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Negative evidence of local adaptation to the establishment conditions in a perennial herb

**José L. Garrido · Pedro J. Rey ·
Carlos M. Herrera · José M. Ramírez**

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Abstract The differential adaptation of populations of the same species to their local environmental conditions through divergent selection, known as local adaptation, is a key step in the process of diversification of species. Here, we explore the local adaptation of the perennial mountain herb *Helleborus foetidus* to variable environmental conditions of seedling emergence and establishment at two different spatial scales (habitats and regions) with special attention to the role of physical and chemical soil properties. The possibility of local adaptation was evaluated under the "local versus foreign" and the "home versus away" criteria. Reciprocal sowing experiments were carried out by cross-sowing seeds among habitats and regions, controlling for maternal effects by means of seed mass, and recording seedling emergence and survival. Several topsoil properties were measured linked to each sowing point. Only partial patterns of local adaptation were found, which were insufficient to eventually state the existence of local adaptation at any spatial scale or under any criteria assessed. Here, we discuss how soil properties and selection on seed size may be related to the non-achievement of local adaptation criteria. Negative evidence of local adaptation seems to be due to a congruency in the selective pressures exerted by the different soil environments on seedling emergence and survival.

Keywords Local adaptation · *Helleborus foetidus* · Topsoil properties · Seedling emergence · Seedling establishment · Divergent selection

Introduction

Studies on spatial variation in plant traits and their related genetic differentiation in natural populations are numerous and continue piling (see Linhart and Grant 1996; Galloway and Fenster 2000; Hereford 2009). Pioneers works by Turesson (1922) and Gregor (1930), as well as the extensive work carried out by Clausen, Keck, and Hiesey (reviewed in Núñez-Farfán and Schlichting 2001), first alerted to the association between the physical heterogeneity of environments and the genetic variability of plants distributed across these environments. The underlying mechanism generating this genetic-ecological association would be that different environments may result in different selective pressures generating genetic and phenotypic variability (Linhart and Grant 1996; Galloway and

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J. L. Garrido (✉) · C. M. Herrera
Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), 41092 Sevilla, Spain
e-mail: jlgarrido@ebd.csic.es

P. J. Rey · J. M. Ramírez
Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, 23071 Jaén, Spain

Fenster 2000). Therefore, genotypes would be locally adapted since, at their local site, they exhibit higher relative fitness than genotypes from other sites (Williams 1966; Kawecki and Ebert 2004).

Local adaptation has been detected to vary in response to both small-scale and broad-scale environmental variations (see Galloway and Fenster 2000 and references therein; Becker et al. 2006, 2008; Hereford 2009). The scale of local adaptation is expected to reflect the scale of the underlying environmental heterogeneity (Galloway and Fenster 2000). Specifically, while at broad spatial scales the climate plays a preponderant role (Macel et al. 2007), at smaller spatial scales, physical and chemical soil properties may represent a key factor for local adaptation (Hufford et al. 2008; Raabová et al. 2011). Particularly, the latter may be the case for local adaptation to the environmental conditions of seedlings' emergence and survival (Shea 1989; Linhart and Grant 1996; Pigliucci 2001), which are two crucial individual fitness components of particular interest in local adaptation processes (see Linhart and Grant 1996; Hereford 2009, and references therein).

Despite its expected relevance as a major factor promoting local adaptation of plants (see Linhart and Grant 1996, and references therein; Herrera 2000; Ortegón-Campos et al. 2011), how the spatial variation in these environmental conditions influences seedling regeneration remains comparatively unexplored (but see, Horvitz and Schemske 1986; Eriksson 1995; Walters and Reich 1997; Stratton and Bennington 1998) relative to the effort dedicated to the study of the influence of other abiotic factors, like light regime (Burton and Mueller-Dombois 1984; Valverde and Silvertown 1995), and biotic ones, like seed predation (Hammond et al. 1999), herbivory and pathogen attack (Packer and Clay 2000; Núñez-Farfán and Dirzo 1988; Ortegón-Campos et al. 2011), plant facilitation and competition (Herrera et al. 1994; Gómez-Aparicio 2009; Siles et al. 2010), and litter (Molofsky and Augspurger 1992).

Many studies on local adaptation, both exploring abiotic and (more recently) biotic factors, have done so by measuring fitness correlates of transplanted individuals (using composite fitness or fecundity), but comparatively less attention has been paid to detecting the signature of local adaptation specifically on early ontogenetic stages, like germination or seedling emergence and survival (using fitness estimates based on early vegetative traits) (see Hereford 2009 and references therein, Bischoff et al. 2006; Raabová et al. 2011). Thus, our study system of local adaptation considers two aspects of local adaptation of plants that remains nowadays insufficiently explored: the influence of spatial variation at different geographic scales on the detection of local adaptation, and exploring this effect on early life history stages.

Studies exploring the influence of soil properties on seedling establishment have traditionally considered just discrete quantitative characteristics (Schupp 1995; Godínez-Álvarez and Valiente-Banuet 1998; but see, Herrera 2002; Rey et al. 2004; Ortegón-Campos et al. 2011), which entail a great loss of environmental variation limiting the analytical power. Instead of doing so, here, we directly measure soil parameters in a continuous way, something we think that may enhance the quality and robustness of the results.

This paper is aimed to evaluate, at different spatial scales [intra-regional (among different habitats within a region) and inter-regional], the local adaptation of the perennial mountain herb *Helleborus foetidus* to the environmental conditions of seedling emergence and establishment, devoting special attention to the role of soil properties. We carry out this assessment by analyzing local adaptation patterns under both "local versus foreign" and "home versus away" criteria (Kawecki and Ebert 2004). The mechanistic basis underlying the potential existence of local adaptation can be reflected by a pattern of differential phenotypic selection among localities on some plant character related with seedling vigor. Here, we shall use the seed mass, the intra- and inter-population variation of which has been shown to be related to variation in seedling emergence and seedling establishment success for many plant species (e.g., Rey et al. 2004, and references therein). Specifically, several questions are addressed: (1) based on the detailed analysis of origin × destination interaction and on the available criteria for detecting local adaptation, does *Helleborus foetidus* exhibit local adaptation to the emergence environment faced by its seedlings? (1.1) Does this result extend to the survival environment? (1.2) Does it vary between the spatial scales considered?; and (2) Which may be the main determinants of the local adaptation pattern found here?

