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RESEARCH ARTICLE

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 indicate 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.

KEY WORDS: Algae, Biomechanics, Cell wall, Corallines, Genicula, Parallel evolution

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^{-1} as waves break, with velocities as high as 25 m s^{-1} 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 of 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.

Most upright algae are generally flexible along their entire thallus, but coralline algae demonstrate 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).

Although an articulated morphology allows upright corallines to bend over and reconfigure in a manner similar 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, 2008). 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 *C. cheilosporioides* 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, 1981; Martone and Denny, 2008; Denny et al., 2013). Amphiroid genicula are often multi-tiered (Fig. 2B), whereas metagoniolithoid genicula lack a tiered structure altogether (Fig. 2C) (Johansen, 1969, 1981; Ducker, 1979). Genicular cells in

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List of symbols and abbreviations

a_{area}	cross-sectional area of cell lumen
b_{area}	combined cross-sectional area of cell lumen and cell wall
c_{area}	combined cross-sectional area of cell lumen, cell wall and half the extracellular matrix
CV	coefficient of variation
CW%	percentage of cell cross-sectional area accounted for by cell wall
E	Young's modulus
l	length
l_o	initial length
ε	tensile strain
σ	tensile stress
σ_{CW}	tensile stress of cell wall
σ_{tissue}	tensile stress of tissue

C. cheilosporioides 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, 1988; 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 ∼3.0 m. *Calliarthron tuberculosum* (Postels & Ruprecht) E. Y. Dawson, *Corallina officinalis* Linnaeus and *Johansenia macmillanii* (Yendo) K. Hind & G. W. Saunders were collected subtidally at a depth of ∼3.0 m from Botanical Beach (48°31′48″ N, 124°27′18″ W) on Vancouver Island, BC, Canada, in June/July 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 4.6–5.2 m, 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 3.0–4.6 m, 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 (K.G.J., unpublished data), and so they have been treated as such in this study. Both *M. stelliferum* 'species' were collected in December 2012 from Point Peron at 3.0–4.6 m, 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 ∼4.6 m, in January 2014.

All plants were collected in their entirety and kept in flowing seawater in the laboratory prior to mechanical testing. Mechanical

