

Geometric morphometrics compared to traditional leaf measurements of *Rubus ulmifolius* (Subgenus *Rubus*) in three different environments in Ireland

Abstract

Plant phenotypic plasticity and genetic recombination during clonal and sexual reproduction can make some plant species identification difficult. Although DNA sequencing has revolutionised species identification, polyploidy, hybridisation and apomixis continue to pose challenges. Subgenus *Rubus* (brambles, blackberries) is one of the most taxonomically challenging groups of plants, and their morphology-based classification which is known not to be consistent with their phylogeny. The definition of bramble species is controversial and is often reliant on leaf and leaflet characters. Here, we compared traditional morphometric methods with geometric morphometrics to look at bramble leaf size and shape change and the role of allometry.

A total of 60 leaves from 30 individual canes were imaged from different environments (woodland, limestone pavement, hedge) in Ireland. We analysed the shape of five-foliolate leaves using landmark-based image analysis. Using Canonical Variate Analysis (CVA) on asymmetrical and symmetrical components, the leaf shapes clustered according to the different environments and were statistically different according to permutation tests ($P < 0.003$). Discrimination Analysis (DA) cross-validated these results ($P < 0.002$). Procrustes ANOVA showed that size, shape and fluctuating asymmetry differed significantly between the three environments ($P < 0.0001$). Allometry explained Principal Component Analysis (PCA) was performed on traditional leaf measurements and failed to separate leaves from the hedge and the limestone pavement suggesting that geometric morphometrics captures more information about leaf shape change. We suggest a statistically robust approach to use geometric morphometrics to understand the variability of leaflet shape which could affect the morphology-based classification of *Rubus*.

Introduction

The survival of plants, unable to migrate to a more favourable environment within a generation, depends on the ability to respond to local conditions and changes. A fine-tuned phenotype to the environment maximises the individual's fitness. Plasticity can be used as an indicator of population fitness, stress, and genetic stability as it acts as a buffer against stress (Bradshaw, 1965; Sultan, 2004; Liao et al., 2016). Phenotypic plasticity most commonly leads to variation in size. For plants, traditional measurements have focused on leaf, stem, flower, root etc. lengths and widths. However, from an evolutionary point of view, changes in shape - rather than in size - can provide more insight regarding local phenotype adaptation. The shape of an object is termed as all of its geometric properties except its size, orientation and position (Kendall, 1977; Klingenberg, 2010; Klingenberg, 2016). It is debated that by using traditional measurements for multivariate statistics, the size of different plant parts is investigated rather than changes in its shape. There can be a high covariation of various measurements of a single organ (e.g. leaf) due to developmental processes (modularity; e.g. one gene is responsible for width and length of a leaf) which is a confounding effect in morphological studies (Klingenberg, 2010; Viscosi and Cardini, 2011).

Geometric morphometrics (GM) provides a mathematical solution to extract only shape information of an object by using well-defined, anatomical landmarks on digital images (Bookstein, 1996; Adams et al., 2013). It has been used for flowers (Shipunov and Bateman, 2005; Savriana et al., 2012; van der Niet et al., 2010; Tucić et al., 2018; Strelin et al., 2018), seeds (Terral et al., 2004; Terral et al., 2012) but mostly on leaves (Jensen et al., 2002; Viscosi et al., 2009a; Viscosi et al., 2009b;

Klingenberg et al., 2012; Silva et al., 2012; Vieira et al., 2014; Viscosi, 2015; Chitwood and Otoni, 2016; Klein et al., 2017; Chitwood and Otoni, 2017). The shape information extraction can be carried out by Procrustes superimposition, which calculates the centroid size of multiple landmarks, scales each object to the same size, positions each to the same centroid centre, and rotates each landmark to an optimal fit (Klingenberg, 2010). The effect of size on the shape (allometry) can also be investigated by using GM (Klingenberg, 2011). There are two main aspects of the relationship between shape and size. The Huxley-Jolicoeur school regards the covariation between traits as a consequence of size variation, while the Gould-Mosimann school regards allometry as a covariation of size and shape (Klingenberg, 2016). We follow the Huxley-Jolicoeur approach, which can be calculated as the regression of shape on size. Allometry can be a major constraint on development and phenotype evolution (Enquist, 2002; Enquist 2003; Weiner, 2004). When a plant is exposed to long-term, extreme or multiple constraints during development, it can result in developmental instability, which has been quantified as fluctuating asymmetry (FA) (Rasmuson, 2002; Nikiforou and Manetas, 2017). FA considers the random small deviations of bilateral characters, which depend on the same gene (Kozlov and Zvereva, 2015) and can be analysed by GM (Klingenberg et al., 2002; Klingenberg, 2011; Martinez et al., 2018).

