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***Rhodenigma contortum*, an obscure new genus and species of
Rhodogorgonales (Rhodophyta) from Western Australia¹**

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ABSTRACT

An unknown microscopic branched filamentous red alga was isolated into culture from coral fragments collected in Coral Bay, Western Australia. It grew well unattached or attached to glass with no reproduction other than fragmentation of filaments. Cells of some branch tips became slightly contorted and digitate, possibly as a substrate-contact-response seen at filament tips of various algae. Attached multicellular compact discs on glass had a very different cellular configuration and size than the free filaments. In culture the filaments did not grow on or in coral fragments. Molecular phylogenies based on four markers (*rbcL*, *cox1*, 18S, 28S) clearly showed it belongs to the order Rhodogorgonales, as a sister clade of *Renouxia*. Based on these results, the alga is described as the new genus and species *Rhodenigma contortum* in the Rhodogorgonaceae. It had no morphological similarity to either of the other genera in Rhodogorgonaceae and illustrates the unknown diversity in cryptic habitats such as tropical coral rubble.

Keywords: Australia, Coral Bay, *cox1*, molecular phylogeny, *psbA*, *rbcL*, *Rhodenigma contortum*, Rhodogorgonales, Rhodophyta, 18S, 28S

INTRODUCTION

Small, branched, filamentous red algae occupy various marine habitats as epiphytes and endophytes of other algae, or on vascular plants or invertebrates, and also include surface dwelling and boring forms in non-living substrates. Reproduction in these diminutive organisms is often inconspicuous or unknown, making identification troublesome. These well-documented forms are primarily in the Florideophyceae (orders Acrochaetiales, Coelaconematales, Palmariales, Rhodochaetales, etc.) as well as in Bangiophyceae, Compsopogonophyceae and Stylonematophyceae (McCarthy and Orchard 2007, Hwang and Kim 2011, Kim and Kim 2011).

The Rhodogorgonales is an order of marine red algae found in coral reef habitats in the Caribbean Sea and the tropical Indo-Pacific (Ogden 1992, Fredericq and Norris 1995, Kraft et al. 1999). Its two known species, *Rhodogorgon ramosissima* J.N.Norris & Bucher and *Renouxia antillana* Fredericq & J.N.Norris, are macrophytes growing on rocky substrates and the order is not known to contain small filamentous forms.

■ *Rhodogorgon ramosissima* is a robust, branched, soft tissue, pale red alga with specialized calciferous cells. It was described from the Caribbean Sea (Antigua, Belize, Bahamas, Lesser Antilles, Martinique and Panama) by Norris and Bucher (1989) and was also reported from Florida (Ballantine 1996), St Croix (Ogden 1992), Colombia (Diaz-Pulido and Díaz-Ruíz 2003) and Venezuela (Vera et al. 2006). It has also been recorded in the Pacific Ocean from the Philippines (Ogden 1992), Papua New Guinea (Coppejans and Millar 2000, Littler and Littler 2003) and New Caledonia (Bittner et al. 2011). *Rhodogorgon carriebowensis* was described by Norris and Bucher (1989) based on different branching patterns but was later placed in synonymy with *R. ramosissima* (Ogden 1992).

■ *Renouxia antillana* was first recorded from the Caribbean Island of Guadeloupe and was also placed in the Rhodogorgonales and family Rhodogorgonaceae (Fredericq and Norris 1995). Like *Rhodogorgon*, it has calciferous cells, uninucleate vegetative cells, no secondary pit connections, separate male and female gametophytes, and subterminal spermatangial parent cells. It lacks auxiliary cells and connecting filaments, and carposporangia develop from branched gonimoblast filaments. *Renouxia* differs from *Rhodogorgon* in having flaccid, lobed and lubricious thalli. Assimilatory filaments are loosely interwoven with dome shaped densely pigmented apical cells. Thalli have thin walled rhizoidal filaments, indeterminate pseudodichotomous growth and sessile calciferous cells. *Renouxia* was also reported in the Caribbean Sea from Puerto Rico (Ballantine et al. 2004) and in the Indo-Pacific: Philippines (Kraft et al. 1999), Papua New Guinea (Coppejans and Millar 2000), French Polynesia (Littler and Littler 2003, N'Yeurt and Payri 2010), Fiji (South and Skelton 2003), New Caledonia (Bittner et al. 2011), Thailand (Liao and Charatsee 2000), Rodrigues and Tanzania (De Clerck et al. 2004).

