Diagnostic Permit 3160: Fungal Pathogen Isolation Update, 2017–2018

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Under Diagnostic Permit 3160, the Mordecai Laboratory (Department of Biology, Stanford University) is identifying the fungal taxa causing disease in the grasslands of Jasper Ridge Biological Preserve. Here, we provide updated information on the fungal pathogen collection since the last report (submitted August 16, 2016 by Dr. Erin Spear).

METHODS

Study Site. Fungi were isolated from grass tissue collected in Jasper Ridge Biological Preserve (Fig. 1), located in San Mateo County, California (37°24′ N, 122°13′30″ W). We sampled tissue from the native grasses *Stipa pulchra* (SP) and *Elymus glaucus* (EG) and the exotic grasses *Avena barbata* (AB), *Avena fatua* (AF), *Brachypodium distachyon* (BRAD), *Bromus hordeaceus* (BH), *Bromus diandrus* (BD), *Festuca perennis* (FP), and *Phalaris aquatica* (PA). All grasses and fungi sampled for our study occur naturally in California.

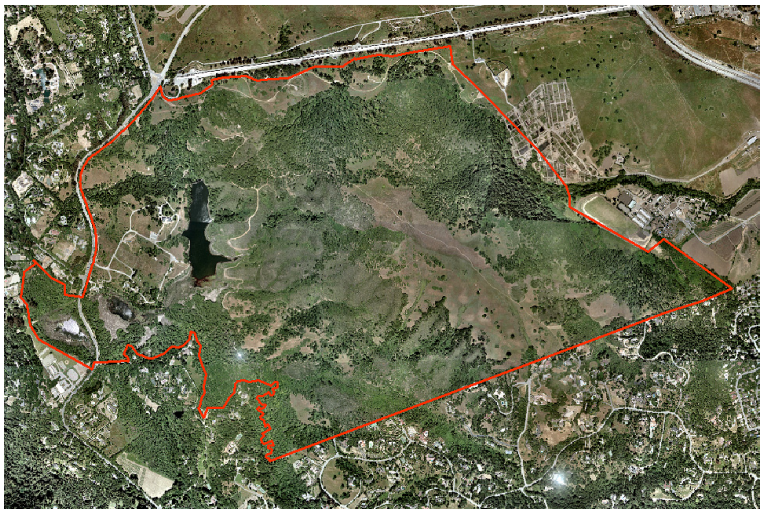

Figure 1: Jasper Ridge Biological Preserve. Preserve boundaries are highlighted in red. © 1998–2015 Jasper Ridge Biological Preserve of Stanford University.

Isolation of fungi. Symptomatic and asymptomatic grass tissue (n = 791 samples) was collected from JRBP between April 29, 2017 and June 15, 2017. All culture work was performed in the Mordecai Lab (Room 402, Gilbert Biological Sciences Laboratory) at Stanford University (Stanford, CA; Santa Clara County), as specified by the Mordecai lab SOP (Appendix 1) and CDFA Diagnostic Permit number 3160. Counters and tools were disinfected after use.

Excisions were made from symptomatic tissue, targeting the advancing margin of disease. We surface sterilized all tissue samples by immersion in 70% ethanol followed by immersion 10% commercial bleach (following Gilbert and Webb 2007). Excised pieces of grass tissue were plated on individual labeled petri dishes using 2% malt extract agar (MEA) with chloramphenicol (to prevent bacterial growth). Dishes were

sealed with Parafilm (Bemis Company, Neenah, WI, US), stored at room temperature (22°C) in the Mordecai Lab (Gilbert 400A) and checked for growth for four weeks. Fungi emerging from the plant tissue were transferred into pure culture (using the same MEA with chloramphenicol culture medium). In some cases, more than one fungal morphotype could be observed emerging from the same tissue sample; in these cases, we transferred each morphotype into pure culture and relabeled these isolates (A, B, etc.). All culture manipulations were performed as specified in the Mordecai Lab SOP (Appendix 1).

