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Amazonocrinis thailandica sp. nov. (Nostocales, Cyanobacteria), a novel species of the previously monotypic *Amazonocrinis* genus from Thailand

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Cyanobacteria are distributed worldwide, and many new cyanobacterial species are discovered in tropical region. The *Nostoc*-like genus *Amazonocrinis* has been separated from the genus *Nostoc* based on polyphasic methods. However, species diversity within this genus remains poorly understood systematically because only one species (*Amazonocrinis nigriterrae*) has been described. In this study, two novel strains (NUACC02 and NUACC03) were isolated from moist rice field soil in Thailand. These two strains were characterized using a polyphasic approach, based on morphology, 16S rRNA phylogenetic analysis, internal transcribed spacer secondary structure and ecology. Phylogenetic analyses based on 16S rRNA gene sequences confirmed that the two novel strains formed a monophyletic clade related to the genus *Amazonocrinis* and were distant from the type species *A. nigriterrae*. The 16S rRNA gene sequence similarity (<98.1%) between novel strains and all other closely related taxa including the *Amazonocrinis* members exceeded the cutoff for species delimitation in bacteriology, reinforcing the presence of a new *Amazonocrinis* species. Furthermore, the novel strains possessed unique phenotypic characteristics such as the presence of the sheath, necridia-like cells, larger cell dimension and akinete cell arrangement in long-chains and the singularity of D1-D1', Box-B, V2, and V3 secondary structures that distinguished them from other *Amazonocrinis* members. Considering all the results, we described our two strains as *Amazonocrinis thailandica* sp. nov. in accordance with the International Code of Nomenclature for Algae, Fungi and Plants.

Keywords: *Amazonocrinis*; cyanobacteria; ITS secondary structure; *Nostoc*; polyphasic approach; Thailand

INTRODUCTION

Cyanobacteria, as one of the earliest microorganisms, are a most important microbial group that contributes to oxygen production through photosynthesis and nutrient cycling (Brocke et al. 2015, 2018). They are commonly distributed in aquatic, terrestrial, and extreme environments (i.e., deserts, polar regions, hot springs, and endolithic

habitats) (Whitton and Potts 2013, Tashyreva and Elster 2015, Bravakos et al. 2016, Strunecky et al. 2020, Dvořák et al. 2021). Cyanobacteria are the most morphologically diverse phyla of prokaryotes (Komárek 2016). Several studies have found that the morphological characteristics of some cyanobacteria do not reflect evolutionary



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relationships (Řeháková et al. 2007, Hašler et al. 2012, Shalygin et al. 2017, McGregor and Sendall 2021, Szubert et al. 2021). Recently, the polyphasic approach combining molecular, morphological, ecological and other data (ultrastructure, biochemical analysis) has been used for cyanobacterial classification to assist the recognition of new taxa (Komárek et al. 2014, Komárek and Johansen 2015, Komárek 2016). In modern cyanobacterial taxonomy, the 16S rRNA gene sequence data are utilized as molecular evidence that can differentiate cyanobacterial diversity at the genus level (Johansen and Casamatta 2005, Komárek and Mareš 2012). However, using 16S rRNA gene sequences alone for species-level identification may not have enough resolution, and other data are required for species evaluation (Dvořák et al. 2015). The 16S–23S rRNA internal transcribed spacer (ITS) region is also recommended for comparative analysis of secondary structures and offers information concerning divergence when the operational taxonomic units of studied strains are more closely related (Piccin-Santos et al. 2014). Thus, the polyphasic approach has become the basis for cyanobacterial taxonomic revision and the establishment of new taxonomic groups.

The cyanobacterial genus *Nostoc*, the type genus of the family Nostocaceae, has a complicated life cycle, with broad ecological diversity and insufficient morphological traits for differentiating this genus from closely related genera (Hrouzek et al. 2005, Řeháková et al. 2007, Papaefthimiou et al. 2008, Suradkar et al. 2017, Saraf et al. 2019). Furthermore, the genus *Nostoc* is polyphyletic in origin and a taxonomic revision of the genus is required (Hrouzek et al. 2005, Rajaniemi et al. 2005a, 2005b, Komárek et al. 2014). Based on the 16S rRNA gene phylogeny, one of the many clades for *Nostoc*-like strains was estimated as containing a representative of the type-species of the genus and defined as corresponding to the true *Nostoc* clade (also called the *Nostoc sensu stricto* clade) (Hrouzek et al. 2005, Řeháková et al. 2007, Papaefthimiou et al. 2008, Lukešová et al. 2009, Komárek et al. 2014). Thereafter, several *Nostoc*-like genera such as *Aliinostoc*, *Compactonostoc*, *Desikacharya*, *Desmonostoc*, *Minunostoc*, *Mojavia*, *Halotia*, *Komareliella*, *Parakomareliella*, *Pseudoaliinostoc*, *Purpureonostoc*, *Violetonostoc*, *Amazonocrinis*, *Atlanticothrix*, and *Dendronalium* have been separated from the genus *Nostoc* by their distant position in phylogenetic analysis, morphological markers and other information including 16S–23S rRNA ITS sequences, ecological traits, genomics and in some cases even physicochemical characteristics in polyphasic approaches (Řeháková et al. 2007, Hrouzek et al. 2013,

Genuário et al. 2015, Bagchi et al. 2017, Hentschke et al. 2017, Cai et al. 2019a, 2019b, 2020, Saraf et al. 2019, Soares et al. 2020, Alvarenga et al. 2021, Lee et al. 2021). The genus *Amazonocrinis* with type species *A. nigriterrae* was described based on the polyphasic study results of *Nostoc*-like strains from Brazil (Alvarenga et al. 2021). This genus is morphologically similar to *Nostoc* and has heterocytous filaments with uniseriate trichomes, without branching or meristematic zones, with single-pored heterocytes at just one end and two-pored heterocytes internally presented in trichomes. However, phylogenetic analysis based on the 16S rRNA gene and phylogenomic reconstruction of cyanobacteria genomes revealed that the genus *Amazonocrinis* was positioned distantly from the *Nostoc sensu stricto* (Alvarenga et al. 2021). Studies on *Amazonocrinis* mostly emanate from soil or freshwater samples collected in Brazil. Furthermore, species diversity within the genus *Amazonocrinis* is poorly understood systematically because only *A. nigriterrae* has been described.

