Range-expanding plant species and their belowground neighbours – digging into novel interactions

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Chapter 1

General introduction

Natural ecosystems are increasingly affected by human activities (Rockström *et al.* 2009). In addition to major anthropogenic pressures such as land use change, pollution, hunting, and fishing, organisms in these ecosystems also have to cope with human-induced climate change (Walther *et al.* 2002; Parmesan & Yohe 2003; Parmesan 2006; Poloczanska *et al.* 2013). Due to, among other factors, rising emission levels of greenhouse gasses and feedbacks between increasing temperatures and greenhouse gas emissions from natural ecosystems (Crowther *et al.* 2016), global temperatures might increase as much as 3^0C by 2100 (Rogelj *et al.* 2016). These rapid changes in global climate are unprecedented in recent evolutionary history. Understanding how natural communities respond to direct and indirect consequences of human-induced climate change therefore has become one of the major research challenges in contemporary ecology (Lavergne *et al.* 2010).

Ecological responses to climate change

Local responses: adaptation & ecological mismatches

To cope with climate change and to avoid local extinction, species have to become adapted to the new local conditions (Jump & Peñuelas 2005; Berg *et al.* 2010). Because of the speed of climate change, adaptation to these new conditions often fails. This can have disastrous consequences for ecosystem function, such as the bleaching of coral reefs (Hoegh-Guldberg *et al.* 2007). Local species persistence is especially difficult when climate warming disrupts key interactions between species. For example, many plant and insect species can show rapid responses to changing temperature regimes, e.g. by advancing their phenology by means of plastic responses to temperature cues (Menzel *et al.* 2006), while other groups of species cannot.

When the offsets of life history events are determined by different environmental cues, long-established interactions between different organisms may become disrupted or imbalanced because of climate change (Visser & Both 2005). For example, due to increasing spring temperatures, the peak of plant growth in the Arctic tundra has advanced, and no longer corresponds with the timing of calve birth of Caribou (*Rangifer tarandus*), as their reproduction is timed in response to day length. This trophic mismatch has resulted in a decline of Caribou numbers (Post & Forchhammer 2008). Similarly, in temperate forests, the timing of reproduction of migratory and non-migratory songbirds

is increasingly disconnected from the peak of moth caterpillar availability for bird chicks (Both *et al.* 2006; Reed *et al.* 2013). Additionally, climate warming can affect host-disease interactions, with potentially disastrous effects. In the Neotropics, the extinction of numerous amphibians has been related to increasingly severe outbreaks of the fungal pathogen *Batrachochytrium dendrobatidis,* that thrives under hot conditions (Pounds *et al.* 2006).

Range boundaries on the move: retractions and expansions

The failure and success to adapt to the new local abiotic and biotic conditions caused by climate change may affect the spatial distribution of species. Under this assumption many plant species have been predicted to go extinct or to strongly decline in distribution, as their niche will no longer be present in places where the species previously occurred (Thomas *et al.* 2004; Thuiller *et al.* 2005). Indeed, range retractions have been reported for many species. For example, several butterfly species are in decline at the southern edge of their original range (Franco *et al.* 2006). In mountains, many plant species are not anymore found at the lower altitudes of their original range (Rumpf *et al.* 2018). In contrast, climate warming also creates opportunities for range expansion, as plant and animal species favouring warm conditions may colonize areas that previously were too cold. For example, in Europe, bird species such as the Great egret, previously associated with Mediterranean areas, have been expanding their range northwards and are now successfully reproducing in north-western Europe (Ławicki 2014). Similarly, many south-European invertebrate species (e.g. Wasp spider and Scarlet dragonfly) show northward range expansions in Europe (Ott 2001; Krehenwinkel & Tautz 2013). Moreover, many plant species have been expanding their range, both to higher latitudes and altitudes (Chen *et al.* 2011).

Globally, tree lines in mountain areas are elevated, and also boreal forests are extending polewards (Gehrig-Fasel, Guisan & Zimmermann 2007; Kelly & Goulden 2008). In North-western Europe, dozens of South-European plant species, e.g. *Dittrichia graveolens, Rorippa austriaca* and *Tragopogon dubius,* have become established or are expanding their range (Tamis *et al.* 2005; van Grunsven *et al.* 2010; Macel *et al.* 2017; Lustenhouwer *et al.* 2018). Also urbanization may facilitate the establishment and expansion of plant species that previously occurred at lower latitudes as urbanized areas are often

relatively warm and stony. For example, in The Netherlands several Mediterranean herb and grass species, such as *Polycarpon tetraphyllum*, *Catapodium rigidum* and *Eragrostis minor,* are almost exclusively found in cities (NDFF 2017), whereas *Dittrichia graveolens* expands its range along highway networks (Lustenhouwer *et al.* 2018). Thus, while climate change has caused extinctions and range retractions of species associated with cold conditions, it has facilitated the range expansion of many species associated with warm conditions.

Variation in rate of range expansion

Although range expansions have been documented for many plant and animal species, the consequences for species interactions and community dynamics are still largely unexplored (Lavergne *et al.* 2010; van der Putten 2012). With range expansion, species interactions could change when associated species are not expanding their range at the same rate, due to varying dispersal abilities (Morriën *et al.* 2010; van der Putten 2012). Importantly, aboveground organisms are expected to disperse faster than belowground organisms (Berg *et al.* 2010). Therefore, especially the interactions between plants and the associated soil organisms from their original range may become disrupted. **In this thesis I explore the consequences of climate change-driven range expansion for interactions between plants and plant-associated soil organisms.**

Plant-soil interactions

Rhizosphere communities

Plants directly and indirectly interact with a wide variety of soil organisms such as bacteria, fungi, protists, nematodes, micro-arthropods and other invertebrates (De Deyn & Van der Putten 2005). The majority of these interactions take place in the rhizosphere, which is the interface of plant roots and the surrounding soil (Philippot *et al.* 2013). Here, plants exude volatile and non-volatile compounds, which in turn directly and indirectly attract soil organisms that together form the rhizosphere community (van Dam & Bouwmeester 2016; Venturi & Keel 2016; Schulz-Bohm *et al.* 2017). As plant species vary in some of the chemical compounds they produce, they also attract and stimulate the growth of different soil organisms, thereby accumulating species-specific rhizosphere communities. Next to differences in root chemistry, variation in root architecture also explains part of these species-

specific rhizosphere communities. For example, plant species with thick roots are more densely colonized by arbuscular mycorrhizal fungi than plant species with thin roots (Cortois *et al.* 2016; Ma *et al.* 2018). Thus, by variation in chemical and structural root traits each plant species shapes a more or less unique rhizosphere community.

Plant-soil feedbacks

Organisms in the rhizosphere have varying functions and may either be beneficial, neutral or harmful for the plant (Bardgett & van der Putten 2014). Roughly, harmful organisms include bacterial, protist and fungal plant pathogens, root-feeding nematodes, and insect larvae, while the group of beneficial organisms mostly consists of arbuscular-mycorrhizal fungi, growth-promoting rhizobacteria and organisms that suppress plant enemies (Berendsen, Pieterse & Bakker 2012; Bardgett & van der Putten 2014). Importantly, the balance between harmful and beneficial organisms in attracted rhizosphere communities can differ between plant species, with important consequences for future generations of plants (Bever, Westover & Antonovics 1997; van der Putten *et al.* 2016). New generations of plants are expected to perform poorly when their conspecific predecessors accumulated considerable amounts of harmful organisms. Alternatively, plants may perform well when especially mutualists are accumulating. Such 'plant-soil feedbacks' (Bever, Westover & Antonovics 1997) are important for the dynamics of plant communities, as poor performance of plant species in soils conditioned by conspecifics allows the establishment of plant species that are either better defended against these harmful organisms (van der Putten, Van Dijk & Peters 1993; Mills & Bever 1998; Kardol, Bezemer & van der Putten 2006). Thus, via the conditioning of soil communities plant species persistence at a micro-scale indirectly may be either promoted or resisted.

Soil nematodes and their functions

The primary group of soil organisms studied in the present thesis are soil nematodes. Nematodes are the most abundant soil animals (Bardgett & van der Putten 2014) and, as a result of fundamental diversification in food sources, occupy a variety of positions in the soil food web (Yeates *et al.* 1993). In addition to root-feeding nematodes, there are bacterivorous and fungivorous nematodes, predatory nematodes that feed on other nematodes or unicellular organisms, and omnivorous nematodes which

feed on multiple groups of organisms (Yeates *et al.* 1993). Because of this wide array of feeding types and the relatively straightforward methods to morphologically quantify and identify nematodes to the level of feeding type and family, nematodes are often used as indicators of soil quality and of the complexity of the soil food web. For example, high numbers of bacterivores indicate a disturbed bacteria-dominated soil, whereas high numbers of predatory and omnivorous nematodes indicate matured, stable soils with a complex soil community (Bongers 1990; Bongers & Ferris 1999).

Root-feeding nematodes

Root-feeding nematodes are especially known as important agricultural pests, as they reduce global food production with an annual damage estimated at 80 billion USD (Nicol *et al.* 2011; Jones *et al.* 2013). Less known is their important function in natural systems, where they play a role in plant-soil feedback interactions that may contribute to the natural succession of vegetation (van der Putten, Van Dijk & Peters 1993; De Deyn *et al.* 2003). Within the group of root-feeding nematodes there is considerable variation in feeding mode, and generally five main feeding types are recognized (Yeates *et al.* 1993). Root-hair feeders, of which most members belong to the family Tylenchidae, have not been shown to be detrimental to plant performance and have received little attention in scientific literature (Bongers 1988). All other groups of root-feeding nematodes contain species that can be detrimental for plant performance (Decraemer *et al.* 2006). Ectoparasites, which pierce plant roots with their stylets, contain several different nematode genera and families, such as *Paratylenchus*, Criconematidae and Dolichodoridae. Semi-endoparasites, primarily species of the genera *Helicotylenchus* and *Rotylenchus*, partly enter the roots and feed on cells on the inside of the roots (Yeates *et al.* 1993; Decraemer *et al.* 2006). Migratory endoparasites, such as the root lesion nematodes *Pratylenchus*, can enter the roots of multiple plants, whereas sedentary endoparasites such as *Meloidogyne* are known to create galls in the roots, thereby disturbing plant growth (Decraemer *et al.* 2006).

Plant defences against root-feeding nematodes

Studies on root-feeding nematodes that occur as agricultural pests have shown that many nematode species may have broad host ranges, but also that there can be strong variation in performance on different host plants (Wood 1973; Santo *et al.* 1980; Starr 1991; Decraemer *et al.* 2006). This indicates that plant species vary in their defence mechanisms against root-feeding nematodes. The production of certain secondary chemicals is considered to be an important bottom-up defence mechanism against root-feeding nematodes (Halbrendt 1996; Potter *et al.* 1999; Soriano *et al.* 2004), suggesting that variation in root chemistry drives host suitability for root-feeding nematodes. In addition to their direct, chemical defence mechanisms, plants possibly also indirectly defend themselves against root-feeding nematodes via the attraction of soil organisms that parasitize nematodes. Such soil-borne nematode-antagonistic organisms include species of protists, fungi and bacteria (Kerry 2000; Piskiewicz *et al.* 2007; Geisen *et al.* 2015). Plants have been shown to attract other beneficial microbes over long distances (Schulz-Bohm *et al.* 2017) and there is also evidence on plant-mediated top-down control of larger root herbivores (Rasmann *et al.* 2005; Turlings, Hiltpold & Rasmann 2012). Therefore, the existence of similar plant-mediated top-down control mechanisms against root-feeding nematodes is not unlikely, although this possibility remains to be tested.

Root-feeding nematodes as model system to test belowground consequences of plant range expansion

Because of the assumed negative effects of root-feeding nematodes on natural plant performance (van der Stoel, van der Putten & Duyts 2002; De Deyn *et al.* 2003), it is important to examine whether plant-nematode interactions change between original and new plant ranges. Theoretically, there are two possible causes of such changes in plant-nematode interactions (Fig. 1.1.). First, nematode species present in the original range of the range-expanding plant species may not be present in the new range. So far, studies have shown that there is geographic and latitudinal variation in nematode community composition which has partly been related to variation in climate (Nielsen *et al.* 2014; Song *et al.* 2017). Although these studies did not examine rhizosphere communities, such latitudinal variation suggests that range-expanding plants indeed may face partly different nematode communities in their new range, provided that they outrun their native nematode community (Berg et al. 2010). Second, even if

the composition of nematode communities in general and root-feeding nematode communities in particular is comparable between the original and the new range, nematodes may be locally adapted to the present community of plants and other organisms. Because gene flow between nematode populations is limited due to the relatively poor dispersal capacities of nematodes (Blouin, Liu & Berry 1999), local adaption of root-feeding nematode populations to native plants seems very well possible but remains untested. As nematodes are relatively well identifiable and quantifiable, and because they can be cultured and used for experimentation under lab conditions, nematodes are considered to be useful for testing proposed effects (Berg et al. 2010, van der Putten 2012) of plant range expansions on plant-soil interactions.

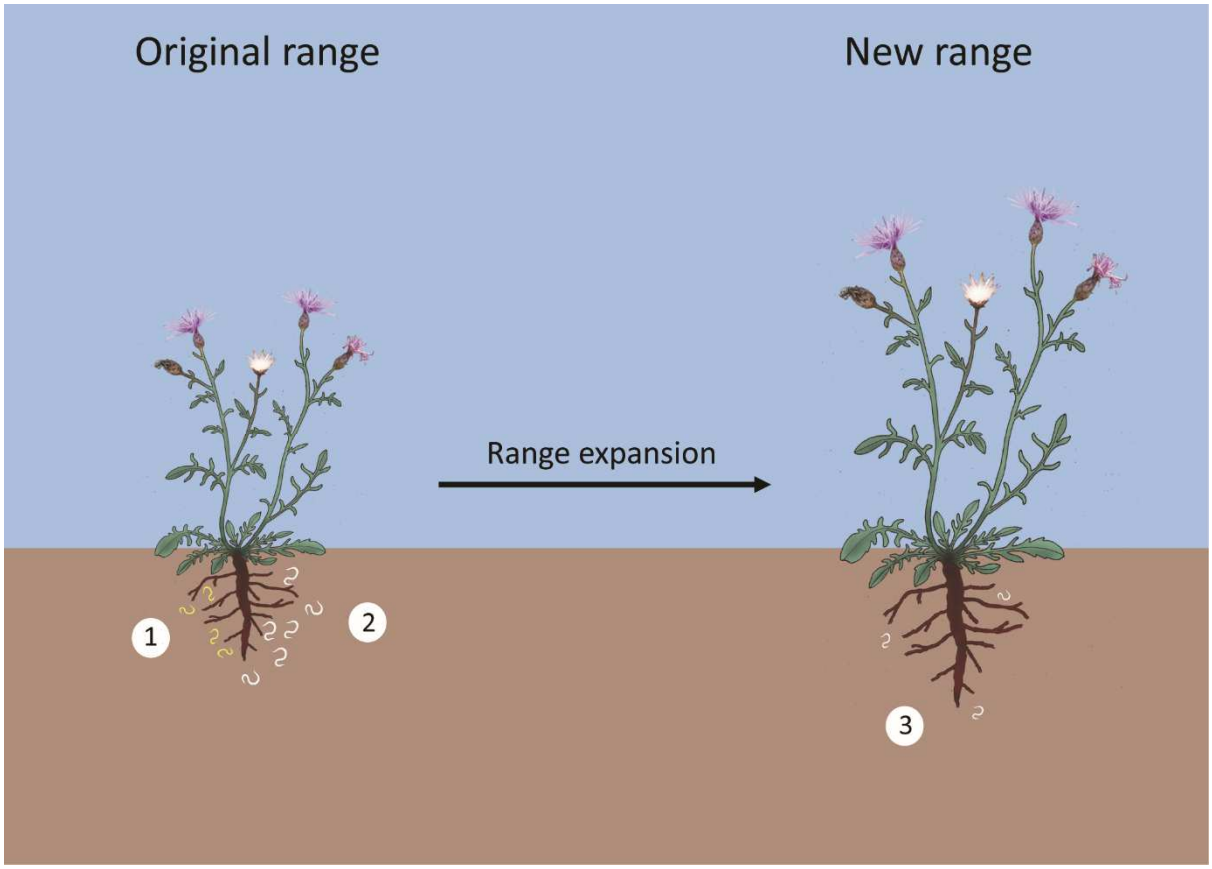

Fig. 1.1 Conceptual overview of changes in the interactions between range-expanding plant species and root-feeding nematodes that may occur due to range expansion. In their original range, range-expanding plant species may be affected by specialized root-feeding nematodes (1) and generalist root-feeding nematodes (2). In the new range, specialized root-feeding nematodes are not likely to be present, whereas generalist root-feeding nematodes may peform poorly on range-expanders due to their local adaptation to plant species in the native community (3). These changes in plant-nematode interactions may benefit range-expanding plant species in their new range.

Lessons from exotic species: losing old, and gaining new belowground interactions

Enemy release

The possibility of disrupted plant-soil interactions has been extensively studied with respect to a different ecological phenomenon in the Anthropocene: the intentional or unintentional introduction of exotic plant species to continents where they previously did not occur (van Kleunen *et al.* 2015). A considerable number of these exotic plant species show invasiveness in their new range: they have become disproportionally abundant after introduction, thereby negatively affecting native biodiversity (Hejda, Pyšek & Jarošík 2009; Powell, Chase & Knight 2013). One of the major hypotheses that has been tested to explain this invasiveness is the loss of co-evolved specialist natural enemies from the new range (Enemy release hypothesis; Keane & Crawley 2002). Indeed, experiments using soils from both the original and the new ranges of exotic plant species have shown that in soils from their new range, exotic plant species are less negatively affected by soil biota (Reinhart *et al.* 2003; Callaway *et al.* 2004) than in soils from their original range. Moreover, pathogens from the original range have been shown to be absent in the new range (Reinhart *et al.* 2003; Blumenthal *et al.* 2009; Reinhart *et al.* 2010). These results indeed suggest that enemy release contributes to the disproportional abundance and invasiveness of some introduced exotic plant species in their non-native range.

Establishment of novel interactions and the role of novel chemistry

Non-native plant species can only strongly benefit from the release of their co-evolved natural enemies when natural enemies in the new range do not affect them as strongly as they affect their native host plant species. The novel interactions established between exotic plant species and non-coevolved native natural enemies therefore play a crucial role in the potential success of exotic plant species in their new range (Verhoeven *et al.* 2009). Whether a plant species will be affected by native natural enemies has been proposed to depend on its ecological similarity to plant species already present in the community (Elton 1958). Non-native plant species that have chemical compounds and defensive strategies similar to native plant species present in the communities in the new range are likely to be similarly good hosts for native pathogens and herbivores, allowing host switching of natural enemies (Parker & Gilbert 2004). These spill-over events are especially likely to happen when non-native plant

species are closely related to members of the native plant community. For example, exotic plant species closely related to native plant species may be negatively affected by soil communities accumulated by these close relatives in their new range (Callaway *et al.* 2013). Contrastingly, when non-native plant species possess defence traits not possessed by members of the native community, they may strongly impact the native community in various ways. Most directly, the production of 'novel defence chemicals' may benefit the non-native plant species when these chemicals deter native herbivores and pathogens. (Schaffner *et al.* 2011; Macel *et al.* 2014). Moreover, the exudation of novel root metabolites may directly or indirectly affect native plant species by allelopathic effects on neighbouring plants or the symbionts of these plants (Callaway & Aschehoug 2000; Stinson *et al.* 2006; Callaway *et al.* 2008). As non-native plant species that are distantly related to the native community are likely to possess the most strongly dissimilar defence traits (Gilbert & Parker 2016), this may explain why on average this group of non-natives is most prone to become invasive (Strauss, Webb & Salamin 2006). However, the chance to successfully establish in a new plant community is lower for distantly related species than for closely related species, as the latter likely are more strongly pre-adapted to the local conditions (Park & Potter 2013; Bezeng *et al.* 2015).

Evolutionary responses to novel biotic conditions – shifting defences

In addition to their novel defence chemicals, non-native plant species may also acquire a superior defence against local herbivores and pathogens due to an evolutionary process following the invasion or range expansion process. The costly defence mechanisms used against specialists are no longer necessary when non-native plant species are liberated from co-evolved specialist natural enemies from their original range. Instead, genotypes that invest more strongly in defence mechanisms against generalist enemies will benefit from the selective pressure against individuals that also invest in defences against specialists (Doorduin & Vrieling 2011; Lin, Klinkhamer & Vrieling 2015). Additionally, as toxic chemicals that defend plants against generalists are expected to be relatively cheap for the plant, non-native plant species may have more resources left to invest in growth than native plants that require costly defences against both specialist and generalist herbivores (Joshi & Vrieling 2005). This may lead to 'evolution of increased competitive ability' (Blossey & Notzold 1995), which is another possible key mechanism explaining the success of some plant species in their non-

native range. However, evidence for this hypothesis is still scarce (Bossdorf 2013; Uesugi & Kessler 2013). Contrary to populations of intercontinentally introduced exotic species, which are genetically isolated from their core population in the native range, populations of intra-continental range-expanders will likely experience continued gene flow from the original range. Due to this continued gene flow, populations in the expanded range may not be able to show strong evolutionary responses in response to their novel environment (Kirkpatrick & Barton 1997). However, recent studies showing natural selection during intracontinental range expansion (Macel *et al.* 2017; Lustenhouwer *et al.* 2018) indicate that evolutionary responses should be considered when examining the novel interactions of climate-driven range-expanding plant species.

State of the art: plant-soil interactions of intracontinental range-expanders

The same mechanisms that may benefit introduced exotic plant species in their novel environments, usually on other continents, might also benefit intra-continental range-expanders in their new range. One of the key questions in current studies on climate-driven range expansion is whether range-expanding plant species can become released from natural enemies when they establish in areas of higher latitude or altitude (van der Putten 2012). Such a disruption of plant-enemy interactions due to intra-continental range expansion may not be as strong when compared to intercontinental introductions, as some natural enemies of range-expanders may have wider distributions and will therefore already be present at higher latitudes (Menendez *et al.* 2008). Yet, an increasing number of studies show that some range-expanding plant species are less affected by soil communities in their new range compared to their original range, suggesting a degree of enemy release (van Grunsven *et al.* 2010; De Frenne *et al.* 2014; Dostálek *et al.* 2015; Van Nuland, Bailey & Schweitzer 2017). However, comparisons between the compositions of soil communities from the original and new range of range-expanding plant species so far have rarely been made (Van Nuland, Bailey & Schweitzer 2017). Nevertheless, within their new range, the few studies published thus far have shown that range-expanding plant species on average accumulate fewer pathogens and root-feeding nematodes, and are less negatively affected by soil communities than related native plant species (van Grunsven *et al.* 2007; Engelkes *et al.* 2008; Morriën, Duyts & Van der Putten 2012; Morriën & van der Putten 2013). Altogether, these studies suggest that intra-continental range-expanders can benefit from either

specialist enemy release or a superior defence against native natural enemies, or a combination of these mechanisms (Fig. 1.1). Currently, the generality of these processes and the actual mechanisms underlying them remains to be established.

Research aim and thesis outline

To predict the potential impact of range-expanding plant species on native communities, it is important to understand how their plant-soil interactions differ from native plant species and how these interactions differ between the original and new range. Therefore, in this thesis, I study the shifts in belowground communities between the original and new range of range-expanding plant species. Moreover, I examine whether there are fundamental differences in plant-soil interactions between range-expanders and plant species that are native in both the original and new range of the range-expanders. I especially focus on the interactions between plants and root-feeding nematodes, but also consider other groups of soil organisms, such as protists and other microbes that might act as natural enemies of the nematodes. I aim to relate differences in plant-soil interactions between range-expanders and native plant species to variation in chemical and morphological root traits, and test whether plant phylogeny can be used as a predictor of these differences in plant-soil interactions.

In Chapter 2 I study nematode communities in the rhizospheres of range-expanding and congeneric native plant species along a latitudinal transect from south-eastern Europe (the original range of the range-expanders studied in this thesis) to north-western Europe (the new range of the range-expanders). In this way, I examine whether latitudinal changes in rhizosphere nematode communities under range-expanders are stronger than under congeneric plant species that are native along this entire latitudinal transect.

To experimentally test whether range-expanding plant species differently affect nematode communities in their new range compared to their old range, I performed an experiment with plant communities of either native or range-expanding plant species on soil from either northern or southern Europe (Chapter 3). Here, I also test whether nematode communities show stronger differences between northern and southern soils when they are conditioned by range-expanding plant

species that lack phylogenetically close relatives in the new range, compared to when they are conditioned by range-expanders that do have phylogenetically close relatives in the new range.

In Chapters 4 and 5, I examine the differences in plant-nematode interactions within different pairs of range-expanding and related native plant species in more detail.

In a greenhouse experiment with multiple belowground trophic levels, I examine whether top-down control (by nematode-antagonistic microbes) and bottom-up control (by plant defences) of root-feeding nematodes differs between range-expanders and natives (Chapter 4). I test the hypotheses that root-feeding nematodes perform more poorly on range-expanders than on natives, but are also less affected by nematode-antagonists as these may be less successfully attracted by range-expanders than by natives.

To gain a more mechanistic understanding of possible bottom-up control of root-feeding nematodes, I examine how root-feeding nematode attraction and performance differs between range-expanding and native plant species and how these patterns relate to differences in plant chemistry between the plant species (Chapter 5).

In the final experiments of this thesis (Chapter 6) I studied effects of plant origin and phylogenetic distance on plant-soil interactions using multiple range-expanding and native plant species that all belong to the same genus. This approach was aimed at answering the question whether nematode reproduction, rhizosphere community composition and plant-soil feedback are phylogenetically determined or explained by plant origin. Additionally, I analysed root traits, such as root chemistry, in order to obtain a mechanistic understanding of the observed plant-soil interaction patterns in this part of my study.

In Chapter 7 I discuss the findings of the different data chapters in relation to the overall research questions and aims of my thesis. I will also discuss linkages between my research on range expansions and the research fields of invasion ecology and plant-soil interaction ecology. Finally, I will propose future research directions.

Chapter 2

Soil nematode community composition and climate warming-induced plant range expansions along a latitudinal transect

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Manuscript

Abstract

Climate change affects the distribution of organisms by enabling latitudinal and altitudinal range expansions. Plant species that expand their range to higher latitude regions have been predicted to become released from belowground natural enemies. However, those predictions have not yet been tested. Here, we combine novel molecular and classical identification methods in order to examine nematode communities in the rhizospheres of four range-expanding and four congeneric native species along a 2000 km transect from Mediterranean to North-Western Europe. Nematode communities consist of functionally diverse species, including important belowground herbivores, bacterivores and fungivores. We tested the hypotheses that 1) nematode communities show a consistent change with increasing latitude, 2) range-expanding plant species experience stronger latitudinal shifts in nematode community composition than related natives and 3) range-expanding plant species accumulate fewer root-feeding nematodes in their new than in their original range. Our results indeed show latitudinal variation in nematode community composition, indicating that range-expanding plant species face different nematode communities when arriving in a novel habitat. However, only one of the examined range-expanding plant species, *Centaurea stoebe*, experienced stronger nematode community shifts than its congeneric native and was partly released from root-feeding nematodes in its new range. All other range-expanding plant species did not experience stronger shifts in nematode community composition, and accumulated comparable root-feeding nematode numbers in their new compared to their original range. We conclude that while nematode communities change with latitude, the release of root-feeding nematodes may not be a general mechanism during climate warming-driven plant range expansion.

Introduction

Anthropogenic climate change directly and indirectly affects natural communities by impacts on the phenology and performance of organisms (Parmesan 2006). To avoid local extinction, species either need to adapt to the novel local conditions, or shift their range towards previously unsuitable areas (Berg *et al.* 2010). As a consequence, many organisms have been recorded to expand their range to higher latitudes or altitudes (Parmesan & Yohe 2003; Rumpf *et al.* 2018; Steinbauer *et al.* 2018).

However, expansion rates are not uniform across organismal groups. Plants, for instance, are suggested to expand at a faster rate than their intimately connected rhizosphere communities (Berg *et al.* 2010). Among those rhizosphere organisms are mutualists and antagonists that can affect plant performance and vegetation dynamics (Kardol, Bezemer & van der Putten 2006; Van Der Heijden, Bardgett & Van Straalen 2008). Through plant range-expansions, interactions between plants and specific rhizosphere communities can be disrupted with functional consequences for plant performance in the new range (Morriën *et al.* 2010; van der Putten 2012). Indeed, several range-expanding plant species seem to be less negatively affected by soil communities in their new than in their original range, suggesting that range expansion enables release from natural enemies from the original range (van Grunsven *et al.* 2010; De Frenne *et al.* 2014; Dostálek *et al.* 2015; Van Nuland, Bailey & Schweitzer 2017).

The actual biotic players that drive plant performance belowground are diverse and include bacteria, archaea, fungi, protists, nematodes and larger animals. Particularly nematodes include functionally diverse taxa including bacterivores, fungivores, root-feeders, omnivores and predators that interact with other members of the soil food web (Yeates *et al.* 1993; de Ruiter, Neutel & Moore 1995). A particular study focus has been on root-feeding nematodes due to their prevalent role as agricultural pests. Similarly, a release from root-feeding nematodes has been suggested as an underlying driver of successful plant range-expansion (Engelkes *et al.* 2008; Morriën, Duyts & Van der Putten 2012). However, shifts in soil nematode community composition along the plant range expansion transects have not been examined yet.

Survey-based sampling, such as transects, can be useful to explore biogeographic patterns of the community structure of soil biota and investigate dependences of organisms to (a)biotic conditions (Tedersoo *et al.* 2014; Thompson *et al.* 2017; Delgado-Baquerizo *et al.* 2018). Sampling along transects has shown that soil bacteria are mainly influenced by pH, and communities of fungi and protists by a combination of moisture and other abiotic factors (Bates *et al.* 2013; Tedersoo *et al.* 2014; Delgado-Baquerizo *et al.* 2018). For nematodes, survey-based approaches have been rarely performed due to methodological constraints, as the time-consuming and expert-dependent morphological identification still dominates nematode community analyses over molecular methods (Geisen *et al.*

2018; Griffiths *et al.* 2018). The few survey- based studies as well as global meta-analyses have shown that climate, vegetation and soil abiotic conditions determine nematode community compositions (Nielsen *et al.* 2014; Sylvain *et al.* 2014; Chen *et al.* 2015; Song *et al.* 2017). However, all these studies have used a limited number of samples, low taxonomic resolution, or different methodology to investigate soil nematode communities.

Here we used a combination of morphological and molecular techniques to perform high-resolution analyses of soil nematode communities along a latitudinal transect of plant range expansion in Europe. Next to the composition of nematode communities, we quantified the abundances of nematode feeding types and different root-feeding nematode groups, which differ in feeding mode and their effects on plant performance (Bongers 1988; Yeates *et al.* 1993). Across a latitudinal gradient of approximately 2000 km long, including six countries in Europe, we collected 356 independent rhizosphere nematode community samples of four range-expanding plant species in their new range in north-western Europe and their original range in south-eastern and central Europe. To disentangle range expansion effects from latitudinal variation in nematode community composition we also collected nematode communities from four congeneric plant species that are native along this latitudinal gradient. We tested the hypotheses that 1) there is an overall latitudinal gradient in nematode community composition, 2) along the latitudinal transect, range-expanding plant species would experience stronger nematode community shifts than related native plant species and 3) root-feeding nematodes in the rhizospheres of range-expanding plant species are less abundant in their new that in their original range.

Methods

Plant species

We sampled nematode communities from four plant species that are expanding their range and four congeneric species that are native in both the original and new range of the range-expanders. These 'climate-driven range-expanders' naturally occur in southern and/or central Europe and have recently expanded their range into north-western Europe (NDFF 2017). The congeneric native plant species are native in southern, central and northern Europe. The range-expanders in our study were

Centaurea stoebe (Asteraceae), *Tragopogon dubius* (Asteraceae), *Geranium pyrenaicum* (Geraniaceae) and *Rorippa austriaca* (Brassicaceae). Congeneric native plant species were *Centaurea jacea*, *Tragopogon pratensis*, *Geranium molle* and *Rorippa sylvestris*, respectively. Such a comparison between range-expanders with congeneric natives is aimed at minimizing the possibility that findings may be obfuscated by e.g. differences in phylogenetic position (Agrawal *et al.* 2005; Engelkes *et al.* 2008). In north-western Europe, all plant species occur in the same riverine ecosystem, although specific habitat requirements may differ.

Field sampling

In the growing seasons of 2013 and 2014 we collected soil around the roots of flowering individuals of all eight plant species along a latitudinal transect from south-eastern to north-western Europe, including Greece, Montenegro, Slovenia, Austria, Germany and The Netherlands. In each country, we aimed to sample 9 individual plants: 3 individuals from one sampling area and three different sampling areas. As *Centaurea stoebe* and *Rorippa austriaca* do not occur in Greece and Montenegro, these species were only sampled from Slovenia northwards. Samples from all other plant species were collected from each country mentioned above. After collection, soils were stored in transportable coolers and, as soon as logistically possible, at 4˚C until nematode extraction. Additionally, for all 2014 samples, we measured soil pH, C/N ratio, soil content of plant available NH_4^+, NO_2^- + NO_3^- and phosphate (P_{olsen}).

Nematode extraction

Before nematode extraction, stones and other large particles were removed from the collected soils, after which we used approximately 100 g of soil for nematode extraction. A separate soil sample of roughly 10 g was used for soil chemical analyses and to determine soil moisture. Nematodes were extracted from a weighed amount of soil using Oostenbrink elutriators (Oostenbrink 1960). Suspensions (10 ml) with extracted nematodes were divided into two halves: 50% of each sample was used for DNA-extraction and amplicon sequencing (see below) while the other 50% was used for nematode quantification. Before nematode counting, these suspensions were concentrated to 2 ml, after which 4 ml hot (90˚ C) and 4 ml cold (20˚ C) formaldehyde was added to heat-kill and fixate the

nematodes. Nematodes were counted using an inverse-light microscope (200x; Olympus CK40) and nematode numbers were expressed per 100 g dry soil.

DNA extraction and amplicon sequencing

DNA from the other subsample was extracted using the Clear Detections Nematode DNA extraction and purification kit™ (Clear Detections, Wageningen, Netherlands). DNA isolates were stored at -20 °C until further use. To obtain taxonomic information on the complete soil nematode community we amplified the most variable part of the 18S rDNA, the V4 region (Pawlowski *et al.* 2012) using the universal eukaryotic primers 3NDf together with 1132rmod as previously described (Geisen *et al.* 2018). For all primers we used pre-tagged primers with Illumina adapters, a 12 bp long barcode to allow demultiplexing of the reads after sequencing, a primer linker and the sequencing primers. All PCRs were conducted in duplicate, product quality was visually verified on agarose gel and duplicates were pooled before PCR cleanup with Agencourt AMPure XP magnetic beads (Beckman Coulter). PCR cycling conditions were as follows: initiation for 5 min at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 1 min at 53 °C and 90 sec at 72° with a final elongation for 10 min at 72 °C). PCR-products were pooled in equimolar ratios after determining concentrations with a fragment analyser (Advanced Analytical) and sent for sequencing to BGI, China.

Bioinformatics

The obtained raw 18S rDNA sequence reads were curated in the Hydra pipeline (de Hollander 2017) implemented in Snakemake (Köster & Rahmann 2012); in short, after filtering contaminants and removing barcodes, the forward reads were used for annotation. Thereafter, vsearch (Rognes *et al.* 2016) was used to cluster all reads into OTUs using the UPARSE strategy by de-replication followed by sequence-sorting by abundance (singletons were removed) and clustering using the UCLUST smallmem algorithm (Edgar 2010). Chimeric sequences were removed using UCHIME (Edgar *et al.* 2011), as implemented in vsearch. To create an OTU table, all reads were mapped to OTUs using the usearch_global method (vsearch). Sequences were aligned to the PR2 database (Guillou *et al.* 2013). Reference sequences were first trimmed with forward and reverse primer using cutadapt (Martin 2011). Moreover, we deleted all reference sequences of environmental nematode DNA, to improve

annotation success. Prior to further analyses, we removed samples with fewer than 1000 reads. We then recalculated read numbers to relative abundances of the OTU's. OTUs that could be assigned to nematode genera allowed estimates of relative abundances of functional groups (Yeates *et al.* 1993).

Statistical analyses

For both multivariate and univariate analyses, nematode communities collected under individual plants were treated as independent replicates. We decided not to include sampling area as a random factor in our models, as within-sampling area variation was variable among the sampling areas, due to strong differences in distance between collected plants.

Multivariate analyses of nematode community composition: Prior to multivariate analyses, we assembled two databases, one containing relative abundance data of nematode OTU's and one with relative abundance data of nematode genera. All multivariate analyses were performed in Canoco 5 (Ter Braak & Smilauer 2012; Šmilauer & Lepš 2014), and all analyses were performed for both the OTU-level and genus-level datasets. With all samples collected in 2014, for which soil characteristics were measured, we first ran forward selection RDA's to estimate the importance of the nominal factor plant species and the continuous factors latitude, soil moisture, pH, soil C/N, NH_4^+, NO_2^- + NO_3^- and available phosphate to the variation in nematode community composition. All factors explaining at least 5% of the variation in the model were included in PCA's to visualize their contribution to the separation of the samples. Subsequently, using the combined 2013 and 2014 data, we tested for each plant pair whether range-expanding plant species showed stronger differences in nematode community composition between the different parts of the range than native plant species. For this, we combined the country data to compose three latitude region: south (Greece and Montenegro), middle (Slovenia and Austria) and north (Germany and The Netherlands). To examine the differences in nematode community shifts, we performed PCA-analyses per plant genus and tested the plant*range interaction using RDA-analyses.

Univariate analyses of functional group abundance: Per nematode genus/functional group, the abundance per 100 g dry soil was determined by combining relative abundances based on 18S rDNA

and total nematode counts per sample. Per plant pair, we then modelled absolute abundances (per 100 g soil), relative abundances of bacterivorous, fungivorous, predatory-omnivorous and root-feeding nematodes, as well as absolute abundances of the four groups of root-feeding nematodes: endoparasites, semi-endoparasites, ectoparasites and root hair feeders. Absolute abundances were treated as count data and converted to integer values, as required in count data analyses. All subsequent analyses were performed in R (R Core Development Team 2012). To account for overdispersion, abundance data were modelled using generalized linear models with a negative binomial distribution, *glm.nb* in MASS (Ripley *et al.* 2013), which included species, range, and the species*range interaction as fixed factors. Models were validated by inspection of residual plots. Relative abundance data were modelled with general linear models (*lm* in the stats package), including the same factors as the models for total abundance data.

Results

Nematode abundances ranged from 12 to 17664 per 100 g dry soil. After removal of samples with fewer than 1,000 reads, our database consisted of 5,368,503 sequences (average of approximately 15,000 sequences per sample). From all 961 detected OTUs, 653 were nematodes. Of these OTUs, 356 could be assigned to 92 known nematode genera with 297 OTUs remaining as unclassified nematodes.

