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Capturing variation in floral shape; a virtual 3D based morphospace for *Pelargonium*

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**Background.** Variation in floral shapes has long fascinated biologists and its modelling enables testing of evolutionary hypotheses. Recent comparative studies that explore floral shape have largely ignored 3D floral shape. We propose quantifying floral shape by using geometric morphometrics on a 3D model based on 2D photographic data and demonstrate its performance in capturing shape variation.

**Methods.** This approach offers unique benefits to complement established imaging techniques i) by enabling adequate coverage of the potential morphospace of large and diverse flowering-plant clades; (ii) by circumventing asynchronicity in anthesis of different floral parts; and (iii) by incorporating variation in copy number of floral organs within structures. We demonstrate our approach by analysing 90 florationally-diverse species of the Southern African genus *Pelargonium* (Geraniaceae). We quantify *Pelargonium* floral shapes using 117 landmarks and show similarities in reconstructed morphospaces for spur, corolla (2D datasets), and a combined 3D dataset.

**Results.** Our results indicate that *Pelargonium* species differ in floral shape, which can also vary extensively within a species. PCA results of the reconstructed 3D floral models are highly congruent with the separate 2D morphospaces, indicating it is an accurate, virtual, representation of floral shape. Through our approach, we find that adding the third dimension to the data is crucial to accurately interpret the manner of, as well as levels of, shape variation in flowers.
Capturing variation in floral shape; a virtual 3D based morphospace for *Pelargonium*

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Abstract

Background. Variation in floral shapes has long fascinated biologists and its modelling enables testing of evolutionary hypotheses. Recent comparative studies that explore floral shape have largely ignored 3D floral shape. We propose quantifying floral shape by using geometric morphometrics on a 3D model based on 2D photographic data and demonstrate its performance in capturing shape variation.

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Introduction

Variation in floral form continues to be an inspiration for a wide variety of research fields, ranging from taxonomy (Linnaeus, 1758), developmental biology (Carr and Fenster, 1994; Coen and Meyerowitz, 1991; Cubas et al., 1999; Fenster et al., 1995; Luo et al., 1995; Mummenhoff et al., 2009; Parenicova et al., 2003), evolution (Darwin, 1877a; Reyes et al., 2016; Sauquet et al., 2017), adaptation, to pollination biology and speciation (Darwin, 1877a, 1877b; Fernández-Mauzeocos et al., 2013; Gómez et al., 2016; Grant, 1949; Van der Niet and Johnson, 2012). The term 'form' refers to a combination of size and shape (Goodall, 1991; Zelditch, 2012). Whereas allometry is the study of the effect of size on the variation in morphological traits (Klingenberg, 2016), shape is defined as “those geometrical attributes that remain unchanged when the figure is translated, rotated and scaled” (Goodall, 1991).

The total variation in shape of a clade after scaling and aligning forms its ‘morphospace’ (Chartier et al., 2014), which can change depending on the taxa included in the study. Traditional versus geometric morphological methods have been the subject of debate (see Adams et al., 2004; Rohlf and Marcus, 1993). In (GMM), landmarks placed on homologous structures capture the geometry of the studied object. Shape is maintained throughout the analyses, preserving the geometric relationships between structures (Adams et al., 2004; Rohlf and Marcus, 1993).

Challenges for any morphometric study are measurement accuracy and precision. For accuracy, including as many taxa as possible seems important in GMM studies, because the aim is to cover variation in shape. Taxonomic coverage is often used here as a proxy to determine inclusiveness or accuracy. However, more important might be to include a broad representation of the expected morphological diversity in the sampling, irrespective of phylogenetic diversity. In general, larger clades are considered to be more informative because more taxa means more data, likely increasing the accuracy in measuring the studied shape variation. But when the taxon- and total potential morphospaces cannot be adequately covered, studying large clades is less meaningful.