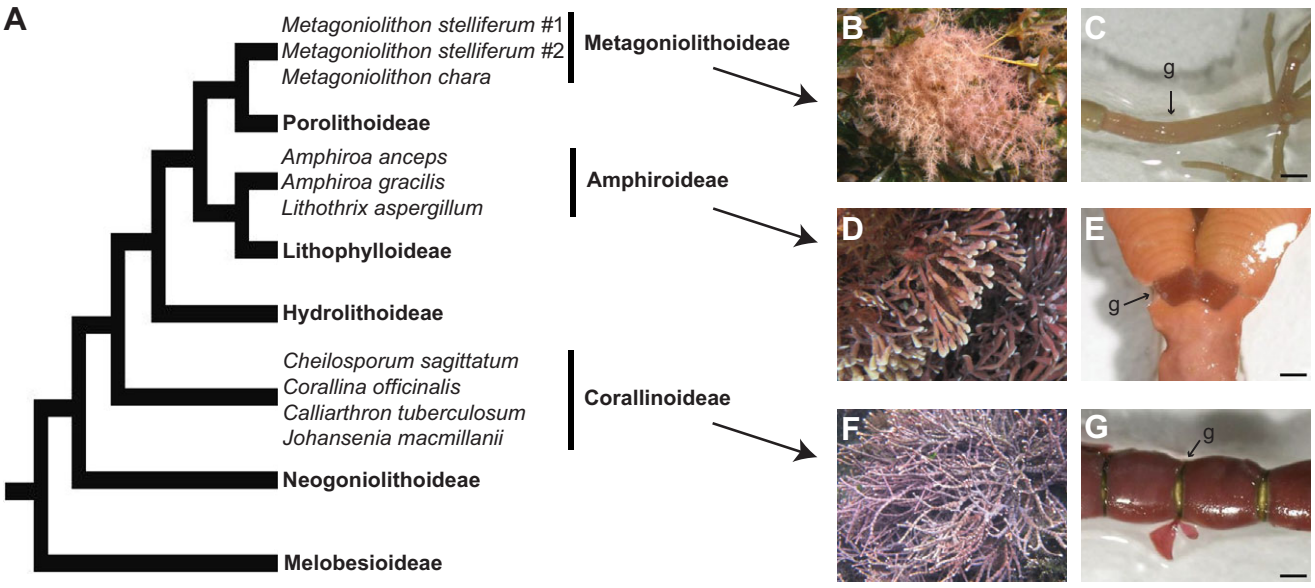

Fig. 1. Evolutionary relationships between articulated coralline subfamilies and select crustose subfamilies, showing similarities in thallus morphology and differences in genicular morphology. (A) Phylogenetic summary of relationships between coralline subfamilies (in bold) (information from Kato et 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 (g) under a dissecting microscope. Scale bars, 700 µm. (B,C) *Calliarthron tuberculosum* (Postels & Ruprecht) E. Y. Dawson. (D,E) *Amphiroa anceps* (Lamarck) Decaisne. (F,G) *Metagoniolithon stelliferum* #1 (Lamarck) Ducker.

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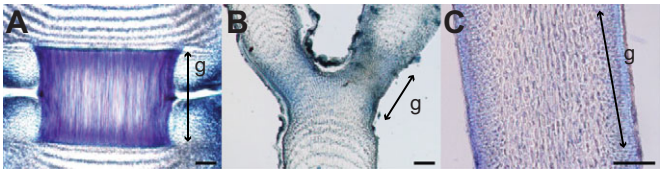

Fig. 2. Long-sections of genicula under light microscopy, dyed with 1% Aniline Blue. Scale bars, 100 µm. Arrows and 'g' labels indicate location of genicular tissue – note that all tissue shown in C is genicular tissue. (A) *Calliarthron tuberculosum* (Postels & Ruprecht) E. Y. Dawson. (B) *Amphiroa gracilis* Harvey. (C) *Metagoniolithon stelliferum* #1 (Lamarck) Ducker.

tests were performed no later than 72 h 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); and *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 that 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, and 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^{-1} until tissue failure, as measured by a sudden drop in force. All data were collected and initially processed using Instron Bluehill 3 software (Instron Corp.).

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 that moved along a tensometer track. Clamps were positioned on the intergenicula, and lined with rubber pads 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, 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^{-1} 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 manner similar 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). Force was measured with a 5 kg beam transducer (model FORT5000, World Precision Instruments, Sarasota, FL, USA), which was amplified through a transducer amplifier (model SYS-TBM4M, World Precision Instruments) and collected in real time using LabVIEW SignalExpress software (National Instruments Canada, Vaudreuil-Dorion, QC, Canada). 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^{-1} until failure.

As this study includes data collected over a span of 5 years, using two different extension rates, we compared results for five specimens of *Calliarthron tuberculosum* that were also tested in the portable tensometer at a rate of 60 mm min^{-1}, as described above. Breaking stress, breaking strain, Young's modulus and breaking energy all fell within the ranges found for *C. tuberculosum* specimens tested in the Instron tensometer.

After testing, samples were dissected under a dissecting microscope (model SZ61, Olympus Canada, ON, Canada) with an attached camera (model DP20, Olympus Canada) 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 min prior to morphometric measurements. Stress (σ, N m^{-2}) 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 (l_o). The resulting stress–strain curve was used to calculate Young's modulus (E, N m^{-2}), a measurement of initial tissue stiffness, by taking the slope of the curve from 0 to 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 values 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

One representative species was chosen to illustrate each subfamily – *Calliarthron tuberculosum* for Corallinoideae, *Amphiroa anceps* for Amphiroideae and *Metagoniolithon stelliferum* for Metagoniolithoideae. One specimen of each representative species was rehydrated for 1 h 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 1 h per treatment. Specimens were left in 100% ethanol overnight, then placed in medium-grade LR White embedding resin overnight. Specimens were placed in gel capsules, immersed in fresh LR White embedding resin, and baked at 62°C for 1.5 h.

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 min and Reynold's lead citrate for 6 min. Sections were visualized and

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photographed on a transmission electron microscope (model H7600, Hitachi High-Technologies Canada, Toronto, ON, Canada).

Cell wall analysis

Five specimens of each species were rehydrated in saltwater for a minimum of 10 min, after which they were decalcified in 1 mol l^{-1} HCl for between 2 and 24 h (the time required for full decalcification varied widely between species). Decalcified samples were placed in ethanol for 10 min, embedded in Tissue Tek OCT compound (Sakura Finetek, Europe), then cross-sectioned within the basal genicular region using a freezing microtome (model CM1850, Leica Biosystems). Thickness of sections varied between 10 and 20 µm. Sections were dyed with 5% Potassium Permanganate for approximately 5 min, then washed with freshwater and viewed under a light microscope (model BX51wi, Olympus Canada). Photos were taken using a camera (DP21, Olympus Canada) attached to the microscope.

