

Reproductive isolation between populations of *Iris atropurpurea* is associated with ecological differentiation

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- **Background and Aims** Speciation is often described as a continuous dynamic process, expressed by different magnitudes of reproductive isolation (RI) among groups in different levels of divergence. Studying intraspecific partial RI can shed light on mechanisms underlying processes of population divergence. Intraspecific divergence can be driven by spatially stochastic accumulation of genetic differences following reduced gene flow, resulting in increased RI with increased geographical distance, or by local adaptation, resulting in increased RI with environmental difference.
- **Methods** We tested for RI as a function of both geographical distance and ecological differentiation in *Iris atropurpurea*, an endemic Israeli coastal plant. We crossed plants in the Netanya Iris Reserve population with plants from 14 populations across the species' full distribution, and calculated RI and reproductive success based on fruit set, seed set and fraction of seed viability.
- **Key Results** We found that total RI was not significantly associated with geographical distance, but significantly increased with ecological distance. Similarly, reproductive success of the crosses, estimated while controlling for the dependency of each component on the previous stage, significantly reduced with increased ecological distance.
- **Conclusions** Our results indicate that the rise of post-pollination reproductive barriers in *I. atropurpurea* is more affected by ecological differentiation between populations than by geographical distance, supporting the hypothesis that ecological differentiation is predominant over isolation by distance and by reduced gene flow in this species. These findings also affect conservation management, such as genetic rescue, in the highly fragmented and endangered *I. atropurpurea*.

Key words: Adaptive divergence, aster modelling, *Iris atropurpurea* Baker, *Iris* section *Oncocyclus*, isolation by distance, isolation by ecology, local adaptation, post-zygotic reproductive barriers.

INTRODUCTION

Speciation is often described as a continuous dynamic process, expressed by different magnitudes of reproductive isolation among groups in different levels of divergence (Nosil, 2012). While partial divergence does not necessarily lead to complete reproductive isolation, it can serve as the first step towards it. Thus, studying partial reproductive isolation can shed light on micro-evolutionary processes (Scopece *et al.*, 2010; Wang and Bradburd, 2014). Moreover, since new species emerge from populations that previously belonged to a single species, studying intraspecific reproductive isolation can aid in identifying the forces that reinforce or diminish reproductive isolation in different scenarios, while avoiding the effect of secondary reproduction barriers that appear in already reproductively isolated populations (Coyne and Orr, 2004; Nosil and Feder, 2013).

Reproductive isolation (RI) is the result of barriers at different life stages that cumulatively prevent gene transfer. In plants, different barriers may act in the pre-pollination stage, such as habitat separation, phenological disjunction (Savolainen *et al.*, 2006) and differential pollinator preference (Schemske and Bradshaw, 1999; Hoballah *et al.*, 2007). Later barriers act in the

post-pollination, pre-zygotic stage and may include pollen–pistil interactions and gamete incompatibility (Ramsey *et al.*, 2003; Kermicle, 2006). Post-zygotic barriers are those affecting life stages following fertilization, and can be further divided into intrinsic and extrinsic barriers. Intrinsic barriers cause low fitness of hybrids due to genetic incompatibility, such as Dobzhansky–Müller incompatibility (Fishman and Willis, 2001; Skrede *et al.*, 2008; Scopece *et al.*, 2010; Lowry *et al.*, 2014) or cytonuclear interactions (Fishman *et al.*, 2006). Extrinsic barriers cause low hybrid fitness in an ecological context as the hybrid is maladapted to the local environment, which is different from both maternal and paternal environments (Emms and Arnold, 1997).

The extent and permeability of various barriers differ among groups and species (Lowry *et al.*, 2008; Scopece *et al.*, 2010). Pre-zygotic barriers, such as pollinator preference and pollen–pistil interactions, have been suggested to be more important in creating reproductive isolation compared with post-zygotic barriers such as seed production and hybrid fitness (Lowry *et al.*, 2008). However, a recent review suggests that post-pollination barriers, either intrinsic or extrinsic, also play a major role in this process (Baack *et al.*, 2015). Post-zygotic barriers expressed

by reduced fitness of the offspring in crosses between plants from different populations of a single species are termed outbreeding depression (Schierup and Christiansen, 1996). Outbreeding depression that is related to intrinsic reproductive barriers can be the underlying mechanism of early stages of reproductive isolation (Etterson *et al.*, 2007; Scopece *et al.*, 2010).

Post-pollination pre-zygotic RI is determined by pollen–pistil interactions resulting from different genetic mechanisms, some of which are directly related to plant fitness (e.g. Levin, 2000, p. 11). The first three life-history stages that allow the examination of RI in the context of pollen–pistil interactions are the production of a fruit (fruit set), the number of ovules fertilized (seed set) and the development of zygotes to mature seeds (seed viability). These reproductive components were previously used to assess post-pollination pre-zygotic reproductive barriers in various studies of species that differ in their ecological niches (Rieseberg and Blackman, 2010; Burge *et al.*, 2013; Melo *et al.*, 2014; Wang *et al.*, 2015). Thus, testing for reproductive success of crosses in these three stages can hint at ecologically driven reproductive barriers.

Intraspecific divergence is the result of several processes that are not mutually exclusive (Thorpe *et al.*, 2008; Nosil, 2012). When spatially stochastic processes are governing divergence, it is expected that genetic differences between populations are negatively associated with pollen and seed dispersal distances and can be expressed in reduced hybrid fitness due to genetic incompatibility associated with the spatial distance between populations (Slatkin, 1993; Sapir and Mazzucco, 2012; Orsini *et al.*, 2013).

Alternatively, intraspecific divergence processes may be driven by divergent selection and local adaptation. Populations in different habitats are exposed to heterogeneous selection, and adaptation to the different habitats leads to differences in allele frequencies among populations. Consequently, local adaptation lowers the rate of gene flow among populations due to selection against maladapted migrants (extrinsic barrier) or to genetic incompatibilities (intrinsic barriers; Rundle and Nosil, 2005; Wang and Bradburd, 2014). This process is expected to be evident in correlations between the ecological distance, a measure of ecological habitat differentiation among populations, and the extent of reduction in hybrid fitness between crossed populations (Rundle and Whitlock, 2001).

