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Evolution in closely adjacent plant populations X: long-term persistence of prereproductive isolation at a mine boundary

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Flowering time differences between metal-tolerant and nontolerant populations of the grass *Anthoxanthum odoratum* growing across a mine boundary have persisted for over 40 years. These flowering time differences result in a high degree of prezygotic genetic isolation (isolation index = 0.43) between the tolerant mine populations and nontolerant pasture populations. Previous work showing genetic determination of flowering time and a high turnover of individual plants argues strongly for the selective maintenance of this difference.
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Introduction

The past 50 years have seen a maturation of theories of speciation (Coyne and Orr, 2004), yet the importance of different speciation modes and the strength of empirical support for particular theories of speciation remains highly contentious. A continuing issue is the relative importance of sympatric speciation, and the genetic and ecological mechanisms that facilitate it (Via, 2001; Waser and Campbell, 2004). This debate has been aggravated by the semantic and operational difficulty of precisely defining 'sympatric'. Various other terms such as 'parapatric' and 'micro-allopatric' have been used for situations such as the one described here, where there are adjacent but not completely intermingled habitats causing disruptive selection. Given that the historical debate was originally fuelled by Mayr (1942, 1963) who considered sympatric speciation to be speciation occurring among populations between which there was substantial gene flow we use the term sympatric speciation in this broader sense. Early theoretical work showed that there were plausible mechanisms for speciation in panmictic populations (Maynard Smith, 1966; Antonovics, 1968b; Dickinson and Antonovics, 1973) as well as in populations spanning adjacent habitats between which there was substantial gene flow (Caisse and Antonovics, 1978). These studies also showed that whereas genetic divergence is very likely to evolve in populations subject to disruptive selection, the evolution of reproductive isolation by assortative mating is likely to be considerably more difficult to achieve (Caisse and Antonovics, 1978; Felsenstein, 1981). More recent studies based on quantitative genetics have

confirmed these earlier theoretical results, particularly in the context of competitive interactions for resources and for different modes of assortative mating (Kondrashov and Shpak, 1998; Gavrilets, 2000; Drossel and McKane, 2000; Doebeli and Dieckmann, 2003; Bolnick, 2004). Empirical evidence that sympatric speciation can occur has lagged behind these theoretical studies, but has gradually accumulated through experimental work (Rice and Hostert, 1993) and detailed genetic analysis of field populations (Hawthorne and Via, 2001). It has been strongly suggested, if not proved unequivocally in every particular instance, that under certain ecological and genetic conditions closely related species have probably speciated in sympatry, especially in phytophagous insects (Berlocher and Feder, 2002), parasites (McCoy, 2003), and the cichlid fishes (Kornfield and Smith, 2000).

The evolution of metal tolerance in plants growing on metal contaminated soils is an early example of the operation of divergent selection between adjacent populations (Antonovics, 1971). Measurement of metal tolerance and other key traits across mine boundaries revealed that high levels of genetic differentiation are possible over short distances even in the face of substantial gene flow (McNeilly, 1968; Antonovics and Bradshaw, 1970). Further studies showed the presence of barriers to gene flow at these boundaries, both through earlier flowering and greater self-fertility of plants on the mine soils (Antonovics, 1968a; McNeilly and Antonovics, 1968). Common garden studies on two different grass species showed that the flowering time differences between the mine and the pasture plants persisted under cultivation in standard garden conditions, and were greater at the mine boundary than away from the boundary. Such 'inverse clines' were consistent with theoretical expectations of speciation along environmental gradients (Caisse and Antonovics, 1978). This and the observation of earlier flowering in plants from smaller mines (McNeilly and Antonovics, 1968), suggested that

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flowering time differences might have evolved due to selection against gene flow between habitats, and that therefore this situation represented the possible beginnings of sympatric divergence and speciation.

In 1979 and again in 2005, I had the opportunity to revisit one of original mine sites where these studies were performed in 1965 and to measure flowering time of the plants along the same transect. Here, I report that the cline in flowering time has persisted since the first study, and that it continues to show an inverse pattern with greater isolation at the mine boundary.

