Convergence of joint mechanics in independently-evolving, articulated coralline algae

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Abstract
Flexible joints are a key innovation in the evolution of upright coralline algae. These structures have evolved in parallel at least three separate times, allowing the otherwise rigid, calcified thalli of upright corallines to achieve flexibility when subjected to hydrodynamic stress. As all bending occurs at the joints, stress is amplified, which necessitates that joints be made of material that is both extensible and strong. Data presented here indicates that coralline joints are in fact often stronger and more extensible, as well as tougher, than fleshy seaweed tissues. Corallinoids are particularly strong and tough, which is largely due to the presence of secondary cell walls that strengthen the joint tissue without adding bulk to the joint itself. Cell wall thickness is shown to be a large contributing factor to strength across all groups, with the exception of the corallinoid Cheilosporum sagittatum, which likely possesses distinct chemical composition in its walls to increase strength beyond that of all other species tested.
Introduction

Wave-swept, rocky shorelines are a place of extreme hydrodynamic stress. Organisms living in these habitats are subject to water velocities that regularly reach 2 m/s as waves break, with velocities as high as 25 m/s being recorded in intertidal surf (Denny, 1988; Denny et al., 2003). Sessile organisms such as seaweeds cannot relocate to avoid high wave action, and must contend with the drag forces imposed upon them. Drag depends upon the size and shape of seaweeds, and flexible seaweeds optimize both these factors by bending over to minimize projected area and reconfiguring branches or blades into more streamlined shapes (Denny and Gaylord, 2002; Harder et al., 2004; Martone, 2006; Martone et al., 2012). This means that they take on a drag-reducing form only when drag is actually imposed.

Unlike upright algae that are generally flexible along their entire thallus, coralline algae provide a unique and interesting exception. Coralline algae are morphologically distinguished from most other algae by their hard thalli, resulting from calcium carbonate that is deposited within their cell walls (Johansen, 1981). How, then, do coralline algae withstand wave-induced drag? Crustose coralline species grow prostrate and thus not only remain in the slower moving water in the boundary layer (Denny and Gaylord, 2002), but also maintain maximum attachment to the substrate. Upright coralline species must find other ways to minimize or withstand the hydrodynamic forces being imposed and they do so, surprisingly, by utilizing the same strategy as fleshy upright algae: flexibility. To achieve flexibility, many upright corallines have evolved uncalcified joints, called genicula, that separate calcified segments, called intergenicula; together, these components make up calcified-yet-flexible upright fronds.

Evidence suggests that upright articulated corallines have evolved from prostrate, crustose coralline ancestors. This evolutionary trajectory is supported both by the fossil record (Aguirre et al., 2010; Kundal 2011) and by molecular phylogenetics (Bailey and Chapman, 1998; Bittner et al., 2011; Kato et al., 2011). Moreover, these data suggest that articulated thalli are polyphyletic, evolving from crustose corallines multiple times and leading to three distinct phylogenetic groups of articulated coralline algae: Corallinoideae, “Amphiroideae”, and Metagoniolithoideae (Fig. 1; Johansen 1981). Genicula in these lineages are non-homologous, suggesting that evolution has iterated this distinct way of achieving flexibility at least three separate times in parallel. In fact, while Corallinoideae and Metagonioilthoideae represent distinct coralline subfamilies, molecular data (Bailey, 1999) have sunk the articulated coralline subfamily “Amphiroideae” into the crustose coralline subfamily Lithophylloideae. This further highlights the repeated evolution of articulated taxa. For simplicity, the term “amphiroids” in this study will refer to Amphiroideae sensu Johansen (1981).

While an articulated morphology allows upright corallines to bend over and reconfigure in a similar manner to fleshy algae, it also presents unique biomechanical challenges. Bending occurs only at discrete joints along articulated thalli, and so joints must be composed of materials that are both extensible enough to retain flexibility, and strong enough to resist amplified bending stress (Martone
and Denny, 2008a). Furthermore, joints must also resist tensile forces associated with drag, after bending has occurred. Genicula in the corallinoid Calliarthron cheilosporioides Manza are composed of tissues that are often more extensible than other red algal tissues (Hale, 2001), as well as 35-400% stronger than other red algal tissue (Hale, 2001; Kitzes and Denny, 2005; Martone, 2006).

The exceptional material properties of Calliarthron likely contribute to its dominant abundance in wave-swept intertidal habitats where it is found, but do other articulated corallines display similar properties? Structural differences between genicula in the corallinoids, amphiroids, and metagoniolithoids previously described could affect the mechanical performance of joints under bending stress. For example, corallinoid genicula are unique in being composed of a single tier of cells that are anchored to adjacent intergenicula, but only loosely connected to one another laterally (Fig 2A; Johansen, 1969; Johansen, 1981; Martone and Denny, 2008a; Denny et al., 2013). Amphiroid genicula are often multi-tiered (Fig. 2B), whereas metagoniolithoid genicula lack a tiered structure altogether (Fig 2C; Johansen, 1969; Ducker, 1979; Johansen, 1981). Genicular cells in Calliarthron cheilosporioides Manza also possess secondary cell walls that likely play a role in strengthening genicular tissue (Martone, 2007; Martone et al., 2009), whereas no similar feature has been documented in either amphiroids or metagoniolithoids.

