ORIGINAL ARTICLE

The biogeographical history of the interaction between mycoheterotrophic *Thismia* (Thismiaceae) plants and mycorrhizal *Rhizophagus* (Glomeraceae) fungi

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ABSTRACT

Aim Achlorophyllous mycoheterotrophic plants and mycorrhizal fungi often have highly specific interactions that potentially limit the plants' distribution and diversification potential. However, specificity in biotic interactions may differ considerably over a species' distribution range and therefore interactions need to be studied over their entire range to assess their evolution in space and time. The present study investigates the biogeographical history of the interaction between five closely related mycoheterotrophic *Thismia* species and arbuscular mycorrhizal fungi over the distribution range of the plant species.

Location Temperate south-east Australia and New Zealand.

Methods Phylogenetic relationships of *Thismia* (nrITS and mt*cob*) and their arbuscular mycorrhizal fungi (partial nrSSU) were reconstructed based on data from 65 plant specimens. The diversification times in *Thismia* were estimated with a Bayesian relaxed clock approach using a Dioscoreales framework (nrSSU, mt*atp1*, mt*matR*, mt*nad1 b-c*). Ancestral geographical ranges were reconstructed using a maximum likelihood approach. The same approach was used to reconstruct ancestral mycorrhizal associations.

Results Our analysis shows that *Thismia* plants have highly specific, phylogenetically conserved and evolutionarily persistent interactions with *Rhizophagus* fungi. Nevertheless, *Thismia* was able to diversify and radiate recently due to the wide geographical distribution of the host fungi. In addition, we find that although the mycorrhizal interactions of this clade of mycoheterotrophs are strictly bound to a fungal lineage, host switches remain possible.

Main conclusions In this clade of closely related mycoheterotrophs, dependency on highly specific fungal interactions is the result of phylogenetic niche conservatism, acting over at least 12 million years. Nevertheless, plants that are dependent on highly specific fungal interactions have ample opportunities to disperse and radiate over the geographical range of their hosts. Our study highlights the need to link the ecology and evolution of species interactions over broad geographical and evolutionary scales for understanding mycorrhizal interactions.

Keywords

Australasia, biogeography, cheating, co-evolution, long-distance dispersal, mycoheterotrophy, mycorrhiza, parasite

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INTRODUCTION

Species diversification usually occurs in a dynamic context. Both abiotic and biotic factors can influence genetic differentiation among populations and result in genetic variation that is strongly structured in space and time (Thompson, 2005; Benton, 2009). For plants, patterns of species diversification are usually investigated in the context of abiotic changes (e.g. Hoorn *et al.*, 2010; Hughes & Atchison, 2015), although it has long been recognized that biotic interactions with animal pollinators can be an important driver for plant diversification (e.g. Ehrlich & Raven, 1964; Van der Niet & Johnson, 2012; Lagomarsino *et al.*, 2016). In general, when plants are dependent on highly specific obligate interactions these interactions have the potential to limit the geographical distribution and influence the diversification of the plant species (Futuyma & Moreno, 1988). For example, in parasitic plants, the distribution of the host plants limits the distribution of the parasites (Norton & Carpenter, 1998; Schneeweiss, 2007) and host adaptation may trigger speciation (Thorogood *et al.*, 2008, 2009).

One particularly interesting group of plants with high biotic specificity are plants that target mycorrhizal fungi; a guild of fungi which have an obligate mutualistic interaction with plant roots. These mycoheterotrophic plants derive all of their carbon from mycorrhizal fungi that are often simultaneously mycorrhizal with surrounding photosynthetic plants. Mycoheterotrophs are thus sometimes referred to as exploiters, cheaters, or epiparasites (Bidartondo, 2005). In general, the interactions between green plants and mycorrhizal fungi are promiscuous (Giovannetti *et al.*, 2004), but the interactions of mycoheterotrophic plants with mycorrhizal fungi often show extreme specificity by being targeted on narrow clades of fungi (Bidartondo, 2005; Merckx *et al.*, 2012). This host specificity pattern parallels that of many parasitic organisms (Page, 2003). In theory, the distribution of their associated fungi can influence the geographical distribution and diversification of these mycoheterotrophic plants, and it has been suggested the scattered distributions and rarity of some mycoheterotrophic plants are the result of high mycorrhizal specificity and the narrow distributions of their fungal partners (e.g. Bidartondo & Bruns, 2001; Bougoure *et al.*, 2009; Yamato *et al.*, 2011; Merckx *et al.*, 2013).

