

Chapter 4

New Perspectives on the Distribution and Roles of Thermophilic Fungi

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4.1 Introduction

The goal of this chapter is twofold. First, we briefly review the history, basic biology, evolution, and industrial relevance of thermophilic fungi. Second, we address ongoing questions concerning the ecology of these organisms. In the past two decades, several excellent reviews have considered one or more of these topics (Oliveira and Rodrigues, this volume; Maheshwari et al. 2000; Mouchacca 2000a, b; Salar and Aneja 2007; Salar 2018). Here, we give particular attention to topics for which there has been some difference of opinion. These include a discussion of the definition of thermophily as it pertains to fungi and an evaluation of the types of microhabitats that are most relevant to the growth and distribution of these organisms. We argue that the microenvironments capable of supporting the growth of thermophilic fungi are widespread and often transient. In the latter context, we present the results of a recent previously unpublished survey of thermophilic fungi in diverse ecosystems of the western United States, Mexico, and Canada.

Definition. While thermophilic fungi do not grow at the extreme temperatures that are optimal for many thermophilic bacteria and archaea, they are the only eukaryotes demonstrated to grow at temperatures up to 60 °C (Tansey and Brock 1972). In practice, the term thermophilic, when applied to fungi, has sometimes been used quite loosely, and there is no universally accepted definition.

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S. M. Tiquia-Arashiro, M. Grube (eds.), *Fungi in Extreme Environments: Ecological Role and Biotechnological Significance*,
https://doi.org/10.1007/978-3-030-19030-9_4

Cooney and Emerson (1964), who wrote the first monograph for thermophilic fungi, considered such fungi to be those that have “a maximum temperature for growth at or above 50 °C and a minimum temperature for growth at or above 20 °C.” We have adopted a simpler working definition (Powell et al. 2012; Hutchinson et al. 2016). Namely, we consider a thermophilic fungus to be one that grows better at 45 °C than at 25 °C. One practical advantage of this latter definition is that it permits easy evaluation of fungal isolates.

Less consistent in the literature is the distinction between thermotolerance and thermophily. Cooney and Emerson considered thermotolerant fungi to be those with a maximum growth temperature near 50 °C while having a minimum growth temperature “well below” 20 °C. This definition is quite restrictive on the high end. Although it permits inclusion of the ubiquitous *Aspergillus fumigatus*, it excludes many fungi, for example, the model organism *Neurospora crassa*, that can grow at temperatures near or above 45 °C while having temperature optima below 50 °C. From a practical point of view, 45 °C is a temperature that is lethal or stress-inducing for most organisms, and we consider fungi that can grow at 45 °C to be thermotolerant.

History. The first reported thermophilic fungus, *Rhizomucor pusillus*, was isolated from bread by Lindt in the 1880s (Lindt 1886). Later, Tsiklinsky (1899) identified another thermophile, *Thermomyces lanuginosus*, growing on potatoes. In the early 1900s, Hugo Miehe (1907a, b; 1930a, b) published a series of papers derived from his investigations regarding the role of living organisms in the self-heating of stored hay. One result was the description of two new thermophiles, *Thermoidium sulfureum* (*Malbranchea cinnamomea*) and *Thermoascus aurantiacus*.

The study of these organisms languished for several decades before they were discovered to be part of the composting process associated with the production of rubber from the desert shrub Guayule (*Parthenium argentatum*). During World War II, the United States and allies lost access to rubber-plant plantations in the Pacific, which hindered the manufacture of rubber badly needed for the war effort. The US Department of Agriculture had a large-scale program aimed at developing Guayule latex as an alternative source of rubber. One of the experimental approaches involved chopping the shrub into pieces and composting it in piles. These “rets” were strongly thermogenic as a result of microbial activity, and the characterization of the organisms involved led to the identification of new and previously recognized thermophilic fungi (Cooney and Emerson 1964). The single publication by Allen and Emerson (1949) that resulted from the study of the effects of microbial activity on rubber quality did not detail the organisms involved in the process. The importance of the Guayule project in the “rediscovery” of thermophilic fungi as the basis for the studies that led to the Cooney and Emerson (1964) monograph of thermophilic fungi was recounted in the latter.

Industry. In recent decades, much of the attention given to thermophilic fungi has been in industry. This interest stems in large part from the ability of these fungi to yield thermostable enzymes, especially those that are cellulose-active. These enzymes function at temperatures high enough to exclude contaminants, and they accelerate reactions that convert cellulose into fermentable sugars for bioethanol

(Beckner et al. 2011; Rubin 2008; van den Brink et al. 2013). To understand the genetic mechanisms of thermophily and thermostability, the genomes of several fungal thermophiles have been sequenced (Berka et al. 2011).

4.2 Evolution

Of the more than 100 thousand described species of fungi, only approximately 50 species are thermophilic, representing a small fraction of the 2.2–3.8 million estimated fungal species (Salar and Aneja 2007; Hawksworth and Lücking 2017). Thermophilic fungi are known from two phyla, the Ascomycota and the Mucoromycota. In the Ascomycota, thermophiles are restricted to the orders Sordariales, Eurotiales, and Onygenales. Thermophiles in the Mucoromycota occur in the Mucorales (Salar 2018) and a recently created order, the Calcarisporiellales (Hirose et al. 2012; Morgenstern et al. 2012; Tedersoo et al. 2018). The order Mucorales contains two families with thermophiles, the Rhizopodaceae and the Lichtheimiaceae (Hoffmann et al. 2013). The Calcarisporiellales contains the thermophilic species *Calcarisporiella thermophile*. In the Sordariales, all known thermophilic species belong to the family Chaetomiaceae, which contains the greatest diversity of thermophilic fungi (Morgenstern et al. 2012). Among the Eurotiales, two families are considered to possess thermophilic members, the Trichocomaceae and the Thermoascaceae (Houbraken et al. 2014, 2016). A sole species of thermophilic fungus, *Malbranchea cinnamomea*, is found in the Onygenales (Morgenstern et al. 2012). Thermophilic Basidiomycota have been described by Straatsma et al. (1994) and Fergus (1971) but these species have either not been confirmed to be thermophilic or, as in the case of *Myriococcum thermophilum*, have been found to belong in the Ascomycota instead (Morgenstern et al. 2012; Koukol 2016).

