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Heteroplasmy in a deep-sea protobranch bivalve suggests an ancient origin of doubly uniparental inheritance of mitochondria in Bivalvia

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Abstract Most metazoan species have strict maternal inheritance of the mitochondrial genome. In bivalves, a unique inheritance pattern called doubly uniparental inheritance (DUI) occurs in at least seven bivalve families. In this system of mitochondrial inheritance, males inherit and carry mtDNA from both parents, while females only carry mtDNA from the mother. Here, we present evidence of mitochondrial heteroplasmy in deep-sea protobranch bivalves. Divergent 16S rRNA and cytochrome *b* sequences were obtained within individuals of *Ledella ultima. Ledella sublevis* also exhibited divergent 16S sequences. Levels of divergence between 16S sequences within individuals were 27 and 15 % for each species, respectively. Ratios of homoplasmic to heteroplasmic individuals were not significantly different from 1:1, in agreement with sex ratios in protobranchs. The results provide the first evidence for mitochondrial heteroplasmy in the protobranchs and suggest DUI might have evolved much earlier in the evolution of the Bivalvia than previously thought.

Introduction

Most eukaryotes have maternal transmission of clonally copied mitochondrial DNA (mtDNA) (Birky 2001). The presence of divergent mtDNA sequences within a single individual, heteroplasmy, occurs in a wide range of taxa and usually results from mutations within an individual, transmission of mutated copies from the mother, paternal leakage or paternal transmission (reviewed in Hurst and Hoekstra 1994; Rand 2001; White et al. 2008). Heteroplasmy is thought to be detrimental leading to selection of selfish deleterious elements (Hurst and Hoekstra 1994, reviewed in Breton et al. 2007) and to cause some human diseases (reviewed in Schapira 2006). In most organisms, heteroplasmy is a transient state, where mutations in some copies of mtDNA are lost by genetic drift in a few generations due to bottlenecks of mtDNA during oogenesis or negative selection on cells carrying a high mtDNA mutation load (Chinnery et al. 2000; Cree et al. 2008; Khrapko 2008). In some taxa, single-nucleotide polymorphisms in the mitochondrial DNA have become fixed (Doublet et al. 2008; McLeod and White 2010). In contrast, a number of bivalve species have highly divergent mitochondrial genomes, with a 20–50 % difference between two types of mtDNA found within a single individual (Passamonti et al. 2003; Mizi et al. 2005; Breton et al. 2006; Theologidis et al. 2008; Cao et al. 2009).

Heteroplasmy in bivalves is due to an unusual type of mitochondrial inheritance, where females inherit mitochondria only from their mothers and males inherit mitochondria from both their mother and father, thus possessing two types of mitochondria: the female (F) mitotype and the male (M) mitotype (Skibinski et al. 1994a, b; Zouros et al. 1994a, b; Zouros 2000). This type of mitochondrial transmission is called doubly uniparental inheritance (DUI) (Zouros 2000) and appears to be widespread throughout the Class Bivalvia, as it has been found in seven bivalve families (Theologidis et al. 2008). Heteroplasmy has been extensively studied in the Mytilidae (Skibinski et al. 1994a,

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b; Zouros et al. 1994a, b; Zouros 2000; Hoeh et al. 1996, 1997), Unionidae (Hoeh et al. 1996; Liu et al. 1996), and Veneridae (Passamonti et al. 2003) where experiments have clearly documented the nature of DUI.

DUI is thought to have evolved early within the bivalves but its origin is unknown (Hoeh et al. 1997; Theologidis et al. 2008). The evolutionary distribution of DUI within and among bivalve families is complex. Phylogenetic analysis of mitochondrial genes in the superfamily Unionidae finds that male types from different families cluster together separately from the female types from those families, suggesting the origins of the male type precedes the origin of the superfamily (Hoeh et al. 1996; Hoeh et al. 2002; Curole and Kocher 2005; Theologidis et al. 2008). In Mytilids, the male and female types of a species or closely related species generally cluster together, but the associations are not as deep as in the Unionidae (Hoeh et al. 1996, 2002; Theologidis et al. 2008). The different patterns in Mytilids may result from the presence of recently "masculinized" M-types where a female type becomes entrained into the male transmission line (Hoeh et al. 1996, 2002; Zouros 2000). There is also recombination between the male and female types in Mytilids (Ladoukakis et al. 2011). In the Unionidae, there is an absence of recombination or masculinization (Hoeh et al. 2002) perhaps because the male mitotype has a longer COII gene, which prevents recombination (Curole and Kocher 2002, 2005; Chakrabarti et al. 2006). These differences initially led to the hypothesis that heteroplasmy has multiple origins in the Bivalvia (Hoeh et al. 1996). However, because heteroplasmy is such a complex and unique phenomena and is widespread in the Bivalvia, this seems unlikely (Hoeh et al. 1997; Theologidis et al. 2008). Although in some taxa, this phenomena is well studied, much remains to be learned about the origins, taxonomic distribution, maintenance and function of DUI (Theologidis et al. 2008; Passamonti and Ghiselli 2009).

