DIGGING DEEPER – HOW SOIL BIOTA DRIVE AND RESPOND TO PLANT INVASIONS
Plant mutualisms with rhizosphere microbiota in introduced versus native ranges

Natasha Shelby[1]*, Richard P. Duncan[2], Wim H. van der Putten[3,4], Kevin J. McGinn[1], Carolin Weser[3] and Philip E. Hulme[1]

[1]Bio-Protection Research Centre, Lincoln University, Lincoln 7647, New Zealand; [2]Institute for Applied Ecology, University of Canberra, Canberra, ACT 2601, Australia; [3]Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen 6708 PB, The Netherlands; and [4]Laboratory of Nematology, Wageningen University, Wageningen 6700 ES, The Netherlands

Summary

1. The performance of introduced plants can be limited by the availability of soil mutualists outside their native range, but how interactions with mutualists differ between ranges is largely unknown. If mutualists are absent, incompatible or parasitic, plants may compensate by investing more in root biomass, adapting to be more selective or by maximizing the benefits associated with the mutualists available.

2. We tested these hypotheses using seven non-agricultural species of *Trifolium* naturalized in New Zealand (NZ). We grew seeds from two native (Spain, UK) and one introduced (NZ) provenance of each species in glasshouse pots inoculated with rhizosphere microbiota collected from conspecifics in each region.

3. We compared how plant biomass, degree of colonization by rhizobia and arbuscular mycorrhizal fungi (AMF), and the growth benefit associated with each mutualist differed between provenances (native and introduced populations) when grown with soil microbiota from each region. We also tested whether the growth benefit of colonization by mutualists was correlated with the extent to which alien plants were distributed in the introduced range.

4. Rhizobia colonization was generally lower among introduced relative to native provenances. In NZ soils, 9% of all plants lacked rhizobia and 16% hosted parasitic nodules, whereas in native-range soils, there was no evidence of parasitism and all but one plant hosted rhizobia. Growth rates as a factor of rhizobia colonization were always highest when plants were grown in soil from their home range. Colonization by AMF was similar for all provenances in all soils but for four out of seven species grown in NZ soils, the level of AMF colonization was negatively correlated with growth rate. In general, introduced provenances did not compensate for lower growth rates or lower mutualist associations by decreasing shoot–root ratios.

5. *Synthesis.* Despite differences between introduced and native provenances in their associations with soil mutualists and substantial evidence of parasitism in the introduced range, neither level of colonization by mutualists nor the growth benefit associated with colonization was correlated with the extent of species' distributions in the introduced range, suggesting mutualist associations are not predictive of invasion success for these species.

Key-words: alien, arbuscular mycorrhizal fungi, invasive, non-native, parasitism, plant–soil (below-ground) interactions, rhizobia, root fungal symbiont

Introduction

Interactions between plants and rhizosphere microbes can strongly affect plant growth and community composition (Bardgett & van der Putten 2014) and consequently can influence the performance and naturalization of plants introduced to regions outside their native ranges (Nuñez, Horton & Simberloff 2009; Wandrag *et al.* 2013). Biogeographical studies show that opposite to the standing paradigm that 'everything is everywhere', most soil microbes are dispersal-limited at

*Correspondence author. E-mail: natasha.shelby17@gmail.com

least to some extent (Andonian *et al.* 2012; Rout & Callaway 2012; Tedersoo *et al.* 2014), implying that plants introduced to new locations will often leave behind microbiota from their native range. Thus, several studies have compared the rhizosphere communities with which plants associate in their native and non-native ranges (Richardson *et al.* 2000a; van der Putten *et al.* 2005; Reinhart & Callaway 2006; Reinhart *et al.* 2010; Callaway *et al.* 2011; Birnbaum *et al.* 2014; Gundale *et al.* 2014; McGinn *et al.* 2016). The microbiome of plant roots includes antagonists such as pests, pathogens and herbivores that suppress plant performance (Agrawal *et al.* 2005; Liu, Stiling & Pemberton 2007; Mendes *et al.* 2011), along with symbiotic mutualists that can facilitate resource uptake (Schulz, Boyle & Sieber 2007), buffer plants from abiotic stress (Abd-Alla *et al.* 2014), protect against natural enemies (Pieterse *et al.* 2014) and increase or decrease competitive ability (van der Putten & Peters 1997; Sabais *et al.* 2012). While leaving behind antagonistic rhizosphere biota has been shown to facilitate some plant invasions, the absence of certain mutualists can hinder establishment (Mitchell *et al.* 2006; Rodríguez-Echeverría & Crisóstomo 2009) or limit the performance of introduced species in their new ranges (Wandrag *et al.* 2013), particularly when plant-soil mutualisms are highly specialized (Klironomos 2000; Popovici *et al.* 2011; Bever, Broadhurst & Thrall 2013).

Legumes (Fabaceae) are one group of plant species where loss of soil biota could impact their ability to establish and spread in new regions due to their reliance on a specialized mutualism with nitrogen-fixing rhizobia (Parker 2001; Rodríguez-Echeverría *et al.* 2012). Species in the genus *Trifolium* (true clovers), for example, associate with only one biovar of nitrogen-fixing rhizobia, *Rhizobium leguminosarum* biovar *trifolii* (Burton 1985). Clover species are specialized to particular strains of this biovar, with substantial differences in growth depending on how well matched a strain is to the species of clover it colonizes (Howieson *et al.* 2005). Consequently, the absence of a specific strain of rhizobia could potentially limit the ability of some clover species to establish and spread in new regions. In addition, clovers frequently form a tripartite association involving the plant, rhizobia and arbuscular mycorrhizal fungi (AMF; Bethlenfalvay, Newton & Regional 1991). AMF colonization can increase the ability of plants to take up water and nutrients, particularly phosphorus (Smith & Read 2010), provided the AMF are a proper match for the species or genotype, and phosphorus is limiting (Lim & Cole 1984). Because nitrogen fixation by rhizobia requires high phosphorus input, rhizobia colonization of clover can fail in phosphorus-limited soils unless the plant is colonized by AMF (Crush 1974), whereas if highly compatible strains of both mutualists are present, and if environmental conditions are conducive to the mutualism (Walder & van der Heijden 2015), plant growth increases substantially (Sprent & James 2007). Both plant–rhizobia pairings and plant–AMF associations are increasingly recognized to be specialized (Klironomos 2000, 2003; Kiers *et al.* 2003), and differences at the level of species or strain can result in substantial performance differences in the host plant (Johnson, Graham &

Smith 1997). Evidence for adaptive divergence has been found as a result of the availability of compatible strains (Seifert, Bever & Maron 2009), the genotypes of the partners available (Howieson *et al.* 2005) and the local environmental conditions (Porter, Stanton & Rice 2011).

