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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.

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

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its native range cheatgrass does not dominate plant communities (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 promotion of both the frequency and size of fires (D'Antonio & Vitousek 1992). Cheatgrass-fire feedback is positive in that once cheatgrass density passes a certain threshold, cheatgrass promotes fire, which in turn promotes cheatgrass (Mosley et al. 1999). In sagebrush steppe before the cheatgrass 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 forested, 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 symbiosis (Redman et al. 2002). One mechanism of endophyte-mediated thermotolerance involves interaction with the heat shock proteins of the host (McLellan et al. 2007). *Paraphaeosphaeria 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 promoting 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 morphologically 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 sequenced at the USDA-ARS Systematic Mycology and Microbiology Laboratory in Beltsville, MD, following protocols described below for *Morchella* DNA isolation, amplification, and sequencing; the ITS of zygomycetous endophytes was sequenced 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 repeated 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. Environmental 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 culture 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 cm^2 pot filled with a potting mixture (Sunshine Mix #1), which prior to planting, was autoclaved for 2 h (121 °C and 15 lb in^2) to ensure sterility. 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 (Mo Bio Laboratories, Solana Beach, CA, USA). The entire nuclear ribosomal ITS region and domains D1 and D2 at the 5′ 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 elata* 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.

Endophyte[a]	GenBank accession no.		No.[b]	Sites[c]	Association with fire or heat[d]	Reference
Absidia sp. (CID 296, 374)	28S	HQ829144, HQ829173, respectively	2 (2)	CO	Identified in the Mt. St. Helen's devastation zone Survival following 2 min at 100 °C	Carpenter *et al.* 1982 Warcup 1951a
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)	28S	HQ829069, HQ829074, HQ829075, HQ829076, HQ829078, HQ829079, HQ829080, HQ829081, HQ829082, HQ829085, HQ829086, HQ829096, HQ829097, HQ829099, HQ829100, HQ829101, HQ829102, HQ829103, HQ829107, HQ829109, HQ829110, HQ829114, HQ829115, HQ829116, HQ829123, HQ829124, HQ829128, HQ829132, HQ829134, HQ829136, HQ829140, HQ829141, HQ829143, HQ829145, respectively	34 (41)	BC, CO, ID, NM, WA	Identified in the Mt. St. Helen's devastation zone Found in recently burned prairie soils	Carpenter *et al.* 1982 Wicklow 1973
Aspergillus sp. (CID 122, 125, 213, 310, 372, 377)	28S	HQ829092, HQ829094, HQ829113, HQ829151, HQ829172, HQ829175, respectively	6 (82)	BC, CO, IA, ID, IL, NM, NV, WA	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 Isolated from compost; spores germinated from 25–50 °C Germination and colony growth stimulated by heat treatment	Carpenter *et al.* 1982 Seaver & Clark 1910 Warcup 1951a Warcup 1951b Fergus 1964 Warcup & Baker 1963
Aureobasidium sp./*A. pullulans* (CID 103, 113, 114, 116, 327, 334)	28S	HQ829084, HQ829087, HQ829088, HQ829090, HQ829153, HQ829155, respectively	6 (20)	CO, ID, WA	Identified in the Mt. St. Helen's devastation zone	Carpenter *et al.* 1982

Table 1 – (*continued*)

