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Nunduva, a new marine genus of Rivulariaceae (Nostocales, Cyanobacteria) from marine rocky shores

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Abstract: Several populations of a non-tapering and tapering, fasciculated, single and geminate false branching heterocytous cyanobacterium were collected from rocky shores in the Pacific Ocean and Gulf of Mexico. The populations were provisionally placed in *Brasilonema* based on morphology, but upon sequencing of both environmental and culture material it was discovered that the populations/cultures belonged to the Rivulariaceae, in a marine subclade of the family containing *Kyrtuthrix huatulcensis*. In culture, the taxon exhibited tapering in isopolar filaments, providing further evidence that it was a member of the rivulariacean clade. Based on molecular data for other cyanobacteria within the rivulariacean clade, we identified at least three more species morphologically distinguishable from the *Brasilonema*-like material, all of which show more pronounced tapering. These cyanobacteria include not only tropical marine strains, but also a strain isolated from the English coastline in the Atlantic Ocean. We propose a new genus and four species for members of this distinctive clade, *Nunduva fasciculata* gen. nov., sp. nov., *N. kania* sp. nov., *N. biania* sp. nov., and *N. britannica* sp. nov. Other strains that others and we have isolated are sister to *Nunduva* and may eventually be placed within this genus, but at present, we consider the evidence for inclusion in *Nunduva* to be insufficient.

Key words: 16S rRNA gene, 16S–23S ITS region, *Calothrix*, geminate branching, intertidal, isopolar growth, marine, Nostocales, Rivulariaceae, Gulf of México, Pacific, Atlantic, European

INTRODUCTION

Despite the large number of papers that have been published in recent years, the taxonomic diversity of cyanobacteria in tropical environments is still poorly known (NABOUT et al. 2013; HAUER et al. 2013). Several special morphotypes representing new species and genera of cyanobacteria have recently been described in tropical and extreme habitats (KOMÁREK & KOMÁRKOVÁ 2007; GAMMA et al. 2014; GOLD–MORGAN et al. 2015; ALVARENGA et al. 2016; HENTSCHKE et al. 2016; MISCOE et al. 2016). The polyphasic approach, which combines morphological and molecular methods, allows greater precision and recognition of taxonomic diversity, as well as a means of revision of the hierarchical classification

of some cyanobacterial taxa to better reflect their evolutionary history (FIORE et al. 2007; HAUER et al. 2014; GENUARIO et al. 2015). Phylogenetic analyses based on molecular markers, especially the 16S rRNA gene, have been the basis for the reorganization of some traditional cyanobacterial families (GEITLER 1932; HAUER et al. 2014; KOMÁREK et al. 2014).

Based on molecular phylogenetic analyses, numerous morphologically well-defined genera have been found to be polyphyletic e.g., *Anabaena*, *Nostoc*, *Tolypothrix*, and *Calothrix* among others (RAJANIEMI et al. 2005; HAUER et al. 2014; KOMÁREK et al. 2014; GENUARIO et al. 2015; BERRENDERO et al. 2016). This problem can only be corrected through a careful taxonomic revision that can result in the description of new genera, or the

unification of different generic entities.

The family Rivulariaceae is comprised presently of 10 genera (*Rivularia*, *Calothrix*, *Dichothrix*, *Gardnerula*, *Isactis*, *Sacconema*, *Microchaete*, *Phyllonema*, *Macrochaete* and *Kyrtuthrix* – see KOMÁREK et al. 2014; ALVARENGA et al. 2016; BERRENDERO et al. 2016; LEÓN–TEJERA et al. 2016). The family includes mainly heteropolar forms with tapered trichomes, which are widest at the base and sometimes end in a hyaline hair with thin, elongated cells, possess an obligately basal heterocyte in mature trichomes, and reproduce by hormogonia (BERRENDERO et al. 2016). Recently *Kyrtuthrix* was placed in the Rivulariaceae, even though it has isopolar trichomes and no basal heterocytes. However, it does possess an attenuated, heteropolar, differentiated growth form (LEÓN–TEJERA et al. 2016). The Scytonemataceae and Symphyonemataceae are similar to *Kyrtuthrix* in that they have tapering isopolar trichomes, but they are widest in the middle of the trichome and are phylogenetically distant (KOMÁREK 2013). These findings show that the traditional morphological concept of the Rivulariaceae has been expanded, but still does not include all tapering heterocytous genera.

During studies of marine cyanobacteria from supra and intertidal fringes of tropical regions of the Mexican coast, we have found that most of the benthic rocky shore mats are dominated by heterocytous cyanobacterial morphotypes (GONZÁLEZ–RESENDIZ et al. 2015). Frequently, a special morphotype was found, resembling *Brasilonema* in the Scytonemataceae in filament morphology, intercalary heterocytes, fasciculated filaments and scarce geminate or single branching. However, our populations differ from *Brasilonema* in ecology and morphology in various characters such as the shape of the apical cell, as well as the development in culture of isopolar and heteropolar hormogonia and markedly tapering trichomes and formation of a long hair not seen in natural material. In addition to the *Brasilonema*-like populations, mucilaginous populations with isopolar tapering trichomes similar to *Scytonematopsis* were also found, isolated, and sequenced as part of our ongoing interest in the cyanobacteria of tropical rocky shores. Through phylogenetic analysis, we found a number of other marine tapering taxa sequenced by others, which apparently shared a close relationship with our tapering and nontapering taxa.

The aim of this study was to characterize these populations, employing a polyphasic approach, incorporating morphological, ecological and molecular information.

MATERIALS AND METHODS

Samples were collected in supratidal and intertidal fringes from San Agustín Bay (15°41'17.41"N, 96°14'15.28"W), Tangolunda bay (15°46'24.79"N, 96°5'28.32"W) and Playa Hermosa Beach (18°40'03.8"N, 95°07'47.7"W) in the Mexican coasts.

Twelve samples were analyzed, eight from San Agustín Bay, three from Tangolunda Bay, and one from Playa Hermosa

Beach. Each sample was divided into three parts: one dried subsample for culturing, one part fixed in formalin, and one part dried in silica gel.

Morphological characterization was performed on formalin-preserved samples of different populations. Semi-permanent slides were prepared and micrographs were acquired with an Olympus DP12 digital camera adapted to an Olympus BX51 microscope. Morphological measurements (n=30) were obtained from micrographs using SigmaScan Pro© automated image analysis software (Jandel Scientific, Sausalito, California). A separate strain, long in culture, was obtained from the CCAP culture collection (*Microchaete grisea* CCAP 1445/1), characterized in the light microscope, and sequenced as part of another study (HAUER et al. 2014). This phylogenetically close strain was added to this study when we realized it belonged to the same clade as our other strains.

Transmission electron microscopy (TEM) images of field material were obtained in a JEOL mod JEM 1010 microscope after fixation in 6% glutaraldehyde in 0.1 M PBS and subsequent post-fixation in 1% osmium tetroxide, dehydration in ethanol series and embedding in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to REYNOLDS (1963).

Morphological identification was done in accordance with recent and traditional reference works (GEITLER 1932; FRÉMY 1934; KOMÁREK 2013), and several papers that report tropical and subtropical rocky shore taxa (SANT'ANNA et al. 1985; SANT'ANNA 1988; SANT'ANNA 1997; LEÓN–TEJERA et al. 2005). Systematic assignment was made according to the system of KOMÁREK et al. (2014).

Material isolation and strains. Dry samples were rehydrated with sterile seawater on a microscopy slide and filaments were isolated through serial dilutions in drops of seawater. Reduction or elimination of sheath was obtained by washing for one hour with Extran® 1%, (in order to get rid of epiphytes), then afterwards rinsed four or five times with liquid SN medium (WATERBURY et al. 1986), followed by an additional two or three rinses with semi-solid SN medium (to eliminate sheath and epiphyte remnants). The seawater was filtered with 0.45 and 0.22 µm Millipore® membranes and sterilized. Strains C58–M5–K83, C458–M16–K66, C694–M8–K148, and C708–M10–K85, were isolated from enrichment media cultures in Petri plates with solid agar SN, the plates were incubated at 25 °C in a 12/12h, light/dark regime in a culture chamber. Additional strains of marine Rivulariaceae were isolated following the same protocols (C1088–M21–K118, C1230–M22–K135, C708–M3–KA), and additional environmental materials characterized and sequenced (but not isolated) include C57–M6, C57–M7, C923–M12, C1290–M9).

Molecular methods and phylogenetic analyses. DNA was extracted from cultures and environmental samples, using the UltraClean® Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. The 16S rRNA gene and 16S–23S ITS region, were amplified using primers 27F after NEILAN et al. (1997), and VRF1 after WILMOTTE et al. (1993). The PCR amplification was performed with an Eppendorf Mastercycler following the conditions described by LEÓN–TEJERA et al. (2016). PCR products were gel purified, quantified, and cloned with the CloneJET PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA). One or two clones with verified long PCR products were then chosen for each strain for sequencing at the Biotechnology Institute, UNAM sequencing facility using an Applied Biosystems (Foster

City, CA) model 3130xl Genetic Analyzer. We assembled and corrected sequences obtained using Bioedit software version 7.0.9.0 (HALL 1999). The GenBank accession numbers for consensus sequences (for those samples with multiple sequences) of *Nunduva* and closely related taxa are HE797730, KT936560–72, and AM230677–78.