Drivers of nematode community composition

All factors included in the RDA-analyses together explained 13.3% of the variation in the nematode community composition on the OTU-level (pseudo-F all axes test = 3.7, $df = 14$, $p < 0.01$) and 15.1% of the variation in nematode community composition based on nematode genus level (pseudo-F all axes test = 2.9, $df = 14$, $p < 0.01$). Of the individual factors, latitude contributed most strongly to the variation in nematode community composition, both on the OTU-level and on the level of genera (Table S2.1). The amount of available phosphate also contributed strongly to the nematode community separation on both the OTU- and genus-level (Table S2.1). In the analysis of the OTU-based communities, the majority of the plant species contributed for at least 5% to the variation explained by the RDA-model (Table S2.1), whereas plant effects were not as strong when the community composition was based on nematode genera (Table S2.1). In the PCA ordination of OTU-

based nematode community composition, both latitude and plant species corresponded with the first PCA-axis, whereas available phosphate and soil moisture corresponded most strongly with the second PCA-axis (Fig. 2.1). Latitude also most strongly corresponded to the first PCA-axis in the ordination of the genus-based nematode community composition, while in this ordination differences in community composition between plant species did not clearly correspond with the first or the second PCA-axis. Instead, the effects of soil available phosphate, $NO_2^- + NO_3^-$ content and soil pH showed the clearest correspondence with the second PCA-axis (Fig. 2.1).

Notably, the four plant genera varied in differences between nematode community composition of the native and congeneric range expander. The two *Geranium* species had similar nematode community compositions, both on the OTU-and genus-level, while the *Tragopogon* species were separated only along the second PCA-axis. The *Rorippa* and *Centaurea* species pairs showed the strongest within-genus separation in nematode community composition (Fig 2.1).

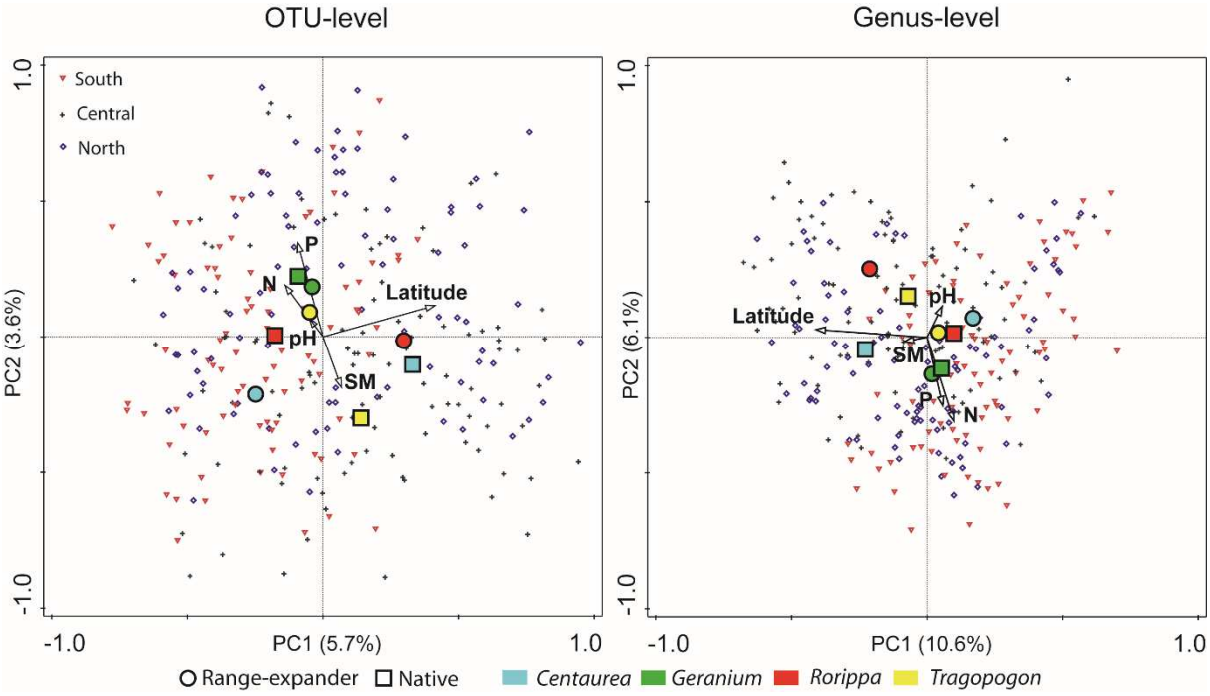

Fig. 2.1 Ordination plots based on principal component analyses (PCA) of the composition of nematode communities, on the level of OTUs (left) or nematode genera (right). Black arrows represent the effects of continuous factors latitude, soil moisture (SM), pH and the available $NO_2^- + NO_3^-$ (N) and phosphate (P). Small signs indicate individual communities from different latitudinal areas. Centroids of plant species are indicated with coloured circles and squares.

Nematode community composition shifts between original and new range

Strength of the differences in nematode communities between the latitude regions varied between plant genera and species, and changes in nematode community composition between the latitude regions were not consistently stronger for range-expanding plant species than for related natives. Within *Centaurea,* OTU-level nematode communities of the two plant species were strongly separated (Fig 2.2A), while the effect of latitude region had opposite directions between the plant species (Fig 2.2A; RDA range*species: explained variation = 14.0%, *df* = 3, pseudo-F all axes test = 3.6, $p < 0.01$). In *Geranium*, OTU-level nematode communities were more strongly separated by latitude region than by plant species, and this separation between latitude regions were stronger for native *G. molle* than for range-expanding *G. pyrenaicum* (Fig. 2.2B; RDA range*species: explained variation = 12.1%, *df* = 5, pseudo-F all axes test = 2.6, $p < 0.01$). In *Rorippa*, OTU-level nematode communities were not strongly separated in the central latitude region, while this was the case in the northern latitude region (Fig. 2.2C; RDA range*species: explained variation = 14.4%, *df* = 3, pseudo-F all axes test = 2.4, $p < 0.01$). OTU-level nematode communities of *Tragopogon* were separated based on latitude region, but there were no overall linear effects of either plant species or latitude region in this genus (Fig. 2.2D; RDA (OTU) interaction species*range: explained variation = 12.9%, *df* = 5, pseudo-F all axes test = 2.6, $p < 0.01$).

Effects of plant species and latitude region on genus-based nematode communities were mostly similar to the effects on OTU-based nematode communities (Fig. S2.1). Most notably, in *Centaurea,* genus-based communities strongly differed between the central and northern latitude regions for range-expanding *C. stoebe*, while they were comparable in the case of congeneric native *C. jacea* (Fig. S2.1), indicating stronger shifts in nematode community composition for the range-expander than for the native. Also for *Rorippa* the separation of genus-based nematode communities between central and northern latitude region appeared to be stronger for range-expanding *R. austriaca* compared to native *R. sylvestris* (Fig. S2.1).

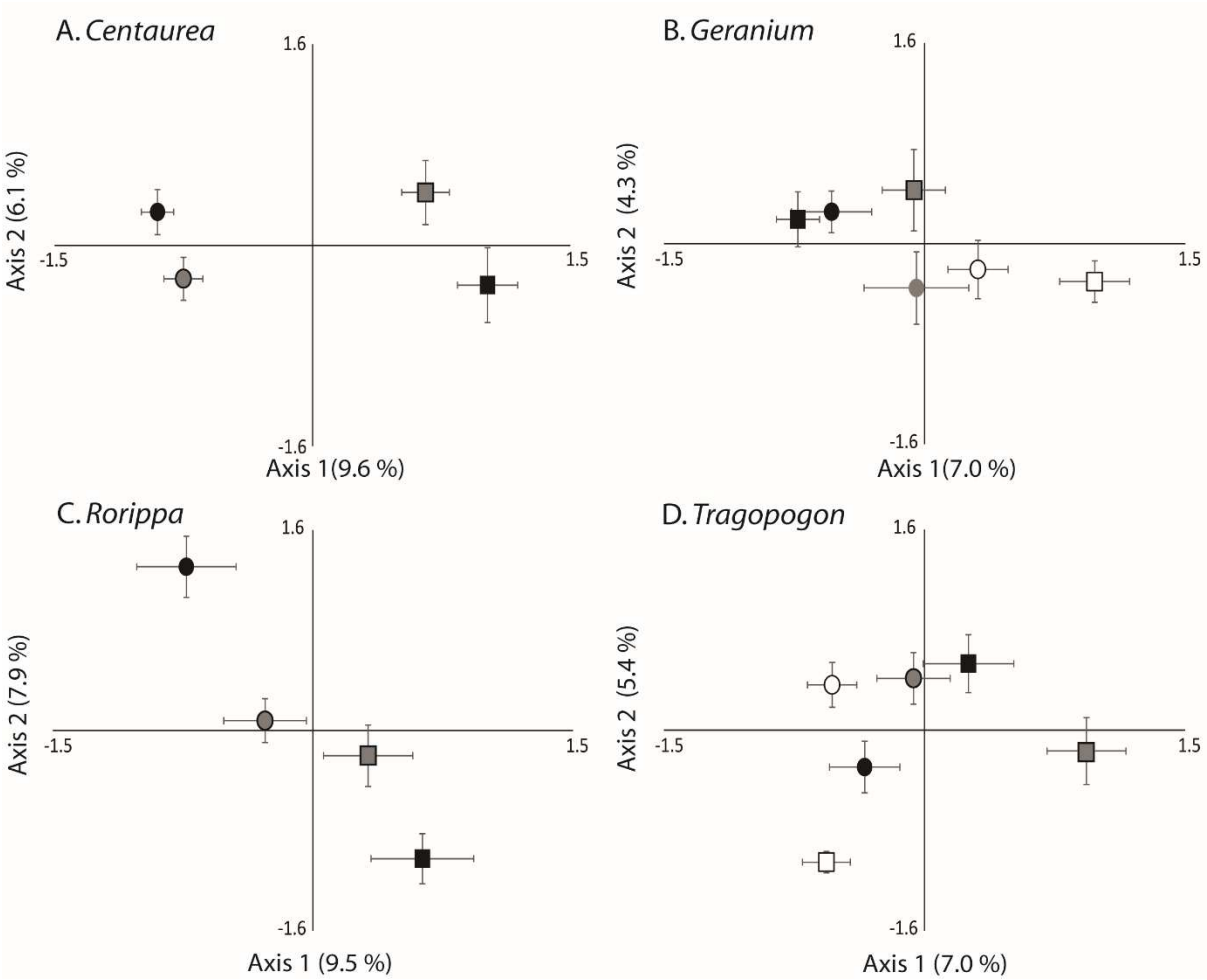

Fig. 2.2 Ordination plots based on principal component analyses (PCA) of OTU-based nematode communities in the rhizospheres of range-expanding and native *Centaurea* (A), *Geranium* (B), *Rorippa* (C), and *Tragopogon* (D). Centroid circles and squares represent range-expanders and natives, respectively. Sign colours represent southern latitude soils (white; Greece and Montenegro), central latitude soils (grey; Slovenia and Austria) and northern latitude soils (black; Central-West Germany and The Netherlands).

Abundances of nematode feeding groups

The abundances of nematode feeding groups depended on the plant species and/or latitude region, and none of the feeding groups showed systematic differences between the range-expanding plant species and the native plant species (Fig. 2.3). In *Centaurea* (Fig. 2.3A), absolute abundances of root-feeding nematodes were consistently higher in native *C. jacea* than in range-expanding *C. stoebe* ($X^2 = 34.2$, $df = 1$, $p < 0.001$) and consistently lower in the northern latitude regions than in the central latitude regions ($X^2 = 4.2$, $df = 1$, $p < 0.05$). Moreover, the latitude effect on absolute abundances of

fungivores depended on the plant species ($X^2 = 10.9$, $df = 1$, $p < 0.001$): whereas nematode communities of native *C. jacea* had more fungivores in northern latitude regions than in central latitude regions, fungivore abundance under *C. stoebe* was lower in northern latitude regions than in central latitude regions (Fig. 2.3A). Independent of plant species, absolute root-feeding nematode abundances in *Geranium* samples were lower at central latitudes than at northern and southern latitudes ($X^2 = 7.8$, $df = 2$, $p < 0.05$; Fig. 2.3B). Rhizospheres of *G. pyrenaicum* contained more predatory-omnivorous and bacterivorous nematodes at southern latitudes than at central and northern latitudes, whereas this was not the case for *G. molle* (Species*Latitude effect predatory-omnivorous nematodes: $X^2 = 17.64$, $df = 2$, $p < 0.001$; Species*Latitude effect bacterivorous nematodes: $X^2 = 13.56$, $df = 2$, $p < 0.01$; Fig. 2.3B). In *G. molle* fungivores were less abundant lower at southern latitudes than at central and northern latitudes, whereas in *G. pyrenaicum* they did not differ in abundance between the latitude regions (Region*Species interaction: $X^2 = 8.24$, $df = 2$, $p < 0.05$; Fig. 2.3B). For *Rorippa*, the absolute abundance of predatory-omnivorous nematodes was lower in samples of the range-expander *R. austriaca* than in samples of native *R. sylvestris* ($X^2 = 10.77$, $df = 1$, $p < 0.01$; Fig. 2.3C), whereas the other nematode feeding groups did not show significant differences in absolute abundance. In samples of *Tragopogon,* species effects on absolute abundances of fungivores and bacterivores depended the latitude region (Region*Species fungivores: $X^2 = 18.94$, $df = 2$, $p < 0.001$; Region*Species interaction bacterivores: $X^2 = 6.30$, $df = 2$, $p < 0.05$; Fig. 2.3D): at southern latitudes fungivores were lowest in *T. pratensis* but highest in *T. dubius*, whereas bacterivores were less numerous at northern latitudes than at central and southern latitudes in *T. dubius*, but not in *T. pratensis*.

Analyses of relative abundances of the different nematode feeding types revealed patterns that were comparable to the analyses of total abundances (Fig. S2.1 *versus* Fig. 2.3). Most importantly, although some nematode groups showed significant differences in relative abundance between plant species or latitude regions, these patterns did not necessarily hold when absolute abundances were analysed.

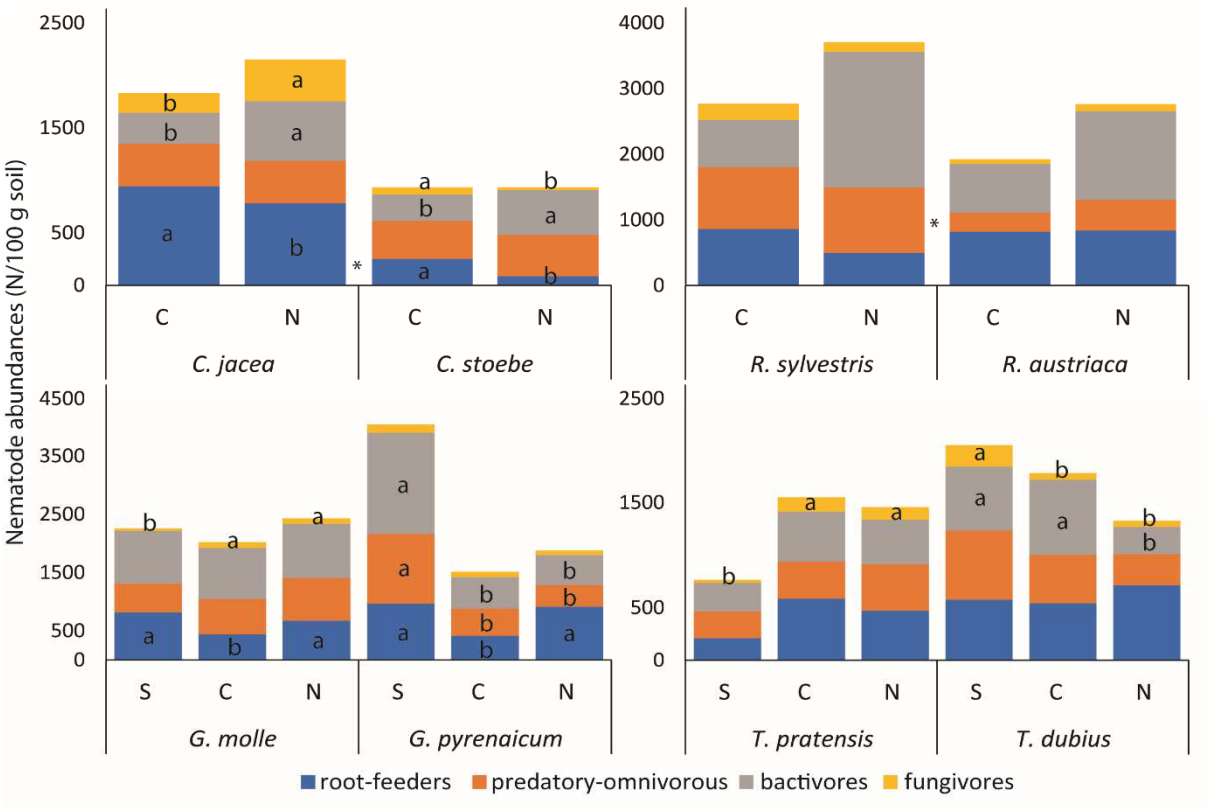

Fig. 2.3 Absolute abundances (N/100 g dry soil; see Methods) of four major nematode feeding groups in rhizosphere samples of range-expanding *Centaurea stoebe*, *Geranium pyrenaicum*, *Rorippa austriaca* and *Tragopogon dubius* and native plant species *Centaurea jacea*, *Geranium molle*, *Rorippa sylvestris* and *Tragopogon pratensis* in southern and central original range soils S (Greece and Montengro; only *Geranium* and *Tragopogon*) and C (Slovenia and Austria) and new range soils N (Central-West Germany and The Netherlands). Small letters indicate significant within-species differences according to post-hoc Wald tests. Significant between-species differences of nematode feeding type abundances are indicated with *. Note that x-axes differ between the genera.

Abundances of root-feeding nematode types

All four root-feeding nematode types were more abundant in native *Centaurea jacea* than in range-expanding *C. stoebe* (Fig. 2.4). In range-expanding *C. stoebe* the abundance of endoparasitic nematodes appeared to be lower in the new range (northern latitude region) than in the original range (central latitude region), while such differences were not found in *C. jacea* (marginally significant interaction effect: $X^2 = 3.60$, $df = 1$, $p = 0.058$; Fig. 2.4). The abundance of semi-endoparasites was also reduced in the northern latitude region compared to the central latitude region, but this effect was not

significantly stronger in *C. stoebe* than in *C. jacea* (Fig. 2.4). Moreover, whereas numbers of ectoparasitic nematodes tended to increase in the new range compared to the original range in *C. jacea* samples, they tended to decrease in *C. stoebe* samples (Fig. 4). In range-expanding *Geranium pyrenaicum* samples, there were significantly fewer endoparasites in northern latitude than in southern latitude samples (Fig. 4), whereas this was not found for native *G. molle*. The abundance of ectoparasites was higher in *G. pyrenaicum* than in *G. molle* samples and higher in northern latitude samples than in central and southern latitude samples. Finally, the latitudinal variation in numbers of root-hair feeders depended on the plant species: in *G. molle,* central and northern samples contained fewer nematodes than southern samples. In *G. pyrenaicum*, root-hair feeders were less abundant in central latitude samples than in northern and southern latitude samples. Between range-expanding *R. austriaca* and native *Rorippa sylvestris* only the abundance of semi-endoparasites differed, as they were more numerous in *R. austriaca* samples (Fig. 2.4). In *Tragopogon*, the absolute abundance of endoparasites was higher in central latitude samples, irrespective of plant species (Fig. 2.4). Latitude region also significantly affected the semi-endoparasite abundance, but post-hoc analyses did not reveal significant differences between southern, central and northern latitude samples. Abundances of semi-endoparasites and ectoparasites were higher in range-expanding *T. dubius* than in native *T. pratensis*. Finally, the abundance of ectoparasites was higher in northern latitude samples than in central and southern latitude samples.

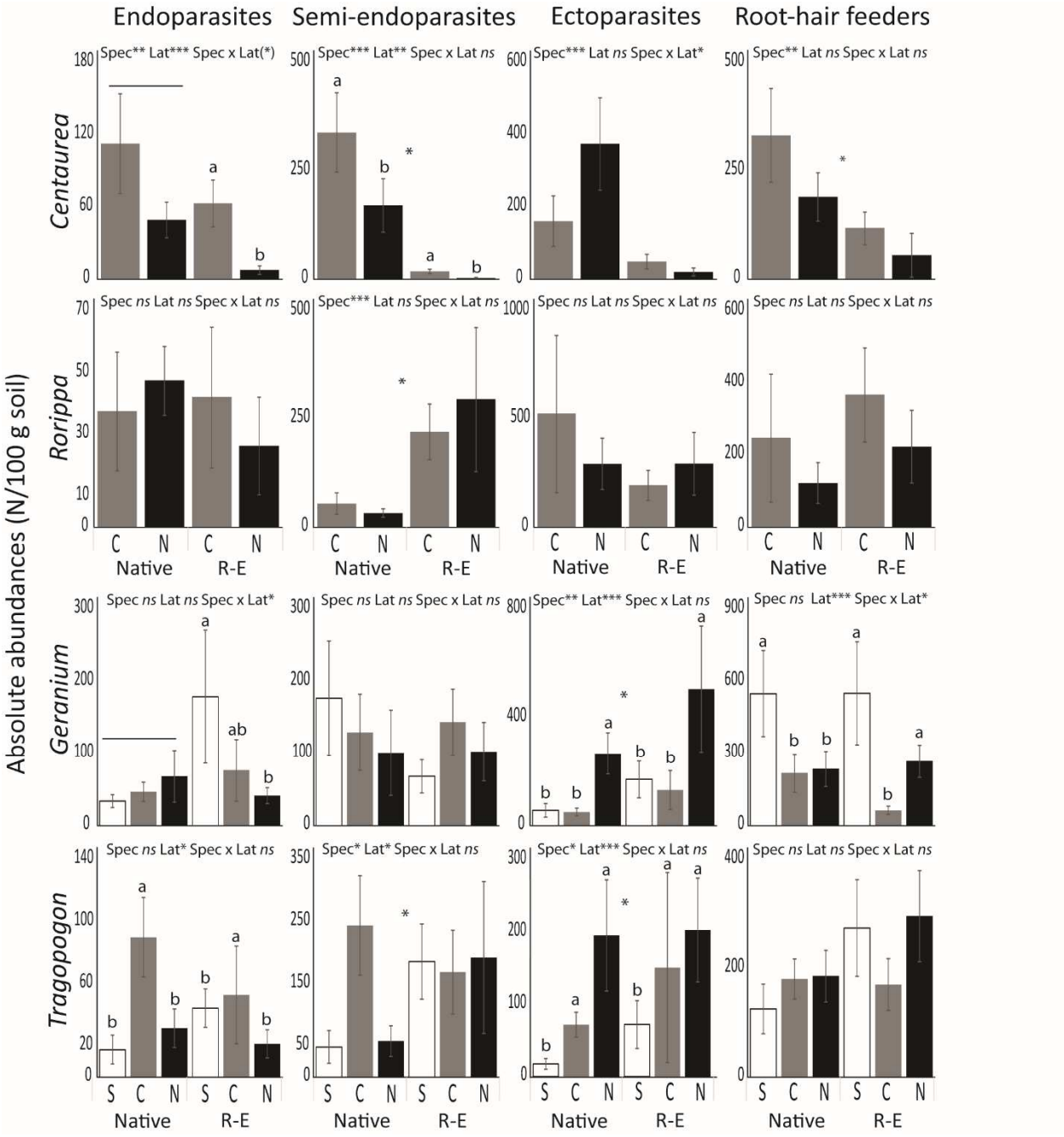

Fig. 2.4 Absolute abundances (N/100 g dry soil; see Methods) of four root-feeding nematode types in rhizosphere samples of four pairs of congeneric range-expanding and native plant species in southern original range soils S (Greece and Montenegro; only *Geranium* and *Tragopogon*) and central original range soils C (Slovenia and Austria; all genera), and in new range soils N (Central-West Germany and The Netherlands; all genera). Statistical significance levels, based on negative binomial GLM, of the effects of plant species (Spec), latitude region (Lat) and the interaction (Spec x Lat) are noted with *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), (*) (marginally significant, $p<0.06$) and *ns* (non-significant). Small letters and bars are used to visualize statistical interactions and range effects based on post-hoc Wald tests. Overall species effects are noted with *. Note that y-axes vary among panels.

Discussion

Our results show latitudinal variation in soil nematode communities along a range expansion gradient, which is in support of our first hypothesis. Such latitudinal variation may include temperature and precipitation, as both have been proposed as drivers of nematode community composition (Nielsen *et al.* 2014; Song *et al.* 2017). Next to latitude, also plant species identity appeared to be an important driver of soil nematode community composition such as shown before (Song *et al.* 2017). Compared to latitude, soil characteristics, such as available phosphate, pH and soil moisture, were less important in explaining nematode community composition. Available phosphate appeared to be more important for nematode community composition than the other soil characteristics, which corresponds with previously observed effects of fertilizers on nematode communities (Hu & Qi 2010; Zhao *et al.* 2014). Our analysis suggests that nematode community composition along such a latitudinal gradient is driven in fundamentally different ways than community compositions of bacteria, that strongly correspond with pH (Fierer *et al.* 2009). Instead, the responses of nematode communities resemble those of fungal and protist communities, for which climatic factors are most important (Bates *et al.* 2013; Tedersoo *et al.* 2014)

The latitudinal variation in nematode community composition confirms our assumption that plant species that are expanding their range northwards in response to climate change will face different nematode communities in their new range. Moreover, it shows that plant species with widespread distributions are associated with different nematode communities in different parts of their native range. We found mixed support for our second hypothesis that across the latitudinal gradient shifts in nematode community composition were stronger for range-expanding plant species than for related natives. Most notably, genus-based nematode communities of range-expanding *Centaurea stoebe* differed between the original range in Central Europe and the new range in North-Western Europe, while nematode communities of *C. jacea* were comparable between these areas. While this result suggest different responses of nematodes in the new compared to the original range, such shifts in nematode communities may also partly be explained by differences in abiotic conditions between the original and new range areas. Nevertheless, the nematode community shift as observed in range-expanding *Centaurea* is in line with differences in seed-associated communities of fungi between

northern and southern *C. stoebe* populations, which were not found between *C. jacea* populations (Geisen *et al.* 2017).

Our third hypothesis that range-expanding plant species are exposed to lower numbers of root-feeding nematodes in the expanded range was only supported by *Centaurea stoebe*. Especially numbers of endoparasites were reduced in the new range, while such a strong decrease was not evident for native *C. jacea*. Overall, however, the reduction of root-feeding nematodes between the new and the original range was not stronger for *C. stoebe* than for *C. jacea*. This indicates that not all root-feeding nematode types will respond similarly to plant range expansions. The low accumulation of endoparasitic root-feeding nematodes in the rhizosphere of *C. stoebe* in the new range is likely linked to its strong chemical repellence of root-feeding nematodes from the new range (Chapter 5). The other three range-expanding plant species did not show lower numbers of root-feeding nematodes in the new compared to the original range, indicating that latitudinal shifts in nematode community composition of range-expanding plant species do not necessarily imply that they are exposed to fewer numbers of root-feeding nematodes. Functional consequences of eventual changes in exposure to root-feeding nematodes need to be examined by performing inoculation experiments.

Effects of range-expanding plant species on numbers of fungivorous nematodes varied more strongly along the latitudinal transect than effects on other nematode feeding types. Both range-expanding *Centaurea* and *Tragopogon* accumulated few fungivorous nematodes in northern latitude sites compared to central and southern latitude sites, respectively, whereas the opposite was found for their congeneric natives. These results correspond with a previous study showing lower abundances of fungivores in the rhizospheres of range-expanders compared to natives (Morriën, Duyts & Van der Putten 2012). Possibly, this effect can be explained by inhibitory effects of the range-expanders on soil fungi in their new range, as has been shown for introduced exotic species (Callaway *et al.* 2008), although this remains to be tested for range-expanders.

We analyzed nematode community composition by a combination of rapid nematode quantification followed by high-taxonomic classification beyond genus-level using high-throughput sequencing. Molecular analyses only would have limited comparisons to relative abundances, rather than enabling

quantitative analyses (Vandeputte *et al.* 2017; Geisen *et al.* 2018). However, we acknowledge that the community composition that we provide might not represent abundances as provided by morphological identifications (Darby, Todd & Herman 2013). For instance, we find high numbers of large-sized omnivores and predators compared to morphological studies (Song *et al.* 2017) suggesting that our data might include information on nematode biomasses rather than only nematode abundances (Zhu *et al.* 2005). While other biases, e.g. those related with PCR amplification, might also affect the resulting nematode community composition (Griffiths *et al.* 2018), these biases are indifferent across all samples.

Conclusion

Our study shows that nematode community composition along a latitudinal transect of plant range expansion varies more strongly with latitude and plant species identity than with soil characteristics. The strength of nematode community shifts between the original and new range of four range-expanding plant species depended on plant species identity, and latitudinal community shifts were not always stronger for range-expanding plant species than for related natives. Only one of the four examined range-expanding plant species accumulated fewer root-feeding nematodes in its new range than in its original range. Therefore, enemy release from root-feeding nematodes may not be a general phenomenon for range-expanding plant species. Nevertheless, our results are among the first to empirically test predictions on belowground community shifts (Berg et al. 2010) suggesting that consequences of plant range shifts for belowground community (re)organization depend on range-expanding plant identity.

Supplementary information

Table S2.1: results of Redundancy analyses (RDA) with forward selection on the composition of nematode communities based on nematode OTUs or nematode genera. Factors contributing more than 5% to the explained variation by the RDA-model are listed.

Database: OTU-level	Explains %	Contribution %	pseudo-F	P	P(adj)
Latitude	2.1	15.6	6	0.002	0.03
Phosphate	1.3	9.5	3.7	0.002	0.03
Plant species: *Centaurea stoebe*	1.2	9	3.5	0.002	0.03
Soil moisture	1.1	8.5	3.4	0.002	0.03
pH	1.1	8.4	3.3	0.002	0.03
Plant species: *Rorippa sylvestris*	1.1	8	3.2	0.002	0.03
Plant species: *Tragopogon pratensis*	0.9	6.9	2.8	0.002	0.03
Plant species: *Centaurea jacea*	0.8	6	2.4	0.002	0.03
Plant species: *Geranium pyrenaicum*	0.8	6	2.4	0.002	0.03
$NO_2^- + NO_3^-$	0.7	5.5	2.2	0.002	0.03
Plant species: *Rorippa austriaca*	0.7	5.2	2.1	0.002	0.03

Database: Genus-level	Explains %	Contribution %	pseudo-F	P	P(adj)
Latitude	3.1	19.1	8.9	0.002	0.03
Phosphate	1.6	10	4.7	0.002	0.03
Soil moisture	1.8	11	5.3	0.002	0.03
Plant species: *Centaurea stoebe*	1.5	9	4.4	0.002	0.03
pH	1.3	8	3.9	0.002	0.03
Plant species: *Rorippa sylvestris*	1.2	7.3	3.6	0.002	0.03
$NO_2^- + NO_3^-$	1.1	6.6	3.3	0.002	0.03
Plant species: *Tragopogon pratensis*	0.8	5.1	2.5	0.002	0.03

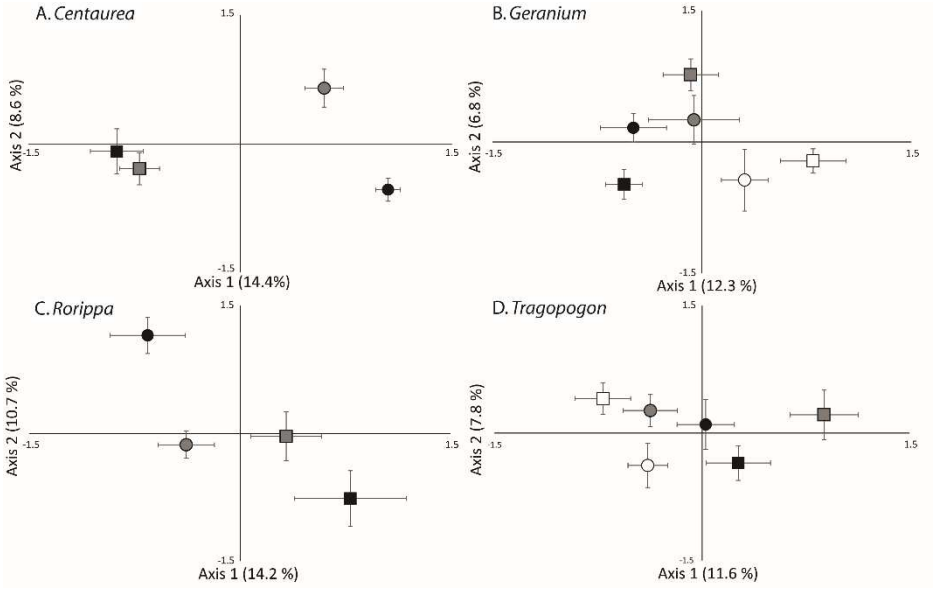

Fig. S2.1 Ordination plots based on principal component analyses (PCA) of genus-based nematode communities in the rhizospheres of range-expanding and native *Centaurea* (A), *Geranium* (B), *Rorippa* (C), and *Tragopogon* (D). Centroid circles and squares represent range-expanders and natives, respectively. Sign colours represent southern latitude soils (white; Greece and Montengro), central latitude soils (grey; Slovenia and Austria) and northern latitude soils (black; Central-West Germany and The Netherlands).

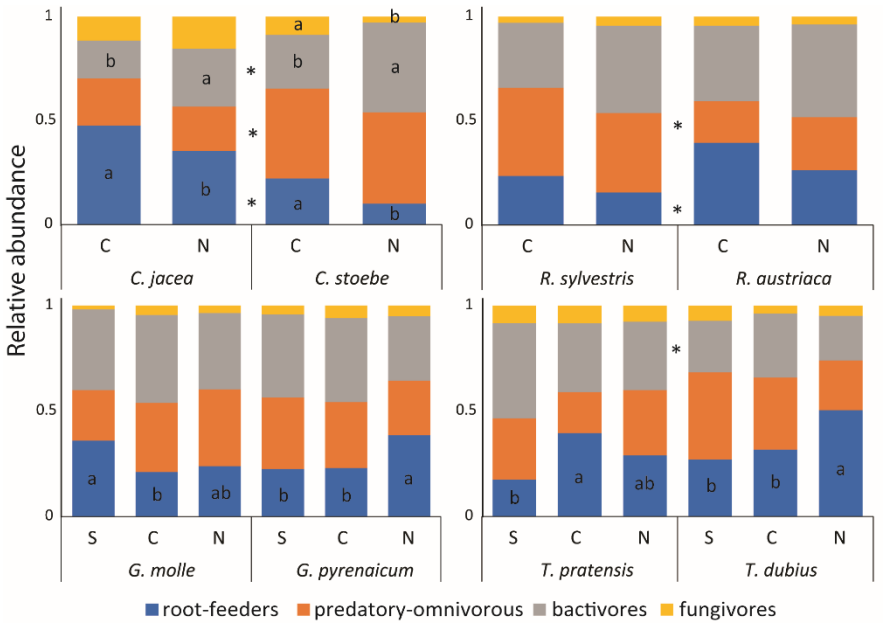

Fig. S2.2 Relative abundances of four major nematode feeding groups in rhizosphere samples of range-expanding *Centaurea stoebe*, *Geranium pyrenaicum*, *Rorippa austriaca* and *Tragopogon dubius*, and of native plant species *Centaurea jacea*, *Geranium molle*, *Rorippa sylvestris* and *Tragopogon pratensis* in southern original range soils S (Greece and Montengro; only *Geranium* and *Tragopogon*) and central original range soils C (Slovenia and Austria) and new range soils N (Central-West Germany and The Netherlands). Small letters indicate significant within-species differences according to post-hoc Wald tests. Significant between-species differences of nematode feeding type abundances are indicated with *.

Chapter 3

Nematode community responses to range-expanding and native plant communities in original and new range soils

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Abstract

Many plant species expand their range to higher latitudes in response to climate change. However, it is poorly understood how biotic interactions in the new range differ from interactions in the original range. Here, in a mesocosm experiment, we analyzed nematode community responses in original and new range soils to plant communities with either 1) species native in the original and new range, 2) range-expanding plant species related to these natives (related range-expanders), or 3) range-expanding plant species without native congeneric species in the new range (unrelated range-expanders). We hypothesized that nematode community shifts between ranges are strongest for unrelated range-expanders and minimal for plant species that are native in both ranges. As a part of these community shifts, we hypothesized that range-expanders, but not natives, would accumulate fewer root-feeding nematodes in their new range compared to their original range. Our study reveals that none of the plant communities experienced evident nematode community shifts between the original and new range. However, in the new range, root-feeding nematode communities of natives and related range-expanders were more comparable than in the original range, whereas the nematode community of unrelated range-expanders was distinct in both ranges. The abundances of root-feeding nematodes were comparable between the original and new range for all plant communities. Remarkably, unrelated range-expanders overall accumulated most root-feeding nematodes, whereas related range-expanders accumulated fewest. We conclude that nematode communities differ between communities of range-expanding and native plant species, but that nematode communities associated to native and range-expanding plant species do not strongly differ between original and new range soils.

Introduction

Worldwide, many native plant communities are invaded by exotic species that have been introduced intentionally or unintentionally by humans (van Kleunen *et al.* 2015). In addition to exotic species that originate from other continents, current climate change enables intra-continental range expansion of plant and animal species to higher latitudes and altitudes (Walther *et al.* 2002; Parmesan 2006). While such range-expanders have become increasingly common (Tamis *et al.* 2005), little is known about

their influence on native above- and belowground plant-associated biota in their novel habitat (Van Nuland, Bailey & Schweitzer 2017). The limited co-evolutionary history may result in naïve responses of either plants or associated biota (Verhoeven *et al.* 2009; Pearse *et al.* 2013), which makes outcomes of such novel interactions difficult to predict.

The success of introduced exotic plant species has often been related to their possession of traits that are not present in the invaded native community. Next to novel traits such as fire resistance (D'Antonio & Vitousek 1992) and nitrogen fixation (Stock, Wienand & Baker 1995), non-native plant species may also benefit from the production of metabolites that are not produced by native plant species (Cappuccino & Arnason 2006). In the new range, such 'novel weapons' may suppress the growth of neighboring plant species (Callaway & Aschehoug 2000), mutualists of native species (Stinson *et al.* 2006; Callaway *et al.* 2008), and natural enemies (Schaffner *et al.* 2011; Macel *et al.* 2014). Because plant traits such as root chemistry are often phylogenetically conserved (Agrawal *et al.* 2009; Pearse & Hipp 2009; Gilbert & Parker 2016), exotic species that are phylogenetically closely related to native flora may host more natural enemies in the invaded range than distantly related range-expanders (Gilbert & Parker 2016). These, so-called spill-over effects of local enemies (Malmstrom *et al.* 2005) are considered as one of the possible explanations why phylogenetically distinct exotic species can become more abundant than exotic species that are strongly related to native species (Strauss, Webb & Salamin 2006).

