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Their Day in the Sun: molecular phylogenetics and origin of photosymbiosis in the 'other' group of photosymbiotic marine bivalves (Cardiidae: Fraginae)

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The subfamily Fraginae (Cardiidae) is a morphologically diverse group of small-bodied marine clams inhabiting shallow seas worldwide. Like the exclusively photosymbiotic giant clams (Cardiidae: Tridacninae), some fragines are known to host zooxanthellae photosymbionts. However, surveys to widely determine photosymbiotic status and the lack of a comprehensive phylogeny have hindered attempts to track the evolution of photosymbiosis in the group. Worldwide sampling of all fragine genera and subgenera with phylogenetic reconstructions based on four gene regions [nuclear (28S) and mtDNA (16S, cytochrome oxidase I, cytochrome *b*)] does not support a monophyletic Fraginae. Sampled taxa form four restructured clades: (1) the '*Fragum*' group, (2) the '*Trigoniocardia*' and '*Ctenocardia*' groups, (3) the '*Parvicardium*' group and (4) the '*Papillicardium*' group. Maximum likelihood analyses strongly support a clade of European cardiids uniting species from three subfamilies. Live examination of > 50% of species reveals that less than half of derived genera and subgenera host photosymbionts, supporting a single and relatively late origin of photosymbiosis in the Fraginae. The evolutionary implications for a small and little modified earliest diverging photosymbiotic lineage are discussed. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **97**, 448–465.

ADDITIONAL KEYWORDS: cardiids – evolution – Fraginae – marine clams – mtDNA – nuclear DNA – photosymbiosis – phylogenetics – zooxanthellae.

INTRODUCTION

The marine bivalve superfamily Cardioidea is composed of a single well-known family, the Cardiidae or cockles, with a fossil record dating to the Late Triassic (Keen, 1980; Coan, Scott & Bernard, 2000; Morton, 2000; Schneider & Carter, 2001). The family consists of least 20 genera and approximately 200 species distributed worldwide, with the bulk of recent species members of the shallow infauna of tropical seas (Vidal, 1994; Schneider, 1995; Vidal, 1997a, b; Schneider, 1998; Schneider & Ó Foighil, 1999; Vidal, 1999; Coan *et al.*, 2000; Morton, 2000; Hylleberg, 2004; Vidal & Kirkendale, 2007, although see Poutiers, 1992, 2006 for deep-water Protocardiinae). Subfamilial classification of the Cardiidae has varied greatly. Although Keen (1980) recognized six subfamilies, including the Fraginae, the most recent study reorganized higher-level cardiid diversity into three clades, with tested representatives distributed among eleven subfamilies (Schneider, 1995).

The subfamily Fraginae was first delineated by Stewart (1930) and originally included five genera and two subgenera, grouped together because they shared two shell characters: (1) a marked umbonal ridge and (2) subequal cardinals (Table 1). Most subsequent work has largely followed Stewart's original delineation (e.g. Kafanov & Popov, 1977; Keen, 1980), except for one of the most recent phylogenetic treatments that recognized twelve genera and subgenera (Schneider, 1998).

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Table 1. Membership in the subfamily Fraginae

Genera	Stewart (1930)	Keen (1980)	Voskuil & Onverwagt (1989, 1991)	Schneider (1998)	Vidal (2000)
Papillicardium	Cardiinae?*	Cardiinae	Cerastodermatiinae†,‡	X	X†
Cerastobyssum§			Cerastodermatiinae‡	X	
Parvicardium	Cardiinae?*	Cardiinae	Cerastodermatiinae‡	X	X
Trigoniocardia	X	X	X	X	X
Apiocardia	X	X	X	X	X
Lunulicardia	X	X	X	X	X
Corculum	X	X	X	X	X
Fragum	X	X	X	X	X
Microfragum		X	X	X	X
Ctenocardia	X	X	X	X	X
Americardia	X	X	X	X	X
Afrocardium		X	X	Cardiinae	X
Goniocardia¶				X	

*The question marks reflect Stewart's uncertainty with placing *Parvicardium* (including *Papillicardium*) in the Cardiinae.
†*Papillicardium* is considered a subgenus of *Parvicardium*.
‡Schneider (1998) recognizes Cerastodermatiinae as a synonym of Lymnocardiinae.
§*Cerastobyssum* is considered a subgenus of *Parvicardium* by some authors (e.g. Schneider, 1998, Aartsen & Goud, 2000, but not Voskuil & Onverwagt, 1989).
¶*Goniocardia* is the only extinct taxon.

Parvicardium: sister to all other fragines?

Conflicting views regarding the higher-level taxonomy of the earliest diverging fragines make tackling these small European bivalves critical. For example, although *Parvicardium* is supported as sister to all fragines by some authors, others place it in different cardiid subfamilies (e.g. compare Keen, 1980 with Voskuil & Onverwagt, 1991 and Stewart, 1930 with Schneider, 1998; Table 1). Membership in, and relationships among, the earliest diverging fragines are either controversial or ill defined. Sampling of just two species by Schneider (1998) yielded a paraphyletic *Parvicardium*, with *P. siculum* Sowerby, 1834 [considered a junior synonym of *P. exiguum* (Gmelin, 1791) by Aartsen & Goud, 2000] sister to *Cerastobyssum hauniense* Petersen & Russell 1971 and *P. exiguum* sister to all remaining fragines (Fig. 1).

The '*Trigoniocardia*' and '*Ctenocardia*' groups

Schneider (1998) recovered the '*Trigoniocardia*' group, composed of *Apiocardia*, extinct *Goniocardia* and *Trigoniocardia*, as a well-supported sister clade to *Corculum*, *Lunulicardia*, *Fragum* and the '*Ctenocardia*' group, comprised of *Ctenocardia*, *Americardia* and *Microfragum* (Fig. 1). Although the bulk of earlier work (Stewart, 1930; Clench & Smith, 1944; Keen, 1951; Olsson, 1961; Popov, 1977; Keen, 1980) recognized the distinction between these two groups, membership within each group differed. In contrast with Schneider (1998) and Voskuil & Onverwagt (1989), geographically proximate but morphologically disparate forms were united, with *Americardia* allied to the *Trigoniocardia* group and not to the *Ctenocardia* group.

Known Photosymbiotic Representatives: *Fragum*, *Lunulicardia* and *Corculum*

Reef-associated species in the genera *Fragum*, *Lunulicardia* and *Corculum* include the most morphologically divergent cardiids (Bartsch, 1947; Kawaguti, 1950, 1968; Trench, Wethey & Porter, 1981; Kawaguti, 1983; Ohno, Katoh & Yamasu, 1995), with photosymbiotic status (Kawaguti, 1950; Ohno *et al.*, 1995; Persselin, 1998; Schneider, 1998; Morton, 2000) and putative morphological adaptations for photosymbiosis (Watson & Signor, 1986; Persselin, 1998; Ohno *et al.*, 1995; Carter & Schneider, 1997; Schneider & Carter, 2001; Farmer, Fitt & Trench, 2001) studied and documented for decades. Since the first accounts of photosymbionts in *Corculum cardissa* (Linné, 1758) [Kawaguti, 1941 (in Japanese); Kawaguti, 1950 (in English)] several other fragine species have been found to possess photosymbionts: *F. fragum* (Linné, 1758) and *F. unedo* (Linné, 1758) (Kawaguti, 1983; Umeshita & Yamasu, 1985), *F. loochoanum* Kira, 1959 (Ohno *et al.*, 1995), *Lunulicardia retusa* (Linné, 1767) (Schneider & Carter, 2001), *F. erugatum* (Tate, 1889) (Morton, 2000) and *L.* sp. 1,

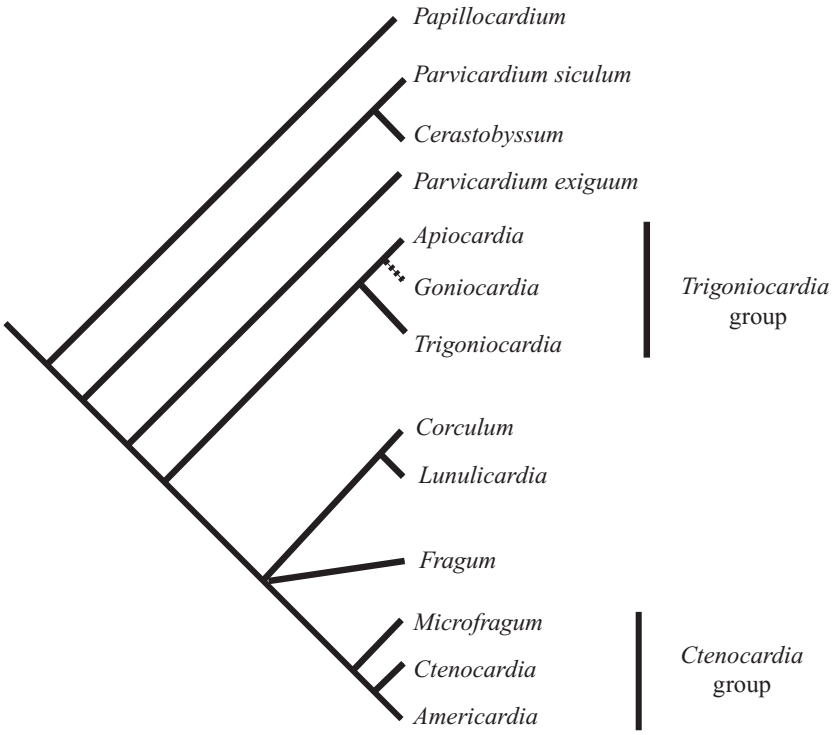

Figure 1. Fraginae phylogeny (Schneider, 1998). Dashed line indicates extinct taxon. The major difference between Schneider (1998) and Persselin (1998) is the position of *Fragum*. Persselin recognizes a paraphyletic *Fragum*, while Schneider (1998) is uncertain of the position of *Fragum*.

