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Within-host speciation events in yoyo clams, obligate commensals with mantis shrimps, including one that involves a change in microhabitat and a loss of specialized traits

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Compared to host shifts, the importance of within-host cladogenesis in the diversification of symbionts remains less well understood in marine systems. Yoyo clams (Galeommatidae: Vasconiellinae) are a clade of marine bivalves that live commensally with burrowing mantis shrimp. Almost all yoyo clams byssally-attach to the host burrow wall via a specialized hanging foot structure bearing a thread-like posterior extension. In contrast, *Parabornia squillina* (Vasconiellinae) byssally-attaches directly to the host shrimp and lacks a hanging foot structure. In this study, we examine phylogenetic relationships among vasconiellines by performing molecular analyses based on five genes (28S and 16S rRNA, *H3*, *COI* and *ANT*). We found evidence for two within-host speciation events among Floridian vasconiellines commensal with the same mantis shrimp host, *Lysiosquilla scabricauda*. One involved a cryptic sister species pair of burrow-wall commensals. The other involved the ectocommensal *P. squillina* and its somewhat unexpected sister taxon, the burrow-wall commensal *Divariscintilla octotentaculata*. This latter result suggests that a habitat shift from host burrow wall to host body surface occurred while retaining the same host species and led to the loss of the specialized hanging foot structure. Our findings suggest that ostensibly modest within-host ecological shifts can lead to major morphological changes in these clams.

ADDITIONAL KEYWORDS: adaptation – burrow – commensalism – Galeommatoidea – habitat shift – host shift – specialization – Stomatopoda – symbiosis.

INTRODUCTION

Symbiotic and parasitic organisms represent a large fraction of the Earth's biodiversity (Windsor, 1998; Poulin & Morand, 2004; Moran, 2006). Host shifting, an evolutionary change in host species, has been recognized as the major driver of speciation in these organisms both in terrestrial (Coyne & Orr, 2004; Matsubayashi, Ohshima & Nosil, 2010) and in marine realms (Duffy, 1996; Munday, van Herwerden & Dudgeon, 2004; Faucci, Toonen & Hadfield, 2007; Tsang *et al.*, 2009; Goto *et al.*, 2012; Hurt *et al.*, 2013). However, the role of host shifts in diversification may be less important than previously thought (Winkler & Mitter, 2008; Imada, Kawakita & Kato, 2011; Nakadai & Kawakita, 2016), and alternative speciation processes, such as allopatric speciation and within-host speciation, can also play an important role in the diversification of parasites and symbionts (Imada *et al.*, 2011; Nakadai & Kawakita, 2016; Jahner *et al.*, 2017). Within-host speciation driven by ecological shifts has been studied mainly in phytophagus insects (e.g. gall-inducing insects) (Cook *et al.*, 2002; Joy & Crespi, 2007). In these systems, speciation is probably initiated by adaptations to different host tissues (Cook *et al.*, 2002; Joy & Crespi, 2007; Althoff, 2014) or different life history stages (Zhang *et al.*, 2015). Similar speciation patterns have also been reported in other systems, such as avian malaria (Pérez-Tris *et al.*, 2007) and freshwater fish parasites (Vanhove

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et al., 2016). However, evidence for within-host speciation driven by ecological shifts remains limited in marine systems.

The superfamily Galeommatoidea is a group of small-bodied bivalves that exhibit high species diversity in shallow-water environments (Bouchet *et al.*, 2002; Paulay, 2003). Many galeommatoidean species are commensal with benthic invertebrates in soft sediments (Boss, 1965a; Morton & Scott, 1989; Goto *et al.*, 2012; Li, Ó Foighil & Middelfart, 2012). Galeommatoideans associate with various animal phyla (e.g. Arthropoda, Echinodermata and Annelida) (Boss, 1965a; Morton & Scott, 1989), with many species demonstrating high fidelity to a particular host species or genus (Sato *et al.*, 2011), with some exceptions (Li & Ó Foighil, 2012). Although recent molecular phylogenies suggest that host shifts between distantly related taxa occurred frequently in some clades of Galeommatoidea (Goto *et al.*, 2012; Li, Ó Foighil & Strong, 2016), some congeneric galeommatoidean species share the same host (Mikkelsen & Bieler, 1989, 1992; Goto & Kato, 2012; Goto *et al.*, 2014; Goto, Ishikawa & Hamamura, 2016), suggesting the possibility of within-host speciation in these lineages.

The subfamily Vasconiellinae is a group of galeommatoideans that includes seven genera (Huber, 2015). Most species have reduced shells covered by hypertrophied mantles with highly developed sensory tentacles (Popham, 1939; Mikkelsen & Bieler, 1989, 1992; Fig. 1). Among them, four genera (*Divariscintilla*, *Phlyctaenachlamys*, *Parabornia* and *Ephippodontomorpha*) are known as symbionts of burrowing mantis shrimp (Stomatopoda: Lysiosquillidae) (Boss, 1965b; Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Simone, 2001; Middelfart, 2005; Yamashita, Haga & Lützen, 2011). Except for *Parabornia* species, these vasconiellines live suspended from the burrow walls of their stomatopod hosts by means of a specialized hanging foot structure having a thread-like posterior extension (Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Middelfart, 2005; Yamashita *et al.*, 2011; Fig. 1). In association with this posture, these clams engage in a characteristic 'yo-yo' up and down motion by contracting and relaxing the posterior foot (Mikkelsen & Bieler, 1989, 1992; Fig. 1B; see Supporting Information, Movie S1), hence the informal 'yoyo clam' name (Mikkelsen & Bieler, 1989).

Divariscintilla includes seven described species that have been recorded from Florida, New Zealand and Japan (Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Yamashita *et al.*, 2011). Interestingly, five species (*D. yoyo* Mikkelsen & Bieler, 1989, *D. troglodytes* Mikkelsen & Bieler, 1989, *D. octotentaculata* Mikkelsen & Bieler, 1992, *D. luteocrinita* Mikkelsen & Bieler, 1992 and *D. cordiformis* Mikkelsen & Bieler, 1992) are at present known only from south-eastern Florida (Mikkelsen & Bieler, 1989, 1992), living exclusively in the burrows of a single stomatopod host, *Lysiosquilla scabricauda* (Lamarck, 1818) (Mikkelsen & Bieler, 1989, 1992). In addition to *Divariscintilla*, the ectocommensal vasconielline *Parabornia squillina* Boss, 1965 also utilizes *L. scabricauda* as a host in Florida (Boss, 1965b; Mikkelsen & Bieler, 1992), although this species has also been recorded from Mississippi (Boss, 1965b) and Panama (Moore & Boss, 1966). Unlike *Divariscintilla* spp., *Parabornia* spp. live attached to the host body surface (Boss, 1965b). Taken together, *L. scabricauda* hosts six vasconielline species in eastern Florida, thereby providing an invaluable opportunity to investigate the possibility of within-host speciation.