Some intra-continental range-expanders are closely related to plant species in the native plant community, but are nonetheless found to be more successful in suppressing generalist insects, fungal pathogens and root-feeding nematodes than their related native species (Engelkes *et al.* 2008; Morriën, Duyts & Van der Putten 2012; Morriën & van der Putten 2013). Range-expanders that are phylogenetically more distinct from native flora can be expected to have even stronger suppressive effects on these native natural enemies, but such evidence is lacking so far. Moreover, it is still largely unknown if the interactions between range-expanding plant species and their natural enemies differ

between their original and new range as only a couple of studies (van Grunsven *et al.* 2010; Dostálek *et al.* 2015; Macel *et al.* 2017) have addressed these questions experimentally.

The aim of the present study was to examine plant-nematode interactions of natives, range-expanders related to these natives (hereafter: related range-expanders) and range-expanders without native species from the same genus in their new range (hereafter: unrelated range-expanders), in soils from the new and original range. We focus on belowground plant-nematode interactions, as nematodes have important roles in the soil food-web (Ferris, Bongers & De Goede 2001) and can affect spatio-temporal dynamics in natural vegetation (De Deyn *et al.* 2003; Brinkman *et al.* 2015). We established mesocosms with soil from either the original or the new range, in which we grew communities of each of the three groups of plant species. We recorded the abundance of root-feeding nematodes, as well as bacterivores, fungivores, omnivores and predators in the root zones of all plant communities growing in soils from the original and the new range.

We tested the hypotheses that 1) range-expanders, but not natives, associate with different nematode communities in the original compared to the new range, mostly by accumulating fewer root-feeding nematodes in soil from their new range, 2) these shifts in nematode communities will be stronger for unrelated than for related range-expanders, and 3) we expect that numbers of bacterivorous, fungivorous, omnivorous and predatory nematodes vary less between the plant communities than root-feeding nematodes, as they are only indirectly interacting with the plants (De Deyn *et al.* 2004; Scherber *et al.* 2010).

Methods

We tested our hypotheses using three types of plant communities consisting either of: 1) four plant species that are native in both South-Eastern Europe, where the range-expanders originate from, and North-Western Europe, where range-expanders have expanded to, 2) four plant species belonging to the same genera as the natives that have expanded their range from South-Eastern Europe to North-Western Europe, or 3) four plant species that have expanded their range from South-Eastern Europe to North-Western Europe and have no native species in the same genus in the new range. In a

greenhouse experiment, we grew all three plant communities in mesocosms with a sterilized background soil, inoculated with individual replicates of soil from either the original or the new range (see below). After a growth period of 14 weeks, we extracted the nematode communities from the soil of each mesocosm for counting and identification.

Plant species and seed collection

All plant species occur in central Netherlands in riparian habitats of the two rivers that are branches of the Rhine. The majority of these plant species can be found in the same nature reserves (Dutch nature observation website: https://www.waarneming.nl). The native plant species were *Centaurea jacea* L. (Asteraceae), *Tragopogon pratensis* L. (Asteraceae), *Geranium molle* L. (Geraniaceae) and *Rorippa sylvestris* (L.) Besser (Brassicaceae). As related range-expanders we used *Centaurea stoebe* L., *Tragopogon dubius* Scop., *Geranium pyrenaicum* Burm. f. and *Rorippa austriaca* Crantz. The four unrelated range-expanders were *Dittrichia graveolens* (L.) Greuter (Asteraceae), *Lactuca serriola* L. (Asteraceae), *Rapistrum rugosum* (L.) All. (Brassicaceae) and *Bunias orientalis* L. (Brassicaceae). *Centaurea stoebe, T. dubius, R. austriaca, D. graveolens and R. rugosum* colonized the Netherlands from the 20th or early 21st century onwards, while *G. pyrenaicum, L. serriola* and *B. orientalis* already occurred in suitable habitats of the Netherlands before the 20th century, but strongly expanded their range during recent decades (NDFF 2017). Seeds of all 12 plant species originated from single, wild populations growing in the Netherlands. For *G. pyrenaicum*, *T. dubius* and *T. pratensis* seeds were supplied by Cruydthoeck, a company that grows plants from field-collected seeds in the Netherlands for seed production. For all other plant species we collected seeds directly from plants growing in natural areas, mainly in riverine systems in eastern Netherlands.

Soil collection

We collected soil from areas in Slovenia and Austria where all plant species occur naturally and from the riverine system in The Netherlands where all the range-expanding plant species have become established. In all three countries, we selected three riverine areas of approximately 30 ha each for soil collection. In each area, soils were collected from three sub-locations separated by a distance of minimally 300 m. First we removed the upper 3 cm soil layer and then collected the soil between 3 and

15 cm depth, where most living roots occur. Thereafter, soil was sieved using a 4 mm mesh and gently homogenized, while keeping sub-locations separate. Half the soil from each sub-location was stored at 4-8 °C, while the other half was sterilized by gamma irradiation (>25 KGray) at Steris AST (Ede, The Netherlands). To compare the effects of soil biota under the same abiotic conditions, we used a common sterilized background soil that was a mixture of soil additionally collected from all sub-locations in the Netherlands. Background soil was sieved, homogenized and then gamma-sterilized as indicated above.

Experimental set-up

We first created nine soil replicates for both the original and the new range. To obtain soil replicates with communities of soil organisms that represented the new and original range in a general and not location-specific way, each of these nine replicate soils consisted of sterilized background soil to which live soil from two sub-locations, originating from two different main areas in either the original or the new range, was inoculated (see Koorem *et al.* 2017). This approach resulted in nine soil mixes that were non-identical, yet partly overlapping in donor soils, and avoided the risk of idiosyncratic differences among individual soil samples. All soils were collected from sites where at least several of the plant species that were used in the experiment occurred. However, we did not collect soil directly beneath our focal plant species to avoid potential experimental biases. The soil mixes representing soils from the original range were a combination of soil from one of the nine Slovenian and one of the nine Austrian sub-locations (See Table S3.1). For the new range, nine soil mixes were created by combining soils from two different locations in the riverine system in the Netherlands (See Table S3.1), so that each sub-location was used in two different soil mixes. Each mesocosm (7L, diameter 26 cm, height 20 cm) in the experiment was filled with 1.5 kg of gravel (4-8 mm particles) at the bottom on top of which 4.2 kg of soil was added, consisting of 80% sterilized background soil and 10% live soil inoculum from the two sub-locations. To avoid potential abiotic differences between soils from the original and the new ranges, we added 10% of sterilized inoculum soil from the complementary range, so that in all cases every mesocosm had 10% of (sterilized or unsterilized, respectively) soil from the original and 10% from the new range.

Per range, each of the nine soil mixes was divided over three different mesocosms, resulting in 54 mesocosms (9 soil mixes × 3 plant communities × 2 soil origins) in total. Each mesocosm was planted with two seedlings of each of the four plant species of the same plant type in the Netherlands, so that on each soil mix all three plant communities were grown. Seedlings were planted in a circle in a fixed order at approximately 4 cm of each other, in such a way that conspecific seedlings were not close neighbours. Mesocosms were placed in a climate-controlled greenhouse of 16 h 21° (day) and 8 h 16° (night) and were watered three times per week in order to keep soil moisture at 60% water holding capacity. After 12 weeks of plant growth two *Mamestra brassicae* L. (Lepidoptera: Noctuidae) caterpillars were introduced to pots with the soil replicates 1-5 in order to test their response to the different plant communities (see Koorem *et al.* 2017). We did not aim to test the effects of aboveground herbivory on nematode community composition. The herbivory treatment was assigned to soil mixes 1-5 (Table S3.1), which due to their origin likely more closely resemble each other than they resemble soil mixes 6-9. Because of this non-random assignment of the herbivory treatment, it is impossible to disentangle herbivory effects from soil mix effects in the presented study.

Harvest

After 14 weeks of growth, shoots of all individual plants were clipped, dried at 70 °C and weighed. As it was not possible to disentangle the roots of each individual plant, roots of all plants were washed from the soil collectively and dried at 70 °C to constant weight. We used 50 g of soil (wet weight) from each pot for nematode extraction, morphological identification, and counting to feeding type. Additionally, soil samples were taken for determining soil moisture content, so that the number of nematodes could be expressed per dry weight of soil. Nematodes were extracted from soil using an Oostenbrink elutriator (Oostenbrink 1960). After extraction, we concentrated the nematode suspensions to 2 ml, after which 4 ml hot (90 ^{0}C) and 4 ml cold (20 ^{0}C) formaldehyde was added to fixate the nematodes before identification and counting.

Nematode identification

Morphological identification and counting of nematodes was done using an inverse-light microscope at 200× magnification. Per sample, all nematodes were classified to one of the five feeding types

(predators, root-feeders, fungivores, omnivores or bacterivores) according to Yeates *et al.* (1993) and counted. Root-feeding nematodes were further identified to either family or genus level using Bongers (1988). Root-feeding nematode genera identified were *Meloidogyne* (Heteroderidae), *Paratylenchus* (Tylenchulidae), *Pratylenchus* (Pratylenchidae), *Psilenchus* (Psilenchidae), and root-feeding nematode families identified were Hoplolaimidae, Tylenchidae, Anguinidae, Dolichodoridae, Criconomatidae, Hemicycliophoridae and Heteroderidae.

Statistical analyses

Prior to statistical analyses, soil moisture percentages were used to calculate nematode numbers per 100 g dry soil. We also calculated the density of root-feeding nematode taxa per gram root, as an indication of the root-feeding nematode density on plant roots. For this, we calculated total number of nematodes of each taxon per mesocosm and divided those numbers by total root dry weight in that mesocosm (Table S3.2; also presented in Koorem *et al.* 2017).

Multivariate analyses: First, we performed a Principal Component Analysis (PCA in Canoco 5; Šmilauer and Lepš 2014) comparing nematode community composition based on the abundances of the five nematode feeding types. Second, in another PCA analysis, we compared community composition of only the root-feeding nematode community, as root-feeding nematodes were expected to show the strongest responses to plant status in the Netherlands. Nematode taxa with fewer than 3 occurrences in the data set were excluded from the analyses to avoid strong effects caused by rare taxa. We used the factors 'plant community' and 'soil origin' to independently classify the mesocosms. In both PCA's we included soil mix as a covariate in order to account for variation between the nine soil mixes. To test the effects of plant community, soil origin and their interaction on the nematode community composition we used redundancy analyses (RDA). The significance of the RDA-models is based on 999 Monte Carlo permutations, which were restricted to incorporate the effect of soil mix.

Univariate analyses: All univariate analyses were performed in R version 3.1.0 (R Core Development Team 2012). We selected four nematode feeding types and four root-feeding nematode genera/families that - based on the PCA - contributed most to the separation of the treatments. We

used generalized linear models with a negative binomial error distribution (Hilbe 2014) to model densities of the nematode feeding types in soil (N/100 g soil), and densities in soil (N/100 g soil) and densities per g root (N/g root) of the selected root-feeding nematode genera and families. Generalized linear models included the fixed factors soil mix (nested in soil origin), plant community, soil origin and the soil origin*plant community interaction. Post-hoc Wald tests were performed using the phia package (De Rosario-Martinez 2013) to individually test differences between plant communities. Densities of predators were low (average 1.27 per sample), and therefore not modelled.

Results

Nematode feeding type community composition

The nematode community composition based on feeding types was significantly affected by the interaction between plant community and soil origin (RDA: total variation explained: 22.1%; pseudo-F = 2.7, *df* = 5, p = 0.003). In particular, the nematode communities accumulated by related range-expanders differed between soils from the original range and soils from the new range, while nematode communities accumulated by natives and unrelated range-expanders did not differ between original and new range soils (Fig. 3.1A).

Root-feeding nematode community composition

The community composition of root-feeding nematodes was affected by the interaction between plant community and soil origin (RDA: total variation explained: 21.4%; pseudo-F = 2.6, *df* = 5, p = 0.001, Fig. 3.1B). In particular, all three plant communities had differently composed root-feeding nematode communities. However, in the original range nematode communities of native and related range-expanders were more strongly separated than in the new range. In contrast, the nematode community of the unrelated range-expanders was more separated from the other nematode communities in the new range compared to the old range. The root-feeding nematode groups that contributed most strongly to the separation between the treatments were *Meloidogyne*, *Paratylenchus,* Hoplolaimidae and Tylenchidae.

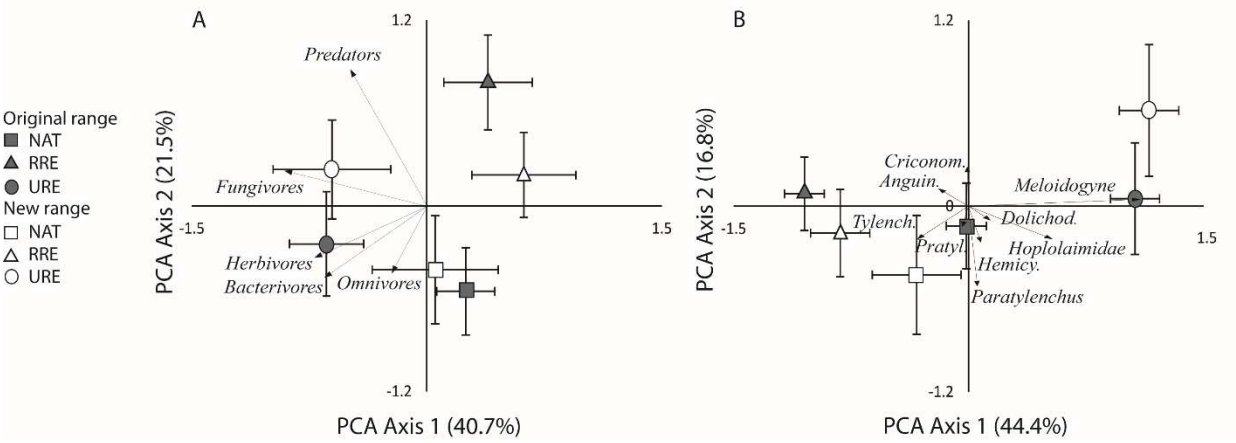

Fig. 3.1 Ordination diagrams of principal component analyses (PCA) showing the centroids of nematode community composition based on nematode feeding types (left) and the community of root-feeding nematodes (right). Centroids represent nematode communities in mesocosms inoculated with soils from the original range (filled signs) or new range (open signs), grown with either natives (NAT; squares), related range-expanders (RRE; triangles) or unrelated range-expanders (URE; circles). Arrows represent the relation between nematode feeding types (a) or root-feeding nematode taxa (b) and the variation in nematode community along the PCA-axes. Horizontal and vertical error bars represent standard errors along the first and second PCA-axes. Percentages of total explained variation by the PCA-axes are shown in the parentheses.

Abundances of the nematode feeding types

Differences in densities of root-feeding nematodes (N/100 g soil) were solely explained by plant community type (X^2 = 44.55, *df* =2, p < 0.0001; Fig. 3.2A). Overall, unrelated range-expanders accumulated more root-feeding nematodes (N/100 g soil) than natives (X^2 = 14.74, p < 0.001) and related range-expanders (X^2 = 43.63, p < 0.0001), whereas natives accumulated more root-feeding nematodes than their related range-expanders (X^2 = 7.69, p < 0.01). Numbers of bacterivorous and omnivorous nematodes (N/100 g soil) differed between original and new range soil: bacterivorous nematodes were most abundant in soil from the new range (X^2 = 22.32, *df* =1, p < 0.0001; Fig. 3.2B), whereas omnivorous nematodes (N/100 g soil) were most abundant in soils from the original range (X^2 = 26.81, *df* =1 p < 0.0001; Fig. 3.2C). The numbers of fungivores (N/100 g soil) depended on the interaction between soil origin and plant community type (X^2 = 6.11, *df* = 2, p < 0.05). In soils from the original range, fungivore densities (N/100 g soil) were higher in mesocosms with unrelated range-

expanders than with native plant species (X^2 = 7.13, p < 0.01; Fig. 3.2D), whereas there were no differences between plant community types in soils from the new range.

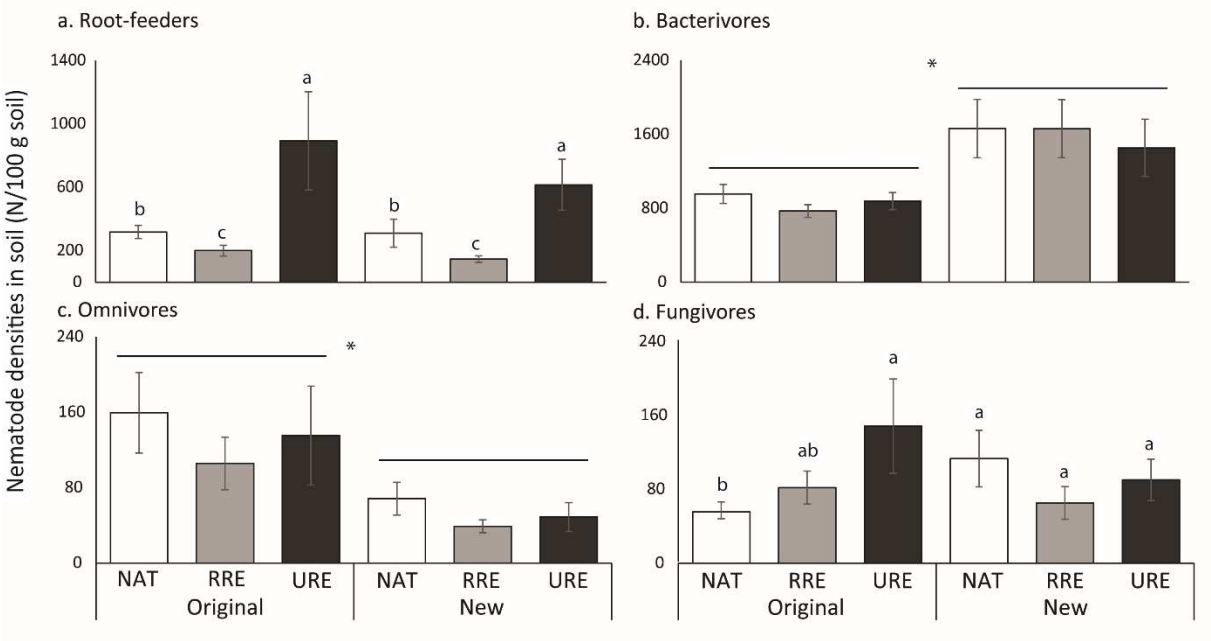

Fig. 3.2 Densities of root-feeding, bacterivorous, fungivorous and omnivorous nematodes in soil (N/100 g dry soil) in mesocosms with native plant species (NAT; white), related range-expanders (RRE; light grey) and unrelated range-expanders (URE; dark grey) in soils from the original range (south) and the new range (north) of the range-expanders. Bars represent averages ± standard errors. Horizontal bars and asterisks indicate significant differences between soil origins and different letters indicate significant (p < 0.05) differences between plant communities within ranges based on Negative binomial GLM and Post-hoc Wald tests.

Abundances of root-feeding nematodes

Responses of all root-feeding nematodes to soil origin and plant community composition depended on genus/family and whether nematode numbers were analysed per 100 g soil or per g root (Fig. 3.3). *Meloidogyne* was the most abundant root-feeder, as 44% of the root-feeding nematodes in the mesocosms with natives, 30% with related and 82% with unrelated range-expanders belonged to this genus. *Meloidogyne* densities were strongly affected by plant community type (N/100 g soil: X^2 = 55.15, *df* =2, p <0.0001; N/g root: X^2 = 99.82, *df* =2, p < 0.0001; Fig. 3.3A, E). Densities of *Meloidogyne* in soil, as well as *Meloidogyne* densities on roots, were higher in mesocosms with unrelated range-expanders

than with natives (N/100 g soil: $X^2 = 21.35$, $p < 0.0001$; N/g root: $X^2 = 53.33$, $p < 0.001$; Fig. 3.3A,E), or related range-expanders (N/100 g soil: $X^2 = 55.49$, $p < 0.0001$; N/g root: $X^2 = 97.99$, $p < 0.0001$; Fig. 3.3B,F). *Meloidogyne* densities in mesocosms with natives were higher than in mesocosms with related range-expanders (N/100 g soil: $X^2 = 8.12$, $p < 0.01$; N/g root: $X^2 = 6.77$, $p < 0.01$; Fig. 3.3A, E).

Soils from the original range contained more Hoplolaimidae (N/100 g soil: $X^2 = 13.12$, $df = 1$, $p < 0.001$; N/g root: $X^2 = 10.64$, $df = 1$ $p < 0.01$; Fig. 3.3B, F) and Tylenchidae (N/100 g soil: $X^2 = 21.06$, $df = 1$, $p < 0.0001$; N/g root: $X^2 = 18.02$, $df = 1$, $p < 0.0001$; Fig. 3.3C, G) than soils from the original range. The densities of Hoplolaimidae on roots differed also between plant communities ($X^2 = 22.83$, $df = 2$, $p < 0.0001$; Fig. 3.3F): unrelated range-expanders had more Hoplolaimidae per g root than natives ($X^2 = 10.83$; $p < 0.001$) and related range-expanders ($X^2 = 18.67$, $p < 0.0001$). Tylenchidae densities in soil were also affected by plant community type ($X^2 = 8.25$, $df = 2$, $p < 0.05$; Fig. 3.3C): both natives ($X^2 = 7.02$, $p < 0.01$) and related range-expanders ($X^2 = 7.92$, $p < 0.01$) had higher Tylenchidae densities than unrelated range-expanders. Neither plant community nor soil origin significantly affected numbers of *Paratylenchus* (Fig. 3.3D, H).

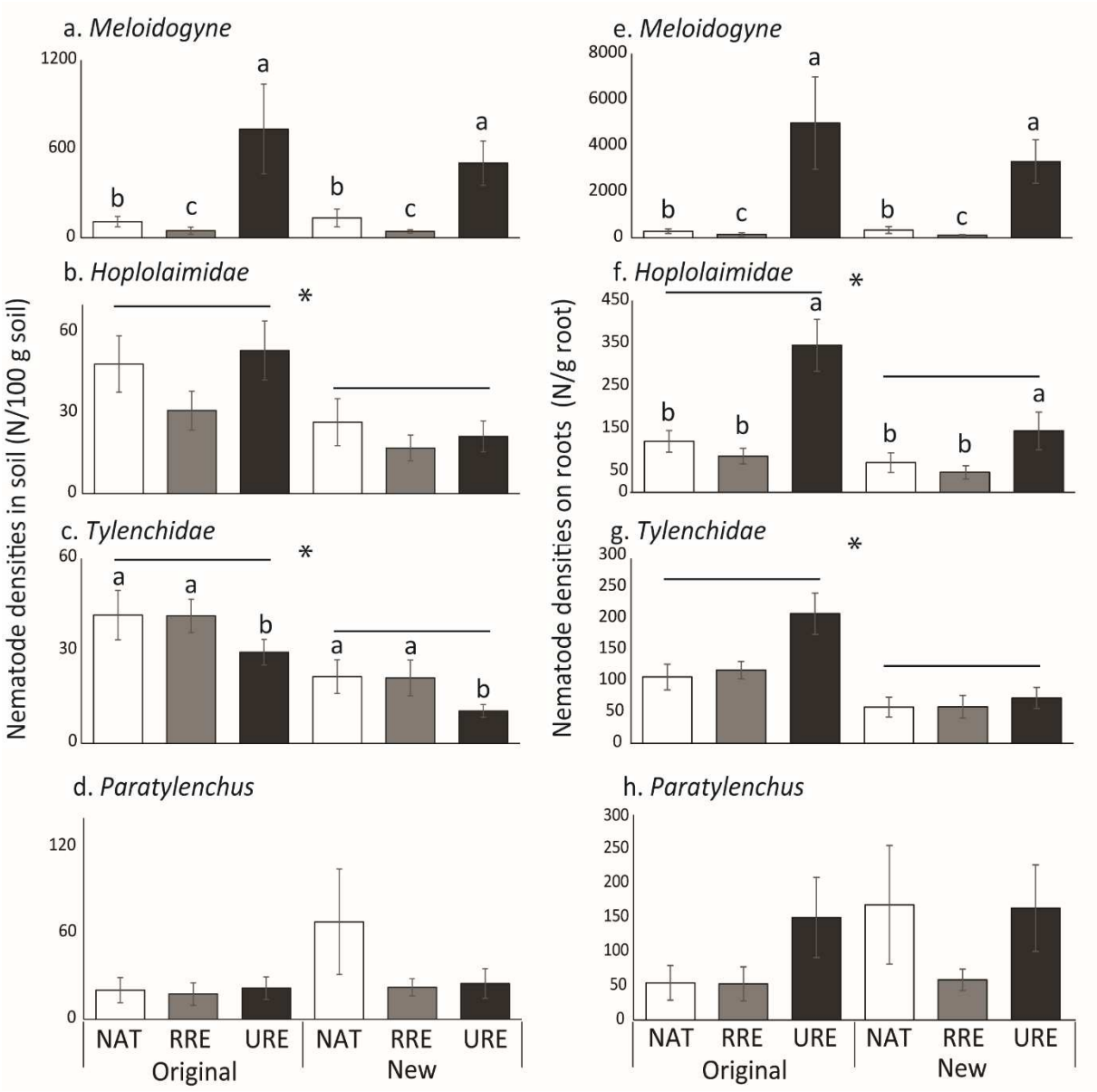

Fig. 3.3 Plant community effects on densities in soil (N/100 g dry soil) and on roots (N/g root) of root-feeding nematode groups *Meloidogyne* (a,e), Hoplolaimidae (b,f), Tylenchidae (c,g) and *Paratylenchus* (d,h) in soils from the original and new range. Different bars represent the communities of native plants (NAT; white), related range-expanders (RRE; light grey) and unrelated range-expanders (URE; dark grey). Bars represent averages ± standard errors. Horizontal bars and asterisks represent significant ($p < 0.05$) differences between soil origins and different letters indicate significant ($p < 0.05$) differences between plant communities within the ranges based on Negative binomial GLM and Post-hoc Wald tests.

Discussion

Climate warming-induced range-expanding plant species can experience weaker negative impact in soil from the new than from the original range (van Grunsven *et al.* 2010; De Frenne *et al.* 2014; Dostálek *et al.* 2015; Van Nuland, Bailey & Schweitzer 2017). This may be caused by the loss of belowground natural enemies, such as root-feeding nematodes and soil-borne pathogens, as a result of plants having higher dispersal capacities than soil biota (Berg *et al.* 2010; Morriën *et al.* 2010). However, biogeographic studies on soil-borne enemies along expansion gradients are scarce (Van Nuland, Bailey & Schweitzer 2017), and to our knowledge such studies are non-existent along intra-continental latitudinal gradients. Our study shows that, differently as hypothesized, for none of the plant communities there were evident differences in root-feeding nematode community composition between original and new range soils, suggesting that range-expanding plant species do not experience strong shifts in root-feeding nematode communities as a consequence of latitudinal range expansion. Between new and original range soils, we did observe differences in the community composition based on nematode feeding types, but only for related range-expanders. Therefore, our hypothesis of stronger nematode community shifts between the original and new range for unrelated range-expanders than for range-expanders with native relatives was not confirmed.

Plant community effects on root-feeding nematode community composition were not the same between the ranges. Most notably, in the new range the root-feeding nematode community composition of unrelated range-expanders was more distinct from the communities of natives and related range-expanders in the original range (Fig. 3.1B), suggesting distinct nematode responses to these phylogenetically distant plant species in the new range. Moreover, root-feeding nematode communities of natives and related range-expanders were more comparable in the new range than in the original range, suggesting nematode spill-over effects from natives to related range-expanders. In spite of this interactive effect between plant community and soil origin on the root-feeding nematode community composition, we did not find such significant interaction effects on densities of root-feeding nematodes or on root-feeding nematode groups (Figs. 3.2 and 3.3, respectively). This may indicate relatively subtle shifts in multiple root-feeding nematode groups that only could be detected when the full root-feeding nematode community was analyzed. Densities of Hoplolaimidae and

Tylenchidae were higher in soils from the original than from the new range, but these effects did not depend on plant community (Fig. 3.3) and therefore do not underlie the observed interactive effect. Also the interactive effect of plant community and soil origin on the nematode community composition based on nematode feeding types could not be explained by differences in in the densities of the different nematode feeding groups. Possibly, densities of predatory nematodes play a role in the statistical separation between the original and new range for the plant community of related range-expanders, but total predatory nematode densities were too low to reliably model in a univariate analysis.

The root-feeding nematode community of unrelated range-expanders differed from those of native and related range-expanders. These differences in nematode community composition may be explained by plant phylogeny, as the unrelated range-expanders belong to different genera than the natives and related range-expanders and therefore have different traits (Gilbert & Webb 2007). However, as the community of unrelated range-expanders was largely dominated by annuals, whereas the other two communities include mostly perennials (Koorem *et al.* 2017), it is also possible that nematode responses were the result of differences in plant life history strategies. Annual plant species are often early-successional colonizers known to develop strongly negative plant-soil feedbacks (Kardol, Bezemer & van der Putten 2006), which corresponds with the strong accumulation of root-feeding nematodes found in the plant community of unrelated range-expanders. While the plant species in the community of unrelated range-expanders had the smallest root systems (Table S3.2; Koorem *et al.* 2017), they accumulated the highest numbers of root-feeding nematodes, suggesting poor defence against nematodes. As a result, differences between plant communities were even stronger when nematode densities were expressed per gram of root (Fig. 3.3).

While intercontinentally exotic early colonizers have been shown to accumulate fewer natural enemies in their new than in their original range (Blumenthal *et al.* 2009), we found no such pattern in our study. Experimental comparisons between the group of unrelated range-expanders and native plant species with an annual life history strategy are needed in order to examine whether there is any benefit for this group of range-expanders over ecologically comparable native plant species in the new range. However, in order to examine the effects of ecological novelty associated with phylogenetic

distinctiveness (Strauss, Webb & Salamin 2006) in the context of climate-driven range expansion, future studies also need to focus on unrelated range-expanders with a perennial life history. Overall, our results emphasize that plant species' life histories need to be taken into account when analyzing effects of biotic interactions on range-expanding and exotic plant species.

As hypothesized, root-feeding nematodes were more clearly affected by the different plant communities than the other nematode feeding groups. The community of related range-expanders accumulated fewer root-feeding nematodes than their congeneric natives, which is in line with a study on range-expanding plant species in their new range soil (Morriën, Duyts & Van der Putten 2012). Our study, which considered responses of nematode communities from both the new and original range, shows that range-expanders also accumulate fewer root-feeding nematodes in soil from their original range than related species native in both areas of soil origin (Fig. 3.2). These results suggest that related range-expanders on average are better defended against root-feeding nematodes than related native species, regardless of the origin of the nematodes. This corresponds with a previous study showing that intracontinental range-expanders were better defended against an aboveground herbivore that was naïve to all of the examined plant species (Engelkes *et al.* 2008). However, all plants used in the study by Engelkes et al. (2008), as well as in the present study originated from seeds that were collected from the new range (The Netherlands). We therefore cannot exclude that the strong defence against root-feeding nematodes by these related range-expanders is the result of natural selection during range expansion for genotypes that are especially well-defended against generalist herbivores (Doorduin & Vrieling 2011; Lin, Klinkhamer & Vrieling 2015). Future experiments using plant populations from both the original and the new ranges of the range-expanders are needed in order to examine whether such shifts in plant defence traits may have occurred during climate-driven range expansion (Macel *et al.* 2017).

The nematode abundances presented in our study are the net effects of bottom-up and top-down control by both the plants and the micro-organisms present in the soils (Chapter 4). While bottom-up effects on nematode numbers are stronger than top-down effects, potential differences between the plant communities in their ability to attract natural enemies of root-feeding nematodes, such as bacteria, fungi and protists (Stirling *et al.* 1998; Piskiewicz *et al.* 2007; Geisen *et al.* 2015) could add

additional variation in root-feeding nematode accumulation. Interestingly, plant effects strongly differed between root-feeding nematode groups: while *Meloidogyne* and Hoplolaimidae densities strongly depended on the plant community, such differences were not found in *Paratylenchus* and Tylenchidae, indicating that the latter may be more generalistic and not strongly responsive to species-specific plant traits, such as root chemistry (Chapter 5). This could be due to their feeding strategy (Yeates *et al.* 1993): while *Meloidogyne* and Hoplolaimidae partly or completely feed inside the roots, *Paratylenchus* and Tylenchidae are ectoparasites or root-hair feeders and therefore may be less affected by defence chemistry.

We conclude that there are no consistent shifts in nematode community composition between the original and new range of range-expanding plant species, and that range-expanders do not accumulate fewer root-feeding nematodes in the new range than in the original range. Unexpectedly, the range-expanders without native congeners accumulated more root-feeding nematodes than the natives and their congeneric related range-expanders, but this might also be due to their annual life-history strategy. The community of congeneric related range-expanders was found to be the most suppressive to root-feeding nematodes compared to the natives, which may have benefitted their range expansion. Subsequent studies are needed where plant populations from both ranges will be included in the analysis, in order to elucidate the impact of range-expanding plant species on native soil communities.

Supplementary information

Table S3.1: overview of soil collections for original and new range soil. Coordinates for the collection sites are given for three sub-locations per collection area in each of the three countries where soil was collected. Non-sterilized soil from each sub-location was used in one of the 9 soil mixtures of either the original range (O) or the new range (N). Note that we accounted for potential abiotic between the new range and original range soil mixes by the addition of sterilized soil from the complementing range (see methods).

Range	Location ID	Sub-location	Coordinates	Mix
	Austria (AU)			
Original	1	AU 1.1	N 48.26557 E 13.24619	O1
		AU 1.2	N 48.30533 E 13.30192	O2
		AU 1.3	N 48.30513 E 13.30614	O3
	2	AU 2.1	N 48.32249 E 14.33575	O4
		AU 2.2	N 48.31162 E 14.33176	O5
		AU 2.3	N 48.32202 E 14.31814	O6
	3	AU 3.1	N 48.31063 E 14.33555	O7
		AU 3.2	N 48.31239 E 14.33491	O8
		AU 3.3	N 48.30348 E 14.33887	O9
	Slovenia (SL)			
Original	1	SL 1.1	N 46.37294 E 14.16777	O6
		SL 1.2	N 46.37294 E 14.16777	O7
		SL 1.3	N 46.37294 E 14.16777	O3
	2	SL 2.1	N 45.92891 E 15.50848	O1
		SL 2.2	N 45.92891 E 15.50848	O5
		SL 2.3	N 45.93038 E 15.49567	O2
	3	SL 3.1	N 46.13559 E 14.60972	O9
		SL 3.2	N 46.16527 E 14,75565	O8
		SL 3.3	N 45.96904 E 14.54572	O4
	The Netherlands (NL)			
New	1	NL 1.1	N 51.87657 E 6.00357	N1, N7
		NL 1.2	N 51.87937 E 6.00413	N2, N8
		NL 1.3	N 51.86766 E 5.99216	N3, N9
	2	NL 2.1	N 51.85399 E 5.88374	N1, N4
		NL 2.2	N 51.85884 E 5.88557	N2, N5
		NL 2.3	N 51.86067 E 5.89020	N3, N6
	3	NL 3.1	N 51.89423 E 5.63424	N4, N7
		NL 3.2	N 51.89265 E 5.64489	N5, N8
		NL 3.3	N 51.89569 E 5.64446	N6, N9

Table S3.2: Shoot and root biomass (g) of the plant communities 'natives' (NAT), 'related range-expanders' (RRE) and 'unrelated range-expanders' (URE) in original and new range soils.

	Shoot biomass (g)		**Root biomass (g)**	
Community (N=9)	Mean	±SE	Mean	±SE
NAT Original	10.93	0.44	16.64	0.76
NAT New	11.81	0.61	17.04	0.94
RRE Original	12.44	0.67	14.86	0.71
RRE New	13.33	0.64	16.22	0.47
URE Original	20.28	0.74	6.19	0.28
URE New	21.53	0.99	6.63	0.56

Chapter 4

Interspecific differences in nematode control between range-expanding plant species and their congeneric natives

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Abstract

Climate change enables range expansions of plants, animals and microbes to higher altitudes and latitudes. Plants may benefit from range expansion when they escape from natural enemies, however, range expansion becomes a disadvantage when plants become disconnected from organisms that control enemies in the new range. Here, we examined nematode control in the root zone of range-expanding plant species and congeneric natives. In a greenhouse, we determined bottom-up (by the plants) and top-down (by natural enemies of the nematodes) control of two root-feeding nematode species (*Helicotylenchus pseudorobustus* and *Meloidogyne hapla*) in the rhizospheres of two range-expanding species, *Centaurea stoebe* and *Geranium pyrenaicum*, and two congeneric natives, *C. jacea* and *G. molle*. Pots with plants growing in sterilized soil were inoculated with either a microbial soil community from the newly colonized natural habitat, or a mixture of native microbial nematode antagonists, or a mixture of these two communities. We tested the hypotheses that bottom-up control of root-feeding nematodes would be strongest in the root zone of range-expanders and that top-down control would be strongest in the root zone of native plant species. We observed profound intra- and interspecific differences in bottom-up and top-down control among all four plant species. Bottom-up control by the range-expanding plant species was either strong or weak. Top-down control by microbes was strongest in native *Centaurea*. The addition of a mixture of both microbial communities reduced control of *M. hapla* in the root zones of the native plant species, and enhanced its control in the root zones of range-expanding plant species. We conclude that there was species-specific bottom-up and top-down control of root-feeding nematodes among the four plant species tested. Range-expanding plant species influence their microbial rhizosphere community differently compared to native plant species, but top-down control in the root zone of natives was not systematically superior to that of range-shifting plant species.

Introduction

Recent climate warming has enabled altitudinal and latitudinal range expansions of many animal and plant species (Parmesan 2006; Chen *et al.* 2011). Such range expansions can lead to disruptions of co-evolved biotic interactions, as individual species shift range at contrasting rates (Berg *et al.* 2010). While some plant species, aboveground vertebrates and invertebrate species may be able to shift range relatively quickly, belowground organisms are likely to lag behind (Berg *et al.* 2010). Eventually, such complex interactions might become re-established in the new range, when slower range-expanding species colonize the new areas. However, it is currently unknown what happens in the initial phases of range-expansion, when plant species are colonizing new areas and encounter novel enemies and their antagonists, which are both non-adapted to the introduced plant species.

Some recent studies have shown that climate warming-induced range-expanding plant species or populations can be less strongly affected by belowground enemies in their new range than in their old range (van Grunsven *et al.* 2010; De Frenne *et al.* 2014). Moreover, these range-expanders may experience less negative effects of soil organisms in their new range than congeneric natives (van Grunsven *et al.* 2007; Engelkes *et al.* 2008). This suggests that range shifts result in a release from natural enemies, which has been proposed as an important cause of invasiveness of introduced exotic species (Keane & Crawley 2002; Mitchell & Power 2003). However, compared to exotic species introduced from geographically isolated areas, plant species expanding their range within a continent are less likely to be completely released from natural enemies as some of these enemies might be widespread in a larger geographical area.

