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LEAF PHYSIOLOGY REFLECTS ENVIRONMENTAL DIFFERENCES AND CYTOPLASMIC BACKGROUND IN *IPOMOPSIS* (POLEMONIACEAE) HYBRIDS[1]

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Natural hybridization can produce individuals that vary widely in fitness, depending upon the performance of particular genotypes in a given environment. In a hybrid zone with habitat heterogeneity, differences in physiological responses to abiotic conditions could influence the fitness and spatial distribution of hybrids and parental species. This study compared gas exchange physiology of *Ipomopsis aggregata*, *I. tenuituba*, and their natural hybrids in situ and assessed whether physiological differences were consistent with their native environmental conditions. We also produced reciprocal F2s in a greenhouse study to test for cytonuclear effects on water-use efficiency (WUE). The relative performance of natural hybrids and parentals was consistent with their native habitats: *I. aggregata* at the coolest, wettest locations had the lowest WUE, while hybrids from the most xeric sites had the highest WUE. In hybrids, the mechanism by which both natural and experimental hybrids achieved this high WUE depended on cytotype: those with *I. tenuituba* cytoplasm had reduced transpiration, while those with *I. aggregata* cytoplasm had an increased photosynthetic rate, consistent with patterns in the cytoplasmic parent. The high WUE in hybrids may contribute to their high survival in the dry center of the natural hybrid zone, consistent with environment-dependent models of hybrid zone dynamics.

Key words: cytoplasmic effects; hybridization; *Ipomopsis*; physiological performance; Rocky Mountains; water-use efficiency.

Natural hybridization is recognized as a significant creative force in the evolution of many species complexes, particularly within the flowering plants (Anderson, 1949; Grant, 1981; Rieseberg and Wendel, 1993). Hybridization can produce a range of new recombinant genotypes, whose phenotypes may resemble that of one parent, be intermediate, or be novel relative to both parents (Arnold and Hodges, 1995). An extensive body of research has addressed the relative fitness of hybrids and their parental taxa, with a primary goal of identifying the evolutionary consequences of hybridization (reviews in Arnold, 1997; Rieseberg and Carney, 1998; Burke and Arnold, 2001). These consequences can include a stable hybrid zone, disappearance or reinforcement of species differences, or production of a new hybrid species (Arnold, 1997). Plant studies have typically estimated fitness components (e.g., survival and seed production) or have examined morphological characters correlated to fitness (e.g., size and shape of vegetative and floral structures, biomass). Only recently has variation in physiological performance been examined in natural hybrid systems (Wang et al., 1997; Johnston et al., 2001; Schwarzbach et al., 2001; Ludwig et al., 2004).

Physiological traits that affect photosynthesis are integral to vegetative growth and thus are likely to be major determinants of fitness (Ackerly et al., 2000). Consequently, hybrids with photosynthetic physiology that differs from their parental species may be better suited to certain habitats than the parents. However, such high relative hybrid performance may not be enjoyed by all hybrid genotypes in a given hybrid system. Because photosynthetic processes require the coordination of numerous proteins encoded in the nuclear and chloroplast genomes (Keegstra and Cline, 1999; Jarvis and Robinson, 2004; Lambrides et al., 2004; Smith, 2006), the expression of these traits in recombinant hybrids may be influenced by both their nuclear genotype and their cytoplasmic background. Evidence for cytoplasmic control of morphological traits has recently been documented in several species (Galloway and Fenster, 1999; Burgess and Husband, 2004; Hausmann et al., 2005), but little is known about cytoplasmic effects on physiological characters in natural hybrids.

Photosynthesis will also depend in part on the environment experienced by the plant. For example, water limitation can exert selection on leaf traits such as water-use efficiency (WUE; Dudley, 1996; Geber and Dawson, 1997). Plants adapted to drought generally have higher WUE, which is expected to increase drought tolerance, and thus fitness, in dry conditions (Geber and Dawson, 1990, 1997; Nilsen and Orcutt, 1996; Heschel and Riginos, 2005). If drought tolerance is critical for survival on dry sites, then populations are expected to vary in WUE in response to environments that vary spatially in water availability (Zangerl and Bazzaz, 1984; Ehleringer, 1993; Dudley, 1996). Indeed, comparisons of related taxa have found greater drought tolerance and higher WUE in taxa that have less access to soil moisture compared to those with greater moisture availability (Dudley, 1996; Williams and Ehleringer, 2000; Heschel et al., 2002; McKay et al., 2003). Similarly, if a hybrid zone occurs where there is habitat heterogeneity, then

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differences in leaf ecophysiological traits such as WUE may influence the spatial distribution of hybrids and parental species.

To test whether the relative physiological performance of hybrids and their parental species reflect differences in their natural habitats, we compared leaf-level ecophysiological traits of the parental species *Ipomopsis aggregata* (Pursh) V. Grant and *I. tenuituba* (Rydb.) V. Grant and their hybrids, with particular focus on photosynthesis and WUE. Previous work in *Ipomopsis* has demonstrated that experimentally produced parental, F1, and F2 plants differ in several physiological traits when grown in common field sites (Campbell et al., 2005), in patterns consistent with survival differences observed under those conditions (Campbell and Waser, 2001). Further, hybrid survival varies with cytoplasmic background and with environment (Campbell and Waser, 2001). Here we extended those studies by comparing the parental species and hybrids from natural populations to examine physiological traits of natural plants in their native environments and determine whether the differences observed previously in common gardens (Campbell et al., 2005) are maintained in the native environments. In addition, we tested for cytonuclear epistastic effects on these physiological traits, using experimentally produced reciprocal F2 hybrids grown in common greenhouse conditions and comparing natural hybrids with different cytoplasmic backgrounds. We asked the following specific questions: (1) How does the expression of ecophysiological traits compare for natural hybrid and parental plants growing in their natural field sites? (2) Are differences in these traits consistent with variation in abiotic factors across the hybrid zone? (3) Does cytoplasmic background influence physiological traits in recombinant *Ipomopsis* hybrids?