In the arbuscular mycorrhizal (AM) mutualism between the majority of land plants and fungi of the phylum Glomeromycota (Smith & Read, 2008) – one of the most ancient and prevalent mutualisms on earth – mycoheterotrophy has over 18 independent evolutionary origins and gave rise to *c*. 230 extant plant species of mycoheterotrophs (Leake, 1994; Merckx, 2013). At a local scale, most of these species show extremely high specificity towards particular AM fungi (Bidartondo *et al.*, 2002; Merckx & Bidartondo, 2008; Yamato *et al.*, 2011; Ogura-Tsujita *et al.*, 2013). In one case, closely related mycoheterotrophic plant species were found to associate with narrow closely related clades of AM fungi, which was hypothesized to result from a delayed cospeciation process (Merckx & Bidartondo, 2008). This process implies host specialization and a lack of host switching, in which the degree of mycorrhizal specificity in diversifying plant lineages increases over time, causing each lineage to 'get stuck' on a particular clade of AM fungi. This may also imply that speciation is driven by resource partitioning, in which specialization to different hosts leads to reproductive isolation of populations, as suggested for parasitic plants (Thorogood *et al.*, 2009). Although little is known about the distribution ranges of AM fungi, and many appear to be widespread, their distribution may be restricted due to specific abiotic habitat requirements and dispersal barriers (Johnson, 2010; Johnson *et al.*, 2010; Davison *et al.*, 2015).

Because specificity in biotic interactions may differ considerably over a species' distribution range, interactions need to be studied over their entire range (Thompson, 2005; Hynson *et al.*, 2015). Thus far, the specificity of AM mycoheterotrophic plants has been assessed over restricted areas only, potentially overlooking the geographical structure of the interactions (e.g. Bidartondo *et al.*, 2002; Yamato *et al.*, 2011). To investigate the geographical structure of mycorrhizal specificity and its role in plant evolutionary and biogeographical history, we studied mycoheterotrophs of the genus *Thismia* Griff. (Thismiaceae J. Agardh) in temperate Australia, Tasmania and New Zealand. Recent molecular analysis of the AM fungi in the roots of *Thismia* plants from two populations in Tasmania showed that both plants were growing with a single Glomeraceae virtual taxon (Merckx *et al.*, 2012). This suggests that the species potentially shows high mycorrhizal specificity similar to mycoheterotrophic plants in the related genus *Afrothismia* Schltr. (Merckx & Bidartondo, 2008). We investigate the mycorrhizal associations of five closely related *Thismia* species and test if this mycorrhizal specificity is constant over the plant species' distributions and their evolutionary and biogeographical history.

MATERIALS AND METHODS

Sampling

We studied *Thismia* species and their mycorrhizal fungi in temperate Australia, Tasmania and New Zealand (see Table S.1.1 in Appendix S1 in Supporting Information). The sampled specimens belong to five species. *T. megalongensis* C.Hunt, G.Steenbeeke & V.Merckx was collected at the type locality in New South Wales (NSW), the only place where this species is known to occur (Hunt *et al.*, 2014). *Thismia clavarioides* Thiele & Jordan (Fig. 1c) was also sampled at the only sites where this species is known to occur: the type locality and a locality close-by, both in Morton National Park (NSW). *Thismia hillii* (Cheeseman) N.Pfeiff., known from the North Island of New Zealand (Cheeseman, 1908; Schlechter, 1921), was sampled at five sites in New Zealand. Our study suggests that specimens from a site in New South Wales also belong to this species. *Thismia rodwayi* F.Muell. was sampled in Tasmania (10 sites) and Victoria (one site).

Figure 1 Species of *Thismia* sampled for this study. (a) *Thismia rodwayi* at Mount Wellington in Tasmania. (b) *Thismia hillii* at the Megalong valley in New South Wales. (c) *Thismia megalongensis* from the Megalong Valley. (d) *Thismia clavarioides* at Morton National Park in New South Wales. (e) *Thismia* sp. from Morton National Park. Photos by V. Merckx and C. Hunt.

According to literature, the species also occurs in New South Wales and south Queensland (Thiele & Jordan, 2002), but specimens from three sites NSW initially identified as *T. rodwayi* were found to belong to *T. hillii* and another species that awaits description (here labelled as *Thismia* sp.; Fig. 1). No samples from Queensland were used in this study. Sites were at least 500 m from each other, but in most case they were several kilometres from the most proximate site. In addition, *Thismia huangii* P.Y.Jiang et. T.H.Hsieh was collected at one site in Taiwan. At each site, one to five *Thismia* specimens were sampled. *Thismia* often grows in clumps, with many flowers appearing above the leaf litter within an area of 1–2 square meter (Roberts *et al.*, 2003). In some case these flowers are attached to the same root. Therefore, plants were considered to belong to different specimens if they were growing at least 3 m from each other with no *Thismia* flowers observed in between. From each *Thismia* specimen flower material was preserved on silica gel and root sections were preserved on 2% CTAB buffer. In total, 66 *Thismia* specimens (65 from Australia and New Zealand and one from Taiwan) were sampled at 20 sites. The reproductive biology of *Thismia* remains unstudied. Roberts *et al.* (2003) speculate that pollen is transported only over short distances (perhaps only metres). Wapstra *et al.* (2005) hypothesize that cross-pollination occurs by flies. Seeds of *Thismia* are minute and well-adapted for dispersal by air or water (Maas-Van de Kamer, 1998). However, wind dispersal of seeds of *T. rodwayi* and related species seems unlikely because the flowers usually mature at the interface of the soil and dense layer of leaf litter, where air movement would be slight (Wapstra *et al.*, 2005).

