Genes, morphology, development and photosynthetic ability support the resurrection of *Elysia cornigera* (Heterobranchia: Plakobranchoidea) as distinct from the ‘solar-powered’ sea slug, *E. timida*

**Patrick J. Krug**, **Katharina Händeler** and **Jann Vendetti**

A Department of Biological Sciences, California State University, Los Angeles, CA 90032-8201, USA.
B Institut für Molekulare Evolution, Heinrich Heine Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany.
C Corresponding author. Email: pkrug@calstatela.edu

**Abstract.** Some groups of marine heterbranch sea slugs (formerly Opisthobranchia) have few discrete characters or hard parts and many ‘cosmopolitan’ species, suggesting an overly conservative taxonomy in need of integrative approaches. Many herbivorous sea slugs in the clade Sacoglossa retain algal chloroplasts that remain functionally photosynthetic for 1–2 weeks, but at least four species can sustain chloroplasts for several months. To better understand the origins of long-term kleptoplasty, we performed an integrative study of the highly photosynthetic species *Elysia timida* from the Mediterranean and Caribbean populations that were described as *E. cornigera* but later synonymised with *E. timida*. Nominal *E. cornigera* were distinct in their anatomy and aspects of larval development, and had dramatically reduced chloroplast retention compared with *E. timida*. Mean divergence at three genetic loci was determined for ten pairs of sister species in the genus *Elysia*, confirming that *E. cornigera* and *E. timida* have species level differences. Both taxa had a high degree of population genetic subdivision, but among-population genetic distances were far less than interspecific divergence. In an integrative taxonomic framework, *E. cornigera* is thus restored to species rank and fully redescribed, and baseline molecular data are presented for evaluating species level differences in the Sacoglossa.

Received 10 June 2011, accepted 4 December 2011, published online 7 May 2012

**Introduction**

Opisthobranchs, or sea slugs, are a traditional but polyphyletic group of ecologically diverse heterobranch gastropods that typically lack an external shell (Jörger *et al*. 2010). Taxonomic study of opisthobranchs has been hampered by the lack of hard parts other than radular teeth, internal shells or penial stylets, where present. Compounding the challenge, parallel adaptation to ecologically similar niches by unrelated species may introduce homoplasy, and selection imposed by visual predators can lead to convergence in external morphology and mimicry (Gosliner and Ghiselin 1984; Gosliner 2001). A shortage of reliable characters and vague descriptions in species-rich groups has led to cycles of splitting and lumping that have impaired our understanding of biodiversity and evolution in marine heterobranchs, as illustrated by the numerous species with ‘circumtropical’ distributions or ranges spanning multiple ocean basins (Gosliner and Draheim 1996; Jensen 2007). Long-lived planktotrophic larvae may disperse enough to connect populations over long distances or permit occasional trans-oceanic colonisation (e.g. Ellingson and Krug 2006), but a seemingly cosmopolitan species may actually comprise a cryptic species complex masked by an overly conservative taxonomy (Klautau *et al*. 1999). Distinguishing between these scenarios would greatly advance our understanding of the biogeography of marine speciation.

Given the inherent challenges of sea slug taxonomy, integrative studies are needed to establish whether widely distributed ‘species’ comprise distinct lineages in different oceans. Data on morphology, behaviour and life history can be compared with molecular phylogenies to identify subtle trait differences that are congruent with genetic breaks, but that may have been previously overlooked or treated as intraspecific variation (Collin 2005; Blanquer and Uriz 2008; Padial *et al*. 2009; Tan *et al*. 2010). Lineages differing in reproductive or ecological traits may offer insight into the speciation process itself (Smith *et al*. 2006, 2007, 2008). Even when geographically isolated lineages remain similar in niche and appearance, genetic divergence may be used as a yardstick to evaluate the likelihood that such lineages have a history of independent evolution and deserve species status (Hebert *et al*. 2003; Halt *et al*. 2009; Cook *et al*. 2010). However, rates of molecular evolution differ widely across loci and taxa, necessitating careful attention to vagaries of the coalescent

We are currently using an integrative approach to identify uncharacterised biodiversity, infer relationships and study trait evolution in the Sacoglossa, a clade of herbivorous sea slugs specialising on siphonaceous algae (Jensen 1996). Most species in the clade Plakobranchioidea maintain undigested chloroplasts for days or weeks in digestive diverticula that ramify throughout their bodies, and benefit from continuing photosynthesis of these hijacked plastids (Händeler et al. 2009; Curtis et al. 2010). Four species remain photosynthetic for many months, long after the nuclear gene-encoded light-harvesting complexes should have burnt out without replacement: *Elysia chlorotica* Gould, 1870, *E. crispa* Morch, 1863, *E. timida* Risso, 1818 and *Plakobranchus ocellatus* van Hasselt, 1824 (Händeler et al. 2009). Ancestral character state reconstructions indicate that long-term chloroplast maintenance arose independently in each of the four highly photosynthetic species; parallel origins present the opportunity to study the repeated evolution of chloroplast symbiosis in animal hosts (Händeler et al. 2009). Lateral transfer of photosynthetic genes from the algal nucleus to the slug genome has been implicated for *Elysia chlorotica* (Rumpho et al. 2008; Pierce et al. 2009; Schwartz et al. 2010), but algal gene expression is yet to be detected in other species exhibiting long-term kleptoplasty (Pelletreau et al. 2011; Wägele et al. 2011).

The phylogenetic relationships of highly photosynthetic species are critical for understanding the evolution of plastid symbiosis in sacoglossans. The Mediterranean species *Elysia timida* Risso, 1818 is the type species of the genus, and has long been studied for its remarkable photosynthetic abilities (Rahat 1976; Marin and Ros 1993; Evertsen et al. 2007). Two Australian species, *E. filicauda* Jensen & Wells, 1990 and *E. thompsoni* Jensen, 1993, feed on the same genus of host algae (*Acetabularia*) and are morphologically similar to *E. timida*, but are clearly distinct in external appearance and/or larval biology (Jensen and Wells 1990; Jensen 1993). A candidate sister species, *E. cornigera* Nuttall, 1889, was described from Florida. Based on Cuban material, Ortega et al. (1997) synonymised *E. cornigera* with *E. timida*, noting similarities in external appearance, radular teeth and flinching movements of the head. Differences in Caribbean specimens, such as more pronounced parapodial wing flaps or lobes, papillae on the rhinophores and brownish–rose indentations across the body, were noted but dismissed. The view that *E. cornigera* was synonymous with *E. timida* based on morphology was shared by Rudman (2004). Bass (2004) asserted that genetic analysis suggested *E. cornigera* and *E. timida* were conspecific, but no data were published to substantiate this claim.

