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A review of taxonomy and phylogenetic relationships in the genus *Costasiella* (Heterobranchia: Sacoglossa), with a description of a new species

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ABSTRACT

The history of the genus *Costasiella* is summarized. A new species of *Costasiella*, *C. arenaria* n. sp., from Lake Worth, Florida, USA is described. For comparative purposes specimens of *C. formicaria* (Baba, 1959) from Japan and serial sections of *C. pallida* Jensen, 1985 from Hong Kong have also been examined. These three species, like the type species *C. virescens* Pruvot-Fol, 1951 and *C. coronata* Swennen, 2007 from Thailand are not associated with the green alga *Avrainvillea*, which is the food of most known species of the genus. They share several morphological characters, i.e. pharynx without muscular pouches, radular teeth with narrow, smooth, blade-shaped cusps and short bases, unarmed penises and branches of albumen gland entering the cerata. A molecular phylogeny of 14 species of *Costasiella* based on four genes (mitochondrial COI and 16S rRNA and nuclear 28S rRNA and H3) supports morphological findings that the species not associated with the green alga *Avrainvillea* form a monophyletic group within the genus.

INTRODUCTION

The first description of the limapontioid genus *Costasiella* was based on a single specimen of the type species *C. virescens* Pruvot-Fol, 1951. The description was very short and no anatomical details were given. Nine years later a second species, *C. nonatoi*, was described by Marcus & Marcus (1960). This description was based on two specimens, one of which was serially sectioned and designated as the holotype. It was also indicated that a much older species, *Doto ocellifera* Simroth, 1895, might be a *Costasiella*. Later, the Marcuses doubted the validity of the genus *Costasiella*, and *C. nonatoi* was first transferred to *Placida* (Marcus & Marcus, 1970) and then back to *Costasiella* (Marcus, 1982). Meanwhile, a species with admedian eyes and long pedal tentacles had been described as *Stiliger formicarius* from Japan (Baba, 1959). This was transferred to *Costasiella* by Baba (1961). The next two species were described as *Stiliger (Ercolania) illus* Marcus, 1965 from a flabellate alga in the Caroline Islands, and *Stiliger (Stiliger) lilianae* Marcus & Marcus, 1969 from Brazil. The former was not transferred to *Costasiella* until 1982 (Marcus, 1982), whereas the latter was first transferred to *Ercolania* (Marcus, 1976) and then to *Costasiella* by Thompson (1977), and finally synonymized with *C. ocellifera* by Clark (1984).

The association of *Costasiella* species with algae of the genus *Avrainvillea* was noted for the two Caribbean species, *C. nonatoi* and *C. ocellifera*, in a series of papers (Clark *et al.*, 1981; Jensen, 1981, 1983; Jensen & Clark, 1983; Clark, 1984) and subsequently several species were described from this alga in Japan (Ichikawa, 1993) and Australia (Jensen, 1997). Some of these species were later recorded from other places in the Indo-West Pacific, including Guam (Carlson & Hoff, 2003), Australia (Händeler *et al.*, 2009) and Singapore (Jensen, 2009). Meanwhile, a new species, *C. pallida* Jensen, 1985, was described from Hong Kong, which fed on *Vaucheria* rather than *Avrainvillea* (Jensen, 1985, 1990). Neither the type species, *C. virescens*, nor the Japanese *C. formicaria* has been found associated with *Avrainvillea*. Finally, Swennen (2007) described *C. coronata* from Thailand, which feeds on *Vaucheria*.

Species of *Costasiella* seem to combine a mixture of limapontioid morphological characters, some species having one or more of the following characters: pedal tentacles, a tail shaped like a ceras, a penial stylet, pharyngeal pouches and albumen gland in the cerata. Therefore a separate family, Costasiellidae Clark, 1984, was eventually created. Phylogenetic analysis based on anatomical characters indicated, however, that this was unwarranted (Jensen, 1996). Subsequent molecular analyses have been inconclusive about the affinity of the genus (Händeler *et al*., 2009; Maeda *et al*., 2010).

In the present study we provide a preliminary review of *Costasiella*. We describe a new species collected in Florida in 2008 and redescribe *C. formicaria* from specimens collected in Japan. The anatomy of the latter species has not been described previously, except for light-microscopic drawings of radular teeth. We further include comparative information from serial sections of *C. pallida* from Hong Kong. The species not associated with *Avrainvillea* are compared anatomically with some *Avrainvillea*-associated species and a molecular phylogeny of the genus is presented.

MATERIAL AND METHODS

Morphological analysis

Specimens from Florida were collected in Lake Worth Lagoon by A. Dupont and J. Townsend and preserved directly in ethanol to allow DNA extraction. Underwater photos, including some of spawning animals, and video sequences were provided by several local divers (see Acknowledgements). Specimens of *Costasiella formicaria* were collected by M. Nishina on 2 March 2008 at Futou, northeastern side of Izu Peninsula, east coast of central Japan, at a depth of 8 m. The animals were photographed alive and likewise preserved in alcohol.

Specimens were studied after fine dissection. Tissues were brittle due to fixation directly in alcohol, and especially the thin reproductive ducts were impossible to trace. Digital photos were taken during dissections to document the position of organs. Radulae were extracted by placing the pharynx in NaOH to dissolve the tissues. They were then rinsed in demineralized water and at least two rinses of 96% ethanol prior to drying and placing on SEM-stubs. Penises were dissected out and photographed under Light Microscopy, after which they were dehydrated in a series of acetone, dried and mounted on SEM-stubs.

For comparisons serial sections of *C. pallida* Jensen, 1985 from Hong Kong (Jensen, 1990), *C. usagi* from Singapore (Jensen, 2009), and *C. ocellifera* from St. Thomas, US Virgin Islands (Jensen, 1991) were re-examined, also including a few specimens of *C. ocellifera* collected by K.B. Clark in 1992 in Florida. In addition, literature information was included for all known species of *Costasiella*.

Molecular phylogenetic analyses

Specimens were provisionally identified by the authors and colleagues and preserved in 95–100% ethanol, or were obtained from museum collections (Table 1). Samples included seven unidentified species, which were given a species number, including five taxa provisionally identified as matching the description of *C. kuroshimae* (labeled 'cf. *kuroshimae*' plus a species number). Sequence data were generated for 14 species, including divergent lineages of *C. ocellifera* from Florida (lecithotrophic development) and Bermuda (planktotrophic development) (Ellingson, 2006). We sequenced portions of four genes: the mitochondrial cytochrome *c* oxidase I (COI) and large ribosomal subunit (16S) rRNA genes, and the nuclear histone III (H3) and large ribosomal subunit (28S) rRNA genes. Amplifications and sequencing reactions used universal primers and reaction conditions as described by Händeler *et al*. (2009) and, for H3, by Krug *et al*. (2013). Sequences have been deposited in the National Center for Bioinformatics (NCBI) database and accession numbers are given in Table 1. Alignments were performed using the MUSCLE multiple sequence alignment option (with default settings) in Geneious v. 6.1.6 and refined by eye for 16S and 28S. Gaps were coded as missing data. Final aligned sequence partitions were 658 bp (COI), 405 bp (16S), 1387 bp (28S) and 328 bp (H3).

Table 1. Provisional species identifications, sample codes, collection details and NCBI accession numbers for Costasiella species.