F. mundum (Reeve, 1845), *F. nivale* (Reeve, 1845), *F. sueziense* (Issel, 1869) and *F.* sp. 11 (Persselin, 1998). Based on observed digestive system simplifications in generic exemplars, coupled with the then ubiquitous occurrence of photosymbiosis in tested fragines, Schneider (1998) proposed that all members of the subfamily Fraginae were likely photosymbiotic. In contrast, Persselin (1998) suggested that photosymbiosis was likely restricted to *Fragum*, *Corculum* and *Lunulicardia*, but absent from other fragine lineages.

The most species-rich and poorly understood group of photosymbiotic fragines, exhibiting the widest range of putative adaptive morphologies for a photosymbiotic lifestyle, is *Fragum* (Ohno *et al.*, 1995; Persselin, 1998). A handful of representatives have been included in molecular phylogenetic treatments (Maruyama *et al.*, 1998; Giribet & Distel, 2003) or have been the focus of microstructural analyses (Persselin, 1998; Schneider & Carter, 2001), but, like all fragine genera, the group has never been revised. The position of two species of *Fragum*, *F. erugatum* and *F. sueziense*, has been especially controversial. Although both are now generally accepted as members of *Fragum*, *F. erugatum* has been placed in five different genera (see Hylleberg, 2004: 502), while *F. sueziense* has been allied with six different genera or subgenera (see Hylleberg, 2004: 793).

This study represents the most comprehensive phylogeny of fragines and, as such, offers a new perspective on difficult systematic questions in the group, including tests of monophyly at multiple phylogenetic levels. Worldwide collection and examination of fresh tissues establishes photosymbiotic status, vital to tracking the evolution of photosymbiosis in the subfamily. Placing the evolution of photosymbiosis in a phylogenetic context permits insight into the origin, distribution and geographic signature of fragine photosymbiosis. The phylogenies presented here lay the foundation for future revisionary work, comparative tests and timing estimates, as well as detailed character trait analyses to examine evidence for putative adaptations.

MATERIAL AND METHODS

SPECIMEN ACQUISITION

Fraginae were collected worldwide, resulting in genetic material of over 60% of recognized ingroup species with representatives sampled from all extant genera and subgenera (Table 2, Fig. 2A and Appendix). Subgeneric names are used throughout to denote ingroup taxa of the *Trigoniocardia* and *Ctenocardia* groups. Outgroups include representatives from four cardiid subfamilies as recognized by Schneider (1998):

Table 2. Provisional checklist of recent Fraginae

Taxa	Biogeographic region*
Fragum fragum (Linné, 1758)	Indo-West Pacific
Fragum scruposum (Deshayes, 1855)	Indo-West Pacific
Fragum loochoanum Kira, 1959	Indo-West Pacific
Fragum carinatum (Lynge, 1909)	Indo-West Pacific
Fragum mundum (Reeve, 1845)	Indo-West Pacific
Fragum nivale (Reeve, 1845)	Indo-West Pacific
Fragum unedo (Linné, 1758)	Indo-West Pacific
Fragum erugatum (Tate, 1889)	Indo-West Pacific
Fragum sueziense (Issel, 1869)	Indo-West Pacific
Lunulicardia retusum (Linné, 1767)	Indo-West Pacific
Lunulicardia hemicardium (Linné, 1758)	Indo-West Pacific
Corculum cardissa (Linné, 1758)	Indo-West Pacific
Trigoniocardia granifera (Broderip & Sowerby, 1829)	East Pacific
Trigoniocardia antillarum (d'Orbigny in Ramon de la Sagra, 1846)	West Atlantic
Apiocardia obovale (Sowerby, 1833)	East Pacific
Americardia biangulata (Broderip & Sowerby, 1829)	East Pacific
Americardia media (Linné, 1758)	West Atlantic
Americardia speciosa (Adams & Reeve, 1850)	East Pacific
Americardia planicostata (Hertlein & Strong, 1947)	East Pacific
Ctenocardia symbolica (Iredale, 1929)	Indo-West Pacific
Ctenocardia fornicata (Sowerby, 1841)	Indo-West Pacific
Ctenocardia translata (Prashad, 1932)	Indo-West Pacific
Ctenocardia virgo (Reeve, 1845)	Indo-West Pacific
Ctenocardia fijianum Vidal & Kirkendale, 2007	Indo-West Pacific
Ctenocardia gustavi Vidal & Kirkendale, 2007	Indo-West Pacific
Ctenocardia victor (Angas, 1872)	Indo-West Pacific
Microfragum subfestivum Vidal & Kirkendale 2007	Indo-West Pacific
Microfragum festivum (Deshayes, 1855)	Indo-West Pacific
Parvicardium exiguum (Gmelin, 1791)	East Atlantic
Parvicardium minimum (Philippi, 1836)	East Atlantic
Parvicardium scriptum (Bucquoy, Dautzenbery & Dollfus, 1892)	East Atlantic
Parvicardium trapezium Cecalupo & Quadri, 1996	East Atlantic
Parvicardium vroomi Aartsen, Menkhorst & Gittenberger, 1984	East Atlantic
Parvicardium scabrum (Philippi, 1844)	East Atlantic
Parvicardium pinnulatum (Conrad, 1831)	East Atlantic
Papillicardium papillosum (Poli, 1791)	East Atlantic
Papillicardium turtoni (Sowerby, 1894)	East Atlantic

*See Hylleberg (2004) for specific distributions.

Laevicardiinae, Protocardiinae, Cardiinae and Lymnocardiinae (Appendix). At least two individuals were sequenced per species, where possible, for each of four gene regions: three mitochondrial [cytochrome oxidase I (COI), 16S, cytochrome *b* (CytB)] and one nuclear (28S rDNA). All samples were fixed in ethanol, and all newly collected material (shells and unextracted tissues) are housed at the Florida Museum of Natural History (UF) (Appendix).

DNA Extraction and PCR

Total genomic DNA was obtained from ethanol-preserved muscle tissue (foot or, if the animal was < 1 cm, entire body) using DNAzol (Chomczynski *et al.*, 1997; Molecular Research Center Inc.) methodologies at one-half suggested volumes with extended digestion times (1 day–1 week). Primers D1F and D6R were used to amplify and sequence the D1–D3 domains of 28S rDNA for most species, but occasionally D2F was used in place of D1F (Park & Ó Foighil, 2000; Table 3). Universal primers were used for COI (Folmer *et al.*, 1994), 16S (Palumbi, 1996) and CytB (Kocher *et al.*, 1989), with specific COI primers designed to target taxa in the genera *Fragum*, *Lunulicardia* and *Corculum* (Table 3). PCR cocktails included 1 μL of genomic DNA template, 5 μL of 10 × buffer, 5 μL 10 mM dNTPs, 2 μL of 10 μM solution

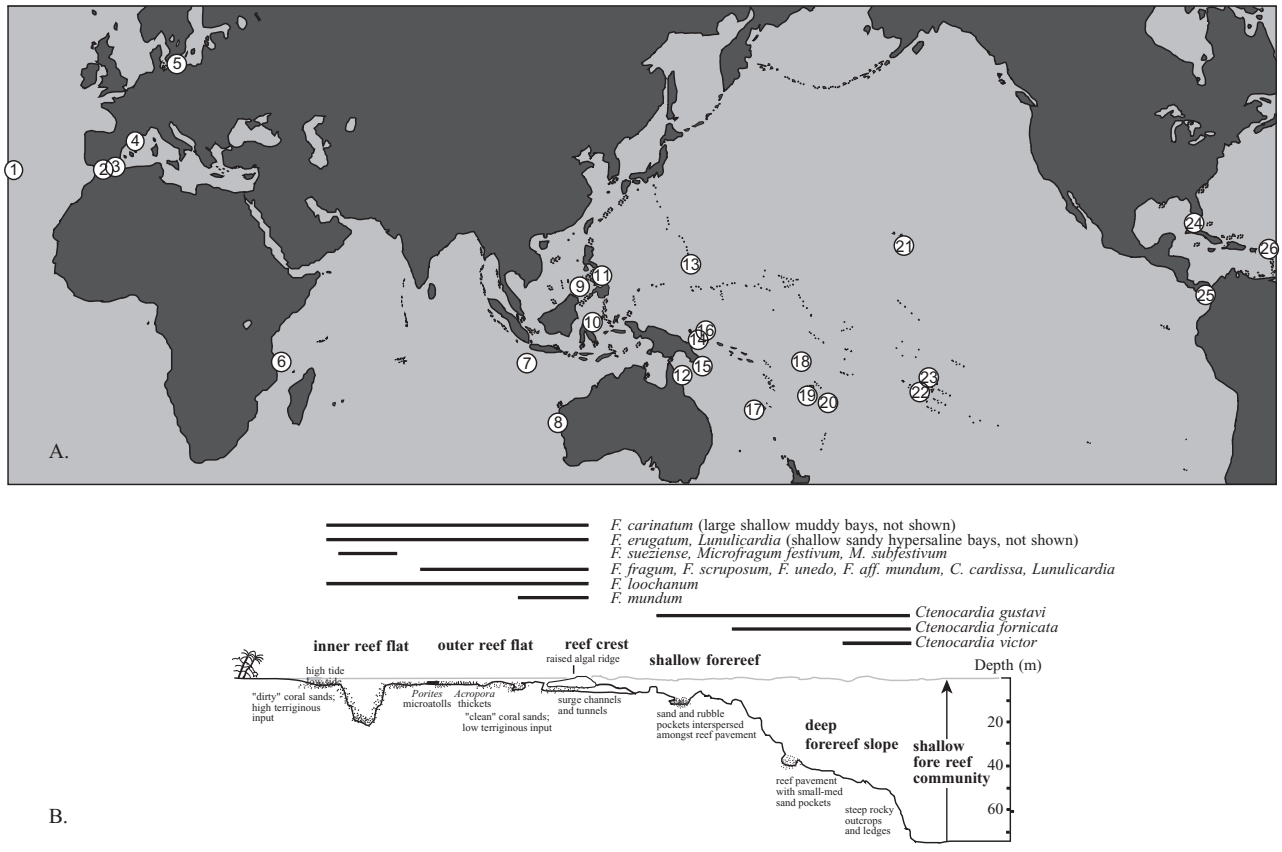

Figure 2. A, collection localities for ingroup and outgroup cardiid taxa included in the core phylogeny (numbers cross-reference to Appendix). B, microhabitats of commonly encountered Indo-West Pacific Fraginae.