So far, heteroplasmy has not been found in the protobranch bivalves, but very few have been analyzed genetically. Protobranch bivalves are the most basal extant group within the Bivalvia (Giribet and Wheeler 2002; Giribet et al. 2006; Smith et al. 2011). Unlike most bivalves, they are deposit feeders and are more diverse and abundant in the deep sea (Allen 1978, 1979). Previous phylogeographic studies with formalin-fixed specimens of a common abyssal protobranch *Ledella ultima* (Etter et al. 2005, 2011) occasionally yielded sequences of the 16S rRNA gene with extensive double-peaked reads indicating overlapping divergent products. Further analyses of this species in fresh specimens utilizing universal primers yielded both previously observed and highly divergent 16S sequences among individuals from the same location. These two results suggested that *L. ultima* might possess more than one type of mitochondrial DNA. Alternative explanations for why divergent 16S sequences might be found in individuals of the same species from the same location include the possibility of cryptic species, the presence of a nuclear copy of mitochondrial DNA (Numt) or contamination. Here, we test whether the divergent haplotypes between individuals of *L. ultima* could be found within individuals and whether this is mitochondrial heteroplasmy. We also explore whether heteroplasmy exists in the congeneric species, *Ledella sublevis*.

The Nuculanidae subfamily Ledellinae is a diverse group of tiny bivalves, endemic to the deep sea, and widespread both geographically and bathymetrically (Filatova and Schileyko 1984, Allen and Hannah 1989). Sequencing of the 16S and cytochrome *b* (cytb) mitochondrial genes yields strong evidence of heteroplasmy in *L. ultima,* and sequencing 16S in the less abundant congener *L. sublevis* is also suggestive of heteroplasmy. The results provide the first evidence of heteroplasmy in the protobranchs (palaeotaxodonta) and suggest a much earlier origin of DUI than previously thought (Theologidis et al. 2008; Doucet-Beaupré et al. 2010).

Methods

Fresh specimens of *L. ultima* (depth range 2,800–5,000 m) and *L. sublevis* (depth range 2,200–3,500 m) were collected from the North American Basin (NAB) in June 2008 (research cruise EN447). Whole DNA was extracted from individuals using a Qiagen minikit with the standard protocol. Additionally, museum samples of *L. ultima* from several other North Atlantic stations (77, 70, 92, 52216, 10148 and 8528 for station data see Etter et al. 2011) were extracted using a modified protocol for formalin-fixed ethanol preserved (FFEP) tissues (Boyle et al. 2004).

To rule out the possibility of nuclear copies of mitochondrial genes (numts or pseudogenes), the mitochondria were isolated from the nucleus of four frozen individuals of *L.ultima* using a modification of the Arnason Differential Centrifugation protocol (Jennings and Halanch 2005). The DNA from the mitochondrial fraction was extracted separately from the nuclear fraction.

For *L. ultima,* the universal primer 16aR (Kocher et al. 1989) and a *Ledella* (or Nuculanidae-specific) primer Lu16r4 (Chase et al. 1998a) yielded divergent fragments from whole DNA extraction approximately 400 bp in length from the 3' half of the 16S gene (List of primers used in this study; Table 1). For *L. sublevis,* the primer combination 16aR and Lu16R4 yielded divergent 400-bp fragments, both of these fragments aligned to the *L. ultima* 16S fragments. To determine if these divergent sequences could be found within an individual,