A total of 260 sequences were chosen for the phylogenetic analysis, including the four consensus sequences obtained in this study as well as representatives of the main groups of heterocytous cyanobacteria available in GenBank, and three outgroup taxa (*Chroococcidiopsis* 9E–07 FR798923, *Chroococcidiopsis cubana* SAG39.79 AJ344558 and *Chroococcidiopsis thermalis* AB039005). All sequences were aligned using default parameters in CLUSTAL W Multiple Sequence Alignment Program (THOMPSON et al. 1994) and manually edited in PhyDE® version 0.9971 (MÜLLER et al. 2010).

Phylogenetic relationships were inferred with maximum parsimony analysis (MP) and maximum likelihood analysis (PhyML) in Seaview v4 (GOUY et al. 2010; GUINDON et al. 2010). Bootstrapping with 1000 replicates was conducted for both analyses. Bayesian analysis (BA) was run in MrBayes 3.2.2 (Ronquist et al. 2012), using two independent runs with eight chains each, for ten million generations with default parameters and first 25% of sampled trees were discarded as burn-in. The evolutionary model GTR+I+ Γ was determined based on the Maximum Likelihood (ML) ratio test implemented by TOPALi version 2 software (MILNE et al. 2009). The BA had a mean estimated sample size (ESS) exceeding 2000 for all parameters (ranging 2,655–7369), well above the average of 200 typically accepted as sufficient by phylogeneticists (DRUMMOND et al. 2006). The final average standard deviation of split frequencies was <0.038. The potential scale reduction factor (PSRF) value for all the estimated parameters in the Bayesian analysis was 1.00, indicating that convergence of the MCMC chains was statistically achieved (GELMAN & RUBIN 1992). The range of 16S rRNA gene divergence values within and among species was calculated using uncorrected p-distance in Mega V6.

Secondary structure of ITS region. The secondary structures of the 16S–23S ITS region were determined using a combination of comparative analysis of the secondary structures of our strains, with additional related taxa from the 16S rRNA phylogeny. In addition to *Nunduva* species, we selected representatives of close related clades *Kyrtuthrix huatulcensis* and “*Rivularia*” PCC 7116 (phylogenetically not in *Rivularia sensu stricto*). The construction of secondary structures of D1–D1', Box–B, V2 and V3 helices were determined separately using Mfold version 2.3 (ZUKER 2003), and illustrated and corrected in Adobe Illustrator.

RESULTS

We obtained ten consensus sequences of the 16S rRNA gene with their associated 16S–23S ITS region (2037 to 2090 bp) for cultured strains and environmental isolates of a new genus within the Rivulariaceae. Morphological characterization of this taxon was previously reported in GONZÁLEZ–RESENDIZ et al. (2015), but is named and formally described here.

Nunduva GONZÁLEZ–RESENDIZ, LEÓN–TEJERA et JOHANSEN gen. nov. (Figs 1A–11O)

Diagnosis: Differing from all other genera in the Rivulariaceae in that trichomes possess geminate and single false branching. Phylogenetically distant from all Scytonemataceae and *Calothrix* spp. from freshwater, soil, and thermal habitats (Fig. 10).

Description: Colonies caespitose on rocks in the marine habitat, formed by filaments arranged in erect fascicles, with heteropolar appearance achieved after basal fragmentation. Sheaths lamellate, thin or wide, becoming diffuent at the end, colorless or yellow–brown, and roundly closed or open at the end. Trichomes are untapered or distinctly tapered at some phase of life–cycle and/or in culture material, sometimes developing a hyaline or semi–hyaline hair, vacuolated or not; with geminate and single false branches, mainly in basal parts of filaments. Trichomes constricted or not at cross walls, in both environmental samples and strains. Cells of variable length and shape, shorter than wide to almost isodiametric in environmental and culture material. Heterocytes are basal or intercalary, solitary or serial (–3), of variable length and shape. Hormogonia, isopolar or heteropolar, produced through trichome fragmentation, with or without necridic cells, cell constriction or heterocyte breakup. Akinetes unobserved.

Etymology: *Nunduva* (Mixtec) = Oaxaca. Mixtec is the language spoken by one of the ethnic groups of Oaxaca, a State located in Southwestern Mexico where some these populations are found.

Type species: *Nunduva fasciculata* GONZÁLEZ–RESENDIZ, LEÓN–TEJERA et JOHANSEN sp. nov.

Nunduva fasciculata GONZÁLEZ–RESENDIZ, LEÓN–TEJERA et JOHANSEN sp. nov. (Figs 1A–5I)

Description: Thallus caespitose, composed of blackish–green erect filaments densely fasciculate (Figs 1A–1B, 3A), 2–3 cm high, filaments with rare geminate false branching, 9–15 μ m wide (Fig. 1C). Sheaths firm, cylindrical, stratified, yellowish brown (Figs 1C, 1G–1H, 2B, 4A) Trichomes cylindrical, constricted or not at cross–walls, 6–10 μ m wide (Figs 1H, 1J, 1K); slightly tapered towards the ends as well as in hormogonia (Figs 2K, 4A); apical cell colorless, distinctively short and spherical–rounded, bead–like (Figs 1D–1G, 5D–5E). Cells with blue–green to olive–green granulated content (Fig. 1K), isodiametric in old filaments, 5 to 8 μ m long, shorter than wide in apical zones, 2–3 μ m long 5.6–7 wide. Heterocytes intercalary, elongated, flattened, discoid or cylindrical 8–15 μ m long, up to 2 to 3 times longer than wide, 4.5–6.7 μ m long, 1.8–4.8 μ m wide, rarely three short heterocytes contiguous or near one another (Figs 1H–J, 2A–2B). Reproduction by isopolar hormogonia attached to filaments, straight or more frequently crescent–shaped (Figs 2G–2J, 3B, 3D–3F, 4C, 5C, 5G, 5I). Hormogonia, produced by necridic cell or heterocytes formation (Figs

2B–2G, 5H) Akinetes not observed in environmental or culture conditions. Transmission electron microscopy (TEM) images obtained from *Nunduva fasciculata* field material show thylakoids disposed in packets (Fig. 4B) with isopolar hormogonia with bead-like terminal cell (Fig. 4C).

Habitat: Supratidal and intertidal fringe; epilithic on granitic rock.

Type locality: MEXICO. Oaxaca: San Agustín Bay, 15°41'17.41"N, 96°14'15.28"W, October 2012. C694 collected by L. González-Resendiz & H. León-Tejera.

Holotype here designated: Dry material FCME–C694 from environmental sample deposited at Herbarium of Faculty of Sciences, Universidad Nacional Autónoma de México, (UNAM), Ciudad de México. Figures 1D–1F; 1H–1K; 2B; 2F–2I; 2K–5L.

Isotypes: FCME–C57, FCME–C58 (environmental samples) and dry material of FCME strain C694–M8–K148. Herbarium of Faculty of Sciences, Universidad Nacional Autónoma de México, UNAM. México.

Paratypes: FCME–C36, –C645, –C719, –C737, –C1313, –C1288, –C1289 and –C1290 (environmental samples). Herbarium of Faculty of Sciences, Universidad Nacional Autónoma de México, UNAM. México.

Reference strain: FCME C694–M8–K148.

Etymology: *fasciculata* (Latin), referring to the fasciculate growth form.

Notes: Some of the important features in populations from environmental material changed in culture, while other traits remained constant. The characteristic smaller rounded apical cell was lost in culture and replaced by a tapering trichome with a long pigmented hair. Environmental material rarely had geminate false branching, but false branching increased markedly in culture material. In young colonies (30 days after the inoculation) populations show mixed characteristics concerning the differentiation of terminal cell and tapering of the trichomes. In old cultures (24 months), the fasciculate growth form did not change and heteropolar hormogonia are more abundant than the isopolar ones (Figs 3A–3H).

***Nunduva kania* JOHANSEN, GONZÁLEZ–RESENDIZ et LEÓN–TEJERA sp. nov. (Figs 6A–6J)**

Description: Thallus filamentous, heteropolar and isopolar (6B, 6D, 6F–6H), with frequent geminate false branching (6A, 6D, 6F). Sheaths diffuent, lamellate, colorless, open at the end. Trichomes constricted at cross walls, 1.7–2.6 µm long, 7.5–9 µm wide, tapered to form long hair composed by semi-hyaline and “vacuolated” cells up to 1–1.5 µm wide (6A, 6E–6F). Cells with olive-green granulated content, 2 to 5 µm long, wider than long; in the apical zone cells are longer than wide, 1.7–2.8 µm long, 1.2–1.5 µm wide. Heterocytes basal and no more than one, conical or hemispherical in shape (4.9–5.4 µm wide 2.5–2.6 µm long) (6F–6H). Reproduction by heteropolar and isopolar hormogonia, produced by

separation of branches and by deep cell constriction without necridic cell (Figs 6B–6D, 6E, 6H–6J arrows). Akinetes not observed in culture conditions.

Habitat: Intertidal fringe; epilithic on basaltic rock.

Type locality: MEXICO. Veracruz: Playa Hermosa, 18°40'03.8"N, 95°07'47.7"W, April 2012. C458 Collected by H. León-Tejera & L. González-Resendiz.

Holotype here designated: Dry material of FCME strain C458–M16–K66 deposited at Herbarium of Faculty of Sciences, Universidad Nacional Autónoma de México, (UNAM), Ciudad de México. Figures 6A–6J.

