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Ecological selection maintains cytonuclear incompatibilities in hybridizing sunflowers

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Abstract

Despite the recent renaissance in studies of ecological speciation, the connection between ecological selection and the evolution of reproductive isolation remains tenuous. We tested whether habitat adaptation of cytoplasmic genomes contributes to the maintenance of reproductive barriers in hybridizing sunflower species, *Helianthus annuus* and *Helianthus petiolaris*. We transplanted genotypes of the parental species, reciprocal F1 hybrids and all eight possible backcross combinations of nuclear and cytoplasmic genomes into the contrasting xeric and mesic habitats of the parental species. Analysis of survivorship across two growing seasons revealed that the parental species' cytoplasm was strongly locally adapted and that cytonuclear interactions (CNIs) significantly affected the fitness and architecture of hybrid plants. A significant fraction of the CNIs have transgenerational effects, perhaps due to divergence in imprinting patterns. Our results suggest a common means by which ecological selection may contribute to speciation and have significant implications for the persistence of hybridizing species.

Keywords

Cytoplasm; ecological divergence; ecological speciation; habitat adaptation; hybridization; natural selection; reciprocal transplant; reproductive isolation

INTRODUCTION

The last decade has seen a resurgence of interest and study of ecological speciation. New models of ecological speciation have been proposed (Dieckmann & Doebeli 1999; Gavrillets & Vose 2007), explicit tests have been developed to distinguish ecological speciation from other speciation modes (Rundle 2002) and considerable empirical evidence for ecological speciation has accumulated (Rundle 2002; Funk *et al.* 2006). Nonetheless, this recent focus on

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ecological speciation has been tempered by reports of the fragility of ecological reproductive barriers (Seehausen 2004) and by increasing evidence of the importance of genetic conflict (as opposed to ecological selection) in the evolution of intrinsic reproductive barriers (Orr *et al.* 2007). Because intrinsic reproductive barriers typically result from higher-order epistatic interactions, they may be more stable than ecological reproductive barriers, which can break down as environments change (Price 2007). Although intrinsic and ecological reproductive barriers are often presented as alternate hypotheses, both forms of isolation often likely contribute to speciation.

Genetic analyses indicate that both nuclear–nuclear and cytonuclear interactions (CNIs) may contribute to hybrid incompatibilities. Most studies have focused on the former as the number of possible interactions among nuclear genes is vast. However, CNIs have received considerable attention recently because of (i) frequent reports of asymmetric post-mating isolation (Tiffin *et al.* 2001), and (ii) increased recognition that conflicts between nuclear and cytoplasmic genes may lead to the rapid evolution of cytonuclear incompatibilities (Wade & Goodnight 2006). For example, many agricultural crops express cytoplasmic male sterility because of failed interactions between mitochondrial and nuclear genes (Chase 2007), hybrids between two species from the plant genus *Mimulus* have deformed anthers due to maladaptive CNIs (Fishman & Willis 2006), and CNIs are responsible for hybrid breakdown and reproductive isolation in interpopulation hybrids of the copepod *Tigriopus californicus* (Burton *et al.* 2006). In sunflowers, which are the focus of the present study, plant weakness, delayed maturity, reduced seed weight and/or pollen inviability have been reported to result from the replacement of the common sunflower's (*Helianthus annuus* L.) cytoplasm by the cytoplasm of *Helianthus petiolaris* Nutt., *Helianthus mollis* Lam., *Helianthus maximilliani* Schrader, *Helianthus divaricatus* L., *Helianthus angustifolius* L. or *Helianthus grosserratus* Martens (Levin 2003). Based on these observations, Levin (2003) argued that maladaptive CNIs may be the earliest kind of intrinsic post-zygotic barrier to arise and may sometimes initiate speciation.