Table 1 List of primers used in this study

Primer name	Sequence 5′ to 3′	Reference
General primers for 16S Nuculanidae		
16Sar	ATGTTTTTGATAAACAGGCG	Kocher et al. (1989)
Lu16R4	GCTGTTATCCCTCCAGTAAC	Chase et al. (1998a)
Ledella ultima M/F specific for 16S- Forward primers also work with Lu16r4		
LedFa16F	AGTTCCTGCTCAATGATAATAA	This study
LedFa16R	CCAGTTGCCCCAACTAAAATT	This study
LedMa16F	TTCTGCTCAATGGTGTRCG	This study
LedMa16R	AAACACACCATAAGCCAAAAC	This study
Ledella sublevis M(b)/F(a) specific for 16S work with Lu16R4		
Lsa116F	TTTTATGAAAGAAGAATTTAACTTTGC	This study
Lsb116F	GTTTATGAAAGTAAAAATTAACCTTGT	This study
Ledella ultima M/F specific for Cytb		
LucytbF (male primer)	TCCCTGAGGTGGGAAATAA	Chase unpublished
LucytbR (male primer)	CCTTCAACACAAAATATTTATAAAACC	Chase unpublished
Lucytb2F	TCYTTTTGAGGSTTAACTGTT	This study
Lucytb3R	TRAARAAACGWGTTAATGTAG	This study
LucytbFFor (female primer)	AACATTGGTACAAGAGTTGC	This study
UALuFcytbR (female primer)	CCTRTTAGATCTTTAAGCGA	This study
18S primers—protobranch specific		
Proto18sA2F	ATGCATGTCTAAGTACANACT	This study
Proto18bb2R	AACCACGGTAGGCATATCA	This study
28S primers		
28sa	GACCCGTCTTGAAACACGGA	Giribet et al. (2006)
28SRA	GAAAAGARAACTCTTCCCGG	Chase unpublished
H3 primers		
H3F	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. (2000)
H3R	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (2000)

"male"- and "female"-specific primers were developed by targeting areas of high divergence between the 2 types from an alignment of sequences for each species (Figures S1 and S2).

Forward and reverse primers for the presumptive male (Led16MaF/Led16MaR) and female lineages (Led16FaF/Led16FaR) were used in separate PCR. PCR was conducted in 50-µl reaction volume consisting of 2 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25 mM $MgCl_2$, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H_2O (for fresh material) or 10 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25 mM $MgCl_2$, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, 0.5 µl Promega Hot start Taq and H_2O (for FFEP specimens). All extractions and PCR amplifications of FFEP samples were conducted with separate equipment and reagents in a separate room to prevent spurious results due to contamination. PCR conditions were initial 2-min denaturation at 94 °C followed by 5 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, then 35 (or 40 for FFEP) cycles 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C.

To confirm the presence of mitochondrial heteroplasmy in *L. ultima,* the mitochondrial gene cytochrome *b* (cytb) was also amplified. The primer pair LucytbF and LucytbR were previously developed in the laboratory (Chase unpublished), PCR resulted in products only in presumptive males. To design primers for the "female" cytb copy, degenerate primers Lucytb2F and Lucytb3R were developed from an alignment of *Nuculana commutata* (GenBank accession number GQ166622), *Nucula nucleus* (GenBank accession number GI 122003918) and the "male" *L. ultima* cytb sequences. Resulting PCR products were cloned using PGEM T easy kit (Promega). These sequences were added to the alignment, and the primers LucytbFFor and UALuFcytbR were designed to overlap the region of cytb amplified by the "male" pair (Table 1). For both primer sets, PCR was conducted in 50-µl reaction volume consisting of 2 µl undiluted DNA, 10 µl Promega flexi buffer, 5 µl 25 mM Mg Cl_2, 2.5 µl BSA, 2 µl each primer, 1 µl

PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H_2O. PCR conditions were initial 2-min denaturation at 94 °C followed by 5 cycles of 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C then 30 cycles 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C followed by a final 5 min at 72 °C.

For *Ledella sublevis*, the "female" Ls16a1F or "male" Ls16b1F primers were used in combination with the reverse primer Lu16R4 in separate reactions. PCR was conducted in 50-µl reaction volume consisting of 2 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25 mM Mg Cl_2, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H_2O. PCR conditions were initial 2-min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C. PCR products were run out on a 1.5 % agarose gel stained with EtBr, and the reactions were scored as positive or negative depending on whether a band was present or not. Positive PCR products were sent to Agencourt (Beverly, MA) or MGH (Cambridge, MA) for direct sequencing. Sequences were submitted to GenBank (Accession numbers 16S HQ907887–HQ907914; cytb JX435273–JX435297).

