

Integrative systematics of *Placida cremoniana* (Trinchese, 1892) (Gastropoda, Heterobranchia, Sacoglossa) reveals multiple pseudocryptic species

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Abstract *Placida cremoniana* (Trinchese, 1892) is a sacoglossan heterobranch sea slug of striking coloration, with a reported range that has increased dramatically in recent years. Described from the Mediterranean in 1892, nominal conspecifics were reported from Japan in 1959, and since the 1990s, additional populations have been reported from across the tropical Atlantic, Pacific, and Indian oceans, plus temperate areas of Japan, Australia, and Europe. This sequence of events is consistent with a dramatic range expansion by one species due to human-mediated introductions, or dispersal from tropical into temperate areas induced by recent climate change. Alternatively, the expanding range of this nominal species could, instead, reflect the gradual discovery of previously unsampled members of a global species complex. These hypotheses were tested using a combination of molecular data from three loci (COI, 16S, and H3), as well as radular morphology and external color patterns, from specimens sampled from most of the reported range. Molecular species delimitation recovered *P. cremoniana* as a complex of four genetically divergent species, further distinguished

by consistent differences in head coloration and radular morphology. As the Mediterranean species retains the name *P. cremoniana*, the three new species are described here, including an Eastern Pacific endemic and two species that are sympatric across large areas of the Central and Western Pacific. Photographic records and literature reports suggest that additional species may occur in the Indian Ocean, tropical Australia, and Caribbean.

Keywords Molecular systematics · Mollusca · New species · Species delineation · Taxonomic revision

Introduction

Placida cremoniana (Trinchese, 1892) is a sacoglossan sea slug currently recognized as having a circumtropical distribution spanning the Atlantic, Pacific, and Indian oceans. This species exhibits a conspicuous yellow-orange and black contrasting color pattern, unlike any other sacoglossan (Fig. 1). Originally described from Naples, Italy (Trinchese 1892, as *Hermaea cremoniana*), *P. cremoniana* was subsequently reported from Mediterranean localities (Pruvot-Fol 1951, as *Ercolania trinchesei* Pruvot-Fol, 1951; Pruvot-Fol 1954; Schmekel 1968; Fez 1962, as *Hermaea carmeni* Fez, 1962; Schmekel and Portman 1982; Sammut and Perrone 1998; Ballesteros 1980, 2007) (Fig. 2). Mediterranean records, albeit uncommon, were consistent across time and space, suggesting that this region is the native range of *P. cremoniana*.

The first record outside the Mediterranean concerned specimens collected from three localities in the Sea of Japan and southern Japan (Baba 1959) (Fig. 2). Reports of this species were restricted to the Mediterranean and temperate Japan for several decades until the 1990s, when records began extending the range of nominal *P. cremoniana* to most tropical and

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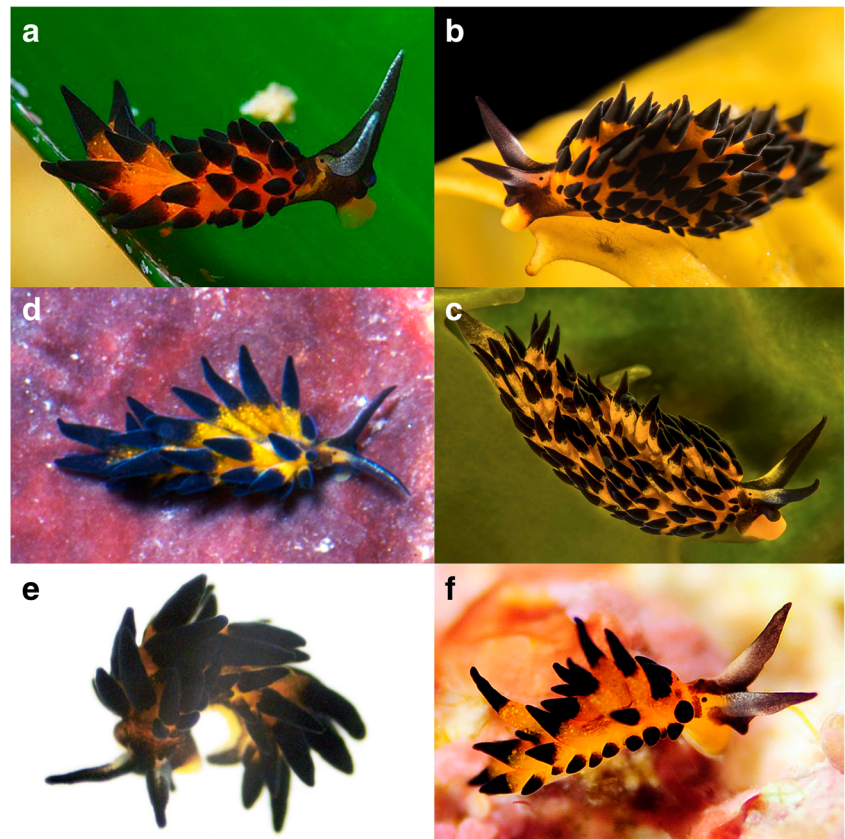
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Fig. 1 Living animals of the four species recognized in this study. **a** *Placida cremoniana* (Trinchese, 1892), from Spain (photo: Josep Lluís Peralta Rebull). **b** *Placida brookae* sp. nov., from Anacapa Island, California (photo: Brook Peterson). **c** *Placida brookae* sp. nov., from Catalina Island, California (photo: Brook Peterson). **d** *Placida barackobamai* sp. nov., from Maui, Hawaiian Is. (photo: Ángel Valdés). **e** *Placida kevinleei* sp. nov., from Maui, Hawaiian Is. (photo: Patrick Krug). **f** *Placida* cf. *kevinleei* sp. nov., from Kenya (photo: Kevin Lee)



temperate seas, including the Eastern Atlantic (Ortea et al. 1998; Fontes et al. 2001; Cortés 2008), the Caribbean (Caballer Gutiérrez et al. 2015), Eastern Pacific (e.g., Camacho-García et al. 2005; Behrens and Hermosillo 2005), western tropical Pacific (e.g., Burn 1998; Warren 2000; Carlson and Hoff 2003; Behrens 2004; Trowbridge et al.

2010; Chang 2012, and numerous photos online), the Indian Ocean and Red Sea (Bidgrain 2010; Yonow 2015), and temperate Australia (Rudman 2001; Schutz 2005) (Fig. 2). This proliferation of records could reflect a global range expansion due to anthropogenic introduction or could be the result of the discovery of new populations of a widespread species whose

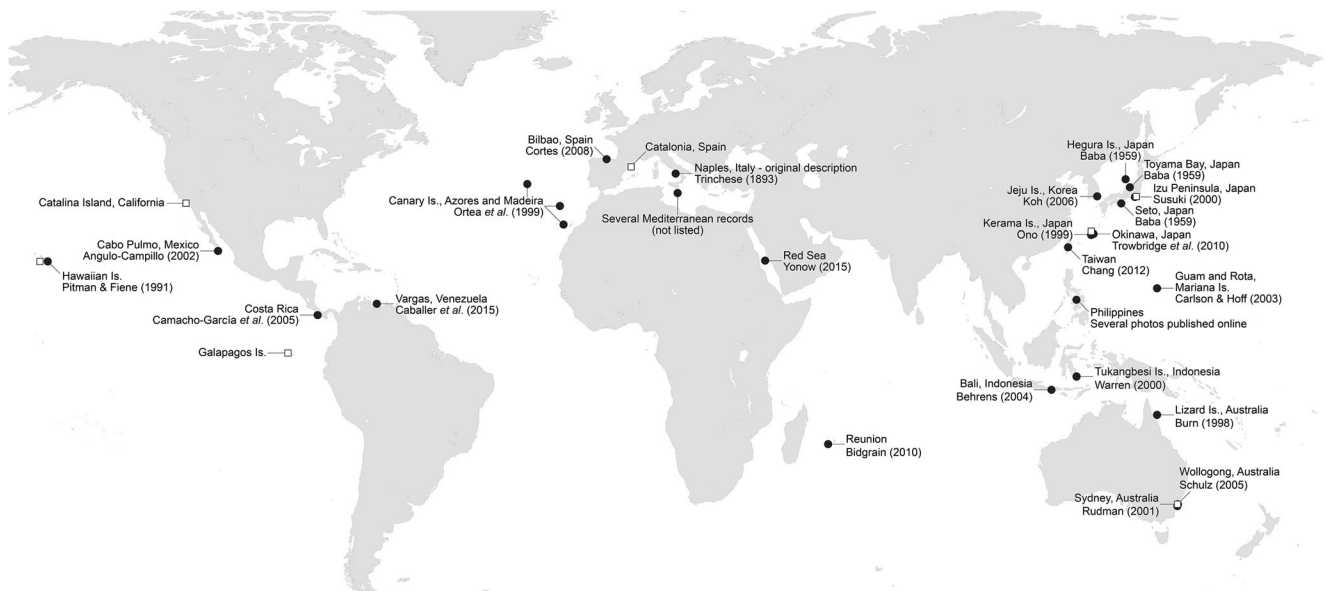


Fig. 2 Distribution map of *Placida cremoniana*. The *black circles* represent previous records of this species and the *white squares* are localities where specimens examined here were collected

range was poorly known. However, subtle external differences between specimens from different ocean basins suggested an alternative hypothesis, that nominal conspecifics comprised members of a pseudocryptic species complex. Recent range expansions of cryptic tropical species into warm-temperate regions may have facilitated their discovery by taxonomists/divers in regions such as Sydney, Australia, and the Channel Islands, California, USA.