MATERIALS AND METHODS

Study system—*Ipomopsis aggregata* and *I. tenuituba* are found throughout the western United States, and hybridization between the species is often extensive. One such hybrid zone is located along Poverty Gulch, a montane valley draining into the Slate River Valley in Gunnison County, Colorado, that is located 10 km from the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Colorado, USA. Populations of *I. aggregata* are found on the gentle, vegetated slopes along the base of the valley up to 2900 m a.s.l., while *I. tenuituba* grows on the steep rocky slopes above 3100 m (Grant and Wilken, 1988; Campbell et al., 1997). The parental populations at the end of this hybrid zone (*I. tenuituba* populations A–C and *I. aggregata* population L in Campbell et al., 1997) are separated by 1.93 km and are linked by a series of discrete hybrid populations (D–K; Grant and Wilken, 1988; Wu and Campbell, 2005). Much of the central region of this contact zone in which natural hybrids are located is characterized by steep talus slopes with relatively low vegetative cover. Largely because of these differences in vegetation and substrate, the conditions are warmer and drier at the *I. tenuituba* sites than the *I. aggregata* sites, and the center of the hybrid zone is even warmer and drier (based on relative humidity and soil moisture) than either parental location (Campbell et al., 2005; Wu and Campbell, 2006). This suggests that the demand for water from leaves is greatest for plants in the center of the hybrid zone and lowest at the *I. aggregata* sites along the valley floor. Light intensity is relatively high at sites across the hybrid zone, typically >1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at midday (Campbell et al., 2005).

Source of natural and experimental plants—Both natural plants growing in situ and experimentally produced hybrids were included in this study. First, to examine natural variation in ecophysiology in this hybrid system, in 2003 we measured individuals from the parental species in populations past the ends of the hybrid zone (*I. aggregata* from site L and *I. tenuituba* from site C) and natural hybrids from the center of the hybrid zone (site I in Campbell et al., 1997; see Table 1).

To further explore how ecophysiology depends on the genetic composition of hybrids, we compared four genotypic classes of experimentally produced F2 hybrids: those with *I. aggregata* ($F2_A$) or *I. tenuituba* ($F2_T$) cytoplasmic background and either the presence or absence of an *I. aggregata* nuclear marker. The reciprocal F2 hybrids were generated through a series of hand-pollinations. In 2002 we reciprocally hand-pollinated 13 pairs of experimental F1 hybrids with alternate cytoplasmic backgrounds that had been maintained in pots at the RMBL (thereby producing 13 reciprocal families), which themselves were the product of crosses made in 2000 between plants from parental sites C and L. Each of these 26 F1 plants was derived from a unique set of parents, except for two F1 individuals that were full sibs. The F2 crosses produced 2630 seeds, half of which were planted in greenhouse pots for this study, with each maternal family equally represented in the planting. These greenhouse F2 hybrids were raised from seed in fiber pots, kept during the summers in WeatherPort portable mesh greenhouses (Hansen WeatherPort, Olathe, Colorado, USA) at the RMBL, and sunk into the ground to overwinter. During the summer months, plants were watered 2–3 times per week and fertilized monthly.

TABLE 1. Source of natural and experimental plants used to examine cytoplasmic effects on water-use efficiency and related traits in *Ipomopsis* from Poverty Gulch, Colorado, USA. The 12 populations in this hybrid zone, labeled A–L, include all populations along a transect separating the high-elevation *I. tenuituba* populations (A–C) from the more widespread *I. aggregata* at population L and beyond.

Plant source	Type of plants
Natural populations	
Site L	*Ipomopsis aggregata*
Site I	Natural hybrids with *I. tenuituba* cytoplasm
Site G	Natural hybrids with *I. aggregata* cytoplasm
Site C	*Ipomopsis tenuituba*
Experimental plants	
$F2_A$	Experimental F2 hybrids with *I. aggregata* cytoplasm
$F2_T$	Experimental F2 hybrids with *I. tenuituba* cytoplasm

Because we found evidence for cytoplasmic effects on some aspects of leaf ecophysiology within the experimental F2 hybrids (see Results), we also tested whether the same patterns held for natural hybrids growing in situ. This required additional field measurements to include hybrids with both cytoplasmic backgrounds. While the vast majority of the natural hybrids have *I. tenuituba* cytoplasm, including those measured for ecophysiological traits in 2003, a single population near the center of the hybrid zone (site G) appears to consist entirely of individuals with *I. aggregata* cytoplasm (Wu and Campbell, 2005). To explore whether natural hybrids from populations with different cytotypes show different physiological performance in the field, in 2004 we measured plants from hybrid population G and compared them to individuals from the nearby population I that have *I. tenuituba* cytoplasm (Table 1). We recognize that any differences found could be due to factors other than cytotype, but our main purpose was to see whether differences between these natural populations were consistent with those found in the greenhouse study. These two sites are located within 0.49 km of one another and superficially appear similar in vegetative cover and slope. To quantify whether these two hybrid populations were growing under similar environmental conditions, temperature and relative humidity were measured every 10 min from 8 July to 23 August 2004, with Hobo data loggers (Onset Computer, Bourne, Massachusetts, USA). These sensors were placed in partial shade in the two hybrid populations, approximately 4 cm above the ground, similar to the height of the vegetative *Ipomopsis* rosettes. Data were downloaded using BoxCar Pro software (Onset Computer) and used to determine daily maxima and minima for temperature and ambient vapor pressure deficit (VPD, calculated from temperature and relative humidity following Murray, 1967).

Hybrid genotyping—Hybrid individuals were assigned to one of four genetic classes using nuclear amplified fragment length polymorphism (AFLP) and cytoplasmic cpDNA markers. Following ecophysiological measurements, whole genomic DNA was extracted from freeze-dried leaf tissue from the same plants using DNeasy Plant Mini Kits (Qiagen, Valencia, California, USA). The cytoplasmic background of each individual was determined using species-diagnostic cpDNA PCR-RFLP markers developed for the two parental species (Wu and Campbell, 2005). In addition, hybrids were scored for the presence of

a single AFLP nuclear marker, *agg1*, that is characteristic of *I. aggregata* (Wu and Campbell, 2005). This is the sole species-diagnostic nuclear genetic marker that has been identified for the two parental species, in spite of rigorous screening using AFLP (Wu and Campbell, 2005) and RAPD (G. Aldridge, University of California, Irvine, personal communication) methods. Because AFLP markers are dominant, we cannot distinguish between the homozygous *agg1* and heterozygous genotypes at this locus. Nonetheless, together these nuclear and cytoplasmic markers allowed the F2 and natural hybrids to be assigned to one of four genetic classes: presence or absence of *agg1*, with either *I. aggregata* or *I. tenuituba* cytoplasmic background.

Ecophysiological measurements—During 23–27 July 2003 and 4–10 July 2004, we measured instantaneous rates of photosynthesis (A), transpiration (E), stomatal conductance (g_s), and internal CO_2 concentration (c_i) for vegetative plants in the natural hybrid zone using a LI-COR 6400 infrared gas analysis system (LI-COR, Lincoln, Nebraska, USA), which controls environmental conditions within the measurement chamber. In 2003, we examined plants from the two parental populations ($N = 15$ *I. aggregata* site L and $N = 14$ *I. tenuituba* site C) and hybrid population I ($N = 16$), which has been used as a representative hybrid site in previous studies (Campbell and Waser, 2001). In 2004, we also sampled from the second hybrid population G ($N = 41$), which is unique in possessing the *I. aggregata* cytotype (Wu and Campbell, 2005), in addition to sampling new individuals from hybrid population I ($N = 33$). These same physiological parameters were measured on the potted experimental F2 hybrids from 28 July–6 August 2003. All F2 seedlings that were large enough to accommodate the standard LI-COR 6400 leaf chamber were included ($N = 342$).

