A novel plant–fungal mutualism associated with fire

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ABSTRACT

Bromus tectorum, or cheatgrass, is native to Eurasia and widely invasive in western North America. By late spring, this annual plant has dispersed its seed and died; its aboveground biomass then becomes fine fuel that burns as frequently as once every 3–5 y in its invaded range. Cheatgrass has proven to be better adapted to fire there than many competing plants, but the contribution of its fungal symbionts to this adaptation had not previously been studied. In sampling cheatgrass endophytes, many fire-associated fungi were found, including Morchella in three western states (New Mexico, Idaho, and Washington). In greenhouse experiments, a New Mexico isolate of Morchella increased both the biomass and fecundity of its local cheatgrass population, thus simultaneously increasing both the probability of fire and survival of that event, via more fuel and a greater, belowground seed bank, respectively. Re-isolation efforts proved that Morchella could infect cheatgrass roots in a non-mycorrhizal manner and then grow up into aboveground tissues. The same Morchella isolate also increased survival of seed exposed to heat typical of that which develops in the seed bank during a cheatgrass fire. Phylogenetic analysis of Eurasian and North American Morchella revealed that this fire-associated mutualism was evolutionarily novel, in that cheatgrass isolates belonged to two phylogenetically distinct species, or phylotypes, designated Mel-6 and Mel-12 whose evolutionary origin appears to be within western North America. Mutualisms with fire-associated fungi may be contributing to the cheatgrass invasion of western North America.

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Introduction

Relatively few plants introduced to new regions become invaders. Attempting to predict the invaders, invasion biologists have considered many hypotheses involving biotic interaction (Mitchell et al. 2006). Novel mutualisms involving seed dispersal and mycorrhization appear to underlie the success of a few invaders (Richardson et al. 2000). However, a general mechanism has proven to be elusive, and even some of the world’s most dramatic invasions remain puzzling in key respects.

Bromus tectorum, or cheatgrass, is an invasive winter annual native to Eurasia that is now widespread throughout the U.S. and Canada (USDA-ARS 2011). It typically flowers and sets seed in the spring. Introduced to North America late in the nineteenth century, cheatgrass rapidly became the dominant plant of the treeless steppes of western North America (Mack 1981); in
its native range cheatgrass does not dominate plant communi-
ties (Hierro et al. 2005). Factors contributing to its domination in
its invaded range have been identified: climatic preadaptation
and habitat alteration are prominent among them (Mack 1981).
Also noted is the ability of cheatgrass to successfully
compete in differing ecological scenarios as either a mycorrhizal
or non-mycorrhizal plant (Richardson et al. 2000).

The ecosystem effects of cheatgrass are tied to its pro-
motion of both the frequency and size of fires (D’Antonio &
Vitousek 1992). Cheatgrass-fire feedback is positive in that
of both the frequency and size of fires (D’Antonio &
Vitousek 1992), but it has now been reduced to 3
invasion, the fire return interval was between 60 and 110 y
Whisenant 1990), but it has now been reduced to 3–5 y in
cheatgrass-dominated communities (D’Antonio & Vitousek
1992). Domination by cheatgrass makes fire 500 times more
likely than it would have been if the community had not
been invaded. As cheatgrass has spread to the edge of for-
ested, higher elevations, its fires have become larger and
more damaging (D’Antonio & Vitousek 1992).

Endophytic fungi can function as obligate mutualists of
plants by contributing essential thermotolerance to a symbio-
sis (Redman et al. 2002). One mechanism of endophyte-
mediated thermotolerance involves interaction with the
heat shock proteins of the host (McLellan et al. 2007). Para-
phaeosphaeria quadriseptata can affect expression of heat shock
proteins and enhance thermotolerance in Arabidopsis thaliana.
This mechanism could possibly apply to fire-adapted plants.

Fungi can also be fire-adapted in their own right, as their
fruiting can be more prolific after a fire (Pilz et al. 2004), or
they can be favoured relative to less tolerant fungi by the soil
heating associated with fire (Peay et al. 2009). Spores of fire-
adapted Neurospora do not germinate without heat or chemical
conditioning associated with fire (Jacobson et al. 2004).

Our objective here was first to determine whether fungi
known to be favoured by fire are endophytic in a dominant,
fire-adapted invader. Once Morchella had been isolated as an
endophyte, our corollary objective became the determination
of the role of this well-known, fire-adapted fungus in promot-
ing its fire-adapted host.

Materials and methods

Endophyte sampling and sequencing

Cheatgrass was sampled during 2009 and 2010 (May–August)
in 63 sites in British Columbia, Colorado, Idaho, Illinois,
Iowa, Nevada, New Mexico, and Washington. Cheatgrass
plants collected in 2008 from two additional sites in Idaho
and New Mexico were also included in the sequencing effort.
Habitats included coniferous forests, sagebrush-grasslands,
desert scrub, agricultural fields, and disturbed roadsides.
Twenty plants at each site were sampled by removing a single
culm (the jointed stem of grasses) from each.