When a floral GMM analysis is performed on a plant clade, maintaining the precision of gathering the data poses an additional challenge. Since plant morphology can be considered “a process” (Sattler, 1996, 1990), i.e. development, it is important to make sure that there is no noise from developmental signals in the data and its resulting morphospace, and hence that comparisons are made for the same ontogenetic stage across individual flowers. Ontogenetic noise can be prevented by deciding on a particular developmental stage for all individuals when measured. Full anthesis of the corolla is an example thereof (Gómez et al., 2016). However, studies have shown that different floral parts are not synchronised in their development (refs) and that species differ in the synchronisation of their floral parts (van de Kerke, unpublished)
data). Therefore, the floral parts of all individuals included in the study should be captured during the same ontogenetic stages, which poses a practical problem in data gathering. Another, practical, challenge in floral GMM is the variation in copy number of included structures. For example, a species can display a range in number of stamens or petals within its flowers. This can be problematic because GMM studies are based on capturing homologous structures and therefore retaining accurate homology assessment is essential. Simply omitting copy number-variable structures from the analysis is not desirable since they represent evidence on shape. Assuming serial homology, and ‘filling in’ missing copies could be one solution but the ensemble shape may be affected. Thus, how to handle such morphs and their varying copy numbers is not straightforward.

We aim to address the GMM challenges outlined above, using the predominantly South African genus *Pelargonium* (Geraniaceae) as a model. The genus is known for its stunning floral and vegetative diversity across its ~280 species (Bakker et al., 2005, 1999, Jones et al., 2009, 2003; Nicotra et al., 2008; Röschenbleck et al., 2014; Struck, 1997, Figure 1) and has been the subject of wide-spread breeding and horticulture (Becher et al., 2000; James, 2002; Miller, 2002). Roughly 70% of the genus occurs in the South African Greater Cape Floristic Region (GCFR; Linder, 2003; Manning and Goldblatt, 2012; Snijman, 2013), other species occur in eastern Africa, Namibia, Asia Minor, the Arabian peninsula, Madagascar and Australia (Bakker et al., 2005). Phylogenetic relationships within the genus are well known (Bakker et al., 2005; Röschenbleck et al., 2014; van de Kerke et al., 2019) and show a pattern of deep splits as well as more recent species radiations (i.e. the geophytic sect. *Hoarea*; Bakker et al., 2005).

*Pelargonium* flowers are specialised when compared with the remainder of the Geraniaceae clade (i.e. *Geranium*, *Erodium*, *Monsonia* and *California*), as they exhibit strongly zygomorphic corollas and possess nectar spurs that are formed adnate to the pedicels (Albers and van der Walt, 2007; Bakker et al., 2005; Goldblatt et al., 2000; Hodges, 1997; Hodges and Arnold, 1995; Manning and Goldblatt, 2012; Tsai et al., 2018; Van der Walt and Vorster, 1988, 1981), which is unique in angiosperms (Hodges, 1997; Tsai et al., 2018). Throughout *Pelargonium*, variation in floral shape occurs in a number of ways. Most strikingly, the orientation of the petals ranges from highly zygomorphic (*P. fulgidum*) to almost actinomorphic (*P. cotyledonis*). Secondly, the variation in petal copy number occurs between and within a species [i.e. *P. caucalifolium*] and alters between five (the ‘standard’ in Geraniaceae), four (*P. tetragonum*), two (*P. dipetalum*), and can even be missing (*P. apetalum*). Third, the shape of the petals varies tremendously: from slender and elongated (*P. paniculatum*) to almost round (*P. inquinans*). *Pelargonium* exhibits a range of pollination syndromes, including species of long-tongued hovering flies (*Tabanidae*, *Bombyliidae*, and *Nemestrinidae*), bees (*Apidae*, *Anthophoridae*, *Megachilidae*), wasps (*Vespidae*), and beetles (*Scarabaeidae*; Struck, 1997). Some syndromes are highly-specialised, as in the oceanic island endemic *P. cotyledonis* (occurring on St. Helena) where the nectar spur is reduced to a few millimetres. Another extreme example is the geophytic *P. appendiculatum* (with a limited distribution range along the South African west coast [see Marais, 1999]) which has a nectar spur of 10 cm long, while no pollinator with a suitable proboscis is known. Spur length in *Pelargonium* appears to be a driver of speciation rate, whereby speciation rate seems to decrease with an increase in spur length and is associated with small clade size (Ringelberg, 2012). The wide variety of known pollinators for *Pelargonium* is reflected in spur length, whereby the spur matches the proboscis of the pollinator species.
The extent to which the pedicel is covered by the spur differs greatly among species (Bakker et al., 2005; Manning and Goldblatt, 2012; Tsai et al., 2018). This could indicate pedicel length is independent from spur length, and thus is a potential constraint on spur length change.

In this study, we infer the floral morphospace for the corolla and the nectar spur across Pelargonium. We use two-dimensional (2D) photographs to form three-dimensional (3D) representations of virtual flowers in order to quantify floral shape in 90 Pelargonium species. We explore the diversity of floral forms within the genus and using this dataset as a case study we apply GMM methods to determine and compare natural variation in floral shape.

