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Integrative species delimitation in photosynthetic sea slugs reveals twenty candidate species in three nominal taxa studied for drug discovery, plastid symbiosis or biological control

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Abstract

DNA barcoding can highlight taxa in which conventional taxonomy underestimates species richness, identifying mitochondrial lineages that may correspond to unrecognized species. However, key assumptions of barcoding remain untested for many groups of soft-bodied marine invertebrates with poorly resolved taxonomy. Here, we applied an integrative approach for species delimitation to herbivorous sea slugs in clade Sacoglossa, in which unrecognized diversity may complicate studies of drug discovery, plastid endosymbiosis, and biological control. Using the mitochondrial barcoding COI gene and the nuclear histone 3 gene, we tested the hypothesis that three widely distributed "species" each comprised a complex of independently evolving lineages. Morphological and reproductive characters were then used to evaluate whether each lineage was distinguishable as a candidate species. The "circumtropical" *Elysia ornata* comprised a Caribbean species and four Indo-Pacific candidate species that are potential sources of kahalalides, anti-cancer compounds. The "monotypic" and highly photosynthetic *Plakobranchus ocellatus*, used for over 60 years to study chloroplast symbiosis, comprised 10 candidate species. Finally, six candidate species were distinguished in the *Elysia tomentosa* complex, including potential biological control agents for invasive green algae (*Caulerpa* spp.). We show that a candidate species approach developed for vertebrates effectively categorizes cryptic diversity in marine invertebrates, and that integrating threshold COI distances with non-molecular character data can delimit species even when common assumptions of DNA barcoding are violated.

Keywords

barcoding; *Caulerpa*; candidate species; *Elysia*; Heterobranchia; kahalalides; kleptoplasty; *Plakobranchus*; Sacoglossa; species delimitation

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Introduction

The pace of traditional taxonomic investigation can be slow relative to the need for accurate species delimitation, especially if a poorly studied taxon suddenly attracts attention due to its applied potential or interesting traits warranting basic study. DNA barcoding emerged as a promising approach to make species discovery faster and more quantitative for speciose groups like insects, or morphologically challenging marine invertebrates; however, it remains contentious whether barcoding will compensate for a diminishing pool of taxonomic experts or further erode expertise in morphological study (DeSalle et al., 2005, DeSalle, 2006; Hebert et al., 2010). Controversies also persist over whether mitochondrial DNA (mtDNA) lineages represent biologically "good" species that can be described using conventional characters (Blaxter, 2004; Abdo and Goulding, 2007; Nielsen and Matz, 2006; Pons et al., 2006). Recent advances in coalescent-based molecular taxonomy hold promise for delimiting species, but may require multilocus genetic data or suffer if rare species are inadequately sampled (Fujita et al., 2012; Lim et al., 2012). For marine invertebrates, range-wide samples may be challenging to obtain for widespread taxa, candidate species are often known from only one location or individual, and technical hurdles remain for obtaining multi-locus sequence data. The mitochondrial cytochrome *c* oxidase I (COI) gene thus remains the workhorse marker for barcoding and species delimitation efforts (Kelly et al., 2007; Grant and Linse, 2009; Plaisance et al., 2009).

Given the heavy reliance on COI datasets, it remains important to define the point at which COI lineages likely represent diagnosable species. Universal thresholds for distinguishing lineages from species have failed to emerge, making the practice of DNA barcoding more idiosyncratic than envisioned (Blaxter et al., 2005; Hebert and Gregory, 2005; Ward, 2009). In any taxon, rapid radiations may lead to an overlap between intra- and inter-specific genetic distances instead of the "barcoding gap" traditionally required for species delimitation (Beltrán et al., 2002; Hebert et al., 2004; Meyer and Paulay, 2005; Meier et al., 2006, 2008). Lineages can be treated as species hypotheses based on taxon-specific divergence thresholds, but hypothesis tests are necessary to justify taxonomic consideration of lineages as species. Recent work supports the value of identifying candidate species based on the concordance of mtDNA lineage divergence with nuclear gene genealogies, and characters drawn from morphology, ecology, reproduction or behavior (Blanquer and Uriz, 2008; Bucklin and Frost, 2009; Cardosa et al., 2009; Halt et al., 2009; Naughton and O"Hara, 2009; Vieites et al., 2009; Barrett and Freudenstein, 2011). However, DNA barcoding has yet to bridge the gap with alpha taxonomy for marine invertebrates, despite adoption by large-scale marine biodiversity inventories (e.g. Census of Marine Life; O'Dor, 2004). Fewer than 10% of known marine species have been barcoded in the Arthropoda, Mollusca and Annelida, which account for over half of marine species, yet a third of marine chordates were barcoded (Bucklin et al., 2011). Key barcoding assumptions also remain untested for most marine taxa, including (1) intra-specific divergence at COI is rarely >2%, and (2) widespread marine animals will show less phylogeographic structure than terrestrial taxa, given the high dispersal potential of planktonic larval stages and/or pelagic adults (Hebert et al., 2010).

Here, we test the utility of barcoding approaches to identify candidate species in a group of marine heterobranchs. Sea slugs lack many shell characters of other gastropods, contributing to an unstable taxonomy and cycles of splitting and lumping; cryptic diversity is likely rampant within most groups, particularly those including "circumtropical" species (Wägele and Klussmann-Kolb, 2005; Gosliner et al., 2008; Jörger et al., 2012). Sacoglossa is a clade of herbivorous sea slugs long studied for their retention of diet-derived chloroplasts (Kawaguti, 1941; Kawaguti and Yamasu, 1965; Greene, 1970; Händeler et al., 2009; Pierce and Curtis, 2012). Some have a fast molecular clock and high intra-specific COI diversity,

possibly due to solar irradiance of exposed body tissues and/or mutagenic radicals released by photosynthetically active plastids (Ellingson and Krug, 2006; Krug et al., 2011; Vendetti et al., 2012). These slugs therefore present a useful contrast with animal groups studied in past barcoding efforts, which generally had low divergence among conspecific COI lineages.

We focused on three putative species complexes, based on their respective importance for drug discovery, studies of plastid symbiosis, and biological control. The “circumtropical” *Elysia ornata* (Swainson, 1840) and related species are sources of kahalalides, anti-cancer drug prospects (Hamann et al., 1996; Horgen et al., 2000; Ashour et al., 2006). The Indo-Pacific *Plakobranchus ocellatus* van Hasselt, 1824 has long been studied for kleptoplasty, the ability to sustain functional algal plastids for months after consumption, and remains a model for studies of early-stage endosymbiosis; recent attention has focused on the role of horizontal gene transfer in long-term plastid maintenance in three species, including *P. ocellatus* (Pelletreau et al., 2011; Wägele et al., 2011; Maeda et al., 2012; Pierce and Curtis, 2012; Pierce et al., 2012). Finally, a putative complex of species collectively termed *E. tomentosa* Jensen, 1997 feeds on chemically defended green algae in the genus *Caulerpa*; some complex members have been proposed as biological control for the invasive aquarium strain of *C. taxifolia* (Coquillard et al., 2000; but see Trowbridge et al., 2012). In all three cases, original descriptions lacked detail, and later taxonomists considered variation in external and radular morphology to represent intra-specific polymorphism (Risbec, 1953; Marcus; 1980; Jensen, 1992).

To test the hypothesis that each “species ” comprised a complex of distinguishable taxa, we applied an iterative approach for delimiting candidate species that has been widely used in biodiversity inventories of terrestrial vertebrates, but not previously applied to marine invertebrates (Vietes et al., 2009; Yeats et al., 2010). For each complex, specimens were barcoded using the front half of COI, and two procedures were used to identify a genetic-distance threshold for delimiting potential candidate species. We then used characters from morphology and development, and allelic variation at the nuclear H3 locus, to distinguish deep conspecific lineages from candidate species. Our results show that species richness in key sea slug groups may be underestimated by an order of magnitude. Further, we show that integrative practices developed for species delimitation of vertebrates can be productively applied to marine invertebrates, including groups in which common assumptions of barcoding studies are invalid.

Materials and Methods

Collection and taxonomy of study organisms

As part of broader efforts to delineate sacoglossan biodiversity, we sampled green algae in the genera *Bryopsis* and *Caulerpa*, respective hosts of the *E. ornata* and *E. tomentosa* complexes (Händeler et al., 2009; Trowbridge et al., 2010). Algae were collected by SCUBA or snorkeling from visited field sites; small slugs were removed in the laboratory, while large specimens of *Elysia* and all *Plakobranchus ocellatus* were collected *in situ* from rocky or sandy substrata. Live specimens were held in aquaria to obtain egg masses, from which the following reproductive characters were recorded: larval development mode (planktotrophic or lecithotrophic), and the pattern and color of extra-capsular yolk (ECY) deposits for *Elysia* spp. (no ECY occurs in *Plakobranchus*) (Krug et al., 2007; Krug, 2009). Such reproductive characters have species-diagnostic value in sacoglossans, and changes may be associated with premating isolation (Ellingson and Krug, unpublished data). After reproducing, slugs were relaxed in $MgCl_2$ isotonic with seawater and photographed. The number and pattern of raised vessels lining the inside of the parapodial flaps was ntoed, and specimens were scored for presence/absence of a pointed tail, color and shape of

rhinophores (anterior sensory extensions), relative height of parapodial side-flaps, and color, shape and texture of parapodial sides and margins. Samples were preserved in 95-100% ethanol. Preserved specimens and any accompanying collection notes or photographs were also obtained from colleagues and museum collections (Table 1).

We focused on three clades within the Plakobranchoidea that were suspected to contain unrecognized species based on circumtropical distributions and/or the taxonomic history of each complex, briefly summarized below. Specimens were collected from sites bounding the western tropical Pacific basin to the east (Hawaii, Palmyra Atoll, Moorea), north (Japan), west (Thailand, Philippines), and south (Australia, Vanuatu), as well as central Pacific locations (Guam, Saipan, New Guinea). Although the range of each nominal species was unknown, sampling sites spanned most of the tropical Pacific and were expected to encompass range boundaries for most taxa (with the exception of western range edges in the Indian Ocean, from which samples and biogeographical information were not available). Caribbean sites were also surveyed for taxa in the two *Elysia* species complexes; *Plakobranchus* does not occur outside the Indo-Pacific.

Elysia ornata—Three large *Elysia* spp. feeding on *Bryopsis* spp. were initially named in tropical oceans, with a black band along the parapodial edge and a submarginal orange band. The description of *E. ornata* (Swainson, 1840) and re-descriptions (Verrill, 1901; Marcus, 1980) were from the Caribbean. A similar Indo-Pacific species, named *Elysia marginata* (Pease, 1860, 1871) after several rounds of systematic transfer, has a white band between the orange and black marginal bands. Another similar species, *E. grandifolia* (Kelaart, 1858) from Sri Lanka, was described as having parapodia that joined with the tail posteriorly, rimmed by black and golden-yellow lines; later authors disagreed on whether *E. grandifolia* differed from *E. ornata* by having denticulate (minutely serrated) radular teeth (Eliot 1904, 1908). Both *E. marginata* and *E. grandifolia* were synonymized with *E. ornata* by workers who found no consistent differences between Pacific and Caribbean material (Marcus, 1980; Heller and Thompson, 1983; Jensen, 1992). A related but morphologically distinct species, *E. rufescens* (Pease, 1860, 1871) was the original source of anti-cancer compounds called kahalalides, which have subsequently been reported from Indo-Pacific "*E. ornata*" (Hamann et al. 1996; Horgen et al. 2000; Ashour et al. 2006). Finally, *E. kushimotoensis* Baba, 1957 was described from Japan but presumed to be conspecific with *E. rufescens* by subsequent workers (Trowbridge et al., 2011a,b); however, this hypothesized synonymy has not been tested.

Plakobranchus ocellatus—The genus *Plakobranchus* is currently monotypic, with *P. ocellatus* described from Indonesia. At least 12 other species were named in the 1800s but were later synonymized by Bergh (1887), Risbec (1953), and Jensen (1992), due to a lack of apparent anatomical differences. However, several authors noted distinct color morphs in Japan that might represent candidate species (Yamasu and Adachi, 1990; Adachi, 1991; Yamasu, 1997; Hamatani, 2000; Hirano et al., 2005; Ono, 2005; Trowbridge et al., 2011a,b).

