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Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America

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ABSTRACT

White-nose syndrome (WNS) of bats, caused by the fungus previously known as *Geomyces destructans*, has decimated populations of insectivorous bats in eastern North America. Recent work on fungi associated with bat hibernacula uncovered a large number of species of *Geomyces* and allies, far exceeding the number of described species. Communication about these species has been hindered by the lack of a modern taxonomic evaluation, and a phylogenetic framework of the group is needed to understand the origin of *G. destructans* and to target closely related species and their genomes for the purposes of understanding mechanisms of pathogenicity. We addressed these issues by generating DNA sequence data for the internal transcribed spacer (ITS) region, nuclear large subunit (LSU) rDNA, *MCM7*, *RPB2*, and *TEF1* from a diverse array of *Geomyces* and allies that included isolates recovered from bat hibernacula as well as those that represent important type species. Phylogenetic analyses indicate *Geomyces* and allies should be classified in the family *Pseudeurotiaceae*, and the genera *Geomyces*, *Gymnostellatospora*, and *Pseudogymnoascus* should be recognized as distinct. True *Geomyces* are restricted to a basal lineage based on phylogenetic placement of the type species, *Geomyces auratus*. Thus, *G. destructans* is placed in genus *Pseudogymnoascus*. The closest relatives of *Pseudogymnoascus destructans* are members of the *Pseudogymnoascus roseus* species complex, however, the isolated and long branch of *P. destructans* indicates that none of the species included in this study are closely related, thus providing further support to the hypothesis that this pathogen is non-native and invasive in eastern North America. Several conidia-producing isolates from bat hibernacula previously identified as members of *Pseudeurotium* are determined to belong to the genus *Leuconeurospora*, which is widespread, especially in colder regions. *Teberdinia hygrophila* is transferred to *Pseudeurotium* as *Pseudeurotium hygrophilum*, comb. nov., in accordance with the one name per fungus system of classification, and two additional combinations are made in *Pseudogymnoascus* including *Pseudogymnoascus carnis* and *Pseudogymnoascus pannorum*. Additional sampling from other regions of the world is needed to better understand the evolution and biogeography of this important and diverse group of fungi.

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Introduction

The genus *Geomyces* is widespread and has been reported from a multitude of substrates, but it is especially common in soil in colder regions (Carmichael 1962; van Oorschot 1980; Rice & Currah 2006; Domsch *et al.* 2007; Loque *et al.* 2009; Gonçalves *et al.* 2012). *Geomyces* was erected by Traaen (1914) to accommodate four species of anamorphic hyphomycetes: namely *Geomyces auratus*, *Geomyces cretaceus*, *Geomyces sulphureus*, and *Geomyces vulgaris*. Carmichael (1962) synonymized these four species and numerous others under the name *Chrysosporium pannorum*, a combination made earlier by Hughes (1958), while noting substantial variation, especially in colony coloration and spore ornamentation. Sigler & Carmichael (1976) recognized *Geomyces* as a genus distinct from *Chrysosporium* Corda and typified the genus with *G. auratus* since it was the only species originally included in the genus that could be associated with a living culture isolated by Traaen; apparently this isolate (CBS 108.14) was used in the Traaen (1914) publication describing the genus. Dal Vesco (1957) was the first to connect a *Geomyces* species to a sexual stage/teleomorph when the author described the new species *Geomyces vinaceus* Dal Vesco, which was linked to and treated as conspecific with *Pseudogymnoascus vinaceus*, an ascomata producing genus and species described by Raillo (1929), as allowed under earlier Codes of nomenclature when naming each stage/morph of a pleomorphic fungus (Norvell 2011; ICN, McNeill *et al.* 2012). Samson (1972) treated the latter as a synonym of *Pseudogymnoascus roseus* and Sigler *et al.* (2000) outlined typification-related issues of *Pseudogymnoascus* associated with the works of others who considered this topic including Kuehn (1958), Samson (1972), and Orr (1979). Sigler & Carmichael (1976) also discussed the anamorph of *P. roseus*, which they accepted as a species separate from *Geomyces pannorum*, and noted that only purplish-red isolates were associated with the sexual stage in spite of the anamorph being indistinguishable from that of *G. pannorum*. Van Oorschot (1980) supported Sigler and Carmichael's (1976) assertion that *Geomyces* is distinct from *Chrysosporium*, listed the additional genus *Glenosporella* Nann. as a synonym, and further reinforced broad species concepts for the genus by regarding all of the treated taxa as varieties of *G. pannorum* with each variety having additional synonyms. *Gymnostellatospora*, based on a teleomorphic type, was erected by Udagawa *et al.* (1993) and was distinguished on the basis of its ascospores with longitudinal ridges. Sigler *et al.* (2000) and Rice & Currah (2006) discussed the relationships of *Geomyces*, *Gymnostellatospora*, and *Pseudogymnoascus* and the validity of earlier documentation that *Gymnostellatospora* possesses *Geomyces* anamorphs. Sigler *et al.* (2000) suggested the anamorph of *Gymnostellatospora canadensis* T.C. Lumley, Sigler & Currah was not a *Geomyces* due to its lack of whorled branching. Sigler *et al.* (2000) and Rice & Currah (2006) maintained *Gymnostellatospora* and *Pseudogymnoascus* as distinct genera, but identified a morphological continuum among the taxa and included species.

Other taxa included in *Geomyces*, *Gymnostellatospora*, and *Pseudogymnoascus* listed in the Index Fungorum (http://www.indexfungorum.org/Names/Names.asp) and MycoBank (http://www.mycobank.org/; Crous *et al.* 2004) have been added over the years, but a complete taxonomic treatment is beyond the scope of this work. Taxonomic activity in this group progressed slowly in recent decades, although it is widely recognized that numerous distinct *Geomyces* species have been lumped under broad concepts, e.g. Williams & Pugh (1974) and Domsch *et al.* (2007). In spite of this, *G. pannorum* and its synonym, *Chrysosporium pannorum*, are utilized commonly and frequently in the literature for almost any isolate belonging to this group, e.g. Hayes (2012), contributing to the confusion and miscommunication about *Geomyces* species and allies.