Polyploidy, hybridisation and asexual reproduction pose challenges to plant species-level identification (Hörandl and Paun, 2007; Soltis et al., 2007; Krahulec and Vašut, 2017). Brambles (subgenus *Rubus*, section *Eubatus*) are distributed worldwide, and numerous species are notorious weeds but also important pollen providers (Whitney, 1984; Nybom, 1985; Brown and McNeil, 2009). They are one of the most taxonomically challenging groups of dicots (Watson, 1958; Edees et al., 1988; Weber, 1996; Holub, 1997; Sochor et al., 2015) and their morphology-based classification does not reflect their molecular phylogeny (Kraft and Nybom, 1995; Nybom, 1998; Alice et al., 2001). The complications of their classification is mainly caused by (i) variable reproduction strategies (sexual, asexual, rapid hybridization) (Kraft and Nybom, 1995; Šarhanová et al., 2012), (ii) nature of polyploidy (diploid to octoploids, Sochor et al., 2015), and (iii) geographic parthenogenesis (Hörandl et al., 2008; Krutto et al., 2010; Haveman et al., 2013a and 2016). In Europe, the number of species based on their latest classification system (Edees et al., 1988) is estimated to be from 200 up to 750 (Krutto et al., 2010; Sochor et al., 2017). In their morphology-based classification system, the species are grouped into sections, subsections, and series. Their identification focuses on characters of their leaves, prickles, flowers, and root systems.

The majority of *Rubus* are tetraploid (Šarhanová et al., 2017) and only four are sexual diploid (*R. ulmifolius*, *R. canescens*, *R. incandescens* and *R. sanctus*; Kurtto et al., 2010) in Europe. Their rapid hybridisation leads to several genotypes within a single patch. A few ecological observations were documented for bramble growth habitat (Beijernick, 1953; Heslop-Harrison, 1959; Taylor, 1980; Walter et al., 2016). The diploid *R. ulmifolius* responded to different light conditions, fertiliser application, and cutting management inside and outside a woodland (Bazely et al., 1991). Unreliable species identification can seriously confound ecological studies, habitat management, and conservation efforts (Hufford and Mazer, 2003; Ouborg et al., 2006; Viscosi and Cardini, 2011). The first step to establish a more reliable classification system is to quantify the morphological variation of a single species with a reliable method.

There is great variability of leaf sizes, even along one cane, which is likely to distort the variance of traditional measurements; therefore, GM is a more appropriate approach to study leaf shape change of brambles in different environments. The asymmetrical nature of blackberry veins, serration and other topological characters make it difficult to define landmarks on a leaf and require

a novel approach. This study proposes the use of the GM approach to investigate the morphology of five-foliolate *Rubus ulmifolius* (series *Discolores*) leaves from three different environments and compares the results with traditional measurements. It aims to provide a statistically robust method to reconsider bramble classification based on plastic traits such as leaves. We hypothesised that bramble (i) leaf size, (ii) shape and (iii) level of asymmetry changes in different but close (>200 m) habitats in Ireland.

Methods

1. Sample collection and imaging

The total of 20 leaves from 10 *R. ulmifolius* plant was collected from three locations in the Burren, area, Ireland. These included limestone pavement (53°02'21.3"N 9°03'40.2"W), woodland (53°02'15.9"N 9°03'35.4"W) and hedge (53°02'19.1"N 9°03'42.9"W) habitats (Figure 1). The distance between the pavement and the hedge were 90 m, and the woodland was 178 m. Between the woodland and the hedge, there was 167 m distance and the altitude difference between these locations was 3 m (112-115 m). The limestone pavement habitat for brambles consisted of shallow soil layer, exposed to sunlight and wind and dominated by calcareous vegetation (GL3A vegetation classification; National Biodiversity Centre, 2018). The dense, shaded woodland consisted of *Corylus avellana*, *Prunus spinosa* on peat (Irish Soil Information System, 2018). The north-facing hedge was situated next to a road and was subject to unknown management practices (likely to be regular cutting). Leaves were taken from the first third section of primocanes, were cut from the cane at the very base of the petiole. They were pressed for 1-2 hours, and before imaging, the compound leaves were laid down and using the cellar tape, they were flattened for imaging. Images were taken by the digital camera (Canon D100), using a ruler as a reference object on the pictures. The images were processed in Adobe Photoshop CS6.

