To mix or not to mix the sources of relocated plants? The case of the endangered *Iris lortetii*

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ABSTRACT

Active management of endangered species is required for the persistence of many rare species. Species translocation, a common practice used to mitigate the negative effects of small population sizes, entails risks of outbreeding depression due to dilution of local adaptations, and therefore must be weighed against the costs of a hands-off conservation approach. *Iris lortetii* is an endangered rhizomatous plant, growing in a small number of isolated populations in northern Israel. We implemented a quasi-in-situ reintroduction program by planting 234 rhizomes from diverse origins in five new sites. All new sites were selected to be as similar as possible to those of the largest natural population. We recorded plant survival and flowering in the field after four years. Flowering plants were artificially crossed either with plants from the same population of origin (within population) or with plants from different origin (between populations). We found no differences in survival between populations of origin and only some indication of local adaptations in the form of increased flowering of the local population. Nonetheless, seed set was significantly higher (a 73% increase) in crosses between populations of origin, compared to within-population crosses, suggesting low genetic diversity within the natural populations. The ability to combine active conservation with rigid testing of theoretical hypothesis, while avoiding all risk to natural populations, highlights the value of the quasi-in-situ approach for restoration. Our results indicate that, in the case of *Iris lortetii,* active relocation of genotypes, seeds or pollen can enhance the survival of natural populations over time.

1. Introduction

The preservation of species diversityis one of the main goals of modern conservation (Dawson, Jackson, House, Prentice, & Mace, 2011; Pimm et al., 2014). Rare species often receive much conservation attention and resources (Early & Thomas, 2007; Sapir, Shmida, & Fragman, 2003), where the conservation strategy is frequently derived from knowledge of the species' biology, level of threat and spatial distribution (Bacchetta, Farris, & Pontecorvo, 2012; Sapir et al., 2003). In many cases, habitat destruction and fragmentation limit the spatial distribution of rare species to a few isolated patches, increasing their probability of extinction, thus prioritizing them for conservation (Sapir et al., 2003).

Small and isolated populations are known to be exposed to a variety of processes that might lead to population decline and local extinction. Among these are genetic drift and fixation of deleterious mutations and inbreeding depression (Elam, Ridley, Goodell, & Ellstrand, 2007; Ellstrand & Elam, 1993), demographic stochasticity (Volis, Bohrer, Oostermeijer, & Van Tienderen, 2005) and habitat loss (González-Varo, Albaladejo, Aizen, Arroyo, & Aparicio, 2015). Mitigating these risks requires the increase of their population sizes, as well as their genetic diversity. Such goals may be achieved by various conservation actions that facilitate gene flow and immigration among populations. The nature of these actions depends on the levels of isolation, species biology and resource availability. For example, in organisms with limited movement or dispersal, such as plants, establishment of protected areas and corridors may prove insufficient, and additional strategies, such as genetic rescue, might be required. This is especially true in fragmented habitats where the distance between patches is large or when the fragmentation is caused by irreversible factors, such as urban development.

Genetic rescue is a strategy to alleviate reduced genetic diversity in

small and fragmented populations (Frankham, 2015; Richards, 2000). Genetic rescue involves enrichment of genetically poor populations by transferring pollen (Christmas, Breed, & Lowe, 2015; Xiao, Jiang, Tong, Hu, & Chen, 2015), seeds or propagules from another population, a method called 'species translocation' or 'assisted migration' (Kramer & Havens, 2009; Richards, 2000). Seeds or pollen used for genetic rescue may come from different geographical or ecological regions, in order to increase the genetic variation in the fragmented population (Ingvarsson, 2001; Richards, 2000; Vandepitte, Honnay, Jacquemyn, & Roldán-Ruiz, 2010). However, in some cases, crosses may result in negative outcomes, due to outbreeding depression in cases of strong divergence following local adaptation (Edmands, 2007).

