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SYMPOSIUM

Poecilogony and Population Genetic Structure in *Elysia pusilla* (Heterobranchia: Sacoglossa), and Reproductive Data for Five Sacoglossans that Express Dimorphisms in Larval Development

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Synopsis Credible cases of poecilogony, the production of two distinct larval morphs within a species, are extremely rare in marine invertebrates, yet peculiarly common in a clade of herbivorous sea slugs, the Sacoglossa. Only five animal species have been reported to express dimorphic egg sizes that result in planktotrophic and lecithotrophic larvae: the spionid polychaete *Streblospio benedicti* and four sacoglossans distributed in temperate estuaries or the Caribbean. Here, we present developmental and genetic evidence for a fifth case of poecilogony via egg-size dimorphism in the Sacoglossa and the first example from the tropical Indo-Pacific. The sea slug *Elysia pusilla* produced both planktotrophic and lecithotrophic larvae in Guam and Japan. Levels of genetic divergence within populations were markedly low and rule out cryptic species. However, divergence among populations was exceptionally high (10–12% at the mitochondrial cytochrome *c* oxidase I locus), illustrating that extensive phylogeographic structure can persist in spite of the dispersal potential of planktotrophic larvae. We review reproductive, developmental, and ecological data for the five known cases of poecilogony in the Sacoglossa, including new data for *Costasiella ocellifera* from the Caribbean. We hypothesize that sacoglossans achieve lecithotrophy at smaller egg sizes than do related clades of marine heterobranchs, which may facilitate developmental plasticity that is otherwise vanishingly rare among animals. Insight into the environmental drivers and evolutionary results of shifts in larval type will continue to be gleaned from population-level studies of poecilogonous taxa like *E. pusilla*, and should inform life-history theory about the causes and consequences of alternative development modes in marine animals.

Introduction

Comparative analyses of diverse clades of marine invertebrate reveal frequent transitions between planktotrophic and lecithotrophic life histories (Hart et al. 1997; Duda and Palumbi 1999; Jeffery et al. 2003; Collin 2004; Byrne 2006). Egg size underlies most transitions in larval type, and is thus highly labile over evolutionary time scales. Egg size can also vary within marine species in response to local selective pressures (Marshall and Koeugh 2003; Marshall et al. 2008). It is therefore an outstanding mystery of marine evolutionary ecology why so few species express dimorphisms in egg size that result in alternative larval types (Hoagland and Robertson 1988; Bouchet 1989; Hart 2000; Collin 2012).

At the time of the 2012 symposium on poecilogony sponsored by the Society for Integrative and Comparative Biology, only five marine taxa were known to express egg-size dimorphisms. In all five cases, conspecific mothers produce two distinct larval morphs: small eggs develop into planktotrophic larvae that require an extended period of feeding, while large eggs develop into lecithotrophic larvae that can metamorphose without feeding in

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the plankton. The well-studied spionid polychaete *Streblospio benedicti* is the only annelid that expresses poecilogony through egg-size dimorphism (Levin 1984; Levin et al. 1991; Levin and Bridges 1994). The remaining four examples all belong to the Sacoglossa, a clade of sea slugs (heterobranch gastropods) that are primarily host-specialized consumers of siphonaceous or coenocytic algae (Jensen 1996). The high frequency with which egg-size dimorphisms occur in the Sacoglossa is a striking anomaly, given the almost complete absence of such reproductive plasticity in the rest of the animal kingdom. Sacoglossans therefore warrant special attention, as this under-studied group may yield important mechanistic insights into the causes and consequences of life-history transitions.

In three sacoglossan species, most populations are planktotrophic but a few demes express lecithotrophy. In the northwestern Atlantic, most *Elysia chlorotica* are planktotrophic but one population in Massachusetts produced lecithotrophic larvae (West et al. 1984). Similarly, in 8 of 10 Caribbean populations, *E. zuleicae* produced only planktotrophic larvae, but some individuals sampled from two Bahamas islands produced lecithotrophic clutches (Krug 2009). Nine of 13 populations of the Caribbean species *Costasiella ocellifera* produced planktotrophic larvae, two demes produced only lecithotrophic larvae, and both developmental modes were expressed in two populations (Ellingson 2006; Ellingson and Krug, unpublished data). In the warm-temperate eastern Pacific, populations of *Alderia willowi* switch development seasonally (Krug and Ellingson 2006; Krug et al. 2012b). All four examples of gastropod poecilogony are supported by data confirming that two truly distinct larval types are produced by one species. In *E. chlorotica* and *C. ocellifera*, breeding crosses established conspecificity of individuals differing in development (West et al. 1984; Ellingson and Krug, unpublished data). In *E. zuleicae* and *C. ocellifera*, molecular data confirmed that all sampled individuals were conspecific (Ellingson 2006; Trathen 2010).

However, unsubstantiated or erroneous claims of poecilogony have also been made for sacoglossans (Clark and Jensen 1981; Marín and Ros 1993) and other heterobranchs (Eyster 1979; Chia et al. 1996; Clemens-Seely and Phillips 2011). Some claims derived from errors in taxonomy (Clark 1984), or from confusing dimorphisms in *larval type* with dimorphisms in *dispersal potential* among lecithotrophic clutches. Lecithotrophic veligers can metamorphose (1) within the egg mass, (2) after hatching and swimming, or (3) a mixture of the two (Krug 2009). Clutches with both hatching larvae and encapsulated metamorphosis represent a dispersal dimorphism but not poecilogony (a dimorphism in egg size and/or larval type). In contrast to some literature assertions, no poecilogonous species expresses both swimming lecithotrophic larvae and ametamorphic or "type 3" development (Thompson 1967), the suppression of larval features; true cases of ametamorphic development are relatively rare in sea slugs (Thompson 1967; Chia 1971; Hadfield and Miller 1987).