Burrow-wall-commensal *Divariscintilla* spp. and ectocommensal *Parabornia* spp. are thought to be closely related because many possess flower-like organs near the base of the foot in addition to morphological similarity in the posterior foot structure (Bieler & Mikkelsen, 1992; Mikkelsen & Bieler, 1992), although the posterior foot extension of the genus *Parabornia* is much shorter than that of the genus *Divariscintilla* (Mikkelsen & Bieler, 1992). Unlike the genus *Parabornia*, the shells of *Divariscintilla* spp. are partially to fully covered by mantle tissue that bears highly developed sensory tentacles (Boss, 1965b; Mikkelsen & Bieler, 1989, 1992; Simone, 2001; Fig. 1). These differences in mantle coverage and foot structure are thought to reflect the differences in host utilization between the two genera (burrow-wall-commensals vs. ectocommensals). However, the phylogenetic relationship of these genera remains unexamined.

In this study, we addressed the following questions: (1) are the six vasconiellines associated with *L. scabricauda* in Florida monophyletic, and if so, (2) how have evolutionary transitions between burrow-wall-commensal and ectocommensal lifestyles occurred in this bivalve clade? We performed molecular analyses of Vasconiellinae based on two nuclear genes (28S rRNA and histone *H3*) and three mitochondrial genes [cytochrome *c* oxidase subunit I (*COI*), 16S rRNA and adenine nucleotide translocator (*ANT*)]. Because the ectocommensal lifestyle of *Parabornia* was only briefly mentioned in previous studies (Boss, 1965b; Mikkelsen & Bieler, 1992; Simone, 2001), we observed living *P. squillina* to further understand its ecological adaptations to an ectocommensal lifestyle. Lastly, morphological characteristics of *Divariscintilla* and *Parabornia* were compared to reveal if morphological differences between genera are associated with ecological shifts.

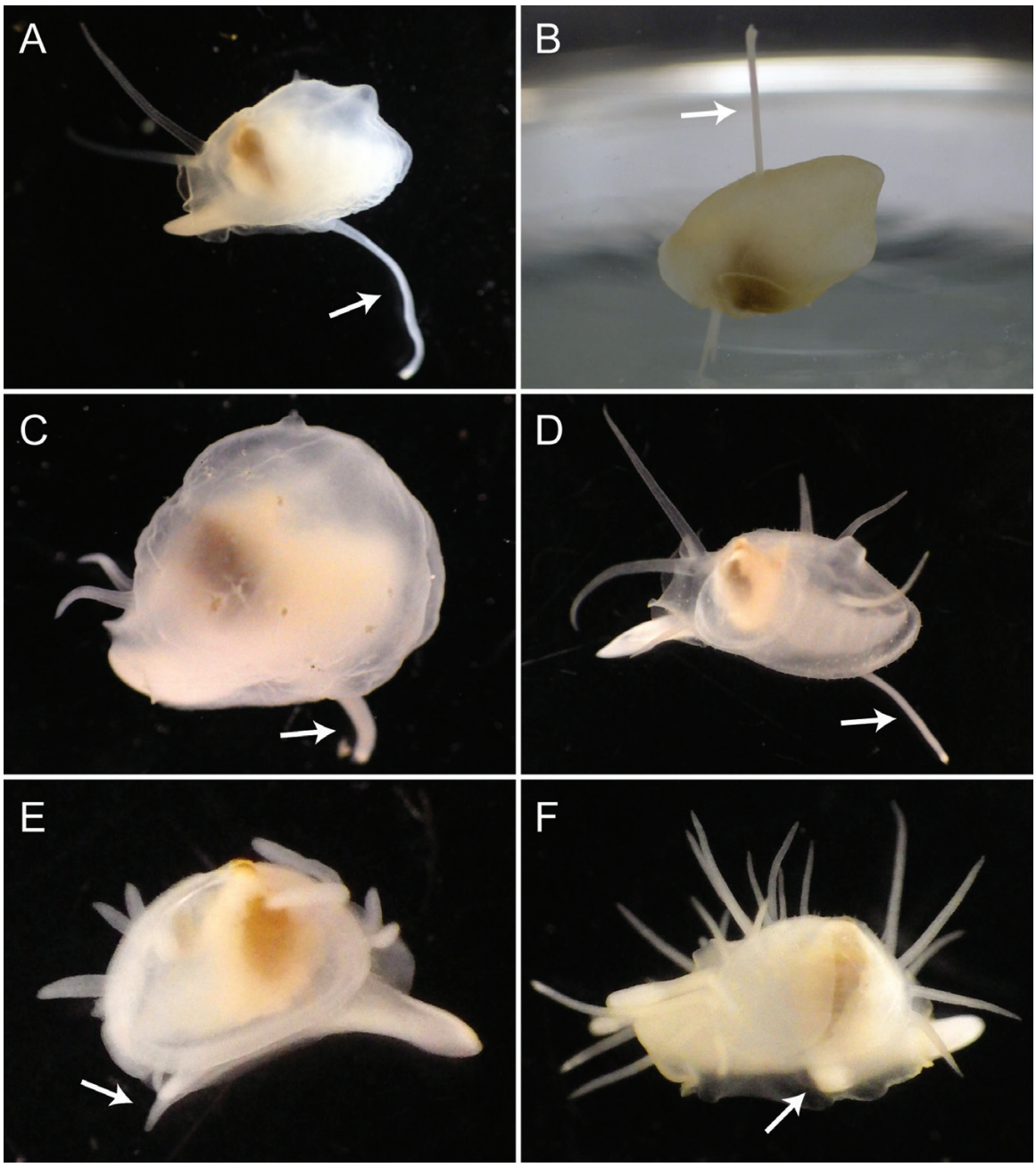

Figure 1. Diversity of Floridian yoyo clams (Galeommatoidea: Galeommatidae: Vasconiellinae: *Divariscintilla*) collected from the burrow of *Lysiosquilla scabricauda*. A. *Divariscintilla yoyo*. B. Hanging behaviour of *D. yoyo*. C. *D.* aff. *yoyo*. D. *D. troglodytes*. E. *D. octotentaculata*. F. *D. luteocrinita.* Arrows indicate posterior foot extension (hanging foot structure).

