# Evolutionary relationships within the *"Bathymodiolus" childressi* group

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**Abstract:** Recent discoveries of deep-sea mussel species from reducing environments have revealed a much broader phylogenetic diversity than previously imagined. In this study, we utilize a commercially available DNA extraction kit to obtain high-quality DNA from two mussel shells collected eight years ago at the Edison Seamount near Papua New Guinea. We include these two species into a comprehensive phylogeny of all available deep-sea mussels. Our analysis of nuclear and mitochondrial DNA sequences supports previous conclusions that deep-sea mussels presently subsumed within the genus *Bathymodiolus* comprise a paraphyletic assemblage. This assemblage is composed of a monophyletic group that might properly be called *Bathymodiolus* and a distinctly parallel grouping that we refer to as the *"Bathymodiolus" childressi* clade. The "childressi" clade itself is diverse containing species from the western Pacific and Atlantic basins.

*Keywords*: *Bathymodiolus* ● Phylogeny ● *Childressi* clade ● Deep-sea ● Mussel

## Introduction

Many new species of mussels (Bivalvia: Mytilidae: Bathymodiolinae) have been discovered during the past two decades of deep ocean exploration. A number of genus names are currently applied to members of this subfamily (e.g., *Adipicola, Bathymodiolus, Benthomodiolus, Gigantidas, Idas, Myrina* and *Tamu*), but diagnostic characters are not well defined morphologically or phylogenetically (Jones et al., 2006). Though systematists recognize the need for revision of the subfamily Bathymodiolinae, new species commonly receive facile assignment to the most diverse genus, *Bathymodiolus*. For

example, Gustafson et al. (1998) noted that *"Bathymodiolus" childressi* Gustafson et al. (their quotes), a newly discovered species from the Gulf of Mexico, differed from other known *Bathymodiolus* for a number of morphological characters: multiple separation of posterior byssal retractors, single posterior byssal retractor scar, and rectum that enters ventricle posterior to level of auricular ostia. They recommended that *"Bathymodiolus"* be used in quotes to imply uncertainty regarding affinity of the new species with other members of this genus. Based on a comparative analysis of soft anatomy, muscle attachment scars, and shell shape, von Cosel (2002) recognized that several recently discovered species (*"B." platifrons*

Hashimoto & Okutani, 1994, "*B.*" *mauritanicus* von Cosel, 2002, "*B.*" *tangaroa* von Cosel & Marshall, 2003, and "*B.*" sp I Barbados von Cosel & Olu, unpublished) are closely allied with "*B.*" *childressi*. A molecular phylogenetic analysis of the bathymodiolin species available to us at the time confirmed the evolutionary affinity of "*B.*" *childressi*, "*B.*" *mauritanicus* and "*B.*" *tangaroa*, and helped to define a clade that warrants recognition as a distinct genus (Jones et al., 2006). Two recent events allow us to extend our characterization of the *childressi* clade. First, we examine recently published DNA sequences (mitochondrial *ND4*) from other new bathymodiolin species that fall into the *childressi* clade. Second, we obtained novel *mtND4* sequences from dried muscle tissue attached to mussel shells, collected from Edison Seamount, ca. Papua Guinea. The ability to obtain reliable mitochondrial DNA sequences from dried adductor muscle tissue adhering to molluscan shells could allow analysis of specimens from museum collections, and thereby extend the scope of systematic studies.

## Materials and Methods

*Bathymodiolus mtND4* sequences were downloaded from GenBank (Table 1). Novel sequences were obtained from dried adductor tissue from two mussel individuals from the Edison Seamount (samples courtesy of the personal collection of V. Tunnicliffe). These new sequences are available in GenBank (DQ317306-7). Single shell valves from two individuals collected on the SO-133 cruise were stored at ambient temperature since collection ("Mussel Scarp," Edison Seamount, 44 GTVA, 1598 m, July 26, 1998). Individually packaged shells were shipped from the University of Victoria (Canada) to our research laboratory in Moss Landing, California (USA) on February 28, 2005.

Upon arrival, shells were visually examined for adhering mantle and adductor tissue. Remnants of adductor muscle tissue were present in the anterior adductor scars of both shells. Sterile forceps and scalpels were used to scrape a small piece of adductor tissue from the individual shells. The dried pieces of tissue were placed in 180 µl of ATL buffer and 20 µl of Proteinase K (Qiagen, Valencia, California, USA). Samples were digested in a 55°C water bath overnight. The remaining DNA extraction followed the "Isolation of Total DNA from Animal Tissues" protocol (Qiagen Handbook #1025017) except for the final step where the DNA was eluted only once from the Qiagen DNAEasy column using 200 µl of autoclaved Nanopure water rather than two times with the Qiagen AE Buffer. DNA purity (i.e., A260/A280) was determined using a NanoDrop ND-1000 Spectrophotometer. Amplification of the mitochondrial *ND4* gene follows the protocol of Jones et al. (2006) with appropriate positive and negative controls as well as use of aerosol-resistant pipette tips to prevent contamination. Sequences were checked and aligned using Sequencher 4.1.

