

**CONTINUOUS WITHIN-PLANT VARIATION AS A SOURCE
OF INTRASPECIFIC FUNCTIONAL DIVERSITY: PATTERNS,
MAGNITUDE, AND GENETIC CORRELATES OF LEAF VARIABILITY
IN *HELLEBORUS FOETIDUS* (RANUNCULACEAE)¹**

CARLOS M. HERRERA², MÓNICA MEDRANO, AND PILAR BAZAGA

Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Américo Vespucio s/n, Isla de La Cartuja, 41092 Sevilla, Spain

- *Premise of the study:* Continuous within-plant variation in quantitative traits of reiterated, homologous structures is a component of intraspecific variation, but its contribution to functional diversity remains largely unexplored. For the perennial *Helleborus foetidus*, we measured functional leaf traits to quantify the contribution of within-plant variation to intraspecific functional variance and evaluate whether within-plant variability itself deserves separate consideration.
- *Methods:* Within-individual variation in eight leaf traits was quantified for 138 plants sampled from 10 widely spaced locations in the Sierra de Cazorla, southeastern Spain. An amplified fragment length polymorphism (AFLP) technique was used to look for associations between within-plant variability and specific AFLP markers.
- *Key results:* Leaflets from basal positions in ramets were longer, heavier, had greater surface area and larger stomata, and lower specific area, stomatal index, and stomatal density than those from distal positions. Continuous variation between leaves from the same ramet was the main source of population-wide variance for most traits. Within-plant variability differed among populations. Individuals differed in within-plant variability, which was largely independent of trait means and associated with genetic characteristics. Up to four AFLP markers were associated with the within-plant variability level of a given leaf trait.
- *Conclusions:* Subindividual variability in continuous leaf traits was independent of plant means and related to genetic features. The within-individual component generally exceeded the between-individual component of intraspecific variance. Within-plant variation may broaden the ecological breadth and enhance stability and persistence of plant populations and communities and may provide novel insights when incorporated in trait-based community ecology models.

Key words: AFLP; amplified fragment length polymorphisms; functional diversity; *Helleborus foetidus*; intraspecific variation; leaf traits; marker-trait association analysis; Ranunculaceae; within-plant variation.

Functional diversity was originally envisaged as the component of biodiversity that is measured, for those species present in an ecosystem, by the range of values for organismal traits that influence ecosystem functioning (Tilman, 2001). This species-centered concept was soon expanded to incorporate intraspecific variability (Violle et al., 2007; Cianciaruso et al., 2009), which is now widely acknowledged as an important contributing factor to community-wide functional diversity (de Bello et al., 2011; Albert et al., 2012; Violle et al., 2012; Auger and Shipley, 2013). Variability in the functional traits of

conspecific individuals is often comparable to variability between species (Medrano et al., 2014; Mitchell and Bakker, 2014), and intraspecific variation represents a significant source of community-wide variance for some traits (Auger and Shipley, 2013; Kang et al., 2014), with important consequences for the ecological breadth and distributional range of species (Sides et al., 2014). Although recognition of the importance of individual differences has significantly improved our understanding of the role of trait variation in species distribution and plant community organization (Violle et al., 2012; Sides et al., 2014), subindividual variation represents another layer of intraspecific variation that remains largely unexplored from the perspective of its contribution to functional diversity in plant communities.

Plant functional traits fall into one of two major categories, depending on whether they refer to the whole plant (e.g., size, life span) or to the homologous structures produced reiteratively by a single individual (e.g., leaves, flowers) (Pérez-Harguindeguy et al., 2013). That homologous structures produced by the same plant sometimes differ markedly has been emphasized many times in the botanical literature since the classical accounts of the Greek philosopher Theophrastus (Herrera, 2009). Historically,

¹Manuscript received 6 October 2014; revision accepted 22 January 2015.

The authors thank L. Cabral, R. Fouce, E. López, C. Molina, and R. Requerey for laboratory assistance, Centro de Supercomputación de Galicia (CESGA) for access to computer resources, and two anonymous reviewers for valuable suggestions. Permission to work in Sierra de Cazorla was provided by the Consejería de Medio Ambiente, Junta de Andalucía. C.M.H. and M.M. contributed equally to this work.

²Author for correspondence (e-mail: herrera@ebd.csic.es)

however, interest on subindividual variation has concentrated on the relatively uncommon instances of discontinuous variation involving morphologically or functionally distinct variants of leaves (heteroblasty, heterophylly), fruits (heterocarpy), or seeds (heterospermy, heteromorphism) (Mandák, 1997; Wells and Pigliucci, 2000; Imbert, 2002; Matilla et al., 2005; Zotz et al., 2011). Rather paradoxically, the magnitude and significance of continuous within-plant variation in quantitative traits remains comparatively unexplored despite being a quintessential plant feature (Herrera, 2009). In the relatively few cases where it has been quantified, continuous subindividual variation usually emerges as a major source of population-wide variance in functionally important traits (Herrera, 2009; Ishii and Harder, 2012; Zywiec et al., 2012; Sobral et al., 2013), yet studies on intraspecific functional diversity have rarely considered the contribution of continuous subindividual variation (Auger and Shipley, 2013; Kang et al., 2014).

Studies of intraspecific functional diversity have customarily assessed intraspecific variability in quantitative traits of reiterated structures using plant means obtained by averaging replicated measurements on structures sampled from the same individual (Boucher et al., 2013; Pérez-Harguindeguy et al., 2013; Mitchell and Bakker, 2014). This widely used procedure has at least two unappreciated drawbacks. First, the within-individual variance of continuously varying traits of reiterated structures is comparable, and often greater, than the variance of individual means (Herrera, 2009). Neglecting subindividual variation in functional traits will thus tend to exaggerate the importance of individual differences as a source of total population- or community-wide functional diversity. And second, as noted long ago by Haldane (1957) and Roy (1963), individual plants not only have their characteristic means, but should also have their characteristic standard deviations that might vary independently of means. Subindividual variability in functional traits may thus behave in itself as a functional trait influencing population persistence and community stability through its effects on individual performance (Herrera, 2009). For example, within-plant gradients in leaf functional features may represent adaptive solutions to small-scale variations in the light environments. By producing leaves with different specific area, nitrogen content, and photosynthetic capacity along within-plant light gradients, individuals can maximize overall photosynthetic performance in the face of environmental heterogeneity, and inequality in the distribution of nitrogen among leaves exposed to different light intensities can enhance photosynthetic gain at the whole-plant level (Givnish, 1988; Hollinger, 1996; Osada et al., 2014).

