

## **Article**



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# A new species of Aglaothamnion (Ceramiales, Rhodophyta) from Korea, Aglaothamnion inkyui sp. nov.

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#### **Abstract**

The genus Aglaothamnion has been separated from Callithamnion based on several characters, most notably the presence of a single nucleus per cell in Aglaothamnion versus multi-nucleate Callithamnion. Few studies have investigated whether these genera are genetically monophyletic. Several species of Aglaothamnion have been reported from Korea, but no phylogenetic studies have been conducted on them. We collected a specimen resembling the genus Aglaothamnion from the east coast of Korea, which is characterized by alternate branching, long axial cells and uninucleate cells. Phylogenetic analyses using rbcL sequences and two DNA-based species delimitation analyses revealed that this specimen is a new species closely related to A. pseudobyssoides. Morphologically, the new species was distinguished by longer axial cells and shorter determinate branches compared to A. pseudobyssoides. The new specimen did not show any reproductive features. Our new species and A. pseudobyssoides are distantly related to other sequences used in the analysis. Our phylogenetic analyses, using our samples and sequences deposited in Genbank, suggests that the named genera, Callithamnion and Aglaothamnion, are not monophyletic, or that the samples were incorrectly named before being submitted to Genbank. While the taxonomy of these two genera requires further analysis, with more markers and type specimens sequenced, it is clear that our new sample is a distinct genetic species. Therefore, we propose the establishment of a new species of Aglaothamnion from Korea, Aglaothamnion inkyui sp. nov.

Key Words: rbcL, red algae, Aglaothamnion, species delimitation, taxonomy

#### Introduction

The genus Aglaothamnion was established by Feldmann-Mazoyer (1941) for species previously placed in the genus Callithamnion, with the designated type species, A. furcellariae (J.Agardh) Feldmann-Mazoyer (1941: 454), which was later synonymized with A. tenuissimum (Bonnemaison) Feldmann-Mazoyer (1941; 469) (see Furnari et al. 1998). The genus was separated mainly based on the following criteria: 1) uninucleate vegetative cells; 2) 'zigzag' orientation of the cells of the carpogonial branches; and 3) 'lobed', non-rounded gonimolobes (Feldmann-Mazoyer 1941). Vegetatively the genera are very similar with alternately branched uniseriate filaments, with long axial cells. This simple morphology is likely to lead to poor species discrimination (Verbruggen 2014).

The separation of the two genera, into monophyletic genera, based on the presence of a single nucleus per cell in *Aglaothamnion* as opposed to multi-nucleate *Callithamnion* (L'Hardy-Halos & Rueness 1990) has not been well tested. A previous study suggested that while it was possible that *Callithamnion* may be monophyletic, this made *Aglaothamnion* paraphyletic (McIvor *et al.* 2002). This study used a limited data set and suggested that other genera (*Aristothamnion collabens* (Rudolphi) Papenfuss (1968: 268) now *Callithamnion collabens* (Rudolphi) L.M.McIvor & Maggs (2002: 443) were nested within *Callithamnion*. These simple algae require molecular studies to determine their taxonomic placement. The number of cell nuclei in all genera and species of the Callithamnieae, especially

species of *Aglaothamnion* and *Callithamnion*, have not comprehensively been examined, and therefore the evolution of this significant cellular difference is not known. The patterns of reproductive morphology are often not reported as these plants are often vegetative when collected (McIvor *et al.* 2002).

The genus *Aglaothamnion* has been accepted by many phycologists (e.g., Kylin 1956, Itono 1977, Abbott & Hollenberg 1976, Chah & Kim 1998). *Aglaothamnion pseudobyssoides* (P.Crouan & H.Crouan) Halos (1965: 117) was originally described by P. Crouan and H. Crouan (1867) from the Atlantic coast of France, but was considered to be a variety of *A. byssoides* (Arnott ex Harvey) C.F.Boudouresque & M.M.Perret\_Boudouresque (1987: 17) by Miranda (1932). Subsequently, Halos (1965) maintained it as a distinct species, indicating the main distinguishing characters (e.g., globose or subspherical carposporophytes versus strongly lobed gonimoblasts, respectively) between this entity and *A. furcellariae*. Hybridization assays, testing reproductive isolation in laboratory culture, between *A. tenuissimum* and *A. byssoides* indicate that they are conspecific with both reproductively isolated from *A. pseudobyssoides* (Furnari *et al.* 1998).