A 2-cm segment centred on the lowest node of each culm
was excised, surface-sterilised in 50 % ethanol for 5 min,
and rinsed in sterile, distilled water for 1 min (Luginbuhl &
Muller 1980; Schulz et al. 1993). Culm segments were plated
on potato dextrose agar (PDA) and incubated at ambient
room temperature (~20 °C).

From the 1260 culm sections sampled, 1064 endophytes
were isolated. Some endophytes were identified morphologi-
cally to genus; the rest were placed into morphotype groups
based on macroscopic and microscopic characteristics. The
nuclear ribosomal internal transcribed (ITS rDNA) region of
representative isolates of 221 morphotype groups was se-
quenced at the USDA-ARS Systematic Mycology and Microbi-
ology Laboratory in Beltsville, MD, following protocols
described below for Morchella DNA isolation, amplification,
and sequencing; the ITS of zygomycetous endophytes was se-
quenced at the Friedrich Schiller University in Jena, Germany.

Endophyte identifications and associations with
thermotolerance

Endophytes were considered matches with recognised species
only if the ITS sequences were identical. Endophytes were
identified to genus only if their ITS sequence identity was
97–99 %. Five of the endophytes were identified as one of
two phylotypes of the Morchella elata clade (Mel-6 and Mel-12;
O’Donnell et al. 2011), as described below. These five isolates
were characterised further using multilocus DNA sequence
data. Associations with thermotolerance were assessed
according to the literature associated with those taxa.

Effects of Morchella on cheatgrass growth and fecundity

The experiment was conducted as a randomised complete
block design with two treatments and 20 replicates of each.
Treatments included the control (M−) and inoculation with
the NM isolate of the Mel-6 phylotype (M+). Over the course
of the study, pots were randomised bimonthly within each
block. The initial experiment was conducted in 2009 and re-
peated in 2010.

Seeds were collected from northeast of Albuquerque, New
Mexico (Zia Indian Reservation) where the NM isolate of the
Mel-6 phylotype had been obtained. Cleaned seeds were
surface-sterilised in 50 % ethanol for 5 min, and then rinsed
for 1 min in sterile distilled water (Luginbuhl & Muller 1980;
Schulz et al. 1993). They were then transferred onto sterile
blotter paper within Petri dishes and vernalised in the dark
at 2 °C for 8 weeks. Following vernalisation, the Petri dishes
were transferred to the greenhouse for germination. Environ-
mental conditions within the greenhouse included an 18:6
hour photoperiod (day:night) with average temperatures of
25 °C (day) and 20 °C (night).

M+ seedlings were placed on the mycelium of a PDA cul-
ture of the NM isolate of Mel-6 and inoculated through direct
contact for 24 h. M− seedlings were placed into Petri dishes
of sterile PDA. Seedlings were planted into a 10 cm2 pot filled
with a potting mixture (Sunshine Mix #1), which prior to plant-
ing, was autoclaved for 2 h (121 °C and 15 lb in2) to ensure ste-
tility. To reduce potential phytotoxic effects from the
sterilisation process, soil was allowed to sit for 2 weeks before
planting (Rovira & Bowen 1966; Koide & Li 1989).

Plants were grown in the greenhouse for 4 m during which
observations were recorded daily with respect to plant health,
appearance, flowering, and seeding. Upon flowering, mesh
bags were placed over the inflorescences and seeds were allowed to mature on the plants. Once ripened, inflorescences were clipped just below the lowest panicle branch on each culm. For each treatment, inflorescences were combined in a paper bag and dried for 72 h at 60 °C. Once seeds were cast, biomass was harvested. The aboveground biomass was clipped to the soil surface, placed into paper bags and dried for 72 h at 60 °C. Following drying, the aboveground biomass for each individual plant was recorded. Any seeds remaining on the dried panicles were collected and the remaining panicle biomass was added to the aboveground biomass for its respective treatment. Pearson correlations among uncleaned seed weight, cleaned seed weight, and seed number were highly positive, ranging from 0.957 to 0.993. Thus, fecundity was determined using the easily determined dry weight of uncleaned seed. Soil was rinsed from the belowground biomass and roots were collected and placed into individual plastic bags for evidence of colonisation.