With a short planktonic period, the lecithotrophic larvae of *E. timida* should be incapable of trans-Atlantic dispersal; however, long-term chloroplast retention may allow adults to disperse long distances by rafting. Given the importance of *E. timida* for studies on chloroplast symbiosis and its suggested amphiplastic distribution, we evaluated the species status of Caribbean and Mediterranean lineages based on external and radular morphology, larval biology, photosynthetic ability and molecular phylogenetic relationships. Our results have important implications for understanding the evolution of functional kleptoplasty in sacoglossans, and highlight the importance of integrating many lines of evidence into tests of taxonomic hypotheses.

**Materials and methods**

**Collection of organisms and egg masses**

Specimens were collected and examined alive from sites in Florida, the Bahamas and Jamaica (*E. cornigera*), and from the Mediterranean coasts of France and Spain (*E. timida*; Table 1). Live specimens were photographed with an Olympus 5060 digital camera (Olympus, Center Valley, PA) through a Zeiss Stemi 3000 stereomicroscope (Carl Zeiss Microscopy LLC, Thornwood, NY) and observed in the laboratory for several weeks.

Larval development mode and hatching behaviour were not documented in the original description of *E. cornigera*; we here expand on the brief description in Krug (2009). Egg masses (*n = 5*) were deposited in the laboratory by specimens collected 5 km from the type locality in August 2007. Egg masses were carefully removed from the substrate and isolated in individual dishes in 4 mL of 0.45 μm filtered seawater, and allowed to develop through larval metamorphosis with daily water changes. Two egg masses were broken apart when larvae had developed eyespots to assess swimming ability of veligers; the remaining three egg masses were left undisturbed to determine whether larvae metamorphosed before or after hatching.

**Radular morphology**

Radulae from multiple Caribbean and Mediterranean individuals were prepared by dissecting out the pharynx and dissolving the tissue in 10% NaOH for >12 h, then rinsing in distilled water followed by ethanol, and mounting on stubs for scanning electron microscopy (SEM). Prepared stubs were sputter-coated with an Emitech K550x sputter coater (Quorum Technologies Ltd, Ashford, Kent, UK) to a thickness of 200 Å and visualised with a Hitachi S-3000N variable pressure scanning microscope (Hitachi High Technologies, San Diego, CA). Specimens were viewed at an accelerating voltage of 10–15 kV.

**Phylogenetic analyses**

Genomic DNA was extracted from specimens preserved in 100% ethanol with a QiAamp DNA mini kit (Qiagen, Valencia, CA) and at −20°C stored in extraction buffer. Polymerase chain reaction (PCR) was used to amplify a 710-base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene from the specimens listed in Table 1, using primers and reaction conditions described in Händeler et al. (2009). Purified PCR products were directly cycle-sequenced in both directions using PCR primers and Big Dye Terminator 3.1 cycle sequencing chemistry (Applied Biosystems, Foster City, CA), either at the High-Throughput Genomics Unit, University of Washington, or on an ABI PrismTM 377 DNA Sequencer (Applied Biosystems). Chromatograms were edited and primer sequences removed in GeneiousPro 4.8 software (Biomatters Ltd, Auckland, New Zealand). Alignments were generated in ClustalX (Thompson et al. 1997) using default settings.

For COI, aligned full-length sequences of 658 bp were obtained for most specimens; the ends of shorter sequences were coded as missing data for phylogenetic analysis.
Table 1. Collection dates, localities and number of sampled specimens

Unless otherwise indicated, NCBI accession numbers are for COI haplotypes with sampling frequency given in parentheses. Sugarloaf Key specimens were used in photosynthetic assays but not genetic analyses. P.K., Patrick Krug; K.H., Katharina Händeler; Y.G., Yvonne Gryzmbowski; A.D., Anne DuPont; R.M., Roland Melzer; S.A., Sven Affeld.

<table>
<thead>
<tr>
<th>Site</th>
<th>E. cornigera (N)</th>
<th>E. timida (N)</th>
<th>Latitude, longitude</th>
<th>Date sampled</th>
<th>Collector</th>
<th>NCBI accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bahamas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Little San Salvador Island (LSS)</td>
<td>1</td>
<td></td>
<td>26°33′43″N, 77°51′15″W</td>
<td>Jun 2007</td>
<td>P.K.</td>
<td>JN819086</td>
</tr>
<tr>
<td>Lee Stocking Island (Lee)</td>
<td>1</td>
<td></td>
<td>25°49′12″N, 77°53′56″W</td>
<td>Jun 2007</td>
<td>A.D.</td>
<td>JN819085, JN819127 (16S)</td>
</tr>
<tr>
<td>San Salvador Island (SSal)</td>
<td>1</td>
<td></td>
<td>24°34′30″N, 75°56′30″W</td>
<td>Jul 2010</td>
<td>P.K.</td>
<td>JN819087, JN819126 (16S)</td>
</tr>
<tr>
<td>Florida Keys, U.S. (FL Keys)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugarloaf Key</td>
<td>13</td>
<td></td>
<td>24°37′10″N, 81°31′18″W</td>
<td>Jul 2007</td>
<td>Y.G.</td>
<td>–</td>
</tr>
<tr>
<td>Geiger Beach, Key West</td>
<td>6</td>
<td></td>
<td>24°33′39″N, 81°40′39″W</td>
<td>Aug 2007</td>
<td>P.K.</td>
<td>JN819079 (2), JN819080, JN819081, JN819082, JN819083, JN819153 (H3)</td>
</tr>
<tr>
<td>Jamaica</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discovery Bay</td>
<td>1</td>
<td></td>
<td>18°28′07″N, 77°24′53″W</td>
<td>Mar 2006</td>
<td>P.K.</td>
<td>JN819084, JN819125 (16S), JN819154 (H3)</td>
</tr>
<tr>
<td>Croatia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bay at Saline, near Vśtar</td>
<td>1</td>
<td></td>
<td>45°08′59″N, 13°36′09″E</td>
<td>2003</td>
<td>R.M.</td>
<td>JN819076, JN819124 (16S)</td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banyuls-sur-Mer (BsM)</td>
<td>11</td>
<td></td>
<td>42°29′18″N, 03°07′47″E</td>
<td>May 2006</td>
<td>K.H. &amp; S.A.</td>
<td>JN819070 (9), JN819071, JN819072, JN819116 (16S), JN819117 (16S), JN819118 (16S), JN819155 (H3)</td>
</tr>
<tr>
<td>Spain (northern)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roses (R)</td>
<td>3</td>
<td></td>
<td>42°14′24″N, 03°11′28″E</td>
<td>May 2006</td>
<td>K.H.</td>
<td>JN819073, JN819075 (2), JN819119 (16S)</td>
</tr>
<tr>
<td>Plaja Caials, near Cadaques (PC)</td>
<td>3</td>
<td></td>
<td>42°17′06″N, 03°17′47″E</td>
<td>May 2006</td>
<td>K.H.</td>
<td>JN819073 (2), JN819074, JN819120 (16S), JN819121 (16S)</td>
</tr>
<tr>
<td>Spain (southern)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baynorth of Cala Giverola (CG)</td>
<td>3</td>
<td></td>
<td>41°44′18″N, 02°57′28″E</td>
<td>May 2006</td>
<td>K.H.</td>
<td>JN819077 (2), JN819078, JN819123 (16S)</td>
</tr>
<tr>
<td>Cala St. Francesc, Blanes (B)</td>
<td>3</td>
<td></td>
<td>41°40′41″N, 02°48′27″E</td>
<td>May 2006</td>
<td>K.H.</td>
<td>JN819077 (3), JN819122 (16S)</td>
</tr>
</tbody>
</table>

