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RESEARCH ARTICLE

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A New Species and a New Record of *Graphium* from Freshwater Environment in Korea

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ABSTRACT

The genus *Graphium* belonging to order *Microascales*, comprises known wood pathogens that cause sapstain in timbers and wood degradation. However, this genus has been scarcely studied in Korea. Therefore, the current study was conducted to investigate the genus *Graphium* in freshwater environments as new habitat in Korea. Three strains, CNUFC PYW4-15, CNUFC BCW49, and CNUFC BCW48 were isolated from freshwater samples. Based on the morphological characteristics and phylogenetic analysis of the internal transcribed spacer (ITS) and translation elongation factor-1 alpha (*TEF-1α*) gene sequences, the isolated strain, CNUFC PYW4-15 was identified as *Graphium carbonarium* as an unrecorded species in Korea. While the strains CNUFC BCW49 and CNUFC BCW48 were discovered as a new species, named *Graphium aquaticum* sp. nov.

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Microascales; phylogeny; morphology; water environment; *Graphium* spp.

1. Introduction

Freshwater is considered a complex and diverse environment, providing various habitats to fungi [1]. The genus *Graphium* belongs to the family *Graphiaceae*, order *Microascales* and was first described in 1837 by Corda, with *G. penicillioides* as the type species among four others [2]. The genus describtion was later revised based on conidial development and lectotype species were chosen [3]. *Graphium* species are mainly ophiostomatoid fungi found on bark beetles, but their presence in freshwater has not yet been studied. They are morphologically characterized bases on the presence of dark synnemata, spreading black colonies, and mostly aerial mycelium [4]. So far, more than 140 *Graphium* species have been detected while only 20 of them have been published from many different hosts, i.e. lumber, live flora including *Salix babylonica* [5], *Salix alba* [6], baobab trunks [7,8], *Pinus radiata*, spruce trees [9,10], vine leaves [11,12] and the fecal matter of *Lamae guanicoe* monkey, and birds [13], bark beetles (Order: *Coleoptera*, Family: *Curculionidae*, Subfamily: *Scolytinae* [5,14], and humans, including patient with leukemia [15] and those who have undergone a heart transplant [16]. *Graphium* species are weak pathogens that cause sapstain on hardwoods and conifers [8].

In Korea, very little is known about the anamorphic synnematous fungi that colonize coniferous trees. *Pinus radiata* is an important source of lumber in Korea that is known to be susceptible to *Graphium* species [17]. To date, only three unknown *Graphium* species have been reported from Pinus and spruce trees in Korea [10] but none of them belongs to freshwater. Fungi belonging to *Microascales* have been isolated from the insect *Platypus koryoensis*, and from pines and oaks in Korea [14,18]. It has been reported that fungal species isolated from bark beetles express wood-decaying enzymes [14]. *Graphium carbonarium* has been isolated from *Salix babylonica* and *Pissodes* spp. samples from China [5] and also from the heads of female polyphagous shot hole borer beetles from Vietnam [19]. *Graphium basitruncatum* is known to cause skin lesions in leukemia patients [15] and subcutaneous infections in heart transplant patients [16]. *Graphium euwallaceae* together with *Fusarium euwallaceae* are the causal agents of *Fusarium* dieback [20]. *Graphium kuroshium* has been used for pathogenicity tests in avocado plants [21]. *Graphium* species can also grow on short-chain alkanes, ethers, and phenols [22].

To date, no antifungal activity has been reported against *Graphium* species, but avocado rhizobacteria have been used as biocontrol agents

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against *G. kuroshium* [20]. *Graphium jumulu* was first isolated from *Ophiostoma eucalyptigena* and *Adansonia gregorii* in Australia [23]. *Graphium ilexiense* was isolated from *Ilex mitis* wounds [24], while other *Graphium* species including *G. adansoniae* and *G. pseudormiticum* were isolated from bark beetles living on exotic pine trees in South Africa. Hence, all literature indicates that *Graphium* species are associated with the tree trunk and are vectored by insects [5]; instead, the strains studied in the present work were found in freshwater, thus exhibiting different habitat. It is important to identify the *Graphium* species in order to prevent the spread of disease. Thus, the objective of this study is to describe and illustrate a new species and a new record of *Graphium* based on the morphology and multi-locus sequence data (ITS, *TEF-1α*).

2. Materials and methods

2.1. Isolation of fungal strains

Freshwater samples were collected from Pungyeongjeongcheon River, and Byeongcheon stream (Chungnam Province) in June, and August 2018. Samples were collected in small sterile falcon tubes and transferred to the laboratory. Malt extract agar (MEA; 20 g malt extract and 20 g agar in 1 L distilled H_2O), potato dextrose agar (PDA; Becton, Dickinson and Co., Sparks, MD) and sabouraud dextrose agar (SDA; 40 g glucose, 15 g agar and 10 g peptone in 1 L distilled H_2O) were used to plate using the serial dilution method [25]. Individual colonies with different morphologies were transferred to the PDA plates and subcultured to obtain pure isolates. The cultures were maintained in PDA slant tubes and in 20% glycerol at −80 °C under numbers: CNUFC PYW4-15, CNUFC BCW49, and CNUFC BCW48. Ex-type living cultures were deposited at Environmental Microbiology Laboratory Fungarium, Chonnam National University (CNUFC), Gwangju, Korea. CNUFC PYW4-15 and CNUFC BCW49 were also deposited at the Collection of the Nakdonggang National Institute of Biological Resources (NNIBR), Sangju, Korea.

