Contrasting covariation of above- and belowground invertebrate species across plant genotypes

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Summary

1. Invertebrate species generally do not respond independently to genotypic variation in plants, giving rise to clusters of species that naturally associate with or avoid certain genotypes. This covariation causes coevolution to be diffuse rather than pairwise. Studies on this topic, however, have never considered the belowground invertebrate community, leaving a critical gap in our understanding.

2. We investigated the covariation among naturally colonising above- and belowground invertebrate species across six genetically distinct populations of the dune grass *Ammophila arenaria*. After having grown from seed in a common garden, plants were randomised in a single field site to exclude all but broad-sense genetic variation.

3. Strong positive covariation across genotypes among both above- and belowground invertebrates was detected, while correlations between these two groups were negative. This clustering of above- and belowground species matched well with order level taxonomy. Host range, trophic level and food type on the other hand did not correspond well with the clusters. Within the cluster of aboveground fauna, subsequent groupings were not related to any phylogenetic or ecological characteristic, although correlations within these subgroups were very high. We furthermore demonstrated significant differences in multiple invertebrate species occurrence between plant genotypes, in general as well as at the above- and belowground level.

4. The observed strong covariation suggests diffuse coevolution between *A. arenaria* and its associated invertebrate species. The trade-off between root and shoot invertebrates could however hamper directional selection on resistance to either group.

5. Our results clearly demonstrate the need for studies of plant–animal interactions to include the belowground fauna, as this might drastically alter our general conception of how plants and their associated animal communities interact and how these interactions shape the process of evolution.

Key-words: aboveground–belowground interactions, community structure, diffuse selection, herbivore, host plant selection, host plant variation, plant–animal interactions, resistance trade-off

Introduction

It is becoming increasingly clear that invertebrate species do not only distinguish between different host-plant species but also select on variation in traits within a particular plant species (Johnson, Lajeunesse & Agrawal 2006). This variation can be a result of the genotype and/or the environment of the plant, or the interaction between both (Falconer & Mackay 1996). If variation in the selected plant traits has a genetic component, plants have the potential to evolve in response to this selection, in turn feeding back to the performance of invertebrates (Toju 2009). Most plant species, however, are host to a plethora of invertebrate herbivores, predators and detritivores (Schoonhoven, van Loon & Dicke 2005). A key question in the evolutionary ecology of plant–invertebrate interactions is whether responses of different invertebrates to plant genotypes are correlated or independent (Leimu & Koricheva 2006). If different invertebrate species select on different plant traits, independent responses should occur (Leimu & Koricheva 2006). Negative correlations between invertebrate responses to plant genotypes point at genetically embedded trade-offs between the plant's susceptibilities to different invertebrate species (Leimu & Koricheva 2006). Positive covariation of invertebrate species across plant genotypes, on the other hand, can be expected if invertebrates depend on similar qualitative or quantitative properties of the plant, or when a plant develops generalised defence traits that are effective against a range of invertebrates (Leimu &

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Koricheva 2006). These different patterns of responses have consequences for the evolution of plant–invertebrate interactions. Genetically uncorrelated responses are a prerequisite to pairwise selection and (co)evolution, while correlated responses imply diffuse selection (Iwao & Rausher 1997; Inouye & Stinchcombe 2001).

Few field studies have investigated the effect of intraspecific genetic variation in plants on the covariation within the associated invertebrate communities (Maddox & Root 1990; Roche & Fritz 1997; Johnson & Agrawal 2007; Newton, Bullock & Hodgson 2009). In general, these studies provide more evidence of positive covariation of invertebrates across plant genotypes than of negative correlations or independent responses, suggesting a prevalence of diffuse coevolution. Moreover, these studies found little evidence for shared phylogeny or ecological aspects of the covarying invertebrate species. All these studies focused solely on the aboveground invertebrate community. Most plants, however, harbour a diverse community of belowground invertebrates that rivals its aboveground counterpart in diversity of functions, interactions and degrees of specificity (Boag & Yeates 1998; Brussaard *et al*. 2007). Although studies on the effect of plant genotypic variation on belowground invertebrates in natural ecosystems are scarce, agricultural systems provide ample evidence of root-associated invertebrates selecting on plant genotypes (Anwar & McKenry 2007). The devastation caused by some root-feeding pest species leaves no doubt about the potential impact of belowground invertebrates on plant fitness (Smiley *et al*. 2005). The 'belowground avoidance, aboveground defence' hypothesis (van der Putten *et al*. 2001) states that dispersal is a more useful mechanism for plants to escape from belowground antagonists, while the development of direct defences is better suited to withstand attack by mobile aboveground animals. However, this hypothesis assumes aggressive soil pathogens and short-lived plants. Therefore, we see no theoretical reason as to why selective pressures imposed by root-associated invertebrates should be less important than those exerted by shoot-dwelling invertebrates, especially for perennial plants lacking obvious direct defences. However, compared to aboveground invertebrates, our current understanding of the distinction between plant genotypes by belowground invertebrates is quite limited. Investigating how different above- and belowground invertebrates covary across plant genotypes would provide a first step towards a more complete understanding of the evolutionary ecology of plants and their invertebrate communities. Our aim was to investigate whether root and shoot invertebrates respond similarly, independently or oppositely to plant genotype. The first option would suggest some generalised defence mechanism in the plant to both above- and belowground invertebrates. The second possibility implies selection of root and shoot invertebrates on different and independent plant traits, while the last option would be indicative of some trade-off between susceptibility towards above- and belowground invertebrates.