Despite the presence of natural enemies, successful range-expanding plant species might have a benefit over native plants, as range-expanders have been shown to be more strongly defended against naïve aboveground herbivores than congeneric natives (Engelkes *et al.* 2008). This stronger defence against generalists by the range-expanding plant species could be due to increased resource allocation to general defence mechanisms, due to reduced specialist herbivore and pathogen pressure (Müller-Schärer, Schaffner & Steinger 2004; Joshi & Vrieling 2005; Oduor *et al.* 2011; Lin, Klinkhamer & Vrieling 2015). Additionally, range-expanders might possess certain allelochemicals in roots or

shoots, to which the native soil community is not well adapted (Cappuccino & Arnason 2006; Schaffner *et al.* 2011). Indeed, range-expanders produce more unique metabolites than related natives (Macel *et al.* 2014). Together, these defence mechanisms may provide the range-expanding plant species with a competitive benefit over native plant species as they suffer less from specialist herbivores and the generalists are not well adapted to their novel defence mechanisms (Bossdorf 2013; Uesugi & Kessler 2013).

Also belowground, range-expanding plants may be better defended against generalist herbivores from the new range than their native congeners. In soil from the new range, range-expanders indeed were shown to accumulate fewer root-feeding nematodes per unit root mass than congeneric species that are native in the new range (Morriën, Duyts & Van der Putten 2012). Such reduced densities of root-feeding nematodes might be due to either enhanced control by the plant roots (also named bottom-up, or resource control) or control by natural enemies (also named top-down or predator control), or a combination of both mechanisms. Previous studies in other systems have shown that bottom-up control by direct plant defence mechanisms (van der Stoel, Duyts & van der Putten 2006) and top-down control by fungi, bacteria, micro-arthropods and protists are all possible (Kerry 2000; Piskiewicz, Duyts & van der Putten 2008; Costa *et al.* 2012; Geisen *et al.* 2015). These control mechanisms can operate on nematodes in species-specific ways (Piskiewicz, Duyts & van der Putten 2008). Range-expanding plant species have been shown to accumulate different microbial communities in their rhizospheres compared to closely related natives (Morriën & van der Putten 2013). However, it is unknown whether these community differences have consequences for root-feeding nematode control, for example due to longer shared co-evolutionary histories of microbial nematode antagonists with native than with range-expanding plant species.

Here, we quantify and compare effects of top-down and bottom-up control of root-feeding nematodes in the rhizosphere of range-expanding plant species and congeneric natives. We tested the hypotheses that 1) if top-down control of nematodes by soil microbes is plant-species specific, we expect this control within congeneric pairs to be stronger in the native than in the range expander and 2) range-expanding plant species exert stronger bottom-up control on root-feeding nematodes than congeneric natives. In order to test the hypotheses, we conducted a greenhouse experiment to examine the

microbial control of two native generalist root-feeding nematode species, *Meloidogyne hapla* and *Helicotylenchus pseudorobustus,* in the rhizospheres of two range-expanding plant species and their native congeners. This experiment will provide insights in how complex multi-trophic interactions may function in the rhizospheres of climate-driven range-expanding plant species in their new range, and how these interactions differ from those of related native plant species. The experimental results will contribute to enhanced insights in how multi-trophic interactions of non-native plant species may become assembled in their new range.

Methods

Plant species and seed collection

We tested our hypotheses using two range-expanding plant species that originate from southern Europe, *Centaurea stoebe* L. and *Geranium pyrenaicum* Burm. f., and two congeneric species that are native in the newly colonized range in north-western Europe, *Centaurea jacea* L. and *Geranium molle* L. *Centaurea stoebe* originates from the Danube area and since the late 1990's invaded the Rhine valley and some suitable habitats in The Netherlands (NDFF 2017). *Geranium pyrenaicum* originally has a more widespread south-European distribution and although it colonized Northwestern Europe already in the 19th century, it only showed a strong expansion in the Netherlands since the 1980's, where it now is common (NDFF 2017). Both congeneric native species *C. jacea* and *G. molle* are also common throughout northern and southern Europe.

All seeds used for the present study originated from plant populations from the Netherlands. Seeds of *C. stoebe* and *G. molle* were collected directly from the field. Seeds of *C. jacea* originated from an experimental garden in Wageningen. They were collected from first generation plants grown from seeds of plants growing in Dutch field sites. Seeds of *G. pyrenaicum* were delivered by the seed production company Cruydthoeck (Nijeberkoop, The Netherlands), where plant species are cultured from seeds collected in Dutch field sites. Seeds of all plant species were surface-sterilized by washing them for 3 min in 10% bleach solution, after which they were rinsed with demineralized water, and germinated on glass beads in a growth cabinet (20/10 °C; 16 h light/8 h dark).

Nematode cultures

Two generalist root-feeding nematodes that commonly occur throughout Europe were extracted from cultures originating from Dutch field sites. An inoculum of the sedentary endoparasite *Meloidogyne hapla* Chitwood (hereafter referred to as *Meloidogyne*) was collected from a field near Bovensmilde (Drenthe, The Netherlands), subsequently cultured on tomato (*Solanum lycopersicum* L.) at PPO-AGV (Lelystad, The Netherlands) and extracted using a mistifier (Funnel-spray method; Oostenbrink 1960). A population of the ectoparasite *Helicotylenchus pseudorobustus* Steiner (hereafter referred to as *Helicotylenchus*), originating from coastal sand dunes, was cultured on Marram grass (*Ammophila arenaria* L.) at NIOO-KNAW (Wageningen, the Netherlands) and extracted using an Oostenbrink elutriator (Oostenbrink 1960).

Microbial inocula

We prepared three different microbial inocula and tested their effects on root-feeding nematode abundance on range-expanders and congeneric natives: a general microbial inoculum obtained from field soil, a specific nematode-antagonist inoculum and a combination of the two. The used field soil was collected from riverine grasslands where most of the plant species used in the present study are present in the immediate surroundings. To obtain the general microbial inoculum, we used a serial wet-sieving approach to establish a community of predominantly microbes <20µm (see: van de Voorde, van der Putten & Bezemer 2012). We used nine batches of 2 kg top soil collected from 3 sites (6 kg per site) in a riverine grassland (Wageningen, The Netherlands; 51˚57'N, 5˚39'E) that were mixed with 1.5 l demineralized water, stirred and left for 15 min. This stirring procedure was then repeated for each batch, after which the supernatant went through sieves with mesh sizes of 1 mm, 180 µm, 75 µm, 45 µm (twice) and 20 µm. Hence, we obtained 12.5 l inoculum with a general microbial wash from 18 kg of field soil.

The inoculum of nematode antagonists included three nematophagous fungi and the nematophagous amoeba *Cryptodifflugia operculata,* which was cultured on a mixed prokaryotic community in a liquid wheat grass medium (Geisen *et al.* 2015). The nematophagous fungi were obtained from field soil from a riverine grassland (Millingerwaard, Netherlands; 51˚52'N, 6 ˚0'E), by adding 0.1 g of soil to three

Petri dishes filled with water that contained a free-living nematode community from different trophic groups, which was collected from the same grassland. After one week, an inverted microscope (Olympus CK40) at 100 and 200 x magnification was used to detect killed or parasitized nematodes. Dead nematodes with hyphae or spores of potentially nematophagous fungal or oomycete origin were transferred individually to 1 % water agar for subsequent cultivation. Three well-growing monoclonal fungal cultures were selected and used for the experiment. We collected spores using a sterile metal cell-scraper after adding 1 ml double-distilled water. Spore numbers were determined using an inverted microscope (Olympus CK40) at 400x magnification. The amoebae were acquired by detaching one week old, well active cultures from the surface of five 10 cm Petri dishes by vigorous shaking. The amoebae-suspension then immediately was transferred to 50 ml centrifuge tubes and carefully centrifuged at 800rpm for 5 min. The supernatant was then decanted, after which the suspensions were pooled and enumerated. The three fungal and the amoebae cultures were combined and named nematode-antagonist inoculum. Each pot inoculated with the nematode-antagonist mixture received 1.4 ml suspension containing 1.6×10^6 *C. operculata* amoebae, as well as 3.4×10^6, 1.3×10^6 and 1.5×10^6 spores of fungal isolates Mil3, Mil4, and Mil5b, respectively.

Experimental set-up

A three-factor pot experiment was set up using 4 plant species (*C. jacea, C. stoebe, G. molle and G. pyrenaicum*), 3 nematode treatments (*Helicotylenchus, Meloidogyne* and a control without root-feeding nematodes), and 4 soil treatments (microbial inoculum, nematode antagonist inoculum, combined microbial and nematode antagonist inoculum and a control without live inoculum), with each treatment replicated 5 times, resulting in 240 pots. Sandy clay soil was collected from a former agricultural field in the riparian area of the same river system as Millingerwaard (Beneden-Leeuwen, The Netherlands; N51° 53.952, E05° 33.670). This soil was homogenized with sand (2:1 soil:sand) and sterilized using gamma-sterilization (McNamara *et al.* 2003; 25 KGray, Syngenta bv, Ede, The Netherlands). Pots of 1 L were filled with 830 g of the sterilized soil. Of each plant species 60 seedlings were planted in individual pots. After 10 days, two thirds of all pots were inoculated with 2 ml water suspension containing 200 juveniles of either *Meloidogyne* or *Helicotylenchus*. One third of all pots did not receive any nematodes. Next, microbial treatments were established: pots received either 50 ml of

the general microbial inoculum, 1.4 ml of the nematode-antagonist inoculum, or a combined inoculum of both the general microbial (50 ml) and the nematode-antagonist inoculum (1.4 ml). Control pots did not receive any live inoculum. To compensate for potential nutrient and moisture effects control pots received 50 ml sterilized general microbial inoculum and 1.4 ml sterilized nematode-antagonist inoculum, pots containing the general microbial community received sterilized 1.4 ml nematode-antagonist inoculum and pots with the nematode-antagonist community received 50 ml sterilized general microbial inoculum. The pots were placed in a greenhouse compartment at 16 h light (20 °C), 8 h dark (15 °C) and 60% relative humidity according to a randomized block design on carts, which were rotated weekly. Throughout the experiment the pots were watered twice per week. Once a week, pots were reset to a weight of 860 g by adding demineralized water, representing a moisture content of approximately 15 %.

Harvest

Fifteen weeks after inoculation, the aboveground plant parts were harvested and dried at 70 °C until constant weight. Subsequently, all soil from every pot was collected for nematode extraction, and 2-ml centrifuge tubes with well-homogenized soil were stored at -20 °C for DNA extraction. To reduce the loss of nematodes from the rhizosphere, roots were first washed in 200 ml water, after which the washout was stored at 4 ˚C until nematode extraction. Root systems from *Helicotylenchus* pots were placed in a mistifier (Funnel-spray method; Oostenbrink 1960) for 24 hours to extract remaining root-attached nematodes of this ectoparasitic species. The roots were dried at 70 °C until they reached constant weight. Root systems from pots containing the endoparasitic *Meloidogyne* were split: one half was placed in a mistifier for 4 weeks in order to extract nematodes from developing eggs inside the roots, and the other half was weighed fresh, dried at 70 °C until constant weight, and weighed again. Once per week nematodes were collected from the mistifier and stored at 4 °C. After 4 weeks, all nematode subsamples harvested from the same root sample were combined into one single pot and concentrated to 10 ml. For both the pots with *Helicotylenchus* or *Meloidogyne*, as well as 3 replicates of the non-nematode treatments, free-living nematodes were extracted from the bulk soil and the rhizosphere soil suspension using an Oostenbrink elutriator (Oostenbrink 1960), and concentrated to 10 ml prior to counting.

Nematode counting

Nematodes were counted alive using an inverted microscope (Olympus CK40, 40x and 100x magnification). Either the full sample was counted or, in case of high densities, 2 subsamples of 1 ml, each diluted 10 times. During nematode counting, all samples were carefully checked for contamination with other root-feeding nematodes. Because of contamination with *Meloidogyne*, 2 samples from pots inoculated with *Helicotylenchus* were excluded from further analysis. In all samples bacterivorous nematodes were found, which could originate from both co-inoculations of bacterivorous nematode eggs with the microbial inocula and natural colonization of the pots via air.

Bacterial and fungal quantification

We quantified bacteria and fungi using quantitative (q)PCR in the pots containing *Meloidogyne*, as we found stronger inoculum effects on this nematode species than on *Helicotylenchus*. Soil DNA was extracted using the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc, Carlsbad, USA) and stored at -20 °C. Bacterial 16s rDNA copy numbers were quantified using the primer combination 515F and 806R (Caporaso *et al.* 2011). The qPCR mastermix contained 0.25 µl BSA (Roche Diagnostics, Basel, Switzerland), 10 µl SensiFAST SYBR® No-ROX (Bioline, Taunton, USA), 0.25 µl 515F (10 uM; Apha DNA, Montréal, Canada), 0.25 µl 806R (10 uM; Apha DNA) and 5 µl DNA template in a total volume of 20µl. Cycling conditions were the following: initiation for 3 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 50 °C, 1 min at 72 °C with a final elongation for 5 min at 72 °C). Fungal ITS copy numbers were quantified using the primer combination ITS4 and ITS9 targeting the fungal ITS2 region (White *et al.* 1990; Ihrmark *et al.* 2012). The qPCR mix contained 1 µl $MgCl_2$ (Roche Diagnostics), 0.25 µl forward ITS4 primer (30 uM; Alpha DNA), 0.25 µl reverse ITS9 primer (30 uM; Alpha DNA), 10 µl SensiFAST SYBR® No-ROX, 5 µl DNA template in a total volume of 20 µl. Cycling conditions were the following: initiation for 3 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 1 min at 72° with a final elongation for 5 min at 72 °C). Both qPCR approaches were replicated twice for each sample. Analyses of the qPCRs were done using Biorad CFX manager (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The average number of PCR-cycles needed to reach a threshold value determined by the software was used to calculate total abundances of bacteria

(16S rDNA copy numbers) and fungi (ITS2 copy numbers) in each sample. The ratio between the inverse of these abundance measures was used to calculate the bacterial/fungal-ratio.

Statistical analyses

All statistical analyses were performed in R Studio (Version 0.98.507; R Core Development Team 2012). Nematode count data were analyzed using negative binomial generalized linear models, as the data were strongly overdispersed (Hilbe 2014). *Helicotylenchus* and *Meloidogyne* counts were analyzed separately. We modeled total numbers per pot and numbers per gram root as the response of each nematode species to the fixed factors block, plant species and inoculum, as well as to the interaction between plant species and inoculum. Because of the use of only 2 species pairs, we did not include the factor origin (range-expander or native). As negative binomial generalized linear models have to be provided with integer values, and *Helicotylenchus* numbers were low, we expressed *Helicotylenchus* numbers per 10 gram root to avoid introduction of zeroes in the model. Model fit was checked using residual plots and AIC-values. Using post-hoc Wald tests performed with the R-package *phia* (De Rosario-Martinez 2013) we determined for each plant species the pairwise differences in nematode numbers between the different inocula and overall differences in nematode numbers between plant species. A general linear model and subsequent post-hoc Wald tests were used to test the effects of nematode species, inoculum and plant species on total plant biomass data. Two-way ANOVA models were used to analyze the effect of plant species and inocula on the relative abundance of bacteria, fungi and the bacterial/fungal ratio for the pots inoculated with *Meloidogyne hapla*. Residual plots and Shapiro-Wilk normality tests were used to confirm that model assumptions were not violated.

Results

Plant biomass

There was a significant main treatment effect of inoculum addition ($F= 2.68$, $p < 0.05$), because plants receiving the combined microbial and nematode antagonist community produced significantly less biomass than plants receiving the nematode-antagonist and the microbial communities alone (Fig. S4.1). However, this effect size was relatively minor.

Root-feeding nematode numbers

Meloidogyne hapla: We found strong differences in total *Meloidogyne* numbers among plant species (X^2 = 111.89, *df* = 3, p < 0.001). Total numbers of *Meloidogyne* were significantly (X^2 = 434.54, *df* = 1, p < 0.01) higher in native *C. jacea* than in range-expanding *C. stoebe* (Fig. 4.1). *Meloidogyne* also performed significantly poorer on *C. stoebe* than on both native and range-expanding *Geranium* species (Fig. 4.1). However, the range-expander *G. pyrenaicum* was a better host for *Meloidogyne* than the native *G. molle* (X^2 = 51.76, *df* = 1, p < 0.01; Fig. 4.1). Effects of plant species on the total numbers of *Meloidogyne* depended on soil inoculum (interaction effect: X^2 = 86.53, *df* = 9, p < 0.01). The nematode-antagonist community significantly reduced *Meloidogyne* numbers in *C. jacea* (X^2 = 4.58, *df* = 1, p < 0.05; Fig. 4.2A). This reduction, however, disappeared when the nematode antagonists were added in combination with the general microbial community; in that case *Meloidogyne* numbers were significantly higher than in pots with only the nematode antagonist community (X^2 = 5.91, *df* = 1, p < 0.05; Fig. 4.2A). There were no strong inoculum effects in the root zone of *C. stoebe*. However, in this species, the combined microbial and nematode antagonist community significantly reduced *Meloidogyne* numbers compared to the general microbial community (X^2 = 8.94, *df* = 1, p < 0.01; Fig. 4.2B). In *G. molle*, pots with nematode antagonists added had significantly lower numbers of *Meloidogyne* than pots with the combined microbial and nematode antagonist community added (X^2 = 4.65, *df* = 1, p < 0.05; Fig. 4.2C). In *G. pyrenaicum* the opposite pattern ocurred: pots with the combined microbial and nematode antagonist community had lower numbers of *Meloidogyne* than pots with only nematode antagonists (total: X^2 = 4.24, *df* = 1, p < 0.05; Fig. 4.2D). Overall, patterns of *Meloidogyne* numbers per gram root strongly corresponded with total *Meloidogyne* numbers per pot with some minor exceptions: while *C. jacea* was found to accumulate the highest *Meloidogyne* numbers per pot, numbers of *Meloidogyne* per gram root were higher in *G. pyrenaicum* than in *C. jacea* (Fig. S4.2). Furthermore, in *G. molle,* pots inoculated with the nematode antagonists did not have lower *Meloidogyne* numbers per gram root than pots inoculated with the combined microbial and nematode antagonist community (Fig. S4.3).

Helicotylenchus pseudorobustus: Numbers of the ectoparasite *Helicotylenchus* in all 4 plant species were substantially lower than numbers of *Meloidogyne* (Fig. 4.1). Nevertheless, we found a significant plant species effect on total *Helicotylenchus* numbers (X^2 = 104.85, *df* = 3, p < 0.01); there were significantly higher numbers of *Helicotylenchus* on *G. pyrenaicum* than on all other plant species (all p-values <0.01). Effects of plant species on total numbers of *Helicotylenchus* depended on soil inoculum (significant species*inoculum interaction; X^2 = 85.11, *df* = 9, p < 0.05). Inoculum type did not have a significant effect on numbers of *Helicotylenchus* in both native species *C. jacea* and *G. molle* (Fig. 4.2E, G). In *C. stoebe*, nematode antagonists significantly reduced total numbers of *Helicotylenchus* compared to adding the combined microbial and nematode antagonist community (X^2 = 5.22, *df* = 1, p < 0.05; Fig. 4.2F). In *G. pyrenaicum*, the total number of *Helicotylenchus* was significantly lower in pots with the combined microbial and nematode antagonist community than in pots with the general microbial inoculum (X^2 = 6.66, *df* = 1, p < 0.01), or in pots with the nematode antagonists (X^2 = 6.01, *df* = 1, p < 0.05; Fig. 4.2H). *Helicotylenchus* densities per gram root were significantly different among plant species (all p-values < 0.05; Fig. S4.2). Both range-expanding plant species contained more *Helicotylenchus* per gram root than their native congenerics (Fig. S4.2), and plant species effects did not depend on inoculum, which differs from the data on total numbers per pot. There was also no main effect of inoculum when *Helicotylenchus* densities were expressed as numbers per g root.

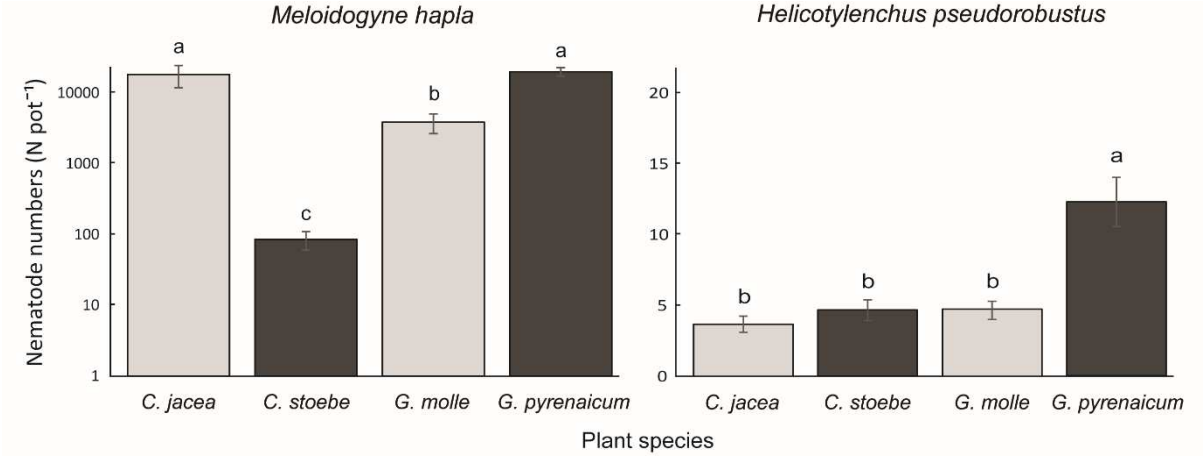

Fig. 4.1 Mean total numbers (N pot^{-1}) of root-feeding nematodes *Meloidogyne hapla* (left; logarithmic scale) and *Helicotylenchus pseudorobustus* (right; linear scale) on range-expanding (black) plants *Centaurea stoebe* and *Geranium pyrenaicum* species and related natives *Centaurea jacea* and *Geranium molle* (grey). Vertical bars show means ± standard errors. Different letters indicate significant (p < 0.05) pairwise post-hoc Wald tests between plant species.

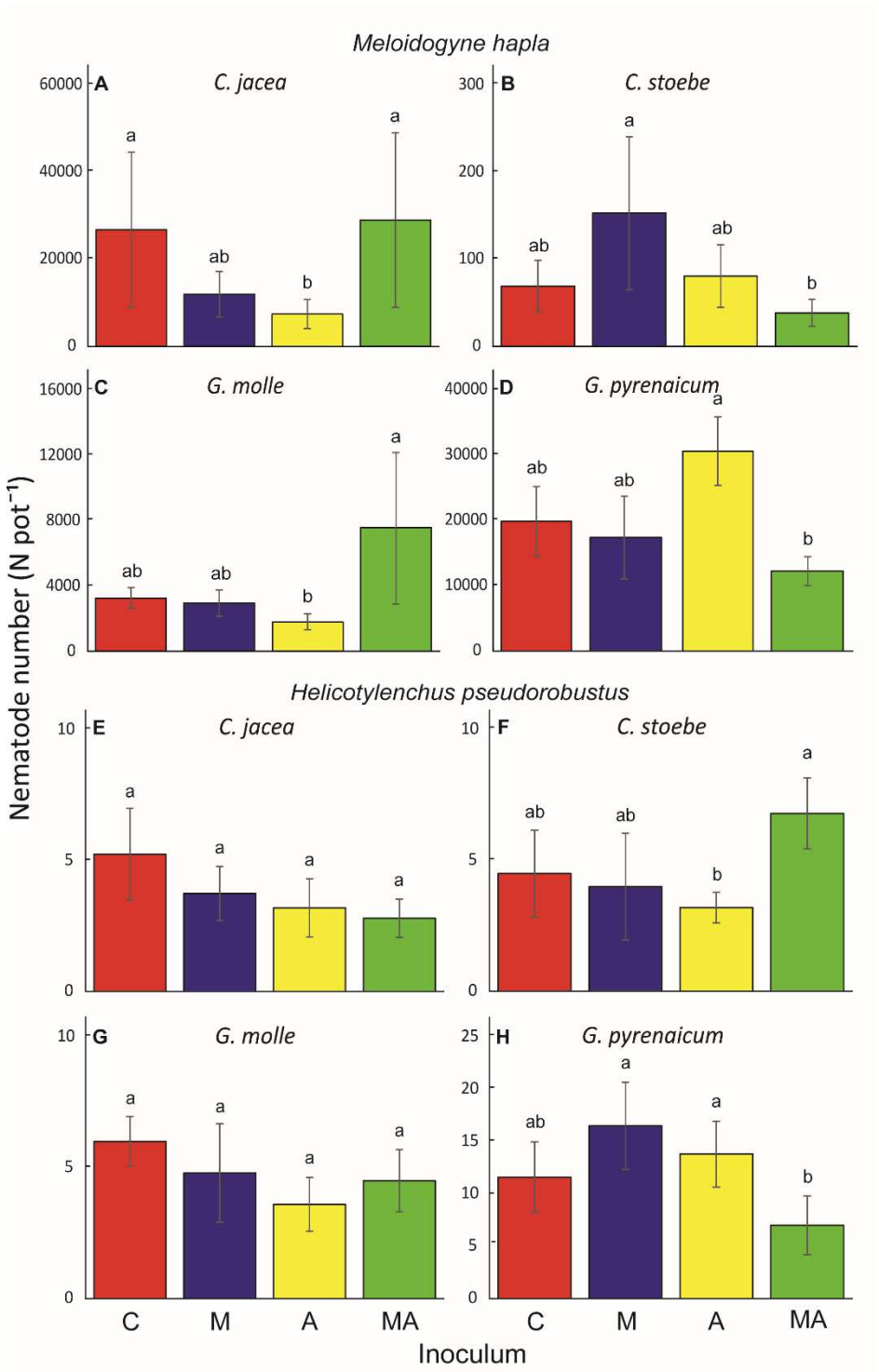

Fig. 4.2 Microbial inoculum effects on mean total numbers of root-feeding nematodes *Meloidogyne hapla* (A,B,C,D) and *Helicotylenchus pseudorobustus* (E,F,G,H) on native plant species *Centaurea jacea* and *Geranium molle* (left) and range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum* (right). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species. Per panel, the four bars represent following inoculum treatments: control (C; red), general microbial community (M; blue), nematode antagonists (A; yellow) and the mixed community (MA; green).

Bacterial and fungal abundances

Between plant species, abundances of soil bacteria, expressed as 16S rDNA copy numbers, were significantly different ($F_{3,63} = 3.18$, $p < 0.05$; Fig. 4.3). *Geranium* species harboured more bacteria than *Centaurea* species, whereas differences within species pairs were not significant. Fungal abundances, based on ITS copy numbers, depended on a combination of plant species and soil inoculum (species*inoculum interaction $F_{9,63} = 2.19$, $p < 0.05$). *Centaurea stoebe* had fewer fungi in the control than in the three soil inoculation treatments (all p-values < 0.05), and fungal abundance was lower in the combined microbial and nematode antagonist community than in the nematode-antagonist community ($F = 4.91$, $df = 1$, $p < 0.05$; Fig. 4.4). The *C. stoebe* control treatment had a lower fungal abundance than the control treatments of *C. jacea* ($F = 8.71$, $df = 1$, $p = 0.052$) and *G. molle* ($F = 11.82$, $df = 1$, $p < 0.01$; Fig. S4.4). Overall, the bacterial/fungal ratio was significantly ($F = 3.45$, $p < 0.05$) influenced by soil inoculation, and the bacterial/fungal ratio in the nematode antagonist treatment was significantly lower than in the control and other inoculum treatments (Fig. 4.5). This change in bacterial/fungal ratio occurred due to both a relatively low bacterial abundance and a relatively high fungal abundance.

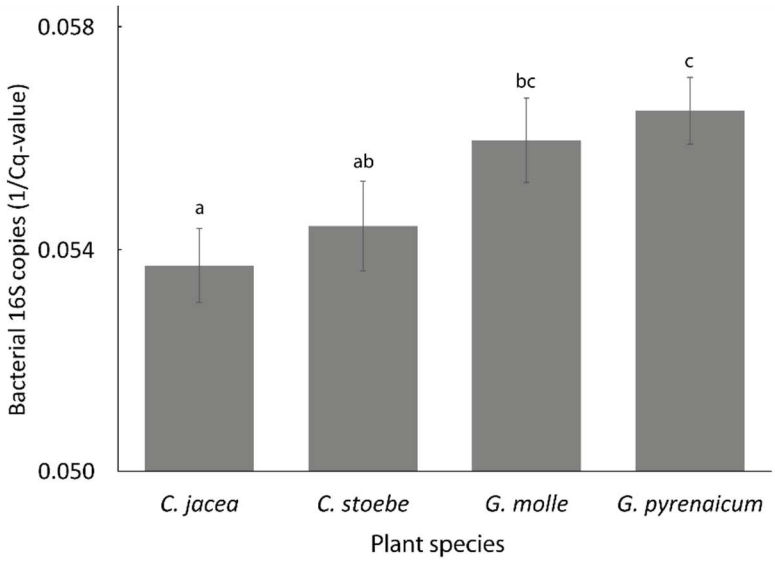

Fig. 4.3 Plant species effects on bacterial abundances (1/qPCR threshold value) of native plant species *Centaurea jacea* and *Geranium molle* and range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum*. Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species per inoculum treatment.

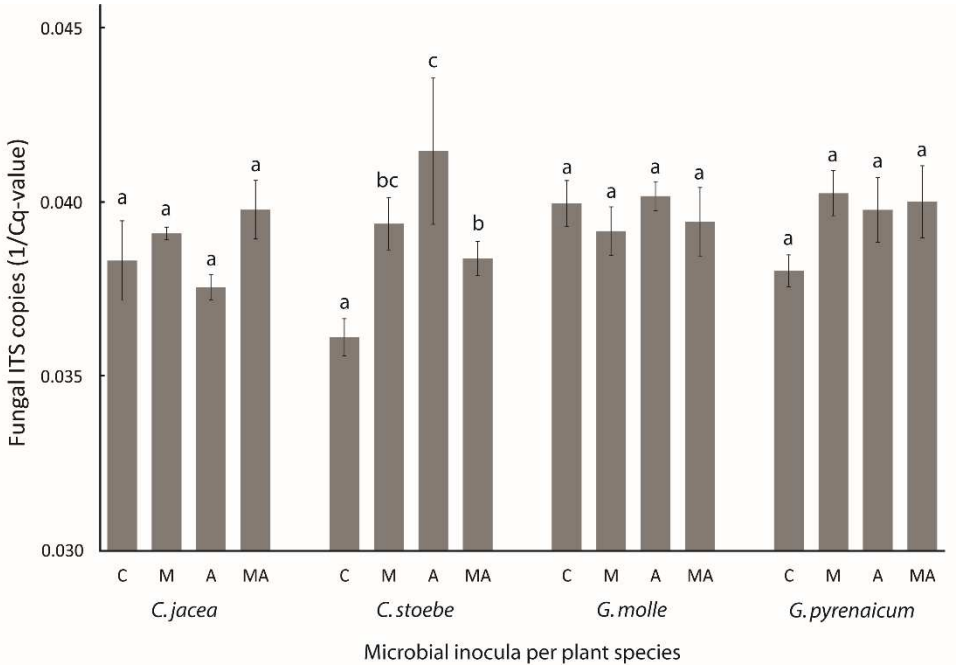

Fig. 4.4 Microbial community effects on fungal abundances (1/qPCR threshold value) per plant species. Vertical bars show means ± standard errors**.** Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between inoculum treatments per plant species. Microbial treatments are abbreviated: control (C), general microbial community (M), nematode antagonists (A) and the combined microbial and nematode antagonist community (MA).

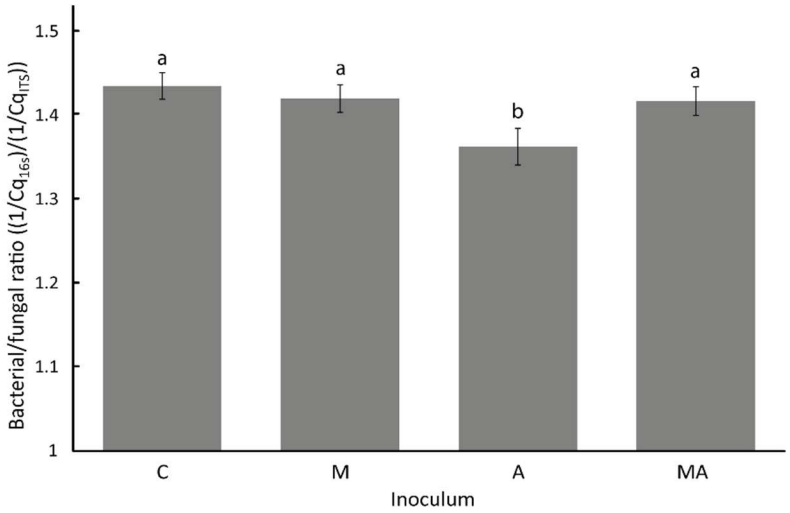

Fig. 4.5 Ratios of bacterial and fungal abundances per inoculum treatment, quantified by the ratio between the inverse qPCR Cq-values of bacterial 16s and fungal ITS copy numbers. Vertical bars show means ± standard errors**.** Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between inoculum treatments. X-axis labels represent inoculum treatments: control (C), general microbial community (M), nematode antagonists (A) and the mixed community (MA).

Discussion

Our results show species-specific patterns of bottom-up and top-down control of generalist root-feeding nematodes, both between and within two pairs of range-expanding and related native plant species. Our hypothesis that bottom-up control of root-feeding nematodes is stronger in the root zone of range-expanding plant species than of their congenerically related natives was supported in the case of the range-expander *C. stoebe*. This plant species had considerably stronger bottom-up defence against the endoparasite *Meloidogyne* than the congeneric native *C. jacea* (Fig. 4.1). However, *Meloidogyne* showed stronger multiplication on roots of the range-expanding *G. pyrenaicum* than on the native *G. molle* (Fig. 4.1). *Geranium pyrenaicum* was also a better host for the ectoparasitic *Helicotylenchus* than *G. molle*. *Helicotylenchus* numbers did not differ between the two *Centaurea* species. When expressed per unit of root weight, *Helicotylenchus* densities tended to be higher on both range-expanders than on related natives (Fig. S4.2), which is not in support of our hypothesis. On all plant species, numbers of *Helicotylenchus* were relatively low.

Although range-expanding plant species are thought to benefit when released from their specialized soil-borne enemies after latitudinal range-expansion (van Grunsven *et al.* 2010; De Frenne *et al.* 2014), plants will still be exposed to natural enemies in the new range, including widespread generalist enemies. Both *Meloidogyne hapla* and *Helicotylenchus pseudorobustus* are widespread throughout Europe (Bongers 1988), which does not exclude a co-evolutionary history with all four plant species. However, the limited dispersal capacity of nematodes and low gene flow between nematode populations (Blouin, Liu & Berry 1999) could have led to local adaptation of the nematodes to native plant species of Northwest European populations. A similar event of local adaptation of a natural enemy was also found for range-expanding butterflies and their parasitoids in Great Britain (Menendez *et al.* 2008). Therefore, plant-nematode interactions that are established when range-expanding plant species encounter individuals of these root-feeding nematodes populations in newly colonized areas, may at least to some extent result in novel interactions when the plants encounter non-adapted populations of the same herbivores in the new range.

Both strong suppression by, or release from aboveground and belowground herbivores has been argued a possible outcome of novel plant-herbivore interactions, as both plant and herbivore might be maladapted to their new host or enemy (Verhoeven *et al.* 2009). The strong bottom-up control of *Meloidogyne* by *C. stoebe* corresponds with low levels of herbivory on this plant species as found in several studies in North America (Cappuccino & Carpenter 2005; Schaffner *et al.* 2011), where *C. stoebe* is an invasive exotic. Native generalist moths grow poorer on *C. stoebe* than European generalists (Schaffner *et al.* 2011). Moreover, *C. stoebe* is less prone to aboveground herbivory than the non-invasive exotic *C. jacea* (Cappuccino & Carpenter 2005), indicating that *C. stoebe* mayproduce secondary compounds to which the native community is not adapted. In our study, the strong bottom-up control of *Meloidogyne* by *C. stoebe* suggests a similar maladaptation of the nematode to the root compounds of this plant species. Interestingly, we also found evidence for lower fungal abundances in the control soils of *C. stoebe* than in control soils grown with the other plant species, suggesting an inhibiting effect of *C. stoebe* root compounds or exudates on fungal growth. In contrast to the strong direct defence of *C. stoebe,* the high *Meloidogyne* numbers found in *G. pyrenaicum* point to a non-existent or weak bottom-up defence of the plant, allowing herbivores associated with related native plants to easily exploit the new host (Louda *et al.* 1997).

We found strong plant species-specific effects on top-down control of both root-feeding nematode species (Fig. 4.2). We expected the microbial communities to have strong nematode control potential in the rhizospheres of the native plant species. However, the nematode antagonist community effectively controlled *Meloidogyne* numbers only in the root zone of the native *C. jacea*. Therefore, we found mixed evidence to support the hypothesis that top-down control of root-feeding nematodes is strongest in native plant species. Remarkably, unlike in other experiments on nematode control by microbial communities (Piskiewicz *et al.* 2007; Viketoft & van der Putten 2014) there was no effective top-down control of the two root-feeding nematode species by the general microbial inoculum. Interestingly, the controlling effect of the nematode antagonists in the root zone of *C. jacea* was lost when they were added in combination with the general microbial community (Fig. 4.2). In both native plant species, numbers of *Meloidogyne* were higher in the presence of the combined microbial and nematode antagonist community than in pots with the nematode-antagonist community alone.

Possibly the nematode antagonists could have been outcompeted by micro-organisms from the general microbial community resulting in a reduced top-down control of the nematodes. Alternatively, as none of the inoculated nematode antagonists are obligatory nematophagous (the three fungi can grow purely saprophytically, the amoeba merely on bacteria and fungi (Geisen *et al.* 2016)), they could predominantly feed on other food sources in the presence of a diverse microbial community, thereby releasing the nematodes from their control. Interestingly, in both range-expanders *Meloidogyne* numbers were found to be reduced by the combined microbial and nematode antagonist community compared to the other microbial communities, suggesting a synergistic effect of potential nematode antagonists from both communities.

Top-down control of *Helicotylenchus* differed from *Meloidogyne*. While there were no top-down control effects in both native plants, *Helicotylenchus* was effectively controlled in the root zone of *G. pyrenaicum*, both by the combined microbial and nematode antagonist community and by the nematode-antagonist community in *C. stoebe* (Fig. 4.2). The overall differences in top-down control patterns of two root-feeding nematode species in four plant species indicate that interactions between soil microbes, nematode antagonists and root-feeding nematodes are strongly plant and also nematode species-specific. Such plant species-specific interactions in the rhizosphere can probably be best explained by plant species-specific root chemistry, influencing rhizosphere communities differently (Shi *et al.* 2011), by which top-down control of nematodes is altered. As bacterial or fungal abundances do not seem to explain differences in root-feeding nematode abundances, it is likely that interspecific differences in top-down control effects on root-feeding nematodes are caused by differences in the microbial rhizosphere community composition rather than sheer microbial abundances (Fig. 4.3-5).

