Reduced genetic diversity and increased reproductive isolation follow population-level loss of larval dispersal in a marine gastropod

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Population-level consequences of dispersal ability remain poorly understood, especially for marine animals in which dispersal is typically considered a species-level trait governed by oceanographic transport of microscopic larvae. Transitions from dispersive (planktotrophic) to nondispersive, aplanktonic larvae are predicted to reduce connectivity, genetic diversity within populations, and the spatial scale at which reproductive isolation evolves. However, larval dimorphism within a species is rare, precluding population-level tests. We show the sea slug Costasiella ocellifera expresses both larval morphs in Florida and the Caribbean, regions with divergent mitochondrial lineages. Planktotrophy predominated at 11 sites, 10 of which formed a highly connected and genetically diverse Caribbean metapopulation. Four populations expressed mainly aplanktonic development and had markedly reduced connectivity, and lower genetic diversity at one mitochondrial and six nuclear loci. Aplanktonic dams showed partial postzygotic isolation in most interpopulation crosses, regardless of genetic or geographic distance to the sire’s source, suggesting that outbreeding depression affects fragmented populations. Dams from genetically isolated and neighboring populations also exhibited premating isolation, consistent with reinforcement contingent on historical interaction. By increasing self-recruitment and genetic drift, the loss of dispersal may thus initiate a feedback loop resulting in the evolution of reproductive isolation over small spatial scales in the sea.

KEY WORDS: Development mode, genetic diversity, lecithotrophy, planktotrophy, poecilogony, reinforcement.

Successful dispersal can depend upon individual-level trait variation, allowing mean distance traveled, or the propensity to leave a natal site, to evolve in response to selection (Haag et al. 2005; Phillips et al. 2006; Van Belleghem et al. 2015). However, dispersal also has emergent properties at the community, species, and lineage level. At ecological time scales, dispersal affects population size and persistence (Hansson 1991), as well as metacommunity structure and diversity (Bie et al. 2012; Jones et al. 2015). Over evolutionary time, dispersal can influence standing genetic diversity (Méndez et al. 2014), range size (Kubisch et al. 2014), and diversification rate of a lineage (Krug et al. 2015). However, there is a dearth of research linking individual variation in dispersal with population-level properties (Lowe and McPeek 2014). Viewing dispersal as a stochastic, externally forced process ignores potentially important feedback loops by which selection on dispersal ability can have profound consequences across higher levels of biological organization.

In benthic marine invertebrates, most dispersal occurs during a microscopic larval stage that develops during either (i) a prolonged period of swimming and feeding (planktotrophy), or (ii) a shorter period where feeding is not required (lecithotrophy), and may occur inside an egg mass or the mother (aplanctic...
development) (Strathmann 1985; Krug 2009). Larval transport has long been studied as a process driven by stochastic forcing due to the slow swimming speeds of most larvae compared to ocean currents (Cowen et al. 2000). Moreover, as development mode is canalized in most species, the role of individual trait variation was historically de-emphasized in studying the consequences of alternative dispersal strategies. Studies generally contrasted species differing in development mode and hence pelagic larval duration (PLD), a trait likely to swamp out individual differences in settlement behavior (Hadfield and Strathmann 1996; Burgess et al. 2009) or swimming speed (Treml et al. 2015).

Planktotrophs generally have a longer PLD and larger expected dispersal kernels than swimming lecithotrophs, which should increase gene flow among demes (Strathmann 1990; Pechenik 1999). Early comparisons found more genetic subdivision in lecithotrophs than related planktotrophs (McMillan et al. 1992; Hoskin 1997, Todd et al. 1998, Collin 2001), but subsequent meta-analyses did not detect a strong correlation between PLD and gene flow among taxa with swimming larvae (Ayre et al. 2009; Weersing and Toonen 2009; Kelly and Palumbi 2010; but see Selkoe and Toonen 2011; Faurbry and Barber 2012). Aplanktonic development generally correlates with smaller population sizes, reduced genetic variation, and/or greater genetic subdivision even at fine spatial scales (Hoskin 1997; Foltz 2003; Lee and Boulding 2009; Kelly and Palumbi 2010). However, correlations between development mode and genetic diversity or gene flow may be confounded by other life-history traits that co-vary among species, such as brooding or self-fertilization (Bradbury et al. 2008; Puritz et al. 2012; Barbosa et al. 2013; Keever et al. 2013), or habitat use (Bird et al. 2007; Ayre et al. 2009). Demographic histories that vary among species can also mimic the predicted effects of alternative larval types (Hart and Marko 2010; McGovern et al. 2010; Dawson et al. 2014). Finally, phylogenetic effects may create apparent correlations between development mode and gene flow, absent a correction for shared evolutionary history (Nakagawa and Santos 2012). Thus, expected dispersal kernels than swimming lecithotrophs, which should increase gene flow among demes (Strathmann 1990; Pechenik 1999). Early comparisons found more genetic subdivision in lecithotrophs than related planktotrophs (McMillan et al. 1992; Hoskin 1997, Todd et al. 1998, Collin 2001), but subsequent meta-analyses did not detect a strong correlation between PLD and gene flow among taxa with swimming larvae (Ayre et al. 2009; Weersing and Toonen 2009; Kelly and Palumbi 2010; but see Selkoe and Toonen 2011; Faurbry and Barber 2012). Aplanktonic development generally correlates with smaller population sizes, reduced genetic variation, and/or greater genetic subdivision even at fine spatial scales (Hoskin 1997; Foltz 2003; Lee and Boulding 2009; Kelly and Palumbi 2010). However, correlations between development mode and genetic diversity or gene flow may be confounded by other life-history traits that co-vary among species, such as brooding or self-fertilization (Bradbury et al. 2008; Puritz et al. 2012; Barbosa et al. 2013; Keever et al. 2013), or habitat use (Bird et al. 2007; Ayre et al. 2009). Demographic histories that vary among species can also mimic the predicted effects of alternative larval types (Hart and Marko 2010; McGovern et al. 2010; Dawson et al. 2014). Finally, phylogenetic effects may create apparent correlations between development mode and gene flow, absent a correction for shared evolutionary history (Nakagawa and Santos 2012). Thus, expected relationships between life history and genetic structure are challenging to test using among-species comparisons, but as larval type is canalized in most species, there are few opportunities to compare population-level effects of dispersal on genetic diversity and migration rates.

Poecilology, or intraspecific variation in development mode, presents the opportunity to test whether shifts in larval type affect diversity within, and gene flow among, populations. Patterns of seasonal or geographic variation in development may also provide insight into the evolutionary drivers that favor life-history transitions (Collin 2012). For instance, lecithotrophy increases survivorship at undisturbed sites in the polychaete Streblospio benedicti (Levin and Huggett 1990), and is expressed during seasonal closure of estuaries in the sea slug Alderia willowi (Krug et al. 2012), suggesting that stable or physically isolated habitats may select against dispersal. While avoiding the confounding effects of interspecific comparisons, studies of poecilogonous taxa may be confounded by oceanographic differences among sites that vary in development (whereas interspecific studies compare populations of different species sampled from the same sites). No prior study has identified a poecilogonous species with multiple populations fixed for different development modes, each found in a range of physical transport regimes (e.g., enclosed lagoon vs. open coast); such a species could provide the level of natural replication needed to test the evolutionary effects of larval type at the population level.

Population-level shifts in larval type may also produce reproductive isolation as a pleiotropic by-product of disruptive selection on egg size, if genetic programs controlling embryonic axis formation are incompatible in interpopulation hybrids. Crosses between echinoid species fail if the maternal species is planktotrophic but the paternal species is lecithotrophic, because axes are determined by the zygotic genome of planktotrophs and are disrupted in hybrids (Raff et al. 2003). In the poecilogonous worm S. benedicti, Zakas and Wares (2012) found partial genetic differentiation between co-occurring adults that differed in larval type, suggesting inhibition of crosses between development modes. Thus, shared development mode may better predict hybrid compatibility than genetic distance, and local shifts in larval type may be an unrecognized driver of speciation in the sea.