An avalanche of new interest in *Geomyces* began, however, when white-nose syndrome (WNS) of bats emerged in North America in 2006. As of Apr. 2013, this condition has spread to 22 states in the USA and five Canadian provinces (http://www.whitenosesyndrome.org/resources/map); the mass mortality of bats associated with WNS has caused concern over the long term survival of bat species in North America (Blehert *et al.* 2009; Frick *et al.* 2010; Turner *et al.* 2011). WNS, named for the signs of fungal growth on bats, is associated with the recently described *Geomyces destructans* (Gargas *et al.* 2009), which has been demonstrated to be the causal agent of WNS (Lorch *et al.* 2011; Warnecke *et al.* 2012). Efforts have been made to document various physiological traits of *G. destructans* (Chaturvedi *et al.* 2010; Verant *et al.* 2012; Shelley *et al.* 2013) and its interaction with bats (Meteyer *et al.* 2009; Warnecke *et al.* 2013).

Interest in the distribution, ecology, and life cycle of *G. destructans* led to an examination of North American bat hibernacula with an existing internal transcribed spacer (ITS)-based diagnostic PCR test for *G. destructans* (Lorch *et al.* 2010), resulting in the discovery of a number of closely related but distinct *Geomyces* ITS sequences in soil (Lindner *et al.* 2011). Lorch *et al.* (2013a) performed a culture survey of fungi associated with bat hibernacula and determined that there were more *Geomyces* species in bat hibernacula of eastern North America than have been described in the genus to date; the cultures were used to generate additional molecular data and a new intergenic spacer (IGS)-based qPCR test for *G. destructans* that is both sensitive and accurate (Muller *et al.* 2013). Lorch *et al.* (2013a) also noted that there were about 17 named species of *Geomyces*, not including heterotypic synonyms, under a broadly defined generic concept based on their ITS phylogeny of *Geomyces* and allies under a one name per fungus system of classification (Norvell 2011; ICN, McNeill *et al.* 2012), though a critical reevaluation of the taxonomy of *Geomyces* was not available and an ITS-based phylogeny was not suitable for determining relationships.

Questions about *Geomyces* and allies addressed here include (1) which species are most closely related to *G. destructans* for comparison of genomic and physiological data? (2) how to communicate about the diversity of *Geomyces* and allies? and (3) what is the origin of *G. destructans* and the biogeography of the group in general? In order to address these issues, we obtained DNA sequence data for five loci to produce a robust phylogenetic and taxonomic overview of the genus and its allies. The loci included the ITS region, nuclear large subunit (LSU) rDNA, DNA replication licensing factor MCM7

Table 1 – Isolates used in *Geomyces* and allies phylogenetic analysis, their origin, substrate, and GenBank numbers. Bold indicates newly generated data.

Isolate	Species	Locality	Substrate/host	GenBank no.				
				ITS region	LSU	*MCM7*	*RPB2*	*TEF1*
CBS 108.14	*Geomyces auratus*	Norway	Soil, filter paper bait	**KF039895**	**KF017864**	**KF017690**	**KF017746**	**KF017805**
23WI05	*Geomyces* sp.	USA, Wisconsin	Hibernacular soil	JX270595	**KF017857**	**KF017683**	**KF017740**	**KF017798**
CBS 343.76/ AFTOL-ID 1397	*Leuconeurospora pulcherrima*	Switzerland	Forest soil	KF049206	FJ176884	na[a]	FJ238367	FJ238409
01NH01	*Leuconeurospora* sp.	USA, New Hampshire	Hibernacular soil	JX270336	**KF017814**	**KF017645**	**KF017699**	**KF017754**
01NH04	*Leuconeurospora* sp.	USA, New Hampshire	Hibernacular soil	JX270339	**KF017815**	**KF017646**	**KF017700**	**KF017755**
02NH04	*Leuconeurospora* sp.	USA, New Hampshire	Hibernacular soil	JX270349	**KF017817**	**KF017648**	**KF017702**	**KF017757**
15PA04	*Leuconeurospora* sp.	USA, Pennsylvania	Hibernacular soil	JX270479	**KF017841**	**KF017669**	**KF017725**	**KF017781**
CBS 329.36/ AFTOL-ID 1912	*Pseudeurotium zonatum*	France	Soil near gas leakage	AY129286	DQ470988	na	DQ470940	DQ471112
20631-21 (ex-type)	*Pseudogymnoascus destructans*	USA, New York	*Myotis lucifugus*	EU884921	**KF017865**	**KF017691**	**KF017747**	**KF017806**
01NH08	*Pseudogymnoascus* sp.	USA, New Hampshire	Hibernacular soil	JX270343	**KF017816**	**KF017647**	**KF017701**	**KF017756**
02NH05	*Pseudogymnoascus* sp.	USA, New Hampshire	Hibernacular soil	JX270350	**KF017818**	**KF017649**	**KF017703**	**KF017758**
02NH11	*Pseudogymnoascus* sp.	USA, New Hampshire	Hibernacular soil	JX270356	**KF017819**	**KF017650**	**KF017704**	**KF017759**
03VT05	*Pseudogymnoascus* sp.	USA, Vermont	Hibernacular soil	**KF039892**	**KF017820**	**KF017651**	**KF017705**	**KF017760**
04NY11	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270375	**KF017821**	**KF017652**	**KF017706**	**KF017761**
04NY16	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270377	**KF017822**	**KF017653**	**KF017707**	**KF017762**
04NY17A	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270378	**KF017823**	**KF017654**	**KF017708**	**KF017763**
05NY06	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270385	**KF017824**	**KF017655**	**KF017709**	**KF017764**
05NY08	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270387	**KF017825**	**KF017656**	**KF017710**	**KF017765**
05NY09	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270388	**KF017826**	**KF017657**	**KF017711**	**KF017766**
07MA02	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	JX270402	**KF017827**	**KF017658**	**KF017712**	**KF017767**
10NY08	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270432	**KF017829**	**KF017659**	**KF017714**	**KF017769**
10NY09	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270433	**KF017830**	**KF017660**	**KF017715**	**KF017770**
10NY10	*Pseudogymnoascus* sp.	USA, New York	Hibernacular soil	JX270434	**KF017831**	na	**KF017716**	**KF017771**
11MA03	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	JX270438	**KF017832**	**KF017661**	**KF017717**	**KF017772**
11MA05	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	JX270440	**KF017833**	**KF017662**	**KF017718**	**KF017773**
11MA07	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	JX270442	**KF017834**	**KF017663**	**KF017719**	**KF017774**
11MA08	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	JX270443	**KF017835**	**KF017664**	**KF017720**	**KF017775**
12NJ13	*Pseudogymnoascus* sp.	USA, New Jersey	Hibernacular soil	JX270459	**KF017838**	**KF017667**	**KF017722**	**KF017778**
14PA06	*Pseudogymnoascus* sp.	USA, Pennsylvania	Hibernacular soil	JX270469	**KF017839**	**KF017668**	**KF017723**	**KF017779**
15PA10B	*Pseudogymnoascus* sp.	USA, Pennsylvania	Hibernacular soil	**KF039894**	**KF017842**	**KF017670**	**KF017726**	**KF017782**
15PA11	*Pseudogymnoascus* sp.	USA, Pennsylvania	Hibernacular soil	JX270486	**KF017843**	**KF017671**	**KF017727**	**KF017783**
17WV03	*Pseudogymnoascus* sp.	USA, West Virginia	Hibernacular soil	JX270510	**KF017844**	**KF017672**	**KF017728**	**KF017784**
17WV06	*Pseudogymnoascus* sp.	USA, West Virginia	Hibernacular soil	JX270513	na	**KF017673**	**KF017729**	**KF017785**
18VA07	*Pseudogymnoascus* sp.	USA, Virginia	Hibernacular soil	JX270527	**KF017847**	**KF017675**	na	**KF017788**
18VA08	*Pseudogymnoascus* sp.	USA, Virginia	Hibernacular soil	JX270528	**KF017848**	**KF017676**	**KF017731**	**KF017789**
18VA12	*Pseudogymnoascus* sp.	USA, Virginia	Hibernacular soil	JX270532	**KF017849**	na	**KF017732**	**KF017790**
18VA13	*Pseudogymnoascus* sp.	USA, Virginia	Hibernacular soil	JX270533	**KF017850**	na	**KF017733**	**KF017791**
20KY08	*Pseudogymnoascus* sp.	USA, Kentucky	Hibernacular soil	JX270562	**KF017851**	**KF017677**	**KF017734**	**KF017792**
20KY10	*Pseudogymnoascus* sp.	USA, Kentucky	Hibernacular soil	JX270563	**KF017852**	**KF017678**	**KF017735**	**KF017793**

