The relationship between functional trait variation and water availability in hybridising Australian alpine buttercups

Julia M. Hammer[1]

Abstract

Australian alpine buttercup (*Ranunculus*) species are capable of interspecific hybridisation, yet remain as largely distinct taxonomic entities exhibiting characteristic leaf morphology, growth form, reproductive architecture, phenology, and alpine microhabitat preferences. Past research suggests that interspecific hybrids are morphologically intermediate. If hybrids are also ecologically intermediate, and if the bounded hybrid superiority model holds, then perhaps parent taxa are separated and purified by their habitat preferences. I examined six buttercup taxa and measured soil moisture and leaf traits relevant to water use: area, dissection, shape complexity, specific leaf area, stomatal density, and vascular bundle area. I found no relationship between soil moisture and leaf area, specific leaf area, stomatal density, or vascular bundle area. I found a significant, positive relationship between soil moisture and leaf dissection and shape complexity. These results indicate that other environmental factors, for example temperature or soil salinity, are at play. This was a small study with a limited sample, but nevertheless provides a good starting point for further research on trait variation and hybridisation in Australian alpine buttercups.

Key Words

Functional traits, hybrid zone, leaf morphology, leaf physiology, soil moisture, speciation

Introduction

The genus *Ranunculus* contains 600 species worldwide (Tamura 1995, cited in Hörandl *et al.* 2005). Roughly 20 of these are found in Australia and New Zealand, especially in high mountain regions (Lockhart *et al.* 2001). Six species in particular – *Ranunculus dissectifolius* Benth., *R. graniticola* Melville, *R. millanii* F. Muell., *R. muelleri* Benth. var. *muelleri*, *R. muelleri* Benth. var. *brevicaulis*, and *R. niphophilus* Briggs – are common around Charlotte Pass in Kosciuszko National Park (Costin *et al.* 2000). Field observations, artificial pollination experiments and transplant studies show that some of these species are interfertile (hybrids and backcrosses are viable) but remain generally distinct in their leaf morphologies and habitat preferences (Table 1; Briggs 1962; Armstrong 2003).

Barbara Briggs (1962) observed that hybrids are morphological intermediates (Figure 1), and suggested that they are also ecological intermediates. Armstrong (2003) observed that hybrids mainly occur in transition zones between parental habitats, or in recently disturbed areas. These findings conform with the bounded hybrid superiority model (BHSM; Moore 1977), in which hybrids are fitter than parents in certain bounded regions, but less fit towards the edges of, or outside, those regions. In this scenario, *Ranunculus* hybrids may be restricted to the narrow soil-moisture transition zones, or ecotones, between parental habitats, or to areas where competition for resources and microhabitat-specific selective pressures have been relaxed through physical disturbance. Here, I aimed to assess whether the BHSM provided a good interpretation of *Ranunculus* distribution with soil moisture. I proposed that functional traits related to water use could be responsible for the hybrid distributions along a soil moisture gradient. Specifically, if parent species show distinct values for water-use traits, and hybrids show intermediate values, then species and hybrid fitness with soil moisture would align with predictions of the BHSM.

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[1] Corresponding author: u5797927@anu.edu.au, Research School of Biology, ANU, Canberra, ACT, Australia

Table 1: Habitat associations and leaf morphologies of Australian alpine buttercups (*Ranunculus* sp.). Species with matching superscripts are known to form hybrids in nature (Armstrong 2003; Briggs 1962). Scale bar = 1 cm.

Species	Habitat association	Morphology
R. dissectifolius[abc]	Raised bogs with *Sphagnum cristatum* and *Empodisma minus*; generally restricted to valley floors	
R. graniticola[ade]	Tall alpine herb-fields found on well-drained, sloping sites	
R. millanii[bdfg]	Fens – permanently waterlogged, shallow depressions subject to frequent flooding	
R. muelleri var. *muelleri*[cefhi]	Sod tussock grassland, on organic soils dominated by *Poa spp.* and *Empodisma minus*	
R. muelleri var. *brevicaulis*[h]	*Epacris* and *Chionohebe* feldmarks	
R. niphophilus[gi]	Short alpine herb-fields and in springs or running water	

Source: Armstrong (2003) and Costin et al. (2000); Author’s photographs.

Some features of leaf morphology and physiology are functional adaptations to water availability. Givnish (1979) proposed that effective leaf size (the largest circle that can be transcribed within the leaf lamina) follows a hyperbolic curve with soil moisture (Figure 2). An initial increase in moisture allows energy to be directed from water transport to leaf growth, but too much moisture impedes root absorption, and energy must be redirected to root development. Another example is dissection. Dissection is considered an adaptation to (or consequence of) dry conditions because it reduces the distance between photosynthetic material and major veins (reviewed in Nicotra *et al.* 2011). Very high dissection, however, is characteristic of aquatic and frequently inundated plants: smaller, more dissected leaves interfere less with the movement of surrounding fluid, improving aquatic diffusion (Lynn and Waldren 2002).