Materials and methods

Study species

Helleborus foetidus L. (Ranunculaceae), hellebore hereafter, is a perennial herb widely distributed in Western Europe (Werner and Ebel 1994). In the Iberian Peninsula, it typically occurs in mountain forests, particularly in clearings and forest edges from 800 to 1,200 m a.s.l. Each plant consists of one or several ramets which develop a terminal inflorescence after several seasons of vegetative growth. Its apocarpous flowers have 1–5 carpels (most commonly 2–3) each containing 8–15 elaiosome-bearing seeds. Fruit maturation and seed shedding take place in June-early July. Seedling germination and emergence occur from late December to early May with a peak in March (Garrido et al. 2007). Flowers, developing fruits, and seeds are consumed by rodents (mainly *Apodemus sylvaticus*) and lepidopteran larvae (*Noctua* spp. and *Trigonophora flammea*, Noctuidae). Although rodents consume hellebore seeds, quick ant removal of released seeds generally prevents extensive seed losses (Fedriani et al. 2004; Garrido et al. 2005; Manzaneda et al. 2007). Seeds on the ground remain dormant for at least 2 years (Garrido et al. 2007). Further information on this species' biology can be found in Garrido et al. (2002, 2005), and Herrera et al. (2001, 2002).

Experimental design and study sites

We conducted two reciprocal seed-sowing experiments to study local adaptation of hellebore to the environmental conditions of seedling establishment at two spatial scales: between different habitats, within the same mountain system (inter-habitat scale), and between different mountain systems (inter-regional scale). The latter experiment was conducted in three localities of South-eastern Iberian Peninsula, namely, "Collado de Góntar" (38°16′N–2°32′W, 1,550 m a.s.l., Mediterranean patchy scrubland, Sierra de Segura; Segura, hereafter), "Aguaderillos" (37°56′N–2°52′W, 1,270 m a.s.l., *Pinus nigra* forest, Sierra de Cazorla; Cazorla, hereafter), and "Puerto de La Mata" (37°44′N–3°28′W, 1,650 m a.s.l., Mediterranean patchy scrubland, Sierra Mágina; Mágina, hereafter). The former experiment was set up in four different locations distributed across the localities of "Puerto de La Mata" and "La Pasailla" (37°43′N–3°30′W, 1,440 m a.s.l., dense oak mixed forest, Sierra Mágina). Sowing sites where chosen further enough from any hellebore population to prevent possible interference of seeds from a natural seed bank. Seeds used were collected during June and July 1999. Seedlings' emergence and subsequent survival were monitored monthly during 29 months.

For the inter-habitat approach, seeds came from 2 origin populations at Sierra Mágina: "Puerto de La Mata" and "La Pasailla" (Open scrubland and Dense Oakwood, hereafter, respectively). Seeds from both origin populations were sown in November 1999 at four different destination habitats at Sierra Mágina: Dense Oakwood, Open Scrubland, Sparse Oakwood (a partially open Oak mixed forest, 1,600 m a.s.l), and Pine Forest (a *Pinus* forest, 1,650 m a.s.l) (see experimental scheme in Fig. 1). Geographic distances between each destination habitat ranged from 666 (OS-SOW) to 1,922 m (OS-DOW). Open Scrubland and Dense Oakwood represent the two main habitats were hellebore occurs at Southern Iberian Peninsula. Sparse Oakwood and Pine Forest represent secondary habitats where hellebore also frequently occurs (Garrido et al. 2005). At each origin population, ten hellebore individuals were selected as mother plants. Within each mother plant, seeds were collected homogeneously among different fruits. Seeds from a single mother plant were randomly assigned to 2 sowing points within each of the 4 destination habitats, i.e., two replicates per mother plant at each destination habitat. At each destination habitat, forty 30 × 30 cm sowing points were homogeneously distributed over 12 m^2 and their mean seed size were measured. Each sowing point was protected with a 1 cm-mesh metal fiber cage to prevent removal by rodents. During the pre-emergence period, this protection was also reinforced with 1-mm mesh fiberglass to prevent seed removal by ants. Forty seeds were placed in each sowing point, which added up 1,600 per habitat and a total of 6,400 seeds. The experiment consisted of 3 factors: Origin population [2 levels: Dense Oakwood (DOW) and Open Scrubland (OS)], Destination habitat [4 levels: Dense Oakwood (DOW), Open Scrubland (OS), Pine Forest (PF), and Sparse Oakwood (SOW)], and Mother plant (10 mother plants from each of the two origin populations at Sierra Mágina).

Ten soil samples were obtained in February 2001 from each destination habitat. Soil samples were

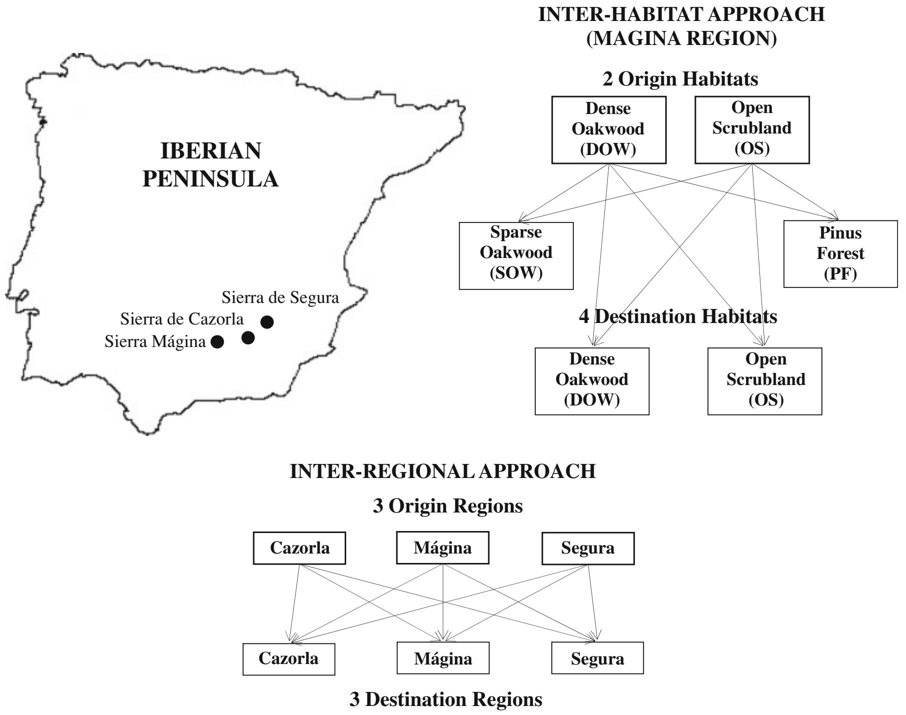

Fig. 1 Geographic location of sampling sites and experimental schemes. Inter-habitat approach was studied by sowing seeds from 2 origin habitats in four different habitats (Pine Forest, Dense Oakwook, Sparse Oakwood, and Open Scrubland) at Sierra Mágina (i.e., Mágina Region). Inter-regional approach was evaluated by sowing seeds from 3 different regions (Sierra de Cazorla, Sierra Mágina, and Sierra de Segura) in each one of these regions

