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Divergence and reproductive isolation between two closely related allopatric *Iris* species

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Speciation involves the establishment of reproductive isolating barriers between diverging populations. Two closely related iris species, *Iris atrofusca* and *Iris mariae*, have non-overlapping geographical distributions characterized by differences in soil type and precipitation. We aimed to obtain a better understanding of pre- and postzygotic isolating barriers between these two species and the possible role of habitat-specific selection in preventing gene flow between them. We examined molecular genetic (AFLP) and phenotypic divergence and conducted species distribution modelling, tests for local adaptation and crossing experiments on the two species. The two species were found to be clearly divergent genetically and phenotypically from each other. Species distribution modelling further showed that each species distribution was largely associated with climate and soil type and that the area predicted to be shared by both species was very narrow, resulting in very high estimates of ecogeographical isolation. In contrast, other isolating barriers examined were either weak (flowering time differences) or absent (related to immigrant inviability and reduced fitness of hybrids generated from reciprocal crosses). Each species showed no home advantage in a reciprocal transplant analysis and little preference for indigenous soil type and water regime in experimental tests of this, thus indicating an absence of local adaptation. Reproductive isolation between the two iris species appears almost entirely attributable to geographical isolation unlinked to local adaptation. However, more detailed studies are required before local adaptation is dismissed as a cause of species divergence and occupation of contrasting habitats.

ADDITIONAL KEYWORDS: *Iris* – natural selection – *Oncocyclus* – reproductive isolation – speciation.

INTRODUCTION

Speciation proceeds through the establishment of spatial and/or biological isolation barriers that limit or prevent gene exchange between populations, leading to the maintenance of genetic and (usually) phenotypic distinctiveness of populations (Coyne & Orr, 2004; Baack *et al.*, 2015). In the classic scenario of allopatric speciation, an ancestral population divides into two spatially isolated populations that subsequently diverge and become reproductively isolated in the absence of gene flow (Mayr, 1942). In contrast, parapatric and sympatric speciation models incorporate some degree of interpopulation gene flow (Maynard Smith, 1966; Endler, 1977), the effect of which is overridden by factors creating or maintaining species boundaries. According to the biological species concept, in all of these models reproductive isolation between populations evolves as a direct or indirect result of several possible mechanisms of genetic divergence (Schluter, 2001). Among the four main mechanisms promoting divergence (divergent natural selection, genetic drift, mutation and polyploidy), ecologically based divergent natural selection leading to so-called ecological speciation is of special importance

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for understanding the evolutionary process (Nosil, 2012). Divergent natural selection, in the absence of other forces and constraints, is expected to result in the formation of adaptive genetic differences between diverging populations, and the process, in which populations evolve towards a phenotype that has the highest fitness in its local environment, is termed local adaptation (Kawecki & Ebert, 2004).

If two taxa have non-overlapping distributions, it is useful to assess, via species distribution modelling (also called ecological niche modelling), how differences in niche translate into ecogeographical isolation (Sobel *et al.*, 2010; Schemske, 2010; Sobel, 2014). However, even in situations where ecogeographical isolation is implied from such modelling, reciprocal transplant analysis is required to confirm that geographical separation and genetic divergence are, in fact, linked to differential adaptation (Sobel, 2014). Although many reciprocal transplant studies have yielded evidence of local adaptation between populations and closely related species that occupy different habitats, a meta-analysis of such studies revealed that in only 45% of cases were local plants favoured at their respective sites (Leimu & Fischer, 2008). Moreover, few studies have simultaneously assessed reproductive isolation via crossing experiments (reviewed by Schluter, 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005; Lowry *et al.*, 2008a; Nosil, 2012).

In the study reported here, we examined genetic and phenotypic divergence and conducted species distribution modelling, tests for local adaptation and crossing experiments to determine distinctiveness and reproductive isolation between two closely related iris species in section *Oncocyclus* (Iridaceae), *Iris atrofusca* Baker and *Iris mariae* Barbey. *Oncocyclus* irises are an ideal system for studying the role of local adaptation at early stages of speciation because reproductive isolation between them is incomplete (Avishai & Zohary, 1980), although generally they are genetically and phenotypically distinct. Based on floral morphology, Avishai & Zohary (1980) subdivided section *Oncocyclus* into seven aggregates and considered each aggregate to represent a single variable species, for which one or more subspecies might be recognized. Both *I. atrofusca* and *I. mariae* were placed by Avishai & Zohary (1980) in the Haynei aggregate and therefore may be considered as subspecies rather than species. Nonetheless, they are readily distinguished by their distinct floral phenotypes (different colour of petals and signal patch), their different geographical distributions in the eastern Mediterranean, and by their occupation of habitats that differ in soil type and climatic conditions (Fig. 1). *Iris atrofusca* occurs on loess and rendzina soils where annual precipitation is > 200 mm, whereas *I. mariae* is found on inland sand dunes where annual precipitation is < 200 mm. These differences have been considered to reflect differences in local adaptation (e.g. Avishai & Zohary, 1980), although experimental support for this is lacking. For the purposes of this study, we consider the two taxa to represent incipient species and refer to them using their given species names. Although natural hybridization between the two taxa has not been observed in the wild, no sterility barrier exists between them according to Avishai & Zohary (1980).

In the absence of any hard evidence on the nature of local adaptation shown by these two irises and apparent absence of early stage postzygotic reproductive isolation, questions remain as to what reproductive barriers might exist between them apart from geographical isolation. These additional barriers might include, for example, limited dispersal ability, temporal isolation, pollinator preference and selection against immigrants and hybrids. To obtain a better understanding of the possible influence of habitat-specific selection on the different distributions of the two taxa we: (1) performed a habitat suitability analysis of the two incipient species via species distribution modelling; (2) tested for local adaptation by conducting a reciprocal transplant analysis and additional experiments measuring plant performance in different environmental conditions (soil type and precipitation) related to differences of habitat; and (3) performed artificial crosses within and between the two species and a comparison of progeny fitness. Before undertaking these studies, we analysed molecular [amplified fragment length polymorphism (AFLP)] and phenotypic variation within and between the two species to assess their distinctiveness. From our results, we calculated estimates of the strength of several pre- and postzygotic isolating barriers to determine their relative importance in preventing gene flow and maintaining divergence between the two taxa.