of each primer, 2–4 μL of 25 mM magnesium chloride ($MgCl_2$) solution, 0.2 μL TAQ, 2.5 μL dimethylsulphoxide (DMSO) brought up to a total volume of 50 μL with double distilled water (ddH_2O). Reactions were run for 35–40 cycles with the following parameters for the mitochondrial genes: an initial 1–2.5 min denaturation at 95 °C; further denaturation at 94–95 °C for 40 s, annealing at 38–44 °C (COI, CytB), 48–55 °C (16S) for 35–45 s and extension at 72 °C for 1–3 min (with larger fragments requiring longer extension times). The 28S profile followed Park & Ó Foighil (2000) with 36 cycles: denaturation for 4 min at 94 °C followed by 40 s at 94 °C, annealing for 40 s at 55 °C and extension for 1.45 at 72 °C and 10 min at

Table 3. Primers used to sequence gene regions for phylogenetic reconstructions

Primer name/gene region	Sequence
28S-D1F	5′-GGAACTACCCCCTGAATTTAAGCAT-3′
28S-D2F	5′-TCAGTAAGCGGAGGAA-3′
28S-D6R	5′-CCAGCTATCCTGAGGGAAACTTCG-3′
LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′
HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′
FRAG1-LCO	5′-TCATTTAGWATYATKATYCGWAC-3′
FRAG2-LCO	5′-TCTTTTAGRRTWATAATYCGWAC-3′
FRAG1-HCO	5′-GACCAAAAAATCARAANARATG-3′
16Sar	5′-CGCCTGTTTATCAAAAACAT-3′
16Sbr	5′-GCCGGTCTGAACTCAGATCACGT-3′
CytBF	5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′
CytBR	5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′

72 °C. The addition of a 'hot start' step before PCR (10 min at 99 °C) was used for trouble taxa and/or gene regions. The PCR product was electrophoresed, stained, and photodocumented. Multiple PCR products, indicated by double bands, were subjected to increased annealing temperatures during subsequent rounds. Successful PCR products were cleaned for cycle sequencing using Wizard Preps (Promega) following described protocol and then visualized. Approximately 95% of sequences were generated using an ABI Prism 377 automated sequencer following manufacturers' recommendations and utilizing ABI Big Dye with DyeDeoxyTermination protocols (Perkin Elmer). A small subset of CytB sequences were generated with a Beckman CEQ 8000 (Beckman-Coulter) automated sequencer following manufacturer's recommendations.

Alignment and molecular analyses

Tier 1

Sequences were initially aligned by eye during editing in Sequencher 3.1.1 (Genes Codes). COI and CytB sequences were translated to amino acids using MacClade v4.08 (Maddison & Maddison, 2005) to assist in alignment, but were easy to align because of an absence of indels. Default parameters in Clustal X v1.81 were used to aid in alignment of the 28S gene region (Thompson *et al.*, 1997). In all analyses, gaps were treated as missing and character states were unordered. Partition–homogeneity tests, implemented in PAUP* 4.0b10 (Swofford, 2002), were run to test for significant differences among all four gene regions. Significant differences were detected among all tested gene regions and, as a result, topologies were generated and compared for each gene region to facilitate visual examination of possible conflicts.

All gene regions sampled from available Fraginae species, as well as outgroup representatives, were concatenated to construct a 'core' phylogeny, largely to test subfamily and generic monophyly. Analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) methods implemented in PAUP* 4.0b10 (Swofford, 2002) and Bayesian analyses (with burn-in excluded after runs) conducted in MrBayes v3.1.1 (Ronquist & Huelsenbeck, 2003). ML and Bayesian analyses were run at the UF Phyloinformatics High Performance Computing Cluster. Analyses occurred on three data sets: (1) concatenated mitochondrial (Bayesian and MP), (2) nuclear (Bayesian, MP and ML) and (3) concatenated nuclear and mitochondrial (Bayesian and MP). Unordered and user-specified 3 : 1, 5 : 1 and 10 : 1 transversion biases were assigned in parsimony to correct for saturation. However, because resultant topologies from variable weighting schemes did not differ from those generated using equally weighted data sets, equal weighting was employed in later analyses. ModelTest v3.7 (Posada & Crandall, 1998) was used to determine the appropriate model of molecular evolution for all other analyses [all GTR + I + G for both Akaike information criterion (AIC) and likelihood ratio test (LRT)]. Tree robustness was assessed using bootstraps (100–1000 replicates, ML and MP, respectively) and posterior probabilities (Bayesian).

Tier 2

A second tier of alignment and analyses was conducted to: (1) refine hypervariable regions of the 16S and 28S data sets and (2) complete ML analyses and estimate branch support for large data sets. These tasks were completed at the phylogeny.fr site (Dereeper *et al.*, 2008). Both 28S and 16S gene regions were aligned in MUSCLE v3.7, configured for highest accuracy using default settings (Edgar, 2004). Conserved, well-aligned regions appropriate for phylogenetic analysis were then identified using Gblocks v0.91b (Castresana, 2000). Ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed following the least stringent settings, in order to retain as much data as possible. This resulted in a final data set of 908 bp for 28S, compared with 1364 bp preprocessing, and a final data set of 379 bp for 16S, compared with 569 bp preprocessing. Individual gene region data sets were concatenated using Mesquite v2.5 (Maddison & Maddison, 2008). Maximum likelihood was implemented in PhyML v3.0 aLRT (Guindon & Gascuel, 2003) to estimate trees for each gene region and for a concatenated nuclear (28S) + mitochondrial (16S, COI, CytB) data set. The GTR substitution model was selected assuming an estimated proportion of invariant sites. Four gamma-distributed rate categories accounted for rate heterogeneity across sites and the gamma shape parameter was estimated directly from the data for each run. Tree robustness was assessed using bootstraps (100 replicates) and the approximate LRT (aLRT) (SH-like) (Anisomova & Gascuel, 2006). Initial annotation and editing of ML trees was carried out in TreeDyn v198.3 (Chevenet *et al.*, 2006).

Determination of photosymbiotic condition

Assessment of photosymbiotic status for sampled Fraginae was undertaken by examining the mantle, gill and foot of live-collected animals in the field. Where possible, live tissue was microscopically examined immediately to confirm the presence of symbionts. When field-based microscopic examination was not possible, the colour of live tissue was noted, photo-

graphs were taken and these animals were then fixed in formalin for later microscopic confirmation of symbiont occurrence. Microscopic examination to confirm symbiont presence involved cutting a small piece of tissue and placing it on a glass slide to find evidence of zooxanthellae, the photosymbiont most common to shallow-water Indo-West Pacific (IWP) corals and giant clams. Zooxanthellae have a characteristic shape (completely spherical), colour (dark or golden brown) and size (approximately 5–8 μm) and cells that fit this description were considered to be zooxanthellae. The taxonomic identity of fragine symbionts was directly determined in one case via restriction fragment length polymorphism (RFLP) analysis of *Fragum fragum* (*Symbiodinium*; L. Kirkendale, unpubl. data). Because most, if not all, photosymbiotic zooxanthellae are dinoflagellates of the genus *Symbiodinium,* it is very likely that all fragine photosymbionts pertain to this algal genus. Tentative photosymbiotic status of specimens supplied by collectors [*Parvicardium exiguum* by the late J. Vidal and *Apiocardia obovale* (Sowerby, 1833) by R. Collin] was established via discussion, as rapid ethanol preservation of specimens for molecular analysis precluded easy photosymbiotic verification.

RESULTS

The bulk of ingroup fragine species, with representatives sampled from all extant genera and subgenera, were included in phylogenetic reconstructions (Table 2, Appendix). An additional 15 species from eight cardiid genera were included as outgroups for rooting purposes (Appendix). Bayesian analyses were conducted on individual gene data sets and a four-gene concatenated data set without exclusion of hypervariable regions (28 sequences of 1364 bp for 28S, 60 sequences of 569 bp for 16S, 64 sequences of 714 bp for COI and 60 sequences of 369 bp for CytB with a total length of 3016 bp). ML analyses were completed for individual gene regions, as well as a four-gene concatenated data set, with exclusion of hypervariable regions via Gblocks (29 sequences of 908 bp for 28S, 60 sequences at 381 bp for 16S, 63 sequences of 714 bp for COI and 60 sequences at 369 bp for CytB with a total length of 2372 bp). These data sets contained 25 (roughly 60%) ingroup species; 72% of ingroup representatives (and multiple individuals of a species) had complete mitochondrial data sets, while approximately 60% had nuclear representation (Appendix).

Individual gene regions were chosen to provide resolution across a broad range of taxonomic levels in a little-studied group. Variable rates, coupled with variable taxonomic coverage, resulted in differing levels of resolution, support and consistency among regions. A total evidence approach utilizing the full, concatenated genetic data set (all four gene regions) was chosen as the best strategy for maximizing signal and resolution. Although discussion of trends for each gene region is beyond the scope of this paper, analyses of individual gene regions are available upon request from the author.