Materials & Methods

Flower data sampling

Floral shape was compared for 90 Pelargonium species growing in living collections in The Netherlands, Germany and in South Africa (see Supplementary Table 1 for an overview of species, numbers of individuals, and location). The sampling covers approximately 32% of known species in the genus and includes 378 individual flowers. We covered the potential morphospace as adequately as possible (based on known extreme floral forms from taxonomic studies (Albers et al., 1995; van der Walt, 1985; van der Walt and Boucher, 1986; van der Walt and van Zyl (nee Hugo), 1988) but not-necessarily representing phylogenetic diversity.

Geometric morphometric data collection

We selected flowers with corollas and flowers with stamens (used a proxy for full anthesis of the spurs, which was confirmed by eye) in full anthesis separately to limit possible ontogenetic effects on measured shape. We digitally photographed each flower using a standardised procedure in front and side view to avoid positional effects on measured shape. For each photograph, we defined a set of landmarks to provide comprehensive coverage of the specimen. We used both primary landmarks on homologous positions as well as sliding landmarks along a curve between two fixed primary landmarks. A datafile was created using tpsUtil (Rohlf, n.d.) and landmarks were placed using tpsDig v. 232 (Rohlf, 2010).

For the side view photograph, covering the spur aspect, we defined a set of 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (Figure 2A [grey labels]). We labelled this data set SPUR (containing 134 individuals, Supplementary Table S1).

For the front view photograph, we followed the corolla shape landmarks as defined by Gómez et al. (2006) and placed 32 landmarks along the outline of the corolla and the opening of the nectar spur using midrib, primary and secondary veins and petal attachment as a guide (Figure 2B [grey labels]). We labelled this data set PETAL (containing 287 individuals, Supplementary
Table S1). For specimens with four petals, we assumed that for the middle anterior petal the meristem is present but does not develop (Ronse De Craene, 2018). Therefore, landmarks allocated for this petal were placed but with zero length from the missing petal base (Figure 2B [pink labels]). A 5 mm scale bar was included in each picture to be able to represent all landmark coordinates on the same interval scale.

Creating 3D virtual representation from two 2d photographs

To be able to understand how shape variation happens at the level of the complete flower we linked individuals from both datasets at the species level. One-on-one pairing of individuals in the separate SPUR and PETAL databases was not possible because the flowers we used are not the same for both datasets (as a result of the separate sampling in order to avoid of asynchronisation), nor were individuals sampled from the same plant. Therefore, we designed a random sampling bootstrapping method based on the SPUR and PETAL datasets (see below, Figure 3).

First, we reoriented all individuals in both SPUR and PETAL dataset in the same position before we connected them to assure a virtual 3D flower that is as congruent with actual morphology as possible. To that extent, we performed an initial Generalised Procrustes Analysis (GPA) on the SPUR and PETAL datasets separately in order to align specimens and remove size components. Subsequently, we reintegrated the size component in order to retain actual size of the individual when coupling them from SPUR and PETAL datasets (via an anchor point, see below and Figure 3D). This was accomplished by multiplying each individual with its calculated centroid size. In this way, we orientated all specimens in the same position based on their landmarks, without removing size information (Figure 3B).

Next, we selected the species present in both SPUR and PETAL datasets. For each species, the number of individuals in each dataset was counted and we recorded at which row in the dataset a new species starts. In a linking step, a random individual from a certain species in dataset SPUR was then drawn and combined with a random individual of the same species from dataset PETAL. This was done six times per species, with replacement (Figure 3C).

To integrate the two 2D datasets into a single 3D dataset, a common anchor point was defined in both the SPUR and the PETAL datasets, corresponding to the top of the opening of the spur. In the SPUR dataset, the first landmark was chosen as anchor and for PETAL we defined the anchor to be the average of landmarks 22 and 23, as these anchors are homologous (Figure 3D).