Elysia tomentosa—Members of the *E. tomentosa* complex are large slugs with wide parapodial flaps and an elongated renopericardial complex; except for *E. pratensis*, all specimens in this complex fed on *Caulerpa* spp., including invasive strains of *C. taxifolia* and *C. racemosa* (Baumgartner et al., 2009). Two described Indo-Pacific species, *E. expansa* (O'Donohue, 1924) and *E. tomentosa* (Jensen, 1997), differ in that *E. expansa* has a black line along the parapodial marginal. Two morphologically distinct species occur in the Caribbean, *E. subornata* (Verrill, 1901) and *E. pratensis* (Ortea and Espinosa, 1996). Perusal of the Sea Slug Forum (http://www.seaslugforum.net) suggests *E. expansa* has been informally synonymized with *E. tomentosa*, together with at least six other morphotypes.

Phylogenetic analyses

Genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen; Valencia, CA) and stored in extraction buffer at −20°C. Polymerase chain reactions (PCR) amplified two gene regions: a 658 base pair (bp) fragment of the mitochondrial COI gene, using primers LCO1490 and HCO2198 (Folmer et al., 1994), and a 328 bp region of the nuclear histone 3 (H3) gene (Colgan et al., 2000). Reaction conditions were as described in Vendetti et al. (2012). Amplification success was 90% for the COI locus and 95% for H3 (Table 1). Purified PCR products were directly cycle-sequenced in both directions using PCR primers and Big Dye Terminator 3.1 Cycle Sequencing chemistry at the High-Throughput Genomics Unit, University of Washington or on an ABI PrismTM 377 DNA Sequencer (Applied Biosystems). Chromatograms were edited and primer sequences removed in GeneiousPro 4.8 software, and alignments generated using default settings in ClustalX (Thompson et al., 1997). Additional COI haplotypes (n = 9) from *Plakobranchus ocellatus* were downloaded from the NCBI database. Most specimens were homozygous at the H3 locus, and inference of allelic phase was straightforward for the few heterozygotes as their alleles differed at only one position. Histone 3 alleles for *E. subornata* and *E. pratensis* were inferred from range-wide population genetic surveys of both species (Rodriguez, 2009). Sequences were deposited in the National Center for Bioinformatics (NCBI) database and accession numbers listed in Table 1.

Evolutionary relationships were inferred separately for the COI haplotypes and H3 alleles of each species complex, using Bayesian Markov-chain Monte Carlo (MCMC) methods. Analyses were run with designated outgroups identified from the three-gene phylogeny in Handeler et al. (2009) or subsequent analysis of a four-gene dataset (Vendetti and Krug, unpublished). Because *Plakobranchus* is monotypic, a basal clade in the sister taxon *Thuridilla* was used as the outgroup (Händeler et al., 2009). Ends of shorter sequences were coded as missing data for phylogenetic analyses. Mixture models of sequence evolution were implemented in the software package BayesPhylogenies, which uses a likelihood criterion to assign the best-fit model to each position in the data alignment; mixture models better capture among-site heterogeneity in mutation rates and base frequencies than conventional partitioning by gene or codon position (Pagel and Meade, 2004). For COI gene trees, two GTR + Γ models with separate base frequencies were parameterized during runs, with four rate multipliers to accommodate among-site heterogeneity; adding a third model did not improve likelihood scores or alter tree topology for any of the three species complexes. One GTR + Γ model was sufficient to model evolution at the H3 locus.

Following Pagel and Meade (2004), four independent Markov chains were run for 5×10^6 generations, saving a tree every 10^3 generations. Log-likelihood scores and model parameter estimates were inspected in Tracer v1.4 (Rambaut and Drummond, 2007) to confirm that individual chains reached stationarity. From the final 10^3 trees of each run, we calculated the (a) harmonic mean of log-likelihood scores, (b) clade support values, and (c) 50% majority-rule consensus tree in BayesTrees (http://www.evolution.reading.ac.uk). For each marker and species complex, all runs converged on equivalent topologies and likelihood scores; the final 10^3 trees of the four runs were therefore pooled, and a consensus tree was generated with mean branch lengths. Posterior probabilities (PP) ≥90% were taken as statistical support that a node was present on the true gene tree (Huelsenbeck and Rannala, 2004; Simmons et al., 2004). For each complex, consensus trees for the COI and H3 loci were compared to determine if the same samples formed supported and distinct clades on each tree, and whether the gene trees were topologically congruent. Trees and alignments were deposited in TreeBASE (www.treebase.org). Photographs of exemplar candidate species were posted to the LifeDesk platform for Sacoglossa (http://sacoglossa.lifedesks.org) using the provisional names assigned in this study.

Delimiting candidate species

An integrative approach was used to determine whether COI clades corresponded to unnamed candidate species or conspecific lineages (Vieites et al., 2009). This approach uses concordance between mtDNA lineages and morphology or reproductive characters to distinguish species, while relying on genetic divergence to identify candidate species for which other forms of data are unavailable. Nuclear sequence data can be incorporated into this framework either as evidence that syntopic mitochondrial lineages share nuclear alleles and may be interbreeding, or alternatively as evidence that specific alleles distinguish candidate species. A confirmed candidate species (CCS) is a genetically divergent mtDNA lineage (no threshold distance implied) that either has a diagnostic morphological or reproductive characteristic, or co-occurs with related species without interbreeding (Vieites et al., 2009); thus, a CCS shows at least some genetic differentiation, a diagnosable trait difference, and either geographical or reproductive isolation from close relatives.

For each of our three putative species complexes, we classified each supported clade on the COI gene tree as a CCS if its members shared one or more diagnostic morphological or reproductive features. Morphological characters were chosen that typically vary little within species, but differ among established species of sacoglossans (Supplemental Table 1). As traits associated with reproduction and development are usually species-specific and may be associated with reproductive isolation, we also scored larval type (planktotrophic or lecithotrophic) as well as the color and shape of extra-embryonic yolk reserves. Distinct H3 alleles sampled in sympatric lineages was taken as evidence against admixture.

Two remaining categories of Vieites et al. (2009) distinguish sampled lineages from other described or candidate species in a complex, using a threshold genetic distance that distinguishes well-defined species from their nearest relatives. Lineages above this threshold are classified as unconfirmed candidate species (UCS) if no other data are available. If sister lineages differ by more than the threshold amount but have no concordant differences in morphology or evidence of isolation (reproductive or geographic), they are classified as deep conspecific lineages (DCL) that reflect the phylogeographic history of a species. This flexible approach can thus recognize young but diagnosable species, without separating ancient yet conspecific mitochondrial lineages.

We used two approaches to determine an appropriate threshold genetic distance for identifying UCS and DCL in each species complex. We first implemented a recursive analytical procedure, Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), which estimates the genetic distance corresponding to the difference between a speciation process versus a coalescent (intra-specific) process of sequence evolution. Using the authors' web interface, a matrix of Tamura-Nei corrected distances was input for each ingroup taxon. Runs were performed using the default range of priors (pmin = 0.001; pmax = 0.10) which bound the range of possible conspecific distances; adjusting pmax within the range of intra-specific variation observed in other sacoglossans did not affect the number of species estimated from our data. Additional COI sequence data were included in the ABGD analysis of the *E. tomentosa* complex for *E. subornata* (n= 21 haplotypes) and *E. pratensis* (n= 17 haplotypes), a subset of the data from Rodriguez (2009). The ABGD procedure was not performed on H3 datasets as most species were represented by only one or two alleles, and ABGD fails to estimate accurately the number of species when few representative sequences are available (Puillandre et al., 2012). Vieites et al. (2009) recommend thresholds based on minimum distances to well-recognized and described species. We therefore also determined the minimum COI distance between a described, morphologically distinctive species in each complex and its closest relative. This distance, which we term the "Relative of Established Species Threshold" (REST), is a cutoff representing the lowest inter-specific distance.

To compare distances within and among clades to ABGD and REST cutoffs, minimum between-clade and maximum within-clade pairwise genetic distances were calculated in Mega 5.0 for both COI and H3 loci (Meier et al., 2008; Tamura et al., 2011). After clades were classified as conspecific lineages or candidate species, frequency distributions of all pairwise COI distances within each complex (excluding outgroup taxa) were plotted to visualize the gap between intra- and inter-specific distances. Multiple substitutions among ingroup sequences were corrected when calculating genetic distances using the Tamura-Nei model (TrN), the best-fit model identified using jModelTest (Posada, 2008) that was also available in Mega 5.0. Although species delimitation studies often report uncorrected p-distances when related taxa show little divergence (especially if a more conserved locus such as 16S was studied), inter-specific COI distances in sacoglossans are typically 10-20%, necessitating a model correction for saturated sites (Krug et al., 2011). To test formally for population genetic subdivision within species, an analysis of molecular variance (AMOVA) was performed on COI haplotypes for select CCSs in Arlequin 3.5 (see Results). The proportion of TrN-corrected genetic distances that was due to among-population differences (Φ_{ST}) was estimated, with significance based on 10^4 permutations of the data (Excoffier et al., 2005).

Results

Elysia ornata *complex*

Every specimen (n = 31) had a unique COI haplotype. The morphologically distinctive *E. rufescens* was also recovered as genetically distinct from the *E. ornata* complex on the Bayesian consensus phylogram (Fig. 1). Japanese material matching the description of *E. kushimotoensis* was genetically conspecific with *E. rufescens* from Hawaii (<0.01 TrN distance), supporting their presumed synonymy. Nominal "*E. ornata*" sequences belonged to one of six divergent COI lineages, including five clades with high support and one divergent haplotype from Japan (Fig. 1A). Caribbean samples formed a clade representing the true *E. ornata*, based on proximity to the type locality. An ABGD analysis detected five distinct ingroup clusters aside from *E. rufescens* and *E. ornata*, and set 3.6% as the upper bound for intra-specific divergence. However, the REST distance for *E. ornata* and its closest relative was 8.1%, consistent with the distribution of pairwise differences among ingroup sequences which supported a threshold of 8% for species-level divergence (Table 1A, Fig. 2A).

Sister to *E. ornata*, we recovered an unsupported group of two mtDNA clades that were >7.5% divergent. The clades had fixed differences in morphology and egg mass traits, and co-occurred in Guam, and were thus considered CCS. As the oldest available name for Indo-Pacific "*E. ornata*"-type slugs was *E. marginata*, these CCS were provisionally termed "*E.* cf. *marginata* sp. 1" and "sp. 2" (Fig. 1A). Maximum intra-specific distance at COI was 0.8% for "sp.1", sampled from Guam and Moorea. Sister to "sp. 1" was a lineage recovered with marginal support (PP = 0.87), which was divided into two highly supported subclades that were maximally 5.9% divergent but co-occurred in Japan (Fig. 1A).

A third candidate species ("*E.* cf. *marginata* sp. 3") comprised specimens from Japan, Moorea, and Palmyra Atoll in the central North Pacific (Fig. 1A). Within "sp. 3", four haplotypes from Moorea were monophyletic with high support (PP = 1.0). An AMOVA revealed significant differentiation among sampled populations of "sp. 3" (Φ_{ST} = 0.6038; P = 0.0005). In pairwise Φ_{ST} comparisons, Moorea was significantly different from Japan (Φ_{ST} = 0.657; P = 0.007) and Palmyra Atoll (Φ_{ST} = 0.775; P = 0.023), but Japan and Palmyra Atoll populations were not differentiated despite being ~7,000 km distant (Φ_{ST} = 0.219; P = 0.108). The maximum pairwise TrN distance between "sp.3" haplotypes was 1.1% (Table 1A). The remaining CCS, "*E.* cf. *marginata* sp. 4", was represented by a single

specimen from Sobe, Japan; “sp. 4” was morphologically distinct and ~12% divergent from all other taxa.

Histone 3 alleles from each of the six major mtDNA clades (*E. ornata, E. rufescens*, and the four CCS) also formed supported clades in BI analyses (Fig. 1B). Topology of the H3 consensus phylogram was not congruent with the COI gene tree, but as most internal nodes were unsupported on the COI tree, neither was there significant conflict. Diversity was lower at the nuclear H3 locus; four alleles were recovered from Caribbean specimens of *E. ornata*, while each species from the Pacific was represented by 1-3 alleles.

As suggested by the COI gene tree, *E. ornata* formed a supported clade (0.99) with “sp. 1” and “sp. 2” in the H3 analysis (Fig. 1B). Two H3 alleles differing at one position were sampled from *E.* cf. *marginata* “sp. 1”, while three alleles from “sp. 2” formed a supported clade (0.93). One H3 allele was fixed in the specimens of “sp. 2” from Guam, Vanuatu and Japan (10Jpn01) that comprised one COI subclade; specimens representing the other COI subclade (08Jpn01,02) were fixed for a distinct H3 allele differing at two positions. A third, more divergent H3 allele was recovered from a specimen from Hawaii for which COI failed to amplify (Fig. 1B). Within a species, alleles were often shared between populations in Japan and distant sites such as Hawaii (*E. rufescens*), Guam and Vanuatu (sp. 2), and Australia and Palmyra atoll (sp. 3). However, no alleles were shared between candidate species, indicating distinct nuclear gene pools. Maximum divergence of H3 alleles within *E. ornata* was 0.9%, the same as the minimum distance between *E. ornata* and “sp. 1” similarly, intra-specific divergence of “sp. 2” alleles was equal to the minimum distance between alleles from “sp. 1” and “sp. 2” (Table 3A). Thus, there was no barcoding gap at H3, although most interspecific distances were >2%.

