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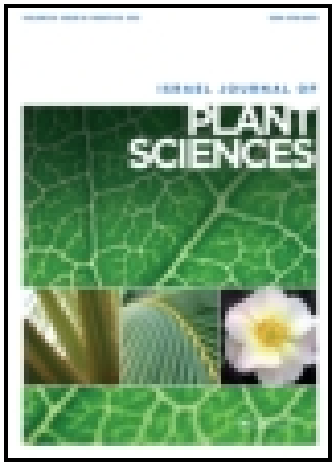

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Iris atrofusca genetic and phenotypic variation, the role of habitat-specific selection in this variation structuring, and conservation implications using quasi in situ guidelines

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Iris atrofusca genetic and phenotypic variation, the role of habitat-specific selection in this variation structuring, and conservation implications using *quasi in situ* guidelines

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Knowing the extent and structure of genetic variation in an endangered species is essential for establishing efficient conservation practices. However, the proper use of this information requires understanding the role of habitat-specific selection in genetic structuring. We present a study of population differentiation in an endangered species that utilizes guidelines of recently a proposed *quasi in situ* conservation approach, i.e. taking into account the scale and spatial pattern of local adaptation since if local adaptation is important, the introduced genotypes must be matched to the local biotic/abiotic conditions. Following this approach, we examined the extent and structure of genetic (AFLP) and phenotypic variation and tested for adaptive significance of this variation in critically endangered *Iris atrofusca* growing in Israel and Jordan. From these results we propose a sampling design that would (i) preserve species adaptive potential and (ii) insure environmental match of the plant material for relocation, reintroduction or enhancement.

Keywords: *Oncocyclus* iris; plant conservation; adaptation; genetic variation; conservation strategy; conservation management; *quasi in situ*

Introduction

Genetic variation at the intraspecific level is a prerequisite for species future adaptive changes and evolution, and has profound implications for species conservation (Schaal et al. 1991). Knowledge of the extent and structure of genetic variation of an endangered species may help in establishing improved conservation practices (Hamrick 1983; Hamrick & Godt 1996b; Woodruff 2001). On the other hand, this knowledge is often insufficient for successful conservation. In many cases it is important to understand the role of habitat-specific selection in structuring of genetic variation. The latter requires testing for local adaptation in natural or simulated environmental settings.

In the recently introduced *quasi in situ* approach (Volis & Blecher 2010), determination of the scale and spatial pattern of local adaptation is an important step in developing a species-specific conservation program. It requires analysis of the species distribution to identify potential locally adapted populations or population groups. A method of choice for experimental determination of a scale of local adaptation is a test for outbreeding depression, in which locally adapted genotypes are crossed with plants originating elsewhere (Hufford & Mazer 2003). In a case of strong local selection, the progeny from crosses with non-adapted genotypes are expected to have lower fitness and a decrease in fitness should be roughly proportional to a level of dissimilarity of the two parent habitats. If local adaptation is important, the introduced genotypes must be matched to the local biotic/abiotic conditions, i.e. they should come from the area defined as that of intensive local selection, or from a habitat with closely similar local conditions, and hence, exerting similar selection regimes.

We present a study of population differentiation in an endangered species using the *quasi in situ* guidelines. We examined the extent and structure of genetic variation and tested for adaptive significance of this variation in *Iris atrofusca* (Siems.) Baker. From these results we proposed a sampling design that would (i) preserve species adaptive potential and (ii) insure environmental match of the plant material for relocation, reintroduction or enhancement.

Material and methods

Study species and sampling

Iris atrofusca (Siems.) Baker is a perennial rhizomatous plant. Population distribution within the species range is discontinuous and comprises many populations with clearly recognizable boundaries and different

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environmental conditions (Arafeh et al. 2002; Sapir et al. 2002; Volis et al. 2010). Plants are clonal and create large patches of leaf fans (ramets) that are connected by the rhizome indicating that they belong to a single genet. Each ramet produces only one flower. Plants flower in late February−early April. Although the species is reported to be self-incompatible (Avishai & Zohary 1980), in fact intra- and inter-flower selfing (geitonogamy) is possible (S. Volis, unpublished data), but only via insect pollinators, which are night-sheltering solitary male bees (Sapir et al. 2005). Upon maturation in May, the seeds are released from a capsule containing 30−50 seeds (Avishai & Zohary 1980). *I. atrofusca* is relatively widely distributed; it grows from the northern Negev desert (ca. 150 mm rainfall), through the eastern slopes of Judean and Samaritan mountains in Israel and the western slopes of Gilead mountains in Jordan, to the southern Golan heights (ca. 500 mm rainfall) (Figure 1 and Table 1).