Figure 1: Leaf morphologies of *R. dissectifolius* (far left), *R. graniticola* (far right), and potential hybrids (centre two). Scale bar = 1 cm.

Kincaid and Schneider (1983) formulated a dissection index (DI; calculated with Equation 1) that compares leaf shape to circles. Leaves with dissection indices greater than 1.0 are more complex than circles with the same area. Another measure of leaf shape is the normalised difference of margin complexity (NDMC), put forward by Leigh *et al.* (2017). NDMC is the difference between leaf perimeter and convex hull (the smallest convex shape that contains the leaf area), normalised by their sum (Equation 2). Leaves with NDMC approaching 1.0 are more dissected (Figure 3). Together, we might expect species adapted to dry or waterlogged soil to have small leaf area, and high DI and NDMC.

$$NDMC = \frac{perimeter - convex\ hull}{perimeter + convex\ hull} \qquad (1)$$

$$DI = \frac{perimeter}{2\sqrt{area \times \pi}} \qquad (2)$$

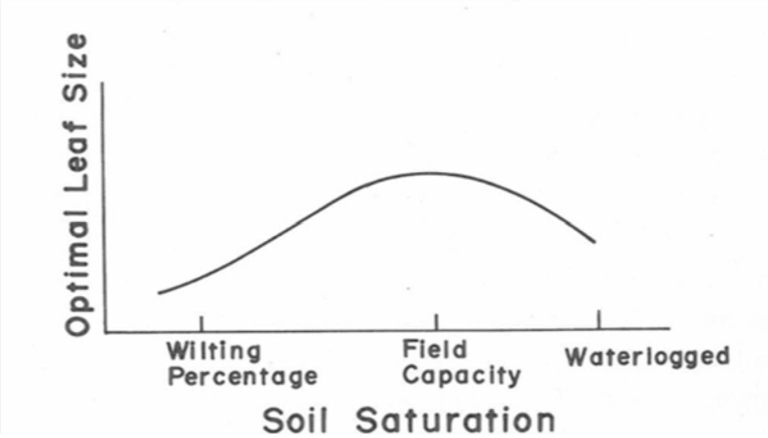

Figure 2: Estimated leaf size with increasing soil moisture. Source: Givnish (1979).

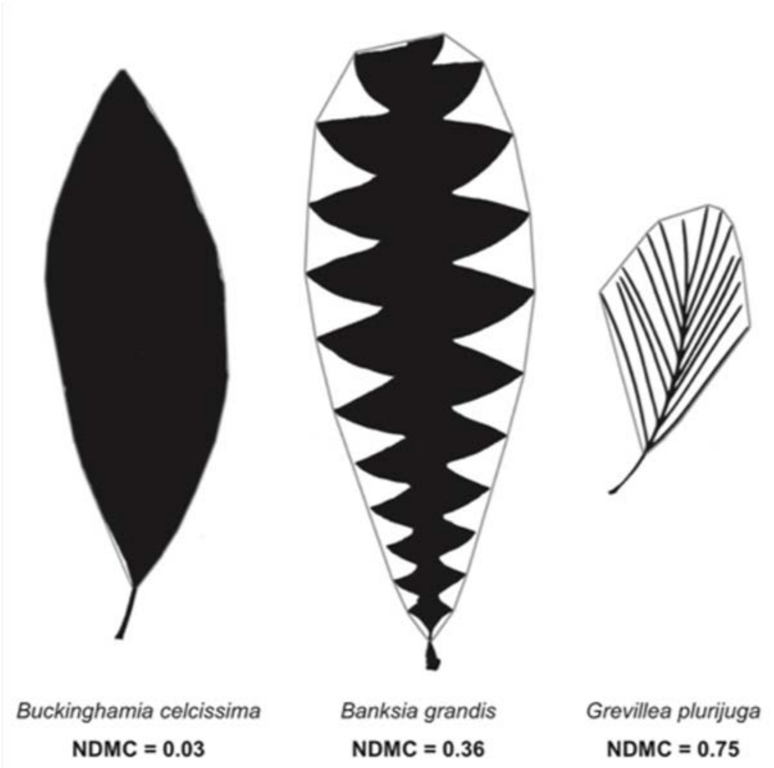

Figure 3: Leaf dissection and complexity increases from left to right, and NDMC approaches 1.0. Source: Leigh et al. (2017).