Morphological characters supported the designation of four CCS in the *E. ornata* complex, including color of the rhinophores (antenna-like sensory structures) and bands running along the parapodial margin, folding of parapodia into siphonal openings, tail shape, and the number and arrangement of dorsal vessels emerging from the renopericardial organ (Fig. 1; Supplemental Table 1). For instance, in contrast to the thin parapodial margin of *E. ornata* (Fig. 1C), most specimens of “sp. 1” had parapodia with thick edges that formed three distinct siphonal openings, and lacked a pointed tail (Fig. 1F). One specimen from Guam had low parapodia (Fig. 1G) and was provisionally identified as a distinct species, but this hypothesis was rejected by molecular analysis; the low-parapodia specimen nested within “sp. 1” in the COI gene tree, and shared the same H3 allele as all other specimens of “sp. 1”, and was therefore a case of intra-specific polymorphism. In contrast, the genetically distinct CCS “sp. 2” had red-tipped rhinophores, thin parapodia, and a pointed, red-edged tail (Fig. 1I; Supplemental Table 1). Morphology did not distinguish the two subclades of “sp. 2” that were split by ABGD analysis; these subclades were also below the 8% REST distance for the *E ornata* complex, and were thus considered conspecific lineages. Morphology also distinguished “sp. 3” (darker body pigmentation, ruffled parapodial margin) and “sp. 4” (thick white submarginal band, enlarged anterior and reduced posterior siphonal openings).

Egg mass characters further distinguished CCS, which differed in the color and arrangement of extra-capsular yolk (ECY) reserves distributed around their planktotrophic embryos. Clutches laid by *E. ornata* from Jamaica and Curacao contained regularly spaced blobs of white ECY (Fig. 1D). In contrast, all specimens of “sp. 1” from Guam (n = 7) laid egg spirals containing a continuous thread of black ECY that gradually broke up into shorter line segments and progressively faded to brownish-grey and then white by the end of the egg strand (Fig. 1H). The low-parapodia morph of “sp. 1” also laid eggs with black ECY, congruent with molecular data supporting its conspecificity with the high-parapodia morph. Egg spirals of “sp. 2” from Guam had regularly spaced blobs of bright yellow ECY (Fig.

1J), while ECY was darker gold in “sp. 3” and orange in “sp. 4” (Supplemental Table 1). Reproductive characters were thus congruent with morphology and both mitochondrial and nuclear gene trees, indicating mtDNA lineages were diagnosable as distinct taxonomic units.

Plakobranchus ocellatus *complex*

At the COI locus, 34 out of 35 specimens yielded unique haplotypes, with one haplotype shared by two specimens from Moorea. Bayesian analysis of COI data revealed 10 genetically distinct lineages, and most internal nodes were supported on the COI gene tree (Fig. 3A). For all lineages sampled more than once, haplotypes formed a supported clade within which the mean COI divergence was <2%, despite the large geographical distances separating some populations (e.g., Hawaii versus Australia). An ABGD analysis of the COI data suggested 10 candidate species of *Plakobranchus*, returning a threshold intra-specific distance of 3.6% (Table 2B). As *Plakobranchus* is monotypic, REST comparisons were performed among basal species in the sister group *Thuridilla*. The minimum inter-specific distance between recognized species was 5.5% for *T. picta* (Caribbean) and *T. hopei* (Mediterranean), while all other species differences were >8.8% (Table 2C). Similarly, the minimum pairwise distance between *Plakobranchus* clades was 6.0%, although inter-clade distances were >8% for all but two comparisons (Table 2B, Fig. 2B).

Six COI lineages were classified as CCS based on genetic divergence, morphology and/or development, and syntopy. A further four lineages were >8% divergent from their sister lineage but no morphological data were available, so were classified as UCS. Five CCS co-occurred in Japan and were distinguished by the size, color and distribution of ocelli, characteristic ringed spots on the head, foot and parapodia. Full details on the morphology and development of these CCS, termed “white”, “black”, “purple”, “spotless”, and “blue”, will be reported elsewhere. One supported clade (PP = 0.99) comprised the CCS “white” and “black,” plus three UCS. “White” was the most widely sampled taxon, occurring in Australia, Guam, Japan, the Philippines, and Thailand; specimens had a single row of large black-ringed, orange ocelli along the body margin, and dark ocelli ringed with white on the ventral surface of the foot. The CCS “black”, sampled only in Japan, was superficially similar and sister to “white” (PP = 0.97). However, “black” had larger ocelli on the foot, and was the only lineage that produced lecithotrophic larvae. Sister to (white + black) was the widely distributed UCS “*Plakobranchus* sp. 2” haplotypes from Hawaii, Australia and the Philippines were a maximum 3.3% divergent. The UCS “sp. 1” and “aff. sp. 1” were sister taxa (PP = 1.0) that were a minimum 8.7% distant, but were sampled only 1-2 times apiece.

The remaining *Plakobranchus* COI haplotypes formed an unresolved polytomy. “Purple” and “spotless” were sympatric in Japan and 8.0% distant at COI. Specimens of “purple” lacked ocelli on parapodia, had faint purple spots with dark centers on the foot, and a purple reticulation on the body margin and rhinophores; “spotless” slugs had a few dark ocelli inside clear rings on otherwise uniformly pigmented parapodia. The morphologically distinctive CCS “aff. purple” was closely related to “spotless” (6.0% minimum divergence), but had parapodia and rhinophores densely peppered with small orange spots; “aff. purple” was sampled from Guam and Moorea but was not found in Japan despite extensive sampling. The CCS “blue” was sampled in Japan, Guam and Vanuatu, with a maximum pairwise distance of 3.2% between geographically distant haplotypes; specimens had dark orange ocelli with a light orange center on the parapodia and head, and tiny orange spots on the foot. The UCS “sp. 3” was represented by a haplotype from the NCBI database.

Allelic differences at the nuclear H3 locus distinguished seven out of nine candidate species of *Plakobranchus* for which data were available (Fig. 3B, Table 3B). One H3 allele (#7) was shared by four “aff. purple” and one “spotless” specimens, the closest relatives by COI distance. Allele #4 was fixed in all “white” and “black” specimens (n = 4 black, 7 white).

Although there was no barcoding gap at H3, minimum H3 distances between most candidate species of *Plakobranchus* (0.6-1.5%) were comparable to H3 distances between recognized *Thuridilla* spp. in the *T. livida* clade (Fig. 3B, Table 3C).

Elysia tomentosa *complex*

Phylogenetic relationships of COI haplotypes were not well resolved by Bayesian Inference within the *E. tomentosa* complex. Nominal outgroup species *E. setoensis* and *E. atroviridis* were not genetically distinguishable (<1% different at the COI locus), forming a lineage >14% divergent from ingroup taxa (Fig. 4A, Table 2D). An ABGD analysis of the COI data distinguished nine clusters, including six candidate species, and returned a threshold of 6.0% for species delimitation. Morphologically distinct Caribbean species *E. pratensis* and *E. subornata* were sister on the COI gene tree, and were a minimum 9.4% divergent; the distinctive but undescribed "*Elysia* sp. 22" from Australia was >15% divergent from all other lineages. The distribution of pairwise distances among COI clades was trimodal, with discontinuous peaks corresponding to inter-specific distances from 8-12% and 14-22%. All data were therefore consistent with a REST value of 8%, the same threshold inter-specific distance as used for the *E. ornata* complex.

The remaining samples comprised six divergent COI lineages, with the minimum distance between a lineage to its nearest relative ranging from 8.6 – 22.9% (Table 2D). Each lineage also had distinctive morphological or developmental features, supporting six CCS in this complex which were termed "*E.* cf. *tomentosa* sp. 1-6". Allopatric sister taxa "sp. 2" (Caribbean) and "sp. 5" (Indo-Pacific) were a minimum 9.1% divergent, while conspecific isolates of "sp. 5" from Australia and Thailand only differed by 0.6%. Both "sp. 2" and "sp. 5" were distinct from all other species, having wide parapodial margins with heavily papillose surfaces and an overall brown coloration; however, larval development for "sp. 2" was lecithotrophic, whereas "sp. 5" was planktotrophic (Supplemental Table 1). Three of the remaining four CCS were sampled only in Japan, and differed in the extent to which parapodia were papillose or laterally undulating (Supplemental Table 1). The final taxon, "sp. 6" from Guam and Australia, had distinctively thickened parapodia held open by the living animal; specimens from Guam and Australia were less than 0.5% divergent.

The H3 gene tree was similar in topology but better resolved than the COI tree (Fig. 4B). Sister relationships were supported for (*E. subornata* + *E. pratensis*) and (sp. 2 + sp. 5), and the sister relationship (sp. 3 + sp. 6) was recovered with borderline support (0.87). The H3 tree also placed "sp. 1" as sister to (sp. 3 + sp. 6), and recovered "sp. 4" as sister to (*E. subornata* + *E. pratensis*). Conspecific H3 alleles were a maximum 1.5% divergent in both *E. subornata* and "sp. 5", whereas most pairwise distances between species were over 1.5%. However, one H3 allele (cf. tom #2) was shared by four specimens of "sp. 3" from Japan and one Japanese specimen identified as "sp. 1" by mtDNA (Fig. 4B). Allele "cf. tom #2" differed at only one position from allele #8, which was homozygous in the specimen of "sp. 6" from Guam (Table 3D).

Discussion

Integrative delimitation of candidate species in photosynthetic sea slugs

Molecular approaches to species discovery have documented unrecognized diversity in an array of marine invertebrates, but were not often tied to parallel studies of morphological, reproductive or ecological characters (Fukami et al., 2004; Gómez et al., 2007; Blanquer and Uriz, 2008; Bucklin and Frost, 2009). The candidate-species approach offers a framework for delimiting evolutionarily independent units by integrating metrics of lineage divergence with characters of taxonomic value, and traits linked to reproductive isolation (Vieites et al.,

2009). A strength of this approach is that divergence thresholds are secondary to trait-based discrimination among species. Lineages that are more divergent than a chosen threshold may be considered conspecific if they co-occur without morphological differences or reproductive isolation; conversely, less divergent lineages are considered candidate species if they are consistently distinguishable by a valid character. Genetic thresholds chiefly serve to define UCS, divergent specimens for which character data are lacking but that are likely to be distinct species based on mtDNA genealogy.

Despite its potential utility, the framework and terminology of Vieites et al. (2009) have not been previously used to delimit marine invertebrate species. In this study, we documented 20 CCS currently masked under three names in the Sacoglossa, a clade of herbivorous sea slugs. Just as the slugs themselves lack discrete characters or hard parts, many species descriptions lack relevant details and associated type specimens, resulting in taxonomic instability. Recent work on marine heterobranchs showcased how molecular characters can be incorporated into an integrative framework for describing species or resurrecting names lost to synonymy (Krug et al., 2007, 2008; Stout et al., 2010; Ornelas-Gatdula et al., 2012; Pola et al., 2012). However, heterobranch taxonomy lacks a common framework for delimiting species and defining threshold genetic distances (Krug et al., 2011; Jörger et al., 2012; Vendetti et al., 2012). Our results defined threshold distances that effectively delimited all candidate species, based on subsequent examination of morphology and reproductive characters, and provide a useful framework for subsequent diversity surveys and taxonomic work (Table 4).

We used two approaches to define a threshold cutoff for candidate species of sacoglossans. The ABGD procedure set an upper limit on intra-specific variation at 3.6% (*E. ornata* + *Plakobranchus* complexes) or 6% (*E. tomentosa* complex). Vieites et al. (2009) recommend setting a threshold distance based on the nearest relative of a well-described species in the complex under scrutiny. Our “Relative of Established Species Threshold” or REST values were 6% in *Plakobranchus* and 8% in *Elysia*, and corresponded well with diagnostic differences in taxonomically useful characters (Table 4). Notably, the lower 6% threshold distinguished all CCS and UCS without subdividing any candidate species into deep conspecific lineages; even a more conservative 7.5% threshold delimited candidate species in all three complexes with a low error rate ($P < 0.004$).