Despite these strain-specialized relationships and tripartite dependencies, there are many widespread and problematic legume invaders, some of which are considered to be among the worst invasive species globally (e.g. *Robinia pseudoacacia, Cytisus scoparius, Ulex* spp.*, Medicago* spp.*, Acacia* spp.*, Trifolium* spp.) (Pysek 1998; Richardson *et al.* 2000b). The success of legumes as invaders despite their reliance on specialized soil mutualists could be attributed to: (i) introduced legumes encountering compatible mutualists outside their native range, either because they are able to form associations with new taxa or because well-matched strains from the native range are co-introduced [e.g. via inoculating economically important species with rhizobia (Lowther & Kerr 2011)]; or (ii) any loss of specialized soil mutualists being outweighed by the benefits of escaping antagonists (Callaway *et al.* 2011).

There is also a third explanation that has not been widely tested: introduced plants may adapt to differences in soil mutualists outside their native ranges (Seifert, Bever & Maron 2009; Porter, Stanton & Rice 2011), such as by diverting energy away from maintaining mutualists and towards other characters that increase fitness (Tawaraya 2003). If introduced plants lack specialized rhizosphere mutualists in their new range, they may adapt by reducing their reliance on these associations and consequently will be less likely to re-associate with those mutualists if they encounter them again. For example, introduced populations of *Hypericum perforatum* (Hypericaceae) in North America benefitted less from inoculation by cosmopolitan AMF compared with plants from the native range in Europe, suggesting a genetic shift resulting in less dependence on AMF following introduction to North America (Seifert, Bever & Maron 2009). North American plants also had lower shoot–root ratios and finer root architecture compared with native conspecifics (Seifert, Bever & Maron 2009), potentially to compensate for the reduced AMF association given that greater allocation to root biomass is common among non-mycorrhizal species (Johnson, Graham & Smith 1997) and fine root systems are an adaptation typical of plants with low AMF responsiveness (Hetrick, Wilson & Todd 1992; but see Maherali 2014).

Our aim in this study was to test the hypothesis that introduced plant provenances have reduced mutualistic associations relative to native-range provenances in response to a lack of specialized soil mutualists in the introduced range. To do this, we compared the performance of introduced and native provenances when grown in soil containing microbiota from the introduced and native ranges. We used level of colonization as a measure of the degree to which plants formed associations with mycorrhizas and rhizobia – a higher level of colonization would indicate a greater degree of association. We expect the degree of association to vary by both provenance and by soil origin. We then compared the growth

benefit of native and introduced seed provenances when grown in native-range soils and in introduced-range soils. We made the following specific predictions:

1. If introduced-range soils lack specialized rhizosphere mutualists (AMF and rhizobia) and introduced plants have consequently reduced their association with these mutualists, we predict that:
   a When grown in introduced-range soils, plants sourced from both the introduced and native ranges will be poorly colonized by mutualists relative to their colonization in native-range soils.
   b When grown in native-range soils, introduced plants will be poorly colonized by mutualists relative to native conspecifics.
2. If introduced plants have reduced mutualistic associations, we predict:
   a Introduced plants will invest more in root biomass (i.e. have lower shoot–root ratios) relative to native conspecifics when grown in both introduced- and native-range soils.
   b Introduced plants will maximize relationships with available mutualists, so that the incremental growth benefit as a factor of mutualist colonization will be greater among introduced plants relative to native conspecifics when grown in introduced-range soils.
3. Among introduced provenances grown in introduced-range soil, the degree of mutualist association and the growth benefit (growth rate as a factor of degree of colonization) will be positively correlated with species' geographic extents in the introduced range (i.e. species that benefit more from available mutualists will be more successful at invading).

We tested these predictions using seven species in the genus *Trifolium* that are native to Europe but were accidentally introduced and have naturalized in New Zealand to varying degrees (Table 1). This is a strong system to investigate potential post-naturalization differences in rhizosphere mutualists for several reasons. First, *Trifolium* can derive great nutritional benefit from associations with rhizobia and AMF (Bethlenfalvay, Newton & Regional 1991), but the degree of benefit can differ among AMF strains or genotypes (Johnson *et al.* 2012; Werner & Kiers 2015), and colonization by rhizobia is strictly limited by species-strain compatibility (Yates *et al.* 2005) and the density of compatible rhizobia in the soil (Yates *et al.* 2008; Drew *et al.* 2011). Second, our previous work in this system supports the prediction that the composition of mutualists that colonize plants differs between ranges: although rhizobia strain richness in the root nodules of *Trifolium* spp. growing in New Zealand was comparable to the levels in plants growing in native UK soils, < 3% of strains were shared between ranges (McGinn *et al.* 2016). Although the effectiveness of rhizobia for non-agricultural clovers in New Zealand has not been tested (Boswell *et al.* 2003), optimal rhizobia for non-agricultural species introduced as seed contaminants (Gravuer 2004) are likely to be rare because rhizobia colonize directly from the soil. Even if compatible soil mutualists from the native range are introduced, they must

compete with widely naturalized agricultural rhizobia in New Zealand (Greenwood 1964; Lowther & Kerr 2011), and naturalized rhizobia are highly competitive (Denton *et al.* 2003). Third, having multiple closely related congeners in the same naturalized range is beneficial because although individual populations would have been subjected to various stochastic factors, they would have encountered similar biotic and abiotic factors. This enables a more direct investigation of post-naturalization differences that may have been shaped by the conditions of the non-native range and it minimizes the role of chance or founder effects in the results (Keller & Taylor 2008). Finally, because these seven species have spread to varying degrees throughout New Zealand, we can use species as a unit of replication to investigate whether differences in mutualist association are correlated with invasion success. For the seven species, there is no correlation between New Zealand naturalization date and countrywide geographic extent (Pearson's correlation = -0.34, $P = 0.45$; Fig. S2 in Supporting Information) eliminating the potentially confounding factor of residence time (Richardson *et al.* 2000b; Gravuer 2004).

We expected some degree of phenotypic plasticity among plants from both provenances in each soil type. However, we predicted that if post-naturalization adaptations have occurred, the difference between provenances would be greater than the plastic responses of these plants and conform to our predicted pattern. Specifically, we predicted that plants from the introduced range would have lower mutualist association than natives in both ranges. We further predicted that introduced plants would have greater performance than natives in the introduced range but poorer performance than natives in the native range. Our goal was to test the specific hypotheses above; however, our experimental design and statistical analyses were set up to allow us to identify and quantify any difference in mutualist association and benefit between provenances – including differences in the opposite direction predicted.

Materials and methods

To test whether native and introduced provenances differ in performance and mutualist association, we performed two glasshouse experiments in which plants were inoculated with soil microbiota from either the native range (Spain or the UK) or the introduced range (New Zealand). Because of logistic and biosecurity issues associated with moving soil between these countries, the glasshouse experiments with native-range soils were conducted at the Netherlands Institute of Ecology in Wageningen, the Netherlands, in Northern Hemisphere summer 2012, while experiments with introduced-range soil were carried out at Lincoln University in Canterbury, New Zealand, in Southern Hemisphere summer 2013.

