Mycobiology

New Records of *Xylaria* Species in Korea: *X. ripicola* sp. nov. and *X. tentaculata*

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Abstract During a Korean mushroom diversity survey from 2011 to 2014, we found one new *Xylaria* species (*X. ripicola* sp. nov.) and one *Xylaria* species that had not been previously observed in Korea (*X. tentaculata*). To confirm the phylogenetic placement of the new species, we conducted a phylogenetic investigation based on internal transcribed spacer regions of ribosomal DNA sequences. Additionally, the new species, *X. ripicola*, was subsequently analyzed for RNA polymerase II subunit sequences. We also evaluated the macroscopic and microscopic features of this species. Herein, *X. ripicola* is described as a new species that was collected from a natural beach habitat and *X. tentaculata* is formally reported as newly found in Korea.

Keywords Ascomycota, Morphology, Phylogeny, Taxonomy, Xylariaceae

Xylaria Hill ex Schrank is the largest genus in Xylariaceae Tul. & Tul. and its members are commonly found on dead wood. Presently, ca. 300 species of *Xylaria* are found worldwide [1, 2]. However, only seven *Xylaria* species were reported in Korea [*X. carpophila* (Pers.) Fr., *X. filiformis* (A. & S. ex Fr.) Fr., *X. hypoxylon* (L.) Grev., *X. longipes* (Nitschke) Dennis, *X. oxyacanthae* Tul., *X. persicaria* (Schwein.) Berk. & M. A. Curtis, and *X. polymorpha* (Pers.) Grev.], identified mainly based on morphological characteristics [3, 4]. Molecular data have now become essential for describing new species [5-7]. Therefore, the modern taxonomy of *Xylaria* species utilizes complex combinations of phylogenetic and morphological data [2, 7].

Herein, we describe two new records of *Xylaria* species (*X. ripicola* sp. nov. and *X. tentaculata*), which were collected during a Korean mushroom diversity survey conducted from 2011 to 2014, mainly based on the results of phylogenetic analysis of internal transcribed spacer of rDNA (ITS; barcode gene for fungi) [8] sequences and morphological investigation. In addition, one of the new species (*X. ripicola*) was subsequently analyzed for its RNA polymerase II subunit (RPB2) sequence to confirm its accurate phylogenetic position.

MATERIALS AND METHODS

Specimens and morphological observations. Eight *Xylaria* specimens were deposited in the Korea National Arboretum Herbarium (KH) and their information is listed in Table 1. Among these, two specimens (KA11-0060-1 and KA11-0060-2) were collected from a saline sand beach (Myeongsasimni Beach, Bigeum-do) in Korea (Fig. 1). Macro-morphological characters were determined based on field notes and colored photographs of fresh fruiting bodies. Micro-morphological characteristics of dried specimens were examined after sectioning and rehydration, which was conducted following the protocols described by Largent *et al.* [9]. Microscopic observations were made using an Olympus BX53 microscope (Olympus, Tokyo, Japan) and Jenoptik ProgRes C14 Plus Camera (Jenoptik Co., Jena, Germany). Measurements of microscopic characteristics

Table 1. *Xylaria* specimens examined in this study

Species	Specimen No.	Locality	Coll. date	GenBank accession Nos.	
				ITS	RPB2
X. ripicola sp. nov.	KA11-0060-1	Myeongsasimni Beach, Bigeum-do, Wando-gun, Jeonnam Prov., Korea	2 Jun 2011	KM817199	KU554696
	KA11-0060-2	Myeongsasimni Beach, Bigeum-do, Wando-gun, Jeonnam Prov., Korea	2 Jun 2011	KM817200	KU554697
X. tentaculata	KA12-0530	Mt. Seonyubong, Seonyu-do, Gunsan-si, Jeonbuk Prov., Korea	11 Jul 2012	KM077162	–
	KA13-1324	Gwangneung Forest, Gyeonggi Prov., Korea	1 Oct 2013	KM077163	–
	KA13-1325	Gwangneung Forest, Gyeonggi Prov., Korea	1 Oct 2013	KM077164	–
	KA14-0263	Mt. Jogye, Suncheon-si, Jeonnam Prov., Korea	25 Jun 2014	–	–
	KA14-0620	Mt. Dongak, Gokseong-gun, Jeonnam Prov., Korea	16 Jul 2014	–	–
	KA14-0730	Gwangneung Forest, Gyeonggi Prov., Korea	29 Jul 2014	–	–

