The leafing intensity premium hypothesis tested across clades, growth forms and altitudes

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Summary

1. Recent reports provide empirical evidence for a negative isometric scaling of (log)leaf number per unit shoot volume (i.e. leafing intensity) to (log)leaf size in woody plants. In this context, a theory of leaf size variation was anticipated, stating that natural selection should have favoured high leafing intensity, given that most tree and shrub species are small-leaved. Leaf size is thus proposed to be a mere correlate of leafing intensity. This was coined the leafing intensity premium hypothesis.

2. I investigated the generality of the leaf size–number trade-off by testing its extension to a set of 60 small-stature species, growing along an altitude gradient. Also, I compiled data from 224 species and re-analysed their leaf size and leafing intensity. Finally, I explored the validity of the leafing intensity premium hypothesis to account for the leaf size–number trade-off, if the latter was sustained, and examined patterns of coordinated evolution of leaf size and leafing intensity across seed plant lineages.

3. Negative isometric scaling was supported in all analyses, except when evergreens were considered in isolation. Frequency distribution of both leaf size and leafing intensity was severely right-skewed in the 224-species data set, which provided no evidence in support of the view that natural selection favoured species with high leafing intensity.

4. Node divergences in the phylogenetic tree showed a tendency for inverse coordinated evolution of leafing intensity and leaf size. However, interactions between environments and divergence patterns, and also node-wise peculiarities, were detected.

5. *Synthesis.* The leaf size–number trade-off is of general scope, although scaling exponents differ among evergreen and deciduous species. In addition, signals of coordinated evolution of both traits across the seed plant tree were detected. Still, the leafing intensity premium hypothesis cannot be supported, since leafing intensity of present-day species is not particularly biased towards high scores.

Key-words: altitude gradient, compilation, leaf size, leafing intensity premium hypothesis, phylogeny

Introduction

Phenotypic diversity in plant form is astonishing at all scales, from the cellular to the individual level, and has long attracted scientific enquiry. One particularly diverse feature is the way plant species display units of foliage in their canopies. Individual leaf size, as the very basic unit of foliage display, spans six orders of magnitude across species (Milla & Reich 2007). Explanations for this remarkable variety of responses to the same ecological problem of harvesting light, while maintaining water balance and competitive capacity, arise from viewpoints as varied as heat dissipation needs, water and carbon balance or competition for light (Parkhurst & Loucks 1972; Givnish 1979). Recently, comprehensive compilations across and

within species have informed on the within-leaf constraints that may govern leaf size variation (Pickup *et al.* 2005; Niinemets *et al.* 2006, 2007; Milla & Reich 2007; Niklas *et al.* 2007; Price & Enquist 2007; Milla *et al.* 2008a). At the twig level, traits such as twig size, wood density or internode length are also reported to account for part of cross-species variation in leaf size (Westoby & Wright 2003; Wright *et al.* 2006; Poorter & Rozendaal 2008). The advent of empirical data calls for further theoretical development. A timely idea was put forward in this regard by Kleiman & Aarssen (2007), namely the leafing intensity premium hypothesis.

Kleiman & Aarssen's (2007) proposal can be summarized as follows. First, the authors detected an isometric trade-off between the number of leaves attached to a single shoot, corrected per twig volume, and the size of individual leaves. This pattern was described in a sample of 24 deciduous temperate

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tree species. Then, after stating that evolution has not favoured large leaves, as reasoned after the fact that leaf size spectrum is significantly right-skewed across 378 species of deciduous trees and shrubs from North America, Kleiman & Aarssen's (2007) argued that most species should possess a high number of nodes and internodes per twig volume. Subsequently, a selective advantage of high leafing intensity is proposed, given its putative preponderance in nature. This was coined the leafing intensity premium hypothesis (Kleiman & Aarssen 2007). The corollary of this hypothesis is that leaf size variation might be severely constrained to small sizes not because of direct adaptive value of small leaves, but merely as a trade-off to the more relevant ability of maintaining a high number of axillary buds per shoot (Kleiman & Aarssen 2007).

The leafing intensity premium is an appealing hypothesis, since it links with other frequently observed size–number compromises in plants, such as seed size–seed number trade-offs (Venable 1992; Westoby *et al.* 2002), trade-offs between plant size and fecundity (Aarssen 2005; Aarssen *et al.* 2006), or even compromises at the population-level between size and number of individuals (Deng *et al.* 2008). In addition, the few empirical studies performed so far have conformed to the pattern of inverse and isometric relationship between leaf size and leafing intensity (Ogawa 2008; Yang *et al.* 2008).

Yet, more comprehensive data and the extension to alternative growth forms and diverse plant families are needed to claim wide generality of this trade-off. More importantly, the adaptive significance of the leafing intensity premium hypothesis needs to be evaluated in depth. First, this hypothesis is based on the assumption that cross-species frequency distribution of leafing intensity is left-skewed, given its tight trade-off with leaf size, the distribution of which is right-skewed. This assumption, however, needs some examination. The trade-off between size and number of leaves was described in a log–log scale, which, in itself, contracts sparse data from the tails of the untransformed data distribution, and expands data from the central stretch of the whole range of variation. Afterwards, right-skewness of leaf size distribution was shown in an untransformed data scale (Kleiman & Aarssen 2007). Frequency distribution of data surely varies when it is log-scaled. However, both patterns, one reported in a log–log scale, and the other in a raw data scale, were combined directly to infer that leafing intensity should be necessarily left-skewed (Kleiman & Aarssen 2007). This inference should be addressed explicitly. Secondly, if the compromise among both traits is tight enough, evolutionary divergence events should have precluded the generation of species with large leaves and highly leaved twigs, or species with small leaves and scarcely leaved twigs. If true, coordinated evolution of leaf size and leafing intensity should be detectable in phylogenetically explicit analyses.

In this study, I made use of two different data sets to advance the testing of the leafing intensity premium hypothesis. First, I investigated whether the leaf size–number trade-off and its constancy across environments can be extended to perennial herbs and low-stature chamaephytes, both of which may confront different selective pressures on the design of their shoots than phanaerophytes (e.g. short life span of stem seg-

ments in perennial herbs should select for low-cost twigs). To carry out this investigation, I measured leaf and twig traits in a group of 60 congeneric altitudinal vicariants from northern Spain (congeneric vicariants data set henceforth). Secondly, I brought together literature data from the references cited in the preceding paragraphs, which were tested simultaneously for homogeneity of regression slopes (compilation data set henceforth). This should help to check for consistency of the leaf size–number trade-off across leaf habits and geographical origins using a large pool of species.

Furthermore, I made use of phylogenetic reconstructions to explore the evolutionary history of this trade-off across the selected sets of species. Primarily, I tested whether evolutionary divergences in leaf number across clades are accompanied by divergences in leaf size in the direction predicted by the leafing intensity premium hypothesis. This was tested separately in the two data sets. Additionally, I investigated whether vicariance of congeneric species along an environmental gradient (altitude) generates disruptions in the negative isometric scaling of leaf size to leaf number. This was tested only in the congeneric vicariants data set.

Methods and materials

COMPILATION OF LITERATURE DATA

Raw data on leaf mass (dry mass, g), and volume-based leafing intensity (number of leaves per unit twig volume, n_L mm^{-3}) were obtained from Kleiman & Aarssen (2007) and Yang *et al.* (2008). Leaf mass data in Kleiman & Aarssen (2007) were reported as fresh mass. To achieve comparability with other data sets, the data were converted to dry mass assuming 50% water content of leaves. Average water content of fresh leaves was estimated from leaf size data in Kleiman & Aarssen (2007), together with relative water content, leaf thickness and leaf mass per area data in Abrams *et al.* (1994). I decided to apply the same correction factor (i.e. 50%), instead of a species-specific parameter, to avoid generating distortion of the original scaling relationships reported in Kleiman & Aarssen (2007). Also, data from Westoby & Wright (2003) were re-worked to obtain volume-based leafing intensity and leaf dry mass data. Each species was categorized as evergreen or deciduous, on the basis of data published in source papers or descriptions in regional floras. The data thus obtained were complemented with data collected as described in the subsections below (congeneric vicariants data set) to build the complete 224-species data base.