Taxonomy for thermophilic fungi is in a state of considerable flux (Mouchacca 2000b; Oliveira et al. 2015; Natvig et al. 2015). This results in part from the fact that under the “One Fungus = One Name” convention recently adopted by *the International Code of Nomenclature for Algae, Fungi, and Plants*, the names for many thermophiles in the fungal kingdom need to be revised (Oliveira et al. 2015). This convention requires that the asexual and sexual nomenclature be unified and that a single name be assigned to a single species. In addition to name changes that have been required by changes in nomenclatural codes, in many cases, thermophilic fungi have simply been misclassified because of the failure to identify correct taxonomic affinities. The genus *Myceliophthora* provides examples of name changes required by new nomenclatural rules and by molecular phylogenetic studies that reveal true relationships (van den Brink et al. 2012). For example, the species recently recognized as *Myceliophthora heterothallica* was previously known under the teleomorphic names *Theilavia heterothallica* and *Corynascus heterothallicus*. To add to the confusion, as *T. heterothallica*, this species was once thought to be the teleomorph of *Chrysosporium thermophilum*, now recognized as *M. thermophila* (von Klopotek 1976; Hutchinson et al. 2016; van den Brink et al. 2012). A similar

case exists for *Rasamsonia*, a genus erected to accommodate teleomorphs of *Geosmithia* and *Talaromyces* species, which were improperly identified (Houbraken et al. 2012). As a final example, the genus *Mycothermus* was recently erected to accommodate fungal strains previously known as *Scytalidium thermophilum*, placed in a genus (*Scytalidium*) that is appropriate for organisms in a different fungal class (Natvig et al. 2015).

4.3 Ecology

Despite advances in industry and genetics, comparatively little is known about the natural role and distribution of thermophilic fungi. Although commonly isolated from compost, these fungi are known to exhibit a variety of lifestyles, including as animal and plant associates and as saprotrophs (Salar 2018). For example, the thermophilic species *Myceliophthora thermophila* was identified as an endophyte of foliar tissue from a desert tree, *Parkinsonia microphylla* (Massimo et al. 2015). Another thermophile, *Rhizomucor pusillus*, has been reported to cause human infections, especially in immune-compromised individuals (St-Germain et al. 1993; Andrey et al. 2017). Cooney and Emerson (1964) noted that thermophilic fungi often remain unrecognized in culture when moderate incubation temperatures are used. As such, it may be that many thermophilic fungi remain undescribed.

A debate exists regarding how broadly distributed are the habitats in which thermophilic fungi can thrive. One hypothesis suggests that most thermophilic fungi are specialists of insulated compost-like substrates and that the presence of these fungi in soil and other non-compost substrates represents dispersal of aerial propagules (Maheshwari et al. 2000). Support for this idea has been presented for *Thermomyces lanuginosus*, which though common in soil was not competitive with mesophilic and thermotolerant fungi in soil microcosm experiments performed under fluctuating temperature regimes, unless temperatures were maintained above 40 °C. In addition, spores of *T. lanuginosus* failed to germinate in soil under conditions favorable for growth (Rajasekaran and Maheshwari 1993).

On the other hand, it is possible to wonder if understanding the role of thermophilic fungi in soil requires consideration of specific microhabitats and substrates suitable for growth. The proportion of physiologically active microorganisms in soil can be small compared to the total microbial biomass, and the level of activity for a microorganism or microbial group is dependent on substrate availability (Blagodatskaya and Kuzyakov 2013). Moreover, microcosm experiments performed with only mesophilic “soil” fungi demonstrate that the performance of one species relative to another is substrate dependent (e.g., Deacon et al. 2006). Therefore, while previous studies have reported thermophiles from diverse compost or pseudo-compost materials such as animal nests, manure compost, mushroom compost, and self-heating hay bales (Fergus and Sinden 1969; Tansey 1971, 1973, 1975, 1977; Tiquia 2005), it is likely that even a small 5-cm mass of leaf litter can be sufficiently insulated, moist and solar-heated to encourage growth of thermophilic fungi

(Subrahmanyam 1999). Indeed, recent studies of arid ecosystems (where sizeable composts are rare, if not absent), including the Sevilleta Long-Term Ecological Research (LTER) site in New Mexico, have demonstrated that thermophilic fungi are common in certain microhabitats (Powell et al. 2012). We recovered isolates from a variety of substrates including soil, biological soil crusts, leaf litter, and herbivore droppings. While these and other previous studies have shed light on microhabitats and distributions, the extent to which thermophilic fungi exhibit habitat specificity is unclear, as is the prevalence of thermophilic fungi on a regional scale.

Microhabitats Suitable for the Growth of Thermophilic Fungi Are Common in Diverse Ecosystems. Although the early studies of thermophilic fungi examined substrates that were self-heating as a result of microbial activity (Miehe 1907a, b; Cooney and Emerson 1964), soil and other substrates can achieve temperature and moisture conditions suitable for thermophiles as a result of solar gain (Tansey and Jack 1976; Powell et al. 2012). In reality, soil, litter, and herbivore droppings in temperate ecosystems often reach temperatures at or above those suitable for thermophilic fungi. In an experiment designed to follow the succession of thermophiles in a natural setting, we monitored temperatures in the droppings of three herbivores (elk, oryx, and rabbit) over a period of approximately 1 year (Fig. 4.1) at the Sevilleta National Wildlife Refuge. Even during winter months, daytime temperatures were often near or above 40 °C. In warmer months, daytime temperatures often reached 60–75 °C, temperatures at which fungal growth has ceased. In a single 24-h period, temperatures could swing from 15 °C to above 60 °C (Fig. 4.1). Droppings in this environment therefore represent an extreme microhabitat with dramatic and rapid changes in temperature and moisture. Thermophilic fungi are common in this microenvironment, and they participate in decomposition along with a complex community of bacteria, non-thermophilic fungi, and microfauna.

