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Selection pressures have caused genome-wide population differentiation of *Anthoxanthum odoratum* despite the potential for high gene flow

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Abstract

The extent to which divergent selection can drive genome-wide population differentiation remains unclear. Theory predicts that in the face of ongoing gene flow, population differentiation should be apparent only at those markers that are directly or indirectly (i.e. through linkage) under selection. However, if reproductive barriers limit gene flow, genome-wide population differentiation may occur even in geographically proximate populations. Some insight into the link between selection and genetic differentiation in the presence of ongoing gene flow can come from long-term experiments such as The Park Grass Experiment, which has been running for over 150 years, and provides a unique example of a heterogeneous environment with a long and detailed history. Fertilizer treatments applied in the Park Grass Experiment have led to rapid evolutionary change in sweet vernal grass *Anthoxanthum odoratum*, but until now, nothing was known of how these changes would be reflected in neutral molecular markers. We have genotyped ten *A. odoratum* populations from the Park Grass Experiment using Amplified Fragment Length Polymorphisms (AFLPs). Our data show that nutrient additions have resulted in genome-wide divergence among plots despite the high potential for ongoing gene flow. This provides a well-documented example of concordance between genomes and environmental conditions that has arisen in continuous populations across a time span of fewer than 75 generations.

Introduction

The genetic differentiation of populations is influenced by a combination of gene flow, natural selection and genetic drift. In general, population differentiation is inversely correlated with gene flow and directly correlated with genetic drift (Frankham *et al.*, 2002; Freeland, 2005). The effects of natural selection on population differentiation are more complicated. Divergent selection may promote differentiation by altering the frequencies of alleles that are under selection, plus alleles at linked loci (Barton, 2000; Endler, 1973); at the same time, neutral alleles that are unlinked to selected loci may experience no constraints in their flow between populations that inhabit contrasting environments (Emelianov *et al.*, 2004; Gavrilets & Vose, 2005). Alternatively, divergent selection may act indirectly on genome-wide allele frequencies by promoting reproductive isolation; this creates a barrier to gene flow that results in the genome-wide differentiation of populations following genetic drift (Grahame *et al.*, 2006; Nosil *et al.*, 2009; Rundle & Nosil, 2005; Schluter, 2000; Thibert-Plante & Hendry, 2009). Under this model, gene flow will be inversely correlated with the adaptive divergence of populations; the result of this is a positive association between the phenotypic divergence and molecular genetic differentiation of populations, a pattern that is known as isolation by adaptation (IBA) (Nosil *et al.*, 2009; Thibert-Plante & Hendry, 2009).

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The Park Grass Experiment (PGE) at Rothamsted Research in Harpenden, United Kingdom, provides a unique forum to investigate the potential effects of divergent selection on the differentiation of proximate populations growing in environmentally heterogeneous conditions. The experiment, which began in 1856, is the oldest ecological experiment in existence (Tilman *et al.*, 1994). It began with approximately 2.8 ha of nearly level land that had been grassland for more than a century prior to the start of the experiment. At that time, the floral species composition appeared to be consistent across the site, which was characterized by slightly acidic (pH 5.4–5.6), nutrient-poor soil (Silvertown *et al.*, 2006). Beginning in 1856, with a staggered introduction over a period of approximately 15 years, the experimental site was divided into 20 plots which are identified by number (Fig. 1). Varying levels and types of nutrients (most notably N, P and K) were added to these plots each year with the exception of three control plots, to which no nutrients were added. In 1903, each plot was further subdivided into two plots, one of which received regular applications of lime, to test the effects that changes in soil pH would have on hay yield. In 1965, most plots were further divided into four plots, three of which are limed to maintain pH values of approximately 7, 6 and 5 ('a'–'c'; Fig. 1), with the fourth plot ('d') receiving no lime and hence maintaining a pH of between 3.5 and 5.7 depending on the fertilizer treatment (Rothamsted Research, 2006).

Microevolutionary changes that have resulted from different nutrient regimes have been identified in sweet vernal grass, *Anthoxanthum odoratum*, a plant that is outcrossing (self-incompatible) and wind-pollinated, and which should therefore experience gene flow across plot boundaries (Silvertown *et al.*, 2006). Despite the potential for considerable gene flow, strong selection pressures have resulted in heritable differences in many traits such as disease resistance and plant height between *A. odoratum* plants in adjacent plots that received different nutrient treatments (Davies & Snaydon, 1973a,b, 1974, 1976; Snaydon, 1970; Snaydon & Davies, 1972, 1976). Reciprocal transplants between plots showed that selection against transplanted genotypes originating from a plot with a different treatment could be as high as 70% in a single season (Davies & Snaydon, 1976). In a later study, Silvertown *et al.* (2005) inferred from a combination of phenotypic and genotypic data that reproductive isolation across plot boundaries had been reinforced by natural selection. These studies collectively demonstrate that local adaptation of *A. odoratum* in the PGE has occurred despite ongoing gene flow between plots.