homogeneously distributed, corresponding each to 4 sowing points (see Herrera 2002 for a similar procedure). Soil samples were analyzed by the Food and Agriculture Laboratory of Junta de Andalucía (Atarfe, Granada, Spain). Standard analytical soil procedures were used to determine texture (percentage of sand, silt and clay), total Nitrogen (TN), oxidable organic matter (OOM), assimilable Phosphorus and Potassium (AP and AK, respectively), pH and water retention capacity at 1/3 atm (WRC1/3; standard value corresponding to Soil Field Capacity), and 15 atm (WRC15; standard value corresponding to Soil Permanent Withering Point).

For the inter-regional approach, the experiment consisted of a completely crossed sowing of seeds from the origin regions of Sierra de Segura, Sierra Mágina and Sierra de Cazorla (Fig. 1). Twelve sowing points (4 from each region) were established at each destination region with 50 seeds each and characterized by their mean seed size. Several mother plants were selected at each origin region. Seeds were collected similarly to the intra-habitat scale. Soil parameters and maternal origin were not considered here. Geographic distances between each destination region ranged from 46.9 km (Cazorla-Segura) to 103.5 km (Segura-Mágina).

Contrasting "local versus foreign" and "home versus away" criteria for local adaptation

Local adaptation should be detected as improved mean relative fitness of each local population at its own habitat. Traditionally, this has been studied by means of reciprocal transplant experiments in which the average relative fitness of several demes is measured across a set of habitats and the genotype × environment (i.e., deme × habitat) interaction assessed. This interaction has been usually analyzed by comparing the performance of a given genotype "at home" and "away," but it has been suggested that the key comparison should be between "local" and "foreign" genotypes within each habitat (Kawecki and Ebert 2004).

Here, origin × destination statistical interaction effects on seedling emergence and survival are used to depict "local versus foreign" and "home versus away" criteria of local adaptation. "Local versus foreign" criteria is represented by the differential fitness, in terms of emergence and survival, of "local" versus "immigrant" origin populations within each destination (habitat or region). "Home versus away" approach is represented by the differential fitness of each origin population at "home" and "away" destinations (habitats or regions). Following the terminology by Kawecki and Ebert (2004), to conclude on the existence of a general pattern of local adaptation, we need to compare "sympatric" versus "allopatric" combinations of the interaction between destinations and origins.

Data analyses

Under both spatial scales, emergence, survival and the potential influence of seed size were analyzed through a Generalized Linear Mixed Model by means of GLIMMIX Procedure of SAS (SAS Institute Inc. 2004), including mean seed size of sowing points as a covariate. Cumulative seedling emergence and survival for three seasons (2000–2002) were adjusted to a binomial distribution ("emerged/total sown seeds per sowing point" and "survived/total emerged seeds per sowing point" at the end of the three seasons) using "logit" as link function. Origin population, Destination habitat/region, and its interaction were treated as fixed factors. In those analyses considering maternal effect, i.e., those regarding inter-habitat scale, Mother (nested within Origin) and its interaction with Destination were considered as random factors. Seed size × destination interaction was also included once tested for homogeneity of slopes of the covariate across factor levels. Slope contrasts among destinations were tested by regressing, at each destination, the predicted values of emergence probability for three reference values of seed mass (first quartile, median, and third quartile). Contrasts were also performed to test differences in emergence probability between destinations at the three reference values of seed mass.

The pattern of variation of seed size among mother plants and origin populations was analyzed by a General Linear Mixed Model by means of MIXED Procedure of SAS with Origin as fixed factor and Mother, nested within Origin, as random.

To assess the pattern of variation in soil parameters and its relation with emergence and survival, we first decomposed the variation of each variable, between and within sites, by means of VARCOMP Procedure of SAS. As reflected by other studies (Webster and Butler 1976; Arp 1984; Arp and Krause 1984), soil parameters were highly correlated. Comparisons among habitat types were carried out both under univariate and multivariate tests. Thus, we secondly performed a Principal Component Analysis to synthesize all soil parameters in independent factors.

To assess the combined influence of soil properties on emergence and survival, we performed an ANCOVA analysis using a Generalized Linear Mixed Model by incorporating factor scores of principal components as covariates. Given their potential combined effects on emergence and survival, destination habitat, and mean seed size of sowing points (the latter as a covariate) were also included in these analyses. All analyses involving covariates were carried out once tested for the homogeneity of slopes of each covariate variation across factors levels.

Results

Inter-habitat approach

Soil properties

All soil parameters varied considerably both between and within habitats (Table 1). OOM, WRC1/3, WRC15, and texture properties (clay, sand, and silt contents) showed higher variation within habitats (with between 55 and 85 % of their variances due to this level) than between habitats. Properties related to soil fertility (AP, AK, TN, and pH) varied mainly between habitats (61–94 % of their variances due to this level) with pH showing a particularly low within-habitats variation. All habitat types differed significantly in mean soil parameters (except for OOM, WRC1/3, and silt content), both considering these parameters individually in univariate tests (Table 1) or combined in a multivariate analysis ($F_{30,\,87} = 14.89$, $P < 0.0001$). PCA analysis performed on correlation matrix of soil variables extracted three principal factors (data not shown) that represented the 82 % of total variation in soil properties: Water-related Soil Properties factor (positively loaded by WRC1/3, WRC15, and OOM), Soil Fertility factor (negatively loaded by N, AP, and AK, and positively by pH), and Soil Texture factor (negatively loaded by silt, the finest soil fraction, and positively by clay).

