Testing relationships of a new form of boring bivalve: the interesting kind

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Abstract

Sunken wood in marine environments supports a diverse community, the stars of the show being the wood-boring bivalves. Shallow versus deep-sea woodfalls tend to only attract bivalves from one of two clades; Teredinidae and Xylophagaidae respectively. A recent deployment of experimental wood substrates returned a new form of boring bivalve that has morphological characteristics (mesoplax and lack of viscera in the siphons) indicating it as a xylophagaid, yet has hard pallet-like structures that had previously only been observed in the shallow water teredinids. This study sought to understand the phylogenetic placement of this new taxon (Xylophagaid A) with consideration of the various siphon-associated hard parts that have been described across boring bivalve groups. I sequenced the 18S and 28S rDNA nuclear genes from Xylophagaid A and *Xylophaga zierenbergi* and aligned these with sequences from Distel et al. (2011) for Maximum Likelihood and Bayesian phylogenetic analyses. The resulting trees confirm Xylophagaid A as a member of Xylophagaidae and indicate a close relationship to *Xyloredo,* a genus that has a calcareous tube. Additional taxonomic sampling and closer morphological investigations are required, but this study indicates preliminary evidence for convergence in siphon-associated hard parts in wood-boring bivalves.

Introduction

Organic falls are patchy reducing habitats that reflect diversification patterns and processes in the marine environment and lend themselves well to manipulative experiments. In the food-poor deep-sea environment, a whale fall, wood fall or any other

relatively large organic input is a valuable resource to those organisms that can take advantage of it as a colonization substrate, or direct or indirect food source (Smith et al. 2006). Past experimental deployments of organic substrates in both tropical and temperate regions have attracted a diverse community of organisms, many of which are specialists on wood and other organic substrates, such as the wood-boring bivalves in Xylophagaeinae (Voight 2007; Samadi et al. 2010). Driftwood is an important habitat and food source for many organisms at every point of its transport from river, to estuary, to ocean surface, and eventually to the benthos across a wide depth range (Maser and Sedell 1994). The primary taxa specializing on sunken wood habitats are Teredinidae and Xylophagaidae wood-boring bivalves (Pholadoidea). These bivalves act as ecosystem engineers that literally carve out space for other animals by consuming wood while enriching the space within the wood and the surrounding sediments with their fecal pellets (Turner, 2002, Voight 2007). Taxonomy based on morphological characters and recent molecular phylogenetic analyses split xylophagous wood-boring bivalves into either Teredinidae or Xylophagaidae (Distel et al. 2011).

These two groups are generally segregated into shallow and deep waters respectively. A key synapomorphy of teredinids is the presence of pallets, a feature on the end of the siphon that is used to block the bore hole; a convenient feature in shallow water when one's bore hole may be exposed to air (Turner 2002). Teredinids also have a smooth calcareous burrow lining. Xylophagaids do not have calcified pallets, but some genera have other hard structures associated with the siphon. The only known xylophagaids to possess a calcareous tube lining are species of *Xyloredo,* yet it differs from typical

teredinid linings in that it has distinct concentric growth lines along the length of the tube (Fig. 1DEF), and some even have lateral blades on the inner wall of the distal end of the tube (Haga and Kase 2008, Turner 1972). *Xylopholas* possesses siphonal plates, which are composed of a chiton-like material that likely offers protection from predators (Fig. 1G). Members of Pholadidae often have a siphonoplax; a chitonous, or more rarely calcareous, extension of the valve which surrounds and protects the siphon (Turner 1954). Thus, it seems that many members of Pholadoidea have the capacity to develop a wide range of structures (Distel et al. 2011). The patterns of evolution for this group and the siphonal characters discussed above are still poorly understood and require additional sampling, inclusion of taxa in phylogenetic analyses, and morphological investigations.

A recent experimental deployment of wood to the deep-sea returned with it several xylophagaid taxa, including one that possesses pallet-like features, previously unobserved in deep wood-borers (hereafter referred to as Xylophagaid A). This taxon has been designated as a xylophagaid because it lacks an apophysis (thin extension of the inside shell), possesses a mesoplax and has the other shell and siphonal features typical of a xylophagaid, except for the pallet-like structures at the end of the siphon (Fig. 1ABC, identified by Janet Voight). The aims of this study were to (1) use molecular markers from nuclear 28S and 18S rDNA genes to phylogenetically place Xylophagaid A, and *Xylophaga zierenbergi* (from the same deployment, Fig. 1HI) with sequences from a recent tree of the Pholadoidea (Distel et al. 2011) and (2) discuss the siphonal features of wood-borers in a phylogenetic context.

