

Research

Disentangling evolutionary, environmental and morphological drivers of plant anatomical adaptations to drought and cold in Himalayan graminoids

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Understanding what determine plants ability to survive drought and cold is crucial for predicting how plants may respond to ongoing climate change. Plant survival strategies are usually characterized by morphological and physiological adaptations, while their underlying anatomical settings are largely unknown. Woody angiosperms and herbaceous dicots have repeatedly evolved small water transporting conduits and large storage parenchyma tissues at colder or drier places to cope with freezing- and drought-induced damages. However, whether these adaptations are also valid for graminoids remains unclear. Here we show that stem anatomical variations in grasses, sedges and rushes dominating in western Himalayan grasslands are driven by elevation and soil moisture via control over aboveground plant stature and belowground clonal growth, while phylogenetic constraints have only a weak effect. Phylogenetic comparative analyses controlling for confounding factors showed that the elevation-related cooling controls the conductive system through reduced vessel diameter and extended assimilatory and storage tissues with more chlorenchyma and less sclerenchyma around vessels. The soil moisture deficit, on the other hand, determines stabilization structures by promoting short-rhizomatous turf graminoids with hollow stems, thicker epidermis and deep adventitious roots in dry steppes and semi-deserts. Saline wetlands and moist alpine pastures promote long-rhizomatous short-stature plants with lower need for mechanical support (absence of hollow stem) and exposure to high evaporative forcing (thinner epidermis). Observed trends of decreasing vessel sizes and lignification rate with elevation supports the existing knowledge that narrower vessels and extensive parenchyma assist plants to grow in cold environments by avoiding freezing-induced cavitation. Our results bring novel information on ecological drivers influencing the evolution of anatomical adaptations in high mountain graminoids. Distinct grassland types, covering elevations from 2650 to 6150 m, harbor unrelated species with different evolutionary histories that have converged towards similar anatomical structures.

Keywords: comparative plant anatomy, evolutionary inertia, gradient analysis, grasses, Himalaya, parenchyma cells, phylogeny, sclerenchyma, vessels

Introduction

During their evolution, vascular plants have developed complex adaptations for survival under various stressful conditions. While their strategies are usually characterized by morphological, ecophysiological and life-history adaptations (Körner 2003, Linder et al. 2017), their anatomy is rarely studied (Ahmad et al. 2018). For example, we know that at high elevations, plants have small stature, are protected against freezing by soluble carbohydrates, have slow growth and adults are long-lived (Nobis and Schweingruber 2013). The detailed knowledge of plant anatomical characters and their variation among different species and habitats is both scarce and restricted to dicotyledonous herbs (Schweingruber et al. 2012). Although the monocot families Poaceae (grasses), Cyperaceae (sedges) and Juncaceae (rushes), represent about one third of all monocotyledons and include many economically significant crops (Linder and Rudall 2005), comprehensive evaluation of their anatomical structures in environmental and evolutionary contexts is still rare (Ahmad et al. 2016, Schweingruber and Berger 2017).

Variations in anatomical structure should reflect differences in physiology (Baas et al. 2004, Hacke et al. 2016), and subsequent distinct tolerance of environmental conditions (Gleason et al. 2016). For instance, smaller conduits (vessels) have repeatedly evolved in colder places to enable plants to cope with freezing-induced embolism and cavitation (Foster and Gifford 1974). Xylem cavitation diminishes a plant's capacity for water transport and impairs carbon fixation by inducing stomatal closure to prevent further cavitation and desiccation of leaf tissues (Davis et al. 1999, Sperry 2003). In response to harsh conditions, herbaceous dicotyledonous plants allocate the majority of their total biomass to below-ground organs and only a small share to aboveground shoots and leaves, resulting in a limited need for stem mechanical support. Most of the plant tissue is composed of small vessels surrounded by unlignified, fiberless parenchyma-like living cells (Spicer 2014) with a high capacity for storing carbohydrates and hence osmoprotection (Doležal et al. 2018). In more favorable environment, taller plants should have larger vessels, embedded in heavily lignified fibers, to minimize hydraulic resistance by their greater path lengths (Meinzer et al. 2009). All these temperature- and size-related differences in vessel sizes and lignification extent have been repeatedly demonstrated in perennial forbs or trees (Morris et al. 2018), but it has not been established if these basic relationships are also valid for grasses and sedges.

Specific anatomical structure is not, however, only the result of adaptation to the prevailing environmental conditions (Carlquist 2012). Other factors can also modify these patterns, such as morphological and evolutionary constraints. For example, elevation effects may indirectly influence stem anatomy via plant size (e.g. smaller plants have smaller vessels regardless of elevation). Phylogenetic relatedness may cause similar 'blue-prints' among taxa that share part of their evolutionary history (Desclaves et al. 2003). Understanding the

adaptations of plant anatomical structures therefore requires considering evolutionary relatedness among species (species are not independent observations from a statistical perspective). This is commonly done by phylogenetic comparative models, which assume that the residual variation has a covariance structure according to species distances in the phylogenetic tree (Adams and Collyer 2018). Discounting all of the variation that could possibly be explained by phylogenetic relatedness of the species studied enables the direct effect of other potential predictors to be considered. While there are numerous examples linking interspecific anatomical variation in trees and forbs to phylogeny, plant size and environmental constraints (Schweingruber et al. 2012, 2014), similar tests in graminoids are missing. Similarly, we are not aware of any study examining the effect of clonal growth form (e.g. rhizomatous versus bunch grasses) on anatomical structure.

Grassland ecosystems, where grasses and other graminoids (*Carex*, *Juncus*, *Kobresia*) dominate the vegetation, make up 25–40 percent of Earth's land surface, including high elevation plateaus in the Himalayas and Tibet (Miehe et al. 2002). Despite long-term interest in grassland ecology, we have little information on anatomical adaptations of high-mountain graminoids. Most studies are based on data from European mountains (Schweingruber and Berger 2017), while the largest and highest mountainous areas located in Asia remain unexplored. On the Tibetan Plateau and the neighboring Himalayan crests, extensive areas of alpine grasslands and steppes host a unique and species-rich flora (Dvorský et al. 2011). This high-altitude region is being strongly impacted by global warming and overgrazing (Schmidt and Nüsser 2017), which leads to rapid changes in biodiversity (Doležal et al. 2016). Decline in vegetation cover and biodiversity loss can alter ecosystem processes and the resilience and resistance of ecosystems to environmental change. Baseline data on plant adaptations would help us track the effects of environmental change, and understanding the drivers of plant adaptations would enable us to predict how climate change may affect these extensive grasslands.

