

First report of leaf spot on *Atractylodes lancea* caused by *Alternaria gaisen* in Japan

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Research Article

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Abstract

Atractylodes lancea is a medicinal plant that has been used in traditional Chinese and Japanese herbal medicines. In 2020, *A. lancea* plants with leaf spot were identified in Ibaraki, Japan. We isolated fungi from the leaf spot symptom, and in inoculation tests, the isolate reproduced these symptoms. The isolate was identified as *Alternaria gaisen* based on conidial morphology and sequences of three genomic regions (*rpb2*, *gapdh*, and *tef1*). To our knowledge, this is the first report of leaf spot of *A. lancea* caused by *A. gaisen*.

Full Text

Atractylodes lancea is a medicinal plant distributed across East Asia (Shiba et al. 2006). It is a perennial plant of the Asteraceae family. Its dried rhizome has been used as a crude drug “So-jutsu” (Chinese Pharmacopoeia Commission 2020; The Society of Japanese Pharmacopoeia 2021) mainly for the treatment of digestive disorders and as a diuretic in traditional Chinese and Japanese herbal medicines (Kitajima et al. 2003; Wang et al. 2008). In 2018, approximately 1000 tons of So-jutsu were used, making it the fifth commonly used crude drug in Japan (Yamamoto et al. 2021). *Atractylodes lancea* is therefore one of the most important medicinal plants; however, in the past, it was entirely imported from China. For a stable supply of So-jutsu, cultivation of *A. lancea* has been attempted in Japan (Matsuno et al. 2018). *Atractylodes lancea* can propagate by both seed propagation and vegetative propagation (Pharmaceutical Affairs Bureau 1995). It is cultivated for several years and harvested in autumn.

In June 2020, concentric-shaped brown lesions were found on the leaves of plants cultivated in a field in Ami-machi, Ibaraki Prefecture, Japan. The lesions enlarged and developed over the entire leaf (Fig. 1a, b). These symptoms differ from those caused by *Sclerotium rolfsii*, which has been reported to infect *Atractylodes* in Japan (Hoshi et al. 2003; Takeuchi et al. 1995). Blighted leaves lead to reduced rhizome yield. The purpose of this study was to identify the fungus causing this disease and confirm its pathogenicity on *A. lancea* leaves.

To isolate the pathogen, pieces of symptomatic leaves were surface disinfested in 1% (w/v) sodium hypochlorite solution for 2 min, rinsed in sterile distilled water, and placed on potato dextrose agar (PDA) containing 100 ppm chloramphenicol in Petri plates. The plates were incubated at 25 °C for three days in darkness. Hyphal tips were aseptically transferred to fresh PDA, and three monoconidial isolates (AI1-2, AI3-1, and AI3-2) were obtained. Isolate AI3-2 was used for species identification and pathogenicity experiments.

Colonies grown on PDA produced pale olive to white aerial mycelia (Fig. 1c). The mycelia grew at 15–30 °C on PDA, with optimum growth at 25 °C. The conidiophores were brown in color (Fig. 2a). Potato carrot agar (PCA) was used for observation of conidial morphology and pathogenicity tests. The conidia on PCA were pale brown to medium brown, ovoid to obclavate, and sometimes ellipsoid with 1–7 transverse and 0–3 longitudinal septa, 15.5–77.6 × 6.9–18.2 µm, often with a short conical or cylindrical beak (0.0–

26.0 µm; Fig. 2c); 3–10 conidia were chained (Fig. 2b). The chains were unbranched, with occasional short lateral branches. The morphological characteristics of the conidia in the isolate resembled those of *Alternaria gaisen* (Table 1).

Table 1
Comparison of morphological characteristics of *Alternaria* species related to this study.

Isolate	Size (µm)	Shape	Number of septa (Transverse)	Number of septa (Longitudinal)	Catenation
AI3-2	15.5–77.6 × 6.9–18.2 (29.5 × 12.8)	Ovoid to obclavate, sometimes ellipsoid, pale brown to medium brown	1–7	0–3	Formed conidial chains of 3–10 conidia. The chains were unbranched, with occasional short lateral branches
<i>Alternaria gaisen</i> ^a	10–67 × 6–18 (27–31 × 11–13)	Ovoid to ellipsoid, sometimes obclavate, pale to olive brown	0–8	0–2	Conidial formation was in chains of 3–13, usually remained unbranched
<i>Alternaria alternata</i> ^b	11–50 × 7–18 (25 × 12)	Ovoid to ellipsoid, pyriform or obclavate, pale brown to brown	1–7	0–5	Long chains of 10–22, commonly with lateral branches
^a Nishikawa and Nakajima (2019)					
^b Nishikawa and Nakajima (2020)					

Pathogenicity tests were performed using conidia obtained from AI3-2 cultures grown on PCA for seven days at 20 °C. A conidial suspension (2.0×10^5 spores/ml) was sprayed on wounded or intact *A. lancea* plants grown in 9cm diameter plastic pots of commercial potting soil for four months. The control plants were sprayed with sterilized distilled water. There were three replicates for each treatment plant. After inoculation, plants were kept in a growth room at 24 °C under a 16-h photoperiod. Seven days after inoculation, leaf spot symptoms were observed on the wounded inoculated plants, and the lesions enlarged thereafter (Fig. 1d). Eighteen days after inoculation, leaf spot symptoms were observed on the intact inoculated plants, and the lesions enlarged thereafter (Fig. 1e). However, no spots appeared on both wounded and intact control plants. The inoculated fungus was successfully isolated again from the artificially infected plants.