Fredericq and Norris (1995) created the order and family (Rhodogorgonales and Rhodogorgonaceae) to include *Rhodogorgon* and *Renouxia*. In molecular phylogenies, Rhodogorgonales are reliably positioned within the subclass Corallinophycideae (Le Gall and Saunders 2007) as sister to the Sporolithales (Le Gall et al. 2010).

In the process of collecting and culturing algae growing in close association with tropical limestone substrates, we obtained an isolate of a filamentous red alga. Our goal in this study is to describe the morphology of the strain and infer its phylogenetic position using DNA data. While the species has no morphological affinities with the macroscopic Rhodogorgonales, the molecular phylogeny shows that it belongs to this group with high confidence and should be recognized as a new genus within the order.

MATERIALS AND METHODS

Collections, cultures and light microscopy. The alga appeared in a water sample containing fragments of *Acropora* sp. and other limestone fragments collected in Coral Bay (23° 08.476'S 113° 46.134'E), Western Australia (WA) in 2013 and transported to the School of Biosciences, University of Melbourne. The filaments were isolated into culture (strain JAW4846) using Modified Provasoli's Medium (MPM; West and McBride 1999), 30 psu sterile seawater, 50 x 70 mm glass culture dishes containing sterile glass cover slips or sterile (bleached) coral fragments for substrates, 10:14 light-dark daily photoperiod, 19-22°C, 4-12 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ cool white LED lighting. Most cultures were maintained on rotary shakers (70 rpm) to optimize growth. All the photographs were taken with a Zeiss GFL bright field microscope (Zeiss Australia, Sydney) and Canon G3 camera (<https://www.canon.com.au/>) using Adobe Photoshop CS4 (<http://www.adobe.com/au/>) to make the figures for publication.

Molecular analyses. DNA was extracted from the culture using a modified CTAB protocol, and an Illumina sequencing library was prepared and sequenced on an Illumina 2000 sequencer as described in Verbruggen and Costa (2015). Sequence assembly was done with MEGAHIT (Li et al. 2015) as described in Verbruggen and

Costa (2015). The nuclear ribosomal RNA genes 18S and 28S, the plastid gene *rbcL* and the mitochondrial *cox1* gene were extracted from the resulting contigs. After preliminary analyses showed an affinity of the sequences to those of Rhodogorgonales, the new sequences were aligned manually with Rhodogorgonales sequences downloaded from Genbank (Table 1). Based on the relationships between Rhodogorgonales and other orders in the Corallinophycidae (Le Gall et al. 2010), the Sporolithales genera *Heydrichia* and *Sporolithon* were included in the alignments as outgroup taxa. We attempted to only combine sequences across different markers if they came from the same specimen, but in a few cases this was not possible. In those cases, sequences from different specimens of the same species were combined after single-gene analyses indicated it was reasonable to do so. Newly generated sequences were submitted to Genbank.

Individual gene trees were found to yield largely similar trees, so the genes were concatenated into a single alignment and analyzed jointly. An analysis to determine suitable partitioning strategy and model of sequence evolution (PMT.pl; Verbruggen 2010) indicated that partitioning by genes and codon positions with uncoupled GTR+ Γ_4 models for the partitions yielded the lowest AICc score and this strategy was used.

Phylogenies were inferred with Maximum Likelihood (ML) and Bayesian Inference (BI). For ML inference, RAxML 7.3.0 was run from 50 starting trees and bootstrap analyses used 1000 pseudoreplicates (Stamatakis 2006). For BI inference, BEAST 1.8.1 was run for 10 million generations using a Yule tree prior and a lognormal relaxed clock model, the nucleotide substitution model determined by PMT.pl and the remaining settings left as default (Drummond and Rambaut 2007). Stationarity of the run was assessed visually and a consensus tree was calculated with a burn-in of 1 million generations.

RESULTS

The molecular data show that the filamentous red alga from WA falls within the Rhodogorgonales (Fig. 1). The four-gene phylogeny very strongly supports a sister relationship with the genus *Renouxia* but the divergence between the two lineages is

large. Based on these results and the unique morphology, we describe the entity as a new genus and species in the family Rhodogorgonaceae.

