Plant root pathogens over 120,000 years of temperate rainforest ecosystem development

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Abstract

The role of pathogens, including oomycetes, in long-term ecosystem development has remained largely unknown, despite hypotheses that pathogens drive primary succession, determine mature ecosystem plant diversity, or dominate in retrogressive, nutrient-limited ecosystems. Using DNA sequencing from roots, we investigated the frequency and host relationships of oomycete communities along a 120 000 year glacial chronosequence. Oomycetes were frequent in early successional sites (5 - 70 yrs), occurring in 38 – 65% of plant roots, but rare (average 3%) in all older ecosystems (280 yrs and older). Oomycetes were highly host specific, and more frequent on plant species that declined most strongly in abundance between ecosystem ages. In contrast, oomycetes were not correlated with plant abundance or plant root traits associated with retrogression. The results support the importance of root pathogens in early succession, but not thereafter, suggesting root pathogen-driven dynamics may be important in driving succession but not long-term diversity maintenance.

50 **Introduction**

51

52 As ecosystems develop, biotic communities undergo succession both in composition and in
53 the nature of mutualistic and antagonistic interactions. Many of these changes have been well
54 studied, including changes in plant communities and traits, competition intensity, and
55 mutualisms (Lambers *et al.* 2008). Nonetheless, long-term successional changes in
56 composition and community interactions of many other organisms, such as pathogens, remain
57 poorly understood. Indeed, plant pathogens are often out-of-sight out-of-mind, until large-
58 scale forest diebacks (Scott & Williams 2014) or major crop losses (Martin & Loper 1999)
59 occur. This lack of information is particularly apparent in forest disease outbreaks, where it is
60 often unclear whether the pathogen is native, from where it may have originated, or what
61 background levels of pathogens are typical in healthy ecosystems (Podger & Newhook 1971).
62 Nonetheless, pathogens occur as part of healthy ecosystems and play an important role in
63 ecosystem processes (Castello *et al.* 1995; Gomez-Aparicio *et al.* 2012; Bever *et al.* 2015).
64 For example, *Pythium* and other oomycetes (Oomycota = Peronosporomycota) can drive
65 negative density dependence in plants (Packer & Clay 2000) and hence are believed to
66 contribute to the maintenance of forest plant diversity (Mangan *et al.* 2010a). Pathogenic
67 organisms, including oomycetes, are also an important component of soil biodiversity, as
68 pathogens of plant roots and soil invertebrates, and as saprotrophs (Arcate *et al.* 2006).
69 However, the ecological literature is highly divided in predicting when pathogens might be
70 important in ecosystem succession and development.

71

72 One view of soil pathogens in relation to ecosystem development is that they play a
73 particularly critical role in driving early succession, which we define as the period of large
74 changes in vegetation composition with time (van der Putten *et al.* 1993; van der Putten

2003). This view is based, in part, on the assumption that newly establishing plant species will encounter only low levels of pathogens in the absence of conspecific host plants during succession. Over time, these established plants accumulate pathogens, which prevent establishment of conspecific seedlings but have less negative effects on other plant species. Part of the role of pathogens in succession is driven by plant age, as pathogens that kill seedlings are often tolerated by established plants (Martin & Loper 1999; van der Putten 2003). This implies that early-successional (*r*-selected) plants may have limited selection for pathogen resistance. Instead, these species may accumulate and tolerate pathogens as mature plants, and rely on seed dispersal into new habitats to escape pathogens during the vulnerable seedling stage (van der Putten 2003). Support for the role of pathogens in early succession comes from studies of invertebrates in grasslands, where dominant early-successional species are more suppressed than subordinate or late-successional species (de Deyn *et al.* 2003) and from studies showing negative plant-soil-feedback early in succession, with mid- and late-successional ecosystems showing neutral or positive feedback, respectively (Kardol *et al.* 2006).

A second view is that pathogens are of little importance early in succession, but increase in importance in mature ecosystems, where vegetation composition is more stable (Reynolds *et al.* 2003; Bardgett *et al.* 2005; Peltzer *et al.* 2010). Soil biotic communities as a whole increase in biomass and species diversity over periods of tens to hundreds of years (Bardgett *et al.* 2005), and pathogen communities may follow similar patterns of increasing biomass and diversity. Further, Gilbert (2002) suggests that increasing nutrient competition in mature ecosystems may stress plants and increase susceptibility to pathogens. Pathogen host-specificity in these systems leads to density-dependent mortality (Augspurger 1984). Density dependence may occur within a cohort, driven by a high density of co-occurring conspecific

100 seedlings near seed sources, or cross-cohort, with mature plants supporting high levels of
101 pathogens on their roots and hence reducing establishment of conspecific seedlings (Connell
102 1970), but most studies do not distinguish these two possibilities. Evidence for negative
103 density dependence has come from both tropical and temperate forests (Packer & Clay 2003;
104 Mangan *et al.* 2010b), although other studies have found positive density dependence or no
105 effect (Reinhart *et al.* 2012).
106
107 One of the signatures of density dependence should be a correlation between plant abundance
108 and plant-soil feedback strength, where soil feedbacks are defined as the growth of seedlings
109 of a species in a soil community developing under con-specific plants relative to other species
110 in the same soil (Bever *et al.* 2010). Several studies suggest that correlation between plant
111 abundance and feedback strength should be negative, with more abundant species showing
112 stronger negative feedback (Queenborough *et al.* 2007). However, positive correlations of
113 plant abundance and the strength of negative soil feedbacks have also been taken as evidence
114 supporting pathogen driven density-dependence as a mechanism for supporting mature forest
115 plant diversity (Xu *et al.* 2015). A logical argument can be made for either direction of
116 correlation, depending on whether you assume that observed abundances are the outcome of
117 prior negative density dependence (Xu *et al.* 2015) as opposed to drivers of current negative
118 density dependence. Direct measurement of pathogen frequency is not typically part of plant-
119 soil feedback studies, but strong negative feedback is often assumed to indicate high
120 pathogen loads. Nonetheless, other soil organisms, including mycorrhizas, can drive similar
121 outcomes (Bever *et al.* 2010).
122
123 Finally, a recently proposed third view of the role of pathogens in ecosystem development is
124 that soil pathogens may be particularly critical in retrogressive ecosystems (Laliberté *et al.*

2015), where declining soil nutrient availability results in declining vegetation stature and diversity (Richardson *et al.* 2004; Porder *et al.* 2007; Peltzer *et al.* 2010). Laliberté and colleagues (2015) suggest that the highly weathered, P-limited soils of retrogressive ecosystems favour species with ephemeral roots to maximize nutrient uptake, but that this imposes a trade-off with increased susceptibility to pathogens. Ecosystem retrogression has been linked to increased specific root length (length per unit mass), thinner roots, and increased root branching (Holdaway *et al.* 2011), all of which may increase susceptibility to root pathogens (Laliberté *et al.* 2015). This hypothesis makes no explicit prediction about how plant abundance will be correlated with pathogens.

