KRAFTIONEMA ALLANTOIDEUM, A NEW GENUS AND FAMILY OF ULOTRICHALES (CHLOROPHYTA) ADAPTED FOR SURVIVAL IN HIGH INTERTIDAL POOLS

Richard Wetherbee and Heroen Verbruggen
School of BioSciences, University of Melbourne, Victoria 3010, Australia

Abstract:
The marine, sand-dwelling green alga Kraftionema allantoideum gen. et sp. nov. is described from clonal cultures established from samples collected in coastal, high intertidal pools from south-eastern Australia. The species forms microscopic, uniseriate, unbranched, 6-8 µm wide filaments surrounded by a gelatinous capsule of varying thickness. Filaments are twisted, knotted and variable in length from 4–50 cells in field samples but straighter and much longer in culture, up to 1.5 mm in length. Cell division occurs in several planes, resulting in daughter cells of varying shape, from square to rectangular to triangular, giving rise to gnarled filaments. Mature cells become allantoid – elongate with rounded ends – before dividing one time to form bicells comprised of two domed cells. Adjacent bicells separate from one another and mature filaments appeared as a string of loosely arranged sausages. A massive, single, banded chloroplast covered ¾ of the wall circumference and contained a single large pyrenoid encased in a starch envelope that measures 1.5 – 2.5 µm. Filaments were not adhesive nor did they produce specialized adhesive cells or structures. Reproduction was by fragmentation with all cells capable of producing a new filament. No motile or reproductive cells were observed. Filaments in culture grew equally well in freshwater or marine media as well as at high salinity, and cells quickly recovered from desiccation. Phylogenetic analysis based on the nuclear-encoded small subunit ribosomal RNA (18S) shows the early-branching nature of the Kraftionema lineage among Ulotrichales, warranting its recognition as a family (Kraftionemaceae).

Key words: 18S nuclear ribosomal DNA, Chlorophyta, Kraftionema, Kraftionemaceae, molecular phylogeny, sand dwelling, Ulotrichales
INTRODUCTION

The Ulvophyceae (Chlorophyta) is a diverse class of 8 orders classically distinguished by their life cycles and ultrastructure, and more recently through molecular phylogenetics using a range of markers. The Ulotrichales and Ulvales form a clear lineage within the Ulvophyceae based on nuclear and plastid multigene data sets (Cocquyt et al. 2010, Fucikova et al. 2014). The two orders were classically distinguished by features of their life histories but molecular phylogenies have not supported the orders as monophyletic (reviewed by Leliaert et al. 2012).

The Ulotrichales-Ulvales are highly diverse in their cytology, morphology and ecology. Their functional types range from single-celled organisms to filaments and colonies to larger multicellular seaweeds such as Ulva and Monostroma. They have marine species as well as species living in freshwater habitats. Some species grow on rock or on sand, while some are subaerial and others burrow into marine limestone.

Molecular phylogenies suggest that the origin of the macroscopic growth forms has been achieved independently in the major orders of the Ulvophyceae from ancestral unicellular and filamentous forms (Cocquyt et al. 2010). The genera Rhizoclonium, Ulothrix and Oedogonium, along with the new genus Kraftionema described here, are some common examples of Ulvophyceae characterized by uniseriate, unbranched filaments that undergo vegetative reproduction by cell division and fragmentation into segments of one or more cells. These features are also found in several lineages of the Chlorophyta, including the Trebouxiophyceae (e.g., Stichococcus, Geminella and Koliellopsis) and Chlorophyceae (e.g., Schizomeris, Cylindrocapsa, Radiofilum and the siphonous Sphaeroplea). The green algae belonging to the Streptophyta also have these growth forms, most noticeably in Klebsormidium (Klebsormidiophyceae) as well as Spirogyra, Zygnema, Mougeotia and other related genera from the Zygnematophyceae.

We have a research program studying the diversity and phylogeny of microalgae adapted to life in marine tide pools, a relatively unexplored, often dynamic habitat where organisms typically require adhesive structures and/or mechanism to survive. Several groups of sand-dwelling algae have been studied in detail, including species of diatoms (e.g., Hay et al. 1993), pelagophytes (Wetherbee et al. 2015) and euglenids (e.g., Kingston 1999) that migrate within sand in response to light, tidal and diurnal rhythms. In addition, benthic diatoms can have a range of adhesive structures to secure their place in benthic surfaces, including adhesive stalks, tubes and pads (for review, Molino & Wetherbee 2008). Unicellular dinoflagellates have also been studied to some degree, and can attach with stalks or pads, or adhere and flatten out over the surface of sand grains (e.g. Tamura & Horiguchi 2005). Benthic cell rafting is another classic mechanism for adhesion to sand. Motile unicells attach adjacent to one another and become
amoeboid and crowd together to form rafts that appear to increase the overall strength of adhesion. Rafting has also been observed for the zoospores of the green seaweed *Ulva* (Callow et al. 1997) as well as unicellular genera of the Prymnesiophyceae (Haptophyta) (Grant et al. 2011) and Raphidophyceae (Heterokonta) (Grant et al. 2013) amongst others. Many species that occupy sand are observed to divide only in the benthic stage, producing motile cells that are short lived but allow for colonization of new surfaces. The shorter the exposure of motile cells in a life history, the seemingly greater chance for survival in a dynamic habitat.