Given the potential role of poecilogony as a transitional stage in life-history shifts, it is important to understand why it evolves more frequently or persists for longer periods in herbivorous sea slugs, compared to all other animal groups. Identifying additional cases from the Sacoglossa should also permit tests for correlated ecological or life-history traits within a phylogenetic context; such patterns may yield insight into the selective regimes or suites of traits that favor the evolution of poecilogony, and allow it to persist as a stable strategy. We report reproductive, ecological, and molecular genetic data for *Elysia pusilla* from sites across the tropical Indo-Pacific, and demonstrate that this species is the fifth case of poecilogony in the Sacoglossa. We also present new data and summarize the literature on reproduction, development, and ecology for all five poecilogonous sacoglossans in an attempt to identify traits potentially correlated with origins of variable larval development.

Materials and methods

Identification and collection of organisms

Elysia pusilla (Bergh 1872) is distributed throughout the tropical Indo-Pacific and has a complex taxonomic history. Macnae (1954) described *E. halimedae* from South Africa, and the species was quickly recognized as widespread. Marcus (1980) recognized that specimens termed *E. halimedae* from Japan by Baba (1957) were morphologically distinct from South African *halimedae* and described the Japanese species as *E. macnaei*. Jensen and Wells (1990) later identified both *E. halimedae* and *E. macnaei* as junior synonyms of *Elysiella pusilla*, a monotypic genus. We found no rationale for why *E. macnaei* was synonymized with *E. pusilla*, and genetic studies indicate that material matching the description of *E. macnaei* is an unrelated species (Händeler et al. 2009). Jensen (1997) subsequently described a second species of *Elysiella* (*E. stylifera*), disregarding Gosliner (1995) who determined that *Elysiella* was a junior synonym of *Elysia*. Molecular phylogenetic studies recovered

E. pusilla and *E. stylifera* as sister taxa within a basal clade of tropical *Elysia* spp. (Händeler et al. 2009), supporting Gosliner (1995); we thus refer both *pusilla* and *stylifera* to *Elysia*.

Adult specimens were obtained by the authors by collecting algae in the host genus *Halimeda* via snorkel in the tropical waters of Guam, Japan, Australia (Table 1). Specimens from Hawaii, Vanuatu, and Costa Rica were obtained as preserved material from colleagues or museum collections. A collection of slugs that superficially resembled *E. pusilla* was made in Moorea by M. Phuong from *Halimeda* spp.; one specimen and two planktotrophic egg masses laid by morphologically similar specimens were obtained as preserved material.

Characteristics of egg masses and larvae

Slugs were collected from host algae in the laboratory, and held in groups to facilitate mating and oviposition. In *Elysia* spp., fertilized ova are deposited within a membranous capsule containing albumen; a string of capsules is embedded within a jelly matrix that is enclosed within a protective outer membrane (Klussmann-Kolb and Wägele 2001). We use ''egg'' to refer to uncleaved ova, ''embryo'' to pre-veliger stages, and ''egg mass'' to the entire structure. Many tropical *Elysia* spp. embed strings or blobs of extra-capsular yolk (ECY) in the jelly matrix, but outside of the individual egg capsules (Krug 2009).

Egg masses from Guam and Australia were carefully removed from the surface of containers using a scalpel blade and were photographed with an Olympus 5060 digital camera (Olympus, Center Valley, PA) through a Zeiss Stemi 3000 stereomicroscope (Carl Zeiss Microscopy LLC, Thornwood, NY). Egg masses from Japan were examined and imaged using an Olympus Mic-D micoscrope. In collections from Guam and Japan, two non-overlapping size classes of eggs were observed; representative egg masses of each type were separated for measurements. Diameters of uncleaved ova were made for putatively planktotrophic ($n = 7$–14 ova per clutch) and lecithotrophic ($n = 16$–20 ova per clutch) egg masses using an ocular micrometer, or from high-resolution digital images calibrated with a hemocytometer. After hatching, larval shell length was measured across the aperture, and mean shell length was determined for two clutches of each development mode. Means are reported ±1 standard error. Distribution and color of ECY were noted for all egg masses. Putatively lecithotrophic egg masses from Guam were transported back to Los Angeles; after hatching, larvae were maintained in 0.45 µm filtered seawater (FSW) for 9 days and scored daily for metamorphosis in the absence of any cue.

Extra-capsular yolk has been proposed to be defensive (Clark et al. 1979) or nutritive (Boucher 1983; Allen et al. 2009; Krug 2009). To determine if feeding on ECY contributes to larval growth in *E. pusilla*, a preliminary trial was run on two planktotrophic clutches of *E. pusilla* from Australia, laid on April 20, 2007. One egg mass was opened when veligers were beginning to emerge from their individual capsules, but were still contained within the egg mass; freed veligers were fed a culture of a local phytoplankton ($\sim 5 \times 10^4$ cells ml^{-1}) for 3 days, until the second egg mass hatched naturally. The veligers in the second egg mass were able to ingest ECY granules for 3 days while the outer casing of their egg mass gradually broke down. Shell length was measured for 24–30 larvae per clutch and compared to determine whether feeding on ECY or phytoplankton resulted in larger larvae.

Phylogenetic analyses

Genomic DNA was extracted from specimens preserved in 100% ethanol with a QIAamp DNA Mini

Table 1 Collection localities, dates, and number of specimens and clutches examined for *Elysia pusilla*

Location	Latitude, longitude	Date sampled	Collector	Number of specimens examined	Development mode (number of clutches)	Sample codes
Coulom, Australia	26°40′45″S, 153°7′59″E	4/2007	P.K.	10	P (3)	Epus_07Aus01-06
Pago Bay, Guam	13°25′38″N, 144°47′56″E	8/2009	P.K.	22	P (11), L (2)	Epus_09Gua01-04, 10,19,20,22
Sobe, Okinawa, Japan	26°13′3″N, 127°39′29″E	7/2004	C.T.	5	P (1), L (1)	Epus_04Jap01-05
Maui, Hawaii	20°43′N, 156°27′W	7/2011	A.V.	2	n.d.	Epus_11Maui01-02
Guanacaste, Punta Carbon, Costa Rica (Pacific coast)	9°50′N, 85°24′W	1/2001	Y.C.-G.	1	n.d.	Epus_01Cos01
Moorea, French Polynesia	17°30′12″S, 149°49′24″W	10/2012	M.P.	3	P (2)	Epus_10Mor01,07,17
Vanuatu	15°8′S, 166°53′E	2006	T.G.	1	n.d.	Epus_06Van01

Note: T.G., Terry Gosliner; P.K., Patrick Krug; M.P., Mark Phuong; C.T., Cynthia Trowbridge; A.V., Ángel Valdés.

