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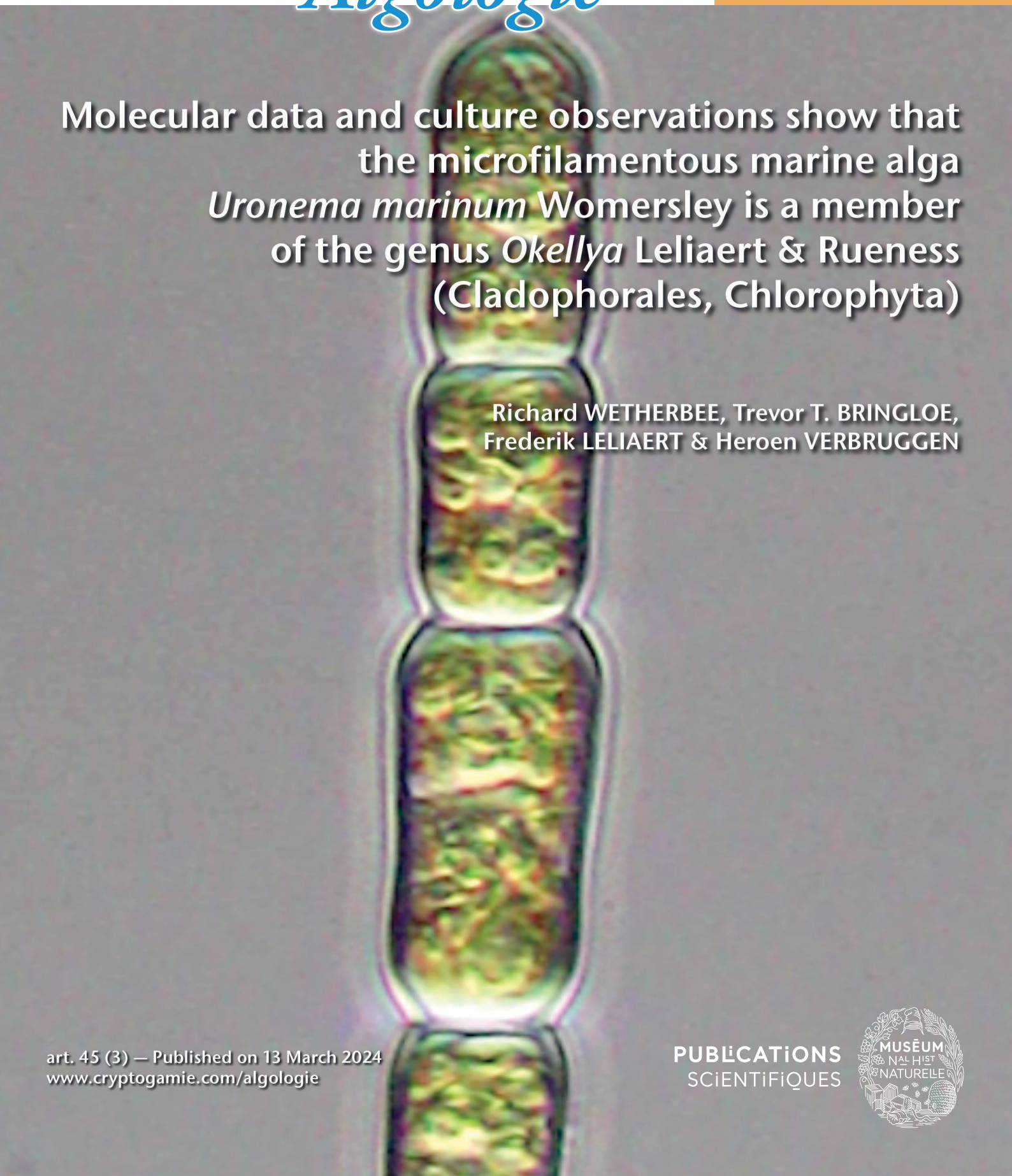

