Polyploid speciation in the Greater Cape Floristic Region: Species limits within *Ehrharta calycina*

Honours Thesis

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ABSTRACT

Ehrharta calycina J. E. Sm. (Poaceae) is a widespread and often abundant species whose core distribution lies within the Greater Cape Floristic Region (GCFR). Importantly, it shows a high degree of morphological and ploidy level variation. Based on observations of distinct forms co-occurring at multiple sites, the hypothesis that *E. calycina* comprises more than one species was tested. Morphological analysis of herbarium specimens using a multivariate approach found strong evidence for the presence of two distinct phenetic clusters among specimens from sites of sympatry, and these were termed 'robust' and 'slender' forms. This translated to a broader sampling of specimens from across the GCFR (n = 516), of which over 91% could be attributed to one or other form with >90% confidence using linear discriminant analysis. Importantly, chromosome counts supported the hypothesis that these phenetic clusters represent distinct cytotypes, with slender forms being diploid (n = 12) and robust forms tetraploid (n = 24), thus providing the primary reproductive isolating mechanism between the forms. Furthermore, differences in climatic niche suggest that these forms represent ecologically independent entities. Finally, evidence that the forms differ in palatability has considerable practical implications given that *E. calycina* is regarded as having high pasture value. Together, our results provide multiple lines of evidence for the existence of at least two morphologically distinct, reproductively isolated species within *E. calycina*, and suggest that the role of polyploidy in generating floral diversity in the GCFR may be more important than previously thought.

INTRODUCTION

By defining species limits, the fields of taxonomy and systematics provide the foundation for many important fields of biology. In addition, they provide insight into the processes that give rise to species and the mechanisms that maintain species isolation. In doing so, these disciplines link the ecology of species to their evolutionary history. However, the practice of delimiting species is often difficult, and has historically been surrounded by much confusion. Part of the problem has stemmed from the search for an all-inclusive 'species concept'. It is generally agreed that species should represent separately evolving lineages (the evolutionary species concept of Simpson [1951] and Wiley [1978]), but that this alone provides no operational means of delimiting species (Hey 2006). As a result, many alternative concepts have been proposed, mostly in an attempt to provide a single criterion or set of related criteria for delimiting species in a way that is both universally applicable and conceptually satisfactory. However, no species concept has achieved this goal. As de Queiroz (2007) notes, there is no single observable property of separately evolving lineages that consistently distinguishes them from one another. Rather, properties such as reproductive isolation or niche differentiation can be viewed as 'contingent properties' of species whose presence depends on history and circumstance. This 'unified species concept' conceptualises species in the Darwinian sense as 'branches in the lines of descent' or 'lineage segments' (Darwin 1859; de Queiroz 2011), whose existence can be critically assessed using various contingent properties of species as lines of evidence (reviewed in Sites and Marshall 2004).

The African grass genus *Ehrharta* Thunb. currently comprises 23 species whose centre of diversity lies in the semi-arid regions of the Greater Cape Floristic Region (GCFR; Born et al. 2006) of South Africa (Verboom et al. 2003). The GCFR itself is a global hotspot of plant diversity and endemism (Myers et al. 2000). Verboom et al. (2003) showed that the GCFR's diversity in *Ehrharta* is primarily due to the rapid radiation of a single clade within the genus concurrent with the onset of a seasonally arid climate in the late Miocene. An important feature of the genus is that it shows very high diversity of morphological and life history traits (Verboom et al. 2004). The high interspecific trait diversity appears to be the result of adaptive radiation facilitated by the high edaphic heterogeneity in the semi-arid regions of the GCFR (Verboom et al. 2004), but considerable trait diversity also exists within some species, notably *Ehrharta calycina* J. E. Sm., which varies from perennial to annual across its range, with correspondingly high variation in growth form (Verboom et al. 2012). Furthermore, there is some evidence that the *E. calycina* complex may in fact comprise more than one species.

The widespread and often abundant *E. calycina* occurs throughout the GCFR but is primarily associated with seasonally arid areas (Gibbs Russell et al. 1990; Verboom et al. 2003). In addition, it is one of the most morphologically variable species in the genus (Gibbs Russell et al. 1990; Verboom et al. 2012). As a result of this variation, several forms and varieties have been described by various authors, both formally and informally. Gibbs Russell et al. (1990) acknowledged two varieties, *E. calycina* var. *angustifolia* Kunth and *E. calycina* var. *versicolor* Stapf. Chippendall (1955, p. 43) noted that the former 'has been recognised as a distinct variety', having, among other features, longer, more rigid leaves and longer spikelets than the type (Stapf 1900). Stapf (1900, p. 676) noted that specimens of this variety had been collected in 'Little Namaqualand', as well as areas further to the east, around Uitenhage and Graaf Reinet. John Acocks described several 'forms' of the species, including a 'long-leaved tufted form [occurring] in the west coast belt and Namaqualand' that may refer to this variety (Zacharias 1990, p.52).

Although these classifications exist, the high degree of variation in *E. calycina* means that there is still much uncertainty surrounding the taxonomy of the complex. Chippendall (1955, p. 43) stated that 'intermediate forms [between the described varieties] necessitate a more detailed study of the group before the variants can be classified accurately', and Gibbs Russell et al. (1990, p. 124) reiterated this, stressing the need for a 'full biosystematic study' to assess the status of these varieties. Furthermore, Chippendall (1955) mentioned the possibility that *E. calycina* may comprise more than one species. These statements have been followed by observations (GA Verboom, pers. comm.) of morphologically distinct 'forms' of *E. calycina* occurring sympatrically at various sites across the GCFR, with one being more typical of the species, and the other fitting the description of *E. calycina* var. *versicolor*. This has led to the hypothesis that these forms represent distinct species.

Accurate delimitation of species within *E. calycina* is likely to have considerable practical significance. The species has important local pasture value, being noted as 'one of the few winter-rainfall [i.e. GCFR] grasses even potentially valuable for grazing' (Gibbs Russell 1990, p. 124). Furthermore, Van Breda and Barnard (1991) noted that, in the semi-arid areas of the GCFR, '[*E. calycina*] is now usually found only inside other nurse crops because it is very palatable and is eaten immediately if it grows outside the protection of other plants.' However, observed differences in growth form and leaf morphology between co-occurring putative species within *E. calycina* suggest that these might differ in palatability. Such a difference would have economic implications due to the importance of livestock farming in many of the areas in which *E. calycina* occurs. In addition, the existence of multiple species within *E. calycina* might imply important, previously unrecognised ecological variation. A greater understanding of this variation might alter our view of how the complex fits into the ecology of the GCFR, with resulting practical implications. Returning to palatability as an example, differences between species within the complex would imply variation in disturbance tolerance, which would in turn have implications for the management of grazing land.