4.4 A Survey of Thermophilic Fungi from Across the Western United States

In a previously unpublished study, we surveyed thermophilic fungi in soils, plant litter, and herbivore droppings from a wide range of latitudes, elevations, and distinct climatic regions across sites from central Mexico to southern Canada. One goal was to evaluate the extent to which these fungi are common in locations where the opportunities for natural compost are rare. A second goal was to evaluate whether there exist geographical, latitudinal, or substrate differences in the distributions of major thermophile groups. Our sampling focused on soil, litter, and herbivore droppings. In addition, deep-frozen (−80 °C) rhizosphere soil samples collected from under blue grama grass (*Bouteloua gracilis*) were tested for the presence of thermophilic fungi.

Experimental Approach. Samples were collected in two phases. From May through June of 2008, 10 samples of rhizosphere soil were collected from each of five stands of *Bouteloua gracilis* in western North America as part of a separate

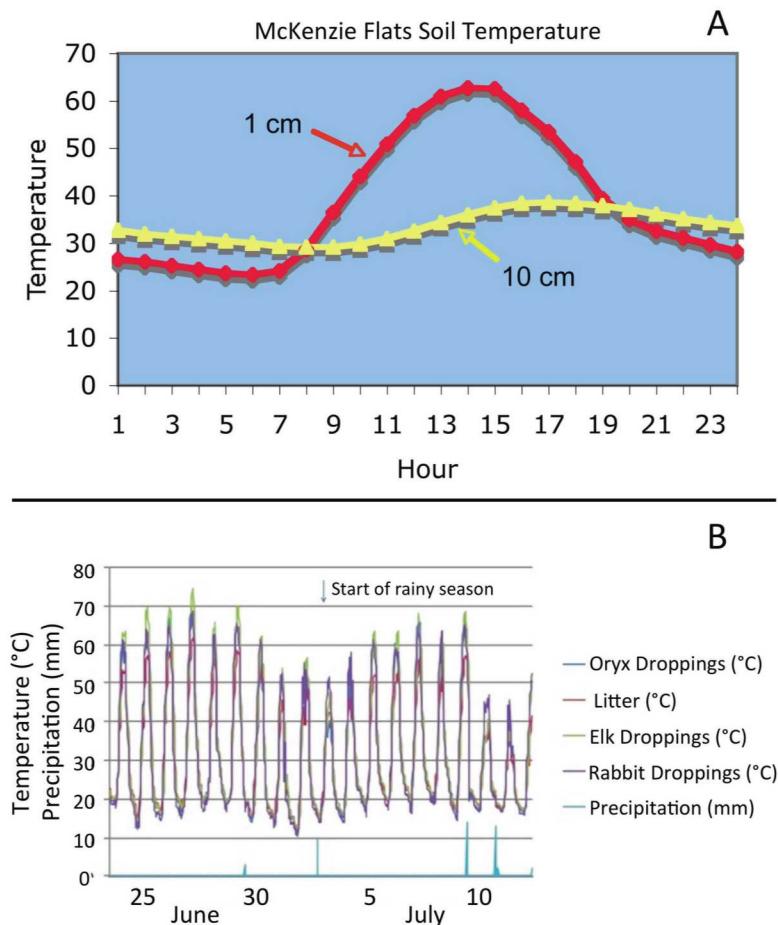


Fig. 4.1 Extreme microenvironments are common in temperate ecosystems. (a) Variation in soil temperature for a typical 23-h period (1:00 AM to midnight) in July at the Sevilleta National Wildlife Refuge in central New Mexico (adapted from Fig. 1 in Powell et al. 2012, copyright © Mycological Society of America, <https://msafungi.org/>, reprinted with permission from Taylor & Francis Ltd., <http://www.tandfonline.com> on behalf of the Mycological Society of America). (b) Dramatic swings in internal temperatures for herbivore droppings and litter in the foothills of the Los Pinos mountains in central New Mexico over 19 days surrounding the transition to the monsoon season in 2013. The temperature swings were frequently from 12 °C to 15 °C in early morning to over 70 °C at midday. The high temperatures were driven by solar gain. Air temperatures did not exceed 35 °C. Temperatures were measured with a small thermocouple and recorded on a Campbell Scientific CR1000 datalogger

study of root-associated fungi (Herrera et al. 2010). Soils were transported from the field on ice within 48 h and ultimately stored at -80°C . These samples were plated in January of 2013. In a second effort, soil, herbivore droppings, and leaf litter samples were collected from each of 10 locations in the western United States



Fig. 4.2 Locations of soil, litter, and herbivore dropping samples employed for the thermophile survey presented here. Details of the samples are given in Table 4.1

between March 2012 and May 2013 (Fig. 4.2, Table 4.1). These samples were stored at 4–5 °C for no more than 4 days before plating.

All samples were plated onto malt extract agar (MEA) with 50 µg/mL ampicillin (to exclude bacteria) and incubated up to 10 days at 50 °C (see Bustamante 2006). Approximately 0.5–1.0 g of substrate was used for each plate. Rhizosphere soils from the Herrera et al. (2010) study were plated in replicates of 3. Resulting colonies from all cultures were then sub-cultured to obtain axenic isolates.

A cetyl trimethylammonium bromide (CTAB) DNA extraction procedure modified from Winnepernincx et al. (1993) was used to isolate DNA from cultures, using methods previously described (Hutchinson et al. 2016). DNA was amplified by PCR of the ribosomal internal transcribed spacer (ITS) region using the fungal-specific primers ITS4 and ITS1F (White et al. 1990; Gardes and Bruns 1993). Each reaction consisted of 6.5 µL ExTaq polymerase (Takara, Mountain View, CA), 1 µL of each (5 µM) primers, 2 µL of 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 2 µL milliQ purified water, and 1 µL of template DNA, for a total of 13.5 µL. The following thermocycler settings were used: 95 °C for 5 min, 30 cycles at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension of 72 °C for 7 min. After PCR, reactions were purified by an enzyme procedure using the ExoSAP-IT kit (Affymetrix, Santa Clara, CA) and manufacturer's specifications.