The extent of divergence among populations can be revealed by correlating the extent of RI with either geographical or ecological distances. Although such correlation does not provide direct evidence of the extent of intraspecific divergence, it may suggest a connection between the rise of reproductive barriers and the process that drives it. In this study we present a test for association of RI with either geographical or ecological distances between populations of *Iris atropurpurea*. *Iris atropurpurea* belongs to the section *Oncocyclus* in the genus *Iris*, which was shown to be in the course of speciation (Sapir and Shmida, 2002; Wilson *et al.*, 2016). Populations of *Oncocyclus* species are highly fragmented and grow in a relatively wide range of ecological conditions, showing morphological correlation with environmental and morphological continuum among species (Sapir *et al.*, 2002). This suggests that *Oncocyclus* irises are incipient species that have not completely diverged. *Iris atropurpurea* is a plant endemic to the Israeli coastal region (Fig. 1A, B) and is listed as Critically Endangered in the IUCN

red list of threatened species (Sapir, 2016). In a preliminary simulation model that considered local adaptation and gene flow among populations of *I. atropurpurea*, it was predicted that there is a non-linear association between geographical distance among populations and RI (Sapir and Mazzucco, 2012). However, data in that study failed to support the theory, which led us to draw new hypotheses concerning spatial distance, ecological selection, connectivity and the speed of colonization.

We considered four possible patterns of population divergence, based on different scenarios of the species origin and historical dispersion (Fig. 2). The first scenario considers a relatively rapid expansion process in a stepping-stone fashion with low connectivity and no selection (Fig. 2A), which should lead to increased RI with geographical distance and is driven by genetic drift. The second scenario assumes slower colonization, which gives time to local divergence to act and create divergence that is related to both spatial and ecological distances (Fig. 2B). The third scenario assumes that the current distribution is the outcome of two groups of previously divergent species, where each group dispersed independently and is currently experiencing a secondary contact (Fig. 2C). Within each group, RI is expected to be low, while far populations should show highly reduced compatibility. Under this scenario, no continuous association is expected with either ecological or geographical distances. The fourth scenario is local adaptation following range expansion (Fig. 2D), which predicts that RI will be associated with ecological distance, where hybrids between populations from different habitats will be less fit. Under this scenario no geographical pattern is expected.

We crossed plants from populations of *I. atropurpurea* across its distribution in order to find the spatial and ecological pattern of reproductive isolations. Our results show that the generation of post-pollination reproductive barriers is affected more by ecological differentiation between populations than by geographical distance, supporting the hypothesis that ecological differentiation is predominant over geographical distance (scenario D in Fig. 2), suggesting that local adaptation, rather than range expansion, is responsible for population divergence in this species.

MATERIALS AND METHODS

Study species and site

Iris atropurpurea (Fig. 1A) is an endemic species occupying a geographical belt of about 70 × 12 km along the coast of Israel between Ashdod and Hadera, a highly populated and thus highly vulnerable habitat (Fig. 1B). It is a perennial rhizomatous plant, bearing a single dark-red flower on each stem. The flower is extremely large, nectarless and self-incompatible (Sapir *et al.*, 2005). The flowering season starts in January and the peak is in mid-February. Flower longevity is 4–7 d, the stigma is receptive throughout all floral life stages, and pollen remains viable even longer (Sapir *et al.*, 2005).

The Netanya population (coded NET) was used as the acceptor population in this study. It is a large and variable population consisting of thousands of individual plants and residing in a nature reserve located in the northern part of the species range (34°84' E, 32°28' N; altitude 27 m above sea level; see map in Fig. 1B), growing on a sandstone ridge covered by semi-stable

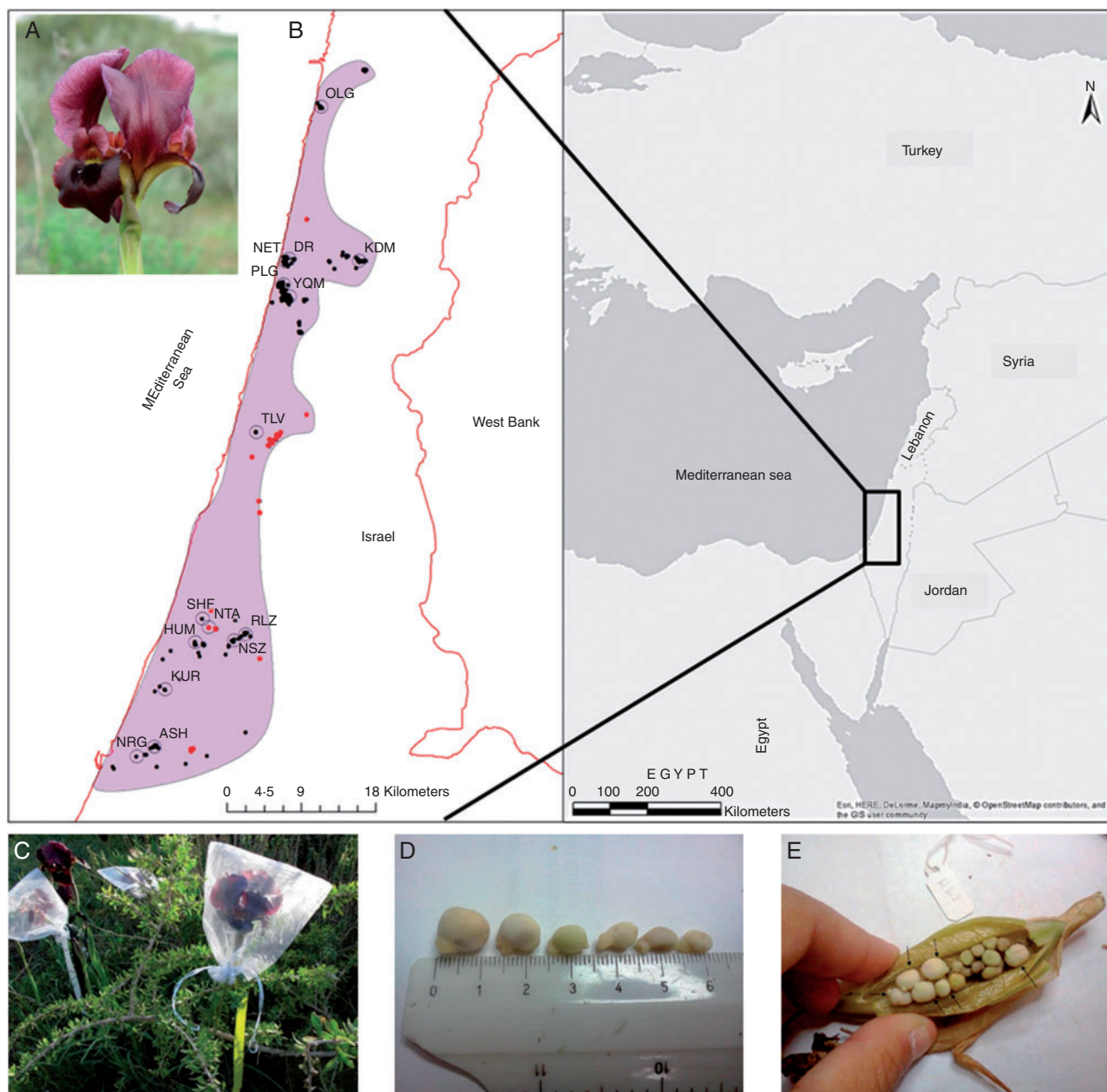


FIG. 1. (A) Flower of *Iris atropurpurea* in NET population. (B) Distribution map of *I. atropurpurea*. All known populations are marked as dots. Red dots represent extinct populations. Circles denote populations used in this study and their codes. (C) Flowers of *I. atropurpurea* covered to prevent natural pollination. (D) Premature seeds of *I. atropurpurea*, representing the range of seed sizes. (E) Premature seeds in a capsule. Six of the seeds (marked with arrows) are viable.