During the summer of 2013, a sand-dwelling green alga comprised of thin, unbranched filaments was isolated from sand samples taken from a high intertidal pool near Point Lonsdale Pier, Victoria, Australia. Another strain was isolated from sand obtained from an upper intertidal tide pond from Bittangabee Bay, NSW, Australia in April, 2014 and again in March, 2015. Not subjected to excessive wave action, this alga does not feature any of the adhesive cells or structures observed in other sand-dwelling algae, but appears to flourish by producing fast growing, entangled filaments that readily reproduce by fragmentation. Our goal in this study is to describe the morphology, ecology and evolutionary relationships of these strains, which turn out to represent a new species, genus and family. Our approach consisted of a combination of culture observations, molecular phylogenetic analysis, light and transmission electron microscopy.

**MATERIALS AND METHODS**

*Sampling, isolation and culture* – Sand samples were collected in December 2013 from deep, high intertidal tide pools in a sandstone platform at the base of a sandstone cliff approximately 100m SSW of the Point Lonsdale Pier, Victoria (38°17’S, 144°36’E). The pools would be replenished at virtually all high tides and on our numerous visits to this site the salinity has always been between 28‰ and 30‰. On two separate occasions, sand samples were also collected from a large tidal pond on the foreshore at Bittangabee Bay, 300m north of the campground, New South Wales (37°12’57S, 150°00’53E). This pond would only be replenished at very high tides and during storm surges. The first samples were collected in March, 2014 when the pond was reduced in size due to dry conditions and the salinity was 45‰. Sand samples were taken from the bottom of the pond as well as the dried out sand on the pond edge. Further samples were collected from the same site in April, 2015 when significant rain and run off from the surrounding area had collected in the pond, and salinity was measured at between 15‰ to 20‰. Samples consisted of approximately 0.5 - 1.0 cm of sand taken from the bottom of the tide pools plus seawater that was placed into 60 ml plastic jars and the salinity determined. The sand only samples taken
in April, 2015 were returned to the lab where sterile filtered seawater (30‰) was added. Clonal cultures of the filamentous green alga that is the focus of this study were established by isolating filaments from field samples by micro-pipetting into either K- enriched seawater medium (30‰) (Keller et al. 1987) or the freshwater media DM (Cohn & Pickett-Heaps 1988), and were maintained in 60 ml plastic containers at 21°C under Sylvania 58 Luxline Plus and Gro-Lux fluorescent lamps with a daily 14:10 hr light:dark cycle; stock cultures were transferred into new K- or DM medium once every two weeks. Two strains (CS-1143, CS-1144) were isolated into culture, one from each sample site. The cultures used for microscopy were taken from the K- culture isolated from Point Lonsdale.

**Salinity and Desiccation experiments** – Filaments in log phase growth in both K- (30‰) and freshwater DM media (2-3‰), were placed in 10ml test tubes and concentrated into a pellet with a hand centrifuge and the media removed. Filaments grown in the K- marine media were resuspended into freshwater DM media and cells grown in the DM media resuspended into K- media. Filaments were then observed immediately and subsequently over 72 hours. Five millilitres of culture medium with a low concentration of *K. allantoideum* filaments were allowed to dry in Greiner 6-well plastic trays and placed in a constant temperature room at 21°C with the lids off and allowed to dry out. Wells were rehydrated after 30 and 60 days by the addition of either K- or DM media and filaments observed during rehydration and over the next 72 hours.

**Light, fluorescence and electron microscopy** – Filaments of both strains were mounted onto microscope slides with coverslips sealed with a 1:1:1 ratio of Vaseline, lanoline, and paraffin wax. Live cells were observed and recorded using a Zeiss AxioPlan 2 microscope (Carl Zeiss, Oberkochen, Germany) and photographs were taken using a Canon EOS 60D digital single-lens reflex camera (Canon USA, Melville, New York, USA). Live cells in K-media were first stained with Hoechst stain (1.0µg of stain per 1.0ml) (Lyndon et al. 1980) for one minute, washed in K- media and then stained with SYBRGreen nucleic acid stain (Suzuki et al. 1997) for another minute, then mounted as above. Confocal images were taken on a Leica SP2, Laser Scanning Confocal Microscope. For transmission electron microscopy, 5 ml of a 2% solution of glutaraldehyde in K- seawater media was mixed with 5 ml of cells from a thick culture and left for one hour, washed 3x with K- media, post fixed in 1% osmium tetroxide in K- media, followed by 3x washes in K- media. Cells were then brought into distilled water in steps: 75:25, 50:50 and 25:70 K-media:distilled water. Cells were washed 3 times in distilled water and placed in a solution of 2% uranyl acetate in distilled water for one hour before gradual dehydration in EtOH (10%, 25%, 50%, 75%, 90% and 3x at 100%). Cells were then gradually embedded in Spurr’s medium (Spurr 1969).

**DNA sequencing and analysis** – DNA was isolated from fresh cultures either using DNeasy Plant Mini Kit (Qiagen) or by following an adapted CTAB, chloroform:isoamyl alcohol protocol (Kooistra et al.
The SSU rRNA (18S) region was amplified by PCR using primers described in Grant et al. (2011). PCR fragments were purified using the Isolate II PCR and Gel Kit (Bioline). Sanger sequencing was outsourced to the Australian Genome Research Facility and reads were obtained with the PCR primers and four additional primers: EukC (5′–GCGGTAATCCGCTCAATAGC–3′), EukD (5′–GTRAGTTTYCCCGGTGTAGTC–3′), BgR (5′–CAAGAAGGTTAGGGG–3′) and ENDF (5′–GCTATTGGACGCGGAATTAC–3′). Individual reads were assembled in Geneious R6 (Kearse et al. 2012) and the consensus sequences submitted to Genbank (KU862658-9).