In a recent study (Viketoft & van der Putten 2014) native microbes showed effective top-down control of root-feeding nematodes in the root zones of both native and range-expanding plant species, although top-down control effects were highly plant species-specific. We show such plant species-specific top-down control effects as well, but we also show that range-expanding plant species interact with their microbial community differently than their related natives. As a result, patterns of top-down (and bottom-up) control turned out to be highly species-specific. As in the experiment of Viketoft and

van der Putten (2014) root-feeding nematodes did not decrease plant biomass. Only plants treated with the combined microbial and nematode antagonist community tended to produce less plant biomass, potentially caused by an increased competition for nutrients between the plants and the microbial community (Clarholm 1985) or by mild pathogenic effects only affecting plant biomass when the combined microbial and nematode antagonist community was added. The absence of a negative effect of *Meloidogyne* on plant biomass might be explained by the low nematode densities in the early phases of the experiment. The strong differences in nematode densities between the root zones of native *C. jacea* and range-expanding *C. stoebe* that build up over the course of time might have strong effects on next generations of conspecifics, but we did not test such feedback effects.

In conclusion, we show that range-expanding plant species influence top-down control of root-feeding nematodes in their root zones differently than related native plant species. Our results add to the findings that range-expanding plant species accumulate different soil microbial communities compared to related native species (Morriën & van der Putten 2013), as we provide novel evidence that these different soil communities affect root-feeding nematodes differently. Furthermore, we show that bottom-up control of root-feeding nematodes can both be strong and weak in the root zones of range-expanding plant species. The root-feeding nematode abundance patterns indicate that range-expanding plant species influence root-feeding nematode populations in a plant species-specific manner, which likely will result in strongly different plant-soil feedback outcomes. Range-expanding plant species that escaped their specialized enemies and have strong defence mechanisms, even against generalist nematodes, could eventually become increasingly abundant. Thereby they might negatively influence the native vegetation, while other range-expanding plant species are more likely to develop similar negative plant-soil feedbacks as related natives.

Supplementary information

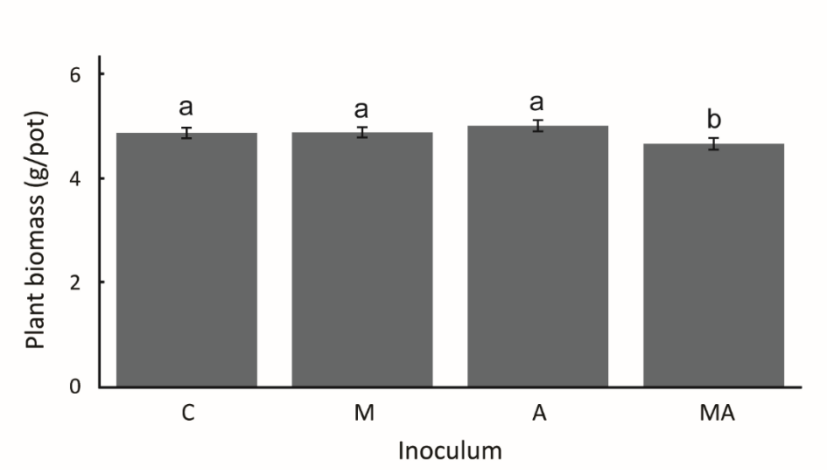

Fig. S4.1 Plant biomass (shoots + roots) in response to the four different inoculum treatments: control (C), general microbial community (M), nematode antagonists (A) and the mixed community (MA). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between inocula.

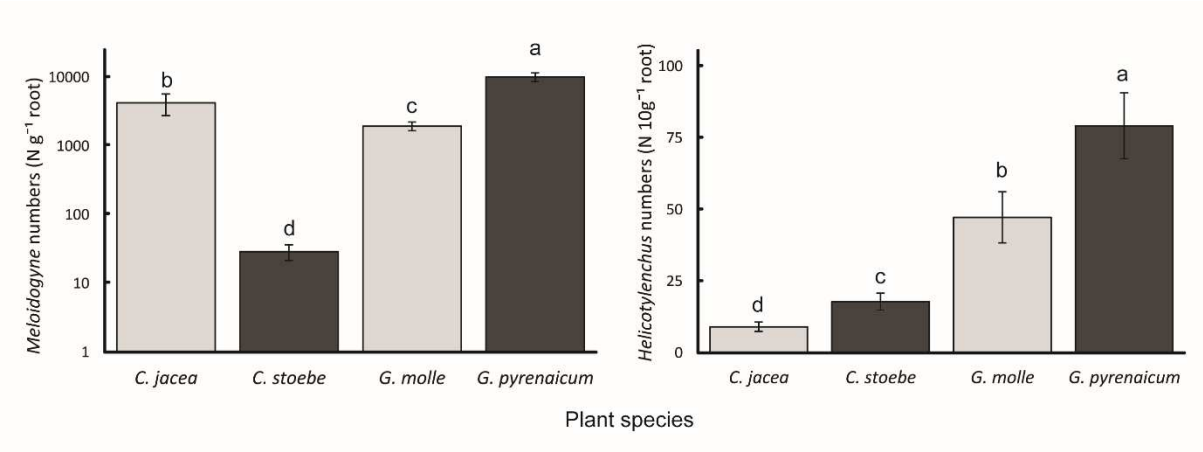

Fig. S4.2 Relative numbers of root-feeding nematodes *Meloidogyne hapla* (left; N g root^{-1}) and *Helicotylenchus pseudorobustus* (right; N 10 g root^{-1}) on range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum* (black) and related natives *Centaurea jacea* and *Geranium molle* (grey). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species.

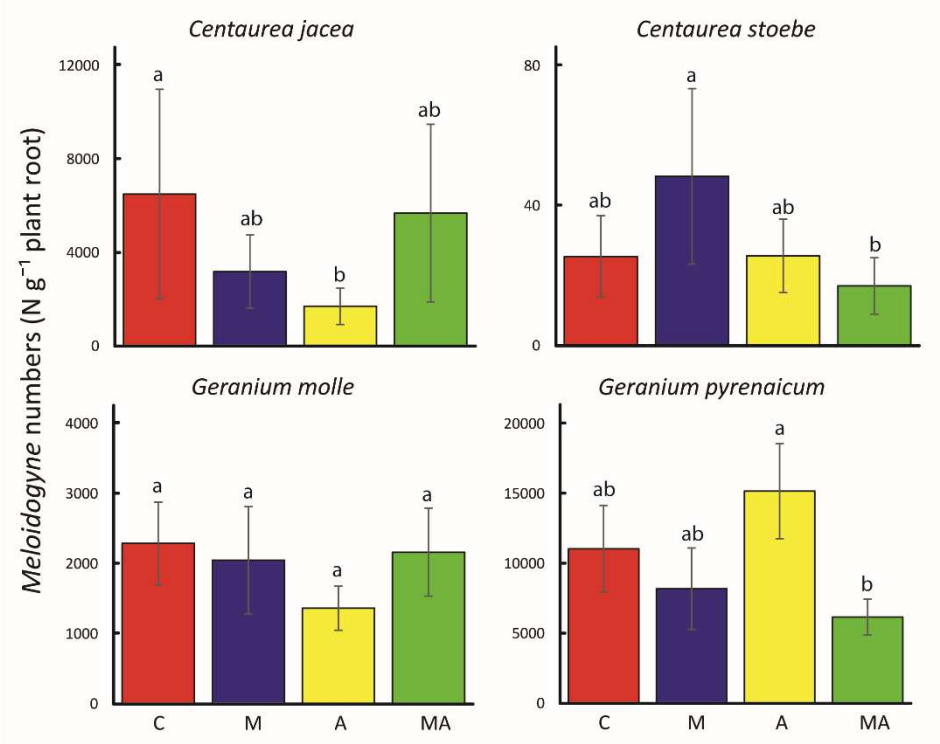

Fig. S4.3 Microbial inoculum effects on numbers of *Meloidogyne hapla* per gram root of native plant species *Centaurea jacea* and *Geranium molle* (left) and range-expanding plant species *Centaurea stoebe* and *Geranium pyrenaicum* (right). Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species. Per panel, the four bars represent following inoculum treatments: control (C; red), general microbial community (M; blue), nematode antagonists (A; yellow) and the mixed community (MA; green).

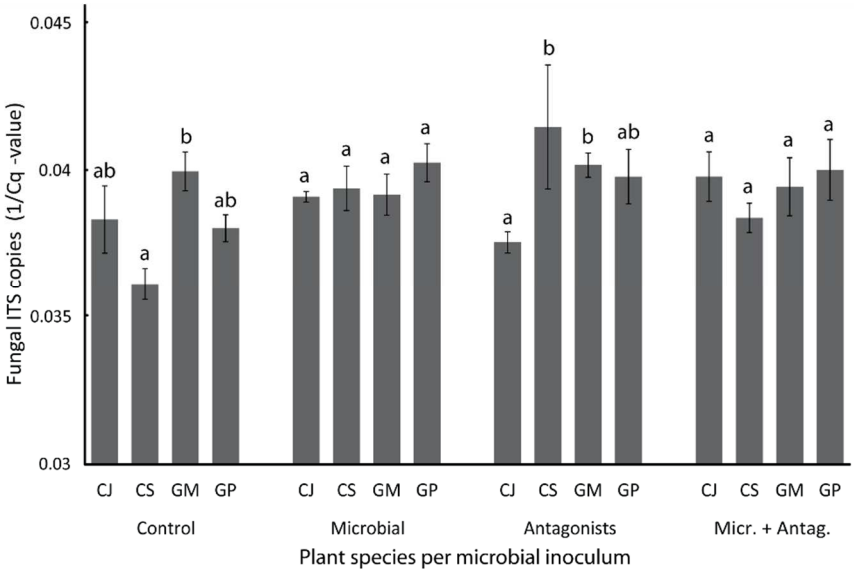

Fig. S4.4 Plant species effects on fungal abundances (1/qPCR threshold value) per inoculum treatment. Vertical bars show means ± standard errors. Different letters indicate significant ($p < 0.05$) pairwise post-hoc Wald tests between plant species per inoculum treatment. Plant species names are abbreviated: *Centaurea jacea* (CJ), *Centaurea stoebe* (CS), *Geranium molle* (GM) and *Geranium pyrenaicum* (GP).

Chapter 5

Belowground plant-herbivore interactions vary among climate-driven range-expanding plant species with different degrees of novel chemistry

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Abstract

An increasing number of studies report plant range expansions to higher latitudes and altitudes in response to global warming. However, consequences for interactions with other species in the novel ranges are poorly understood. Here, we examine how range-expanding plant species interact with root-feeding nematodes from the new range. Root-feeding nematodes are ubiquitous belowground herbivores that may impact the structure and composition of natural vegetation. Because of their ecological novelty, we hypothesized that range-expanding plant species will be less suitable hosts for root-feeding nematodes than native congeneric plant species. In greenhouse and lab trials we compared nematode preference and performance of two root-feeding nematode species between range-expanding plant species and their congeneric natives. In order to understand differences in nematode preferences, we compared root volatile profiles of all range-expanders and congeneric natives. Nematode preferences and performances differed substantially among the pairs of range-expanders and natives. The range-expander that had the most unique volatile profile compared to its related native was unattractive and a poor host for nematodes. Other range-expanding plant species that differed less in root chemistry from native congeners, also differed less in nematode attraction and performance. We conclude that the three climate-driven range-expanding plant species studied varied considerably in their chemical novelty compared to their congeneric natives, and therefore affected native root-feeding nematodes in species-specific ways. Our data suggest that through variation in chemical novelty, range-expanding plant species may vary in their impacts on belowground herbivores in the new range.

Introduction

One of the most evident ecological consequences of current climate change is the latitudinal and altitudinal range expansion of many plant and animal species (Walther *et al.* 2002; Parmesan 2006; Le Roux & McGeoch 2008). As not all species expand their range at similar rates (Berg *et al.* 2010), coevolved interactions between plants, aboveground and belowground organisms are likely to become disrupted, whereas novel interactions can be developed in the new range (Lavergne *et al.* 2010; van der Putten 2012). Range-expanding plant species might benefit from these new biotic conditions when

they do not encounter coevolved natural enemies in the expanded range (De Frenne *et al.* 2014; Dostálek *et al.* 2015). At the same time, range-expanders will become exposed to non-coevolved natural enemies that are native to these new areas. The strength of the enemy release effect will be largely determined by the inability of the novel natural enemies to exploit the range-expanders and the ability of the range-expanders to successfully defend themselves (Verhoeven *et al.* 2009). The present study was initiated in order to examine how root herbivores in the new range respond to range-expanding plant species.

Range-expanding plant species could benefit from the lack of coevolved novel natural enemies when they produce chemicals to which these enemies are not adapted. Such novel chemicals make the plants either less attractive or less digestible. For intercontinental introductions of exotic plant species, this possibility has been investigated under the "novel weapon hypothesis" (Callaway & Ridenour 2004; Schaffner *et al.* 2011). Several studies have shown that invasive exotic plant species produce more unique shoot compounds than native plant species in the invaded range (Cappuccino & Arnason 2006; Macel *et al.* 2014), thereby negatively affecting the performance of native aboveground invertebrate herbivores (Macel *et al.* 2014). The strength of novel weapon effects could differ between introduced exotic plant species and intra-continental range-expanders as more natural enemies may be shared between the original range and the new range of intra-continental range-expanders than of intercontinentally introduced exotic species. Yet, aboveground herbivores that lack a co-evolutionary history with both the range-expanding and the related native plant species performed less well on some successful range-expanders than on related natives (Engelkes *et al.* 2008). This suggests a role for plant chemistry in the success of range-expanding plants. However, the novel weapon hypothesis so far has not been tested in studies on intracontinental range-expanding plant species. Moreover, there is a paucity of studies testing the effects of novel chemistry on belowground herbivores, both for introduced exotics and intra-continental range-expanders.

In their new range, successful range-expanding plant species on average are less negatively affected by soil communities than congeneric natives (van Grunsven *et al.* 2007; Engelkes *et al.* 2008). This effect has been explained by the on average lower accumulation of soil-borne fungal pathogens (Morriën & van der Putten 2013) and root-feeding nematodes (Morriën, Duyts & Van der Putten 2012) on the

roots of range-expanding plant species than on congeneric natives. However, there is considerable variation in the outcome of plant-nematode interactions among range-expanding plant species (Chapter 4, Morriën, Duyts & Van der Putten 2012; Viketoft & van der Putten 2014). A likely explanation for this variation that has not yet been studied is the role of novel plant chemistry. Therefore, the aim of the present study was to examine how differences in plant-nematode interactions between range-expanding and native plant species relate to differences in root chemistries. We compared preference and reproductive performance of root herbivores on range-expanders with congeneric plant species that are native in the new range, in order to confound our tests as minimal as possible with general differences in plant chemistry.

We tested the hypotheses that native generalist root-feeding nematodes (1) are more strongly attracted to native than to range-expanding plant species, (2) prefer native plant species over range-expanding plant species and (3) show higher reproduction on native than on range-expanding plant species. We studied differences in nematode attraction to single plants of all tested plant species (hypothesis 1), differences in nematode preference between range-expanders and related natives (hypothesis 2) and differences in nematode performance between range-expanders and related natives (hypothesis 3) under both lab and greenhouse conditions. As root volatiles are known to influence attraction of entomo-pathogenic nematodes (Rolfe, Barrett & Perry 2000; Rasmann *et al.* 2005; Turlings, Hiltpold & Rasmann 2012), we examined volatile profiles of all 6 plant species as they also may explain patterns in root-feeding nematode attraction and preference. Together, our results will contribute to the understanding of how novel chemistry might affect belowground plant-herbivore interactions of range-expanding plant species.

Methods

Plant species and seed collections

We selected three plant species that recently expanded their range naturally from lower latitude areas to higher latitude areas in North-Western Europe and that have a related native species in their new range. Range-expanding plant species that were examined in the experiments were *Centaurea stoebe* L., *Geranium pyrenaicum* Burm. f., and *Rorippa austriaca* Crantz and their congeneric native species

were *Centaurea jacea* L., *Geranium molle* L. and *Rorippa sylvestris* (L.) Besser. All six plant species now co-occur in riparian grassland areas in the eastern part of the Rhine-Waal area in The Netherlands. Therefore, these plant species are subjected to at least partly overlapping abiotic and biotic conditions. Range-expanding *R. austriaca* and *G. pyrenaicum* naturally established in the Netherlands at the end of the 19th century and are now widespread, while the first population of range-expanding *C. stoebe* in the Netherlands was recorded in the last decade of the 20th century (NDFF 2017). Seeds of all six plant species originate from natural areas in the Netherlands. Seeds of *C. stoebe*, *G. molle*, *R. austriaca* and *R. sylvestris* were directly collected from single populations the field. Seeds of *C. jacea* were collected from mother plants that were grown in an outside experiment at NIOO-KNAW (Wageningen, The Netherlands) from seeds collected in a natural population. Seeds of *G. pyrenaicum* were delivered by the company Cruydthoeck (Nijeberkoop, The Netherlands), that grows wild plants under field conditions from seeds that originate from natural field sites. For all experiments, seeds of *Centaurea* and *Geranium* species were surface-sterilized by washing for 3 min in a 10% bleach solution, followed by rinsing with demineralized water, after which they were germinated on glass beads. Due to their small size, seeds of both *Rorippa* species were not surface-sterilized, but directly germinated on sterilized soil. Seeds were germinated in a climate cabinet at 20/10 °C and 16 h light/8 h darkness.

Nematodes

We used cultures of two root-feeding nematode species, the ectoparasitic *Helicotylenchus pseudorobustus* Steiner (hereafter *Helicotylenchus*) and the sedentary endoparasitic *Meloidogyne hapla* Chitwood (hereafter *Meloidogyne*), originating from populations in The Netherlands. We selected these species as they both have a wide host range, are common and widely distributed throughout Europe (Bongers 1988). Both used cultures were previously established in a greenhouse at NIOO-KNAW. The culture of *Helicotylenchus* on Marram grass (*Ammophila arenaria* L.) originates from nematodes collected from coastal dunes. The culture of *Meloidogyne* originates from nematodes collected from a field near Bovensmilde (Drenthe, The Netherlands) which were subsequently cultured on tomato (*Solanum lycopersicum* L.).

Nematode choice experiments

To study differences in nematode attraction and preference, we performed choice experiments on agar and in soil, where nematodes could move to one of two opposing treatments. To examine nematode *attraction* to a plant species, we planted one seedling of a species at one side and left the other side unplanted. To examine nematode *preference* for either natives or range-expanders we planted single seedlings of congeneric native and range-expanding plant species at opposing sides of the test units. As a control for attraction and preference, we examined nematode movement in test units without seedlings. We calculated the percentage of nematodes moving to either one of the sides of the test units.

Choice experiment on agar: to examine nematode choice *in vitro*, we used Petri dishes of 9 cm diameter filled with 20 ml 0.5% microbial agar (Merck kGaA, Germany) (Piskiewicz *et al.* 2009). We used eight independent replicates for each treatment. We placed 20-days-old seedlings 4 cm from the center of the Petri dish. Thereafter, the Petri dishes were placed in a climatized chamber at 16/8 h light/dark and 20 °C. After two days, 20 µl of tap water suspension containing 40 juveniles of either *Helicotylenchus* or *Meloidogyne* was pipetted at the center of the Petri dishes. Nematode choice was examined two days after inoculation by counting using a stereo-microscope (200× magnification). We considered a nematode to be significantly attracted to one treatment when it moved at least 0.5 cm into the half of the Petri dish oriented towards that treatment.

Choice experiment in soil: To examine nematode choices under more natural conditions than on agar, we performed a choice experiment in soil-filled Y-tubes (van Tol *et al.* 2001; Piskiewicz *et al.* 2009) in a greenhouse at 16/8 h light/dark and 20/15 °C. We used 6 independent replicates for each treatment. Each Y-tube consisted of a core piece and two removable arms, which were all filled with gamma-sterilized soil (25 KGray, Syngenta bv, Ede, The Netherlands). The soil originated from a former agricultural field (Beneden-Leeuwen, The Netherlands; N51° 53.952, E05° 33.670) in a riparian system where all plant species can occur. Prior to sterilization, the field soil was homogenized with sand at a rate of 2:1 (w:w) in order to reduce the relative clay content. Seedlings of 20 days old were planted in the Y-tube arms. Soil moisture was adjusted to 10% (w:w) and maintained at this level until nematode

inoculation. Five days after planting the seedlings, two ml of water suspension with 200 *Helicotylenchus* or *Meloidogyne* juveniles was inoculated two cm deep in both sides of the core piece, to have an equal distribution of nematodes throughout the core piece. Then, both units with the planted seedlings were placed on the Y-tube and for the remaining experimental time the arms were moistened daily with five ml of demineralized water. After that, nematodes could enter an arm in which the roots were growing. Four days after inoculation, the two arms of the Y-tube were separated and nematodes from each arm and the core piece were extracted by Cobb's decantation (Cobb 1918) and counted using an inverted light microscope (200x magnification).

Nematode reproduction experiment

For each plant species, ten 12-days-old seedlings were planted separately in 11x11x12 cm pots filled with soil homogenized and sterilized as explained above. The pots were placed in a greenhouse in a randomized block design with five replicate blocks. After 12 days, pots were inoculated with two ml water suspension with either 200 *Meloidogyne* or 200 *Helicotylenchus* juveniles. During the subsequent 16 weeks the pots were watered twice a week and kept on the same weight of approximately 870 g, of appr. 15% (w:w) soil moisture content. Thereafter, roots and soils were separated and used for nematode extraction. All roots were washed in 200 ml tap water, after which the washing water containing nematodes that were present in the rhizosphere was stored. Nematodes of each individual replicate were combined into a single sample by extracting all nematodes from the wash and soil using an Oostenbrink elutriator (Oostenbrink 1960). Roots collected from pots inoculated with the ectoparasite *Helicotylenchus* were dried at 70 ˚C. Roots from pots inoculated with *Meloidogyne* were split and both halves were weighed fresh. One half of the roots was dried at 70 ˚C until constant weight, whereas the other half was cut into pieces of 1-2 cm and placed for four weeks in a mistifier to extract nematodes from the inside of the roots (Funnel-spray method; Oostenbrink 1960). Total dry root biomass was assessed using total fresh weight and fresh/dry weight ratio of each sample. Nematode suspensions were harvested from the mistifier after two and four weeks, combined, and concentrated to 10 ml. Nematodes were counted using an inverted light microscope (200x magnification).

Root volatile analysis

To relate nematode attraction, preference, and performance to root chemistry, we analyzed root volatile profiles by Gas Chromatography Quadrupole Time of Flight (GC-QTOF) analysis.

Volatile trapping: Four 20-days-old seedlings of each plant species were placed in individual 70 ml glass pots filled with sterilized soil (see choice experiment in soil). After 15 days, steel traps containing the volatile absorbants Tenax TA (150 mg) and Carbopack B (150 mg; Markes International Ltd., Llantrisant, United Kingdom) were attached at both sides of the glass pots. After 24 hours of incubation the traps were removed, capped and stored at 4 ˚C until GC-QTOF analysis.

GC-QTOF analysis of volatiles compounds: The volatiles were collected from the traps using an automated thermos desorption unit (Unity TD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (Helium flow 50 ml/min) and trapped on a cold trap at -10 °C. The volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap for 3 min to 280 °C. Split ratio was set to 1:10, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 μm (Restek 13424-6850, Bellefonte, PA, USA). The following temperature program was used: 39 °C for 2 min, from 39 °C to 95 °C at 3.5 °C/min, then to 165 °C at 6 °C/min, to 250 °C at 15 °C/min and finally to 300 °C at 40 °C/min and 20 min at 300 °C. The volatiles were detected by a mass spectrometer (MS) operating at 70 eV in EI mode. Mass spectra were acquired in full-scan mode (30–400AMU, 4 scans/s). GC-MS-data were collected and converted to a mzData file using the Chemstation B.06.00 (Agilent Technologies, USA). Data were further processed with MZmine 2.14.2 (Pluskal *et al.* 2010) with the tools mass detection (centroid mode, noise level = 1000), chromatogram builder (min time span = 0.05 min, min height = 1.5E03, m/z tolerance of 1 m/z or 5 ppm), and chromatogram deconvolution (local minimum search, chromatographic threshold = 40%, Min in RT range = 0.1 min, Min relative height = 2.0%, Min absolute height = 1.5E03, Min ratio of peak top/edge = 2, peak duration = 0.0–0.5 min). Detected and deconvoluted peaks were identified by their mass spectra using NIST MS Search and NIST 2014 (National Institute of Standards and Technology, USA) and aligned using Random Sample Consensus (RANSAC) aligner (mz tolerance = 1 m/z or 5 ppm, RT tolerance = 0.1, RT tolerance after correction

= 0.05, RANSAC iteration = 10000, Min number of points = 60%, threshold value = 0.1). Processed data were exported for further statistical analysis as explained under 'Statistical analysis'. The identification of detected compounds was further evaluated using the software AMDIS 2.72 (Stein 1999; http://chemdata.nist.gov/). The retention indexes were calculated for each compound and compared with those found in NIST 2014 and in-house databases.

Statistical analyses

Differences in nematode attraction and preference were tested by pair-wise t-tests in SigmaPlot (Systat software, Inc). Overall differences in nematode attraction between natives and range-expanders were tested using general linear models with origin as fixed factor and plant species as random factor (packages *lme4* and *lmerTest*; Bates *et al.* 2014; Kuznetsova, Brockhoff & Christensen 2015) using R studio (version 0.98.507; R Core Development Team 2012). Differences in nematode numbers between plant species were tested for each nematode species separately using generalized linear models with a negative binomial error distribution (*MASS* package; Venables & Ripley 2013) modeling fixed factors 'plant species' and 'experimental block'. Wald post-hoc tests were then used to test for differences between plant species using the *phia* package (De Rosario-Martinez 2013). Using Pearson correlation tests, we examined whether nematode reproduction corresponded with nematode attraction in the y-tubes. Analyses on volatile data were performed using MetaboAnalyst V3.0 (www.metaboanalyst.ca; Xia *et al.* 2015). Prior to One-way ANOVA and multivariate analyses (PLS-DA) data were normalized via log-transformation and auto scaling. To identify mass features significantly differing between plant species, a one-way-ANOVA with post-hoc Tukey HSD-tests was performed. Mass features were considered to be statistically relevant when p- and FDR-values were ≤ 0.05.

Results

Nematode attraction

First, we confirmed that the controls in the nematode attraction experiments were effective. Indeed, when the tests were performed in the absence of plants both on agar and in soil neither *Helicotylenchus* nor *Meloidogyne* showed significant movement away from the point of addition (Fig. 5.1, Fig. S5.1).

Meloidogyne: On average, there was a trend of stronger attraction of *Meloidogyne* to natives than to range-expanding plant species on agar (natives: 25.3 ± 3.6 %, range-expanders: 10.9 ± 3.9%; $F = 7.56$, $p = 0.051$), but this was not significant in soil (natives: 21.9 ± 4.4 %, range-expanders: 9.0 ± 3.8%; $F = 4.86$, $p = 0.09$). On agar, all natives significantly attracted *Meloidogyne* away from the empty control (all t-values > 3.48, all p-values < 0.05; Fig. S5.1A), whereas none of the range-expanders did so (Fig. S5.1A). In soil, all three native species significantly attracted *Meloidogyne* away from the empty controls (all t-values > 6.65, all p-values < 0.01; Fig. 5.1A). Both range-expanding *Geranium* and *Rorippa* also attracted *Meloidogyne* away from the empty control in soil (t-values > 4.84, p-values <0.01; Fig. 5.1A). Interestingly, the range-expanding *Centaurea* significantly repelled *Meloidogyne* towards the empty control in both agar and soil (t-values < -3.21, p-values < 0.05; Fig. 5.1a, Fig. S5.1A). Thus, all natives significantly attracted *Meloidogyne*, whereas range-expanders either repelled *Meloidogyne* or attracted *Meloidogyne* only in one of the two test units.

Helicotylenchus: On average, native plant species did not attract *Helicotylenchus* more strongly than range-expanders on agar (natives: 21.9 ± 8.0%, range-expanders: 13.6 ± 2.4%; $F = 0.99$, $p = 0.38$), while they did so in soil (natives: 17.2 ± 0.8%, range-expanders: 7.4 ± 3.3%; $F = 7.83$, $p < 0.05$). Individually, all native plant species significantly attracted *Helicotylenchus* in both test units, when compared to empty controls (all t-values > 3.2, all p-values < 0.05; Fig. 5.1A, Fig. S5.1A). On agar only range-expanding *Geranium* significantly attracted *Helicotylenchus* away from the empty control ($t = 4.34$, $p < 0.01$; Fig. S5.1A), while in soil both range-expanding *Geranium* and *Rorippa* did so (t-values > 6.57, p-values <0.01; Fig. 5.1A). Range-expanding *Centaurea* significantly repelled *Helicotylenchus* towards the empty control on agar ($t = -2.83$, $p < 0.05$; Fig. S5.1A), but not in soil ($t = -1.98$, $p = 0.10$; Fig. 5.1A). Overall, native plant species always significantly attracted *Helicotylenchus*, whereas attraction and repellence by range-expanding plant were species-specific and depended on test unit.

Nematode preference

Meloidogyne and *Helicotylenchus* preferred native *Centaurea* and *Rorippa* over their congeneric range-expanders (t-values > 3.68, p-values < 0.05; Fig. 5.1B; Fig. S5.1B), although the preference of *Helicotylenchus* for native *Rorippa* was not significant on agar (t = 1.47, p = 0.19). Both *Meloidogyne* and *Helicotylenchus* did not show a preference for either native or range-expanding *Geranium* on either agar or in soil (all t-values < 1.59, all p-values > 0.15; Fig. 5.1B, Fig. S5.1B). Therefore, our results show that two out of three native plant species were preferred over related range-expanding plant species by both nematode species, whereas in the third plant pair both nematode species did not show a preference for either the native or the range-expander.

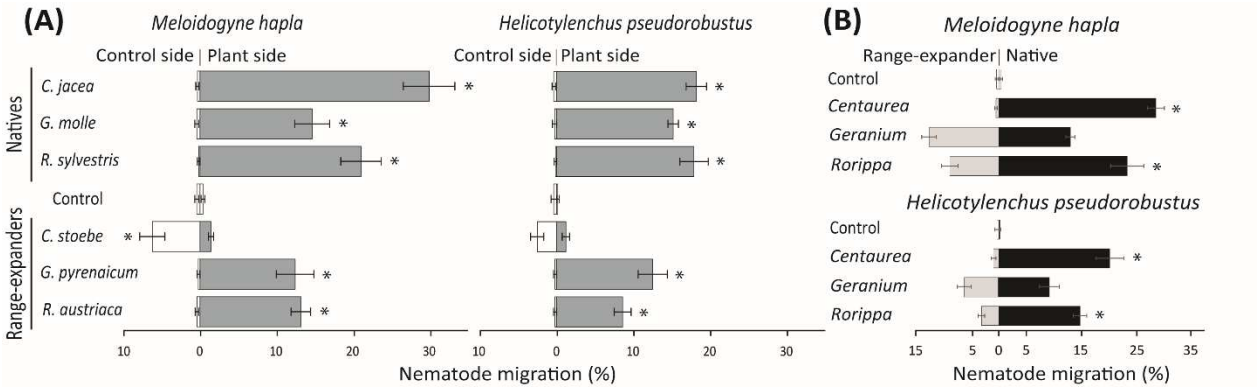

Fig. 5.1 (A) Attraction or repellence (% individuals migrated) of the nematode species *Meloidogyne hapla* and *Helicotylenchus pseudorobustus* by native and range-expanding plant species in sterilized soil. (B) Nematode preference between native plant species *Centaurea jacea*, *Geranium molle* and *Rorippa sylvestris* and congeneric range-expanders *Centaurea stoebe*, *Geranium pyrenaicum* and *Rorippa austriaca*. In both panels horizontal bars show averages ± standard errors and asterisks represent significant paired t-test values ($p < 0.05$) between empty control and plant (A) or between native and range-expanding plant species (B).

Nematode reproductive performance

Meloidogyne reproduction differed significantly among plant species ($X^2 = 182.45$, $df = 5$, $p < 0.0001$). *Meloidogyne* numbers were higher on native *C. jacea* than on range-expanding *C. stoebe* ($X^2 = 251.94$, $df = 1$, $p < 0.0001$; Fig. 5.2) and higher on native *R. sylvestris* than on range-expanding *R. austriaca* ($X^2 = 12.18$, $df = 1$, $p < 0.001$; Fig. 5.2). However, in *Geranium*, *Meloidogyne* numbers were higher on the range-expander *G. pyrenaicum* than on the native *G. molle* ($X^2 = 5.87$, $df = 1$, $p < 0.05$; Fig. 5.2). *Helicotylenchus* numbers also differed significantly among plant species ($X^2 = 114.05$, $df = 5$, $p < 0.0001$; Fig. 5.2). There were significantly more *Helicotylenchus* on native *C. jacea* than on range-expander *C. stoebe* ($X^2 = 10.10$, $df = 1$, $p < 0.05$; Fig. 5.2). However, post-hoc analysis of the other two plant pairs did not reveal any significant differences in *Helicotylenchus* numbers between range-expanders and congeneric natives. *Meloidogyne* numbers per plant species strongly correlated with the attraction by these plant species in y-tubes ($R^2 = 0.92$, $p < 0.01$; Fig. 5.3A), while this correlation was not significant for *Helicotylenchus* ($R^2 = 0.11$, $p = 0.52$; Fig. 5.3B).

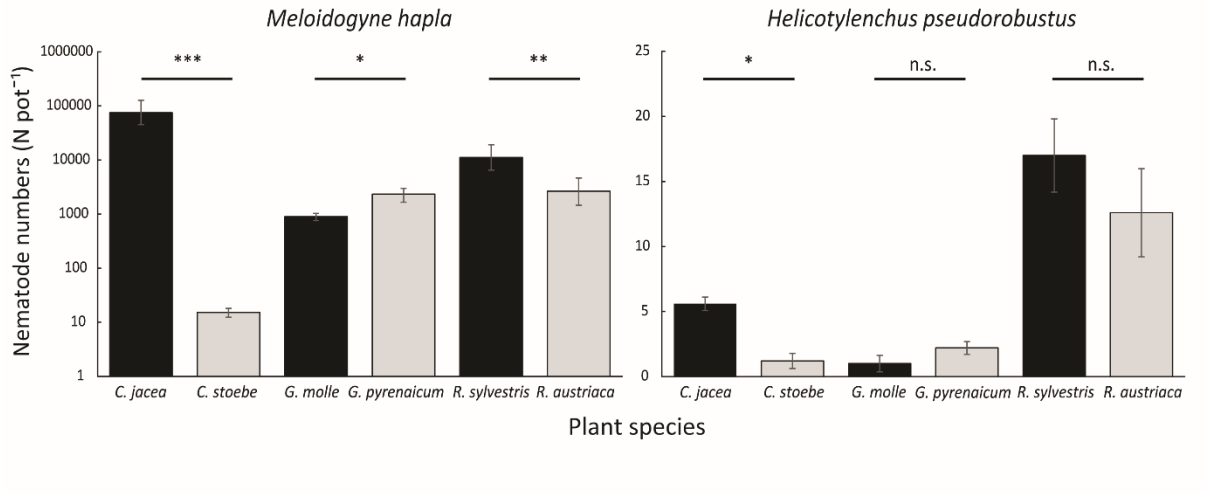

Fig. 5.2 Mean total numbers (N pot^{-1}) of root-feeding nematodes *Meloidogyne hapla* (left; logarithmic scale) and *Helicotylenchus pseudorobustus* (right; linear scale) on range-expanding plant species *Centaurea stoebe*, *Geranium pyrenaicum* and *Rorippa austriaca* (grey), and congeneric natives *Centaurea jacea*, *Geranium molle* and *Rorippa sylvestris* (black). Vertical bars show means ± standard errors. Asterisks indicate levels of significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant) of pairwise post-hoc Wald tests within plant pairs.

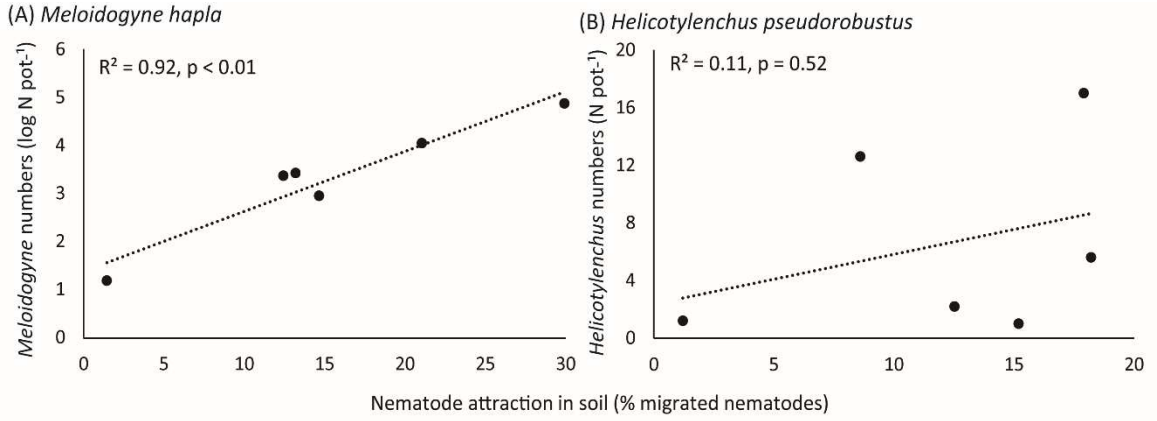

Fig. 5.3 Correlation plots between nematode attraction (x-axis) and nematode reproduction (y-axis), for root-feeding nematodes (A) *Melodoigyne hapla* and (B) *Helicotylenchus pseudorobustus*. Dots represent the 6 different plant species tested. R^2-values and p-values of the Pearson correlation tests are given.

Root volatiles

We detected 1964 putative volatile compounds in all samples, of which approximately 25 % (491 volatile compounds) were produced by plants (Fig. S5.2). The other 1473 volatile compounds were detected in the tubes containing only gamma-sterilized soil. When the root volatiles of all six plant species were analyzed together, the strongest overlap between species was found within the pairs of congeneric species, indicating that chemistry varies more strongly between genera than within genera (Fig. S5.3). Within the *Centaurea* pair 21 volatile compounds were significantly different between the native and range-expander, resulting in a clear separation of their volatile profiles (Fig. 5.4A). Five of these compounds were detected only in the headspace of *C. stoebe*: indene, tridecane and nonadecane (alkanes), 1,2-benzisothiazole (benzenoids/ketone) and alpha-gurjunene (sesquiterpene), and three volatiles were detected only in the headspace of the native *C. jacea*: petasitene (sesquiterpene), benzophenone (benzenoids/ketone), and an unknown terpene (Table 5.1). Thirteen compounds where found in both *Centaurea* species, but in different abundances (Table 5.1). Volatile profiles from native and range-expanding *Geranium* and *Rorippa* were less clearly separated in the PLS-DA score plots, although samples from controls, native and range-expanding plants could still be divided into three distinct groups with 95% confidence intervals (Fig. 5.4B,C). There were 11 volatiles that showed significant differences between the *Geranium* species and 6 between the *Rorippa* species (all p-values <0.05). Native *G. molle* produced five unique volatile compounds, compared to four by range-expanding *G. pyrenaicum,* while two volatiles differed in production levels between the species. The

native *R. sylvestris* produced four unique compounds compared to two unique compounds that were produced exclusively by the range-expander *R. austriaca*. Therefore, differences in volatile profiles between range-expanders and congeneric natives depended on the species pair; in two out of three cases, the range-expander produced fewer unique volatiles than the congeneric native.