Table 4.1 Isolate identifications and collection sites

OTU (putative species)	Isolate(s) represented	Substrate type	Collection date	GPS coordinates	Location	Elevation (m)
OTU 1 (<i>Thermomyces lanuginosus</i>)	6II	Litter	May 2013	44°56.593'N 110°38.397'W	Undine Falls, WY	2033
	10B_I	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	12I	Soil	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	13F	Litter	May 2013	44°24.653'N 108°59.557'W	Cody, WY	2579
	24B	Litter	May 2013	38°51.423'N 105°03.795'W	Pike's Peak, CO	3951
	28C	Droppings	May 2013	38°54.032'N 105°04.058'W	Pike's Peak, CO	3033
	32D_I	Droppings	May 2013	36°33.535'N 104°34.692'W	Maxwell Wildlife Refuge, NM	1835
	OBg1_1	Rhizosphere soil	May 2008	21°46.860'N 101°36.721'W	Ojuelos, JAL, Mexico	2230
	OBg2_3	Rhizosphere soil	May 2008	21°46.860'N 101°36.721'W	Ojuelos, JAL, Mexico	2230
	OBg3_1	Rhizosphere soil	May 2008	21°46.860'N 101°36.721'W	Ojuelos, JAL, Mexico	2230
	SBg8_2	Rhizosphere soil	May 2008	34°24.094'N 106°40.662'W	Sevilleta, NM	1544
	Th002	Litter	March 2012	34°15.267'N 109°24.267'W	Apache County, AZ	1958
	Th047	Soil	March 2012	34°49.183'N 118°56.683'W	Los Padres National Forest, CA	1413
	ThUS015	Litter	September 2012	44°30.753'N 110°15.897'W	Lake Butte, WY	2679
	ThUS028	Litter	September 2012	43°31.117'N 108°10.917'W	Wind River Canyon, WY	1451
	ThUS057	Litter	September 2012	45°00.183'N 109°24.867'W	Beartooth Highway, WY	315

OTU 2 (<i>Chaetomium thermophilum</i> var. <i>dissitum</i>)	3I	Soil	May 2013	45°45.950'N 109°47.583'W	Grey Cliff Prairie Dog State Park, MT	1208
	10B_II	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	12II	Soil	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	16_I	Litter	May 2013	43°44.752'N 108°23.502'W	Thermopolis, WY	2580
	32A_II	Droppings	May 2013	36°33.535'N 104°34.692'W	Maxwell Wildlike Refuge, NM	1835
	WBg1_MH1	Rhizosphere soil	May 2008	43°34.236'N 103°23.210'W	Wind Cave, SD	1121
OTU 3 (<i>Thielavia arenaria</i>)	15C_L1	Droppings	May 2013	44°24.653'N 108°59.557'W	Cody, WY	2579
	18E_L1	Droppings	May 2013	43°44.752'N 108°23.502'W	Thermopolis, WY	2580
	GBg10_1	Soil	June 2008	49°10.705'N 107°33.634'W	Grasslands, SK, Canada	785
	Th044-2	Soil	March 2012	34°05.484'N 110°10.632'W	Gila National Forest, AZ	1793
OTU 4 (<i>Myceliophthora heterothallica</i>)	Th021	Soil	March 2012	34°27.833'N 118°41.017'W	Val Verde, CA	554
	Th022	Litter	March 2012	34°05.484'N 110°10.632'W	Gila National Forest, AZ	1793
	Th041	Litter	March 2012	37°00.117'N 120°50.367'W	Central Valley, CA	40
	Th044	Soil	March 2012	34°05.484'N 110°10.632'W	Gila National Forest, AZ	1793

(continued)

Table 4.1 (continued)

OTU (putative species)	Isolate(s) represented	Substrate type	Collection date	GPS coordinates	Location	Elevation (m)
OTU 5 (<i>Talaromyces thermophilus</i>)	5B	Droppings	May 2013	45°45.950'N 109°47.583'W	Grey Cliff Prairie Dog State Park, MT	1208
	10A	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	13B	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	19I	Litter	May 2013	42°34.898'N 106°41.133'W	Grey Reef, WY	1648
	ThUS017	Litter	September 2012	45°00.183'N 109°24.867'W	Beartooth Highway, WY	315
OTU 6 (<i>Aspergillus fumigatus</i>)	1E	Dropping	May 2013	45°45.950'N 109°47.583'W	Grey Cliff Prairie Dog State Park, MT	1208
	9	Soil	May 2013	44°50.328'N 110°26.528'W	Mount Washburn, WY	2529
	13D	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
	17	Soil	May 2013	43°44.752'N 108°23.502'W	Thermopolis, WY	2580
	GBg6_1	Rhizosphere soil	June 2008	49°10.705'N 107°33.634'W	Grasslands, SK, Canada	785
	GBg9_1	Rhizosphere soil	June 2008	49°10.705'N 107°33.634'W	Grasslands, SK, Canada	785
	JBg11_2	Rhizosphere soil	May 2008	30°53.878'N 108°26.057'W	Janos, CHH, Mexico	1391
	OBg6_1	Rhizosphere soil	May 2008	21°46.860'N 101°36.721'W	Ojuelos, JAL, Mexico	2230

OTU 7 (<i>Rasamsonia emersonii</i>)	2 Pike's Peak	Soil	August 2013	38°51.292'N 105°05.253'W	Pike's Peak, CO	3041
	6I	Litter	May 2013	44°56.593'N 110°38.397'W	Undine Falls, WY	2033
	Th008	Droppings	March 2012	37°44.300'N 121°36.7'W	Altamont Pass, CA	160
OTU 8 (<i>Rhizopus microsporus</i>)	JBg17_2	Rhizosphere soil	May 2008	30°53.878'N 108°26.057'W	Janos, CHH, Mexico	1391
	SBg6_3	Rhizosphere soil	May 2008	34°24.094'N 106°40.662'W	Sevilleta NWR, NM	1544
	SBg10_2	Rhizosphere soil	May 2008	34°24.094'N 106°40.662'W	Sevilleta NWR, NM	1544
OTU 9 (<i>Aspergillus nidulans</i>)	SBg3_3	Rhizosphere soil	May 2008	34°24.094'N 106°40.662'W	Sevilleta NWR, NM	1544
OTU 10 (<i>Thielavia gigaspora</i>)	WBg9_2	Rhizosphere soil	May 2008	43°34.236'N 103°23.210'W	Wind Cave, SD	1121
OTU 11 (<i>Thermoascus aurantiacus</i> var. <i>levisporus</i>)	16II	Litter	May 2013	43°44.752'N 108°23.502'W	Thermopolis, WY	2580
OTU 12 (<i>Mycothermus thermophilus</i>)	13C	Droppings	May 2013	44°30.693'N 110°16.338'W	Lake Butte, WY	2614
OTU 13 (<i>Chaetomium jodhpurens</i>)	WBg10_2	Rhizosphere soil	May 2008	43°34.236'N 103°23.210'W	Wind Cave, SD	1121
OTU 14 (<i>Rhizopus microsporus</i>)	Th040	Soil	March 2012	33°15.6'N 111°17.317 W	Near Phoenix, AZ	1740

