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Host shift and speciation in a coral-feeding nudibranch

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While the role of host preference in ecological speciation has been investigated extensively in terrestrial systems, very little is known in marine environments. Host preference combined with mate choice on the preferred host can lead to population subdivision and adaptation leading to host shifts. We use a phylogenetic approach based on two mitochondrial genetic markers to disentangle the taxonomic status and to investigate the role of host specificity in the speciation of the nudibranch genus *Phestilla* (Gastropoda, Opisthobranchia) from Guam, Palau and Hawaii. Species of the genus *Phestilla* complete their life cycle almost entirely on their specific host coral (species of *Porites*, *Goniopora* and *Tubastrea*). They reproduce on their host coral and their planktonic larvae require a host-specific chemical cue to metamorphose and settle onto their host. The phylogenetic trees of the combined cytochrome oxidase I and ribosomal 16S gene sequences clarify the relationship among species of *Phestilla* identifying most of the nominal species as monophyletic clades. We found a possible case of host shift from *Porites* to *Goniopora* and *Tubastrea* in sympatric *Phestilla* spp. This represents one of the first documented cases of host shift as a mechanism underlying speciation in a marine invertebrate. Furthermore, we found highly divergent clades within *Phestilla* sp. 1 and *Phestilla minor* (8.1–11.1%), suggesting cryptic speciation. The presence of a strong phylogenetic signal for the coral host confirms that the tight link between species of *Phestilla* and their host coral probably played an important role in speciation within this genus.

Keywords: cryptic species; host shift; nudibranchs; *Phestilla*; speciation

1. INTRODUCTION

The requirements for ecological speciation (i.e. reproductive isolation via selection for an alternative environment or food source) have been defined using mathematical models and simulations (Coyne & Orr 2004; Doebeli *et al.* 2005). The general consensus is that ecological speciation is plausible and may be more common than believed formerly, and that the most likely scenarios involve habitat or host preference, as previously stated by Bush (1969) and Mayr (1976). When host preference and local adaptation are combined with mate choice on the preferred habitat or host, assortative mating can lead to population subdivision, adaptation and divergence (Diehl & Bush 1989; Via 2001). Under these conditions, subdivision and reduction in gene flow arise from reproductive rather than geographic barriers (Bush 1994). Host preferences leading to host shifts have been most extensively investigated in phytophagous insects (reviewed in Coyne & Orr 2004). Such shifts may allow incipient species to escape from direct competition for resources or to enjoy benefits from enemy-free space (Gross & Price 1988; Lill *et al.* 2002).

The assumption that widespread marine species with larval dispersal should show little spatial variation in genetic structure, given their high potential for dispersal via ocean currents, has been questioned (e.g. Hellberg 1998; Carlon & Budd 2002; Rocha *et al.* 2005). Recent research has also shown that many nominal marine species consist of a number of cryptic taxa (reviewed in Knowlton 2000; Hart

et al. 2003; Landry *et al.* 2003; Meyer *et al.* 2005). The presence of many cryptic species (i.e. difficult or impossible to distinguish by morphology) in the oceans may be related to the role of chemical recognition systems, which are involved in many aspects of reproduction and settlement in the marine environment (Knowlton 1993). Differences in substrate specificity provide the potential for niche partitioning, adaptive shifts and the formation of marine sibling species complexes (Knowlton 1993, 2000).

Host preference is a powerful factor in promoting ecological speciation in plant–insect associations (Bush 1969; Coyne & Orr 2004). The apple maggot fly *Rhagoletis pomonella* is the most studied and best understood example of speciation by host shift (Bush 1969, 1994; Via 2001). Recent work has revealed that *R. pomonella* has many characteristics that are thought to be necessary for sympatric speciation (Feder 1998; Via 2001). For example, fruit odour discrimination led to pre-mating reproductive isolation in *R. pomonella*, which then resulted in host-race formation and incipient sympatric speciation (Linn *et al.* 2003). However, evidence from genetic data suggests that allopatry and secondary introgression may have acted in conjunction with host shifts to facilitate sympatric speciation in *R. pomonella* (Feder *et al.* 2003). Although there are still few such studies in marine communities (reviewed in Sotka 2005), several marine organisms show local adaptation to their hosts, suggesting that host use could play a fundamental role in the differentiation and speciation of groups, such as herbivorous amphipods (Stanhope *et al.* 1992; Sotka *et al.* 2003), sponge-dwelling alpheid shrimps (*Synalpheus brooksi*; Duffy 1996),

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coral-inhabiting barnacles (*Savignium milleporum*; Mokady & Brickner 2001), a predatory whelk (*Nucella caniculata*; Sanford *et al.* 2003), herbivorous saccoglossan sea slugs (Jensen 1997; Trowbridge & Todd 2001) and coral-dwelling gobies (Munday *et al.* 2004).

