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Physoderma, not *Olpidium*, is the true cause of faba bean gall disease of *Vicia faba* in Ethiopia

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Abstract

Faba bean gall (FBG) is a devastating disease of faba bean (*Vicia faba*) in Ethiopia. Studies were undertaken first to compare and contrast similarities between FBG disease symptoms and morphology in Ethiopia with those reported earlier in China and, secondly, to identify definitively the FBG causal agent, previously considered as *Olpidium viciae*, through molecular studies. Morphological studies confirmed an epibiotic phase of zoosporangia for dispersing zoospores, characteristic of *Physoderma* but not *Olpidium*, and did not show critical diagnostic characteristics of *Olpidium* such as presence of numerous short zoosporangial discharging tubes, or binucleate resting sporangia. Recognizing this epibiotic phase is a foundation for comprehending FBG epidemiology and will allow forecasting of zoospore release to highlight best timings for applications of chemical sprays to reduce reinfection cycles. Sequences of partial ITS1-5.8S-partial ITS2, the 18S-ITS1-5.8S-ITS2-part of 28S rRNA, and LSU (28S rRNA) derived from tissue with symptoms confirmed *Physoderma*, and not *Olpidium*, as the causal agent. Sample sequences were either close to *Physoderma* or the contaminant ascochyta pathogen *Didymella*. From symptom, morphological, and molecular data, the causal agent of FBG disease in Ethiopia is *Physoderma*. From observations of symptoms that *Physoderma* can cause, it was determined that this *Physoderma* crosses over between different legume host genera (e.g., *Vicia*, *Pisum*, *Trifolium*), highlighting the significant biosecurity risk for countries currently free of FBG.

KEYWORDS
faba bean, faba bean gall, *Olpidium viciae*, *Physoderma*, *Vicia faba*

1 | INTRODUCTION

In Ethiopia, pulses are cultivated on roughly 1.5 million ha annually, of which faba bean constitutes approximately 0.49 million ha (Central Statistical Agency – Federal Democratic Republic of Ethiopia, 2019), making faba bean the most important pulse crop in the country. However, the growing environment for faba bean in Ethiopia is highly conducive to a wide range of plant diseases, particularly in high rainfall years that are common across the highland faba bean production areas. Some 26 different diseases attack faba bean in Ethiopia, the most important of which include leaf blights (chocolate spot [*Botrytis fabae*], Ascochyta blight [*Ascochyta fabae*]), rust (*Uromyces viciae-fabae*), black root rot (*Fusarium solani* and *F. avenaceum*), and viruses like faba bean necrotic yellows virus (FBNYV). Each of these diseases can reach severities that severely impact yield and, in some cases, can cause complete crop failure (Abraham et al., 2000; Berhanu et al., 2003; Tadesse et al., 2008).

Until recently, chocolate spot was considered the most destructive faba bean disease in Ethiopia (Sahile et al., 2008). However, in 2010, a previously unknown disease, faba bean gall (FBG), was

reported causing galling and distortion of faba bean foliage in the North Shoa region (Gorfu et al., 2012). It then spread rapidly through farmers' fields in the central highlands and became increasingly severe (Hailu et al., 2014). Surveys highlighted the rapid spread and devastating impact of FBG across the different regions of Ethiopia in subsequent years. Examples include surveys in 2012 and 2013 in the highlands of Wollo that showed FBG incidence of 50%–100% and with severity 10%–59% (Hailemariam et al., 2016). Surveys by Abebe et al. (2014) showed severity ranging from 30% in the Emba-Alaje district to as high as 100% in Ofla, Enda-Mekoni, and Raya-Alamata districts, highlighting the importance of FBG in Tigray. Hailu et al. (2014) showed FBG severity in the Amhara region was 22%, but was lower at that time in Tigray and Oromiya regions at 11% and 8%, respectively. Bitew (2015) highlighted the importance of FBG in surveys across the nine different districts of North Shoa. Furthermore, Debela et al. (2017) reported FBG in the Western Highlands of Oromiya with severity of 4%–80% and with yield losses up to 100% in some crops. Similarly, Debela et al. (2017) also highlighted up to 100% losses from FBG, particularly in higher altitudes of 2,000-4,000 m above sea level where rainfall is greatest (Abebe et al., 2014; Bitew & Kebede, 2012). Not only did FBG quickly become established across all main faba bean-growing regions of Ethiopia, but at severities surpassing all other diseases (Hailu et al., 2014). Currently, farmers are using fungicide sprays to manage the disease, but the requirement of frequent applications prohibits poor farmers from saving their crops from FBG (Teklay et al., 2018).

Olpidium viciae (Kusano, 1912), belonging to the Olpidiaceae, was accepted as the causal agent of FBG in Ethiopia (Gorfu et al., 2012; International Food Policy Research Institute, 2010), the same pathogen first reported in Japan in 1912 on *Vicia unijugae* (Kusano, 1912) and reported subsequently as the cause of "blister disease" of faba bean in China by Liang (1986), Lang et al. (1993), Yan (2012), and Yan and Ye (2012). The Ethiopian identification was based on similarity of symptoms (Earecho, 2019) and observation of resting spores within the galls of affected leaves, as reported in China for morphological studies by Xing (1984), Liang (1986), Yan (2012), and Yand and Ye (2012). While there have been several attempts to confirm molecularly the identity of the FBG pathogen, these have not been successful, only showing similarities with unrelated pathogens such as *Phoma*, *Peyronellaea pinodella* (i.e., syn. *Mycosphaerella pinodes*, *Didymella pinodes*), *Aureobasidium/Kabatiella lini*, *Cryptococcus victoriae*, or *Albugo laibachii*, none of which are known to produce the typical FBG symptoms on faba bean. These molecular investigations on Ethiopian isolates of the FBG pathogen contrast to those in China, where Yan (2012) used the primer pair ITS1 and ITS4 to amplify the rRNA internal transcribed spacer (ITS) region of the pathogen and "confirmed" the identity of "faba bean blister" causal pathogen in China as *O. viciae* according to pathogen sequence. However, this would be the first ever *Olpidium* species to operate via foliar disease cycles rather than root infection. Here, we report studies undertaken first to compare and contrast similarities between the FBG disease symptoms and morphology in Ethiopia with those reported earlier in China and Japan and, secondly, to identity definitively the FBG pathogen causal agent through molecular studies.

2 | MATERIALS AND METHODS

2.1 | Disease symptoms, and pathogen morphology and histology

Investigations of FBG field symptoms, and pathogen morphology and histology were undertaken in Ethiopia, in September 2019 in the Debre Birhan Agricultural Research Centre laboratory and later in March 2020 in the International Livestock Research Institute laboratories in Addis Ababa. These included comparisons with descriptions made earlier, in Japan, by Kusano (1912) (and with those later reproduced and redrawn by Alexopoulos, 1962) and in China by Liang (1986), and, in particular, the detailed descriptions and photographs provided in the PhD thesis of Yan (2012) and those in Yan and Ye (2012). In-field symptoms were similar to those described in these earlier studies and, as we have shown in Figure 1a–g, taken during field visits to affected areas in Ethiopia in August 2017, and in September 2018 and 2019. Most samples used for histological investigations (Figure 2a–f) were collected from the field in early March 2020. These observations were made on hand-sectioned wet mounts of infected and healthy faba bean leaves and stems using an Olympus CX33 microscope, with images captured using an Olympus EP50 digital photographic system.