New 18S sequences were aligned with those from previous taxonomic work on the order Ulotrichales (Friedl & O'Kelly 2002, O'Kelly et al. 2004a, 2004b, Škaloud et al. 2013, Schmidt & Darcy 2015), and sequences of four Ulvales species were used as outgroups. Alignment was done with MUSCLE v3.8.31 using default settings (Edgar 2004) and the result visually verified. Phylogenetic trees were inferred from the 1,823 positions long alignment using maximum likelihood (ML) and Bayesian inference (BI). Analyses were done with a GTR+Γ model. For ML, we performed 20 likelihood searches starting from different starting trees and 1,000 replicates of the rapid bootstrapping algorithm in RAxML v7.3.0 (Stamatakis 2006). For BI, we used the birth-death tree prior and the log-normal relaxed clock model implemented in BEAST v1.8.2 (Drummond et al. 2012). The chain was run for 10 million generations, saving every 1,000th generation. Convergence of the chain was visually assessed in Tracer and a burn-in of one million generations was used to calculate a maximum clade credibility consensus tree with median node heights in TreeAnnotator.

We opted not to present other gene phylogenies because there are very few Ulotrichales sequences available for comparison. Nonetheless, we have generated rbcL and tufA sequences for the species using high-throughput sequencing of a total DNA extract using previously described methods (Verbruggen & Costa 2015). These sequences are available on Genbank (KX268523-4) for future multi-gene studies.

RESULTS

The phylogenetic analyses of 18S sequence data show that the green alga we isolated forms a lineage distinct from other sequenced Ulotrichales (Fig. 1, our new lineage flagged with a star). The families of Ulotrichales that were recognized in recent phylogenetic studies are indicated with different shading, along with other family-level lineages (Škaloud et al. 2013, Schmidt & Darcy 2015). This comparison with other family-level lineages shows that our alga is distinct at the family level. The combination of this distinctive molecular phylogenetic nature and its unique morphology among Ulotrichales leads us to recognize our alga as a new species, genus and family, Kraftionema allantoideum in the Kraftionemaceae. The new family is situated in the "core Ulotrichales" (O'Kelly et al 2004a) indicated
with a hexagon in the phylogeny (Fig. 1). While it is found as a sister clade to Gomontiaceae in the Bayesian and ML trees, this position is not statistically supported in either of the analyses.

**Kraftionema allantoideum** Wetherbee & Verbruggen, *gen. et sp. nov.*

*Description* – Filaments thin, uniseriate, unbranched, twisted and knotted, variable in length from a few cells in field samples up to 1.5 mm in culture, 6-8 µm in width surrounded by a mucilagenous capsule between 1-3 µm thick (Figs 2,3). Cells highly variable in shape when actively dividing, from square to rectangular to triangular 2-6 µm x 6-8 µm wide. Following division, all cells enlarge and become allantoid (sausage-shaped) with rounded edges, 10-18 µm x 6-8 µm wide. Allantoid cells typically divide once and produce bicells consisting of two dome-shaped cells. During most of the light/dark cycle, filaments comprised largely of loosely associated allantoid cells and bicells that had the appearance of a string of sausages. A single, dark green, broad band chloroplast covered ¾ of the wall circumference and contained a single pyrenoid encased in a starch envelope with an overall diameter of 1.5-2.5 µm (Fig 4). Filaments have no adhesive cells or structures. Reproduction was by fragmentation only with all cells capable of producing new filaments. No motile zoospores or gametes were observed. The species is characterized by a substantial divergence of the 18S ribosomal RNA gene from that of other known lineages of Ulotrichales, and its 18S sequence can be consulted on Genbank (accession KU862658).

*Holotype* – MELU A 120812A, a mounted specimen derived from strain CS-1143.

*Type locality* – Approximately 100 m SSW of the Point Lonsdale Pier, Victoria, Australia (38°17’S, 144°36’E) in a small (2m x 1m), deep (1.5m) tide pool lined by sand in the upper intertidal; collected by Heroen Verbruggen in December 2013.

*Genbank accession numbers* – Strain CS-1143: KU862658; CS-1144: KU862659.

*Etymology* – The genus is named in honour of Dr Gerald Thompson Kraft for his outstanding contributions to our knowledge of seaweeds from Australia and around the world, including the green algae of which *Kraftionema* is a new genus and family. Gerry was a legendary teacher of marine botany at the University of Melbourne for three decades, and inspired generations of undergraduate, postgraduate and post-doctoral students with his unparalleled scholarship, passion and expertise in marine phycology. The specific epithet indicates the allantoid shape of the mature cells, plus the appearance of the filaments as a loose string of sausages.

*Habitat* – marine, sand-dwelling.
**Culture lodgement** – ANACC code: Point Lonsdale strain CS-1143; New South Wales strain CS-1144; CSIRO, Hobart, Tasmania, Australia

**Kraftionemaceae Wetherbee & Verbruggen, fam. nov.**

*Type species* – *Kraftionema allantoideum* Wetherbee & Verbruggen

*Description* – Family forms a distinct lineage in molecular phylogenies of 18S sequences, with the sequence of the type strain of the type species serving as a reference (Genbank accession KU862658). Only known representative of the family forms microscopic, uniseriate, unbranched, knotted filaments variable in length surrounded by a mucilagenous capsule. Cells are allantoid and have a single, dark green, broad band chloroplast contained a single, large pyrenoid encased in a starch envelope.