Fig. 1. Map of Bigeum-do, Korea, and the collection locality of *Xylaria ripicola* (inverted triangle).

were taken using ProgRes Capture Pro v.2.8.8 (Jenoptik Co.). Cultures were obtained by placing the tissue of freshly collected stomata on potato dextrose agar (Difco, Detroit, MI, USA). The resulting colonies were transferred to 9-cm Petri dishes containing 2% Difco oatmeal agar (OA) and yeast-malt-glucose agar (YMG) and were incubated at 23°C under 12 hr of fluorescent light.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses. Total DNA extraction was conducted based on the protocols described by Kim *et al.* [10]. PCR mixtures contained 0.5 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 2.5 U of Taq DNA polymerase, and 15 ng of template DNA. PCR conditions for ITS were as follows: an initial denaturation step at 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and a final elongation step at 72°C for 10 min. Only the ITS

regions of ribosomal DNA sequences from five specimens (KA11-0060-1, KA11-0060-2, KA12-0530, KA13-1324, and KA13-1325) were deposited in GenBank, because amplification failed for the other specimens (Table 1). For amplification of RPB2 regions of the new species (KA11-0060-1 and KA11-0060-2), we used the fRPB2-5F and fRPB2-7cR primers (annealing temperature 50°C) [11]. PCR conditions for RPB2 region were similar to those used for the ITS region with the exception of annealing temperature. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea).

We edited raw sequences using PHYDIT 3.2 [12]. For phylogenetic analyses, DNA sequences were aligned using ClustalX 1.81 [13] with manual adjustment using PHYDIT. MrBayes 3.1 [14] was used to construct phylogenies under Bayesian inference. For Bayesian analyses, data were first analyzed with jModelTest2 software [15] to determine the most appropriate model of DNA substitution using the
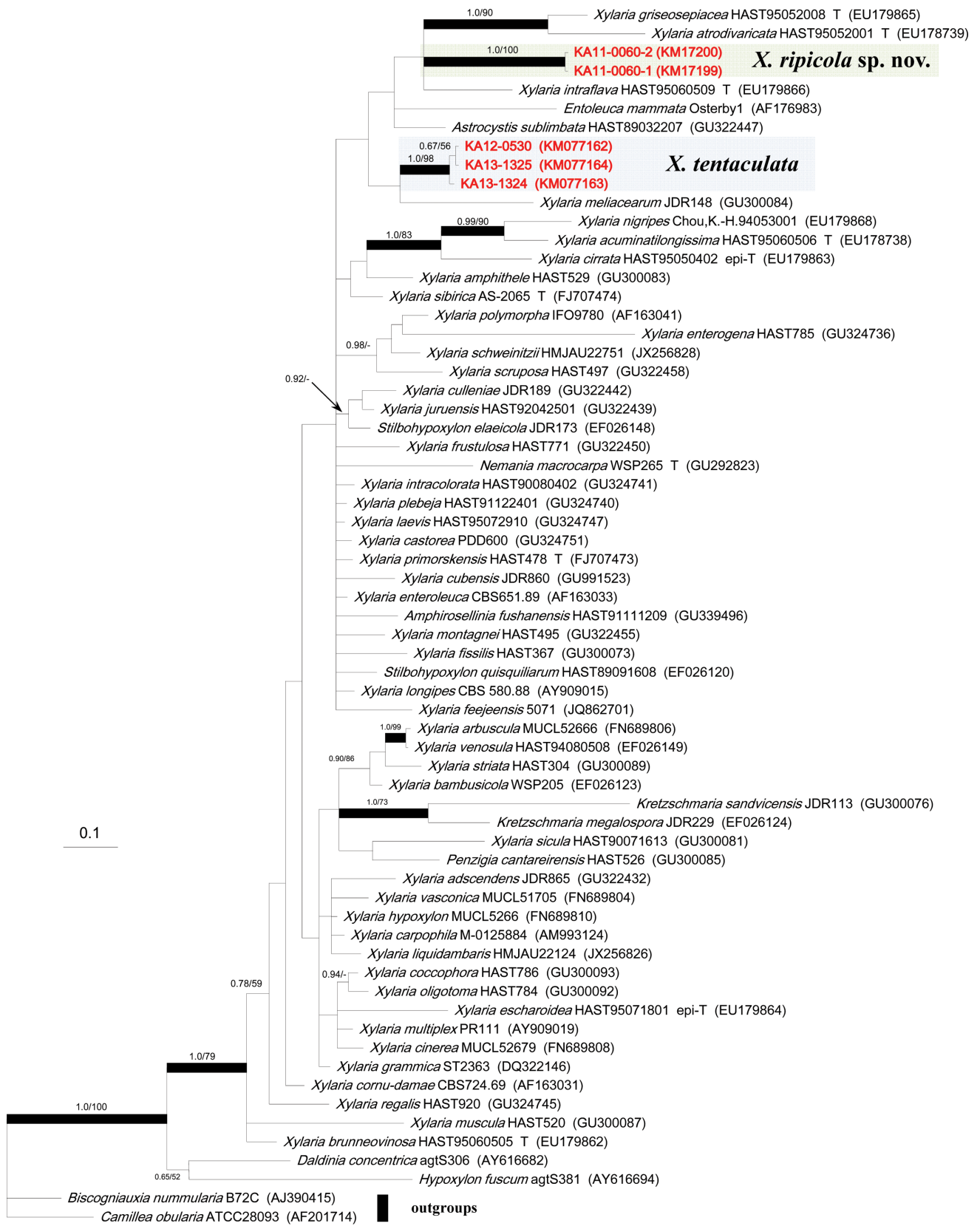
Fig. 2. Bayesian 50% majority-rule consensus topology based on internal transcribed spacer of rDNA sequence data. Broad black branches indicate posterior probabilities > 0.95 and maximum parsimony bootstrap support > 60% (posterior probabilities/ maximum parsimony). *Biscogniauxia nummularia* B72C (AJ390415) and *Camillea obularia* ATCC28093 (AF201714) were used as outgroups. 'T' and 'epi-T' indicate sequences of the holotype and epi-type, respectively.