Bayesian and ML analyses provided the greatest resolution and support for the same four major fragine clades (Figs 3, 4). Relationships differed between methods almost exclusively in regions of the phylogeny where branch support was low (compare Figs 3, 4). This was likely a consequence of: (1) inclusion of hypervariable regions in Bayesian analyses and exclusion of these regions in ML analyses, (2) incomplete taxon sampling and (3) poor signal/marker choice for higher-level reconstructions. Given these concerns, phylogenetic inference focused on well-supported nodes common to both ML and Bayesian analyses.

Higher-Level Phylogenetics and Fraginae monophyly

Four major clades of fragines were resolved and well supported in Bayesian and ML analyses: (1) the '*Fragum*' group composed of all species in the genera *Fragum*, *Corculum* and *Lunulicardia* (Clade I); (2) Schneider's (1998) '*Trigoniocardia*' and '*Ctenocardia*' groups except *C. victor* (Clade II) (Fig. 1); (3) the '*Parvicardium*' group uniting the majority of tested members from *Parvicardium* (Clade III); (4) the '*Papillicardium*' group, joining two highly divergent species, *Papillicardium papillosum* (Poli, 1791) and *Parvicardium minimum* (Philippi, 1836) (Clade IV) (Figs 3, 4).

These four well-supported clades were not reconstructed as monophyletic in any analyses (Figs 3, 4). *Papillicardium papillosum* and *Parvicardium minimum* were consistently recovered as sisters, but were highly divergent from most other tested ingroup and outgroup cardiids; no analyses recovered these two species as sister to tested congeners. To test the hypothesis of long-branch attraction, *Parvicardium minimum* and *Papillicardium papillosum* were analysed in isolation (L. Kirkendale, unpubl. data). Each species fell in the same position as when jointly analysed, falsifying the hypothesis that long-branch attraction was a factor determining their original, sister–taxon relationship. *Ctenocardia victor* (Angas, 1872) was similarly divergent and consistently fell with distantly related outgroup species instead of with congeners. Sequence quality and alignments were verified for multiple individuals of these three aberrant species, confirming that highly divergent sequences were not artifacts.

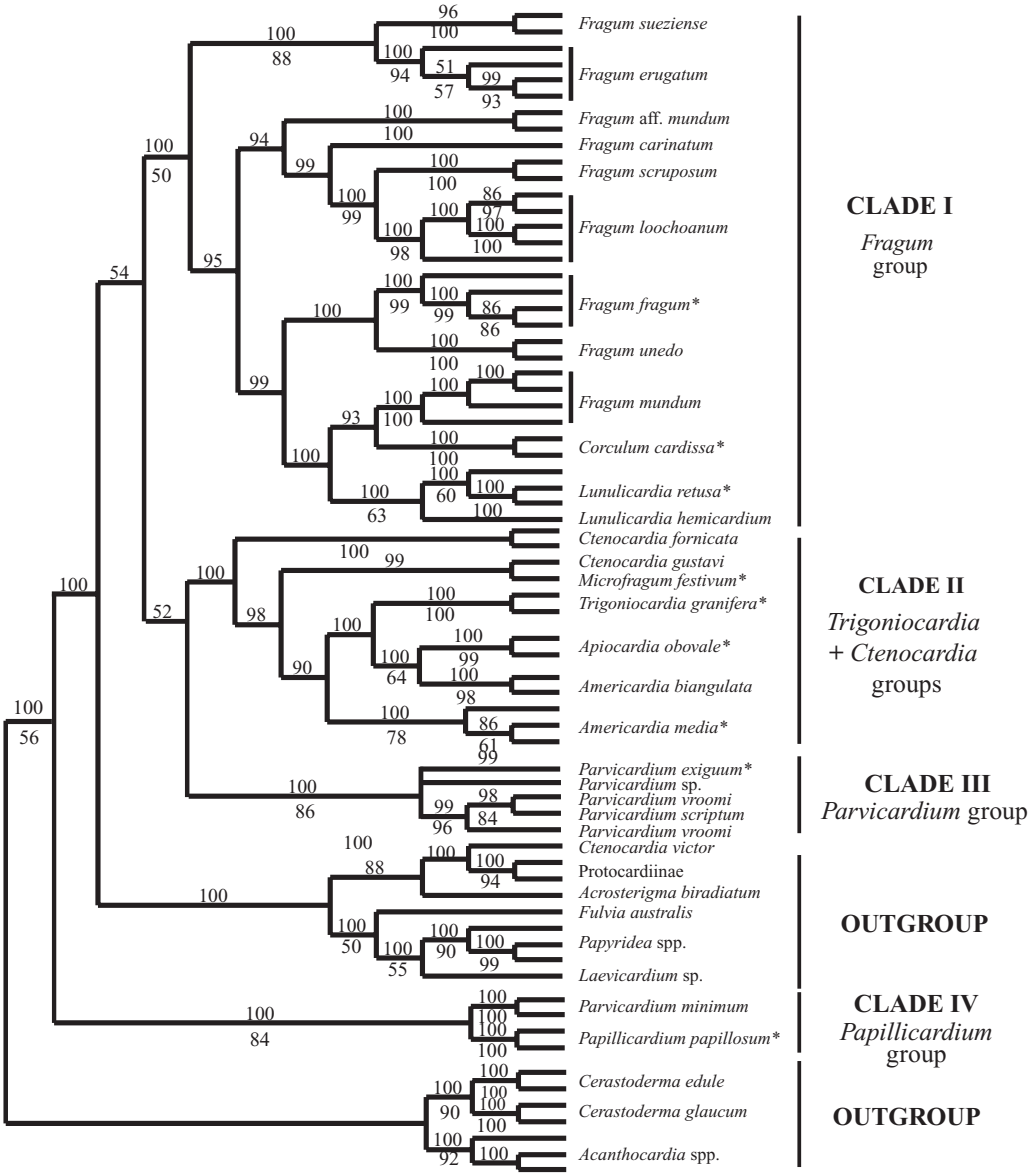

Figure 3. Bayesian 50% majority topology of the concatenated data set of all four gene regions (28S, 16S, COI and CytB). Branch support as posterior probabilities above branches, maximum parsimony bootstrap values below branches. An asterisk denotes the type species of sampled Fraginae genera and subgenera. COI, cytochrome oxidase I; CytB, cytochrome *b*.

PARVICARDIUM AND *PAPILLICARDIUM*

Bayesian and ML analyses recovered high support for a clade composed of most *Parvicardium* species (Clade III) and another of *Papillicardium* + *Parvicardium* (Clade IV) (Figs 3, 4). Four species of *Parvicardium* were included in the '*Parvicardium* group' (Clade III): an undescribed but divergent taxon (*P.* sp. 1 LaHerra), *P. vroomi* Aartsen, Menkhorst & Gittenberger, 1984, *P. scriptum* (Bucquoy, Dautzenbery & Dollfus, 1892) and *P. exiguum*. Species boundaries between *P. vroomi* and *P. scriptum* were unclear; both Bayesian and ML analyses recovered a paraphyletic *P. vroomi* (Figs 3, 4). Bayesian analyses weakly supported the '*Parvicardium* group' as sister to the *Ctenocardia* and *Trigoniocardia* groups (Clade II) (Fig. 3), while ML analyses recovered this clade as a well-supported sister to a clade uniting the '*Papillicardium* group' (Clade IV) with members from two genera of outgroup cardiids, *Acanthocardia* and *Cerastoderma* (Fig. 4).

THE '*CTENOCARDIA*' AND '*TRIGONIOCARDIA*' GROUPS

Clade II, composed of all '*Ctenocardia*' and '*Trigoniocardia*' group members, was recovered as a