*Light microscopic and TEM observations* – Uniseriate, unbranched, twisted and knotted filaments were found growing among sand grains in high intertidal tide pools. Filaments were not sticky nor were they attached to the substratum by any differentiated cell and/or adhesive structure. In field samples, the knotted filaments were relatively short, between 4-50 cells and individual cells were rarely in focus (Fig 2, a-c). The longer, entangled filaments from cultures were best observed when tightly pressed between the slide and coverslip where sequences of cells could be observed in clear focus (Fig 2d, e). In field samples, filaments of strain CS-1143 from Point Lonsdale were consistently longer, up to 50 cells in length, while strain CS-1144 from Bittangabee Bay had shorter filaments to a maximum of 8-15 cells. No other differences were observed in the sequencing data, light or electron microscopy, so the strains were regarded as the same species.

Cell shape and size varied considerably, depending on the stage of the cell cycle and the age of the culture. In log phase cultures, most cells divided during the first 2-6 hours of the light period, the resulting cells being square, slightly rectangular or triangular in shape measuring 2-6 μm x 6-8 μm wide (Figs 2d, f, g and 3 a, b). The planes of cell division often varied considerably, accounting for the twisted, knotted filaments (Fig. 2g and 3a, b). The large banded chloroplast occupied much of the cell volume and contained a large pyrenoid surrounded by a starch envelope that was obvious in the light microscope (Figs 2e-g and 3a-c).

Following the division cycle, cell growth resulted in elongate cells with rounded ends that eventually pulled away from adjacent cells in the filament (Figs 2e,f and 3c). These allantoid cells (sausage-shaped) were 10-18 μm x 6-8 μm wide and typically divided once, forming bicells consisting of two dome-shaped cells that shared a cross wall (Figs 2e, f and 3c). For most of the cell cycle, allantoid cells and bicells were aligned in a haphazard way along each filament, looking like a string of sausages (Figs 2e, f and 3c,d and
Filaments often fragmented at this stage and formed fragments of one or more cells/bicells retaining a portion of the parental capsule (Fig. 3b). During the next division cycle, the allantoid cells and bicells from each fragment divided and developed into the next generation of filaments, with the rounded ends of the parental dome cells defining the ends of each new filament (Figs 2f, g and 3a, b). The 1-3 µm thick capsule could be observed at any stage of development (Figs 2g and 3a,b), but was most obvious during fragmentation (Fig. 3b).

Stains-All (MP Biomedicals, Cole & Narine 1984) showed two distinct extracellular layers, the cell wall adjacent the plasma membrane and the capsule that encased filaments (Figs 3d-f). The cell wall was best observed by staining live cells with Hoechst stain (Lyndon et al. 1980). The dye did not enter the cells or stain the capsule material, but accumulated at the cell surface (blue in Figs 3g-i), presumably within the fibrous cell wall observed in transmission EM images (Figs 4a-c). The same cells were then stained with SYBRGreen nucleic acid stain (Suzuki et al. 1997) that penetrated the live cells and stained the nuclei (yellow green). Red auto fluorescence revealed the chloroplast, which occupied most of the cell cytoplasm, plus the pyrenoid, which was surrounded by large starch grains that do not autoflouresce.

Allantoid bicells are seen as a loose chain, surrounded by a cell wall that does not abut adjacent bicells in the filament (Fig. 3g). However, as the allantoid cells divide, the cell walls of recently divided cells are appressed against one another (Figs 3h, i), and the progeny of a single allantoid bicell can be discerned by the rounded dome cells that defined the parental bicell. An 8 cell fragment resulting from two divisions of a bicell was observed (Figure 3i), with 6 square cells sandwiched between the two parental dome cells.

Transmission electron micrographs further revealed the chloroplast and pyrenoid surrounded by a starch envelope (Figs 4a-d). Chloroplast thylakoids intersected the pyrenoid matrix from several locations through the starch envelope (Fig 4d). Starch grains were also common within the chloroplast matrix (Figs 4a-d). The fibrous cell walls are observed while the capsules are electron opaque. When the filament orientation is correct, the nuclei and starch coated pyrenoids in daughter cells had opposite polarity. This was most obvious in recently divided cells (Figs 3h) and was also observed in electron microscope images (Fig 4c).

Salinity and Desiccation trials – Filaments of K. allantoideum grew well in both K- marine media at 30‰ or in freshwater media DM (2-3‰). In order to assess resistance to changes in salinity, filaments grown in K- marine media (30‰) were concentrated by centrifugation and resuspended immediately into DM freshwater media (2-3‰), or the reverse with filaments grown in DM concentrated and resuspended into K- media. No obvious impact on filament/cell survival or growth was observed, as cells continued to divide in subsequent division cycles. Filaments grown in both DM and K- media were allowed to dry out in open petri dishes and left uncovered in a constant temperature room with a 10/14 hours light/dark cycle
at 21°C for 30 and 60 days. Filaments were surrounded by enlarged capsules and never appeared to shrivel or lose colour. On rehydration the filaments regained their normal morphology and within 24 hours dividing cells were obvious as the filaments resumed growth.