Reference strain: FCME C458–M16–K66.

Etymology: *kani* (Mixtec) = “Large”, due to long hair.

Notes: The description is based on material cultivated in SN medium. The long hair and basal heterocyte are the main morphological traits that differentiate this species from *N. fasciculata*, *N. biania* and *N. britannica*. The heteropolar trichome developed by hormogonia is released by constriction of cells from the mature filament; the isopolar trichomes develop from branch separation. Apical ends may differentiate, one becomes slightly wider and produces a single terminal heterocyte, and the other extreme cells become elongated, tapering, forming a long hair or sometimes hormogonia.

***Nunduva biania* LEÓN–TEJERA, GONZÁLEZ–RESENDIZ et JOHANSEN sp. nov. (7A–8O)**

Description: Thallus caespitose, composed of yellowish-brown erect heteropolar filaments, 3–8 µm wide (Fig. 7A), with rare single false branching. Sheaths diffuent, lamellate, colorless to yellowish, and flared at the apex (Figs 7B–7D, 8B, 8L–8O arrows). Trichomes cylindrical, constricted or not at cross-walls, 2–7 µm wide, slightly swollen at base and tapered without hair (Figs 7A, 7D–7E, 7H, 8A, 8–8F, 8L–8O). Cells shorter than wide, with olive-green granulated content, 1–2.7 µm long in basal zone; 0.5–0.8 µm long in apical zone. Heterocytes intercalary or basal, elongated, discoid or cylindrical, 4–4.5 µm, long 4–6 µm wide, rarely two short heterocytes contiguous or near one another (Figs 7B–7C, 7F–7G, 8D arrow). Reproduction by isopolar hormogonia that may remain temporarily within the sheaths (Fig. 7H), or heteropolar hormogonia produced by cell constriction, or heterocyte formation and by separation of branches (Figs 8F, 8L, 8J, 8O arrows). Akinetes not observed in environmental or culture conditions.

Habitat: Intertidal fringe; epilithic on granitic rock

Type locality: MEXICO. Oaxaca: San Agustín Bay, 15°41'17.41"N, 96°14'15.28"W, October 2012. C708 Collected by L. González-Resendiz & H. León-Tejera.

Holotype here designated: Dry material of FCME strain C708–M–10–K85 deposited at Herbarium of Faculty of Sciences, Universidad Nacional Autónoma de México, (UNAM), Ciudad de México. Figures: 8A–8O.

Isotype: FCME–C708 (environmental sample).



Fig. 1. *Nunduva fasciculata* environmental populations: (A–B) filaments disposed in fascicles; (C) geminate branching; (D–G) bead-like apical cell development in slightly attenuated trichomes; (H–J) variable dimensions and disposition of heterocytes: single or in series; (J–K) variable cell dimensions. Scale bars 30 μm (A–B), 20 μm (C), 10 μm (D–K).

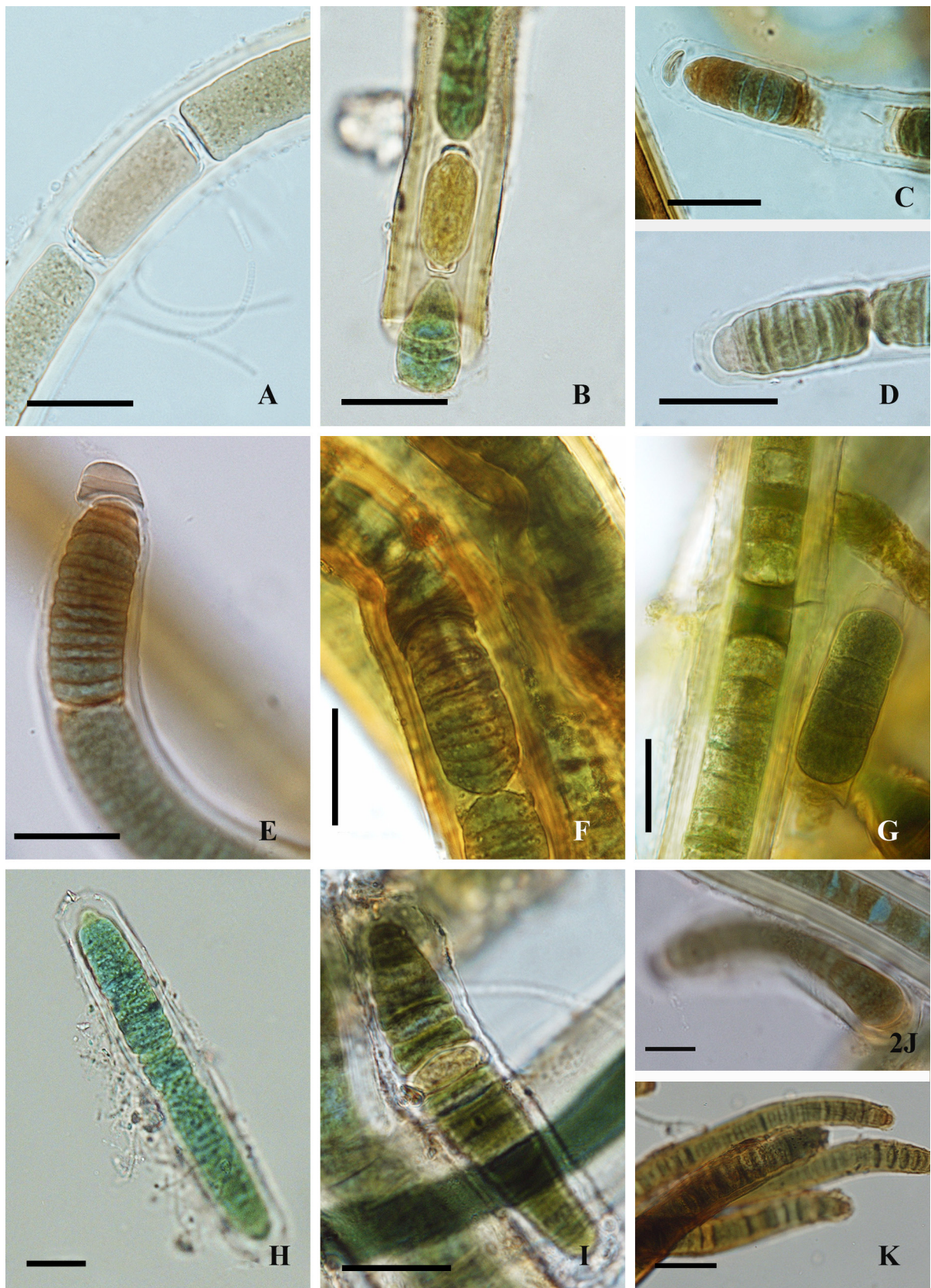


Fig. 2. *Nunduva fasciculata* environmental populations: (A–B) wide sheath, hyaline or pigmented; (C–E) stages of terminal hormogonia development; (F) non-terminal hormogonia development; (G–I) different stages of isopolar hormogonial development, some with slightly attenuated terminal part; (J) heteropolar curved hormogonia; (K) filaments arranged in fascicles. Scale bars 10 μm (A–J), 20 μm (K).