Placida cremoniana has been used as a model in feeding experiments (Trowbridge et al. 2010) and has been included in a few sacoglossan-wide phylogenetic studies (e.g., Krug et al. 2015), but detailed molecular analyses for this taxon are lacking. Here, we investigate the taxonomic status of *P. cremoniana* using an integrative approach. DNA sequence data from three loci, two mitochondrial [cytochrome *c* oxidase I (COI) and 16S] and one nuclear (histone H3) gene, were analyzed from specimens covering most of the known range of this nominal species. The resulting species hypotheses were then tested by comparing fixed differences in morphological

characteristics, including head coloration and the morphology of microscopic radular teeth.

Materials and methods

Source of specimens

Data for 19 specimens of nominal *P. cremoniana* were obtained, representing the majority of the reported range (Fig. 2). Most specimens (16) were collected in the field by the authors or colleagues, or were obtained from the California Academy of Sciences (CASIZ) and the Australian Museum (AM) (Table 1). Type specimens are deposited at the Natural History Museum of Los Angeles County (LACM) or the California State Polytechnic University Invertebrate Collection (CPIC). Specimens were preserved in 95% or 70% ethanol. The sequences of three additional specimens were obtained from the NCBI database (Table 1).

Table 1 List of specimens sequenced for this study, including locality, museum voucher numbers, and GenBank accession numbers

Species	Locality	Isolate	Voucher number	GenBank accession numbers		
				COI	16S	H3
<i>P. cremoniana</i>	Illa de Fitor, Begur, Girona, Spain	JCP07	CPIC 01631	MF773465	MF773451	MF773476
<i>P. cremoniana</i>	Punta del Xeric, L'Escala, Girona, Spain	JCP08	CPIC 01633	MF773466	MF773452	MF773477
<i>P. brookae</i> sp. nov.	Catalina Island, California, USA	JCP02	CPIC 01265	MF773467	MF773453	MF773478
<i>P. brookae</i> sp. nov.	Catalina Island, California, USA	JCP04	CPIC 01962	MF773468	MF773454	MF773479
<i>P. brookae</i> sp. nov.	Catalina Island, California, USA	JCP05	LACM 3469	MF773469	MF773455	MF773480
<i>P. brookae</i> sp. nov.	Catalina Island, California, USA	JCP06	CPIC 01962	MF773470	MF773456	MF773481
<i>P. brookae</i> sp. nov.	Isla Española, Galápagos Is., Ecuador	JCP10	CASIZ 173619	MF773471	MF773457	MF773482
<i>P. barackobamai</i> sp. nov.	Maui, Hawaiian Is., USA	JCP03	CPIC 00400	MF773472	MF773458	MF773483
<i>P. barackobamai</i> sp. nov.	Sydney, Australia	JCP12	AM C.469568.3	–	MF773459	MF773484
<i>P. barackobamai</i> sp. nov.	Sydney, Australia	JCP13	AM C.469568.5	–	–	MF773485
<i>P. barackobamai</i> sp. nov.	Sydney, Australia	JCP14	AM C.469568.7	–	MF773460	MF773486
<i>P. barackobamai</i> sp. nov.	Sydney, Australia	11Syd01	AM C.469568.1	MF773473	–	–
<i>P. barackobamai</i> sp. nov.*	Kanagawa, Japan	–	–	AB501312	AB501312	–
<i>P. barackobamai</i> sp. nov.*	Guam, USA	862	–	HQ380201	HQ380184	KJ138301
<i>P. barackobamai</i> sp. nov.	Maui, Hawaiian Islands, USA	JCP15	CPIC 02037	MF773474	MF773461	MF773487
<i>P. barackobamai</i> sp. nov.	Maui, Hawaiian Islands, USA	JCP17	CPIC 02038	MF773475	MF773462	MF773488
<i>P. kevinleei</i> sp. nov.*	Okinawa, Japan	–	–	KM086427	KM204286	–
<i>P. kevinleei</i> sp. nov.	Maui, Hawaiian Islands, USA	JCP16	–	–	MF773463	MF773489
<i>P. kevinleei</i> sp. nov.	Sobe, Japan	JCP18	LACM 3471	–	MF773464	MF773490
<i>P. sp. 3**</i>	Lord Howe Island, Australia	11How01	–	KM086429	KM204288	KM040899
<i>P. dendritica**</i>	San Diego, California, USA	06SD02	–	KM086428	KM204287	KM040897
<i>P. verticillata**</i>	San Salvador, Bahamas	07Sal01	–	GU191064	GU191045	KM040900
<i>P. kingstoni**</i>	Dry Tortugas, Florida, USA	07FL06	–	GU191063	GU191044	KM040898

AM Australian Museum; CASIZ California Academy of Sciences Invertebrate Zoology; CPIC California State Polytechnic University Invertebrate Collection

*Denotes specimen sequences obtained from GenBank

**Denotes outgroup

DNA extraction, amplification, and sequencing

Depending on the size of the specimen, either 1 mg of tissue from the foot or two cerata were used for DNA extraction with QIAGEN DNeasy Blood & Tissue kits, following standard protocols but with only one elution step. Portions of two mitochondrial (COI, 16S) and one nuclear gene (H3) were amplified by polymerase chain reaction (PCR) using universal primers for COI (Folmer et al. 1994; LCOI490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3', HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'), 16S (Palumbi 1996; 16Sar-L 5'-CGC CTG TTTATC AAA AAC AT-3', 16S-br-H 5'-CCG GTC TGA ACT CAG ATC ACG T'3') and histone H3 (Colgan et al. 2000; HexAF 5'-ATG GCT CGT ACC AAG CAG ACG GC-3', HexAR 5'-ATA TCC TTG GGC ATG ATG GTG AC-3'), respectively. The PCR master mix volume was 50 μ L per reaction, containing: 33.75 μ L of H₂O, 0.25 μ L of DreamTaq, 5 μ L of 10 \times DreamTaq Buffer, 5 μ L of 2 mM dNTPs, 3 μ L of 25 nM MgCl₂, 1.5 μ L of each forward and reverse primer, and 2 μ L of DNA template. The reaction conditions for COI were as follows: initial 95 °C denaturation for 3 min, 35 cycles of 94 °C for 45 s (denaturation step), 45 °C for 45 s (annealing step), 72 °C for 45 s (elongation step), followed by a final elongation step at 72 °C for 10 min. The reaction conditions for 16S and H3 were as follows: initial 94 °C denaturation for 2 min, 35 cycles of 94 °C for 30 s (denaturation step), 50 °C for 30 s (annealing step), 72 °C for 1 min (elongation step), followed by a final elongation step at 72 °C for 7 min. Amplicons were verified by agarose gel electrophoresis and PCR products purified using GeneJET kits. Sanger sequencing was performed by Source BioScience (Santa Fe Springs, California). Sequences were assembled and edited in Geneious Pro R8 (Kearse et al. 2012). Phase of H3 alleles was easily resolved from the chromatograms of heterozygotes, which showed only one mixed base and reflected a combination of alleles identified from homozygotes.