MATERIAL AND METHODS

Study species

Iris atrofusca and *I. mariae* are perennial herbs that produce underground rhizomes, creating patches of leaf fans comprising interconnected, genotypically identical ramets. Each ramet produces a single flower pollinated by night-sheltering male solitary bees of *Eucera* and *Synhalonia* (Sapir *et al.*, 2005) that show no species preference when visiting plants (Y. Sapir, personal communication). Flowers last for ≤ 5 days and produce fruits (capsules), which at maturation are dry and split open to release 30–50 globose seeds each of 3–5 mm in diameter (Avishai & Zohary, 1980). Seeds have fleshy

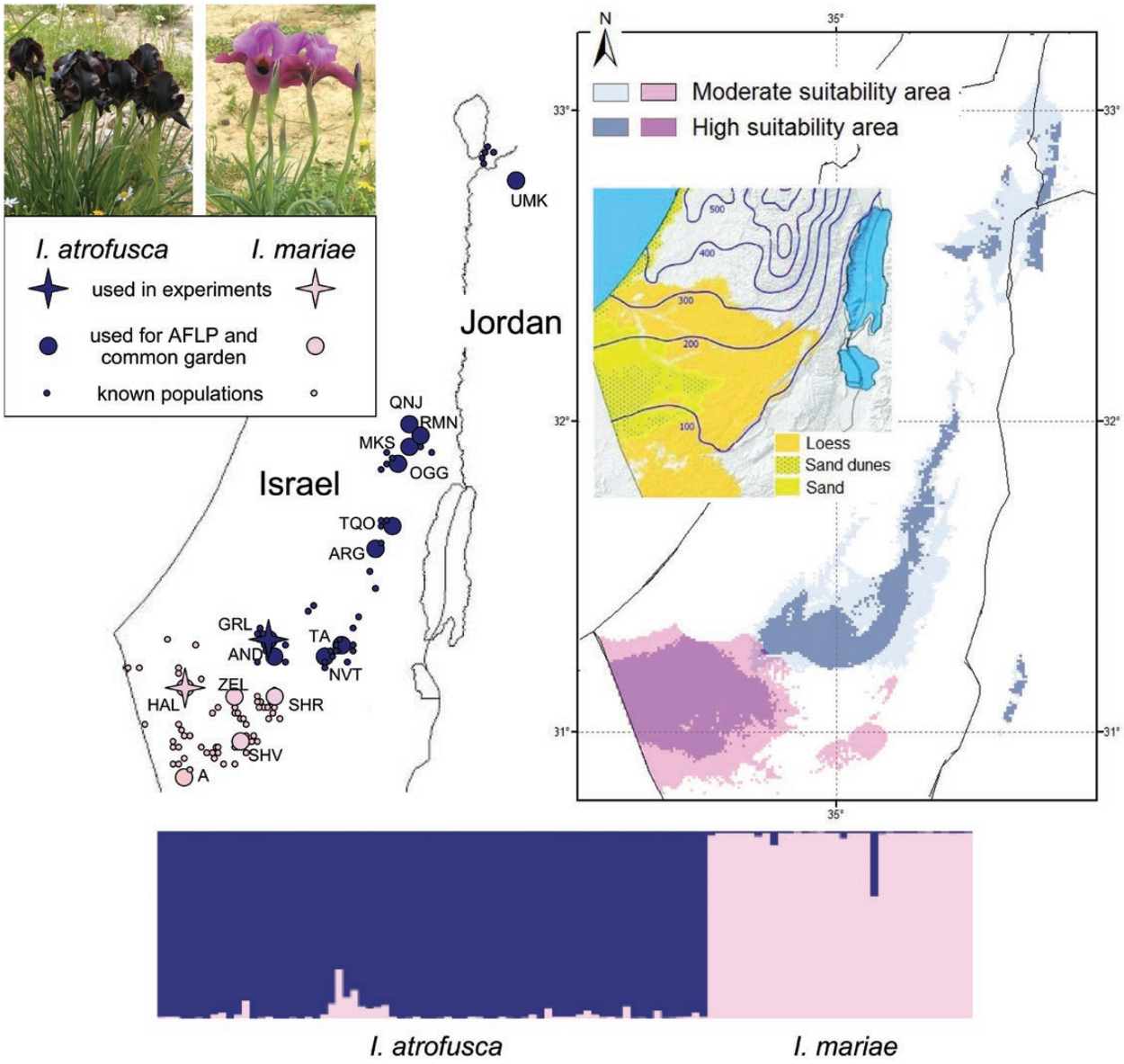

Figure 1. Top left panel, map showing the distribution of *Iris atrofusca* (dark purple) and *Iris mariae* (pink) populations in Israel (after Shmida & Pollak, 2007); the populations sampled for experiments and analyses of genetic and phenotypic variation are indicated. Top right panel, predicted ranges of the two species based on climate + soil data according to an ecological niche modelling analysis; inset shows the distribution of loess, sand dunes and sand in south and southwest Israel where the two iris species occur (isohyet lines indicate rainfall in millimetres per annum). Bottom panel, model-based ancestry, assuming admixture for each individual, with each individual represented by a vertical line partitioned into two coloured segments that represent the individual's assignment to two genetic groups.

appendages called arils that are assumed to attract ants (as a food source), although there is currently no observational evidence for the latter in the literature.

The two species grow in different environmental conditions with respect to rainfall and soil type, with populations distributed discontinuously. Relatively large and dense populations of *I. atrofusca* are located on loess in the northern Negev Desert, where annual rainfall is 200–300 mm, with some small and isolated populations occurring on loess or rendzina soils eastwards into the Judean Desert and northwards into eastern Samaria and southern Golan, where annual rainfall is 200–500 mm (Fig. 1) (Volis *et al.*, 2016). In contrast, *I. mariae* grows in more arid conditions on stabilized desert sand dunes in the western Negev Desert, where annual rainfall is 100–200 mm (Fig. 1).