To rule out the possibility of cryptic species or contamination, the nuclear genes 18S, 28S and H3 were also amplified from a subset of *L. ultima* and *L. sublevis* individuals where divergent 16S sequences had been obtained. For 18S, the protobranch-specific primers Proto18sA2F and Proto18bb2R (Boyle in prep) were used with the following PCR conditions: initial 2-min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, then one cycle of 72 °C for 5 min. To amplify the 28S gene, the primers 28a (Giribet et al. 2006) and 28SRA (Chase unpublished) were used with the following PCR conditions initial 2-min denaturation at 94 °C followed by 5 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min 30 s at 72 °C, then 30 cycles of 1 min at 94 °C, 1 min at 59 °C, 1 min 30 s at 72 °C, followed by one cycle of 72 °C for 10 min. For the H3 gene, we used the primers H3F and H3R (Colgan et al. 2000) with the following PCR conditions: initial 2-min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, then one cycle of 72 °C for 10 min. Positive PCR results were submitted for direct sequencing at Agencourt (Beverly, MA) or MGH (Cambridge, MA) to confirm identification.

Data analysis

Sequences were edited and initially aligned in Sequencer ver 4.8. (Gene Codes); within-species alignments for each gene were adjusted by eye within MacClade4 (Maddison and Maddison 2003). MEGA 5.5 was used to calculate K-2P distance between "male" and "female" sequences, transition/transversion ratios and neighbor-joining trees (Tamura et al. 2011) with *Nuculana minuta* (GenBank accession number DQ280030) as the outgroup for 16S and *Nuculana commutata* (GenBank accession number GQ166622) as the outgroup for cytb. A Homogenity test of base pair composition using χ^2 was used to test for differences in base frequencies between "male" and "female" sequences. Maximum likelihood (in PhyML (Guindon and Gascuel 2003)) and Bayesian phylogenies (in BEAST (Drummond and Rambaut 2007)) were inferred from the cytb sequences for *L. ultima* using the HKY+G model as determined by jModelTest (Posada 2008) and *Nuculana commutata* as an outgroup.

Alignments 16S sequences were submitted to the RNAz webserver (Gruber et al. 2010) to determine if the male and female 16S sequences produced functional products, using the longest fragments obtained from each type and species. The "male" and "female" cytb sequences were aligned to cytb sequences from *Nuculana commutata* in MacClade 4 and translated using the invertebrate mitochondrial genetic code to determine if the sequences coded for amino acids.

To evaluate the phylogenetic distribution of heteroplasmic sequences within *Ledella*, we estimated a phylogeny for the "male" and "female" 16S sequences of *L. ultima* and *L. sublevis* combined with sequences from additional congeners *L. pustulosa pustulosa*, *L. p. marshalli* and *L. ecaudata* (GB accession numbers HQ907911–HQ907913). Because the male and female primers for *L. ultima* and *L. sublevis* did not have a large region of overlap, we used a subset of individuals for which we had a larger fragment of 16S. All sequences were aligned using MUSCLE (Edgar 2004) and adjusted by eye within MacClade4 resulting in a trimmed 216 bp alignment. Maximum likelihood (in PhyML (Guindon and Gascuel 2003)) and Bayesian phylogenies (in BEAST (Drummond and Rambaut 2007)) were inferred from the 16S sequences using the HKY+G model as determined by jModelTest (Posada 2008) with *Yoldiella inconspicua inconspicua* as the outgroup.

Results

Ledella ultima

Both "male" and "female" primers successfully amplified in *L. ultima* producing fragments of 289 bp and 319 bp, respectively, for 16S and 240 bp and 210 bp for cytb. The presumptive male and female primers yielded PCR products in 29 individuals, while in 23 individuals only the presumptive female primers yielded PCR products. This was consistent for both 16S and cytb. The ratio of homoplasmic to heteroplasmic individuals for both genes is 23/29, suggesting a sex ratio that is not statistically different from 1:1 (Exact test of goodness of fit $p = 0.44$).

When mtDNA was separated from the nDNA, two individuals yielded both "male" and "female" 16S fragments and two individuals yielded only female 16S fragments. "Male" and "female" primers generated sequenceable PCR products from the FFEP samples (three were heteroplasmic; six were homoplasmic), indicating the secondary 16S sequence was not nuclear in origin. Because the formalin-fixed specimens were somewhat unreliable for PCR, we did not include them in the counts. Nuclear genes (18S, 28S and H3) were not divergent between a subsample of individuals with divergent 16S sequences.