Leaf physiology can also be adapted to water availability. Plants in arid regions exhibit xeromorphic characteristics, which include small intercellular air spaces, large proportions of mechanical tissue and sunken stomata (Kirkham 2014). Specific leaf area (SLA) is the ratio of leaf area to dry mass. SLA is an estimate of investment in structural tissue over photosynthetic tissue: plants with high SLA have thin, weak leaves, and plants with low SLA have thick, dense leaves (Cornelissen *et al.* 2003). Wright et al. (2004) found that plants with low SLA exhibit low rates of photosynthesis, and inferred that thick leaves have longer diffusion paths and internal chloroplast shading. This suggests that low SLA plants use less water. Indeed, Wright *et al.* (2004) also found that SLA increases with rainfall, and decreases with vapour pressure deficit. We might expect species adapted to dry soil to have low SLA and slow photosynthesis, and species adapted to wet soil to show the opposite.

Water transport systems control water conductivity from roots to shoots. These systems include stomata and xylem vessels. Stomatal density, size and regulation determine water loss via transpiration. According to Brown and Escombe's (1900) 'diameter law', more water vapour diffuses through many small stomatal openings compared to a single large pore of the same area (unless the small openings are very close together). Since water loss is costlier for plants in dry conditions, we might expect species adapted to dry soil to have lower stomatal densities than those adapted to moist soil (Kirkham 2014). However, stomatal density is often plastic. For example, Xu and Zhou (2008) found that stomatal density in perennial grass *Leymus chinensis* increased with moderate drought, but decreased as drought became more severe. This suggests that stomatal density measured at one point in time may only reflect water availability shortly before that time. Nevertheless, large differences in stomatal density may indicate a difference in soil moisture preference between species.

Xylem vessels are similarly sensitive to water availability. According to the Poisuelle Hagen Law, the volume of fluid moving per unit time through a cylinder is proportional to the fourth power of its radius, and to the difference in pressure between the ends of the cylinder (Kirkham 2014). In other words: wider xylem vessels conduct more water. However, Hargrave *et al.* (1994) found that wider vessels increase the likelihood of drought-induced embolism. We might expect plants adapted to dry soil to have smaller diameter xylem vessels compared to plants adapted to wet soil, to avoid cavitation. Indeed, Lovisolo and Schubert (1998) showed that water-stressed grape vines had smaller xylem cross-sectional areas compared to well-watered vines. They also showed that vessel area changed as vines grew, so xylem vessels, like stomata, may be plastic.

I hypothesised that those *Ranunculus* species capable of hybridisation (genetically and spatially) would exist on a micro-environmental cline following a soil moisture gradient, where species are separated by hybrid zones. I further hypothesised that this cline would correlate with leaf area and shape, SLA, stomatal density, and xylem area, because those traits could restrict species and hybrids to certain soil moistures. Support for these hypotheses would substantiate the presence of a Bounded Hybrid Superiority Model (BHSM). Specifically, I predict that as soil moisture increases, leaf area will increase until soils reach field capacity, then decrease as the soil becomes waterlogged, and that DI and NDMC will produce the opposite pattern. I also predict that as soil moisture increases, SLA, stomatal density and the ratio of vascular bundle cross-sectional area to petiole cross-sectional area will also increase.

Methods

Sampling design

Sampling was carried out in Kosciuszko National Park, New South Wales, Australia, in early December (Summer) 2016. I took most samples from three sites near the Kosciuszko Road, between Charlotte Pass and Betts Creek (SC1, SC2, SC3; Figure 4). Additional samples came from the feldmark near Carruthers Peak (FM), and along the Main Range track from Charlotte Pass to Carruthers Peak via Blue Lake (MR; Figure 4). See Table 2 for which species were found in which sites. For SC sites, I ran two transects (70–130 m in length) perpendicular to the creek and up a slope to capture a soil moisture gradient and a good range of species. I expected soil moisture to decrease with distance from water. Whole plants were collected every 5 m (or more, if samples were sparse) along each transect. Samples from the feldmark and Main Range were collected opportunistically. Each sample was placed in a ziplock bag for transport back to the laboratory at the Southern Alps Ski Lodge, Charlotte Pass. Samples and hybrids were identified by their leaves using visual aids from Armstrong (2003), Briggs (1962), and personal communication with Armstrong. A total of 90 samples were collected.