This study aims to investigate the precision with which joints have evolved in parallel in articulated coralline algae by comparing their material properties, which are integral to the functioning of those joints under hydrodynamic stress. Given the unique mechanical challenges posed by possessing a jointed morphology, we hypothesized that genicular tissue in all three groups would be both stronger and more extensible than other fleshy red algal tissues. The maximum material stress and strain required to break joint tissue was measured, as well as the stiffness of joint tissue during loading in tension. Tensile toughness (strain energy density, i.e. the energy absorbed before breaking) was calculated from the area under a stress-strain curve, with an alga achieving toughness by being very strong, very extensible, or both. This property has been widely reported for marine plant tissues (Koehl and Wainwright, 1977; Armstrong, 1987; Patterson et al., 2001; Harder et al., 2006), however the biological significance is unclear (Denny and Gaylord, 2002; Denny and Hale, 2003). Finally, we explored whether any apparent differences in those properties among the three subfamilies could be attributed to differences in cellular structure or cell wall thickness.

Materials and Methods

Specimen Collection

Cheilosporum sagittatum (J.V. Lamouroux) Areschoug was collected from Glaneuse Reef, Point Lonsdale, Victoria, Australia (38°17’37”S, 144°36’47”E) in January 2009, from a depth of about 10 ft. Calliarthron tuberculatum (Postels & Ruprecht) E.Y. Dawson, Corallina officinalis Linnaeus, and Johansenia macmillanii (Yendo) K.Hind & G.W. Saunders were collected subtidally at approximately
10 ft. from Botanical Beach (48°31’48” N, 124°27’18” W) on Vancouver Island, BC, Canada, in June/July of 2012.

*Lithothrix aspergillum* J.E. Gray was collected from Potato Harbor (32°02’52” N, 119°35’31” W) on Santa Cruz Island, CA, USA, at depths of 15-17 ft., in September 2006. *Amphiroa anceps* (Lamarck) Decaisne and *Amphiroa gracilis* Harvey were collected in Point Peron (32°16’01” S, 115°41’14” E), Perth, Western Australia at depths of 10-15 ft., in December 2012.

*Metagoniolithon stelliferum* #1 and #2 indicate specimens that currently fall under the name *Metagoniolithon stelliferum* (Lamarck) Ducker but that appeared morphologically distinct in the field. Sequencing of psbA, CO1 and rbcL genes indicates that these two groups represent distinct species (Janot, unpublished data), and so they have been treated as such in this study. Both *Metagoniolithon stelliferum* “species” were collected in December 2012 from Point Peron at 10-15 ft., where they were growing epiphytically side by side on seagrass. *Metagoniolithon chara* (Lamarck) Ducker was collected off of Carnac Island (32°07’07” S, 115°39’52” E) near Perth, Western Australia, at depths of about 15 ft., in January 2014.

All plants were collected in their entirety and kept in flowing seawater in the lab prior to mechanical testing. Mechanical tests were performed no later than 72 hours after collection, and remaining specimens were air dried for later microscopic analysis. Representative vouchers for each species were deposited into the University of British Columbia Herbarium for future taxonomic reference: *Cheilosporum sagittatum* (A88599) *Calliarthron tuberculosum* (A91564); *Corallina officinalis* (A91563); *Johansenia macmillanii* (A91561); *Lithothrix aspergillum* (A88575); *Amphiroa anceps* (A91566); *Amphiroa gracilis* (A91572); *Metagoniolithon stelliferum* #1 (as *Metagoniolithon stelliferum*, A91576); *Metagoniolithon stelliferum* #2 (as *Metagoniolithon sp.*, A91579); *Metagoniolithon chara* (A91464).

**Pull-to-Break Tests**

*Calliarthron tuberculosum* (n=15), *Corallina officinalis* (n=15), and *Johansenia macmillanii* (n=12) were tested using a standard tensile method in a computer-interface tensometer (model 5500R, Instron Corp, Canton, MA, USA). Basal 2-3 cm segments were held in pneumatic clamps lined with neoprene and sandpaper, which provided both cushioning and friction. Each segment included multiple genicula which floated between the clamps – the exact number varied depending on the species. Samples were wetted with seawater after being mounted in the clamps and before testing. Extension was continuously measured via movement of the crosshead, while force was measured via a 50 kg tension load cell. Specimens were directly observed during testing to monitor slippage in the clamps, and tests in which slippage occurred were not included in analysis. The crosshead was set to move at a rate of 10 mm/min until tissue failure, as measured by a sudden drop in force. All data was collected and initially processed using the Instron Bluehill 3 software (Instron Corp., Canton, MA, USA).
Lithothrix aspergillum (n=9) was tested with the same custom built, portable tensometer described in Martone (2006). In short, fronds were held between two sets of aluminum clamps which moved along a tensometer track. Clamps were positioned on the intergenicula, and lined with rubber pads in order to prevent the calcified tissue from being crushed. Force was quantified as the deflection of a stationary clamp mounted to two steel beams, measured by a linearly variable differential transformer (LVDT; model 100HR, Schaevitz Engineering, Pennsauken, NJ, USA). Strain was measured directly using a video camera (model TMC-S14, Pulnix Sensors Inc., Sunnyvale, CA, USA) and video dimension analyser (model V94, Living Systems Instrumentation, Burlington, VT, USA), which tracked the relative position of intergenicula flanking individual joints in each stretched specimen. Specimens were pulled at a rate of 60 mm/min until failure.