CONGENERIC VICARIANTS DATA SET: STUDY SPECIES AND SITES

The study area was located along a sharp altitude gradient in northern Spain (Picos de Europa, Cantabrian mountain range). The climate in the lowlands is humid oceanic, with mild winters and warm summers, which turns colder and more humid as altitude increases. However, given its oceanic character, typical Mediterranean summer drought is absent even at the lowland sites. The area has an abrupt topography, with altitudes ranging from 0 to 2640 m a.s.l. across only 40 km.

Careful attention was paid in this study to the selection of a set of congeneric pairs of altitudinal vicariants suitable to address evolutionary patterns of trait variation. To accomplish this, I established

separate criteria for the group of genera as a whole, and for each single pair of vicariants. The criteria that the whole set of genera should meet were: (i) that most of the high-order taxonomic diversity of seed plants of temperate latitudes was included in it and (ii) that the group of species comprised as many representatives of low-stature perennial herbs and chamaephytes as possible. The criteria that each single pair of congeneric vicariants should meet was that both species in a pair share growth form, leaf habit and microsite affinities. A set of 30 pairs of congeneric vicariants was thus selected (Table 1, note that all vicariants were congeneric except the two representatives of *Orchidaceae*). This set was taxonomically diverse, including 24 families that encompass most seed plant lineages from temperate latitudes (Fig. 1).

CONGENERIC VICARIANTS DATA SET: SAMPLING AND MEASUREMENT OF TWIGS AND LEAVES

For each single species, a site representative of its typical habitat was located during spring–summer 2005 and 2006. Date of sampling varied depending on each species' phenology, and occurred roughly from May to August 2006 in the lowlands, and from July to August 2006 in the highlands. Twig sampling was performed by randomly selecting five plants per population, located at some distance from each other, and harvesting two current-year fully developed and foliated twigs from each plant. This sampling strategy is generally suitable for studies screening an ample number of species, as the one undertaken in this project, although not recommendable for detecting intraspecific variability (Cornelissen *et al.* 2003). Individual and twig selection in the field avoided very large and small plants and twigs, and standardized canopy exposure and type of twig in case of twig heteromorphism.

Twigs were taken to the laboratory, and the following measurements were performed. First, a representative leaf lamina was cut from each twig, avoiding cataphylls, hypsophylls and particularly small nomophylls from the distal and proximal short-internode ends of the twig axis. Leaves were oven-dried at 70 °C and weighed to the nearest 0.00001 g using a microbalance (MT XP6; Mettler-Toledo Inc., Westerville, OH, USA) (leaf mass, g, LM hereafter). All mature-sized nomophylles on the twig were subsequently counted (leaf number, n_L hereafter) and detached. Then, the basal diameter of the stem segment was measured to the nearest 0.01 mm using a dial thickness gauge (Mitutoyo Co., Aurora, IL, USA), and the length of that stem was measured to the nearest 0.1 mm with a digital calliper.

Leaf number per shoot was standardized as a volume-based 'leafing intensity' index (number of leaves per unit of shoot volume), following Kleiman & Aarssen (2007). This provides a metric comparable among species, representing a measure of relative investment in leaf number. Stem volume was assumed to approximate to a cylinder shape, with basal stem diameter as the cylinder width and stem length as cylinder height. Total leaf number on a twig was divided by total volume of the twig's sustaining stem (volume-based leafing intensity, n_L mm^{-3}, LI_V hereafter). I also measured (and show in Table 1) raw data on mass-based leafing intensity, as calculated in Kleiman & Aarssen (2007), but only show and discuss analyses on LI_V because I lacked LI_M data for many of the compiled species.

DATA ANALYSIS

Analysis of the congeneric vicariants data set

Data were log-transformed before analyses. Three hierarchical ANOVAS, including (log)LM or (log)LI_V as response variables, were conducted to decompose trait variance among altitudes, species, individuals and twig effects. Altitude (either highland or lowland) and species (nested within altitude) were included as fixed-effect factors. Individuals were specified as a random-effect factor, and residual variance was assumed to represent inter-twig variation within individuals. Type I sum of squares was specified, which is more adequate for variance partitioning analyses. Also, to evaluate the effect of altitude over each pair of congeneric vicariants, I plotted the mean values of each trait of the lowland species against that of their highland congeners. Significance of differences between lowland and highland congeners was assessed with a Wilcoxon signed-rank test for each trait.

Leaf mass and LI_V were assumed to be related to each other as defined by the following power function:

$$LI_V = \alpha LM^\beta \qquad \text{eqn 1}$$

which was log-linearized, thus converting the power exponents to linear slope scores:

$$\log(LI_V) = \log(\alpha) + \beta \log(LM), \qquad \text{eqn 2}$$

where $\beta > 1$ indicates that larger-leaved species tend to show disproportionally higher LI_V, whereas $\beta < 1$ means that larger-leaved species tend to show disproportionally lower LI_V. If LM and LI_V scale isometrically, then the relationship remains constant across the full range of leaf sizes.

I used Type II linear regression procedures to fit regressions between (log)LM and (log)LI_V (Sokal & Rohlf 1995). Type I regression calculates parameters so that the sum of the residuals to the regression line is minimum at the *y*-axis. Type II regression, or standardized major axis (SMA) regression, minimizes the error associated with variation in both dependent and independent variables. Type II regression is more appropriate when knowledge on scaling exponents, instead of prediction of *y*-values, is the focus of the study (Warton *et al.* 2006). Type II regression procedures were carried out using (S)MATR (Version 2.0, Falster DS, Warton DI & Wright IJ, http://www.bio.mq.edu.au/ecology/SMATR). Cross-species regressions were fitted separately for highland and lowland vicariants. Heterogeneity of regression slopes among altitudes and against slope = −1 was tested through re-sampling and permutation procedures (Warton *et al.* 2006). When slopes did not differ among altitudes, differences in the elevation of regression slopes (*y*-intercept) and in shifting along the common slope were tested by Wald tests as available in (S)MATR.

Detailed descriptions of the methods used to implement phylogenetically explicit analyses are provided in Appendix S1 (see Supporting Information). In short, a phylogeny of the study species was built. Then I performed correlated divergence analyses of LM versus LI_V, separately for highland and lowland species. These analyses investigate whether divergence in leafing intensity was consistently correlated with divergence in leaf size across all the radiations along the phylogenetic tree. Secondly, I investigated whether the divergence events that contributed the most (or the least) to explain total trait variance at the tips of the tree in the lowlands were the same ones that contributed the most (or the least) to that in the highlands (see Appendix S1, for methods employed).