Although the sex of individuals cannot be directly confirmed, for the sake of clarity, and because the reverse is unlikely (Zouros 2000), we refer to heteroplasmic individuals as males and homoplasmic individuals as females. BLAST searches to Genbank identified the confamial *Nuculana minuta,* as the closest match to both 16S types. "Male" and "female" 16S sequences from *L. ultima* were quite divergent (K2P distance of 0.210) and clustered separately on a neighbor-joining tree with "female" haplotypes more distant (K2P distance of 0.248) from the outgroup *Nuculana minuta* (Figure S3). They also clustered separately on a Bayesian tree with high support values (Fig. 1). "Male" and "female" cytb sequences were even more divergent (K2P distance 0.41) with the female type more similar to the outgroup (K2P distances 0.478 M vs. 0.374 F) and the "male" and "female" sequences forming distinct clades on a Bayseian with high support values (Fig. 2). There was no significant change in base pair composition between the 16S fragment of "males" and "females" (M vs. F $\chi^2 = 0.585$, $p > 0.05$, $df = 3$). In all cases, the sequences were AT rich, as is typical of molluscan mtDNA. Transition/transversion bias for16S was $R = 3.108$ and for cytb was $R = 2.72$. "Male" and "female" 16S fragments were predicted to be RNA (SVM RNA-class probability: male 0.998; female 0.993) while "male" and "female" cytb sequences coded for amino acids with no stop codons and with 70 and 73 % amino acid similarity to *Nuculana commatula*.

Ledella sublevis

For some individuals of *Ledella sublevis,* two sets of primers yielded 16S sequences. Males were considered those individuals where both the Ls16b1F primer and the Ls16a1F primer produced sequences (both paired with Lu16R4), while those in which only the Ls16a1F primer produced sequences were considered "females". Male and female 16S sequences (166 bp) were less divergent (K2P distance of 0.162) than those from *L. ultima*, but still formed separate clades (Fig. 1; S4). As with *L. ultima*, the male type was more similar to the 16S sequence from the

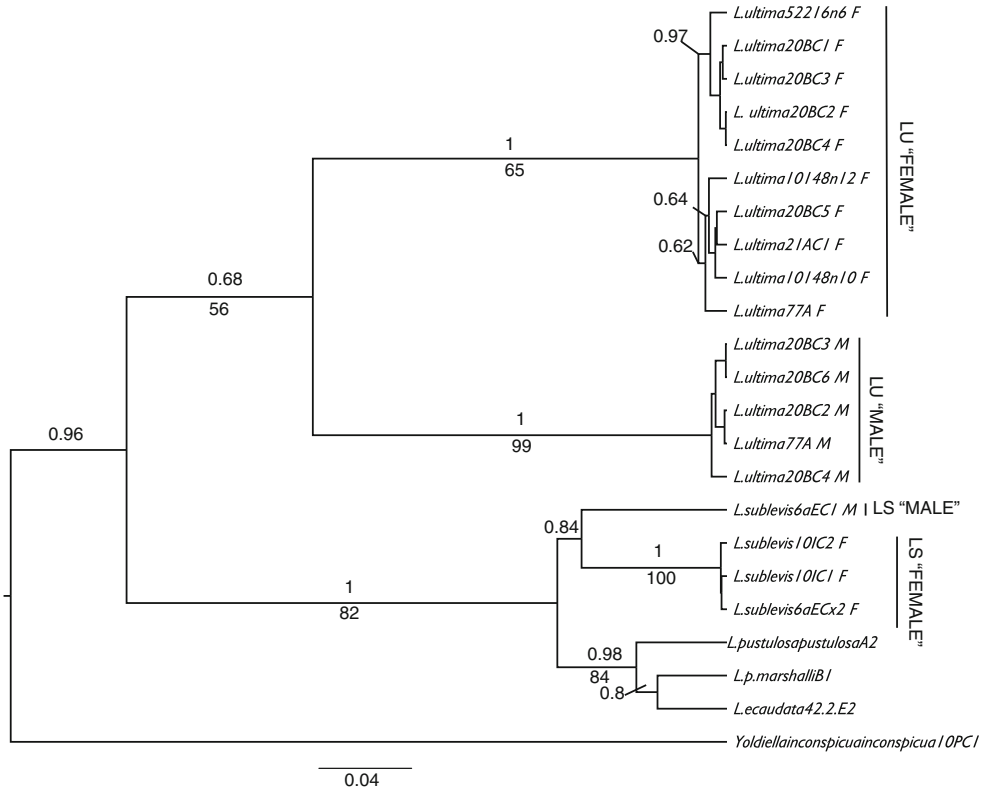

Fig. 1 Bayesian tree of *Ledella* spp. based on 216 base pairs of 16S sequences. Labels *above* the branch indicate Bayesian posterior values >0.50, labels *below* the branch indicate bootstrap supported branches in ML trees. Clades of putative "male" and "female" 16S types are highlighted