Opisthobranch molluscs have undergone major evolutionary radiations in connection with habitat and diet (Jensen 1997; Rudman 1998), and represent an excellent group to investigate speciation and host shifts. Opisthobranchs, including nudibranchs, are among the best examples of marine specialists; approximately, 50% of the species for which feeding preference is established appear to consume only one prey species, and many others prefer just one or two (Todd *et al.* 2001). Studies on opisthobranchs have also shown high specificity among settling larvae (Hadfield 1977; Havenhand 1991; Ritson-Williams *et al.* 2003), and that settlement cues originate from the host (Hadfield 1977; Lambert & Todd 1994; Krug & Manzi 1999).

Parallels between terrestrial, herbivorous insects and 'insect-like' marine invertebrates (i.e. those that are relatively immobile, small in size compared to their biotic hosts and use hosts for both food and shelter) have been discussed previously by Hay *et al.* (1987) and Sotka (2005). Herein, we use the term 'host' to describe the association between the tropical nudibranch *Phestilla* and its coral prey in order to make the comparison between this association and that described extensively in the insect literature (reviewed in Coyne & Orr 2004).

Species of the aeolid nudibranch genus *Phestilla* (class Gastropoda, subclass Opisthobranchia and family Tergipedidae) feed and reproduce on specific scleractinian corals (table 1). The planktonic larvae of *Phestilla* spp. require a coral-specific chemical cue to settle onto their host coral, metamorphose (Hadfield 1977; Hadfield & Pennington 1990) and complete their life cycle almost entirely on their specific coral host. Ritson-Williams *et al.* (2003) studied the host specificity of four species of *Phestilla* (*Phestilla minor*, *Phestilla sibogae*, *Phestilla* sp. 1 and *Phestilla* sp. 2) in Guam. They investigated host preference of adults in choice and no-choice feeding assays, and tested larval host specificity by measuring percentage of metamorphosis in response to different coral species. Their data reveal that larvae of *Phestilla* spp. can generally distinguish among coral species within a host genus and have highest rates of metamorphosis on their preferred coral hosts (Ritson-Williams *et al.* 2003). Furthermore, Hadfield & Koehl (2004) showed that larvae of *P. sibogae* display distinct behavioural reactions to their specific host in the water column, which affects the location of their settlement.

All species of *Phestilla* have planktonic larvae, but species differ in larval type (i.e. feeding or non-feeding larvae) and therefore in the minimum time spent in the plankton, ranging from a few hours for species with non-feeding larvae (*Phestilla* sp. 1 and *P. minor*; Ritson-Williams *et al.* 2003) to 8 days for species with feeding larvae (*Phestilla melanobranchia*; Harris 1975). *Phestilla* sp. 1 and *P. minor* are competent to metamorphose at hatching (Ritson-Williams *et al.* 2003). Reduced dispersal potential of *Phestilla* sp. 1 and *P. minor* could facilitate local adaptation and eventually, together with assortative mating on the host, ecological speciation on different coral hosts.

Thus, the genus *Phestilla* represents an excellent system to investigate host shifts and the potential for ecological speciation. The goal of this study was to construct a molecular phylogeny of the genus *Phestilla* using mitochondrial DNA (mtDNA) and to investigate the possible role of host shifts in speciation.

2. MATERIAL AND METHODS

(a) *Species studied, specimen collection and DNA extraction*

Nudibranchs were collected from corals in Hawaii, Guam and Palau (table 1) and were preserved in >70% ethanol. Species were identified according to Bergh (1905), Rudman (1981) and Ritson-Williams *et al.* (2003). In addition to the nominal species *Phestilla sibogae* Bergh, 1905 (type locality: Indonesia), *P. lugubris* Bergh, 1870 (Philippines), *P. melanobranchia* Bergh, 1874 (Philippines) and *P. minor* Rudman, 1981 (Tanzania), two undescribed species (*Phestilla* sp. 1 and sp. 2 of Ritson-Williams *et al.* 2003) and two different morphotypes for *P. minor* were distinguished. As *P. sibogae* and *P. lugubris* have been synonymized without rationale (Rudman 1981), we treat them as two different species due to their differences in larval development and adult morphology. *Phestilla* sp. 2 was identified based on distinct morphology, larval biology and food source (Ritson-Williams *et al.* 2003). Furthermore, we consider *Phestilla* sp. 1 and *P. minor* morphotype II (*P. minor* II) as distinct from *P. minor* morphotype I (*P. minor*) due to identifiably different cerata (Ritson-Williams *et al.* 2003). The only known species in the genus *Phestilla* not included in this study is the rare *Phestilla panamica* from Panama. *Phestilla panamica* resembles *P. sibogae* in morphology, but we were not able to obtain any specimens. For each specimen collected, the morphology, location and coral species on which it was found were recorded. Total genomic DNA was extracted from muscle (or whole animal if smaller than 2 mm in length) using the QIAGEN DNeasy tissue kit.