Sequences were deposited in the National Center for Bioinformatics (NCBI) database (accession numbers given in Table 1). Evolutionary relationships among haplotypes were inferred using the Bayesian Markov-chain Monte Carlo (MCMC) method. Trees were rooted on the Indo-Pacific species *Elysia thompsoni*, which is morphologically similar and feeds on the same host (*Acetabularia*). Mixture models of sequence evolution were implemented in the software package Bayes Phylogenies (Pagel and Meade 2004). From a user-specified number of models, a likelihood criterion was used to assign the best-fit model to each position in the data alignment; this approach contrasts with conventional *a priori* data partitioning, which forces the same model to apply to all nucleotides at a given position. Base frequencies, rate parameters and a gamma shape parameter governing among-site rate heterogeneity were independently estimated during runs, with four rate classes drawn from the gamma distribution. Two GTR + Γ models were parameterised during runs, as adding a third model did not improve likelihood scores or alter the topology. Following Pagel and Meade (2004), we ran four replicate Markov chains each for 5 × 10⁶ generations, saving a tree every 10⁴ generations. For each run, the harmonic mean of log-likelihood scores was calculated for the final 10⁴ trees, and nodal support plotted on a 50% majority-rule consensus tree in BayesTrees (http://www.evolution.reading.ac.uk). As all runs converged on equivalent topologies and likelihoods, the final 10⁴ trees of all four runs were pooled and one consensus tree generated with mean branch lengths and posterior probabilities. Support values ≥90% were taken as statistically meaningful (Douady et al. 2003; Huelsenbeck and Rannala 2004).

Interpreting genetic distance between nominal species is best done in conjunction with an estimation of genetic subdivision among populations within a species. We thus separately tested the degree of population structure in *E. timida* and *E. cornigera* using COI data. Sampling sites that yielded only one specimen were excluded from analysis. Based on inspection of the COI gene tree and the geographic sampling performed, *E. timida* was divided into three regional populations: France (all samples collected near Banyuls-sur-Mer), northern Spain (Roses and Plaja Caials) and southern Spain (Blanes and Cala Giverola). Due to the limited samples available, specimens of *E. cornigera* were grouped into two populations: the Bahamas (one individual each from Little San Salvador, San Salvador and Lee Stocking islands) and Florida Keys. Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed in Arlequin 3.0 (Excoffier et al. 2005) to assess the proportion of genetic covariance partitioned among
demes, computing $\Phi_{ST}$ as a fixation index using Tamura-Nei distances between haplotypes. Pairwise $\Phi_{ST}$ was also computed among the three populations of *E. timida*. Significance was assessed using 10^4 permutations of the data.

Mean pairwise divergence among COI haplotypes was calculated (a) between *E. cornigera* and *E. timida*, and (b) among haplotypes within each species, in Mega 5.0 (Tamura et al. 2011). The COI dataset for each species was collapsed so each haplotype was represented once before distance calculations. Distances were corrected for multiple substitutions per site using the Tamura-Nei model, chosen under the Bayesian information criterion, using a maximum likelihood optimised base tree and default parameters (Posada 2008). Mean between-species pairwise distances were also calculated for five sister species of *Elysia* identified in Händeler et al. (2009), plus four pairs identified in a subsequent analysis of a larger 4-gene dataset, each including one undescribed species (collection location in parentheses): *E. abei* – *Elysia* sp. 5 (Hawaii); *E. tuca* – *Elysia* sp. 6 (Mexico); *E. ornata* – *E. cf. marginata* (Guam, Moorea); and *E. crispata* – *Elysia* sp. 19 (Bahamas) (P. J. Krug and J. E. Vendetti, unpubl. data). For all nine reference pairs of sister species, we obtained 1–4 unique haplotypes per species.

To compare the magnitude of between-species divergence in *Elysia* at additional loci, we also amplified portions of the mitochondrial large ribosomal subunit rRNA (16S) gene and the nuclear histone 3 (H3) gene from select specimens of *E. timida*, *E. cornigera* and the nine pairs of sister species described above (*n*=1–4 individuals per species; accession numbers given in Supplementary Table 1, available on the Invertebrate Systematics website). Primers and reaction conditions are described in Händeler et al. (2009) for 16S, and Rodriguez (2009) for H3. Sequences for each pair were aligned separately in Clustal to minimise artefacts from hyper-variable loops in 16S. Ends of alignments were trimmed to exclude regions with data for only a single specimen. When H3 genotypes were heterozygous, individual alleles were inferred by comparison with common homozygote sequences and mean distances between alleles were computed; all known H3 alleles for *E. subornata* and *E. pratensis* were included in analyses (Rodriguez 2009). Mean between-species distance was then calculated in Mega 5.0 for 16S using the Tamura-Nei model (gaps coded as missing data), and for H3 using uncorrected p-distances as the low divergence between alleles obviated the need to correct for multiple hits.

**Photosynthetic ability**

Photosynthetic activity and longevity of diet-derived chloroplasts were assessed using a pulse amplitude modulated fluorometer (Walz, Germany) to measure the maximum quantum yield of chlorophyll a fluorescence from photosystem II. Data from Händeler et al. (2009) were reanalysed here for our two focal species. Briefly, slugs were collected from host algae and held without food in outdoor aquaria; *E. cornigera* ranged from 3 to 8 mm, while all *E. timida* were ~1 cm long. From a fibre optic probe held ~5 mm above a dark-acclimated slug, a burst of actinic light induced closure of reaction centres and maximum emission of fluorescence. Measurements were taken for ten replicate specimens per species until animals began to die (11 days for *E. cornigera*, 35 days for *E. timida*). Three replicate measurements were recorded per slug each day, at 15 min intervals to allow re-acclimation. If inconsistent values were obtained across measurements, no data were recorded for that individual that day. Mean values per species per day were calculated as the mean of means for daily measurements from each specimen.