Phylogenetic analyses

Alignments for each gene were generated with MUSCLE (Edgar 2004), as implemented in Geneious. The concatenated mitochondrial DNA (mtDNA) dataset (COI + 16S), and H3 alleles, were analyzed separately using Markov chain Monte Carlo (MCMC) methods implementing mixture models in BayesPhylogenies; this approach captures heterogeneity in mutation rates and base frequencies without a priori partitioning (Pagel and Meade 2004). For mtDNA, four chains were run for 10⁷ generations each, using two GTR + Γ models; the best-fit model was assigned to each position (adding models did not improve *L* scores). Trees were saved every 5000 generations and *L* scores and parameter estimates were inspected in Tracer v1.6 (Rambaut et al. 2013) to confirm stationarity within runs, and convergence between runs. Trees were then pooled from all runs, discarding the first 20%

as burn-in, and a 50% consensus tree generated. Posterior probabilities (PP) ≥ 0.9 were treated as significant (Huelsenbeck and Rannala 2004). Maximum likelihood (ML) analyses were run with raxmlGUI 1.0 (Silvestro and Michalak 2012) using the bootstrap + consensus option, implementing a GTR + Γ model with four rate multipliers. Nodal support was assessed from 10,000 bootstrap pseudoreplicates (BS), taking values $\geq 70\%$ as significant (Hillis and Bull 1993). Similar analyses were performed for H3 alleles, except that a single GTR + Γ model was used in BayesPhylogenies. Phylogenetic analyses were conducted with four species of the genus *Placida* designated as outgroups: *Placida verticilata* Ortea, 1982; *P. dendritica* (Alder & Hancock, 1843) from the temperate Eastern Pacific; *P. kingstoni* Thompson, 1977; and *Placida* sp. 3 (from Krug et al. 2015), an unidentified species from Lord Howe Island, Australia.

Species delimitation analysis

Automatic Barcode Gap Discovery (ABGD) was used to delimit species on separate COI and 16S datasets. Pairwise distances were calculated in MEGA 7.0.16 (Kumar et al. 2016) using the Tamura–Nei model with $\Gamma = 1$. The distance matrix was analyzed via the ABGD webtool (Puillandre et al. 2012) using the default settings.

Morphological analyses

Color patterns were documented from photographs of freshly collected live specimens, using either a camera attached to a dissecting microscope or with a macro lens. Patterns on the dorsal view of the head, rhinophores, cerata, and posterior end of the foot, as well as the color of the ventral side of the foot, were compared among specimens. The radula of at least two representative specimens from each delimited candidate species was examined, as subtle differences in tooth shape are important taxonomic criteria for sacoglossans (Krug et al. 2016). Buccal masses were dissected from specimens and placed in 10 mM NaOH for 48 h to dissolve excess tissue. Radulae were rinsed in distilled water and placed on glass slides, then mounted on scanning electron microscope (SEM) stubs for sputter coating. Radulae were visualized using a JEOL JSM-6010 variable pressure SEM. All specimens were too small for detailed examination of the reproductive anatomy.

Results

Phylogenetic analyses

Both ML and Bayesian analysis of mtDNA supported two main clades in the *P. cremoniana* complex, each divided into

two divergent subclades (Fig. 3). ML analysis of mtDNA strongly supported all four subclades, distinguishing specimens from the native range (Mediterranean) from three Pacific lineages (Fig. 3). Bayesian analysis also returned complete support for each of the three Pacific lineages. Sister to *P. cremoniana* from the Mediterranean was a clade receiving full support in both analyses, comprising sequences from warm-temperate Australia, Japan, Guam, and the Hawaiian Islands. In the other main clade, one subclade included all specimens from the Eastern Pacific (BS = 98, PP = 1.0), which was sister to a fully supported clade comprising samples from southern Japan and Hawaii (Fig. 3).

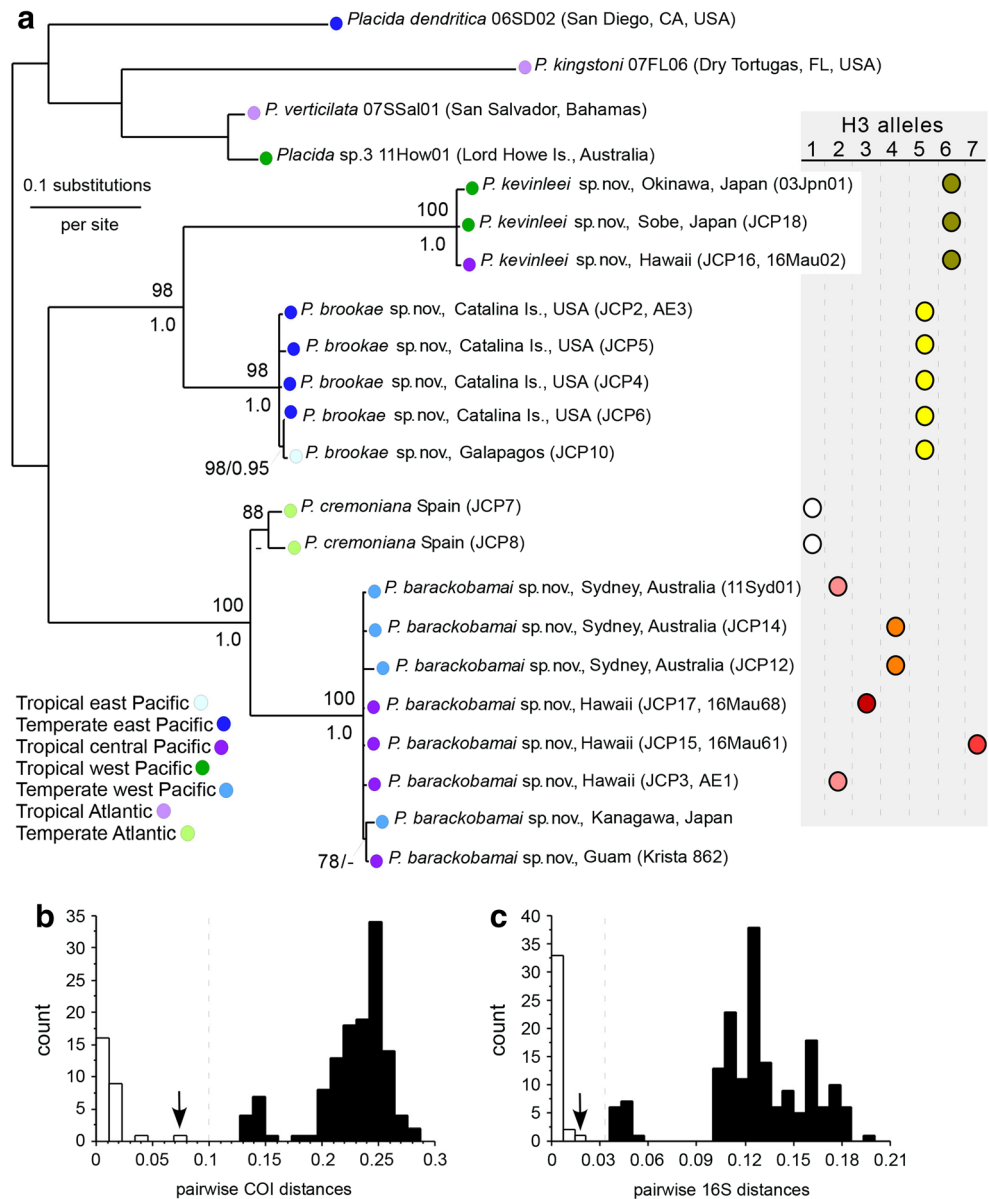
Analyses of H3 alleles yielded roughly equivalent topologies despite the short length of the gene fragments, again recovering two main clades with full support in Bayesian inference

(BI) analyses and > 90% BS support (Fig. S1). One subclade included two divergent alleles, one fixed in the Eastern Pacific samples and the other fixed in members of the mtDNA clade sampled only from Japan and Hawaii. The second main clade (PP = 1.0, BS = 97%) contained one allele sampled from Mediterranean *P. cremoniana* and four alleles sampled from specimens belonging to the remaining Western and Central Pacific mtDNA clade; relationships among the alleles in this clade were not resolved in either analysis, but most Pacific alleles formed an unsupported subclade.

Molecular species delimitation

For COI, ABGD recovered seven species at all pmax values, including four candidate species in the *P. cremoniana*

Fig. 3 Evolutionary relationships among mitochondrial DNA (mtDNA) haplotypes from the *Placida cremoniana* complex and four congeneric outgroups. **a** Topology shown is from the Bayesian consensus phylogram; maximum likelihood (ML) bootstrap support values are above branches, posterior probabilities below branches. The colored dots on the branch tips indicate the geographic region of origin for the specimen. The distribution of seven alleles sampled from the nuclear H3 locus is shown to the right of the corresponding terminals (one circle indicating a homozygous genotype; color is arbitrary). **b, c** Distributions of pairwise distances between sequences and Automatic Barcode Gap Discovery (ABGD) results for (b) COI and (c) 16S alignments. Intraspecific distances are indicated by white bars and interspecific distances by black bars; the dashed line denotes the threshold for intraspecific divergence estimated by ABGD. The arrow indicates the genetic distance between outgroup taxa *P. verticilata* (Caribbean) and *Placida* sp. 3 (Australia)



complex corresponding to the mtDNA subclades (Fig. 3b). A lower bound of 10% was recovered for the threshold interspecific COI divergence. Such a bound is likely conservative, as ABGD analysis lumped outgroup taxa *P. verticilata* (Caribbean) and *Placida* sp. 3 (Australia), which were 7.1% divergent (Fig. 3b, arrow). Given their allopatric distributions (west Atlantic versus South Pacific), those taxa likely represent distinct species, barring a recent introduction to Australia. However, even at a conservative 10% threshold, all four candidate species in the *P. cremoniana* complex were fully delimited. Pairwise distances between the closest relatives, Mediterranean *P. cremoniana* and the most widespread Pacific lineage, ranged from 13 to 15%, while all other complex members different by > 20% at COI, comparable to most distances among allopatric outgroup taxa. The maximum intraspecific divergence within a subclade was 2.2% for specimens from Hawaii and Japan.