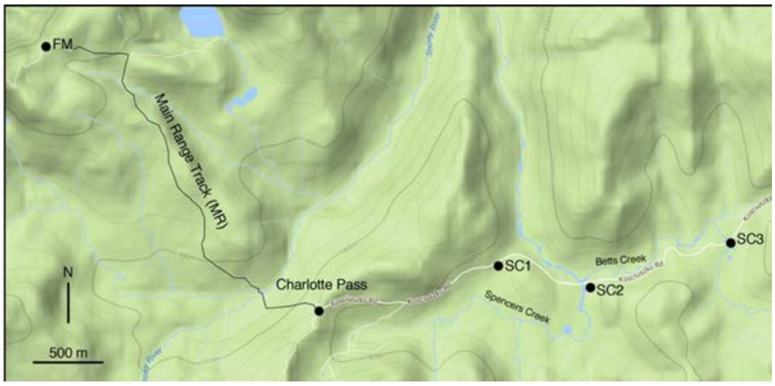

Figure 4: Map of Charlotte Pass, Kosciuszko National Park, containing sites SC1 (36.427508°S, 148.349709°E), SC2 (36.429546°S, 148.360515°E), SC3 (36.425324°S, 148.376982°E), FM (36.40680°S, 148.29660°E), and the Main Range Track (MR). Source: Taken with Google Maps.

I measured soil moisture using a Delta-T HH2 with ML2x ThetaProbe at least three times around each sample and averaged the values. Notes were taken on surrounding vegetation and topography. Soil moisture was not recorded for the feldmark (soil was too rocky for the probe) or where plants were inundated (100 per cent). The soil moisture measures reflect only early spring conditions. Note that wind exposure and substrate are likely to impact the traits and distribution of species but were beyond the scope of this project.

Leaf morphology

I chose one young, fully expanded, undamaged leaf from each sample and scanned it without its petiole on a flatbed scanner. Leaves were allowed to curl while scanned to capture convex hull. Leaves were then flattened, and lobes and teeth separated, to capture area and perimeter. These measurements were taken by ImageJ software version 11.2.1 and used to calculate DI and NDMC.

Leaf and petiole physiology

Leaves were oven-dried at 30°C for at least 48 hours then weighed. Area and dry weight were used to calculate SLA. Between one and seven samples were chosen per site for vascular bundle measurements (SC1 was excluded due to logistical constraints). These included representatives of each of the morphologies and soil moisture conditions observed. I hand sectioned one petiole per sample and stained it with 0.1 per cent toluidine blue (see Peterson *et al.* 2008). Sections were viewed under a compound microscope (Olympus BH2) at 100x magnification and photographed. One section per petiole was used to measure the proportion of vascular bundle area to total section area (VB ratio: a proxy for xylem area; see Discussion)) via ImageJ. At least one leaf (young, fully expanded, undamaged) from each sample was preserved in 70 per cent ethanol for transport back to the laboratory at The Australian National University, Canberra for stomatal impressions. Preserved leaves were dabbed dry, then spotted with superglue (approximately 5 mm × 15 mm) on either the abaxial or adaxial surface. Superglued leaves were pressed onto a glass slide with a weight for seven minutes, then peeled off with forceps. Impressions were viewed under a compound microscope at 200x magnification and photographed. Stomatal density was measured by counting the pores inside, or partially inside, a grid (etched on the eyepiece), then dividing that number by the grid area.

Statistical analyses

Data from all sites were combined, and analyses were performed with JMP statistical software version 13.1 (SAS Institute) and Microsoft Excel (version 15.19.1). I first tested for differences in soil moisture preference between species with an analysis of variance (ANOVA). I then performed simple linear regressions between soil moisture and distance from open water (to visualise species and hybrid distribution with soil moisture), and between soil moisture and each functional trait (area, DI, NDMC, SLA, stomatal density and VB ratio). I did not perform analyses of variance for functional traits between species because I was sometimes unable to distinguish species from hybrids.

Results

Soil moisture preference and the BHSM

Soil moisture preference was significantly different between species (n = 56, $R^2 = 0.573$, $p < 0.0001$). *Ranunculus millanii* preferred the highest soil moisture (n = 6, $\bar{x}$ = 96.4%, SE = 7.9), followed by *R. niphophilus* (n = 5, $\bar{x}$ = 86.4%, SE = 8.7), *R. dissectifolius* (n = 26, $\bar{x}$ = 72.3%, SE = 3.8), *R. muelleri* var. *muelleri* (n = 8, $\bar{x}$ = 45.1%, SE = 6.9), and *R. graniticola* (n = 11, $\bar{x}$ = 30.6%, SE = 5.8; Figure 5). Soil moisture significantly decreased with distance from water (n = 76, $R^2 = 0.425$, $p < 0.05$), and species distribution with distance was consistent with soil moisture preference (Figure 6). *Ranunculus millanii* was always either submerged or waterlogged directly at the water source, whereas *R. graniticola* was always found furthest from the water, on well-drained soil. *Ranunculus dissectifolius* was found 5–49 m from the water, sometimes on beds of *Sphagnum*; this species appeared to hybridise often with *R. graniticola*. *Ranunculus muelleri* var. *muelleri* was found sporadically, usually in dry soil, but frequently hybridised with the more dissected species. *Ranunculus muelleri* var. *brevicaulis* was only found on the feldmark, and *R. niphophilus* was only found at high elevations with running water. Hybrids were found throughout the transect – that is, they did not show preference for certain zones.