The region between ABGD and REST cutoffs should correspond to the barcoding gap, but may include distances between divergent conspecific lineages that are separated by ABGD yet fall below the REST distance for candidate species (Fig. 2A, grey bars). Out of 17 described or candidate species with multiple COI haplotypes, ABGD separated only the subclades of “*E.* cf. *marginata* sp. 2”. Divergent lineages of “sp. 2” did not have obvious morphological or developmental differences, and were only 5.9% distant, below the REST for *E. ornata* (~8%) and within the range of intra-specific COI variation noted for other sacoglossans (Ellingson and Krug, 2006; Trathen, 2010; Rico, 2012; Vendetti et al., 2012; Vo, 2013). Syntopic specimens from Japan that belonged to different subclades did not share H3 alleles, however, suggesting lineages may be reproductively isolated and therefore candidate species. Alternatively, outbreeding depression might inhibit admixture between historically isolated lineages that recently entered secondary contact (e.g., Edmands, 1999). We provisionally regard the subclades of “sp. 2” as conspecific lineages, but future work should test for minor diagnostic differences that may yet distinguish these as CCS.

Notably, different threshold COI distances for species delimitation were returned for the two *Elysia* complexes (ABGD), or for *Elysia* versus *Plakobranchus* (REST method). Universal or phylum-specific cutoffs will often fail when the coalescence process varies among closely related lineages, as in our three species complexes. Witt et al. (2006) proposed a relative

inter-specific threshold distance of tenfold greater than the mean intra-population divergence, but for some sacoglossans that would lump distinct species separated by distances under 20%. Even a 10% threshold proposed to delimit mollusc species (Hebert et al., 2003; Malaquias and Reid, 2009) would be too high for all three complexes we studied, in which young species were often 6-9% divergent from close relatives. Marine invertebrates are thus likely to require taxon-specific standards that depart from recommendations based on terrestrial taxa or vertebrates, and uniform thresholds may not apply across a family or genus (Dawson and Jacobs, 2001; Shearer et al., 2002; Govindarajan et al., 2005; Wörheide, 2006). The idiosyncratic nature of barcoding gaps should not be surprising given that coalescent times vary even among related taxa, due the degree of heterotachy (variation in mutation rates), the extent of phylogeographic structure, and the size of populations relative to the timing of speciation (Hebert et al., 2004; Hickerson et al., 2006; Meier et al., 2006). The distribution of genetic distances within and among species must be carefully evaluated for poorly studied groups to identify a threshold distance congruent with other diagnosable differences, providing clade-specific criteria for delimiting species without relying on a generic cutoff (e.g., Lefébure et al., 2006).

DNA barcoding assumptions and marine phylogeography

DNA barcoding emerged as a powerful tool with which to inventory biological diversity, but most barcoding applications make assumptions about how COI variation is likely to be distributed within versus among species. For instance, a 2% COI distance threshold was proposed to distinguish terrestrial species and aquatic vertebrates (Hebert et al., 2010), and embraced by the Marine Barcoding of Life initiative (www.marinebarcoding.org). However, the suitability of this cutoff remains untested for most marine invertebrates. Low thresholds risk erroneously diagnosing conspecifics as separate species, or creating a false impression of mtDNA paraphyly. We found that conspecific COI haplotypes were 3-6% divergent in five of 17 species sampled from geographically distant sites; thus, intra-specific divergence in sacoglossans can be markedly greater than 2%. Standard barcoding criteria would advocate splitting our samples into five additional “species” that were not distinct by morphology, development, or ecology – i.e., provisional species that could not be described. Splitting lineages using a 2% threshold would also create non-monophyly of COI haplotypes in some species, whereas prior phylogeographic work on 19 sacoglossan taxa found only a single case of COI paraphyly due to localized introgression in a few populations of *E. pratensis* (Ellingson and Krug, 2006; Rodriguez, 2009; Trathen, 2010; Krug et al., 2011; Rico, 2012; Vendetti et al., 2012; Vo, 2013).

Effective barcoding may also require range-wide estimates of intra-specific variation to model the coalescent process within a species complex (Nielsen and Matz, 2006; Bond and Stockman, 2008; Fujita et al., 2012). For most studied species, we detected little sequence variation at the COI locus across the Indo-Pacific, suggesting typical barcoding thresholds might apply to our study taxa: maximum intra-specific COI distances were below 2% in 16 of 21 species for which multiple haplotypes were recovered (including data for *E. pratensis* and *E. subornata* from Rodriguez, 2009). However, low apparent intra-specific divergence can be an artifact of limited geographic sampling or species rarity (Bergsten et al., 2012; Lim et al., 2013). Indeed, out of 21 species sampled multiple times, four were found at only one site and nine at only two sites. Conversely, 5 of 7 taxa sampled from at least three distant locations yielded intra-specific COI distances >3%. Thus, adequate sampling poses a special challenge for delimitating marine species that are locally rare but have extensive ranges. Further sampling should improve estimates of intra-specific variation for candidate species uncovered in this study, and better define their geographic ranges.

Low intra-specific variation in well-sampled taxa may alternatively result from high connectivity via larval exchange among demes. In fact, some barcoding studies presume that

benthic marine species will be genetically homogenous due to their dispersive planktonic larvae (e.g., Hebert et al., 2009), although recent meta-analyses questioned the link between larval period and population subdivision (Shanks, 2009; Weersing and Toonen, 2009; Kelly and Palumbi, 2010). Most species in our study produced planktotrophic larvae capable of long-distance dispersal, which may explain the genetic similarity of conspecifics separated by over 6,000 km in taxa such as *E. rufescens* and *Plakobranchus* sp. 2. However, a few planktotrophic species nevertheless had differentiated populations (cf. *marginata* sp. 3) or divergent COI lineages (*Plakobranchus* "white" from Thailand). Broader geographic sampling may be needed to capture the evolutionary history of fast-evolving markers like COI in more genetically structured species, and to test whether taxa with similar larval development have congruent phylogeographic patterns.

Congruence of mtDNA with nuclear gene sequences, morphology and development

Barcoding studies of non-marine taxa found that including a nuclear locus can greatly increase the power to identify cryptic species (Monaghan et al., 2005; Elias et al., 2007; Sonnenberg et al., 2007; Smith et al., 2008). In the present study, the range of intra-specific divergence among H3 alleles overlapped with the inter-specific distances. Nevertheless, in our three species complexes, H3 alleles were only shared between species in two pairs of recently diverged *Plakobranchus* spp., and one pair of candidate species in the *E.* cf. *tomentosa* complex. In the case of *Plakobranchus*, a single allele was fixed in all "white" and "black" specimens; a different allele was fixed in five specimens of "aff. purple" and the lone "spotless" specimen. The lack of H3 diversity is consistent with recent speciation and incomplete lineage sorting at this conserved nuclear locus. Further, in all but one case, hypothesized relationships among H3 alleles were consistent with those inferred from COI data, supporting group membership for 80 individuals in 25 candidate species. The sole exception was that H3 alleles of *Plakobranchus* sp. 1 from Sulawesi did not form a clade with alleles from a conspecific sampled in the Philippines; however, the absence of support for internal nodes indicates a lack of phylogenetic signal in the H3 data. Genetic data from both loci also failed to distinguish *E. atroviridis* and *E. setoensis*, consistent with recent findings that these traditional taxa are conspecific endpoints on a spectrum of varying morphology (Takano et al., in press). Thus, the H3 data strongly corroborate provisional species delimitation based on COI sequences and morphology.

Nuclear alleles shared between candidate species could reflect hybrid introgression or incomplete sorting of ancestral polymorphism, but may also result if conspecific COI lineages were treated as different "species" despite a common nuclear gene pool. In the *E. tomentosa* complex, there was little variation among *E.* cf. *tomentosa* allele #1 (sp. 1), allele #2 (sp. 1 + sp. 3), and allele #8 (sp. 6), suggesting recent shared ancestry. The COI distances separating these three putative species (~9-11%) were at the low end of the distribution for this complex, also consistent with a recent radiation. We therefore hypothesize that allele #2 was shared between *E.* cf. *tomentosa* sp. 1 and sp. 3 due to incomplete lineage sorting. However, further work is necessary to confirm that the "sp.1" and "sp. 3" in fact represent distinguishable taxa, and not divergent COI lineages that have entered secondary contact in Japan, as documented for some Caribbean sacoglossans (Trathen, 2010; Rico, 2012).

External morphology was highly consistent with molecular analyses across all three complexes. The principle exception was the low-parapodia morph of "*E.* cf. *marginata* sp. 1", which was initially presumed to be a distinct species from the high-parapodia morph. Genetic similarity of COI haplotypes and a shared H3 allele instead indicated that all specimens with three parapodial siphons and no marginal bands of color were conspecific, and that differences in parapodial height reflect intra-specific variation. Reproductive characters provided additional characters with which to delimit candidate species, including larval development mode (*Plakobranchus, E. tomentosa* complex) or distribution and color

of yolk reserves (*E. ornata* complex). Notably, *Plakobranchus* "white" and "black" shared an H3 allele yet differed in both external morphology (pattern of ocelli) and larval type, confirming they are distinct species as suggested by Yamasu and Adachi (1990), Adachi (1991), and Yamasu (1997). Patterns of yolk deposition in egg masses supported the DNA-based separation of "*E.* cf. *marginata* sp. 1" and "sp. 2", and distinguished both cryptic species from the Caribbean *E. ornata*, with which all Indo-Pacific material had previously been synonymized. Reproductive and developmental characters are therefore taxonomically valuable and should be included wherever possible in species descriptions of sacoglossans (e.g., Krug et al., 2007).

Implications for drug discovery, plastid symbiosis research, and biological control

We focused on species complexes of broad significance to highlight the importance of accurately assessing sacoglossan diversity. Kahalalide F, a cytotoxic depsipeptide, is a natural product in clinical trials as an anti-tumor drug (Faircloth and Cuevas, 2006), and also has anti-fungal and anti-parasite activity (Shilabin et al., 2007; Cruz et al., 2009). Kahalalides were originally isolated from *E. rufescens* and its host alga *Bryopsis* sp. (Hamann et al., 1996), but related molecules have been isolated from Indo-Pacific material described as "*E. grandifolia*" (Ashour et al., 2006) or "*E. ornata*" (Horgen et al., 2000). The importance of correctly identifying sources of anti-cancer metabolites spurred us to investigate the number of cryptic lineages masquerading as *Elysia* "*ornata*" and kin, given the taxonomic ambiguity of these taxa. Our work suggests up to five Indo-Pacific species may be sources of kahalalides: *E. rufescens*, and *E.* cf. *marginata* sp. 1-4. Given the need for novel drug candidates, the four candidate species of the *E. marginata* complex should be surveyed for diet-derived depsipeptides, and their algal hosts properly characterized and vouchered (Trowbridge et al., 2010). Finding new kahalalides may facilitate studies of structure-activity relationships and biosynthesis of these medically important compounds. Our data underscore the importance of characterizing biodiversity in groups that harbor drug candidates, to identify the source of known metabolites as well as targets for future bioprospecting efforts.

We urge natural-product chemists to deposit sacoglossan and algal voucher material (and if possible, a COI barcode) in conjunction with drug discovery work, to ensure that the biological source of important molecules can be determined. Sending material to a taxonomist for identification may be inadequate in groups where cryptic species are common. Voucher samples suitably preserved for molecular work should be archived in museum collections that are accessible to the community engaged in phylogenetic systematics. Notably, DNA is well-preserved in tissue that has been dehydrated by nonpolar solvents such as acetone or methanol, commonly used to extract secondary metabolites from samples; we therefore recommend that natural products chemists preserve vouchers in ethanol after extraction. We also advocate greater integration of taxonomy and systematics into the training that students of natural-products chemistry receive, to ensure that vouchers for morphological and molecular analysis are properly archived to facilitate both taxonomic practice and future drug discovery work.