Outbreeding depression is expressed as reduced cross success with increasing ecological differentiation, due to divergent selection (Ruane, Dickens, & Wall, 2015; Sapir & Mazzucco, 2012; Schierup & Christiansen, 1996). Thus, genetic rescue with source population of intermediate ecological distances is likely to maximize success (Grindeland, 2008; Hufford, Krauss, & Veneklaas, 2012; Pélabon, Carlson, Hansen, & Armbruster, 2005; Price & Waser, 1979; Sapir & Mazzucco, 2012; Waddington, 1983). However, when populations have been separated for longer time periods, possible local adaptations may result in an advantage to local gene pools (Anderson, Willis, & Mitchell-Olds, 2011; Griswold, 2006). Conceivably, in some cases, the benefit of increased genetic diversity due to genetic rescue might be outweighed by the dilution of local adaptations. This trade-off confronts conservationists with a dilemma - whether to mix or not to mix plants from different sources in relocation practice (Edmands, 2007; Frankham, 2015; Ouborg, Vergeer, & Mix, 2006).

Quasi in-situ conservation offers a way to partially circumvent this dilemma: new populations are established in habitats similar to natural sites, but not within the natural populations, in order to prevent genetic contamination and consequent outbreeding depression (Volis & Blecher, 2010; Volis, Blecher, & Sapir, 2010). Use of a comparison of plants from multiple populations, grown under the same ecological conditions could help quantify the relative effects of local adaptations and lack of genetic diversity, without jeopardizing the natural populations. To the best of our knowledge, while numerous studies tested for local adaptation per se, only a few empirical studies explicitly tested the relative roles of diluting local adaptations and increasing genetic diversity (Volis et al., 2010).

Adding to the "mixing or not mixing" dilemma are the long-term effects. While the first generation after assisted gene flow may be viable, the second generation may experience introgression of deleterious (or beneficial) mutations among the mixed populations (Campbell, Snow, & Ridley, 2006; Oakley, Agren, & Schemske, 2015). As a first proxy for long-term effect, it is hence important to elaborate on the outcome of crossing among plants from different populations when testing for the advantages and disadvantages of mixing sources in relocating endangered plants.

Iris lortetii Barbey (*Iris* section *Oncocyclus*; Fig. 1a) is a Mediterranean rhizomatous endangered species, endemic to the Upper Galilee in northern Israel and southern Lebanon (var. *lortetii*), with a disjunctive population in central Palestine (var. *Samaria*; (Feinbrun-Dothan, 1986; Sapir, 2016). Different populations are found in sites with notably diverse environmental conditions, in terms of bedrock (either chalk or limestone with brown rendzina or shallow terra-rossa soils), slope (12–30 degrees), aspect, elevation (525–718 m a.s.l) and surrounding woody vegetation cover (4–53%). Most populations are found on stony steep slopes, perhaps because other microhabitats were target for either collection by 19th century commercials or porcupine herbivory (Sapir, 2016). Due to its limited distribution (about 220 km^2 area of occupancy), accompanied by a severe decline in population size and number of sites, it is considered "endangered", according to the International Union for Conservation of Nature categories (Sapir, 2016), and is listed in the Red Data Book of the Israeli Endangered Plants (Shmida & Pollak, 2008). Major threats to *I. lortetii* include collection of rhizomes for

commercial use and habitat transformation and fragmentation due to agriculture and afforestation (Shmida & Pollak, 2008). The species has experienced significant population decline and fragmentation in northern Israel, and has probably become extinct in southern Lebanon (Sapir, 2016; M. Semaan, per. comm.). In the Upper Galilee the species is currently limited to three populations (a few hundred individuals each) and a handful of satellite sites (a few dozen individuals in each). The total number of individual plants in all populations is estimated at between 2000–2500 (Sapir, 2016). Like all other species in the section *Oncocyclus*, *I. lortetii* is self-incompatible and is obligatorily pollinated by males of a solitary bee (Eucera spp.; Sapir, Shmida, & Ne'eman, 2005). Some evidence for inbreeding depression in crosses within populations was shown in two other related species, *Iris bismarckiana* Regel (Segal, Sapir, & Carmel, 2007) and *I. atropurpurea* Dinsm. (Sapir & Mazzucco, 2012), suggesting low within-population genetic diversity resulting in reduced seed production. Evidences from other species suggest that seed dispersal is limited to a few tens of meters, and pollen flow is restricted to a few hundreds of meters (Sapir, un-published). The low dispersal and pollination distances of *I. lortetii* and the isolation among subpopulations are likely to minimize the effectiveness of ecological corridors and highlight the need for an active conservation strategy.