Field measurements were made between 0930 and 1300 hours under full sun, using the following settings for the LI-COR 6400: air temperature at 27°C, ambient CO_2 concentration at 350 ppm, and photosynthetically active radiation (PAR) at 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Vapor pressure deficit (VPD) remained at 2.7 ± 0.2 kPa (mean ± SE) during all measurements, and leaf temperature was monitored with a thin wire thermocouple inside the chamber. Greenhouse-grown F2 plants were measured using similar parameters, but within the WeatherPort greenhouses. Differences in photosynthetic rates between the hybrids and parental species are similar at CO_2 levels of 350 and 375 ppm (Wu and Campbell, 2006), and PAR of 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is typical of sunny conditions at these sites (Campbell et al., 2005). Following gas exchange measurements, leaves were collected in coin envelopes and their areas determined within 6 h using a scanner with ROOTEDGE Software (2002, National Soil Tilth Laboratory, Ames, Iowa, USA). Reported gas exchange parameters have been corrected for leaf area.

We measured intrinsic WUE as A/g_s, the ratio of photosynthetic rate to stomatal conductance. In our study, intrinsic WUE was very tightly correlated with other potential measures of WUE, including both A/E and c_i (correlation coefficients $r = 0.99$, $P < 0.0001$; see Results). Because of these tight correlations, we report further only on the analyses using intrinsic WUE.

For the plants examined from natural populations, the instantaneous gas exchange measurements were supplemented with a measure of leaf nitrogen content (%N). Following LI-COR measurements and leaf area determinations, leaves were air dried for at least 48 h. These leaves were then combined with additional dried leaves collected from the same plants, ground using a Wig-L-Bug (Bratt Technologies, East Orange, New Jersey, USA), and sent to the UC Berkeley Center for Stable Isotope Biogeochemistry for elemental analysis with a gas phase isotope ratio mass spectrometer.

Statistical analyses—Leaf physiological traits were compared among the parental species and natural hybrids measured in 2003 with one-way analyses of variance (ANOVAs), followed by Tukey-Kramer a posteriori comparisons (SPSS 11.0.2, SPSS, Chicago, Illinois, USA). Because VPD was not explicitly controlled in our measurements, we initially included it as a covariate in the model testing for differences in WUE among these natural populations. However, we removed VPD from our final model because it had no significant effect on WUE ($P > 0.6$).

We tested for cytonuclear effects on gas exchange parameters separately in F2 hybrids and in natural hybrids from sites G and I. In each case we used a two-way fixed effects ANOVA for each parameter (A, g_s, WUE, %N), with the fixed factors cytoplasmic background and presence/absence of the nuclear marker *agg1*. Additionally, for the experimental F2 hybrids, WUE, A, and g_s were analyzed with a mixed-model two-way ANOVA using PROC MIXED (SAS 6.12, SAS Institute, Cary, North Carolina, USA) to test for effects of family on these physiological traits. For these analyses, cytoplasmic background was fixed, whereas family and family × cytotype interactions were considered random effects. Initial analyses found no effect of day or time of day when included as covariates in the models, and therefore we present the analyses for the experimental F2s with these variables removed from the model. Of the original 13 families, only 10 had >1 individual from each of the two cytotypes represented ($N = 296$); measurements from the other three families were excluded from these analyses. The significance of the main effect of family was determined by running a second model with this random effect removed; the difference in the residual likelihood values from the two models follows a χ^2 distribution.

Using all natural plants measured from Poverty Gulch, we calculated Pearson correlation coefficients between WUE and %N, because higher photosynthetic rates are often accompanied by a similar increase in leaf nitrogen (Field and Mooney, 1986; McDowell, 2002; Wright et al., 2003). We also explored the consistency of physiological traits in the natural hybrids across the two years by comparing WUE, A, and g_s between years with a one-way ANOVA. This analysis included only hybrids from site I, because hybrid plants from G were only measured in 2004.

RESULTS

Natural hybrids and parental Ipomopsis plants in the field—During field measurements, both the parental *Ipomopsis* species and natural hybrids maintained leaf temperatures within 0.5°C of the ambient chamber temperature (*I. aggregata*, 27.2 ± 0.1°C; *I. tenuituba*, 27.4 ± 0.1°C; hybrids, 27.3 ± 0.1°C; $F_{2,42} = 1.30$, $P = 0.283$). At these temperatures, the hybrid plants exhibited only a slightly greater VPD (2.86 ± 0.04 kPa; $F_{2,42} = 27.39$, $P < 0.001$) than the parental species *I. aggregata* (2.51 ± 0.04 kPa) and *I. tenuituba* (2.61 ± 0.03 kPa), which did not differ significantly from one another. These differences in VPD for leaves under experimentally controlled conditions are extremely small compared to the range in ambient VPD seen naturally across these sites (maximum = 5.3 vs. 2.3 kPa for hybrid and *I. aggregata* sites in 2002; Campbell et al., 2005) and are typical for leaf to air values observed in similar comparative studies of herbaceous plants (e.g., McDowell, 2002; Sherrard and Maherali, 2006).

When growing in their native sites in 2003, *I. tenuituba* had higher intrinsic WUE (A/g_s) than *I. aggregata*, and WUE of natural hybrids significantly exceeded both parental types (ANOVA $P < 0.001$; Fig. 1A). As expected with the controlled conditions in the leaf chamber, all estimates of water use efficiency (A/E, A/g_s, and c_i) were very tightly correlated (absolute value of $r = 0.99$, $N = 45$, $P < 0.0001$), averaging 2.73 μmol/mmol, 103 μmol/mol, and 170 ppm, respectively. Consequently, only values for A/g_s are presented further as indicators of WUE. Both the *I. tenuituba* and hybrids maintained their high WUE via reduced g_s ($P < 0.0001$) relative to *I. aggregata*, rather than increased photosynthetic rates ($P = 0.06$; Fig. 1). Leaf N showed a similar pattern, differing significantly among the three groups in Poverty Gulch ($F_{2,42} = 27.96$, $P < 0.001$), with *I. aggregata* having significantly lower N (2.01% ± 0.05%) than both *I. tenuituba* (2.48% ± 0.05%) and natural hybrids (2.58% ± 0.07%). Among all parental and hybrid plants measured in the natural hybrid zone in 2003 and 2004, instantaneous WUE also correlated positively with leaf N ($r = 0.313$, $P < 0.01$).