The erroneous consolidation of at least 10 *Plakobranchus* spp. under the name "*P. ocellatus*" similarly poses fundamental problems for understanding the literature on plastid symbiosis. Sacoglossans in clade Plakobranchoidea are famed for kleptoplasty, the ability to retain functional, diet-derived chloroplasts in cells lining their branched digestive gland. Kleptoplasty has long been considered a model for early-stage endosymbiosis. Chloroplasts photosynthesize for a few weeks in most plakobranchoidean taxa, but remain functional for more than a month in four highly photosynthetic species including "*P. ocellatus*", long after nuclear-encoded components of the light-harvesting complex should burn out (Händeler et al., 2009; Rumpho et al., 2011). Recent work on *Elysia chlorotica* demonstrated horizontal

transfer of over 50 nuclear genes from the host alga to the slug genome, allowing chloroplasts to survive up to nine months (Pierce et al., 2012); however, it remains contentious whether lateral gene transfer has occurred in other species (Wägele et al., 2011; Pelletreau et al., 2011). A range of estimates exist for chloroplast longevity and its importance to survival under food-limiting conditions in *Plakobranchus*, but it is unclear which species, and how many, were the focus of prior studies (Dunlap, 1975; Hirose, 2005; Händeler et al., 2009; Wägele et al., 2011; Maeda et al., 2012; Pierce and Curtis 2012). Only Adachi (1991) and Yamamoto et al. (2012) specified the morphs (i.e., species) studied from Japan. Similarly, the preferred host alga(e) and degree of diet specificity may vary across the 10 or more *Plakobranchus* spp., obscuring our understanding of how host association affects plastid longevity in this complex of photosynthetic animal species (Christa et al., in press).

Lastly, invasive strains of the green algae *Caulerpa taxifolia* and *C. racemosa* are economically and ecologically disruptive in the Mediterranean and parts of the Pacific (Balata et al., 2004). Repellent terpenoid metabolites render *Caulerpa* spp. unpalatable to generalist herbivores, leaving sacoglossans as the major consumers of these algae. In the genus *Elysia*, only members of the *E. tomentosa* complex specialize on *Caulerpa*, although other species may include *Caulerpa* spp. in their diet (Baumgartner et al., 2009; Händeler et al., 2009; Takano et al., in press). At least one candidate species, *E.* cf. *tomentosa* sp.1, causes extensive damage to *Caulerpa* spp. in Okinawa; larvae of sp. 1 are planktotrophic and dispersive (Trowbridge et al., 2012). The Caribbean species *E. subornata* was proposed as a biological control agent for non-indigenous *Caulerpa* spp. in the Mediterranean, due to its rapid feeding rate and non-dispersive larval development (Coquillard et al., 2000). Members of the *E. tomentosa* complex are therefore potentially important as native consumers of highly invasive algae, or as potential biological control agents. However, relevant species-specific characteristics must be determined for each taxon to evaluate its potential for biological control of algal outbreaks. Species that differ in larval dispersal will also differ in their potential for post-introduction spread, and in other parameter such as feeding rates, lifespan, and fecundity. Further, given the potential for hybridization and introgression between related species in the *E. tomentosa* complex (e.g., Rodriguez, 2009), the species identities and native distributions of specialized *Caulerpa* consumers must be addressed as part of any responsible biocontrol project.

Conclusions

Most marine heterobranch groups are in need of systematic overhaul, and alpha taxonomy lags well behind molecular surveys of diversity. Using an integrative approach, we delimited 20 candidate species based on congruence between mtDNA lineages and morphological or reproductive characters. A “local” barcoding threshold was required to delineate evolutionarily independent units, highlighting the idiosyncratic nature of the coalescent process. However, nuclear gene trees supported nearly all candidate species assignments, indicating broad congruence among loci evolving at different rates. Diversity in our three focal taxa was underestimated up to tenfold by overly conservative taxonomic practices, suggesting sea slug diversity could be far higher than current estimates. The historical failure to delimit species in these complexes has compromised the literature on drug discovery and plastid endosymbiosis, and raises concerns about the biological control of *Caulerpa*. Taxonomic work will now focus on describing all confirmed candidate species, while the existence of unconfirmed candidate species will guide future collecting efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DNA barcoding revealed three “species” of photosynthetic sea slugs comprised a total of 20 candidate species
- Mitochondrial lineages were largely congruent with the distribution of nuclear histone III alleles
- Morphological and reproductive characters distinguished 14 of 20 sampled lineages as confirmed candidate species
- Unrecognized species complicate efforts in drug discovery, biological control, and studies of early-stage endosymbiosis
- Minimum inter-specific divergence was 6-8% at COI, requiring taxon-specific thresholds for effective species delimitation

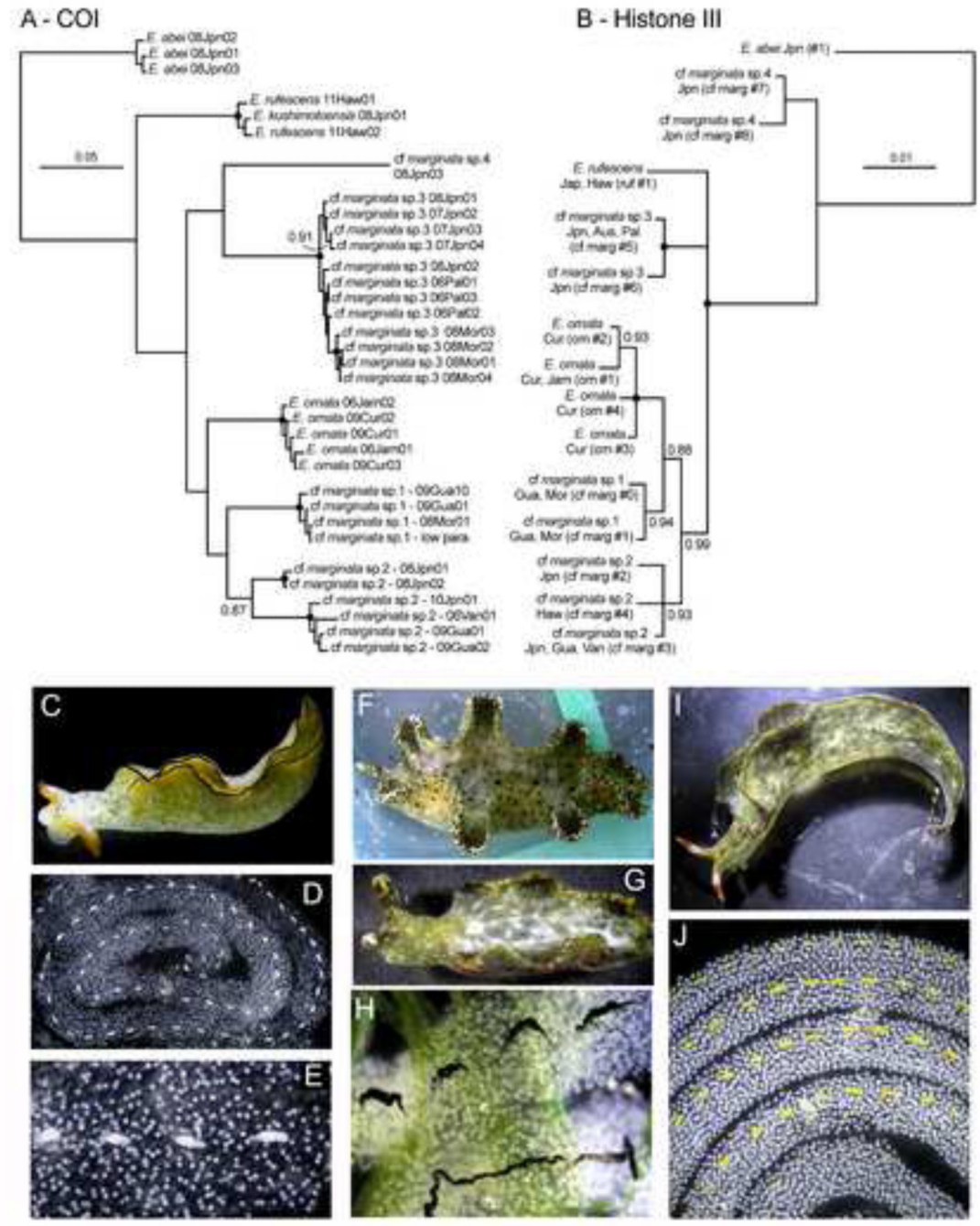

Figure 1.

Molecular, morphological and development characters support four confirmed candidate species (CCS) in the "*Elysia ornata*" complex, termed "*E.* cf. *marginata* sp. 1-4". **A.** Consensus phylogram based on Bayesian analysis of partial COI gene sequences, with mean branch lengths given in substitutions per site. Posterior probabilities of key nodes are given numerically (PP = 0.87 – 0.99) or indicated with a solid circle (PP = 1.0). Haplotypes are coded by date-location-specimen number as given in Table 1. **B.** Consensus phylogram from Bayesian analysis of H3 gene fragments, after resolving allelic phase for heterozogytes. Alleles are indicated by a species-number code, and the locations where that allele was sampled. **C.** Representative specimen of *E. ornata* (20 mm long) from Jamaica, showing

characteristics including pointed tail, black and orange bands along the parapodial margin, and tiny black spots. **D.** Egg mass (15 mm across) spawned by *E. ornata*, showing regularly spaced ovals of white extra-capsular yolk (ECY) deposited within the egg mass. **E.** Close-up of egg mass from D showing individual capsules, each containing one to three uncleaved ova, and embedded ECY. Field = 3 mm across. **F.** Specimen of *E.* cf. *marginata* sp. 1 (30 mm long) from Guam, showing the three orange-edged siphonal openings, blunt-ended body, and large black spots typical of the high-parapodia morph. **G.** Low-parapodia morph of *E.* cf. *marginata* sp. 1 (25 mm long) from Guam. **H.** Close-up of egg mass spawned by specimen from F. A continuous ribbon of black ECY gradually breaks up into regularly spaced strands in outer whorls of the egg spiral. Field = 6 mm across. **I.** Specimen of *E.* cf. *marginata* sp. 2 (25 mm long) from Guam, showing the characteristic red rhinophores, narrow black marginal band and reddish submarginal line, and pointed tail. **J.** Egg mass of *E.* cf. *marginata* sp. 2, showing regularly spaced ovals of yellow ECY deposited throughout. Field = 4.7 mm across.

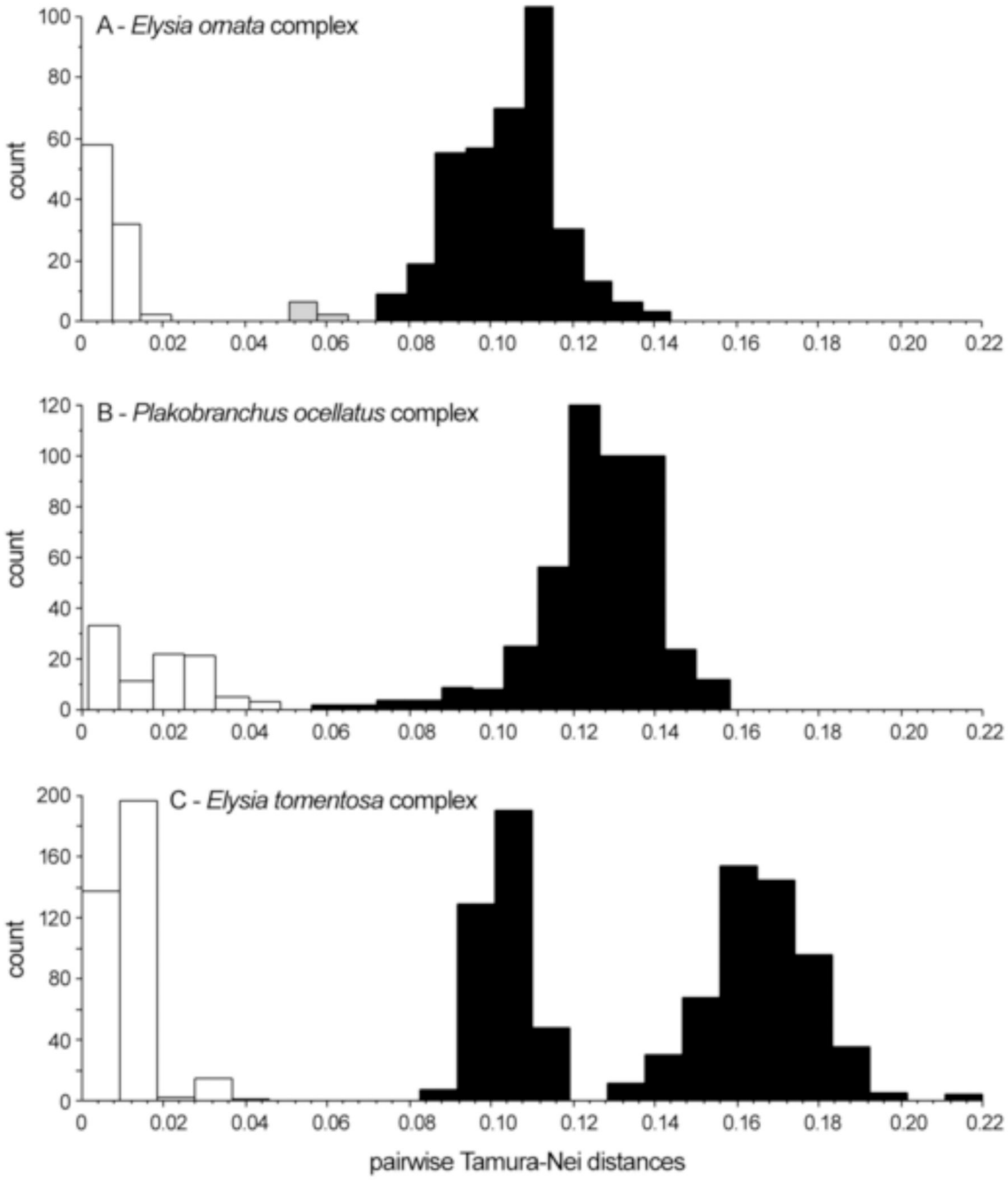

Figure 2.