Species	Sample code	Collection locality	Date	Collector/museum reg. no.	COI	16S	28S	H3
Costasiella arenaria n. sp.	Cos_are_08FL01	Lake Worth, Florida, U.S.	26 Aug 2008	A. DuPont/ ZMUC GAS-326	KJ610066	KJ610026	KJ610039	KJ610053
C. coronata	Ccor_11HK01	Hong Kong	6 Apr 2011	C. Swennen	KJ610067	KJ610027	KJ610040	KJ610054
C. formicaria	Cfor_08Jap03	Futou, Izu Peninsula, Japan	2 May 2008	M. Nishina	KJ610068	KJ610028	KJ610041	KJ610055
C. ocellifera	Coce_06LKey02L	Florida Keys, U.S.	Oct 2006	P. Krug	KF438671	KF438669	–	KF438673
C. ocellifera	Coce_06Ber05P	Bermuda	Jun 2006	P. Krug	KF438672	KF438670	KJ610042	KF438674
C. nonatoi	Cnon_04Pan01	Bocas del Toro, Panama	Dec 2004	P. Krug	KJ610069	KJ610029	KJ610043	KJ610056
C. paweli	Cpaw_8Mor01	Moorea, French Polynesia	7 May 08	R. Ellingson	KJ610070	KJ610030	KJ610044	KJ610057
C. usagi	Cusa_09Gua01	Cocos lagoon, Guam	Aug 2009	P. Krug	KJ610071	KJ610031	KJ610045	KJ610058
C. sp.1, cf. *kuroshimae*	Cos_sp1_09Gua05	Cocos lagoon, Guam	Aug 2009	P. Krug	KJ610072	KJ610032	KJ610046	KJ610059
C. sp.2, cf. *kuroshimae*	Cos_sp2_09Gua05	Cocos lagoon, Guam	Aug 2009	P. Krug	KJ610073	KJ610033	KJ610047	KJ610060
C. sp.3, cf. *kuroshimae*	Cos_sp3_09Gua01	Cocos lagoon, Guam	Aug 2009	P. Krug	KJ610074	KJ610034	KJ610048	KJ610061
C. sp.4	Cos_sp4_09Gua01	Cocos lagoon, Guam	Aug 2009	P. Krug	KJ610075	KJ610035	KJ610049	KJ610062
C. sp.5	Cos_sp5_06Van01	Espiritu Santo Island, Vanuatu	4 Oct 2006	Y. Camacho/ CAS 176872	KJ610076	KJ610036	KJ610050	KJ610063
C. sp. 6, cf. *kuroshimae*	Cos_sp6_05Jap01	Sobe, Okinawa, Japan	30 Nov 2005	C. Trowbridge, Y. Hirano, Y. Hirano	KJ610077	KJ610037	KJ610051	KJ610064
C. sp. 7, cf. *kuroshimae*	Cos_sp7_05Jap02	Zanpa, Japan	2 Dec 2005	C. Trowbridge, Y. Hirano, Y. Hirano	KJ610078	KJ610038	KJ610052	KJ610065

Abbreviations: CAS, California Academy of Science Invertebrate Zoology collection; ZMUC, Zoological Museum of Copenhagen University.
dash = unsuccessful amplification.

As individual gene trees showed no conflict among loci, a concatenated alignment was analysed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods. A phylogenetic analysis of 202 sacoglossan species (Krug *et al.*, submitted) failed to recover a sister group for *Costasiella*; however, *C. nonatoi* and *C.* sp. 4 were basal and highly divergent from all other *Costasiella* species, and were therefore used as outgroups in the following analyses. For BI analyses, mixted models of sequence evolution were parameterized during Markov-chain Monte Carlo (MCMC) runs in *BayesPhylogenies* (Pagel & Meade, 2004). The best-fit out of three GTR + Γ models was assigned to each nucleotide position, avoiding a priori partitioning by gene; a fourth model did not improve likelihood scores. Four independent Markov chains were run for 10^8 generations, saving a tree every 1,000 generations, and discarding the first 80% as burn-in. Each run converged on the same topology and yielded nearly identical post-burn-in harmonic mean log-likelihood (L) scores (maximum difference of L scores = 2.7). Post-burn-in tree samples were therefore pooled for all four runs to estimate posterior probability (PP) support for nodes.

ML analyses were performed using RAxML v. 7.6.6 (Stamatakis, 2006) through the CIPRES Science Gateway v. 3.3 (Miller, Pfeiffer & Schwartz, 2010, http://www.phylo.org). A single GTR + Γ model of sequence evolution was parameterized using 25 rate multipliers and 250 bootstrap pseudoreplicates were used to assess nodal support, taking values ≥70% as significant (Hillis & Bull, 1993).

For identification of the diet, several cerata from four specimens of *C. arenaria* n. sp. were sent to Dr Gregor Christa from the University of Bonn, Germany, who kindly analysed the contents of the digestive gland tubules using molecular methods.

SYSTEMATIC DESCRIPTIONS

Order: Sacoglossa von Ihering, 1876
Suborder: Plakobranchacea Gray, 1840
Superfamily: Limapontioidea Gray, 1847
Costasiellidae Clark, 1984
***Costasiella* Pruvot-Fol, 1951**

Type species: C. virescens Pruvot-Fol, 1951 by monotypy.

Costasiella arenaria new species
(Figs 1, 2, 3)

Types: Holotype 2 mm long × 1.5 mm wide preserved, Lake Worth Lagoon, Florida (USA), 26 August, 2008 (Zoological Museum of

Figure 1. *Costasiella arenaria* n. sp.: live animals in the field. **A.** Light green specimen with brown dorsal body and stripes on rhinophores (photo by J. Townsend). **B.** Dark brown specimen with dark pigment on cerata as well as body surface (photo by J. Townsend). **C.** Spawning specimen, light green colored with almost no brown pigment (photo by S. Edwards). **D.** Spawning specimen, pale with many red and white dots (photo by L. Ianniello).

