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Genetic differentiation of *Agrostis capillaris* in a grassland system with stable heterogeneity due to terricolous ants

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Summary

1 We examined whether two different grassland microhabitats forming a stable mosaic (anthills and surrounding grassland) are colonized by different genotypes of a common grass, *Agrostis capillaris*, and thus maintain genetic variation in the species.
2 The potential of individual *Agrostis* genotypes, coming from different microhabitats, to withstand burial by ants was examined under experimental burial conditions. The same genotypes were then planted into these microhabitats in a reciprocal fashion to test whether their performance differs according to their source microhabitat. Isozymes were used to identify genet diversities and mean genet sizes at the microhabitats. They were also used to determine whether there is any indication that different genets occupy each of these microhabitats.
3 The burial experiment showed a high genetic variation between clones, both in overall performance (mean over both environments) and in their plastic response to burial. Variation in the way individual clones respond differently to changes in their environment was observed. In contrast to the response in the burial experiment, no clone–origin interaction was found in the transplant experiment. The performance of all plants on the anthills was significantly lower than on other habitats.
4 Isozyme analyses showed that the number of genets (per ramet sampled) on anthills is significantly lower than in the surrounding grassland. It also indicated a partial separation of genets into these microhabitats compared with homogeneous grassland conditions.
5 The harsh environment of anthills therefore acts as a filter for the total pool of *Agrostis* genets in the grassland, with only the fastest growing clones able to survive under these conditions. As *Agrostis* is capable of extensive clonal growth, it is able to cross microhabitats given sufficient time. Isozyme data showed that the contribution from generative reproduction is very low. Such a system indicates some 'genet sorting' through vegetative growth instead of genetic differentiation.

Key-words: clonal growth, functional differentiation, genetic variation, isozyme markers, *Lasius flavus*, microhabitats, reciprocal transplant

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Introduction

Habitat heterogeneity is a ubiquitous feature of ecological systems. It typically enables species of different habitat requirements to coexist and thus it is assumed to be one of the major factors accounting for species richness in ecological communities (Palmer 1994). In addition, it has dynamical consequences: depending on the grain size and the mobility rates, spatial heterogeneity may be perceived either as 'fine-grained' (i.e. the same individual can access different microhabitats) or 'coarse grained' by the plants. Owing to the migration of organisms in heterogeneous landscapes, non-trivial spatial structures may arise from habitat heterogeneity depending on its grain relative to the size and migration rate

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of the organism in question (Bell 1984; Muko & Iwasa 2003).

At the population level, habitat heterogeneity in space accounts for genetic differentiation of subpopulations (Heywood 1991). Plant population studies show that genetic differentiation may occur over short gradients if selective forces along such gradients are sufficiently strong and gene flow is low (Prati & Schmid 2000; Van Kleunen & Fischer 2001). However, in such studies, the geometric size of the environmental gradient is always larger than the size of individual plants, and therefore they have to rely on generative reproduction in order to respond to the gradient. An intriguing question is whether even smaller scale heterogeneity would still produce a similar response at the plant level.

An excellent example of strong, persistent and regular fine-scale heterogeneity is found in grassland systems with abundant anthills ('antscapes'), which occur in various types of temperate, extensively managed grazing systems (Elmes 1991; Dean *et al*. 1997; Blomqvist *et al*. 2000). In these grasslands, ants (typically the terricolous species *Lasius flavus*) act as ecosystem engineers and build regularly spaced mounds 50–100 cm in diameter and 10–50 cm in height. Thus, these systems possess considerable environmental heterogeneity, which is known to be stable over time, at least over several decades (King 1981; Woodell & King 1991; P. Kovář and M. Kovářová unpublished data). This heterogeneity causes floristic differentiation between mound vegetation and the rest of the grassland (King 1977a; Dean *et al*. 1997; Kovář *et al*. 2001). This is due to two reasons: (i) different soil environments in the mounds; and (ii) disturbances caused by the activity of the ants (King 1977b,c; Petal 1980; Elmes 1991). The different soil environments in the mounds are a consequence of the soils possessing more potassium and available phosphorus, and are particularly pronounced when the ant colony is active in the mound (Petal 1980; Dostál *et al*. 2005). Disturbances by soil transport are due to the ant's nest-building activities; they pile-up large amounts of material, open the soil surface and bury the plants that are growing on the top of the mounds. As a result, plants that are not able to cope with continuous burial (such as rosette plants without the capacity for clonal growth) are typically absent from the mounds (King 1977b; Kovář *et al.* 2001).

In contrast to specialist species restricted to only one microhabitat within these grasslands, other plant species (e.g. *Agrostis capillaris*, *Festuca rubra*, *Anthoxanthum odoratum*) are regularly found in both of these very different microhabitats. An analysis of *Agrostis capillaris* has shown that the architectural parameters of its rhizomes and roots in the anthills, are considerably different to those growing in the open grassland (Dostál *et al.* 2005). In particular, large rhizome structures are found in the anthills. Burial by the ants enforces a specific response in the plant, as is known from other systems with strong burial regimes (Brown 1997; Maun 1998). If there is any population-level variation in the ability to build such rhizome structures, then differences between microhabitats, both in the burial mounds and other parameters, may lead to a concentration of adapted genotypes in each of these microhabitats. *Agrostis capillaris* is known to quickly adapt to different environments by forming specific ecotypes, for example along gradients of heavy metal or salt concentrations (Kik 1989; Kik *et al*. 1990; Rapson & Wilson 1992). In a spatially heterogeneous system, this capacity could be augmented by the fact that the species is clonal and therefore may exploit its space, and spread in to the most favourable microsites (Oborny *et al*. 2000).

