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Phylogenetic position reevaluation of *Kyrtuthrix* and description of a new species *K. huatulcensis* from Mexico´s Pacific coast

HILDA LEÓN-TEJERA[1]*, LAURA GONZÁLEZ-RESENDIZ[1], JEFFREY R. JOHANSEN[2], CLAUDIA SEGAL-KISCHINEVZKY[3], VIVIANA ESCOBAR-SÁNCHEZ[3] & LUISA ALBA-LOIS[3]
[1]Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México (UNAM). Coyoacán, Código Postal 04510, P.O. Box 70–474, México, Ciudad de México, México.
[2]John Carroll University, Cleveland, Ohio, USA
[3]Departamento de Biología Celular, Facultad de Ciencias, UNAM, Ciudad de México, México.
**Corresponding author (hlt@fciencias.unam.mx)*

ABSTRACT

Benthic marine heterocytous cyanoprokaryotes of Mexico´s tropical coast are being recognized as an important and conspicuous component of the supralittoral and intertidal zones usually described as an extreme and low diversity biotope. Although *Kyrtuthrix* has been reported from different coasts worldwide, its complex morphology has led to differing taxonomic interpretations and positioning. Ten marine supra and intertidal populations of *Kyrtuthrix* were analyzed using a detailed morphological approach, complemented with ecological and geographical information as well as DNA sequence data of the 16S rRNA gene and associated 16S–23S ITS. *Kyrtuthrix huatulcensis* is described as a new species, different from *K. dalmatica* Ercegovic and *K. maculans* (Gomont) Umezaki based primarily on morphological data. Our material has smaller dimensions in thalli, filaments, trichomes and cells, and possesses differences in qualitative characters as well. Our material is epilithic as in *K. maculans* but not endolithic as in *K. dalmatica*. The analysis of molecular data, mainly 16S rRNA gene sequence, show that this genus is within the Rivulariaceae clade, and outside of the Scytonemataceae clade. ITS secondary structure comparison with some of the closer representatives of Rivulariaceae shows considerable differences with these related genera. Based on the evidence gathered in this study, inclusion of *Kyrtuthrix* within the Rivulariaceae, rather than the Scytonemataceae, is fully supported.

Key words: Rivulariaceae, Scytonemataceae, 16S rRNA gene, 16S–23S ITS

INTRODUCTION

Recently, cyanoprokaryotes in general and heterocytous taxa in particular have been reevaluated taxonomically with molecular methods. This has been necessitated in part by the discovery of morphologically novel populations that have been characterized from more habitats and regions outside of Europe where the bulk of species and genera were originally described. Not only are new species and genera being described, but new taxa are also being created as existing species are being transferred to different genera, families or orders with the advent of the polyphasic approach which incorporates morphological, ecological, and molecular data into our understanding of what constitutes cyanobacterial taxa (Komárek *et al.* 2014). Molecular markers such as the 16S rRNA gene have been proven to give valuable information for comparing and helping to solve phylogenetic relationships at generic and family levels (Fiore *et al.* 2007, Hauer *et al.* 2014, Mareš *et al.* 2015), and the characterization of the secondary structure of the 16S–23S (ITS) region has been useful in separation of morphologically similar species (Perkerson *et al.* 2011, Johansen *et al.* 2014, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014, Bohunická *et al.* 2015).

Kyrtuthrix was erected with *K. dalmatica* Ercegovic (1929a: 173) as the type species, described from intertidal endolithic populations from the Adriatic coast. In Ercegovic's subsequent more detailed treatment (1929b), he described a special type of branching that supported placement of *Kyrtuthrix* within the family Mastigocladaceae. Presently, three species have been described—the endolithic generitype *K. dalmatica,* the epilithic *K. maculans* (Gomont 1901: 201) Umezaki (1958:64), and *K. sinensis* Chu H. J., & B. T. Wu (1984: 227), the latter being considered invalid

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(Komárek 2013). *K. maculans* (Gomont) Umezaki was originally described as *Brachytrichia maculans* Gomont in Schmidt (1901, as a member of Rivulariaceae). Umezaki (1958) assigned *B. maculans* to *Kyrtuthrix*, and concluded that there was only a single species, which he erroneously assigned to *K. maculans*. *K. dalmatica* is the type species for the genus as it was the type species under which the genus was described. The species epithet *maculans* is older than *dalmatica*, but the combination *K. maculans* is more recent than *K. dalmatica*. If these two taxa truly were synonyms, the valid name would have to be *K. dalmatica*. Umezaki's synonymization of the two taxa and his use of *K. maculans* as the name for both likely contributed to its reportedly wide geographic range (Umezaki 1961; Ramírez & Parra 1975; Sant'Anna *et al*. 1985; Sant'Anna 1997; Silva & Pienaar 2000; Montoya 2003; León-Tejera *et al*. 2005; Crispino & Sant'Anna 2006; González-Resendiz *et al*. 2015). Komárek (2013) has called for revision of this genus, including molecular evaluation as well as better evaluation of the nature of the branching.

Kyrtuthrix is a peculiar marine heterocytous genus characterized by the presence of uniseriate filaments arrayed in parallel arrangement to form firm, thick mats. Its isopolar trichomes are bent, with a regular growth pattern in which their attenuated extremes are directed upwards while at the base they are in tight loops or sometimes lateral. The isopolarity of attenuated trichomes as well as varied interpretations of their bent and sometimes tortuous filaments forming loops have been the source of diverging interpretations as to the type and even presence/absence of branching, resulting in the placement of *Kyrtuthrix* in various families including Mastigocladaceae (Ercegovic 1929a, Geitler 1932), Rivulariaceae (Gomont 1901, as *Brachytrychia maculans* Gomont 1901: 201), Stigonemataceae (Umezaki 1958), and Scytonemataceae (Fritsch 1945, Komárek & Anagnostidis 1989, Komárek 2013).

The endolithic species *K. dalmatica* has been described from diverse coastal regions but mainly from the Mediterranean Sea—Greece (Pantazidou 1991), France and Morocco (as *Brachytrichia dalmatica* Fremy 1934: 162). Epilithic populations described as *K. maculans* from coasts of South America were reported from Chile (González & Parra 1975), Peru (Montoya 2003) and Brazil (Sant´Anna *et al.* 1985; Sant'Anna 1997). In Mexico, populations of *Kyrtuthrix* cf. *maculans* were recorded previously from Barra Santa Elena and San Agustin (León-Tejera *et al.* 2005, González-Resendiz *et al.* 2015). It has also been described by Silva & Pienaar (2000) from South Africa, and included in checklists or floristic studies from other geographic regions such as Australia (Phillips 2002, Bostock & Holland 2010), Andalucia in the Mediterranean (De la Rosa & Sánchez Castillo 2009, De la Rosa 2012), East Asian coasts (Gomont 1901, Lobban 2006, Liu 2008), and Japan (Umezaki 1958).

Our records are from a region in the Tropical Pacific marine littoral that has been considered as a hotspot for marine littoral biodiversity (Bastida-Zavala *et al.* 2013). Concerning marine benthic cyanoprokaryotes, specially from intertidal and supratidal zones, this region´s previous studies (González-Resendiz *et al.* 2013, 2015, Gold-Morgan *et al.* 2015) have also shown a high potential for the discovery of new taxa to science. This is mainly due to the fact that this taxonomic group and the tidal zone in the region have been unattended by previous phycologists. The aim of this paper is to describe the morphologically unique *K. huatulcencis* as a new species and to evaluate the phylogenetic position of *Kyrtuthrix* within the Nostocales.