Slopes representing change in photosynthetic activity over the first 11 days were compared using ANCOVA, with PAM yield as the response variable, species as a fixed factor and day as a covariate. Yield data (percentages) were normalised by arcsine (square-root)-transformation before statistical analysis. A significant main effect indicates that species differ in their y-intercept value; a significant interaction term indicates a difference in slope between species (Quinn and Keough 2002). Curve fitting was also performed in SPSS version 17.0 (SPSS Inc., Chicago, IL) to model decay of yield values for each species over the full experimental period.

**Results**

**External and radular morphology of Elysia cornigera versus E. timida**

All specimens of *Elysia cornigera* examined were <8 mm in body length, smaller than most adult *E. timida*, which ranged from 6 to 13 mm. The head and parapodial flaps of *E. cornigera* are tinted grey and highly papillose, contrasting with the smooth, almost pure white epithelium covering the head, rhinophores and external parapodial surface of *E. timida* (Fig. 1A–C, E, F). Numerous large, golden-brown spherical inclusions in the epithelium of *E. cornigera* give the sides of the body an overall brownish tinge. Although both species have bright red spots, those on *E. cornigera* are proportionally smaller. In *E. cornigera*, the rhinophores have an uneven edge caused by many extended papillae, and taper to a point, whereas the rhinophores of *E. timida* are smooth-edged and end in a wide, rounded tip.

Nuttall (1989) presented SEM of radulae for *E. cornigera* showing tri-keeled teeth with two serrated cutting edges and one smooth edge. Published SEM of radulae of *E. timida* did not clearly show an unserrated edge (Bouchet 1984), so we compared radulae of *E. timida* from France with published SEM for *E. cornigera*. Radulae of *E. timida* also had two serrated cutting edges and a third, unserrated edge visible in lateral views (Fig. 1G, H). There was considerable variation in the degree to which leading teeth were pointed or blunt in radulae from different specimens, suggesting plasticity in radular development.

**Larval development**

A ribbon of colourless, semi-transparent extra-capsular yolk (ECY) was deposited inside the casing on the upper face of each egg mass (Fig. 1D). Encapsulated development time was 15–19 days at ~22°C. Embryos developed into veliger larvae capable of swimming if artificially liberated from their egg capsules, but all larvae from two of three undisturbed egg masses underwent intra-capsular metamorphosis and emerged.
as crawl-away juveniles. In the third egg mass, 3 of 24 larvae hatched as veligers and swam for 1–2 days before undergoing spontaneous metamorphosis in the absence of any habitat cues. This confirms lecithotrophic development for *E. cornigera*. Final larval shell size was 248.4 ± 4.8 µm (n = 2 clutches).
Molecular phylogenetic analysis

To test whether the two nominal species were genetically distinct, we inferred evolutionary relationships of haplotypes of the fast-evolving mitochondrial COI gene using Bayesian inference. Mediterranean (timida) and Caribbean (cornigera) haplotypes were reciprocally monophyletic with high posterior probability support (Fig. 2). Mean divergence between the two species was 11.24% (Tamura-Nei distance) at the COI locus, comparable to interspecific distances for sister species of Elysia (Table 2). Maximum genetic distance between haplotypes within each species was substantially lower, indicating a clear barcoding gap (Table 2).

Mean divergence of haplotypes at the more conserved 16S locus was 6.28% between timida and cornigera, whereas the maximum within-species distance was only 1.86% for E. timida and 2.47% for E. cornigera. At 16S, timida–cornigera were more divergent than four other sister species pairs (Table 2). Two specimens of E. cornigera sampled in Florida were homozygous for the same allele of the nuclear histone H3 gene, while the specimen from Jamaica was homozygous for a different allele that was 0.61% divergent (p-distance). In contrast, one specimen from France was homozygous for an allele that was 1.52% divergent from both E. cornigera alleles. Mean genetic distance between H3 alleles of timida–cornigera was commensurate with interspecific divergence in Elysia (Table 2).

Within each species there was significant population genetic structure at the COI locus indicating barriers to gene flow. In E. timida there were significant genetic differences between three regional populations: France, northern Spain (Roses and Plaja Caials) and southern Spain (Blanes and Cala Giverola) (Table 3A). No haplotypes were shared between the three populations, each population had a common private haplotype shared by three to nine sampled individuals, and haplotypes from each population were phylogenetically distant; combined, these factors resulted in an exceptionally high overall $\Phi_{ST}$ value. All pairwise comparisons between populations were highly significant ($P < 0.0001$) with $\Phi_{ST}$ ranging from 0.725 to 0.977. Although fewer sites and samples were available for E. cornigera, AMOVA revealed significant genetic differentiation between Florida and the Bahamas (Table 3B).

Photosynthetic ability of Elysia cornigera versus E. timida

Yield values after one day of starvation did not differ between E. timida (0.68 ± 0.07, n = 10) and E. cornigera (0.65 ± 0.09, n = 10). However, over the next 10 days there was a significant difference in the slopes of regression lines fit to yield data from
Table 2. Divergence at three genetic loci for ten pairs of sister species of *Elysia*

Data are genetic distances corrected with the Tamura-Nei model (COI, 16S) or uncorrected p-distances (H3). (A) Mean interspecific genetic distance between haplotypes (COI, 16S) or alleles (H3) for sister species of *Elysia*. Values for *E. timida* and *E. cornigera* are bolded. Dash indicates data were not available for one species in that pair. (B) Mean and maximum pairwise genetic distances for unique COI haplotypes within *E. timida* versus *E. cornigera*.

