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Evolutionary vestigialization of sex in a clonal plant: selection versus neutral mutation in geographically peripheral populations

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The loss of traits that no longer contribute to fitness is widespread; however, the causative evolutionary mechanisms are poorly understood. Vestigialization could proceed through the fixation of selectively neutral degenerative mutations via genetic drift. Alternatively, selection may facilitate vestigialization if trait loss results in enhanced fitness. We tested these hypotheses using *Decodon verticillatus*, a clonal plant in which sexual sterility has arisen repeatedly in populations across the northern geographical range limit. We compared growth and survival of replicated genotypes from 7 sexually fertile and 18 sterile populations, over 3 years in a common environment. Survival of sterile genotypes was 53% greater than for fertile genotypes, but there was no difference in biomass accumulation. Almost all mortality, and hence increased performance of sterile genotypes, occurred during simulated overwinter dormancy. These observations suggest that selection has facilitated the vestigialization of sex, and thus do not support the neutral mutation hypothesis. The selective mechanism probably involves the relaxation of a genetic trade-off between sexual reproduction and survival: alleles that increase vegetative performance at the expense of sexual fertility are selected in geographically peripheral populations where sexual reproduction is suppressed by adverse environmental conditions.

Keywords: antagonistic pleiotropy; asexuality; clonal reproduction; *Decodon verticillatus*; neutral mutation; selection

1. INTRODUCTION

The loss or vestigialization of traits that no longer contribute to fitness is a widespread phenomenon that highlights the importance of natural selection for the maintenance of adaptive traits (Fong *et al.* 1995; Wiens 2001; Porter & Crandall 2003). However, except for a few well-studied cases, such as the loss of eyes and pigments in cave-dwelling organisms, the evolutionary and genetic mechanisms underlying trait loss remain poorly understood (Fong *et al.* 1995; Culver & Wilkens 2000). Trait reduction could occur through the fixation, via genetic drift, of selectively neutral mutations that cause degeneration of the non-functional trait (Haldane 1933; Muller 1949; Emerson 1961; Brace 1963; Wilkens 1988). Although this simple mechanism is generally supported by a wealth of evidence from the molecular evolution of pseudogenes and non-coding DNA (Wilkens 1988), the random fixation of degenerative mutations is liable to be a slow process and seems an unlikely explanation for cases where vestigialization has occurred repeatedly and rapidly (Porter & Crandall 2003).

Vestigialization could proceed more quickly if trait reduction was associated with increased fitness. Reduction of the vestigial trait may be selected directly if the trait becomes a fitness liability, like some sexually selected traits (Wiens 2001). Trait loss could also be selected indirectly if the reduction in the non-functional trait frees resources that can be reallocated to traits or functions that increase fitness (Regal 1977). Indirect selection could also play a key role if degenerative mutations that reduced the non-functional trait had positive pleiotropic effects on, or genetic correlations with, functional traits that contribute to fitness (Prout 1964; Wright 1964). Negative genetic correlations between fitness-enhancing traits are generally expected (Williams 1966), so that if one trait ceases to function, alleles that increase fitness at the expense of the non-functional trait become advantageous.

The relative importance of neutral versus selective processes in evolutionary vestigialization is largely unknown. Early work on the best-studied systems, such as the loss of eyes and pigments in cave-dwelling organisms, produced results that could be accounted for by the neutral mutation hypothesis. Moreover, complex traits, like eyes, that are governed by many loci, present large targets for mutational degeneration, and molecular estimates of divergence times between cave- and surface-dwelling populations appeared for sufficient long for degenerative mutations to have become randomly fixed (reviewed in Wilkens 1988; Fong *et al.* 1995; Culver & Wilkens 2000). However, recent selection, genetic mapping and developmental studies suggest that selection may play an important role in trait loss (reviewed in Jeffery 2001; Culver & Wilkens 2000; Porter & Crandall 2003).