Cheilosporum sagittatum (n=10), Amphiroa anceps (n=15), Amphiroa gracilis (n=12), Lithothrix aspergillum (n=9), Metagoniolithon stelliferum #1 (n=20), Metagoniolithon stelliferum #2 (n=15), and Metagoniolithon chara (n=14) were tested with a second custom built, portable tensometer. Fronds were clamped in a similar manner to that described in Martone (2006), with the aid of a motor (model SM2315D, Moog Animatics, Milpitas, CA, USA) controlled via the SmartMotor Interface (Moog Animatics, Milpitas, CA, USA). Force was measured with a 5 kg beam transducer (model FORT5000, World Precision Instruments Inc., Sarasota, FL, USA), which was amplified through a transducer amplifier (model SYS-TBM4M, World Precision Instruments Inc., Sarasota, FL, USA) and collected in real time using LabVIEW SignalExpress software (National Instruments Canada, Vaudreuil-Dorion, QC, CA). Extension was measured as the displacement of the mobile clamp, calculated from the number of rotations of the motor. Specimens were pulled at a rate of 60 mm/min until failure.

As this study includes data collected over a span of five years, using two different extension rates, we compared results for 5 specimens of Calliarthron tuberculosum were also tested in the portable tensometer at a rate of 60 mm/min, as described above. Breaking stress, breaking strain, Young’s modulus and breaking energy all fell within the ranges found for Calliarthron specimens tested in the Instron tensometer.

After testing, samples were dissected under a dissecting microscope (model SZ61, Olympus Canada Inc., ON, CA) with an attached camera (model DP20, Olympus Canada Inc., ON, CA) to measure cross-sectional area of the broken interface (estimated as elliptical) and cumulative genicular length (i.e. length of all genicula in the testing area added together). All specimens tested with the custom portable tensometer were first dried for transport, then rehydrated in saltwater for at least 10 minutes prior to morphometric measurements. Stress (σ, N/m²) was obtained by dividing force measurements by cross-sectional area of the broken geniculum, and strain (ε) was calculated by dividing extension (l) by initial cumulative genicular length (l₀). The resulting stress-strain curve was used to calculate Young’s modulus (E, N/m²), a measurement of initial tissue stiffness, by taking the
slope of the curve from 0-0.1 strain. Breaking strain energy density (MJ/m$^3$), or toughness, was calculated from the total area under the stress-strain curve when specimens were pulled to break.

Data from fleshy red, green, and brown algal tissues were compiled from Hale (2001). Three species from each group were selected to represent a large range of breaking stress, breaking strain, Young’s modulus, and breaking energy. These species were graphed alongside data from this study for comparative purposes.

**Transmission Electron Microscopy (TEM)**

One representative species was chosen to illustrate each subfamily - *Calliarthron tuberculinosum* for Corallinoideae, *Amphiroa anceps* for Amphiroideae, and *Metagoniolithon stelliferum* for Metagoniolithoideae. One specimen of each representative species was rehydrated for 1 hour in seawater, and fixed overnight in 5% formalin seawater. Fixed specimens were decalcified overnight in HCl, and then dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, and 100%) for an hour per treatment. Specimens were left in 100% ethanol overnight, then placed medium grade LR White embedding resin overnight. Specimens were placed in gel capsules, immersed in fresh LR White, and baked at 62 °C for 1.5 hours.

Resin blocks were sectioned using a diamond knife mounted on an ultramicrotome (model Ultracut T, Leica Biosystems, Nussloch, Germany). Sections were mounted on formvar coated 100 mesh copper grids, and stained with uranyl acetate for 17 minutes and Reynold’s lead citrate for 6 minutes. Sections were visualized and photographed on a transmission electron microscope (model H7600, Hitachi High-Technologies Canada Inc., Toronto, ON, CA).

**Cell Wall Analysis**

Five specimens of each species were rehydrated in saltwater for a minimum of ten minutes, after which they were decalcified in 1M HCl between 2-24 hours (the time required for full decalcification varied widely between species). Decalcified samples were placed in ethanol for ten minutes, embedded in Tissue Tek O.C.T. compound (Sakura Finetek, Europe), then cross-sectioned within the basal genicular region using a freezing microtome (model CM1850, Leica Biosystems, Nussloch, Germany). Thickness of sections varied between 10-20 µm. Sections were dyed with 5% potassium permanganate for approximately five minutes, then washed with freshwater and viewed under a light microscope (model BX51wi, Olympus Canada Inc., ON, CA). Photos were taken using a camera (DP21, Olympus Canada Inc., ON, CA) attached to the microscope.