2. Image analysis

TpsUtil software (Rohlf, 2004) was used to create a TPS file from all the images and was further processed in TpsDig software (Rohlf, 2006). After scaling each image using the reference object, landmarks were selected. All analyses were carried out in MorphoJ software (Klingenberg, 2011). The total of 32 landmarks was selected anti-clockwise on the five-foliolate leaves. The basal (1) and end tip of the petiole (2), the basal point of the basal leaf of on the right (3), the top (4) and bottom (5) side of the first fourth of the basal leaflet's midvein, the top (6) and bottom side (7) of the third fourth section along the midvein of the same basal leaflet. The same six landmarks (2-7) were selected on the other four leaflets. The landmarks on leaflet sides were selected in the sinus of serrations.

The software ImageJ2 (Rueden et al., 2017) was used for recording traditional measurements after scaling each image using the reference object. The length between end tip of the petiole and the tip of the terminal leaflet (a), the length of the petiole (b), the length of the terminal leaflet (c), the length of the second and third leaflets (d and e), and the widest point of the terminal leaflet (f) were recorded to 0.01 cm accuracy (Figure 1). Measurements were entered into a Microsoft Excel (2016) sheet. Analyses were carried out in R version 3.4.3 (R Development Core Team, 2008).

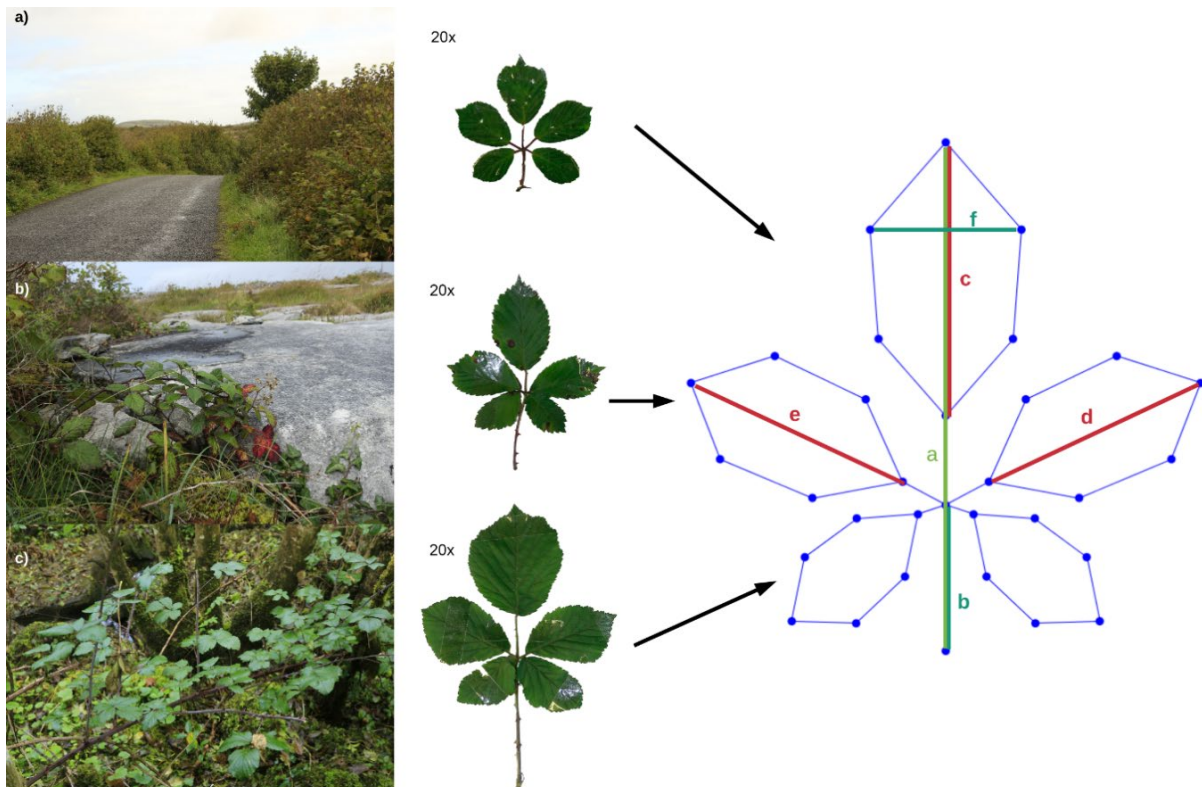


Figure 1. The collection sites consisted of a hedge (a), limestone pavement (b) and woodland (c). Twenty leaves per site were collected for landmark (blue wireframe graph on left) and traditional image analysis (a-f).