2.2. Morphological studies

All the cultures were three-point inoculated on PDA, oatmeal agar (OA; 30 g oatmeal and 15 g agar in 1 L distilled H_2O), and MEA. All the plates were kept at 25 °C for 8 days in the dark. For morphological comparisons, fungal structures were mounted in lactic acid (60%) and fungal structures were observed and measured under an Olympus BX51 compound microscope with differential interference contrast optics (Olympus, Tokyo, Japan).

2.3. DNA extraction, PCR, and sequencing

The isolated strains were cultured at 25 °C for 8 days on PDA covered with cellophane. Genomic DNA extraction was extracted using the Slog TM Genomic DNA preparation Kit (Solgent Co. Ltd., Daejeon, Korea). ITS and *TEF-1α* sequences were amplified using the primer pairs ITS1/ITS4 [5,25,26] and EF1-728F/EF2 [19,21,27], respectively. PCR reaction for ITS gene was carried out using following parameters: 5 min at 94 °C for initial denaturation, followed by 28 cycles of 30 sec at 94 °C for denaturation, 30 sec at 55 °C for annealing, and 30 sec at 72 °C for extension, with 7 min at 72 °C for terminal extension. PCR reaction to amplify *TEF-1α* gene (primers EF1-728F and EF2) was conducted using the following conditions: 5 min at 94 °C for initial denaturation, followed by 35 cycles of 30 sec at 94 °C for denaturation, 50 sec at 48 °C for annealing, and 2 min at 72 °C for extension, with 5 min at 72 °C for terminal extension. PCR was performed and the products were purified using the Accuprep PCR purification Kit (Bioneer Corp., Daejeon, South Korea). The purified PCR products were sequenced using the same primers pairs on an ABI PRISM 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Phylogenetic analysis

Phylogenetic analyses were conducted using Clustal_X version 2.1 and aligning the fungal sequences with reference sequences obtained from GenBank [28]; the results were edited manually using the Bioedit version 7.2.6.0 [29]. All maximum likelihood (ML) phylogenies were assessed by employing programs available in the MEGA6 software [30]. The sequences of *Ambrosiella xylebori* was used as an outgroup. All the sequences of the isolates in the current study were deposited in the NCBI database under the accession numbers shown in Table 1.

3. Results

3.1. Phylogenetic analysis

The strains were identified based on morphological characteristics and ITS and *TEF-1α* gene sequences. BLASTn analysis revealed that the rDNA ITS and *TEF-1α* sequences of the isolates CNUFC PYW4-15,

Table 1. Accession numbers of fungal strains used for the phylogenetic analysis.

Species	Collection no.	GenBank accession no.	
		ITS	*TEF-1α*
Ambrosiella xylebori	CBS 110.61 ^{T}	NR_144921	KT318385
Graphium adansoniae	CBS 124917 ^{T}	NR_137038	HM630598
G. adansoniae	CMW 30617	GQ200610	HM630596
G. basitruncatum	JCM 9300 ^{T}	NR_111015	KJ131248
G. basitruncatum	JCM 8083	AB038425	n.a.
G. brachiatum	CBS 147987 ^{T}	MF782695	n.a.
G. brachiatum	CBS 147966	ON531996	n.a.
G. carbonarium	CBS 123610 ^{T}	MH863310	HM630603
G. carbonarium	CBS 123611	MH863311	HM630602
G. carbonarium	CXY1701	KM245113	KM245121
G. carbonarium	CXY1700	KM245114	KM245122
G. carbonarium	**CNUFC PYW4-15**	**PQ655121**	**PQ662606**
G. euwallaceae	CBS 140035 ^{T}	KF540224	KF534805
G. euwallaceae	UCR2308	KM592371	KM592363
G. fabiforme	CBS 124921 ^{T}	NR_172291	HM630592
G. fabiforme	CBS 127181	GQ200617	HM630593
G. fimbriasporum	CMW 5605 ^{T}	AY148177	HM630590
G. fimbriasporum	CMW 43240	MH144137	MH124412
G. fructicola	CBS 107.68	MH859078	n.a.
G. fructicola	CBS 217.72	MH860451	n.a.
G. fructicola	CBS 234.48	MH856321	n.a.
G. jumulu	CBS 139898 ^{T}	NR_137980	n.a.
G. kuroshium	CBS 142643 ^{T}	KX262276	KX262286
G. kuroshium	UCR4594	KX262277	KX262287
G. kuroshium	UCR4606	KX262278	KX262288
G. laricis	CMW 5601 ^{T}	AY148183	HM630588
G. laricis	CXY1501	KM245109	KM245117
G. longistipitatum	CBS 147984 ^{T}	MH283079	MH283395
G. longistipitatum	CBS 147985	MH283080	MH283394
G. madagascariense	CMW 30628 ^{T}	GQ200619	HM630595
G. madagascariense	CMW 30629	GQ200620	HM630594
G. penicillioides	JCM 10498 ^{T}	NR_111009	HM630600
G. penicillioides	CMW 5295	HQ335311	HM630601
G. polonicum	CBS 147982 ^{T}	MH283078	MH283393
G. polonicum	CBS 147983	MH283077	MH283392
G. pseudormiticum	CMW 503 ^{T}	AY148186	HM630586
G. pseudormiticum	CMW 41665	MG205680	MG205781
G. putredinis	CMW 352	HQ335312	n.a.
G. radicatum	CBS 147981 ^{T}	MH283071	MH283388
G. radicatum	CBS 147979	MF782698	ON532012
G. radicatum	CBS 147978	MH283072	ON532009
G. scolytodis	CCF 3566 ^{T}	NR_155107	n.a.
G. scolytodis	CCF 3570	AM267265	n.a.
G. aquaticum	**CNUFC BCW49** ^{T}	**PQ655122**	**PQ784402**
G. aquaticum	**CNUFC BCW48**	**PQ655123**	**PQ784403**
G. trypophloei	CBS 147988 ^{T}	ON531997	ON532013
G. trypophloei	CBS 147989	ON531998	ON532014