Methods

Experimental deployments and sample recovery

Material from ten phylogenetically diverse plant species including representatives of tree fern (*Cyathea sp*), *Ginkgo*, pine (*Pinus pinea*), redwood (*Sequoiadendron sempervirens*), yew (*Torreya californica*), monocot (*Washingtonia filifera,* palm), early eudicot (*Dendromecon rigida,* tree poppy), and other representative angiosperms (*Calycanthus occidentalis* spice bush*, Quercus agrifolia* coast live oak*, and Lyonothamnus floribundus*) was bundled separately in 3mm mesh, tagged with numbered polyurethane tags, weighed, and measured. Plant bundles were deployed in triplicate at 3200m at the "Deadwood 2" site (36° 15.6768' N, 122° 40.6790' W) by ROV *Doc Ricketts* on an MBARI cruise aboard the *R/V Western Flyer* organized by Jim Barry from October 17-22, 2011.

Bundles were placed arbitrarily every 3m in three rows, each row separated by 5m. Bundles were retrieved after 2 years, and animals and pieces of wood containing animals were preserved in ethanol onboard the ship. Subsequently, animals were removed from the wood and sorted to morphotypes. Bivalve morphotypes were identified by Janet Voight, and the following are included in this study: *Xylophaga zierenbergi* (Voight, 2007)*,* and an undescribed taxon hereafter referred to as "Xylophagaid A". Photographs were taken for comparision and discussion of siphonal features.
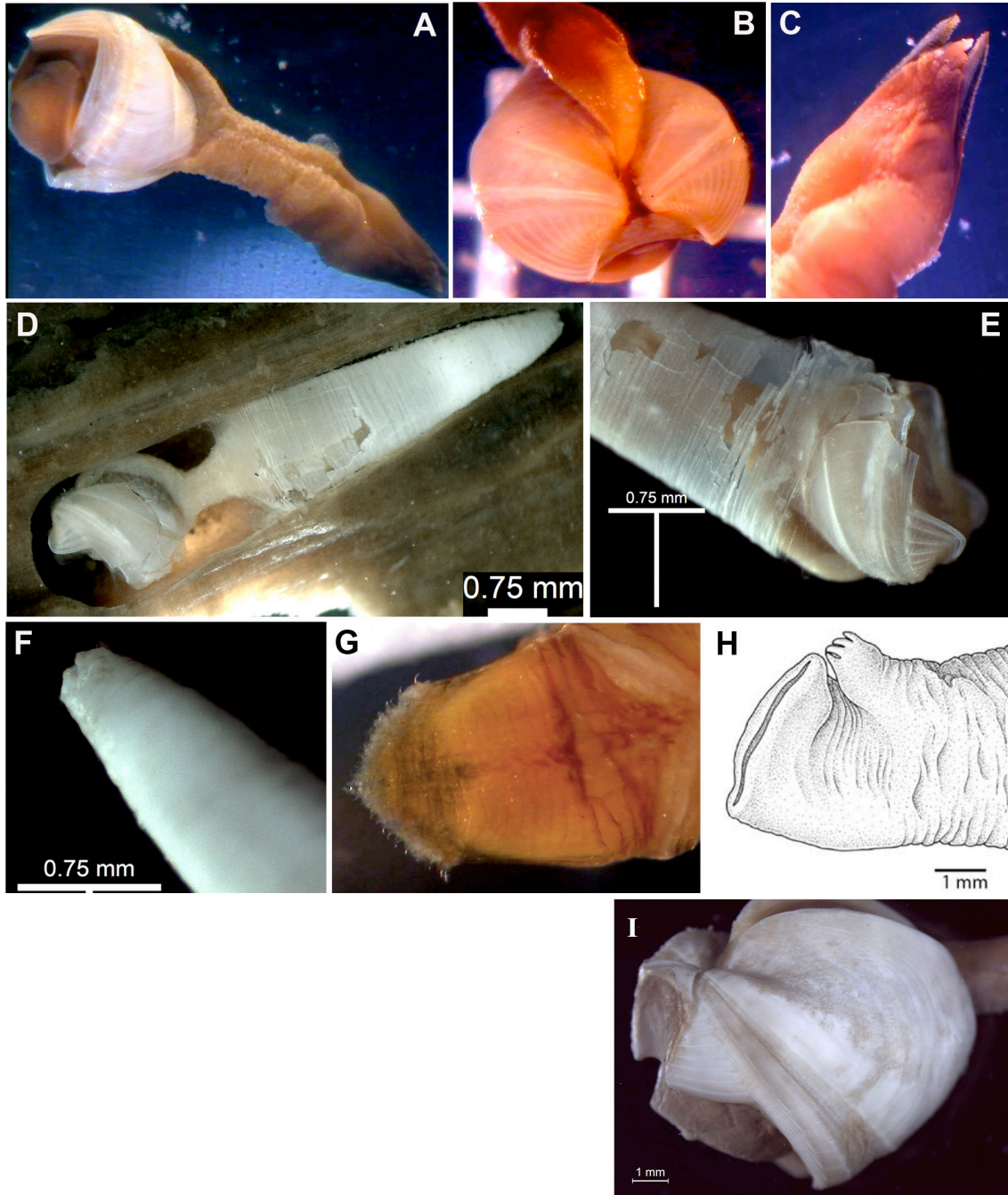
Figure 1: Xylophagaid shell morphology and siphonal details. A. Xylophagaid A B. Xylopagaid A, shell with mesoplax in view. C. Xylophagaid A, siphon end. D. *Xyloredo sp.* from Judge (unpublished). E. *Xyloredo sp.* shell and calcareous siphon tube detail. F. *Xyloredo sp.*, detail of siphon end covered with calcareous tube. G. *Xylopholas crooki* from Judge (unpublished), detail of siphon end. H. *Xylophaga zierenbergi* siphon detail (modified from Voight 2007). I. *Xylophaga zierenbergi* from Judge (unpublished).

