

First report of chocolate spot disease on broad bean (*Vicia faba*) caused by *Botrytis fabiopsis* in Japan, and the draft genome sequence of the pathogen.

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Abstract

Broad bean chocolate spot (BCS) is an economically important disease of broad bean (*Vicia faba*). In 2022, we collected broad bean leaves showing disease symptoms from a field where the typical BCS observed. Isolated fungus exhibited pathogenicity on broad bean leaves, producing with typical BCS symptoms. Whole genome sequencing and phylogenetic analysis using *G3PDH*, *HSP60* and *RPB2* gene sequences identified the isolated pathogen as *Botrytis fabiopsis*. This is the first report of *B. fabiopsis* causing BCS on broad bean in Japan, and also the first report of its draft genome sequence.

Main text

Broad bean (syn. faba bean, *Vicia faba* L.) is cultivated worldwide as an important food and feed crop, yet its production is often constrained by fungal diseases. Among these, broad bean chocolate spot (BCS) is one of the most severe diseases, caused by various *Botrytis* species, and has been reported in temperate regions worldwide. Infected leaves, stems, and pods of broad bean exhibit numerous circular lesions ranging from red to dark brown, which sometimes merge into large necrotic areas (Wilson 1937). To date, *B. cinerea*, *B. fabae*, *B. elliptica*, *B. fabiopsis*, and *B. eucalypti* have been reported as causal agents of BCS (Harrison 1988; Ran et al. 2024; Tsukamoto and Okubo 1997; Wilson 1937; Zhang et al. 2010), although only *B. fabae*, *B. cinerea*, and *B. elliptica* have been reported in Japan (Nonaka 1976; Tsukamoto and Okubo 1997). In May 2023, we observed the emergence of BCS on broad bean leaves, characterized by small red spots (Fig. 1a) in field located in Nishio, Aichi Prefecture, Japan. Infected leaf pieces (cut into 5 mm x 5 mm squares) were surface sterilized by sequentially immersing them in 70% ethanol, and 1% sodium hypochlorite for 1 min each. The sections were then rinsed with sterilized distilled water, placed on 1.5% agar, and incubated at 23°C for 2 days in the dark. The elongated mycelial tips were transferred to potato dextrose agar (PDA). Genomic DNA was extracted from mycelia grown on PDA using the Extract-N-Amp plant PCR kit (Sigma-Aldrich, Burlington, MA, USA), and used as template for PCR amplification of the partial internal transcribed spacer (ITS) region. PCR amplification of ITS was performed for two isolates (Vfpl3, Vfpl5) using primer sets ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). The sequence of PCR products from both isolates were identical, which were analyzed using BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the NCBI nucleotide database, revealing greater than 99% identity with multiple *Botrytis* species. When both isolates were cultured on PDA for one week, they produced numerous sclerotia (Fig. 1b, c). However, they did not produce any conidia on PDA or on broad bean leaves under our experimental conditions. We then evaluated the pathogenicity of isolates Vfpl3 and Vfpl5 on broad bean. Agar plugs (Approx. 5 x 5 mm) of mycelia grown on PDA were placed on broad bean leaves and covered with 3-cm squares of lens paper, moistened with distilled water, followed by incubation at 23°C for 3 days. Both isolates exhibited pathogenicity to broad bean, but not to other plant species tested, such as tomato and *Nicotiana benthamiana* (data not shown). Figure 1d shows representative symptoms caused by isolate Vfpl5. The inoculated fungus was re-isolated from surface-sterilized leaves, thereby fulfilling Koch's postulates.