Reciprocal transplants using controlled crosses are powerful tools for elucidating the relationship between ecological and genetic factors that contribute to reproductive isolation. If interspecific ecological barriers dominate, hybrid fitness is contingent on the habitat into which they are planted, with performance depending on the proportion of locally adapted alleles. Alternatively, if intrinsic genetic barriers dominate, then hybrid fitness will be independent of habitat (Rundle & Whitlock 2001). To study the contribution of CNIs to both intrinsic and extrinsic reproductive isolation, we conducted reciprocal transplants of first generation and backcross hybrids between the naturally hybridizing sunflowers, *H. annuus* and *H. petiolaris* that represent all possible interspecific combinations of nuclear and cytoplasmic genomes (Table 1).

Helianthus annuus and *H. petiolaris* constitute an excellent study system to assess the relationship between ecological and intrinsic genetic factors in speciation. They inhabit contrasting and contiguous habitats across North America, with *H. petiolaris* found in dry sandy soils, and *H. annuus* in wetter, clay-based soils (Heiser 1947). The species have overlapping flowering times and are visited by the same pollinators (Rieseberg *et al.* 1998). The opportunities for hybridization are immense, and sympatric populations of the species share alleles at many loci despite having diverged more c. 1 million years bp (Yatabe *et al.* 2007). Pollen sterility in early generation hybrids appears to be the strongest reproductive barrier between them, apparently due to underdominant effects caused by chromosomal rearrangements and linked Bateson–Dobzhansky–Muller (BDM) incompatibilities (Lai *et al.* 2005). However, other experiments have shown that the two species are differentially adapted to salt and drought conditions, suggesting that ecological selection could also play a major role in reproductive isolation (Gross *et al.* 2004; Karrenberg *et al.* 2006). In addition to abiotic

tolerances, these species are distinguishable in many morphological features and this distinction remains across their geographic ranges (Rosenthal *et al.* 2002). The consistent interspecific differences seen in ecological adaptation and morphology, combined with observations of introgression at many loci across the genome, suggests that interspecific gene flow is only strongly impeded near loci underlying local adaptation and plant architecture, or near chromosomal breakpoints (Yatabe *et al.* 2007).

We planted several thousand seedlings (2006) or seeds (2007) of the eight possible backcross combinations of nuclear and cytoplasmic genomes, as well as the two parental populations, and reciprocal F₁ hybrids (2007 only) into replicated xeric and mesic sites inhabited by the two parental species, and measured a suite of fitness and plant architecture-related traits. We asked whether species-specific cytoplasms could affect both fitness and architectural differences between *H. annuus* and *H. petiolaris*. Our results indicate that the cytoplasms characteristic of these species are not only locally adapted, but also contribute to species-specific plant architectures and maladaptive CNIs in hybrids. Thus, in sunflowers, ecological selection on cytoplasmic variation plays a clear role in maintaining species-specific ecological and morphological differences despite hybridization. Ecological interactions with organelles could prove to be an important driver of speciation.

MATERIALS AND METHODS

Crosses

Achenes (one-seeded fruits) of *H. annuus* and *H. petiolaris* were collected from natural populations along Hwy 91, c. 9 and 12 km south of Santa Rosa, New Mexico respectively. Reciprocal F₁ hybrids were generated in the Indiana University greenhouse by crossing 172 individuals from 75 families of *H. annuus* with 184 individuals from 79 families of *H. petiolaris*. Sixty-three F₁s (30 *F1a* and 33 *F1p*) were reciprocally crossed with 50–60 plants of either parental species to generate 238 backcross families, which were sorted into eight backcross populations that differ in the combination of ovule and pollen parents. This reciprocal backcross design controls for maternal environment and endosperm nuclear effects. While most families were employed in field experiments, a few families were too small to be useful. Table 1 reports the number of families, per cross, used in this experiment.