METHODS

Sample collection:—Sampling of intertidal and supralittoral zones was carried over a period of several years in the coast of Oaxaca in the Mexican Pacific littoral. The number of samples analyzed was twelve from four localities: Barra Santa Elena (15° 44' 00" N, 96° 46' 48"W), San Agustín Bay (15° 41' 17.41" N, 96° 14' 15.28" W), Cacaluta Bay (15° 43' 09.00" N, 96° 09' 59" W), and Panteones Beach at Puerto Angel (15° 39' 50.00" N, 96° 29' 43.93" W). The samples were subdivided according to differing means of preservation: formalin, direct drying in paper and drying in silica gel.

Culture isolation:—The strains used in the molecular study were isolated from conspicuous microbial soft crustal growths, collected from a granitic coastal cliff on the east side of San Agustín bay, within Huatulco National Park. Strains C695 and C708 were isolated from enrichment in Petri plates with solid agar SN (Waterbury *et al*. 1986), the plates were incubated at 25 ˚C in a 12/12h, light/dark regime in a culture chamber.

TABLE 1. Phenotypical characters of *Kyrtuthix maculans* (Type description and other materials) and *K. dalmatica* ((Ercegovic, 1929, Type description*)*: only differential characters. Information gathered from Central and South America: Mexico (León-Tejera *et al*. 2005; González Resendiz *et al*. 2015; Brazil (Crispino & Sant'Anna 2006; Sant'Anna 1997; Sant'Anna *et al*. 1985); Chile (González & Parra, 1975); Perú (Montoya, 2003); Asia: Japan (Umezaki, 1961); Thailand (Gomont 1901 as *Brachytrichia maculans, K. maculans* type); Africa (Silva & Pienaar 2000); EUROPE: (Ercegovic 1929a) NA: Not available. *: Data not included in the descriptions but obtained from images of cited references; (extreme values). PO: Pacific Ocean; AO: Atlantic Ocean; IO: Indian Ocean; ME: Mediterranean Sea.

Taxon Region	***Kyrtuthrix huatulcensis*** **México** **PO**	*K. maculans* Brasil AO	*K. maculans* Chile PO	*K. maculans* Perú PO	*K. maculans* Japan PO	*K. maculans* Thailand (type) IO	*K. maculans* South Africa IO	*Kyrtuthrix dalmatica* Dalmatian coast (type) ME
	Central & South America				**Asia**		**Africa**	**Europe**
Ecology	**Sunny or shaded granitic rock in the supratidal and intertidal fringes**	On supralittoral granitic rocks or intertidal mass on shells	Tidal pool margins	Supratidal and intertidal fringes	On calcareous rocks in the upper littoral or supralittoral belt	Strongly epilithic	Epilithic on littoral zone rocks. Material described from culture material	On calcareous rocks of the intertidal zone
"Growth" description	**Epilithic, soft spoted irregular crust**	Epilithic round,vesiculous, gelatinous cushion or crustous mass	Ample, crustose, irregularly expanded	Crustaceous laminar, roseta, round to irregular patches.	Widely expanded epilithic or endolithic	Plane, thin circular to confluent crust	Flat and gelatinous	Endolithic crust
Tallus height (µm)	**50–120**	450–500*	100–400	NA	100–400 (500)	NA	300*	–400

...Continued on next page

TABLE 1. (Continued)

Taxon Region	***Kyrtuthrix huatulcensis*** **México** **PO**	*K. maculans* Brasil AO	*K. maculans* Chile PO	*K. maculans* Perú PO	*K. maculans* Japan PO	*K. maculans* Thailand (type) IO	*K. maculans* South Africa IO	*Kyrtuthrix dalmática* Dalmatian coast (type) ME
Filaments description	**Parallelly disposed, bent at the base and attenuated at their upper terminal parts**	"U" form filaments, parallel, "fan" like disposition crustose mass of confluent	Layer of compact parallel filaments, sometimes the middle part adhered. Geminated	Gemminate type false branched	Parallely disposed. V-shaped branching	One layer of filaments tightly apressed	NA, "U" form straight to contorted*	Parallel bent gemminate, some flexuous, terminally attenuated, forming a hair; "gemminate false branches"
Filament total lenght)/t width (µm)	**Single 60–100. Folded 120–300/7–9**	NA/18–23 or NA/9–13	100–400/10–22	NA/6–18	100–400 (500)/10–20*	NA/NA	300*/–21	–400/15–20 (pair)
Sheath	**Firm, hyaline, lamellate and darker or basally yellowish. Individual, paired filaments and colonial sheath.**	Firm, wide, anastomosed, hyaline to yellowish brown at the upper part, homogeneous	Firm, hyaline, yellow to yellowish brown at the extremes.	Thick stratified hyaline to brownish to 15 µ thick	Hyaline, above yellowish or light brown	Wide*	Wide*	Firm, hyaline, yellowish in the surface
Cell shape	**Isodiametrical in middle parts, larger cells cylindrical to irregular; some truncated cone to irregular, thinner at apex**	Irregular, rounded to quadratic barrel	Variable in size shorter than wide or wider than long	Discoidal, clindrical to elongated; frequent biconcave cells	Variable in shape, quadrate, cylindrical or barrel-shaped	Quadrate, cylindrical or barrel-shaped	Usually shorter than wider cells, square or longer than wide	Cylinder-barrel

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TABLE 1. (Continued)

Taxon Region	***Kyrtuthrix huatulcensis*** **México** **PO**	*K. maculans* Brasil AO	*K. maculans* Chile PO	*K. maculans* Perú PO	*K. maculans* Japan PO	*K. maculans* Thailand (type) IO	*K. maculans* South Africa IO	*Kyrtuthrix dalmática* Dalmatian coast (type) ME
Cell length/width (µm)	**(1) 1.5–3 (6) /(1.2) 2–3 (5) Hair cells 3–5/1–2 (3)**	2–5/3–5.5 Hair cells NA/3–5	3–10/3.5–6	2–7/4–9 Apical cells elongated	3–10/4–9	6–8/4	1.8–4.6/5–8.4	5–9/4–7
Heterocyte shape	**Cylindrical to polygonal some subspherical**	Flattened, quadratic, to rounded quadratic, conical, rounded or oblong	Variable in form and shape, subquadrate to cilindrical	Obvoid or cylindrical, in photo 5, 8 very large	Nearly quadrate or a little longer	Nearly quadrate or a little longer	Shorter than wide or square with yellowish and homogeneous protoplasm	Oval
Heterocyte length × width (µm)	**(1.5) 3–4 (10)/1.8–3.1** **2–3 (4)/2 –3**	2–5 (8.5)/ 3–9 1–2 pores	10/5	(5) 9–15/7–12 20–25/7–12*	4.5–15.5/ 6–11	NA	2.8–8.4/4.6–12	9 (avg.)
Hormogonies length × diam (µm)	**6–12/1.8–2.1**	NA/NA	NA/NA	Yes	17–35/NA. Single within sheath	Up to 70 long	9–38.7/3.1–7.5	25–40/NA

Morphology:—Characterization was performed primarily on environmental samples. We analyzed morphological traits from populations of six samples collected in San Agustín (different years), three from Panteones Beach in Puerto Angel, and one sample from Cacaluta. The data from these populations was compared with those of a population described by León Tejera *et al.* (2005) as *K.* cf. *maculans* as well as with other reports for species of this genus in different parts of the world (Table 1). Semi-permanent slides were prepared from each sample and deposited in the FCME collection. Micrographs were acquired with an Olympus DP12 digital camera adapted to an Olympus CX51 microscope (DIC and bright-field). Morphological measurements (20–30 per population per character) were obtained from micrographs using SigmaScan© automated image analysis software (Jandel Scientific, Sausalito, California). Morphological description and identification was done in accordance with new and traditional reference works (Ercegovic 1929a, b; Frémy 1934, Geitler 1932, Komárek 2013), along with additional studies that describe populations of epilithic *Kyrtuthrix* species (Umezaki 1958, Kosinskaja 1948, Sant'Anna *et al.* 1985, Silva & Pienaar 2000, León-Tejera *et al.* 2005). The diagnosis and description follow the format recommended by Stearn (1992), which is based on Linnean precedents.