We formalized each of these views from the literature into three testable, non-mutually exclusive, sets of hypotheses. Each set makes specific predictions about the occurrence of pathogens at different ecosystem stages (H1.1, H2.1, H3.1) and the occurrence of pathogens on different hosts based on plant strategies and root traits (H1.2, H2.2, H3.2).

H1. Early-successional ecosystem hypotheses

H1.1 Pathogen frequency and diversity are high in early-successional ecosystems relative to later stages.

H1.2 Pathogen-driven succession will be evident as plant species that decline in abundance having higher pathogen loads than plant species that increase or maintain their abundance.

H2. Mature ecosystem hypotheses

H2.1 Pathogen frequency and diversity are high in mature ecosystems relative to other stages.

149 H2.2 Negative density-dependent mortality in mature ecosystems will be evident as a
150 correlation between plant abundance with pathogen frequency, either positively
151 (Queenborough *et al.* 2007; Bever *et al.* 2015), or negatively (Xu *et al.* 2015).
152 H3. Retrogression hypotheses:
153 H3.1 Pathogen frequency is high in retrogressive ecosystems relative to earlier stages.
154 H3.2 Trade-offs between nutrient acquisition and pathogen resistance will be evident as
155 plant species with high specific root length, thinner roots, and abundant root hairs having
156 higher pathogen loads than species without these traits.
157
158 In addition, we tested the hypothesis (H4) that oomycete communities are host-specific in
159 their plant associations, as host specificity is requisite for pathogens to either drive early-
160 successional vegetation change (H1) or to contribute to maintaining plant diversity in mature
161 ecosystems (H2).
162
163 We tested our hypotheses using an extensively studied soil and ecosystem development
164 chronosequence created by the Franz Josef glacier in New Zealand, where glacial advances
165 and retreats have created a series of soils of varying age in close proximity (Walker & Syers
166 1976; Richardson *et al.* 2004). We focused on oomycetes as widespread pathogens that have
167 been directly linked to plant succession and negative density-dependence in forest
168 ecosystems, as well as forest die-back (Packer & Clay 2000). Using direct DNA sequencing
169 from roots, we provide the first study of pathogen dynamics throughout ecosystem
170 development from early-succession to retrogression, and find strong support for the role of
171 oomycetes in early-succession but not in later stages of ecosystem development.
172
173 **Methods**

174

175 Study site and sampling

176

177 The Franz Josef chronosequence includes early successional (5, 15, 70 years of
178 development), mature (280, 500, 1000, 5000, 12 000 years), and retrogressive (60 000, 120
179 000 years) sites. As soils age, there are dramatic changes in nutrient availability (declining P,
180 increasing and then decreasing N), pH, and physical properties (Walker & Syers 1976). These
181 changes are linked to changes in plant communities, with plant biomass increasing through
182 succession to mature stages, and then entering retrogression where declining soil nutrients
183 result in a concomitant decline in plant biomass, stature, and diversity (Richardson *et al.*
184 2004) and shifts in root traits (Holdaway *et al.* 2011). Vegetation shifts from a rock field with
185 sparse herbaceous plants and sub-shrubs (5 years) to shrub land (15 years) to angiosperm
186 forest (70 years), followed by an increasing dominance of large gymnosperm trees
187 (Podocarpaceae) through mature stages, with an eventual decline in plant biomass, canopy
188 height, and canopy closure in retrogression. Rainfall is high along the entire chronosequence,
189 ranging from 3.5 to 6.5 m, and all sites are below 365 m elevation (Richardson *et al.* 2004).

190

191 In a previous study we collected 510 individual plant roots from ten sites along the
192 chronosequence and characterized both plant identity and arbuscular mycorrhizal fungal
193 communities in those roots (Martinez-Garcia *et al.* 2015). Here we use these same samples
194 but used taxon-specific primers to amplify DNA from oomycetes. The collection of samples
195 is described in full in Martinez-Garcia *et al.* (2015). In brief, we collected 51 individual root
196 fragments, taking a single root fragment (approx. 15 mg dry weight) every 2 m along two
197 parallel transects with equal sampling from three depths. For the 5-year-old site, which
198 comprised sparse vegetation in rocks, we collected the nearest plant to the sample point and

199 sampled one root from that plant. This was a necessary given that most of the soil at the 5-
200 year-old site was root free. Roots from all sites were thoroughly rinsed in water and dried
201 before DNA extraction. DNA was extracted using a MoBio soil DNA kit, and plant species
202 identified by PCR amplification and DNA sequencing of the tRNL gene region, except for
203 the 5-year-old site, where the plant was already known due to collection method. The one
204 difference in the sampling between this study and the prior study (Martinez-Garcia *et al.*
205 2015) was that in the earlier paper a spare ("B") sample was used if the first ("A") sample
206 failed to yield both plant and arbuscular-mycorrhizal fungal PCR products. In the present
207 study we did not use the "B" samples, as it would have made quantification difficult.
208
209 Oomycete PCR and identification
210
211 We primarily based our oomycete detection, operational taxonomic unit (OTU) clustering,
212 and identification on a nested PCR of large-subunit (28S) DNA (supplementary methods). A
213 negative (omitting DNA template) and a positive control (including genomic DNA from a
214 *Pythium* species) were included in every PCR. PCR products were purified using DNA Clean
215 and Concentrator Kit (Zymo Research Corporation) prior to performing Sanger sequencing
216 (Canterbury Sequencing and Genotyping, University of Canterbury, New Zealand).
217
218 Some ecosystem ages had a high proportion of samples that failed to amplify a product of the
219 expected size in the first-round PCR. In order to ascertain whether these were true absences
220 of oomycetes or due to PCR inhibition, nine samples from each of these sites with the highest
221 failure rate (500, 100, 1200, 60 000 and 120 000 yrs) were tested for PCR inhibitors by
222 repeating PCR reactions in duplicate for each sample, with one of the duplicate reactions
223 spiked with 20 ng positive control DNA. Only one out of the 45 samples spiked with positive

control DNA failed to amplify, suggesting PCR inhibitors were not likely to be causing low detection rates.

To confirm identities of sequenced oomycetes, samples that produced positive large subunit PCR products were sequenced for the internal transcribed spacer (ITS) 1 region (supplementary material). As DNA sequences were obtained from environmental samples (root fragments) potentially containing multiple oomycetes, the ITS sequence may or may not represent the same species as the 28S sequence. ITS sequences were therefore used to help inform identification, but all analyses were based on the 28S results.