Of the seven species confirmed to exhibit egg-size dimorphism, four are found in Sacoglossa, a clade of herbivorous sea slugs (Vendetti et al. 2012). A fifth candidate species is Costasiella ocellifera from Florida (FL), United States and the Caribbean. Populations assigned to this species were planktotrophic in Lake Surprise, FL but aplanktonic at Geiger Beach near Key West, FL. As preliminary crosses suggested these populations did not interbreed, Miles and Clark (2002) suggested the Lake Surprise population was a cryptic species. New Costasiella spp. were recently described from FL (Jensen et al. 2014) and Caribbean sites (Espinoza et al. 2014), so more unrecognized species may exist; alternatively, C. ocellifera could be poecilogonous, with partial reproductive isolation between populations differing in larval type. Here, we determined development mode for C. ocellifera from Florida and the Caribbean, and tested whether aplanktonic development reduced genetic diversity and connectivity at the population level. We then performed crosses to test for pre- or postzygotic isolation between populations differing in development, proximity, or genetic distance. Our results indicate that developmental transitions can accelerate evolutionary processes that promote reproductive isolation at small spatial scales, relative to the dispersal potential of most marine animals.
Table 1. Sampling sites (codes given in parentheses) and number of specimens (N) of Costasiella ocellifera sequenced for molecular analyses.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>N</th>
<th>Latitude, longitude</th>
<th>Date sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Surprise, Key Largo, Florida, USA (LAK)</td>
<td>12</td>
<td>25°10.88’N, 80°23.10’W</td>
<td>March 1996, October 2009, October 2010</td>
</tr>
<tr>
<td>Geiger Beach, Key West, FL (GEI)</td>
<td>11</td>
<td>24°33.98’N, 81°40.22’W</td>
<td>October 2006, June 2007, October 2009, June 2010</td>
</tr>
<tr>
<td>Bahamas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweeting’s Cay, Grand Bahama Island (SWE)</td>
<td>17</td>
<td>26°37.10’N, 77°53.07’W</td>
<td>June 2004, July 2007</td>
</tr>
<tr>
<td>Stirrup Cay (STIR)</td>
<td>12</td>
<td>25°49.18’N, 77°53.93’W</td>
<td>June 2007</td>
</tr>
<tr>
<td>Little San Salvador (LSS)</td>
<td>15</td>
<td>24°34.50’N, 75°56.52’W</td>
<td>June 2007</td>
</tr>
<tr>
<td>San Salvador (SSAL)</td>
<td>15</td>
<td>24°08.50’N, 74°28.30’W</td>
<td>June 2004, July 2007</td>
</tr>
<tr>
<td>Plana Cays (PLA)</td>
<td>10</td>
<td>22°36.67’N, 73°33.87’W</td>
<td>June 2004, July 2007</td>
</tr>
<tr>
<td>Compass Cay (COMP)</td>
<td>6</td>
<td>24°16.48’N, 76°30.58’W</td>
<td>July 2010</td>
</tr>
<tr>
<td>Northern Exumas (NEX)</td>
<td>13</td>
<td>24°46.00’N, 76°49.02’W</td>
<td>July 2010</td>
</tr>
<tr>
<td>Bimini (BIM)</td>
<td>8</td>
<td>25°44.32’N, 79°16.33’W</td>
<td>July 2010</td>
</tr>
<tr>
<td>Biological Station for Research, Bermuda (BER)</td>
<td>9</td>
<td>32°22.20’N, 64°41.87’W</td>
<td>June 2006</td>
</tr>
<tr>
<td>Discovery Bay, Jamaica (JAM)</td>
<td>7</td>
<td>18°28.15’N, 77°24.90’W</td>
<td>March 2006</td>
</tr>
<tr>
<td>Turneffe Atoll, Belize (BLZ)</td>
<td>8</td>
<td>17°10.50’N, 87°54.98’W</td>
<td>June 1991</td>
</tr>
<tr>
<td>Spanish Waters, Curacao (CUR)</td>
<td>16</td>
<td>12°04.85’N, 68°51.25’W</td>
<td>January 2009</td>
</tr>
</tbody>
</table>

1Specimens included in msat, but not mtDNA, analyses.

Materials and Methods

STUDY TAXA AND COLLECTION

Specimens of C. ocellifera were collected from 15 sites in FL, Bermuda and the Caribbean encompassing most of the species’ range (Table 1). Slugs feed and oviposit on the host alga Avrainvilia nigricans (Marcus and Marcus 1969; Jensen et al. 2014). Embryos develop within a gelatinous egg mass; planktotrophic larvae have a month-long swimming period after hatching, whereas aplanktonic larvae metamorphose within the egg mass (Miles and Clark 2002). At each site, ~1 kg of A. nigricans was collected and aerated. Slugs were removed and isolated in dishes with 4 ml of 0.22 μm filtered seawater (FSW) for oviposition; development mode was scored according to egg size. Specimens were preserved in 100% EtOH after egg laying, or after two to three weeks if no eggs were laid. Specimens from Belize were not individually typed for development, but egg masses collected with slugs were planktotrophic (M. Hellberg, pers. comm.). Slugs from Stirrup Cay, Bahamas were below reproductive size.

To estimate relative productivity among sites, chlorophyll a concentrations were obtained from data collected by the NASA Earth Observing System using Moderate Resolution Imaging Spectroradiometer (MODIS), downloaded from the Goddard Space Flight Center’s OceanColor website (http://oceandata.sci.gsfc.nasa.gov/). Annual mean daily measurements for 2003–2013 were downloaded, and mean annual concentrations were visualized with ArcGIS.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Mitochondrial DNA

Genomic DNA was extracted from tissue or larvae using DNeasy kits (Qiagen, Valencia, CA) and stored in buffer at ~20°C. For polymerase chain reactions (PCR), 50–200 ng of DNA was added to 50 μl of buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100) containing 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM of each primer, and 1 U Promega Taq DNA polymerase. A 710 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994) and the following profile: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 45 sec, and 72°C for 1 min. For each primer, and 1 U Promega Taq DNA polymerase. A 710 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994) and the following profile: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 40°C for 45 sec, and 72°C for 60 sec. Products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and both strands cycle sequenced using amplification primers. A 501 bp segment (positions 127–627 of the Elysia chlorotica COI gene; NCBI accession #EU599581) was obtained for 151 specimens of C. ocellifera (accession #s KP116117–KP116238), and aligned with Geneious version 7.1.9 (Kearse et al. 2012).

Nuclear microsatellites

Microsatellite (msat) loci were identified via sequencing by synthesis through the MiSeq platform (Illumina, Inc., San Diego, CA). DNA was extracted from four Bahamian slugs, pooled, tagged with a unique barcode during Illumina library preparation,
and sequenced at the UCLA Genotyping and Sequencing Core. Automated screening of sequences for tetranucleotide repeats and primer design were performed simultaneously in msatcommander version 1.0.8 (Faircloth 2008). Of 24 primer pairs tested, six polymorphic loci amplified reliably, allowing 134 individuals to be genotyped with 6.6% missing data in the final dataset. Genotypes were scored in Geneious version 7.1.9 (Kearse et al. 2012). Summary statistics and primers for each locus are given in Table S.1. Due to inconsistent amplification success for samples from Compass Cay, msat alleles were instead scored for the nearby planktotrophic population from Bimini to keep the number of populations the same for mtDNA and nuclear msat analyses.