<table>
<thead>
<tr>
<th>A</th>
<th>Sister species pair</th>
<th>Genetic distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. timida</em>–<em>E. marginata</em></td>
<td>COI</td>
</tr>
<tr>
<td></td>
<td><em>E. cornigera</em></td>
<td>11.24</td>
</tr>
<tr>
<td></td>
<td><em>E. pratensis</em>–<em>E. ornata</em></td>
<td>11.33</td>
</tr>
<tr>
<td></td>
<td><em>E. abei</em>–<em>E. sp.5</em></td>
<td>14.84</td>
</tr>
<tr>
<td></td>
<td><em>E. crispata</em>–sp.19</td>
<td>15.17</td>
</tr>
<tr>
<td></td>
<td><em>E. australis</em>–<em>E. hamatani</em></td>
<td>16.39</td>
</tr>
<tr>
<td></td>
<td><em>E. papillosa</em>–<em>E. zuleiaceae</em></td>
<td>18.50</td>
</tr>
<tr>
<td></td>
<td><em>E. serca</em>–<em>E. chlorotica</em></td>
<td>18.93</td>
</tr>
<tr>
<td></td>
<td><em>E. styllera</em>–<em>E. pusilla</em></td>
<td>18.96</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>14.46</td>
</tr>
<tr>
<td></td>
<td>s.d.</td>
<td>3.53</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>COI distance</th>
<th><em>timida</em></th>
<th><em>cornigera</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-group mean</td>
<td>2.23</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>Within-group maximum</td>
<td>4.47</td>
<td>2.22</td>
</tr>
</tbody>
</table>

each species (Table 4). Values decreased linearly for *E. cornigera* over 11 days without food (Fig. 3, and results of a simple linear regression: $F_{1,76} = 86.16$, $r^2 = 0.53$, $P < 0.00001$). Photosynthetic activity did not decrease over the first 11 days for *E. timida*; a linear regression of the effect of days of starvation on yield showed the slope did not differ from zero ($F_{1,81} = 1.43$, $P = 0.235$). After three weeks the yield values for *E. timida* gradually decayed; the best-fit curve for transformed yield data for *E. timida* was a cubic function that underwent a slight initial decline, levelled out, then decayed at the end of the experimental period (Fig. 3, and results of a cubic regression: $F_{3,154} = 23.04$, $r^2 = 0.30$, $P < 0.00001$).

Discussion

Photosynthesis by sacoglossans has been recognised for over 40 years (Trench et al. 1969), but a recent resurgence of interest in this phenomenon was born from molecular studies implicating lateral gene transfer between algal hosts and the slugs that maintain their chloroplasts (Rumpho et al. 2008; Pierce et al. 2009; Schwartz et al. 2010; Rumpho et al. 2011; Wägele et al. 2011). Short-term chloroplast retention, for 1–2 weeks, is a potential key character behind the plakobranchioidean radiation (Händeler et al. 2009). Phylogenetic studies suggest that chloroplast retention on the order of several months had four independent origins in the Plakobranchioidea, raising questions about how and when long-term kleptoplasty arose in each lineage (Händeler et al. 2009). Clarifying the evolutionary relationships of highly photosynthetic species and their relatives that lack long-term chloroplast function is critical for our understanding of kleptoplasty, a phenomenon that straddles the gulf between endosymbiosis and herbivory.

As putative sister taxon to the highly photosynthetic *E. timida*, the taxonomic status of the Caribbean species *E. cornigera* is of evolutionary importance. We thus examined its morphology, photosynthetic ability, reproductive attributes, and divergence at three genetic loci. Multiple lines of evidence demonstrate that *E. cornigera* is a distinct species that cannot maintain chloroplasts long term. Morphologically, *E. cornigera* has several apomorphies that were disregarded by Ortea et al. (1997) in their synonymy: pointed, bumpy rhinophores; highly papillose parapodia bearing large golden-brown granules; and an overall greyish-brown body colour. Reproductive traits including egg size, larval size and appearance of ECY also differed between the species, highlighting the utility of developmental characters in sacoglossan taxonomy (Krug et al. 2007; Krug, 2009). Radular morphology was similar between *E. cornigera* and *E. timida*; a conservative radular morphology may result from purifying selection, given that both species feed on the algal host *Acetabularia* and hence occupy the same ecological niche, or could be a shared plesiomorphy.

The limited photosynthetic ability of *E. cornigera* clearly distinguishes it from *E. timida*. Stored chloroplasts in both species were highly photosynthetic at the time of collection, but yield values declined linearly in *E. cornigera*, indicating rapid digestion of chloroplasts. In contrast, photosynthetic activity showed no decline in *E. timida* until after four weeks of starvation. Duration of chloroplast activity is thus a useful taxonomic character for sacoglossans.

Table 3. Population genetic structure at the COI locus for *Elysia timida* and *E. cornigera*

Results from analysis of molecular variation are given for (A) *E. timida* populations in the Mediterranean, and (B) *E. cornigera* populations in the Caribbean. Sites were grouped as indicated in Table 1. Values of $\Phi_{ST}$ were computed from genetic distances corrected with the Tamura-Nei model. Significance was tested with 10 000 permutations of the data.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>% of variation</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among populations</td>
<td>2</td>
<td>128.038</td>
<td>8.682</td>
<td>93.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Within populations</td>
<td>20</td>
<td>12.074</td>
<td>0.604</td>
<td>6.50</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>140.113</td>
<td>9.286</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixation index</td>
<td>$\Phi_{ST}$</td>
<td>0.935</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among populations</td>
<td>1</td>
<td>12.434</td>
<td>2.630</td>
<td>57.86</td>
<td>0.0116</td>
</tr>
<tr>
<td>Within populations</td>
<td>7</td>
<td>13.406</td>
<td>1.915</td>
<td>42.14</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>24.840</td>
<td>4.545</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixation index</td>
<td>$\Phi_{ST}$</td>
<td>0.5786</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Species descriptions are hypotheses that can be rigorously tested in an integrative framework using all available characters (Dayrat 2005; DeSalle et al. 2005). Reproductive traits (Hart et al. 2003; Collin 2005; Krug et al. 2007; Naughton and O’Hara 2009) and ecological characters (Hebert et al. 2004; Smith et al. 2006, 2007, 2008) can be critically informative for soft-bodied or poorly known taxa, but generally require access to live material. Molecular characters have revolutionised our ability to recognise independently evolving lineages but there are important caveats to the use of genetic data in a taxonomic context (Lipscomb et al. 2003; Tautz et al. 2003; DeSalle et al. 2005; Hebert and Gregory 2005; Cook et al. 2010).

Genetic distances are a quantitative metric that can identify thresholds of divergence representing species level differences. A molecular approach therefore offers the chance to alleviate some measure of the subjectivity that has characterised taxonomic practice in many groups. However, to properly interpret genetic data, one must estimate levels of divergence both within a species (population genetics) and also between related species (phylogenetics). Divergence between conspecific lineages must be less than the genetic distance between species for DNA-based taxonomy to work (Hebert et al. 2003; Meyer and Paulay 2005; Pons et al. 2006). Levels of divergence that are congruent with species boundaries are also necessarily locus-specific, as mutation rates vary across the genome, and will vary among taxa as a function of life history, demography and the myriad factors that influence fixation by drift and selection. Thus, while holding promise for making species delineation more quantitative, genetic data must be carefully interpreted given the idiosyncratic properties of any locus–taxon combination, and the hazards of subjective or universal thresholds for species delimitation (e.g. Hebert et al. 2010).