Despite its widest distribution, compared with other *Oncocyclus* species in Israel (all of them are Red-listed in the country), *I. atrofusca* currently is probably one of the most threatened species of the group in Israel. For example, its habitats in the Northern Negev are highly vulnerable and in the last decade many populations in this region dramatically decreased in size or became extinct due to anthropogenic disturbances. These disturbances included urbanization, infrastructure works, intensive and extensive agriculture, overgrazing, forestry works, and illegal Bedouin settlements (Volis et al. 2010). The situation in Judea and Samaria is not different principally: the majority of *I. atrofusca* populations, as in the Northern Negev, are not situated in protected areas (Shmida & Pollak 2007).

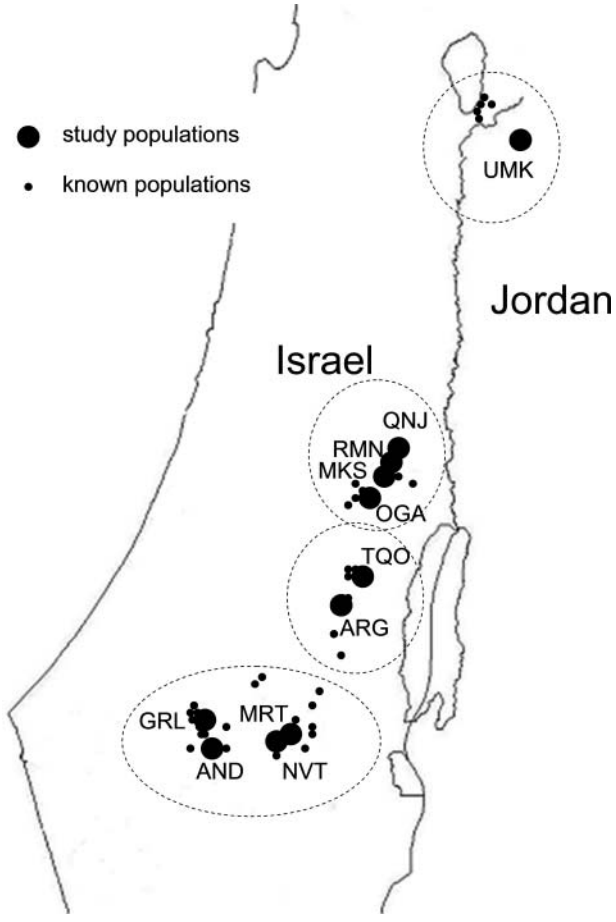

Figure 1. A map showing the distribution of *I. atrofusca* in Israel and Jordan (after Shmida & Pollak 2007), four recognized regions, and study populations (see supplementary Table S1 for additional information).

Sampling was conducted from 2006 to 2009 in 11 *I. atrofusca* populations covering the species range (Figure 1) with sampled individuals being separated by at least 10 m. The fresh leaf material collected in the field was stored under −80°C and the plants were maintained in the living collection at the Bergman Campus, Ben-Gurion University. The information about sampled populations, including their geographic coordinates and population sizes is presented in supplementary Table S1. In estimating population sizes a genet (= a patch of leaf fans) was treated as an individual plant.