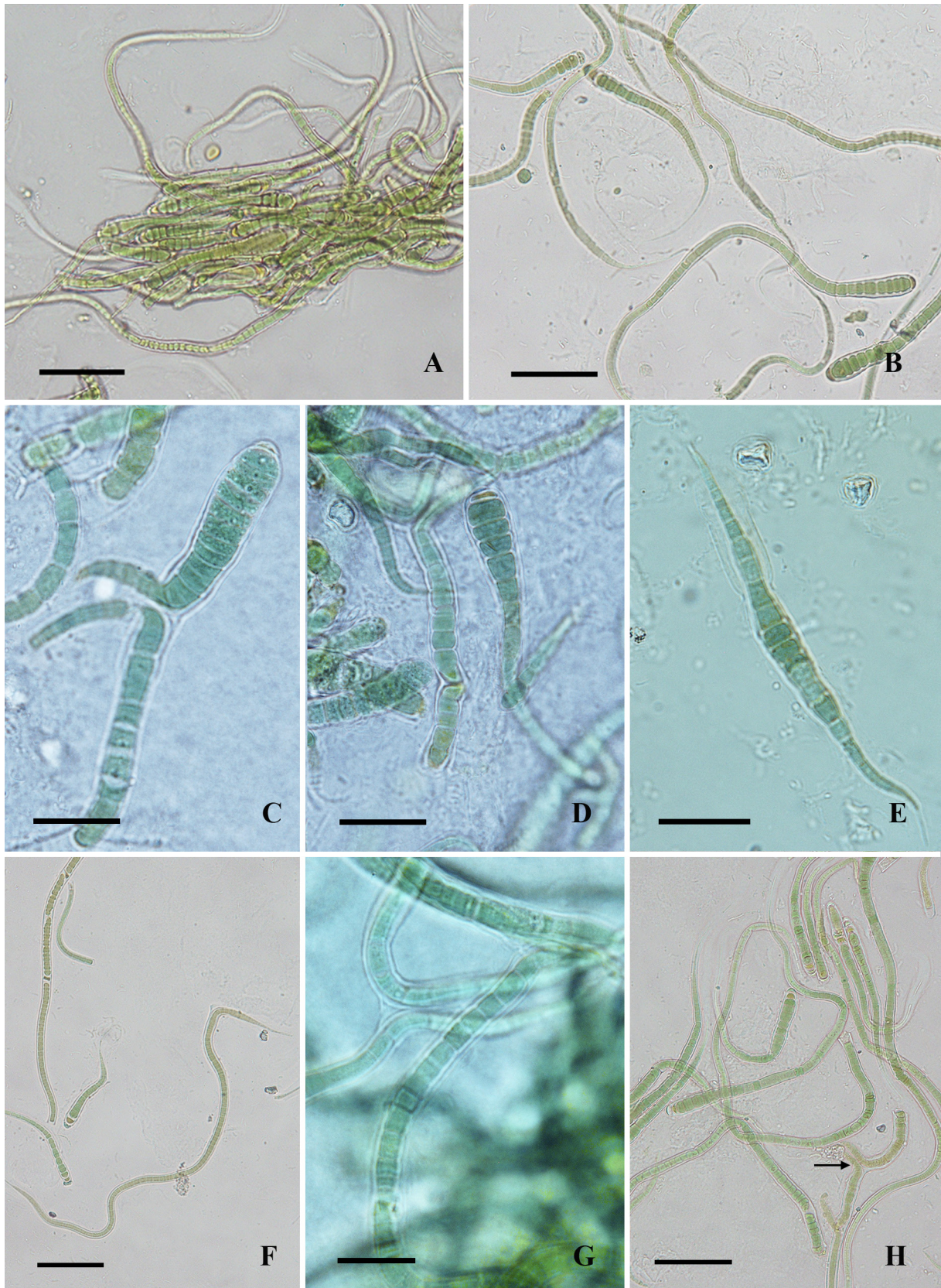


Fig. 3. *Nunduva fasciculata* culture populations: (A) filaments arranged in fascicles; (B, D) heteropolar development of hormogonia into non-mature filaments; (C) geminate branching of hormogonia; (E) isopolar development of hormogonia; (F) isopolar mature filaments; (G–H) mature filaments with geminate branches. Scale bars 30 μm (A, B, F, H), 10 μm (C–E, G).

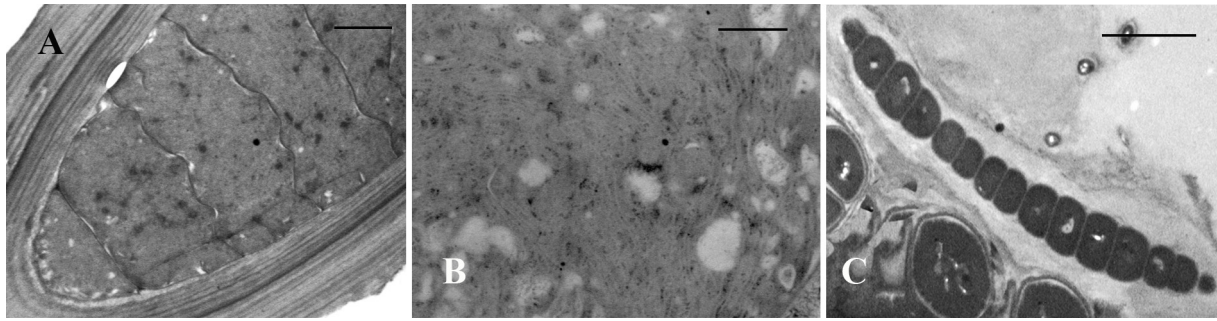


Fig. 4. *Nunduva fasciculata*, TEM micrographs of environmental populations: (A) stratified sheath of terminally attenuated filament; (B) thylakoids arranged in irregular packets; (C) isopolar hormogonia with thickened sheath. Scale bars 3 μm (A), 1 μm (B), 4 μm (C).

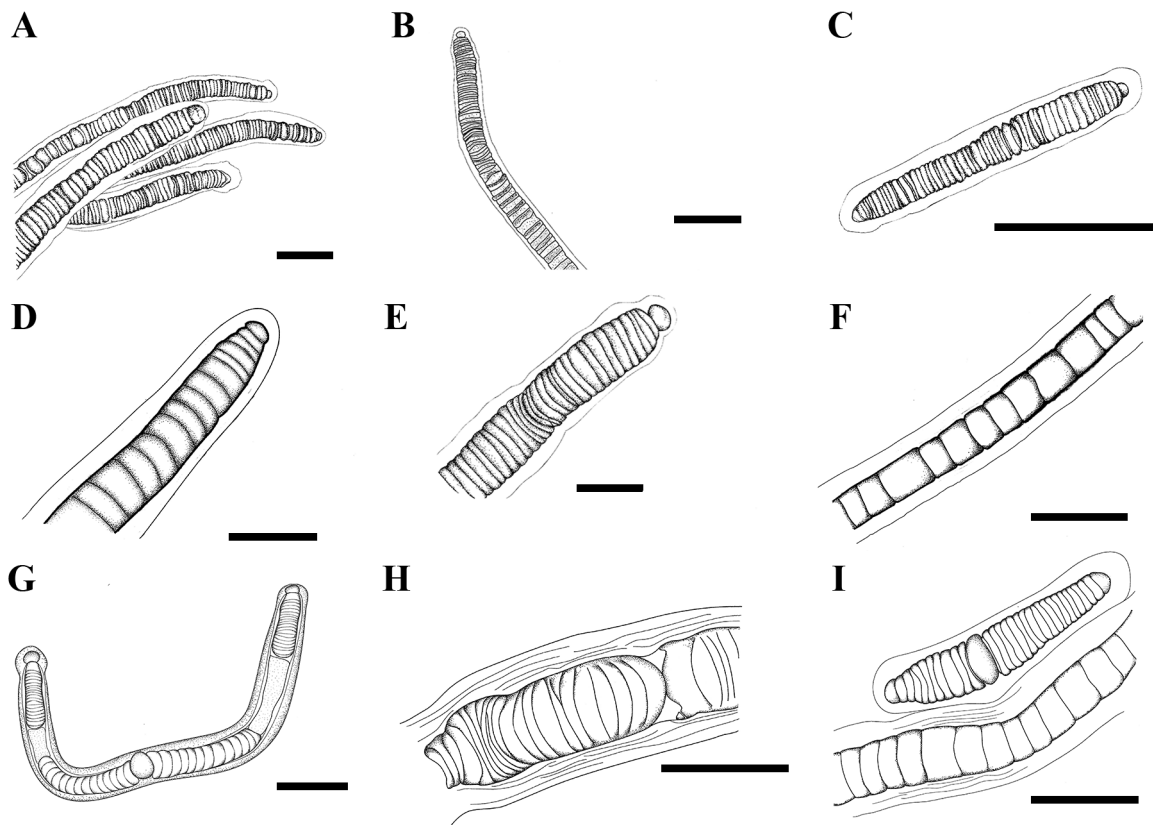


Fig. 5. *Nunduva fasciculata*, drawings of environmental populations: (A) filaments arranged in fascicles; (B–E) bead-like apical cell development in slightly attenuated trichomes; (F) trichome with variable cell dimensions; (G) isopolar curved hormogonia with enlarged heterocytes; (H) non-terminal hormogonial development; (I) isopolar hormogonia adherent to mature filament. Scale bars 20 μm (A, B, G), 10 μm (C–F, H–I).

Reference strain: FCME C708–M10–K85.

Etymology: *biani* (Zapotec) = “lamp” referring to the apical form of sheath in culture that seems as oil lamp

Notes: The heteropolar growth form does not change in culture and environmental conditions. In culture, sheath is straight to undulate, thin to sometimes prominent, stratified, becoming diffuent and opens to form a flared “oil lamp” shape at the apex. Environmental material does not have geminate false branching, but in culture simple or geminate branches are common. In culture cells are wider than long, with diameter more or less

homogeneous, variously constricted, becoming attenuated and longer towards terminal parts. Intercalary or basal heterocytes are few and small in SN media with supplied nitrogen. Cytoplasm with small to big granules. Hormogonia isopolar, developing mainly intercalarly (Figs 8H–8I, 8O), those heteropolar upon release are terminal, strongly tapered, and proximal to the widened lamp-shaped end of the sheath (Figs 8B, 8M–8O).

***Nunduva britannica* Hauer sp. nov. (Figs 9A–K)**

Description: Young cultures with heteropolar filaments, straight or wavy, taper towards the end, 5–11 μm wide,

with thin, colorless unstratified sheath, with geminate false branching (Fig. 9A). Trichomes 5–9 µm wide, clearly constricted at cross walls, swollen at basal part, without a terminal hair (Figs 9B–9D). Cells distinctly shorter than wide. Heterocytes basal, sometimes in pairs, hemispherical or trapezoid, up to 10 µm wide. Reproduction by trichome disintegration. Old cultures with heteropolar or isopolar filaments (Fig. 9G), straight or wavy, not tapering towards the end, 5–13 µm wide, with colorless sheath, up to 4 µm thick, stratified, frayed when old (Figs 9E–9F, 9J). Trichomes 5–9 µm wide, width slightly varying along the length of trichome. Cells distinctly shorter than wide up to isodiametric, well developed terminal cell almost spherical (Figs 9H, 9I, 9G). Heterocytes basal and intercalary, cylindrical (Figs 9I–9J). Reproduction by isopolar hormogonia (Fig. 9K) and by trichome disintegration. Akinetes not observed.