2.2 | Collection of FBG DNA samples

From two separate collections of plants in Ethiopia showing FBG symptoms, one in September 2019 (127 samples), followed by a second in March 2020 (60 samples), crude extracts of DNA from pathogen-infected materials were applied onto Whatman FTA cards. Field sampling at each location was undertaken where at least two plants showing obvious gall symptoms were selected. When returned to the laboratory, an area of galled fresh tissue, mostly on leaves, was excised from each plant sample. For the 2020 collection, diseased tissue surfaces were first wiped with 70% ethanol for initial sterilization before excision. For both sampling collections, fresh galled pieces were placed into a 1.5 ml Eppendorf tube until the tube was approximately half-filled, with a separate Eppendorf tube for each sample. Gall tissues were thoroughly macerated in these tubes using a plastic pestle. Additionally, for the 2020 collection, what remained of the upper and lower epidermal layers of infected leaf tissues were separately and carefully peeled off by hand and treated similarly. Then macerated gall extract samples were placed onto separate FTA cards and air-dried. Additionally, the 2020 gall samples were first processed in Ethiopia to extract DNA as described in 2.3 below, and extracted DNA was applied to FTA cards. Impregnated FTA cards were exported out of Ethiopia under an Ethiopian Biodiversity Institute Material Export Permit (reference no. EBI71/2553/2011) and imported into Australia under an Australian

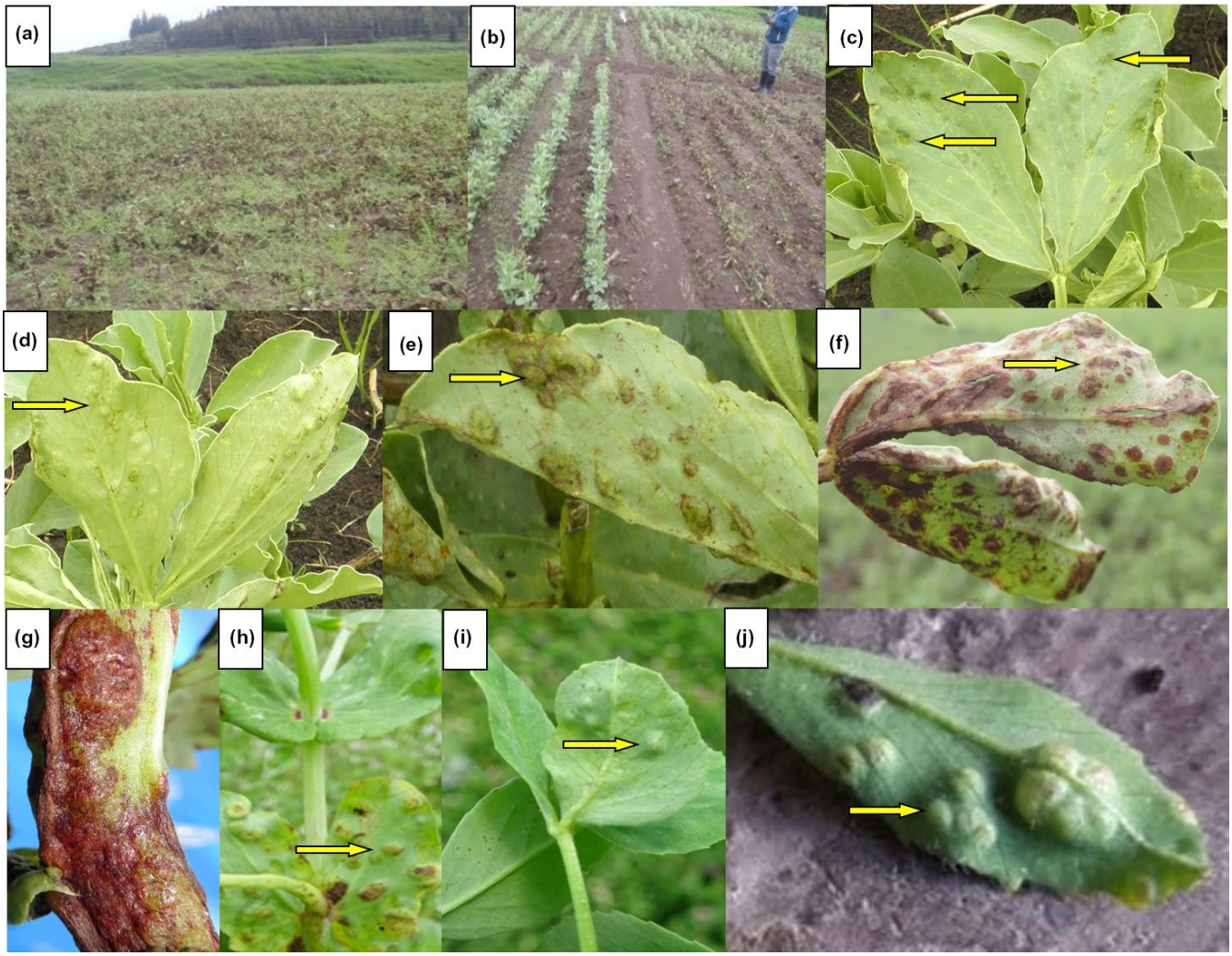

FIGURE 1 Field and plant host symptoms for faba bean gall (FBG) disease in Ethiopia (a–g) showing poor plant growth and major foliage death in a severely infested farm field (a); impact of disease demonstrated by fungicide control on left and no fungicides on right of photograph (b); "sunken-well" indentations on upper leaf surface (arrows), within which zoosporangia quickly develop and release zoospores enhancing their dispersal (c); areas of bulging (i.e., galling) of lower leaf surface (arrows) in early symptom development (d), and darkening of such tissues as symptoms develop into more pronounced galls over time (arrows) (e, f); typical galling on stem (g). Symptoms (arrowed) were also found, to a much lesser degree, on field pea sown together with faba bean (h), and on two different unidentified clover species growing in close proximity to infested faba bean crops (i, j)

Government, Department of Agriculture and Water Resources Import Permit specifically for this purpose (permit no. 0002826465).

2.3 | DNA extraction and PCR conditions

In Australia, DNA was extracted from FTA cards carrying pathogen DNA by cutting two or three pieces each 2 mm square into a 2 ml Eppendorf tube and washing to remove inhibitors using a modified method of Ahmed et al. (2011). Briefly FTA card pieces in 2 ml tubes were washed twice in 200 µl of TENT (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 12 mM NaCl, 2.5% Triton X-100) and incubated for 5 min each time with gentle agitation at 1,000 rpm on an agitator (TTS 3digital Yellow line). The FTA cards were then washed twice in 200 µl of TE 0.1 (10 mM Tris-Cl, pH 8, 0.1 mM EDTA) buffer and agitated for 5 min each time at 1,000 rpm and supernatant removed each time. Subsequently, the FTA card pieces were left to dry at room temperature (laminar flow for at least 2 hr). Dried FTA card pieces were then ready for subsequent DNA extraction undertaken as follows. Two ceramic beads (2 mm) and 300 µl extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, 0.5% wt/vol sodium dodecyl sulphate [SDS]) were added into the tube and homogenized using a Precellys Evolution homogenizer at 12,208 × g for 3 × 60 s cycles, each cycle with a 30 s pause between cycles. Then, 150 µl of 3 M sodium acetate (pH 5.2) was added, mixed well by pipetting, and left at room temperature for 10 min before centrifuging at 15,871 × g for 10 min. Subsequently, supernatant was transferred to a fresh tube containing an equal volume of ice cold isopropanol (450 µl), mixed well by pipetting, and then left to stand at least 15 min at room temperature (22°C) or in a fridge overnight