The preceding considerations suggest that incorporating the within-plant component of continuous variability into studies of functional leaf traits may lead to a more biologically realistic assessment of the contribution of intraspecific variation to the functional diversity of communities, particularly if within-plant variability of most traits varies independently of the corresponding individual means. This paper examines continuous within-plant variation in eight quantitative leaf features in a large sample of wild-growing plants of the perennial herb *Helleborus foetidus* (Ranunculaceae). The objectives of the study are to identify predictable patterns of continuous within-plant variation in quantitative functional leaf traits and evaluate their contribution to total functional variance. In addition, the proposition that within-plant variability level is in itself a trait deserving separate consideration will be explored. To this end, we will test whether trait variability varied among populations and also

whether individual variation in variability was unrelated to individual trait means and related to genetic characteristics, as implied by recent models of adaptive subindividual variation (Herrera, 2009; Sobral et al., 2013; Hudson et al., 2014; Austen et al., 2015; Schreiber et al., 2015).

MATERIALS AND METHODS

Study species and field sampling—*Helleborus foetidus* L. (Ranunculaceae) is an evergreen herb widely distributed in western and southwestern Europe (Mathew, 1989). Adult plants generally consist of vegetative and reproductive ramets arising from a small rhizome (Werner and Ebel, 1994). After several seasons of vegetative growth, each ramet produces a single terminal inflorescence and dies following fruit maturation and seed shedding. *Helleborus foetidus* bear pedate, deeply divided leaves arranged along the unbranched ramets (Fig. 1). During the seasonal growth period (late autumn-early summer), each ramet typically produces 6–12 leaves sequentially as it elongates. The number, size, and shape of leaf segments (“leaflets” hereafter) vary slightly among leaves of the same plant (Mathew, 1989; Werner and Ebel, 1994). To obtain comparable figures for foliar measurements considered in this study, we measured the central leaflets of leaves from different ramets and positions within ramets, which were sampled using a stratified scheme (see below).

Sampling for this study was conducted during the spring of 2013 in the Sierra de Cazorla mountains (Jaén Province, southeastern Spain). Patterns and amount of within-plant variation in eight quantitative functional leaf traits were studied on 138 adult plants sampled from 10 widely spaced locations (range = 4–19 plants/site, mean = 13.8) encompassing the whole ecological (evergreen oak forest through mixed forest to open pine woodlands) and elevational (700–1800 m a.s.l.) ranges occupied by the species in the region. This sampling scheme was designed to obtain representative data from the species’ distribution range in the region. Mean (\pm SE) pairwise distances between sampling sites was 9.3 ± 0.7 km (range = 1–19 km). Plants included in this study were the same studied by Medrano et al. (2014), where full details on sampling locations and habitat features are provided. The two longest vegetative ramets were selected from each sampled plant. From each ramet, we collected the central leaflets of one of the basal (produced early in the growing season) and one of the distal (produced near the end of the growing season) undamaged, fully expanded, first-year mature leaves (Fig. 1). Collected leaflets were labeled and kept in sealed plastic bags in a cooler until processed in the laboratory.

Laboratory methods—All collected leaflets were mounted on paper sheets while still fresh, digitally scanned, then desiccated. Total area, maximum length and maximum width of each leaflet were measured using SigmaScan Pro (version 5.0; Systat Software, San Jose, CA, USA). Dried leaflets were weighed on an analytical balance and specific area calculated as the area to dry mass ratio (Pérez-Harguindeguy et al., 2013). The impression approach (e.g., Peterson et al., 2012) was used to measure stomatal traits. Transparent impressions of the widest portion of the abaxial surface of each leaflet were created using clear nail polish and mounted on microscope slides. Stomatal density (number of stomata per mm^2) was estimated for five fields of view widely spaced across each impression at 400 \times magnification. Two photomicrographs were taken from haphazardly selected, nonoverlapping areas of each impression at 100 \times magnification using transmitted differential interference contrast microscopy. Stomata and epidermal cells were counted on two nonadjacent quadrats of a 0.0625- mm^2 grid overimposed on each image, and the area-independent stomatal index (SI) was calculated as $SI = [s/(e + s)] \times 100$, where s is the number of stomata and e is the number of epidermal cells (Salisbury, 1927). Guard cells were not included in the number of epidermal cells. Stomatal length, defined as the distance between the junctions of guard cells at opposite ends of the stomata, was measured on 20 randomly chosen, open stomata per impression (10 per photomicrograph). For stomatal traits, replicate measurements from the same leaflet were averaged to obtain a single value per trait and leaflet.

All plants included in this study were characterized genetically and by their mean leaf traits in a previous investigation (Medrano et al., 2014), and these data will be used here to look for correlations between subindividual variability in leaf traits, on one side, and mean trait values and genetic features of individual plants, on the other. Plants were characterized genetically using the amplified fragment length polymorphisms technique (AFLP; Weising et al., 2005; Meudt and Clarke, 2007). AFLP analyses were conducted using standard protocols involving the use of fluorescent dye-labeled selective primers (Weising