akaike information criterion. Posterior probabilities (PP) were approximated using the metropolis-coupled Markov chain Monte Carlo method. Two parallel runs were conducted one cold and three heated chains for 10 million generations in the ITS data set and for 3 million generations in the RPB2 data set, starting with a random tree. The trees were
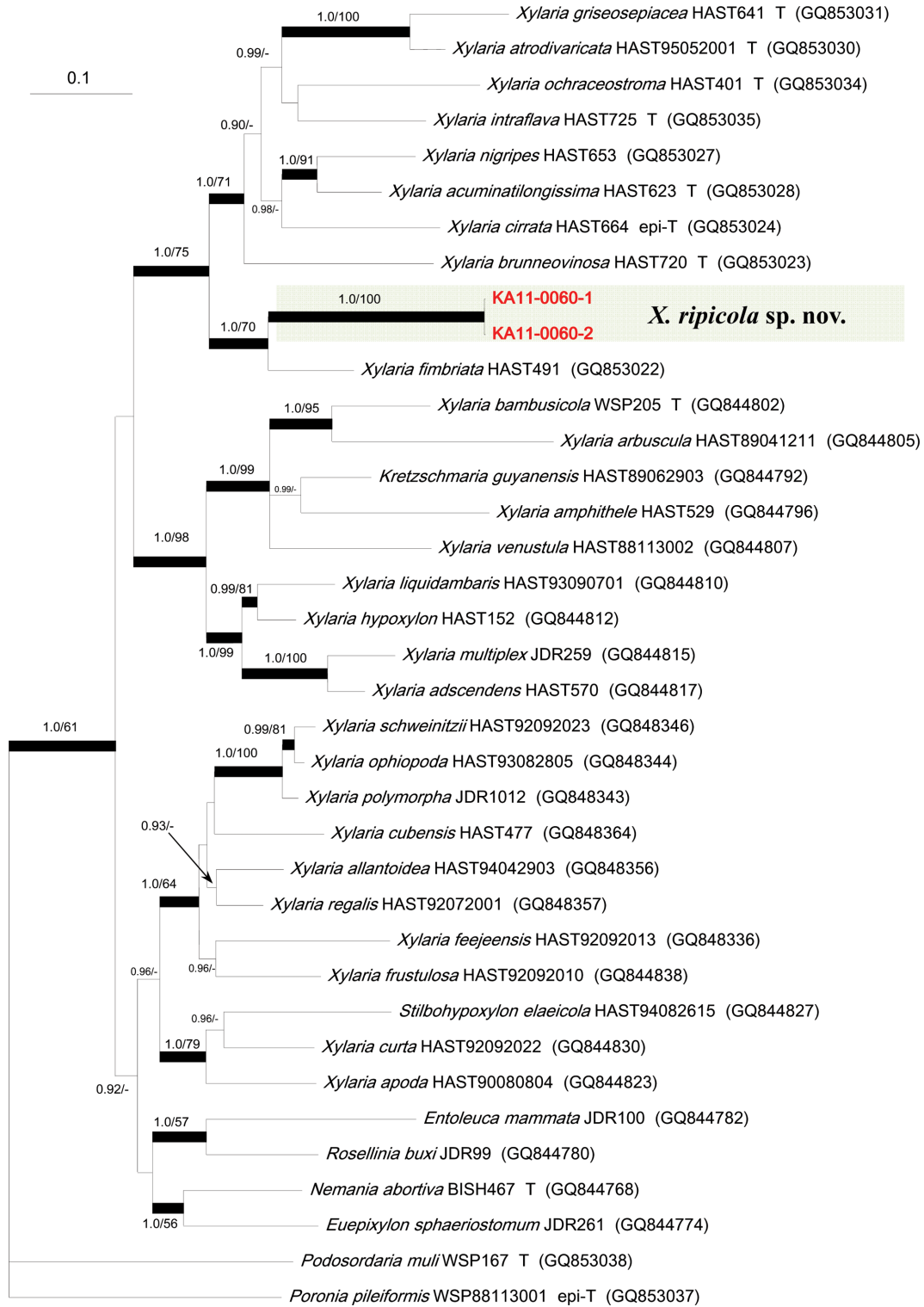