Photos were analysed using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). Cell wall proportion in a cross-section was estimated by drawing and measuring the area of polygons around the a) lumen, b) lumen + cell wall, c) lumen + cell wall + half the extracellular matrix/middle lamella (Fig. 8). Cell wall percent was calculated as:
\[
\text{CW}\% = \frac{(b_{\text{area}} - a_{\text{area}})}{c_{\text{area}}} \times 100
\]

(1)

Cell wall percent was calculated for 20 randomly selected cells per cross-section, and then averaged to obtain one value per specimen. In the case of the metagoniolithoids, both cortex and medulla cells were visible and had slightly different morphologies; measured cells were split evenly between the two layers, and the final average weighted these measurements depending on proportion of each tissue layer in the overall cross-section.

Given that coefficients of variation (\(c_v\)) for CW\% were generally low, under 0.1 for all species except *Cheilosporum sagittatum* (\(c_v = 0.17\)), an average cell wall percentage was calculated for each species. This average was used to correct breaking stress values obtained from pull-to-break tests, calculated as:

\[
\sigma_{\text{CW}} = \frac{(\sigma_{\text{tissue}} \times 100)}{\text{CW}\%}
\]

(2)

One specimen of *Cheilosporum sagittatum* was embedded in LR White resin using the same protocol described for TEM. 10 µm sections were obtained with an ultramicrotome ("Porter-Blum" MT-2, Sorvall Products, New Castle, Delaware, USA) and stained and visualized with the same methods used for the cryosections.

**Statistics**

As unequal variances between species could not be solved with either logarithmic or square root transformations, non-parametric Kruskal-Wallis tests and post-hoc Dunn’s tests were performed to compare breaking stress, breaking strain, Young’s Modulus and breaking energy, as well as cell wall stress. Statistical comparisons were made at the species level only. This was done in R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria) using the RStudio interface (version 0.98.1056, RStudio Inc., Boston, Massachusetts, USA), using the dunn.test() function from the dunn.test package (dunn.test: Dunn’s Test of Multiple Comparisons Using Rank Sums, version 1.2.3, Alexis Dinno 2015). The relationship between tissue stress and cell wall percent in cross-section was tested in R 3.0.1 with a one-way ANOVA using the lm() and anova() functions from the base stats package.

Averages and standard errors reported for each subfamily were calculated by pooling all data from all species within each subfamily. Statistics were not performed at the subfamily level.
Results

Tissue Breaking Stress

Average tissue breaking stress (mean ± s.e.m.) was 31.9 ± 2.0 MPa for the corallinoids, 10.7 ± 0.6 MPa for the amphiroids, and 5.6 ± 0.5 MPa for the metagoniolithoids. Average stress varied significantly between species (Kruskal-Wallis test, p<0.001), and these differences were consistently segregated among subfamilies (Dunn’s test, Fig. 3A). With the exception of Johansenia macmillanii, all corallinoid species were significantly stronger than all amphiroid and metagoniolithoid species tested. Cheilosporum sagittatum was the strongest of the corallinoids, with an average breaking stress of 56.3 ± 2.4 MPa, over 75% stronger than the next strongest species, Corallina officinalis, with an average breaking stress of 31.7 ± 2.0 MPa.

All species tested appeared stronger than typical green and brown algal tissues (Fig. 3A). While corallinoid and amphiroid species were consistently stronger than other red algal tissues, metagoniolithoid species fell within the range for fleshy red algae reported by Hale (2001).

Breaking Strain

Average breaking strain (mean ± s.e.m.) was 0.77 ± 0.04 for the corallinoids, 0.84 ± 0.06 for the amphiroids, and 0.56 ± 0.07 for the metagoniolithoids. Average breaking strain was significantly different among species (Kruskal-Wallis test, p<0.001), although this variation was unrelated to subfamily (Dunn’s test, Fig. 3B). Both amphiroids and metagoniolithoids contained species with some of the highest average breaking strains (e.g. Amphiroa anceps: 1.02 ± 0.09, and Metagoniolithon chara: 1.07 ± 0.14) and some of the lowest (e.g. Lithothrix aspergillum: 0.35 ± 0.04, and Metagoniolithon stelliferum #1: 0.32 ± 0.02).

Many of the species tested had breaking strains that were double or more that of other red, green, and brown algal tissues (Fig. 3B). Even the lowest strains (i.e. that of Lithothrix aspergillum and Metagoniolithon stelliferum #1) were on the higher end of the range for other algal tissues.

Young’s Modulus

Average Young’s modulus (initial stiffness, mean ± s.e.m.) was 51.7 ± 4.6 MPa for the corallinoids, 19.5 ± 3.7 MPa for the amphiroids, and 15.8 ± 2.2 MPa for the metagoniolithoids. Although average Young’s modulus was significantly different among species (Kruskal-Wallis test, p<0.001), modulus was highly variable for all species, and did not consistently differ among subfamilies (Dunn’s test, Fig. 3C). Cheilosporum sagittatum had the highest average stiffness, with a Young’s modulus of 92.1 ± 14.4 MPa, over 75% higher than the modulus of Corallina officinalis at 51.8 ± 6.5 MPa. While the stiffest species came from the corallinoids, high modulus values were also
found in the amphiroids and metagoniolithoids – *Lithothrix aspergillum* had a modulus of 43.6 ± 11.6 MPa, while *Metagoniolithon stelliferum* #1 had a modulus of 30.3 ± 3.3 MPa.