In this study, we examined the anatomical structure of monocot species in Indian northwestern Himalaya, including the westernmost part of Tibetan Plateau, in order to understand how microscopic anatomical features vary with plant size, elevation, hydrological conditions and taxonomic origin. The anatomical characteristics serve as a proxy for physiological adaptations that are more difficult to study for a large set of species. The study is based on more than 60 anatomical features described in cross sections of stems from 155 monocots. The studied taxa come from all dominant habitats of this vast territory including cold deserts, alpine steppes, saline wetlands, alpine grasslands, ruderal vegetation, alpine scree and subnival zones, covering elevations from 2650 to 6150 m. Our major goals were: 1) describing stem anatomical structures of monocotyledonous plants, 2) constructing a phylogenetic tree for target species from nucleotide sequences, 3) relating stem anatomical features to species distribution, phylogeny and morphology.

Material and methods

Study area

The study was conducted in Ladakh, Jammu and Kashmir State, Indian northwestern Himalaya (34° N, 77° E). The elevational extent of the study region is 2600–7650 m, area 80 000 km². Ladakh is situated in a double rain-shadow, and receives only little precipitation; with winter rains coming from the west and summer monsoons coming from the south (Pant et al. 2018). The climate of Ladakh is therefore predominantly arid, with annual precipitation between 50 and 300 mm, and cold due to high elevation, with an annual mean temperature well below 10°C. At low elevation deserts, intense drought prevails due to lower precipitation and relatively high summer temperatures. On the other hand, higher elevation steppes are more humid because of a decrease in evapotranspiration and an increase in precipitation. Annual precipitation is about 50 mm in deserts and semi-deserts (falling mostly during summer season), 100 mm in the alpine steppes and 150–250 mm in the alpine and subnival zones. While winter westerlies bring about 65% of annual precipitation, the highest monthly precipitation (around 40 mm) falls in August and the lowest (<10 mm) in October and November. Precipitation above 5000 m falls mostly as snow. The length of the growing season decreases linearly with increasing elevation, from 270 to 30 frost-free days, while the mean growing season temperature decreases from 16 to 3°C between 2650 and 6150 m (Dvorský et al. 2017). Temperatures in the study area vary from 0 to 30°C in summer and from –40 to –10°C in winter.

Vegetation description

Desert occupies only a small part of the arid gorge section in the lower Indus valley close to the Pakistani border and up to approximately 2900 m; the relatively high summer temperatures provide occasional habitats for species typical of adjacent lower Baltistan and other taxa of subtropical affinities, usually not ascending to much higher elevations. Much of the main Indus valley stretching beyond the capital Leh, and reaching far into the tributary valleys, including Dras, Suru, Zaskar and the Shyok valley system up to Nubra hosts a semi-desert vegetation, extending up to 4200 m in the west and 4400 m in the east. Alpine steppe represents the zonal vegetation and prevails widely, extending up to 4700 m in the west, and to 5400 m in the east. Higher up, a narrow belt of alpine grasslands occurs, including the characteristic moist alpine turf of *Kobresia pygmaea*. Above between 5200 m in the west and south, and 5700 m in the east, a very sparse subnival vegetation is developed; the upper vegetation limit lies at 6150 m (Dvorský et al. 2015). Vegetation of snow beds and springs can be found near the snow line and on slopes with surface water. Vegetation of screes is frequent throughout the region. Azonal vegetation includes wetlands, occupying relatively large areas of flat and broad valley bottoms in the whole area; these habitats are often saline. Vegetation

of river banks and river beds often supports large woody species, and can be found along major rivers and streams. Synanthropic ruderal vegetation includes plant assemblages developed on eutrophicated ground by stables of domestic animals and near villages up to 5400 m; weedy assemblages grow in arable fields up to 4700 m.

Anatomical description

Altogether, we collected the 155 species, the majority of monocots growing naturally in Ladakh (Doležal et al. 2018), belonging to seven families and 43 genera. The most species-rich family was Poaceae (109 taxa, 70%), followed by Cyperaceae (35 species, 23%), Juncaceae (5 taxa, 3%), Liliaceae (3, 2%), Juncaginaceae (1), Potamogetonaceae (1) and Zannichelliaceae (1). The most species-rich genera were *Carex* (20), *Stipa* (16), *Poa* (13), *Elymus* (12), *Kobresia* (11), *Bromus* (10), *Festuca* (10), *Puccinellia* (8), *Juncus* (5) and *Agrostis* (4). To describe plant anatomy, the transverse sections were cut from all studied individuals using a sliding microtome and double stained by Astrablue and Safranin (Gärtner and Schweingruber 2013). Each section was located at internodes above leaf sheaths, approximately in the middle part of the total length of the aboveground vertical stem (i.e. the culm in graminoids). The whole set of analysed material is comparable by the constant internal location of the section within the stem/culm. Double staining gave information about the distribution of vessels and lignified and unlignified (parenchyma) parts.

Anatomical analysis evaluated 67 variables grouped into 12 anatomical parameters including stem/culm stem diameter, surface outline (smooth, wavy etc.), stem center construction (hollow, full, aerenchyma), epidermis (thin-walled, thick-walled), the structure of the conductive system, chlorenchymatic assimilatory tissue, sclerenchymatic sheaths and aerenchymatic air spaces (for details see Supplementary material Appendix 1 Fig. A1). All analyses were based on at least two specimens collected in Ladakh. Finally, we analysed double-stained cross sections to gather quantitative information on proportion of three basic tissues: parenchymatic (storage), water conductive (transport) and mechanical (support), which deploy key functions for plants. By randomly drawing 100 polygons, each covering ¼ of the stem cross-sectional area, we calculated average conduits area (white), fiber area (red-stained), and parenchyma (blue-stained) using ImageJ image analysis software (Crivellaro et al. 2012).

Clonal growth forms

In addition to anatomical traits, each species was measured for plant height (ranging from <10 cm to 120 cm) and classified into clonal growth forms according to Klimešová et al. (2011a). The examined monocotyledonous species had bulbs, stolons, hypogeogenous rhizomes and epigeogenous rhizomes, or were annual. Rhizomatous plants were classified into one of two rhizome categories: 1) long (>10 cm between mother and daughter ramet) hypogeogenous rhizomes typical

for rhizomatous grasses, i.e. perennating and clonal growth organ of stem origin and formed below-ground. These rhizomes usually grow at a species-specific depth, periodically become orthotropic and form above-ground shoots; the horizontal parts of the rhizome bear bracts and adventitious roots, characterized by long internodes; they spread quickly vegetatively and their persistence differs considerably among species. 2) Short (<10 cm between mother and daughter ramet) epigeogenous rhizomes typical for turf graminoids, i.e. a perennating and clonal growth organ of stem origin formed above-ground; its distal part is covered by soil and litter or pulled into the soil by contraction of roots. They have nodes bearing green leaves, the internodes are usually short, rhizomes bear roots and serve as a bud bank and storage organ (Klimešová et al. 2011a).