Kit (Qiagen, Inc., Valencia, CA) and at −20°C stored in extraction buffer. Using polymerase chain reactions (PCR), portions of three loci were amplified from selected specimens (Table 1): (1) 658 bp of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene; (2) ~420 bp of the mitochondrial large ribosomal subunit rRNA (16S) gene, using primers and reaction conditions described by Händeler et al. (2009); and (3) 328 bp of the nuclear histone 3 (H3) gene using conditions described by Colgan et al. (2000). Two different-sized H3 products persistently amplified from Guam slugs; a modified forward primer (AAGTCTACCGGAGGCAAGG) paired with the Colgan et al. (2000) reverse primer amplified only the correct product, but resulted in shorter sequences (300 bp). Purified PCR products were directly cycle-sequenced in both directions using PCR primers and Big Dye Terminator 3.1 Cycle Sequencing chemistry at the High-Throughput Genomics Unit, University of Washington. Chromatograms were edited and primer sequences removed in GeneiousPro 5.1.4 software.

Sequences for each locus were aligned separately in ClustalX v1.83 (Thompson et al. 1997) using default settings. One variable loop region present only in the 16S sequence from the out-group *E. stylifera* was deleted prior to phylogenetic analysis; indels were otherwise treated as missing data. Alignments were deposited in TreeBase. When H3 genotypes were heterozygous, gametic phase was inferred if alleles differed by a single base. Phase of H3 alleles could not be determined for most specimens from Australia without cloning, which was not attempted. All H3 sequences were truncated to the 300 bp region obtained for Guam samples. Sequences will be deposited in the National Center for Bioinformatics (NCBI) database and accession numbers included in Supplementary Table 1 upon acceptance.

Evolutionary relationships among mitochondrial haplotypes were inferred using Bayesian Markov-chain Monte Carlo (MCMC) methods on concatenated COI + 16S sequences, using mixture models implemented in the software package BayesPhylogenies (Pagel and Meade 2004). For specimens, for which only one of the two loci amplified, the other was filled in as missing data. Two GTR + Γ models were parameterized during runs as adding a third model did not substantially improve likelihood scores. The better-fit model was assigned to each position in the data alignment during runs, to avoid forcing one model to apply to all positions in a given gene partition. Base frequencies, rate parameters, and a γ-shape parameter were estimated during runs, with four rate classes drawn from the gamma distribution to allow rate heterogeneity among positions.

Based on prior analysis of evolutionary relationships among sacoglossans (Händeler et al. 2009; Vendetti and Krug, unpublished data), four taxa were included as out-groups in phylogenetic analyses. Within *Elysia*, the most basal clades are (1) *E. pusilla* and its sister taxon *E. stylifera*, and (2) the Caribbean species *Elysia patina*, *E. zuleicae*, and *E. papillosa*. The three Caribbean species were designated as the out-group for Bayesian analyses. Four independent Markov chains were run for 5×10^6 generations, saving a tree every 10^3 generations. The first 80% of trees from each run was discarded as burnin, and the final 10^3 trees of all runs were combined into one tree sample. A 50% majority-rule consensus tree with mean branch lengths was generated in BayesTrees (http://www.evolution.reading.ac.uk); posterior probability (PP)** support values ≥0.9 were interpreted as statistically significant (Douady et al. 2003; Huelsenbeck and Ranalla 2004).

Mean genetic distances within and among populations were calculated in Mega 5.0 for each locus. For COI and 16S, the Tamura–Nei (TrN) model was used to correct for multiple substitutions per site and gaps were coded as missing data; the TrN model was the best-fit model of sequence evolution available in Mega 5.0, based on jModeltest runs under the Bayesian Information Criterion (Posada 2008). For the slowly evolving H3 gene, p-distances were calculated between alleles. For population-level comparisons, two samples from Maui (Hawaii) and one from Costa Rica were grouped as a single northeastern Pacific population, due to phylogenetic affinity (see "Results" section). An analysis of molecular variance (AMOVA) was also performed on COI haplotypes in Arlequin 3.0 (Excoffier et al. 1992, 2005) to determine how genetic covariance was partitioned among, versus within, populations, using 10^4 permutations of the data for significance tests and TrN-corrected genetic distances. Haplotypes were edited to a uniform 650 bp, and Vanuatu was excluded as only one sample was available.

Reproductive and ecological data for four other poecilogonous sacoglossans

To screen for reproductive, geographical, or ecological traits that might be correlated with poecilogony, we compiled data for the five sacoglossans known to express variable development. We used published data for *Elysia chlorotica* (West et al. 1984) and *E. zuleicae* (Krug 2009). Raw data for *Alderia willowi*

summarized by Krug (1998) were re-analyzed, calculating mean egg, and larval sizes for individual clutches and then calculating grand means and errors from the per-clutch mean values. Original data were collected for *Costasiella ocellifera* from the Bahamas and Florida (FL) Keys (Table 1). Miles and Clark (2002) proposed that a planktotrophic population in Lake Surprise (upper Keys) was a cryptic species, distinct from the lecithotrophic population near Key West, FL. However, molecular analyses indicated both demes were conspecific with Caribbean *C. ocellifera* populations, which also differ in development (Ellingson 2006). Slugs producing lecithotrophic offspring were collected from Sweetings Cay, Bahamas in June 2004, and from Key West, Florida in October 2009 and 2010. Slugs producing planktotrophic larvae were collected from San Salvador, Bahamas in June 2004, and from Lake Surprise, FL in October 2009 and 2010. Slugs were isolated in Petri dishes for oviposition; egg masses were transferred to FSW until planktotrophic larvae hatched, or lecithotrophic larvae completed intra-capsular metamorphosis. Egg sizes were taken from Miles and Clark (2002). Shell size was measured for planktotrophic larvae at hatching ($n = 2$ clutches), and for lecithotrophic larvae at metamorphosis ($n = 5$ clutches), and body length was measured for newly metamorphosed juveniles ($n = 3$ lecithotrophic clutches).