Sequence bioinformatics

All DNA sequences were matched against GenBank using BLAST to find the closest matching sequence, and, where the closest match had no reliable taxonomic identity, the closest matching sequence associated with a taxonomic identity. Sequences that matched non-oomycete specimens were discarded. Three sequences had a closest match to a Cercozoan sequence, but only at 78% identity. The phylogeny of Cercozoans remains unclear, but some classifications place this group within the same kingdom (Chromista) as oomycetes (Cavalier-Smith 2010). We therefore considered these three sequences to be potentially basal oomycetes and retained them in the analysis. Out of 510 root samples, 122 had positive PCR products with oomycete primers. After filtering for sequence quality and matching to an oomycete sequence, the final dataset contained 91 DNA sequences of oomycetes with a mean sequence length of 310 bp. Plant IDs were obtained for 458 samples overall and 86 of the 91 samples with oomycete products through DNA sequencing of the trnL gene (Martinez-Garcia *et al.* 2015). We clustered sequences into OTUs using BLASTn

249 to merge any sequences with > 97% similarity over at least 95% of the shorter sequence
250 length based on single-linkage clustering. Finally, we assembled all of our environmental
251 DNA sequences and a selection of named sequences from GenBank to examine how our
252 OTUs were reflected in phylogenetic clustering. Phylogenetic clustering was determined
253 using Clustal Omega for alignment and ClustalW2 phylogeny based on both UPGMA
254 clustering (ebi.ac.uk). The single-linkage BLAST clustering and phylogenetic clustering were
255 broadly consistent.
256
257 *Root traits*
258
259 Root trait data (root diameter, root hairs per mm, and specific root length (SRL)) had been
260 previously obtained for most of our plant species in Holdaway *et al.* (2011). Intraspecific root
261 trait variability across sites is minimal (Holdaway *et al.* 2011). We collected new trait data
262 for two species that were abundant in our data but missing from Holdaway *et al.* (2011),
263 *Raoulia hookeri* and *Epilobium glabellum*. Additional trait data were obtained for *Nertera*
264 *depressa* root diameter from Johnson (1976). Roots that could not be identified to species
265 (e.g., *Coprosma* spp.) were excluded from root trait analysis due to high variability of traits
266 within genus (Holdaway *et al.* 2011).
267
268 *Vegetation cover*
269
270 The percent cover of all vascular plants within sites was measured following standard
271 protocols (Hurst & Allen 2007) in a 20 x 20 m area located within 50 m of the root sample
272 transects. For testing H1.2 and 2.2 we used the mid-point of vegetation cover classes summed
273 across height tiers as a measure of the abundance of each plant species. Vegetation data are

274 archived in the New Zealand National Vegetation Survey Databank
275 (https://nvs.landcareresearch.co.nz; last accessed 8 Feb 2016).
276
277 *Statistics*
278 Changes in the frequency of oomycetes as a function of site age were tested by
279 treating site age as a factor, and as a function of successional stage were tested by treating age
280 as a three-level factor. Sites were allocated to successional stage levels on the basis of soil
281 nutrient concentrations, vegetation height, species richness and biomass, and plant traits
282 (Walker & Syers 1976; Richardson *et al.* 2004). Early-successional sites (5, 15, 70 years) had
283 abundant N-fixing trees; vascular plant traits of high foliar nutrient concentrations, low leaf
284 mass per unit area; very high soil P, rapidly accumulating biomass and species richness of
285 vascular plants, and increasing nitrate-N. Mature ecosystems (280 to 12 000 years) had slow
286 rates of change in species richness, height and biomass among sites. These sites also had the
287 highest biomass and plant diversity. Retrogressive sites (60 000, 120 000 years) had a decline
288 in vegetation height, richness and biomass and exceptionally proficient phosphorus resorption
289 (Richardson *et al.* 2005; Peltzer *et al.* 2010; Vitousek *et al.* 2010).
290 The significance of changes in the frequency of oomycetes as a function of site age
291 (H1.1, 1.2 and 1.3) was tested using a chi-square test, treating site age as a factor. We tested
292 changes in the frequency of oomycetes as a function of successional stage using a binomial
293 glm, fitted with quasi-likelihood to account for over-dispersion. To test whether declining
294 plant species had higher than random oomycete levels (H1.2), we calculated the change in
295 cover of each plant species from one time point (t_0) to the next (t_1) as a log ratio (log((cover t_0
296 +1) / (cover t_1 +1))) and then used a binomial mixed effects model to test whether the
297 presence / absence of oomycetes in root fragments at t_0 could be predicted by change in cover
298 from t_0 to t_1 across the nine intervals between site ages. This test was carried out using lmer in

299 the R package lme4, with site age and plant species (to account for species occuring across
300 multiple sites) as random effects and the canonical logit link function. For trait correlations,
301 only observations with more than three root samples were included in order to have some
302 degree of confidence in oomycete frequency estimation. We similarly tested the correlation
303 between oomycete frequency and vegetation abundance as cover scores (H2.2) and root traits
304 (H3.2), again using mixed effects models and including random terms for plant species and
305 site. For all binary tests, over dispersion was tested using the Pearson residuals divided by
306 degrees of freedom, tested against a chi-square distribution (from
307 http://glmm.wikidot.com/start; accessed 10 July 2015). Host-specificity was tested within
308 ecosystem age using chi-square tests.
309
310 **Results**
311
312 *Oomycete frequency as a function of stage of ecosystem development (H1.1, 2.1 and 3.1)*
313
314 In early-successional ecosystems (5, 15 and 70 years), 38 to 65% of root samples had
315 oomycete DNA detected (Figure 1). In contrast, in mature and retrogressive ecosystems (280
316 to 120 000 years) an average of only 2% and never more than 8% of roots had oomycete
317 DNA found (Figure 1; $\chi^2 = 194$, df = 9, $P < 2.2 \times 10^{-16}$). Treating the sites as representing
318 three stages following our hypotheses, the early successional stages had a significantly higher
319 frequency of oomycetes than mature stages (quasibinomial family glm; $t = -6.6$, $P = 0.00050$)
320 or retrogressive stages ($t = -4.6$, $P = 0.0025$), and the difference between mature and
321 retrogressive ecosystems was not significant ($t = -1.1$, $P = 0.32$).
322
323 *Oomycete frequency as a function of plant strategies and traits (H1.2, 2.2, 3.2)*

The frequency of oomycetes was significantly correlated with the decline of vegetation cover between ecosystem ages (Figure 2a, z = -2.4, P = 0.018), supporting hypothesis 1.2. There was no correlation between oomycete frequency and plant abundance (z = 0.84, P = 0.40). Of the root traits tested, root hair abundance was significantly correlated with oomycete frequency (Figure 2b, z = 3.0, P = 0.003), providing partial support for hypothesis 3.2, while other root traits were not significant (SRL, z = 0.013, P = 0.99; root diameter, z = 0.82, P = 0.42).

