranchs (the Nuculoidea and Nuculanoidea, both placed in the Nuculoida), and 14 of the 55 accepted genera in WoRMS (Appeltans *et al*. 2010). The majority of these specimens were collected on a research cruise to the deep Northwest Atlantic, and one to the Skagerrak Straits (Scandinavia). Additional material was obtained on cruises to the Angola Basin (SW Atlantic) and the Southern Ocean; the shallow species *Yoldia limatula* and *Nucula tenuis* were collected locally from the NW Atlantic (Boston area), and the shallow species *Nuculana fossa* and *Yoldia scissurata* were obtained locally from the NE Pacific (Friday Harbor area; Table 3).

Results and discussion

Table 4 contains PCR protocols that best amplified the targeted gene segments, with success rates across protobranch species and genera. The given amplicon lengths are approximate and do not account for introns, whose lengths were highly variable. Detection and annotation of exons and introns was done manually using BioEdit (Hall 1997). For most genes, a single primer pair or PCR

Table 3 Protobranch species on which primers were tested and PCR optimized

Higher taxonomy	Family	Species	Ocean region
Nuculanoidea	Lametilidae	*Lametila abyssorum*	NW Atlantic
	Malletiidae	*Malletia abyssorum*	NW Atlantic
		Malletia cuneata	NW Atlantic
		Malletia johnsoni	NW Atlantic
		Malletia polita	NW Atlantic
	Malletiidae*	*Neilonella salicensis*	NW Atlantic
		Neilonella whoii	NW Atlantic
	Nuculanidae	*Ledella sublevis*	NW Atlantic
		Ledella ultima	NW Atlantic
		Nuculana fossa	NE Pacific
		Nuculana minuta	Skagerrak Straits
		Nuculana pernula	Skagerrak Straits
		Spinula filatova	Angola Basin
		Spinula hilleri	Southern Ocean
	Siliculidae	*Silicula rouchi*	Southern Ocean
	Yoldiidae	*Yoldia limatula*	NW Atlantic
		Yoldia scissurata	NE Pacific
		Yoldia valettei	Southern Ocean
		Yoldia cf. valettei	Southern Ocean
		Yoldiella inconspicua	NW Atlantic
		Yoldiella lucida	Skagerrak Straits
		Yoldiella nana	Skagerrak Straits
		Yoldiella subcircularis	NW Atlantic
Nuculoidea	Nuculidae	*Brevinucula verrilli*	NW Atlantic
		Deminucula atacellana	NW Atlantic
		Nucula proxima	NW Atlantic
		Nucula tenuis	Skagerrak Straits
		Nuculoma granulosa	NW Atlantic
	Pristiglomidae†	*Pristigloma alba*	NW Atlantic
		Pristigloma nitens	NW Atlantic

*Accepted as Neilonellidae in WoRMS.
†Accepted as Sareptidae in WoRMS.

protocol could not be used across all species (cf. TPI), which was probably due in part to sequence diversity at the priming sites, and in part to gene structure variation. In most species, one or a few strong bands were reliably amplified, accompanied by fainter, more numerous and more variably present bands. In trying to balance comprehensive examination with economy, most of these fainter bands were not pursued further.

The CAL primers (CAL1/CAL2) developed by Duda & Palumbi (1999) proved highly successful in protobranchs (Table 4). The fate of PCR bands, number of sequence types detected and GenBank accessions for sequences generated here are given in Table 5. When downstream reverse primers were successful, the longer exon segments obtained were quite useful in separating putative functional from pseudogenous sequences, and in implying that multiple CAL copies existed. Across protobranchs, CAL appears to be present in multiple copies, as it is in other mollusks (Côrte-Real *et al*. 1994; Duda & Kohn 2005; Hare & Weinberg 2005). As further implied by these studies, CAL contained an intron in this location for every protobranch species for which useable sequence was obtained – the only gene to exhibit such high structural conservation. When species-specific primers were developed within the introns of *Deminucula atacellana* and *Malletia abyssorum*, CAL exhibited *p*-distances of 0.021 in the former species (73 individuals) and 0.152 in the latter (23 individuals). By comparison, 65 individuals of *D. atacellana* exhibited a *p*-distance of 0.016 in the mitochondrial COI gene, demonstrating high polymorphism in CAL suitable for population genetic analysis.

