Ecology and Evolution Open Access

Dispersal Limitation and the Assembly of Soil Actinobacteria Communities in a Long-Term Chronosequence

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Journal:	*Ecology and Evolution*
Manuscript ID:	ECE-2011-12-0156.R1
Wiley - Manuscript type:	Original Research
Date Submitted by the Author:	23-Dec-2011
Complete List of Authors:	Eisenlord, Sarah; University of Michigan, School of Natural Resources and Environment Zak, Donald; University of Michigan, Ecology and Evolutionary Biology Upchurch, Rima; University of Michigan, School of Natural Resources and Environment
Search Terms:	Molecular Ecology, Bacteria
Abstract:	It is uncertain whether the same ecological forces that structure plant and animal communities also shape microbial communities, especially those residing in soil. We sought to uncover the relative importance of present-day environmental characteristics, climatic variation, and historical contingencies in shaping soil actinobacterial communities in a long-term chronosequence. Actinobacteria communities were characterized in surface soil samples from four replicate forest stands with nearly identical edaphic and ecological properties, which range from 9,500 to 14,000 years following glacial retreat in Michigan, USA. TRFLP profiles and clone libraries of the actinobacterial 16S rRNA gene were constructed in each site for phenetic and phylogenetic analysis to determine whether dispersal limitation occurred following glacial retreat, or if community composition was determined by environmental heterogeneity. At every level of examination, actinobacterial community composition most closely correlated with distance, a surrogate for time, than with biogeochemical, plant community, or climatic characteristics. Despite correlation with leaf litter C:N and annual temperature, the significant and consistent relationship of biological communities with time since glacial retreat provides evidence that dispersal limitation is an ecological force structuring actinobacterial communities in soil over long periods of time.

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Dispersal Limitation and the Assembly of Soil *Actinobacteria* Communities in a Long-Term Chronosequence

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Running Title:
Actinobacterial Phylogeography

Key Words:
Actinobacteria, chronosequence, microbial biogeography

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1 **ABSTRACT**

2 It is uncertain whether the same ecological forces that structure plant and animal communities
3 also shape microbial communities, especially those residing in soil. We sought to uncover the
4 relative importance of present-day environmental characteristics, climatic variation, and
5 historical contingencies in shaping soil actinobacterial communities in a long-term
6 chronosequence. *Actinobacteria* communities were characterized in surface soil samples from
7 four replicate forest stands with nearly identical edaphic and ecological properties, which range
8 from 9,500 to 14,000 years following glacial retreat in Michigan, USA. TRFLP profiles and clone
9 libraries of the actinobacterial 16S rRNA gene were constructed in each site for phenetic and
10 phylogenetic analysis to determine whether dispersal limitation occurred following glacial
11 retreat, or if community composition was determined by environmental heterogeneity. At
12 every level of examination, actinobacterial community composition most closely correlated
13 with distance, a surrogate for time, than with biogeochemical, plant community, or climatic
14 characteristics. Despite correlation with leaf litter C:N and annual temperature, the significant
15 and consistent relationship of biological communities with time since glacial retreat provides
16 evidence that dispersal limitation is an ecological force structuring actinobacterial communities
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19 **Acknowledgements**

20 Our research was supported by grants from the National Science Foundation and the US

21 Department of Energy Office of Biological and Environmental Research.

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23 **INRODUCTION**

24 Biogeography is the study of geographical distribution of organisms over the Earth in
25 both time and space. Ecologists seek to understand how biological diversity is generated and
26 maintained, especially in the light of a changing environment. For microbial biogeography, the
27 traditional view has held that "Everything is everywhere, but the environment selects" (Baas
28 Becking 1934). The large population size and short generation times typical of microbial
29 communities lead to rapid genetic divergence, potentially resulting in biogeographic patterns
30 (Green & Bohannan 2006). However, it has been assumed that unlimited microbial dispersal
31 leads to constant input of new members, increasing gene flow and overwhelming the forces of
32 genetic drift (Roberts & Cohan 1995; Ramette & Tiedje 2007b). Global studies of microbial
33 diversity in aquatic and soil communities support this theory (Fierer & Jackson 2006, Van der
34 Gucht *et al*. 2007); however, evidence is accumulating that some microorganisms do exhibit
35 biogeographical patterns across time and space (Fulthorpe *et al.* 1998; Cho & Tiedje 2000;
36 Whitaker *et al.* 2003). It is currently under debate whether variation in microbial communities
37 over space results from environmental filtering, or if geographic barriers and other historical
38 contingencies contribute to spatial structure in community composition through limiting
39 dispersal (Horner-Devine *et al.* 2004; Martiny *et al.* 2006; Ramette & Tiedje 2007a; Ge *et al.*
40 2008). If not all microbes are equally and evenly dispersed over time, it would suggest that
41 forces structuring microbial communities are more complex than adaptive evolution through
42 natural selection. Historical contingencies could give rise to compositional patterns through
43 isolation and genetic divergence.

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44 We address this issue by examining the community patterns of a deeply diverse and
45 divergent phylum, the *Actinobacteria*, in a northern hardwood forest chronosequence.
46 *Actinobacteria* are important organisms mediating plant litter decay and the subsequent
47 formation of soil organic matter in terrestrial ecosystems (DeAngelis *et al.* 2011, Paul & Clark
48 1996). This phylum is phylogenetically divergent and the closest prokaryotic relative has yet to
49 be identified (Ventura *et al*. 2007; Embley & Stackebrandt, 1994). *Actinobacteria* express a
50 variety of morphologies and life-history traits, including sporulation, which could be
51 advantageous for long-distance dispersal. There is no consensus whether *Actinobacteria*
52 exhibit endemism or have a cosmopolitan distribution (Gløckner *et al.* 2000; Wawrik *et al.*
53 2007). Here, we evaluate whether dispersal limitation is a factor structuring the community of
54 soil *Actinobacteria* following glacial retreat in a present-day forest ecosystem in northeastern
55 North America.

56 Previous work provides evidence that soil actinobacterial communities exhibit regional
57 biogeography, wherein community membership changes across the north-south distribution of
58 a northern hardwood ecosystem in the Upper Great Lakes region of the U.S. (Eisenlord & Zak
59 2010). Across this geographic region, the periodic retreat of glaciation *ca.* 14,000 years ago
60 occurred in a south to north direction. Over a period of 5,000 years, new landscapes were
61 revealed forming a chronosequence, in which soils were formed from similar parent material,
62 yet differ in time since deglaciation set in motion the process of soil formation. According to
63 pollen records, forests dominated by *Acer saccharum* Marsh. (sugar maple) established at the
64 beginning of the Holocene in the Upper Lake States region. These pollen records indicate sugar

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65 maple became dominant *ca.* 4,000 years following the retreat of glacial ice (Davis 1983), leaving
66 behind a long-term chronosequence.

67 Along this chronosequence, we previously located ecologically and edaphically matched
68 sugar maple stands which provides a unique opportunity to study the structuring force of time
69 on the assembly of soil microbial communities. Replicate sampling of *Actinobacteria*
70 communities within the same habitat type in four different geographic locations allows us to
71 determine if there is a "distance effect" (Martiny *et al.* 2006). Because each geographic
72 location corresponds with time elapsed following glacial retreat, we considered distance to be a
73 surrogate for time. Due to the periodic nature of glacial retreat, distance and time do not
74 follow a linear relationship. If dispersal limitation was a force structuring soil microbial
75 communities over long time frames, dispersal of actinobacterial propagules would be limited in
76 the more northern sites because they are the youngest. Therefore, differences in community
77 composition should correlate with distance, after controlling for present day environmental
78 variability. Furthermore, if the source of actinobacterial communities originated from the older
79 sites, then the distribution of these communities should be clustered on a phylogenetic tree.
80 That is, younger actinobacterial communities in the north should be a phylogenetic subset of
81 older communities in the south. Moreover, if dispersal limitation is not a factor shaping these
82 communities as the Baas-Becking theory predicts, then we would expect similar communities in
83 all sites. This alternative predicts that variation in actinobacterial community composition
84 should be structured by environmental factors such as overstory plant community composition
85 and biogeochemical characteristics of the soil.

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86 To test these alternatives, we initially characterized actinobacterial communities using
87 16S rRNA gene terminal restriction fragment length polymorphism (TRFLP) fingerprints. Using
88 this information, we further refined the test of our hypothesis via cloning and sequencing of the
89 actinobacterial 16S rRNA gene and subsequent taxonomic and phylogenetic analysis.
90 Actinobacterial community composition in our four study sites was assessed by examining
91 community similarity, identifying a distance-decay relationship, and testing the relatedness of
92 community patterns to environmental variation, climatic factors, and geographic distance, as a
93 proxy for site age, through multivariate statistics. Here, we provide evidence that dispersal
94 limitation is a mechanism shaping *Actinobacteria* communities in a northern hardwood forest
95 ecosystem over a relatively long time frame (i.e., *ca.* 5,000 yrs).

96 **METHODS:**

97 ***Study Sites and Sampling***

98 The biogeography of *Actinobacteria* was examined in the surface soil of four sugar
99 maple dominated forests on the Lower and Upper Peninsula of Michigan (Fig. 1). These sites
100 were selected from 31 candidate sites based on their ecological and edaphic similarity, which
101 were assessed by multivariate analyses of plant community composition, stand age, and soil
102 properties (Burton et al. 1991). Soils are well-drained sandy, typic haplorthod of the Kalkaska
103 series and overstory biomass is dominated by sugar maple (~70-85%). These sites form a long-
104 term chronosequence due to their similarity of environmental, ecological, and edaphic
105 characteristics, yet thousands of years elapsed following deglaciation and establishment of
106 forests at each site. The southernmost site D was ice free approximately 13,500 years before

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107 present (BP) followed by maple forest establishment 3,500 years later (Evenson *et al.* 1976;
108 Drexler *et al.* 1983; Davis 1983). Site C is located 83 km north of site D and was deglaciated
109 approximately 13,000 years BP, followed by maple forest establishment 4,000 later (Evenson *et*
110 *al.* 1976; Drexler *et al.* 1983; Davis 1983). Site B, located 150 km north of Site C, was uncovered
111 approximately 11,000 years BP, and pollen records indicate maple forest establishment 4,000
112 years later. Finally, the northernmost site A, 343 km North West of site B, was ice free 9,500
113 years BP, with maple forest establishing 3,500 years later (Evenson *et al.* 1976; Drexler *et al.*
114 1983; Davis 1983).

115 These sites are well characterized in terms of their climate, plant community and
116 biogeochemical characteristics. For example, daily air temperature, soil moisture and soil
117 temperature along with annual measurements of tree species, diameter and height, leaf litter
118 biomass, water balance, and leaf litter production have been recorded since 1994 and are
119 available at the Michigan Gradient website (http://www.webpages.uidaho.edu/nitrogen-
120 gradient/Default.htm). Forests on our study sites were harvested *ca.* 1900-1910 and have not
121 experienced human disturbance since that time; to the best of our knowledge, they all have
122 been exposed to the same disturbance regime and share the same land-use history.

123 We collected surface soil horizons (Oe, Oa, and A horizons) on three separate dates
124 (June 2006, October 2006 and May 2007) to characterize actinobacterial communities. In each
125 of the four sites, there are three randomly located 30-m x 30-m replicate plots ranging 15 to
126 150 meters apart. We have previously and continuously quantified ecological, edaphic, and
127 biogeochemical characteristics for each plot in all four study sites (Burton *et al.* 1993; Reed *et*

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128 *al.* 1994; Pregitzer *et al.* 2004; Burton *et al.* 2004). In each 30-m x 30-m plot, we collected 10
129 soil samples using a 2.5-cm diameter soil core, which extended to a depth of 5 cm. The 10
130 surface soil samples in each plot were composited and passed through a 2-mm sieve in the
131 field. From the sieved composite sample, a 5-g sub-sample was removed for DNA extraction.
132 By pooling the 10 soil cores, our sampling scheme aggregated small-scale spatial heterogeneity
133 at the scale of individual plots. We did so because our goal was to characterize the
134 actinobacterial community at the scale of entire forest stands, and to explore regional trends in
135 community similarity that many be structured by historical contingences and environmental
136 factors.

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137 Samples were placed on ice in DNA extraction vials and immediately transport to the
138 University of Michigan, where they were held at -80°C prior to DNA extraction. In May of 2007,
139 Site B was defoliated by the canopy consuming insect, *Operophtera bruceata,* which deposited
140 large amounts of insect frass and green-leaf fragments on the forest floor (D.R. Zak, *personal*
141 *observation*). Because these insects dramatically altered the biochemical constituents, the
142 amount, and timing of leaf litter fall, we eliminated the May 2007 Site B samples from our
143 analyses.

144 **DNA extraction and PCR protocol**

145 Soils sampled in 2006 were used to characterize the actinobacterial community using
146 TRFLP, whereas we used cloning and sequencing to further characterize the community from
147 samples collected in the subsequent year. Microbial DNA extraction and actinobacterial 16S
148 rRNA gene amplification followed similar protocols to those previously described in Eisenlord

149 and Zak (2010). Briefly, microbial community DNA was extracted from our 2006 samples in
150 triplicate from 0.25 to 1 g of soil using the Ultraclean Soil DNA extraction kit (Mo Bio
151 Laboratories) for TRFLP analysis. Microbial community DNA was extracted from our 2007 soil
152 samples using 5-g surface soil subsamples with MoBio PowerMax Soil DNA isolation kits (Mo
153 Bio Laboratories) within one week of field collection for clone analysis. Actinobacterial 16S
154 rRNA genes were amplified from total community DNA with primers Eub338F-
155 ACGGGCGGTGTGTACA and Act1159R – TCCGAGTTRACCCCGGC (Blackwood *et al.* 2005). The
156 PCR protocol followed 95 °C for 5 min for initial denaturing, then 25 rounds of amplification (94
157 °C for 30 sec, 57 °C for 30 sec, 72 °C for 90 sec) followed by 10 min at 72 °C for elongation, and
158 finally held at 6 °C before removal (adapted from Blackwood *et al.* 2005). All PCRs were
159 conducted in duplicate and products were pooled before purification with MoBio Ultra Clean
160 PCR Clean up Kit according to manufacturer's instruction. For TRFLP the PCR reaction differed
161 from above by having a 6-Carboxyflurescein (6-FAM) attached the Eub338F primer.

162 **Community Characterization using Terminal fragment length polymorphism (TRFLP)**

163 Following PCR clean up of the actinobacterial 16S rRNA gene amplicon, approximately
164 200-500 ng of purified PCR product, as determined by Picogreen® analysis (Invitrogen; as
165 instructed by the manufacturer) was digested with 5U of TaqI (Promega) at 65 ^{o}C for 1 h.
166 Passing digests through a Microcon YM-30 filter (Millipore) desalted them and removed
167 enzymes from the reaction. Each sample was submitted in duplicate for genotyping conducted
168 at the University of Michigan's Core Sequencing Facility using an ABI 3730XL DNA Sequencer
169 with a 96 capillary array. Rox 1000 (Bioventures) was used as a standard to determine

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170 restriction fragment lengths. Electropherograms were inspected using Genemarker 1.60
171 (SoftGenetics). We required a peak height 50 fluorescence units and the appearance of each
172 restriction fragment in both duplicates for our subsequent analyses. Each TRF with a peak
173 height that of 1% or greater of the total intensity were scored into the presence-absence matrix
174 (Hassett *et al* 2009).

175 **16S rRNA Gene Cloning and Phylogenetic Analysis**

176 Actinobacterial 16S rRNA genes were cloned with the Invitrogen TOPO TA cloning kit
177 using TOP10 chemically competent cells (Invitrogen). Inserts were sequenced at the Georgia
178 Genomics Facility at the University of Georgia (Athens, GA). This study expanded our previous
179 sequencing efforts of 33 clones in each plot (Eisenlord & Zak, 2010; Genbank accession
180 FJ661107-FJ662388) to include an additional 63 clones from each of the twelve samples (i.e., 3
181 plots in each of 4 study sites), totaling 1152 sequences (Genbank accession HQ845548-
182 HQ845603).