Seeds and rhizomes of *I. mariae* were collected in 2007 from plants taken from Halutsiot dunes in the western Negev Desert (HAL population), where the natural habitat of the iris has been destroyed for agricultural development (Fig. 1). Seeds and rhizomes of *I. atrofusca* were also collected in 2007 from an area of ground to be used as a road-building strip for new railroad tracks, in the Goral hills, northern Negev (GRL population) (Fig. 1). Seeds were kept in paper bags at room temperature before being used along with rhizomes in reciprocal transplant, germination, soil type and soil type × water availability experiments. In addition, rhizomes from the live collection of *Oncocyclus* irises maintained at the Bergman Campus, Ben-Gurion University of the Negev, Beer Sheva, were used for genetic analysis, a common garden experiment and as pollen donors in a crossing experiment.

Molecular genetic divergence

Molecular genetic divergence between the species was examined using AFLPs. Leaf samples were collected from individuals in 11 *I. atrofusca* populations and five *I. mariae* populations covering the distributions of each species (Fig. 1; Supporting Information, Table S1). In total, 105 individuals were sampled, with individuals separated by ≥ 5 m at each location. DNA was extracted from fresh leaves using the protocol of Doyle & Doyle (1987). DNA quality and concentrations were checked on 1% agarose gels by comparison to a known standard. AFLP analysis was conducted using the protocol described by Volis *et al.* (2016). In total, 302 AFLP loci were identified and used in analysis.

The Bayesian clustering program STRUCTURE (Pritchard *et al.* 2000) was used to estimate the number of genetic groups (K) and to assign individuals to such groups. The admixture model was used, with allele frequencies correlated among populations for K values from one to ten, with ten runs for each value of K. Each run comprised a burn-in of 100 000 iterations followed by 100 000 Markov chain Monte Carlo (MCMC) iterations. The optimal number of genetic groups was determined from a plot of ΔK against K (Evanno *et al.*, 2005) generated using Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) (Supporting Information, Fig. S1). A bar graph showing the assignment of individuals to genetic groups and levels of admixture was produced by STRUCTURE and visualized and edited using ILLUSTRATOR software (https://www.adobe.com/illustrator).

To examine the partitioning of genetic variation further, GENALEX v.6.0 (Peakall & Smouse, 2006) was used to perform an analysis of molecular variance (AMOVA) to assess the hierarchical genetic structure within and between the two species. In addition, the relationship between population pairwise geographical and Nei's unbiased genetic distance was analysed by the Mantel test, for each species separately, and a unweighted pair group method with arithmetic mean (UPGMA) cluster dendrogram was produced from Nei's unbiased genetic distance using the NTSYS program (Rohlf, 1998).

Phenotypic divergence

A common garden experiment was conducted to examine phenotypic divergence between the two species. In September 2008, 126 plants, representing eight and three populations of *I. atrofusca* and *I. mariae*, respectively (90 plants of *I. atrofusca* and 36 of *I. mariae*), were planted individually in 3 L pots placed randomly on tables inside a net house on the Bergman Campus, Beer Sheva. Pots were filled with a soil mix comprising equal parts of gravel, loess and sand. Plants were watered regularly via drip lines throughout the experiment (from September to April).

Leaf length, thickness, width and curvature (b/a ratio; Fig. 2A) were measured on two leaf fans of most plants (72 *I. atrofusca* and 30 *I. mariae*) and on one leaf fan of each remaining plant. Data for plants in which two leaf fans were measured and at least four individuals were present in a population (thus representing 202 leaf fans of 101 individual plants) were subjected to nested ANOVA. Independent variables were species, population (nested within species) and individual (nested within population and species). These were designated as random effects in the REML method for fitting models with random effects. In addition, a principal components analysis and a discriminant analysis (DA) were conducted on data of all plants after averaging the two records per leaf trait for individuals in which two leaf fans were measured.

For 35 plants that had flowered by the end of the experiment (20 *I. atrofusca* and 15 *I. mariae* plants), the time to first flowering was recorded and analysed by Cox proportional hazards regression, with species treated as an independent variable. All statistical analyses were conducted using STATISTICA software (StatSoft Inc., 2004).

Species distribution modelling

We used species distribution modelling to predict the geographical distribution of suitable habitat for each species. For climate, we used the 19 'Bioclim' variables from WorldClim v.1.4 (Hijmans *et al.*, 2005) with a resolution of 30″ latitude/longitude (~1 km^2 at the ground level). For soil variables, i.e. soil bulk density, pH, soil depth, total organic carbon, texture, sand fraction, silt fraction, clay fraction and cation exchange capacity of soil, we accessed the Harmonized World Soil Database (Fischer et al., 2008).