In this study, we examined whether individuals of *Agrostis capillaris* occurring at different microhabitats in ant-inhabited grasslands show any functional differentiation. We first examined the potential of individual *Agrostis* genotypes to withstand burial in a common garden experiment. We exposed clones sampled from three different environments (mounds, grassland between mounds, and mown grassland without mounds) to simulated burial conditions. We recorded both above-ground and below-ground growth, and compared their performance between individual clones and between the microhabitats they came from. Then we replanted the clones coming from different microhabitats back into the grassland in a reciprocal fashion and observed their survival under field conditions. Finally, we used molecular marker techniques (isozymes) to identify genet diversity and mean genet size, at the three different microhabitats (mound, grassland between the mounds, and mown grassland without mounds), and to determine whether there is any indication of different genets occupying different microhabitats. We also used the data to determine the frequency with which a single genet occurs in both microhabitats and to obtain estimates of genet recruitment rates.

Methods

STUDY SITE

The study was conducted in an area of grazed and mown grasslands in the Slovenské Rudohorie mountain region of Slovakia, where grasslands with anthills are abundant. It is located at an altitude of *c*. 950 m in the summit area of the Slovenské Rudohorie mountains, which is a part of the Western Carpathians range and lies *c*. 0.3 km SE of Obrubovanec (1020 m a.s.l.; 48°41′ N, 19°39′ E). The bedrock is formed by migmatites and amphibolites; the soils are therefore acidic and the grasslands consist of only a few species. The study site is under regular, low-intensity grazing by sheep and other livestock; some areas are also mown. The grasslands can be classified as the alliance Nardo-Agrostion tenuis Sillinger 1933; dominant grass species are *Festuca rubra*, *F. pratensis*, *Agrostis capillaris* and *Dactylis glomerata*. Further data on the locality are reported by Kovář & Kovářová (1998) and Kovář *et al*. (2001).

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Two habitat types were studied at this locality: grassland with mounds/anthills, and mown grassland with no visible mounds. The grasslands with mounds typically occur in extensively grazed areas. In the absence of regular mowing, ant colonies form pronounced mounds, which make a fine-scale mosaic within the surrounding grassland. The mounds are typically 20–40 cm tall, arranged fairly regularly, with a mean density of *c*. 12 mounds per 25 m^2 (J. Raabová, unpublished data). The grasslands with mounds are densely inhabited by colonies of several different ant species. The most common species is *Lasius flavus* (F), which forms dense and long-lasting (*c*. 20 years or more; M. Kovářová *et al*., unpublished data) mounds. Other species include *Tetramorium caespitum* (L.), *Formica fusca* L., *F. lemani* Bondroit, *F. pratensis* Retz., *F. rufa* (L.), *Lasius niger* (L.) and *Myrmica ruginodis* Nyl.

At these sites, we studied three separate types of habitats: two microhabitats in the grazed grassland, the mounds, and the grassland between mounds; and in addition, mown grassland without mounds for comparative purposes. These habitats are further called (i) anthills, (ii) grassland between anthills, and (iii) mown grassland.

CLONE SAMPLING FOR RECIPROCAL TRANSPLANT AND BURIAL EXPERIMENTS

We sampled 15 plants of *Agrostis capillaris* (further called *Agrostis*) using a stratified random design from two grasslands with mounds and two grasslands without mounds. Five clones were taken from anthills, five clones from grassland between anthills, and five from a mown meadow without anthills. The minimum distance between samples was 6 m and care was taken to sample a single genetic individual at each sampling point. The plants were vegetatively multiplied and maintained at the experimental garden of the Institute of Botany at Prùhonice for 1.5 years to remove any environmentally induced effects from their original habitats. These plants are further termed clones.

BURIAL EXPERIMENT

Individual shoots taken from source plants maintained in culture were planted into a soil layer 6 cm deep in 16 × 16 cm pots, in mid-May 2000. Ten plants of each clone were used. Half of the plants (i.e. untreated, control plants) were planted into shallower pots (7 cm high) in order that the rim of the pot did not shade the growing plants; plants to be treated were planted into 15 cm high pots (to hold the additional soil later used for burying) (Fig. 1). After rooting, plant height and the number of tillers were recorded for each plant. During the experiment, garden soil was added to the treated pots to simulate burial. Soil was added five times at approximately 10-day intervals. For the first two burial events, 2 cm of garden substratum was added to the treated pots, whilst in the later burial events, only

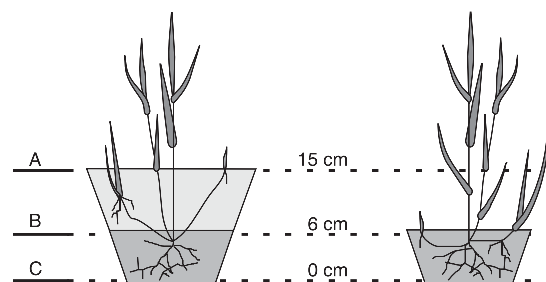

Fig. 1 Design of the burial experiment with *Agrostis capillaris* in the experimental garden. Left = treated plants; right = non-treated plants. A, B, C refer to the layers from which the biomass was collected.

1.5 cm substratum was added. This amount is in the same order of magnitude as the amount transported by the ants under field conditions (typically several centimetres per growing season; Petr Dostál and Jan Rothanzl, unpublished data). A layer of sand 0.5 cm thick was spread on to the surface of the treated pots before the first burial to indicate the depth of the original substratum. Standard garden substratum soil, sieved with a 5-mm sieve, was used in the experiment. The pots were kept in the experimental garden of the Institute of Botany at Prùhonice, and were watered twice a day.