The primer combination mac1F/mac1R amplified approximately half (17/30) of the suite of protobranchs, although three of five species amplified with a 48° annealing temperature were not amplified with annealing at 50°. This implication of annealing temperature sensitivity was reflected in the differential success of nested PCR strategies for MAC (Table 4). Although attempts at amplifying longer sections of MAC in a single round of PCR had very

Table 4 PCR protocols, including single-round and nested strategies for amplification of selected loci

Gene	PCR name	Primers	Cycle conditions	Intron-less length	Successes
CAL	C1	CAL1/CAL2	ann 50° ext 72°, 1:30	54 bp	17 spp.
	C2	CAL5/CAL6	ann 50° ext 72°, 2:30	216 bp	3 spp.
	C3	CAL5/CAL7	ann 52° ext 72°, 1:30	294 bp	
	[C4]	CAL1/CAL7	ann 57° ext 72°, 1:30	213 bp	
	[C5]	CAL1/CAL2	ann 55° ext 72°, 2:30		
	[C6]	CAL1/CAL2	ann 56° ext 72°, 1:30		
	Nested C2, C5				7 spp.
	Nested C2, C6				3 spp.
	Nested C3, C4				8 spp.
MAC	M1	mac1F/mac1R	ann 50° ext 72°, 1:15	186 bp	14 spp.
	M2	mac1F/mac1R	ann 48° ext 72°, 1:15		5 spp.
	[M3]	mac1F/macR4	ann 55° ext 72°, 1:30	300 bp	
	[M4]	mac1F/macR3	ann 45° ext 72°, 2:30	300 bp	
	[M6]	mac1F/macR4	ann 45° ext 72°, 2:00		
	Nested M3, M4				10 spp.
	Nested M4, M1				4 spp.
	Nested M4, M6				8 spp.
BTUB					
3′ segment	Bp1	BtubF2/BtubR2	ann 55° ext 72°, 1:15	114 bp	23 spp.
5′ segment	B1	BtubF1/BtubR1	ann 45° ext 72°, 1:30	84 bp	6 spp.
	B2	BtubF1/BtubR1b	ann 55° ext 72°, 1:30	84 bp	8 spp.
	[B3]	BtubF1/BtubR1b	ann 50° ext 72°, 2:00		
	[B4]	BtubF1/BtubR1c	ann 57° ext 72°, 2:00	84 bp	
	[B5]	BtubF1/BtubR1b	ann 50° ext 72°, 1:15		
	Nested B3, B4				6 spp.
	Nested B2, B5				4 spp.
Long segment	[BL1]	BtubF1/ABtub4r	ann 58° ext 72°, 2:30	1152 bp	
	[BL2]	BtubF1/ABtub2r	ann 45° ext 72°, 2:30	1134 bp	
	Nested BL1, BL2				21 spp.
TPI	T1	TPIf/TPIr	ann 57° ext 72°, 1:15	486 bp	20 spp.
	T2	TPIf/TPIr	ann 45° ext 72°, 1:15		9 spp.
	T3	TPIf/TPIr	ann 55° ext 72°, 1:15		17 spp.

Table 4 Continued

Gene	PCR name	Primers	Cycle conditions	Intron-less length	Successes
ANT	A1	ANTf1/ANTr1	ann 50° ext 72°, 1:30	60 bp	1 spp.
	A2	ANT f3/r4	ann 52° ext 72°, 2:30	690 bp	4 spp.
	[A3]	ANT f4/r4	ann 52° ext 72°, 2:30	498 bp	
	Nested A2, A3				13 spp.

Protocols in brackets were used only as the second PCR of a nested strategy. All PCR protocols included an initial denaturation at 90 °C for 3 min, and each cycle began with denaturation at 90 °C for 30 s; all protocols ended with a final extension at 72 °C for 3 min and a final hold at 4 °C. Species were counted under 'Successes' for multiple protocols if they amplified under multiple sets of conditions. ann, annealing temperature (for 45 s in all protocols); ext, extension temperature and time; CAL, calmodulin; MAC, actin; BTUB, β-tubulin; TPI, triose phosphate isomerase; ANT, adenosine nucleotide transporter.

low success, the downstream reverse primers were suitable as the first round in nested strategies. Intron presence was more variable in MAC than in CAL, but on average fewer sequence types were detected per species (Table 5). Screening *D. atacellana* and *M. abyssorum* using intron-based species-specific primers yielded *p*-distances of 0.051 (11 individuals) and 0.047 (10 individuals), respectively; these levels of polymorphism are again higher than in COI, as desired for population-level analysis.