20KY12	*Pseudogymnoascus* sp.	USA, Kentucky	Hibernacular soil	JX270565	**KF017853**	**KF017679**	**KF017736**	**KF017794**
21IN01	*Pseudogymnoascus* sp.	USA, Indiana	Hibernacular soil	JX270568	**KF017854**	**KF017680**	**KF017737**	**KF017795**
21IN05	*Pseudogymnoascus* sp.	USA, Indiana	Hibernacular soil	JX270572	**KF017855**	**KF017681**	**KF017738**	**KF017796**
21IN10	*Pseudogymnoascus* sp.	USA, Indiana	Hibernacular soil	JX270577	**KF017856**	**KF017682**	**KF017739**	**KF017797**
22984-1-I1	*Pseudogymnoascus* sp.	USA, Tennessee	*Perimyotis subflavus*	JX415262	**KF017866**	**KF017692**	na	**KF017807**
23014-1-I6	*Pseudogymnoascus* sp.	USA, Tennessee	*Lasionycteris noctivagans*	JX512256	**KF017867**	**KF017693**	**KF017748**	**KF017808**
23342-1-I1	*Pseudogymnoascus* sp.	USA, Wisconsin	*Perimyotis subflavus*	JX415266	**KF017868**	**KF017694**	**KF017749**	**KF017809**
24MN04	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	JX270612	**KF017859**	**KF017685**	**KF017741**	**KF017800**
24MN06	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	JX270614	**KF017860**	**KF017686**	**KF017742**	**KF017801**
24MN13	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	JX270621	**KF017861**	**KF017687**	**KF017743**	**KF017802**
24MN14	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	JX270622	**KF017862**	**KF017688**	**KF017744**	**KF017803**
24MN18	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	JX270626	**KF017863**	**KF017689**	**KF017745**	**KF017804**
A07MA10	*Pseudogymnoascus* sp.	USA, Massachusetts	Hibernacular soil	**KF039893**	**KF017828**	na	**KF017713**	**KF017768**
MN-Mycosel-7	*Pseudogymnoascus* sp.	USA, Minnesota	Hibernacular soil	**KF039899**	**KF017872**	**KF017698**	**KF017753**	**KF017813**
RMF 7792	*Pseudogymnoascus* sp.	USA, Wyoming	Periglacial soil	**KF039898**	**KF017871**	**KF017697**	**KF017752**	**KF017812**
RMF C 101	*Pseudogymnoascus* sp.	USA, Utah	Soil from desert grasslands	**KF039896**	**KF017869**	**KF017695**	**KF017750**	**KF017810**
WSF 3629	*Pseudogymnoascus* sp.	USA, Wisconsin	Amorphus peat	**KF039897**	**KF017870**	**KF017696**	**KF017751**	**KF017811**
12NJ08	Undetermined	USA, New Jersey	Hibernacular soil	JX270454	**KF017836**	**KF017665**	na	**KF017776**
12NJ10	Undetermined	USA, New Jersey	Hibernacular soil	JX270456	**KF017837**	**KF017666**	**KF017721**	**KF017777**
15PA02	Undetermined	USA, Pennsylvania	Hibernacular soil	JX270477	**KF017840**	na	**KF017724**	**KF017780**
17WV02	Undetermined	USA, West Virginia	Hibernacular soil	JX270509	**KF017845**	na	**KF017730**	**KF017786**
17WV09	Undetermined	USA, West Virginia	Hibernacular soil	JX270515	**KF017846**	**KF017674**	na	**KF017787**
23WI08	Undetermined	USA, Wisconsin	Hibernacular soil	JX270598	**KF017858**	na[b]	na	**KF017799**
23WI14	Undetermined	USA, Wisconsin	Hibernacular soil	JX270604	na	**KF017684**	na	na

a na = not available.
b 23WI14 *MCM7* used for 23WI08.

(*MCM7*), RNA Polymerase II second largest subunit (*RPB2*), and translation elongation factor EF-1α (*TEF1*).