Numbers of progeny tillers were recorded for all plants at both the soil surface level (which was lower for the non-treated plants), and for non-treated plants, at an identical height from the bottom of the pot (at the transition between layers A and B, see Fig. 1). At the end of the experiment, treated plants were clipped at the soil surface and the above-ground biomass of the non-treated plants separated into two samples, corresponding to the height of the soil surface of the treated plants (Fig. 1). In addition, the roots and rhizomes were removed from the pots, washed, sorted, dried and weighed. Rhizomes and roots of treated plants were separated into two samples, depending on their position relative to the original soil surface marked by the sand layer. This gave three layers of biomass: biomass above the soil level in the buried plants (above-ground in both treated and non-treated plants, denoted A); the biomass between the soil levels of buried and non-buried plants (below-ground for treated plants and above-ground for non-treated plants, B); and the biomass in the original soil (C). Rhizomes and buried stems (in the layer B) were sorted from the rest of the biomass and weighed separately.

RECIPROCAL TRANSPLANT EXPERIMENT

Two identical reciprocal experiments were set up in May 2001 and May 2002. First, well-developed single shoots were taken from each of the 15 clones used in the artificial burial experiment. These shoots were allowed to root in water and after a week were individually planted into filter paper tubes (6 cm × 1 cm) filled with garden soil. These were planted in two microhabitats,

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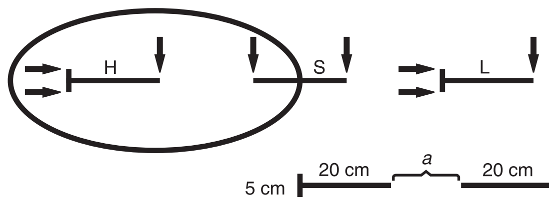

Fig. 2 (A) Sampling design for isozyme analysis. The ellipse represents an anthill. Arrows indicate sampling points. Distances between ramets indicated by thick lines were constant for all transects. (B) Distance in between the thick lines (*a*) was 10–30 cm and changed according to the size of anthill.

anthills and the grassland between anthills, in a block design. Twelve active mounds (i.e. having discernible colony mounding activity) were chosen at random. At each of these mounds, one plot was established on its summit (i.e. mound environment) and one plot in the surrounding grassland (*c.* 1 m away) (i.e. grassland environment). All 15 clones were planted (without replicates) into both plots with the aid of a steel corer of the same diameter as the paper tubes. The shoots were planted in a grid arrangement, with 6 cm between neighbouring shoots. The order of the clones in the grid within each plot was randomly shifted to avoid systematic positioning effects. All ramets were identified with coloured plastic rings and measured. Their performance (mean length of individual shoots, number of leaves, number of live shoots and number of flowering shoots) was recorded twice a year (early May and late September) over 1½ years following planting. All newly formed shoots were marked separately.

The first reciprocal experiment started in May 2001. Altogether 342 plants were planted, but only 127 ramets survived till the next recording date. Recording took place in September 2001, early May 2002 and late September 2002. The second experiment was set up in May 2002 at the same locality, using the same design and the same clones. Altogether 360 plants were planted, but on this occasion only 61 ramets survived till the next recording date. Plant performance was recorded four times (again in early May and late September) during 2002 and 2003.

CLONE SAMPLING FOR ISOZYME ANALYSES

Agrostis ramets for isozyme analyses were sampled at three plots (15 × 15 m) of grassland with mounds (referred to as ML1, ML2 and ML3) and three plots at the mown grassland (referred to as SL1, SL2 and SL3). At each plot, nine anthills that were taller than 20 cm were chosen by random coordinates. One transect was lain over the edge of each anthill in to the surrounding meadow. The length of each transect was 0.8–1.2 m; variable length was used to enable sampling at larger anthills. Eight sampling points were set up along each transect (Fig. 2) and the *Agrostis* ramet closest to the sampling point was taken. If no *Agrostis* ramet occurred within 2.5 cm of any sampling point, the whole transect was discarded and a new one chosen, again by random coordinates. The same procedure was also used to sample the mown meadow, where the starting points of the transects were established using random coordinates and the transects were always 1 m long. Altogether 368 ramets on 46 transects were sampled.

The ramets were sampled in May 2000 (plot ML1), May 2001 (plots ML2, ML3, SL1 and SL2) and May 2002 (plot SL3). All ramets were taken to the experimental garden of the Institute of Botany at Pruhonice and grown in pots containing sterilized soil. Here they were cultivated for a few weeks to acclimatize and grow new leaves. Transects where two or more ramets did not survive were excluded from the analysis. As a result, 299 ramets in 38 transects were analysed.

ISOZYME ANALYSES

In a pilot study, 11 enzyme systems were screened on 10–11 samples: AAT (EC 2.6.1.1), aspartateaminotransferase; ADH (EC 1.1.1.1), alcoholdehydrogenase; EST (EC 3.1.1.1), esterase; G6PDH (EC 1.1.1.49), glucose-6-phosphate dehydrogenase; GDH (EC 1.4.1.2), glutamatedehydrogenase; LAP (EC 3.4.11.1), leucinaminopeptidase; PGI (EC 5.3.1.9), phosphoglucoisomerase; PGM (EC 2.7.5.1), phosphoglucomutase; SHDH (EC 1.1.1.25), shikimatedehydrogenase; 6PGDH (EC 1.1.1.44), 6-phosphogluconatedehydrogenase; SOD (EC 1.15.1.1), superoxidedismutase. On the basis of the screening procedure, four enzyme systems (AAT, ADH, EST and LAP) having sufficient polymorphic banding patterns of significant resolution were selected for the final study.