**Root colonisation**

Since some species of *Morchella* have been reported to form mycorrhizal-like associations with plants (Dahlstrom et al. 2000; Pilz et al. 2004) and mycorrhization could explain effects on growth and fecundity, roots from five M+ and five M− greenhouse-grown cheatgrass plants were cleared, stained, and examined microscopically for structural evidence of mycorrhization. Both M+ and M− roots were cut into 2-cm segments from three sections of root (near the crown, centre, and at the tips). Following procedures developed by Brundrett et al. (1996), roots were cleared in test tubes in a 10 % KOH solution at 85 °C for 2 h. Once cleared, roots were rinsed with sterile distilled water, stained with 0.03 % w/v chlorazol black E in lactoglycerol at 85 °C for 2 h, and placed in 50 % glycerol for 2 d to remove excess stain. Slide mounts were observed with a Zeiss Axioskop.

**Re-isolation of *Morchella* from culms after inoculation of roots**

Since, to our knowledge, *Morchella* has never been reported to grow endophytically in aboveground plant tissues, colonisation of culms from roots by the NM isolate of Mel-6 was investigated. Segments for re-isolation were centred on surface-sterilised, basal nodes of culms of both inoculated M+ and M− cheatgrass plants. Of the 40 plants, five from each treatment type (M+ and M−) were randomly selected and a single stem from each was selected and clipped at the base. To adjust for the loss in biomass from the clipped stem, fresh weight was recorded for each, and its dry weight estimated on the basis of the ratio of fresh:dry weight.

**Thermotolerance**

During a typical cheatgrass fire, temperatures reach approximately 63 °C at depths of 1–2 cm below the soil surface (Beckstead et al. 2011). Buried seeds that survive these temperatures are likely not current-season but seeds from the year before. To determine whether *Morchella* conferred thermotolerance to cheatgrass, 800 seeds were placed in Petri dishes, half on sterile PDA (M−) and half in contact with a culture of the NM isolate of Mel-6 on PDA (M+). Petri dishes were sealed with parafilm and left for 3 d. Laboratory conditions included a 10:14 hour photoperiod (day:night) with an average temperature of 20 °C. Seeds were then placed onto sterile filter paper in new Petri dishes and subjected to one of four heat treatments: 20 °C (positive control), 55 °C, 60 °C, or 65 °C for 1 h. One hundred seeds were included in each Morchella/temperature combination. Seeds from each treatment combination were then placed into separate 10 mL tubes containing sterile distilled water. Seed imbibed for 24 h and then were placed into separate 10 mL tubes containing 1 % tetrazolium blue for another 24 h. Seeds with stained and unstained embryos were recorded as viable and non-viable, respectively (Patil & Dadlani 2009).

**Statistical analyses**

Biomass, fecundity, and thermotolerance data were analysed with SysStat 12.02.00. In the first two cases, Student’s two-sample t-test was employed. Thermotolerance data were analysed with a GLM model, nesting temperature within treatment (i.e., M+ vs. M−).

**Morchella DNA isolation, amplification, and sequencing**

Total genomic DNA was extracted with the UltraClean Plant DNA Isolation Kit (Qbiogene, Carlsbad, CA, USA). The entire nuclear ribosomal ITS region and domains D1 and D2 at the S′ end of the nuclear large subunit (LSU) ribosomal RNA, and portions of the tef1-α and rpb2 genes were amplified (Taşkin et al. 2010; O’Donnell et al. 2011). PCR products were cleaned and sequenced using PCR primers. Sequences were assembled and edited in Sequencher v. 4.8 (Gene Codes, Ann Arbor, MI, USA) and deposited in GenBank (accession numbers tef1-α: HM756733–HM756737; rpb2: HM756738–HM756742; and LSU rDNA: HM756728–HM756732).

**Morchella phylogenetic analysis**

Edited sequences from each locus were aligned individually with Morchella sequences retrieved from GenBank using ClustalX 2.0.11 and the alignments were adjusted manually in MacClade 4.08 (Maddison & Maddison 2001). The final combined data set used for analysis contained 2622 characters (tef1-α:1180 bp, rpb2:873 bp, and LSU rDNA:569 bp). Sequences from the ITS rDNA region were not included in this analysis because they cannot be aligned across the breadth of the *Morchella* clade. A partition-homogeneity test (incongruence length-difference test) was implemented to evaluate the homogeneity of different data partition subsets using PAUP* v.4.0d10 (Swofford 2002). The test implemented 1000 replicates (heuristic search; random simple sequence additions; tree-bisection reconnection (TBR); max-trees = 1000). Comparisons were evaluated using a threshold of P < 0.001 and were made between all data partitions.

Maximum likelihood (ML) analysis was implemented in PAUP* using the best fitting model for each data partition, as determined using Akaike Information Criterion, as implemented by Modeltest 3.7 (Posada & Crandall 1998). The general time reversible model incorporating a proportion of invariable sites and a distribution of rates at variable sites modelled on...
Table 1 – Thermotolerant fungi isolated as endophytes from cheatgrass.