To identify the isolate by phylogenetic analysis, genomic regions of the RNA polymerase second largest subunit (*rpb2*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and translation elongation factor 1- α (*tef1*) were sequenced (Woudenberg et al. 2013). To sequence the genomic regions, DNA was extracted from a seven-day-old PDA colony of AI3-2 using a DNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA). The *rpb2* region was amplified using primers RPB2-5F2 (Sung et al. 2007) and fRPB2-7cR (Liu et al. 1999), the *gapdh* region using primers gpd1 and gpd2 (Berbee et al. 1999), and the *tef1* gene using primers TEF1_F and TEF1_R (Carbone and Kohn 1999). Each region was amplified using the respective primer sets and a 50 μ l reaction mixture containing 1 μ l template DNA, 0.5 μ M of each primer pair, 200 μ M dNTP mixture, 1.25 U ExTaq (Takara, Kusatsu, Siga, Japan), and 5 μ l reaction buffer in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The cycling conditions consisted of 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 40 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Sequencing reactions were performed in a BioRad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Single-pass sequencing was performed for each template using the same primers used for amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators using either the ethanol precipitation method or the BigDye XTerminator™ Purification Kit (Applied Biosystems). The samples were analyzed using a 3730xl DNA Analyzer (Applied Biosystems), and the sequences were deposited in the DNA Data Bank of Japan (DDBJ; accession numbers *rpb2*: LC750711, *gapdh*: LC750712, and *tef1*: LC749824). Homology searches were performed using the NCBI BLAST server, in which the analysis showed that the sequence data for all loci were highly similar to those of *A. gaisen*.

Clustal W was used for sequence alignment. A phylogenetic tree was constructed in MEGA version 11 (Tamura et al. 2021) using the neighbor-joining method with maximum composite likelihood based on the concatenated *rpb2*, *gapdh*, and *tef1* sequences of isolate AI3-2 and *Alternaria* reference sequences obtained from GenBank. Confidence values for individual branches were determined by 1000 replication bootstrap analyses. The phylogeny showed that isolate AI3-2, obtained from *A. lancea*, clustered with authentic isolates of *A. gaisen* (Fig. 3). Based on the morphology and DNA sequences, isolate AI3-2 was therefore identified as *A. gaisen*. The isolate AI3-2 was deposited in the National Agriculture and Food Research Organization (NARO) Genebank as MAFF 247770.

Although a disease caused by *Alternaria* has been reported on *A. lancea* in China and Korea (Romain et al. 2022; Tan et al. 2013), an *Alternaria* disease of *A. lancea* had not been previously reported in Japan. Therefore, we refer to this new disease as black spot disease of *A. lancea* (Kokuhan-byo in Japanese). Further studies are necessary to develop control methods to protect *A. lancea* from this disease in the future.

Declaration

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Author contributions

Both authors contributed to the study conception and design. Sampling and material preparation were performed by Miki Sakurai and Midori Kawashimo. Data collection and analysis were performed by Midori Kawashimo. The first draft of the manuscript was written by Midori Kawashimo and Miki Sakurai commented on previous versions of the manuscript. Both authors read and approved the final manuscript.