Approximately 60–80 mg of tissue from several intact leaves from each cultivated ramet were sampled, and ground in a chilled mortar with 750 µL of extraction buffer ‘Luzula’ (Kaplan *et al.* 2002) and with *c.* 1/8 cm^3 Dowex.Cl (1-X8); when only a small amount of tissue was available then only 500 µL of extraction buffer was used. The extracts were centrifuged for 10 min at 15 000 ***g*** in a pre-chilled centrifuge. The supernatants were stored at −75 °C for subsequent electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was performed using a vertical HOEFER SE 600 (Amersham plc., Amersham, UK); 30–35 µL samples were analysed on polyacrylamide gels (4% stacking gel, 8% resolving gel, discontinuous tris-glycine buffer system, pH 8.3). Electrophoresis was carried out for 4 hours at 4 °C (20 mA per gel for the fist hour and then increased to 25 mA). Staining methods followed Peckert *et al.* (2005) for EST, LAP and AAT and Vallejos (1983) for ADH (with the following modification: 20 mL ethanol). If the whole enzyme system was missing from one extraction run, the process was repeated until enzyme activity was observed.

We used the band patterns of the four enzyme systems to identify individual ‘enzyme phenotypes’, which can be reasonably assumed to represent clones. Unfortunately, this could not be supported by the allelic interpretation of the bands as *Agrostis* is tetraploid and

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allelic interpretation of the enzymatic systems is virtually impossible. While this approach may combine two different genets into one enzyme phenotype, the likelihood was clearly very low as two different transects never shared similar phenotypes (i.e. over a distance of 120 cm, while many identical phenotypes were found over shorter distances).

AGROSTIS RAMET DENSITY

The data for *Agrostis* ramet density was collected in May 2002. All ramets were counted in 13.5 cm diameter circular plots, i.e. 143.1 cm^2, located in grassland with anthills (three plots with 30 data points each, half of the samples taken from anthills and half from the grassland in between the anthills) or in mown grassland (three plots of 15, 16 and 9 data points). The collecting sites corresponded with the plots used for genet sampling.

DATA ANALYSIS

Data (biomasses in the three layers, and number of shoots at the end of the experiment) from the garden experiment were analysed using a mixed-model of analysis of variance with burial and clone origin as fixed factors and clone as random factor using procedures **`aov`** and **`lme`** in Splus (MathSoft 2000). The effect of clone origin and burial were tested using the error sum of squares of clone × treatment, and effects of treatment (burial), clones and clone × treatments were tested using the error sum of squares of pots. Data were not transformed as they did not deviate strongly from a Guassian distribution. Two types of analyses were run: (i) without covariates; and (ii) with total biomass as covariate to express differences in proportional allocation into individual layers.

Data from both reciprocal transplant experiments were analysed together at the level of individual plants. Not enough replicates were available to perform the analysis at the clone level. Data on shoot size during the experiment were analysed using analysis of variance with clone origin, treatment and origin × treatment as tested independent factors, and initial shoot length, experiment and block (nested in experiment) as block ('nuisance') independent factors. Data were not transformed. Number of shoots per transplant was tested with analysis of deviance with Poisson distribution and log link using the same model as in the analysis of variance. A same analysis with binomial distribution and logit link was used for the number of flowering shoots per transplant and survival (number of surviving transplants per plot). Splus (MathSoft 2000) was used to perform all statistical tests. Survival prior to the first recording was assumed to be due to 'transplanting shock' and only plants that survived till the first recording were included into the analysis.

The simultaneous use of the clones for both experiments made it possible to link the behaviour of individual clones in culture with their growth in the field. However, because of the high mortality in the field, only a subset of clones could be analysed (nine in the mounds and 14 in the surrounding grassland). Therefore no formal analysis of the results was undertaken.

Number of genets found by isozyme analyses was compared between the habitats using analysis of deviance with binomial distribution and logit link. In order to satisfy the assumptions of binomial distribution, number of genets – 1 was taken as dependent variable (number of positive responses) and number of ramets – 1 as the weight variable (total number of responses). Numbers of genets found in the 'mound' and 'grassland' sections of the transects were analysed separately.

To calculate the probability of seedling establishment, we used the approach of Harada *et al*. (1997), where the probability that two ramets belong to the same genet over a distance r is $ke^{cr}\sqrt{r}$, k is a positive constant (corresponding to ramet size), and $c = \sqrt{2u/v\sigma^2}$, where σ^2 is the variance of distance between mother and daughter ramets and u and v are the respective probabilities that a newly formed ramet is of seedling and vegetative origin. First, distances between all ramet pairs were calculated and the genetic identity of these ramets was determined within each transect. The data were binned into intervals 10 cm wide (5–15 cm, 15–25 cm, etc.), with the exception of distance 0–5 cm, which was kept as a separate category. Then the formula above was fitted by means of non-linear regression (Marquardt algorithm, SPSS 2000); a weighted form of the regression was used, where each point was assigned a weight proportional to the number of observations on which it was based. The regression coefficient c was then used to estimate the ratio of vegetatively and sexually formed ramets, which were used to estimate the number of recruiting seedlings per area. To obtain absolute numbers, we estimated the number of vegetatively formed ramets per area using the mean number of ramets per unit area and their turnover, known to be approximately 50% annually (R. Wildová, unpublished data). The mean distance between a vegetatively formed ramet and its parent was assumed to be 3 cm. Finally, the mean genet size was estimated as $k(\pi/c)^{1.5}$ (Schläpfer & Fischer 1998).

