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The Functional and Distributional Ecology of Mycetozoans under Changing Edaphic and Climatic Dynamics

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The Functional and Distributional Ecology of Mycetozoans under Changing Edaphic and Climatic Dynamics

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by

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ABSTRACT

Investigations into the distribution and ecosystem functions of fruiting amoebae revealed that local-scale environmental conditions can largely explain broad biogeographical patterns in species assemblage, the way in which amoeboid predators shape bacterial communities and how this top-down influence may affect global biogeochemical processes in a changing climate. The distribution and assemblage of protosteloid amoebae on the islands of New Zealand and Hawaii did not yield any expected patterns of island biogeography, and conformed to other global regions studied. The strongest predictor of species richness in a given region was sampling effort and these species do not appear to have any extant barriers to global dispersal. It is proposed that morphological adaptations such as tiny resilient spores contribute to their ability to disperse widely. In addition, the role of soil amoebae in stimulating the mineralization of soil nutrients was examined using a series of microcosm experiments. It was confirmed that amoeboid predators are causative for large increases in carbon and nitrogen mineralization but that the magnitude of this effect depends on complex interactions between climate and edaphic variables. In particular, land management practices such as no-till agriculture determine the nature of predator responses to climate change with regard to biogeochemical cycling. Subsequently, soil amoebae were shown to have a strong influence on the composition of bacterial communities. This influence was also dependent on climate factors. The predation-induced changes to bacterial taxa was different when incubation temperatures were increased, suggesting that even if protists are considered in models of nutrient dynamics, the parameters describing their influence on decomposer communities will depend on environmental factors. Future work should focus on testing hypotheses concerning the importance of morphology and anthropogenic vectors to amoebal dispersal and on further quantifying the interaction between a changing environment and predator-mediated control of bacterial communities for a wider range of predator taxa.

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Finally, though not inclusively, my thanks go out for the support given to me by the staff, colleagues, and friends I have made at the University of Arkansas. Their moral support and intellectual contributions are not directly quantifiable, but are very large.

DEDICATION

This dissertation is dedicated to the collected works of Pink Floyd and Claude Debussy, which were constant companions while I wrote, and to the one person who earned this more than any other, and without whom I never even would have begun… for Kelly, with love and squalor.

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Chapter 3. Zahn, G. L., Shadwick, J. D., Hemmes, D. E., &. Spiegel, F. W. Protosteloid amoebae as a flagship group for investigating the global distribution of naked amoebae. Submitted as a Short Communication to *The ISME Journal.*

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CHAPTER 1

INTRODUCTION

Introduction to mycetozoan ecology

Mycetozoans exist only in the periphery of most biologists' awareness, and those to whom the term has any familiarity will likely reflect only on the famous model organisms *Dictyostelium discoideum* and *Physarum polycephalum*. Yet to one who has gotten to know these organisms just a little bit, Mycetozoans represent a sublimely diverse group which are important and ubiquitous members of the microbial community in many habitats. They are categorized into three main groups: two monophyletic sister groups known as Myxomycetes and Dictyostelids, and a paraphyletic assemblage known as protosteloid amoebae (Shadwick *et al.*, 2009). First described in the mid-1800s, consensus on their phylogenetic affiliation has varied considerably (Baldauf and Doolittle, 1997), but now places these main groups unambiguously within the eukaryotic supergroup of Amoebozoa.

Mycetozoans' life cycle details vary considerably between taxa, but in general are characterized by an amoeboid or flagellated trophic stage followed by a dispersal stage in which spores are born on or inside a fruiting body. Some of these fruiting bodies can by very conspicuous (even beautiful) and often superficially resemble fungal morphologies, a fact that contributed greatly to the initial confusion surrounding their classification (Olive, 1975).

Mycetozoans can, in theory, be isolated from any habitat where decaying plant material is present (Rollins, 2008). They have been found in melting snowbanks (Ronikier and Ronikier, 2009), in freshwater ponds (Lindley *et al.*, 2007), and on the bark of trees (Schnittler, 2001). They occur in tropical, temperate, grassland and desert habitats, on remote islands, in dense urban centers and, of course, in soils where they often represent a full 25% of all protists in the community (Geisen *et al.*, 2015; Urich *et al.*, 2008).

Despite their ubiquity, abundance, and environmental diversity, the detailed study of their distributional and functional ecologies is still a young discipline. This is true to varying degrees between the three groups. Dictyostelid ecology has perhaps received the most attention, spurred by early work by Cavender and Raper (1965) and which has now accumulated into a large body of literature examining the genetic ecology of dictyostelid populations (eg. Fortunato *et al.*, 2003; Cavender, 2013; Landolt *et al.*, 2014). Work regarding the functional ecology of this group, however, has largely been limited to one species, the model organism *Dictyostelium discoideum* (Montagnes *et al.*, 2012). The ecology of myxomycetes has received less attention, though major efforts during the past two decades (eg. Liu *et al.*, 2015; Stephenson *et al.*, 2011; Stephenson and Feest, 2012; Stephenson, 2011) have begun to form a sharpening image of the distribution of this charismatic group. Lastly, and described most recently (Olive, 1967), the paraphyletic assemblage of protosteloid amoebae has received the least attention, likely due to their relatively inconspicuous fruiting bodies and lack of a current "model" member. Still, species in this diminutive group have been found in virtually every location and habitat where myxomycetes and dictyostelids have been observed (though they are less common in soils) and recent efforts have shown them to be globally ubiquitous (Chapter 2).

The greatest scarcity of information with all three of these groups concerns the environmental factors that influence their distributions (particularly at scales relevant to microbes) and the functional roles that they play in the systems where they are found. It has been shown that, at a broad-scale, precipitation patterns (Rollins *et al.*, 2010; Ogata *et al.*, 1996), latitude (Zahn *et al.*, 2014; Stephenson *et al.*, 2000; Perrigo *et al.*, 2012), and elevation (Landolt *et al.*, 2006; Rojas *et al.*, 2012) influence mycetozoan abundance, and that some taxa seem to show limited occurrence consistent with Foissner's "moderate endemism" hypothesis

(Stephenson *et al.*, 2007). It is yet to be accounted for, though, what specific mechanisms are the driving factors behind these patterns or whether they account for any effective functional differences in the overall makeup of the mycetozoan community.

Mycetozoans are largely bacterivorous predators and as such abundant members of the predatory protist community they undoubtedly play a significant role in shaping bacterial communities on a global scale. The specifics of these interactions with bacterial decomposers and the extent to which this interaction influences large-scale biogeochemical processes remains elusive. Thus, the focus of this dissertation is two-fold: 1) To increase our understanding of the global distribution of the least-studied mycetozoans (the protosteloid amoebae) and the broad factors that influence their local diversity and abundance, and 2) To investigate, mechanistically, the roles that mycetozoan predators play in shaping soil bacterial communities and the biogeochemical processes associated with soils.

Microbial distributions

There are two major competing hypotheses regarding the global distributions of microbes. The first is known as the Baas-Becking hypothesis: "Everything is everywhere but the environment selects," (EiE) (Baas-Becking, 1934; Finlay, 2002) and insists that the small size of microbes lends them to worldwide dispersal. It is suggested that the reason a given microbe does not occur in a given location is not due to lack of dispersal but to lack of a suitable habitat. The main alternative hypothesis is known as the "moderate endemism model" (ME) (Foissner, 2006) which claims that, for perhaps a full third of extant protist taxa, historical or morphological limitations act as barriers to dispersal, generating endemic groups.

For the EiE model to be accurate some necessary conditions must be met by the taxa in question: 1) High dispersal rates, 2) Small size, 3) Availability of appropriate dispersal vectors, 4) Morphological adaptations for resilience and dispersal such as spores or cysts, and 5)

Sufficient time to have achieved ubiquitous dispersal. It seems clear that many protist taxa fail to meet some or all of these criteria (those lacking spores or cysts, for example), and in fact, the literature shows that some protists do appear to exhibit endemism or patchy distributions (Smith and Wilkinson, 2007; Kooistra *et al.*, 2008). The key to this hypothesis, however, is more likely to be found in the second clause of the slogan, "…but the environment selects."

Proponents of the ME model often cite evidence from macro-organisms as evidence that small size and dispersal vectors do not equate ubiquitous dispersal. Ferns are a favorite example, as many fern species have very patchy distributions though they disperse via large numbers of tiny resilient spores and have been extant for hundreds of millions of years. Thus they seem to fulfill the requirements of the EiE model yet exhibit clear biogeographical patterns (Foissner, 2006). Of course, crucial to this example is the assertion by Foissner and other proponents of ME that suitable habitats exists for these widely dispersed propagules (Foissner, 2007). This claim is then extrapolated to protists and becomes something akin to: "Here is a suitable habitat for protist X, yet protist X does not occur here, therefore there must be a barrier to its dispersal." In light of how little we know about what actually constitutes a suitable habitat for any given species, this claim seems absurd and has been experimentally debunked, at least in the favorite fern example (Frahm, 2007).

This illustrative argument against the ME model is not intended to refute the hypothesis. Both models (ME and EiE) may turn out to be correct, just not for the same species (Caron, 2009). It is clear that some protist species do exhibit endemism or patchy distributions but it is still entirely plausible that the main reason for this observation is that "the environment selects." Perhaps it just selects in ways we currently do not understand. We know so little about so many protist taxa that we simply cannot assume what constitutes a "suitable habitat." This can be seen

as a problem of scale, particularly is some of the extremely complex environments where protists are abundant, such as soil.

Introduction to soil habitats, communities, and biogeochemistry

Soils are some of the most complex environments on earth with the most diverse biota (Tringe *et al.*, 2005) and most versatile biochemistry (White, 1995). They are extremely heterogeneous at scales from continents to micrometers, making quantitative extrapolations difficult, and most of our current knowledge about the microbes living in them comes only from environmental DNA. Soil complexity is difficult to overstate and an adequate treatise is beyond the scope of this work (for thorough reviews see Paul, 2006; Tan, 1998; Marshall *et al.*, 1996) but it is crucial that we work to understand it because three-fourths of Earth's terrestrial carbon (Whitman *et al.*, 1998) and a substantial portion of Earth's labile nitrogen (Söderlund and Svensson, 1976) are tied up in soils.

The fluxes of carbon and nitrogen into and out of soils are controlled largely by biotic processes such as microbial decomposition, but microbial processes are highly dependent on abiotic factors. These factors, such as water availability, temperature, cation exchange capacity, and physical structure are in turn, highly interdependent and thus present a difficult challenge to untangle. Still, accurate predictions of biogeochemical cycling in a changing global climate hinge on understanding the myriad interactions between the abiotic environment and the diverse biotic components of soils.

One important type of relationship that has received comparatively little attention is the interaction between the soil organisms themselves (Wardle, 2006). Until fairly recently soil systems have been treated as a "black box" where large-scale abiotic inputs and geochemical outputs were measured without regard to the mechanisms behind the observed trends (Tiedje *et*

al., 1999). For example, it has been noted that a linear increase in soil temperature leads to exponential increases in total respiration (Lloyd and Taylor, 1994).

This black box approach was taken out of necessity since an estimated 99% of microbial taxa are not amenable to traditional culture-based study (Pham and Kim, 2012) and though it has been useful in generating rough predictions, improvements in environmental molecular methods such as high-throughput nucleic acid sequencing are now enabling more detailed mechanistic research into the biotic processes at work (Whiteley *et al.*, 2006). Incorporating measures of microbial community structure and biochemical potential has already proven useful as it has led to improved predictive power in nutrient flux modeling efforts (Ali *et al.*, 2015). With growing concern over global climate change, accurate modeling of the fate of soil carbon and nitrogen is becoming more important, but this goal cannot be realized if mechanistic data about the organisms responsible for these processes are lacking. Examples of such data include quantifying the influences of temperature, precipitation variation, and management strategies on soil bacteria, fungi and protists, and the way in which these groups interact with each other under predicted climate scenarios.

Fortunately, there is a substantial body of work investigating the direct influences of environmental parameters on soil microbes, though most of these efforts have focused on bacterial and fungal members of the community (eg. Williams *et al.*, 1972; Hayden *et al.*, 2012; Cregger *et al.*, 2012; Evans and Wallenstein, 2014; Zogg *et al.*, 1997). Considerably less effort has been made to quantify the same effects on protists, though many research groups are currently attempting to eliminate this gap in our knowledge (eg. Tsyganov *et al.*, 2013; Stefan *et al.*, 2014; Domonell *et al.*, 2013). Additionally, there has been a small but steady interest in dissecting the relationships between bacterial and protistan taxa and in measuring the emergent

biogeochemical changes that result from these species interactions. It has been shown, for example, that bacterivorous protists significantly change bacterial community compositions (Rønn *et al.*, 2002), increase rates of organic carbon and nitrogen decomposition (Kuikman *et al.*, 1990), and stimulate plant growth (Bonkowski, 2004), but surprisingly little is known about the environmental factors that influence these interactions (Rosenberg *et al.*, 2009) or of the identity of bacterial groups that are affected by protist grazing (Murase *et al.*, 2006).

Driving questions behind this research

The motivations behind this dissertation work were driven by the aforementioned gaps in knowledge concerning the distributional and ecological function of protists. Working to fill those gaps in such an abundant and widely distributed group as myectozoans is necessary in order to better predict global-scale biogeochemical processes. The questions that drove this research were:

1. Do mycetozoan taxa exhibit biogeographical patterns despite traits that lend themselves to widespread dispersal, and if so, what factors might explain these patterns?
2. What specific taxonomic and functional changes do mycetozoans exert on soil bacterial communities?
3. How will climate change affect the influence of mycetozoan predators on carbon and nitrogen cycling in soils?
4. Do these community- or functional-level changes to bacterial communities explain any or all of the changes to nutrient dynamics?

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CHAPTER 2

ECOLOGICAL DISTRIBUTION OF PROTOSTELOID AMOEBAE IN NEW ZEALAND

Abstract

During the period of March 2004 to December 2007, samples of aerial litter (dead but still attached plant parts) and ground litter (dead plant material on the ground) were collected from 81 study sites representing a wide range of latitudes (34°S to 50°S) and a variety of different types of habitats throughout New Zealand (including Stewart Island and the Auckland Islands). The objective was to survey the assemblages of protosteloid amoebae present in this region of the world. Twenty-nine described species of protosteloid amoebae were recorded by making morphological identifications of protosteloid amoebae fruiting bodies on cultured substrates. Of the species observed, *Protostelium mycophaga* was by far the most abundant and was found in more than half of all samples. Most species were found in fewer than 10% of the samples collected. Seven abundant or common species were found to display significantly increased likelihood for detection in aerial litter or ground litter microhabitats. There was some evidence of a general correlation between environmental factors - annual precipitation, elevation, and distance from the equator (latitude) - and the abundance and richness of protosteloid amoebae. An increase in each of these three factors correlated with a decrease in both abundance and richness. This study provides a thorough survey of the protosteloid amoebae present in New Zealand and adds to a growing body of evidence which suggests several correlations between their broad distributional patterns and environmental factors.

Introduction

The term “protosteloid amoebae” refers to a paraphyletic assemblage of unicellular eukaryotes within the supergroup Amoebozoa that exhibit spore dispersal via sporocarpic fruiting (Figure 2.1). For most of their life cycle, protosteloid amoebae exist as single amoeboid cells that

may or may not possess flagella (Shadwick et al., 2009). These organisms are thought to be important consumers of bacteria and other microorganisms (Adl & Gupta, 2006). Although global inventories carried out thus far suggest that protosteloid amoebae occur in every type of terrestrial system (Ndiritu et al., 2009), very little is known about their ecology. The results obtained from previous studies (Moore et al., 2000; Spiegel & Stephenson, 2000; Stephenson et al., 2004) have provided some evidence that ecosystems located at higher latitudes support fewer species and show a decline in species abundance. Because of its location, size, and isolation, New Zealand provided an excellent opportunity to investigate these patterns.

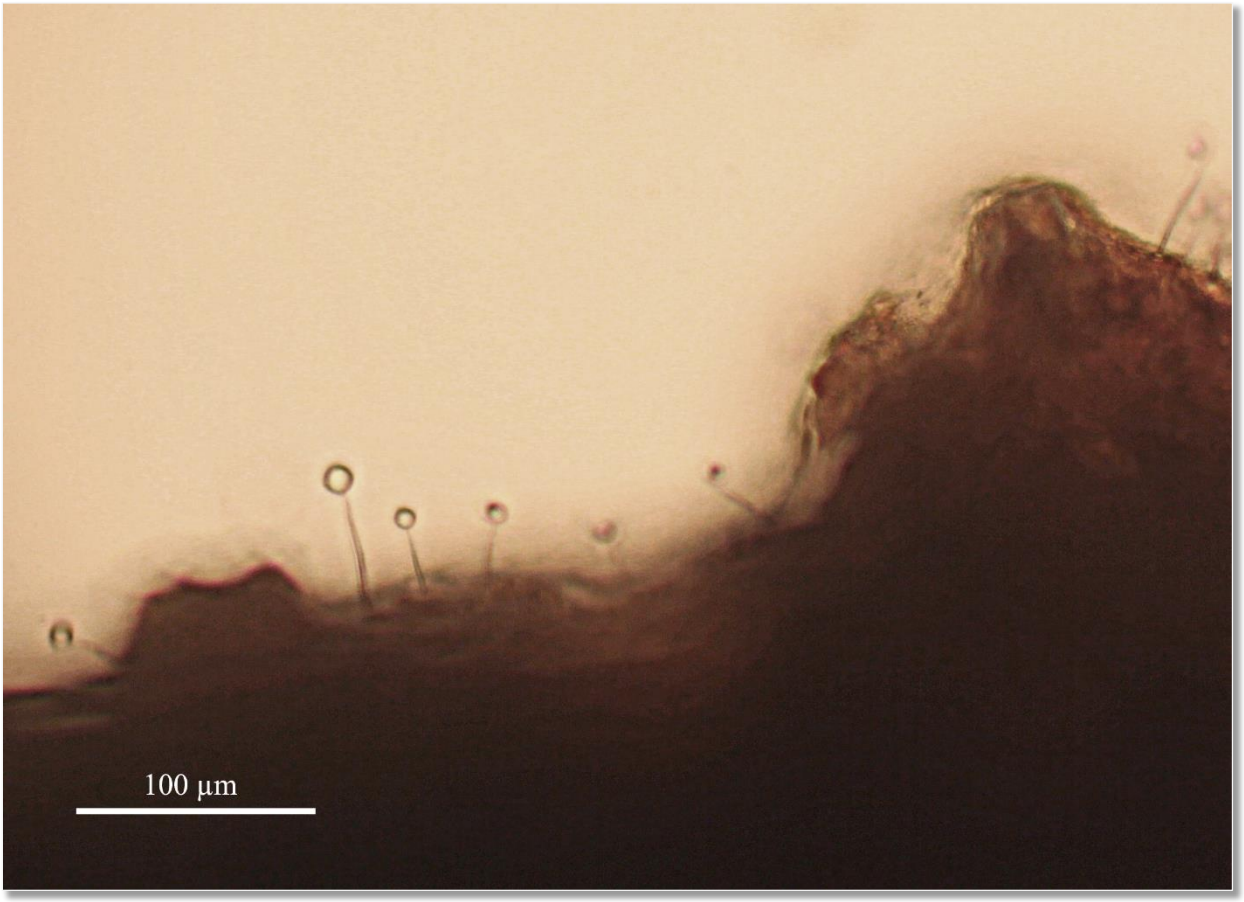

Figure 2.1. Fruiting bodies of protosteloid amoebae in situ. A cluster of sporocarps of the protosteloid amoeba *Tychsporium acutostipes* fruiting on a leaf. This image was taken at a total magnification of 100X. The scale bar is 100 µm. For high quality images of all species discussed in this paper, see Spiegel et al. (2007) online.

New Zealand is the most isolated land mass of its size in the world (Cavender et al., 2002) and represents a unique collection of ecosystems with highly endemic flora (Fleet, 1986). Protosteloid amoebae have been known from New Zealand (Olive & Stoianovitch, 1969), and is the location from which the type specimen of *Schizoplasmodium cavostelioides* was originally isolated (Olive, 1967). The primary focus of the present study was to exhaustively sample as much of this range as possible in order to characterize the ecological distribution of the protosteloid amoebae present.

Materials and Methods

During the period of March 2004 to December 2007, three separate collecting trips were made to 81 sites on the North Island (113,729 km^2), South Island (151,215 km^2) and the Auckland Islands (625 km^2) (Figure 2.2 and Table 2.1). Samples were obtained from Stewart Island (1,746 km^2) in 2006, but yielded no observations of protosteloid amoebae. Collectively, the study sites sampled represent a well-characterized and diverse array of habitats encompassing a variety of elevations (extending from 0 m to 1636 m), every major vegetation type found in New Zealand, and a rather wide range of latitudes, from 34.44° S to 50.85° S. A total of 247 samples of aerial litter and 234 samples of ground litter were taken collected from 81 different study sites. These samples were placed in small paper bags, air dried, and transported to the laboratory for processing. In order to achieve a broad coverage of many different types of dead plant material (substrates), sampling efforts did not include systematic replications of substrate types or habitats, but multiple samples from many habitats were collected. Ecosystem types ranged from beaches and open roadsides to tree fern forests and alpine tundra (see Table 2.1).

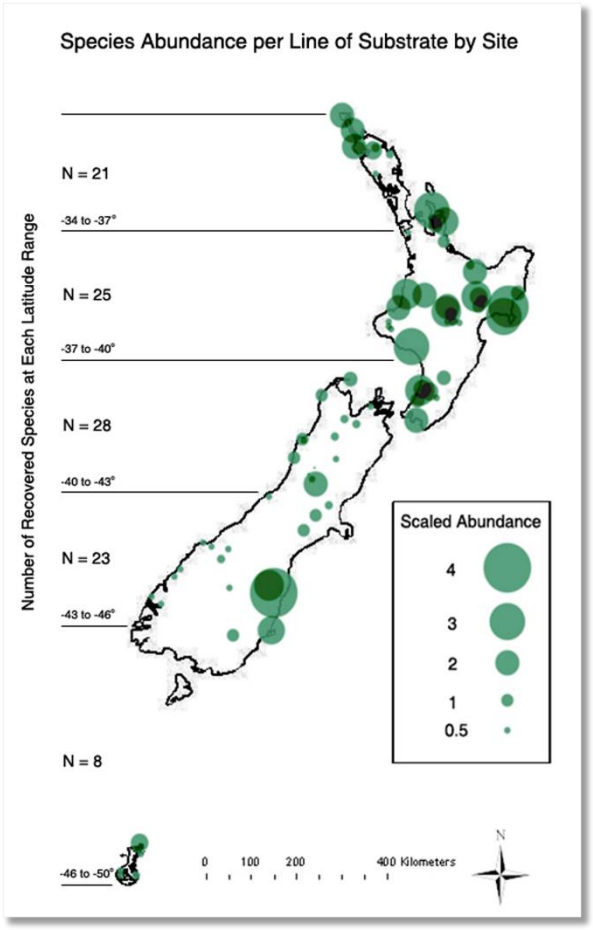

Figure 2.2. Map of sampling locations. Sample site markers are scaled to represent the mean number of protosteloid amoebae fruiting bodies encountered for each line of substrate observed from that site. N = species richness observed at each major latitudinal range

Table 2.1. Study site locations and information

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Tairoa Head Albatross Colony (263)	45°46'30.1000"S, 170°43'41.4998"E	67	Grassland	3/2004	218	10
West of Dunback (264)	45°19'13.3000"S, 170°34'34.2001"E	130	Grassland	3/2004	306	13
West of Morrisons (265)	45°13'16.1000"S, 170°25'24.3001"E	561	Scrub	3/2004	192	11
Boundry Creek Rest Area (266)	44°21'13.5000"S, 169°10'07.7002"E	277	Mixed Dry Forest	3/2004	194	7
Blue Pools (267)	44°09'00.8640"S, 169°16'00.6100"E	277	Beech	3/2004	160	1
Haast Pass (268)	45°06'00.4380"S, 169°21'00.2830"E	716	Beech	3/2004	188	1
South of Haast (269)	44°03'21.1000"S, 168°42'35.3999"E	716	Rainforest	3/2004	320	7
Jacksons Head (270)	43°57'52.6000"S, 168°36'19.4000"E	1	Podocarp/Beech	3/2004	320	11
Road to Hokitika (271)	42°59'00.0790"S, 170°40'00.7961"E	30	Rainforest	3/2004	162	5
Port Elizabeth (272)	42°22'00.5920"S, 171°14'00.3862"E	0	Beach	3/2004	156	18

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Punakaiki (273)	42°06'00.9560"S, 171°19'00.7741"E	0	Beach/Nileau	3/2004	336	9
Temple Basin Trail (274)	42°54'44.1000"S, 171°33'32.1001"E	876	Scrub	3/2004	160	7
The Chasin Trail (276)	42°55'09.3000"S, 171°33'30.4999"E	842	Beech	3/2004	162	1
U of Canterbury (277)	43°02'09.0000"S, 171°45'25.9999"E	561	Grassland	3/2004	168	6
Eastern Beech (278)	43°17'28.8000"S, 171°55'01.2000"E	493	Beech	3/2004	158	8
Sharplin Falls (279)	43°37'41.2000"S, 171°25'04.5998"E	463	Beech	3/2004	154	8
Peel Forest (280)	43°53'34.7000"S, 171°15'42.0001"E	289	Podocarp/Beech	3/2004	443	12
Te Anau (281)	45°26'38.0000"S, 167°41'03.0998"E	218	Beech	3/2004	229	3
Mirror Lake (282)	45°01'44.2000"S, 168°00'46.8000"E	350	Beech/Wetland	3/2004	239	2
Lake Gunn (283)	44°53'26.4000"S, 168°05'06.7999"E	485	Beech	3/2004	164	1

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Red Tussock Conservation Area (284)	45°33'38.0000"S, 168°02'07.4000"E	480	Native Grassland	3/2004	162	6
Taputaputa Bay (302)	34°26'13.7400"S, 172°42'48.4200"E	5	Teatree	5/2005	40	10
Pine Block Road (303)	34°44'57.7800"S, 173°01'05.8800"E	70	Pine	5/2005	52	12
Ahipara Gum Lands (305)	35°11'40.6800"S, 173°08'06.5400"E	178	Teatree	5/2005	40	9
Herekino Forest Tracks (306)	35°12'35.5200"S, 173°11'27.2400"E	154	Teatree	5/2005	40	10
Mangamuka Forest (304)	35°11'24.2400"S, 173°27'18.7801"E	379	Broadleaf	5/2005	30	10
Puketi Forest (307)	35°16'32.6400"S, 173°41'09.9600"E	16	Podocarp	5/2005	40	13
Harrison Scenic Reserve (308)	35°18'37.2600"S, 174°06'24.7799"E	79	Forest (Coastal)	5/2005	40	9
Trounson Kauri Park (309)	35°43'13.5000"S, 173°39'00.1199"E	234	Podocarp	5/2005	40	1
Mill Bay (310)	36°59'30.7800"S, 174°36'11.2201"E	17	Rainforest	5/2005	44	5

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Aratoro Scenic Reserve (359)	38°30'14.7420"S, 175°15'10.8000"E	129	Podocarp	12/2005	40	7
TongariroNP1 (360)	39°14'16.8540"S, 175°33'26.5680"E	1636	Scrub	12/2005	20	1
TongariroNP2 (361)	39°12'08.9820"S, 175°32'25.8720"E	1134	Beech	12/2005	40	6
DesertRoad (362)	39°18'59.4180"S, 175°43'49.7280"E	1015	Grassland	12/2005	40	2
TongariroNP3 (363)	39°10'10.6140"S, 175°31'26.5440"E	930	Flax/Scrub	12/2005	40	1
AraokiGorge (364)	38°40'16.8240"S, 174°41'40.1028"E	8	Tree Fern/Podocarp	12/2005	40	14
GorgePulloff (365)	38°53'45.9240"S, 174°35'56.4360"E	214	Tree Fern	12/2005	40	11
EgmontNp1 (366)	39°16'45.1560"S, 174°05'05.9280"E	1199	Scrub	12/2005	40	1
EgmontNP2 (367)	39°14'20.6880"S, 174°06'46.1160"E	941	Podocarp/Broadleaved	12/2005	40	2
EgmontNP3 (368)	39°18'28.4760"S, 174°05'50.2800"E	1159	scrub	12/2005	40	1

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Wanganui1 (369)	39°49'08.7600"S, 174°50'22.2360"E	120	Mixed Broadleaf	12/2005	60	13
Wanganui2 (370)	39°45'54.2160"S, 175°10'15.1680"E	24	Beech	12/2005	40	10
Manawata (371)	40°20'22.5600"S, 175°49'05.3760"E	76	Broadleaf	12/2005	40	9
Waihini (372)	40°59'46.1760"S, 175°23'22.8120"E	166	Podocarp/Broadleaved	12/2005	40	3
Rimutaka (373)	41°20'56.3280"S, 174°56'15.9000"E	70	Podocarp/Broadleaved	12/2005	40	6
Titahi (374)	41°05'58.8840"S, 174°50'06.5760"E	0	Scrub (Coastal)	12/2005	40	9
QEPark (375)	40°58'19.5600"S, 174°57'36.5400"E	0	Scrub (Coastal)	12/2005	40	15
Otaki (376)	40°51'14.2920"S, 175°14'06.6480"E	128	Secondary Growth	12/2005	40	11
Mahia (377)	39°04'18.0480"S, 177°48'39.4920"E	34	Scrub	12/2005	40	10
Bush (378)	38°52'34.1040"S, 177°51'20.4480"E	543	Secondary Growth	12/2005	40	14

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Okita (379)	38°39'53.5320"S, 178°10'49.4040"E	37	Mixed Broadleaf	12/2005	40	10
TeUruwera1 (380)	38°47'56.6880"S, 177°07'22.9440"E	607	Beech/Fern	12/2005	40	8
TeUruwera2 (381)	38°47'02.3280"S, 177°08'04.0200"E	609	Scrub	12/2005	40	14
TeUruwera3 (382)	38°43'43.8240"S, 177°05'11.0760"E	653	Beech/Podocarp	12/2005	40	11
TeUruwera4 (383)	38°39'51.3000"S, 177°02'13.3440"E	661	Beech	12/2005	40	6
HukaFalls (384)	38°38'57.3720"S, 176°05'20.6160"E	580	Broadleaf	12/2005	40	10
LakeTaupo (385)	38°44'41.7840"S, 176°04'07.5000"E	367	Grassland	12/2005	40	7
HinaKapu (386)	38°02'14.6400"S, 176°33'00.0000"E	350	Podocarp	12/2005	40	9
BayPlenty (387)	37°52'15.2400"S, 176°42'32.0400"E	2	Dunes	12/2005	40	4
Hiwy25 (388)	37°18'16.9920"S, 175°53'29.7600"E	65	broadleaf	12/2005	40	9

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
TwinKauri (389)	36°58'44.6520"S, 175°50'30.9120"E	117	Tree Fern/Kauri	12/2005	40	10
Maungataururu (390)	36°44'54.7440"S, 175°32'15.2520"E	370	Tree Fern/Nikau	12/2005	40	12
SquareKauri (391)	36°59'23.0640"S, 175°34'19.3080"E	306	Kauri/Broadleaved	12/2005	40	9
Hihi (392)	37°06'43.5600"S, 175°38'02.2920"E	59	Nikau/Broadleaved	12/2005	40	11
AUK06-1 (422)	50°50'20.6412"S, 165°55'15.2400"E	9	Forest (Coastal)	3/2006	4	2
AUK06-2 (423)	50°50'20.6412"S, 165°55'15.2400"E	9	Forest (Coastal)	3/2006	4	2
AUK06-4 (425)	50°51'11.0412"S, 165°55'12.9000"E	324	Forest (Coastal)	3/2006	4	1
AUK06-9 (430)	50°48'58.6188"S, 166°12'02.5200"E	20	Forest (Coastal)	3/2006	4	2
AUK06-16 (437)	50°32'43.8612"S, 166°12'45.7812"E	11	Forest (Coastal)	3/2006	4	1
AUK06-17 (438)	50°29'34.3788"S, 166°16'51.9600"E	35	Scrub (Coastal)	3/2006	4	3

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
AUK06-19 (440)	50°31'51.4812"S, 166°18'05.1588"E	6	Scrub (Coastal)	3/2006	4	1
AUK06-20 (441)	50°31'51.4812"S, 166°18'05.1588"E	6	Scrub (Coastal)	3/2006	4	1
Charming Creek (1188)	41°44'24.0000"S, 171°35'42.0000"E	3	Forest (Native)	5/2006	24	1
Truman Track (1187)	42°00'38.8800"S, 171°20'09.6000"E	0	Scrub (Coastal)	5/2006	20	2
Knight's Bush (1281)	45°54'44.1000"S, 169°29'42.5004"E	152	Beech/Broadleaved	5/2007	20	8
Route 6 Nelson (1282)	41°09'47.4984"S, 173°32'55.3992"E	84	Scrub	5/2007	20	1
Kowhai Point (1284)	41°42'44.2008"S, 173°06'46.2996"E	420	Scrub	5/2007	20	5
Lewis Pass (1286)	42°22'26.4000"S, 172°23'46.7988"E	914	Beech	5/2007	16	1
Route 63 (1287)	42°01'52.1004"S, 172°14'35.8008"E	479	Beech	5/2007	16	3
Kahurangi (1288)	41°41'07.5984"S, 172°26'37.1004"E	259	Beech/Broadleaved	5/2007	16	4

Table 2.1. (Cont.)

Site	Latitude/longitude	Elev. (m)	Habitat	Month/year collected	Lines plated	Site richness
Pigeon Saddle (1289)	40°49'57.2988"S, 172°58'08.5008"E	244	Tree Fern/Broadleaved	5/2007	32	6

Note. Table of study sites. Habitat types are generalizations. No significant correlations between habitat type and abundance were found, either generally or by species. At some sites dead vegetation suitable as a substrate was very limited and at others it was highly abundant. Thus, the number of lines plated at each site varies from 4 to 443.