In this study we estimated the relative importance of local ecological adaptations and genetic diversity, in order to answer the question – should we use mixed or non-mixed seed sources for establishing new populations of *I. lortetii*. This question is an extension of the quasi in-situ conservation framework, proposed by Volis et al. (2010, 2015). In light of the small population sizes and the results of previous studies (Segal, Sapir, & Carmel, 2006), we hypothesized that cross-pollination between plants that differ in their population of origin will increase seed production due to increased genetic diversity. Due to the large environmental variability between the sites of the natural populations, as well as the short dispersal and pollination distances of iris species, we further hypothesized that plants growing in their habitat of origin would show higher survival and flowering rates due to a possible home advantage. Briefly, we created new populations of *I. lortetii* and tested whether local adaptations have developed in plants from one of the three major populations, and whether mixing genotypes (by crosses) between populations is beneficial or maladapted, by measuring the reproductive success of crosses.

2. Materials and methods

2.1. Seed collection and germination

We collected the seeds in April 2011 at five sites throughout the distribution of the species in the Upper Galilee. Fruits (pods) were collected haphazardly at each site in order to represent the entire population. Largely, in cases where genets were clearly defined one pod was collected per genet (see below). The sites (and their codes) were as follows: Avivim (AVI; 37 pods, 943 seeds); Malkiya (MLK; 50 pods, 1236 seeds); Mt. Pua (PUA; 37 pods, 934 seeds); Ayelet Hashachar (ALT; 11 pods, 253 seeds); and, Dishon (DSH; 3 pods, 47 seeds). Fig. 1b shows the location of these sites. Altogether, 138 pods containing 3413 seeds were collected. Of these five sites, the populations of Pua, Avivim and Malkiya were substantially larger (more than 50 clearly distinguishable individuals). In Malkiya the population is relatively dense and defining the exact population size was practically impossible because genets were growing continuously. Hence, pods in the MLK population were collected without defining the genets. The small number of fruit collected in DSH was the result of lack of fruit production.