Results

EXPERIMENTS

The garden burial experiment showed a strong effect of burial on all plant parameters. The buried plants produced more shoots and roots and more total biomass than the plants that were not buried (Fig. 3). As expected, buried plants produced more below-ground structures (both roots and rhizomes) than the plants that were not buried. There were strong differences between the clones used in the experiment, both in the mean of their responses and in the interaction with the burial treatment (Table 1, Fig. 4). The differences between clones were significant for almost all parameters.

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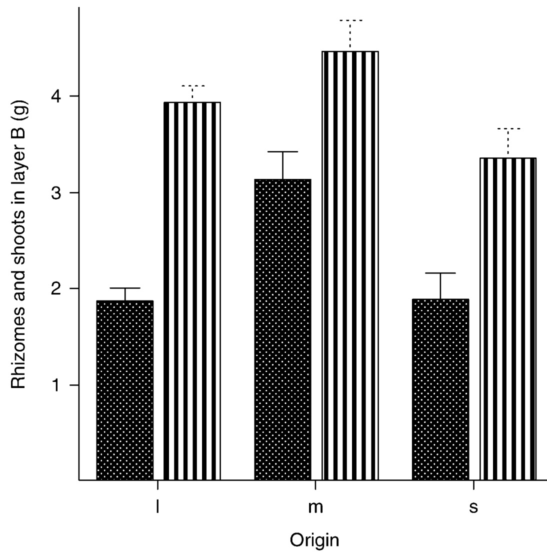

Fig. 3 Biomass of rhizomes and shoots in the layer B in the garden burial experiment. Fifteen clones of *Agrostis capillaris* differing in their origin were tested (10 plants of each clone). G = grassland between anthills; A = anthill; MG = mown grassland without anthills. Dotted bars = not buried; hatched bars = buried. Bars indicate standard errors.

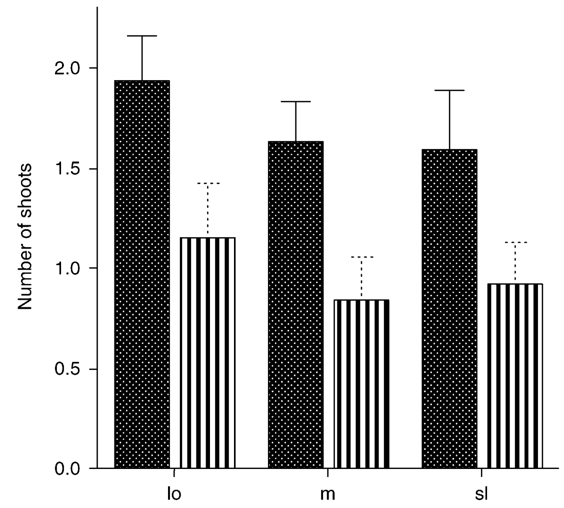

Fig. 5 Number of daughter shoots of *Agrostis capillaris* formed in the reciprocal transplant experiment with clones differing in their origin. All clones were planted into mounds (hatched bars) and grassland off the mounds (dotted bars). Habitat = microhabitat into which the clone was planted; origin = microhabitat where the clone was sampled. G = grassland between anthills; A = anthill; MG = mown grassland without anthills. Bars indicate standard errors.

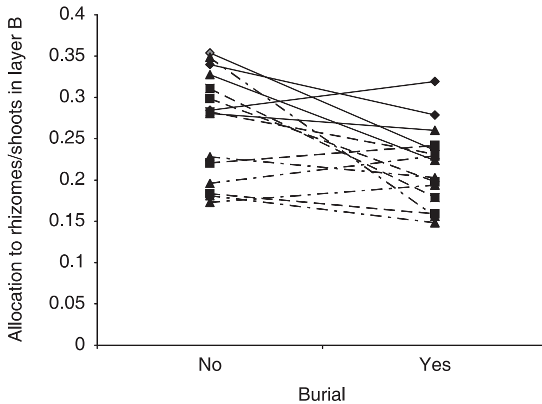

Fig. 4 Allocation to rhizomes and shoots in layer B of clones of different origin in response to the burial treatment. Full lines = clones from anthills; broken lines = clones from grassland between anthills; thick broken lines = clones from mown grassland without anthills. Diamonds = clones from mounds; squares = clones from grassland between mounds; triangles = clones from mown grassland.

Relative effects (i.e. allocation) were also strong, both between clones and in their response to burial (interaction).

Clones from different habitats (anthill, grassland and mown grassland) differed significantly in several growth parameters (namely allocation to rhizomes in layers B and C; see Table 1). Clones originating from anthills produced on average the largest plants with most rhizomes. However, their relative response to burial was weaker than the response of the clones from grassland, grassland between anthills and mown grassland. Response to burial (i.e. interaction between burial and clone origin) was marginally significant for the number of shoots, and allocation to layer C rhizomes (Table 1).

In the reciprocal transplant experiments, the major factor affecting performance of the transplants was microhabitat (anthill vs. grassland between anthills); plants planted at anthills showed reduced growth and lower survival than plants planted into the grassland between anthills (Tables 2 and 3, Fig. 5). In contrast, there were no significant effects of clone or clone origin on plant survival, size parameters or flowering (Tables 2 and 3); the only weak effect was on mean height in the first autumn of the experiment. Interaction with clone origin was never significant. In addition, flowering did not differ between levels of any of these factors.