Various factors may explain the genesis and maintenance of distinct species within *E. calycina*. As the two putative species within the complex appear to regularly occur in sympatry, intrinsic reproductive barriers are likely to be important. One such barrier may be polyploidy. The importance of polyploidy as a reproductive isolating mechanism is well-established (Soltis et al. 2007), as not only are crosses between different ploidy levels likely to fail, but the progeny resulting from such crosses also often exhibit lower fitness or inviability (Ramsey and Schemske 1998; Husband and Sabara 2004). Consequently, genome multiplication has long been regarded as an important mechanism for sympatric speciation, as it instantaneously gives rise to post-zygotic reproductive isolation (Levin 1975) and is often associated with novel traits that can result in increased fitness and pre-zygotic reproductive isolation (Levin 1983; Ramsey and Schemske 2002). Despite this, taxonomists have traditionally tended to overlook the case for polyploid species, particularly when it does not involve hybridization (i.e. autopolyploidy). However, recent evidence for multiple cytotypes within species each fulfilling several species criteria suggests that polyploid speciation is far more common in nature than was previously thought (Soltis et al. 2007). Spies et al. (1989) found evidence of polyploidy in *E. calycina*, with diploids having the basic *Ehrharta* chromosome number (n) of 12 and tetraploids having n = 24. Further, these authors noted (p. 127) that the tetraploid state ‘appears to be restricted to densely tufted plants with erect, narrow, often rolled leaf blades’, which may refer to *E. calycina* var. *versicolor*. This, combined with the fact that the putative species co-occurring at various sites include one fitting the description of this variety, suggests that these putative species may represent diploid and tetraploid cytotypes.

Aside from polyploidy, ecological factors may also contribute to the isolation of species within the complex. Differences in flowering time would represent an important intrinsic, pre-zygotic isolating barrier, given that the putative species occur in sympatry, and that *E. calycina* is wind-pollinated (which excludes the possibility of pollinator-mediated isolation). Spatial-ecological differences within *E. calycina* might also be important as extrinsic isolating mechanisms between species. The complex is part of a major clade within the *Ehrharta* that underwent rapid adaptive radiation in the late Miocene, seemingly in response to an increase in the ecological heterogeneity of the landscape following the onset of a seasonally arid climate in the GCFR (Verboom et al. 2003). This emphasises the potential for ecologically driven speciation within the complex. In fact, it is probably only very rarely that speciation does not involve ecological factors (Sobel et al. 2010). As a pertinent example, despite the involvement of strong post-zygotic isolation, polyploid speciation is unlikely to occur in the absence of ecological factors counteracting the minority cytotype disadvantage of the polyploid (Levin 1975; Ramsey and Schemske 2002).

The aim of this study was therefore to evaluate species limits within *E. calycina* using multiple lines of evidence, in keeping with the approach of the unified species concept. The primary means of delimiting species was based on a multivariate analysis of morphological variation within the complex. Various possible mechanisms of reproductive isolation between phenetic groups were then evaluated. Finally, the practical significance of delineating multiple species within the complex was explored. To that end, the following set of hypotheses were tested:

1. *E. calycina* comprises at least two morphologically distinct species.

2. These species are reproductively isolated.

3. The origin and maintenance of species integrity within the complex is attributable to a ploidy level difference.

4. The species show ecological divergence in the form of geographical range, climatic niche and/or flowering time differences.

5. The species differ in terms of their palatability and thus their pasture value.

METHODS

Morphology

For the morphological analysis, the approach of Mallet (1995) was followed, which delimits species as phenetic clusters based on morphology and genotype. This approach assumes that genetic and morphological variation is greater between species than within them, such that discrete clusters identified by multivariate statistical approaches will indicate phenetic gaps denoting species boundaries (Sites and Marshall 2004). It was first investigated whether phenetic clusters could be resolved for individuals occurring in sympatry. A similar approach was then applied to over 500 herbarium specimens to determine whether the same distinctions held true for the whole of the GCFR.

Twenty-five specimens (housed at the Bolus Herbarium [BOL], University of Cape Town) of each of two putative species within *E. calycina* that occurred sympatrically at 12 sites in the GCFR (primarily in the Namaqualand region; see Table 1) were assessed for morphological differences, using both continuous and discrete traits. Continuous traits are summarised in Table 2. Each measurement was taken for a single representative example of the relevant character. Discrete traits were: whether or not the leaf margins were sinuated (i.e. having a wave-like structure, creating indentations where the edge of the wave troughs have become folded); and whether the culms at the base of the specimen were spreading (i.e. remaining close to the ground and spreading away from each other before becoming upright) or upright (i.e. all being perpendicular to the ground at the base).

The existence of morphologically distinct groups (or phenetic clusters) across these sites of sympatry was assessed using principal components analysis (PCA) of the 10 continuous trait variables. To determine the degree to which the discrete traits correlated with the continuous variation, both discrete traits were plotted onto the resulting PCA plots, and differences in mean PC 1 and PC 2 values for each were assessed using Wilcoxon's rank-sum tests.

Once the phenetic clusters had been identified, the individual continuous traits that discriminated most strongly between them were identified using Wilcoxon's rank-sum test. Based on these tests a subset of the continuous traits was selected for assessing the existence of comparable morphometric groups across a broader sampling of *E. calycina* specimens from the western GCFR. In total this analysis included 491 specimens, in addition to the 25 used in the sympatric analysis, all of which are housed at either BOL or the Compton (NBG) and South African Museum (SAM) herbaria, Kirstenbosch National Botanical Gardens, Cape Town. Base angle and the presence or absence of sinuated leaf margins were also noted for the additional specimens.

PCA was performed on this expanded data set to determine whether phenetic clusters could be discerned without any *a priori* grouping of specimens. In order to determine the extent to which all specimens across the western GCFR could be assigned to the morphometric groups identified in the analysis of sympatric specimens, a linear discriminant analysis (LDA) was applied to the continuous trait data for all specimens, using the sympatric specimen data (with the reduced set of continuous variables) as the training set. Group assignments based on the LDA results were used in all later analyses, with specimens that could not be assigned to one or other form with greater than 90% confidence being excluded. Variation in individual continuous traits between these groups was assessed using either Welch's t-test or Wilcoxon's rank-sum test. In addition, the degree to which these groupings associated with each of the discrete traits (base angle and sinuated leaf margins) was assessed using Pearson's Chi-squared test for independence with Yates' continuity correction.

The two phenetic clusters identified were termed 'slender' and 'robust' forms of *E. calycina*. The slender forms are characterised, among other features, by: thin (< ~0.15 mm) leaves which feel soft to the touch; generally having sinuations along the leaf margin; and generally having culms spreading at the base. In contrast, the robust forms generally have thick (> ~0.15 mm) and relatively long (> ~15 cm) leaves which feel coarse to the touch; a lack of sinuated leaf margins; and upright culms at the base (see RESULTS). Based on these characters, all individuals could be identified as one of these forms without difficulty in the field during data collection for further analyses (see below).

Chromosome counts

Suitable cytogenetic material was collected from immature spikelets of twelve specimens (two of each form at each of four localities, two where the forms occurred sympatrically, and one for each where they did not) during August 2013 in the Namaqualand region of the GCFR (see Table 1). The material was collected between 09h00 and 12h00, and fixed in the field using Carnoy's (1886) fixative (100% ethanol: chloroform: glacial acetic acid; 6: 3: 1, by volume). Material was kept in the fixative for three to five days before being transferred to 70% ethanol and stored at 4 ˚C. Voucher specimens representing all collections are deposited at BOL. For meiotic analysis, anthers were dissected out under a dissecting microscope and the sporogenous material squashed out in a drop of acetic carmine on a clean microscope slide, and squashed under a cover slip. A deep violet stain was produced as the acetic carmine reacted with oxides on the forceps (Darlington and LaCour 1976). Chromosome counts were done on a Leica DM 750 microscope. A minimum of eight chromosome counts (number of bivalents or 'gametic numbers' [Spies et al. 2002]) were obtained for each specimen from cells undergoing meiotic division I and the ploidy level determined on the basis of these counts.