STUDY SPECIES

We selected seven non-agricultural *Trifolium* species that were introduced accidentally to New Zealand (Gravuer 2004): *T. arvense, T. campestre, T. glomeratum, T. micranthum, T. ornithopodioides, T. striatum* and *T. tomentosum* (Table 1). All seven are annual species recorded as being naturalized in New Zealand for between 84

Table 1. Summary information for the seven species of *Trifolium* used in this study

Trifolium species	Year naturalized in New Zealand	New Zealand distribution*	Shoot : root ratio	AMF (%)	Rhizobia score (0–3)
T. arvense L.	1880	83	NZ: 3.3 ± 0.2	4.0 ± 1.0	0.95 ± 0.08
			SP: 3.5 ± 0.2	9.0 ± 2.5	2.00 ± 0.00
			UK: 3.9 ± 0.2	10.4 ± 3.3	2.00 ± 0.18
T. campestre Schreb.	1867	46	NZ: 3.3 ± 0.4	10.9 ± 3.1	1.85 ± 0.08
			SP: 5.2 ± 1.4	11.2 ± 4.0	1.69 ± 0.13
			UK: 4.1 ± 0.5	5.9 ± 1.6	1.60 ± 0.13
T. glomeratum L.	1870	44	NZ: 5.3 ± 0.9	4.5 ± 0.9	0.79 ± 0.24
			SP: 8.0 ± 1.2	8.0 ± 2.3	1.69 ± 0.27
			UK: 6.5 ± 1.1	7.8 ± 1.9	1.60 ± 0.27
T. micranthum Viv.	1854	23	NZ: 4.7 ± 0.4	14.8 ± 4.8	1.60 ± 0.16
			UK: 3.3 ± 0.2	13.1 ± 5.0	1.90 ± 0.31
T. ornithopodioides L.	1930	26	NZ: 3.6 ± 0.2	13.8 ± 3.8	1.57 ± 0.31
			UK: 3.5 ± 0.4	17.9 ± 5.3	2.23 ± 0.30
T. striatum L.	1878	44	NZ: 2.1 ± 0.3	10.3 ± 2.3	1.60 ± 0.16
			SP: 2.3 ± 0.2	8.0 ± 2.2	1.60 ± 0.22
			UK: 2.1 ± 0.2	9.4 ± 3.2	1.60 ± 0.27
T. tomentosum L.	1948	21	NZ: 7.0 ± 1.4	12.4 ± 3.2	2.00 ± 0.17
			SP: 5.6 ± 1.4	9.0 ± 2.6	1.50 ± 0.34

Colonization by arbuscular mycorrhizal fungi (AMF) were calculated as a percentage of 'hits' from 100 intersects (see Materials and methods for details). Rhizobia scoring was based on a 0–3 score that accounts for the size, abundance and colour of root nodules (Table S3). Shoot–root ratios, mean percentage colonization by arbuscular mycorrhizal fungi (AMF) and mean rhizobia score are given as means ± SE, calculated separately by seed provenance. NZ = New Zealand, SP = Spain, UK = United Kingdom. Distribution data are from Gravuer (2004).
*Number of 10 km × 10 km NZMS260 grids occupied by at least one population.

and 160 years, suggesting sufficient time to adapt to the novel rhizosphere communities of the introduced range (Atwood & Meyerson 2011). Estimates of species distributions in New Zealand were taken from Gravuer (2004). In each inter-provenance comparison, species served as the unit of replication.

STUDY LOCATIONS

We collected New Zealand seed and soil from Banks Peninsula, a region comprising a variety of habitats broadly representative of the naturalized range of *Trifolium* in the South Island (Boswell *et al.* 2003). We selected two locations in the native range (northern Spain and southern United Kingdom) that broadly match the latitude of New Zealand and encompass a wide range of climates and soil communities (Tables S1 and S2). Ideally, tests comparing native and introduced provenances would use the source provenance in the native range (Gundale *et al.* 2014), but this is rarely possible as the origins of most founding populations are unknown. The native ranges of these *Trifolium* species encompass much of Europe, but most of New Zealand's agricultural clovers were imported from the UK (Gravuer 2004), making the UK a likely source location for accidental introductions and an appropriate native-range comparison. We also sampled from northern Spain to include a region that more closely matched the sampling latitude in New Zealand (Colautti, Maron & Barrett 2009) and to use an additional species, *T. tomentosum,* that is not native to the UK but widely naturalized in New Zealand. At all sites in both ranges, the study species co-occurred with other *Trifolium* species, particularly *T. repens*. Although there are native legumes and native rhizobia in New Zealand (Weir 2006), *Trifolium* species only associate with one rhizobia biovar, *Rhizobium leguminosarum* bv. *trifolii*, and the only nitrogen-fixing plants identified at the New Zealand study sites were *Trifolium* and *Medicago* species.

RHIZOSPHERE SOIL COLLECTION

Soil collection and storage methods were designed to maintain the viability of the rhizosphere microbiota sampled in each range. We used this approach with the goal of inoculating experimental pots with a representative mixture of rhizosphere microbiota, including the rhizobia and mycorrhizas associated with these plants growing in the wild in each location. While some studies have inoculated experimental pots with specific soil biota to test for the effects of individual biota on plant performance, our goal was to capture the net effect of the rhizosphere microbiota on plant growth. In each country and for each species, we collected soil at five sites – at least 1 km apart – and sampled a wide range of soils and therefore soil communities. At each site, we collected approximately 100 mL of rhizosphere soil from beneath 10 plants of the target species located at least 1 m apart and placed these soils in separate bags. We sterilized our digging equipment with Dettol or bleach between sites to avoid cross-contamination of soil biota. The soil samples collected from each of the 10 individual plants of each species at each site were air-dried (Reinhart *et al.* 2003), bulked within each of the five sites (i.e. the five soil site replicates) and sieved to 4 mm. Keeping the five sites separated enabled us to preserve the spatial variation within each range; bulking within a single site allowed us to cover the spatial within-site variation properly. We removed all visible macrobiota and roots before storing the soils in sealed bags in cool storage rooms (16–22 °C).

SEED TREATMENT

For each species, seed was hand-collected from a minimum of 12 plants at one site in each country (New Zealand, Spain and the UK). Field plants of *T. arvense* in the UK were not setting seed at the time of collection so we sourced UK seed from a germplasm centre in the UK (Herbiseed, Reading, UK); *T. tomentosum* was only sampled in

New Zealand and Spain as it is not native to the UK; and *T. micranthum* and *T. ornithopodioides* were only sampled in New Zealand and the UK because we could not locate sufficient populations in Spain. Seed from a single site is not expected to capture all of the within-species variation in a given range (Erfmeier & Bruelheide 2005); however, we made species our unit of replication. If, as a general rule, introduced legumes tend to lose mutualistic associations, then most populations in the introduced range should show this pattern and we should observe it, on average, across a group of replicate species. We intend our study to function somewhat like a meta-analysis, enabling us to detect trends that are common to a group of functionally similar species. All seed was treated to remove existing microbiota from the seed coat by sterilizing in a 10% solution of household bleach for 2 min and rinsing thoroughly in de-ionized water. Seeds were then scarified gently with a scalpel to perforate the testa and germinated on sterile glass beads under species-specific temperature and day length requirements in a germination cabinet.