DNA extraction and AFLP analysis

Genomic DNA was extracted using the CTAB protocol of Doyle (1990). DNA quality and concentrations were checked on 1% agarose gels by comparison to a known standard. Amplified fragment length polymorphism (AFLP) analysis generally adhered to the protocol described by Vos et al. (1995) with minor modification. Genomic DNA (about 200 ng) was digested and ligated to adaptors (*Eco*RI adaptor: 5'-CTCGTAGACTGCGTACC-3'/5'-AATTGGTACGCAGTCTAC-3'; *Mse*I adaptor: 5'-GACGATGAGTCCTGAG-3'/5'-TACTCAGGACTCAT-3') in one step, using 1U *Mse*I and 5U *Eco*RI restriction enzymes and 1.2 U of T4DNA Ligase (New England Biolabs). Restriction-ligation was carried out over 3 h at 37°C, using ligation buffer provided by the supplier (New England Biolabs). Pre-amplification of the diluted (10-fold) restriction-ligation was carried out with primers (one selective nucleotide with each primer, A for *Eco*RI primer, and C for *Mse*I primer) in the thermal cycler (Eppendorf, Germany) using the following cycling parameters: 30 cycles set at 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. The pre-amplified products were diluted 10-fold and used as the template for selective amplification. Based on a preliminary assignment, where 16 selective primer combinations were tested three times for polymorphism and reproducibility, the whole sample set was genotyped using five primer combinations (fluorescent dye in parentheses): *Eco*RI ACT (FAM)−*Mse*I CTG; *Eco*RI AAG (6-NED)−*Mse*I CTG; *Eco*RI ACT (FAM)−*Mse*I CAG; *Eco*RI AGC (6-VIC)−*Mse*I CAG; *Eco*RI AAG (6-NED)−*Mse*I CAG. The polymerase chain

Table 1. Environmental parameters for the four recognized regions (A. Danin, personal communication).

Region	Altitude (m)	Rainfall (mm)	Soil	Vegetation
Negev	250–600	150–250	Brown lithosol	Shrub-steppe
Judea	300–800	300–400	Brown rendzina	Semi-steppe batha
Samaria	100–650	400–500	Brown rendzina	Semi-steppe batha
Gilead and Southern Golan	50–350	400–500	Brown rendzina	Batha

reaction (PCR) program consisted of two segments. The first segment comprised 12 cycles with one cycle at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The annealing temperature was lowered by 0.7°C per cycle. The second segment comprised of 25 cycles at 94°C for 30 s, 56°C for 60 s and 72°C at 60 s. For each individual, 0.5 μl of selective PCR products were combined with 0.25 μl GeneScan ROX 500 (PE Applied Biosytems) internal size standard and 8.25 μl formamide and run on a capillary sequencer ABI 3130XL (Applied Biosystems). Fragments in the ranges 50–500 bp were scored and exported as binary presence/absence matrix using GeneMapper version 4.0 software (Applied Biosystems) with manual check.

The hierarchical genetic structure in *I. atrofusca* was investigated by an analysis of molecular variance (AMOVA; Excoffier et al. 2005) and using Nei's G_{ST} statistic (Nei 1973). The number of permutations for significance testing was set at 1000 for analysis. The AMOVA was performed with GENALEX version 6.5 (Peakall & Smouse 2006) and the G_{ST} statistic was calculated with POPGENE version 1.31 (Yeh et al. 1998). A relationship between the population pairwise geographic and Nei unbaised genetic distances was analyzed by Mantel test.

Common garden experiment

In September 2008 a total of 90 plants from eight populations of *I. atrofusca* were planted in 3 l pots placed randomly on tables inside a net house in the Bergman Campus, Beer Sheva. The pots were filled with a mixture comprising equal parts of gravel, loess and sand. Plants were watered through drip lines, with one dripper placed in each pot. During the experiment (September–April), the plants were watered regularly to compensate for the high evaporation rate and to keep them in good condition without any signs of stress. On each plant that reached the six-leaves stage, the third and fourth fully-developed leaves were measured for leaf length, thickness, width and curvature. These measurements were taken between the middle of February and the middle of March. Vegetative traits were measured on two leaf fans per plant in most plants (72 in total), one leaf fan was measured for the rest. Vegetative traits' measurements were averaged per plant per trait.

Phenotypic differences in leaf shape among individuals having different population origin were analyzed by Principal Components Analysis (PCA) using the STATISTICA program (StatSoft Inc. 2004).

Cross-breeding experiment

We performed during 2007–2011 cross-pollinations of *I. atrofusca* plants from the Tel Arad (TA) population (located 5.5 km east from the MRT population; Figure 1) with *I. atrofusca* plants from (i) the same population; (ii) another population (GRL) from the same region (Negev); and (iii) other regions. Prior to the experiment, large rhizomes comprising many connected genetically identical ramets were dug out in 2007 in the TA population and planted separately in isolated sections 50 cm (length) × 50 cm (width) × 30 cm (height) of plastic boxes filled with the original loess soil. The boxes were placed in a net house in the Bergman Campus, Beer Sheva. Plants were watered regularly during the growing season (from October until April). The amount and timing of water supplied depended on ambient conditions and varied from two to three times a week. During four years, the emerging flowers were pollinated by deposition of removed anthers on a stigma of the recipient plant. The seed set and weight of the seeds were documented for each performed pollination. The seeds had been stored in a refrigerator until September 2011, when they were sown in 3 l pots filled with a mixture of sand and loess and the number of emerging seedlings was recorded over two consecutive seasons.