DNA extraction, amplification, and sequencing

DNA was extracted from tissue from specimens identified as *Xylophaga zierenbergi* and

Xylophagaid A using the DNAeasy Blood and Tissue Extraction Kit (Qiagen,

www.qiagen.com). The nuclear 28S rDNA and 18S rDNA genes (hereafter 28S and 18S)

were amplified by the polymerase chain reaction using primers indicated in Table 1.

Using reagents from the Promega PCR kit (Promega, www.promega.com), each PCR

reaction contained a 50 µl reaction volume: 31.75 µl milliQ water, 10 µl 5X Green

GoTaq® Flexi Buffer, 5 µl 25 mM $MgCl_2$ solution, 1 µl PCR Nucleotide Mix (10mM

each), 0.5 µl of each primer (10µM), 0.25 µl GoTaq® G2 Flexi DNA Polymerase, and 1

µl template DNA (*X. zierenbergi* extract was diluted 1:10 and extract from Xylophagaid

A was used undiluted due to a low concentration of DNA from the initial extraction). The

following PCR program was performed to amplify 18S on a heated-lid thermal cycler:

one initial step at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 94°C, 1

min annealing at 42°C, and 1.5 min extension at 72°C, and a final extension step at 72°C

for 4 min. The program for 28S differed from 18S as follows: 94°C initial denaturation

followed by 34 cycles of 1 min denaturation at 94C, 1.5 min annealing at 45°C, and

extension for 2 min at 72°C, with a final 4 min extension at 72°C. A QIAquick gel

extraction kit (Qiagen, www.qiagen.com) was used to isolate and purify PCR products

for sequencing. Purified PCR products were sent to GENEWIZ, Inc.

(www.genewiz.com) for bidirectional sequencing using sequencing primers listed in

Table 1.

Table 1: Oligonucleotides used for priming polymerase chain reaction (PCR) amplification and sequencing of 18S and 28S nuclear genes.