MATERIAL AND METHODS

SAMPLE COLLECTION AND OBSERVATIONS

Sampling was performed in intertidal sand flats in the Indian River lagoon (Fort Pierce, FL, USA), the type locality of the five *Divariscintilla* species (*D. yoyo*, *D. troglodytes*, *D. octotentaculata*, *D. luteocrinita* and *D. cordiformis*) (Mikkelsen & Bieler, 1989, 1992) during 30 May–4 June 2016 and 26–31 January 2017. We collected *Divariscintilla* species from *L. scabricauda* burrows using stainless steel bait pumps ('yabby pumps') and 1–2-mm mesh sieves. With the exception of *D. cordiformis*, a very rare species at this site (Mikkelsen & Bieler, 1992), all known Floridian *Divariscintilla* species were collected. We also collected *P. squillina* from the ventral body surface of it host *L. scabricauda*, which were captured

manually using fish bait. The bivalves were kept for several days in aquaria for observations and then preserved in 100% ethanol for DNA analyses. Additionally, alcohol-fixed museum specimens of *Divariscintilla* spp. and close relatives were loaned from the Muséum National d'Histoire Naturelle, Paris, Field Museum, Florida Museum of Natural History and Museum of New Zealand, Te Papa Togarewa for DNA analyses (Table 1). The DNA

Table 1. Species used for molecular phylogenetic analyses with museum catalogue number or private specimen ID, sampling localities and GenBank accession numbers

Species	Specimen ID	Sampling locality	28S rRNA	16S rRNA	*COI*	*H3*	*ANT*
Divariscintilla luteocrinita Mikkelsen & Bieler, 1992	FMNH F318896	Fort Pierce, FL, USA	**LC375966**	KX376063	**LC375982**	KX375835	KX361301
Divariscintilla octotentaculata Mikkelsen & Bieler, 1992	SMBL Mol2001	Fort Pierce, FL, USA	**LC375967**	**LC375976**	**LC375983**	**LC375991**	**LC375999**
Divariscintilla toyohiwakensis Yamashita, Haga & Lützen, 2011	SMBL Mol2002	Nakatsu, Oita, Japan	AB714788	**LC375977**	AB714869	AB714831	–
Divariscintilla troglodytes Mikkelsen & Bieler, 1989	SMBL Mol2003	Fort Pierce, FL, USA	**LC375968**	**LC375978**	**LC375984**	**LC375992**	**LC376000**
Divariscintilla yoyo Mikkelsen & Bieler, 1989	SMBL Mol2004	Fort Pierce, FL, USA	**LC375969**	**LC375979**	**LC375985**	**LC375993**	**LC376001**
Divariscintilla aff. *yoyo*	SMBL Mol2005	Fort Pierce, FL, USA	**LC375970**	**LC375980**	**LC375986**	**LC375994**	**LC376002**
Divariscintilla aff. *maoria* Powell, 1992	NMNZ M301615	Off Otago Peninsula, South Island, New Zealand	**LC375971**	KX376064	**LC375987**	**LC375995**	–
Ephippodontomorpha hirsuta Middelfart, 2005	AM C452337	Magnetic Island, Queensland, Australia	**LC375972**	KX376066	**LC375988**	KX375935	**LC376003**
Parabornia squillina Boss, 1965	FLMNH 446286	Rattle Snake Island, FL, USA	**LC375973**	**LC375981**	**LC375989**	**LC375996**	–
Phlyctaenachlamys lysiosquillina Popham, 1939	FLMNH 436851	Moorea Island, French Polynesia	**LC375974**	KX367605	**LC375990**	**LC375997**	KX361304
Phlyctaenachlamys sp.	FLMNH 436804	Moorea Island, French Polynesia	**LC375975**	KX376062	–	**LC375998**	KX361303
Outgroup							
Lasaea adansoni (Gmelin, 1791)	GenBank	–	KC429472	KC429282	KC429124	KC429203	–
Galeommatoidea sp. 1	MNHN 16650	Off Aurora, Philippines	KX376127	KX376027	–	–	KX361300
Galeommatoidea sp. 2	MNHN 7676	Off Vella Lavella Island, Solomon Islands	KX376191	KX376057	–	–	–

Abbreviations: AM, Australian Museum; FMNH, Field Museum of Natural History; NMNZ, Museum of New Zealand, Te Papa Tongarewa; MNHN, Muséum National d'Histoire Naturelle; SMBL, Seto Marine Laboratory; and FLMNH, Florida Museum of Natural History.

sequences of *Divariscintilla* and closely related species used in previous phylogenetic studies were obtained from GenBank (Table 1). For outgroups, we used several galeommatoideans that were identified to be closely related to Vasconiellinae by Li *et al.* (2016).

DNA EXTRACTION, PCR AND SEQUENCING

Total genomic DNA was isolated from the mantle or foot tissue of each bivalve specimen, including museum specimens, with the Omega Bio-Tek E.Z.N.A. Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA, USA). We sequenced fragments of 28S, 16S, *COI* and *ANT* genes. Polymerase chain reactions (PCRs) were used to amplify ~1030 bp of 28S, ~480 bp of 16S, ~690 bp of *COI*, ~330 bp of *H3* and ~580 bp of *ANT*. Amplifications were performed in 12.5-μL mixtures consisting of 1.0 μL of forward and reverse primers (10 μM each; Table 2), 0.5 μL of template DNA, 6.25 μL of GoTaq Green master mix (Promega, Madison, WI, USA) and 3.75 μL of distilled water. Thermal cycling was performed with an initial denaturation of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at a gene-specific annealing temperature (50–55 °C) and 2 min at 72 °C, with a final 3 min extension at 72 °C. All PCR products were directly sequenced at the University of Michigan Sequencing Core using PCR primers and internal primers (Table 2). The obtained sequences were deposited in the DDBJ/EMBL/GenBank databases with accession numbers LC375966–LC376003 (Table 1).