***Rhodenigma* gen. nov. J.A.West, Verbruggen & Loiseaux**

Description – In laboratory culture this small red alga forms unattached uniseriate branched filaments, either in unattached aggregate clumps or attached to glass, producing compact crusts with conspicuous vein-like, narrow filaments and laterals, many short-celled branches filling open free space and becoming tightly coherent between main filaments. Cells have a single narrow or variously shaped peripheral red chloroplast without pyrenoids. Apical and subapical cells are usually a very pale pink color, often becoming contorted and digitate, either attaching to glass or to other adjacent filaments forming nodules. Pit connections are visible under the compound microscope. No sporic reproduction was observed. In culture the alga attached to glass but not to bleached coral fragments.

***Rhodenigma contortum* sp. nov. J.A.West, Verbruggen & Loiseaux**

Description – Species description as for genus.

Holotypus – Strain JAW4846, collected in Coral Bay, Western Australia. The holotype specimen is deposited in the Royal Botanic Gardens Melbourne, National Herbarium of Victoria, Australia (MEL 2389688). The culture from which the holotype derives (JAW4846) has been accessioned (CCMP3453) by the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratory for Ocean Sciences, P.O. Box 380, 60 Bigelow Drive, East Boothbay, ME 04544, USA.

Isotypus – Isotype specimens are also deposited in the University of Michigan Herbarium (MICH 701679) and University/ Jepson Herbaria, University of California, Berkeley (UC 2050600).

Etymology – *Rhodo* for red, *enigma* for its puzzling appearance, *contortum* for twisted or convoluted, referring to the branch tips when they attach to substrate and when they cohere to form a nodule or attachment disc.

Anatomical observations – On shakers, cells of actively growing unattached filaments are 3.5-5.5 μm diam. and variable in length (14-50 μm ; Figs. 2, A and B; 3A). Apical and subapical cells of actively growing unattached filaments are usually pale pink with

thin elongate spiral peripheral plastids lacking pyrenoids. Growth is by apical cell divisions apparently without intercalary cell divisions. Branching is usually first evident near the distal end of the 3rd to 5th subapical cells of filaments (Fig. 2A). In older cells the plastids become darker red occupying the entire cell periphery with a single central vacuole along the cell (Fig. 3B). The nucleus is small (2.5-3.0 μm diam, not shown) and becomes obscured in older cells. Numerous small spherical bodies of unknown composition are evident in the cytoplasm of older cells (Fig. 3B). Pit connections were faintly visible in fixed specimens stained with aniline blue (Fig. 3C inset).

Many free filaments have cells with uniform cell dimensions but there are some with apical and subapical cells that become irregularly shaped and contorted (Figs. 2A and 3C). Some of these contorted filaments twist around adjacent filaments forming nodules (Figs. 2, A-C and 3C). Unattached filaments are either randomly oriented or form parallel rows (Fig. 3B).

Those filaments that adhere to the glass substrate form flat discs with tightly adherent, coalescent, much branched filaments. Discs initially appear one cell layer thick but become thicker, polystromatic and nodular with age (Fig. 2A). Clear main and lateral filaments with elongate narrow cells (4-5 μm wide by 30-45 μm long) formed conspicuous vein-like patterns in the flat discs adherent to glass (Fig. 3C). Numerous smaller cells (4-9 μm overall) branch from the elongate filaments and densely fill the interspace. Aniline blue stained the protoplasts very well but pit connections were only faintly visible between cells of discs (Fig. 3C). Although the filament cell walls usually cohere tightly, no protoplast fusions between cells of adjacent filaments were seen. While this isolate was obtained from an *Acropora* fragment in the field, it never attached to coral fragments in the laboratory or produced a macroscopic thallus like *Rhodogorgon* or *Renouxia*. Fragmentation of vegetative filaments is the only way that new thalli formed in culture.

DISCUSSION

Culturing from environmental samples or substrates has revealed many new and interesting algae (e.g., West et al. 2007a, West et al. 2007b, Zuccarello et al. 2010) and

before the advent of environmental DNA sequencing was the only way to discover cryptic algal species. *Rhodenigma contortum* while clearly part of the Rhodogorgonales shares no obvious, or diagnostic, vegetative or reproductive anatomical features with the other two macroscopic genera in the order. Vegetative cell fusion is supposedly recorded in the *Rhodogorgon* and *Renouxia* (Bittner et al. 2011) but it has not been confirmed in *Rhodenigma contortum*. Cell fusion is a complex process seen in many orders, such as the Ceramiales, involving secondary pit connection formation between the fusing cells. Cell fusion in some red algae is direct, i.e. without formation of pit connections, in the basal system of some genera in the Palmariales (Clayden and Saunders 2010). Pit connections between cells were seen in *Rhodenigma*. The Rhodogorgonales and other orders are placed in a clade with two-cap layered pit plugs (Pueschel et al. 1992, Saunders and Bailey 1997, Le Gall and Saunders 2007), and it would be worthwhile to carry out a TEM study to investigate if *R. contortum* also has this pit plug structure.