Two studies have investigated the effect of variation in the dune grass *Ammophila arenaria* (L.) Link on the performance of root-feeding nematodes (Reis, Freitas & van der Putten 2008; de la Peña, Bonte & Moens 2009). In both cases, at least some of the tested nematode populations showed different multiplication on different host plant populations. Genetic differences between the populations of *A. arenaria* that were used in these studies were confirmed by ISSR markers (Rodriguez-Echeverria, Freitas & van der Putten 2008). Combined, these studies demonstrate that a broad-sense genetic component of variation between populations affected nematode performance. Given the evidence for effects of plant genotype on the belowground invertebrate fauna and the genetic differentiation between populations of *A. arenaria*, we assessed natural colonisation by above- and belowground invertebrates of different populations of this plant species in a field experiment. We specifically addressed the following questions: (i) Does plant genotype affect colonisation by above- and belowground invertebrate species and as such determine the composition of the resulting above- and belowground invertebrate community? (ii) To what extent do above- and belowground invertebrate species covary in their responses to plant genotype, and more specifically, are aboveground invertebrates' responses similar to, opposite to or independent from belowground invertebrate responses? (iii) Is this covariation related to phylogenetical or ecological characteristics of the involved species?

Materials and methods

STUDY SITE AND SYSTEM

The experiment was conducted at the Flemish nature reserve 'Westhoek' in De Panne, Belgium (51° 5′ N, 2° 34′ O). This nature reserve is located at the North Sea coast and comprises a mixture of dynamic sand dunes, moss dunes, grasslands and shrub thickets. In the dynamic sand dunes, *A. arenaria* is the dominant species, often occurring in large monospecific stands that alternate with open patches of bare sand (Huiskes 1979).

Ammophila arenaria is a native perennial grass, which depends on regular sand burial to maintain vigorous growth (Huiskes 1979). It is distributed along all European coasts south of latitude 63° N. Previous study in the field site has demonstrated that the most abundant invertebrates associated with roots of *A. arenaria* are different root-feeding nematodes belonging to the genera *Heterodera*, *Pratylenchus* and *Meloidogyne* (Vandegehuchte, de la Peña & Bonte 2010). All these genera have been proven to reduce plant performance in at least some cases (Brinkman, Duyts & Van Der Putten 2005a,b; de la Peña *et al*. 2008).

Although less well studied, the aboveground invertebrate community of *A. arenaria* vegetations is a diverse one comprising woodlice, molluscs and a range of arthropod species. The community includes herbivores, detritivores and predators, with different herbivores feeding on different plant parts and varying in host range (Huiskes 1979; Weeda *et al*. 1994).

SETUP OF FIELD EXPERIMENT

Seeds of *A. arenaria* were collected each summer from 2002 until 2005 from single populations at six locations in Europe (Fig. S1, Supporting information), namely Oostvoorne (the Netherlands: 51° 53′ N, 4° 02′ O), Het Zwin (Belgium: 51° 21′ N, 3° 21′ O), De Panne

(Belgium: 51° 5′ N, 2° 34′ O), Westende (Belgium: 51° 9′ N, 2° 45′ O), Comporta (Portugal: 38° 22′ N, 8° 48′ W) and São Jacinto (Portugal: 40° 39′ N, 8° 44′ W). At each location, 5–10 spikes were collected from four plants, standing 25 m apart. The genotype of the populations from Het Zwin, Oostvoorne, São Jacinto and Comporta has previously been characterised by means of ISSR markers (Rodriguez-Echeverria, Freitas & van der Putten 2008) revealing genetic differences between all these populations that corresponded to geographical distance. Although the distance between Westende and De Panne is somewhat smaller (17 km) than average distances in the cited study, a study by Hol *et al.* (2008) demonstrated genetic differentiation between stands of *A. arenaria* in coastal and inland dunes located a mere 200 m apart. Analysis of seedlings of these six populations with AFLP markers confirmed both differentiation between populations and isolation by distance (Vandegehuchte M.L., Breyne P., de la Peña E. & Bonte D., unpublished data).

Seeds were germinated and plants were grown according to a standardised procedure (see Appendix S1, Supporting information). Seventeen months after germination, 10 mature tussocks of each population were transferred to the field. This length of time in a common environment should effectively minimise maternal effects (Johnson, Lajeunesse & Agrawal 2006). Plants were placed in a randomised design of six rows of 10 tussocks at a large open sandy patch in a dynamic dune area (51° 5′ 29·79″ N, 2° 34′ 8·73″ O). Each tussock was carefully removed from its pot, without damaging the roots, and transplanted into the dune soil with the root system freely accessible to soil organisms. Plants were in the field from 22 May until 25 September, 2008.

Plants of each population were clones neither half or full sibs. Additive genetic variation in the plants' associations with different invertebrates could therefore not be estimated. We deliberately chose geographically distant populations in order to maximise the genetic variation between, relative to within, populations. The low variation within populations is probably due to the almost exclusively clonal reproduction of *A. arenaria* in the field (Huiskes 1979; Hol *et al.* 2008). It therefore seems reasonable to consider each population as a distinct genotype. Moreover, we chose our field site because its uniformity should eliminate environmental differences and interactions with wild stands of *A. arenaria*. Therefore, all observed differences between plant populations should be because of broad-sense genetic variation. For ease of reading, we used genotype and population as exchangeable terms throughout the text. Our aim was in first instance to determine the variation in the associations of these genotypes with different invertebrate species as such, not the particular plant trait causing this variation. Only the first invertebrate species to arrive is known with certainty to select a plant independently, because subsequent colonisation might be affected by the presence of other invertebrates. These interactions, however, can be considered as part of the natural seasonal assembly process of the community on each plant. The effect of plant genotype on this process hence forms the focus of this study.