Analysis of the compilation data set

The procedures described earlier in this study were also used to address trait relationships in the wider compilation data set. Type II regressions were fitted separately for deciduous and evergreen species to check for consistency of scaling exponents between leaf habits. The phylogenetic tree for the compilation data set was built as described in Appendix S1, but the seed plant tree was pruned for the 224 species

Table 1. List of study species of the congeneric vicariants data set, together with their taxonomical and growth form affiliation, and altitude of study sites. Also, species arithmetic mean values of LM (leaf mass, mg), LI_V (volume-based leafing intensity, n_L mm^{-3}) and LI_m (mass-based leafing intensity, n_L g^{-1}). Growth form categories – H, hemicryptophyte; C, chamaephyte; P, phanaerophyte; T, terophytes

Species	Family	Growth form	high/low vicariant	Altitude (m a.s.l.)	LM (mg)	LI_V (n_L mm^{-3})	LI_M (n_L g^{-1})
Aquilegia pyrenaica	Ranunculaceae	H	h	1998	16.37	0.115	0.362
Aquilegia vulgaris	Ranunculaceae	H	l	350	469.00	0.001	0.004
Arenaria purpurascens	Caryophyllaceae	C	h	2090	0.75	1.029	2.568
Arenaria montana	Caryophyllaceae	C	l	200	1.68	0.440	0.591
Campanula arvatica	Campanulaceae	H	h	2100	0.67	1.190	2.058
Campanula patula	Campanulaceae	H	l	100	7.81	0.006	0.046
Chenopodium bonus-henricus	Chenopodiaceae	H	h	1900	9.05	0.183	1.143
Chenopodium album	Chenopodiaceae	H	l	30	66.44	0.003	0.015
Epilobium alsinifolium	Onagraceae	H	h	2110	1.77	0.446	–
Epilobium parviflorum	Onagraceae	H	l	175	18.59	0.004	0.034
Orchis mascula	Orchidaceae	H	h	100	79.22	0.001	0.024
Epipactis atrorubens	Orchidaceae	H	l	1875	57.25	0.006	0.083
Erigeron alpinus	Asteraceae	H	h	2110	6.00	1.533	4.055
Erigeron karvinskianus	Asteraceae	H	l	100	7.55	0.028	0.101
Erysimum mayorii	Brassicaceae	H	h	2000	36.89	0.044	0.196
Erysimum duriaei	Brassicaceae	H	l	15	6.83	0.027	0.140
Euphorbia pyrenaica	Euphorbiaceae	C	h	2090	2.31	0.272	1.257
Euphorbia flavicoma	Euphorbiaceae	C	l	350	3.06	0.161	0.582
Helianthemum urrielense	Cistaceae	C	h	2100	1.84	0.551	1.598
Helianthemum nummularium	Cistaceae	C	l	100	2.96	0.138	0.454
Hypericum nummularium	Clusiaceae	H	h	2090	2.03	2.425	4.331
Hypericum perforatum	Clusiaceae	H	l	1060	1.52	0.018	0.195
Linaria supina	Scrophulariaceae	H	h	2110	0.48	2.213	–
Linaria alpina ssp. *faucicola*	Scrophulariaceae	H	l	100	0.51	0.439	0.936
Lonicera xylosteum	Caprifoliaceae	P	h	1400	57.08	0.052	0.145
Lonicera peryclimenum	Caprifoliaceae	P	l	5	87.65	0.015	0.066
Lotus alpinus	Fabaceae	H	h	2090	3.03	0.989	1.642
Lotus corniculatus	Fabaceae	H	l	100	7.24	0.066	0.256
Mathiola perennis	Brassicaceae	H	h	1900	7.96	0.604	1.841
Mathiola incana	Brassicaceae	H	l	40	26.34	0.007	0.052
Myosotis alpestris	Boraginaceae	H	h	2110	7.02	0.054	0.252
Myosotis lamottiana	Boraginaceae	H	l	200	9.81	0.006	0.056
Pedicularis pyrenaica	Scrophulariaceae	H	h	1875	3.29	0.039	0.105
Pedicularis sylvatica	Scrophulariaceae	H	l	830	3.12	0.078	0.557
Pimpinella siifolia	Apiaceae	H	h	1875	35.40	0.022	–
Pimpinella major	Apiaceae	H	l	175	111.75	0.001	0.006
Pinus sylvestris	Pinaceae	P	h	1650	15.33	0.141	0.506
Pinus pinaster	Pinaceae	P	l	200	70.14	0.026	0.110
Polygonum viviparum	Polygonaceae	H	h	2110	11.12	0.023	0.064
Polygonum salicifolium	Polygonaceae	H	l	30	56.88	0.002	0.012
Quercus petraea	Fagaceae	P	h	1392	282.99	0.016	0.031
Quercus robur	Fagaceae	P	l	40	218.43	0.027	0.044
Reseda glauca	Resedaceae	H	h	2090	3.34	0.245	0.504
Reseda phyteuma	Resedaceae	H	l	19	7.99	0.034	0.140
Rinanthus serotinus	Scrophulariaceae	T	h	1875	3.28	0.133	0.508
Rinanthus angustifolium	Scrophulariaceae	T	l	100	13.84	0.008	0.058
Rosa pendulina	Rosaceae	P	h	1534	26.18	3.508	0.929
Rosa canina	Rosaceae	P	l	404	120.18	0.031	0.083
Salix hastata	Salicaceae	C	h	1866	12.58	0.124	0.354
Salix repens	Salicaceae	C	l	200	6.14	0.252	0.759
Silene vulgaris ssp. *glareosa*	Caryophyllaceae	H	h	1850	7.26	0.075	0.249
Silene vulgaris ssp. *conmutata*	Caryophyllaceae	H	l	350	19.17	0.021	0.101
Sorbus aucuparia	Rosaceae	P	h	1560	653.99	0.007	0.025
Sorbus torminalis	Rosaceae	P	l	520	431.75	0.008	0.017
Trifolium thalii	Fabaceae	H	h	1875	3.93	0.317	–
Trifolium pratense	Fabaceae	H	l	15	38.38	0.002	0.019
Vaccinium gaultheroides	Ericaceae	C	h	2110	3.39	1.105	–
Vaccinium myrtillus	Ericaceae	C	l	200	4.89	0.201	0.538
Viola biflora	Violaceae	H	h	2110	3.29	0.328	1.145
Viola riviniana	Violaceae	H	l	583	1.78	0.305	1.167

compiled. Correlated divergence analysis, calculation of node-wise contribution indexes and sensitiveness to phylogenetic uncertainty were elaborated as explained for the congeneric vicariants data set (see Appendix S1). Similar to the earlier data analyses, results did not change with input tree.

Results

CONGENERIC ALTITUDINAL VICARIANTS FROM NORTHERN SPAIN

Leaf mass spanned three orders of magnitude among the 507 twigs sampled in northern Spain, from 0.00034 g of the smallest *Linaria supina* leaf, to 0.76275 g of the largest *Sorbus aucuparia* leaf (Table 1). Variation in LI_V was also large, spanning four orders of magnitude. The majority of this variation was accounted for by differences among species (Table 2). Altitude explained a fair amount of variation in LI_V, although its effect over LM was modest (Table 2). Variance absorbed by among-twig variation within canopies was comparatively negligible, while inter-individual differences accounted only for 2–5% of variation in the target traits (Table 2). Highland vicariants displayed comparatively higher LI_V than their lowland congeners, as shown in the bisector plots of Fig. 2. LM was significantly lower in the highland vicariants, although differences were closer to the 1 : 1 isocline (Fig. 2).

Neither the highland nor the lowland log–log SMA regressions differed significantly from slope = −1 (Fig. 3a). Goodness-of-fit, however, was poorer than that reported in Kleiman & Aarssen (2007) and Yang *et al.* (2008). Slopes did not differ among altitudes; thus differences in elevation and in shifting along the common slope were tested. Elevation was significantly higher for regression lines fitted to highland species. Shifts along the common slope also occurred, with highland species displaced towards the high LI_V–low LM side of the trade-off.

Regarding phylogenetically independent contrasts, overall, the degree of divergence in LM at a given internal node of the phylogenetic tree was accompanied by similar divergence in LI_V, but in the opposite direction (Fig. 3b). However, remarkable scatter around this significant tendency was evident. This is reflected, for instance, in the poor fit of this relationship at the highlands. Contribution of internal nodes to the partition of tree-wide divergence in traits (contribution index) also showed significant correlation between LM and LI_V (result not shown).