3. Statistical analysis

The Procrustes fit was superimposed on 32 landmarks from the 60 leaves in MophoJ. This removed the effect of size, position and orientation from the landmark configurations. Canonical Variate Analysis was used to investigate the leaf shape of the 60 leaves. The classifier for the analyses were the three habitats. The CVA reduces within-group variance, which is appropriate for bramble leaves and creates a shape space on a graph that represents all possible shapes. Pairwise differences in mean shapes using Mahalanobis distance was tested by permutation test (10,000 permutations per test). To cross-validate these results, Discrimination Analysis (DA) was used. The presence of FA was investigated by forcing bilateral symmetry on landmark pairs and calculating the deviation of the original shape. Procrustes ANOVA (Klingenberg, 2011) was used to test the effect of FA on size and shape in between habitats which assumes that the variation of landmarks is isotropic (there is the same amount of variation at each landmark and in every direction). Allometry was investigated by transforming the centroid size to its natural logarithm and regressing onto size, which was pooled within groups. The traditional measurements were analysed by Principal Component Analysis (PCA) with 'FactoMiner' package (Lê et al., 2008) in R version 3.4.3 (R Development Core Team, 2008), and the contribution of each measurement to the variance in the data was calculated and visualised by 'factoextra' package (Kassambara and Mundt, 2016). A natural logarithm transformation increased the linearity assumed by the PCA.

Results

The CVA on the asymmetrical component showed statistically different leaf shapes (Figure 1a; Table 1) where CV1 explained 81.07%, and CV2 explained 18.93% of the total variation. Procrustes

distance varied from 0.079 to 0.122, and the scatter plot of the CV scores showed no overlap between groups. DA cross-validated these results (Figure 1b; Table 1). CV1 represented the change in petiole length and how to spread out the leaflets were arranged. In the woodland, the petiole was longer, and the petiolules of the basal leaflets were much shorter, almost sessile. The leaf shape at the limestone pavement was intermediate compared to the woodland and hedge shapes. CV2 represented the variation of the basal leaflets' petiolules and acuteness of the basal leaflets and the terminal leaflet. The landmarks representing the leaf area of each leaflet were wider at the woodland compared to the hedge, and the leaves were smaller at the limestone pavement, compared to the hedge.

In comparison, the CVA on the symmetrical component showed CV1 explains 76.18% while CV2 23.82% of the total variation (Figure 1c). The CV2 axis explained more variation compared to the CV2 of the asymmetrical component. The permutation tests showed that leaf shape differences of the three groups were statistically significant ($P \leq 0.001$) and these results were confirmed by DA ($P \leq 0.003$) (Figure 1c; Table 1). While the Procrustes distances between groups were similar to the asymmetrical component (0.076 to 0.117), the individual groups showed more spread on the scatter plot. CV1 represented leaf shapes from shorter petiole and longer petiolule lengths at the hedge to longer petiole and shorter petiolule lengths at the woodland. The terminal leaflets also appeared to have wider bases (cordate to ovate) at the woodland.

Similarly to the asymmetrical component, the leaf shapes at the limestone pavement were intermediate compared to the hedge and woodland ones. CV2 showed the variation of the leaflet acuteness, and interestingly the petiolules of the four basal leaflets seemed to originate from the same