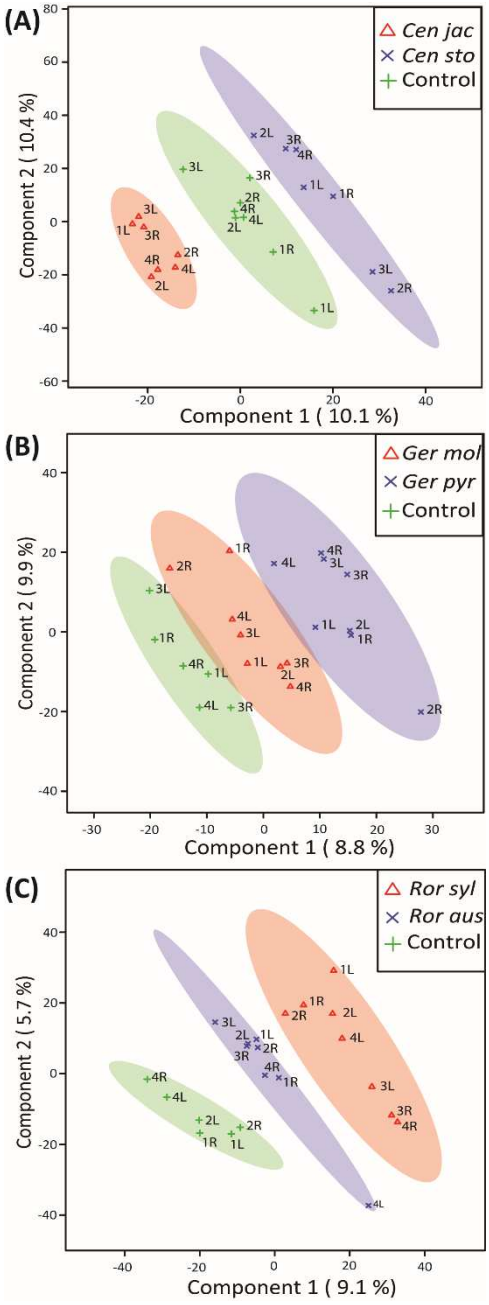

Fig. 5.4 Partial least square-discriminant analysis (PLS-DA) score plots of root volatile profiles measured with GC-QTOF-MS. The semi-transparent ovals outline the 95% confidence intervals of natives (red triangles), range-expanders (blue crosses) and sterilized control soils (green crosses) for *Centaurea* (A), *Geranium* (B) and *Rorippa* (C). Sample numbers and position of the volatile trap (left or right) are given.

Table 5.1 Volatile organic compounds produced by native *Centaurea jacea* and range-expanding *Centaurea stoebe*. Tentative compound names are shown, which are based on retention time (RT) and ELRI (Experimental linear retention index) values, measured with GC-QTOF-MS. All compounds are significantly more produced by either *C. jacea* (CJ) or *C. stoebe* (CS). Compounds that are produced solely by *C. jacea* are indicated with '*' and compounds produced solely by *C. stoebe* with '**'.

Compound name	RT	ELRI	Plant
sulfur dioxide	2.04	488	CJ
dimethylsulfide	2.4	529	CS
carbondisulfide	2.5	541	CJ
furan, 2-methyl	2.9	583	CJ
1,3-dioxolane, 2-methyl-	3.4	639	CS
benzene 1,2 dimethyl	10.1	890	CJ
dimethylsulfone	10.9	916	CS
dimethyl-trisulfide	13.1	963	CS
mesitylene	14.3	990	CJ
*indene***	15.7	1023	CS
acetophenone	17.4	1062	CS
*1,2-benzisothiazol***	23.9	1229	CS
*tridecane***	26.8	1299	CS
*petasitene**	30.1	1398	CJ
*alpha-gurjunene***	30.4	1407	CS
*unknown terpene**	32.73	1448	CJ
phenyl maleic anhydride	34.29	1534	CJ
*benzophenone**	36.9	1620	CJ
pentadecanoic acid	40.02	1867	CS
*nonadecane***	40.4	1901	CS
diphenylsulfone	40.7	1934	CS

Discussion

Several studies have proposed that invasiveness of intercontinentally introduced exotic plant species can be enhanced by their novel chemistry, e.g. through allelopathy (Callaway & Aschehoug 2000; Zheng *et al.* 2015), or by the suppression of the local natural enemies (Schaffner *et al.* 2011). Yet, little is known about the effects of novel chemistry of intra-continental climate-driven range-expanders on communities in the new range. Moreover, empirical studies testing novel chemistry effects on belowground plant-herbivore interactions in the novel range are lacking. Here, we show that root-feeding nematodes from the novel range were strongly attracted to native plant species, while, in support of our hypothesis, the average attraction by range-expanders mostly was less strong. Yet, we also found substantial differences in nematode attraction among range-expanding plant species: while the range-expanding *C. stoebe* repelled both nematode species in at least one of the attraction experiments, range-expanding *G. pyrenaicum* and *R. austriaca* attracted nematodes. Therefore, we show that some range-expanding plant species will attract considerable amounts of root-feeding nematodes in their new range, while other species will repel them, potentially leading to profound differences in herbivore pressure between range-expanders in their new range.

In test units with both natives and congeneric range-expanders, both nematode species preferred native *Centaurea* and *Rorippa* over their congeneric range-expanders, while our hypothesis of stronger nematode preference for natives was not confirmed when comparing the *Geranium* species. In plant communities in the new range, the preference for native plant species could lead to apparent competition (Holt 1977), when natives experience stronger herbivore pressure (Orrock, Witter & Reichman 2008), leading to indirect competitive benefits for the range-expanders. For *Meloidogyne,* reproduction strongly corresponded with the attraction to the different plant species, as we found that *Meloidogyne* reproduction was significantly higher in the roots of native *Centaurea* and *Rorippa* than in the roots of their congeneric range-expanders. Notably, the differences in *Meloidogyne* reproduction between the *Centaurea* species were more substantial than between the *Rorippa* species. This was especially due to poor nematode reproduction on the range-expanding *Centaurea stoebe*, which is in line with a previous study (Chapter 4). *Helicotylenchus* numbers did not fully correspond with the attraction to the different plant species. Although they were lower in the rhizosphere of range-

expanding *Centaurea* than in that of native *Centaura*, no differences were found in the other two plant pairs. The overall very low *Helicotylenchus* numbers indicate that no – or hardly any – reproduction of this species took place in this experiment. While the species did show profound chemical attraction to some of the plant species, we could therefore not properly estimate differences in performance on these different plant species.

Contrary to our hypothesis, but in line with a previous study (Chapter 4), the range-expanding *Geranium* hosted slightly higher numbers of *Meloidogyne* than the native *Geranium*, indicating that not all range-expanding plant species are poorer nematode hosts than congeneric natives. Depending on naivety of either the host plant species or the herbivore in a novel plant-herbivore novel interaction, herbivore performance can be found to be strong or weak (Verhoeven *et al.* 2009). We did not perform experiments using *Meloidogyne* and *Helicotylenchus* populations from the original range of the range-expanding plant species, so our data do not allow to draw conclusions on nematode preference and performance of the range-expanding plant species in their native range. However, as gene flow between soil-born nematode populations is expected to be low (Blouin, Liu & Berry 1999), a certain degree of local adaptation is well possible, so that it may well be that the nematode populations in the new range differ, at least to some extent, from populations in the original range. The use of nematode populations originating from natural areas in the new range and the subsequent culturing on plant species that is phylogenetically unrelated to the examined plant species allowed a phylogenetically unbiased test of the effects of the natural co-evolutionary histories between the nematode and plant species on nematode attraction and performance.

We expected that the patterns in nematode attraction, preference and reproduction found in the present study would be caused by differences in root chemistry between native and range-expanding plant species. Indeed, the analyses of volatile compounds revealed that range-expanding *C. stoebe* produced more unique volatile compounds than native *C. jacea*. These results correspond with a study on aboveground herbivores, in which herbivore performance was also shown to be low on range-expanding and exotic plants with more unique chemistry than their related natives (Macel *et al.* 2014). In addition to higher numbers of unique compounds, our study also reveals differences in the production levels of several shared volatile compounds between the *Centaurea* species. Therefore, the

nematode repellence and the poor nematode reproduction on the range-expanding *C. stoebe*, compared to the native *C. jacea,* might be explained by both the production of higher numbers of unique compounds and by different production levels of shared compounds. Interestingly, novel chemistry of *C. stoebe* has also been related to the poor performance of aboveground generalist herbivores in North America (Schaffner *et al.* 2011), where this plant species is invasive. In contrast to range-expanding *Centaurea*, both range-expanding *Rorippa* and *Geranium* produced fewer unique volatiles than their congeneric natives. Differences in volatile profiles were stronger in *Geranium* than in *Rorippa*, which was not reflected in the patterns of nematode preference and reproduction. Native *Rorippa* hosted higher nematode numbers and was more attractive to both nematode species than range-expanding *Rorippa*, while in *Geranium* there was no clear nematode preference for either the native or the range-expander, and nematode reproduction levels were higher in the range-expander than in the native. These results suggest that when unique volatile compounds play a role in nematode attraction or distraction, the identity, rather than the number of unique compounds may influence the outcome of plant-nematode interactions. Interestingly, but not unexpectedly, the differences in volatile profiles between all three pairs of congeneric native and range-expanding plant species were smaller than the differences among the three genera. This suggests that while root-feeding nematode species such as *Meloidogyne* have adapted to plant species with strongly different root chemistries, they may still perform poorly on range-expanding plant species that possess root chemistries slightly deviating from that of the plant species the nematodes are adapted to.

Our volatile analyses revealed, next to many plant volatiles, a large diversity of volatiles emitted by gamma-sterilized soils, which is in line with earlier studies (Schulz-Bohm *et al.* 2015; Kai, Effmert & Piechulla 2016). Possibly, the chemical background of the soil caused the differences in nematode attraction between the tests on agar and soil, namely the higher numbers of nematodes moving to the unplanted side on agar. Alternatively, this effect could be caused by a stronger diffusion of root metabolites in the Petri dishes than in the soil-filled Y-tubes, resulting in a more equal distribution of root metabolites throughout the Petri dishes. Based on the differences between the two choice experiments we therefore conclude that choice experiments with root-feeding nematodes should preferably be performed in soil.

The application of GC-QTOF for volatile analysis allowed to obtain the tentative identification of the measured root volatiles. We identified several volatile compounds that were only detected in range-expanding *C. stoebe*, and therefore could cause the nematode-repelling effect found for this plant species. Root-emitted volatiles are known to play versatile roles in long distance below-ground interactions (Erb *et al.* 2013; van Dam & Bouwmeester 2016) and some of the volatile compounds identified in the present study have been shown to negatively affect nematodes (Piluk, Hartel & Hanies 1998). Future studies testing the identified metabolites in different combinations and ratios could reveal which compounds cause the nematode-repelling effect found in *C. stoebe*. Yet, pin-pointing of the observed effects to a single volatile compound can be complicated, because nematodes might react to a blend of volatiles, rather than to single compounds (McCormick, Unsicker & Gershenzon 2012).

Successful range-expanding plant species have been shown to be better defended against naïve aboveground generalist herbivores than congeneric native plant species (Engelkes *et al.* 2008), indicating that they may possess superior defence mechanisms compared to related native species in the new range. Such defence mechanisms may especially be effective when they are novel to the natural enemies in the new range. Our results suggest that together with the release of soil enemies from the original range (van Grunsven *et al.* 2007), the possession of novel chemistry could explain why range-expanding plant species are less negatively affected by soil communities than related native plant species (van Grunsven *et al.* 2007; Engelkes *et al.* 2008). As range-expanding plant species without closely related species in the new range are likely to possess the most unique root chemistries compared to native species present in the community, a phylogenetic approach (as in Strauss, Webb & Salamin 2006) may be considered to forecast which range-expanding plant species have the strongest potential to affect native communities in their novel range (Gilbert & Parker 2016).

In conclusion, we provide evidence that novel belowground chemistry of the root system of range-expanding plant species may suppress root herbivores in the new range. A range-expander that had the most different root chemistry compared to its related native suppressed root-feeding nematodes more strongly than range-expanders with root chemistries that were more comparable to those of related natives. However, our study included six plant species from three genera. Therefore, while our results elucidate the variation in potential impact of range-expanding plant species on native

communities in their novel range, further studies are needed in order to be able to generalize these results and predict which range-expanding plant species may have strong impacts on native communities in the future.

Supplementary information

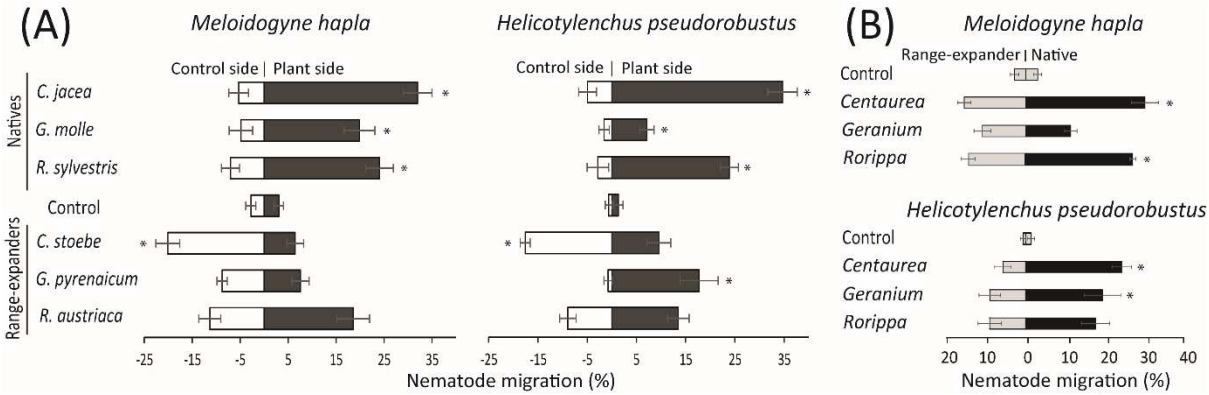

Fig. S5.1 (A) Attraction or repellence (% individuals migrated) of the nematode species *Meloidogyne hapla* and *Helicotylenchus pseudorobustus* by native and range-expanding plant species on agar. Grey bars represent the plant sides and white bars the control sides of the agar plates. (B) Nematode choice between native plant species *Centaurea jacea*, *Geranium molle* and *Rorippa sylvestris* (dark grey) and congeneric range-expanders *Centaurea stoebe*, *Geranium pyrenaicum* and *Rorippa austriaca* (light grey). In both panels horizontal bars show averages ± standard errors and asterisks represent significant paired t-test values ($p < 0.05$) between empty control and plant (a) or between native and range-expanding plant species (B).

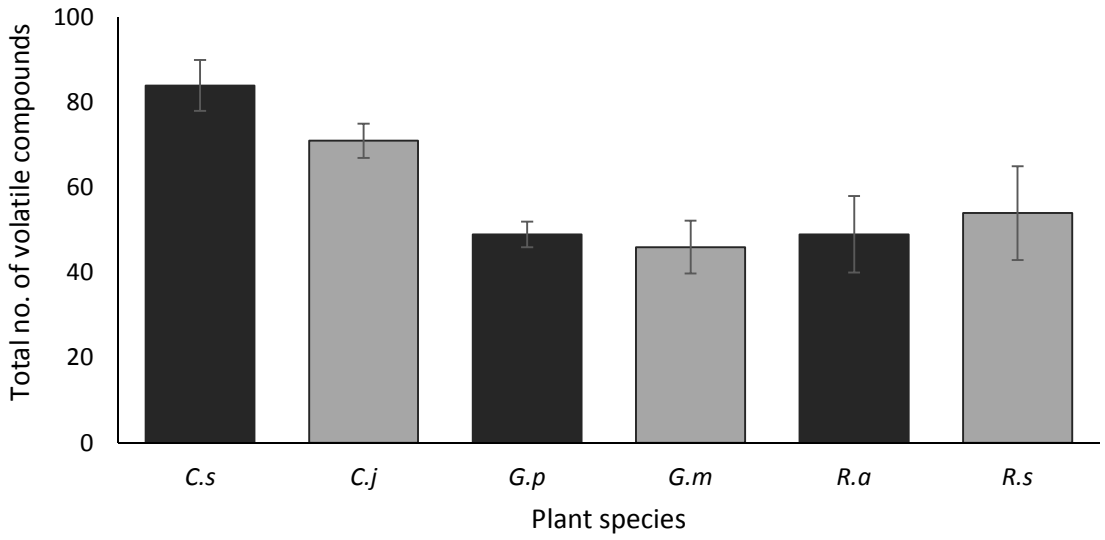

Fig. S5.2 Total volatile numbers found in pots grown with range-expanding plant species *Centaurea stoebe* (C.s), *Geranium pyrenaicum* (G.p) and *Rorippa austriaca* (R.a) (black) and related native species *Centaurea jacea* (C.j), *Geranium molle* (G.m) and *Rorippa sylvestris* (R.s) (grey). Vertical bars show averages ± standard errors.

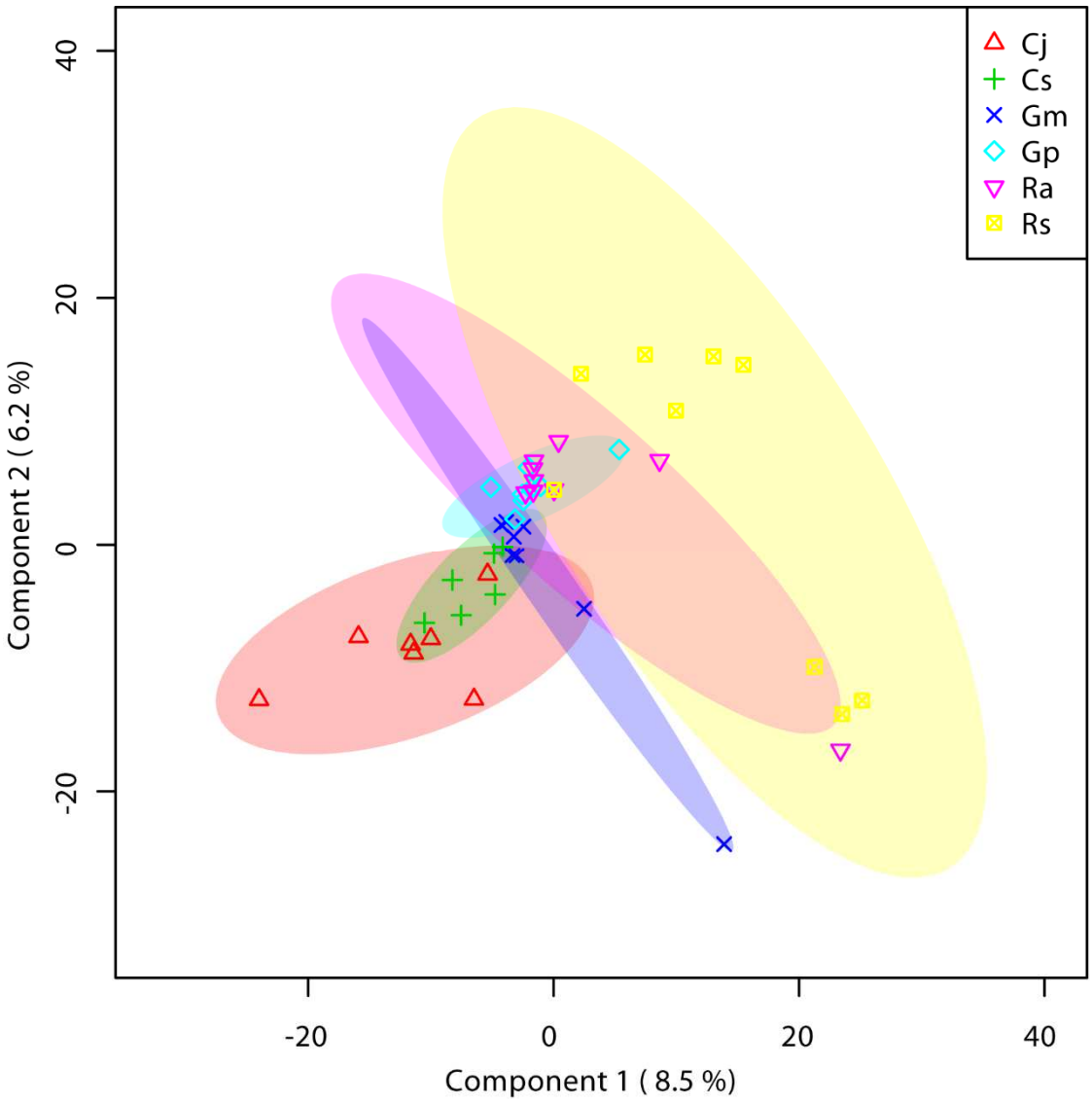

Fig. S5.3 Partial least square-discriminant analysis (PLS-DA) score plots of root volatile profiles measured with GC-QTOF-MS. The semi-transparent ovals outline the 95% confidence intervals of native plant species *Centaurea jacea* (Cj), *Geranium molle* (Gm) and *Rorippa sylvestris* (Rs), and congeneric range-expanding plant species *Centaurea stoebe* (Cs), *Geranium pyrenaicum* (Gp) and *Rorippa austriaca* (Ra).

Chapter 6

Root traits and root herbivores explain plant-soil feedback variation among congeners

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Abstract

Soil biota play important roles in plant community dynamics by causing positive or negative plant-soil feedback effects (Kardol, Bezemer & van der Putten 2006; Van Der Heijden, Bardgett & Van Straalen 2008), which differ among plant species (Cortois *et al.* 2016). Plant-soil feedbacks on conspecifics vary with phylogenetic distance (Anacker *et al.* 2014) and are affected by plant origin (Klironomos 2002) when phylogenetically diverse plant species were examined. However, it remains unknown if these explanatory variables also predict plant soil feedback differences between closely related plant species. Here, we show that among eight congeneric native and non-native, range-expanding plant species, differences in belowground community composition are predicted more by root trait variation than by plant origin or phylogenetic distance. After conditioning soil from the new range with each of the eight plant species, fungal community composition correlated with variation in specific root length, whereas bacterial community variation was associated with differences in root chemical profiles. Protist and nematode communities co-varied with bacterial communities. Plant performance in soil conditioned by conspecifics was independent of plant origin, and did not vary with phylogenetic distance. Plant performance strongly correlated with root-feeding nematode abundance, and not with microbial rhizosphere community composition. We conclude that plant-soil interactions of closely related species may be explained more strongly by root traits than by phylogenetic distance or plant origin, and that the strength of plant-soil feedback may be predicted by root-feeding nematode abundances.

Introduction

Soil biota play an important role in controlling species diversity in plant communities (Van Der Heijden, Bardgett & Van Straalen 2008; Mangan *et al.* 2010). Over time, many plant species accumulate plant species-specific antagonistic and symbiotic-mutualistic soil organisms, thereby reducing or promoting themselves, while often having the opposite effect on neighbouring plant species (van der Putten *et al.* 2013). Plant species differentially shape soil communities (Bais *et al.* 2006; Burns *et al.* 2015), for example by producing different root exudates and volatiles (van Dam & Bouwmeester 2016; Venturi & Keel 2016). As phylogenetically closely related plant species are more

likely to share such chemical traits than distantly related species (Gilbert & Parker 2016; Senior *et al.* 2016), it may be expected that close relatives condition soil communities more similarly than distantly related species. In turn, natural enemies in the attracted soil communities will likely have comparable effects on closely related plant species (Gilbert & Webb 2007; Parker *et al.* 2015). Therefore, conspecific plant-soil feedback as a whole may, at least in part be phylogenetically determined. While plant-soil feedback differences among taxonomically diverse plant species might be explained by phylogeny (Anacker *et al.* 2014), very few -if any- studies have tested whether closely related congeneric species, such as sister species, have a more similar pattern of plant-soil feedback than distantly related congeneric species. Likewise, only few studies have studied the whole rhizosphere microbiome including nematodes among plant species (Leff *et al.* 2018), while differences of only distinct parts of the microbiome were compared between congeneric plant species (Bouffaud *et al.* 2014; Schlaeppi *et al.* 2014).

Reduced negative plant-soil feedbacks have been proposed to determine the success of non-native plant species in their new range (Klironomos 2002; Engelkes *et al.* 2008). However, the mechanisms underlying plant-soil feedback differences between non-natives and natives, such as interactions between plants and root-feeding nematodes, vary with the degree of chemical differences between congeneric non-native and native plant species (Chapter 5). Therefore, the differences in plant-soil feedback outcomes between natives and non-natives may largely be predicted by phylogenetic distance. However, it is unknown whether plant origin itself, in addition to phylogenetic distance, may predict plant-soil interactions of non-native plant species in their new range, for example by the consistent absence of co-evolved specialists (Reinhart *et al.* 2010). Here, we tested the hypothesis that differences in rhizosphere community composition and plant-soil feedback among range-expanding and congeneric native plant species, and the traits underlying this variation, can be explained by their phylogenetic distances.

We tested our hypothesis using eight congeneric *Geranium* species that all occur in north-western Europe. Four of these species are native, whereas the other four recently have become established in the last century (see methods), most likely as a consequence of climate warming (Parmesan 2006). We were able to test of plant origin effects irrespective of phylogenetic distance, because native and range-

expanding plant species were not phylogenetically clustered (Fig. S6.1). We combined a plant-soil feedback experiment with sequencing of the communities of prokaryotes and eukaryotes conditioned by the different plant species, and performed analyses of root chemical profiles and structural root traits, allowing us to relate plant-soil feedback effects to soil communities and plant traits.

Methods

Plant species and germination

Seeds of native *Geranium* species *G. dissectum* L., *G. molle* L., *G. pusillum* L. and *G. robertianum* L. and range-expanding species *G. lucidum* L., *G. purpureum* Vill., *G. pyrenaicum* Burm.f. and *G. rotundifolium* L. were collected from single natural populations in The Netherlands. All natives naturally occur in The Netherlands, whereas the range-expanders established populations in north-western Europe in the late 20th century (*G. lucidum* and *G. purpureum*), or were already present in restricted areas and strongly expanded their range in the last decades of the 20th century (NDFF 2017; BRC 2018). For each experiment, seeds were surface-sterilized by washing them for 3 min in a 10% bleach solution, followed by rinsing with demineralized water, after which they were germinated on glass beads.

Phylogeny reconstruction

We concatenated three barcoding regions commonly used to infer plant phylogenies: rbcL (Wolf, Soltis & Soltis 1994), the trnL gene (Fangan *et al.* 1994) and the intergeneric spacer trnL-trnF (Bortiri *et al.* 2001). Due to multiple sequences for rbcL present in GenBank we decided to re-amplify the rbcL gene for all plants used in our experiment. For this, root DNA was extracted from all *Geranium* species using the PowerSoil DNA Isolation kit (Qiagen, USA), which was adjusted by using iron beads to increase physical impact. We amplified the large chain of the ribulose bisphosphate carboxylase (rbcL) using the primers 1F(Wolf, Soltis & Soltis 1994) and the newly designed primer 1361rMod (5'-TATCCGTAAGGCTTGCAAGTGGAGT-3') modified from a previously described primer (Schuettpelz & Pryer 2007), with PCR cycling conditions as follows: initiation for 5 min at 95 °C, followed by 30 cycles of 30 sec at 95 °C, 1 min at 59 °C and 75 sec at 72° with a final elongation for 5 min at 72 °C). DNA sequencing was performed by LGC Limited (Middlesex, United Kingdom).

Obtained sequence chromatograms were manually curated in Chromas Lite v 2.11 (http://chromas-lite.software.informer.com/2.1/; Technelysium, Queensland, Australia). Curated sequences were aligned using MAFFT (Katoh & Standley 2013) and visualized in Seaview v4.6.3 (Gouy, Guindon & Gascuel 2009). Maximum likelihood analyses were run directly in Seaview using PhyML using the GTR model with four rate categories based on 2251 nucleotide sites. The stability of the branches in the resulting phylogenetic tree was assessed based on 1000 bootstrap replicates.

Soil conditioning experiment

The plant-soil feedback experiment consisted of two phases. For the conditioning phase, we prepared a common background soil by homogenizing sandy clay soil from a former agricultural field (Beneden-Leeuwen, Netherlands; N51° 53.952, E05° 33.670) with sand, after which it was sterilized using gamma-sterilization (25 KGray, Syngenta bv, Ede, Netherlands). To establish independent replicates, we collected field soil from five different sites in the same river valley in the region of Wageningen (The Netherlands), each with 4 different sub-samples. These subsamples were pooled, sieved and homogenized, after which the mixtures were kept separate throughout the experiment as 5 replicate soils. Per replicate soil, 16 2.5L pots were filled with a mixture of 1.8 kg of sterilized background soil and 200 g of sieved (1 cm) alive field soil. For each replicate soil, 8 pots were planted with one of the eight different *Geranium* species, while the other pots were left unplanted. All pots were then positioned in a randomized block design with 5 replicate blocks in a climatised greenhouse (16/8 h light/dark and 20/15°C). For the next 14 weeks the pots were watered twice per week and kept at the same soil moisture content (~15%). Thereafter, shoots were clipped, dried and weighed, while roots were washed, dried and weighed. Soils from each pot were collected and kept separate and a sub-sample was stored at -4 ˚C for DNA-extraction.

Feedback experiment

In the second phase, soils from each of the first phase pots were individually transferred to 1L pots, which were filled with 830 g soil (moisture content ~15%) and put in the same randomized block design as in the first experimental phase. Soils that were conditioned by a plant in the first phase, were planted with a seedling of the same species in the second phase. Each pot with unconditioned soil was

also planted with one of the eight species. The same watering regime was applied as in the first phase. To compensate for differences in nutrient availability originating from the conditioning phase, all pots each week received 10 ml of 25% Hoagland solution from the second week onwards, so that all plants had ample available nutrients. After seven weeks of plant growth, shoots and roots were harvested as described above.

Soil DNA extraction

For each pot with conditioned soil, DNA was isolated from 1 gram of soil based on the principle of the MoBio PowerSoil DNA isolation kit. Ceramic beads were replaced by iron spheres (ø 3mm) in order to have a higher physical impact. Instead of spin filters a vacuum manifold was used.

16S and 18S rDNA amplicon sequencing

The community structure of prokaryotes (bacteria and archaea) was determined using the prokaryote-wide primers 515F/806R targeting the V4 region of the 16S rDNA gene (Caporaso *et al.* 2012). The eukaryotic community structure was assessed using the general eukaryotic primers 3NDf (Cavalier-Smith *et al.* 2009) and 1132rmod (Geisen *et al.* 2018) targeting the most variable V4 region of the 18S rDNA (Pawlowski *et al.* 2012). In short, all primers were pre-tagged with Illumina adapters, a 12 bp long barcode to allow demultiplexing of the reads after sequencing, a primer linker and the sequencing primers. All PCRs were performed in duplicates, before quality assessment on 1.5% agarose gel. PCR duplicates were pooled and cleaned using Agencourt AMPure XP magnetic beads (Beckman Coulter). DNA concentrations were assessed with a fragment analyser (Advanced Analytical), pooled in equimolar ratios and sent for sequencing to BGI, China.

Bioinformatics

The obtained raw 16S and 18S rDNA sequence reads were curated in the Hydra pipeline (de Hollander 2017) implemented in Snakemake (Köster & Rahmann 2012); in short, after filtering contaminants and removing barcodes, 16S rDNA reads were merged with the fastq_mergepairs option of vsearch (Rognes *et al.* 2016), while for the 18S data the forward reads were used. Thereafter, for both 16S and 18S rDNA reads VSEARCH was used to cluster all reads into OTUs using the UPARSE strategy by de-

replication followed by sequence-sorting by abundance (singletons were removed) and clustering using the UCLUST smallmem algorithm (Edgar 2010). Chimeric sequences were removed using UCHIME(Edgar *et al.* 2011), implemented in VSEARCH. To create an OTU table, all reads were mapped to OTUs using the usearch_global method (VSEARCH). OTUs obtained from 16S rDNA sequences were taxonomically assigned by aligning them to the SILVA database (Yilmaz *et al.* 2014), 18S rDNA sequences were aligned to the PR2 database (Guillou *et al.* 2013). Reference sequences were first trimmed with forward and reverse primer using cutadapt (Martin 2011). Prior to the analyses, we deleted all OTUs present in less than 25% of the samples. Moreover, we removed samples with fewer than 3,000 18S rDNA reads from further analyses. All 16S rDNA samples contained at least 17,000 reads and therefore none were discarded from further analyses. We then recalculated read numbers to relative abundances of the OTUs. OTUs were then manually assigned into the functional groups, allowing estimates of relative abundances of root-feeding nematodes (Yeates *et al.* 1993), arbuscular mycorrhizal fungi (*Glomeromycota*), and plant pathogens (Plasmodiophorida, Oomycetes and *Rhizoctonia* sp.).

Nematode reproduction experiment

A sterilized background soil was prepared as described above. Forty 1L pots were filled with 830 g of sterilized background soil, and were assigned to one of the eight plant species. After planting of single seedlings per pots, the pots were placed in a randomized block design under the same greenhouse conditions as described above. After two weeks of plant growth, a suspension containing approximately 400 *Meloidogyne hapla* juveniles was inoculated near the main root of each of the plants. The same watering regime was applied as in the feedback experiment. After twelve weeks, shoots were clipped and dried and root systems were carefully separated from the soil. All soil from each pot was individually bagged and stored at 4 ˚C until nematode extraction. Nematodes were subsequently extracted using an Oostenbrink elutriator (Oostenbrink 1960) and concentrated to 10 ml. Subsequently, we extracted nematodes from the roots. For this, roots from all plants were separated in two parts, which both were weighed fresh. One part of the roots then was dried at 70°C until constant weight, while the other half was cut into pieces of 1–2 cm and placed in a mistifier for 4 weeks to extract nematodes from the inside of the roots (Oostenbrink 1960). Nematode suspensions were

harvested from the mistifier after 2 and 4 weeks, combined, and concentrated to 10 ml. Both nematode samples were then counted using an inverse light microscope (200x; Olympus CK40)). Using the total fresh weight and the dry-fresh root weight ratio, total nematode numbers inside the roots were estimated.

Structural root traits

For each plant species, three seedlings were grown individually in sterilized soil as described above. After four weeks of growth, all plants were stored at 4 °C until root trait analyses. Prior to this analysis, shoots were clipped and dried at 70°C until constant weight, whereas root systems were carefully washed. Individual root systems then were fragmented and scanned using an Epson Perfection V850 Pro scanner (Epson America, Inc). Scans were subsequently analysed using WINRHIZO Pro v.2005b(Arsenault *et al.* 1995) for total root lengths and mean diameters. After scanning, root systems were dried until constant weight and weighed, after which the root/shoot ratio was determined.

Root chemistry analysis

For all plant species, four 5-week old plants were harvested from sterilized soil, after which their root systems were carefully washed. Thereafter, we used Direct Analysis in Real Time mass spectrometry (DART-HRMS) to determine the root chemical profile of all plant species. The DART mass spectrometry set-up consists of a DART ion source (model DART-SVP, IonSence, Saugus, USA) coupled with Q Exactive Focus high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was calibrated prior to the samples measurements. The Xcalibur software (v.3.0) was used for instrument control and data acquisition. The distance between mass inlet and the DART outlet was kept at ~3cm. To standardize the measurements, root samples were placed on glass plates, and automatically moved (0.4 mm/s) along the ion source. DART settings were: Helium as ionizing gas, fixed flow of ~3.5L/min; gas beam temperature set at 450 ^{0}C; grid electrode voltage +350V. The resolution was set at ultrahigh and a scan rate of 1Hz was used. The mass spectra were recorded in the *m/z* range 100-1500 at acquisition rate of 2 spectra s^{-1}.

Mass spectrometry data processing

The DART-MS spectra were acquired and converted from their respective raw data formats to open-source mzXML file format using MSConvertGUI (64-bit) available from ProteoWizard (Kessner *et al.* 2008). For further mass spectral data processing, the open-source software package MZmine 2.20 (Pluskal *et al.* 2010) was used. Acquired mass spectrometry data from the samples was imported in MZmine 2.20 and the total ion current (TIC) chromatographic data was evaluated. Based on the evaluation, mass detection, chromatogram building and chromatogram deconvolution was performed in a step-wise manner using the available functionalities in the software. The detected and deconvoluted peaklists containing mass features for each sample were aligned using the RANSAC aligner available in MZmine. The aligned peaklists were exported in .csv format for subsequent chemometric analysis.

Chemometric analysis was performed using MetaboAnalyst 3.0 (Xia *et al.* 2015). Prior to applying chemometrics, the uploaded data was filtered and normalized. Thereafter, differences of ion abundances within the samples were investigated by applying Partial Least Square Discriminate Analysis (PLS-DA). To visualize the degree of relatedness amongst different samples, hierarchical clustering was performed using complete linkage and Euclidean distance. Dendrograms were constructed using the *stats* package in R(R Core Development Team 2012) (version 3.3.3). To generate the distance matrix and dendrogram, the resulting peaklists exported from MZmine were averaged over the four replicates for each sample, giving an average peaklist per sample.

Statistical analyses

Variations in prokaryotic and eukaryotic communities were explored by running separate PCA analyses in Canoco 5 (Ter Braak & Smilauer 2012), comparing the communities between plant origins and plant species, while including soil replicate as a covariate. We then performed partial RDA-analyses to individually test the effect of plant origin and plant species on variation in prokaryotic and eukaryotic communities, while partialling out the variation explained by the different soils. Similar analyses were performed to test plant origin and species effects on variation in the major subgroups of the eukaryotic communities: fungi, protists and nematodes. We then used 'vegdist' in the R *vegan*

package (Oksanen *et al.* 2007) to calculate pairwise community dissimilarities of prokaryotes and all eukaryotes and the eukaryotic subgroups fungi, protists and nematodes between all eight plant species in each independent soil. Overall pairwise community dissimilarities were calculated by averaging the pairwise dissimilarities in the five independent soil replicates.

We examined the phylogenetic effects on community composition by testing the correlation between pairwise phylogenetic distances and community dissimilarities using Mantel tests in *vegan*, with correlation method 'pearson' and 999 permutations. To determine whether closely related species had more similar root traits than distantly related species, we similarly tested the correlations between pairwise phylogenetic distances and absolute differences in specific root length, average root diameter and chemical dissimilarity based on the DART-analysis. Subsequently, also the correlations between rhizosphere community dissimilarities and trait dissimilarities were tested.