A primer set targeting a short segment of BTUB near the 3′ end (protocol Bp1) gave robust and consistent amplification (Table 4); however, an intron was not found in any protobranch at this location. Unfortunately, primers targeting a BTUB intron near the 5′ end were more successful in species without introns than in species with introns. Counterintuitively, nested protocols targeting just the 5′ region exhibited much lower success than a nested protocol designed to span the 5′ and 3′ regions (amplifying most of the gene). Among the 21 spp. for which these long BTUB sequences were generated, the only intron location detected was in the targeted 5′ region. Although this intron was consistently present like the CAL intron, in many species, it contained simple dinucleotide repeats and/or more complex repeats, which probably lowered PCR and sequencing success.

Only a single set of primers was designed for TPI, but it amplified very robustly and consistently across protobranchs (Table 4). Unfortunately, TPI's most prominent feature was a lack of introns in any of the eight species for which sequence was obtained, again indicating an inverse correlation between PCR success and intron presence. The high similarity of band sizes in successful amplifications most probably indicates that no protobranch possesses an intron in this region. Successful PCR amplifications from which few bands could be sequenced cleanly were much more common in TPI than in other genes tested here with similar strategies. Although it is difficult to determine the cause of such 'negative results', possible explanations include the presence of nucleotide repeats, suboptimal sequencing reaction annealing temperature and complications from using the same degenerate primers for both PCR and sequencing. Alignment of the eight sequences obtained indicated high nucleotide and amino acid variability, suggesting its potential as a shallow phylogenetic marker.

Amplification of ANT was similar to that of BTUB, where nested PCR was required for success; however, only one (*M. abyssorum*) generated even passably useable sequence for ANT. As with BTUB, the presence in this sequence of dinucleotide repeats upstream of a small, clean region of 3′ exon sequence implies that microsatellites reduced sequencing success. No clear 5′ exonic sequence or intron splice sites could be detected, making it unclear whether the amplified fragment is a pseudogene, or a portion of a functional ANT copy with an intron >400 bp.

Conclusion

The primers developed here have proven useful in amplifying several protein-coding genes, most of which contain promising EPIC loci in protobranchs. Because the primers were designed based on alignments of mollusk and/or other invertebrate species, they will probably amplify these genes from other invertebrates as well. Although some care must be taken to optimize PCR and reduce or eliminate spurious sequences, the protocols suggested herein should greatly speed the most difficult step, obtaining sequences *de novo* from nonmodel organisms. Researchers will probably use these primers to obtain preliminary sequence for the design of species-specific primers. Several of the loci tested here contained the high polymorphism needed to provide powerful markers for population genetics, and the intron-less TPI could potentially be developed for shallow phylogenies.

Table 5 Successfully amplified and/or sequenced genes for the suite of test species