Oomycete diversity, host specificity and ecosystem age (H4)

A total of 37 different OTUs were found (Figure 3). Five OTUs were found more than 5 times. The most abundant OTU occurred 17 times, and had DNA-sequence affinities to *Lagena radicicola* and an uncultured oomycete from New York, USA agricultural soils (17 occurrences). Other abundant OTUs included an OTU matching *Pythium dissotocum* and *Pythium aquatile* (13 occurrences); *Pythium tracheiphilum* (6 occurrences); *Pythium volutum* (6 occurrences); and *Phytophthora hedraiandra* (28S) / *cactorum* (ITS) (5 occurrences).

Within ecosystem age there was a significantly non-random distribution of oomycete OTU identity across plant species at 5 (χ^2 = 42.96, df = 12, p-value = 0.000022) and 15 years (χ^2 = 225.87, df = 182, p-value = 0.015), but only marginally significant at 70 years (χ^2 = 109.85, df = 90, p-value = 0.076; Figure 4). There were too few observations at the ecosystem stages after 70 years to meaningfully test for plant by OTU host-specificity at later ecosystem stages.

Discussion

Our results strongly support the importance of pathogens in early succession, with high frequencies observed in the three earliest ecosystem stages (5, 15, and 70 years) but oomycetes being present only at very low levels thereafter. Although the importance of pathogens is often discussed in the ecological literature (Bagchi *et al.* 2014), relatively few studies have actually quantified pathogen abundance in natural ecosystems (Gomez-Aparicio *et al.* 2012) and we believe our results are the first study of pathogenic organisms throughout a complete ecosystem development sequence. The community of oomycetes was diverse, including multiple *Pythium* and *Phytophthora* species and, surprisingly, a very abundant sequence matching *Lagena,* a pathogen previously only reported on grass, on *Raolia hookeri* (Asteraceae) and *Epilobium glabellum* (Onograceae).

Pathogens as drivers in early successional ecosystems

The early succession hypothesis postulates that pathogens drive early-successional change in plant communities (Kardol *et al.* 2006). The high frequency of oomycetes in early successional ecosystems strongly supports this hypothesis. In addition, those plant species that had the highest frequency of oomycetes at a given ecosystem stage declined in percent cover most strongly before the next stage. The higher frequency of oomycetes on plant roots in early-successional ecosystems compared to mature and retrogressive ecosystems was not due to PCR inhibition in older sites, as samples spiked with positive control oomycete DNA showed no evidence of inhibition.

Oomycetes, particularly *Pythium*, are often tolerated by established plants, but prevent seedling establishment (Martin & Loper 1999; van der Putten 2003). The correlation of oomycete frequency with decline in cover may not, therefore, reflect a direct negative effect

374 of the pathogens on established plants. Instead, we suggest that these pathogens are primarily
375 preventing re-establishment of plants and hence contributing to vegetation change across
376 cohorts (van der Putten 2003).

377 Our definition of "early succession" comprises the major transition from rock field (5
378 years) to shrub land (15 years) to forest (70 years). Therefore our finding of high pathogen
379 levels is potentially consistent with Packer & Clay (2000), who found strong negative
380 feedback in a shade-intolerant, seral species (*Prunus serotina*) in a forest described as "at
381 least 70 years old" (Packer & Clay 2000).

382 Changes in oomycete frequency may, in part, reflect a substantial site-age effect and
383 direct effects of changing soil environments on oomycete populations, independent of
384 changing plant communities (akin to the “habitat” hypothesis for mycorrhizal fungi of Zobel
385 & Opik (2014). Meaningful testing of soil variables as predictors of oomycetes was not
386 possible, as the three early-successional sites had both uniformly high oomycete frequency
387 and uniformly high (pH, total P) or low (total C) soil measures. Thus, while we can conclude
388 that oomycetes were particularly abundant in early-successional ecosystems and on early-
389 successional plants, our data are insufficient to determine whether this was due to the
390 presence of particular plant hosts, or to soil abiotic conditions driving both host plants and
391 oomycete frequency (Martin & Loper 1999).

392

393 *Oomycetes in mature ecosystems and forest diversity*

394 The mature ecosystem hypothesis suggests that pathogens are important drivers of
395 diversity in mature forest ecosystems, with evidence from tropical and temperate forests
396 (Bagchi *et al.* 2010; Bagchi *et al.* 2014), although other studies have failed to support a role
397 of pathogens in mature ecosystems (Reinhart *et al.* 2012). Our finding of only low levels of
398 oomycetes on roots in mature ecosystems suggest that any role of pathogens in maintaining

399 mature forest diversity is unlikely to be driven by oomycete populations on the roots of
400 established plants. Further, we found no significant correlation between the abundance of a
401 particular tree species and its pathogen abundance, whereas the mature ecosystems
402 hypothesis would predict either a positive (Queenborough *et al.* 2007) or negative correlation
403 (Xu *et al.* 2015) depending on interpretation.

404 Our findings do not rule out negative density dependent mechanisms linking pathogen
405 infection to forest diversity, but suggest an important restriction of potential mechanisms. In
406 the original formulation, negative density dependent theories distinguished between distance-
407 dependent and density-dependent agents of seed and seedling mortality (Janzen 1970).
408 Distance-dependent agents are primarily driven by the presence of the parent tree itself, while
409 density-dependent agents are attracted by the density of seeds and seedlings around that tree.
410 While it was previously known that established plant individuals are less susceptible to
411 oomycetes than seedlings, we had limited prior knowledge of whether roots in mature
412 ecosystems tolerated oomycetes, hence supporting populations that could cause distance-
413 dependent mortality, or resisted oomycete infection altogether (c.f., Spear *et al.* 2015). Our
414 results suggest that roots of established plants in mature ecosystems are not supporting high
415 oomycete populations, implying that distance-dependent oomycete infection is not an
416 important driver of diversity, at least in this temperate rainforest. This is consistent with a
417 number of studies showing positive, rather than negative, effects of established plants on
418 conspecific seedlings (Simard & Durall 2004). On the other hand, our results do not rule out
419 density-dependent mechanisms driven by oomycetes on seedlings. A high density of
420 conspecific seedlings may still lead to increased risk of oomycete driven mortality, as has
421 been found in other studies (Bagchi *et al.* 2010).