With the exception of *Cheilosporum sagittatum*, which was almost twice as stiff as the stiffest fleshy red species tested by Hale (2001), most coralline species tested fell within the range of stiffness reported for other red algal tissues (Fig 3C). Consistent with fleshy red algae, all coralline species were stiffer than green algal tissues, though not notably different from brown algal tissues.

**Toughness**

Average toughness (breaking strain energy density, mean ± s.e.m.) was 15.7 ± 1.5 MJ/m$^3$ for the corallinoids, 5.0 ± 0.5 MJ/m$^3$ for the amphiroids, and 2.2 ± 0.6 MJ/m$^3$ for the metagoniolithoids. Average toughness differed among species (Kruskal-Wallis test, p<0.001). All corallinoid species were tougher than all metagoniolithoid species, while amphiroid species were not significantly different from either corallinoids or metagoniolithoids (Dunn’s test, Fig. 3D). *Cheilosporum sagittatum* had the highest breaking energy at 31.6 ± 3.8 MJ/m$^3$ - more than double that of the next toughest species, *Corallina officinalis*, which had a breaking energy of 12.1 ± 1.2 MJ/m$^3$.

Almost all species tested had a higher toughness than the fleshy red, green, and brown algal tissues tested by Hale (2001). *Metagoniolithon stelliferum* #2 fell within the range of other red algae.

**Cell Wall Thickness and Stress**

With the exception of *Cheilosporum sagittatum*, corallinoid species had genicula with proportionally thicker cell walls than genicula in amphiroid and metagoniolithoid species (Fig. 4). This was largely due to the presence of a secondary cell wall, which roughly doubled the total cell wall area in cross-section. Secondary walls were not consistently visible in *Cheilosporum*, with the exception of the most basal tissue (Fig. 9). In addition, all amphiroids tested as well as one metagoniolithoid species (*Metagoniolithon stelliferum* #1) had large non-fibrillar extracellular spaces/middle lamellae that may have been either not present, or not visible to the naked eye in other metagoniolithoids or in any of the corallinoids (see Fig. 5 for examples).

Correcting cross-sectional area with approximate cell wall percentage yields breaking stresses that are much closer across species and articulated groups (Fig. 6), with the exception of *Cheilosporum sagittatum*. Cell wall breaking stress (mean ± s.e.m.) was 50.1 ± 4.3MPa for the corallinoids (but 32.6 ± 1.6 MPa if we exclude *Cheilosporum*), 24.8 ± 1.4 MPa for the amphiroids, and 15.2 ± 1.2 MPa for the metagoniolithoids. While cross-sectional area did not account for all of the variation in breaking stress among species (Kruskal-Wallis test, p<0.001), many differences among individual species are lost after accounting for the cell wall (Dunn’s test, Fig. 6), so that the previously clear relationship between subfamily and strength is blurred. Cell walls in the corallinoid *Johansenia macmillanii* are statistically indistinguishable from any of the amphiroids, as well as
**Discussion**

Results presented here support the hypothesis that unique challenges faced by articulated corallines contribute to extraordinary mechanical properties of joint tissue. Genicula were generally tougher, and often stronger and more extensible, than fleshy algal tissues. This is particularly striking given the evolutionary and structural differences of the joints among the three subfamilies. Corallinoids were much stronger and tougher than both fleshy algae and other articulated corallines, as well as much more extensible than fleshy species. Amphiroid species were stronger and tougher than fleshy algae, and either exceeded or fell at the high end of the range for extensibility in fleshy species. *Metagoniolithon stelliferum* #1 and *Metagoniolithon chara* were tougher than fleshy algae, while *Metagoniolithon chara* was also more extensible than fleshy algae. In all other instances, metagoniolithoid species fell within the ranges of strength/extensibility/toughness for fleshy algae.

Tensile toughness is measured as the area under the stress-strain curve, and high breaking stress or high breaking strain can both result in “tough” biological materials. In the case of articulated corallines, both properties appear to play a role – this is most apparent when comparing amphiroid and metagoniolithoid species to other red algal tissues. *Metagoniolithon stelliferum* #1, for example, is neither obviously stronger nor more extensible than fleshy red algal tissues, but moderate performance in both traits results in a comparably high toughness. Toughness in corallinoids is also related to both high stress and strain relative to fleshy algae, however it is the high strength of this group that pushes its toughness past that of other coralline subfamilies. Although the almost universally high toughness of articulated corallines distinguishes their genicular material from other algal tissues, it is not clear whether this ability to absorb energy is beneficial to survival in wave-swept environments. The amount of energy actually absorbed by an alga in flow is likely negligible compared to the vast kinetic energy available in a given wave (Denny and Gaylord, 2002). Furthermore, energy that is absorbed by seaweeds in this way could be released via propagation of cracks through the tissue being loaded, ultimately leading to catastrophic failure (Denny and Hale,
Thus, the biological significance of high toughness in algal tissues is unclear and deserves more study.