Molecular methods:—Genomic DNA was extracted using the UltraClean® Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene and 16S–23S ITS region, were amplified using primers 27F after Neilan *et al*. (1997), (5'-AGA GTT TGA TCC TGG CTC AG-3') and VRF1 after Wilmotte *et al*. (1993) (5'-CTC TGT GTG CCT AGG TAT CC-3'). Amplification reactions were setup as follows: 50 µL PCR reaction mix containing 1x reaction buffer, 1.5 mM $MgCl_2$, 2.5U of *Taq* DNA polymerase (Amplificasa, Biogenica®, Mexico), 0.2 µM of each primer, 0.2 mM dNTPs (ThermoFisher Scientific, Waltham, MA), and 10 ng of genomic DNA. PCR was performed with an Eppendorf Mastercycler under the following conditions: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 10 min. PCR products around 2.2 kb were gel purified using the Zymoclean® Gel DNA Recovery Kit (Zymo Research, Irvine, CA), quantified and cloned with the CloneJET PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA). The size of the cloned products from three to five clones was examined by PCR, using pJET1.2 Forward and Reverse sequencing primers (ThermoFisher Scientific, Waltham, MA). Three clones with long PCR products were sequenced at the Biotechnology Institute, UNAM sequencing facility using an Applied Biosystems (Foster City, CA) model 3130xl Genetic Analyzer. Sequences were obtained for both DNA strands, assembled and corrected into 2016 bp fragments using Bioedit software version 7.0.9.0 (Hall 1999). The GenBank accession number for a consensus sequence from three clones is KT936560.

Phylogenetic analyses:—A total of 181 OTUs were chosen for analysis, including the one sequence newly obtained by this work together with representatives of the main groups of heterocytous cyanobacteria available in GenBank, and three outgroup taxa (*Chroococcidiopsis* 9E-07 FR798923, *Chroococcidiopsis cubana* Komárek et Hindák (1975: 320) SAG39.79 AJ344558 and *Chrococcidiopsis thermalis* Geitler (1933: 625) AB039005). All sequences were aligned using the ClustalW 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) in Mega version 6 (Tamura *et al*. 2013) and maximum likelihood analysis (ML) in PhyML 3.0 (Guindon *et al*. 2010), bootstrapping with 1000 replicates was conducted for both analyses. The Bayesian analysis (B) was run in MrBayes 3.2.2 (Ronquist *et al*. 2012), using two independent runs with eight chains each, for five million generations with default parameters. The evolutionary model GTR+I+G was determined based on the Maximum Likelihood (ML) ratio test implemented by TOPALi version 2 software (Milne *et al*. 2009). The range of 16S rRNA gene divergence values within and among species was calculated using uncorrected "p" distances using Mega V6.

The secondary structures of the 16S–23S ITS region, were determined using a combination of comparative analysis of the secondary structures of our strain, with related taxa in the topology of the tree based on 16S rRNA. In addition to *K. huatulcensis,* we selected representatives of clades designated as Marine Rivulariaceae I (MRI) and Marine Rivulariaceae II (MRII), Marine Rivulariaceae III (MRIII) and *Microchaete grisea* Thuret ex Bornet et Flahault (1887: 84). 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

Class Cyanophyceae

Subclass Nostocophycideae
Order Nostocales
Family Rivulariaceae (non Scytonemataceae sensu Komárek *et al*. 2014)