ITS molecular barcoding of fungi. To identify the fungi isolated from grass tissue, we determined the sequence of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene by PCR amplification followed by Sanger sequencing. DNA was extracted from fungal cultures using REDExtract-N-Amp™ (Sigma-Aldrich, Saint Louis, MO, USA). We amplified the ITS1 region, 5.8S rRNA gene, ITS2 region, and part of the rRNA LSU gene using the primers ITS1F (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990). In some cases, we chose to amplify a shorter region (ITS1, 5.8S, ITS2) using the primers ITS1F and ITS4 (White et al. 1990). We used a thermal cycling program consisting of the following steps: 3 minutes at 95°C; then, 35 cycles of three stages: 30 s at 95°C, 30 s at 54°C, and 60 s at 72°C; and finally, 10 minutes at 72°C. The resulting amplicons were sent to MCLAB (South San Francisco, CA) to be cleaned and sequenced using ABI 3730XL sequencers.

Taxonomic identification. Using Geneious 7.1.9 (http://www.geneious.com; Kearse 2012), we trimmed forward and reverse Sanger sequencing reads to a maximum error rate of 5%, then assembled corresponding reads (De Novo Assemble tool, “Highest Sensitivity” option). Based on estimated quality scores from the output, we trimmed the assembled sequences to a maximum error rate of 1%. If automatic assembly was not possible, we manually assembled the reads in Geneious; when this failed, we instead selected the longest trimmed individual read, provided it was over 100 bp. After screening out low-quality sequences, we exported consensus sequences for further processing.

To group ITS sequences into operational taxonomic units (OTUs), we clustered ITS sequences for all three sampling years (2015–2017) at the 97% sequence identity level USEARCH 10.0.240 (https://www.drive5.com/usearch/; Edgar 2010). We selected USEARCH parameters (`cluster_smallmem` with options: `-id 0.97 -sortedby other`) and sorted input sequences so that long, high-quality ITS1F/ITS4 sequences would preferentially be selected as cluster centroids (followed by lower-quality ITS1F/ITS4 sequences, ITS1/LR3 sequences, and then single reads). If the different morphotypes (A, B, etc.) from the same sample were clustered into the same OTU, we considered these to represent the same isolate.

We used the curated UNITE database (https://unite.ut.ee/repository.php; UNITE Community 2017a) to estimate the taxonomic placement of our ITS OTUs. Taxonomy was assigned in mothur v.1.40.5 (http://mothur.org/; Schloss 2009) using the naïve Bayes classification approach (`classify.seqs` with options: `method='wang', cutoff=0, iters=200`). We required a bootstrapping confidence score of at least 80% to assign a species name to each OTU (though we note that these assignments should be interpreted with caution) and at least 60% to assign taxonomy at any other rank.

In order to facilitate comparison of our isolates to other published sequences, we also matched each OTU to the nearest species hypothesis (SH) from the UNITE database, version 01.12.2017 (https://unite.ut.ee/repository.php; UNITE Community 2017b) using USEARCH (options: `-evalue 1e-2 -strand both -top_hits_only`).

Culture storage and disposal. Isolates from 2017 are stored in parafilm-sealed 2% malt agar extract plates with chloramphenicol in room 400A. All successful cultures are also stored agar plugs in cryogenic vials of sterile water at 5°C (room 405) and on autoclaved barley seeds frozen at −80°C (405), as detailed in the Mordecai Lab SOP (Appendix 1). Access to rooms 400A and 405 is restricted to trained personnel and both rooms are locked when not in use. Tissue pieces which did not display fungal growth and fungal cultures no longer needed were destroyed and disposed of following the Mordecai lab SOP (Appendix 1).

RESULTS

2017 cultures. In 2017, we obtained 545 distinct fungal isolates in pure culture (out of 791 grass tissue samples). These cultures represent 54 operational taxonomic units (OTUs) at the 97% ITS sequence identity level (Table 1). The most common OTU (OTU 1, putatively identified as *Alternaria*) accounts for nearly a quarter (24.6%) of isolates, whereas 24 OTUs have only one representative isolate from 2017. Thus, fungal diversity is dominated by a few frequent species: for instance, the most common 10 OTUs represent 80.6% of the year's isolates.