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Molecular data and culture observations show that the microfilamentous marine alga *Uronema marinum* Womersley is a member of the genus *Okellya* Leliaert & Rueness (Cladophorales, Chlorophyta)

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ABSTRACT
The marine microfilamentous green algal genus *Okellya* Leliaert & Rueness forms a distinct lineage of Cladophorales Haeckel, and currently contains only a single species, *Okellya curvata* (Printz) Leliaert & Rueness, originally described as *Uronema curvatum* Printz from Norway. Based on phylogenetic analyses of SSU and LSU rDNA sequences, we show that the Australian *Uronema marinum* Womersley also belongs to *Okellya*, and the species is transferred there as *O. marina* (Womersley) Wetherbee, comb. nov. We provide detailed observations of cultured material and show that *O. marina* (Womersley) Wetherbee, comb. nov. differs from *O. curvata* in the presence of pyrenoids, necessitating a revised circumscription of the genus *Okellya* and family Okellyaceae Leliaert & Rueness.

KEY WORDS
Australia, Chlorophyta, *Okellya*, *Uronema*, molecular phylogeny, new combination.

RÉSUMÉ
Les données moléculaires et l'observation des cultures montrent que l'algue verte microfilamenteuse marine Uronema marinum *Womersley est membre du genre* Okellya *Leliaert & Rueness (Cladophorales, Chlorophyta).*
Le genre d'algue verte microfilamenteuse marine *Okellya* Leliaert & Rueness forme une lignée distincte des Cladophorales Haeckel, et contient actuellement une seule espèce, *Okellya curvata* (Printz) Leliaert & Rueness, originellement décrite comme *Uronema curvatum* Printz en Norvège. Sur la base d'analyses phylogénétiques des séquences SSU et LSU rDNA, nous montrons que l'espèce australienne *Uronema marinum* Womersley appartient aussi à *Okellya*, et l'espèce est transférée comme *O. marina* (Womersley) Wetherbee, comb. nov. Nous fournissons des observations détaillées de matériel en culture et montrons que *O. marina* (Womersley) Wetherbee, comb. nov. diffère de *O. curvata* dans la présence de pyrénoïdes, appelant une délimitation révisée du genre *Okellya* et de la famille Okellyaceae Leliaert & Rueness.

MOTS CLÉS
Australie, Chlorophyta, *Okellya*, *Uronema*, phylogénie moléculaire, combinaison nouvelle.

INTRODUCTION

Marine microfilamentous green algae occur in different lineages of Ulvophyceae K.R.Mattox & K.D.Stewart, including the Bryopsidales J.H.Schaffner, Ulvales Blackman & Tansley, Ulotrichales Borzi, Cladophorales Haeckel and *Blastophysa* Reinke (Verbruggen *et al.* 2009; Leliaert *et al.* 2011; Gunnarsson & Nielsen 2016; Bartolo *et al.* 2022). Despite being common and widespread in marine benthic environments, they are poorly studied and are often overlooked in biodiversity surveys. Because of their diminutive size and cryptic morphology, their identification requires cultivation and molecular studies.

Okellya Leliaert & Rueness is a genus of marine microfilamentous green algae in the order Cladophorales. It forms the monogeneric family Okellyaceae Leliaert & Rueness, which is the sister lineage to all other Cladophorales (Leliaert *et al.* 2009). At present, the genus has only a single species –*Okellya curvata* (Printz) Leliaert & Rueness– which was originally described as *Uronema curvatum* Printz (1926). The genus *Okellya* was described when molecular phylogenies showed *O. curvata* to be unrelated to other *Uronema* Lagerheim species but rather forming a separate lineage in the Cladophorales (Leliaert *et al.* 2009). The genus *Uronema* contains several other species, mostly freshwater, but also the species *U. marinum* Womersley originally described from marine habitats in southern Australia (Womersley 1984, as *U. marina*). Leliaert *et al.* (2009) did not have access to material from this species and its taxonomic status has remained uncertain.