422 While Packer and Clay (2000) clearly demonstrated oomycetes driving seedling
423 dynamics in a relatively young temperate forest, a more recent study by Bagchi and

424 colleagues (2014) in a tropical seasonal forest found that eliminating fungi alone had a
425 stronger effect on negative density-dependence than eliminating both fungi and oomycetes.
426 Further research is needed, but this may suggest that fungal pathogens are more important
427 drivers of forest diversity in mature ecosystems than oomycetes.
428
429 *Oomycetes in retrogression*
430 Laliberté and colleagues (2015) suggested that root traits adapted to highly P-limited
431 retrogressive ecosystems would increase susceptibility to root pathogens. Of the root traits we
432 tested, only root hair abundance was correlated with a high frequency of oomycetes in roots.
433 While this supports the general concept that root traits can determine pathogen susceptibility,
434 species with root hair abundance shows no increase with ecosystem age at this site
435 (Holdaway et al. 2011) and hence this correlation did not drive an increase in oomycete
436 frequency during retrogression. Studies of oomycete populations in other retrogressive
437 ecosystems will be needed to confirm our findings, but at present the suggestion of Laliberté
438 and colleagues (2015) of increasing pathogen loads in retrogression is not supported by our
439 findings, despite part of the mechanistic basis of that hypothesis (root traits determining
440 pathogen loads) was supported at least in the case of root hairs.
441 In comparing our results to other studies, it may be important to distinguish between
442 ecosystem age, as in the pedogenic sequence studied here, and response of pathogens to
443 disturbance or secondary succession (e.g., Reinhart *et al.* 2010). None of our older sites
444 showed any sign of stand-replacing disturbance in the past hundreds to thousand years, but
445 smaller-scale disturbance is a normal part of ecosystem development (Peltzer *et al.* 2010).
446 The retrogressive sites, in particular, have much younger vegetation (the oldest trees being no
447 more than 1000 years old) than underlying soils, and were unlikely to have supported tall
448 forests during the Pleistocene when these sites were exposed by glacial retreat.

449

450 *Novel organisms or native?*

451 Having baseline data on oomycete ecology may be important for understanding and
452 managing future pathogen outbreaks. For example, *Phytophthora agathidicida* (=
453 *Phytophthora* taxon *Agathis*) has been implicated as a cause of forest dieback of the iconic
454 New Zealand-endemic *Agathis australis*, but, like other oomycete disease outbreaks, the
455 origin of the species remains uncertain (Weir *et al.* 2015). The single-direction sequencing
456 we performed directly from environmental samples was not designed for accurate
457 phylogenetic classification, even with two gene regions sequenced per sample, but it does
458 suggest considerable diversity of *Phytophthora*. We found sequences with affinities to a
459 number of species already known to be present in New Zealand, including *P. cinnamomi*, *P.*
460 *cactorum*, *P. infestans* and *P. kernovieae* (Scott & Williams 2014). Given that our samples
461 came from relatively pristine ecosystems, we believe these OTUs are likely to be from native
462 *Phytophthora*.

463 More surprising was that the most common OTU in our data had affinities to *Lagena*
464 *radicicola* in both 28S and many of the ITS sequence results. *Lagena* is a monotypic genus
465 widespread as a pathogen of grasses in North America (Blackwell 2011), but Barr and
466 Désaulniers (1990) suggest it may be under-reported due to being morphologically very
467 similar to *Pythium*, and not being easily culturable. Spores described as "resembling
468 *Lagenocystis* [syn. *Lagena*] spp." were noted by Skipp and Christensen (1989) in New
469 Zealand *Lolium perenne* pastures, but our finding is the first report of a putative *Lagena*
470 species outside of grasses. We believe this most likely reflects a lack of prior knowledge, as
471 very few prior studies have used molecular methods to detect oomycetes from healthy forest
472 ecosystems, and none of those have taken place in temperate southern hemisphere rainforest.

473 Our sampling of washed plant roots and choice of primers that preferentially amplify
474 the Peronosporales focused our analysis on putative plant pathogens within the Oomycota
475 occurring in soils. This is reflected in the affinity of most of our sequences to known plant
476 pathogens. Nonetheless, we note that the Oomycota also includes animal pathogens and free-
477 living saprotrophs as well as foliar pathogens; potential pools of species that were not likely
478 to be included in our sampling.
479
480 *Conclusions*
481 Much of the Earth's surface is covered in young, early successional ecosystems
482 comparable to our early successional sites of 5, 15, and 70 years (Vitousek *et al.* 1997;
483 Haddad *et al.* 2015). Our results support the concept that oomycete pathogens are a diverse
484 part of these ecosystems. Further, the strong plant-host specificity and correlation of plant
485 decline and oomycete frequency is consistent with the suggestion that these pathogens
486 contribute to vegetation succession. While we do not find abundant oomycetes in older
487 ecosystems, oomycete DNA was detected in all but one site. These low levels of oomycetes
488 in roots, along with oospores in soil, may still be sufficient to prevent establishment of
489 susceptible plant species or to drive pathogen outbreaks following external climatic stressors,
490 but seem incompatible with density dependent mortality being driven by oomycetes on
491 established plant roots.
492
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Figure 1. Frequency of oomycetes on plant roots (%) as a function of ecosystem age. The 5, 15, and 70-year-old sites represent a successional sere from rock field through shrubland to angiosperm forest. Sites 280 to 5000 years of age are mature forest with high biomass and plant diversity, while after 12 000 to 60 000 years plant biomass, stature, and diversity decline due to retrogressive P-limitation.

Figure 2. The two significant predictors of oomycete frequency on roots: vegetation cover (a) and the abundance of root hairs (b), with circles representing each plant species between site transitions (a) or within site (b). Lines indicate random effects to account for site in a binary mixed effects model with random intercepts for site and plant species. Color coding in (a) reflects the site age at t_0 for each transition, hence no points for 120 000 years are shown.

Figure 3. Mapping of DNA sequences into OTUs and against known species. Phylogenetic clustering (at left) based on 28S gene of samples and a selection of named sequences from GenBank, using Clustal Omega 1.2.1 for alignment and phylogeny based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering. OTUs were based on pairwise BLASTn of 28S sequences at 97% similarity; resulting clusters shown as colored squares, with singletons shown as black squares. Bold indicates representative sequence from each non-singleton OTU accessioned into GenBank (numbers KU863588-KU863606). For each collection, the best matching 28S sequence name in GenBank is shown, followed by the

sequence similarity (%) / percent of query matched and the actual length of the query sequence (in bp). ITS1 sequences were obtained from most samples, best match in GenBank shown, again with % identity over % of query and query length. Where the ITS1 sequence is likely to be a different species sequenced from the same sample, this is indicated by parentheses.

Figure 4. Plant by site age visualisation of oomycete frequency within sampled roots. Each square represents a single root sample, with coloured squares indicating the presence of oomycete pathogens with black indicating an OTU found only once and other colours indicating the OTU groupings identified in figure 3. Grey dots indicate species not found in root samples for that site.

Supplemental files S1: Detailed molecular methods and discussion.

