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Evolutionary reversals in *Bossiella* (Corallinales, Rhodophyta): first report of a coralline genus with both geniculate and nongeniculate species<sup>1</sup>

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## ABSTRACT

This is the first report of a coralline genus with both geniculate (upright fronds with non-calcified joints) and nongeniculate species that has been verified by DNA sequence data. Two nongeniculate (crustose) species of *Bossiella* are recognized, *B. mayae* sp. nov. and *B. exarticulata* sp. nov. DNA sequencing of the lectotype specimen of *Pseudolithophyllum whidbeyense* revealed that this name had been misapplied and instead belongs to an undescribed coralline species in the Hapalidiales. Phylogenetic analyses of concatenated DNA sequences (*psbA*, *rbcL*, COI-5P) indicate that *B. mayae* and *B. exarticulata* represent phenotypic reversals from the geniculate character state back to the nongeniculate character state. Secondary loss of genicula has occurred three times in the subfamily Corallinoideae, once to generate the entirely nongeniculate genus *Crusticorallina* and twice in the now morphologically heterotypic *Bossiella*. Since phenotypic reversals have occurred several times during the evolution of coralline algae, we speculate about the putative mechanism and adaptive significance of this phenomenon.

**Key Words:** *Bossiella exarticulata*, *Bossiella mayae*, COI-5P, crustose coralline algae, cryptic species, *psbA*, *Pseudolithophyllum whidbeyense*, *rbcL*, secondary loss, sequencing type specimens

**Abbreviations:** BC British Columbia, BI Bayesian inference, BS bootstrap, CA California, COI-5P cytochrome c oxidase subunit 1-five prime, mya million years ago, ML maximum likelihood, PP posterior probability, WA Washington

## INTRODUCTION

Evolutionary reversals occur when a derived character state reflects the phenotype of an ancestral lineage (Porter and Crandall 2003). The mechanism for evolutionary reversals and the most parsimonious explanation for their occurrence is the secondary loss of a trait that was previously gained by an ancestral lineage. Secondary loss is the most likely explanation for an evolutionary reversal when character state changes are infrequent and when the lineage exhibiting secondary loss is embedded within a clade of organisms where the trait is present (Wiens 2001). With the advent of molecular systematics, we now have an independent data set, DNA sequence data, to recognize and assess what appear to be phenotypic reversals (Porter and Crandall 2003).

Coralline algae (orders Corallinales, Hapalidiales and Sporolithales) are a group of red seaweeds within subclass Corallinophycidae that have calcium carbonate deposited in their cell walls, primarily as high Mg-calcite (Nash et al. 2011, 2016, Diaz-Pulido et al. 2014). Two morphological character states occur in coralline red algae: geniculate corallines are typically erect with short, non-calcified segments (genicula) between larger calcified segments (intergenicula). Nongeniculate corallines are encrusting in habit or form rhodoliths and are predominately calcified (Johansen 1981). The Sporolithales and Hapalidiales are comprised entirely of nongeniculate corallines, whereas Corallinales contains both geniculate and nongeniculate taxa (Nelson et al. 2015). The fossil record has long suggested that nongeniculate corallines are ancestral to geniculate corallines (Johnson 1961, Wray 1977). Johansen (1969) and Cabioch (1971) hypothesized that geniculate corallines arose from nongeniculate corallines several times because different groups of geniculate corallines have different genicular anatomies. Johansen (1969: 43-44) suggested that genicula evolved three times giving rise to the subfamilies Metagoniolithoideae, Amphiroideae, and Corallinoideae, but did not speculate from which nongeniculate ancestors the three subfamilies of geniculate corallines arose.

Molecular phylogenies (Aguirre et al. 2010, Bittner et al. 2011) have been congruent with the fossil record indicating that nongeniculate corallines evolved earlier than geniculate corallines. DNA sequence data have been used to infer which nongeniculate lineages share a common ancestor with geniculate lineages, lending insight into this repeated evolutionary trend. Within Metagoniolithoideae the geniculate genus *Metagoniolithon* is sister to the nongeniculate genus *Porolithon* (Bittner et al. 2011, Kato et al. 2011, Röser et al. 2016) and within Lithophylloideae the geniculate genera *Amphiroa* and *Lithothrix* are sister to the nongeniculate *Lithophyllum* (Bailey and Chapman 1998, Bailey 1999, Gabrielson et al. 2011), but which nongeniculate taxon is sister to Corallinoideae is unclear.

