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RESEARCH ARTICLE

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Bremia itoana (Oomycota, Peronosporales), a Specialized Downy Mildew Pathogen on an East Asian Plant, *Crepidiastrum sonchifolium* (Asteraceae)

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ABSTRACT

Crepidiastrum sonchifolium, a flowering plant in the daisy family (Asteraceae), is native to East Asia. In Korea, this plant is a locally cultivated vegetable, and its market size is gradually growing. Since the plants with downy mildew infection were initially found at a private farm of Chuncheon city, the occurrences have continued in commercial farms of other regions, highlighting that this disease is spreading throughout Korea. The pathogen was attributed to a member of the genus *Bremia* that contains many specialized species, each of which displays a narrow host spectrum on Asteraceae. Based on morphological and molecular phylogenetic analyses, along with the high host specificity recently proven for *Bremia* species, the identity of the causal agent was confirmed as a so far undescribed species of *Bremia*. Here, we introduce *Bremia itoana* sp. nov., specific to *C. sonchifolium*.

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1. Introduction

Crepidiastrum sonchifolium (Maxim.) Pak & Kawano, called "sonchus-leaf crepidiastrum", is native to East Asia. In Korea, it is an economically important vegetable, which is consumed to make kimchi, and its cultivated area is gradually expanding throughout the country [1–4]. In addition to its culinary value, this plant has been traditionally used as a folk medicine because of its digestive, diuretic, and anti-inflammatory activities [5].

Since downy mildew disease caused by the genus *Bremia* (Oomycota, Peronosporaceae) has been initially found on *C. sonchifolium* at a private farm in Chuncheon city, Korea in 1998, this disease has been continuously spreading to other regions, although it does not seem to have reached outside of Korea. On a large number of genera or species of the family Asteraceae, including this crop, downy mildew is a notorious disease, among which *Bremia lactucae* is one of the most well-known species, which causes devastating damage in the cultivation of lettuce (*Lactuca sativa*) [6]. According to the broad species concept that a downy mildew species is responsible for all infections occurring on a host family [7], the causal pathogen affecting *C. sonchifolium* has been presumed to be *B. lactucae*. However, recent phylogenetic studies with multigene sequences have found several well-supported groups in *Bremia* but also a better resolution power for discriminating them at the species level [8–13]. As a result, it revealed that *Bremia* is not monotypic, which consists of a dozen of highly host-specific species, and in addition, Choi et al. [10] found several previously overlooked lineages of *Bremia*, including *Bremia* sp. affecting *C. sonchifolium*, which was distant from other East Asian species of *Bremia*, parasitic to *Crepidiastrum* and two allied genera, *Ixeris* and *Youngia*. In the present study, we aimed to clarify the identity of the downy mildew pathogen, parasitic to *C. sonchifolium*, using both morphological and molecular phylogenetic approaches.

2. Materials and methods

2.1. Morphological analysis

C. sonchifolium plants with downy mildew symptoms were collected from various regions of Korea. For morphological investigation, conidiophores and conidia formed from the lower surface of the infected leaves were transferred onto a drop of lactic acid on a slide glass, covered with a coverslip, and gently warmed up using an alcohol lamp. A detailed microscopic examination was performed using an

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Table 1. Herbarium specimens of *Bremia* sp. parasitic to *Crepidiastrum sonchifolium*.

				GenBank Acc. No.	
Herb. No. (KUS-F)	Seq. ID	Collection year	Geographic origin	18S + ITS1/LSU D1-3/*BrRxLR11* nDNA	*cox2/cox1*/cox2-1 spacer mtDNA
19257	D216	2002	Korea; Hongcheon	KT249120/ KT249315/ KT249705	KT249510/ KP684694/ KP684894
19490	D217	2003	Korea; Chuncheon	KT249121/ KT249316/ KT249706	KT249511/ KP684695/ KP684895
23946	D543	2008	Korea; Yangpyeong	MH665654/MH665655/MH665656	MH665652/MH665651/MH665653

Olympus BX53F microscope (Olympus, Tokyo, Japan) equipped with a DigiRetina 16 M digital camera (Tucsen, Fuzhou, China). The following morphological characteristics were observed at 100–200× for conidiophores and at 400× for conidia and ultimate branchlets. Measurements were reported as maxima and minima in parentheses and the mean plus and minus the standard deviation of the number of measurements given in parentheses. Among the specimens examined morphologically, three specimens were selected for phylogenetic analyses (Table 1), and deposited at the Korea University Herbarium (KUS-F, Seoul, Korea) and the National Institute of Biological Resources (NIBR, Incheon, Korea).