2006 Field experiment

We carried out our field experiments at the Sevilleta Wildlife Refuge near Socorro, New Mexico, where we chose two xeric and two mesic sites that were previously inhabited by *H. petiolaris* and *H. annuus* respectively. On 14 March 2006, we scarified c. 3250 seeds from the 238 backcross and 111 parental species' families and placed them on damp filter paper. Seed coats were removed 2 days later, and seeds were planted in expandable peat pellets (72Ct by Jiffy Products®, Jiffy International Lorain, OH, USA) and propagated in greenhouses located at the Sevilleta Wild Life Refuge until most of them reached a two true-leaf stage. To account for variation in germination time (developmental stage hereafter), we qualitatively scored each seedling before transplanting, noting if they had two or four true leaves. We transplanted 2720 seedlings representing the parental and all backcross types (Table 1) into the two xeric and the two mesic sites (12 × 5 m each). Because natural germination in 2006 was minimal in the xeric habitat, sites were chosen based on the presence of dead *H. petiolaris* plants from the previous season. Each site was fenced to prevent herbivory and contained five blocks, each with 136 seedlings. Seedlings were planted 15 cm apart from each other, tagged and mapped.

Sites were irrigated with deionized water for 5 consecutive days after planting. We continued to irrigate the xeric sites three times per week during the first half of June because otherwise all plants would have died immediately; 2006 was an unusually dry year, and the soil had failed

to accumulate moisture during the winter. We reduced the frequency of irrigation to twice weekly in the second half of June 2006, and to once a week in July until the arrival of the monsoon season. At this point, we halted irrigation completely.

Measurements

Survivorship was scored every other day, starting immediately after the initial five irrigations, and continuing until plants flowered. In October, 2006, surviving plants were harvested and measured for the following traits: plant dry weight, number of nodes to the apical head, branch number, number of heads and mean head diameter. We also measured head diameter in terms of the number of seed insertions across the head. Estimated fecundity was estimated as the product of number of heads by seed-insertion area ($1/4 \times \pi \times \text{head diameter squared}$). Note that estimated fecundity may not be equivalent to reproductive fitness because the backcross populations segregate for chromosomal rearrangements and linked BDM incompatibilities that affect fertility (Lai *et al.* 2005).

Survivorship analyses

Using the 2006 experiment data, we first asked whether progeny from the different genotypic classes, including both parental and backcross populations, varied in survivorship across the two different habitats (Fig. 1). A linear model was employed to test for the effects of genotypic class, habitat and genotypic class $\times$ habitat interactions on the log days to mortality for each individual, with families nested within genotypic class and blocks nested within habitats as random effects. The 205 plants that survived to reproduction were excluded to eliminate the differences in survival times between lines due solely to differences in flowering time. This analysis was performed in *JMP* (SAS 2000).

Next, we asked whether survivorship (days to mortality) among backcross families was affected by the cytoplasm (*annuus* vs. *petiolaris*) of the ovule parent, nuclear genome composition (1/4 or 3/4 *annuus*), habitat and the interactions among these factors. After removing the 205 surviving individuals from the data set, we confirmed that the mean was very close to the variance and used a Poisson model with a log-link function; the Poisson model becomes more attractive in this case, in which residuals do not fit a normal distribution. We first analysed the whole data set, with each individual treated as a data point. We then performed a second analysis to account for possible imbalances caused by variation in the number of individuals per family. In this analysis, we removed families with fewer than five individuals and randomly sampled 15 individuals from families with more than 15 individuals. Both analyses were performed using PROC Glimmix in SAS 9.1 (SAS 2002), with block as a random effect nested within habitat.

Analyses of estimated fecundity and total fitness

Only two plants survived to reproduction in the xeric habitat, so total fitness (survivorship $\times$ estimated fecundity) was analysed for plants in the mesic habitat only, where more than 200 plants reproduced. Dead individuals were taken into account and scored with zero fecundity. Over-dispersion of the data precluded the use of a regular linear model or a Poisson generalized linear model. Therefore, we employed a generalized linear model with a negative binomial family. The parameter θ , which relates the mean and variance, was visually estimated using the equation $\text{Var} = \text{mean} + \text{mean}^2/\theta$, as in Venables & Ripley (2002), and included in the model to reduce the over-dispersion effect.