The four performance traits seed set, total and individual seed weight and percent germination were calculated for each type of cross-pollination per mother plant and converted into relative performance.

In November 2012, before the beginning of the rainy season, the one-year old rhizomes grown from germinated seeds were planted in a small field with natural soil and vegetation typical for the northern Negev desert located within the Bergman Campus, Ben-Gurion University. The 12 one-year-old rhizomes representing each of six groups of different geographic origin were individually weighed and randomly placed into cells of the 24-cell plastic tray (35 × 26 × 16 cm). The groups were F1 that resulted from crosses between mother TA plants and pollen donors

from: (i) TA population and (ii) other *I. atrofusca* populations. There were 2−5 mother plants and pollen donors in each group. Three trays were filled with the sieved local loess soil and buried adjacent to each other to the surface level. The plants were allowed to grow under natural precipitation throughout the season and to remain dormant after drying out the aboveground biomass during summer. In September 2013, just before the beginning of the next season, the trays were dug out, and brought to the laboratory for examination and weighing of the survived rhizomes.

The individual rhizome growth rate was used as a performance measure that approximates, through direct positive relationship, plant fitness (e.g. Johnston et al. 2001; Taylor et al. 2011). We used one-way ANOVA for analysis of origin effect on rhizome growth rate and Dunnett's test for mean comparisons.

Results

AFLP variation

In total, 302 AFLP loci were identified and used in the analysis. Among these, 274 (94.5%) were polymorphic. Measures of regional and population genetic diversity are summarized in Table 2. For no estimate of genetic diversity used, neither regions nor populations differed in the extent of variation. The average within-population diversity was 1.52 for the observed number of alleles (Na), 1.34 for the effective number of alleles (Ne), 0.20 for the expected heterozygosity (He), 0.29 for Shannon's index and the mean proportion of loci polymorphic (P) was 54.2%.

Table 2. Regional and population genetic variation in *I. atrofusca*.

Region	Population	Na	Ne	He	I	P (%)
Negev	AND (7)	1.53	1.34	0.20	0.29	53.1
	GRL (9)	1.55	1.34	0.20	0.30	55.2
	MRT (10)	1.61	1.37	0.22	0.33	61.0
	NVT (6)	1.50	1.33	0.19	0.28	50.3
Judea	ARG (5)	1.52	1.35	0.20	0.30	52.1
	TQO (9)	1.65	1.39	0.23	0.34	64.8
Samaria	OGA (5)	1.49	1.32	0.19	0.28	48.6
	RMN (7)	1.56	1.34	0.20	0.30	55.9
	MKS (7)	1.51	1.32	0.19	0.28	51.4
	QNJ (3)	1.39	1.31	0.17	0.25	39.0
Gilead	UMK (3)	1.38	1.31	0.17	0.24	38.3

Note: The numbers in the parentheses after the population code represent the number of sampled individuals. Na, observed number of alleles; Ne, effective number of alleles; He, expected heterozygosity; I, Shannon's Information index; P, the percentage of polymorphic loci.