Photos were analysed using ImageJ (US National Institutes of Health, Bethesda, MD, USA). Cell wall proportion in a cross-section was estimated by drawing and measuring the area of polygons around the lumen (*a*), lumen+
cell wall (*b*), and lumen+cell wall+half the extracellular matrix/middle lamella (*c*) (Fig. 3). Cell wall percent was calculated as:

$$\mathrm{CW\%} = \left(\frac{(b_{\mathrm{area}} - a_{\mathrm{area}})}{c_{\mathrm{area}}}\right) \times 100. \quad (1)$$

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 the proportion of each tissue layer in the overall cross-section.

Given that coefficients of variation (CV) for CW% were generally low, under 0.1 for all species except *Cheilosporum sagittatum* (CV=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:

$$\sigma\mathrm{CW} = (\sigma_{\mathrm{tissue}} \times 100)/\mathrm{CW\%}. \quad (2)$$

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

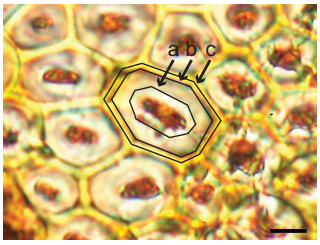

Fig. 3. Cross-section of *Amphiroa anceps* geniculum. Letters indicate polygons used to measure different cell layers: a, cell lumen; b, cell wall; and c, extracellular matrix (halved to account for portion associated with other cells). Scale bar, 5 µm.

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, Boston, MA, USA)

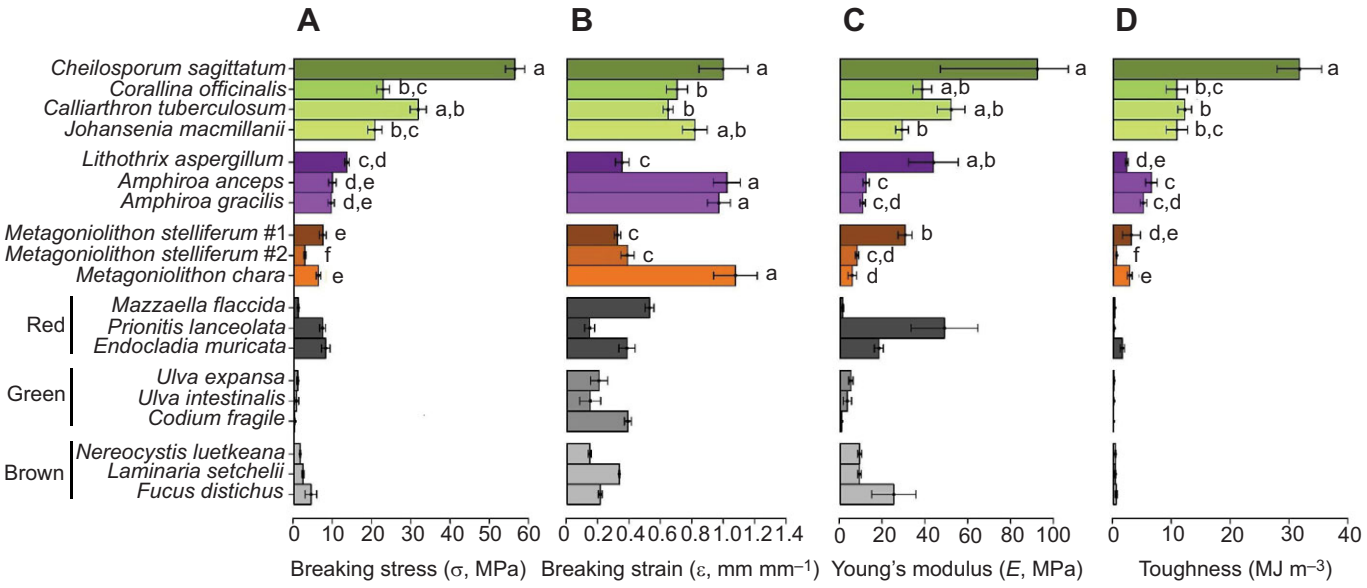

Fig. 4. Material properties of genicular tissue in tension. (A) Breaking stress, (B) breaking strain, (C) Young's modulus and (D) breaking energy of each species. Corallinoid species are in green, amphiroid species are in purple and metagoniolithoid species are in orange. Significant differences between 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 non-parametric *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 s.e.m.