PHYLOGENETIC ANALYSES

In addition to the sequences obtained in this study, we also accessed sequence data of other galeommatoideans and outgroups from GenBank (Table 1). Sequences of the 28S and 16S genes were aligned using the Muscle program (Edgar, 2004) with default settings in the software Seaview (Galtier, Gouy & Gautier, 1996; Gouy, Guindon & Gascuel, 2010). We employed Gblocks v0.91b (Castresana, 2000; Talavera & Castresana, 2007) to eliminate the ambiguously aligned regions in the 28S and 16S genes. The sizes of 28S and 16S sequences prior to treatment with Gblocks were 1042 and 481 bp, respectively, whereas those after Gblocks treatment were 1032 and 343 bp, respectively. Phylogenetic trees were constructed using Bayesian and maximum likelihood (ML) methods. Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with substitution models chosen by Kakusan 4 (Tanabe, 2011). In the combined data set, substitution parameters were estimated separately

Table 2. Information on primers used in this study

Primer	Direction	Sequence 5′–3′	References
28S rRNA			
PCR amplification and sequencing			
D1	Forward	ACCCSCTGAAYTTAAGCAT	Colgan *et al.* (2003)
D3	Reverse	GACGATCGATTTGCACGTCA	Vonnemann *et al.* (2005)
Sequencing			
D2F	Forward	CCCGTCTTGAAACACGGACCAAGG	Vonnemann *et al.* (2005)
C2R	Reverse	ACTCTCTCTTCAAAGTTCTTTTC	Dayrat *et al.* (2001)
16S rRNA			
PCR amplification and sequencing			
16SarL	Forward	CGCCTGTTTATCAAAAACAT	Palumbi *et al.* (1991)
16SbrH	Reverse	CCGGTCTGAACTCAGATCACGT	Palumbi *et al.* (1991)
H3			
PCR amplification and sequencing			
H3F	Forward	ATGGCTCGTACCAAGCAGACVGC	Colgan *et al.* (1998)
H3R	Reverse	ATATCCTTRGGCATRATRGTGAC	Colgan *et al.* (1998)
COI			
PCR amplification and sequencing			
LCO1490	Forward	GGTCAACAATCATAAAGATATTGG	Folmer *et al.* (1994)
HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATC	Folmer *et al.* (1994)
ANT			
PCR amplification and sequencing			
ANTGF1	Forward	GCCAACTGCATTCGGTATTTCCC	Audzijonyte & Vrijenhoek (2010)
ANTR1	Reverse	TTCATCAAMGACATRAAMCCYTC	Audzijonyte & Vrijenhoek (2010)

for each gene partition [28S: GTR + Gamma, 16S: HKY85 + Gamma, *COI*: HKY85 + Gamma, GTR + Gamma, and F81 + Homogeneous (for each codon partition), *H3*: GTR + Gamma, K80 + Homogeneous, and JC69 + Homogeneous (for each codon partition), *ANT*: HKY85 + Gamma, F81 + Gamma, and JC69 + Homogeneous (for each codon partition)]. Two independent Metropolis-coupled Markov chain Monte Carlo runs were carried out simultaneously, sampling trees every 100 generations and calculating the average standard deviation of split frequencies (ASDSFs) every 1000 generations. Analyses were continued until ASDSF dropped below 0.01, at which point the two chains were considered to have achieved convergence. Because ASDSF was calculated based on the last 75% of the samples, we discarded the initial 25% of the sampled trees as burn-in. We confirmed that analyses reached stationarity well before the burn-in period by plotting the ln-likelihood of the sampled trees against generation time. ML analyses were performed using RAxML (Stamatakis, 2006) as implemented in raxmlGUI 1.31 (Silvestro & Michalak, 2012). The robustness of the ML tree was evaluated based on 1000 bootstrap replications. Datasets were partitioned by gene and the GTR + GAMMA model was implemented.

RESULTS

Observation of living *Parabornia squillina*

Three individuals of *P. squillina* were collected from one male individual of *L. scabricauda* (Fig. 2). Each individual was attached by byssal threads to the host abdomen, specifically the lateral portion of the pleonal sternite (Fig. 2E, F). Two individuals were found between the 1st and 2nd pleopods, and one was found between 2nd and 3rd pleopods. We detached the bivalves from the host to observe the extension of the foot and mantle in the living state. The bivalves have numerous short papillae extended along the ventral and posterior–dorsal margins (Fig. 2A–C). One pair of longer papillae was observed anterodorsally (Fig. 2A). The clams were placed with their host in an aquarium to test if they would reattach after removal (Movie S2). They directly approached the host by crawling, and once below the host pleopods, each clam waved its foot upward towards the host (Fig. 2G). Once their foot touched the host pleon, the bivalves attached using newly secreted byssal threads. The bivalves then crawled across the host until they reached their original position on the lateral portion of the pleonal sternite.

Molecular phylogenetic analyses

Our results suggest that Vasconiellinae is monophyletic [Bayesian posterior probability (PP) = 1.00, bootstrap percentage (BS) = 92] (Fig. 3). *Divariscintilla* aff. *maoria* Powell, 1932 was sister to all of the remaining vasconiellines (PP = 1.00, BS = 80), including the other species of *Divariscintilla*, *Phlyctaenachlamys*, *Ephippodontomorpha* and *Parabornia*. The ectocommensal *P. squillina* was nested within the burrow-wall-commensal vasconiellines and was sister to *D. octotentaculata* (Fig. 3). *Divariscintilla yoyo* included one cryptic sister species (*D.* aff. *yoyo*) (Fig. 3). Floridian vasconiellines were not monophyletic; *D. troglodytes* was sister to a clade of Pacific and Floridian species, whereas all of the other Floridian taxa formed a crown clade that was well supported in Bayesian (PP = 0.99) but not in ML (BS = 28) phylogenetic analyses.