CNUFC BCW49, and CNUFC BCW48 matched those of *G. carbonarium* (GenBank accession no. KM245114 and HM630602), *G. basitruncatum* JCM 9300, *G. kuroshium* UCR4618, and *G. carbonarium* CMW12418 (GenBank accession no. NR_111015, KX262284, and HM630602) with similarity values of 100% (528/528 bp and 406/406 bp), 98.7% (458/464 bp), 99.1% (448/452 bp), and 90.9% (369/406 bp), respectively. The maximum likelihood gene tree for the ITS and *TEF-1α* showed that the strain CNUFC PYW4-15 was identical to *G. carbonarium*, while the strains CNUFC BCW49 and CNUFC BCW48 as new species to sciense (Figures 1 and 2).

3.2. Taxonomy

3.2.1. Taxonomy of CNUFC BCW49

Graphium aquaticum Hyang B. Lee ***sp. nov.***

Index Fungorum number: IF 903142; Table 2, Figure 3.

Etymology: Refers to the aquatic environment from which the species was isolated.

Typus: Republic of Korea, Chungnam Province, Cheonan, Byeongcheon (36°45'59"N 127°18'09"E), from freshwater, August 2018, holotype CNUFC HT2449, ex-type culture CNUFC BCW49.

Colony characteristics: On PDA, colonies olive to dark brown, evenly spread, central immersed mycelium, aerial mycelium at the periphery, hyaline, septate hyphae, reverse light brown, reaching 15 mm in diameter after 7 d at 25 °C. On MEA, colonies flat, smooth, spreading evenly, centrally light olivaceous, white at the periphery, reaching 18 mm in diameter after 7 d at 25 °C. On OA, colonies flat, dark olivaceous green, immersed mycelium, reverse dark green, reaching 13 mm in diameter after 7 d at 25 °C. Slow growth was observed at 37 °C on PDA and MEA. No growth was observed at 37 °C on OA.

Micromorphology: Two type of synnemata, long elongated slender synnemata, light, dark brown to black, becoming pale at the apex, comprised of interwoven packed parallel filamentous hyphae, 98–528 µm long and 9.5–18 µm wide at the top, 5–9.5 µm wide at the center, and 6.5–13 µm wide at the base, others observed as short tree-like synnemata structures, mostly branched, measured 75.5–335.5 µm long and 14–21.5 µm wide at the top, 3–8 µm wide at the center, and 7–10 µm wide at the base. Conidia observed two types, hyaline, cylindrical with truncated ends, thin-walled, 2–6 × 1–2 µm, and obovoid, thick-walled, dark brown, 3–4.5 × 1.5–2.5 µm. Conidiogenous cells were annelidic, septate, monoblastic, filiform, hyaline, sympodial holoblastic conidium ontogeny, terminally arise on synnematal hyphae, and measured 10–33 × 1.5–2 µm.

Notes: Phylogenetic analysis of ITS and *TEF-1α* sequence dataset indicates that *Graphium aquaticum* forms a distinct lineage (Figures 1 and 2). *Graphium aquaticum* differs from other *Graphium* spp. by forming bigger synnemata and smaller conidia.

Additional material examined: Republic of Korea, Chungnam Province, Cheonan, Byeongcheon stream (36°45'59"N 127°18'09"E), from freshwater, August 2018, culture CNUFC BCW48.

3.2.2. Taxonomy of CNUFC PYW4-15

Graphium carbonarium Paciura, Z.W. de Beer, X.D. Zhou and M.J. Wingf., Fungal Diversity 40(1): 85 (2010); Figure 4.