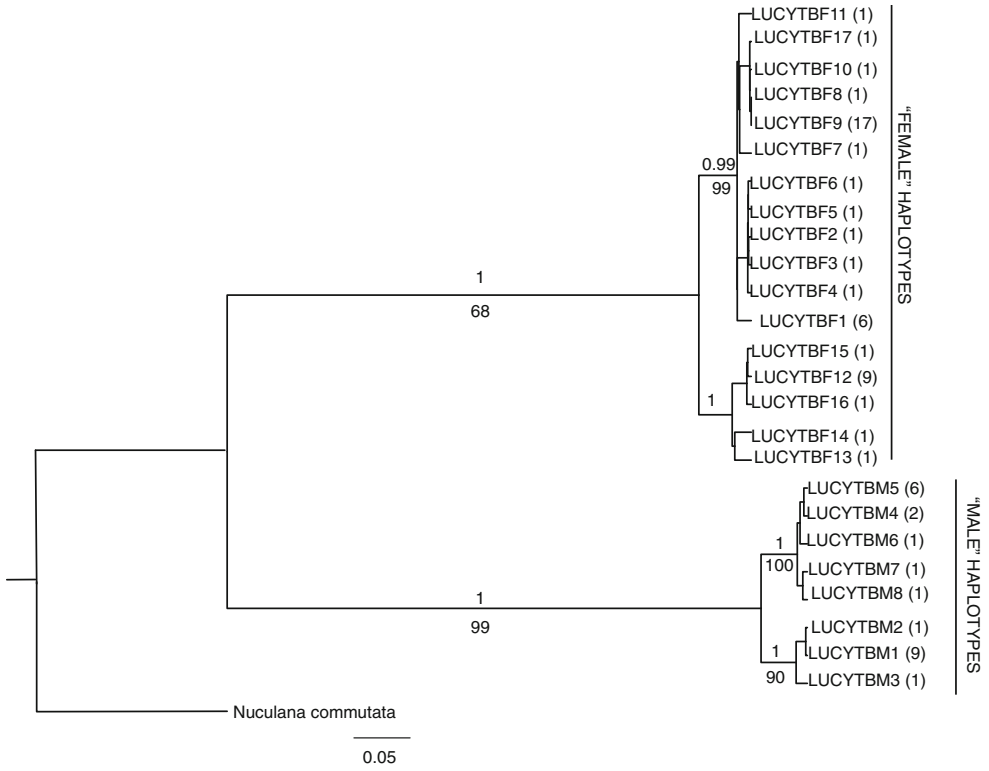

Fig. 2 Bayesian tree of *Ledella ultima* based on 187 base pairs of the mitochondrial cytb gene showing the separate clades for putative "male" and "female" cytb haplotypes of *Ledella ultima* in relation to the outgroup *Nuculana commutata.* Labels *above* the branch indicate Bayesian posterior values >0.50, labels *below* the branch indicate bootstrap supported branches in ML trees. Number in parenthesis indicates how many individuals had that haplotype

outgroup *Nuculana minuta* (K2P distance of 0.787). The ratio of individuals with both types (males) to those with one type (females) was 4/3, which was not significantly different from a 1:1 ratio (exact test of goodness of fit $p = 0.5$). There was no significant change in base pair composition between the 16S fragment of males and females (M vs F $\chi^2 = 0$ .0972, $p > 0.05$, $df = 3$). Transition/transversion bias was $R = 16.354$ which is considerably greater than *L. ultima*. The RNAz model predicted both male and female *L. sublevis* 16S sequences were RNA (SVM RNA-class probability: male 0.904; female 0.76). There were fewer longer 16S sequences available for RNAz analysis of the *Ledella sublevis* "female" which might explain the lower probability value. Nuclear genes (18S, 28S, and H3) were identical between individuals with divergent 16S sequences.

Phylogenetic analyses produced trees where "male" and "female" 16S haplotypes formed distinct intraspecific clades (Fig. 1) for both species. Only the Baysian tree is shown because both methods produced similar overall topology. We indicate significantly supported branches from the maximum likelihood analysis on the Bayesian tree.