Flowering time

Flowering time variation between the two forms was determined using both herbarium specimen and field-sampled data. As specimens are usually collected when flowering, collection date was used as a proxy for flowering time, and differences between the forms were tested for using Welch's t-test. Because *E. calycina* flowers throughout the summer (Gibbs Russell et al. 1990), specimens collected in the first half of the year were regarded as late- rather than early-flowering. Thus the dates of specimens collected in the second half of the year were recoded as [-1*(number of days before December 31)]. Specimens with collection dates in March-May were excluded as these dates are likely to be incorrect. To determine whether (i) collection date differed significantly between forms, (ii) whether differences in collection date between forms correlated with climate, and (iii) whether the latter relationship differed between forms, ANCOVA was used to predict collection date based on bioclimatic PC 1 score (identified in the climatic niche analysis [see below]) and form.

As another means of assessing flowering time differences between forms, field surveys were conducted at two localities in Namaqualand where both forms co-occurred: 'Skilpad' in the Namaqua National Park and 'Farm' along the Meselpad road south of Springbok (see Table 3). At each site, ten 25 m x 2 m transects were run. Each individual along each transect was identified as either robust or slender, and its flowering status noted as either 'in anthesis' (one or more spikelets in anthesis) or 'pre-anthesis'. The effect of site and form on proportion of individuals in anthesis was assessed using a generalised linear model (GLM) with a binomial error distribution. Proportions were only included in the analysis if more than five individuals of either form had been recorded in a given transect.

Geographic range and climatic niche

The geographic distributions of herbarium specimens assigned to each form were plotted in order to visually assess range differences. To test for differences in climatic niche, a PCA was conducted on 19 bioclimatic variables plus altitude from the Worldclim v1.4 dataset (Hijmans et al. 2005) for the GCFR, and the PC scores extracted for each specimen locality. Differences in climatic niche occupancy between forms were assessed by plotting the climatic PC 1 against PC 2 for all localities of each form. Welch's t-test was used to test for significant differences in PC 1 and PC 2 scores between the forms. Significant differences in key climatic variables between the forms were also tested for, using Welch's t-test or Wilcoxon's rank-sum test.

Palatability

To test for differences in palatability between the forms, field surveys were conducted at two sites where the forms occurred sympatrically, one situated within ('Skilpad') and one outside ('Farm') the Namaqua National Park. Because of protection from domestic livestock within the national park, the Skilpad site was assumed to have relatively low grazing pressure. In contrast, conversation with the landowner at the Farm site suggested that it was subject to frequent grazing by livestock, and thus the grazing regime at the two sites was assumed to differ. The same transects were used as for the flowering time field survey (see above). Every individual within each transect was identified to form and the distance from the centre of the grass tuft to the edge of the nearest live shrub was measured. If the individual was inside the shrub, its distance was coded as negative. For statistical analysis, distances were positivised by adding a constant to all values, and then square root transformed to improve normality. The transformed data were analysed using MANOVA, with form and site as predictor variables. In addition, differences in location (outside or on the edge of shrubs vs. within shrubs) between forms were assessed for each site separately, using Pearson's Chi-squared test for independence with Yates' continuity correction.

All statistical analyses were performed in R v3.0.2 (R Development Core Team 2013). The package 'raster' (Hijmans 2013) was used for geographical analyses, and 'MASS' (Venables and Ripley 2002) was used for the LDA of morphological data.

RESULTS

Morphology

Sympatric specimens

Amongst specimens sampled from sites of sympatry, PCA identified two distinct phenetic clusters (termed 'robust' and 'slender') which separated along the first PC axis, this explaining 49% of the variance in the data, compared to 20% for PC 2 (Figure 1). Important traits separating the clusters included, in order of importance: leaf thickness; spikelet length; length of the fertile lemma; leaf length; and glume length/spikelet length (Table 4; Figure 2). The robust cluster was associated with high values for all traits except the last, which was generally greater in slender plants. Except for fertile lemma length, which correlates very closely with spikelet length (Figure 3), and whose measurement requires a time-consuming spikelet dissection, all of the above mentioned traits were chosen for morphological analysis of a broader sampling of western GCFR specimens of *E. calycina*.

Considering only material from sites of sympatry, the presence or absence of leaf sinuations separated the two forms perfectly, with robust forms always lacking sinuations. Base angle performed slightly less well in separating the two forms, with two slender forms and all robust forms having upright bases (Figure 1). Both of these traits divided the total specimen set into groups whose PC 1 scores differed significantly (leaf sinuations present vs. absent: W = 156, $p < 0.0001$; base spreading vs. upright: W = 144, $p < 0.0001$).

All specimens

The PCA of continuous traits for all specimens suggested the presence of two phenetic clusters that separated primarily along PC 1, which explained 51% of the variance in the data, compared to 23% for PC 2, although there is some ambiguity (Figure 4). These phenetic clusters corresponded with those identified for the sympatric specimens, with robust forms having higher PC 1 values (greater than ~1), corresponding to longer spikelets, thicker and longer leaves, and lower glume length/spikelet length (Table 5).

The LDA, which used prior groupings based on the analysis of specimens in sites of sympatry, identified over 91% of specimens as either robust or slender with more than 90% confidence. This figure decreased to 79% at the 99% confidence level (Table 6). The high level of confidence in assigning specimens to forms is reflected in the histogram of posterior probabilities, which highlights the low frequency of specimens that could not be assigned with confidence to either form (Figure 5). In addition, all four continuous traits differed significantly between forms (Figure 6).

In terms of the discrete traits, the forms separated most strongly according to the presence or absence of sinuated leaf margins (Table 5), but both traits showed a significant association with the groups identified in the LDA with greater than 90% confidence (leaf sinuations present vs. absent: $\chi^2 = 149.9$, df = 1, $p < 0.0001$; base spreading vs. upright: $\chi^2 = 112.0$, df = 1, $p < 0.0001$).

Isolating mechanisms

Ploidy level

Chromosome counts (n = 3 per form) confirmed that specimens belonging to the robust form of *E. calycina* are consistently tetraploid (n = 24), whereas slender form specimens are consistently diploid (n = 12), although there was potential evidence of aneuploidy, with mean chromosome numbers ranging up to 14 for slender and 30 for robust form specimens (Table 8).

Flowering time

The ANCOVA revealed a significant difference in herbarium specimen collection dates between the forms (Table 9). Furthermore, collection date correlated positively with PC 1 from the climate analysis, and the interaction term between PC 1 and form was not significant, suggesting that both forms are collected earlier in areas with drier climates relative to areas with wetter climates (Figure 7). Thus, irrespective of climate, slender forms are collected later than robust forms. On average, slender forms are collected 21.1 days later than robust forms (slender mean [± SD] = 78.0 [± 41.6] days before Dec 31; robust mean [± SD] = 99.1 [± 39.9] days before Dec 31).

