Morphology and molecular evaluation of *Iphinoe spelaeobios* gen. nov., sp. nov. and *Loriellopsis cavernicola* gen. nov., sp. nov., two stigonematalean cyanobacteria from Greek and Spanish caves

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Caves have generally been found to host phototrophic micro-organisms from various taxonomic groups, with cyanobacteria comprising an important group that have adapted to these stable and highly specific environments. A polyphasic study based on aspects of classical morphology and molecular data revealed two new monospecific genera from fresh material of Greek and Spanish caves. Both taxa are characterized by obligatory true branching (T-type, V-type and false branching), the presence of heterocysts, and reproduction by hormocysts and akinetes. They shared some similarities in their morphological characteristics as revealed by light, scanning electron and transmission electron microscopy, but phylogenetic analysis based on 16S rRNA gene sequences showed that the two phylotypes were different (89.8 % similarity); this represents an example of shared morphology in genetically different strains of cave-adapted species. Phenotypic and genetic traits strongly support classification of the phylotypes as independent taxa in the order Stigonematales (the most differentiated and complicated group of cyanobacteria), family Loriellaceae Geitl 1925. Hence, the names *Iphinoe spelaeobios* Lamprinou and Pantazidou gen. nov., sp. nov. and *Loriellopsis cavernicola* Hernández-Mariné and Canals gen. nov., sp. nov. are proposed.

Caves represent a stable ecosystem where light controlling the growth of photosynthetic organisms is the limiting factor (Hernández-Mariné & Canals, 1994; Asencio & Aboal, 1996). However, a variety of microhabitats for autotrophic growth is created when adequate natural light reaches the inner part of the caves or when artificial lights are installed.

There have been many studies concerning the photosynthetic microflora of these unique environments worldwide (Borzi, 1917; Chu, 1952; Chu *et al.*, 1991; Friedmann, 1955, 1964; Şerbãnescu & Decu, 1962; Claus, 1962, 1964; Hajdu, 1966; Golubić, 1967; Skuja, 1970; Bourrelly & Dupuy, 1973; Dobat, 1977; Leclerc *et al.*, 1983; Abdelahad, 1989; Sant'Anna *et al.*, 1991; Aboal *et al.*, 1994; Asencio & Aboal, 1996, 2000; Ariño *et al.*, 1997; Vinogradova *et al.*, 1998; Dor & Dor, 1999; Hernández-Mariné *et al.*, 2001; Roldán *et al.*, 2004; Pantazidou & Roussomoustakaki, 2005; Lamprinou *et al.*, 2009; Roldán & Hernández-Mariné, 2009). Despite the fact that Greece has more than 8000 karstic caves, since limestone is the dominant rock over the land area, studies on cave microflora are scarce (Anagnostidis *et al.*, 1982; Iliopoulou-Georgoudaki *et al.*, 1993; Pantazidou, 1996, 1997; Pantazidou & Roussomoustakaki, 2005; Lamprinou *et al.*, 2009). A similar situation is observed in Spain, even though several caves are known, mainly in the Mediterranean area (Gracia-Alonso, 1974; Asencio & Aboal, 1996, 2000; Beltrán & Asencio, 2009; Hernández-Mariné *et al.*, 2001; Roldán *et al.*, 2004; Roldán & Hernández-Mariné, 2009).

Abbreviations: LM, light microscopy; PAR, photosynthetically active radiation; RH, relative humidity; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Iphinoe spelaeobios* LO2-B1 and *Loriellopsis cavernicola* LF-B5 are HM748317 and HM748318, respectively.

New genera of the Stigonematales (*Spelaeopogon*, *Geitleria*) and Chroococcales (*Asterocapsa*), as well as a number of novel species, have been established from cave ecosystems including *Spelaeopogon sommierii* Borzi 1917, *Asterocapsa gloeotheceformis* Chu 1952, *Asterocapsa hyalina* Chu 1952, *Asterocapsa trochiscioides* (Jao) Chu 1952, *Chroococcidiopsis kashaii* Friedmann 1961, *Geitleria calcarea* Friedmann 1955, *Geitleria floridana* Friedmann 1979, *Asterocapsa longipapilla* Liu 1985, *Gloeothece filiformis* Sant'Anna *et al.* 1991, *Herpyzonema pulverulentum* Hernández-Mariné and Canals 1994, and *Symphyonema cavernicolum* Asencio *et al.* 1996.

Species identification within the cyanobacteria is based mainly on aspects of morphology, according to classical literature (Geitler, 1932; Desikachary, 1959; Anagnostidis & Komárek, 1985, 1988, 1990; Komárek & Anagnostidis, 1986, 1989, 1998, 2005). However, the recent application of molecular tools offers the potential to resolve much of the controversy over cyanobacterial taxonomy, evolution and the species concept (Wilmotte, 1994; Hoffmann *et al.*, 2005). As a result, a polyphasic approach to taxonomic identification is currently being adopted.