Comparisons between clone performance in the garden experiment and the reciprocal transplant experiment showed that the only consistent relationship was between performance in non-buried treatments and shoot height in the field (no formal analysis done because there were only nine clones available with full data from the field).

ISOZYME ANALYSES

Isozyme analysis identified 217 genets in 299 ramets. Most genets were found as a single ramet (148, i.e. 49.5%); the highest number of ramets in a single genet was seven. Ramets identified to belong to the same genet were always found in one transect; no genet was found in two transects. The probability that two ramets sampled along a single transect belonged to the same genet predictably decreased with distance (Fig. 6). The greatest distance between two ramets belonging to one genet was 100 cm in the mown grassland and 83 cm in the grassland with anthills.

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Table 1 The garden burial experiment with 15 clones of *Agrostis capillaris* (differing in their origin). Total biomass was used as a covariate for all biomass variables. The number of shoots at the start of the experiment was used as a covariate for the number of shoots. Effects significant at α = 0.05 (except for the effect of burial) are shown in bold, whilst those significant at α = 0.10 are in italics

		No covariate		Covariate	
Source of variation	d.f.	*F*-statistic	*P*	*F*-statistic	*P*
Number of shoots at the soil surface					
Burial	1, 110			33.94	< 0.001
Origin	2, 12			2.56	0.119
Burial × origin	2, 12			2.52	0.122
Clone	12, 110			**2.90**	**0.001**
Burial × clone	12, 110			1.96	0.183
Number of shoots in the layer A					
Burial	1, 110			302.20	< 0.001
Origin	2, 12			3.26	0.092
Burial × origin	2, 12			*3.40*	*0.067*
Clone	12, 110			**3.10**	**< 0.001**
Burial × clone	12, 110			**3.19**	**< 0.001**
Total biomass					
Burial	1, 110	134.61	< 0.001	NA	
Origin	2, 12	0.05	0.948	NA	
Burial × origin	2, 12	1.23	0.326	NA	
Clone	12, 110	**3.77**	**< 0.001**	NA	
Burial × clone	12, 110	**3.62**	**< 0.001**	NA	
Above-ground biomass (layer A)					
Origin	2, 12	0.45	0.647	1.62	0.237
Burial × origin	2, 12	1.86	0.198	2.18	0.116
Clone	12, 110	**6.16**	**< 0.001**	**8.75**	**< 0.001**
Burial × clone	12, 110	**3.07**	**< 0.001**	**3.12**	**< 0.001**
Layer B total biomass					
Burial	1, 110	154.30	< 0.001	5.74	0.018
Origin	2, 12	**4.19**	**0.042**	**5.30**	**0.022**
Burial × origin	2, 12	0.42	0.660	1.97	0.182
Clone	12, 110	**3.75**	**< 0.001**	**5.12**	**< 0.001**
Burial	1, 110	327.42	< 0.001	92.83	< 0.001
Burial × clone	12, 110	**2.84**	**0.0015**	**2.79**	**0.002**
Stems and rhizomes in layer B					
Burial	1, 110	66.17	< 0.001	**5.33**	**0.022**
Origin	2, 12	**5.46**	**0.021**	**6.67**	**0.011**
Burial × origin	2, 12	0.68	0.525	0.40	0.681
Clone	12, 110	**2.56**	**0.005**	**3.25**	**< 0.001**
Burial × clone	12, 110	**2.07**	**0.024**	**2.55**	**0.005**
Total biomass in layer C					
Burial	1, 110	10.91	0.001	241.48	< 0.001
Origin	2, 12	0.42	0.666	*2.98*	*0.089*
Burial × origin	2, 12	0.75	0.490	3.06	*0.085*
Clone	12, 110	**5.28**	**< 0.001**	**5.52**	**< 0.001**
Burial × clone	12, 110	**4.43**	**< 0.001**	**1.97**	**0.033**
Roots in layer C					
Burial	1, 110	2.01	0.159	146.76	< 0.001
Origin	2, 12	0.16	0.857	0.09	0.916
Burial × origin	2, 12	0.33	0.726	*3.00*	*0.088*
Clone	12, 110	**8.04**	**< 0.001**	**8.76**	**< 0.001**
Burial × clone	12, 110	**5.28**	**< 0.001**	**3.12**	**< 0.001**
Rhizomes in layer C					
Burial	1, 110	47.3	< 0.001	209.25	< 0.001
Origin	2, 12	2.24	0.149	**4.54**	**0.034**
Burial × origin	2, 12	*2.80*	*0.100*	0.65	0.542
Clone	12, 110	**2.41**	**0.007**	**4.10**	**< 0.001**
Burial × clone	12, 110	**2.56**	**0.005**	1.35	0.202

The number of ramets per genet decreased in the order: anthill, mown grassland and grassland between anthills (Table 4). The difference between the three habitats together was not significant using analysis of deviance (deviance = 4.93, d.f. = 2, residual deviance = 112.73, d.f. = 73, $F = 2.12$, $P = 0.128$). Differences between the two microhabitats within the grassland with anthills (i.e. anthills proper and grassland between anthills)

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Table 2 Number of daughter shoots and their height in the reciprocal transplant experiments with 15 *Agrostis capillaris* clones differing in their origin. The clones were planted into the mounds and surrounding grassland. Block (categorical, d.f. = 22, also includes experiment) and initial size (quantitative, d.f. = 1) are used as independent factors. Effects significant at α = 0.05 are shown in bold