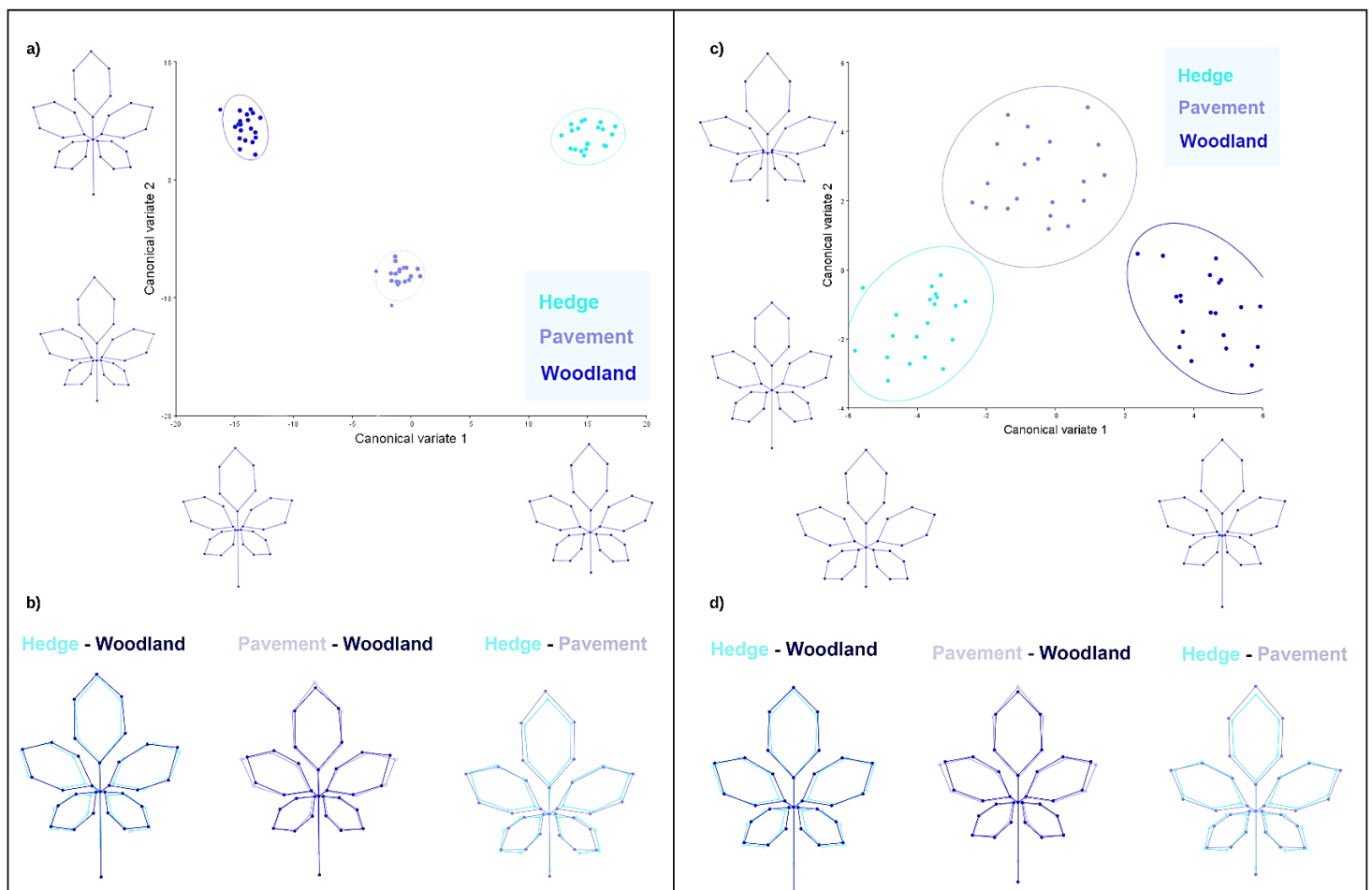


Figure 2. Comparison between the CVA (a) and DA (b) of asymmetrical component and the on the symmetrical component (c and d). Wireframe graphs on the CVAs are scaled according to their position underneath the axes and 95% confidence ellipses are illustrated on the graphs.

insertion point at the pavement. The leaflets appeared to be more spread out and more elliptical at the hedge and the woodland according to this axis.

The significance of allometry was investigated by multivariate regression and was not statistically significant on both the asymmetric ($P = 0.110$) and symmetric component ($P = 0.112$), explaining 3.07% and 2.75% of the total variance (Figure 3). Procrustes ANOVA showed that both the size and shape were highly statistically different ($P < 0.0001$) (Table 2) and while 55.95% variance of size was explained by the habitat, only 23.17% variance of shape change was due to the habitat. The effect of FA was reported as the 'side' effect, which explained 2.27% variance and was statistically significant ($P < 0.0001$) on leaf shape. The PCA on length and width measurements (Appendix 1) showed that the leaf measurements at the limestone pavement and the hedge, as well as at the pavement and woodland were overlapping (Figure 3) where PC1 axis explained 90.98% of the total variance and PC2 explained 4.21% and separated the woodland and hedge leaf measurements. Along PC1, all measurements contributed almost equally while on PC2, the petiole and terminal leaf widest width explained the most variance (Appendix 2).