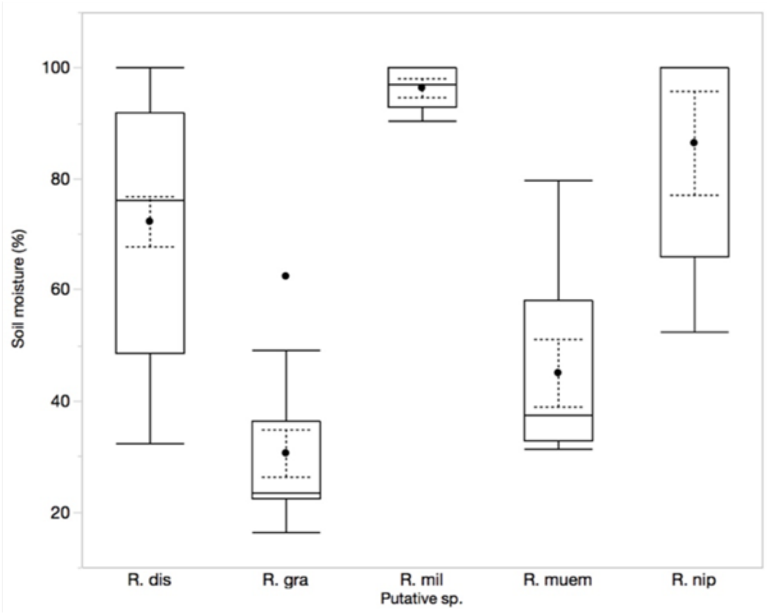

Figure 5: Box plots of soil moisture preferences for *R. dissectifolius* (R. dis), *R. graniticola* (R. gra), *R. millanii* (R. mil), *R. muelleri* var. *muelleri* (R. meum), and *R. niphophilus* (R. nip). We did not measure soil moisture for *R. muelleri* var. *brevicaulis*. Dashed error bars indicate standard error. Circles (●) indicate means. Dots outside the boxes indicate outliers.

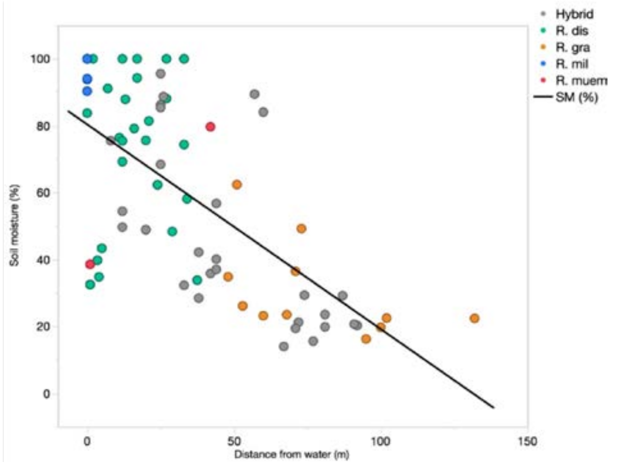

Figure 6: Regression for soil moisture and distance from water. See Figure 5 for species abbreviations.

Leaf area, shape and dissection

There was no relationship between soil moisture and leaf area (n = 86, $R^2 = 0.028$, $p > 0.99$): all species and hybrids shared the same range in leaf area (Figure 7a). DI significantly increased with soil moisture (n = 71, $R^2 = 0.342$, $p < 0.0001$): in general, *R. dissectifolius and R. millanii* showed higher DI than *R. graniticola* and *R. muelleri* var. *muelleri* (Figure 7b). NDMC also significantly increased with soil moisture, though the relationship was much weaker (n = 70, $R^2 = 0.074$, $p < 0.05$). Interestingly, *R. dissectifolius* showed the highest NDMC, *R. graniticola* and *R. millanii* showed similar, intermediate NDMC, and *R. muelleri* var. *muelleri* showed the lowest NDMC (Figure 7c).