DISCUSSION

Within-host speciation in Floridian yoyo clams

Our analysis discovered one previously unknown cryptic species (*D.* aff. *yoyo*) that is sister to *D. yoyo* (Fig. 3). They differed by 14.8% in their mitochondrial *COI* gene sequences, which is much higher than intraspecific variation levels reported for galeommatoideans [e.g. ~2% in Sato *et al.* (2011); ~5% in Li & Ó Foighil (2012)], or in our preliminary results for these two taxa [1.5% in *D. yoyo* (N = 2) and 0–0.2% in *D.* aff. *yoyo* (N = 3)] (unpublished data). They are superficially identical in external appearance but can be morphologically distinguished by their shell outlines: an angulate anterior shell margin is present in *D. yoyo* but not in *D.* aff. *yoyo* (our unpublished data). This means that in Florida, *L. scabricauda* hosts no fewer than seven vasconielline species including six burrow-wall-commensal species (*Divariscintilla* spp.) and one ectocommensal species (*P. squillina*). Our phylogenetic analyses included six Floridian vasconiellines except for *D. cordiformis*. Bayesian analyses suggested that Floridian vasconiellines are not monophyletic but are divided into two groups: *D. troglodytes* and the remaining five species (Fig. 3). The monophyly of five Floridian vasconielline species, except for *D. troglodytes* (Fig. 3), was supported by Bayesian posterior probabilities, suggesting that the diversity of Floridian vasconiellines is caused both by secondary contact of a distantly related linage (*D. troglodytes*) and by local diversification. However, bootstrap values supporting this topology are low (Fig. 3). Thus, a molecular analysis with more genetic data should be conducted in the future.

Our phylogenetic analyses identified two sister-group pairs among Floridian yoyo clams: (1) *D. octotentaculata* and *P. squillina*, and (2) *D. yoyo* and *D.* aff. yoyo. In Florida, all of these species use a single host, *L. scabricauda* (Mikkelsen & Bieler, 1989, 1992; this

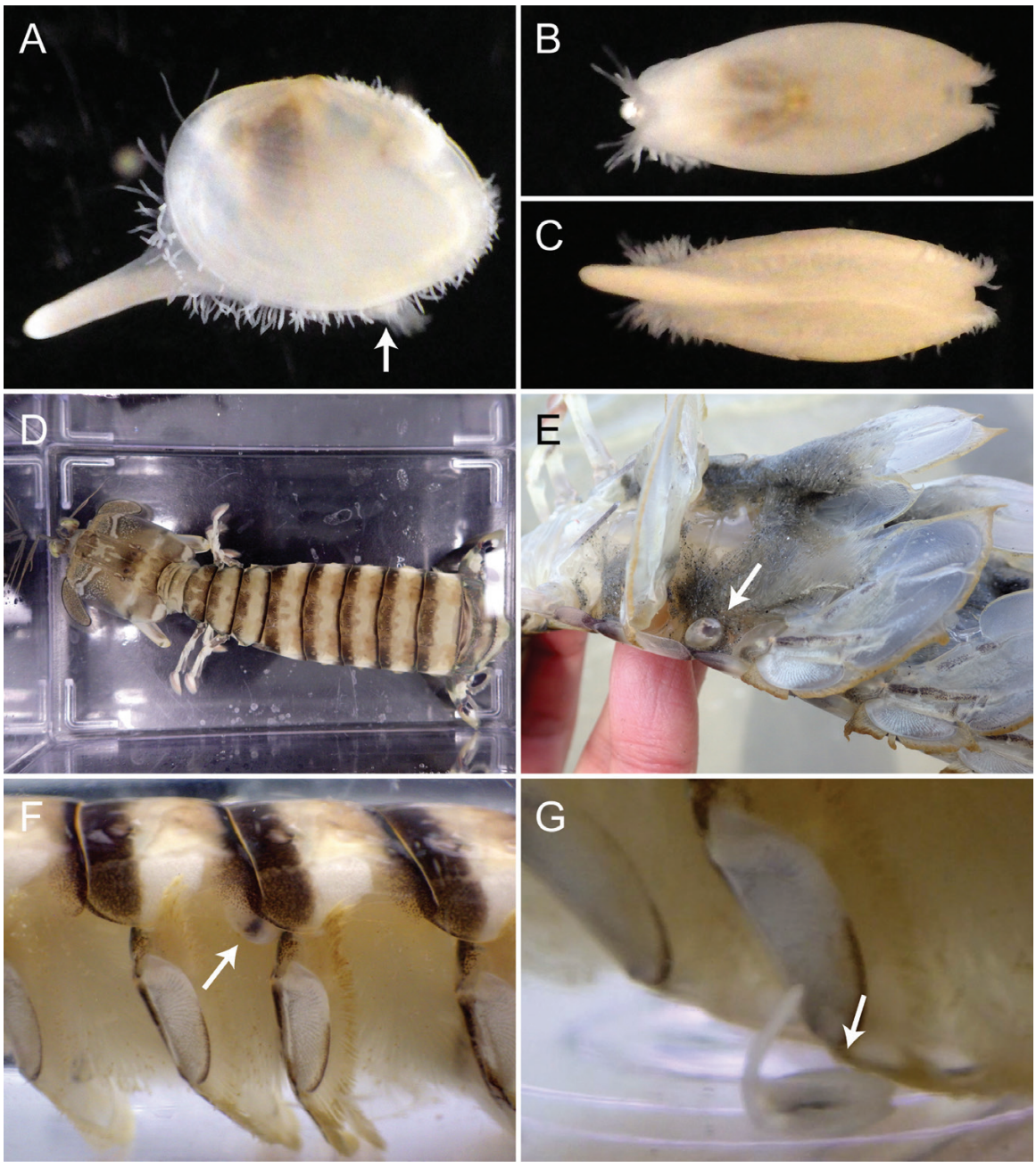

Figure 2. *Parabornia squillina* and its host *Lysiosquilla scabricauda.* A–C. A crawling individual of *P. squillina* (A, lateral side; B, dorsal view; C, ventral view). D. *L. scabricauda.* E and F. *P. squillina* attached to the lateral portion of the pleonal sternite. G. *P. squillina* extending its foot to attach to the host pleon. Arrows indicate the heel of *P. squillina* without posterior extension or hanging foot structure (A) and *P. squillina* (E–G).

study), suggesting that within-host speciation may have occurred in these two cases. Interestingly, these sister-group pairs have contrasting characteristics. *Divariscintilla octotentaculata* and *P. squillina* are ecologically and morphologically quite distinct. The former lives on host burrow walls, whereas the latter lives on the host body surface. There are differences in morphological characteristics between these two species as well, possibly corresponding to differences in host use patterns (see details below). Ecological shifts associated with host use mode probably played a key role in speciation events and led to dramatic