Using a polyphasic approach, members of two new monospecific genera of Stigonematales, *Iphinoe spelaeobios* gen. nov., sp. nov. and *Loriellopsis cavernicola* gen. nov., sp. nov., are proposed based on examination of natural and cultured material from Greek caves ('Koutouki' and 'Kastria') and a Spanish cave ('Papellona') by light (LM) and electron [transmission (TEM) and scanning (SEM)] microscopy, as well as by 16S rRNA gene sequencing. Both phenotypic and 16S rRNA gene-based phylogenetic analyses confirm that the two new genera belong to Stigonematales, the most differentiated and complicated order of the cyanobacteria (Anagnostidis & Komárek, 1990).

Cave 'Koutouki' (37° 56′ 45.84″ N 23° 49′ 43.46″ E), a popular tourist spot near Paeania, is on the eastern side of Mount Hymettus (Attica, Greece). It is a vertical cave with an entry shaft 38.5 m deep, opening at the bottom into a single huge cavern (60 × 60 m). Material was collected from dimly lit sites illuminated with artificial light. Cave 'Kastria' (37° 57′ 37.54″ N 22° 08′ 26.91″ E) is located in Peloponnese, Greece, and is an old subterranean river with an explored length of 1980 m. The part of the cave that is open to tourists is currently 500 m long with a separate entrance. Material was collected from part of the cave that is not open to tourists near the physical entrance. Cave 'Papellona' (41° 17′ 10″ N 1° 54′ 49.5″ E), near Barcelona, Spain, is a sinkhole with a wide and shallow entrance (about 3 m) that is bifurcated at its end into two wings (Papellona in Catalan means butterfly); one wing is practically horizontal and the other one is a well of 3 m depth.

Air temperature (°C), relative humidity (RH; %) and photosynthetically active radiation (PAR; μmol m^{-2} s^{-1}) were measured in Greek caves using a LI-1400 data logger (LI-COR Biosciences) and these parameters were determined in the Spanish cave using a Squirrel Data Logger (Grant).

In cave 'Koutouki' during a 4 month survey, the mean air temperature was 15.7–16.5 °C, the mean PAR was 0.0203–0.0354 μmol m^{-2} s^{-1} and the RH was 92.3–95.1 %. In cave 'Kastria' during a 1 year survey, mean air temperature was 6.31–18.50 °C, PAR was 0.0100–0.7750 μmol m^{-2} s^{-1} and RH was 88.06–97.76 %.

In cave 'Papellona', air temperature gradually stabilized to around 10 °C up to the deepest zone of the cavity (>6 m), RH increased up to 60 % and PAR decreased to below the threshold of the sensor (<1 μmol m^{-2} s^{-1}) (Roldán *et al.*, 2004).

Samples from all caves were taken at different distances from the physical cave entrances and from selected sites inside the caves representing distinct environmental conditions and hosting various cyanobacteria. Sampling from the Greek caves was conducted monthly from May to August 2008 ('Koutouki') or seasonally in 2009 ('Kastria'). Samples from cave 'Papellona' have been collected at irregular intervals since 1988 up to the present.

Collected material was partly fixed with formaldehyde solution at a final concentration of 2.5 % and partly kept alive for culturing. Enrichment cultures were obtained in flasks and Petri dishes with BG11 (Stanier *et al.*, 1971) and BBM (Bischoff & Bold, 1963) liquid media. Cultures were maintained in an incubator (Gallenkamp; Sanyo) under stable conditions and in daylight (north-facing window) at room temperature. Cultures were kept alive for 3 months only; attempts to grow and maintain the species under study in cultures are in progress.

For LM, natural and cultured material was observed on glass slides under a high-resolution light microscope (Photomicroscope III; Zeiss). In order to dissolve sheath encrustation, material was examined after treatment with Pereny's solution (10 % HNO_3, 0.5 % Cr_2O_3, 95 % C_2H_5OH at 4 : 3 : 3) (Bornet & Flahault, 1889).

For SEM, specimens from Greece were dehydrated in an alcohol series (30–100 %), critical-point dried and spray-coated in gold/palladium. They were observed under a JEOL JSM 35 scanning microscope. Specimens from Spain were fixed for 2 days with osmium tetroxide vapour (starting from a 1 % solution), freeze-dried in an Edwards ETD4 tissue drier, gold-coated and observed using a Hitachi-2300 microscope.

For TEM, samples were fixed in a mixture of glutaraldehyde (2.5 %) in 0.1 M cacodylate buffer for 2–4 h, washed three times in this buffer, decalcified by adding Pereny's solution and post-fixed in 1 % OsO_4 in the same buffer. The organisms were dehydrated by a graded acetone series and washed twice in propylenoxide. They were then embedded in three mixtures of propylenoxide and resins (1/0.5, 1/1 and 0.5/1), and finally in Spurr's resins. Surface sections were stained with 2 % uranyl acetate and lead citrate as described

by Reynolds (1963). The sections were examined using a JEOL 1010 TEM at 100 kV accelerating voltage.