(b) *PCR, sequencing and sequence alignment*

A fragment of the mitochondrial cytochrome oxidase I gene (*COI*) was amplified using the universal *COI* primers (Folmer *et al.* 1994) under the following PCR conditions: 2 min at 94°C, 35 cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 30 s with a final extension at 72°C for 7 min. Amplification of a portion of the mitochondrial 16S ribosomal RNA gene (16S) was performed using the primers 16sar-L and 16sbr-H (Palumbi *et al.* 1991) using the same PCR conditions, but with an annealing temperature of 50°C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) prior to cycle sequencing. DNA sequencing was performed using an ABI 377 automated DNA sequencer.

The sequences were aligned and edited using SEQUENCHER v. 2.4 (Gene Codes). Alignments were confirmed and edited by eye in MACCLADE v. 4.05 (Maddison & Maddison 2000). Sequences were deposited in GenBank under accession numbers DQ417228–DQ417325.

(c) *Phylogenetic analysis*

Phylogenetic analyses were conducted with maximum-parsimony (MP), maximum-likelihood (ML), neighbour-joining (NJ) and Bayesian methods using all 49 sequences. MP, ML and NJ analyses were performed with PAUP* v. 4.0b10 (Swofford 2002). Support for individual nodes was assessed using 100 (ML) or 1000 (MP and NJ) bootstrap

Table 1. Species investigated, with larval types, collection sites, host coral, number of specimens included, collector with year of collection and GenBank accession numbers for sequences from *COI* and *16S*. (Larval types: feeding (planktotrophic), non-feeding (lecithotrophic); collectors: AF, Anuschka Faucci; GP, Gustav Paulay; MH, Michael Hadfield and RR, Raphael Ritson-Williams.)

taxon	larval type	collection locality	host coral	n	collection	GenBank accession no.	
						<i>COI</i>	<i>16S</i>
<i>Phestilla lugubris</i>	planktotrophic	Guam, Pago Bay	<i>Porites rus</i>	1	RR, 2002	DQ417298	DQ417252
		Guam, Apra Harbor	<i>Porites rus</i>	1	GP, 1996	DQ417299	DQ417253
		Guam	<i>Porites rus</i>	1	MH, 1991	DQ417300	DQ417254
<i>Phestilla melanobrachia</i>	planktotrophic	Oahu/HI, Kaneohe Bay	<i>Tubastrea coccinea</i>	3	AF, 2002	DQ417274-76	DQ417228-30
		Guam, Apra Harbor	<i>Tubastrea coccinea</i>	3	RR, 2002	DQ417277-79	DQ417231-33
		Palau, Western Channel	<i>Tubastrea micrantha</i>	3	RR, 2003	DQ417280-82	DQ417234-36
<i>Phestilla minor</i> , morphotype I	lecithotrophic	Oahu/HI, Kaneohe Bay	<i>Porites compressa</i>	3	AF, 2002	DQ417301-03	DQ417255
		Guam, Pago Bay	<i>Porites annae</i>	2	RR, 2003	DQ417304-05	DQ417256-57
		Guam, Tangrissan Reef	<i>Porites annae</i>	3	RR, 2003	DQ417306-08	DQ417258-59
		Palau, Ngel Channel	<i>Porites lutea</i>	2	RR, 2003	DQ417309-10	DQ417260-61
<i>Phestilla minor</i> , morphotype II (= <i>Phestilla minor</i> II)	lecithotrophic	Palau, N of Ngel Channel	<i>Porites lutea</i>	2	RR, 2003	DQ417312-13	DQ417263-64
		Palau, Western Channel	<i>Porites lutea</i>	1	RR, 2003	DQ417311	DQ417262
<i>Phestilla sibogae</i>	facultative planktotrophic	Oahu/HI, Kaneohe Bay	<i>Porites compressa</i>	3	AF, 2002	DQ417287-89	DQ417241-43
		French Frigate Shoals/HI	<i>Porites compressa</i>	1	AF, 2002	DQ417290	DQ417244
<i>Phestilla</i> sp. 1	lecithotrophic	Guam, Pago Bay	<i>Porites lutea</i>	3	RR, 2003	DQ417291-93	DQ417245-47
		Palau, N of Ngel Channel	<i>Porites rus</i>	2	RR, 2003	DQ417294-95	DQ417248-49
		Palau, Western Channel	<i>Porites cylindrica</i>	2	RR, 2003	DQ417296-97	DQ417250-51
		Guam, Luminao Reef	<i>Porites cylindrica</i>	3	RR, 2003	DQ417314-16	DQ417265-67
		Palau, N of Ngel Channel	<i>Porites cylindrica</i>	4	RR, 2003	DQ417319-20,23-24	DQ417269-72
<i>Phestilla</i> sp. 2	lecithotrophic	Palau, N of Ngel Channel	<i>Porites rus</i>	4	RR, 2003	DQ417317-18,21-22	DQ417268
		Guam	<i>Goniopora fruticosa</i>	3	RR, 2003	DQ417283-85	DQ417237-39
<i>Caloria indica</i> (outgroup)		Palau, Lighthouse Reef	<i>Goniopora djiboutiensis</i>	1	RR, 2003	DQ417286	DQ417240
		Oahu/HI, Kaneohe Bay		1	AF, 2002	DQ417325	DQ417273