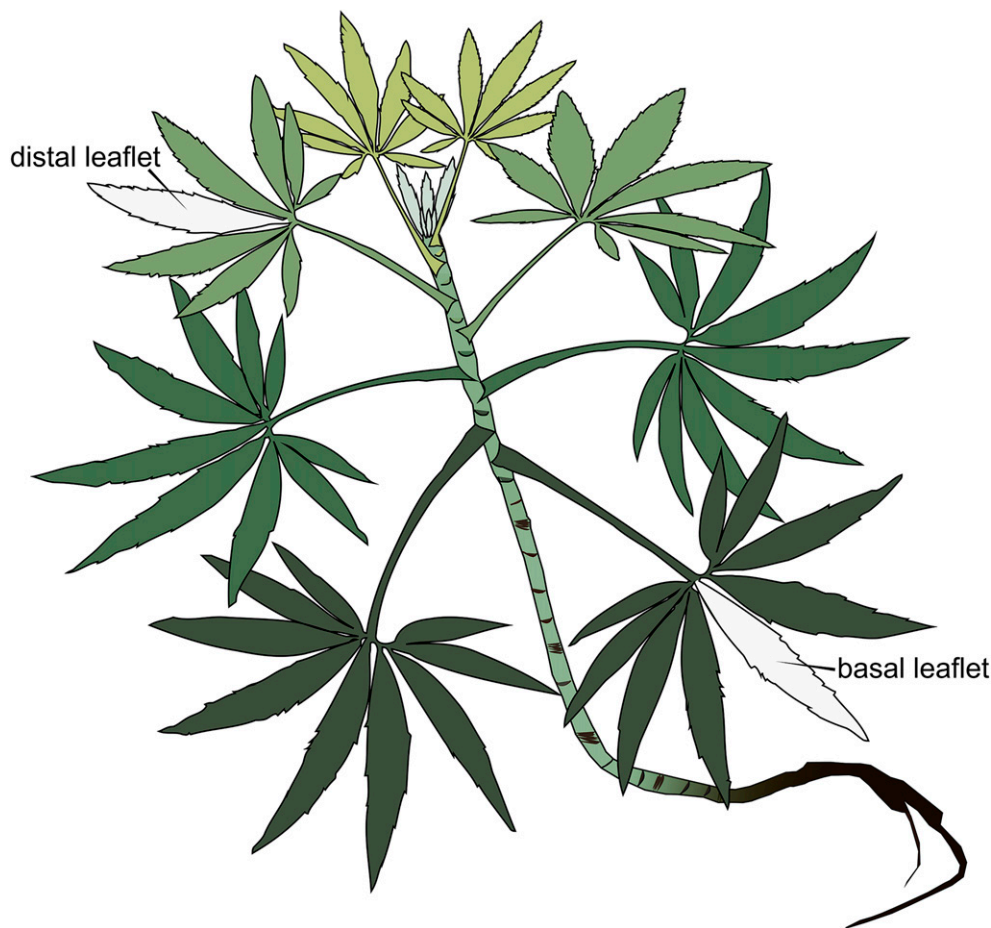


Fig. 1. Drawing of an idealized vegetative ramet of *Helleborus foetidus* arising from the short rhizome, with indication of the basal and distal leaflets sampled for this study. Note that, to aid legibility, we omitted some leaves along the aerial stem and size differences between basal and distal leaves are slightly exaggerated.

et al., 2005). A total of eight *Pst*I + 2 / *Mse*I + 3 primer pairs were chosen that provided reliable, consistently scorable results. Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of each AFLP fragment in each individual plant was scored manually by visualizing electropherograms with GeneMapper 3.7 software. See Medrano et al. (2014) for additional details on AFLP laboratory procedures, including primer combinations and scoring error estimates. A total of 251 AFLP markers were polymorphic (at least 2% of plants showed a variant score) in the sample of 138 plants considered here, and were included in the association analyses for identifying genetic features associated with within-plant leaf variability.

Data analysis—All statistical analyses were conducted using the R 3.1.0 computing environment (R Core Team, 2014). The effect of position on ramet on leaf traits was tested by fitting linear mixed-effects models to the data, with nodal position (basal, distal) as a two-level fixed effect, and sampling site, plant nested within site, and ramet nested within plant, as random effects. The contributions of different sources to total local variance in leaf traits were estimated by fitting intercept-only random effects models to the data separately for each sampling site, with plants, ramets within plants and position within ramets as hierarchically nested random effects. The proportional variance contribution by each nested level was then averaged across sites. Mixed-effects and random-effects models were fitted with the *lme* function of the *nlme* package using REML estimation.

Within-plant variability in leaf traits was assessed by computing for each plant the coefficient of variation (= standard deviation/mean) for each trait. To test for individual heterogeneity in leaf variability levels, we characterized plants by the mean coefficient of variation averaged over traits and then used a

nonparametric analysis of variance to test for between-plant heterogeneity in within-plant variability level. Although the small number of sampling sites precluded a detailed analysis of geographical variation, it was possible to test whether within-plant variability for a given leaf trait varied across sites and also whether such variation was similar for all traits considered. The latter was tested by applying principal components analysis to the 10 sites \times 8 traits matrix whose elements were population means of the within-plant coefficients of variation for each trait.

To assess whether observed differences between *H. foetidus* plants in leaf trait variability were related to their genetic features, we looked for statistically significant associations across plants between AFLP markers and the within-plant coefficients of variation for each leaf trait. For each trait, separate linear mixed-effects models were fit for each AFLP marker using REML estimation. In each model, the within-plant coefficient of variation was the dependent variable and marker presence-absence the single fixed-effect, two-level factor. A previous study has shown that the *H. foetidus* plants sampled are genetically structured, falling into one of two genetically distinct clusters (Medrano et al., 2014). Since genetic stratification and possible cryptic relatedness can produce spurious marker-trait associations in genome-wide association studies if not properly corrected, we incorporated genetic cluster and the sampling site nested within genetic cluster as random effects in the mixed models (Price et al., 2010). *P*-values for the effect of marker presence-absence on variability were used to identify significant associations. The *q*-value method of Storey and Tibshirani (2003) was applied to estimate false discovery rates. Statistical significance thresholds were established by finding the largest *q*-value leading to an expectation of less than one falsely significant model [i.e., $q\text{-value} \times (\text{number of models accepted as significant}) < 1$].

RESULTS

Patterns and magnitude of subindividual leaf variation—Plants of *Helleborus foetidus* exhibited a predictable pattern of subindividual variation in quantitative leaf traits, with leaves at basal and distal positions on the same ramet differing significantly in all traits considered except leaflet width (Table 1). Leaflets from basal leaves were longer, heavier, had greater surface area and larger stomata, and lower specific area, stomatal index and stomatal density, than leaflets from distal leaves (Table 1).

Partitions of trait variance into hierarchically nested components (among plants, between ramets within plants, between positions within ramets), conducted separately for each sampling site, revealed that variation between leaves from the same ramet was the main source of local sample-wide variance for all traits examined except specific area (Fig. 2). The quantitative importance of within-ramet, positional variation as a source of local functional diversity of leaves was greatest for stomatal index, stomata length, and stomatal density. For each of these traits, leaf position within ramets accounted, on average, for $\geq 70\%$ of local sample-wide variance (Fig. 2). Depending on the trait, differences between plants accounted, on average, for an additional 22–50% of total local variance, while differences between ramets of the same plant accounted for only 1–28% of total.

Variation in leaf variability—*Helleborus foetidus* plants differed with regard to the level of subindividual leaf variability, as revealed by significant individual heterogeneity in the set of coefficients of variation for the eight leaf traits considered ($\chi^2 = 212.4$, $df = 137$, $P = 0.00004$; Kruskal–Wallis rank sum test). Sampled plants exhibited broad differences in within-plant leaf variability levels, as revealed by the 4-fold difference between the mean coefficients of variation of the most and least variable individuals (Fig. 3).

