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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.



## 1. 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 and Gregory, 2005). Controversies also persist over whether mitochondrial DNA (mtDNA) lineages represent biologically "good" species that can be described using conventional characters (Blaxter, 2004; Abdo and Golding, 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; Ward, 2009). In any taxon, rapid radiations may lead to an overlap

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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., 2013). 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 (Vieites et al., 2009; Yeates et al., 2011). 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.

## 2. Materials and methods

### 2.1. 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 noted, 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.

#### 2.1.1. 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,