DNA was extracted from scraped material from two natural samples in Greece and Spain, using the UltraClean Soil DNA kit (MoBio Laboratories) following the manufacturer's protocol with minor modifications: bead beating was reduced from 10 to 5 min, and this step was immediately followed by three freeze–thaw cycles (−80 °C for 3 min and then immediately transferred to a 65 °C water bath for 5 min) after addition of the inhibitor removal solution. Bacterial 16S rRNA genes were amplified using the bacterial primers GM3 (5′-AGAGTTTGATCMTGGC-3′) (Muyzer *et al.*, 1995) and GM4 (5′-TACCTTGTTACGACTT-3′) (Kane *et al.*, 1993). The PCR included an initial denaturation step at 94 °C for 1 min followed by *n* cycles (see below) consisting of 94 °C for 1 min, 44 °C for 1 min and 72 °C for 3 min, and a final extension at 72 °C for 5 min. The number of cycles was determined after cycle optimization. PCRs were repeated with different cycle numbers and the lowest number of cycles that gave a positive signal (n=22 cycles) was then used for cloning and sequencing in order to minimize PCR bias (Spiegelman *et al.*, 2005). Eight tubes of PCR products were pooled for clean-up and cloning to reduce the bias of each individual reaction.

The combined PCR product was purified using the Montage purification kit (Millipore) and then immediately cloned using the TOPO XL PCR cloning kit (Invitrogen) using electrocompetent cells according to the manufacturer's specifications. A maximum of 24 clones was analysed from each library. These clones were grown in liquid LB medium with kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel) for DNA sequencing. Sequence data were obtained by capillary electrophoresis (Macrogen) using the BigDye Terminator kit (Applied Biosystems) with primers M13F (5′-GTAAAACG-ACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTAT-GAC-3′). Each sequence read was approximately 850 bp. For each individual clone, forward and reverse reads were assembled and then the assembled sequences were checked for chimeras using the CHIMERA-CHECK function of the Ribosomal Database Project II (Maidak *et al.*, 2001). After excluding chimeras and non-cyanobacterial sequences, there were a total of 10 and 18 sequences for LO2 (Greece) and LF (Spain) samples, respectively. The dominant phylotypes were LO2-B1^T (9/10 clones) and LF-B5^T (11/18 clones). Due to this high dominance, no further clones were analysed. The two sequences were compared with the BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for detection of closest relatives. Sequence data were compiled using the software MEGA4 (Kumar *et al.*, 2008) and aligned with sequences obtained from GenBank (www.ncbi.nlm.nih.gov) using CLUSTAL_X. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in MEGA4 (Kumar *et al.*, 2008). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates per dataset, each followed by tree bisection-reconnection topological rearrangements. Tree topology was based on neighbour-joining according to Jukes–Cantor. Bootstrapping under parsimony criteria was performed with 1000 replicates.

Description of *Iphinoe spelaeobios* Lamprinou and Pantazidou gen. nov., sp. nov.

Iphinoe spelaeobios (I.phi.no′e. N.L. fem. n. *Iphinoe* named after a Greek nymph inhabiting the cave 'Kastria' in Greek mythology; spe.lae.o′bi.os. Gr. n. *spelaion* cave, cavern, grotto; Gr. n. *bios* life, dweller; N.L. n. *spelaeobios* inhabitant in caves).

The thallus creeps on the calcareous substrate as a white-silver to purple coating. Filaments are 7–10 (15) μm wide, interwoven with the nostocalean cyanobacterium *Scytonema julianum* (Kütz) Menegh. Colour of cytoplasm is purple-brown; cell content has granulation. Cells are rather cylindrical or doliform, 5–7 μm wide, 6–10 μm long. Apical cells are rounded. Branching is mainly of T-type, rarely of V-type. Heterocysts are intercalary and cylindrical. Reproduces by hormocysts and akinetes.

The phylotype strain is LO2-B1, isolated from a Greek cave.

Herbarium of Greece: ATHU-CY 3313M.

Herbarium of Philadelphia (PH), Academy of Natural Sciences: 1088600.

Latin diagnosis of *Iphinoe spelaeobios* Lamprinou and Pantazidou gen. nov., sp. nov.

Thallus tectis calce incrustata crassis adsuper serpit plerumque fulvus et purpureus. Fila 7–10 (15) μm, *colour cytoplasmatis purpureus-fuscus, cellula interiore granulosa. Cellulis praecipue cylindricis vel torulosis*, 5–7 μm *latis*, 6–10 μm *longis*, *apice rotundata. Divergentibus suffultis plerumque modi* T, *rariter modi* V. *Heterocystis intercalaribus cylindricis. Multiplicatione hormocystis et akinetibus. Fila intertexta nostocaleano cyanobacterio Scytonema julianum* (Kütz) Menegh.