The majority of pathogens we isolated are ascomycete fungi from the class Dothideomycetes (39 out of 54 OTUs, representing 95.8% of isolates). Common genera represented in the 2017 isolates include *Pyrenophora (Drechslera), Phaeosphaeria (Parastagonospora),* and *Lewia (Alternaria)*.

The majority of common pathogens infect multiple grass hosts. One OTU, however, was isolated only from the perennial native grass *Stipa pulchra* (OTU 6, *Keissleriella* sp.; isolated 33 times in 2017). While most of the abundant pathogens from 2017 were also isolated in previous years, one relatively abundant OTU (OTU 16, *Alternaria* sp.) from 2017 was not present in previous years.

Overview of culture collection. As of August 2018, our fungal culture collection contains 1555 unique isolates (sampled over three years, from 2015 to 2017). These isolates make up 164 OTUs (Table 2), and the majority belong to the ascomycete class Dothideomycetes (95.3% of isolates and 70 out of 112 OTUs). Sordariomycetes (Ascomycota; 2.3% of isolates and 16 OTUs) are the next most common class.

Full dataset. Host plant, isolation date, and OTU assignment for all 1555 isolates can be found in Data File 1. Species codes follow those described in the Methods.

Additionally, we provide centroid sequences, taxonomic information, and data on the best-matching UNITE species hypothesis for each OTU in Data File 2. Column definitions for this spreadsheet can be found in Table 3.

In Data File 3 we provide full information on plant tissue samples from all three years. Definitions of the columns used in this sheet can be found in Table 4.

Table 1: Operational taxonomic units (OTUs) detected in 2017. Numbers in parentheses represent isolates obtained in previous years (2015, 2016). We omit 17 OTUs from 2017 which were observed only once across all years of sampling.

				host abundance: 2017 (2015 + 2016)					
ID[1]	estimated taxonomy	UNITE SH[2]	isolates	AB	BD	BH	EG	SP	other
1	*Alternaria* cf. *infectoria*	SH434793	162 (134)	24 (26)	3 (18)	3 (9)	41 (16)	80 (58)	10 (6)
5	*Pyrenophora* cf. *lolii*	SH193150	62 (62)	32 (15)	1 (13)	1 (5)	11 (11)	17 (14)	0 (4)
4	*Pyrenophora* cf. *chaetomioides*	SH206786	57 (71)	49 (44)	0 (8)	0 (1)	5 (2)	3 (11)	0 (5)
3	*Ramularia* cf. *proteae*	SH193143	42 (76)	3	5 (10)	4 (2)	2 (1)	27 (58)	1 (5)
6	*Keissleriella* sp.	SH193145	34 (30)					33 (30)	
16	*Alternaria* sp.	SH207018	23	3	1		8	11	
8	*Pyrenophora* cf. *tritici-repentis*	SH526155	16 (27)		0 (1)		14 (25)	2 (1)	
11	*Stemphylium* sp.	SH193139	16 (14)	1 (1)		0 (1)		14 (11)	1 (1)
12	Phaeosphaeriaceae[4]	SH010247	16 (13)	2 (1)			3	11 (12)	
10	Pleosporaceae[4]	SH193141	11 (26)	1 (9)	0 (4)	1 (4)	2	5 (9)	1
14	*Cladosporium* sp.	SH387143	10 (18)	0 (1)	1 (3)	1 (2)	1 (1)	6 (9)	1 (2)
13	*Neoascochyta* sp.	SH526155	9 (19)				0 (1)	9 (18)	
2	*Drechslera* sp.	SH215818	7 (243)	0 (28)	0 (41)	0 (40)	1 (72)	6 (58)	0 (4)
7	*Parastagonospora* cf. *avenae*	SH216250	7 (55)	1	0 (2)	0 (5)	1 (22)	5 (26)	
9	*Drechslera* cf. *fugax*	SH199529	5 (36)	0 (1)	0 (1)		1 (4)	4 (25)	0 (5)
25	*Alternaria* cf. *thalictrigena*	SH434793	5				1	4	
15	*Alternaria* cf. *eureka*	SH182993	4 (20)	0 (5)	0 (3)	1 (4)	0 (2)	3 (6)	
22	*Nemania* cf. *serpens*	SH189948	4 (6)		2 (1)		1 (1)	1 (3)	0 (1)
29	*Trametes* cf. *versicolor*	SH183029	4	2				2	
20	*Drechslera* cf. *nobleae*	SH405223	3 (8)	0 (1)				2 (5)	1 (2)
23	*Phaeosphaeria* sp.	SH208464	3 (5)					3 (5)	
31	*Cladosporium* sp.	—	3		1		1	1	
38	*Pyrenophora* cf. *erythrospila*	SH215377	3	2				1	
40	*Loratospora* sp.	SH198821	3					3	
18	*Phaeosphaeria* sp.	SH185074	2 (13)		1 (5)	0 (1)	0 (1)	1 (5)	0 (1)
21	*Parastagonospora* cf. *cumpignensis*	SH217942	2 (9)	2 (6)			0 (1)	0 (2)	
24	*Epicoccum* cf. *nigrum*	—	2 (3)	0 (3)				2	
47	*Heyderia* sp.	SH193707	2					2	
52	*Plectania* sp.	SH215377	2					2	
55	*Loratospora* sp.	SH215377	2					2	
28	*Phaeophlebiopsis* sp.	SH203320	1 (3)				1 (2)	0 (1)	
34	Phaeosphaeriaceae[4]	SH196429	1 (2)	1			0 (1)	0 (1)	
36	*Phlebia* cf. *acerina*	SH013036	1 (2)		0 (1)		1 (1)		
49	*Stereum* cf. *hirsutum*	SH494992	1 (1)	0 (1)				1	
53	*Alternaria* sp.	SH182991	1 (1)					1 (1)	
54	*Loratospora* sp.	SH238525	1 (1)					1	0 (1)