In the laboratory, within 3 months of collection, samples were cut into small pieces, wetted with sterile water, and plated in lines on minimal nutrient agar (0.002 g malt extract, 0.002 g yeast extract, 0.75 g K_2HPO_4, 15.0 g Difco Bacto Agar, 1.0 L deionized [DI] H_2O) as described by Spiegel et al. (2004), yielding 6,533 lines of substrate that were examined in 1,175 plates. Lines of substrate consisted of approximately 2cm x 0.5cm wetted strips of dead plant matter gently pressed to the surface of the agar (see Figure 2.3). Daily observations were made for a minimum of seven days using bright-field microscopy with the 10X objective lens on a Zeiss Axioskop 2 microscope. Species were identified based on sporocarp morphology according to Olive (1967, 1970) and Spiegel et al. (2010). Observations of amoeboid and prespore stages were carried out to corroborate sporocarp identifications when necessary. This method provides a quick way to assess presence/absence of these amoebae since sporocarps are easy to detect and morphologically distinct from each other.

Species observations were recorded as presence or absence for each plated line of substrate and this resolution was used for comparisons between sites. Since sites were surveyed with varying numbers of lines of substrate, abundance and richness data were scaled by dividing by the total number of lines from a specific sample to represent abundance and richness per line of substrate observed. Precipitation data were extracted from the New Zealand National Climate Database (http://cliflo.niwa.co.nz/) and consisted of absolute precipitation amounts from the nearest weather station in the year samples were taken. A sample-based rarefaction curve (Figure 2.4) was generated using Ecosim 7 (Gotelli & Entsminger, 2009). Since data were not normally distributed, the individual effects of latitude, elevation, and precipitation gradients, and microhabitat (aerial vs. ground litter) on scaled species richness and abundance were tested with

the Kruskal-Wallis test, and R^2 values for linear correlations were calculated using the Pearson correlation statistic in Minitab® Statistical Software version 16.

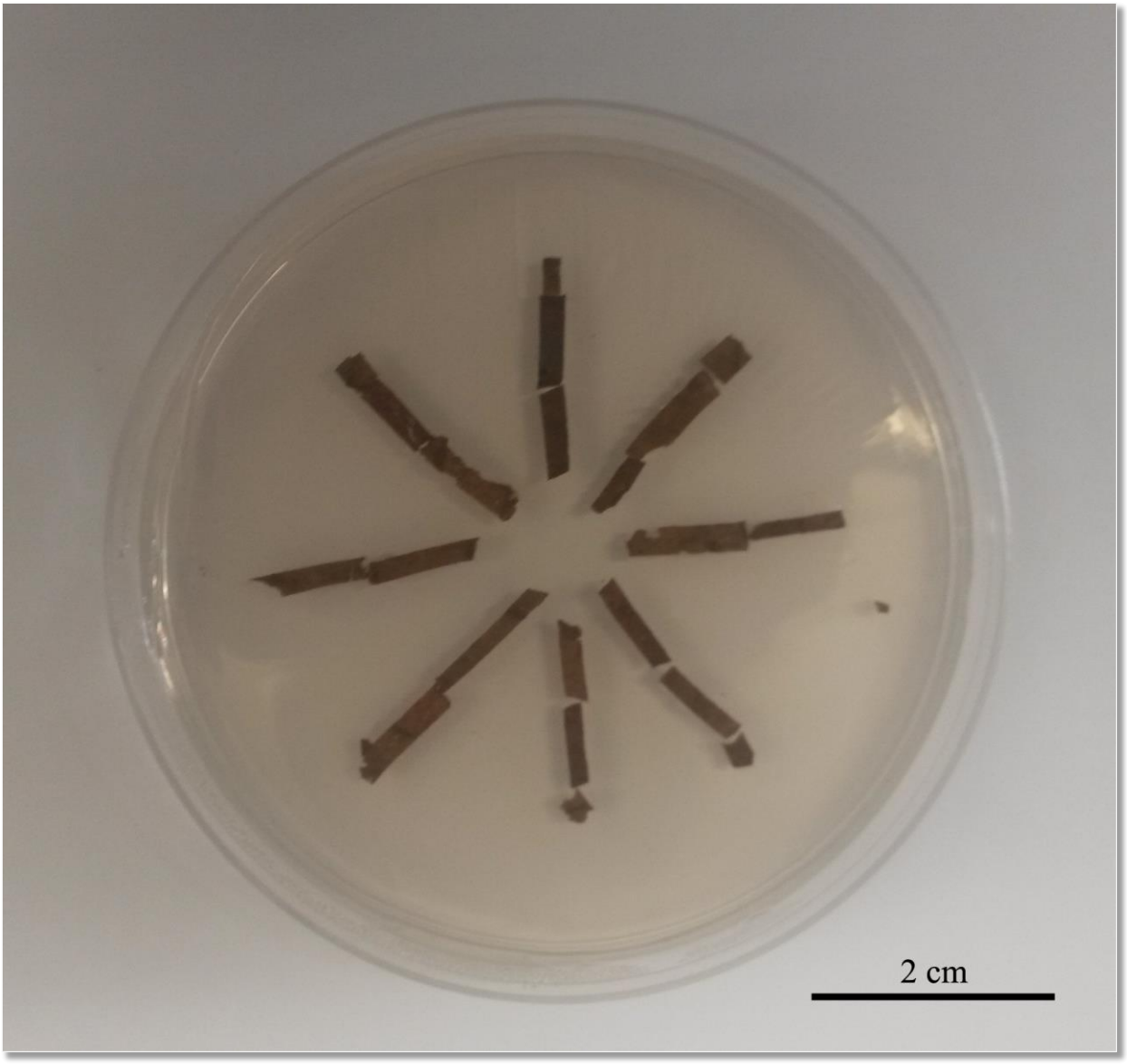

Figure 2.3. Primary isolation plate for protosteloid amoebae. A primary isolation plate with 8 lines of substrate arranged in a circle. Each line of substrate is labeled and observations of protosteloid amoebae are labeled according to which line they occurred on.

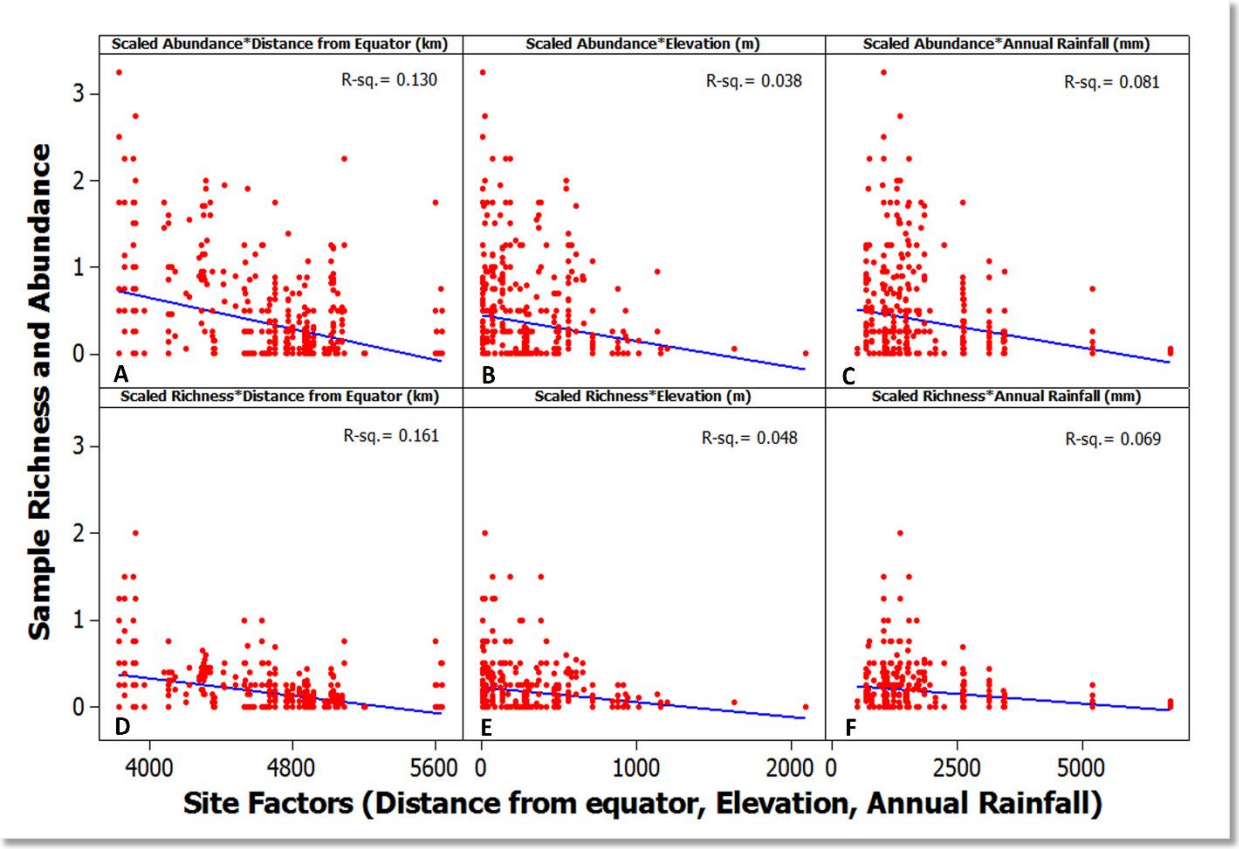

Figure 2.4. Rarefaction curve of species richness and sampling effort. Sampling effort appears sufficient to uncover the diversity of protosteloid amoebae. An increase in random sub-sampling from 200 to 300 collections only yielded an additional 2 species.

Results

Twenty-nine species of protosteloid amoebae, including the minuscule myxomycete *Echinostelium bisporum*, were recovered in the present study. The sample-based rarefaction curve (Figure 2.4) reached a clear asymptote at this species richness. While not traditionally grouped together with the now defunct "Protostelids" (Shadwick et al., 2009), the small fruiting bodies of *E. bisporum* display a protosteloid growth form and are commonly encountered using the current methods, so it has been included in this study. Species were grouped into abundance categories consistent with similar studies (Aguilar et al., 2011; Ndiritu et al., 2009) such that species recovered from: >10% of samples = abundant; 5-10% = common; 1-5% = occasional; <1% = rare. Seven species were found to be abundant across all study site locations while ten were considered commonly occurring (Table 2.2). *Protostelium mycophaga* was by far the most commonly encountered species, accounting for twenty-five percent of all fruiting body observations. Eighty out of eighty-one sites were positive for fruiting bodies of protosteloid amoebae (99%). The only site that did not yield any observations of protosteloid amoebae, located on Stewart Island, was left out of subsequent analyses.

Table 2.2. Observed species

Species name	Abbreviation	Total encounters	Frequency per sample	Category	Aerial encounters	Ground encounters
Protostelium mycophaga[a]**	Pm	598	2.06	A	398	200
Schizoplasmodiopsis pseudoendospora[b]*	Sps	323	1.20	A	119	204
Nematostelium gracile[a]*	Ng	239	1.05	A	83	156
Soliformovum irregularis[c]	Si	213	1.14	A	130	83
Schizoplasmodiopsis vulgare[a]***	Sv	197	0.95	A	40	157
Protostelium nocturnum[c]***	Pn	182	0.98	A	136	46
Schizoplasmodiopsis amoeboidea[d]	Sa	174	1.06	A	92	82
Protostelium arachisporum[b]	Pa	73	0.33	C	43	30
Protostelium pyriformis[a]	Ppyr	57	0.41	C	27	30
Schizoplasmodium cavostelioides[a]	Sc	51	0.28	C	38	13
Tychosporium acutostipes[e]	Ta	49	0.42	C	29	20
Cavostelium apophysatum[b]	Ca	43	0.25	C	15	28
Nematostelium ovatum[a]	No	41	0.31	C	14	27
Protostelium mycophaga[a] var. little***	lilPm	34	0.25	C	33	1

Table 2.2. (Cont.)

Species name	Abbreviation	Total encounters	Frequency per sample	Category	Aerial encounters	Ground encounters
Endostelium zonatum[f]	Ez	31	0.19	C	17	14
Echinosteliopsis oligospora[g]	Eo	28	0.20	C	14	14
Soliformovum expulsum[c]*	Se	27	0.30	C	21	6
Echinostelium bisporum[d]	Eb	16	0.16	O	7	9
Protosteliopsis fimicola[a]	Pf	12	0.12	O	7	5
Microglomus paxillus[a]	Mp	9	0.07	O	1	8
Clastostelium recurvatum[a]	Cr	8	0.09	O	3	5
Protostelium mycophaga[a] var. repeater	Pmrep	7	0.05	O	7	0
Schizoplasmodiopsis micropunctata[a]	Sm	5	0.05	O	5	0
Protostelium okumukumu[h]	Po	5	0.05	O	1	4
Schizoplasmodiopsis reticulata[a]	Sr	4	0.01	R	2	2
Ceratiomyxa hemisphaerica[a]	Ch	2	0.01	R	0	2
Protosporangium articulatum[a]	Partic	1	0.01	R	1	0
Protosporangium bisporum[a]	Pbisp	1	0.01	R	1	0
Schizoplasmodium obovatum[a]	So	1	0.01	R	0	1

Note. Total observed species from all sites. A: abundant, C: common, O: occasional, R: rare.

[a] Olive and Stoianovich

[b] Olive,

[c] Spiegel

[d] Olive and Whitney

[e] Spiegel, Moore, and Feldman

[f] Olive, Bennet, and Deasey

[g] Reinhardt and Olive

[h] Spiegel, Shadwick, and Hemmes

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; All tests: significant difference between aerial and ground litter abundance, Kruskal-Wallis test; Superscript numbers refer to naming authorities.

The number of samples varied at each site due to local conditions, such as a lack of suitable standing plant material, but of the 481 total samples, 299 of them yielded identifiable fruiting bodies of protosteloid amoebae (62%). These numbers are consistent with previous studies (Aguilar et al., 2011; Ndiritu et al., 2009; Stephensonet al., 1999). While no studies have previously examined the protosteloid amoebae of New Zealand, the methods we used for collection and observation in the previous surveys were very similar.

Microhabitat (aerial vs. ground litter) did not have a significant influence on either the abundance or species richness of fruiting amoebae as a whole (P=0.888, Kruskal-Wallis; P=0.746; Kruskal-Wallis, respectively), but several species displayed a significantly increased likelihood of being observed in a specific microhabitat. Of these, *Protostelium mycophaga*, *Protostelium nocturnum*, *Protostelium mycophaga var. little*, and *Soliformovum expulsum* were significantly more likely to be found on aerial litter, while *Schizoplasmodiopsis pseudoendospora*, *Nematostelium gracile*, *and Schizoplasmodiopsis vulgare* were more likely to be found on ground litter (Table 2.2). Microhabitat also made no difference to the significance of correlations between broader environmental factors (i.e. latitude, elevation, and annual precipitation) and community richness or abundance. Ecosystem type did not have any significant effect on richness or abundance, with most species displaying a cosmopolitan distribution among the different ecosystems. Species occurring in only one ecosystem type were uncommon or rare, thus it could not be determined whether these patterns were significant.

The most important factors related to protosteloid amoeba richness and abundance were elevation, precipitation and latitude (distance from the equator) (Table 2.3). Increases in all three factors led to perceived declines in protosteloid amoebae community measures though R^2 values for linear correlations were weak (Figure 2.5). The most abundant and diverse communities were

typically found in drier, more northerly locations close to sea level (See Figure 2.2 and Table 2.1).

Table 2.3. Statistical test values

Model	Test statistic (H)	P-value
Abundance × Distance from equator	341.38	<.0005
Abundance × Elevation	264.68	<.0005
Abundance × Precipitation	275.23	<.0005
Richness × Distance from equator	298.86	<.0005
Richness × Elevation	248.29	<.0005
Richness × Precipitation	259.39	<.0005

Note. Kruskal-Wallis test statistics and *P*-values for the influence of environmental factors on protosteloid abundance and richness. Model = Response × Factor. Abundance refers to scaled abundance per line of substrate. Richness refers to scaled richness per line of substrate. Test statistics are corrected for ties. All models showed significant effects of environmental gradients on scaled abundance and richness.

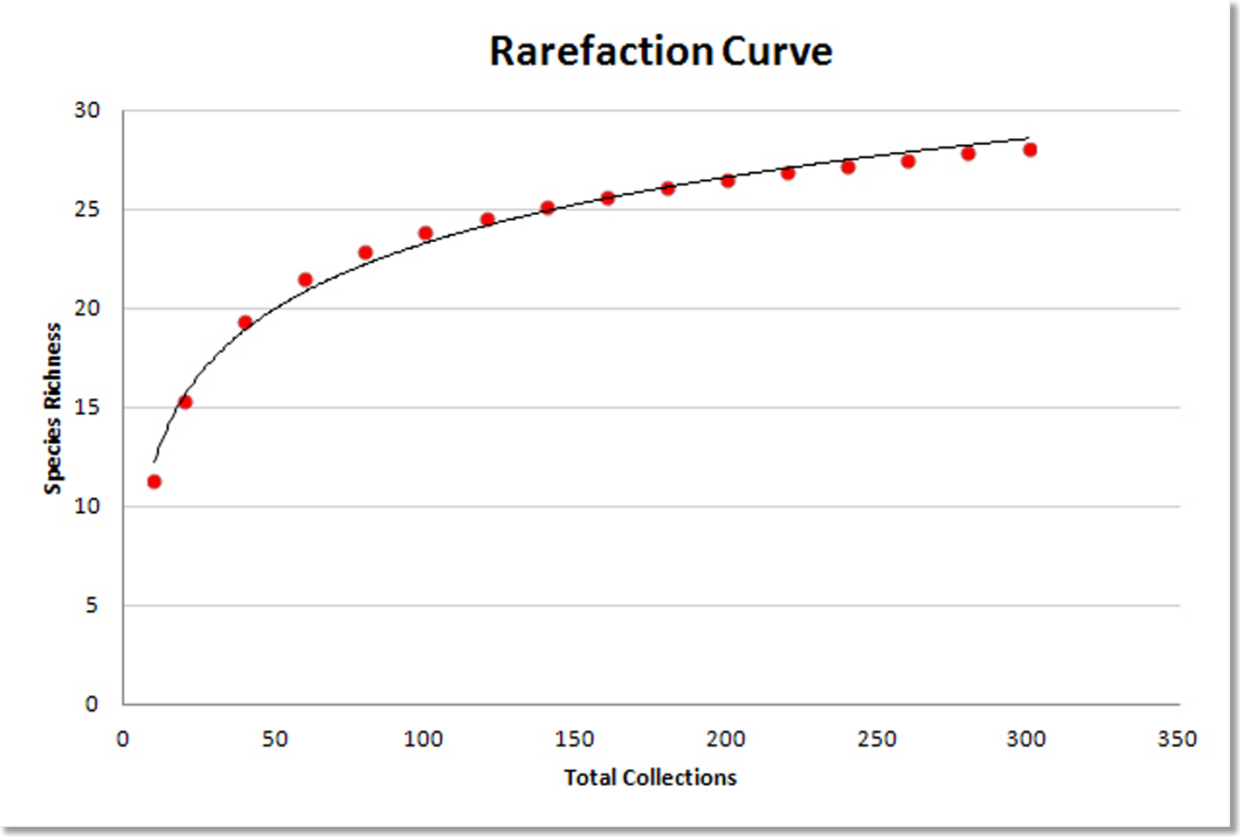

Figure 2.5. Species encounters along environmental gradients. (A–C): The scaled abundance (abundance per line of substrate observed) of protosteloid amoebae (all species). (D–F): The scaled species richness (richness per line of substrate observed). X-axis factors: Gradients of distance from equator (km, A and D), elevation (m above sea level, B and E), and annual rainfall (mm, C and F). R squared values for the linear regression are given in each panel.

Discussion

The main focus of this study was to provide a comprehensive survey of the protosteloid amoebae of New Zealand and to investigate the distribution of these species along gradients of precipitation, elevation, and latitude. A sample-based rarefaction curve (Figure 2.4 suggests that sampling effort was sufficient to recover the bulk of the known and described species richness present. Broadly, we were able demonstrate that the abundance and richness of protosteloid amoebae in New Zealand were correlated with latitude, elevation, and precipitation (Table 2.3). However, ecosystem type did not appear to influence these relationships. Moore et al. (2000) initially suggested that latitude may play a role in the presence/absence of protosteloid amoebae when only 6 species were recovered from 80 samples in the arctic tundra. Shadwick et al. (2009) had results more consistent with the present study, recovering 26 species from 205 samples in Great Smoky Mountains National Park, TN. In the current study microhabitat was a significant predictor of presence/absence for several species (Table 2.2), but the extent of this effect was far less than was reported by Aguilar et al. (2011) in which only 3 out of 18 species recovered from 100 samples did not display significant differences in presence/absence between microhabitats.

The sampling method varied somewhat between collecting trips. The first and last samples collected (sampling years 2004 and 2007, Table 2.1) were physically separated by substrate type (i.e. a separate bag for each species of litter collected), whereas the other samples were pooled together (i.e. all aerial litter in one bag and all ground litter in another bag). This change was made for convenience, since many study sites had limited amounts of litter present and it was difficult to find substrate species that yielded both aerial and ground litter of the same species in the same general area. Cursory analysis of the two sampling methods suggested that species observations were not affected by initial pooling of samples and thus sampling methods were treated as equal for all subsequent analyses. Briefly, data from the 2004 and 2007 samples

were artificially pooled within sites and randomly resampled to resemble what physically occurred in pooled sample collections. These resampled data were not significantly different from a random selection of the original unpooled data (P=0.420, Kruskal-Wallis test). The sampling protocol did not allow for further rigorous testing of this assumption, and this is beyond the scope of the present study. Additionally, the number of plated lines of substrate per study location varied from 4 to 443 as shown in Table 2.1. For most sites (68%), at least forty lines of substrate were plated for observation.

These heavily observed sites may display a bias toward an increase in the observations of rare species when compared with sampling locations such as the Auckland Island sites, in which only four lines of substrate were observed. Of the five rare species identified, two (*Ceratiomyxa hemisphaerica* and *Protosporangium bisporum*) were only found at the sample location from which 443 lines were plated (Peel Forest) and none were found at any locations from which less than 32 lines were plated. These rare species account for only nine distinct observations, and excluding them from further analyses had no impact on the significance of results, so they have been left in.

The effectiveness of various levels of observational effort for the detection of protosteloid amoebae was quantified by Aguilar *et al.* (2011) and it was found that four lines of substrate per sample was enough to detect 80% of species present, while eight lines per sample was able to yield 90% of the species present. Substantial increases in observational effort yielded only one or two additional rare species. In the present study, site richness was not significantly correlated with the number of plated lines per study location (R^2=0.033; P=0.103, Kruskal-Wallis test). Interestingly, six of the nine observations of rare species occurred at sites in which forty lines of substrate were plated, further suggesting that sampling efforts greater than that did little to

increase the effectiveness of ecological surveys for rare species of protosteloid amoebae. It is apparent that comparisons between abundant, common, and occasional species may be safely made using the current study's sampling and observation protocol.

This study took place over several years and samples were collected during different seasons. Though there is little evidence for true seasonality in protosteloid amoeba presence/absence (Spiegel, unpublished data) this must be considered when drawing conclusions from the present study. Moore and Spiegel (2000) showed that protosteloid amoebae spore dispersal was dramatically reduced in winter using artificial substrates, but on native *in situ* substrates, dormant stages of these amoebae persist throughout the year. Protosteloid amoebae are very tolerant of adverse conditions (drying out, etc.) and have been recovered from dried substrate at least as long as 12 years after collection (Zahn, unpublished data) so it is likely that seasonal changes in the *in situ* activity of the amoebae are not reflected in the current sampling protocol, which inherently encourages encysted or dormant amoebae to reactivate and fruit. Further, in the present study, North Island sites were sampled primarily in the early austral fall and South Island sites were sampled primarily in the late austral spring. Corresponding seasons in temperate North America are excellent times to sample for protosteloid amoebae. Still, seasonal changes to substrate quality, type, and abundance are likely to have an impact on the amoebae present and may affect our results.

Acknowledgements

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Appendix 2.1: Lead Author Confirmation Letter for Paper 1

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Chapter 2, titled "Ecological Distribution of Protosteloid Amoebae in New Zealand," of G. L. Zahn's dissertation was published in *PeerJ* in 2014 with coauthors S. L. Stephenson & F. W. Spiegel.

I, Dr. Frederick W. Spiegel, advisor of Geoffrey Lloyd Zahn, confirm Geoffrey Lloyd Zahn was first author and completed at least 51% of the work for this manuscript.

Dr. Frederick W. Spiegel
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CHAPTER 3

PROTOSTELOID AMOEBAE AS A FLAGSHIP GROUP FOR INVESTIGATING THE GLOBAL DISTRIBUTION OF NAKED AMOEBAE

Abstract

Protosteloid amoebae offer an excellent "flagship" group for investigating biogeography and dispersal within the naked amoebae. The historically isolated islands of Hawaii were extensively sampled over a period of eight years (the most intensive survey of protosteloid amoebae yet reported) but did not show any evidence of classical island biogeographical patterns. Here we present results from this survey and previously unreported global distributions to suggest that protosteloid amoebae do not have any extant barriers to dispersal. Their global occurrences are briefly discussed within the context of competing models of microbial distribution.

Body

The ongoing debate over the global distribution of microbes features two main paradigms: “everything is everywhere” (EiE), referring to cosmopolitan distributions of microbes selected only by local environmental variables (Fenchel and Finlay, 2004) and “moderate endemism” (ME), with the contrasting claim that many microbial species display patchy distributions even within suitable environments (Foissner, 2006). Much effort has been devoted to testing these models and it seems clear that some protist species do appear to have limited geographic ranges (Foissner and Hawksworth, 2009) though it remains unclear as to which factors (species age, availability of dispersal vectors, adaptations for dispersal, or availability of local habitats) are lacking in suitability to facilitate EiE distributions for these species. The use of “flagship” species that exhibit “conspicuous size, morphology, and/or colour” has been proposed as an effective way to test the EiE model in specific cases such as

testate amoebae (Foissner, 2006), but little attention has been given to distributions of non-testate (naked) amoebae, largely due to the difficulty associated with their accurate identification.

Protosteloid amoebae, formerly known as protostelids, are a paraphyletic assemblage of non-testate amoebae scattered widely across the Amoebozoa supergroup and are characterized by a shared ability to form distinctive fruiting bodies consisting of one or a few spores on an acellular stalk (Lora L. Shadwick *et al.*, 2009). They fit the qualifications of a "flagship" group since the fruiting bodies are conspicuous (from 10 to >100 µm), morphologically distinctive (Spiegel *et al.*, 2007), and have varied microhabitat requirements (Aguilar *et al.*, 2011). Additionally, nearly one third of the 31 described morphospecies exhibit ballistosporous dispersal and the most common species, Protostelium mycophaga, is known to readily and successfully disperse via airborne spores (Tesmer et al., 2005) in spite of claims by Foissner (2006) that adaptations for air dispersal were unknown in protists. Here, we present results from the most intensive local survey of protosteloid amoebae within the context of previously unreported global distributions (see the Appendix to this chapter) to suggest that no distributional barriers currently exist within this morphological grouping of non-testate amoebae.

Selected for their unique geologic isolation, the Hawaiian Islands were repeatedly sampled for protosteloid amoebae, over a period of 8 years, in order to look for classical patterns of island biogeography such as limited richness, endemism, and radiation. Sampling and observation methods were comparable to methods described in Zahn et al. (2014) but, briefly, they consisted of plating out collections of dead plant material from different microhabitats at each site onto weak nutrient agar dishes and microscopically observing fruiting bodies after 3-7 days of incubation. Basic site information collected included elevation, mean annual rainfall, and dominant vegetation.

When compared to other global observations, Hawaii showed no expected signs of island biogeographical patterns, emerging instead as the richest region yet studied. The six observed islands contained every described species with generally above-average abundance, and there was no correlation between island size and species richness (Figure 3.1). Several undescribed species were observed, but these have been recorded either previously or subsequently from other regions (data not shown). Sites dominated by alien (recently introduced) vegetation had greater richness (ANOVA, $P<0.001$) and relative abundance (ANOVA, $P=0.032$) of protosteloid amoebae than those dominated by native vegetation. This observation is consistent with the ME model prediction that human influences can be expected to play a key role in microbial distributions and it cannot be ruled out that protosteloid amoebae have been recently introduced to Hawaii, possibly transported with human-introduced vegetation.

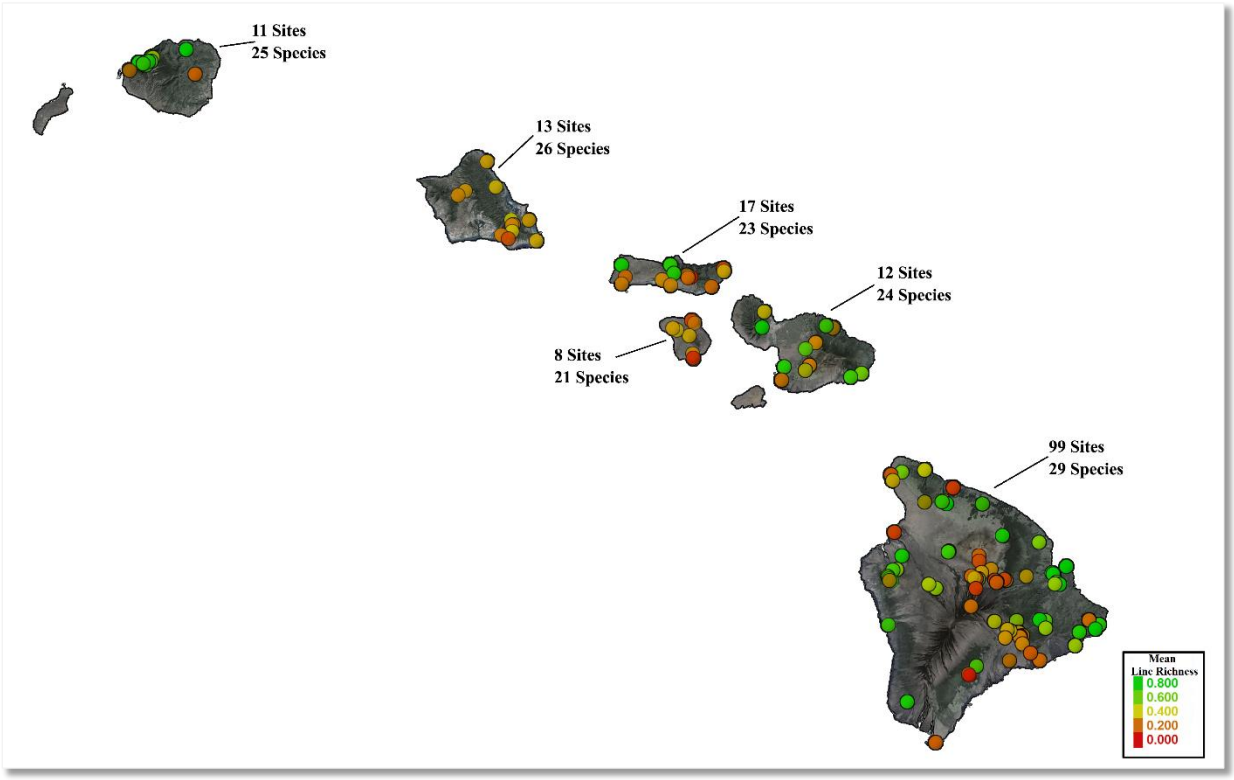

Figure 3.1. Map of study sites within the Hawaiian Islands. Site locations colored by mean species richness per line of substrate observed at the site.

The species assemblage in Hawaii was remarkably similar to the majority of regions surveyed globally (Table 3.1). It appears that the relative abundance classes (see Ndiritu *et al.*, 2009) of morphological species remain nearly the same regardless of geographic region, but site-specific microhabitat and environmental variables have a significant influence on species compositions in Hawaii and around the globe (Zahn *et al.*, 2014; Aguilar *et al.*, 2011; Ndiritu *et al.*, 2009; John D. L. Shadwick *et al.*, 2009). The strongest predictor of regional richness was sampling effort (Univariate linear regression on box-cox transformed data; $r^2=0.528$, $P<0.0005$) which seems to imply that regions with low observed richness simply need to be more intensively studied. It is obvious however, that similar sampling effort can yield very dissimilar richness from different regions (*eg*. Central United States vs. Patagonia, Table 3.1).

Table 3.1. Global protosteloid amoeba richness and species distributions

Global region	Sample		*Protostelium mycophaga*	*Schizoplasmodiopsis pseudoendospora*	*Schizoplasmodiopsis amoeboidea*	*Soliformovum irregularis*
	Effort	Richness				
Hawaii	11658	31	0.216	0.150	0.088	0.084
New Zealand	6251	27	0.176	0.065	0.046	0.064
Carribean	1908	24	0.496	0.339	0.240	0.151
Central USA	3387	27	0.422	0.103	0.059	0.164
Eastern Africa	2128	23	0.599	0.174	0.184	0.135
Kazakstan/Russia	468	26	0.114	0.195	0.059	0.131
Australia	1140	24	0.242	0.068	0.068	0.059
Northern Thailand	264	20	0.353	0.108	0.037	0.167
Western USA	920	21	0.255	0.119	0.197	0.110
Ukraine	204	18	0.424	0.068	0.136	0.295
NE Canada	260	16	0.578	0.029	0.025	0.211
China/Mongolia	1314	18	0.113	0.245	0.491	0.005
Ascension Island	200	14			0.029	
Northern Africa	120	13	0.171	0.198	0.028	0.036
Oman	344	12	0.071	0.136	0.087	0.016
Patagonia	4086	13	0.064	0.045	0.012	0.023
Bermuda	64	10	0.396	0.042	0.021	
Southern Mexico	428	10	0.213	0.238	0.038	0.080
France	64	7	0.056	0.250		
Germany	119	7	0.295		0.045	0.152
U.K./Norway	122	7	0.282		0.050	0.075
Antarctica	264	1				
Total	35713	31				

Table 3.1. (Cont.)