In our surveys of sand-dwelling algae of the Australian coastline (Wetherbee & Verbruggen 2016; Wetherbee *et al.* 2019a, b, 2021, 2023), we came across the microfilamentous green alga *Uronema marinum*. Our goal in this paper is to provide detailed observations of cultured material of this species and infer its phylogenetic position.

MATERIAL AND METHODS

A sand sample containing *Uronema marinum* was collected from a shallow, high intertidal pool at Narooma Inlet, New South Wales (36°12'27.8"S, 150°07'30.4"E) in March 2019 by R. Wetherbee. The sample consisted of approximately 0.5-1.0 cm³ of sand plus seawater that was placed into a 60 ml plastic jar and returned to the lab. A clonal culture (56a) was established by isolating single filaments of *U. marinum* by micro-pipetting it into K enriched seawater medium (Keller *et al.* 1987). The culture was maintained in 60 ml plastic containers at 21°C under Sylvania 58 Luxline Plus and Gro-Lux fluorescent lamps with a daily 10:14 hours light:dark cycle; a stock culture was transferred into new K medium once a month. The culture was deposited in the Australian National Algal Culture Collection (ANACC) with accession CS-1486.

To observe filaments of *U. marinum*, a drop of cell culture containing filaments was taken and mounted onto microscope slides with coverslips sealed with a 1:1:1 ratio of Vaseline, lanoline, and paraffin wax. Filaments 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, Melville, New York, United States). To observe the presence and location of pyrenoids, filaments were briefly stained with iodine as described in Leliaert *et al.* (2009). A voucher with representative material was deposited in MELU with accession MELUA132472a.

DNA was extracted from the culture using a CTAB-based method (Cremen *et al.* 2016). Illumina library preparation (VAHTS Universal DNA Library prep kit) and sequencing (NovaSeq, 150bp PE, *c.* 4Gb of data) was carried out by Azenta (Suzhou, China). Reads were trimmed (Bolger *et al.* 2014) and assembled (Bankevich *et al.* 2012) as previously described (Bringloe *et al.* 2021) and the 18S and 28S genes were extracted from the assembly and submitted to GenBank (OR405984 and OR416167).

The phylogenetic position of the Australian *Okellya* isolate 56a was verified based on analysis of 18S and 28S nrDNA sequences. A selection of species of Cladophorales (based on Leliaert *et al.* 2009; Boedeker *et al.* 2016) and two outgroup taxa (*Ulva* L. and *Trentepohlia* Martius) were selected (Table 1). The 18S and 28S datasets were aligned separately using MUSCLE (Edgar 2004) and concatenated, yielding an alignment of 2376 positions (18S: 1689 positions, and 28S: 687 positions). A ML analysis was conducted using RAxML v.8 (Stamatakis 2014) under a GTR + CAT model. The robustness of the resulting phylogenies was tested using 1000 replicates of a rapid bootstrap heuristic.

RESULTS AND DISCUSSION

Our culture strain of *Uronema marinum* initially showed a benthic green alga forming minute tufts (Fig. 1A) of slightly curved, erect, unbranched, uniseriate filaments, attached by a basal discoid holdfast (Fig. 1B, C) or a "basal, gelatinous conical attachment" as described by Womersley (1984). Filaments were 3-12 cells in length, with cylindrical cells 5-8 µm in diameter and a rounded apical cell tip. Over time in ideal culture conditions, isolated filaments had seemingly indeterminate growth by intercalary cell divisions with occasionally up to *c.* 100 cells in a filament and filaments up to *c.* 1 mm long. Individual cells were often thicker in diameter at their apical ends, but that was not always the case in long cultured filaments. Settled zoospores produced thick, grainy cells (Fig. 1B, C) that eventually elongate into cells where the parietal chloroplast and single, central pyrenoid are obvious (Fig. 1E, F: arrowheads), more so after staining with iodine (Fig. 1G, H: arrowheads).

All cells in a filament appeared to have the capability of transforming into a zoosporangium containing 8-16 zoospores (Fig. 1I, K, L). Spores were elongate, generally 10-12 µm in diameter and 18-20 µm in length, and escaped through an apical pore in the mother cell wall (Fig. 1J-M: arrows). Zoospores occasionally fail to escape and germinate inside the mother cell wall (Fig. 1K-M), some appear stuck in the pore (Fig. 1K-M: arrows) and grow up to four or more cells in length with an established holdfast (Fig. 1M: asterisk).