Discussion

The presence of two distinct haplotypes within some, but not all individuals of *L. ultima* for both 16S and cytb is strongly indicative of mitochondrial heteroplasmy. In the congener *L. sublevis* divergent 16S within some individuals but not all is suggestive of mitochondrial heteroplasmy, but due to the lack of specimens for this deep-sea species, there is less data to support this finding. The ratios of heteroplasmic/homoplasmic individuals in both species were also similar to known sex ratios of protobranchs, which are generally even (Zardus 2002). These findings add to the growing phylogenetic and taxonomic occurrence of mitochondrial heteroplasmy reported from seven other bivalve families (Theologidis et al. 2008) and represent the first record in the protobranchs, the most basal lineage within the bivalves (Giribet and Wheeler 2002; Smith et al. 2011).

Evidence for heteroplasmy

The recovery of divergent and functional 16S and cytb sequences from individual clams combined with the lack of variation at nuclear loci (18S, 28S, and H3) is consistent

with heteroplasmy and rules out alternative explanations involving contamination or cryptic species. Contamination is unlikely because the "male" sequences are unique, meaning they are not identical to any of our other amplified sequences within the laboratory or to those deposited in GenBank, they were retrieved independently from samples processed with separate equipment and reagents in a different room, and our negative controls were blank. The consistent amplification of divergent 16S and cytb sequences from individual clams, and the lack of variation at nuclear loci suggests the divergent sequences do not represent cryptic species. The closest match in BLAST searches to both the female and male types was *Nuculana minuta,* suggesting the mtDNA was Nuculanidae in origin and not parasitic or exogenous DNA.

Several lines of evidence suggest the divergent sequences are not nuclear mitochondrial pseudogenes. The presence of divergent functional 16S and cytb genes within individuals is consistent with heteroplasmy. The RNAz model predicts the 16S male and female sequences are RNA in function for both species and both male and female types of cytb sequences coded for amino acids in *L. ultima*. The functionality of both male and female types from both genes strongly suggests neither is a numt. Transition/Transversion bias (*R*) is greater than 0.5 in both species indicating a clear transitional bias and suggesting male and female forms might be under selection (Yang and Yoder 1999; Lü et al 2002) and more likely to be functional. In addition, if one of the two 16S sequences was a Numt, we would expect the sequence to amplify in most individuals, but be less likely to amplify in FFEP specimens, or from individuals where the mitochondria were isolated. We found neither of these expectations to be true. Furthermore, the incidence of reported pseudogenes from Mollusca is low (Bensasson et al. 2001). A recent BLAST search using the terms "Mollusca and pseudogene" yielded relatively few hits (30) compared to other taxa (e.g., crustaceans (Buhay 2009)), and most of these matched COI or tRNAs.

Divergence of male and female mitotypes

The degree of divergence between the "male" and "female" 16S haplotypes (27 % in *L. ultima* and 15 % in *L. sublevis*) is within the range of that found for bivalves with DUI, but far exceeds what we typically find among conspecifics for deep-sea protobranchs throughout the Atlantic (Chase et al. 1998b; Etter et al. 2005; 2011; Zardus et al. 2006). Geographic and bathymetric divergence of 16S haplotypes among conspecifics vary with depth, but divergence is generally less than 10 % throughout the Atlantic (Chase et al. 1998b; Etter et al. 2005; 2011; Zardus et al. 2006). Levels of divergence in whole mitochondrial analyses of the two gender-associated mitotypes in other species was similar to that for the protobranchs (e.g., 50 % in *Inversidae japanensis* Unionidae; 34 % in *Venerupis philippinarum* Veneridae; 37 % in *Donax trunculus* Donacidae (Theologidis et al. 2008). The average in Mytilids was about 20 % with the difference in 16S being 16 % (Mizi et al. 2005; Breton et al. 2006, Cao et al. 2009), while in *Venerupis philippinarum* the 16S divergence was 15 % (Passamonti et al. 2003).

The high rate of divergence between male and female mitotypes in bivalves suggests separation over evolutionary time scales and possibly relaxed selective constraints, particularly in males, where its function is primarily restricted to gonad tissues (Stewart et al. 1996; Zouros 2000; Passamonti et al. 2003; Breton et al. 2006). A variety of mechanisms have been suggested for faster evolution of male mitotypes including relaxed selection, higher mutation rates, the smaller population size of the M mtDNA and the higher rate of M mtDNA duplication during spermatogenesis (Zouros 2000; Passamonti and Ghiselli 2009). A faster rate of evolution in the male mtDNA would enhance the rate of divergence from female mtDNA (Liu et al. 1996; Stewart et al. 1995; 1996; Rawson and Hilbish 1995; Quesada et al. 1998; Passamonti et al. 2003).

Is this heteroplasmy due to doubly uniparental inheritance?