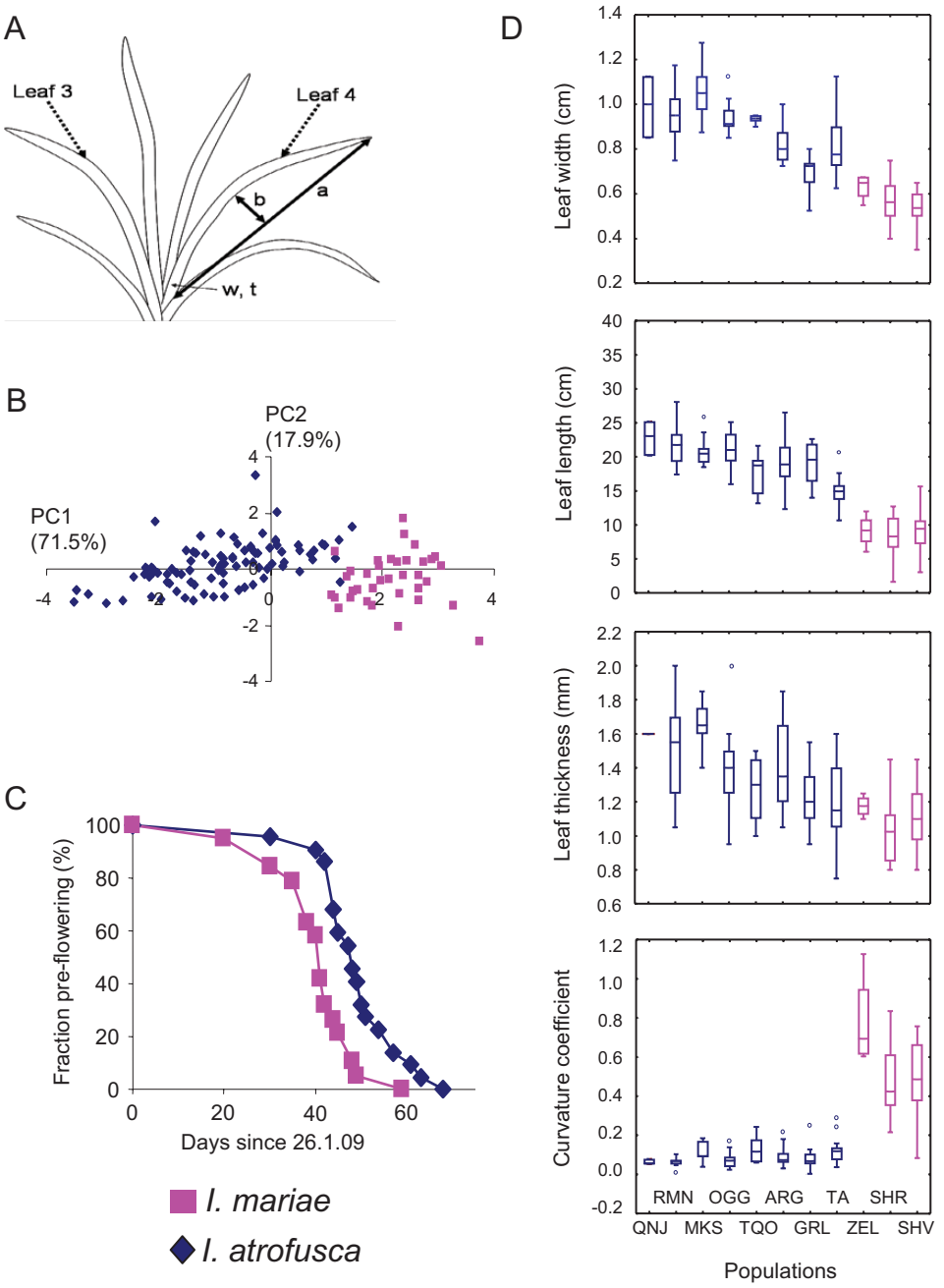

Figure 2. Phenotypic variation in common garden conditions. A, the four leaf characters recorded on a leaf fan: *a*, length; *b*/*a* ratio, leaf curvature coefficient; *t*, thickness; and *w*, width. B, plots of principal components analysis scores per individual for principal components (PC) 1 and 2, based on leaf traits. C, days to onset of flowering in the two species. D, box-and-whiskers plots of eight and three populations of *Iris atrofusca* and *Iris mariae*, respectively, for the four leaf traits. Populations are arranged with decreasing latitude.

A total of 504 and 489 occurrence points for *I. atrofusca* and *I. mariae*, respectively, were compiled using records from the Israel Nature and Parks Authority database (2013). To predict the potential niche for each species, we used MAXENT v.3.3.3 (Phillips *et al.*, 2006; Phillips & Dudik, 2008), withholding 25% of the occurrence data for model evaluation and setting the number of iterations to 500. Cross-validation through jackknifing was used to calculate the contribution of each environmental variable in the final model. Model predictions in ASCII grid layer format were loaded into ArcGIS v.10.2 to produce the species predicted

distribution maps. Suitability values of map grid cells produced by MAXENT and ranging between zero and one were converted into Boolean values of 'suitable habitat' and 'unsuitable habitat', with two thresholds of suitability: moderate (0.4) and high (0.6).

Reciprocal transplant analysis

In September 2011, three sets of 24 rhizomes of each of *I. atrofusca* and *I. mariae* produced from seed of plants originally collected from the Halutsiot dunes (HAL) and Goral hills (GRL) sites, respectively, were reciprocally planted into the populations at these two sites. The 24 rhizomes represented 1-year-old plants classified into seven size classes: 0.2–0.3 g (12), 0.3–0.4 g (2), 0.5 g (2), 0.6–0.8 g (3), 1 g (2), 1.2–1.7 g (2) and 2 g (1). Rhizome size in these species was previously shown to be directly related to fitness, i.e. probability of survival and flowering (Volis *et al.*, 2010; S. Volis, unpublished data). Over-representation of small rhizomes and the small sample size used in the study were attributable to limitations of available material. Rhizomes were randomly placed into cells of 24-cell plastic trays (35 cm × 26 cm × 16 cm) filled with local soil. Two trays, one with *I. atrofusca* and another containing *I. mariae*, were then buried adjacent to each other, to surface level. Three replicate pairs of such trays were buried at each site, with replicate pairs positioned ≥ 10 m apart. In May 2012, after aboveground biomass had dried out, trays were dug out and surviving rhizomes counted and weighed.

A split-plot ANOVA and a Kolmogorov–Smirnov test were used to analyse rhizome weight. In the ANOVA, plant origin and transplant location were taken as fixed effects, whereas replicate pairs were treated as a random effect owing to them experiencing different microenvironments within sites. The number of surviving plants was analysed by a test of independence.

Effect of soil type and water availability on plant performance

Several experiments were conducted to test the effects of differences in soil type and water availability on the relative performance of both iris species at different life-history stages. Taken overall, these experiments provided no firm evidence of differences in performance that reflected local adaptations to the respective habitats of the species. A full description of these experiments, their results and interpretation is provided in the Supporting Information.

Crossing experiment

To test for intrinsic postzygotic reproductive isolation between the two species, a crossing experiment was conducted. Before the experiment, large rhizomes comprising many connected genetically identical ramets were dug out in 2007 from two populations, HAL and GRL (representing *I. mariae* and *I. atrofusca*, respectively), and planted separately in isolated sections 50 cm (length) × 50 cm (width) × 30 cm (height) of plastic boxes filled with the original soil (sand for HAL and loess for GRL). These plants acted as mother plants in the experiment. The boxes were placed in a net house on the Bergman Campus, Beer Sheva, and plants were watered regularly during the growing season (from October until April) depending on ambient conditions. Over 4 years, flowers produced on these mother plants were pollinated with pollen from other plants originating from: (1) the same population; (2) a different population of the same species; or (3) the other species. Pollination was performed by depositing anthers on a stigma of a mother plant. Seed set and weight were recorded for each pollination. Seeds were stored at 4 °C in a refrigerator until September 2011, before sowing in 3 L pots filled with a mixture of sand and loess. Over three consecutive seasons, the number of emerging seedlings was recorded.