183 Sequences were manually edited in Geneious v.5.0.2 (Biomatters Ltd.) and 727 high
184 quality contiguous sequences were generated from forward and reverse sequences. The top-
185 type species matches were retrieved from the Ribosomal Database Project (RDP; Cole *et al.*
186 2009) for all sequences, and 50 representative sequences from every major group of the
187 *Actinobacteria* phyla were retrieved from the NCBI Taxonomy Browser for use as references.
188 Clone and reference sequences were aligned using ClustalW (Thompson *et al.* 1994) in the
189 program Geneious. Reference sequences were included in the alignment to build phylogenetic
190 backbone support by preserving spatial heterogeneity in the 16S sequences. Alignments were

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191 manually edited to remove gaps and ambiguously aligned sequences. Reference sequences
192 were removed from the clone alignment before operational taxonomic units were determined.

193 The clone sequence alignment was used to generate a distance matrix in Phylogeny
194 Inference Package (PHYLIP) version 3.69 (Felsenstein 2005), using the Jukes Cantor algorithm of
195 substitution. Mothur (Schloss *et al.* 2009) was then employed to assign operational taxonomic
196 units at 90, 93, 95, 97 and 99% similarity using the average neighbor algorithm. The relative
197 abundance of operational taxonomic units (OTUs) at each similarity level was examined to
198 address the argument that the resolution at which microbial communities are analyzed
199 influences results and subsequently their interpretation (Cho & Tiedje 2000). At 97% similarity,
200 Mothur was used for taxa-based alpha and beta diversity estimates within and across sites and
201 to run ʃ-LIBSHUFF (Schloss *et al.* 2004), a program which uses coverage curves to statistically
202 detect if two or more microbial communities are similar using the Cramer-von Mises test
203 statistic (Schloss *et al.* 2009). OTU sequences at 97% similarity were generated by consensus of
204 clone sequences in Geneious.

205 Reference sequences and 56 actinobacterial OTUs defined at 97% similarity were then
206 re-aligned in Geneious with ClustalW for phylogenetic analysis. Because phylogenetic analyses
207 are sensitive to tree topology, RaxML was used to select the best-fit tree with the Maximum
208 Likelihood algorithm; *Staphylococcus aureus* was used to root the tree. Differences in the
209 phylogenetic patterns in each study site were quantified with the online statistical tool UniFrac
210 (Lozupone *et al.* 2006). Phylogenetic distances matrices reported by UniFrac, along with the

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211 relative abundances of OTUs at all five similarity levels, were used for multivariate statistics
212 described below.

213 **Environmental Variables**

214 Environmental characteristics were assembled into four data sets: i) a biogeochemical
215 data set composed of factors which we selected *a priori* that are relevant to soil microbial
216 communities, ii) plant community composition, iii) climatic characteristics, and iv) distance
217 which represented time since glacial retreat. The biogeochemical data matrix included: soil pH
218 and moisture content (measured from our 2007 samples), and previously collected values for
219 leaf litter C content, leaf litter C:N ratio, total leaf litter mass, C:N ratio of soil organic matter,
220 and extractable soil NO_3^- (Table 1; Burton *et al.* 1993; Reed *et al*. 1994; Pregitzer *et al.* 2004;
221 Burton *et al.* 2004). All metadata are available at http://www.webpages.uidaho.edu/nitrogen-
222 gradient/Default.htm. All environmental data used in this study were averages over growing
223 season from the years 2005 to 2008. The second matrix represented the plant community
224 based on the relative importance (i.e., basal area of a species/basal area of all species) of
225 overstory and understory species (Supplemental Table A1). Our third data matrix characterized
226 climatic variation by including temperature, precipitation, and ambient N deposition, to identify
227 the role of climate in shaping these actinobacterial communities (Table 1). The primary
228 historical event taken into consideration for this study is the periodic retreat of the Wisconsin
229 ice sheet across lower and upper Michigan. Because distance between sites overlays time since
230 de-glaciation in our chronosequence, we used distance-time as our fourth data set; it was
231 composed of GPS coordinates taken at the center of each sample plot (Supplemental Table A2).

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232 The chosen variables for each set of data were assigned to biogeochemical, plant community,
233 climatic, and distance-time data sets for multivariate statistical analysis.

234 **Multivariate Statistical Analysis** It is plausible that soil *Actinobacteria* biogeography is
235 shaped by local environmental conditions, historical factors, or by both. Following the
236 framework of Martiny *et al.* (2006), we used multivariate analyses (PRIMER v6; Plymouth, UK),
237 in order to identify significant correlations between factors composing biogeochemical, plant
238 composition, climatic, and distance-time data matrices.

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239 TRFLP fingerprint data matrices, OTU relative abundance matrices, and phylogenetic
240 distances were treated similarly as 'biological' data. Similarity matrices for TRFLP fingerprints
241 were generated using the Bray-Curtis similarity metric (Bray & Curtis 1957) on non-transformed
242 presence-absence data. Relative abundances of OTUs were square root transformed to lessen
243 the emphasis of the most abundant species prior to the generation of similarity matrices with
244 the Bray-Curtis coefficient. Phylogenetic distances were generated with the online package
245 UniFrac (Louzapone *et al.* 2006).

246 Biochemical and climatic similarity matrices were generated with Euclidian distances of
247 standardized data, whereas the distance-time matrix was generated with the great circle
248 distance (Vincety 1975). The plant community similarity matrix was generated with the Bray-
249 Curtis metric (Bray & Curtis 1957). Biogeochemical, plant community, climatic, and distance-
250 time data were visualized with non-metric multidimensional scaling (nMDS; Fig. 2).

251 With site as the main factor, an analysis of similarity (ANOSIM) test was used to
252 compare communities across sites (n = 4), with individual plots as replicates within each site (n

253 = 3). The Mantel-type test, RELATE, was used in conjunction with the Spearman rank
254 correlation coefficient to determine if there were significant correlations between the biological
255 data (TRFLP, OTU, and Phylogenetic distances) and the biogeochemical, plant community,
256 climatic, and distance-time data sets. The RELATE test is similar to the Mantel test in that it
257 uses element-by-element correlations of similarity matrices. Though instead of Pearson
258 correlations used by the Mantel test, RELATE uses Spearman rank correlation coefficients, as is
259 more appropriate for the interpretation of our data (Clark & Gorley 2006). Rank similarities
260 between site averages were used in this analysis to correct for the different scaling of each
261 correlation coefficient. The distance-decay relationship was explored with the 2006 TRFLP data
262 by plotting the log transformed average Sørensen community similarity metric (gained from
263 PRIMER), against the log transformed geographic distance between the plots.

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264 Additional statistics were conducted in the R (R Development Core Team 2011) package
265 vegan (Oksanen *et al* 2011). Environmental vectors, of biogeochemical, climatic, and distance-
266 time data sets, were fit to nMDS ordinations of biological data, which identified the individual
267 variables correlated with community patterns. Redundancy analysis (RDA) was used to
268 examine the correlations between species patterns and environmental variables to evaluate
269 which variables explained significant proportions of variation in *Actinobacteria* community
270 composition. Distance-based redundancy analysis (db-RDA; Legandre & Anderson, 1999) was
271 applied using the Bray-Curtis distance metric to determine if distance-time significantly
272 accounted additional biological variation, after the variation due to environmental variables
273 was held constant.

274 **RESULTS**

275 At all levels of examination, actinobacterial communities were compositionally different
276 in each of our four forest sites, and variation in the communities was significantly correlated
277 with time since glacial retreat (i.e., distance; Table 2).

278 **TRFLP community comparison**

279 Based on greater than 1% contribution to total TRFs, there were 27 unique TRFs in July,
280 and 26 in October. Rarefaction curves generated in EstimateS (Colwell 2009) approached an
281 asymptote and can be viewed online in supplemental information (Fig. A1). TRFLP
282 actinobacterial community similarity, based on the Sørensen metric, had a significant negative
283 relationship with time since glacial retreat (z-score=-0.094, P = 0.02, Fig. 3). This distance-decay,
284 or time-decay, relationship held true for both July and October sampling dates; as distance
285 increased, community similarity decreased (July slope= -0.11 October slope= -0.03). There was
286 no such relationship present when biogeochemical characteristics were regressed against
287 geographic distance (P = 0.29). RELATE results, based on the Bray-Curtis similarity matrices of
288 July and October TRFLP data, indicate actinobacterial communities are more similar in
289 composition the closer they are geographically and in age (Table 2). There was no evidence in
290 the July samples that communities with similar biogeochemical or climatic characteristics have
291 similar actinobacterial TRFLP profiles (P = 0.115, P = 0.436). The fitting of all environmental
292 vectors to the July nMDS, displayed in Figure 4, revealed distance-time to be significantly
293 correlated with community patterns (P=0.014), none of the other variables were significant (P
294 values range 0.174-0.671). The db-RDA analysis revealed distance-time accounted for an

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295 additional 17% of community variation, after variation correlated with environmental and
296 climatic factors was held constant ($P = 0.048$).

297 In contrast, based on RELATE analysis, October samples with similar biogeochemical
298 characteristics did have similar actinobacterial profiles (Spearman = 0.309, $P = 0.015$).
299 Environmental vector fitting revealed both distance-time ($P = 0.030$) and litter C:N ($P = 0.043$)
300 were correlated with community patterns; no other variables were significant ($P = 0.174$-0.797).
301 The RDA found distance-time significantly accounted for 18% of community variation (P =
302 0.026) and litter C:N accounts for 18% of the variation ($P = 0.015$). However, dbRDA revealed,
303 when variation due to litter C:N was held constant, distance-time did not significantly explain
304 any more of the variation in the communities ($P = 0.665$), and the same was true for litter C:N
305 after variation due to distance was held constant ($P = 0.263$). There was no relation of
306 actinobacterial communities to the plant community in either July (Spearman = 0.04, $P = 0.544$)
307 or October (Spearman = 0.03, $P = 0.524$).

308 **Taxonomic Alpha and Beta diversity**

309 Analysis of 727 cloned actinobacterial sequences from May 2007 sample sites A, C, and
310 D resulted in 56 OTUs grouped at 97% similarity. We identified OTUs in 16 out of 39
311 actinobacterial families , classified with the Ribosomal Database Project (Table 3). For the most
312 abundant OTUs, the closest similarity to known organisms was 90% to members of the
313 *Thermomonosporaceae* family. ʃ-LIBSHUFF results revealed significant differences in
314 community membership between sites A and D ($P < 0.001$), sites C and D ($P = 0.007$), and
315 between sites A and C ($P = 0.015$). Diversity estimates, Ace and Chao1, indicated that the oldest

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316 site D was more diverse than the two northern and younger sites, but this difference was not
317 resolved when the 95% confidence intervals were considered. Rarefaction curves (see Fig. A1
318 in Supporting Information) also indicate the oldest (Site D) contained a greater richness than
319 the younger sites. Although the rarefaction curves approached an asymptote, we did not
320 capture the full diversity of the actinobacterial community. When examining the families found
321 at each site, the oldest site (D) contained thirteen unique OTUs from four families not found in
322 any of the younger sites. The next oldest site (C) contained one unique family, whereas the
323 youngest site (A) contained no unique families (Table 3). We individually regressed the most
324 abundant and diverse groups of *Actinobacteria*, the *Micromonospora, Pseudonocardia,*
325 *Thermomonopsora*¸and *Acidimicrobium* against pH, DOC, SOM N content, leaf litter mass, and
326 C:N ratio and found no significant relationship in any case (data not shown).

327 **Phylogenetic Community Analysis**

328 The UniFrac metric was used to identify unique phylogenetic branch length belonging to
329 actinobacterial communities within each site when compared with each other site, as well as
330 when compared to the entire community. The youngest site (A) and the oldest site (D) each
331 had significantly unique lineages when compared against the entire phylogenetic tree (P = 0.03,
332 P = 0.02, respectively); however the site of intermediate age (C) did not (P = 0.42). UniFrac also
333 revealed that the oldest site (D) had unique lineages when compared with the two younger
334 sites (D-C P = 0.06; D-A P = 0.03). The P test further revealed that phylogenetic clustering of
335 sites within the phylogenetic tree did not occur (P = 0.15), and upon visual inspection of our

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336 tree, there was no evidence that actinobacterial communities in the younger sites were a
337 subset of those in the oldest site.

338 The UniFrac distance matrices were analyzed with PRIMER to determine if samples with
339 similar biogeochemical, plant community, climatic, or distance-time characteristics also
340 contained closely related communities. The ANOSIM, with site as a main factor, indicated that
341 plots within a particular site were more similar than plots between different sites ($P = 0.004$);
342 the nMDS ordination of actinobacterial phylogenetic distances is displayed in Figure 2. As with
343 the TRFLP fingerprint data, the 97% similarity phylogenetic distances were significantly
344 correlated with distance-time (Spearman = 0.24 $P = 0.064$), but not with biogeochemical
345 (Spearman = 0.17 $P = 0.223$), plant community (Spearman = 0.15 $P = 0.293$) or climatic
346 (Spearman = 0.17 $P = 0.159$) variables. Fitting of all environmental variables to the nMDS
347 demonstated leaf litter C:N ($P = 0.028$), temperature ($P = 0.062$), and distance-time ($P = 0.081$)
348 all correlated with patterns of phylogenetic distance when considered independently. When
349 variation due to environmental variables was held constant in the db-RDA, distance-time did
350 not explain additional variation in the communities ($P = 0.33$). Interestingly, when variation due
351 to distance-time was held constant, none of the environmental variables could account for
352 variation in the communities either (P values range 0.16-0.25).

353 Because there is much debate about the scale at which to examine relationships
354 between microbial communities and environmental characteristics (Cho & Tiedje 2000; Bissett
355 *et al*. 2010), we examined the relative abundance of OTUs at 90, 95, 97, and 99% similarity and
356 their relationship to biogeochemical, plant community, climactic and distance-time data sets

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357 (Table 2). Relative abundances were square root transformed to minimize the impact of
358 abundant species and allow for higher contribution of the more rare species. When the
359 actinobacterial sequences were examined at 90% similarity, there were no significant
360 differences in the relative abundances of these phylotypes between sites, as detected by
361 ANOSIM; however, at each of the higher similarities, plots grouped more closely within sites
362 than between sites. Furthermore, there were no instances in which biogeochemical or plant
363 community characteristics significantly correlated with OTU relative abundances (*P* = 0.068-
364 0.13; Table 2). Climate characteristics, specifically annual temperature, were correlated with
365 actinobacterial relative abundance at 97 and 99% similarity and distance-time significantly
366 correlated with relative abundances of *Actinobacteria* at 95, 97, and 99% similarity (Table 2).

367 **DISCUSSION**

368 Microorganisms are believed to be globally distributed by prevailing winds (Griffin *et al.*
369 2002) and community patterns in space and time are thought to result from barriers to
370 dispersal, physiological requirements, resource availability, competition, or some combination
371 thereof (Whitaker *et al.* 2003; Papke & Ward 2004). Several factors lead us to reason that the
372 regional species pool of *Actinobacteria* lies to the west of our study sites and provided
373 propagules in a consistent manner as each site was freed from glacial ice over the past *ca.*
374 14,000 yrs. First, the prevailing winds at each study site come from the west, across large
375 bodies of water (i.e., Lake Michigan and Lake Superior). Wind can be an agent of long-distance
376 dispersal for *Actinobacteria,* as well as other bacteria (Pearce *et al.* 2009), and each study site
377 should have received wind-blown propagules from the same regional species pool due to their
378 perpendicular orientation to prevailing winds. If indeed 'everything is everywhere' and there

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379 are no dispersal limitations within the *Actinobacteria*, theory follows that each ecologically
380 equivalent study site will have similar actinobacterial communities due to near identical
381 environmental variables, which eliminate environmental filtering as well as constant additions
382 by the regional species pool. Conversely, Bissett *et al.* (2010) described a hypothesis "wherever
383 you go, that's where you are" implying that beyond strong environmental selection, other
384 factors (i.e. dispersal or colonization limitation and evolutionary events) play a significant role in
385 shaping microbial communities. If this is true, and not all sites received constant additions of
386 *Actinobacteria* as glaciers receded from the region due to dispersal limitation, then distance-
387 time would be detectable as a significant force in structuring the assembly of these
388 communities. Consistent with this expectation, our analyses revealed that distance, a surrogate
389 for time, was a significant factor shaping actinobacterial communities in soil, thereby providing
390 evidence that dispersal limitation was an ecological force structuring these communities.