A third coordinate was then added to the two 2D coordinate datasets (PETAL and SPUR), effectively making a virtual 3D image (dataset VIRTUAL3D). For the PETAL data set we kept the original x and y values and add a z = 0 coordinate to all landmarks. In this way, we ‘forced’ the corolla of the flower to be flat because we do not have data on the curvature of the petals. For SPUR the coordinate system was altered from x,y to z,y, which effectively becomes the depth of the flower. This alteration is relative to the coordinate combination of the anchor point defined previously, landmark SPUR 1 and landmarks PETAL 22-23, i.e. they are placed perpendicular to each other, around the anchor. Therefore the coordinates became negative for the spur and positive for the stamens. The value x = 0 was added for all SPUR landmarks, again resulting in a flat object. The new x and y SPUR coordinates were then transposed
relative to the landmarks 22-23 anchor point of PETAL (Figure 3D), with which they were
subsequently combined. The new 3D coordinates were written to a file using a format that is
suitable for later analysis with Geomorph. This process was repeated for all combinations of
individuals in the set selected in the linking step described above (Figure 3E).
This process is repeated 20 times to assess the structure in the virtual 3D flower data, and
hence its stability, resulting in 20 bootstrap pseudoreplicate datasets containing $6 \times 68 = 408$ virtual flowers, which we label VIRTUAL3D, (with $i = 1, \ldots, 20$). We combined all resulting
8160 virtual flowers in VIRTUAL3D, a dataset which we use for further analyses.

Morphometric analysis
Landmark coordinates in the SPUR, PETAL, VIRTUAL3D, and all VIRTUAL3D, datasets were
each aligned using a final Generalised Procrustes Analysis, extracting the shape information
(Rohlf and Slice, 1990). Results were projected into tangent space to summarise and explore
actual (SPUR, PETAL) and virtual (VIRTUAL3D) floral shape variation across Pelargonium
species. Shape changes associated with principal components where illustrated using thin-plate
spline deformation plots.
We conducted a Principal Component Analysis (PCA) on the GPA-aligned coordinates for each
of the VIRTUAL3Di datasets. PCA results for the 20 VIRTUAL3Di datasets are highly congruent
(results not shown, data will be made available). This indicates that there is high consistency in
our data and that bootstrap subsampling seems justified for connecting the differently samples
SPUR and PETAL datasets. We therefore decided to continue our analyses with the
VIRTUAL3D dataset including all 8160 virtual flowers, as this dataset assures an even coverage
of all included species and is the most inclusive.
Spurs occur adnate to the pedicel in Pelargonium species and pedicels can be ‘occupied’ by
spurs to varying degrees. As this may in fact present limits to spur length it could constrain spur
evolution and be relevant to floral shape exploration. We therefore decided to extract ‘spur-
filling’ levels from our data in the following way: for each individual in the SPUR dataset, we
extracted relative spur and pedicel length from the SPUR dataset using the function
‘interlmkdist’. We calculated the ratio between the spur and pedicel length as a measure for the
‘filling’ of the pedicel by the spur. This ratio is visualised in the SPUR PCA plot as the
transparency of the individual.
All analyses were performed in R v.3.2.2 (R Core Team, 2015) using the Geomorph library v.3.0
(Adams and Otárola-Castillo, 2013). All R scripts can be found in Supplementary R scripts S2,
S3, and S4.

Results
Our analysis on 68 Pelargonium species identified a wide variety of floral shapes across and
within the species examined (see also Fig. 1). Supplementary Figure S5 shows the mean
consensus configuration and Procrustes residuals (i.e. differences between observed and
estimated value) calculated for the SPUR and PETAL datasets using the generalised
Procrustes analysis (GPA). The figure illustrates the variability in landmarks around the
calculated mean shape (in blue). What is striking is that halfway through the spur we see a constrained area where variation is limited compared with the base of the pedicel (Sup. Fig S5A). In addition, in Figure S 5B it is conspicuous that the anterior petals are more restricted in shape variation that the posterior petals.