Results

Reproduction and development in *Elysia pusilla*

Initial observations of Japanese specimens suggested that *E. pusilla* was poecilogonous. Specimens collected from *Halimeda macroloba* and *H. opuntia* had a mean body length of 3.9 mm (range: 2–8 mm). One collection of slugs from Sobe, Okinawa (July 2004) produced two egg masses, both containing yellow–orange ECY but differing greatly in embryonic size. The first clutch of embryos developed into small veliger larvae, which ingested ECY granules after emerging from their individual capsules, but prior to hatching from the egg mass. Hatching veligers had eyespots, normally a sign of competence; however, pre-competent planktotrophic larvae hatch with eyespots in some heterobranchs including the sacoglossan *Costasiella nonatoi* (Goddard 2001; Ellingson and Krug, unpublished data). At hatching, mean length of larval shells was 140.3 μm (±7.1 SE; $n = 20$), typical for planktotrophic larvae. The second egg mass contained larger embryos that developed into hatching larvae with a mean shell length of 267.3 μm (±4.3 SE; $n = 7$), large by comparison with other lecithotrophic larvae (Krug 2009). No attempts were made to induce settlement and metamorphosis. Egg sizes for the two morphs were not recorded, but Baba et al. (1956) reported uncleaved ova were 114 μm in diameter for *E. pusilla* (as *E. halimedae*) from Japan, consistent with sizes of lecithotrophic eggs measured in other populations (see below).

Specimens from Queensland, Australia (April 2007) were collected from *H. discoidea* in <5 m depth. Three egg masses laid by 10 pooled specimens were planktotrophic and contained a winding ribbon of orange ECY. The ECY gradually disappeared as larvae completed intracapsular development and the larval digestive gland turned orange, suggesting larvae were absorbing yolk or ingesting particles that entered their capsules through minute tears. Larvae that fed on ECY for the final 3 days of development achieved a significantly larger mean shell size (146.2 μm ± 1.3 SE; $n = 30$) than did larvae that were hatched prematurely and fed concentrated phytoplankton for 3 days (120.2 μm ± 1.6 SE; $n = 25$) (two-tailed *t*-test: d.f. = 53, $t = 12.68$, $P < 0.0001$). Larvae that hatched naturally from a second clutch had a mean shell size of 153.8 μm (±1.5 SE; $n = 13$).

Specimens of *E. pusilla* ($n = 22$) from Guam were collected from *H. taenicola* in the shallow subtidal zone (August 2009; Fig. 1A). Of 13 deposited egg masses, 11 were planktotrophic. For two such clutches, mean egg diameter was 71.4 μm (±0.4 SE; $n = 15$) and 68.8 μm (±0.5 SE; $n = 16$). However, the remaining two clutches had substantially larger eggs; mean ovum diameter in one clutch was 97.0 μm (±0.9 SE, $n = 14$), much larger than mean egg size for the planktotrophic clutches (Table 2). Hatching larvae had a mean shell length of 218.8 μm (±2.3 SE, $n = 26$) and 217.6 μm (±2.5 SE, $n = 16$), respectively, significantly larger than planktotrophic larvae. The larger larval morph developed in 15–16 days at ~22°C, and hatched with eyespots and a well-developed propodium, consistent with metamorphic competence. In the first clutch, one larva metamorphosed in FSW after 2 days of swimming; a further seven larvae metamorphosed 1 week later, in the absence of any inductive algal cue. The larger larvae were therefore lecithotrophic, as all nutrients needed to complete metamorphosis were maternally provided. An extended swimming phase prior to metamorphosis suggested that newly hatched larvae delay settlement in the absence of the host alga (Krug 2001, Trowbridge and Todd 2001; Krug 2009).

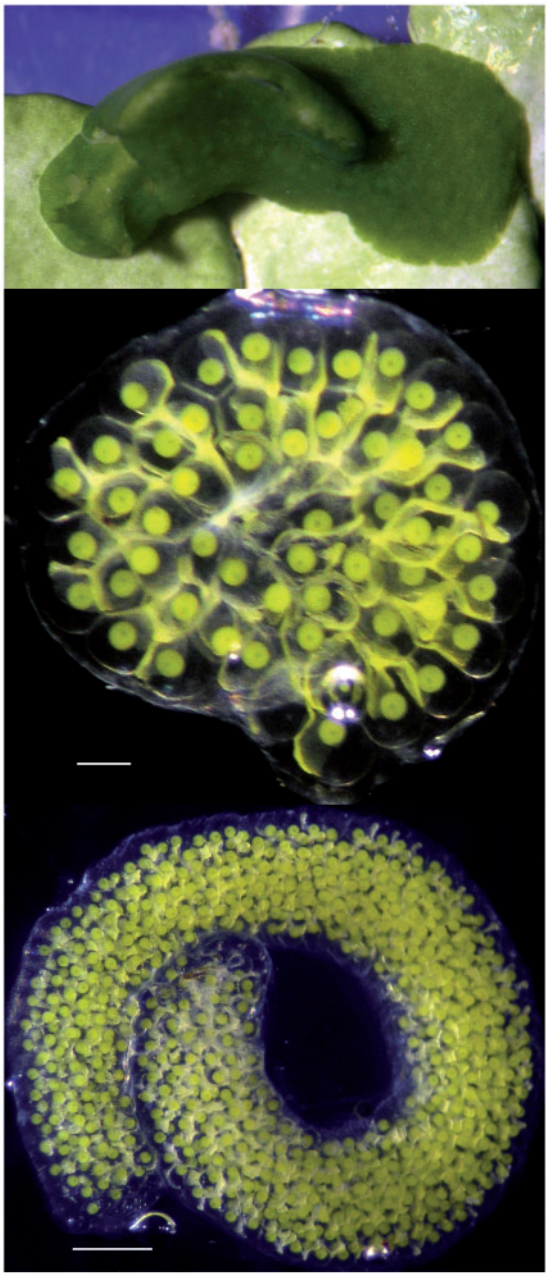

Fig. 1 *Elysia pusilla* from Guam, and egg masses of both developmental morphs. (**A**) Representative specimen of *E. pusilla* (6 mm) resting on a piece of the host alga *Halimeda taenicola*. The flattened foot and reduced parapodial lobes are characteristic adaptations to adhering to *Halimeda* in high-surge environments. (**B**) Lecithotrophic egg mass showing encapsulated uncleaved ova, with orange ribbon of ECY winding among the egg capsules. Scale bar = 200 μm. (**C**) Planktotrophic egg mass with uncleaved ova and ECY, laid by a specimen collected on the same clump of *Halimeda* as the slug that deposited the lecithotrophic clutch in (**B**). Scale bar = 500 μm.