Source	d.f.	Mean height autumn *F*	Mean height autumn *P*	Mean height next spring *F*	Mean height next spring *P*	Mean height next autumn *F*	Mean height next autumn *P*
Habitat	1	1.04	0.309	**9.24**	**0.003**	0.297	0.588
Origin	2	2.58	0.078	0.424	0.655	0.01	0.991
Habitat × origin	2	0.108	0.897	0.917	0.406	0.11	0.89
Residual							
Residual d.f.		153		117		54	

Table 3 Survival and flowering in the reciprocal transplant experiments with 15 *Agrostis capillaris* clones differing in their origin. All clones were planted into mounds and surrounding grassland. Block (categorical, d.f. = 22) and initial size (quantitative, d.f. = 22) are used as independent factors. Effects significant at α = 0.05 are shown in bold

		Number of shoots autumn		Number of shoots next spring		Flowering		Survival next spring		Survival next autumn	
		Deviance	*P*	Deviance	*P*	Deviance	*P*	Deviance	*P*	Deviance	*P*
Habitat	1	**12.2**	**< 0.001**	**13.38**	**< 0.001**	2.07	0.151	1.58	0.208	**13.57**	**< 0.001**
Origin	2	3.05	0.218	0.48	0.785	4.17	0.124	2.32	0.312	0.34	0.844
Habitat × origin	2	0.81	0.666	4.58	0.101	4	0.135	0.3	0.861	0.29	0.862
Residual		236.49		292.03		52.31		95.33		112.28	
Residual d.f.		158		159			60	82		82	

were marginally significant (deviance = 4.39, d.f. = 1, residual deviance = 57.21, d.f. = 36, $F = 3.94$, $P = 0.054$). Consequently, the relationship of the probability of genet identity with distance was different across the microhabitats (Fig. 6). Probability of genet identity over a given distance was always greater in the (homogeneous) mown grassland without anthills than in the grassland with anthills (Fig. 6). Further, probability of genet identity over short distances was much higher on anthills than in grassland surrounding anthills.

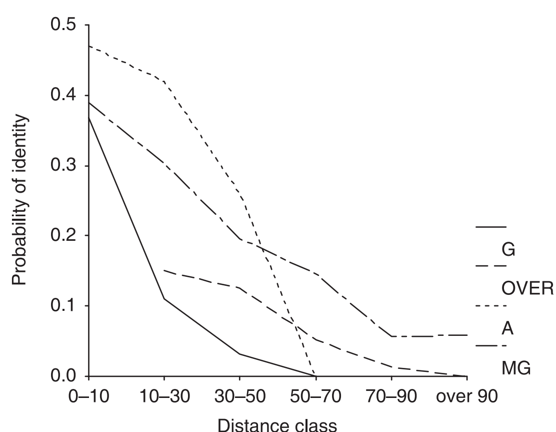

Fig. 6 Probability that two *Agrostis capillaris* ramets belong to the same genet as a function of their distance in different microhabitats. Genetic identity was estimated by isozyme markers. MG = mown grassland without anthills; A = anthill; G = grassland surrounding anthills; OVER = one ramet on an anthill, the other in the surrounding grassland. Distances in centimetres. Due to the sampling design used, OVER is not available for distances shorter than 20 cm; within-microhabitats comparisons (A and G) are not available for distances greater than 50 cm.

The density of ramets in the field was high in all three microhabitats. Mean density decreased in the order: anthill, grassland between anthills and mown grassland (Table 4). The difference is highly significant using ANOVA ($F = 8.19$, d.f. = 2, 127, $P < 0.001$).

The ratio between generative and vegetative reproduction was estimated by the non-linear regression of the genet identity-distance data and found to be 7.32×10^{-6} for the mown grassland and 0.0074 for the grassland between anthills with R^2 values of 0.76 and 0.98, respectively. No estimate could be made for the anthill itself because of the low number of genets and the decrease of probability of genet identity with distance. Generative reproduction thus occurs in the order of one genet per several square meters per year in the mown grassland, and several genets per square meter per year in the grassland surrounding anthills (Table 4). Corresponding genet sizes are in the order of several decimetres (in the grassland between anthills) and several metres (in the mown grassland; Table 4).

Discussion

RESPONSE TO BURIAL AND GXE INTERACTIONS

Clearly, burial had a strong effect on the performance of *Agrostis capillaris* plants; buried plants grew significantly better, most likely due to both stimulation of clonal

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Table 4 Number of genets determined by isozyme analyses of *Agrostis capillaris* and population-level genet parameters at the three microhabitats. Estimated values of frequency of generative recruitment and mean genet size using the fomulas of Harada *et al.* (1997) and Schläpfer & Fischer (1998). NA = data not available

Microhabitat	Anthill	Grassland between anthills	Mown grassland
Total number of ramets examined	77	79	143
Number of genets found by isozyme genotyping	52	64	101
Ramets per genet	1.48	1.23	1.41
Mean ramet density m^{-2} (mean ± SE)	2215 ± 286	1397 ± 154	1055 ± 133
Ratio between vegetative and generative ramet natality (v/u)	NA	135	136000
Number of seedlings m^{-2} $year^{-1}$	NA	5.17	0.0038
Genet diameter in cm^2	NA	20.8	295

growth by burial (Brown 1997; Yu *et al.* 2004) and to the addition of extra nutrients in the soil used for burial.