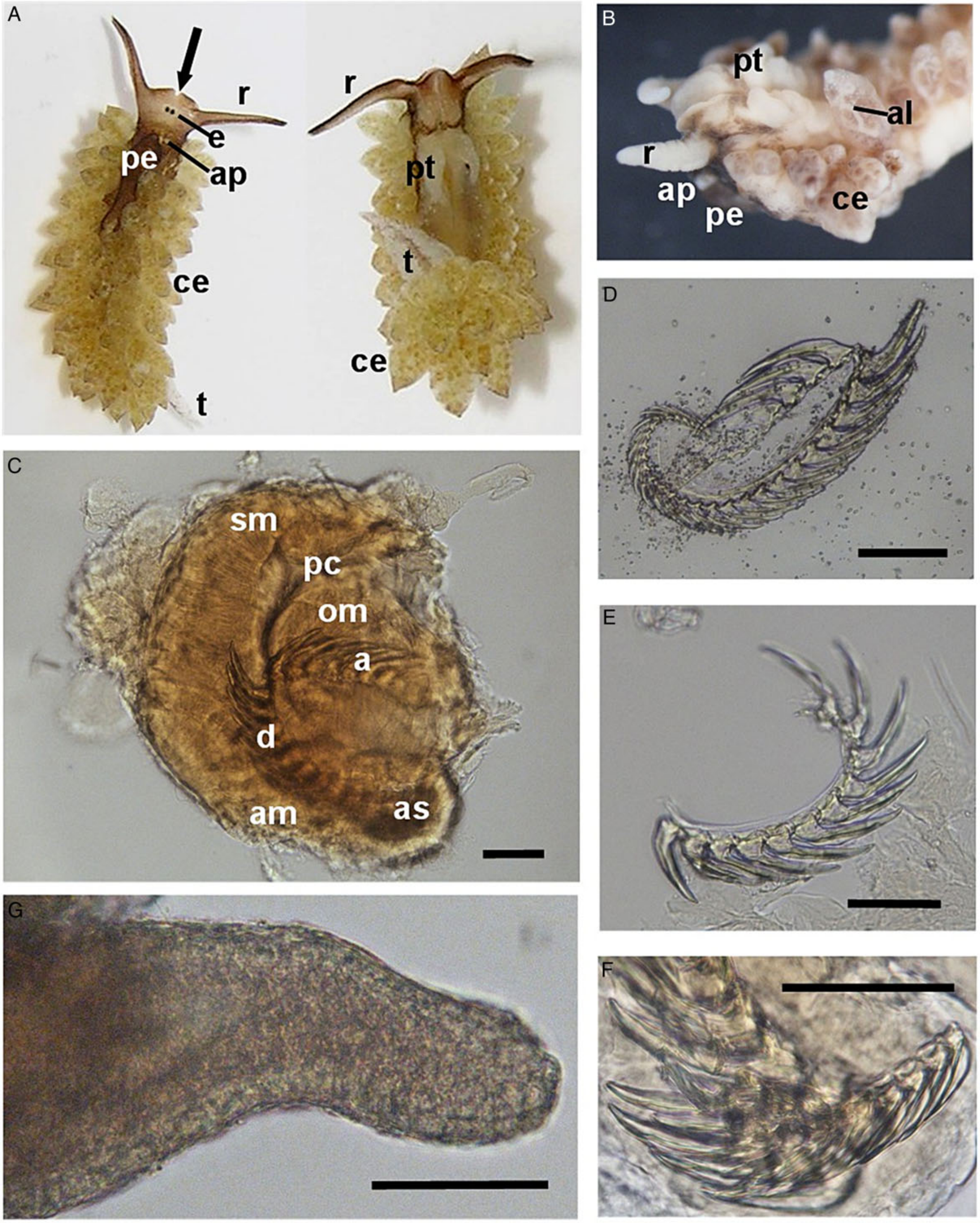

Figure 2. *Costasiella arenaria* n. sp. **A.** Live specimens photographed in the laboratory, dorsal and ventral view (photos by A. Dupont). **B.** Lateral view of preserved specimen. **C.** Pharynx of specimen no. 2, partly cut sagittally. **D.** Radula of specimen no. 6. **E.** Descending limb of radula of specimen no. 2. **F.** Teeth in ascus of specimen no. 2. **G.** Penis of specimen no. 1. Abbreviations: a, ascending limb; al, albumen gland tubule in ceras; am, ascus muscle; ap, anal papilla; as, ascus; ce, cerata; d, descending limb; e, eyes; om, odontophore muscle; pc, pharyngeal cavity; pe, pericardium; pt, pedal tentacle; r, rhinophore; sm, dorsal septate muscle (partly cut off); t, tail. Arrow points to anterior concavity in head. Scale bars: **C–E** = 100 μm; **F, G** = 50 μm.

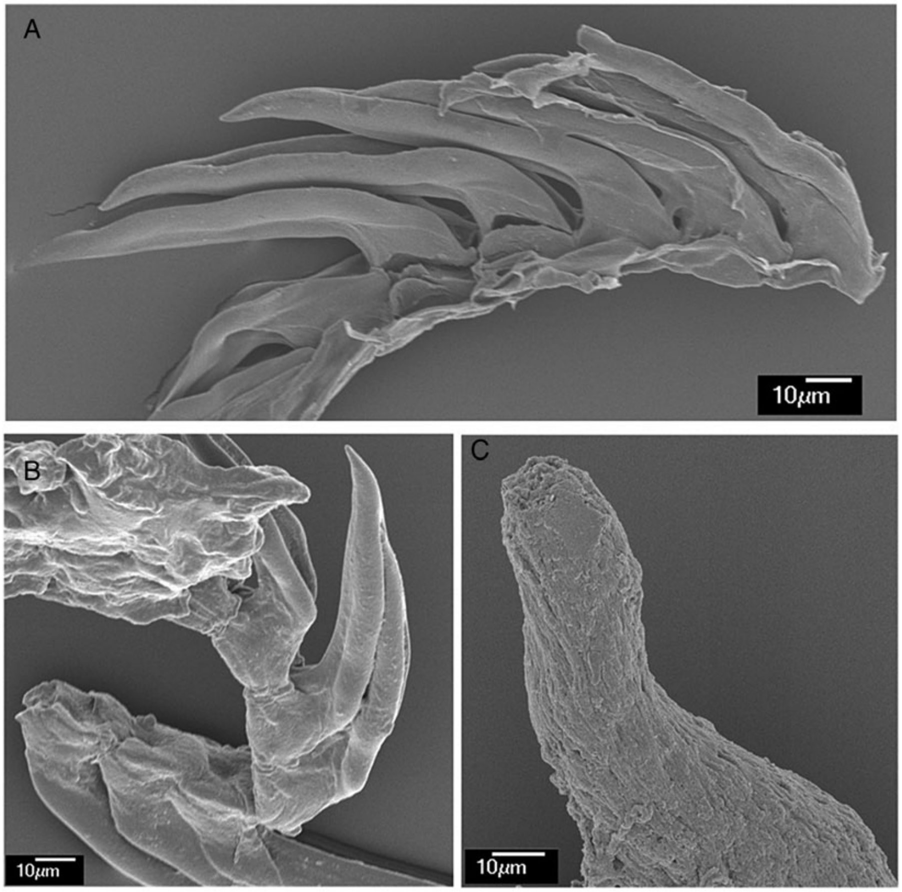

Figure 3. *Costasiella arenaria* n. sp.: SEMs**. A, B.** Radular teeth of specimen no. 2. **C.** Penis of specimen no. 1.

Copenhagen University, ZMUC GAS-324). Paratypes: 5 specimens, same locality and date (ZMUC GAS-325-329); 4 of them dissected (ZMUC GAS-326-329).

ZooBank registration: urn:lsid:zoobank.org:act:2689CD48-E91D-4926-B0B1-60B2389625F3.

Etymology: The specific epithet *arenaria* means sand-loving, referring to the habitat where the species was collected.

Colour of live animals: Most specimens light green based on photographs (Fig. 1A); some pigmented dark brown on dorsal surfaces and rhinophores (Fig. 1B); others completely transparent, without any green contents of digestive gland tubules, only a few light brown epidermal patches. Most peppered with white dots, particularly dense at the cerata; some also with red dots on cerata (Fig. 1D).

External morphology: Total length 5–8 mm crawling, 2–3 mm preserved. Rhinophores simple with flattened bases. Anterior edge of head deeply notched, corresponding to thick ventral lips surrounding mouth (Fig. 2A, B). Eyes admedian, between rhinophores, elongate, 100 µm long by 65 µm wide. Pericardium tall, elongate, slightly wider anteriorly. Anus on small papilla to right of anterior margin of pericardium. Cerata in 3–4 longitudinal rows on either side of body, inflated, but pointed tips not prominent. Sole of foot undivided posteriorly, with conspicuous pedal tentacles at anterior corners. Genital openings indistinct externally.

Internal anatomy: Thick layer of glandular tissue fills fleshy lips and surrounds pharynx anteriorly. Pharynx tall with thick ventral ascus-muscle, but no pharyngeal pouches (Fig. 2C). Radular teeth narrowly blade-shaped with relatively short bases (Figs 2D–F, 3A, B). Pharynx 473 µm long, 567 µm high including ascus muscle (in 2.5 mm preserved specimen). Radula (2.5 mm preserved specimen): leading tooth 100 µm, of which base 26 µm; of 7 fully formed teeth plus one 'ghost' tooth in ascending limb, 11 teeth in descending limb, at least 24 teeth in ascus; no preradular teeth seen in ascus, but possibly several of smallest teeth lost during preparation.

Description of reproductive structures incomplete due to preservation state of specimens. Branches of albumen gland visible in cerata (Fig. 2B). Penis narrow, tubular, unarmed (Figs 2G, 3C). Hermaphroditic follicles ventral behind stomach and mucus gland. Large, spherical genital receptacle on left side, behind pharynx, thick-walled and containing dense lump of presumed surplus sperm and possibly other discarded reproductive material.

Spawn and spawning: Eggs irregularly shaped immediately after leaving oviduct (Fig. 1C, D), capsules only become distinct after some time. Egg and capsule diameters could not be estimated from photos, but judging from number of eggs in one egg mass and capsule diameter being less than twice egg diameter (Jensen, 2001), we judge that development is planktotrophic.

