

RESEARCH NOTE

First Report of *Rhytisma* sp. Associated with Tar Spots on *Ilex cornuta*

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ABSTRACT

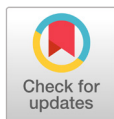
Tar spots have been observed on *Ilex cornuta* in Korea since 2015. Based on morphological and molecular analyses, the fungus responsible was determined to be a *Rhytisma* species, with characteristics similar to *R. ilicis-latifoliae* observed on *Ilex latifolia* from Japan. Herein, we document the first report of *Rhytisma* sp. on *I. cornuta*.

Keywords: Identification, *Ilex cornuta*, Phylogenetic analysis, *Rhytisma*

Ilex cornuta Lindl. & Paxton is native to Korea and China and is predominantly distributed in southern coastal areas and the island Jeju in Korea [1]. It is widely used as an ornamental plant in gardens and parks, as a bonsai plant, and as a Christmas tree owing to its glossy, spiny leaves, and red berries [2]. Tens of cultivars and hybrids (Avery Island, Dwarf Burford, etc.) have been developed based on this species [3]. In addition, its leaves, bark, and berries contain useful chemical compounds, and thus, have long been utilized for medicinal purposes in Asia [4–6].

Tar spots, caused by *Rhytisma* fungal species, are a serious disease, which decreases the aesthetic value of *Ilex* spp. Worldwide, seven species of *Rhytisma* have been recorded on *Ilex* spp.: *R. bontocense* on *Ilex buergeri* from the Philippines [7]; *R. concavum* on *I. verticillata* from North America [8]; *R. himalense* on *I. dipyrena*, and *I. fargesii* from India, Pakistan, and China [9,10]; *R. loeseneriana* on *I. dumosa* from Uruguay [11]; *R. ilicis-integrae* on *I. integra*, *R. ilicis-latifoliae* on *I. latifolia*, and *R. ilicis-pedunculosa* on *I. pedunculosa* from Japan [13]. However, there have been no previous reports of *Rhytisma* spp. on *I. cornuta*. Typical symptoms of tar spots have been observed on the leaves and stems of *I. cornuta* in Naju and Jeju in Korea since 2015. Voucher specimens of the leaves have been housed in the Korea University Herbarium: KUS-F28926 (13 Oct 2015, Naju), F30014 (11 Nov 2016, Jeju), F29730 (20 Apr 2017, Jeju), and F29682 (14 Sep 2017, Jeju).

For morphological observation, leaves with tar spots were immersed in 3% potassium hydroxide solution for 1 day, washed 3 times in distilled water, and soaked in sterile distilled water for a week to



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allow the apothecia to open. For the observation of the ascomata, the leaves were vertically sectioned to a 10 µm thickness using a cryomicrotome (Leica CM 3050 S, Leica Biosystems, Wetzlar, Germany) and the sections were mounted in water on glass slides. Microscopic observation of stromata, ascomata, asci, paraphyses, and ascospores was performed under compound light microscopes (Olympus BX51, Olympus, Tokyo, Japan; Zeiss AX10 equipped with an AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

Tar spots on leaf surfaces and stems were roughly circular, with irregular outlines. The spots were uniformly surrounded by a yellow halo that became pale gray then dark brown towards the outer periphery (Fig. 1A, 1B). Stromata were amphigenous, black, sometimes confluent, and 0.5-2 mm in diameter (n=15). Ascomata on the abaxial surfaces matured in late spring and exposed a yellow hymenium (Fig. 1C). In the median vertical sections, outer layers of the stromata grew to 70-95 µm thick on the abaxial surface (n=20) and their inside was tightly packed with hyphae and thick-walled hyaline cells (Fig. 1D). Ascomata were confined to the abaxial part of the stromata and were 180-260 µm deep with two to three loculi (n=20). Paraphyses were filiform, simple, 150-260 × 0.5-1 µm (n=30), slightly swollen, and not coiled at the apex. Asci were 8-spored, elongated clavate, long-stalked, rounded or slightly rostrate at apex, and 160-220 × 9-14 µm (n=30). Ascospores were hyaline, long clavate, or fusoid without gelatinous sheaths, and 26-40 × 2.5-4 µm (n=30) (Fig. 1E-1I). *Rhytisma* species are known to enter the spermatogonial stage before the ascomatal stage. However, no spermatia were observed in our samples. Among the seven *Rhytisma* species reported on *Ilex*, the anamorph stage has only been described for *R. ilicis-integrae*, *R. ilicis-latifoliae*, and *R. ilicis-pedunculosa* [13]. The morphological characteristics of stromata, ascomata, and paraphyses were most similar to *R. ilicis-latifoliae* on *I. latifolia* described in Japan in 2009 [13], with the exception of slightly wider asci and ascospores approximately 1.5-2 times longer. Asci and ascospores were similar to those of *R. himalense* in shape and size. However, the stromata and paraphyses were distinctly different (Table 1).