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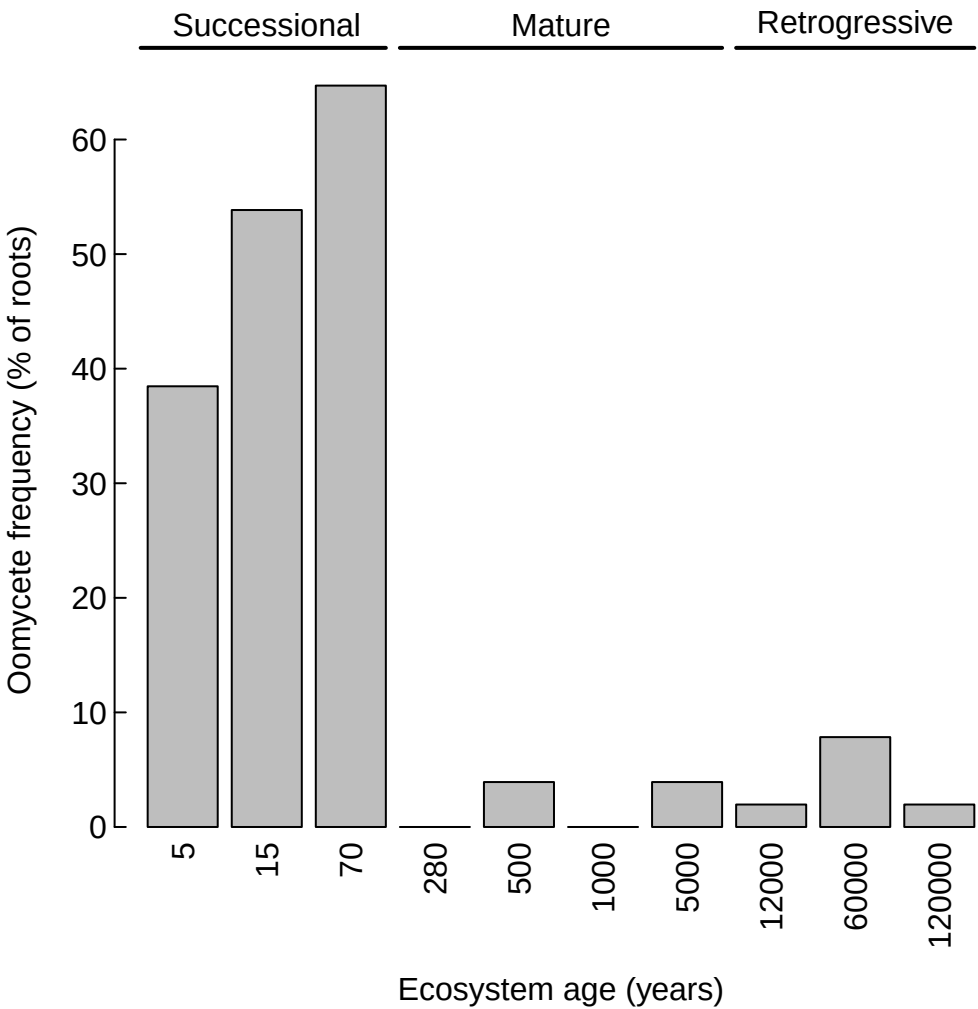