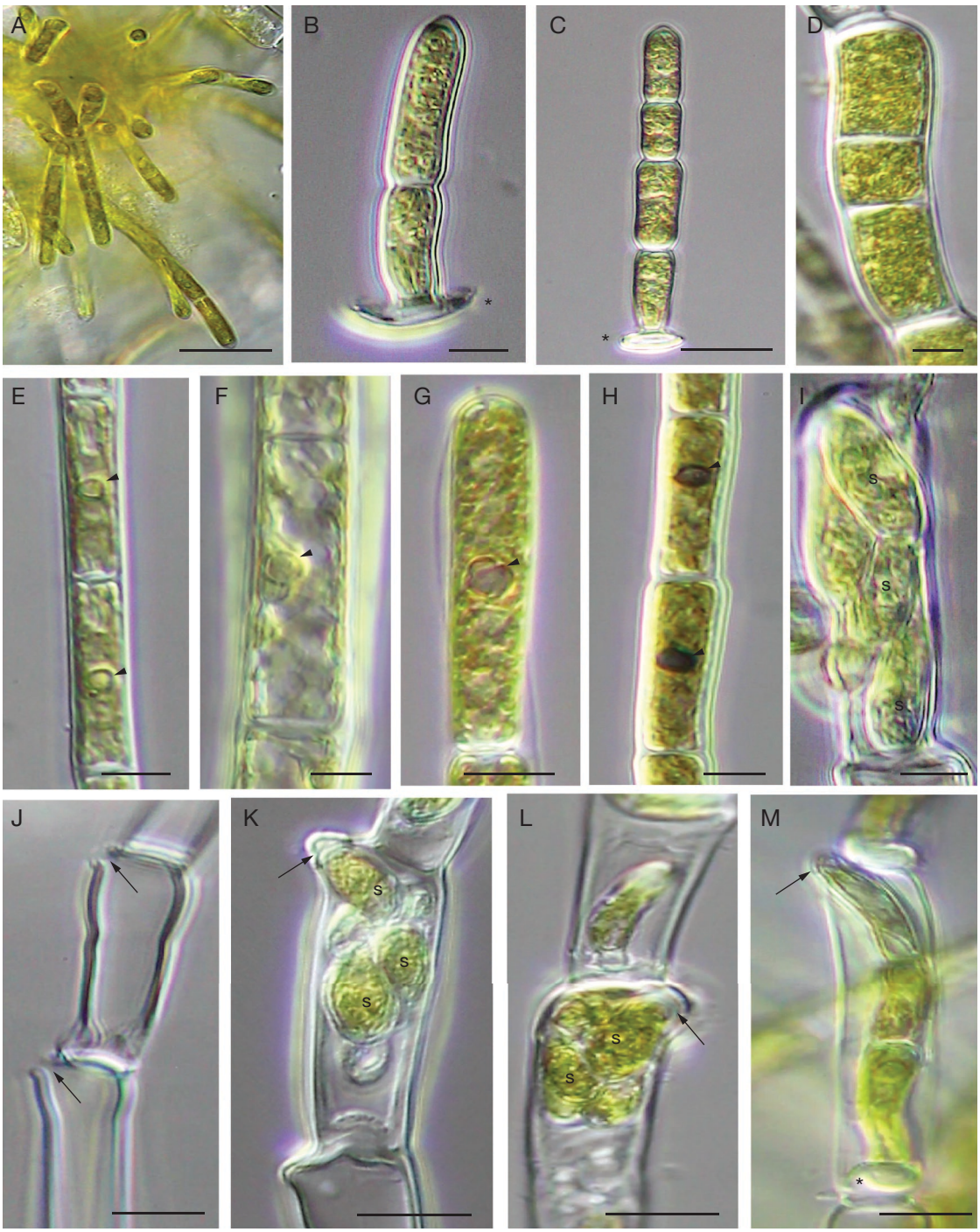

FIG. 1. — *Okellya marina* (Womersley) Wetherbee, comb. nov. (strain 56a) from New South Wales: **A**, tuft of filaments; **B**, **C**, newly formed filaments of two and four cells attached by a discoid holdfast (**asterisks**), apical cells rounded; **D**, larger filaments occur with dense cytoplasm; **E**, **F**, filaments showing a single pyrenoid at the center of cells (**arrowheads**); **G**, **H**, pyrenoids stained with iodine solution (**arrowheads**); **I**-**M**, elongate zoospores (**s**) have differentiated in an intercalary cell (**I**) and escape from a pore at the apical end of cells (**J**-**M**: **arrows**); zoospores often fail to escape through the pore and germinate inside the sporangium (**K**-**M**), occasionally even forming holdfasts (**asterisk** in **M**). Scale bars: A, 50 µm; B, D-I, 10 µm; C, J-M, 20 µm.

The ML phylogeny inferred from the rDNA alignment placed our *Uronema marinum* strain (56a, listed as *Okellya marina* (Womersley) Wetherbee, comb. nov.) with *Okellya curvata* in the family Okellyaceae of the Cladophorales (Fig. 2). The Okellyaceae were recovered as sister to the remaining Cladophorales with maximal bootstrap support, concordant to previous phylogenetic analysis (Leliaert *et al.* 2009). Uncorrected p-distances between *O. marina* (Womersley) Wetherbee, comb. nov. and *O. curvata* were 0.032 and 0.111 for 18S and 28S, respectively, which is considerably higher than intraspecific distances typically found within cladophoralean species (e.g. Leliaert *et al.* 2007), and on par with divergences

TABLE 1. — Information on DNA sequences and specimens used in the rDNA phylogenetic analysis.