We conducted a Principal Component Analysis (PCA) on the GPA aligned coordinates for each of the SPUR, PETAL, and VIRTUAL3D datasets in order to assess variation in shape. For the SPUR dataset, the first PC accounts for 47% of the total variation present across the species and the first four axes explaining more than 90% of the data (Figure 4A, Supplementary Figure S6). The first two PCs and corresponding shape outlines of the extremes are plotted in Figure 4A and 4B, respectively. The variation in shape explained by the first PC corresponds with the coverage of the spur relative to the pedicel. On the negative extreme of the axis, spurs are elongated and are the same length as the pedicel. On the positive extreme, spurs are much shorter than the length of the pedicel and, in addition, the opening of the spur is wide. PC2 corresponds with the curve of the stamens. Individuals on the negative extreme of the PC have stamens that are so curved they are doubled up on themselves, while those on the positive side have elongated stamens (Fig 4B, PC2). In Fig 4A some species, represented by multiple samples, are spread in varying degrees around the morphospace, such as *P. mutans* (in green) and *P. crithmifolium* (in red) along both PC1 and PC2. Other species appear to be much more clustered, such as *P. triste* (in brown) and *P. pseudoglutinosum* (in orange). Overall, a clear pattern emerges of individuals distributed along a trajectory corresponding with the ratio between the length of the spur and pedicel (indicated by transparency of the markers in Fig 4A). with individuals with a low spur-pedicel ratio occupying the lower region of the PC plot while individuals towards the top have an increasingly higher spur-pedicel ratio, i.e. each having nearly the same length, towards a boundary reflecting a physical barrier. This boundary is also reflected in the ‘avoided area’ in the *Pelargonium* SPUR morphospace just above it. In this area, the spur of a hypothetical flower would be longer than the pedicel of that individual, and this is not possible for *Pelargonium* flowers as spurs and pedicels are adnate.

Compared with the results of the SPUR dataset, the PCA results of the PETAL dataset are more centralised. In Figure 4C and 4D, the first two PCs and shape outlines are plotted. The first PC (explaining 40% variation, Supplementary Figure S6) corresponds with the position and number of petals in the flower. On the negative extreme of the axis, flowers consist of five petals with the two posterior ones close together and the three anterior petals spread out. On the positive extreme, the two posterior petals are enlarged and only two anterior petals appear to be present. PC2 (13%) corresponds with the distribution of the petals over the corolla. On the positive extreme of the PC, the posterior petals are narrow and overlap, while on the negative side the posterior petals are rounded. Overall, individuals cluster around the mean shape (as *P. multibracteatum* [yellow]) while other species show within-species variation with individuals that spread toward the positive extreme of PC1 (*P. myrrhifolium* [darkgreen]). A few species (as *P. mutans* [green]), with high within-species variation, found across the entire PCA spectrum.

For the VIRTUAL3D dataset, containing 8160 virtual flowers, the first PC accounts for 41% of the total variation present across species, with the first 5 axes collectively explaining > 80% of the data (Figure 5A, Figure 5B, and Supplementary Figure S6). Shape outlines illustrating the extreme forms are shown in Figure 5C. The variation in shape explained by the first PC corresponds with a zygomorphic flower, with corolla size varying with regards to pedicel length.
On the positive extreme, individuals have a short pedicel and spur and a large corolla while flowers on the negative extreme show a more elongated spur and a relatively small corolla. Individuals from all species are spread along this axis, showing a high variability in spur and pedicel elongation and no clustering. PC2 (19%) corresponds with the length and curvature in stamens, with virtual flowers on the negative extreme showing straight stamens and those on the positive extreme showing highly curved ones. More importantly, this PC appears to correspond with the ‘filling’ of the pedicel by the spur, whereby we either see a long pedicel and relatively short spur (positive side) or a spur that ‘spills over’ the pedicel (negative side).

Individuals from all species are spread along the axis but with an emphasis toward the negative extreme, suggesting a trend towards individuals with a high filling ratio. PC3 (14%) again (as PC1) appears to correspond with the filling of the pedicel by the spur as well as the length and orientation of the stamens. In individuals toward the positive end of this axis, the spur completely fills the pedicel and stamens are stretched out. On the negative side, only a small part of the pedicel is taken up by the spur and stamens are small. No clustering is observed and individuals are spread along the axis but with a strong emphasis on the negative end of the spectrum. Individuals within species are spread in varying degrees around the morphospace, such as *P. mutans* (in green) along PC1, PC2, and PC3. Other species vary along a number of PC axes, as *P. crithmifolium* (in dark blue) is variable along PC1 and PC3, but not along PC2. Lastly, some species are overall much more clustered, such as *P. pseudoglutosum* (in orange).

**Discussion**

In this study, we explore the potential of combining two 2D photograph-based datasets of floral morphology into a single 3D virtual flower giving us the opportunity to bring together multiple layers of shape variation. Using this method, we are able to investigate the tremendous floral diversity of *Pelargonium* species using 3D geometric morphometrics based on the spur plus corolla perspective. Our virtual 3D dataset gives a more nuanced view on shape variation in *Pelargonium* than the separate SPUR and PETAL perspectives, as we find the corolla perspective to be of less importance (see below). Our approach can serve as a low-cost alternative to emerging high-tech robotic and photogrammetry-based approaches to 3D geometric morphometrics.