GLM results from the field survey data showed evidence for a difference in flowering time between the forms, but this was dependent on locality, with the robust form having a smaller proportion of individuals in anthesis than the slender form at Skilpad, but both forms having a similar proportion in anthesis at the Farm (Table 10, Figure 8).

Geographic range

The two forms of *E. calycina* show a high degree of overlap in geographic range (Figure 9), although there are some important differences. The robust form is largely absent from the Overberg region to the south and east of the Cape Peninsula, whereas the slender form is widespread there (Figure 9, A). In addition, the slender form is absent from the more arid areas of the GCFR, including along the interior escarpment (Figure 9, B) and northern Namaqualand (Figure 9, C), where the robust form occurs.

Climatic niche

The first and second principal components of bioclimatic variables explained 39% and 27% of the variance in the data, respectively. PC 1 is most strongly associated with variation in the seasonality of both temperature and precipitation and differences in aridity, such that areas with low PC 1 scores have, among other features, higher mean temperatures especially during the dry summer months, greater seasonal variation in temperature, and lower precipitation over the whole year and during the driest quarter (Table 11). PC 2, by contrast, is more strongly associated with altitude, distinguishing the higher altitude summer-rainfall interior of South Africa from the generally lower altitude, winter-rainfall GCFR (Figure 10). The robust and slender forms showed a high degree of overlap in climatic niches, although the robust form appears to be more strongly associated with low PC 1 scores, indicating an association with conditions of greater aridity. In contrast, the slender form is distributed across a broad range of PC 1 scores, and instead is clustered somewhat towards higher PC 2 scores (Figure 11). Mean PC 1 scores differed significantly between forms (robust mean [±SD] = 0.38 [±2.86], slender mean [±SD] = 2.94 [±3.56]; t = -7.78, edf = 373.4, $p < 0.0001$), whereas PC 2 scores did not (robust mean [±SD] = 0.84 [±2.00], slender mean [±SD] = 1.11 [±2.03]; t = -1.31, edf = 334.0, p = 0.191).

In addition, slender and robust forms differed significantly in selected bioclimatic variables, with robust forms occurring at sites characterized by higher seasonality, higher maximum temperature of the warmest month, lower annual precipitation and lower precipitation during the driest quarter (Figure 12).

Palatability

The final model indicated significant differences in the distribution of the two forms in relation to shrubs, irrespective of site, and hence the interaction term was not included in the final model (Table 12). Slender forms occurred further inside shrubs than robust forms, which, on average, occurred ~10 cm outside shrubs at the Farm (presumed to be grazed), but ~13 cm within shrubs at Skilpad (which was presumed to have little to no grazing) (Figure 14). Despite occurring within shrubs on average, at Skilpad 25% of robust forms occurred outside shrubs, and 50% occurred either outside or on the edge of the nearest shrub (Figure 14; Table 13). In contrast, over 75% of slender form individuals occurred within shrubs at Skilpad. These differences were more pronounced at the Farm site, but at both sites the location of individuals with respect to shrubs was significantly associated with form (Farm: $\chi2 = 25.44$, df = 1, $p < 0.0001$; Skilpad: $\chi2 = 6.924$, df = 1, p = 0.0085).

DISCUSSION

This study provides evidence for the existence of two distinct species within *E. calycina*. These species show clear differences in morphology based on several traits at sites where they occur sympatrically. Furthermore, analysis of a smaller set of traits from a broader sampling of specimens across the GCFR corroborated this finding. Evidence for morphological differences being maintained in sympatry suggests the existence of intrinsic reproductive isolating barriers between the species. A difference in ploidy level provided evidence for this, with the robust species being tetraploid and the slender species being diploid, while evidence for significant bioclimatic differences suggests that the species also represent ecologically independent entities. Finally, there is evidence that these species differ in palatability. Overall, aside from having obvious taxonomic implications, these results have practical implications for the management of grazing land with regard to this widespread and economically important species complex.

Two species within *E. calycina*

Multiple lines of evidence support the hypothesis of at least two distinct, reproductively isolated species existing within *E. calycina*. Importantly, there is clear morphological distinctness between two forms occurring in sympatry, and these can be distinguished according to both discrete and continuous trait variation (the latter including both single and multivariate traits) (Figures 1 and 2). The fact that morphological distinctness is maintained in sympatry suggests that the two forms are reproductively isolated. Although this distinctness is less pronounced when all GCFR specimens are considered (Figure 4), 91% of all specimens can be assigned to one of the sympatrically occurring forms with greater than 90% confidence on the basis of just four continuous traits (Table 6). Furthermore, variation in the two discrete traits considered conforms well to this classification (Table 7).

Further support for the existence of reproductive isolating barriers between these forms is provided by evidence for a difference in ploidy, with the robust form being tetraploid (n = 24) and the slender form being diploid (n = 12, the base chromosome number of *Ehrharta* [Spies et al. 2002]) (Table 8). This difference represents a strong intrinsic post-zygotic isolating mechanism due to the fact that crosses between diploid and tetraploid cytotypes in plants often fail to produce viable seed, and if triploid progeny do form, they are usually inviable (Ramsey and Schemske 1998). This is supported by the fact that a triploid specimen of *E. calycina* has never been observed despite numerous chromosome counts having been done for the complex (Spies et al. 2002).

The two forms also show significant differences in climatic niche, with the robust form being primarily associated with drier areas and the slender form having a relatively broad climatic range (Figure 11). Importantly, this suggests that in addition to showing reproductive isolation through polyploidy, the two forms represent ecologically independent entities.

Overall, these findings suggest that two morphologically and ecologically distinct, reproductively isolated species exist within *E. calycina*. However, there remains the possibility that more than two species may exist within the complex, particularly considering the high degree of variation in morphology and life history within the slender species.

Implications

These results have obvious taxonomic implications. One of the species will have to be named and formally described, probably taking the name of a previously described variety of *E. calycina*.

However, the identities of the relevant type specimens remain uncertain, thus precluding the naming of each species for the time being. Nevertheless, some predictions can be made based on the available material. The robust species resembles the type specimen of *Ehrharta versicolor* Schrad., which Schrader (1821) described as a distinct species, having (among other features) erect culms, flat leaves, and the glumes shorter than the outer (infertile) lemmas/valves. Stapf (1900, p.675) preferred to view *E. versicolor* as a variety of *E. calycina*, and refined its description, noting its rigid leaves up to one foot long as well as long (3–4.5 lin.) spikelets with 'the lower empty valve slightly exceeding the lower glume'. It is not known whether the type of *E. calycina* fits the description of the robust or slender species, as neither the material nor the original description can be accessed at present. However, the slender species fits Stapf's (1900, p. 675) description of *E. calycina*, which he notes as having (among other features) 'culms usually geniculate'; 'blades… long and gradually tapering or shortly acute…, 1–4 lin. by 1–3 lin.'; '[blade] margins sometimes wavy'; 'spikelets… 2.5–3 lin. long'; and the lower lemma 'as long as the lower glume or shorter'. Overall, this suggests that the slender form is likely to retain the name *E. calycina*, while the robust form will probably take the name *E. versicolor*.