2.2. Molecular phylogenetic analysis

About 10–20 mg of infected leaves from herbarium specimens were disrupted with homogenizing pestles in 1.5-mL tube. Genomic DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Three nuclear (ITS1, LSU D1-3 rDNA, *BrRxLR11*) and three mitochondrial (*cox1*, *cox2*, cox2-1 spacer mtDNA) markers were amplified by PCR as outlined previously [10–12]. The PCR products were purified and sequenced by a DNA sequencing service (Macrogen Inc., Seoul, Korea). For phylogenetic analysis, multigene sequences of *Bremia* species used in the previous studies [10–12] were retrieved from GenBank. Alignments for each of multigene datasets were done using the MAFFT 7 [14], by choosing the Q-INS-i algorithm [15]. After ensuring no strongly supported conflicting topologies among the trees inferred from individual loci, the alignments were concatenated using SequenceMatrix [16]. Two different phylogenetic inference methods were performed on the concatenated alignment. Minimum Evolution (ME) was done in MEGA6.0 [17] using the Tamura-Nei substitution model, and the robustness of internal branches was evaluated by performing 10,000 bootstrap replicates. Maximum Likelihood (ML) inference was computed using RAxML 7.0.3 [18], with default settings on the RAxML BlackBox web server [19].

3. Results and discussion

Trees based on each alignment of three nuclear (ITS, LSU D1-3 rDNA, *BrRxLR11*) and three mitochondrial (*cox*1, *cox*2, cox2-1 spacer mtDNA) loci showed no strong conflicting support with a phylogeny based on the concatenated alignment of all six loci. Thus, only the phylogeny based on concatenation of all loci was used for phylogenetic reconstruction. The final concatenated alignment displayed 4148 total characters, including 903 variable characters, 800 of which were parsimony-informative for *Bremia* species. Because the dataset revealed no significant conflicts in the topologies derived from ME and ML analyses, only the tree from the ME inference was shown in Figure 1. The overall topology and major groupings were in line with those of the previous studies [8–12], with unsupported replacements of a few branches. Three specimens affecting *C. sonchifolium* formed an independent lineage with maximum support and was separated from all previously accepted species of *Bremia*. Interestingly, this lineage is quite distant from another well-supported group consisting of four East Asian species, *B. microspora*, *B. ovata*, *B. polycephala*, and *B. sawadae*, despite their host plant affinity within the tribe Cichorieae (subfamily Cichorioideae). Instead, *Bremia* sp. affecting *C. sonchifolium* grouped with other *Bremia* species, parasitic to two subfamilies Asteroideae and Carduoideae, with moderate support of 70% in ME and 78% in ML analyses.

In agreement with the previous studies [8,11,20,21] that all *Bremia* species, parasitic to Cichorieae, have smaller conidia than other accepted species of *Bremia*, the present pathogen affecting *C. sonchifolium* could be characterized by small conidia of av. 15.9 × 14.1 µm, which is similar to *B. microspora* (av. 15.9 × 13.7 µm) and *B. polycephala* (av. 15.6 × 14.0 µm), but somewhat larger than *B. ovata* (av. 13.2 × 11.2 µm) and *B. microspora* (av. 13.4 × 12.7 µm). However, in line with the present phylogenetic results, this species exhibits several morphological differences. First, the length of conidiophores was markedly shorter as 200–410 (av. 305) µm than other four species (at least more than av. 400 µm). About 20–40% of conidiophores in *Bremia* sp. are shorter than 250 µm, but in other species, such short ones were <5%. As a related feature, the length of trunks was also shorter; av. 150 µm in *Bremia* sp. versus at least av. 237 µm in other species. In addition, the position of the first branching in *Bremia* sp. was somewhat lower as 4/10–6/10, thus often rendering the branching part occupying over half of the conidiophore's length,