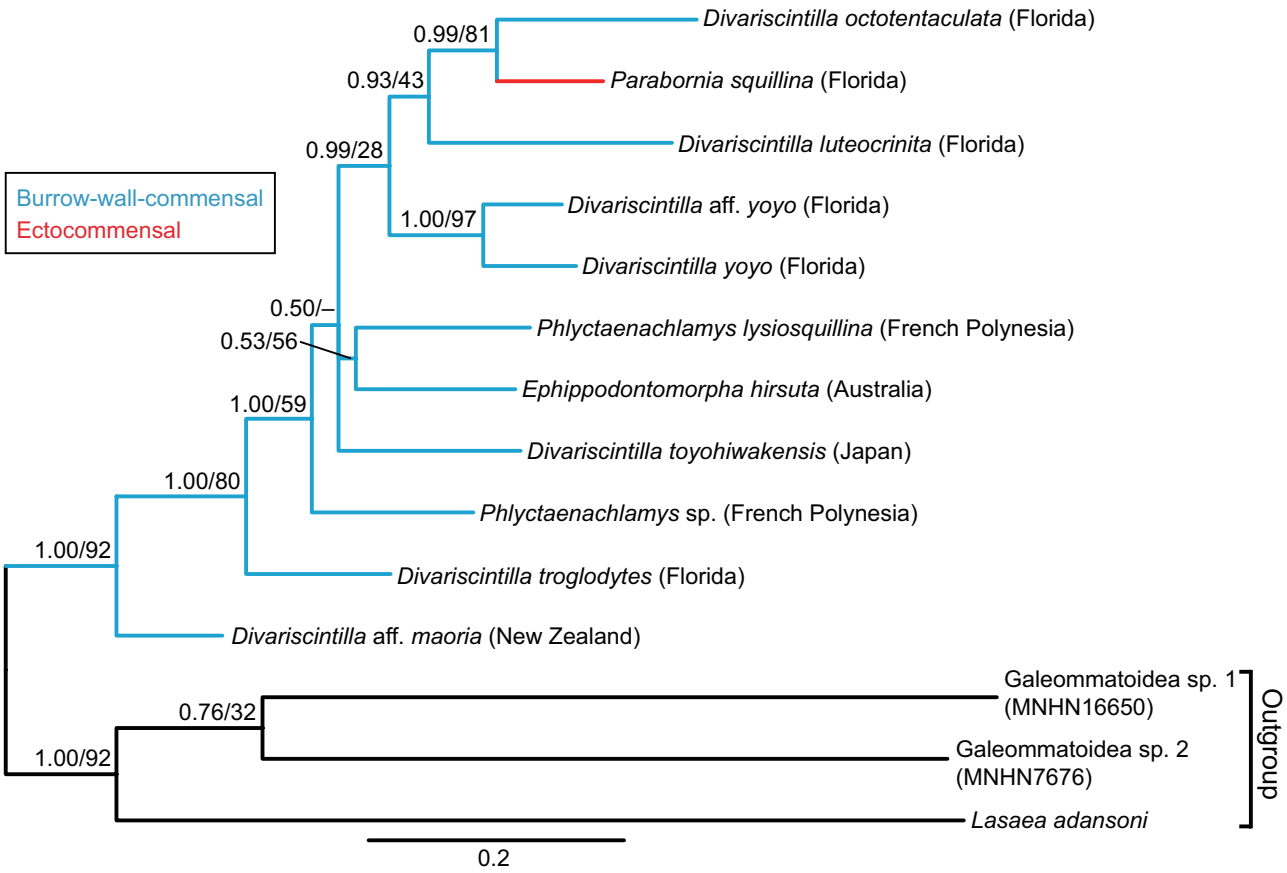

Figure 3. Bayesian phylogenetic tree of yoyo clams (Galeommatoidea: Galeommatidae: Vasconiellinae) based on the combined data set of 28S, 16S, *H3*, *COI* and *ANT* genes. Numbers above branches indicate Bayesian posterior probabilities followed by maximum likelihood bootstrap support values. Six species collected from Florida are associated with *Lysiosquilla scabricauda* and *Divariscintilla toyohiwakensis* in Japan is associated with *Bigelowina phalangium*, whereas the other species were collected from mantis-shrimp burrows but the host species were not identified. Abbreviation: MNHN, Muséum National d'Histoire Naturelle.

morphological change. *Divariscintilla yoyo* and *D.* aff. *yoyo*, by contrast, are ecologically and morphologically very similar: both live on the host's burrow walls and have two elongated anterior tentacle pairs (Fig. 1A, C). Lastly, an ecological shift is not apparent in this speciation event.

Sympatrically distributed sister species are common among marine benthic invertebrates (Knowlton, 1993) and stem from either sympatric ecological speciation or allopatric speciation with subsequent range expansion and secondary contact (Bowen *et al.*, 2013). These two mechanisms can be difficult to distinguish based upon existing patterns, and it is unclear whether sympatric speciation occurred in our two Floridian sister-group pairs (Fig. 3). If *D. yoyo* and *D.* aff. *yoyo* are not ecologically differentiated, it may be more likely that they speciated in allopatry prior to secondary contact. While Floridian *Divariscintilla* species have been recorded only from the Indian River Lagoon and areas nearby (Mikkelsen & Bieler, 1992; Mikkelsen, Mikkelsen & Karlen, 1995), this may be due to insufficient sampling (Mikkelsen & Bieler, 1992). Considering that *L. scabricauda* is distributed broadly from the Atlantic coast of the United States to Brazil (Reaka *et al.*, 2009), allopatric speciation of yoyo clams within the host distribution range is plausible. To explore this question, further investigation of the distribution of each vasconielline species is necessary.

Divariscintilla species are simultaneous hermaphrodites that brood their young to a straight-hinge 'D' veliger stage in the suprabranchial chamber as well as in the outer demibranch, and then release them to the water column through their exhalant siphon (Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Yamashita *et al.*, 2011). It remains unknown how long the planktotrophic larval stage endures in these species prior to metamorphosis and settlement in the host burrows. The shorter the duration of the planktonic stage for

sedentary or sessile marine invertebrates, the lower the rate of gene flow among discontinuously distributed populations, and the greater the probability of allopatric speciation (but see Weersing & Toonen, 2009).