Methods

Study system
Studies were carried out on the grass *Anthoxanthum odoratum* L. (sweet vernal grass) growing at Trelogan Mine in Trelogan, Flintshire, North Wales, UK (National Grid reference: SJ 123805). The soil on the mine tailings is contaminated with very high levels of zinc and lead (Antonovics, 1972). Historical data (Smith, 1921) indicate that the part of the mine site on which this study was carried out was originally an area of ore tailings established in the mid 1800s and then re-worked again between 1873 and 1898. It is likely therefore that the evolutionary processes on the mine have largely occurred within the last 100 years. Although metal concentrations were not re-measured in 1979 and 2005, the general appearance of the contaminated area and its plant community has remained unchanged, indicating that the soil there remains highly contaminated with heavy metals. There were still large areas of bare soil, and the metal indicator species *Minuartia verna* and *Viola calaminaria* are still present. Moreover, several individuals of *A. odoratum* showed clear evidence of metal-induced chlorosis on the mine area but not on the pasture.

Assessment of flowering time
The study transect was oriented east (mine) to west (pasture) and crossed at right angles to the mine boundary at N 53° 18.839′ and W 03° 19.217′. The area of the mine where the study was carried out has been designated a conservation site and is fenced. Outside this fence on the western side there is a stone wall, ca. 25 cm thick and 0.5–1 m high, separating the area of mine tailings from a pasture. The wall marks the boundary of the mine tailings (and is the 0 m point in Figures 1 and 2 below). However, although the vegetation beyond the wall is a dense sward of mixed pasture grasses, there are elevated metal concentrations up to ca. 1–2 m beyond the wall (Jain and Bradshaw, 1966), presumably due to leaching from the adjacent area of mine tailings which is higher (ca. 1 m) relative to the pasture. The mine tailings area is very heterogeneous with mounds of tailings interspersed with wetter low areas.

In 1979, flowering stage of the most advanced inflorescence was recorded on all plants (829 on the mine, and 228 in the pasture) within 1 m segments of a belt transect 3 m wide that extended 30 m either side of the mine boundary (marked by a low stone wall separating the field from the mine).

In 2005, flowering stage of every inflorescence (1647 inflorescences on the mine, and 1056 in the pasture) on
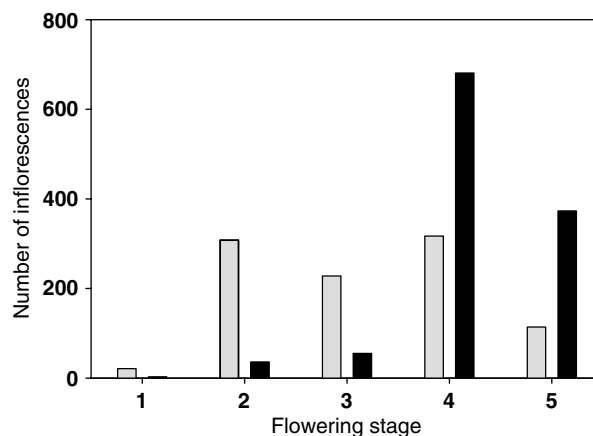

Figure 1 Number of inflorescences in each of the flowering stages in 2005 on the mine (black) and pasture (grey), excluding the transitional region (−6 to +6 m).
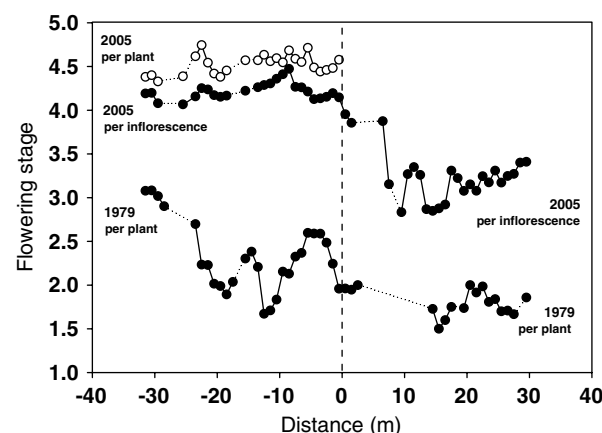

Figure 2 Mean flowering stage along a transect across the mine boundary in 1979 and 2005. Negative distances on the *X*-axis represent metal contaminated soil and positive values represent pasture, with zero being the mine-pasture boundary indicated by dashed vertical line. The 'per plant' data are based on the flowering stage of the most advanced inflorescence on a plant and were available for 2005 only on the mine. The 'per inflorescence' data are based on all inflorescences within a quadrat and were available only for 2005. To compensate for small sample sizes, values are running means for three successive quadrats (all means based on >10). Dotted lines indicate regions with no plants. Vertical dashed line is the position of the mine boundary.

every plant was recorded in an $80 \times 80 \, cm^2$ quadrat placed at every meter along the transect, south of the center line. Individual plants were distinguishable only on the mine portion of the transect (433 plants), but not in the dense sward of the pasture sites. The data for 2005 taken on the mine on a per inflorescence basis, correlate well with the data taken on a per plant basis ($r = 0.702$, $P < 0.0001$), as was performed for the entire transect in 1979.