Molecular phylogenetic analysis of the fast-evolving COI gene resolved haplotypes of *E. cornigera* and *E. timida* as reciprocally monophyletic and 3–5 times more divergent than the maximum intraspecific genetic distance. Further, mean divergence between *timida–cornigera* at three loci was comparable to the genetic distance between other sister species of *Elysia*. Genetic data thus strongly support the resurrection of *E. cornigera*. The *timida–cornigera* distance was on the low end of the spectrum, compared with the nine other sister species pairs; however, *timida–cornigera* were more divergent than 1–4 other species pairs at each locus. At COI and 16S the *timida–cornigera* distance was greater than the divergence between *E. ornata* from the Caribbean and a species provisionally identified as *E. marginata* (one of several species in an Indo-Pacific complex, and sister to Caribbean *ornata* in preliminary analyses). Young sister species with one member in the Caribbean and the other in the Pacific are likely gerniates isolated when the Isthmus of Panama arose 3.1 million years ago (mya) (Coates and Obando 1996). Genetic data therefore suggest that *E. cornigera* and *E. timida* speciated over 3 mya.

During the Messinian salinity crisis (5.6–5.33 mya), the Mediterranean was intermittently cut off from the world’s oceans and became highly desiccated (Clauzon et al. 1996; Hsü et al. 1973; Krijgsman et al. 1999). An ancestor of *Elysia timida* may have colonised the Mediterranean after the Zanclean flooding, an explosive inundation of the basin by Atlantic waters (Blanc 2002; Garcia-Castellanos et al. 2009). During subsequent divergence, *E. timida* evolved long-term retention of chloroplasts, becoming that rare anomaly: a highly photosynthetic animal. However, despite prolonged isolation, *E. timida* and *E. cornigera* display enough morphological conservation to have been placed in synonymy. Occupying a similar niche (*Acetabularia* feeder) may impose stabilising selection and impede divergence in appearance and radular characters, necessitating a careful consideration of other characters.

Within each species, genetic subdivision indicated limited gene flow over small distances. In *E. cornigera*, populations in Florida and the central Bahamas (~600 km distance) were substantially isolated. Spanish populations of *E. timida* separated by only ~80 km of coast were highly differentiated, showing deep phylogeographic structure in the COI gene tree and exceptional isolation by pairwise *θ*ST. Such divergence in the absence of an obvious dispersal barrier suggests that a persistent feature of the environment may retard gene flow, either by differential selection (an ecological mechanism) or oceanographic transport. The Palmiõs (~ Fonera) submarine canyon incises the continental margin close to shore along the region of coast separating the divergent Spanish populations of *E. timida* (Palanques et al. 2005). Coastal waters extending ~150 km north of the canyon tend to be colder and fresher than those farther north or south of the canyon (Palanques et al. 2005); persistent environmental differences on either side.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>2</td>
<td>0.003</td>
<td>0.277</td>
<td>0.7582</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>0.635</td>
<td>61.160</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Species × day</td>
<td>1</td>
<td>0.424</td>
<td>42.836</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Residual</td>
<td>156</td>
<td>0.010</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Comparative photosynthetic activity of *Elysia timida* versus *E. cornigera*

Results of an analysis of covariance comparing photosynthetic yield over 11 days of starvation; data were arcsine(square-root) transformed before statistical analysis. d.f. = degrees of freedom, MS = mean square.

![Fig. 3. Duration of chloroplast retention in *Elysia timida* (diamonds) versus *E. cornigera* (squares). Data are mean yield values ± one standard deviation from PAM measurements of ten specimens of each species held without food. The decay in yield was modelled using the best-fit curves shown for *E. timida* (dashed, cubic) and *E. cornigera* (solid, linear).](image)
of the canyon may select against migrants and hasten genetic divergence. A deflection in the south-flowing surface current caused by the canyon may also contribute to population subdivision across this region, leading to larval retention on either side of the canyon. Further study of *E. timida* across the Mediterranean could determine whether phylogeographic boundaries are broadly consistent with current patterns or regional differences in temperature and salinity.

At even smaller spatial scales, populations ~45 km distant across the France–Spain border were also differentiated at the COI locus. An allozyme survey yielded comparable results for populations in southern Spain; allele frequencies were significantly different for coastal populations 120 km distant, and even less gene flow was inferred between open coast and lagoon populations 60 km apart (González-Wangüemert et al. 2006). Constraints on dispersal imposed by intra-capsular metamorphosis may result in a positive feedback loop, allowing local adaptation that puts immigrants at a competitive disadvantage, further reducing gene flow (Marshall et al. 2010). Divergent natural selection can thus lead to genetic differences between nearby sites that differ in their physical environment, such as enclosed lagoons versus open coastlines. Alternatively, local populations may be physically isolated by flow regimes such that they receive few immigrants, leading to divergence by genetic drift. Fine-scale sampling and ecological study would be necessary to unravel the link between selective landscape, oceanography and gene flow.

Regardless of the driving force, the high level of population subdivision in both *E. timida* and *E. cornigera* highlights the need to evaluate within-species genetic distances for integrative taxonomy. If specimens A and B are 10% divergent at a particular locus, is that typical of co-occurring individuals, of isolated populations or of sister species? The maximum divergence among conspecific lineages sets a lower limit on what can be considered a ‘species level’ genetic difference for closely related taxa, but such limits have rarely been defined and utilised in the taxonomic study of molluscs. Our data on species level genetic distances at three commonly studied loci should be of general utility for further studies of sacoglossan diversity and evolution. Ultimately, molecular characters should provide a quantitative framework for systematists to use in delineating what constitutes variation at the individual, population or species level for a given taxon.

**Developmental characters and dispersal dimorphisms**

Developmental characters, such as egg size, features of extra-embryonic yolk reserves or hatching and settlement behaviour of larvae are often informative but under-utilised characters in gastropod taxonomy (Collin 2005; Krug et al. 2007). The ECY of *E. timida* was described as bright yellow (Marin and Ros 1993). In contrast, ECY of *E. cornigera* is distinctively semi-transparent; in 28 other *Elysia* species studied to date, ECY was composed of opaque coloured granules (white, yellow, orange or red) (Krug 2009; and unpubl. data). Eggs were much larger in *E. cornigera* than the 70 µm egg diameter reported for *E. timida* by Rahat (1976) and Marin and Ros (1993). Although 70 µm is very small for a lecithotrophic ovum, some sacoglossans achieve lecithotrophy at exceptionally small egg sizes for marine heterobranchs (Hadfield and Miller 1987). However, Clark and Jensen (1981) stated that Rahat corrected the egg size for *E. timida* to 120 µm in an unpublished letter. Adding further confusion, Clark and Jensen listed *E. timida* as present in Florida, suggesting they were actually discussing *E. cornigera* but using Rahat’s revised data for *E. timida*. Egg size is thus either substantially greater (120 µm) or smaller (70 µm) in *E. timida* than in *E. cornigera* (105 µm), but clarification is needed for egg size in *E. timida*.