We initially asked whether genotypic class, including both parental and backcross populations (Table 1), affected total fitness, using the developmental stage of transplanted seedlings as a covariate. We fitted a negative binomial model to our data using the *r* package glm function (R_Development_Core_Team 2008) and iterated the initial estimated value of $\theta = 0.012$ until

the residual deviance was closest to the residual degrees of freedom ($\theta = 0.074$) and the dispersion parameter was close to one.

We then tested the following specific hypotheses: (i) heterospecific CNIs are maladaptive; and (ii) nuclear genes are affected by the cytoplasm of the pollen parent, and become incompatible with ovules carrying the alternative cytoplasm. Hypothesis 1 was evaluated by comparing backcross populations that differ only in terms of the cytoplasm for the ovule parent: BCA2 vs. BCA3 and BCP2 vs. BCP3, whereas hypothesis 2 was assessed by comparing backcross populations that differ only in the cytoplasm of the pollen parent: BCA1 vs. BCA4 and BCP1 vs. BCP4. Comparisons were made using the same negative binomial model approach described above: a new θ was estimated as before and P -values were obtained for between-cross t -tests.

To evaluate the relative contributions of survivorship and estimated fecundity to variation in total fitness, we repeated these comparisons with estimated fecundity only, using a negative binomial model as before. Results were graphically displayed by plotting the log of total fitness (Fig. 2) and log of expected fecundity (Fig. 3) for the relevant backcross populations using the estimates provided by the R 's `predict.glm` function. Results were confirmed using an ANOVA approach with 5000 permutations.

Morphological analyses

MANOVAS were performed to test for maternal and paternal cytoplasmic effects on morphological traits associated with differences between *H. annuus* and *H. petiolaris* in plant architecture (Heiser 1947), with plant dry weight, plant height/number of nodes, plant height/number of nodes (as a measure of mean internode length), average disk diameter, and the number of heads as response variables and cross-type as the independent variable. Because these species occupy contrasting habitats, phenotypic differences between them may also be involved in ecological adaptation (Gross *et al.* 2004). Usually *H. petiolaris* plants are short with many small heads, whereas *H. annuus* plants tend to be taller and larger with a variable number of larger heads (Fig. S1). The number of nodes and internode length define the plant height. Because flower heads are at the ends of branches, head number is both an important fitness component and a surrogate for branching architecture. Likewise stem diameter is an excellent predictor of plant biomass: $r^2 = 0.8$ between stem diameter and above-ground dry weight. Prior to the MANOVAS, a univariate linear model was employed to analyse the residuals of each trait. Based on this analysis, data were Box–Cox-transformed as necessary to conform to the parametric assumptions of the MANOVA (see Item S1, Supporting Information). Likewise, phenotypic correlations were calculated, and a single trait was arbitrarily chosen to represent pairs of highly correlated traits ($r > 0.8$) to minimize collinearity. To test for paternal cytoplasmic effects, we first compared all relevant backcross populations (BCA1, BCA4, BCP1 and BCP4), and then performed the appropriate contrasts (BCA1 vs. BCA4 and BCP1 vs. BCP4). We used the same approach to test for the maternal cytoplasmic effects using backcross families BCA2, BCA3, BCP2 and BCP3. We excluded block effects from both analyses because not all genotypic classes were represented in each block. A canonical correlation table was generated to identify the traits that contributed most strongly to significant MANOVA effects. A univariate comparison for the traits used in the MANOVA among all backcrosses and paternal genotypes is provided graphically in Fig. S1.

2007 Field experiment

Results from the 2006 experiment implied that germination stage might play a critical role in seedling establishment and local adaptation (see *Results and Discussion*). Therefore, we repeated the early stages of the 2006 experiment, except that (i) pre-germinated seeds were planted directly into the natural sites rather than into peat pellets and (ii) reciprocal F1 hybrids