391 To better understand the importance of dispersal limitation as an ecological force, we
392 sought to identify the degree to which environmental heterogeneity, climatic variation, and
393 distance influenced actinobacterial communities in soil. We purposely held ecological and
394 edaphic factors as constant as possible across our study sites to minimize differences in habitat
395 characteristics. Distance was used as a proxy of the time since glacial retreat exposed new
396 landscapes for colonization. As distance increased, the community similarity of *Actinobacteria*
397 significantly decreased with a z-score similar to those found in a study by Martiny *et al.* (2011)
398 of salt marsh microbial communities. If environmental conditions became increasingly different
399 over distance as well, the most logical explanation for this distance-decay relationship would be
400 that species are adapted to, and structured by, their niche requirements. However, our forest

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401 sites were chosen to constrain differences in edaphic and ecological characteristics, and as
402 distance increased between sites, these properties did not become increasingly different.
403 Furthermore, July actinobacterial community composition was not correlated with any of our
404 measured environmental characteristics when considered together or independently, despite
405 the fact that these edaphic characteristics can shape soil microbial communities (Bååth &
406 Anderson 2003; Van der Guch 2007; Lauber *et al.* 2008). When variation related to these
407 variables was held constant, distance-time still accounted for close to 20% of total variation in
408 our communities.

409 We also can dispel the notion that subtle variation in plant community composition
410 influences soil actinobacterial communities, because we found no relationship between the
411 plant community and actinobacterial communities at every level of investigation. When we
412 examined the relation of actinobacterial communities at 90, 93, 95, and 99% DNA similarity,
413 there were no detectable differences between the sites at the coarsest level (90%) of similarity.
414 Patterns emerged only when the communities were considered at finer phylogenetic
415 resolutions. This implies all of our stands have community members from the same pool of
416 actinobacterial sub-orders, and the differences in the communities occur at the family and
417 genus levels, represented by our analysis of higher DNA similarity percentages. The variation in
418 communities at these finer levels of genetic resolution was consistently related to distance-
419 time, litter C:N, and temperature. Despite our best efforts to hold edaphic properties constant
420 to minimize the effect of environmental filtering and allow for the detection of possible
421 dispersal limitation, changes in the communities over the growing season lead to correlations
422 with the minor changes leaf litter C:N ratio and temperature in our May communities, and litter

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423 C:N in October biological data set. In both cases, when variation due to distance-time (17-18%)
424 was held constant, litter C:N and temperature no longer explained a significant proportion of
425 the variation. Regardless, by examining these communities at many levels of resolution, we
426 have revealed distance (i.e., time) was consistently correlated with variation in actinobacterial
427 community composition, indicating both environmental heterogeneity and historical
428 contingencies play a role in shaping these microbial communities

429 Although the *Actinobacteria* communities in the younger sites did not phylogenetically
430 cluster as a sub-set within the older sites, the oldest site D had the highest species richness
431 estimates and rarefaction curves based on TRFLP and taxonomic data sets. Furthermore,
432 phylogenetic analysis revealed this oldest site contained members of four families and one sub-
433 order, which did not occur in the other sites. Our oldest site also has the highest proportion of
434 unique community members, and this trend was further supported by a significant amount of
435 unique phylogenetic lineage when compared to the younger sites. It is plausible that the higher
436 diversity in our oldest site could result from longer time elapsing since de-glaciation, allowing
437 more time to accumulate additional species from the regional species pool, as well as more
438 time for local adaptation or drift to occur. Although we concede a more in-depth and thorough
439 evaluation of these communities is needed before we can draw firm conclusions from these
440 taxonomic and phylogenetic community patterns, the consistent nature of our results indicate
441 historical contingencies do influence *Actinobacteria* community composition over long periods
442 of time regardless of the high amount of unexplained variation.

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443 This study highlights the importance of examining the identity of organisms as well as
444 how related they are to one another when studying microbial biogeography. Based on
445 presence-absence and relative abundance of 16S rRNA actinobacterial genes, multivariate
446 statistics indicate the observed distance-decay relationship was best explained by distance-
447 time, providing evidence that dispersal limitation structures actinobacterial communities
448 (Whitaker *et al.* 2003). Furthermore, after variation due to distance-time was held constant,
449 biogeochemical and climatic variation could not account for further variation in these
450 communities. However, further multivariate and phylogenetic analyses revealed a significant
451 amount of unique lineage at the youngest site, lack of clustering along the phylogenetic tree,
452 and the correlation of genetic distance to leaf litter C:N and temperature as well as distance-
453 time, all indicate that a simple mechanism of time and dispersal limitation may not be the only
454 ecological factor shaping these communities. Therefore, we suspect other mechanisms
455 contribute to the spatial patterns of soil *Actinobacteria* in our study: such as the lasting imprint
456 of "priority effects" on microbial community assembly (Fukami *et al.* 2010), the possibility that
457 subtle difference in leaf litter biochemistry could alter community composition, or changes in
458 unmeasured variables such as the community composition of other bacteria and fungi which
459 share a similar niche, all could impact actinobacterial communities. Regardless, we have strong
460 evidence on many levels of resolution establishing that time since glacial retreat leads to
461 decreased community similarity without decreasing environmental homogeneity across this
462 chronosequence of sugar maple forest ecosystems, and that distance, a surrogate for time, has
463 consistent and significant impacts on these *Actinobacteria* communities.

For Review Only

464 References

465 Baas Becking LGM (1934) Geobiologie of inleiding tot de milieukunde. *The Hague, the*
466 *Netherlands: W.P. Van Stockum & Zoon (in Dutch).*

467 Bååth E, Anderson TH (2003) Comparison of soil fungal/bacterial ratios in a pH gradient using
468 physiological and PLFA-based techniques. *Soil Biology and Biochemistry,* **35**, 955-963.

469 Bissett A, Richardson E, Baker G, Wakelin S, Thrall PH (2010) Life history determines
470 biogeographical patterns of soil bacterial communities over multiple spatial scales.
471 *Molecular Ecol*ogy, **69**, 134-157.

472 Blackwood CB, Oaks A, Buyer JS (2005) Phylum- and class-specific PCR primers for general
473 microbial community analysis. *Applied Environmental Microbiology*, **71**, 6193-6198.

474 Brady NC, Weil RR (2002) The nature and properties of soils: Thirteenth edition. Pearson
475 Education, Inc., Upper Saddle River, New Jersey. ISBN 0-13-016763-0.

476 Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin.
477 *Ecology Monog*raphs, **27**, 325-349.

478 Burton AJ, Ramm CW, Pregitzer KS, Reed DD (1991) Use of multivariate methods in forest
479 research site selection. *Canadian Journal of Forest Research,* **21**, 1573-1580.

480 Burton AJ, Pregitzer KS, MacDonald NW (1993) Foliar nutrients in sugar maple forests along a
481 regional pollution-climate gradient. *Soil Science Society of America,* **57**, 1619-1628.

482 Burton AJ, Pregitzer KS, Crawford JN, Zogg GP, Zak DR (2004) Simulated chronic

For Review Only

483 NO_3^- addition reduces soil respiration in northern hardwood forests. *Global Change*
484 *Biology,* **10**, 1080-1091.

485 Cho JC, Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent Pseudomonas
486 strains in soil. *Applied Environmental Microbiology,* **66**, 5448-56.

487 Clark KR, Warwick RM (2001) *Change in marine communities: an approach to statistical analysis*
488 *and interpretation, 2nd edition.* PRIMER-E Ltd: Plymouth, UK.

489 Clark KR, Gorley RN (2006) *PRIMER v6: User Manual/Tutoria.l* PRIMER-E Ltd: Plymouth, UK.

490 Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, *et al.* (2009) The Ribosomal Database
491 Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*
492 37 (Database issue): D141-D145; doi: 10.1093/nar/gkn879.

493 Colwell RK (2009) EstimateS: Statistical estimation of species richness and shared species from
494 samples. Version 8.2. User's Guide and application published at:
495 http://purl.oclc.org/estimat

496 Davis MB (1983) Quaternary history of deciduous forests of eastern North America and Europe.
497 *Garden Journal*, **70**, 550-563.

498 DeAngelis KM, Allgaier M, Charvarria Y, Fortney JL, Hugenholtz P, Simmons B et al. (2011)
499 Characterization of trapped lignin-degrading microbes in tropical forest soil. PLoS One
500 doi: 10.1371/journal.pone.0019306

For Review Only

501 Drexler CW, Farrand WR, Hughes JD (1983) Correlation of glacial lakes in the Superior Basin
502 with eastward discharge events from Lake Agassiz. in Teller, J.T. and L. Clayton (eds.)
503 Glacial Lake Agassiz. *Geology Association of Canada,* Special Paper **26**, 309-329.

504 Eisenlord SD, Zak DR (2010) Simulated atmospheric nitrogen deposition alters Actinobacterial
505 community composition in forest soils. *Soil Science Society of America,* **74**, 1157-1166.

506 Embley TM, Stackebrandt E (1994) The molecular phylogeny and systematic of the
507 actinomycetes. *Annual Review of Microbiology*, **48**, 257-289.

508 Evenson E, Farrand W, Eschman D, Mickelson D, Maher L (1976) Greatlakean substage: A
509 replacement for valderan substage in the lake Michigan basin. *Quaternary Research,* **6**,
510 411-424.

511 Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Dep. of Genome Sci.,
512 Univ. of Washington, Seattle.
513 Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities.
514 *Proceedings of the National Academy of Sciences,* **103,** 3, 626-631.

515 Fulthorpe R, Rhodes AN, Tiedje J (1998) High levels of endemicity of 3-chlorobenzoate-
516 degrading soil bacteria. *Applied Environmental Microbiology,* **64**, 1620-1627.

517 Fukami T, Dickie IA, Wilkie P, Paulus BC, Park D, Robers A, *et al*. (2010) Assembly history
518 dictates ecosystem functioning: evidence from wood decomposer communities. *Ecology*
519 *Letters*, **13**, 675-684.

For Review Only

520 Ge Y, He J, Zhu Y, Zhang J, Xu Z, Zhang L, Zheng Y (2008) Differences in soil bacterial diversity:
521 driven by contemporary disturbances or historical contingencies? *International Society*
522 *of Microbial Ecology,* **2**, 254-64.

523 Green J, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. *Trends in Ecology and*
524 *Evolution*, **21**, 501-507.

525 Griffin DW, Kellogg CA, Garrison VH, Shinn EA (2002) The global transport of dust: an
526 intercontinental river of dust, microorganisms and toxic chemicals flows through the
527 Earth's atmosphere. *American Scientist,* **90**, 228-235.

528 Hassett JE, Zak DR, Blackwood CB, Pregitzer KS (2009) Are basidiomycete laccase gene
529 abundance and composition related to reduced lignolytic activity under elevated
530 atmospheric NO3- deposition in a northern hardwood forest. *Microbial Ecology,* **57**,
531 728-739.

532 Horner-Devine MC, Carney KM, Bohannan BJM (2004) An ecological perspective on bacterial
533 biodiversity. *Proceedings of the Royal Society of London B*, **271**, 113-122.

534 Lauber CL, Strickland MS, Bradford MA, Fierer N (2008) The influence of soil properties on the
535 structure of bacterial and fungal communities across land-use types. *Soil Biology and*
536 *Biochemistry*, **40**, 2407-2415.

537 Legandre P, Anderson MJ (1999) Distance-based redundancy analysis: testing multispecies
538 responses in multifactorial ecological experiments. *Ecological Monographs,* **69**, 1–24.

For Review Only

539 Lozupone C, Hamady M, Knight R, (2006) UniFrac: An online tool for comparing microbial
540 community diversity in a phylogenetic context. *BMC Bioinformatics,* **7**, 371.

541 Martiny JBH, Bohannan BJM, Brown JH, Colwell R, Fuhrman J, *et al.* (2006) Microbial
542 biogeography: putting microorganisms on the map. *National Review of Microbiology,* **4**,
543 102-112.

544 Oksanen J, Blanchet G, Kindt R, Legendre P, O'Hara RB, Simpson GL1, Solymos P, Henry H.
545 Stevens and Helene Wagner (2011). vegan: Community Ecology Package. R package
546 version 1.17-11. http://CRAN.R-project.org/package=vegan

547 Papke RT, Ward DM (2004) The importance of physical isolation to microbial diversification.
548 *FEMS Microbial Ecology*, **48**, 293-303.

549 Martiny JBH, Eisen JA, Penn K, Allison SD, Honer-Devine MC (2011) Drivers of bacterial B-
550 diversity depend on spatial scale. *Proceedings of the National Academy of Science*, **108,**
551 7850-7854.

552 Paul EA, Clark FE (1996) *Soil Microbiology and Biochemistry*. Second Edition. Academic Press,
553 Inc. Sand Diego, CA. Chapter 6. 130-152.

554 Pearce D, Bridge PD, Hughes KA, Sattler B, Psenner R, Russell N (2009) Microorganisms in the
555 atmosphere over Antarctica. *FEMS Microbial. Ecology,* **69**, 143-57.

For Review Only

556 Pregitzer KS, Zak DR, Burton AJ, Ashby JA, MacDonald NW (2004) Chronic nitrate additions
557 dramatically increase the export of carbon and nitrogen from northern hardwood
558 ecosystems. *Biogeochemistry*, **68**, 179-197.
559 R Development Core Team (2008) R: A language and environment for statistical computing. R
560 Foundation for statistical computing, Vienna, Austria. ISBN 3-900051-07-0, URL
561 http://www.R-progect.org

562 Roberts MS, Cohan FM (1995) Recombination and migration rates in natural populations of
563 *Bacillus subtilis* and *Bacillus mojavensis. Evolution* **49**, 1081-1094

564 Ramette A, Tiedje JM (2007a) Multiscale responses of microbial life to spatial distance and
565 environmental heterogeneity in a patchy ecosystem. *Proceedings of the National*
566 *Academy of Science*, **4**, 2761-2766.

567 Ramette A, Tiedje JM (2007b) Biogeography: an emerging cornerstone for understanding
568 prokaryotic diversity, ecology, and evolution. *Microbial Ecology*, **53**, 197-207.

569 Reed DD, Pregitzer KS, Liechty HO, Burton AJ, Mroz GD (1994) Productivity and growth
570 efficiency in sugar maple forests. *Forest Ecology Management*, **70**, 319-327.

571 Schloss PD, Westcott S, Rayabin T, Hall J, Harmann M, Hollister E, *et al*, (2009) Introducing
572 mothur: Open-source, platform-independent, community-supported software for
573 describing and comparing microbial communities. *Applied Environmental Microbiology,*
574 **75**, 7537-7541.

For Review Only

575 Schloss PD, Larget BR, Handelsman J (2004) Integration of microbial ecology and statistics: a
576 test to compare gene libraries. *Applied Environmental Microbiology,* **70**, 5485-92.
577 (http://www.plantpath.wisc.edu/joh/s-libshuff.html)

578 Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive
579 multiple sequence alignment through sequence weighting, position-specific gap
580 penalties and weight matrix choice. *Nucleic Acids Research,* **22,** 4673-4680.