<table>
<thead>
<tr>
<th>Endophyte*</th>
<th>GenBank accession no.</th>
<th>No.</th>
<th>Sites*</th>
<th>Association with fire or heat†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absidia sp. (CID 296, 374)</td>
<td>28S</td>
<td>HQ829144, HQ829173, respectively</td>
<td>2 (2)</td>
<td>CO</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
</tr>
<tr>
<td>Acremonium sp. (CID 35, 55, 60, 83, 87, 89, 93, 99, 100, 105, 110, 138, 139, 147, 156, 165, 166, 167, 198, 200, 201, 216, 217, 218, 237, 238, 254, 264, 268, 272, 283, 285, 295, 298)</td>
<td>28S</td>
<td>HQ829069, HQ829074, HQ829075, HQ829076, HQ829078, HQ829079, HQ829080, HQ829081, HQ829082, HQ829085, HQ829086, HQ829097, HQ829099, HQ829100, HQ829101, HQ829102, HQ829103, HQ829107, HQ829109, HQ829110, HQ829114, HQ829115, HQ829116, HQ829123, HQ829124, HQ829128, HQ829132, HQ829134, HQ829136, HQ829140, HQ829141, HQ829143, HQ829145, HQ829145, respectively</td>
<td>34 (41)</td>
<td>BC, CO, ID, NM, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
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<tr>
<td>Aspergillus sp. (CID 122, 125, 213, 310, 372, 377)</td>
<td>28S</td>
<td>HQ829092, HQ829094, HQ829113, HQ829151, HQ829172, HQ829175, respectively</td>
<td>6 (82)</td>
<td>BC, CO, IA, ID, IL, NM, NV, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
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<tr>
<td>Aureobasidium sp./A. pullulans (CID 103, 113, 114, 116, 327, 334)</td>
<td>28S</td>
<td>HQ829084, HQ829087, HQ829088, HQ829090, HQ829153, HQ829155, respectively</td>
<td>6 (20)</td>
<td>CO, ID, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
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<tr>
<td>Endophytea</td>
<td>GenBank accession no.</td>
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<td>Chaetomium sp. (CID 236, 241, 280)</td>
<td>HQ829122, HQ829126, HQ829138, respectively</td>
<td>3 (17)</td>
<td>CO, ID, NM, WA</td>
<td>Higher frequency following exposure to 40–100 °C in soil samples vs. nonsteamed soil</td>
<td>Zak &amp; Wicklow 1978</td>
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<td>Isolated from burned prairie soils</td>
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<td>Rapidly grew on straw in compost at 50 °C identified in the Mt. St. Helen’s devastation zone</td>
<td>Wicklow 1975</td>
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<td>Found in recently burned prairie soils</td>
<td>Wicklow 1973</td>
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<tr>
<td>Chromelosporium sp. (CID 312)</td>
<td>HQ829063, HQ829064, HQ829067, HQ829073, HQ829120, HQ829139, HQ829150, respectively</td>
<td>7 (19)</td>
<td>CO, ID, NV, WA</td>
<td>Found in recently burned prairie soils</td>
<td>Zak &amp; Wicklow 1978</td>
</tr>
<tr>
<td>Cladosporium sp. (CID 28, 29, 32, 54, 228, 281, 308)</td>
<td>HQ829070, HQ829104, respectively</td>
<td>2 (6)</td>
<td>ID, NM</td>
<td>Higher frequency following exposure to 70–85 °C in soil samples vs. nonsteamed soil</td>
<td>Wicklow 1973</td>
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<td>Ascospores germinated after 2 weeks following exposure to aerated steam at 55 °C for 60 s isolated from burned prairie soils</td>
<td>Wicklow &amp; Hirschfield 1979</td>
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<td>Thermotolerance conferred to Dichanthelium lanuginosum</td>
<td>Redman et al. 2002</td>
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<td>Growth more abundant on heated than unheated soil</td>
<td>Seaver &amp; Clark 1910</td>
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<td>Survival following 6 min at 100 °C</td>
<td>Warcup 1951a</td>
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<td>Recolonisation successful following steam-treatment of soil</td>
<td>Warcup 1951b</td>
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<td>Prolific fruiting after burns</td>
<td>Pilz et al. 2004</td>
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<td>Curvularia sp. (CID 31)</td>
<td>HQ829066, respectively</td>
<td>1 (6)</td>
<td>CO</td>
<td>Thermotolerance conferred to Dichanthelium lanuginosum</td>
<td>Redman et al. 2002</td>
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<tr>
<td>Fusarium oxysporum (CID 84, 199, 207, 208, 220, 261, 270, 375)</td>
<td>HQ829077, HQ829108, HQ829111, HQ829112, HQ829117, HQ829131, HQ829135, HQ829174, respectively</td>
<td>8 (84)</td>
<td>BC, CO, ID, NM, NV, WA</td>
<td>Growth more abundant on heated than unheated soil</td>
<td>Seaver &amp; Clark 1910</td>
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<td>Found in recently burned prairie soils</td>
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<td>Thermotolerance conferred to Dichanthelium lanuginosum</td>
<td>Redman et al. 2002</td>
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<td>Morchella phytotypes within M. elata complex (CID 2, 5, 16, 40, 121)</td>
<td>HM756729, HM756730, HM756728, HM756731, HM756732, respectively</td>
<td>5 (6)</td>
<td>ID, WA, NM</td>
<td>Fruiting within first year following fires associated with eruption of Mt. St. Helen’s volcano</td>
<td>Claridge et al. 2009</td>
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<td>efla</td>
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<td>Fruiting within Mt. St. Helen’s volcanic devastation zone</td>
<td>Carpenter et al. 1982</td>
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<td>RPB2</td>
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<td>Fruiting within Mt. St. Helen’s volcanic devastation zone</td>
<td>Carpenter et al. 1982</td>
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Table 1 – (continued)