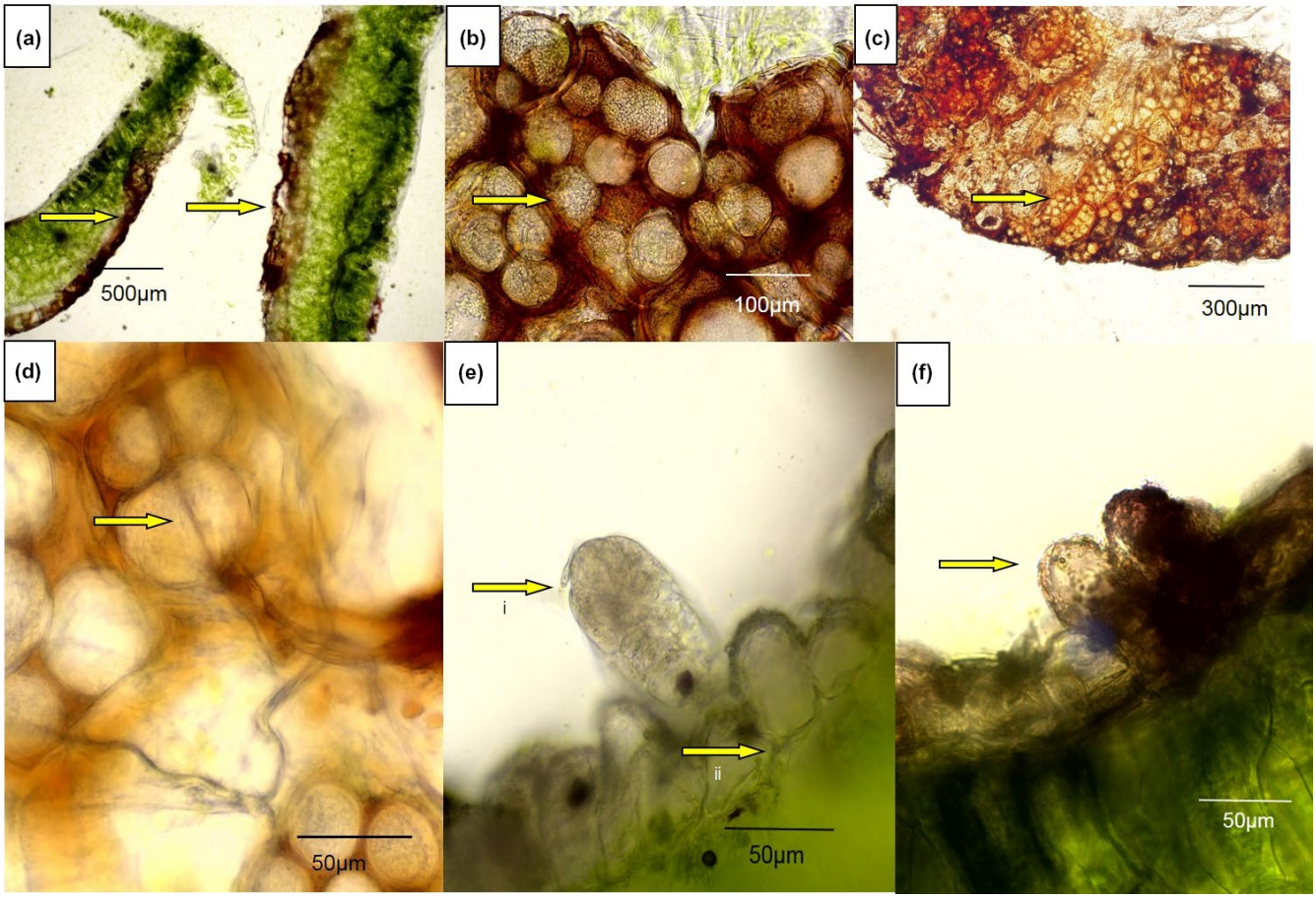

FIGURE 2 Typical cross section of a bean leaf with symptoms of faba bean gall disease showing dark brown regions (arrows) on underside of leaf gall (a), in which large masses of resting spores are produced (arrow) (b), and typical masses of resting spores in end-of-season dried residues (c). Turbinate cells with two segments were also observed (d). Zoosporangium with opening (e, arrow i) and base of zoosporangium (e, arrow ii). Zoosporangia develop internal zoospores masses (arrow) (f)

for best results. Precipitated DNA was collected by centrifugation at 15,871 × g for 10 min and removal of the supernatant. The pellet was washed with 70% (vol/vol) ethanol, dried (air dry or vacuum dry), and then resuspended in 50 µl of TE buffer. The quantity and quality of extracted DNA was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific). DNA was stored at 4°C. The DNA was subjected to PCR in a total volume of (25 µl) that contained 0.5 µM of each primer, 1 µl test DNA, 12.5 µl Dream Taq Green PCR Master Mix (2×) (ThermoFisher Scientific), and 10.5 µl nuclease-free water (details of primers used and PCR conditions are listed in Table 1). PCR products were subjected to agarose gel electrophoresis at 60 mV for ≥1 hr (dependent on size of the PCR products) on a 1% (wt/vol) agarose gel containing 0.1% GelRed (Biotium Inc.) and then visualized under UV light. PCR products were then sequenced by outsourcing via Macrogen Inc. (Korea).

2.4 | Primer pairs

Primer pairs (as listed in Table 1) were used to amplify small subunit rRNA (*SSU*) (James et al., 2006), the ITS region (ITS1 to ITS4) (White et al., 1990), and the nuclear large ribosomal subunit *LSU* (Rehner & Samuels, 1994; Stielow et al., 2015; Vilgalys & Hester, 1990). Primers EF and RPB2 were also tried, but without success. Initially, assuming the pathogen was *O. viciae*, general *Olpidium*-specific reverse primers (Herrera-Vásquez et al., 2009) for amplifying the ITS region were also used in combination with ITS1 (and with the specific primer pair OLPv4F and OLPv4R developed in the current study; Table 1) for *O. viciae*, designed according to the sequence of *O. viciae* available in GenBank (HQ677595) that had been submitted in December 2010 by Yan, J., Dai, H., & Shi, X., College of Veterinary Medicine, Animal Biotechnology Center, Sichuan Agricultural University, Sichuan Province, China, 2012 .

2.5 | Phylogenetic tree building

All successfully sequenced sample DNAs were aligned using Geneious Prime v. 2020.03 and the resulting consensus sequences were submitted to a BLAST search in GenBank (NCBI). If sequences had low percentage identity (≤97%) or had low coverage (≤85%), the most frequent and closest matching pathogens from GenBank