Comparing natural hybrids across years, plants at site I had higher WUE in 2003 (mean = 174.5 μmol CO_2/mol H_2O, $N = 16$ plants) than in 2004 (mean = 51.74 μmol CO_2/mol H_2O, $N = 41$, ANOVA $P < 0.001$). This difference in WUE reflected changes in g_s, which increased from an average of 0.10 to 0.46 mol $H_2O \cdot m^{-2} \cdot s^{-1}$ ($P < 0.001$), while there was no significant

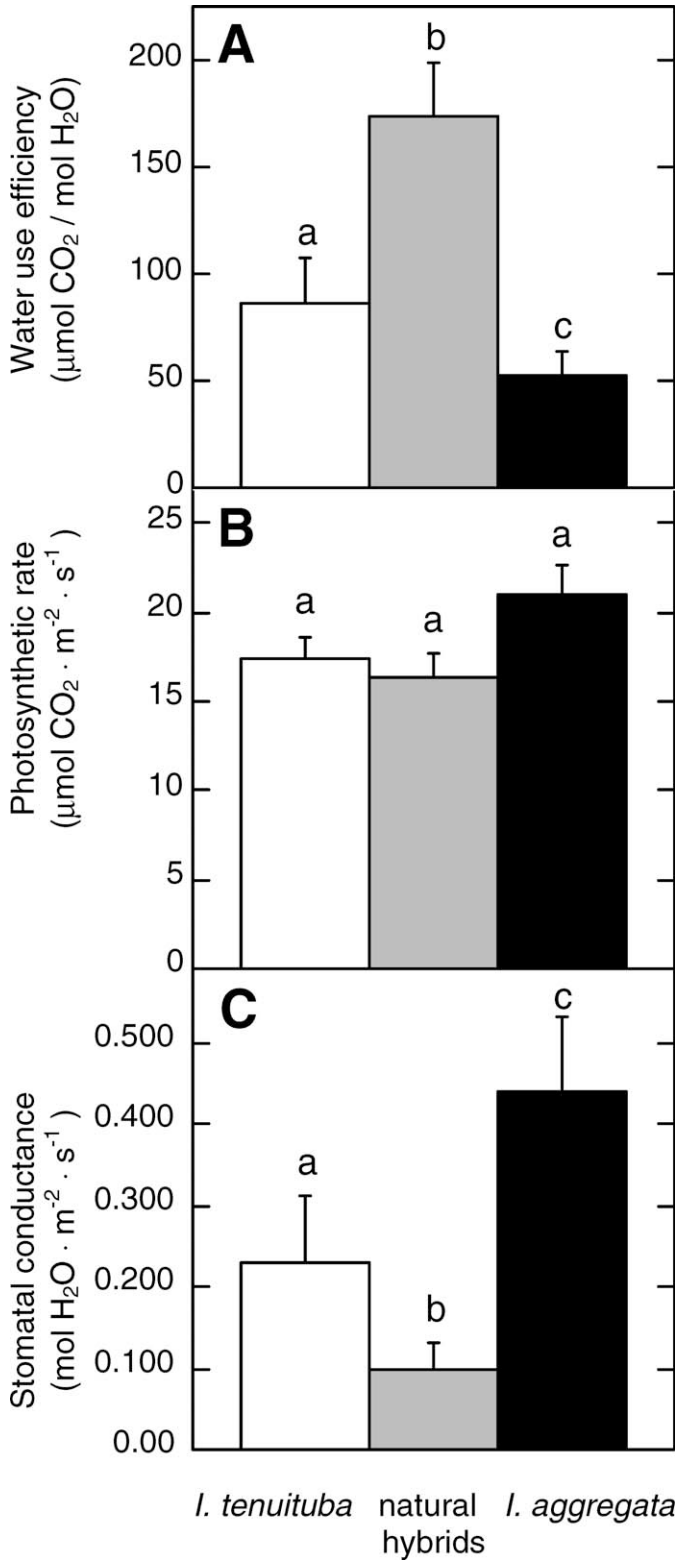

Fig. 1. (A) Intrinsic water-use efficiency, (B) photosynthetic rate, and (C) stomatal conductance for natural hybrids from site I and two parental *Ipomopsis* species growing in situ at a natural hybrid zone in Poverty Gulch, Colorado, USA, in 2003. Bars are means + SE ($N = 14$–16), and letters indicate means that differ from one another based on Tukey a posteriori comparisons.

TABLE 2. Two-way analysis of variance (ANOVA) for cytonuclear effects on gas exchange characteristics in experimental F2 *Ipomopsis* hybrids ($N = 342$) with alternate cytoplasmic backgrounds grown under common greenhouse conditions. P values in boldface type are significant at the $\alpha = 0.05$ level.

	A		g_s		A/g_s	
Source of variance	$F_{1,338}$	P	$F_{1,338}$	P	$F_{1,338}$	P
Cytotype	10.124	**0.002**	34.535	**<0.001**	2.805	0.095
agg1	1.968	0.162	1.333	0.249	0.016	0.898
Cytotype × *agg1*	3.608	0.068	3.230	0.073	0.020	0.888

Note: A, photosynthetic rate (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$); g_s, stomatal conductance (mol $H_2O \cdot m^{-2} \cdot s^{-1}$); A/g_s, intrinsic water-use efficiency (μmol CO_2/mol H_2O).

change in photosynthetic rate between the two years (mean = 16.3 vs. 16.4 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ in 2003 and 2004, $P = 0.998$).

Reciprocal F2 hybrids—When grown under common conditions, experimentally produced F2 hybrids did not differ significantly in WUE, regardless of cytoplasmic background or the presence of AFLP marker *agg1* ($P > 0.05$; Table 2). However, the mechanism by which the WUE was achieved depended upon the cytoplasmic background. The $F2_T$ hybrids had reduced g_s ($P < 0.001$), while $F2_A$ hybrids had increased photosynthetic rates ($P = 0.002$; Fig. 2). Overall, the WUE of these potted F2 hybrids was lower than that of natural hybrids growing in the field (compare Figs. 1 and 2; t test with unequal variances, $P < 0.001$), likely due in part to the relatively well-watered conditions in the greenhouse compared to the natural hybrid zone.