Fig. 3. Bayesian 50% majority-rule consensus topology based on RNA polymerase II subunit sequence data. Broad black branches indicate posterior probabilities > 0.95 and maximum parsimony bootstrap support > 60% (posterior probabilities/ maximum parsimony). *Poronia pileiformis* WSP88113001 and *Podosordaria muli* WSP167 were used as outgroups. 'T' and 'epi-T' indicate sequences of the holotype and epi-type, respectively.

sampled every 100 generations. We determined that both independent runs had converged when the average standard deviation of the split frequencies dropped below 0.01. PP values below 0.95 were not considered significant, and values below 0.90 were indicated on the resulting phylograms. Maximum parsimony (MP) analysis was performed using the heuristic search option in PAUP* 4.0 [16] with the following settings: all characters were equally weighted, gaps were treated as missing characters, starting trees were obtained by random addition with 1,000 replicates, and tree bisection-reconnection branch swapping algorithm was used.

RESULTS

Phylogenetic analyses. Fig. 2 shows the results of Bayesian analysis using a GTR + I + R model of evolution for 10 million generations that was performed on the ITS dataset (including 65 taxa, 533 characters; burninfrac = 0.40). MP analysis of the ITS data resulted in four most-parsimonious trees of 1,111 steps (consistency index = 0.4365, retention index = 0.5207, homoplasy index = 0.5635, and 205 parsimony-informative). Fig. 3 shows the results of Bayesian analysis using a GTR + I + R model of evolution
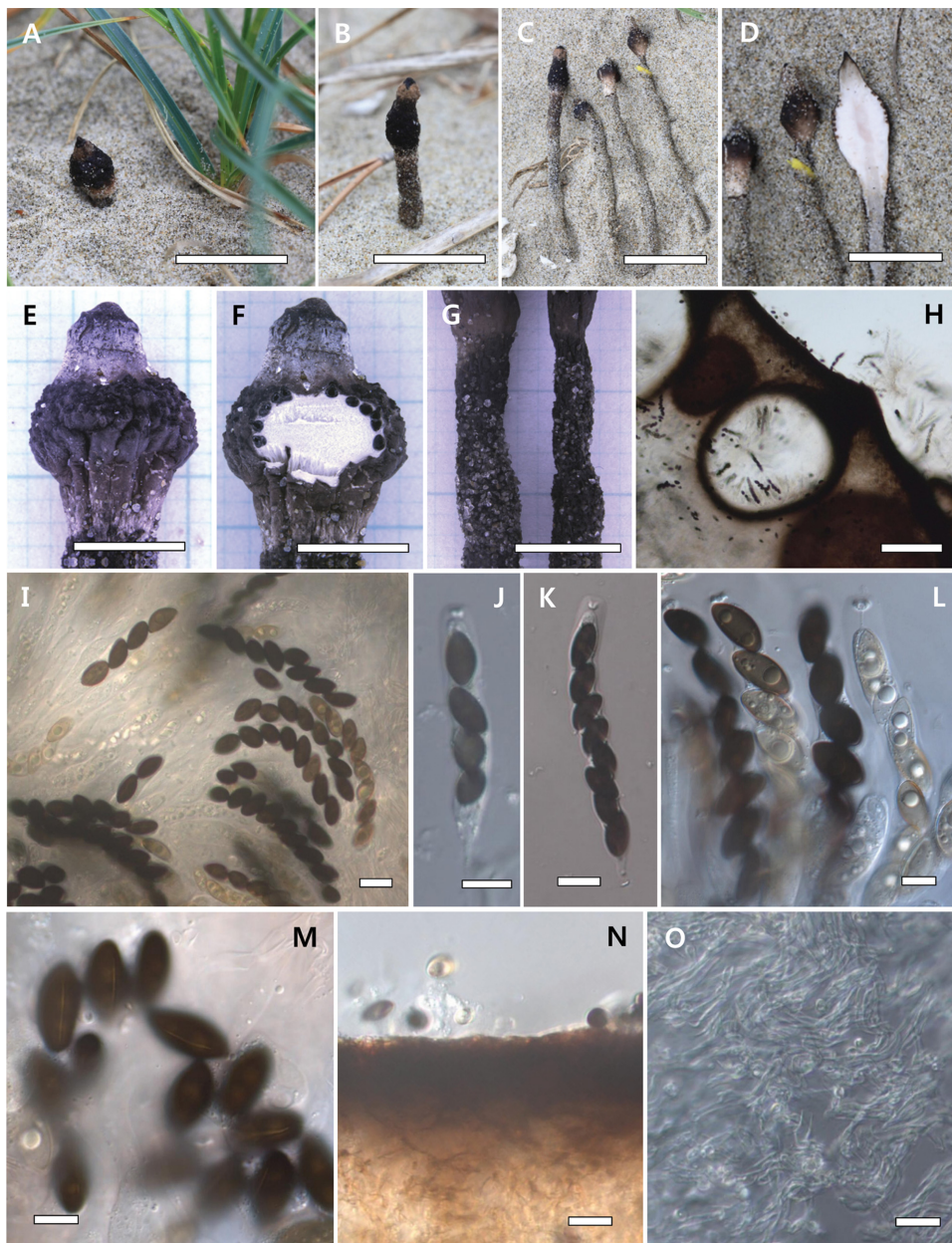

Fig. 4. *Xylaria ripicola* sp. nov. A~D, Natural habit and stromata of *X. ripicola*; E, Stromata surface with conspicuous perithecia and ostioles (dried specimen); F, Longitudinal section showing immersed perithecia (dried specimen); G, Stromata surface; H, Perithecia section; I~L, Asci with ascospores; M, Ascospores; N, Cortical and subcortical tissue section; O, Subperithecial tissue section (scale bars: A~D = 2 cm, E~G = 1 cm, H = 200 μm, I~K, N, O = 20 μm, L, M = 10 mm).

for 3 million generations that was performed on the RPB2 dataset (including 37 taxa, 1,046 characters; burninfrac = 0.25). MP analysis of the RPB2 data resulted in two most-parsimonious trees of 1,353 steps (consistency index = 0.2647, retention index = 0.4264, homoplasy index = 0.7353, and 435 parsimony-informative).