Amplicons were Sanger sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) in 10 μ L reactions containing 0.5 μ L BigDye Terminator v3.1, 2 μ L of 5X Sequencing Buffer (Life Technologies/Applied Biosystems, Carlsbad, CA) 1 μ L of 3 μ M primer, and 5.5 μ L of milliQ water. A BigDye STeP protocol was used with the following parameters: 96 °C for 60 s followed by 15 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 1 min 15 s; then 5 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 1 min 30 s; and a final 5 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 2 min/s (Platt et al. 2007).

Chromatogram files for the forward and reverse reads were edited and assembled into contigs using Sequencher v5.1 (Gene Codes, Ann Arbor MI). To determine the overall species richness among the isolates, ITS sequences were assembled into Operational Taxonomic Units (OTUs) using UPARSE 9.0 (Edgar 2013). OTU cut-offs were set to 97% identity. To obtain taxonomic information, the resulting OTUs were then queried at National Center for Biotechnology Information (NCBI) GenBank with Basic Local Alignment Search Tool Nucleotide (BLASTN) searches using the option to exclude uncultured and environmental samples.

Phylogenetic Analyses. ITS sequences were aligned in MUSCLE implemented through the European Bioinformatics Institute web interface (Edgar 2004; Li et al. 2015). Alignments were then visualized and trimmed in AliView v1.2.1 (Larsson 2014). Reference sequences from GenBank were included as a comparison to the newly acquired sequences, and type strains were selected as references when possible (Tables 4.2 and 4.3). Trees were constructed with the Randomized Axelerated Maximum Likelihood (RaxML) program v7.3.2 using 1000 bootstrap replicates (Stamatakis 2006). Because ITS sequences align poorly across distant phylogenetic groups, we built separate trees for each of the three orders to which the sequences were classified. Trees were visualized and edited with Mesquite v2.75 (Maddison and Maddison 2010).

Results. Thermophilic and thermotolerant fungi were recovered from every substrate type and nearly every location. Notably, propagules of thermophilic fungi from the rhizosphere soil were also able to survive storage at -80 °C for nearly 5 years. Sixty-two total isolates were recovered. After excluding duplicates from the same sample, 55 isolates were characterized at the sequence level, resulting in 14 putative OTUs, 10 genera, and 13 known species. The identity of each of the OTUs is summarized in Table 4.2, and relationships among the isolates are shown in Fig. 4.3. Most isolates fell into the fungal orders Eurotiales (34 isolates) and Sordariales (17 isolates). Only 4 isolates belonged to the Mucorales, and no isolates from the Onygenales were identified. The lack of isolates from the Onygenales may owe to the types of substrates and media used, as this group of fungi is known to be keratinophilic (Sharpton et al. 2009). The most common species was *Thermomyces lanuginosus*, represented by 16 isolates, followed by *Aspergillus fumigatus*, represented by 8 isolates, and *Chaetomium thermophilum* var. *dissitum*, represented by 6 isolates.

Several of the isolates were from species viewed as thermotolerant rather than thermophilic. Mouchacca (2000a) suggests that *A. fumigatus*, *A. nidulans*, and *C. jodhpurensis* have been erroneously reported as thermophiles when they actually

Table 4.2 Isolate abundance and best BLAST hits

OTU	Abundance	Best blast hit (species)	Order	Family	Accession number
OTU 1	16	<i>Thermomyces lanuginosus</i> isolate TCSB341	Eurotiales	Trichocomaceae	KT365217.1
OTU 2	6	<i>Chaetomium thermophilum</i> var. <i>dissitum</i> strain: NBRC 31807	Sordariales	Chaetomiaceae	AB746179.1
OTU 3	4	<i>Thielavia arenaria</i> strain CBS 507.74	Sordariales	Chaetomiaceae	JN709489.1
OTU 4	4	<i>Myceliophthora heterothallica</i> CBS 202.75	Sordariales	Chaetomiaceae	JN659478.1
OTU 5	5	<i>Talaromyces thermophilus</i> strain NRRL 2155	Eurotiales	Trichocomaceae	JF412001.1
OTU 6	8	<i>Aspergillus fumigatus</i> strain IHEM 13935 isolate ISHAM-ITS_ID MITS168	Eurotiales	Aspergillaceae	KP131565.1
OTU 7	3	<i>Rasamonia emersonii</i> strain CBS 396.64	Eurotiales	Trichocomaceae	JF417479.1
OTU 8	3	<i>Rhizopus microsporus</i> strain: TISTR 3518	Mucorales	Rhizopodaceae	AB381937.1
OTU 9	1	<i>Aspergillus nidulans</i> isolate KZR-132	Eurotiales	Aspergillaceae	KX878986.1
OTU 10	1	<i>Thielavia gigaspora</i> strain CBS 112062	Sordariales	Chaetomiaceae	MH862888.1
OTU 11	1	<i>Thermoascus aurantiacus</i> var. <i>levisporus</i> strain T81	Eurotiales	Thermoascaceae	FJ548834.1
OTU 12	1	<i>Mycothermus thermophilus</i> isolate A74	Sordariales	Chaetomiaceae	KX611046.1
OTU 13	1	<i>Chaetomium jodhpurense</i> strain CBS 602.69	Sordariales	Chaetomiaceae	MH859386.1
OTU 14	1	<i>Rhizopus microsporus</i> isolate VPCI 128/P/10	Mucorales	Rhizopodaceae	KJ417570.1

possess lower temperature optima than true thermophiles. Additionally, *Thielavia gigaspora* is a thermotolerant species previously isolated in Egypt (Moustafa and Abdel-Azeem 2008). Mouchacca (2000a) also reported *Rhizopus microsporus* as a misattributed thermophile, but (Peixoto-Nogueira et al. 2008) demonstrated that isolates grow optimally at 45 °C. Overall, thermotolerant species represented 29% of all of our isolates. Excluding the thermotolerant species, there were 25 isolates from the Eurotiales and 14 from the Sordariales.