Thailand is a tropical hotspot that contains unique biodiversity (Tantipisanuh et al. 2016) and novel cyanobacterial species have previously been described in this area (Komárek et al. 2010, Chatchawan et al. 2012, Kozlíková-Zapomělová et al. 2016, Tawong et al. 2019, 2022). This study presented the novel species within the genus *Amazonocrinis* from Thailand. Two novel *Amazonocrinis* strains isolated from a rice field in the central region of Thailand were characterized using a polyphasic approach. Based on their morphology, 16S rRNA gene sequence, 16S–23S rRNA ITS region secondary structures, the two novel strains presented dissimilar characteristics to the currently described species of the genus *Amazonocrinis*. Thus, we proposed the description of a novel *Amazonocrinis* species as *Amazonocrinis thailandica* sp. nov.

MATERIALS AND METHODS

Isolation and cultivation

A soil sample was collected from a rice field in Manorom District, Chai Nat Province, Thailand in June 2016. The sampling location was a rice field near the confluence of the Chao Phraya and Sakae Krang Rivers. The air temperature while sampling was 32°C. Aliquots of 0.1 mL of collected soil sample dilutions (10^3 – 10^5) were streaked into CT solid medium (Andersen 2005) following the slightly modification using NaNO_3 ($2,260 \mu\text{mol L}^{-1}$) as a nitrogen source until a single cyanobacterial colony ap-

peared. Two strains that displayed *Nostoc*-like morphology were obtained and further cultured in screw-capped glass tubes containing 10 mL of modified liquid CT medium to investigate morphological features and life cycle. The strains were maintained at 25°C under light intensity of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ from white fluorescent lamps with a 12 : 12 h (light : dark) cycle at the Naresuan University Algal and Cyanobacterial Collection, Naresuan University, Phitsanulok and labeled NUACC02 and NUACC03.

Morphological analyses

In both exponential and stationary phase cultures, morphological dimensions (e.g., length and width of the vegetative cells, heterocytes and akinetes) of the studied strains (NUACC02 and NUACC03) were investigated from >50 individuals using an optical photomicroscope BX53/DP72 (Olympus, Tokyo, Japan) with 400–1,000 \times magnification. The microphotographs were analyzed using an AxioVs40x64 v 4.9.10 imaging system (Carl Zeiss, Jena, Germany).

DNA extraction and polymerase chain reaction amplification

Unialgal cultures of strains used in this study were harvested and then rinsed at least three times in distilled water. To obtain the total genomic DNA, the rinsed pellet cell was extracted using the methods described previously by Tawong et al. (2019). Polymerase chain reaction (PCR) amplifications were conducted with the primer set 27F1/1494Rc (Neilan et al. 1997) for the 16S rRNA gene region and the primers 322F/340R (Iteman et al. 2000) for the 16S–23S rRNA ITS regions. The PCR reaction was performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). The amplification program was carried out as follows: one cycle of 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C and then a final 5 min elongation step at 72°C. The PCR products were purified using PureDireX PCR Clean-Up & Gel Extraction Kit (Bio-Helix, Keelung, Taiwan) following the manufacturer's instructions. The purified PCR products were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3730XL DNA analyzer (both from Applied Biosystems, Foster City, CA, USA). All primer sets used in the amplification were used for sequencing, while additional primers CYA359F, 809R, and 740F were also used for the 16S rRNA gene sequencing, as mentioned in Tawong et al. (2019). The nucleotide se-

quences of each primer set were assembled into contigs with 100% overlapping regions, and then the obtained consensus sequence was used for molecular analyses. All sequences in this study were deposited in GenBank under accession numbers LC657918–LC657919 for the 16S rRNA gene sequences and LC657920–LC657921 for the 16S–23S rRNA ITS region sequences.

Phylogenetic analyses

For phylogenetic analyses, the novel 16S rRNA gene sequences obtained in this study were aligned with the reference sequences from the GenBank database based on a BLAST search and previous studies (i.e., Cai et al. 2019a, 2019b, 2020, Alvarenga et al. 2021) using a MAFFT 7.309 (Katoh and Standley 2013). The GTR + G + I as the best-fit model of DNA nucleotide substitution based on the Akaike Information Criterion in MrModeltest v2.4 (Nylander 2004) was obtained for the 16S rRNA gene dataset. The final phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) methods. The ML analysis with 1,000 bootstrap replications was run via RAxML 8.2.9 (Stamatakis 2014). The BI analysis was performed using MrBayes 3.2.7a (Ronquist et al. 2012). Two runs of four Markov chains were executed for 2.0×10^7 generations, sampling every 100th generation. In the BI analysis, the convergence of the runs was assessed using the average standard deviation of split frequencies, potential scale reduction factor (PSRF) and effective sample size (ESS) of all parameters in MrBayes 3.2.7a. The final average standard deviation of split frequencies below 0.01, a value of PSRF close to 1.00 and ESS values of all parameters above 200 were considered as good indicators of convergence. A 25% generation of each run was then discarded as burn-in and the remaining trees were used to calculate a 50% majority-rule consensus tree as well as posterior probabilities. For 16S rRNA gene sequences, genetic distance values (p-distance) were estimated using Mega X (Kumar et al. 2018) and then similarity values [$100 \times (1 - p)$] were also calculated.

16S–23S rRNA ITS structure folding

Secondary structures of the ITS regions of our strains and their related phylogenetical taxa were predicted based on sequence regions of D1–D1', V2, Box-B, and V3 helices. The putative regions found in the sequence were determined using RNA Folding Form V2.3 in the UNAFold (Markham and Zuker 2008).

Pigment spectral characterization

Phycobiliproteins as water-soluble protein pigments were extracted from cultures in 2 mL of 0.01 M potassium phosphate buffer pH 7.0, following Sciuto et al. (2017). The harvested cells were also extracted in 2 mL of methanol to obtain methanol-soluble pigments following Rastogi and Incharoensakdi (2014). Then, all samples were disrupted by freezing (-20°C) for 24 h and thawing at room temperature for 24 h. Cell debris was removed by centrifugation at 4°C and $10,000 \times g$ for 10 min. All absorbance spectra of each sample were recorded using a UV-1800PC Spectrophotometer (Shimadzu Corp., Kyoto, Japan) in the range 250–750 nm. Absorption spectra of the two different extracts were compared with the reference information described by Pagels et al. (2019) and Roshan et al. (2015).