Remarks: Only one species of *Costasiella*, *C. ocellifera* had been recorded from Florida (Jensen & Clark, 1983), but recently *C. nonatoi* or a sibling species also has been found (Rico, 2012; Christa *et al.*, 2014). Miles & Clark (2002) described the occurrence of a possibly sibling species (or else poecilogony) in Florida

Costasiella, referred to as *C. ocellifera* and *C.* cf. *ocellifera*. The latter had previously been called '*Costasiella* n. sp.' (Clark, 1994). However, both populations were collected from *Avrainvillea* and thus cannot be identical to the species here described. The egg mass of *C. arenaria* differs from both *C. ocellifera* and *C.* cf. *ocellifera*. The capsule diameter of *C. ocellifera* is about three times that of the egg diameter, and eggs of the planktotrophic *C.* cf. *ocellifera* are spirally arranged inside the egg mass (Miles & Clark, 2002), whereas capsules in *C. arenaria* are less than twice the egg diameter and appear to be irregularly arranged inside the egg mass (Fig. 1C, D). The eyes of *C. arenaria* do not appear particularly big in live specimens. However, they are bigger than those of *C. nonatoi* (65–100 μm *vs.* 70–80 μm) (Marcus & Marcus, 1960; present study). *Costasiella arenaria* was not seen associated with any recognizable algae in the field. Single strands of green siphonalean filaments appear on several photos (Fig. 1), but are not distinct enough to identify even to genus. Molecular analysis of the contents of the digestive gland of several cerata combined from 4 specimens, identified the algal source as *Bryopsis hypnoides* and an unidentified Pseudocodiaceae species (G. Christa, pers. comm.). Attempts to obtain algal samples from the type locality were unsuccessful and *Bryopsis* was not visible on any of the photos.

***Costasiella formicaria* (Baba, 1959)**

(Figs 4, 5, 6)

Material examined: 13 preserved specimens, of which 4 dissected. One voucher specimen deposited in ZMUC (unregistered).

Colour of live animals: Animals dark green, peppered with small white dots (Fig. 4A); green colour restricted to cerata, which in living specimens cover most of dorsal surface (Fig. 4B–D); white

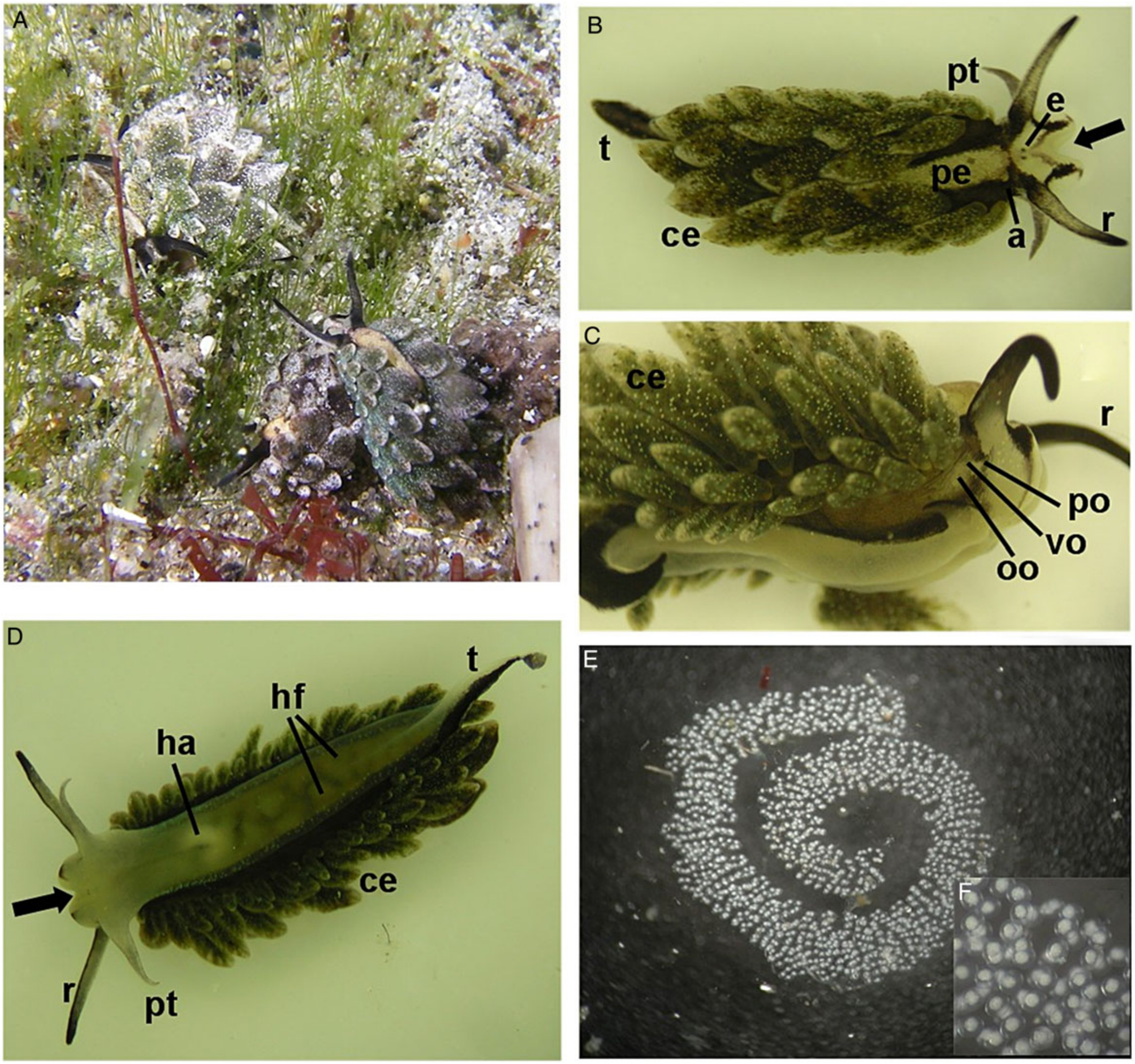

Figure 4. *Costasiella formicaria*. **A.** Three specimens photographed in the field. **B–D.** Specimens photographed in the laboratory. **B.** Dorsal view. **C.** Lateral view. **D.** Ventral view. **E.** Egg mass produced in the laboratory. **F.** Close-up of section of egg mass (photos by M. Nishina). Abbreviations: a, anal papilla; ce, cerata; e, eyes; ha, hermaphrodite ampulla; hf, hermaphrodite follicles; oo, oviducal opening; pe, pericardium; po, penial opening; pt, pedal tentacles; r, rhinophore; t, tail; vo, vaginal opening (?). Arrow points to anterior concavity in head.