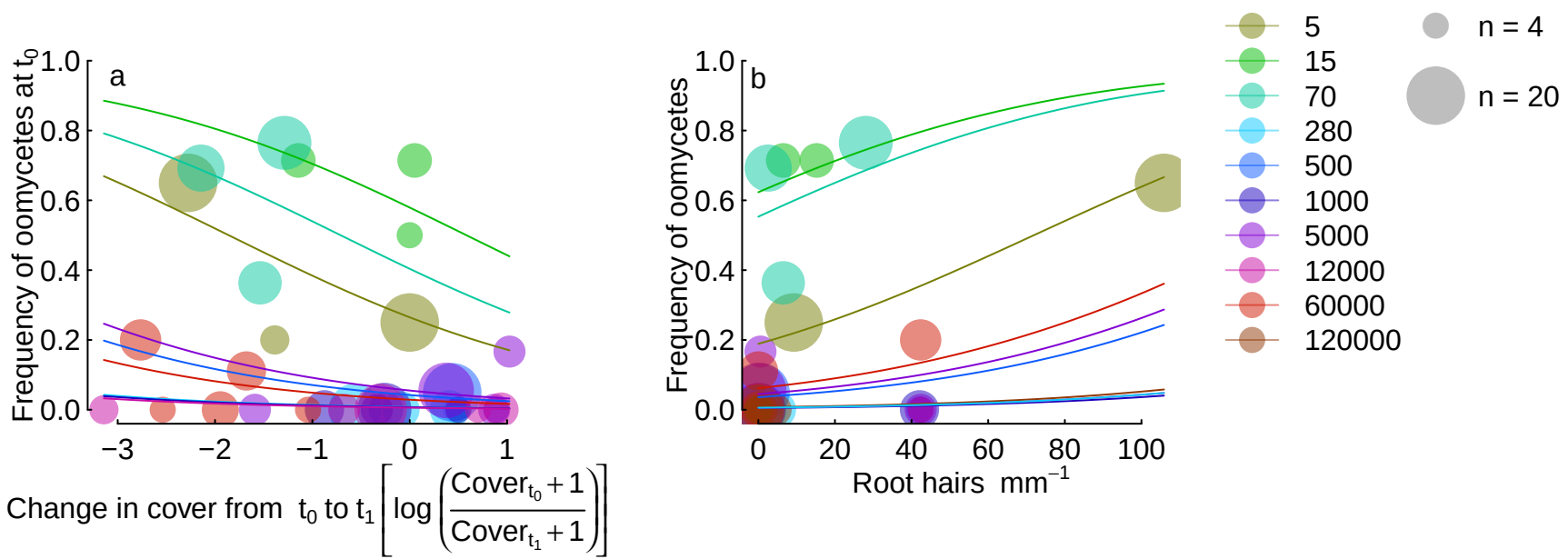
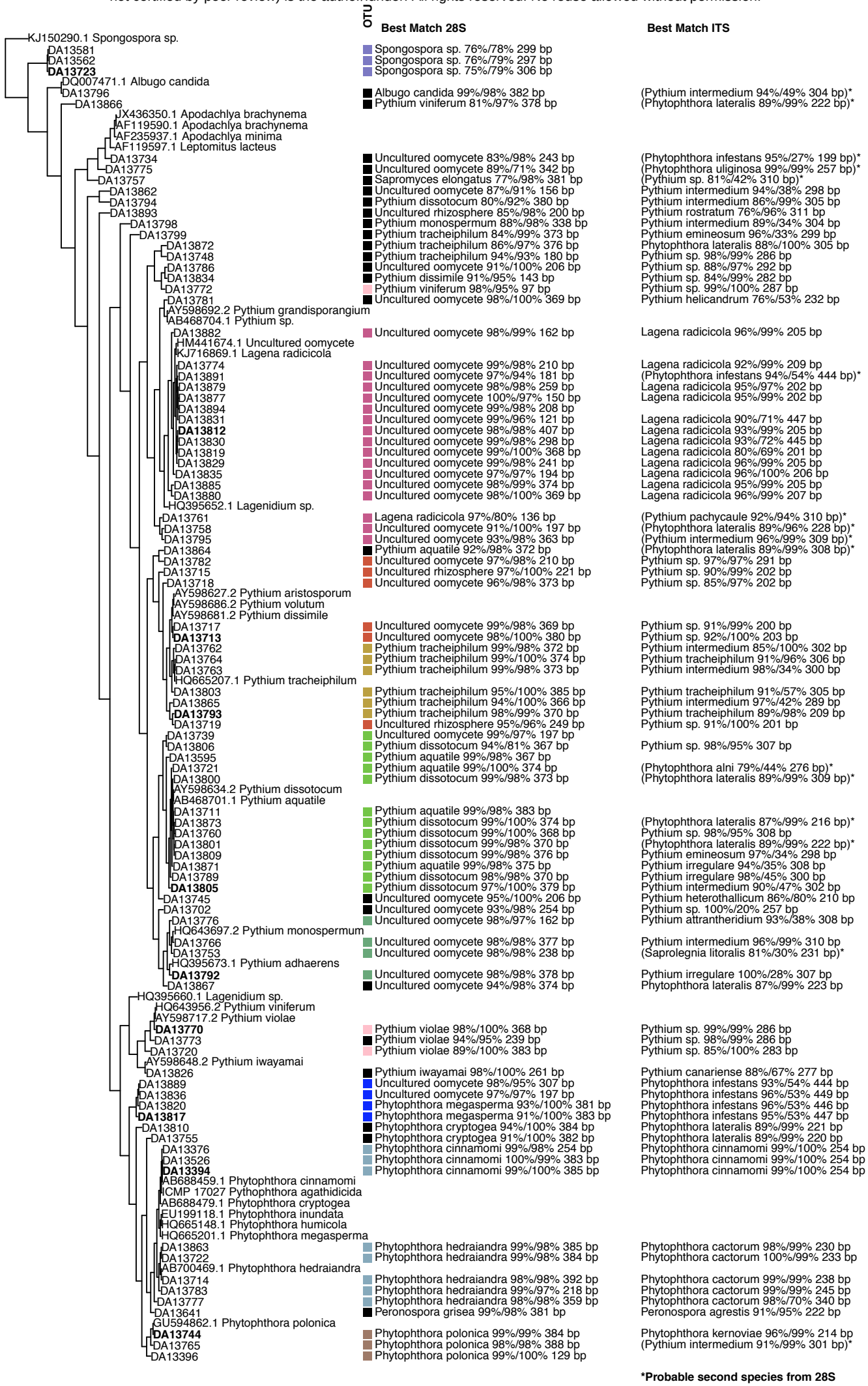
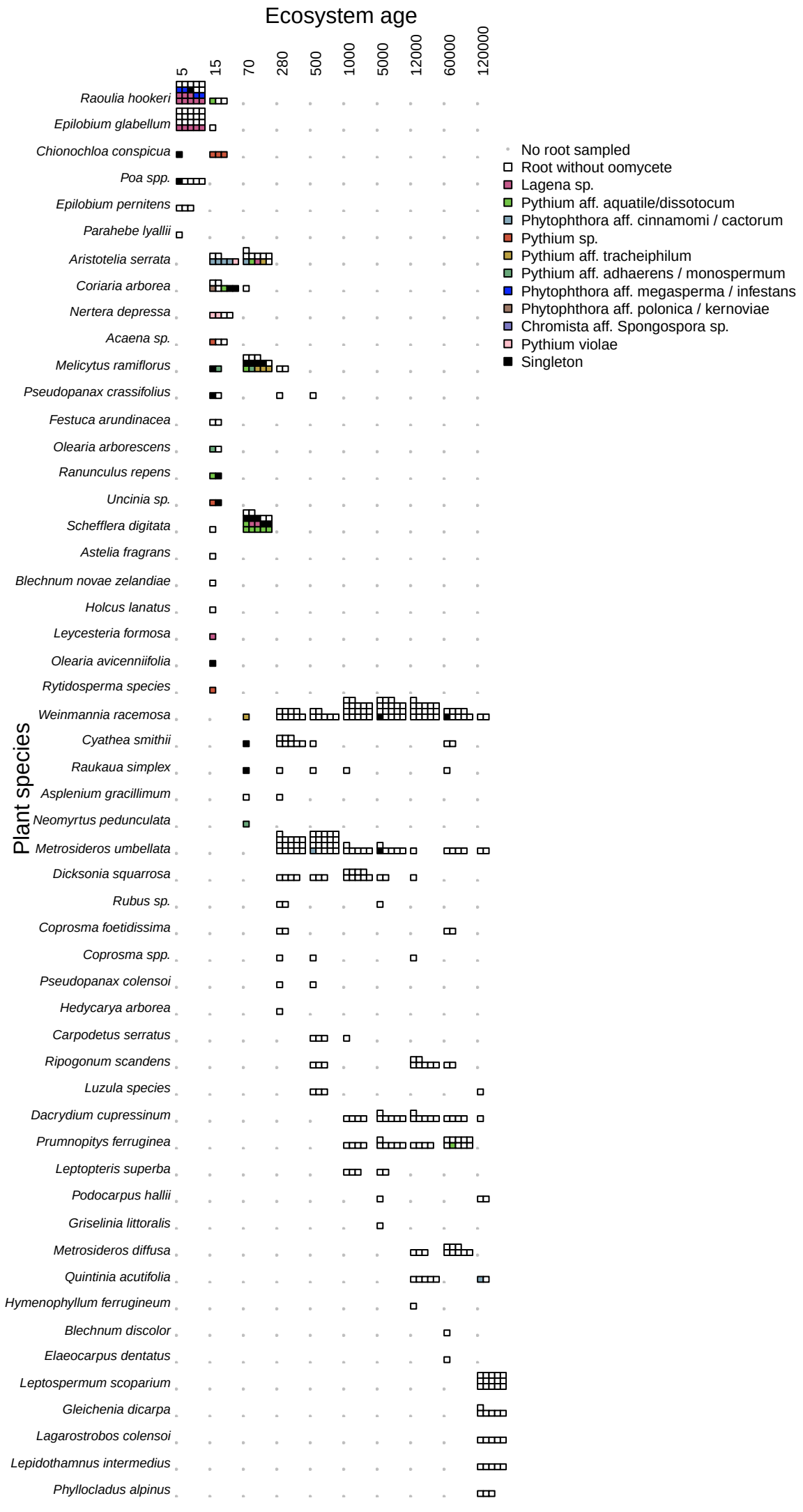