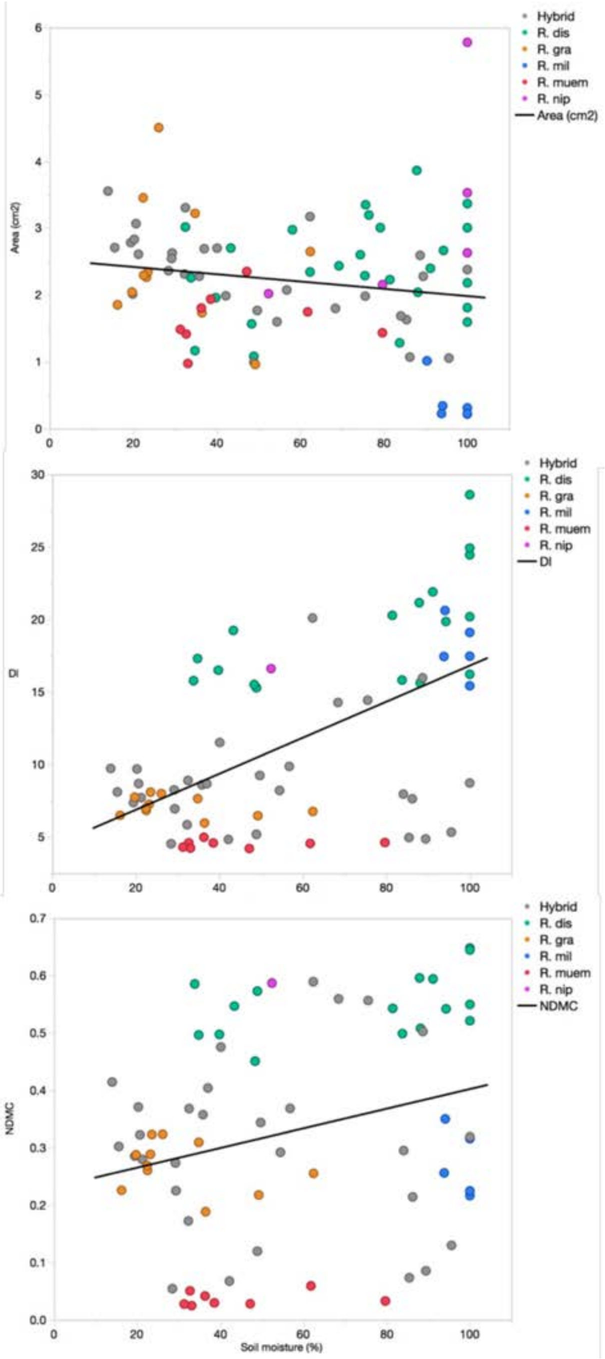

Figure 7: Regressions for a) soil moisture and leaf area, b) dissection index (DI), and c) normalised difference of margin complexity (NDMC). See Figure 5 for species abbreviations.

Specific leaf area, stomatal density and VB ratio

There was no relationship between soil moisture and SLA (n = 82, $R^2 = 0.005$, $p > 0.5$; Figure 8a), abaxial (n = 57, $R^2 = 0.031$, $p > 0.18$; Figure 8b) or adaxial stomatal densities (n = 57, $R^2 = 0.005$, $p > 0.58$; Figure 8c), or VB ratio (n = 14, $R^2 = 0.0005$, $p > 0.94$; Figure 8d). In general, *R. niphophilus* showed the highest SLA, followed by *R. graniticola* and *R. millanii*, *R. dissectifolius*, and finally *R. muelleri* var. *muelleri* (Figure 8a). All species shared the same range of stomatal densities for both abaxial and adaxial surfaces (Figure 8b and 8c). *Ranunculus dissectifolius*, *R. graniticola* and *R. niphophilus* all shared similarly high VB ratios, whereas *R. muelleri* var. *muelleri* and *R. millanii* showed low VB ratios (Figure 8d).