For 16S, across the broadest range of pmax values (0.0017–0.0359), ABGD again recovered seven species, including four candidate species in the *P. cremoniana* complex (Fig. 3c). Analysis of the 16S data also lumped the mildly divergent outgroup species, *P. verticilata* and *Placida* sp. 3. Only above implausibly high pmax values (> 6%) did ABGD lump Mediterranean *P. cremoniana* with Pacific specimens.

Morphological analyses

External coloration and radular tooth morphology revealed consistent differences among delimited species in the *P. cremoniana* complex (Figs. 1 and 4–8). These lineages were, thus, recognized as distinct species and are formally described (or redescribed) in the “Systematics” section below.

Systematics

Family LIMAPONTIIDAE

Genus *Placida* Trinchese, 1876

Placida cremoniana (Trinchese, 1892)

(Figs. 1a, 3a, 4a, b, 5)

Hermaea cremoniana Trinchese, 1892: 155 (original combination)

Ercolania trinchessii Pruvot-Fol, 1951: 70–72, pl. 1, figs. 12–13

Hermaea carmeni Fez, 1962: 107–109, figs. 3–4

Material examined

Illa de Fitor, Begur, Girona, Spain, 12 Sep 2015, 1 specimen 3 mm preserved length, leg. Irene Figueroa (CPIC 01631). Punta del Xeric, L’Escala, Girona, Spain, 26 Aug 2015, 1 specimen 3 mm preserved length, leg. Robert Fernández (CPIC 01633).

Description

Maximum length 10 mm. Rhinophores cylindrical, en-rolled, black, with posterior white stripes from base to tip (Fig. 1a). Oral tentacles completely black; foot corners yellow. Head black with two yellow-orange to red patches, eyespots visible near center of yellow-orange patches (Fig. 4a). Lateral yellow-orange line on head connects yellow-orange eye patches to dorsum (Fig. 4b). Body elongate, with numerous cylindrical cerata with pointed apices covering most of dorsum. Proximal one-third of cerata yellow-orange to red, distal two-thirds black. Foot yellow-orange from head to tail ventrally. Posterior end of foot is pointed, black dorsally.

Radula of a 3 mm long specimen from Spain (CPIC 01633) composed of 4 teeth in ascending row and 11 teeth in descending row (Fig. 5a). Leading tooth smooth, lacking denticles, with a cylindrical, slightly bowed, pointed cusp (Fig. 5b). Base of teeth short, slightly concave ventrally. Ascus not examined.

Fig. 4 Schematic representation of diagnostic color pattern differences among species. **a, b** *Placida cremoniana* (Trinchese, 1892). **c, d** *Placida kevinleei* sp. nov. **e, f** *Placida barackobamai* sp. nov. **g, h** *Placida brookae* sp. nov.

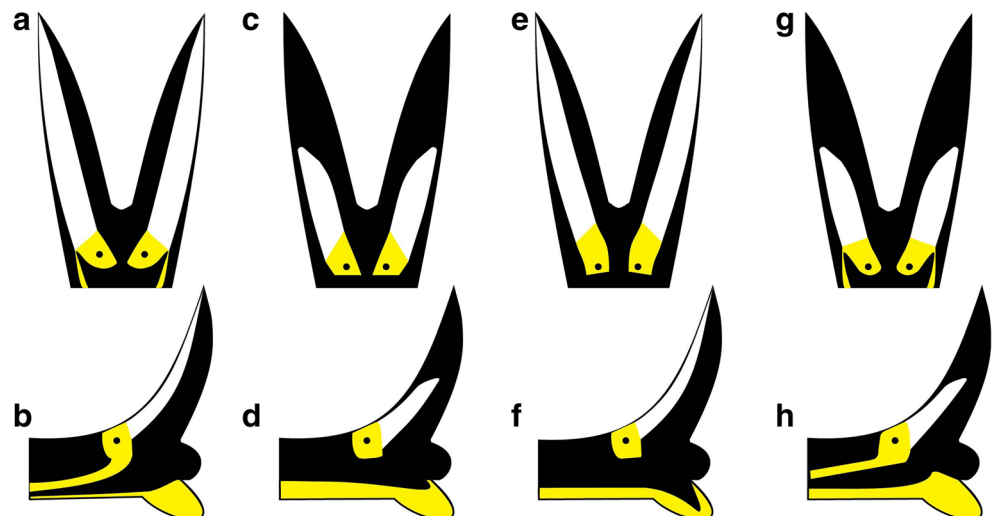
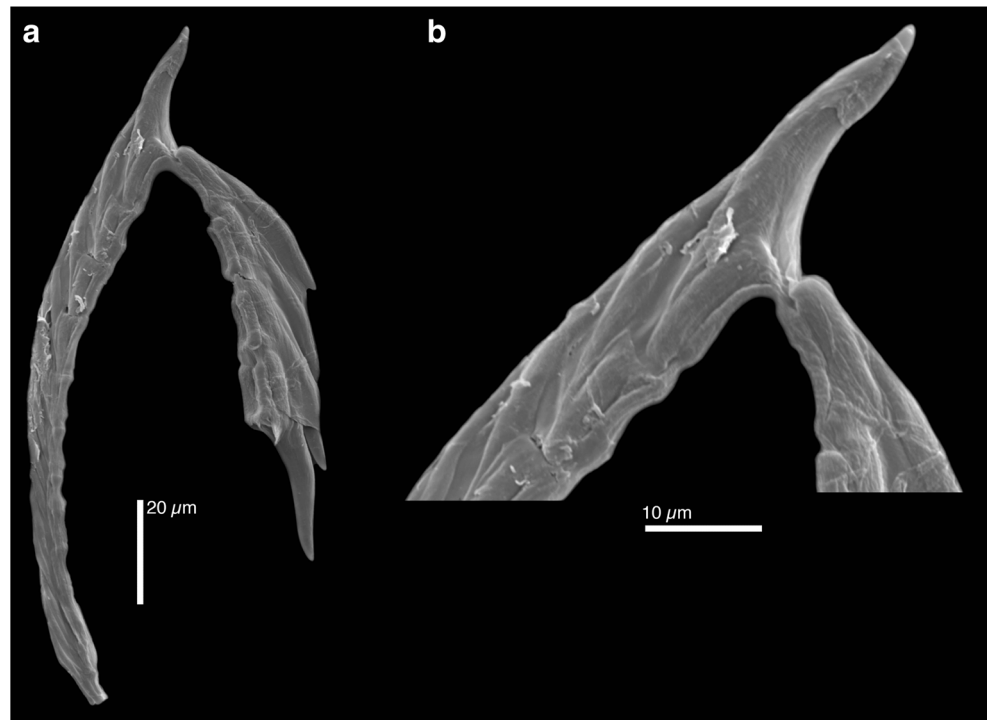


Fig. 5 *Placida cremoniana* (Trinchese, 1892). Scanning electron microscope (SEM) images of the radula of a specimen from Spain (CPIC 01633). **a** View of the entire radula. **b** Leading tooth



Geographic range

Eastern Atlantic Ocean, from Northern Spain (Cortés 2008) to the Canary Islands (Ortea et al. 1998) and the Mediterranean (see references cited in the Introduction; Fig. 2). Records from the Caribbean (Caballer Gutiérrez et al. 2015) could belong to *Placida cremoniana*, but this hypothesis needs to be tested with molecular data.

Phylogenetic relationships

The mtDNA lineage from European specimens was recovered as sister to the widespread Pacific lineage, *P. barackobamai* sp. nov. (see below). No H3 alleles were shared between Mediterranean samples and other species described herein.

Host ecology

We found no record for the algal diet of *P. cremoniana*.