Table 1 General statistics and variance partition between and within habitats in soil parameters ($n = 40$)

Soil parameter	Range	Median	CV (%)	Habitats variation (%)		$F_{(3,\ 36)}$
				Between	Within	
Physical and chemical properties						
AP	4–47	16.50	64.81	61.97	38.02	17.30***
AK	225–900	600	25.83	61.96	38.03	17.29***
N	0.11–1.06	0.64	35.27	70.74	29.25	25.18***
pH	6.50–8.10	7.60	06.09	94.78	05.21	182.84***
OOM	2.77–16.24	9.73	29.48	23.25	76.74	4.03*
WRC1/3	28.59–60.48	42.30	17.09	22.97	77.02	3.98*
WRC15	20.40–55.47	35.86	23.60	31.03	68.96	5.50**
Texture properties						
Clay (%)	7.60–61.85	44.90	32.67	44.41	55.58	8.99***
Sand (%)	5.25–74.60	11.81	75.26	18.84	81.15	3.32
Silt (%)	26.65–73.70	44.92	21.66	14.98	85.01	2.74

AP assimilable phosphorus (ppm), *AK* assimilable potassium (ppm), *N* total sodium (%), *OOM* oxidable organic matter (%), *WRC* water retention capacity (at 1/3 and 15 standard atmospheres)

Significance levels (Bonferroni Sequential Correction applied): * ≤0.05, ** ≤0.001, *** ≤0.0001

Emergence and survival

Emergence and survival probabilities differed between destination habitats. Emergence probability was higher at Sparse Oakwood habitat, followed by Open Scrubland and Dense Oakwood (Mean ± SE: $0.28 \pm 0.04 > 0.16 \pm 0.03 = 0.14 \pm 0.02$, respectively), while the lowest was recorded at Pine Forest (0.04 ± 0.01). Survival probability at Dense Oakwood, Sparse Oakwood, and Pine Forest was higher than at Open Scrubland ($0.51 \pm 0.07 = 0.50 \pm 0.06 = 0.64 \pm 0.09 > 0.18 \pm 0.04$, respectively). Destination effect on emergence and survival was independent from the origin population. Maternal effects on emergence and survival depended on the destination habitat (Table 2a).

Mean seed size varied significantly among mother plants within each origin ($F = 2.97$, $P = 0.0015$), but not among different origin populations ($F = 0.45$, $P = 0.5123$). Seed size effect differed between destinations only for seedling emergence (Table 2a, Fig. 2). Despite a punctual negative relationship at Pine Forest ($b = -0.51$, $P = 0.0015$), seed size was positively related to emergence probability at the rest destination habitats ($b = 0.01$–0.37, all $P < 0.0072$). Slope contrasts determined that at Pine Forest, except for low values of seed mass where no clear differences were found, seed mass was significant, and negatively related to emergence probability, while at the rest of destinations the relationship was significantly positive (Fig. 2).

Excluding the covariate seed size from the model determined the appearing of a maternal effect ($Z = 1.79$, $P = 0.0368$), thus suggesting that maternal effects can be appropriately explained by seed size differences.

Soil properties, emergence, and survival

ANCOVA analyses including soil properties factors, seed size, and destination habitat confirmed the influence of seed size on emergence (Table 3). There was also an important effect of destination habitat on emergence through different water-related and texture soil properties. In the case of survival, the most marked effect was water retention properties. Destination effect on survival was slightly mediated by soil textural properties (as shown by the marginally significant effect on Table 3). Fertility properties did not have any effect either on emergence or on survival.

Inter-regional approach

Seedling emergence differed between origin and destination regions and (Table 2b). Thus, emergence probability of seedlings coming from Segura was

Table 2 Generalized Linear Mixed Model performed to explore at (a) inter-habitat scale and (b) inter-regional scale, the variation between origins, destinations and mother plants in emergence and survival probabilities. Seed size is included as a covariate

Fixed factors	(a) Inter-habitat scale							(b) Inter-regional scale					
	Emergence				Survival			Emergence				Survival	
	dfn	*dfd*	*F*	*P*	*dfd*	*F*	*P*	*dfn*	*dfd*	*F*	*P*	*F*	*P*
Origin (O)	1	15.33	2.18	0.1602	57.72	1.23	0.2716	2	26	8.03	**0.0019**	0.80	0.4622
Destination (D)	3	64.55	5.32	**0.0024**	54.67	7.43	**0.0003**	2	26	36.85	**<0.0001**	16.17	**<0.0001**
O × D	3	43.38	0.83	0.4863	54.71	0.30	0.8287	4	26	1.59	0.2060	1.17	0.3472
Seed size	1	37.64	0.06	0.8042	58.65	3.57	0.0638	1	26	0.25	0.6226	1.67	0.2077
Seed size × D	3	64.99	7.71	**0.0002**	(*)			(*)				(*)	
Random factors			Wald χ^2	*P*		Wald χ^2	*P*			Wald χ^2	*P*	Wald χ^2	*P*
Mother (O)			1.43	0.0760		(**)				(***)		(***)	
Mother (O) × D			3.82	**<0.0001**		3.74	**<0.0001**						

Destination showed an overwhelming effect independently from the spatial scale and the life cycle stage considered. Significant values are shown in bold. (*) The covariate (seed size) exhibited homogeneous slopes across factor (destination) levels (see "Methods"). (**) Non estimable variation for this factor. (***) Factors not included in the model (see "Methods")

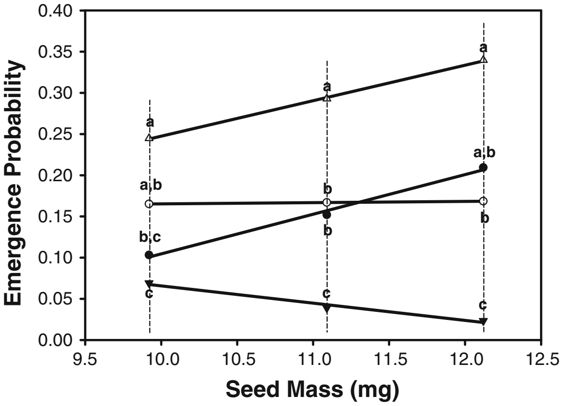

Fig. 2 ANCOVA-predicted variation of seed size effect on emergence across the four destination habitats: Open Scrubland (*open circles*), Dense Oakwood (*black circles*), Sparse Oakwood (*open triangles*), Pine Forest (*black triangles*). Regression lines at each destination habitat are represented for values predicted by the model at the first quartile (9.92 mg), median (11.09) and third quartile (12.12) of seed mass variable (*vertical dashed lines*). Slopes and significance of the effect of seed mass at SOW, DOW, OS, and PF were $b = 0.21$ ($P = 0.0003$), 0.01 (0.0072), 0.37 (<0.0001), and −0.51 (0.0015), respectively. Differences among predicted emergence probability at each reference value of seed mass are also shown with same letters indicating no significant differences at $P < 0.05$

higher than that of seedlings from Mágina and Cazorla ($0.27 \pm 0.04 > 0.14 \pm 0.02$ and 0.17 ± 0.03, respectively). Emergence probability of seedlings sowed at Cazorla was higher than that of seedlings at Mágina and Segura ($0.32 \pm 0.01 > 0.12 \pm 0.01$ and 0.15 ± 0.01, respectively). Seedling survival differed between destination regions. Thus, seedlings at Segura presented higher survival probabilities than at Cazorla, where, in turn, survival was higher than at Mágina ($0.93 \pm 0.03 > 0.60 \pm 0.05 > 0.43 \pm 0.02$, respectively). There was no origin × destination interaction effects on emergence or survival at this scale. Thus, the destination effect on emergence and survival was independent from origin population. Seed size had no effect on emergence at this spatial scale, and no variation in the effect of seed size was detected among destination regions.