In both species, embryos develop through a veliger stage, with a variable proportion of larvae undergoing encapsulated metamorphosis within the egg mass and emerging as juvenile slugs (Marin and Ros 1993; Krug 2009). Encapsulated metamorphosis in *E. timida* was misinterpreted as ‘direct’ or ‘Type 3’ development, a widespread mistake in the opisthobranch literature (Rahat 1976; Marin and Ros 1993). Attribution of ‘direct’ development is incorrect when a fully formed veliger larva develops (Krug 2009). In marine heterobranchs, direct or ametamorphic development occurs in a few species that suppress most larval features; in the Sacoglossa, ametamorphic development is known only for *Limapontia senestra* (Thompson 1967; Chia 1971).

Both *E. timida* and *E. cornigera* express a mixture of intra-capular and post-hatching metamorphosis, a dispersal dimorphism common among lecithotrophic sea slugs (Marin and Ros 1993; Krug 2009). Dispersal dimorphisms were often misinterpreted as poecilology in the literature, but comprise only one ‘type’ of larval development (Krug 2009). In our study, most or all larvae of *E. cornigera* metamorphosed before hatching and quickly emerged from the egg mass. Encapsulated development time was comparable (15–19 days) between *E. timida* and *E. cornigera*, but release of newly metamorphosed juveniles from the egg mass took ~10 days longer for *E. timida* than for *E. cornigera* clutches held at similar temperatures (~20–24°C; Marin and Ros 1993; Krug 2009). Juveniles remain in the egg mass post-metamorphosis to consume ECY in other sacoglossans (Krug 2009), suggesting a link between greater maternal investment in ECY and a prolonged post-metamorphic period spent in the egg mass for *E. timida*.

Although a seasonal shift in dispersal patterns has been repeatedly asserted for *E. timida*, no data support this. Marin and Ros (1989) and Rahat (1976) report only encapsulated metamorphosis in *E. timida*. Marin and Ros (1992) reported in their abstract and discussion that development varied seasonally in *E. timida*, with all larvae undergoing encapsulated metamorphosis from November to March and all larvae hatching in October and April to June; however, no data on hatching or development were presented to substantiate this claim. Confoundingly, Marin and Ros (1993) stated in their results section that 100% of larvae metamorphosed within the egg mass (their ‘direct’ development) from December to April, yet their figures show 100% of larvae hatching and swimming (their ‘lecithotrophic’) from mid-February onwards. No methods or quantitative data were provided to support assertions about developmental trends. It thus remains unclear whether there is any seasonal pattern to the dispersal dimorphism in *E. timida*; we expect that both pre- and post-hatching metamorphosis occur in some egg masses of *E. timida*, as in *E. cornigera* and other sacoglossans (Krug 2009).
Taxonomy

Family PLAKOBANCHIDAE Gray, 1840
Genus Elysia Risso, 1818, by monotypy
Elysia cornigera, stat. rev. Nuttall, 1989 (Figs 1, 2)

Types
Holotype (USNM 859144) and paratypes (USNM 859145) were designated by T.R. Nuttall in 1989, from Spanish Harbor on West Summerland Key, Florida, USA, and reside in the National Museum of Natural History, Smithsonian Institution.

Material examined
Re-description is based on live material corresponding to E. cornigera from Jamaica, three Bahamas islands, and two sites in the Florida Keys (Table 1); Sugarloaf Key is ~5 km from the type locality of Summerland Key, FL. Live specimens of E. timida were collected and examined from sites in the Mediterranean along the coasts of France, Spain and Croatia (Table 1).

Description
External anatomy. We supplement the anatomical description in Nuttall (1989). Body is small (to 8 mm). Outer colour of live animal is white to grey on parapodia and head, due to small (15 μm) and large (46 μm) white pigment granules on the epithelium. Numerous papillae give body an overall warty appearance (Fig. 1A). Inside lining of parapodia is densely enervated with green digestive diverticula ~30 μm across, conferring a light green tinge to outside of fed specimens where visible through white pigment. Foot, sides of head and anterior juncture of the parapodia are all green. Red granules (45 μm across; Nuttall 1989) dot head and rhinophores; smaller red spots are scattered over outer parapodial surface. Curled rhinophores widen medially, then taper to a point. Rhinophores are long relative to body size for an elysid, reaching up to 216 μm in length, although typically ranging from 120 to 160 μm. Teeth have three keels, or cutting edges. Two keels have serrated edges with denticles pointing forward; the unserrated keel is short and smooth. The tooth usually narrows to a pointed tip.

Radular anatomy. Nuttall (1989) reported 2–5 teeth on the ascending limb and 6–7 teeth on the descending limb, with >12 discarded teeth in the ascus. Teeth are unusually long for an elysid, reaching up to 216 μm in length, although typically ranging from 120 to 160 μm. Teeth have three keels, or cutting edges. Two keels have serrated edges with denticles pointing forward; the unserrated keel is short and smooth. The tooth usually narrows to a pointed tip.

Reproduction and development. Gelatinous egg spirals contain a string of 20–137 ova within a clear outer casing (Nuttall 1989; Krug 2009). Ova were reported as 105 μm in diameter (Nuttall 1989), but no data on variance within or among clutches were published. A ribbon of colourless, semi-transparent ECY is deposited inside the casing on the upper face of the spawn mass (Fig. 1D). Embryos develop into lecithotrophic veliger larvae over 15–19 days at ~22°C, but most or all larvae undergo intra-capsular metamorphosis and emerge as crawling juveniles soon after metamorphosis (Krug 2009). Larval shells measured 248.4 ± 4.8 μm at metamorphosis, the second smallest among Caribbean sacoglossans with lecithotrophic development (Krug 2009). Nuttall (1989) noted a distinctive extension of the shell aperture for veligers of E. cornigera.

Host use
This species has only been observed to feed on Acetabularia crenulata, and is normally affiliated with A. crenulata in the field. Laboratory observations confirmed that the utricles forming the ‘flower’ atop a stipe of Acetabularia are drained of cytoplasm one at a time until all are emptied. Nuttall (1989) states that feeding involved piercing one or two utricles at a time, with fecal material simultaneously ejected while feeding.