In November 2011 the seeds were planted into small pots (~1 L), in a mixture of commercial potting soil mixed with ~5% natural soil inoculum collected from Pua site, in order to provide possible mycorrhizal symbiosis, if exists. The seeds were planted in the nursery of Tel Aviv University Botanical Garden and watered twice daily by misters
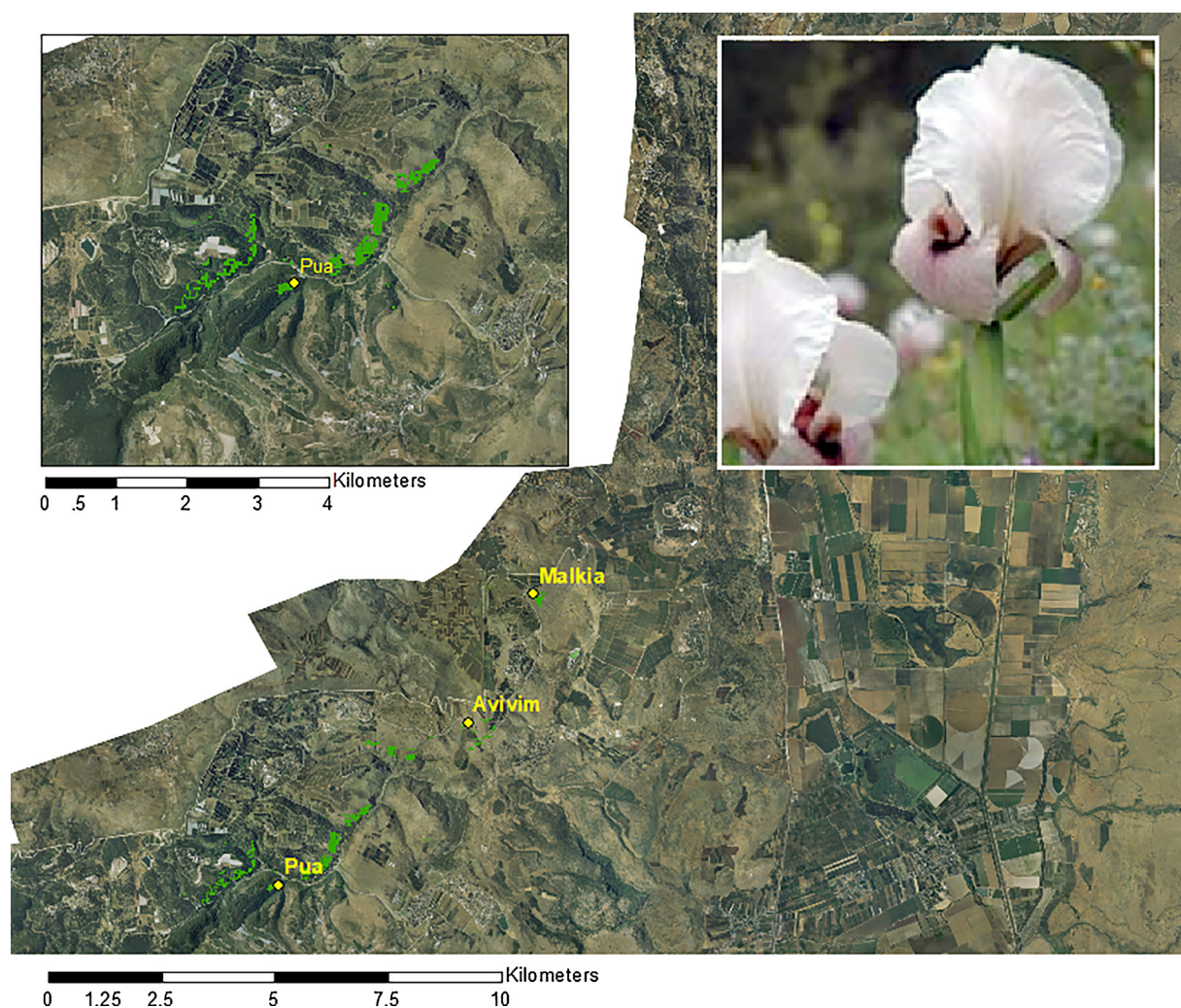
Fig. 1. (a) *Iris lortetii* in its natural habitat, Upper Galilee, Israel; (b) The locations of the three *I. lortetii* populations in the Upper Galilee. Areas colored in green within the insert are sites that the model identified as having the same conditions as in the Pua population (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for 20 min. Due to the low germination rate in *Oncocyclus* irises regardless of seed treatment and environmental conditions (Dorman, Melnikov et al., 2009; Dorman, Sapir et al., 2009), seed germination was consecutively monitored over a few years. Indeed, in the first year, only 12 seeds (0.35%) germinated. In the second year, 291 seeds (8.4%) germinated. Germination varied between populations ranging from 6 to 12% germination. Upon germination, we transferred the seedlings to larger pots and allowed them to grow until the end of the growing season. In late April we terminated the irrigation and left the seedlings to dry. The young rhizomes were collected and kept in paper bags until planting in the experimental sites. Before planting, we weighed them all to estimate the dry mass.

2.2. Experimental design

We started the reintroduction experiment in a habitat similar to the Pua site, because this population is the largest among the natural populations, and because seedlings from this population were the most abundant. We selected five reintroduction sites for planting new populations, determined by an environmental envelope model using seven environmental variables, including edaphic, vegetation cover, precipitation, and topography (see Supporting information). We used a simple envelope model, with relatively narrow boundaries around the values of the natural occurring populations (Table ESM1), in order to

choose reintroduction sites with the highest resemblance to the original Pua site. The model was constructed in ArcGIS software (ESRI, 2011).