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and 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.

Means 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. 4A). 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. 4A). 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. 4B). 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. 4B). 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.

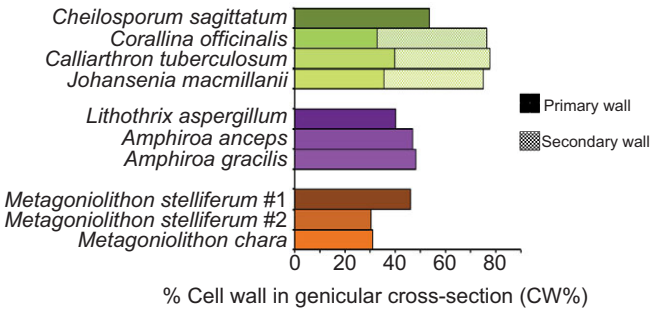

Fig. 5. Average percent of genicular area taken up by primary and secondary cell wall. The remaining percentage (not shown) represents cell lumen and extracellular matrix/middle lamella. Corallinoid species are in green, amphiroid species are in purple and metagoniolithoid species are in orange.

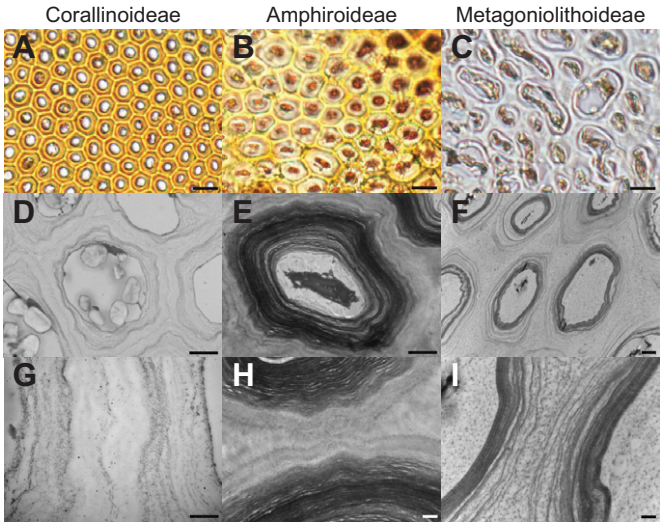

Fig. 6. Cross-sections of genicula showing differences in cell wall structure between the three articulated coralline subfamilies. (A–C) Cross-sections of genicula under light microscopy, dyed with 5% Potassium Permanganate. Scale bars, 10 µm. (D–F) Cross-sections of genicular cells under transmission electron microscopy (TEM). Scale bars, 2 µm. (G–I) TEM of cell wall layers and extracellular matrix between genicular cells in cross-section. Scale bars, 500 nm. (A,D,G) *Calliarthron tuberculosum* (Postels & Ruprecht) E. Y. Dawson. (B,E,H) *Amphiroa anceps* (Lamarck) Decaisne. (C,F,I) *Metagoniolithon stelliferum* #1 (Lamarck) Ducker.

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, $P<0.001$), it was highly variable for all species, and did not differ consistently among subfamilies (Dunn's test; Fig. 4C). *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, and *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. 4C). Consistent with fleshy

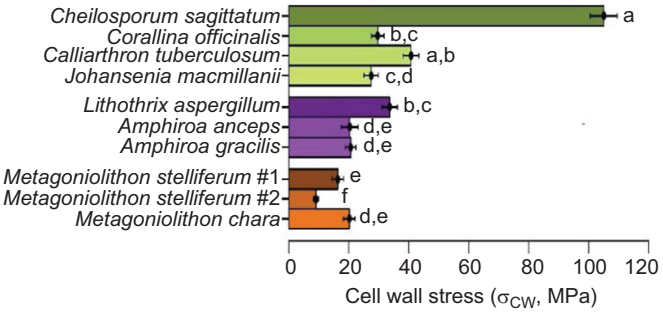

Fig. 7. Cell wall material breaking stress of each species. Corallinoid species are in green, amphiroid species are in purple and metagoniolithoid species are in orange. Lowercase letters indicate results of a non-parametric *post hoc* Dunn's test ($P<0.05$). Error bars represent s.e.m.