sand dunes. The local vegetation is dominated by the shrubs *Retama raetam* and *Lycium schweinfurthii*, alongside many annual species. Fourteen populations of *I. atropurpurea* were used as pollen donors (Fig. 1B, Table 1). These populations represent the entire range of the species' distribution, putatively representing the various ecological conditions across the full distribution along the coast.

Experimental design

Crosses were conducted in February 2013. Flowers in the acceptor population NET were covered prior to anthesis with fine

mesh bags to prevent pollination by insects (Fig. 1C). Flowers were then assigned randomly to be pollinated by pollen from any of the other 14 populations or by pollen from flowers within the NET population. As a control, additional flowers were marked and left uncovered for natural pollination. Anthers were collected from all 14 donor populations during several trips lasting 1 d, several times during the flowering season. The entire stamen was collected from the flowers with fine forceps, and kept in 1.5-mL plastic tubes with tissue paper scraps to reduce moisture and to prevent mould. Tubes were kept at 4 °C until used, usually up to 2–3 d later. At the onset of flowering, the covered flowers were pollinated with pollen collected from

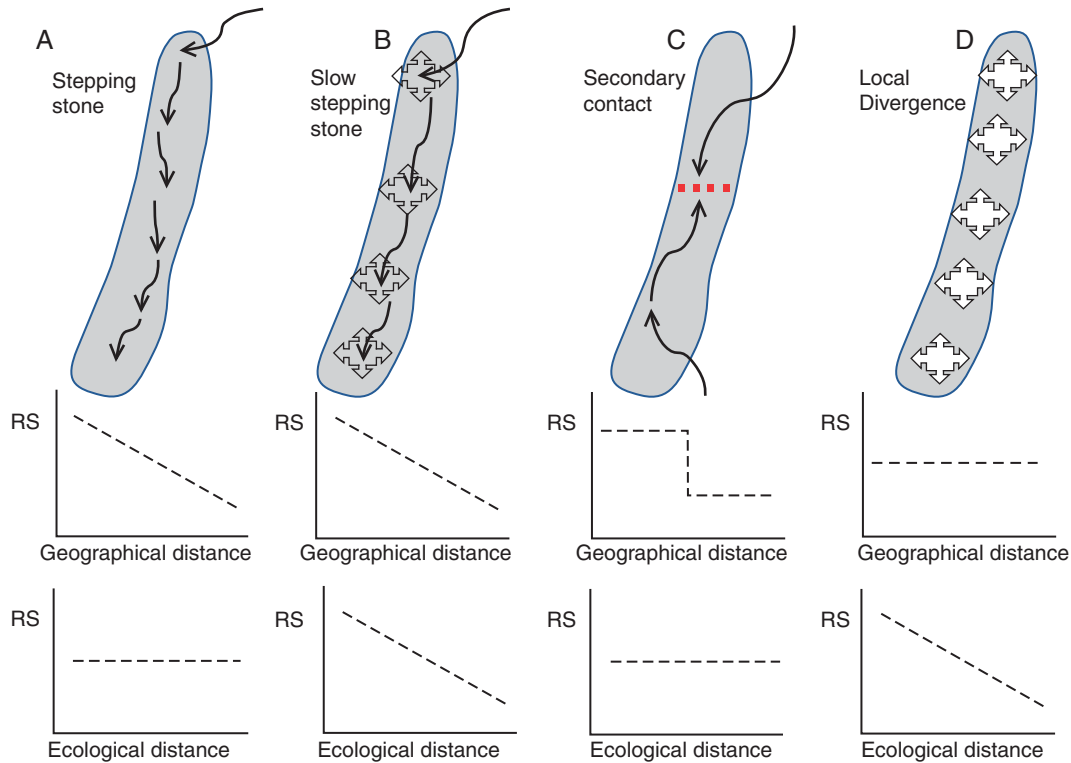


Fig. 2. Four hypothetical scenarios of the origin and expansion range of *Iris atropurpurea* along the coastal region of Israel. The bottom graphs present the expected effect of either geographical or ecological distance on reproductive success of crosses between population pairs.

TABLE 1. Populations of *Iris atropurpurea* used for crosses, and values of reproductive components measured. Population size is total size of the population based on estimation (#) or exact count (‡); Ecological distance is the ecological distance between populations, based on distance on the first ordination axis (see Materials and methods); n is the number of flowers treated in the population/treatment; Fruit set is the fraction of flowers that produced fruits; Seed set is the mean number of seeds produced in a capsule; Seed viability is the mean fraction of mature seeds out of the total seeds fertilized within a capsule