Species elevational optima and ecological indicator values

To obtain a robust estimate of the elevational optima and species range, we calculated response curves through Huisman–Olff–Fresco models (Huisman et al. 1993). Species response curves were derived from 4150 vegetation plots (each 100 × 100 m) sampled over the entire Ladakh between 1999 and 2014 (Dvorský et al. 2017). The dataset contains more than 122 000 records of occurrence of vascular plant species along the elevational gradient from 2650 m to 6150 m. Ecological indicator values (species' optima on soil moisture gradient) were derived from the vegetation composition of 369 plots (each 100 m²) sampled in a stratified design to cover major vegetation types over the study area (Dvorský et al. 2011).

Species phylogeny

The phylogenetic relationships between the species studied were constructed based on three molecular markers: *matK*, *rbcL* and *ITS*. Combined, these three loci cover protein-coding, RNA-coding and noncoding sequences, as well as both plastid (*matK*, *rbcL*) and nuclear DNA (*ITS*). Varying mutational rates between loci maximize the ability to discern major lineages together with species-level phylogenies; this threesome of markers was therefore suggested as a standard for phylogenetic analyses and barcoding of angiosperms in the past (Li et al. 2011).

As a starting point, we acquired all relevant sequences from NCBI GenBank (about one third of the species was represented at least by one marker). Secondly, for species not present in GenBank, we performed sequencing on material collected in the field. DNA was isolated from silica-gel dried leaf tissue using a Qiagen DNeasy Plant Mini Kit. The classical chain-termination method (Sanger sequencing) was used, whereas the marker regions were amplified using following primers: *rbcLa-f* + *rbcLa-rev* (Levin et al. 2003), *matK-413f-2* + *matK-1227r-1* + *matK-1227r-3* (Heckenhauer et al. 2016) and *ITS5* + *ITS4* (White et al. 1990). Finally, we searched the NCBI GenBank again, looking for taxa closely

related to species for which molecular data were still missing (e.g. due to the material degradation). Where it was possible to get relevant sequences of an unequivocally sister taxon, these data were used as a stand-in. Otherwise, species groups (sections, genera) containing one or more unknown species were modeled using at least five different sets of intrinsic sequences (these substituted nodes were polytomized further in the analysis).

Data obtained by sequencing were edited and trimmed in Sequencher ver. 5.2.3 prior to merging with GenBank sequences. All three combined matrices (one dataset per locus) were afterwards aligned in MAFFT 6 (Katoh and Toh 2008), using the L-INS-i algorithm. Partial alignments were concatenated, manually adjusted in BioEdit (Hall 1999) and subdued to the automated1 algorithm in trimAll software (Capella-Gutiérrez et al. 2009) to exclude highly divergent and gap-rich regions. The best-fit model for phylogenetic inference was selected according to the Bayesian information criterion (Schwarz 1978) using the Baseml core of Kakusan4 package (Adachi and Hagesawa 1996, Tanabe 2011), resulting in the choice of the GTR model with rate variation across locations simulated by discrete gamma distribution (Γ8), autocorrelated by the AdGamma rates prior, and unlinked for particular gene partitions. This model was afterwards submitted to MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) as the basis for MCMC analysis, encompassing two independent runs with four Metropolis-coupled MCMC chains of 10⁷ generations sampled after every 1000th generation. In each run, one Markov chain was cold and three were incrementally heated by a parameter of 0.3. The first 25% of entries were discarded as burn-in to eliminate trees sampled before reaching apparent stationarity, and the rest was used to compute the majority-rule consensus. The resulting tree contained nodes supported only by substituted sequences and hence had to be edited: internal phylogeny of these groups was collapsed to polytomy and branch lengths were averaged.

Data analysis

To evaluate the effect of species elevation optima, soil moisture indicator values, clonal growth form and height of plants on anatomical traits we used a distance based phylogenetic generalized least squares model (Adams 2014b). This model can deal with a high dimensionality of dependent variables in a phylogenetic context, which is problematic for R-mode alternatives (sensu Legendre and Legendre 1998) facing Rao's paradox (i.e. statistical power decreases with increasing dimensionality of dependent variables; Adams 2014b, Adams and Collyer 2018). Anatomical traits are categorical variables, thus we had to compute a distance matrix from them prior to the analysis. We used distance derived from a simple matching coefficient (Legendre and Legendre 1998):

$$\text{Distance} = 1 - \frac{\text{Number of agreements}}{\text{Number of variables}}$$

We ran the model with phylogeny transformed using Pagel's lambda (Pagel 1999) which scales tree branches leading to the Brownian motion model as the value approaches 1 and star-like phylogeny with a value of 0. We evaluated models with lambda from 0 to 1 by 0.01 steps and selected the model with the highest explained variability by our four predictors. This way, we accounted for an appropriate degree of phylogenetic relatedness and avoid phylogenetic overcorrection.

The model sequentially evaluates all terms, so to estimate the explained variability by each of our explanatory variables, we ran the model repeatedly with permutation of the explanatory variables. We obtained for each explanatory variable *r*-squared and *p*-values after and before accounting for all other variables. All runs of the model were carried out with 999 iterations. All analyses were based on 149 rhizomatous and stoloniferous grass, sedge and rush species, both perennial and annual, for which we gathered complete information on anatomy, phylogeny, soil moisture and elevation optima. The three monocot species having bulbs were omitted from analyses.

Individual models

Since our four explanatory variables (elevation, moisture, growth form and height of plants) had a significant effect on the anatomical traits, we explored those traits separately to get more insight into the relation. We used the same model as in the previous case. Each anatomical trait was evaluated with elevation, moisture, growth form and height of plants and as explanatory variables. The order of variables was again permuted to obtain *r*-squared and *p*-values for each variable after and before accounting for all other variables. The lambda parameter was estimated for each model as per the main model.

Phylogenetic signal

To evaluate the strength of the phylogenetic signal in our anatomical traits we used multivariate generalization of Blomberg's *K* (Blomberg et al. 2003, Adams 2014a). For analyses we used R (<www.r-project.org>), implementation of distance based phylogenetic generalized least squares model in package *geomorph* (Adams et al. 2018) and package *geiger* (Harmon et al. 2008) for tree transformations.