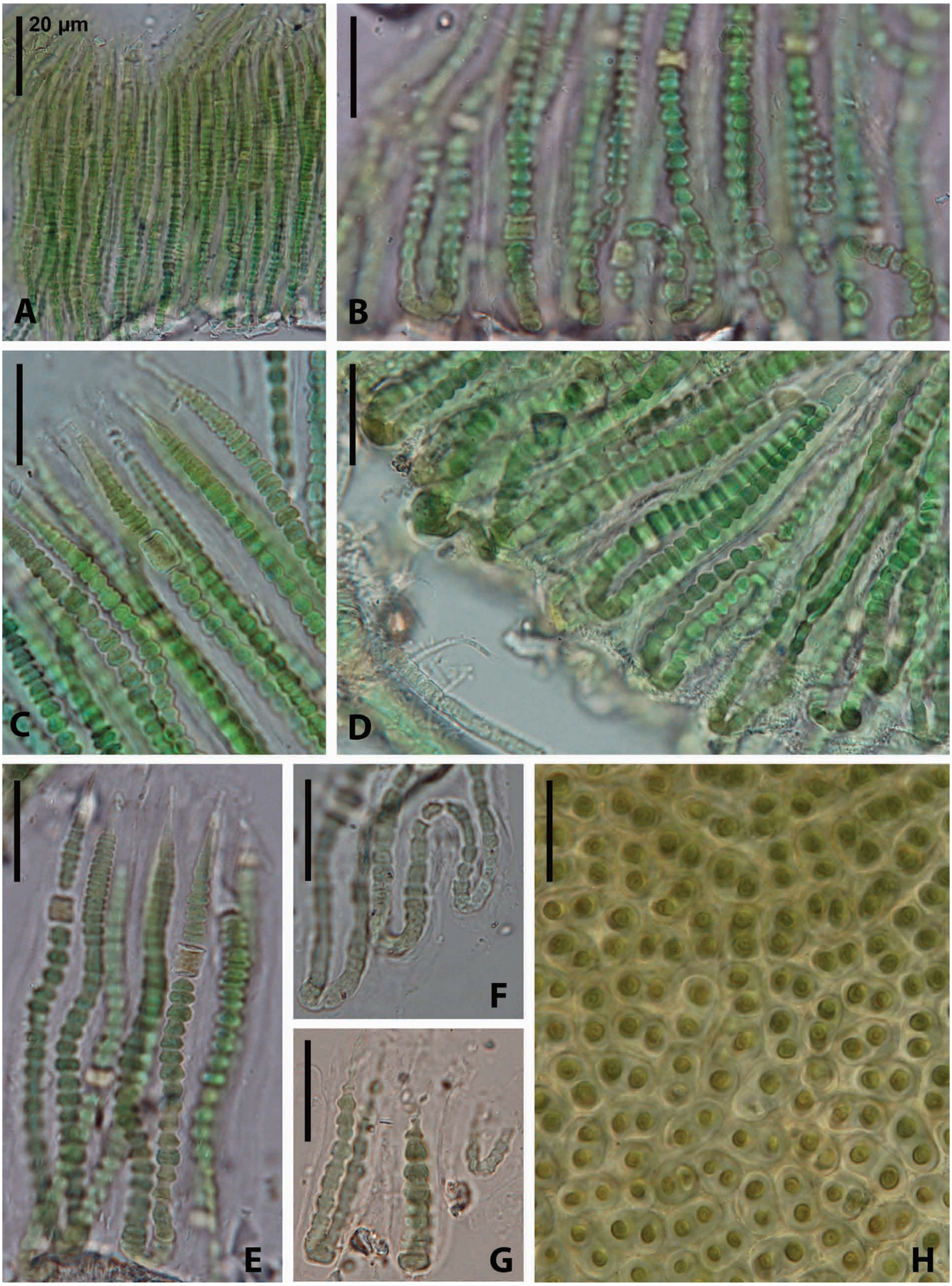

FIGURE 1. A–H. *Kyrtuthrix huatulcensis* field material. A. Lateral section of thallus showing parallel arrangement of filaments. B, D–F Loops formed at the base of the thallus. C, E. Filaments tapering towards the upper surface of the thallus. G. Young filaments with loops at the base and tapering apices. H. Cross section of thallus parallel to the surface plane showing paired cells of bent trichomes surrounded by individual and paired sheaths. Scale bar A=20 µm, B–H 10 µm.

Kyrtuthrix huatulcensis **León-Tejera, González-Resendiz & Johansen *sp. nov*., Figs. 1–3.**

Diagnosis:—Most similar to *K. maculans*, from which it differs in having a stratified sheath, thinner thalli, thinner trichomes, and shorter cells (Table 1). Differing from *K. dalmatica* by its epilithic habit and stratified sheath (Table 1). Differing from both species in the absence of false branches and thalli and smaller cell dimensions (Table 1).

Description:—In natural populations—thalli blue-green, crustaceous, flat, soft, forming neatly delimited and commonly abundant freckle or mole-type colonies 0.5 to 1.0 cm in diameter, less frequently forming longer mat-like colonies up to a few centimeters long, 60–80 μm high. Filaments arranged in tight parallel series, isopolar, forming sharply bent loops in the lower part (Fig. 1 A, B, D, F), with distal ends always directed and attenuated towards the upper part of the thallus (Fig. 1 A, C, E), to 60 μm long from the basal loop to the terminus. Colonial mucilage firm, generally colorless (Fig. 1 E–G), but sometimes yellowish or brownish in the upper surface of the crust (Fig. 1 A), lamellated (Fig. 1 H, Fig. 2 A, B, H), maintaining the integrity of crustose thalli in intertidal populations subject to battering waves at high tide and drought at low tide. Individual sheaths enclosing and binding both parts of a single bent trichome, producing an evident external layer for each paired portion of a single trichome (Fig. 1 H, 2 H, 3 C, H), embedded in the common mucilaginous matrix (Fig. 3 A–C, H). Trichomes constricted at cross walls (Fig. 2 A–G), (1.2) 2–3 (5) μm wide. Cells variable in form, commonly cylindrical or subspherical, sometimes having the shape of a truncated cone, or extremely irregular with different values of length and diameter within a single cell (Fig. 1 B–F. Fig. 2 B, C, D, E), (1) 1.5–3 (6.) μm long. Hormogonia isopolar or heteropolar (Fig. 3 D–G, L), liberated through the upper surface, after release soon becoming bent and somewhat attenuated before heterocytes are differentiated (Fig. 1 G. Fig. 2 A, B. Fig. 3 F–G, K). Heterocytes solitary, intercalary, mostly cylindrical to quadrate, 1.8–3.1 μm in diameter (Fig. 1 B–E), shorter to longer than wide ((1.5) 3–4 (10) μm long); near the apices and sometimes after release of attenuated trichome segments (heteropolar hormogonia) have subspherical shape, 3 μm in diameter (Fig. 2 C–F).

In cultures (C708, C695)—thalli soon (1–2 weeks) losing typical parallel disposition of filaments (Fig. 3 A–B). Hyaline sheaths surrounding single trichomes difficult to distinguish, gelatinized, remaining evident around pairs of trichomes (Fig. 3 A–C, H). Trichomes contorted, conserving constricted cells of a wide range of cell shape and sizes (3–10 μm wide), terminally attenuated (Fig. 3 A, C–D, G). Hormogonia commonly short, one to few-celled (4–10 μm long), straight or curved or folded, attenuated or not (Fig. 3 D–G). Cells with big pigmented granules (Fig. 3 A–H).

Notes on hormogonial development:—Occasionally segments of trichomes become detached, mainly in the upper part of the thalli, and then appear heteropolar. These are likely attenuated hormogonia prior to liberation (Fig. 3 I). Other hormogonia appeared to be isopolar (Fig. 3 E in culture, 3 L), possibly produced below a heteropolar hormogonium, or as the posterior stage of a heteropolar hormogonia after detachment of the attenuated part. Evidence of these types of hormogonia was found in field material; some were straight and others curved, but all were several cells in length. In culture curved cells were seen from a one-celled stage to several-celled segments. Culture conditions likely cause the physiological response for attachment to a substratum to occur earlier. In some instances truncated cone cells in contact with other cells favors fragmentation and the subsequent detachment of a hormogonium, due to the presence of nodes with narrower points of contact between cells (Fig. 3 F).

Holotype here designated:—FCME-PTM! C708 deposited in the herbarium FCME-C Facultad de Ciencias, UNAM, preserved in 4% marine formaldehyde with dry material duplicate.

Isotypes:—FCME! material included in C695, C700. Monoclonal population of the reference strain used for molecular data was originated from C708. Additional reference materials are samples PTM 6300, PTM-C59/C61/C707/C1319/ C1339/C1340/C1341.

TYPE LOCALITY: Mexico. Oaxaca: Supralittoral zone of San Agustín Bay, Huatulco, 15° 41’ 17.41” N, 96° 14’ 15.28” W, August 2014.

Etymology:—Named for the Huatulco region, a natural reserve zone of great biological diversity, from which the species was collected.

Habitat:—Supratidal and intertidal fringes; epilithic on granitic rock. Some supratidal populations exposed, others partially shaded by rock or other Cyanoprokaryotes. Intertidal populations exposed to insolation and various conditions of humidity.

Occurrence:—MEXICO. Oaxaca: Barra Santa Elena 15° 44’ 00” N, 96° 46’ 48”W, September 1997 *H. León-Tejera* (PTM 6300); San Agustín Bay 15° 41’ 17.41” N, 96° 14’ 15.28” W, *December 2010*, *October 2012*, *August 2014 L. González-Resendiz & H. León-Tejera* (C59, C61, C695, C700, C707, C708); Cacaluta Bay, 15° 43’ 09.00” N, 96° 09’ 59” W *August 2014 H. León-Tejera L. & González-Resendiz* (C1319); Panteones beach at Puerto Angel, 15° 39’ 50.00” N, 96° 29’ 43.93” W, *August 2014 González-Resendiz & H. León-Tejera* (C1339, C1340, C1341).