| Code | Site description | GPS coordinates | Population size | Geographical distance (km) | Ecological distance | n | Fruit set | Seed set | Seed viability |
|-------|---|------------------------|--------------------|----------------------------|---------------------|-----|-----------|----------|----------------|
| C | Netanya Iris Reserve, control flowers | 32°17'11 N, 34°50'27 E | >1000 [#] | 0 | 0 | 64 | 0.703 | 29.62 | 0.9 |
| NET | Netanya Iris Reserve, crosses within population | 32°17'11 N, 34°50'27 E | >1000 [#] | 0 | 0 | 57 | 0.842 | 38.21 | 0.896 |
| DR | Dora winter pond Nature Park | 32°17'19 N, 34°50'40 E | 500 [#] | 0.5 | 0.7 | 33 | 0.848 | 40.71 | 0.89 |
| PLG | Poleg Nature Reserve | 32°15'52 N, 34°50'17 E | 580 [‡] | 2.4 | 0.8 | 27 | 0.963 | 37.58 | 0.86 |
| YQM | Kibbutz Yaquim | 32°15'21 N, 34°50'40 E | 95 [‡] | 3.3 | 2.2 | 27 | 0.889 | 28.12 | 0.874 |
| KDM | Kadima Oak Nature Reserve | 32°17'14 N, 34°55'16 E | 227 [‡] | 7.6 | 3.8 | 30 | 0.867 | 28.27 | 0.908 |
| OLG | Givat-Olga, Gdor Nature Reserve | 32°25'44 N, 34°52'47 E | 66 [‡] | 16.3 | 0.01 | 29 | 0.69 | 30.00 | 0.872 |
| TLV | Tel Aviv, Afeka Caves | 32°07'45 N, 34°48'33 E | 3 [‡] | 17.6 | 2.3 | 10 | 1.00 | 36.30 | 0.784 |
| SHF | Shafdan Dunes | 31°57'25 N, 34°45'04 E | 350 [#] | 37.4 | 3.1 | 31 | 0.71 | 37.77 | 0.858 |
| NTA | Netaim ^A | 31°56'48 N, 34°45'55 E | 77 [‡] | 38.3 | 3.6 | 38 | 0.658 | 36.40 | 0.92 |
| RLZ | Rishon Le-Zion Iris Hill | 31°56'34 N, 34°47'49 E | 103 [‡] | 38.2 | 3.9 | 34 | 0.676 | 24.87 | 0.9 |
| NSZ | Nes Ziona Kurkar National Park | 31°56'12 N, 34°47'06 E | 182 [‡] | 39.1 | 3.8 | 34 | 0.794 | 27.15 | 0.903 |
| HUM | Humra Hill | 31°56'06 N, 34°44'35 E | 255 [‡] | 40.0 | 3.0 | 14 | 0.643 | 30.00 | 0.847 |
| KUR | Soreq Nuclear Research Center | 31°53'25 N, 34°42'36 E | 216 [‡] | 45.6 | 2.7 | 34 | 0.706 | 32.50 | 0.852 |
| ASH | Ashdod industrial area | 31°50'13 N, 34°41'40 E | 100 [#] | 51.6 | 3.1 | 12 | 0.75 | 31.00 | 0.938 |
| NRG | Nir Galim industrial area ^A | 31°49'43 N, 34°40'45 E | 10 [#] | 53.0 | 2.9 | 20 | 0.70 | 49.79 | 0.871 |
| Total | | | | | | 494 | 0.769 | 22.93 | 0.88 |

^APopulation extinct in the wild; plants maintained in Tel Aviv University Botanical Garden.

another donor population. Hand pollination was performed by brushing the anthers from the donor flowers against all three stigmas of the acceptor flower, with each acceptor flower receiving pollen from one donor plant. In the rare case that pollen of one donor plant was insufficient, extra pollen was added from another plant of the same population. All three stigmas were brushed with one donor anther, ensuring that >800 grains were deposited on each stigma to guarantee sufficient pollen for maximal fertilization (Watts *et al.*, 2013). Each pollinated flower was individually marked using a coded paper tag and with a coloured ribbon to ensure locating the plants after flowering. All treated flowers were covered immediately after the pollination treatment and left covered until full wilting, to prevent uncontrolled natural pollination.

A total of 494 flowers were used in Netanya for the experiment, with an average of 27 (range 10–38) flowers for each intra-population cross treatment. Fifty-seven flowers were crossed with pollen from flowers within the NET population, and 64 flowers were untreated and left open for natural pollination as the control (Table 1).

Components of reproductive success

All flowers were surveyed about 4 weeks after executing the pollination experiment at the end of March 2013. Flowers were recorded for whether they set a fruit or not. Capsules were collected and kept in paper bags at room temperature. Seeds were counted 2 weeks after collection. Non-viable or aborted seeds were identified based on size, colour and whether or not endosperm had developed (Fig. 1D, E). For each fruit the total number of seeds and the proportion of viable seeds were recorded.

Geographical and ecological distance

Spatial distances between donor and acceptor populations were estimated using Google Earth. Distance was measured between the centres of the two populations to a resolution of 0.1 km. To estimate ecological distance, we used climatological, topographic and edaphic data collected for all *I. atropurpurea* sampling sites. Climatological and topographic data of each site were obtained from the GIS unit at the Hebrew University of Jerusalem. Soil samples were collected from all sites at five random spots within the range of the *Iris* population, from depths of 5 and 10 cm. About 300 g of soil was collected from each spot. Soil chemistry and structure were analysed in the soil laboratory of the Ministry of Agriculture, Hadera. Soil parameters included soil chemical properties, such as pH, ion concentrations and conductivity (Supplementary Data Table S1). For most soil parameters, samples from the two soil depths differed significantly and were used as separate parameters for the multivariate analysis.

We used an ordination technique to obtain a multivariate measure of ecological distance, using CANOCO for Windows v.4.5 (Microcomputer Power, Ithaca, NY, USA), following the previously proposed recommendations (ter Braak and Šmilauer, 2002; Lepš and Šmilauer, 2003). We used the detrended correspondence analysis technique developed to relate community composition to known variation in the environment (ter Braak, 1983; Cañadas *et al.*, 2010). The ecological distance between

each donor population and the NET acceptor population was calculated as the difference between the values of the sites on the first axis of the ordination, which explained 91.1 % of the ecological variation among sites (Supplementary Data Fig. S1).

Data analysis

All statistical analyses were performed using R (R Development Core Team, 2014). Cross treatment was defined as a single combination of acceptor (NET) and donor populations. Fruit set for each treatment was calculated as the proportion of flowers that set fruit within that cross treatment. Seed set for each treatment was calculated as the average number of seeds per capsule across all flowers of the same treatment. Only flowers that produced fruit were included in the seed-set count. The proportion of viable seeds (i.e. non-aborted) in each capsule was defined as the ratio between mature seeds and all the seeds in the capsule, both viable and aborted. This ratio was then averaged across all capsules for each cross treatment.

The distribution of *I. atropurpurea* along the coastal plain of Israel is highly fragmented, with a clear clustering of populations north or south to Tel Aviv (Fig. 1B). We used a generalized linear model (GLM) to test for the effect of treatment (i.e. donor population) on each of the above three components of reproductive success. We tested for the effect of donor population, compared means of the NET (within population) treatment against all other treatments, and compared the means of all populations north of Tel Aviv (which we termed ‘near’) with the means of populations south of Tel Aviv (‘far’). Significance was obtained assuming a binominal distribution for fruit set and seed set, and with an arcsine transformation for seed viability.

Reproductive isolation, the absolute contribution of a single RI component and total RI were calculated following Sobel and Chen (2014) as follows: First, RI for each of the three reproductive success components was calculated as:

$$RI = 1 - 2 \times \left(\frac{X_{\text{crosses between populations}}}{X_{\text{crosses within populations}} + X_{\text{crosses between populations}}} \right)$$

where X is the value of the fitness component measured: fruit set, average number of seeds or proportion of viable seeds. Second, following Ramsey *et al.* (2003) we calculated the absolute contribution (AC) of each component to the total reproductive isolation as (1) $AC_1 = RI_1$, (2) $AC_2 = RI_2(1 - AC_1)$ and (3) $AC_3 = RI_3[1 - (AC_1 + AC_2)]$, to account for the effect of previous stages on accumulating RI. Finally, total RI was calculated as the sum of all absolute contributions of the individual RIs: $RI_{\text{total}} = [\Sigma(AC_i)]$ (Ramsey *et al.*, 2003).