Materials and methods

Taxon sampling (Table 1) was based on a selection of *Geomyces* and allies cultures obtained by Lorch *et al.* (2013a) from bat hibernacula in eastern North America. We included at least one '*Geomyces*' and '*Pseudeurotium*' isolate of each ITS lineage from that study and only one isolate apparently representing the same lineage from the same location was included for most lineages. A culture representing the type species of *Geomyces*, *Geomyces auratus*, and the ex-type of *Geomyces destructans* were included along with a number of other isolates obtained from Martha Christensen's published (Christensen & Whittingham 1965; States & Christensen 2001) and unpublished studies and isolates recovered, especially from bats, by National Wildlife Health Center associated-personnel (Lorch *et al.* 2013a; Muller *et al.* 2013). GenBank data from AFTOL isolates of *Leuconeurospora pulcherrima* and *Pseudeurotium zonatum*, representing the types of each genus, were also included on the basis of earlier phylogenetic analyses (Sogonov *et al.* 2005; Wang *et al.* 2006a,b; Lorch *et al.* 2013a) to serve as outgroup taxa and aid in the determination of basal lineages. All cultures are preserved in the culture collection of the Center for Forest Mycology Research (CFMR).

Genomic DNA from each isolate was obtained from fungal cultures grown in plastic test tubes with Difco™ Bacto® Potato Dextrose Broth (PDB) or in plastic Petri dishes with Difco™ Oatmeal Agar (OA) according to the methods of Lindner & Banik (2009). PCR through DNA sequencing, and sequence editing, etc., of ITS region DNA sequences followed Lorch *et al.* (2013a) using ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) primers with the PCR program modified as follows: denaturing 40 s, annealing at 53 °C for 40 s, and extension for 90 s in the 30 PCR cycles with a final extension of 5 min. The LSU was sequenced identically with the LROR (Moncalvo *et al.* 2000) and LR7 (Vilgalys & Hester 1990) primers for initial PCR and LROR, LR5 (Vilgalys & Hester 1990), and LR7 primers for sequencing. *MCM7* was amplified and sequenced with the *Mcm7*-709for/*Mcm7*-1348 primer combination (Schmitt *et al.* 2009) except with all inosine (I) bases substituted with N, and *TEF1* was with the 983F/2218R primer combination (Rehner & Buckley 2005). PCR conditions consisted of 2 min initial denaturation at 94 °C, 47 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min and 40 s, and a final extension at 72 °C for 10 min. *RPB2* was amplified and sequenced with the fRPB2-7cF (Liu *et al.* 1999)/RPB2-3053bR (Reeb *et al.* 2004) primers; PCR conditions differed from *MCM7* and *TEF1* in 5 min initial denaturation, annealing at 50 °C for 2 min and extension for 130 s. For all three protein coding loci, the concentration of each primer was doubled and 33 % more *Taq* polymerase was used than in the ITS and LSU PCR reactions. Otherwise, all methods were identical to those of Lorch *et al.* (2013a) for these loci.

DNA sequences of each of the five loci were aligned individually using the default settings of the online version (http://mafft.cbrc.jp/alignment/server/) of MAFFT version 7 (Katoh & Standley 2013). Further adjustments and concatenation were performed with Rambaut's Se-Al v2.0a9, and all homologous gaps including areas with introns and other inserts were deleted from the final concatenated alignment. Positions with missing data were included. Only a portion of the ITS region that includes the entire 5.8 S (approx. 49 % of portion) and immediately surrounding areas of the ITS1 and ITS2 was included in the final alignment due to the alignment difficulties with this region in *Geomyces* and allies. All new sequences (Table 1) were deposited into GenBank, and the final alignment was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S14417).

Conflicts among the partitions of the five loci, ITS region, LSU, *MCM7*, *RPB2*, and *TEF1*, were determined by several methods. An incongruence length difference test (ILD; Farris *et al.* 1994) was executed as implemented in PAUP* (Swofford 2003) with the partition homogeneity test. Taxa lacking any data for one of the five partitions and uninformative characters were excluded. One thousand heuristic search homogeneity replicates were included, maxtrees was set and limited to 1000 with 100 random taxon-addition replicates and ten trees held at each step; and all other parameters were left at the default settings. A $P \leq 0.05$ was used as the level of significance in comparisons. Additionally, maximum parsimony (MP) analyses were performed on individual partitions with taxa having any data for that partition, the complete final concatenated alignment with all taxa included, and on individual partitions and the final concatenated alignment with all taxa lacking any data for one of the five partitions excluded with PAUP* (Swofford 2003). MP searches had maxtrees set and limited to 1000 with 1000 random taxon-addition sequence replicates and ten trees held at each step, and other parameters left as default. Bootstrap (BS) analyses (Felsenstein 1985) with 1000 BS replicates with maxtrees set and limited to 1000 with 100 random taxon-addition sequences and ten trees held at each step were performed to address clade support. Partitions were evaluated for incongruence with 70 % BS support as the level necessary to indicate noteworthy conflict (Mason-Gamer & Kellogg 1996). Lastly, maximum likelihood (ML) analyses via RAxML (Stamatakis 2006; Stamatakis *et al.* 2008) of individual partitions with all taxa having any data for that partition and with taxa lacking any data for one of the five partitions excluded were conducted via the RAxML BlackBox (http://phylobench.vital-it.ch/raxml-bb/index.php) using default settings with gamma and ML boxes checked; the resulting trees with the BS values from 100 iterations were compared. An additional RAxML analysis using the same settings was also made of the final concatenated dataset with taxa lacking any data for one of the five partitions excluded.

Bayesian analysis of the final alignment was performed with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) via the Cipres Science Gateway (Miller *et al.* 2010). Analysis included two independent runs of two million generations with four chains each, a sampling frequency of 1000 generations, and stop early set to no, nst = 6 and rates = invgamma, partitioned by locus with the parameters statefreq, revmat, shape, and pinvar unlinked and allowed to vary across partitions, the first 25 % of samples and trees discarded as burnin, and all other parameters left as default. The trees from each independent run that were remaining after discarding the burnin were pooled and used to construct a 50 % majority rule consensus tree.