Seed set, weight and percentage germination were calculated for each type of cross-pollination per mother plant and converted into a measure of relative performance using the equation $RP = w_i / \max(w_{w\text{-}p}, w_{a\text{-}p}, w_{i\text{-}s})$, where w_i stands for a performance trait attributed to a fruit of a particular mother plant that resulted from a particular type of cross. For each species separately, a Wilcoxon matched pairs test examined differences for within-population ($w_{w\text{-}p}$), among-population ($w_{a\text{-}p}$) and interspecific ($w_{i\text{-}s}$) crosses in seed set, total weight of the seeds and cumulative percentage of germinated seeds over three consecutive seasons.

In addition, a comparison was made of the performance of 12 1-year-old rhizomes of parent plants from each of the GRL and HAL populations and their F1 offspring. These rhizomes were planted before the rainy season began, in November 2012, on the Bergman Campus, Beer Sheva, at a location with natural soil and vegetation typical of the northern Negev Desert. At the start of the experiment, rhizomes were individually weighed and randomly placed into cells of 12-cell plastic trays (26 cm × 17 cm × 16 cm) filled with sieved local loess soil and buried adjacent to each other to surface level. Plants grew under natural precipitation throughout the growing season and remained dormant during the summer. In September 2013, rhizomes were extracted from trays and weighed, and individual rhizome growth rate was calculated and used as a measure of fitness (e.g. Johnston *et al.*, 2001).

Strength of reproductive isolating barriers

The strength of reproductive isolating (*RI*) barriers exhibited by each species was calculated for: (1)

ecogeographical isolation calculated from the proportion of a species distribution predicted to be shared with the other species based on climate and soil variation in the species distribution modelling analysis; (2) temporal isolation based on flowering time differences recorded in the common garden phenotypic divergence experiment; (3) immigrant inviability measured in the reciprocal transplant experiment; and (4) intrinsic postzygotic incompatibility measured in terms of F1 hybrid seed set and viability (assessed by germination). Methods of calculation were based on those described by Sobel (2014) and Sobel & Chen (2014).

RESULTS

Molecular genetic divergence

AMOVA revealed that 19% of AFLP variation was distributed between species, 10% between populations within species and 71% within populations. Clear genetic divergence between the two species was apparent from both the UPGMA dendrogram generated from Nei's unbiased genetic distances between populations (Supporting Information, Fig. S2) and the STRUCTURE analysis assuming admixture (Fig. 1). One individual of *I. mariae* and two of *I. atrofusca* exhibited mixed ancestry (Q values < 0.9), indicating either a hybrid origin or incomplete lineage sorting of some AFLP markers. Mantel tests revealed a significant correlation between genetic and geographical interpopulation distances only in *I. atrofusca* ($r = 0.462$, $t = 2.24$, $P = 0.018$).

Common garden phenotypic divergence

Nested ANOVAs (Table 1) showed that the two species differed greatly in leaf width, length and curvature and, to a lesser extent, in leaf thickness. Discriminant analysis (Table 1) and principal components analysis (Fig. 2B) also showed that the two species were clearly differentiated by these four traits, with 125 of the 126 plants correctly classified to species according to DA. Plants of *I. atrofusca* possessed leaves that were larger (both longer and wider), thicker and less curved than leaves of *I. mariae* plants (Fig. 2D). The decrease in leaf size and thickness with latitude observed from comparing *I. mariae* with *I. atrofusca* was also evident at the intraspecific level within *I. atrofusca* (Fig. 2D).

In addition to leaf differences, the two species differed in flowering time (Wald statistics 11.55, $P < 0.001$), with *I. mariae* flowering earlier and having a shorter flowering season than *I. atrofusca*. However, there was considerable overlap in the flowering times of the two species (Fig. 2C).

Species distribution modelling

The accuracy of species distribution model prediction based on climate and soil, and on climate only, was high (area under the receiver operating characteristic curve > 0.99) and showed that predicted and current ranges largely coincided for *I. mariae*, whereas the predicted range of *I. atrofusca* extended beyond its current range, indicating that its realized niche is smaller than its potential one (Fig. 1). The area predicted to be shared by both species based on climate plus soil variation was narrow, largely in agreement with their current allopatric distributions. Thus, MAXENT predicted that shared highly suitable habitat composed 0.05 and 0.08% of the *I. atrofusca* and *I. mariae* distributions, respectively, whereas for climate only, it composed 0.58 and 0.66% of each species' range, respectively (Fig. 1).

The contributions of climate and soil variables to the climate and soil niche prediction of each species are presented in the Supporting Information, Table S2. Among soil variables, silt fraction (18.4%), bulk density of soil (9.0%), pH (3.2%) and total organic carbon (1.0%) were the most important in defining the predicted niche of *I. mariae*, whereas in *I. atrofusca*, sand (2.4%) and clay (1.2%) were the most important, although of

Table 1. Results of a discriminant analysis and nested ANOVA, showing differences between and within species for each of four leaf traits measured on plants raised in common garden conditions

Trait	Discriminant analysis		Variance components in nested ANOVA			
	Partial Wilks' Λ	F-remove (4,121)	Species	Population	Individual	Residual
Width	0.68	57.4***	60.0	15.4	4.4	20.2
Thickness	0.87	17.6***	16.2	28.7	19.5	35.6
Length	0.89	14.5***	73.7	8.9	10.7	6.7
Curvature	0.75	39.5***	79.5	0.9	7.6	12.0

For each trait, the contribution (partial Wilks' Λ) and its significance in the model that best discriminates the two species in the discriminant analysis, together with variance components (as a percentage of the total) for species, population, individual and residual effects extracted from nested ANOVAs are presented.
***$P < 0.001$.

minor overall effect. For both species, the contributions of climate variables were much greater than those of soil variables, with temperature seasonality (26.2%), mean temperature of the warmest quarter (15.1%) and precipitation of the coldest quarter (11.8%) being the most important for *I. mariae*, whereas precipitation of the wettest quarter (38.8%), precipitation seasonality (17.3%) and minimum temperature of the coldest month (11.7%) were the most important for *I. atrofusca*.