Species	Origin	Voucher	SSU	LSU
Aegagropila linnaei Kützing	Iceland	L: 0793580	FR719925	EU655697
Anadyomene stellata (Wulfen) C.Agardh	Philippines	GENT: PH209	AF510147	AJ544746
Arnoldiella kosterae (C.Hoek) C.Boedeker	Netherlands	L: 0793302, 0793303	FR719933	FR719945
Boergesenia forbesii (Harvey) Feldmann	Tanzania, Zanzibar	GENT: FL1009	AF510164	AJ544742
Boodlea composita (Harvey) F.Brand	Tanzania, Zanzibar	GENT: FL950	AF510157	AJ544731
Chaetomorpha brachygona Harvey	Caribbean, Lesser Antilles	WELT: A033200	LT607332	LT607064
Chaetomorpha coliformis (Montagne) Kützing	New Zealand, Otago	WELT: A033166	LT607335	LT607067
Chaetomorpha linum (Muller) Kützing	Germany, North Sea	WELT: A033188	LT607341	LT607073
Chaetomorpha moniligera Kjellman	Japan	WELT: A033156	LT607343	LT607075
Chaetomorpha vieillardii (Kützing) M.J.Wynne	Philippines, Siquijor	GENT: FL1132	LT607347	LT607079
Cladophora aokii Yamada	Japan	GENT: C ryu 1 = CryHJ	AM498747	AM503434
Cladophora glomerata (L.) Kützing	Kazakhstan, Caspian Sea	WELT: A033196	LT607358	LT607090
Cladophora ruchingeri (C.Agardh) Kützing	–	GENT: Cruch3 = Ru84.60	LT607374	LN679070
Cladophora sericea (Huds.) Kützing	Scotland, Stonehaven	WELT: A033243	LT607379	LT607108
Cladophora vagabunda (L.) C.Hoek	France, Brittany	WELT: A033276	LT607354	LT607086
Dictyosphaeria cavernosa (Forsskål) Børgesen	Tanzania, Zanzibar	GENT: FL913	AM498756	AJ544745
Lurbica zinkwasii Boedeker	South Africa, Eastern Cape	WELT: A033222	LT607405	LT607135
Lychaete herpestica (Montagne) M.J.Wynne	Japan	GENT: C herp4 = Cloz51d2HJ	LT607361	LT607093
Lychaete mirabilis (C.Agardh) J.Agardh	South Africa, Cape Town	L: 0793561	FM205050	FM205034
Lychaete pellucida (Hudson) M.J.Wynne	France, Brittany	L: 0793562	LT607370	FM205037
Lychaete rhodolithicola (Leliaert) M.J.Wynne	Wales, Milford Haven	GENT: Bunker1	FM205053	FM205044
Okellya curvata (Printz) Leliaert & Rueness	Norway	CCAP455/1	FN257508	FN257507
Okellya marina (Womersley) Wetherbee, comb. nov.	Australia, New South Wales	MELUA132472a	OR405984	OR416167
Pithophora roettleri (Roth) Wittrock	Portugal, Montemor-o-Velho	L: 0793289	FR719930	FR719942
Pseudocladophora conchopheria (Sakai) Boedeker & Leliaert	Japan, Ishikawa	L: 0793297	AB062705	FR719951
Pseudocladophora horii (C.Hoek & Chihara) Boedeker & Leliaert	South Africa, KwaZulu-Natal; Japan, Ishigaki	GENT: HEC10983	AB078731	AJ544728