In order to test the effect of geographical and ecological distances between crossed populations on each of the three RI components and on total RI, we used GLMs with ecological or geographic distance as an explanatory variable. Significance was obtained assuming a binominal distribution for fruit set and seed set and an arcsine transformation for seed viability.

To further estimate the differences among cross treatments we used an aster model for life history analysis (Geyer *et al.*,

2007), which allows a joint analysis of several fitness components, accounting for their different probability distributions and the dependence of each stage on the earlier stages (Shaw *et al.*, 2008). Analysis was performed using the R package ‘aster’. We combined the three components and compared models with geographical or ecological distances, or both, as explanatory variables, and used likelihood ratio tests to compare model fit. We then used the aster model to compare the crosses by predicting reproductive success, computed as the unconditional expected number of viable seeds for a typical individual in each cross (Shaw *et al.*, 2008).

In addition to the analysis using the multivariate ecological distance, individual climatic, topographic and edaphic factors were used as explanatory variables to test hypotheses on the influence of specific ecological components on total reproductive success, as obtained using the aster model. To avoid bias due to multiple tests, the significance of these models was adjusted by Benjamini–Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995).

Finally, to simultaneously test for the effect of both geographical and ecological distances on total reproductive success we performed a spatial mixed model using the R package ‘spaMM’, which accounts for spatial autocorrelation between populations (Rousset and Ferdy, 2014).

RESULTS

Reproductive components

Three reproductive components were examined for each of the crosses (Table 1). Fruit set, seed set and seed viability were not significantly different between control flowers and flowers pollinated within the NET population ($\chi^2_1 = 2.54$, $P = 0.11$; $\chi^2_1 = 0.17$, $P = 0.68$; $t_{90.98} = 0.032$, $P = 0.98$, respectively). Fruit set and seed set from hand crosses were slightly greater than the control treatment, likely due to the common pollen limitation in this population (Lavi and Sapir, 2015). These differences are probably not due to differences in pollen quality because pollen viability was the same in both kinds of treatment.

Fruit set was significantly affected by the identity of the donor population (GLM with binomial-distributed residual error, $\chi^2_{14} = 28.6$, $P = 0.012$). Crosses within the NET population did not significantly differ in fruit set from all other cross treatments combined ($\chi^2_1 = 1.62$, $P = 0.203$). Fruit set in crosses of populations from the northern part of the distribution (near populations, mean fruit set = 0.902) was significantly higher than that of crosses of populations from the southern part of the distribution (far populations; mean fruit set = 0.705; $\chi^2_1 = 14.2$, $P < 0.001$).

Seed set was also affected by the identity of the donor population (GLM with correction for quasi-Poisson-distributed residual error due to overdispersion, $F_{14,319} = 1.84$, $P = 0.032$). Mean seed set in crosses within the NET population was not significantly different from all other cross treatments combined ($F_{1,333} = 1.96$, $P = 0.19$). No significant difference was found between mean seed set of near and far populations ($F_{1,333} = 0.34$, $P = 0.56$).

No significant effect of donor population on seed viability was found (GLM with binomial-distributed residual error,

$F_{14,318} = 1.10$, $P = 0.35$). The proportion of viable seeds was also not significantly different between crosses within the Netanya population and crosses with other populations ($F_{1,331} = 0.28$, $P = 0.59$). No significant difference was found between the proportion of viable seeds between near and far populations ($t = -0.732$, d.f. = 316.2, $P = 0.47$).

Reproductive isolation

Throughout analysis the OLG population was an extreme outlier in the model of reproductive isolation regressed against ecological distance (Supplementary Data Fig. S3). The OLG population is the only one positioned north of the NET population, and may be exceptional in its biological/genetic composition. In addition, based on field observation we suspected that pollen was heavily consumed by florivores in the OLG site, and was inviable. Therefore, analyses were performed twice: once including the datum and a second time excluding the datum from the analyses (Supplementary Data Fig. S2).

For analysis excluding the OLG population, reproductive isolation was significantly associated with both geographical distance and ecological distance when fruit set was the basis for calculating RI (slope = 0.003, $P = 0.001$ and slope = 0.040, $P = 0.043$, respectively; Fig. 3A, B). Reproductive isolation based on seed set or seed viability was not significantly associated with either geographical or ecological distances (slope = -0.001, $P = 0.82$ and slope = 0.044, $P = 0.091$, respectively; Fig. 3C, D; slope = -0.001, $P = 0.52$ and slope = -0.007, $P = 0.26$, respectively; Fig. 3E, F). Total RI was not significantly associated with geographical distance (slope = 0.021, $P = 0.19$; Fig. 3G), but increased significantly with ecological distance (slope = 0.074, $P = 0.005$; Fig. 3H).

Reproductive success

The full aster model, which contained both geographical and ecological distances as explanatory variables of unconditional mean fitness, did not differ significantly from the model that contained only the effect of ecological distance (ANOVA for aster, deviance = 2.35, $P = 0.13$), while showing a significant difference from the model that contained only the effect of geographical distance (deviance = 6.21, $P = 0.013$), thus suggesting ecological distance to be the major explanatory factor. We found no significant effect of geographical distance or ecological distance on reproductive success (slope = -0.122, $P = 0.099$ and slope = -1.955, $P = 0.065$, respectively; Fig. S2). In an analysis excluding OLG from the data, no significant effect of geographical distance was found (slope = -0.136, $P = 0.061$; Fig. 4A) but a significant effect of ecological distance was found (slope = -3.279, $P = 0.002$; Fig. 4B).