Higher	Family	Species	CAL results	Accessions	MAC results	Accessions	BTUB results	Accessions	TPI results	Accessions	ANT results
Nuculanoidea	Lametilidae	*Lametila abyssorum*	1 i+	JF410881	1 i+	JF410916	3p 1 i−	JF410956	1 i−	JF410996	PCR
	Malletiidae	*Malletia abyssorum*	1 i+	JF410882	3; 2 i+, 1 i−	JF410917-JF410919	5p 2 i+, 3p 1 i−	JF410957-JF410959	PCR		PCR
		Malletia cuneata	1 i+	JF410883	3 i−	JF410920-JF410922	3p 1 i−	JF410960	PCR		
		Malletia johnsoni	1 i+	JF410884	3 i+	JF410923-JF410925	5p 1 i+, 3p 1 i−	JF410961-JF410962	PCR		PCR
		Malletia polita	1 i+	JF410885	3 i−	JF410926-JF410929	5p 1 i+, 3p 1 i−	JF410963-JF410964	PCR		PCR
	Malletiidae*	*Neilonella salicensis*	3 i+	JF410886-JF410888	1 i+	JF410930	5p 3 i+	JF410965-JF410967	PCR		
		Neilonella whoii	4 i+	JF410889-JF410892	1 i+	JF410931			1 i−	JF410997	PCR
	Nuculanidae	*Ledella sublevis*	3 i+	JF410893-JF410895	1 i−	JF410932	3p PCR		PCR		
		Ledella ultima	1 i+	JF410896	1 i+	JF410933			PCR		
		Nuculana fossa	1 i+	JF410897	1 i+	JF410934	3p 1 i−	JF410968	1 i−	JF410998	PCR
		Nuculana minuta	3 i+	JF410898-JF410900	1 i+	JF410935	5p 4 i+, 3p 1 i−	JF410969-JF410973	1 i−	JF410999	PCR
		Nuculana pernula	2 i+	JF410901-JF410902	1 i−	JF410936	3p 1 i−	JF410974	PCR		
		Spinula filatova							PCR		
		Spinula hilleri			1 i+	JF410937	3p 1 i−	JF410975	1 i−	JF411000	
	Siliculidae	*Silicula rouchi*	PCR		1 i+	JF410938	5p 1 i+, 3p 1 i−	JF410976-JF410977	PCR		
	Yoldiidae	*Yoldia limatula*	3 i+	JF410903-JF410905	1 i+	JF410939	3p 1 i−	JF410978	PCR		PCR
		Yoldia scissurata	1 i+	JF410906	2 i+	JF410940-JF410941	5p 2 i+, 3p 1 i−	JF410979-JF410981	PCR		PCR
		Yoldia valleri	1 i+	JF410907	1 i+	JF410942	3p 1 i−	JF410982	PCR		
		Yoldia cf. valleri			3; 2 i+, 1 i−	JF410943-JF410945	3p 1 i−	JF410983	PCR		
		Yoldiella inconspicua	PCR		1 i+	JF410946	3p 1 i−	JF410984	PCR		PCR

Table 5 Continued

Higher	Family	Species	CAL results	Accessions	MAC results	Accessions	BTUB results	Accessions	TPI results	Accessions	ANT results
		Yoldiella lucida	1 i+	JF410908	1 i−	JF410947	3p 1 i−	JF410985			PCR
		Yoldiella nana	1 i+	JF410909	2 i+	JF410948-JF410949	5p 1 i+, 3p 1 i−	JF410986-JF410987	PCR		PCR
		Yoldiella subcircularis	1 i+	JF410910	1 i+	JF410950			1 i−	JF411001	
Nuculoidea	Nuculidae	*Brevinucula verrilli*	2 i+	JF410911-JF410912	1 i+	JF410951	3p 1 i−	JF410988	1 i−	JF411002	
		Deminucula atacellana	1 i+	JF410913	1 i+	JF410952	5p 1 i+	JF410989	PCR		
		Nucula proxima					3p 1 i−	JF410990	PCR		PCR
		Nucula tenuis			1 i−	JF410953	3p 1 i−	JF410991			PCR
		Nuculoma granulosa	1 i+	JF410914	1 i+	JF410954	5p 3 i+	JF410992-JF410994	PCR		PCR
	Pristiglomidae†	*Pristigloma alba*					3p 1 i−	JF410995			
		Pristigloma nitens	1 i+	JF410915	1 i−	JF410955			1 i−	JF411003	PCR
Total species = 30		Success species	24		27		25		27 (8)		16 (0)
Total genera = 14		Success genera	12		14		14		14 (7)		9 (0)
Total families = 7											

For successful sequences, the number of sequence types encountered is listed, followed by the number of intron-containing (i+) and/or intron-lacking (i−) sequences, respectively. PCR, successful PCR but sequencing failed. For BTUB, 5p reflects amplification/sequencing of the 5′ region, and 3p the downstream 3′ region. Accession numbers in GenBank are given. CAL, calmodulin; MAC, actin; BTUB, β-tubulin; TPI, triose phosphate isomerase; ANT, adenosine nucleotide transporter.
*Accepted as Neilonellidae in WoRMS.
†Accepted as Sareptidae in WoRMS.