Morphology – LM observations (Fig. 1a–g). Thallus creeps on the calcareous substrate as a white-silver to purple coating. Filaments are usually finely calcified (rarely heavily calcified), variously bent and entangled, 7–10 (15) μm wide (mean ± SD is 7.77 ± 0.935 μm; n=35). Sheath is colourless, gelatinous, firm and not lamellated. Trichome is mainly cylindrical, slightly constricted at the cross-walls, or torulose. The colour of the cytoplasm is purple-brown and the cell content is granulated. Cells are rather cylindrical or doliform, 5–7 μm (5.87 ± 0.68 μm; n=35) wide and 6–10 μm (8.43 ± 1.194 μm; n=35) long. Heterocysts are mainly intercalary and solitary in the main axis, and rarely terminal at the ends or at the base of branches; they are 5–8 μm (6.32 ± 0.97 μm; n=35) wide and 5–10 μm (7.47 ± 1.38 μm; n=35) long. Branching is mainly of T-type (lateral), rarely of V-type (dichotomous); false branching is rare, Y-type (reverse-V) is absent, and

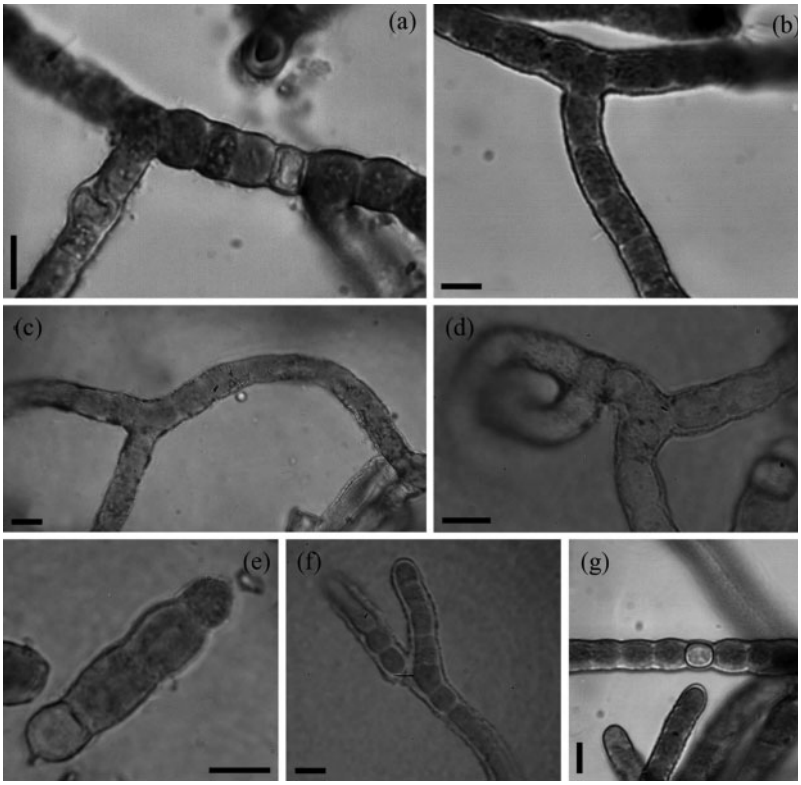

Fig. 1. LM images of *Iphinoe spelaeobios* gen. nov., sp. nov. showing filaments and type of branching (a–d), hormocyst with terminal heterocyst (e), filament with evidence of the presence of false branching (f) and filament with intercalary heterocyst (g). Bars, 10 μm.

secondary branching is found in both directions with abnormal frequency. Reproduction occurs by uncalcified hormocysts and akinetes. Hormocysts are commonly formed at the end of branches, consisting of 3–8 cells (20–75 μm long) with terminal heterocysts (5 μm wide and 5–6 μm long); they have the same granulation and colour as the filaments but the sheath is slightly wider. Akinetes are spherical to oblong–spherical, up to 14 μm wide, 1–3 in a row and bigger than ordinary cells. Hormogonia are not observed.

TEM observations (Fig. 2a–g). Thylakoids occupy the whole cell area forming blocks of lamellae, sometimes concentrically arranged. No intercellular connections between vegetative cells are present. Trichome disintegration by necridic cells accompanied by mucilaginous biconcave lamellae formation between cells is observed, leading to trichome breakage and false branching (Fig. 2b).

SEM observations (Fig. 2h–k). The sheath is built of small calcite crystals. SEM examination confirms the morphology of the trichomes as being cylindrical or torulose, the dominance of T-type branching (Fig. 2h, i) and the scarce presence of dichotomous V-type branching (Fig. 2k).

Description of *Loriellopsis cavernicola* Hernández-Mariné and Canals gen. nov., sp. nov.

Loriellopsis cavernicola [Lo.ri.el.lop′sis. N.L. n. *Loriella* name of a genus; Gr. fem. n. *opsis* aspect, appearance; N.L. fem. n. *Loriellopsis* that which appears similar to *Loriella*; ca.ver.ni′co.la. L. n. *caverna* a hollow, cavity, cave, cavern, grotto; L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. *cavernicola* inhabitant in caves].