A ML analysis of the complete final alignment with all taxa included was conducted with RAxML (Stamatakis 2006; Stamatakis *et al*. 2008) as implemented with RAxML-HPC2 v. 7.4.4 via the Cipres Science Gateway (Miller *et al*. 2010). All parameters were left on their default settings except search for the best scoring ML tree and BS analysis were made in a single run, 1000 BS iterations were included, and GTRGAMMA was used for the bootstrapping phase and the final tree.

Results

The final alignment included five loci, 62 isolates, and 3649 characters (ITS region: 324, LSU: 1198, *MCM7*: 564, *RPB2*: 711, *TEF1*: 852) after the exclusion of all homologous gaps including introns and other inserts. Character and MP tree statistics for individual loci (taxa lacking any data for that locus excluded) and the concatenated final alignment (TreeBASE http://purl.org/phylo/treebase/phylows/study/TB2:S14417) from the dataset including all 62 isolates are summarized in Table 2. MP analysis of ITS region stopped early due to tree number. Data were available for at least four loci for all included taxa, but 14 taxa did not have any data for one locus (1 missing LSU, 8 *MCM7*, 5 *RPB2*); further work may benefit from development of internal primers as well as optimized PCR primers and conditions as explored by Rehner *et al*. (2011) for *Beauveria*. Thus, the reduced dataset used to examine incongruence contained 48 taxa. The ILD test indicated significant incongruence, $P = 0.001$, but the relevance of this test has been questioned due to a high type I error rate (Barker & Lutzoni 2002). Comparison of the MP analyses and BS support values for each individual locus of the reduced dataset revealed minor incongruences with none being strongly supported. This included relationships among long branched basal lineages, especially in the LSU phylogeny, and the differential placement of members of clade C and 20KY10 (Fig 1) in the large well-supported clade consisting of clades A, B, and C in the protein coding phylogenies with this larger clade being poorly resolved in general relative to the mostly unresolved backbone in the *TEF1* phylogeny. This suggests that homoplasy in datasets with few informative characters and insufficient sampling might be associated with at least a large portion of the detected incongruence; though it is also possible that individual loci are not tracking the species phylogeny, possibly in relation to an explosive and relatively recent radiation of crown group diversity and incomplete lineage sorting. Comparison of MP and RAxML analyses of individual loci and the concatenated alignment in all of the various treatments with and without the 14 taxa that lack any data for one locus excluded did not indicate any conflicting or surprising phylogenetic placements of these 14 taxa; missing data and incomplete taxa can be safely included in Bayesian analyses without affecting phylogenetic accuracy (Wiens & Moen 2008). Hence, we performed phylogenetic analyses on the complete concatenated final alignment in spite of potential risks associated with accuracy and incongruence, especially since analyses of different combinations and numbers of loci converged upon and supported the same major conclusions (analyses not shown). The 50 % majority rule consensus tree of the combined results of the two independent Bayesian runs is presented in Fig 1 with posterior probabilities (PPs). The presence of homologous gaps of various types including introns and other inserts is shown as these are informative for some groups. No conflicts were observed among the results of the Bayesian, MP, and RAxML analyses of the complete final alignment. The RAxML tree with BS values from 1000 iterations and one of six equally parsimonious trees with BS values from 1000 replicates resulting from the analysis of the complete final alignment are included in the supplemental material.

Phylogenetic diversity and relationships of Geomyces and allies

Nineteen distinct clades/lineages were revealed by the phylogenetic analysis, a number of these include multiple species, and named species were placed in seven of the clades/lineages, suggesting a large amount of undescribed diversity. The genera *Pseudeurotium*, represented by *Pseudeurotium zonatum*, and *Leuconeurospora*, represented by *Leuconeurospora pulcherrima* and other isolates (PP = 1), are supported as two distinct taxa basal to the remaining

Table 2 – Character, MP tree statistics for ITS region, LSU, *MCM7*, *RPB2*, *TEF1*, and combined concatenated for datasets of 62 taxa.[a]

Locus	No. characters	Number PIC[b]	PIC/bp[c]	MPTs[d]			
				Number	Length	CI[e]	RI[f]
ITS region	324	39	0.12	1000	106	0.585	0.849
LSU	1198	40	0.03	696	77	0.649	0.907
MCM7	564	232	0.41	758	858	0.438	0.759
RPB2	711	206	0.29	40	843	0.406	0.733
TEF1	852	148	0.17	729	500	0.48	0.779
Combined	3649	665	0.18	6	2481	0.431	0.75

a Taxa lacking any data for an individual locus were excluded from its analysis, combined included all 62 taxa.
b PIC, parsimony-informative base pairs.
c PIC/bp, parsimony-informative base pairs/total base pairs.
d MPTs, most parsimonious trees.
e CI, consistency index.
f RI, retention index.