Global region	Sample		*Nematostelium gracile*	*Schizoplasmodiopsis vulgare*	*Cavostelium apophysatum*	*Echinostelium bisporum*
	Effort	Richness				
Hawaii	11658	31	0.089	0.027	0.051	0.016
New Zealand	6251	27	0.052	0.044	0.011	0.005
Carribean	1908	24	0.336	0.026	0.221	0.081
Central USA	3387	27	0.050	0.045	0.016	0.009
Eastern Africa	2128	23	0.081	0.009	0.040	0.141
Kazakstan/Russia	468	26	0.051	0.010	0.294	0.129
Australia	1140	24	0.038	0.021	0.023	0.013
Northern Thailand	264	20	0.105	0.005	0.039	0.017
Western USA	920	21	0.023	0.044	0.122	0.057
Ukraine	204	18	0.076	0.083		0.068
NE Canada	260	16	0.010	0.049		
China/Mongolia	1314	18	0.077	0.028	0.094	0.033
Ascension Island	200	14		0.066		
Northern Africa	120	13	0.036	0.036	0.083	0.143
Oman	344	12	0.136	0.011	0.016	0.005
Patagonia	4086	13	0.034	0.078	0.002	
Bermuda	64	10	0.208			
Southern Mexico	428	10	0.076	0.312		
France	64	7			0.028	0.028
Germany	119	7	0.009	0.091		
U.K./Norway	122	7	0.000	0.052		
Antarctica	264	1		0.521		
Total	35713	31				

Table 3.1. (Cont.)

Global region	Sample		*Echinosteliopsis oligospora*	*Protostelium arachisporum*	*Tychosporium acutostipes*	*Endostelium zonatum*	*Protostelium nocturnum*
	Effort	Richness					
Hawaii	11658	31	0.036	0.048	0.015	0.024	0.031
New Zealand	6251	27	0.009	0.014	0.014	0.004	0.047
Carribean	1908	24	0.103	0.190	0.004	0.143	0.086
Central USA	3387	27	0.044		0.033	0.029	0.026
Eastern Africa	2128	23	0.081	0.027	0.029	0.076	0.030
Kazakstan/Russia	468	26	0.121	0.024	0.161	0.007	0.068
Australia	1140	24	0.013	0.024	0.005	0.018	0.022
Northern Thailand	264	20	0.191	0.069	0.005	0.034	0.074
Western USA	920	21	0.021			0.037	0.021
Ukraine	204	18	0.061	0.015	0.159	0.015	0.038
NE Canada	260	16	0.054	0.025	0.098	0.010	0.020
China/Mongolia	1314	18		0.059	0.012	0.003	0.008
Ascension Island	200	14		0.015	0.022		0.228
Northern Africa	120	13				0.036	0.036
Oman	344	12		0.005			
Patagonia	4086	13		0.002	0.004		0.002
Bermuda	64	10	0.104			0.042	0.021
Southern Mexico	428	10	0.010				0.020
France	64	7			0.083	0.028	0.000
Germany	119	7			0.009	0.000	0.009
U.K./Norway	122	7			0.038		
Antarctica	264	1					
Total	35713	31					

Table 3.1. (Cont.)

Global region	Sample		*Nematostelium ovatum*	*Schizoplasmodium cavostelioides*	*Protostelium pyriformis*	*Soliformovum expulsum*	*Ceratiomyxella tahitiensis*
	Effort	Richness					
Hawaii	11658	31	0.037	0.024	0.022	0.036	0.002
New Zealand	6251	27	0.007	0.010	0.018	0.009	
Carribean	1908	24	0.070	0.013	0.035	0.051	
Central USA	3387	27	0.019	0.057	0.015	0.019	
Eastern Africa	2128	23	0.019	0.016	0.015	0.051	
Kazakstan/Russia	468	26	0.030		0.025	0.004	0.120
Australia	1140	24	0.039	0.009	0.040	0.012	0.000
Northern Thailand	264	20	0.039	0.108	0.005	0.010	0.002
Western USA	920	21	0.011	0.011	0.002		0.025
Ukraine	204	18	0.015	0.008	0.008	0.023	
NE Canada	260	16	0.005	0.025	0.029	0.005	
China/Mongolia	1314	18	0.014	0.003		0.002	
Ascension Island	200	14	0.015		0.044		0.044
Northern Africa	120	13	0.028		0.028		
Oman	344	12	0.071	0.005		0.011	
Patagonia	4086	13			0.004		
Bermuda	64	10	0.063	0.042	0.021	0.000	
Southern Mexico	428	10	0.000	0.000	0.010		
France	64	7	0.000	0.000	0.000		0.083
Germany	119	7	0.000	0.000	0.000		
U.K./Norway	122	7	0.029			0.013	
Antarctica	264	1					
Total	35713	31					

Table 3.1. (Cont.)

Global region	Sample		*Protosporangium articulatum*	*Microglomus paxillus*	*Clastostelium recurvatum*	*Schizoplasmodium seychellarum*	*Protostelium okumukumu*
	Effort	Richness					
Hawaii	11658	31	0.010	0.014	0.007	0.001	0.007
New Zealand	6251	27	0.000	0.004	0.002	0.002	0.000
Carribean	1908	24		0.011	0.029	0.004	0.028
Central USA	3387	27	0.005	0.001	0.001		0.000
Eastern Africa	2128	23	0.013	0.007	0.008		
Kazakstan/Russia	468	26	0.034			0.026	0.050
Australia	1140	24	0.015	0.000	0.003	0.000	0.003
Northern Thailand	264	20		0.010	0.000	0.034	0.000
Western USA	920	21	0.045		0.007		
Ukraine	204	18	0.044	0.008			
NE Canada	260	16					
China/Mongolia	1314	18	0.008				
Ascension Island	200	14		0.029	0.022	0.088	
Northern Africa	120	13		0.028			
Oman	344	12		0.000	0.000	0.000	0.000
Patagonia	4086	13	0.000				
Bermuda	64	10					
Southern Mexico	428	10	0.002				
France	64	7					
Germany	119	7					
U.K./Norway	122	7					
Antarctica	264	1					
Total	35713	31					

Table 3.1. (Cont.)

Global region	Sample		*Protosporangium bisporum*	*Protosporangium conicum*	*Ceratiomyxa hemisphaerica*	*Endostelium amerosporum*
	Effort	Richness				
Hawaii	11658	31	0.004	0.000	0.001	0.004
New Zealand	6251	27	0.000		0.001	
Carribean	1908	24	0.001			0.006
Central USA	3387	27	0.006	0.001	0.001	0.001
Eastern Africa	2128	23	0.004	0.001		
Kazakstan/Russia	468	26	0.003	0.009	0.180	
Australia	1140	24	0.001	0.004	0.000	0.001
Northern Thailand	264	20	0.000		0.000	0.000
Western USA	920	21		0.008		0.018
Ukraine	204	18				
NE Canada	260	16			0.010	
China/Mongolia	1314	18	0.005	0.002		
Ascension Island	200	14			0.066	
Northern Africa	120	13				
Oman	344	12	0.000		0.000	0.000
Patagonia	4086	13				
Bermuda	64	10				
Southern Mexico	428	10				
France	64	7				
Germany	119	7				
U.K./Norway	122	7				
Antarctica	264	1				
Total	35713	31				

Table 3.1. (Cont.)

Global region	Sample		*Protosporangium fragile*	*Schizoplasmodiopsis micropunctata*	*Schizoplasmodiopsis reticulata*	*Schizoplasmodium obovatum*
	Effort	Richness				
Hawaii	11658	31	0.002	0.001	0.002	0.000
New Zealand	6251	27		0.001	0.001	0.001
Carribean	1908	24				0.001
Central USA	3387	27	0.002	0.004	0.002	
Eastern Africa	2128	23	0.001			
Kazakstan/Russia	468	26	0.002		0.082	0.031
Australia	1140	24	0.001	0.000	0.000	0.000
Northern Thailand	264	20		0.000	0.000	0.000
Western USA	920	21	0.012	0.011		
Ukraine	204	18				
NE Canada	260	16				
China/Mongolia	1314	18				
Ascension Island	200	14			0.015	0.022
Northern Africa	120	13				
Oman	344	12		0.000	0.000	0.000
Patagonia	4086	13		0.002		
Bermuda	64	10				
Southern Mexico	428	10				
France	64	7				
Germany	119	7				
U.K./Norway	122	7				
Antarctica	264	1				
Total	35713	31				

Note. Table of relative species abundances for each observed global region (proportion of observed lines on which each species was seen at least once). Sampling effort refers to the number of observed lines of substrate from that region. Missing values indicate absence of that species in a given region.

Since so little is known about the detailed ecological requirements of most microbes, the variables that constitute a suitable habitat are best not assumed. In fact, it is probable that a large portion of the factors that shape microbial diversity occur at scales that have not yet been addressed (Vos *et al.*, 2013). Fruiting amoebae have been extant for 1-1.5 billion years (Eme *et al.*, 2014), have adaptations (cysts and spores) that facilitate dispersal, and are likely capable of exploiting anthropogenic vectors. Thus, it cannot be ruled out that environmental factors and the availability of suitable local microhabitat are the main drivers of regional differences in protosteloid richness as opposed to dispersal barriers. Testing this hypothesis is currently impossible since it would require extensive knowledge of species-specific microhabitat requirements, including biotic and abiotic factors, at scales for which there is currently a paucity of data. It would be theoretically possible, however, to test hypotheses regarding the importance of anthropogenic vectors for dispersal of this group.

The results from global distributions and this intensive survey of Hawaii make it clear that, even with flagship species, increased sampling effort may alter our previous assumptions of microbial distributions. The protosteloid amoebae are a useful system for testing hypotheses regarding the biogeography of non-testate amoebae, but it must be remembered that these findings cannot be carelessly applied to all non-testate amoebae since the traits that define this group also lend themselves to environmental resilience (Aguilar and Lado, 2012) and widespread dispersal. Genetic data generated thus far seem to agree with this implication. Preliminary data from one species, *Protosteium mycophaga,* has not yielded any geographic patterns in genetic haplotypes (Shadwick, JD and Spiegel FW, unpublished). With this in mind, future research should be directed toward investigating the importance of the various factors that might explain

the universal distributions of these species (i.e. anthropogenic vectors, spore viability, and air dispersal).

Acknowledgements

We would like to thank Helen Hemmes for hosting visits to Hawaii, Martin Schnittler, Carlos Lado, Aguilar, Maria Romeromo, Jens Tesmer, Steven L. Stephenon, Diana Wrigley de Basanta, Randy Darrah, Andy Swanson, Matthew Brown, Arturo Estrada-Torres, and numerous others in the mycetozoan community for assisting with collections. This research was supported by the following funding sources: National Science Foundation Grant #INT-9902175, National Science Foundation Grant #INT-0139547, Australian Biological Resources Study (ABRS) Program Grant #202-027, National Science Foundation Grant #DEB-0316284, National Science Foundation Grant #DEB-03029192, National Science Foundation Grant #INT-0244329, National Geographic Society Grant #7925-05, National Science Foundation Grant #OISE-0701464, National Science Foundation Grant #OISE-0934084, National Science Foundation #OISE-1042602, Australian Biological Resources Study (ABRS) Program Grant #211-06

Supplementary information is available at The ISME Journal's website.

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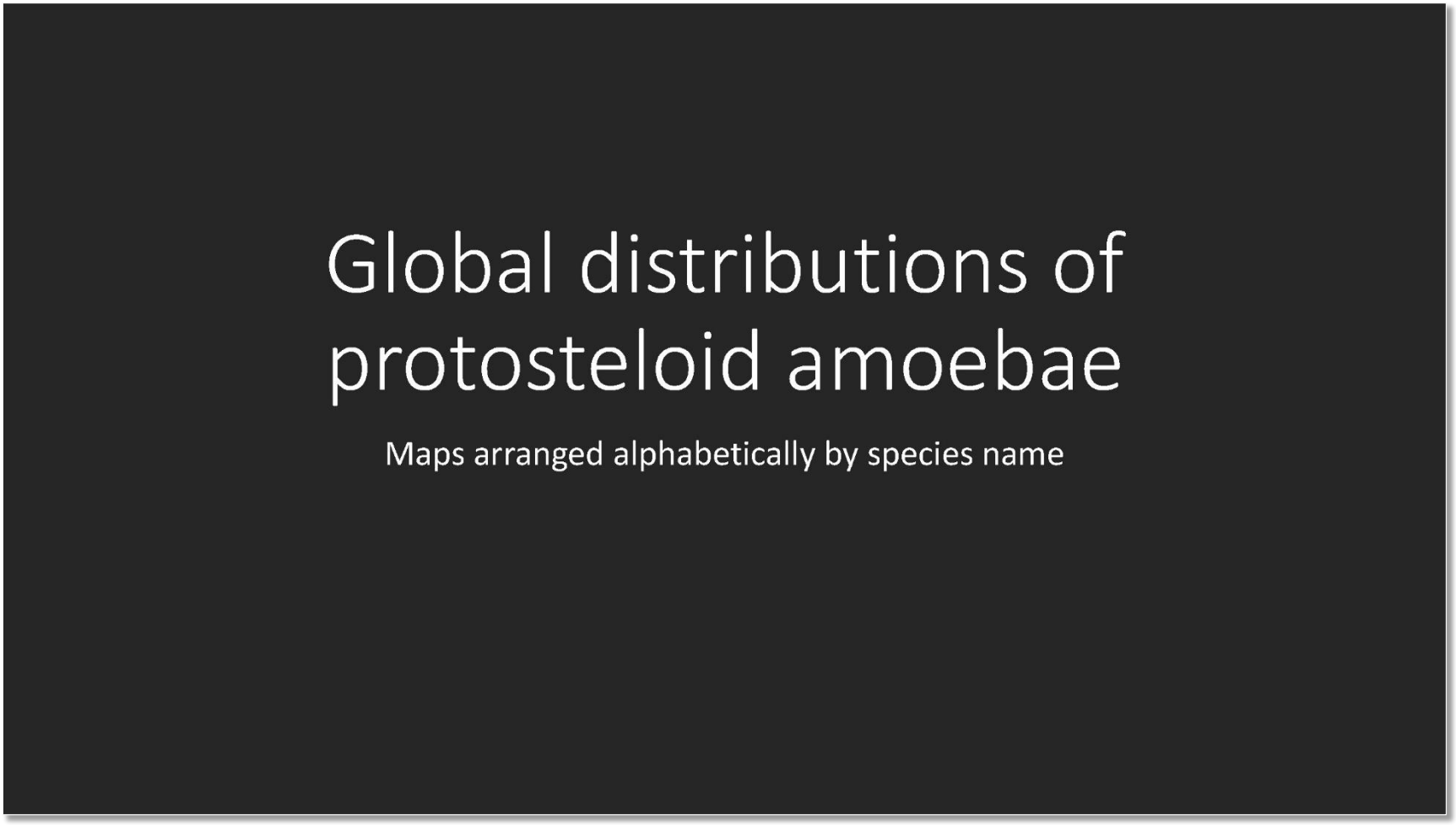
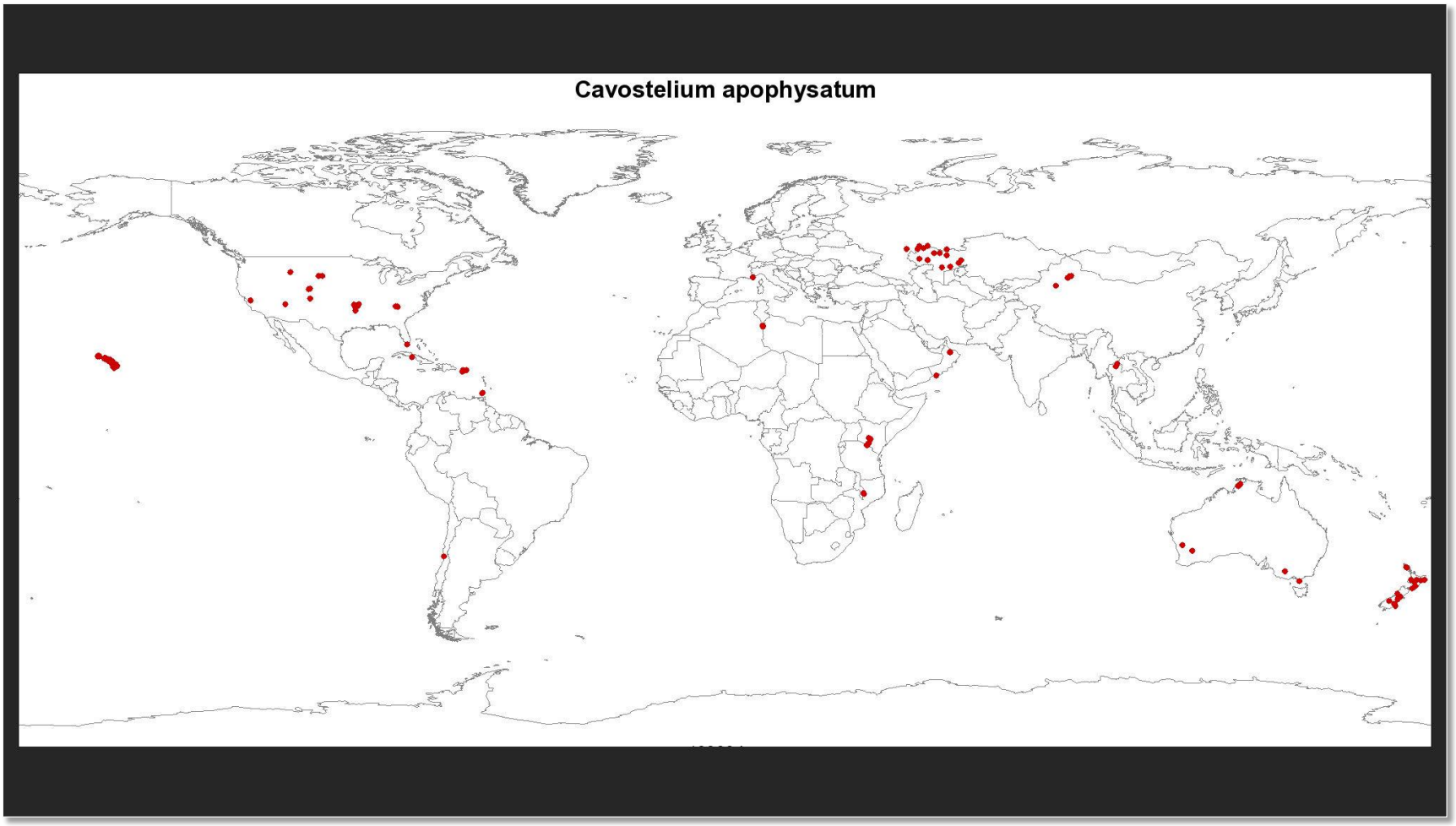
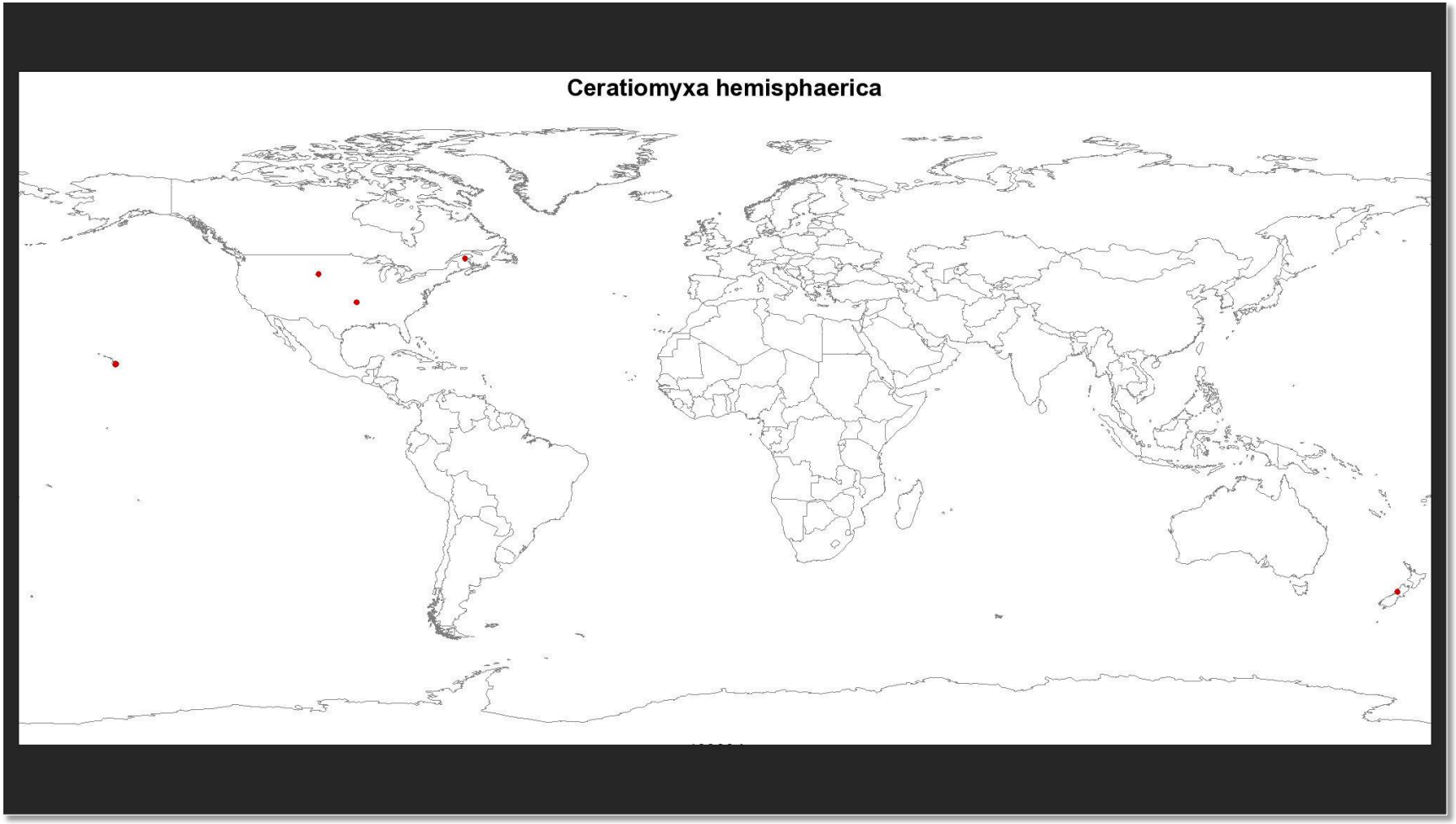
Appendix: Species Distribution Maps

Distribution maps for each species of described protosteloid amoeba are presented below. Dots indicate the presence of protosteloid amoeba in a given location.




Cavostelium apophysatum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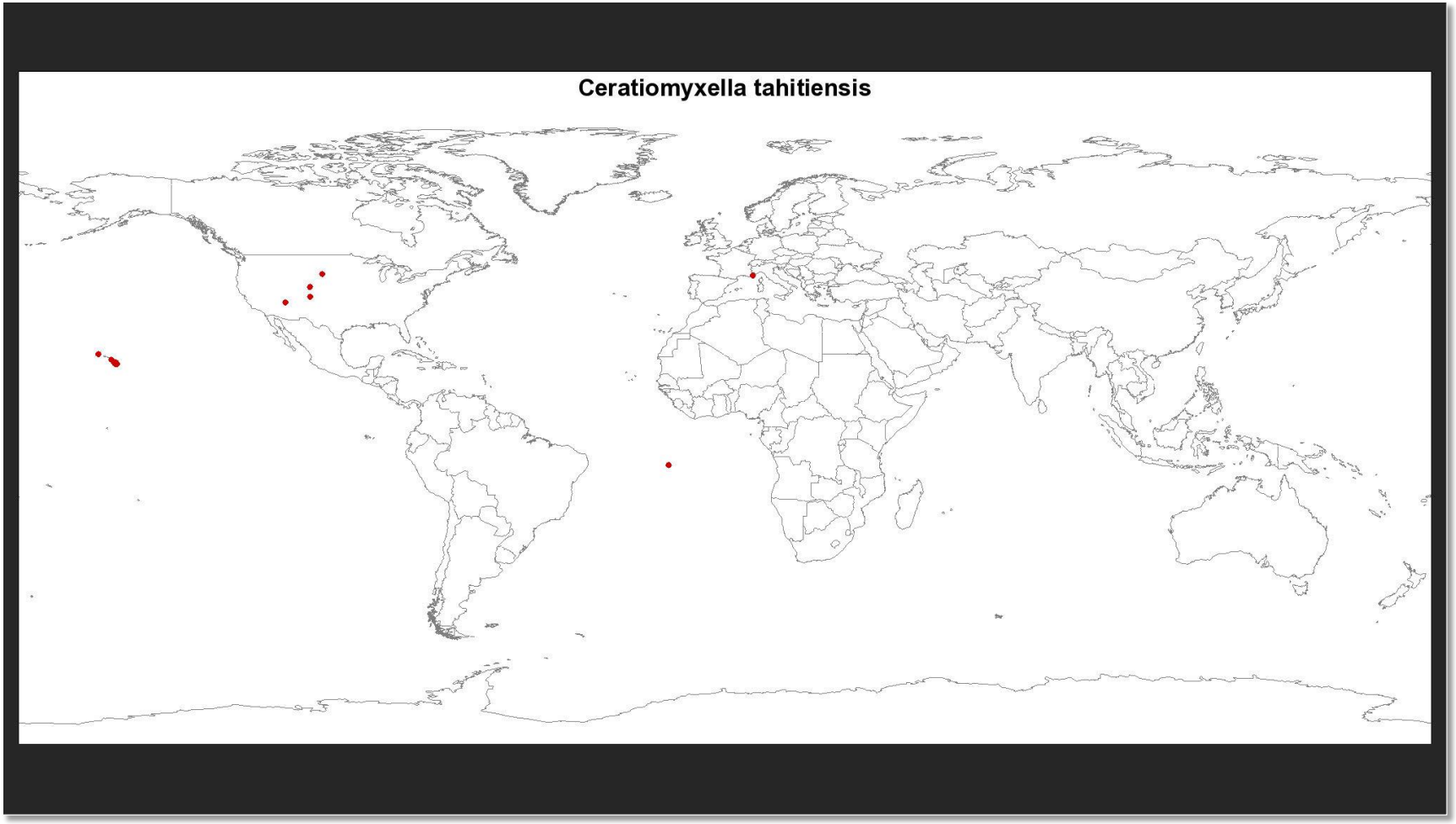
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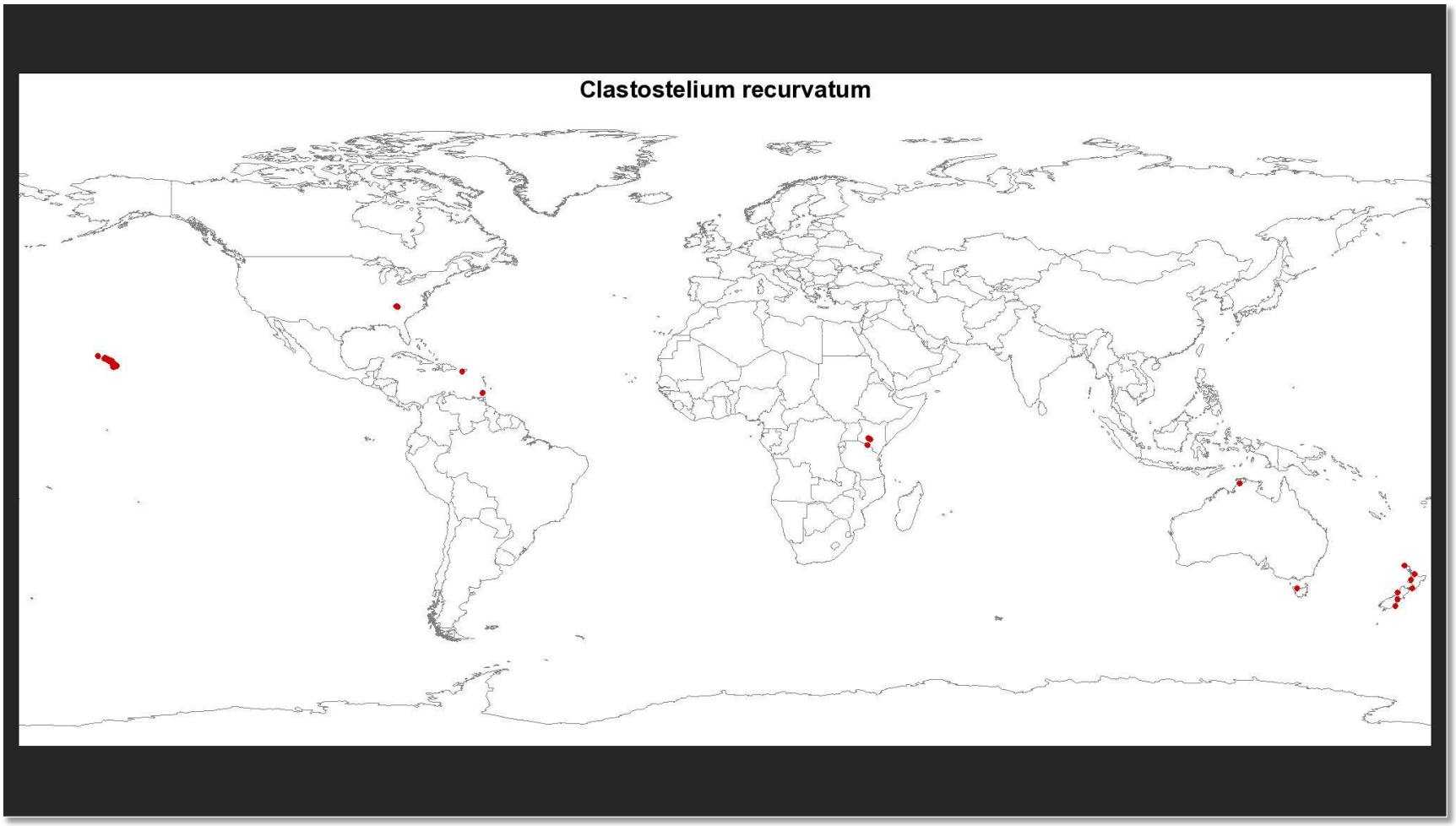
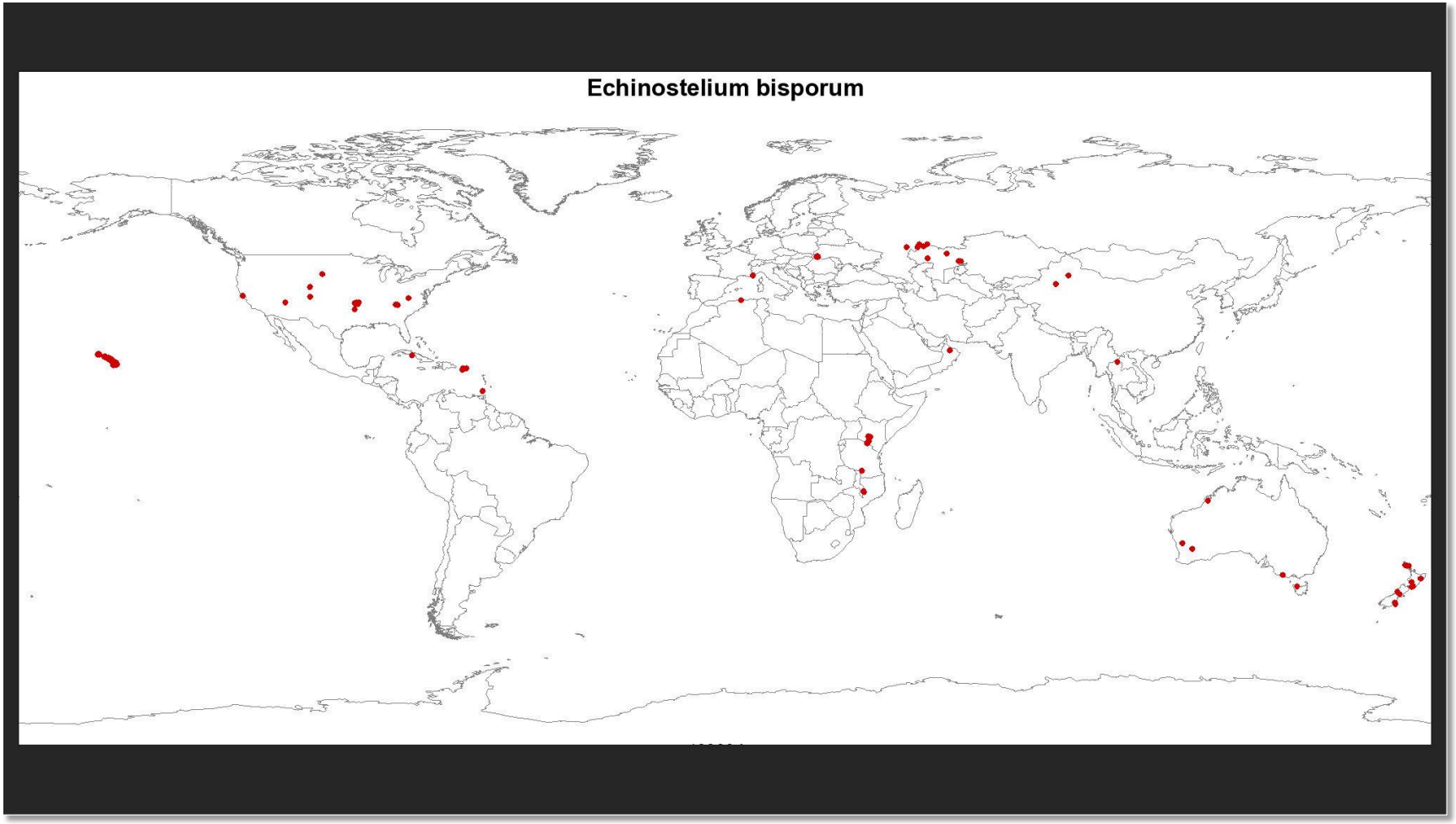
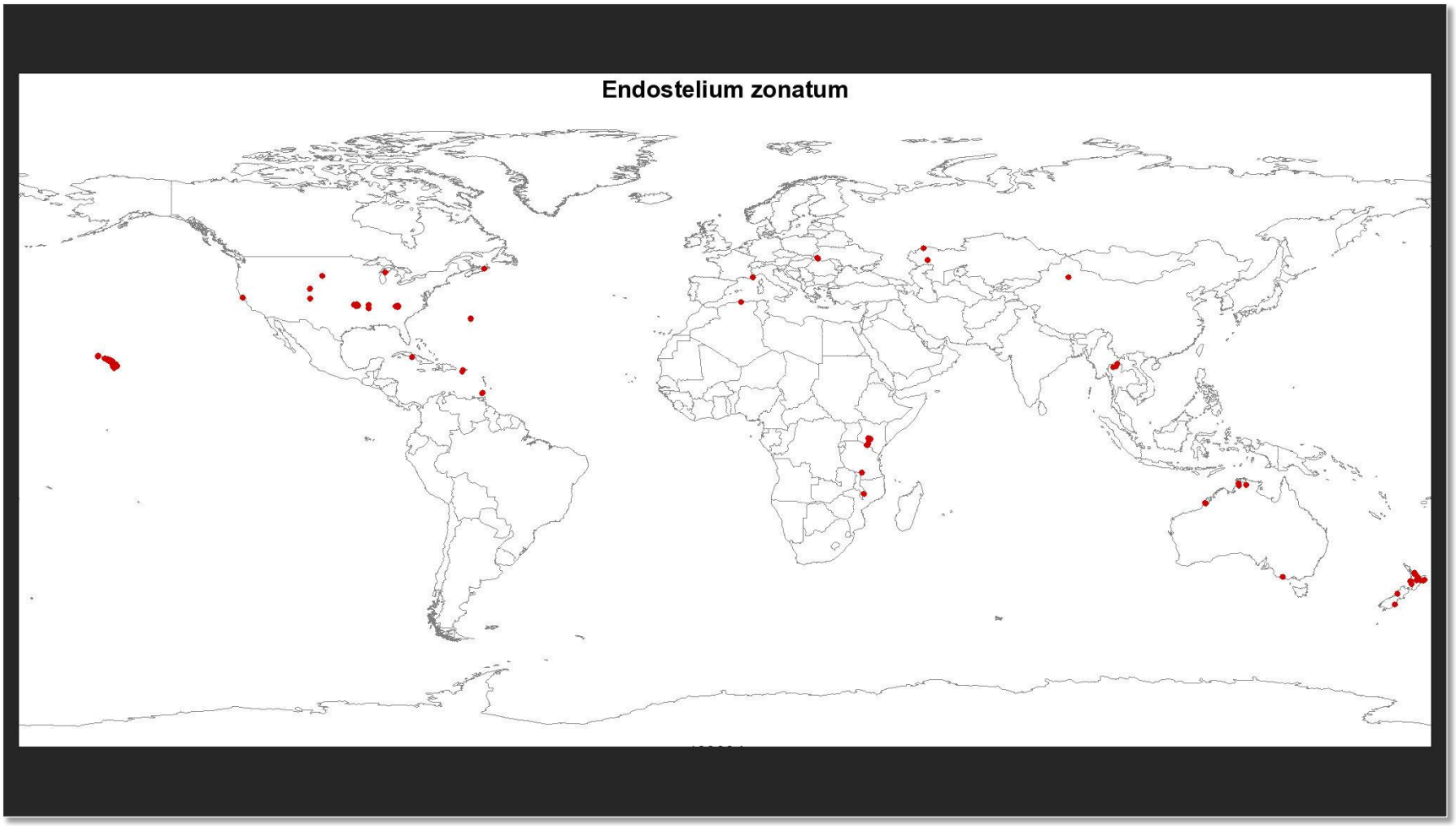
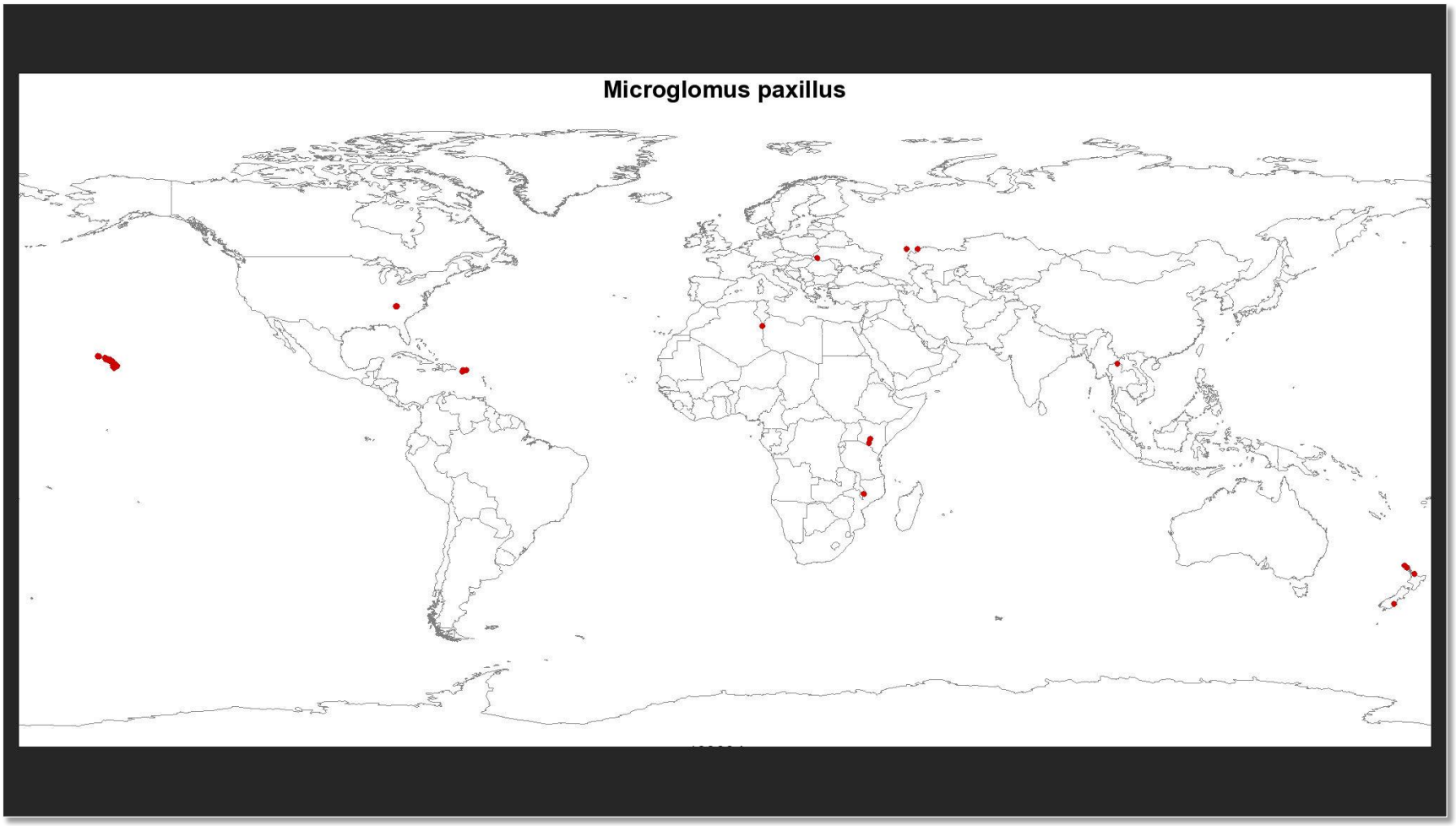