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<thead>
<tr>
<th>Endophytea</th>
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<th>No.</th>
<th>Sites</th>
<th>Association with fire or heat&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucor sp. (CID 351, 352, 357, 358, 359, 360, 361, 362, 364, 365, 366, 368, 369, 371)</td>
<td>HQ829158, HQ829159, HQ829160, HQ829161, HQ829162, HQ829163, HQ829164, HQ829165, HQ829166, HQ829167, HQ829168, HQ829169, HQ829170, HQ829171, respectively</td>
<td>14 (20)</td>
<td>BC, CO, ID, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone Growth more abundant on heated than unheated soil Survival following 2 min at 100 °C Isolated from surface sample of compost; grew well at 25–50 °C</td>
<td>Carpenter et al. 1982 Seaver &amp; Clark 1910 Warcup 1951a Fergus 1964</td>
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<tr>
<td>Paeilomycys sp./ P. lilacinus (CID 4, 101, 135, 306)</td>
<td>HQ829056, HQ829083, HQ829095, HQ829149, respectively</td>
<td>4 (5)</td>
<td>CO, ID, WA</td>
<td>Found in recently burned prairie soils</td>
<td>Wicklow 1973</td>
</tr>
<tr>
<td>Penicillium sp. (CID 6, 42, 184, 195, 221, 240, 259, 260, 265, 299)</td>
<td>HQ829057, HQ829071, HQ829105, HQ829106, HQ829118, HQ829125, HQ829129, HQ829130, HQ829133, HQ829146, respectively</td>
<td>10 (21)</td>
<td>BC, CO, ID, NM, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone Found in recently burned prairie soils Survival following 6 min at 100 °C Recolonisation successful following steam-treatment of soil Germination and colony growth stimulated by heat treatment</td>
<td>Carpenter et al. 1982 Wicklow 1973 Warcup 1951a Warcup 1951b</td>
</tr>
<tr>
<td>Peziza ostracoderma (CID 8, 12)</td>
<td>HQ829059, HQ829060, respectively</td>
<td>2 (2)</td>
<td>ID, WA</td>
<td>Found developing most abundantly on steamed soil</td>
<td>Fergus 1960</td>
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<tr>
<td>Phoma sp. (CID 30, 115, 117, 277)</td>
<td>HQ829065, HQ829089, HQ829091, HQ829137, respectively</td>
<td>4 (5)</td>
<td>ID, NM, WA</td>
<td>Found developing most abundantly on steamed soil</td>
<td>Fergus 1960</td>
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<tr>
<td>Podospora sp. (CID 52, 337)</td>
<td>HQ829072, HQ829156, respectively</td>
<td>2 (3)</td>
<td>ID, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
<td>Carpenter et al. 1982 Wicklow 1973 Warcup 1951b</td>
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<td>Pyronema sp. (CID 7, 124)</td>
<td>HQ829058, HQ829093, respectively</td>
<td>2 (3)</td>
<td>ID, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone Found in recently burned prairie soils Recolonisation successful following steam-treatment of soil</td>
<td>Carpenter et al. 1982 Wicklow 1973 Warcup 1951b</td>
</tr>
</tbody>
</table>

<sup>a</sup> The endophytes are presented in decreasing order of occurrence reported in the literature.

<sup>b</sup> No. = Number of isolates.

<sup>c</sup> Sites = Number of sites within a state.