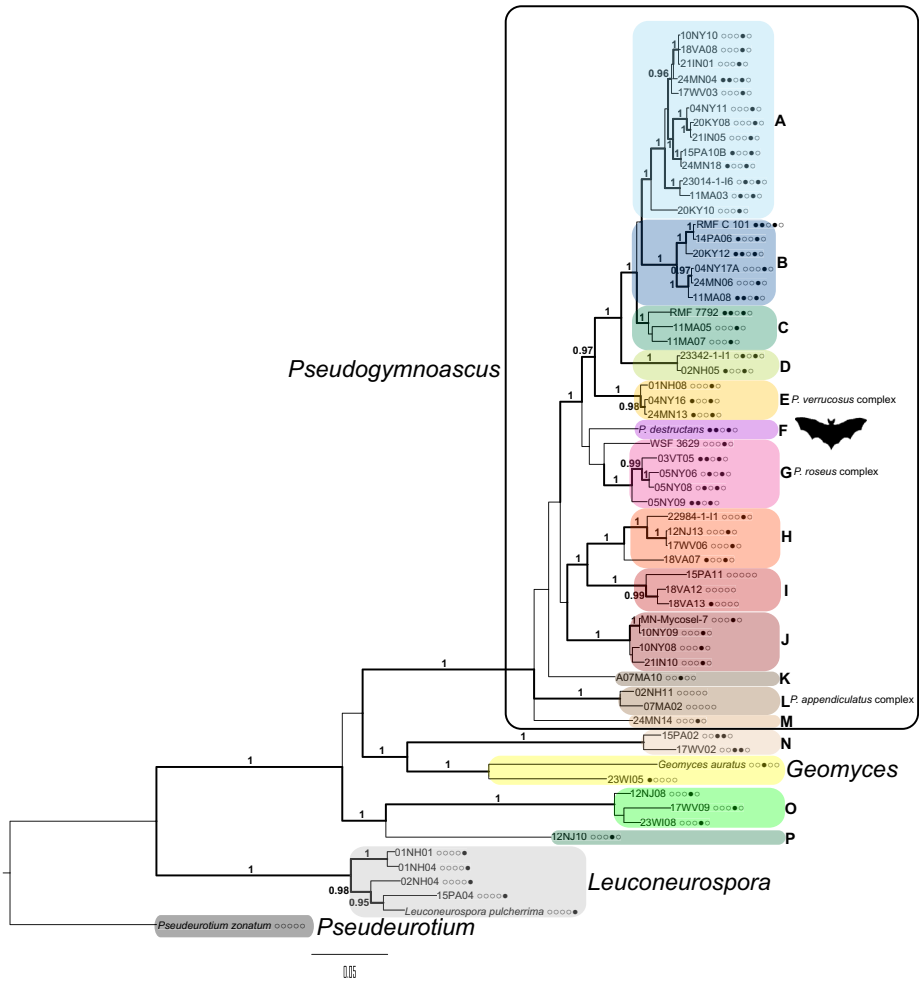

Fig 1 – 50 % majority rule consensus tree resulting from the Bayesian analysis of the complete final alignment of *Geomyces* and allies. Branches supported by PP values ≥ 0.95 are thickened and the value for the branch is presented. Closed and opened circles indicate the presence/absence of the ITS region intron, LSU intron, *TEF1* amino acid insert, *TEF1* intron, and *TEF1 Leuconeurospora* intron, respectively and in that order.

diversity (Fig 1). *Leuconeurospora* possesses an intron in the *TEF1* that is not shared by the other taxa. The remaining diversity consists of a clade (PP = 1) that includes three well-supported clades (PP = 1). The clade labeled *Pseudogymnoascus* contains most of the examined isolates as well as isolates that represent the type species of the genus (e.g. WSF 3629/*Pseudogymnoascus roseus* complex). A second clade is composed of *Geomyces*, represented by *Geomyces auratus* and 23WI05, and clade N. All in this clade except 23WI05 possess an extra amino acid in the same position in the *TEF1* protein; only A07MA10 in *Pseudogymnoascus* shares this trait at the same position, but the associated DNA sequence codes for tryptophan (TGG) in A07MA10 while that in the second clade codes for glycine (GGG). The third includes clades O and P, which may be sister to *Gymnostellatospora* based on Lorch *et al.* (2013a). An additional *TEF1* intron is shared by many, but not all of the members of these three clades. The members of the basal two clades (*Geomyces*, N, O, P) are genetically distinct from one another and *Pseudogymnoascus* and their relationships to one another are not fully resolved. *Pseudogymnoascus* (PP = 1) is composed of a number of clades/lineages of relatively closely related species. A well-supported crown group (PP = 1) includes clades/lineages A–G. Basal to the crown group are a number of clades/lineages (H–M) whose relationships are not fully resolved. Within *Pseudogymnoascus*, clade A (PP = 1) possesses a number of the most geographically widespread lineages. Both isolates in clade D (PP = 1) were observed to produce ascomata and ascospores in culture. Clades E (PP = 1) and G are the most closely related to *Pseudogymnoascus destructans*, the fungus associated with WNS. Clade E has proximity to *P. destructans* based on some loci, but overall it is rather distant. *Pseudogymnoascus destructans* and WSF 3629, which produces ascomata and ascospores typical of *P. roseus* and is associated with the remainder of the isolates (PP = 1) in clade G, both represent relatively isolated and long branched taxa. The two share a similar *TEF1*, but WSF 3629 is more closely related to the rest of clade G than *P. destructans* in spite of its many distinguishing genetic features. *Pseudogymnoascus destructans* and most members of the *P. roseus* complex possess an LSU intron, but this has limited phylogenetic value as it is present and scattered

among a number of unrelated members of the crown group of *Pseudogymnoascus*. Similarly, the ITS region intron possessed by many members of *Pseudogymnoascus* is scattered among distantly related members of *Pseudogymnoascus* and 23WI05.

Taxonomy and accepted taxa

Pseudeurotiaceae Malloch & Cain, Canad. J. Bot. **48**: 1815. 1970.
Type genus: *Pseudeurotium* J.F.H. Beyma.
Geomyces Traaen, Nytt Mag. Naturvidensk. **52**: 28. 1914.
Type species: *Geomyces auratus* Traaen; 'lectotype' by analogy (ICN Art. 10) designated by Sigler & Carmichael, Mycotaxon **4**: 376. 1976.

The genus *Geomyces* is distinct from other genera in *Pseudeurotiaceae* based on the phylogenetic placement of its type species in a distinct basal lineage, and the species described as *Geomyces destructans* is not congeneric with the type species of *Geomyces*.
Geomyces auratus Traaen, Nytt Mag. Naturvidensk. **52**: 30. 1914.
Gymnostellatospora Udagawa, Uchiy. & Kamiya, Mycotaxon **48**: 158. 1993.
Type species: *Gymnostellatospora japonica* Udagawa, Uchiy. & Kamiya.

According to Index Fungorum, five species of *Gymnostellatospora* are included in the genus, and Rice & Currah (2006) provided ITS region sequence data for them. Phylogenetic analysis presented by Lorch *et al.* (2013a) suggested a sister-relationship with clade O of this study. Our own neighbor-joining analysis of ITS region data (results not shown) with a reduced number of distant outgroup taxa than that of Lorch *et al.* (2013a) showed 72 % BS support for this relationship. We have no bona fide isolates of this genus at the time of this study; thus its final placement is unresolved. However, we expect the five species in *Gymnostellatospora* to remain members of a distinct lineage basal to *Pseudogymnoascus*.
Leuconeurospora Malloch & Cain, Canad. J. Bot. **48**: 1820. 1970.
Type species: *Leuconeurospora pulcherrima* (G. Winter) Malloch & Cain.