were included in the 2007 experiment. In all, 2880 seeds representing 12 different genotypic classes (two parental, two F1 and eight backcross populations) were planted. The total number of seeds per genotypic class varied from 238 to 242. In each habitat type, we haphazardly chose 15 different sites where 96 seeds were planted in a grid of eight rows and 12 columns with a spacing of 15 cm between seeds. Seeds were planted on 20–21 March 2007. Because of abundant rain, the sites were watered only twice – just before planting and 1 week later. The presence and absence of seedlings was monitored three times a week until the last week of April 2007. To test for maternal cytoplasmic effects on seedling establishment, we performed a logistic regression of presence (1) or absence (0) of seedlings for cytoplasm, habitat and cytoplasm by habitat interaction. Parental and F1 genotypes were excluded from the analyses to avoid confounding cytoplasmic effects with maternal nuclear or maternal environmental effects. Families were nested within cytoplasm and blocks nested within habitat as random effects. Analyses were performed using PROC Glimmix in SAS 9.1 (SAS 2002).

RESULTS

Survivorship

Analyses of the survivorship of 2720 seedlings (2006) and 2880 seeds (2007) revealed that all classes of genotypes left more survivors in the mesic than in the xeric habitat in both growing seasons ($P < 0.0001$ each year). Both genotypic class ($P = 0.02$) and genotypic class by habitat interaction ($P = 0.03$) had a significant effect on survivorship (measured as time to death) in 2006. In addition, there was a significant interaction between the cytoplasm of the ovule parent and habitat in both 2006 and 2007 when tested along with fraction of *H. annuus* in the nuclear genome (Fig. 1; Table 2). In both years, the *H. annuus* cytoplasm significantly increased survivorship in the mesic habitat. Likewise, the *H. petiolaris* cytoplasm was favoured in the xeric habitat in both years (Table 2), although significance was lost in 2006 if a balanced design was employed, in which small families were removed from the analysis and the number of genotypes from the remaining families was equalized (see *Materials and methods*). This pattern indicates that the cytoplasm is adapted to the local habitat, and that selection for the locally adapted cytoplasm is possibly stronger in the mesic habitat. A significant interaction was found between fraction of *H. annuus* in the nuclear genome and habitat in the 2006 experiment ($P = 0.051$), implying local adaptation of nuclear genes as well.

Fitness

Our analyses of total fitness (i.e. survivorship $\times$ estimated fecundity) were restricted to the 2006 data set and to the mesic habitat because few plants survived to reproduction in the xeric habitat. Although the parental species did not differ significantly in total fitness (Item S2, Supporting Information), differences were found among backcross populations (Fig. 2a). In particular, large differences in fitness were found between backcross populations that vary only in the cytoplasm of the pollen parent (BCA1 vs. BCA4 and BCP1 vs. BCP4; Table 1, Fig. 2a). Because cytoplasmic genomes are strictly maternally inherited in crosses between *H. annuus* and *H. petiolaris* (Rieseberg *et al.* 1994), we refer to the paternal cytoplasmic effects as ‘transgenerational effects’.

Fitness differences were also found between backcross populations that varied only in the cytoplasm of the ovule parent (BCA2 vs. BCA3 and BCP2 vs. BCP3; Table 1). We predicted that backcrosses in the direction of the maternal parent would have greater total fitness than backcross plants with a mismatch between nuclear and cytoplasmic genomes. This is the pattern observed for backcrosses towards *H. annuus* (Fig. 2b). However, no differences in fitness were observed in backcrosses towards *H. petiolaris* (Fig. 2b).

When we repeated the analysis for estimated fecundity rather than total fitness, the results were similar, but weaker (Fig. 3), indicating that overall fitness differences, which include the habitat advantage of the *H. annuus* cytoplasm, have a strong survivorship component.