581 Van der Gucht K, Cottenie K, Muylaert K, Muylaert K, Vloemans N, Cousin S, *et al*. (2007) The
582 power of species sorting: local factors drive bacterial community composition over a
583 wide range of spatial scales. *Proceedings of the National Academy of Science*, **104**,
584 20404-20409.

585 Wawrik B, Kutliev D, Abdivasievna UA, Kukor JJ, Zylstra GJ Kerkhof L (2007) Biogeography of
586 actinomycete communities and type II polyketide synthase genes in soils collected in
587 New Jersey and Central Asia. *Applied and Environmental Microbiol*ogy, **73**, 2982-2989.

588 Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of
589 hyperthermophilic archaea. *Science,* **301**, 976-8.

590 Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald FG, Chater KF, van Sinderen D (2007)
591 Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum.
592 *Microbiology and Molecular Biology Reviews,* **71**, 495-548.

593 Vincety T (1975) Direct and inverse solutions of geodesics on the ellipsoid with application of
594 nested equations. Survey Review (Kingston Road, Tolworth, Surrey), **23**, 88-93.

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595

596 DATA ACCESSIBILITY

597 DNA sequences: Genbank accessions FJ661107-FJ662388; HQ845548-HQ845603

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FIGURE LEGENDS

Figure 1. Forest sites composing a long-term chronosequences following glacial retreat. Southernmost Site D is the oldest, whereas northernmost Site A is the youngest. Details regarding site age and environmental characteristics can be found in the text, along with our methods for identifying ecologically similar sites across this region.

Figure 2. Non Metric Multi-Dimensional Scaling averaged by site (stress < 0.05), error bars represent standard error of three biological replicates within each site. (a,b) Biogeochemical and climatic data sets separate sites A, B, C and D based on Euclidian distances (c) distance data, as a proxy for site age, is represented by the great circle distance between sites (d,e) Biological data matrices, July TRFLP and October TRFLP, were generated using the Bray-Curtis similarity metric (f) Phylogenetic distances between sites A, C, and D based on UniFrac genetic distances. All images were generated in PRIMER v.6 and edited in Excel 2007.

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Figure 3. Distance-decay relationship displaying the log transformed community similarity based on the Sørensen metric of averaged July and October TRFLP profiles plotted against the log transformed distance between sites. Slope is 0.0946, Y intercept is 2.07, and P=0.02.

Figure 4. Environmental vector fitting on a non-metric multidimentional scaling (nMDS) plot of July 2006 TRFLP *Actinobacteria* communities calculated with the Bray-Curtis dissimilarity metric. Environmental variables included were *a priori* decided biogeochemical factors, climatic variable temperature, and distance-time. Distance-time was the only variable significantly correlated with community composition, designated by *(P=0.014).

Table 1. Site averages for age, climate, and environmental characteristics.

	A	B	C	D
Glacial Retreat (years BP)	9500	11000	13000	13500
Climate				
*Mean Temp (C)	4.82	6.06	6.49	7.65
Mean Precipitation (cm)	91.87	93.28	92.81	86.63
*Ambient N dep (kg/ha)	5.89	6.07	7.37	7.37
Environment				
*Leaf Litter [C] (g/kg)	458	456	453	455
*Leaf Litter C:N	63.68	57.06	52.91	43.41
*Leaf Litter Mass (g)	412.7	396.3	591	550.2
Extractable DOC (mg/L)	5.5	2.79	5.85	9.95
Extractable NO_3^- (mg/L)	0.08	0.55	0.86	0.92
SOM C:N	13.12	22.55	15.9	11.42
SOM [N] (mg/g)	1.84	1.36	1.83	1.73
*pH	4.55	4.7	4.41	4.61
*Moisture Content (%)	23	24	18	14

* Paramaters included in data sets for RDA Analysis

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Table 2. Analysis of similarity (ANOSIM) results with site as a main factor and Mantel-type test RELATE results for TRFLP, phylogenetic distance, and OTU relative abundances of five levels of similarity. TRFLP data is presence absence from 2006, phylogenetic distances based on 97% similarity, and five levels of OTU relative abundances were square root transformed before analysis. Spearman metric statistic is related as Rho.

			RELATE							
	ANOSIM		Biogeochemical		Plant community		Climate		Distance-time	
Data	R Statistic	P-value	Rho	P-Value	Rho	P-value	Rho	P-value	Rho	P-value
TRFLP June	0.45	**0.002**	0.18	0.115	0.04	0.54	0.02	0.436	0.35	**0.022**
TRFLP Oct	0.29	**0.015**	0.31	**0.015**	0.03	0.52	0.14	0.144	0.32	**0.027**
Phylogenetic Distance	0.58	**0.004**	0.17	0.223	0.15	0.29	0.17	0.159	0.24	*0.064*
OTU 90%	0.06	0.320	-0.10	0.680	0.5	0.51	0.08	0.672	0.00	0.479
OTU 93%	0.33	**0.020**	0.19	0.155	0.5	0.52	0.15	0.165	0.15	0.186
OTU 95%	0.35	**0.050**	0.02	0.474	0.5	0.48	0.16	0.133	0.36	**0.043**
OTU 97%	0.69	**0.004**	0.21	0.138	1	0.18	0.33	**0.032**	0.35	**0.036**
OTU 99%	0.56	**0.001**	0.12	0.222	0.5	0.51	0.38	**0.041**	0.32	**0.031**

Bold designates significant *P* values as less than 0.050. Italicized *P*-values are considered suggestive as less than 0.075.

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Table 3. A total of 727 clones from three sites (A, C and D) grouped into 56 OTUs at 97% similarity. We identified *Actinobacteria* in 16 out of 39 Actinobacterial families based on RDP values and phylogenetic analysis. All groupings except the *Acidimicrobiales* are within the Order *Actinomycetales*. We report total number of clones and their abundance at each site as well as the total number of OTUS and their abundance at each site.

	Clones:				OTUs:			
Family	Total	A	C	D	Total	A	C	D
Micromonospora	42	20	6	16	5	4	2	3
Actinospicaceae	8	5	1	2	4	3	1	0
Catenulisporaceae	5	0	0	5	1	0	0	1
Pseudonocardiaceae	56	26	16	14	4	4	3	3
*Corynibacterineae**	87	29	27	31	4	2	2	4
Thermomonosporaceae	334	109	119	106	6	4	4	5
Streptosprangeaceae	1	0	0	1	1	0	0	1
Nocardioidaceae	5	1	1	3	3	1	1	3
Microbacteriaceae	17	8	6	3	3	1	2	3
Micrococaceae	3	0	0	3	1	0	0	1
Streptomycetaceae	11	4	2	5	3	2	1	3
Nakamureliaceae	8	0	3	5	1	0	1	1
*Frankia**	1	0	0	1	1	0	0	1
Kinosproaceae	1	0	1	0	1	0	1	0
Geodermaceae	5	1	2	2	2	1	1	1
Acidimicrobium†	143	42	59	42	17	9	11	11

*Designates grouping to Sub-order †Designates Order *Acidimicrobiales*

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Appendices

Figure A1. Rarefaction curves for (a) TRFLP based on greater than 1% contribution, and (b) Phylogenetic analysis based on 97% DNA similarity for each site A, C and D.

Table A1. Geographic coordinates for Michigan Gradient sample plots.

Table A2. Basal area for the over-story tree species.

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Figure A1.

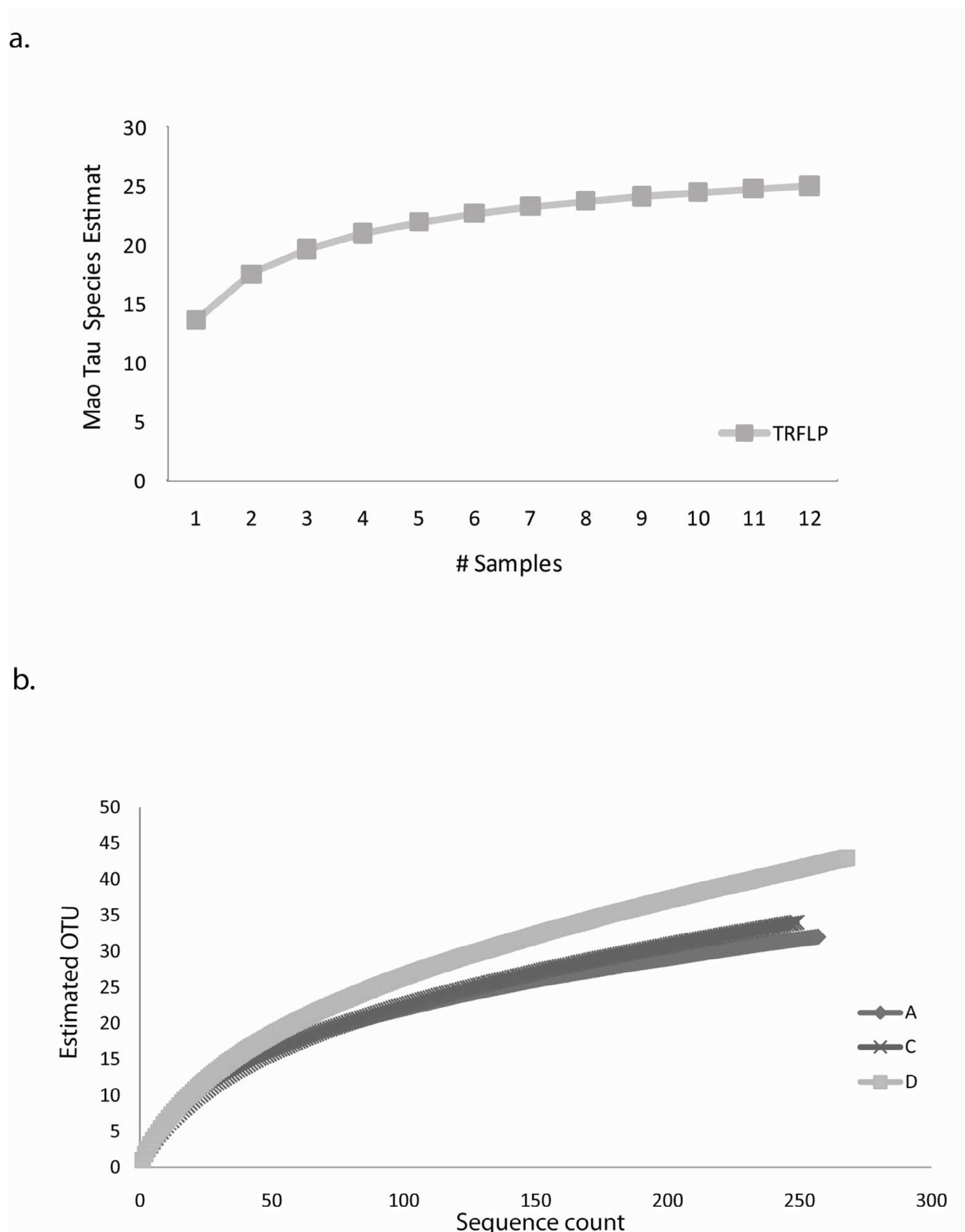

Table A1. Geographic coordinates for each sample plot (1-3), within each site (A-D)

	Lattitude	Longitude
A1	46:51.6642	88:52.8793
A2	46:51.7075	88:52.9198
A3	46:51.6996	88:52.9634
B1	45:32.7536	84:51.5597
B2	45:32.7153	84:51.5292
B3	45:32.6869	84:51.5473
C1	44:22.8473	85:49.9909
C2	44:22.7751	85:50.0526
C3	44:22.8381	85:49.9910
D1	43:40.0910	86:08.6249
D2	43:40.1556	86:08.7045
D3	43:40.1729	86:08.7750

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Table A2. Basal area for over-story tree species. AB = American Beach, BC = Black Cherry, BF= Balsam fir, BO= Black Oak, BW = American Basswood, HM = Hard Maple (Sugar Maple), RM = Red Maple, RO= Red Oak, IW = Iron Wood, YB= Yellow Birch, WA = White ash.

		Basal Area / Species										
Site	Total	AB	BC	BF	BO	BW	HM	RM	RO	IW	YB	WA
A1	35.42	-	-	-	-	-	29.27	5.65	-	0.06	0.44	-
A2	33.93	-	-	-	-	-	33.93	-	-	-	-	-
A3	31.49	-	-	0.03	-	1.40	29.66	-	-	-	0.40	-
B1	31.23	0.18	-	-	-	-	26.93	-	-	-	-	4.12
B2	33.71	-	-	-	-	1.14	30.44	-	-	-	-	2.13
B3	34.59	-	-	-	-	-	29.01	-	-	-	-	5.58
C1	37.05	-	1.29	-	-	-	26.02	6.44	3.30	-	-	-
C2	32.41	0.13	-	-	-	-	29.21	3.07	-	-	-	-
C3	35.51	-	-	-	4.78	-	26.79	0.58	3.36	-	-	-
D1	35.11	-	-	-	-	-	30.55	1.42	3.14	-	-	-
D2	40.4	1.06	2.54	-	-	-	25.02	11.78	-	-	-	-
D3	34.64	1.17	5.29	-	-	-	22.71	3.97	1.39	0.11	-	-

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Dispersal Limitation and the Assembly of Soil *Actinobacteria* Communities in a Long-Term Chronosequence

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Running Title:
Actinobacterial Phylogeography

Key Words:
Actinobacteria, chronosequence, microbial biogeography

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1 **ABSTRACT**

2 It is uncertain whether the same ecological forces that structure plant and animal communities
3 also shape microbial communities, especially those residing in soil. We sought to uncover the
4 relative importance of present-day environmental characteristics, climatic variation, and
5 historical contingencies in shaping soil actinobacterial communities in a long-term
6 chronosequence. *Actinobacteria* communities were characterized in surface soil samples from
7 four replicate forest stands with nearly identical edaphic and ecological properties, which range
8 from 9,500 to 14,000 years following glacial retreat in Michigan, USA. TRFLP profiles and clone
9 libraries of the actinobacterial 16S rRNA gene were constructed in each site for phenetic and
10 phylogenetic analysis to determine whether dispersal limitation occurred following glacial
11 retreat, or if community composition was determined by environmental heterogeneity. At
12 every level of examination, actinobacterial community composition most closely correlated
13 with distance, a surrogate for time, than with biogeochemical, plant community, or climatic
14 characteristics. Despite correlation with leaf litter C:N and annual temperature, the significant
15 and consistent relationship of biological communities with time since glacial retreat provides
16 evidence that dispersal limitation is an ecological force structuring actinobacterial communities
17 in soil over long periods of time.

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19 **Acknowledgements**

20 Our research was supported by grants from the National Science Foundation and the US

21 Department of Energy Office of Biological and Environmental Research.

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23 **INRODUCTION**

24 Biogeography is the study of geographical distribution of organisms over the Earth in
25 both time and space. Ecologists seek to understand how biological diversity is generated and
26 maintained, especially in the light of a changing environment. For microbial biogeography, the
27 traditional view has held that "Everything is everywhere, but the environment selects" (Baas
28 Becking 1934). The large population size and short generation times typical of microbial
29 communities lead to rapid genetic divergence, potentially resulting in biogeographic patterns
30 (Green & Bohannan 2006). However, it has been assumed that unlimited microbial dispersal
31 leads to constant input of new members, increasing gene flow and overwhelming the forces of
32 genetic drift (Roberts & Cohan 1995; Ramette & Tiedje 2007b). Global studies of microbial
33 diversity in aquatic and soil communities support this theory (Fierer & Jackson 2006, Van der
34 Gucht *et al*. 2007); however, evidence is accumulating that some microorganisms do exhibit
35 biogeographical patterns across time and space (Fulthorpe *et al.* 1998; Cho & Tiedje 2000;
36 Whitaker *et al.* 2003). It is currently under debate whether variation in microbial communities
37 over space results from environmental filtering, or if geographic barriers and other historical
38 contingencies contribute to spatial structure in community composition through limiting
39 dispersal (Horner-Devine *et al.* 2004; Martiny *et al.* 2006; Ramette & Tiedje 2007a; Ge *et al.*
40 2008). If not all microbes are equally and evenly dispersed over time, it would suggest that
41 forces structuring microbial communities are more complex than adaptive evolution through
42 natural selection. Historical contingencies could give rise to compositional patterns through
43 isolation and genetic divergence.