Reciprocal transplant analysis

Split-plot ANOVA revealed a significant effect of site, plant origin and their interaction on final rhizome weight of surviving plants in the transplant experiment ($F_{1,187}$ = 142, 20 and 18, $P < 0.001$, 0.01 and 0.01, respectively). The mean rhizome weight of surviving plants of *I. atrofusca* (2.1 ± 0.3 g) was greater than that of *I. mariae* (1.4 ± 0.1 g) in the native *I. atrofusca* site, where large rhizomes were produced by both species, but there was no difference between species for the same trait in the native site of *I. mariae*, where both species produced much smaller rhizomes (0.4 ± 0.1 g for both species). This apparent advantage of *I. atrofusca* in its native site was offset, however, by its poorer survival there, relative to *I. mariae*. *Iris atrofusca* also showed lower survivorship than *I. mariae* in the native site of *I. mariae.* Here, the survivorship of both species increased proportionally, and the species × site interaction was non-significant ($\chi^2 = 0.18$, $P > 0.10$). A Kolmogorov–Smirnov test showed no differences in the size (rhizome weight) distributions of surviving plants of the two species within either site ($D = 0.375$ and 0.500, $P > 0.10$; Fig. 3). Taken overall, these results provide no convincing evidence of home advantage for either species, nor reproductive isolation attributable to immigrant inviability (see 'Strength of reproductive isolating barriers' subsection, below).

Crossing experiment

The Wilcoxon matched pairs test revealed no difference between within-population, among-population and interspecific crosses for seed set, total weight of seeds produced or cumulative percentage germination over three consecutive seasons for *I. mariae* (Fig. 4). For *I. atrofusca*, although there was no difference for germination percentage, the seed set and total weight of seeds were higher for interspecific than within-population crosses ($Z = 2.27$, $P = 0.02$ for both). In addition, the growth rate of F1 rhizomes was not inferior to those of either parent species (0.06 ± 0.23, 0.01 ± 0.30 and 0.27 ± 0.30 for *I. atrofusca*, *I. mariae* and F1 progeny, respectively). In fact, the opposite (possibly suggesting hybrid vigour) was indicated, although the difference lacked significance. These results show that the two species are interfertile and that there is no evidence of intrinsic postzygotic incompatibility existing between them, at least at the F1 stage.

Strength of reproductive isolating barriers

Estimates of *RI* calculated for each species in turn (Table 2) showed that although ecogeographical isolation was very strong for both species (*RI* > 0.9), other isolating barriers were either weak or absent. Thus, estimates of immigrant inviability calculated from survivorship data in the reciprocal transplant analysis indicated that there was no barrier to *I. mariae* immigrants surviving in the native site of *I. atrofusca* (*RI* = –0.026), whereas that for *I. atrofusca* immigrants surviving in the native site of *I. mariae* was weak (*RI* = 0.289). Although there was a difference in the start and duration of flowering time between the two species, estimated *RI* values for temporal isolation were low for each taxon (0.237 and 0.256 for *I. atrofusca* and *I. mariae*, respectively). Finally, for neither species was there evidence for intrinsic postzygotic isolation based on F1 seed set and germination. For both these traits, very low or negative *RI* values were estimated.

DISCUSSION

Speciation involves the establishment of isolating barriers between diverging populations that may be spatial and/or biological, thus limiting or preventing gene exchange and favouring the maintenance and further development of genetic divergence (Coyne & Orr, 2004). Our analyses of variation showed a clear genetic separation between a pair of nascent, morphologically distinct iris species, *I. atrofusca* and *I. mariae*. Although a few admixed individuals were detected by STRUCTURE analysis of AFLP variation, their occurrence could reflect incomplete lineage sorting of some AFLP markers rather than hybridization and introgression. Based on morphology, no hybrids between the two species have been observed in the wild (Avishai & Zohary, 1980; S. Volis, personal observations). The clear phenotypic and genetic divergence of the two species, plus the lack of hybrids between them, indicate that they show strong reproductive isolation from each other. The two species have allopatric distributions and, consequently, show strong geographical isolation from each other. However, our analyses failed to detect further strong isolating barriers between the species. Thus, we obtained evidence for weak temporal isolation attributable to differences in flowering time, no evidence for local adaptation and immigrant inviability, and no evidence for intrinsic postzygotic isolation based on measures of F1 hybrid