Habitat: Brackish, estuarine.

Type locality: Newtown, Isle of Wright, England, United Kingdom. Collected by R. W. Butcher. Original collection date unspecified, likely in early 1960's.

Holotype here designated: Dry material CBFS A–081–1 is based on strain CCAP 1445/1 deposited at Herbarium of Faculty of Sciences, University of South Bohemia, České Budějovice, Czech Republic.

Reference strain: CCAP 1445/1.

Etymology: *britannica* (Latin), referring to the geographical area of the original sample from which strain CCAP 1445/1 was isolated.

Notes: There is no information concerning the morphology of this species in nature. It is very possible, even likely, that the morphology in culture differs from material in nature. The strain was originally identified as *Microchaete grisea*. It differs from *Microchaete grisea* by intercalary heterocytes, isodiametric cells, stratified and frayed sheath, and double and single false branching. Contrary to the original description of *M. grisea*, the trichomes in young cultures taper towards the end. In older, senescent cultures, the conical end cells and tapering disappear, and are replaced by rounded cells more similar to *M. grisea*, but the other differences between this strain and *M. grisea* provide evidence that the strain is not conspecific with this species or congeneric with *Microchaete*.

***Nunduva* species LEGE 07159 (KC989702)**

Description: This strain was originally placed in *Rivularia* by the isolators, who sequenced the majority of the 16S rRNA gene (1354 bp), but not the ITS region. It was isolated from the intertidal zone on the coast of Portugal, and so matches in ecology to other members of the genus. It is sister to *Nunduva biania*, although its position is poorly supported (Fig. 10). We have fairly high confidence that it belongs in the genus *Nunduva*, but without the ITS sequence, it is difficult to give it a specific epithet. We hope other workers will characterize this strain more

fully, as it is from a coastline environmentally unique from all other origin sites for the genus, and could very well represent another new species.

Phylogenetic analysis: The Bayesian analysis based on molecular sequences obtained in this study shows two major clades of tapering forms (Fig. 10). The *Calothrix* cluster contains three paraphyletic strains at the base of the clade and two subclades, one with mostly brackish strains, the other with mostly freshwater and soil strains. These two clades are bisected by the *Macrochaete* clade (Fig. 10), suggesting that once the brackish/marine species *C. confervicola* Agardh ex Bornet et Flahault (the type species of *Calothrix*) is sequenced, the subclade not containing the type will require reassignment to a new or existing genus. The other major clade contains the type species of *Rivularia* (*R. atra* Roth ex Bornet et Flahault), and has been consequently designated *Rivulariaceae sensu stricto*. *Rivulariaceae sensu stricto* has two major subclades, one a group of marine strains, and the second a group of brackish strains that includes the type for *Rivularia*. The *Rivulariaceae sensu stricto* has the recently described *Phyllonema* as a sister taxon to the whole group, and this comprises a third minor subclade in this family level cluster.

Nunduva belongs to the “marine *Rivulariaceae*”, a well defined and delimited marine cluster that includes strains assigned to *Calothrix* or *Rivularia*, and a number of uncertain genera referred to as Marine *Rivulariaceae* III–V. Since the type species for *Calothrix* and *Rivularia* fall outside of this clade, all of the strains so identified will need to be assigned to other existing or new genera. This cluster of marine, tapering, heterocytous forms is sister to the one formed by *Kyrtuthrix huatulcensis* and “*Rivularia*” from the Gulf of México (Fig. 10). The strains assigned to *Rivulariaceae* III–V were isolated by our research group and are morphologically diverse (very different from *Nunduva*). All strains/populations in this cluster are found in the supratidal and intertidal waters growing tightly adhered to rocks and battered by wave action; except for *Nunduva*, their taxonomic identity is uncertain at this time.

Examination of sequence identity of the *Rivulariaceae* from this study revealed that all taxa were highly similar in their 16S rRNA gene sequence (Table 1). *Nunduva* species were 98.6–100.0% similar to each other; as high as 98.6% similar to some other strains lacking the morphology of *Nunduva*, and above 97.6% similarity to all members of *Rivulariaceae sensu stricto* (compare Fig. 10 with Table 1).

16S–23S ITS region (Figs 11A–12L): A comparison of the sequence data derived for the ITS region of seven clones representing four populations of *Nunduva fasciculata* (generitype) showed very minor differences among sequences. These differences were too minor to come to a conclusion as to whether the differences were due to possession of multiple operons or just sequencing error. The p-distances among ITS regions of *Nunduva*

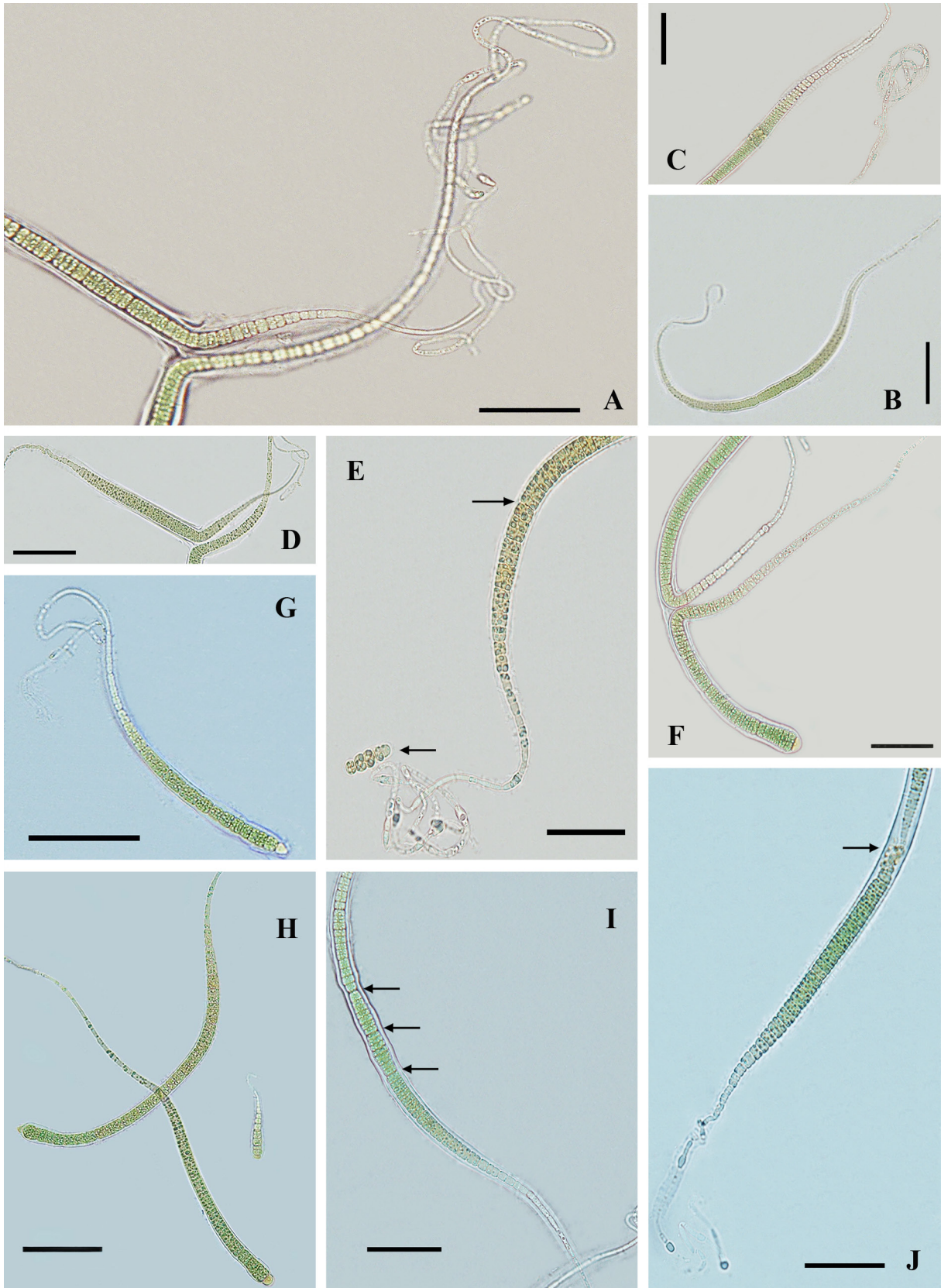


Fig. 6. *Nunduva kania*, culture population: (A, C–D) gemitate branch and long semi-hyaline hair; (B–D) isopolar filament formation; (G–H) heteropolar hormogonia development; (E) isopolar hormogonia (arrow), constriction (arrow), and semi-hyaline long hair; (G, E) heteropolar mature filament and basal heterocyte; (F) basal heterocyte and gemitate branching; (I) isopolar hormogonia formation (arrows); (J) apical heteropolar hormogonia formation. Scale bars 20 μm (A, C, E–F, I–J), 30 μm (B, D, G–H).