Until 2013, Corallinoideae was characterized as containing only geniculate taxa, but Hind and Saunders (2013) documented the first evidence of secondary loss of genicula in that subfamily. Using a molecular phylogenetic approach, they demonstrated that the Northeast Pacific nongeniculate species then known as *Pseudolithophyllum muricatum* belonged in the Corallinoideae. Recently,

Hind et al. (2016) erected a new genus, *Crusticorallina*, for *P. muricatum* and related species. Although Metagoniolithoideae, Lithophylloideae and Corallinoideae all contain geniculate and nongeniculate species, genera within each subfamily are defined as possessing one or the other morphological character state (Hind et al. 2016). The Corallinoideae offers an excellent system for studying secondary loss of genicula because this phenotypic reversion is relatively infrequent and *Crusticorallina* is embedded within a large clade of geniculate species.

In the early 2000s KRH and PWG collected nongeniculate corallines in BC, Canada and in northern WA, USA, respectively, matching the description of *Pseudolithophyllum whidbeyense* (Corallinoideae). *Pseudolithophyllum whidbeyense* is a thin, adherent, epilithic crust, smooth or with small, flat-topped protuberances, and sometimes tessellate with semi-circular swirls (Steneck and Paine 1986). Upon sequencing, these specimens proved to belong in *Bossiella* (Corallinoideae), a genus of geniculate species with lateral conceptacles. We first assumed that we sequenced the encrusting base of one of the geniculate species of *Bossiella*, as several species (notably *B. frondifera*) have conspicuous and extensive crustose bases from which numerous erect axes arise. However, the sequences did not match any geniculate *Bossiella* in our databases, then or now. Most convincing, however, were conceptacles found within the crusts we had sequenced, clearly distinguishing these specimens from other *Bossiella* species. Conceptacles have never been recorded from the crustose bases of geniculate corallines, including *Bossiella*, demonstrating that these specimens most likely represented nongeniculate species. Using DNA sequences from two plastid (*rbcL* and *psbA*) and one mitochondrial (COI-5P) marker, we demonstrate that two species belonging in *Bossiella* have lost their genicula; these nongeniculate species are described here as *B. mayae* sp. nov. and *B. exarticulata* sp. nov. We also explain why the name *Pseudolithophyllum whidbeyense* applies to a species of Hapalidiales and cannot be used for any *Bossiella* species.

#### *History of Pseudolithophyllum whidbeyense*

The basionym of *Pseudolithophyllum whidbeyense* is *Lithophyllum whidbeyense*, originally described by Foslíe (1906). Foslíe (1906) based his description of *Lithophyllum whidbeyense* on two collections sent to him by Nathaniel Lyon Gardner (NLG 655 and NLG 656), both from the west coast of Whidbey Island, WA, USA and all on limpet shells (*Acmea mitra*). Foslíe (1906) described this new species as crustose, 0.3-1 mm thick, smooth, and weakly glossy with a hypothallus occupying 1/3-2/3 of the crust thickness and those cells 2-4 times longer (12-25 up to 30  $\mu\text{m}$ ) than wide (6-10  $\mu\text{m}$ ). Perithallial cells were subquadrate to vertically elongate (7-14 up to 18  $\mu\text{m}$  long) and 6-10  $\mu\text{m}$  wide. Cystocarpic conceptacles were tightly packed, weakly convex and 350-600  $\mu\text{m}$  diameter.

Mason (1953) added to the description that the crust was "crowded with numerous closely crowded excrescences 2 mm high and 1-1.5 mm thick" and that specimens were also found on stones. Adey (1970) transferred the species to *Mesophyllum* as *M. whidbeyense*, but expressed "considerable doubt" about its generic placement as he had not seen bi-tetrasporangial conceptacles (Adey's asexual conceptacles) and specimens had a *Mesophyllum*-like epithallium and upper perithallium. Steneck and Paine (1986), based on numerous field collected specimens as well as an isoelectotype specimen (UC 739464), provided a detailed description of the vegetative and reproductive morpho-anatomy of the species, commented on its ecology, and reviewed its taxonomy. They transferred the species to *Pseudolithophyllum*, as *P. whidbeyense*, based on the following features: 1) elongate intercalary meristematic and perithallial cells, 2) thin, parallel, hypothallus and 3) the tendency for conceptacles to become buried. These features corresponded to Adey's concept of *Pseudolithophyllum* based on a

spurious lectotype (Silva et al. 1996: 269) and not Lemoine's (1978) revised concept. Prophetically, Steneck and Paine (1986) wrote, "Morphologically, it (*P. whidbeyense*) resembles the basal systems of articulated corallines in *Bossiella* and *Corallina* but can be distinguished from them by its thicker crust and buried conceptacles."