There are currently 35 accepted names of *Aglaothamnion* (Guiry & Guiry 2024), of these 3 are recorded from Korea: *A. callophyllidicola* (Yamada) Boo, I.K.Lee, Rueness & Yoshida (1991: 305), *A. chejuense* (G.H.Kim & I.K.Lee), *and A. oosumiense* Itono (1971: 221), No new species have been reported since the 2010s. In this study, we examined a new collection of a filamentous red algae tentatively identified as *Aglaothamnion* from Korea. We chose to sequence the *rbc*L gene because previous studies, and available data from other red algae, have shown this gene to be useful for interspecific relationships in other families of the Ceramiales (Kamiya & West 2014, Zuccarello *et al.* 2015, Won *et al.* 2021).

## **Material and Methods**

## Algal sampling cultures

Samples were collected from the rocky intertidal zone of Sungeut Beach (37°49′2.6″N, 128°53′40.5″E), Gangneungsi, Gangwon-do, Korea, on January 6, 2024. Samples were cultured in sterilized seawater with Institute of Marine Resources (IMR) medium (Klochkova *et al.* 2006) at 20 °C in a 16 h light/8 h dark cycle with illumination of >20 µmol photons m<sup>-2</sup>·s<sup>-1</sup> provided by cool white fluorescent lighting. Samples were transferred to fresh medium every 1-2 weeks. Samples including the type specimen were deposited at the National Marine Biodiversity Institute of Korea (MABIK: AL00100606).

## Preparations for microscopy

Samples were examined live on an Olympus BX54 research microscope equipped with differential interference contrast (DIC) optics and Samsung *iPolis* camera (Samsung, Suwon, Korea). For nuclear staining, cells were fixed in culture media by microwave irradiation for a few seconds, and then 1 µl/mL Hoeschst 33342 in IMR medium was added for 15 min in the dark before observation under the microscope. By nuclear staining, we measured the size of 500 nuclei of main axis cells 5-10 cells away from the apex and 500 nuclei of apical cells. Light and fluorescence microscopy images were merged using Adobe Photoshop CS6 software. The images were then analyzed using ImageJ software (NIH, http://imagej.nih.gov/ij/) to quantify the length of the cells and the diameter of the nuclei.

## DNA extraction, amplification and sequencing

Total DNA was extracted from fresh or dried samples using the Chelex method (Zuccarello *et al.* 1999). The partial sequence of the plastid-encoded ribulose-1, 5-bisphophate carboxylase/oxygenase large subunit gene (*rbc*L) was amplified using the primer pairs F321 and R1150 (Kang *et al.* 2020). PCR conditions followed a touchdown PCR as follows: initial denaturation at 94°C for 4 min, followed by 10 cycles of 94°C for 1 min, 55°C for 30sec, and a decrease in annealing temperature by 1°C per cycle, and 72°C for 1 min; followed by 25 cycles of 94°C 1min/45°C, 30sec /72°C for 1 min and a final step at 72°C for 5 min. PCR products were purified using Zymoclean<sup>TM</sup> Gel DNA recovery Kit (Zymoresearch, USA). PCR products were subjected to commercial Sanger sequencing using both sets of PCR primers (Cosmogene Tech, Daejeon, Korea). Sequences were edited and assembled in Geneious® Prime 2024.0.5 (https://www.geneious.com).