Phylogenies were inferred using the maximum-parsimony (MP) search as implemented in PAUP 4.0b10 (Swofford, 1998). A heuristic approach with 10 random additions of the input taxa was used to search for the shortest parsimony trees. The robustness of parsimony trees was assessed by bootstrap analysis (Felsenstein, 1985), using the heuristic search procedure in PAUP 4.0b10 with 1000 replicates and 10 random additions of the input taxa.

Bayesian phylogenetic trees were estimated using MrBayes version 3.0b4 (Huelsenbeck & Ronquist, 2001) using the codon model for *ND4*. The Monte Carlo Markov chain (MCMC) length was $1.1 \times 10^6$ generations with 6 Markov chains, and we sampled the chain every 100 generations to minimize autocorrelation. MCMC conver-

**Table 1.** Specimen collection sites, species identifications, and GenBank accession numbers for bathymodiolin mussels.
**Tableau 1.** Sites de prélèvement, identification des espèces et numéros d'identification GenBank des échantillons.

| OTU | Location/Reference | Ocean Basin | Latitude, Longitude | Depth | GenBank # |
|---|---|---|---|---|---|
| *B. platifrons* | Hatoma Knoll | W. Pacific | 24°51'N; 123°50'E | 1468 | AB175290 |
| *B. tangaroa* | Edison Seamount | W. Pacific | 03°19'S; 152°35'E | 1598 | DQ317306* |
| *B. securiformis* | Atsumi Knoll | W. Pacific | 33°54' N; 37°20' E | 1050 | AB175298 |
| *B. japonicus* | Sagami Bay | W. Pacific | 34°58'N; 139°11'E | 1100 | AB175285 |
| *B. hirtus* | Kuroshima Knoll | W. Pacific | 24°05'N; 124°10'E | 600 | AB175299 |
| *B. sp. nov.* "Edison Seamount" | Edison Seamount | W. Pacific | 03°19'S; 152°35'E | 1598 | DQ317307* |
| *B. childressi* | Alamiños Canyon | Atlantic | 26°21'N; 94°29'W | 540-2222 | AY130248 |
| *B. mauritanicus* | West Africa | Atlantic | 00°53'N; 05°28'W | 1000-1267 | AY649810 |
| *Gigantidas gladius* | Rumble III | W. Pacific | 35°44'S; 178°30'E | 300-460 | AY649813 |
| *B. tangaroa* | Cape Turnagain | W. Pacific | 40°26'S; 178°58'E | 920-1205 | AY649811 |
| *Tamu fisheri* | Garden Banks | Atlantic | 27°50'N; 92°10'W | 546-650 | AY649814 |

\* new sequences obtained in this study

gence was assessed by visually inspecting the sample paths of model parameters (to determine an appropriate burn-in period) and by repeating the analysis at least three times with random initial parameter values (to assess the dependence of posterior distributions on initial conditions). Parameter estimates were graphically analyzed to assess stability. Log-likelihood values and associated parameters for sampled trees stabilized after approximately 5-6 x $10^5$ generations for *ND4*. Therefore, we conservatively used the last 5000 sampled trees to estimate Bayesian posterior probabilities. If ≥ 95% of the sampled trees contained a given clade, we considered it to be significantly supported by our data (Jones et al., 2006).

## Results

DNA extracted from the dried adductor mussel provided high quality DNA (A260/A280 "*B.*" *tangaroa*: 0.8, A260/A280 "*Bathymodiolus*" new species, Edison Seamount: 2.12). The full length PCR of *ND4* was about 780 base pairs (bp), which included two transfer RNAs (~ 200 bp) and the first third of the coding region for *ND4*. Confident sequence alignments were obtained for 516 bp of the *ND4* coding sequence for the novel Edison Seamount mussel samples. For "*B.*" *securiformis*, "*B.*" *platifrons*, "*B.*" *japonicus*, and "*B.*" *hirtus*, only 423 bp of *ND4* sequence was available from GenBank. All analyses reported in this paper are based on the 423 bp fragment.