Results

Taxonomic composition

Species of the Poaceae constituted the majority of the studied monocots at lower elevations (Table 1), and their abundance decreased with increasing elevation from 100% below 3000 m to 30% above 5000 m. Cyperaceae showed the opposite elevational pattern, making up 70% above 5000 m. They were most abundant in alpine grasslands (48%) and wetlands

(29%), while Poaceae dominated (86–100%) in all dry habitats (deserts, steppes and screes). Annuals were exclusively in Poaceae (94% of all species) and Juncaceae (6%), short-rhizomatous turf graminoids in Poaceae (78%) and less so in Cyperaceae (20%). Plants with long hypogeogenous rhizomes were rather equally distributed among Poaceae (52%) and Cyperaceae (44%). Most Cyperaceae were short species, being less than 40 cm tall, while Poaceae dominated among taller graminoids. Alpine grasslands were dominated by *Carex* (31%), *Kobresia* (28%) and *Poa* (16%), ruderal vegetation by *Bromus* (67%), steppes by *Elymus* (21%) and *Stipa* (34%), saline wetlands by *Carex* (30%), *Puccinellia* (26%) and *Juncus* (22%) and screes by *Poa* (21%), *Stipa* (21%) and *Elymus* (28%). The proportion of *Carex* and *Kobresia* species increased significantly towards higher elevation belts, while that of *Stipa* and *Elymus* decreased.

Effect of environment and morphological constraints

The main anatomical differences among the studied species were associated with habitat preferences along elevational and aridity gradients from cold deserts to the alpine and subnival zones, but also with variations in plant size and growth form. Ordination of plant anatomical features, using non-metric multidimensional scaling, with projection of predictors, showed that the first (horizontal) ordination axis was associated with the aridity gradient. This separated short-rhizomatous species of low- to mid-elevation deserts and steppes from long-rhizomatous grasses and sedges more typical of saline wetlands and high alpine moist pastures (Fig. 1). The anatomical changes along the second axis seemed to be associated with decreasing plant height as the elevation increased. The low-elevation desert and steppe plants had anatomical structures characterized by large culm height and diameter, a circular culm outline, a full culm center, a thick-walled epidermis, large sclerenchymatic sheaths around vascular bundles, large vessels distributed in the whole stem and thick-walled peripheral sclerenchymatic belts (Fig. 2). The high-elevation alpine plants typically had smaller culm diameters, a triangular cross-section, a hollow culm center, a thin-walled epidermis, small sclerenchymatic sheaths around vascular bundles, only peripheral sclerenchymatic girders, often large intercellular spaces and smaller vessels. The high-elevation alpine plants had high proportion of storage-related tissues (parenchyma, chlorenchyma) and lower proportion of conductive and mechanical tissues (Fig. 3).

Phylogenetic trait–environment analyses

The analysis evaluated the effect of elevation, soil moisture, growth form and height of plants on anatomical traits. The model selected to account for the appropriate degree of phylogenetic relatedness was the one with lambda 0. All explanatory variables had a significant effect (all *p*-values <0.02) on anatomical traits, even after accounting for all others and together they explained 18.65% of the variability (Fig. 4). Individual models revealed that each of the

Table 1. Frequency of species of main plant taxonomic families and growth forms in individual elevation belts in Ladakh, NW Himalayas.

Elevation belts		2650–3000	3000–3500	3500–4000	4000–4500	4500–5000	5000–6000
Plant families	total sp. number	6	31	35	32	40	10
	Poaceae	6	27	26	20	27	3
	Cyperaceae		2	6	10	9	7
	Juncaceae		1	1	1	2	
	Juncaginaceae					1	
	Liliaceae			1	1	1	
	Potamogetonaceae		1				
Growth forms	Zanichelliaceae			1			
	annuals	1	11	4	1		
	with bulbs			1	1	1	
	long-rhizomatous	2	3	6	5	8	2
	short-rhizomatous	3	15	22	21	29	7
	stoloniferous		1	2	4	2	1

explanatory variables had an effect on some of the anatomical traits (Fig. 5). In the anatomical data we found a phylogenetic signal of K_{mult} 0.0888, which was significantly different from 1 (p -value: 0.001 with 1000 permutations). K_{mult} <1 means a phylogenetic signal lower than expected under Brownian motion.

Individual anatomical features and their ecological context

The structure of the conductive system

Elevation accounted for the most variation in vessel size and unlike plant height, retained significance after controlling for other confounding explanatory variables. The harsh environment homogenized the structure of the hydrological conductive system among the studied monocotyledons, with 62% of species having small vessels with a diameter <20 μ m. The fraction of species with small vessels increased with elevation from 16% below 3000 m to 90% above 5000 m. Small vessels were mostly found in small *Kobresia*, *Festuca* and *Poa* species from higher-elevation alpine grasslands, while larger vessels with a diameter of 20–50 μ m were found in taller grasses (*Bromus*, *Elymus*), tall sedges (*Carex stenocarpa*, *C. nivalis*) and other monocots (*Allium* spp.) from drier lower-elevation habitats (Supplementary material Appendix 1 Fig. A2). Vessels made up vascular bundles which are usually positioned in 2–3 peripheral rows in most species (82%), or in one peripheral row (16%), rarely in the whole stem (*Juncus himalensis*). Vascular bundles in one peripheral row were found in *Allium* spp., short-rhizomatous (*Puccinellia*, *Eleocharis*, *Triglochin*, *Carex microglochin*) and annual ruderal and wetland taxa (*Bromus oxyodon*, *Digitaria stewartiana*, *Schismus arabicus*). Vascular bundles were missing in water plants (*Stuckenia amblyophylla*, *Zannichellia palustris*).

In 96% of species, vascular bundles were surrounded with a fibrous lignified sheath. Soil moisture accounted for the most variation in the fibrous sheath structure around vascular bundles and unlike plant height and elevation, retained significance after controlling for other confounding predictors. A sclerenchymatic sheath composed of one-sided small, 1–2 cells prevailed (64%, typical of *Agrostis*, *Bromus*, *Elymus*,

Festuca, *Puccinellia*), followed by a one-sided large 2–4 centripetal cell sheath (19%, *Carex* and *Kobresia*), and one-sided large 2–4 centrifugal cells (8%, *Stipa* and *Juncus*). The large centripetal sheaths are more represented in long-rhizomatous (*Carex*, *Kobresia*) and stoloniferous (e.g. *Juncus membranaceus*, *Juncus himalensis*, *Carex montis-everesti*) species typical of wetter habitats (Fig. 1). The large centrifugal sheaths are typical of most *Stipa* species from dry steppes.

The sclerenchymatic sheath was absent in aquatic plants studied (*Stuckenia amblyophylla*, *Zannichellia palustris*).

Chlorenchymatic assimilatory and storing tissue

The presence of chlorenchymatic assimilatory tissue was a widespread feature. Large tangentially enlarged groups of assimilating chlorenchyma tissues predominated (46%), increasing with elevation from 17% below 3000 m to 90% above 5000 m, and were characteristic of small *Carex* and *Kobresia* species. Assuming that the amount of leaves in relation to culms is smaller in high-alpine graminoids, the assimilating surface can be compensated by a large amount of chlorenchyma in culms of small *Carex* and *Kobresia* species that dominate in high-elevation alpine grasslands above 5000 m. Round oval chlorenchyma groups (27%) occurred mainly in short-lived and perennial short-rhizomatous grasses of dry low-elevation habitats, while continuous chlorenchymatic belts of unligified round cells occurred in 8% of species (*Allium*, *Juncus*, *Triglochin*).