Reproduction

Schmekel and Portmann (1982) described the egg mass as a ring or a short flat spiral-shaped band with white eggs, 50–60 μm in diameter; eggs of that size indicate planktotrophic development for sacoglossans (Krug et al. 2015).

Remarks

Trinchese (1892) described *Hermaea cremoniana* based on specimens collected in Naples, Italy, with a short description and no illustrations. The animal was described as black with the foot, the bases of the cerata, and longitudinal lines on the rhinophores brick red. This description was later expanded (Trinchese 1896) to include illustrations of the live animal (Fig. S2a) and the radular teeth. Pruvot-Fol (1951) described *Ercolania trinchessii* from two specimens collected in Banyuls, Mediterranean coast of France, and

Monaco. The animals were described as having a black head, dorsum, and rhinophores, with white longitudinal lines on the latter, extending into the head and body and surrounding the eyes; cerata with black apices and bright yellow bases (Fig. S2d). Pruvot-Fol (1951) made no reference to the work of Trinchese, suggesting that she was unaware of the early description of *Hermaea cremoniana* or that she did not consider the obvious similarities between the two species. Subsequently, Pruvot-Fol (1954) transferred *Hermaea cremoniana* to the genus *Placida*, but maintained *Ercolania trinchessii* as a distinct and valid species in the genus *Ercolania*. Based on the striking morphological and color similarities between *P. cremoniana* and *E. trinchessii*, Schmekel (1968) considered these species as synonyms, with *P. cremoniana* having priority. Earlier, Fez (1962) described *Hermaea carmeni* based on three specimens collected in the Valencia Harbor, Spain. A live animal was illustrated in dorsal and lateral views (Fez 1962: figs. 3–4; Figs. S2b, c), which clearly matches the characteristics of *P. cremoniana*, currently considered the senior synonym (Ballesteros 2014).

Our molecular and morphological analyses indicate that Mediterranean animals are distinct from Pacific specimens assigned to *P. cremoniana*. Because the type locality of *P. cremoniana* is Naples, Italy, we retain this name for the Eastern Atlantic and Mediterranean species. *Placida cremoniana* is the only species in the complex with a posterior white stripe from the base to the tip of the rhinophores, as well as a yellow-orange line connecting the yellow-orange eye patches to the dorsum (Figs. 1 and 4).

Because we did not have access to specimens from the Caribbean, we cannot determine whether animals from Venezuela assigned to *P. cremoniana* by Caballer Gutiérrez et al. (2015) belong to this species. However, the illustrations of the live animal provided by Caballer Gutiérrez et al. (2015: fig. 5o, p) are very similar to Mediterranean specimens studied herein.

Placida brookae sp. nov.

(Figs. 1b, c, 3a, 4g, h, 6)

ZooBank registration

urn:lsid:zoobank.org:act:60027293-FDCC-4ED1-8C11-670A2224D8DF

Holotype

Catalina Island, California, USA, 5 Nov 2015, 4 mm preserved length, LACM 3469.

Other material examined

Catalina Island, California, 10 Oct 2014, 1 specimen 2.5 mm preserved length, leg. Brook Peterson (CPIC 01265), paratype 1; 5 Nov 2015, 2 specimens 3–4 mm preserved length, leg. Brook Peterson (CPIC 01962), paratypes 2–3. Bahía Balandra, near La Paz, Baja California Sur, Mexico, 4 Jul 1987, tissue only, leg. Terry Gosliner (CASIZ 087217), paratype 4. Gardner Bay, Isla Española, Galápagos Is., Ecuador, 25 May 2005, tissue only, leg. Terry Gosliner (CASIZ 173619), paratype 5.

Derivatio nominis

Named after underwater photographer Brook Peterson, who first collected this species in Catalina Island and drew attention to its possible spread from the tropical to the temperate Eastern Pacific.

Description

Maximum length 6 mm. Rhinophores cylindrical, enrolled, black with posterior white stripes from base to half-way up, leaving apices completely black (Fig. 1b, c). Oral tentacles completely black; foot corners yellow. Head black with two yellow-orange patches, eyespots at base of yellow-orange patches (Fig. 4g). Lateral yellow-orange line on head connects yellow-orange eye patches to dorsum (Fig. 4h). Body elongate, with numerous cylindrical cerata covering most of the dorsum. Proximal half of cerata yellow-orange, distal half black. Foot yellow-orange ventrally from head to tail. Posterior end of foot is pointed, black dorsally.

Radula of a 4 mm long specimen from Catalina Island, California (CPIC 01962) composed of 7 teeth in ascending row, 12 teeth in descending row, and 2–4 teeth in ascus (Fig. 6a). Leading tooth smooth, lacking denticles, with a cylindrical, short, slightly curved, pointed cusp (Fig. 6b). Base of teeth short, triangular, slightly concave ventrally.

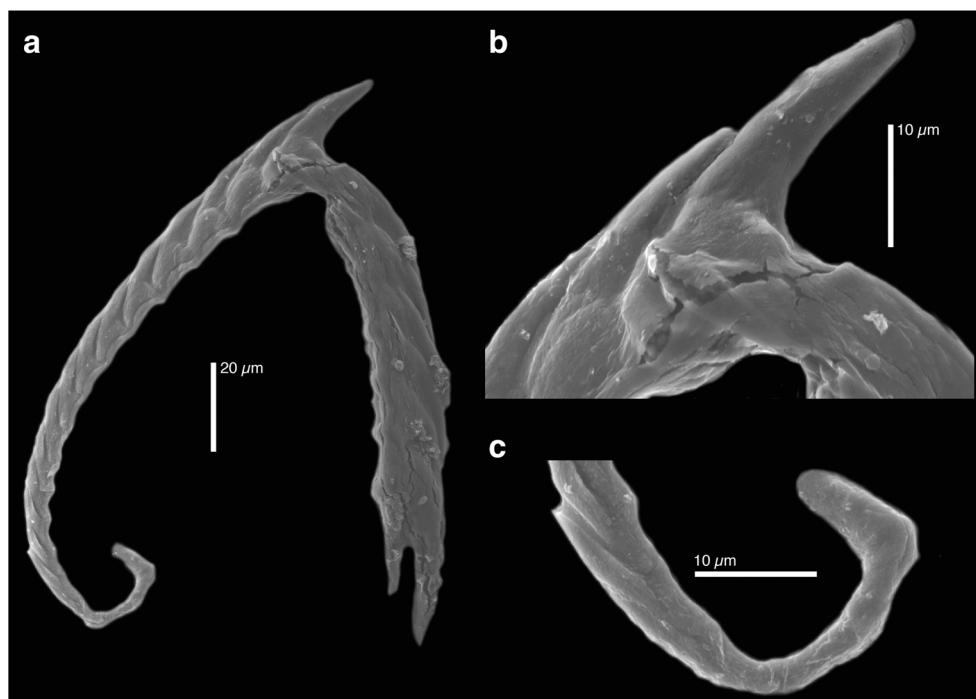
Geographic range

Eastern Pacific from Anacapa Island, California to the Galapagos Islands (present study). Specimens resembling *P. cremoniana* were not previously reported from California, and may represent a recent range expansion.

Phylogenetic relationships

Specimens of *P. brookae* sp. nov. from the Galapagos Islands, in the southern East Pacific, were closely related to specimens from Catalina Island, California, USA, from the northern East Pacific; intraspecific pairwise COI distances between Galapagos and Catalina samples ranged from 0.5 to 1.5%. Analyses of mtDNA relationships indicated that

Fig. 6 *Placida brookae* sp. nov. SEM images of the radula of a specimen from Catalina Is., California (CPIC 01962). **a** View of the entire radula. **b** Leading tooth. **c** Ascus



P. brookae sp. nov. was sister to the Pacific species *P. kevinleei* sp. nov. (see below). Those sister species were each fixed for different H3 alleles, which also showed a sister relationship.

Host ecology

Specimens collected in Catalina Island were found on an unidentified filamentous green alga.

Reproduction

No data on development mode were available for this species.

Remarks

Because there are no available names for orange and black species of the genus *Placida* from the Eastern Pacific, the new name *Placida brookae* sp. nov. is formally introduced here. *Placida brookae* sp. nov. is the only complex member with a posterior stripe of white running from the base only half-way up the rhinophores, and with a lateral yellow-orange line running from the head to the dorsum. The radula of *P. brookae* sp. nov. is characterized by having teeth with relatively short and straight cusps and elongate bases; this is different from the tooth morphology of *P. cremoniana*, in which teeth have more elongated, slightly bowed cusps. The other two new species described here (*P. barackobamai* sp. nov. and *P. kevinleei* sp. nov.) have shorter and more triangular teeth bases.