This finding also has significance of a practical nature. Based on their fine-scale distribution in relation to shrubs, the two species appear to differ in palatability (Figures 13 and 14; Table 13). However, an unexpected finding was that both species were found, on average, further within shrubs at the site that was presumed to have low grazing levels (Skilpad). The shrubs at Skilpad were larger and more closely spaced (SM pers. obs.) than at the Farm, which might explain this result, as the grasses would be more likely to occur within shrubs if they were distributed randomly, and more likely to occur further within shrubs simply due to the shrubs' larger size. Furthermore, the grazing regimes at the two sites may have differed from what was presumed. This might explain why a greater proportion of slender individuals was found outside of shrubs at the Farm than at Skilpad. Despite these uncertainties, the fact that the binary distribution of the species in relation to the edge of shrubs was non-random at both sites still suggests that the robust species is less prone to herbivory than the slender species.

There is also the possibility that the observed distribution pattern might be explained by the recruitment patterns of the two species, rather than differences in palatability. Verboom et al. (2012), considering only the slender species of *E. calycina*, showed that it varies in life history such that in arid areas it is either functionally-annual or summer deciduous. In contrast, based on various traits – including relatively thick, long and rigid leaves and a generally robust growth habit – the robust species appears to be perennial. If both species are more likely to recruit under shrubs (De Villiers et al. 2001), then the higher frequency of seedling recruitment of the annual slender species may explain its high probability of occurrence within shrubs. In contrast, the robust species may outlive its nurse plant and hence occur in the open more often (Zacharias 1990). In this regard, feeding preference experiments (e.g. Prince et al. 2004) using ungulate herbivores would be useful in confirming whether the species differ in palatability. However, if the robust species is palatable, it is unlikely that it would persist in the open as frequently as was observed.

Thus, overall, the two species are likely to differ in palatability, and this suggests that they differ in ecological function. Whereas the slender species is palatable and may avoid herbivory by being functionally-annual or summer deciduous in more arid regions (Verboom et al. 2012), the robust species' frequent distribution outside shrubs and perennial life history suggests that it is capable of tolerating herbivory, thus having little to no pasture value. This has implications for the management of grazing land in relation to the *E. calycina* complex (Briske 1996), which has previously only been reported as having high pasture value in general (Van Breda and Barnard 1991; Gibbs Russell et al. 1990).

Polyploidy: isolation and speciation

Evidence for extensive overlap in both flowering time (Figure 7) and geographic range (Figure 9) suggests that ecological factors probably play only a limited role in maintaining the reproductive isolation of the two species. This points to polyploidization as a likely isolating mechanism. Soltis et al. (2007) identified autopolyploidy as an important, and perhaps underappreciated, mode of speciation in plants, particularly in angiosperms. Along with others (e.g. Husband and Sabara 2004), they have found that autopolyploid forms of many angiosperm species, which are often regarded merely as cytotypes, exhibit properties which identify them as distinct species according to many of the traditional species concepts. Critically, in contrast to the traditional view – which emphasised the possibility of gene flow between cytotypes via the formation of unreduced gametes in the diploid – pre-zygotic reproductive isolating barriers often exist between autopolyploids and their progenitors (Soltis et al. 2007). In the case of *E. calycina*, ecological isolation through climatic niche differentiation appears to be an important pre-zygotic barrier in the context of polyploidy.

For polyploid speciation to occur, the neopolyploid must establish a viable population (Sobel et al. 2010). Because autopolyploids originate in sympatry or parapatry with their progenitors, this requires them to overcome their minority cytotype disadvantage (Levin 1975). This may be facilitated by various processes, including assortative mating arising from ecological differentiation (Sobel et al. 2010), and recurrent polyploid formation (Soltis and Soltis 1999).

In the context of *E. calycina*, this raises the question of what facilitated the establishment of a viable population of the robust species following polyploidization. Assortative mating may arise through habitat isolation (Sobel et al. 2010). Analysis of climatic niche differences suggests that the robust species is capable of surviving in more arid areas than the slender species (Figure 11). It is possible, therefore, that the robust species initially became established by occupying drier, hotter sites within or at the edge of the range of its progenitor (Ramsey and Schemske 2002), with subsequent dispersal back into current sites of sympatry.

Assortative mating might also have resulted from a difference in flowering time between the species. However, the two analyses of flowering phenology in this study present conflicting results. According to collection date, the robust species flowers earlier than the gracile species, irrespective of climatic variation (Table 9; Figure 7), whereas field survey data suggest that the robust species flowers significantly later than the slender species, although this varies between sites (Table 10; Figure 8). Regardless, neither analysis suggests that the flowering times of the two species are exclusive. To the contrary, there appears to be extensive overlap. Thus it seems that a flowering time difference contributes little to the overall reproductive isolation of the two species at present, and therefore may not have been involved in the initial establishment of the robust species.

The chance of a polyploid establishing a viable population can also be increased by recurrent polyploidy (Soltis and Soltis 1999). There may be some evidence for this in *E. calycina*, as some specimens conforming to the robust species have been collected in uncharacteristic climatic areas (Figure 11). Although this is circumstantial evidence, it does support the possibility that polyploidization occurs relatively frequently within the complex, suggesting that recurrent polyploidy might have contributed to the establishment of the robust species.

Polyploidy and novelty in *E. calycina*

That speciation in *E. calycina* appears to have proceeded via polyploidy raises the question of which key traits (i.e. those important in facilitating the establishment of the robust species and in maintaining the isolation of the two species) were directly precipitated by polyploidization, and which arose subsequent to the establishment of independently evolving lineages. Considering that speciation within *E. calycina* most likely occurred in sympatry or parapatry, the most important traits involved in the establishment of the robust species would have been those that arose earliest (Sobel et al. 2010). Thus, the novelty directly associated with polyploidization in *E. calycina* is likely to have been crucial to the speciation process. Such novelty is well-documented in neopolyploids, which often exhibit ecological characteristics that may result in pre-zygotic reproductive isolation (reviewed in Ramsey and Schemske 2002). In a pertinent example, Stebbins (1949; 1985) conducted a long term study on the success of populations of the diploid versus artificially produced tetraploid progeny of *Ehrharta erecta* plants, when introduced to several sites in central California. Tetraploid populations only survived in two of 22 original sites, and occupied a markedly narrower habitat range than the diploids, which often spread to new areas and colonised a diversity of habitats. Differences in climatic niche between the species within *E. calycina* are consistent with this, with the robust species occupying a narrower climatic range than the slender species. Importantly, though, the robust species appears to be capable of occupying areas that the slender species cannot. Thus, immediate habitat isolation following polyploidization seems a plausible mechanism for the initial establishment of the robust species.

The different morphology of the robust species may also have been directly precipitated by polyploidization. Stebbins (1949, p. 465) found that, in *E. erecta*, artificial autotetraploids are 'taller, coarser… [and the] spikelets are characteristically larger' than their diploid progenitors, which is consistent with general findings regarding phenotypic changes in relation to polyploidization in plants (Ramsey and Schemske 2002). The results of this study thus conform to both general and *Ehrharta*-specific findings in this regard.

Experiments involving artificially produced autotetraploids of the slender species would be useful in assessing the significance of polyploidization in the initial divergence of the two species (Ramsey and Schemske 2002). Genotypic differences in flowering time and climatic tolerance between diploid and tetraploid offspring of the same plants would allow the importance of each in the initial pre-zygotic isolation of the robust and slender species to be inferred. Levels of initial post-zygotic isolation could also be inferred using fertilization experiments involving these plants. The artificial production of autopolyploids should take into account variation within the slender species, which itself may comprise multiple diploid species. Fertilization experiments would also be useful to determine the current extent of post-zygotic isolation between the species.