Reproductive isolation mechanisms have been often considered to be necessary for the maintenance of coexistence of closely related species, because otherwise, hybridization may lead to the breakdown of species boundaries (Shine et al., 2002; Muthiga, 2003). Thus, how Floridian yoyo clams achieve reproductive isolation among co-occurring species is an intriguing question. Mikkelsen & Bieler (1992) observed an interesting copulatory-like behaviour in *D. yoyo* and *D. octotentaculata*. If this behaviour is actually copulatory in function and common in *Divariscintilla* species, it may allow them to engage in species-specific selective mating that can prevent interspecific hybridization.

Ectocommensal lifestyle of *Parabornia*

The genus *Parabornia* comprises two species, *P. squillina* and *P. palliopapillata* Simone, 2001 (Boss, 1965b; Simone, 2001). They are very similar in morphology (Simone, 2001) and both are ectocommensal on the same host, *L. scabricauda* (Boss, 1965b; Mikkelsen & Bieler, 1992; Simone, 2001). The former is distributed from Florida to Panama (Boss, 1965b; Moore & Boss, 1966; Mikkelsen & Bieler, 1992), whereas the latter is known only from Brazilian coasts (Simone, 2001). Previous studies briefly describe *P. squillina* as attached to the inner surface of the abdominal sclera of *L. scabricauda* (Mikkelsen & Bieler, 1992), whereas Simone (2001) mentioned that the other species, *P. palliopapillata*, lives attached to the pleopod base of the host. In this study, we found *P. squillina* attached to the lateral portion of the pleonal sternite of the host (Fig. 2). This is consistent with previous descriptions of *P. squillina* and *P. palliopapillana*, and suggests that these two species use the host in the same way. Simone (2001) mentioned that young individuals of *P. palliopapillata* occur on maxilipedal bases and under the carapace. This may be characteristic of *P. squillina* as well, although it was not confirmed in this study.

During behavioural trials, *P. squillina* actively moved back to the lateral portion of the pleonal sternite after being detached from the host (Movie S2), suggesting that this species has a strong habitat preference for a specific part of the host abdomen. Habitat preference for a specific part of the host abdomen is common among galeommatoideans that are ectocommensal with mantis shrimp and mud shrimp (Morton, 1972; Ó Foighil, 1985; Kato & Itani, 1995), but these shared preferences in microhabitat are the result of convergent evolution (Goto *et al.*, 2012).

Gage (1968) suggested that some galeommatoideans detect hosts by using chemicals emitted from the host. We found that *P. squillina* can home back to a specific part of host body when it is detached from the host. Thus, it is probable that *P. squillina* recognizes *L. scabricauda* based on chemotaxis to host-emitted chemicals. Most members of Vasconiellinae, including *Parabornia*, have a flower-like organ near the foot, which is suggested to be a receptor of host chemicals (Mikkelsen & Bieler, 1989, 1992; Middelfart, 2005). Additionally, we found that this bivalve has numerous short papillae that occur densely along the ventral and dorsal edges of the mantle, and the former directly touch the host abdominal body surface (Fig. 2A–C). Such papillae are not known in other burrow-wall-commensal yoyo clams (Fig. 1). It is probable that these papillae have a sensory function, and that *P. squillina* uses them to locate its preferred position on the host body.

Habitat shift from host burrow wall to host body surface

Ectosymbionts that live on the body surface of burrowing invertebrates have evolved in various marine invertebrate lineages (Funch & Christensen, 1995; Kobayashi & Kato, 2003). However, the evolutionary processes that produce an ectosymbiotic lifestyle are not well understood. In this study, we show that ectocommensal *Parabornia* evolved from burrow-wall-commensal ancestors (Fig. 3), indicating that the burrow-wall-commensal lifestyle was an evolutionary stepping stone for an ectocommensal lifestyle in this case. Other than the genus *Parabornia*, ectocommensalism has evolved multiple times in Galeommatoidea (Goto *et al.*, 2012). Evolutionary transitions from free-living to commensal lifestyles have occurred multiple times in Galeommatoidea, most of which are transitions from a free-living to burrow-wall-commensal lifestyle (Goto *et al.*, 2012; Li *et al.*, 2016). However, transitions from a free-living to an ectocommensal lifestyle have not previously been reported (Goto *et al.*, 2012; Li *et al.*, 2016), indicating that a burrow-wall-commensal lifestyle may be a prerequisite to attaining an ectocommensal lifestyle in these clams. Future in-depth phylogenetic studies of commensal galeommatoideans are likely to uncover additional cases of such evolutionary transitions.

Competition for limited resources is frequently recognized as a selective pressure that promotes habitat shifts (Schluter, 2000; Munday *et al.*, 2004; Losos, 2011; Hurt *et al.*, 2013). However, whether resource competition has influenced the habitat shift from host burrow wall to host body surface in *P. squillina* is unclear, and

based on the evidence to date, *P. squillina* and burrow-wall-commensal species do not co-occur (Mikkelsen & Bieler, 1992). While more evidence is needed, evolving an ectocommensal lifestyle may benefit *P. squillina* in several ways. For instance, in the case of burrow abandonment of one or both host shrimps, an ectocommensal can move to a new burrow with its host, although lysiosquillids may stay in the same burrow in monogamous pairs for up to 15 years (R. L. Caldwell, pers. comm.), making it unclear how large a factor this is in the ecology of *P. squillina*. Attachment to the host body may also add another level of protection. *Parabornia squillina* are hidden within the host's pleopods (Fig. 2), whereas burrow-wall-commensals may be exposed to small predators accessing the burrow. Additionally, attachment to the host may provide additional food resources and a more consistent flow of oxygenated water due to the constant movement of the pleopods (Movie S2), as known in the galeommatoidean *Borniopsis subsinuata* ectocommensal with mantis shrimps (Morton, 1981). This positioning could be particularly useful when the burrow opening is capped during moulting or during low tides. However, there are potential disadvantages to the ectocommensal lifestyle including predation on the host, especially when outside of its burrow, being lethal to the *P. squillina* and the requirement for *P. squillina* to successfully reattach to the host after moulting events, as known in the galeommatoidean *Peregrinamor* ectocommensal with mud shrimps (Itani, Kato & Shirayama, 2002). Further research is required to evaluate the relative importance of these factors.