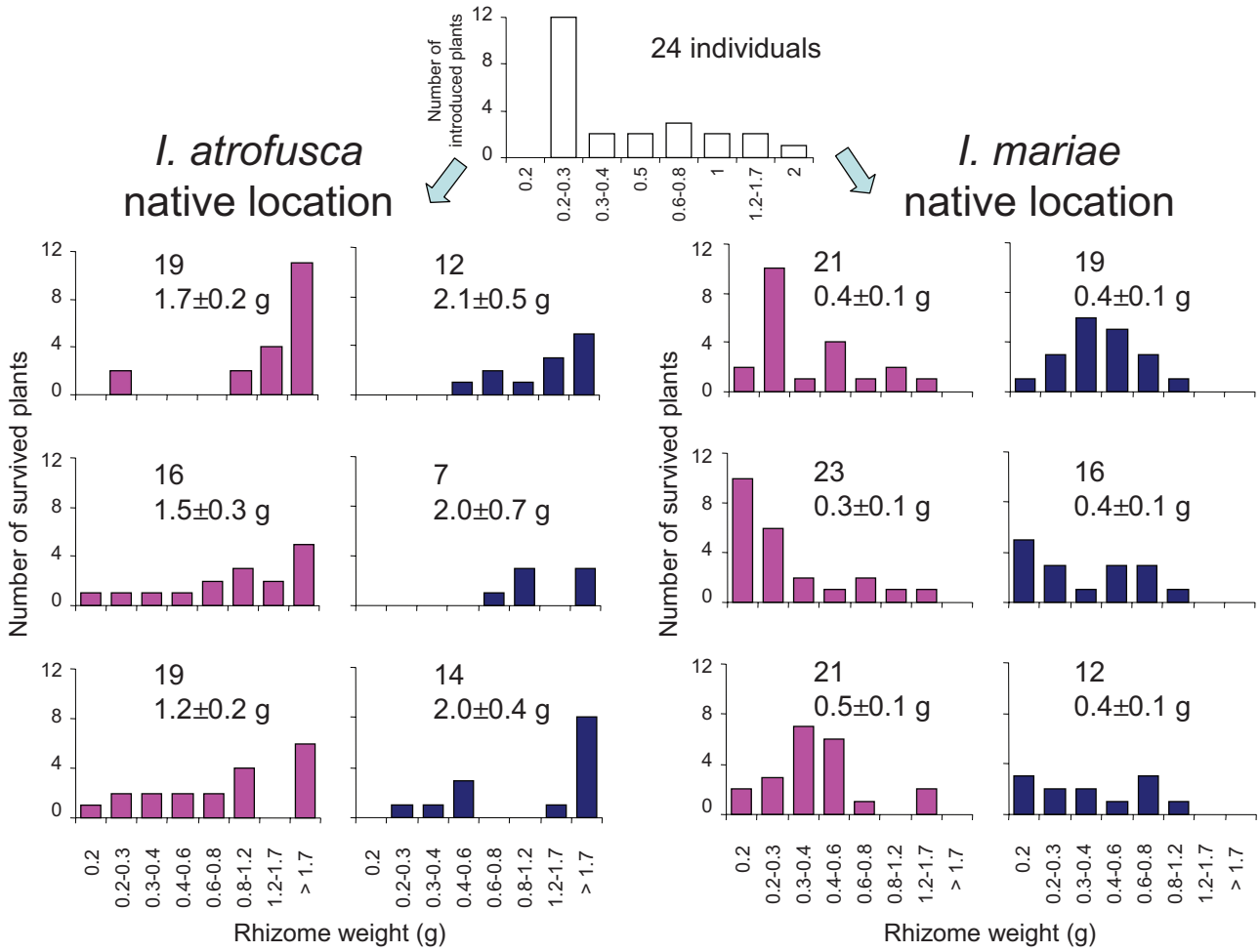

Figure 3. Histograms for rhizome weight of surviving plants of *Iris atrofusca* (dark blue) and *Iris mariae* (pink) within each of three pairs of trays transplanted into the native sites of each species. Each tray of a pair initially contained 24 rhizomes of either *I. atrofusca* or *I. mariae* representing seven different size classes. For each histogram, the total number of surviving plants and mean weight of their rhizomes ±SE are given.

seed set and germination. The lack of evidence for local adaptation was particularly surprising given that the species occupy habitats that differ markedly in climate and soil type.

Strong ecogeographical isolation but absence of local adaptation

Sobel (2014) emphasized that closely related species are often allopatrically distributed owing to the effects of historical or ecological factors, or a combination of both. He also advocated the use of species distribution modelling to indicate potential ecological factors that influence species distributions and, in turn, how such variation might affect potential gene flow between species. Using this approach, he showed that several recently diverged pairs of *Mimulus* species exhibit substantial levels of ecogeographical isolation. However, although species distribution modelling may indicate that species are differentially adapted to the particular habitats that characterize their different distributions, experiments are required to prove this.

Our species distribution modelling analysis indicated that the area predicted to be shared by both iris species based on climate plus soil variation was very narrow. From this, it was estimated that both species exhibited strong ecogeographical isolation from each other ($RI > 0.9$). To test for possible ecological isolation owing to local adaptation, we conducted a series of experiments in which a range of environmental conditions in terms of soil type and water supply were varied and examined the performance of each species at different stages of the plant life cycle (Supporting Information). All these experiments failed to show convincing evidence of local adaptation with regard to: seed germination;

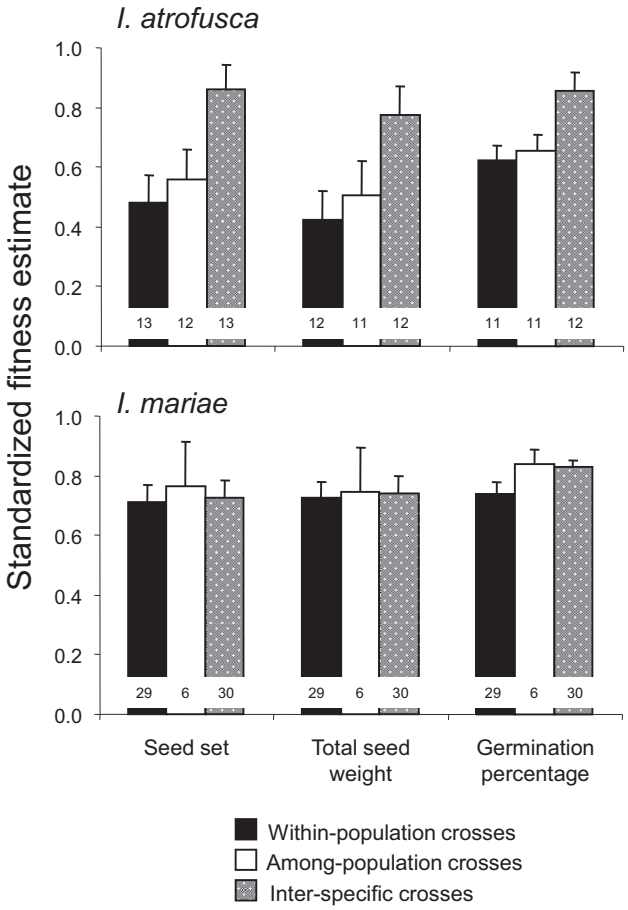

Figure 4. Standardized fitness estimates for seed set, total seed weight and germination percentage with SE for within-population, among-population and interspecific crosses performed on *Iris atrofusca* and *Iris mariae* mother plants. The numbers inside bars indicate the number of pollinations performed.

rhizome growth, leaf fans, and survival of plants raised from rhizomes produced by adult plants; and rhizome weight and survival of plants raised from rhizomes of 1- and 2-year-old plants. We also tested for home advantage in the field by conducting a reciprocal transplant experiment and detected no overall home advantage for *I. atrofusca* (reflected in a negative *RI* for immigrant inviability in its habitat) and only a small home advantage for *I. mariae* (reflected in a positive but relatively small *RI* for immigrant inviability in its habitat; Table 2).

A surprising finding of our experiments that examined the effects of soil type and water availability (Supporting Information) was the better performance of young plants of both species on rendzina soil, i.e. a soil type that is not the primary one on which *I. atrofusca* is found or that *I. mariae* ever occurs on. It is possible that in the wild there is a trade-off between optimal plant growth achieved on rendzina vs. optimal biotic interaction (e.g. competition) on the other soil types on which the two species normally occur. This might exclude the species from occurring either often (in the case of *I. atrofusca*) or entirely (in the case of *I. mariae*) on rendzina soils in the wild. The two species we studied represent the extreme southern edge of the distribution of *Oncocyclus* irises, which extends from Central Asia, Iran, the Caucasus and Turkey to the Near East, reaching its southwestern limit in the Negev Desert. It is likely, therefore, that the two species originated from ancestral forms native to the more favourable northern environment, having higher precipitation and a soil type often of a rendzina kind. Their high performance on rendzina soils noted in our study might, therefore, be a legacy of their origin from northern ancestral stock well adapted to rendzina soils.