replicates. Bootstrap replicates for the ML tree were obtained using a reduced dataset (all replicate haplotypes were removed) with 32 sequences for computational reasons. The overall tree topology of the ML tree from this reduced dataset was identical to the ML tree including all 49 sequences. For ML and NJ analyses, the model for best nucleotide substitution was selected using the Akaike Information Criterion as implemented in MODELTEST v. 3.7 (Posada & Crandall 1998). The best fit to our combined dataset was provided by the general time reversible (GTR) model of substitution, with a gamma parameter (G) of 0.466 and a proportion of invariant sites (I) of 0.488.

Bayesian analyses were conducted with MRBAYES v. 3.1.1p (Huelsenbeck & Ronquist 2001). Analyses were performed with uninformative priors. Four chains were used per run (three heated and one cold), and each analysis was repeated at least three times, twice with two million generations, and a final analysis running for 10 million generations. The *COI* and combined datasets were run as unpartitioned and partitioned datasets (i.e. the combined dataset was partitioned into the two genes and the *COI* dataset was partitioned according to the three different codon positions). All runs of the same dataset (partitioned or unpartitioned) produced nearly identical tree topologies, with the only exceptions being some minor rearrangements of sequences within a species or among clades with low support in all analyses.

Analysis of the two datasets (*COI* and *16S*) produced almost identical tree topologies when analysed individually; differences were all minor rearrangements of sequences within a species or among clades with low support. Therefore, the *16S* and *COI* data were combined, and the combined dataset was used for all analyses presented here.

Several nudibranch species within the Aeolidae were included as outgroup taxa based on the most recent nudibranch phylogeny in Wollscheid *et al.* (2001); *Eubranchus exiguus* of the family Tergipedidae and the aeolids *Caloria indica*, *Flabellina verrucosa* and *Flabellina pedata* (for phylogeny and GenBank accession number, see Wollscheid *et al.* 2001 and table 1 for *C. indica*). Genetic distances were calculated through a pairwise distance matrix in MEGA v. 3.1 (Kumar *et al.* 2004).

Monophyly of the nominal species *Phestilla* sp. 1 and *P. minor* were tested using the Shimodaira–Hasegawa test (SHT). An unconstrained ML tree was compared to an ML tree where monophyly was constrained for *Phestilla* sp. 1 or *P. minor*. The SHT was performed under the likelihood model resulting from Modeltest and using 1000 Resampling of Estimated Log Likelihood (RELL) replicates.

To test the presence of a phylogenetic signal in the characters host-coral specificity and geographic location, we used the difference in the number of steps for a character on random trees compared to the observed number of steps on the MP tree. If the number of steps for a character is less in the observed data than in at least 95% of the randomized trees, we conclude that the evolution of this character is most likely associated with this tree (i.e. there is a phylogenetic signal in this character). MACCLADE was used to determine the presence of a phylogenetic signal in both host-coral specificity and geographic location. Taxa were coded according to host genus and species as well as geographic location. All four multistate characters: (i) host genus, (ii) host species, (iii) island and (iv) locality were then mapped onto 1000 MP and 1000 randomized trees. The thousand most parsimonious trees (created in PAUP*) were randomized in MACCLADE using random joining and splitting. For all

characters, the distribution of the total number of steps (i.e. host switches or dispersal among locations) on each tree was then compared between the MP and the randomized trees.

3. RESULTS

A 615 base pair (bp) fragment of the mitochondrial gene *COI* and a 404 bp fragment of the ribosomal *16S* gene were sequenced in each of six species of the nudibranch genus *Phestilla* from Guam, Palau and Hawaii. Overall, 45 specimens of *Phestilla* from nine localities and nine different species of host corals (table 1) and four outgroups were included. Within the *16S* gene sequences, two indels were present. Specifically, all sequences of *Phestilla* sp. 1 from Guam showed a 1 bp deletion at position 192 and all sequences of *P. melanobrachia* showed a 1 bp deletion at position 194. The combined dataset included 1019 bp out of which 649 sites were constant. Of the remaining variable sites, 321 were parsimony informative.