We further explored the effect of individual environmental parameters on reproductive success of crosses among populations. Three environmental parameters were significantly associated with reproductive success: distance from the sea and maximum summer temperature decreased reproductive success, while sodium content at 10 cm soil depth increased reproductive success (Supplementary Data Table S2). However, after controlling for inflated P value in multiple tests using Benjamini–Hochberg correction (Benjamini and

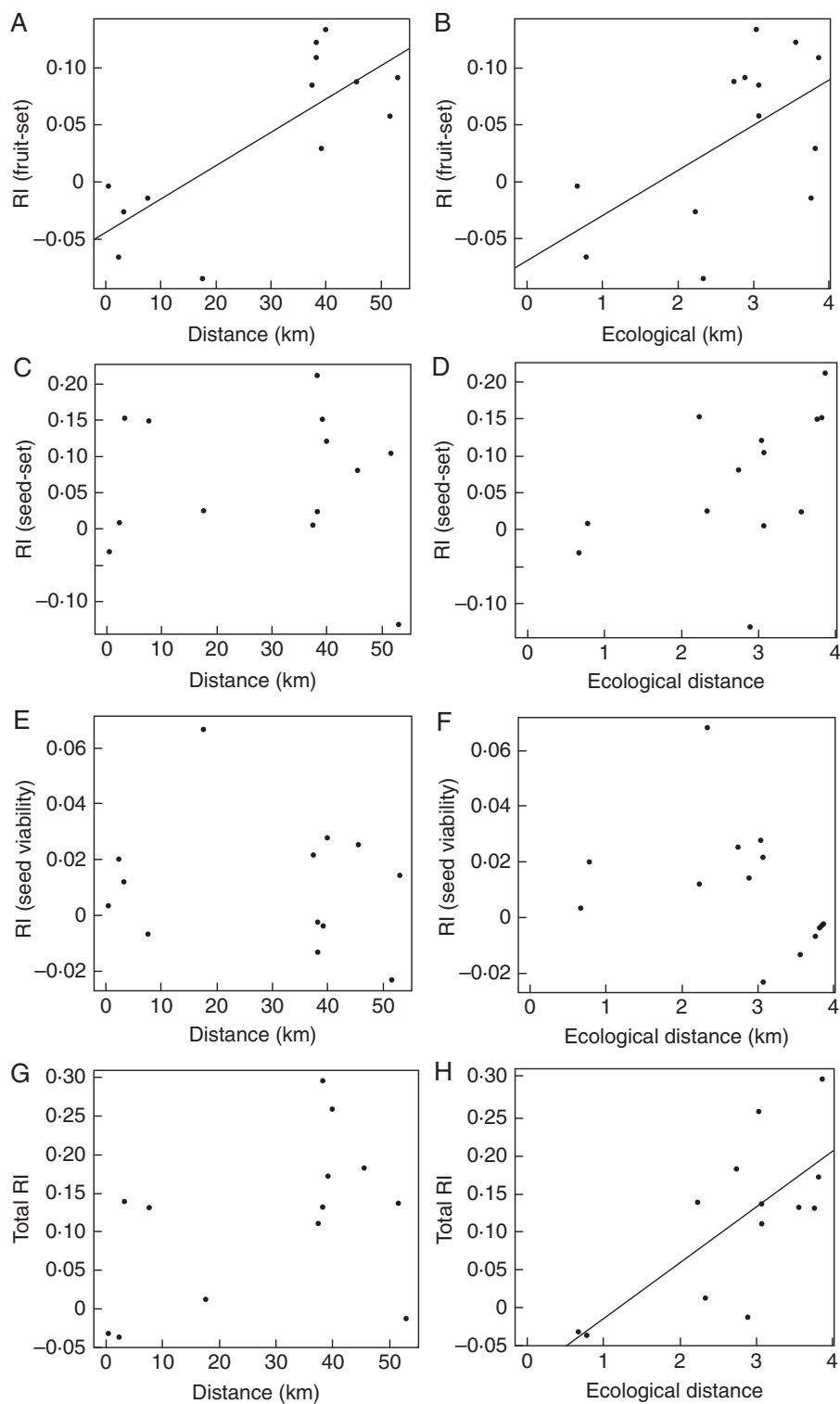


FIG. 3. Effect of the geographical and ecological distances on RI based on three fitness components. Left column, geographical distance; right column, ecological distances. (A, B) RI based on fruit set; (C, D) RI based on seed set; (E, F) RI based on proportion of seed viability; (G, H) Total RI. For calculation of RI and total RI see Materials and methods. Each point represents one donor population in the cross experiment.

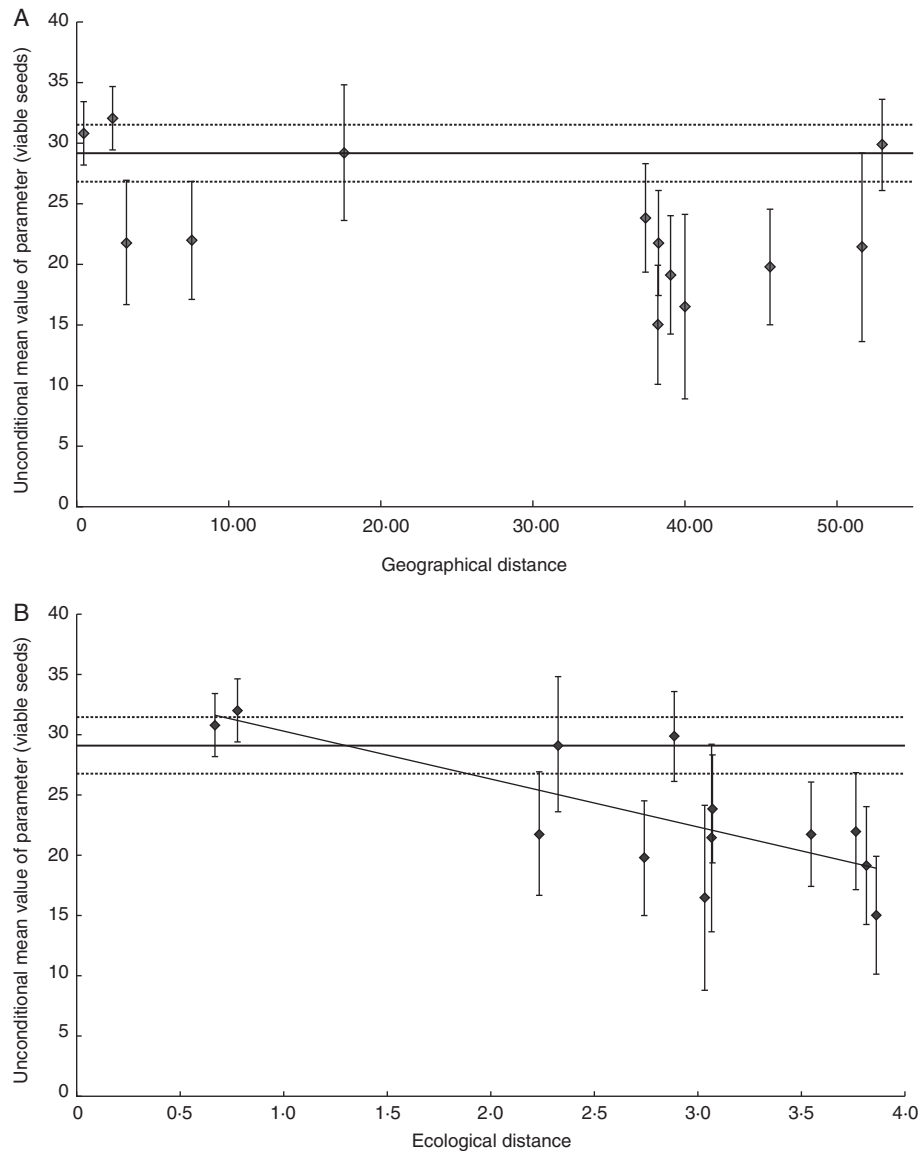


FIG. 4. Predicted number of viable seeds for a typical individual in crosses between populations of *Iris atropurpurea* as a function of (A) geographical distance and (B) ecological distance between populations. Each point represents a single population with 95 % confidence interval. Dotted lines indicate the 95 % confidence interval for crosses within the acceptor population (NET). A significant linear decrease in reproductive success with increase ecological distance was found ($P = 0.002$).