The mechanisms potentially responsible for trait loss can also be differentiated using relatively straightforward experiments. The selective hypothesis predicts that trait loss should be associated with an overall increase in fitness, whereas there should be no increase in fitness when

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vestigialization occurs through the accumulation of selectively neutral mutations. These predictions have very rarely been tested (Culver & Wilkens 2000).

Evolutionary vestigialization is of particular significance when it involves major life-history or reproductive traits. For instance, most perennial flowering plants combine sexual reproduction via seed with some form of asexual, clonal reproduction via vegetative propagation (Klimes *et al.* 1997). Although sexual reproduction is usually a major component of fitness, it is generally thought that clonal reproduction is successful over a wider range of environmental conditions than sexual reproduction (Eckert 2002). Under some conditions, therefore, sex may contribute very little to population recruitment and hence to fitness. At the extreme, traits involved in sex may cease to be maintained by selection, and might, as a result, become lost or dysfunctional (Klekowski 1988). Sexual reproduction by flowering plants involves an intricate series of developmental and morphological traits, from the formation of flowers and gametophytes to the production of fruits and seeds. Accordingly, many loci are involved in the expression of traits associated with sexual reproduction (O'Neill & Roberts 2002), and hence sex may be vulnerable to vestigialization via mutation when it no longer contributes to fitness. The evolutionary vestigialization of sex may thus help to explain the many anecdotal reports of marked reduction in seed production in a wide range of clonal plants in some habitats or parts of their geographical distribution (Eckert 2002). Our studies of *Decodon verticillatus* provide, to our knowledge, the first clear example of the evolutionary vestigialization of sexual reproduction in flowering plants (Eckert & Barrett 1993; Eckert *et al.* 1999; Dorken & Eckert 2001).

Decodon verticillatus (L.) Ell. (Lythraceae) is a diploid, clonal, perennial, emergent, aquatic plant common to wetland habitats throughout eastern central North America. Sexual reproduction involves insect-pollinated, self-compatible, tristylous flowers. Asexual reproduction occurs through adventitious rooting of branch tips making contact with wet substrate, and is therefore closely associated with vegetative growth. Across the northern periphery of the geographical range of *D. verticillatus*, there is a steep reduction in sexual fertility. At the range limit, populations rarely, if ever, produce seed and almost always consist of single clonal genotypes. A few hundred kilometres to the south, populations produce abundant viable seed and are genetically diverse with genotype frequencies indicative of regular sexual reproduction (Eckert & Barrett 1992; Dorken & Eckert 2001). Common environment experiments demonstrate that this geographical variation in sexual fertility has a strong genetic basis: plants from sterile populations produce little or no seed even when hand-pollinated under benign greenhouse conditions (Dorken & Eckert 2001). Patterns of allozyme variation indicate that infertility appears to have arisen independently in most populations (Eckert & Barrett 1993; Dorken & Eckert 2001). Sex is usually impaired at different stages of flower and fruit development in different sterile populations, further supporting the independent origin of mutations causing the loss of sex (Eckert *et al.* 1999).

If the loss of sex has been facilitated by selection, then we predicted that sexually sterile genotypes should exhibit enhanced vegetative fitness compared with sexually fertile genotypes. By contrast, no differences in performance are expected under the neutral mutation hypothesis. Here, we test these predictions by comparing the vegetative growth and survival of replicated genotypes sampled from a large number of sexually fertile versus sterile populations, and grown in a common greenhouse environment over 3 years.

2. MATERIAL AND METHODS

We used all 18 of the sexually sterile populations and 7 of the 10 sexually fertile populations studied by Eckert *et al.* (1999) and Dorken & Eckert (2001). They are located across a 500 km latitudinal transect in the New England region of the United States, with sterile and fertile populations predominating at the northern and southern portions of the study area, respectively (see fig. 1 in Eckert *et al.* 1999). During June 1997, we randomly collected 9–13 ramets (mean of 12) from throughout each population, and grew them in a randomized array under standard greenhouse conditions for the summer and autumn, then transferred them to a dark cold room at 4 °C for four months (details in Eckert *et al.* 1999).