**Geometric Morphometrics**

*Pelargonium* flowers exhibit high variability in their floral shape with species ranging between zygomorphic to near-actinomorphic corolla shape (*P. cotyledon/rn*), varying in petal copy number (between five [most common in Geraniaceae], four [i.e. *P. caucalifolium*], two (in *P. dipetalum*; not included) and zero (in *P. apetalum*; not included), and with lengths of nectar spurs varying between zero to ten cm (*P. appendiculatum*; not included). The variation in floral shape present in the VIRTUAL3D dataset as depicted in Figure 5 corresponds with this known variation in *Pelargonium* flowers, as well as with the separate PETAL and SPUR datasets (Figure 3 and 4). Findings of the separate PETAL and SPUR datasets have now been put into perspective, giving us a better understanding of which changes in *Pelargonium* floral shape are relevant.
Resembling the results of the SPUR dataset, the elongation of the spur and size of the corolla are the most variable traits among the species included in the VIRTUAL3D morphospace (PC1, 41%). This trait corresponds with the unique spur pollinator syndrome featured in *Pelargonium* and correlates with their highly variable pollinator types (Struck, 1997, 1994). We know in some species the spur is almost completely missing (Figure 5C, as for example in the oceanic island endemic *P. cotyledonis*, probably pollinated by bees) or in *P. hirtum* with 3 mm short spurs. The latter is closely related to *P. appendiculatum* (probably pollinated by long-tongued hovering flies) where the spur is elongated to almost ten cm length (Struck, 1997).

Corresponding to PC2 (19%), and linked to inferred shifts in pollinators, is the curvature of the stamens. Along the PC, we find a shift of stamen shape ranging from short and straight to long and curved. For some hovering pollinator species, the stamens are thought to ‘move out of the way’ of the spur entrance by means of a large curve in the filament, both increasing accessibility to the flower (Goldblatt and Manning, 1999; Manning and Goldblatt, 1996) and enhancing contact of anthers and insect abdomen and head (Goldblatt and Manning, 1999). This would correspond to the long and curved stamens of *Pelargonium* species pollinated by long-tongued, hovering insects such as species from the *Tabanidae*, *Bombyliidae*, and *Nemestrinidae* (Struck, 1997). The short and straight stamens on the other end of the spectrum would then correspond with the association with short-proboscid, landing pollinator species, such as *Anthophoridae*, *Megachilidae*, and *Vespidae* to increase potential pollen transfer.

The ‘filling’ of the pedicel by the spur, which corresponds to both the second as well as the third PC (11%) in the VIRTUAL3D as well as the SPUR dataset is a relatively unexplored trait in *Pelargonium* literature. Recent studies found spur length to be dependent on both rate of cell division and duration of spur growth (Tsai et al., 2018). As the authors indicate, these mechanisms do not fully account for differences in spur length, suggesting other evolutionary influences. Ringelberg (2012) found spur length to be significantly correlated with speciation rate, whereby speciation rate appeared to decrease with increased spur length.

The distribution of virtual flowers over the first three PCs of the VIRTUAL3D morphospace varies and appears to be the results of interaction between the SPUR and PETAL morphospaces. In the SPUR morphospace, we see a clear boundary limiting the distribution of individuals based on the ratio of spur and pedicel length (Figure 4A). In the PETAL morphospace on the other hand, the majority of species cluster together around the mean shape, indicating that there is variation to a limited extend. Some species in the VIRTUAL3D morphospace are highly variable and occur throughout large areas of the morphospace (for instance *P. mutans* [grey]) while others occupy a much smaller area (e.g. *P. multibracteatum* [light blue]). The former pattern does not directly correspond with a high individual count in PETAL and SPUR datasets. Certainly, in cases as *P. crispum* the low variability is the consequence of there being only one individual in the PETAL and SPUR datasets. As a result, over all the bootstrap iterations, only a single virtual-flower is included in the final analysis. But in other cases, as for instance with *P. multibracteatum*, multiple individuals are included in the separate datasets and still we find a narrow distribution in the morphospace.

Surprisingly, the results of the VIRTUAL3D dataset as discussed above are highly congruent with the results of the SPUR dataset while the PETAL dataset does not appear to have much influence since we do not find the variation in shape along PC1 in the PETAL dataset (variability in length of the fifth petal) until the third PC (14%). Rather the size of the corolla relative to the
length of the spur is found to be of more influence in the VIRTUAL3D dataset. The variability in spur and stamens, combined with this relative size difference of the corolla, thus seem to be more relevant for distinguishing different shapes and presumably for attraction.