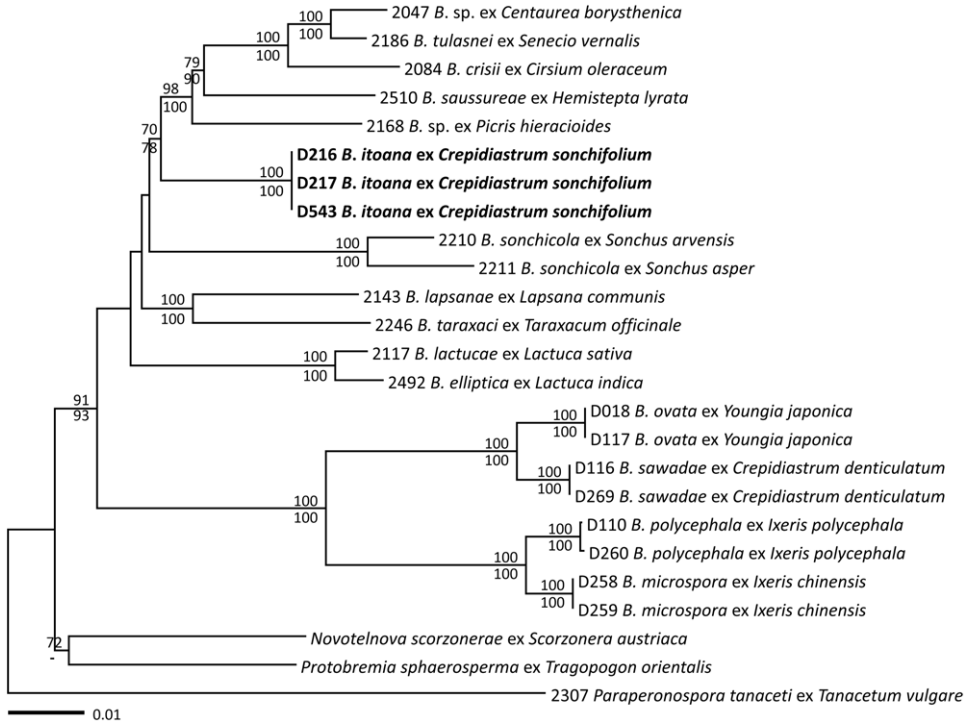

Figure 1. Minimum evolution tree based on a concatenated alignment of ITS1, LSU nrDNA, *cox*2, *cox*1, the spacer region between *cox*2 and *cox*1 mtDNA, and *BrRxLR11* sequences. Bootstrap support values higher than 70%, are displayed above (minimum evolution) or below (maximum likelihood) the corresponding branches. Branch lengths are proportional to the estimated number of nucleotide substitutions.

Table 2. Comparison of morphological characteristics of *Bremia* species parasitic to *Crepidiastrum* species.