Independent-samples Welch's t-tests were employed to compare elevation and latitude specificity for thermophilic isolates in the Eurotiales and Sordariales. Because the Mucorales were comparatively rare, they were not included in statistical analyses. For elevation, there was no significant difference between the distributions of Eurotiales and Sordariales ($M_{EUROTIALES} = 2038.28$ m, $SD = 900.51$; $M_{SORDARIALES} = 1765$ m, $SD = 823.66$; $t(29) = 0.96$, $p = 0.05$). For latitude, again,

Table 4.3 Reference strains used for phylogenetic analyses

Order	Strain	Species	Thermophile?	Accession number
Eurotiales	CBS 525.65	<i>Aspergillus fischeri</i>	No	MH858698.1
	CBS 139343	<i>Aspergillus fumigatus</i>	No	KU296268.1
	CBS 467.88	<i>Aspergillus nidulans</i>	No	KU866630.1
	CBS DTO_283-D3	<i>Aspergillus udagawae</i>	No	KY808744.1
	CBS 393.64	<i>Rasamsonia emersonii</i> ^a	Yes	JF417478.1
	CBS 398.64	<i>Thermoascus aurantiacus</i>	Yes	MH858464.1
	CBS 181.67	<i>Thermoascus crustaceus</i> ^a	Yes	FJ389925.1
	CBS 236.58	<i>Thermomyces dupontii</i>	Yes	MH857768.1
	CBS 632.91	<i>Thermomyces lanuginosus</i>	Yes	MH862287.1
Onygenales	CBS 120936	<i>Coccidioides immitis</i> ^a	No	NR_157446.1
Mucorales	ATCC 36186	<i>Pilobolus crystallinus</i>	No	FJ160949.1
	CBS 130158	<i>Rhizopus microsporus</i>	No	MH865595.1
	CBS 182.67	<i>Rhizomucor miehei</i> ^a	Yes	JF412011.1
Sordariales	CBS 160.62	<i>Chaetomium globosum</i> ^a	No	MH858130.1
	CBS 602.69	<i>Chaetomium jodhpurens</i>	No	MH859386.1
	LC4128	<i>Chaetomium thermophilum</i> var. <i>dissitum</i>	Yes	KP336781.1
	NBRC 31807	<i>Chaetomium thermophilum</i> var. <i>dissitum</i>	Yes	AB746179.1
	CBS 202.75	<i>Myceliophthora heterothallica</i> ^a	Yes	JN659478.1
	CBS 629.91	<i>Mycothermus thermophilus</i>	Yes	MH862286.1
	CBS 709.71	<i>Neurospora crassa</i>	No	MH860307.1
	CBS 507.74	<i>Thielavia arenaria</i> ^a	Yes	JN709489.1
	CBS 112062	<i>Thielavia gigaspora</i> ^a	No	MH862888.1
	CBS 125981	<i>Thielavia subthermophila</i>	No	MH863860.1

^aType strain

there was no significant difference between the distributions of Eurotiales and Sordariales ($M_{EUROTIALES} = 38.79^\circ$, $SD = 16.35$; $M_{SORDARIALES} = 41.44^\circ$, $SD = 28.92$; $t(18) = -0.32$, $p = 0.05$).

In terms of substrate preference, thermophilic samples in Eurotiales were most frequently isolated from litter (44%), while for samples in the Sordariales, the top sources were droppings (35.71%) and top soil (35.71%). Overall, the most thermophilic isolates originated from litter substrates (35.9%), followed by droppings (30.7%), soil (20%), and finally rhizosphere, which represented 12.8% of the samples.

For the soils collected in 2008 and stored at -80°C , there appeared to be a latitudinal gradient in terms of the success of platings. Just over half (62.5%) of soils collected in Saskatchewan, Canada, were positive for thermophiles, compared to 80% of soils from Custer, South Dakota; 86.7% from Socorro, New Mexico; 93.9% from Janos (Chihuahua), Mexico; and 89.7% from Ojuelos (Jalisco), Mexico. With the exception of the soils from Janos (which showed a higher percentage than