Phylogenetic analyses and species delimitation

For phylogenetic analyses, the newly determined *rbcL* sequences and *rbcL* sequences of *Callithamnion* and *Aglaothamnion*, plus related genera were downloaded from GenBank. The data set was aligned using MAFFT in Geneious® Prime 2024.0.5 (https://www.geneious.com) together with our new sequences of *Aglaothamnion oosumiense* collected from Korea (PQ115088), *Aglaothamnion callophyllidicola* collected from Korea (PQ110138), *Agalothamnion tenuissimum* collected from Norway (PQ110137) and *Callithamnion corymbosum* from Japan (PQ110136). Phylogenetic analyses were performed using the maximum likelihood (ML) method with IQ-TREE 2.3.4 (Minh *et al.* 2020). Codon positions were partitioned and the best-fit model was determined using ModelFinder (Kalyaanamoorthy *et al.* 2017) and partition model (Chernomor *et al* 2016). The *rbcL* DNA substitution models were selected for each codon (i.e., first codon: TN+F+I+G4; second codon: JC+I+G4; third codon: TPM3u+F+I+G4) were selected. Support for each internal branch was determined by non-parametric bootstrap (500 replicates) (Felsenstein 1985). Bayesian inference analysis was conducted with MrBayes v. 3.2 (Ronquist *et al.* 2012) with partitioned codons using variable rates and six rate categories. Two parallel runs of Markov chain Monte Carlo were performed for 5,000,000 generations, sampling every 1,000 generations. Estimated sample sizes, split frequencies, and stationarity were checked after each run. After analysis, 10% of generations were removed as a burn-in and the posterior probabilities were visualized in Figtree v1.1.4 (Rambaut 2009).

Two DNA-based species delimitation methods were used to determine species status. For the Assemble Species by Automatic Partitioning (ASAP) method, the Kimura (K80) Ts/Tv 2.0 model was selected and analyzed from the online ASAP online site (https://bioinfo.mnhn.fr/abi/public/asap/). A tree-based method (Poisson-Tree processes, PTP; Zhang *et al.* 2013) was used online using the ML tree topology. The final tree was then edited using Adobe Illustrator 2024 (Adobe, San Jose, CA, USA).

## Results

Phylogenetic analysis

Based on 903 bp of *rbc*L sequences, we performed phylogenetic analyses of 37 species in the order Ceramiales (Table 1, Fig. 1). The analyses of our newly collected *Aglaothamnion* samples revealed it to be well supported sister to *A. pseudobyssoides* (USA sample, AF439308). The interspecific difference between our new specimen and *A. pseudobyssoides* was 2.8 %. This clade of these two species was sister to all the other *Aglaothamnion* and *Callithamnion* in our data set. Both genetic species delimitation methods tested suggest that these two samples are distinct species (Fig. 1).

**Table 1.** Collections of *Callithamnieae* and outgroup taxa from which *rbc*L sequences were obtained.

Species	Location of collection and/or source of culture; collector or depositor	Date	GeneBank Accession NO.
Callithamnieae			
Aglaothamnion bipinnatum	Wembury Bay, Devon, England, United Kingdom	28.07.2003	EU195048
Aglaothamnion byssoides	NA	22.07.2016	AF259489
Aglaothamnion callophyllidicola	Jungdori, Wando, South Korea	28.06.2005	DQ110891
Aglaothamnion callophyllidicola	Eocheongdo Island, South Korea	NA	PQ110138
Aglaothamnion gallicum (Nägeli) Halos ex Ardré	Fanore, Co. Clare, Ireland; CAM	07.02.1999	AF439299
Aglaothamnion halliae (Collins) Aponte, Ballantine & J.N. Norris	Wrightsville Beach, NC, USA; UTEX culture 2294; D. Kapraun, as <i>C. byssoides</i>	NA	AF439305
Aglaothamnion halliae	UTEX culture LB1411; F.D. Ott	NA	DQ110892
Aglaothamnion halliae	Wrightsville Beach, New Hanover Co., North Carolina USA	06.05.1996	DQ022820
Aglaothamnion hookeri (Dillwyn) Maggs & Hommersand	Donaghadee, Co. Donegal, Ireland; R. Wattier	15.31999	AF439296
Aglaothamnion hookeri	Castilo San Cristobal, Canary, Spain	23.04.2004	EU195049

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**TABLE 1.** (Continued)