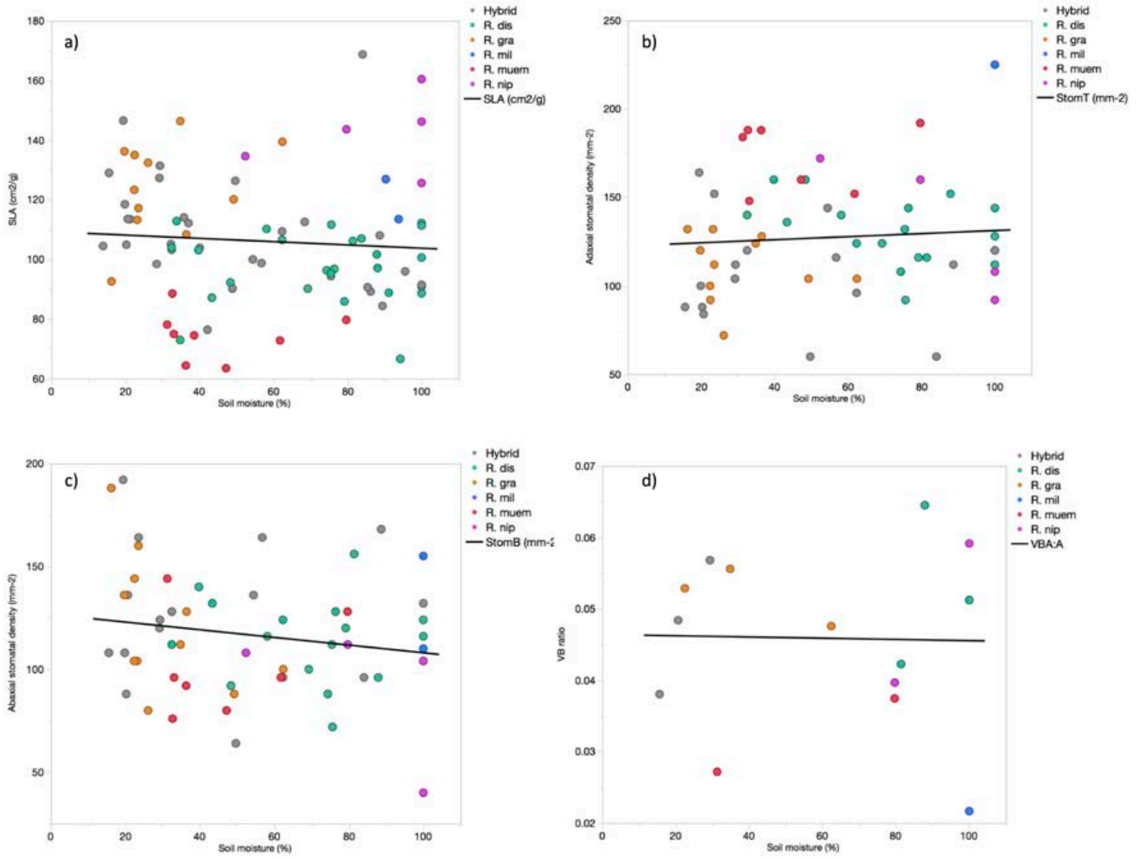

Figure 8: Regressions for a) soil moisture and specific leaf area (SLA), b) adaxial stomatal density, c) abaxial stomatal density, and d) vascular bundle area to total petiole cross-sectional area (VB ratio). See Figure 5 for species abbreviations.

Discussion

The current study aimed to investigate the distribution of *Ranunculus* species and hybrids with respect to soil moisture, functional traits and the BHSM. I hypothesised that species capable of hybridisation would exist on a soil moisture cline, where parent species are separated by hybrid zones. I further hypothesised that soil moisture would correlate with leaf area, DI, NDMC, SLA, stomatal density and VB ratio. I expected species to show distinct ranges for each trait, and hybrids to show intermediate values. My hypotheses were mostly not supported, which suggests other environmental factors at play, or the need for improved methods.

Soil moisture preference and the BHSM

The results confirm that *Ranunculus* species have distinct soil moisture preferences. These preferences were consistent with the habitat associations observed by Armstrong (2003) and Costin *et al.* (2000). I also found that soil moisture decreases with distance from a water source, and that *Ranunculus* species are spatially distributed along this gradient according to their soil moisture preferences. These findings conform to the BHSM. However, the BHSM also predicts that hybrids are restricted to narrow transition zones between parent habitats, and this was not supported by observations: hybrids were found within species ranges. Notably, distinguishing hybrids is not straightforward, so it is possible that I mistook some species for hybrids and vice versa. In future, I recommend using an identification guide that includes floral and seed traits, as well as leaf traits. Alternatively, known hybrids could be bred in the greenhouse and grown under different soil moisture regimes to establish proper preference ranges.

Leaf morphology

Leaf area was not related to soil moisture, and species and hybrids showed similar ranges in leaf area. In contrast, both DI and NDMC increased with soil moisture, and species differences were clear. This implies that leaf shape, rather than area, is adaptive to soil moisture in *Ranunculus*. Givnish (1979) proposed a direct relationship between water availability and growth to leaf area. This may be true within species, but adaptations in different species, like dissection, can maintain leaf area at different soil moistures: effective leaf width may be a more informative metric. My prediction that DI and NDMC would decrease with soil moisture until field capacity is reached, then increase as soils become waterlogged, was not supported. It is possible that field capacity was actually near the lowest soil moisture measurements I made (14 per cent), and that dissection was increasing towards inundation. This explanation is consistent with findings from Lynn and Waldren (2001), and Kołodziejek and Michlewska (2015): leaves from *Ranunculus repens* and *R. acris* demonstrated smaller, but more dissected forms in wet conditions. These authors suggest that wet populations simply speed up leaf development to achieve high dissection before inundation sets in. It is also possible that leaf shape is regulated by thermal regime, rather than soil moisture. Leaf lobing is known to increase airflow across the lamina, encouraging convective heat loss; though, unlobed leaves can also lose heat via transpiration if enough water is available (Nicotra *et al.* 2011). I suggest that future research include air or leaf temperature measurements to clarify this issue. In any case, DI and NDMC are useful features for distinguishing *Ranunculus* species.