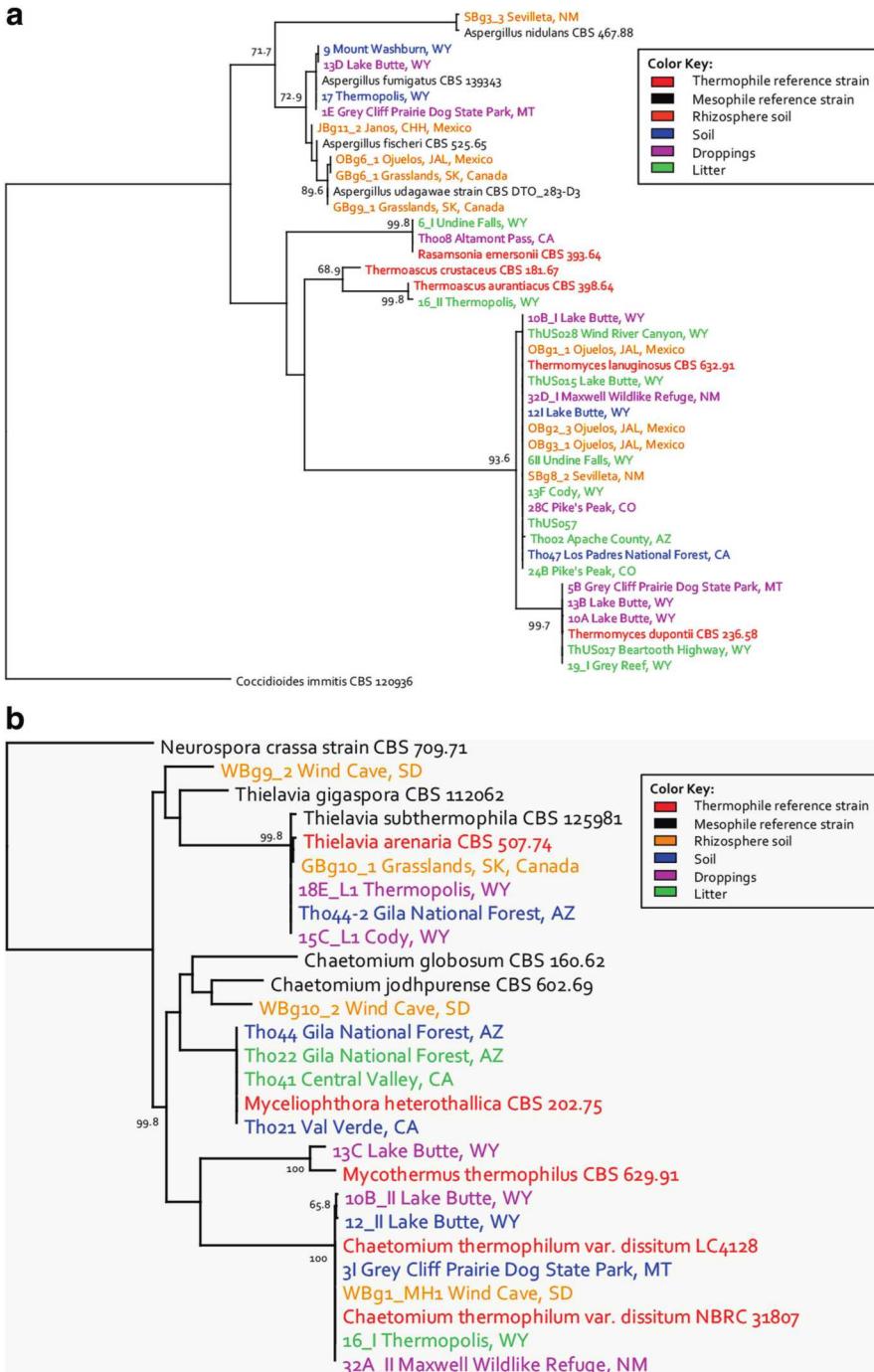


Fig. 4.3 Ribosomal RNA ITS gene trees for three orders of thermophilic fungi recovered from a recent survey (collection sites are presented in Fig. 4.2 and Table 4.1): Eurotiales (a), Sordariales (b), Mucorales (c). Trees were rooted with *Coccidioides immitis*, *Neurospora crassa*, and *Pilobolus crystallinus*, respectively. New isolates are color coded by substrate type, while reference strains are colored by temperature optimum. Bootstrap values (1000 replicates) are displayed for all nodes receiving 65% or greater support. All new isolates form well-supported clades with previously identified species, and represent diverse substrate types and locations

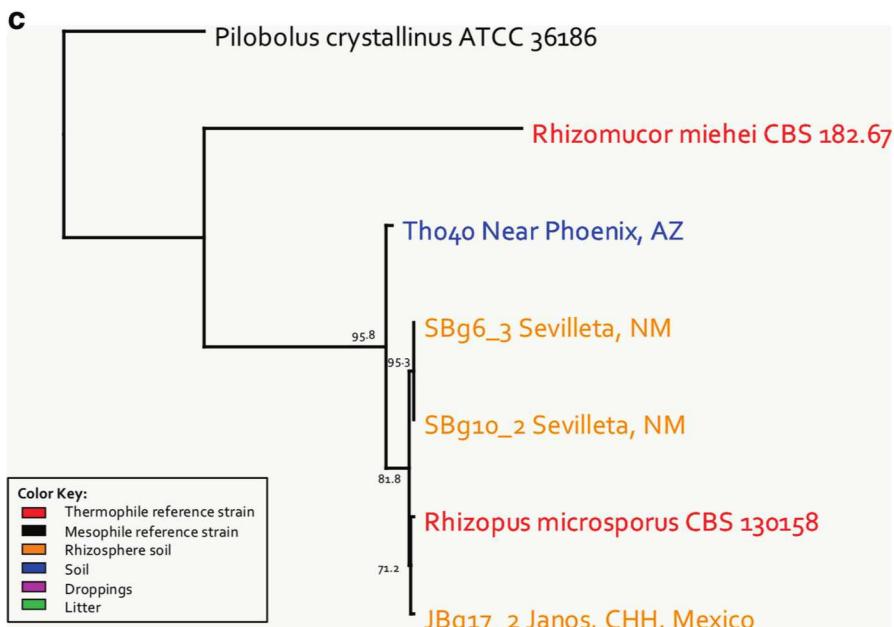


Fig. 4.3 (continued)

Ojuelos to the south), plating success declined with increasing latitude. In pairwise comparisons, plating success for Saskatchewan was an outlier, and significantly different from all other locations except South Dakota according to a Pearson's N-1 chi-square test [$\chi^2_{\text{SOUTHDAKOTA}}(1, N=54) = 2.00, p = 0.16$; $\chi^2_{\text{NEWMEXICO}}(1, N=54) = 4.20, p = 0.04$; $\chi^2_{\text{JALISCO}}(1, N = 63) = 6.60, p = 0.003$; $\chi^2_{\text{CHIHUAHUA}}(1, N = 57) = 8.63, p = 0.01$]. No other pairwise comparisons were significantly different.

Discussion. Our results indicate that thermophilic fungi are readily isolated from various substrates, from elevations as low as 40 m above sea level to as high as 3951 m and from a great range of latitudes between Mexico and Canada. We observed no correlation between phylogeny and environment. Specifically, isolates from the Eurotiales and Sordariales did not differ significantly for substrate preference, elevation, or latitude. Even within a single OTU cluster, constituent sequences were derived from diverse locations and substrates. For example, OTU1 (*Thermomyces lanuginosus*) represents isolates from as far south as Ojuelos, Jalisco, to as far north as the Beartooth Highway in Wyoming. This cluster also consisted of multiple isolates from every substrate type and of elevations from 315 m to above timberline at 3951 m. Indeed, at the resolution of OTUs at the 97% level, there appears to be no specificity of thermophilic fungi to a particular habitat. It is possible, however, that the 97% cutoff is too generous and blurs the finer distinctions among the isolates. To develop a better sense of the phylogenetic relationships between the isolates, one might also collect data for functional DNA regions that are less variable and more reliable at predicting deeper levels of taxonomy.