Species	Location of collection and/or source of culture; collector or depositor	Date	GeneBank Accession NO
Aglaothamnion inkyi sp. nov.	Sungeut beach, Gangwon-do, South Korea	06.01.2024	PQ110135
Aglaothamnion oosumiense	Jungdori, Wando, South Korea	07.01.2010	PQ115088
Aglaothamnion pseudobyssoides (P. Crouan & H. Crouan) Halos	Woods Hole, MA, USA; UTEX culture 2293; M.J. Wynne as	NA	AF439308
Aglaothamnion roseum (Roth) Maggs & Hommersand	Norway; UTEX culture 2292; J. & M. Rueness	NA	AF439297
Aglaothamnion seposium	Port Salon, Donegal, Ireland; CAM	31.01.1999	AF439295
Aglaothamnion seposium	Ona Isl, Mare og Romdal, Norway	NA	DQ110893
Aglaothamnion sp. Spain	Cadiz City, Spain; cultured; J.A. Berges	04.05.1998	AF439298
Aglaothamnion sp. USA	Shark River inlet, Belmar, NJ, USA; UTEX culture 2306; D. Shevlin as <i>C. baileyi</i>	NA	AF439306
Aglaothamnion tenuissimum (Bonnemaison) Feldmann-Mazoyer	Cudillero Harbor, Asturias, Spain; cultured; CAM	06.03.1999	AF439307
Aglaothamnion tenuissimum	Hakonsund, Norway	10.06.2016	MN184538
Aglaothamnion tenuissimum	Norway	NA	PQ110137
Aglaothamnion tripinnatum (C. Agardh) Feldmann-Mazoyer	Milford Haven, Pembrokeshire, Wales; CAM	19.03.2000	AF439309
Callithamnion acutum	Sitka, Alaska, USA	21.06.2005	FJ415860
Callithamnion baileyi	Belmar, New Jersey, USA; UTEX culture 2306; D. Shevlin	10.17.1981	EU195050
Callithamnion consanguineum	Breaker Bay, Wellington, New Zealand	11.09.2002	DQ787565
Callithamnion corymbosum	Oregon, USA	14.05.2001	EU195051
Callithamnion corymbosum	UTEX LB1950	NA	DQ110896
Callithamnion corymbosum	Honcho, Muroran, Hokkaido, Japan	21.06.2024	PQ110136
Callithamnion sp.	Hansuri, Jejudo, Korea	19.11.2004	C1226
Callithamnion sp.	La Boca, Valparaiso, Chile	04.02.2003	C762
Callithamnion collabens (Rudolphi) Papenfuss	S. Africa, sequence courtesy of S. Fredericq and M. Hommersand	NA	AF439303
Callithamnion collabens	Western Cape Province, Yzerfontein, South Africa	25.01.2001	DQ022818
Callithamnion tetragonum (Withering) S.F. Gray	Port Salon, Co. Donegal, Ireland; CAM	31.01.1999	AF439301
Callithamnion granulatum (Ducluzeau) C. Agardh	Cudillero Harbor, Asturias, Spain; CAM	03.06.1999	AF439304
Callithamnion pikeanum	Lone Ranch Beach, Oregon, Josephine Co., USA	18.06.1999	DQ022819
Outgroup			
Cerntroceras micracanthum NA		NA	KP222804
Seirospora interrupta	NA	NA	AF439310

There was no strong support for either of the two genera being monophyletic based on the names of the sequences in Genbank. In fact, nearly identical sequences were listed as the two different genera (*Aglaothamnion* sp. AF439306 and *Callithamnion baileyi* EU195050) (Fig. 1).

Our collection of *Callithamnion* from Japan grouped with *Callithamnion corymbosum* from North America. *Aglaothamnion callophyllidicola* from Korea is closely related to *A. ooosumiense* and neither species delimitation method indicated that they are distinct species although they differ in morphology by having curved branches in the male gametophyte of *A. ooosumiense* (Chah & Kim 1998).

Using our two species delimitation methods, our new *Aglaothamnion* samples from Korea are distinct at the species level from *A. pseudobyssoides*. Therefore, based on the species delimitation methods, variation in *rbc*L and slight morphological differences we propose that our collection is a new species from Korea.