Rhodenigma has features in common with several unrelated species that attach to and penetrate substrates and form distinct attachment discs, for example the brown alga *Myrionema* and the red algae *Meiodiscus* (Nemaliophycidae) and *Sahlingia* (Compsopogonophyceae). Cells of the branch tips in these taxa are similar in shape, feature lateral cell adhesion, and do not have protoplast fusions between adjacent cells. In *Rhodenigma*, cells of the attachment discs are completely different in shape and smaller in size compared to cells of the unattached filaments. A similar cell differentiation pattern is seen in *Rubrointrusa membranacea* (Palmariales), endozoic in hydroids, with distinctly shaped cells penetrating the host tissues. However, most of these epi- and endozoic species grow upright filaments that produce reproductive structures (West 1979, Clayden and Saunders 2010). Despite our careful review of the literature on simple filamentous red algae, we have not found any previously described taxa providing a morphological match to *Rhodenigma*.

At this stage of our knowledge, we can only speculate as to whether *Rhodenigma* has a macroscopic stage. There are no published reports of any species in the Rhodogorgonales from Western Australia, but there is a collection of a yet to be named *Rhodogorgon* species from Dixon Island that shows the order is present there (John Huisman, pers. comm., August 2015). Considering that *Rhodenigma* is more closely related to *Renouxia* than it is to *Rhodogorgon*, it would seem unlikely that the sample

from Dixon Island represents the macroscopic stage of our alga. Sequence data would be needed to be definitive about this. It cannot be dismissed that our taxon is a microscopic life stage of an unknown *Renouxia* species, but this seems unlikely considering the large sequence divergence from the only known *Renouxia* species. To be conclusive about this, two resource-intensive approaches could be followed. First, one could embark on a search for a corresponding macroscopic stage in the field without the knowledge of what it looks like or whether it even exists. Second, one could subject our strain to a wider range of culture conditions hoping that it will grow into a macroscopic structure or that it reproduces to form a macroscopic structure in the next life stage.

Endo- and epilithic filamentous algae have complex associations and environmental roles in coral habitats. They are diverse and difficult to identify (Laborel and Le Campion-Alsumard 1979, Tribollet 2008, Gutner-Hoch and Fine 2011). The red alga considered in this paper is a puzzle particularly because while it was isolated from limestone fragments in seawater, it did not grow over bleached coral skeleton in culture conditions, and it is possible that the culture originated from propagules in the seawater rather than the collected limestone fragments themselves. This source of contamination is almost inevitable when collecting limestone fragments from the field. What is certain is that cryptic habitats such as the surfaces as well as the interior of tropical limestone rubble and coral fragments are a treasure trove of small algae that have been studied in much less detail than macrophytes. A similar situation is evident with crustose algae, with a recent survey of Peyssonneliaceae using DNA barcodes discovering and describing one new genus and 11 new species (Dixon and Saunders 2013).

Our discovery indicates that the species diversity of the Rhodogorgonales is not yet completely known. This assertion is indirectly corroborated by work done by Bittner et al. (2011). They included an unnamed species of *Renouxia* and an unnamed species of *Rhodogorgon* as outgroups in their study of the Corallinales. The *cox1* sequences of their samples from New Caledonia are related to, but quite divergent from those of the described species *Renouxia antillana* and *Rhodogorgon ramosissima* from the Caribbean Sea, suggesting that there is additional species diversity to be discovered in both of the macroscopic genera as well.

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Table 1. Sequences used in the phylogenetic analyses.