Thallus is expanded, partially endolithic. Creeps on the substrata, sometimes interwoven with other cyanobacteria. Trichomes are uniseriate, surrounded by a mucilaginous sheath, embedded with calcareous deposits at the base. Trichomes are irregularly branched, at the tips (true V-branching) and laterally (true T-branching). Cells are cylindrical or torulose, 4.5–6.5 μm long and violet. Heterocysts are intercalary. Reproduces by hormocysts. A ring of mucilaginous material helps separation.

The phylotype strain is LF-B5, isolated from a Spanish cave.

Herbarium of Spain: BCN-Phyc 6000.

Herbarium of Philadelphia (PH), Academy of Natural Sciences: 1089025.

Latin diagnosis of *Loriellopsis cavernicola* Hernández-Mariné and Canals gen. nov., sp. nov.

Thallus indefinitus, partim endolithicus. Stratum saxis calcareis adpressum, vel inter cyanophyceae. Filamenta cum serie singulari cellularum et cum vaginis mucilaginis incoloratis basi calcareo incrustata. Trichomata irregulariter ramose, terminalia dichotoma et lateralia (T). *Cellulae cylindricae vel torulosae*, 4.5–6.5 μm *longae, violaceae. Heterocystae intercalares. Multiplicatio hormocystis inter quas et filamenta corona mucilagina inflate divisionem fovens.*

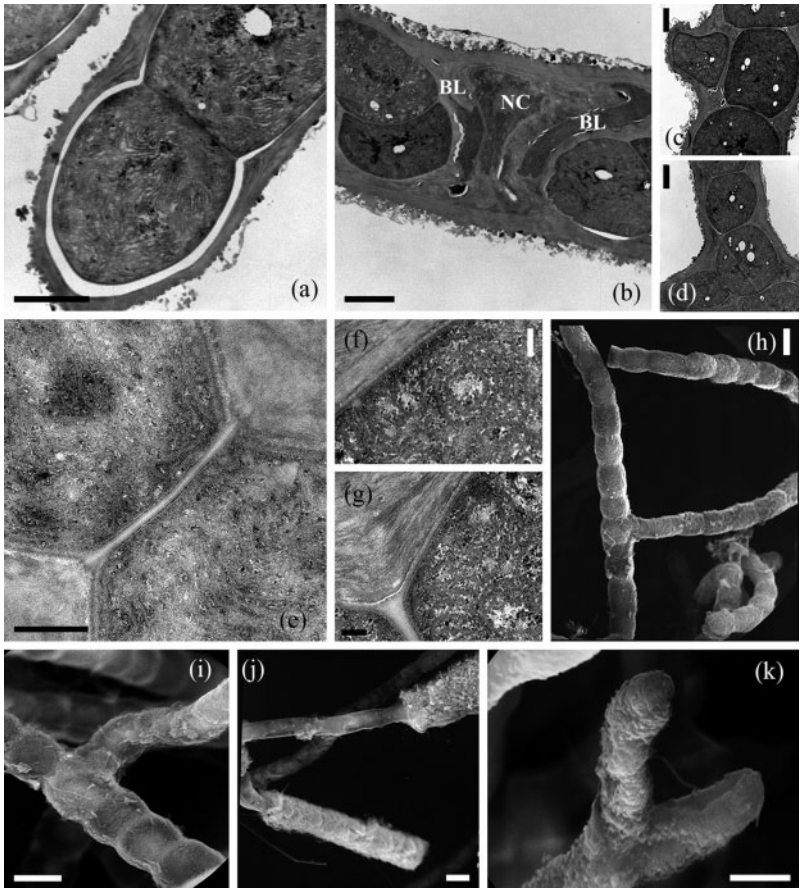

Fig. 2. Micrographs of *Iphinoe spelaeobios* gen. nov., sp. nov. obtained by TEM (a–g) and SEM (h–k): (a) longitudinal section showing thylakoids occupying the whole cell area; (b) section of a filament showing trichome disintegration with the help of a necridic cell (NC), accompanied by mucilaginous biconcave lamellae formation (BL); (c, d) sections of filaments indicating generation of T type of branching; (e–g) septum without intercellular connections between vegetative cells; (h–k) calcified sheaths, cylindrical or torulose filaments and type of branching. Bars: (a–d) 2 μm; (e) 0.5 μm; (f, g) 0.2 μm; (h–k) 10 μm.