TABLE 1 Primers and PCR conditions used in this study

Gene/locus amplified	Primer	Sequence (5′–3′)	Initial T (°C)	Duration (min)	Denature T (°C)	Duration (s)	Annealing T (°C)	Duration (s)	Elong. T (°C)	Duration (s)	Cycle	Extension T (°C)	Duration (min)
ITS	ITS1	TCCGTAGGTGAACCTGCGG	94	1	94	30	60	30	72	60	35	72	10
	ITS4	TCCTCCGCTTATTGATATGC											
	ITS1	TCCGTAGGTGAACCTGCGG	94	5	94	45	55	60	72	60	35	72	10
	OLPR	TCCTCCGCTTATTGATATGCTTA											
	OLPvic4F	ATCGATGAAGAACGCAGCGA	95	5	95	45	55	40	72	45	35	72	10
	OLPvic4R	TTCAGCGGGTATCCCTACCT											
	TW81	GTTTCCGTAGGTGAACCTGC	94	2	94	60	55	60	72	90	35	72	10
	OLPvic4R	TTCAGCGGGTATCCCTACCT											
SSU rRNA	SR1-R	TACCTGGTTGATTCTGC	95	2	94	60	55	60	72	210	38	72	10
	NS4	CTTCCGTCAATTCCTTTAAG											
	SR1-R	TACCTGGTTGATTCTGC	95	2	94	60	55	60	72	210	38	72	10
	SR6	TGTTACGACTTTTACTT											
	SR1-R	TACCTGGTTGATTCTGC	94	1	94	30	55	30	72	300	35	72	10
	LR12	GACTTAGAGGCGTTCAG											
	18S-Cs-1F	GAGGCCTACCATGGTGAT	95	2	94	60	55	60	72	210	38	72	10
	NS6	GCATCACAGACCTGTTATTGCCTC											
LSU	LROR	ACCCGCTGAACTTAAGC	95	2	94	60	55	60	72	60	38	72	10
	LR5	TCCTGAGGGAAACTTCG											
RPB1	RPB1af	GAATGTCCAGGACATTTCGG	94	1	94	30	52	30	72	90	38	72	10
	RPB1cr	CCTGCAATTTCATTATCCATGTA											
RPB2[a]	RPB2-5f	GATGATAGAGATCACTTTGG	94	5	95	30	55	60	72	60	35	72	7
	RPB2-7cr	CCCATAGCTTGTTTACCCAT											
	RPB2-7cF	ATGGGTAAGCAAGCCATGGG											
TEF1α[b]	EF1-1018F (Al33F)	GACTTCATCAAGAACATGAT	94	5	94	30	48	50	72	50	40	72	7
	EF1-1620R (Al33R)	GACGTTGAATCCAACATTGTC											
	EF1-1002F (Al34F)	TTCATCAAGAACATGAT	94	5	94	30	48	50	72	50	40	72	7
	EF1-1688R (Al34R)	CTATCATCACAATGGACGTTCTTGGAG											

Abbreviations: ITS, internal transcribed spacer region; *SSU rRNA*, small subunit rRNA gene; *LSU*, nuclear large ribosomal subunit gene; *RPB1* and *RPB2*, DNA-directed RNA polymerase II subunit genes; *TEF1α*, translation elongation factor 1α.

[a]RPB2-5f can be used with RPB2-7cr.

[b]If not successful then repeated using (Al34F/R).

were selected as references for building phylogenetic trees, again using Geneious Prime v. 2020.03. Sequences of morphologically similar and taxonomically related pathogens *Olpidium*, *Physoderma*, *Synchytrium*, and *Urophlyctis* were also selected from GenBank and included in building phylogenetic trees for comparison.

A phylogenetic tree was built for each amplification region: ITS1-5.8S-ITS2, partial 18S-ITS1-5.8S-ITS2-partial 28S, and sequences from *LSU* (28S rRNA).

Pairwise sequence alignment (alignment type: global alignment with free end gaps; cost matrix: 65% similarity [5.0/−4.0]) was used with the Tamura–Nei genetic distance model and neighbour-joining for tree building.

3 | RESULTS

3.1 | Disease symptoms and pathogen morphology and histology

Field and plant host symptoms for FBG disease in Ethiopia (see Figure 1a–g) included poor plant growth and major foliage death in severely affected farm fields (Figure 1a); a severe impact of disease as demonstrated by comparing fungicide-treated plots with untreated plots (Figure 1b); "sunken-well cupping" indentation areas on upper leaf surface within which zoosporangia quickly developed and released zoospores into these "contained" water pools that maximized leaf wetness and enhanced dispersal of zoospores (Figure 1c); areas of bulging (i.e., galling) of lower leaf surface in early symptom development (Figure 1d); darkening of such tissues as symptoms developed into more pronounced galls over time (Figure 1e,f); and typical galling on stems (Figure 1g). Symptoms were also found, but to a much lesser degree, on field pea sown together with faba bean (Figure 1h), and on two different but unidentified clover species growing in close proximity to affected faba bean crops (Figure 1i,j).

When histological studies were conducted, we observed a range of morphological symptoms that included dark brown regions on the underside of the leaf gall seen in typical leaf cross sections (Figure 2a); here, large masses of resting spores were produced (Figure 2b) and typically, masses of resting spores in the end-of-season dried residues (Figure 2c). We also observed turbinate cells (Sparrow et al., 1961) with two segments (Figure 2d) and zoosporangia with openings, inside which zoospore masses developed (Figure 2e–f). Importantly, in our leaf sections, we consistently observed the epibiotic phase of zoosporangia for dispersing zoospores (Figure 2e,f). However, a thorough search of sections of affected leaves did not reveal either the presence of numerous short zoosporangia discharging tubes nor the binucleate resting sporangia characteristic of *Olpidium* spp.

3.2 | Sequence BLAST results

A total of 173 sequences were obtained, 95 from ITS, 55 from *LSU*, 20 from *SSU*, 1 from *TEF1α* and 2 from *RPB2*. Nearly 40% of all sequences were close to *Didymella*, with percentage identity from 83% to 99% and coverage from 27% to 99% (Table 2). Sequences of 11 isolates from the 2020 collection gave a result of "uncultured fungus" and there was a high similarity between them for the *LSU* sequences.

Within sequences from the ITS region, approximately 39% were close to the genus *Didymella*, 20% close to *Phoma*, 14% close to *Mycosphaerella*, and 19% close to the host *Vicia*. Within sequences from the *LSU* gene, approximately 53% were close to the genus *Didymella*, 20% were close to "uncultured fungus", 5% close to *Olpidium*, and 4% that were close to each of *Cladosporium*, *Cymadothea*, "no significant result obtained", and *Vicia*. For the *SSU* gene, 30% were close to *Leucosporidium*, 10% (two samples) were close to *Phoma*, 5% (one sample) each were close to *Boeremia*, *Cryptococcus*, *Filobasidium*, and *Phaseoleae* (plant), and 35% close to the host plant *Vicia* (Table 2). A large portion of sequences identified as genera *Didymella*, *Olpidium*, and "uncultured fungus" included those showing a low percentage query coverage and identity from either or both the ITS and *LSU* locus/gene (Figure S1; Figure 2). Genera sequenced from *SSU* showed only small variations both in percentage query coverage and in identity (Figure S3). None of the genera identified from BLAST results with above 97% identity have previously been recorded as causing a "faba bean blister" type symptom. Despite being recorded as either producing a possible symptom with some similarity to "faba bean blister" and/or showing at least some morphological similarity, we did not find *Physoderma*, *Synchytrium*, or *Urophlyctis* in these BLAST results.

3.3 | Phylogenetic analysis

The phylogenetic tree constructed from the ITS1-5.8S-ITS2 sequences (38 test sample sequences) obtained in this study and from related isolates from the NCBI database showed that all test isolates formed a group (group 3) with *Physoderma maydis* (HB683909). *O. viciae* (HQ677595) from China was close to two *Didymella* isolates from NCBI, forming group 1. Another four *Physoderma* isolates and one *Synchytrium* from NCBI formed group 2 (Figure 3).

When sequences from partial 18S-ITS1-5.8S-ITS2-partial 28S (27 test sample sequences) were used to construct a phylogenetic tree, one test sequence was close to *Physoderma* in group 1, three test sequences in group 3 were close to *Physoderma*, 22 test sequences were close to *Didymella* in group 6, group 5 included *Physoderma* and *Synchytrium*, and group 4 included *Olpidium* from NCBI (Figure 4).