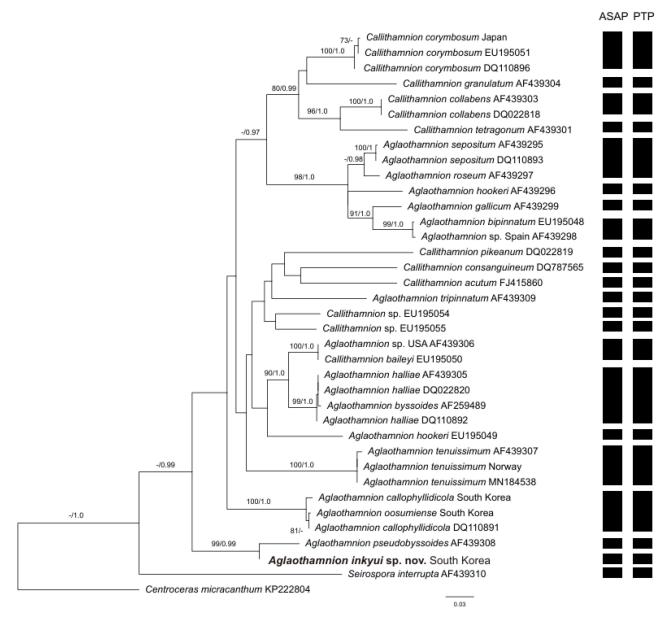
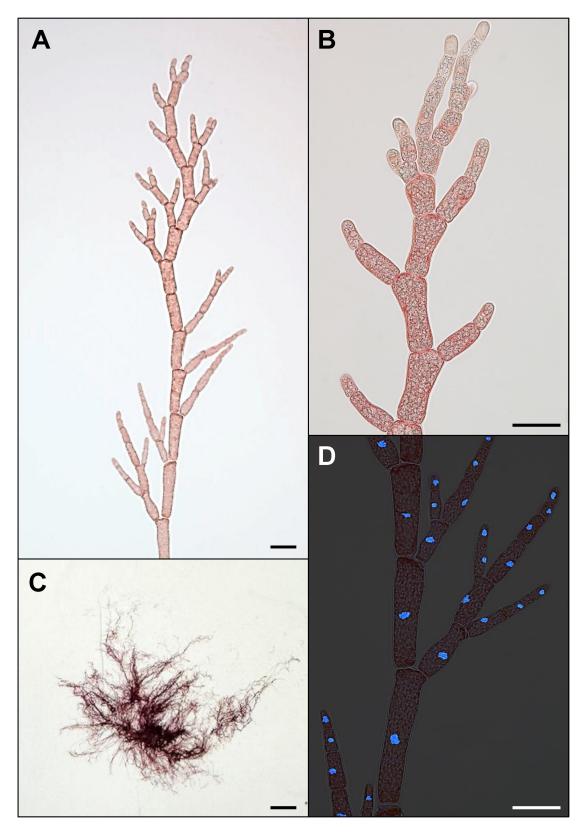


FIGURE 1. Maximum likelihood phylogenetic tree of rbcL from Aglaothamnion inkyui sp. nov. and other species. Only ML bootstrap values  $\geq$ 70% or Bayesian posterior probabilities over >0.90 are shown. The new species is highlighted in bold. Outgroup selected is Centroceras micracanthum.

## Morphological observation

Our culture sample from Sungeut beach grew well in laboratory culture for over 6 months, but never reproduced sexually (Fig. 2). The main axis produced indeterminate branches at the distal end of axial cells alternately, but these were often produced irregularly, missing on cells or on the same side (Fig. 2A). Determinate branches were also produced irregularly. Lateral branches produced branchlets two to three times but did not overgrow the main axis. Samples cultured for more than 2 weeks were observed to grow up to 3-4 cm long, from which voucher specimens were made (Fig. 2C). The apical one is bluntly shaped (Fig. 2B). The cells of the main axis were 30-60  $\mu$ m wide and 300-500  $\mu$ m long and the L/B ratio of axial cells in the central part was 2-8. The cells were uninucleate, with an average nuclear size of 10x15  $\mu$ m in the main axis, while the newly growing cells at the tip had smaller nuclei, averaging 4x6  $\mu$ m (Fig. 2D).