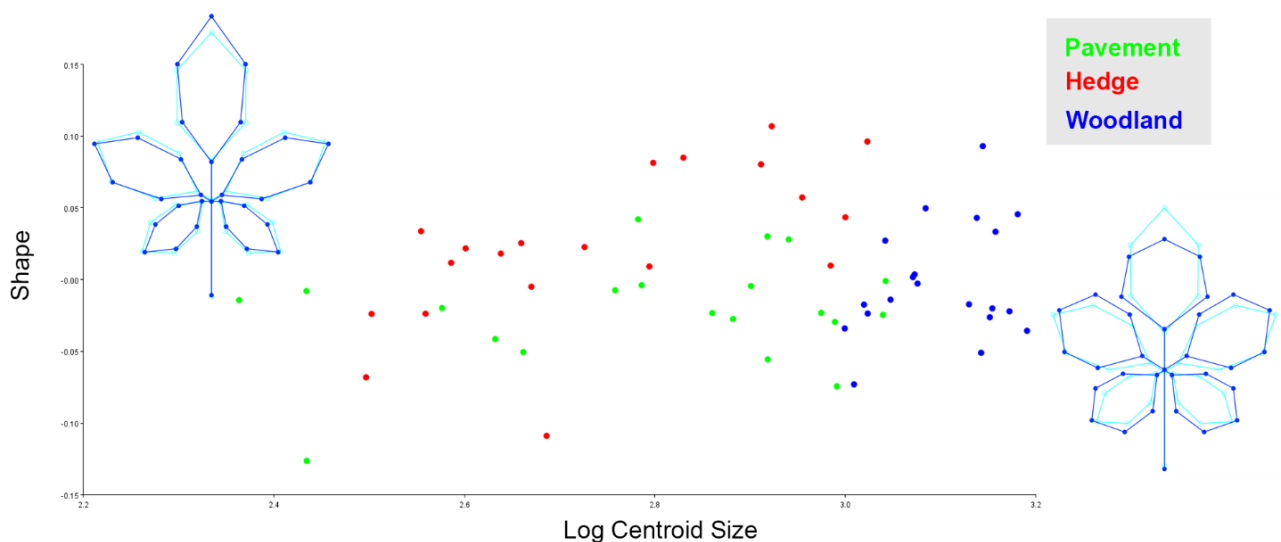


Figure 3. Allometry of the symmetrical component of bramble leaf shapes from the three different habitats. Wireframe graphs shows the shape change the predicted leaf shape from 2.1 to 3.2 on the horizontal axis.

Table 1. The results of the CVAs and DAs of the asymmetrical and symmetrical components, including the Procrustes distances and P-values from 10,000 permutation tests.

	P.distance	CVA P-value	DA P-value
Asym			
Pavement - Hedge	0.079	0.002	0.002
Woodland - Hedge	0.122	<.0001	<.0001
Woodland - Pavement	0.095	<.0001	<.0001
Symmetry			
Pavement - Hedge	0.076	0.001	0.003
Woodland - Hedge	0.117	<.0001	<.0001
Woodland - Pavement	0.090	<.0001	<.0001

Table 2. Procrustes ANOVA results of size and shape variation in between habitats, individuals and sides. Side refers to FA's effect on shape.

	Var%	SS	MS	df	F	P
Size						
Habitat	55.95	489.167	244.583	2	36.2	<.0001
Individual	44.05	385.087	6.756	57		
Shape						
Habitat	23.17	0.184	0.003	60	8.85	<.0001
Individual	74.56	0.592	0.000	1710	2.97	<.0001
Side	2.27	0.018	0.001	30	5.19	<.0001