Flowering time was assessed 'cross-sectionally' by determining the flowering stage of inflorescences on plants at one point in time. The flowering stages were scored as follows: 1-Flowering tiller swollen, but inflorescence not visible; 2-Inflorescence visible; 3-Inflorescence fully exserted with inflorescence stalk visible; 4-Stigmas exserted; 5-Anthers exserted. Longitudinal

studies under garden conditions showed that a difference of one flowering stage represents approximately 7–8 days of difference in flowering time (McNeilly and Antonovics, 1968). In 1965 (May) and 1979 (May 21st), flowering time was assessed by determining the flowering stage of the most fully developed inflorescence on every plant. In 2005 (May 24th), the flowering stage was determined for every inflorescence on every plant, and on a per plant basis on the mine where individuals were clearly distinguishable.

In 1965, flowering stages had been assessed at eight general transect positions in the field and plants were taken as single tillers from these same positions for the common garden experiments (McNeilly and Antonovics, 1968; Antonovics and Bradshaw, 1970). The 1979 and 2005 transects both excluded the extreme transect positions recorded in 1965; the furthest position on the mine had been covered by dumping of building waste, and study could not be extended to the furthest point in the pasture because of time constraints. Statistical comparisons among all three years therefore used only transect distances corresponding to the following 1965 positions (position 2 = −32 to −29; 3 = −9 to −7; 4 = −3 to −1; 5 = +1 to +2; 6 = +15 to +17; and 7 = +30 m; here and in the figures the boundary is at 0 m, with negative values on the mine, and positive values on the pasture).

Isolation index

An isolation index was calculated using the 2005 data gathered on a per inflorescence basis, excluding the quadrats in the middle fifth of the transect (at −6 to +6 m), where there was a steep gradient in flowering time between the mine and pasture. The observed distribution of the frequency of the different stages (Figure 1) was assumed to represent the time course of flowering, and the duration of each stage was assumed to be the same on the mine and pasture. The isolation index (Moyle et al, 2004) was calculated as $I = 1 - (B/(1-B))$, where the frequency of between population matings, $B = \sum_i X_i Y_i / (X_i + Y_i)$, with X_i and Y_i being the frequencies of the ith flowering stage ($i = 1$–5) on the pasture and mine, respectively. The isolation index ranges from 1 for complete isolation to 0 for panmixis.

Results

Detailed plots of the data by each meter section of the transect showed that plants flowered earlier on the mine in both 1979 and 2005 (Figure 1). The differences were highly significant statistically (using four inflorescence classes, 1979: $\chi^2 = 33.3$, df = 3, $P < 0.001$; 2005: $\chi^2 = 79.6$, df = 3, $P < 0.0001$). In 1979, at the time the data were recorded, 20.0% of the mine plants had exserted their stigmas, but only 2.6% of the pasture plants had done so; 15 plants on the mine were in anthesis, whereas none were in anthesis on the pasture. In 2005, 62.9% of the inflorescences of the mine plants had exserted their stigmas, but only 33.8% of the pasture plants had done so; 29.8% of the inflorescences of the plants on the mine were in anthesis, whereas 11.9% were in anthesis on the pasture. The isolation index between the tolerant and nontolerant populations in 2005 was 0.43.

In 1979 there was substantial heterogeneity in the flowering times on the mine. In 2005, there was much less heterogeneity within the mine, and visually the cline showed an inverse pattern (Figure 2), with flowering times being less different at the transect extremes than towards the mine boundary. To test for this, I divided the transect arbitrarily into five nearly equal sections (−32 to −19 m, −18 to −7 m; −6 to +6 m, +7 to +18 m, +19 to +30 m), and estimated the regression of mean flowering stage (weighted by number measured) on distance in the two outermost sections both on the pasture and on the mine. In 1979, there was no evidence of inverseness in the cline: on the mine, flowering stage was significantly more advanced away from the boundary ($P < 0.001$), and on the pasture there was no significant relationship with distance ($P < 0.564$). In 2005, there was suggestive evidence of an inverse cline: on the mine, flowering stage was significantly more advanced towards the boundary ($P < 0.031$), and while the trend was in the same direction on the pasture, it was not significant ($P < 0.165$).