Stem stabilizing elements: culm diameter, construction of stem center, culm epidermis and cavities

Variation in culm diameter was primarily related to plant height and growth form, while the net elevation effect on culm size variation was non-significant. The majority of species had culm diameters under 0.5 mm (46%) or 1–2 mm (46% taxa). The fraction of species with smaller diameters (<0.5 mm) increased with increasing elevation from 16 to 60%, being most represented in short (<20 cm) species with short rhizomes (e.g. *Kobresia pygmaea*, *Carex microglochin*, *Trikeriaia oreophila*, most *Stipa* and *Puccinellia* spp.) while the species with thicker culms of 1–2 mm were taller, often stoloniferous (e.g. *Agrostis stolonifera*, *Catabrosa aquatica*, *Poa*

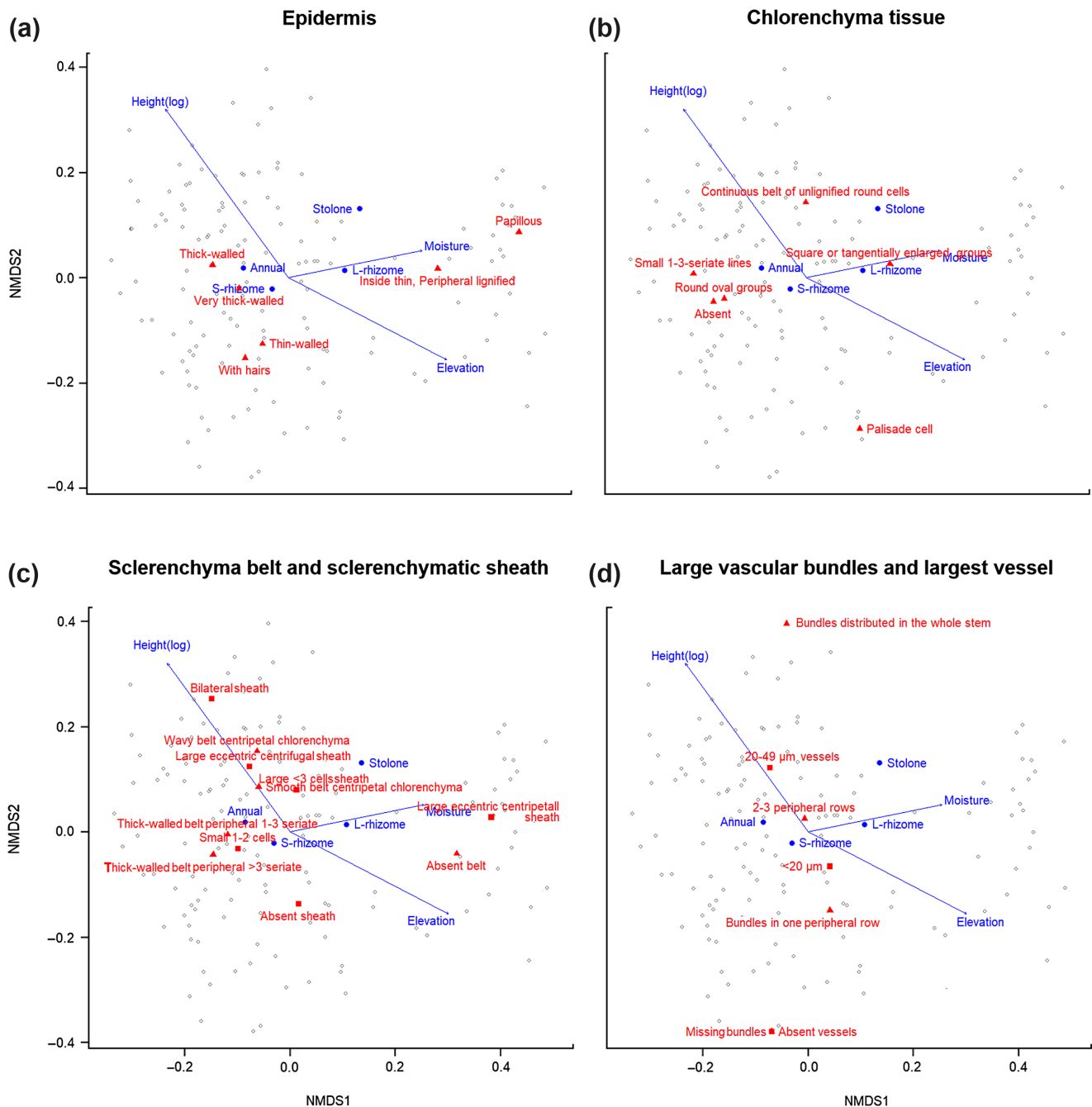


Figure 1. Ordination of main plant anatomical features with projection of predictors for (a) epidermis, (b) chlorenchyma tissue, (c) sclerenchyma belt and sclerenchymatic sheath and (d) large vascular bundles and largest vessel. Non-metric multidimensional scaling (since the lambda parameter of the main model was 0, we have not incorporated phylogeny) of the distance matrix among species was used with passively projected predictors and selected response variable. Stress measure of the scaling with three axes (plotted only first two) was 0.1347.

sikkimensis, *Carex heterostachya*) or long-rhizomatous (e.g. *Alopecurus arundinaceus*, *Blasmus compressus*, *Elymus repens*, *Leymus secalinus*) species occupying productive low-elevation wetlands.

Anatomical changes in culm epidermis were mostly driven by plant soil moisture preferences. Reflecting soil water deficit tolerance, epidermis cells had mostly thick walls (63%), and were mainly in lower-elevation ruderal, scree, steppe and desert species. A very thick-walled epidermis was found in

Bromus, *Colpodium*, *Elymus*, *Piptatherum* and *Leymus* species. Thin epidermis cells (5%, found in water plants) or cells, which were thin inside and lignified on the periphery (25%, typical for *Kobresia* and *Carex*) were found in higher elevation grassland and wetland taxa.