**PHYLGENETIC AND POPULATION GENETIC ANALYSES**

**Mitochondrial DNA**

Haplotypes from *Costasiella* sp. 3 (n = 5) and *C. paweli* were designated as outliers in phylogenetic analyses of COI sequences from *C. ocellifera*, based on a four-locus phylogeny that included 14 congeners (Jensen et al. 2014). Bayesian inference (BI) and maximum-likelihood (ML) methods were used to infer COI gene trees. For BI, Markov chain Monte Carlo (MCMC) methods were implemented in *BayesPhylogenies* to capture heterogeneity in base frequencies and mutation rates without an a priori partitioning (Pagel and Meade 2004). Four independent chains were run for $10^5$ generations, each using two GTR + $\Gamma$ models from which the best-fit model was assigned to each position. Trees were saved every $10^5$ generations, and harmonic mean log-likelihood ($L$) scores and parameter estimates inspected to confirm that runs reached stationarity. The final 200 trees from each run were pooled into a posterior sample and a 50% consensus tree generated; posterior probabilities (PP) $\geq 95\%$ were taken as significant support (Huelsenbeck and Rannala 2004). ML analyses were run with *RAxML* version 7.6.6 (Stamatakis 2006) through the CIPRES Science Gateway version 3.3 (Miller et al. 2010), using a GTR + $\Gamma$ model with four rate multipliers. Nodal support was assessed from 100 bootstrap pseudoreplicates, taking values $\geq 70\%$ as significant (Hillis and Bull 1993).

A null hypothesis of panmixia among demes (genetically homogenous groups within a subdivided metapopulation) was tested by analysis of molecular variance (AMOVA), using $\Phi_{ST}$ (genetic distance based) and conventional $F_{ST}$ (frequency based) statistics in *arlequin* version 3.5 (Excoffier and Lischer 2010). The Kimura two-parameter model of sequence evolution was used for $\Phi_{ST}$ analyses, based on *ModelTest* results. Phylogenetic analysis indicated that most COI haplotypes sampled in Florida were genetically distinct from Caribbean populations (see Results); we therefore tested a nested hypothesis that (a) Florida and the Caribbean were differentiated regional populations, and (b) within regions, each sampled site was genetically distinct.

Data for Sweeting’s Cay from 2004 and 2007 were pooled for AMOVA comparisons.

**Nuclear microsatellites**

Population subdivision in the nuclear genome was inferred using *STRUCTURE* version 2.3.4 (Pritchard et al. 2000). The admixture model with independent allele frequencies was employed with 10 replicates for each number of clusters ($K$), from two to 14. For each replicate, the MCMC chain ran for 500,000 iterations following a burn-in period of 50,000. The web-based version of *STRUCTURE HARVESTER* (Earl and vonHoldt 2012) was used to determine the best-fit $K$ value, plotting all four steps of the “Evanno” method used to calculate $\Delta K$ (equal to the absolute value of the second order rate of change of the likelihood distribution $L''(K)$) across all replicates for each successive value of $K$ (Evanno et al. 2005).

**EFFECTS OF DEVELOPMENT MODE ON GENE FLOW AND GENETIC DIVERSITY**

Compared to planktotrophic demes, we expected aplanktonic populations to have reduced genetic diversity due to smaller effective population sizes, and reduced gene flow due to the loss of a swimming stage. Nine population samples were classified as exclusively ($n = 7$) or predominantly ($\geq 75\%$ of clutches) planktotrophic, including Sweeting’s Cay 2007–2010. Four populations were classified as exclusively ($n = 2$) or substantially aplanktonic ($\geq 50\%$ of clutches in at least one sample; Plana Cays and Sweeting’s Cay 2004) (see Results). Data from Sweeting’s 2004 were treated separately from later samples when testing effects of development mode on genetic diversity. Stirrup Cay was omitted from analyses involving larval type.

**Mitochondrial DNA**

For each site, haplotype ($h$) and nucleotide ($\pi$) diversities were calculated from COI data in *arlequin*, and reported $\pm 1$ standard error. A one-tailed, nonparametric Mann–Whitney test was used to determine whether aplanktonic populations had lower haplotype diversity, given the a priori expectation that drift would act more strongly on small, isolated populations. A nonparametric test was used because data could not be transformed to meet assumptions of normality and heteroscedasticity. To determine if development mode affected gene flow, we calculated the mean pairwise $\Phi_{ST}$ and $F_{ST}$ value for each sampling site, based on AMOVA results. We then used a one-tailed Mann–Whitney test to determine if aplanktonic populations had higher mean $\theta_{ST}$ and $F_{ST}$ values than planktotrophic populations, again based on longstanding theory predicting increased subdivision following loss of dispersal. Analyses were performed by (i) coding pooled data from Sweeting’s Cay as aplanktonic, or (ii) excluding this population. For all demes, we also determined the test statistics Tajima’s $D$ (Tajima 1989).
Effects of larval type on (a) mtDNA polymorphism and (b) effective population size ($N_e$) were visualized separately for three aplanctonic populations (GEI, LSS, PLA); the isolated planktotrophic population in Lake Surprise; and a pooled sample of all Caribbean planktotrophic demes (BER, JAM, CUR, BEL, SSAL, SWE 2007, COMP, and NEX), which comprised one metapopulation without subdivision (see Results). Mismatch distributions were plotted based on pairwise differences among haplotypes. To estimate $N_e$ through time, demographic history was also inferred by constructing Bayesian skyline plots (BSPs) in BEAST version 1.8.2 (Drummond et al. 2012). BSPs estimate $N_e$ based on branch density in coalescent trees over a given number of time intervals from the tips to the root, testing a range of demographic models (Drummond et al. 2005). By coestimating genealogy, demographic history and substitution parameters from sequence data in a Bayesian framework, BSPs account for phylogenetic uncertainty and provide credible intervals for all parameters (Ho and Shapiro 2011). To avoid violating assumptions of panmixia (Heller et al. 2013), each population was analyzed separately, and immigrant Caribbean haplotypes were excluded from GEI and LAK datasets.

A lineage-specific mutation rate was calculated by halving the mean divergence (12.4% TrN + Γ) between the only strongly supported pair of geminate sacoglossan species ($E. tuca$ and $Elysia$ sp. 6) recovered by Krug et al. (2015), and dividing by the age of the Isthmus of Panama (3.1 MY), yielding $2.00 \times 10^{-8}$ substitutions per site per year. This mutation rate was converted into substitutions per site per generation for each development mode, estimating generation time as the encapsulated period (5 days, planktotrophic; 15 days, aplanctonic; Vendetti et al. 2012) plus P Lod (30 days, planktotrophic; 0 days, aplanctonic) plus 60 days of postmetamorphic maturation (both), yielding mutation rates of $5.20 \times 10^{-9}$ substitutions per site per generation (planktotrophic) and $4.10 \times 10^{-9}$ substitutions per site per generation (aplanctonic). A strict clock model was then calibrated using the appropriate rate for each population, so population sizes output by BEAST were in units of $N_e$. To ensure convergence, each data subset was run twice for 100 million generations under the GTR + Γ model of sequence evolution, a piecewise-constant skyline model, and default priors. Log and tree files from two independent runs were combined, discarding the first 50% as burn-in. Tracer version 1.6 was used to check for sufficient mixing within and convergence between runs, and to generate skyline plots (Rambaut et al. 2014).

**Nuclear microsatellites**

To test the effects of larval type on allelic richness at msat loci, we calculated three summary statistics for each population in ARLEQUIN: (i) expected heterozygosity, (ii) mean number of alleles, and (iii) the G-W index corrected for potential monomorphy (Garza and Williamson 2001; Excoffier et al. 2005). The G-W index is a modified ratio of the number of alleles in a population sample to the allelic range at a given locus, and can detect bottlenecks or other demographic processes that cause loss of alleles without reducing the size range (i.e., number of allelic repeats). For each metric, a one-tailed, nonparametric Mann–Whitney test was used to determine whether aplanctonic populations had reduced diversity. Means for each development mode are reported in the text ± 1 standard error.