DISCUSSION

The phylogeny clearly shows that Kraftionema is an early-branching lineage within the Ulotrichales. It also shows very limited statistical support for many of the deeper relationships among Ulotrichales lineages. This includes the sister relationship between Kraftionema and the Gomontiaceae shown in the figure, which has very low statistical support and should not be interpreted as indicative of these two families forming a natural lineage.

The lack of support in our phylogenetic tree points to the 18S gene not providing much information about early relationships among Ulotrichales lineages. For future studies of the phylogenetic history of the order, a different source of data will thus be needed. In the related class Trebouxiophyceae, chloroplast phylogenomics have drastically improved resolution at the level of relationships among genera and families, and in many cases also among the orders (Lemieux et al. 2014, Turmel et al. 2015), and we anticipate that this would also improve the resolution of the relationships among the higher-level taxa of the Ulvales-Ulotrichales lineage.

Our choice to recognize Kraftionemaceae as a family is primarily based on the early-branching nature of the lineage in the phylogeny. The level of molecular divergence between Kraftionema and the Gomontiaceae is similar to that between Ulotrichaceae and Monostromataceae (Fig. 1). A second argument to recognize the family is that not doing so would result in the genus not being assigned to any family, because the low support for early branches does not allow its assignment to any of the other families. We hope that future studies based on larger datasets will resolve Ulotrichales radiation and discover how Kraftionema relates to other families. Such studies would also facilitate subdividing the order into monophyletic lineages at the suborder and family level. We have not presented other gene phylogenies because few Ulotrichales sequences are available for comparison, but deposited rbcL and tufA sequences of Kraftionema for future work.

Besides the molecular data, a combination of morphological features delineates K. allantoideum from other green algae with uniseriate, unbranched filaments that undergo fragmentation as a form of asexual reproduction (examples in Table 1). Features include twisted and knotted filaments, a distinct capsule, cells with a massive, single banded chloroplast containing one large pyrenoid encased in a starch envelope and allantoid cells and bicells loosely arranged in mature filaments. Unusual for a sand-dwelling
protist, filaments lack any obvious mechanism for adhesion, neither a sticky surface nor a specialized basal cell/adhesive pad for attachment. In addition to the algae listed in Table 1, there are a range of macroscopic algae (seaweeds) that have uniseriate, unbranched filaments reproducing by fragmentation, but we have not included them Table 1 as their size clearly sets them apart from the microscopic species we need to compare *Kraftionema* to. These macroscopic algae lack the combination of fine details found in *K. allantoideum* and generally possess complex morphologies and life histories.

Based on morphology alone, the new genus has characteristics that might place it in a number of classes in two different divisions of green algae. Of all those genera investigated, including those listed in Table 1, some species of the freshwater genus *Geminella* Turpin (Tsarenko 2011) are known to produce somewhat bent, twisted filaments encased in mucilage with a single pyrenoid, thereby showing a morphological resemblance to *Kraftionema*. However, a combination of morphological features, including the absence of a pyrenoid starch envelope, make *Kraftionema* distinctive. Only a few species of *Geminella* have been sequenced with all now belonging to the Trebouxiophyceae. The single species *Geminella terricola* J.B.Petersen was subsequently found to be *Interfilum terricola* (J.B. Petersen) Mikhailiyuk, Sluiman, Massalski, Mudimu, Demchenko, Friedl & Kondratyuk and transferred to the Klebsormidiophyceae (Mikhailiyuk et al. 2008).

Some species of *Klebsormidium* P.C.Silva, Mattox & W.H.Blackwell and *Stichococcus* Nägeli show a resemblance to *Kraftionema*, particularly in old cultures where the their filaments are more constricted at the cross walls, yet are quite distinct. Pyrenoid morphology is a major difference, as the pyrenoid of *Klebsormidium* is coated by a large number of small starch grains, so the large, distinct starch envelop seen in *Kraftionema* is not formed. *Stichococcus* has a small, naked pyrenoid in all but one species, where a number of starch grains surround the pyrenoid (Neustupa et al. 2007). Interestingly, *Klebsormidium* and *Stichococcus* were once considered to be closely related in classifications based on their similar vegetative morphology and by ultrastructural characteristics such as the presence of open mitosis and cytokinesis by a cleavage furrow (e.g., Mattox & Stewart 1984, Ettl & Gärtner 1995). Originally, *Klebsormidium* along with *Stichococcus* were placed in the charophycean evolutionary lineage based on ultrastructural features, including the structure of the flagellar apparatus of motile zoospores, though motile cells were not observed in *Stichococcus*. Despite all the similarities, molecular phylogenies constructed mainly on 18S rDNA gene sequences have shown these two genera to be only distantly related, *Klebsormidium* was confirmed within the charophycean lineage (Division Streptophyta) and *Stichococcus* in the Trebouxiophyceae of the chlorophycean lineage (Division Chlorophyta).

In terms of pyrenoid morphology, *Ulothrix* (Ulvophyceae) most closely resembles *Kraftionema*. However, *Ulothrix* filaments consist of relatively large, cylindrical cells with no constriction of the cross
walls, and filaments are never twisted and knotted like observed in the thin filaments of *Kraftionema*. A mucilaginous sheath is absent from *Ulothrix* and filaments are anchored to the substratum by an adhesive basal cell, additional features that distinguish it from *Kraftionema*.