Mechanical support in the monocotyledons was achieved through the formation of a hollow stem and stabilizing sclerenchymatic elements at the periphery of the culms in the form of fairly thick continuous belts or girders composed of

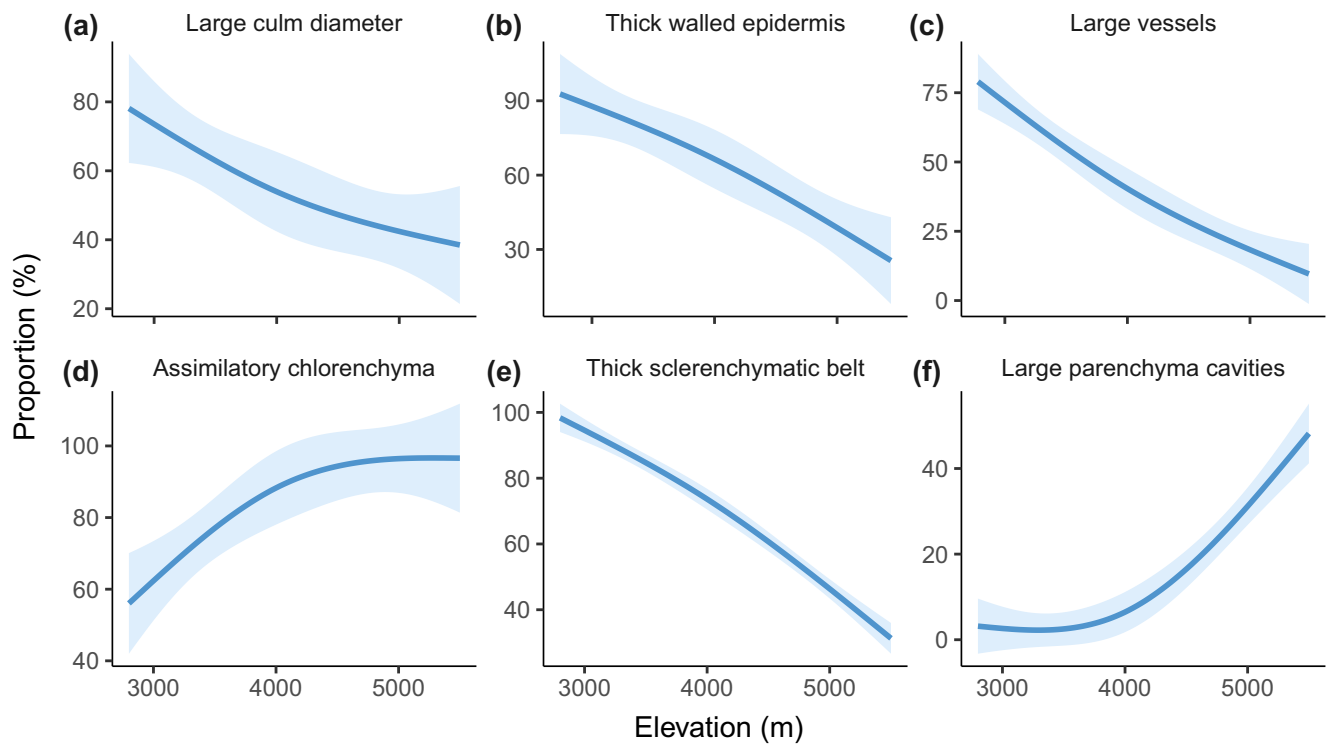


Figure 2. Major elevation-related trends in selected anatomical stem features of monocotyledonous species in Ladakh, NW Himalayas, based on 155 taxa and their elevation optima between 2650 and 6150 m. Depicted is (a) culm diameter, (b) thick-walled epidermis, (c) large vessels, (d) chlorenchyma, (e) thick sclerenchymatic belt, and (f) large parenchyma cavities. The influence of elevation was analysed using the RLM (robust fitting of linear models, thick lines) with 95% confidence intervals. All RLM models were significant at $p < 0.05$.

thick-walled lignified cells (Schweingruber and Berger 2017). A ring of supporting or mechanical tissue was often found just beneath the epidermis, or forming part of the outer or inner cortex (sclerenchymatic girders). Anatomical changes in composition of sclerenchymatic belts showed strong environmental controls that remained significant after accounting for phylogeny and plant size. Thick-walled multiseriate peripheral sclerenchymatic belts prevailed among the studied taxa (52%), especially in tall-turf, short-rhizomatous

and annual grasses of lower-elevation dry habitats (*Agrostis*, *Bromus*, *Stipa*, *Puccinellia*). A wavy peripheral belt of the sclerenchyma located inside of the chlorenchyma was found in 13% of the species (*Juncus* and *Elymus* spp., *Leymus secalinus*, *Phalaris canariensis*), while a smooth peripheral centripetal belt of chlorenchyma was found in 9% (e.g. *Allium* spp., *Triglochin palustris*, *Poa tibetica*). Peripheral sclerenchymatic belts were missing in 26% of the species (all *Carex* and *Kobresia* species, *Blysmus compressus*, *Colpodium* and

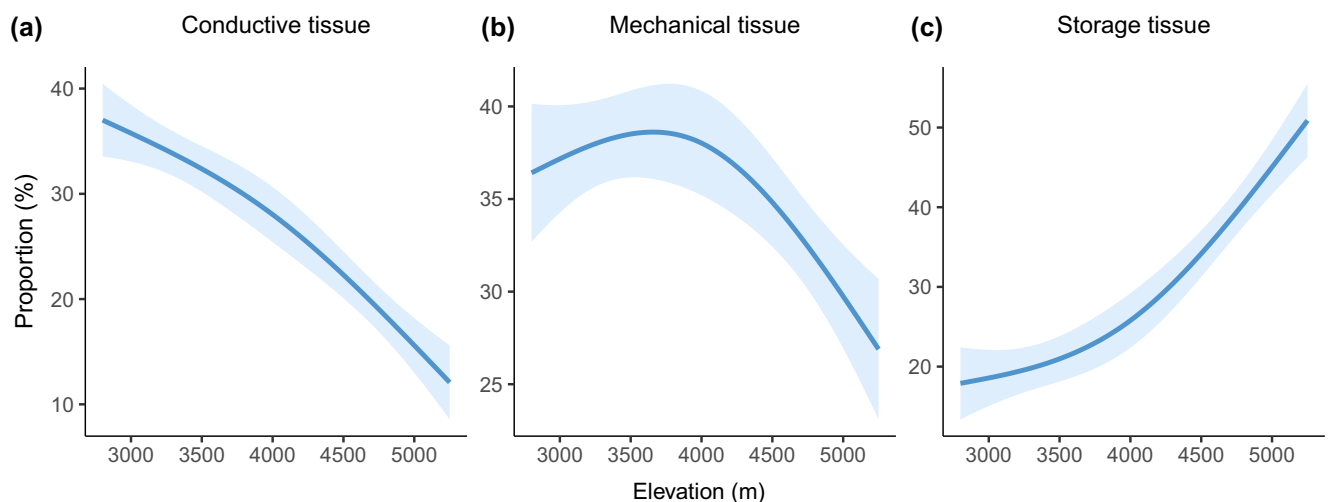


Figure 3. Elevation-related changes in proportion of three basic tissues: (a) water conductive (transport), (b) mechanical (support) and (c) parenchymatic (storage). All RLM models were significant at $p < 0.05$.