Lorch *et al.* (2013a) tentatively labeled a clade of unusual anamorphic fungi as '*Pseudeurotium*'. Analyses of additional data herein reveal that these represent asexual stages of *Leuconeurospora*, though we have not observed any sexual stages to date in our cultures. Based on GenBank BLAST searches of available ITS data, *Leuconeurospora* is a diverse and poorly documented genus, especially in colder regions, and frequently mislabeled as *Pseudeurotium*. In addition to the type species, Malloch (2013) recently added two species names, which brings the accepted total to three. The data presented by Malloch (2013) and co-authors of the two names, Hambleton and Sigler, are insufficient for identification of our isolates at this time. A large number of species in this genus remain to be isolated and described.
Leuconeurospora pulcherrima (G. Winter) Malloch & Cain, Canad. J. Bot. **48**: 1820. 1970 as '*pulcherrimum*'.
Pseudeurotium J.F.H. Beyma, Zentrabl. Bakteriol., 2 Abt. **96**: 415. 1937.
Type species: *Pseudeurotium zonatum* J.F.H. Beyma.
Synonym: *Teberdinia* Sogonov, W. Gams, Summerb. & Schroers, Mycologia **97**: 698. 2005.
Type species: *Teberdinia hygrophila* Sogonov, W. Gams, Summerb. & Schroers.
These two genera are synonyms based on teleomorphic (Beyma 1937) and anamorphic (Sogonov *et al.* 2005) types, respectively, since separate names for different stages of pleomorphic fungi are no longer allowed as they used to be under earlier Codes of nomenclature (Norvell 2011; ICN, McNeill *et al.* 2012). Sogonov *et al.* (2005) provided a recent treatment of species in this group with a substantial amount of molecular data for a number of them, and Lorch *et al.* (2013a) demonstrated the phylogenetic position of species with available molecular data as sister to the other '*Pseudeurotium*' clade recognized as *Leuconeurospora* herein and *Geomyces* in a broad sense. The following new combination is required under a one name per fungus system of classification:
Pseudeurotium hygrophilum (Sogonov, W. Gams, Summerb. & Schroers) Minnis & D.L. Lindner, **comb. nov**.
MycoBank No.: MB804766
Basionym: *Teberdinia hygrophila* Sogonov, W. Gams., Summerb. & Schroers, Mycologia **97**: 703. 2005.
Pseudeurotium zonatum J.F.H. Beyma, Zentrabl. Bakteriol., 2 Abt. **96**: 416. 1937.
Pseudogymnoascus Raillo, Centrabl. Bakteriol., 2 Abth. **78**: 520. 1929.
Type species: *Pseudogymnoascus vinaceus* Raillo; 'lectotype' by analogy (ICN Art. 10) designated by Kuehn, Mycologia **50**: 432. 1958.

Raillo (1929) did not designate a type for the genus and Kuehn (1958) was the first to designate one, *P. vinaceus*. Though *Pseudogymnoascus roseus* is widely considered to be the type, Samson (1972) designated *P. roseus* as the type of the genus at a later date than Kuehn (1958), and ICN Art. 10.5 is clear that Kuehn's designation must be followed as previously noted by Orr (1979) unless formal procedures are enacted to change the type species.
Pseudogymnoascus appendiculatus A.V. Rice & Currah, Mycologia **98**: 309. 2006.

The phylogenetic analysis of Lorch *et al.* (2013a) and our own examination of available ITS region sequence data place this species in clade L. We do not have additional data for a representative isolate than that provided by Rice & Currah (2006).
Pseudogymnoascus carnis (F.T. Brooks & Hansf.) Minnis & D.L. Lindner, **comb. nov.**
MycoBank No.: MB804768
Basionym: *Sporotrichum carnis* F.T. Brooks & Hansf., Trans. Brit. Mycol. Soc. **8**: 131. 1923.
Additional synonym: *Aleurisma carnis* (F.T. Brooks & Hansf.) Bisby, Trans. Brit. Mycol. Soc. 27: 111. 1945.

This species was found to be associated with meat spoilage (Brooks & Hansford 1923) and was considered taxonomically by Bisby (1945). The phylogenetic analysis of Lorch *et al.* (2013a) and our own examination of available ITS region sequence data place this species in clade L. We do not have additional data for the original isolate than that made available in GenBank (ITS: FJ545236) by the ATCC (American Type Culture Collection). It appears to be distinct from *P. appendiculatus* based on the ITS region sequences of this

latter species that were provided by Rice & Currah (2006), but more molecular data are needed from isolates in this complex. *Pseudogymnoascus carnis* has priority if the two taxa are later found to be synonyms.

Pseudogymnoascus destructans (Blehert & Gargas) Minnis & D.L. Lindner, **comb. nov.**

MycoBank No.: MB804767

Basionym: *Geomyces destructans* Blehert & Gargas, Mycotaxon **108**: 151. 2009.

Based on phylogenetic placement of this species in *Pseudogymnoascus* near the isolates/lineage representing the type species of *Pseudogymnoascus* (Clade G), this species does not belong in the same genus as true *Geomyces,* which is restricted to a distinct basal lineage based on the phylogenetic placement of its type species, *G. auratus*.

Pseudogymnoascus pannorum (Link) Minnis & D.L. Lindner, **comb. nov**.

MycoBank No.: MB804769

Basionym: *Sporotrichum pannorum* Link, Species Plantarum, 4th ed. **6**: 13. 1824.

Additional synonyms: *Chrysosporium pannorum* (Link) S. Hughes, Canad. J. Bot. **36**: 749. 1958.

Geomyces pannorum (Link) Sigler & J.W. Carmich., Mycotaxon **4**: 377. 1976 as '*pannorus*'.

Original material of *S. pannorum* housed in Berlin (B) was not available at the time of this study, but the original description by Link (1824) clearly indicates this is a rose-colored species found on rotting clothes. We suspect this is a member of clade G (*P. roseus* species complex), but more taxonomic work is needed.

Pseudogymnoascus roseus Raillo, Centrabl. Bakteriol., 2 Abth. **78**: 520. 1929.