## MATERIALS AND METHODS

Specimens were collected from bedrock using hammer and chisel or from live mollusk shell and placed in silica gel. Vouchers were deposited in NCU, UBC, UNB or TRH; herbarium acronyms follow Thiers (2018). Specimen information, including collection data, GenBank numbers and herbarium accessions, is included in Table S1 in the Supporting Information. Total genomic DNA was extracted according to either Gabrielson et al. (2011) or Saunders (2008) with modifications from Saunders and McDevit (2012b). Amplification and sequencing of COI-5P (664 bp), *psbA* (853 bp) and *rbcL* (1401 bp) gene fragments followed Hind et al. (2016). DNA was extracted from historical specimens following the protocol of Hughey et al. (2001). Sequence data was edited and aligned using Geneious 7.1.8 (Kearse et al. 2012). Maximum likelihood (ML) analyses were conducted using the RAxML (Biomatters Ltd.) plugin (V 1.0) in Geneious on a concatenated alignment of COI-5P, *psbA* and *rbcL* gene fragments (2596 bp) and partitioned by gene. The GTR+I+G GAMMA nucleotide model of evolution was obtained using jModelTest version 2.1.10 (Darriba et al. 2012). Five hundred bootstrap (BS) replicates of the 'rapid bootstrapping and search for best scoring ML tree' algorithm were conducted. Bayesian posterior probabilities (PP) were generated using the MrBayes Plugin v2.2.4 (Huelsenbeck and Ronquist 2001) in Geneious 7.1.8 using the GTR+I+G model. Bayesian analysis parameters followed Hind et al. (2016). Morpho-anatomical assessments of non-geniculate corallines followed methods used for *Crusticorallina* species in Hind et al. (2016). Historical specimens from TRH were sampled, namely NLG 655 (TRH A6-315) and NLG 656 (TRH A6-316). Mason (1953) designated NLG 655 in TRH as the lectotype, thereby excluding NLG 656, and designated specimens in UC as isotypes (UC 739464 and UC 745688). NLG 655 comprises two coralline covered *Acmea mitra* shells that Foslie treated as separate, making a preparation of each, and designated as 802 and 803. We sequenced the specimens on each shell of samples 802 and 803 (Figs. S1-S3 in the Supporting Information).

## RESULTS

**Molecular data.** DNA sequence data from specimens that morpho-anatomically conformed to Steneck and Paine's (1986) description of *Pseudolithophyllum whidbeyense* clearly indicated that two nongeniculate coralline species were present. There was 4.4-5.8%, 1.6-1.9%, 0.7-1.3% DNA sequence divergence between these two species for the COI-5P, *rbcL* and *psbA* markers respectively (Table S2 in the Supporting Information). The divergence values for these markers were consistent with DNA sequence divergence values between other species of coralline algae (Broom et al. 2008, Hind and Saunders 2013, Nelson et al. 2015, Hind et al. 2016). One outlier in the COI-5P distance matrix (UNB GWS030783) had 2.9-3.2% intraspecific DNA sequence divergence within *B. exarticulata* specimens. This was the most northern record for *B. exarticulata* and could represent a divergent haplotype, but more specimen collections from northern waters are needed. Specimens UBC A91401 and UBC A91402, had an intraspecific DNA sequence divergence of 0.9-1.3% among other *B. exarticulata* specimens for the *psbA* gene region (Table S2). These collections were our

deepest collections of *B. exarticulata* (Table S1) and could represent a divergent deep-water haplotype, however additional collections are warranted.