Clastostelium recurvatum

Echinosteliopsis oligospora


Echinostelium bisporum


Endostelium zonatum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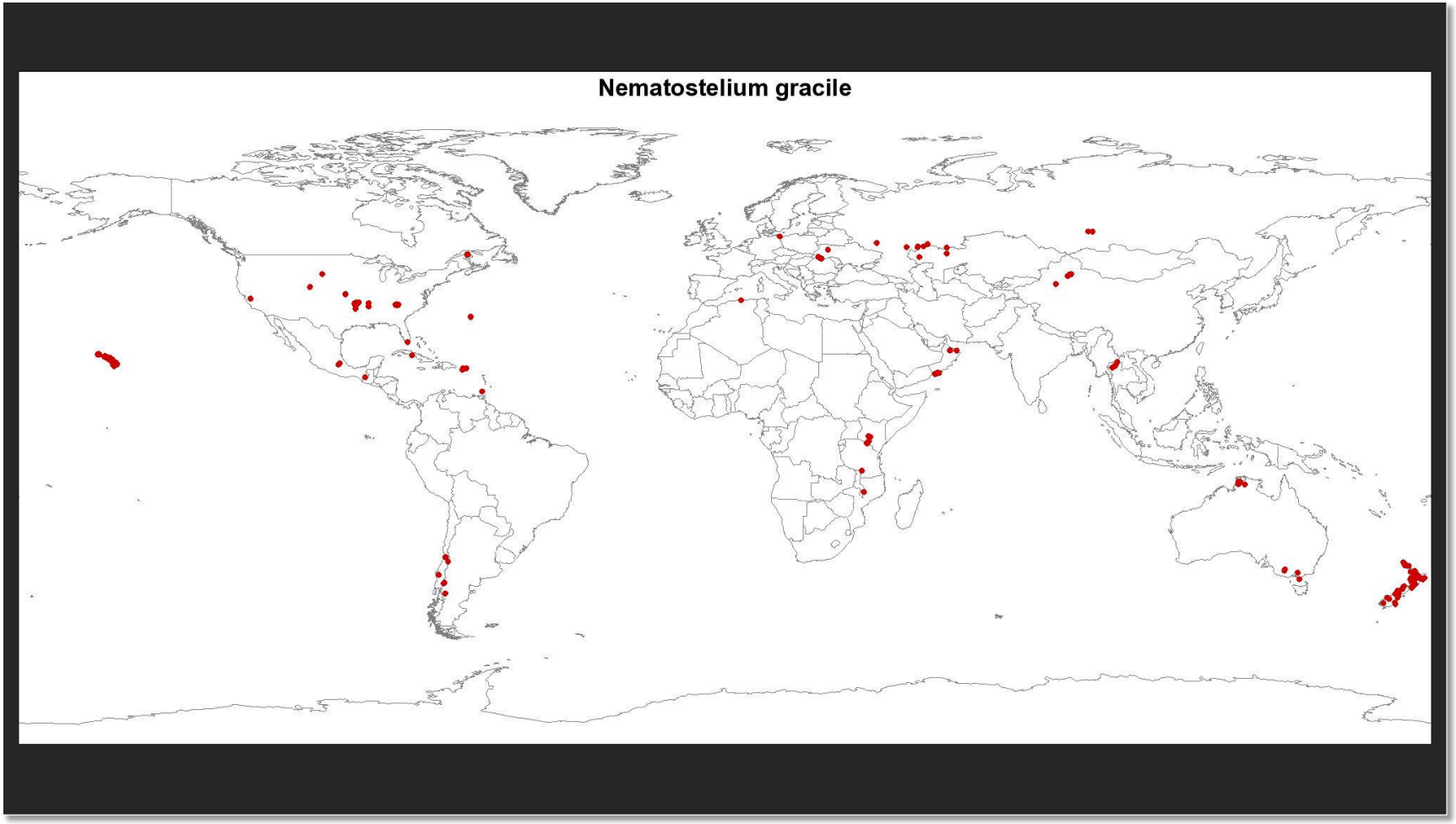
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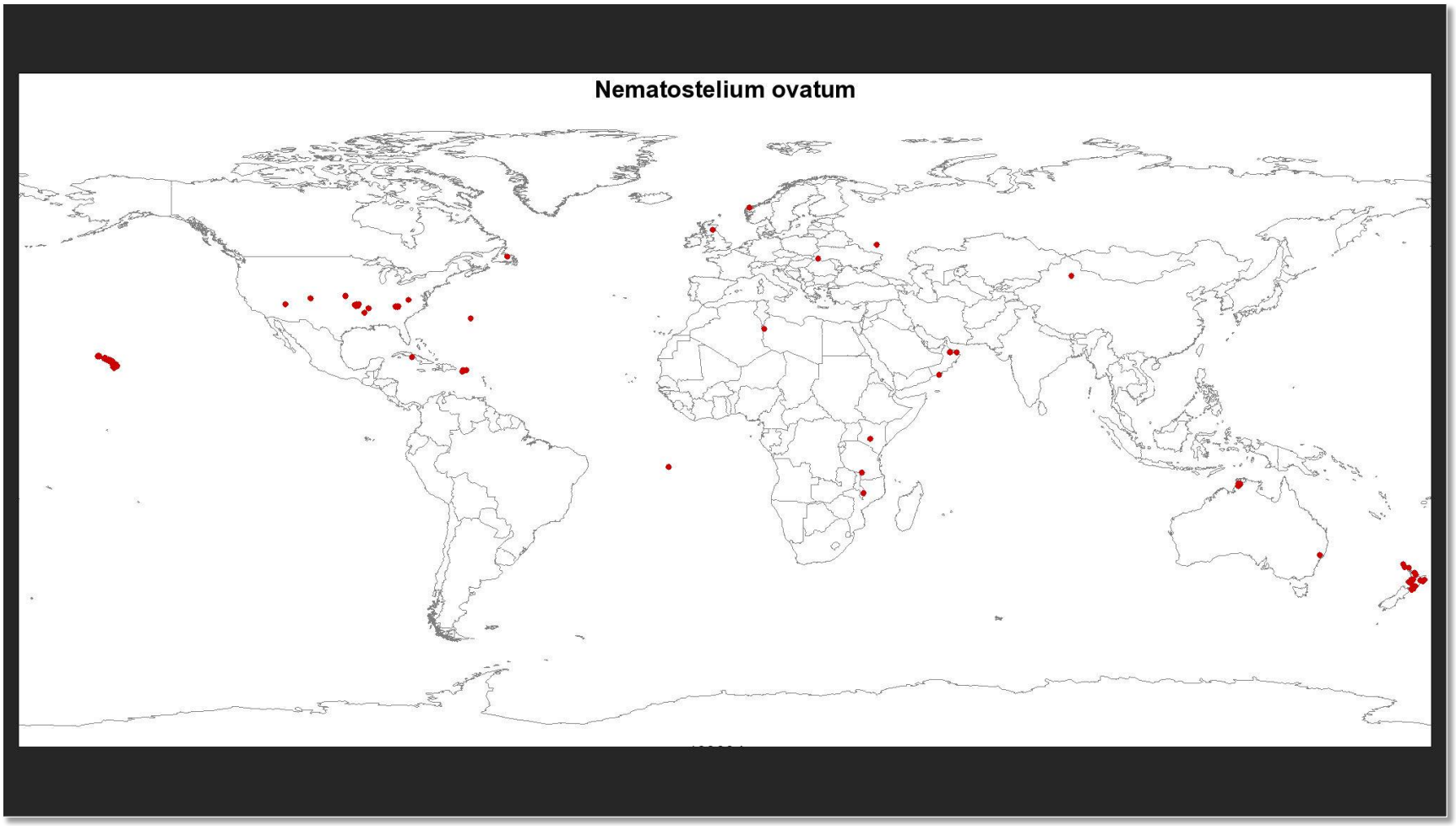
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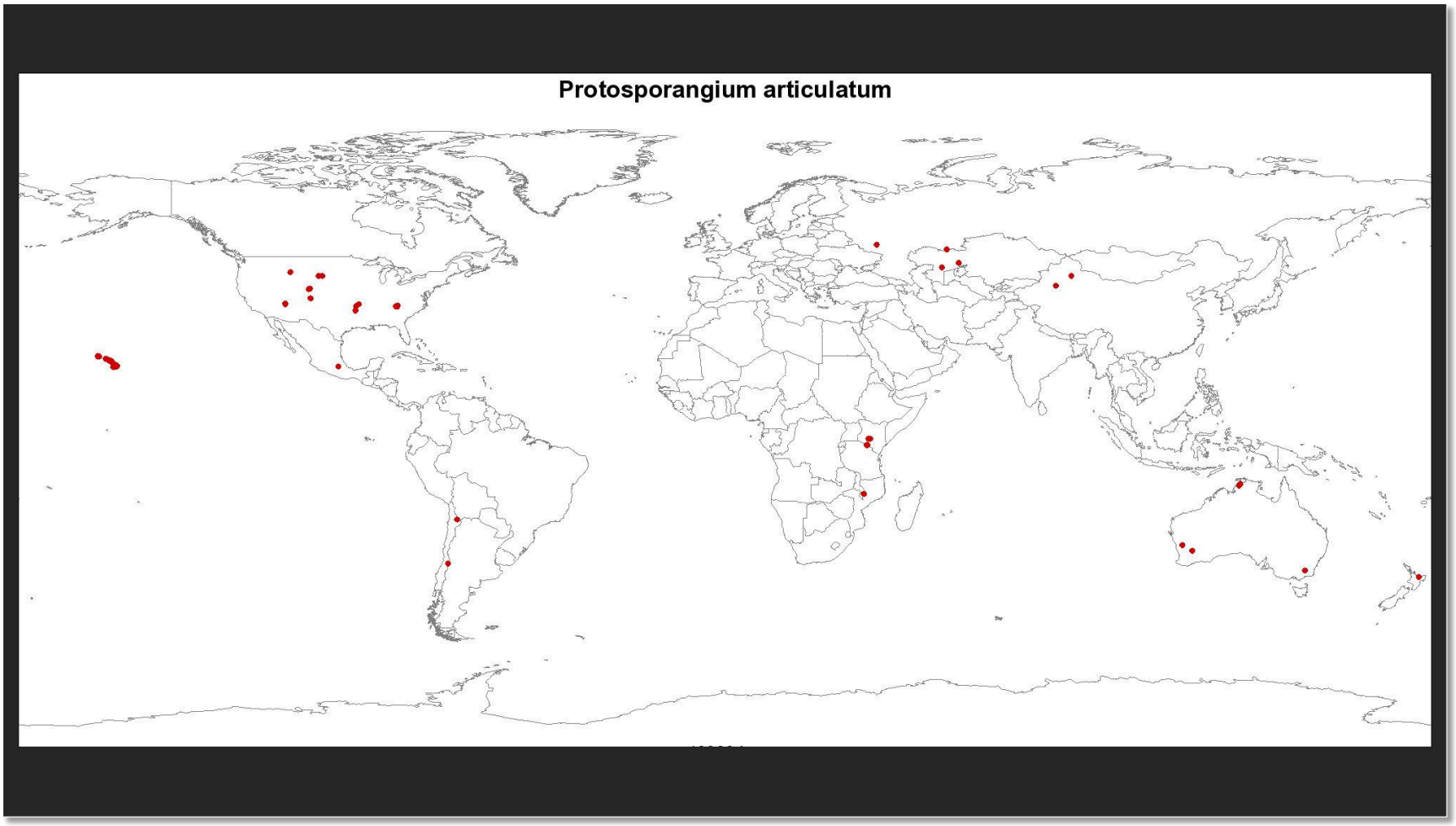
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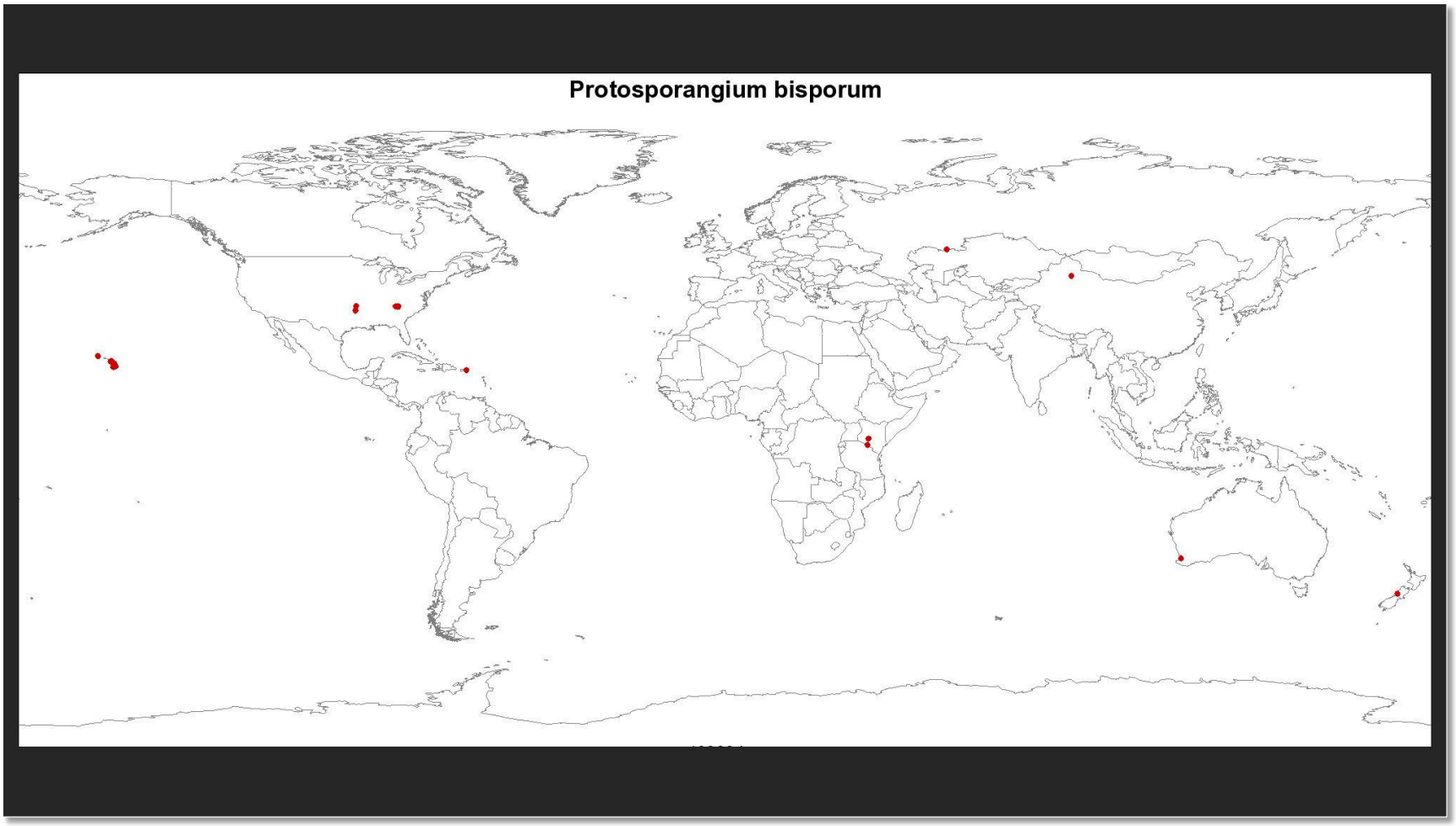
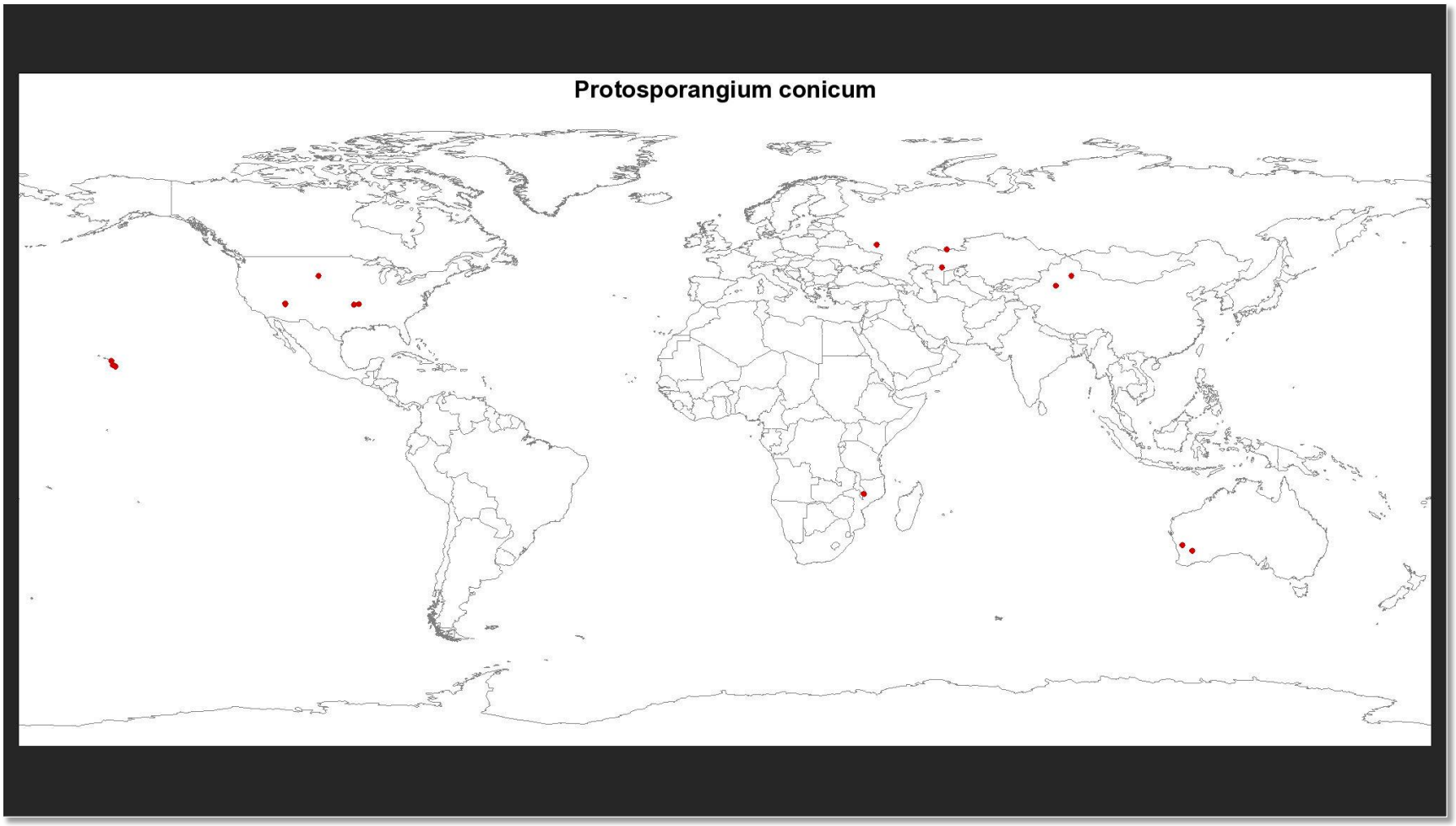