Next, whole genome sequencing was performed to determine the species of the isolate Vfpl5. Extraction and sequencing of genomic DNA were performed as previously reported (Ashida et al. 2023). Genomic DNA was extracted from mycelia grown in potato dextrose broth (PDB) using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Whole-genome sequencing libraries were prepared with MGIEasy FS PCR-Free DNA Library Prep Set (MGI, Yantian, Shenzhen, China), and sequenced on the DNBSEQ-G400RS (MGI) with the DNBSEQ-G400 High-Throughput Sequencing Set. After quality filtering and trimming with fastp v0.12.4 to remove adaptors and low-quality reads (Chen et al. 2018), paired-end datasets were assembled de novo using SPAdes v3.15.5 with -careful option (Bankevich et al. 2012). Assembly quality was assessed using QUAST v4.4 (Gurevich et al. 2013). The assembly was assessed and curated for potential contamination based on taxonomic identification. Specifically, we calculated the mapping coverage of genomic sequencing reads for each contig using BWA-MEM v0.7.17 (Vasimuddin et al. 2019) and samtools v1.21 (Danecek et al. 2021), conducted taxonomic annotation by querying all contigs against the UniProt Reference Proteomes database (version 2024_02) using DIAMOND blastx v2.1.9 with --evaluate 1e-25 --sensitive option (Buchfink et al. 2021), and visualized the results with Blobtools v1.1.1 (Laetsch and Blaxter 2017). Contigs assigned to Ascomycota exhibited a unimodal coverage peak (median $\approx 250\times$), whereas non-Ascomycota contigs clustered below $100\times$ (Supplementary Fig. S1). Contigs with mean coverage $< 100\times$ were removed, and the retained sequences constitute the decontaminated assembly. A summary of genome assemblies is shown in Table 1. Gene prediction was performed using BRAKER3 version 3.0.8 (Gabriel et al. 2024), incorporating the annotated single copy orthologs from ascomycota_odb10 (Kriventseva et al. 2019) as protein hints. The completeness of the assembled genome was estimated with BUSCO version 5.7.0 (Manni et al. 2021) using the ascomycota_odb10 database. Using the Ascomycota dataset, the protein sets of Vfpl5 were found to have BUSCO scores of 98.9% and a total of 1687 complete BUSCOs (Table 1).

For species identification, sequences of the *G3PDH*, *HSP60*, and *RPB2* genes were extracted from the assembled genome and subjected to phylogenetic analysis. A maximum-likelihood phylogenetic tree of Vfpl5, along with representative strains of *Botrytis* spp. (Li et al. 2012; Liu et al. 2016; Staats et al. 2005; Zhang et al. 2010) was constructed using the combined *G3PDH*, *HSP60*, and *RPB2* sequences by phyML (Guindon and Gascuel 2003) with the GTR model option and 1,000 bootstrap replicates. The sequence data of fungi used for phylogenetic analysis are shown in Supplementary Table S1. The analysis indicated that strains Vfpl5 had the closest relationship (100 and 99.9% identities, respectively) with the representative strains of *B. fabiopsis* BC-2 and BC-13 (Fig. 2). Therefore, strain Vfpl5 was identified as *B. fabiopsis*. This is the first report of *B. fabiopsis* being isolated from domestically grown broad beans in Japan.

B. fabiopsis strain Vfpl5 was deposited at the Research Center of Genetic Resources, National Agriculture and Food Research Organization (NARO), Japan, under accession No. MAFF247882. The DNA sequence used for phylogenetic analysis were registered in the DDBJ/EMBL/GenBank databases under the following accession Nos; LC866877 (*G3PDH*), LC866878 (*HSP60*), and LC866879 (*RPB2*). The draft genome sequence of *B. fabiopsis* strain Vfpl5 was deposited in DDBJ/EMBL/GenBank under

accession number GCA_965283105.1

(https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_965283105.1/).

Collectively with previous studies, this study further suggested that BCS outbreaks in Japan are caused by a variety of *Botrytis* spp. Identifying the specific *Botrytis* species responsible for the disease in BCS-infected broad bean fields is probably crucial for effective disease management. *B. cinerea*, a typical polyxenous pathogen, has a range of strategies to infect various plant species (Bi et al. 2023; Bulasag et al. 2023, 2024; Kuroyanagi et al. 2022), and the emergence of fungicide-resistant *B. cinerea* strains has long been a significant challenge in disease control (Fillinger and Walker 2016). BCS caused by *B. cinerea* poses a risk of transmission to neighboring crops, particularly in the area with high fungicide resistance. Given that *B. fabiopsis* has been identified as the causal agent of grey mold on *Bletilla striata* (hyacinth orchid) in Japan (Kawarazaki and Kato 2025), the occurrence of BCS caused by *B. fabiopsis* on broad bean also pose a risk of disease development in other crops. The infection of multiple *Botrytis* species on a single host potentially raises concerns about the emergence of fungicide resistance in this complex species. In combination with ecological analysis in the field, understanding the virulence mechanisms of closely related *Botrytis* species by comparative genome sequence analysis would be important for developing effective long-term strategies for controlling crop diseases caused by *Botrytis* spp.