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44 We address this issue by examining the community patterns of a deeply diverse and
45 divergent phylum, the *Actinobacteria*, in a northern hardwood forest chronosequence.
46 *Actinobacteria* are important organisms mediating plant litter decay and the subsequent
47 formation of soil organic matter in terrestrial ecosystems (DeAngelis *et al.* 2011, Paul & Clark
48 1996). This phylum is phylogenetically divergent and the closest prokaryotic relative has yet to
49 be identified (Ventura *et al*. 2007; Embley & Stackebrandt, 1994). *Actinobacteria* express a
50 variety of morphologies and life-history traits, including sporulation, which could be
51 advantageous for long-distance dispersal. There is no consensus whether *Actinobacteria*
52 exhibit endemism or have a cosmopolitan distribution (Gløckner *et al.* 2000; Wawrik *et al.*
53 2007). Here, we evaluate whether dispersal limitation is a factor structuring the community of
54 soil *Actinobacteria* following glacial retreat in a present-day forest ecosystem in northeastern
55 North America.

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56 Previous work provides evidence that soil actinobacterial communities exhibit regional
57 biogeography, wherein community membership changes across the north-south distribution of
58 a northern hardwood ecosystem in the Upper Great Lakes region of the U.S. (Eisenlord & Zak
59 2010). Across this geographic region, the periodic retreat of glaciation *ca.* 14,000 years ago
60 occurred in a south to north direction. Over a period of 5,000 years, new landscapes were
61 revealed forming a chronosequence, in which soils were formed from similar parent material,
62 yet differ in time since deglaciation set in motion the process of soil formation. According to
63 pollen records, forests dominated by *Acer saccharum* Marsh. (sugar maple) established at the
64 beginning of the Holocene in the Upper Lake States region. These pollen records indicate sugar

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65 maple became dominant *ca.* 4,000 years following the retreat of glacial ice (Davis 1983), leaving

66 behind a long-term chronosequence.

67 Along this chronosequence, we previously located ecologically and edaphically matched

68 sugar maple stands which provides a unique opportunity to study the structuring force of time

69 on the assembly of soil microbial communities. Replicate sampling of *Actinobacteria*

70 communities within the same habitat type in four different geographic locations allows us to

71 determine if there is a "distance effect" (Martiny *et al.* 2006). Because each geographic

72 location corresponds with time elapsed following glacial retreat, we considered distance to be a

73 surrogate for time. Due to the periodic nature of glacial retreat, distance and time do not

74 follow a linear relationship. If dispersal limitation was a force structuring soil microbial

75 communities over long time frames, dispersal of actinobacterial propagules would be limited in

76 the more northern sites because they are the youngest. Therefore, differences in community

77 composition should correlate with distance, after controlling for present day environmental

78 variability. Furthermore, if the source of actinobacterial communities originated from the older

79 sites, then the distribution of these communities should be clustered on a phylogenetic tree.

80 That is, younger actinobacterial communities in the north should be a phylogenetic subset of

81 older communities in the south. Moreover, if dispersal limitation is not a factor shaping these

82 communities as the Baas-Becking theory predicts, then we would expect similar communities in

83 all sites. This alternative predicts that variation in actinobacterial community composition

84 should be structured by environmental factors such as overstory plant community composition

85 and biogeochemical characteristics of the soil.

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86 To test these alternatives, we initially characterized actinobacterial communities using
87 16S rRNA gene terminal restriction fragment length polymorphism (TRFLP) fingerprints. Using
88 this information, we further refined the test of our hypothesis via cloning and sequencing of the
89 actinobacterial 16S rRNA gene and subsequent taxonomic and phylogenetic analysis.
90 Actinobacterial community composition in our four study sites was assessed by examining
91 community similarity, identifying a distance-decay relationship, and testing the relatedness of
92 community patterns to environmental variation, climatic factors, and geographic distance, as a
93 proxy for site age, through multivariate statistics. Here, we provide evidence that dispersal
94 limitation is a mechanism shaping *Actinobacteria* communities in a northern hardwood forest
95 ecosystem over a relatively long time frame (i.e., *ca.* 5,000 yrs).

96 **METHODS:**

97 ***Study Sites and Sampling***

98 The biogeography of *Actinobacteria* was examined in the surface soil of four sugar
99 maple dominated forests on the Lower and Upper Peninsula of Michigan (Fig. 1). These sites
100 were selected from 31 candidate sites based on their ecological and edaphic similarity, which
101 were assessed by multivariate analyses of plant community composition, stand age, and soil
102 properties (Burton et al. 1991). Soils are well-drained sandy, typic haplorthod of the Kalkaska
103 series and overstory biomass is dominated by sugar maple (~70-85%). These sites form a long-
104 term chronosequence due to their similarity of environmental, ecological, and edaphic
105 characteristics, yet thousands of years elapsed following deglaciation and establishment of
106 forests at each site. The southernmost site D was ice free approximately 13,500 years before

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107 present (BP) followed by maple forest establishment 3,500 years later (Evenson *et al.* 1976;
108 Drexler *et al.* 1983; Davis 1983). Site C is located 83 km north of site D and was deglaciated
109 approximately 13,000 years BP, followed by maple forest establishment 4,000 later (Evenson *et*
110 *al.* 1976; Drexler *et al.* 1983; Davis 1983). Site B, located 150 km north of Site C, was uncovered
111 approximately 11,000 years BP, and pollen records indicate maple forest establishment 4,000
112 years later. Finally, the northernmost site A, 343 km North West of site B, was ice free 9,500
113 years BP, with maple forest establishing 3,500 years later (Evenson *et al.* 1976; Drexler *et al.*
114 1983; Davis 1983).

115 These sites are well characterized in terms of their climate, plant community and
116 biogeochemical characteristics. For example, daily air temperature, soil moisture and soil
117 temperature along with annual measurements of tree species, diameter and height, leaf litter
118 biomass, water balance, and leaf litter production have been recorded since 1994 and are
119 available at the Michigan Gradient website (http://www.webpages.uidaho.edu/nitrogen-
120 gradient/Default.htm). Forests on our study sites were harvested *ca.* 1900-1910 and have not
121 experienced human disturbance since that time; to the best of our knowledge, they all have
122 been exposed to the same disturbance regime and share the same land-use history.

123 We collected surface soil horizons (Oe, Oa, and A horizons) on three separate dates
124 (June 2006, October 2006 and May 2007) to characterize actinobacterial communities. In each
125 of the four sites, there are three randomly located 30-m x 30-m replicate plots ranging 15 to
126 150 meters apart. We have previously and continuously quantified ecological, edaphic, and
127 biogeochemical characteristics for each plot in all four study sites (Burton *et al.* 1993; Reed *et*

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128 *al.* 1994; Pregitzer *et al.* 2004; Burton *et al.* 2004). In each 30-m x 30-m plot, we collected 10
129 soil samples using a 2.5-cm diameter soil core, which extended to a depth of 5 cm. The 10
130 surface soil samples in each plot were composited and passed through a 2-mm sieve in the
131 field. From the sieved composite sample, a 5-g sub-sample was removed for DNA extraction.
132 By pooling the 10 soil cores, our sampling scheme aggregated small-scale spatial heterogeneity
133 at the scale of individual plots. We did so because our goal was to characterize the
134 actinobacterial community at the scale of entire forest stands, and to explore regional trends in
135 community similarity that many be structured by historical contingences and environmental
136 factors.

137 Samples were placed on ice in DNA extraction vials and immediately transport to the
138 University of Michigan, where they were held at -80°C prior to DNA extraction. In May of 2007,
139 Site B was defoliated by the canopy consuming insect, *Operophtera bruceata,* which deposited
140 large amounts of insect frass and green-leaf fragments on the forest floor (D.R. Zak, *personal*
141 *observation*). Because these insects dramatically altered the biochemical constituents, the
142 amount, and timing of leaf litter fall, we eliminated the May 2007 Site B samples from our
143 analyses.

144 **DNA extraction and PCR protocol**

145 Soils sampled in 2006 were used to characterize the actinobacterial community using
146 TRFLP, whereas we used cloning and sequencing to further characterize the community from
147 samples collected in the subsequent year. Microbial DNA extraction and actinobacterial 16S
148 rRNA gene amplification followed similar protocols to those previously described in Eisenlord

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149 and Zak (2010). Briefly, microbial community DNA was extracted from our 2006 samples in
150 triplicate from 0.25 to 1 g of soil using the Ultraclean Soil DNA extraction kit (Mo Bio
151 Laboratories) for TRFLP analysis. Microbial community DNA was extracted from our 2007 soil
152 samples using 5-g surface soil subsamples with MoBio PowerMax Soil DNA isolation kits (Mo
153 Bio Laboratories) within one week of field collection for clone analysis. Actinobacterial 16S
154 rRNA genes were amplified from total community DNA with primers Eub338F-
155 ACGGGCGGTGTGTACA and Act1159R – TCCGAGTTRACCCCGGC (Blackwood *et al.* 2005). The
156 PCR protocol followed 95 °C for 5 min for initial denaturing, then 25 rounds of amplification (94
157 °C for 30 sec, 57 °C for 30 sec, 72 °C for 90 sec) followed by 10 min at 72 °C for elongation, and
158 finally held at 6 °C before removal (adapted from Blackwood *et al.* 2005). All PCRs were
159 conducted in duplicate and products were pooled before purification with MoBio Ultra Clean
160 PCR Clean up Kit according to manufacturer's instruction. For TRFLP the PCR reaction differed
161 from above by having a 6-Carboxyflurescein (6-FAM) attached the Eub338F primer.

162 **Community Characterization using Terminal fragment length polymorphism (TRFLP)**

163 Following PCR clean up of the actinobacterial 16S rRNA gene amplicon, approximately
164 200-500 ng of purified PCR product, as determined by Picogreen® analysis (Invitrogen; as
165 instructed by the manufacturer) was digested with 5U of TaqI (Promega) at 65 °C for 1 h.
166 Passing digests through a Microcon YM-30 filter (Millipore) desalted them and removed
167 enzymes from the reaction. Each sample was submitted in duplicate for genotyping conducted
168 at the University of Michigan's Core Sequencing Facility using an ABI 3730XL DNA Sequencer
169 with a 96 capillary array. Rox 1000 (Bioventures) was used as a standard to determine

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170 restriction fragment lengths. Electropherograms were inspected using Genemarker 1.60
171 (SoftGenetics). We required a peak height 50 fluorescence units and the appearance of each
172 restriction fragment in both duplicates for our subsequent analyses. Each TRF with a peak
173 height that of 1% or greater of the total intensity were scored into the presence-absence matrix
174 (Hassett *et al* 2009).

175 **16S rRNA Gene Cloning and Phylogenetic Analysis**

176 Actinobacterial 16S rRNA genes were cloned with the Invitrogen TOPO TA cloning kit
177 using TOP10 chemically competent cells (Invitrogen). Inserts were sequenced at the Georgia
178 Genomics Facility at the University of Georgia (Athens, GA). This study expanded our previous
179 sequencing efforts of 33 clones in each plot (Eisenlord & Zak, 2010; Genbank accession
180 FJ661107-FJ662388) to include an additional 63 clones from each of the twelve samples (i.e., 3
181 plots in each of 4 study sites), totaling 1152 sequences (Genbank accession HQ845548-
182 HQ845603).

183 Sequences were manually edited in Geneious v.5.0.2 (Biomatters Ltd.) and 727 high
184 quality contiguous sequences were generated from forward and reverse sequences. The top-
185 type species matches were retrieved from the Ribosomal Database Project (RDP; Cole *et al.*
186 2009) for all sequences, and 50 representative sequences from every major group of the
187 *Actinobacteria* phyla were retrieved from the NCBI Taxonomy Browser for use as references.
188 Clone and reference sequences were aligned using ClustalW (Thompson *et al.* 1994) in the
189 program Geneious. Reference sequences were included in the alignment to build phylogenetic
190 backbone support by preserving spatial heterogeneity in the 16S sequences. Alignments were

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191 manually edited to remove gaps and ambiguously aligned sequences. Reference sequences
192 were removed from the clone alignment before operational taxonomic units were determined.

193 The clone sequence alignment was used to generate a distance matrix in Phylogeny
194 Inference Package (PHYLIP) version 3.69 (Felsenstein 2005), using the Jukes Cantor algorithm of
195 substitution. Mothur (Schloss *et al.* 2009) was then employed to assign operational taxonomic
196 units at 90, 93, 95, 97 and 99% similarity using the average neighbor algorithm. The relative
197 abundance of operational taxonomic units (OTUs) at each similarity level was examined to
198 address the argument that the resolution at which microbial communities are analyzed
199 influences results and subsequently their interpretation (Cho & Tiedje 2000). At 97% similarity,
200 Mothur was used for taxa-based alpha and beta diversity estimates within and across sites and
201 to run ∫-LIBSHUFF (Schloss *et al.* 2004), a program which uses coverage curves to statistically
202 detect if two or more microbial communities are similar using the Cramer-von Mises test
203 statistic (Schloss *et al.* 2009). OTU sequences at 97% similarity were generated by consensus of
204 clone sequences in Geneious.

205 Reference sequences and 56 actinobacterial OTUs defined at 97% similarity were then
206 re-aligned in Geneious with ClustalW for phylogenetic analysis. Because phylogenetic analyses
207 are sensitive to tree topology, RaxML was used to select the best-fit tree with the Maximum
208 Likelihood algorithm; *Staphylococcus aureus* was used to root the tree. Differences in the
209 phylogenetic patterns in each study site were quantified with the online statistical tool UniFrac
210 (Lozupone *et al.* 2006). Phylogenetic distances matrices reported by UniFrac, along with the

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211 relative abundances of OTUs at all five similarity levels, were used for multivariate statistics
212 described below.

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213 **Environmental Variables**

214 Environmental characteristics were assembled into four data sets: i) a biogeochemical
215 data set composed of factors which we selected *a priori* that are relevant to soil microbial
216 communities, ii) plant community composition, iii) climatic characteristics, and iv) distance
217 which represented time since glacial retreat. The biogeochemical data matrix included: soil pH
218 and moisture content (measured from our 2007 samples), and previously collected values for
219 leaf litter C content, leaf litter C:N ratio, total leaf litter mass, C:N ratio of soil organic matter,
220 and extractable soil NO_3^- (Table 1; Burton *et al.* 1993; Reed *et al*. 1994; Pregitzer *et al.* 2004;
221 Burton *et al.* 2004). All metadata are available at http://www.webpages.uidaho.edu/nitrogen-
222 gradient/Default.htm. All environmental data used in this study were averages over growing
223 season from the years 2005 to 2008. The second matrix represented the plant community
224 based on the relative importance (i.e., basal area of a species/basal area of all species) of
225 overstory and understory species (Supplemental Table A1). Our third data matrix characterized
226 climatic variation by including temperature, precipitation, and ambient N deposition, to identify
227 the role of climate in shaping these actinobacterial communities (Table 1). The primary
228 historical event taken into consideration for this study is the periodic retreat of the Wisconsin
229 ice sheet across lower and upper Michigan. Because distance between sites overlays time since
230 de-glaciation in our chronosequence, we used distance-time as our fourth data set; it was
231 composed of GPS coordinates taken at the center of each sample plot (Supplemental Table A2).

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232 The chosen variables for each set of data were assigned to biogeochemical, plant community,

233 climatic, and distance-time data sets for multivariate statistical analysis.