Phylograms were constructed using bayesian inference (BI) and maximum likelihood (ML) analyses on a concatenated dataset of three gene fragments (COI-5P, *psbA*, *rbcL*; 2596 bp) (Fig. 1). The resulting tree topologies were identical. Posterior probabilities (PP) and BS support were appended to the BI tree (Fig. 1). *Bossiella mayae* and *B. exarticulata* resolved with full support in a monophyletic lineage including the generitype *B. plumosa* (Fig. 1). The phylogenetic analyses further suggested that these two nongeniculate species are not sister species and that each arose independently from a geniculate ancestor (Fig. 1). Three *Bossiella* species, provisionally named *B. chiloensis* were included in the analyses because their inclusion in the tree vastly changed the relationships among *B. mayae*, *B. exarticulata*, and the remaining *Bossiella* species. In addition, these unidentified species (and their provisional names) were included here because they have previously been deposited under these names in the Barcode of Life Database (BOLD) and GenBank (see Table S3 in the Supporting Information). Relationships between geniculate and nongeniculate sister species were not strongly supported (e.g., *Bossiella mayae* and *Bossiella* cf. *2chiloensis*; PP=0.74, BS<62; Figs. 1 and S4 in the Supporting Information).

#### *Historical DNA Analysis.*

We sequenced the lectotype specimen (NLG 655) that consists of two crusts on two different *Acmea mitra* shells (preparations 802 and 803; Figs. S1-S3). We found that sample 802 is one of the nongeniculate *Bossiella* species (described below) and that sample 803 is an undescribed species belonging in Hapalidiales. We determined that the 802 specimen cannot serve as the lectotype because on the "Prep. 802" label Foslie has written "Lithoph.whidbeyense? cfr. 803" (Fig. S1) and because according to Article 9.14 of the ICN "...the name must remain attached to the part ... that corresponds most nearly with the original description or diagnosis." (McNeill et al. 2012). The most defining feature in the protologue of *L. whidbeyense* (Foslie 1906) is the presence of tightly packed, weakly convex and 350-600  $\mu$ m diameter gametangial conceptacles. Only the Prep. 803 specimen bears conceptacles measuring (300) 400-600  $\mu$ m that correspond to the original description (Fig. S2). Thus, the epithet "*whidbeyense*" will apply to an otherwise undescribed species belonging to Hapalidiales in the NE Pacific, and both of the nongeniculate *Bossiella* species require new names that we provide below along with an amended description of *Bossiella*.

#### *Taxonomic Revisions.*

***Bossiella*** P.C. Silva 1957: 46

Thalli epilithic or epizoic; multiaxial, cells of adjacent filaments sometimes united by open fusions but never by secondary pit connections; main growth by elongation and nearly simultaneous divisions of cells terminating filaments at margin of crusts and at apices of branches; all pigmented tissue covered by an epithallium of 1-3 cell layers; conceptacles

superficial uniporate, central or acentric, 300-1000  $\mu\text{m}$  outside diam.; gametophytes dioecious; male conceptacles beaked, sometimes markedly so, roofs low over fertile areas that extend up sides of chamber, canals usually more than 200  $\mu\text{m}$  long; female conceptacles with uni- or bicarpogonial procarps; some carpogonia not developing completely and apparently non-functional; where known, carposporangial filaments arising from anywhere on upper surfaces of fusion cells. Bi-tetrasporangial conceptacles 35-100  $\mu\text{m}$  wide, 90-200  $\mu\text{m}$  long, containing up to 60 zonately divided bisporangia or tetrasporangia, rarely both, spores uninucleate.

Geniculate species consisting of more or less erect fronds of calcified intergenicular segments separated by non-calcified genicula; genicula non-calcified (except where they join calcified intergenicula) composed of one tier of narrow, thick-walled cells 150-350  $\mu\text{m}$  long; one to several fronds arising from an adherent basal crust; fronds branching dichotomously or pinnately or irregularly in one plane when two or three genicula are produced by an intergeniculum, lower parts of fronds unbranched, comprising a stipe with terete or subterete intergenicula; upper intergenicula with two flat processes, or wings, projecting laterally from a midrib that sometimes protrudes as a ridge; secondarily produced cortices sometimes bearing secondary branches and conceptacles; apical growth of fronds generating intergenicula with medulla of straight, unpigmented cells in arching tiers about 45-90  $\mu\text{m}$  high, peripheral medullary filaments arching outward producing cortex of short, pigmented cells; epithallial cell walls partly uncalcified; basal crusts, if present, always lack conceptacles; conceptacles originating and developing in cortical tissue on wings of subterminal intergenicula; 1 to more than 50 per intergeniculum.