There was no significant family × cytotype interaction for WUE ($P = 0.1123$), A ($P = 0.1446$), or g_s ($P = 0.0817$), suggesting that as a whole, the relative performance of the reciprocal F2 hybrids was similar for each of the 10 families considered. Neither did any of these three physiological traits depend significantly on family (each $P > 0.5$). As in the full set of F2s, WUE did not depend on cytotype ($P = 0.3262$), but g_s was higher in $F2_A$ hybrids ($P = 0.0048$), and those plants also tended to have higher photosynthetic rates ($P = 0.0617$). However, in three of these 10 families, transpiration rate followed the reverse pattern but did not differ significantly between the reciprocal cytotypes (Fig. 3), indicating that individual genotypes or families may have responses that diverge from the remainder of the genotypic class.

Natural reciprocal hybrids growing in situ—The two natural hybrid sites in the center of the hybrid zone experienced large daily temperature fluctuations, with ranges similar to those observed within this hybrid zone in previous years (Campbell et al., 2005; Wu and Campbell, 2006). Compared to hybrid site G, site I had significantly higher daily maximum temperatures and greater ambient VPD ($P = 0.003$ for each; Table 3), placing more demand for water from leaves on plants. Nonetheless, hybrid plants from both populations maintained leaf temperatures near ambient chamber levels during field measurements (site G, 26.6° ± 0.21°C; site I, 27.0° ± 0.04°C; t test, $P > 0.05$), although plants from site I had slightly greater VPD (2.32 ± 0.03 vs. 2.06 ± 0.02 kPa; t test, $P < 0.001$). Minimum nighttime temperatures and VPD were similar at the two sites (Table 3). Because of the environmental differences

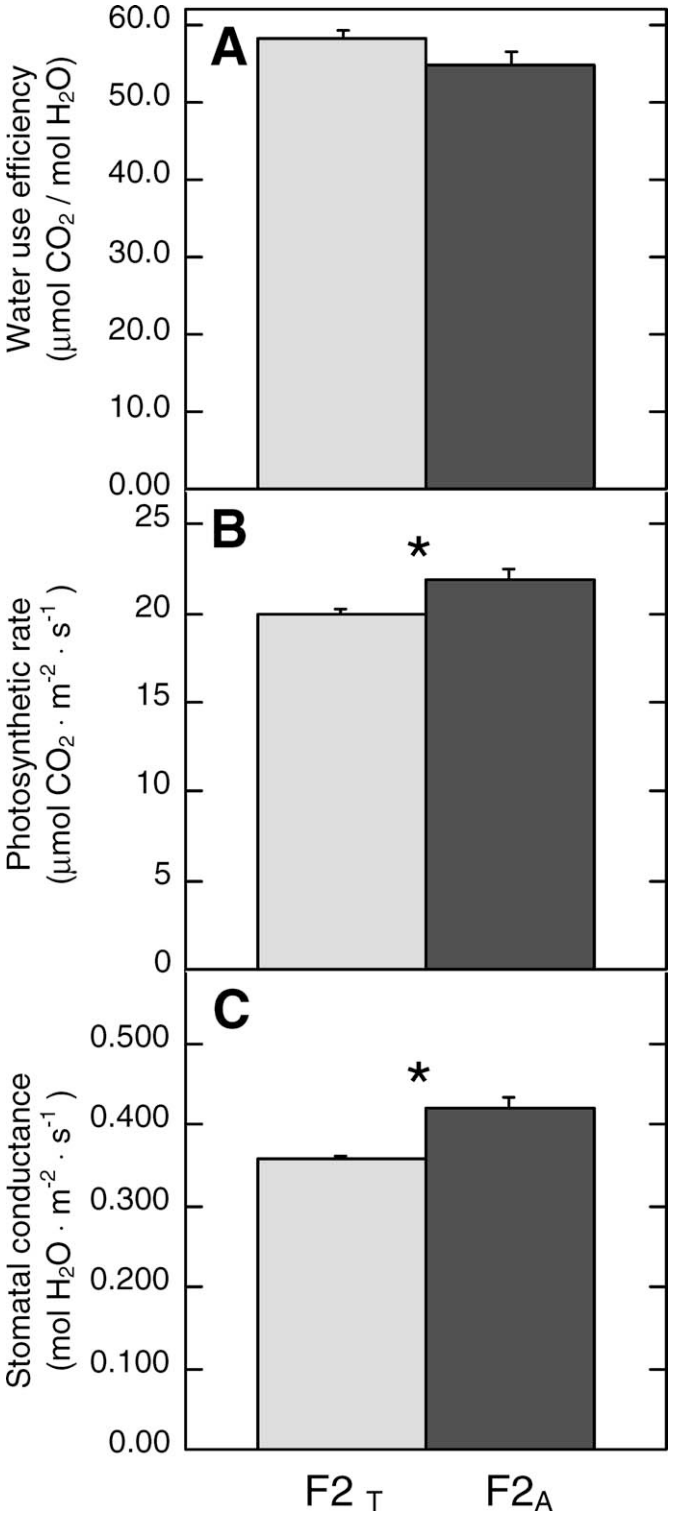

Fig. 2. (A) Intrinsic water-use efficiency, (B) photosynthetic rate, and (C) stomatal conductance for experimental F2 hybrids with alternate cytoplasmic backgrounds grown in common greenhouse conditions at the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA. Bars are means + SE ($F2_T$ $N = 109$; $F2_A$ $N = 233$). An asterisk (*) indicates significant differences in means between the two groups, based on Tukey a posteriori comparisons.