Morphological changes associated with ecological shift

Our results show that ectocommensal *Parabornia* evolved from burrow-wall-commensal ancestors (Fig. 3). The morphologies of *P. squillina* and *Divariscintilla* species have been well described in previous studies (Boss, 1965b; Mikkelsen & Bieler, 1989, 1992; Simone, 2001). By comparing their morphological characters, we detected four major morphological changes associated with the ecological shift in *Parabornia*: (1) loss of the thread-like posterior foot extension and associated hanging behaviour, (2) loss of covering of the shell by mantle tissue (i.e. shell externalization), (3) loss of developed sensory tentacles, and (4) acquisition of dense papillae along the mantle margin. We discuss these in detail below.

Many members of Vasconiellinae, including *Divariscintilla*, *Phlyctaenachlamys* and *Ephippodontomorpha*, have a specialized foot with a thread-like posterior extension (Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Middelfart, 2005; Yamashita *et al.*, 2011; Fig. 1). This specialized foot, associated with the yoyo motion, is only known in Vasconiellinae and is suggested to be an adaptation to life on mantis shrimp burrow walls (Popham, 1939; Mikkelsen & Bieler, 1989). On the other hand, *P. squillina* has a more typical galeommatoidean foot lacking a thread-like posterior extension (Mikkelsen & Bieler, 1992; this study; Fig. 2A). Additionally, *Parabornia* has never been observed hanging from vertical wall surfaces or the host body in aquaria nor engaging in yoyo saltatory behaviour typical of *Divariscintilla* (R. Goto & T. A. Harrison, personal observations). While extreme morphological specialization of the foot has been reported in some bivalves (e.g. Dufour & Felbeck, 2003), significant morphological change in bivalve foot structure associated with a microhabitat shift is documented here for the first time.

Burrow-wall-commensal yoyo clams have reduced shells fully or partially internalized by mantle tissue bearing highly developed tentacles (Popham, 1939; Judd, 1971; Mikkelsen & Bieler, 1989, 1992; Middelfart, 2005) (Fig. 1). *Parabornia squillina* has an externalized shell as is typical in most bivalve species (Boss, 1965b; Simone, 2001; Fig. 2A). Unlike typical bivalves, burrow-wall-commensal yoyo clams actively crawl on the burrow wall surfaces using their foot. The soft mantle and tentacles are probably useful in perceiving the surrounding environment during crawling behaviour as well as in perceiving and reacting to host movement within the burrow (Judd, 1971). Unlike burrow-wall-commensal yoyo clams, *Parabornia* spp. are basically sessile on the host body surface (Fig. 2E, F), probably reducing the necessity for sensory and defensive structures and hence the loss of a mantle shell covering, although *P. squillina* does have numerous short mantle papillae (Fig. 2), which touch the host body surface. Our results suggest that evolution from (semi)internalized to externalized shells occurred as a consequence of a change in microhabitat. Evolution of shell internalization is known in several molluscan lineages (e.g. Oposthobranchia and cephalopods) (Wägele & Klussmann-Kolb, 2005; Tanner *et al.*, 2017). However, as far as we know, the evolution of shell externalization in molluscs has not been previously reported.

The internalized shells, developed tentacles and specialized foot of burrow-wall-commensal yoyo clams are considered adaptations to the unique habitat of living on walls of mantis shrimp burrows (Popham, 1939; Mikkelsen & Bieler, 1989, 1992; this study). Our study suggests that these specialized morphological traits are lost as a consequence of colonization of the host body surface. Host shifts and subsequent specialization to different host taxa have been considered drivers of morphological evolution in Galeommatoidea

(Goto *et al.*, 2012; Li *et al.*, 2016). Our study suggests that a microhabitat shift within a single host can also lead to significant morphological change. Goto *et al.* (2014) found that sister galeommatoidean species ectocommensal on *Lingula* brachiopods have significantly different shell shapes (elongated triangular shape vs. ovate shape) and suggested that this difference is due to adaptations for different posture on the host body. Goto *et al.* (2014) and the present study suggest that not only a host shift but also a ecological shift in association with the same host can play an important role in morphological evolution. However, burrow-wall-commensal yoyo clams show great morphological diversity, especially in their number of tentacles (Mikkelsen & Bieler, 1989, 1992; Fig. 1). The degree and functional significance of this tentacle diversity remains unknown. To answer this question, further examination of ecological differences among these species (e.g. niche partitioning within the host burrow) and of tentacle function is required.

Taxonomic implications and remaining issues in Vasconiellinae

Li *et al.* (2016) found that the genera *Divariscintilla*, *Ephippodontomorpha* and *Phlyctaenachlamys* formed a clade although their inter-relationships were not fully resolved. Our molecular analysis based on five genes showed that *Ephippodontmorpha*, *Phlyctaenachlamys* and *Parabornia* are nested within *Divariscintilla* (Fig. 3). According to Huber (2015), these genera are assigned to the same subfamily Vasconiellinae. The other four genera within this subfamily (i.e. *Vasconiella*, *Bellascintilla*, *Ceratobornia* and *Aclistothyra*) were not included in the present analysis. *Ceratobornia* also has hanging foot structure, but lives attached to the burrow walls of ghost shrimp on western Atlantic coasts (Dall, 1899; Narchi, 1966). Morphologically similar species with different hosts imply that host shifts have occurred in Vasconiellinae, although the hanging foot structure may have evolved multiple times in this group. Lastly, our results show that *D.* aff. *maoria* is sister to the remaining vasconiellines. *Divariscintilla* aff. *maoria* is distinguished from the other species used in this study in having a notch in the ventral side of shells. A ventral shell notch is also known in some other vasconiellines (i.e. *Vasconiella*, *Bellascintilla* and *D. cordiformis*) (Mikkelsen & Bieler, 1992; Huber, 2015). It is possible that Vasconiellinae may prove to be separable into two major groups discernible by the presence or absence of a notch in the ventral side of shells. To resolve these remaining issues, a further molecular analysis based on more taxon sampling is required.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Movie S1. Hanging behaviour of *Divariscintilla yoyo*.
Movie S2. *Parabornia squillina* moving back to the abdomen of the mantis shrimp *Lysiosquilla scabricauda*.