Nongeniculate species monomerous or dimerous in construction up to 1.6mm thick; marginal growth of crusts resulting in thin hypothallus 75-240  $\mu\text{m}$  thick (comprising no more than 25% total thallus thickness) of non-pigmented, horizontally oriented filaments (cells 10-



40  $\mu\text{m}$  long); uppermost hypothallial cells arch upward to form a perithallus of vertically oriented cells 425-1475  $\mu\text{m}$ ; intercalary meristematic cells dividing to produce distally unpigmented epithallial cells with flared walls and proximally photosynthetic perithallial cells; young conceptacles superficial; older conceptacles buried and rarely filled in.

*Type species: Bossiella plumosa* (Manza) P.C.Silva

***Bossiella mayae*** P.W.Gabrielson, K.R.Hind, Martone, & C.P.Jensen *sp. nov.*

*Holotypus*: NCU 591286, leg. P. W. Gabrielson & K. Britton-Simmons, 20.vii.2009, on bedrock in low intertidal zone.

*Type Locality*: Cattle Point, San Juan Island, WA, USA.

*Etymology*: The species is named for Mary Love May, who has provided financial and loving support for coralline research and for Paul W. Gabrielson.

*Description*: Thallus to 1.6 mm thick and tightly adherent to substratum, encrusting, smooth or with pronounced bumps and ridges (Fig. 2, A-D); white thin margin often present (Fig. 2B); tessellations and white swirls commonly present, especially noticeable when dry (Fig. 2, A, C and D). Epithallial cells 4.2-8.3  $\mu\text{m}$  tall, always flared distally to 6.1-11.8  $\mu\text{m}$  wide (Fig. 2E); perithallus 424-1474  $\mu\text{m}$  thick; hypothallus 76-240  $\mu\text{m}$  thick; uniporate conceptacles flush with thallus surface to barely raised, scattered (Fig. 2D) or crowded (Fig. 2F), rounded chambers 244-309  $\mu\text{m}$  wide x 214-248  $\mu\text{m}$  tall (Fig. 2, G and H) with elongated canals 52-103  $\mu\text{m}$  long (Fig. 2, G and H; Table S4 in the Supporting Information); *rbcL*, *psbA* and COI-5P sequences diagnostic (Table S1).

*Habit and Habitat*: Epilithic on bedrock, no records from cobble; epizoid on barnacles and shells of *Acmaea mitra*. Common in mid- and low intertidal zones; no subtidal specimens were collected.

*Distribution*: Gwaii Haanas, BC, Canada south to Mill Creek Beach, Monterey Co., CA, USA.

***Bossiella exarticulata*** K.R.Hind, Martone, C.P.Jensen & P.W.Gabrielson *sp. nov.*

*Holotypus*: UBC A91389, leg. K. R. Hind, 31.i.2014, on bedrock in low intertidal zone.

*Type Locality*: Brady's Beach blowhole, Bamfield, Vancouver Island, BC, Canada.

*Etymology*: *Bossiella exarticulata* is named for the absence of articulated uprights in this species.



*Description:* Thallus encrusting, sometimes with scattered white swirls or tessellations, visible especially when dry (Fig. 3, A and B), and other times smooth (Fig. 3C), up to 1.6 mm thick, thin white margin sometimes present (Fig. 3C); epithallial cells 5.1-7.6  $\mu\text{m}$  tall, always flared distally to 5.0-8.2  $\mu\text{m}$  wide (Fig. 3D); perithallus (585-1370  $\mu\text{m}$ ) thicker than hypothallus (120-295  $\mu\text{m}$ ) (Fig. 3E); tetrasporangial conceptacles crowded together in fields (Fig. 3F), slightly convex to flush with thallus surface, spherical (Fig. 3, G and H), chambers ~220  $\mu\text{m}$  wide x 184  $\mu\text{m}$  tall, with short canals ~57  $\mu\text{m}$  long (Fig. 3, G and H; Table S2); *rbcL*, *psbA* and COI-5P sequences diagnostic (Table S1).

*Habitat and Habit:* Epilithic on bedrock and cobble and epizoic on molluscs (snails and mussels) and cup coral (*Balanophyllia elegans*); primarily found at moderate to fully exposed sites; uncommon in the mid-intertidal; common in the low intertidal zone and subtidally to 13.5 m depth.