3D connection of 2D data sets

The combining of separate 2D datasets into a single 3D dataset by creating virtual flowers as we demonstrate here complements existing 3D approaches (van der Niet et al., 2010). We find the main PCs of the VIRTUAL3D dataset summarise the variability in shapes as presented in the separate SPUR and PETAL datasets and accurately portray the natural variation found in Pelargonium flowers (based on visual inspection). Having rendered the flower in 3D, we can now investigate the interaction between floral parts in more detail.

Our method enables us to circumvent a main issue in morphometric studies on flowers and thus to increase the precision of the data: asynchronicity in anthesis of floral parts. The moment of anthesis of floral parts differs both between and within species. This makes it impossible to pinpoint an ontogenetic stage for the entire flower that is the same for all species. We argue that anthesis is the most relevant ontogenetic stage for reproduction as well as pollinator attraction and thus is the most meaningful stage to include in our study. Following other plant studies (Gomez et al., 2014; Gómez et al., 2006; Savriama et al., 2012; Savriama and Klingenberg, 2011), we decided to include all floral parts at their own, separate, anthesis. This results in the separate datasets of the SPUR (containing the spur and stamens) and the PETAL (containing the corolla). We consider the combination of spur and stamen floral parts in the SPUR dataset plausible since we suspect the flower’s reward system to develop approximately in concert with the contact apparatus, in order to ‘fit’ the visiting pollinator.

A drawback of combining the different floral parts each at their own anthesis is that we construct ‘virtual-flowers’ from our data. As a result, the morphospace is arguably not biologically and temporally accurate. However, we argue that gathering the data in the same ontogenetic stage gives us the advantage of not polluting our data with unwanted developmental signal and enables the testing of evolutionary hypotheses regarding dynamic (un)coupling of compartments (van de Kerke et al., unpublished data).

Another problematic issue in plant geometric morphometrics is the variability in copy number within floral parts. A striking example of this phenomenon in Pelargonium is the variability in petal number, varying from four to the symplesiomorphic five. This variability makes it seemingly impossible to include all intended landmarks since they have to be placed on homologous structures. Not including these landmarks in the study is not desirable as they represent an important difference in shape between species. Likewise, it is not an option to treat these landmarks as ‘missing’ or ‘NA’ since the flower did not drop the petal by accident, but it is simply not present. Ideally, we would like to confirm the presence of petal primordia in an electron microscopy study. Based on literature describing the occasional loss of petals (Ronse De Craene, 2018, 2015), we now chose to simulate this ‘missing’ petal as if it is present, but with a length of zero (Figure X). The influence of this simulation on morphospace results is limited since the variation between four and five petals is only visible on the fifth PC (4%) of the VIRTUAL3D dataset. We admit this approach is conceptually problematic because we assume
the petal to be present, but operationally warranted because we find the corresponding
difference in shape to be rather unimportant. Therefore we conclude this is a justifiable decision
that can be put into practise for other similar cases that will occur in plant morphometric studies.
Unfortunately, we were not able to achieve complete matching in taxonomic coverage between
the separate SPUR and PETAL datasets because sometimes there were no flowers in anthesis
available for both datasets. The separate morphospaces therefore have a higher taxonomic
sampling than the VIRTUAL3D dataset (68 for the VIRTUAL3D compared with 82 in SPUR and
90 in PETAL). This is an insurmountable drawback in combining the datasets, since in
morphometric studies all landmarks need to be present in all included specimens. Estimating
missing landmarks, as is available in the geomorph package, is not desirable when a large part
of the studied shape of an entire species is missing because then the average *Pelargonium*
shape is superimposed on a set of individuals and their unique shape is lost.

More important than high taxonomic coverage in the VIRTUAL3D dataset is to ensure accuracy
of the data by good coverage of morphological extremes in the morphospace, which is not
driven by the number of species included but by the shapes. In the case of *Pelargonium*, we
have several 'missing' shapes that we were not able to include in the sampling (we did not
encounter them while flowering) that will probably change the morphospace were they to be
included. For example, we did not have the opportunity to include species such as *P.
endlicherianum* and *P. dipetalum*, that only have two posterior petals. Likewise, we could not
include species showing highly reflexed petals (for example *P. luridum*) as well as the peculiar,
keel-flowered shaped *P. rapaceum* and the allopolyploid *P. quercetorum*. Notwithstanding these
gaps in the prospective morphospace, we are confident we reconstructed a fair representation
of overall variability in floral shape found in *Pelargonium* and therefore provide a solid base for
exploring floral shape in this clade.