Placida barackobamai sp. nov.

(Figs. 1d, 3a, 4e, f, 7)

ZooBank registration

urn:lsid:zoobank.org:act:1BB2333D-87F7-462F-AC55-0E7DD266BA06

Holotype

Maliko Bay, Maui, Hawaiian Islands, 18 Jun 2011, 1 mm preserved length, LACM 3470.

Other material examined

Maliko Bay, Maui, Hawaiian Islands, 18 Jun 2011, 1 specimen destroyed during dissection (CPIC 00367). Kapalua Bay, Maui, Hawaiian Islands, 21 Jun 2011, 1 specimen 1 mm preserved length (CPIC 00400), paratype 1. Maliko Bay, Maui, Hawaiian Islands, 22 Jun 2016, 1 specimen 1.5 mm preserved length (CPIC 02037), paratype 2; 22 Jun 2016, 1 specimen 1.5 mm preserved length (CPIC 02038), paratype 3. Sydney, Australia, 2003, size unknown (AM C.469568.1), paratype 4.

Derivatio nominis

This species is named in honor of the 44th President of the United States of America, Barack Hussein Obama, born in the State of Hawaii, where the holotype of this species was collected. With this name, we would like to recognize President Obama for his efforts to reduce carbon emissions globally and lessen the effects of climate change, as well as for his Proclamation on August 26, 2016 that dramatically expanded the Papahānaumokuākea Marine National Monument, in order to preserve this culturally and ecologically important region for future generations.

Description

Maximum length 3 mm. Rhinophores cylindrical, enrolled, black with posterior white stripes from base to tip (Fig. 1d). Oral tentacles black; foot corners yellow with black intrusions (Fig. 4f). Head black with two yellow-orange patches, eye-spots visible near base of yellow-orange patches (Fig. 4e). Body elongate, with numerous cylindrical cerata with pointed apices covering most of the dorsum. Proximal half of cerata yellow-orange, distal half black. Cerata swelling at the middle then tapering to pointed tip. Foot yellow-orange from head to tail ventrally, lined with black on Sydney specimens. Posterior end of foot pointed or blunt-ended (Sydney specimens), black dorsally with orange edges.

Pericardium black, raised. Black renal extension running from pericardium along dorsum approximately one-third of body length. Pronounced anal papilla extending anterior to the pericardium. Everted penis orange, slightly conical, with white rounded tip; stylet visible by light microscopy.

Radula of a 1.5 mm long specimen from the Hawaiian Islands (CPIC 02038) with 8 teeth in ascending row and 12 teeth in descending row (Fig. 7a). Leading tooth smooth, lacking denticles, with cylindrical, needle-like pointed cusp, slightly curved towards tip (Fig. 7b). Base of teeth short, slightly concave.

Geographic range

Hawaiian Islands, to temperate Eastern Australia, and temperate Japan (present study). Literature records from New South Wales, Australia (Rudman 2001; Schulz 2005) could belong to this species.

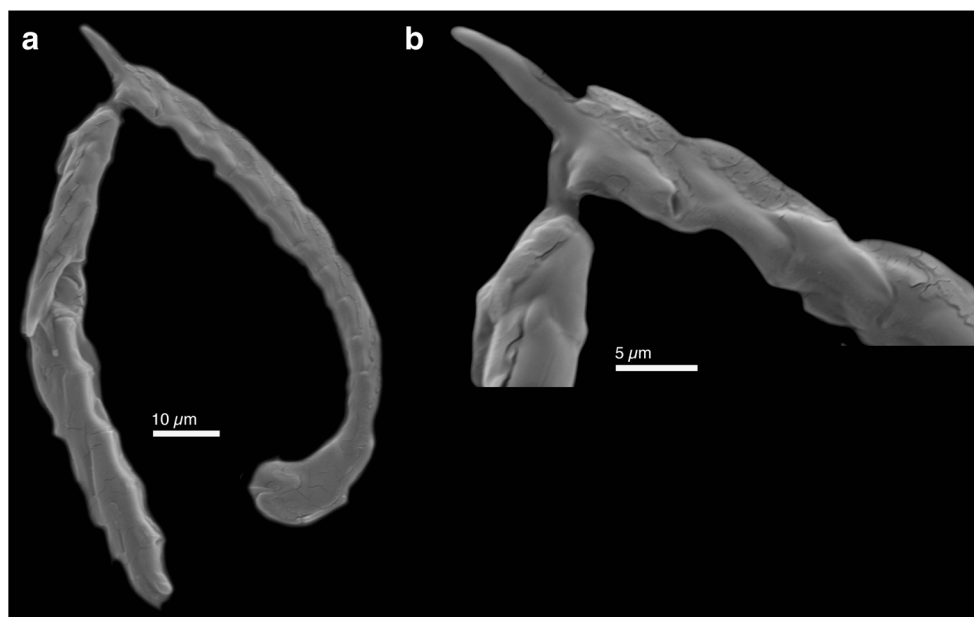
Phylogenetic relationships

Despite the wide distribution of this species across the Central and Western Pacific, the maximum divergence among COI haplotypes was only 2.3% (TrN distance), and some haplotypes from Australia and Hawaii were only 0.3% divergent. There was mild support in the ML analysis (BS = 78) for a subclade of mtDNA haplotypes comprising samples from Japan and Guam, suggesting some geographical structuring within this species. Evolutionarily, *P. barackobamai* sp. nov. was sister to European specimens of *P. cremoniana* in analyses of mtDNA (Fig. 3a), and H3 alleles in *P. barackobamai* sp. nov. were closely related to the lone sampled Mediterranean allele.

Host ecology

Specimens were collected intertidally beneath rock overhangs at Long Reef near Sydney, Australia, in association with a filamentous green alga identified as *Derbesia* sp. The alga had irregularly spaced side branches emerging from the main axis, which was partitioned by widely spaced septa; no sporangia were evident. *Derbesia* sp. was readily consumed by specimens of *P. barackobamai* sp. nov. in the laboratory; when slugs were starved for 2 days and offered *Codium fragile* (Suringar) Hariot 1889, they remained on *C. fragile* and were observed to feed, but not as readily as they consumed *Derbesia* sp.

Fig. 7 *Placida barackobamai* sp. nov., SEM images of the radula of a specimen from Maui, Hawaiian Is. (CPIC 02038). **a** View of the entire radula. **b** Leading tooth



Some small specimens of a species identified as *Placida* cf. *dendritica* were collected near Sydney, Australia from *Codium fragile* and *Codium arabicum* Kützing, 1856 (Bare Island), and morphologically similar specimens were collected from *Derbesia* sp. (Long Reef), together with *P. barackobamai* sp. nov. When specimens of *Placida* cf. *dendritica* from *Codium* sp. were starved for 2 days and offered *Derbesia* sp., the slugs fed immediately; their diverticula flushed green within an hour, and frequent excretion was observed from the dorsal anal papilla while feeding.

Reproduction

Two egg masses were laid by specimens from Sydney, Australia, with mean egg diameters of $54.1 \pm 1.3 \mu\text{m}$ SD ($n = 7$) and $54.0 \pm 1.0 \mu\text{m}$ SD ($n = 8$), respectively, egg sizes indicating planktotrophic development (Krug et al. 2015).

Remarks

Placida barackobamai sp. nov. is the only species of the complex with a posterior white stripe running from the base to the tip of the rhinophores, as well as a completely black head, with no lateral yellow-orange line. The radular teeth of *P. barackobamai* sp. nov. are distinguishable from those of *P. cremoniana* and *P. brookae* sp. nov. by having more triangular, shorter bases, while *P. kevinleei* sp. nov. (see below) also has triangular bases but with more elongate cusps than in *P. barackobamai* sp. nov.

Placida kevinleei sp. nov.

(Figs. 1e, 3a, 4c, d, 8)

ZooBank registration

urn:lsid:zoobank.org:act:2A91A53D-2291-48E3-BBC4-0878D4E67408

Holotype

Sobe, Okinawa Prefecture, Japan 04 Nov 2003, 5 mm preserved length, LACM 3471.

Other material examined

Maliko Bay, Maui, Hawaiian Islands, 22 Jun 2016, 1 specimen 1 mm preserved length, destroyed during dissection.

Derivatio nominis

This species is named in honor of Kevin Lee, adventurer, naturalist, and photographer. Kevin Lee has generously collected and contributed important specimens, photographs, and other data for scientific research over the years. His capacity to overcome adversity and his resilience are an inspiration to many.