Evidence for rapid adaptive change of *A. odoratum* on the PGE has so far been based primarily on quantitative genetics, and there is little understanding of whether these microevolutionary changes are reflected in patterns of population differentiation at neutral loci. We have therefore investigated the extent to which divergence of

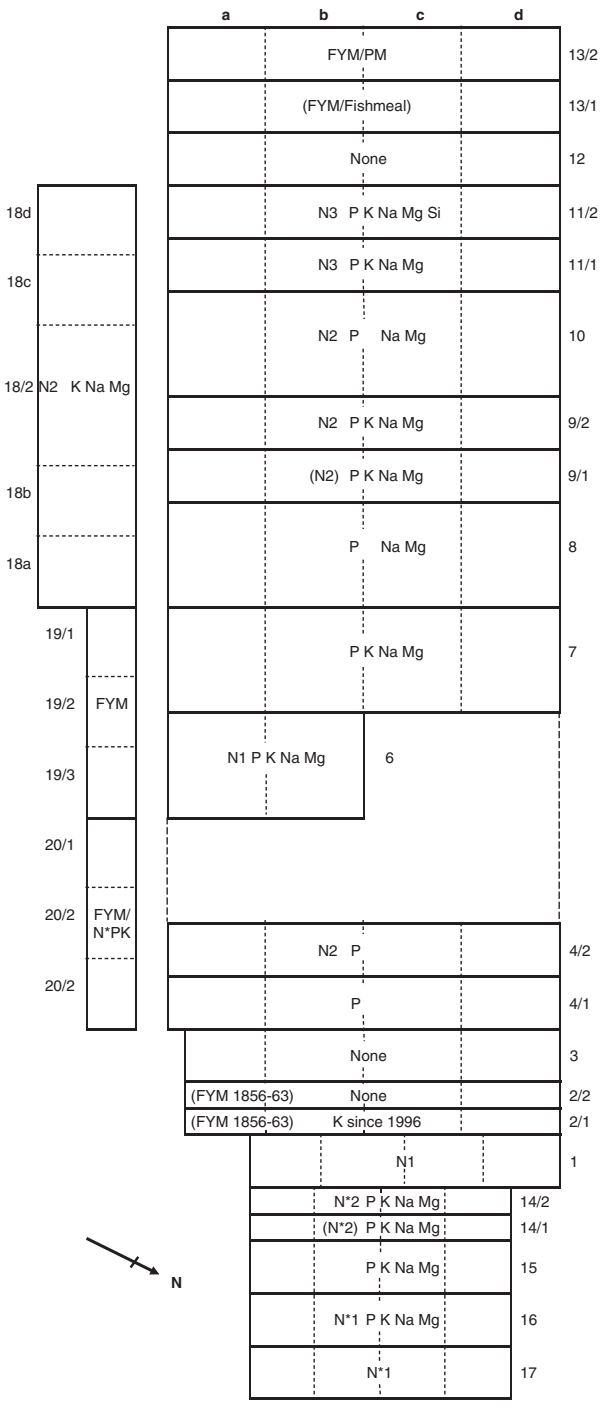

Fig. 1 Plan of the Park Grass Experiment at Rothamsted showing the inputs to the plots. 'a' plots are limed to pH 7, 'b' to pH 6, 'c' to pH 5 and 'd' are unlimed. FYM = Farmyard manure; N1–N3 = lowest to highest levels of nitrogen addition, other symbols indicate which element is being added but not in which form; N* = nitrogen added as nitrate rather than ammonium salt: (N) = nitrogen addition that was withdrawn in 1990. Plots 1–13 were started in 1856, 14–17 added in 1858, and 18–20 in 1905. Figure reproduced with permission of Rothamsted Research.

populations based on quantitative traits is reflected in the differentiation of putatively neutral markers. We did this by genotyping populations of *A. odoratum*, using Amplified Fragment Length Polymorphisms (AFLPs) markers, from a subset of PGE plots. The unique facility of the PGE, in which both temporal and spatial scales of edaphic change have been well-documented for 150 years, has provided us with a valuable model for investigating whether or not rapid microevolutionary change, in response to a heterogeneous environment, can be strong enough to leave population-level signatures despite the opposing forces of gene flow and genetic drift.