The relative contribution of a given node to total variation at the highlands was unrelated to the contribution of that node to variation at lowlands (Fig. 4). Note that the phylogenetic tree is exactly the same for lowlands and highlands, and thus internal nodes are directly comparable among altitudes.

224-SPECIES COMPILATION

Frequency distributions of LM and LI_V scores for the 224 species compiled in this study were significantly right-skewed. Both traits showed similar degrees of leptokurtosis and asymmetry (Fig. 5). Also, when data were scaled to logarithms, both variables conformed to a normal distribution, with low asymmetry and kurtosis indexes (Fig. 5).

When the whole set of species was considered to elaborate (log)LM versus (log)LI_V Type II regressions, deciduous species strictly conformed to the isometric negative scaling of (log)LM to (log)LI_V, while evergreen species showed an allometric scaling slope, significantly higher than −1.

Phylogenetic analyses of the compilation data set confirmed the patterns outlined by the smaller congeneric vicariants data set. Evolutionary divergences produced, as a whole, coordinated changes in LM and LI_V (Fig. 6). Several node divergences showed a tendency towards exacerbated increases in leafing intensity accompanying moderate decreases in LM (mainly *Trifolium*, Monocot–Eudicot, *Hypericum*, *Rosales* and *Mirbelieae*). Moreover, relative contribution of each single node divergence to present-day variation in the target traits was similar for LM and LI_V (not shown).
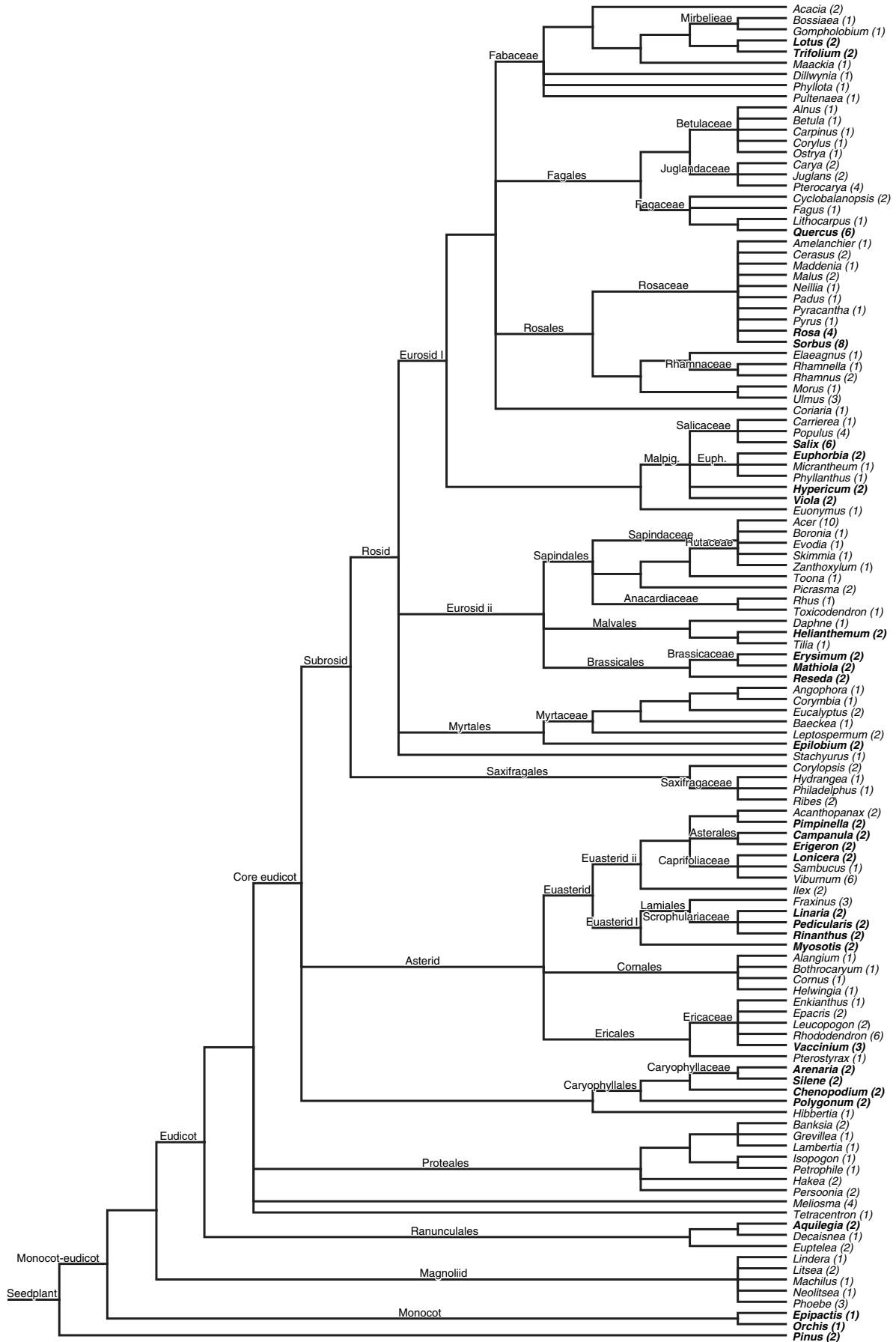
Discussion

IS THE LEAF SIZE–NUMBER TRADE-OFF OF GENERAL SCOPE?

The results shown in this study support the existence of a cross-species trade-off between the number of leaves attached to a unit of yearly shoot and the size of individual leaves, which conforms to the pattern described in Kleiman & Aarssen (2007). The realm of this trade-off is thus expanded to low-stature plants, to a new geographical area and to a set of species representative of high-order phylogenetic diversity of seed plants. Hence, adding to previous reports on phanaerophytes (Kleiman & Aarssen 2007; Ogawa 2008; Yang *et al.* 2008), overall generality of the leaf size–number trade-off is reinforced.

The isometric nature of the leaf size–number trade-off was disrupted only when the evergreen species of the complete compilation data set were considered in isolation. This contrasts with regression slopes reported separately for the evergreen species in Yang *et al.* (2008), which were built with part of the species pool used in this study. Deciduous species, on the other hand, conformed to the isometric trade-off in a very accurate manner. It is thus stated that, per unit of crown volume, leaf biomass should remain relatively constant across deciduous species, which has been demonstrated by Ogawa (2008) for trees. However, why should the rate of decrease in leafing intensity as LM

Fig. 1. Phylogenetic tree of the seed plants, pruned for the list of genera of the whole compilation data set. The topology displayed was obtained from the maximally resolved seed plant tree available in Phylomatic (http://www.phylodiversity.net/phylomatic). For simplicity, branch lengths are not proportional to time since evolutionary divergence. Genera in bold are those that include species of the congeneric vicariants data set of northern Spain. Numbers in parentheses are number of species contributed by each genus to the compilation data set.