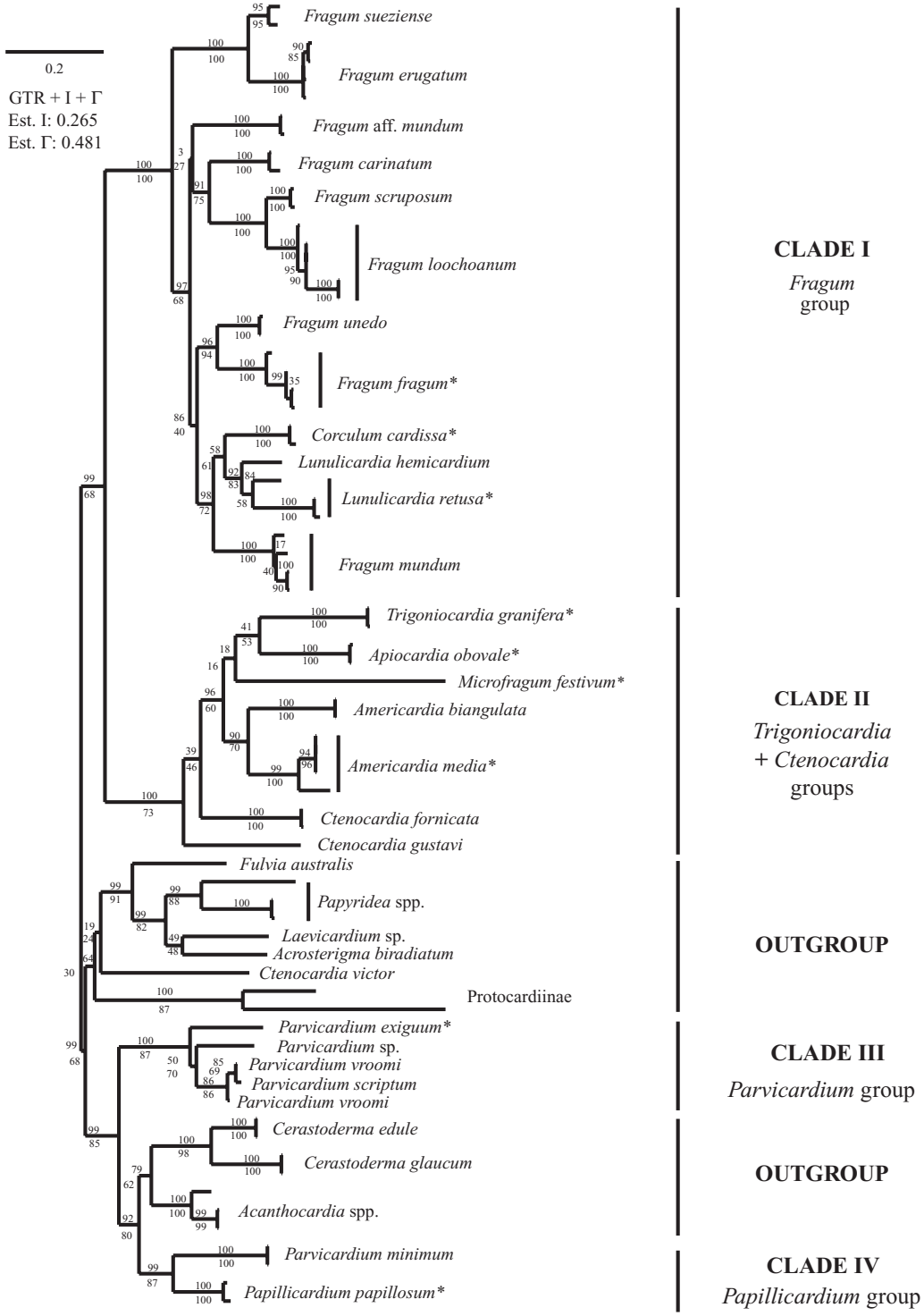

Figure 4. ML phylogram of the concatenated data set of all four gene regions (28S, 16S, COI and CytB) with Gblock for 28S and 16S. Approximate LRT (SH-like) bootstrap values above branches, ML bootstrap values below branches (both rounded to two decimal places from three and converted to percentage). An asterisk denotes the type species of sampled Fraginae genera and subgenera. COI, cytochrome oxidase I; CytB, cytochrome *b*; LRT, likelihood ratio text; ML, maximum likelihood.

well-supported fragine subclade, except for the abbarent *C. victor* that consistently fell with outgroups (Figs 3, 4). Excluding *C. victor*, the genus *Ctenocardia* was not recovered as monophyletic in any analyses (Figs 3, 4). ML analyses supported a monophyletic *Americardia*, while Bayesian analyses recovered a paraphyletic *Americardia* (compare Figs 3, 4, respectively). *Trigoniocardia* was supported as sister to *Apiocardia* in ML analyses, whereas Bayesian analyses recovered *Apiocardia* sister to *Americardia biangulatum* (Broderip & Sowerby, 1829) (compare Figs 3, 4, respectively). Boundaries in subgenus *Apiocardia, Microfragum* and *Trigoniocardia* could not be tested; only one species of each was included in the study.

Fragum, *Lunulicardia* and *Corculum*

The '*Fragum*' group, composed of the IWP genera *Fragum*, *Lunulicardia* and *Corculum*, was highly supported and well resolved in all analyses (Clade I, Figs 3, 4). *Corculum* and *Lunulicardia* were consistently recovered as monophyletic, while *Fragum* was paraphyletic. Within the *Fragum* clade, three well-supported subclades were recovered: (1) a subclade of earliest diverging members; *F. sueziense* and *F. erugatum*; (2) the morphologically impenetratable '25-rib' subclade; (3) a subclade uniting *Fragum mundum*, *Corculum*, *Lunulicardia*, *F. fragum* and *F. unedo*. Within the third subclade, two additional subclades were resolved in both ML and Bayesian analyses: (1) *F. fragum* and *F. unedo* and (2) a group including *F. mundum*, *Corculum* and *Lunulicardia* (Figs 3, 4). Subtle differences in the latter subclade were evident between ML and Bayesian methodologies. ML recovered a poorly supported subclade that united *Corculum* and *Lunulicardia* to the exclusion of *F. mundum* (Fig. 4), while Bayesian analyses recovered *F. mundum* and *Corculum* as sisters, to the exclusion of *Lunulicardia* (Fig. 3).

The second major *Fragum* complex recovered, the '25-rib' group, includes *F. loochoanum*, *F. scruposum* (Deshayes, 1855), *F. carinatum* (Lynge, 1909) and *F.* aff. *mundum*. In Bayesian analyses, *F.* aff. *mundum* was recovered as sister to *F. carinatum*, which was, in turn, sister to the remaining 25-rib members (Fig. 3). The ML topology differed from Bayesian analyses with respect to relationships in this subclade, with *F.* aff. *mundum* recovered as sister to *F. carinatum* and *F. scruposum* + *F. loochoanum* (Fig. 4).

Photosymbiotic status

All tested representatives of three fragine genera (*Corculum, Lunulicardia* and *Fragum*) were entirely photosymbiotic, while no sampled members of the other seven fragine genera and subgenera surveyed (*Trigoniocardia, Apiocardia, Americardia, Ctenocardia, Microfragum, Parvicardium* and *Papillicardium*) were found to host photosymbionts (Table 4 and Fig. 5).

Table 4. Photosymbiotic status of sampled Fraginae

Species	*N*	Photosymbionts*†‡§
Fragum fragum	50	Present†
Fragum scruposum	20	Present†
Fragum loochoanum	20	Present†
Fragum carinatum	13	Present‡
Fragum mundum	3	Present†
Fragum aff. mundum	3	Present‡
Fragum nivale		Present*
Fragum unedo	5	Present†
Fragum erugatum	20	Present‡
Fragum sueziense	6	Present†
Fragum sp. 11 (Persselin, 1998)		Present*
Lunulicardia retusa		Present*
Lunulicardia hemicardia	2	Present‡
Lunulicardia sp. 1 (Persselin, 1998)		Present*
Corculum cardissa	3	Present*
Trigoniocardia granifera	10	Absent†
Apiocardia obovale		Absent§
Americardia biangulata	2	Absent†
Americardia media	3	Absent†
Ctenocardia fornicata		Absent*
Ctenocardia victor		Absent*
Ctenocardia gustavi	1	Absent‡
Microfragum subfestivum	3	Absent‡
Microfragum festivum	10	Absent†
Parvicardium exiguum		Absent§
Parvicardium scriptum	10	Absent†
Parvicardium vroomi	10	Absent†
Papillicardium papillosum	8	Absent†

*Status previously known (see text for literature-based references).
†Microscopic examination of live animals.
‡Microscopic examination of formalin-fixed animals.
§Microscopic examination of ethanol-fixed animals.

DISCUSSION

European Fraginae and evidence for subfamilial monophyly

The phylogenies recovered here well illustrate the difficulties faced by previous workers in tackling membership in the Fraginae. The relationships within, and position of, the earliest diverging fragine lineages, *Parvicardium* and *Papillicardium*, remain at the crux of this issue.