Plant phylogenetics

Nuclear ITS and mitochondrial *cob* sequences were used to investigate the relationships among all sampled *Thismia* specimens. A six-gene dataset (18S rDNA, ITS, *atpA, nad1b-c, cob, matR*) of 12 *Thismia* taxa was used to infer species-level relationships. Alignments were produced with MAFFT 6.814b (Katoh *et al.*, 2002). Phylogenetic inference on the aligned datasets, separate and combined, were performed with RAXMLHPC-SSE3 (Stamatakis, 2014). Branch support

was calculated by nonparametric bootstrapping using 500 pseudoreplicates. Bayesian inferences were performed on the combined dataset with MrBayes 3.2.3 (Ronquist *et al.*, 2012). The analysis was run for 10^7 generations, sampling every 5000th generation. A majority-rule consensus trees were calculated excluding the first 200 sampled trees. See Appendix S1 for detailed information.

Plant diversification and biogeography

To estimate the divergence times between the *Thismia* species we compiled a combined 7681 bp dataset of 71 taxa, 62 Dioscoreales and 9 Pandanales outgroup taxa using 18S rDNA (1743 bp), *atpA* (1153 bp), *matR* (1308 bp) and *nad1 b-c* data (3477 bp; Table S1.3). A relaxed molecular clock analysis was performed with beast 2.1.3 (Bouckaert *et al.*, 2014), using a partitioned substitution model and a relaxed lognormal clock. Based on the results of Merckx *et al.* (2008) two secondary calibration points were set. Posterior distributions of parameters were approximated with two analyses of 10^8 generations each, sampling every 5000th generation. See Appendix S1 for detailed information.

The ancestral geographical ranges of *Thismia* in Australia and New Zealand were inferred using the dispersal-extinction-cladogenesis (DEC) and DEC+J models implemented in 'BioGeoBears' (Ree & Smith, 2008; Matzke, 2013). We used optimized ancestral ranges on two phylogenies: (1) a tree with the mean dated divergences of the studied *Thismia* species, and (2) the same tree but for which the populations of *Thismia hillii* in Australia and New Zealand, and *T. rodwayi* in Victoria and Tasmania were treated as separate taxa. The following geographical areas were used: New South Wales, Victoria, Tasmania and New Zealand. No range or dispersal constraints were set. A likelihood ratio test was used to test if the DEC and DEC+J models converged to similar likelihoods.

Arbuscular mycorrhizal fungi

Fungal DNA was extracted from the CTAB preserved roots with the KingFisher Flex Magnetic Particle Processors (Thermo Scientific, Waltham, MA, USA) using the NucleoMag 96 Plant Kit (Machery-Nagel, Düren, Germany). Partial 18S rDNA fragments from the associated AM fungi of all 66 *Thismia* roots were amplified following previously described methods (Schechter & Bruns, 2008). The PCR products were sequenced directly by Macrogen (Amsterdam, the Netherlands). To infer the phylogenetic position of AM fungal taxa a maximum likelihood (ML) analysis was performed on sequences representing all 238 virtual taxa of Glomeraceae currently represented in the Maarj*AM* database (Öpik *et al.*, 2010), including three Claroideoglomeraceae sequences as outgroups. An alignment was obtained with MAFFT 6.814b and the highest likelihood tree was calculated with raxmlHPC-SSE3 using the GTR+I+G model of substitution as obtained with jModeltest 2.1.5 (Darriba *et al.*, 2012). Complete 18S sequences were amplified from six *Thismia* root DNA extractions and subsequently cloned. Amplification was done with JumpStart (Sigma, St. Louis, MO, USA) as described by Bidartondo *et al.* (2011). All product amplifications were cloned with a TOPO TA cloning kit for sequencing (Invitrogen, San Diego, CA, USA), and at least four clones for each sample were sequenced with BigDye 3.1 on an ABI3730 genetic analyser (Applied Biosystems). The obtained sequences were aligned with MAFFT with 18S sequences of Glomeraceae representatives and two Claroideoglomeraceae sequences as outgroup. The highest likelihood tree was calculated with raxmlHPC-SSE3 using the GTR+I+G model of substitution as obtained with jModeltest. Branch support was calculated by nonparametric bootstrapping using 500 pseudoreplicates.