The gross tree topology from the ML analysis of the combined dataset (figure 1) is identical to the most parsimonious tree, the NJ tree, as well as the 50% majority consensus tree resulting from the Bayesian analysis. All trees differed only in a few minor rearrangements of sequences within a species or among clades with low support (bootstrap supports (BS) and Bayesian posterior probabilities (PP) lower than 60). *Phestilla melanobrachia* and *Phestilla* sp. 2 are well distinguished by *COI* and *16S* sequence comparisons, as shown by the highly supported and reciprocally monophyletic clades. Therefore, the mitochondrial phylogeny agrees with the morphological taxonomy of these species. In contrast, *P. lugubris* and *P. sibogae*, which occur on different host species of *Porites*, cannot be distinguished by *COI* and *16S* sequence comparisons. They form a single highly supported monophyletic clade with no obvious structure corresponding to the two putative species. *Phestilla minor* morphotype II forms a monophyletic clade distinct from other sequences of *P. minor*. However, *Phestilla* sp. 1 and *P. minor* form a single monophyletic clade, where individuals of the two nominal species are intermixed. Support for some branches is low within this clade (lower than 60 for BS and PP) and monophyly was rejected for either species (SHT: $p < 0.001$). Each of the sub-clades (figure 1) within the clade including *P. minor* I and *Phestilla* sp. 1 is highly supported (both BS and PP higher than 84) and represents specimens of one species from one location and from a single species of host coral.

A total of six specimens, three from *Phestilla* sp. 1 (from Palau on *Porites rus*) and three from *P. minor* (one from Guam and two from Oahu), did not produce unambiguous *16S* sequences and were therefore included only in the *COI* dataset. The inclusion or exclusion of these individuals did not change the overall tree topology. Both sub-clades, *Phestilla* sp. 1 from Palau on *Porites rus* and *P. minor* from Oahu on *Porites compressa*, were highly supported (BS and PP higher than 89) in all *COI* trees (tree not shown).

For the combined dataset, the average pairwise sequence divergence (uncorrected p ; table 2) between the outgroup taxa and each of the nominal species of *Phestilla* (considering *P. minor* II as a distinct species) was between 17.2 and 19.9%. Among nominal species of *Phestilla*, the divergence ranged from 7.3 to 15.8%, with the exception of the divergence between *P. sibogae* and