In the phylogenetic tree constructed from the *LSU* (28S rRNA) sequences, the 47 test sequences formed four groups, with 13 sequences grouped with *Physoderma* (group 2); 11 sequences grouped with *Physoderma* in group 3, two sequences were close to *Cymadothea* in group 4, 21 sequences grouped with *Didymella* in group 5, and group 1 was formed by *Olpidium* sequences from NCBI (Figure 5). Sample sequences that grouped with *Physoderma* have been deposited in GenBank (accession numbers from MW414613 to MW414631, from MW448404 to 448414, from MW497579 to MW497587, and from MW587325 to MW587329).

TABLE 2 Genera obtained from BLAST search results using successfully sequenced genes/loci from isolates obtained from plants with faba bean gall disease in this study

	Gene/locus					Percentage of successfully sequenced isolates per genus			
Genus	ITS	*LSU*	*SSU*	*TEF1α*	*RPB2*	Total[a] (%)	Within ITS (%)	Within *LSU* (%)	Within *SSU* (%)
Abrothrix	0	1	0	0	0	0.6	0	1.8	0
Ascochyta	1	0	0	0	0	0.6	1.0	0	0
Aspergillus	1	0	0	0	0	0.6	1.0	0	0
Boeremia	0	0	1	0	0	0.6	0	0	5
Cladosporium	0	2	0	0	0	1.2	0	3.6	0
Coniothyrium	1	0	0	0	0	0.6	1.0	0	0
Cryptococcus	1	1	1	0	0	1.7	1.0	1.8	5
Cymadothea	0	2	0	0	0	1.2	0	3.6	0
Didymella	37	29	0	0	0	38.2	39.0	52.7	0
Didymosphaeria	1	0	0	0	0	0.6	1.0	0	0
Epicoccum	1	0	0	0	0	0.6	1.0	0	0
Filobasidium	0	0	1	0	0	0.6	0	0	5
Leucosporidium	0	0	6	0	0	3.5	0	0	30
Mycosphaerella	13	0	0	0	0	7.5	13.7	0	0
No significant	1	2	0	0	1	2.3	1.0	3.6	0
Olpidium	0	3	0	0	0	1.7	0	5.4	0
Penicillium	0	1	0	1	1	1.7	0	1.8	0
Phaseoleae	0	0	1	0	0	0.6	0	0	5
Phoma	19	1	2	0	0	12.7	20.0	1.8	10
Uncultured	1	11	1	0	0	7.5	1.0	20.0	5
Vicia	18	2	7	0	0	15.6	19.0	3.6	35
Total	95	55	20	1	2	100	100	100	100

[a]Sequences from all gene regions considered together.

4 | DISCUSSION

It was evident that, in general, disease symptom development in fields of both Ethiopia (e.g., Hailemariam et al., 2016) and China (e.g., Yan, 2012; Yan & Ye, 2012) are similar, and included typical galling of leaves and stems, and browning of the affected tissues over time. However, there were some contrasts; for example, in the current study, we highlight how the upper leaf surfaces show distinct sunken-well cupping indentations before any leaf browning occurs. At the margins of these indentations, we observed prolific zoosporangia formation and zoospore release. These indentations naturally retain moisture, fostering multiple cycles of zoosporangia production and zoospore dispersal by rain-splash to progress the disease epidemic rapidly under conducive conditions. This was quickly followed by production of masses of resting sporangia, particularly on the undersides of leaves, ensuring pathogen survival both across seasons and when seasonal conditions become less favourable for epidemic spread.

Importantly, in the current studies, there were a number of morphological characteristics observed that either supported an identification of the causal pathogen as *Physoderma* or were contrary to an identification of it as *Olpidium*. First, in our leaf sections, although we observed the epibiotic phase of zoosporangia for dispersing zoospores, a diagnostic characteristic of *Physoderma*, we did not observe the endobiotic zoosporangia characteristic of *Olpidium* (Gould & Schaechter, 2009; Johns, 1966); *Physoderma*, but not *Olpidium*, has two phases (epibiotic monocentric phase and an endobiotic polycentric phase) (Johns, 1966). Secondly, we did not observe the presence of numerous short zoosporangia discharge tubes or the binucleate resting sporangia so characteristic of *Olpidium* (Hiruki & Alderson, 2011), or the much wider variation in size and frequency of discharge tubes that has been reported for *Olpidium* (Garrett & Tomlinson, 1967) compared to *Physoderma*. Thirdly, while we observed masses of large resting spores, it appears that the true identity as *Physoderma* was missed from mistakenly assuming that the resting spores observed in FBG disease belonged to the holocarpic genus *Olpidium*, particularly when observed at the advanced stages of infections where the rhizomycelial elements would not have been apparent (Johns, 1966).

While our morphological studies did not support any consensus that the causal pathogen could be an *Olpidium* species, the symptoms and presence of resting spores in leaves clearly indicates it to be a chytridiomycete pathogen. Chytridiomycetes are pathogens associated with aquatic habitats, have a flagellated stage (zoospores), with plants generally reacting to infection by forming gall structures