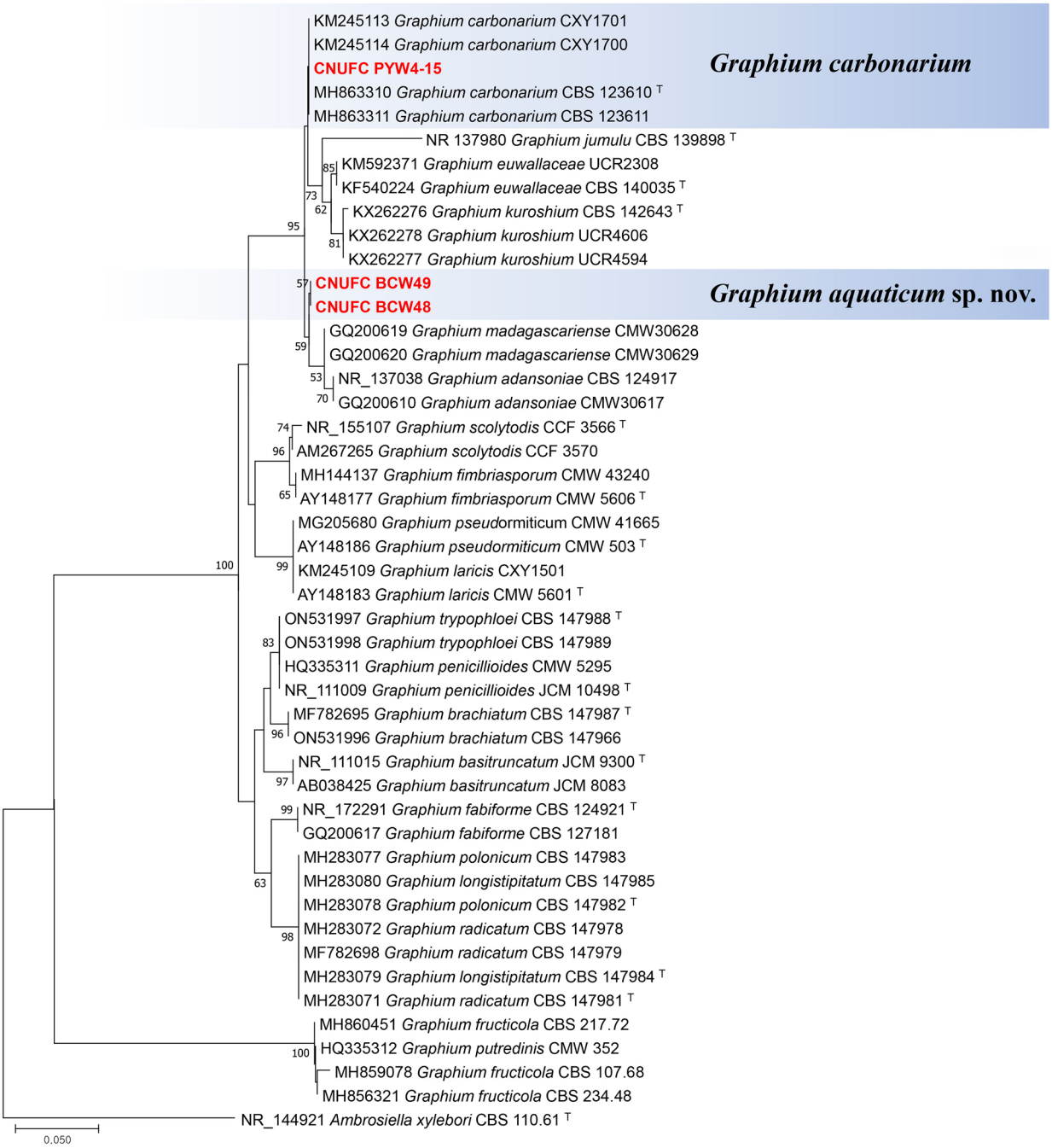

Figure 1. Phylogenetic tree based on maximum likelihood analysis for rDNA ITS sequence data of *Graphium* species. The numbers at nodes show the bootstrap values (>50%) from 1000 replicates. Sequence of *Ambrosiella xylebori* CBS 110.61 was used as an outgroup. Newly generated sequences in this study are in bold red. T = ex-type strain.

Colony characteristics: On PDA, colonies light buff with abundant central aerial and immersed mycelium. Mycelia were septate, hyphae were hyaline, 2–3 µm wide, no soluble pigments were observed, reverse was buff yellowish; the diameter reached 32–34 mm after 7 d of incubation at 25 °C. On MEA, colonies were light green at the center, but turned white at the corners and the reverse was greenish-white; the diameter reached 34–36 mm after 7 d at 25 °C. On OA, colonies were dark grayish olive and the reverse was dark mouse grey; the colonies consisted of synnemata, pigmentation was not observed, and grow at 20 °C to 37 °C, and reached 19–21 mm in diameter after 7 d at 25 °C.

Micromorphology: Conidiophores are present in groups sometimes arising singly or in groups in form of synnemata; consist of dark grayish brown or black stipes similar to coal at the base that become hyaline and; hazel and wide at the apex forming slimy masses; 133.5–229 µm (length) and 32–34.5 µm (width at the center) and 41–59 µm (width at the apex). Per branch of conidiophores, 2–3 conidiogenous cells are present; each branch was 13–16 µm long and 1–3 µm wide, nodular annelation was present. Conidia were present at the apices of the synnemata, and aggregated in a hyaline mucilaginous mass. Conidia were 4–6.5 µm long and 1–3 µm wide; cylindrical, aseptate, and curved, and darkened with