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234 **Multivariate Statistical Analysis** It is plausible that soil *Actinobacteria* biogeography is

235 shaped by local environmental conditions, historical factors, or by both. Following the

236 framework of Martiny *et al.* (2006), we used multivariate analyses (PRIMER v6; Plymouth, UK),

237 in order to identify significant correlations between factors composing biogeochemical, plant

238 composition, climatic, and distance-time data matrices.

239 TRFLP fingerprint data matrices, OTU relative abundance matrices, and phylogenetic

240 distances were treated similarly as 'biological' data. Similarity matrices for TRFLP fingerprints

241 were generated using the Bray-Curtis similarity metric (Bray & Curtis 1957) on non-transformed

242 presence-absence data. Relative abundances of OTUs were square root transformed to lessen

243 the emphasis of the most abundant species prior to the generation of similarity matrices with

244 the Bray-Curtis coefficient. Phylogenetic distances were generated with the online package

245 UniFrac (Louzapone *et al.* 2006).

246 Biochemical and climatic similarity matrices were generated with Euclidian distances of

247 standardized data, whereas the distance-time matrix was generated with the great circle

248 distance (Vincety 1975). The plant community similarity matrix was generated with the Bray-

249 Curtis metric (Bray & Curtis 1957). Biogeochemical, plant community, climatic, and distance-

250 time data were visualized with non-metric multidimensional scaling (nMDS; Fig. 2).

251 With site as the main factor, an analysis of similarity (ANOSIM) test was used to

252 compare communities across sites (n = 4), with individual plots as replicates within each site (n

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253 = 3). The Mantel-type test, RELATE, was used in conjunction with the Spearman rank
254 correlation coefficient to determine if there were significant correlations between the biological
255 data (TRFLP, OTU, and Phylogenetic distances) and the biogeochemical, plant community,
256 climatic, and distance-time data sets. The RELATE test is similar to the Mantel test in that it
257 uses element-by-element correlations of similarity matrices. Though instead of Pearson
258 correlations used by the Mantel test, RELATE uses Spearman rank correlation coefficients, as is
259 more appropriate for the interpretation of our data (Clark & Gorley 2006). Rank similarities
260 between site averages were used in this analysis to correct for the different scaling of each
261 correlation coefficient. The distance-decay relationship was explored with the 2006 TRFLP data
262 by plotting the log transformed average Sørensen community similarity metric (gained from
263 PRIMER), against the log transformed geographic distance between the plots.

264 Additional statistics were conducted in the R (R Development Core Team 2011) package
265 vegan (Oksanen *et al* 2011). Environmental vectors, of biogeochemical, climatic, and distance-
266 time data sets, were fit to nMDS ordinations of biological data, which identified the individual
267 variables correlated with community patterns. Redundancy analysis (RDA) was used to
268 examine the correlations between species patterns and environmental variables to evaluate
269 which variables explained significant proportions of variation in *Actinobacteria* community
270 composition. Distance-based redundancy analysis (db-RDA; Legandre & Anderson, 1999) was
271 applied using the Bray-Curtis distance metric to determine if distance-time significantly
272 accounted additional biological variation, after the variation due to environmental variables
273 was held constant.

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275 **RESULTS**

276 At all levels of examination, actinobacterial communities were compositionally different

277 in each of our four forest sites, and variation in the communities was significantly correlated

278 with time since glacial retreat (i.e., distance; Table 2).

279 **TRFLP community comparison**

280 Based on greater than 1% contribution to total TRFs, there were 27 unique TRFs in July,

281 and 26 in October. Rarefaction curves generated in EstimateS (Colwell 2009) approached an

282 asymptote and can be viewed online in supplemental information (Fig. A1). TRFLP

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283 actinobacterial community similarity, based on the Sørensen metric, had a significant negative

284 relationship with time since glacial retreat (z-score=-0.094, *P* = 0.02, Fig. 3). This distance-decay,

285 or time-decay, relationship held true for both July and October sampling dates; as distance

286 increased, community similarity decreased (July slope= -0.11 October slope= -0.03). There was

287 no such relationship present when biogeochemical characteristics were regressed against

288 geographic distance (*P* = 0.29). RELATE results, based on the Bray-Curtis similarity matrices of

289 July and October TRFLP data, indicate actinobacterial communities are more similar in

290 composition the closer they are geographically and in age (Table 2). There was no evidence in

291 the July samples that communities with similar biogeochemical or climatic characteristics have

292 similar actinobacterial TRFLP profiles (*P* = 0.115, *P* = 0.436). The fitting of all environmental

293 vectors to the July nMDS, displayed in Figure 4, revealed distance-time to be significantly

294 correlated with community patterns (*P*=0.014), none of the other variables were significant (*P*

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295 values range 0.174-0.671). The db-RDA analysis revealed distance-time accounted for an
296 additional 17% of community variation, after variation correlated with environmental and
297 climatic factors was held constant (P = 0.048).

298 In contrast, based on RELATE analysis, October samples with similar biogeochemical
299 characteristics did have similar actinobacterial profiles (Spearman = 0.309, P = 0.015).
300 Environmental vector fitting revealed both distance-time (P = 0.030) and litter C:N (P = 0.043)
301 were correlated with community patterns; no other variables were significant (P = 0.174-0.797).
302 The RDA found distance-time significantly accounted for 18% of community variation (P =
303 0.026) and litter C:N accounts for 18% of the variation (P = 0.015). However, dbRDA revealed,
304 when variation due to litter C:N was held constant, distance-time did not significantly explain
305 any more of the variation in the communities (P = 0.665), and the same was true for litter C:N
306 after variation due to distance was held constant (P = 0.263). There was no relation of
307 actinobacterial communities to the plant community in either July (Spearman = 0.04, P = 0.544)
308 or October (Spearman = 0.03, P = 0.524).

309 **Taxonomic Alpha and Beta diversity**

310 Analysis of 727 cloned actinobacterial sequences from May 2007 sample sites A, C, and
311 D resulted in 56 OTUs grouped at 97% similarity. We identified OTUs in 16 out of 39
312 actinobacterial families , classified with the Ribosomal Database Project (Table 3). For the most
313 abundant OTUs, the closest similarity to known organisms was 90% to members of the
314 *Thermomonosporaceae* family. ʃ-LIBSHUFF results revealed significant differences in
315 community membership between sites A and D (P < 0.001), sites C and D (P = 0.007), and

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316 between sites A and C (*P* = 0.015). Diversity estimates, Ace and Chao1, indicated that the oldest
317 site D was more diverse than the two northern and younger sites, but this difference was not
318 resolved when the 95% confidence intervals were considered. Rarefaction curves (see Fig. A1
319 in Supporting Information) also indicate the oldest (Site D) contained a greater richness than
320 the younger sites. Although the rarefaction curves approached an asymptote, we did not
321 capture the full diversity of the actinobacterial community. When examining the families found
322 at each site, the oldest site (D) contained thirteen unique OTUs from four families not found in
323 any of the younger sites. The next oldest site (C) contained one unique family, whereas the
324 youngest site (A) contained no unique families (Table 3). We individually regressed the most
325 abundant and diverse groups of *Actinobacteria*, the *Micromonospora, Pseudonocardia,*
326 *Thermomonopsora*¸and *Acidimicrobium* against pH, DOC, SOM N content, leaf litter mass, and
327 C:N ratio and found no significant relationship in any case (data not shown).

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328 **Phylogenetic Community Analysis**

329 The UniFrac metric was used to identify unique phylogenetic branch length belonging to
330 actinobacterial communities within each site when compared with each other site, as well as
331 when compared to the entire community. The youngest site (A) and the oldest site (D) each
332 had significantly unique lineages when compared against the entire phylogenetic tree (*P* = 0.03,
333 *P* = *0*.02, respectively); however the site of intermediate age (C) did not (*P* = 0.42). UniFrac also
334 revealed that the oldest site (D) had unique lineages when compared with the two younger
335 sites (D-C *P* = 0.06; D-A *P* = 0.03). The P test further revealed that phylogenetic clustering of
336 sites within the phylogenetic tree did not occur (*P* = 0.15), and upon visual inspection of our

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337 tree, there was no evidence that actinobacterial communities in the younger sites were a
338 subset of those in the oldest site.

339 The UniFrac distance matrices were analyzed with PRIMER to determine if samples with
340 similar biogeochemical, plant community, climatic, or distance-time characteristics also
341 contained closely related communities. The ANOSIM, with site as a main factor, indicated that
342 plots within a particular site were more similar than plots between different sites (P = 0.004);
343 the nMDS ordination of actinobacterial phylogenetic distances is displayed in Figure 2. As with
344 the TRFLP fingerprint data, the 97% similarity phylogenetic distances were significantly
345 correlated with distance-time (Spearman = 0.24 P = 0.064), but not with biogeochemical
346 (Spearman = 0.17 P = 0.223), plant community (Spearman = 0.15 P = 0.293) or climatic
347 (Spearman = 0.17 P = 0.159) variables. Fitting of all environmental variables to the nMDS
348 demonstated leaf litter C:N (P = 0.028), temperature (P = 0.062), and distance-time (P = 0.081)
349 all correlated with patterns of phylogenetic distance when considered independently. When
350 variation due to environmental variables was held constant in the db-RDA, distance-time did
351 not explain additional variation in the communities (P = 0.33). Interestingly, when variation due
352 to distance-time was held constant, none of the environmental variables could account for
353 variation in the communities either (P values range 0.16-0.25).

354 Because there is much debate about the scale at which to examine relationships
355 between microbial communities and environmental characteristics (Cho & Tiedje 2000; Bissett
356 *et al*. 2010), we examined the relative abundance of OTUs at 90, 95, 97, and 99% similarity and
357 their relationship to biogeochemical, plant community, climactic and distance-time data sets

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358 (Table 2). Relative abundances were square root transformed to minimize the impact of
359 abundant species and allow for higher contribution of the more rare species. When the
360 actinobacterial sequences were examined at 90% similarity, there were no significant
361 differences in the relative abundances of these phylotypes between sites, as detected by
362 ANOSIM; however, at each of the higher similarities, plots grouped more closely within sites
363 than between sites. Furthermore, there were no instances in which biogeochemical or plant
364 community characteristics significantly correlated with OTU relative abundances (P = 0.068-
365 0.13; Table 2). Climate characteristics, specifically annual temperature, were correlated with
366 actinobacterial relative abundance at 97 and 99% similarity and distance-time significantly
367 correlated with relative abundances of *Actinobacteria* at 95, 97, and 99% similarity (Table 2).

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368 **DISCUSSION**

369 Microorganisms are believed to be globally distributed by prevailing winds (Griffin *et al.*
370 2002) and community patterns in space and time are thought to result from barriers to
371 dispersal, physiological requirements, resource availability, competition, or some combination
372 thereof (Whitaker *et al.* 2003; Papke & Ward 2004). Several factors lead us to reason that the
373 regional species pool of *Actinobacteria* lies to the west of our study sites and provided
374 propagules in a consistent manner as each site was freed from glacial ice over the past *ca.*
375 14,000 yrs. First, the prevailing winds at each study site come from the west, across large
376 bodies of water (i.e., Lake Michigan and Lake Superior). Wind can be an agent of long-distance
377 dispersal for *Actinobacteria,* as well as other bacteria (Pearce *et al.* 2009), and each study site
378 should have received wind-blown propagules from the same regional species pool due to their
379 perpendicular orientation to prevailing winds. If indeed 'everything is everywhere' and there

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380 are no dispersal limitations within the *Actinobacteria*, theory follows that each ecologically
381 equivalent study site will have similar actinobacterial communities due to near identical
382 environmental variables, which eliminate environmental filtering as well as constant additions
383 by the regional species pool. Conversely, Bissett *et al.* (2010) described a hypothesis "wherever
384 you go, that's where you are" implying that beyond strong environmental selection, other
385 factors (i.e. dispersal or colonization limitation and evolutionary events) play a significant role in
386 shaping microbial communities. If this is true, and not all sites received constant additions of
387 *Actinobacteria* as glaciers receded from the region due to dispersal limitation, then distance-
388 time would be detectable as a significant force in structuring the assembly of these
389 communities. Consistent with this expectation, our analyses revealed that distance, a surrogate
390 for time, was a significant factor shaping actinobacterial communities in soil, thereby providing
391 evidence that dispersal limitation was an ecological force structuring these communities.

392 To better understand the importance of dispersal limitation as an ecological force, we
393 sought to identify the degree to which environmental heterogeneity, climatic variation, and
394 distance influenced actinobacterial communities in soil. We purposely held ecological and
395 edaphic factors as constant as possible across our study sites to minimize differences in habitat
396 characteristics. Distance was used as a proxy of the time since glacial retreat exposed new
397 landscapes for colonization. As distance increased, the community similarity of *Actinobacteria*
398 significantly decreased with a z-score similar to those found in a study by Martiny *et al.* (2011)
399 of salt marsh microbial communities. If environmental conditions became increasingly different
400 over distance as well, the most logical explanation for this distance-decay relationship would be
401 that species are adapted to, and structured by, their niche requirements. However, our forest

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402 sites were chosen to constrain differences in edaphic and ecological characteristics, and as
403 distance increased between sites, these properties did not become increasingly different.
404 Furthermore, July actinobacterial community composition was not correlated with any of our
405 measured environmental characteristics when considered together or independently, despite
406 the fact that these edaphic characteristics can shape soil microbial communities (Bååth &
407 Anderson 2003; Van der Guch 2007; Lauber *et al.* 2008). When variation related to these
408 variables was held constant, distance-time still accounted for close to 20% of total variation in
409 our communities.

410 We also can dispel the notion that subtle variation in plant community composition
411 influences soil actinobacterial communities, because we found no relationship between the
412 plant community and actinobacterial communities at every level of investigation. When we
413 examined the relation of actinobacterial communities at 90, 93, 95, and 99% DNA similarity,
414 there were no detectable differences between the sites at the coarsest level (90%) of similarity.
415 Patterns emerged only when the communities were considered at finer phylogenetic
416 resolutions. This implies all of our stands have community members from the same pool of
417 actinobacterial sub-orders, and the differences in the communities occur at the family and
418 genus levels, represented by our analysis of higher DNA similarity percentages. The variation in
419 communities at these finer levels of genetic resolution was consistently related to distance-
420 time, litter C:N, and temperature. Despite our best efforts to hold edaphic properties constant
421 to minimize the effect of environmental filtering and allow for the detection of possible
422 dispersal limitation, changes in the communities over the growing season lead to correlations
423 with the minor changes leaf litter C:N ratio and temperature in our May communities, and litter

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424 C:N in October biological data set. In both cases, when variation due to distance-time (17-18%)
425 was held constant, litter C:N and temperature no longer explained a significant proportion of
426 the variation. Regardless, by examining these communities at many levels of resolution, we
427 have revealed distance (i.e., time) was consistently correlated with variation in actinobacterial
428 community composition, indicating both environmental heterogeneity and historical
429 contingencies play a role in shaping these microbial communities

430 Although the *Actinobacteria* communities in the younger sites did not phylogenetically
431 cluster as a sub-set within the older sites, the oldest site D had the highest species richness
432 estimates and rarefaction curves based on TRFLP and taxonomic data sets. Furthermore,
433 phylogenetic analysis revealed this oldest site contained members of four families and one sub-
434 order, which did not occur in the other sites. Our oldest site also has the highest proportion of
435 unique community members, and this trend was further supported by a significant amount of
436 unique phylogenetic lineage when compared to the younger sites. It is plausible that the higher
437 diversity in our oldest site could result from longer time elapsing since de-glaciation, allowing
438 more time to accumulate additional species from the regional species pool, as well as more
439 time for local adaptation or drift to occur. Although we concede a more in-depth and thorough
440 evaluation of these communities is needed before we can draw firm conclusions from these
441 taxonomic and phylogenetic community patterns, the consistent nature of our results indicate
442 historical contingencies do influence *Actinobacteria* community composition over long periods
443 of time regardless of the high amount of unexplained variation.