RECORDING OF ABOVE- AND BELOWGROUND INVERTEBRATE COLONISATION

All plants were carefully hand searched at eight times throughout the growing season (from 4 July to 24 September, 2008) to assess the occurrence (presence/absence) of aboveground invertebrate species. Occurrence seemed more appropriate than abundance as the majority of observations of species on plants concerned single individuals, leaving a large proportion of plants unoccupied. Only aphids established large numbers. These colonies, however, were likely founded through parthenogenesis by a single fundatrix that selected a host plant. We thus decided to record the occurrence of aphid species too, because it allowed objective comparison with the other recorded species. At harvest, all plants were cut off at ground level and the entire root system was dug out. Samples were transferred to the laboratory. Leaves were fresh weighed, oven-dried overnight at 65 °C and weighed again. The relative water content of the shoots was calculated as the difference between fresh and dry weight divided by the fresh weight. Roots were washed with tap water, fresh weighed and separated into three equal parts, one of which was stained with acid fuchsin (Baker & Gowen 1996) to visualise root-feeding nematodes in radicula. Stained roots were cut into pieces of *c.* 1 cm. From each plant, 30 randomly chosen root fragments were inspected under a microscope and nematodes were counted and identified. A fragment that had lost more than half of its cortex was categorised as degenerate, otherwise as vital. A vitality measure for the root system was calculated as the proportion of vital fragments in each sample.

SELECTION OF INVERTEBRATE SPECIES FOR ANALYSES

A diverse array of aboveground invertebrates was observed on the experimental plants over the course of the experiment. The 10 most encountered species were the aphids *Schizaphis rufula* (Walker 1849), *Rhopalosiphum padi* (Linnaeus 1758) and *Laingia psammae* Theobald 1922, the froghopper *Neophilaenus lineatus* (Linnaeus 1758), the leafhopper *Psammotettix maritimus* (Perris 1857), the snails *Theba pisana* (O.F. Muller 1774) and *Candidula intersecta* (Poiret 1801), the woodlouse *Porcellio scaber* Latreille 1804, the harvestman *Phalangium opilio* Linnaeus 1761 and the weevil *Philopedon plagiatum* (Schaller 1783). The majority of the root-feeding nematodes belonged to the species *Meloidogyne duytsi* Karssen *et al.* 1998, *Heterodera* sp. and *Pratylenchus* sp. (presumably *Heterodera arenaria* Cooper 1955 and *Pratylenchus brzeskii* Karssen *et al.* 2000). These 13 species were chosen for further analyses. They cover a wide spectrum of taxa, functional groups and degrees of specificity and include herbivores, a predator and a detritivore (Fig. 4). Species identifications and ecological characteristics were based on the available literature (Ribaut 1952; Sutton 1972; Freude, Harde & Lohse 1981; Heie 1982, 1986; Gittenberger, Backhuys & Ripken 1984; Baker & Vogelzang 1988; Brzeski 1998; Karssen, van Aelst & van der Putten 1998; Sternberg 2000; Holzinger, Kammerlander & Nickel 2003; Nickel 2003; van der Stoel & van der Putten 2006; de la Peña *et al.* 2008; Holman 2009; Wijnhoven 2009).

STATISTICAL ANALYSES

Details on the differences in plant characteristics between populations can be found in Appendix S1, Fig. S2 and Fig. S3, Supporting information.

To investigate whether plant genotype affected the colonisation by aboveground invertebrate species, the effect of plant population on the occurrence of each of the 10 aboveground species was modelled by means of a generalised linear mixed model with a binomial distribution and a logit link function. Dates before the first or after the last date of presence of each species were excluded to avoid zero inflation. To account for temporal autocorrelation of occurrences on individual plants, the date effect for each plant was modelled as a residual random effect with individual plant as subject. A first-order autoregressive covariance structure was used, as it could be assumed that the correlation between occurrences got higher as dates were situated closer to each other in time. To evaluate the extent to which effects

were because of differences in biomass or relative water content, each model was run both with and without these two variables as covariates. We know from previous work that in this system both above- and belowground plant biomass can affect aboveground invertebrates (Vandegehuchte, de la Peña & Bonte 2010). Because of the high correlation between root and shoot fresh weight in this case (Pearson's r: 0·78379), only shoot fresh weight was however included as a biomass covariate. Non-significant covariates ($P > 0·05$) were excluded from the model to obtain robust P-values for the other predictors. The Kenward–Roger approximation was used in all cases to calculate the denominator degrees of freedom.

To assess whether plant genotype affected the colonisation by belowground invertebrate species, the effect of plant population on the occurrence of each of the root-feeding nematodes was modelled by means of a generalised linear model with a binomial distribution and a logit link function. Root fresh weight and root vitality are known to potentially affect nematode densities (Vandegehuchte, de la Peña & Bonte 2010) and were hence included as covariates in a second model. Non-significant covariates ($P > 0·05$) were excluded from the model.

To investigate to what extent the above- and belowground invertebrate species covaried among plant genotypes, we calculated all 78 pairwise Pearson product–moment correlations between the occurrences of species over the six plant genotypes. We used the mean occurrence on each genotype as estimated by the previously described models without covariates. Because it can be expected that 5% of all correlations are significant at the $P = 0·05$ level by chance, a binomial test was used to assess whether the proportion of significant correlations significantly differed from 0·05 (Zar 1996). To assess whether groups of invertebrates covaried in their occurrence, we used hierarchical cluster analysis based on the species x species matrix of across-genotype correlation coefficients. Prior to analysis, correlation coefficients were transformed to Euclidean distance by the formula $\sqrt{(1-r)}$. Ward's minimum variance method was used to create a dendrogram that hierarchically groups the 13 invertebrate species, minimising the variance in correlation coefficients within clusters and maximising the variance between clusters. We cut the dendrogram at the level where groups retain 75% of the information compared to individual species in order to obtain robust clusters.

