SHORT COMMUNICATION

Mechanisms of constraints: the contributions of selection and genetic variance to the maintenance of cotyledon number in wild radish

J. K. CONNER* & A. A. AGRAWAL†

*W. K. Kellogg Biological Station, and Department of Plant Biology, Michigan State University, MI, USA
†Department of Ecology and Evolutionary Biology, Cornell University, NY, USA

Abstract

The evolutionary mechanisms underlying the maintenance of invariant traits are poorly understood, partly because the lack of variance makes these mechanisms difficult to study. Although the number of cotyledons that plant species produce is highly canalized, populations of plants frequently contain individuals with abnormal cotyledon numbers. In a garden study with 1857 wild radish plants from 75 paternal half-sibling families, 89 (almost 5%) had cotyledon numbers less or greater than two. We found evidence for direct selection on cotyledon number, but no evidence for additive genetic variation for cotyledon number. In spite of the very large sample size, our power to detect variation and selection was hampered by the small number of individuals (10) producing more than two cotyledons. Thus, our results provide support for both a lack of genetic variation and selection as reasons for the current lack of variation in wild radish cotyledon number.

As far as I know, there are hardly any references to tricotylous races in botanical literature, and the possibility of the existence of tricotylous intermediate races seems never to have been discussed. In this part, however, I shall describe some instances of such races in order to demonstrate their existence and study their characters. In the period from 1892–1897 I succeeded in producing such races from half a dozen very different species. – De Vries (1909–1910).

Introduction

The maintenance of apparently fixed characteristics of organisms is little-studied in evolutionary biology (Stearns, 1994; Karoly & Conner, 2000). The obvious difficulty of addressing questions relating to characteristics that are largely fixed within species (or higher taxonomic groups) is that the lack of variation itself becomes a barrier to investigation. Nonetheless, fixed

Correspondence: Jeffrey K. Conner, Kellogg Biological Station, 3700 East Gull Lake Drive, Hickory Corners, MI 49060, USA.
Tel.: 269 671 2269; fax: 269 671 2104;
e-mail: connerj@msu.edu

traits may represent important adaptations in which strong stabilizing selection has eliminated most variation. Waddington (1957) proposed that such traits are canalized, that is, they are buffered against phenotypic changes caused by mutations of small effect or environmental variation during development. Traits may also lack phenotypic variation for nonadaptive reasons, including a loss of genetic variation through drift, or constraints imposed by development or genetic correlations with other traits under strong stabilizing selection.

One of the least variable traits in plants is the number of cotyledons produced, but the potential adaptive value of cotyledon number has rarely been considered. Most angiosperms are mono- or dicotyledonous, that is, within a species plants typically produce either one or two seed leaves or cotyledons. However, there is some interspecific variation in this trait. Within the monophyletic group of eudicots, species of *Degenaria* (Mabberley, 1989), *Szymgium* (Shavalli Khan *et al.*, 1997), and *Psittacanthus* (Kuijt, 1967) typically produce more than two cotyledons. In addition, among conifers, especially in the Pinaceae, higher cotyledon numbers are the rule in several genera, ranging from three to nine cotyledons in *Tsuga*, *Sequoia*, *Larix*, and *Cedrus* (Butts & Buchholz, 1940; Haskell, 1954;

Mabberley, 1989; von Aderkas, 2002). Still, the vast majority of plant species produce one or two cotyledons.

Intraspecific variation in cotyledon number also occurs. The phenomenon of typically dicotyledonous plants producing three cotyledons has been referred to as tricotyledony or tricotyly and has been reported from hundreds of species in over 15 families of plants (Gates, 1910; Went, 1944; Haskell, 1954; Harrison, 1964; Dessureaux, 1967; Rajora & Zsuffa, 1986; Graz, 2001). More generally, the production of an abnormal number of cotyledons has been referred to as pleiocotyly. Early research on pleiocotyly was focused on the potential use of abnormal cotyledons as a genetic marker and other uses in crop breeding (Haskell, 1961; Rajora & Zsuffa, 1986). Several previous experiments have examined the genetic basis of pleiocotyly (Holthorp, 1944; Haskell, 1949, 1962; Harrison, 1964; Dessureaux, 1967; Rajora & Zsuffa, 1986). Although many of these studies were not well-replicated, and few data are actually presented in the papers, apparently statistically significant responses to artificial selection were demonstrated in some species, suggesting that lack of genetic variation is not the cause of the highly conserved nature of cotyledon number. For example, Hugo De Vries described experiments with *Antirrhinum majus* in which six successive generations of artificial selection for pleiocotyly resulted in the increase in its frequency from 5 to 64% (De Vries, 1909–1910; Haskell, 1954). However, in many other species artificial selection was ineffective (De Vries, 1909–1910; Haskell, 1954). In addition, there is limited evidence that the frequency of pleiocotyly may be affected by maternal environment, including temperature, light availability, and soil conditions (Haskell, 1954; Harrison, 1964), but it is unclear whether pleiocotyly is consistently increased by stressful conditions.