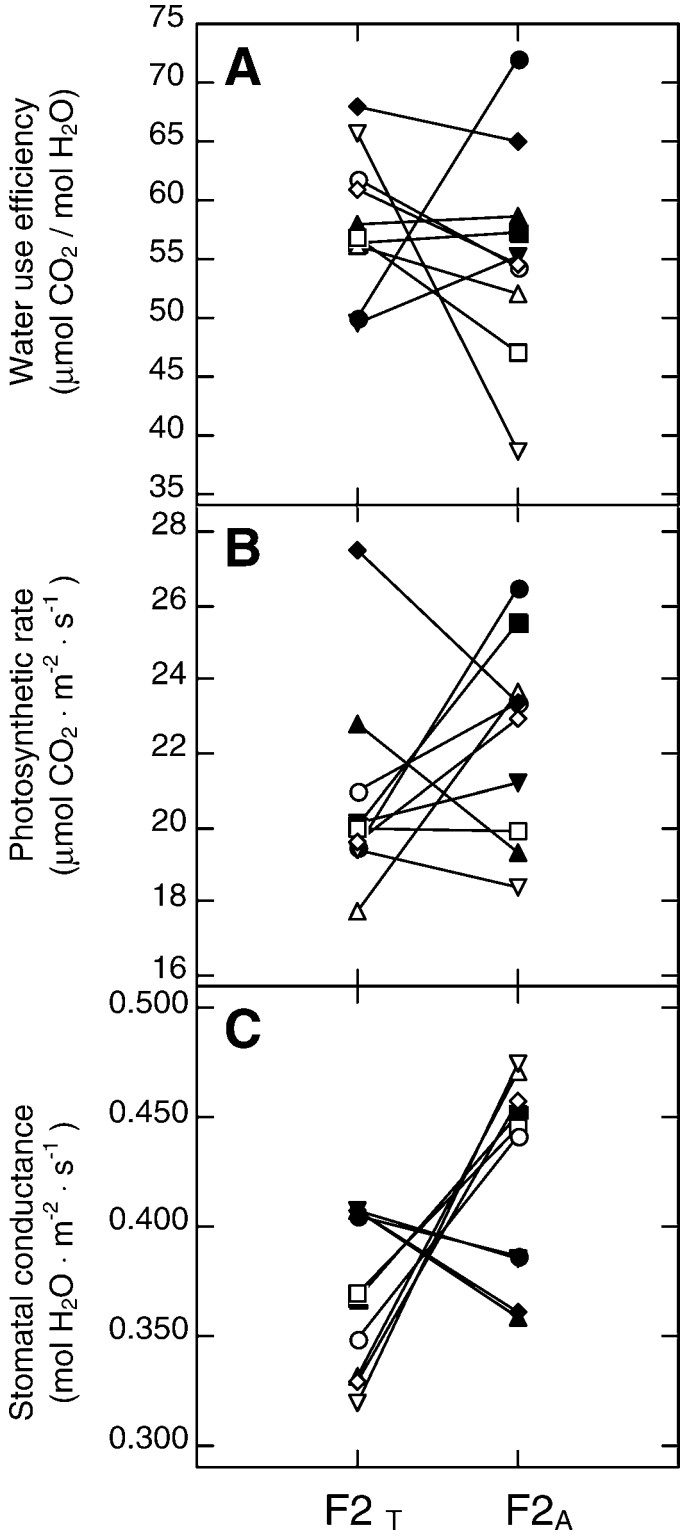

Fig. 3. Mean (A) intrinsic water-use efficiency, (B) photosynthetic rate, and (C) stomatal conductance in a common greenhouse environment for each of 10 experimental, reciprocal F2 hybrid families. Symbols are family means ($N = 5$–73 plants per family; 296 plants total). Error bars are not included in the figure for clarity, but for each data point, 1 SE ranges from 2.17 to 5.25 for water-use efficiency, 0.8 to 2.5 for photosynthetic rate, and 0.012 to 0.022 for stomatal conductance.

TABLE 3. Environmental conditions at vegetative rosette height at two sites within the natural *Ipomopsis* hybrid zone in Poverty Gulch, Colorado, USA. Plants in site G have the *I. aggregata* cytoplasmic background, while those in site I have the *I. tenuituba* cytotype. Temperatures (T) and vapor pressure deficits (VPD) are average daily maximum and minimum values (±SE) from 8 July to 23 August 2004, determined from Hobo data logger measurements. Each variable was analyzed in a separate one-way ANOVA, with total $N = 94$. P values in boldface type are significant at the $\alpha = 0.05$ level.

Variable	Hybrid site G	Hybrid site I	P
Max T (°C)	26.6 ± 0.8	30.3 ± 0.9	**0.003**
Min T (°C)	5.8 ± 0.3	6.3 ± 0.3	0.166
Max VPD (kPa)	2.75 ± 0.19	3.67 ± 0.23	**0.003**
Min VPD (kPa)	0.13 ± 0.02	0.16 ± 0.02	0.308

between the two hybrid sites, we cannot directly test for cytoplasmic effects in the natural hybrids but instead refer to the effect of "site" rather than "cytotype" when comparing plants from G and I.

Overall, there was no effect of the nuclear marker *agg1*, or *agg1* × site interaction, on WUE, A, or g_s (Table 4). However, there were strongly significant differences between plants from the two hybrid sites for both A and g_s. Natural hybrids from G and I had similar WUE (two-way ANOVA with site and *agg1*, $P = 0.155$; Fig. 4), but as in the experimental F2 hybrids, achieved it in different ways. Hybrids with the *I. aggregata* cytoplasmic background from G had higher photosynthetic rates, while hybrids with the *I. tenuituba* cytoplasmic background from site I had significantly lower g_s (Fig. 4, Table 4).

Likewise, %N did not vary with presence of the *agg1* marker in the natural hybrids, nor was there an *agg1* × site interaction (Table 4). However, hybrids from site I had higher leaf nitrogen content (2.94% ± 0.06%) compared to those from site G (2.61% ± 0.07%, $P < 0.05$).

DISCUSSION

This study compared aspects of the leaf-level ecophysiology of *Ipomopsis aggregata*, *I. tenuituba*, and their natural hybrids as a step toward understanding differences in the habitat distribution of hybrids and their parental species. We examined plants growing in their native sites in Poverty Gulch, Colorado, and tested for cytonuclear effects on gas exchange parameters in experimentally produced F2 hybrids grown under common garden conditions. In their natural habitats within the hybrid zone, *I. tenuituba* had significantly higher WUE than *I. aggregata*, and hybrids were even more efficient than either of the parental species. These results are consistent with previous studies of these taxa (Campbell et al., 2005), which found that hybrids had higher WUE than the parental species when plants were grown in two different common garden locations that spanned environmental conditions found within the natural hybrid zone. This relationship held both when using instantaneous measures of WUE, as in this study, and when using leaf carbon isotope ratios ($\delta^{13}C$, Campbell et al., 2005), which provide an integrated measure of WUE over the lifetime of the leaf (Farquhar et al., 1989; Donovan and Ehleringer, 1994). Although increased WUE is generally expected to confer a fitness advantage to plants growing in water-limited sites, greater leaf-level WUE does not necessarily equate to less water used because high WUE can be achieved by increased photosynthetic gain, by decreasing water loss, or some combination of these responses (Donovan and Ehleringer, 1994). In this study, the greater WUE in hybrids and *I. tenuituba* relative to *I. aggregata* resulted from lowered g_s, a strategy that is likely to be favorable in the more xeric regions of the hybrid zone where *I. tenuituba* and hybrids are found (Ludwig et al., 2004; Sherrard and Maherali, 2006; Wu and Campbell, 2006). Indeed, initial studies of plant water potentials suggest that $F1_T$ hybrids and *I. tenuituba* are under less water stress in the field than *I. aggregata* during the midday heat (Campbell et al., 2005).