That the metagoniolithoids were weaker than other articulated corallines is perhaps not surprising, given the difference in substrates and tissue composition. First, while the corallinoids and amphirhoids tested in this study were found growing predominantly on rock, all three metagoniolithoid species tested were growing as epiphytes on seagrass (mainly *Amphibolus* sp.). There are two potential biomechanical consequences of this epiphytic habit: 1) dislodgement is partially dependent on how much force is resisted by the host seagrass, and 2) drag may be lessened by growing epiphytically, due to both the potential “drafting” effect of the host as well as the host’s reconfiguration capabilities (see Anderson and Martone, 2014). This means that an epiphytic metagoniolithoid might not require tissue strength as high as an epilithic corallinoid or amphirhoid – indeed, having the capability to withstand more force than that of the host seagrass would be superfluous. It should be noted that the only known epilithic metagoniolithoid species, *Metagoniolithon radiatum* (Lamarck) Ducker, was not included in this study due to failure to procure fresh samples. Data from this species would be necessary in order to start disentangling the effects of taxonomy and environment.

Additionally, the unique biomechanical challenges faced by articulated corallines may not apply to metagoniolithoids. The segmented body plan of articulated corallines can result in amplification of bending stress at the joints, the degree of which is affected by a variety of morphological factors (Martone and Denny, 2008). Shorter joints, as well as joints that are flanked by long calcified “lips” (see Fig. 2A), experience more tissue stress. All of the corallinoid species tested possess calcified lips. All corallinoid and amphirhoid species had much shorter joints than any of the metagoniolithoid species (Table S1). In contrast, joints up to 6-8 mm are seen in *Metagoniolithon stelliferum* #1 (Fig.1C, Fig. 1F). By having long joints that are unhindered by calcified lips, metagoniolithoids may experience drag in a way that is more similar to fleshy algae than it is to other articulated corallines, making such high strength unnecessary.

While all of the corallinoid species tested possess large breaking stresses compared to other articulated groups, *Cheilosporum sagittatum* was particularly impressive. High material strength in this species may be necessary to offset its slender genicula; with an average cross-sectional area of 0.04 mm², joints in *Cheilosporum sagittatum* were anywhere from 3-15x skinnier than joints of other species (Table S1). Across both red and brown algal species, there is a tendency for algae with more slender thalli to be composed of stronger tissues than algae with thicker thalli (Martone, 2007). By increasing the quality, rather than the quantity, of joint tissue, *Cheilosporum sagittatum* may withstand forces similar to seaweeds of much larger sizes. This may mean that *Cheilosporum sagittatum* is over-designed for the drag forces it encounters – frond area affects drag in flow, and *Cheilosporum* is a diminutive species relative to the others tested. Frond area was not measured in this study, but would be a key factor to consider in future mechanical comparisons.
One factor that we were unable to control for in this study was the mechanical history of the specimens tested. Algae in wave-swept environments are subject to constant, repetitive stress that, over time, can lead to breakage at stresses far below the maximum strength of the tissue (Hale, 2001; Mach, 2009; Mach et al., 2011). This phenomenon, known as fatigue, is due to the accumulation of small imperfections in the tissue that can increase the likelihood of a crack propagating, ultimately leading to tissue failure (Vincent, 1990). While it is impossible to determine the degree to which fatigue played a role for each species in this study, it is likely that some species are more resistant to fatigue than others. For example, the genicula of Calliarthron cheilosporioides are known to be highly resistant to fatigue, due to the loose connection between genicular cells which minimizes propagation of cracks (Denny et al., 2013). While other corallinoids have a joint structure similar to Calliarthron, genicular cells in amphiroids and metagoniolithoids appear to be much more adherent to one another, potentially allowing for more energy transfer between adjacent cell walls. Amphiroid and metagoniolithoid species may be more susceptible to fatigue, thereby breaking at lower stresses that reflect imperfections accumulated during previous wave impacts in the field. Additionally, all non-corallinoid species except for Lithothrix aspergillum had multi-tiered joints – this could increase the number of weak points in the tissue, allowing cracks to propagate around the cells (through the middle lamella) rather than through the cell wall. The combination of differences in cell-cell adherence and tier structure could help explain the comparatively high strength of the corallinoids as a group, though these differences did not correlate with breaking strain.