	18S	28S	cox1	rbcL
<i>Heydrichia woelkerlingii</i>	GenBank: U61253 strain: HWU61253 length: 1758 nt			GenBank: KM369120 strain: WELT.A029119 length: 1419 nt
<i>Renouxia antillana</i>				GenBank: U04181 strain: n/a length: 1419 nt
<i>Renouxia</i> sp. HV508	GenBank: EF033584 strain: HV508 length: 1901 nt	GenBank: EF033601 strain: HV508 length: 2917 nt	GenBank: KC130151 strain: HV508A length: 660 nt	GenBank: KC134345 strain: HV508A length: 1358 nt
<i>Rhodenigma contortum</i>	GenBank: KU762333 strain: JAW4846 length: 1916 nt	GenBank: KU762334 strain: JAW4846 length: 2946 nt	GenBank: KU762335 strain: JAW4846 length: 660 nt	GenBank: KU762336 strain: JAW4846 length: 1419 nt
<i>Rhodogorgon carriebowensis</i>	GenBank: AF006089 strain: n/a length: 1841 nt	GenBank: AF419142 strain: n/a length: 2968 nt		GenBank: U04183 strain: n/a length: 1419 nt
<i>Rhodogorgon ramosissima</i>		GenBank: EF033602 strain: HV434 length: 2985 nt	GenBank: KC130149 strain: HV434 length: 660 nt	GenBank: KC134341 strain: HV434 length: 1358 nt
<i>Sporolithon</i> spp.	GenBank: KM369010 strain: LTB21140 length: 1700 nt	GenBank: HQ421906 strain: ARS02349 length: 657 nt	GenBank: KF186230 strain: n/a length: 660 nt	GenBank: KM369121 strain: LTB21140 length: 1337 nt

FIGURE LEGENDS

Fig. 1. Phylogenetic tree resulting from BEAST analysis of the four-gene dataset, with the new genus *Rhodenigma* highlighted. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values.

Fig. 2. A. Habit of free filaments partially growing unattached with contorted branch tips and partially attached to glass forming flattened adherent irregular crusts on glass (arrowheads). Scale bar is 50 μm . Inset shows straight filaments with branching at 2nd-3rd subapical cell. Scale bar is 30 μm . **B.** Free growing unattached filament tips and sub-apical branches aggregating to form a nodule (arrowhead). Scale bar is 15 μm . **C.** Flat adherent crust with unattached emergent branching filament tips growing downward to contact substrate (arrowheads). Scale bar is 15 μm .

Fig. 3. A. Unattached filaments with many randomly oriented filaments and some parallel filaments (bar). Scale bar is 25 μm . **B.** Filaments attached to glass, branching irregularly and forming lateral adhesion to other filaments. Some branch tips making contact with adjacent filaments and possibly fusing (arrowhead). Scale bar is 5 μm . **C.** Formalin-fixed and aniline-blue-stained specimen. Crust adherent on glass showing veins of single or multiple filaments with narrow elongate cells and lateral complex of shorter more irregular cells and branches. Scale bar is 10 μm . Inset shows faintly visible pit connections (arrowheads) between some cells. Scale bar is 4 μm .