We further investigated whether the covariation captured by the obtained clusters was related to each of five phylogenetical or ecological species traits: taxonomy at the order level, host range (generalist, oligophagous and specialist), food type (leaf tissue, phloem, xylem, prey, detritus and root), trophic group (herbivore, predator and detritivore) and location on the plant (aboveground and belowground). We considered herbivores that fed on *A. arenaria* and maximum four other related grass species as specialists. Species were considered oligophagous if they mainly fed on grasses and to some extent on Juncaceae or Cyperaceae. Generalist herbivores are species that can feed on a multitude of plant families. The detritivore *P. scaber* is a generalist, because it feeds on detritus in almost any habitat type. The predator *P. opilio* is also a generalist, feeding on a wide range of small soft-bodied invertebrates. For each of these five traits, we assessed whether the average correlation coefficient among species within groups was greater than the average correlation coefficient among species between groups. The same was carried out for the statistically derived clusters that served as a control, where we expected the maximum difference between within- and between-group correlations. The significance of differences in correlation coefficient within vs. between groups was determined by means of permutational analysis of variance based on Euclidean distance and 99 999 permutations of raw data (Anderson, Gorley & Clarke 2008). Because we specifically

addressed the question whether above- and belowground invertebrates responded in similar, opposite or independent ways to plant genetic variation, we calculated the average correlation and a one-sided 95% confidence interval (CI) for aboveground invertebrate pairs, belowground invertebrate pairs and pairs composed of an above- and belowground invertebrate.

To assess whether plant genotype affected the overall composition of the above- and belowground invertebrate community, we performed a permutational multivariate analysis of variance. As occurrence of the aboveground invertebrate species was measured on several dates, we estimated least squares mean occurrence on each plant using individual plant as a predictor in the previously described generalised linear mixed models. We used Bray–Curtis distance and 99 999 permutations of raw data. Analogously we calculated pairwise differences between genotypes. To assess whether effects of plant genotype were detectable within the above- and belowground invertebrate community, we performed two similar analyses with only the above- or belowground invertebrate occurrences as dependent values.

Results

INVERTEBRATE RESPONSES TO GENOTYPE

Considering overall differences in aboveground invertebrate species occurrence on plants from different populations, seven of 10 tested species responded significantly to plant population (Table 1). For *L. psammae*, *N. lineatus*, *P. opilio*, *P. scaber*, *P. maritimus* and *R. padi*, there was a similar trend of higher occurrence on more local plant populations (Fig. 1). Shoot fresh weight proved to be a significant covariate for *P. opilio*, *P. scaber*, *P. maritimus* and *T. pisana*, while both shoot fresh weight and shoot relative water content significantly affected the occurrence of *L. psammae* and *N. lineatus*. Inclusion of these covariates only altered the population effect for *T. pisana*, where after correction for biomass differences the population effect disappeared. Note that the population effect for the beetle *P. plagiatum* ($F_{5,174}$: 2·07, P: 0·07) concurs with the observed trend of higher occurrence on more local plant populations. This species too was not affected by shoot biomass or water content. Two species, *S. rufula* and *C. intersecta*, did not respond to plant population, a result that did not change after correcting for the effect of shoot mass on *C. intersecta* and the effect of shoot mass and water content on *S. rufula* (Table 1 and Fig. 1). For all species whose occurrence responded significantly to shoot fresh weight, the effect was positive. However, the occurrence of *N. lineatus* and *S. rufula* increased, while that of *L. psammae* decreased with increased water content of the shoot.

None of the root-feeding nematodes' occurrences was significantly influenced by plant genotype (Fig. 2). Inclusion of root biomass and/or root vitality did not significantly improve any of the models.

COVARIATION OF INVERTEBRATES ACROSS PLANT GENOTYPES

Correlations between species across plant genotypes varied from −0·73 to 0·97, with an average of 0·31 (SE: 0·055, n: 78).

Table 1. Results of the generalised linear mixed models (*F*- and *P*-values) of the effects of *Ammophila arenaria* population of origin on the occurrence of the ten most abundant aboveground invertebrate species, without (left panel) or with (right panel) shoot fresh weight and shoot relative water content as covariates. NS: not significant ($P > 0.05$), and hence excluded from the model

Source of variation	d.f.	F	P	d.f.	F	P
Candidula intersecta						
Population	5, 171·9	1·32	0·2581	5, 171·4	0·77	0·5737
Shoot mass				1, 171·4	4·79	0·0300
Shoot water content						NS
Laingia psammae						
Population	5, 181·4	3·10	0·0104	5, 189·5	2·13	0·0641
Shoot mass				1, 189·5	4·33	0·0389
Shoot water content				1, 189·5	4·48	0·0357
Neophilaenus lineatus						
Population	5, 134·3	3·32	0·0074	5, 136	3·53	0·0050
Shoot mass				1, 136	6·09	0·0148
Shoot water content				1, 136	3·95	0·0489
Phalangium opilio						
Population	5, 149·2	2·90	0·0157	5, 153·8	2·35	0·0436
Shoot mass				1, 153·8	14·67	0·0002
Shoot water content						NS
Philopedon plagiatum						
Population	5, 174	2·07	0·0708	5, 174	2·07	0·0708
Shoot mass						NS
Shoot water content						NS
Porcellio scaber						
Population	5, 181·6	3·42	0·0056	5, 188·3	4·34	0·0009
Shoot mass				1, 188·3	33·86	< 0·0001
Shoot water content						NS
Psammotettix maritimus						
Population	5, 220·7	4·11	0·0014	5, 225·5	2·86	0·0159
Shoot mass				1, 176·5	3·74	0·0547
Shoot water content						NS
Rhopalosiphum padi						
Population	5, 150·3	3·09	0·0110	5, 150·3	3·09	0·0110
Shoot mass						NS
Shoot water content						NS
Schizaphis rufula						
Population	5, 157·4	1·21	0·3061	5, 167·5	1·94	0·0902
Shoot mass				1, 167·5	10·85	0·0012
Shoot water content				1, 167·5	5·14	0·0246
Theba pisana						
Population	5, 174·2	3·38	0·0061	5, 174·6	1·41	0·2241
Shoot mass				1, 174·6	5·81	0·0170
Shoot water content						NS