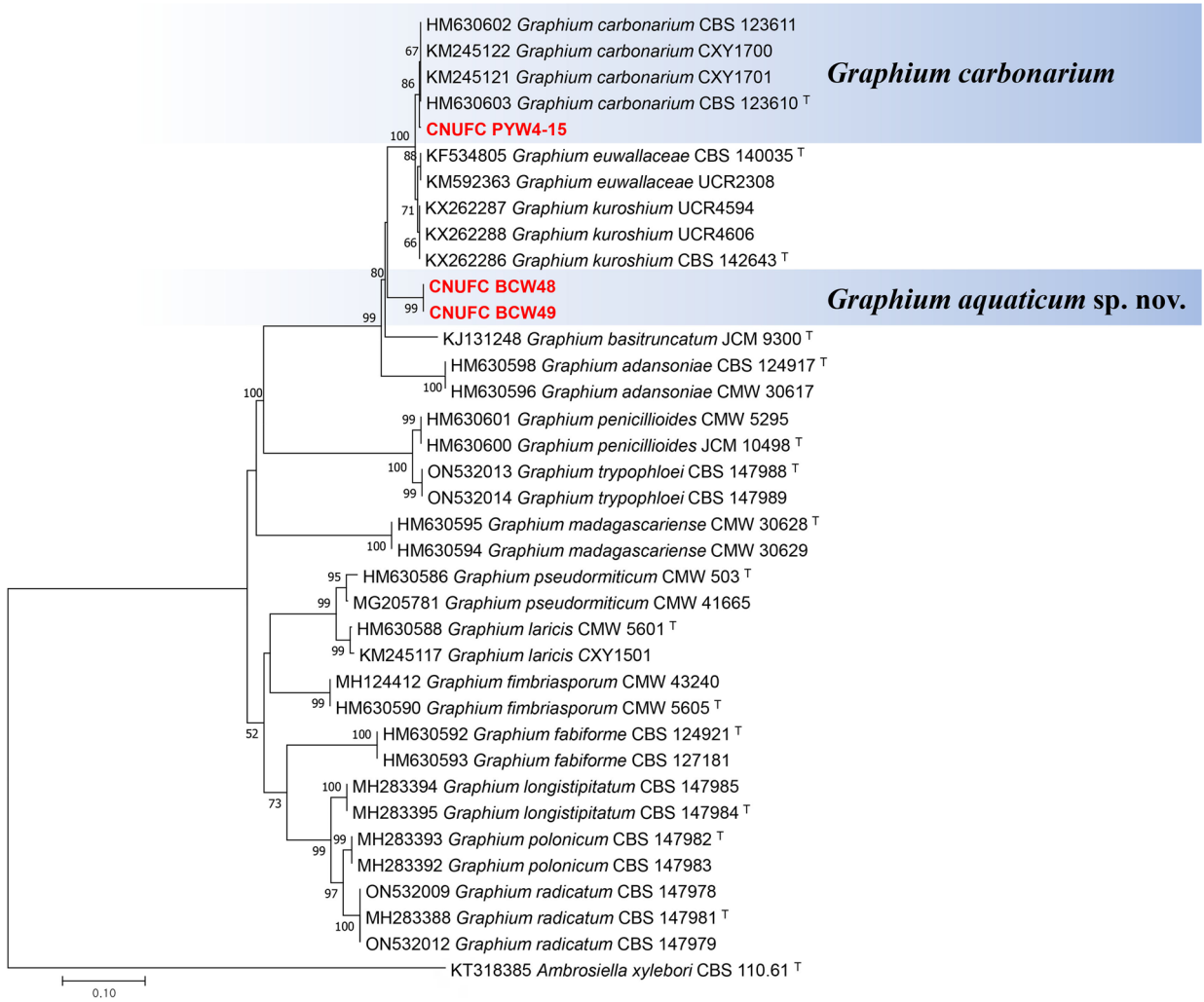

Figure 2. Phylogenetic tree based on maximum likelihood analysis for *TEF-1α* sequence data of *Graphium* species. The numbers at nodes show the bootstrap values (>50%) from 1000 replicates. Sequence of *Ambrosiella xylebori* CBS 110.61 was used as an outgroup. Newly generated sequences in this study are in bold red. T = ex-type strain.

age. Teleomorphs not observed but *Scedosporium*-like synanamorphs were observed both on PDA and 2% MEA as thick-walled, dark brown to olive, obovoid, with erect, simple or branched conidiophores at 25 °C; they were 6–8.5 µm long and 4.5–6.5 µm wide.

Material examined: Republic of Korea, Gwangju, Pungyeongjeongcheon stream (35°11'3.93"N 126°48' 55.97"E), from freshwater, June 2018, culture CNUFC PYW4-15=NNIBRFG9342.

4. Discussion

In this study, we describe the novel species *Graphium aquaticum* and a new record, *G. carbonarium* from freshwater environment. In previous studies, *Graphium* species were associated only with bark beetles and wood staining [5,7, 27,31–33]. To the best of our knowledge, this is the first report of these species from freshwater environment.

Graphium carbonarium was first reported from *Salix babylonica* in China and also reported from *Pissodes* spp. samples and from the heads of female polyphagous beetles from China and Vietnam [5,19]. The species is closely related to *G. basitruncatum*, but the former had larger synnemata, conidia, and *Scedosporium*-like conidia, and was associated with bark beetles. Instead, *G. basitruncatum* was first isolated from forest soil as *Stilbum basitruncatum* in the Solomon Islands [34]; later it was moved to the *G. penicillioides* complex based on the presence of *Scedosporium*-like synanamorphs and no known teleomorphs, and its name was changed into *G. basitruncatum*. It is a well-known human pathogen, as confirmed by isolation from leukemia patients in Canada [15]. Whereas, compare to this, the present study isolate *G. carbonarium* CNUFC PYW4-15 was isolated from freshwater niche in Korea.

Moreover, *Graphium euwallaceae* was first described after isolation from 36 macerated female heads of polyphagous shot hole borers and their gallery walls in *Acer negundo*, *Persea americana*, *Platanus racemosa*, and *Ricinus communis*. It was reported as a pathogen on avocado and box elder. Its conidia are narrower and shorter than those of *G. basitruncatum* and *G. carbonarium* [19]. *Graphum*

Table 2. Comparison of morphological characteristics of *Graphium aquaticum* with those of *G. basitruncatum*, *G. carbonarium*, *G. euwallaceae*, *G. jumulu*, and *G. kuroshium*.