Plant architecture

MANOVA revealed significant differences in plant architecture among the four backcross populations tested for paternal cytoplasmic effects: BCA1, BCA4, BCP1 and BCP4 ($P < 0.0001$; Fig. 4). While the BCA1 vs. BCA4 populations differed strongly in plant architecture for essentially all measured traits ($P = 0.001$; Fig. 4), the BCP1 vs. BCP4 populations differed only in the number of flowering heads (Fig. 4b). Canonical correlation analyses indicated that disk head diameter correlated with the first canonical axis ($r = 0.85$), number of heads (Box-Cox-transformed) and stem diameter correlated with the second canonical axis ($r = 0.84$ and $r = 0.67$ respectively), and the number of nodes correlated with the third canonical axis ($r = 0.85$). Other traits exhibited smaller correlations with canonical axes ($r < 0.43$). In contrast to the strong effects of the paternal cytoplasm on plant architecture, MANOVA failed to detect significant architectural differences among the four backcross populations (BCA2, BCA3, BCP2 and BCP3) tested for maternal cytoplasmic effects. Interestingly, only average head diameter and head number differed significantly between parental populations of *H. petiolaris* and *H. annuus*, and the means of the backcross populations were often equivalent to or transgressive when compared with the parental populations, rather than intermediate as expected (Fig. S1).

DISCUSSION

Cytonuclear interactions may play a special role in the evolution of reproductive isolation for several reasons. As alluded to earlier, nuclear and cytoplasmic loci in separate species are likely to diverge because of intergenomic conflict and (possibly) genetic drift. If cytoplasmic loci were involved in local adaptation, the linkage of cytoplasmic genes would create associations between genes involved in ecological divergence and those that contribute to maladaptive CNIs in hybrids. These associations may facilitate the origin and maintenance of species differences in the presence of gene flow.

The results from the large-scale transplant experiments presented here support the importance of CNIs in preserving species differences by demonstrating that (i) the cytoplasm of the two sunflower species are locally adapted; (ii) maladaptive CNIs affect multiple components of fitness in hybrids and may have transgenerational effects and (iii) CNIs affect architectural traits that differentiate the two species. Combined with previous studies of introgression between the two species (Yatabe *et al.* 2007), our results imply that ecological selection on cytoplasm should limit their introgression, as well as the introgression of nuclear alleles they interact with or modify. This process should maintain the species' phenotypic identities despite gene flow.

The cause(s) of transgenerational effects in *Helianthus*

A possible explanation for the appearance of transgenerational effects is transmission ratio distortion (TRD), in which nuclear alleles that are compatible with the cytoplasm are favoured. There is evidence for TRD resulting from cytonuclear interactions in crosses involving *H. annuus* and more distant species (Faure *et al.* 2002). However, this explanation seems inconsistent with the phenotypic patterns observed, in which the architectural traits of the paternal grandmother are recovered for some BC populations, despite having a complete copy of the nuclear genome from the alternate species (Fig. S1).

An alternative hypothesis is that epigenetic changes are altering fitness and morphology. Under this hypothesis, cytoplasmic alterations of nuclear expression – perhaps through the imprinting of multiple loci – contribute to both fitness and plant architecture. At this time, we have no data on methylation or acetylation to directly address the imprinting hypothesis. Nonetheless, several lines of evidence suggest that genomic imprinting is a plausible hypothesis. Genomic imprinting appears to be the most common cause of parent-of-origin effects in mammals and flowering plants (Tilghman 1999; Scott & Spielman 2006). Also, as discussed above, BC plants often are similar to one or the other parental species in architectural traits (Fig. S1) rather than intermediate as predicted on the basis of nuclear genome composition. Imprinting patterns have been hypothesized to evolve due to conflicts of interest between parents (Haig 1997) or to increase the integration of offspring and parental genomes (Wolf & Hager 2006). Species may diverge in their imprinting patterns, leading to incompatibilities in hybrids (Josefsson *et al.* 2006; Kinoshita 2007). We are unaware of other putative examples in which cytoplasmic epigenetically modify nuclear genes. In most other cases, such as hybrid dysgenesis in *Drosophila* (e.g. Haley *et al.* 2005), nuclear factors with maternal effects appear to underlie the differential behaviour of alternative cytotypes. Note that while our experiment was designed to control for maternal nuclear effects, if imprinting was disrupted in interspecific sunflower hybrids as reported for *Arabidopsis* (Josefsson *et al.* 2006), we could not distinguish between cytoplasmic and nuclear epigenetic effects. That is, disruption of imprinting in the F1s could potentially leave intact imprinting from the parental generation.