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444 This study highlights the importance of examining the identity of organisms as well as
445 how related they are to one another when studying microbial biogeography. Based on
446 presence-absence and relative abundance of 16S rRNA actinobacterial genes, multivariate
447 statistics indicate the observed distance-decay relationship was best explained by distance-
448 time, providing evidence that dispersal limitation structures actinobacterial communities
449 (Whitaker *et al.* 2003). Furthermore, after variation due to distance-time was held constant,
450 biogeochemical and climatic variation could not account for further variation in these
451 communities. However, further multivariate and phylogenetic analyses revealed a significant
452 amount of unique lineage at the youngest site, lack of clustering along the phylogenetic tree,
453 and the correlation of genetic distance to leaf litter C:N and temperature as well as distance-
454 time, all indicate that a simple mechanism of time and dispersal limitation may not be the only
455 ecological factor shaping these communities. Therefore, we suspect other mechanisms
456 contribute to the spatial patterns of soil *Actinobacteria* in our study: such as the lasting imprint
457 of "priority effects" on microbial community assembly (Fukami *et al.* 2010), the possibility that
458 subtle difference in leaf litter biochemistry could alter community composition, or changes in
459 unmeasured variables such as the community composition of other bacteria and fungi which
460 share a similar niche, all could impact actinobacterial communities. Regardless, we have strong
461 evidence on many levels of resolution establishing that time since glacial retreat leads to
462 decreased community similarity without decreasing environmental homogeneity across this
463 chronosequence of sugar maple forest ecosystems, and that distance, a surrogate for time, has
464 consistent and significant impacts on these *Actinobacteria* communities.

For Review Only

465 References

466 Baas Becking LGM (1934) Geobiologie of inleiding tot de milieukunde. *The Hague, the*
467 *Netherlands: W.P. Van Stockum & Zoon (in Dutch).*

468 Bååth E, Anderson TH (2003) Comparison of soil fungal/bacterial ratios in a pH gradient using
469 physiological and PLFA-based techniques. *Soil Biology and Biochemistry,* **35**, 955-963.

470 Bissett A, Richardson E, Baker G, Wakelin S, Thrall PH (2010) Life history determines
471 biogeographical patterns of soil bacterial communities over multiple spatial scales.
472 *Molecular Ecol*ogy, **69**, 134-157.

473 Blackwood CB, Oaks A, Buyer JS (2005) Phylum- and class-specific PCR primers for general
474 microbial community analysis. *Applied Environmental Microbiology*, **71**, 6193-6198.

475 Brady NC, Weil RR (2002) The nature and properties of soils: Thirteenth edition. Pearson
476 Education, Inc., Upper Saddle River, New Jersey. ISBN 0-13-016763-0.

477 Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin.
478 *Ecology Monog*raphs, **27**, 325-349.

479 Burton AJ, Ramm CW, Pregitzer KS, Reed DD (1991) Use of multivariate methods in forest
480 research site selection. *Canadian Journal of Forest Research,* **21**, 1573-1580.

481 Burton AJ, Pregitzer KS, MacDonald NW (1993) Foliar nutrients in sugar maple forests along a
482 regional pollution-climate gradient. *Soil Science Society of America,* **57**, 1619-1628.

483 Burton AJ, Pregitzer KS, Crawford JN, Zogg GP, Zak DR (2004) Simulated chronic

For Review Only

484 NO_3^- addition reduces soil respiration in northern hardwood forests. *Global Change*
485 *Biology,* **10**, 1080-1091.

486 Cho JC, Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent Pseudomonas
487 strains in soil. *Applied Environmental Microbiology,* **66**, 5448-56.

488 Clark KR, Warwick RM (2001) *Change in marine communities: an approach to statistical analysis*
489 *and interpretation, 2nd edition.* PRIMER-E Ltd: Plymouth, UK.

490 Clark KR, Gorley RN (2006) *PRIMER v6: User Manual/Tutoria.l* PRIMER-E Ltd: Plymouth, UK.

491 Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, *et al.* (2009) The Ribosomal Database
492 Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*
493 37 (Database issue): D141-D145; doi: 10.1093/nar/gkn879.

494 Colwell RK (2009) EstimateS: Statistical estimation of species richness and shared species from
495 samples. Version 8.2. User's Guide and application published at:
496 http://purl.oclc.org/estimat

497 Davis MB (1983) Quaternary history of deciduous forests of eastern North America and Europe.
498 *Garden Journal*, **70**, 550-563.

499 DeAngelis KM, Allgaier M, Charvarria Y, Fortney JL, Hugenholtz P, Simmons B et al. (2011)
500 Characterization of trapped lignin-degrading microbes in tropical forest soil. PLoS One
501 doi: 10.1371/journal.pone.0019306

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For Review Only

502 Drexler CW, Farrand WR, Hughes JD (1983) Correlation of glacial lakes in the Superior Basin
503 with eastward discharge events from Lake Agassiz. in Teller, J.T. and L. Clayton (eds.)
504 Glacial Lake Agassiz. *Geology Association of Canada,* Special Paper **26**, 309-329.

505 Eisenlord SD, Zak DR (2010) Simulated atmospheric nitrogen deposition alters Actinobacterial
506 community composition in forest soils. *Soil Science Society of America,* **74**, 1157-1166.

507 Embley TM, Stackebrandt E (1994) The molecular phylogeny and systematic of the
508 actinomycetes. *Annual Review of Microbiology*, **48**, 257-289.

509 Evenson E, Farrand W, Eschman D, Mickelson D, Maher L (1976) Greatlakean substage: A
510 replacement for valderan substage in the lake Michigan basin. *Quaternary Research,* **6**,
511 411-424.

512 Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Dep. of Genome Sci.,
513 Univ. of Washington, Seattle.
514 Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities.
515 *Proceedings of the National Academy of Sciences,* **103,** 3, 626-631.

516 Fulthorpe R, Rhodes AN, Tiedje J (1998) High levels of endemicity of 3-chlorobenzoate-
517 degrading soil bacteria. *Applied Environmental Microbiology,* **64**, 1620-1627.

518 Fukami T, Dickie IA, Wilkie P, Paulus BC, Park D, Robers A, *et al*. (2010) Assembly history
519 dictates ecosystem functioning: evidence from wood decomposer communities. *Ecology*
520 *Letters*, **13**, 675-684.

For Review Only

521 Ge Y, He J, Zhu Y, Zhang J, Xu Z, Zhang L, Zheng Y (2008) Differences in soil bacterial diversity:
522 driven by contemporary disturbances or historical contingencies? *International Society*
523 *of Microbial Ecology,* **2**, 254-64.

524 Green J, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. *Trends in Ecology and*
525 *Evolution*, **21**, 501-507.

526 Griffin DW, Kellogg CA, Garrison VH, Shinn EA (2002) The global transport of dust: an
527 intercontinental river of dust, microorganisms and toxic chemicals flows through the
528 Earth's atmosphere. *American Scientist,* **90**, 228-235.

529 Hassett JE, Zak DR, Blackwood CB, Pregitzer KS (2009) Are basidiomycete laccase gene
530 abundance and composition related to reduced lignolytic activity under elevated
531 atmospheric NO3- deposition in a northern hardwood forest. *Microbial Ecology,* **57**,
532 728-739.

533 Horner-Devine MC, Carney KM, Bohannan BJM (2004) An ecological perspective on bacterial
534 biodiversity. *Proceedings of the Royal Society of London B*, **271**, 113-122.

535 Lauber CL, Strickland MS, Bradford MA, Fierer N (2008) The influence of soil properties on the
536 structure of bacterial and fungal communities across land-use types. *Soil Biology and*
537 *Biochemistry*, **40**, 2407-2415.

538 Legandre P, Anderson MJ (1999) Distance-based redundancy analysis: testing multispecies
539 responses in multifactorial ecological experiments. *Ecological Monographs,* **69**, 1–24.

For Review Only

540 Lozupone C, Hamady M, Knight R, (2006) UniFrac: An online tool for comparing microbial
541 community diversity in a phylogenetic context. *BMC Bioinformatics,* **7**, 371.

542 Martiny JBH, Bohannan BJM, Brown JH, Colwell R, Fuhrman J, *et al.* (2006) Microbial
543 biogeography: putting microorganisms on the map. *National Review of Microbiology,* **4**,
544 102-112.

545 Oksanen J, Blanchet G, Kindt R, Legendre P, O'Hara RB, Simpson GL1, Solymos P, Henry H.
546 Stevens and Helene Wagner (2011). vegan: Community Ecology Package. R package
547 version 1.17-11. http://CRAN.R-project.org/package=vegan

548 Papke RT, Ward DM (2004) The importance of physical isolation to microbial diversification.
549 *FEMS Microbial Ecology*, **48**, 293-303.

550 Martiny JBH, Eisen JA, Penn K, Allison SD, Honer-Devine MC (2011) Drivers of bacterial B-
551 diversity depend on spatial scale. *Proceedings of the National Academy of Science*, **108,**
552 7850-7854.

553 Paul EA, Clark FE (1996) *Soil Microbiology and Biochemistry*. Second Edition. Academic Press,
554 Inc. Sand Diego, CA. Chapter 6. 130-152.

555 Pearce D, Bridge PD, Hughes KA, Sattler B, Psenner R, Russell N (2009) Microorganisms in the
556 atmosphere over Antarctica. *FEMS Microbial. Ecology,* **69**, 143-57.

For Review Only

557 Pregitzer KS, Zak DR, Burton AJ, Ashby JA, MacDonald NW (2004) Chronic nitrate additions
558 dramatically increase the export of carbon and nitrogen from northern hardwood
559 ecosystems. *Biogeochemistry*, **68**, 179-197.
560 R Development Core Team (2008) R: A language and environment for statistical computing. R
561 Foundation for statistical computing, Vienna, Austria. ISBN 3-900051-07-0, URL
562 http://www.R-progect.org

563 Roberts MS, Cohan FM (1995) Recombination and migration rates in natural populations of
564 *Bacillus subtilis* and *Bacillus mojavensis. Evolution* **49**, 1081-1094

565 Ramette A, Tiedje JM (2007a) Multiscale responses of microbial life to spatial distance and
566 environmental heterogeneity in a patchy ecosystem. *Proceedings of the National*
567 *Academy of Science*, **4**, 2761-2766.

568 Ramette A, Tiedje JM (2007b) Biogeography: an emerging cornerstone for understanding
569 prokaryotic diversity, ecology, and evolution. *Microbial Ecology*, **53**, 197-207.

570 Reed DD, Pregitzer KS, Liechty HO, Burton AJ, Mroz GD (1994) Productivity and growth
571 efficiency in sugar maple forests. *Forest Ecology Management*, **70**, 319-327.

572 Schloss PD, Westcott S, Rayabin T, Hall J, Harmann M, Hollister E, *et al*, (2009) Introducing
573 mothur: Open-source, platform-independent, community-supported software for
574 describing and comparing microbial communities. *Applied Environmental Microbiology,*
575 **75**, 7537-7541.

For Review Only

576 Schloss PD, Larget BR, Handelsman J (2004) Integration of microbial ecology and statistics: a
577 test to compare gene libraries. *Applied Environmental Microbiology,* **70**, 5485-92.
578 (http://www.plantpath.wisc.edu/joh/s-libshuff.html)

579 Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive
580 multiple sequence alignment through sequence weighting, position-specific gap
581 penalties and weight matrix choice. *Nucleic Acids Research,* **22,** 4673-4680.

582 Van der Gucht K, Cottenie K, Muylaert K, Muylaert K, Vloemans N, Cousin S, *et al*. (2007) The
583 power of species sorting: local factors drive bacterial community composition over a
584 wide range of spatial scales. *Proceedings of the National Academy of Science*, **104**,
585 20404-20409.

586 Wawrik B, Kutliev D, Abdivasievna UA, Kukor JJ, Zylstra GJ Kerkhof L (2007) Biogeography of
587 actinomycete communities and type II polyketide synthase genes in soils collected in
588 New Jersey and Central Asia. *Applied and Environmental Microbiol*ogy, **73**, 2982-2989.

589 Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of
590 hyperthermophilic archaea. *Science,* **301**, 976-8.
591 Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald FG, Chater KF, van Sinderen D (2007)
592 Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum.
593 *Microbiology and Molecular Biology Reviews,* **71**, 495-548.

594 Vincety T (1975) Direct and inverse solutions of geodesics on the ellipsoid with application of
595 nested equations. Survey Review (Kingston Road, Tolworth, Surrey), **23**, 88-93.

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597 DATA ACCESSIBILITY

598 DNA sequences: Genbank accessions FJ661107-FJ662388; HQ845548-HQ845603

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FIGURE LEGENDS

Figure 1. Forest sites composing a long-term chronosequences following glacial retreat. Southernmost Site D is the oldest, whereas northernmost Site A is the youngest. Details regarding site age and environmental characteristics can be found in the text, along with our methods for identifying ecologically similar sites across this region.

Figure 2. Non Metric Multi-Dimensional Scaling averaged by site (stress < 0.05), error bars represent standard error of three biological replicates within each site. (a,b) Biogeochemical and climatic data sets separate sites A, B, C and D based on Euclidian distances (c) distance data, as a proxy for site age, is represented by the great circle distance between sites (d,e) Biological data matrices, July TRFLP and October TRFLP, were generated using the Bray-Curtis similarity metric (f) Phylogenetic distances between sites A, C, and D based on UniFrac genetic distances. All images were generated in PRIMER v.6 and edited in Excel 2007.

Figure 3. Distance-decay relationship displaying the log transformed community similarity based on the Sørensen metric of averaged July and October TRFLP profiles plotted against the log transformed distance between sites. Slope is 0.0946, Y intercept is 2.07, and P=0.02.

Figure 4. Environmental vector fitting on a non-metric multidimentional scaling (nMDS) plot of July 2006 TRFLP *Actinobacteria* communities calculated with the Bray-Curtis dissimilarity metric. Environmental variables included were *a priori* decided biogeochemical factors, climatic variable temperature, and distance-time. Distance-time was the only variable significantly correlated with community composition, designated by *(P=0.014).

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Table 1. Site averages for age, climate, and environmental characteristics.

	A	B	C	D
Glacial Retreat (years BP)	9500	11000	13000	13500
Climate				
*Mean Temp (C)	4.82	6.06	6.49	7.65
Mean Precipitation (cm)	91.87	93.28	92.81	86.63
*Ambient N dep (kg/ha)	5.89	6.07	7.37	7.37
Environment				
*Leaf Litter [C] (g/kg)	458	456	453	455
*Leaf Litter C:N	63.68	57.06	52.91	43.41
*Leaf Litter Mass (g)	412.7	396.3	591	550.2
Extractable DOC (mg/L)	5.5	2.79	5.85	9.95
Extractable NO_3^- (mg/L)	0.08	0.55	0.86	0.92
SOM C:N	13.12	22.55	15.9	11.42
SOM [N] (mg/g)	1.84	1.36	1.83	1.73
*pH	4.55	4.7	4.41	4.61
*Moisture Content (%)	23	24	18	14

* Paramaters included in data sets for RDA Analysis

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Table 2. Analysis of similarity (ANOSIM) results with site as a main factor and Mantel-type test RELATE results for TRFLP, phylogenetic distance, and OTU relative abundances of five levels of similarity. TRFLP data is presence absence from 2006, phylogenetic distances based on 97% similarity, and five levels of OTU relative abundances were square root transformed before analysis. Spearman metric statistic is related as Rho.