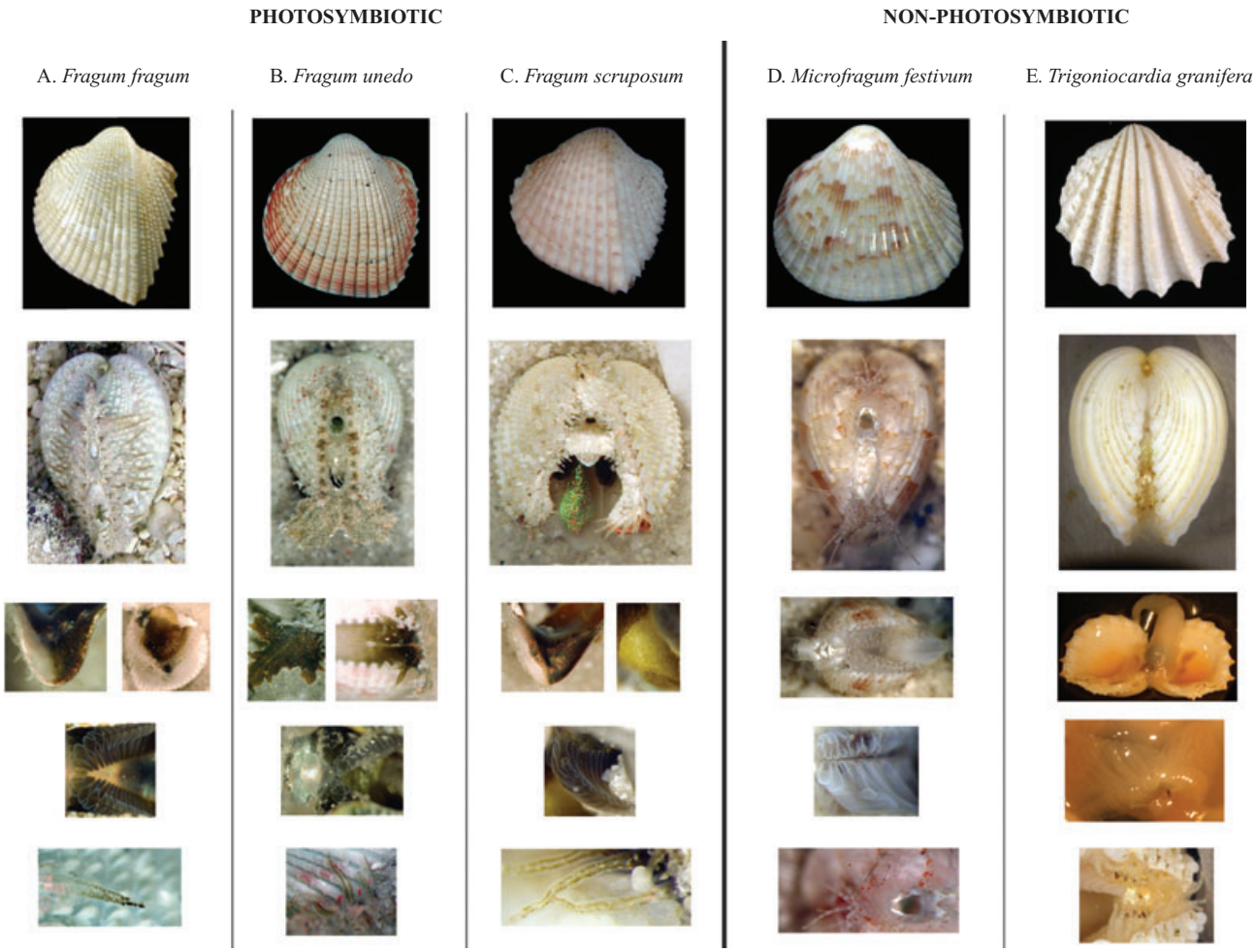

Figure 5. Photosymbiotic (A, *Fragum fragum*; B, *Fragum unedo*; C, *Fragum scruposum*) and non-photosymbiotic (D, *Microfragum festivum*; E, *Trigoniocardia granifera*) Fraginae featuring from top: 1, external shell (right valve figured); 2, live animal in typical orientation with posterior side incidental to sediment–water interface; 3, mantle; 4, gill; 5, mantle tentacle(s).

Parvicardium is not supported as monophyletic by the results of this study (Figs 3, 4). Two distantly related lineages, both comprised of members of this genus, are consistently recovered (Figs 3, 4, Clades III and IV). The '*Parvicardium* group' (Clade III) is so recognized because the type species of the genus *Parvicardium*, *P. exiguum*, is a member. Other sampled *Parvicardium* recovered in the '*Parvicardium*' group are *P.* sp LaHerra, *P. vroomi* and *P. scriptum*. The second clade composed of fragines is referred to as the '*Papillicardium*' group because the type species of the genus *Papillicardium*, *P. papillosum*, is a member. *Parvicardium minimum* is the other member of this small clade. *Papillicardium papillosum* has long been recognized as distinct from other small *Parvicardium*, with *Papillicardium* afforded subgeneric (Kafanov & Popov, 1977; Keen, 1980; Voskuil & Onverwagt, 1989, 1991; Aartsen & Goud, 2000) or generic status (Schneider, 1998) to reflect these differences.

Bayesian methods recover low support for the '*Parvicardium*' group as sister to Clade II (Fig. 3), while ML methods recover a well-supported larger subclade of European cardiids that includes the '*Parvicardium*' and '*Papillicardium*' groups (Clades III and IV), but not as sisters. This divergence among the European fragines is not without precedence. Schneider (1998) also recovered a paraphyletic *Parvicardium* (Fig. 1). Although sampling of *Parvicardium* differs between the two studies, the type species of *Parvicardium*, *P. exiguum*, was common to both. *P. exiguum* was recovered as the most likely sister to the rest of the fragines by Schneider (1998), a finding that is only weakly supported here (Fig. 3). Although cardiid sampling was sparse, *Parvicardium exiguum* was recovered as sister to non-fragine cardiids, not sister to the three fragine representatives included in the study (in the genera *Corculum* and *Fragum*) (Giribet & Distel, 2003). Redefining a new Fraginae

will require additional markers and taxa to confirm the relationships recovered here. That the '*Trigoniocardia*' + '*Ctenocardia*' (Clade II) and '*Fragum*' (Clade I) groups are well-supported sisters (Fig. 4) and represent the Fraginae of many previous authors (Stewart, 1930; Keen, 1980; Voskuil & Onverwagt, 1989) will be an important consideration in this regard.

Maximum likelihood analyses recovered high support for a morphologically divergent European clade uniting species distributed amongst three different cardiid subfamilies: Cardiinae (*Acanthocardia*), Fraginae (*Parvicardium* and *Papillicardium*) and Lymnocardiinae (*Cerastoderma*) (Fig. 4). This finding contrasts with recent phylogenetic work (Schneider, 1998), but bears resemblance to earlier taxonomic work (Stewart, 1930; Keen, 1980; Voskuil & Onverwagt, 1989, 1991) that placed *Parvicardium* and *Papillicardium*, not in the Fraginae, but in either the Cardiinae or Cerastodermatiinae (now Lymnocardiinae) (Table 1). Similar to recent findings of Caribbean reef corals (Fukami *et al.*, 2004), this finding supports a history of intraregional morphological radiation in European cardiids and suggests that the retention of plesiomorphic shell characters among distantly related, geographically disjunct species (e.g. *Parvicardium* and *Papillicardium* + fragines from IWP and the Americas) may have confused taxonomic affinities in the Fraginae. Increased taxon sampling of European cardiids, as well as the inclusion of additional genetic data for sampled members, will permit further tests of this trend.

Schneider (1998), in contrast with others, supported an inclusive Fraginae uniting *Parvicardium* and *Papillicardium*, as well as more derived members (Table 1 and Fig. 1). Review of morphological characters reveals that the lack of a perisiphonal suture, which results in confluence of the incurrent siphonal aperture and pedal gape, is the strongest synapomorphy uniting the Fraginae. As stated firmly by Schneider (1998: 326), 'all fragines and only fragines lack a perisiphonal suture'. However, live fragines effectively have a separate incurrent aperture, as they hold the two mantle edges together muscularly at the ventral margin of the incurrent aperture (L. Kirkendale, pers. observ.). Similar separation of incurrent or excurrent apertures is common in many lineages of bivalves (e.g. mytilids, thyasirids: Bernard, 1972; Payne & Allen, 1991, respectively). The absence of mantle fusion at the ventral margin of the incurrent aperture is clearly secondary in fragines, as it is present in all other cardiids, as well as almost all members of the Heterodonta, the order to which cardiids belong. Moreover, the results presented here now support a threefold loss of perisiphonal fusion within cardiids, given the distant relationship of (1) *Papillicardium papillosum* + *Parvicardium minimum* and (2) *Ctenocardia victor* to (3) other fragines. Only a few other heterodonts lack mantle fusion around the incurrent apertures, most notably members of the Galeommatoidea. The absence of such mantle fusion in galeommatoids was thought to be plesiomorphic, but new work suggests that galeommatoids may be secondarily simplified from higher heterodonts (Giribet & Wheeler, 2002), implying that they may also have lost mantle fusion, a finding that resonates in the fragines.

THE '*CTENOCARDIA*' AND '*TRIGONIOCARDIA*' GROUPS: CLADE II

The '*Trigoniocardia*' and '*Ctenocardia*' groups were recovered as a well-supported clade in Bayesian and ML analyses (Figs 3, 4). This contrasts with others who recognized two separate fragine subclades (Stewart, 1930; Clench & Smith, 1944; Keen, 1951; Olsson, 1961; Popov, 1977; Keen, 1980; Voskuil & Onverwagt, 1989; Schneider, 1998). The most recent phylogenetic appraisal split these groups into two divergent fragine subclades and recovered the '*Trigoniocardia*' group (members of the subgenera *Trigoniocardia*, *Apiocardia* and extinct *Goniocardia*) as sister to the remaining fragines (*Ctenocardia*, *Microfragum*, *Americardia*, *Fragum*, *Corculum* and *Lunulicardia*) (Fig. 1) (Schneider, 1998). Although Clade II is well supported by both ML and Bayesian methods, relationships within this clade are poorly supported and differ between ML and Bayesian methods (Figs 3, 4). This highlights the need for increased taxon sampling and more complete and additional sequence data sets to clarify relationships within and between genera and subgenera.

The second most diverse fragine lineage, *Ctenocardia,* is paraphyletic (Figs 3, 4) and several newly discovered species indicate it is also poorly known (Vidal & Kirkendale, 2007). Although *C. victor* has been previously allied to this genus, it consistently falls within a clade of outgroup cardiids and is clearly more closely related to other cardiids than to other *Ctenocardia* or other Fraginae (Figs 3, 4). The morphological disparity of *C. victor*, relative to other *Ctenocardia*, has long been appreciated. Wilson & Stevenson (1977) did not support placement of *C. victor* in *Ctenocardia*; instead this species was allied to the genus '*Cardium*' because of significant differences in hinge morphology relative to other conspecifics. For example, *C. victor* has a single right posterior lateral tooth, whereas all other *Ctenocardia* species have two posterior lateral teeth. Although no *Cardium* representatives were included in the study, two members of the Cardiinae, the subfamily to which *Cardium* belongs, were sampled. If *C. victor* was a member of the genus *Cardium*, it should fall sister to

other subfamily members, in this case, *Acanthocardia*. However, *C. victor* was not recovered as closely related to *Acanthocardia,* suggesting that *C. victor* is even more taxonomically elusive than expected.

Systematic implications and the evolution of photosymbiosis

All sampled representatives from the genera *Fragum*, *Lunulicardia* and *Corculum* were recovered in a single clade, the '*Fragum*' group (Clade I, Figs 3, 4). *Fragum* was strongly supported as paraphyletic, with all congeners, as well as monophyletic *Corculum* and *Lunulicardia*, distributed amongst three well-supported subclades. The recovery of a paraphyletic *Fragum* confirms the results of Persselin (1998) and one possibility postulated by Schneider (1998) (Fig. 1).