Percent of nucleotide divergence, *d*, between sequences within the *childressi* clade following the GTR + gamma model with shape parameter α = 1.1361, ranged from 0.2% to 60.6% (mean of 29.1%). The inferred number of amino acid substitutions ranged from 0 to 26 of the 141 amino acid sites. "*Bathymodiolus*" *tangaroa* from the Kermadec Arc was nearly identical (*d* = 0.20) to "*B.*" *tangaroa* from the Edison Seamount 4,700 km away. "*Bathymodiolus*" *platifrons* is closely related to "*B.*" *mauritanicus* and "*B.*" *childressi* (*d* = 7.2 and 9.0, respectively). *Gigantidas gladius* and the undescribed species from Edison Seamount (Fig. 1) are clearly the most divergent species examined in the *childressi* clade.

Of 423 nucleotide positions, 171 (40.4%) were variable and 104 (24.6%) were parsimony informative. An exhaustive search identified a single most parsimonious tree of 329 steps. The Bayesian phylogenetic tree was identical in topology to the MP tree. Therefore, we show the Bayesian phylogenetic tree (Fig. 1) with Bayesian posterior probabilities (BPP) and maximum-parsimony bootstrap support.

Within the *childressi* clade, four species are from the reducing environments near Japan, two are from the Edison Seamount, two are from the Kermadec Arc, and two are from the Atlantic Ocean basin (Table 1). *Gigantidas gladius*

is found on shallow hydrothermal seamounts while two of the members of the *childressi* clade, "*B.*" *japonicus* and "*B.*" *platifrons*, occur in both seep and vent environments off Japan (Miyazaki et al., 2003). The remaining known members of the *childressi* clade are known only from seep environments, though this may change with discovery of new populations in the western Pacific. The present results are consistent with the conclusion made by Won et al. (2002) and Jones et al. (2006) that mussel species are capable of habitat reversals.

## Discussion

The "*Bathymodiolus*" *childressi* clade is comprised of the most geographically diverse assemblage of deep-sea mussel species. It is the only clade of deep-sea mussels that has representatives in the Gulf of Mexico, the Atlantic Ocean, and the western Pacific Ocean. This group also is particularly interesting because of the diversity of habitats and environments in which these mussels are found. Especially important to the understanding of gene flow in deep-sea mussels is the discovery of species such as "*B.*" *platifrons* and "*B.*" *japonicus* that occur in cold seep and hydrothermal vent areas (Miyazaki et al., 2003). Species such as these provide further evidence that deep-sea mussels are habitat opportunists.

Additionally, discovery of new species and populations such as those from the Edison Seamount fills gaps in our growing knowledge of dispersal and connectivity between vent fields and ocean basins. Surveys of shallow-water seamounts in the Kermadec Arc have revealed an amazing amount of diversity in species and habitat composition (Rowden et al., 2005). Increased sampling of reducing environments in the western Pacific may reveal hitherto unknown connections between ocean basins as well as increase the phylogenetic breadth of known mussel species.