Pseudorhizoclonium africanum (Kützing) Boedeker	Philippines, Negros Oriental	GENT: FL1164	LT607390	LT607119
Pseudorhizoclonium africanum	Tanzania	GENT: TZ0781	LT607392	LT607121
Pseudorhizoclonium sp.	Madagascar, Isle St. Marie	WELT: A033187	LT607401	LT607131
Rhizoclonium hieroglyphicum (C.Agardh) Kützing	Netherlands	WELT: A033168	LT607394	LT607124
Rhizoclonium riparium (Roth) Harvey	Germany, North Sea	WELT: A033189	LT607398	LT607128
Siphonocladus tropicus (P.Crouan & H.Crouan) J.Agardh	Dominican Republic	GENT: O. Dargent (no number)	AM498761	AJ544744
Struvea sp.	Papua New Guinea	GENT: HEC10437	AF510149	AJ544737
Valonia aegagropila C.Agardh	Tanzania, Zanzibar	GENT: FL960	AM498762	AJ544748
Valoniopsis pachynema (G.Martens) Børgesen	Tanzania, Zanzibar	GENT: FL1006	AM498765	AJ544741
Willeella montagneana (Kützing) Boedeker	New Zealand, North Island	WELT: A033289	LT607365	LT607097
Willeella ordinata Børgesen	Panama, Isla Escudo de Veraguas	US: DML 64271	LT607368	LT607099
Wittrockiella amphibia (Collins) C.Boedeker & G.I.Hansen	United States, Washington	CCMP1674	GU384872	GU384873
Wittrockiella lyallii (Harvey) C.Hoek, Ducker & Womersley	Chile, Aysén	SGO: 158361	GU198502	GU198503
Outgroup				
Trentepohlia sp.	Belgium	L0793309	FR719938	FR719952
Ulva mutabilis Foyn	Portugal	Friedrich Schiller University Jena, Germany	GCA_900538255	GCA_900538255

observed between species within other genera (e.g. *Chaetomorpha* Kütz., *Lychaete* J.Agardh; see Fig. 2).

In its morphological appearance, our strain corresponds very well with descriptions given for *Uronema marinum*. The type description lists cells 4-6 µm in diameter, but other reports of *U. marinum* report larger cells (Kraft 2007; Huisman 2015). Observations on our original collections was 5-8 µm but cells became larger (up to 20 µm) in ideal culture conditions. The phylogenetic placement of our strain clearly indicates that it belongs to the genus *Okellya*. The high genetic divergence with *O. curvata*, the only species that currently exists in the genus *Okellya*, indicates these two are separate species. Considering these observations, we formally transfer *Uronema marinum* to the genus *Okellya* in the family Okellyaceae of the order Cladophorales.