Conclusions

This study appears to be the first to develop a case for polyploid speciation in the GCFR. Krejčíková et al. (2013a, b) found evidence for both broad- and fine-scale ecological differentiation within the widespread *Oxalis obtusa* (Oxalidaceae) in relation to cytotype, but did not ascribe cytotypes to species due to the high degree of ploidy level variation and the complexity of the ecological patterns. In contrast, the *E. calycina* complex shows just two cytotypes with distinct morphological and ecological characteristics, and thus each merits full species status. Aside from the obvious taxonomic implications, this finding also has considerable practical significance due to probable differences in palatability between the species.

Considering the high degree of ploidy level variation across *Ehrharta* as a whole (Spies et al. 2002), this study should stimulate similar investigations into species limits within other multiple-cytotype complexes in the genus. In addition, it is surprising that polyploidy has been widely disregarded as a driver of diversification in the super-diverse GCFR (e.g. Linder 2005; Schnitzler et al. 2011), despite widespread recognition of its importance in generating plant diversity in general (Soltis et al. 2009). As Krejčíková et al. (2013a) note, this has probably been precipitated by findings suggesting low karyotypic diversity in many species-rich groups within the GCFR (Goldblatt 1978). However these authors also note that the assumption of low rates of intraspecific ploidy diversity in the Cape flora might simply be a consequence of insufficient sampling, with the majority of published chromosome counts being based on single specimens. This study thus highlights the need for more extensive research into the rate of intraspecific polyploidy in the GCFR, and, echoing the sentiments of Krejčíková et al. (2013a) and Soltis et al. (2007), suggests that polyploid speciation may be more common, both globally and locally, than current taxonomic classifications indicate.

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TABLES and FIGURES

Table 1: Localities of sympatrically occurring specimens used for morphological analysis

Locality	Voucher no.	Latitude	Longitude
NORTHERN CAPE - Kamiesberg, top of Kamiesberg Pass, Ou Tuin Farm	*Verboom 574*	30˚ 11′ S	18˚ 1′ E
	Verboom 575		
NORTHERN CAPE - Skilpad Nature Reserve, about 15 km W of Kamieskroon	*Verboom 577*	30˚ 10′ S	17˚ 47′ E
	Verboom 578		
NORTHERN CAPE - Kamieskroon area, SW of Skilpad Nature Reserve, Farm Brandkop	*Verboom 579*	30˚ 12′ S	17˚ 51′ E
	Verboom 580		
NORTHERN CAPE - Kamiesberg, top of Studer's Pass, near Wolfhoek	*Verboom 581*	30˚ 23′ S	18˚ 7′ E
	Verboom 582		
NORTHERN CAPE - Nieuwoudtville Wildflower Reserve	*Verboom 584*	31˚ 22′ S	19˚ 8′ E
	Verboom 585		
NORTHERN CAPE - N7, N of Garies	*Verboom 736*	30˚ 24′ 22" S	17˚ 55′ 47" E
	Verboom 737		
NORTHERN CAPE - Along N7, S of Kamieskroon	*Verboom 738*	30˚ 18′ 12" S	17˚ 53′ 39" E
	Verboom 739		
NORTHERN CAPE - SW of Springbok along road from N7 to Koingnaas, near Koereeberg	*Verboom 743*	29˚ 49′ 43" S	17˚ 46′ 36" E
	Verboom 744		
NORTHERN CAPE - SE of Garies, about 2km E of N7 along Buffelsfontein road / SE of Garies, about 3km E of N7 along Buffelsfontein road	*Verboom 747*	30˚ 33′ 45" S	18˚ 4′ 45" E
	Verboom 749	30˚ 38′ 29" S	18˚ 5′ 36" E
WESTERN CAPE - Kamannassieberg, base of mountain at Rietvlei, along road to Mannetjiesberg	*Verboom 1151*	33˚ 40′ S	22˚ 58′ E
	Verboom 1152		
	Verboom 1154		
NORTHERN CAPE - Top of Spektakel Pass, W of Springbok	*Verboom 1200*	29˚ 41′ 35" S	17˚ 39′ 4" E
	Verboom 1201		
NORTHERN CAPE - Top of Grootvlei pass, west of Kamieskroon / Between Kamieskroon and Grootvlei pass	*Verboom 1203*	30˚ 12′ 59" S	17˚ 46′ 4" E
	Verboom 1204	30˚ 12′ 35" S	17˚ 47′ 17" E

Table 2: Summary of continuous traits used in both morphological analyses. Traits in bold are those used in the analysis of all specimens

Trait	Mechanism	Precision
Awn length	Eyepiece graticule	0.1 mm
Spikelet length (excluding awn length)	Eyepiece graticule	0.1 mm
Lower glume length/Spikelet length	Eyepiece graticule	0.1 mm
Length of the fertile lemma	Eyepiece graticule	0.1 mm
Inflorescence length (from first node)	Ruler	5 mm
Plant height (including inflorescence)	Ruler	1 cm
Leaf length	Ruler	1 cm
Leaf width	Ruler	1 mm
Leaf thickness	Electronic digital vernier caliper	0.01 mm
No. of spikelets per inflorescence		~ 5 spikelets

Table 3: Summary of field activities in Namaqualand

Site	Co-ordinates	Altitude (m asl)	Activities/collections	Presumed grazing regime
Skilpad	30˚ 10' 10.5'' S, 17˚ 46' 25.8'' E	668	Palatability/flowering transects, cytological material (both forms)	Ungrazed
Meselpad road ('Farm')	29˚ 50' 09.6'' S, 17˚ 46' 35.8'' E	828	Palatability/flowering transects, cytological material (both forms)	Grazed
Kamieskroon roadside	30˚ 08' 21.1'' S, 17˚ 51' 51.1'' E	690	Cytological material of slender form	
Wildeperdehoek pass	29˚ 56' 36.6'' S, 17˚ 37' 43.3'' E	516	Cytological material of robust form	

Morphological analyses

Sympatric specimens

Table 4: First principal component scores for the PCA of sympatric specimens

Variable	PC 1 score
Leaf thickness	-0.403
Spikelet length	-0.381
Third lemma length	-0.381
Leaf length	-0.356
Glume length/Spikelet length	0.352
Plant height	-0.327
Inflorescence length	-0.322
Spikelets per inflorescence	-0.250
Leaf width	0.148
Awn length	-0.031