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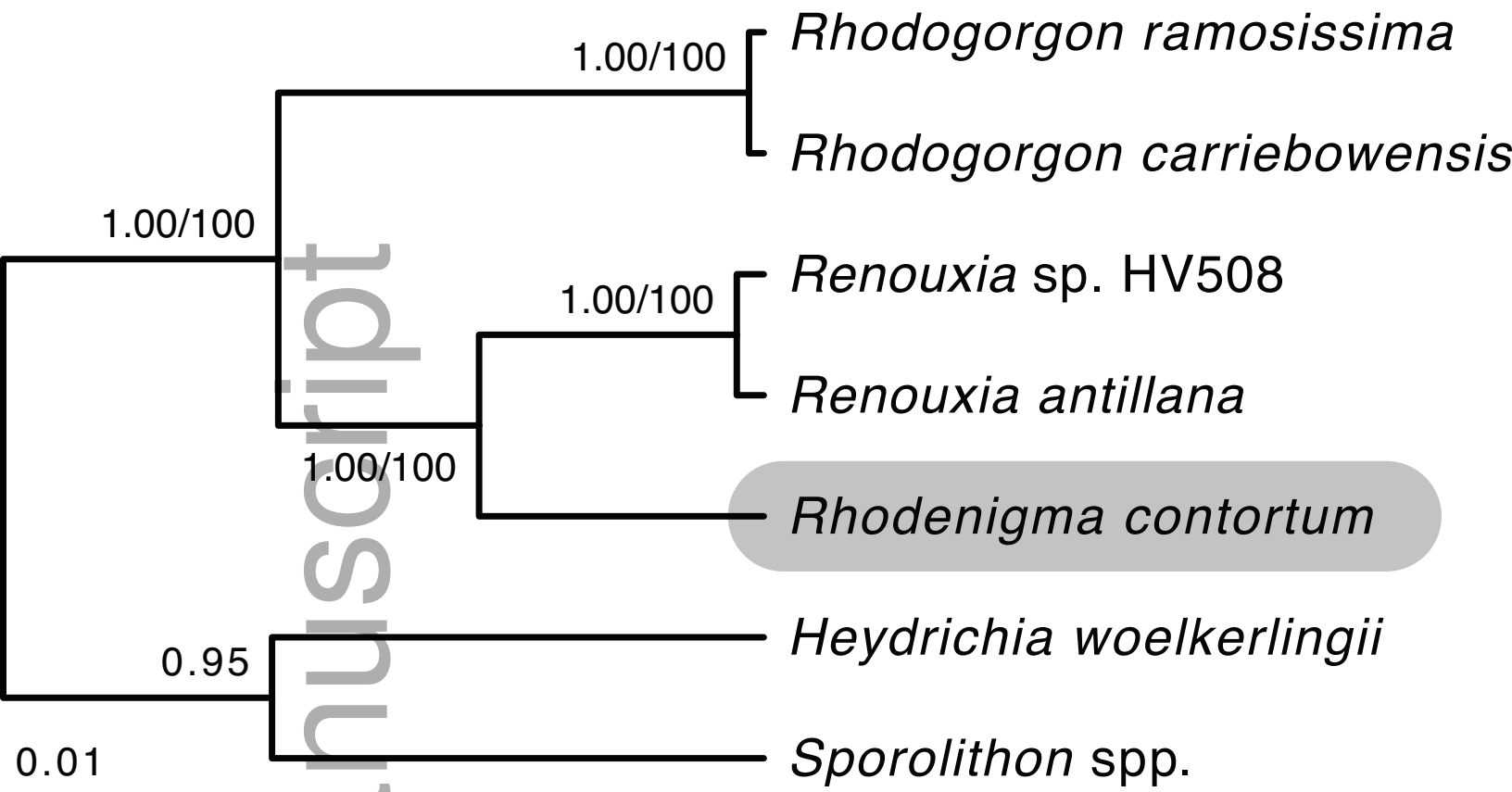
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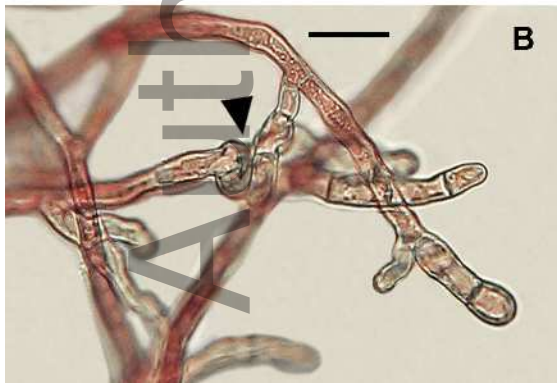
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