Reconstruction of ancestral mycorrhizal range

In general, photosynthetic plants are able to associate simultaneously with multiple lineages of mycorrhizal fungi. This shows parallels with biogeographical inference in which taxa can occupy multiple areas (Page & Charleston, 1998). We reconstructed the ancestral mycorrhizal association of the most recent common ancestor of the Australia-New Zealand *Thismia* species studied here by using DEC models and a likelihood version of BayArea (BAYAREALIKE; Landis *et al.*, 2013) implemented in the package 'BioGeoBears' for R 3.3.1 (R Development Core Team, 2008). The *Thismia* species-level tree used for the ancestral geographical range reconstruction (see above) was used as input. For the 'geographical ranges' we coded for each species the presence–absence of the association with the four fungal genotypes detected: three in fungal lineage A (labelled A1–A3) and one genotype in fungal lineage B. For both the DEC and BAYAREALIKE models, we compared the fit with and without a founder-event parameter, j, which describes a speciation event where a "jump dispersal" event quickly results in an evolutionarily independent lineage (Matzke, 2014). This is similar to a sudden switch in mycorrhizal partners. In addition, we tested distance-based dispersal models (+x) where dispersal probability is multiplied by distance to the power x (Van Dam & Matzke, 2016). In this case, we used the phylogenetic distance between the four fungal genotypes as a proxy for distance, assuming that the probability of acquiring a new mycorrhizal partner reduces with the phylogenetic distance towards this new fungal lineage. Model comparisons were evaluated using Akaike Information Criterion (AIC) scores calculated from each model's log-likelihood. Pairwise comparison of nested models was done using the likelihood ratio test.

RESULTS

Plant phylogenetics

The ITS and *cob* phylogenetic trees do not show any well-supported incongruence (Figs S1.1–2). The tree obtained from the combined analysis is shown in Fig. 2. All species

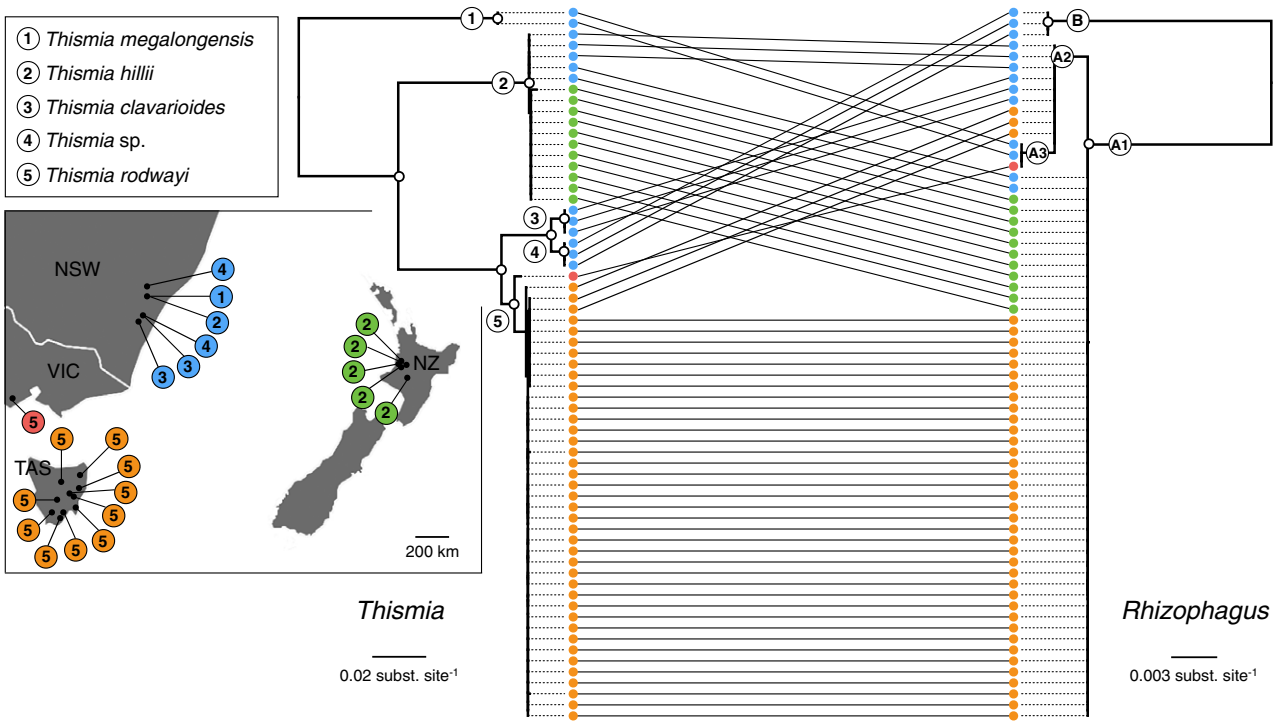

Figure 2 Geographical and phylogenetic variation of sampled *Thismia* plants (left) and associated AM fungi (right). Highest likelihood trees are shown, based on ITS and *cob* data (*Thismia*) and partial SSU (*Rhizophagus*). Open dots highlight nodes with bootstrap support of >90%. Colours refer to geographical origin of the sampled plants, shown on the map. Plant clades are labelled with numbers referring to species identifications. The two distinct fungal clades are labelled A and B respectively. The tanglegram with taxon labels is shown in Fig. S1.9.