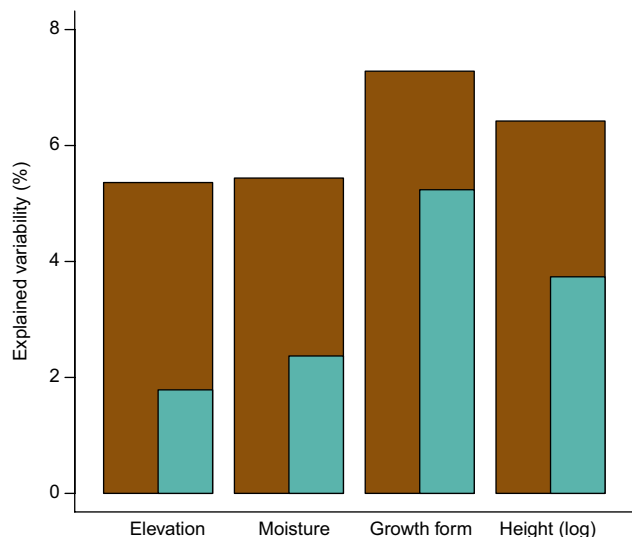


Figure 4. Explained variability of anatomical traits. Brown columns show explained variability by each explanatory variable without accounting for other explanatory variables. Green columns show explained variability after accounting for all other explanatory variables.

Eleocharis spp.). Sedges instead contained peripheral sclerenchymatic girders, found in 48% of the studied taxa. Square or radial rectangular girders (28%) were found almost exclusively in *Kobresia*, *Elymus* and *Poa* species, while conic girders (18%) were found in all *Carex* and *Colpodium* species. Tangentially enlarged girders occurred in *Catabrosa aquatica*, and round girders in *Bromus confinis* and *B. danthoniae*.

Additional mechanical support was achieved through the formation of a hollow stem. Most of the interspecific variation in the culm center construction was explained by growth form and plant height and less by elevation; their net effects, however, remained significant after accounting for all other explanatory variables. A hollow culm surrounded with many large and thin-walled unligified cells was a prevailing feature in 43% of the studied taxa, mainly in tall turf graminoids from steppes and deserts (majority of *Elymus*, *Festuca*, *Piptatherum*, *Poa* spp.). Species with hollow culms surrounded with a few thin-walled (and unligified) cells formed 21%, mostly among the low-elevation saline wetland (*Puccinellia*) and alpine grassland (*Carex* and *Poa*) species at elevation below 4500 m. Species with hollow culms surrounded with a few thin-walled lignified cells formed 19% and included most *Kobresia* and *Bromus* taxa. Full culms containing unligified cells were found in 9% of the studied species, mostly in low-elevation wetland and ruderal species (*Juncus*, *Eleocharis*, *Digitaria* and *Pennisetum* spp., *Chloris virgata* and *Danthonia cachenmyriana*). Full culms containing lignified cells made up 5% of species from lower-elevation steppe and ruderal habitats (*Allium* spp, *Cymbopogon jwarancusa*, *Bothriochloa ischaemum* and some *Stipa* spp.). Culms with net-like aerenchyma were typical for water plants (*Stuckenia amblyophylla*, *Triglochin palustris*, *Zannichellia palustris*).

Additional mechanical support and aeration was achieved through the formation of conspicuous intercellular spaces (cavities) in parenchymatic tissue, present in 66% of the studied taxa, or in the protoxylem of vascular bundles (99% of cases). Small triangular cavities prevailed (39%), typically in tall grasses of dry habitats (*Elymus*, *Bromus*, *Stipa*,

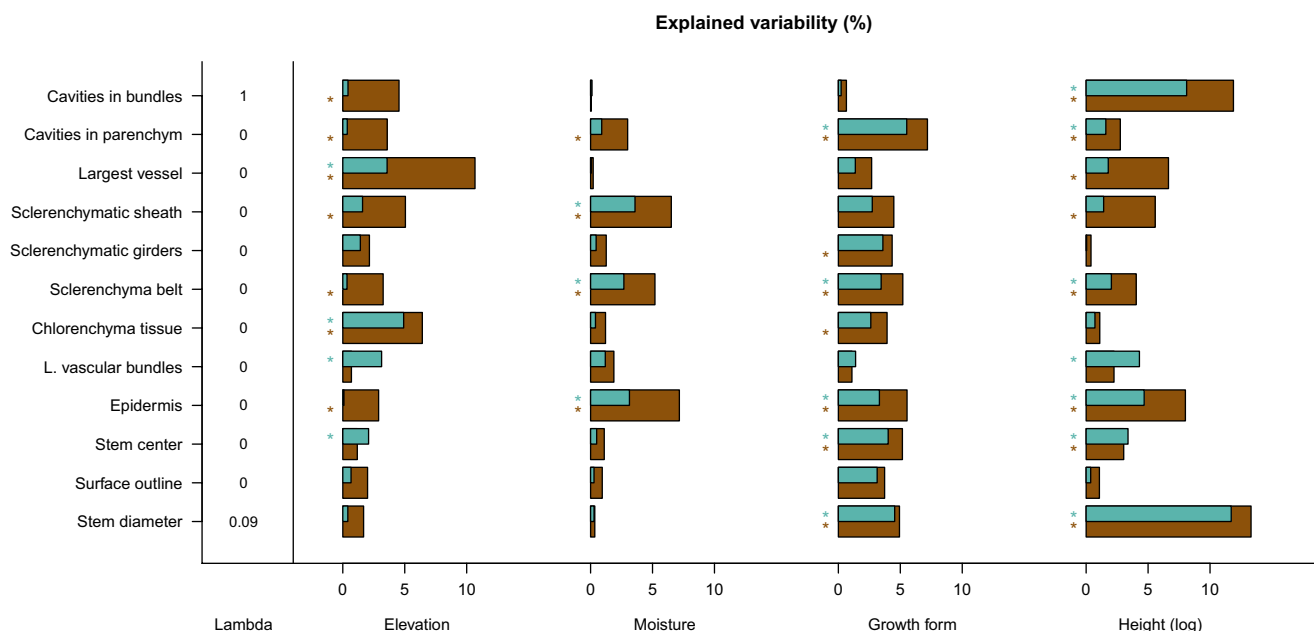


Figure 5. Explained variability and significance of individual models. Brown bars show explained variability by particular variable before accounting for all others. Green bars show explained variability by particular variable after accounting for all other explanatory variables. Corresponding p-values are indicated by '*' in case of p-value lower than 0.05. Lambda of each individual model is written in the left column.