Individual differences in within-plant trait variability, as measured with the coefficient of variation, were largely unrelated to differences in trait means. Correlations across plants between means and coefficients of variation were statistically nonsignificant for six of eight traits and weakly significant for the other two traits (Table 2). No relationship would reach statistical

TABLE 1. Characteristics of central leaflets of *Helleborus foetidus* leaves in basal and distal positions on vegetative ramets.

Leaflet trait (units)	Nodal position in ramet (trait mean \pm SE)		Significance of position effect	
	Basal	Distal	$F_{1,275}$	P value
Length (mm)	121.0 \pm 7.0	100.3 \pm 7.0	310.8	$<<0.0001$
Width (mm)	16.5 \pm 1.1	16.7 \pm 1.1	0.54	0.46
Area (cm ²)	13.7 \pm 1.6	11.3 \pm 1.6	70.6	$<<0.0001$
Dry mass (mg)	101.9 \pm 0.8	81.9 \pm 0.8	73.4	$<<0.0001$
Specific area (mm ² ·mg ⁻¹)	13.8 \pm 0.9	14.1 \pm 0.9	8.3	0.0043
Stomatal index (%)	24.6 \pm 0.3	25.8 \pm 0.3	13.5	0.00028
Stomatal density (mm ⁻²)	88.1 \pm 2.7	101.2 \pm 2.7	102.8	$<<0.0001$
Stomata length (μ m)	40.8 \pm 0.4	37.9 \pm 0.4	223.7	$<<0.0001$

Notes: The effect of nodal position on leaf traits was tested by fitting linear mixed-effects models to the data, with position as the single fixed effect, and sampling site, plants nested within sites, and ramets nested within plants, as random effects. Mean values shown are least-squares, model-adjusted means.

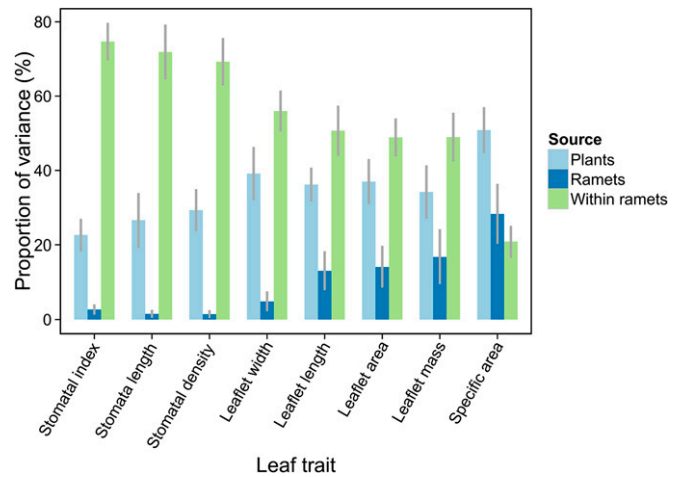


Fig. 2. Proportion of local sample-wide variance explained by variation among plants, between ramets within plants, and between nodal positions within ramets (basal vs. distal), for each of the eight leaf traits considered. Shown are mean values for the 10 sites sampled (bars) and associated standard errors (vertical segments). Traits are ranked in decreasing order of within-ramet variance component.

significance if the P -value threshold were adjusted for multiplicity of tests (e.g., by applying a Bonferroni correction, $P < 0.006$ would be required for significance at a nominal $P = 0.05$). Irrespective of statistical significance, the R^2 for the relationships between means and coefficients of variation was extremely low for all traits ($R^2 \leq 0.039$), thus strengthening the conclusion that variation across plants in means and subindividual variability levels of leaf traits were two essentially independent phenomena.

For most leaf traits considered, within-plant variability (coefficient of variation) was significantly heterogeneous across sampling sites (Table 3). Variation among sites in variability was not consistent across all traits, as revealed by the principal components analysis of the sites \times traits mean variability matrix. Two well-defined sets of traits emerged whose within-plant variabilities varied across sites independently of each

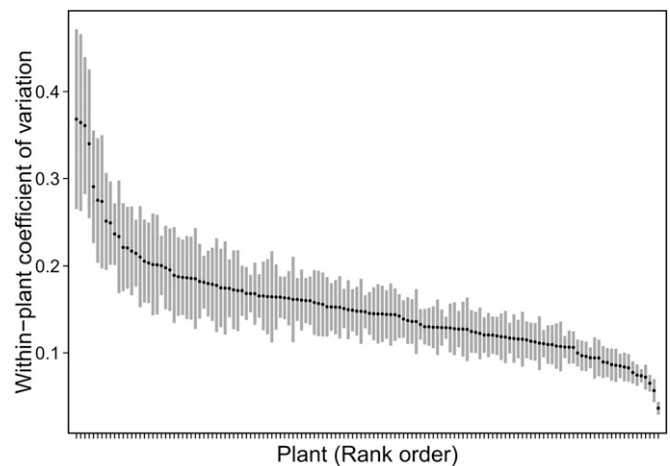


Fig. 3. Individual differences in within-plant variability in functional leaf traits in the sample of 138 plants of *Helleborus foetidus* studied, as measured by the mean coefficient of variation for each plant for the eight leaf traits considered (filled dots). Vertical segments denote ± 1 SE.

TABLE 2. Correlations across sampled plants of *Helleborus foetidus* ($N = 138$) between individual means and coefficients of variation for the eight leaflet traits considered.

Leaflet trait	Correlation coefficient (Pearson's r)	P value	R^2
Length	-0.049	0.57	0.0024
Width	0.018	0.83	0.00032
Area	0.064	0.46	0.0041
Dry mass	0.031	0.72	0.00096
Specific area	0.142	0.096	0.020
Stomatal index	-0.197	0.020	0.039
Stomatal density	0.182	0.033	0.033
Stomata length	-0.067	0.44	0.0045

other, namely stomatal density, stomata length and specific area, on one side, and area, length and dry mass, on the other (Fig. 4).

Genetic correlates of individual variation in leaf variability—After statistically controlling for possible nonindependence of data due to genetic stratification (genetic cluster) and cryptic relatedness (sampling site) of sampled plants, linear mixed-effects models revealed significant relationships between the magnitude of subindividual variability in leaf traits, assessed using the coefficient of variation, and several genetic markers. A total of 251 polymorphic AFLP markers were tested for significant associations with subindividual variability in each of the eight leaf traits considered, and 14 significant instances involving 11 distinct markers were found (Table 4). Within-plant variability in every leaf trait considered was significantly associated with at least one AFLP marker (mean \pm SE = 1.75 ± 0.41 associated markers per trait, or 0.69% of markers assayed per trait).