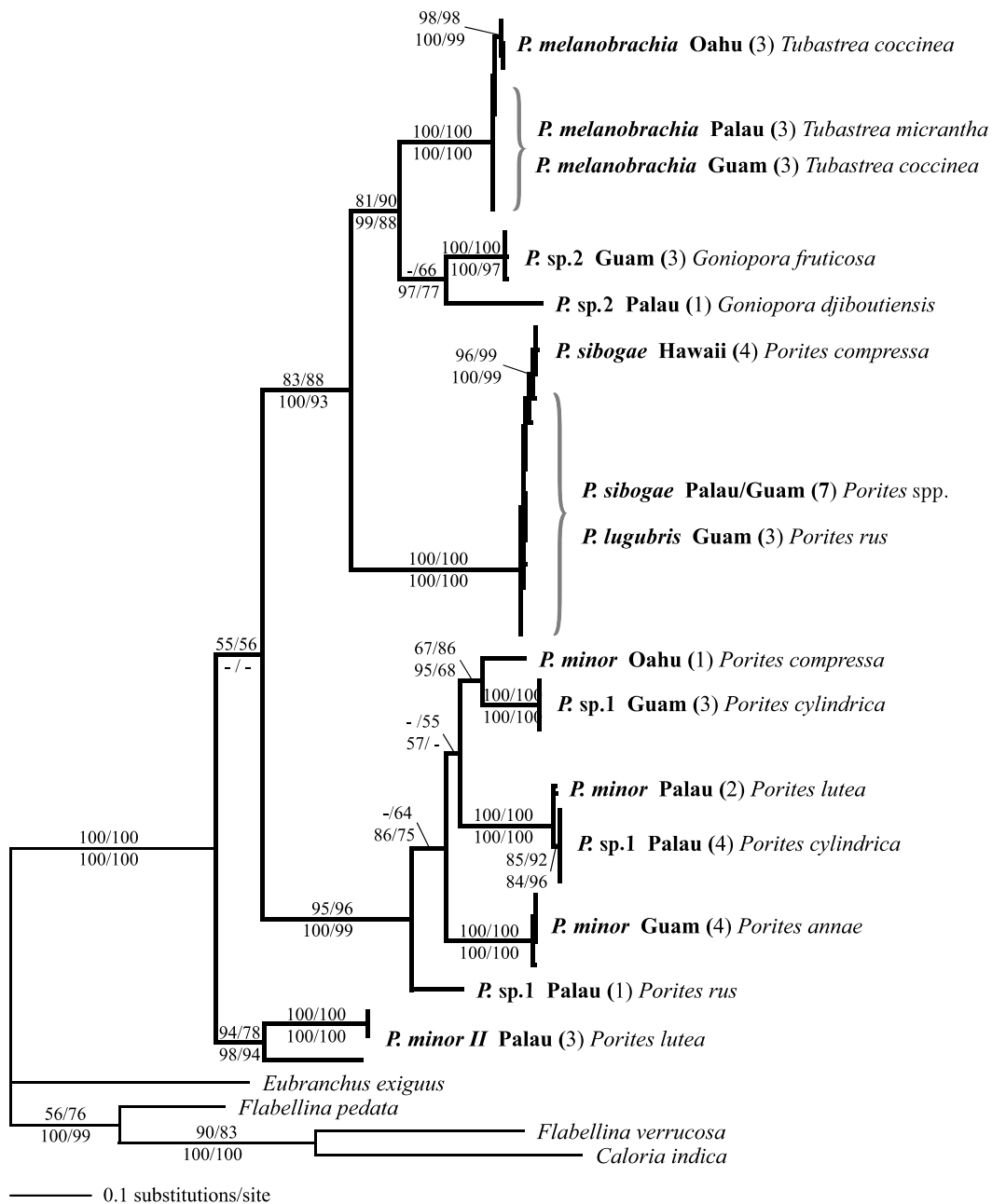


Figure 1. Maximum-likelihood tree (GTR+I+G) of the combined *COI* and *16S* dataset. Branch support is as follows: MP and NJ bootstrap support of 1000 replications are above; Bayesian posterior probabilities and ML bootstrap support of 100 replicates are below branches. Species of *Phestilla* are in bold italics, host coral species in italic and number of sequences in parentheses.

Table 2. Average genetic distances (uncorrected *p*) of combined *COI* and *16S* sequences for within (diagonal) and among (below diagonal) species of *Phestilla* and outgroup.

		1	2	3	4	5	6	7	8
1	<i>P. melanobrachia</i>	0.005							
2	<i>P. sp. 2</i>	0.102	0.044						
3	<i>P. sibogae</i>	0.148	0.14	0.009					
4	<i>P. lugubris</i>	0.148	0.139	0.008	0.006				
5	<i>P. minor</i>	0.134	0.136	0.153	0.153	0.055			
6	<i>P. minor II</i>	0.149	0.141	0.154	0.153	0.14	0.061		
7	<i>P. sp. 1</i>	0.132	0.139	0.158	0.158	0.073	0.138	0.059	
8	outgroup	0.183	0.187	0.199	0.199	0.172	0.181	0.174	0.164

P. lugubris, which was only 0.8%. The average divergence within nominal species of *Phestilla* was between 0.5 and 0.9% for *P. melanobrachia*, *P. sibogae* and *P. lugubris*, and between 4.4 and 6.1% for *Phestilla* sp. 2, *P. minor II*,

P. minor and *Phestilla* sp. 1. The average divergence for *COI* (uncorrected *p*; data not shown) among locations within *P. melanobrachia* and *P. sibogae/lugubris* is 0.2–2.3%. In comparison, the average divergence among locations

within *Phestilla* sp. 1 and *P. minor* is much higher, ranging from 8.1 to 11.1%, with the exception of *Phestilla* sp. 1 from Palau on *Porites cylindrica*, which was only 0.8% divergent from *P. minor* from Palau on *Porites lutea*. However, out of the 10 variable sites of the six combined *COI* and *16S* sequences between the two latter clades, five sites are fixed (i.e. sites at which all of the sequences in one clade are different from all of the sequences in the other clade). The most surprising result was the high sequence divergence (11.1%) between specimens of *Phestilla* sp. 1, which were collected on the same day and at the same locality in Palau but from different species of coral (*Porites rus* and *P. cylindrica*). The high divergence was also reflected in the high number of fixed differences between the eight *COI* sequences of these two clades (66 fixed sites).

Randomization tests in *MACCLADE* found a strong phylogenetic signal of both host-coral genus and species use by species of *Phestilla*. For the combined dataset, the character 'host genus' involved two steps in the MP trees and 8–13 steps in the randomized trees, whereas 'host species' involved 15–18 steps in the MP trees and 26–35 steps in the randomized trees. Therefore, the number of steps for the character 'host' (i.e. changes in host genus or species within the trees) was roughly two to six times less and non-overlapping for the MP trees as compared to the randomized trees (data not shown). There was also a phylogenetic signal for geographic location. For the combined dataset, the character 'island' involved 8–12 steps in the MP trees and 14–25 steps in the randomized trees, and 'locality' involved 16–19 steps in the MP trees and 25–35 steps in the randomized trees. Thus, the number of steps for the character 'geography' was roughly 1/2 for the MP trees as compared to the randomized trees, and for the character 'host coral genus' was always less than one-quarter of the randomized trees (data not shown).

4. DISCUSSION

Few host shifts have been reported for marine invertebrates. Some of these adaptations to different hosts or different feeding preferences evolved in different geographic populations within a species (Sanford *et al.* 2003; Sotka *et al.* 2003; Sotka 2005). In other cases, there is no genetic evidence for a host shift (Jensen 1997; Trowbridge & Todd 2001), or host races have not yet diverged, and differences may be due to phenotypic plasticity (Duffy 1996). Our data (figure 1) show a host switch from the coral genus *Porites* to *Goniopora* and *Tubastrea* in sympatric *Phestilla* spp. Host shift seems to be the likely mechanism underlying speciation in this nudibranch genus. The differentiation apparent in the mtDNA sequence data agrees with the data on host use in the field and host preference and specificity of adults and larvae in laboratory experiments (Ritson-Williams *et al.* 2003). For example, adults of *Phestilla* sp. 2 ate only polyps of species of *Goniopora* and its larvae had by far the highest rate of metamorphosis in response to *Goniopora* species (Ritson-Williams *et al.* 2003). Likewise, adults of *Phestilla* sp. 1, *P. minor* and *P. sibogae* ate only polyps of *Porites* spp., preferring the species on which they have been found most frequently in the field, and on which the larvae had the highest rate of metamorphosis (Ritson-Williams *et al.* 2003). In *Phestilla*, both host coral and island location show a