Protosporangium bisporum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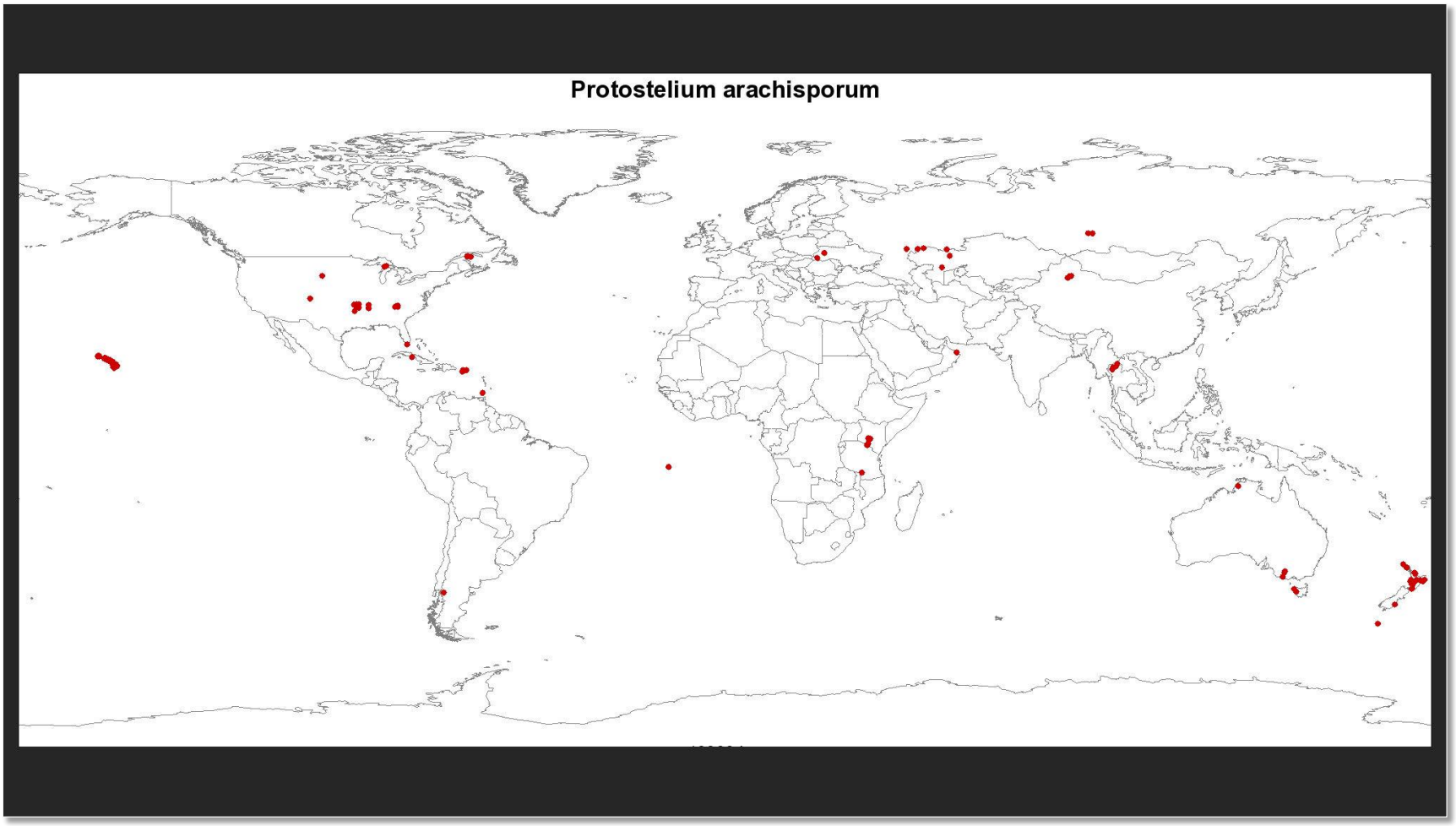
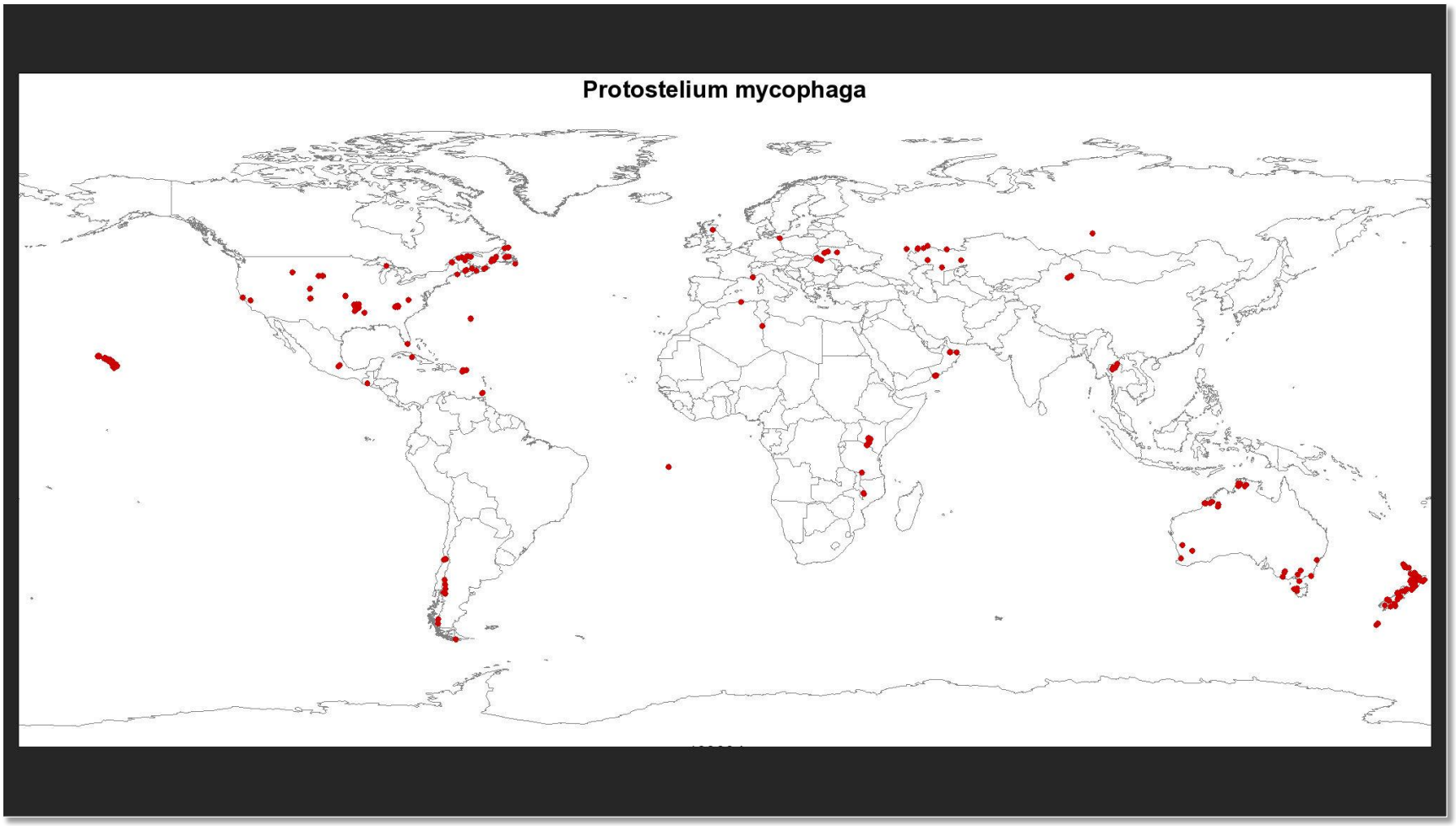
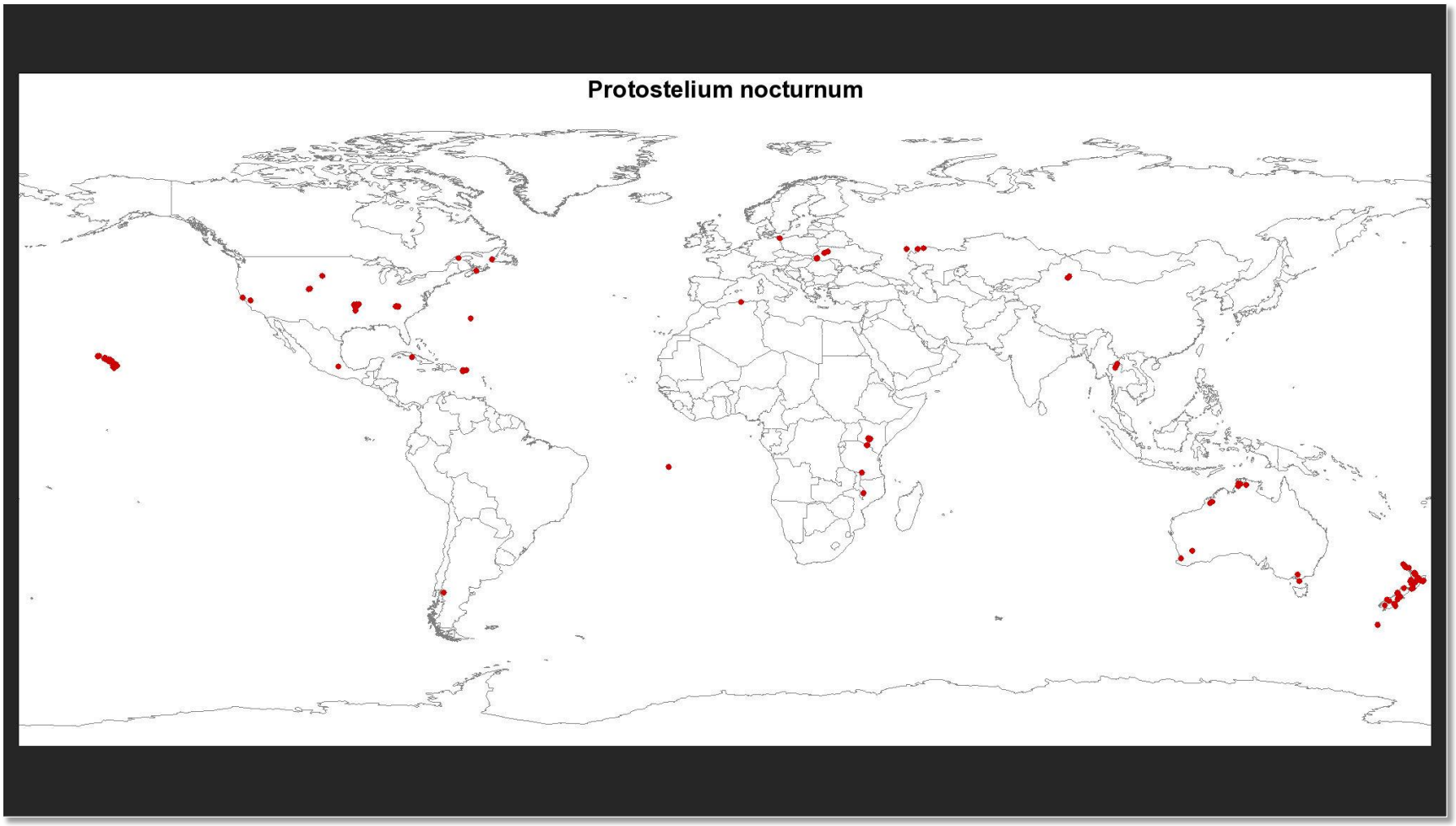
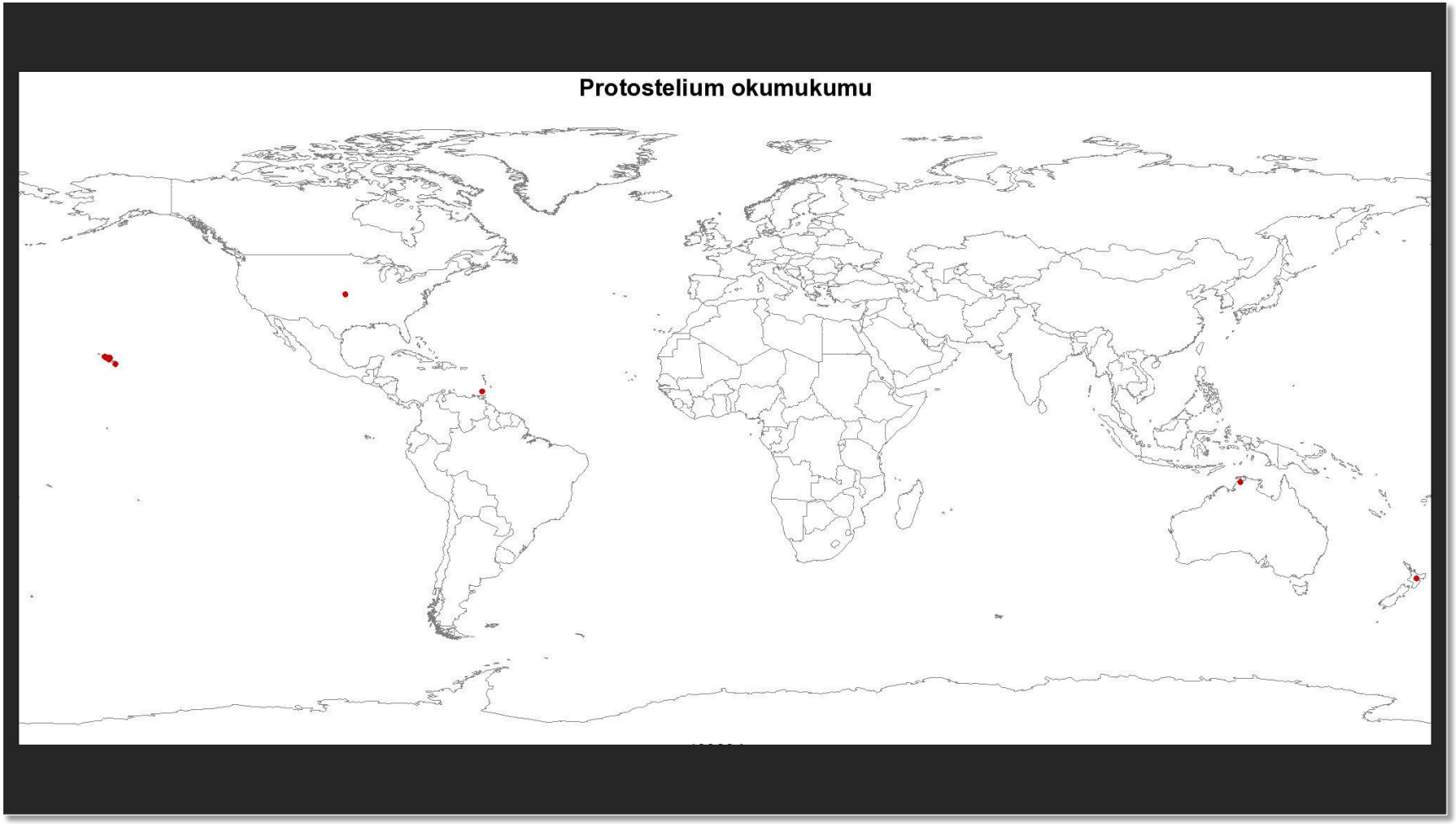
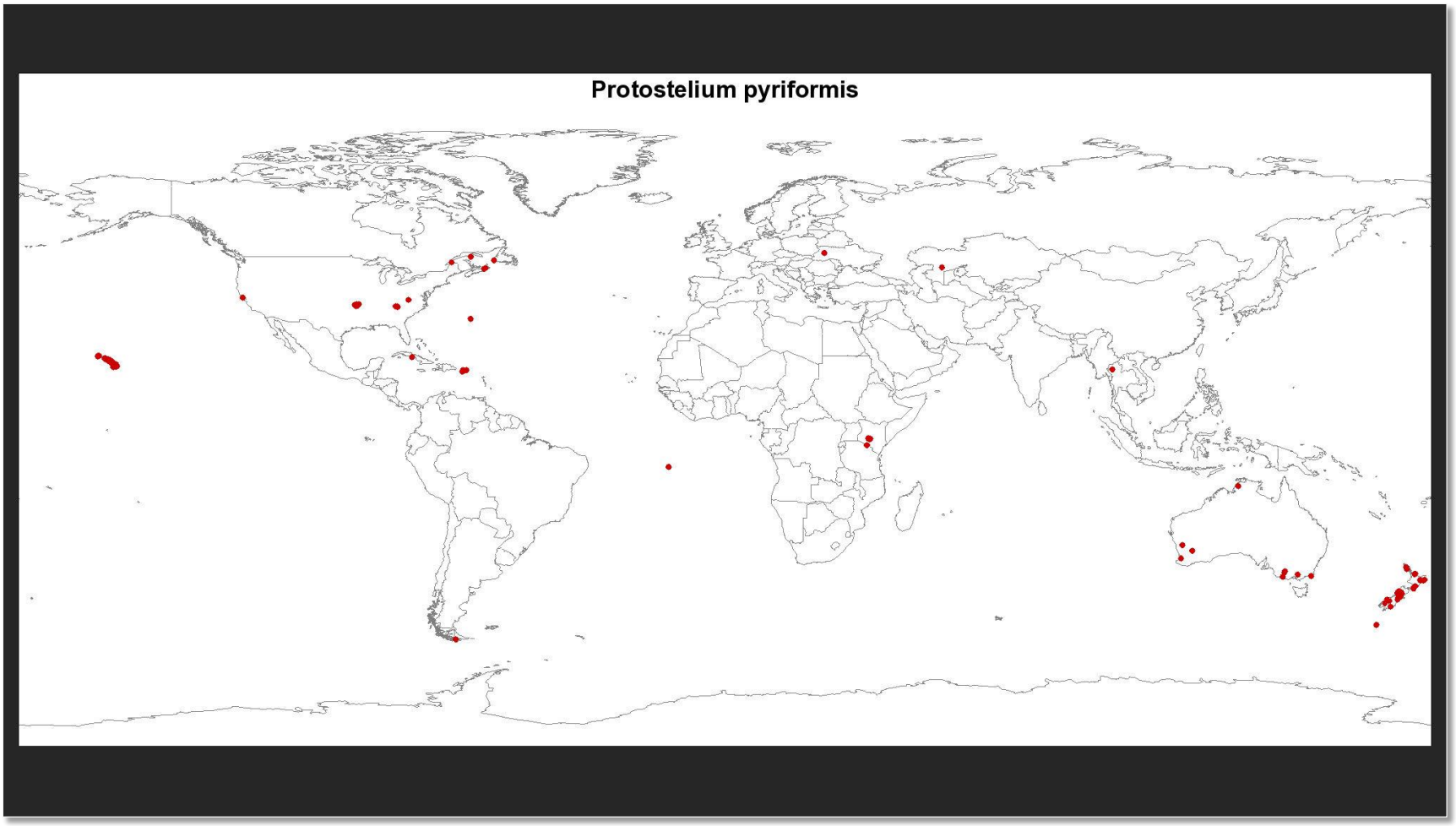
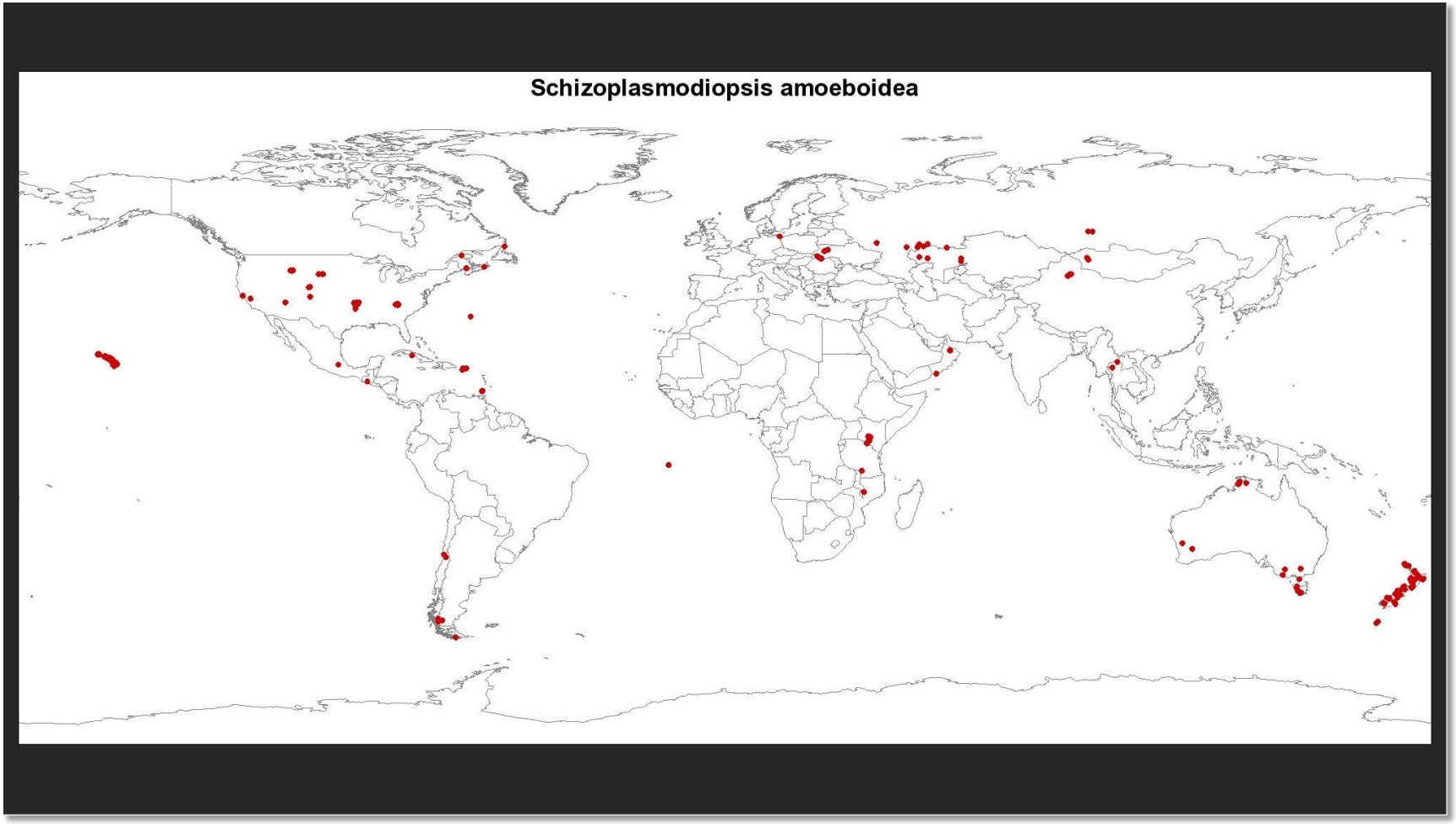

Protostelium arachisporum

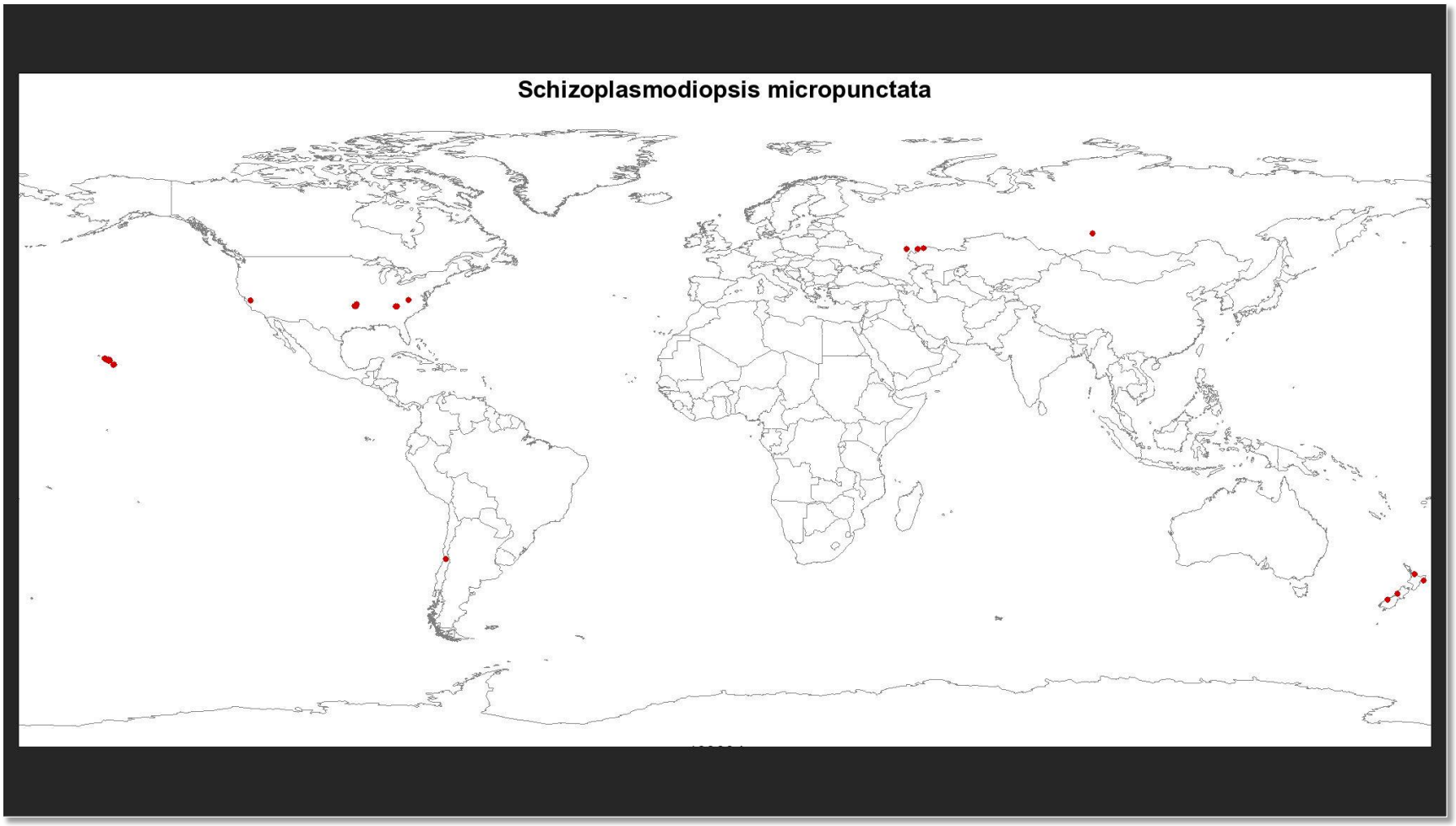

Protostelium mycophaga


Protostelium nocturnum


Protostelium okumukumu


Protostelium pyriformis


Schizoplasmodiopsis amoeboidea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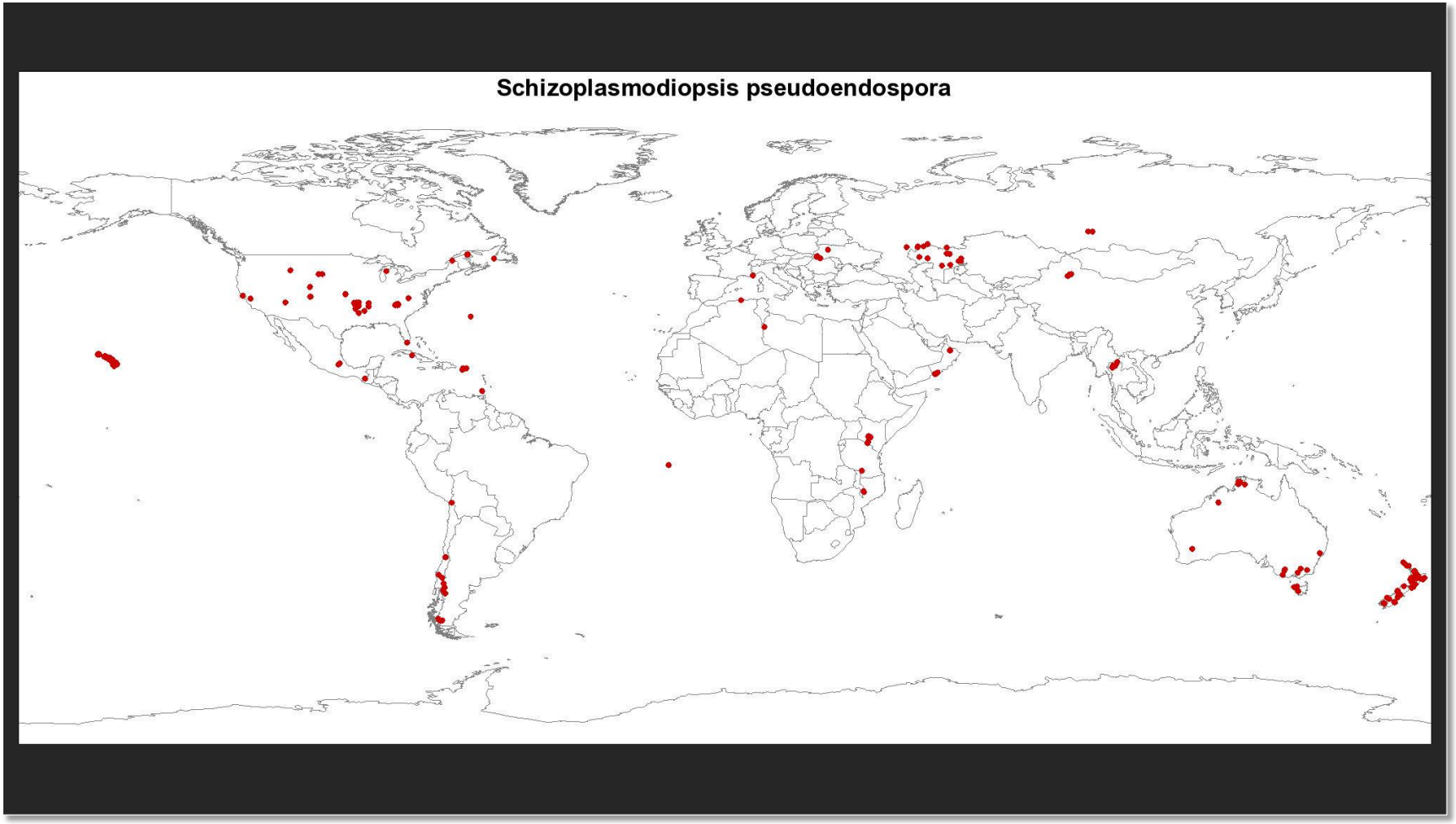
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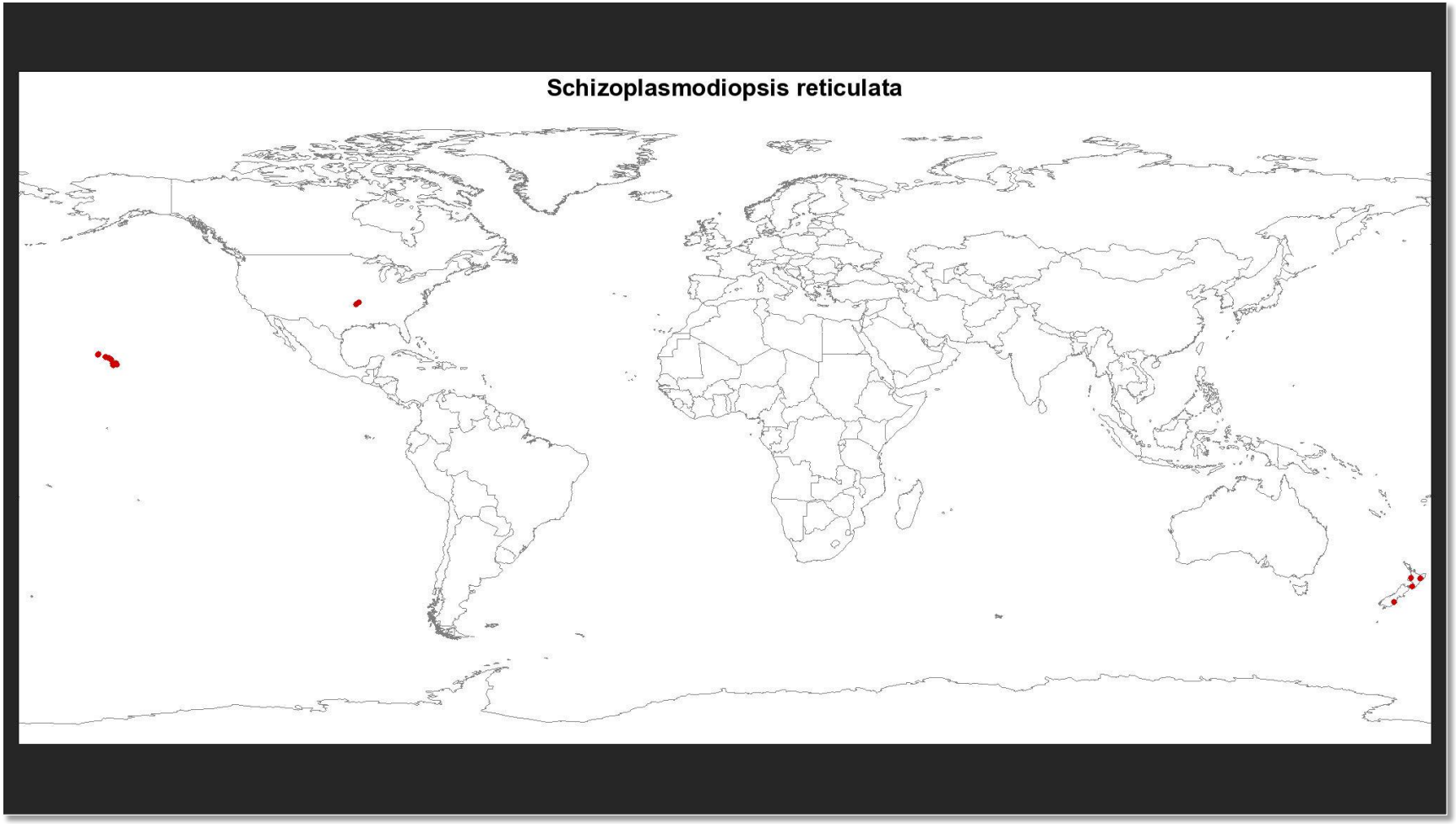
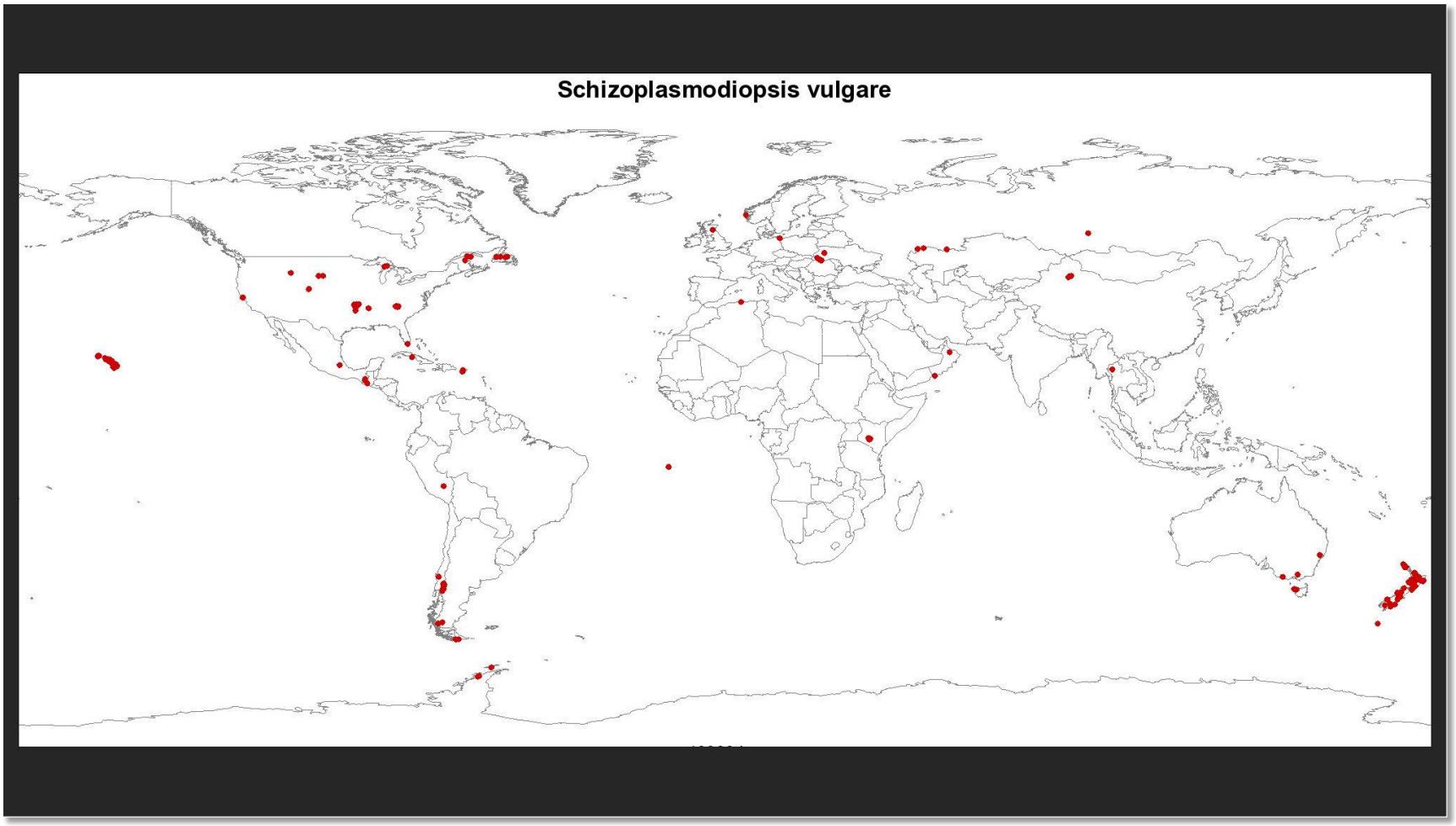
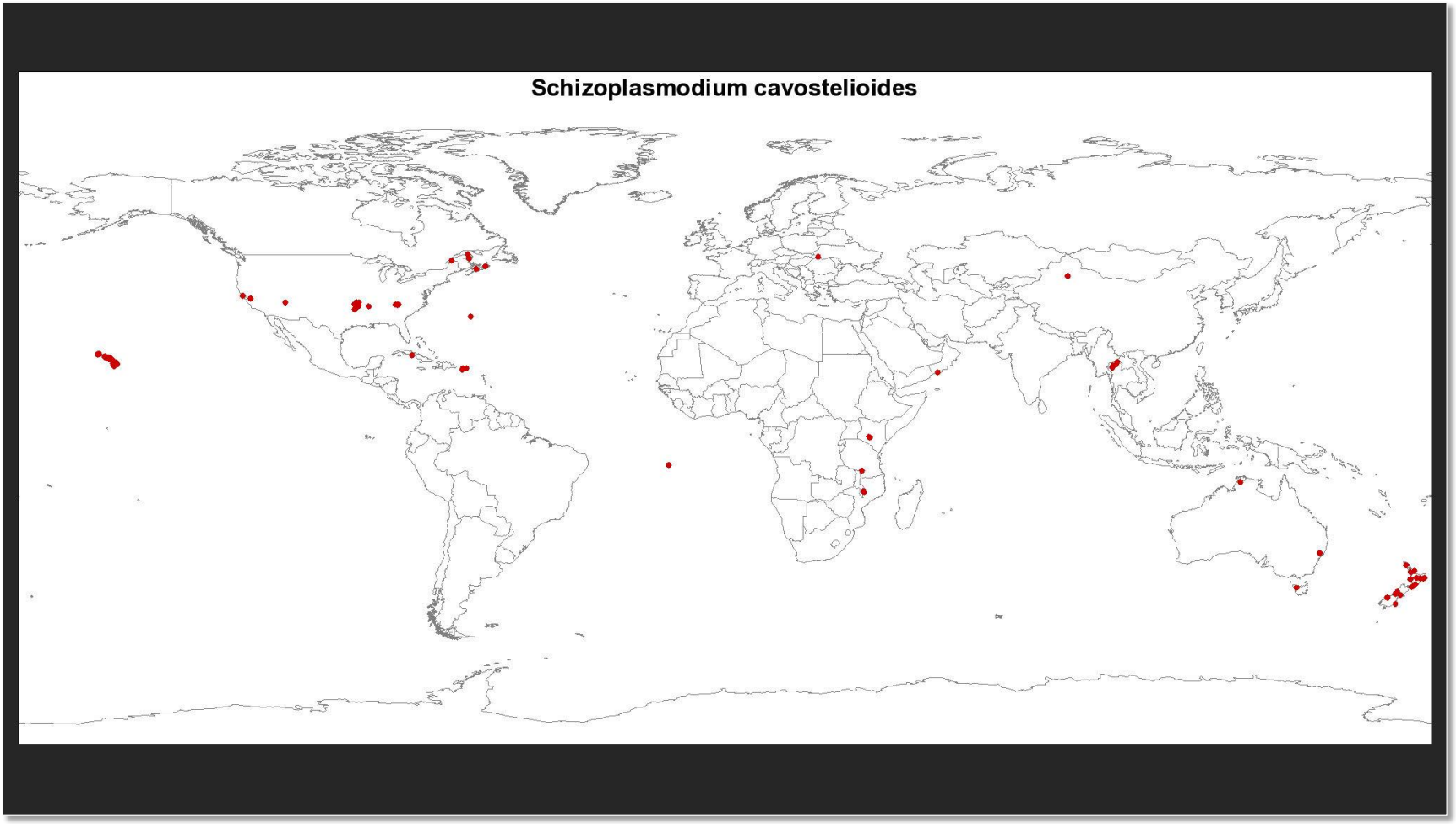