Our ITS phylogenetic trees were similar to those reported by previous Xylariaceae phylogenetic studies, indicating that this genus is paraphyletic (Fig. 2) [17, 18]. Fig. 2 shows that our specimens of *X. ripicola* and *X. tentaculata* form a clade with high PP and maximum parsimony bootstrap support (MPBS) values. In case of the RPB2 tree, *X. ripicola* is related to *X. fimbriata* but is clearly distinct and is supported by PP and MPBS (1.0/70) (Fig. 3).

Taxonomic description.
Xylaria ripicola C. S. Kim & S.-K. Han, sp. nov. (Fig. 4).

Etymology: Latin '*ripa*' = beach, shore; '*cola*' = inhabitant. MycoBank No.: MB810483.

Stromata subcylindrical-fusoid, upright, solitary, scattered to gregarious but not fasciculate, usually 5~7 cm high, sometimes grows up to 10 cm, 3~7 mm thick; stipe plane to wrinkled and cracking into polyhedral plates, pale gray to blackish; head wrinkled with ostioles, unbranched, mostly spathulate but sometimes acuminate at apex, dark brown to black. Cortical layer was thick-walled and did not change color in 3% KOH. Perithecia was observed at the swollen head, immersed, subglobose to ellipsoidal, ca. 280~500 μm × 250~400 μm; ostioles conspicuously papillate, canal length ca. 70~95 μm, width ca. 60~80 μm; subperithecial tissue of *textura intricate*, hyaline, unchanging color in 3% KOH. Asci cylindrical, eight spores or sometimes four spores uniseriately arranged in the ascus, cuneate to tubular apical apparatus, 116~133 μm × 15.2~18.7 μm, length/width ratio
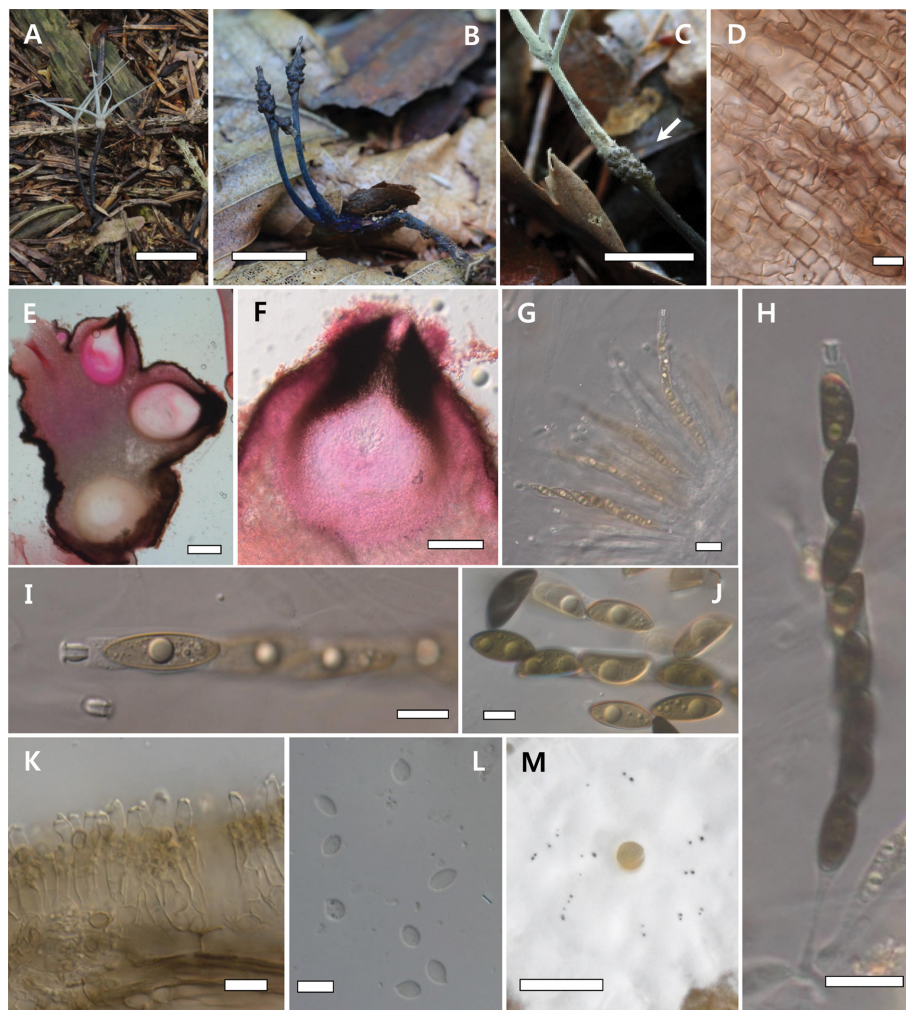