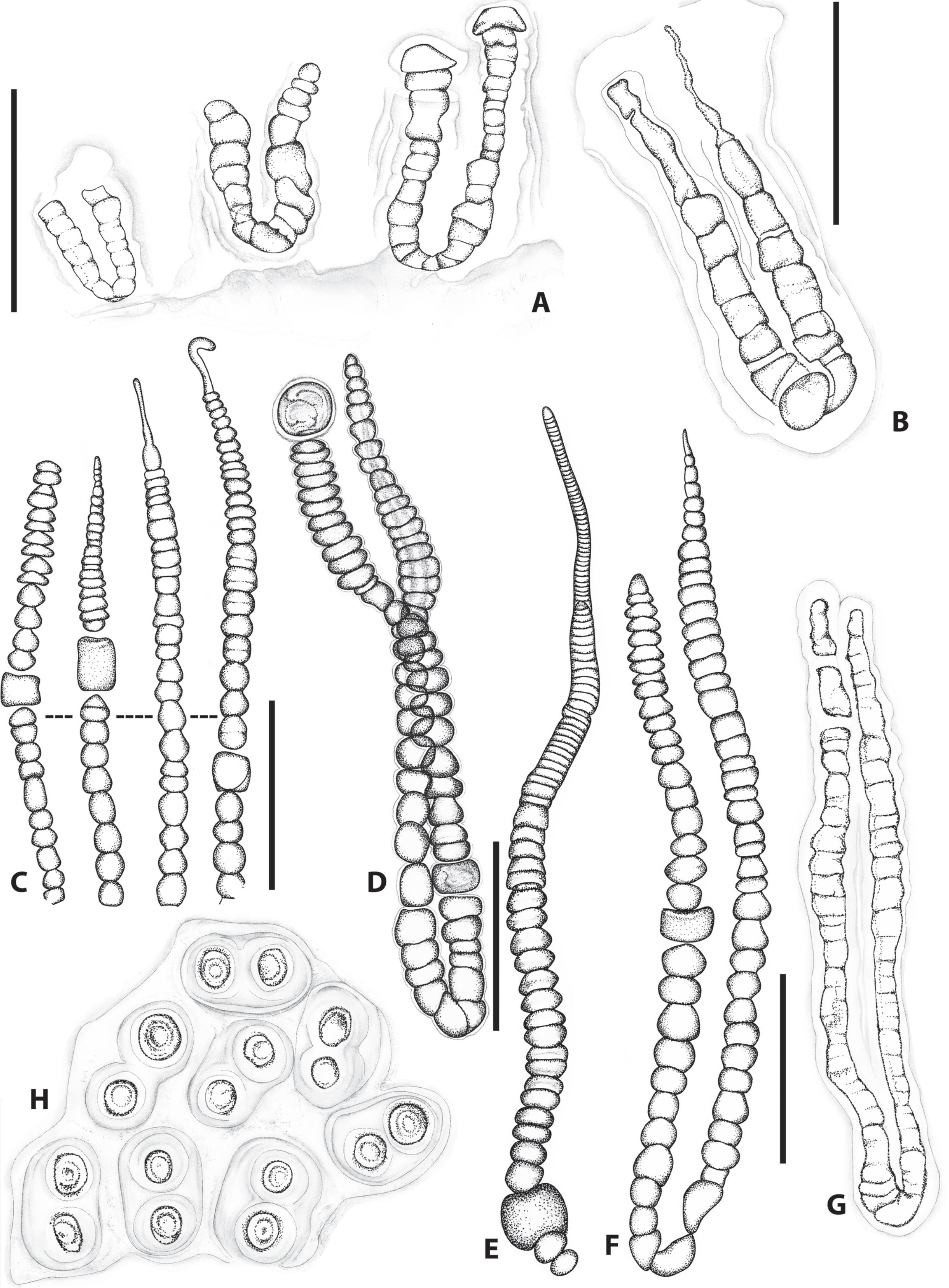

FIGURE 2. A–H. Drawings of *Kyrtuthrix huatulcensis* field material. A. Different stages of young already bent filaments in lateral view. B. Young bent attenuated filament. C–G. Varied examples of the diversity of heterocyte and cell shape, size and disposition. D, F, G. Filaments with loop at the base, tapering upwards. C, E. Filaments tapering towards the upper surface of the thallus. H. Cross section of thallus showing paired cells of bent trichomes surrounded by individual and paired sheaths. A. Scale bar A=20 µm, B–H 10 µm.