Materials and methods

Sampling

Sampled plots included control plots 3b and 3d, to which no nutrients have been added, plus four other pairs of plots that collectively provide a range of pH values, and of nutrient additions (Fig. 1, Table 1). Samples of leaf tissue from *A. odoratum* were taken along four transects placed the length of each sampled plot, evenly spaced across the plot and at least 1 m from the plot boundaries. Individually sampled plants were at least 0.5 m apart from one another. Two leaf samples approximately 1.5 cm long were taken from each plant, placed onto ice until the transect was complete, and then snap-frozen into liquid nitrogen in the field. These were then stored at −80 °C in the lab until DNA was extracted.

DNA extraction and genotyping

The Qiagen™ Tissue Lyser with 2-mm-diameter tungsten beads was used at 25 Hz for 2 × 1 min to disrupt the leaf samples which were then extracted with the Qiagen™ DNeasy96 plant kit (Qiagen Ltd, Crawley, UK), according to the manufacturer's instructions, and eluted with 100 + 50 uL of elution buffer.

DNA extractions were digested and ligated according to the method of Vos *et al.* (1995) except that *Hind*III was used as the rare-cutter enzyme in place of *Eco*R1, and digestions were carried out in 96-well plates as 20 μL reactions containing 250 ng DNA, 2.5 U of each enzyme and correspondingly reduced amounts of other reagents. The pre-amplification protocol followed Vos *et al.* (1995), except that reactions were carried out in 96-well plates and scaled down to a total volume of 25 μL, and Hi-Di formamide (Applied Biosystems) was added to the PCR mix to a final concentration of 2%. The amplified products were diluted with ddH_2O to 200 μL, and 5 μL of this was used for the selective amplifications. These were carried out with three +3 primer combinations (+AAC/+CAC; +AAC/+CAG; +AGT/+CAC), again according to the method of Vos *et al.* (1995) except scaled down to 25 μL reactions in 96-well plates, and with the addition of formamide as earlier. Forward primers were labelled with 6-FAM, NED or PET (Applied Biosystems) and PCR products visualized on an Applied Biosystems 3730 DNA Analyser. Repeatability was checked using two replicates for each primer combination for each of 11 samples taken from three different plots. PCR products were sized using GeneMarker v. 1.6 (SoftGenetics, State College, PA, USA).

Table 1 Plot treatments and sample sizes of *A. odoratum* included in this study. N1 = low level of nitrogen added, N2 higher level of nitrogen added. Each pair of 'b' and 'd' plots is considered, for the purposes of this study, to be a 'group', based on the fact that they have received the same nutrient inputs.

Group	Plot	pH	Fertilizer additions	No. of AFLP genotypes	Nei's genetic diversity
1	1b	6.2	N1 + lime	40	0.1166
	1d	4.1	N1	45	0.1659
2	3b	6.3	None + lime	42	0.1322
	3d	5.3	None	39	0.1396
3	4/2b	6.3	N2, P + lime	40	0.1706
	4/2d	3.7	N2, P	39	0.1658
4	8b	6.1	P, Na, Mg + lime	42	0.1785
	8d	5.1	P, Na, Mg	37	0.1685
5	9/1b	6.4	(N2)*, P, K, Na, Mg + lime	44	0.2057
	9/2d	3.6	N2, P, K, Na, Mg	45	0.1952

*(N2) = nitrogen treatment was discontinued in 1990.

AFLP analysis

We used Popgene (Yeh *et al.*, 1997) to calculate Nei's genetic diversity, allele frequencies, and Nei's unbiased measures of genetic distance between plots. Pairwise F_{ST} values were calculated in Arlequin (Excoffier *et al.*, 2005), and the significance value tested using a non-parametric permutation test following the method of Excoffier *et al.* (1992). Bootstrapped neighbour-joining trees based on these distances were reconstructed in Phylip v. 3.5 (Felsenstein, 2005) using 5000 randomizations. Geographical distances between plots were calculated from the centre of each plot, and Mantel tests were performed using the IBDWS programme (Jensen *et al.*, 2005) to compare genetic distances (Nei's D and F_{ST}) with geographical distances. Arlequin (Excoffier *et al.*, 2005) was used to calculate an AMOVA to apportion genetic variation to levels of hierarchical organization. Our organizational hierarchy in this case was based on five plot pairs (= groups) that had the greatest environmental similarity to each other, having received the same nutrient additions but different liming treatments: 1b and 1d, 3b and 3d, 4/2b and 4/2d, 8b and 8d, 9/1b and 9/2d. This organization was supported by the genetic distances within and among plots (see Results).