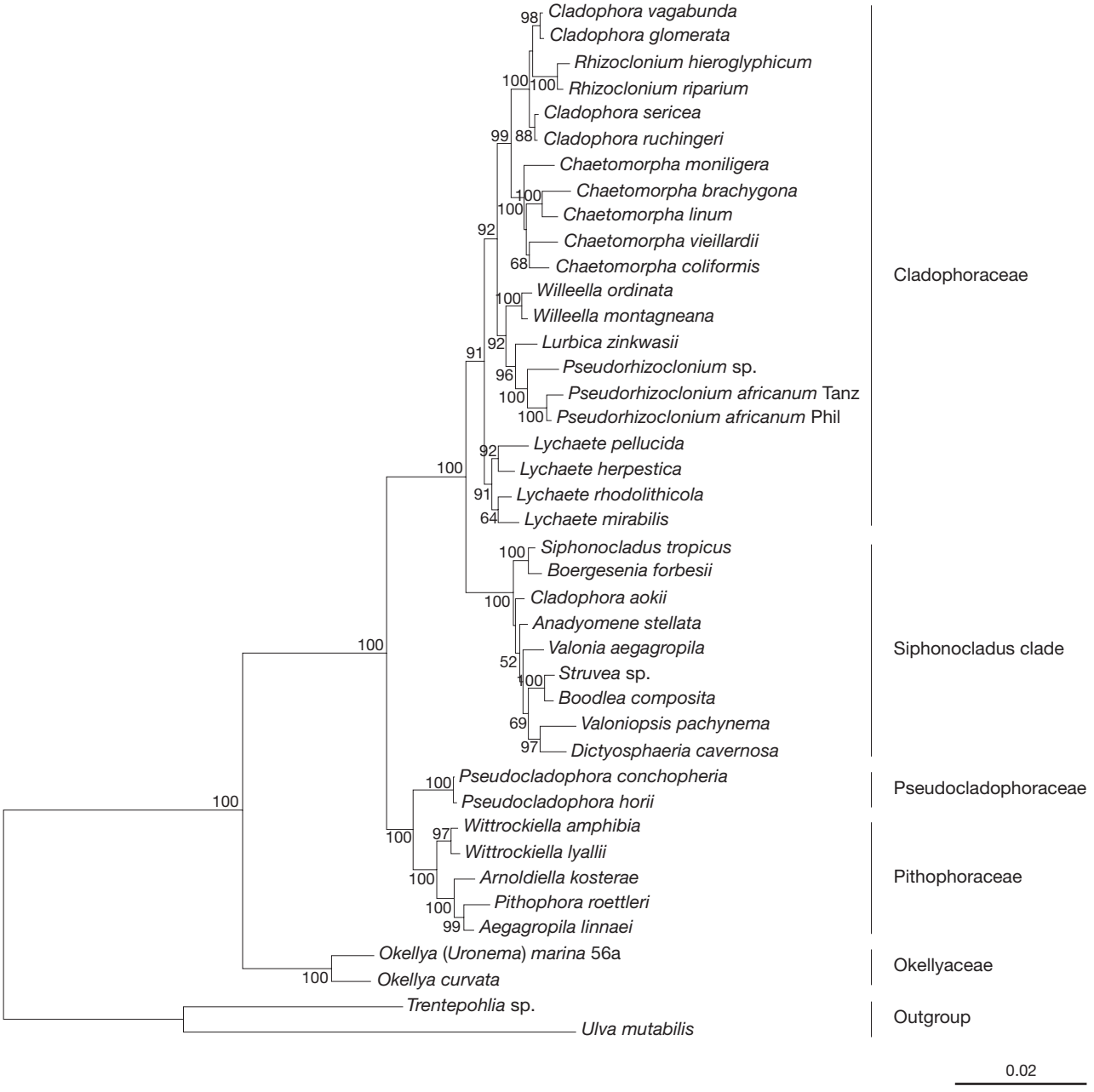

Fig. 2. — Maximum likelihood phylogenetic tree of selected Cladophorales Haeckel, showing the position of the species *Okellya marina* (Womersley) Wetherbee, comb. nov. as sister to *Okellya curvata* (Printz) Leliaert & Rueness in the Okellyaceae Leliaert & Rueness family. Numbers shown at nodes represent RAxML rapid bootstrap values. The scale is in estimated substitutions per site in the concatenated 18S and 28S alignment.