Description

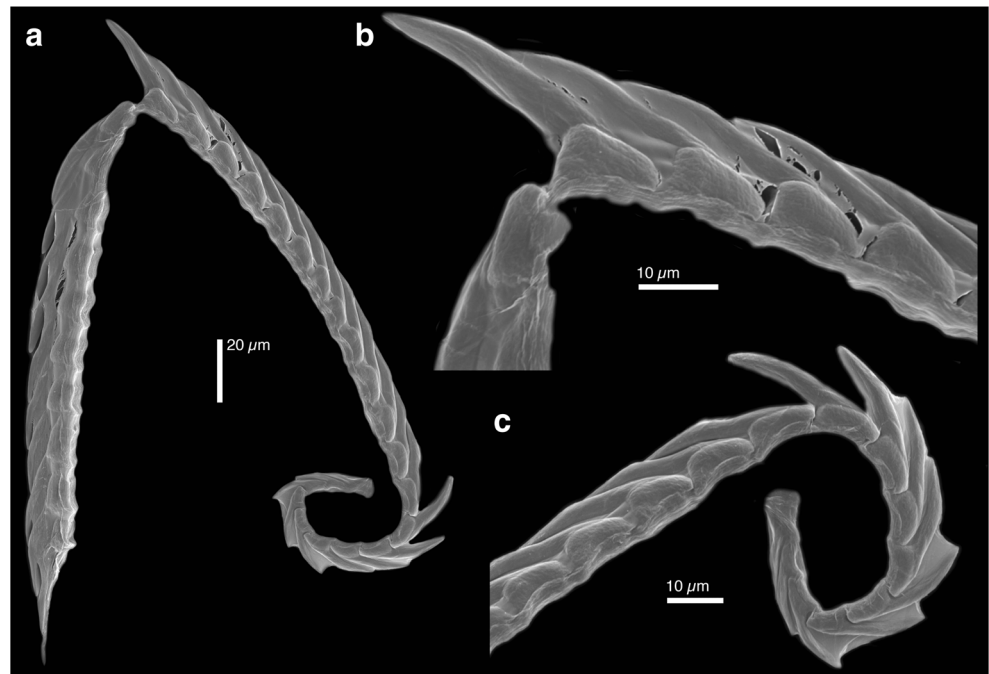
Maximum length 8 mm. Rhinophores are cylindrical, en-rolled, black with posterior white stripes from base to half-way up, leaving apices completely black (Fig. 1e). Oral tentacles black; foot corners yellow (Fig. 4f). Head black with two yellow-orange patches, eyespots visible near base of yellow-orange patches (Fig. 4e). Body elongate, with numerous cylindrical cerata with pointed apices covering most of the dorsum. Proximal half of cerata yellow-orange, distal half black. The foot is yellow-orange from head to tail ventrally. Foot yellow-orange from head to tail ventrally. Posterior end of foot is pointed, black dorsally.

Radula of a 5 mm long holotype from Sobe, Japan (LACM 3471) composed of 10 teeth in ascending row, 12 teeth in descending row, and 10 in the ascus (Fig. 8a). Leading tooth smooth, lacking denticles, with cylindrical, needle-like cusp, slightly curved towards tip (Fig. 8b). Base of teeth robust, triangular, slightly concave. Ascus with teeth organized in single row (Fig. 8c).

Geographic range

Hawaiian Islands and tropical Japan (present study). Other records in the literature from Korea (Koh 2006), the Kerama Islands, Japan (Ono 1999; Nakano 2004; Gosliner et al. 2008,

Fig. 8 *Placida kevinleei* sp. nov., SEM images of the radula of the holotype from Okinawa, Japan (LACM 3471). **a** View of the entire radula. **b** Leading tooth. **c** Ascus



2015), and Indonesia (Warren 2000; Behrens 2004) could belong to this species.

Phylogenetic relationships

Placida kevinleei sp. nov. was sister to *P. brookae* sp. nov. in analyses of mtDNA (Fig. 3a). Each species was fixed for a different H3 allele, which also showed a fully supported sister relationship (Figs. 3a and S1).

Host ecology

Trowbridge et al. (2010) studied the algal diet of specimens identified as *P. cremoniana* in Sobe, Japan, from which location we sampled *P. kevinleei* sp. nov. They reported specimens likely representing *P. kevinleei* sp. nov. as associated in the field with sporophytes of the filamentous green alga *Derbesia* sp., which commonly fouled other branching algae (Trowbridge et al. 2010). In pairwise choice assays, slugs rejected two species of the genus *Codium*, *C. geppiorum* O.C. Schmidt, 1923 and *C. arabicum*, both as food and as hosts to rest upon (Trowbridge et al. 2010). One juvenile specimen fed and grew on *Bryopsis harveyana* J. Agardh, 1887, whereas two adult slugs fed selectively on *Derbesia* sp.; slugs preferentially affiliated with *Derbesia* sp. in laboratory trials (56% occupancy) versus *Bryopsis* sp. (17% occupancy).

Reproduction

No data available.

Remarks

Placida kevinleei sp. nov. is the only species of the complex that has a posterior white patch on the rhinophores that occupies only the proximal half as well as a completely black head, with no lateral yellow-orange lines. The radular teeth of *P. kevinleei* sp. nov. are distinct from those of the other species by having short triangular bases and very elongate cusps.

Discussion

Divergence and speciation in the *P. cremoniana* complex

Molecular species delimitation and congruent morphological differences indicated that the nominal species *P. cremoniana* constituted a global species complex of at least four distinct taxa. Phylogenetic analyses revealed two deep splits reflecting allopatric origins of the two pairs of sister species: one split between Eastern Atlantic and Mediterranean *P. cremoniana* and Western Pacific *P. barackobamai* sp. nov., and a second split between Western Pacific *P. kevinleei* sp. nov. and Eastern Pacific *P. brookae* sp. nov. Although *P. barackobamai* sp. nov. and *P. kevinleei* sp. nov. are sympatric at least in Hawaii and Japan, they are not sister taxa. Other studies of species complexes in benthic heterobranchs revealed cryptic allopatric taxa in different biogeographic regions (e.g., Krug et al. 2007; Ornelas-Gatdula et al. 2012; Alexander and Valdés 2013; Carmona et al. 2014). Allopatric divergence driven by vicariance or environmental differences between biogeographic regions has been suggested as the predominant mechanism of speciation in the sea (Mayr 1954). Divergence between *P. kevinleei* sp. nov. and *P. brookae* sp. nov. was likely promoted by the Eastern Pacific Barrier, a 5000-km expanse of deep water that inhibits gene flow in diverse marine lineages (Baums et al. 2012; Cowman and Bellwood 2013). The geographical divide between Mediterranean and East Atlantic *P. cremoniana* and Indo-Pacific *P. barackobamai* sp. nov. could have arisen through dispersal and vicariance, but greater sampling around the

coast of Africa and in the Caribbean is needed to reconstruct the biogeographic history of this complex.

In contrast to the historical attention paid to dispersal barriers, studies of pelagic heterobranchs unveiled sympatric species in which sexual selection on reproductive anatomy was proposed as the driver of reproductive isolation (Churchill et al. 2013, 2014). Other studies have recovered similar cases of sympatric sister species in benthic sea slugs that differ in reproductive anatomy, suggesting a role for sexual selection in speciation (Krug et al. 2016). Further studies on the reproductive systems of *P. cremoniana* complex members are needed to assess whether sexual selection is a strong evolutionary force in this group.

Shifts to non-dispersive larvae have also been suggested to drive reproductive isolation in sacoglossans (Krug et al. 2015; Ellingson and Krug 2016); however, the small egg sizes of two of the four *P. cremoniana* complex members (*P. cremoniana* and *P. barackobamai* sp. nov.) indicate that they produce dispersive, planktotrophic larvae (the egg size and development for *P. kevinleei* sp. nov. and *P. brookae* sp. nov. are unknown). Planktotrophy typically confers substantial dispersal abilities during the larval stage, consistent with the low genetic divergence within the species described here, despite their large geographical ranges. Development mode is, therefore, likely to inhibit local adaptation and divergence in the *P. cremoniana* complex, rather than facilitating speciation.

Finally, disruptive selection on host use may promote ecological speciation in sacoglossans, due to the coevolutionary dynamics between slugs and their obligate host algae (Krug 2011; Krug et al. 2016). In the *P. cremoniana* complex, *P. kevinleei* sp. nov. and *P. barackobamai* sp. nov. both fed on *Derbesia* sp., while an unidentified filamentous green alga was the host of *P. brookae* sp. nov. on Catalina. Further study is needed to assess the host of *P. brookae* sp. nov. in the tropical Eastern Pacific and for *P. cremoniana* in the Mediterranean, but there is no evidence that differential host use contributed to recent speciation within the complex. Differences in radular morphology among species may reflect drift or local adaptation to different species of the genus *Derbesia*.