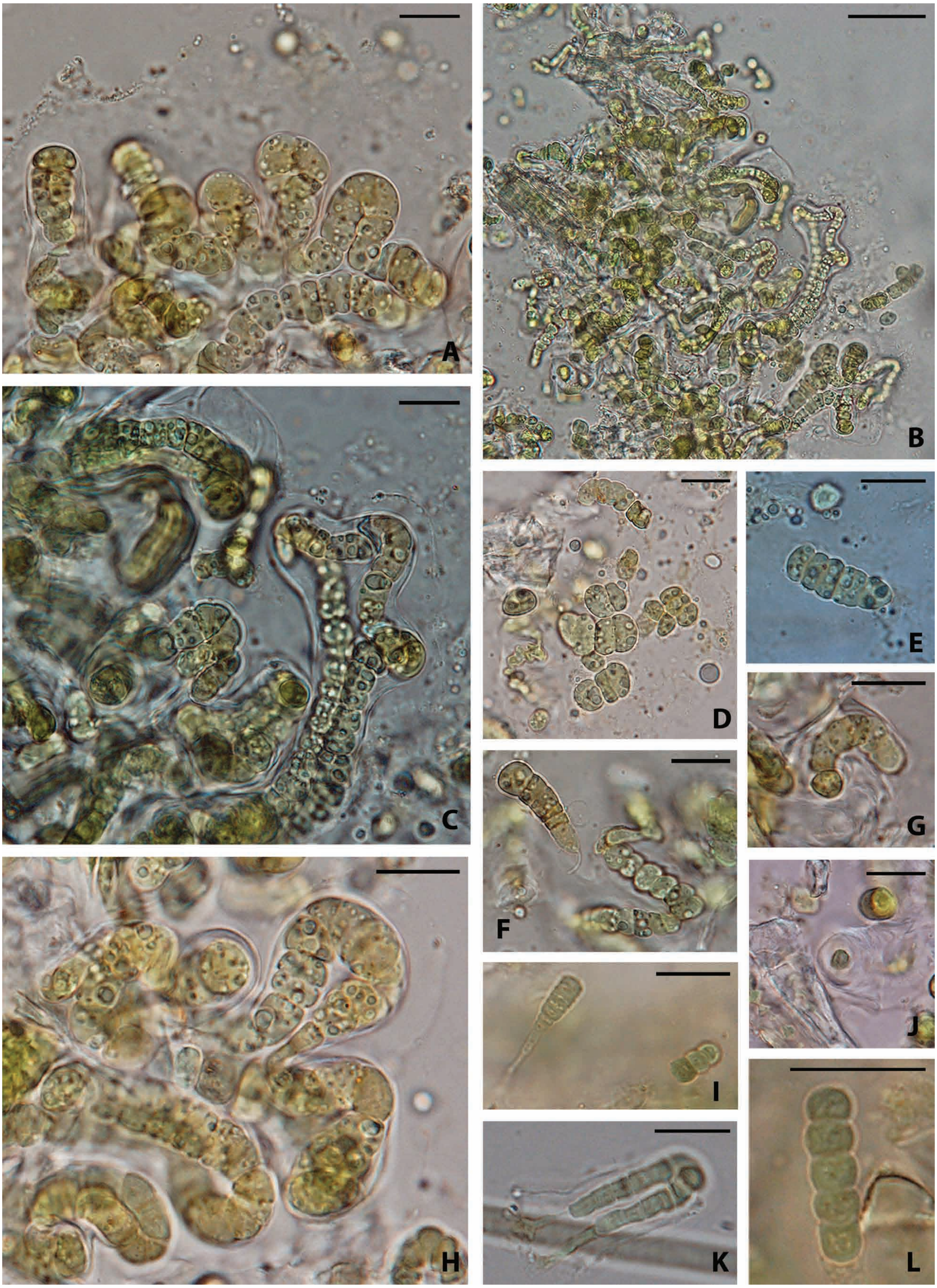

FIGURE 3. A–L. *Kyrtuthrix huatulcensis* A–J culture material. K, L field material. A–C, H. Lateral view of growth form in culture showing both individual and paired sheaths as well as tortuous filaments with loops, surrounded by common mucilaginous matrix. D, E. Two to few celled isopolar hormogonia in culture. F, G, I. Short heteropolar hormogonia. K. Young filament already bent. L. Isopolar hormogonia from field material. Scale bar A=20 µm, B–H 10 µm.

Phylogenetic analyses:—*K. huatulcensis* clearly belongs to the Rivulariaceae (Fig. 4). This clade contains tapering heterocytous forms that in our analysis are all marine in origin. This clade had good support (92/98/0.63, MP/ML/BI, respectively). Rivulariaceae in its historical sense includes *Calothrix* (Komárek *et al*. 2014), but in our analysis the clade that includes both marine Rivulariaceae and *Calothrix* is not supported (-/-/0.55), even though *Calothrix* is shown in a position sister to the Rivulariaceae. However, regardless of the eventual circumscription of the Rivulariaceae, we have clear evidence from the molecular data that *Kyrtuthrix* belongs in the Rivulariaceae *sensu stricto*, and certainly is not in the Scytonemataceae, Stigonemataceae or Mastigocladaceae (=Hapalosiphonaceae in modern taxonomy, see Komárek *et al*. 2014) as proposed by earlier workers (Ercegovic 1929a, Umezaki 1958, Komárek & Anagnostidis 1989, Komárek 2013). The taxonomic identity of some of the strains in our clade designated "Rivulariaceae" is uncertain. There is apparent confusion in differentiation of *Calothrix, Rivularia*, and *Microchaete*. The strains in the three lineages labeled "Marine Rivulariaceae I, II and III" are based on our material, and they correspond morphologically to "*Brasilonema*" (MR I) and "*Scytonematopsis*" (MRII and MRIII), both of which are very morphologically dissimilar to *Rivularia* (Fig. 4). This clade likely has undescribed species and genera within it, and highlights the need for sequence data for a morphologically well-established European population of the type species of *Rivularia*, *R. dura* Roth ex Bornet et Flahault (1887: 347), so that the other morphologically similar but phylogenetically distinct genera can be properly classified.

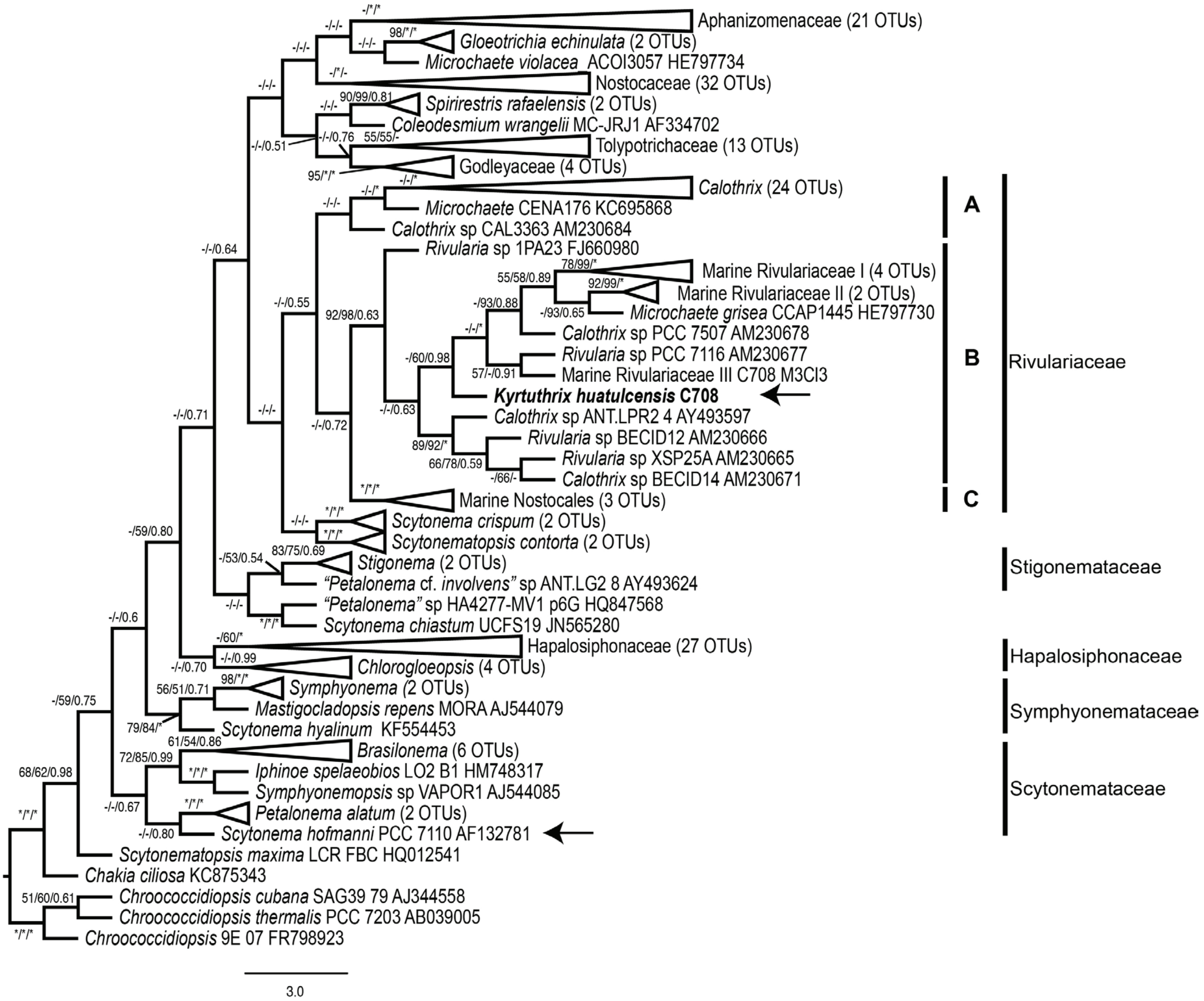

FIGURE 4. Phylogenetic analysis based on 16S rRNA sequences of 181 OTUs showing position of *Kyrtuthrix huatulcensis*. The tree is based on Maximun parsimony (MP) and the support values are MP/ML/ BI posterior probabilities. The cut-off values for bootstrap and probability are 50 and 0.5, respectively, * 100/1; - lower 50 or 0.5. Clades represent different taxonomic groups at family level. Clade Rivulariaceae divided by their environment; A: Soil, Freshwater and marine (brackish), B and C: Marine. Arrows mark both Scytonemataceae, the most recently designated family of *Kyrtuthrix* (Komárek *et al*. 2014) and its present position within the Rivulariaceae clade according to our analysis.