Frequency distribution of pairwise genetic distances (Tamura-Nei corrected) for all COI haplotypes recovered for each of three species complexes. Unoccupied bins between modes (the "barcoding gap") typically distinguish intra-specific (white bars) from inter-specific (black bars) divergence, although distances between deep conspecific lineages distinguished by the ABGD procedure (grey bars) may fall in this region. **A**. *Elysia ornata* complex, including four candidate species and the morphologically distinctive taxa *E. ornata* and *E. rufescens*. **B**. *Plakobranchus ocellatus* complex of 10 candidate species. **C**. *Elysia tomentosa*

complex, including six candidate species and the morphologically distinctive taxa *E. subornata, E. pratensis* and *Elysia* sp. 22.

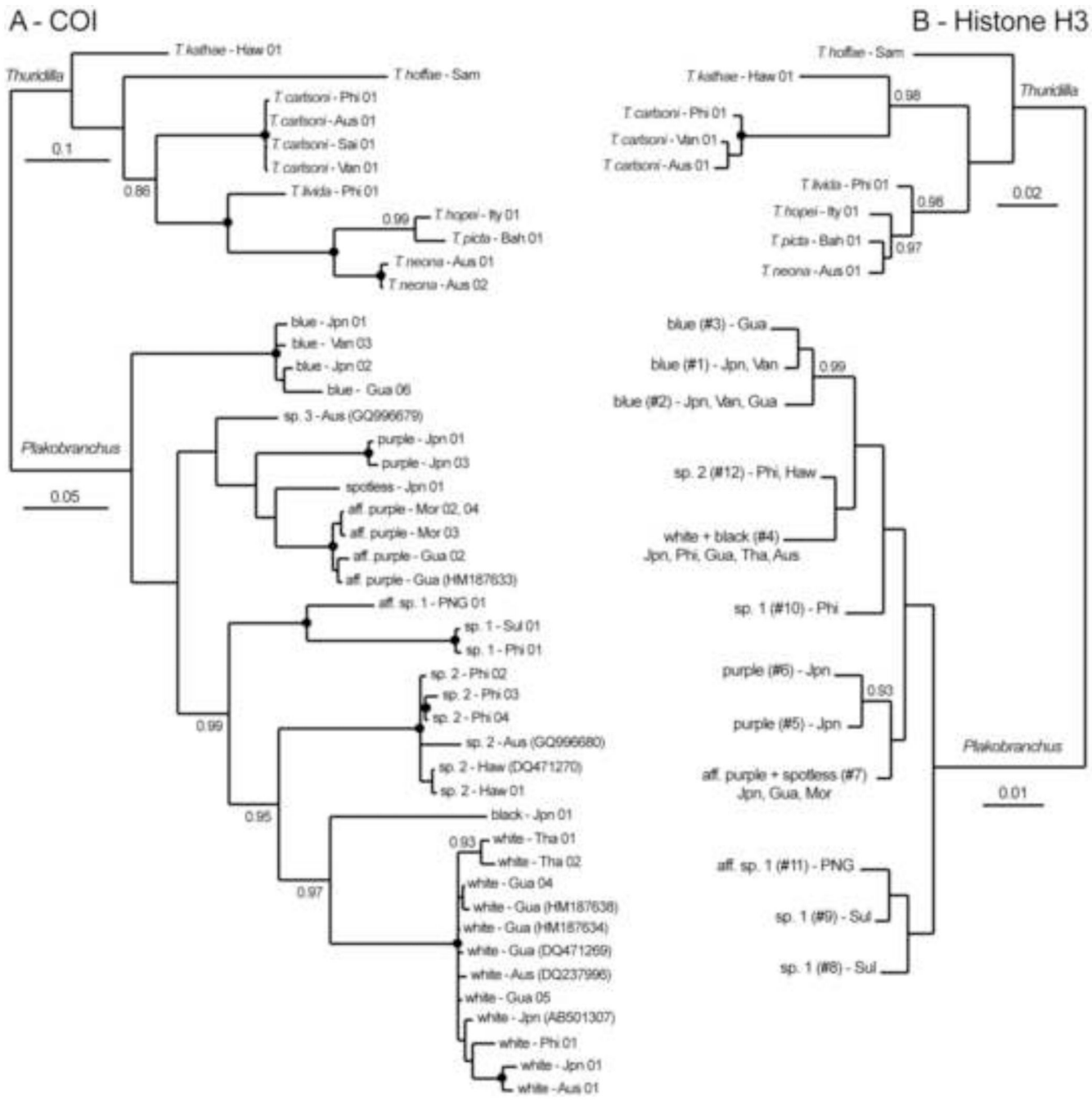

Figure 3.

Molecular phylogenetic evidence for 10 candidate species in the "*Plakobranchus ocellatus*" complex, rooted on a basal clade of species from the sister taxon *Thuridilla* due to the monotypic status of *Plakobranchus*. **A.** Consensus phylogram based on Bayesian analysis of partial COI gene sequences, with mean branch lengths given in substitutions per site. Posterior probabilities are given numerically (PP = 0.86 – 0.99) or indicated with a solid circle (PP = 1.0). Haplotypes are coded by location-specimen number, or by location-accession number when obtained from a public database. **B.** Consensus phylogram from Bayesian analysis of H3 gene fragments, after resolving allelic phase for heterozygotes.

Alleles are indicated by a species-number code, and the locations where that allele was sampled.

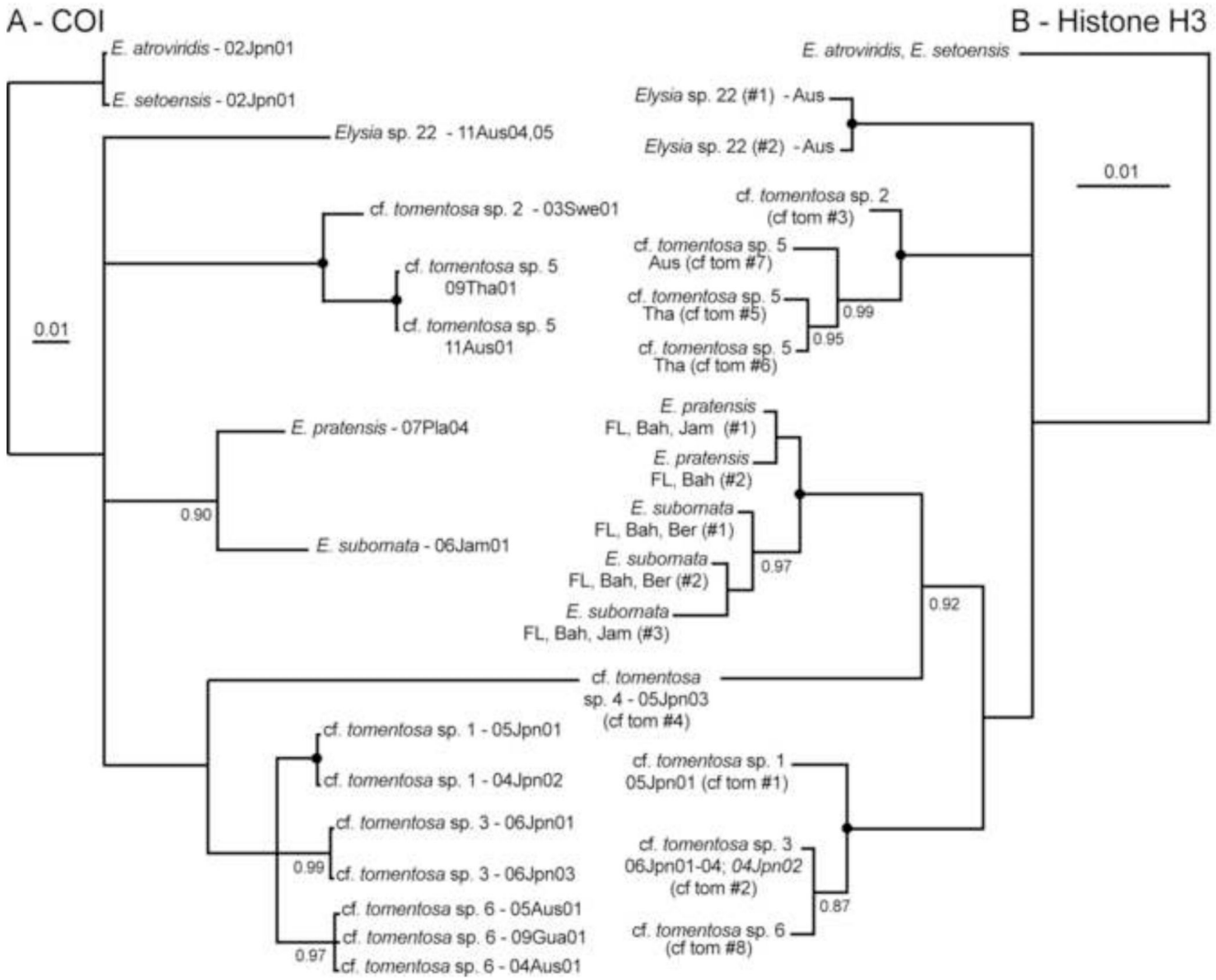

Figure 4.

Molecular phylogenetic evidence for six candidate species in the "*Elysia tomentosa*" complex. **A.** Consensus phylogram based on Bayesian analysis of partial COI gene sequences, with mean branch lengths given in substitutions per site. Significant posterior probabilities are given numerically, or indicated with a solid circle (PP = 1.0). Haplotypes are coded by date-location-specimen number. **B.** Consensus phylogram from Bayesian analysis of H3 gene fragments, after resolving allelic phase for heterozogytes. Alleles are indicated by a species-number code, and the locations where that allele was sampled.

Table 1

Provisional species identifications, sample codes, collection details and accession numbers for outgroup taxa and cryptic species in the (A) *Elysia ornata* complex, (B) *Plakobranchus ocellatus* complex, (C) basal clade of *Thuridilla*, and (D) *Elysia tomentosa* complex

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
A	*E. abei*	Eabei_07Jap01	Kanagawa, Japan	10/27/07	Y. Hirano	not used	Eabei_H3_01	JN819171	
	(on *Codium*)	Eabei_08Jap01	Shirahama, Japan	8/4/08	”	KC573711	n.a		
	(on *Codium*)	Eabei_08Jap02	”	”	”	KC573712	n.a		
	(on *Bryopsis*)	Eabei_08Jap03	”	”	”	KC573713	n.a		
	E. rufescens	Eruf_08Jap01	Sobe, Okinawa, Japan	12/10/08	Y. Hirano	KC573688	Eruf_H3_01	KC597152	
		Eruf_11Haw01	Kahului Harbor, Maui,	6/20/11	Á. Valdés	KC573689	Eruf_H3_01		
		Eruf_11Haw02	Hawaii	”	”	KC573690	Eruf_H3_01		
	Elysia ornata	Eorn_06Jam01	Discovery Bay, Jamaica	3/1/07	P. Krug	JN819093	Eorn_H3_01	JN819157	
		Eorn_06Jam02	”	”	”	JN819094	-		
		Eorn_09Cur01	Playa Kanoa, Curacao	1/4/09	P. Krug	JN819096	Eorn_H3_02	JN819158	
		Eorn_09Cur02	”	”	”	JN819097	Eorn_H3_01		
		Eorn_09Cur03	Spanish Waters inlet, Curacao	1/9/09	P. Krug	JN819095	Eorn_H3_03	KC614690	
		”	”	”	”		Eorn_H3_04	JN819159	
	“*E* cf. *marginata*”	cf marginata sp.1_09Gua00	Pago Bay, Guam	8/2009	P. Krug	KC573691	cf_marg_sp1_H3_00	KF322025	
	sp.1, “3 siphons”	”	”	”	”		cf_marg_sp1_H3_01	JN819156	
		cf-marginata_sp.1_09Gua01	”	”	”	JN819099	cf_marg_sp1_H3_01		
		cf_marginata_sp.1_09Gua10	”	”	”	JN819100	cf_marg_sp1_H3_01		
		cf_marginata_sp.1_08Mor01	Moorea, French Polynesia	5/7/08	R. Ellingson	JN819098	cf_marg_sp1_H3_01		
	sp. 2, “red tips”	cf_marginata_sp.2_08Jap01	Sobe, Okinawa, Japan	12/10/08	Y. Hirano	KC573692	cf_marg_sp2_H3_02		
		cf_marginata_sp.2_08Jap02	”	”	”	KC573693	cf_marg_sp2_H3_02	KC597153	
		cf_marginata_sp.2_09Gua01	Piti Bay, Guam	8/2009	P. Krug	KC573694	cf_marg_sp2_H3_03	KC597154	
		cf_marginata_sp.2_09Gua02	Pago Bay, Guam	”	K. Händeler	KC573695	cf_marg_sp2_H3_03		