of *Thismia* as recognized here form well-supported clades. Maximum likelihood and Bayesian analyses on the *Thismia* species-level dataset converged to the same well-supported phylogeny (Fig. S1.3). The *Thismia* species from the Southern Hemisphere are sister to species from Malaysia. *Thismia megalongensis* was found to be the sister group to the rest of the Australia-New Zealand *Thismia* species. The inferred species relationships are identical to those at the population level.

Plant diversification and biogeography

The majority-rule consensus tree from the Dioscoreales Bayesian relaxed clock analysis is shown in Fig. S1.4. The crown age of the clade consisting of *Thismia* in Australia and New Zealand is estimated at 12.02 Ma (95% CI 7.09 – 32.52 Ma). The phylogenetic split between *T. hillii* from Australia and New Zealand is estimated at 0.18 Ma (0–1.65 Ma). The split between *T. rodwayi* from Victoria and Tasmania is estimated at 0.06 Ma (0–1.00 Ma). Regardless of the phylogenetic tree (species vs. geographically separated populations) or model used (DEC vs. DEC+J), the most recent common ancestor of the Australia and New Zealand *Thismia* species was reconstructed to occur in New South Wales (Fig. 3, S1.5). In the species-level analysis the DEC+J and DEC model reconstructions did not have significantly different likelihood as determined by a likelihood ratio test. In the analysis with split taxa for *Thismia hillii* and *T. rodwayi*, the DEC+J model received a significantly better likelihood than the DEC model ($P < 0.01$).

Arbuscular mycorrhizal fungi

We obtained a single unambiguous DNA sequence for each *Thismia* root extraction with direct Sanger sequencing of partial 18S rDNA using Glomeromycota primers AM1-NS41. The majority of the obtained sequences were identical to Genbank accession JQ246072, a sequence obtained from *Thismia rodwayi* roots in an earlier study (Merckx *et al.*, 2012). From nine roots, a single sequence was obtained that showed a difference of only one base with this sequence (Genbank KY711229). The sequences obtained from the two *T. megalongensis* samples and the *T. rodwayi* specimen from Victoria differed by two bases from JQ246072 (Genbank KY711228). A BLAST search against the Maarj*AM* database showed that all these sequences are part of virtual taxon VTX00345. In all sampled *Thismia* sp. roots from sites NSW2 and NSW4, a sequence was obtained (Genbank KY711230) that is 100% identical to HE799235, which belongs to VTX00092 in the Maarj*AM* database. From samples of *T. huangii* we obtained a sequence (Genbank KY711231) that is 99% identical to sequences of VTX00113.