Target-Primer Name	Primer Sequence (5^0 ? 3^0)	Sense	References
18S-EukF[*]	WAYCTGGTTGATCCTGCCAGT	Forward	(Medlin et al., 1988)
18S-EukR[*]	TGATCCTTCYGCAGGTTCACCTAC	Reverse	(Medlin et al., 1988)
18S-581F	CAAGTCTGGTGCCAGCAGCCGC	Forward	(Distel, 2000)
18S-560R	GCGGCTGCTGGCACCAGACTTG	Reverse	(Distel, 2000)
18S-926F	AAACTYAAAKGAATTGACGG	Forward	(Lane, 1991)
18S-907R	CCGTCAATTCMTTTRAGTTT	Reverse	(Distel, 2000)
28S-NLF184-21[*]	ACCCGCTGAAYTTAAGCATAT	Forward	www.psb.ugent.be/rRNA
28S-1600R[*]	AGCGCCATCCATTTTCAGG	Reverse	(Distel et al. 2011)
28S-D23F	GAGAGTTCAAGAGTACGTG	Forward	(Park and O' Foighil, 2000)
28S-D24R	CACGTACTCTTGAACTCTC	Reverse	(Park and O' Foighil, 2000)
28S-D5CF	ACACGGACCAAGGAGTCT	Forward	(Park and O' Foighil, 2000)
28S-D4RB	TGTTAGACTCCTTGGTCCGTGT	Reverse	(Park and O' Foighil, 2000)
28S-D6R	CCAGCTATCCTGAGGGAAACTTCG	Reverse	(Park and O' Foighil, 2000)
28S-NLF105-22	CCGAAGTTTCCCTCAGGATAGC	Forward	www.psb.ugent.be/rRNA

[*] Denotes PCR primer pairs.

Table 2: taxa examined in this study (modified from Distel et al. 2011)

Species name	Voucher number[*]	GenBank 18S	GenBank 28S	Collection site
Bankia australis	S00494	JF899202	JF899174	Manado Bay, Indonesia
Bankia carinata	S00492	JF899203	JF899175	Lac Bay, Bonaire, Netherlands Antilles
Bankia gouldi		JF899204	JF899176	Newport River, Beaufort, NC
Bankia setacea	S00489	JF899205	JF899177	Brown's Bay, WA
Barnea candida		AM774541	AM779715	
Barnea parva		AM774542	AM779716	
Barnea truncata	S00484	JF899206	JF899178	Wachapreague, VA
Dicyathifer manni	S00500	JF899208	JF899180	West coast of Minahasa Peninsula, Indonesia
Kuphus polythalamia	S00487	JF899210	JF899182	Zamboanga del Sur, Mindanao, Philippines
Lyrodus massa	S00495	JF899212	JF899184	Manado Bay, Indonesia
Lyrodus pedicellatus	S00469	AM774540	AM779714	Long Beach, CA
Martesia striata	S00501	JF899213	JF899185	West coast of Minahasa Peninsula, Indonesia
Nausitora dunlopei	S00499	JF899215	JF899187	West coast of Minahasa Peninsula, Indonesia
Nausitora fusticula	S00491	AY192697	JF899188	Praia Dura, Ubatuba, Brazil
Neoteredo reynei	S00490	JF899217	JF899189	Praia Dura, Ubatuba, Brazil
Pholas dactylus	S00483	JF899220	JF899192	Rocky shore, Charmouth, Dorset, UK
Spathoteredo obtusa	S00493	JF899221	JF899193	Manado Bay, Indonesia
Teredo navalis	S00486	JF899222	JF899194	Collection panels, Belfast pier, Belfast, ME
Teredora malleolus	S00497	JF899223	JF899195	Lagoen, Bonaire, NA, driftwood
Teredothyra dominicensis	S00496	JF899225	JF899197	Bachelor's Beach, Bonaire, NA, 3 m
Xylophaga atlantica	S00472	AY070123	AY070132	12 miles east of Southwest Harbor, ME, 100 m
Xylophaga sp.	S00488	JF899226	JF899198	SE of Port Dunford ($29°02.2^0$S, $32°19.6^0$E)800 m
Xylophaga washingtona	S00481	JF899227	JF899199	Dredged wood, Friday Harbor, WA
Xylopholas sp.	S00504	JF899228	JF899200	Gulf of Mexico ($27°44.75^0$N, 91°13.31'W) 540 m
Xyloredo sp.	S00503	JF899229	JF899201	Gulf of Mexico ($27°44.75^0$N, $91°13.31^0$W) 540 m
Xylophagaid A.		This study	This study	Monterey Bay (36° 15.68'N, 122° 40.68'W) 3200m
Xylophaga zierenbergi		This study	This study	Monterey Bay (36° 15.68'N, 122° 40.68'W) 3200m

Molecular Analyses

Sequences were trimmed and de novo assembled to make consensus sequences in

Geneious (BioMatters, New Zealand). 18S and 28S sequences for each individual were

concatenated and aligned with a subset of pre-concatenated sequences from Distel et al.