**POPULATION CROSSES AND TESTS FOR PRE- AND POSTZYGOTIC ISOLATION**

When populations with partial postzygotic isolation frequently encounter each other, selection favors prezygotic mechanisms that inhibit hybridization (Coyne and Orr 2004). We expected prezygotic isolation to manifest as reduced egg production in population crosses, either because slugs did not mate or allosperm did not remain viable. We scored the percentage of embryos that failed to hatch as a measure of postzygotic isolation, potentially resulting from (i) developmental differences between sites, or (ii) outbreeding depression (OD) due either to historical isolation of FL versus Caribbean lineages, or drift acting on small aplanctonic populations. To distinguish among these hypotheses, we crossed the divergent FL and Caribbean lineages, each represented by a pair of populations fixed for different development modes (see Results). In 2010, slugs and algae were collected from two FL sites: Lake Surprise (LAK, planktotrophic) and Geiger Beach (GEI, aplanctonic), 145 km apart; and two Bahamas sites: San Salvador (SSAL, planktotrophic) and Little San Salvador (LSS, aplanctonic), 155 km apart. Interpopulation crosses paired slugs from (1) divergent populations but same development (LAK × SSAL, planktotrophic; GEI × LSS, aplanctonic); (2) adjacent sites differing in development (LAK × GEI, SSAL × LSS); and (3) divergent populations differing in development (LAK × GEI, SSAL × SSAL). Intrapopulation control pairs were maintained in parallel.

Prior to crosses, slugs were held on algae from their home site. Replicate dishes ($n = 7$–$10$ per cross) were established for each cross or control by haphazardly pairing two slugs in a bin ($15 \times 10 \times 6$ cm) with 500 ml FSW and algae from their source population, cleaned of any egg masses. Sacoglossans are outcrossing hermaphrodites that store allosperm from prior matings for up to two weeks (Smolensky et al. 2009). Pairs were initially held for 16–19 days to exhaust allosperm reserves, with daily water
changes and algae added as needed; no data were collected. Then, pairs were transferred to new bins every 2 days for ~4 weeks, replacing algae weekly. Each egg mass was removed from the substrate with a scalpel, placed in a dish with 4 ml FSW, typed for development mode, and held at 25°C with daily water changes. Numbers of eggs laid, and of embryos completing development to hatching, were scored under a dissecting microscope, after which offspring were preserved in 100% ethanol; adults were preserved after the experiment.

For interpopulation crosses, mean daily egg production per slug was determined by assigning each clutch to its mother, and dividing total eggs laid by the number of experimental days. For crosses of different development modes, maternal identity was unambiguous. For LAK×SSAL and GEI×LSS crosses, clutches were matched to dams by extracting DNA from offspring and parents, and sequencing the COI gene as described. For intrapopulation controls, the level of replication was the dish, so egg production per pair per day was halved to obtain per-slug output. Total percentage of hatching success was calculated for all offspring of each individual (or control pair); no data on hatching success were available for replicates in which no eggs were laid, reducing sample sizes. In a few replicates involving LAK slugs, the Caribbean partner laid aberrant egg masses containing a yolk string instead of eggs (SSAL, n = 2; LSS, n = 3); to be conservative, data for those Caribbean mothers were excluded from analyses.

For prezygotic isolation, the null hypothesis was that dams from a given site would reproduce at an equivalent rate regardless of the sire’s source population; reduced egg production would indicate partial isolation. Because slugs laying planktotrophic versus aplanktonic eggs differ greatly in fecundity, a two-way ANOVA could not be performed to test for interactive effects of maternal and paternal population on egg output. Instead, a separate one-way ANOVA was run for dams from each site, comparing daily egg production according to the sire’s population of origin. Post-hoc Dunnett’s t-tests were used for unplanned comparisons of means against intrapopulation controls when the overall ANOVA result was significant.

For postzygotic isolation, the null hypothesis was that offspring of dams from a given site would have the same hatching success regardless of the sire. A two-way ANOVA was run on all percent hatching data with two fixed factors, maternal and paternal source population, and their interaction. Percentages were arcsine (square root) transformed prior to analysis. If populations differing in larval type produced hybrids in which embryonic axes did not form properly, hatching success would be higher for LSS×GEI pairs (aplanktonic) and LAK×SSAL pairs (planktotrophic) than for within-region crosses. Alternatively, if postzygotic isolation scaled with divergence, hatching success would be reduced for FL×Caribbean crosses compared to within-region crosses and control pairs. If instead aplanktonic populations suffer disproportionately from OD, hatching success would be reduced for GEI and/or LSS dams in some or all crosses.

Results

**PHYLOGEOGRAPHY AND DEVELOPMENT MODE**

From 2006 to 2010, all specimens collected from Lake Surprise, FL laid planktotrophic eggs, whereas all slugs from Geiger Beach, FL expressed aplanktonic development (Fig. 1). Six Caribbean populations expressed only planktotrophy: Bermuda (type locality of *C. ocellifera*), Jamaica, Curaçao, and three Bahamian islands (San Salvador, Bimini, Compass Cay). All slugs from Little San Salvador had aplanktonic development, whereas three other Bahamas sites had mixed development. Planktotrophy predominated in Northern Exumas, whereas both larval types were equally prevalent in Plana Cays in 2004 and 2007 (although few slugs laid eggs in either year). A temporal shift in development occurred at Sweeting’s Cay, where clutches were mostly lecithotrophic in 2004 yet mostly planktotrophic in 2007 and 2010.

ML and BI returned the same basic topology for the COI gene tree, based on 121 ingroup haplotypes (Fig. 2). Haplotypes from aplanktonic egg layers were not monophyletic, and one haplotype was shared by two aplanktonic specimens from Sweeting’s Cay and a planktotrophic specimen from San Salvador. Haplotypes from Caribbean sites formed a poorly resolved polytomy with little phylogeographic structure. Of three haplotypes sampled at Little San Salvador, two were shared by seven specimens apiece and formed a highly supported clade (BP = 76; PP = 0.92). Clades receiving full support in both analyses (BP = 100; PP = 1.0) included (1) three haplotypes from Northern Exumas, including one haplotype sampled twice; (2) two haplotypes from Plana Cays, including a private haplotype sampled four times; and (3) a mildly divergent clade including haplotypes sampled in Sweeting’s Cay, Belize, Jamaica, Curaçao, and Geiger Beach, FL. The few other supported clades contained representatives of distant sampling sites, such as a clade of three haplotypes from Stirrup Cay and one haplotype each from Jamaica, Belize, and Curaçao (BP = 92, PP = 0.96). A few haplotypes from FL (one from Lake Surprise, two from Geiger Beach) were presumably of recent Caribbean origin, grouping within the main grade of Caribbean haplotypes.

The Caribbean grade was paraphyletic with respect to a divergent, supported clade comprising most FL haplotypes (BP = 78, PP = 0.97) (Fig. 2). Net K2P sequence divergence between the FL clade and the grade of remaining *C. ocellifera* haplotypes was 5.2%. No haplotype was recovered as sister to the FL clade. All haplotypes from Lake Surprise and one from Geiger Beach formed a grade paraphyletic with respect to a highly supported clade comprising all remaining haplotypes from Geiger Beach.
Figure 1. Distribution of larval development modes in Caribbean populations of *Costasiella ocellifera*. Pie charts indicate the proportion of larval type (gray = planktotrophic; black = aplanktonic development) among specimens that laid egg masses, with the corresponding sample size and survey year. Bottom panel is inset (gray box) from top panel showing Bahamas sites.

(Fig. 2; BP = 98, PP = 1.0). A pair of Lake Surprise haplotypes grouped with the Geiger Beach clade (BP = 97; PP = 1.0), and was recovered as its sister group by ML analysis (BP = 89). Mean sequence divergence between the Geiger Beach clade and the rest of the Florida clade was 2.4%.