In the fall of 2013, after the first significant rainfall event (30 mm), 234 rhizomes were planted in the five reintroduction sites. In each of the five reintroduction sites, three 4 × 4 m plots were established with a distance of 20 m between neighboring plots. In each plot, 15 or 16 rhizomes were planted at intervals of approximately 1 m apart. The existing woody vegetation (typically dwarf shrubs) was manually removed to reduce shading. Rhizomes were assigned to plots using the following scheme: one plot was composed entirely of rhizomes originating from the Pua population; the second was composed of rhizomes from the Malkiya population; and, the third was composed of a mixture of rhizomes from all five natural populations. Rhizomes were tagged individually and mapped.

2.3. Plant survival and flowering

Plant survival and flowering were recorded in the third growing seasons after planting (2016). Survival was recorded in February when herbaceous vegetation is still low and enables visual detection of the *Iris* leaf fans. Flowering was recorded during two weeks in late March to early April 2016.

2.4. Crosses

In late March and early April 2016, during the flowering season, 124 flowers (on 77 plants) were hand-pollinated. Half of the flowers (n = 61) were pollinated with pollen from plants that originated from the same source population (hereafter termed "within population") while the other half (n = 63) received pollen from plants originating from different source populations (hereafter termed "between population"). When possible, pollination was conducted within a plot. When no appropriate flowers were found, pollinations were done at the within and between site levels. A table depicting the frequency of different pollination combinations is presented in the Supplementary materials (Table ESM2). In order to rule out the possibility that cross-pollination between siblings biased the seed set results, we excluded nine pollination cases (three from PUA, one from AVI and five from ALT) where both plants originated from the same fruit (reducing the number of "within population" pollinations from 61 to 52). We further compared the seed set of the nine excluded cases to that of the remaining 52.

To avoid natural pollination, all flowers were covered by a mesh bag prior to anthesis. We then carried out pollination by collecting the three anthers of each donor flower and scraping the pollen off with a fine brush, before applying it to the three stigmas of a different flower. Extra care was taken so that all stigmas received the same amount of pollen. Crosses were performed on the same day of collecting the pollen, and none was kept overnight. In cases where a plant had more than one flower, the pollination treatments (i.e., crosses within/between population) were assigned randomly to the different flowers. After artificial pollination was implemented, the flowers were once again covered. Pollination was conducted on six different days (1–3 days apart from each other) in order to accommodate for natural variation in flowering time. We distributed both treatments (within and between populations crosses) evenly throughout time, in order to avoid a confounding effect of pollination timing. Thus, we pollinated all flowers found open on a certain day on the same or consecutive day. All tools were sterilized in 70% ethanol after each cross, to avoid pollen contamination.

2.5. Fruit and seed set

Three weeks after performing the last pollination, we recorded whether a fruit was produced for each flower. The fruits of all crosses were collected after seed maturation and before seed dispersal. Fruits were collected in paper bags, brought to the laboratory and stored at room temperature. The number of seeds was recorded for each fruit and they were allowed to dehydrate naturally.

Statistical analyses: We conducted statistical analyses using R (R Development Core Team, 2014) and SPSS 19. Differences among source populations in seedling growth after germination were tested using analysis of variance (ANOVA) with rhizome dry mass as the explained variable and source population (categorical) as the explanatory variable. We tested the probability of survival of a seedling as a function of initial rhizome weight using a generalized linear model (GLM) with correction for binomial distribution (*logit* link function). We also used a generalized linear mixed model (GLMM) with correction for binomial distribution to test for the effects of source population (fixed variable) and its interaction with the reintroduction site and plot (random variables) on probability of survival. To account for initial rhizome mass at planting, we analyzed survival with rhizome mass as a covariate.

We used a generalized linear mixed model (GLMM) with correction for Poisson distribution (*ln* link function) to test for the effect of source population and its interaction with the reintroduction site and plot on number of flowers per flowering plant, with initial rhizome mass as covariate.

In order to test for the effect of pollen origin, whether within or between populations, on cross success we used GLM with number of seeds (corrected for Poisson distribution) as explained variable, and

source population of the maternal plant and cross type (within/between population) and their interaction as explanatory variables. Crosses that did not produce fruit were considered as zero seeds. We used only maternal success because of the relatively small number of potential paternal plants. Moreover, partitioning the effect of paternal population revealed small sample size that prevented convergence of the model (see table ESM2 for a seed production per cross combination).