DISCUSSION

Intraspecific variation in functionally relevant traits contributes to broaden the ecological breadth of species (Sides et al., 2014). Insofar as such traits refer to reiterated plant structures (e.g., leaves, flowers, seeds, fruits), such intraspecific variation will consist of a between-individual and a within-individual component, the latter contributing to extend the range of environmental conditions that can be successfully exploited by single individuals (Herrera, 2009). While the between-individual component has frequently occupied the focus of recent trait-based ecological studies (references in the Introduction), the

TABLE 3. Results of Kruskal–Wallis rank sum tests for heterogeneity among the 10 sampling sites in within-plant variability (coefficient of variation) for each leaf trait considered.

Leaflet trait	χ^2	P value
Length	30.20	0.00041
Width	10.34	0.32
Area	23.16	0.0058
Dry mass	13.77	0.13
Specific area	26.88	0.0015
Stomatal index	8.95	0.44
Stomatal density	29.85	0.00046
Stomata length	22.97	0.0062

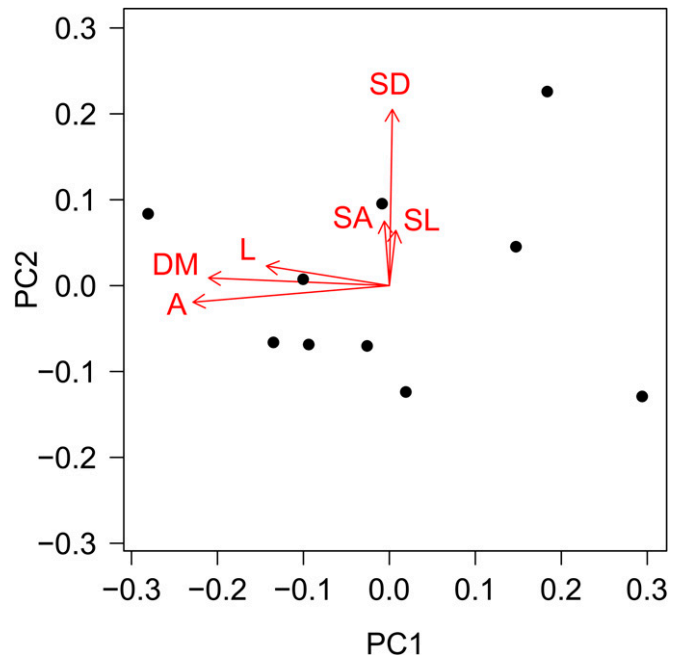


Fig. 4. Biplot representation of the principal components analysis of the site \times trait matrix of mean within-plant variability. Axes represent the first two principal components (PC1 and PC2), leaf traits are represented as red vectors and sites as black dots. Trait codes: A, area; DM, dry mass; L, length; SA, specific area; SD, stomatal density; SL, stomata length. Vectors corresponding to width and stomatal index, which fell very close to the origin and whose variabilities did not vary significantly across sites (Table 3), were omitted to avoid cluttering.

within-individual component has been rarely taken into consideration (Auger and Shipley, 2013; Kang et al., 2014). This neglect is somewhat paradoxical, given that the within-plant variance for quantitative traits of reiterated structures is often considerably greater than the between-individual variance, frequently emerging as the predominant source of intraspecific

TABLE 4. Genetic (AFLP) markers exhibiting statistically significant associations with subindividual variability in functional leaf traits in the studied sample of plants of *Helleborus foetidus*.

Leaflet trait	Marker	P -value
Length	AA-CAC_275	0.0041
	AA-CAC_249	0.0028
	AG-CTA_203	0.0052
Area	AA-CAC_275	0.0074
	AA-CAC_275	0.0080
Dry mass	AC-CTA_155	0.0012
	AT-CTC_327	0.0033
	AT-CTC_371	0.0065
Specific area	AC-CAT_494	0.00051
	AC-CTA_293	0.0013
	AC-CTA_290	0.0021
	AG-CTA_472	0.0043
Stomatal index	AT-CTC_327	0.0028
Stomatal density	AC-CTA_348	0.0055
Stomata length		

Notes: Within-plant variability was assessed by computing for each plant the coefficient of variation (= standard deviation / mean) for each trait. AFLP markers were identified by primer combination (*PstI/MseI*) and fragment size (base pairs). Marker(s) significantly associated with variability in a given trait had P values that, when considered simultaneously, led to an expected number of false positives < 1 .

variability in plant populations (references in the introduction). Such a pattern applies particularly to structural and chemical features of leaves, for which previous studies on nonheterophyllous trees and shrubs have shown that nearly all population-level trait variance (>90% of total) can take place within the confines of individual plants (Herrera, 2009: table 3.1; see also Auger and Shipley, 2013; Kang et al., 2014). Although herbaceous species have been investigated less frequently than trees or shrubs from the perspective of continuous subindividual variation in leaf features, extensive variability and steep gradients in quantitative functional leaf traits can also occur even within the restricted spatial scales of herbs (Lemaire et al., 1991; Williams et al., 1993). The present investigation on *H. foetidus* has shown that, with the single exception of leaflet width, all functional leaf traits considered varied predictably from basal to distal positions within ramets. Furthermore, the within-ramet gradient in leaf traits was steep enough to become the main source of population-level variance for most traits considered, although its quantitative importance differed between trait classes. The contribution of within-ramet variation to population-wide variance declined from stomatal features (69–75% of total) through size-related traits (49–56% of total) to specific area (21%). The predominance of within-plant variation as a source of population-wide variance in stomatal features is a remarkable result, given the functional importance of these traits due to their influence on water economy, gas exchange, and carbon assimilation (Pérez-Harguindeguy et al., 2013). This study has shown that stomatal features of leaves produced by *H. foetidus* plants at different positions along ramets, corresponding to different moments in the seasonal growth period, encompass nearly the whole range of variation for the population as a whole. This finding supports our contention, noted in the introduction, that neglecting the within-plant component may grossly underestimate intraspecific functional diversity of plant populations.