Morphology – LM observations (Fig. 3a, b). Thallus is partially endolithic and creeps on the substrata at the cave deep zone, sometimes interwoven with *Geitleria calcarea*. The colour of the trichome is reddish-purple and the cell content bears yellow-green granulation. Trichomes are uniseriate, laterally true-branched and not differentiated into main and lateral trichomes. Cells are of varying shapes and sizes, short barrel shaped [12.3–6.3 μm (mean ± SD is 8.29 ± 1.17 μm; n=30) wide and 6.7–12.6 μm (9.27 ± 1.61 μm; n=30) long] or slightly thinner, doliform to irregular cylindrical cells [3.64–5.9 μm (4.56 ± 0.63 μm; n=30) wide and 4.5–8.5 μm [6.55 ± 2.13 μm; n=30) long]. Terminal cells do not present clear differences in size or shape compared with the rest of the cells. Heterocysts are intercalary and very scarce; they are subspherical or slightly cylindrical, 6 μm wide and 6–8 μm long. Branching is mainly of T-type (lateral), rarely of V-type (dichotomous); false branching is rare, Y-type (reverse-V) is absent and secondary branching is found in both directions with abnormal frequency. Reproduction occurs by fragmentation of tips or branches, helped by a ring of mucilaginous material, giving rise to hormocysts of 2 to 10 cells, with sheaths slightly or not calcified, and akinetes that are isolated or forming chains.

TEM observations (Fig. 3c, d). Distribution of thylakoids is similar to that observed in *I. spelaeobios*. Trichome breakage and release of hormocysts occur by enlargement of sheaths in between neighbouring cells. Necridic cells are not observed. Presence of intercellular or pit connections in between some old cells but not at the septum of the thinner terminal ones.

SEM observations (Fig. 3e, f). No calcification is found on the sheath of terminal cells.

Phylogenetic analysis (Fig. 4)

Phylogenetic analysis showed that the two phylotypes (*Iphinoe spelaeobios* and *Loriellopsis cavernicola*) were different (89.8 % similarity). *Iphinoe spelaeobios* was affiliated within the Stigonematales and was closely related (98.8 % similarity) to an environmental clone (HAVOmat34) from a cyanobacterial mat from a lava cave in Hawaii Volcanoes National Park and also to *Symphyonemopsis* sp. VAPOR1 (98.9 % similarity), isolated from the El Vapor cave, Spain (Gugger & Hoffmann, 2004). *Loriellopsis cavernicola* was not affiliated to any known cyanobacterial taxonomic group and was distantly related (95.4 % similarity) to a different phylotype (HAVOmat106) from the same Hawaii Volcanoes National Park library as above.

Discussion

The described novel taxa of the new genera *Iphinoe* and *Loriellopsis* definitely belong to the order Stigonematales

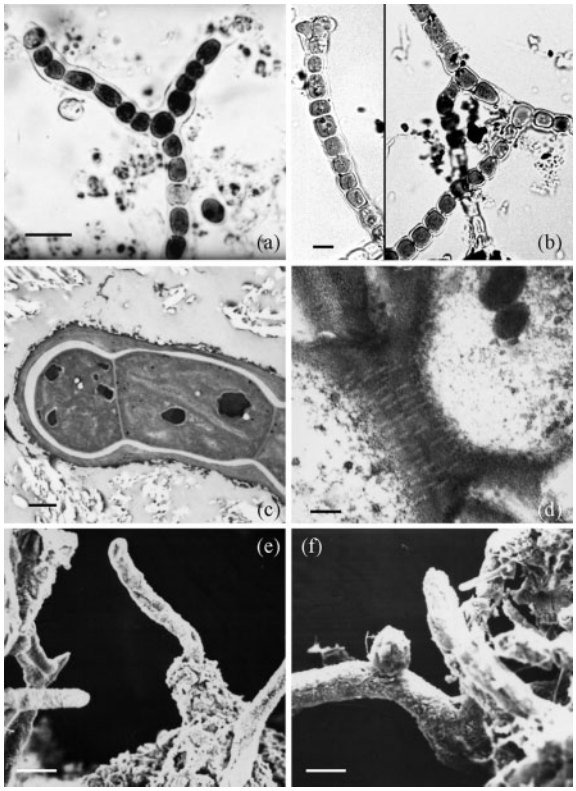

Fig. 3. Micrographs of *Loriellopsis cavernicola* gen. nov., sp. nov. LM images of decalcified material (a, b) showing filament with intercalary heterocyst and V-branching (a) and filaments with a terminal branch division, generating V-branching, and lateral true T and false branching (b). TEM images (c, d) of longitudinal section showing arrays of parallel thylakoids and cyanophycin granules (c) and septum showing intercellular connections (d). SEM images (e, f) showing filaments calcified at the base (e) and a weakly calcified sheath, with development of a lateral branch (f). Bars: (a, b) 10 μm; (c) 2 μm; (d) 0.1 μm; (e, f) 10 μm.

since they display true branching and heterocysts. It is noted that Stigonematales is the most differentiated and complicated group of cyanobacteria, divided into eight families according to Anagnostidis & Komárek (1990); apart from the presence of heterocysts, this order is characterized by various types of true branching (X-, T-, V-, Y-type) (Anagnostidis & Komárek, 1990; Golubić *et al.*, 1996), an obligatory character sometimes combined with false branching.