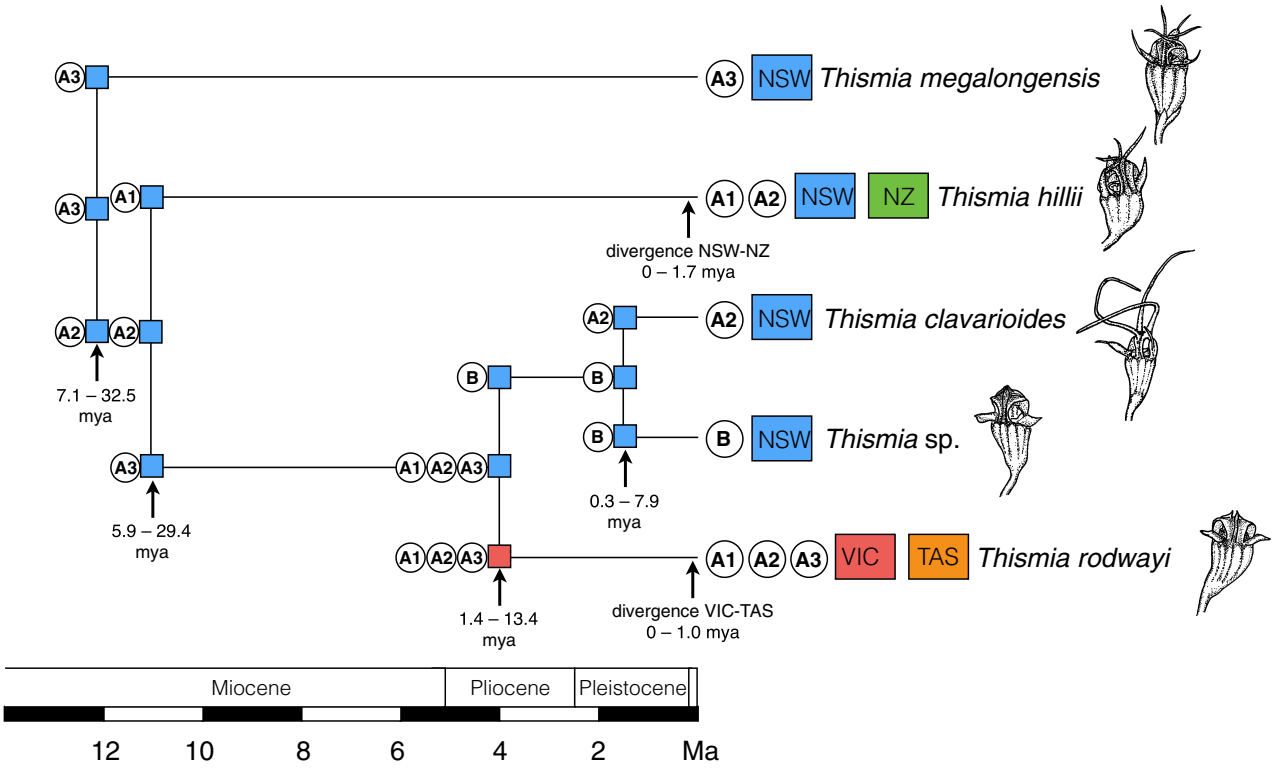

Figure 3 Dated species-level phylogeny of the analysed *Thismia* species isolated from the Bayesian relaxed clock analysis of Dioscoreales. Coloured boxes show the single-most-probable ancestral geographical range estimation using the DEC+j model (blue = New South Wales, green = New Zealand, red = Victoria, orange = Tasmania). Circles represent the single-most-probable ancestral mycorrhizal range estimation using the favoured DEC+x+j and BAYAREALIKE+x+j models.

Figure S1.6 shows the highest likelihood tree of all Glomeraceae virtual taxa highlighting the phylogenetic position of these three detected virtual taxa. A phylogeny based on full 18S rDNA sequences obtained from the cloning reactions also recovered three clades of *Thismia* AM fungi (Fig. S1.7). One clade (A) includes sequences of fungi from the roots of all Australian *Thismia* species, except for fungi of *Thismia* sp. which were found to belong to a separate clade (B). The third clade (C) included fungi detected in *T. huangii*. All of these clades were found to be most closely related with sequences that belong to the genus *Rhizophagus* Herbst.

Reconstruction of ancestral mycorrhizal range

Model comparisons based on AIC using the dated *Thismia* phylogeny consistently favoured models with a founder effect (j) and a constrained-dispersal matrix (x), but found no difference between DEC and BAYAREALIKE (Table 1). Pairwise likelihood ratio test detected a significant ($P = 0.014$) better fit of models with a founder effect (j). No significant differences were found between DEC vs. DEC+x ($P = 0.35$), and BAYAREALIKE vs. BAYAREALIKE+x ($P = 0.30$). Differences between '+j' and '+x+j' models were significant in favour of

Table 1 Summary of data likelihoods under each model for the ancestral mycorrhizal range reconstructions, and results of statistical model choice. The +x models used phylogenetic distance between the fungal genotypes areas to implement 'dispersal' constraints.

	Log-likelihood	Number of free parameters	d	e	j	x	AIC	Relative model probability
DEC	−12.12	2	0.05	0.03	0	0	28.25	2%
DEC+J	−9.09	3	0.02	0	1.71	0	24.18	12%
DEC+x	−11.69	3	0.10	0.05	0	−0.46	29.38	1%
DEC+x+j	−6.99	4	0.07	0	3.00	−2.18	21.99	35%
BAYAREALIKE	−13.68	2	0.10	0.15	0	0	31.35	0%
BAYAREALIKE+j	−8.86	3	0.01	0	0.51	0	23.71	15%
BAYAREALIKE+x	−13.15	3	0.27	0.19	0	−0.54	32.30	0%
BAYAREALIKE+x+j	−6.99	4	0.07	0	1.00	−2.29	21.98	35%

the more complex models: DEC+j vs. DEC+x+j ($P = 0.04$), and BAYAREALIKE+j vs. BAYAREALIKE+x+j ($P = 0.05$). The best-fitting models DEC+x+j and BAYAREALIKE+x+j inferred similar ancestral mycorrhizal associations, in which the most recent common ancestor of *Thismia* was associated with a single fungal genotype; A3 (Fig. 3). In these models x is estimated to be around −2, indicating that the probability of a switch of mycorrhizal partners drops off as the phylogenetic distance increases. All reconstructions are shown in Fig. S1.8.

DISCUSSION

Thismia diversification and biogeography

The population-level phylogeny supports the recognition of five species of *Thismia* among our samples from Australia and New Zealand (Fig. 2). We recognize a cryptic species from New South Wales ('*Thismia* sp.'; Fig. 1e) that interacts with a different clade of AM fungi from that of *T. rodwayi* and a detailed study is currently underway examining any morphological differences that distinguish it from that species. More species may await discovery, and further sampling in mainland Australia and New Zealand is necessary to uncover the total diversity of *Thismia* in the region.