<sup>d</sup> Association with fire or heat: Identified in the Mt. St. Helen’s devastation zone, Growth more abundant on heated than unheated soil, Survival following 2 min at 100 °C, Isolated from surface sample of compost; grew well at 25–50 °C, Identified in the Mt. St. Helen’s devastation zone, Growth more abundant on heated than unheated soil, Survival following 6 min at 100 °C, Recolonisation successful following steam-treatment of soil, Germination and colony growth stimulated by heat treatment, Found developing most abundantly on steamed soil, Found developing most abundantly on steamed soil, Found developing most abundantly on steamed soil, Fruiting within first year following fires associated with eruption of Mt. St. Helen’s volcano, Collected in fireplaces, Spore germination higher at 50 °C than 20 °C following a 2-min heat treatment, Growth more abundant on heated than unheated soil, Fruits more abundantly on soil sterilised at 135–145 °C than at 95–110 °C, Cultured from bonfire cinders.
Table 1 – (continued)

<table>
<thead>
<tr>
<th>Endophytea</th>
<th>GenBank accession no.</th>
<th>No.</th>
<th>Sites</th>
<th>Association with fire or heat</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrenophora semeniperda (CID 22, 23)</td>
<td>28S HQ829061, HQ829062, respectively</td>
<td>2 (2)</td>
<td>ID</td>
<td>Higher thermal death point than cheatgrass</td>
<td>Beckstead et al. 2011</td>
</tr>
<tr>
<td>Rhizopus sp. (CID 348)</td>
<td>28S HQ829157</td>
<td>1 (15)</td>
<td>BC, CO, ID, NM, CO, ID, NM</td>
<td>Exhibits optimal growth at 50 ºC</td>
<td>Peixoto et al. 2003</td>
</tr>
<tr>
<td>Sordaria sp. (CID 33)</td>
<td>28S HQ829068</td>
<td>1 (5)</td>
<td>Higher frequency following exposure to 55–100 ºC in soil samples vs. nonsteamed soil</td>
<td>Zak &amp; Wicklow 1978</td>
<td></td>
</tr>
<tr>
<td>Sporormiella sp. (CID 329)</td>
<td>28S HQ829154</td>
<td>1 (1)</td>
<td>NM</td>
<td>Higher frequency following exposure to 35–85 ºC in soil samples vs. nonsteamed soil</td>
<td>Zak &amp; Wicklow 1978</td>
</tr>
<tr>
<td>Trichoderma sp. (CID 143, 229, 286)</td>
<td>28S HQ829098, HQ829121, HQ829142, respectively</td>
<td>3 (44)</td>
<td>CO, ID, WA</td>
<td>Survival following 4 min at 100 ºC Recolonisation successful following steam-treatment of soil</td>
<td>Wicklow 1951a</td>
</tr>
<tr>
<td>Ulocladium sp. (CID 225, 324)</td>
<td>28S HQ829119, HQ829152, respectively</td>
<td>2 (3)</td>
<td>CO, ID, WA</td>
<td>Identified in the Mt. St. Helen’s devastation zone</td>
<td>Carpenter et al. 1982</td>
</tr>
<tr>
<td>Verticillium sp. (CID 245, 301, 302)</td>
<td>28S HQ829127, HQ829147, HQ829148, respectively</td>
<td>3 (5)</td>
<td>CO, ID, NM, NV, WA</td>
<td>Growth more abundant on heated than unheated soil</td>
<td>Seaver &amp; Clark 1910</td>
</tr>
</tbody>
</table>

a Endophytes were considered tentative matches with recognised species only if their ITS sequences were identical. From 97 % up to but not including 100 % identity endophytes were identified only to genus. Based on the discovery of two phylotypes of Morchella as cheatgrass endophytes (i.e., Mel-6 and Mel-12), five isolates were characterised further using multilocus DNA sequence data (Fig 5). CID refers to culture identification numbers at the University of Idaho.

b Number of morphotypes sequenced as this taxon (total number of isolates of this taxon). Thus, of 1064 isolates, 419 (39 %) were equal to, or closely related to, taxa that are known as fire followers and/or thermotolerant fungi.

c Sites where endophyte was isolated from cheatgrass (BC = British Columbia, CO = Colorado, IA = Iowa, ID = Idaho, IL = Illinois, NM = New Mexico, NV = Nevada, and WA = Washington).

d Association with fire and/or heat, as reported for this taxon in the linked reference.

e Endophyte identified based on morphological characteristics rather than DNA sequence data.

a discrete gamma distribution was selected by Modeltest for each data partition. Heuristic searches were performed with 1000 random addition sequence replicates and tree-bisection-reconnection branch swapping, collapse and MulTrees (saving all optimal trees) options in effect. Characters were equally weighted and unordered with gaps treated as missing data. Outgroups were defined as Disciotis venosa M504, Morchella cf. esculenta Mes-17 (M98) and Mes-8 (M218), and Verpa bohemica M197 (Taşkin et al. 2010; O’Donnell et al. 2011). Maximum parsimony bootstrap support was calculated using the same settings as above with 1000 replicates, each with 100 random taxon addition replicates (Felsenstein 1985). Bayesian (BI) analyses were conducted with MrBayes 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) using the appropriate model with four incrementally heated Markov chains and two concurrent runs of 1000000 generations sampled every 10000 generations for a total of 1000 trees saved. The outgroup taxon was defined as Morchella cf. esculenta Mes-17 (M98). The initial 25 % of trees sampled was discarded as burn-in. A majority rule consensus tree was calculated from the remaining pool of trees.