Survival of *Ipomopsis* F1 hybrids depends strongly on both the environment and on which species serves as the maternal parent (Campbell and Waser, 2001), suggesting that the cytoplasmic background is important to at least some aspects of hybrid fitness. We explored this possibility using reciprocal F2 hybrids in a common garden to test for cytonuclear effects on physiological performance. Water-use efficiency was not affected by cytoplasmic background or the presence of an *I. aggregata* nuclear marker, but the mechanism by which the F2s maintained their WUE did depend on cytoplasmic background. The F2 hybrids with *I. tenuituba* cytoplasm maintained high WUE by reducing g_s, while those with *I. aggregata* cytoplasm had an increased photosynthetic rate. The strategies employed by these reciprocal hybrids were consistent with that of their cytoplasmic parent when the pure species were grown in their natural habitats. This suggests the importance of maternally inherited cytoplasmic genes, such as those in chloroplast DNA, to photosynthetic performance. The contributions of cytoplasmic genes to plant phenotype have recently begun to receive substantial attention (Galloway and Fenster, 1999, 2001; Hausmann et al., 2005), and the specific inclusion of cytotype in future studies will improve our understanding of the importance of cytoplasmic genes to the relative performance of hybrids and their parents.

TABLE 4. Two-way ANOVAs comparing physiological characteristics of natural *Ipomopsis* hybrids from two sites in Poverty Gulch, Colorado, USA, where plants have alternate cytoplasmic backgrounds. The factor *agg1* refers to the presence of an AFLP nuclear marker specific to the parental species *I. aggregata*. P values in boldface type are significant at the $\alpha = 0.05$ level.

	A		g_s		A/g_s		Leaf N	
Source of variance	$F_{1,70}$	P	$F_{1,70}$	P	$F_{1,70}$	P	$F_{1,69}$	P
Site	18.51	**<0.001**	16.30	**<0.001**	1.63	0.206	14.44	**<0.001**
agg1	0.22	0.644	0.42	0.518	2.38	0.127	0.159	0.691
Site × *agg1*	0.14	0.707	0.117	0.733	0.41	0.840	1.707	0.196

Note: A, photosynthetic rate (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$); g_s, stomatal conductance (mol $H_2O \cdot m^{-2} \cdot s^{-1}$); A/g_s, intrinsic water-use efficiency (μmol CO_2/mol H_2O); leaf N, leaf nitrogen content (%N).

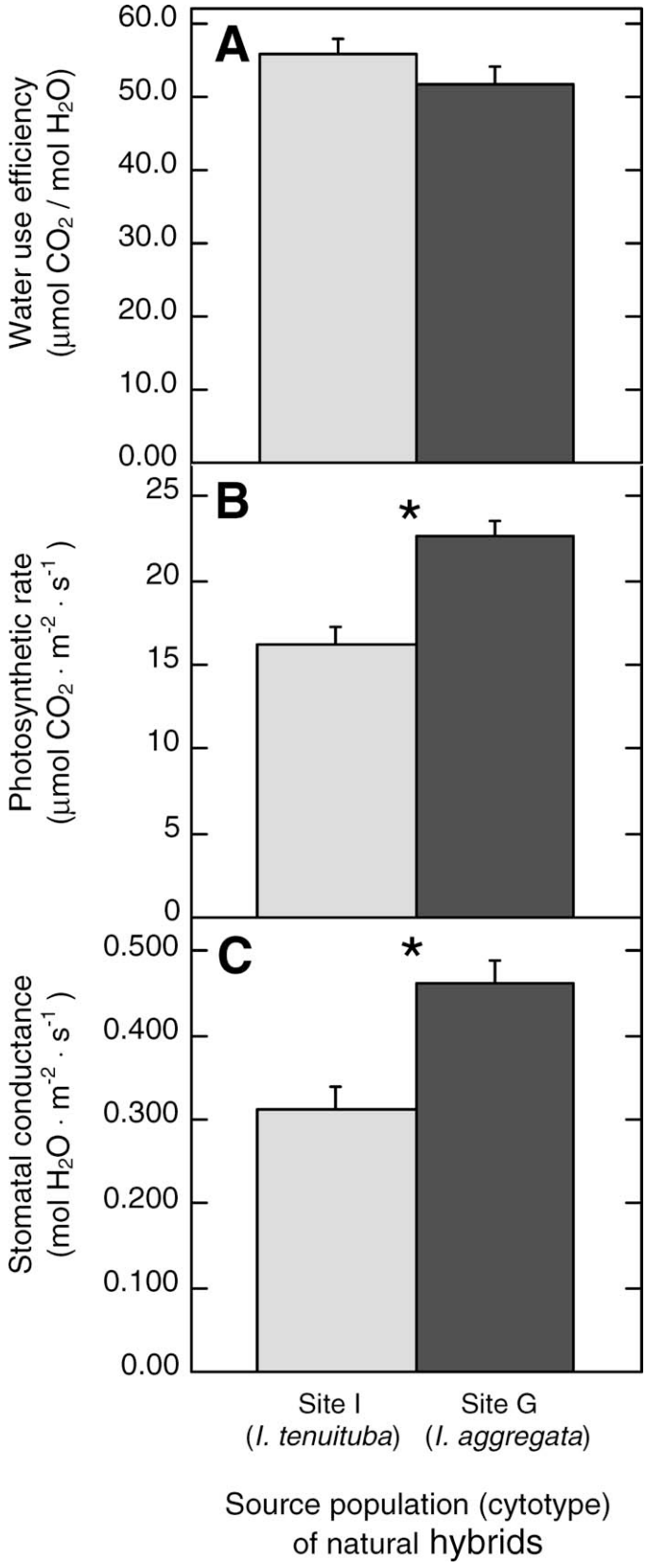

Fig. 4. (A) Intrinsic water-use efficiency, (B) photosynthetic rate, and (C) stomatal conductance for natural hybrids from two populations in Poverty Gulch, Colorado, USA, that contain plants with alternate cytoplasmic backgrounds, measured in summer 2004. Bars are means + SE (*Ipomopsis tenuituba* cytotype from site I, $N = 33$; *I. aggregata* cytotype from site G, $N = 41$). An asterisk (*) indicates significant differences in means between the two groups, based on Tukey a posteriori comparisons.