Only five seedlings from the Dishon population were planted in the field. Due to these low numbers, the Dishon plants were excluded from analyses in which population of origin was an independent variable.

3. Results

3.1. Survival

Rhizome mass at planting was significantly different between plants from different populations (ANOVA: $F_{5,270} = 4.38$, $P < 0.001$). Rhizomes from Ayelet Hashahar and Avivim were significantly larger than rhizomes of seeds from Malkiya (Tukey: $P < 0.05$). We used GLMM with correction for binomial data to test for the effects of source population and its interaction with reintroduction site and plot on probability of survival (Fig. 2). Only the effect of reintroduction site was significant ($F_{5,239} = 49.27$, $P < 0.001$). The probability of survival was positively associated with rhizome mass, but this effect was only marginally significant ($F_{1,239} = 3.56$, $P = 0.060$).

3.2. Flowering

In the spring of 2016, three years after planting in the reintroduction sites, 87 plants (46% of all surviving plants) set flowers. Population of origin had an effect on the probability of a plant to flower, with the Ayelet Hashahar and Malkiya source populations showing the highest and lowest probabilities, respectively ($F_{5,164} = 2.40$, $P = 0.039$, Fig. 2). Number of flowers per flowering plant was significantly affected by the origin of the population ($F_{3,63} = 3.07$, $P = 0.034$). Specifically, Pua had the highest (2.18) and Malkiya had the lowest (1.35) mean number of flowers per flowering plant (Fig. 3). Inclusion of the non-flowering plants did not affect the results or their significance.

3.3. Crosses

Overall, between-population crosses resulted in higher fruit sets (fruits: $\chi^2_1 = 14.903$, $P < 0.001$, n = 115; Fig. 4a). Both maternal origin and cross type significantly affected the number of seeds ($F_{3,94} = 6.8$, $P < 0.001$ and $F_{1,94} = 14.9$, $P < 0.001$, respectively; Figs. 4b, 5, Table ESM2), but the interaction between them was not
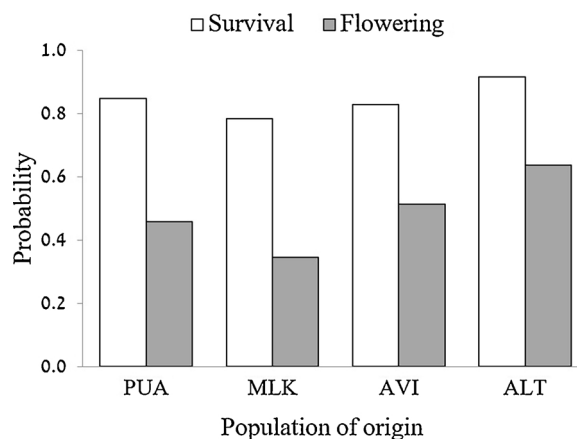
Fig. 2. Effect of population of origin on the probability of survival and flowering.
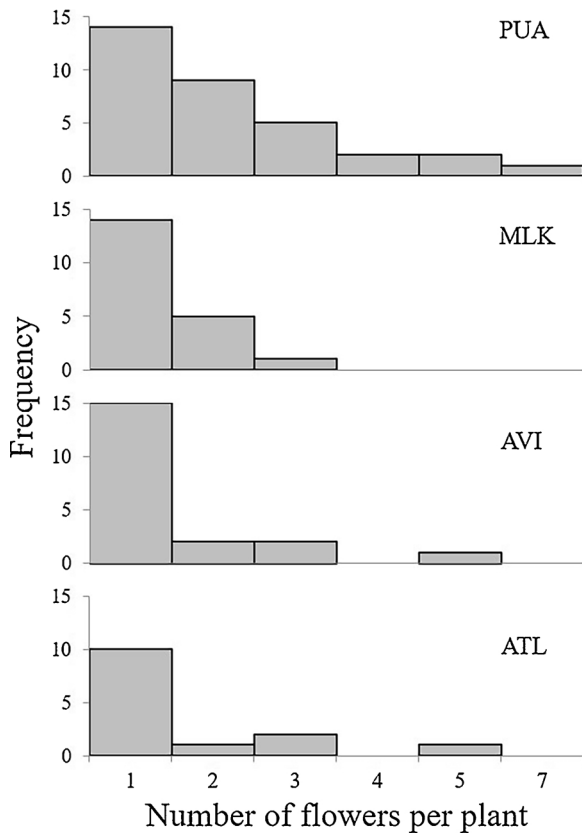
Fig. 3. Distribution of the number of flowers per flowering plant, presented by population of origin.