Structure	*G. basitruncatum*	*G. carbonarium*	*G. euwallaceae*	*G. jumulu*	*G. kuroshium*	*G. aquaticum*
Synnemata	(70–)72–131(–158) µm in length	134–225(–300) µm long, 31–36(–43) µm wide at the center and (40–)46–58(–60) µm wide at the apex	155 ± 35 µm long, 36 ± 3.5 µm wide at the mid region, and 44 ± 4.5 µm wide at the apex	100–150 µm long, 5–15 µm wide at the base and 20–60 µm wide at apex	Length n.a., 9.88 µm wide at the top and 6.12 µm wide at the base	Two type of synnemata, long elongated slender synnemata, light, dark brown to black, becoming pale at the apex, comprised of interwoven packed parallel filamentous hyphae, 98–528 µm long and 9.5–18 µm wide at the top, 5–9.5 µm wide at the center, and 6.5–13 µm wide at the base; short synnemata, 75.5–335.5 µm long, and 14–21.5 µm wide at the top, 3–8 µm wide at the center, and 7–10 µm wide at the base
Scedosporium-like synanamorph	5–6(–7) × 3–5(–7) µm	7–8 × 4–6 µm, obovoid	n.a.	Developing on SNA	n.a.	Observed on PDA, obovoid, thin-walled, 3–4.5 × 1.5–2.5 µm
Conidia	5–6 × 1–2 µm, obovoid	4–6(–7) × 1–3 µm, cylindrical	Two types of conidia: (i) hyaline cylindrical, 4.0–6.0 × 1.0–2.0 µm (ii) obovoid, 5.0–6.0 × 3.0–4.0 µm	(3–)4–5(–7) × 2(–2.5) µm, subcylindrical	Two types of conidia: (i) hyaline cylindrical, 3.0–7.2 × 0.8–2.3 µm (ii) obovoid, 3.4–5.6 × 1.8–3.6 µm	Two types of conidia, hyaline cylindrical 2–6 × 1–2 µm, and obovoid thick-walled dark brown conidia, 3–4.5 × 1.5–2.5 µm
Conidiogenous cell	n.a.	15–18(–24) × 1–3 µm, nodular annelation	15 ± 3.5 × 1.0–3.0 µm, annelated, or as sympodial proliferation	20–35 × 1.52 µm	Forming on synnemata	10–33 × 1.5–2 µm
Origin	The Solomon Islands	China	United States, Vietnam	Australia	United States	South Korea
Maximum growth temperature	37 °C	35 °C	35 °C	n.a.	25 °C	37 °C
Host	Forest soil	*Tsuga dumosa*	*Acer negundo*, *Persea americana*, *Platanus racemosa*, *Ricinus communis*	*Adansonia gregorii*	Kuroshio shot hole borer	Freshwater
References	Okada et al. 2000 [33]	Paciura et al. 2010 [5]	Lynch et al. 2016 [20]	Crous et al. 2015 [24]	Na et al. 2018 [22]	This study