Hochberg, 1995), none of the effects remained significant ($P > 0.05$).

We used mixed models that implemented spatial autocorrelation as a random effect to test the effect of either geographical or ecological distance, or both, on reproductive success, expressed as unconditional mean number of viable seeds, as revealed by the aster model. We compared three models: one with both ecological and geographical distances (the saturated model); one with only ecological distance; and a null model without any fixed effect. Likelihood ratio tests showed no significant difference between the saturated model and the model with ecological distance alone (i.e. removing the fixed effect due to geographical distance; $P = 0.96$). A significant difference was found between the model with ecological distance and the null model ($P < 0.001$).

We used a qualitative presentation of the combined effect of geographical and ecological distances on reproductive success (Fig. 5). Qualitatively, it seems that geographical and ecological distances jointly affect reproductive success in a non-linear fashion. While the small sample size prevents thorough analyses of these relationships, it can be appreciated that ecological distance has a slightly more substantial contribution to the differences in reproductive success among crosses.

DISCUSSION

Studying intraspecific partial reproductive isolation can facilitate our understanding of the mechanisms underlying divergence and incipient speciation. The concept of speciation continuum

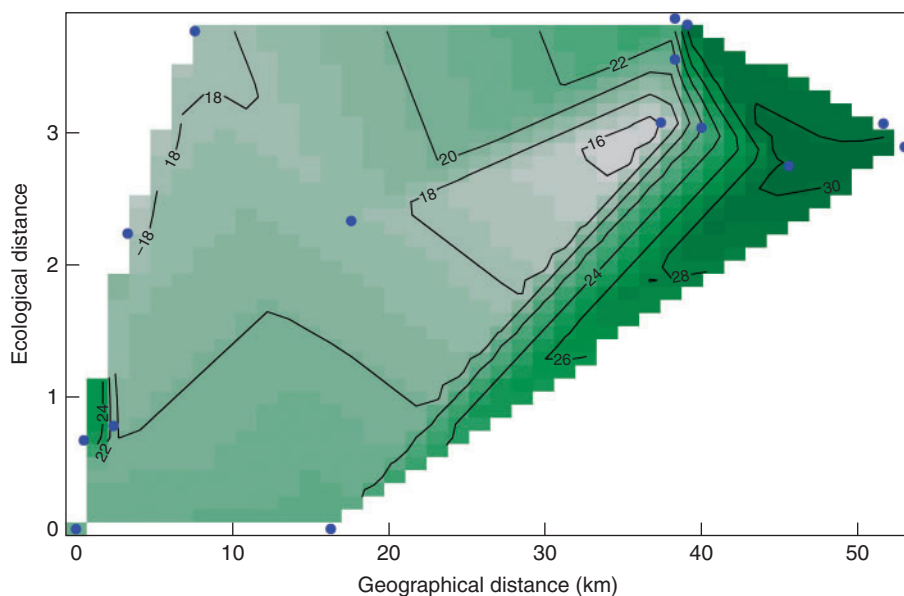


FIG. 5. Combined effect of ecological and geographical distances on total reproductive success, presented as the expected number of viable seeds for a typical individual in each cross. Expected number of viable seeds is represented by a colour gradient, where darker colour represents larger numbers. Each blue point represents a single population.

(Nosil, 2012) provides a framework for treating partial reproductive isolation among populations as an intermediate phase in the divergence of species. Our results show that partial intraspecific reproductive isolation in *I. atropurpurea* is mostly associated with ecological divergence. This was shown using two approaches to quantify population divergence, either by calculating reproductive isolation (Fig. 3H) or by calculating unconditional cross reproductive success using aster modelling (Fig. 4B).

The pattern observed in *I. atropurpurea* is similar to that expected in cases of isolation by ecology (IBE), where divergent selection and local adaptation drive genetic differentiation among populations (Bradburd *et al.*, 2013; Shafer and Wolf, 2013). In *I. atropurpurea*, this pattern is more pronounced than the alternative of isolation by distance (IBD), a pattern in which genetic differentiation among populations is driven by reduced gene flow due to geographical distance (Wright, 1943; Slatkin, 1993; Bradburd *et al.*, 2013). Moreover, the pattern revealed supports the local divergence hypothesis (Fig. 2D), while the hypotheses of stepping stone dispersion and secondary contact (Fig. 2A and C, respectively) were not supported. While our data do not allow a definite conclusion about the relative roles of stochastic genetic drift and local adaptation, the effect of ecological divergence is evidently stronger than the putative effect of spatially driven reduced gene flow in *I. atropurpurea*. These findings are in agreement with the prevalence of IBE across species (Shafer and Wolf, 2013) and its dominance over IBD, although not particularly in plants (Sexton *et al.*, 2014). Our results on the non-linear correlations of RI with both distances are also similar to the simulated genetic distance plotted against these two distances in Wang and Bradburd (2014). Further studies in *I. atropurpurea* will shed more light on the relationships between RI and genetic distance, to assess whether IBD and IBE observed here indirectly are reflected at the genetic level.

Reproductive success in crosses among populations was also subjected to theoretical considerations regarding the combined effect of inbreeding and outbreeding depression, predicting a hump-shaped pattern with a peak of highest success at an intermediate distance (Waddington, 1983; Waser and Price, 1989; Sapir and Mazzucco, 2012). We found no spatial pattern of reproductive success in *I. atropurpurea*, in contrast to the above optimal crossing distance hypothesis. Sapir and Mazzucco (2012) provided explicit predictions for *I. atropurpurea*, considering seeds and pollen dispersal, in addition to the effects of inbreeding and outbreeding depression, and showed that the extent of environmental heterogeneity affects the combined spatial effect of inbreeding and outbreeding. Here, however, the negative association between reproductive success and ecological distance (Fig. 4B) does not support this model.