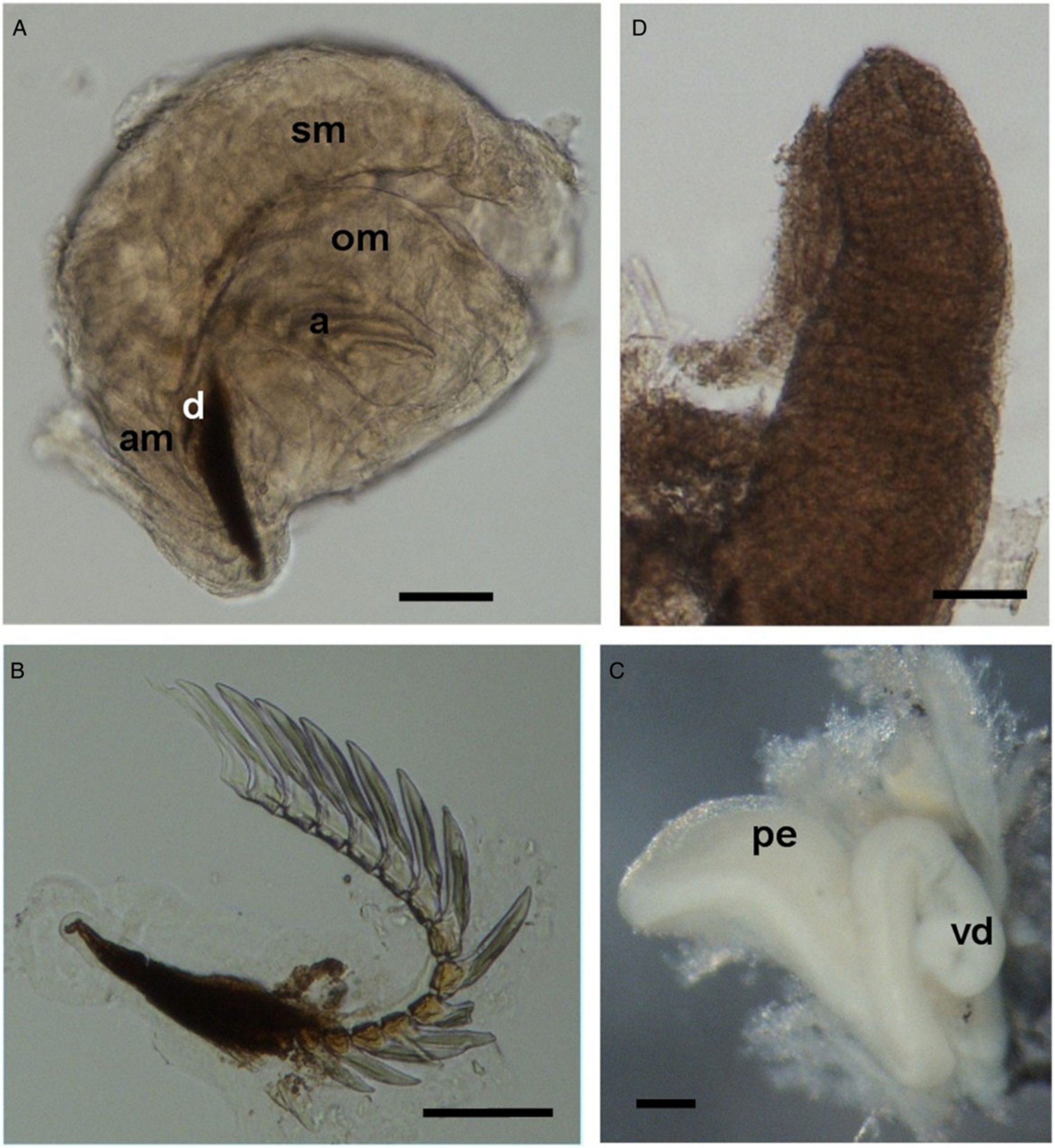

Figure 5. *Costasiella formicaria*: anatomy. **A.** Pharynx of specimen no. 2. **B.** Radula of specimen no. 3. **C, D.** Penis of specimen no. 3. Abbreviations: a, ascending limb; am, ascus muscle; d, descending limb; om, odontophore muscle; pe, penis; sm, dorsal septate muscle; vd, vas deferens. Scale bars = 100 μm.

dots dense at tips of cerata. Rhinophores black dorsally and pale on ventral side (Fig. 4C); black pigment continuing anteriorly as a line down thick crests of anterior head; some specimens also with a dark pigmented line running along dorsal edges of crests, forming a V with tip near eyes (Fig. 4B). Dorsal surface of body black behind white patch surrounding eyes. Renopericardium transparent in most specimens; yellowish renal ducts visible through epidermis (Fig. 4B). Black pigment also on dorsal surface of pedal tentacles and on lateral surfaces of body below cerata (Fig. 4C).

External morphology: Live specimens 4–12 mm long. Cerata slightly inflated, in 4–5 longitudinal rows, almost covering dorsal surface of body. Eyes close together, located between rhinophores; eye diameter 150 μm. Anterior margin of head deeply cleft, forming two fleshy crests surrounding ventral mouth (Fig. 4B–D). Rhinophores simple, but look flattened due to colour difference between dorsal and ventral sides; flattened only at bases. Preserved specimens had lost several cerata, but at least 25–30 cerata present on either side. Pericardium tall, elongate, slightly wider anteriorly (Fig. 4B). Anus on small

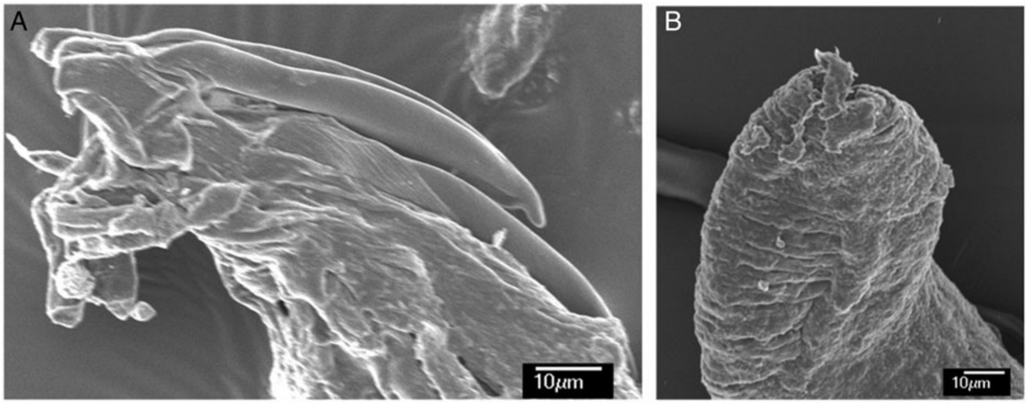

Figure 6. *Costasiella formicaria*: SEMs. **A.** Radular teeth of specimen no. 2. **B.** Penis of same specimen.

papilla to right of anterior pericardium. Renal pore possibly in small white patch medial to anal papilla. Penial opening below base of right rhinophore; female genital aperture closely behind and below this. Vaginal opening may lie between other genital openings (Fig. 4C), but not possible to determine if vaginal duct actually opened to outside. Sole of foot undivided posteriorly, with longitudinal groove formed by locomotory musculature; with long, thin tentacles and median notch anteriorly (Fig. 4D); posterior tip long, narrow.

Internal anatomy: Anterior crests of head filled with branching oral glands opening into oral tube in front of pharynx. Pharynx without muscular pharyngeal pouch, nearly spherical. Large part of descending limb of radula and ascus surrounded by dense rust-coloured tissue, visible as dark line extending almost vertically on ventral surface of pharynx (Fig. 5A). Radula: teeth narrow, blade-shaped with smooth cutting edge (Figs 5B, 6A), with short, rust-coloured bases in fully formed teeth; length of fully formed tooth 110–118 μm; teeth of descending limb difficult to observe due to the rust-coloured tissue. Digestive gland branched in cerata; branches also extend into lateral and posterior parts of renopericardial ridge.

Reproductive structures difficult to distinguish. Cerata containing distinct branches of albumen gland. Penis cylindrical, unarmed (Figs 5C, D, 6B). Prostate conical, composed of several lobes enclosed in common membrane. Prominent genital receptacle on left side, but its connection(s?) to remaining reproductive system could not be traced; containing dense ball of sperm (probably surplus allosperm). Hermaphroditic ampulla long, coiled; like the gonadal follicles, visible through semitransparent sole of foot in live animals (Fig. 4D).

Spawn: One specimen spawned in laboratory of Mr Nishina. Egg mass spirally coiled with diameter of 4.5 mm (Fig. 4E, F). Neither eggs nor capsules were measured, but relatively small diameter of egg capsules (<2 times egg diameter judged from photos) indicates that development is planktotrophic (Jensen, 2001).