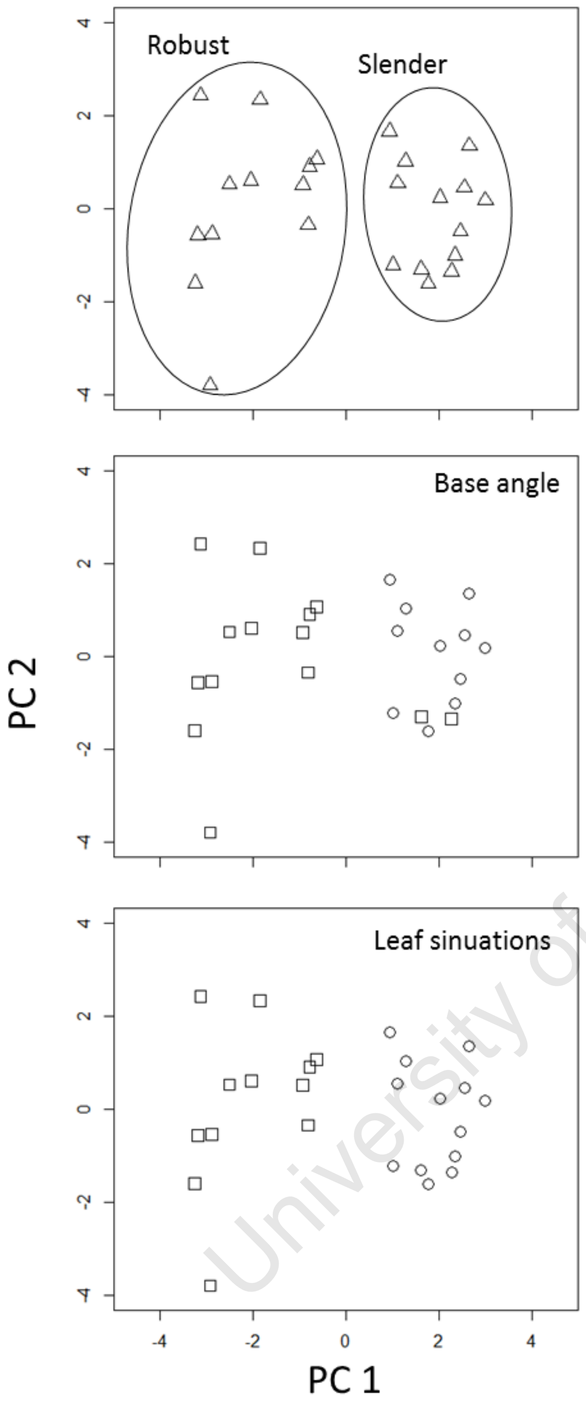

Figure 1: Plot comparing PCA scores based on morphological traits for sympatric specimens (top; two phenetic clusters circled [see text]), and with specimens distinguished by base angle (middle; squares = upright, circles = spreading) and by leaf sinuations (bottom; squares = absent, circles = present)

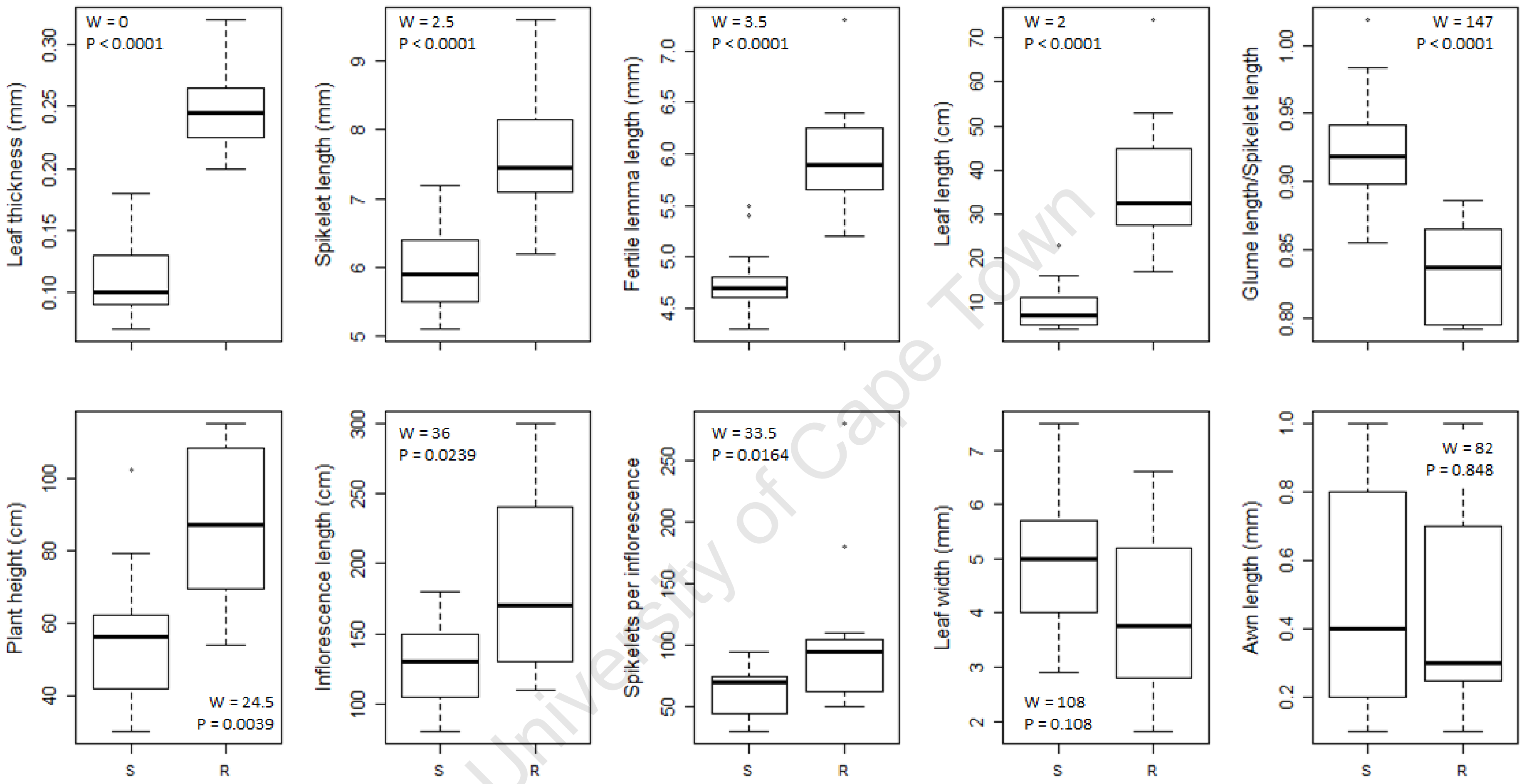

Figure 2: Boxplots comparing variation in morphological traits between phenetic clusters 'slender' (S) and 'robust' (R) identified in the multivariate analysis. Results for Wilcoxon's rank-sum test are shown for each trait. Traits are ordered by PCA score (see Table 2)

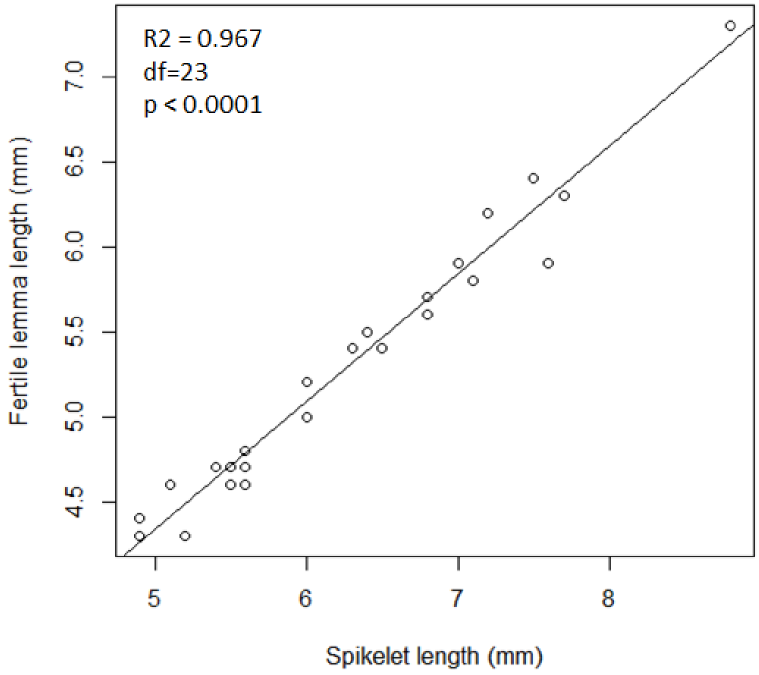

Figure 3: Correlation between spikelet length and fertile lemma length for the specimens in sympatry