Var% = Explained variance

SS = Sums of squares

MS = Mean sums of squares

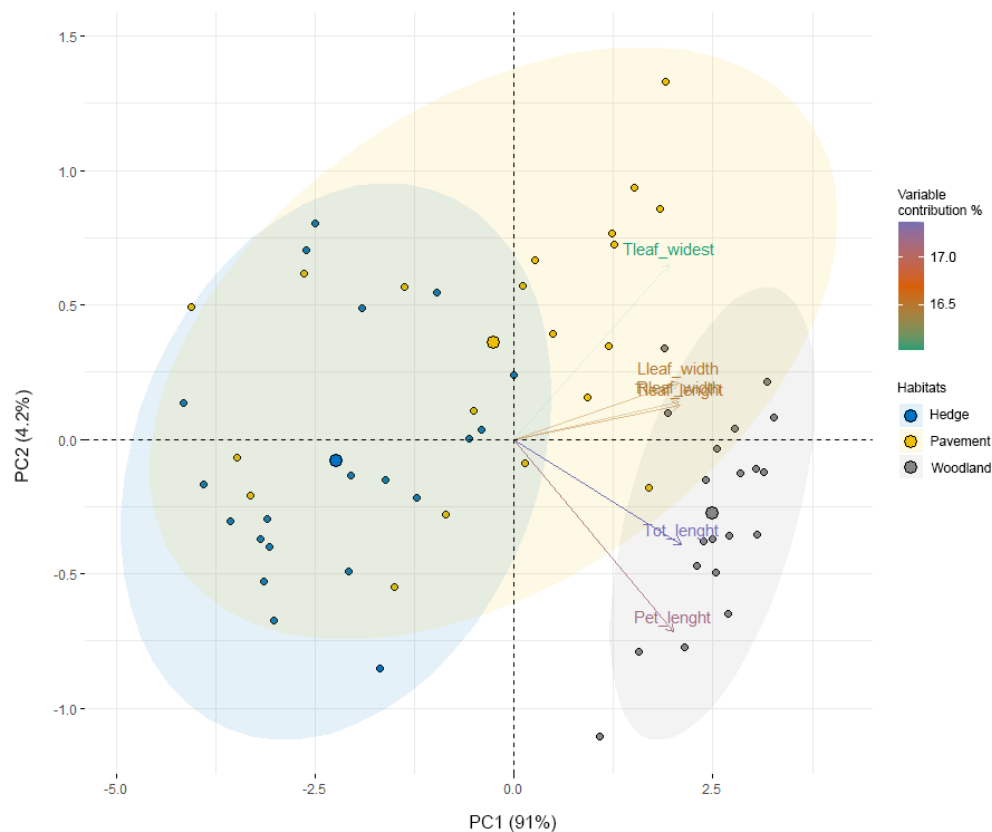


Figure 4. Scatter plot of PCA on leaf widths and lengths showing overlapping groups on PC1 and PC2. The total variable contribution shows that the petiole length and total length variables contributed the most to the variance within the data.

Discussion

In this study, *R. ulmifolius* leaf shape and size was shown to vary between woodland, hedge and limestone habitats. Geometric morphometrics provided more information than traditional measurements about how the leaf shapes differ. The PCA of traditional leaf width and length measurements failed to separate the three groups without any overlaps and provided less information about the contribution of the different variables.

The CVA of both asymmetrical and symmetrical components of leaf shape clustered according to habitats. The clusters were more tight and further apart for the asymmetrical component, and Procrustes ANOVA confirmed that FA was significant on shape, between sites. FA could be reflecting localised phenotypic plasticity induced by the different stresses (i.e. shade at woodland, exposure to light and wind at the limestone pavement and management at the hedges) (Vogel, 2009; Tucić et al., 2018). There is a high error rate and hence unreliability of FA measurements of the traditional width and length measurements (Heard et al., 1999; Kozlov, 2015; Kozlov et al., 2017; Alves-Silva et al., 2018). The use of GM is suggested to be a more reliable method for FA (Klingenberg et al., 2002; Klingenberg et al., 2012) and for larger datasets, MANOVA test could be used with multiple classifiers.

By visualising the shape change on the CVA graphs, the length and position of petiolules of the basal leaflets vary greatly between habitats for both asymmetrical and symmetrical components. The

arrangement of leaflets is one of the main characters used in bramble classification (e.g. the section *Corylifolii* is distinguished by the imbricate leaflets) (Edees et al., 1988). Another character is whether a leaf is digitate or pedate and the variability of one species' morphology suggests it to be unreliable. The terminal leaflets' acuteness is also an important descriptor for different species, and it was represented mostly on CV2, distinguishing the leaf shapes at the limestone pavement.

More variance was explained by the habitat for the size variation than the shape and allometry explained a small proportion of the total variation, and was not statistically significant. These findings suggest that *R. ulmifolius* leaf shapes vary in between environments and the shape does not change with the size. Interestingly, the multivariate regression separated the regression scores from the woodland habitat. The leaf measurements only separated the leaves at the hedge from the woodland ones along PC1 while the groups did not cluster along PC2 to which the petiole length and the terminal leaf width contributed the most. It is interesting that petiole shape was represented along CV1 when using landmark data. The longer petiole in woodlands has shown to increase light capture efficiency as it decreases leaf overlap in shaded habitat (Takenaka, 1994; Balandier et al., 2012; Gallagher et al., 2015). When GM was compared to traditional measurements of four oak species (Viscosi et al., 2009b), they concluded that GM was a faster and reliable method to distinguish different species and hybrids using PCA, CVA and DA. Another study by Viscosi (2015) focusing on oak leaves found that PCA and CVA separated one species while allometry clearly distinguished two species according to regression scores. Although these studies were carried out on different species, Silva et al. (2012) and Vieira et al. (2014) distinguished different populations of plants based on leaf shapes.