Plant-soil feedback variation among plant species was tested by modelling the biomass response in a general linear model including fixed factors block, soil treatment and plant species, and the plant species*treatment interaction (*lm* in R). A significant plant species*treatment interaction would indicate that plant species differ in their biomass response to soil conditioning. Overall feedback differences between native and range-expanding plant species were tested by the specification of a contrast. Significant differences in biomass in the conditioning and control treatments were tested using with the package *lsmeans*. Average plant-soil feedback values per plant species were calculated by averaging the feedback value ($\ln(\text{biomass}_{\text{conditioned}}/\text{biomass}_{\text{control}})$) in each of the five independent soil replicates. To test whether feedback differences were stronger between more distantly related species than between closely related species, we tested the correlation between pairwise phylogenetic distance and pairwise feedback differences using a Mantel test. Moreover, correlations between plant-soil feedback outcome and the relative abundance of root-feeding nematodes (genera), plant pathogens (genera/families) and arbuscular mycorrhizal fungi were tested to examine whether these groups may have determined the observed plant-soil feedback patterns. The reproduction of *Meloidogyne hapla* was modelled using a generalized linear model with a negative binomial distribution (Hilbe 2014) that included the fixed factors species and soil replicate. Between-species

differences were tested using post-hoc Wald tests with the package *phia* (De Rosario-Martinez 2013). Finally, we tested the correlation between *Meloidogyne* numbers and plant-soil feedback.

Results

The conditioned prokaryotic (16S rDNA) and eukaryotic (18S rDNA) rhizosphere communities varied between the eight plant species (Fig. 6.1A,B; Table S6.1). The composition of the three distinct taxonomic groups composing the 18s rDNA, fungi, protists and nematodes, also differed between the plant species (Table S6.1, Fig. S6.2). Compositional differences in any of these communities were not explained by plant origin, indicating that soil communities in general were not differently conditioned by natives than by related range-expanders (Table S6.1). Between-species dissimilarity of the full prokaryotic and eukaryotic rhizosphere communities did not correlate with the phylogenetic distance between the plant species (Fig. 6.1C, Table S6.1). However, distantly related plant species had more dissimilar fungal communities than closely related plant species ($R^2 = 0.15$, $p < 0.05$; Fig. 6.1D).

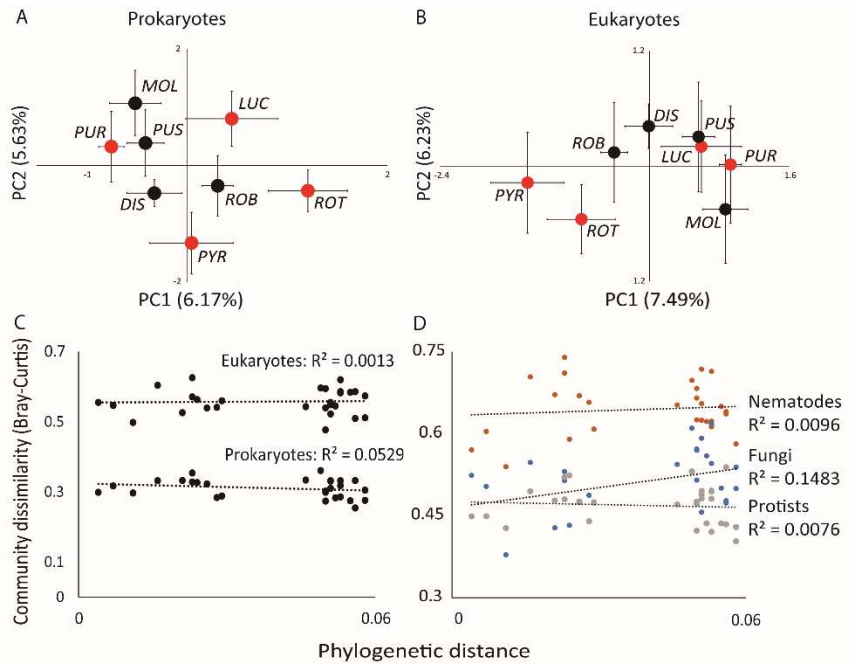

Fig. 6.1 Compositional variation in rhizosphere communities of A) prokaryotes (16S rDNA reads) and B) eukaryotes (18S rDNA reads) among native (black: *G. dissectum* (*DIS*), *G. molle* (*MOL*), *G. pusillum* (PUS) and *G.robertianum* (*ROB*)) and range-expanding plant species (red: *G. lucidum* (*LUC*), *G. pyrenaicum* (*PYR*), *G. purpureum* (*PUR*) and *G. rotundifolium* (*ROT*)). Correlations of phylogenetic distance with community dissimilarity of prokaryotes and eukaryotes (C) and eukaryotic groups nematodes (orange), fungi (blue) and protists (grey) (D). The Pearson R^2 for each correlation is shown.

Further analyses showed that variation in fungal community composition correlated with differences in specific root length, whereas differences in bacterial community composition appeared to correspond with variation in root metabolic profiles (Fig. 6.2, Fig. S6.3). Average root diameter did not explain variation in any of the groups in the rhizosphere community (Fig. S6.3). Between-species variation in root chemical profiles could not be explained by phylogenetic distance, whereas differences in specific root length and average root diameter marginally significantly correlated with phylogenetic distance (Fig. S6.4). Among plant species, protist and nematode communities co-varied with the community composition of bacteria, whereas the composition of nematode communities also co-varied with the composition of fungal communities (Fig. 6.2).

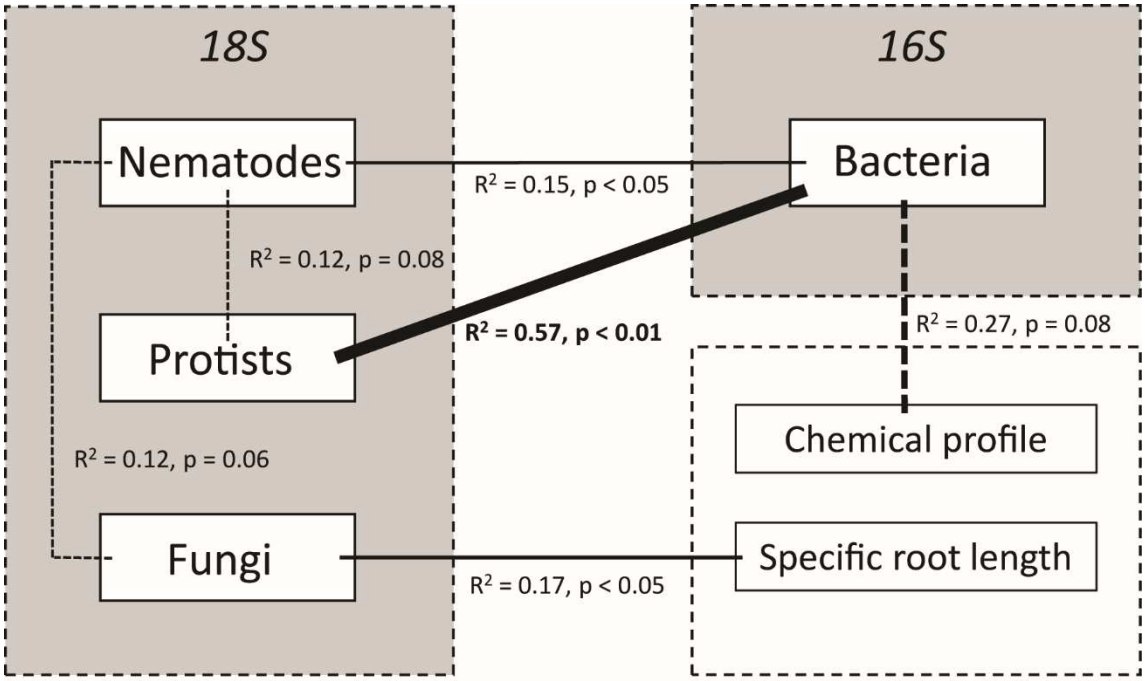

Fig. 6.2 Overview of correlational links (correlations with $p < 0.1$ are shown) between rhizosphere community dissimilarities of bacteria, nematodes, protists and fungi (all based on 18S and 16S rDNA OTUs) and between root trait variation (Root chemical profile, Specific root length) and dissimilarities of rhizosphere communities. Significant correlations (based on Mantel-tests) are depicted with solid lines, whereas trends ($p > 0.05, <0.10$) are depicted with dashed lines. Line thickness represents the relative strength of the correlational link based on R^2.

There was an overall effect of soil conditioning ($F_{1,60}$ = 435.6, p < 0.001) on plant performance in the feedback phase, and all species grew equally poor in soil conditioned by their conspecifics (Fig. 6.3A). However, the species differed profoundly in their proportional loss of biomass in response to soil conditioning and thus in their plant-soil feedback responses (conditioning*species: $F_{7,60}$ = 6.20, p < 0.001). On average, range-expanding plant species responded more negatively to soil conditioning than natives (contrast range-expanders-natives: F: 13.81, p < 0.001), which was likely mainly due to the high biomass of the range-expander *G. purpureum* in unconditioned soils and its low biomass in conditioned soils (Fig. 6.3A). Pairwise comparisons of plant-soil feedback strength did not reveal that plant-soil feedback is phylogenetically determined (R^2 = 0.001, p = 0.49) in this group of plant species (Fig. 6.3B).

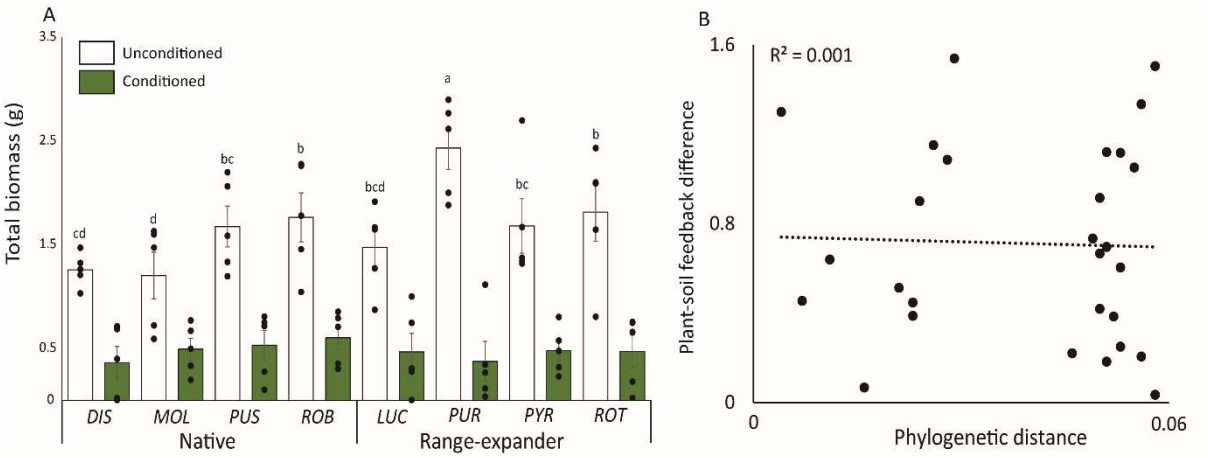

Fig. 6.3 A) Plant biomass of eight *Geranium* species in soils conditioned by conspecifics (green) or in unconditioned soils (white). Native plant species are *G. dissectum* (*DIS*), *G. molle* (*MOL*), *G. pusillum* (PUS) and *G.robertianum* (*ROB*) and range-expanding plant species are *G. lucidum* (*LUC*), *G. pyrenaicum* (*PYR*), *G. purpureum* (*PUR*) and *G. rotundifolium* (*ROT*). Bars and whiskers represent average biomass ± standard errors. Small letters show post-hoc test results between plant species in unconditioned soils. B) Between species average differences in plant-soil feedback (Ln(own/control)) do not correlate with pairwise phylogenetic distance.

Plant-soil feedback variation was neither correlated with dissimilarity in complete 16S and 18S communities, nor with the dissimilarity in fungal, nematode or protist communities (Fig. S6.5). These results motivated us to explore the relationship between specific organismal groups and the strength of plant-soil feedbacks. We tested the correlation between the relative abundances of potential

mutualistic, arbuscular mycorrhizal fungi (AMF) and eukaryotic plant pathogens and root-feeding nematodes (see methods). Relative abundances of root-feeding nematodes correlated with plant-soil feedback strength ($R^2 = 0.67$, $p < 0.05$; Fig. 6.4). There was a weak trend that also the relative abundance of plant pathogens correlated with the strength of negative plant-soil feedback, whereas there was no correlation between plant-soil feedback and AMF abundance (Fig. 6.4). Relative abundances of root-feeding nematodes did not correlate with plant biomass in the conditioning phase, indicating that root traits other than biomass determine the accumulation of these organisms (Fig. S6.6).

To test whether plant-soil feedback is also related to absolute root-feeding nematode abundance, we correlated plant-soil feedback with the reproduction of a species of *Meloidogyne* (Fig. S6.7). This was the most dominant root-feeding nematode genus in the conditioned soils (68% of root-feeding nematodes reads per sample). Plant species that developed the most negative feedbacks indeed were the best hosts for *Meloidogyne* ($R^2 = 0.80$, $p < 0.01$; Fig. 6.4).

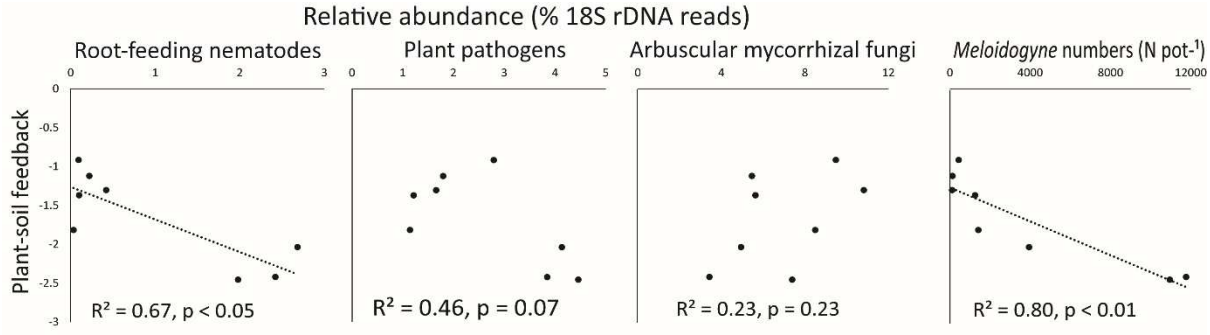

Fig. 6.4 Correlations between plant-soil feedback ($\ln(biomass_{conditioned}/biomass_{control})$; average of five independent replicate soils) of eight *Geranium* species and the relative abundances (% 18S rDNA reads) of root-feeding nematodes, plant pathogens (see Methods) and arbuscular mycorrhizal fungi (AMF) in the conditioned soils, and the absolute abundance of *Meloidogyne hapla* in a nematode reproduction experiment with each of the eight plant species. Pearson R^2 and p-values of Pearson correlation tests are given.

Discussion

The results show that variation in rhizosphere community composition among eight congeneric native and climate warming-driven range-expanding plant species could be explained most strongly by variation in their root chemical profiles and morphological root traits. Only differences in fungal community composition could be explained, at least in part, by phylogenetic distance, likely underlain by the phylogenetic signal in specific root length. Communities of fungi varied with specific root length, which is possibly explained by plant interactions with root-associated fungi, such as AMF (Smith & Read 2010). The direct link between root chemistry and bacterial community composition is in line with previous research (Schulz-Bohm *et al.* 2017). Interestingly, there was evident co-variation in the composition of the different rhizosphere groups among plant species, especially between bacteria and protists. This is likely explained by feeding relationships between these two groups (Xiong *et al.* 2017). Variation in plant-soil feedback could not be directly linked to the composition of the rhizosphere community, but rather to the abundances of groups of antagonistic soil organisms, especially root-feeding nematodes. Such root-feeding nematodes have been widely acknowledged as major agricultural pests (Nicol *et al.* 2011), but here we add evidence that they also function as drivers of natural succession (De Deyn *et al.* 2003).

Our experiment revealed no plant origin effect on rhizosphere community composition, unlike previously assumed (Morriën & van der Putten 2013). In contrast to previous work (Engelkes *et al.* 2008), we found that range-expanding plant species on average developed a more negative plant-soil feedback than native species. However, this effect was likely mainly driven by one single range-expanding plant species (*Geranium purpureum*; Fig. 6.3A), and plant-soil feedback outcomes differed within both native and range-expanding plant species. The present study shows that the negative plant-soil feedbacks of the range-expanding *Geranium* species have not hampered them from successful establishment in the new range.

Phylogenetic distance has been successfully used as a measure of ecological (dis)similarity and as a predictor of biotic interaction outcomes in studies that included plant species from multiple families (Anacker *et al.* 2014; Parker *et al.* 2015). It is likely that ecological differences in such studies may be

influenced by deeply conserved traits that vary between families and therefore are phylogenetically determined. Our study shows that among a group of congeneric species, even the most closely related species (e.g., *G. robertianum* and *G. purpureum*) can have more strongly different rhizosphere communities and plant-soil feedback than less closely related species. Therefore, our study challenges the use of phylogenetic distance as a measure to explain plant-soil interaction patterns in the case of closely related plant species. Instead, we show that non-phylogenetically conserved root traits may help to understand plant-soil interaction variation between closely related plant species, such as congeners.

Our results raise the question under which conditions phylogenetic distance will explain variations in plant-soil interactions among closely related species. The examined *Geranium* species show notable variation in abiotic niche conditions (Table S6.2), and do not all grow together in the same plant communities, suggesting that their root traits have been selected in the presence of different soil communities, which co-vary with abiotic soil conditions (de Vries *et al.* 2012). Congeneric plant species that have limited abiotic niche differences may face more similar selection pressures (Parker *et al.* 2015) and in turn have root traits that more strongly resemble their phylogenetic history. We conclude that root traits are good predictors of rhizosphere community composition variation among congeneric plant species, and propose root-feeding nematodes as potential drivers of plant-soil feedback in this this study system. We propose that an integrated approach of root traits and phylogeny may need to be taken to fully understand variation in plant-soil interactions among plant species.

Supplementary information

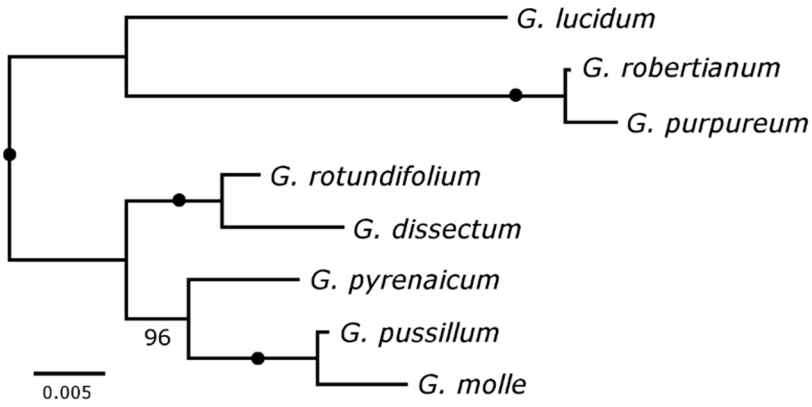

Fig. S6.1 Phylogenetic relationships among native plant species *G. dissectum, G. molle, G. pusillum* and *G.robertianum,* and range-expanding plant species *G. lucidum*, *G. pyrenaicum*, *G. purpureum* and *G. rotundifolium* (*ROT*), based on DNA-regions rbcL, the trnL gene and the trnL-trnF intergeneric spacer. Bootstrap support is visualized with black dots (100% support) or numbers indicating the percentage support. Branch lengths indicate average nucleotide substitution rates per base.

Table S6.1 RDA-results of plant species and plant origin effects on the composition of the components of the *Geranium* rhizosphere communities, and results of Mantel test on the correlations between pairwise phylogenetic distances and pairwise community dissimilarity among the eight *Geranium* species.

	Species effect		**Origin effect**		**Phylogeny**
	Expl. Var.	Permutation test	Expl. Var.	Permutation test	Mantel cor, p
16S (all OTUs)	23.2%	Pseudo-F: 1.2, p<0.01	3.1%	Pseudo-F: 1.1, p = 0.22	-0.23, p = 0.84
18S (all OTUs)	24.5%	Pseudo-F: 1.2, p<0.01	3.2%	Pseudo-F: 1.1, p = 0.27	0.04, p = 0.42
Fungi	26.2%	Pseudo-F: 1.3, p<0.01	3.4%	Pseudo-F: 1.1, p = 0.21	0.39, p < 0.05
Protists	23.9%	Pseudo-F: 1.2, p<0.01	3.1%	Pseudo-F: 1.0, p = 0.44	-0.09, p = 0.63
Nematodes	28.2%	Pseudo-F: 1.5, p<0.01	3.8%	Pseudo-F: 1.3, p = 0.16	0.10, p = 0.31

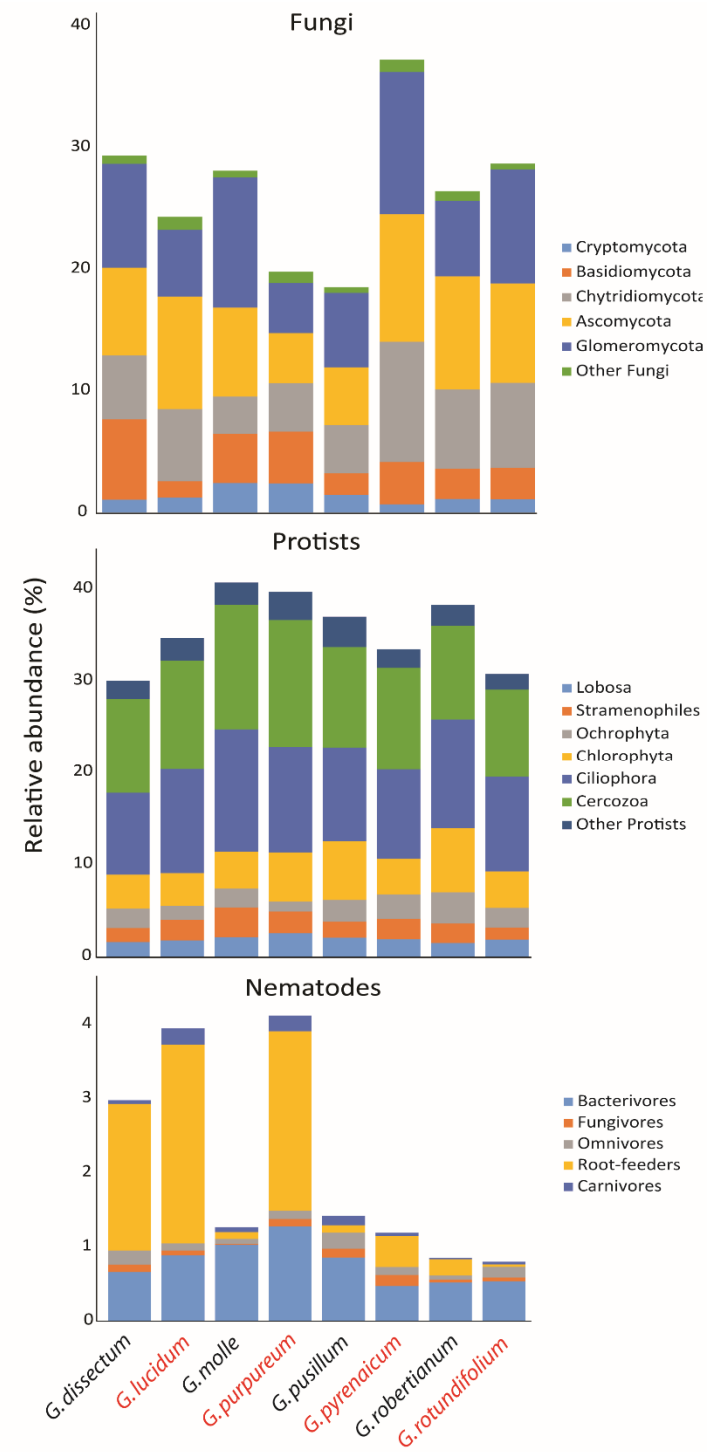

Fig. S6.2 Average relative abundances (% 18S reads) of taxonomic groups of fungi and protists and functional groups of nematodes in the rhizospheres of native (black) and range-expanding (red) *Geranium* species.

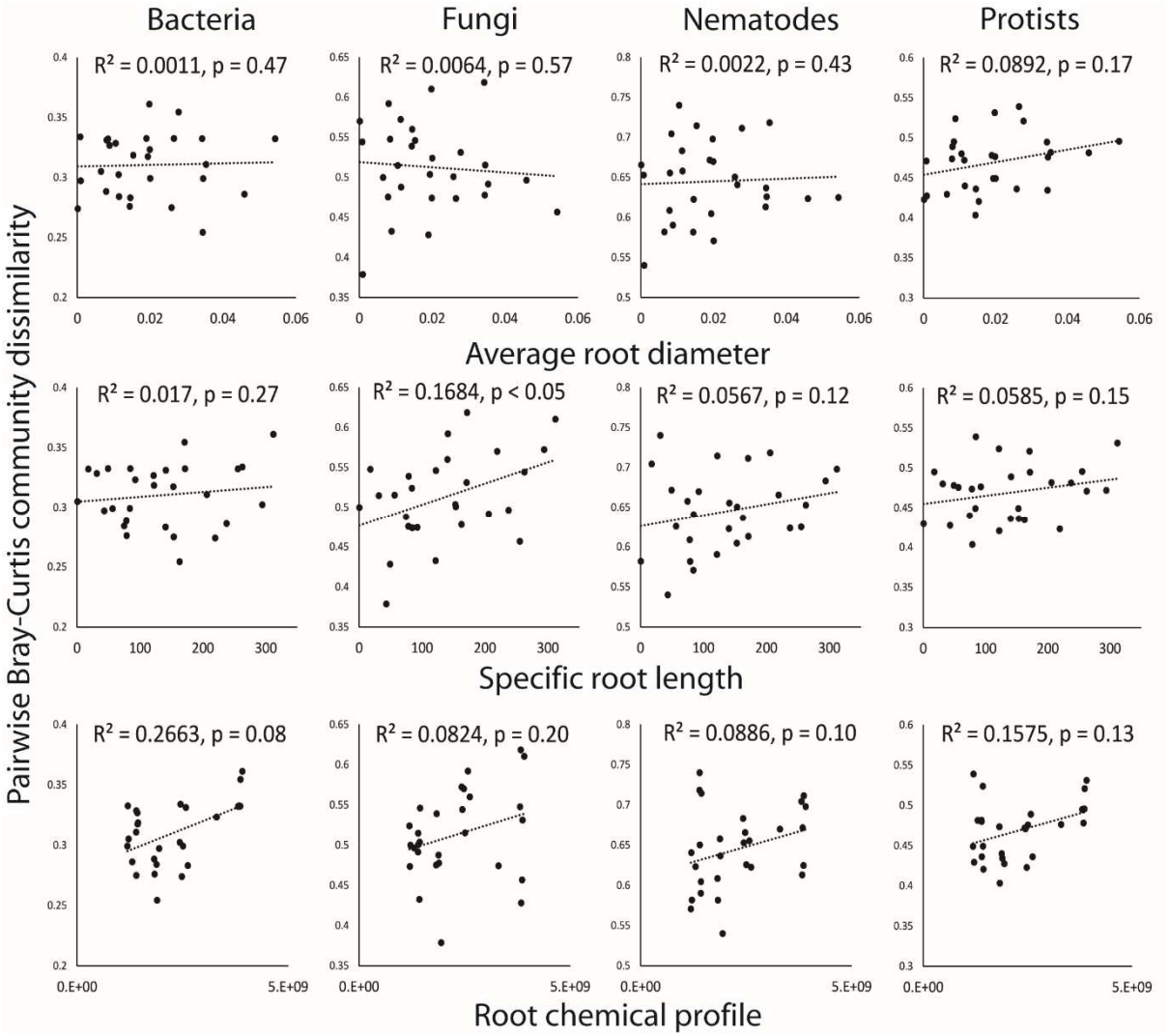

Fig. S6.3 Correlations between root traits (root chemical profile, specific root length and average root diameter) and Bray-Curtis community dissimilarities (16S, 18S, Fungi, Nematode, Protists; also see Fig. 6.2). R^2 and p-values based on Mantel tests are shown.

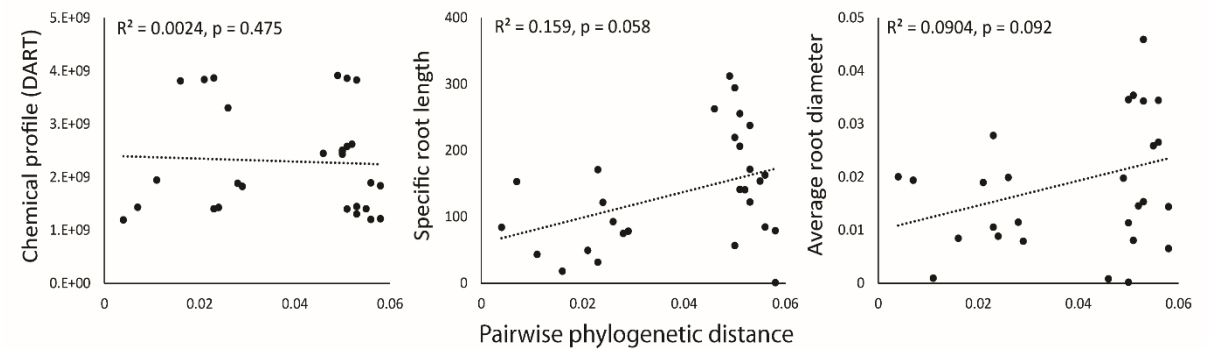

Fig. S6.4 Correlations between phylogenetic distance and variation in root traits (root chemical profile, specific root length and average root diameter). R^2 and p-values based on Mantel tests are shown.

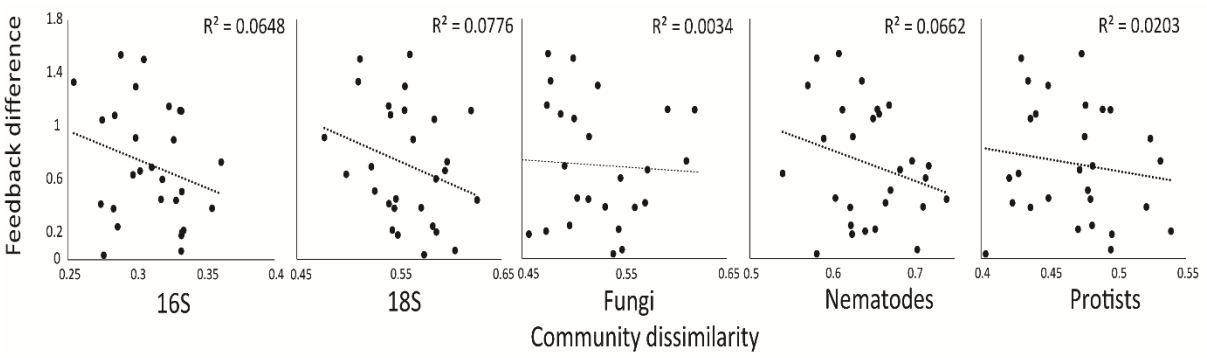

Fig. S6.5 Correlations between Bray-Curtis community dissimilarities (16S, 18S, Fungi, Nematode, Protists) and plant-soil feedback differences show no association between community dissimilarity of different *Geranium* species and their pairwise differences in plant-soil feedback.

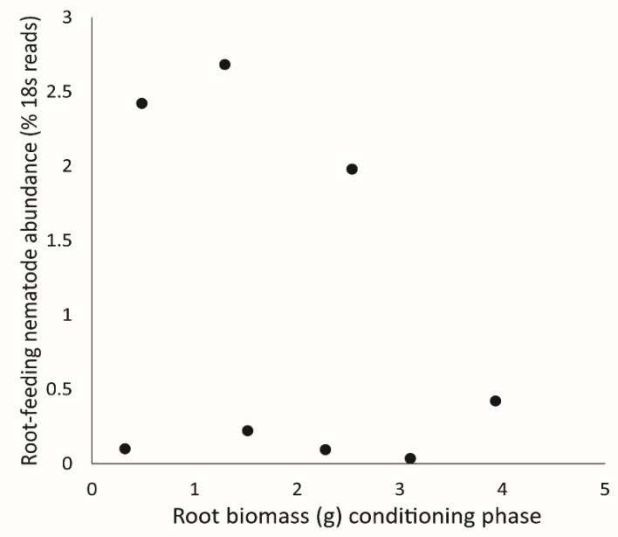

Fig. S6.6: Correlation between plant root biomass and the relative abundance of root-feeding nematodes at the end of the soil conditioning phase shows that high relative abundances of root-feeding nematodes are not associated with high root biomass.

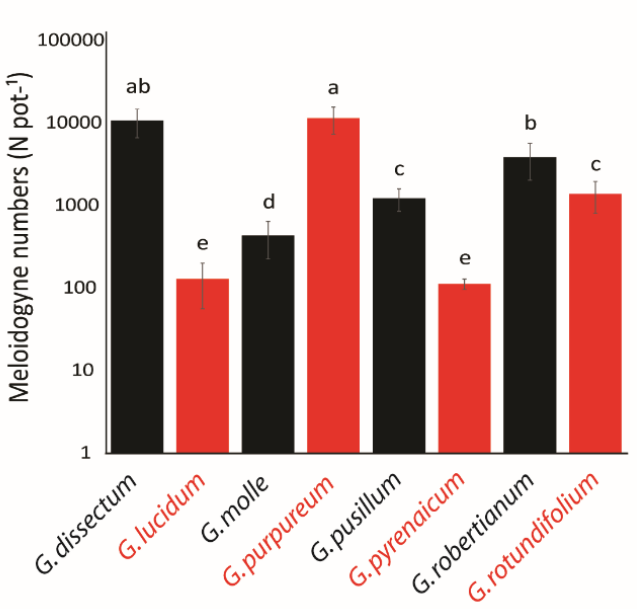

Fig. S6.7 Abundances of root-feeding nematode *Meloidogyne hapla* on native (black) and range-expanding (red) *Geranium* species in a nematode reproduction experiment. Bars represent average nematode numbers ± standard errors. Letters indicate the significant differences based on negative binomial GLM and post-hoc Wald tests. Note that the x-axis has a logarithmic scale.

Table S6.2 Ellenberg indicator values for all eight *Geranium* species (Hill *et al.* 1999). Ellenberg values (scaled from 1 to 9) indicate species preferences for light, moisture, pH and nitrogen conditions. Conditions associated with the values are given in brackets (low numbers-high numbers).

	Light (shaded-light)	Moisture (dry-wet)	Reaction (acidic-alkaline)	Nitrogen (low-high)
G. dissectum	7	5	7	6
G. lucidum	6	4	7	6
G. molle	7	5	6	5
G. purpureum	7	3	6	3
G. pusillum	7	4	7	7
G. pyrenaicum	8	4	7	6
G. robertianum	5	6	6	6
G. rotundifolium	7	4	7	6

Chapter 7

General discussion

The ecological consequences of climate change-driven range expansions are only starting to be explored. For plants, potential changes in their interactions with belowground organisms, such as soil nematodes, could alter their performance in the new range. However, changes in these interactions between the original and new range have not yet been examined, and plant-soil interactions of range-expanders in the new range still are poorly studied. The main aims of my thesis were to examine whether range-expanding plants indeed experience shifts in nematode community composition between the original and new ranges, and to study how plant-nematode interactions differ between range-expanding and native species in the new range. Most often, I compared the interactions of range-expanding plant species with those of congeneric native plant species, so that comparisons were phylogenetically constrained. However, I also studied a group of range-expanding plant species that don't have congeneric species in the new range. Here, I discuss the results of my thesis and place them in the broader context of invasion ecology and plant-soil interaction research.

Latitudinal shifts in rhizosphere nematode communities

Range-expanding plant species have been shown to perform better in soils from their new range than in soils from their original range (van Grunsven *et al.* 2010; Dostálek *et al.* 2015; Van Nuland, Bailey & Schweitzer 2017). These results suggest that range-expanding plant species experience shifts in soil communities during the range expansion process, and suffer less from belowground natural enemies in the new range. Using a latitudinal survey approach, I showed that the composition of nematode communities to some extent varies with latitude for both range-expanding and native plant species (Chapter 2). Out of four studied species, only one range-expanding plant species, *Centaurea stoebe*, clearly experienced a more substantial shift in nematode community composition between central and northern Europe than a congeneric native species. *Centaurea stoebe* also was the only of all examined range-expanders that accumulated fewer root-feeding nematodes in its new range than in its original range; especially numbers of endoparasitic nematodes in the rhizosphere were reduced towards the north. Thereby, this study provides the first direct evidence for 'enemy release' from feeding-specialist root herbivores due to latitudinal range expansion. However, range-expanding *Geranium, Rorippa* and *Tragopogon* did not accumulate fewer root-feeding nematodes in their new range than in their original range. Therefore, the increased performance of range-expanders *Rorippa austriaca* and

Tragopogon dubius in new range soils compared to original range soils (van Grunsven *et al.* 2010; Dostálek *et al.* 2015) does not seem be attributable to changes in root-feeding nematode abundance.

In a greenhouse experiment (Chapter 3), an experimental plant community consisting of the same four range-expanding plant species as used for the field survey did not accumulate fewer root-feeding nematodes in soils from the new range compared to soils from the original range. However, due to the community approach of this study, single-species effects may have been masked: *Centaurea stoebe* may still have had different effects on nematode communities and root-feeding nematode numbers in new range compared to original range soils, as observed in the transect study. Moreover, it must be noted that the morphology-based identification approach used in the plant community experiment did not provide an equally high taxonomic resolution of the nematode community as the molecular analyses of the transect study did (Geisen *et al.* 2018), thereby decreasing the detection probability of nematode community shifts between plant species and ranges. Nevertheless, this community approach showed that range-expanding plant species on average did not accumulate functionally different nematode communities in the new compared to the original range.

True enemy release?

The low numbers of endoparasitic root-feeding nematodes found in the rhizosphere of *C. stoebe* in the new range correspond with findings in a study on native and exotic populations of marram grass *Ammophila arenaria (van der Putten et al. 2005)*. Whereas native range populations of this grass harbour several specialized endoparasitic nematode species (Karssen, Aelst & Putten 1998; de la Pena *et al.* 2006; Van der Stoel & Van der Putten 2006), none or only few specialized taxa were found in exotic range populations (van der Putten *et al.* 2005), indicating that the release from natural enemies of non-native plant species may apply to these specialized root-feeding nematodes as well. However, in the transect study, the reduction of endoparasitic nematodes could not be traced back to a certain nematode species, as most OTUs could not be assigned to species level. Nevertheless, given the low number of described root-feeding nematode species, e.g. approximately 90 species of *Meloidogyne* (Karssen, Wesemael & Moens 2013), it also seems unlikely that there are many taxa truly specialized on a single or a small set of plant species. Therefore, variation in performance of generalistic and

widespread, but locally adapted, nematode taxa on *C. stoebe* between the new and the original range may be a more likely mechanism underlying the observed differences in endoparasite numbers.