(2011) (see Table 2 for complete list of specimens included in this study) using MUSCLE (Edgar 2004) implemented in Geneious and further aligned by eye. Gblocks (Castresana 2000) was applied to the alignment selecting parameters that permitted gaps and smaller blocks in the final alignment. Maximum likelihood and Bayesian phylogenetic analyses were run using the RAxML BlackBox (Stamatakis et al. 2008) and MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) in the CIPRES Science Gateway (www.phylo.org). The parameters used in both analyses were not rigorous (minimal defaults on web-based servers were used), thus trees produced are preliminary trees for the future development of this study. For the RAxML analysis, defaults were used and the boxes for 'Gamma model of rate heterogeneity' and 'Maximum likelihood search' were checked and an outgroup was designated (*Pholas dactylus, Martesia striata, Barnea truncata, Barnea candida, Barnea parva*). The MrBayes run had a burn in of 10 with 500 generations.

Results

Maximum likelihood (ML) and Bayesian (BI) phylogenetic analyses returned phylograms with similar topologies that had two regions of variability (indicated by pink stars in Figure 2). The bootstrap support values in the ML tree are generally lower and more variable than in the BI tree. A well-supported clade containing all Xylophagaids contains Xylphagaid A in a sister relationship with *Xyloredo sp.* in both trees (72 bootstrap and 100 posterior probability). *Xylophaga zierenbergi* comes out sister to *Xylophaga atlantica* in both analyses with high support (100/100) (Fig. 2).
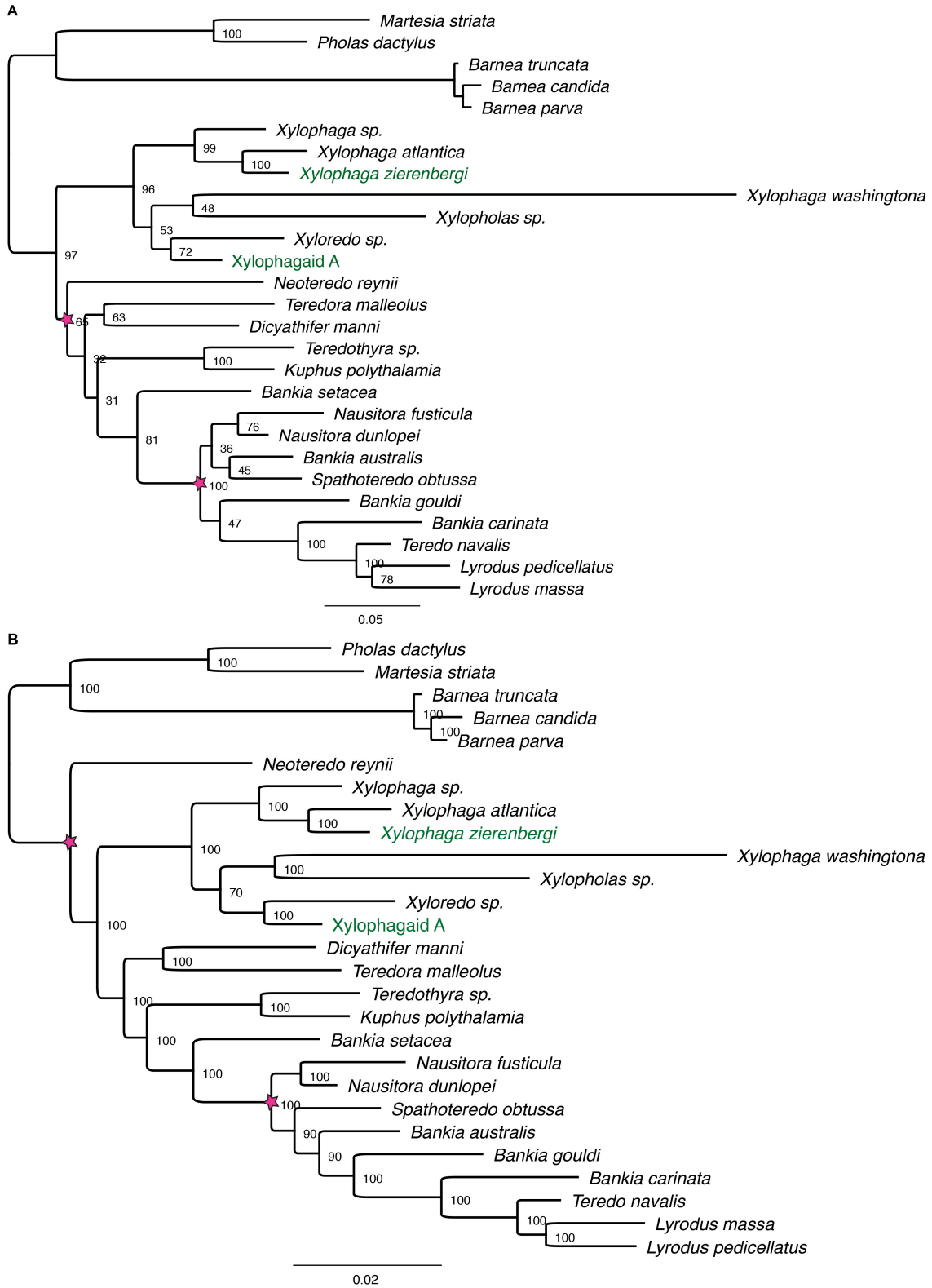
Figure 2: Phylogenetic trees. A. Maximum Likelihood phylogram B. Bayesian inference phylogram. New sequences from this study are indicated in green. Pink stars denote clades with differing topologies between trees.