**DEVELOPMENT MODE AND DISTRIBUTION OF GENETIC VARIANCE**

**Mitochondrial DNA**

The sequenced COI fragment was highly polymorphic in *C. ocellifera*, as in most sacoglossans. At seven sites plus Sweeting’s Cay 2007, every slug had a unique haplotype (Table 2). Only three haplotypes were shared among sites (Sweeting’s Cay and San Salvador, Curacao and Jamaica, and Curacao and Belize). Five populations had private, common haplotypes, including Lake Surprise (two haplotypes, sampled two and four times) and all four demes with predominantly aplanktonic development: Geiger Beach (one haplotype, sampled five times), Plana Cays (one haplotype, sampled four times), Little San Salvador (two haplotypes, each sampled seven times), and Sweeting’s Cay 2004 (two haplotypes, each sampled twice) (Fig. 2, Table 2). Nucleotide diversity was notably five to 10 times lower at Little San Salvador than any other site.
Figure 2. Gene tree for COI haplotypes from Costasiella ocellifera, based on BI and ML analyses. Circle fill denotes haplotypes sampled from specimens expressing planktotrophy (white) or aplanktonic development (black). Tip labels indicate population of origin for a given haplotype (see Table 1), with the number of individuals sharing that haplotype in parentheses following the population label. Support values are given as BI posterior probabilities (before slash) and ML bootstrap percentages (after slash). Branches marked with a horizontal double slash are not shown to scale.
Table 2. Molecular diversity indices in *Costasiella ocellifera* for one mitochondrial locus (COI), and mean values for six nuclear microsatellite loci, with standard deviations given in parentheses; bold = predominantly aplanktonic populations.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>mtDNA</th>
<th>Microsatellites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>k^1</td>
</tr>
<tr>
<td>Geiger Beach, FL</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Sweetings Cay '04</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Little San Salvador</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Plana Cays</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Lake Surprise, FL</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Sweetings Cay '07</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Stirrup Cay</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>San Salvador</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Northern Exumas</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Compass Cay</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Bimini</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Bermuda</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Jamaica</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Curacao</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

1 Number of distinct haplotypes present.
2 Number of variable sites out of 501 bp.
3 Nucleotide diversity based on K2P distances.

Haplotype diversity was significantly lower in four predominantly or exclusively aplanktonic populations (0.816 ± 0.079) compared to planktotrophic populations (0.988 ± 0.011) (Table 2, and results of a one-tailed Mann–Whitney test: $z = -2.85$, $P = 0.002$). In contrast, mean nucleotide diversity was similar between aplanktonic (0.020 ± 0.007) and planktotrophic (0.022 ± 0.002) populations. Fu’s test of selective neutrality revealed deviations from equilibrium in two planktotrophic demes in which all sampled individuals had unique haplotypes, San Salvador ($F_S = -8.07$; $P < 0.0001$) and Curaçao ($F_S = -8.49$; $P < 0.05$), and in Stirrup Cay (development mode unknown) ($F_S = -4.71$; $P < 0.05$). Tajima’s $D$ was also significant for San Salvador ($D = -1.74$, $P < 0.05$) and Curaçao ($D = -2.03$, $P < 0.01$).

**Nuclear microsatellites**

For each metric of microsatellite diversity, the lowest mean values were recorded for the two strictly aplanktonic populations, Geiger Beach and Little San Salvador, with low to moderate values for the partially (Plana Cays) or transiently (Sweetings Cay ‘04) aplanktonic sites (Tables 2, S2). Averaged across loci, expected heterozygosity was significantly lower for aplanktonic populations (0.508 ± 0.093), which included three of the four lowest values, compared with planktotrophic populations (0.701 ± 0.009) (Table 2, and results of a one-tailed Mann–Whitney test: $z = -1.98$, $P = 0.024$). The mean number of alleles per locus was also smaller for aplanktonic (3.877 ± 0.614) than planktotrophic populations (5.650 ± 0.257) (Table 2, and results of a one-tailed Mann–Whitney test: $z = -2.13$, $P = 0.017$). Lastly, the Garza–Williamson index was lower for aplanktonic (0.091 ± 0.041) than planktotrophic populations (0.130 ± 0.007) (Table 2, and results of a one-tailed Mann–Whitney test: $z = -1.98$, $P = 0.024$).

**POPULATION STRUCTURE AND GENE FLOW**

**Mitochondrial DNA**

Nested AMOVA ($\Phi_{ST}$) significantly supported differentiation between Florida and Caribbean regions ($P = 0.013$), within which genetic covariance was significantly partitioned among subpopulations ($P < 0.00001$; Table 3). Pairwise $\Phi_{ST}$ values were significant for all comparisons involving FL populations (LAK, GEI) and Little San Salvador ($P < 0.001$), and for all but two comparisons involving Plana Cays ($P < 0.05$; Table 4). Only one other pairwise comparison among Caribbean demes was weakly significant (Northern Exumas × Curaçao). Mean pairwise $\Phi_{ST}$ values were significantly higher for populations with aplanktonic larvae, whether Sweeting’s Cay was coded as aplanktonic (one-tailed Mann–Whitney test: $z = -1.70$, $P = 0.045$) or excluded ($z = -2.45$, $P = 0.007$).

Nested AMOVA using haplotype frequencies did not support regional differentiation between FL and the Caribbean, but a
conventional $F_{ST}$ analysis treating all demes as distinct populations revealed significant subdivision ($F_{ST} = 0.073, P < 0.00001$). All pairwise $F_{ST}$ comparisons involving Little San Salvador, most involving the two FL populations, and some involving Plana Cays were significant (Table 4). No pairwise comparison between planktotrophic demes in the Caribbean was significant. Mean pairwise $F_{ST}$ values were significantly higher for populations with aplanktonic larvae, whether Sweeting’s was coded as $F_{ST} = 0.635, P = 0.013$ or excluded ($F_{ST} = 0.681, P < 0.00001$).

Mismatch distributions for the planktotrophic Caribbean metapopulation showed a wave-like pattern consistent with demographic expansion (Fig. 3A, left panel), whereas distributions for the isolated planktotrophic Lake Surprise population and three aplanktonic populations were significantly left skewed (Fig. 3B–E). BSPs also supported a late-Pleistocene population expansion (Fig. 3A, left panel), whereas distributions with aplanktonic larvae, whether Sweeting’s was coded as $z = -2.37, P = 0.005$ or excluded ($z = -2.46, P = 0.007$).

Nuclear microsatellites

All four metrics derived from STRUCTURE outputs supported $K = 3$ populations. Most genotypes from the four aplanktonic populations clustered together (blue), whereas most planktotrophic specimens belonged to one of two clusters sampled predominantly in the Caribbean (yellow) or Lake Surprise, FL (red) (Fig. 4). At $K = 2$, aplanktonic genotypes remained distinct from Caribbean planktotrophic genotypes, while assignment probabilities for LAK genotypes were ambiguously split. However, neither geography nor development mode correlated perfectly with population assignment. Although most planktotrophic slugs from the Caribbean grouped together, one or two slugs from each of five Caribbean sites were assigned to the Florida planktotrophic cluster (red) with probability $\geq 0.5$. Two planktotrophic egg layers from Jamaica grouped with aplanktonic genotypes ($\geq 0.9$), as did two to three individuals of unconfirmed development mode sampled from planktotrophic populations in Belize, Bermuda, and Bimini (assignment probability $\geq 0.5$). No confirmed aplanktonic specimens clustered with Caribbean planktotrophic (yellow) genotypes, but one slug of unconfirmed development from each of three predominantly aplanktonic populations (Plana, Sweetings 2004, and Little San Salvador) were assigned to the Caribbean planktotrophic cluster with $\geq 0.5$ probability.

Assignment probabilities for genotypes changed dramatically at Sweetings Cay from 2004 to 2007, congruent with the shift in predominant development mode (Fig. 4). In 2004, all but two planktotrophic slugs were assigned to the aplanktonic genotype cluster (blue). However, by 2007, aplanktonic (blue) genotypes were almost completely displaced by Caribbean planktotrophic genotypes (yellow), except for a lone specimen. This turnover in the nuclear gene pool coincident with a shift in larval type indicates that the aplanktonic population was successfully invaded and displaced by planktotrophic genotypes between 2004 and 2007.