Distribution
Previously reported from the Florida Keys (Nuttall 1989) and Cuba (Ortea et al. 1997). The range is here extended to include the Bahamas and Jamaica.

Remarks
Despite similarities in appearance, E. timida and E. cornigera differ consistently in external morphology, development and behaviour. In general, Elysia timida is a larger species, ranging from 6–13 mm long, whereas E. cornigera is <8 mm. The parapodia, head and rhinophores of E. timida are almost pure white with red dots, in stark contrast to the deep green diverticula lining the insides of the parapodia (Fig. 1D, E). In contrast, the surface of the parapodia and head in E. cornigera is much more

Pericardium is rounded, white with red spots. Renopericardial extension, an opaque, white tube, extends from posterior end of pericardium for over half the body length, then narrows and descends into tissue of dorsum where it is surrounded by digestive diverticula. One pair of short dorsal vessels emerges laterally from pericardium just anterior to junction with renal extension. Vessels are opaque, covered in dense white spots, bending towards tail then forking at a point approximately even with posterior edge of elevated parapodial wing flap. From fork, finer vessels extend along anterior and posterior edge of parapodium with many lateral vessels branching off and extending up towards parapodial margin.

Radular anatomy. Nuttall (1989) reported 2–5 teeth on the ascending limb and 6–7 teeth on the descending limb, with >12 discarded teeth in the ascus. Teeth are unusually long for an elysid, reaching up to 216 μm in length, although typically ranging from 120 to 160 μm. Teeth have three keels, or cutting edges. Two keels have serrated edges with denticles pointing forward; the unserrated keel is short and smooth. The tooth usually narrows to a pointed tip.

Reproduction and development. Gelatinous egg spirals contain a string of 20–137 ova within a clear outer casing (Nuttall 1989; Krug 2009). Ova were reported as 105 μm in diameter (Nuttall 1989), but no data on variance within or among clutches were published. A ribbon of colourless, semi-transparent ECY is deposited inside the casing on the upper face of the spawn mass (Fig. 1D). Embryos develop into lecithotrophic veliger larvae over 15–19 days at ~22°C, but most or all larvae undergo intra-capsular metamorphosis and emerge as crawling juveniles soon after metamorphosis (Krug 2009). Larval shells measured 248.4 ± 4.8 μm at metamorphosis, the second smallest among Caribbean sacoglossans with lecithotrophic development (Krug 2009). Nuttall (1989) noted a distinctive extension of the shell aperture for veligers of E. cornigera.

Host use
This species has only been observed to feed on Acetabularia crenulata, and is normally affiliated with A. crenulata in the field. Laboratory observations confirmed that the utricles forming the ‘flower’ atop a stipe of Acetabularia are drained of cytoplasm one at a time until all are emptied. Nuttall (1989) states that feeding involved piercing one or two utricles at a time, with fecal material simultaneously ejected while feeding.

Distribution
Previously reported from the Florida Keys (Nuttall 1989) and Cuba (Ortea et al. 1997). The range is here extended to include the Bahamas and Jamaica.

Remarks
Despite similarities in appearance, E. timida and E. cornigera differ consistently in external morphology, development and behaviour. In general, Elysia timida is a larger species, ranging from 6–13 mm long, whereas E. cornigera is <8 mm. The parapodia, head and rhinophores of E. timida are almost pure white with red dots, in stark contrast to the deep green diverticula lining the insides of the parapodia (Fig. 1D, E). In contrast, the surface of the parapodia and head in E. cornigera is much more

Pericardium is rounded, white with red spots. Renopericardial extension, an opaque, white tube, extends from posterior end of pericardium for over half the body length, then narrows and descends into tissue of dorsum where it is surrounded by digestive diverticula. One pair of short dorsal vessels emerges laterally from pericardium just anterior to junction with renal extension. Vessels are opaque, covered in dense white spots, bending towards tail then forking at a point approximately even with posterior edge of elevated parapodial wing flap. From fork, finer vessels extend along anterior and posterior edge of parapodium with many lateral vessels branching off and extending up towards parapodial margin.
papillose, and the overall colour more grey. On *E. cornigera* the red spots are proportionally smaller. Only *E. cornigera* has large and numerous golden-brown spherical inclusions in its epithelium, often giving the live animal an overall brownish tint. The rhinophores of *E. timida* are smooth-edged, and end in a wide, rounded tip; in *E. cornigera* the rhinophores have an uneven edge caused by many extended papillae, and taper to a point. We did not observe *E. cornigera* expressing the characteristic ‘head bobbing’ motion of *E. timida*, in which the rhinophores contract and the body hunches forward rhythmically (up to once per second) when crawling (Thompson and Jaklin 1988; Wirtz and Anker 2009).

Both species have lecithotrophic development with ECY, but the ECY of *E. cornigera* is distinctively semi-transparent, whereas that of *E. timida* is bright yellow (Marin and Ros 1993). Egg size in *E. cornigera* (105 μm) is different from the two sizes reported for *E. timida*: 70 μm (Rahat 1976; Marin and Ros 1993) and 120 μm (M. Rahat, pers. comm. referenced in Clark and Jensen 1981). In both species, embryos develop through a veliger stage, with a variable proportion of larvae undergoing encapsulated metamorphosis within the egg mass and emerging as juvenile slugs. Encapsulated development time was comparable between *E. timida* and *E. cornigera*, but newly metamorphosed juveniles emerge from the egg mass ~10 days faster in *E. cornigera* (Marin and Ros 1993; Krug 2009).

Acknowledgements

We thank Heike Wägele for logistical and financial support, and Ángel Valdés for assistance with SEM and critical feedback. Anne DuPont, Enrico Schwabe and Cynthia Trowbridge provided specimens and collection information. Comments from three anonymous reviewers greatly improved the paper. Material was collected with permission of the host countries or states, including Special Activity Licence 07SR-1034 from the State of Florida, USA. This study was supported by awards to P. J. K. from the USA National Science Foundation program in Biological Oceanography (OCE 06–48606 and OCE 11–30072) and Systematics (award DEB-0817084). J. E. V. was supported in part by a postdoctoral fellowship from the Encyclopedia of Life.

References


Wirtz, P., and Anker, A. (2009). Range extension for Elysia timida (Opisthobranchia: Sacoglossa) to Sō Tomé Island (eastern central Atlantic), with a film showing the curious locomotion of the species. Marine Biodiversity Records 2, e144. doi:10.1017/S1755267209001171