Order CLADOPHORALES Haeckel
Family Okellyaceae Leliaert & Rueness
Genus *Okellya* Leliaert & Rueness

Okellya marina (Womersley) Wetherbee, comb. nov.

Basionym: *Uronema marinum* ('*marina*') Womersley, *The Marine Benthic Flora of Southern Australia*. Part I: 131, figs 41D, E (Womersley 1984).

Type material. — **Australia** • South Australia, Coffin Bay, Kellidie Bay; holotype: AD-A31827.

Type locality. — Australia, South Australia, Coffin Bay, Kellidie Bay.

Notes

Nineteen species have been described or transferred to *Uronema* (Guiry & Guiry 2023), all but two restricted to freshwater habitats. The only marine species are the North Atlantic *U. curvatum* (*Okellya curvata*) and the Australian *U. marinum* (*Okellya marina* (Womersley) Wetherbee, comb. nov.). The two former marine *Uronema* species differ from the freshwater representatives of the genus in zoosporangial and apical cell morphology (usually tapering). The generic placement of *U. curvatum* has

been debated by Rueness (1992) and Kornmann & Sahling (1994), who already suggested a relationship with the Cladophorales or Ulotrichales. Womersley (1984) provisionally referred his new species to *Uronema*, but commented that this placement needed investigation from living material.

The presence of pyrenoids in *O. marina* (Womersley) Wetherbee, comb. nov. is noteworthy, considering the lack of pyrenoids in *O. curvata*. The absence of pyrenoids was seen as an exceptional and defining trait of *Okellya* and Okellyaeae within the Cladophorales (Leliaert *et al.* 2009). Nevertheless, with the observation of pyrenoids in *O. marina* (Womersley) Wetherbee, comb. nov., it is necessary to revise the morphological circumscription of the genus and family.

Microfilamentous species like those discussed in this paper are rarely if ever observed in the field. *Okellya curvata* is best known as an epiphyte of various other seaweeds, most often only seen upon microscopic observation of larger seaweeds. It has also been found on pebbles, but we would argue that its niche is probably broader than currently appreciated. The available information for *O. marina* (Womersley) Wetherbee, comb. nov. suggests a similar overall habitat. Our culture grew up from a sand sample, and we observed filaments attached to sand grains. Other Australian reports of *Okellya marina* (Womersley) Wetherbee, comb. nov. (as *U. marinum*) show it growing as an epiphyte on other macroalgae.

The biodiversity of microfilamentous algae and small turf algae is likely strongly underestimated, in part due to them being understudied and in part due to the morphologically simple forms found in these groups of algae showing prevalent cryptic diversity (e.g. Verbruggen *et al.* 2009; Verbruggen 2014; Díaz-Tapia *et al.* 2020). Besides our strain from New South Wales on the Australian East coast (*c.* 1340 km sea distance from the type locality) and the sites listed in the original description of the genus (Coffin Bay in South Australia and Rottnest Island in Western Australia), *Uronema marinum* has been reported from Ningaloo Reef in Western Australia (Huisman 2015), Lord Howe Island (Kraft 2000) and the Great Barrier Reef (Kraft 2007), as well as several locations in the Pacific Ocean, the Indian Ocean and the Mediterranean Sea (Guiry & Guiry 2023). As more molecular data are gathered for this apparently widespread species, it will be interesting to see what we can learn about the level of species diversity in this entity, as reportedly widespread seaweed species tend to exhibit cryptic diversity (Díaz-Tapia *et al.* 2018). In addition, we may learn whether or not these *Uronema* records should also, in fact, belong in the genus *Okellya*, and how the diversity of this genus is structured biogeographically.

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