Characteristics	*B. sawadae*	*B. itoana*
Host plant	*Crepidiastrum denticulatum*	*Crepidiastrum sonchifolium*
Conidiophores ($n = 50$)		
Length	(355–)424–536–648(–718) μm	(150–)200–305–410(–500) μm
length of trunk	(224–)288–374–460(–514) μm	(60–)100–150–200(–300) μm
Width of trunk	(5.4–)6.2–7.1–8.0(–9.4) μm	(4.9–)6.5–7.7–9.3(–10.9) μm
Position of the first branch	2/10–4/10	4/10–6/10
Branching type	Dichotomous	Dichotomous
No. of branch orders	4–6	3–5(–6)
Callose plugs	Often present in trunk, rarely in branches	Often present in trunk and branches
Ultimate branchlets ($n = 50$)		
Shape	Curved	Slightly curved to substraight
Length	8–20(–30) μm	6–17 μm
Vesicles ($n = 50$)		
Shape	Spherical	Spherical or confluent
Size (diam.)	8–10 μm	8–12 μm
No. of extensions	4–6(–7)	4–6
Length of extensions	5–7 μm	6–8 μm
Shape of tips	Obtuse to somewhat swollen	Obtuse to somewhat swollen
Width of tips	1.3–1.8 μm	1.4–1.9 μm
Conidia ($n = 100$)		
Shape	Ovoid	Oblong to ovoid
Colour	Hyaline	Hyaline
Length (μm)	(11.5–)12.5–13.4–14.3(–14.7) μm	(14.0–)15.1–15.9–16.7(–17.7) μm
Width (μm)	(10.6–)11.1–11.7–12.4(–14.1) μm	(13.0–)13.4–14.1–14.8(–15.7) μm
l/*w* ratio	(1.06–)1.1–1.14–1.19(–1.26) μm	(1.05–)1.09–1.13–1.17(–1.22) μm
Pedicel	Mostly present	Mostly present
Reference	Park et al. [12]	The present study

while in other species they branched mostly at 3/10. As *Bremia* sp. and *B. sawadae* have the close host affinity [22], both parasitic to the genus *Crepidiastrum*, their morphological characteristics were compared in detail (Table 2). In addition to the differences listed above, the shapes of conidia and ultimate branchlets can discriminate between the two species; oblong to ovoid conidia in *Bremia* sp. versus ovoid conidia in *B. sawadae*, and often confluent vesicles at the end of ultimate branchlets in *Bremia* sp. versus mostly spherical vesicles in *B. sawadae*.

The host range of *Bremia* species is restricted to the plant family Asteraceae, favouring three subfamilies, Asteroideae, Carduoideae, and Cichorioideae [10]. Especially, small conidia-possessing species of *Bremia*, including the present species, all colonize the three East Asian genera of the subtribe

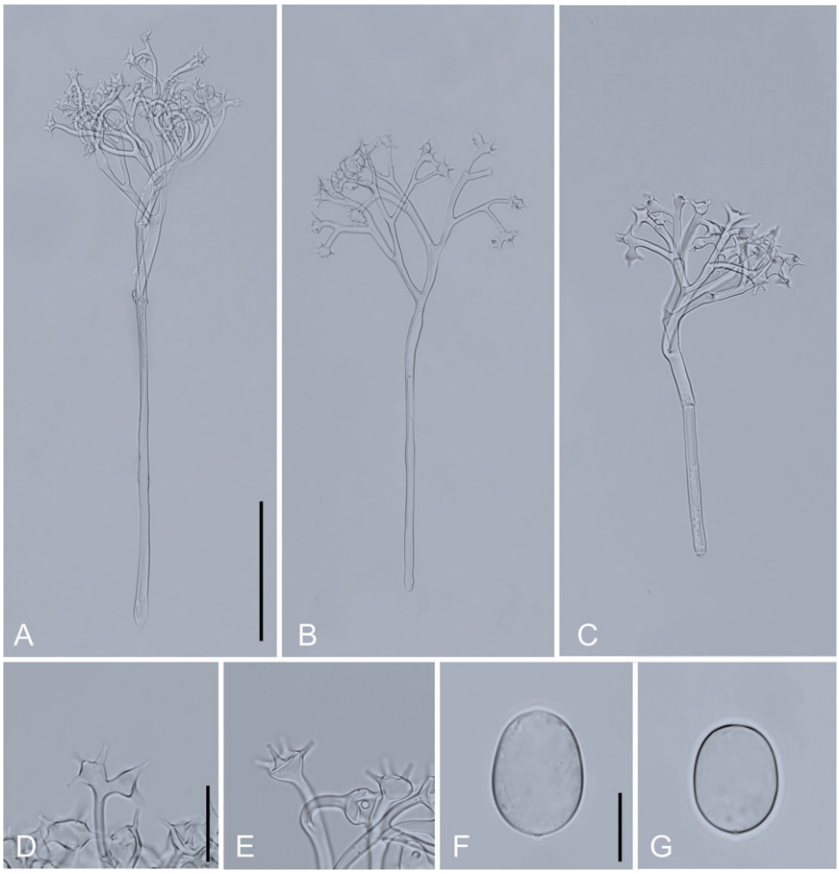

Figure 2. Morphological characteristics of *Bremia itoana* sp. nov. parasitic on *Crepidiastrum sonchifolium*. (A–C) Conidiophores; (D–E) Ultimate branchlets; (F–G) Conidia. Scale bars: 100 µm for conidiophores, 20 µm for ultimate branchlets, and 10 µm for conidia. Source: NIBRFG0000501456.