RESULTS

Amazonocrinis thailandica Tawong, Pongcharoen, Pongpadung, Ponza & Saijuntha sp. nov. (Fig. 1)

Description. Macroscopic colonies on solid agar are brown and amorphous with a smooth surface enclosed by mucilage. In liquid cultivation, they grow as gelatinous biomass on the bottom of the test tube or attached to the tube walls as irregular clusters enclosed by diffluent mucilage. The filaments are uniseriate, densely or loosely agglomerated in the diffluent mucilaginous matter, irregularly flexuous with akinete-like cells, blue-green to brown-reddish, sometimes interleave with purple cell. The sheath surrounding the filaments is mucilaginous, hyaline, and thick or thin, sometimes absent. The trichomes are cylindrical, flexuous and distinctly constricted at cross-walls. Vegetative cells are mostly barrel-shaped to spherical, blue-green to brown, granular in the central region, with distinct chromoplasm and centropoplasm, with dimensions of $3.33\text{--}9.38$ (mean 6.00) μm in length and $3.68\text{--}7.05$ (5.12) μm in width with, $0.59\text{--}2.26$ (1.17) length/width ratio. Heterocytes are terminal and intercalary, blue-green, solitary, very rarely in pairs, variable in shape and size as oblong to spherical and irregularly compressed in flexuous filament or old trichome. Single-pored heterocytes are usually isolated, mostly at the terminal or basal areas and originating from the cells at both sides of dying or releasing two-pored heterocytes cells, with dimensions $3.68\text{--}10.72$ (6.49) μm in length and

$3.80\text{--}7.76$ (5.57) μm in width with $0.74\text{--}2.00$ (1.17) length/width ratio. Two-pored heterocytes are mostly intercalary, $4.90\text{--}10.28$ (7.16) μm in length, $4.53\text{--}7.15$ (5.91) μm in width and $0.80\text{--}1.96$ (1.23) length/width ratio. Akinetes are produced solitary or apoheterocytically in long-chains, intercalary or apical in position with a smooth surface, mostly spherical or subspherical, irregularly compressed in compact old trichomes, bright green or purple, showing high granulation in young akinete cells that developed from vegetative cells in trichomes, larger than the vegetative cells at $4.79\text{--}11.58$ (7.92) μm length, $4.76\text{--}9.92$ (7.15) μm width and $0.64\text{--}2.24$ (1.14) in length/width ratio. Necrotic cells are similar to disc or necridia cells present in the filament. Cell divisions are in one plane forming uniseriate and elongated trichomes surrounded with mucilage layer or forming larger spherical and encapsulated colonies. Reproduction is by germination of akinete cells with releasing new trichomes and by hormogonia, formed by fragmentation at the junction of the necrotic cell. Hormogonia are common, straight, with rounded or irregularly compressed cells.

Diagnosis. Species are delimited by strong molecular and phylogenetic analyses using 16S rRNA gene sequences. The morphology differs from the type species *A. nigriterrae* and *Amazonocrinis*-related cyanobacteria by having large vegetative cells and heterocytes, apoheterocytical akinetes and purple vegetative cells inserted in the trichomes with the presence of sheath and necridia-like cells. The distinctive structures of D1–D1', V2, Box-B, and V3 helices from the 16S–23S rRNA ITS region sequence are also evidence for establishment as a new species of the genus *Amazonocrinis*.

Holotype (here designated). A formaldehyde-fixed specimen of strain NUACC02 was deposited at the Queen Sirikit Botanic Garden Herbarium (QBG), the Botanical Garden Organization, Chiang Mai, Thailand under the designation QBG No. 132169.

Reference strain. *Amazonocrinis thailandica* NUACC02, deposited at the Thailand Institute of Scientific and Technological Research (TISTR) Culture collection, Thailand under the designation TISTR 9505.

Type locality. Found in moist rice field soil 500 m from Chao Phraya River ($15^{\circ}18'33.6''\text{N}$, $100^{\circ}05'12.8''\text{E}$), Manorom District, Chai Nat Province, Central Thailand in June 2016 by Tawong, W.

Etymology. From Latin *thailandica* as the modern name of Thailand related to the location where the new species of the genus *Amazonocrinis* was isolated.

Gene sequences. LC657918 for the 16S rRNA gene region and LC657920 for the 16S–23S rRNA ITS region.