There is no general superiority of any single method for identifying outlier loci in all settings, and it is therefore

considered appropriate to use at least two different tests when looking for outliers (Bonin *et al.*, 2006; Oetjen & Reusch, 2007; Tsumura *et al.*, 2007; Meyer *et al.*, 2009). We therefore looked for outlier loci in two ways: (i) based on all population pairwise comparisons using the version of DetSel (Vitalis *et al.*, 2003) that has been modified to accommodate dominant data (distributed by Vitalis, pers. comm.) and (ii) using multiple univariate logistic regressions to test for associations between marker frequencies and environmental variables, using the spatial analysis method (SAM) of Joost *et al.* (2007). For the latter analysis, we included as environmental variables nitrogen, phosphorus, potassium, the total amount of added nutrients and the pH of a plot (which depended on whether or not it had been limed). We considered as outliers only those markers which were identified as such by both methods. All of our analyses were carried out with and without outlier loci to determine whether or not our results were being unduly influenced by a subset of our markers which may be under selection. We also reconstructed an additional neighbour-joining tree based solely on outlier loci to see if its topology differed from that of the tree based on putatively neutral loci.

The genetic similarity of individuals within and between plots was visualized using a principal coordinates analysis (PCoA). Pairwise similarities between individuals were calculated using Jaccard's index (Jaccard, 1908), and a PCoA was applied to the resulting matrix of similarities (Gower, 1966).

Results

The three primer combinations generated a total of 225 highly repeatable (> 97% repeatability) independent AFLP alleles in 413 samples that were collected from the ten plots; markers with repeatability values lower than 97% were discarded. Levels of genetic diversity within plots are shown in Table 1; these values are proportional to the numbers of nutrient additions that the plots had received (Silvertown *et al.*, 2009). The neighbour-joining tree shows that populations of *A. odoratum* from plots with similar fertilizer treatments group together (with the exception of plot 1) with high bootstrap support (86–100%; Fig. 2). The grouping of the b and d plots is not because of their geographical proximity to each other because plot c separates plots b and d, so that adjacent plots (e.g. 3d and 4/2d, 8b and 9/1b) are closer to each other than to the other plot receiving the same fertilizer treatment (Fig. 1).

A total of 21 outlier loci were identified by both DetSel and SAM. This translates into 9.3% of our markers, which is comparable with other studies: a recent review of fifteen studies reported that a mean of 8.5% of markers were identified as outliers following genome scans (Nosil *et al.*, 2009). The outliers that we identified in DetSel came from comparisons of all pairwise

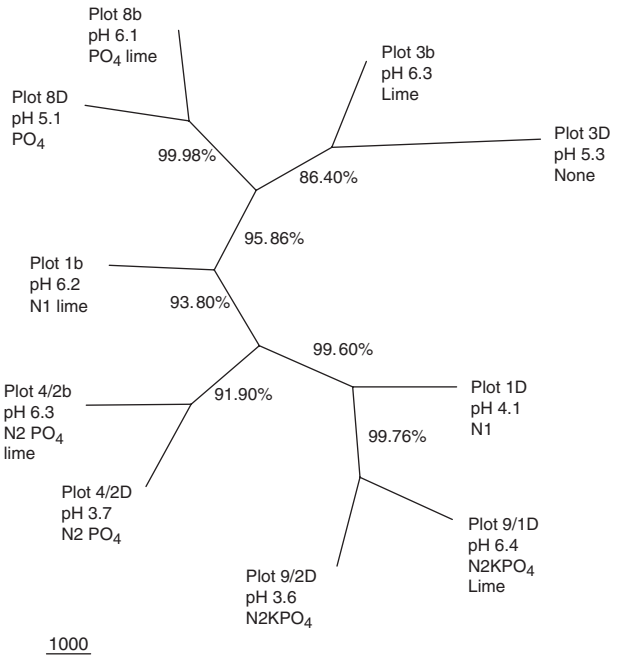

Fig. 2 Bootstrapped neighbour-joining tree of populations of *A. odoratum* from 10 Park Grass plots based on Nei's genetic distance.

plot/group comparisons with the exception of three pairs of plots: 3b and 9/1b, 3d and 8d, and 4/2d and 9/2d. The plot frequencies of these same outliers were identified in SAM as being influenced by the addition of one or more nutrients (P, K and N were all implicated), but there was no influence of pH. The outliers that we identified may be linked to genes that are under selection, although their distribution among plots may also be influenced by other factors including drift, linkage equilibrium, homoplasy, epistasis and pleiotropy (Caballero *et al.*, 2008; Holderegger *et al.*, 2006; Latta, 2006; Nordborg, 2001; Streisfeld & Kohn, 2005). Regardless, none of our results were altered by the removal of outlier loci from the

Table 2 Matrix showing the pairwise F_{ST} values for all plot comparisons. All values are significant with the exception of the F_{ST} value between plots 3b and 3d.