Acacia (2)
Bossiaea (1)
Gompholobium (1)
Lotus (2)
Trifolium (2)
Maackia (1)
Dillwynia (1)
Phyllota (1)
Pultenaea (1)
Alnus (1)
Betula (1)
Carpinus (1)
Corylus (1)
Ostrya (1)
Carya (2)
Juglans (2)
Pterocarya (4)
Cyclobalanopsis (2)
Fagus (1)
Lithocarpus (1)
Quercus (6)
Amelanchier (1)
Cerasus (2)
Maddenia (1)
Malus (2)
Neillia (1)
Padus (1)
Pyracantha (1)
Pyrus (1)
Rosa (4)
Sorbus (8)
Elaeagnus (1)
Rhamnella (1)
Rhamnus (2)
Morus (1)
Ulmus (3)
Coriaria (1)
Carrierea (1)
Populus (4)
Salix (6)
Euphorbia (2)
Micrantheum (1)
Phyllanthus (1)
Hypericum (2)
Viola (2)
Euonymus (1)
Acer (10)
Boronia (1)
Evodia (1)
Skimmia (1)
Zanthoxylum (1)
Toona (1)
Picrasma (2)
Rhus (1)
Toxicodendron (1)
Daphne (1)
Helianthemum (2)
Tilia (1)
Erysimum (2)
Mathiola (2)
Reseda (2)
Angophora (1)
Corymbia (1)
Eucalyptus (2)
Baeckea (1)
Leptospermum (2)
Epilobium (2)
Stachyurus (1)
Corylopsis (2)
Hydrangea (1)
Philadelphus (1)
Ribes (2)
Acanthopanax (2)
Pimpinella (2)
Campanula (2)
Erigeron (2)
Lonicera (2)
Sambucus (1)
Viburnum (6)
Ilex (2)
Fraxinus (3)
Linaria (2)
Pedicularis (2)
Rinanthus (2)
Myosotis (2)
Alangium (1)
Bothrocaryum (1)
Cornus (1)
Helwingia (1)
Enkianthus (1)
Epacris (2)
Leucopogon (2)
Rhododendron (6)
Vaccinium (3)
Pterostyrax (1)
Arenaria (2)
Silene (2)
Chenopodium (2)
Polygonum (2)
Hibbertia (1)
Banksia (2)
Grevillea (1)
Lambertia (1)
Isopogon (1)
Petrophile (1)
Hakea (2)
Persoonia (2)
Meliosma (4)
Tetracentron (1)
Aquilegia (2)
Decaisnea (1)
Euptelea (2)
Lindera (1)
Litsea (2)
Machilus (1)
Neolitsea (1)
Phoebe (3)
Epipactis (1)
Orchis (1)
Pinus (2)

Internal clade labels: Mirbelieae, Fabaceae, Fagales, Betulaceae, Juglandaceae, Fagaceae, Rosales, Rosaceae, Rhamnaceae, Eurosid I, Salicaceae, Malpig., Euph., Rosid, Sapindales, Sapindaceae, Rutaceae, Anacardiaceae, Eurosid ii, Malvales, Brassicales, Brassicaceae, Subrosid, Myrtales, Myrtaceae, Saxifragales, Saxifragaceae, Core eudicot, Euasterid ii, Asterales, Caprifoliaceae, Euasterid, Euasterid I, Lamiales, Scrophulariaceae, Asterid, Cornales, Ericales, Ericaceae, Caryophyllales, Caryophyllaceae, Eudicot, Proteales, Ranunculales, Monocot-eudicot, Magnoliid, Monocot, Seedplant

Table 2. Percentage of total variance of LM, LI_V and LI_M explained by altitude, species, individual and twig. Differences among twigs of the same individual were assumed to approximate to residual unexplained Type I sums of squares of ANOVA analyses. Variables were log-transformed before analyses. See text for ANOVA model specifications. LM is leaf mass (dry mass, g) and LI_V is volume-based leafing intensity (number of leaves per unit twig volume, n_L mm^{-3})

	Altitude	Species (within altitudes)	Individuals (within species)	Twigs (within individuals)
LM	4.60	92.25	2.31	0.85
LI_V	25.44	69.03	3.46	2.08
LI_M	17.97	74.84	5.07	2.11

increases be different between deciduous and evergreens? In evergreens, stem sections are foliated up to the oldest segment with green leaves. Thus, integration of foliage functioning in evergreens does not occur at the yearly twig level, but at a larger scale (Sprugel *et al.* 1991). The size of foliated stems is rather variable, provided that leaf longevity varies 22-fold among evergreens (Wright *et al.* 2004). Compromises at branch sizes larger than the yearly shoot, which is the basic unit of the leaf size–number trade-off, may have disrupted evergreens from the clearly isometric scaling detected for deciduous species.

The trade-off is maintained when analysed separately for two subsets of species living at contrasting altitudes (i.e. no slope heterogeneity between altitudes). This is remarkable and, along with results of Yang *et al.* (2008), supports the fact that the nature of the leaf size–number trade-off holds even under very contrasting environmental scenarios. However, highland species showed higher leafing intensity at any given LM (i.e. higher elevation of regression line at highlands). Yang *et al.* (2008), in contrast, found that sets of species living at different altitudes shared elevation of LM versus leafing intensity regression lines. Thus, as far as the little evidence available can support, it seems that high altitude selects for particularly high leafing intensity in herbs and low-stature woody plants, but not in phanaerophytes. This may relate to the different biotic and abiotic challenges that both growth forms have to deal with. Competition for light is of moderate relevance in alpine communities dominated by low-stature plants, as compared with that in forests and more fertile sites (Körner 1999). The fact that stems of low-stature plants are designed for functions other than over-shading neighbours probably allows for the preponderance of more intensively leafed twigs with short internodes. This is in accordance with the whole-plant architecture syndrome of high-altitude species, which tend to show prostrate and compact habits (Körner 1999; Milla *et al.* 2008b).

The leaf size–number trade-off is in line with the several well-known compromises between allocation to size and number of organs in plant bodies, or of individuals in plant populations (Venable 1992; Deng *et al.* 2008). Size–number trade-offs may add to the typical characteristics of modular organisms,
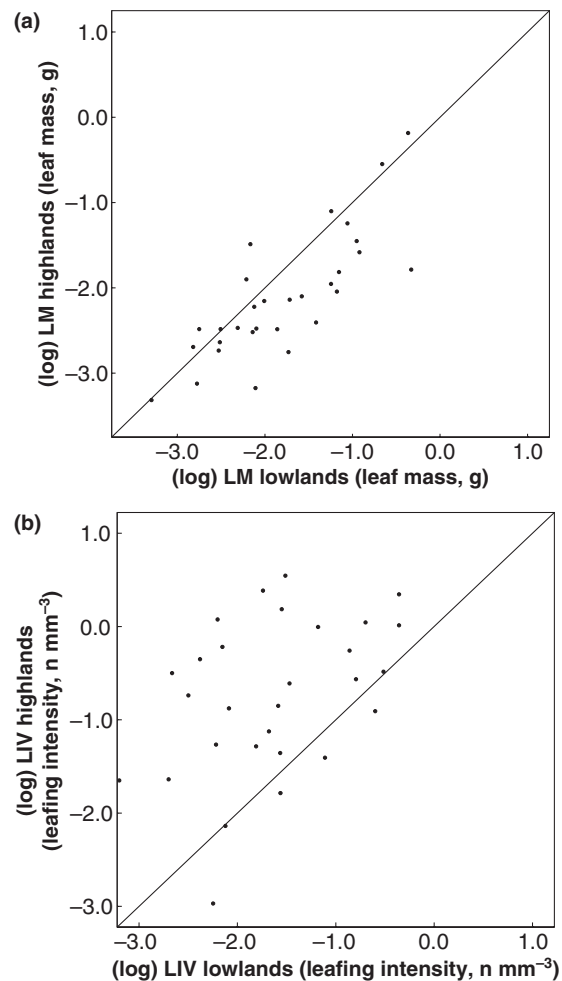
Fig. 2. LM (a) and LI_V (b) scores of lowland species (*x*-axis) and their highland congeners (*y*-axis). The bi-sector represents the portion of the graph where lowland and highland congeners have equal values for the studied trait. *P*-values were obtained from Wilcoxon signed-rank tests to assess the significance of a general difference in the studied traits between lowland and highland congeners. LM is leaf mass (dry mass, g) and LI_V is volume-based leafing intensity (number of leaves per unit twig volume, n_L mm^{-3}).