Molecular phylogenetic analysis

To test for differences among regional populations and the potential for cryptic species within populations, we inferred evolutionary relationships of mitochondrial haplotypes based on concatenated COI and 16S partial gene sequences. Bayesian Inference revealed a high degree of phylogeographic structure among tropical Pacific populations of nominal *E. pusilla* (Fig. 2). All sampled populations of *E. pusilla* were 19.7–23.0% divergent from the sister taxon, *E. stylifera*. A basal clade of three haplotypes from Moorea, French Polynesia, was highly divergent from all other *E. pusilla* populations, with pairwise TrN distances ranging from 14.4% to 18.4% (Table 3A). This magnitude of divergence is comparable to interspecific distances observed between other pairs of sister species in *Elysia* (Krug et al. 2012a), suggesting the Moorea population might comprise a cryptic species.

The remaining mtDNA sequences comprised five divergent and highly supported (PP = 1.0) clades (Fig. 2). Only one clade was sampled at each site except for Guam, which contained representatives of three divergent clades. One significantly supported clade (PP = 0.96) comprised two reciprocally monophyletic groups, with a clade of Australian haplotypes that was sister to a clade containing the lone Vanuatu sample and specimen Guam_10. A second large clade (PP = 0.97) comprised a polytomy of three strongly supported subclades (PP = 1.0). Six of eight Guam haplotypes formed one subclade. In the second subclade, both samples from Maui, Hawaii, grouped with the lone sample from the Pacific coast of Costa Rica. In the third subclade, all samples from Japan formed a clade with weak support (0.88) that was sister to, but 7% divergent from one haplotype from Guam. Pairwise TrN distances between populations at the COI locus ranged from 9.8% to 12.8%, excluding Moorea (Table 3). Mean divergence among haplotypes was low within populations other than Guam, even when grouping samples from Hawaii and Costa Rica as one population (0.3–2.3%). High among-population divergence was also evident at the more conserved 16S locus (Table 3B). However, the Moorean population was no more divergent at 16S than any other population, in contrast to patterns at the COI locus.

Including Moorea as a putative population of *E. pusilla*, AMOVA revealed highly significant differentiation among populations at the COI locus ($\Phi_{ST} = 0.7833$, $P < 0.00001$; Table 4A). Omitting the Moorean population had little effect on estimates of genetic structure ($\Phi_{ST} = 0.7245$, $P < 0.00001$; Table 4B), and all populations were significantly different in pairwise Φ_{ST} comparisons when Moorea was excluded ($P < 0.02$). Thus, *E. pusilla* has an exceptionally high degree of genetic subdivision, and this

Table 2 Summary reproductive data for five poecilogonous sea slugs in clade Sacoglossa

	Egg diameter (μm)		Number of eggs per clutch		Larval shell size (μm)	
Species	**Planktotrophic**	**Lecithotrophic**	**Planktotrophic**	**Lecithotrophic**	**Planktotrophic**	**Lecithotrophic**
E. pusilla[a]	70 ± 1.2 (2)	97.0 ± 0.9 (1)	n.d.	59 (1)	150.0 ± 3.8 (2)	218.2 ± 0.6 (2)
E. zuleicae[b]	66.1 ± 1.6 (2)	n.d.	n.d.	104.0 ± 10.4 (4)	109.5 ± 6.2 (1)	253.9 ± 4.5 (4)
E. chlorotica[c]	79.3 ± 3.7 (5)	96.0 ± 0.7 (15)	8091.5 ± 1814.5 (16)	175.7 ± 45.9 (6)	145.8 ± 1.2 (80)	216.7 ± 0.8 (100)
A. willowi[d]	67.9 ± 1.1 (6)	106.0 ± 1.2 (11)	310.7 ± 24.5 (30)	32.4 ± 2.3 (30)	116.0 ± 2.8 (4)	186.0 ± 0.8 (11)
C. ocellifera[e]	76.6 ± 0.4 (10)	106.0 ± 1.4 (10)	219.0 ± 19.6 (31)	24.0 ± 3.5 (26)	120.8 ± 7.2 (2)	267.8 ± 5.6 (4)

	Time to hatching (d)		Percentage of intracapsular metamorphosis	Juvenile size (μm)	Extra-capsular yolk?	Habitat (region)	Host algal genus
	Planktotrophic	**Lecithotrophic**					
E. pusilla	n.d.	15 ± 1.0 (2)	0% (2)	241.7 ± 7.2 (1)	Orange ribbon	Reef (IP)	*Halimeda*
E. zuleicae	5.7 ± 0.7 (3)	18.5 ± 0.5 (2)	0% (4)	n.d.	White ribbon	Mangrove, reef (Car)	*Udotea*
E. chlorotica	5.8 ± 0.3 (10)	10.7 ± 0.2 (10)	~100% (93)	n.d.	None	Estuarine (NW Atl)	*Vaucheria*
A. willowi	3.0 ± 0.1 (30)	5.4 ± 0.1 (30)	5.5% ± 1.6 (30)	n.d.	None	Estuarine (NE Pac)	*Vaucheria*
C. ocellifera	5.4 ± 0.4 (7)	14.6 ± 0.5 (7)	100% (>50)	511.6 ± 28.1 (4)	None	Mangrove (Car)	*Avrainvillea*

Note: Data given as mean ± standard error, with number of clutches in parentheses. IP, Indo-Pacific; Car, Caribbean; Atl, Atlantic; Pac, Pacific.
[a]This study.
[b]Krug (2009).
[c]West et al. (1984).
[d]Krug (1998, 2001). Raw data from Krug (1998) were re-analyzed to yield grand mean values based on individual clutch means.
[e]Egg size data from Miles and Clark (2002); all other data are from this study.

pattern is not driven by the divergence of the Moorean samples.