A combination of features distinguish the filaments of *K. allantoideum*, but nothing is as compelling as the clear delineation determined by the molecular phylogeny. Morphologically, the uniseriate, unbranched filaments result from cells dividing in several different planes, resulting in cells that are variously shaped and therefore assembled into markedly twisted and knotted filaments. Following a cycle of divisions, the cells enlarge and become allantoid, and often divide into bicells that remain as such until the next division cycle. Allantoid cells and bicells do no share adjacent walls, and have the unusual appearance of a string of sausages. The large pyrenoid with thylakoids intersecting the matrix and surrounded by an envelope of large starch grains is a distinctive feature of cells, and these features may be considered a deeply conserved character in the evolutionary lineages of the Trebouxiophyceae and Chlorophyceae.

Wave action would be limited in the high intertidal pools where *K. allantoidium* was collected, except during extreme tides or storm surges where some disturbance would occur. Adhesion is an important mechanism for survival in dynamic environments such as sand subjected to constant wave action. However, *K. allantoideum* filaments have no specialized cells or mechanism for adhesion to a surface, nor do they have any known motile stage in their life history in order to populate new sand. Filament capsules were never observed to be sticky, not surprising as that feature would have left them clogged with sand and detritus in their habitat. Likewise, filaments were never observed adhered to sand, and therefore there was no evidence to suggest that the capsule of growing filaments became momentarily adhesive on contact before then curing. Direct adhesion to a substratum appears unnecessary in a sand dwelling organism that is not subjected to routine wave action, but rather maintains itself by producing fast growing, knotted and entangled filaments that penetrate throughout the substratum. Filaments routinely fragment, particularly if subjected to any disturbance, increasing their chances of establishing and maintaining themselves in sand.

The ability of filaments to grow successfully in a range of salinities, as well as periods of desiccation, are consistent with a sand organism living in high intertidal tide pools or ponds just above the intertidal where only extreme tides or storm surges would replenish the pools. Such ponds may occasionally dry out, or the salinity might increase with evaporation or decrease drastically with the addition of rainwater. The protective capsule of *K. allantoideum* appears to allow the filaments to successfully survive periods of osmotic stress and/or drying out. All of these features together would appear to enhance the survival of the species in its natural habitat.
Acknowledgements

We thank Joana Costa for generating the 18S sequences and Fabio Rindi for nomenclatural advice. Funding during the preparation of this study was provided by the Australian Biological Resources Study (RFL213-08) and the Australian Research Council (FT110100585, DP150100705).