Successional
Mature
Retrogressive
Oomycete frequency (% of roots)
0
10
20
30
40
50
60
5
15
70
280
500
1000
5000
12000
60000
120000
Ecosystem age (years)


a
b
Frequency of oomycetes at t_0
Frequency of oomycetes
1.0
0.8
0.6
0.4
0.2
0.0
−3
−2
−1
0
1
0
20
40
60
80
100
Change in cover from t_0 to t_1 $\left[\log\left(\frac{\text{Cover}_{t_0}+1}{\text{Cover}_{t_1}+1}\right)\right]$
Root hairs mm^{-1}
5
15
70
280
500
1000
5000
12000
60000
120000
n = 4
n = 20




Ecosystem age
5
15
70
280
500
1000
5000
12000
60000
120000
Plant species
Raoulia hookeri
Epilobium glabellum
Chionochloa conspicua
Poa spp.
Epilobium pernitens
Parahebe lyallii
Aristotelia serrata
Coriaria arborea
Nertera depressa
Acaena sp.
Melicytus ramiflorus
Pseudopanax crassifolius
Festuca arundinacea
Olearia arborescens
Ranunculus repens
Uncinia sp.
Schefflera digitata
Astelia fragrans
Blechnum novae zelandiae
Holcus lanatus
Leycesteria formosa
Olearia avicenniifolia
Rytidosperma species
Weinmannia racemosa
Cyathea smithii
Raukaua simplex
Asplenium gracillimum
Neomyrtus pedunculata
Metrosideros umbellata
Dicksonia squarrosa
Rubus sp.
Coprosma foetidissima
Coprosma spp.
Pseudopanax colensoi
Hedycarya arborea
Carpodetus serratus
Ripogonum scandens
Luzula species
Dacrydium cupressinum
Prumnopitys ferruginea
Leptopteris superba
Podocarpus hallii
Griselinia littoralis
Metrosideros diffusa
Quintinia acutifolia
Hymenophyllum ferrugineum
Blechnum discolor
Elaeocarpus dentatus
Leptospermum scoparium
Gleichenia dicarpa
Lagarostrobos colensoi
Lepidothamnus intermedius
Phyllocladus alpinus
No root sampled
Root without oomycete
Lagena sp.
Pythium aff. aquatile/dissotocum
Phytophthora aff. cinnamomi / cactorum
Pythium sp.
Pythium aff. tracheiphilum
Pythium aff. adhaerens / monospermum
Phytophthora aff. megasperma / infestans
Phytophthora aff. polonica / kernoviae
Chromista aff. Spongospora sp.
Pythium violae
Singleton

Detailed molecular methods and discussion

Molecular methods

We primarily based our oomycete detection, operational taxonomic unit clustering, and identification on a nested PCR of large-subunit (28S) DNA. The primer pair LR0R: ACCCGCTGAACTTAAGC (LoBuglio *et al.* 1991) and LR5: TCCTGAGGGAAACTTCG (Vilgalys & Hester 1990) was used for first-round PCR amplification, followed by using 1µL of a 10-fold dilution from this PCR product as the template for the second-round PCR amplification with Oom1F: GTGCGAGACCGATAGCGAACA and Oom1R: TCAAAGTCCCGAACAGCAACAA (Arcate *et al.* 2006) primers, fluorochrome-labelled with VIC and 6FAM respectively. PCR reactions for LR0R-LR5 were carried out in a 20 µl volume containing 2 µl 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.2 mg ml^{-1} BSA, 0.8 U Roche Faststart Taq DNA Polymerase, and 1 µl DNA extract; with thermocycling of 94°C for 1 min, then 35 cycles at 94 °C for 30s, 47 °C for 30s and 72 °C for 60s, final extension at 72 °C for 7 min. PCR reactions for the Oom1F-Oom1R primer pair were the same, with the exception that $MgCl_2$ was 2.5 mM, annealing temperature of 57 °C, and extension time was 45 s with final extension of 10 min. A negative (omitting DNA template) and a positive control (including genomic DNA from a *Pythium* species) were included in every PCR. Our intention in using fluorochrome-labelled primers was to provide the option of performing tRFLP analysis. However, initial results suggested that most samples contained a single tRFLP type. We therefore moved to a direct-sequencing technique. PCR products were purified using DNA Clean and Concentrator Kit (Zymo Research Corporation) prior to performing Sanger sequencing (Canterbury Sequencing and Genotyping, University of Canterbury, New Zealand).

To confirm identities of sequenced oomycetes, samples that produced positive large subunit PCR products were sequenced for the ITS region. The oomycete specific primer pair OOMUP18Sc: TGCGGAAGGATCATTACCACAC (Lievens *et al.* 2004) and ITS2-OOM: GCAGCGTTCTTCATCGATGT (Lievens *et al.* 2006) was used to amplify the ITS1 gene region (~ 240 bp). PCR reactions were carried out in a 25 µl volume containing 2.5 µl 10X Ex Taq Buffer, 2 µl Ex Taq dNTP Mixture (2.5 mM each), 0.5 µl of 10 µM of each primer, 5 µl of 10 mg ml-1 RSA, 1 U TaKaRa Ex Taq DNA Polymerase, and 1 µl DNA extract, and sterilized distilled water up to 25 µl; with thermocycling of 94 °C for 2 min, then 35 cycles at 94 °C for 30s, 60 °C for 30s and 72 °C for 60s, final extension at 72 °C for 7 min.

Additional root trait data

Two species were present on the 5 year site that had no previously measured root traits: *Epilobium glabellum* and *Raoulia hookeri*. Root traits were measured using the same methods as for the earlier data set (Holdaway *et al.* 2011). Diameters = 0.22 ± 0.015 mm and 0.28 ± 0.018 mm; specific root lengths = 1437 ± 147 and 2354 ± 387 cm g^{-1}; and root hairs = 9.3 ± 4.8 and 106 ± 3.4 hairs cm^{-1} for *Epilobium glabellum* and *Raoulia hookeri,* respectively.

Supplemental discussion of methods

Using direct Sanger sequencing from environmental samples results in somewhat short and lower quality sequences than might be achievable with other methods, such as clone libraries. Nonetheless, direct sequencing is a cost-effective and efficient way of detecting and

identifying the oomycetes in samples with a single dominant DNA sequence. This greater cost-effecitveness allows greater investment in replication; a critical factor in ecological studies of cryptic organisms (Prosser 2010).

Most samples that produced a positive PCR product in large subunit sequencing also produced a positive PCR product with ITS primers. In most cases, the two sequences appear to represent the same species (Figure 3). The exceptions tended to be samples at the base of the phylogeny, matching *Spongospora, Albugo*, and *Sapromyces* in 28S and either failing to produce ITS PCR products at all, or producing ITS sequences that were distantly related. This suggests that the ITS primers were more specific to a subset of the Oomycota, and may omit some basal groups; while the 28S primers better amplify basal Oomycota but potentially amplify other Chromalveolata as well. Neither gene region was sufficient to identify all OTUs to species, largely reflecting the relative paucity of oomycota sequences in GenBank compared to better studied groups, such as fungi. Large subunit sequences also lacked resolution within *Phytophythora* compared to ITS sequences.

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