All substitutions at the nuclear H3 locus were silent, including outgroup alleles. Within *E. pusilla*, H3 alleles were <2% divergent except for two alleles from Moorea that were 6.6–7.3% distant from other *E. pusilla* alleles. Pairwise distances between alleles from *E. stylifera* and non-Moorean *E. pusilla* ranged from 6.6% to 9.6%, further suggesting that the Moorean samples comprised a cryptic species (Table 3C). All five specimens from Japan were homozygous for an H3 allele, as was the Guam_20 specimen that grouped with Japan by mtDNA. The Japanese allele was distinguished from other alleles by two fixed differences. The remaining H3 alleles from Guam ($n = 6$) had two fixed differences with all other populations, and three additional polymorphic sites (although gametic phase could not be resolved for two specimens). The Guam_20 individual was likely a recent immigrant to Guam from a western Pacific population, as its mtDNA and H3 alleles had no affinity with other Guam samples. The Guam_10 specimen, which had divergent mtDNA that grouped with Vanuatu, was homozygous for the H3 allele common in Guam, but H3 alleles were not available for the Vanuatu specimen for comparison. The Costa Rican specimen was homozygous for one of two alleles sampled in Maui, Hawaii, consistent with their mtDNA similarity. Only one H3 allele could be resolved from Australian samples, but all Australian H3 alleles shared one fixed difference. An additional eight sites were polymorphic in Australia, which harbored the highest level of allelic and nucleotide diversity.

Developmental and ecological data for five poecilogonous sacoglossans

Egg sizes for planktotrophic morphs of all five poecilogonous sacoglossans fell within a narrow range of 13 μm, and lecithotrophic egg sizes differed by a maximum of 10 μm among species (Table 2). Mean egg diameter for the lecithotrophic morph was 38.6% ± 14.3 SE greater than the corresponding planktotrophic morph (range: 21.1–56.1%, $n = 4$ species). Clutch size of the lecithotrophic morph was an order of magnitude lower than the planktotrophic morph in *A. willowi* and *C. ocellifera*, whereas in the larger species *E. chlorotica*, mothers produced >40 times more planktotrophic larvae than lecithotroph-producing conspecifics.

Planktotrophic larvae of *E. pusilla* were slightly larger than those of *E. chlorotica*, which feed for only ~2 weeks prior to metamorphosis, but were much larger than larvae of the other three species. Lecithotrophic larvae of *E. pusilla* were larger in Japan than Guam, and comparable to lecithotrophic larvae of *C. ocellifera* (Table 2). There was no obvious pattern in egg or larval size among habitat types.

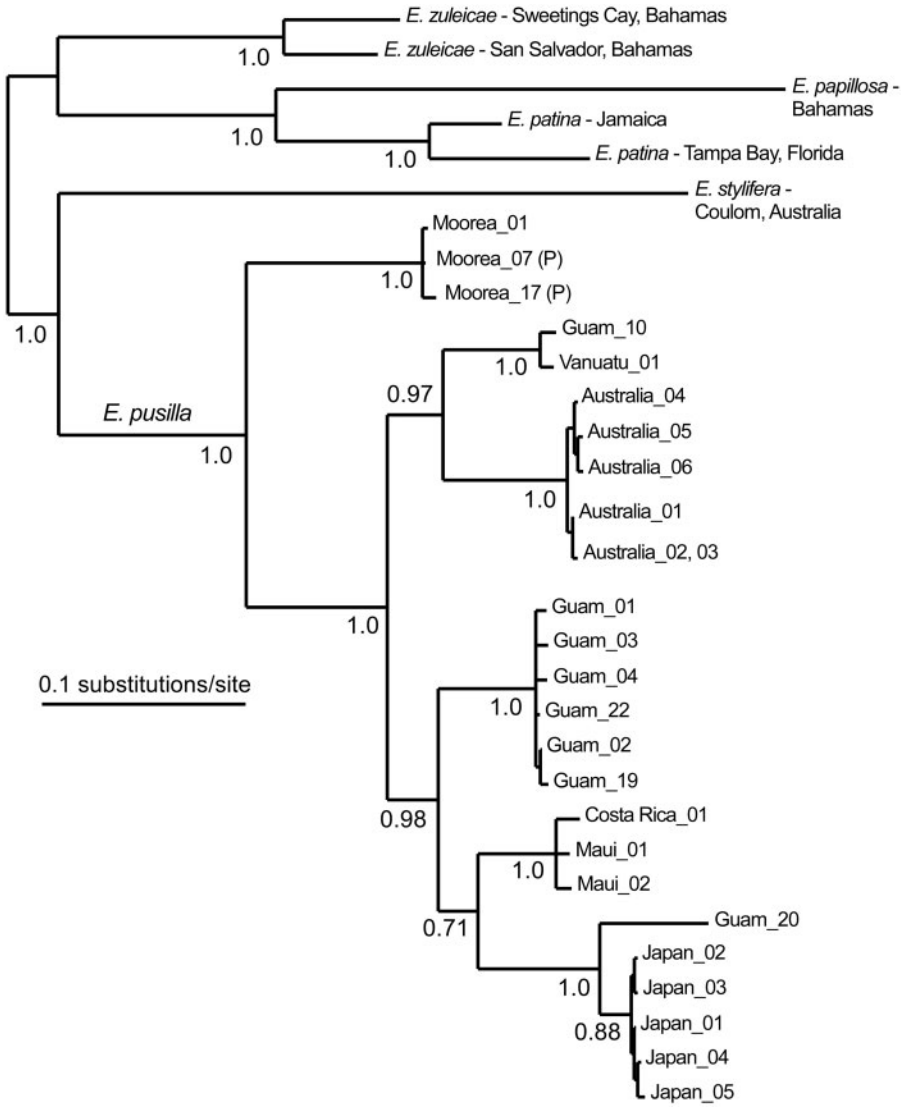

Fig. 2 Phylogenetic relationships among mitochondrial haplotypes of *Elysia pusilla* based on Bayesian analysis of concatenated COI and 16S gene fragments. A 50% consensus phylogram was generated with mean branch lengths from a tree sample derived by pooling the final 10^3 trees from four independent MCMC analyses. Posterior probabilities are given before key nodes, with values $\geq$0.9 taken as significant. "P" denotes specimens from Moorea that were individually typed as planktotrophic.