Predictable variation in functional leaf features along nodal positions on stems may arise from programmed developmental change, phenotypic plasticity in response to short-term changes in the environment, or some combination of these (Zotz et al., 2011; Dang-Le et al., 2013). Regardless of its mechanistic basis, subindividual variation in functional leaf traits may be advantageous to individuals by optimizing the exploitation of environmental variation in time and/or space and enhancing whole-plant photosynthetic performance (Givnish, 1988; Hollinger, 1996; Mulkey et al., 1992; Winn, 1996b, 1999; Osada et al., 2014). In our study region, ramet elongation and production of new leaves by *H. foetidus* extends from late autumn to early summer. The succession of contrasting environmental conditions along this protracted period may have favored the production of leaves with different functional features at different times, as reported for other species from seasonal habitats (Mulkey et al., 1992; Aronne and de Micco, 2001; Lianopoulou et al., 2014). No information is available on photosynthetic parameters and water-use efficiency of leaves at different positions along *H. foetidus* ramets; hence, the net advantage accrued to plants from producing functionally heterogeneous leaves at different moments of the growing season can only be speculated. In the high-elevation mountain ecosystems sampled for this study, the lower stomatal index and stomatal density of basal, early-season leaves of *H. foetidus* may be advantageous during winter and early spring, when low ambient temperatures will chronically limit the access of plants to soil water (Mayr et al., 2012). Leaf size reduction from early to late season might

reflect adjustments to increased solar irradiance and ambient temperature from winter through early summer (Parkhurst and Loucks, 1972; Ackerly et al., 2002). Variation between plant parts in stomatal density is directly related to photosynthetic performance in *Helleborus viridis* (Aschan et al., 2005); hence, the higher stomatal index and stomatal density of late-season leaves could likewise enhance carbon assimilation by plants of *H. foetidus* at a time of year when water and irradiance limitations on photosynthesis are possibly at their seasonal minima. Correspondences between seasonally variable leaf traits and changing environmental conditions, however, may be purely fortuitous, as shown by Winn (1999) for the annual plant *Dicerandra linearifolia*, a species that produces anatomically and morphologically distinct leaves at different times of the growing season. In the case of *H. foetidus*, limited observations in the study area suggest that variation across individuals in level of within-plant variability in leaf stomatal traits are positively related to fecundity (number of seeds produced), which points to the adaptiveness of seasonally variable leaves in this species (M. Medrano and C. M. Herrera, unpublished data). Although only experimental tests could properly assess the hypothesis that within-plant variation in leaf traits represents an adaptive response of *H. foetidus* plants to seasonal environmental changes, further circumstantial evidence is provided by some results of this study, as discussed below.

Adaptive interpretations of within-plant variation in characteristics of reiterated structures will make biological sense only if within-plant variability in a given trait represents a distinctive feature of individual plants whose variation is independent of variation in trait means (named the “Haldane–Roy conjecture” by Herrera [2009]). In this way, differences between conspecific individuals in level of intraplant variability will provide an opportunity for selection to act on such variability, as envisaged by models of adaptive subindividual variation (Winn, 1996a; Herrera, 2009; Austen et al., 2015; Schreiber et al., 2015). The Haldane–Roy conjecture has been shown to hold true for functional leaf traits in the few species where it has been explicitly examined to date (Herrera, 2009: table 7.1), and results of the present study further confirm it. Subindividual variability varied among populations and individuals, and individual variation was unrelated to differences in corresponding trait means but related to genetic characteristics (AFLP markers). None of the AFLP markers found to be associated with within-plant variability here were found to be related to trait means in a previous study (Medrano et al., 2014), which further supports the independent variation across plants of trait means and variabilities. Keeping in mind the caveat that genetic marker–trait associations do not provide conclusive proof of causality (Platt et al., 2010), our results provide plausible grounds for advancing the hypothesis that differences between plants of *H. foetidus* in subindividual variability might have a (causal) genetic basis. Some circumstantial evidence is compatible with this idea. Although AFLP markers are generally considered to be neutral (i.e., not subject to selection), in plant genomes a certain proportion of AFLP markers are positioned within gene sequences or linked to quantitative trait loci (QTLs) of known phenotypic effects potentially subject to selection, including some traits considered here such as leaf size and specific leaf area (Scafì et al., 2004; Caballero et al., 2013). AFLP marker–variability associations such as those found here might arise if the markers involved were linked to, or positioned within, genes directly controlling seasonal leaf development. Quantitative genetics studies have shown that subindividual variability of continuous

traits may have a significant hereditary component (Seyffert, 1983; Biere, 1991; Winn, 1996a), and developmental changes associated with leaf dimorphism are under genetic control in heteroblastic plants (Hamilton et al., 2011; Hudson et al., 2014).

Recent investigations have highlighted the quantitative importance in wild plant populations of individual variation in functional traits, the magnitude of which is often comparable to interspecific variation (Iannetta et al., 2007; Boucher et al., 2013; Laforest-Lapointe et al., 2014; Medrano et al., 2014; Mitchell and Bakker, 2014). In *H. foetidus*, for instance, ranges of plant means for some leaf traits are broad enough to almost encompass the corresponding ranges for interspecific variation in large multispecies samples worldwide (Medrano et al., 2014). Increasing evidence that functional differences between individuals of the same species can be nearly as large as differences between species provided motivation for the incorporation of intraspecific variation into trait-based plant community studies (de Bello et al., 2011; Violle et al., 2012; Sides et al., 2014). These studies, however, have generally tended to equate intraspecific functional diversity with variation between individual means, hence neglecting that, in the case of reiterated structures of immediate functional value (e.g., leaves), total population-wide trait variance (i.e., intraspecific functional diversity) is actually made up of within- and between-individual components. The main conclusion arising from the present study on *H. foetidus* (see also Herrera, 2009; Auger and Shipley, 2013) is that, for some functionally important leaf traits, the within-individual component of intraspecific variance can vastly exceed the between-individual component, thus becoming a major source of intraspecific functional diversity in populations of this species. Continuous within-plant variation in quantitative functional traits is an individual feature that can improve fitness by broadening the range of environmental conditions, either abiotic or biotic, that plants can successfully exploit or cope with, seasonally or in a longer term (Herrera, 2009). Through this mechanism, within-plant variation may contribute significantly to broaden the ecological breadth and enhance the stability and persistence of plant populations and communities. Furthermore, our finding that subindividual variability varies among populations in trait-specific ways prompts the hypothesis that the contribution of subindividual variation to local functional diversity might perhaps respond adaptively to variable ecological conditions across a species' range, although our data were insufficient to examine this possibility. Novel insights are therefore to be gained from the incorporation to trait-based community ecology models of within-individual variation as a distinct component of intraspecific diversity in functional traits.