Though *P. vinaceus* was designated as the type of *Pseudogymnoascus* by Kuehn (1958), *P. roseus* has priority as the correct name of this species if *P. roseus* and *P. vinaceus* are treated as synonyms since Samson (1972) was the first to effectively publish a choice (ICN Art. 11.5) when he treated them as synonyms. Given the diversity of clade G, a detailed taxonomic investigation is needed to sort out the identities and relationships of *G. vinaceus*, *P. roseus*, *P. vinaceus*, and probably *P. pannorum*. Lorch *et al.* (2013a) also showed the available ITS region sequence (GenBank DQ117444, Rice & Currah 2006) places the ex-type of *Geomyces asperulatus* Sigler & J.W. Carmich. (Sigler & Carmichael 1976) in clade G. Isolate WSF 3629 produces ascomata and ascospores typical of *P. roseus*, and its LSU sequence is identical to that of the 'neotype' culture (CBS 395.65 = IMI 114651, Samson 1972) that is available in GenBank (AB040690).

Pseudogymnoascus verrucosus A.V. Rice & Currah, Mycologia **98**: 311. 2006.

The phylogenetic analysis of Lorch *et al.* (2013a) and our examination of available ITS region sequence data place this species in clade E. We do not have additional data for the ex-type than that provided by Rice & Currah (2006).

Discussion

Geomyces, *Gymnostellatospora*, and *Pseudogymnoascus* have historically been considered to be members of the family *Myxotrichaceae* Locq. ex Currah (Currah 1985; Udagawa *et al.* 1993; Rice & Currah 2006). However, numerous phylogenetic studies indicate that *Myxotrichum* and these members of *Geomyces* and allies are not closely related (Sogonov *et al.* 2005; Wang *et al.* 2006a, 2006b; Lorch *et al.* 2013a). Malloch & Cain (1970) erected the family *Pseudeurotiaceae* to accommodate five genera of cleistothecial ascomycetes. Suh & Blackwell (1999) examined the phylogeny of these five genera and found three were distantly related, which left *Leuconeurospora* and *Pseudeurotium* as residual members of the family. Given the close relationships of *Geomyces* and allies, including *Geomyces*, *Gymnostellatospora*, *Leuconeurospora*, *Pseudeurotium*, and *Pseudogymnoascus* in the context of existing phylogenetic studies (Suh & Blackwell 1999; Sogonov *et al.* 2005; Rice & Currah 2006; Wang *et al.* 2006a,b; Lorch *et al.* 2013a; this study), they should be considered members of the *Pseudeurotiaceae*; we see no reason for an additional name at the familial rank at this time. Though, a case could be made for erecting *Pseudogymnoascaceae* based on ascomatal differences, e.g. cleistothecia in *Pseudeurotiaceae* and gymnothecia/open peridial networks in *Pseudogymnoascaceae*. Phylogenetic analyses by Schoch *et al.* (2009) support the placement of members of *Geomyces* and allies as sister to the order *Thelebolales* P.F. Cannon.

Geomyces as defined here based on the phylogenetic placement of the type species, *G. auratus*, is genetically distinct from *Pseudogymnoascus* and the two genera should not be considered to be congeneric. *Geomyces*, *Gymnostellatospora*, and a number of undetermined lineages basal to *Pseudogymnoascus* including clades N, O, and P are under sampled. The phylogeny presented here indicates *Pseudogymnoascus* is well supported phylogenetically and extremely diverse, with its type species seemingly affiliated with clade G, *Pseudogymnoascus roseus* complex. Further study of *Pseudogymnoascus* is needed to identify and name included species, a large number of which are undescribed. This naming should include existing species and many heterotypic synonyms (Carmichael 1962; van Oorschot 1980; Stalpers 1984; Domsch *et al.* 2007). The phylogenetic placement of *Glenosporella* also remains uncertain, but this genus associated with skin disease (Agostini 1931) would be a later synonym, priority 1931 according to Index Fungorum, of both *Geomyces* and *Pseudogymnoascus*. The diversity of *Geomyces* and allies represented here is only a fraction of their total diversity.

Significant interest on the topic of WNS relates to the origin of the pathogen in North America. A number of studies and accumulating evidence suggest that *Pseudogymnoascus destructans* is an introduced exotic species (Warnecke *et al.* 2012; Lorch *et al.* 2013b); this hypothesis is supported by the widespread occurrence of *P. destructans* in Europe without mass mortality of bats (Puechmaille *et al.* 2010; Martínková *et al.* 2010; Wibbelt *et al.* 2010; Puechmaille *et al.* 2011). Furthermore, the center of origin for biological groups is generally known to be where they display the most genetic diversity. Ren *et al.* (2012) provided evidence that *P. destructans* is genetically homogenous and clonal in North America. *Fusarium* isolates associated with soybean sudden death syndrome (SDS) were also shown to be clonal in North America (Achenbach *et al.* 1997) and Aoki *et al.* (2005) discovered and named several closely related species capable of causing this disease while noting their probable origin in South

America. WNS appears to be following a similar epidemical trajectory, and we hypothesize that if *P. destructans* were native, closely related species would occur in hibernacula of eastern North America. Our results suggest that there are no closely related sister taxa in hibernacula of eastern North America, which provides further support for the exotic invasive hypothesis. Additional screening of data from North American isolates shared by Porras-Alfarro, Barton, and Reynolds revealed a small number of lineages not addressed herein, but we have included most of the diversity that has been isolated to date from bats and their hibernacula and these other isolates were not more closely related to *P. destructans* than anything we studied. The general phylogenetic placement of *P. destructans* among a number of lineages with known sexual stages including clades D, E, and G suggests the possibility that *P. destructans* may produce a sexual stage under suitable conditions. We have not observed a sexual stage of *P. destructans* and have no knowledge of any reports of this at this time, but the potential of interbreeding isolates that could overcome host resistance that may be found in North American bat populations is of great concern if any further introductions of *P. destructans* occur. Vanderwolf *et al.* (2013a) recently reviewed the worldwide knowledge of fungi found in caves and Vanderwolf *et al.* (2013b) obtained a number of Canadian isolates of *Geomyces* and allies from bats prior to the emergence of WNS. The impact of *P. destructans* on the diversity of cave fungi in North America and its ability to persist over long periods of time remain unanswered questions. This study should prove to be useful in determining which taxa are most similar to *P. destructans* as ongoing studies address mechanisms of pathogenicity of and control methods for this deadly fungus. Future work will address the taxonomy of species in greater detail and the global diversity and biogeography of *Geomyces* and allies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2013.07.001.

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