GLASSHOUSE EXPERIMENTS

We grew plants in 1-L pots in glasshouses; each pot was composed of a background soil that had been sterilized either by successive autoclaving (two cycles of 20 min at 121 °C) in New Zealand or by gamma irradiation (> 25 kGray) in the Netherlands (Table S5). To inoculate each pot with rhizosphere microbiota cultured by conspecifics from each range, we added a 10% (v/v) inoculum of unsterilized soil from a single site from each country. This inoculation approach serves to minimize differences in abiotic soil properties (pH, macro- and micronutrient content, etc.) and standardize the effects of nutrient flushes that occur after soil sterilization. Seedlings of each species were transplanted into pots after all had developed their first true leaves. Seedlings that died within the first week were replaced. Pots were assigned random locations in the glasshouses, rotated every 2 weeks and watered to species-standardized weights on a weekly or twice-weekly basis as needed. During the experiments, we responded to outbreaks of thrips by releasing biocontrol mites *Amblyseius cucumeris* (twice in New Zealand and once in the Netherlands) and we applied a topical, non-systemic fungicide (Chlorotek; Taranaki NuChem, New Plymouth, New Zealand) equally to all *T. campestre* plants in New Zealand to combat powdery mildew. Plants of the same species were harvested on the same day after approximately 3 months when plants began forming flower buds, indicating an energetic switch from growth to reproduction. Roots were washed gently, scored for rhizobia colonization (details below and in Table S3), and each plant was separated into roots and shoots before being oven-dried at 65 °C. We used growth rate (dry biomass in g/number of glasshouse-grown days) to standardize comparisons (McKenney *et al.* 2007). Roots and shoots were weighed separately to provide a shoot–root ratio (S : R ratio).

QUANTIFYING COLONIZATION BY MUTUALISTS

Colonization by rhizobia was scored during root washing. We followed a modified protocol from Corbin, Brockwell & Gault (1977), using a 0 to 3 scale that takes into account the number, size, location and colour of root nodules (Table S3). Briefly, nodules that are pink or purple indicate the presence of leghaemoglobin, an oxygen-binding protein synthesized when rhizobia are actively fixing nitrogen to maintain anoxic conditions in the nodule. White or pale-coloured nodules indicate parasitism – bacteria acquiring plant photosynthate without fixing nitrogen (Melino *et al.* 2012). In *Trifolium*, pink or purple nodules larger than 1 mm near the root crown indicate the presence of highly beneficial nitrogen-fixing strains (Greenwood & Pankhurst 1977). Each nodule might host as many as 10 or more strains, each of differing productivity (Denison & Kiers 2004a,b), so it is not possible to quantify the efficacy of nodules visually; however, nodule scores provide a proxy to characterize rhizobial association and also to calculate an estimated degree of benefit: plant growth as a function of association (Corbin, Brockwell & Gault 1977; Wandrag *et al.* 2013).

Arbuscular mycorrhizal fungi colonization was quantified using a protocol adapted from Vierheilig *et al.* (1998). First, we sampled the distal 2 cm of each plant's roots and rehydrated them in separate Eppendorf tubes in 70% ethanol. Next, roots from each plant were placed in separate histology cassettes and heated in a 90 °C bath of 10% KOH for 11 min to clear the cytoplasm. After rinsing with DI water, cassettes were transferred to a 5% solution of black Schaeffer ink in white vinegar and stained for 7 min at 80 °C. To de-stain, we rinsed the cassettes several times in tap water and bathed them for at least 1 h in a room-temperature water bath acidified with a few drops of vinegar. Roots from a single plant were plated on a microscope slide using lactic acid and glycerol and examined at 100× magnification. By moving the microscope stage in a horizontal plane, we scanned each slide at 1-mm intervals. When the centre of the viewing area intersected with any material, the 'hit' was scored as an arbuscule, vesicle, internal hyphae, external hyphae or root. Only arbuscules and vesicles were classified as AMF. Passes were made until we had 100 observations from each plant. Plant growth response as a function of percentage AMF colonization has been examined previously (Clark, Zeto & Zobel 1999; Fahey *et al.* 2016).

STATISTICAL ANALYSES

To test the prediction that rhizobia nodulation scores and degree of AMF colonization differ between introduced and native provenances in native-range soil but not in introduced-range soil, in each experimental test, we ran separate analyses for each soil origin (New Zealand, Spain and the UK). We fitted a generalized linear mixed-effects (GLME) model with *seed provenance origin* (introduced or native) as a fixed effect and *seed provenance origin* × *species* as a random effect. This random effect allowed for differences among species in degree of colonization and allowed the effect of provenance on colonization to differ among species, with the overall effect of provenance on degree of colonization (i.e. the average effect across all seven species) captured by the fixed-effect term. We also included as a random effect a reference factor *ref* that incorporates both *species* and the *site* from which soil was collected as an additional random effect in the model to account for potential non-independence due to site-specific soil factors or *species* × *soil* interactions. We fitted models of the same form, but with different response variables (e.g. *colonization*, *shoot–root ratios*, *growth rate*), to directly test for performance differences between native and introduced provenances in each of these response variables.

We also tested for differences among introduced and native provenances in the calculated growth benefit as a factor of rhizobia and AMF colonization by running GLME models of *growth rate* as a function of *nodulation score* and *AMF score*. We fitted three regression models to examine the relationship between growth rate and mutualist colonization (percentage root infection for AMF and nodulation score for rhizobia), one for each soil origin (NZ, Spain and the UK). In NZ soil, the regression model included data from 89 pots: 7 species × 3 seed provenances × 5 replicates (with three species lacking provenances in one region and one missing value). In Spain and UK soils, the regression models included data from 47 and 60 pots, respectively. We used these data to test for differences between

provenances in the slope of the relationship between growth rate and nodulation score. This meant that for each soil type, each provenance (NZ, Spain or UK) had between 23 and 34 observations on which the regression was fitted (Table S4). These observations comprised replicates of each species, and we allowed for differences among species to vary by fitting a mixed-effects model in which both the slope and intercept terms for the relationship between growth rate and colonization score was allowed to vary by species. In each case, the slope of this regression estimates the increase in growth rate associated with increasing the nodulation or AMF score by one, which measures the benefit a plant gains from a given level of colonization. Plants that derive more benefit given the same level of colonization could be viewed as having a more effective mutualistic association. We further predicted that the absolute level of association with available mutualists and the calculated growth benefit associated with mutualist colonization is greater among New Zealand species that are more widely naturalized in New Zealand. We tested this by correlating the random effect estimates describing, for each species, the growth benefit for New Zealand provenances grown in New Zealand soil against the distribution of each species in New Zealand.