The primary evidence for establishing heteroplasmy in early studies was the difference in mtDNA sequences between males and females (e.g., Fisher and Skibinski 1990; Hoeh et al. 1991; Liu et al. 1996), suggesting something unusual occurred in these bivalve species. Subsequent experiments where males and females with known mitotypes were crossed confirmed the presence and nature of DUI, especially in Mytilids, for example (Skibinski et al. 1994a; Zouros et al. 1994a; Garrido-Ramos et al. 1998; Cogswell et al. 2006; Kenchington et al. 2002; 2009). Unfortunately, because deep-sea protobranchs are difficult to keep alive and probably have very long generation times (Turekian et al. 1975), experimental confirmation of DUI is impractical at this time.

This is the first report of mitochondrial heteroplasmy in the protobranchs. The cause of it is unknown, but we suggest the most likely source is DUI. The known causes of heteroplasmy are mutation, paternal leakage and paternal transmission (i.e., DUI) (White et al. 2008). Both the presence of heteroplasmy in approximately half the individuals and the high divergence between types are consistent with doubly uniparental inheritance. Other explanations for divergent types of mitochondrial genes within some individuals but not all are less plausible because they would result in relatively uncommon haplotypes with low levels of divergence (White et al 2008).

Neither of these expectations is consistent with the patterns we documented for these two protobranch species. The divergent types are found in numerous individuals from distant populations (distributed between 3,500–5,000 m and over 4,000 km for *L. ultima* and between 2,200–3,500 m over 164 km for *Ledella sublevis*), suggesting the presumptive male type is consistently inherited through the germ line. The widespread and consistent occurrence of the divergent types is not characteristic of paternal leakage (White et al. 2008) and also conflicts with being transmitted through the maternal line where mutations are often lost via a bottleneck (reviewed in White et al. 2008). The high rate of divergence between the male and female types is also inconsistent with maternal inheritance of mitochondrial mutations (e.g., isopods Doublet et al. 2008; whales McLeod and White 2010), where the divergence between types is typically a single base pair, not 15–27 % of the base pairs.

Phylogenetic implications

Mitochondrial heteroplasmy is widely distributed within the Bivalvia, occurring in seven families and five superfamilies (Theologidis et al. 2008). Phylogenetic patterns provide information about the taxonomic distribution of heteroplasmy, but remain ambiguous in deciphering its origins and evolutionary spread because of the complexities in mtDNA transmission, potential masculinization events and the limited taxa that have been carefully screened (Theologidis et al 2008; Passamonti and Ghiselli 2009). Nevertheless, its presence in the basal branch of bivalves suggests DUI is more widespread and may have originated much earlier than previously thought.

Although we cannot experimentally confirm the mitochondrial heteroplasmy found in *L. ultima* and *L. sublevis* results from doubly uniparental inheritance, the evidence presented here strongly suggests male/female mitochondrial heteroplasmy occurs in *L. ultima*. The evidence for heteroplasmy is not as strong in *L. sublevis* because it is typically less abundant than *L. ultima*, limiting sample size; however, the data available are indicative of heteroplasmy as well. If this phenomena within protobranchs is due to DUI it would support the hypothesis that DUI evolved early in Bivalvia with subsequent loss from some families. The presence of heteroplasmy in the Nuculanids significantly predates prior estimates of the origin of DUI (Theologidis et al. 2008; Doucet-Beaupré et al. 2010). Previous work documented DUI in both the Paleoheterodonta and the Pteriomorpha suggesting that it might have evolved in the branch leading to the Autolamellibranchia, about 460 Mya (Little and Vrijenhoek 2003). The occurrence of heteroplasmy in the protobranchs (palaeotaxodonta) suggests a much earlier evolution, perhaps in the early Cambrian when the bivalves are thought to have evolved from rostroconch molluscan ancestors (Waller 1998).

The nature of how DUI evolved and why it persists remains unknown (Passamonti and Ghiselli 2009). There is some suggestion that the different mitochondrial types play a role in sex determination (Saavedra et al. 1997; Zouros 2000; Passamonti and Ghiselli 2009; Breton et al. 2011; but see Kenchington et al. 2002, 2009), and if true protobranchs may be important for uncovering the nature of sex determination in bivalves. Another hypothesis for the evolution of DUI is that the M-type mitochondria affect sperm function. However, recent experiments in Mytilids have shown the M-type sperm are slower than recently masculinized M-types (Jha et al. 2008). Whatever its function, the detection of heteroplasmy in protobranchs provides an opportunity to study the early evolution of DUI.

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