We returned the plants to the greenhouse in spring 1998, and generated four clonal replicates for each of 6–12 ramets per population (mean of 9) by rooting tiny axillary shoots consisting of the apical bud and one pair of expanding leaves. After the cuttings rooted, they were individually weighed (to an accuracy of ±0.001 g) and transplanted into 5.8 cm pots filled with Sunshine Mix no. 3 growth medium, and grown in a randomized array on a single greenhouse bench under ambient light and 20–30 °C. Between one and five clonal replicates for each ramet survived transplanting (mean ± s.d. $= 3.5 \pm 0.9$ replicates per ramet; two or more replicates for 97.5% of ramets; $n = 780$ plants in total). In October, just as the foliage began to senesce, we scored each plant as alive or dead and harvested its above-ground biomass. Plants were then subjected to a four-month cold period (as above). In spring 1999, we transferred each plant to a 7.75 cm pot and grew them until October in a new randomized array under the same conditions as during 1998. Above-ground biomass was harvested, as above, and plants were then subjected to a six-month cold period. In early summer 2000, plants were again returned to the greenhouse, allowed to grow for two months and then scored as alive or dead.

All above-ground biomass harvested in 1998 and 1999 was dried to constant mass at 70 °C, stored for several months at room temperature, and weighed to ±0.001 g. In 1999, 13 surviving plants were damaged before harvest and thus did not yield dry mass data. Some plants produced flowers in 1999 and, for these, we counted the number of leaf axils bearing flowers. Although flowers of *D. verticillatus* are self-compatible, they do not self-fertilize autonomously (Eckert & Barrett 1994), hence none of the flowering plants set fruit.

Holding the ramets for a year under uniform conditions before the experiment and then subsequently generating each experimental plant from a relatively small standard length of axillary shoot should have greatly reduced variation in performance because of clonal maternal effects resulting from environmental variation among the original populations (Schwaegerle *et al.* 2000). Accordingly, we assume that most of the variation among populations and ramets within populations has a genetic basis.

We compared total survival, from transplant in early summer 1998 until June 2000, among clonal replicates from sterile ($n = 562$ plants) and fertile populations ($n = 218$). We also compared survival during each of the three phases of the experiment: transplant to autumn harvest 1998, the 1998 harvest to the 1999 harvest, and the 1999 harvest to summer 2000. To account for the fact that clonal replicates and even individual ramets within populations (especially sterile populations) were not genetically and thus statistically independent, we also compared mean total survival between the 18 sterile and 7 fertile populations.

We analysed dry mass production by averaging the clonal replicates for each ramet, and testing for variation between sterile and fertile populations using a nested analysis of covariance (ANCOVA) with population type as the main fixed effect, populations nested within type as a random effect and ramets nested within populations as the residual variance (Neter *et al.* 1990). *F*-tests were performed using restricted maximum likelihood. For dry mass at the 1998 harvest, we used the initial fresh mass of the clonal cuttings as a covariate, which correlated with dry mass at harvest ($r = +0.56$, $F_{1,196} = 117.6$, $p < 0.0001$) but did not differ between sterile and fertile populations ($F_{1,197} = 0.3$, $p = 0.61$) or exhibit heterogeneity in slope between population types (interaction $F_{1,196} = 1.8$, $p = 0.18$). To analyse growth during 1999 independently of growth during 1998, we used dry mass at the end of 1998 as a covariate. Again, this covariate correlated with dry mass at harvest in 1999 ($r = +0.28$, $F_{1,179} = 10.8$, $p = 0.001$), but did not differ between population types (see § 3) or exhibit heterogeneity in slopes ($F_{1,178} = 0.012$, $p = 0.91$).