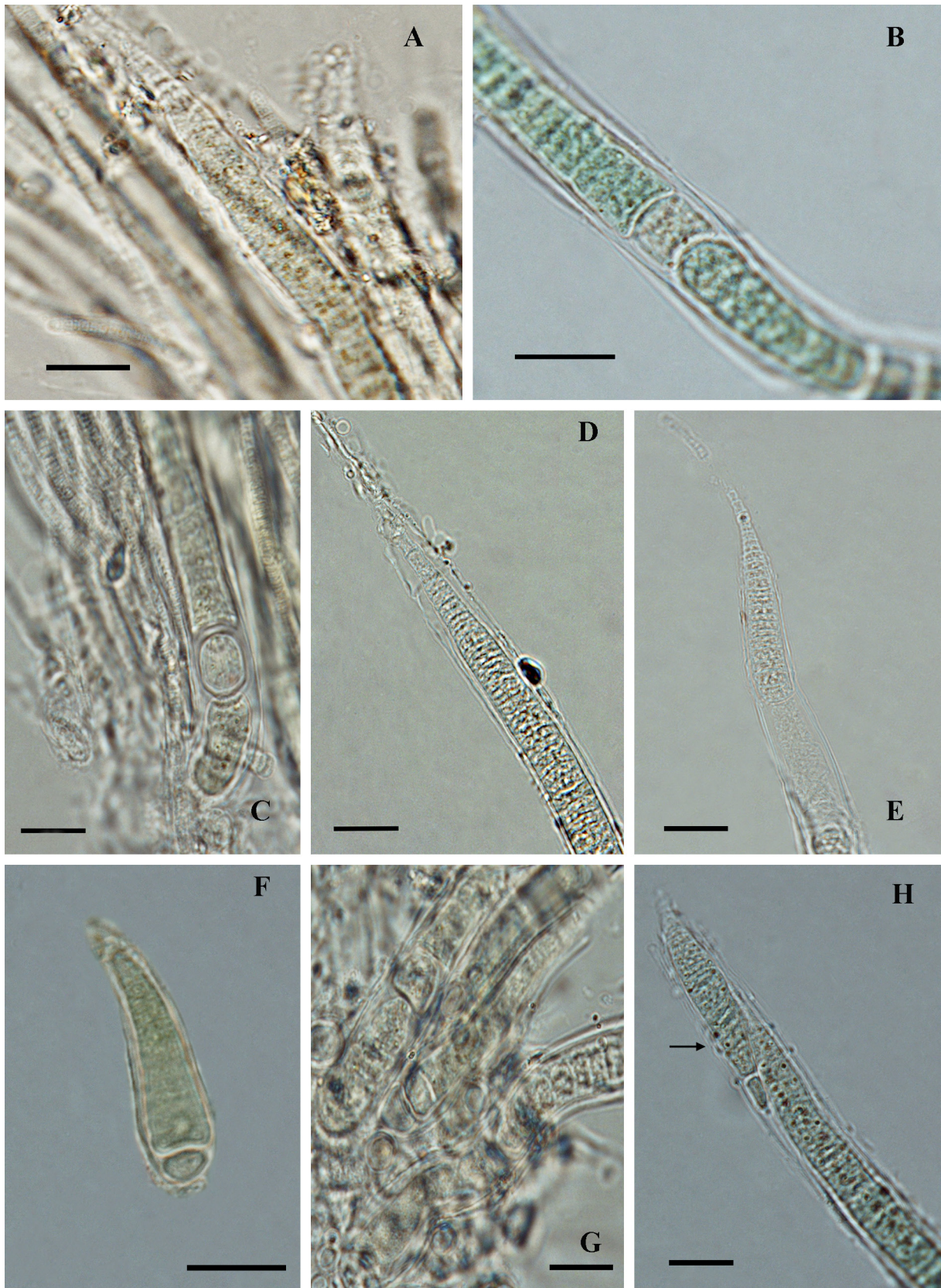


Fig. 7. *Nunduva biana*, environmental population: (A) filaments in fascicles; (B–C) intercalary heterocytes; (D–E) filaments tapering; (F) heteropolar hormogonia; (G) base of filaments showing basal and intercalary heterocytes; (H) development of isopolar hormogonia. Scale bars 10 μ m (A–H).

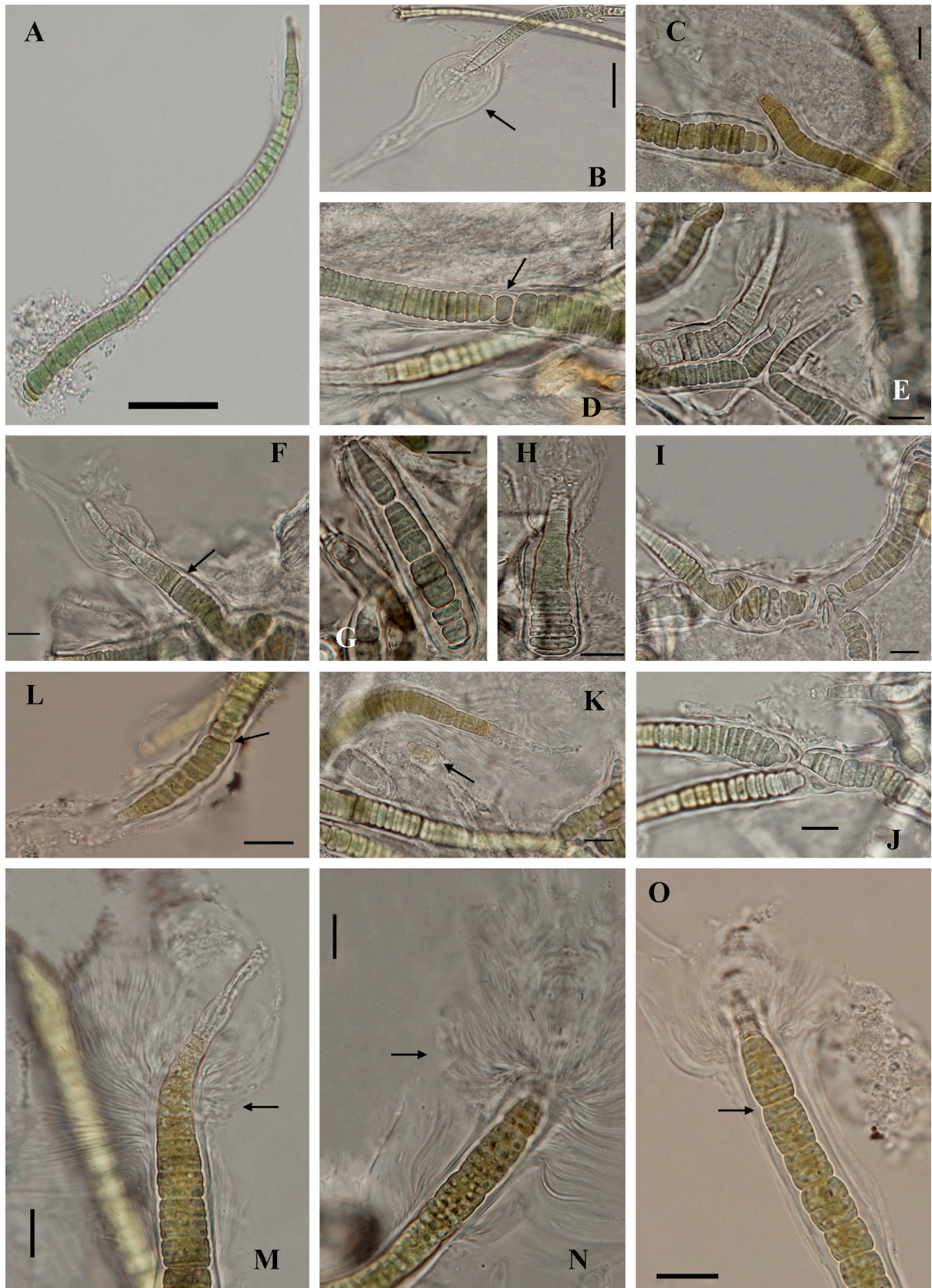


Fig. 8. *Nunduva biania*, culture population: (A) heteropolar mature filament; (B, M–O) lamellate, diffluent and stratified sheath; (C, E–F, I–J) single and geminate branching; (D) intercalary heterocyst; (F, L, O) heteropolar hormogonia formation by constriction (arrows); (G–H) heteropolar hormogonia; (K) heteropolar hormogonia liberation (arrow). Scale bars 20 μm (A–B), 10 μm (C–O).



Fig. 9. *Nunduva britannica*, culture population, with young (A–D) and old (E–K) cultures: (A, G) single and geminate branching; (B) straight filaments; (C–F) trichomes not tapering towards ends; (J) frayed sheath; (I–J) intercalary heterocytes; (K) isopolar hormogonia. Scale bars 50 μm (A); 20 μm (B); 10 μm (C–K).



Fig. 10. Phylogenetic analysis based on 16S rRNA gene sequence data showing position of *Nunduva*, gen. nov. in the Rivulariaceae. The tree is based on Bayesian topology, with support values given for maximum parsimony, maximum likelihood, and Bayesian posterior probabilities analysis (MP/ML/BA). The cut-off values for bootstrap and posterior probability are 50 and 0.5, respectively.

Fig. 12. Secondary structures of the 16S–23S ITS region, V2 helix and V3 helix in marine related sequences. V2 helix: (A) *Nunduva fasciculata*; (B) *Nunduva kania* C458; (C) *Nunduva biana* C708; (D) *Nunduva britannica* CCAP 1445/1; (E) *Kyrthutrix huatulcensis*; (F) “*Rivularia*” PCC7116. V3 helix: (G) *Nunduva fasciculata*; (H) *Nunduva kania* C458–K66; (I) *Nunduva biana* C708; (J) *Nunduva britannica* CCAP 1445/1; (K) *Kyrthutrix huatulcensis*; (L) “*Rivularia*” PCC7116. Variability in sequence between strains or between operons in a single strain is indicated by showing alternate bases within in circles.