Results

Endophyte identifications and associations with thermotolerance

Many known fire-adapted or thermotolerant fungi (39 % of 1064 isolates — Table 1) were isolated as endophytes from cheatgrass. These included 419 isolates belonged to 25 taxa that are associated with fire or heat tolerance (Table 1).
Prominent among these was *Morchella*; five endophytic isolates from three states (New Mexico, Washington, and Idaho) were obtained from culm tissue. The remaining 645 isolates belonged to 46 taxa that were not associated with fire or heat tolerance according to the literature: *Allantophomopsis* sp., *Alternaria alternata*, *Alternaria* sp., *Apiosporaceae*, *Ascochyta* sp., *Aspergillus vadensis*, *Bipolaris spicifera*, *Ceratobasidiaceae*, *Ceratobasidium* sp., *Chalastospora* sp., *Cochliobolus* sp., *Cytospora* sp., *Didymella fabae*, *Drechslera poae*, *Drechslera* sp., *Epicoccum nigrum*, *Epicoccum* sp., *Fusarium acuminatum*, *Fusarium equiseti*, *Fusarium flocciferum*, *Fusarium proliferatum*, *Fusarium* sp., *Hypocreales*, *Leotiomycetes*, *Leptodontidium* sp., *Lewia infectoria*, *Magnaporthe* sp., *Microdochium nivale*, *Microdochium* sp., *Monographella* sp., *Myrothecium roridum*, *Myrothecium* sp., *Nectria* sp., *Penicillium canescens*, *Penicillium expansum*, *Penicillium olsonii*, *Penicillium raistrickii*, *Pestalotiopsis* sp., *Pezizales*, *Pezizomycete*, *Pezizomycotina*, *Phaeosphaeria nodorum*, *Phaeosphaeria* sp., *Preussia intermedia*, *Preussia* sp., *Pyrenophora lolii*, *Pyrnomataceae*, *Sigmoidea* sp., *Thelavia* sp., *Trichothecium roseum*, *Truncatella* sp., unknown 1 (fungal sp.), unknown 2 (basidiomycete), unknown 3 (basidiomycete), and unknown 4 (ascomycete).

**Effects of Morchella on cheatgrass growth and fecundity**

The effects of the NM isolate of Mel-6 on fecundity and aboveground biomass were measured as dry biomass after the plants had completed their life cycle in each of two, 4-m greenhouse studies. Increases in fecundity due to this *Morchella* isolate were 35% ($t = 6.80, P < 0.001$) and 18% ($t = 2.07, P = 0.05$) in the two experiments (Fig 1). In the same two experiments, increases in growth due to *Morchella* inoculation were 15% ($t = 2.39, P = 0.03$) and 24% ($t = 2.16, P = 0.04$), respectively (Fig 2).

**Root colonisation**

Mycorrhization with *Morchella* can result in a mantle, and a Hartig net (Dahlstrom et al. 2000) but these structures were

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not observed in cleared and stained roots of M+ plants. However, extensive fungal colonisation of M+ roots was evident (Fig 3). Fungal colonisation was absent in the M− roots.

**Re-isolation of Morchella from culms after inoculation of roots**

Via re-isolation from five stem nodes, we confirmed that the NM isolate was capable of growing from inoculated roots into aboveground tissue. In contrast, Morchella was not re-isolated from five sampled M− stem nodes.

**Thermotolerance**

At 20 °C and 55 °C, M+ and M− seed viability did not differ (Fig 4). However, at both 60 °C and 65 °C, the NM isolate increased seed viability in both the first experiment ($F = 5.49$, $P < 0.001$) and its repeat ($F = 5.74$, $P < 0.001$).

**Morchella phylogenetic analysis**

Based on phylogenetic analyses of three nuclear genes (translation elongation factor-1 alpha, DNA-dependent RNA
polymerase II, and 28S ribosomal RNA), all five, endophytic isolates of Morchella belonged to one of two Morchella elata phylotypes, Mel-6 and Mel-12; the evolutionary origin of these phylotypes appears to have been within western North America (Fig 5).