As first suggested by Persselin (1998) and confirmed in this study, the majority of fragines are not photosymbiotic (Table 4). Only three genera, *Fragum, Lunulicardia* and *Corculum*, corresponding to the '*Fragum'* group (Clade I), are photosymbiotic (Figs 3–5). Placing photosymbiosis in a phylogenetic context reveals one large, wholly photosymbiotic lineage, supporting a single origin of photosymbiosis in the group (Fig. 6). All members are exclusively known from the Indo-West Pacific and all but two are most commonly found at depths of 0–3 m on clear, coral reef flats or shallow lagoons. With this information in hand, basic research focusing on the photosymbionts is ripe for examination. Estimates of (1) symbiont diversity, population size and turnover, as well as, (2) nutrient transfer between host and symbiont may serve to clarify the patterns of morphological, behavioural and ecological diversity in the group.

Anatomical examination of Fraginae representatives revealed gut simplification trends (e.g. gut simplification in *Trigonicardia* and *Apiocardia* from Type V to Type IV, reductions in crystalline style and style sac in *Corculum* and the loss of ridges on the labial palps of *Microfragum*) that were interpreted as early evidence to support an hypothesis of a wholly photosymbiotic Fraginae (Schneider, 1998). Given the presence of photosymbionts in only three Fraginae genera, gut simplification trends in confirmed photosymbiotic members should be carefully re-examined.

The divergent morphologies exhibited by confirmed photosymbiotic species of fragines (Kawaguti, 1950, 1968; Trench *et al.*, 1981; Kawaguti, 1983; Ohno *et al.*, 1995; Carter & Schneider, 1997; Persselin, 1998; Schneider, 1998; Morton, 2000; Schneider & Carter, 2001) as well as the giant clams (Yonge, 1936, 1981) have long been appreciated, but little has been known about the earliest diverging photosymbiotic fragine lineages until now. *Fragum erugatum* and *F. sueziense* are strongly supported as the earliest diverging lineage of the photosymbiotic clade (Clade I) and sister to all other tested *Fragum*, *Lunulicardia* and *Corculum* (Figs 3, 4). These two species, unlike many other photosymbiotic bivalves that often exhibit bizarre shell forms (e.g. *Corculum, Lunulicardia*, see Fig. 6) and unique microstructural features (Carter & Schneider, 1997; Schneider & Carter, 2001), are quite conservative, sharing shell characters with a diversity of cardiid genera and confounding early attempts at their taxonomic placement (see Hylleberg, 2004) (see shell profiles, Fig. 6). Photosymbiotic status of these two species was only confirmed relatively recently (*F. erugatum* by Morton, 2000 and *F. sueziense* by Persselin, 1998), as few obvious external shell features, often the first line of evidence, suggested a relationship with photosymbionts.

Although these two species share a number of gross morphological characteristics, they are quite distinct ecologically, both from each other and from other fragines. *Fragum erugatum* is endemic to Shark Bay, Western Australia, where it is the dominant infauna of many shallow, hypersaline reaches (e.g. Shell Beach) (Fig. 2A). It is a morphologically variable species, with conspecifics exhibiting differences in shell shape, dentition and features of the hinge often recovered among different classes of bivalves (L. Kirkendale, unpubl. data). In contrast, *F. sueziense* is more morphologically conservative than *F. erugatum*, but more widespread geographically (IWP-wide based on collections made in this study). *Fragum sueziense*, like *F. erugatum*, occupies a unique environment compared with all other known *Fragum* species; it is entirely restricted to relatively turbid, subtidal environments typical of lagoons and large bays throughout its range, an unlikely environment for a photosymbiotic species (Persselin, 1998) (Fig. 2A).

As suggested by the small, morphologically simple photosymbiotic clams reconstructed as the earliest diverging lineage in this study, perhaps the early evolutionary stages of photosymbiosis in fragines were quite modest. A small clam would have been pre-adapted to photosymbiosis, as small size elevates surface area to volume ratios, increases shell translucency in thin, small shells and, together with short siphons, limits burrowing to shallow depths within the sediment. Within the span of < 20 mya (*Fragum* has a fossil record that dates back to the Miocene-Holocene) (Keen, 1980), photosymbiotic fragines have evolved into a wide diversity of morphological forms; from simple, little modified species (e.g. *F. carinatum*, *F. scruposum*, *F. loochoanum*) to living solar panels (*Corculum cardissa*) with sophisticated window shell microstructure (*C. cardissa*, *F. mundum*, juvenile *Lunulicardia*) to 'mini' giant clams, with hypertrophied mantles splayed out on the sediment surface and valve gaping behaviour (*Fragum unedo*). This

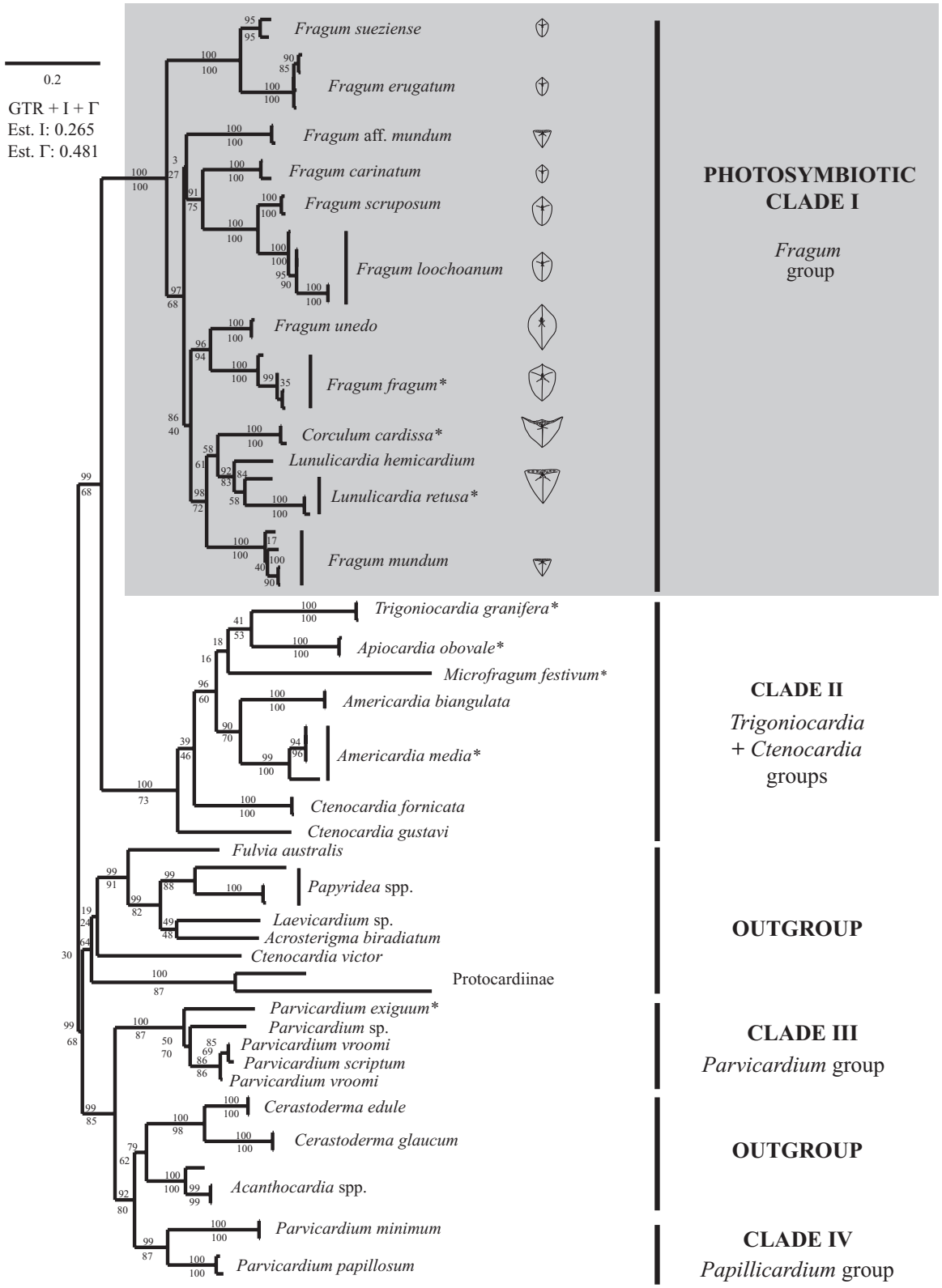

Figure 6. Origin of photosymbiosis in the Fraginae traced onto Figure 4.

broad spectrum of solutions to photosymbiosis in such a small, young clade evokes the early stages of giant clam evolution and provides evidence for the role of historical contingency in the evolution of form.

SUMMARY

The subfamily Fraginae is not monophyletic and significant restructuring is supported at multiple phylogenetic levels, considering: (1) a polyphyletic *Parvicardium*, (2) union of the '*Trigionicardia*' and '*Ctenocardia*' groups and (3) a paraphyletic *Fragum*. The most conservative phylogeny recovers a well-supported European clade composed of three different cardiid subfamily members, including *Parvicardium* and *Papillicardium*. Morphologically disparate *C. victor*, long recognized as distinct from other congeners, is distantly related to all other fragines. The lack of a perisiphonal suture, a key morphological character of the Fraginae championed by Schneider (1998), is homoplastic.

Within Clade I, all and only members of the '*Fragum*' group, composed of members of the genera *Fragum*, *Lunulicardia* and *Corculum*, bear photosymbionts. This finding, in contrast with earlier predictions that all derived fragines would host algal symbionts, supports a single and relatively late origin of photosymbiosis in the Fraginae. Gut simplification trends, previously used as evidence of a wider occurrence of photosymbiosis in the group, need re-evaluation. The earliest diverging lineage of photosymbiotic fragines is small and little modified in contrast with many of the highly-modified photosymbiotic fragines, such as *Corculum* and *Lunulicardia*.