While the relative WUE of hybrids and the parental *Ipomopsis* species observed here were consistent with previous work that employed plants in experimental field plantings (Campbell et al., 2005), our findings on cytoplasmic background were not as consistent. In the earlier transplants (Campbell et al., 2005), there was no significant cytoplasmic effect on WUE in hybrids, and the trend for how WUE was achieved went in the opposite direction than that found here: hybrids with *I. tenuituba* mothers had unusually high photosynthetic rates. There are at least two potential explanations for differences in cytoplasmic effect between the two studies. First, the studies tested for cytoplasmic effects in different hybrid classes (F2 hybrids here vs. F1 hybrids in Campbell et al., 2005), which can differ greatly in fitness (Arnold and Hodges, 1995; Arnold, 1997, and references therein). Epistatic interactions between nuclear genes that become more apparent in the post-F1 recombinant generations may also depend on the cytoplasm in which the nuclear genes are expressed (Fenster et al., 1997; Burke et al., 1998). The consistency of the overall cytoplasmic effect in both the experimental F2s and natural hybrids reported here strongly indicates that cytoplasmic factors do influence gas exchange trait expression in these plants. Second, experimental plants in the two studies were grown under different environmental conditions, which may have led to genotype × environment effects on physiological performance. However, this explanation seems less tenable, because the natural hybrids examined here and the greenhouse-grown F2s showed similar cytoplasmic effects. There may well be additional nuclear or cytonuclear effects on gas exchange physiology that went undetected in this study, because we were constrained to coarse-level genetic classifications with a single nuclear marker. Cytonuclear interactions on fitness traits have been documented in several recent studies of plants and animals (Breewuwer and Werren, 1995; Li et al., 1997; Galloway and Fenster, 1999; Willet and Burton, 2001), though few have examined cytonuclear effects at the species level in natural hybrid populations (but see Burke et al., 1998).

Natural hybrids with alternate cytoplasmic backgrounds also had similar WUE. But, as observed with the reciprocal F2 hybrids grown in the greenhouse, natural hybrids with *I. tenuituba* cytoplasm (from site I) had lower g_s than those with *I. aggregata* cytoplasm (from site G). However, we cannot definitively distinguish between site and cytoplasm effects on WUE strategies in these natural hybrids, because in the center of the hybrid zone the *I. aggregata* cytoplasm is only found in a single hybrid population (Wu and Campbell, 2005). The observed physiological differences could instead largely be due to environmental variation between the two locations where hybrids were examined. In addition, the lower g_s of hybrids from site I relative to hybrids from site G is consistent with abiotic differences between the two sites, as site I was both warmer and drier than site G in 2004 (Table 2). Teasing apart cytoplasmic from local environmental effects requires that both cytotypes be present in populations of hybrid plants at each site, which unfortunately does not appear to occur in the natural

populations at Poverty Gulch (Wu and Campbell, 2005). Future studies in which advanced-generation hybrids are planted into hybrid sites could determine whether physiological differences between the cytotypes persist under field conditions where natural hybrids occur.

Water-use efficiency changed dramatically from 2003 to 2004 in the natural hybrids from site I. The large reduction in WUE across the period was due to increased rates of conductance and transpiration, while photosynthetic rates did not change. Because altering stomatal conductance in lieu of photosynthetic capacity to change WUE will affect water loss, the reduction in WUE may reflect a response to changes in environmental conditions between the years: in 2003, daily maximum temperatures averaged ~7°C higher than in 2004 and were accompanied by a nearly twofold greater VPD (Wu and Campbell, 2006). In perennial plants such as *Ipomopsis*, the ability to respond to interannual changes in moisture conditions by adjusting traits governing resource use could provide a fitness advantage, especially if there is a cost for having a conservative WUE in wet years (Zangerl and Bazzaz, 1984; Cowan, 1986; Givnish, 1986; Arntz and Delph, 2001). If so, this plasticity could conceivably confer an advantage over a simple strategy of maintaining drought tolerance via consistently high WUE.

Leaf nitrogen was positively correlated with WUE in the natural *Ipomopsis* plants. This is a common finding, because much leaf N is allocated to photosynthetic pigments and enzymes, making nitrogen a major determinant of the biochemical capacity for photosynthesis (Field and Mooney, 1986). However, we found no significant difference in photosynthetic rate among the parental and hybrid populations, even though they differed in leaf N. In these plants, *I. aggregata* had the lowest leaf N, while *I. tenuituba* and hybrids had similar leaf N, as also seen in a common garden in the field (Campbell et al., 2005). In 2004, hybrids from site I had higher leaf N than any of the taxa sampled in 2003. The higher N in hybrids and *I. tenuituba* could, in principle, reflect a greater soil N availability or higher success at gaining this resource. Indeed, alpine plants have been found to have higher leaf N concentrations than those from lower altitudes (Körner et al., 1986; Körner, 1989). As the *I. aggregata* site does not seem to have lower soil N (Campbell and Waser, 2001; D. R. Campbell, unpublished data), hybrids may be experiencing selection for altered uptake or may have different patterns of nitrogen partitioning than the parents, perhaps allocating more to structural proteins that may improve long-term persistence of overwintering leaves in the more xeric hybrid sites (Field and Mooney, 1986; Hikosaka, 2004; Takashima et al., 2004; Katahata et al., 2007).

This study revealed that ecophysiological traits in *Ipomopsis* can be both intermediate and transgressive relative to those in the parental species. The few studies of physiology conducted in other natural hybrid systems show similar trends, with hybrids not necessarily demonstrating reduced performance. Instead, as with morphological traits, hybrids can comprise a mosaic of parental-like and transgressive traits. For example, the putative hybrid sunflower species *Helianthus anomalus* did not differ from its parental species for leaf-lifetime, integrated WUE but was positively transgressive for leaf succulence (Schwarzbach et al., 2001; Rosenthal et al., 2002; Ludwig et al., 2004). Natural hybrids between *Picea glauca* and *P. sitchensis* had higher WUE than either of the parents (Silim et al., 2001). Another sunflower hybrid species, the halophyte *H. paradoxus* that is restricted to brackish marshes in New Mexico and Texas, was transgressive for concentrations of four leaf nutrients (Rosenthal et al., 2002). However, many of these studies were conducted under controlled conditions, and the relative performance of the hybrids and parents may differ in the natural habitats because of genotype × environment interactions (Ludwig et al., 2004). Here we found that the relative WUE of natural parental and hybrid *Ipomopsis* plants seen in common gardens (Campbell et al., 2005) is also observed in the natural populations.

In sum, *Ipomopsis* hybrids and their parental species differed in WUE in patterns consistent with differences in environmental conditions across the natural hybrid zone. *Ipomopsis tenuituba*, which is found in more xeric sites in this hybrid zone, had more conservative water use than *I. aggregata*. Hybrids were generally *tenuituba*-like or extreme for the ecophysiological traits measured in this study. Overall, the high WUE of hybrids could provide these plants with a survival advantage, at least relative to parental *I. aggregata* plants, at the dry sites located at the center of the natural hybrid zone.

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