<u>Discussion</u>

The primary outcomes of the phylogenetic analyses were firstly that Xylophagaidae is monophyletic (as in Distel et al 2011) with *Xylophaga zierenbergi* sharing a most recent common ancestor with *Xylophaga atlantica.* Secondly, Xylophagaid A shared a most recent common ancestor with *Xyloredo sp.* within a clade also containing *Xylopholas sp.* and *Xylophaga washingtona* (Fig. 2). The topologies found in the trees presented here do not differ significantly from that presented in the taxonomically overlapping portion of Distel et al. (2011). Thus, for the purposes here of confirming Xylophagaid A as a Xylophagaid and hypothesizing its placement, these analyses will be discussed at face value. Subsequent analyses implementing models of evolution confirmed to be adequate (rather than using minimum default settings through web-based servers and portals) with larger taxonomic sampling will be applied in a future, more thorough study.

 Within a morphological context, it is interesting and logical that Xylophagaid A be closely related to *Xyloredo sp.* and *Xylopholas sp.* because all three of these genera have hard structures associated with their siphons. When *Xyloredo* was described, it was noted by Turner (1972) that it was very similar to teredinids in that it has a calcareous tube lining the burrow, and actually looks like a teredinid superficially except that it lacks pallets and has no viscera in the siphons. Molecular analyses from Distel et al (2011) confirm her taxonomic placement of *Xyloredo* within Xylophagaidae. *Xylopholas* was also described by Ruth Turner in 1972 (republished in Turner 2002), and she notes the siphonal plates and periostracal siphon covering in comparison to the calcareous lining of *Xyloredo.* She notes that the siphonal plates are possibly derived from the siphonoplax

found in other pholadids, which also function to close the end of the burrow as pallets do in teredinids (Turner, 1954). When Xylophagaid A was examined, it presented a similar superficial similarity as noted above to teredinids, in its well developed pallet-like structures (Fig. 1C). A full investigation and description of the morphology of Xylophagaid A will hopefully provide insight into the affinities of these distinct pallet-like structures to other siphon associated features.

The wood-boring bivalves in woodfall ecosystems may be the best known of the fauna, yet there is still little known of the global diversity, and taxa continue to be discovered with nearly every new deployment or woodfall focused cruise (Voight 2007, 2008, 2009, 2012, Romano et al. 2014). Thus, hypotheses of xylophagaid evolution and history of characters will require additional sampling, phylogenetic analyses, and morphological characterizations to integrate knowledge of these interesting boring bivalves.

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