All statistical analyses were performed in R ver. 3.2.3 (R Development Core Team 2013). Linear mixed-effects models were fit using the 'lmer' function, which uses restricted maximum likelihood, in the R package 'arm' ver. 1.6.10 (Gelman & Su 2014).

Results

COLONIZATION BY RHIZOBIA

Degree of rhizobia colonization was generally higher in native provenances relative to introduced provenances regardless of soil type (Fig. 1), which did not match our predictions. Averaged across species and provenances, and consistent with the prediction that introduced-range soils lack specialized mutualists, the mean rhizobia nodulation scores were lowest in New Zealand soil and higher in Spanish and UK soils (Fig. S1a).

In New Zealand soils, Spanish and UK provenances had substantially higher levels of nodulation than New Zealand provenances, with 95% confidence intervals for the difference between provenances not overlapping zero (Fig. 1a). In native-range soils, only a single plant was not colonized by rhizobia (a *T. glomeratum* plant from New Zealand), whereas when grown in New Zealand soil, 9% of plants completely lacked rhizobia (15% of plants sourced from New Zealand, 8% of plants from Spain and 2% of plants from the UK; Fig. 2a). Also in New Zealand soil, 16% (14/89) of plants hosted nodules characteristic of parasitism (evenly spread across introduced and native provenances) while none of the plants grown in either of the native-range soils had parasitic nodules (Spain = 47 and the UK = 60 plants; Fig. 2b), a difference unlikely to be due to chance (Pearson χ^2 = 18.1, d.f. = 2, $P < 0.001$).

COLONIZATION BY AMF

Colonization by AMF varied among species and seed origin, with average colonization levels ranging between 4.0% ± 1.0 (SE) and 17.9% ± 5.3 (Table 1). We predicted all provenances should behave similarly and be poorly colonized in New Zealand soils, but here UK provenances had higher mean AMF colonization relative to New Zealand provenances (Fig. 1b). When grown in native-range soils, we expected native provenances to be more strongly colonized than introduced provenances, but here there were no significant differences in AMF colonization between native and introduced provenances (Fig. 1b).

GROWTH RATE AND SHOOT–ROOT (S : R) RATIOS

Native plants grew faster, on average, than introduced plants in all soils (Fig. 3a). Shoot–root ratios showed all plants allocated more to above-ground biomass (Table 1). As evidence of compensation for lower mutualist associations, we expected lower S : R ratios in introduced relative to native provenances in all soils, but this was not supported. In UK soil, UK plants had significantly lower S : R ratios compared with NZ conspecifics (Fig. 3b); in all other soils, S : R ratios did not differ significantly between native and introduced provenances (Fig. 3b).

CALCULATED GROWTH BENEFIT OF MUTUALIST ASSOCIATION

Plants colonized by rhizobia from the same geographic range derived more growth benefit per level of rhizobia nodulation compared with plants colonized by rhizobia from a different source range (Fig. 1c). When grown with soil microbiota from the introduced range, New Zealand provenances had significantly greater growth rates per nodule score compared with native provenances, while the opposite was true when plants were grown in soil inoculated with native-range biota (Fig. 1c). The same was not true for AMF, as there were no clear differences in calculated growth benefits of AMF association between provenances, regardless of whether the AMF originated from the same or a different range (Fig. 1d).

Among introduced provenances grown in New Zealand soil, colonization by rhizobia (Fig. 4a) and AMF (Fig. 4b) varied among species, but the growth benefit as a factor of rhizobia colonization was always positive (Fig. 4c), whereas the growth benefit as a factor of AMF colonization was negative on average for four of the seven species (Fig. 4d).

GEOGRAPHIC EXTENT

In contrast to our predictions, the degree of growth benefit (growth rate as a factor of mutualist colonization) among plants from introduced provenances was not correlated with species' geographic distributions (Fig. 4). Although species differed substantially in their calculated growth benefit as a factor of association with rhizobia (Fig. 4c) and AMF (Fig. 4d) in New Zealand soil, these values were not predictive of the extent of their distribution.

Discussion

We tested the prediction that alien plants differ in their relationship with rhizosphere mutualists compared with native
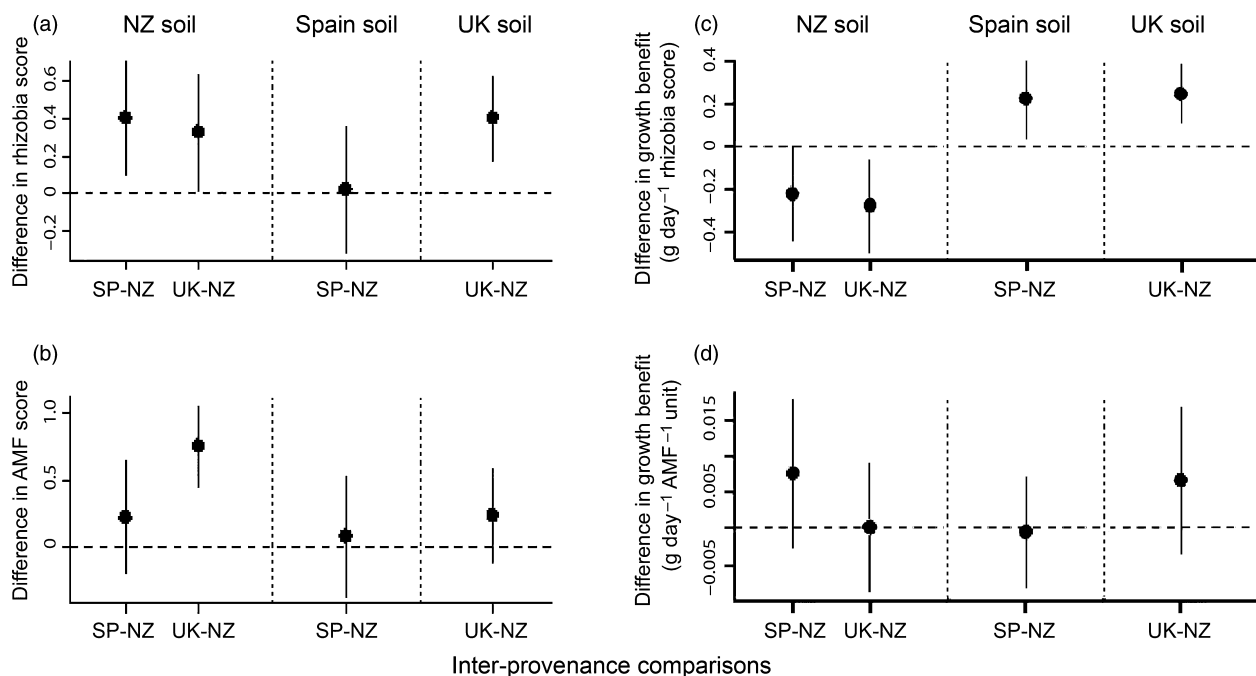
Fig. 1. The mean difference in rhizobia nodulation score (a), growth benefit as a factor of rhizobia nodulation (b), colonization by arbuscular mycorrhizal fungi (AMF) (c) and growth benefit as a factor of AMF colonization (d) between introduced (New Zealand, NZ) and native (Spain, SP and United Kingdom, UK) provenances, averaged across seven species of *Trifolium*. Plants were grown in glasshouse pots inoculated with microbiota sourced from NZ, SP or UK soils. In each panel, circles show the mean difference between NZ (the reference class, which is set to zero) and either SP or UK provenances. A value of zero indicates no difference between introduced and native provenances averaged across all species, and a positive value indicates native provenances had, on average, greater colonization or greater growth benefit compared with introduced provenances. Solid lines are 95% confidence intervals.