Remarks: This species was originally described as *Stiliger formicarius* Baba, 1959, but was transferred to *Costasiella* soon afterwards (Baba, 1961). The original description was based on specimens from two localities in Japan, but apparently no type material exists today. Ichikawa (1993) described six species of *Costasiella* from the Ryukyu Islands, but none was identical to *C. formicaria*. Prior to the collection of the present specimens, photos of this species from Tatsunokuchi, near Nagasaki have been posted on the internet (http://www.umimogura.com/cool/ooarimo.htm) from March 2004, and (http://www.geocities.co.jp/Outdoors-Mountain/3860/ooarimo.htm) from April 2006. This locality is not very far from one of the original localities where *C. formicaria* was collected (Baba, 1959). Specimens tentatively identified as *C. formicaria* have been photographed several times from different localities in the Indo-West Pacific region, e.g. Hawaii (http://seaslugsofhawaii.com/species/Costasiella-formicaria-a.html), Bali (Indonesia) (http://www.underwaterkwaj.com/nudi/bali/b091.htm) and Guam (http://guamreeflife.com/htm/inverts/opistos/limapontiids.htm). The admedian eyes do not appear very large, which caused Marcus (1982) not to accept this species in the genus *Costasiella*. However, the eyes of this species are larger than any other known species. The animals are also bigger than other species, so the eyes seem relatively small. The original specimens were collected intertidally, whereas the present specimens were collected from a depth of 8 m, on a fine filamentous green algae, subsequently identified by Dr J. Tanaka (Tokyo University of Marine Science and Technology) as *Pseudochlorodesmis furcellata*.

Costasiella pallida Jensen, 1985

(Fig. 7)

Remarks: The external morphology has been described by Jensen (1985, 1990) and several features of the internal anatomy by Jensen (1990, 1993). In summary, the pharynx does not have muscular pouches. Radular teeth are blade-shaped with smooth cutting edges and short bases (see SEM micrograph in Jensen, 1993: fig. 9). The anterior corners of the foot protrude slightly to give a spatulate appearance (Fig. 7A, arrow), but do not form real pedal tentacles. The penis is long, cylindrical and unarmed (Fig. 7C, E). The albumen gland extends into the cerata. A few further remarks can be added from the serial sections (Fig. 7E, F). A vaginal opening was said to be absent in the original description (Jensen, 1985), but in the sections a vaginal opening is located a short distance behind the penial papilla and the oviduct opening is located shortly behind this. The seminal receptacle is rather small and located close to a large, spherical genital receptacle. The renal shield, as seen in live specimens (Fig. 7B), contains tubules of kidney (Fig. 7E). *Costasiella pallida* is most similar to *C. coronata*. Both species lack pedal tentacles and have the peculiar renal shield, though it is smaller (or less extensible) in *C. pallida* than in *C. coronata*. Also, both species feed on *Vaucheria* sp. and are found intertidally, and both have pigmented pharyngeal cavities, more prominent in *C. coronata* than in *C. pallida*. Preradular teeth have not been described previously for *C. pallida*, but re-examination of a dissected pharynx shows three or possibly four preradular teeth (Fig. 7D). The fourth

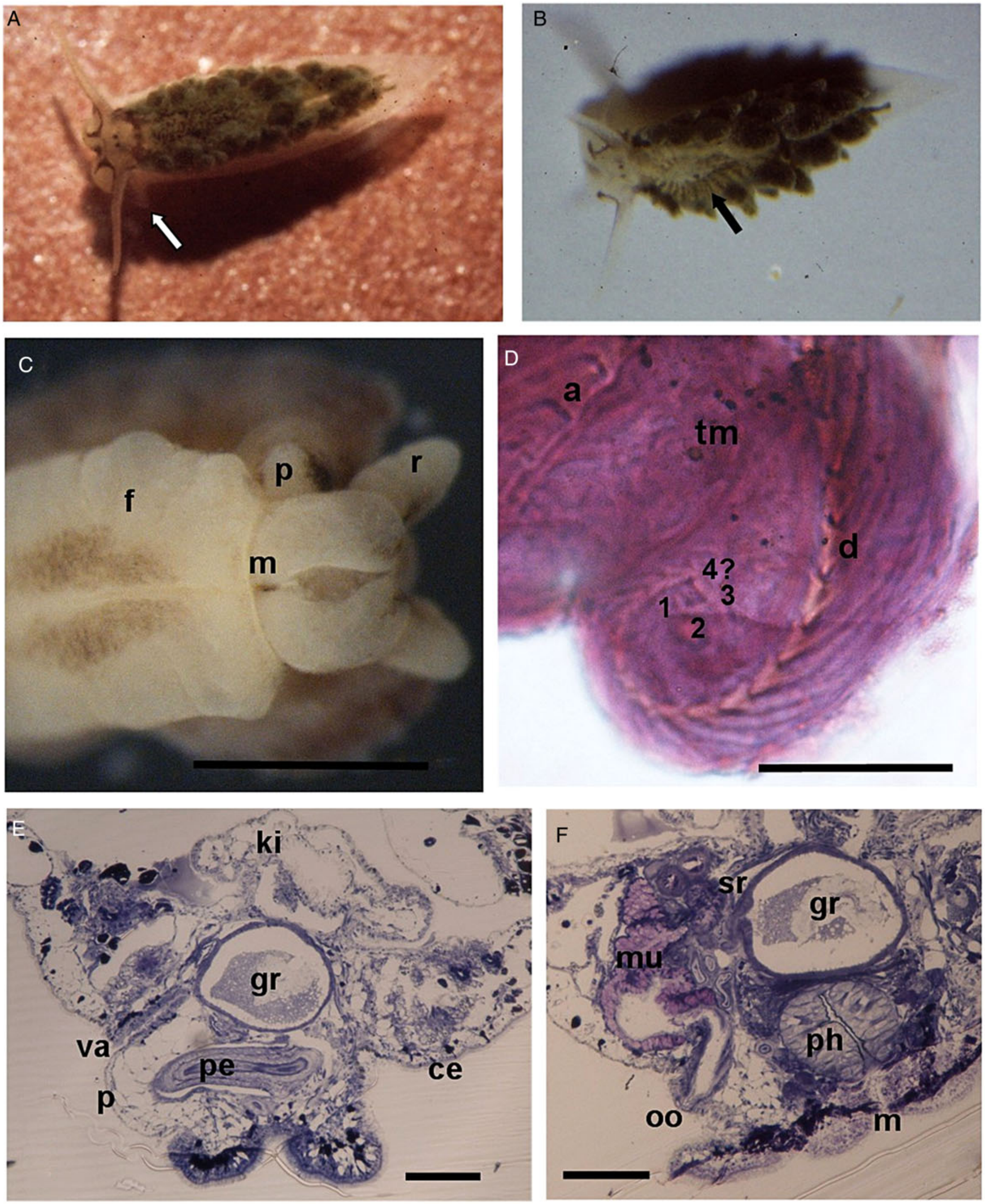

Figure 7. *Costasiella pallida* from Hong Kong, 1986. **A.** Live specimen with pericardial shield contracted; arrow points to extended foot corners (photo by K.R. Jensen). **B.** Live specimen with pericardial shield extended so renal ducts are visible (arrow) (photo by B. Darvell). **C.** Ventral view of preserved specimen. **D.** Ventral part of pharynx (stained with acetocarmine). **E.** Section at level of penis (stained with toluidine blue). **F.** Section at level of anterior pharynx (stained with toluidine blue). Abbreviations: a, ascending limb; ce, ceras; d, descending limb; f, foot; gr, genital receptacle; ki, kidney; m, mouth; mu, mucus gland; oo, oviducal opening; p, penial papilla; pe, penis; ph, pharynx; r, rhinophore; sr, seminal receptacle; tm, transverse muscles; va, vaginal opening. Scale bars: **C** = 1 mm; **D, E, F** = 100 μm.

tooth has a cusp, but it is not possible to see through the stained musculature whether the cusp is a fully formed blade.