Specifically, the observed type of branching (mainly T-type, rarely V-type) and the mode of reproduction (hormocysts and akinetes) in *Iphinoe spelaeobios* and *Loriellopsis cavernicola* indicate that they are closely related to the families Loriellaceae Geitler 1925 and Mastigocladaceae Geitler 1925 of the order Stigonematales (Anagnostidis & Komárek, 1990). However, Mastigocladaceae is characterized by Y-type of branching and by differentiation of end trichomes, characters that definitely were not observed in our specimens, indicating that the new taxa should be classified in the family Loriellaceae.

From genera included in the Loriellaceae, the monospecific genus *Loriella* Borzi 1892 (*Loriella osteophila* Borzi 1892), which is characterized by the formation of hormogonia and by terminal, regularly dichotomous branching, is the most related genus to *Iphinoe spelaeobios* and *Loriellopsis cavernicola*. As well as being isolated from material growing on human skulls in Papua New Guinea (Borzi 1892 in Hoffmann, 1990), *Loriella osteophila* was rediscovered in a neighbouring location on limestone material, specifically in low light environments either on fossil coral debris or at the entry of a small limestone cave, and was considered to be endemic to Melanesia (Hoffmann, 1990). It is noted that a second species, *Loriella racovitzae* Şerbănescu in Şerbănescu & Decu (1962) described from caves of Oltenia (Romania), is considered to be a synonym of *Geitleria calcarea* since it lacks heterocysts (Bourrelly & Dupuy, 1973).

Geitleria is another stigonematalean genus described from limestone caves, colonizing the least illuminated areas of the photic zone (type species *Geitleria calcarea* Friedmann 1955). Two species are classified under this genus, *Geitleria calcarea* and *Geitleria floridana*. *Geitleria calcarea* shares some common morphological characteristics with our specimen of *Iphinoe spelaeobios* but the absence of heterocysts is a strong evidence for its classification under a different genus.

It is noted that *Loriella* populations have also been described by Hernández-Mariné *et al.* (1999) from Spanish limestone caves. These populations share some common characteristics with the type species *Loriella osteophila,* i.e. dichotomous terminal branching (V-type of branching) and intercalary heterocysts, but differ in other taxonomic features (presence of T-type branching, reproduction by hormocysts) and habitat. Therefore, these populations were not assigned to *Loriella osteophila* as further molecular investigation is needed. These differences were also observed in the Greek material in this study. Phylogenetic analysis (Fig. 4) based on 16S rRNA gene sequences showed that the two phylotypes (Greek and Spanish) were different (89.8 % similarity).

In conclusion, the type of branching (mostly T-type, rarely V-type and also false branching), the mode of reproduction (hormocysts and akinetes) and the absence of dichotomous terminal branching, as well as the absence of intercellular or pit-connections, clearly differentiate the isolates obtained from the Greek caves from the genus *Loriella*, thus warranting the establishment of a new genus, *Iphinoe* gen. nov.

Furthermore, 16S rRNA gene sequencing of *Iphinoe spelaeobios* revealed high similarity (98.8 %) to an environmental clone (HAVOmat34) and 98 % similarity with a strain (VAPOR1) of the order Stigonematales (Hoffmann *et al.*, 2003), subsequently named *Symphyonemopsis* sp.

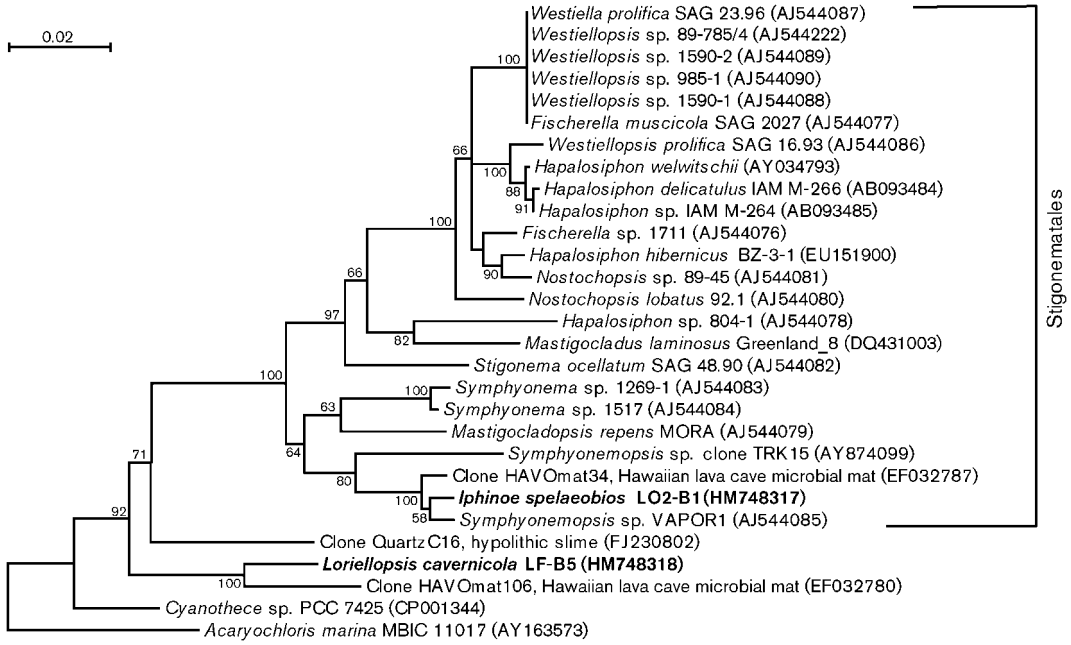

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences showing the distances between the phylotypes *Iphinoe spelaeobios* LO2-B1 (HM748317) and *Loriellopsis cavernicola* LF-B5 (HM748318), and related strains. Bar, 0.02 substitutions per site.