significant phylogenetic signal. However, the number of additional steps between observed and randomized host trees was at least twice (and often much more) than between observed and randomized location trees. Therefore, we hypothesize that speciation in allopatry with subsequent dispersal, which results in currently overlapping ranges, played a smaller role than adaptation to different hosts in driving the speciation of *Phestilla*.

Shifts to a new host may provide release from direct competition for resources and/or enemy-free space (Gross & Price 1988; Lill *et al.* 2002). Most nudibranchs have diverse chemical and other defence mechanisms and are rarely victims of predation (Todd 1981). However, besides camouflage and hiding, *P. sibogae* has no recognized defence mechanism (Haramaty 1991). *Phestilla sibogae* is rarely encountered in the field, but becomes very abundant when coral heads of *Porites* spp. are kept in predator-free seawater tanks (Haramaty 1991). Further, it has been shown that *P. sibogae* is heavily preyed upon by reef fish and crustaceans in the field (Gochfeld & Aeby 1997). Harris (1975) suggested that rapid growth and shorter time to sexual reproduction in *P. sibogae* compared to *P. melanobranchia* evolved as a result of intense predation pressure. Therefore, looking at our phylogeny of *Phestilla*, where feeding on the coral genus *Porites* is ancestral, it appears that the slower growth and longer time to reproduction of *P. melanobranchia* may be the result of a host switch from *Porites* to *Tubastrea* and release from predation pressure. This host switch is remarkable considering the substantial chemical and morphological differences between the solitary cupcoral *Tubastrea* and the reef building colonial coral *Porites*.

Differences in larval host preference can promote host-race formation (Linn *et al.* 2003). There can be differences among batches of larvae in the proportion of metamorphosis of larvae of *Phestilla* spp. in response to settlement cues (Hadfield 1984; Ritson-Williams *et al.* 2003), which might suggest a genetic component (Toonen & Pawlik 2001) or plasticity (Hadfield & Strathmann 1996) to the response of settlement cues. Furthermore, larval response to host corals appears to be less specific than adult feeding preference (Ritson-Williams *et al.* 2003), suggesting that host shifts are most likely to occur at the larval stage. Since the larval stage is also the stage at which dispersal occurs for *Phestilla* species, it seems most plausible for this to be the stage at which the host shift occurs. However, these host-switching events must be rare to prevent hybridization between already isolated and specialized populations or new host races or species could not arise. *Phestilla* sp. 1 and *P. minor* appear to comprise a cryptic species complex. The short or non-existent planktonic period of these species implies reduced potential for dispersal, which could be one reason for this divergence, because restricted gene flow via restricted dispersal or active habitat choice can lead to local adaptation (Kawecki & Ebert 2004).

Phestilla lugubris and *P. sibogae* cannot be distinguished using *COI* and *16S* sequence comparisons. In Guam, where both the species occur, they are distinguished by morphological differences (i.e. shape of adult head, size of adults and size of egg masses) as well as by the mode of larval development. However, *P. sibogae* has been observed to occasionally lay eggs with feeding (planktotrophic) larvae on Guam, but when these feeding larvae were raised to adults, they laid eggs containing non-feeding

(lecithotrophic) larvae (V. Paul 2005, personal communication). However, we (MGH) have never seen planktotrophic larvae emerge from egg masses of *P. sibogae*, despite 37 years of studying this species in Hawaii. The opisthobranch mollusc, *Alderia modesta*, can switch from producing only lecithotrophic larvae to producing a mix of lecithotrophic and planktotrophic larvae under starvation (Krug 1998). In the same study, Krug (1998) showed that the two reproductive morphs were not distinct based on *COI* sequences. Furthermore, even though larvae of *P. sibogae* are lecithotrophic, they retain all feeding structures and are actually able to feed; i.e. the larvae are facultative planktotrophic (Kempf & Hadfield 1985). Thus, the main difference between the larvae is presence or absence of yolk. Consequently, two possibilities emerge: (i) the divergence between *P. lugubris* and *P. sibogae* is relatively recent and not yet reflected in the mtDNA sequences or (ii) the two nominal species represent different morphs of the same species and the differences in morphology, adult and egg mass sizes and mode of larval development may be triggered by environmental factors. However, we do not want to base species designation solely on 1019 bp of mtDNA (Rubinoff 2006). Therefore, until additional investigation adds clarity to these questions, it is useful to retain the names *P. lugubris* for the form with planktotrophic larvae and *P. sibogae* for the better studied form with lecithotrophic development.