Planktotrophic eggs of two species that lacked ECY (*C. ocellifera* and *E. chlorotica*) were the largest among these five species, but those of *A. willowi* (which also lacks ECY) were the second smallest. There were no evident phylogenetic effects, as the three *Elysia* spp. were not more similar to each other in any respect than they were to the more distantly related sacoglossans.

No trends in the dispersal potential of lecithotrophic larvae were evident (Table 2). In two species, all larvae complete metamorphosis within the egg mass. In *A. willowi*, a small percentage of larvae undergo intracapsular metamorphosis, but a larger fraction settles spontaneously within 1–2 days of hatching (Krug 2001). In *E. pusilla* and *E. zuleicae*, all lecithotrophic larvae hatched and little metamorphosis occurred soon after hatching. Juveniles of *E. pusilla* were less than half the length of emerging juveniles of *C. ocellifera*, and smaller than four Caribbean elysiids for which juvenile sizes are known (Krug 2009).

Discussion

Genetic divergence and species status

Despite high divergence in mtDNA, all samples of *E. pusilla* collected by the authors were morphologically and ecologically indistinguishable. Most sequenced specimens were not typed for mode of development, except for planktotrophic samples

Table 3 Genetic distances within and among populations of *Elysia pusilla* at mitochondrial and nuclear loci

	Vanuatu	Australia	Guam	Maui + Costa Rica	Japan	Moorea
(A) COI						
Australia	0.0974	**0.0059**				
Guam	0.1041	0.1137	**0.0736**			
Maui + Costa Rica	0.1190	0.1256	0.1118	**0.0239**		
Japan	0.1282	0.1236	0.1084	0.1039	**0.0043**	
Moorea	0.1442	0.1828	0.1785	0.1590	0.1768	**0.0051**
E. stylifera	0.1972	0.2298	0.2183	0.2215	0.2036	0.2281
(B) 16S						
Australia	0.0654	**0.0028**				
Guam	0.0685	0.0822	**0.0233**			
Maui + Costa Rica	0.0565	0.0783	0.0610	**0.0062**		
Japan	0.0741	0.0996	0.0685	0.0654	**0.0019**	
Moorea	0.0538	0.0726	0.0678	0.0655	0.0695	**0.0062**
E. stylifera	0.1383	0.1291	0.1345	0.1444	0.1325	0.1311
(C) H3						
Australia	–					
Guam	0.0143	**0.0066**				
Maui + Costa Rica	0.0050	0.0127	**0.0033**			
Japan	0.0099	0.0176	0.0050	–		
Moorea	0.0677	0.0688	0.0660	0.0710	**0.0033**	
E. stylifera	0.0726	0.0825	0.0776	0.0825	0.1139	

Note: Data are mean pairwise distances between populations, corrected with the Tamura–Nei model of sequence evolution (A, B) or uncorrected p-distances (C). Bolded values on the diagonal are mean within-population distances. COI, mitochondrial cytochrome *c* oxidase I gene; 16S, mitochondrial large-ribosomal subunit rRNA gene; H3, nuclear histone 3 gene. Dashes indicate that only one H3 allele could be resolved for that population.

Table 4 Population genetic structure at the COI locus for *Elysia pusilla*, (A) including all sampled populations or (B) excluding the Moorean population

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*
(A)					
Among populations	4	640.62	33.53	78.33	<0.0001
Within populations	18	166.99	9.28	21.67	
Total	22	807.61	42.81		
Fixation index	Φ_{ST}:	0.7833			
(B)					
Among populations	3	452.98	26.89	72.45	<0.0001
within Populations	16	163.61	10.23	27.55	
Total	19	589.59	37.12		
Fixation index	Φ_{ST}:	0.7245			

Note: Results are from an Analysis of Molecular Variation using genetic distances corrected with the Tamura–Nei model. Significance was tested with 10,000 permutations of the data.

from Moorea; however, the pool of specimens from Guam and Japan included individuals that differed in their larval type. Genetic data revealed no evidence of cryptic species within Japan or Guam, although the Moorean sample may comprise a cryptic species. Our data thus demonstrate that *E. pusilla* is the fifth case of poecilogony in the Sacoglossa, and the first from the tropical Pacific.

Few molecular studies have previously examined marine heterobranchs with ranges spanning the Indo-Pacific. Proper interpretation of molecular data requires estimating how genetic variance is distributed within versus among populations, and between related species (Krug et al. 2012a). In *E. pusilla*, the magnitude of among-population genetic divergence at the COI locus (10–13%) exceeds most inter-specific distances reported in bar-coding studies (Hebert et al. 2003, 2010). However, sacoglossans likely have a faster molecular clock than do other molluscs, as sister species typically differ by 15–20% at COI (Ellingson and Krug 2006; Krug et al. 2012a). Thus, high divergence in mtDNA among populations does not necessarily indicate cryptic species in the Sacoglossa. In *E. pusilla*, divergence among haplotypes and alleles within populations was less than comparable distances between sister species, arguing for conspecificity despite a high degree of population structure.

Despite the high dispersal potential of planktotrophic larvae, little migration occurs among populations of *E. pusilla*, at least over the geographic scale sampled in this study. Indeed, genetic exchange among sampled populations has likely been minimal for >2 million years, based on differences for geminate species split by the Isthmus of Panama (Ellingson and Krug 2006; Krug et al. 2012a). The expression of lecithotrophy by some slugs in a deme may favor local recruitment, accelerating the effects of drift and local adaptation and promoting divergence. Further studies of gene flow in *E. pusilla* are warranted to examine whether the divergence we report here is a consequence of the distances separating our sampling sites, or if poecilogony and larval behavior truly reduce migration relative to that in co-occurring species with solely planktotrophic development.