To explore the contributions of cell wall composition and thickness to tissue strength, we corrected breaking stress measurements by the amount of cell wall. As much of the tensile load is likely to be taken up by the cell wall, this essentially calculated the breaking stress of the wall itself. For most species, cell wall quantity appeared to account for much of the difference in strength between groups. That is, articulated corallines appear to strengthen primarily by increasing the amount of cell wall within their tissues (Fig. 7). This is a very different strategy from that documented in other algae; kelps increase breaking force by adding cells near the stipe surface to increase girth (Martone, 2007), while fleshy red algae add cells to medullary tissue to increase blade thickness (Demes et al., 2011). Corallinoids generally had more cell wall than amphiroids and metagoniolithoids. However, Cheilosporum sagittatum was a notable exception (Fig. 4). While all other corallinoid species tested had clear secondary cell walls that accounted for roughly half of the cell wall volume - consistent with previous findings in Calliarthron cheilosporioides Manza (Martone, 2007; Martone et al., 2009) - none were immediately visible in the Cheilosporum sections investigated. Closer inspection of resin embedded specimens (as opposed to the cryosections used for cell wall measurements) revealed a layer within the primary cell wall that may represent a secondary cell wall (Fig. 9). If this is the case, this layer is chemically and mechanically distinct from the secondary walls present in other corallinoids – not only did it not stain with potassium permanganate, indicating a chemical composition differing from that of the primary wall, it also appeared to pull
away from the primary wall in some cells. Ultimately, cell wall strength of *Cheilosporum* was even greater than other articulated corallines, suggesting that the cell walls in *Cheilosporum* may be doing something unique at the chemical level.

Remaining differences in strength after accounting for the amount of cell wall may be due to differences in the types and quantities of different polysaccharides within the wall. Cell walls in most red algae are characterized by skeletal polysaccharides such as cellulose, as well as an amorphous matrix composed mostly of sulfated galactans (Frei and Preston, 1961; Usov, 1992; Tsekos, 1999; Vreeland and Kloareg 2000). In land plants, variation in the proportion of cellulose to matrix has been found to affect tensile strength (Genet et al., 2005; Girault et al., 1997). Furthermore, angle of the cellulose microfibrils may affect stiffness (Koehl and Wainright, 1977; Kohler and Spatz, 2002), as less steeply angled cellulose will take more time to reorient in the direction of the applied force. Strength, extensibility, and stiffness may depend on the type of sulfated galactans produced by different life stages of red algae (Carrington and Grace, 2001). The high material strength of corallinoids, in particular *Cheilosporum sagittatum*, could be due to either high levels of cellulose relative to other corallines, or a unique set of matrix polysaccharides linking the cellulose together.

Articulated corallines represent an interesting example of parallel evolution, in which multiple calcified algal groups have come to the same general solution for mitigating drag: growing upright flexible thalli via segmentation. Given the mechanical challenges inherent in a jointed morphology, articulated corallines have converged on a similar set of mechanical properties. Coralline joints are generally stronger and tougher than tissues of fleshy algae, while maintaining high strains comparable to fleshy algae. Tensile stiffness is highly variable among corallines. Differences in the cellular structure of joints, such as cell-to-cell adherence and the number of cell tiers, likely contribute to the slight remaining differences in mechanical behaviour between subfamilies. Data suggest that articulated corallines universally strengthen joints by augmenting the quantity of cell wall, with remaining differences in strength pointing to a potential contribution of cell wall composition. This is particularly evident in the unusual strength and toughness of the corallinoid *Cheilosporum sagittatum*, which warrants further investigation.
### Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$a_{\text{area}}$</td>
<td>cross-sectional area of cell lumen</td>
</tr>
<tr>
<td>$b_{\text{area}}$</td>
<td>combined cross-sectional area of cell lumen and cell wall</td>
</tr>
<tr>
<td>$c_{\text{area}}$</td>
<td>combined cross-sectional area of cell lumen, cell wall, and $\frac{1}{2}$ the extracellular matrix</td>
</tr>
<tr>
<td>CW$%$</td>
<td>percentage of cell cross-sectional area accounted for by cell wall</td>
</tr>
<tr>
<td>$E$</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>$l$</td>
<td>length</td>
</tr>
<tr>
<td>$l_0$</td>
<td>initial length</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>tensile strain</td>
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<tr>
<td>$\sigma$</td>
<td>tensile stress</td>
</tr>
<tr>
<td>$\sigma_{CW}$</td>
<td>tensile stress of cell wall</td>
</tr>
<tr>
<td>$\sigma_{\text{tissue}}$</td>
<td>tensile stress of tissue</td>
</tr>
</tbody>
</table>
Acknowledgements

We are immensely thankful towards Mark Denny, who aided in the collection of corallines in California and Western Australia and provided fantastic advice during the preparation of this manuscript. We would also like to thank Gerry Kraft and Rebecca Martone, who helped locate and collect Cheilosporum in Point Lonsdale, Australia, as well as Kathy Ann Miller, for her collection of Lithothrix in California. John Statton was invaluable in hunting down the elusive Metagoniolithon chara. John Huisman and Gary Kendrick both provided lab space in Australia, for which we are grateful. Derrick Horne of the UBC BioImaging Facility patiently guided us through TEM process. Finally, this manuscript benefited from input of Samuel Starko, Lauran Liggan, Laura Borden, and Liam Coleman, all of whom deserve thanks.

Competing Interests

None

Author Contributions

This study represents part of K.J.’s PhD dissertation. K.J. collected algae, conducted biomechanical experiments, analyzed the data, and wrote the manuscript. P.T.M. collected algae and provided raw data that had gone unused in his previous research. PTM also contributed ideas and guidance for the research and provided lab equipment and funding.