**FIGURE 2.** Morphology of *Aglaothamnion inkyui sp. nov.* in laboratory culture. A. The upper axis with branches arising in zig-zag form. B. The apical cells of the main axis are blunt. C. Pressed holotype specimen. D. One nucleus in each vegetative cell under fluorescence. Scale bars:  $A=50 \mu m$ ; B=1 cm;  $C-D=50 \mu m$ .

## Aglaothamnion inkyui E.Shim & G.H.Kim sp. nov. (Fig. 2)

Description:—The thallus is uniseriate, thin, up to 4 cm high, attached at the base by multicellular rhizoids. No reproductive structures were seen. Branching is dichotomously alternate, sometimes branches appear at irregular intervals at the bottom of the axial cells (Fig. 2A). Freshly collected thalli are reddish in color with a light brown hue (Fig. 2C). Near the base of the thallus, the initial regular dichotomous pattern is sometimes less easy to observe. Branching results in a marked zig-zag arrangement of the axial cells in the apical parts of the thallus. Mature axial cells, ca. 10 cells below the apex, are longer than wide (30-60 mm by 300-500 mm; L/W: 2–8:1). Lateral branching is of 2-3 orders; the apical cell is blunt (Fig. 2B). All cells are uninucleate (Fig. 2D). Th average nuclear size is 10x15 μm in the main axial cells, while the newly growing cells at the tip had smaller nuclei, averaging 4x6 μm.

Distribution in Korea:—Sungeut beach, Gangneung-si, Gangwon-do, Korea (37°49'2.6"N, 128°53'40.5"E)

Habitat:—Mostly found in intertidal rocky substrate. Filamentous thallus attaches to hard substrates, i.e. rocks.

Remarks:—This is a new species record of South Korea (*rbc*L GenBank Accession number: PQ110135). *Aglaothamnion inkyui* was only collected once.

Holotype:—Type specimens are deposited in National Marine Biodiversity Institute of Korea (MABIK: AL00100606).

Isotypes:—Isotypes are deposited in National Marine Biodiversity Institute of Korea (MABIK: AL00100611, AL00100612).

Etymology:—Named in honor of Professor In kyu Lee for his many years of phycological research in South Korea.

## **Discussion**

The new species *Aglaothamnion inkyui sp. nov.* from South Korea is established based on genetic species delimitation methods using *rbc*L sequences. These species delimitation methods are widely used in red algae and other algal groups for species delimitation and/or confirmation (e.g., Muangmai *et al.* 2014, Zuccarello & D'Archino 2022, Wen *et al.* 2023). These species designations lead to further studies of the biology of these newly designated, possibly cryptic, species revealing differences that would not have been studied without this molecular insight (Muangmai *et al.* 2015). While *Aglaothamnion inkyui* is morphologically very similar to *A. pseudobyssoides* further collections many reveal morphological differences between these two species, especially when reproductive stages are found.

We compared some morphological and taxonomic characters between A. inkyui and related species (Table 2). In addition, we have summarised the distribution of Aglaothamnion and Callitamnion species reported from Korea and key bibliographic references (Table 3). In all five species of Aglaothamnion, most characters overlap and there is no character that can unequivocally designate species differences. The only character that seems to be unique in A. inkyui, is the maximum length of the lower axial cells, whether this character is due to the culture conditions or a stable distinguishing character needs to be explored further. The inability to distinguish algal species is a well-known phenomenon, especially in algae with such simple morphologies (uniseriate, alternate branching, naked carposporophytes etc.), and why molecular methods are the only way to determine species diversity (Schneider et al. 2017, Twist et al. 2020, Preuss et al. 2022). We hope that this study will ignite interest in these very beautiful red algae.