Discussion

Comparison of these results with those of 1965 and 1966 in the field, and with the common garden experiment in 1966 (McNeilly and Antonovics, 1968; Antonovics and Bradshaw, 1970) shows that the differences in flowering time between metal tolerant and nontolerant populations have been maintained for over 40 years (Figure 3).

Flowering time differences will result in strong assortative mating and therefore promote reproductive isolation between tolerant mine populations and nontolerant pasture populations, thus promoting divergence in the face of gene flow. In 2005, where measurements were made on individual inflorescences, the isolation index suggested that even if the populations were intermixed, there would be about 40% less mating between tolerant and nontolerant populations than within those populations. A substantial number of
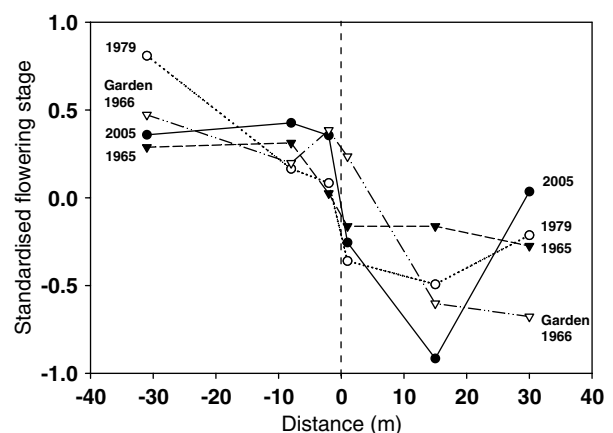

Figure 3 Mean flowering stage at specific transect positions across the mine boundary in 1965, in common garden conditions in 1966 (McNeilly and Antonovics, 1968; Antonovics and Bradshaw, 1970), and in the field in 1979 and 2005. Values are standardized as deviations from the overall mean flowering stage within each year. Negative distances on the X-axis represent metal contaminated soil and positive values represent pasture, with zero being at the mine-pasture boundary represented by the vertical dashed line.

well-recognized plant and animal species show prezygotic isolation indices of this magnitude (Coyne and Orr, 1989, 1997; Moyle *et al*, 2004).

There was no opportunity to assess if the differences observed across the transects in 1979 and 2005 were genetic in origin, but given that in 1966 these differences were shown to have a highly significant genetic basis, it is highly likely that the reproductive isolation seen in 1979 and 2005 is also genetically based. Antonovics and Bradshaw (1970) had shown that genotypic correlations between flowering time and metal tolerance were low and nonsignificant, indicating that the flowering time differences are unlikely to be a pleiotropic consequence of the evolution of metal tolerance. However, they might still be the result of direct selection for both earlier flowering and seed maturation, because the mine site habitat is different from the pasture habitat in many respects other than metal concentration of the soil. Alternatively, such differences could come about as a result of differential mating success of genotypes if there are environmentally induced differences in flowering time between the mine and pasture habitats (Stam, 1983). Evidence that these differences could at least in part be the result of indirect selection for assortative mating so as to minimize gene flow at the mine boundary comes from the fact that in this study and in previous ones (McNeilly and Antonovics, 1968) the flowering time differences tend to be greater towards the mine boundary, although this trend was only significant in 2005 on the mine. It is clear that further studies are needed to quantify the relative magnitude of direct and indirect selection for assortative mating.

Mine and pasture plants differ not only in metal tolerance and flowering time, but also in several conspicuous morphological characters and in levels of self-fertility (Antonovics and Bradshaw, 1970). Such differences are often used in species level taxonomic characterizations. The presence of the cline in the early 1960s and its persistence for over 40 years is therefore consistent with the idea that genetic divergence and the beginnings of the speciation process can occur rapidly in clinal situations (Jain and Bradshaw, 1966; Caisse and Antonovics, 1978). As this study was carried out only three times and at widely separated time intervals using somewhat different sampling methods, interpretation of the data as a time series should be performed with caution. However, the consistency of the pattern cannot be explained by the longevity of the plants on the mine. Antonovics (1972) showed that flowering individuals on the mine have a high turnover, with half-life of only 1.98 years; after even a 10 year period only about 0.1% of the original population would be predicted to be still present on the mine. Further studies that incorporate experimental approaches and include direct estimates of gene flow and selection are clearly needed to gauge the nature of the evolutionary forces that have maintained the consistent long-term pattern of reproductive isolation observed in this study.

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