*Distribution:* Gwaii Haanas, BC, Canada south to Mill Creek Beach, Monterey County, CA, USA.

## DISCUSSION

*Importance of DNA sequence data in understanding the evolution of geniculate corallines.*

Based on morpho-anatomy alone, no one would have hypothesized that the nongeniculate corallines formerly called *Pseudolithophyllum muricatum* or *P. whidbeyense* belonged in Corallinoideae, a subfamily characterized by the presence of genicula. Hind and Saunders (2013) first demonstrated this for *P. muricatum* using DNA sequence data and, subsequently, Hind et al. (2016) erected the genus *Crusticorallina* for *P. muricatum* and related species. DNA sequence data was required to understand the phylogenetic placement of these taxa whose morpho-anatomical characters were not indicative of their evolutionary relationships. The on-going re-evaluation of coralline species using DNA sequence data provides greater insight into which morphological synapomorphic characters are informative in this morphologically diverse assemblage of species.

In addition to problems inherent in relying solely on morpho-anatomical characters to understand the generic placement of taxa, the type material of *Lithophyllum whidbeyense* was heterotypic. The type material of *L. whidbeyense* examined by Adey (1970) in TRH was placed in *Mesophyllum*, a genus of Melobesioideae (Hapalidiales), whereas material examined by Steneck and Paine (1986) from the same collection in UC was placed in *Pseudolithophyllum*, a genus at that time in Mastophoroideae (Corallinales). Thus, the type collection of *Lithophyllum whidbeyense* included

material from two different coralline orders. Steneck and Paine's (1986) concept of their field-collected material clearly was the nongeniculate *Bossiella* species treated herein, but what they thought was a single species was very likely a mix of both *B. mayae* and *B. exarticulata* specimens from low intertidal habitats. DNA sequence data was essential to resolve these relationships--from orders, to genera, to species, to individual specimens.

#### *Evolutionary reversals.*

In subfamily Corallinoideae we have two clear examples of secondary loss of a derived character, one at the generic rank, *Crusticorallina* (Hind et al. 2016), and the other among *Bossiella* species. Both examples represent phenotypic reversions as defined by Porter and Crandall (2003), when a derived state reverts to an ancestral state that is still present in extant lineages. The character state here is the presence or absence of non-calcified genicula enabling prostrate calcified crusts to produce jointed, upright fronds. Coupled to the production of genicula and erect axes in these taxa is the re-positioning of conceptacles onto upright segments of erect thalli, even in species with extensive crustose bases and few, scattered miniscule uprights (e.g., *Chiharaea americana* [Martone et al. 2012]). Within subfamily Corallinoideae, *Crusticorallina* species are not basal and, thus, are likely not indicative of the crustose ancestors that gave rise to geniculate corallinoids – rather the genus evolved from geniculate ancestors and represents a complete loss of upright fronds (Hind et al. 2016). The same is true for the two encrusting species of *Bossiella*: they are not basal within the genus, but rather each independently evolved back to the crustose state from closely-related, geniculate ancestors (see strong statistical support, Fig. 1). This suggests, at least in Corallinoideae, that the evolutionary loss of genicula and reversion to the crustose state is not rare and that morphological transitions may happen rapidly. According to Aguirre et al. (2010), *Bossiella* diverged approximately 8-11 mya, suggesting that the reemergence of the crustose phenotype evolved during the rapid radiation of *Bossiella* species. We speculate that rapid and repeated transitions between character states are unlikely to be genetically complex, perhaps requiring few genetic mutations. Controlled decalcification occurs in all nongeniculate species to produce reproductive gametangia and sporangia in conceptacles, and we speculate that this pre-existing decalcification process may be co-opted to produce genicula in geniculate corallines.

#### *Link between geniculate fronds and conceptacle position.*

Hind et al. (2016) first noted that all geniculate corallines, even those with extensive crustose bases and with sparse uprights (e.g., *Chiharaea americana*, *C. americana* f. *bodegensis*) bear their conceptacles on upright intergenicula. We have never observed conceptacles in basal crusts of geniculate corallines, including *Bossiella*, the first coralline genus to include both geniculate and nongeniculate species. There are, however, two reports in the literature of geniculate corallines bearing conceptacles in their crusts, *Amphiroa crustiformis* (Dawson 1963) and *Amphiroa currae* (Ganesan 1971). Both of these need to be re-examined as, particularly with tropical corallines, geniculate taxa can appear to arise from crusts, but upon extracting and sequencing erect axes and crusts separately, they prove to be unrelated taxa (P. Gabrielson, pers. obs.).