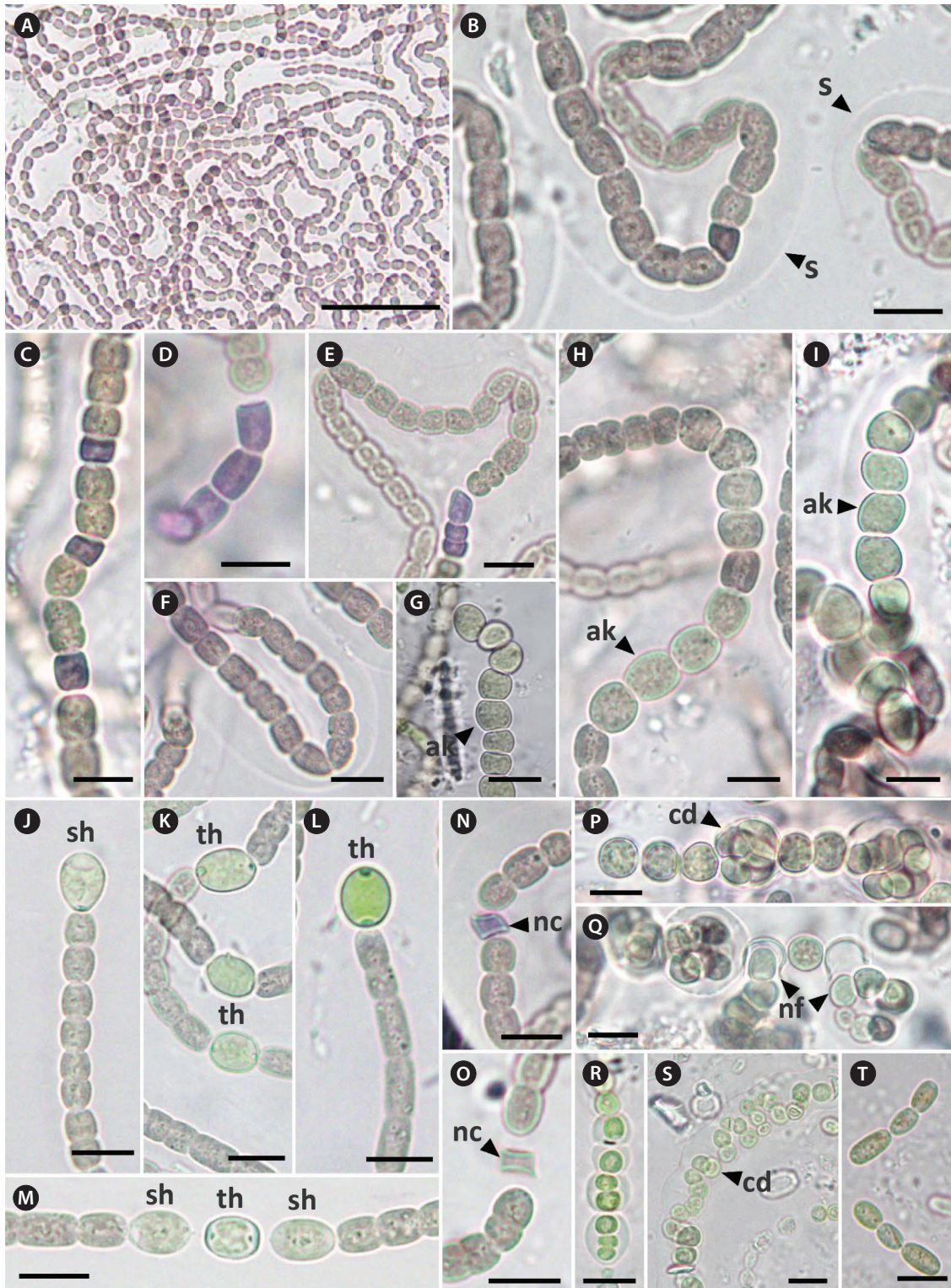


Fig. 1. Micrographs of *Amazonocrinis thailandica*. (A) Filament agglomerate. (B) Filament in mucilaginous sheath (s). (C–F) Filament characteristics showing barrel-shaped vegetative cells interleaved with purple cells. (G–I) Akinete cells (ak). (J) Single-pored heterocyte (sh) in terminal position. (K & L) Two-pored heterocytes (th) in intercalary or terminal position. (M) Single-pored heterocyte (sh) originating from vegetative cells adjacent to both sides of dying or releasing two-pored heterocytes cells. (N & O) Necrotic cells (nc) similar to disc or necridia present in the trichomes. (P) Cell division (cd) forming larger spherical colonies. (Q) New trichome (nf) releasing from akinete cells. (R & S) Cell division (cd) forming elongated trichomes surrounded with mucilage layer. (T) Hormogonia. Scale bars represent: A, 50 µm; B–T, 10 µm.