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Figures

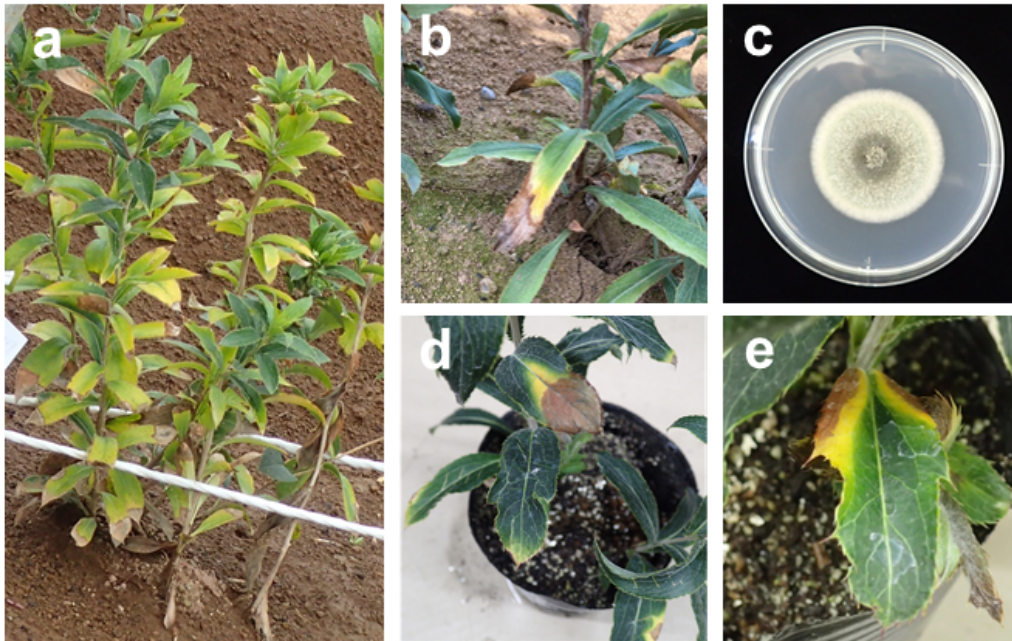


Figure 1

Symptoms of leaf spot disease on *Atractylodes lancea* and culture of the pathogen, *Alternaria gaisen*. **a**, **b** Symptoms on *A. lancea* after natural infection with *A. gaisen*. **c** Six-day-old potato dextrose agar (PDA) culture of isolate AI3-2. **d** Leaf spot caused by *A. gaisen* on wounded leaves of *A. lancea* in the growth room 35 days after inoculation with *A. gaisen*. **e** Leaf spot caused by *A. gaisen* on intact leaves of *A. lancea* in the growth room 35 days after inoculation with *A. gaisen*

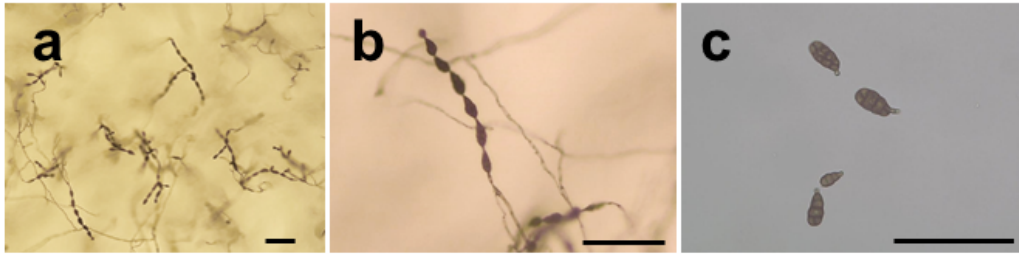


Figure 2

Morphology of the isolate Al3-2. **a, b** Conidiophores and conidia produced simple or branched chains. *Bar* 100 μ m. **c** Conidia on potato-carrot agar. *Bar* 100 μ m

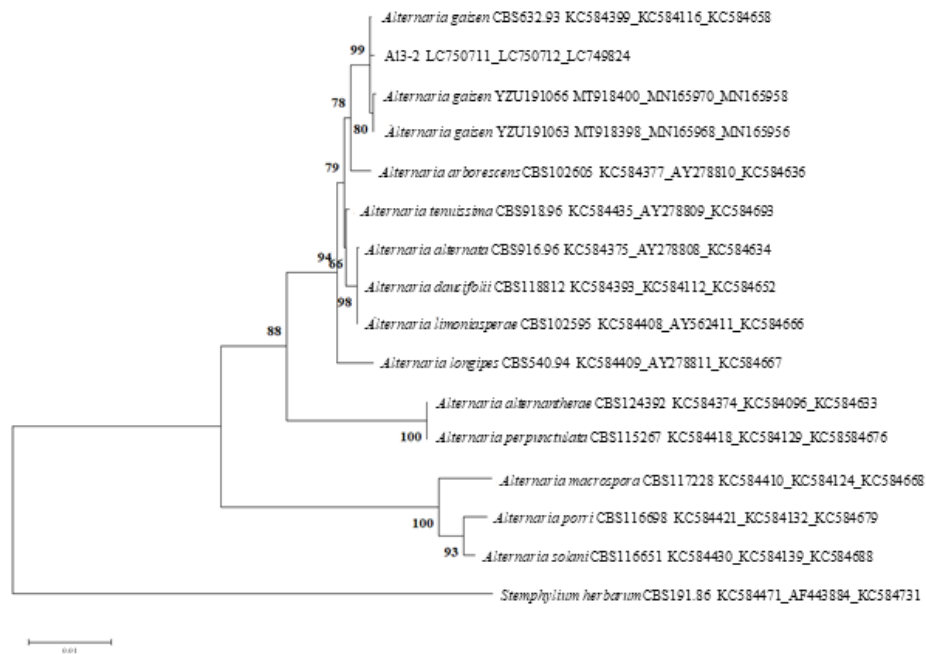


Figure 3

Phylogenetic tree based on concatenated *rpb2*, *gapdh*, and *tef1* sequences of isolate Al3-2 and representative species in the genus *Alternaria* obtained from GenBank constructed using the neighbor-joining method in Clustal W. The maximum composite likelihood method was used as a submission model. Bootstrap values obtained for 1000 replicates are given at the branches. The isolate and accession (*rpb2*, *gapdh*, and *tef1*) numbers are given in parentheses behind the species names. The sequence alignment consisted of 1722 characters (*rpb2* 865, *gapdh* 590, *tef1* 267). Evolutionary analyses were conducted in MEGA 11. The bar indicates 1% sequence dissimilarity