"Local versus foreign" and "home versus away" criteria for local adaptation

Figures 3 and 4 show assessments of local adaptation under "local versus foreign" and "home versus away" criteria for the inter-habitat and inter-regional scales, respectively.

Table 3 ANCOVA analysis performed to explore the effect on emergence and survival of the water-related properties (PC1), fertility properties (PC2), texture properties (PC3), seed size, and destination habitat

	Emergence			Survival	
	df	Wald χ^2	*P*	Wald χ^2	*P*
Destination	3	8.21	**0.0418**	8.21	**0.0418**
Seed Size	1	10.35	**0.0013**	0.02	0.8872
PC1	1	2.14	0.1438	5.04	**0.0248**
PC2	1	0.48	0.4882	0.95	0.3300
PC3	1	0.01	0.9067	2.12	0.1458
PC1 × Destination	3	7.87	**0.0488**	6.58	0.0866
PC2 × Destination	3	5.31	0.1587	3.09	0.3799
PC3 × Destination	3	8.38	**0.0387**	7.30	0.0628

Significant values are shown in bold

At the inter-habitat scale, "local versus foreign" criterion determined for both fitness outcomes that no "local" seeds/seedlings outperformed significantly "foreign" ones (Fig. 3a). Under the "home versus away" criterion, seeds/seedlings at "home" habitat only outperformed partially fitness outcomes at "foreign" destination habitats. Thus, OS origin exhibited higher emergence probability at "home" habitat than at Pine Forest, and lower survival probability than at DOW and PF. On its part, DOW origin showed (a) higher emergence at "home" than at PF, and lower than at SOW; and (b) higher survival probability than at OS destination habitat.

At the inter-regional scale, "local versus foreign" criterion determined for both fitness outcomes that "local" seeds/seedlings outperformed only partially the "foreign" ones (Fig. 4a). At Cazorla, "local" seeds presented lower emergence than "foreign" seeds from Segura. At Mágina, there were no significant differences in emergence or survival. At Segura destination, "local" seeds presented higher survival probability than "foreign" seeds from Cazorla. Under "home versus away" approach, seeds from Cazorla exhibited higher emergence probability at "home" than at Mágina and Segura. Seeds from Mágina origin presented lower emergence probability at "home" than at Cazorla and lower survival probability than at Segura. Seeds from Segura origin showed lower emergence at "home" than at Cazorla and higher survival at "home" than at Cazorla and Segura.

Discussion

Results from this study suggest an overall lack of local adaptation of *H. foetidus* to the conditions of seedling establishment. Such generalization is primarily based in the lack of statistical significance of the interaction between origin and destination effects on seedling emergence and survival. In addition, despite some weak and partial evidence, both the optimal "local versus foreign" and the "home versus away" criteria did not provide support for local adaptation. Instead, we detected at both scales an overwhelming destination effect that much probably will be limiting the possible detection of local adaptation. Similarly, other studies have shown origin and destination effects, but no origin × destination effect (Rendon and Núñez-Farfán 2001), or not consistent patterns toward local adaptation (Macel et al. 2007). Overall, our results agree with the general expectation from the last available meta-analysis by Leimu and Fischer (2008) highlighting that of local adaptation is less common in plant populations than generally assumed.

It is to be noticed that the use of plant traits related to early life history stages instead of reproductive ones may limit the detection of local adaptation (Bischoff et al. 2006; Hereford 2009; Raabová et al. 2011), but, due to the long time period that *Helleborus foetidus* takes to reach the first reproductive event (if reached) (Garrido 2003), the use of reproductive traits may be limiting.

Studies that do not demonstrate local adaptation should not be considered as failed experiments, but as demonstrations that populations may be prevented from reaching adaptive optima (Hereford 2009). Therefore, it has been strongly demanded studies explicitly investigating the mechanisms that may potentially inhibit local adaptation (Hereford 2009). Here, we discuss how environmental factors, particularly water, fertility, and textural soil properties, and selection on seed size may be related to the non-achievement of local adaptation criteria.

Achievement of local adaptation criteria

Inter-habitat scale

In addition to the lack of a significant origin × destination interaction effect, both criteria failed to detect local adaptation at this scale. No origin population