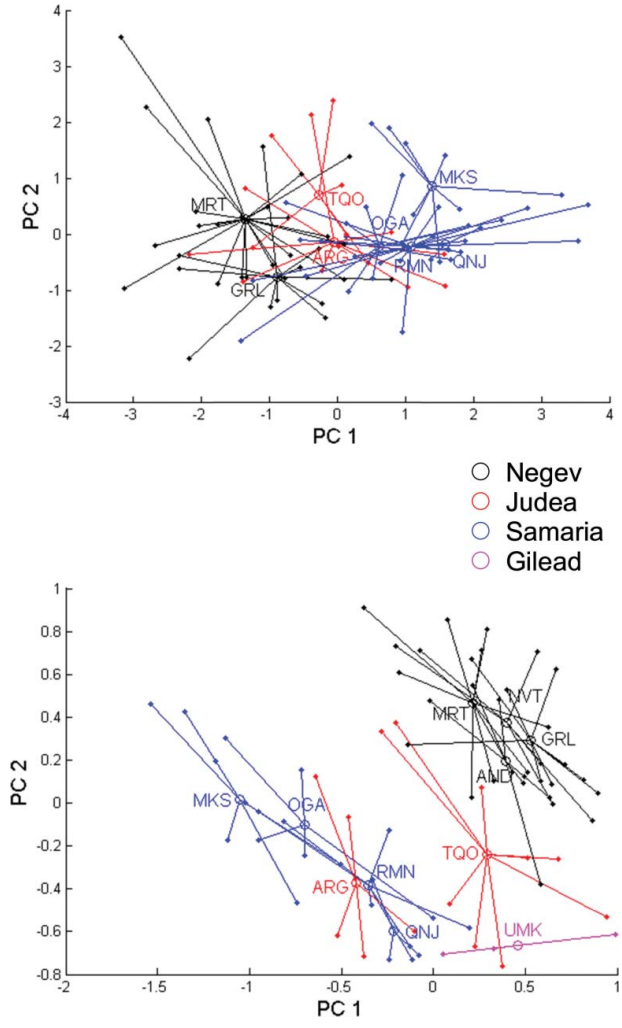

Figure 2. Principal Components Analysis of the four vegetative traits (top) and Principal Coordinates Analysis of the AFLP data (bottom). Small dots represent the individuals connected via a population centroid. The first two axes explained 55.9% and 25.4% (top) and 26.0% and 15.5% of variation (bottom).

The genetic differentiation among populations estimated by G_{ST} and Φ_{ST} (AMOVA) was 0.28 and 13.2, respectively. Subdivision of genetic variation into three levels, among regions, among populations within regions and within populations revealed by AMOVA was 6.6%, 8.1% and 85.3%, respectively.

The principal coordinate analysis revealed a clear separation of the Negev region from the other three (Figure 2). The plants from the three Samarian populations were clustered together, but the two Judean populations were not. Although one Judean population (TQO) was relatively different from those of the other regions, the second one (ARG) did not differ from the Samarian populations. The only Gilead population was similar to the Samarian and Judean populations.

A Mantel test revealed a significant correlation between genetic and geographic interpopulation distances

($r = 0.462$, $t = 2.24$, $p = 0.018$). Population genetic diversity estimated by either N, Ne, He or P was not related to population location latitude.

Phenotypic variation

The PCA analysis did not reveal clear separation of the regions by the overall leaf shape (Figure 2). However, in two traits (leaf length and leaf thickness) Samaria differed from the other two regions, and in leaf width three regions, Samaria, Judea and the Negev, differed from each other (Figure 3).

Flower color exhibited high variation at all three levels, within population, among populations and among regions (supplementary Figure S1).

Cross-breeding experiment

There was no significant difference in either seed set, total or individual seed weight, or percent germination between F1 progeny that resulted from within-population (i.e. both parents of TA origin), within-region and between region crosses (ANOVA, $F_{4,45} = 1.7$, 2.1, 0.2 and 1.9, $p > 0.05$; Figure 4).

The plants in the field experiment received 175.6 mm of natural precipitation which is close to the interannual average (190 mm, observation years 1949−2013). There was relatively high mortality of one-year rhizomes in all genetic groups except F1 TA × MKS (Figure 5). There was no significant difference in plant performance measured as the rhizome growth rate among the groups (ANOVA, $F_{5,68} = 0.8$, $p > 0.05$) and between F1 plants of TA origin and any F1 genetic group that resulted from interpopulation cross (Figure 5).

Discussion

The maintenance of genetic variation is one of the major objectives for conserving endangered and threatened species (Avise & Hamrick 1996). Assessment of genetic diversity is important for designing conservation strategies for threatened and endangered species (Hamrick 1983; Hedrick & Miller 1992; Hamrick & Godt 1996a), but this knowledge is often insufficient to allow better conservation (Hedrick 2001; Pertoldi et al. 2007). We combined this knowledge with the results of testing for local adaptation to understand the role of habitat-specific selection in structuring of genetic variation and to work out detailed recommendations for species conservation.