Funding

Funding for this project was provided by a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant to P.T.M. and NSERC graduate research fellowships to K.J (CGS M, CGS D2). Travel funding was provided by a grant-in-aid of research from the Phycological Society of America and the UBC Beaty Biodiversity Museum. Additional travel funding was provided by a National Science Foundation grant to Mark W. Denny (#IOS- 1052161).
References


Fig. 1. (A) Relationships between articulated coralline subfamilies and select crustose subfamilies (in bold) (phylogenetic information from Kato et al., 2011). “Amphiroideae” refers to Amphiroideae sensu Johansen (1981), and is separated from the rest of Lithophylloideae here for clarity. (B, D, F) Coralline fronds in situ. (C, E, G) Close up of genicula (labeled “g”) under a dissecting microscope. Scales = 700 µm. (B, C) Calliarthron tuberculatum (Postels & Ruprecht) E.Y. Dawson. (D, E) Amphiroa anceps (Lamarck) Decaisne. (F, G) Metagoniolithon stelliferum “#1” (Lamarck) Ducker.
Fig. 2. Long-sections of genicula under light microscopy, dyed with 1% Aniline blue. Scales = 100 µm. Arrows and “g” labels indicate location of genicular tissue – note that all tissue shown in C is genicular tissue. (A) Calliarthron tuberculatum (Postels and Ruprecht) E.Y. Dawson. (B) Amphiroa gracilis Harvey. (C) Metagoniolithon stelliferum “#1” (Lamarck) Ducker.

Fig. 3. Material properties of genicular tissue in tension. (A, B, C, D) Breaking stress, breaking strain, Young’s modulus, and breaking energy of each species. Corallinoid species are green, amphiproid species are purple, and metagoniolithoid species are orange. Significant differences between species were found for breaking stress, breaking strain, Young’s modulus, and breaking energy (Kruskal-Wallis tests, p<0.001 in all cases). Lowercase letters indicate results of a nonparametric post-hoc Dunn’s test (p<0.05). Grey bars show comparative data for fleshy algae: Red (=Rhodophyta), Brown (=Ochrophyta, Phaeophyceae), and Green (=Chlorophyta) from Hale (2001). Error bars represent standard error (s.e.m.).
Fig. 4. Average percent of genicular area taken up by primary cell wall and secondary cell wall. The remaining percentage not shown represents cell lumen and extracellular matrix/middle lamella. Corallinoid species are green, amphiproid species are purple, and metagoniolithoid species are orange.

Fig. 6. Cell wall material breaking stress of each species. Corallinoid species are green, amphiroid species are purple, and metagoniolithoid species are orange. Lowercase letters indicate results of a nonparametric post-hoc Dunn’s test (p<0.05). Error bars represent standard error (s.e.m.).

Fig. 7. Whole tissue breaking stress in genicula increases with an increase in the percentage of cross-section taken up by cell wall. Each point represents species averages. Corallinoid species (excluding *Cheilosporum sagittatum*) are green, amphiroid species are purple, and metagoniolithoid species are orange. *Cheilosporum sagittatum* is shown as an outlier in red. The trend line represents the line of best fit through all points excluding *Cheilosporum sagittatum* (y=0.45-9.79, R²=0.8419). Error bars represent standard error (s.e.m.).
Fig. 8. Cross-section of *Amphiroa anceps* geniculum. Letters indicate polygons used to measure different cell layers – a) cell lumen, b) cell wall, c) extracellular matrix (halved to account for portion associated with other cells). Scale = 5 µm.

Fig. 9. Resin embedded cross-section of *Cheilosporum sagittatum* geniculum under light microscopy, dyed with 5% potassium permanganate. Arrows indicates location of unidentified layer peeling away from inside the primary cell wall, which may represent a secondary wall of distinct chemical composition. Scale = 2 µm.
**Table S1.** Area, length, and breaking force of joints in experimental species (mean±s.e.m). All measurements apply specifically to joints found within the most basal 2 cm of each plant - dimensions may vary for more distal joints.

<table>
<thead>
<tr>
<th>Species</th>
<th>Area (mm²)</th>
<th>Length (mm)</th>
<th>Force (N)</th>
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</thead>
<tbody>
<tr>
<td>Cheilosporum sagittatum</td>
<td>0.04±0.01</td>
<td>0.21±0.01</td>
<td>2.2±0.1</td>
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<tr>
<td>Calliarthron tuberculansum</td>
<td>0.39±0.05</td>
<td>0.49±0.01</td>
<td>8.3±0.9</td>
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<td>Corallina officinalis</td>
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<td>0.43±0.02</td>
<td>12.82±0.87</td>
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<td>Johansenia macmillanii</td>
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<td>Lithothrix aspergillum</td>
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<td>Amphiroa anceps</td>
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<td>0.92±0.08</td>
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<td>Amphiroa gracilis</td>
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<td>Metagoniolithon stelliferum #1</td>
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<td>6.53±0.26</td>
<td>0.83±0.05</td>
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<td>Metagoniolithon stelliferum #2</td>
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<td>Metagoniolithon chara</td>
<td>0.19±0.02</td>
<td>1.31±0.16</td>
<td>1.06±0.07</td>
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