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References

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) *Molecular Biology of the Cell*, 3rd edn. Garland Publishing, Inc, New York.

Allen JA, Sanders HL (1996) The zoogeography, diversity and origin of the deep-sea protobranch bivalves of the Atlantic: The epilogue. *Progress in Oceanography*, **38**, 95–153.

Appeltans W, Bouchet P, Boxshall GA *et al*., (eds) (2010) *World Register of Marine Species*. Available from: http://www.marinespecies.org. Accessed on 27th January 2010.

Bierne N, Daguin C, Bonhomme F, David P, Borsa P (2003) Direct selection on allozymes is not required to explain heterogeneity among marker loci across a *Mytilus* hybrid zone. *Molecular Ecology*, **12**, 2505–2510.

Bouchet P, Rocroi J-P, Bieler R, Carter JG, Coan EV (2010) Nomenclator of bivalve families with a classification of bivalve families. *Malacologia*, **52**, 1–184.

Brito P, Edwards S (2009) Multilocus phylogeography and phylogenetics using sequence-based markers. *Genetica*, **135**, 439–455.

Burke MK, Dunham JP, Shahrestani P, Thornton KR, Rose MR, Long AD (2010) Genome-wide analysis of a long-term evolution experiment with *Drosophila*. *Nature*, **467**, 587–U111.

Carney RS (2005) Zonation of deep biota on continental margins. *Oceanography and Marine Biology–An Annual Review*, **43**, 211–278.

Chin D, Means AR (2000) Calmodulin: a prototypical calcium sensor. *Trends in Cell Biology*, **10**, 322–328.

Côrte-Real HBSM, Dixon DR, Holland PWH (1994) Intron-targeted PCR: a new approach to survey neutral DNA polymorphism in bivalve populations. *Marine Biology*, **120**, 407–413.

Daguin C, Bonhomme F, Borsa P (2001) The zone of sympatry and hybridization of *Mytilus edulis* and *M. galloprovincialis*, as described by intron length polymorphism at locus mac-1. *Heredity*, **86**, 342–354.

Danovaro R, Gambi C, Dell'Anno A *et al.* (2008) Exponential decline of deep-sea ecosystem functioning linked to benthic biodiversity loss. *Current Biology*, **18**, 1–8.

Danovaro R, Corinaldesi C, Luna GM *et al.* (2009) Prokaryote diversity and viral production in deep-sea sediments and seamounts. *Deep-Sea Research II*, **56**, 738–747.

Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology & Evolution*, **24**, 332–340.

DeJong RJ, Morgan JAT, Wilson WD *et al*. (2003) Phylogeography of *Biomphalaria glabrata* and *B. pfeifferi*, important intermediate hosts of *Schistosoma mansoni* in the New and Old World tropics. *Molecular Ecology*, **12**, 3041–3056.

Duda TF, Kohn AJ (2005) Species-level phylogeography and evolutionary history of the hyperdiverse marine gastropod genus *Conus*. *Molecular Phylogenetics and Evolution*, **34**, 257–272.

Duda TF, Palumbi SR (1999) Developmental shifts and species selection in gastropods. *Proceedings of the National Academy of Sciences USA*, **96**, 10272–10277.

Edwards SV, Beerli P (2000) Perspective: gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution*, **54**, 1839–1854.

Einax E, Voigt K (2003) Oligonucleotide primers for the universal amplification of beta-tubulin genes facilitate phylogenetic analyses in the regnum fungi. *Organisms Diversity & Evolution*, **3**, 185–194.

Elder D (2000) Split gene origin and periodic introns. *Journal of Theoretical Biology*, **207**, 455–472.

Etter RJ, Mullineaux L (2001) Deep-sea communities. In: *Marine Ecology* (eds Bertness MD, Gaines S, Hay M), pp. 367–393. Sinauer Associates, Sunderland, Massachusetts.

Etter RJ, Rex MA, Chase MR, Quattro JM (2005) Population differentiation decreases with depth in deep-sea bivalves. *Evolution*, **59**, 1479–1491.

Friedberg F, Rhoads AR (2001) Evolutionary aspects of calmodulin. *Iubmb Life*, **51**, 215–221.