The burial experiment showed a rather high genetic variation between clones, both in their overall performance (mean over both environments) and in their plastic response to burial. This corresponds well to the existing information on genetic variation in clonal plants (Prati & Schmid 2000). As far as we are aware, no evidence of genetic differentiation in response to burial has been shown, although burial is a common stress factor in many ecosystems such as sand dunes or riverbanks (Maun 1998). As genetic variation has been shown in respect of many factors, it would be surprising if burial were an exception, in particular given the ability of *Agrostis* to adapt to different environments by forming genetically distinct lines (Kik 1989; Rapson & Wilson 1993). In particular, clones from anthills grew fastest in the burial experiment and showed the highest allocation to rhizomes. Interactions between burial and clone origin in the burial experiment are weak and only marginally significant, but not completely absent. This may indicate that the fastest growing clones are able to survive and eventually become dominant in the anthill environment. In spite of the rather low number of clones, the experiment indicates variation in the capacity of individual clones to respond differently to changes in their environments.

With only one exception, no significant clone–origin interaction was found in the transplant experiment. Although the plants initially suffered high mortality, the number of surviving plants was still relatively high and the power of the test was therefore acceptable. Therefore we lack a direct test of the relevance of the traits affecting plant performance in the field. However, a comparison at the clone level shows that mean values of performance parameters in culture are correlated with performance of individual clones in the field (data not shown). This indicates that the traits determined in the garden experiment may have relevance for behaviour in the field.

ANTHILLS AS FILTER FOR *AGROSTIS* GENOTYPES

Importantly, reciprocal transplants also showed that plants planted into the anthills grew less well than the plants in the surrounding grassland. This cannot be due to differences in soil quality alone, as the anthill soil has been shown to increase *Agrostis* growth fourfold in culture (Dostál *et al.* 2005; V. Kozlíčková unpublished data). The most likely reasons are: lower soil humidity in the anthills (which might also account for the higher initial mortality in this location); direct damage by the ants; and burial by mounding (Blomqvist *et al.* 2000). The density of *Agrostis* is greater on the mounds (by a factor of approximately 1.5), probably due to lower competition, as many of its potential competitors are unable to survive in this location and thus, the overall plant cover is much lower (Kovář *et al.* 2001).

This harsh environment of anthills is thus likely to filter the total pool of *Agrostis* genets in the grassland to some extent. This is supported by the results of isozyme analyses, which showed that the number of genets (per ramet sampled) on anthills is significantly lower than in the surrounding grassland. In addition, the identity-distance relationship as detected by isozymes further confirms fine-scale differentiation between microhabitats. It shows that the probability of two ramets being identical is higher when ramets from the homogeneous (without anthills, i.e. mown) grassland are compared. In contrast, the probability for the same distance class is much lower when a ramet from an anthill and a ramet from the surrounding grassland are compared (Fig. 6). This indicates a partial separation of genets between anthills and the surrounding grassland. Thus, *Agrostis* clones show a similar differentiation in response to environmental heterogeneity by ants as found at the interspecific level, where some species are restricted to anthills, and other species restricted to the surrounding grassland (King 1977a; Dean *et al.* 1997; Kovář *et al.* 2001). The former species are often those capable of faster (clonal) growth and form abundant rhizomes, as found in the *Agrostis* clones. This separation is most likely due to the direct response of the plants to the heterogeneous environment. Direct dispersal by the ants can be ruled out as *Lasius flavus* do not carry seeds (Pontin 1978; Kovář *et al.* 2001). In this respect the differentiation found is very different from genetic differentiation of plants dispersed by ants, such as *Trillium grandiflorum* (Kalisz *et al.* 1999).

Agrostis can disperse between the microhabitats by both clonal growth and by seed reproduction, but vegetative growth largely prevails. Isozyme data show that

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mean genet size is several metres in diameter. This is due to the extensive rhizome system that the species is known to have (Dostál *et al.* 2005; R. Wildová, unpublished data; J. Rothanzl, unpublished data). Most new ramets are thus formed by vegetative processes and the genet sizes are greater than the size of individual anthills (Table 4). The mean distance that a plant can cover in 1 year by clonal growth is up to 2 decimetres (R. Wildová, unpublished data). Over longer periods of several years, individual genets may reach both habitat types by clonal growth (several cases were detected where a single clone spread from an anthill to the surrounding grassland).

In contrast to clonal growth, generative reproduction generates new genets with (at least, potentially) different growth parameters. However, seed dispersal is also likely to spread the plants over a wide distance (Tackenberg 2003). Thus, it may also contribute to genet separation. However, seedling recruitment of *Agrostis* in grasslands is quite rare: isozyme data on number of clones and their sizes indicate that frequency of seedling recruitment is of several orders of magnitude lower than ramet formation by clonal growth. Over several decades (the duration of an anthill), there may perhaps be one successful establishment event, while the total number of genets on an anthill is much higher. These genets could have established in a grassland between mounds and reached the mound by clonal growth.

It is therefore likely that the major process accounting for the genet separation takes place at the level of vegetative plants. Such a system undergoes partial 'genet sorting', with a vegetative process of spatial spreading instead of genetic differentiation. In such a process, there is a low turnover of existing genets and few recombination events take place. As pollination distances in grasses are typically much greater than the distance between anthills (Wang *et al*. 2004; Watrud *et al*. 2004), both generative reproduction and seed dispersal will act against differentiation between microhabitats. In spite of their effects, the existing fine-scale heterogeneity is sufficient to maintain non-random genetic structure between microhabitats through genet sorting according to their capacity to survive at the mounds. While grasslands containing anthills are an extreme case of environment heterogeneity, plants possessing sufficient genetic variation may show similar levels of separation at small scales, at different habitats as well (Huber *et al*. 2004).

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