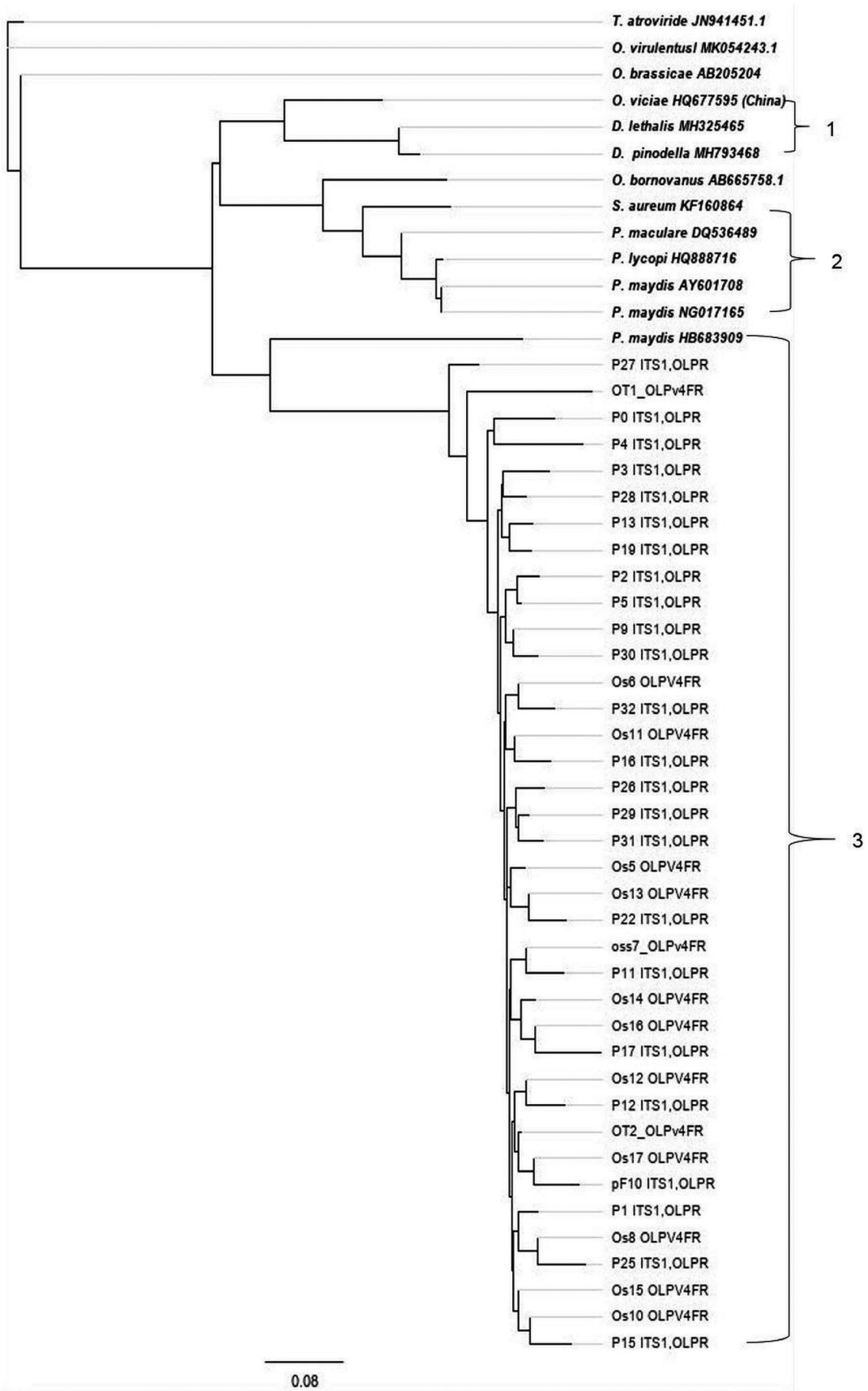

FIGURE 3 Phylogram based on sequences of the ITS1-5.8S-ITS2 region, showing the relationship between isolates obtained from plants with faba bean gall disease in this study and other related isolates from the NCBI database. Reference isolates from NCBI are shown in bold type

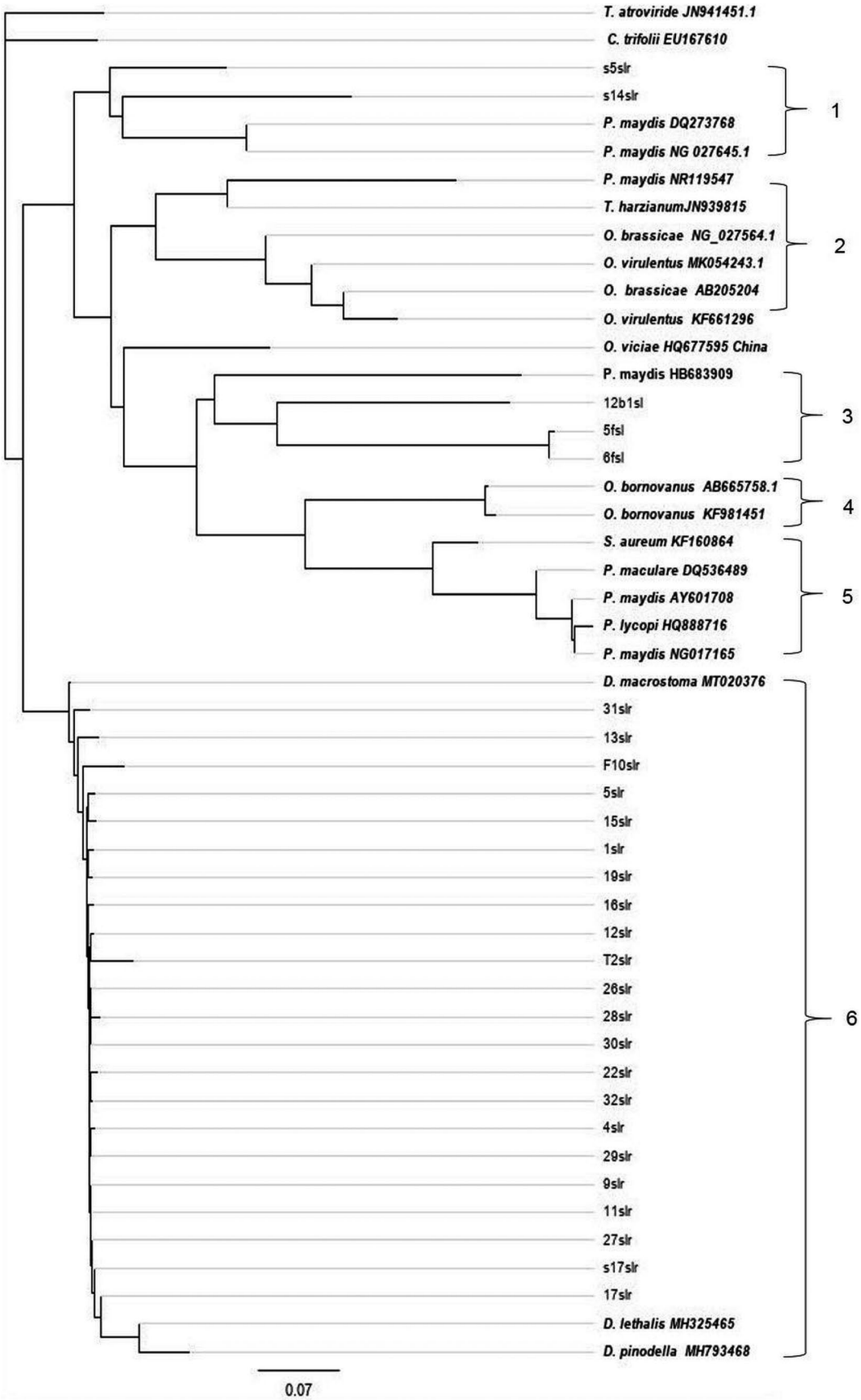

FIGURE 4 Phylogram based on sequences from partial 18S-ITS1-5.8S-ITS2-partial 28S, showing the relationship between isolates obtained from plants with faba bean gall disease in this study and other related isolates from the NCBI database. Reference isolates from NCBI are shown in bold type