[1] OTU IDs reflect abundance rank across the entire (2015–2017) dataset.
[2] All SH codes are version 07FU (e.g. SH434793 indicates SH434793.*07FU*).
[3] Species codes: AB = *Avena barbata,* BD = *Bromus diandrus,* BH = *Bromus hordeaceus* [exotic species]; EG = *Elymus glaucus*, SP = *Stipa pulchra* [native species]. "Other" represents remaining exotic species.
[4] These OTUs were not confidently identified to the genus level; we report the family instead.

Table 2: All fungal isolates, 2015–2017, by year and class. The first number represents the number of individual isolates from a given year, while the second (parenthesized) number represents the number of distinct OTUs. Total for each year and for all three years is given in the final row.

		# isolates (# OTUs)			
phylum	**class**	**2015**	**2016**	**2017**	**all years**
Ascomycota	Dothideomycetes	298 (35)	662 (41)	522 (39)	1482 (70)
	Sordariomycetes	12 (7)	17 (9)	7 (4)	36 (16)
	Leotiomycetes	—	3 (3)	3 (2)	6 (5)
	Eurotiomycetes	—	3 (3)	1 (1)	4 (4)
	Pezizomycetes	—	—	2 (1)	2 (1)
	(unknown)	—	1 (1)	—	1 (1)
Basidiomycota	Agaricomycetes	1 (1)	10 (7)	10 (7)	21 (12)
	Ustilaginomycetes	2 (2)	—	—	2 (2)
(unknown)	(unknown)	1 (1)	—	—	1 (1)
	total:	314 (46)	696 (64)	545 (54)	1555 (112)

Table 3: Column descriptions for Data File 2 (OTU Information).