The start of *Thismia* diversification in the region is estimated to date to the Oligocene or Miocene in mainland Australia. This includes the mid-Miocene period of global warmth, in which parts of Australia were covered by rain forest (Morley, 2000). The current distribution of *Thismia* in mainland Australia is scattered and restricted to small pockets of temperate rain forest (Thiele & Jordan, 2002; Hunt *et al.*, 2014). These forests are species poor, especially in trees, but fossil evidence indicates that many of their current tree species have been present at least since the early Pleistocene (2.5 to 0.788 Ma; for example, Worth *et al.*, 2009, 2010, 2011), and therefore have remained relatively unchanged through many, or perhaps all, of the glacial-interglacial cycles of the Quaternary (Jordan, 1997). Although our dating estimates do not allow sketching a precise scenario, the deepest diversification events within the *Thismia* clade are estimated to precede these glaciations, while the more recent evolutionary splits may have been influenced by global cooling events. The dispersal and subsequent spread of *Thismia* into Tasmania and New Zealand occurred in the Pleistocene or Holocene. During the Pleistocene Tasmania was repeatedly glaciated and linked to mainland Australia, causing many plant taxa to become (at least locally) extinct or survive in wet forest refugia (e.g. McKinnon *et al.*, 2004; Worth *et al.*, 2009, 2010, 2011). Based on the mean divergence age estimate it is possible that the current distribution of *Thismia* in Tasmania is the result of dispersal that occurred after the Last Glacial Maximum, *c.* 23–17 ka. Notwithstanding the uncertainty on this age estimate, our results demonstrate that the current broad distribution of *Thismia* in Tasmania is the result of a recent, and thus relatively rapid spread. As for many New Zealand species (McGlone *et al.*, 2001; Winkworth *et al.*, 2005; Gibbs, 2006), we also infer a recent long-distance dispersal event from Australia to explain the occurrence of *Thismia* in New Zealand. Thus, the arrival of *Thismia* in New Zealand may have followed a route similar to that of many other New Zealand taxa (Gibbs, 2006). The disjunct distribution of *T. hillii* on both sides of the Tasman Sea is remarkable but not unique; the mycoheterotrophic orchids *Danhatchia australis* and *Gastrodia sesamoides*, for example, have a similar distribution pattern (Banks, 2012; Merckx *et al.*, 2013). Our inferences of the dispersal history of *Thismia* are well in accordance with meta-analyses, which indicate that herbs with minute seeds are over-represented among trans-Tasman disjunctions (Jordan, 2001; Higgins *et al.*, 2003). In conclusion, our analyses suggest that the distribution of *Thismia* in temperate Australia and New Zealand is mainly explained by relatively recent dispersal events from mainland Australia into Tasmania and New Zealand. Speciation events preceded these dispersals and occurred within mainland Australia.

Mycorrhizal interactions of *Thismia*

DNA-based characterization of the AM fungi in the roots of the sampled *Thismia* plants reveals that each plant species is associated with a narrow phylogenetic lineage of *Rhizophagus* fungi (Fig. 2), each corresponding to a single virtual taxon of the Maarj*AM* database. Associations with narrow lineages of AM fungi (e.g. a single virtual taxon) have been found in mycoheterotrophic species of Burmanniaceae, Corsiaceae, Gentianaceae and Triuridaceae, from Africa and South America (Merckx *et al.*, 2012). High mycorrhizal specificity, therefore, is not restricted to the geographical area of this study. The observation that each investigated species of *Thismia* is consistently associated with a specific narrow range of AM fungi, even when the plant species occur in different plant communities or are separated by wide dispersal barriers, proves that these mycoheterotrophs are true specialists in their interactions with AM fungi (see also Gomes *et al.*, 2017). High mycorrhizal specificity is also a hallmark of ectomycorrhizal mycoheterotrophic plants (Merckx, 2013), which suggests that both systems are under similar ecological and evolutionary pressures.