Table 1. Morphological comparisons of *Rhytisma* species known on *Ilex*

Species	Host	Ascus (µm)	Ascospore		Paraphysis (µm)	Spermatium (µm)
			Size (µm)	Shape		
<i>Rhytisma</i> sp.	<i>Ilex cornuta</i>	160-220×9-14	26-40×3	Elongated clavate	150-260×0.5-1	Not seen
<i>R. bontocense</i> ^a	<i>I. buergeri</i> var. <i>rolfei</i>	60-100×13-16	25-32×3	Cylindrical to cylindrical fusoid	Not described	Not described
<i>R. concavum</i> ^b	<i>I. verticillata</i>	80-100×8-10	20-35×2-3	Elongated clavate	Not described	Not described
<i>R. himalense</i> ^c	<i>I. dipyrena</i> , <i>Ilex</i> sp.	145-215×10.2-14	25-27.5×3-4	Elongated clavate	Not described	Not described
<i>R. himalense</i> ^d	<i>I. fargesii</i>	90-160×9-13	26-40×3-4.5	Clavate to cylindrical clavate	80-175×1	Not described
<i>R. ilicis</i> ^e	<i>I. micrococca</i> var. <i>longifolia</i>	67-73×10-13	20-31×3.5-4.5	Long-cylindrical clavate	Not described	Not described
<i>R. ilicis-integrae</i> ^f	<i>I. integra</i>	95-180×11-12	16-29×2.5-3	Elongated fusiform, tapering toward the base	170-190×0.5	2-4×1
<i>R. ilicis-latifoliae</i> ^f	<i>I. latifolia</i>	90-150×9-12	15-17×4-5	Elongated fusoid	200-230×0.5	2.5-3×1.5-2
<i>R. ilicis-pedunculosa</i> ^f	<i>I. pedunculosa</i>	120-155×10.5-12	24-43×2.5-4	Elongated clavate, tumidiuscule at the base	170-200×0.5	3-5×1
<i>R. loeseneriana</i> ^g	<i>I. dumosa</i>	70-87×18-21	21-24×4-6	Not described (1-septate)	Not described	Not described

^aSydow, 1932 [7], ^bKellerman, 1902 [8], ^cCannon and Minter, 1986 [9], ^dHou and Piepenbring, 2005 [10], ^eSawada, 1959 [12], ^fSuto, 2009 [13], ^gSaccardo, 1889 [16].

Bold type font: The Korean sample used in this study.