Fig. 5. Effect of crossing treatment (within/between populations) on seed set, divided by origin of maternal plants. Error bars represent ± S.E.

significant ($F_{3,94}$ = 1.9, P = 0.137). Malkiya plants produced the smallest number of seeds (Tukey post-hoc test, P < 0.05; Fig. 5).

Pollination between plants originating from the same fruit did not differ from pollination between plants from different fruits within the same populations ($\bar{x}$ = 27.22, S. E. = 9.83, n = 9) and ($\bar{x}$ = 29.22, S. E. = 4.09, n = 51) respectively (t_{58} = 0.189, P = 0.851).

4. Discussion

Our experimental test of the effect of mixing pollen sources in re-introduction of *I. lortetii* provides support for the approach of mixing genetic resources to facilitate reintroduction success. Studies considering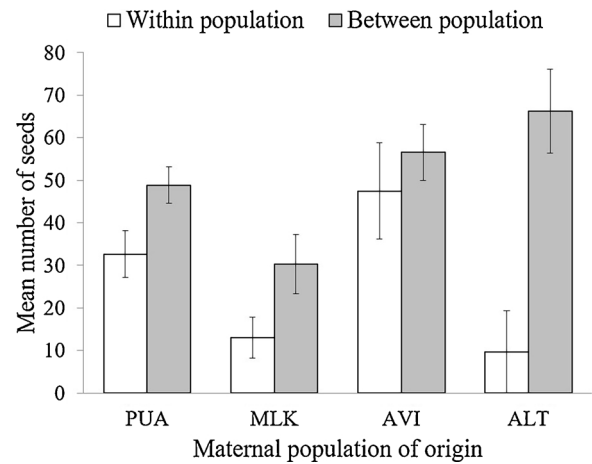 strategies for establishing new populations of endangered species have often found conflicting evidence for the desired source of plants. On the one hand, local adaptation and outbreeding depression jeopardize reintroduction of plant materials from mixed sources or mixing populations via crosses (Frankham, 2010; Leimu & Fischer, 2008; Waller, 2015). On the other hand, inbreeding depression may increase extinction risk if a single source is used for small isolated populations (Ellstrand & Elam, 1993; Morgan, Meyer, & Young, 2013; Waller, 2015). While our experimental design was not sufficient to conclusively rule out local adaptation and outbreeding depression, we do provide a clear indication that mixing genetic sources in *I. lortetii* increases reproductive output and can therefore facilitate successful establishment.

4.1. Local adaptations

To answer the question of whether or not to mix genetic sources, we tested the effect of mixing by comparing both the performance of plants in home/away habitats and the success of within/between population crosses. Survival rate in this study (83.04%) is similar to the mean survival rate reported globally for reintroduction in plants (78% on average; Godefroid et al., 2011), and plants from all populations exhibited similar survival rates (Fig. 2). As a component of fitness, this provides support for the mixing approach, and suggests that local adaptation does not threaten non-local genotypes in *I. lortetii*. Although plants from Pua (i.e., "local") had the highest mean number of flowers per plant, the number of seeds per flower in that population was relatively low, which cancels out this putative "home advantage" (Figs. 3 &

Fig. 4. Overall effect of crossing treatment (within/between populations) on the frequency of fruit set (a) and on the average seed set (b). *** represent significant differences (P < 0.001) in fruit set frequency (a) and seed set (b). Error bars represent ± S.E.