Nuclear data also provided insight into immigration events. A single specimen sampled in Lake Surprise, FL had a mitochondrial genotype of Caribbean origin (Fig. 2, single arrow), and a nuclear genotype assigned to the Caribbean cluster with 0.67 probability (Fig. 4); this specimen was likely either an immigrant into Lake Surprise originating as a planktotrophic larva in a Caribbean population, or an FL hybrid that recruited back into the Lake Surprise population. Two specimens from Geiger Beach, FL had mtDNA belonging to the Caribbean clade (Fig. 2, double arrows). One was the only Geiger Beach slug with a nuclear genotype that showed slight affinity with the Caribbean planktotrophs (0.06 assignment probability vs. $<0.02$ for all other specimens). The second slug had a typical Geiger Beach genotype ($>0.98$ assignment probability). A third Geiger Beach specimen had mtDNA that grouped with the Lake Surprise grade (Fig. 2, double arrow, derived clade) despite a resident Geiger Beach genotype ($P = 0.98$). The cytonuclear discordance of these three slugs is consistent with introgression of mtDNA from planktotrophic immigrants that recruited into, and interbred with, the Geiger Beach population, with immigrant nuclear alleles lost during subsequent generations of backcrossing.

### Table 3. Population genetic structure in the Caribbean sea slug _Costasiella ocellifera_ based on AMOVA of COI haplotypes, testing populations nested within regions (Florida vs. Caribbean).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
<th>$\Phi$-statistics$^1$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>1</td>
<td>406.28</td>
<td>10.07</td>
<td>63.50</td>
<td>$\Phi_{CT} = 0.635$</td>
<td>0.013</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>11</td>
<td>147.61</td>
<td>0.73</td>
<td>4.57</td>
<td>$\Phi_{SC} = 0.125$</td>
<td>$&lt;0.00001$</td>
</tr>
<tr>
<td>Within populations</td>
<td>138</td>
<td>698.92</td>
<td>5.06</td>
<td>31.93</td>
<td>$\Phi_{ST} = 0.681$</td>
<td>$&lt;0.00001$</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>1252.80</td>
<td>15.86</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^1$-statistics based on genetic distances corrected with Kimura two-parameter model of sequence evolution.

$F_{ST}$ = $-\frac{\text{var}(N_e)}{\text{var}(N_e) + \text{var}(N_m)}$, where $N_m$ and $N_e$ are the number of migrants and the effective population size, respectively.
Figure 3. Mismatch distributions (left) and Bayesian skyline plots (BSP; right) for each of five distinct populations identified by AMOVA. Caribbean planktotrophic demes (A) were pooled into one panmictic metapopulation for analysis; one remaining population was planktotrophic (B: Lake Surprise, FL), whereas the others were aplanktonic: Geiger Beach, FL (C); Little San Salvador (D); and Plana Cays (E). For BSPs, dark lines are mean effective population size ($N_e$) plotted on a log-scaled Y-axis, with gray intervals indicating 95% confidence intervals; X-axes show time before present, converted from generations to $10^3$ years, up to the 95% confidence limit on minimum population age. Mean present-day $N_e$ is given in the lower (A) or upper (B–D) left corner of BSPs.
LOS OF DISPERSAL REDUCES GENETIC DIVERSITY

Figure 4. Population subdivision in Costasiella ocellifera, according to STRUCTURE (K = 3) using a nuclear microsatellite dataset of 134 individuals across six loci. Abbreviations for sampling sites are given in Table 1. See online version for reference to colors.

For percent hatching success, there was a significant interaction between population of origin for dam and sire (Table 5; $P = 0.034$), driven largely by elevated failure rates for aplanktonic embryos in interpopulation crosses (Fig. 5, right panels). Dams from both planktotrophic populations generally had >95% hatching success, regardless of the sire’s source. In contrast, dams from GEI had a roughly fourfold increase in embryonic failure when paired with a LAK sire, and an almost sevenfold increase in failure when paired with an LSS sire (Fig. 5B, right panel). Hatching failure was similarly four- to sevenfold higher for LSS dams paired with a sire from any other population, regardless of development mode (Fig. 5D, right panel).

Discussion

LARVAL DISPERSAL AFFECTS GENETIC DIVERSITY

Patterns of diversity in the nuclear and cytoplasmic genomes of C. ocellifera reveal strong population-level effects of dispersal ability. Phylogeographic analyses found three divergent mtDNA lineages, with an older division between FL and the Caribbean, and a more recent split between FL populations differing in development. Divergence between Caribbean and FL mtDNA was well below interspecific COI distances in Costasiella (Jensen et al. 2014), and our crosses show that FL and Caribbean lineages can interbreed and represent one biological species. Microsatellites also distinguished planktotrophs from FL versus the Caribbean, but grouped aplanktonic populations together. Analyses thus sug-
Figure 5. Reproductive output from experimental crosses within and among populations of *Costasiella ocellifera* that differ in development mode and/or degree of relatedness. Planktotrophic populations were from Lake Surprise, FL (LAK) or San Salvador, Bahamas (SSAL); aplanktonic populations were from Geiger Beach, FL (GEI) or Little San Salvador, Bahamas (LSS). The mode of development for dams from a given site is noted by “P” (planktotrophic) or “A” (aplanktonic) after the site abbreviation. Left panels show mean (±SE) daily egg production per dam from four source populations: (A) LAK, (B) GEI, (C) SSAL, and (D) LSS. Sire’s site of origin is indicated on the X-axis for each cross. Results from a one-way ANOVA are given. Right panels show the mean (±SE) percentage of unhatched offspring for each cross, with N replicates given on each bar. Note different Y-axis scales due to differences in fecundity and development success for the two development modes. *P < 0.01; **P < 0.005; ***P < 0.0005.
Table 4. Pairwise tests of population differentiation among sampling localities for *Costasiella ocellifera* based on $\Phi_{ST}$ (below diagonal; K2P distances) and $F_{ST}$ (above diagonal) analyses; $P < 0.05$ for bolded values, based on 10,000 permutations of the data.

<table>
<thead>
<tr>
<th></th>
<th>Lake Surprise</th>
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</thead>
<tbody>
<tr>
<td><strong>LKS</strong></td>
<td>0.143</td>
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<tr>
<td><strong>GEI</strong></td>
<td>0.094</td>
</tr>
<tr>
<td><strong>BER</strong></td>
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</tr>
<tr>
<td><strong>SWE</strong></td>
<td>0.030</td>
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<tr>
<td><strong>STIR</strong></td>
<td>0.044</td>
</tr>
<tr>
<td><strong>SSAL</strong></td>
<td>0.141</td>
</tr>
<tr>
<td><strong>PLA</strong></td>
<td>0.015</td>
</tr>
<tr>
<td><strong>COMP</strong></td>
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</tr>
<tr>
<td><strong>NEX</strong></td>
<td>0.058</td>
</tr>
<tr>
<td><strong>JAM</strong></td>
<td>0.066</td>
</tr>
<tr>
<td><strong>CUR</strong></td>
<td>0.066</td>
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<tr>
<td><strong>BLZ</strong></td>
<td>0.066</td>
</tr>
</tbody>
</table>

**Table 5.** Results of a two-way ANOVA testing the effects of maternal and paternal source population on the percentage of hatching success in *Costasiella ocellifera*.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Male × paternal</th>
<th>Female × maternal</th>
<th>Female × paternal</th>
<th>Male × maternal</th>
<th>Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal source</td>
<td>1</td>
<td>0.036</td>
<td>0.458</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0044</td>
</tr>
<tr>
<td>Paternal source</td>
<td>1</td>
<td>0.052</td>
<td>0.621</td>
<td>0.046</td>
<td>0.046</td>
<td>0.0014</td>
</tr>
<tr>
<td>Maternal × paternal</td>
<td>9</td>
<td>2.165</td>
<td>0.0008</td>
<td>0.0008</td>
<td>0.0008</td>
<td>0.0008</td>
</tr>
<tr>
<td>Female × maternal</td>
<td>3</td>
<td>10.219</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Female × paternal</td>
<td>3</td>
<td>0.498</td>
<td>0.621</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Male × maternal</td>
<td>3</td>
<td>0.026</td>
<td>0.458</td>
<td>0.052</td>
<td>0.052</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td></td>
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<td></td>
<td></td>
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<td>0.0008</td>
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</tbody>
</table>