Over the course of studying natural populations of wild radish plants (*Raphanus raphanistrum* and *R. sativus*) from upstate New York (Conner & Via, 1993; Agrawal *et al.*, 1999, 2002;), northern California (Agrawal, 1998, 1999, 2000, 2001;), and Silwood Park, England (A.A. Agrawal, unpublished data) for over a decade, we have observed pleiocotyly in 0–5% of plants (Fig. 1). De Vries (1909–1910, pp. 381) also noted pleiocotyly in *R. raphanistrum*. Here we test whether cotyledon number in wild radish is an adaptation maintained by stabilizing selection, or whether the low phenotypic variation for this trait is caused by a lack of additive genetic variation.

Cotyledon number was analysed as a threshold trait (Roff, 1996, 1997;). Threshold traits have a few discrete phenotypic classes, but are not inherited in a simple Mendelian manner; rather, a discrete change in phenotype occurs when the value of an underlying continuous variable (the liability) crosses a threshold value (Falconer & Mackay, 1996). The liability is a typical quantitative trait, affected by multiple gene loci and the environment. Traits that are canalized should often be inherited as threshold traits, because changes in the liability that do

Fig. 1 Wild radish seedling with three cotyledons.

not cross a threshold do not cause phenotypic change; therefore, the trait is buffered against a certain amount of genetic and environmental change.

Materials and methods

Raphanus raphanistrum (Brassicaceae) is a widely distributed, self-incompatible annual plant found in disturbed sites on six continents. We bred paternal half-sib families of *R. raphanistrum* from a single wild population in upstate New York (Conner & Via, 1993). To examine genetic variation in cotyledon number of wild radish, we crossed each of 75 Sire plants with three different Dam plants, resulting in 225 full-sib families nested within 75 paternal half-sib families. We removed seeds from dried siliques using needle nose pliers and weighed each of approximately nine seeds from each of the full sib families (total 2014). This seed mass measure was used as a covariate in the tests for selection on cotyledon number (see below). On 1 May 2001, seeds were planted in 150 mL peat pots filled with unfertilized Pro-Mix soil (Red Hill, PA, USA) and placed in completely randomized positions on the out of doors roof of the Earth Sciences Centre, University of Toronto. On 3 May, we planted the peat pots in a completely randomized design in a plowed old field at Koffler Scientific Reserve at Jokers Hill in southern Ontario, Canada (44°03′N, 79°29′W, http://www.zoo.utoronto.ca/jokershill). Plants were not watered in the field.