Phenotypic plasticity and mutations can lead to dramatic changes in compound leaf morphology, which brambles have (Niinemets, 1998; Rozendaal et al., 2006; Klingenberg et al., 2011). The current study only captured a snapshot of bramble leaf morphology, and to quantify the level of phenotypic plasticity in different environments, GM should be used to follow the leaf shape change during plant development (Baret et al., 2003 and 2004). Habitats with higher fluctuations in abiotic factors (e.g. limestone pavement and hedge) are likely to have higher plasticity during development (Little et al., 2010). In the shaded woodland habitat, more serrated and spread out leaves are likely to be fixed characters (Boyce, 2008; Peppe et al., 2011) but the cane diameter and length can rapidly increase (e.g. by sevenfold) when space becomes available (Pancer-Koteja et al., 1998; Gorchov et al., 2011). The evolution of leaf shapes reveals that no one ecological strategy acting on the shape (Givnish, 1987; Nicotra et al., 2011) but numerous abiotic, biotic factors and genetic networks lead to a species' phenotype (Tsukaya, 2004; Ichihashi et al., 2014). Thermoregulation (Vogel, 2009), hydraulic constraints (Brodribb et al., 2010), heteroblasty (Chitwood et al., 2014), adaptations to herbivory (Brown and Lawton, 1991), light optimisation (Falster and Westoby, 2003) and resource allocation (Givnish, 1988) are just some of the examples of strategies to consider. The recent advancements in imaging and molecular methods lead to a new era of big data acquisition (Bucksch et al., 2017) which is currently transforming the field of ecology (Peters et al., 2012; Hampton et al., 2013; Rügge et al., 2014) and conservation (Schimel and Keller, 2015).

Conclusions

Geometric morphometrics was shown to be a useful tool to understand bramble leaf shape and size variation in different habitats in Ireland. This method should be extensively carried out for the most common species on five-foliolate, three-foliolate leaves as well as prickly morphology to work toward a

new classification system for brambles in Europe. By also genotyping these individuals, the MorphoJ software allows the projection of phylogenetic data into the morphospace created by either CVA or PCA and testing for a phylogenetic signal in bramble leaf morphology (Rohlf, 2002; Klingenberg, 2011; Klingenberg et al., 2012). This method would reveal genotype x environment interactions which can be cross-validated by experimental growth in controlled environments. Moreover, the newest image analysis tool, persistent homology can provide 3.2 times more shape information compared to traditional methods by extracting a 2D data point cloud of digital images (Li et al., 2017a, Li et al., 2017b, Li et al., 2018). By understanding these patterns, a new, reliable bramble classification could be established which will help management and conservation efforts of brambles worldwide.

Word count (excluding abstract and within-text references): 3,122

Number of group members: 1

Appendix

Appendix 1. The descriptive statistics of the traditional measurements from the three different habitats.

	Hedge	Pavement	Woodland
Total length	8.35 ± 1.2	9.98 ± 1.79	14.46 ± 0.92
Petiole length	2.69 ± 0.53	3.34 ± 0.81	6.05 ± 0.61
Terminal leaf length	4.06 ± 0.4	5.13 ± 1.02	6.25 ± 0.39
Right leaf width	3.59 ± 0.48	4.47 ± 0.9	5.59 ± 0.47
Left leaf width	3.57 ± 0.48	4.46 ± 0.97	5.58 ± 0.41
Terminal leaf width	2.27 ± 0.52	3.15 ± 0.74	3.99 ± 0.70

Appendix 2. Variable contributions (%) on PC1 and PC2 axes. While the six variables contributed equally to PC1, the total leaf length and terminal leaf length contributed the most. These measurements are highly co-vary. To PC2, mostly the petiole length and terminal leaf width contributed.

	Total length	Petiole length	Terminal leaf length	Right leaf width	Left leaf width	Terminal leaf width
PC1	17.58	15.92	17.26	17.06	17.08	15.09
PC2	13.10	43.88	1.40	1.64	3.79	36.19

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