**Conclusions**

This study provides a new approach for geometric morphometrics to analyse floral shape in 3D.
Our method uses a semi-automated approach to combine 2D shape data of various data sets to
include multiple morphological modules. It offers unique benefits to complement established
imaging techniques by i) providing a bootstrapping method to help acquire adequate coverage
of the potential morphospace of diverse flowering-plant clades when sampling of individual parts
is unequal; (ii) by circumventing asynchronicity in anthesis of different floral parts; and (iii) by
incorporating variation in copy number of parts within structures. This approach, for which the
code is available as supplementary material, can be used for any flower as well as numerous
plant structures and can be used to form an appropriate basis for future geometric
morphometric and related studies starting from 2D pictures.

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Figure 1 (on next page)

Overview of variation in floral shape in *Pelargonium*

Variation in *Pelargonium* floral shape. (A) *P. caucalifolium*, (B) *P. sidoides*, (C) *P. caffrum*, (D) *P. cotyledonis*, (E) *P. columbinum*, (F) *P. tricolor*. Pictures by F.T. Bakker and S.J. van de Kerke.
Landmark placement for the SPUR (A) and PETAL (B) datasets. For the SPUR dataset 10 landmarks and 75 sliding landmarks covering the spur outline and tracking its curvature, as well as that of the shortest, longest and an average stamen (grey labels) are defined. For the PETAL dataset 32 landmarks were placed along the outline of the corolla and the opening of the nectar spur using midrib, primary and secondary veins and petal attachment as a guide (grey labels). For specimens with four petals, we assumed that for the middle anterior petal the meristem is present but does not develop and landmarks allocated for this petal were placed with zero length from the petal base (red labels).
Creating one 3D virtual flower from two 2d photographs.

(A) The two separate datasets (SPUR and PETAL), with limited overlap between and within species. (B) Generalised Procrustes Analysis is performed on the SPUR and PETAL datasets separately in order to filter out all non-shape variation. Size component is then reintegrated by multiplying each individual with its calculated centroid size. In this way, all specimens are aligned based on their landmarks, without removing size information. (C) Species present in both SPUR and PETAL datasets are selected. In order to link species in both data sets, a random individual from dataset SPUR is then drawn for the first species and combined with a random individual of the same species from dataset SPUR. This was done six times per species, with replacement. (D) To integrate the two 2D datasets into a single 3D dataset, a common anchor point is defined in both the SPUR and the PETAL datasets, corresponding here with the top of the opening of the spur. A third coordinate is then added to the coordinate data, effectively making it 3D. See text for further details. (E) This process is repeated for all individuals in the set selected in the linking step.
PCA analysis on SPUR and PETAL datasets. (A) PC1 and PC2 of PCA on SPUR dataset. Colours correspond with selected species: *P. triste* (brown), *P. mutans* (blue), *P. patulum* (green), *P. crithmifolium* (red), and *P. pseudoglutinosum* (orange). (B) Shape outlines corresponding to extremes on axes for PC1 and PC2 of SPUR dataset showing calculated mean shape (grey) and warped extreme shape (black). (C) PC1 and PC2 of PCA on PETAL dataset. Colours correspond with selected species: *P. multibracteatum* (yellow), *P. myrrhifolium* (darkgreen), and *P. mutans* (green). (D) Shape outlines corresponding to extremes on axes for PC1 and PC2 of PETAL dataset showing calculated mean shape (grey) and warped extreme shape (black).
PCA analysis on VIRTUAL3D datasets. (A) PC1 and PC2 and (B) PC3 and PC2. Colours correspond with selected species: *P. multibracteatum* (light blue) *P. triste* (brown), *P. mutans* (grey), *P. myrrhifolium* (yellow), *P. patulum* (brown), *P. crithmifolium* (dark blue), and *P. pseudoglutinosum* (orange). Intensity of colours indicated number of individuals stacked. (C) Shape outlines corresponding to extremes on axes for PC1, PC2, and PC3 of VIRTUAL3D dataset showing calculated mean shape (grey) and warped extreme shape (black).