Of all 78 correlations, 14 were significant at the 0·05 level (Fig. 3). To obtain such a proportion of significant correlations by chance is highly unlikely (binomial test: $P < 0.0001$). Based on cluster analysis, we defined four groups of covarying species (Fig. 4). Consistent with our expectations, the mean correlation between species occurrences across plant genotypes within clusters was significantly higher than the mean correlation between clusters (Table 2). A very large mean correlation coefficient of 0·81 (SE: 0·034, *n*: 17) was observed within clusters. The dendrogram displays a first separation between above- and belowground invertebrates. If species are grouped according to their location with respect to the soil surface, this leads to a significant difference between the average correlation of 0·60 (SE: 0·038, *n*: 48) within groups and the average correlation of −0·16 (SE: 0·070, *n*: 30) between groups (Table 2). The

average correlation between aboveground invertebrates was 0·60 (SE: 0·040, *n*: 45, 95% CI: 0·54–0·67), while that between belowground invertebrates was 0·65 (SE: 0·12, *n*: 3, 95% CI: 0·45–0·85), two highly significant results. The average correlation of −0·16 between above- and belowground invertebrate occurrences also turned out to be significant (95% CI: −0·27 to −0·04). This indicates that on average, belowground invertebrates were negatively correlated with the aboveground ones across plant genotypes. Mean correlations within the different trophic groups were actually significantly lower than between groups (Table 2), implying that trophic level did not contribute to the observed clustering. The mean correlation between invertebrates with the same food type was not significantly different from the correlation between food types (Table 2), although a trend of higher within-group correlations was observed. The host range of species poorly
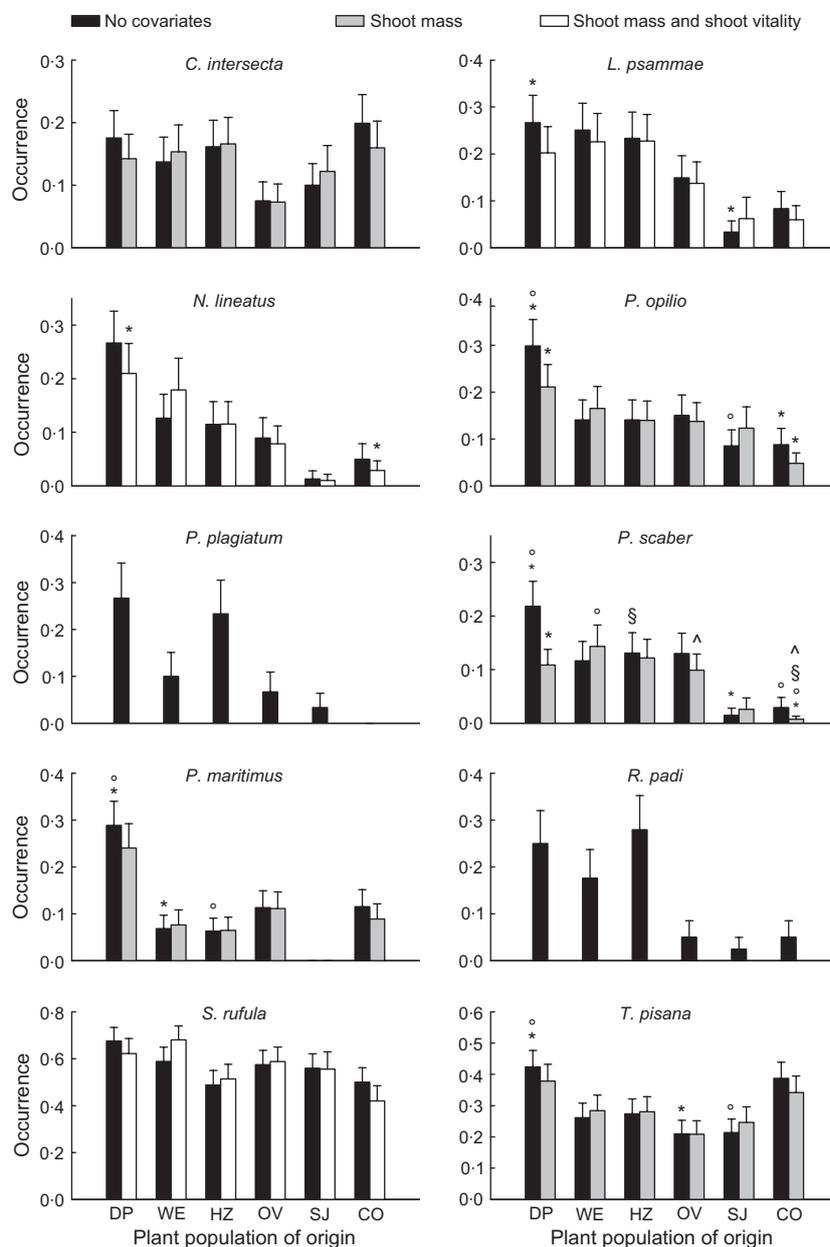
Fig. 1. Chance of occurrence of the ten most abundant aboveground invertebrate species on *Ammophila arenaria* from different populations. In order of ascending distance to the local population: DP, De Panne – Belgium; WE, Westende – Belgium; HZ, Het Zwin – Belgium; OV, Oostvoorne – Netherlands; SJ, São Jacinto – Portugal; CO, Comporta – Portugal. Black bars: overall population effect, grey bars: population effect, modelled with shoot fresh weight as a covariate, white bars: population effect modelled with shoot fresh weight and relative water content as covariates (LS means + SE). Significant differences are indicated by same symbols above the bars following Tukey's hsd post hoc test ($P < 0.05$).

matched the clusters (Table 2), while correlations were significantly higher within than between-order level taxa (Table 2). In summary, the hierarchical clustering first split off the belowground invertebrates (Fig. 4, cluster D), all root-feeding Tylenchida, and within the remaining group of aboveground invertebrates then separated a group of two leaf-feeding Pulmonata (cluster A). The remaining species, all arthropods, were grouped in two clusters (B and C), which did not correspond well with any of the phylogenetic or ecological groupings.