Plant-nematode interactions in the new range

The transect study showed that not all range-expanding plant species accumulated fewer root-feeding nematodes in their new range when compared to related native plant species. On average, however, a community of related range-expanders accumulated fewer root-feeding nematodes than a community of congeneric natives (Chapter 3). Root-feeding nematodes on average were also more strongly attracted to native than to range-expanding plant species (Chapter 5). These findings seem to confirm the results of previous studies that on average, range-expanding plant species are better defended against generalist herbivores than related native plant species (Engelkes *et al.* 2008; Morriën, Duyts & Van der Putten 2012). However, the variation in both attraction and reproduction patterns among range-expanders of different genera suggests that ability of native root-feeding nematodes to exploit these plants will be strongly plant species-specific (Chapters 4 & 5). In line, also within a group of eight plant species from a single genus, root-feeding nematode abundance markedly differed within the group of range-expanders and the group of natives, and was not higher on natives than on range-expanders (Chapter 6). This result also highlights that, in studies comparing pairs of congeneric native and range-expanding plant species, rejection or conformation of the tested hypothesis will strongly depend on which native species have been selected for comparison. Therefore, studies with high numbers of species pairs will be needed to reliably examine average effects of plant origin on, for example, plant-soil feedbacks.

The species-specific nature of plant-nematode interactions of range-expanding plant species may be explained by the strength of chemical differences between the range-expanders and plant species in the native community. Of the three examined range-expanders, *Centaurea stoebe* chemically was strongly divergent from its congeneric native *Centaurea jacea*, whereas the root chemical profiles of range-expanding *Geranium* and *Rorippa* species largely overlapped with those of congeneric natives. In addition to patterns of nematode attraction and reproduction, the chemical variation between range-expanders and natives may also explain the variation in the even more complex, multi-trophic

interactions examined in Chapter 4. Here, range-expanders appeared to be less well able to attract nematode-antagonistic microbes than congeneric natives. Yet, given the complexity of the tested belowground communities, it could not be established which microbes were exactly responsible for the observed differences in the top-down control of root-feeding nematodes. More concise experiments, possibly with single nematode-antagonists, are needed to examine how differences in plant chemistry could affect such belowground multi-trophic interactions (Rasmann *et al.* 2005; Schulz-Bohm *et al.* 2017). Alternatively, studies using labelled CO_2 could target the question whether soil food webs show divergent functioning under range-expanding plant species that possess novel chemistry (Morriën *et al.* 2017).

In line with other studies (e.g. Santo *et al.* 1980), the results of Chapter 5 show that root-feeding nematodes like *Meloidogyne hapla* can exploit a wide and chemically diverse range of native plant species. It may therefore not be surprising that native root-feeding nematodes will only show naïve responses to range-expanding plant species that, compared to native plant species, strongly differ in root chemistry (Verhoeven *et al.* 2009). Therefore, the number of range-expanding plant species to which root-feeding nematodes will show naïve responses may be very low. Altogether, these experiments showed that there are no fundamental differences between the interactions of range-expanding and native plant species with native root-feeding nematodes; root-feeding nematodes only seem to show responses to differences in traits and not to differences in plant origin.

The curious case of Centaurea stoebe

The combination of the reduced numbers of -often highly detrimental- endoparasitic nematodes in the new compared to the original range and the poor performance of native root-feeding nematodes on this plant species, suggests that range-expanding populations of *Centaurea stoebe* benefit from their possession of 'novel' chemistry. Also in its non-native range in North America, where the species was accidentally introduced and now is strongly invasive in natural grassland systems (Tyser & Key 1988; Marrs, Sforza & Hufbauer 2008), *C. stoebe* appears to benefit from novel chemistry (Schaffner *et al.* 2011). Whether the plant species will affect the native community in its new range in North-Western Europe as negatively as the community in its invaded range in North America, will largely depend on

how naïve the native plant and soil community are regarding their interactions with this species (Callaway *et al.* 2004; Callaway *et al.* 2011). In the Netherlands, the occurrence of *C. stoebe* so far is limited to a small, but increasing, number of populations (NDFF 2017). Although it is difficult to forecast the future, the easily dispersing seeds of *C. stoebe* (Sheley, James & Michael 1998) make a further increase of the species quite likely.

Phylogenetic distance as a predictor of plant-soil interaction variation

In my thesis I selected pairs of congeneric species to make a phylogenetically-constrained comparison between range-expanding and native plant species. However, the differences in root chemistry between the range-expanding plant species and congeneric natives may nevertheless be linked to phylogeny, as phylogenetic distance has been shown to explain variation in root chemistry among congeners (Senior *et al.* 2016). Based on root chemistry, *Centaurea stoebe* might therefore be less closely related to *C. jacea* than *G. pyrenaicum* is related to *G. molle*. In line, the species in the community of 'unrelated range-expanders' (Chapter 3), range-expanders without a native relative, were expected to be chemically very dissimilar from native plant species. Therefore, I expected them to be poor hosts of native root-feeding nematodes. Unexpectedly, these unrelated range-expanding plant species on average were the best hosts for root-feeding nematodes of all plant species examined in my study. I proposed that these weak defences may relate to their annual life history, in which fast growth is favoured over strong defences. Importantly, this result indicates that not all non-native plant species that are distantly related to plant species in the native community have the traits to become invasive (Strauss, Webb & Salamin 2006). Moreover, it shows that the life history of non-native plant species should be considered to understand the response of native natural enemies in the new range: only plant species that have evolved strong defence mechanisms in their native range, are likely to benefit from these mechanisms when they are facing non-coevolved herbivores or pathogens in their new range (Verhoeven *et al.* 2009; Gilbert & Parker 2016).

With the study system of eight congeneric native and range-expanding plant species I aimed to further disentangle the effects of phylogenetic distance and plant origin on plant-nematode interactions and plant-soil feedback (Chapter 6). Firstly, and in line with the results of Chapters 3, 4 and 5, patterns of

rhizosphere community composition and plant-soil feedback showed no general differences between intracontinental range-expanding and native plant species. These results are in contrast with previous studies that examined other range-expanding plant species (van Grunsven *et al.* 2007; Engelkes *et al.* 2008; Morriën & van der Putten 2013). Importantly, my study indicates that despite accumulating a negative plant-soil feedback in new range soils, the examined range-expanding plant species have successfully established and have become widespread in North-Western Europe (NDFF 2017). Successful range expansion therefore does not necessarily require a reduced enemy impact (Engelkes *et al.* 2008).

Secondly, the results of Chapter 6 showed that of all groups of rhizosphere organisms, only the community composition of fungi was more similar in closely than in distantly related plant species, which is in line with a previous study showing that plant phylogeny is a better predictor of community variation of fungi than of bacteria (Barberán *et al.* 2015). The apparent phylogenetic conservation of specific root length may be the underlying mechanism of the relation between phylogenetic distance and fungal community dissimilarity. In contrast to fungi, communities of bacteria appeared to vary with chemical dissimilarity among plant species. However, closely related congeners did not have more similar root chemical profiles than distantly related congeners, and therefore phylogenetic distance did not predict variation in bacterial community composition between the plant species. Apparently, and unlike previously shown (Senior *et al.* 2016), root chemistry can be highly variable between closely related plant species. In line with the results of Chapter 3, the results of Chapter 6 therefore show that phylogenetic distance cannot be easily used to estimate differences in chemical profiles between plant species.

The observed differences in plant-soil interactions between the eight *Geranium* species may be linked to the differences in habitat between the plant species. For example, *Geranium purpureum*, which developed the strongest negative feedback and accumulated the highest numbers of root-feeding nematode, of all species occurs in the driest habitats (Hill *et al.* 1999). In The Netherlands, this species usually grows between stones along railways (NDFF 2017). As a result it may have evolved weak chemical defences, due to, for example, the absence of strong root herbivory in its native habitat. Instead, *G. purpureum* might have invested in fast growth, as demonstrated by my observation that

this species was the first to flower in the greenhouse experiment. Contrastingly, its sister species, *G. robertianum* that grows in moist and shaded soils, might experience a completely different soil community in its natural habitat (Brockett, Prescott & Grayston 2012). This could have resulted in different selective pressures during the evolution of root chemistry profiles. Future studies are needed to point out whether root traits and plant-soil interactions of congeneric species indeed have diverged in response to changes in abiotic niche.

Root-feeding nematodes – drivers or predictors of plant-soil feedback?

Variation in plant-soil feedback between the eight *Geranium* species was strongly correlated with the relative abundance of root-feeding nematodes in the conditioned soils, as well as with the multiplication of the endoparasitic nematode *Meloidogyne hapla*. These results have several possible implications that require further experimental testing. Either root-feeding nematodes drive the observed plant-soil feedbacks, or they indicate the strength of belowground defences against a wide array of natural enemies. Few studies have tested the effect of root-feeding nematodes on plant performance, and the majority of these studies show that the effect of nematodes alone cannot fully explain reductions in plant performance (De Rooij-Van der Goes 1995; Zoon 1995; Brussaard, Kuyper & de Goede 2001; van der Stoel, van der Putten & Duyts 2002). As also the group of OTUs identified as root pathogens appeared to co-vary in relative abundance with the negative plant-soil feedback strength, root-feeding nematode abundance may be hypothesized to be one of the drivers, rather than the only driver of plant-soil feedback. Although my study contributes to developing further hypotheses on how root-feeding nematodes may affect plant performance in natural communities, their impact on plants, both alone and in combination with other biotic and abiotic factors, needs further testing.

Future directions

The results of my thesis show that the reduction of negative plant-soil feedback that some range-expanding plant species experience in their new compared to their original range (van Grunsven *et al.* 2010; Dostálek *et al.* 2015), cannot be attributed to a reduction of root-feeding nematode numbers. Other taxonomic groups also need to be examined to understand in which ways biotic interactions of range-expanding plant species may change between their original and new range (Morriën *et al.* 2010;

Geisen *et al.* 2017). For example, the virulence of certain groups of pathogens may differ between the original and new range of the range-expanders, as has been demonstrated for the intercontinentally introduced exotic invader *Prunus serotina* (Reinhart *et al.* 2010). Moreover, studies on changes in plant-soil feedbacks along range expansion trajectories are still very rare (van Grunsven *et al.* 2010; Van Nuland, Bailey & Schweitzer 2017) and it is so far not yet examined whether range-expanders actually experience a gradual or a sudden reduction of negative plant-soil feedback as they expand their range northwards.

In their new range, range-expanding plant species will not only face different soil communities, but will also enter communities of plant species that may be different to the communities in their original range. In turn, native plant species will experience these range-expanding plant species as novel competitors that may be able to become dominant (Alexander, Diez & Levine 2015). Such a superior competitive ability could have multiple causes. First, range-expanders might benefit from novel root chemistry due to allelopathic effects on neighbouring native plant species (Callaway & Aschehoug 2000; Hierro & Callaway 2003) or on the mutualists of these plant species (Stinson *et al.* 2006). Second, based on the diversity and densities of aboveground herbivores and pathogens, plants from low latitude areas are predicted to have stronger defences than plants from higher latitude areas (Rasmann & Agrawal 2011; Baskett, Schemske & Novotny 2018). In the new range, differences in defence strength between plant species may result in enhanced apparent competition, when well-defended plants benefit indirectly from higher levels of herbivory on less well-defended neighbours (Holt 1977). Altogether, these mechanisms could result in enhanced dominance of range-expanding plant species in their new compared to their original range, even when they are still exposed to new, or co-migrating, belowground natural enemies. However, the competitive ability of latitudinal range-expanders has rarely been examined (Koorem *et al.* 2017) and additional experiments are needed to fully understand the potential impact of range-expanding plant species in their new range.

Finally, the considerable variation in plant-soil interactions and plant-soil feedback observed between congeneric species triggers the question how variation in plant-soil interactions has evolved and how patterns in non-studied species may be predicted from information on studied species. Recent research has shown that some structural root traits show a global organization, as plants adapted to

dry and seasonal conditions generally have thinner roots than plants adapted to humid, non-seasonal conditions (Ma *et al.* 2018). In turn, this variation in structural root traits will also affect mycorrhizal dependency and colonization of the plant species, as thin roots can replace the functions otherwise performed by mycorrhizal fungi (Cortois *et al.* 2016; Ma *et al.* 2018). Potentially, adaptations to specific environments and soil communities may also, at least in part, explain differences in plant-soil interactions observed in Chapter 6. In grassland communities, aboveground pathogen pressure is determined by the phylogenetic structure of the plant community (Parker *et al.* 2015). Whether belowground pathogen pressure is explained in a similar way has not yet been studied (but see Leff *et al.* 2018). However, in case of such a phylogenetically structured belowground pathogen community, related plant species will experience similar pathogen pressures and therefore will likely show similar defence mechanisms. In this way, plant-soil feedback may be phylogenetically conserved among co-occurring grassland species (Parker *et al.* 2015). Alternatively, deeply conserved traits also can underlie the explanatory power of phylogeny in this study, and future experiments are needed to point out how niche differentiation may explain plant-soil interaction variation between related plant species.

Conclusions

In my thesis, I show that range-expanding plant species will face different nematode communities in their new range compared to their original range, as nematode community composition varied with latitude. However, in their new range, plant-nematode interactions and plant-soil feedbacks of range-expanding plant species did not fundamentally differ from the interactions and plant-soil feedbacks of related native plant species. Yet, in case the root chemistry of range-expanding plant species differs substantially from the root chemistry of native plant species, as is the case with *Centaurea stoebe*, range-expanders may benefit from a reduced performance of native root-feeding nematodes. In my study, this reduced performance was predictable from the chemical attractiveness of the plant to the root-feeding nematodes. Importantly, the phylogenetic distance between range-expanding plant species and the native community cannot be used as a proxy for such a reduced performance of root-feeding nematodes, and thus does not predict the ecological novelty of range-expanding plant species in terms of their belowground defences. Therefore, the potential impact of range-expanding plant

species on the native community will depend on their species-specific traits and interactions with organisms that are native in the new range.

My results also show that among congeneric native and range-expanding plant species, variation in rhizosphere communities can be explained by a combination of structural and chemical root traits. Differences in these traits between congeneric plant species are not all explained by phylogeny, and instead may have originated from niche differentiation of closely related species. As a result, both rhizosphere communities and plant-soil feedback can be highly species-specific. Within the genus *Geranium*, abundances of root-feeding nematodes were a significant predictor of plant-soil feedback outcomes. Altogether, my results show that naïve responses of native soil communities to range-expanding plant species are likely to be relatively rare, as a minority of the range-expanding plant species seemed to be strongly chemically distinct from the native congener. Although patterns of plant competition and aboveground interactions may show otherwise, the ability of native soil communities to negatively affect range-expanders therefore suggests that very few range-expanding plant species will become disproportionally abundant in the future. In that respect, invasiveness among range-expanders may not differ largely from invasiveness among inter-continental introduced exotics, of which only one out of a hundred to a thousand becomes invasive (Williamson 1996)

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Summary

Human-induced climate change is causing strong pressure on plant and animal species, community interactions, and the functioning of natural ecosystems. Therefore, understanding and predicting ecological responses to climate change has become a key challenge in ecology. In order to survive as a species, organisms need to become adapted to new local conditions, or to migrate to higher latitude and altitude areas that were previously unsuitable. Whereas such range expansions of plant and animal species are increasingly acknowledged, ecological consequences are still largely unknown.

It has been predicted that during range expansion plant species outrun their associated soil organisms, when the latter are constrained in dispersal. Such soil organisms, like microbial pathogens and root-feeding nematodes, play important roles in the dynamics of vegetation, because of their plant species-specific effects. Consequently, the disruption of interactions between range-expanding plant species and co-evolved soil organisms may affect plant performance in their newly colonized range, where native soil organisms may show naïve responses to the new hosts. However, neither the shifts in soil communities between the original and new range, nor the establishment of novel interactions in the new range have been studied in detail.

In this thesis, I examined whether range-expanding plant species indeed experience shifts in soil community composition between their original and new ranges. I also studied whether plant interactions with soil organisms in the new range may differ from interactions of these soil organisms with native plant species. In my thesis, I particularly focussed on the interactions between plants and root-feeding nematodes, as these are ubiquitous belowground herbivores that can be relatively easily identified and quantified.

In Chapter 2, I used a combination of molecular and morphological identification methods to study nematode community composition and nematode abundances along a 2000 km long latitudinal transect from Greece to The Netherlands. Nematode communities were collected from four range-expanding plant species that are native in the southern and/or the central parts of this transect, and from four congeneric plant species that are native along the entire transect. I expected that 1) nematode community composition would vary with latitude, 2) range-expanding plant species would experience

stronger shifts in nematode community composition than native plant species and 3) that numbers of root-feeding nematodes in the rhizospheres of range-expanding plants would be lower in the new compared to the original range.

Overall, the composition of nematode communities indeed varied with latitude, indicating that nematode communities in northern Europe differ from those in southern Europe. This also showed that range-expanding plant species will face different nematode communities during range expansion. However, only one range-expanding plant species, *Centaurea stoebe*, experienced stronger shifts in nematode community composition than its congeneric native species, and accumulated fewer root-feeding nematodes in its new compared to its original range. The other range-expanding plant species did not experience stronger shifts in nematode community composition than their related natives, and accumulated similar numbers of root-feeding nematodes in their new and original range.

The hypotheses of Chapter 2 were also tested in a greenhouse experiment (Chapter 3). Here, the same four range-expanding and congeneric native species were grown in separate plant communities. In addition, communities of four range-expanding plant species that do not have congeneric natives in their new range were established. I hypothesized that, because of their potential ecological novelty, these 'unrelated range-expanders' would cause more strongly naïve responses of nematodes in the new range than 'related range-expanders'. Consequently, I expected unrelated range-expanders to show the strongest shifts in nematode community composition between original and new soils and to accumulate fewest root-feeding nematodes. However, the results showed that there were no strong nematode community shifts between soil from the original and soil from the new range irrespective of the plant communities being natives, related, or unrelated range-expanders. Moreover, the communities of range-expanders did not accumulate fewer root-feeding nematodes in new range soils than in original range soils. Instead, the community of unrelated range-expanders accumulated the highest numbers of root-feeding nematodes. I proposed that this may be explained by their annual life history, in which fast growth might have been traded off with poor defences. While individual plant species effects may have been masked by the community approach, and the taxonomic resolution of the nematode data was considerably lower than in Chapter 2, these results show that on average range-

expanding plant species will not experience major shifts in the functional composition of rhizosphere nematode communities between their original and new range.

In Chapters 4 and 5, I examined the interactions between range-expanding and congeneric native plant species and two specific root-feeding nematode species from the new range to test whether these nematodes would show lower performance on range-expanders than on related natives. In Chapter 4, I set up a greenhouse experiment to examine nematode reproduction on individuals of two range-expanding plant species and congeneric natives, in the presence or absence of communities of native nematode-antagonistic microbes. Range-expanders were expected to be poor hosts for root-feeding nematodes, but also to be unable to attract the enemies of these nematodes. In line with Chapter 2, range-expanding *Centaurea stoebe* was found to be a very poor host for both root-feeding nematode species. However, range-expanding *Geranium pyrenaicum* accumulated more root-feeding nematodes than its native congener. In support of my expectation, native plant species appeared to be better capable of attracting the natural enemies of root-feeding nematodes than range-expanding plant species, but these belowground multi-trophic interactions turned out to be highly plant species-specific.

The experiments described in Chapter 5 were aimed at unravelling potential chemical mechanisms underlying the differences in root-feeding nematode reproduction on range-expanders and related natives. Attraction experiments showed that root-feeding nematodes on average were less strongly attracted by three range-expanding plant species than by three congeneric natives. Interestingly, range-expanding *Centaurea stoebe* was found to chemically repel root-feeding nematodes, whereas the other range-expanders did attract nematodes - though less strongly than native plant species.

Comparisons of the blends of root volatiles, which are gaseous compounds, produced by the different plant species, showed that *Centaurea stoebe* produced considerably more unique volatile compounds compared to its related native than the other range-expanding plant species. This suggests that the degree of chemical novelty of range-expanding plant species will determine the chemical attraction of root-feeding nematodes in the new range. As the chemical attraction appeared to be a good predictor

of nematode reproduction, range-expanding plant species may benefit from the possession of root chemistry unknown to native root-feeding nematodes.

The variation in chemical dissimilarity between range-expanding and native species observed in Chapter 5 might be explained by differences in phylogenetic distance between the examined range-expanding and native plant species. In order to further disentangle the effects of plant origin and phylogenetic distance on plant-soil interactions, I developed a series of experiments using four range-expanding and four native plant species that all belong to the same genus (Chapter 6). In this way I tested the hypothesis that closely related congeners have more similar morphological and chemical root traits, and condition soil communities more comparably than distantly related congeners. I also tested whether variation in plant-soil feedback - the performance of the plant species in soil conditioned by a conspecific plant - was explained by plant origin or by phylogenetic distance.

The results showed that plant origin did not explain variation in rhizosphere community composition or plant-soil feedback. I found that closely related plant species did not have more similar root chemistries than distantly related species within the same genus, but that phylogenetic proximity correlated with similarity in structural root traits. The community composition of fungi was related to specific root length, which is a measure of root system architecture. However, the composition of bacterial community related to variation in root chemistry, whereas communities of protists and nematodes appeared to co-vary with bacterial communities. Therefore, within the genus *Geranium* only differences in fungal community composition could be explained by the phylogenetic distances among plant species.

Finally, in the same *Geranium* study, I showed that differences in plant-soil feedback related to the numbers of root-feeding nematodes in the rhizospheres of the different species. Altogether, these results show that variation in plant-soil interactions among closely related species neither resembles their evolutionary history, nor their origin, thereby suggesting that the (chemical) root traits of these species have evolved in response to species-specific conditions.

Theory predicts that range-expanding plant species may escape their belowground natural enemies, and benefit from naïve responses of natural enemies in the new range. In my thesis, I show that in the

new range only range-expanding plant species that have a strongly different root chemistry compared to native plant species may be less affected by root-feeding nematodes. My work emphasizes that phylogenetic distance does not necessarily indicate ecological novelty, and that very closely related species do not necessarily have comparable plant-soil interactions. Although other groups of soil organisms may show different responses to range-expanding plant species, my results suggest that it is unlikely that range-expanders will become highly dominant in their new range, unless they bring in traits that are not present in the native community.

Samenvatting

De recente, door de mens veroorzaakte, klimaatverandering heeft sterke invloeden op plant- en diersoorten, hun onderlinge interacties, en het functioneren van natuurlijke ecosystemen. Het begrijpen en voorspellen van ecologische veranderingen veroorzaakt door klimaatverandering is daarom een van de speerpunten van de hedendaagse ecologie. Om te overleven als soort zullen organismen zich ofwel moeten aanpassen aan de nieuwe omstandigheden in hun leefgebied, of hun leefgebied moeten verplaatsen of uitbreiden naar gebieden die in het verleden nog niet geschikt waren. Dit soort areaal-uitbreidingen, vaak naar gebieden die noordelijker of hoger liggen, worden steeds vaker waargenomen, maar hun ecologische consequenties zijn grotendeels onbekend.

Een belangrijke voorspelling omtrent areaal-uitbreidingen is dat plantensoorten tijdens hun areaal-uitbreiding hun geassocieerde bodemorganismen te snel af zijn, doordat veel bodemorganismen zich slecht verspreiden. Deze bodemorganismen, zoals microbiële pathogenen en wortel-etende nematoden, spelen een belangrijke rol in de dynamiek van natuurlijke vegetatie, omdat ze niet elke plantensoort in dezelfde mate aantasten. Als gevolg van de disruptie van de interacties tussen plantensoorten en hun geassocieerde bodemorganismen zouden areaal-uitbreidende plantensoorten in hun nieuwe leefgebied minder onderdrukt kunnen worden, doordat de hoeveelheden ondergrondse natuurlijke vijanden hier laag zijn. Deze voorspellingen omtrent de verschuivingen in gemeenschappen van bodemorganismen als gevolg van areaal-uitbreiding zijn echter nog niet getest.

In mijn promotie-onderzoek heb ik gekeken of areaal-uitbreidende planten inderdaad veranderingen ervaren in de gemeenschappen van bodemorganismen tussen gebieden in hun oorspronkelijke en nieuwe areaal. Daarnaast heb ik gekeken of de interacties tussen bodemorganismen en areaal-uitbreidende planten in het nieuwe leefgebied verschillen van dergelijke interacties bij meer of minder nauw verwante inheemse plantensoorten. Wortel-etende nematoden hebben een hoofdrol in mijn experimenten, omdat dit wijdverbreide en belangrijke ondergrondse herbivoren zijn, die bovendien relatief makkelijk te identificeren en kwantificeren zijn.

In hoofdstuk 2 gebruikte ik een combinatie van moleculaire en morfologische identificatiemiddelen om nematodegemeenschappen kwalitatief en kwantitatief in kaart te brengen langs een 2000 kilometer

lang transect van Griekenland tot in Nederland. Nematoden werden verzameld in de wortelgrond van areaal-uitbreidende planten die inheems zijn in de zuidelijke gebieden langs dit transect en die zich pas recentelijk in de meer noordelijk gelegen gebieden hebben gevestigd. Daarnaast werden ook nematodegemeenschappen verzameld uit de wortelgrond van verwante plantensoorten die inheems zijn langs het gehele transect. Ik verwachtte dat langs het transect een geleidelijke verandering in de compositie van nematodengemeenschappen waarneembaar zou zijn door onderliggende veranderingen in abiotische omstandigheden. Daarnaast verwachtte ik dat de veranderingen in de compositie van de nematodengemeenschappen sterker zouden zijn voor areaal-uitbreidende planten dan voor inheemse planten, en dat areaal-uitbreidende planten in hun nieuwe leefgebied minder wortel-etende nematoden in hun wortelgrond zouden hebben dan in hun oorspronkelijke leefgebied.

Globaal gezien veranderde de samenstelling van de nematode-gemeenschappen langs het transect, hetgeen betekent dat nematodegemeenschappen in Zuid-Europa verschillen van die in Noord-Europa. Dit laat zien dat areaal-uitbreidende planten in hun nieuwe leefgebied inderdaad met een andere nematodegemeenschap in aanraking komen dan in hun oorspronkelijke leefgebied. Er was echter maar één areaal-uitbreidende plantensoort die langs het transect een sterkere verandering in nematodegemeenschappen ondervond dan de nauw verwante inheemse soort: *Centaurea stoebe*. Deze areaal-uitbreider was ook de enige soort die minder wortel-etende nematoden had in wortelgrond in het nieuwe dan in het oorspronkelijke leefgebied. Alle andere areaal-uitbreidende soorten ervoeren geen sterkere veranderingen in nematodegemeenschappen dan verwante inheemse soorten en hadden net zoveel nematoden in wortelgrond van hun nieuwe als van hun oorspronkelijke leefgebied.

De hypothesen uit hoofdstuk 2 heb ik ook getest in een kasexperiment (Hoofdstuk 3). In dit experiment groeiden dezelfde areaal-uitbreidende en verwante inheemse plantensoorten in afzonderlijke plantengemeenschappen. Daarnaast werd een plantengemeenschap van areaal-uitbreiders zonder verwante inheemse soorten gecreëerd. Ik verwachtte dat, vanwege hun potentiele ecologische noviteit, deze 'niet-verwante areaal-uitbreiders' nog sterker naïeve responsen van inheemse nematoden zouden veroorzaken dan areaal-uitbreiders met verwante soorten in hun nieuwe leefgebied. Daarom verwachtte ik dat de niet-verwante areaal-uitbreiders de sterkste verschillen in de compositie van nematodegemeenschappen tussen bodems uit hun oorspronkelijke en nieuwe

verspreidingsgebieden zouden laten zien en de laagste aantallen wortel-etende nematoden zouden opbouwen in bodem uit het nieuwe verspreidingsgebied.

Ongeacht aan welke plantengemeenschap de bodems werden blootgesteld (conditionering genoemd), waren er echter geen duidelijke verschillen in nematode-gemeenschappen te zien tussen de bodems uit Zuid- en Noord-Europa. Daarnaast waren er geen verschillen tussen deze bodems in de aantallen wortel-etende nematoden. Verrassend was dat de bodems van niet-verwante areaal-uitbreiders de hoogste aantallen wortel-etende nematoden bevatten. De eenjarige levenswijze van de meeste soorten in deze gemeenschap zou hier een verklaring voor kunnen zijn, aangezien snelle groei vaak ten koste gaat van een goede verdediging tegen natuurlijke vijanden. Hoewel de individuele effecten van de plantensoorten gemaskeerd zijn in deze proef en de taxonomische resolutie lager was dan in het werk beschreven in hoofdstuk 2, wijst deze proef wel uit dat areaal-uitbreidende plantensoorten gemiddeld gezien geen sterke verschuivingen in nematode-gemeenschappen ervaren tussen hun oorspronkelijke en nieuwe verspreidingsgebied.

In de hoofdstukken 4 en 5 heb ik gekeken naar de interacties die areaal-uitbreidende en verwante inheemse plantensoorten aangaan met twee wortel-etende nematodensoorten uit het nieuwe leefgebied van de areaal-uitbreiders. In hoofdstuk 4 beschrijf ik de resultaten van een reproductieproef van deze nematoden op twee areaal-uitbreiders en twee verwante plantensoorten, in de aan- en afwezigheid van gemeenschappen van bacteriën. Ik verwachtte dat areaal-uitbreiders slechte gastheren zijn voor inheemse wortel-etende nematoden, maar dat ze ook slecht in staat zijn om natuurlijke vijanden van de nematoden uit de bodemgemeenschap aan te trekken. In overeenstemming met de resultaten van hoofdstuk 2 vond ik dat *Centaurea stoebe* een zeer slechte gastheer is voor beide nematodesoorten. De andere areaal-uitbreider, *Geranium pyrenaicum*, was echter een betere gastheer dan de verwante inheemse soort. In lijn met mijn hypothese vond ik dat inheemse plantensoorten inderdaad beter leken te zijn in het aantrekken van de vijanden van wortel-etende nematoden, maar ook dat dit soort ondergrondse multitrofe interacties zeer soort-specifiek zijn.

De experimenten in hoofdstuk 5 werden opgezet om de chemische mechanismen te ontrafelen die de verschillen in de reproductie van wortel-etende nematoden tussen areaal-uitbreiders en verwante

inheemse plantensoorten zouden kunnen verklaren. Experimenten waarmee de aantrekkingskracht van planten op nematoden kan worden getest, lieten zien dat de wortel-etende nematoden gemiddeld minder sterk werden aangetrokken door areaal-uitbreiders dan door de verwante inheemse soorten. Een interessante observatie was dat de areaal-uitbreidende plantensoort *Centaurea stoebe* de geteste nematodensoorten afstootte, in plaats van aantrok. De andere areaal-uitbreidende planten trokken nematoden weliswaar aan, maar niet zo sterk als de verwante inheemse plantensoorten.

Vergelijkingen tussen de mixen van vluchtige stoffen die in de wortels van de verschillende plantensoorten worden geproduceerd, lieten zien dat, wanneer vergeleken met de verwante inheemse soorten, *Centaurea stoebe* aanzienlijk meer unieke vluchtige stoffen produceert dan de andere areaal-uitbreidende plantensoorten. Dit suggereert dat de mate van chemische noviteit van areaal-uitbreidende plantensoorten de aantrekking van wortel-etende nematoden zou kunnen bepalen. Aangezien de chemische aantrekkingskracht van nematoden een goede voorspeller van nematoden-reproductie was, lijkt het erop dat areaal-uitbreidende plantensoorten profiteren van de aanmaak van wortelstoffen waar inheemse wortel-etende nematoden niet eerder mee in aanraking zijn geweest.

De variatie in de mate van chemische overeenkomst tussen areaal-uitbreidende en verwante inheemse soorten zou kunnen worden verklaard door onderliggende verschillen in fylogenetische afstanden tussen de verwante soorten. Om de effecten van geografische oorsprong en fylogenetische afstand op plant-bodeminteracties van elkaar te kunnen onderscheiden, ontwikkelde ik een serie experimenten met vier areaal-uitbreidende en vier inheemse soorten, die allemaal tot hetzelfde geslacht behoren (Hoofdstuk 6). Op deze manier kon ik de hypothese testen dat de meest nauw verwante soorten sterker overeenkomen in morfologische en chemische worteleigenschappen, en hun invloed op bodemgemeenschappen, dan minder nauw verwante soorten. Daarnaast testte ik of variatie in plant-bodem-terugkoppelingen - de groei van planten in door soortgenoten geconditioneerde grond - verklaard kon worden door de geografische oorsprong van de planten of door onderlinge fylogenetische afstanden.

De resultaten lieten zien dat de verschillen in geografische oorsprong tussen de plantensoorten niet de variatie in bodemgemeenschappen of plant-bodem-terugkoppelingen konden verklaren. Daarnaast

vond ik dat de meest nauw verwante soorten niet méér overeenkwamen in de chemische wortelsamenstelling dan minder nauw verwante soorten uit hetzelfde geslacht, maar dat fylogenetische afstand wel correleerde met de variatie in sommige morfologische worteleigenschappen. De variatie in schimmelgemeenschappen was gerelateerd aan de variatie in specifieke wortellengte, een maat voor de structuur van het wortelsysteem. Variatie in gemeenschappen van bacteriën correleerden aan verschillen in de chemische wortelsamenstelling tussen de planten, terwijl variatie in de gemeenschappen van protisten en nematoden co-varieerden met de bacteriegemeenschappen. Daarom konden alleen verschillen in schimmelgemeenschappen verklaard worden door onderlinge fylogenetische afstanden tussen de plantensoorten.

Tenslotte liet ik in hetzelfde experiment met *Geranium*-soorten zien dat verschillen in plant-bodem-terugkoppelingen correleerden met de aantallen wortel-etende nematoden in de bodems van de verschillende planten. Samengevat laten deze resultaten zien dat variatie in plant-bodem-interacties tussen nauw verwante soorten noch hun evolutionaire geschiedenis, noch hun geografische oorsprong weerspiegelen, wat suggereert dat de (chemische) worteleigenschappen van deze soort zijn geëvolueerd in respons op soort-specifieke condities.

Ecologische theorie voorspelt dat plantensoorten door areaal-uitbreiding aan hun ondergrondse natuurlijke vijanden kunnen ontsnappen, en in hun nieuwe areaal profiteren van een naïve respons van inheemse natuurlijke vijanden. In mijn proefschrift laat ik zien dat alleen areaal-uitbreidende plantensoorten die sterk afwijken in wortelchemie ten opzichte van inheemse soorten minder worden aangetast door inheemse wortel-etende nematoden. Mijn studies laten zien dat fylogenetische afstand geen goede maat is voor ecologische noviteit en dat sterk verwante soorten niet per se vergelijkbare plant-bodeminteracties hebben. Hoewel andere groepen bodemorganismen anders zouden kunnen reageren op areaal-uitbreidende planten, suggereren mijn resultaten dat het niet aannemelijk is dat de meeste areaal-uitbreiders zeer dominant zullen worden in hun nieuwe verspreidingsgebied, tenzij ze eigenschappen hebben die niet aanwezig zijn in de inheemse gemeenschap en kunnen bijdragen aan het gaan woekeren van deze soorten.

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Rutger

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About the author

Rutger Wilschut was born on the 2nd of February, 1989. At the age of nine, he started to develop an ever-growing interest in nature, and birds in particular, which was broadened when he joined the Dutch youth society for nature studies (NJN). During his BSc and MSc Biology studies in Wageningen he specialized in plant ecology and phylogenetics. In his first MSc-thesis he studied the evolutionary history and biogeography of the Neotropical tree genus *Mosannona* at the Department of Biosystematics, under the supervision of Dr. Lars Chatrou. Thereafter, he moved to the the Department of Terrestrial Ecology at the Netherlands Institute of Ecology for a thesis on ecological epigenetics in *Taraxacum*, therein supervised by Dr. Koen Verhoeven. During this project he got familiarized with experimental plant ecology. In November 2013, soon after his MSc graduation, he started his PhD-project on plant-soil interactions of climate change-driven range-expanding plant species, under the supervision of Prof. Dr. Wim van der Putten. In much of his free time Rutger compensates for the lack of birds in his research, most preferably by birding on his favourite Wadden Sea Island, Vlieland.

List of publications

Pirie, M.D., Maas, P.J.M., Wilschut, R.A., Melchers-Sharrott, H. & Chatrou, L.W. (2018) Parallel diversifications of *Cremastosperma* and *Mosannona* (Annonaceae), tropical rainforest trees tracking Neogene upheaval of South America. *Royal Society Open Science,* **5**.

Koorem, K., Kostenko, O., Snoek, L.B., Weser, C., Ramirez, K.S., Wilschut, R.A. & van der Putten, W.H. (2018) Relatedness with plant species in native community influences ecological consequences of range expansions. *Oikos*, **127,** 981-990.

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Wilschut, R.A., Geisen, S., Ten Hooven, F. & van der Putten, W. (2016) Interspecific differences in nematode control between range-expanding plant species and their congeneric natives. *Soil Biology and Biochemistry,* **100,** 233-241.

Wilschut, R.A., Oplaat, C., Snoek, L.B., Kirschner, J. & Verhoeven, K.J. (2016) Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage. *Molecular Ecology,* **25**, 1759-1768.

Kirschner, J., Oplaat, C., Verhoeven, K., Zeisek, V., Uhlemann, I., Trávníček, B., Räsänen, J., Wilschut, R.A. & Štěpánek, J. (2016) Identification of oligoclonal agamospermous microspecies: taxonomic specialists versus microsatellites. *Preslia,* **88,** 1-17.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Linking invasion ecology and multi-trophic interaction research to predict root-feeding nematode responses to range-expanding plant species

Writing of project proposal (4.5 ECTS)

- Multitrophic plant-nematode interactions in changing communities

Post-graduate courses (6.6 ECTS)

- Introduction to R for statistical analyses; PE&RC (2014)
- Identification of terrestrial and freshwater nematodes; NIOO/Nematology (2014)
- Multivariate analysis; PE&RC (2015)
- Soil Ecology and planetary boundaries; PE&RC (2016)

Invited review of (unpublished) journal manuscript (7 ECTS)

- Oikos: Plant-plant interactions along an elevational gradient (2016)
- Geoderma: Invasive plant effects on soil nutrients (2016)
- Plant ecology & diversity: Plant dioecy and nematode communities (2017)
- Biological journal of the Linnaean Society: Biogeography of a Neotropical plant species (2017)
- Oikos: Herbivory and nitrogen effects on nematode communities (2017)
- Journal of Applied Ecology: Litter and nitrogen effects on nematode communities (2017)
- Journal of Applied Ecology: Phylogeny effects on plant-soil feedback of a crop species (2018)

Competence strengthening / skills courses (1.9 ECTS)

- Growing a PhD; KNAW (2014)
- Career perspectives; WGS (2017)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.6 ECTS)

- PE&RC First years weekend (2014)
- Soil-plant interactions symposium (2014)
- PE&RC Day (2014, 2015, 2016)
- Current themes in ecology (2015)
- Microbe-plant interactions symposium (2016)
- PE&RC Last years weekend (2017)

Discussion groups / local seminars / other scientific meetings (5.5 ECTS)

- WEES (2013-2017)
- NIOO Terrestrial ecology PhD discussion groups (2014-2016)
- PSI Discussion group (2016-2017)

International symposia, workshops and conferences (18.4 ECTS)

- GSBI; poster presentation; Dijon (2014)
- NAEM; Poster presentation: 2014, 2015, 2017; oral presentation: 2016,2018
- Rhizo4; poster presentation; Maastricht (2015)
- ESA Annual meeting; oral presentation; Fort Lauderdale (2016)
- BES Annual meeting; oral presentation; Liverpool (2016)
- Ecology across borders; poster presentation; Gent (2017)

Supervision of a MSc student

- Plant-nematode interactions of a range-expanding plant species (M. Rutten)

Colophon

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