Finally, our study illustrates the utility and methodology whereby DNA can be obtained from museum preserved mussel shells. DNA extractions from museum samples often fail for two main reasons. First, tissues have been exposed to enzymes and bacteria that degraded the DNA. As noted in Paabo et al. (2004), the main type of DNA damage is fragmentation to a small average size (i.e., 100-500 bp). Second, as a result of fragmentation, PCR amplification of DNA strands is difficult, taking significant effort to obtain even short fragments of sequence information. We have used dried adductor tissue from mussel shells that are 7 years old. While not ancient DNA, our study illustrates the utility of a standard, commercially available kit to extract sufficient quantities of high-quality DNA. In addition to *ND4*, we were also able to amplify larger fragments (18S *rRNA*, ~ 2000 bp; *mtCOI* 708 bp) using the same extraction methods. In the case of deep-sea mussels, the
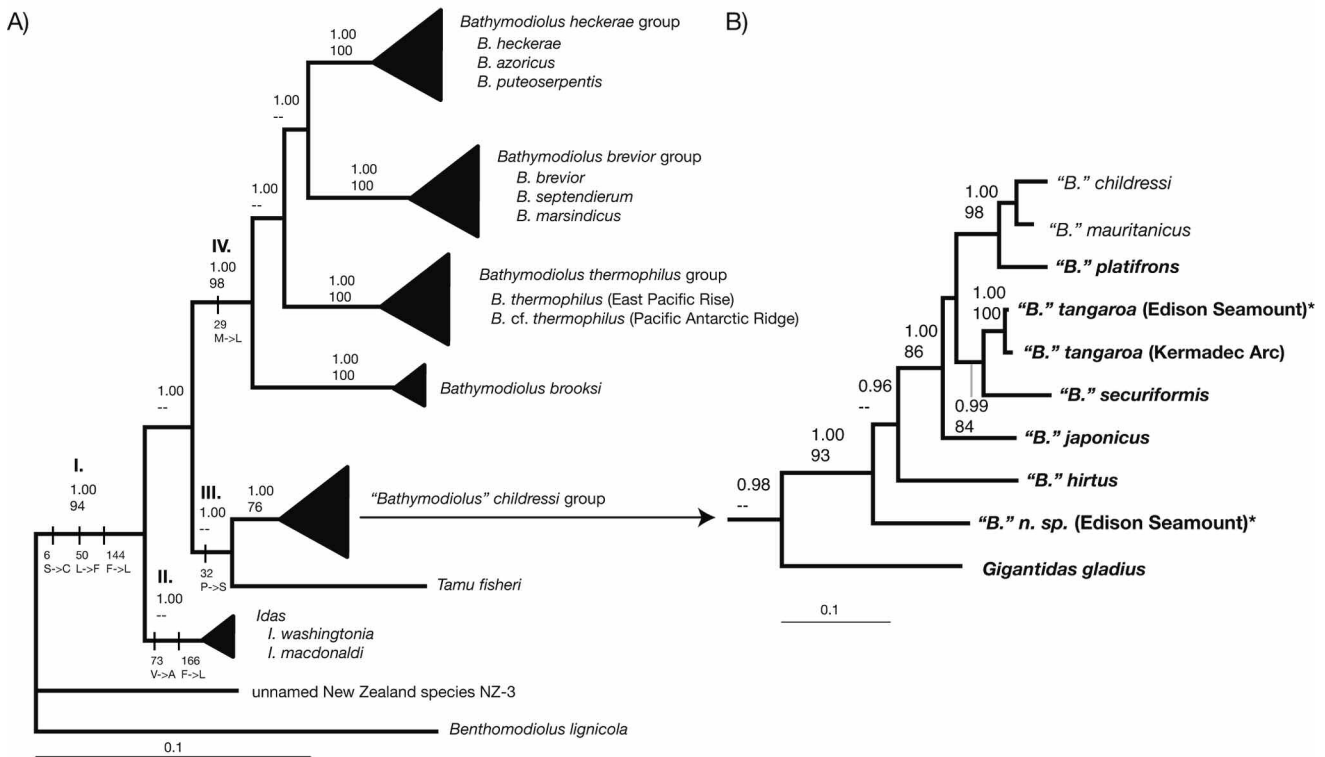
**Figure 1.** Bathymodiolin phylogenetic trees. **A.** Bayesian tree of the combined 28S *rRNA*, *ND4* and *COI* sequence data, as modified from Jones et al. (2006). Four well-supported clades (nodes I-IV) are indicated. Synapomorphic amino acid substitutions in *ND4* are indicated by hash marks and numbers that denote relative positions of the amino acids in the sequenced fragments. **B.** Bayesian tree of *ND4* for "*Bathymodiolus*" *childressi* clade rooted with *Tamu fisheri*. Western Pacific taxa are indicated in boldface. Novel sequences for this study are indicated with *. Scale bars indicate percent sequence divergence. Bayesian posterior probabilities (BPP; upper number) greater than 0.95 and maximum-parsimony bootstrap support greater than 70% are shown (lower number).

**Figure 1.** Arbres phylogénétiques des bathymodiolines. **A.** Arbre bayesien des données combinées des séquences *ND4*, *COI* et ARNr28, tel que modifié de Jones et al. (2006). Quatre clades bien identifiés (noeuds I-IV) sont indiqués. Les substitutions synapomorphiques d'acides aminés du *ND4* sont indiquées par des tirets et des nombres qui donnent les positions relatives des acides aminés dans les fragments séquencés. **B.** Arbre bayesien fondé sur les séquences de *ND4* pour le clade "*Bathymodiolus*" *childressi* enraciné avec *Tamu fisheri*. Les taxons originaires de l'ouest du Pacifique sont en gras. Les nouvelles séquences de cette étude sont indiquées par *. Les barres d'échelle indiquent le pourcentage de divergence des séquences. Les probabilités bayesiennes a posteriori (BPP ; valeur du haut) supérieures à 0,95 et les valeurs de bootstrap (maximum de parcimonie ) supérieures à 70% (valeur du bas) sont données.

DNA may be preserved in adhering tissue because of the rapid desiccation or the DNA adhesion to the mineral matrix in the shell (Paabo et al., 2004). This method advances our ability to examine DNA sequences from earlier oceanographic expeditions that did not focus efforts on preservation of tissues for genetic analysis.

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