EFFECT OF PLANT GENOTYPE ON COMMUNITY COMPOSITION

The effect of plant genotype on the composition of the 13-species invertebrate community was highly significant (pseudo-$F_{5,54}$: 3·07, P: 0·00001). Pairwise differences between plant populations in their invertebrate community composition were significant at the $P < 0.05$ level in 10 of 15 cases. The 10-species aboveground invertebrate community was also significantly affected by plant genotype (pseudo-$F_{5,54}$: 3·48, P: 0·00001) with 10 of 15 pairwise differences between plant genotypes being significant. Although the effect of plant genotype was not significant for any of the individual nematode species, the effect on the group of nematodes was significant (pseudo-$F_{5,54}$: 2·71, P: 0·0124). The nematode community differed significantly for 3 of 15 pairs of plant genotypes. We visualised the relationship between plant genotype and the invertebrate community by means of a Nonmetric Multidimensional Scaling ordination (see Appendix S2 and Fig. S4, Supporting information).
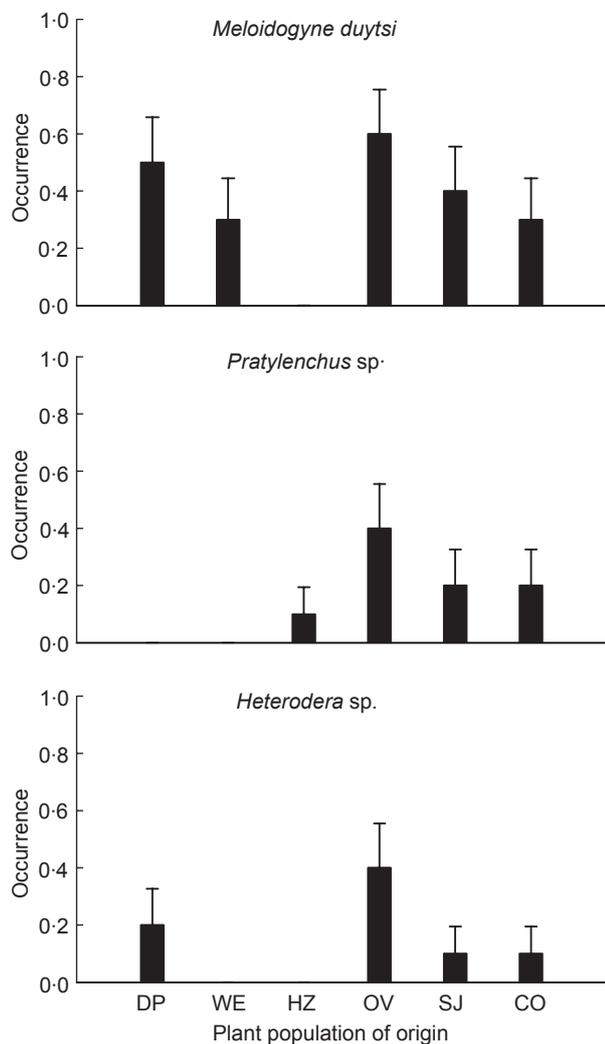
Fig. 2. Chance of occurrence of the three most abundant root-feeding nematodes on *Ammophila arenaria* from different populations. In order of ascending distance to the local population: DP, De Panne – Belgium; WE, Westende – Belgium; HZ, Het Zwin – Belgium; OV, Oostvoorne – Netherlands; SJ, São Jacinto – Portugal; CO, Comporta – Portugal. Bars represent LS means + SE. Significant differences are indicated by same symbols above the bars following Tukey's hsd post hoc test ($P < 0.05$).

Discussion

RESPONSE OF INVERTEBRATE SPECIES TO PLANT GENOTYPE

Our results demonstrate that genetic variation in *A. arenaria* can affect the colonisation by invertebrate species. At the individual species level, aboveground invertebrates tended to differentiate more strongly between plant genotypes than belowground invertebrates. For only one of the aboveground species the effect could be attributed to biomass differences, while differences in relative water content did not affect the genotype effect. This implies that most of the aboveground invertebrates selected on differences of a more subtle physiological nature, such as in nutritional quality or leaf tough-

ness. Although the occurrence of each of the nematode species did not significantly differ between plant genotypes, their combined occurrence did. This can be explained by the positive covariation between the three nematode species and the enhanced statistical power of the multivariate analysis. Considering the large number of aboveground species that responded significantly to plant genotype, it was not surprising that the effect of plant genotype was highly significant on the aboveground community of invertebrate species, as well as on the entire community. This is the first study to demonstrate that intraspecific genetic variation in plants has the potential to structure both the above- and belowground invertebrate community.

Differential colonisation of naturally occurring plant genotypes by aboveground arthropod species is widely documented (Fritz & Price 1988; Fritz 1990; Orians & Fritz 1996; Shen & Bach 1997; Pilson 2000; Stinchcombe & Rausher 2001; Tikkanen *et al.* 2003). One study by Crutsinger *et al.* (2008) demonstrated that the foliage-based arthropod diversity and abundance on *Solidago altissima* L. clones were strongly affected by plant genotypic identity, while the litter-based community only showed weak responses. Interestingly, the species showing the strongest response to *A. arenaria* genotype was *P. scaber*, a detritivore. Therefore, more plant species should be investigated before generalisations can be made about the responses of litter feeders to intraspecific genetic variation in plants.

A large body of literature documents on the genetic base of differences between cultivars of crop species in their resistance to root-associated invertebrates, nematodes in particular (Fuller, Lilley & Urwin 2008). Similar results for non-crop plant species are however limited. In contrast to the laboratory multiplication experiments by Reis, Freitas & van der Putten (2008) and de la Peña, Bonte & Moens (2009), we could not detect species level responses of nematodes to plant genotypic identity. However, because we considered occurrence instead of abundance, and because nematodes in the field rarely reach laboratory densities, the detection level of responses in our study could have been considerably lower. This conjecture is further confirmed by the fact that taken together, the community of three nematode species showed significant differences in occurrence between genotypes.