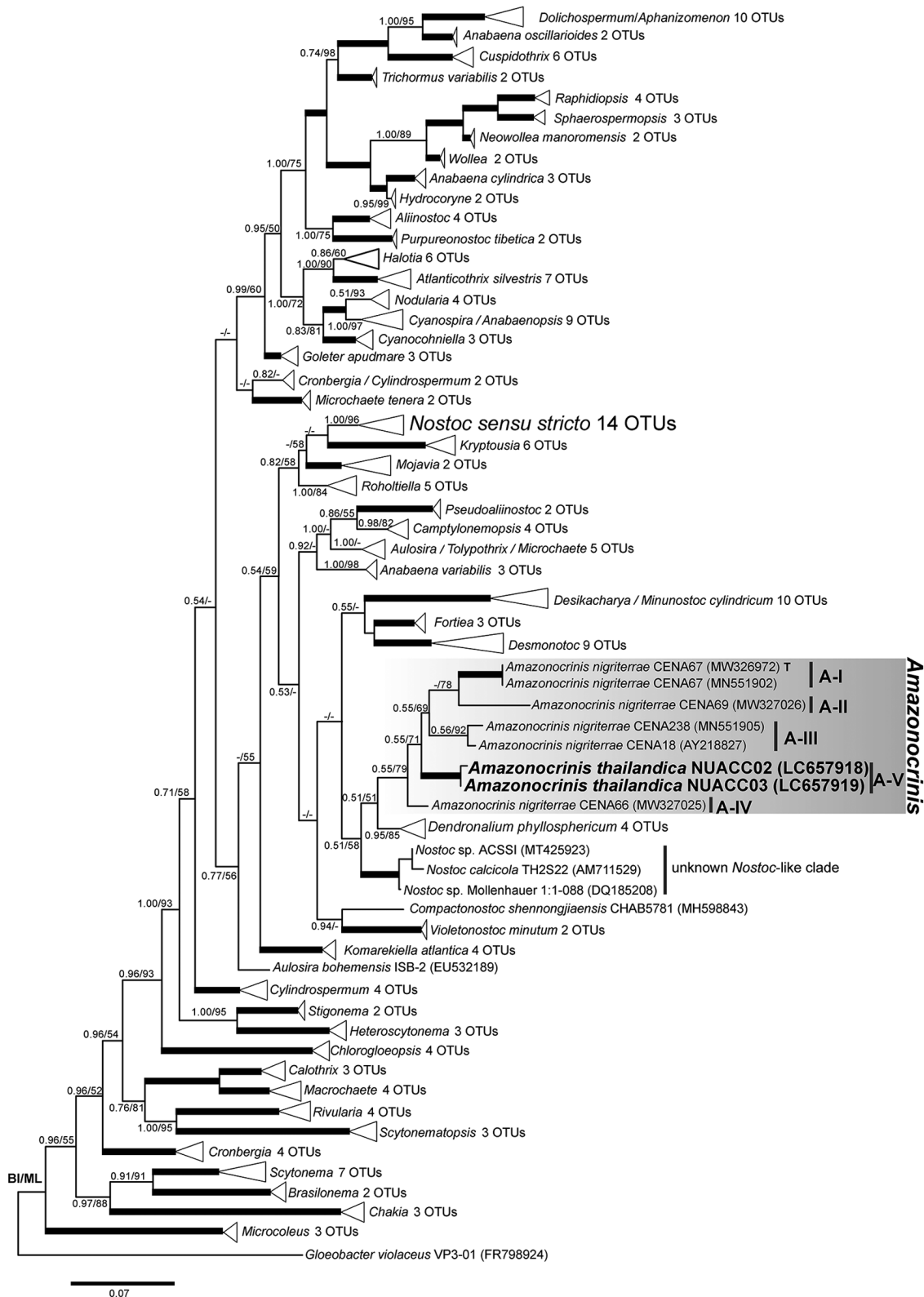


Fig. 2. Maximum likelihood (ML) tree based on 16S rRNA gene sequences showing the phylogenetic position of *Amazonocrinis thailandica*. Numbers at nodes indicate posterior probability (>0.50) and bootstrap values (>50%) obtained from Bayesian inference and ML analyses, respectively. OTU, operational taxonomic unit. Black bars indicate posterior probability of 1.0 and bootstrap values of 100% at the same node. Bold font indicates the strain sequences studied.

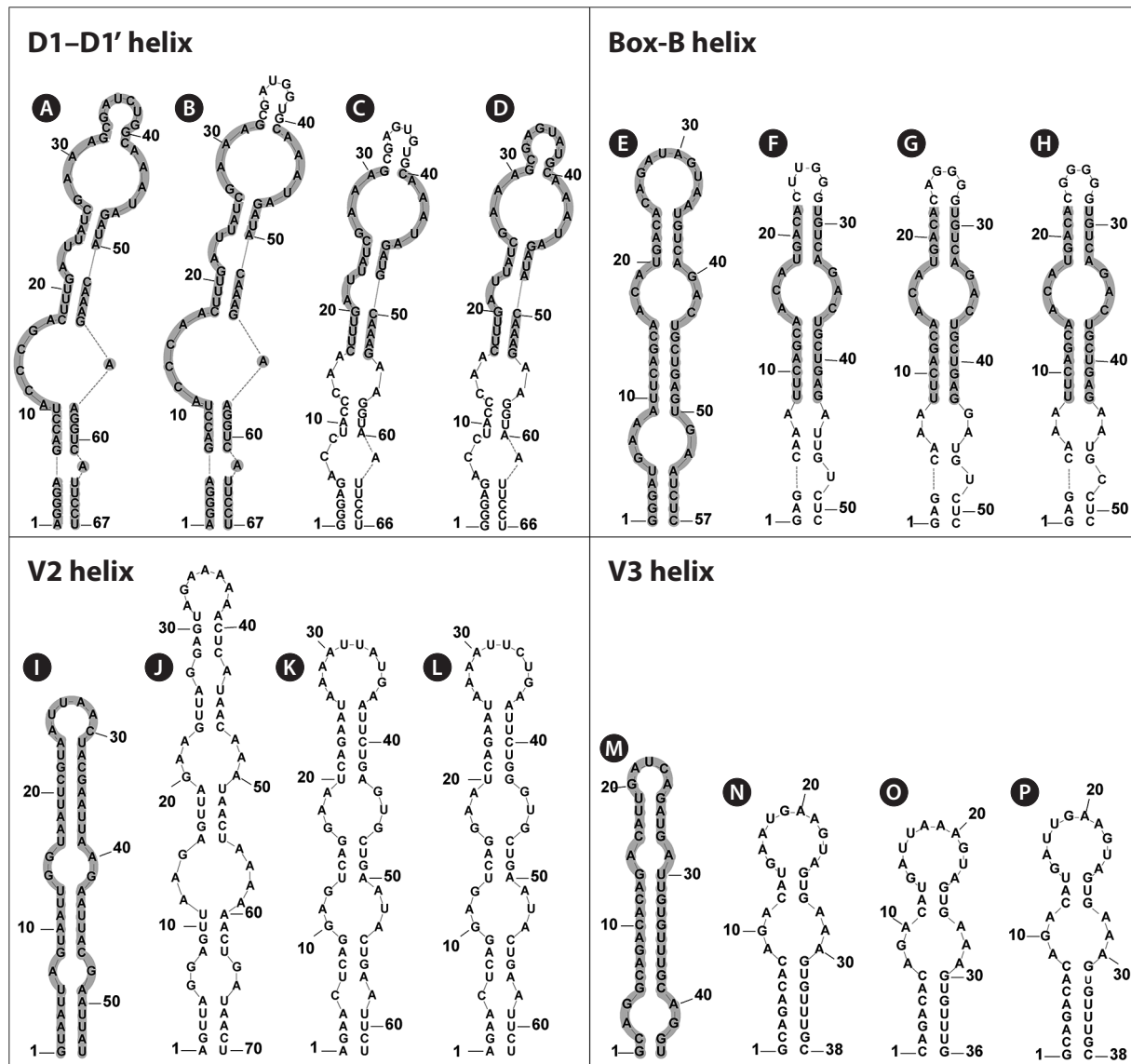


Fig. 3. Reconstruction of secondary structures of D1–D1', Box-B, V2, and V3 helices within the 16S–23S rRNA internal transcribed spacer (ITS) region of *Amazonocrinis thailandica* and related taxa: (A, E, I & M) *Amazonocrinis thailandica* NUACC02. (B, F, J & N) *Amazonocrinis nigriterrae* CENA67. (C, G, K & O) *Amazonocrinis nigriterrae* CENA18. (D, H, L & P) *Amazonocrinis nigriterrae* CENA238. Gray highlights indicate similar structures among *Amazonocrinis thailandica* (A) and other members of the genus *Amazonocrinis* (B–D) for which ITS sequence data are available.