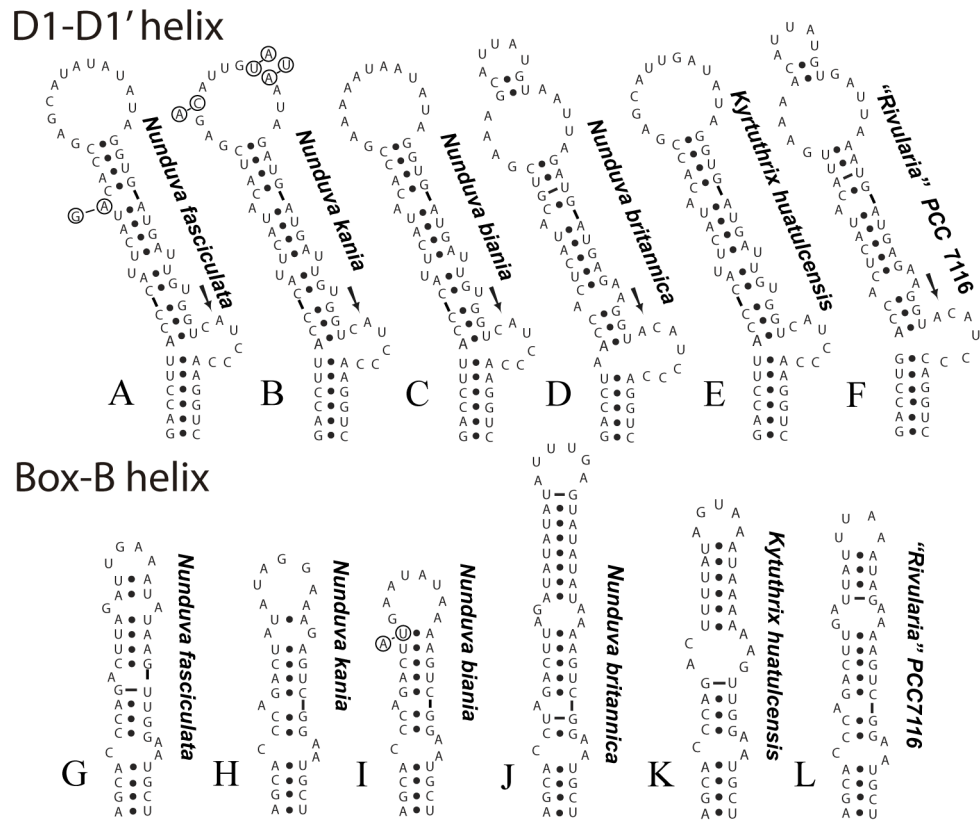


Fig. 11. Secondary structures of the 16S–23S ITS region, D–D1' helix and Box–B helix in *Nunduva* and related sequences. D–D1' helix: (11) *Nunduva fasciculata*; (11) *Nunduva kania* C458–K66; (11) *Nunduva biana* C708; (11) *Nunduva britannica* CCAP 1445/1; (11) *Kyrthutrix huatulcensis*; (11) “*Rivularia*” PCC7116. Box–B helix: (11) *Nunduva fasciculata*; (11) *Nunduva kania* C458; (11) *Nunduva biana* C708; (11) *Nunduva britannica* CCAP 1445/1; (11) *Kyrthutrix huatulcensis*; (11) “*Rivularia*” PCC7116. Variability in sequence between strains or between operons in a single strain is indicated by showing alternate bases within circles.

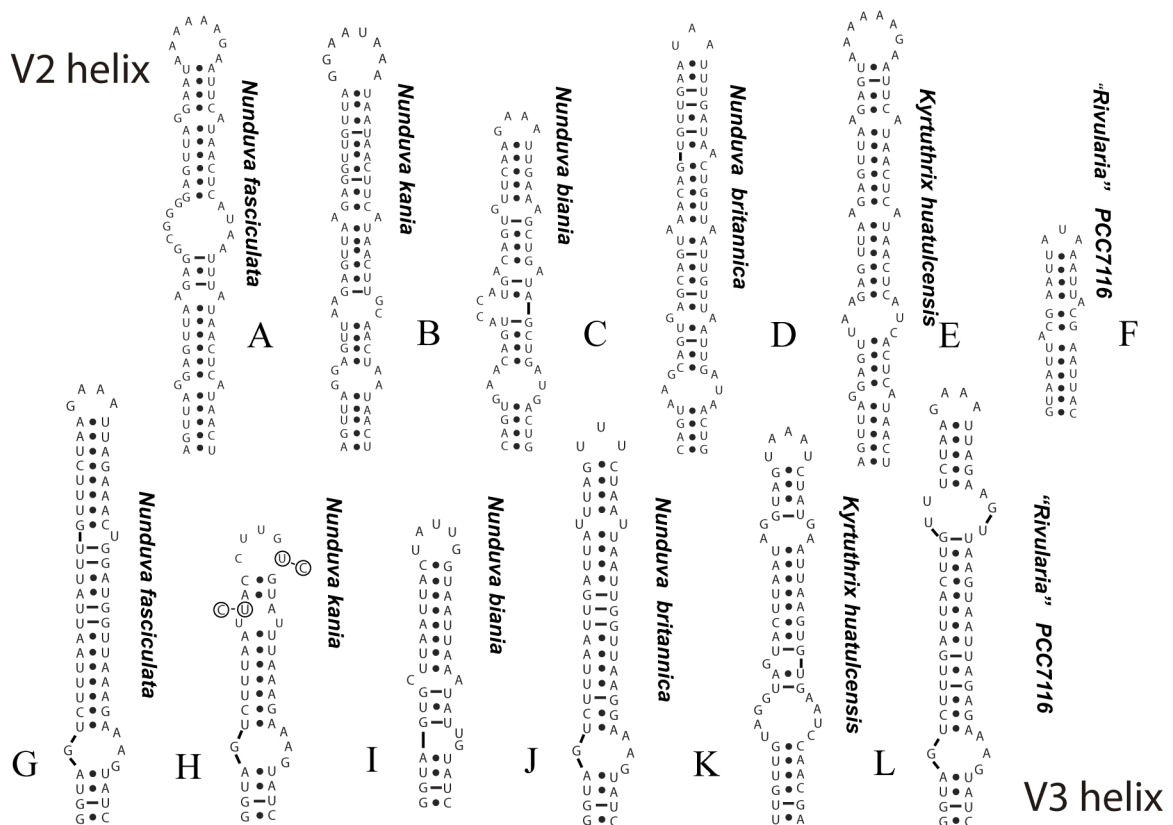


Table 1. Sequence identities between 16S rRNA gene sequences from environmental and culture populations of *Nunduva* spp. and closest relatives in the Rivulariaceae and two representatives from the *Calothrix/Macrochaete* clade.

	1	2	3	4	5	6	7	8	8	10	11	12	13
1 <i>Nunduva fasciculata</i> C58–M5–K83 Culture													
2 <i>Nunduva fasciculata</i> C57–M6, M7 Environmental	99.7												
3 <i>Nunduva fasciculata</i> C694–M8–K148 Culture	99.7	100											
4 <i>Nunduva fasciculata</i> C1290–M9 Environmental	99.7	100	100										
5 <i>Nunduva britannica</i> CCAP1445/1 Culture	98.9	99.2	99.2	99.2									
6 <i>Nunduva biana</i> C708–M10–K85 Culture	99.0	99.3	99.3	99.3	98.6								
7 <i>Nunduva kania</i> C458–16–K66 Culture	98.9	99.2	99.2	99.2	98.6	98.9							
8 Marine Rivulariaceae IV C1088–M21–K118 MF383375	98.3	98.6	98.6	98.6	98.6	98.1	98.0						
9 Marine Rivulariaceae V C1230–M22–K135 MF383373	98.9	99.2	99.2	99.2	98.6	98.5	98.4	99.2					
10 <i>Kyrtuthrix huatulcensis</i> C708 KT936560	98.1	98.4	98.4	98.4	98.0	98.3	98.0	98.0	98.0				
11 <i>Rivularia</i> ERIVALH2 EU009142	97.6	97.9	97.9	97.9	98.0	97.7	97.4	97.5	97.5	98.5			
12 <i>Rivularia atra</i> BIR KRIV1 AM230674	97.7	98.0	98.0	98.0	98.1	97.8	97.5	97.4	97.4	98.4	99.9		
13 <i>Calothrix parietina</i> CCAP1410/11 HE974991	92.2	92.5	92.5	92.5	91.8	92.1	92.0	92.7	93.0	93.4	93.0	93.0	
14 <i>Macrochaete psychrophila</i> ANT SPH13 96 KT336438	92.1	92.4	92.4	92.4	92.3	92.4	92.1	92.7	92.7	93.1	93.4	93.4	95.3

Table 2. P-distances for 16S–23S ITS sequences among *Nunduva fasciculata* populations and close relatives. Distances <0.03 (bold font) are indicative of members of the same species, while distances >0.07 are taken as strong evidence of different species (ERWIN & THACKER 2008; OSORIO–SANTOS et al. 2014).