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
		cf_marginata_sp.2_10Jap01	Zanpa, Okinawa, Japan	4/15/10	Y. Hirano	KC573696	cf_marg_sp2_H3_03		
		cf_marginata_sp.2_06Van01	Espiritu Santo Is., Vanuatu	10/15/06	M. Pola-Perez	KC573697	cf_marg_sp2_H3_03		
		cf_marginata_sp.2_11Haw02	Maliko Bay, Maui, Hawaii	6/18/11	Á. Valdés	-	cf_marg_sp2_H3_04	KC597182	
	sp. 3, “dark”	cf_marginata_sp.3_08Jap01	Sobe, Okinawa, Japan	12/10/08	Y. Hirano	KC573698	cf_marg_sp3_H3_05	KC597155	
		cf_marginata_sp.3_08Jap02	”	”	”	KC573699	cf_marg_sp3_H3_06	KC597156	
		cf_marginata_sp.3_07Jap02	Kanagawa, Japan	10/24/07	Y. Hirano	KC573700	n.a		
		cf_marginata_sp.3_07Jap03	”	”	”	KC573701	n.a		
		cf_marginata_sp.3_07Jap04	”	”	”	KC573702	n.a		
		cf_marginata_sp.3_07Aus01	Lizard Is., Australia	2/12/06	K. Cheney		cf_marg_sp3_H3_05		
		cf_marginata_sp.3_08Mor01	Moorea, French Polynesia	5/7/08	R. Ellingson	KC573703	n.a		
		cf_marginata_sp.3_08Mor02	”	”	”	KC573704	n.a		
		cf_marginata_sp.3_08Mor03	”	”	”	KC573705	n.a		
		cf_marginata_sp.3_08Mor04	”	”	”	KC573706	n.a		
		cf_marginata_sp.3_06Pal01	Palmyra Atoll, Line Islands	9/13/06	T. Gosliner	KC573707	cf_marg_sp3_H3_05		CASIZ 174223
		cf_marginata_sp.3_06Pal02	”	9/27/06	”	KC573708	cf_marg_sp3_H3_05		CASIZ 174208
		cf_marginata_sp.3_06Pal03	”	10/4/06	”	KC573709	cf_marg_sp3_H3_05		CASIZ 174230
	sp. 4, “white”	cf_marginata_sp.4_08Jap03	Sobe, Okinawa, Japan	12/10/08	Y. Hirano	KC573710	cf_marg_sp4_H3_07	KC597157	
		”	”	”	”		cf_marg_sp4_H3_08	KC614691	
B	“ *P. occelatus* ”								
	blue	Plk_blue_04Jap01	Okinawa, Japan	7/2004	C. Trowbridge	KC573714	-		
		Plk_blue_04Jap02	”	”	”	KC573715	Plk_H3_01	KC597158	
		”	”	”	”		Plk_H3_02	KC614692	
		Plk_blue_06Van01	Espiritu Santo Is., Vanuatu	9/12/06	M. Pola-Perez		Plk_H3_01		CASIZ 178311

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
		”	”	”	”		Plk_H3_02		
		Plk_blue_06Van03	”	10/12/06	”	KC573716	Plk_H3_02		CASIZ 179797
		Plk_blue_09Gua06	Bile Bay, Guam	6/16/09	Á. Valdés	KC573717	Plk_H3_02		
		Plk_blue_09Gua08	”	6/17/09	”	-	Plk_H3_02		
		”	”	”	”		Plk_H3_03	KC614693	
	black	Plk_blk_04Jap01	Okinawa, Japan	7/2004	C. Trowbridge	KC573718	Plk_H3_04	KC597159	
		Plk_blk_04Jap04	”	”	”	-	Plk_H3_04		
	white	Plk_wht_05Jap01	Sobe, Okinawa, Japan	12/2/05	C. Trowbridge	KC573719	Plk_H3_04		
		Plk_wht_04Phi01	Panglao, Philippines	6/30/04	T. Gosliner	KC573720	Plk_H3_04		CASIZ 174893
		Plk_wht_09Gua03	Bile Bay, Guam	8/2009	P. J. Krug &	not used	Plk_H3_04		
		Plk_wht_09Gua04	”	”	K. Händeler	KC573721	Plk_H3_04		
		Plk_wht_09Gua05	”	”	”	KC573722	Plk_H3_04		
		Plk_wht_09Gua07	Bile Bay, Guam	6/16/09	Á. Valdés	-	Plk_H3_04		
		Plk_wht_11Tha01	Krabi, Andaman Sea,	11/2011	C. Swennen	KC573723	Plk_H3_04		
		Plk_wht_11Tha02	Thailand	”	”	KC573724	-		
		Plk_wht_11Qld01	Queensland, Australia	9/6/02	Australian museum coll.	KC573725	Plk_H3_04		AM C-415141
		Plk_wht_11Qld02	Queensland, Australia	8/11/06	”				AM C-202726
		Plk_wht_06Van02	Espiritu Santo Is., Vanuatu	9/24/06	M. Pola-Perez		Plk_H3_04		CASIZ 179920
		Plk_wht_Guam	Guam	8/1/09	K. Händeler	HM187638	n.a		
		Plk_wht_Guam	”	”	”	HM187634	n.a		
		Plk_wht_Guam	”	”	”	DQ471269	n.a		
		Plk_wht_Aus	Lizard Is., Australia	unknown	H. Wägele	DQ237996	n.a		
		Plk_wht_Japan	Okinawa, Japan	unknown	T. Maeda	AB501307	n.a		
	purple	Plk_pur_05Jap01	Sobe, Okinawa, Japan	11/27/05	Y. Hirano	KC573726	Plk_H3_05	KC597160	

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
		Plk_pur_05Jap03	”	”	”	KC573727	Plk_H3_06	KC597161	
	aff. purple	Plk_aff_pur_08Mor01	Moorea	5/7/08	R. Ellingson	not used	n.a		
		Plk_aff_pur_08Mor02	”	”	”	KC573728	Plk_H3_07	KC597162	
		Plk_aff_pur_08Mor03	”	”	”	KC573729	Plk_H3_07		
		Plk_aff_pur_08Mor04	”	”	”	KC573728	Plk_H3_07		
		Plk_aff_pur_09Gua01	Bile Bay, Guam	8/2009	P. J. Krug &	not used	Plk_H3_07		
		Plk_aff_pur_09Gua02	”	”	K. Händeler	KC573730	Plk_H3_07		
		Plk_aff_pur_Guam	Guam	8/2009	K. Händeler	HM187633	n.a		
	spotless	Plk_spot_05Jap01	Okinawa, Japan	12/2/05	C. Trowbridge	KC573731	Plk_H3_07		
	sp.1	Plk_sp1_07Sul01	Sulawesi	2007	N. Wilson	KC573732	Plk_H3_08	KC597163	
		”	”	”	”		Plk_H3_09	KC614694	
		Plk_sp1_04Phi01	Panglao Is., Philippines	6/18/04	T. Gosliner	KC573733	Plk_H3_10	KC597164	CASIZ 175533
	aff. sp. 1	Plk_aff_sp1_PNG01	Papua New Guinea	unknown	N. Wilson	KC573734	Plk_H3_11	KC597165	
	sp.2	Plk_sp2_04Phi02	Panglao Is., Philippines	6/18/04	T. Gosliner	KC573735	Plk_H3_12	KC597166	CASIZ 175534
		Plk_sp2_04Phi03	Bohol Island, Philippines	6/3/04	T. Gosliner	KC573736	Plk_H3_12		CASIZ 176603
		Plk_sp2_04Phi04	Panglao Is., Philippines	7/1/04	T. Gosliner	KC573737	-		CASIZ 173494
		Plk_sp2_11Haw01	Hekili Point, Maui, Hawaii	6/13/11	Á. Valdés	KC573738	Plk_H3_12		
		Plk_sp2_Haw	Hawaii	unknown	A. Bass	DQ471270	n.a		
		Plk_sp2_Aus	Lizard Is., Australia	7/25/05	H. Wägele	GQ996680	n.a		
	sp.3	Plk_sp3_Aus	Lizard Is., Australia	3/21/05	H. Wägele	GQ996679	n.a		
C	*T. carlsoni*	Th_car_07Mal01	Tokong Kamundi, Malaysia	9/29/07	T. Gosliner	KC573739	Th_car_H3_01	KC597167	CASIZ 177120
		Th_car_06Van01	Espiritu Santo Is., Vanuatu	10/3/06	M. Pola-Perez	KC573740	Th_car_H3_02	KC597168	CASIZ 179150
		Th_car_10Sai01	Saipan	5/2010	J. Fraser	KC573741	Th_car_H3_01		

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
		Th_car_11How01	Lord Howe Is., Australia	4/9/11	P. Krug	KC573742	Th_car_H3_03	KC597169	
	T. hoffae	Th_hoff_NCBI				GQ996670	n.a	DQ534803	
	T. hopeii	Th_hop_07Ity01	Gigliol, Italy	7/26/07	H. Wägele	KC573743	Th_hop_H3_01	KC597170	
		Th_hop_07Ity02	”	”	”	-	Th_hop_H3_01		
	T. kathae	Th_kath_03Haw01	Maui, Hawaii	6/5/03	C. Pittman	KC573744	Th_kath_H3_01	KC597171	CASIZ 166754
	T. livida	Th_liv_07Mal01	Pulau Aur, Malaysia	10/3/07	T. Gosliner	KC573745	Th_liv_H3_01	KC597172	CASIZ 177122
	T. neona	Th_neo_11How01	Lord Howe Is., Australia	4/13/11	P. Krug	KC573746	Th_neo_H3_01	KC597173	
		Th_neo_11How02	”	”	”	KC573747	Th_neo_H3_01		
	T. picta	Th_pic_10NEx01	Exumas, Bahamas	6/11/10	P. Krug	KC573748	Th_pic_H3_01	KC597174	
D	*E. atroviridis*	Eatr_02Jap01	Choshi, Japan	6/2002	C. Trowbridge	KC573760	Eatr_H3_01	KC597184	
	E. setoensis *	Eset_02Jap01	Kanagawa, Japan	6/2002	C. Trowbridge	KC573761	Eatr_H3_01		
	E. pratensis	Epra_07Pla04	Plana, Bahamas	7/2007	P. Krug	JN819112	Epra_H3_01	JN819169	
			”	”	”		Epra_H3_02	JN819170	
	E. subornata	Esub_06Jam01	Discovery Bay, Jamaica	3/2006	P. Krug	JN819111	Esub_H3_01	JN819166	
		”	”	”	”		Esub_H3_03	JN819168	
		Esub_07Swe02	Sweetings Cay, Bahamas	7/2003	P. Krug	not used	Esub_H3_01		
		”	”	”	”		Esub_H3_02	JN819167	
	Elysia sp. 22	Elysia_sp.22_11How04	Lord Howe Is., Australia	4/5/11	P. Krug	KC573758	Elysia_sp.22 H3_01	KC597181	
		”	”	”	”		Elysia_sp.22_H3_02	KC614696	
		Elysia_sp.22_11How05	Lord Howe Is., Australia	”	P. Krug	KC573759	Elysia_sp.22_H3_01		
	“E. cf. *tomentosa*”								
	sp.1	cf_tomentosa sp.1_05Jap01	Sobe, Japan	11/29/05	C. Trowbridge	KC573749	cf_tom_sp.1_H3_01	KC597175	
		cf_tomentosa_sp.1_04Jap01	Zanpa, Okinawa, Japan	7/3/04	C. Trowbridge		cf_tom_sp.3_H3_03		
		cf_tomentosa_sp.1_04Jap02	”	”	”	KC573750	cf_tom_sp.3_H3_03	KC597185	