	1b	1d	3b	3d	4/2b	4/2d	8b	8d	9/1b
1b									
1d	0.093								
3b	0.08	0.117							
3d	0.058	0.094	0.007						
4/2b	0.103	0.063	0.125	0.116					
4/2d	0.081	0.073	0.105	0.09	0.026				
8b	0.101	0.109	0.078	0.054	0.152	0.128			
8d	0.083	0.122	0.07	0.042	0.151	0.123	0.015		
9/1b	0.18	0.048	0.175	0.152	0.086	0.123	0.137	0.156	
9/2d	0.127	0.032	0.123	0.099	0.059	0.079	0.113	0.118	0.019

analyses, although the branch lengths on the neighbour-joining tree were slightly reduced. The neighbour-joining tree that we reconstructed solely from outlier loci showed exactly the same plot groupings as the other trees, i.e. the one based on all markers, and the one based on putatively neutral markers; the only difference in topology was a substantially reduction in branch lengths, as would be expected from a tree based on only 21 markers.

Pairwise F_{ST} values between plots were consistently low (< 0.085), but significant in all pairwise comparisons with the exception of the F_{ST} value between plots 3b and 3d. For all plots except 1b and 1d, the nearest F_{ST} neighbour was the corresponding 'b' or 'd' plot, i.e. the paired plot which had received the same nutrient additions (Table 2). A Mantel test was not significant using either Nei's genetic distance ($P = 0.1148$, $R^2 = 0.0314$) or F_{ST} ($P = 0.1193$, $R^2 = 0.0326$). The analysis of molecular variance showed that most of the variation (89.5%) is found within each population; however, groups (with each group consisting of plots b and d from the same plot number; see Table 1) are separated by 7.5%, whereas the differences among plots within groups are noticeably smaller (3%; Table 3).

PCoA first was applied to all individuals from all plots. However, as might be expected among individuals from a single species in such close proximity, similarities were high and clusters were not easily discernable along the first two coordinate axes, a result compounded by the difficulty plotting the large sample size. To reduce the visual clutter in the initial analysis, we removed the control plots, 3b and 3d, and also plots 1b and 1d, which did not show any evidence of genetic similarity based on the neighbour-joining tree. Although the resultant PCoA does not reveal substantial differentiation into clusters, there is nevertheless an apparent distribution of samples such that the 'b' and 'd' of each group (i.e. 4/2b and 4/2d, 8b and 8d, 9/1b and 9/2d) are more closely associated with each other than with the plots of other groups, even after outlier loci have been removed (Fig. 3). This indicates a greater genetic similarity within than between groups.

Table 3 Design and results of AMOVA analysis of AFLP data. Groups refer to the 'b' and 'd' of each plot number, e.g. 1b and 1d, 3b and 3d (see Table 1).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation*
Among groups	4	938.361	2.117	7.53
Among populations within groups	5	298.385	0.837	2.98
Within populations	403	10136.548	25.153	89.49
Total	412	11373.293	28.108	100

*In all cases, $P < 0.0001$.