Phylogenetic and molecular analyses

Phylogenetic analyses based on the 16S rRNA gene sequences (1,394 bp) from 211 cyanobacterial taxa including *A. thailandica* strains (NUACC02 and NUACC03) were performed using the BI and ML methods. Because the BI and ML trees have the same topology, only the ML tree is shown in Fig. 2 and Supplementary Fig. S1. The new phylogenetic analyses based on the 16S rRNA gene sequences showed that two *A. thailandica* strains were grouped

within the genus *Amazonocrinis* clade. In this genus clade, five subclades (A-I to A-V) were distinctly subdivided. The first subclade A-I formed a robust clade containing the type species *A. nigriterrae* CENA 67 (MW326972 and MN551902) (BI = 1.00 and ML = 100). This subclade was a sister clade with the second subclade A-II formed by a single strain *A. nigriterrae* CENA69 (MW326026). The third subclade A-III consisted of *A. nigriterrae* CENA18 (AY218827) and CENA238 (MN551905). The *A. thailandica* strains formed a distant and distinct clade as

the subclade A-V with robustly supported values from BI (1.00) and ML (100) methods (Fig. 2, Supplementary Fig. S1). The subclade A-IV containing *A. nigriterrae* CENA66 (MW327025) formed as the earlier divergent species of the genus *Amazonocrinis* clade.

The evolutionary distance matrix based on the 16S rRNA gene sequences of two *A. thailandica* strains shared 99.7% similarity with each other, 96.1–98.1% similarity with other members of the *Amazonocrinis* clade (i.e., *A. nigriterrae* strains CENA66, CENA18, CENA238, CENA67, and CENA238), 96.1% similarity with *Nostoc punctiforme* PCC73102, and 94.4–97.7% similarity with the representative strains of other *Nostoc*-like genera (Table 1).

Characterization of ITS secondary structures

The complete ITS region between 16S and 23S rRNA genes of *A. thailandica* was sequenced and compared with other phylogenetically related taxa for which ITS sequences were available. In this study, only one operon (782 bp) was obtained from both novel strains. Based on Iteman et al. (2000), we identified typical sequence blocks, including conserved domains (D1–D5), major variable stems (V2 and V3), antiterminator (Box-B and Box-A). Furthermore, the 16S–23S rRNA ITS region sequences of *A. thailandica* strains also contained both isoleucine and alanine tRNAs. Putative secondary structures of D1–D1', V2, Box-B, and V3 helices in *A. thailandica* were different and presented unique structures compared with the other strains (Fig. 3A–P). The D1–D1' helix structure of *A. thailandica* was composed of a 5-bp helix basal stem (5'-AGGGA-UUCCG-3'), followed by a single base right

bulge, a 6 : 1 bp bilateral bulge, a 2 base left bulge, a 4 : 5 bp internal loop and a 6 bp terminal hairpin loop (Fig. 3A). The D1–D1' helix structures of *A. thailandica* and *A. nigriterrae* CENA 67 were almost identical but differed in the shape of the terminal hairpin loop. The D1–D1' helix structure of *A. nigriterrae* CENA18 was very similar to *A. nigriterrae* CENA238 but slightly differed in the size of the terminal hairpin loop (Fig. 3C & D). The Box-B helix of *A. thailandica* was comprised of a 5-bp helix (5'-GGGAU-AUCUC-3'), followed by two large internal loops (3 : 2 bp and 3 : 3 bp, respectively) and a terminal hairpin loop with 10 bases (Fig. 3E). Besides differences in nucleotide length, the Box-B structure of *A. thailandica*, particularly the 3 : 2 bp large internal loop and terminal hairpin loop, showed unique characteristics compared to *Amazonocrinis* clade members (Fig. 3E–H). The V2 helix of *A. thailandica* consisted of a 6 bp helix in the base of the stem (5'-GUAAUU-AAUUAU-3') followed by 1 : 1 and 2 : 2 bp internal loops, as well as the terminal hairpin loop, contained 6 bp, which was very different from other phylogenetically related taxa (Fig. 3I). The V2-helix structures of the two *A. nigriterrae* strains CENA18 and CENA238 belonging to the *Amazonocrinis* clade were identical. The V2-helix structure of *A. thailandica* was also very different from *Amazonocrinis* members including *A. nigriterrae* CENA67, CENA18, and CENA238 (Fig. 3I–L). The V3 helix structure of *A. thailandica* was also distinct from all the other taxa (Fig. 3M–P). In detail, the V3 helix of *A. thailandica* consisted of very short stems (5'-GC-GU-3'), followed by a 1 : 1 bp internal loop and further followed by a terminal hairpin loop with 5 bases.

Table 1. Similarity percentage based on 16S rRNA gene sequences for members within the genus *Amazonocrinis* and representative strains of their phylogenetically nearest taxa

Species	1	2	3	4	5	6	7	8	9	10	11	12
1 <i>Amazonocrinis thailandica</i> NUACC02												
2 <i>Amazonocrinis nigriterrae</i> CENA66	98.1											
3 <i>Amazonocrinis nigriterrae</i> CENA18	97.8	99.0										
4 <i>Amazonocrinis nigriterrae</i> CENA238	97.7	98.9	99.3									
5 <i>Amazonocrinis nigriterrae</i> CENA67	96.8	96.7	97.5	97.7								
6 <i>Amazonocrinis nigriterrae</i> CENA69	96.1	97.0	97.5	97.6	97.5							
7 <i>Nostoc</i> sp. Mollenhauer1:1-088	97.7	98.1	98.2	98.1	96.9	96.5						
8 <i>Dendronium phyllosphericum</i> CENA369	97.6	97.9	97.7	97.8	96.8	96.8	97.8					
9 <i>Desmonostoc muscorum</i> Lukesova 2/91	96.8	96.6	96.7	96.6	96.9	97.4	96.9	96.9				
10 <i>Fortia laiensis</i> HA4221-MV2	96.1	96.6	96.1	96.3	95.2	95.4	96.9	97.6	95.9			
11 <i>Nostoc punctiforme</i> PCC73102	96.1	96.5	96.4	96.2	96.5	95.6	97.2	96.0	96.5	96.3		
12 <i>Desikacharya nostocoides</i> BHU1-PS	94.9	95.1	95.0	95.3	95.7	95.4	94.9	95.1	95.2	94.5	95.3	
13 <i>Minunostoc cylindricum</i> CHAB5844-1	94.4	95.3	95.0	94.9	94.7	95.4	93.9	94.5	94.2	94.1	93.9	95.9

16S rRNA gene sequence similarities between *Amazonocrinis thailandica* and phylogenetically nearest taxa are indicated in bold font.