That conceptacle position is so tightly correlated with the presence of geniculate fronds suggests that there may be a developmental link between the two processes and, perhaps, an evolutionary advantage to bearing conceptacles on upright intergenicula. Even in *C. americana*,

where uprights are sparse and are commonly reduced to only one intergeniculum (about 1 mm tall), conceptacles are only found in the minute uprights and never in the extensive crustose base that may be 10-30 cm in diameter. Moreover, conceptacle formation is restricted to the intergenicula of geniculate corallines in all three lineages of Corallinales that independently evolved genicula, suggesting that elevated conceptacles may be one of the selective advantages driving the repeated evolution of upright fronds. This argument fails to explain the several evolutionary reversals that led to the crustose *Bossiella* species presented here and the crustose genus *Crusticorallina*, which not only survived without elevated conceptacles but radiated into four species (Hind et al. 2016). The adaptive significance and ecological costs associated with transitions back to the crustose state warrant further investigation.

#### *The genus Bossiella.*

*Bossiella* is the only genus of coralline algae that contains both geniculate and nongeniculate species. *Bossiella* species are antipodal in the eastern Pacific, ranging from Alaska, USA to Baja California, Mexico in the northern hemisphere (Hind et al. 2014, 2015) and reported from Chile (Ramírez and Santelices 1991, Hind et al. 2014) and around Cape Horn into Argentina (Boraso de Zaiuso 2013) in the southern hemisphere. *Bossiella compressa* Klochcova (1979) from the northwest Pacific requires confirmation of its generic placement by DNA sequencing. Just as it is difficult and frequently impossible to use morpho-anatomical characters to distinguish geniculate species of *Bossiella* in the Northeast Pacific (Hind et al. 2014, 2015), it is difficult to distinguish the nongeniculate species, *B. mayae* and *B. exarticulata*. No morpho-anatomical character reliably distinguishes the two species (Table S2). Reproductive characters are generally uninformative at the species rank, partly because reproductive specimens of *B. exarticulata* were rare. Biogeography is not helpful either, as the ranges of both species overlap from Haida Gwaii, northern BC, Canada to Monterey Co., CA. Both species occur on the same substrata, primarily bedrock (*B. exarticulata* found once on large cobble), but also commonly on shells of molluscs and once on the cup coral *Balanophyllia elegans*. Only *B. exarticulata* occurs subtidally, but both are common in the low intertidal and infrequent in mid-intertidal pools or on exposed bedrock.

*Bossiella mayae* and *B. exarticulata* are readily identified from all other crustose corallines in their range when tessellations and swirls are present (Figs. 2A and 3A), surface texture that arises when crusts grow over old conceptacles (e.g., Fig. 2C) or recover from grazer damage (Steneck and Paine 1986). However, when these features are absent (~50% of collections; Figs. 2B and 3C), crustose *Bossiella* species may be confused with other nongeniculate corallines, including *Lithophyllum impressum*, *Spongites decipiens*, and *Crusticorallina* spp. in the intertidal zone and many undescribed species in the shallow subtidal zone (<15 m). Thus, despite their complex evolutionary history, crustose *Bossiella* species have relatively simple morphologies and require DNA sequencing to be reliably identified.

#### CONCLUSIONS

Molecular phylogenetics has convincingly shown that geniculate corallines have arisen from nongeniculate corallines multiple times in family Corallinaceae. In subfamily Corallinoideae the reverse has also occurred: geniculate corallines have given rise to nongeniculate corallines. We demonstrated that the previously geniculate genus *Bossiella* includes two nongeniculate species, *B.*

*mayae* and *B. exarticulata*, and that each evolved independently and with complete loss of geniculate fronds. The discovery of a coralline genus with both geniculate and nongeniculate species suggests that such evolutionary reversals may occur rapidly and may not be genetically complex. Given the repeated gain and loss of geniculate fronds throughout the Corallinaceae, *Bossiella* is a likely model taxon in which to explore the mechanisms and adaptive significance of evolutionary reversals.