Schizoplasmodiopsis reticulata


Schizoplasmodiopsis vulgare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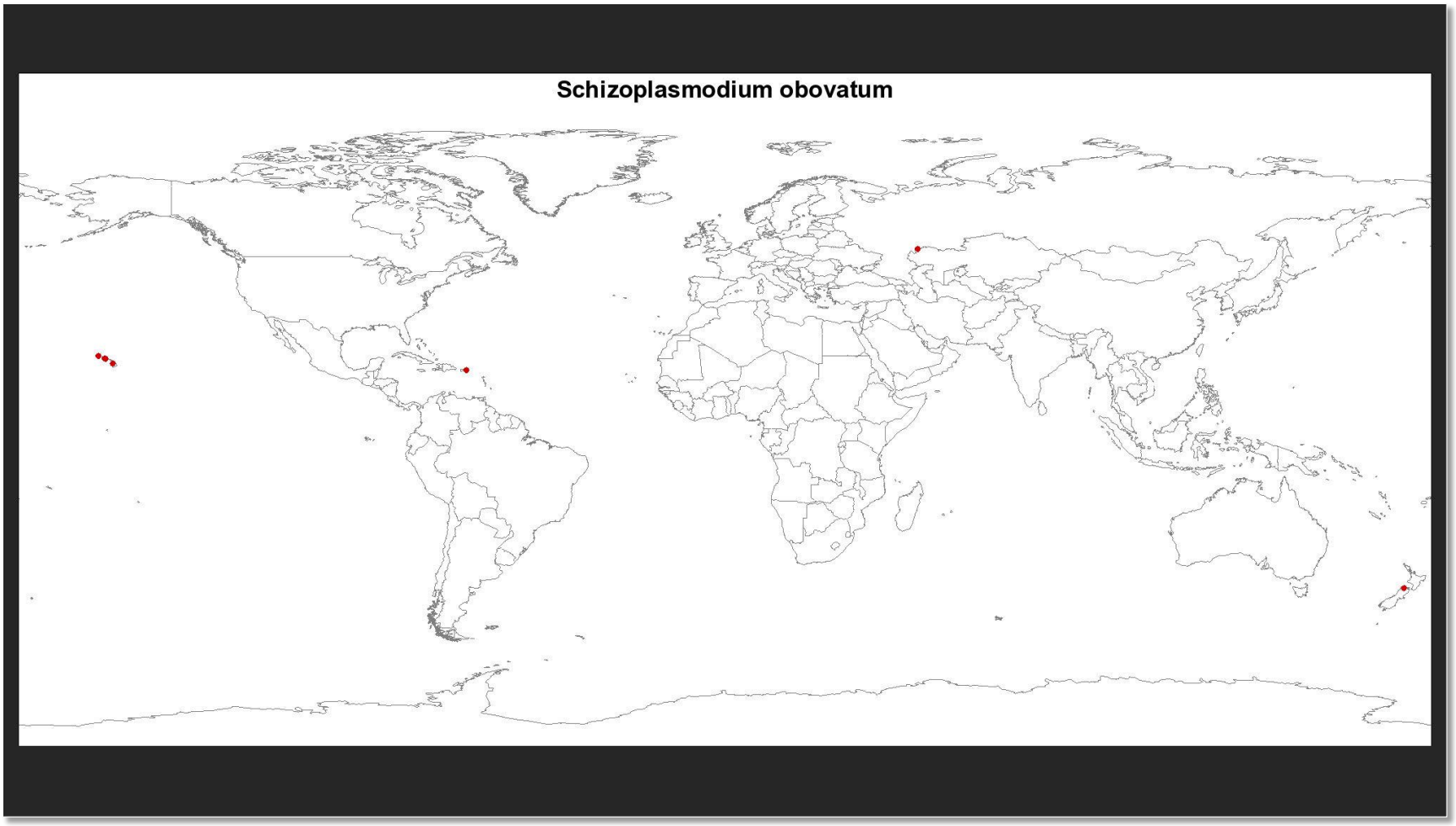
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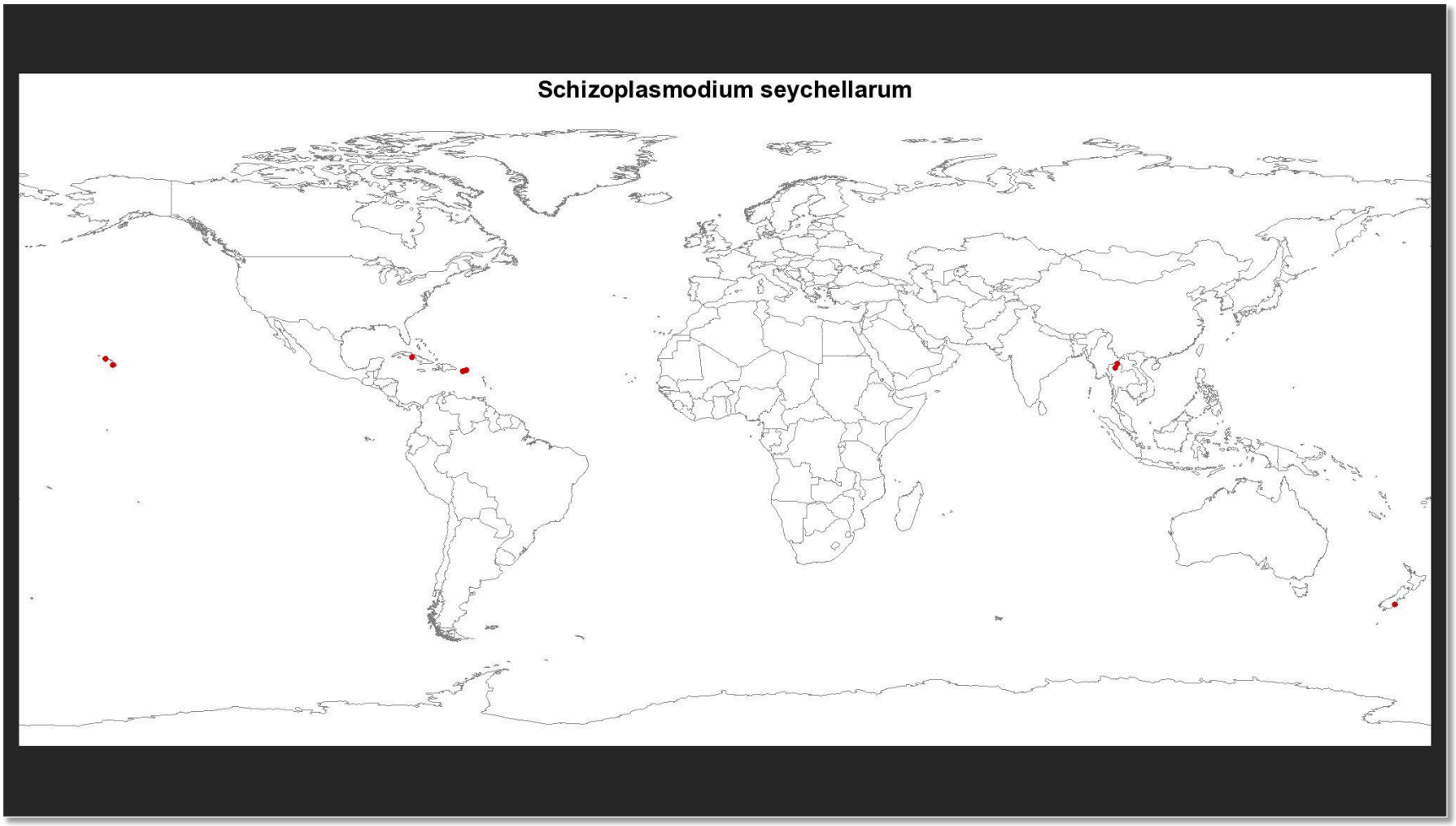
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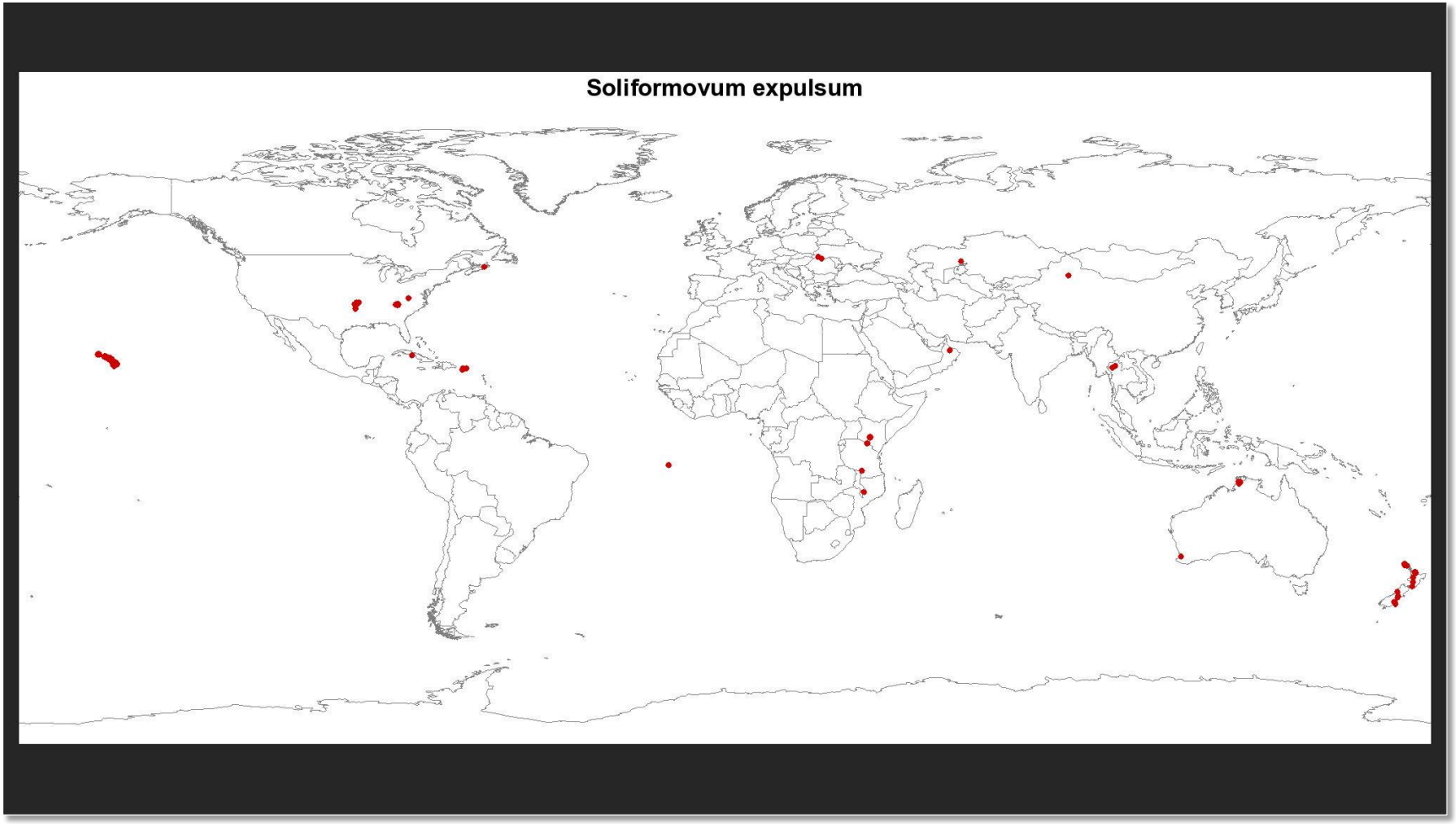
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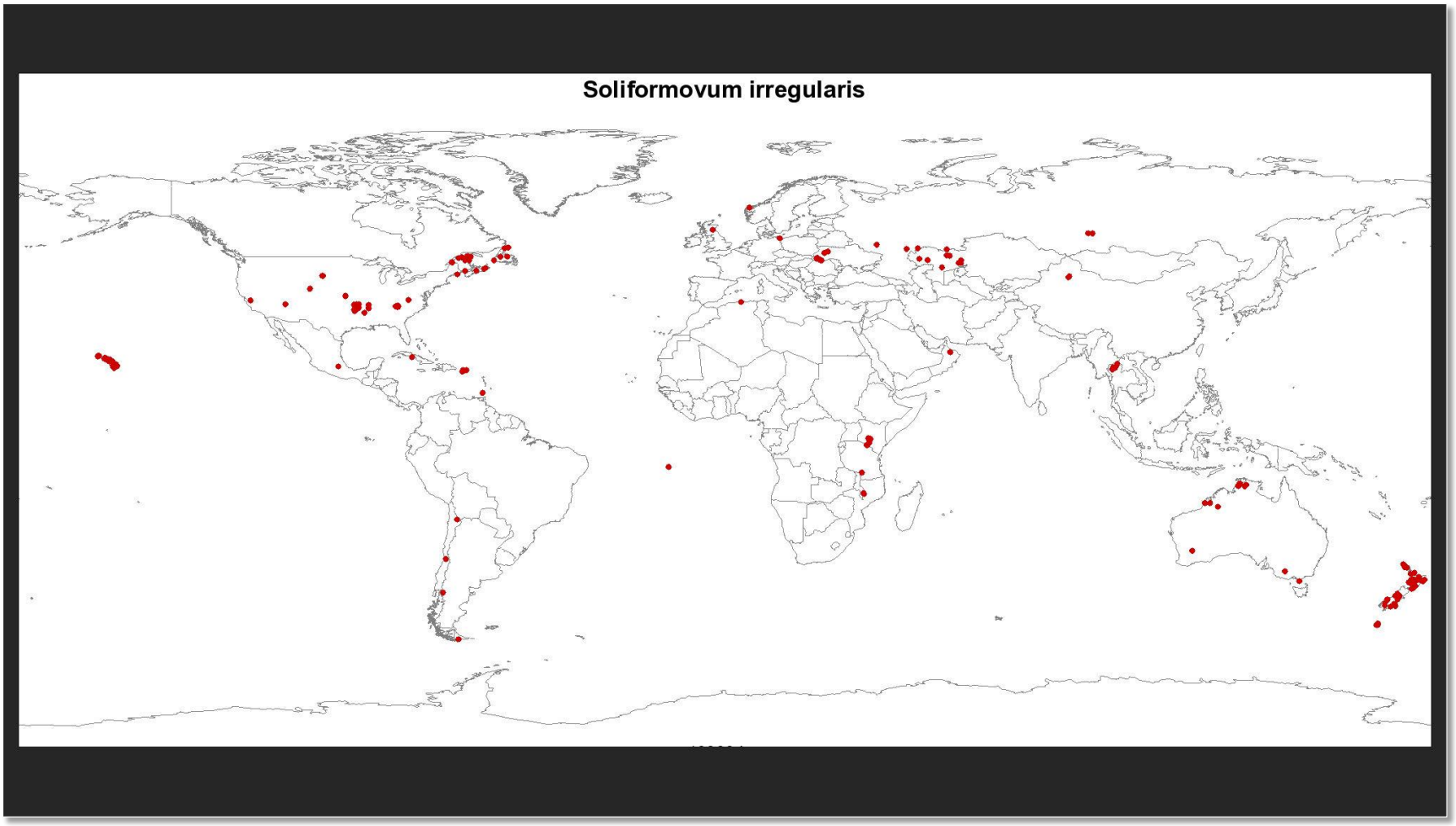
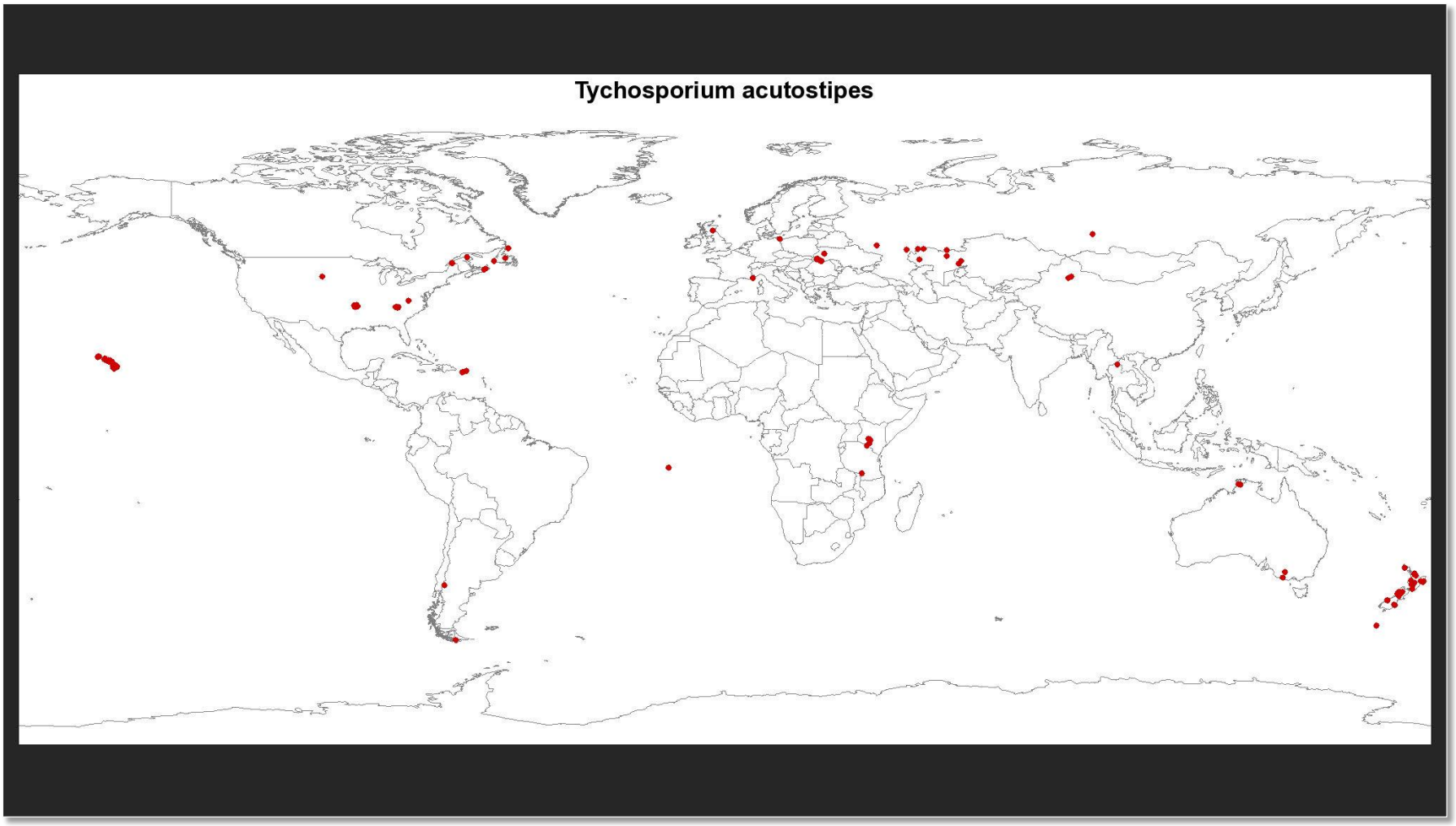

Soliformovum irregularis


Tychosporium acutostipes

Appendix 3.2: Lead Author Confirmation Letter for Paper 2

UNIVERSITY OF ARKANSAS

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter 3, titled "Protosteloid Amoebae as a Flagship Group for Investigating the Global Distribution of Naked Amoebae," of G. L. Zahn's dissertation was submitted as a short communication to *The ISME Journal* in 2015 with coauthors J. D. Shadwick, D. E. Hemmes, & F. W. Spiegel.

I, Dr. Frederick W. Spiegel, advisor of Geoffrey Lloyd Zahn, confirm Geoffrey Lloyd Zahn was first author and completed at least 51% of the work for this manuscript.

Dr. Frederick W. Spiegel
Professor
Department of Biological Sciences
University of Arkansas

Date

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CHAPTER 4

THE EFFECTS OF AMOEBOID PREDATORS ON CARBON AND NITROGEN DYNAMICS DEPEND ON TEMPERATURE AND SOIL STRUCTURE INTERACTIONS

Abstract

Little is known about the role of protozoan predators in regulating soil carbon and nitrogen cycling and, in particular, how these organisms interact with physical and chemical factors to influence a soil community's responses to increased temperature. Using microcosms of simplified bacterial communities, we investigated the net and interactive effects of amoebal predation, soil aggregate structure, agricultural management, and temperature on carbon (C) and nitrogen (N) dynamics. Amoebal predation significantly increased C and N mineralization in all treatments and the magnitude of this effect was significantly influenced by management practices, aggregate structure and temperature. Our findings further confirm the importance of protozoan predation to nutrient dynamics and highlight the importance of further study of these interactions in more natural systems.

Body

Soils cover most of the Earth's terrestrial surface and have an indispensable function in carbon (C) and nutrient cycling. A key component of soils is the assemblage of organisms present, members of which are responsible for carrying out many small scale processes that underlie important biogeochemical functions (Urich et al., 2008). Protozoan predation on bacteria has been shown to be an important factor affecting soil nutrient turnover rates (Coleman et al., 1977; Frey, et al., 1985; Stout, 1980), but the effect of physical and environmental factors on this relationship is poorly understood. This experiment examined the interactive influences of soil physical structure, tillage practices, and warming temperatures on the role that bacterial

predators play in respiration, N-mineralization, and respiratory Q_{10} using one of the most abundant groups of bacterial predators in the soil (Ekelund and Rønn, 1994), the amoebae.

Two well-characterized adjacent allophanic Andisols (Table 4.1) were selected from experimental fields in Tsukuba, Japan (36.024045° N, 140.111558° E) with a mean annual air temperature of 13.7 °C and rainfall of 1300 mm yr^{-1}. One soil received annual tilling (till) and the other received no tilling, but an addition of green manure each year (no-till). More site and soil characteristics have been described elsewhere (Wagai et al., 2013). Each soil was sieved on site to retain aggregates between 4mm and 8mm and then air dried. Plant detritus was manually removed and half of the no-till aggregates were finely crushed by motor and pestle. The three soil treatments (Till [T], Intact no-till [NT], Crushed no-till [NTC]) (Figure 4.1) were then sterilized using ≥36 kGy of gamma radiation. Simple bacterial communities for re-inoculation were obtained by culturing *Escherichia coli* (ATCC #47076) and *Klebsiella pneumoniae* (ATCC #13882) on weak malt-yeast agar (Shadwick et al., 2009), which were then centrifugally washed (10,000 RCF for 10 minutes) three times in Page's Amoeba Saline (PAS) (Page, 1988). These strains were previously shown in a pilot study to grow effectively in both soils and at both temperatures through observation of respiration. Amoeboid predators were obtained by culturing *Dictyostelium discoideum* (strain V12, NBRP, www.nbrp.jp), *Acanthamoeba polyphaga* (ATCC #50372 – originally isolated from Japan) and *Endostelium zonatum* (cultured from the no-till soil *in situ*; identified morphologically using Spiegel et al., (2007); axenized from spores onto heat-killed *E. coli*) on weak malt-yeast agar with the same bacterial inoculum *E. coli* strain (ATCC #47076) as a food source. *D. discoideum* and *A. polyphaga* were originally obtained as axenic cultures and thus did not pose a risk of unwanted bacterial contamination, however, the culture of *E. zonatum* was obtained from the local soil. It is difficult to remove all concomitant bacteria

from natural amoeba isolates and though no bacterial endosymbionts are known from *Endostelium* it is theoretically possible that some contaminants were not removed during axenization. No bacterial growth was observed near axenized *Endostelium* isolates prior to culture with *E. coli* and, barring any undetected contamination, each microcosm treatment received an equivalent inoculum of only the two desired species of bacteria. Cultured amoebae were centrifugally washed (at 500 RCF for 10 minutes) three times in PAS to remove as many *E. coli* cells as possible and all resultant cells in suspension were quantified visually using a hemocytometer. At the conclusion of incubations cultures of soil suspensions were evaluated by bacterial colony morphology on soil agar and only *K. pneumonia* and *E. coli* were observed.

The experiment was factorially designed to interactively investigate temperature, soil type, and predation, such that there were 5 replicate microcosms for each combination of factor levels, and 3 replicates of sterile and unsterilized (natural soil community) controls for each treatment. For each experimental unit, 3g of each sterilized soil was carefully mixed with 12g of fully-combusted sand inside 50 mL septum-sealed microcosm jars under aseptic conditions. Bacterial inoculum (to equal 2.0×10^7 mixed cells $\cdot$ g^{-1} dry soil) was added to all jars except natural community and sterile controls, which received equivalent amounts of sterile PAS, and all jars were incubated in the dark at 15°C for four days to allow bacteria to colonize the substrate (Altenburger et al., 2010). At the end of this initial incubation, soils were brought to 60% water holding capacity using either live amoebal inoculum (to equal 2.9×10^5 cells g^{-1} dry soil) or an equivalent amount of autoclaved amoebal inoculum. Initial CO_2 readings were immediately made using a Li-Cor 7000 Infrared Gas Analyzer (Li-Cor, Lincoln, Nebraska, USA). Half of the jars were then moved to incubate in the dark at 25°C. Subsequent headspace gas measurements were made at 2- or 3-day intervals for 24 days.

Table 4.1. Soil characteristics

Soil (0-5cm)	%C	%N	C:N	pH
Till	5.20	0.42	12.40	6.16
No-till	14.20	0.99	14.30	6.10

Note. Total C and N content, C to N ratio, and pH of each soil. Soils were sampled in May 2013 from long-term experimental plots in the experimental agricultural field at the National Institute for Agro-Environmental Science, Ibaraki, Japan. The no-till plot has been under no-till management for 28 years, including annual addition of green manure at roughly 7 ton C ha^{-1}. The till plot has been under conventional tillage practice.

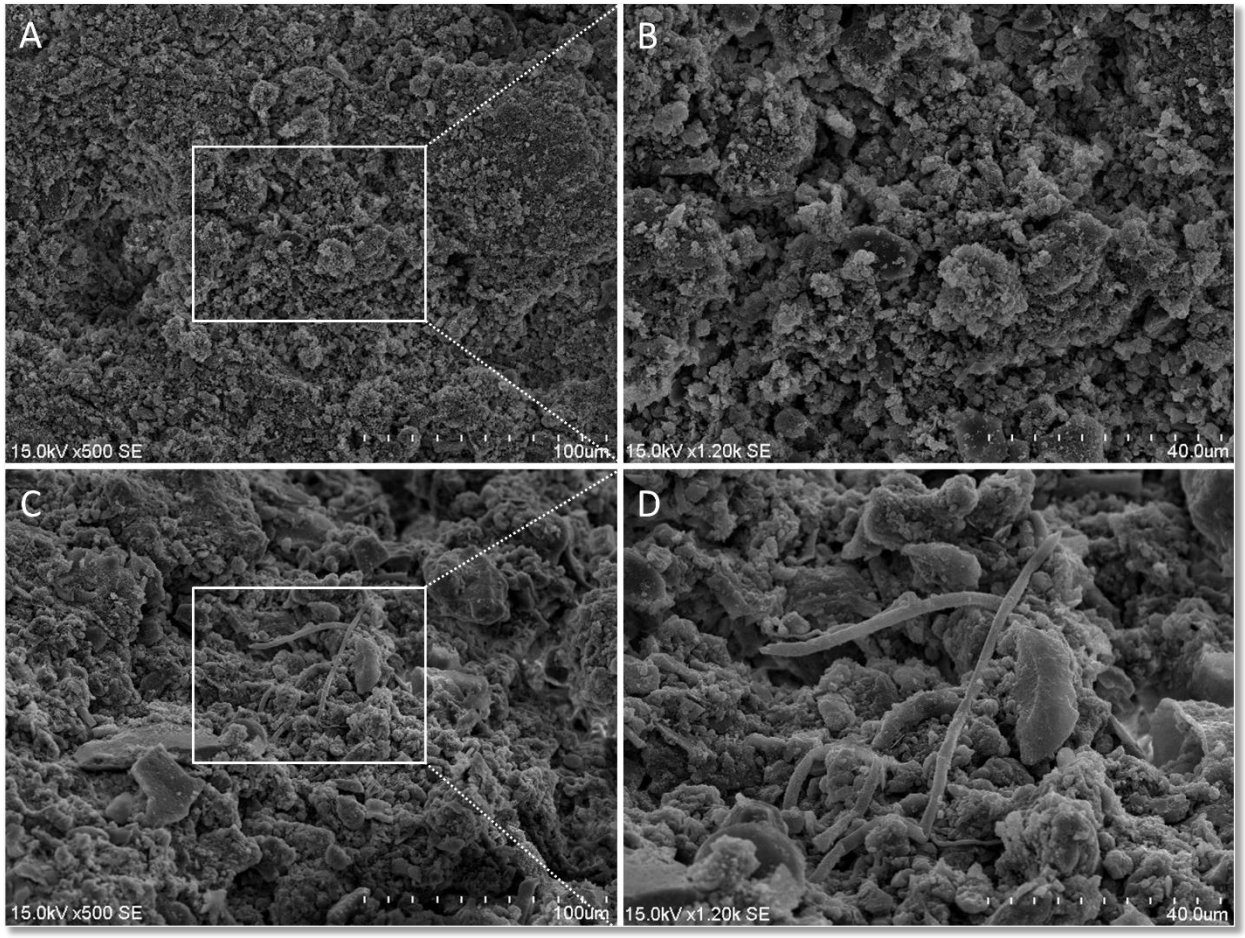

Figure 4.1. SEM of Aggregate surfaces. Scanning electron micrographs of the aggregate surfaces of both soils. A=No-till soil at 500X magnification; B=No-till at 1200X; C=Till soil at 500X; D=Till at 1200X.

The presence of amoebae significantly increased the amount of cumulative C respired in all treatments (Figure 4.2) ($P \leq 0.01$; General linear model ANOVA). These results are consistent with others' (Clarholm, 1981; Frey et al., 2001; Murase et al., 2006; Rønn et al., 2012) and offer further support for the applicability of the "microbial loop" concept to nutrient mineralization in soil systems (Adl and Gupta, 2006). This increase in respiration was interactive with management practice, temperature, and soil structure ($P=0.023$; Figure 4.3). At the higher temperature, an effect of aggregate structure became apparent, with predation contributing to a greater increase in respiration in crushed soil than in intact aggregates ($P<0.0005$; General linear model ANOVA). This suggests that the efficiency of amoebal predation is influenced by an interaction between temperature and soil physical structure, and implies that the distribution of habitable pore space may be limiting to predators (eg. Griffiths and Young, 1994; Rutherford and Juma, 1992).

The influence of amoebal predators on respiratory Q_{10} was inconsistent between two soil types in artificial communities (Figure 4.4). All three natural community treatments, however, showed a consistent Q_{10} of around 2, the value most commonly used as a constant in ecosystem models (Chen and Tian, 2005), illustrating a potential danger of extrapolating inferences from artificial communities to complex natural systems.

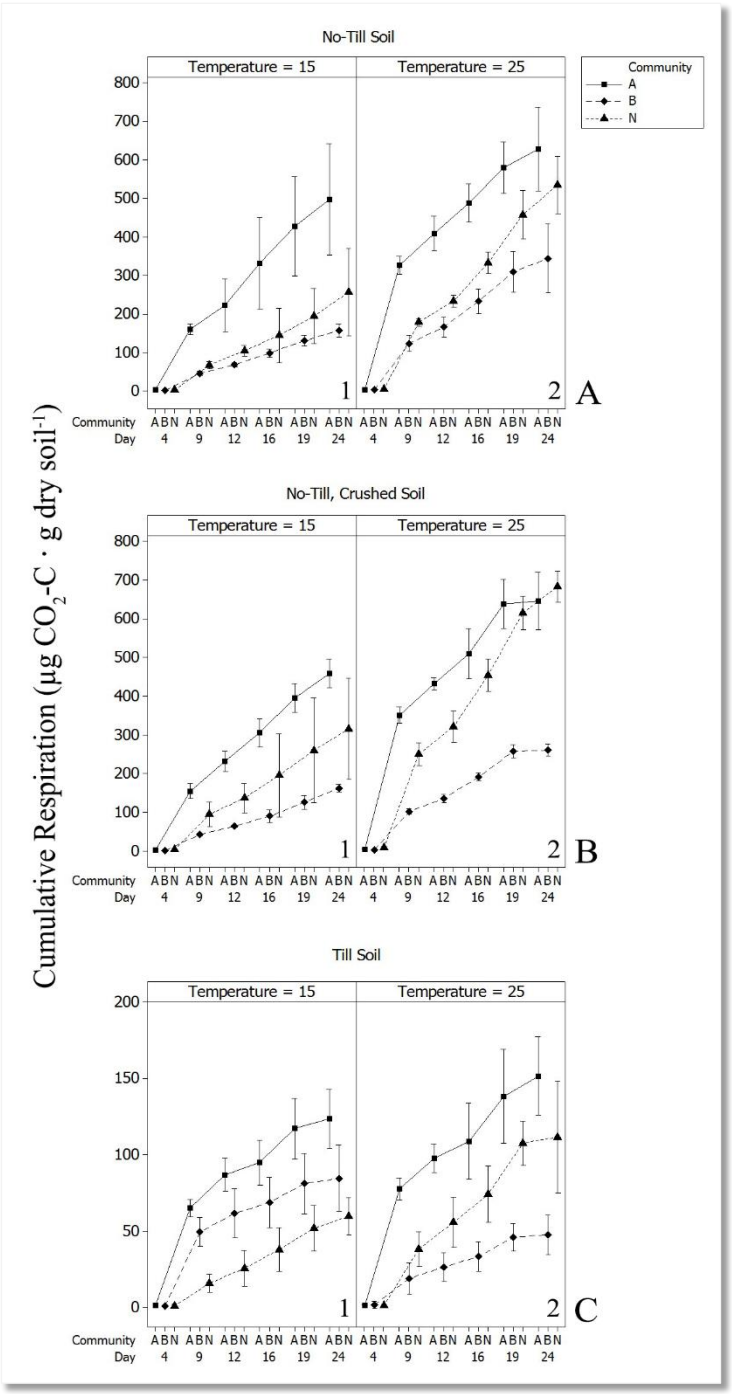

Figure 4.2. Cumulative respiration throughout incubations. Cumulative respiration (µg CO_2-C · g dry $soil^{-1}$) for each treatment group (sterile controls not included), with soil treatments separated into three main panels: A=Intact no-till soil, B=Crushed no-till soil, C=Intact till soil; Temperature treatments as sub-panels: 1=15°C, 2=25°C; Community inoculum treatments: A (square symbols)=artificial community + predators (amoebae), B (diamond symbols)=artificial community with no predators (bacteria only), N (triangle symbols)=natural community controls. Error bars denote 95% confidence intervals for the mean. Note y-axis scale in panel C.