which show little functional differentiation among organs, as compared with that of unitary organisms (see Hall & Hughes (1996), for an example in Cnidaria). Seed plants, irrespective of species-specific differences in clonality, are certainly modular organisms, with recognized capabilities to regulate size, number and shape of organs at the module scale (de Kroon *et al.* 2005). The leaf size–number trade-off is also linked to Corner's Rules (Halle *et al.* 1978; Ackerly & Donoghue 1998). As predicted by Corner's Rules, thin stems bear scarcely separated nodes, with many small leaves per stem unit, and vice versa for thick stems. Isometric negative scaling of leaf size to leafing intensity can be viewed as a quantitative add-on to Corner's Rules. Leafing intensity is negatively related to twig size by definition (*R*-Pearson = 0.93, *P* < 0.01, *n* = 200, for the subset of compiled species with twig diameter or twig volume data available). As highlighted by Yang *et al.* (2008), leafing
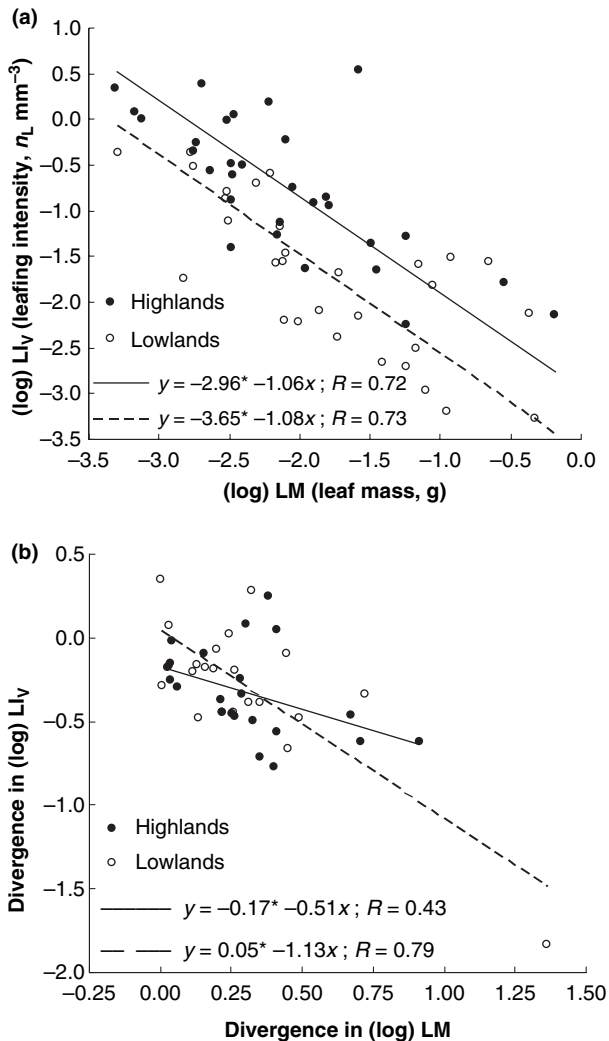
Fig. 3. Standardized major axis (SMA) regressions and correlation of evolutionary divergences in the congeneric vicariants data set (60 species). (a) Scatter plot of (log)LM versus (log)LI_V. Lines are SMA regressions. Slope, intercept and goodness-of-fit are inside the chart. Slopes neither differed between altitudes, nor from slope = −1 at $P = 0.05$. Dots are species. (b) Correlated change of evolutionary divergences in (log)LM against (log)LI_V. Dots are internal nodes of the phylogenetic tree depicted in Fig. 1, but pruned for the genera of the congeneric vicariants data set. All SMA regressions and correlations significant at $P = 0.05$.

intensity, twig size and leaf size are so closely related across species that distinguishing which is(are) the element(s) under selection, and which is(are) a mere correlate(s) will be difficult to achieve (see next section).

RE-EVALUATION OF THE LEAFING INTENSITY PREMIUM HYPOTHESIS

The recurrent prevalence of a cross-species correlation among traits informs on very relevant ecological aspects, from species autecology to ecosystem functioning (Westoby *et al.* 1995; see Ogawa 2008, for relevance of the leaf size–number trade-off to ecosystem level carbon balance). However, more in-depth investigations are needed to assess

adaptive significance of trait correlations and their evolutionary routes. As a first step, Kleiman & Aarssen (2007) proposed the leafing intensity premium hypothesis after reasoning that high leafing intensity should be rewarded by natural selection. This inference was based on the patterns that (i) most species are small-leaved and (ii) both variables scale negatively and isometrically. High leafing intensity may indeed bring about selective advantages. Having more nodes per branch unit entails a larger bud bank for recovery from physical disturbance or browsing. It also implies higher plasticity in meristem allocation to vegetative growth, reproductive display or pre-formation functions, and enhanced ability to react to changes in light availability (Bonser & Aarssen 1996; Vesk & Westoby 2004; Milla *et al.* 2008b). Other putative advantages of high leafing intensity, given its trade-off with leaf size, include higher hydraulic assurance by means of more individual connections to the vascular system (Kleiman & Aarssen 2007).

As claimed in the Introduction, violations of the assumption that leafing intensity is left-skewed would challenge the leafing intensity premium hypothesis. Thus, explicit examination of frequency distribution of the leafing intensity is necessary. Distribution of LI_V scores of the 24 species in Kleiman & Aarssen (2007) was certainly left-skewed, with most species showing relatively high leafing intensity, which supports the arguments in the preceding paragraph (data extracted from Fig. 3 of Kleiman & Aarssen 2007, result not shown). However, leafing intensity data of the whole set of 224 species compiled in this study were clearly right-skewed. Thus, in opposition to the leafing intensity premium hypothesis, the frequency distribution of LI_V of the species of this compilation did not show a left-skewed shape. Instead, distribution of leafing intensity data yielded high right-asymmetry and kurtosis indexes, similarly to leaf size. Consequently, the premium for high leafing intensity was lower than that expected and did not promote preponderance of highly leaved twigs in nature.

It may seem contradictory that, if the log–log relationship between LI_v and LM is negative, both variables are right-skewed in an extremely similar fashion. As stated in the Introduction, log-transformation contracts sparse data from the tails of an untransformed data distribution, and expands data from the central stretch of the whole range of variation. When frequency distributions are built from log-scaled data, right-skewedness disappears (see Fig. 5). Also, when data in Fig. 6a are not plotted in log–log scale, but as untransformed LI_v versus LM, most species cluster near the $x = 0, y = 0$ coordinate (not shown). This means that the majority of compromises between leaf size and leafing intensity occur within a group of relatively small-leaved species bearing relatively scarcely leaved twigs, which simply appear in a more central position in the (log)LI_v–(log)LM space, because of log-transformation.