	species or morph	sample code	location	date	collector	COI NCBI accession #	H3 allele code	H3 NCBI access. #	voucher
	sp.2	cf_tomentosa_sp.2_03Swe01	Sweetings Cay, Bahamas	7/2003	P. Krug	KC573751	cf_tom_sp.2_H3_02	KC597176	
	sp.3	cf_tomentosa_sp.3_06Jap01	Toguchi, Okinawa, Japan	8/26/06	C. Trowbridge	KC573752	cf_tom_sp.3_H3_03		
		cf_tomentosa_sp.3_06Jap02	”	”	”	-	cf_tom_sp.3_H3_03		
		cf_tomentosa_sp.3_06Jap03	”	”	”	KC573753	cf_tom_sp.3_H3_03		
		cf_tomentosa_sp.3_06Jap04	”	”	”	-	cf_tom_sp.3_H3_03		
	sp.4	cf_tomentosa_sp.4_05Jap03	Sobe, Japan	11/27/05	C. Trowbridge	KC573754	cf_tom_sp.4_H3_04	KC597177	
	sp.5	cf_tomentosa_sp.5_09Tha01	Krabi, Andaman Sea, Thailand	9/2009	C. Swennen	KC573755	cf_tom_sp.5_H3_05	KC597178	
		”		”	”		cf_tom_sp.5_H3_06	KC614695	
		cf_tomentosa_sp.5_11How01	Lord Howe Is., Australia	4/6/11	P. Krug	KC573756	cf_tom_sp.5_H3_07	KC597179	
	sp.6	cf_tomentosa_sp.6_09Gua01	Guam	8/17/09	P. Krug	KC573757	cf_tom_sp.6_H3_08	KC597180	
		cf_tomentosa_sp.6_04Aus01	Lizard Is., Australia	9/13/04	I. Burghart	HM187630			
		cf_tomentosa_sp.6_05Aus01	”	3/21/05	H. Waegele	GQ996692	-		

AM = Australian Museum; AU = Australia; CAS-IZ = California Academy of Science-Invertebrate Zoology collection

n.a. = sample amplification of a given locus was not attempted

dash = unsuccessful amplification

not used = sequence confirmed species identity of specimen, but was excluded from analysis due to poor quality

* newly recognized as conspecific with *E. atroviridis* (Takano et al., 2013; present study)

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Table 2

Maximum intra-specific (bold) and minimum inter-specific pairwise Tamura-Nei distances at the COI locus for four clades.

A - *E. ornata* complex

			cf. "*marginata*"				
	rufescens	***ornata***	**sp.1, 3 siphons**	**sp. 2, red tips**	**sp.3, dark pigment**	**sp.4, intermediate**	***abei***
rufescens	**0.0108**						
ornata	0.0988	**0.0109**					
***marginata* sp.1**	0.1087	0.0890	**0.0077**				
***marginata* sp.2**	0.0865	0.0810	0.0754	**0.0592**			
***marginata* sp.3**	0.1092	0.1015	0.0933	0.0923	**0.0107**		
***marginata* sp.4**	0.1375	0.1253	0.1193	0.1201	0.1222	-	
abei	0.1434	0.1444	0.1441	0.1276	0.1324	0.1523	**0.0077**

B - *Plakobranchus ocellatus* complex

	white	**black**	**blue**	**spotless**	**purple**	**aff. purple**	**sp.1**	**aff. sp.1**	**sp.2**	**sp.3**
white	**0.0459**									
black	0.1125	-								
blue	0.1303	0.1223	**0.0323**							
spotless	0.1145	0.1212	0.1189	-						
purple	0.1283	0.1431	0.1008	0.0802	**0.0062**					
aff. purple	0.1093	0.1256	0.1104	0.0604	0.0887	**0.0127**				
sp.1	0.1204	0.1279	0.1320	0.1244	0.1239	0.1274	**0.0031**			
aff. sp.1	0.1149	0.1257	0.1166	0.1246	0.1200	0.1211	0.0869	-		
sp2	0.1158	0.1078	0.1188	0.1011	0.1098	0.1085	0.1178	0.1091	**0.0332**	
sp.3	0.1249	0.1438	0.1054	0.0806	0.0892	0.0780	0.1162	0.0945	0.1031	-

C - *Thuridilla* spp.

	T. kathae	***T. hoffae***	***T. carlsoni***	***T. livida***	***T. hopei***	***T. picta***	***T. neona***
T. kathae	-						
T. hoffae	0.1894	-					
T. carlsoni	0.1374	0.1827	**0.0056**				
T. livida	0.1528	0.2065	0.1474	-			
T. hopei	0.1375	0.1952	0.1560	0.1286	-		

T. picta	0.1459	0.2088	0.1734	0.1449	0.0550	-				
T. neona	0.1612	0.2216	0.1772	0.1359	0.0843	0.0898	**0.0092**			
D - *E. tomentosa* complex				**cf. “ *tomentosa*”**						
	subornata [1]	***pratensis*** [1]	**sp. 22**	**sp.1**	**sp.2**	**sp.3**	**sp.4**	**sp.5**	**sp.6**	***atroviridis* (= *setoensis*)**
subornata	**0.0169**									
pratensis	0.0938 [2]	**0.0425**								
***Elysia* sp.22**	0.1642	0.1783	-							
***tomentosa* sp. 1**	0.1691	0.1577	0.1765	**0.0015**						
***tomentosa* sp. 2**	0.1793	0.1638	0.1699	0.1774	-					
***tomentosa* sp. 3**	0.1685	0.1460	0.1711	0.0857	0.1610	**0.0015**				
***tomentosa* sp. 4**	0.1769	0.2000	0.1841	0.1903	0.2292	0.1705	-			
***tomentosa* sp. 5**	0.1634	0.1492	0.1583	0.1744	0.0907	0.1660	0.2164	**0.0063**		
***tomentosa* sp. 6**	0.1652	0.1567	0.1784	0.0916	0.1706	0.1047	0.1766	0.1643	**0.0046**	
atroviridis	0.1621	0.1488	0.1733	0.1442	0.1644	0.1408	0.1638	0.1708	0.1564	**0.0077**

[1] Maximum within-species genetic distances from Rodriguez (2009).

[2] Minimum between-species pairwise distance from Rodriguez (2009).

Table 3

Maximum intra-specific (bold) and minimum inter-specific pairwise Tamura-Nei distances at the H3 locus.

A - *E. ornata* complex			**cf. *marginata* spp.**				
	rufescens	***ornata***	**sp. 1, 3 siphons**	**sp. 2, red tips**	**sp. 3, dark pigment**	**sp. 4, intermediate**	***abei***
rufescens	**0.0000 (3)**						
ornata	0.0345	**0.0093**					
***marginata* sp. 1**	0.0313	0.0092	**0.0031**				
***marginata* sp. 2**	0.0280	0.0123	0.0092	**0.0123**			
***marginata* sp. 3**	0.0314	0.0281	0.0249	0.0217	**0.0031**		
***marginata* sp. 4**	0.0614	0.0543	0.0543	0.0509	0.0514	**0.0061**	
abei	0.1376	0.1100	0.1106	0.1141	0.1157	0.0916	-
B - *Plakobranchus ocellatus* complex							
	white + black	**blue**	**spotless + aff. purple**	**purple**	**sp. 1**	**aff. sp. 1**	**sp. 2**
white + black	**0.0000 (11)**						
blue	0.0155	**0.0031**					
spotless + aff. purple	0.0124	0.0092	**0.0000 (5)**				
purple	0.0155	0.0123	0.0031	**0.0031**			
sp. 1	0.0156	0.0092	0.0031	0.0061	**0.0031**		
aff. sp. 1	0.0187	0.0155	0.0061	0.0092	0.0031	**0.0000 (2)**	
sp. 2	0.0092	0.0061	0.0031	0.0061	0.0062	0.0093	**0.0000 (3)**
C - *Thuridilla* spp.							
	T. kathae	***T. hoffae***	***T. carlsoni***	***T. livida***	***T. hopei***	***T. picta***	***T. neona***
T. kathae	-						
T. hoffae	0.0847	-					
T. carlsoni	0.0814	0.1028	**0.0062**				
T. livida	0.0742	0.0681	0.0820	-			
T. hopei	0.0851	0.0721	0.0937	0.0155	**0.0000 (2)**		
T. picta	0.0811	0.0676	0.0892	0.0123	0.0093	-	
T. neona	0.0814	0.0682	0.0897	0.0123	0.0093	0.0061	**0.0000 (2)**

D - *E. tomentosa* complex				cf. "*tomentosa*"						*atroviridis*/ *setoensis*
	subornata	*pratensis*	sp. 22	sp. 1	sp. 2	sp. 3	sp. 4	sp. 5	sp. 6	
subornata	**0.0155**									
pratensis	0.0124	**0.0031**								
***Elysia* sp.22**	0.0713	0.0681	**0.0031**							
***tomentosa* sp. 1**	0.0779	0.0713	0.0753	-						
***tomentosa* sp. 2**	0.0777	0.0711	0.0714	0.0785	-					
***tomentosa* sp. 3**	0.0714	0.0685	0.0725	0.0155	0.0683	**0.0000 (4)**				
***tomentosa* sp. 4**	0.0673	0.0710	0.0928	0.0963	0.0852	0.0857	-			
***tomentosa* sp. 5**	0.0887	0.0820	0.0793	0.0864	0.0251	0.0761	0.0933	**0.0154**		
***tomentosa* sp. 6**	0.0750	0.0722	0.0725	0.0187	0.0720	0.0031	0.0895	0.0799	-	
atroviridis/seto.	0.1132	0.1132	0.1024	0.1073	0.0923	0.1082	0.1326	0.1072	0.1125	-

Zero values for intra-specific distances indicate only one allele was sampled (number of specimens given in parentheses); dashes indicate sequence data were only obtained for a single homozygote.

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Table 4

Definition of candidate-species categories for photosynthetic sea slugs, modified from Vieites et al. (2009).

Category	General definition	Definition used in photosynthetic sea slugs	
		Elysia	***Plakobranchus***
Unconfirmed Candidate Species (UCS)	Default classification for divergent mtDNA lineage, absent data on morphology, reproductive biology and/or distribution. Distance between a lineage and its closest relative exceeds a pre-determined threshold, based on clearly distinct and closely related species in the studied group	Pairwise COI distance (model-corrected) >8% to all described species; no data on morphology or reproductive traits	Pairwise COI distance (model-corrected) >6% to all candidate species (genus currently monotypic); no data on morphology or reproductive traits
Confirmed Candidate Species (CCS)	Lineage that is genetically divergent from all described species (no minimum distance required), and concordant with any of the following: **i.** a diagnostic morphological difference in a character of taxonomic value for that group; **ii.** evidence of isolation based on a reproductive character difference; **iii.** syntopy with related lineages, without admixture or loss of lineage-specific characteristics	Pairwise COI distance (model-corrected) usually >8% to described species, but sometimes 5-7%; mtDNA lineage concordant with a diagnostic difference in any of the following: **i.** morphological character known to be species-specific in elysiids: shape and color of rhinophores, parapodial margins, pericardium, tail, or pattern of dorsal vessels; **ii.** reproductive character: larval development mode, or color and placement of extra-capsular yolk reserves	Pairwise COI distance (model-corrected) usually >6% to other candidate species (but may be less); mtDNA lineage concordant with a diagnostic difference in any of the following: **i.** morphological character known to be species-specific: color and arrangement of ocelli, color and shape of rhinophores; **ii.** larval development mode
Deep Conspecific Lineage (DCL)	Divergent mtDNA lineages above a typical inter-specific distance between distinct sister taxa in the studied group, together with any of the following: **i.** no consistent phenotypic difference distinguishes the lineage from specimens of a characterized species; **ii.** no evidence of reproductive isolation from related lineages; **iii.** evidence of admixture with related lineages	Pairwise COI distance (model-corrected) >8% to described species, together with any of the following: **i.** no diagnostic morphological difference in traits listed above; **ii.** no consistent differences in larval development mode or pattern/color of ECY; **iii.** syntopy with divergent mtDNA lineages that cannot be distinguished morphologically	Pairwise COI distance (model-corrected) >6% to described species, together with any of the following: **i.** no diagnostic difference in ocelli or rhinophores; **ii.** no consistent differences in larval development mode; **iii.** syntopy with divergent mtDNA lineages that cannot be distinguished morphologically