PHYLOGENETIC ANALYSIS

Both BI and ML analyses yielded congruent and almost fully resolved phylogenetic hypotheses for *Costasiella* species (Fig. 8). All nodes were fully supported (PP = 1.0) in the BI consensus tree, except that the position of *C.* sp. 5 was not resolved. All nodes received 100% bootstrap support in the ML analysis except two nodes receiving 97% and 99% support, respectively.

The designated outgroups *C. nonatoi* and *C.* sp. 4. were notably divergent from other *Costasiella* species, but retain key synapomorphies of the genus including close-set eyes and putatively defensive vesicles in a jelly layer on the outside face of their egg masses. Among the other (ingroup) *Costasiella* species, the three species known to feed on hosts other than *Avrainvillea* (*C. arenaria* n. sp., *C. formicaria* and *C. coronata*) formed a fully supported clade that was sister to the remaining *Costasiella* species, which were all collected from *Avrainvillea* (Fig. 8). Molecular analyses therefore support the hypothesis that *Costasiella* species feeding on filamentous algae form a monophyletic group. Our phylogenetic results also suggest that a single evolutionary shift from the ancestral host *Avrainvillea* occurred in *Costasiella*, although members of the *C. arenaria* clade may have undergone subsequent shifts among filamentous hosts.

DISCUSSION

The original description of the genus *Costasiella* was brief and without any anatomical features (Pruvot-Fol, 1951). The type species, *C. virescens*, appears to be rare; the total number of specimens known from the literature is 11. Marcus (1982) described the anatomy based on three specimens from the Mediterranean coast of France; later, one specimen was collected from Tenerife, Canary Islands and 6 specimens from the Cape Verde Islands (Ortea *et al*., 1998), but although no anatomical description was given a colour photo was provided. The species appears similar to *C. arenaria*, but paler and with fewer cerata. However, specimens were all less than 3 mm long and may have been juveniles.

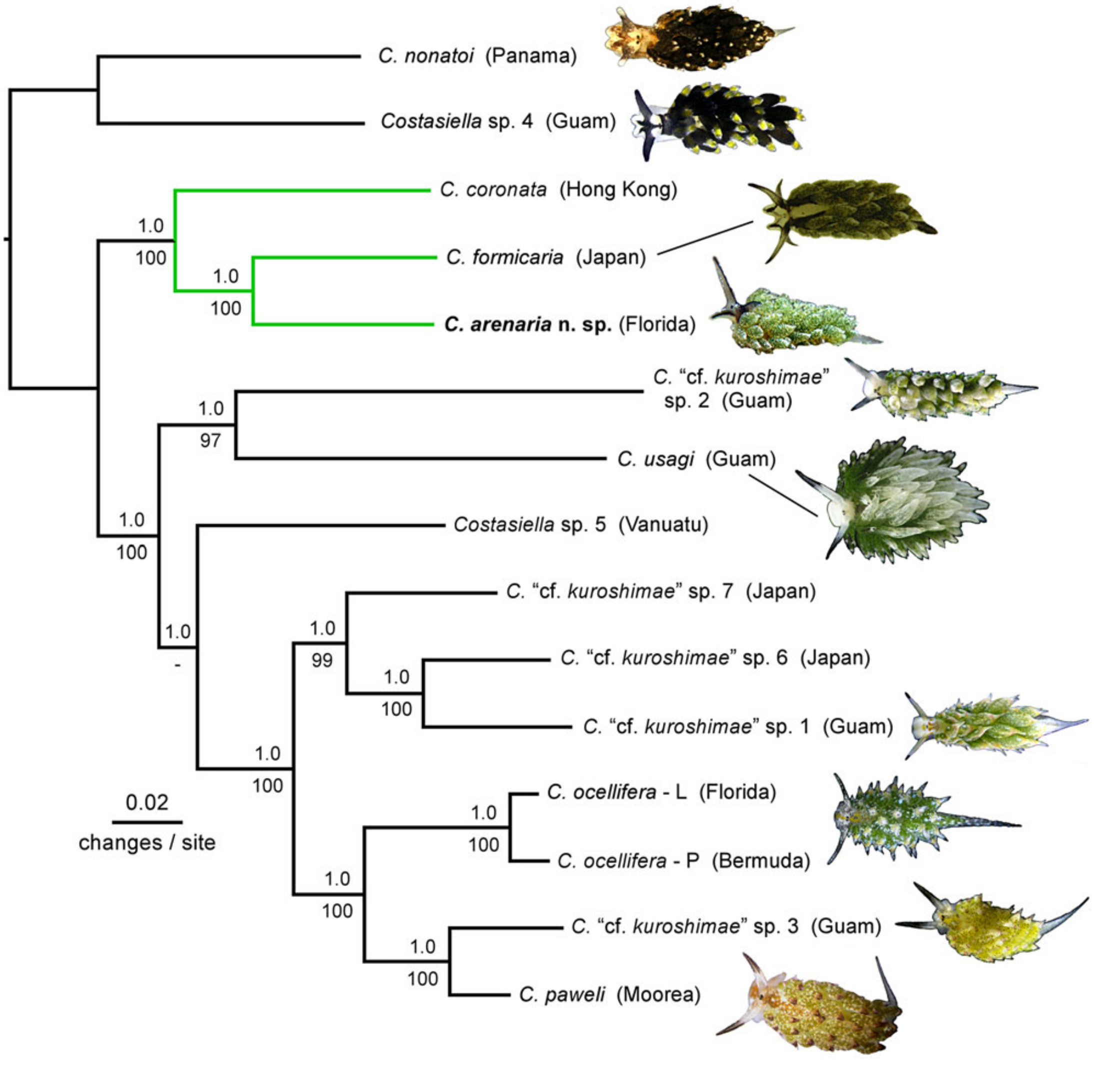

Figure 8. Evolutionary relationships among *Costasiella* species based on analyses of 2,778 base pairs of DNA from four genetic loci. The ML phylogram is shown, with posterior probabilities from BI analyses given above, and corresponding bootstrap support values below, each branch. *Costasiella nonatoi* and *Costasiella* sp. 4 constituted the outgroup. Species provisionally identified as '*C. kuroshimae*' are labelled 'cf. *kuroshimae*' with an unidentified species number. Host alga indicated by branch colour (*Avrainvillea*, black; *Vaucheria*, *Pseudochlorodesmis* or unknown filamentous alga, green).

Table 2. Comparison of anatomical characters of all known species of Costasiella.