Declarations

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Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Table

Table 1 is available in the Supplementary Files section.

Figures



Figure 1

Disease symptoms caused by *Botrytis fabiopsis* on broad bean. **a** Chocolate spot disease of broad bean (*Vicia faba*) resulting from natural infection with *B. fabiopsis*. **b,c** Colonies of *B. fabiopsis* strain Vfpl5 isolated from broad bean grown on PDA at 23°C. **d** Disease symptoms on broad bean leaves inoculated with *B. fabiopsis* strain Vfpl5 and incubated at 23°C for 3 days. **e** Sclerotia produced by *B. fabiopsis* strain Vfpl5 grown on PDA at 23°C for 2 weeks. Bar = 1 mm.

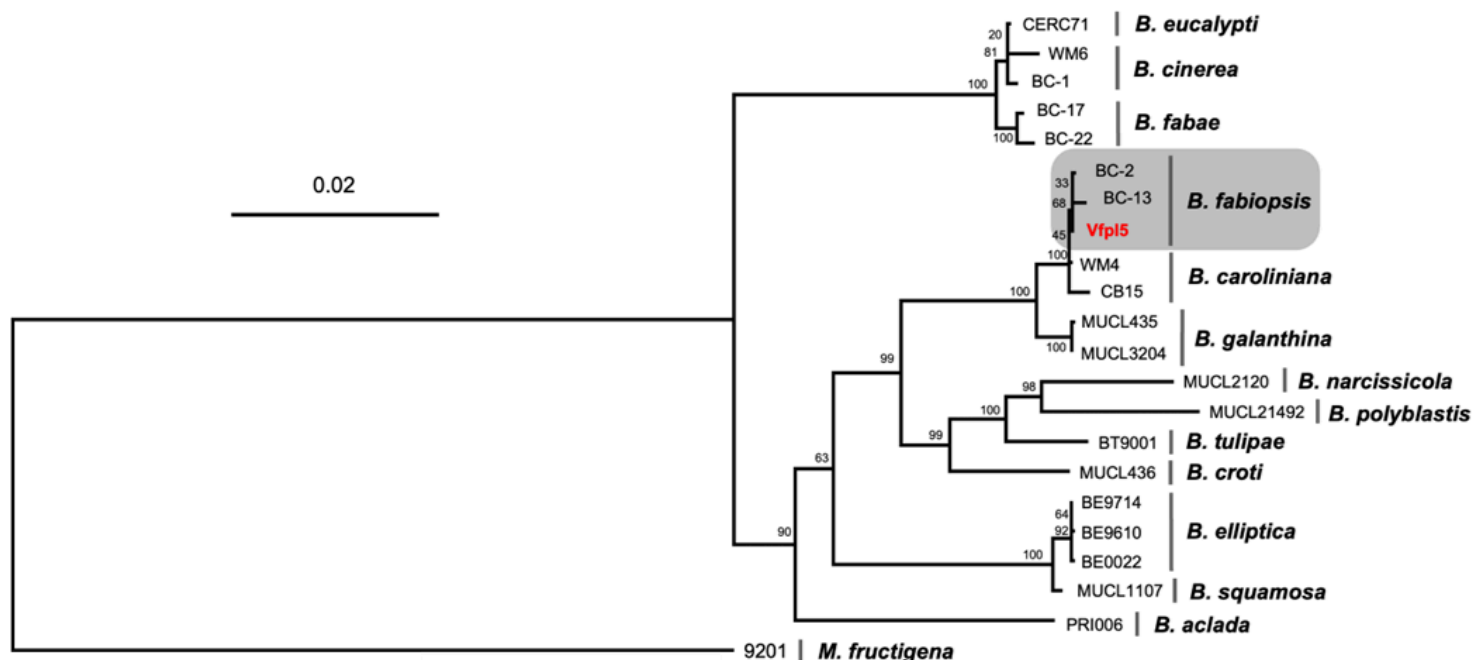


Figure 2

A molecular phylogenetic tree comprising 22 fungal taxa was constructed using the maximum likelihood method, based on combined sequences of the *G3PDH*, *HSP60*, and *RPB2* genes. *Monilinia fructigena* (strain 9201) was used as the outgroup. Bootstrap values are indicated as percentages on the branches and were inferred from 1,000 replicates. Species identified in this study are shown in red.

Supplementary Files

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