LITERATURE CITED

- ACKERLY, D. D., C. A. KNIGHT, S. B. WEISS, K. BARTON, AND K. P. STARMER. 2002. Leaf size, specific leaf area and microhabitat distribution of chaparral woody plants: Contrasting patterns in species level and community level analyses. *Oecologia* 130: 449–457.
- ALBERT, C. H., F. DE BELLO, I. BOULANGEAT, G. PELLET, S. LAVOREL, AND W. THULLER. 2012. On the importance of intraspecific variability for the quantification of functional diversity. *Oikos* 121: 116–126.
- ARONNE, G., AND V. DE MICCO. 2001. Seasonal dimorphism in the Mediterranean *Cistus incanus* L. subsp. *incanus*. *Annals of Botany* 87: 789–794.
- ASCHAN, G., H. PFANZ, D. VODNIK, AND F. BATIC. 2005. Photosynthetic performance of vegetative and reproductive structures of green hellebore (*Helleborus viridis* L. agg.). *Photosynthetica* 43: 55–64.
- AUGER, S., AND B. SHIPLEY. 2013. Inter-specific and intra-specific trait variation along short environmental gradients in an old-growth temperate forest. *Journal of Vegetation Science* 24: 419–428.
- AUSTEN, E. J., J. R. K. FORREST, AND A. E. WEIS. 2015. Within-plant variation in reproductive investment: Consequences for selection on flowering time. *Journal of Evolutionary Biology* 28: 65–79.
- BIERE, A. 1991. Parental effects in *Lychnis flos-cuculi*. I: Seed size, germination and seedling performance in a controlled environment. *Journal of Evolutionary Biology* 3: 447–465.
- BOUCHER, F. C., W. THULLER, C. ARNOLDI, C. H. ALBERT, AND S. LAVERGNE. 2013. Unravelling the architecture of functional variability in wild populations of *Polygonum viviparum* L. *Functional Ecology* 27: 382–391.
- CABALLERO, A., M. J. GARCÍA-PEREIRA, AND H. QUESADA. 2013. Genomic distribution of AFLP markers relative to gene locations for different eukaryotic species. *BMC Genomics* 14: 528.
- CIANCARUSO, M. V., M. A. BATALHA, K. J. GASTON, AND O. L. PETCHEY. 2009. Including intraspecific variability in functional diversity. *Ecology* 90: 81–89.
- DANG-LE, A. T., C. EDELIN, AND K. LE-CONG. 2013. Ontogenetic variations in leaf morphology of the tropical rain forest species *Dipterocarpus alatus* Roxb. ex G. Don. *Trees* 27: 773–786.
- DE BELLO, F., S. LAVOREL, C. H. ALBERT, W. THULLER, K. GRIGULIS, J. DOLEZAL, S. JANECEK, ET AL. 2011. Quantifying the relevance of intraspecific trait variability for functional diversity. *Methods in Ecology and Evolution* 2: 163–174.
- GIVNISH, T. J. 1988. Adaptation to sun and shade: A whole-plant perspective. *Australian Journal of Plant Physiology* 15: 63–92.
- HALDANE, J. B. S. 1957. Karl Pearson, 1857–1957. *Biometrika* 44: 303–313.
- HAMILTON, M. G., P. A. TILYARD, D. R. WILLIAMS, R. E. VAILLANCOURT, T. J. WARDLAW, AND B. M. POTTS. 2011. The genetic variation in the timing of heteroblastic transition in *Eucalyptus globulus* is stable across environments. *Australian Journal of Botany* 59: 170–175.
- HERRERA, C. M. 2009. Multiplicity in unity. Plant subindividual variation and interactions with animals. University of Chicago Press, Chicago, Illinois, USA.
- HOLLINGER, D. Y. 1996. Optimality and nitrogen allocation in a tree canopy. *Tree Physiology* 16: 627–634.
- HUDSON, C. J., J. S. FREEMAN, R. C. JONES, B. M. POTTS, M. M. L. WONG, J. L. WELLER, V. F. G. HECHT, ET AL. 2014. Genetic control of heterochrony in *Eucalyptus globulus*. *G3 – Genes Genomes Genetics* 4: 1235–1245.
- IANNETTA, P. P. M., G. BEGG, C. HAWES, M. YOUNG, J. RUSSELL, AND G. R. SQUIRE. 2007. Variation in *Capsella* (shepherd's purse): An example of intraspecific functional diversity. *Physiologia Plantarum* 129: 542–554.
- IMBERT, E. 2002. Ecological consequences and ontogeny of seed heteromorphism. *Perspectives in Plant Ecology, Evolution and Systematics* 5: 13–36.
- ISHII, H. S., AND L. D. HARDER. 2012. Phenological associations of within- and among-plant variation in gender with floral morphology and integration in protandrous *Delphinium glaucum*. *Journal of Ecology* 100: 1029–1038.
- KANG, M., S. X. CHANG, E. R. YAN, AND X. H. WANG. 2014. Trait variability differs between leaf and wood tissues across ecological scales in subtropical forests. *Journal of Vegetation Science* 25: 703–714.
- LAFORREST-LAPOINTE, I., J. MARTÍNEZ-VILALTA, AND J. RETANA. 2014. Intraspecific variability in functional traits matters: Case study of Scots pine. *Oecologia* 175: 1337–1348.
- LEMAIRE, G., B. ONILLON, G. GOSSE, M. CHARTIER, AND J. M. ALLIRAND. 1991. Nitrogen distribution within a lucerne canopy during regrowth: Relation with light distribution. *Annals of Botany* 68: 483–488.
- LIANOPOULOU, V., A. BOSBALIDIS, A. PATAKAS, D. LAZARI, AND E. PANTERIS. 2014. Effects of chilling stress on leaf morphology, anatomy, ultrastructure, gas exchange, and essential oils in the seasonally dimorphic plant *Teucrium polium* (Lamiaceae). *Acta Physiologiae Plantarum* 36: 2271–2281.
- MANDÁK, B. 1997. Seed heteromorphism and the life cycle of plants: A literature review. *Preslia* 69: 129–159.