Endophyte[a]	GenBank accession no.		No.[b]	Sites[c]	Association with fire or heat[d]	Reference
Chaetomium sp. (CID 236, 241, 280)	28S	HQ829122, HQ829126, HQ829138, respectively	3 (17)	CO, ID, NM, WA	Higher frequency following exposure to 40–100 °C in soil samples *vs.* nonsteamed soil	Zak & Wicklow 1978
					Isolated from burned prairie soils	Wicklow 1975
					Rapidly grew on straw in compost at 50 °C	Fergus 1964
Chromelosporium sp. (CID 312)	n/a[e]		1 (2)	ID	Identified in the Mt. St. Helen's devastation zone	Carpenter *et al.* 1982
Cladosporium sp. (CID 28, 29, 32, 54, 228, 281, 308)	28S	HQ829063, HQ829064, HQ829067, HQ829073, HQ829120, HQ829139, HQ829150, respectively	7 (19)	CO, ID, NV, WA	Found in recently burned prairie soils	Wicklow 1973
Coniochaeta sp. (CID 36, 170)	28S	HQ829070, HQ829104, respectively	2 (6)	ID, NM	Found in recently burned prairie soils	Wicklow 1973
					Higher frequency following exposure to 70–85 °C in soil samples *vs.* nonsteamed soil	Zak & Wicklow 1978
					Ascospores germinated after 2 weeks following exposure to aerated steam at 55 °C for 60 s	Wicklow & Hirschfield 1979
					Isolated from burned prairie soils	Wicklow 1975
Curvularia sp. (CID 31)	28S	HQ829066, respectively	1 (6)	CO	Thermotolerance conferred to *Dichanthelium lanuginosum*	Redman *et al.* 2002
Fusarium oxysporum (CID 84, 199, 207, 208, 220, 261, 270, 375)	28S	HQ829077, HQ829108, HQ829111, HQ829112, HQ829117, HQ829131, HQ829135, HQ829174, respectively	8 (84)	BC, CO, ID, NM, NV, WA	Growth more abundant on heated than unheated soil	Seaver & Clark 1910
					Found in recently burned prairie soils	Wicklow 1973
					Survival following 6 min at 100 °C	Warcup 1951a
					Recolonisation successful following steam-treatment of soil	Warcup 1951b
Morchella phylotypes within *M. elata* complex (CID 2, 5, 16, 40, 121)	28S	HM756729, HM756730, HM756728, HM756731, HM756732, respectively	5 (6)	ID, WA, NM	Prolific fruiting after burns	Pilz *et al.* 2004
	efla	HM756734, HM756735, HM756733, HM756736, HM756737, respectively			Fruiting within first year following fires associated with eruption of Mt. St. Helen's volcano	Claridge *et al.* 2009
	RPB2	HM756739, HM756740, HM756738, HM756741, HM756742, respectively			Fruiting within Mt. St. Helen's volcanic devastation zone	Carpenter *et al.* 1982

(*continued on next page*)

Table 1 – (continued)

Endophyte[a]	GenBank accession no.		No.[b]	Sites[c]	Association with fire or heat[d]	Reference
Mucor sp. (CID 351, 352, 357, 358, 359, 360, 361, 362, 364, 365, 367, 368, 369, 371)	28S	HQ829158, HQ829159, HQ829160, HQ829161, HQ829162, HQ829163, HQ829164, HQ829165, HQ829166, HQ829167, HQ829168, HQ829169, HQ829170, HQ829171, respectively	14 (20)	BC, CO, ID, WA	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	Carpenter *et al.* 1982 Seaver & Clark 1910 Warcup 1951a Fergus 1964
Paecilomyces sp./ *P. lilacinus* (CID 4, 101, 135, 306)	28S	HQ829056, HQ829083, HQ829095, HQ829149, respectively	4 (5)	CO, ID, WA	Found in recently burned prairie soils	Wicklow 1973
Penicillium sp. (CID 6, 42, 184, 195, 221, 240, 259, 260, 265, 299)	28S	HQ829057, HQ829071, HQ829105, HQ829106, HQ829118, HQ829125, HQ829129, HQ829130, HQ829133, HQ829146, respectively	10 (21)	BC, CO, ID, NM, WA	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	Carpenter *et al.* 1982 Wicklow 1973 Warcup 1951a Warcup 1951b Warcup & Baker 1963
Peziza ostracoderma (CID 8, 12)	28S	HQ829059, HQ829060, respectively	2 (2)	ID, WA	Found developing most abundantly on steamed soil	Fergus 1960
Phoma sp. (CID 30, 115, 117, 277)	28S	HQ829065, HQ829089, HQ829091, HQ829137, respectively	4 (5)	ID, NM, WA	Identified in the Mt. St. Helen's devastation zone Found in recently burned prairie soils Recolonisation successful following steam-treatment of soil	Carpenter *et al.* 1982 Wicklow 1973 Warcup 1951b
Podospora sp. (CID 52, 337)	28S	HQ829072, HQ829156, respectively	2 (3)	ID, WA	Higher frequency following exposure to 40 °C in soil samples *vs.* nonsteamed soil	Zak & Wicklow 1978
Pyronema sp. (CID 7, 124)	28S	HQ829058, HQ829093, respectively	2 (3)	ID, WA	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	Claridge *et al.* 2009 Moore & Korf 1963 El-Abyad & Webster 1968 Seaver & Clark 1910 Seaver 1909 Moore-Landecker 1975