TABLE 2. P distance matrix of *Kyrtuthrix* huatulcensis and related taxa of Rivulariaceae.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Kyrtutrhix huatulcensis* C708 M11														
2	Marine Rivulariaceae III C708 M3CL3	**97.1**													
3	*Rivularia* sp PCC 7116 AM230677	**97.0**	97.6												
4	*Calothrix* sp PCC 7507 AM230678	**97.5**	96.9	96.6											
5	*Microchaete grisea* CCAP1445 HE797730	**98.0**	97.5	97.3	97.7										
6	Marine Riviulariaceae II C708 M10	**98.4**	98.0	97.7	98.1	99.0									
7	Marine Rivulariaceae I	**98.2**	98.0	97.5	98.0	99.1	99.3								
8	*Calothrix* sp ANT LPR2 4 AY493597	**98.1**	96.9	97.2	96.7	98.0	98.0	97.8							
9	*Rivularia* sp BECID12 AM230666	**96.9**	95.9	96.0	95.7	96.9	96.7	96.7	98.3						
10	*Rivularia* sp XSP25A AM230665	**97.6**	96.6	96.7	96.4	97.7	97.5	97.5	99.1	98.2					
11	*Calothrix* sp BECID14 AM230671	**98.1**	97.1	97.2	96.9	98.2	98.0	98.0	99.6	98.7	99.5				
12	*Rivularia* sp 1PA23 FJ660980	**97.0**	95.8	96.1	96.2	96.7	96.5	96.6	96.8	95.6	96.4	96.9			
13	*Rivularia* sp MU24 UAM305 EU009149	**93.9**	92.4	92.8	93.3	92.5	93.1	93.2	92.5	91.4	92.0	92.5	92.0		
14	*Calothrix parietina* 1441A4 AF334695	**92.6**	91.4	92.2	92.3	91.4	92.1	92.1	91.9	90.9	91.5	91.9	91.0	97.2	
15	*Scytonema hofmanni* PCC 7110 AF132781	**91.2**	90.9	92.3	91.3	90.8	91.0	91.0	91.5	90.2	91.0	91.3	91.3	91.3	91.4

The strain of *K. huatulcensis* had 16S rRNA genetic identities of 98% based on p-distance determinations (Table 2) to Marine Rivulariaceae I, Marine Rivulariaceae II, *Calothrix* ANT.LPR2.4, and *Calothrix* BECID 14, and *M. grisea*. These taxa are scattered throughout the Rivulariaceae clade, and in order to consider them the same genus, the entire morphologically disparate clade of Rivulariaceae would need to be placed in a single genus (*Rivularia*?). Since we know a number of these taxa and the morphological differences that separate them, we find this conclusion untenable. Examples of genera in the Nostocales that have genetic identities >98% are known (Flechtner *et al.* 2002, Kaštovský *et al.* 2014). The genetic identities do support our conclusion that *Kyrtuthrix* belongs in the Rivulariaceae and not to other families (Fig. 4), such as the Scytonemataceae (Table 2).

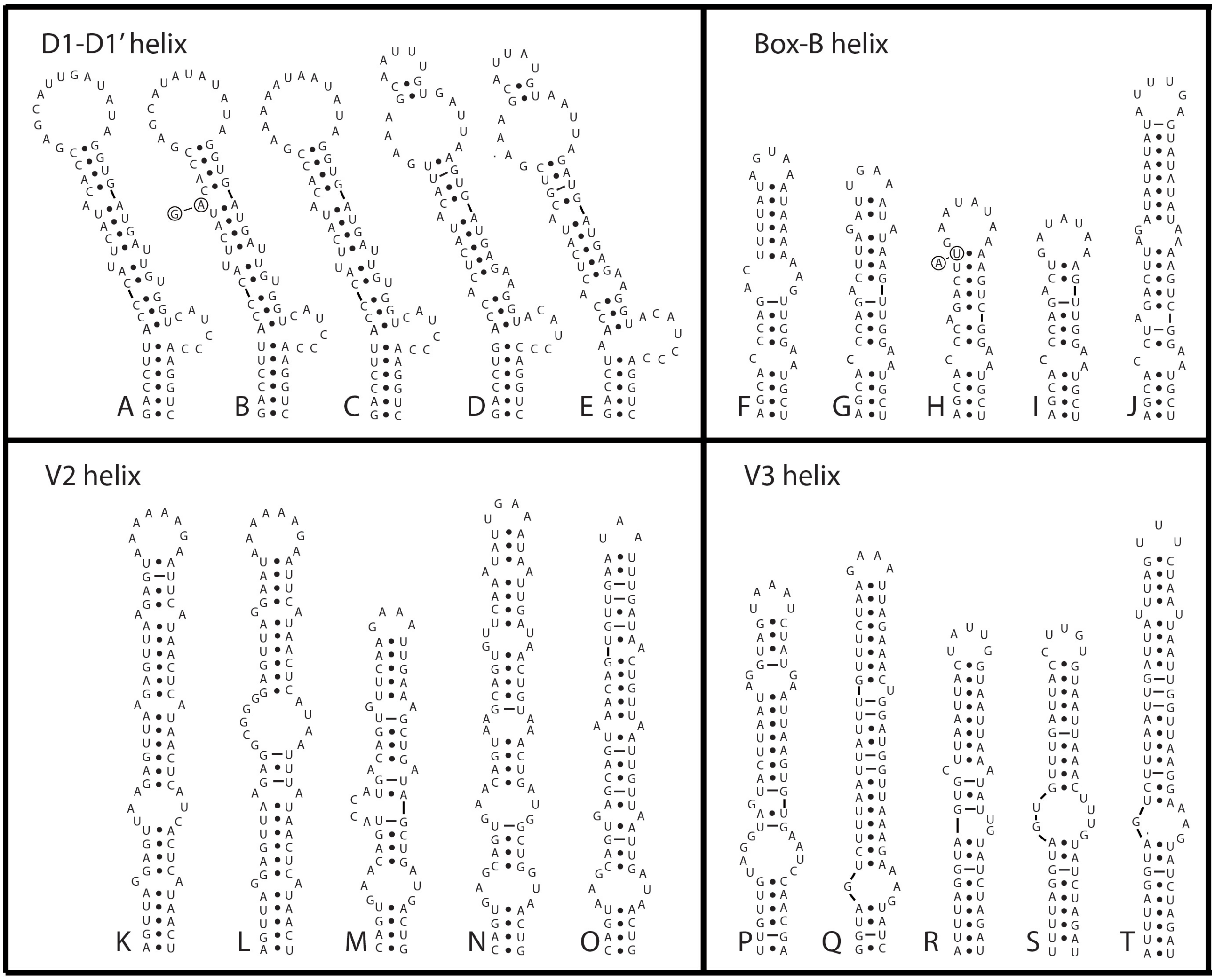

FIGURE 5. Secondary structures for the D1–D1', Box-B, V2 and V3 helices in the conserved regions of the 16S–23S ITS region: A, F, K, P: *Kyrtuthrix huatulcensis*; B, G, L, Q: Marine Rivulariaceae I (with variability in two operons shown); C, H, M, R: Marine Rivulariaceae II; D, I, N, S: Marine Rivulariaceae III; E, J, O, T: *Microchaete grisea* CCAP1445.