Local adaptation of the cytoplasm

Is local adaptation of the cytoplasm common? Unfortunately, there are only a handful of explicit tests using a reciprocal transplant backcross design that controls for maternal environment and endosperm nuclear effects. Galloway & Fenster (2001) reported weak local adaptation of the cytoplasm of an annual legume, whereas no evidence for local cytoplasmic adaptation was observed in the gynodioecious species, *Thymus vulgaris* (Gigord *et al.* 1998). However, the cytoplasm frequently affects phenotypic differences that are likely to be associated with fitness (Levin 2003), mitochondrial haplotypes are known to differentially affect fitness in both animals (Ballard & Whitlock 2004) and plants (McCauley & Olson 2003), and strong maternal effects on fitness are often reported in hybrids (e.g. Burgess & Husband 2004; Campbell & Waser 2007), many of which may be caused by cytoplasmic differences. Thus, while we suspect that local cytoplasmic adaptation is common, this remains to be proven.

Cytoplasmic genes could contribute to ecological adaptation in various ways. For instance, the chloroplast genome houses many of the genes required for photosynthesis, including the large subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco; EC 4.1.1.39), which incorporates CO₂ into plants during photosynthesis (note: the small subunit of Rubisco is nuclear, which could lead to the formation of CNIs in hybrids). Variation in Rubisco can have large effects on photosynthetic rates and on the efficiency of photosynthesis under temperature or drought stress (Andersson & Backlund 2008). Adaptive changes in the large subunit of Rubisco have been associated with the evolution of photosynthetic performance and colonization of new habitats in the Hawaiian endemic genus *Schiedea* (Kapralov & Filatov 2006). In *Ipomopsis*, cytoplasmic genes (probably chloroplast genes) have a significant effect on stomatal conductance and photosynthetic rates in reciprocal F₂ hybrids of *Ipomopsis aggregata* and *Ipomopsis tenuituba* (Wu & Campbell 2007) and likely contribute to differential drought adaptation of the parental species. Similar arguments can be made for the mitochondrial genome, which contains many of the key genes required for respiration. In *Drosophila simulans*, for example, mitochondrial haplotypes vary in development rate, activity levels and survivorship, and these life-history trait differences influence the geographic subdivision of the species (James & Ballard 2003).

In the sunflowers studied here, we speculate that changes in the chloroplast genome may contribute to ecological differentiation (i.e. drought adaptation), whereas mismatches between mitochondrial and nuclear genes are responsible for CNIs. Respiration defects caused by CNIs are common and can affect many aspects of plant development and fertility (Levin 2003; Chase 2007). Under this scenario, the strict maternal inheritance of cytoplasmic genomes in crosses between *H. annuus* and *H. petiolaris* maintains associations between ecologically selected cytoplasmic differences and deleterious CNIs.

Cytoplasmic Introgression

Our argument for a special role of the cytoplasm in speciation appears to run counter to widespread reports of cytoplasmic introgression in plants (e.g. Rieseberg *et al.* 1991; Belahbib *et al.* 2001) and animals (Ballard & Whitlock 2004). Cytoplasmic introgression has even been reported in the two sunflower species that are the focus of the present study (Rieseberg *et al.* 1991; Dorado *et al.* 1992). However, lineage sorting is probably responsible for some putative examples of cytoplasmic introgression. Also, recent descriptions of genome-wide patterns of introgression in a wide range of plants and animals indicate that the introgression of nuclear markers is more pervasive than previously realized (reviewed in Baack & Rieseberg 2007). For example, cpDNA variation shows greater species-specificity in *H. annuus* and *H. petiolaris* than does variation at most nuclear loci (Welch & Rieseberg 2002; Yatabe *et al.* 2007). Thus, it may be that the extent of cytoplasmic introgression (relative to nuclear introgression) has been overestimated. Also, seed dispersal is generally an order of magnitude lower than pollen dispersal (Petit *et al.* 2005), so even weak divergent natural selection on cytoplasmic genomes should be sufficient to drive local adaptation. More generally, the evidence for local cytoplasmic adaptation in this experiment suggests a need to re-examine the patterns of cytoplasmic variation in the context of ecology. A major question is whether our results are common in other plant and animal species, even in other sunflower species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cytoplasm by habitat interaction for survivorship. (a) Survivorship of individuals is expressed as the mean $\pm$ SE of the natural log of days to mortality of individuals sharing the same cytoplasm in each habitat based on the full data set from the 2006 experiment. Numbers displayed beside each square or diamond indicate the untransformed mean of days to mortality and, in parentheses, the percentage of the individuals that did not survive to reproduce and were thus included in the analysis. (b) Logit of the survival probability of individuals sharing the same cytoplasm in each habitat from the 2007 experiment.