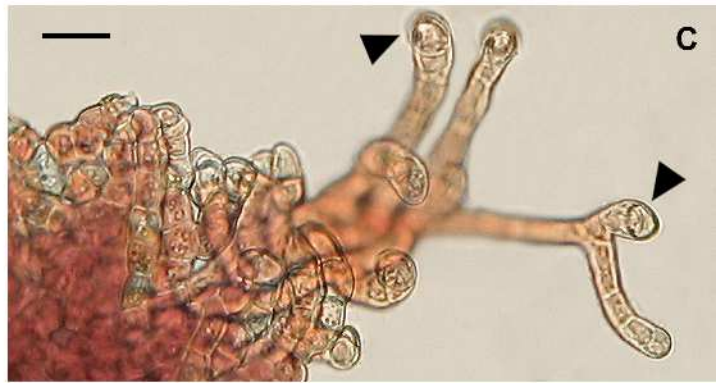
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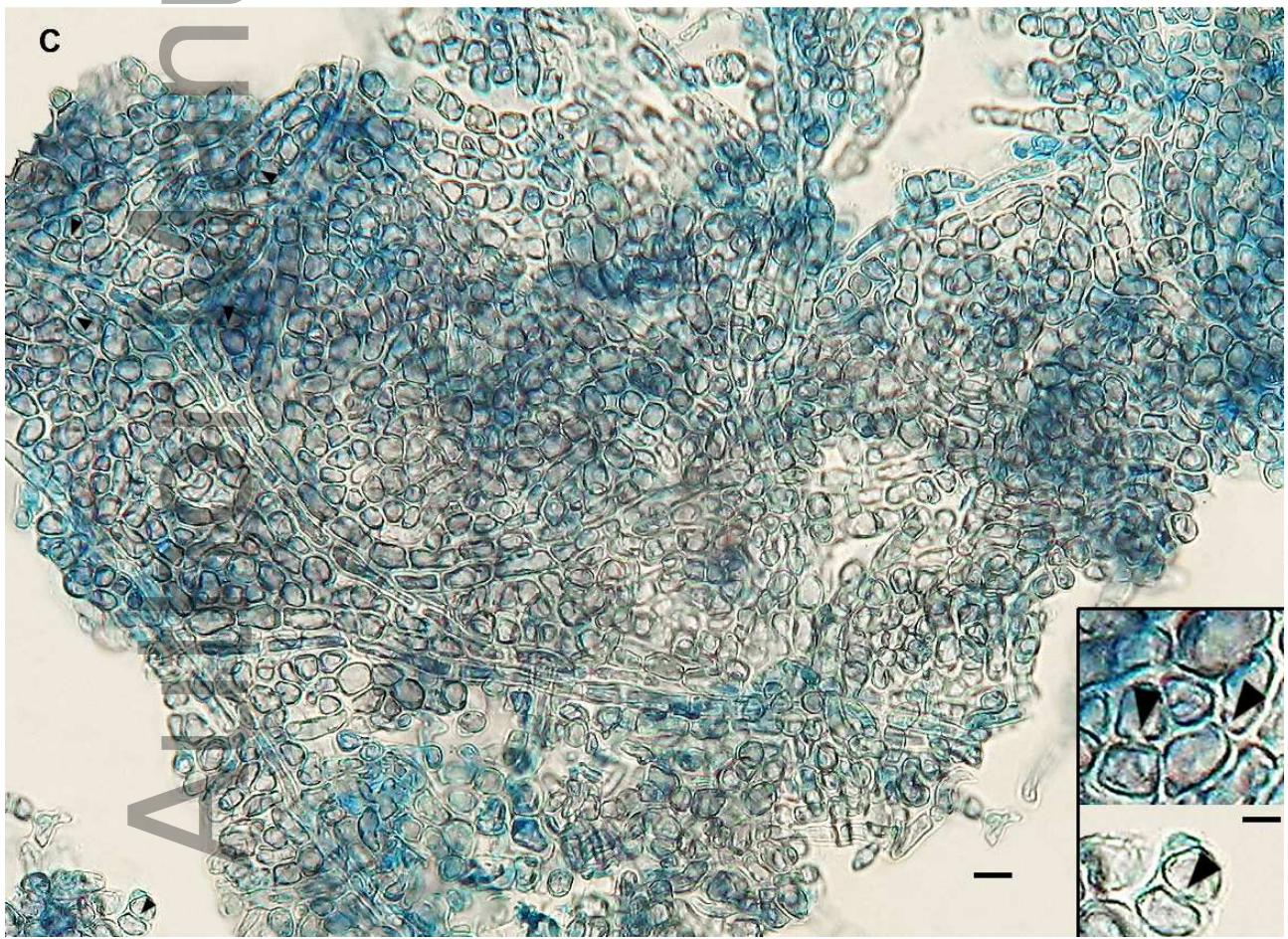
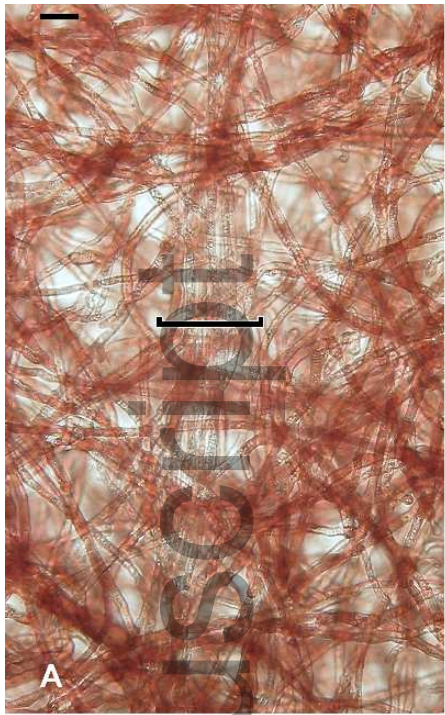


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