conspecifics. By inoculating glasshouse pots with soil microbiota cultivated by conspecific plants growing in each range, we were able to compare native and introduced plants' performance, degree of root colonization by AMF and rhizobia, and mutualist benefit (growth rate as a factor of colonization by each mutualist). We used species as our unit of replication to test for a common response among introduced *Trifolium* species. Furthermore, using species that have naturalized to differing degrees in the introduced range allowed us to ask whether mutualist colonization or benefit correlated with invasion success.

EVIDENCE FOR REDUCED MUTUALIST ASSOCIATION

Our first hypothesis – that *Trifolium* will be poorly colonized in the introduced range and that introduced provenances would have lower colonization by AMF and rhizobia compared to native conspecifics in native-range soils – was only partially supported, and only in the mutualism with rhizobia. The lower rhizobia colonization levels among all plants in introduced-range soil support the assumption that strains associated with New Zealand's non-agricultural *Trifolium* species are less compatible or available than those in the native range. Nevertheless, this comparison could be confounded by differences in glasshouse conditions and so we restrict our inferences to comparisons between provenances within each range. New Zealand provenances had significantly lower rhizobia

colonization compared to native conspecifics in UK soils, but in Spanish soils, nodulation scores were similar. Rhizobia and AMF colonization were also lower among introduced provenances compared with native conspecifics in New Zealand soil, which was contrary to our predictions. Provenances from the introduced range may be exhibiting decreased or more selective associations with rhizobia mutualists, but the similar nodulation scores of introduced and native plants in Spanish soils (grown in parallel with the UK soil treatment) suggest that other factors are affecting colonization levels, such as differences in antagonist biota among the soil origins, a factor that we did not study here.

EVIDENCE FOR POST-NATURALIZATION DIFFERENCES

Our second hypothesis was that alien plants compensate for reduced mutualist associations by either allocating more energy to below-ground biomass or by forming more effective mutualist associations. We reject this hypothesis. First, there was no consistent evidence that introduced plants compensate physiologically. Shoot–root ratios of introduced provenances were significantly higher compared with native conspecifics in UK soil (where introduced provenances also had significantly lower rhizobia colonization compared to natives). In New Zealand soils, despite several significant inter-provenance differences in both rhizobia and AMF colonization, shoot–root ratios of all provenances were similar. Compensation may
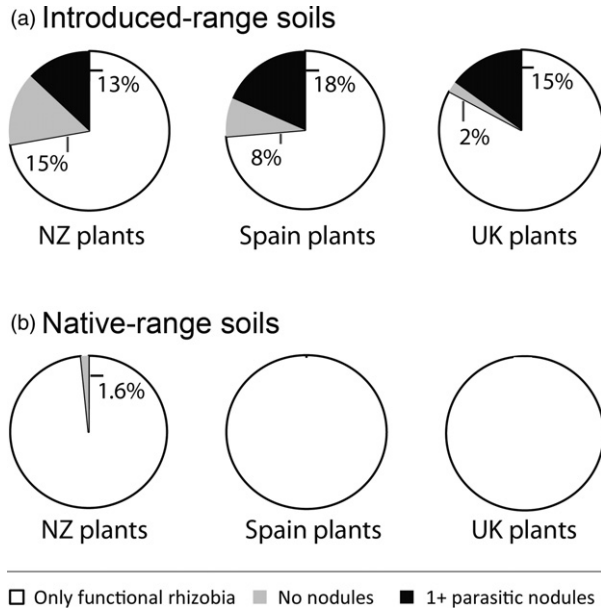
(a) Introduced-range soils

NZ plants   Spain plants   UK plants

(b) Native-range soils

NZ plants   Spain plants   UK plants

☐ Only functional rhizobia   ▨ No nodules   ■ 1+ parasitic nodules

Fig. 2. Rhizobia colonization of *Trifolium* species grown in soils inoculated with rhizosphere microbiota from their introduced (a) or native (b) ranges. Colonization type is given as a percentage and divided by seed provenance (New Zealand, Spain and the UK). Each plant's rhizobia colonization was classified as either only functional rhizobia (white), lack of colonization (grey) or having the presence of one or more parasitic rhizobia nodules (black). In panel B, the native-range soils (Spain and the UK) are grouped; all plants but one (a *Trifolium glomeratum* plant from New Zealand) were colonized by functional rhizobia, and there was no evidence of parasitism.

Fig. 3. Differences in growth rates (a) and shoot–root (S : R) ratios (b) between introduced (New Zealand, NZ) and native (Spain, SP, and United Kingdom, UK) provenances, averaged across seven species of *Trifolium* when grown with soil microbiota sourced from New Zealand, Spain and the UK. Circles show the mean difference in growth rate or S : R ratio between NZ (the reference class, which is set to zero) and either SP or UK provenances. A value of zero indicates no difference between introduced and native provenances averaged across all species, and a positive value indicates native provenances had, on average, higher growth rates or higher S : R ratios than introduced provenances. Solid lines are 95% confidence intervals.

have been manifested in other traits, such as thinner, more branched root architecture or greater specific area of roots (Seifert, Bever & Maron 2009). Root architecture was not assessed in this study and if this form of compensation occurs in the *Trifolium* system, growth rate results could have been biased during root washing as the most delicate sections of root systems may have been lost. It is also possible that compensation was occurring and shoot–root ratios were smaller for non-native provenances, but that the effect was confounded by the contribution of rhizobia nodule weight among plants with high nodulation scores, as we did not weigh nodules separately (Agren & Franklin 2003). Secondly, although the growth benefit per level of rhizobia nodulation was greater among New Zealand provenances compared to native conspecifics in New Zealand soil, growth benefit was also greater for each of the native-range provenances grown in soil from their countries of origin. This suggests that inter-provenance differences may not be attributable to post-naturalization adaptations but simply evidence that *Trifolium* provenances can maximize their associations with available soil mutualists.