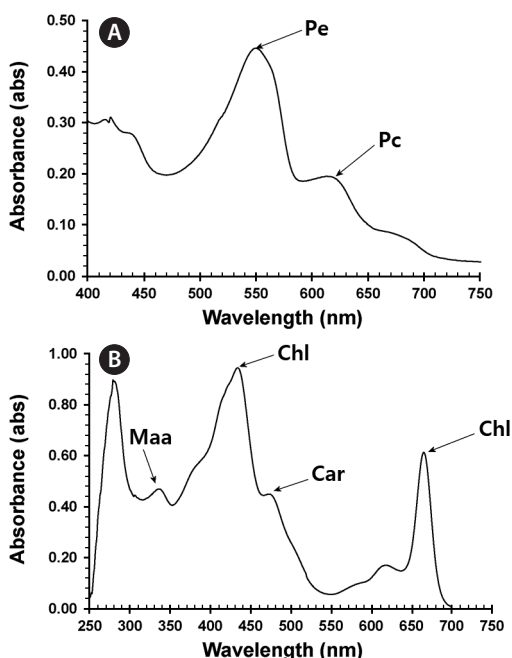


Fig. 4. Absorption spectrum of water-soluble (A) and methanolic extracts (B) of *Amazonocrinis thailandica*, showing the peaks for phycocyanin (Pc, 615 nm), phycoerythrin (Pe, 550 nm), chlorophyll (Chl, 436 and 665 nm), carotenoid (Car, 473 nm), mycosporine-like amino acids (Maa, 337 nm).

Pigment characterization

In the water-soluble extract, *A. thailandica* contained phycoerythrin as the dominant pigment with a high spectrum peak at 550 nm (Fig. 4A). In the methanolic extract, our strains showed general chlorophyll (436 and 665 nm) and carotenoid (473 nm) and also a peak indicating the likely presence of ultraviolet sunscreen pigment such as mycosporine-like amino acids at 337 nm (Fig 4B).

DISCUSSION

This study was carried out to ascertain the taxonomic status of novel *Amazonocrinis* species, named *Amazonocrinis thailandica*, from Thailand using an extensive polyphasic approach. Fiore et al. (2005) first indicated the possibility that an unknown clade containing *Nostoc muscorum* CENA18 (currently named as *Amazonocrinis nigriterrae* CENA18 in this study) would undergo reclassification based on its phylogenetic position. The taxonomic status of this clade was questionable because all contained strains were identified as *Nostoc* species. This unknown clade was later designated as the genus *Ama-*

zonocrinis, and all Brazilian strains in this genus were described and named *A. nigriterrae* as a single species (Alvarenga et al. 2021). However, new phylogenetic analyses based on 16S rRNA gene sequences in this study revealed genetic divergence within the genus *Amazonocrinis*, with five distinct subclades (A-I to A-V). It has now been accepted that cyanobacterial species / genera exist as monophyletic clusters in the newest cyanobacterial taxonomy (Komárek et al. 2014, Dvořák et al. 2015, Komárek 2016, Johansen et al. 2017, 2021, Saraf et al. 2019, Alvarenga et al. 2021). Corresponding to the descriptions above, our results, confirmed by the 16S rRNA gene trees, indicated that *A. thailandica* formed a monophyletic clade that was distant from the type species *A. nigriterrae* CENA67. Findings suggested that *A. thailandica* had unique genetic traits and could be considered as a new species within the genus *Amazonocrinis*. Moreover, our 16S rRNA gene phylogenetic analyses corresponded to previous studies presenting that some sequences identified as *Nostoc* sp. also formed as a monophyletic clade (named as an unknown *Nostoc*-like clade in this study) (Fig. 2, Supplementary Fig. S1); however, this clade has not been investigated to define its taxonomic status (Fiore et al. 2007, Genuário et al. 2007).

The 16S rRNA gene similarity value has been utilized to represent evidence for separate genera or species of prokaryotic organisms including bacteria and cyanobacteria. Several authors suggested that the 16S rRNA gene sequence similarity considered for species delimitation should be below 98.7% (Stackebrandt and Ebers 2006, Kim et al. 2014, Yarza et al. 2014). Later, a <99% 16S rRNA gene similarity cutoff values was suggested to delimit species for nostoclean strains (Kaštovský et al. 2014). In this study, *A. thailandica* shared <98.1% 16S rRNA gene similarity with the closest phylogenetical taxa within the *Amazonocrinis* clade, and less than the species cutoff proposed by Stackebrandt and Ebers (2006), Kim et al. (2014), Yarza et al. (2014), and Kaštovský et al. (2014) (Table 1). This provided strong evidence supporting a species separation of *A. thailandica* from all other *Amazonocrinis* members. Therefore, the evolutionary distance based on the 16S rRNA gene sequence provided molecular evidence supporting the existence of *A. thailandica* as a new species of the genus *Amazonocrinis*.