Table 1 – (continued)

Endophyte[a]	GenBank accession no.		No.[b]	Sites[c]	Association with fire or heat[d]	Reference
Pyrenophora semeniperda (CID 22, 23)	28S	HQ829061, HQ829062, respectively	2 (2)	ID	Higher thermal death point than cheatgrass	Beckstead *et al.* 2011
Rhizopus sp. (CID 348)	28S	HQ829157	1 (15)	BC, CO, ID, NM	Exhibits optimal growth at 50 °C	Peixoto *et al.* 2003
Sordaria sp. (CID 33)	28S	HQ829068	1 (5)	CO, ID, NM	Survival following 4 min at 100 °C	Warcup 1951a
					Higher frequency following exposure to 55–100 °C in soil samples *vs.* nonsteamed soil	Zak & Wicklow 1978
Sporormiella sp. (CID 329)	28S	HQ829154	1 (1)	NM	Higher frequency following exposure to 35–85 °C in soil samples *vs.* nonsteamed soil	Zak & Wicklow 1978
					Ascospores germinated after 6 weeks following exposure to aerated steam at 55 °C for 60 s	Wicklow & Hirschfield 1979
					Isolated from burned prairie soils	Wicklow 1975
Trichoderma sp. (CID 143, 229, 286)	28S	HQ829098, HQ829121, HQ829142, respectively	3 (44)	CO, ID, WA	Survival following 4 min at 100 °C	Warcup 1951a
					Recolonisation successful following steam-treatment of soil	Warcup 1951b
Ulocladium sp. (CID 225, 324)	28S	HQ829119, HQ829152, respectively	2 (3)	CO, ID, WA	Identified in the Mt. St. Helen's devastation zone	Carpenter *et al.* 1982
Verticillium sp. (CID 245, 301, 302)	28S	HQ829127, HQ829147, HQ829148, respectively	3 (5)	CO, ID, NM, NV, WA	Growth more abundant on heated than unheated soil	Seaver & Clark 1910

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 10 000 000 generations sampled every 10 000 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).

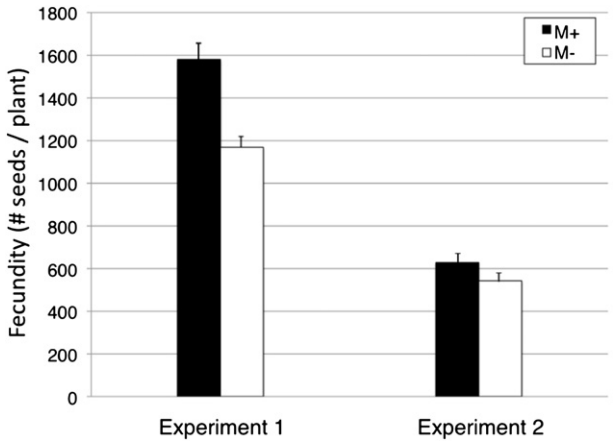

Fig 1 – *Morchella* increased cheatgrass fecundity in both experiments ($t = 6.80$, $P < 0.001$ and $t = 2.07$, $P = 0.05$, respectively).