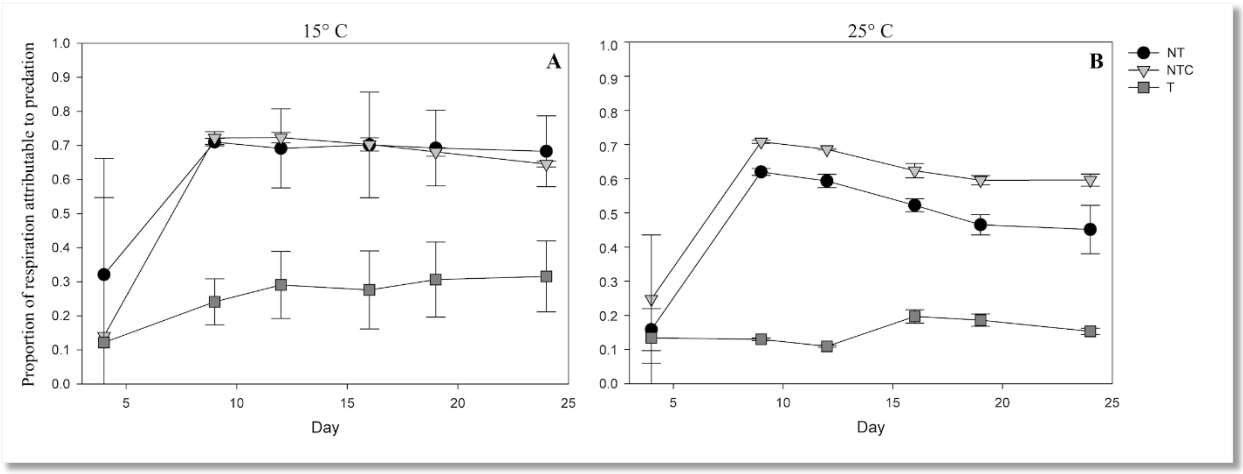

Figure 4.3. Proportion of respiration attributable to predation. The proportionate increase in respiration due to the introduction of predators (the difference between the two predator treatments divided by the maximum respiration at each sample period) for each soil (circle=intact no-till aggregates, triangle=crushed no-till aggregates, square=till aggregates) and temperature (panel A=15°C, panel B=25°C). The proportion of respiration attributable to predation was higher in no-till soils than tilled soil and was influenced by crushing, but only at the higher temperature. Error bars represent propagated 95% confidence intervals about the mean of cumulative respiration.

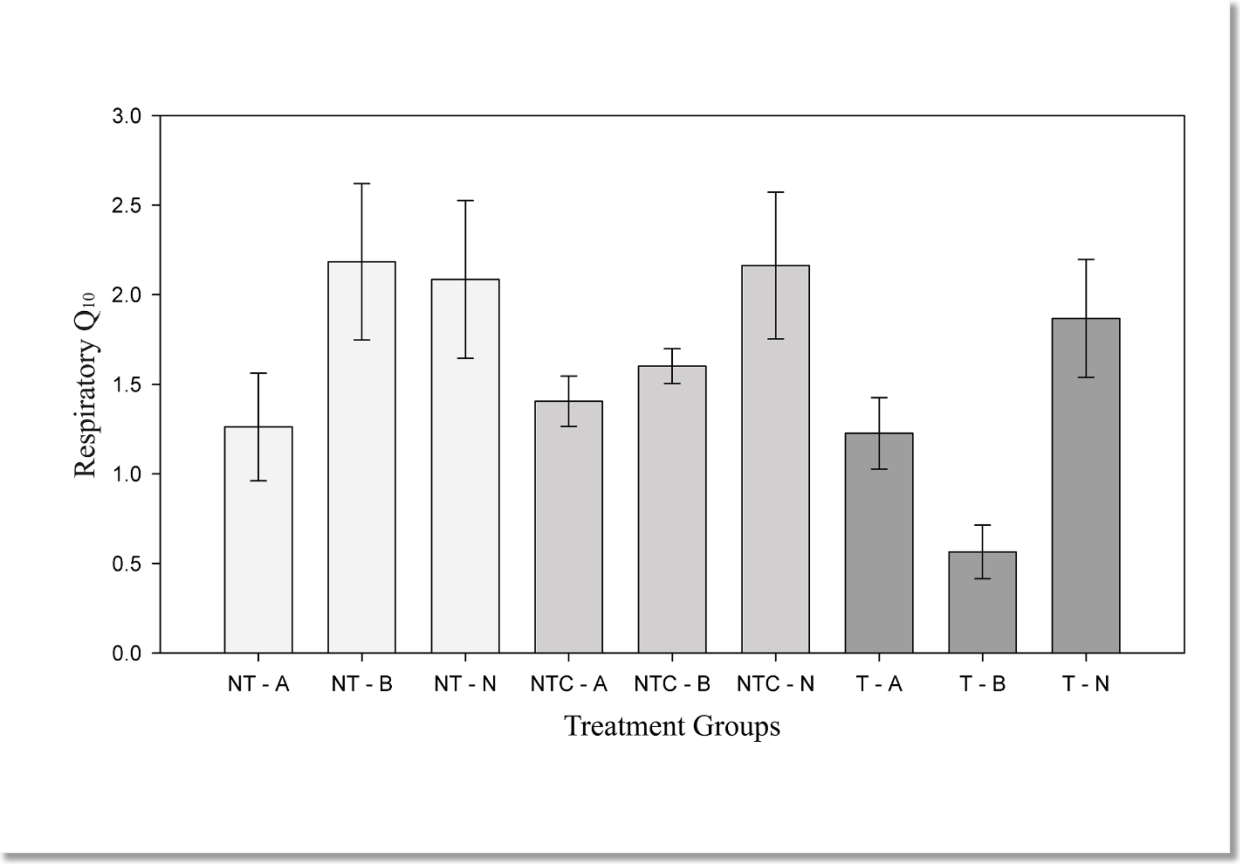

Figure 4.4. Temperature coefficient (Q_{10}) of treatments. The respiration Q10 for each treatment group. NT=Intact no-till soil, NTC=Crushed no-till soil, T=Intact till soil; A=artificial community + predators (amoebae), B=artificial community with no predators (bacteria only), N=natural soil community. Error bars represent propagated 95% confidence intervals for the mean.

Net nitrogen transformations displayed a similar discrepancy between natural and artificial communities. After incubation, both simplified communities showed a net loss of NO_3-N (this was expected due to the absence of any nitrifying taxa) while the natural controls showed significant gains, except for in tilled soils. Natural controls showed a substantial net decrease in NH_4-N while both simplified communities displayed a net increase. In artificial communities, the presence of predators resulted in significantly higher net ammonification of N ($P<0.0005$, One-way ANOVA). These results are consistent with previous work (Frey et al., 1985; Weekers et al., 1993; Woods et al., 1982) which demonstrated the ability of amoebae to stimulate N mineralization by stimulating the turnover of bacterial biomass. Our study further showed that the magnitude of this effect depends on soil structure and temperature. The disaggregation effect on predation-induced mineralization in the no-till soils was greater under the warmer condition for both C (Figure 4.3) and N mineralization (Figure 4.5), suggesting the coupling of C and N mineralization.

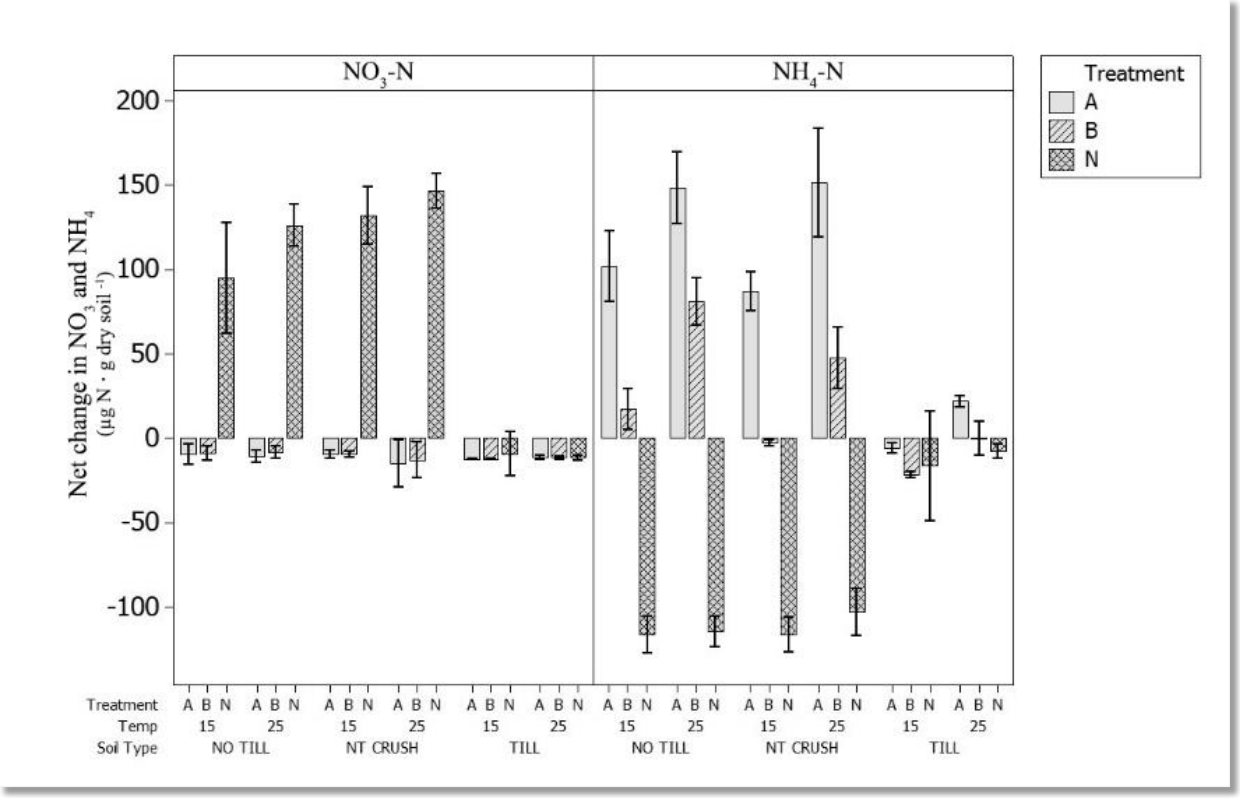

Figure 4.5. Net nitrification and ammonification. The net change (µg N · g dry soil^{-1}) in NO_3 and NH_4 for each treatment after incubation. NT=Intact no-till soil, NTC=Crushed no-till soil, T=Intact till soil; A=artificial community + predators (amoebae), B=artificial community with no predators (bacteria only), N=natural soil community; Temperature in °C.

When studying complex systems such as soil, a trade-off exists between the ability to control factors and the applicability of results to real world situations. This discrepancy was apparent in the Q_{10} values of the current study, but the ability to precisely control climate and community variables allowed us to detect an interaction between temperature, soil structure, and the effect of predator-prey interactions on C and N mineralization. Ours and other studies (Anderson, 2012; Wilkinson, 2008) highlight the need to obtain and incorporate these community-structure data into models of nutrient cycling, but care should be given to the environmental factors such as soil structure and temperature that influence species interactions. The microcosm methods presented here (and within the referenced literature) provide a useful system for mechanistically investigating the factors responsible for changes in C and N cycling dynamics. Further work should focus on testing the interactions between soil structure, temperature, and predation with more complex, natural community assemblages. Incorporating similar microcosm methods with high-throughput sequencing would bring a deeper understanding of the ways in which protist predators interact with environmental parameters to influence complex and uncultured bacterial communities.

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Appendix 4.1: Lead Author Confirmation Letter for Paper 3

UNIVERSITY OF ARKANSAS

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter 4, titled "The Effects of Amoeboid Predators on Carbon and Nitrogen Dynamics Depend on Temperature and Soil Structure Interactions," of G. L. Zahn's dissertation was submitted as a short communication to *Soil Biology and Biochemistry* in 2015 with coauthors S. Yonemura, & R. Wagai.

I, Dr. Frederick W. Spiegel, advisor of Geoffrey Lloyd Zahn, confirm Geoffrey Lloyd Zahn was first author and completed at least 51% of the work for this manuscript.

Dr. Frederick W. Spiegel
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Department of Biological Sciences
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Date

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CHAPTER 5

TOP-DOWN CONTROL OF SOIL BACTERIAL COMMUNITIES BY AMOEBAL PREDATION IS INFLUENCED BY TEMPERATURE

Abstract

The interactive roles of protist predation and increasing temperature in shaping bacterial communities and soil respiration were investigated using a combined microcosm and high-throughput sequencing approach. Protist predators were successfully filtered from soil suspensions and sterile soils were re-inoculated with and without amoeboid predators. Microcosms were incubated at 15°C and 20°C for 30 days and community composition was determined both before and after incubation by the sequencing of 16S SSU rDNA amplicons. Cumulative respiration was also observed. Soils containing amoebae had significantly higher respiration and significantly altered bacterial communities. The effect of predation on bacterial taxa was dependent on temperature. Efforts to model the effects of climate change on bacterial communities should not overlook the protist components of those communities.

Introduction and Background

Understanding the local processes that govern global patterns in carbon (C) cycling is a central goal in biogeochemistry. Beneath our feet, three fourths of the earth's terrestrial C (Whitman, 1998) is tied up in a dynamic web of microbial interactions as part of complex ecosystems called soils. Soils play a critical role in the regulation of the global carbon budget and predicting the fate of this carbon in a warming climate has become a major objective of recent research efforts.

The immense complexity of soil communities (Bailly *et al.*, 2007) and extreme difficulty of obtaining unequivocal data from field studies has historically encouraged the use of biogeochemical models that treat soil ecosystem processes as a "black box," with relatively little

attention paid to community or organism level dynamics (Kennedy and Smith, 1995). More recently, efforts have been made to escape from this black box approach and mechanistically investigate microbe-mediated processes in relation to the members present in the community. Working to understand the interactions of these individuals is helping to shine a light into the black box of soil systems and illuminate some of the fundamental processes occurring there.

One of the most important processes occurring in soils is the C cycle. Soil organic C is the largest reservoir of C in the so-called "fast C cycle" on earth (Ciais et al., 2014) and soils are the ultimate destination of the vast majority of photosynthetically-fixed C in terrestrial ecosystems (Rodriguez-Murillo 2001). Eventually, this fixed C (organic matter) is decomposed by soil biota (mainly bacteria and fungi) and returned to the atmosphere as CO2 (a greenhouse gas) but decomposition rates are controlled by a variety of factors including microbial activity and climate (Trumbore, 1997). As global climate change has become a pressing reality, there has been much concern over the fate of soil C under warming conditions. It has been established that any increase in temperature leads to exponentially greater rates of CO2 losses from soil to the atmosphere (Fang and Moncrieff, 2001) and there is much debate over whether soils may enter a positive feedback loop and become a net source of greenhouse gasses globally (Kirschbaum, 1995; Zhou, et al., 2009) leading to strengthened global warming scenarios (Davidson, et al., 2000). A comparison among global C cycle models revealed a severe discrepancy in terms of future warming effects on soil C decomposition rates (Friedlingstein *et al.*, 2006), suggesting a strong need to better understand the decomposition process, its temperature sensitivity, and the factors that influence decomposer community structure.

Despite active research in the past two decades (Yuste et al., 2007; Fang & Moncrieff, 2001; Monson et al., 2006; Schlesinger & Andrews, 2000; Trumbore, 1997) the factors

controlling temperature sensitivity of soil C decomposition (often expressed as Q_{10} – proportional increase in CO2 released by soil heterotrophic microbes for a 10°C increase in temperature) remain poorly understood (Conant et al., 2011; Davidson, et al., 2006). While soil C quality, soil temperature/moisture, and carbon input rates have been shown to affect Q_{10}, how soil fauna and their predation on bacteria and fungi affects overall soil C decomposition Q_{10} is understudied. Specifically, while the direct roles of certain bacterial and fungal groups have been given considerable attention (Yiqi and Zhou, 2010), far fewer studies (e.g. Adl & Gupta, 2006; Fitter et al., 2005; Roger Anderson, 2008; Stout, 1980) have addressed the influence of protists within these models.

Among the protist predators in soils, amoebae are typically the most abundant bacterivores globally (Anderson, 2010; Clarholm, 1981; Urich *et al.*, 2008) and commonly range in number from 10^4 (Clarholm, 1981) to 10^5 (Ekelund and Rønn, 1994) cells per gram of soil. Their specialized motility and feeding modes give them access to the majority of bacteria in soil, in contrast to other groups which are more restricted by pore size limitations (Elliott *et al.*, 1980) or decreased soil water potential (Young and Ritz, 2000). When active, amoebae can act as a major selective influence on bacterial communities (Rønn et al., 2002) and are, perhaps, a major underlying mechanism of the stimulation of nitrogen and C mineralization as suggested by Bonkowski (2004).

There has been a fair amount of research on the net effects of protist predation in soil systems. For example, amoebal predation has been correlated to increased ammonification in field studies (Weekers, et al., 1993) and has been shown to be causative for this effect in microcosm experiments (Rutherford and Juma, 1992). Additionally, microcosm studies have shown that amoebae strongly increase C mineralization rates (Clarholm, 1981) and that this

effect is interactive with temperature (Zahn, et al., Unpublished – In review) which may have implications for climate change modeling. The possible specific mechanisms for these effects (*eg.* selective grazing, sloppy feeding, etc.) and their interactions with environmental conditions have received considerably less attention.

Microcosms are a useful method for addressing mechanistic questions about complex processes, but they come with major tradeoffs in their applicability to real-world systems since it is not possible to accurately replicate field conditions in the lab. These controlled systems often rely on grossly simplified communities and/or artificial "soils," which limit their applicability to field predictions. One major hurdle to investigating the role of protist predation on natural bacterial populations has been in obtaining undefined natural populations of bacteria *sans* protists. Frey et al. (1985) developed a simple filtration method that progressively size-excluded eukaryotic predators from soil suspensions to examine the effect of protist morphotypes on nutrient dynamics and bacterial abundance. Here we adapt this filtration method and apply high-throughput sequencing to observe the effects of common soil amoebae on "natural" bacterial communities and soil respiration under warming conditions in a structurally-intact soil.

This study focused on testing three main hypotheses: 1) Amoebal predation would increase cumulative respiration over the course of the incubation; 2) Predation would exert a significant top-down control on bacterial community composition; and 3) This predator-induced effect on bacteria would interact with temperature. Additionally, this study sought to generate hypotheses regarding the mechanisms for expected increases to respiration. The prediction that predation would enhance soil respiration has been widely supported in the literature but this aspect was included for confirmation and to provide additional proof-of-concept for the filtration method of obtaining predator-free bacterial communities. Linking this community manipulation

method with high-throughput sequencing of the bacterial 16S marker gene enables an investigation into which bacterial taxa are differentially influenced by predation without the known limitations of culture-based observations and may provide some insight into the mechanisms responsible for the observed increases in C and N mineralization associated with protist predation.

Methods

Soil selection and preparation

The soil used in this study was from the O-horizon (0-10cm) of a riparian forest site in Northwestern Arkansas (35.994654, -94.131481; Table 5.1). The soil was sieved on site and 2-4mm aggregates were brought back to the lab for processing. Aggregates were oven-dried at 85°C, extraneous organic material was manually removed and then aggregates were subjected to three rounds of autoclaving (60 min, 121 °C, 15 PSI). Between each autoclave treatment, soils were brought to 50% water holding capacity (WHC) with sterile water and allowed to incubate for three days at 20°C. After the second round of autoclaving, 5g equivalent dry weight of autoclaved soil was added to 48 sterilized 125ml septa-sealed microcosm jars (company) and allowed to incubate for five days. Jars containing this soil were then autoclaved again and oven dried at 100°C for three days.

Table 5.1. Soil chemical properties

Property	Value
Total exchange capacity (meq/100 g)	23.20
pH	6.30
Organic Matter (%)	6.61
NO3-N (ppm)	2.20
NH4-N (ppm)	31.00
Carbon (%)	4.32
Nitrogen (%)	0.28
C/N Ratio	15.43
Anions	
S[a] (ppm)	29.00
P[a] (ppm)	29.00
Exchangeable cations	
Ca[a] (ppm)	3463.00
Mg[a] (ppm)	219.00
K[a] (mg/kg)	139.00
Na (mg/kg)	20.00
Base saturation	
Ca[b] (%)	74.63
Mg[b] (%)	7.87
K[b] (%)	1.54
Na[b] (%)	0.37
Other bases[b] (%)	5.10
H[b] (%)	10.50
Extractable minors	
B[a] (mg/kg)	0.83
Fe[a] (mg/kg)	124.00
Mn[a] (mg/kg)	331.00
Cu[a] (mg/kg)	2.66
Zn[b] (mg/kg)	4.49
Al[a] (mg/kg)	542.00

[a] Mehlich III extractable elements

[b] Percent of a given element found in the soil's total exchange capacity. Soil was analyzed after autoclave treatments.

Microbial inoculum preparation

Bacterial inoculum was obtained by shaking 50g of unsterilized soil in sterile water for 6 hours. This suspension was allowed to settle for 24 hours and then the supernatant was filtered through 10µm nuclepore filter membranes (Whatman, Piscataway, NJ) to remove extraneous organic matter and larger particles. This inoculum was used for the "natural community" control. Frey et al. (1985) noted that filtration through 3µm pores effectively reduced protist numbers to undetectable levels for up to 80 days of incubation but, in order to more confidently remove predators, we subjected portions of our "natural community" filtrate to further filtration through 1.5µm nuclepore membranes. This 1.5µm filtrate was observed microscopically and subjected to PCR amplifications with the F-566 and R-1200 primer pair from Hadziavik et al. (2014) to ensure that no significant contamination with eukaryotic predators was present and then used as a "predator-free" bacterial inoculum. This filtered inoculum represented, as closely as possible, a natural undefined bacterial community *sans* protist predators.

Amoebae were isolated from the unsterilized soil aggregates using a modified version of Cavender's method (Cavender and Raper, 1965), axenized over several generations on dead *E. coli* cells and then cultured on weak malt yeast agar (Shadwick et al., 2009) with live *E. coli* as a food source. Three distinct isolates of dictyostelid amoebae, identified morphologically and phylogenetically via sanger sequencing of the 18S rDNA marker (Table 5.2), were used. Once there was sufficient growth to obtain enough cells for inoculation into microcosms, amoebae were centrifugally washed three times with sterile Page's amoeba saline (Page, 1988) to remove *E. coli* cells, quantified using a hemocytometer, and mixed together. *E. coli* cells remaining after washing were also quantified so that equivalent amounts could be added to microcosms receiving no live amoebae. Half of the pooled amoebal inoculum was autoclaved and then mixed

with equivalent amounts of live *E.* coli, for use as secondary inoculum in the predator-free treatments.

Experimental design and incubation

Under aseptic conditions, each sterilized microcosm (N=28) was brought to 40% WHC with 1.5µm filtrate, except the natural controls (N=18) which were inoculated with an equivalent amount of 10µm filtrate and sterile controls (N=2) which were inoculated with autoclaved 1.5µm filtrate (T=Day -4). All microcosms were sealed and incubated (half at 15°C, and half at 20°C) for four days to allow bacteria to colonize the soil (Altenburger et al., 2010). Temperatures were chosen to reflect the current and predicted 100-year mean annual temperatures for the region (Barros et al., 2014).

After this initial incubation, four replicates of each group (N=4) were pooled and destructively sampled for nucleic acids to obtain a snapshot of the initial bacterial communities for 10µm and 1.5µm treatments at each temperature (referred to hereafter as the initial communities). For the remaining jars, half of the "predator-free" units (N=10) were brought to 50% WHC with inoculum containing viable amoebae and the other half (N=10) along with natural controls (N=10) were given autoclaved amoebae. Each jar was immediately sealed after inoculation and kept in the dark at its respective temperature except for brief headspace gas sampling.

Table 5.2. Amoebal predators

Species name	Inoculum concentration (cells · g soil-1)	Relative starting abundance	DDBJ Accession No. (18S)
Dictyostelium purpureum	45866	0.47	LC056032
Dictyostelium aureostipes	29498	0.31	LC056033
Dictyostelium mucoroides	21495	0.22	LC056034

Note. Quantification of amoebal cells in inoculum along with DDBJ accession numbers; Number of cells quantified microscopically using a hemocytometer; Relative abundance indicates the proportion of each species in the final mixed inoculum. Equal amounts of inoculum was added to each jar, but the inoculum added to natural controls and predator-free treatments was autoclaved; No viable amoebae were found in autoclaved inoculum

Headspace gas analyses

Immediately after sealing (T=0), 5ml of headspace gas was removed and injected into a helium-purged vacuum vial using a gastight syringe. Subsequent samples were taken on days 4, 9, 14, and 30. The syringe was sterilized with ethanol between treatment groups. Vials containing headspace samples (N=160) were analyzed on a GasBench II gas chromatograph (ThermoScientific). CO_2 concentrations were converted to μg CO_2-C · g dry $soil^{-1}$ using ideal gas law. Q_{10} values for cumulative respiration were calculated from final headspace readings according to Lloyd and Taylor (1994) and 95% confidence intervals were calculated by error propagation.

DNA extraction and amplicon generation

DNA was destructively extracted from soil just after the initial incubation (T=0 days) and just after the final headspace measurement (T=30 days) using the same protocol. Each of the four replicates in the initial extractions and each of the five replicates in the final extraction were pooled together, homogenized, and then a dry weight equivalent of 4.85g of soil was subsampled for extraction using the PowerMax Soil DNA Isolation Kit (MoBio, USA), and eluted into 5ml of 10mM TRIS. Due to low concentrations, DNA from each group was then concentrated via ethanol precipitation, eluted into 100μl of deionized water, and quantified using a PicoGreen® assay on a NanoDrop 3300 Fluorospectrometer (Thermoscientific).

Genomic DNA was used to generate amplicons of the third hypervariable region (V3) of the 16S rDNA gene via PCR using the HotStar HiFidelity Polymerase Kit (Qiagen, USA) with 1μg of DNA template, and primers 338F* and 533R* (Ong et al., 2013). Reaction conditions were as follows: initial denaturing step at 94°C for 5 min, 30 cycles of denaturing at 94°C for 30 sec, primer annealing at 59° for 30 sec and extension at 72° for 45 sec, followed by a final

extension step at 72°C for 5 min. Amplicons from three separate reactions were pooled for each sample.

Library preparation and sequencing

Pooled amplicons from each sample were purified with the Wizard® SV Gel and PCR Cleanup System (Promega, Madison, WI) and quantified using a PicoGreen® assay as before. Libraries were prepared using the NEBNext® Fast DNA Library Prep Kit (New England BioLabs, Inc.). Each sample library was barcoded and randomly assigned to one of two sequencing chips on the IonTorrent PGM platform (Life Technologies, Grand Island, NY). Samples for each chip were mixed in equimolar concentrations and sequencing templates were prepared on the IonTorrent OneTouch 2™ system. Sequencing runs were carried out on Ion 314™ V2 chips and, subsequently, identical runs were carried out on Ion 316™ V2 chips (4 chips total). Both runs were concatenated by sample before downstream processing.

Taxonomic structure

Raw sequences were uploaded into the Metagenome Rapid Annotation using Subsystem Technology (MG-RAST v3.5) bioinformatics server (Meyer et al., 2008), where they are also publicly available (Table 5.3) for initial analyses. Reads were filtered based on length and quality, with a minimum size of 120bp and minimum quality score of 15 (Blankenberg et al., 2010). All reads with ambiguous bases were removed. All analyses within MG-RAST were conducted using the following parameter settings: the Greengenes 13_5 (McDonald *et al.*, 2012) annotation source, maximum e-value = 1.0^{-20}, minimum identity cutoff = 97%, minimum alignment length cutoff = 50 bp. Quality-screened sequences were also exported for taxonomic assignment and downstream analyses within QIIME (Caporaso et al., 2010) for comparison. To ensure conservative estimates of taxonomic and functional diversity, singleton OTUs were removed prior to all downstream analyses. Within QIIME, OTUs were picked using the

Table 5.3. MG-RAST project IDs and stats

MG-RAST ID	Metagenome name	bp count	Raw sequence count	Post QC sequence count	MG-RAST GG species	MG-RAST M5RNA species	QIIME GG species
4636404.3	15_nat3	64,181,395	355,303	220,272	204	359	774
4636405.3	15_no_pred3	68,018,167	373,167	259,104	206	360	645
4636406.3	15_plus_pred3	85,227,814	480,203	395,433	249	440	829
4636407.3	20_nat3	148,137,858	865,283	712,390	271	449	928
4636408.3	20_no_pred3	84,699,698	468,364	390,240	250	421	744
4636409.3	20_plus_pred3	110,046,641	600,559	409,636	275	453	837
4636410.3	init_15_filt3	72,980,164	401,246	348,459	198	350	643
4636411.3	init_15_unfilt3	123,526,461	678,879	563,412	261	437	786
4636412.3	init_20_filt3	63,034,159	348,307	261,162	206	370	604
4636413.3	init_20_unfilt3	58,018,295	318,885	210,781	213	369	719
Total		877,870,652	4,890,196	3,770,889	539	1021	2101

Note. Species richness and Phylogenetic Distance measures represent the mean values from 10 iterations of rarefied data at depth of 131533 reads.

UCLUST algorithm (Edgar, 2010) and assigned to the GreenGenes 13_8 taxonomy at 97% similarity with a maximum e-value of 1.0^{-20}.

Statistical comparisons of taxonomic structure were performed on assigned taxa within STAMP (Parks et al., 2014) using Fisher's exact test and p-values were false discovery rate adjusted for multiple comparisons (Storey, 2002).

Functional predictions

Picked OTUs assigned to taxonomy via the Greengenes 13_8 database were normalized by copy number and used to predict metagenomes using the PICRUSt tool (Langille et al., 2013). Predicted metegenomes were categorized by function to the KEGG hierarchy within PICRUSt and analyzed using STAMP. All between-sample comparisons in STAMP used Fisher's exact test (Rivals et al., 2007) and p-values were false discovery rate adjusted for multiple comparisons (Storey, 2002).

Results

Sequencing stats and completeness

Sequencing produced a total of 4,890,196 raw reads. After filtering for quality and removing singletons 3,770,889 high quality observations of the V3 16S ribosomal RNA gene region remained. These observations resulted in varying numbers of database hits depending on the analysis pipeline used. Within MG-RAST there were 2,070,499 hits against the Greengenes database and 2,436,577 hits against the M5RNA database. Within QIIME there were 3,078,244 hits against the Greengenes database. This variation in OTU assignment efficiency also resulted in different taxonomic richness (Table 5.3). All further analyses were carried out using the QIIME assignments since they have been shown to be more accurate than MG-RAST for 16S amplicon data (D'Argenio *et al.*, 2014).

Overall sequencing thoroughness was examined for each sample via species-level rarefactions within QIIME (Figure 5.1). Nine families contained nearly 62% of all observed OTUs. The most dominant taxa in each sample were consistently Pseudomonadaceae, Flavobacteriaceae, Caulobacteraceae, and Paenibacillaceae.

Effects of filtration

Filtration was highly effective at removing protist predators. No protist cells were microscopically observed in the 1.5µm filtrate, and no 18S rRNA bands were detected in PCR observations. Serially-diluted filtrate plated on nutrient agar showed bacterial colony growth but no fungal or protist observations were noted. This culture-based assessment is limited in its usefulness, but in conjunction with direct visual inspection of the filtrate and 18S amplification, suggests that protist predators were at least effectively reduced to undetectable levels in the "predator-free" inoculum, an outcome consistent with others who have applied similar filtration methods to remove protists (Rosenberg et al., 2009; Sauret et al., 2015). Protists were noted by all observation methods in the 10µm "natural community" filtrate.

Filtration had a strong impact on bacterial community composition (Figure 5.2). Notably (difference of ≥3%; $P<0.005$), reducing filter pore size from 10µm to 1.5µm increased the relative abundance of Flavobacteriaceae and Bacillaceae and decreased the relative abundance of Sphingobacteriaceae at both temperatures. Filtration also decreased the relative abundance of Pseudomonadaceae, but only in the 20°C treatment.

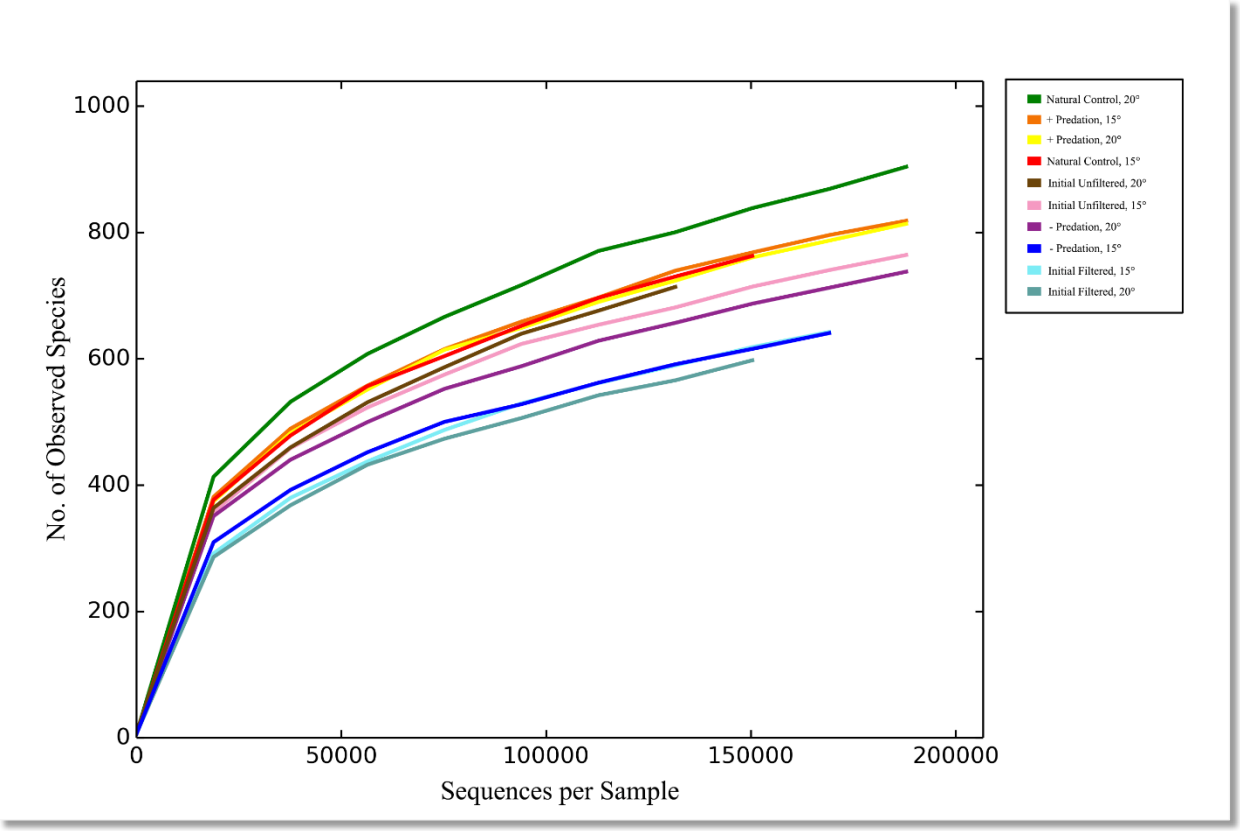

Figure 5.1. Species-level rarefactions for each sample. Rarefactions were performed from a minimum of 10 sequences to 187900 sequences (the median number of sequences observed across all samples, as per the default parameters in QIIME for the *alpha_rarefaction.py* script).