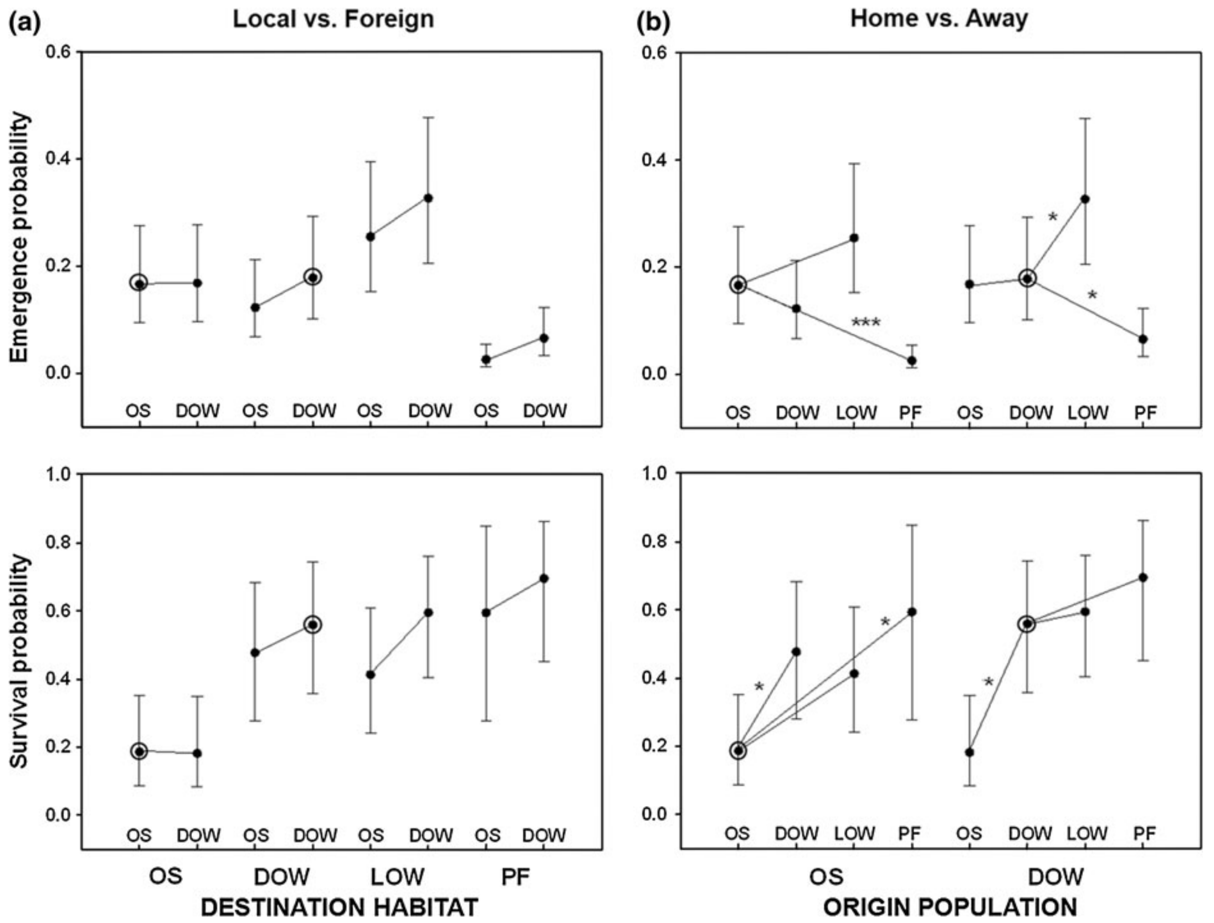

Fig. 3 "Local versus foreign" (**a**) and "home versus away" (**b**) criteria assessing local adaptation of hellebore to the environmental conditions of emergence (*upper panel*) and survival (*lower panel*) of seedlings at the inter-habitat scale. Figure 2a represents emergence and survival fitness outputs for four different destination habitats (x-axis: Open Scrubland, OS; Dense Oakwood, DOW; Sparse Oakwood, SOW; and Pine Forest, PF), each one sowed with seeds from two different origins (OS and DOW in secondary x-axis). Figure 2b represents the emergence and fitness outputs of seedlings from two origins (x-axis: OS and DOW) across the four different destination habitats (secondary x-axis: OS, DOW, SOW, and PF). *Lines* indicate the sense of the comparisons as required for "local versus foreign" and "home versus away" approaches (i.e., the "sympatric" combination versus the "allopatric" ones, see text). (Comparisons significance: *<0.05, **<0.001, ***<0.0001). At this spatial scale, "Local versus foreign" criterion was not satisfied for emergence or survival, since, at no destination habitat, any "local" origin population (circumferences) obtained the highest relative fitness results, outperforming "foreign" ones. "Home versus away" criterion was not satisfied either, since no origin population exhibited the best fitness results at "home" habitat (circumferences), outperforming the outcome at "away" habitats

outperformed at its local habitat every "foreign" one. Similarly, no origin population exhibited its best fitness results at "home" habitat.

No detection of local adaptation is directly related to the overwhelming effect of the destination site since it may limit any origin effect (Sáenz-Romero and Guries 2002; Zamora 2002; Rendon and Núñez-Farfán 2001; Núñez-Farfán and Schlichting 2001). This overwhelming effect is described as one of the main drawbacks attributed to the traditional "home versus away" criterion for local adaptation, since it confounds the effects of divergent selection with intrinsic differences in habitat quality (Kawecki and Ebert 2004).

With regards to maternal effects, our results suggest that, at specific locations, certain mother plants obtained better seedling emergence and survival than others. Thus, mother quality depended on the destination habitat, denoting that environmental conditions of certain habitats may hide differences in mother quality