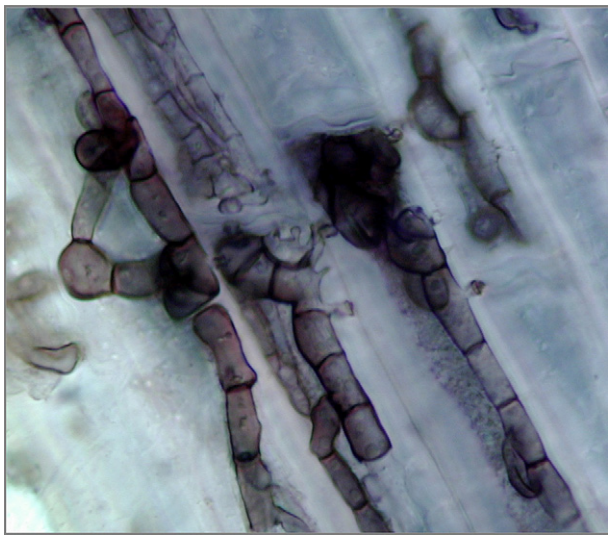

Fig 3 – Non-mycorrhizal colonisation of roots of M+ plants.

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*, Pyronemataceae, *Sigmoidea* sp., *Thelavia* sp., *Trichothecium roseum*, *Truncatella* sp., unknown 1 (fungal sp.), unknown 2 (basidiomycete), unknown 3 (basidiomycete), and unknown 4 (ascomycete).

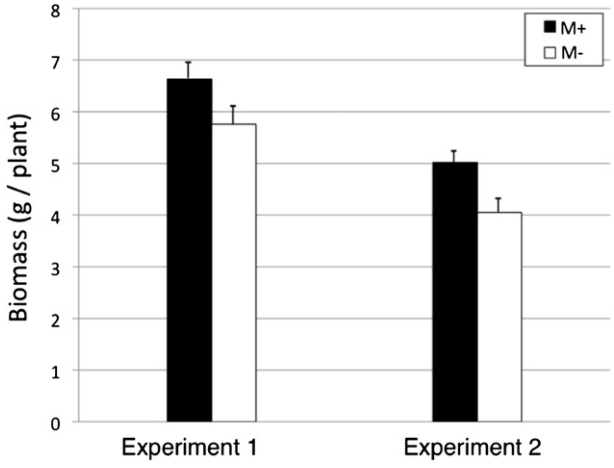

Fig 2 – *Morchella* increased cheatgrass biomass in both experiments ($t = 2.39$, $P = 0.03$ and $t = 2.16$, $P = 0.04$, respectively).

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

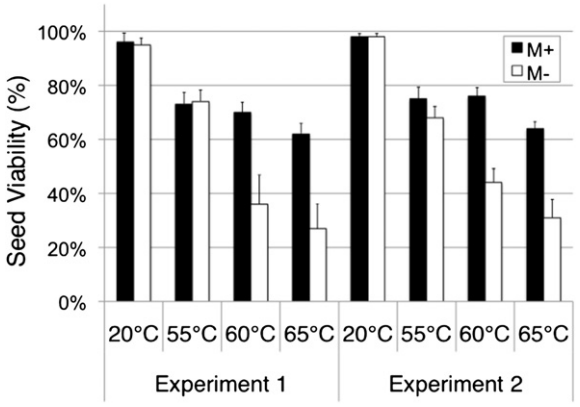

Fig 4 – Temperatures reached during typical cheatgrass fires (60 °C and 65 °C) were more lethal to M− than M+ seed (Experiment 1, $F = 5.49$, $P < 0.001$; Experiment 2, $F = 5.74$, $P < 0.001$).