Piptatherum), round oval to radial intercellulars (14%) were present in *Carex* and *Blasmus*, and large irregular cavities in *Kobresia* species occupying regularly waterlogged riverbanks. Intercellulars in the protoxylem of vascular bundles were either smaller canals surrounded by epithelial cells (58%) found in sedges, rushes and tall grasses (*Stipa*, *Bromus*, *Elymus*, *Pennisetum*, *Leymus secalinus*, *Muhlenbergia huegelii*), or large intercellulars around protoxylem in vascular bundles (41%) characteristic of smaller graminoids.

Discussion

In this study, we assessed different stem anatomical features across most monocotyledons growing in Ladakh and representing key components of the western Tibetan and the western arid Himalayan cold desert and alpine steppe habitats. This enabled us to disentangle the direct contributions of different biotic (plant phylogeny, plant height and clonal growth form) and abiotic (moisture and temperature gradients) drivers affecting interspecific variation in stem structural adaptations that may assist plants to thrive under the prevailing cold and dry conditions. The stems of studied species were essentially composed of conductive, stabilizing and storage tissues providing efficient structural support and water transport, with their proportions varying according to plant morphology, elevation, hydrological conditions and taxonomic origin. Due to the stature variation of the plants (10–120 cm), many anatomical features were related to plant height (culm diameter, vascular bundle cavities) or clonal growth form (sclerenchymatic belts), while others were directly related to elevation (vessel size, chlorenchyma bends) or soil moisture (epidermis thickness, presence of stem hollows) gradients.

Being distributed along elevation and soil moisture gradients, the major mountain habitats studied differed in energy and water availability (Miehe et al. 2002). They harbor relatively unrelated species with different evolutionary histories that converged towards similar anatomical and hence morphological structures (Le Bagousse-Pinguet et al. 2018). The environmental gradients had a significant net effect on anatomical divergence and were not confounded by evolutionary inertia, as indicated by the weak phylogenetic signal. The environmental gradients correlated with plant morphology, namely the soil moisture level with the length of rhizomes, and elevation with plant size, and hence both exhibited a direct effect on stem anatomical variation as well as an indirect one mediated by altering plant height and rhizome length.

Decreasing plant size with increasing elevation is a well described phenomenon related to the incapacity of apical meristems to produce new shoot tissue under low temperatures and strong desiccant winds (sink limitation, Körner 2003). The mean growing season temperatures in the alpine and subnival zones of Ladakh ranged from only 2 to 5°C (Dvorský et al. 2015) with fewer than 60 days

of growing season available. This resulted in small plant sizes, often less than few centimeters, such as alpine grassland dominants *Kobresia pygmaea*, *Poa attenuata* and *Carex pseudofoetida*. Such sheltered stature enabled aerodynamical decoupling of plant canopies from the free cold atmosphere, and hence maintained a positive energy balance with higher leaf canopy temperatures than ambient (Wilson et al. 1987).

Elevation related cooling not only affected the plant size, but also exerted strong control over anatomical structures, similarly as was observed in several grass species in the western Himalayas (Ahmad et al. 2016). This was particularly evident in the conductive system through reduced vessel sizes and in the assimilatory and storage tissues with more chlorenchyma and less sclerenchyma around vessels. On the other hand, the soil moisture deficit exhibited significant control over additional stabilization structures (hollow stems with thick epidermis) by promoting short-rhizomatous tall plants with deep adventitious roots in drier soils (Klimešová et al. 2011b). Moreover, our complex analysis controlling for all possible confounding factors revealed that elevation and soil moisture had direct effects on plant anatomy. This was not mediated by reduced plant size or rhizome length, so was presumably through direct low temperature/water scarcity restrictions of cell division and differentiation in developing tissues (Körner 2015).

These restrictions at high elevations selected for a less lignified construction with smaller vessels surrounded only by small sclerenchymatic sheaths but thick chlorenchyma belts, similarly as in alpine dicots (Schweingruber et al. 2014, Doležal et al. 2018). This is possibly an active adjustment to 1) maximize the rate of photosynthesis during short periods of favorable conditions and 2) to enhance carbohydrate storage capacity (Körner 2003). Osmotic adjustment by accumulation of starch, fructans and other important cryoprotective substance in large parenchyma tissues can lower the freezing point (Linder et al. 2017), and thus increase the freezing resistance of tissues (Valluru and van den Ende 2008, Liu and Osborne 2013).

The moisture gradient had a direct effect on stem anatomical variation as well as an indirect one mediated by altering rhizome length; this pattern is rarely reported (Klimešová et al. 2011b) and never in relation to anatomical features. The long-rhizomatous plants preferred wet alpine meadows and valley bottom wetlands, while short-rhizomatous plants dominated in steppes. Frequent disturbances (landslides, solifluction) are the likely detrimental factors behind the absence of plants with long and slender rhizomes on steep mountain slopes, similarly in deserts and steppes with relatively impermeable stony substrates (Dvorský et al. 2011). As they thrive in wet soils with more total biomass allocated in below-ground rhizomes, long-rhizomatous plants exhibited a lower need for mechanical support (absence of hollow stem) as well as lower exposure to high evaporative forcing (thinner epidermis) compared to short-rhizomatous turf graminoids typical of dry habitats.

In conclusion, we assessed different stem anatomical features across most grasses, sedges and rushes growing in western Himalayan cold desert and alpine steppe habitats and disentangled the contributions of phylogeny, elevation and aridity gradients as well as plant morphology to interspecific variation in stem structural adaptations. Environmental gradients in particular had strong control over plant morphology, namely the soil moisture determined the length of rhizomes, while elevation controlled plant height, which was in both cases reflected in different anatomical settings. Phylogeny, on the other hand, has no effect on the studied anatomical features. The observed trends of decreasing vessel size and lignification rates, and increasing shares of parenchyma-like living cells with elevation-related temperature decline in studied Himalayan monocots supports the existing generalization built previously on dicots (Morris et al. 2018). This generalization is that narrower vessels and extensive parenchyma tissues assist plants to grow in colder places by avoiding/tolerating freezing-induced damages. An additional important finding is that the soil moisture gradient exhibited significant control over stabilization structures, including stem center construction and epidermis thickness by promoting long-rhizomatous shorter plants with lower mechanical support in wetter soils and short-rhizomatous tall plants with well-developed stabilizing sclerenchymatic elements in drier soils. However, further comparative studies based on large number of taxa will be necessary to fully understand the evolution of plant anatomical adaptations in major grasslands worldwide.

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Supplementary material (available online as Appendix oik-06451 at <www.oikosjournal.org/appendix/oik-06451>).
Appendix 1.