This does not rule out the existence of the leaf size–number trade-off. What is definitely challenged by the right-skewed distribution of untransformed data is the validity of the leafing intensity premium hypothesis. Cross-species variation in leaf size is certainly right-skewed (Kleiman & Aarssen 2007; Milla & Reich 2007; Niklas *et al.* 2007). This variation, however,
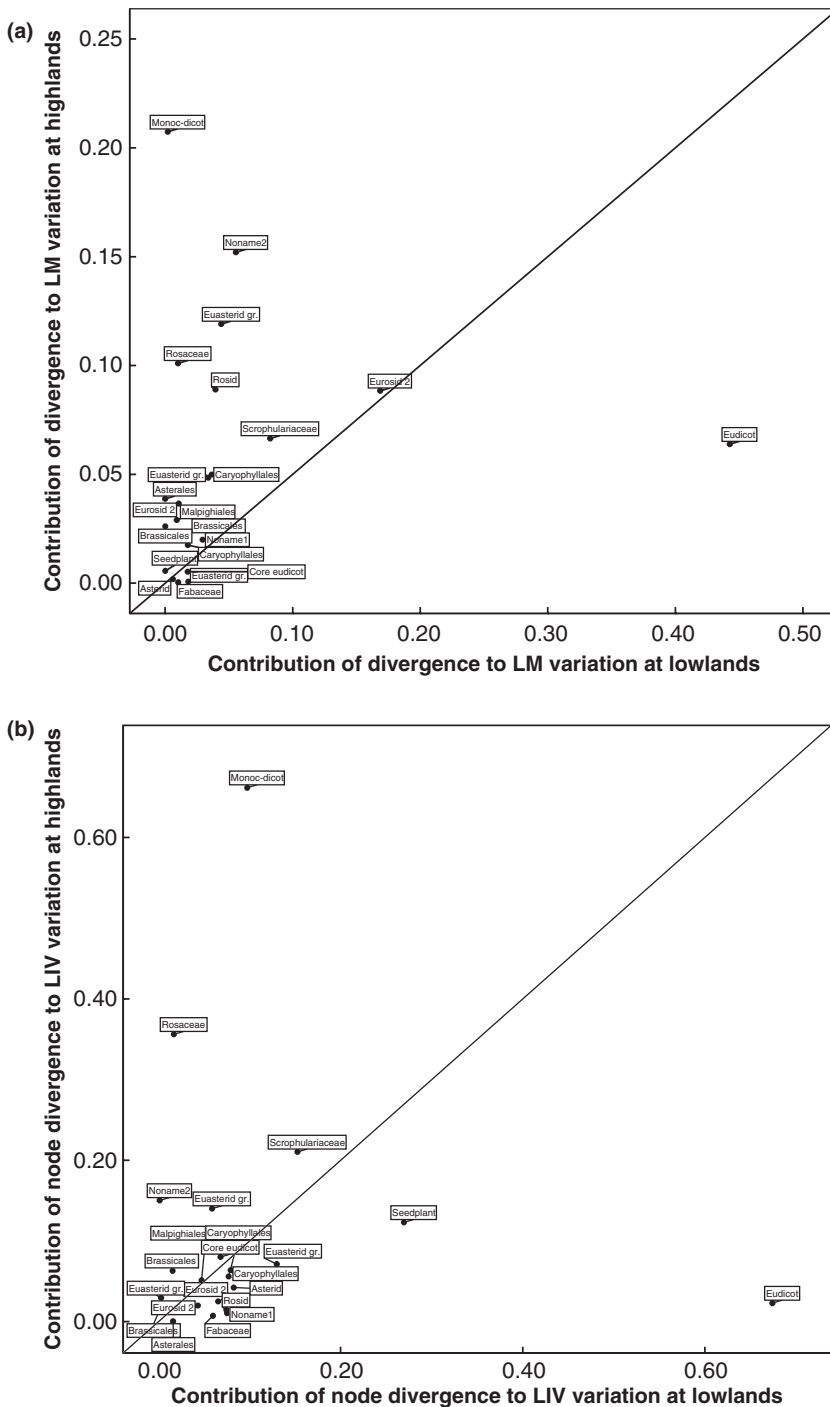
Fig. 4. Scatter plots of the contribution index of node divergences to trait variation – LM (a), LI$_V$ (b) – of the lowland species versus contribution of the same nodes in the highland species. See Appendix S1 and Moles (2005), for more details on the contribution index. Dots are internal nodes of the phylogenetic tree of Fig. 1, pruned for the species of the congeneric vicariants data set. Reference lines are $y = x$. Noname1 is the node separating Polygonaceae and Caryophyllaceae-Amaranthaceae clades. Noname2 is the polytomy separating Fabaceae, Fagales and Rosaceae. No simple regression model fitted the data.

cannot be solely explained as a mere consequence of high leafing intensity being rewarded by natural selection, as proposed by the leafing intensity premium hypothesis. Recently, a mechanism based on whole-tree scaling and universal metabolic relationships has been proposed to account for Corner's Rules, which constitute another example of pervasive pattern of trait correlations in plant twigs (Olson *et al.* 2009). If we are to understand leaf size variation, we need synthetic approaches similar to that undertaken by Olson *et al*.

Another step towards understanding the evolutionary significance of the leaf size–number trade-off involves the assess-

ment of whether both traits changed coordinately during major divergence events of the seed plants, even if most of this variation occurred only at the low-end stretch of the range of both variables, as highlighted earlier. I found significant coordinated evolution of leaf size and leafing intensity, which was particularly strong when all species compiled were included in the analysis; thus increasing the number of node divergences that inform on the pattern. This supports the view that the present-day constancy of the leaf size–number trade-off reflects mutual constraints in the evolution of both traits in the past (Kleiman & Aarssen 2007). Coordinated evolution of other
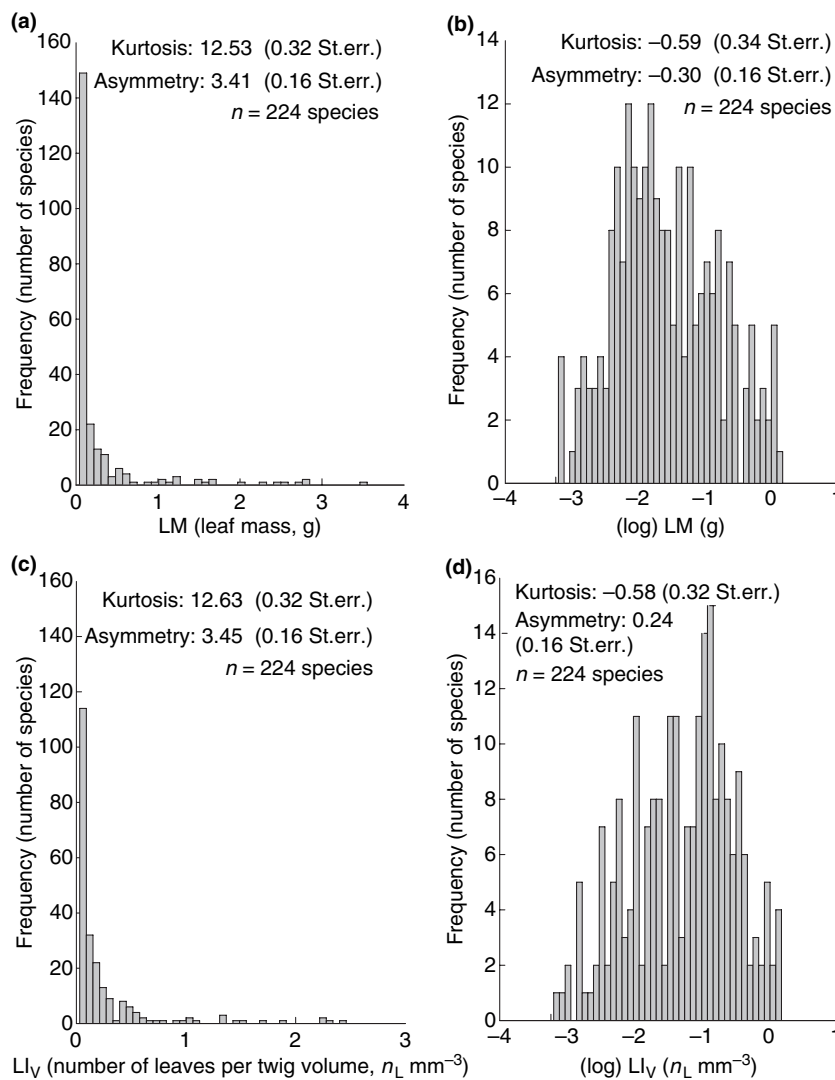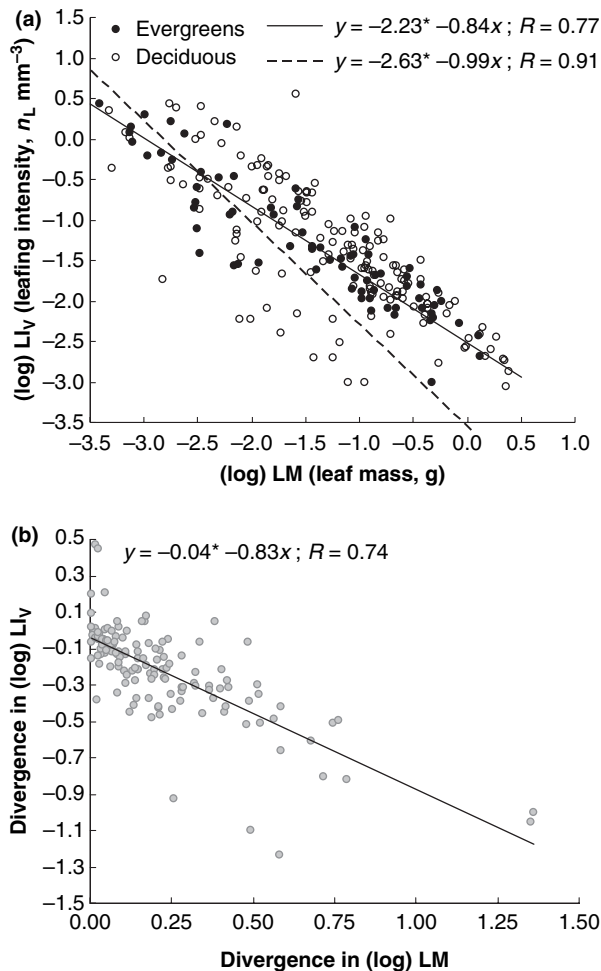
Fig. 5. Frequency distribution of species mean values of LM (a), and LI_V (c), and of (log)LM (b) and (log)LI_V (d), for the whole compilation data set (224 species). Indexes of asymmetry and kurtosis, along with their standard errors, are shown in the insets. LM is leaf mass (dry mass, g) and LI_V is volume-based leafing intensity (number of leaves per unit twig volume, n_L mm^{-3}).