16S–23S ITS structure analysis:—The secondary structure of the conserved regions of the ITS for *K. huatulcensis* were most similar to those for strains representing Marine Rivulariaceae I (MRI) and Marine Rivulariaceae II and III (MRII, MRIII) in the phylogenetic analysis (Fig. 4). This was unusual given that these strains appeared more phylogenetically distant based on both the phylogenetic analysis and morphology than the possibly phylogenetically closer strains *Rivularia* PCC 7116 and *Microchaete grisea* CCAP 1445. The D1-D1' helices in *K. huatulcensis* were identical in structure to those for MRI and MRII, but differed markedly from PCC 7116 and CCAP 1445 in both the structure of the apices of the helices and the presence of the A-AA mismatch near the base of the helices (Fig. 5 A–E). The Box-B helices were similar in sequence in the base, but differed markedly in sequence and length for all five taxa in our comparator group (Fig. 5 F–J). The V2 helices were even more divergent in sequence and structure among the five taxa, although it was clear that *K. huatulcensis* had sequence and structural commonalities with MRI that demonstrated possibly recent phyletic separation (Fig. 5 K–O). The V3 helix was also divergent in the five strains, with that of *K. huatulcensis* being very distinct in nucleotide sequence and structure. It is interesting that *Kyrtuthrix* showed greatest similarity to MRI and MRII. MRI morphologically is most similar to "*Brasilonema*", although it is marine in origin, while MRII and MRIII morphologically are closest to "*Scytonematopsis*". Both of these taxa are very morphologically distant from *Kyrtuthix* (González-Resendiz *et al.* 2015).

DISCUSSION

From the analysis of the phylogeny obtained, Rivulariaceae forms a clade with three subgroups that have low support, similar to what occurs in other families of Nostocales, including Scytonemataceae (Fig. 4). It seems that *Rivularia* and *Calothrix* are polyphyletic, as different strains are included in at least two subclades of the family (Fig. 4). This has been reported also by Hauer *et al.* (2014) and Berrendero *et al.* (2016). The type species of both genera: *Rivularia dura* and *Calothrix confervicola* Agardh ex Bornet et Flahault (1886: 349), have not been sequenced. Furthermore, there is insufficient morphological information on many of the sequenced strains to be certain that they are correctly identified. We question the identification particularly for the strains designated *Rivularia* and *Calothrix* in subclade B (Fig. 4), containing *K. huatulcensis*. Clade A, containing 24 OTUs of *Calothrix* is expanded and discussed in Berrendero *et al.* (2016) as three subclades of *Calothrix*, including: Marine I, Marine II, and Freshwater and Soil (Berrendero *et al.* 2016, Fig. 5). Some of the strains identified as *Rivularia* in our phylogeny may well belong in genera other than *Rivularia*, given their proximity and dispersion among other taxa. However, it is likely that *Rivularia* BECID12, *Rivularia* XSP25A, and *Calothrix* BECID14 all belong to *Rivularia* based on morphology documented for these strains. The phylogenetic position of *Kyrtuthrix* among these Rivulariaceae thus indicates that it is in Rivulariaceae *sensu stricto*. According to 16S rRNA data, *K. huatulcensis* is a sister taxon to the group of strains containing *M. grisea* (Fig. 4). As it is the only sequence of *Kyrtuthrix* presently available, and as other populations are putative new but undescribed genera (Marine Rivulariaceae I, II, III) (Fig. 4), examination and characterization of additional Rivulariaceae populations with molecular as well as morphological data is imperative for resolution of the taxa within this family.

The use of 16S and ITS sequences has been demonstrated to be a good tool for differentiating species (Perkerson *et al.* 2011, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014, Bohunicka *et al.* 2015). Our ITS secondary structure results show that the five close comparison taxa certainly represent different species, and based on our experience in other Nostocales (Řeháková *et al*. 2007, Lukešová *et al*. 2009, Kaštovský *et al*. 2014), the differences are sufficiently large to support recognition of different genera. However, greater taxon sampling is needed, as well as more ITS sequences for the strains represented so far only by 16S rRNA gene sequences. Such sequence data for the type species, *K. dalmatica*, is especially critical, but more sampling within the putative new genera (Marine Rivulariceae I, II, III) is also needed in order to establish the degree of difference among species of the same genus as opposed to the degree of difference between species of other genera. We do know that the morphology of the new genera (Marine Rivulariceae I, II, III) is very divergent from *Kyrtuthrix*, and so placing these as species within that genus would destroy the currently clear concept of the genus.

Kyrtuthrix species demonstrate a complex morphology that involves variable cell shape and trichome disposition according to the life form (endolithic *vs* epilithic), habitat (supralittoral to mesolittoral), substratum (calcareous *vs* granite) and region of distribution (temperate *vs* tropical). There are representatives where irregular disposition of contorted filaments is very common and has been associated with the endolithic species *K. dalmatica*. In other populations, parallel disposition of filaments is predominant and can probably be related to an epilithic habit; this is the case for *K. maculans* and *K. huatulcensis.* We consider these three species to be separate taxa, and disagree with Umezaki (1958) in his synonymization of *K. dalmatica* and *K. maculans*.

According to Ercegovic (1929a,b), the bent part of the trichome is the portion that grows and penetrates the rock, forming the meristematic part of the thalli, where cell division in the confined space constrains filaments to create lateral loops. This assumption may be true for endolithic populations but epilithic taxa such as *K. maculans* and *K. huatulcensis* do not have such pressure on the growth form. So in these two latter species, the loop forming growth form (Fig. 1 A–B, D, F) could generate colonial expansion to the adjacent space through lateral growth whereas colonization of a different space or substratum could be produced by upper surface release of hormogonia and subsequent attachment on uncolonized substratum.

K. huatulcensis clearly represents a new species as it can be distinguished morphologically and ecologically. At a higher rank, some morphological traits at the genus level can be considered diacritical, such as attenuated trichomes, intercalary heterocytes, and loop formation without branching. Parallel disposition of filaments can be also considered diacritical in field material of epilithic types, but it is lost soon in culture material. Concerning the diacritical character of total absence of false branches found in *K. huatulcensis* (field and culture), compared to the original description of both *K. dalmatica* Ercegovic (gemminate) and *K. maculans* Umezaki (V branching) and some reports of *K. maculans* from South America (Chile, Peru, Brasil), this character has to be examined more thoroughly in the future in order to confirm or counterdict records of false branching, which may just represent a morphological misinterpretation of the lateral growth expansion of flexuous trichomes. Phylogenetic results are consistent with inclusion of *K. huatulcensis* within the Rivulariaceae (Fig. 4).

With respect to morphology, the benthic form of life, attenuation of trichomes, and lack of branching agrees with Komarek´s (2013) observation that false branching is apparently facultative within Rivulariaceae. However some important differences with the actual concept of Rivulariaceae (Komarek 2013) are these: filaments are supposedly obligately heteropolar and this genus has isopolar attenuated trichomes that bend in half and are disposed parallely, producing a heteropolar thallus structure. In Rivulariaceae, formation of basal heterocytes is obligate, although some taxa can form intercalary heterocytes as well. In *Kyrtuthrix*, heterocytes are only intercalary. Although we found heterocytes in some hormogonia in culture that appeared terminal, these "terminal" heterocytes were formed in intercalary position, and appear terminal only due to post-formation fragmentation of trichomes. This indicates that some of these diacritical characters within Rivulariaceae should be re-evaluated.

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