5). Such lack of home advantage is not surprising, given the considerable number of cases recorded in the literature with no home advantage (Hereford, 2009; Leimu & Fischer, 2008). Interestingly, home advantage due to local adaptation has been proposed to be more prominent when using seeds (or seedlings) as used here (Raabová, Münzbergová, & Fischer, 2007), while in irises, using rhizome of adult plants in a common garden experiment masked local adaptation, if it existed (Dorman, Melnikov, Sapir, & Volis, 2009; Dorman, Sapir, & Volis, 2009).

Our results support the hypothesis that local adaptation in *I. lortetii* is not affecting its performance outside its (micro-) habitat, and suggests that mixing populations for reintroduction is possible in this species. Nonetheless, local adaptation and mixing effects were tested in one habitat only, constraining this conclusion. In order to achieve a more complete picture of local adaptation in this species, we are currently performing two additional experiments in both the Malkiya and Avivim habitats; this will improve evaluation of local adaptation across the Northern distribution of *I. lortetii*.

4.2. Advantages of mixing in crosses between populations

Testing for the effect of within/between population crosses on reproductive success, we found higher fruit-set in crosses between populations compared to within-population crosses (a 37% increase, Fig. 4a). In other *Iris* species, there are contrasting results regarding the effect of pollen origin on fruit and seed sets. For example, crosses between populations of *I. bismarckiana* significantly increased fruit-set and number of seeds, compared to crosses within populations (Segal et al., 2007), possibly reflecting inbreeding depression due to small effective population size. In contrast, fruit-set in crosses between populations of *I. atropurpurea* reduced with increased ecological distance, hypothesized to reflect outbreeding depression (Yardeni, Tessler, Imbert, & Sapir, 2016). Furthermore, no differences were found among within- and between-population crosses in *I. atrofusca* (Volis, Zhang, Dorman, & Blecher, 2015). Despite the close phylogenetic relations between the *Oncocyclus* irises (Sapir & Shmida, 2002; Wilson, Padiernos, & Sapir, 2016), the contrasting results revealed from four *Iris* species suggest that there is no universal rule for mixing populations.

A possible explanation for the contrasting results between *Iris* species might be related to differences in genetic structure and population sizes of the source populations used for the different species. Although population size is known to affect reproductive success in crosses between populations (Godefroid, Le Pajolec, & Van Rossum, 2016), it was not incorporated into our analysis for a few reasons. First, population size is very hard to estimate for a clonal plant such as *I. lortetii*. Second, population size is confounded with population identity, used here as categorical explanatory factor. Last, the genetic structure of the plants used in this experiment might not represent the genetic structure of the populations of origin due to non-random seed germination.

The reduced fitness of within-population crosses in this experiment, suggests that in *I. lortetii* genetic mixing between populations is advantageous, and is thus recommended in conservation efforts for this species. However, we tested cross success as fruit-set and seed-set only, and did not test for the vigor of the mixed F1 offspring, where outbreeding depression may be also expressed (Oakley et al., 2015; Schierup & Christiansen, 1996). Note that we have evaluated fitness only for a single growing season, while perennial plants may compromise fitness over multiple years (Barrett, 1998; Ehrlén & Münzbergová, 2009; Peterson, Kay, & Angert, 2016). This limits our interpretation regarding cross success. However, because these are experimental results, we believe that also the long-term fitness will be higher in patches of mixed populations in our experimental design. Annual monitoring of flowering and fruiting in the introduced plots is currently ongoing, in order to test this hypothesis over multiple years.

5. Conclusions

Overall, our results clearly indicate that mixing genetic pools increases reproductive output in *I. lortetii*. We conclude that in the scale at which this experiment was performed, mixing of genetic resources is not only harmless, but even beneficial to *I. lortetii*. However, possible effects of outbreeding and inbreeding depression in genetic rescue can be influenced by the genetic structure of populations of origin. In a follow-up study we are currently genotyping populations of *I. lortetii* from its northern distribution in the Galilee. This will enable to associate nucleotide variation across the genome with the relative success of various population-site combinations and crosses. This will complete the picture and facilitate conservation decisions for preserving the endangered *Iris lortetii*.

Declaration of authorship

All authors conceived the ideas and designed methodology; GS and HS collected the data; all authors analyzed the data; HS led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jnc.2018.08.002.

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