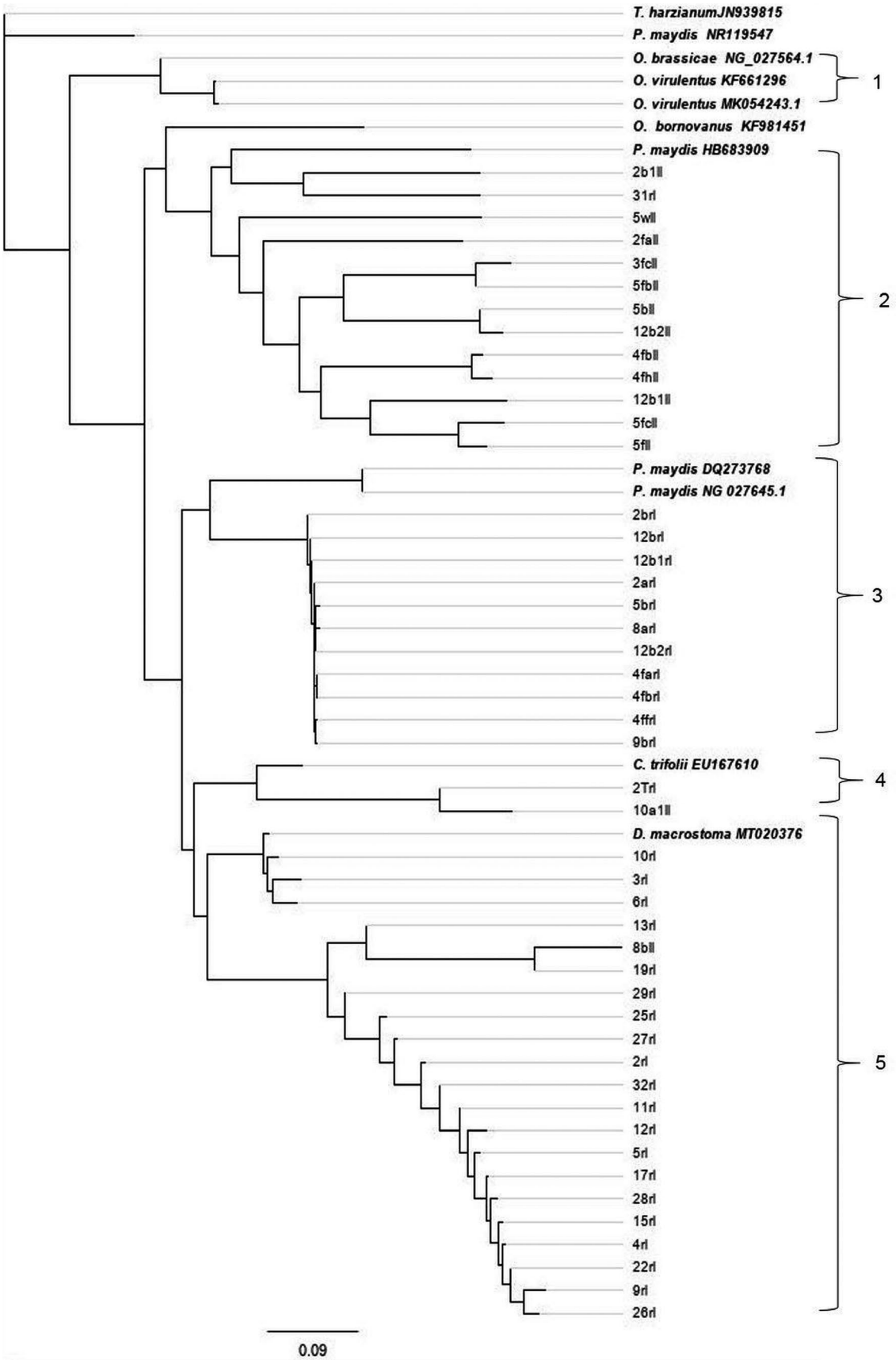

FIGURE 5 Phylogram based on sequences from the large subunit rRNA gene (*LSU* [28S rRNA]) showing the relationship between isolates obtained from plants with faba bean gall disease in this study and other related isolates from the NCBI database. Reference isolates from NCBI are shown in bold type

around the zoosporangia, and with zoospore dissemination depending on flooding or (heavy) rainfall. Chrytridiomycete genera, while often misidentified and reclassified, contain some obligate plant pathogens of worldwide importance (e.g., *Synchytrium endobioticum*, potato wart disease), with resting spores persisting and remaining infective in soil for very long periods (e.g., potato wart disease >40 years), as assumed for the FBG pathogen surviving on infested residues in soil in China (Lang et al., 1993). Plant pathogens *Olpidium*, *Physoderma*, and *Synchytrium* were all previously classified as chytridiomycetes (Agrios, 2005; Alexopoulos, 1962), and all three genera produce thick-walled resting spores and/or zoosporangia, with typical infection caused by zoospores produced from zoosporangia (within a growing season) and from carryover of resting spores between seasons. However, only *Physoderma* and *Synchytrium* cause plant diseases above ground, generally as a gall symptom that occurs from cells in affected tissues being stimulated to divide repeatedly and enlarge excessively (Agrios, 2005), as also observed in the current study. In contrast, *Olpidium* spp. are obligate plant root pathogens found commonly throughout the world infecting the roots, but not foliage, of wild and domesticated plants (Maccarone et al., 2010). Furthermore, the FBG pathogen in Ethiopia and elsewhere attacks host stems and leaves but is not known to cause disease on roots (Alexopoulos, 1962; Kusano, 1912; Lay et al., 2018). There are only three *Olpidium* species recognized as plant pathogens because of their ability to vector viruses, the first two being *O. brassicae* and *O. bornovanus* (=*O. radicale*) (Campbell, 1985, 1996; Herrera-Vásquez et al., 2010; Rochon et al., 2004). However, Sahtiyanci (1962) had earlier separated *O. brassicae* (formerly *Pleotrachelus brassicae*) into two species (*O. brassicae* being crucifer-infecting and *O. virulentus* non-crucifer-infecting). All three of these *Olpidium* species show morphological features clearly distinct from those of *Physoderma*.

In our molecular identity studies, phylograms were used for further investigation. Because multiple sequence alignment resulted in high likelihood topology for our isolates, we used pairwise sequence alignment as it best generates optimal alignment (Xia, 2016). An interesting group from the *LSU* sequences that matched NCBI isolates, "uncultured fungi", showed very high similarity between themselves and grouped with the *Physoderma* isolate from NCBI in group 3. Genera resulting from analysis of *SSU* sequences were of high percentage identity with isolates in NCBI and were therefore considered true genera; however, these genera were clearly not related to the disease symptom or pathogen morphology. Genera *Mycosphaerella* and *Phoma* obtained from ITS sequences, and *Phoma* also from *SSU* sequences, matched with NCBI isolates with a high level of percentage identity and query coverage (>97% percentage identity and >93% query coverage) and, therefore, were assumed to be true matches (Hibbett et al., 2016). All above-mentioned non-*Olpidium* and non-*Physoderma* genera were also isolated and identified by sequencing in our preliminary investigations (including our initial samplings, data not shown).

Olpidium belongs to the Zygomycota while *Physoderma* belongs to the Blastocladiomycota (James et al., 2006; Money et al., 2016). In our phylograms, analysis of the ITS1-5.8S-ITS2 region placed *Physoderma* with *Synchytrium* in group 2 while *Olpidium* was further away; a similar result was found in groups 2, 4, and 5 of the tree constructed from the partial 18S-ITS1-5.8S-ITS2-partial 28S sequences. The findings of the current study are in contrast to those on spring-sown faba beans in high altitude regions of south-west China, where Yan (2012) used primer pair ITS1 and ITS4 to amplify the ITS region of the pathogen and concluded that the causal pathogen of faba bean blister in China was *O. viciae*. Importantly, in our study, *O. viciae* (HQ677595) from China did not group with any other *Olpidium* from NCBI, but instead grouped with *Didymella* in group 1, while sequences of our test isolates were grouped in group 3 with *Physoderma*. It is clear that this isolate of *O. viciae* (HQ677595) from China is neither close to *Olpidium* nor *Physoderma*, but is close to *Didymella*.

The partial 18S-ITS1-5.8S-ITS2-partial 28S sequences amplified from our test isolates placed most of the isolates with *Didymella* in group 5. However, critically, the isolate sequence in group 1 and three isolates in group 3 grouped with *Physoderma*; in contrast, all *Olpidium* isolates from NCBI were grouped in groups 2 and 4. The phylogram constructed from *LSU* sequences showed the test isolates distributed through three main groups (2, 3, and 5), where groups 2 and 3 included *Physoderma* from NCBI; isolates were quite diverse within group 2, but group 3 included isolates with very small differences that constituted the "uncultured fungi" group; and isolates in group 5 grouped with *Didymella* from NCBI. Group 1 were *Olpidium* isolates from NCBI and one of the isolates (2Trl) in group 4 was from *Trifolium*.