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## FIGURE LEGENDS

Figure 1. Phylogram inferred by bayesian inference (BI) analysis of concatenated COI-5P, *rbcL*, and *psbA* sequence data (2596 bp) demonstrating the placement of *B. exarticulata* and *B. mayae* within the genus *Bossiella*. Support values listed as posterior probabilities (PP) and bootstrap for BI and ML analyses respectively. Asterisks denote strongly supported nodes (PP  $\geq 0.98$ , bootstrap  $> 98$ ; dash denotes nodes with PP  $< 0.54$ , bootstrap  $< 62$ ). Scale bar refers to substitutions per site. Only branches of interest in the *Bossiella* clade labeled with support values.

Figure 2. *Bossiella mayae*, D and H holotype specimen. (A) Habit showing tessellations and swirls (UBC A91398, scale = 1 cm); (B) Habit showing thin white margin (UBC A91393, scale = 1 cm); (C) Thallus with swirls surrounding flat-topped protuberances (UBC A91394, scale = 1 mm); (D) Thallus with swirls and uniporate tetrasporangial conceptacles (NCU 591286, scale = 1 mm); (E) Cross-section of thallus showing flared epithallial cells (UBC A91393, scale = 10  $\mu$ m); (F) Surface view of uniporate conceptacles (UBC A91385, scale = 1 mm); (G) Cross-section showing shape of conceptacle chamber (UBC A91384, scale = 100  $\mu$ m).

µm)); (H) Fracture showing cross-section of conceptacle chambers with pigmented tetrasporangia (NCU 591286, scale = 500 µm).

Figure 3. *Bossiella exarticulata*, D, F, G and H holotype specimen. (A) Habit showing tessellations and swirls (UBC A91397, scale = 1 cm); (B) Close-up of tessellations and swirls (UBC A91397, scale = 1 mm); (C) Habit showing smooth surface and thin white margin (UBC A91386, scale = 1 cm); (D) Cross-section of thallus showing flared epithallial cells (UBC A91389, scale = 10 µm); (E) Cross-section of thallus showing thick perithallus (UBC A91397, scale = 100 µm); (F) Surface view of uniporate conceptacles (UBC A91389, scale = 1 mm); (G) Cross-section showing shape of conceptacle chamber (UBC A91389, scale = 100 µm); (H) Fracture showing cross-section of conceptacle chambers with pigmented tetrasporangia (UBC A91389, scale = 500 µm).

Figure S1. Material in TRH (NLG, Nathaniel Lyon Gardner 655) designated by Mason (1953) as lectotype of *Lithophyllum whidbeyense* comprised of two *Acmaea mitra* shells. Shell on left is preparation 802 that bears no conceptacles; shell on right is preparation 803 that bears conceptacles.

Figure S2. Preparation 802 (left hand shell in Fig. S1) with cell measurements in Foslie's hand and annotation "*Lithoph. whidbeyense?* cfr. 803" indicating Foslie's uncertainty of its identity.

Figure S3. Preparation 803 (right hand shell in Fig. S1) with cell measurements in Foslie's hand and "*Lithoph. whidbeyense*" along with conceptacle size range. Lectotype of *Lithophyllum whidbeyense* narrowed to crust with conceptacles on this shell.

Figure S4. Phylogram inferred by maximum likelihood analysis of concatenated COI-5P, *rbcL*, and *psbA* sequence data (2596 bp). Support values listed as bootstrap. Only clades with bootstrap >61 are indicated. Scale bar refers to substitutions per site.

Table S1. Collection information, herbarium accession numbers, and Genbank numbers for *Bossiella mayae* and *Bossiella exarticulata*. Bold indicates type specimens.

Table S2. Distance matrix indicating inter- and intra-specific percent divergence of A) COI-5P, B) rbcL, and C) psbA gene regions for *Bossiella mayae* and *Bossiella exarticulata* specimens in this study.

Table S3. List of COI-5P, psbA, and rbcL sequence fragments used in the concatenated phylogenetic tree of select coralline species.

Table S4. Summary of morphology and habitat assessment of crustose *Bossiella* species examined in this study. Measurement ranges reported for internal anatomy. n/a = not available. n=the number of measurements, not the number of individuals.