Evolution January 2016

Loss of dispersal reduces genetic diversity

Table 5. Results of a two-way ANOVA testing the effects of maternal and paternal source on the percentage of hatching success in *Costasiella ocellifera*.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Male × paternal</th>
<th>Female × maternal</th>
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<td>0.036</td>
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<tr>
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<td>2.165</td>
<td>0.0008</td>
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<tr>
<td>Female × maternal</td>
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<td>10.219</td>
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<tr>
<td>Residual</td>
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<td>0.0008</td>
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</tbody>
</table>
An alternative explanation is that strong balancing selection maintained an aplanktonic genotype in both FL and Caribbean populations, reducing diversity at linked msat loci. In this scenario, selection would be responsible for both the Bayesian clustering of aplanktonic individuals into one gene pool, and also for reduced nuclear diversity. However, it is unlikely that the same msat alleles would remain tightly linked to alleles controlling larval type over the independent histories of FL and Caribbean populations (inferred from divergent mtDNA). It is also unlikely that we isolated multiple msat loci linked to alleles of large effect on development mode, particularly given the lack of evidence for such loci in transcriptomic analyses of alternative larval morphs in *S. benedicti* (Zakas and Rockman 2015). Moreover, selection on nuclear genes does not explain reduced mtDNA diversity at all aplanktonic sites.

Another possibility is that aplanktonic genotypes spread from Geiger Beach, FL to the Bahamas by rafting via the Gulf Stream. In this case, reduced nuclear diversity at aplanktonic sites in the Bahamas could reflect founder effects. However, msat diversity was far lower at Geiger Beach than at the aplanktonic Caribbean sites, making Geiger Beach an unlikely source population. Moreover, the Geiger Beach population had a common allele at msat locus Coce770 (82% frequency) that was absent from aplanktonic Caribbean populations. Spread from the more diverse aplanktonic populations of the Bahamas into Geiger Beach is unlikely given current directions, and the presence of a distinct mtDNA lineage in FL. Thus, low msat diversity in all aplanktonic populations is most likely the result of intensified drift rather than founder effects.

This “Out-of-Florida” hypothesis is also hard to reconcile with aplanktonic populations in the Bahamas having Caribbean mtDNA, requiring introgression of Caribbean mtDNA that then displaced all FL mtDNA from introduced aplanktonic genotypes. Limited introgression into aplanktonic populations is consistent with evidence that three Geiger Beach slugs retained mtDNA from a planktotrophic maternal ancestor despite extensive backcrossing. However, planktotrophic eggs fertilized by sperm carrying aplanktonic alleles would develop into dispersive larvae, which would then have to be locally retained for a month for aplanktonic genotypes (linked to introgressed mtDNA) to recruit locally, and rise in frequency, at the three Bahamas sites. A simpler explanation is that aplanktonic genotypes became locally abundant at each Bahamas sites against a native Caribbean mtDNA background, without colonization from FL, and demographic processes led to a drop in diversity for both mtDNA and msat loci.

Notably, each aplanktonic site had one or two private COI haplotypes each sampled 5.0 ± 2.1 times apiece, whereas 99% of Caribbean planktotrophs had a unique haplotype. Thus, different COI haplotypes became relatively common in each aplanktonic population, whereas planktotrophic demes lacked common alleles (except Lake Surprise, FL). Purifying selection acting anywhere on the mitochondrial genome reduces overall mtDNA diversity, but cannot explain the loss of diversity (a) only in, and always in, aplanktonic populations; and (b) at both mitochondrial and nuclear loci. Given the role of larval type in dispersal, the most parsimonious explanation is that intensified genetic drift in aplanktonic populations consistently reduces diversity. Our findings constitute the first empirical confirmation within a species that the loss of larval dispersal reduces gene flow and diversity, strengthening inferences made from among-species comparisons that control for demographic history and physical transport regime (Ayre et al. 2009; Marko and Hart 2010; McGovern et al. 2010; Barbosa et al. 2013; Dawson et al. 2014).

**POECILOGONY AS A TIPPING POINT: WHAT DRIVES THE LOSS OF DISPERAL?**

Poecilogeny may be an evolutionary tipping point, with dimorphism allowing a locally beneficial strategy like aplanktonic development to fix in a population (and ultimately sweep through a species). Both theory and empirical work indicate that planktotrophic populations can be invaded by less-dispersive strategies, but conditions that favor lecithotrophy remain debated (Marshall et al. 2012). The rarity of poecilogeny suggests that larval dimorphisms are evolutionarily unstable (Collin 2012; Vendetti et al. 2012). However, theoretical treatments indicate that dispersal dimorphisms can be selectively maintained when habitat quality (Mathias et al. 2001; Ronce 2007) or population size (Massol et al. 2011) varies in space or time. In marine animals, poecilogeny may also be maintained by asymmetric dispersal success among patches, resulting from the location of spawning sites in unidirectional current regimes (Zakas and Hall 2012). Planktotrophy was usually displaced by pelagic lecithotrophy in simulations across most parameter combinations, consistent with work showing that unidirectional current regimes generally select against dispersive larvae (Pringle et al. 2014). However, poecilogeny was evolutionarily stable and maintained in 3–5% of simulations, without requiring variable habitat quality, stability, or carrying capacity (Zakas and Hall 2012).

Theory thus predicts that aplanktonic development can be selectively maintained at sites from which dispersal is unlikely to succeed, while planktotrophy remains favored in patches upstream of suitable settlement sites in unidirectional currents. Isolation of the FL Keys by the Gulf Stream may favor aplanktonic development at Geiger Beach, where locally spawned planktotrophic larvae would be advected into the North Atlantic. Despite evidence of immigration *into* FL, no FL mtDNA haplotypes were sampled in Caribbean populations, indicating little emigration. Such an oceanographic barrier to dispersal would also explain mtDNA divergence between FL and Caribbean populations. Emigration from Plan Cays could be similarly disfavored
bility among planktotrophic demes, representing the only genetically isolated population with dispersive larvae. Differentiation in Lake Surprise may reflect isolating effect(s) of the enclosed site itself, the offshore Gulf Stream, and/or reproductive isolation with Geiger Beach. Feeding larvae could be favored in Lake Surprise due to the high productivity of its enclosed coastal waters, in contrast to oligotrophic lagoons in the Bahamas: over 11 years, mean chlorophyll $a$ levels were about twice as high in Lake Surprise as Sweeting’s Cay, and an order of magnitude higher than Little San Salvador (Fig. S1). As feeding and PLD are inherently correlated for marine larvae, high-productivity coastal embayments may favor the higher fecundity associated with planktotrophy despite costs of a prolonged larval period without benefits of occasional long-distance dispersal.

If circulation at four sites favored aplanktonic development, oceanography alone might explain reduced diversity in those populations of *C. ocellifera*, undercutting the proposed link between larval type and genetic polymorphism. We therefore analyzed data from M.S. theses (supplemented with unpubl. data) for 12 co-occurring sacoglossan species, six lecithotrophs, and six planktotrophs; full details will be published elsewhere. Each taxon was sampled from up to 14 sites (mean no. of sites per species: 7.8 ± 1.0 SE) out of 16 localities. We first calculated mean $\Phi_{ST}$ and COI haplotype diversity ($h$) for each site within each species as described for *C. ocellifera*, and then computed the grand mean-of-mean $\Phi_{ST}$ and $h$ for each site among species (Table S3). Values for the four putative “low-diversity” sites (GEI, SWE, LSS, PLA) were compared with the other 12 sites using Mann–Whitney tests (excluding data from *C. ocellifera*). Across 12 species, mean $\Phi_{ST}$ for “low-diversity” sites (0.39 ± 0.07 SE) did not differ from the remaining sites (0.35 ± 0.02 SE) (one-tailed Mann–Whitney test: $z = -0.73$; $P = 0.23$). Mean $h$ also showed no difference ($P = 0.85$) between “low-diversity” ($h = 0.89 ± 0.02$ SE) and other sites ($h = 0.88 ± 0.02$ SE), in marked contrast to the reduced diversity in aplanktonic populations of *C. ocellifera* at these four locations. We also performed one-tailed Mann–Whitney tests on each of six species sampled from ≥9 sites, but again found (i) no significant reduction in $h$ for the four “low-diversity” sites in any species; and (ii) a borderline increase ($P = 0.045$) in mean $\Phi_{ST}$ for aplanktonic sites in just one of six species (a possible Type 1 error, given 12 comparisons).