To summarize growth, we calculated cumulative dry mass by summing the dry masses in 1998 and 1999 for each clonal replicate. Plants that died between harvests were assigned a mass of zero for 1999 because almost all mortality occurred during the overwinter period. Variation in cumulative dry mass was analysed as above, using initial fresh mass as a covariate. Again, the covariate correlated positively with cumulative dry mass ($r = +0.21$, $F_{1,195} = 7.3$, $p = 0.007$) and there was no heterogeneity in slopes ($F_{1,194} = 1.4$, $p = 0.24$). Analysis of residuals confirmed that the data met the assumptions of ANCOVA for all analyses. We used JMP v. 5.0 (SAS Institute 2002) for all analyses.

3. RESULTS

Overall, 38.6% of the 780 plants survived from summer 1998 until summer 2000. Out of the 479 that died, only 28 plants died during the first growing season in 1998, whereas 170 died between 1998 and 1999, and 281 died between 1999 and 2000. Almost all of this mortality occurred during the overwinter periods. Overall survival was much higher for plants from sterile (43%) than fertile populations (28%; table 1). This trend was observed throughout the experiment: during the 1998 growing season, between the 1998 and 1999 harvests, and between the 1999 harvest and the summer of 2000. Comparison of population means (figure 1*a*) also revealed significantly higher overall survival for sterile (range = 11.1–84.2%, mean ± s.e. = 43.6 ± 4.1%) than fertile populations (range = 17.1–33.3%, mean = 28.3 ± 2.1%; Wilcoxon test: $p = 0.01$).

No plants flowered during 1998, and only 49 out of 582 surviving plants flowered during 1999. Though small, the percentage of plants flowering was significantly higher for sterile than for fertile plants (table 1). Among the plants that flowered, there was no difference in the number of nodes bearing flowers per plant between sterile (mean ± s.e. = 10.7 ± 1.2, $n = 44$) and fertile populations (13.0 ± 6.6, $n = 5$; Wilcoxon test: $p = 0.8$).

For both harvests, above-ground dry mass varied greatly among plants (1998 range = 0.02–19.8 g, coefficient of variation (CV) = 52%; 1999 range = 0.05–54.5 g, CV = 70%). Population means varied almost threefold within each year (1998 range = 2.1–5.6 g, CV = 22%; 1999 range = 6.5–17.3 g, CV = 29%) and were correlated between years ($r = +0.44$, $p = 0.03$). However, there was no difference between plants from sterile versus fertile populations in dry mass at the end of 1998, dry mass at the end of 1999, or cumulative dry mass (table 1, figure 1*b*).

4. DISCUSSION

Our results show that genotypes of *D. verticillatus* from a broad sample of sexually sterile populations exhibited substantially higher survival than those from sexually fertile populations. For all but 4 of the 18 sterile populations, mean survival was above the maximum of the 7 fertile populations, indicating that the loss of sexual reproduction in *D. verticillatus* is generally associated with increased vegetative fitness. Thus, our results are consistent with selection facilitating the vestigialization of sex and depart significantly from the null expectation of the neutral mutation hypothesis.

Accepting a role for selection in trait vestigialization assumes that the observed difference in performance between genotypes with and without trait reduction has adaptive significance in natural populations. However, such differences are usually evaluated in a single artificial environment (e.g. Culver & Wilkens 2000), such as the greenhouse environment that we used. Although field transplant studies would provide the most direct evidence that increased survival of sterile genotypes of *D. verticillatus* observed in the greenhouse yields higher fitness in natural populations, our data are entirely consistent with this possibility. Almost all of the survival differential between fertile and sterile genotypes that we observed occurred during simulated overwinter periods. Enhanced overwinter survival should provide an advantage to sterile genotypes at the northern periphery of the species' range. Moreover, enhanced overwinter survival of sterile genotypes was not simply a by-product of more rapid vegetative growth in the preceding summer, as neither seasonal nor total accumulation of above-ground biomass differed between fertile and sterile populations. Sterile genotypes exhibited a survival advantage above and beyond that which might be expected on the basis of plant size alone. This is likely to be adaptive in northern populations of *D. verticillatus* because vegetative growth appears to be strongly suppressed by environmental factors at the limit of the species' range (Dorken & Eckert 2001), and good overwinter survival in spite of limited growth would seem advantageous, especially for new clonal progeny.