References


Table 1. Morphological characteristics for some uniseriate, unbranched, filamentous microscopic green algae from the Ulvophyceae and Trebouxiophyceae (Chlorophyta) and Klebsormidiophyceae (Streptophyta). All listed species undergo fragmentation as a form of asexual reproduction and were selected based on possessing a number of morphological features observed in *Kraftionema*. In appearance only, the genus *Geminella*, as well as *Klebsormidium* and *Stichococcus*, most closely resemble *Kraftionema*, but they are in different classes.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Reference</th>
<th>Filament diameter</th>
<th>Filaments knotted</th>
<th>Basal attachment</th>
<th>Cell size (µm)</th>
<th>Nuclei per cell</th>
<th>Capsule/sheath</th>
<th>Plastid no. and type</th>
<th>Pyrenoids</th>
<th>Pyrenoid starch envelope</th>
<th>Motile asex. stage</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulvophyceae (Chlorophyta)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Kraftionema allantoidaeum</em></td>
<td>This paper</td>
<td>6-8 µm</td>
<td>yes</td>
<td>none</td>
<td>6-8 × 2-18</td>
<td>1</td>
<td>yes</td>
<td>1 banded, massive</td>
<td>1</td>
<td>yes</td>
<td>no</td>
<td>marine sand</td>
</tr>
<tr>
<td><em>Binuclearia tatraana</em></td>
<td>Day et al. 1995</td>
<td>8-12 µm</td>
<td>no</td>
<td>basal cell holdfast</td>
<td>8-12 × 20-35</td>
<td>1</td>
<td>yes</td>
<td>1 girdle shaped</td>
<td>1</td>
<td>yes</td>
<td>zoospires</td>
<td>freshwater</td>
</tr>
<tr>
<td><em>Coccothrix chlorolobata</em></td>
<td>Broady &amp; Lokhorst 2008</td>
<td>3.5-5µm</td>
<td>no</td>
<td>none</td>
<td>3.5-5 × 2-7</td>
<td>1</td>
<td>no</td>
<td>1 lobed, parietal</td>
<td>1</td>
<td>yes</td>
<td>no</td>
<td>terrestrial</td>
</tr>
<tr>
<td><em>Fottea pyrenoides</em></td>
<td>Broady 1976</td>
<td>6 µm</td>
<td>no</td>
<td>none</td>
<td>6 × 9-18</td>
<td>1</td>
<td>mucilagenous colonies</td>
<td>1 parietal plate-like</td>
<td>1</td>
<td>no</td>
<td>no</td>
<td>terrestrial</td>
</tr>
<tr>
<td><em>Gloeotilopsis planctonica</em></td>
<td>Deason 1969</td>
<td>3-7 µm</td>
<td>no</td>
<td>none</td>
<td>3-7 µm × 8-24</td>
<td>1</td>
<td>no</td>
<td>1 plate like</td>
<td>1 or 2</td>
<td>no</td>
<td>zoospires</td>
<td>freshwater and planktonic; terrestrial</td>
</tr>
<tr>
<td><em>Heterothrichopsis viridis</em></td>
<td>Iyengar &amp; Kanthamma 1941</td>
<td>6-8 µm</td>
<td>no</td>
<td>none</td>
<td>6-8 × 14-16</td>
<td>1</td>
<td>no</td>
<td>1-8 plate-like or discoid</td>
<td>1 or more</td>
<td>yes</td>
<td>no</td>
<td>freshwater and terrestrial</td>
</tr>
<tr>
<td><em>Hormidiospora crenulata</em></td>
<td>Burova et al. 2011</td>
<td>14-16 µm</td>
<td>no</td>
<td>none</td>
<td>8-10 × 8-10</td>
<td>1</td>
<td>yes</td>
<td>1 plate-like</td>
<td>none</td>
<td>no</td>
<td>no</td>
<td>freshwater and terrestrial</td>
</tr>
<tr>
<td><em>Hormidiospora verrucosa</em></td>
<td>Lokhorst AlgaeBase</td>
<td>4-6 µm</td>
<td>no</td>
<td>none</td>
<td>4-6 × 15-30</td>
<td>1</td>
<td>no</td>
<td>1-2 parietal, laminate</td>
<td>none</td>
<td>no</td>
<td>zoospires</td>
<td>freshwater</td>
</tr>
<tr>
<td><em>Okellya curvata</em></td>
<td>Lefaert et al. 2009</td>
<td>7-10 µm</td>
<td>no</td>
<td>basal discoid holdfast</td>
<td>4-10 × 40-80</td>
<td>1,2,4-8</td>
<td>no</td>
<td>1 parietal</td>
<td>none</td>
<td>no</td>
<td>zoospires</td>
<td>marine</td>
</tr>
<tr>
<td><em>Pearsoniella variabilis</em></td>
<td>Sarma &amp; Keshri 1995</td>
<td>20-40 µm</td>
<td>no</td>
<td>lobed holdfast</td>
<td>20-40 × 20-90</td>
<td>1</td>
<td>no</td>
<td>1 ring-shaped</td>
<td>many</td>
<td>no</td>
<td>zoospires</td>
<td>freshwater</td>
</tr>
<tr>
<td><em>Rhizoclonium tortuosium</em></td>
<td>Koster 1955</td>
<td>50-60 µm</td>
<td>no</td>
<td>basal cells or holdfast</td>
<td>40-60 × 80-120</td>
<td>multiple</td>
<td>yes</td>
<td>1 parietal, reticulate</td>
<td>2-3 many</td>
<td>no</td>
<td>zoospires</td>
<td>freshwater and marine</td>
</tr>
<tr>
<td><em>Ulothrix zonata</em></td>
<td>John 2002</td>
<td>25-40 µm</td>
<td>no</td>
<td>simple or rhizoidal basal cells</td>
<td>25-40 × 10-20</td>
<td>1</td>
<td>no</td>
<td>1 girdle-like, parietal</td>
<td>1-3 many</td>
<td>occasionally</td>
<td>zoospires</td>
<td>freshwater and marine</td>
</tr>
</tbody>
</table>

Trebouxiophyceae

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Reference</th>
<th>Filament diameter</th>
<th>Filaments knotted</th>
<th>Basal attachment</th>
<th>Cell size (µm)</th>
<th>Nuclei per cell</th>
<th>Capsule/sheath</th>
<th>Plastid no. and type</th>
<th>Pyrenoids</th>
<th>Pyrenoid starch envelope</th>
<th>Motile asex. stage</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Catena viridis</em></td>
<td>Chodat 1900</td>
<td>6-10 µm</td>
<td>no</td>
<td>none</td>
<td>6-10 × 14-20</td>
<td>1</td>
<td>yes</td>
<td>1 parietal, band-shaped</td>
<td>none</td>
<td>no</td>
<td>no</td>
<td>freshwater</td>
</tr>
<tr>
<td><em>Geminella interrupta</em></td>
<td>Tsarenko 2011</td>
<td>10-20 µm</td>
<td>some species</td>
<td>sessile or free floating</td>
<td>4-8 × 4-10</td>
<td>1</td>
<td>yes</td>
<td>1 parietal, girdle shaped</td>
<td>1</td>
<td>no</td>
<td>no</td>
<td>freshwater and terrestrial</td>
</tr>
</tbody>
</table>

*Notes:*
- Pyrenoids: 1 ring-shaped, 1-2 parietal, laminate, 1-3 many occasionally
- Pyrenoid starch envelope: 1 plate-like, none
- Motile asex. stage: 1-2 parietal, laminate, 1-3 many occasionally
- Habitat: freshwater, freshwater and terrestrial, marine, freshwater and planktonic.
<table>
<thead>
<tr>
<th>Species</th>
<th>Authors</th>
<th>Length (μm)</th>
<th>Attachment</th>
<th>Dimensions (μm)</th>
<th>Presence</th>
<th>Shape</th>
<th>Parietal</th>
<th>Zoosporogenesis</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gloeotila vesiculosa</td>
<td>Tsarenko 2011</td>
<td>10-20</td>
<td>no</td>
<td>none</td>
<td>6-10 × 15-30</td>
<td>l</td>
<td>yes</td>
<td>1 band shaped</td>
<td>1 no no freshwater</td>
</tr>
<tr>
<td>Koliellopsis inundata</td>
<td>Lokhorst et al. 2004</td>
<td>3-4</td>
<td>no</td>
<td>none</td>
<td>3-4 × 12-32</td>
<td>l</td>
<td>no</td>
<td>1 parietal</td>
<td>none no zoospores terrestrial and freshwater</td>
</tr>
<tr>
<td>Stichococcus jenerensis</td>
<td>Neustupa 2007</td>
<td>4-6</td>
<td>no</td>
<td>none</td>
<td>3-5 × 4-12</td>
<td>l</td>
<td>no</td>
<td>1 parietal</td>
<td>1 (none in most species) yes no terrestrial for this species</td>
</tr>
</tbody>
</table>