Fig. 5. *Xylaria tentaculata*. A, Natural habitat of *X. tentaculata*; B, Mature specimen (KA14-0730); C, Swollen region at the base of the tentacle-like appendages when immature (indicated by arrow); D, Stromata surface under a microscope; E, F, Perithecia (stained with 1% phloxine and 1% Congo red solutions); G~I, Asci with ascospores; J, Ascospores; K, Conidiophores; L, Conidia; M, Sclerotium-like structures on oatmeal agar media (after ca. 3 months; KA13-1324C) (scale bars: A, B = 1 cm, C = 5 mm, D, I~L = 10 μm, E = 200 μm, F = 100 μm, G, H = 20 μm, M = 15 mm).

6.5~8.4 (n = 30). Ascospores brown to dark brown, smooth, ellipsoid, 18.3~21.9 μm × 10.5~12.5 μm, length/width ratio 1.5~2.0 (n = 30); germ slit with globose.

Anamorph not observed on stromata. Culture not obtained.

Habitat: Sandy beach near *Carex pumila* Thunb.

Known distribution: Korea.

Holotype: Korea, Jeonnam Province, Wando-gun, Bigeum-do, Myeongsasimni Beach, coll. Han & Kwag, 2 Jun 2011, deposited to KH (KA11-0060-1).

Isotype: Korea, Jeonnam Province, Wando-gun, Bigeum-do, Myeongsasimni Beach, coll. Han & Kwag, 2 Jun 2011, deposited to KH (KA11-0060-2).

Xylaria tentaculata Ravenel ex Berk., J. Linn. Soc., Bot. 10: 381 (1869) (Fig. 5).

Stromata subcylindrical, solitary to scattered, usually 2~3 cm high, sometimes grows up to 11 cm, *ca.* 1 mm thick, stipe dark brown to blackish but head pale gray to grayish with tentacle-like appendages (0.5~1.5 cm long). Head bearing the conidiophores, velvety; conidiophores subhyaline, lageniform to cylindrical, 11.8~16.6 μm × 3.5~4.3 μm, length/width ratio 3.0~4.3 (n = 30); conidia hyaline to subhyaline, subglobose to ellipsoid, slightly echinate, 6.2~7.6 μm × 4.8~5.5 μm, length/width ratio 1.2~1.5 (n = 30). Perithecia observed at swollen base of tentacles, immersed, globose to subglobose, ca. 500~600 μm × 350~400 μm; ostioles conspicuously papillate. Asci cylindrical, eight spores uniseriately arranged in the ascus, rectangular to urn-shaped apical apparatus, 137~162 μm × 10.2~13.1 μm, length/width ratio 11.6~14.4 (n = 15). Ascospores brown to dark brown, smooth, ellipsoid to ellipsoid-inequilateral, 20.6~24.4 μm × 8.9~10.8 μm, length/width ratio 2.1~2.6 (n = 30); germ slit with globose.

Colonies on OA and YMG usually covering a 9-cm plate after 2 months, sometimes never reaching the edge of the agar plate, and do not produce stromata, even after prolonged incubation times; mycelia on OA and YMG white, azonate, not pigmented. Sclerotium-like structure observed on OA media after ca. 3 months incubation. No conidiogenous structures observed.

Habitat: on leaf litter or on decaying wood.

Known distribution: Cuba, Korea, Mexico, Sri Lanka, and USA.