Genetic diversity

Overall, our results indicate a moderate level of within-population genetic diversity in *I. atrofusca* (He = 0.20) that is close to the average value over 13 species in a review of Nybom (2004) (He = 0.23±0.08). Our estimate is lower than what was earlier detected with random amplified polymorphic DNA (RAPD) (He = 0.39, Arafeh et al. 2002). Nevertheless, the observed diversity and the fact that the studied populations, including those with very small sizes, exhibited a similar level of AFLP variation is difficult to reconcile with the rapid disappearance of the species' natural environment and direct distraction (partial or full) of many populations. Habitat fragmentation and deterioration under human disturbance is a key

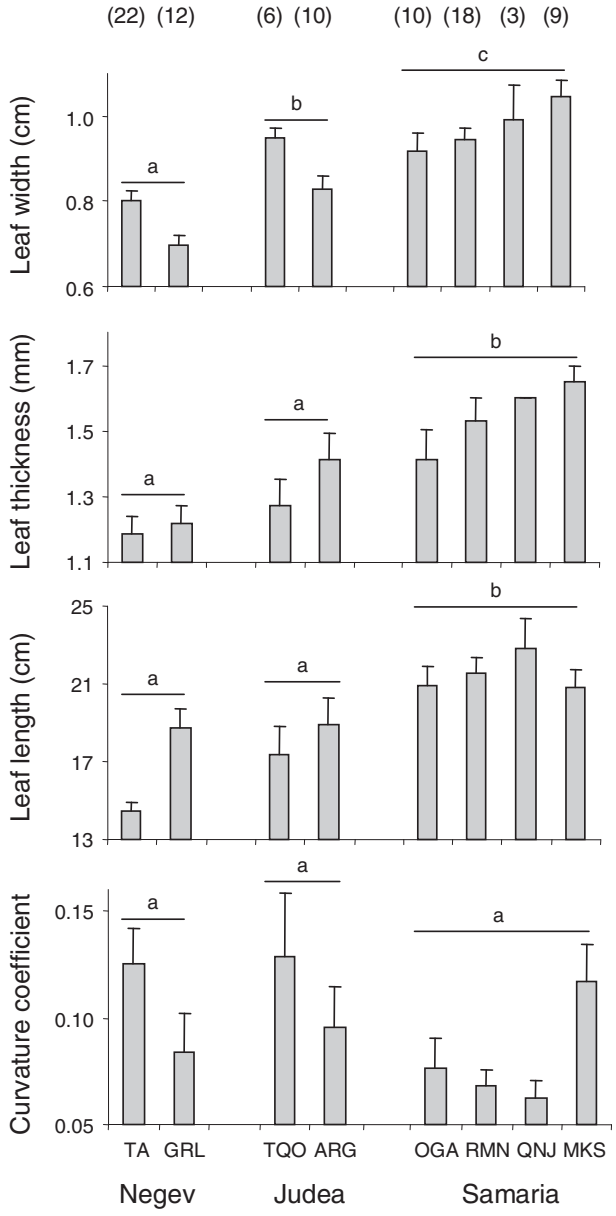

Figure 3. Population means and SE for four vegetative traits that were measured in eight populations representing three regions, and results of Tukey−Kramer test after one-way ANOVA. Population sample sizes are in parentheses. Different letters denote significantly different regions.