Studies show that members of the Chaetomiaceae (Sordariales) are proficient in decomposing cellulosic biomass, so they are thought to associate with plant-based substrates in nature (Ames 1963; Mehrotra and Aneja 1990). They have been previously isolated from herbivore droppings, leaf litter, and even from live plants (Kerekes et al. 2013; Richardson 2001; Abou Alhamed and Shebany 2012). Chaetomiaceae are also prevalent in composts (Cooney and Emerson 1964; Kane and Mullins 1973; Straatsma et al. 1994). For example, using an ITS barcoding approach, Neher et al. (2013) showed *Chaetomium* species to be dominant members of the fungal OTUs across all of the compost recipes they tested, especially in the earlier stages of composting. As discussed previously, composts have been proposed as the primary habitats for thermophilic fungi, with the suggestion that specimens found on other substrates are likely inactive propagules dispersed from compost (Rajasekaran and Maheshwari 1993). However, soil is also sufficiently rich in cellulose as it is one of the top sources of complex carbon polymers (Kögel-Knabner 2002; López-Mondéjar et al. 2016). Thus, it is perhaps unsurprising that many thermophilic species in the Chaetomiaceae have been identified from soil (Tansey and Jack 1976; Pan et al. 2010; Powell et al. 2012). Mesophilic Chaetomiaceae have been demonstrated to be both present and active in the soil. Using Stable Isotope Probing with ¹³C cellulose substrate, Eichorst and Kuske (2012) showed that species of *Chaetomium* actively decay cellulose added to soil. It is reasonable to believe that thermophilic members of the Chaetomiaceae do the same.

Species in the order Eurotiales are also commonly associated with decaying plant material. For example, the well-known fungus *Aspergillus fumigatus* is cited as one of the most frequent species recovered from composts and other plant debris (Taylor et al. 2015). *A. fumigatus* also shows a pan-global distribution, which Pringle et al. (2005) have suggested may be due to the role of humans in expanding composting processes. Another member of the Eurotiales, the thermophilic fungus *Thermomyces lanuginosus* also shows seemingly ubiquitous distribution. In our present study, it was the most frequently isolated taxon and derived from a variety of substrates and locations. Langarica-Fuentes et al. (2014) also found that along with *Talaromyces thermophilus* (another species in the Eurotiales), *T. lanuginosus* accounted for 65% of sequences obtained via 454' barcoding of the fungal community in the middle and center of an in-vessel compost system. Similarly, it was the top isolate in studies of thermophilic fungi from soils in India (Maheshwari et al. 1987; Rajasekaran and Maheshwari 1993). Still, Rajasekaran and Maheshwari (1993) were unable to detect actively growing *T. lanuginosus* in soil with immunofluorescence assays. However, Hedger and Hudson (1974) reported that *T. lanuginosus* shows commensal interactions with cellulolytic fungal thermophiles (*Chaetomium thermophile* and *Humicola insolens*) and subsists on the sugar byproducts from cellulose decomposition. Thus, it may be that this species performs best in a consortium with cellulolytic thermophiles and requires other fungal partners to grow. If there is adequate cellulose in a given substrate, cellulose degrading fungi can likely support commensal fungi, thus providing a niche in soil for species such as *T. lanuginosus*.

Soils undergo diurnal temperature fluctuations to upwards of 70 °C, so soil is a suitably hot substrate for thermophilic fungi (Powell et al. 2012). Leaf litter and

herbivore droppings also experience similar swings in temperature (Fig. 4.1). In addition, thermophilic fungi are more readily isolated from soil after precipitation events, indicating that they are responsive to changes in the soil environment (Powell et al. 2012). Taken together, these factors suggest that thermophilic fungi can inhabit many microhabitats, including soil, provided that they have access to moisture and appropriate temperatures.

4.5 Conclusions

Much remains to be learned about the ecology of thermophilic fungi. Although it has long been known that these fungi can be isolated from soil, herbivore droppings, and other substrates, most studies have focused on composted plant materials in either natural or anthropogenic settings. In contrast, our surveys have shown that nearly all ecosystems provide thermophilic fungi with at least transient access to decomposing plant material, and sufficiently high temperature and moisture (see Fig. 4.1). Our results suggest that such transient microenvironments might be the primary habitats. At the level of resolution provided by ribosomal ITS sequences, there is little evidence for habitat specialization or geographical restrictions among thermophiles. Thermophiles in the Ascomycota are distributed across three orders, with several phylogenetic lineages within each order. We found members of most lineages across wide latitudes, elevations, substrate, and ecosystem types, ranging from desert shrublands and grasslands to montane forests to northern grasslands.

4.6 Future Perspectives

Thermophilic fungi have provided many contributions to science, both in their utility to industry and in the advancement of basic understanding in biology. Information on the distribution of thermophilic fungi, and a better grasp on their natural diversity and roles in the environment, will help further the field of microbial ecology and will aid in bioprospecting new, potentially useful organisms for biotechnology. Although next-generation sequencing methods can detect thermophilic fungi in environmental samples, many thermophiles have close mesophilic relatives, and as a result, the assessment of thermophily often requires evaluation based on growth in the laboratory rather than on sequence analysis alone. Accordingly, it is likely that fungal thermophiles are overlooked in environmental sequencing data. Similarly, culture-based methods of community analysis often employ only temperatures suitable for mesophiles, and temperatures optimal for the growth of thermophiles or psychrophiles are not considered. Moreover, it is possible that certain fungal thermophiles are uncultivable and are only detected as DNA in environmental surveys. These circumstances thereby result in a need for a unified, comprehensive approach to appraising and understanding not only the biology of thermophilic fungi, but also the ecology of non-thermophilic microbes that share environments with thermophiles.

Acknowledgements This research was supported in part by a National Science Foundation award to the University of New Mexico (UNM) for the Sevilleta Long-Term Ecological Research program. We acknowledge support for DNA sequencing from the UNM Department of Biology's Molecular Biology Facility. Data analysis was aided by computing resources of the UNM Center for Evolutionary & Theoretical Immunology (CETI) under National Institutes of Health grant P30GM110907, and the UNM Center for Advanced Research Computing, supported in part by the National Science Foundation.

Funding statement Sandia National Laboratories is a multimission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525.

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