Gadberry MD, Malcomber ST, Doust AN, Kellogg EA (2005) Primaclade—a flexible tool to find conserved PCR primers across multiple species. *Bioinformatics*, **21**, 1263–1264.

Giribet G, Wheeler W (2002) On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. *Invertebrate Biology*, **121**, 271–324.

Hall T (1997) BioEdit software version 7.0.9. Copyright 1997–2007.

Hare MP, Weinberg JR (2005) Phylogeography of surfclams, *Spisula solidissima*, in the western North Atlantic based on mitochondrial and nuclear DNA sequences. *Marine Biology*, **146**, 707–716.

Jarman SN, Ward RD, Elliott NG (2002) Oligonucleotide primers for PCR amplification of coelomate introns. *Marine Biotechnology*, **4**, 347–355.

Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research*, **35** (Suppl. 2), W43–W46 (webserver issue), 25 May.

Knowles LL (2009a) Statistical phylogeography. *Annual Review of Ecology Evolution and Systematics*, **40**, 593–612.

Knowles LL (2009b) Estimating species trees: methods of phylogenetic analysis when there is incongruence across genes. *Systematic Biology*, **58**, 463–467.

Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology*, **11**, 2623–2635.

Lehninger AL, Nelson DL, Cox MM (2008) *Principles of Biochemistry*. W.H. Freeman, Gordonsville, Virginia.

Levin LA, Dayton PK (2009) Ecological theory and continental margins: where shallow meets deep. *Trends in Ecology and Evolution*, **24**, 606–617.

Levin LA, Etter RJ, Rex MA *et al.* (2001) Environmental influences on regional deep-sea species diversity. *Annual Review of Ecology and Systematics*, **32**, 51–93.

Maddison WP (1997) Gene trees in species trees. *Systematic Biology*, **46**, 523–536.

McKean PG, Vaughan S, Gull K (2001) The extended tubulin superfamily. *Journal of Cell Science*, **114**, 2723–2733.

Oakley BR (2000) An abundance of tubulins. *Trends in Cell Biology*, **10**, 537–542.

Palumbi SR (1996) The polymerase chain reaction. In: *Molecular Systematics* (eds Hillis DM, Moritz C, Mable BK), pp. 205–247. Sinnauer Associates, Sunderland, Massachusetts.

Pollard TD (1990) Actin. *Current Opinion in Cell Biology*, **2**, 33–40.

Reagon M, Thurber CS, Gross BL, Olsen KM, Jia YL, Caicedo AL (2010) Genomic patterns of nucleotide diversity in divergent populations of US weedy rice. *BMC Evolutionary Biology*, **10**, doi: 10.1186/1471-2148-10-180.

Rex MA, McClain CR, Johnson NA *et al.* (2005) A source-sink hypothesis for abyssal biodiversity. *American Naturalist*, **165**, 163–178.

Simpson RJ, Wilding CS, Grahame J (2005) Intron analyses reveal multiple calmodulin copies in *Littorina*. *Journal of Molecular Evolution*, **60**, 505–512.

Smith CR, De Leo FC, Bernardino AF, Sweetman AK, Arbizu PM (2008) Abyssal food limitation, ecosystem structure and climate change. *Trends in Ecology & Evolution*, **23**, 518–528.

Smith KL, Ruhl HA, Bett BJ, Billett DSM, Lampitt RS, Kaufmann RS (2009) Climate, carbon cycling, and deep-ocean ecosystems. *Proceedings of the National Academy of Sciences USA*, **106**, 19211–19218.

Snelgrove PVR, Smith CR (2002) A riot of species in an environmental calm: the paradox of the species-rich deep-sea floor. *Oceanography and Marine Biology*, **40**, 311–342.

Zardus JD (2002) Protobranch bivalves. *Advances in Marine Biology*, **42**, 1–65.

Zardus JD, Etter RJ, Chase MR, Rex MA, Boyle EE (2006) Bathymetric and geographic population structure in the pan-Atlantic deep-sea bivalve *Deminucula atacellana* (Schenck, 1939). *Molecular Ecology*, **15**, 639–651.

Data Accessibility

DNA Sequences: GenBank Accessions JF410881–JF411003.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Individual species' sequences obtained for gene alignment and primer design, with GenBank accession nos.

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