In addition to the two mtDNA markers commonly used in phylogeographic studies, we also examined patterns of genetic diversity at the nuclear H3 locus. Results are preliminary, as not all alleles were resolved in this study, but *E. pusilla* alleles from most populations were <2% divergent from one another. The exception was Moorea, which held alleles that were ~7% divergent from the rest of *E. pusilla*, comparable to the divergence of H3 alleles sampled from the nominal sister taxon *E. stylifera*. Together with the magnitude of COI divergence, the H3 data suggest that Moorean specimens comprised a cryptic species sister to *pusilla*, and potentially endemic to French Polynesia. Alternatively, lying ~4600 km away from the nearest sampling site (Vanuatu), the Moorean population may simply be an extreme case of physical isolation resulting in genetic divergence without speciation. All populations of *E. pusilla* are highly divergent at mtDNA and distinguished by multiple fixed differences over a 300-bp stretch of H3, indicating little connectivity among populations. Future studies should test whether Moorean *pusilla* have diverged in ecology, morphology, or reproductive characters that would warrant description as a new species.

Developmental and ecological comparisons of five poecilogonous sacoglossans

Developmental and reproductive data for five poecilogonous sacoglossans shed remarkably little light on the evolution of developmental dimorphism. Grouping species as *Elysia* versus non-elysiids, or temperate versus tropical, revealed few trends in egg or larval size except that lecithotrophic larvae of temperate species developed faster and hatched at a smaller size than did larvae of the tropical species. Lecithotrophic larvae either had no, some, or entirely intra-capsular metamorphosis, indicating that selection against dispersal *per se* is not a plausible explanation for the evolution or persistence of poecilogony. Presence of ECY reserves was not correlated with the hatching size of larvae for either morph.

Ecologically, it has been suggested that environmental heterogeneity may select for dispersal dimorphisms in estuaries (Chia et al. 1996). Both *A. willowi* and *E. chlorotica* feed on the heterokont *Vaucheria* on temperate mudflats, and co-occur with the poecilogonous polychaete *S. benedicti*. However, two other poecilogonous sacoglossans are endemic to the Caribbean, and *E. pusilla* is widespread throughout tropical and subtropical Indo-Pacific environments that presumably vary less than temperate estuaries; these cases argue against environmental heterogeneity as a driver of variable development. It is perhaps surprising that *E. pusilla* is the first poecilogonous species reported from the Indo-Pacific, given the concentration of biodiversity in this region. However, limited access to many field sites has impeded research on larval development across the wide ranges that characterize many tropical Pacific taxa; we anticipate new examples will come from this region with greater sampling effort and increased accessibility of field populations to the scientific community.

Host use also provided no insight into the evolution of poecilogony; although *A. willowi* and *E. chlorotica* share the same host, two tropical species feed on different udotacean host genera, and *C. ocellifera*

feeds on a distantly related chlorophyte. Photosynthetic ability was similarly uncorrelated with poecilogony. In *E. chlorotica*, chloroplasts can function for 9 months, due to lateral transfer of photosynthetic genes from the algal nucleus to the slug genome (Pierce et al. 2009; Schwartz et al. 2010). In contrast, *E. pusilla* and *E. zuleicae* retain plastids for only a few weeks, while *A. willowi* has no photosynthetic ability (Händeler et al. 2009; P. Krug and H. Wägele, unpublished data). Thus, the five poecilogonous sacoglossans have no correlated traits that explain why these taxa have such reproductive versatility.

Phylogenetically, all five poecilogonous sacoglossans are distantly related and represent independent origins. The genus *Elysia* comprises about a fifth of sacoglossan diversity, and contains three cases of poecilogony, but each species belongs to a divergent clade; moreover, *Alderia* and *Costasiella* are distantly related to each other as well as to *Elysia* (Händeler et al. 2009). Even the form of poecilogony is highly variable among sacoglossans. In *E. chlorotica* and *C. ocellifera*, spatially distinct populations express one predominant mode of development, suggesting a genetic polymorphism that is locally fixed. In *E. pusilla* and *E. zuleicae*, planktotrophy is more common but a few individuals in some populations express lecithotrophy, which could reflect either a polymorphism maintained by balancing selection, or genotype × environment effects. In *A. willowi*, development is an environmentally cued polyphenism that varies over the life of an individual (Smolensky et al. 2009; Krug et al. 2012b). Thus, intra-specific studies may provide insight into the conditions that favor planktotrophy versus lecithotrophy, but more examples of poecilogony are needed before comparative studies can yield insight into the circumstances that favor its expression.

The frequency of dimorphism in egg-size in the Sacoglossa suggests an escape from constraints that limit most animals to one mode of development. Mechanical constraints may generally inhibit reproductive tracts from simultaneously producing and packaging eggs of two different size classes. Sacoglossans may achieve lecithotrophy at smaller egg sizes than other heterobranchs, however (Clark and Jensen 1981; Hadfield and Miller 1987; Jensen 2001). We hypothesize that poecilogony is favored when the difference in absolute size between planktotrophic and lecithotrophic eggs is small, as in the Sacoglossa. Testing this hypothesis will require reliable data on egg sizes and evolutionary relationships across the Heterobranchia, however. There are often dramatic inconsistencies in egg size and developmental mode reported for a given species in the literature (e.g., Clark and Goetzfried 1978 versus DeFreese and Clark 1983), and taxonomic uncertainties and misevaluations of larval developmental mode greatly complicate the literature for sacoglossans (Krug 2009). If our hypothesis is correct, then future studies of other groups with small lecithotrophic eggs may identify additional cases of poecilogony, and help to identify potential constraints on life-history flexibility and bet-hedging in the sea.

Conclusions

Developmental and genetic data confirm that *E. pusilla* is the fifth species in clade Sacoglossa that expresses poecilogony, and only the sixth case of egg-size dimorphism among marine animals. As the first Indo-Pacific taxon to be recognized with variable development, *E. pusilla* highlights the need for further study of larval development in biodiverse but under-sampled tropical regions. No correlated traits emerged from a comparison of five poecilogonous sacoglossans, leaving the origins of variable development enticing but still obscure. However, further studies of the phylogeny, development, and ecology of sacoglossans may yet illuminate why this small clade of specialized herbivores so readily achieve a level of life-history flexibility that is otherwise vanishingly rare. Studies of populations within poecilogonous species that differ in their primary mode of development may also identify the environmental drivers that influence marine life-history evolution, and the ecological consequences of alternative dispersal strategies.

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Supplementary Data

Supplementary Data are available at *ICB* online.

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