Several scenarios that are not mutually exclusive can explain the observed pattern. First, intrinsic selection against unfit migrants, i.e. outbreeding depression between ecologically dissimilar populations, might be more effective than inbreeding depression and obscure the effect of the latter. Nevertheless, the environmental basis of such a selection is not very clear, given that none of the environmental parameters was significantly associated with reproductive success.

A second possibility is the continuous distribution of *I. atropurpurea* in the past, constantly homogenized by gene flow (Fig. 2D), while local adaptation created genomic ‘islands’, partially linked to loci affecting reproductive isolation/success (Nosil *et al.*, 2009). Currently, lack of molecular tools for the *Oncocylus* irises is preventing examination of this hypothesis at the single species level. Nonetheless, recent phylogenetic analysis using one low-copy nuclear marker and six plastid markers (Wilson *et al.*, 2016) suggests that there is no spatial genetic segregation of species in the group, hinting at past continuous distribution followed by local divergence.

Third, lack of evidence for reduced reproductive success due to inbreeding depression can be explained by counterbalancing gene flow from genetically divergent populations (Frankham, 2010; Sexton *et al.*, 2014). In *Oncocylus* irises the available evidence is inconclusive. On the one hand, a similar study of crosses between pairs of neighbouring populations of *I. atropurpurea* revealed fruit set and seed set similar to crosses within the population in ten out of 11 population pairs (Y. Sapir, unpubl. res.). On the other hand, a study in *Iris bismarckiana* showed significantly reduced reproductive success in crosses within the population compared with crosses with a neighbouring population (Segal *et al.*, 2007). It may be concluded that inbreeding depression has a limited effect at the population level, and is species-specific in section *Oncocylus*, while local adaptation accompanied by outbreeding depression is hypothesized to be more common in this group.

We examined three reproductive components to measure reproductive success/isolation, namely fruit set, seed set and seed viability. Of these, only fruit set was linearly associated with both distances (Fig. 3A, B). Fruit set depends on pollen–pistil interaction (Lewis and Crowe, 1958; Pandey, 1981), and thus could be considered as a pre-zygotic reproductive component. Pre-zygotic barriers have been shown to have a prominent part in creating partial or complete reproductive isolation, relative to post-zygotic components (Baack *et al.*, 2015). Fruit development is initiated by the physiological processes initiated by pollen germination on the stigma (e.g. Carbonell-Bejerano *et al.*, 2010; Brito *et al.*, 2015), and thus the development of a fruit is a signal for positive pollen–pistil interactions. This is the rationale for looking specifically at fruit set as a fitness measure of the cross, where pollen identity matters. Indeed, we show that populations differed in their fruit set, regardless of the next step of ovule maturation to seeds. Although the mechanism governing this pollen–pistil interaction in irises is unknown, the results of this work suggest that pollen origin can cause reduced fitness and act as a reproductive barrier. Reproductive isolation at the stage of fruit production can be attributed to outbreeding depression that leads to low fecundity of crosses between ecologically distant populations through pollen–pistil interaction (Travers, 1999). Nonetheless, in *I. atropurpurea* reduced fruit set is also linked to spatial distance, and the hypothesis of an ecologically driven pre-zygotic barrier through pollen–pistil interactions requires further experiments.

Implications for conservation

In addition to its possible contribution to the understanding of speciation processes, studying reproductive success/isolation by artificial gene flow among populations is also highly relevant to conservation management. Small plant populations, especially in fragmented habitats, frequently experience loss of individuals, pollinator decline and reduced genetic variation (Ellstrand and Elam, 1993; Ouborg *et al.*, 2006). The loss of genetic variation can cause reduced mean fitness by reduction of fruit and seed production and reduction of offspring fitness due to inbreeding depression, leading to a higher risk of extinction (Bossuyt, 2007; Mannouris and Byers, 2013). Genetic rescue of populations at risk is proposed by crossing plants from different geographical or ecological regions, with the aim of increasing

the genetic variation within a fragmented population, which in turn increases population fitness and decreases the probability of extinction (Richards, 2000; Ingvarsson, 2001). However, outbreeding depression may result in offspring with low fitness or a decreased ability to reproduce, and lower the population fitness (Edmands, 2007). Introducing alleles that are poorly adapted to local conditions can put the population at risk because of low performance of the offspring or F_2 generations. General heterotic effects boosting the fitness of F_1 hybrids were found in assisted gene flow between isolated populations (Frankham, 2015). However, this is not necessarily the outcome in crosses with different populations of the same species. To choose the appropriate population for genetic rescue one should take into account both inbreeding and outbreeding effects, which may be reflected in the distance of the target population from the donor one (Ouborg *et al.*, 2006; Edmands, 2007; Frankham, 2010). So far, surveys of genetic rescue have usually considered population structure (Bossuyt, 2007; Sinclair *et al.*, 2013) or geographical distance (Holmes *et al.*, 2008) as predictors of appropriate populations to avoid outbreeding depression. However, our results indicate that geographical distance provides limited prediction of the possible effect of outbreeding depression. Instead, we argue that conservation management should include a survey of ecological differences between populations and consider a minimal ecological distance as an indicator to facilitate genetic rescue. Specifically, genetic rescue in *I. atropurpurea*, if required, should be performed based on considerations of ecological distance. A preferable source for genetic rescue would be a population that is growing in the most similar ecological conditions. In *I. atropurpurea*, it may be sufficient to estimate ecological distance focusing on the ecological parameters that are most correlated with reproductive success.

To conclude, our study provides evidence that geographical distance is not necessarily a predictor of reproductive isolation. Rather, our results suggest that ecological factors determine the extent of reproductive isolation between populations of *I. atropurpurea*, creating a complex, non-linear pattern of spatial divergence of its populations. We propose that divergence is also linked to pollinator isolation, seed dispersal and genetic mechanisms, which are the subject of future analyses.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: environmental variables of topography, climate and soil from *Iris atropurpurea* sites used in this study. Table S2: regression parameters of RI, or aster-based fitness, as a function of environmental variables. Figure S1: ordination of *Iris atropurpurea* populations studied based on environmental variables of topography, climate and soil. Figure S2: reproductive isolation graphs including the OLG population. (A) RI based on fruit set as a function of geographical distance; (B) RI based on fruit set as a function of ecological distance; (C) RI based on seed set as a function of geographical distance; (D) RI based on seed set as a function of ecological distance; (E) RI based on seed viability as a function of geographical distance; (F) RI based on seed viability as a function of ecological distance; (G) total RI as a function of

geographical distance; (H) total RI as a function of ecological distance. Figure S3: predicted number of viable seeds for a typical individual in crosses between populations of *Iris atropurpurea* as a function of (A) geographical distance and (B) ecological distance between populations.

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