COVARIATION OF INVERTEBRATE SPECIES ACROSS PLANT GENOTYPES

Cluster analysis based on a matrix of across-genotype correlations between invertebrate species' occurrences revealed a large degree of covariation between species across plant genotypes. Interestingly, the first division in the dendrogram was that between above- and belowground invertebrates, indicating that, as groups, they selected differently on plant genotype. Not surprisingly, of all tested ecological and phylogenetic groupings of invertebrate species, the location with respect to the soil surface matched the clusters best. Invertebrate species belonging to the same order also clustered together, and there was a tendency for species within clusters
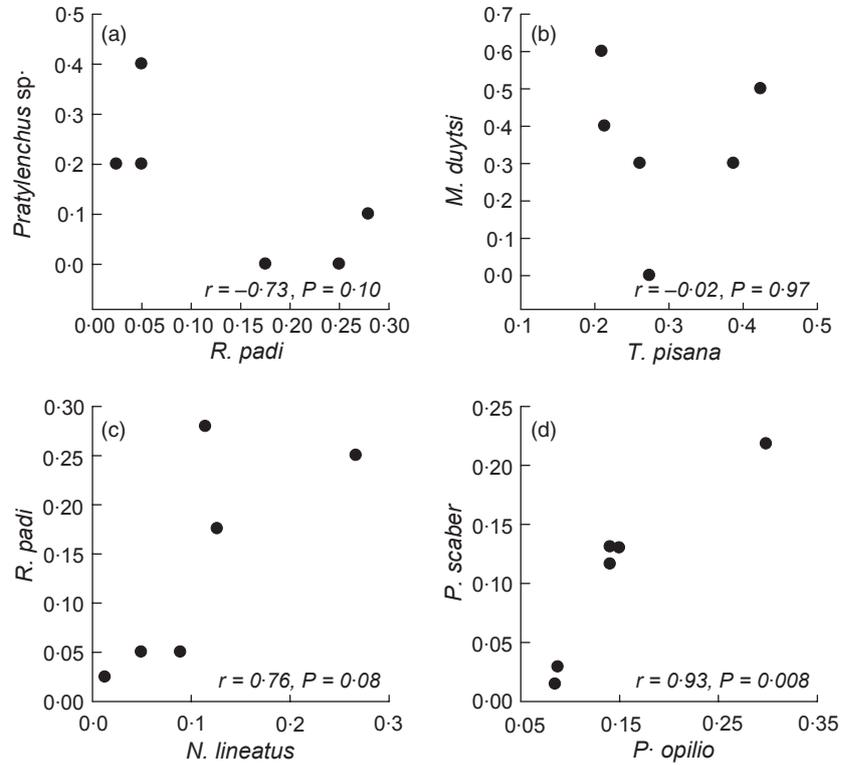
Fig. 3. Examples of pairwise Pearson product–moment correlations between the occurrences of invertebrate species across the six genotypes of *Ammophila arenaria*. These examples demonstrate the range of observed correlations.
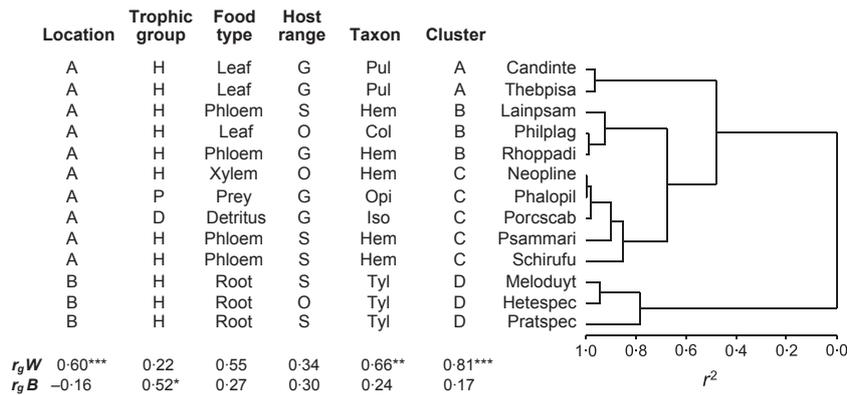

Location	Trophic group	Food type	Host range	Taxon	Cluster	
A	H	Leaf	G	Pul	A	Candinte
A	H	Leaf	G	Pul	A	Thebpisa
A	H	Phloem	S	Hem	B	Lainpsam
A	H	Leaf	O	Col	B	Philplag
A	H	Phloem	G	Hem	B	Rhoppadi
A	H	Xylem	O	Hem	C	Neopline
A	P	Prey	G	Opi	C	Phalopil
A	D	Detritus	G	Iso	C	Porcscab
A	H	Phloem	S	Hem	C	Psammari
A	H	Phloem	S	Hem	C	Schirufu
B	H	Root	S	Tyl	D	Meloduyt
B	H	Root	O	Tyl	D	Hetespec
B	H	Root	S	Tyl	D	Pratspec
r_g**W** 0·60***	0·22	0·55	0·34	0·66**	0·81***	
r_g**B** −0·16	0·52*	0·27	0·30	0·24	0·17	

Fig. 4. Dendrogram depicting the covariation of the 13 most abundant invertebrate species across the six genotypes of *Ammophila arenaria*, based on hierarchical cluster analysis. We considered clusters at the 75% information level. Species are furthermore grouped by order level taxonomy (Pulmonata: Pul, Hemiptera: Hem, Coleoptera: Col, Opiliones: Opi, Isopoda: Iso, Tylenchida: Tyl), host range (generalist: G, oligophagous: O, specialist: S), food type (leaf tissue, phloem, xylem, prey, detritus, root) and trophic level (herbivore: H, predator: P, detritivore: D). We compared the average across-genotype correlation of species occurrences within the clusters/groups (r_g W) with the average correlation of species occurrences between the clusters/groups (r_g B). Significant differences: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Species names on the dendrogram are a combination of the first four letters of the genus followed by the first four letters of the species epithet or spec if the species was uncertain.

to forage on the same food type. Our conclusions therefore would have been drastically different if we had only considered the aboveground component of the invertebrate community. If we remove the cluster of nematodes (D) from the dendrogram (Fig. 4), the average correlations within and between groups only differ for the statistically derived clusters (A, B and C). This implies that the matching with order level taxonomy and food type was because of the first separa-

tion of a cluster of root-feeding nematodes of the order of Tylenchida.