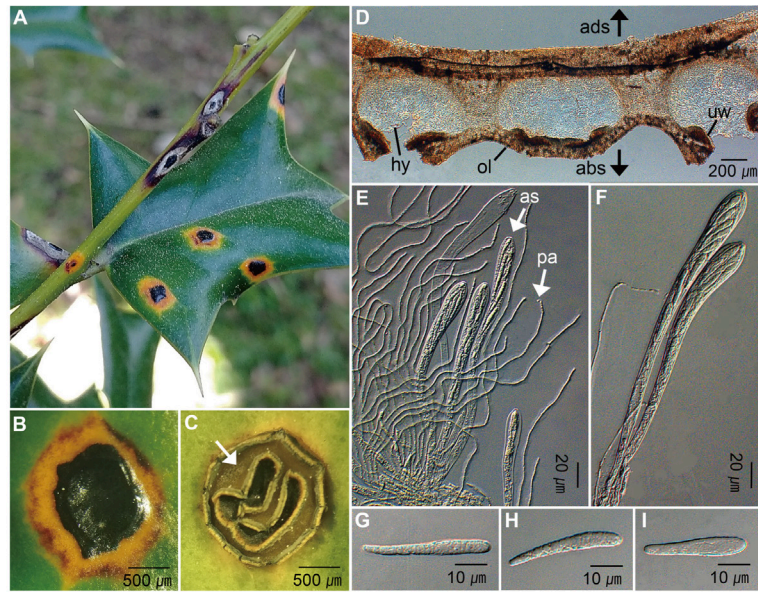


Fig. 1. Tar spots of *Ilex cornuta* infected with a *Rhytisma* sp. **A**, tar spots on an adaxial leaf surface and a herbaceous stem in May 2019; **B**, ascostromata developing on the amphigenous surface of living leaves, surrounded by a yellow halo; **C**, ascostroma on the abaxial leaf surface, exposing a yellow hymenium (arrow head); **D**, vertical section of ascostomata; **E**, hymenial layer of an ascostroma; **F**, asci with ascospores; **G-I**, ascospores. abs, abaxial leaf surface; ol, outer layer of stroma; uw, upper wall of ascostroma; hy, hymenium; as, ascus; pa, paraphysis.

For further characterization, genomic DNA was extracted from the stromal tissues of infected leaves. The internal transcribed spacer (ITS) region of fungal rDNA was amplified and sequenced using the primer pair ITS1F/ITS4. For phylogenetic analyses, ITS sequences from eight *Rhytisma* species were retrieved from the GenBank database. *Lophodermium pinastri* (FJ861986) was used as an outgroup taxon. A phylogenetic tree was constructed with the ITS sequences based on neighbor-joining (NJ) analysis using MEGA7 software with 1,000 bootstrap replicates [14].

The ITS sequences obtained from the four Korean samples (Accession No. MN558946 for KUS-F28926, MN558950 for KUS-F29682, MN560104 for KUS-F29730, and MN560103 for KUS-F30014) were 100% identical. However, there was no *Rhytisma* species with a sequence similarity greater than 90% in the GenBank database. Phylogenetic analysis indicated that the Korean specimens formed a separate clade, distinct from the other *Rhytisma* species in NJ tree analysis (Fig. 2). Unfortunately, our analysis was unable to include *R. ilicis-latifolia* and *R. himalense*, which were morphologically close to our fungus, because sequence data for these fungi were unavailable.

R. ilicis-latifoliae was reported as a new species causing tar spots on *I. latifolia* in Japan in 2009 [13]. Interestingly, *I. latifolia* is phylogenetically close to *I. cornuta*, which has only recently been found to be a host for the tar spot disease in the genus *Ilex*, in Korea [15]. Thus, further examination and comparison with *R. ilicis-latifoliae* specimens from Japan is needed for accurate identification of the *Rhytisma* sp. examined in this study, at the species level.

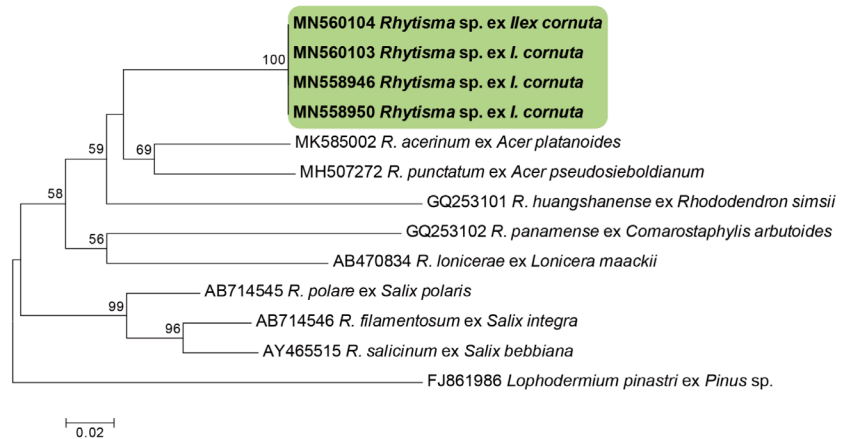


Fig. 2. Phylogenetic relationship of *Rhytisma* sp. between reference sequences retrieved from GenBank, inferred from neighbor-joining analysis using sequences of the internal transcribed spacer (ITS) region. Bootstrap values (1,000 replicates) higher than 50% are presented above the branches. Korean specimens are shown in bold. The scale bar represents 0.02 nucleotide substitutions per site.

To our knowledge, this is the first report of a *Rhytisma* sp. occurring on *I. cornuta* and the first description of its morphological characteristics in detail. *Rhytisma* fungi have generally been thought to have a narrow host range. Thus, this identification of a *Rhytisma* species on *Ilex* is noteworthy and warrants further investigation.

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