Klebsormidiophyceae (Streptophyta)

<table>
<thead>
<tr>
<th>Species</th>
<th>Authors</th>
<th>Length (μm)</th>
<th>Attachment</th>
<th>Dimensions (μm)</th>
<th>Presence</th>
<th>Shape</th>
<th>Parietal</th>
<th>Zoosporogenesis</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsormidium flaccidium</td>
<td>Silva et al. 1972</td>
<td>4-6</td>
<td>no</td>
<td>unattached or basal cell hold fast</td>
<td>4-6 × 8-10</td>
<td>l</td>
<td>no</td>
<td>1 parietal</td>
<td>1 yes zoospores terrestrial</td>
</tr>
</tbody>
</table>
Fig. 1. Phylogenetic tree of Ulotrichales inferred from a dataset of 18S ribosomal RNA sequences. The new species Kraftionema allantoideum – indicated with a star – is shown to be an early-branching lineage of the "core Ulotrichales" (indicated with a hexagon). The tree was inferred by BI and shows Bayesian posterior probabilities and ML bootstrap values.
Fig. 2. Light micrographs in DIC of *Kraftionema allantoideum* gen. *et* sp. nov during stages of its cell cycle. (a) Several knotted, entangled filaments during the division cycle as seen in field samples. (b, c) Single, knotted filaments of between 30-50 cells as seen in field samples. (d) Entangled filaments from cultures during the division cycle, flattened between the slide and coverslip to get the filaments more in focus. Cells appear mainly square to rectangular. (e) Following the division cycle, maturing filaments are twisted and entangled and comprised of allantoid cells (A) and bicells (Bi) held in a loose association within the capsule. Pyrenoids and starch envelopes are observed when cells are in focus (arrows). (f) A maturing filament shows a string of allantoid cells (A) and bicells (Bi) at the bottom of the image. During the division stage, allantoid cells and bicells divide further (arrowheads at the top of the image) to produce short fragments within the parental filament. Pyrenoids are encased in a starch envelope (arrows). (g) Short filament resulting from fragmentation is surrounded by the capsule (arrows) and dividing in several planes (arrowheads) resulting in cells of various shape. Scale bars = 40 µm (a), 25 µm (b-d), 15 µm (e), 10 µm (f, g).
Fig. 3. Light micrographs of filaments of *Kraftionema allantoideum* gen. et sp. nov. in DIC (a-c), stained with Stains-All (d-f) and fluorescently stained (g-i). (a) Dividing fragment with different division planes (arrowheads) resulting in cells of different shape. Pyrenoids plus starch envelopes (p) and the capsule (arrow) are observed. (b) Recently fragmented, dividing filaments, revealing division planes (arrowheads) resulting in cells of different size and shape. The capsule (arrows) is obvious. (c) Several hours after the division cycle, mature allantoid bicells are loosely associated within the capsule. (d-f) DIC images following staining with StainsAll showing the cell wall (cw) and capsule (arrows). (g-i) Fluorescent images showing the cell wall (blue) from Hoechst stain, the nuclei (yellow/green) from SYBRGreen stain and chloroplast auto-fluorescence (red). (g) Filament of bicells showing their loose arrangement in the filament. (h) Dividing filament with blue walls and showing the opposing polarity of pyrenoids (p) and nuclei (n) in adjacent daughter cells. (i) An allantoid bicell has undergone 2 division cycles to produce an 8-celled fragment of 6 square cells and the two terminal dome cells (stars) that defined the original cell. Scale bars = 5 μm (a, c, f, h, i), 10 μm (b, c, g), 15 μm (d, g).
Fig. 4. Transmission electron microscope images (a-d) of cells from *Kraftionema allantoideum* gen. et sp. nov. showing the pyrenoid (p) and surrounding starch envelope. The chloroplast (c) dominates the cell cytoplasm, and contains many small starch grains. (a) Section through a mature allantoid cell with closely appressed cell wall (cw). (b, c) Recently divided cells showing their cell walls (cw) and the opposite polarity of the pyrenoids (p). (d) Chloroplast thylakoids (t) intersected the pyrenoid matrix through the starch envelope. Scale bars = 1 µm (a, d), 1.5 µm (b, c) Scale bars = 5 µm (a, c, f, h), 10 µm (b, c, g), 15 µm (d).
Author/s:  
Wetherbee, R; Verbruggen, H

Title:  
Krafftonema allantoideum, a new genus and family of Ulotrichales (Chlorophyta) adapted for survival in high intertidal pools

Date:  
2016-10-01

Citation:  

Persistent Link:  
http://hdl.handle.net/11343/233615

File Description:  
Accepted version