Additional molecular data from the utilization of ITS secondary structures together with the 16S rRNA gene sequences have been advocated as essential for the recognition of cyanobacterial species (Osorio-Santos et al. 2014, Mai et al. 2018, Cai et al. 2021, Pietrasiak et al. 2021). In particular, the D1–D1', Box-B, V2, and V3 helices in

the ITS region between 16S–23S rRNA sequences are important for the separation of strains in different species (Johansen et al. 2011). Noticeably, among *Amazonocrinis* strains, the presence of a large bilateral bulge in the D1–D1' and the middle bilateral bulge in Box-B were found to be consistent in all four strains studied (Fig. 3A–H). These structures may be unique characteristics of the genus *Amazonocrinis* and can be used to confirm the correction of ITS sequence data. The ITS secondary folding patterns of *A. thailandica* were significantly different from other *Amazonocrinis* taxa and closely related taxa within Nostocaceae reported in previous studies (Osorio-Santos et al. 2014, Mai et al. 2018, Alvarenga et al. 2021, Cai et al. 2021, Pietrasiak et al. 2021), supporting the establishment of this novel *Amazonocrinis* species. *A. thailandica* showed distinctly different structures of the V2 and V3 helices from all other *Amazonocrinis* taxa, highlighting the novelty of our species and supporting the phylogenetic analyses (Fig. 3I–P). Furthermore, the ITS secondary structures exhibited different folding patterns between *A. nigriterrae* strains CENA18, CENA238, and CENA67 indicating a separate species (Fig. 3). When considering the phylogenetic position and lower than the species-level threshold (<98.7%) of 16S rRNA gene sequence similarity (Kaštrovský et al. 2014, Yarza et al. 2014), the four strains

CENA 69, CEN18, CENA238, and CENA66 may be different species within the genus *Amazonocrinis*. Thus, we expect that other *Amazonocrinis* species could be discovered when extra morphological and molecular evidence is available. Increased sampling within the putative new species (A-II to A-IV) would help to observe and identify the divergence within this group.

Morphologically, *A. thailandica* is very similar to *Nostoc sensu stricto* and *Nostoc*-like genera including *Desmonostoc*, *Aliinostoc*, *Minunostoc*, *Halotia*, and *Komarekiella*, because of the overlapping features in all these genera. For example, *A. thailandica* presents diffuent mucilaginous colonies similar to *Minunostoc*, *Halotia*, and *Komarekiella* that differ from typical *Nostoc*, *Aliinostoc*, *Desmonostoc*, and *Mojavia*. Furthermore, cell division in *A. thailandica* occurs by dividing into one plane in trichomes resembling *Nostoc* and *Mojavia*, whereas *Halotia* and *Komarekiella* divide into two planes. *A. thailandica* and *Minunostoc* follow the same type of cell division in trichomes with colonies wrapped by a diffuent mucilaginous sheath but *Minunostoc* does not produce heterocytes and akinete cells throughout the life cycle. Furthermore, our new species also present necrotic cells resembling discs or necridia that may relate to sporogenous cycle stage of *Nostoc* (Caiola and Pellegrini 1979).

Table 2. Comparisons of morphological features of members within the genus *Amazonocrinis*

Strain	Subclade	Filament	Sheath	Cell color	Cell shape / Average size	Heterocytes	Akinetes	Habitat
<i>A. nigriterrae</i> CENA67 (Type species)	A-I	Loosely entangled, flexuous	Absent	Light green, blue-green to brownish blue-green	Rectangular, 4.0 × 2.7 µm	Single-pored usually isolated, basal; two-pored frequent	Absent	Soil
<i>A. nigriterrae</i> CENA69	A-II	Loosely entangled, sometimes circinate	Absent	Blue-green to green	Cylindrical, 2.6 × 1.9 µm	Single-pored isolated, rarely in pairs, basal; two-pored common	Elliptical, up to three in a row, episporic thick striated	Soil
<i>A. nigriterrae</i> CENA18	A-III	Loosely entangled	Absent	Light blue-green to brownish blue-green	Rectangular, 3.8 × 2.9 µm	Single-pored isolated, sometimes in pairs, basal; two-pored common	Absent	Flood plain
<i>A. nigriterrae</i> CENA238	A-III	Loosely entangled, sinuous	Absent	Blue-green to green	Elliptical or cylindrical, 4.5 × 2.7 µm	Single-pored isolated, rarely in pairs, basal; two-pored common	Absent	Fresh-water
<i>A. nigriterrae</i> CENA 66	A-IV	Entangled, flexuous, sometimes circinate	Absent	Blue-green to brownish blue-green	Rectangular, 3.5 × 2.4 µm	Single-pored usually isolated, basal; two-pored occasional	Absent	Soil
<i>A. thailandica</i> NUACC02	A-V	Loosely entangled, flexuous, sometimes circinate	Colorless, mucilaginous, thin or thick	Blue-green to brownish, purple	Barrel-shaped, 6.0 × 5.12 µm	Single-pored usually isolated, basal; two-pored occasional	Elliptical, long chain, green or brown, smooth surface	Soil

Differences in distinguishing *A. thailandica* from the type species *Amazonocrinis* and *Amazonocrinis*-like cyanobacteria include the presence of distinctly large cell dimensions in vegetative cells, purple vegetative cells inserted in trichomes, diffuent mucilaginous sheaths and the presence of large akinete cells (Table 2).

Results of pigment spectra analyses were also in accordance with the type species of *Amazonocrinis* showing purple or brown filaments containing Pe pigment (Alvarenga et al. 2021). Furthermore, the Pe found in our species is a valuable compound that can be applied in various industries such as aquaculture, food, cosmetics, medicine, biotechnology, and pharmacology (Pagels et al. 2019). However, the quality and quantity of light play a vital role in growth and pigment accumulation in cyanobacteria that adapt to different light environments as complementary chromatic adaptation (Hirose et al. 2019). Thus, light influence on phenotype color should be determined.

In terms of ecological information, most members in the genus *Amazonocrinis* occur in soil or sediments, except for *A. nigriterrae* CENA238 which is found in freshwater tropical areas of Brazil. In our study, new species of this genus were found in a rice field in Thailand more than 15,000 km from Brazil. This indicated that the genus *Amazonocrinis* might be found in different tropical zone areas. However, the observed morphological characteristics are insufficient and comparisons between *A. thailandica* and the other members of the genus *Amazonocrinis* remain difficult. Therefore, molecular evidence is an important criterion for distinguishing separate species.

Results revealed intra-species genetic diversity in the genus *Amazonocrinis*. A novel species called *A. thailandica* sp. nov. was described based on molecular and morphological evidence. However, cyanobacterial diversity in tropical regions requires further detailed investigation. Future studies using the polyphasic approach are necessary, with increased sample numbers in this region.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary Fig. S1. Uncollapsed maximum likelihood (ML) phylogenetic analysis based on 16S rRNA gene sequences, with all sequences used to construct the collapsed phylogeny shown in Fig. 2 (<https://www.e-algae.org>).

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