Species	Rhinophores	Eyes	Cerata	Pericardium	Tail	Anterior foot	Pharyngeal pouch	Radular teeth	Oesophageal pouch	CNS	Penis	No. of genital openings	Development type
C. virescens Pruvot-Fol, 1951[*]	Slightly flattened	Close, between rhinophores	Fusiform; alb. gland present	Tall, oval	Undivided	Tentacles present	?	Smooth, blade-shaped	?	3 visc.	Unarmed	2	?
C. formicaria (Baba, 1959)	Flattened at bases	Close, between rhinophores	Fusiform; alb. gland present[†]	Long, narrow	Undivided	Tentacles present	Absent[†]	Smooth, blade-shaped; bases short[†]	?	?	Unarmed[†]	3[†]	P?
C. nonatoi Marcus & Marcus, 1960	Grooved; with d.g.	Behind rhinophores?	Fusiform; alb. gland present; d.g. knobby	Small; dorsal vessels present	Undivided	Rounded	Present?	Smooth, blade-shaped; 14	Present	3 visc.	Unarmed	3	
C. illa (Marcus, 1965)	Flattened with basal lobe	Close, between rhinophores	Tips mammillary; no alb. gland	Elongate/ oval	?		?	Smooth, blade-shaped; 8a, 7d, ?as	?	?	Unarmed		?
C. ocellifera (Simroth, 1895), = *C. lilianae* (Marcus & Marcus, 1969)	Simple	Close, between rhinophores	Fusiform; tips white with orange subterminal band; alb. gland present	Short, round; dorsal vessels present	Like ceras	No tentacles	Present	Smooth, blade-shaped; 6–10a, 8–10d; 65 μm	Absent	3 visc.	Cuticular tip?	4	P/L
C. pallida Jensen, 1985	Long, simple	Close, between rhinophores	Inflated; alb. gland present	Elongate/ oval; with marginal fringe	Undivided	Pointed corners	Absent	Smooth, blade-shaped, narrow; 8a, 14d, × as; 74 μm	Absent?	?	Unarmed	(2)3[†]	P?
C. paweli Ichikawa, 1993[‡]	Simple, ± flattened base	Big, close, between rhinophores	Inflated, tips narrow with subterminal white & orange; alb. gland present	Long	Like ceras	Spatulate tentacles	Large	Smooth, blade-shaped; 6–8a, 5–6d, × as	Present	?	Unarmed	2?	?
C.usagi Ichikawa, 1993[‡]	Simple, thick	Big, in protuberance between rhinophores	Fusiform w. thin longitudinal lines; no albumen gland	Short, tall	Undivided	With thin tentacles	Small	Smooth, blade-shaped; 7a, 8d, ? as	?	2? visc.	Spiral stylet	2? Vaginal duct apparently closed	?
C. kuroshimae Ichikawa, 1993[‡]	Simple	Big, close, between rhinophores	Fusiform with orange & iridescent blue spots; alb. gland present	Tall, oval; dorsal vessels present	Like ceras	Rounded	Large	Smooth, blade-shaped; 7a, 6d, × as	?	?	Unarmed	?	?
C. vegae Ichikawa, 1993	Simple	Close, between rhinophores	With ocellate spot; tips pointed	?	?	Rounded	?	?	?	?	?	?	?
C. rubrolineata Ichikawa, 1993	Simple?	Close between rhinophores	Subterminal orange band; tips pointed	?	?	Rounded	?	?	?	?	?	?	?
C. iridophora Ichikawa, 1993	Simple?	Close, between rhinophores	With iridescent green dots	?	?	?	?	?	?	?	?	?	?
C. mandorahae Jensen, 1997	Simple or flattened with basal groove	Close, between rhinophores	Fusiform, tips narrow, white with subterminal orange; no albumen gland	Tall, rounded	Like ceras	Rounded	Present	Smooth, blade-shaped; 7(8)a, 7d, × as; 34 μm	Large	2? visc.	Unarmed	3 (no vaginal opening, but genital receptacle opens on left side)	P?
C. coronata Swennen, 2007	Simple, flattened at base	Close, between rhinophores	Flattened; alb. gland present	Small; with large renal shield	Undivided	Pointed or rounded corners	Absent	Smooth, blade-shaped; 5–8a, 18–27d, 3 prerad; 85–100μm	Present	?	Unarmed	3	P
C. arenaria n.sp.	Simple	Close, between rhinophores	Fusiform; alb. gland present	Tall, elongate	Undivided	Long tentacles	Absent	Smooth, blade-shaped; short bases; 105 μm	?	?	Unarmed, thin	?	P?

Abbreviations: a, ascending limb of radula; alb., albumen; as, ascus of radula; CNS, central nervous system; d, descending limb of radula; d.g., digestive gland; L, lecithotrophic development; P, planktotrophic development; visc., visceral.

[*] Based on Marcus (1982); [†] present study; [‡] based on specimens from Singapore.

Swennen (2007) compared all species of *Costasiella* morphologically, but the present description of another new species and the examination of the anatomy of *C. formicaria* and serial sections of *C. pallida* have added some new information. Also, the description of the anatomy of three of the species of *Costasiella* described by Ichikawa (1993) and associated with *Avrainvillea* from Singapore (Jensen, 2009) has provided additional information about the genus. Comparison of anatomical characters is given in Table 2. Almost all species have an unarmed penis and therefore presumably a separate vaginal opening. Marcus & Marcus (1969) described a cuticularized tip of the penis of *C. ocellifera* (as *Stiliger lilianae*), but this could not be confirmed in the present study.

The species known to be associated with algae other than *Avrainvillea* share a number of anatomical characters, i.e. no pharyngeal pouches, albumen gland in cerata, long and simple rhinophores, and a tail that is not shaped like a ceras. Three of the five species also have pedal tentacles and the two remaining ones, which both feed on *Vaucheria*, have a 'renal shield' surrounding the pericardium. This morphological resemblance is reflected in the molecular analysis in which the three species form a monophyletic clade (Fig. 8). Genetic data indicate that two cryptic species superficially similar to '*C. nonatoi*' occur in Florida (Rico, 2012), at least one of which may feed on filamentous algae other than *Avrainvillea* (Christa *et al.*, 2014); further study is thus needed to determine if more than one radiation onto algae other than *Avrainvillea* has occurred within *Costasiella*. Only one of the *Avrainvillea*-associated species, *C. usagi*, has a tail not shaped like a ceras. This species also has pedal tentacles, but also has a small pharyngeal pouch and a conspicuous, coiled penial stylet (Jensen, 2009). In the molecular analysis, *C. usagi* is basal to most of the *Avrainvillea*-associated species (Fig. 8).

A prostate gland has only been described in *C. ocellifera* (as *Stiliger lilianae*) (Marcus & Marcus, 1969), *C. mandorahae* (Jensen, 1997) and *C. coronata* (Swennen, 2007). In all species the prostate is composed of a cluster of finger-like lobes, shorter and more numerous in *C. coronata* than in *C. ocellifera*, and with only three lobes in *C. mandorahae*. In *C. paweli* the prostate consists of several lobes, but no information was given on number or size (Jensen, 2009). In *C. formicaria* the lobes appear to be enclosed within a thin common membrane.

Five genetically and morphologically distinct species were previously identified as superficially similar to *C. kuroshimae* (e.g. Gosliner, Behrens & Valdés, 2008: 76; Jensen, 2009: fig. 5D). The true identity of this species may be impossible to determine from the original description, which entirely lacks the anatomical details needed to identify sacoglossans (Ichikawa, 1993). Several of the other species described by Ichikawa (1993) apparently differ from *C. kuroshimae* only in colour pattern. The high proportion of undescribed species highlights the need for future studies focused on the alpha-taxonomy of *Costasiella*.

There is a distinct correlation between size of egg capsules relative to egg diameter, species with planktotrophic larvae having capsule diameters less than twice the egg diameter (Jensen, 2001). *Costasiella arenaria* n. sp. and *C. formicaria* both fit into this category (present study), judged from the photos, and *C. pallida* and *C. coronata* also have planktotrophic larvae (Jensen, 2001; Swennen, 2007). Poecilogony exists in *C. ocellifera*; different populations in both Florida and the Caribbean produced either planktotrophic larvae, or lecithotrophic larvae with 100% encapsulated metamorphosis. Thus, larval development mode may not be a reliable species-specific character in this genus, unless used in careful combination with other morphological, reproductive, ecological and molecular data (Krug, Händeler & Vendetti, 2011; Krug *et al.*, 2013). Other reproductive traits may be more informative in *Costasiella*; for instance, most species have refractile vesicles embedded in a thick jelly covering the outer surface of the egg mass, but some derived species (e.g. *C. ocellifera* and *C.* sp. 3) lack these vesicles. Future work should determine whether there are additional cases of poecilogony in *Costasiella*, to add to the five presently known examples of variable development in Sacoglossa (Vendetti, Trowbridge & Krug, 2012).

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