Crepidinae of Cichorioideae, *Crepidiastrum*, *Ixeris*, and *Youngia* [8,10,11]. However, although they infect the close host plants in the same area, *Bremia* sp. affecting *C. sonchifolium* seems to have evolved independently from other species. It is most likely that long after branching of the main group infecting Crepidinae, *Bremia* sp. has host-jumped from another lineage infecting Cichorioideae onto *C. sonchifolium*. Despite the similar morphology to Cichorioideae-infecting species but the close phylogenetic relationship to Asteroideae- and Carduoideae-infecting species, it is unlikely that this species served as an evolutionary bridge connecting the two groups because the host plant was always limited in East Asia.

The present study provided evidence that the causal agent of downy mildew occurring on the cultivated crop, *Crepidiastrum sonchifolium*, is independent from all previously known species of *Bremia*, for which we introduce a new species here.

Taxonomy

Bremia itoana Y.J. Choi & H.D. Shin, sp. nov. [MB827100] Figure 2.

Etymology: named in honour of Seiya Ito for his outstanding studies on East Asian species of *Bremia*.

Lesions commonly causing discolouration of the tissues, pale green or yellow, later becoming dark brown, vein-limited, poly-angular, frequently covering larger areas by coalescing; infected tissues become necrotic. Down present on the under the surface of host leaf, whitish, consisting of scattered conidiophores, only one or two in a fascicle, sparse. Conidiophores emerging through stomata, colourless, straight, (150–)200–305–410(–500) µm; trunk straight, (60–)100–150–200(–300) µm long, (4.9–)6.5–7.7–9.3(–10.9)µm wide below the first branch; basal end not differentiated to slightly bulbous, (6.4–)8.6–10–11.4(–12.2) µm wide; callose plugs often present in trunk and branches; branches dichotomous, 3–5(–6) orders. Ultimate branchlets in pairs in most branchlets, rarely single, slightly curved to substraight, 6–17 µm long, obtuse to somewhat swollen; vesicles spherical or confluent, 8–12 µm diam., bearing 4–6 extensions with lengths of 6–8 µm. Conidia colourless, oblong to ovoid, almost symmetrical at the equatorial plane, (14.0–)15.1–15.9–16.7(–17.7) µm long, (13.0–)13.4–14.1–14.8(–15.7) µm wide, a l/w ratio of (1.05–)1.09–1.13–1.17(–1.22), greatest width median to submedian, tip round, base broadly round; pedicel mostly present, having thin-walled papilla. Germination directly with a germ tube only at the tip,

up to 215 µm long, rarely branched. Resting organs not seen.

Typus: KOREA; Chuncheon-si, Dongnae-myeon, Goeun-ri (37°50'19"N 127°46'58"E), 21 May 2003, Y.J. Choi & H.D. Shin, NIBRFG0000501456 (holotypus).

Habitat: On living leaves of *Crepidiastrum sonchifolium* (Asteraceae).

Additional specimens examined for morphological investigation: KUS-F15622 (Nov. 1 1998, Chang-ri, Nam-myeon, Yanggu, Korea), 20946 (Nov. 4 2004, Goeun-ri, Dongnae-myeon, Chuncheon, Korea), 21516 (Oct. 17 2005, Jangjeonpyeong-ri, Hongcheon-eup, Hongcheon, Korea), 21684 (Nov. 11 2005, Goeun-ri, Dongnae-myeon, Chuncheon, Korea), 21688 (Nov. 12 2005, Seosang-ri, Seo-myeon, Chuncheon, Korea).

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