leaf versus leaf and leaf versus twig traits has been described previously elsewhere (Ackerly & Reich 1999; Sun *et al.* 2006). These results underpin the notion that genotypes have to undergo coordinated evolution of multiple traits to generate sufficiently integrated phenotypes that are functional (Pugliucci 2003). The tendency found in this study for coordinated evolution, however, was not obligate. First, noticeable scatters in Figs 3b and 6b indicate that manifest divergences in leafing intensity were in some cases accompanied by small divergences in leaf size, e.g. *Mirbelieae* divergence, and reversely for large leaf size divergences. Secondly, it was shown that the same evolutionary divergences may imply contrasting consequences in leaf size and number under contrasting environments, in this study lowlands and highlands (note, for instance, high-order divergences such as Monocot–Eudicot, or Eudicot, in Fig. 4). Interpreting evolution of a trait as a function of coordinated evolution of a single other trait seems difficult, given the multiple-trait constraints that a plant has to accommodate to become functionally viable. More research on multivariate patterns of coordinated evolution of plant traits, together with causal models of trait interrelationships (Shipley 2004), should provide further insight in this area in the near future.

In conclusion, the existence of a cross-species trade-off between the size of individual leaves and the number of leaves per yearly stem unit seems to be of broad scope, although deciduous and evergreen species differ in the scaling factor. Also, as a general pattern, evolutionary divergences tended to produce co-evolving reductions in leafing intensity as a response to increases in leaf size, and vice versa. I found, however, little evidence supporting the proposal that the leaf size spectrum is remarkably right-skewed across species because of recurrent selection of species with high leafing intensity (namely the leafing intensity premium hypothesis).

Fig. 6. Standardized major axis (SMA) regressions and correlation of evolutionary divergences in the compilation data set (224 species). (a) Scatter plot of (log)LM versus (log)LI$_V$. Lines and regression parameters as in Fig. 4. Dots are species. (b) Correlated change of evolutionary divergences in (log)LM against (log)LI$_V$. Dots are internal nodes of the phylogenetic tree depicted in Fig. 1, but pruned for the genera of the compilation data set. All SMA regressions and correlations significant at $P = 0.05$. LM is leaf mass (dry mass, g) and LI$_V$ is volume-based leafing intensity (number of leaves per unit twig volume, n_L mm^{-3}).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Appendix S1. Phylogenetically explicit analyses.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Electronic supplementary material Appendix S1: Phylogenetically explicit analyses.

For implementing phylogenetically explicit analyses, a phylogeny of the study species was built as follows. Nomenclature and family affiliation of our study genera were checked against the Missouri Botanical Garden's VAST nomenclatural database (W3Tropicos,http://mobot.mobot.org/W3T/Search/vast.html). I then built a pruned phylogenetic tree of the seed plants with the study species as terminal tips using the maximally resolved seed plant tree available in Phylomatic (http://www.phylodiversity.net/phylomatic/). The resulting tree lacks an estimation of accurate distances between related nodes (*i.e.*, assumes equal branch lengths), which can result in biased estimates of phylogenetic distances between taxa when intervening terminal taxa are missing. Thus, I calibrated the tree by dating the nodes with the Branch Length ADJustment function (BLADJ) of the PHYLOCOM package (Webb *et al.* 2006). BLADJ assigns branch lengths on the basis of clade age estimates (Wikstrom *et al.* 2001), with undated nodes assigned equal branch lengths between nodes for which age estimates are available. Thus, I obtained a node-dated tree, calibrated in millions of years.

In the Introduction section I claimed that coordinated evolution of leaf size and number should be detectable across a phylogeny, provided that leaf size and leafing intensity mutually constrain the divergence of each other. Also, I aimed to test whether, in spite of vicariance of congeneric species along an altitude gradient, which forces adaptation

to very contrasting environments, isometric scaling of LM to LI_V is maintained. To investigate the effect of common ancestry on LM and LI_V variation, and in their coordinated evolution, I undertook two different approaches as described below.

First, I performed correlated divergence analyses of LM *vs*. LI_V, separately for highland and lowland species. These analyses ask whether divergence in leaf number was consistently correlated with divergence in leaf size across all the radiations along the phylogenetic tree. This was done with the independent contrasts analysis module of AOT. This module calculates, for each trait, contrast size at a given node as the estimated trait value at one daughter node, minus the estimated trait value of the second daughter node, divided by the square root of the total branch length between the two nodes. First and second daughter nodes are assigned arbitrarily for trait 1 involved in the analysis, and kept as assigned for trait 2. Contrast sizes of LM at each internal node were further correlated to contrast sizes of LI_V to estimate the degree of correlated divergence of leaf size and number.

Second, I investigated whether the divergence events that contributed the most (or the less) to explain total trait variance at the tips of the tree at the lowlands, were the same ones that contributed the most (or the less) at the highlands. For doing so, a node-based contribution index was calculated as explained in Moles *et al*. (2005). In brief, this index ranks node divergences as a function of how much present-day variation in the target trait they explain. This is analogous to a partitioning of sums of squares in a nested Anova model. The data base of this study is composed of congeneric vicariants, and thus the phylogenetic tree is the same for both altitudes. Therefore, the Contribution

index was calculated separately for lowlands and highlands, and was regressed for each trait using paired lowland-highland node-specific scores of the Contribution index.

Phylogenetic uncertainty and the presence of polytomies in the input tree may cause biases in phylogenetically explicit analyses (Butler & King 2004). Therefore, although AOT procedures are assumed to be robust to the presence of 'soft' polytomies (Webb *et al.* 2006), I resolved polytomies in the tree by randomly generating 100 fully resolved trees using MESQUITE (Maddison & Maddison 2008). I then re-sampled 50 of the 100 fully resolved trees randomly and run the analyses described above again separately for each of those 50 trees. None of the results described in the following section changed with input tree (data not shown), which supports the robustness of the analyses above to phylogenetic uncertainty.

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