Given that selection is likely to have played a role in the degeneration of sex in *D. verticillatus*, what selective mechanism might have caused the association between sexual sterility and increased vegetative performance? Unfortunately, there has been little theoretical investigation of how sexual sterility might evolve in conjunction with other life-history traits. A simulation model by Peck *et al.* (1998) predicts strong disequilibrium between sexual sterility and increased fitness in populations at geographical range

Table 1. Comparison of survival, flowering and vegetative growth over a 3-year period for clonally replicated ramets from 18 sexually sterile populations ($n = 562$ plants) and seven sexually fertile populations ($n = 218$ plants) of *Decodon verticillatus*.
(The percentage of plants surviving or flowering was compared with 2×2 contingency table likelihood-ratio χ^2 tests (d.f. = 1). The dry mass was compared with ANCOVA (d.f. = 1, 23) using the initial mass of clonal cutting as the covariate for dry mass at the end of 1998 and for cumulative dry mass, and dry mass at the end of 1998 as the covariate for dry mass at the end of 1999. Population means for total survival and cumulative dry mass are shown in figure 1.)

	population type means		statistical comparison	
trait	sterile	fertile	χ^2/F^*	p
survival (%)				
summer 1998	98.9	89.9	32.3	< 0.0001
autumn 1998 to autumn 1999	79.9	70.4	7.1	0.008
autumn 1999 to summer 2000	54.0	44.2	4.1	0.04
total	42.7	28.0	14.8	0.0001
flowering (%)				
1998	0.0	0.0	—	—
1999	9.9	3.6	6.4	0.01
above-ground dry mass (g)				
1998	4.1	3.9	0.4^*	0.5
1999	10.4	11.0	0.4^*	0.5
cumulative	12.4	11.4	0.5^*	0.5

margins, a situation that we have observed in *D. verticillatus*. The model is based on the premise that only rare gene combinations provide tolerance to the extreme environments in peripheral populations (Antonovics 1976; Bradshaw 1991). Sterility mutations protect these high fitness genotypes from being broken up by sexual recombination (e.g. Kaltz & Bell 2002). As a result, only genotypes that combine high fitness and sexual sterility persist in peripheral populations (Peck *et al.* 1998). However, this evolutionary scenario requires that sexual and asexual reproduction be mutually exclusive. By contrast, clonal plants typically combine both modes of reproduction using different developmental mechanisms (Klimes *et al.* 1997). Sexual reproduction in *D. verticillatus* involves flowers, whereas clonal reproduction occurs through adventitious rooting of branch tips. As a result, high fitness genotypes are preserved through clonal propagation regardless of whether they make seed. There is no necessary association between sexual sterility and high fitness, and clonal genotypes with high vegetative fitness could spread in any population.

A more likely scenario is that genetic trade-offs between sexual reproduction and vegetative performance promote the loss of sex in *D. verticillatus*. Such trade-offs may arise via pleiotropy, which is widely viewed as a pervasive aspect of genetic architecture, and expected to generate trade-offs between life-history traits (Williams 1966; Roff 1992). Mounting evidence suggests that pleiotropy plays a role in the coevolution of vestigial and constructive traits in cave organisms (Borowsky & Wilkens 2002; Porter & Crandall 2003). The large number of loci involved in sexual reproduction in plants may facilitate the development antagonistic pleiotropy with other life-history functions. Also, recent selection experiments have demonstrated significant genetic trade-offs between sexual reproduction and components of plant growth (e.g. van Kleunen *et al.* 2003). Detailed quantitative genetic experiments (e.g. Conner 2002) are required to test whether antagonistic pleiotropy is responsible for the consistent association between sterility and enhanced vegetative performance among populations of *D. verticillatus*.