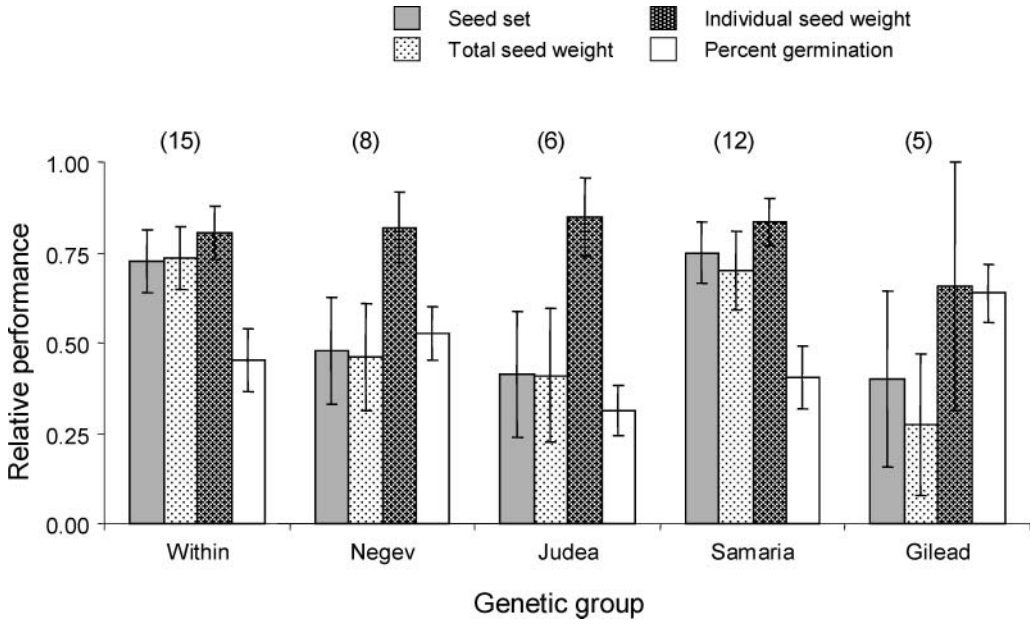

Figure 4. Relative plant performance for five types of cross-pollinations that differed in origin of the pollen donor: seed set, total and individual seed weight, and percent germination (means ± SE) in crosses of TA plants with plants from the same population (Within), another population from the same region (Negev) and other regions (Judea, Samaria, Gilead). The number above the bars denotes sample size, namely number of mother plants used in each cross-pollination type.

factor leading to low genetic diversity within a population (Hunter 1996).

A plausible explanation was proposed by Arafeh et al. (2002): "if populations have only recently experienced a large reduction in size, then the negative effects of isolation, genetic drift and elevated inbreeding may not have become manifested in contemporary levels of genetic diversity (Levin 2000)." We can add to this that most of the plants in populations of *I. atrofusca* are established old clones while young plants and seedlings are very rare

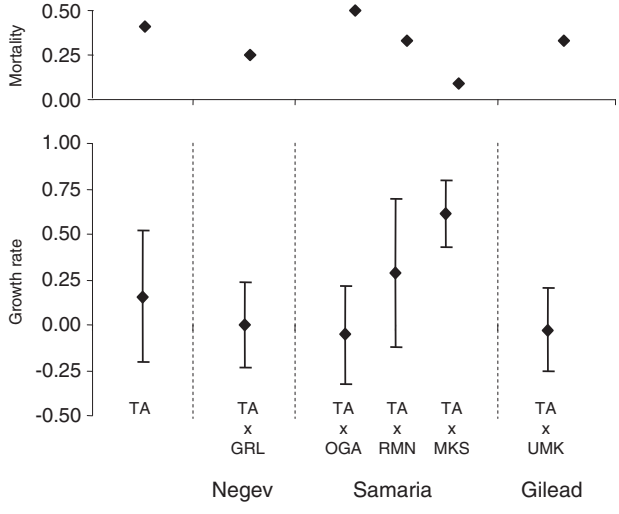

Figure 5. Plant performance in the cross-breeding experiment measured as percent mortality (top) and growth rate (means ± SE) (bottom) of one-year-old field-transplanted plants.

(S. Volis and M. Blecher, personal observations). Although seeds are produced every year, the lack of seedlings and young plants suggests that germination and/or survival of seedlings are rare events and vegetative propagation is a much more common mechanism of propagation in this species. Thus negative genetic consequences of anthropogenic disturbance and direct population destruction will inevitably become evident, but after a time lag of unknown duration. It should be noted, however, that with the current rate of population disappearance and without immediate conservation actions, some populations may disappear before this time lag.

For conservation management the above considerations mean that decision-making concerning *I. atrofusca* should not be based on the extent of population genetic variation revealed by neutral markers.

Genetic structure

Strong population differentiation is expected in *I. atrofusca* because: (i) seed dispersal is by gravity and upon maturation and release from the capsule most of the seeds are dispersed near the mother plant, and (ii) there is a limited pollen movement due to low pollinator visitation rates (Sapir et al. 2005). Indeed, the observed high level of population differentiation ($G_{ST} = 0.28$) is comparable with analogous estimates for this species using RAPDs ($G_{ST} = 0.21$, Arafeh et al. 2002). Similar results were reported for *I. aphylla*, an endangered species in Poland analyzed with RAPDs ($G_{ST} = 0.23$) (Wróblewska et al. 2003).