Higher performance in association with mutualists from a plant population's home range could be due to plants developing more efficient interactions with available soil mutualists and/or their ability to cultivate a subset of available soil organisms that are the most beneficial to themselves. This trend has been observed previously; Porter, Stanton & Rice
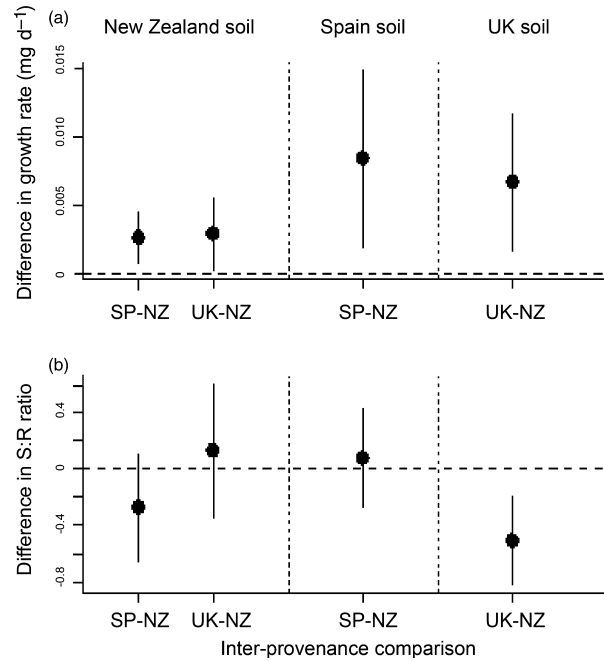
(2011) found that populations of *Medicago* (Fabaceae) had higher seed set when inoculated with rhizobia from the plant's home soil and attributed this to adaptation to local rhizobia communities. Plants may develop increasingly positive associations with locally available mutualists by rewarding beneficial strains with more photosynthate (Werner & Kiers 2015), by signalling selectively to optimal strains in the soil (Cooper 2007) or by levying sanctions against poor performers after nodulation (Kiers *et al.* 2003). Agricultural clover populations are well known to perform better with local coexisting strains compared to strains isolated from conspecifics in other locations (Sherwood & Masterson 1974), but how such differences in partner effectiveness may affect plant performance in the context of invasion has not yet been considered.

CORRELATION WITH DISTRIBUTION

In New Zealand soils, despite introduced provenances having significantly greater growth benefit from rhizobia compared with native conspecifics, our third hypothesis was not supported. Among introduced provenances, species varied in both the level of colonization by AMF and rhizobia and they varied in the degree of growth benefit incurred by association – yet none of these factors were correlated with
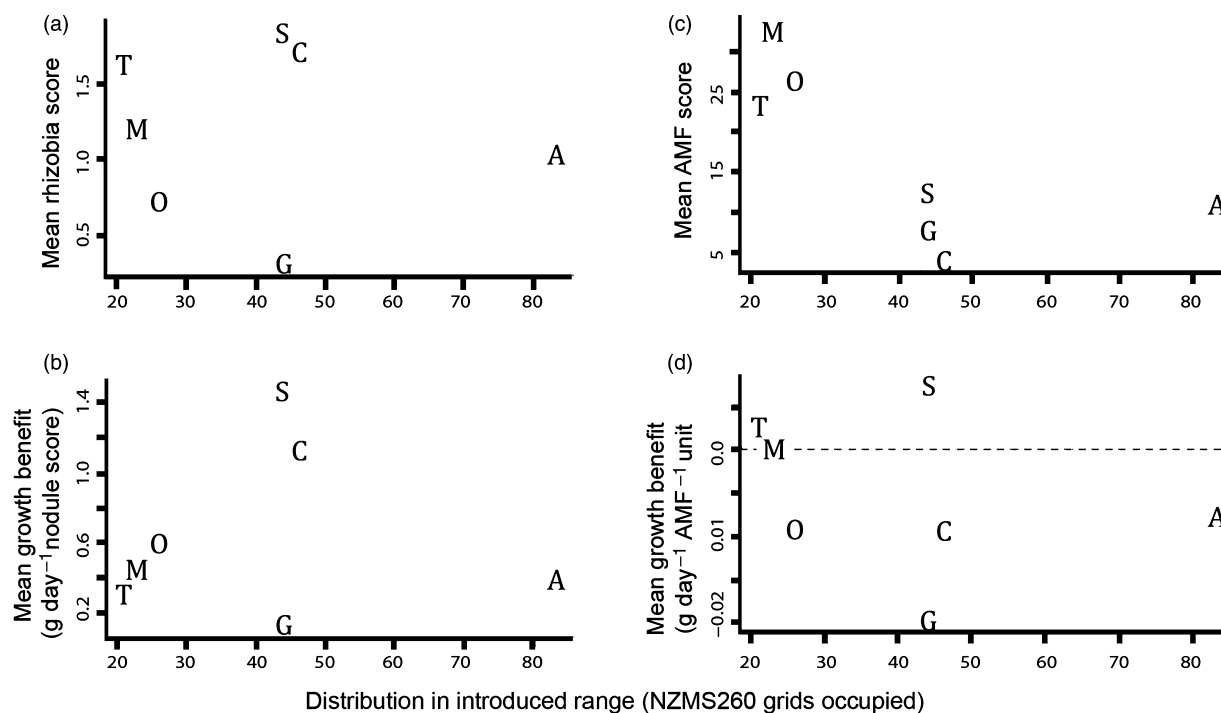
Fig. 4. Mean levels of colonization by rhizobia (a) and arbuscular mycorrhizal fungi (AMF) (b) and the mean levels of benefit for rhizobia (c) and AMF (d) for the New Zealand provenances of seven species of *Trifolium* as a function of the species geographic distribution in New Zealand (number of 10 km × 10 km NZMS260 grids occupied by at least one population). Means were extracted from the linear mixed-effects models; distribution data are from Gravuer (2004). All Pearson's correlation coefficients were < 0.3, and none were statistically significant (all *P* values > 0.1). Letters indicate species' names: *Trifolium arvense* (A); *T. campestre* (C); *T. glomeratum* (G); *T. micranthum* (M); *T. ornithopodioides* (O); *T. striatum* (S); *T. tomentosum* (T).

species' distributions in the introduced range. Although our ability to generalize is limited because seed was sampled from only one population of each species in each range, our results suggest that neither the availability nor the degree of benefit from associating with mutualists are factors affecting invasion success in these species. It is possible that the differences in these species' distributions in New Zealand can be explained by other factors, such as differences in their rate of introduction as seed contaminants (Gravuer *et al.* 2008) or differing degrees of escape from antagonistic biota in the soil, as the strength of enemy release has been shown to be stronger than the benefit of mutualists in the naturalization of another legume, *Robinia pseudoacacia* (Callaway *et al.* 2011). Another important consideration is that we may have omitted important variables in our models. Nitrogen and phosphorus levels of the collected soils could be unmeasured factors determining which mutualists were present (and therefore harvested) from those soils.