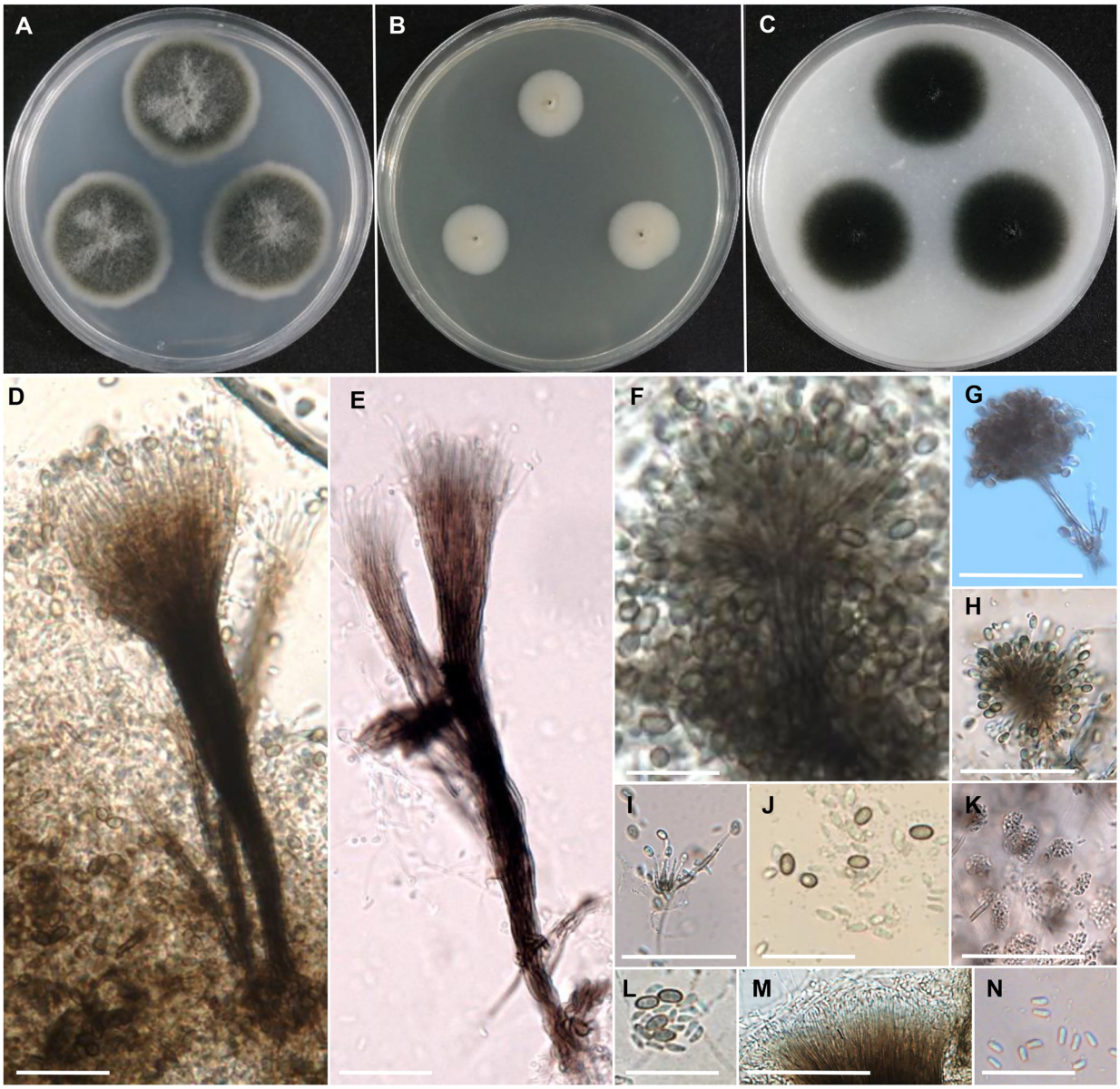

Figure 3. Morphology of *Graphium aquaticum* CNUFC BCW49. A, colonies on PDA. B, colonies on MEA. C, colonies on OA. (A–C: Obverse view). D, E, elongated slender synnemata bearing obovoid and cylindrical conidia observed on PDA. F, G, short synnemata forming tree-like structures observed on PDA. H, short synnemata head bearing obovoid conidia. I, *Scedosporium*-like synanamorph observed on PDA. J, obovoid conidia together with cylindrical conidia. K, L, cylindrical and obovoid conidia in form of circles observed on PDA. M, conidiogenous apparatus. N, cylindrical conidia. Scale bars: G-I, K, M = 20 µm, D-F, J, L, N = 10 µm.

fabiforme is known to grow on dead and wounded baobab trees in Madagascar [7]. The first reported *Graphium* species from *Ilex mitis*, indigenous to South Africa, is *G. ilexiense*; it was first isolated from the wounds on this tree in the Cape Floristic Region [24]. The optimum temperature for the growth of *G. ilexiense* was at 35 °C. Based on ITS sequences, it is grouped with *G. carbonarium* and *G. basitruncatum*, but the ITS and *TEF-1α* data strongly support a separate clade consisting of *G. carbonarium* and *G. basitruncatum* in another group: their colonies and morphology are also completely different from those of *G. ilexiense*. *Graphium jumulu* was first isolated from the damaged trunk of *Adansonia gregorii* in King's Park Botanic Gardens, Perth. The term "jumulu' means a baobab tree.

In 1837, Corda discovered the genus *Graphium* and described it based on the type species *G. penicillioides*, as having a dark synnematous stipe and conidiogenous cells annellations. The species *G. penicillioides* was first observed on *Populus italica* in Prague [2]. It was also described using samples of elm bark and its associated beetle *Hylurgopinus rufipes*, and diseased elms in the Netherland [35]. The species have also been reported on *Populus nigra* trees in Czech Republic [36].

Graphium pseudormiticum was first described in South Africa on the pine-associated bark beetle