In addition to among-population differentiation (8.1%) there was a similar level of differentiation among regions (6.6%). This differentiation is not surprising given the disjunct distribution of regional population groups and apparently no contemporary gene flow between the regions. We do not know the role of region-specific environmental conditions in this differentiation, but the observed phenotypic differences in leaf traits suggest its importance.

The known populations of *I. atrofusca* are distributed in a strip-wise fashion which, together with detected significant association of genetic and geographic interpopulation distances, suggests very limited routes for among-population gene exchange in this species. Although we do not know the exact causes of this distribution pattern, the pattern itself emphasizes the importance of every population that is on a line as a "stepping stone" for maintaining the gene flow in the species. For *in situ* conservation management this means that the small and highly fragmented Samarian and Judean populations are vital and must be given the highest priority.

Adaptive significance of genetic/phenotypic variation

The controlled cross-pollination followed by transplant experiment revealed no superior performance of F1 plants originated from parents of local origin over those with fathers of non-local origin. However, this fact should not be interpreted as either lack of local adaptation or uniform selection regime across the species range. Because of the high mortality of one-year-old rhizomes and high variation in growth rate within genetic groups, the detection of local adaptation in *I. atrofusca* may require much larger sample size than in the current study. It is also plausible that local advantage in this species can be detected only at older plant age, in experimental settings closer to the species native conditions or over longer period embracing a range of environmental fluctuations.

Recommended conservation strategy

The *quasi in situ* strategy is especially applicable to plant species undergoing rapid destruction of their native environment and represented by a small number of existing populations (Volis & Blecher 2010). This is the situation with *I. atrofusca*.

The combined results of genetic and phenotypic variation suggest the importance of all three hierarchical levels, namely within-population, among-population and among-region ones, in sampling, *ex situ* and *in situ* actions in *I. atrofusca*. Earlier, Arafeh et al. (2002) recommended that existing geographically differentiated sets of populations require conservation attention as separate management units. Although we failed to detect the involvement of specific agents of natural selection in this variation, we base our recommendations not only on recognition of regional groups as management units, but also on an assumption of adaptive significance of their variation.

To preserve the species genetic variation and potential to adapt to future climate changes, we recommend that each region is sampled with a representative number of populations (at least five, which for some regions is close to the number of extant populations). As population destruction and decrease in size occur in an accelerating rate, sampling must be done immediately, starting from the smallest and most endangered populations such as MKS, UMK and AND. The seeds and not the rhizomes must be collected. The number of sampled randomly selected individuals will vary from all fruiting plants to 50 depending on the population size. One capsule per plant should be collected. The sampling procedure is easy compared with other endangered species. As the capsule opens immediately upon maturation releasing seeds, we recommend sampling capsules while green and storing in the shade to mature. The seeds should not be bulked, but preserved as separately bagged and labeled accessions. From each accession, the larger part of the seeds is stored in long-term seed storage under low temperature. The smaller part is used for propagation. This step involves germinating and planting under favorable greenhouse/net-house conditions.

As plants reach the size necessary for successful establishment *in situ* (above 4 g; Volis et al. 2007), they should be planted as living collections in appropriate natural or seminatural protected sites protected by law and regular inspection. One such site should be established in each of the four regions Negev, Judea, Samaria and Gilead and harbor plants originating in the same region. The living collections are used for natural or assisted pollination and obtaining large quantities of seeds. These seeds can be balked and sown in mass *ex situ*, grown to 4 g weight and used for some (but not all) actions *in situ*. They can be used for the creation of new populations within the corresponding region (regional match), but cannot be used for the reinforcement of existing populations, which requires an exact population match.

One of the major threats to all existing population of *I. atrofusca* is grazing. Even moderate levels of grazing during vegetation season completely prevents flowering in this species (S. Volis and M. Blecher, unpublished data). Therefore, an important part of *I. atrofusca* conservation strategy is prohibition of access by sheep, goats, camels or cattle to the population locations throughout winter. Only in May, after maturation of seeds, should the herds be allowed into *I. atrofusca* locations.

Once again, we would like to stress that a conservation plan including both *ex situ* and *in situ* components has to be approved and started immediately in light of the disappearance or dramatic decrease in size of many populations in the last 10 years.

Acknowledgments

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