Our *rbc*L phylogeny of *Aglaothamnion* and the related genus *Callithamnion*, clearly shows that further taxonomic work is needed in this group. Based on the sequences submitted to Genbank it is unclear whether either genus is monophyletic. The distinction between *Aglaothamnion* and *Callithamnion* is largely based on the uninucleate versus multinucleate nature of the vegetative cell, respectively (Feldmann-Mazoyer 1941). It is not clear whether this character, or the other diagnostic reproductive characters, has been examined in all of the named sequences submitted to Genbank. It is possible that uninucleate or multinucleate cells are not a defining character of any clade. A previous study suggested that at least the multinucleate state could define a clade, although their sampling of species was limited (McIvor *et al.* 2002). The separation of the two genera based on the number of nuclei per cell is not unanimously accepted (Stegenga 1986, Stegenga *et al.* 1997; Womersley & Wollaston 1998). *Aglaothamnion inkyui* and *A. pseudobyssoides* are a sister clade to the other species sampled, and it is possible that reexamination of taxonomy will place these two species in a separate genus. Clearly more work is required.

**TABLE 2.** A comparison of some characters between *Aglaothamnion inkyui sp. nov.* and other related species.

Characters	A. callophyllidicola	A. oosumiense	A. tenuissimum	A. pseudobyssoides	A. inkyui sp. nov.
Type locality	Enoshima, Sagami Province, Japan	Jungdori, Wando, South Korea	Cannizzaro, Catania, Italy	Brittany, France	Sungeut beach, Gangneung, South Korea
Thallus	erect (0.5-2 cm)	erect (2.5 cm)	erect (2-2.5 cm)	erect (2-3 cm)	erect (3-4 cm)
Axis	naked	naked	naked	naked	naked
Lower axial cell	70-100 μm x 200 μm 22.8:1 L/B	80-100 μm x 200 μm 2.5-4.5:1 L/B	60-75 μm x 210 μm 3-3.5:1 L/B	40-60 μm x 200 μm 3.5-5:1 L/B	30-60 μm x 300- 500 μm 2-8:1 L/B
Branching pattern	alternate to subdichotomous	alternate to dichotomous	alternate to subdichotomous	alternate to subdichotomous	alternate to subdichotomous
Lateral branching	3-4 order	3-4 order	2-3 order	2-3 order	2-3 order
Apex	blunt	blunt	blunt	blunt	blunt
Spermatangia	seriate	seriate	seriate	seriate	-
Carposporophyte	lobed	lobed to subspherical	lobed to irregular	subspherical	-
Tetrasporangia	40-55 μm x 60-70 μm tetrahedral sessile	75 μm x 50 μm tetrahedral sessile	75-80 µm x 35-45 µm tetrahedral sessile	40-55 μm x 35-50 μm tetrahedral sessile	-
References	Yamada 1932 Boo <i>et al.</i> (1991)	Chah & Kim (1998)	Feldmann-Mazoyer (1998)	L'Hardy-Halos & Rueness (1990)	This study

**TABLE 3.** Aglaothamnion and Callithamnion species reported from Korea with references to their distribution and main bibliographic references.

Species	Authority	Reference in South Korea	Other Reference
Aglaothamnion inkyui	Shim & Kim	This study	
Aglaothamnion calloph yllidicola	(Yamada) Boo, I.K.Lee, Rueness & Yoshida, 1991	In Kyu Lee 1986, A check list of marine algae in Korea	Boo et al (1991) Zheng, B., Liu, J. & Chen, Z. (2001) Shim et al (2012) Kim, HS. & Lee, I.K. (2012)
Aglaothamnion oosum iense	Itono, 1971	In Kyu Lee 1989, Notes on Marine algae from Korea (III)	Chah & Kim (1998) Kim & Kim (1999) Han <i>et al</i> (2012)
Callithamnion corymbosum	(Smith) Lyngbye, 1819	In Kyu Lee 1986, A check list of marine algae in Korea	Maggs & L'Hardy-Halos (1993)
Callithamnion pinnatum	Womersley, 1998	Yong Pil Lee, 2008, Marine algae of Jeju (Book)	Britain (Dixon & Price 1981), Sweden (Kylin 1944)

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**Author contributions:** All authors conceived the project idea and developed the taxonomic work, interpreted the data, and contributed to the draft and critical review of the final manuscript. All authors discussed the results and contributed to the final manuscript.

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