	ANOSIM		RELATE							
			Biogeochemical		Plant community		Climate		Distance-time	
Data	R Statistic	P-value	Rho	P-Value	Rho	P-value	Rho	P-value	Rho	P-value
TRFLP June	0.45	**0.002**	0.18	0.115	0.04	0.54	0.02	0.436	0.35	**0.022**
TRFLP Oct	0.29	**0.015**	0.31	**0.015**	0.03	0.52	0.14	0.144	0.32	**0.027**
Phylogenetic Distance	0.58	**0.004**	0.17	0.223	0.15	0.29	0.17	0.159	0.24	*0.064*
OTU 90%	0.06	0.320	-0.10	0.680	0.5	0.51	0.08	0.672	0.00	0.479
OTU 93%	0.33	**0.020**	0.19	0.155	0.5	0.52	0.15	0.165	0.15	0.186
OTU 95%	0.35	**0.050**	0.02	0.474	0.5	0.48	0.16	0.133	0.36	**0.043**
OTU 97%	0.69	**0.004**	0.21	0.138	1	0.18	0.33	**0.032**	0.35	**0.036**
OTU 99%	0.56	**0.001**	0.12	0.222	0.5	0.51	0.38	**0.041**	0.32	**0.031**

Bold designates significant *P* values as less than 0.050. Italicized *P*-values are considered suggestive as less than 0.075.

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Table 3. A total of 727 clones from three sites (A, C and D) grouped into 56 OTUs at 97% similarity. We identified *Actinobacteria* in 16 out of 39 Actinobacterial families based on RDP values and phylogenetic analysis. All groupings except the *Acidimicrobiales* are within the Order *Actinomycetales*. We report total number of clones and their abundance at each site as well as the total number of OTUS and their abundance at each site.

	Clones:				OTUs:			
Family	Total	A	C	D	Total	A	C	D
Micromonospora	42	20	6	16	5	4	2	3
Actinospicaceae	8	5	1	2	4	3	1	0
Catenulisporaceae	5	0	0	5	1	0	0	1
Pseudonocardiaceae	56	26	16	14	4	4	3	3
*Corynibacterineae**	87	29	27	31	4	2	2	4
Thermomonosporaceae	334	109	119	106	6	4	4	5
Streptosprangeaceae	1	0	0	1	1	0	0	1
Nocardioidaceae	5	1	1	3	3	1	1	3
Microbacteriaceae	17	8	6	3	3	1	2	3
Micrococaceae	3	0	0	3	1	0	0	1
Streptomycetaceae	11	4	2	5	3	2	1	3
Nakamureliaceae	8	0	3	5	1	0	1	1
*Frankia**	1	0	0	1	1	0	0	1
Kinosproaceae	1	0	1	0	1	0	1	0
Geodermaceae	5	1	2	2	2	1	1	1
Acidimicrobium†	143	42	59	42	17	9	11	11

*Designates grouping to Sub-order †Designates Order *Acidimicrobiales*

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Appendices

Figure A1. Rarefaction curves for (a) TRFLP based on greater than 1% contribution, and (b) Phylogenetic analysis based on 97% DNA similarity for each site A, C and D.

Table A1. Geographic coordinates for Michigan Gradient sample plots.

Table A2. Basal area for the over-story tree species.

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Figure A1.

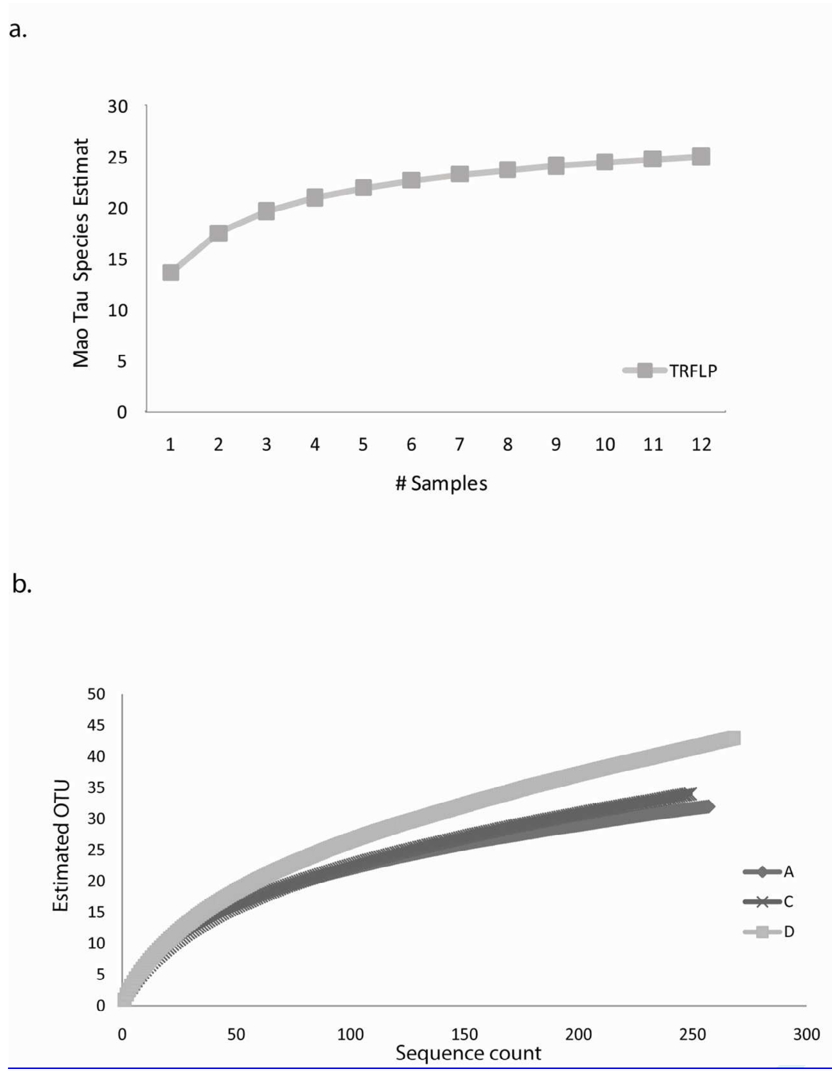

Table A1. Geographic coordinates for each sample plot (1-3), within each site (A-D)

	Lattitude	Longitude
A1	46:51.6642	88:52.8793
A2	46:51.7075	88:52.9198
A3	46:51.6996	88:52.9634
B1	45:32.7536	84:51.5597
B2	45:32.7153	84:51.5292
B3	45:32.6869	84:51.5473
C1	44:22.8473	85:49.9909
C2	44:22.7751	85:50.0526
C3	44:22.8381	85:49.9910
D1	43:40.0910	86:08.6249
D2	43:40.1556	86:08.7045
D3	43:40.1729	86:08.7750

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Table A2. Basal area for over-story tree species. AB = American Beach, BC = Black Cherry, BF= Balsam fir, BO= Black Oak, BW = American Basswood, HM = Hard Maple (Sugar Maple), RM = Red Maple, RO= Red Oak, IW = Iron Wood, YB= Yellow Birch, WA = White ash.

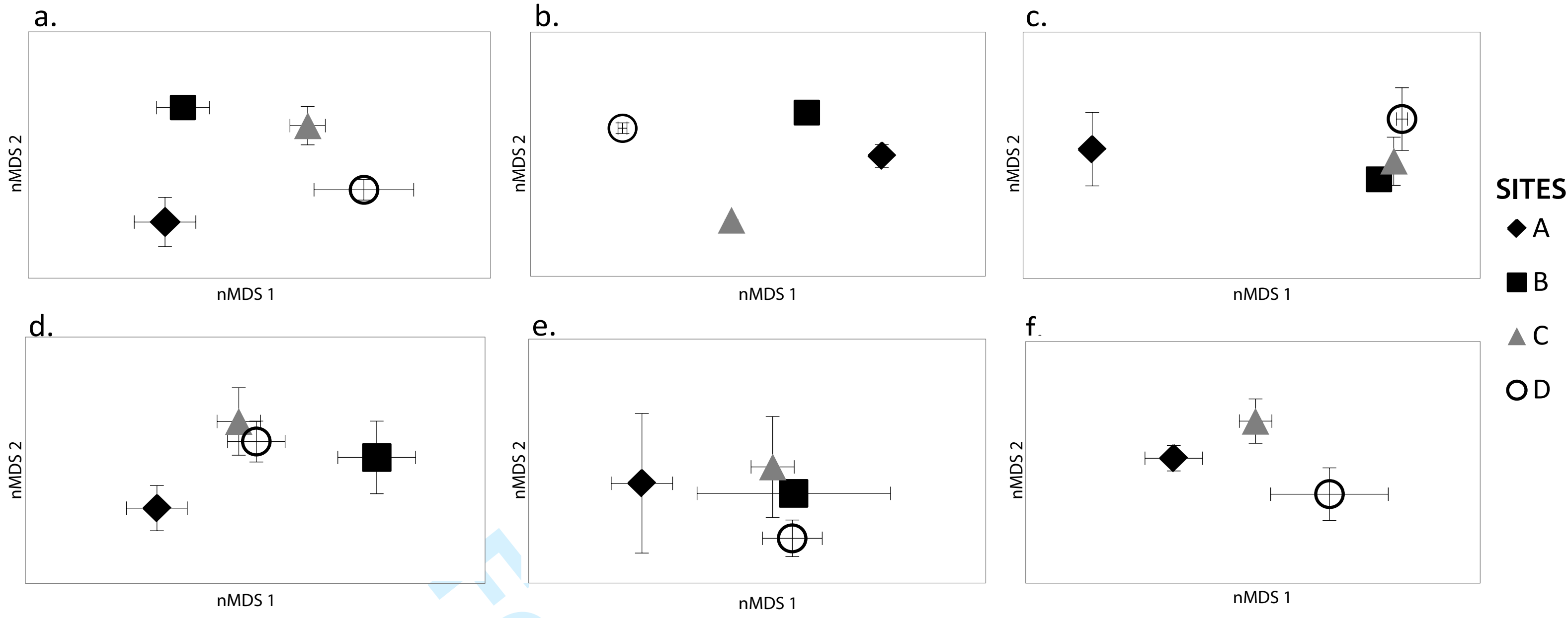
		Basal Area / Species										
Site	Total	AB	BC	BF	BO	BW	HM	RM	RO	IW	YB	WA
A1	35.42	-	-	-	-	-	29.27	5.65	-	0.06	0.44	-
A2	33.93	-	-	-	-	-	33.93	-	-	-	-	-
A3	31.49	-	-	0.03	-	1.40	29.66	-	-	-	0.40	-
B1	31.23	0.18	-	-	-	-	26.93	-	-	-	-	4.12
B2	33.71	-	-	-	-	1.14	30.44	-	-	-	-	2.13
B3	34.59	-	-	-	-	-	29.01	-	-	-	-	5.58
C1	37.05	-	1.29	-	-	-	26.02	6.44	3.30	-	-	-
C2	32.41	0.13	-	-	-	-	29.21	3.07	-	-	-	-
C3	35.51	-	-	-	4.78	-	26.79	0.58	3.36	-	-	-
D1	35.11	-	-	-	-	-	30.55	1.42	3.14	-	-	-
D2	40.4	1.06	2.54	-	-	-	25.02	11.78	-	-	-	-
D3	34.64	1.17	5.29	-	-	-	22.71	3.97	1.39	0.11	-	-

For Review Only

Figure 1.

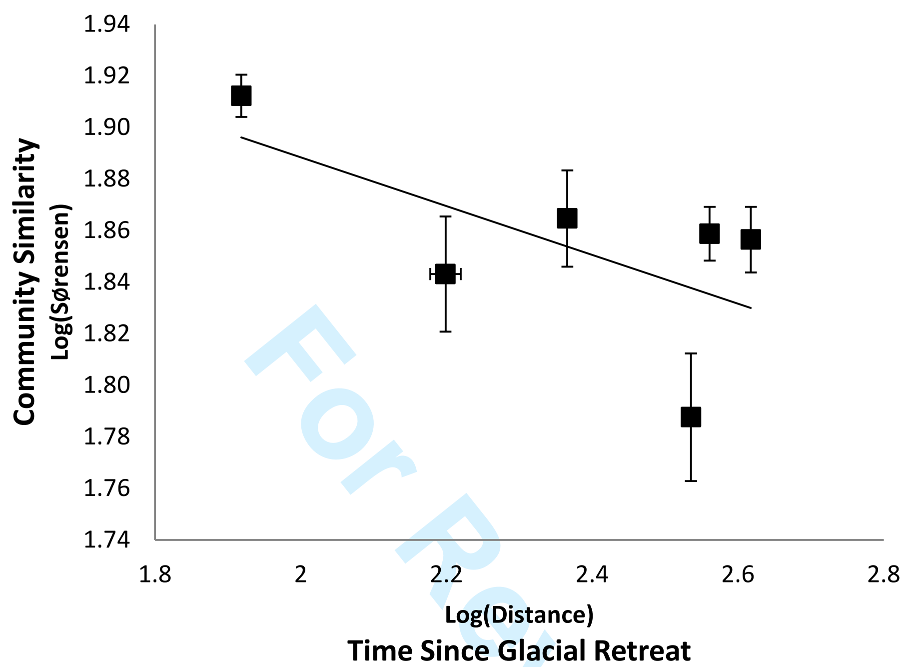
Review Only

Figure 2.



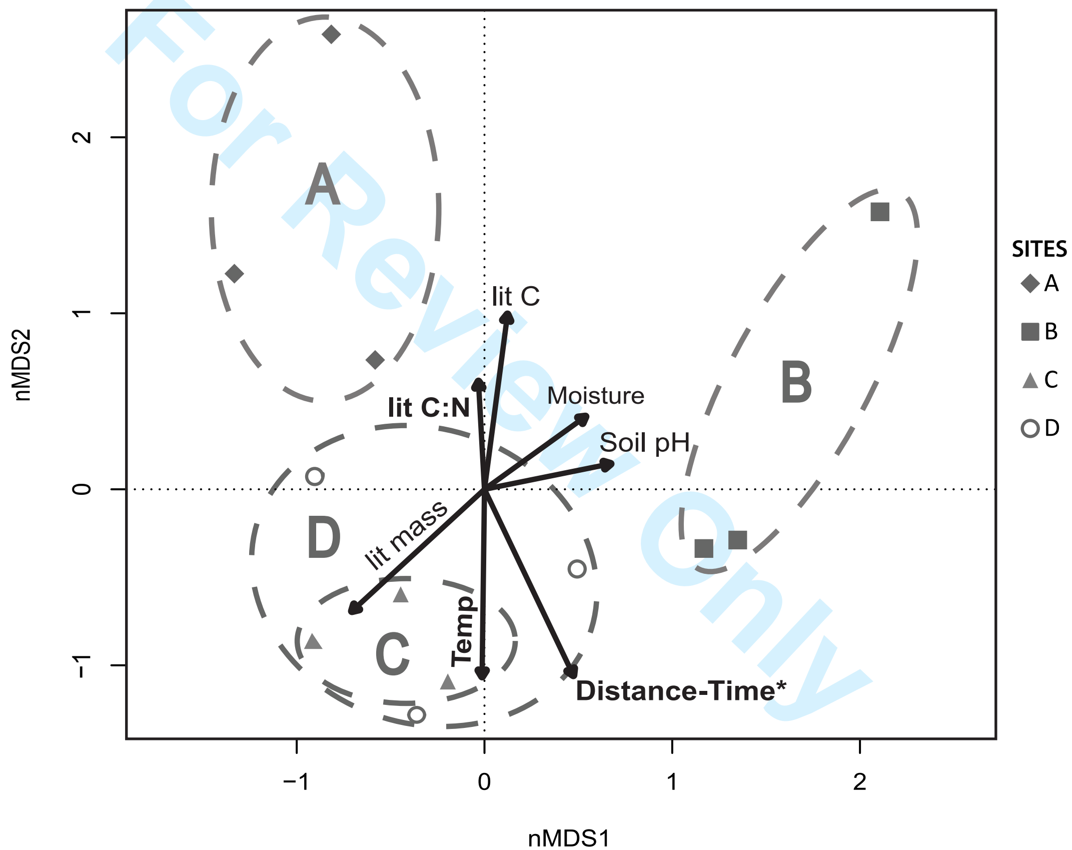
For Review Only

Figure 3.



For Review Only

Figure 4.



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