While patterns of field association and laboratory feeding observations indicate that *Derbesia* sp. is the preferred host of *P. kevinleei* sp. nov. and *P. barackobamai* sp. nov., our observations and those of Trowbridge et al. (2010) demonstrate that these species retain the ability to feed on species of the genera *Bryopsis* or *Codium*, respectively. Similarly, we found a species resembling the widespread *P. dendritica* on both *Codium* sp. and *Derbesia* sp. in Australia. Thus, there is greater ecological and evolutionary plasticity for *Placida* spp. to switch between the genera *Derbesia* and *Codium/Bryopsis* than is generally recognized. However, *P. cremoniana* complex members have never been reported to associate with *Codium* spp. in the field, indicating some degree of ecological

specialization on *Derbesia* spp. or morphologically similar filamentous algae. Because species of the genera *Codium* and *Bryopsis* are the prevailing host algae for all other well-studied *Placida* spp. (Trowbridge et al. 2010), it is possible that the *P. cremoniana* complex may have originated via disruptive selection on host use favoring *Derbesia* sp. in the last common ancestor of the lineage. However, based on the available data, this is speculative and will require further research.

Host use may also be relevant to understanding the evolution of bright orange coloration in the *P. cremoniana* complex. *Derbesia* spp. can be chemically defended by secondary metabolites, including acetylated diterpenoids with antifeedant properties that are stored by other sacoglossans (Gavagnin et al. 1993, 1994). Sequestration of algal secondary metabolites has not been reported for the *P. cremoniana* complex. However, a host shift from *Codium* spp. onto the more chemically rich algal genus *Derbesia* in the ancestor of the *P. cremoniana* complex may have favored the evolution of aposematic coloration if complex members store algal toxins and employ them as a chemical defense against predators.

Biogeography and evolutionary history

The biogeography of complex members and their phylogenetic relationships together offer insights into the general patterns of vicariant speciation proposed for marine animals. In many heterobranch groups examined to date (Valdés 2004; Ornelas-Gatdula et al. 2012), as well as diverse other benthic invertebrate groups (Marko 2002), Eastern Pacific taxa are typically sister to Caribbean (Atlantic) taxa, suggesting that the formation of the Isthmus of Panama split the range of a common ancestor (Knowlton et al. 1993). However, recent phylogenetic studies of sea slugs have more commonly found sister relationships between Western and Eastern Pacific taxa, or between Atlantic and tropical Pacific lineages (Camacho-García et al. 2014; Krug et al. 2015, 2016; Goodheart et al. 2015). Indeed, the *P. cremoniana* species complex presents yet another example of this emerging paradigm for heterobranch biogeography, as both the Eastern Pacific and the Atlantic species were each recovered as sister to a Western Pacific species. This suggests more complex biogeographic scenarios, including the importance of the Eastern Pacific Barrier to dispersal and speciation. However, comprehensive sampling of additional undescribed complex members from the Indian Ocean, tropical Australia, and Caribbean are needed to elucidate fully the evolutionary history of diversification in this group.

Taxonomic history and real versus apparent range expansions

The putative existence of pantropical species complexes in sacoglossans has typically been documented over extended

periods of time across their range [e.g., *Elysia ornata* (Swainson, 1840), Jensen 1992]. Until 1959, specimens resembling *P. cremoniana* were unknown outside the Mediterranean Sea. Because species in the *P. cremoniana* complex have a distinctive color pattern, it is unlikely that these animals were detected earlier but misidentified. Also, species in the *P. cremoniana* complex are conspicuous on green algal hosts, and difficult to overlook by experienced collectors. An apparent rapid range expansion of *P. cremoniana* starting in the 1990s and continuing in the 2000s could be consistent with anthropogenic introduction. Alternatively, earlier detection of complex members may have simply gone unreported or awaited more recent investigation of remote areas by taxonomic experts.

Our findings suggest that, rather than a global expansion, the recent reports of animals identified as *P. cremoniana* from tropical and subtropical waters across the Pacific reflect the recent documentation of members of a pseudocryptic species complex. We term the new species described here as pseudocryptic because they can be distinguished by subtle differences in external appearance, as well as differences in radular morphology and gene sequences. While *P. cremoniana* from the Mediterranean was historically the subject of extensive research, regions such as Japan, California, and Australia harbor numerous inconspicuous species of sea slugs that were, nevertheless, documented much earlier than the first records of an animal identified as *P. cremoniana* from those areas. It may be impossible to determine whether the absence of historical records for any of the newly described species reflects their prior absence from a given region, lack of collection effort, or failure to report a species known to occur locally. Contributing to the lack of earlier records may be the small size of complex members in some areas, low abundance, and/or their affiliation with patches of filamentous microalgae that are difficult to collect, compared with a large thallus of *Codium* spp. or clumps of *Bryopsis* spp.

An alternative possibility is that the species described here were formerly restricted to tropical regions that are understudied by taxonomic experts, but that these taxa recently experienced range expansions into temperate regions as a consequence of climate change (or, less likely, the introduction of their algal host). Movement into warm-temperate waters of Australia, Japan, and California, USA, may have brought the new species to areas where experts reside and perform field studies, including Hirano and Trowbridge in Japan, Rudman and Wilson in Sydney, Australia, and the present authors in Los Angeles, California. We hypothesize that warming trends and a recent El Niño event explain the expansion of *P. brookae* sp. nov. northward to Catalina Island off California, an area well surveyed for decades by sea slug experts and enthusiasts from which this species was not previously reported. A related hypothesis is that the newly described species in the complex

have become increasingly abundant due to increased algal density in tropical systems as a consequence of climate change (Hughes et al. 2003; Mumby et al. 2007; Hoegh-Guldberg et al. 2007).

Color pattern and additional cryptic diversity

Historically, authors were unable to distinguish species in the *P. cremoniana* species complex. However, guided by molecular data, small differences in color pattern became evident. The main traits that distinguish species are the distribution of white pigment on the rhinophores (either occupying the entire rhinophores, as in *P. cremoniana* and *P. barackobamai* sp. nov., or the basal half only, as in *P. brookae* sp. nov. and *P. kevinleei* sp. nov.) and the presence of yellow-orange lines emerging from the dorsal yellow-orange patches on the head (present in *P. cremoniana* and *P. brookae* sp. nov., absent in the other two species). These color traits are consistent in the specimens we sequenced, and, thus, were used to infer the range of each species from available photographic records.

In addition to the four species here recognized, a review of the literature and online photographs suggests that there could be additional species in areas not sampled in our study. For example, specimens from the Indian Ocean have white lines on the rhinophores occupying only the proximal half, as in *P. kevinleei* sp. nov., but also have a connection between the yellow areas on the head and the foot (see Bidgrain 2010 and Fig. 1f), which is not present in other specimens of *P. kevinleei* sp. nov. from the Central Pacific. Specimens from tropical Australia documented by Coleman (2008: 77) have an almost completely yellow-orange head and pericardium, and a translucent white posterior end of the foot. These animals are very different from any of the other species here documented; thus, they may constitute further distinct species. Finally, specimens of *P. cremoniana* from the Caribbean are externally similar to those from the Eastern Atlantic, but due to their geographic separation, they may also comprise a distinct species in the tropical Western Atlantic.

Conclusions

The colorful and nominally circumtropical species *Placida cremoniana* was found to comprise four distinct species, three of which were previously undescribed. Including potentially unsampled members, the eventual diversity in the *P. cremoniana* complex may be six or more species. Notably, the diversity of all other well-recognized species in the genus *Placida* is only six taxa: the three named outgroup species in the present study, plus *Placida babai* Marcus, 1982, *Placida daguilarensis* Jensen, 1990, and *Placida fralila* Burn, 1966 (Jensen 2007; Chichvarkhin et al. 2016). Although *P. dendritica* likely also represents a species complex, the

failure to recognize pseudocryptic species in the *P. cremoniana* complex historically resulted in a significant underestimate of the true species richness in the genus *Placida*, in which complex members may eventually comprise up to 50% of named species. Integrative studies thus continue to expand our understanding of sacoglossan diversity and, by increasing taxon sampling for phylogenetic and biogeographic studies, yield new insights into the evolutionary mechanisms driving diversification. Our findings suggest that, as a lineage, the *P. cremoniana* complex may have originated via ecological factors favoring a host shift from the genera *Codium/Bryopsis* onto *Derbesia* spp., potentially leading to the evolution of aposematic coloration associated with chemical defenses acquired from the algal diet. However, recent divergence appears to have been primarily allopatric, including a significant role for the Eastern Pacific Barrier limiting trans-Pacific dispersal.

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