BIOGEOGRAPHICAL STUDIES OF RHIZOSPHERE MICROBIOTA

Several aspects of this work make it both strong and a logical extension of previous biogeographical studies. These include using multiple congeners, inoculating plants with rhizosphere microbial communities from both the native and invaded range and performing quantitative assessments of both AMF

colonization and rhizobia nodulation on the same plants. This combination of approaches is completely novel in the published literature. Although the limitations inherent in our study prevent us from making conclusions about specific interactions (e.g. we do not know whether the identities of the colonizing taxa differed by seed provenance), our work clearly shows that plant origin plays a role in how plants perform in response to rhizosphere microbial communities. We hope this work stimulates further examination into the interactions between plants and rhizosphere microbiota in the native and introduces ranges. An important next step in plant–rhizosphere research will be to interrogate these rhizosphere communities to see which taxa compose the rhizosphere microbiome in the native and introduced ranges to test whether there are taxonomic or functional trends related to plant communities and abiotic factors. Future designs might also consider collecting data on a broad suite of abiotic soil properties, including phosphorus and nitrogen levels, which might be relevant to rhizosphere community composition and enable statistical models to account for variation related to specific soil characters. This would enable designs to account for plant adaption to local conditions and 'maternal effects', and it could inform on abiotic correlates of rhizosphere communities. A further way to generalize findings and minimize maternal effects would be to incorporate seed from multiple locations within each range. Also, because the effect of a particular mutualist on plant performance will be intimately

related to the local environmental conditions, future studies should integrate both genotype and local conditions in their analyses.

THE SHIFTING MUTUALISM–PARASITISM CONTINUUM

Our study revealed a potential impetus for alien plants to alter their relationship with mutualists in the introduced range: in New Zealand soils, there was visual evidence of parasitic rhizobia and we observed a negative relationship between growth rate and degree of AMF colonization. The presence of parasitic strains could provide selective pressure for plants to maximize beneficial associations, be more selective to colonizers or reduce associations with soil mutualists overall. Although the incidence of parasitic rhizobia nodules was distributed evenly among provenances, the highest rates of failed nodulation (plants containing no rhizobia nodules) were among New Zealand provenances, suggesting plants may be reducing associations with rhizobia. Four of the five *T. glomeratum* plants from New Zealand grown in New Zealand soil lacked nodules, while *T. glomeratum* plants sourced from Spain and the UK were colonized by rhizobia from these same soils. Failed nodulation should theoretically limit plant performance, given rhizobia's substantial contribution to growth, yet *T. glomeratum* is the third most widespread non-agricultural *Trifolium* in New Zealand and the most common of the seven study species in our sampling region in New Zealand (Table 1). Similarly, among New Zealand provenances, we found a negative correlation between AMF colonization and growth benefit for more than half of the species when grown in New Zealand soils. Parasitism in AMF cannot be assessed visually, but the negative correlation between degree of colonization and growth rate suggests that strains of AMF associating with some introduced *Trifolium* in New Zealand are generally detrimental to plant growth. Our finding concurs with a recent meta-analysis of AMF interactions showing that absolute AMF colonization is similar between native and invasive plants, but the positive correlation between AMF colonization and growth response is generally absent among invasive plants, suggesting invaders are less responsive to mycorrhizas (Bunn, Ramsey & Lekberg 2015).

Understanding how relationships between plants and their soil mutualists differ between their introduced and native ranges could provide an important mechanism for why some plants fail to naturalize and others become invasive. The prevalence of parasitism is largely unknown even in agriculturally managed fields, let alone in natural communities or invaded systems (Johnson, Graham & Smith 1997; Denison & Kiers 2004a; Paszkowski 2006), and genetic similarity does not always reliably predict symbiotic effectiveness (Yates *et al.* 2008). For example, two OTUs with > 97% similarity (based on ribosomal RNA) can have genes driving substantially different ecological functions (Rout & Callaway 2012). Investigating how the mutualism–parasitism continuum may differ between native and introduced ranges could also reveal new patterns of adaptation among alien plant provenances and

the microbes they host. Rhizosphere mutualists such as AMF have been shown to alter plant morphology, allometry, phenology, the production of secondary metabolites, and fitness – including reallocation of reproductive strategies (Johnson, Graham & Smith 1997), suggesting great potential for plants to respond and potentially adapt to differences when they encounter different AMF communities outside their native ranges. It has been suggested that invasive plants frequently face a higher incidence of parasitism during naturalization (Thrall *et al.* 2007) but that the phenomenon goes unnoticed simply because it is masked by concurrently higher resource levels (Karst *et al.* 2008), enemy release (Joshi & Vrieling 2005) or reduced competition (van der Putten & Peters 1997). Alternatively, escape from parasitic co-evolved mutualists in the native range could constitute an unexplored form of enemy release for invasive plants, with concurrent potential adaptations in line with EICA predictions of resource reallocation. Indeed, higher rates of parasitism among rhizosphere biota could help to explain why plant–soil feedbacks are generally more negative in the native range (Beckstead & Parker 2003; Reinhart & Callaway 2004; van Grunsven *et al.* 2009). In summary, while rhizosphere mutualist absence or the presence of non-beneficial/parasitic strains can determine naturalization success or failure, such altered mutualisms can alternatively stimulate post-naturalization shifts in physiology that may result in physiological compensations or adaptations.

Conclusion

This study tested whether native and introduced plant provenances differ in performance and mutualist association and whether the benefit of mutualist association could be a predictor for invasion success. Our work shows that plant origin plays a role in how plants perform in response to rhizosphere microbial communities. We found some evidence that introduced provenances have reduced mutualist association with rhizobia, but provenances from both ranges grew better with rhizobia from their own range, suggesting local adaptation instead of post-naturalization differences. Despite evidence for negative associations with two key mutualists in the introduced range, the lack of correlation between the extent of species' distributions and either mutualist colonization or calculated mutualist benefit suggests neither are predictive of invasion success in these species.

Acknowledgements

Data accessibility

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.j9390 (Shelby *et al.* 2016).

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Supporting Information

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

Figure S1. Mean levels of colonisation by mutualists in each soil type.

Figure S2. Species distribution plotted as a factor of naturalisation date in NZ.

Table S1. Geographic locations of the seed used in the experiments.

Table S2. Geographic locations of the soil used in the experiments.

Table S3. Scoring key for rhizobia nodulation.

Table S4. Experimental replicates for the experiments.

Table S5. Soil properties for background soils used in glasshouse experiments.