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

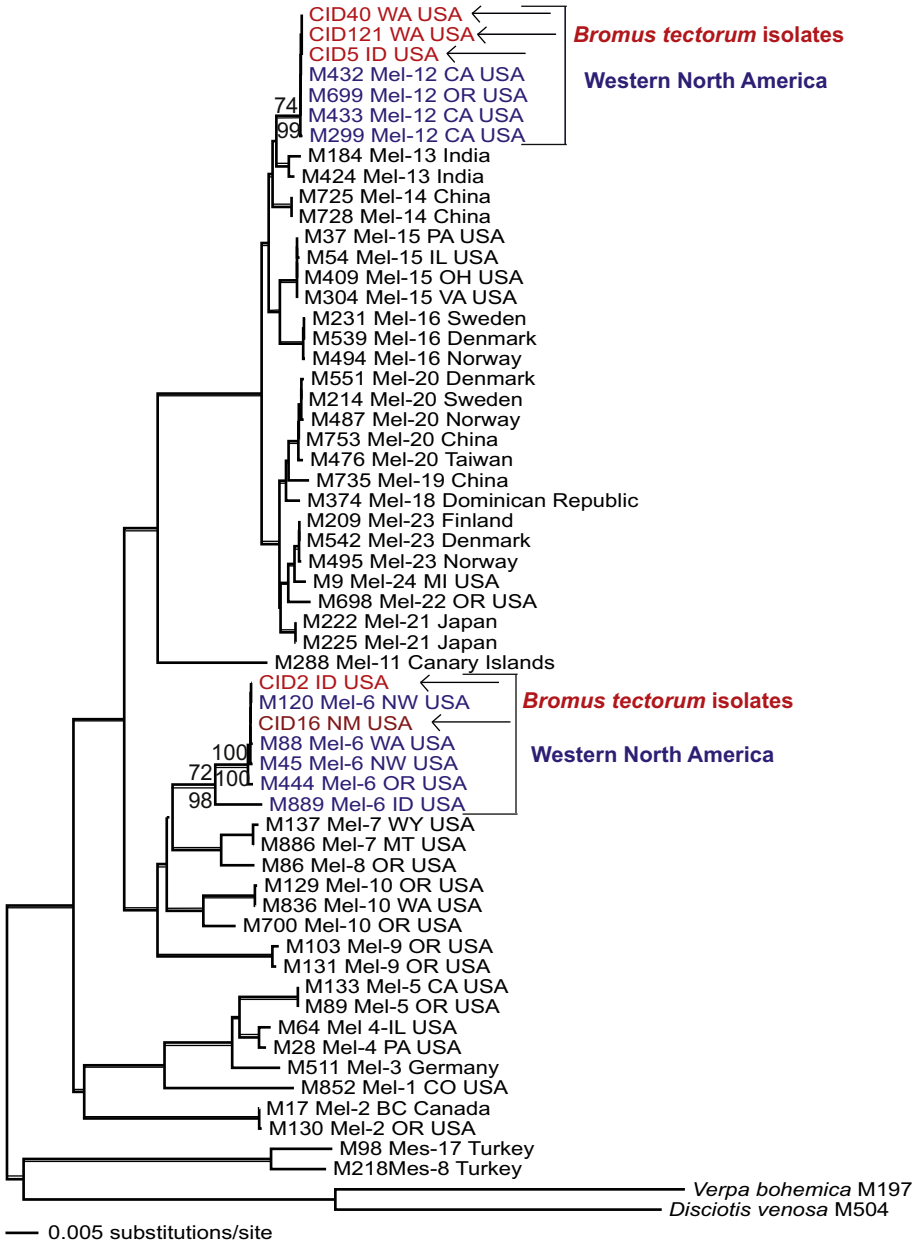

Fig 5 – Maximum likelihood (ML) phylogenetic tree inferred from *tef1-α*, *rpb2*, and 28S nrLSU genes for phylogenetic species in the *Morchella elata* clade (black morel mushrooms). *Bromus tectorum* isolates are highlighted in red and identified with arrows. MP bootstrap support is shown above and Bayesian PP are shown below the branches for nodes of interest. Thickened branches indicate support >95 % PP and >70 % MP bootstraps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 American (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.

Fig 6 – Cheatgrass in morel habitat above the Weiser River of ID in July 2008. Photographed site was not sampled but provides an example of proximity between cheatgrass and morels.

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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