column name(s)	description
`otu.id`	ID representing the OTU
`otu.name`	Name representing the OTU ("97OTU" + `otu.id`)
`taxonomy`	Estimated taxonomic placement for OTU from naïve Bayesian classifier
`count.2015`, `count.2016`, `count.2017`	Number of isolates contained in the OTU for each sampling year
`count.total`	Total number of isolates contained in the OTU (from all years)
`centroid`	ID of the isolate selected as centroid sequence
`kingdom`, `kingdom.conf`, ..., `species`, `species.conf`	Taxonomic identifications from naïve Bayesian classifer and bootstrap confidence scores (% of 200 replicates). Confidence of 80% required for species identification and 60% for all other levels.
`sh`	UNITE species hypothesis ID for closest match in UNITE database
`sh.ref`	Accession number (GenBank, UNITE, etc.) of representative sequence of best UNITE SH
`sh.id`	Sequence identity (%) of OTU centroid sequence to best UNITE SH
`sh.hits`	Number of equally-close hits to UNITE database (if > 1, one SH was arbitrarily selected)
`sh.genus`	Genus name for best UNITE SH (NA if taxonomy not available)
`sh.species`	Species name for best UNITE SH (NA if taxonomy not available)
`sequence`	Full nucleotide sequence of OTU centroid

Table 4: Column descriptions for Data File 3 (Full Dataset).

column name(s)	description
isolate.id	ID of the fungal isolate
sample.id	ID of the plant tissue sample for culturing
year	Year of sampling
date.collected	Date of sample collection and culturing
host	Host species (see main text for codes)
experiment	GCE: global change experiment; JEF transect: serpentine transects; toothpick: seed germination trials; others: as described
plot	Grouping indicating sample location (definition varies by experiment)
subplot	Finer-level grouping under plots (or identical to plot, if not applicable)
environmental.treatment	Indicated environmental treatment (ambient: no treatment)
soil.type	Type of soil (serpentine or non-serpentine)
plant.data.source	File with original plant data
notes	Notes on plant experiment or isolation
fungicide.treated	TRUE if fungicide was applied to the plant
marked.plant.id, perennial.tag	Indicates plant of origin, if it was marked for the experiment
toothpick.plot, toothpick.position	Information for toothpick seed germination trials
sentinel.site, sentinel.bin, site.type	Information for sentinel plant experiments
transect, transect.density	Information for transect experiment
gce.combo, gce.plot, gce.treatment	Information for global change experiment
plant.tissue	Tissure from which the sample was taken
damage.type	Observation of damage type for plant tissue sample
culture.morphotype	Morphotype assigned to culture based on appearance
bg.species, competition.plot, competition.type, competition.density	Information for competion experiment
jef.site, jef.transect, jef.plot	Information for serpentine transect experiment
otu.id	ID representing the OTU
taxonomy	Estimated taxonomic placement for OTU
sequence	Full nucleotide sequence of assembled sequence (using IUPAC ambiguity codes)
sequence.length	Length of ITS sequence
method	Method used to assemble Sanger reads
bin	Quality bin for sequence (assigned by Geneious)
sequencing.set	Batch of samples sent for sequencing
orig.name	Original name of the isolate
has.ITS1F, has.ITS4, has.LR3	Reads used for assembly (TRUE: used, FALSE: not used)
perc.hq, perc.mq, perc.lq	Percentage of bases in each quality category (assigned by Geneious)
number.sources	Number of reads assembled to produce consensus
perc.identical.sites	Percentage of sites identical during assembly
number.ambiguities	Number of ambiguous bases
forward.read, reverse.read	Names of Sanger trace file containing forward and reverse reads
sequencing.run	Sequencing run code (from MCLAB)

ATTACHMENTS

Appendix 1: Mordecai Laboratory SOPs for fungal cultures.

[see attachment: `Appendix 1 - Fungal isolation and culture maintenance.csv`]

Data File 1: All 1555 isolates collected under Diagnostic Permit 3160. For each isolate (`isolate.id`), we list the originating sample (`sample.id`), year (`year`), date of collection (`date.collected`), plant host (`host`), OTU assignment (`otu.id`), and estimated taxonomy (`taxonomy`). For further detail, see the full dataset in Data File 3.

[see attachment: `Data File 1 - Isolate Overview.csv`]

Data File 2: OTUs identified in our culture collection. We list the 112 OTUs identified from all three years and report centroid ITS sequences, taxonomic assignments, and UNITE database matches. See Table 3 for description of the columns.

[see attachment: `Data File 2 - OTU Information.csv`]

Data File 3: Full dataset. All plant samples and resulting fungal isolates. See description of columns in Table 4.

[see attachment: `Data File 3 – Full Dataset.csv`]

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