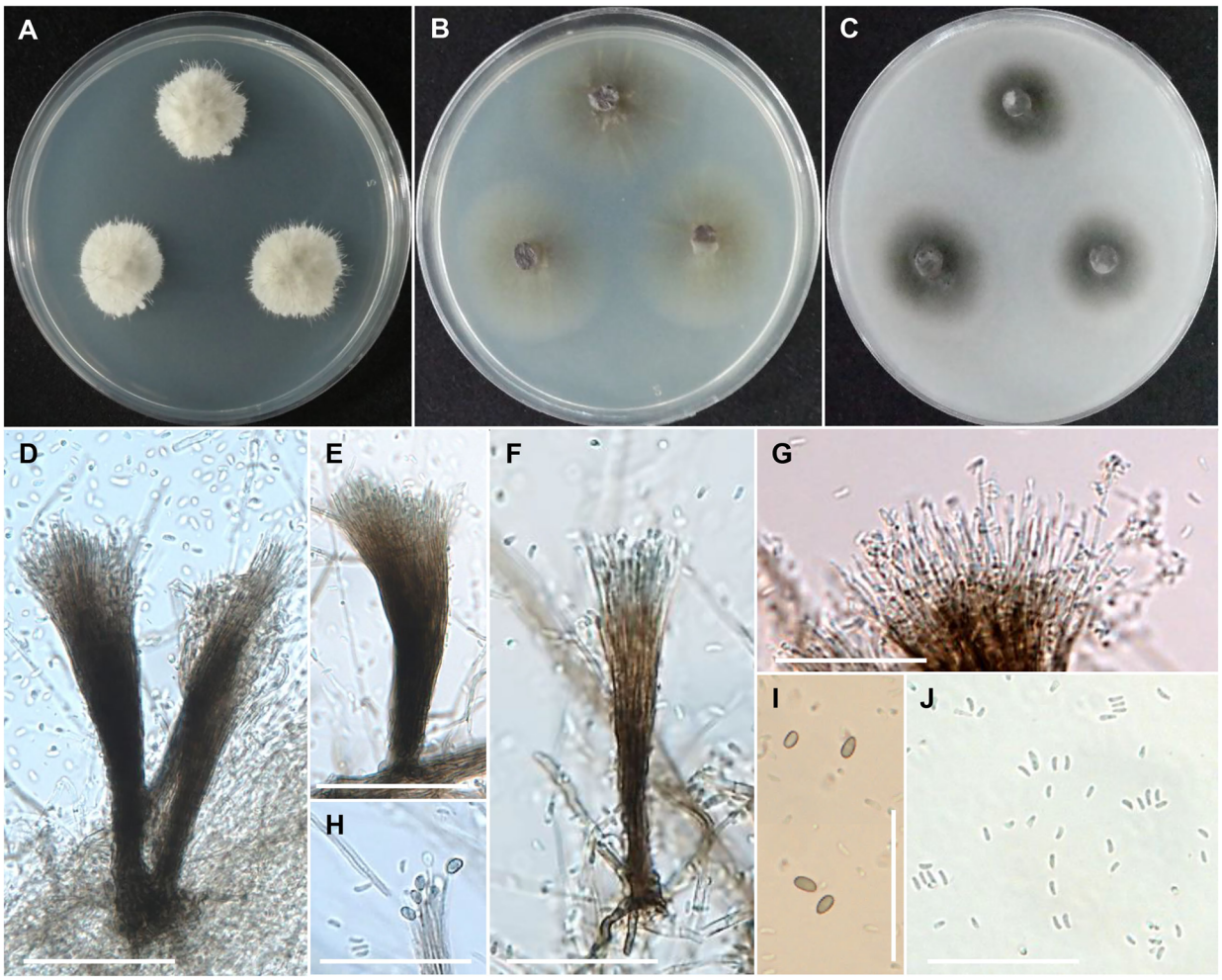

Figure 4. Morphology of *Graphium carbonarium* CNUFC PYW4-15. A, colonies on PDA. B, colonies on MEA. C, colonies on OA. (A–C: Obverse view). D-F, synnemata observed on PDA. G, conidiogenous apparatus. H, I, *Scedosporium*-like synanamorph observed on PDA. J, cylindrical conidia. Scale bars: D-J = 10 µm.

Orthotomicus erosus and recognized as fungus native to Europe [37]. The fungal species was then also isolated from several bark beetles associated with spruce and pine trees in Germany [38]. Jacobs et al. 2003 [39] argued that *G. pseudormiticum* is restricted to pines and has host preferences, but this study reported it for the first time in Korean freshwater.

Phylogenetically, ML analysis of the ITS and *TEF-1α* regions show that the new species introduced here are genetically distinct from all other species in the genus *Graphium*. Morphologically, *Graphium aquaticum* differs from *Graphium basitruncatum*, *G. adansoniae*, *G. madagascariense*, *G. kuroshium*, and *G. euwallaceae* by two synnemata rather than single, bigger synnemata, and smaller conidia. By compairing *Graphium aquaticum* with morphologically related *G. kuroshium*, the former has longer synnemata (98–528 µm and 75.72–335.54 µm), larger synnematal dimensions (14–21.5 µm wide at the top, 7–10 µm wide at the base vs 9.88 µm wide at the top, 6.12 µm wide at the base), conidial dimensions as lower cylindrical (2–6 × 1–2 µm vs 3.0–7.2 × 0.8–2.3) and lower obovoid conidia (3–4.5 × 1.5–2.5 µm vs 3.4–5.6 × 1.8–3.6 µm), smaller average growth rate on PDA (2.2 mm in diameter vs 3.1 mm per day after 7 d at 25 °C), maximum growth temperature (37 °C vs 25 °C), and *Scedosporium*-like synanamorphs were observed on PDA at 25 °C as compared to *G. kuroshium*. *Graphium aquaticum* is isolated from freshwater habitat while other nearest *Graphium* species are isolated from leukemia patient, diseased wood, or bark bettles. Morphologically overlapping the *G. kuroshium* was first reported from the Kuroshio shot hole borer in San Diego County together with *Fusarium kuroshium* as a symbiont [21]. This species was also isolated in the USA from the tree *Picea americana* [36]. Research on *Graphium* fungi from freshwater habitat is lacking worldwide. Therefore, it is reasonable that more isolations, pathogenicity, potential metabolites and their activities are still demandable to be reported from freshwater.

The major aspect of numerous *Graphium* species ecology is their association with subcortical insects.

Their diverse detection in Korean freshwaters may represent independent introductions of their source of isolation. All substrates hosting *Graphium* species, such as freshwater, soils, or living hosts, show similarities; this raises interesting questions about the movement of fungi between substrates. Probably some symbiotic relations between the genus *Graphium* and insects that serve as vector are constant, but interestingly, an outcome of the present study about *Graphium* spp. isolation from freshwater; this is particularly important as the genus is famously considered insect-vectored.

However, the isolation of *Graphium* species from freshwater, might lead to the confirmation of a new habitat for the genus and might increase our understanding of the fungal biodiversity in Korea. Ophiostomatoid fungi are limited in Korea, but interestingly, many *Graphium* species including the present were detected in unusual habitats by surveys such as the one described here. Further studies and sampling may report other novel species in this group from other areas of Korea. More studies are required to better understand the ecological roles of *Graphium* species on diverse substrates. Acknowledging and understanding this group of fungi is important to better define the quarantine measures that will be required for timber trade in Korea in the future. Isolation and description of novel taxa and records of new specific habitat like freshwater will further increase our knowledge in terms of regional biogeography, and ecology, leading to strategies regarding biodiversity protection. More studies on the activities of the enzymes and secondary metabolites produced by these fungi will be needed.

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