Emergence and cotyledon number were checked on each plant approximately every 3 days. Plants were scored in three categories: less than two full cotyledons (incomplete cotyledons were typically <10% of the size of a normal cotyledon and were characterized by a light green colour and a thick, waxy appearance), two full
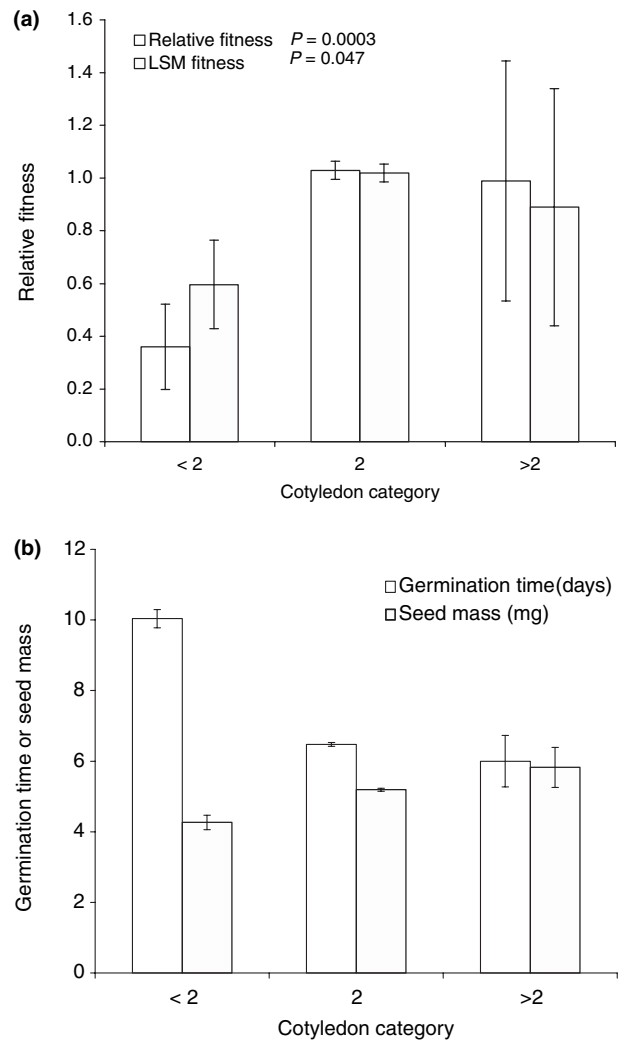
cotyledons, and greater than two cotyledons. At the end of the growing season, when plants began to shed seeds, we harvested all fruits and dried them in paper bags. The total mass of all fruits produced over each plant's lifetime was measured as an indicator of female fitness. Lifetime fruit mass is a good predictor of the number of seeds produced in *R. raphanistrum* ($n = 624$, $R^2 = 0.80$, $P < 0.05$; combined data from Agrawal, 1998; Agrawal *et al.*, 1999).

To determine whether selection was acting on cotyledon number, a one-way ANOVA was performed using relative fitness (each individual fitness estimate divided by overall mean fitness) as the response variable and cotyledon category as the predictor. This tests the total selection on cotyledon number due to both direct selection and indirect selection from correlated traits, analogous to a selection differential. Because plants in the three cotyledon categories differed significantly in both the mass of the seed they germinated from as well as their germination time (see Results), an ANCOVA was also performed by adding seed mass and germination time as predictor variables to the original model. This tests for direct selection on cotyledon number after correcting for correlations with the two covariates, analogous to a selection gradient analysis. These analyses were carried out using JMP (SAS, 2002).

To test for additive genetic variance for cotyledon number we used Proc Mixed in SAS, using restricted maximum likelihood (REML) estimation methods (Littell *et al.*, 1996). Cotyledon category was the dependent variable, and Sire and Dam nested within Sire were the random predictors. Significance was tested using the one-tailed likelihood-ratio chi-square test for variance components (Littell *et al.*, 1996, page 44), which is robust to departures from normality (Roff, 1997; T. Mackay, pers. comm.).

Results

Of the 2014 seeds planted, 1857 (92%) germinated and 89 (4.8%) of the germinated seedlings had an abnormal number of cotyledons ranging from 0 to 4. Most of these (79) had less than two cotyledons whereas only 10 had greater than two. The simple ANOVA testing for total selection on cotyledon number was highly significant. Plants with less than two cotyledons had much lower fitness than plants with two or more cotyledons (Fig. 2a), and although this difference was highly significant ($F_{2,1854} = 8.2$, $P < 0.001$), cotyledon number explained only 0.9% of the variance in relative fitness. Plants with fewer than two cotyledons germinated from significantly lighter seeds and germinated significantly later (Fig. 2b) than plants with two or more cotyledons. Some, but not all, of the selection against plants with fewer than two cotyledons is indirect, caused by the relationships between cotyledon number and these other two traits. When the other two traits are included in the

Fig. 2 Relationships between cotyledon number and other traits; shown are mean ± 1 SE. (a) Selection on cotyledon number – open bars are mean relative fitness with *P*-value from a simple ANOVA; shaded bars are least-squares mean relative fitness after correcting for the seed mass and germination time. Note that the seed weighed was the individual seed that produced the plants under study, not the seeds that the plants produced. (b) Mean seed mass and germination time for seeds producing different numbers of cotyledons; $P < 0.0001$ for both.

model, the lower fitness of plants with less than two cotyledons was less pronounced and less significant ($P = 0.047$; Fig. 2a).