In general, planktotrophic development is considered to be ancestral and easier to lose than gain (Strathmann 1978). Strathmann (1978) stated that the potential for gaining a feeding from a non-feeding stage depends on the original mechanism of larval feeding and the degree of reorganization at metamorphosis and adult structure. Some gastropods appear to have more flexibility in reacquiring planktotrophic larvae because encapsulated or lecithotrophic larvae often retain the structures used in feeding (Strathmann 1978). Most phylogenetic studies on the evolution of different larval types confirm the ancestral character state of planktotrophy and its irreversibility once lost (reviewed in Hart 2000). However, Reid (1989) argued that reversal from non-feeding to feeding larvae was most parsimonious for littorinid snails based on ancestral (feeding larvae) and derived (shell form) traits. Likewise, within calyptraeid gastropods, planktotrophy has been regained possibly three times from direct development with nurse eggs (Collin 2004). Some species with nurse eggs appear to have fewer embryonic modifications than those with large yolky eggs, and therefore retain the greatest possibility of subsequent evolution of a different mode of development (Collin 2004). In *Phestilla*, our results suggest that planktotrophy arose twice (in both *P. melanobranchia* and *P. lugubris/sibogae*) from a lecithotrophic ancestor (figure 1). However, it also appears that lecithotrophic species examined to date (*P. sibogae* and *P. minor*) retain the ancestral feeding structures. Furthermore, larvae of *P. sibogae* have the capacity to feed on phytoplankton (Kempf & Hadfield 1985), and other lecithotrophic species of *Phestilla* may share this ability. In order to clarify the evolution of larval types within the genus *Phestilla*, more experiments and observations of all species within the genus and its closest relatives would be needed.

Our data indicate that a taxonomic revision of the genus *Phestilla* is needed. The two nominal species *Phestilla* sp. 1 and *P. minor* form a polyphyletic clade, where individuals of the two species from different locations and host corals are intermixed (figure 1). Furthermore, *P. minor* II from Palau on *Porites lutea* is very distinct from the rest of the specimens. In addition to the molecular divergence, this morphotype also has identifiably different cerata, suggesting that it may be a cryptic species. We call this species *P. minor* II, but further studies are clearly needed to resolve its taxonomic status.

Although *Phestilla* sp. 1 and *P. minor* occur in sympatry and are morphologically similar, they feed on different species of host corals. In Guam, *Phestilla* sp. 1 has been observed to feed on *Porites cylindrica* and *P. rus* in the field as well as in the laboratory, whereas *P. minor* feeds on *Porites annae* and *P. lutea* in the field, but only *P. annae* in the laboratory (Ritson-Williams *et al.* 2003). Each of the highly divergent sub-clades within the *P. minor*/sp. 1 clade includes individuals of a species from one location and from a single species of host coral. Even between the least divergent sub-clades, *Phestilla* sp. 1 on *Porites cylindrica* and *P. minor* on *Porites lutea* from Palau (figure 1), there are fixed genetic differences. In the most extreme case, specimens of *Phestilla* sp. 1 from Palau collected from two different species of *Porites* are as divergent (11.1%) as recognized nominal species within the genus of *Phestilla* (7.3–15.8%) and show a high number of fixed differences (66 sites within *COI*). The most parsimonious explanation for this divergence appears to be speciation by host shift. We suggest that each of these distinct lineages probably represents a cryptic species; if true, we would have to rename four of these sub-clades and retain *P. minor* for just one of them.

The relationship between clades of *Phestilla* sp. 1 and *P. minor* and their specific hosts may be obscured by the unresolved taxonomy of the host-coral genus *Porites*. Taxonomy at the species level is still problematic in most coral genera due to plasticity and absence of fixed morphological characters among species and the lack of appropriate molecular markers for fine-scale phylogenies (Forsman *et al.* 2006). The genus *Porites* is no exception and its species taxonomy is still largely unresolved (Forsman *et al.* 2006), which raises the possibility that cryptic species within *Porites* are confusing the relationship between nudibranchs and their host species. Since larvae of *Phestilla* are better able to distinguish slight chemical differences among species of *Porites* than we are, the phylogeny of *Phestilla* may also shed light on the species classification of *Porites* corals.

Our data comparing *COI* and *16S* sequences, and the presence of a strong phylogenetic signal for the coral host, confirm that the tight link between species of *Phestilla* and their host coral likely played an important role in the speciation of this genus. To further resolve the relationships within *Phestilla*, we would need to expand collection sites to encompass the entire range of the genus. Furthermore, it would be most useful to compare the complete phylogeny of *Phestilla* with a similarly detailed phylogeny of the respective host corals. Rocha *et al.* (2005) argue that ecological speciation can explain high diversity in marine habitats where physical barriers to gene flow are rare, but many more studies are still needed to understand the mechanisms of speciation in the sea.

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