Taken overall, our transplant experiment, and other studies that examined the performance of each species in relation to soil types and precipitation, yielded no strong evidence for local adaptation. Consequently, our results indicate that the allopatric distributions of these iris species are not attributable to differences in adaptation to local conditions. Other possible explanations for their allopatric distributions are that historical demographic effects and/or selection that no longer operates might have generated them. For example, it is feasible that both species expanded their ranges southwards from more northerly locations in the past and, for a time, occurred sympatrically. However, owing to the differential effects of competition with other species or extirpation by human activities, or as a result of the direct or indirect effects of historical climate change, they have eventually come to occupy different areas in southern Israel. Currently, we have no information on possible differences between the species in competition with other species that might result in them having highly divergent geographical distributions. This is clearly a gap in our knowledge that requires filling. With regard to the possibility of differential extirpation owing to human activities, although humans appear to contribute to the current distribution of at least one species (*I. atrofusca*; Volis *et al.*, 2010), large-scale extirpation, resulting from human activities, of each species from areas occupied by the other species seems highly improbable.

Finally, it is possible that during a recent Pleistocene glacial period the two species diverged from each other in different glacial refugia in the eastern Mediteranean and, although they have expanded their ranges since that time, they are yet to become sympatric owing to low dispersal ability. Further analyses are required to test this possibility.

Table 2. The strength of reproductive isolating barriers between the two iris species

Isolating barrier	Strength of isolation (*RI*)		Method of calculation
	I. atrofusca	*I. mariae*	
Ecogeographical isolation:			
Overlap of predicted suitable area	0.950	0.921	$RI = 1 - \left(\frac{S}{S+U}\right)$; where $S + U = 1$
Ecological isolation:			
Immigrant inviability (Reciprocal transplant experiment)	-0.026	0.289	$RI = 1 - 2 \times \left(\frac{H}{H+C}\right)$
Temporal flowering isolation (Phenotypic divergence experiment)	0.237	0.256	$RI = 1 - \left(\frac{S}{S+U}\right)$; where $S + U = 1$
Intrinsic postzygotic isolation:			
F1 seed set	-0.268	0.014	$RI = 1 - 2 \times \left(\frac{H}{H+C}\right)$
F1 seed germination	-0.107	-0.024	$RI = 1 - 2 \times \left(\frac{H}{H+C}\right)$

The first column under 'Strength of isolation (*RI*)' concerns isolation of *I. atrofusca* from *I. mariae* gene flow, and the second column concerns isolation of *I. mariae* from *I. atrofusca* gene flow.
Abbreviations: C, fitness of resident or offspring of conspecific cross; H, fitness of immigrant or offspring of heterospecific cross; S, shared attribute (area or flowering time); U, unshared attribute (area or flowering time).

Absence of intrinsic postzygotic incompatibility

Using in vitro embryo culture, Avishai & Zohary (1980) previously showed that *Oncocyclus* irises can be crossed to produce fully fertile interspecific hybrids. Our crossing experiment confirmed a lack of intrinsic incompatibility between *I. atrofusca* and *I. mariae* in that there was no reduction in seed set and seed viability (assessed by germination) resulting from interspecific crosses as compared with intraspecific crosses. In fact, when *I. atrofusca* was the female parent, interspecific crosses resulted in higher seed set relative to within-species crosses, yielding a negative *RI* value for intrinsic postzygotic incompatibility based on F1 seed set of *I. atrofusca* plants (Table 2). Thus, speciation and divergence between these two iris species does not appear to have involved selection against the formation of hybrids after fertilization up to and including the seed germination stage. Furthermore, our comparison of the growth rate of rhizomes of parental and F1 origin indicated no reduced growth rate of F1 hybrids relative to that of the parental species during the first year of growth. Our tests of intrinsic postzygotic incompatibility between the two species did not go beyond the F1 hybrid generation, and it is possible, therefore, that reductions in hybrid fitness might become apparent in subsequent generations (for example, see Brennan *et al.*, 2014). Future studies should test this.

Some caveats concerning the absence of local adaptation

Despite our experiments providing little or no evidence of local adaptation and ecological isolation between the two iris species, species distribution modelling strongly indicated that the two species were likely to be ecologically differentiated. This, together with the limitations of our experiments that tested for local adaptation, in terms of sample sizes and number of populations tested, suggests that we cannot rule out the possibility that the allopatric distributions of the two species are governed by their different ecologies and adaptation to divergent climate and soil type. Our experimental analyses of local adaptation may thus be regarded as only preliminary, and it is possible that if other ecological variables were examined, for example, related to herbivory and competitive interactions with other plants, evidence for local adaptation and ecological isolation might be detected.

Conclusions and future directions

Our study suggests that in the case of the two iris species investigated, genetic and phenotypic divergence has occurred in the absence of any strong genetic incompatibility or ecological isolating barrier revealed by our analyses. Despite this, the two species have different geographical distributions that reflect contrasting ecological conditions. Future work might involve more extensive reciprocal transplant analyses over more sites

and years than were attempted in the present study, to examine more comprehensively the possibility of local adaptation. In addition, it would be valuable to conduct a population genomic analysis on both species, which, coupled with phylogenetic analyses and coalescent simulations, would provide information on the origin of the two species, their demographic histories and the possibility that they have diverged at candidate loci for adaptation to the particular climates and soil types that characterize their distinctive habitats.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

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Table S2. Utilized environmental variables and their percent contribution in Maxent model climate + soil for the two study species
Fig. S1. Results of the Structure Harvester. The optimal number of genetic groups corresponds to the largest delta K value
Fig. S2. UPGMA cluster dendrogram based on Nei's genetic distance:
2. SUPPLEMENTARY EXPERIMENTS ON THE EFFECT OF SOIL TYPE AND WATER AVAILABILITY ON PLANT PERFORMANCE