Discussion

Discoveries of symbiont-based thermotolerance in plants (Redman et al. 2002; McLellan et al. 2007) have potentially far-reaching implications. In this study, we found a diverse guild of fungi associated with fire and heat tolerance in a fire-adapted plant invader that is transforming western North America. To begin the study of the nature of cheatgrass symbioses, Morchella was chosen as an initial focus. We determined the effects of Morchella on traits that must be central to cheatgrass fitness: fecundity, biomass, and seed thermotolerance. Via these traits, we found that Morchella can contribute to cheatgrass fitness that in turn should benefit both symbionts due to their adaptations to fire. However, we have not ascertained the direct benefits to Morchella. Likewise, we have not yet determined whether the other 24 taxa of fire-adapted or thermotolerant fungi function in a similar way. Thus, the other taxa may or may not represent mutualisms that, in turn, may or may not be evolutionarily novel (i.e., native fungi symbiotic with an alien plant) as the Morchella isolates proved to be (Fig 5).

Morchella, the genus of true morels, produces highly-prized edible fruiting bodies in temperate and boreal forests following fire, and other disturbances (Pilz et al. 2007). Although some species of Morchella can form mycorrhizal-like associations (Dahlstrom et al. 2000; Pilz et al. 2007), we recovered Morchella as an endophyte in the aboveground stem tissue of cheatgrass.

Morels are more commonly observed fruiting in forested areas (Weber 1997; Pilz et al. 2007) than in the dry, treeless parts of the Intermountain West now dominated by cheatgrass (Mack 1981). This may be linked to the facultatively mycorrhizal-like nature of tree-morel associations (Dahlstrom et al. 2000). Morels and cheatgrass are among the leading examples of fire-adapted fungi (Pilz et al. 2007) and plants (D’Antonio & Vitousek 1992), respectively, yet they have not been ecologically linked. This may be due to their limited distributional overlap or lack of sampling in cheatgrass-infested regions. Furthermore, in areas of overlap, it is unknown whether cheatgrass can suppress or enhance morel fruiting.

During the course of this study we did observe that the margins of burned forest are frequently invaded by cheatgrass (Fig 6). Given that Morchella commonly fruits in such habitat, it is possible that the novel mutualism developed initially in such an area. Two of our endophytic isolates were obtained from coniferous forest (i.e., Weiser, ID and H99, ID), whereas the other three were from desert scrub (North Zia, NM), and bunchgrass scrub (Hagenah, WA and White Bird, ID). With the exception of Hagenah, all collections were made within 35 m of roads within their respective habitats. At the time of collection, there was no visible evidence of recent fire at the H99, North Zia, and White Bird. However, the Weiser and Hagenah sites burned in 2007, 2 y prior to sampling. Morchella sporocarps were not observed at any of the sampling sites except Weiser. Although there are some reports of Morchella fruiting in forested and non-forested communities at lower elevation (Horn et al. 1993; Pilz et al. 2007), it is not known whether the North Zia, Hagenah, and White Bird isolates represent asexual populations.

Dispersal of endophytic Morchella could be mediated by its host. In plants producing tillers, rhizomes, tubers and plantlets, endophytes can be dispersed in these host organs (Clay 1986a, b; Rodriguez et al. 2009); Neotyphodium endophytes are vertically transmitted in seeds of their hosts (Clay & Jones 1984; Clay 1990). Dispersal of cheatgrass itself is entirely via seed that can be transported long distances in or on fur, hooves, feces, clothing, and vehicles (Young et al. 1987; Mosley et al. 1999; Young 2000). But the ability of Morchella to infect cheatgrass seed has not been determined. Nor is it yet known whether herbivores disperse Morchella in feces after consuming infected host tissue, or whether Morchella remains viable in dead, vegetative tissues of cheatgrass that could be then be dispersed by the wind.

According to the enhanced mutualism hypothesis, plant invasions may benefit from novel symbionts (Hoffman & Mitchell 1986; Richardson et al. 2000). Nijjer et al. (2008) found that the Chinese tallow tree (Triadica sebifera) benefited to a greater degree from local soil biota in Texas than did co-occurring native trees. Effects were attributed to arbuscular mycorrhizal fungi that might have been native to the site. Likewise, a mutually beneficial symbiosis was demonstrated between Canada goldenrod (Solidago canadensis), an invasive forb in Asia, and microbes from soil collected in China that, again, may or may not have been native to the site. Relative to a native Chinese grass (Stipa bungeana) grown in the same soil, seedling emergence, growth, and competitive ability of the invasive goldenrod were microbially enhanced (Sun & He 2010). Our findings are similar except that our demonstrated mutualism is shown here to be evolutionarily novel.
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References


Claridge AW, Trappe JM, Hansen K, 2009. Do fungi have a role as endophytic or aborally-mycorrhizal sequences. The mention of trade names or commercial products. 32nd Oregon Academy of Sciences Proceedings. Working with mycorrhizas in forestry and agriculture. ACIAR (Clavicipitaceae) by cleistogamous seed of hypoxylon. Balansia cyperi (Clavicipitaceae).