SPECIES/STRAIN	<i>N. fasciculata</i> C58–M5–K83	<i>N. fasciculata</i> C694–M8–K148	<i>N. fasciculata</i> C1290–M9	<i>N. britannica</i> CCAP1445/1	<i>N. kania</i> C458–M16–K66	<i>N. biana</i> C708–M10–K85	MR IV C923–M12	MR IV C1088–M21–K118	MRV C1230–M22–K135	MR III C708–M3–KA	<i>Rivularia</i> , PCC 7116
<i>N. fasciculata</i> C57–M7	0.49										
<i>N. fasciculata</i> C58–M5–K83	0.33	0.16									
<i>N. fasciculata</i> C694–M8–K148	0.49	0.33	0.16								
<i>N. fasciculata</i> C1290–M9	16.23	16.05	15.87	16.05							
<i>N. britannica</i> CCAP 1445/1	10.90	10.73	10.55	10.73	15.72						
<i>N. kania</i> C458–M16–K66	15.21	15.21	15.01	15.21	13.94	14.69					
<i>N. biana</i> C708–M10–K85	15.83	15.66	15.48	15.66	20.21	13.08	15.08				
MR IV C923–M12 MF383374	15.99	15.82	15.65	15.82	19.68	13.78	15.58	1.62			
MR IV C1088–M21–K118 MF383375	16.73	16.56	16.39	16.56	18.49	11.40	15.48	16.50	17.20		
MRV C1230–M22–K135 MF383373	18.80	18.61	18.43	18.61	14.16	16.16	11.91	16.20	16.11	17.55	
MR III C708–M3–KA KT936564	17.20	17.20	17.00	17.20	17.01	14.37	15.49	17.73	17.56	13.64	13.28

fasciculata were all <1.0% (Table 2), and consequently indicated that all belonged to the same species (ERWIN & THACKER 2008; OSORIO–SANTOS et al. 2014). The p-distances among ITS regions of all *Nunduva* species were all greater >7%, strong evidence that they belong to other species. It was possible to align the ITS regions of other related but unidentified strains with *Nunduva* species, but intergeneric limits have not been set based on ITS p-distance, so this is at present uninformative for taxonomy.

The secondary structure of the ITS region showed that *N. fasciculata* has identical structure in the D–D1' helix to the C458–M16–K66 *N. kania* and to C708–M10–K85 *N. biana*, with differences among the taxa only occurring in the sequence of the terminal loop. *N. britannica* had more substantial sequence differences in both the terminus and in the helix sequence between the subterminal loop and the basal unilateral bulge (Figs 11A–11F). All sequences of *Nunduva* show a strong similitude in their basal unilateral bulge sequence 5'–CAUCCC–3' (Figs 11A–11F see arrows), with one additional adenosine in *N. britannica* (5'–ACAUCCC–3' Fig. 11D).

The Box–B helices (Figs 11G–11L) were consistent in the basal clamp of the helix in all *Nunduva* species, but differed significantly in the terminal part of the helix and the sequence and size of the terminal loop. The sequence length of the Box–B helices also differed notably among the four species compared (Figs 11G–11J).

The *Nunduva* species consistently have operons with both tRNA genes, and consequently have V2 helices which often are formed between the tRNA genes (Figs 12A–12D). The V2 helices were highly divergent in primary sequence and secondary structure among the four *Nunduva* sequences compared, with *Nunduva fasciculata* having the longest sequence (Fig. 12A). The V3 helices (Figs 12G–12J) were similar in sequence in the basal clamp of the helix, but differed markedly in sequence and length for all four taxa, with *Nunduva fasciculata* having the longest V3 helix (Fig. 12G).

DISCUSSION

With the onset of molecular sequencing and phylogenetics, there have recently been substantial changes in the systematics of the Rivulariaceae, including the description of new genera in the family such as *Phyllonema* (ALVARENGA et al. 2016) and *Macrochaete* (BERRENDERO et al. 2016). The genera within the Rivulariaceae have been morphologically defined by their heteropolar growth, basal heterocytes and tapered trichomes. More recently, with the inclusion of the marine genus *Kyrtuthrix* in the family based on molecular phylogeny, the morphological definition of the Rivulariaceae had to be expanded to include genera with isopolar trichomes disposed in a heteropolar growth form (LEÓN–TEJERA et al. 2016).

The *Nunduva fasciculata* environmental populations were described originally as *Brasilonema* sp. (currently

included in Scytonemataceae) by GONZÁLEZ–RESENDIZ et al. (2015), based on morphological features such as intercalary heterocytes, development of isopolar hormogonia, occasional geminate false branches, and fasciculated filaments. Although, some morphological traits are close to the *Brasilonema* genus circumscription, distinctive traits such as the peculiar “bead-like” apical cell, heterocyte size and serial disposition, and distribution in supra and intertidal marine biotopes, in contrast to the freshwater and mainly subaerial habitat of typical *Brasilonema* species (FIORE et al. 2007; SANTA'ANNA et al. 2011; VACCARINO et al. 2012; RODARTE et al. 2014), suggested that it belonged to a separate taxon. Even though the environmental populations have morphological traits that differ from typical Rivulariaceae, phylogenetically they clearly belong to this family and are distant from *Brasilonema* and other members of the Scytonemataceae. However, culture material developed more characteristic features of the Rivulariaceae, including marked trichome attenuation, pigmented terminal hairs, and frequent heteropolar hormogonia formation.

The molecular phylogenetic information obtained, supports the inclusion of *Nunduva* genus into the Rivulariaceae lineage. Our sequences form a well-delimited clade (Fig. 10), sister to the lineage that includes the strains assigned to Rivulariaceae sensu stricto (with type species *R. atra*, see Fig. 10). The topology of our 16S rRNA phylogeny agrees with previous results with slightly different taxon sampling (BERRENDERO et al. 2016, fig. 5), where the Rivulariaceae form a well-defined cluster paraphyletic to the *Calothrix* group, which contains two subclades (marine and freshwater/soil).

The *Nunduva* 16S rRNA gene sequences had a high similarity (over 98%) to other marine sequences (Table 1) including *Kyrtuthrix huatulcensis*, “*Rivularia*” PCC 7116, Marine Rivulariaceae IV, and Marine Rivulariaceae V. Even *Rivularia sensu stricto*, as represented by *Rivularia atra* BIR KRIV1, was ±98% similar to *Nunduva* species. The thresholds set as clear evidence for recognition of new species or genera (STACKEBRANDT & GOEBEL 1994; STACKEBRANDT & EBERS 2006; KIM et al. 2014; YARZA et al. 2014), are not met in the case of *Nunduva fasciculata*. Indeed, all 16S rRNA sequences of members of Rivulariaceae sensu stricto are above 94.5% similar, suggesting that this very morphologically and ecologically diverse group could all belong to the same genus if only this criterion was used. However, previous studies demonstrated that Nostocales are highly similar in the 16S rRNA gene sequence (FLECHTNER et al. 2002; LUKEŠOVÁ et al. 2009; KAŠTOVSKÝ et al. 2014) and that genera in this order frequently are more similar than 98% to at least some other genera. According to KAŠTOVSKÝ et al. (2014), to discriminate between genus and species, the similarity threshold in the 16S rRNA gene can be very close (<99% for species, and < 98% for genera). We conclude that the 16S rRNA gene sequences were too similar to serve as a reliable or sole criterion for genus or species recognition in the Rivulariaceae. We agree

with DVOŘÁK et al. (2015) that similarity values should not be used as a primary criterion to define taxon levels in the Cyanobacteria.

The morphological differences, divergence in ITS secondary structures, and p-distances among ITS sequences among the Marine Rivulariaceae (Fig. 10) provide fairly clear evidence that this group contains multiple different species. However, the group of Marine Rivulariaceae that includes the lineages sister to the node containing *Kyrtuthrix* and “*Rivularia*” Gulf of Mexico are molecularly very similar, and it is conceivable that these undetermined taxa belong to *Nunduva*, despite their morphological divergence. Additional taxon sampling and sequencing of other loci could conceivably provide clarity as to whether these taxa should be members of the same genus or recognized in additional, yet-to-be described genera. The differences in 16S–23S ITS rRNA primary sequence (Table 2) and secondary structure in all marine sequences compared (Figs 11, 12), suggest to us that multiple distinct species exist in this clade.

Isopolar growth seen in *Kyrtuthrix* and *Nunduva* together with geminate false branching evident in *Nunduva* set these genera off from the rest of the Rivulariaceae. Tapering trichomes are typical to at least some life-cycle stages in all Rivulariaceae, but the heteropolar or isopolar nature of trichomes appears to be a less phylogenetically congruent character. Polyphasic evaluation (16S rRNA gene-based phylogeny, 16S–23S ITS secondary structure, ecology and morphology) shows clear differences between the closely related marine taxa and *Nunduva* environmental/culture material, providing support for this newly erected genus.

We recommend, that a complete evaluation of new taxa primarily should include if possible, the morphological description of natural and culture populations based on light and electron microscopy, the inclusion of ecological information, and molecular analysis of 16S rRNA gene and other markers, such as conserved domain structure in the ITS region. Additionally, greater taxon sampling of marine Rivulariaceae is required, including the type species of *Calothrix* and *Rivularia*, as well as the analysis of a more informative multilocus data set to solve the extant polyphyly within this family as it is now conceived.

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