In a meta-analysis on genetic correlations between plant resistances to different aboveground enemies, Leimu & Koricheva (2006) found that across 29 studies, the average correlation was 0·1470 and ranged between −0·833 and +1. From this they concluded that positive covariation between enemies of plants is likely common, suggesting a prevalence of

Table 2. Difference in correlation coefficient across six plant genotypes of pairs of invertebrate species within- vs. between groups. Invertebrate species were grouped according to ecological characteristics as well as based on hierarchical cluster analysis. Significance of differences was tested by means of permutational analysis of variance based on Euclidean distance and 99 999 permutations of raw data

Grouping variable	d.f.	pseudo-F	P
Location (above/below)	1, 76	107·69	0·00001
Trophic group	1, 76	6·62	0·012
Food type	1, 76	3·70	0·058
Host range	1, 76	0·088	0·77
Taxon	1, 76	9·52	0·0029
Statistical cluster	1, 76	31·14	0·00002

generalised defences against enemies. We found positive covariation at the higher end of this spectrum within both the above- and belowground invertebrate community. However, taking into account the large amount of negative correlations between above- and belowground invertebrates lowers the average correlation to 0·31 and broadens the range to levels more comparable with those found by Leimu & Koricheva (2006). It therefore seems that responses to genetic variation in *A. arenaria* are strongly correlated within both the above- and belowground invertebrate fauna, while aboveground invertebrates respond to this variation oppositely to their belowground counterparts.

Leimu & Koricheva (2006) furthermore found no relationship between genetic correlations and the feeding guild of herbivores, but found an overall positive genetic correlation for pairs of specialists or generalists, while specialists were on average not correlated with generalists. This conclusion is nuanced by Johnson & Agrawal (2007) who conclude that in the case of arthropods, host specificity is likely to be a weak predictor of covariation across plant genotypes (Maddox & Root 1990; Roche & Fritz 1997). Indeed, also in our study, host range is not explicative of the observed covariation in invertebrate responses, whether or not the belowground fauna is included.

Implications

The observed large amount of significant correlations between invertebrate species across plant genotypes suggests that selection imposed by these species on *A. arenaria* is diffuse rather than pairwise. Moreover, the positive covariation within the above- and belowground component of the invertebrate fauna suggests selection for generalised resistance traits both at the root and shoot level. However, the observed trade-off between above- and belowground invertebrate occurrence can be an ecological cost constraining the directional evolution of either type of resistance. This can cause stabilising selection to preserve intermediate levels of resistance to both groups of invertebrates and explains why some studies fail to detect the costs associated with resistance (Strauss *et al.* 2002). The cost of increased aboveground

resistance might be an increased vulnerability to root herbivores, rather than some reduced allocation of resources to other plant functions or resistance to other aboveground enemies.

We did not experimentally exclude the different invertebrate species in a factorial design. Our measure of across-genotype correlation thus could be based on ecological interactions where one invertebrate species affects the occurrence of another. For example, herbivores might avoid plants already attacked by other herbivores (Leimu & Koricheva 2006). As the more mobile shoot herbivores arrived first on the plants, they might even have affected subsequent colonisation by root-feeders in this system (Vandegehuchte, de la Peña & Bonte 2010), causing the observed trade-off. Indeed, there is a large body of literature documenting on possible interactions between the above- and belowground fauna (van der Putten *et al.* 2001; Wardle *et al.* 2004; Vandegehuchte, de la Peña & Bonte 2010). We furthermore did not assess fitness effects of the different invertebrates. However, each of the observed nematode species has proven to be able to reduce plant biomass. The most common aboveground species, the aphid *S. rufula*, has proven detrimental for the plant in laboratory conditions (Vandegehuchte, de la Peña & Bonte 2010). The assumption that at least some of the investigated species have the potential to affect plant fitness therefore seems reasonable. Moreover, whether correlated responses of invertebrates are caused by truly genetic traits, such as linkage disequilibrium or pleiotropy, or by some invertebrate species selecting on certain genotypes and subsequently altering the effect on plant fitness by other species, both imply that potential coevolution will be diffuse (Iwao & Rausher 1997; Inouye & Stinchcombe 2001; Stinchcombe & Rausher 2001). If the aim is to gain understanding in the natural selection on variation in plants by invertebrates, associations with these invertebrates therefore have to be measured under natural circumstances, including the natural interactions between species (Roche & Fritz 1997). It furthermore seems practically unfeasible to selectively exclude each of 13 different above- and belowground species.

Our conclusions hold consequences for the study of coevolution in general. The issue of pairwise vs. diffuse coevolution has until now only been investigated aboveground. Our results, however, indicate that the selection imposed by invertebrates associated with plant roots could very well be operating in the opposite direction of selection by aboveground invertebrates. Research on other plant–invertebrate systems could elucidate the generality of our results and add to the understanding of the role of belowground invertebrates in the coevolution between plants and their invertebrate communities.

From a conservational point of view, our results stress the necessity to choose *A. arenaria* populations for sand fixation carefully (van der Putten 1990). As *A. arenaria* is a keystone species, changes in the genetic variation underlying its associated biotic interactions might have ecosystem consequences (Whitham *et al.* 2003). A more holistic approach of the study of ecological communities, including the belowground

component, could thus greatly enhance our understanding of the mechanisms and trade-offs in plant defence and the process of coevolution and provide more effective tools for species conservation.

Acknowledgements

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Supporting Information

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

Appendix S1. Plant characteristics.

Appendix S2. Nonmetric Multidimensional Scaling (NMS).

Fig. S1. Map of locations of plant populations.

Fig. S2. Number of tillers, number of leaves, leaf length.

Fig. S3. Root and shoot fresh weight, shoot relative water content.

Fig. S4. NMS joint plot.

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