Evolution of plant–fungus associations

The observation that four out of five closely related species of *Thismia* in Australia and New Zealand target the same narrow clade of *Rhizophagus* AM fungi suggests that this association is phylogenetically conserved, and thus presents us with an extreme example of niche conservatism (Wiens *et al.*, 2010). Although our reconstruction of the ancestral mycorrhizal partner of *Thismia* cannot take into account potential loss of AM fungal lineages not present among the fungal associates of the extant plant species, the results suggest that the most recent common ancestor of *Thismia* in

the region was probably growing with at least the same AM fungi as the extant species. This also means that none of the deep diversification events within *Thismia* led to a major shift in fungal host associates. Thus, the range and the identity of the exploited fungi have remained largely stable over a time span of c. 12 million years, that saw drastic climate change (Worth *et al.*, 2009, 2010, 2011), and several speciation and colonization events in the mycoheterotroph plant lineage. Despite this host specificity, dispersal and subsequent radiation of *Thismia rodwayi* into Tasmania and *T. hilli* in New Zealand occurred recently, and thus relatively rapidly, and they were aided by the occurrence of the same AM fungal lineages in these newly invaded areas, or possibly through co-invasion of *Thismia* and *Rhizophagus*. According to the Maarj*AM* database, all three fungal taxa associated with the investigated *Thismia* species have been detected in many temperate and tropical habitats around the world (Öpik *et al.*, 2010). Thus, globally, the distributions of *Thismia* species appear narrower than those of their associated fungi. This may indicate that the occurrence of the host fungi does not limit the dispersal potential of these specialized mycoheterotrophic plants. However, there is currently no evidence that virtual taxa represent 'real' AM fungal species. Virtual taxa may be merely 'collections of fairly distantly related taxa' (Bruns & Taylor, 2016) and specificity can act well below the level of the virtual taxon concept.

The tendency of closely related plant species to target closely related AM fungi has been observed previously in *Afrothismia* (Thismiaceae; Merckx & Bidartondo, 2008), and to some extent in *Voyria* (Gentianaceae; Bidartondo *et al.*, 2002) and *Epirixanthes* (Polygalaceae; Mennes *et al.*, 2015). In addition, the mycoheterotrophic species *Petrosavia sakuraii* (Petrosaviaceae) grows with a subset of AM fungi of those found in its closest photosynthetic relative, *Japonolirion osense* (Yamato *et al.*, 2014). Similar to AM interactions, phylogenetic conservatism has also been detected in orchid and ectomycorrhizal systems, particularly in cases where specificity is high (e.g. Shefferson *et al.*, 2007, 2010; Barrett *et al.*, 2010; Jacquemyn *et al.*, 2011; Dowie *et al.*, 2016). Thus, in mycorrhizal interactions phylogenetic conservatism potentially constrains adaptability, due to the inheritance of traits conferring adaptation to particular fungal lineages. These observations highlight the importance of plant phylogeny in determining mycorrhizal interactions, at least in some plant groups, and show that biotic interactions can have ancient roots that go far deeper than the species and ecological conditions seen today (Wiens *et al.*, 2010).

While the *Thismia–Rhizophagus* interaction is evolutionarily conserved, we also find evidence for host switching: a recent host range extension to a different *Rhizophagus* lineage occurred in the lineage leading to *Thismia* sp. from NSW. Remarkably, this *Rhizophagus* lineage belongs to VT00092 that has also been detected in mycoheterotrophic *Afrothismia* species in West Africa (Merckx & Bidartondo, 2008). This suggests that some AM fungi have functional traits that make them more attractive to mycoheterotrophic plants than others. At one of the two sites where *Thismia* sp. is known to occur, it co-exists and co-flowers with *T. clavarioides* (Hunt *et al.*, 2014), its sister species in our phylogeny. Their distinct fungal hosts may facilitate this co-existence, as proposed for some orchids (Waterman *et al.*, 2011; Jacquemyn *et al.*, 2014).

CONCLUSIONS

Species with highly specific ecological interactions are hypothesized to be more vulnerable to current anthropogenic change (Kiers *et al.*, 2010; Ellers *et al.*, 2012) and they are expected to have lower diversification potential than generalist species (Poisot *et al.*, 2011). However, our analysis of the most comprehensive and detailed plant and fungal dataset for any mycoheterotrophic system so far, shows that *Thismia*, despite highly specific and phylogenetically conserved AM interactions which persist over evolutionary time, was able to diversify and radiate recently due to the wide geographical distribution of the host fungi. In addition, we find that although the mycorrhizal interactions of these mycoheterotrophs are strictly bound to a fungal lineage, host switches remain possible. This process may lead to new ecological opportunities for the plants, and demonstrate that taxa that are dependent on highly specific biotic interactions have ample opportunities to radiate and diversify over the geographical range of their hosts.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Additional figures and tables.

BIOSKETCH

Vincent Merckx is the leader of the Understanding Evolution research group at Naturalis Biodiversity Center. His research focuses on the evolution of the interactions between plants and mycorrhizal fungi, with emphasis on mycoheterotrophic plants which obtain all their carbon from associated mycorrhizal fungi.

Author contributions: V.S.F.T.M. initiated the research and wrote the paper; V.S.F.T.M., S.I.F.G. and M.I.B. conceived the ideas; V.S.F.T.M., S.I.F.G., M.W., C.H., G.S., C.B.M., N.W. T.H.H. and M.I.B. acquired the data; V.S.F.T.M. and S.I.F.G. analysed the data; all authors discussed the ideas and commented on the manuscript

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