There was no evidence for additive genetic variance for cotyledon number – the restricted maximum likelihood estimate for the sire variance component was zero. In contrast, the Dam variance component was highly significant ($\chi^2 = 19.3$, 1 d.f., one-tailed $P < 0.0001$). The lack of any Sire effect combined with a large Dam effect is strong evidence for maternal environmental or nonadditive genetic effects on cotyledon number. In a separate

greenhouse study, we attempted unsuccessfully to artificially select for increased cotyledon number. Of 996 plants, the four that had three cotyledons were crossed among themselves. However, of 93 offspring that germinated, all had two cotyledons. Although the sample sizes here are small, these results also suggest a lack of additive variance for cotyledon number in *R. raphanistrum*.

Discussion

Although naturalists, quantitative geneticists, and developmental biologists have long noted that plants frequently have abnormal numbers of cotyledons, this phenomenon is poorly understood. Our results strongly suggest that cotyledon numbers less than two are selected against. Some of this selection is indirect, caused by correlations with seed mass and emergence time, but there was still significant selection after these correlations were accounted for. Whether the remaining selection against cotyledon numbers less than two is direct selection or indirect selection through some unmeasured trait(s) is unknown. Although cotyledon number explained slightly <1% of the variance in relative fitness, over a number of generations this selection could deplete additive variance and/or create canalization in cotyledon number.

We had little power to test for stabilizing selection around an optimum of two cotyledons, given the very low numbers of plants with greater than two cotyledons (10 of 1857). It seems that extremely large sample sizes are needed to obtain reasonable numbers of plants with more than two cotyledons to rigorously test the selective hypothesis. Stressful environments could be used in an attempt to increase variation in cotyledon number, but it might be difficult to control for the effects of stress on other fitness-related traits. Another possibility would be to somehow manipulate cotyledon number to determine its effect on fitness. Perhaps genetic or developmental manipulation could produce larger numbers of plants with abnormal cotyledon number, but again, it might be difficult to isolate this trait from others that would be affected by such manipulation.

Our half-sibling field study produced no evidence for additive genetic variance for cotyledon number, in contrast to earlier artificial selection studies that did report evidence for additive variance (Holtorp, 1944; Haskell, 1949, 1954; Dessureaux, 1967). Artificial selection is a more powerful approach than sibling analysis for uncovering additive variance, particularly for traits such as pleiocotyly for which there is little phenotypic variance. Therefore, more rigorous artificial selection studies on pleiocotyly are needed to resolve this discrepancy and to determine whether the lack of variance in cotyledon number is because of a lack of genetic variation. Given our attempt to artificially select on cotyledon number, it is clear that these kinds of studies will require very large initial sample sizes and perhaps stress to increase the numbers of plants with more than two cotyledons.

If stress did produce increased numbers of plants with cotyledon numbers other than two, and artificial selection applied to the stressed plants caused a genetic change such that the frequency of abnormal cotyledon numbers was increased in the absence of stress, then this would be an example of genetic assimilation (Waddington, 1957). If artificial selection for abnormal cotyledon numbers was successful, then these plants with expanded phenotypic variation could be used for more powerful field tests for stabilizing selection on cotyledon number. Artificial selection would also be the best way to examine constraints on cotyledon number because of genetic correlations, because unfavourable correlated responses to the selection on cotyledon number should be detectable (Conner, 2003).

A better understanding of developmental mechanisms may also shed light on the lack of variation in cotyledon number. Holtorp (1944), working with *Brassica*, suggested that tricotyledons can be produced by fission, twinning, or supplementation. Fission and twinning are the result of one of the normal cotyledons being divided or copied to produce a total of three. Conversely, supplementation refers to the first true leaf being produced in cotyledonary form, with structure, venation, and shape being largely indistinguishable from true cotyledons. Supplementation seems to be caused by heterochrony, that is, changes in the timing of developmental events (Gupta & Jain, 1980; Conway & Poethig, 1997; Fernandez, 1997). These distinct developmental mechanisms for production of tricotyledons imply that there are multiple mechanisms of canalization for cotyledon number.

In conclusion, our results provide some support for both a lack of genetic variation and selection as reasons for the current canalization of cotyledon number in wild radish. Additional studies measuring natural selection on cotyledon number and rigorously selecting for increased and decreased cotyledon number, using very large sample sizes, stress to increase variation, or experimental manipulation of cotyledon number, are necessary to shed further light on the causes of canalization of this trait.

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