Modularity and clonal propagation are likely to promote the selection of alleles that increase vegetative performance at the expense of sexual fertility, when selection for sexual reproduction is relaxed. It is generally thought that clonal reproduction is successful over a broader range of ecological conditions than sexual reproduction in plants, and there are many reports of reduced sexual fertility in geographically peripheral populations that are likely to experience extreme environments (Eckert 2002). Experimental comparison of the sexual fertility of *D. verticillatus* between field and greenhouse environments verifies that seed production is significantly depressed by environmental factors in northern but not in geographically central populations (Dorken & Eckert 2001). Thus, sterility and increased ramet survival could be selected jointly because the contribution of sex to fitness is reduced in peripheral populations.

Two alternative scenarios are unlikely to explain our results. First, if resources allocated to sexual reproduction reduce the amount of resources available for vegetative growth and survival, alleles causing sterility may be favoured in populations with reduced opportunities for sexual recruitment. Studies on a wide variety of plants have demonstrated negative phenotypic correlations between sexual reproduction and other components of fitness, including ramet survival (Bazzaz 1997). It is likely that mutations inhibiting seed production would free resources for investment in vegetative growth and survival. However, this mechanism does not explain our results because we intentionally grew experimental plants at a density that almost entirely suppressed flowering, so that we could quantify vegetative performance in the absence of resource competition with sexual reproduction. Very few plants flowered, none produced fruit, and there was no tendency for fertile genotypes to flower more frequently or invest more in flowering than sterile genotypes. Hence, the increased vegetative survival of sterile genotypes could not have resulted from resource reallocation. Release from the