Leaf and petiole physiology

There was no correlation between soil moisture and SLA, VB ratio, or abaxial or adaxial stomatal densities. It is possible that variation in soil chemistry, in combination with water availability, determined SLA. For example, salinity decreases the osmotic potential of soil water, making it more difficult for plants to pull water upward. As a result, turgor pressure in leaf cells, and therefore cell expansion to leaf area, decreases; reduced leaf area with no change in thickness equates to lower SLAs. Indeed, Curtis and Läuchli (1987) found no relation between salinity and thickness, but did observe a decrease in leaf area. Species differences in SLA follow soil moisture preferences for *Ranunculus niphophilus* (high soil moisture preference and high SLA) and *R. muelleri* var. *muelleri* (low soil moisture preference and lowest SLA), but ranges overlapped for *R. graniticola*, *R. millanii* and *R. dissectifolius*. I recommend including soil chemistry measurements in future research. My results for VB ratio may be confounded by xylem vessel quantity. Existing literature describes a positive relationship between soil moisture and xylem cross-sectional area (e.g. Lovisolo and Schubert 1998). But it is possible that dry soil species use many thin xylem vessels to conduct the same water volume as wet soil species with few wide xylem vessels (see Bacelar *et al.* 2007. Since both species would show the same total VB area, and since the resolution of my petiole sections only allowed the measurement of total VB area, my data may obscure patterns and thus not demonstrate any difference with soil moisture. Future research would benefit from improved techniques: perhaps microtome sectioning would produce clearer sections. Results for stomatal densities did not support my hypothesis: there was no relation between stomatal density and soil moisture. This may be because stomatal density is also correlated with net CO_2 assimilation rate and water use efficiency (Xu and Zhou 2008), which can vary by CO_2 level and humidity. Stomatal density also varies by leaf age, with younger leaves showing higher density than older leaves (Meidner and Mansfield 1968). It may benefit future studies to measure stomatal density as leaves develop, or to examine other stomatal features, such as size and guard cell length.

Limitations

Logistical constraints may explain some unexpected results. The sampling design (transects from a water source up a slope) seemed appropriate and captured a good range of *Ranunculus* species and transient soil moistures, but could not account for temporal – e.g. within and between season – or small-scale spatial variation in soil moisture. Sampling was performed shortly after an unusually late snowmelt, which left puddles in what may have otherwise been well-drained soil. Future studies would benefit from sampling throughout the growing season, and at more than three sites. Leaf area, DI and

NDMC analysis could benefit from different imaging software. For this study, clear adhesive tape was necessary to flatten highly dissected or undulate leaves, introducing texture that confused ImageJ. One suggestion is the Delta-T WinDIAS system, which uses colour to define leaf margins and area. Finally, *Ranunculus* habitats cannot be reduced to differences in soil moisture: other factors including wind exposure and substrate composition could influence species and hybrid distributions. Future research should aspire to compare functional traits along a variety of environmental gradients.

Avenues for further research

I found many points worth expanding in the future. For example, *R. dissectifolius* was sometimes found growing on *Sphagnum*. It would be interesting to explore whether interactions between *Ranunculus* species and associated flora, like *Sphagnum*, affect water-use traits or distribution. It is also worth comparing parent and hybrid genomes with DNA sequencing. Armstrong (2003) found that independently segregating, unlinked genes with additive effects are responsible for variation in *Ranunculus* leaf morphology. If this is true for other traits, then perhaps these species are purely defined by disruptive selection. Another functional trait for investigation is leaf pubescence: Kołodziejek and Michlewska (2015) found that *R. acris* in dry conditions showed higher leaf hairiness than in wet conditions.

Conclusions

Australian alpine buttercups are reportedly distinguished by their soil moisture preferences and leaf morphologies (Briggs 1962; Armstrong 2003). Species capable of hybridisation (genetically and spatially) produce morphological, and possibly ecological, intermediates. I hypothesised that such species would hybridise along a soil moisture cline, whereby parents are separated by narrow hybrid zones. I also hypothesised that traits for water use would change predictably and gradually with the cline. It was difficult to identify hybrids in the field, so the presence of defined hybrid zones is a topic for future research. My hypotheses were mostly unsupported: likely a result of the combination of poor resolution methods, and confounding abiotic effects. In summary, I am not yet confident whether *Ranunculus* spp. are distributed according to the BHSM, nor am I confident that hybrids are ecologically intermediate in terms of water use, or that leaf anatomy is functionally divergent in this system; nevertheless, I hope that the present study sheds light on the complexities of species distinctions and the sensitivity of certain leaf traits to external conditions.

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