From our three phylograms, FBG isolates mostly belonged to two groups, *Physoderma* or *Didymella*. *Physoderma* is clearly the main causal pathogen of faba bean gall, while *Didymella* is present as an accompanying pathogen and/or from secondary infection. *Didymella*, *Mycosphaerella*, and *Phoma* can survive saprophytically, and *Didymella* was present in the faba bean fields causing blight (recorded as *Ascochyta fabae*). There is no available comparative sequence data for *Physoderma* as a legume pathogen. Some other obligate pathogens, such as those causing downy mildew and white rust diseases, produce an abundance of spores on the plant surface, making it easier to identify them morphologically and to extract DNA from spores for molecular identification. However, *Physoderma*, while it produces an abundance of resting spores inside the host, only produces epibiotic zoosporangia to release zoospores for a short period. This characteristic not only makes identification more difficult but allows secondary fungal pathogens to contaminate morphological and molecular identification procedures; this has led previous attempts to determine the causal agent of FBG to identify either secondary pathogens or other contaminating organisms. This is illustrated by two previous unsuccessful attempts to identify molecularly the causal agent of FBG in Ethiopia. The first was an investigation by CABI in 2012, where ITS rDNA analysis with FASTA showed >99% similarity to sequences assigned to *Phoma* and *Peyronellaea*, with 100% match to *Peyronellaea pinodella* (i.e., syn. *Mycosphaerella pinodes*, *Didymella pinodes*), with a strong match with *Aureobasidium*/*Kabatiella lini*; they also used ITS rDNA analysis

with BLAST to highlight similarity with *Cryptococcus victoriae* (CABI, 2012). The second was in 2016, with FBG samples sent from Ambo University to Wageningen Plant Research International, where next-generation sequencing analysis showed a low homology but best fit to *Albugo laibachii* (Wageningen Plant Research International, 2016). However, neither of these organisms could possibly be the cause of FBG disease as both have significantly different morphology from that observed for FBG. For example, *Albugo* species are generally not known to infect Fabaceae and produce different symptoms to FBG, and *Olpidium* and *Albugo* are unrelated genera despite both being favoured by cool and wet environmental conditions. Thus, PCR has limitations as a means of obtaining sequences for identification, but it is an important method for confirmation of morphological observations.

Physoderma has been reported on faba bean and other legume hosts. Importantly, in Japan, *Physoderma fabae* Syd. (1928) was reported on *V. faba* in 1927; however, it did not produce a gall symptom, but instead caused rusty to reddish-brown orbicular or irregular spots on leaves (Watson, 1971). Subsequently, again in Japan, *Physoderma leproides* (Trab.) Lagerh (1950) (basionym: *Entyloma leproideum* Trab. [1894]) was reported as parasitic in leaves and stems of *V. faba*, again without a gall symptom, causing rusty to reddish-brown orbicular or irregular spots (Watson, 1971).

Outside of Ethiopia, *Physoderma* is reported across a wide range of different legume hosts. For example, *P. trifolii* (syn. *S. trifolii*, *O. trifolii*, *Urophlyctis trifolii*) has been reported in China on *Astragalus sinicus*; in Australia on *Swainsona occidentalis*, *Trifolium glomeratum*, *T. subterraneum*, *T. tomentosum*, *T. repens*; and in India on *T. alexandrium*, *T. resupinatum*, *T. carolinianum*, *T. medium*, *T. montanum*, *T. pratense*, *T. repens*, *T. resupinatum* (Allison et al., 1952; Anonymous, 1960; Butler & Hall, 1966; Cook & Dubé, 1989; Cunnington, 2003; Fajardo et al., 2017; Farr & Rossman, 2019; French, 1989; Pande & Rao, 1998; Sampson & Walker, 1982; Shivas, 1989; Tai, 1979; Watson, 1971). Such infections by *P. trifolii* are relevant for Ethiopia, as it is clear that in this country the FBG pathogen not only additionally infects field pea, but also, as we found in the current study, at least two different *Trifolium* spp. A significant number of *Trifolium* species occur in Ethiopia, including *T. subterraneum*, *T. repens*, *T. fragiferum*, *T. pratense*, *T. baccarinii*, *T. polystachyum*, *T. semipilosum*, *T. simense*, *T. tembense*, *T. usambarense*, along with unknown *Trifolium* spp. (ILRI Genetic Resources, unpublished data). That the FBG pathogen can cross over between different host genera and species increases the biosecurity risk of accidental introduction of FBG disease for countries growing faba bean and field pea crops that are currently free of FBG. In addition, as *Trifolium* spp. can be a host, this also has significant biosecurity implications. *T. subterraneum* is an important annual forage legume in Mediterranean-type climatic regions of southern Australia and parts of Africa, Asia, Europe, and North and South America (Nichols et al., 2007, 2014), with an estimated 29 million ha sown in Australia alone (Hill & Donald, 1998; Nichols et al., 2013).

Distinguishing *Physoderma* from *Olpidium* has a number of challenges. It is noteworthy that on analysis of sequences from the ITS region, our *Physoderma* isolates were grouped with *Synchytrium* in group 2 while *Olpidium* were further away; the same applied to the partial 18S-ITS1-5.8S-ITS2-partial sequences in groups 2, 4, and 5. In Japan, on *T. repens*, swellings like blisters on leaves, and galls occurring in the stems and petioles, have been mistakenly reported as caused by *O. trifolii*, but in fact are caused by *P. trifolii* (Anonymous). This is not surprising, as *Olpidium* spp. have frequently been misreported to cause various gall-type symptoms on leaves. Other Physodermataceae, *S. trifolii* and *Urophlyctis trifolii*, have also been misidentified as *O. trifolii* instead of the correct name *P. trifolii*. Thus, it is not unexpected that the original description of the FBG pathogen in Japan was given as *O. viciae*.

As already noted above, our *Physoderma* isolates were closely grouped with *Synchytrium*. *S. aureum* has long been known to attack and cause galls on various *Trifolium* spp. (e.g., Chilton et al., 1943; Oudemans, 1921; Saccardo, 1898). It has been widely reported on *T. subterraneum* and other clovers in eastern Australia (White et al., 1956), and in Western Australia in the 1980s (authors' unpublished observations). In eastern Australia, Walker (1957) actually reported both *S. aureum* and *P. trifolii* occurring together on the same *T. subterraneum* plants. Other *Synchytrium* spp. occurring in Australia include *S. decipiens* as false rust on *Amphicarpaea*, *S. desmodii* as wart disease on tropical legumes, and *S. psophocarpi* as false rust on winged bean (*Psophocarpus tetragonolobus*) (Price, 1987; Price & Lenne, 1987, 1988).

Although FBG had previously been identified as *O. viciae*, *Olpidium* has only an endobiotic phase inside the plant with no epibiotic phase, is a genus generally restricted to roots that does not show symptoms on above-ground parts of plants, and is not primarily spread by rain splash. In contrast, for *Physoderma* we observed both epibiotic and endobiotic phases, with zoosporangia epibiotic in preparation for release of zoospores, and resting spores endobiotic residing inside the plant cells. *Physoderma* is a genus that generally attacks and shows symptoms on above-ground parts of plants and is a pathogen that is primarily spread by rain splash (as occurs in Ethiopia). Recognizing the epibiotic phase is an important foundation, not only for comprehending the disease epidemiology, but also for achieving future disease forecasting; it would enable prediction of zoospore release and consequent best timings for application of chemical sprays to reduce reinfection following the initial infection cycle of faba bean. Our phylogram analyses of sequences from the ITS1-5.8S-ITS2, partial 18S-ITS1-5.8S-ITS2-partial 28S, and *LSU* (28S rRNA) regions all confirmed *Physoderma*, not *Olpidium*, as the causal agent. Sample sequences were either close to *Physoderma* or the ascochyta pathogen *Didymella*. It is clearly evident from symptoms, morphological observations, and molecular data that the true causal agent of FBG disease in Ethiopia is *Physoderma* and not *O. viciae*. That this *Physoderma* causal agent of FBG can cross over between different legume host genera (e.g., *Vicia*, *Pisum*, *Trifolium*) highlights both challenges for its management in Ethiopia and an increased biosecurity risk of its accidental introduction for countries, currently free of FBG, that grow faba bean and field pea crops; this particularly applies where extensive areas of *Trifolium* spp. forages occur.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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