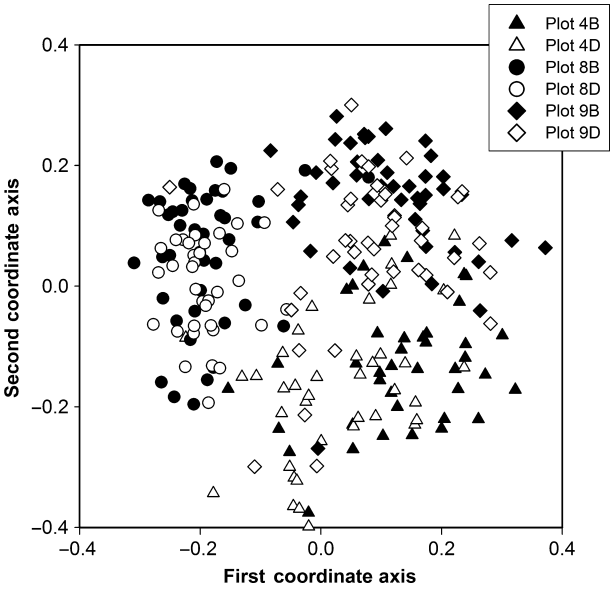

Fig. 3 Coordinate scores on the first two coordinate axes from a PCoA of the genetic similarity of individuals from plot groups 4 (4/2b and 4/2d), 8 (8b and 8d) and 9 (9/1b and 9/2d), based on all AFLP markers with the exception of the 16 'outlier' loci. Individuals from the same group (see Table 1) are represented by the same symbol shape, whereas different plots ('b' and 'd') within each group are coloured black and white.

Discussion

The Park Grass Experiment has been running for more than 150 years, during which time detailed records have been kept regarding edaphic conditions; it thus provides a remarkable opportunity for quantifying the effects that changing environments can have on the population structure of putatively neutral molecular markers. The generation time of *A. odoratum* on the Park Grass Experiment is at least 2 years, and so the maximum amount of time for which any pair of populations can have been separated is 75 years. Considering this relatively short time frame, the proximity of the populations to one another, and the fact that this species is wind-pollinated, we may expect to find little evidence of neutral genomic differentiation between populations. However, our results (neighbour-joining tree, principal coordinates analysis, F_{ST}, AMOVA) collectively show that groups of populations have become differentiated from one another; the neighbour-joining tree, the PCoA and the F_{ST} values all reflected greater similarity within than between groups (a group being the 'b' and 'd' of each plot number), and the AMOVA showed that differences between groups were higher than differences between plots within groups. The patterns revealed by our data are not overwhelmingly strong, for example the AMOVA shows that separation between groups is only 4.5% higher than within groups, and the PCoA showed that

despite some clustering, the genetic dissimilarities between groups were not pronounced; however, our results consistently show stronger genetic similarity within vs. between most of the groups of b and d plots; in addition, the pattern that is emerging is one that provides a plausible link between genetic differentiation and environmental conditions, specifically nutrient additions (see Table 1). Overall, our results suggest that it is nutrient additions, and not pH, that is driving local adaptation in *A. odoratum*.

As noted in the introduction, genome-wide effects of divergent selection can vary. In some cases, gene flow can override genetic differentiation at all loci except those that are directly under selection or are linked to selection (e.g. Bonin *et al.*, 2006; Makinen *et al.*, 2008). However, a recent review by Nosil *et al.* (2009) suggested that natural selection has a potentially important role in neutral genomic differentiation between populations, although this may occur only if certain conditions are met. For example, Thibert-Plante & Hendry (2009) showed through a series of simulations that genome-wide divergence could arise if natural selection against immigrants was sufficient to substantially reduce matings between residents and immigrants against a backdrop of large environmental differences. This has been empirically demonstrated in number of studies (reviewed in Nosil *et al.*, 2005; Schluter, 2000) and is a condition that has likely been met in *A. odoratum* on the PGE, as demonstrated by the reciprocal transplant studies of Davies & Snaydon (1976), which revealed very strong selection pressures (as high as 70%) against immigrant plants. In addition, differences in flowering time between adjacent *A. odoratum* on the PGE have been quantified (Silvertown *et al.*, 2005), and these differences provide a plausible mechanism for reproductive isolation. It is also worth noting that in general, neutral genetic divergence among populations should be more likely in small populations, in which genetic drift will relatively rapidly alter allele frequencies (Thibert-Plante & Hendry, 2009). Populations of *A. odoratum* on the PGE have likely experienced periodic bottlenecks (Rothamsted Research, 2006) and this, combined with the relatively small sizes of the plots, suggests small effective population sizes that could be rapidly influenced by genetic drift if gene flow is negligible.

Our data are unique in that they allow us to infer a genome-wide response of population differentiation across a selection mosaic that comprises a series of plots with known environmental heterogeneity; our findings therefore make an important contribution to the growing set of empirical data that seeks to explain the relationship between adaptive population change and neutral genetic markers. In addition, our work has shown that if environmental conditions change inconsistently over a particular region (e.g. more nutrients in some areas than in others, as is often seen in agricultural regions), populations may become effectively fragmented despite being geographically contiguous; this effective fragmentation can impact population dynamics, genetic diversity and microevolutionary change and may therefore be of growing concern in this time of accelerating anthropogenic environmental change.

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