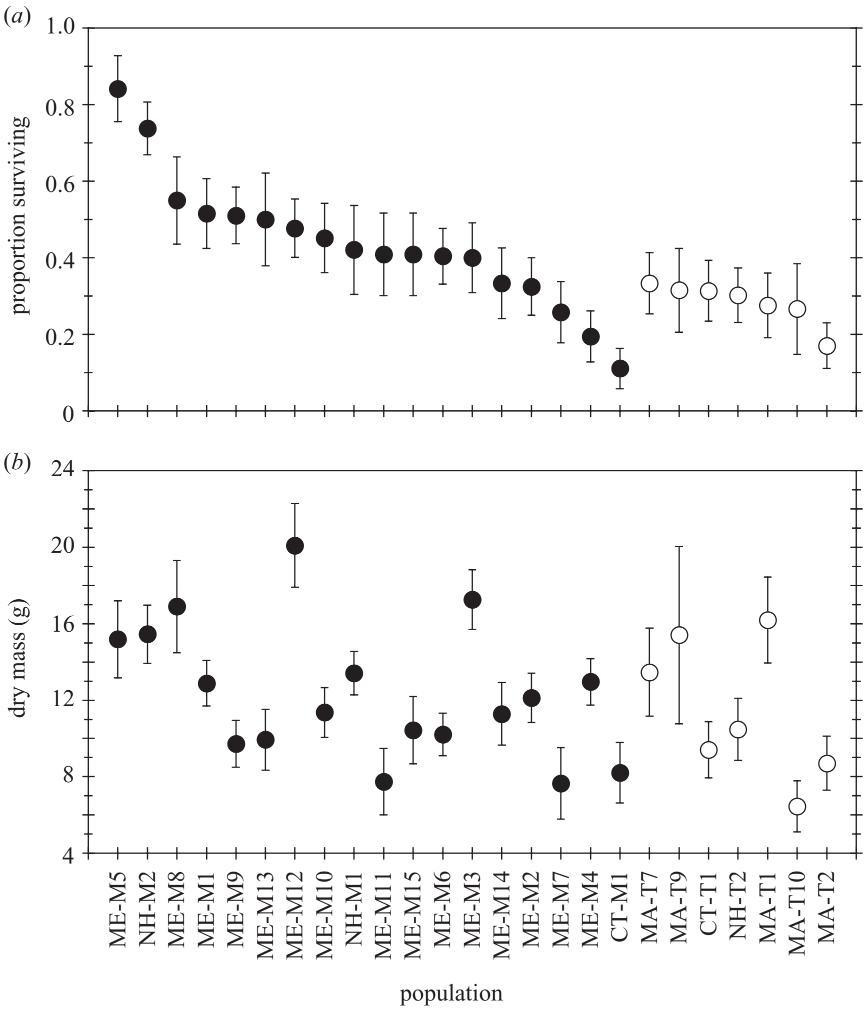

Figure 1. Comparison of (*a*) survival and (*b*) biomass production over a 3-year period for clonally replicated ramets from 18 sexually sterile populations (filled circles) and seven sexually fertile populations (open circles) of *Decodon verticillatus*. For survival (*a*), points are the proportion of *ca*. 31 clonal replicates per population surviving from the summer of 1998 to the summer of 2000 (±1 binomial s.e.m.). For biomass (*b*), points are population means for the cumulative production of above-ground dry mass during the 1998 and 1999 growing seasons, based on data from *ca*. four clonal replicates for each of *ca*. nine ramets from each population (±1 s.e.m. based on ramet means).

resource cost of sexual reproduction could further promote the spread of sterility mutations in natural populations. However, this hypothesis needs to be tested by manipulating resource investment in sex via flower bud removal.

The second alternative scenario is that both neutral mutation accumulation and selection, acting sequentially, might generate an association between sexual sterility and increased vegetative fitness. It is conceivable that increased vegetative fitness of sterile genotypes arose after the loss of sex via neutral mutation accumulation. However, we think this unlikely. First, this scenario requires enough evolutionary time for sterility mutations to have become fixed via genetic drift and vegetative fitness to have been subsequently increased via selection (Culver & Wilkens 2000). However, all the populations of *D. verticillatus* in our study occur well within a recently deglaciated region and, based on dates for the oldest fossil seeds, have only returned to these northern latitudes *ca*. 8000 years before the present (Eckert & Barrett 1995). The long lifespan and clonal reproduction characteristic of *D. verticillatus* should greatly slow the fixation of neutral mutations. Moreover, the loss of genetic recombination caused by sexual sterility in northern populations of *D. verticillatus* (see Dorken & Eckert 2001; Eckert 2002) would greatly slow the rate of subsequent adaptive evolution (e.g. Kaltz & Bell 2002; see also simulations in Eckert & Barrett 1995). Our second objection to this scenario is that it fails to explain why an improvement in vegetative fitness would not be selected in sexually fertile populations as well, unless there was some sort of evolutionary trade-off with sexual reproduction. Third, a pleiotropic link between enhanced vegetative performance and reduced sexual fertility would facilitate the evolution of sexual sterility in environments where sexual reproduction was still marginally functional, and thus better explain the widespread occurrence of sexual sterility across the northern range limit of *D. verticillatus* (Eckert & Barrett 1992, 1993).

The joint evolution of increased vegetative performance and sexual sterility in populations where extreme environments relax selection for sexual reproduction may potentially allow the northward expansion of the geographical range. However, the resulting increased tolerance to environmental extremes comes with extremely low population genetic diversity and a very limited capacity for dispersal (Dorken &

Eckert 2001) owing to the large size and vulnerability of clonal propagules compared with seed (Starfinger & Stöcklin 1996). The evolutionary vestigialization of sex may allow a species to occur where it otherwise would not, but ultimately limits further expansion of the species' range. Studies investigating the causes and consequences of the widespread reductions in sexual fertility of plant populations at range margins (Eckert 2002) may lead to a better understanding of the evolution of range limits in plants and other organisms with mixed reproductive strategies.

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