Because sites are inherently subject to distinct current regimes, local oceanography may potentially confound population-level comparisons in a poecilogonous species. However, in the present work, we found no consistent effect of oceanography alone. Some enclosed lagoon populations were lecithotrophic (SWE ’04, LSS), whereas others were planktotrophic (LKS); some coastal populations exposed to strong currents were lecithotrophic (GEI), whereas other were planktotrophic (SSAL). Moreover, diversity and connectivity were dramatically reduced at the four aplanktonic sites in *C. ocellifera*, but were not consistently lower at those same sites in 12 codistributed, related, and ecologically similar sacoglossan species. Thus, population-level dispersal phenotype better explains the genetic effects observed in *C. ocellifera* at these locations than any co-varying aspect of the physical or biological environment.

Our work therefore compliments studies on co-occurring species that differ in larval type, which may be confounded by factors other than geography, by showing that aplanktonic development in *C. ocellifera* has consistent effects in “replicate” populations of independent origin and different physical settings.

**POECILOGONY AS A BRANCHING POINT: EVOLUTION OF REPRODUCTIVE ISOLATION**

Poecilogony may also be an evolutionary branching point, where different larval types fix in populations representing incipient species. Life-history shifts could contribute to reproductive isolation if zygotes fail to develop when maternal and paternal genomes direct the development of different embryonic morphs (Raff et al. 2003), potentially explaining why lecithotrophs often have planktotrophic sister species (Duda and Palumbi 1999; Hart 2000; Collin 2004). However, Krug et al. (2015) found no evidence for cladogenetic change in development across Sacoglossa. Moreover, recent genomic evidence shows extensive gene flow between larval morphs in the poecilogonous worm *S. benedicti* (Zakas and Rockman 2015), so embryonic differences do not produce strong isolation in this species. Our breeding experiments also rejected the hypothesis that differences in development drive mating incompatibility among populations. Dams from planktotrophic populations had high hatching success in all crosses, whereas aplanktonic dams had reduced hatching success in most crosses, except for GEI (aplanktonic) x SSAL (planktotrophic). Thus, embryonic failure did not generally result from differences in larval type between parents. The hypothesis that reproduc-
tive compatibility would be inversely related to genetic distance (Edmands 2002) was also rejected by the high failure rate in most crosses between related neighboring sites, and the general success of SSAL slugs.

Instead, our results suggest that intrinsic factors partially isolate the two studied aplanktonic populations. OD is a predicted result of self-recruitment and smaller effective size in fragmented populations that receive immigrants (Jourdan-Pineau et al. 2012), which aptly characterizes aplanktonic populations of *C. ocellifera*. Although OD usually manifests in the F2 generation via breakdown in coadapted gene complexes (Ellison and Burton 2008), admixture of low-diversity populations can also reduce F1 hybrid fitness due to breakup of favorable epistatic interactions, underdominance, or gene × environment interactions (Burton 1997; Edmands 2002). Hatching failure in our crosses was also consistent with studies on freshwater snails showing OD in early stages of F1 hybrids, nonadditive effects of maternal and paternal sources, and asymmetric isolation among sites (Escobar et al. 2008). Our assay (hatching success) would not detect fitness costs of OD manifesting later in life for F1 hybrids due to phenotype–environment mismatches (Lynch 1991; Edmands 1999). Given that most planktotrophic demes formed a metapopulation without subdivision, population fragmentation in *C. ocellifera* results primarily from shifts in development, allowing positive epistatic interactions to accumulate in small aplanktonic populations. Shifts in development may thus contribute to incipient speciation by accelerating evolutionary processes that would proceed more slowly in large populations linked by dispersal.

We interpret reduced mating between neighboring sites that differ in development as evidence of reinforcement, driven by reduced hatching success of interpopulation hybrids. Behaviors promoting assortative mating evolve rapidly during secondary contact between populations with some degree of postzygotic isolation (Coyne and Orr 2004; Ortíz-Barrientos et al. 2009). Reinforcement requires interaction between individuals from different sources, such that selection can favor discrimination against immigrants (Coyne and Orr 1989). We therefore predicted that prezygotic barriers should evolve between neighboring populations that had reduced hatching success in crosses, and possibly be more pronounced for aplanktonic populations that receive planktotrophic immigrants, and have the opportunity to evolve local mate discrimination.

As expected, little mating occurred between slugs from adjacent FL populations, and hatching success was markedly reduced for aplanktonic dams in those crosses. Reduced egg production by planktotrophic (LAK) slugs could result from aplanktonic (GEI) slugs refusing to mate, producing a prezygotic barrier in both directions, or if LAK dams also discriminate against GEI sperm donors. Consistent with reinforcement contingent upon historical contact, all FL dams had normal egg production when paired with a Caribbean partner, despite low hatching success in some crosses (e.g., GEI × LSS sperm). Aplanktonic Caribbean dams similarly laid fewer eggs when crossed with a neighboring planktotrophic site than when paired with an aplanktonic slug from FL, despite a greater reduction in hatching success when paired with FL sires. Although based on a limited number of population crosses, these results suggest that slugs from low-diversity populations at risk of OD must encounter immigrants often enough for selection to favor assortative mating. Surprisingly, dams from all populations laid the most eggs when paired with a sire from LSS, possibly indicating that LSS slugs produce a higher quality or quantity of sperm, or inseminate more aggressively in the male role.

Caribbean planktotrophs formed a widespread metapopulation with high connectivity among demes. Thus, selection for premating isolation was not predicted to act on the SSAL population, as offspring must interbreed with whatever subpopulation they recruit into. Consistent with this expectation, SSAL dams had comparable egg output regardless of the sire’s source population. Vulnerability to OD may thus strongly hinge on development mode, with implications for management of marine species (Baums 2008). Given the high dispersal ability and lack of preferential mating exhibited by planktotrophs, intrinsic reproductive barriers that accumulate in aplanktonic populations likely impede planktotrophic immigrants from reintroducing genetic diversity lost to drift, a positive feedback loop driving isolation and differentiation following loss of a dispersive stage.

**CONCLUSIONS**

Poecilogonous species represent a window into transitions that are rarely observed, but must occur at the population level for a derived mode of development to fix in a species. Aplanktonic populations of *C. ocellifera* had reduced connectivity and genetic diversity at both mitochondrial and nuclear loci compared to planktotrophic demes, as predicted following loss of a dispersive larval stage. Moreover, aplanktonic populations exhibited reduced hatching success in most interpopulation crosses but planktotrophic populations did not, suggesting that smaller effective population size may lead to OD. Aplanktonic dams also showed premating isolation when paired with sires from neighboring, but not distant, populations, consistent with reinforcement driven by frequent immigration and partial postzygotic isolation. The loss of dispersive larvae may thus accelerate population-genetic processes that contribute to reproductive isolation, while shrinking the spatial scale over which speciation can occur.

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DATA ARCHIVING
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LITERATURE CITED


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Figure S1.** Chlorophyll a concentrations in the northern Caribbean Sea, showing mean annual productivity of coastal water from 2003 to 2013 visualized in ArcGIS.

**Table S1.** Primers and summary statistics for nuclear microsatellite loci used in this study.

**Table S2.** Three molecular diversity indices for each of six microsatellite loci; bold = predominantly aplanktonic populations.

**Table S3.** Haplotype diversity (A) and mean ΦST values (B) based on sequence data for the mitochondrial COI gene from 12 sacoglossan species; shaded values represent four putatively “low-diversity” sites where C. ocellifera expressed aplanktonic development.