- MATHEW, B. 1989. Hellebores. Alpine Garden Society, St. John's Woking, Surrey, UK.
- MATILLA, A., M. GALLARDO, AND M. I. PUGA-HERMIDA. 2005. Structural, physiological and molecular aspects of heterogeneity in seeds: A review. *Seed Science Research* 15: 63–76.
- MAYR, S., P. SCHMID, AND B. BEIKIRCHER. 2012. Plant water relations in alpine winter. In C. Lütz [ed.], *Plants in alpine regions*, 153–162. Springer, Vienna, Austria.
- MEDRANO, M., C. M. HERRERA, AND P. BAZAGA. 2014. Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Molecular Ecology* 23: 4926–4938.
- MEUDT, H. M., AND A. C. CLARKE. 2007. Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science* 12: 106–117.
- MITCHELL, R. M., AND J. D. BAKKER. 2014. Quantifying and comparing intraspecific functional trait variability: A case study with *Hypochaeris radicata*. *Functional Ecology* 28: 258–269.
- MULKEY, S. S., A. P. SMITH, S. J. WRIGHT, J. L. MACHADO, AND R. DUDLEY. 1992. Contrasting leaf phenotypes control seasonal variation in water loss in a tropical forest shrub. *Proceedings of the National Academy of Sciences, USA* 89: 9084–9088.
- OSADA, N., Y. YASUMURA, AND A. ISHIDA. 2014. Leaf nitrogen distribution in relation to crown architecture in the tall canopy species, *Fagus crenata*. *Oecologia* 175: 1093–1106.
- PARKHURST, D. F., AND O. L. LOUCKS. 1972. Optimal leaf size in relation to environment. *Journal of Ecology* 60: 505–537.
- PÉREZ-HARGUINDEGUY, N., S. DÍAZ, E. GARNIER, S. LAVOREL, H. POORTER, P. JAUREGUIBERRY, M. S. BRET-HARTE, ET AL. 2013. New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany* 61: 167–234.
- PETERSON, C. A., N. FETCHER, J. B. MCGRAW, AND C. C. BENNINGTON. 2012. Clinal variation in stomatal characteristics of an arctic sedge, *Eriophorum vaginatum* (Cyperaceae). *American Journal of Botany* 99: 1562–1571.
- PLATT, A., B. J. VILHJÁLMSOHN, AND M. NORDBORG. 2010. Conditions under which genome-wide association studies will be positively misleading. *Genetics* 186: 1045–1052.
- PRICE, A. L., N. A. ZAITLEN, D. REICH, AND N. PATTERSON. 2010. New approaches to population stratification in genome-wide association studies. *Nature Reviews, Genetics* 11: 459–463.
- R CORE TEAM. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at <http://www.r-project.org>.
- ROY, S. K. 1963. The variation of organs of individual plants. *Journal of Genetics* 58: 147–176.
- SALISBURY, E. J. 1927. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 216: 1–65.
- SCALFI, M., M. TROGGIO, P. PIOVANI, S. LEONARDI, G. MAGNASCHI, G. G. VENDRAMIN, AND P. MENOZZI. 2004. A RAPD, AFLP and SSR linkage map, and QTL analysis in european beech (*Fagus sylvatica* L.). *Theoretical and Applied Genetics* 108: 433–441.
- SCHREIBER, S. J., J. A. ROSENHEIM, N. W. WILLIAMS, AND L. D. HARDER. 2015. Evolutionary and ecological consequences of multiscale variation in pollen receipt for seed production. *American Naturalist* 185: E14–E29.
- SEYFFERT, W. 1983. Homeostasis in defined genotypes of *Matthiola incana*. *Theoretical and Applied Genetics* 64: 205–212.
- SIDES, C. B., B. J. ENQUIST, J. J. EBERSOLE, M. N. SMITH, A. N. HENDERSON, AND L. L. SLOAT. 2014. Revisiting Darwin's hypothesis: Does greater intraspecific variability increase species' ecological breadth? *American Journal of Botany* 101: 56–62.
- SOBRAL, M., J. GUTIÁN, P. GUTIÁN, AND A. R. LARRINAGA. 2013. Selective pressure along a latitudinal gradient affects subindividual variation in plants. *PLOS ONE* 8: e74356.
- STOREY, J. D., AND R. TIBSHIRANI. 2003. Statistical significance for genome-wide studies. *Proceedings of the National Academy of Sciences, USA* 100: 9440–9445.
- TILMAN, D. 2001. Functional diversity. In S. Levin [ed.], *Encyclopedia of biodiversity*, vol. 3, 109–120. Academic Press, Waltham, Massachusetts, USA.
- VIOLLE, C., B. J. ENQUIST, B. J. MCGILL, L. JIANG, C. H. ALBERT, C. HULSHOF, V. JUNG, ET AL. 2012. The return of the variance: Intraspecific variability in community ecology. *Trends in Ecology & Evolution* 27: 244–252.
- VIOLLE, C., M. L. NAVAS, D. VILE, E. KAZAKOU, C. FORTUNEL, I. HUMMEL, AND E. GARNIER. 2007. Let the concept of trait be functional! *Oikos* 116: 882–892.
- WEISING, K., H. NYBOM, K. WOLFF, AND G. KAHL. 2005. DNA fingerprinting in plants. Principles, methods, and applications, 2nd ed. CRC Press, Boca Raton, Florida, USA.
- WELLS, C. L., AND M. PIGLIUCCI. 2000. Adaptive phenotypic plasticity: The case of heterophylly in aquatic plants. *Perspectives in Plant Ecology, Evolution and Systematics* 3: 1–18.
- WERNER, K., AND F. EBEL. 1994. Zur Lebensgeschichte der Gattung *Helleborus* L. (Ranunculaceae). *Flora* 189: 97–130.
- WILLIAMS, J. H. H., B. E. COLLIS, C. J. POLLOCK, AND M. L. WILLIAMS. 1993. Variability in the distribution of photoassimilates among leaves of temperate Gramineae. *New Phytologist* 123: 699–703.
- WINN, A. A. 1996a. Adaptation to fine-grained environmental variation: An analysis of within-individual leaf variation in an annual plant. *Evolution; International Journal of Organic Evolution* 50: 1111–1118.
- WINN, A. A. 1996b. The contributions of programmed developmental change and phenotypic plasticity to within-individual variation in leaf traits in *Dicerandra linearifolia*. *Journal of Evolutionary Biology* 9: 737–752.
- WINN, A. A. 1999. Is seasonal variation in leaf traits adaptive for the annual plant *Dicerandra linearifolia*? *Journal of Evolutionary Biology* 12: 306–313.
- ZOTZ, G., K. WILHELM, AND A. BECKER. 2011. Heteroblasty—A review. *Botanical Review* 77: 109–151.
- ZYWIEC, M., M. DELIBES, AND J. M. FEDRIANI. 2012. Microgeographical, inter-individual, and intra-individual variation in the flower characters of Iberian pear *Pyrus bourgaeana* (Rosaceae). *Oecologia* 169: 713–722.