(Gugger & Hoffmann, 2004), that was not similar morphologically to members of any existing genera within the Stigonematales. There are two species within the genus *Symphyonemopsis* Tiwari and Mitra 1968, *Symphyonemopsis katniensis* Tiwari and Mitra 1968 and *Symphyonemopsis pantii* Chadha and Pandey 1978, both established from soil material; this genus is characterized by Y-type branching, multiseriate filaments in all stages, diversification of the main axis from the branches and reproduction by hormocysts and akinetes. Hence, it is a well-defined genus with a morphology that clearly differs from that observed in our material.

Symphyonemopsis sp. VAPOR1 has previously been used in various comparisons (Gugger & Hoffmann, 2004; Korelusová, 2008), but is not considered to be a typical or reference strain of *Symphyonemopsis* and, according to Korelusová (2008), is grouped with the false branched *Brasilonema*, showing possibly the similarities of true and false branching. Due to the absence of fresh and cultured material, it is difficult to adopt a stance on this issue.

The Spanish specimen is established as *Loriellopsis cavernicola*, a name suggesting the growth environment and also a similarity to *Loriella*, although in Borzi's description this genus is characterized only by terminal, regularly dichotomous branching ('*rami terminales regulariter dichotomi*'). Gene sequencing distinguishes this taxon from previously described genera of cyanobacteria. Comparison with database sequences indicated that the 16S rRNA gene of the Spanish material exhibits highest sequence similarity to that of HAVOmat106, the latter described solely on the basis of environmental DNA sequences. The lack of environmental information in the database prevents a further detailed comparison.

The establishment of two new genera in the order Stigonematales, *Iphinoe* and *Loriellopsis*, is based on both the molecular evaluation of genotypes and phenotypic characters, as an integral part of generic definition (Komárek, 2010b) which must be continually corrected and updated (Hoffmann *et al.*, 2005). The species *Iphinoe spelaeobios* and *Loriellopsis cavernicola* represent an example of shared morphology in genetically different strains. Both are components of natural populations in a highly specialized environment, with a disjointed and worldwide distribution (Hernández-Mariné *et al.*, 1999), where dim light seems to be a major stress factor. The morphological similarity with previously reported morphospecies could be attributed to highly convergent forms that can obscure taxonomic relationships among cave-adapted species (Porter, 2007). Additional molecular characters could help in the identification of those morphologically similar types that are indistinguishable or barely distinguishable, known as cryptic species or pseudocryptic species (Casamatta *et al.*, 2003; Komárek, 2010b). In the case of *Loriellopsis cavernicola*, one of those markers could be the presence of intercellular pit-connections. Moreover, the new taxa must have different microclimatic requirements as revealed by the measured physical parameters and the accompanied species. *Scytonema julianum* is an ubiquitous species, able to bear strong fluctuations, whereas *Geitleria calcarea* colonizes the

surface of stone only under highly specific conditions, including dim light (Ariño *et al.*, 1997). At this time, it is not possible to deduce whether the two novel species are ecologically restricted geographical genotypes or ubiquitous ones, as is the case for *Geitleria calcarea* which, in spite of its need for a specialized environment, has been found almost worldwide.

Being aware of the new trends in modern classification of 'Nostocales' and 'Stigonematales', we tried to compare our new taxa with other heterozygous and true branched cyanobacteria. It seems that nowadays, according to the molecular evaluation, heterocystous cyanobacteria represent a monophyletic clade (Gugger & Hoffmann, 2004; Hoffmann *et al.*, 2005; Korelusová, 2008; Komárek 2010a, b; Zammit *et al.*, 2010). However, morphological characters, especially the type of branching, are very important and, as a result, we accept the term 'Stigonematales' according to the botanical code.

Although molecular data seem to be very important for taxonomy, phylogenetic investigation and separation of cryptogenic genera, the use of the 16S rRNA gene alone – particularly in view of the fact that cyanobacteria can contain multiple *rrn* operons and intra-genomic sequence heterogeneity of the 16S rRNA gene – seems to be precarious and, thus, multilocus approaches should be applied.

In conclusion, our knowledge of the taxonomy of cyanobacteria, especially the heterocystous cyanobacteria, is still in its infancy and a polyphasic approach using sequences from many genes combined with cytomorphological, ecophysiological and biochemical markers is necessary for the development of cyanobacterial taxonomy.

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