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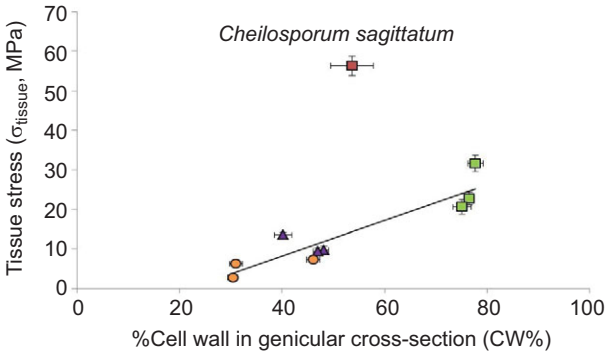

Fig. 8. Whole tissue breaking stress in genicula increases with an increase in the percentage of cross-section taken up by cell wall. Each point represents species averages. Corallinoid species (excluding *Cheilosporum sagittatum*) are in green, amphiroid species are in purple and metagoniolithoid species are in orange. *Cheilosporum sagittatum* is shown as an outlier in red. The trend line represents the line of best fit through all points excluding *C. sagittatum* (y=0.45–9.79, R^2=0.8419). Error bars represent s.e.m.

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. 4D). *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. 5). 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 *C. sagittatum*, 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. 6 for examples).

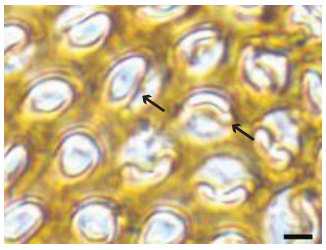

Fig. 9. Resin-embedded cross-section of *Cheilosporum sagittatum* geniculum under light microscopy, dyed with 5% Potassium Permanganate. Arrows indicate location of unidentified layer peeling away from inside the primary cell wall, which may represent a secondary wall of distinct chemical composition. Scale bar, 2 µm.

Correcting cross-sectional area with approximate cell wall percentage yields breaking stresses that are much closer across species and articulated groups (Fig. 7), with the exception of *Cheilosporum sagittatum*. Cell wall breaking stress (mean±s.e.m.) was 50.1±4.3 MPa for the corallinoids (but 32.6±1.6 MPa if we exclude *C. sagittatum*), 24.8±1.4 MPa for the amphiroids and 15.2± 1.2 MPa for the metagoniolithoids. Although 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. 7), 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 *Metagoniolithon chara*. Cell walls in *Lithothrix aspergillum* are comparable in strength to all of the corallinoids except for *Cheilosporum sagittatum*. Some differences are maintained – *Metagoniolithon stelliferum* #2 is still weaker than all other species tested, with a cell wall stress of 9.1±0.9 MPa. Cell walls in *Cheilosporum sagittatum* are still the strongest of all articulated species by a large margin, with a cell wall stress of 102.4±14.2 MPa, over twice that of *Corallina officinalis* at 40.7± 2.4 MPa.

Cell wall proportion and tissue stress do not show any correlation when all species are included in the analysis (ANOVA, P=0.1231). However, after excluding *Cheilosporum sagittatum* as an outlier, tissue stress increased significantly with cell wall proportion across all other species (ANOVA, P<0.001, R^2=0.84; Fig. 8).

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 *M. 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 tissues of amphiroid and metagoniolithoid species with 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

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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 with 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, 2003). 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 amphiroids 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, because of 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 amphiroid – 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 because of the failure to procure fresh samples. Data from this species would be necessary 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 amphiroid 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,F). 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.

Although all of the corallinoid species tested possess large breaking stresses compared with 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 mm^2, joints in *C. sagittatum* were anywhere from three to 15 times 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, *C. sagittatum* may withstand forces similar to seaweeds of much larger sizes. This may mean that *C. sagittatum* is over-designed for the drag forces it encounters: frond area affects drag in flow, and it 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). Although 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, because of the loose connection between genicular cells, which minimizes propagation of cracks (Denny et al., 2013). Although other corallinoids have a joint structure similar to that of *C. cheilosporioides*, 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. 8). 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), whereas 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). Although 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* (Martone, 2007; Martone et al., 2009) – none were immediately visible in the *C. sagittatum* 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 *C. sagittatum* was even greater than that of other articulated corallines, suggesting that the cell walls in *C. sagittatum* 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 Wainwright, 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 et al., 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.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
This study represents part of K.G.J.'s PhD dissertation. K.G.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. P.T.M. also contributed ideas and guidance for the research and provided laboratory equipment and funding.

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Supplementary information
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