Figure 2.

Total fitness of backcross populations. (a) Comparison of BC populations that differ only in the cytoplasm of the pollen parent (BCA1 vs. BCA4 and BCP1 vs. BCP4). (b) Comparison of BC populations that differ only in the cytoplasm of the ovule parent (BCA2 vs. BCA3 and BCP2 vs. BCP3). Total fitness is expressed as the transformed mean $\pm$ SE of survivorship $\times$ estimated fecundity for families from the same BC population. Pollen parent as shown in Table 1.

Figure 3.

Estimated fecundity of backcross populations. (a) Comparison of BC populations that differ only in the cytoplasm of the pollen parent (BCA1 vs. BCA4 and BCP1 vs. BCP4). (b) Comparison of BC populations that differ only in the cytoplasm of the ovule parent (BCA2 vs. BCA3 and BCP2 vs. BCP3). Estimated fecundity is expressed as the transformed mean $\pm$ SE of number of heads by seed-insertion area for surviving individuals from the same BC population. Pollen parent as shown in Table 1.

Figure 4. Comparison of plant architecture in backcross plants differing in paternal cytoplasm. Number of heads are Box–Cox-transformed values (see Supporting Information).

Table 1

Parental (*Helianthus annuus* and *Helianthus petiolaris*) and backcross (BCP1–4, BCA1–4) populations employed in field experiment (bold)

Pollen parent	Ovule parent			
	<i>Helianthus annuus</i>	<i>Helianthus petiolaris</i>	F1 female ann (F1a)	F1 female pet (F1p)
<i>H. annuus</i>	ANN 54 Families <i>n</i> = 172	<i>F1</i>	BCA2 15 Families <i>n</i> = 330	BCA3 36 Families <i>n</i> = 288
<i>H. petiolaris</i>	<i>F1</i>	PET 57 Families <i>n</i> = 192	BCP2 20 Families <i>n</i> = 110	BCP3 52 Families <i>n</i> = 238
F1 female ann (F1a)	BCA1 17 Families <i>n</i> = 488	BCP1 30 Families <i>n</i> = 102	<i>F2 self</i>	<i>F2 outcross</i>
F1 female pet (F1p)	BCA4 28 Families <i>n</i> = 334	BCP4 40 Families <i>n</i> = 466	<i>F2 outcross</i>	<i>F2 self</i>

BCA populations result from backcrosses in the direction of *H. annuus*, whereas BCP populations result from backcrosses in the direction of *H. petiolaris*. Text in italics represent F1 or F2 genotypic classes not used in the 2006 experiment (F1s were included in the 2007 experiments; see text). The number of families and the total number of individuals used in the 2006 experiment are provided for each genotypic class.

Table 2*P*-values for survivorship analyses in 2006 and 2007

Factor	2006 (Poisson model)		2007 (Ordinal logistic) <i>n</i> = 1922
	Whole data (<i>n</i> = 2164)	Balanced design (<i>n</i> = 1323)	
Cytoplasm by habitat interaction	0.0001	0.0003	0.0007
Ann vs. pet/xeric habitat	0.0005	0.15	0.03
Ann vs. pet/mesic habitat	< 0.0001	0.003	0.007