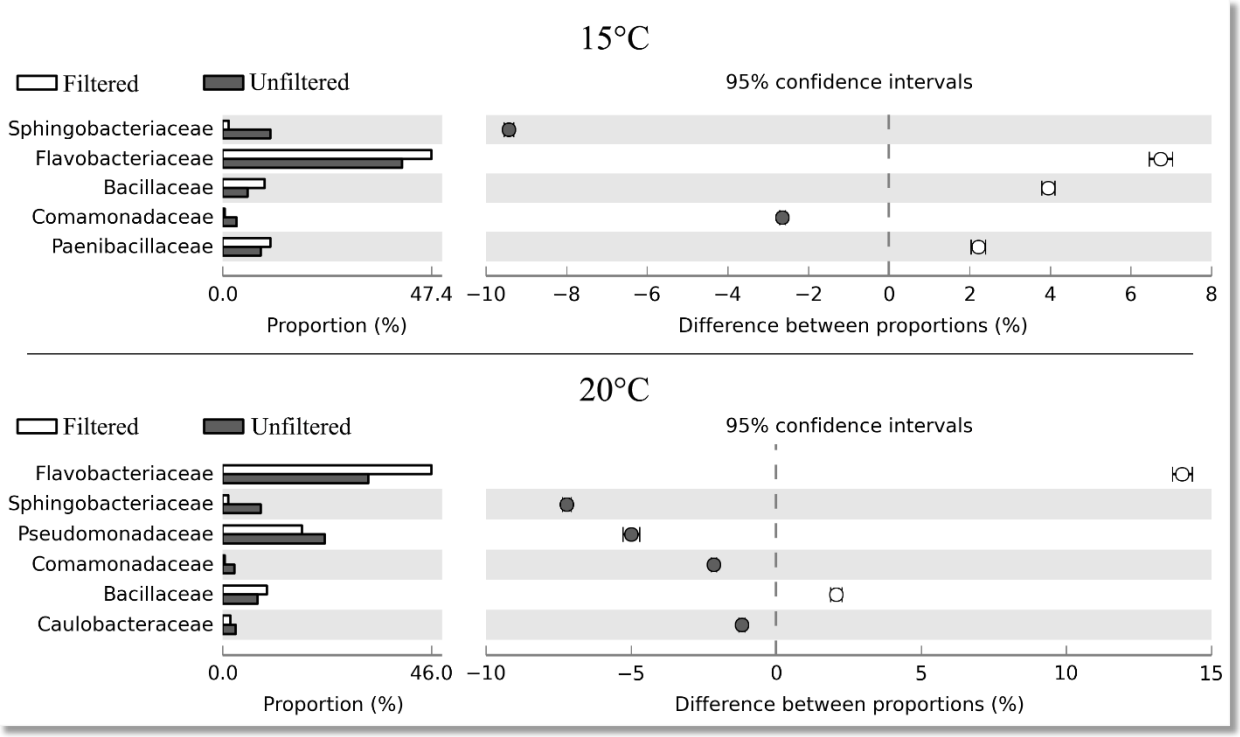

Figure 5.2. Effect of filtration on bacterial taxa. Extended error bar plot showing all significant differences (Fisher's exact test; Storey's FDR correction; $p<0.05$) between family-level taxa with an effect size of at least 2% difference in normalized abundance.

Cumulative respiration

As predicted, cumulative respiration was significantly greater in treatments containing protist predators. Warming significantly increased respiration in all community treatments (Figure 5.3), but this effect was interactive with the presence of predation, with predator treatments and natural controls responding more strongly to warming than predator-free treatments (General Linear Model ANOVA; $P<0.005$). The treatments with added amoebae did not differ significantly from natural controls.

Q_{10} values were not significantly different between community treatments (One-way ANOVA; $P=0.821$; Figure 5.4). All Q_{10} values were within range of 2.0, the most commonly used value of for models of soil respiration (Chen and Tian, 2005) and are consistent with values reached from previous microcosm work using simplified bacterial communities (Zahn et al., Unpublished).

Taxonomic changes in the communities

Samples at both temperatures grouped together well by treatment in the PCoA analysis, with marked differences between communities before and after incubation **(**Figure 5.5**)**. This temporal effect had the largest magnitude, followed by filtration, and then predation. Temperature had a relatively small effect on communities.

There were no direct significant effects of warming on relative abundance of bacterial taxa. This has been seen before in another study where it took 20 years for soil communities to noticeably respond to warming (DeAngelis et al., 2015), though temperature did show an interaction with both temporal change and predation on several key taxa.

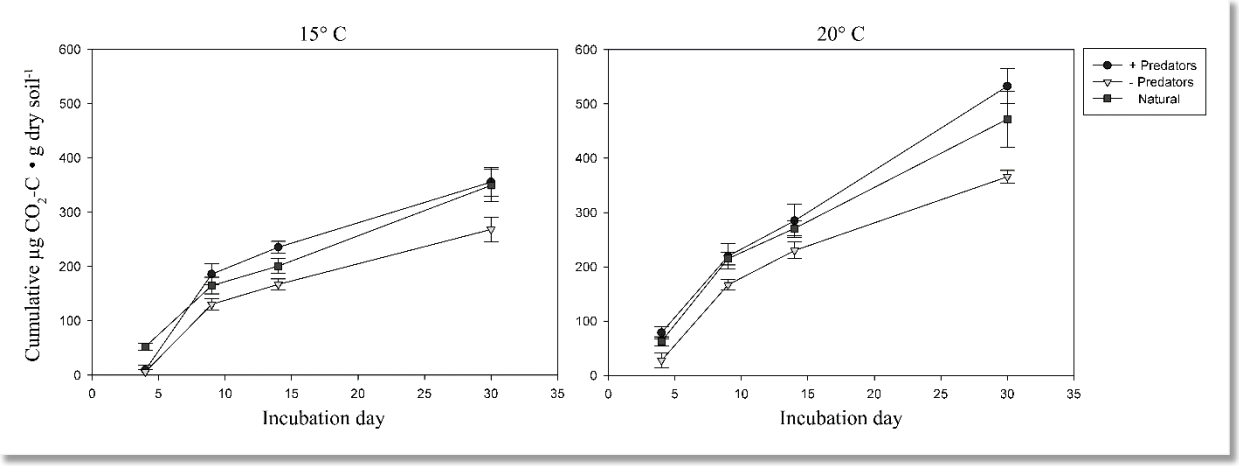

Figure 5.3. Cumulative respiration graphs at both temperatures. Error bars represent 95% C.I. for the mean; Circles represent mean values for predator treatments, Inverted triangles represent predator-free treatments, Squares represent natural controls.

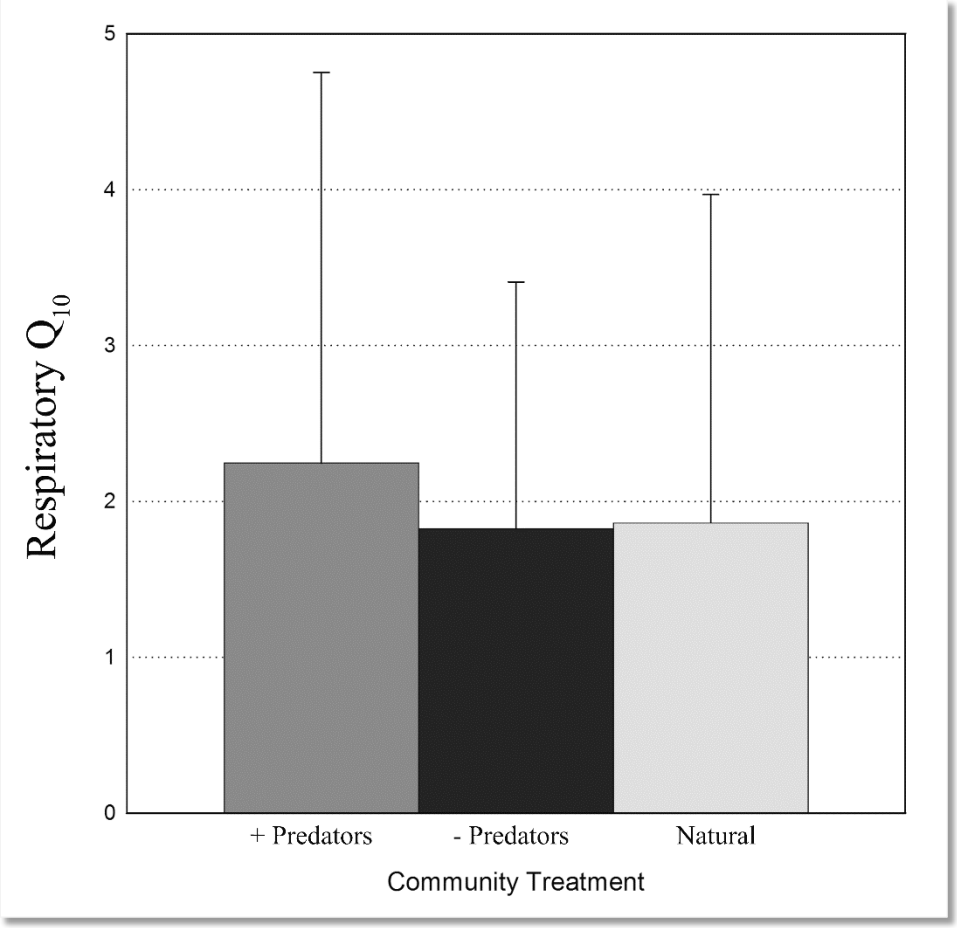

Figure 5.4. Respiratory Q_{10}. The proportionate increase in cumulative respiration for each community treatment due to a 10°C increase in temperature. Error bars represent the propagated 95% C.I. for the mean.

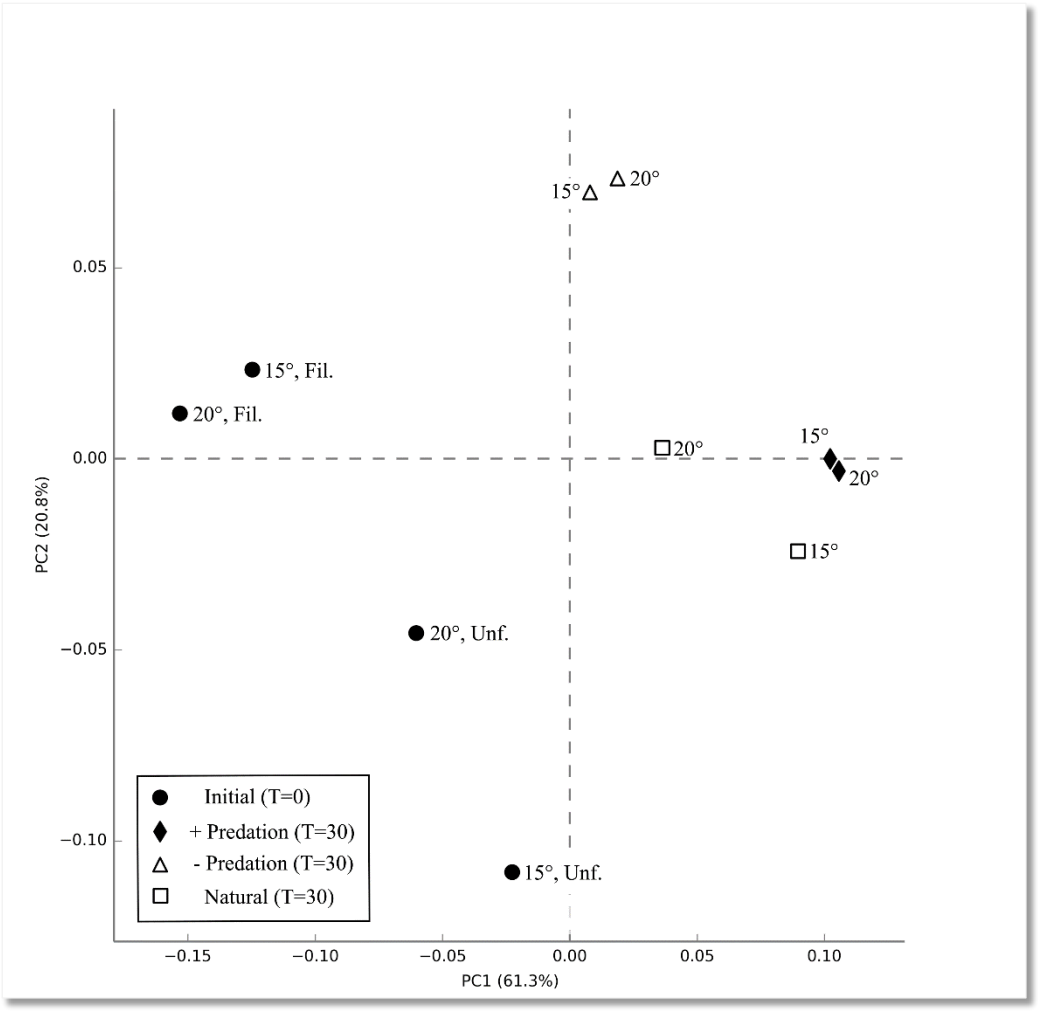

Figure 5.5. PCoA projection. Two-dimensional projection of metagenomic samples using PCoA of the weighted UniFrac distance matrices of their bacterial communities. Closed circles denote communities before incubation for each temperature and filtration treatment (Initial communities, Time=day 0; Fil.=1.5µm filtration, Unf.=10µm filtration); Open squares denote natural community controls after incubation (Time=day 30; Filtration=10µm) at each temperature; Open triangles denote predator-free treatments (Time=Day 30; Filtration=1.5µm; + dead amoebae) for each temperature; Closed diamonds denote predator treatments (Time=Day 30; Filtration=1.5µm; + live amoebae) for each temperature.

Incubation for 30 days resulted in remarkably similar community changes at both temperatures, with significant (Fisher's exact test, Storey's FDR; $p<0.05$) increases in the relative abundance of Symbiobacteriaceae, Sphingobacteriaceae, and Caulobacteraceae, and decreases in Bacillaceae, Paenibacillaceae, and Flavobacteriaceae. At 20°C, however, the 30-day incubation also led to a significant decrease in Pseudomonadaceae that was not observed at 15°C (Figure 5.6).

The presence of amoebal predation also had a significant (Fisher's exact test; Storey's FDR; $p<0.05$) influence on several key taxa (Figure 5.7). Predation resulted in a significant increase in the relative abundance of Flavobacteriaceae, Sphingobacteriaceae, Caulobacteraceae, Oxalobacteraceae, and Cryomorphaceae regardless of temperature, but additional taxa (Sphingomonadaceae, and Paenibacillaceae) were increased in the 15°C treatment. One taxon, Symbiobacteriaceae was differentially affected by predation, with a significant increase in relative abundance at 15°C but a decrease at 20°C. Predation also reduced the abundance of Pseudomonadaceae at 15°C and consistently led to a decrease in the relative abundance of Flavobacteriaceae at both temperatures.

Functional structure of the communities (potential and partial)

There were several significant changes to the predicted functional potential of the communities, but effect sizes were uniformly small. No treatment effect led to any greater than a 0.45% change in the relative abundance of any KEGG orthologs. The greatest significant change (Fisher's exact test; $p<0.05$) was an increase in genes involved with cell motility in soils amended with amoebal predators, though the magnitude of increase was higher at 15°C (0.45%) than at 20°C (0.28%).

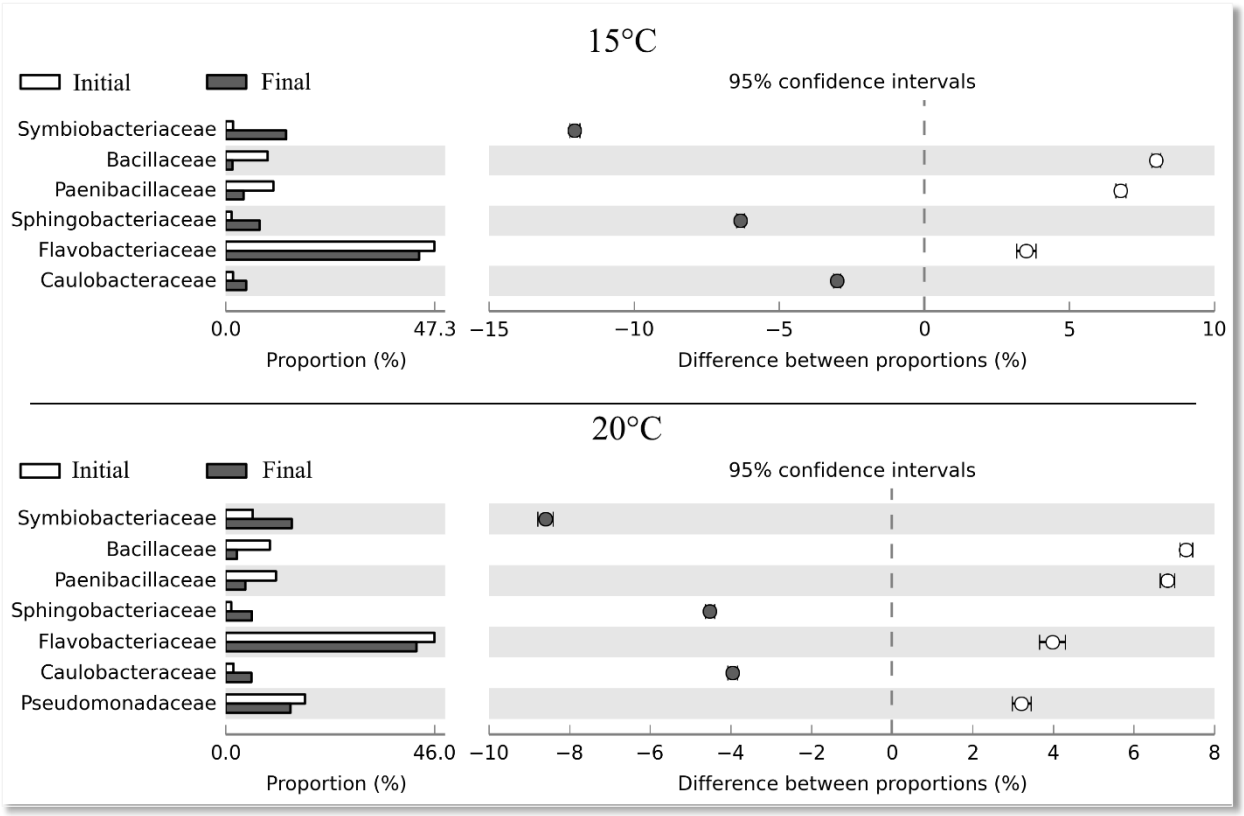

Figure 5.6. Effect of 30-day incubation on bacterial taxa. Extended error bar plot showing all significant differences (Fisher's exact test; Storey's FDR correction; $p<0.05$) between family-level taxa with an effect size of at least 2% difference in normalized abundance.

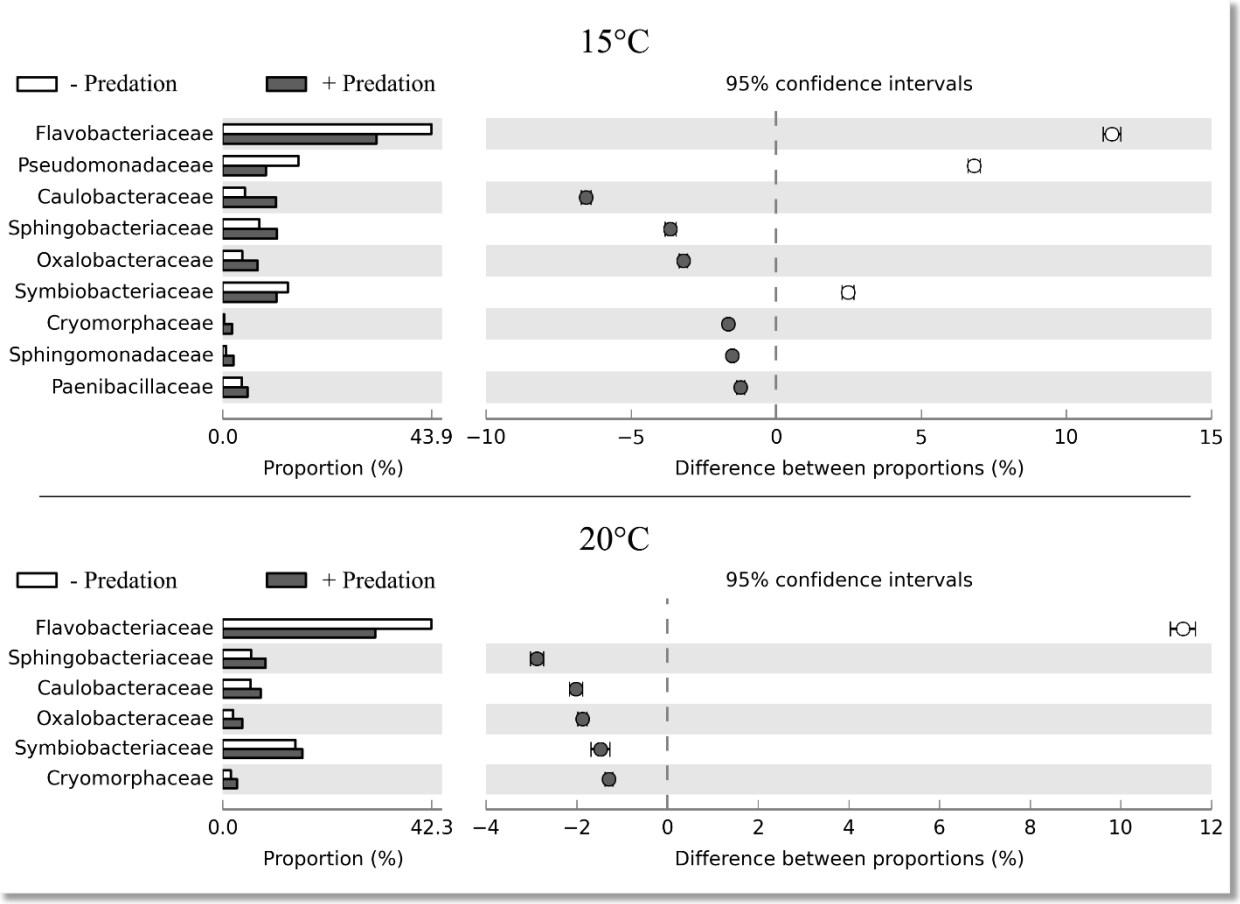

Figure 5.7. Effect of predation on bacterial taxa. Extended error bar plot showing all significant differences (Fisher's exact test; Storey's FDR correction; $p<0.05$) between family-level taxa with an effect size of at least 2% difference in normalized abundance.

Discussion

The influence of protist predation on microbial mineralization of soil C and N has been well documented, but the mechanisms behind these observations are still somewhat uncertain. Two likely scenarios are "sloppy feeding" and selective grazing of protists. Sloppy feeding refers to differential C:N composition between predators and prey leading to inefficient incorporation of bacterial prey into predator biomass. This "extra" prey C or N biomass, along with protists' digestive wastes, is readily bioavailable and can stimulate the growth of bacterial decomposers. This mechanism is also intrinsically linked with predator-mediated top-down control of bacterial populations. Protist predators have been linked to morphological (Corno and Jürgens, 2006) and taxonomic (Hahn and Höfle, 2001) shifts in bacterial community composition.

These taxonomic shifts due to selective feeding depend on the taxonomic identity of the predators and the complex environmental factors that influence them (Bell et al., 2010). Here, we have shown this effect in a soil system with common amoebal predators and shown that increasing temperature led to differential outcomes in grazing on several dominant members of the bacterial community.

The dictyosteloid amoebae in this system exhibited a strong selective force against members of the Flavobacteriaceae family, particularly the genus *Flavobacterium*. We cannot determine if this is the result of amoebae directly grazing on *Flavobacterium*, but it is consistent with others who have shown *Flavobacterium* to be readily consumed by a variety of protists in aquatic systems (Jürgens et al., 1999; Sherr and Sherr, 2002). It differs from at least one study though, where *Flavobacterium* was hardly consumed at all by nanoflagellate protists (Massana et al., 2009), but this discrepancy reinforces the idea that different protist taxa will exert varying selective pressures on bacterial communities.

The predation pressure on Pseudomonadaceae, particularly the genus *Pseudomonas*, was less easily explained. At 20°C, there was no detectable influence of predators, but at 15°C predation resulted in a significant reduction in relative abundance (Fisher's exact test; $p<0.05$; effect size=7.1%). *Pseudomonas* is a known food source for amoebae (Jousset et al., 2010) and it seems likely that it was used as prey by dictyosteloid amoebae in this study regardless of temperature, but it is possible that *Pseudomonas* was simply better able to recover from gazing pressure at the warmer temperature, which is closer to its ideal growth conditions.

It was clear, in any case, that temperature had a much smaller effect of bacterial community structure than any other variable tested. This was an interesting result, but not without precedent since various studies have pointed out the relatively large temperature variations (Chin et al., 1999; Zogg et al., 1997) or long time scales (DeAngelis et al., 2015) needed to detect taxonomic-level changes in soil microbial communities. This does not preclude the notion that relatively small and short-term environmental changes may profoundly affect the biochemical expression (metatranscriptome) of bacterial populations (Gilbert et al., 2010).

The use of shotgun metagenomic profiling when trying to infer community function carries the same limitations as predictive metagenomic profiling from 16S amplicons (as with PICRUSt). That is, DNA-based methods can only ascertain the *potential* biogeochemical function of a community. To obtain *actual* expression profiles RNA-based methods are needed, but these are so far seldom used due to the difficulty of extracting suitable amounts of mRNA from soil environments (Wang et al., 2012).

Conclusion

The methods presented here are an effective way of investigating the interactive roles of environmental parameters and protist predation in shaping bacterial communities and biogeochemical processes. We have shown that amoebal grazing effects are temperature

dependent and have given additional support to the hypothesis that soil bacterial communities are strongly influenced by top-down controls. Predators can shape the taxonomic structure of these communities which can, in turn, affect broader biogeochemical processes such as respiration. If we are to make accurate predictions about the fate of soil carbon in a changing climate, the protist component of the community should not be overlooked.

Acknowledgements

We would like to acknowledge Frederick W. Spiegel for valuable discussions and input about amoebal biology, Erik Pollack and Lindsey Conaway at the University of Arkansas Stable Isotope Laboratory for their help with CO_2 measurements, and members of the Evans-White lab at the University of Arkansas for assistance with filtration of soil suspensions. This research was partially funded by the University of Arkansas Division of Agriculture and The Japan Society for the Promotion of Science (SP-13063).

All raw sequence data are available through the DNA Database of Japan (DDBJ) under the BioProject Accession ID: PRJDB3955.

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Appendix 5.1: Lead Author Confirmation Letter for Paper 4

UNIVERSITY OF ARKANSAS

J. William Fulbright College of Arts and Sciences
Department of Biological Sciences

Chapter 5, titled "Top-Down Control of Soil Bacterial Communities by Amoebal Predation Is Influenced by Temperature," of G. L. Zahn's dissertation is in preparation for submission to *The ISME Journal* with coauthors S. Sharma & B. H. Bluhm.

I, Dr. Frederick W. Spiegel, advisor of Geoffrey Lloyd Zahn, confirm Geoffrey Lloyd Zahn was first author and completed at least 51% of the work for this manuscript.

Dr. Frederick W. Spiegel
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Department of Biological Sciences
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Date

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CHAPTER 6

CONCLUSION

The findings of these studies increase our knowledge of the large-scale ecological distribution of protosteloid amoebae and the local-scale functional services of amoeboid predators. The first two studies have shown that although broad ecological variables influence the abundance and diversity of protosteloid amoebae, no biogeographical patterns seem to exist. The second two studies, focusing on the functional ecology of soil amoebae, have demonstrated that amoebal predation plays a large role in regulating both bacterial communities and the emergent biogeochemical cycles driven by these bacteria.

Distribution of protosteloid amoebae

Our knowledge of the distribution of mycetozoans has increased exponentially over the past few decades, with the majority of this effort being dedicated to dictyostelids and myxomycetes. Less is known about the paraphyletic group known as the protosteloid amoebae. This group provides an excellent system for investigating the biogeography of non-testate (naked) amoebae as they conform to the standards of a "flagship" group set out by Foissner (2006). They are morphologically conspicuous, span a wide breadth of the Amoebozoan phylogeny, and are partially defined by their ability to disperse via tiny propagules. Recently a concerted effort has been made to uncover the global distribution of these organisms. The most intensive regional survey was carried out on the islands of Hawaii, and was undertaken specifically to look for signs of conventional island biogeography such as endemism, low diversity, and adaptive radiation. None of these signs were found.

In comparing the intensive survey of Hawaii to the second most intensively surveyed region, New Zealand, we have seen that no endemism appears to be present at either location, the overall extant community is surprisingly similar, and that landscape-scale variables such as

moisture and elevation have comparable effects on the community assemblage. Further comparisons between all global regions indicate that the most important predictor of whether a given species will be found in a given area is sampling effort. It seems that rare species are rare and common species are common in most cases, regardless of location.

Microhabitat variation was a more important determining factor on a species-by-species level. It is clear that different species of protosteloid amoebae have different habitat requirements, but these relationships between microhabitat and species presence are still unclear. Part of this issue is that we still have not been able to accurately define, in detail, what a habitat consists of, especially for organisms at this size scale.

For example, it was shown repeatedly that certain species seem to have "preferences" for habitats that consist of plant litter that remains aerial (not in contact with the ground), while others seem to "prefer" that the same substrate be in contact with the ground. This observation is clear and repeatable but it has a couple of issues. The first issue is that it is a probabilistic "preference;" the amoebae in question will almost certainly be found in grounded and aerial instances of the same substrate type in a given location. The second issue is the corollary that we do not currently have any robust explanation regarding what causative factors are significantly different between these two microhabitat habitat types. This example serves to illustrate the need for more detailed and painstaking investigations into the biotic and abiotic conditions that constitute "suitable" microhabitats for these organisms.

The notion that microhabitat is the main mechanism selecting the assemblage of protosteloid amoebae fits in with the biogeographic model known as "everything is everywhere, but the environment selects." This seems to be the case for this group of amoebae, but it is possible that the very features which make them a useful flagship group also lend themselves to

widespread dispersal. They have morphological adaptations for dispersal (including many with airborne spores), can utilize dispersal vectors such as anthropogenic movements, and have had sufficient geological time to disperse more or less uniformly. The variation we see in their local assemblages is perfectly consistent with micro-scale habitat variation and the limits of our observational methods to readily detect less-common members.

At this point it seems prudent to thoroughly test these assumptions. In particular, it will be necessary to test whether anthropogenic vectors appear to be responsible for (or at least capable of) the widespread dispersal of protosteloid amoebae. Evidence from Hawaii suggests that the introduction of non-native plants may be a driving factor in the high abundance and diversity of species found there. This suggestion could and should be tested, possibly by extending the same intensity of observation effort further up into the Northwest Hawaiian Islands.

Functional ecology of soil amoebae

A large body of research exists that points to the significant role that protist predators play in driving bacterial community composition and biogeochemical processes. The studies included in this dissertation confirm this conclusion and expound upon the ways by which changing environmental factors influence predator prey interactions in the soil microbiome.

Using simplified soil microcosms, it was demonstrated that increasing temperature interacted with the physical and chemical structure of soil to shape the influence of amoeboid predators on total carbon (C) and nitrogen (N) mineralization. Briefly, soil structure mattered, but only in the warmer temperature. In all cases, predation by amoebae increased the rate of C and N mineralization, but the magnitude of this effect was determined by a combination of temperature and soil aggregate structure. These findings have important implications for efforts to predict the fate of soil nutrients in a changing climate.

Models of processes mediated by soil biota should not neglect the protist component of microbial communities or the extent to which predator-prey interactions are linked to environmental conditions. Differing land management strategies, especially, were shown to affect this trophic interaction, with conventional farming practices exhibiting less amoebal control over biogeochemical cycling than soils under a no-till regime. Thus, the importance of protists in large-scale climate change predictive efforts depends on the soil conditions under consideration.

These results were expanded to investigate whether the same processes can be observed in more natural, undefined communities. Protists were successfully removed from soil communities while still retaining a complex representative assemblage of the bacterial members and these communities were analyzed via high-throughput sequencing to reveal specific influences of soil dictyostelids. It was observed that the addition of amoebae to these complex systems resulted in the same pattern of increased soil respiration and that amoebae exerted a strong top-down control of bacterial community structure. More interestingly, the specific changes to bacterial communities were dependent on temperature.

Again we see that incorporating protists into predictions about soils in a changing climate is essential, and yet a one-size-fits-all approach is likely to be unsuccessful. Amoebal predators (likely each protist taxon will behave differently) have different impacts on bacterial prey at different temperatures. This method of DNA-based surveys paired with microcosms is useful for addressing similar questions, but is limited in that it is never possible to fully replicate the complex conditions and interactions found in the field. Furthermore, this method is incapable of detecting functional transcriptomic changes which may have a profound influence on biogeochemical processes. Further work should attempt to incorporate measurements of the

metatranscriptome as well as testing other predator taxa to see if any broadly applicable principles can be resolved.

References

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