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APPENDIX

Sampling localities and voucher information for Fraginae representatives. Collection localities (CL below) cross-reference to the distribution map (Fig. 2A) with three exceptions (G* refers to *Parvicardium exiguum* sequence from Genbank, U refers to unknown and S* refers to *Cerastoderma glaucum* from a Spanish fish market).

Taxa	CL	Accession numbers (**A**, Voucher; **B**, 28S; **C**, COI; **D**, 16S; **E**, CytB)
Ingroup		
Parvicardium exiguum	G*	**C,** AF120664
*Papillicardium papillosum*278	4	**A**, UF374115; **B**, EU733020; **C**, EU733112; **D**, EU733052; **E**, EU733178
*Papillicardium papillosum*279	4	**A**, UF374115; **B**, EU733019; **C**, EU733111; **D**, EU733051; **E**, EU733177
*Parvicardium minimum*194	5	**A**, UMICH265486; **C**, EU733108; **D**, EU733048; **E**, EU733171
*Parvicardium minimum*195	5	**A**, UMICH265486; **C**, EU733109; **D**, EU733049; **E**, EU733172
*Parvicardium vroomi*294	2	**A**, UF374116; **B**, EU733016; **E**, EU733174
*Parvicardium vroomi*296	2	**A**, UF374116; **B**, EU733017; **E**, EU733175
*Parvicardium scriptum*283	4	**A**, UF374117; **B**, EU733018; **E**, EU733176
Parvicardium sp. LaHerra299	3	**A**, UF374118; **B**, EU733015; **C**, EU733110; **D**, EU733050; **E**, EU733173
*Americardia media*115	25	**A**, UF298641; **B**, EU733026; **D**, EU733058; **E**, EU733184
*Americardia media*387	26	**A**, UF347556; **B**, EU733027; **D**, EU733059; **E**, EU733185
*Americardia media*388	26	**A**, UF347556; **E**, EU733214
*Americardia biangulata*331	24	**A**, UF351615; **C**, EU733148; **D**, EU733090
*Americardia biangulata*332	24	**A**, UF351615; **C**, EU733149; **D**, EU733091
*Trigoniocardia granifera*333	24	**A**, UF359687; **B**, EU733024; **C**, EU733116; **D**, EU733056; **E**, EU733182
*Trigoniocardia granifera*334	24	**A**, UF359687; **B**, EU733025; **C**, EU733117; **D**, EU733057; **E**, EU733183
*Apiocardia obovale*398	24	**A**, UF351671; **C**, EU733146; **D**, EU733088
*Apiocardia obovale*399	24	**A**, UF351671; **C**, EU733147; **D**, EU733089
*Ctenocardia victor*3	13	**A,** UF288935; **B**, EU733022; **C**, EU733114; **D**, EU733054; **E**, EU733180
*Ctenocardia fornicata*17	6	**A**, UF286471; **C**, EU733170; **D**, EU733107; **E**, EU733230

APPENDIX *Continued*

Taxa	CL	Accession numbers (**A**, Voucher; **B**, 28S; **C**, COI; **D**, 16S; **E**, CytB)
*Ctenocardia fornicata*18	6	**A**, UF286471; **B**, EU733021; **C**, EU733113; **D**, EU733053; **E**, EU733179
*Ctenocardia gustavi*311	16	**A**, UF351689; **B**, EU733023; **C**, EU733115; **D**, EU733055; **E**, EU733181
*Microfragum festivum*201	U	**A**, UMICH300091; **C**, EU733150; **D**, EU733092; **E**, EU733215
*Fragum sueziense*31	13	**A**, UF299280; **B**, EU733028; **C**, EU733119; **D**, EU733061; **E**, EU733187
*Fragum sueziense*56	17	**A**, UF299263; **B**, EU733029; **C**, EU733118; **D**, EU733060; **E**, EU733186
*Fragum erugatum*376	8	**A**, UF347869; **C**, EU733151
*Fragum erugatum*419	8	**A**, UF347689; **C**, EU733152
*Fragum erugatum*133	8	**A**, UF299293; **C**, EU733160; **D**, EU733100; **E**, EU733223
*Fragum erugatum*134	8	**A**, UF299293; **C**, EU733161; **D**, EU733101; **E**, EU733224
*Fragum fragum*24	19	**A**, UF299283; **C**, EU733155; **D**, EU733095; **E**, EU733218
*Fragum fragum*48	22	**A**, UF301756; **C**, EU733154; **D**, EU733094; **E**, EU733217
*Fragum fragum*60	6	**A**, UF299259; **B**, EU733033; **C**, EU733130; **D**, EU733072; **E**, EU733198
*Fragum fragum*61	23	**A**, UF299282; **C**, EU733153; **D**, EU733093; **E**, EU733216
*Fragum unedo*129	8	**A**, UF299291; **B**, EU733034; **C**, EU733131; **D**, EU733073; **E**, EU733199
*Fragum unedo*131	8	**A**, UF299291; **B**, EU733035; **C**, EU733132; **D**, EU733074; **E**, EU733200
*Fragum carinatum*318	14	**A**, UF351691; **B**, EU733030; **C**, EU733122; **D**, EU733064; **E**, EU733190
*Fragum loochoanum*382	16	**A**, UF351692; **C**, EU733127; **D**, EU733069; **E**, EU733195
*Fragum loochoanum*383	16	**A**, UF351692; **C**, EU733128; **D**, EU733070; **E**, EU733196
*Fragum loochoanum*385	18	**A**, UF348016; **C**, EU733125; **D**, EU733067; **E**, EU733193
*Fragum loochoanum*386	18	**A**, UF348016; **C**, EU733126; **D**, EU733068; **E**, EU733194
*Fragum loochoanum*121	13	**A**, UF299448; **C**, EU733129; **D**, EU733071; **E**, EU733197
*Fragum scruposum*315	12	**A**, UF374114; **B**, EU733032; **C**, EU733124; **D**, EU733066; **E**, EU733192
*Fragum scruposum*316	12	**A**, UF374114; **B**, EU733031; **C**, EU733123; **D**, EU733065; **E**, EU733191
Fragum aff. *mundum*375	18	**A**, UF374156; **C**, EU733120; **D**, EU733062; **E**, EU733188
Fragum aff. *mundum*377	18	**A**, UF374157; **C**, EU733121; **D**, EU733063; **E**, EU733189
*Fragum mundum*78	21	**A**, UF296894; **B**, EU733036; **C**, EU733133; **D**, EU733075; **E**, EU733201
*Fragum mundum*116	13	**A**, UF298635; **C**, EU733162; **D**, EU733102; **E**, EU733225
*Fragum mundum*379	20	**A**, UF374155; **B**, EU733037; **C**, EU733134; **D**, EU733076; **E**, EU733202
*Fragum mundum*381	7	**A**, UF337833; **B**, EU733038; **C**, EU733135; **D**, EU733077; **E**, EU733203
*Corculum cardissa*9	10	**A**, UF280389; **B**, EU733039; **C**, EU733136; **D**, EU733078; **E**, EU733204
*Corculum cardissa*67	11	**A**, UF286449; **B**, EU733040; **C**, EU733137; **D**, EU733079; **E**, EU733205
*Lunulicardia retusa*21	8	**A**, UF291497; **C**, EU733157; **D**, EU733097; **E**, EU733220
*Lunulicardia retusa*22	8	**A**, UF291497; **C**, EU733158; **D**, EU733098; **E**, EU733221
*Lunulicardia retusa*29	6	**A**, UF287603; **C**, EU733156; **D**, EU733096; **E**, EU733219
*Lunulicardia hemicardia*136	15	**A**, UF299269; **B**, EU733047; **C**, EU733159; **D**, EU733099; **E**, EU733222
Outgroup		
Laevicardium sp.502	1	**A**, Field Museum306536; **C**, EU733164
*Acrosterigma biradiatum*79	6	**A**, UF285613; **C**, EU733163; **D**, EU733103; **E**, EU733226
*Papyridea semisulcata*80	25	**A**, UF286647; **B**, EU733045; **C**, EU733142; **D**, EU733084; **E**, EU733210
Papyridea sp.335	24	**A**, UF351597; **C**, EU733165; **D**, EU733104; **E**, EU733227
*Papyridea aspera*336	24	**A**, UF351597; **B**, EU733046; **C**, EU733143; **D**, EU733085; **E**, EU733211
*Fulvia australis*110	9	**A**, UF286335; **B**, EU733044; **C**, EU733141; **D**, EU733083; **E**, EU733209
*Microcardium tinctum*138	25	**A**, UF294008; **C**, EU733169; **D**, EU733106; **E**, EU733229
*Acanthocardia echinata*204	5	**A**, UMICH265485; **C**, EU733166; **D**, EU733105; **E**, EU733228
*Acanthocardia echinata*491	4	**A**, UF380498; **C**, EU733167
*Acanthocardia tuberculata*492	4	**A**, UF382863; **C**, EU733168
*Cerastoderma edule*300	S*	**A**, UF374113; **B**, EU733042; **C**, EU733139; **D**, EU733081; **E**, EU733207
*Cerastoderma edule*301	S*	**A**, UF374113; **B**, EU733041; **C**, EU733138; **D**, EU733080; **E**, EU733206
*Nemocardium pazianum*341	24	**A**, UF351592; **B**, EU733043; **C**, EU733140; **D**, EU733082; **E**, EU733208
*Cerastoderma glaucum*345	5	**A**, UMICH265488; **C**, EU733144; **D**, EU733086; **E**, EU733212
*Cerastoderma glaucum*346	5	**A**, UMICH265488; **C**, EU733145; **D**, EU733087; **E**, EU733213

COI, cytochrome oxidase I; CytB, cytochrome *b*.