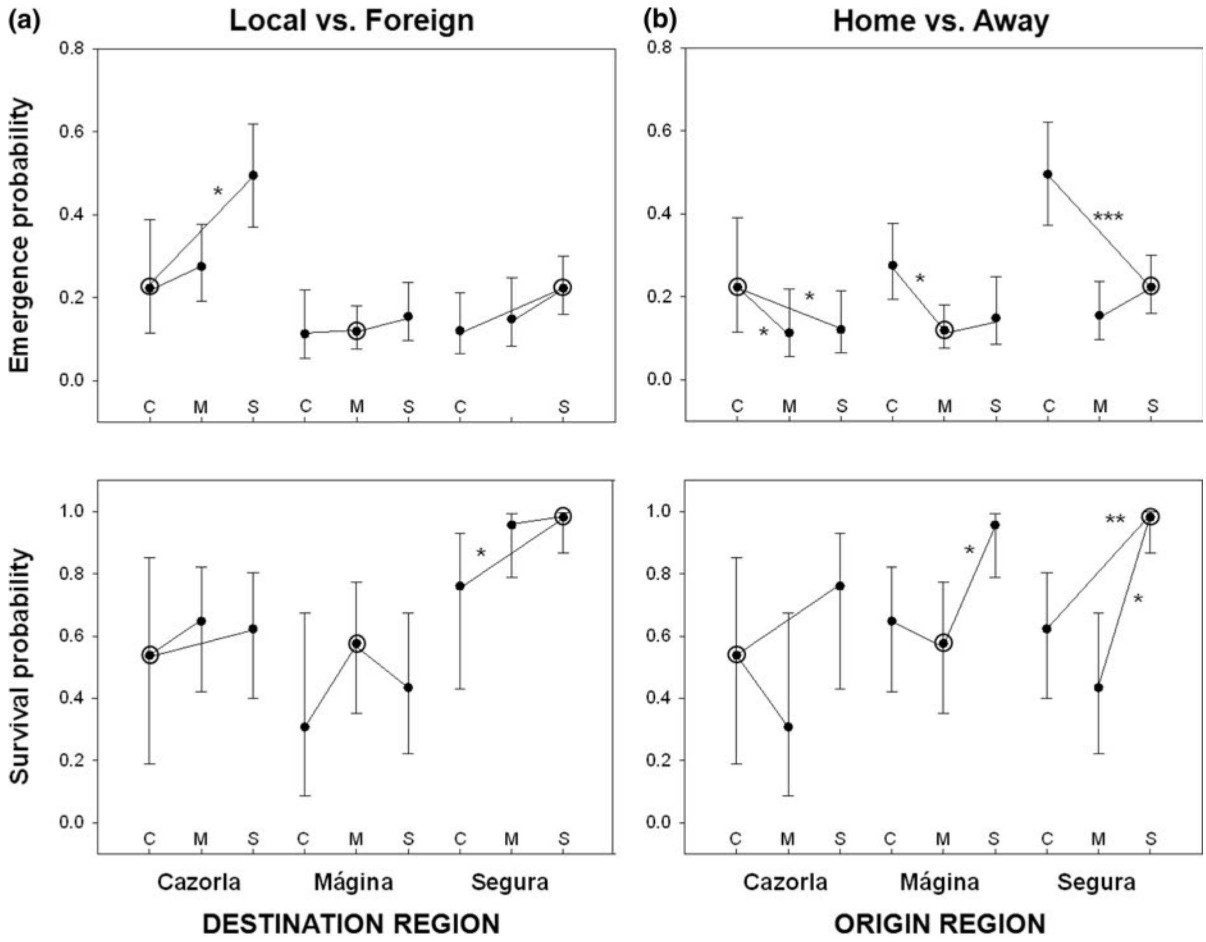

Fig. 4 "Local versus foreign" (**a**) and "home versus away" (**b**) criteria for assessing local adaptation of hellebore to the environmental conditions of emergence (*upper panel*) and survival (*lower panel*) of seedlings at the inter-region scale. Figure 3a represents emergence and survival fitness outputs for three different destination regions (x-axis: Cazorla, C; Mágina, M; and Segura, S), each one sowed with seeds from the three regions (origin regions; secondary x-axis: C, M, and S). Figure 3b represents emergence and fitness outputs of seedlings from the three origin regions (x-axis) across these three regions (destination regions; secondary x-axis). *Lines* indicate the sense of the comparisons as required for "local versus foreign" and "home versus away" approaches (i.e., the "sympatric" combination versus the "allopatric" ones, see text). (Comparisons significance: *<0.05, **<0.001, ***<0.0001). "Local versus foreign" criteria indicated that Segura and Cazorla seedlings were locally adapted to their local environmental conditions of emergence and survival, respectively, since "local" (circumferences) performed better than "foreign" ones. Under the "Home versus away" criteria, Cazorla and Segura seedlings could be locally adapted to the local environmental conditions of emergence and survival, respectively

(see Rendon and Núñez-Farfán 2001). Interestingly, we found that maternal effects on seedling emergence and survival were to large extent mediated by differences in seed size, which were more pronounced among plants within habitats than among habitats. Few studies have explored maternal effects on progeny viability in natural plant populations (Hereford 2009), but the available information suggests that maternal effects may induce differences in both seedlings emergence and survival (Herrera 2000) and that they are frequently mediated by seed size (Gómez 2004; see also Manzaneda et al. 2009 for *H. foetidus*).

Inter-regional scale

Similar to the inter-habitat scale, in addition to the no significant origin × destination interaction effect, we found that both criteria failed to detect local adaptation despite some weak and partial evidence. Partial evidence was mostly found under the “home versus away” criteria and not under the more proper “local versus foreign” one. Remarkably, independent of seeds’ origin, Cazorla and Segura destinations were always the best regional environments for emergence and survival, respectively, again constraining the possibility of local adaptation and divergent selection (Kawecki and Ebert 2004).

Possible determinants of lack of local adaptation

Destination and mother plant identity were the main factors generating variation in emergence and survival at both spatial scales. Destination habitats differed in their edaphic environments and could generate different selective pressures eventually leading to local adaptation. Besides, our data revealed that, at both spatial scales, seed size was a phenotypic trait linked to fitness through emergence and survival successes (marginally in the latter case); since it varied significantly among maternal plants within populations, it may be subject to a differential selection pattern, potentially leading to local adaptation. Nevertheless, selection pressures resulted eventually similar among different habitats, which could act constraining chances for local adaptation.

Destination habitat environment as constraint of local adaptation

Destination habitats differed in their edaphic environments. Water Retention Capacity (WRC hereafter) at 1/3 and 15 atm (standard measures for soil Field Capacity and Permanent Withering Point, respectively) often vary widely depending on soil types. Difference between these two parameters defines Water Availability for plants (WRC1/3–WRC15, Larcher 1977). Here, the effect of WRC (represented by PC1) on emergence differed between habitats although such variation was hardly significant. While at OS and SOW, there was no significant effect, at Pine Forest high values of WRC were related to low emergence ($b = -1.30 \pm 0.43$; $Z = 9.01$, $P = 0.0027$), and at Dense Oakwood high values of WRC were related to high emergence probabilities ($b = 0.36 \pm 0.41$; $Z = 7.75$, $P = 0.0054$).

Seedling survival was also affected by water soil properties although in this case similarly at all destination habitats. An increase of WRC was related to higher survival expectancy. Since water stress is the main seedling mortality cause in hellebore (Garrido et al. 2005, 2007), higher WRC may be beneficial for seedling survival because insuring long-term moisture. This predominant effect of water soil properties on seedling emergence and survival (Evans and Etherington 1990) has been frequently shown in Mediterranean ecosystems (Verdú and García-Fayos 1996; Pugnaire et al. 1996a, b; Herrera 2002; Garrido et al. 2007).

Soil texture also affected emergence and its effect differed between habitats. While main effect of texture on emergence was positive although not significant ($b = 0.47$, $P = 0.13$), its unique significant influence on emergence was negative and occurred at DOW ($b = -0.75$, $P = 0.003$), indicating that higher contents on fine textures (clays) decrease seedling emergence. Since soils of different habitats differed in textures (more fine in the sense $\mathrm{MA} > \mathrm{EL} > \mathrm{P} > \mathrm{ED}$), where clay content was low, emergence was more sensitive to its presence. Influence of texture on emergence in wild plant populations in Mediterranean habitats has been highlighted (Herrera 2002). Furthermore, it has also been stressed the adverse effect of clays on germination and emergence, particularly linked to higher resistance to the penetration of radicle (Townend et al. 1996; Vleeshouwers 1997; Pabin et al. 1998; Herrera 2002).