Examined specimens: Korea, Gyeonggi Province, Gwangneung Forest, coll. Han *et al.*, 1 Oct 2013 (KA13-1324, KA13-1325; ITS GenBank Nos. KM077163, KM077164), 29 Jul 2014 (KA14-0730), deposited to KH; Jeonbuk Province, Gunsan-si, Seonyu-do, Mt. Seonyubong, coll. Han *et al.*, 11 Jul 2012 (KA12-0530; ITS GenBank No. KM077162), deposited to KH; Jeonnam Province, Suncheon-si, Mt. Jogye, coll. Han *et al.*, 25 Jun 2014 (KA14-0263), Gokseong-gun, Mt. Dongak, coll. Han *et al.*, 16 Jul 2014 (KA14-0620) deposited to KH.

Culture strain: KA13-1324C (isolated from KA13-1324; deposited to KH).

DISCUSSION

We collected two unknown *Xylaria* species during the Korean mushroom diversity survey conducted from 2011 to 2014. One species was found on a sand beach (Myeongsasimni Beach) in Bigeum-do, Korea (Fig. 1). Previously reported *Xylaria* species from marine environments are mainly endophytes in trees or herbaceous plants [19, 20]. Interestingly, the fruiting body of *X. ripicola* was collected from a sandy beach and is not endophytic. To the best of our knowledge, it is quite rare that fruiting body-producing fungi can inhabit a beach despite the saline sand. Morphologically, this species resembles *X. longipes* and *X. polymorpha*. However, *X. ripicola* is easily distinguished from these two species, especially by size of the ascospores, which are larger than those of *X. longipes* (ascospores, 13~16 μm × 5.5~7.5 μm) but smaller than those of *X. polymorpha* (ascospores, 22~29 μm × 7~9 μm) (refer to Breitenbach and Kränzlin [21]). In addition, the species is clearly distinguished by the ITS sequences (Fig. 2). In the RPB2 tree (Fig. 3), *X. ripicola* is related to *X. fimbiriata* (numerous fimbriate conidial branches on the top of immature stromata; ascospores: 7~8 μm × 3.5~4 μm) [22, 23] but could be easily distinguished based on the size of its ascospores and its macro-morphological characters.

The second *Xylaria* species, *X. tentaculata*, was reported unofficially by several Korean mycologists through poster presentations based on macro-morphological characteristics alone. In addition, they did not provide its sequence information (Han SK, personal communication). The morphological characters of our specimens were almost identical to the descriptions provided by Callan and Rogers [5] and Roger *et al.* [24]. We also confirmed the phylogenetic placement of *X. tentaculata* based on ITS sequences for the first time. This species is related to *X. comosa* (Mont.) Fr. (with morphologically similar branchlets of tentacle-like structures and anamorphic structures) and *X. meliacearum* Læssøe (phylogenetically related, with morphologically similar anamorphic structures) [25], but is clearly distinct in the context of morphology and phylogeny (Fig. 2) [25-28]. Interestingly, anamorphic structures are difficult to find in mature *X. tentaculata*, which was also mentioned by Callan and Rogers [5] and Roger *et al.* [24]. Additionally, this species produces sclerotium-like structures in OA medium after ca. 3 months (see Fig. 5), but we did not observe anamorphic and teleomorphic structures in culture, even after prolonged incubation times.

Morphological identification of *Xylaria* species is difficult, mainly because the stromata varies greatly in color, size, and sometimes in general shape [29]. However, the most recorded Korean *Xylaria* species were identified by macro-morphological investigation alone, with the exception of *X. persicaria* [3, 4]. Therefore, six Korean *Xylaria* species (*X. carpophila, X. filiformis, X. hypoxylon, X. longipes, X. oxyacanthae,* and *X. polymorpha*) need to be re-evaluated in the near future.

Xylaria species play an important ecological role because of their long co-evolution with vascular plants [2, 30]. Moreover, *Xylaria* species are able to produce ca. 200 useful secondary metabolites [30]. Therefore, the molecular and morphological investigation of Korean *Xylaria* species is necessary to secure a genetic resource for utilization through accurate identification. In the near future, more intensive morphological observations, sequences analysis such as ITS sequences (barcode marker for fungi), and protein coding sequences, and additional specimen sampling is needed to improve our understanding and to determine the accurate number of Korean *Xylaria* species.

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