Contrasting to other studies, soil fertility exerted no influence either in emergence or in survival of seedlings. This was probably due to the overwhelming effect of soil water properties since water stress is by far the main mortality cause of seedlings of this species at the region (Garrido et al. 2005, 2007). It is probable that when soil water properties are more adverse, soil fertility lose weight in favor of water resources.

Selection on seed size as a constraint for local adaptation

Seed size exclusively accounted for the maternal effect on emergence since it exclusion from ANCOVA analyses entailed a significant maternal effect.

Despite a punctual negative effect of seed size on emergence at Pine Forest (that determined a significant seed size × destination effect), at inter-habitat scale, we have found an overall positive effect of seed size on emergence and marginally on survival with heavier seeds obtaining higher emergence success and marginally higher survival than lighter ones. At regional scale, seed size did not have a significant effect although it exhibited a positive tendency with regions with heavier seeds obtaining higher emergence and survival success, independently from destination region.

The effect of seed size on emergence at Pine Forest can be attributed to the particularities of these habitat concerning to the relationship between WRC and Water Availability. At this habitat, high values of WRC were related to low Water Availability (see Appendix 1). Furthermore, Pine Forest was the only habitat where high values of WRC were related to low emergence probabilities, particularly low on seedlings from larger seeds. Therefore, at Pine Forest, low values of Water Availability were related to low emergence probabilities. Similar results have been reported in several studies where low levels of Water Availability induced differences on germination success due to seed size (Saxena et al. 1993; Kikuzawa and Koyama 1999; Vessal et al. 2012). Thus, at low levels of Water Availability, while smaller seeds germinated successfully and entered into the early stages of seedling growth, germination of larger seeds resulted inhibited. This effect has been attributed to a higher efficiency of smaller seeds in re-mobilizing reserves to seedling tissues (Vessal et al. 2012).

Therefore, excepting the punctual case of Pine Forest habitat, selective pressures exerted by the germination and emergence environments always favoured heavier seeds, something frequently reported by several studies (Howe and Richter 1982; Kitajima 1992; Kitajima and Fenner 2000; Alcántara and Rey 2003). This selective pressure acts exclusively on emergence, and does not extend to seedling survival neither intra nor inter-regionally. The fact that seed size effect affects emergence, but not survival is relatively frequent in small seeds with reduced endosperm, as this is the case. Immediately after germination, seedlings start to depend on environment, since nutrient reserves get lost during seed germination and seedling emergence (Howe and Richter 1982; Kitajima 1992; Cornelissen et al. 1998; Kitajima and Fenner 2000). This generalized maintenance of a selective pressure in a unique direction is probably a main determinant of the lack of local adaptation to local/regional environmental conditions of emergence and survival.

Spatial scale and environmental heterogeneity

The effect of the spatial scale (i.e., geographic distance) on local adaptation depends on its specific association with the environmental differences that will be acting as selective agents (Leimu and Fischer 2008). This association makes difficult to determine the spatial scale to which plants may be locally adapted (Vander Mijnsbrugge et al. 2010).

It has been suggested that the likelihood of detecting local adaptation increases with geographic distance (Galloway and Fenster 2000); however, when reviewed, this association could not be corroborated, resulting the magnitude of environmental variation comparable both at large and short geographic distances (Leimu and Fischer 2008). Although recent studies have downplayed the relevance of environmental variation on local adaptation (Leimu and Fischer 2008, Hereford 2009), it has been traditionally assumed that the scale of environmental heterogeneity determines to some extent the geographic scale of local adaptation (Galloway and Fenster 2000).

Here, we have explored intra-regional (among habitats within the same region) and inter-regional scales and we have detected some signs of local adaptation at the greater one, among regions, but not among habitats. Similar results were found in several other studies that only found local adaptation at greater spatial scales (Galloway and Fenster 2000; Becker et al. 2006; Raabová et al. 2011), which highlights the importance of covering different spatial scales in local adaptation or reciprocal transplant/sowing studies.

Nevertheless, assessing the spatial scale of local adaptation requires to clearly delimiting the effects of geographic distance and environmental heterogeneity. Our both spatial scales (although our experiments at different scales are not fully comparable), further than reflecting differences in geographic distance, may be mainly reflecting environmental variation (PF, OS, SOW, and DOW at inter-habitat scale; and *Pinus nigra* forest and Mediterranean open scrubland at inter-regional scale). Therefore, and given the high destination environmental effect found here, it must be noticed that differences between both scales may be due mainly to environmental differences rather than to geographic distances. This agrees with the independence of local adaptation of geographic distance reviewed in Leimu and Fischer (2008), and enhances the relevance recently given to the environmental heterogeneity (Leimu and Fischer 2008; Hereford 2009).

Concluding remarks: why habitat edaphic characteristics and selection on seed size have not promoted local adaptation?

Determinants of lack of local adaptation here studied are, in fact, intimately related. Despite the case of PF habitat, heavier seeds develop better response abilities to the stressing environments of early emergence and survival. The overall maintenance of the same selective pressure on seed size across all habitats was congruent with the fact that selective pressures imposed by the edaphic environment were, overall, in the same direction through all these habitats.

An assumption for local adaptation is that different habitats exert different selective pressures or of different intensity. Except for the effect of texture at DOW, soil water properties affected equally to emergence and survival (case of texture and water availability), or simply did not have any effect (case of fertility). Results suggest that part of the overall lack of local adaptation of seeds and seedlings is due to the preponderant effect of soil water properties on emergence and survival, which acted in the same way at all habitats.

Here, we have proposed the homogeneity of selective pressures as a plausible ecological explanation for the general absence of local adaptation, but there are other alternative, and not mutually exclusive, possibilities. In the case of the inter-habitat scale, a high level of gene flow among habitats, via pollen or seeds, may prevent local adaptation by homogenizing allele frequencies and limiting the response to selection (Hereford 2009). In addition, other probable genetic explanation would be a low level of additive genetic variance for the traits involved. At higher spatial scales, genetic drift can also make small populations be not well adapted to their native environment by not letting advantageous alleles to reach a high frequency (Whitlock 2003) or because genetic load of deleterious alleles may lead to low fitness or even extinction (Hereford 2009 and references therein). The effects of population size on local adaptation are summarized in Leimu and Fischer (2008). These alternative possibilities to the general ecological explanation here developed would provide a highly valuable understanding on the underlying factors involved in the absence of plant adaptation to local environments, a circumstance the enlightenment of which may be as challenging as its existence.

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