All specimens

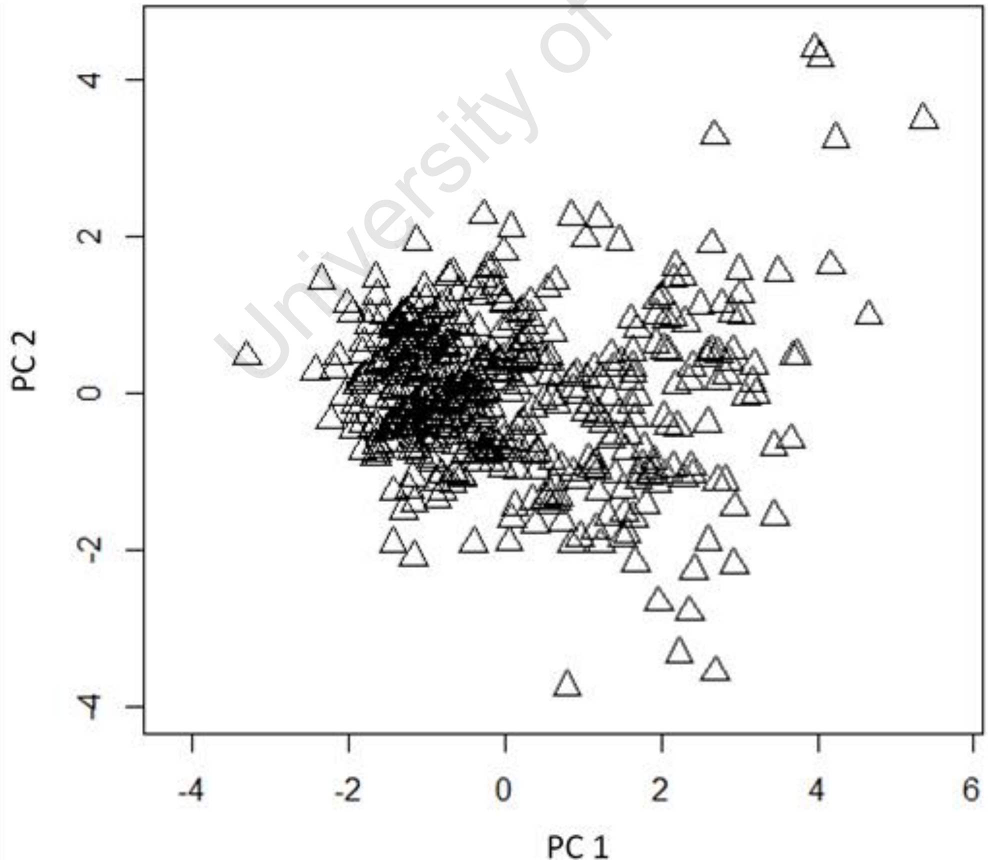

Figure 4: Plot comparing principal components of continuous traits for all specimens

Table 5: PCA scores for traits used in the analysis of all specimens

Variable	**PC1**	**PC2**
Spikelet length	0.564	-0.254
Leaf thickness	0.510	0.453
Leaf length	0.493	0.448
Glume length/Spikelet length	-0.424	0.728

Table 6: Percentage of specimens identified as either slender or robust by the LDA

Species	**Confidence level**		
	90%	**95%**	**99%**
Slender	56.2	54.7	49.8
Robust	34.9	33.1	29.5
Total	91.1	87.8	79.3

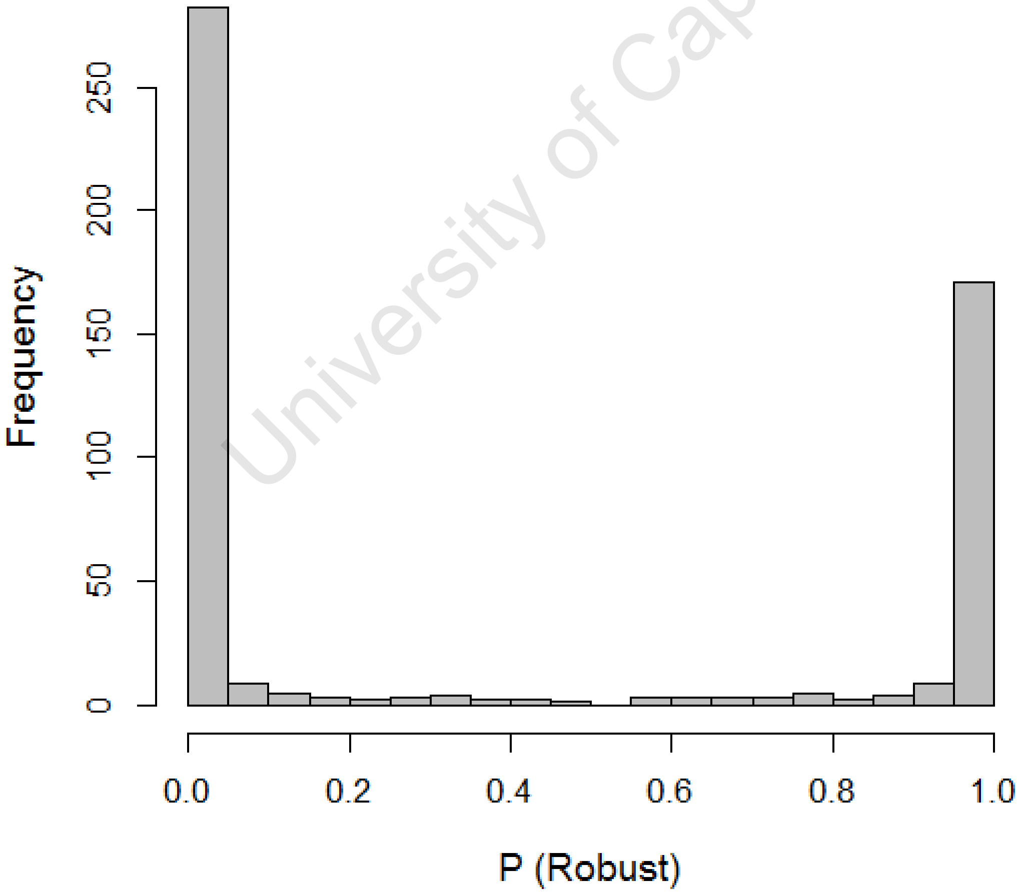

Figure 5: Histogram of probability of being robust as determined by the LDA

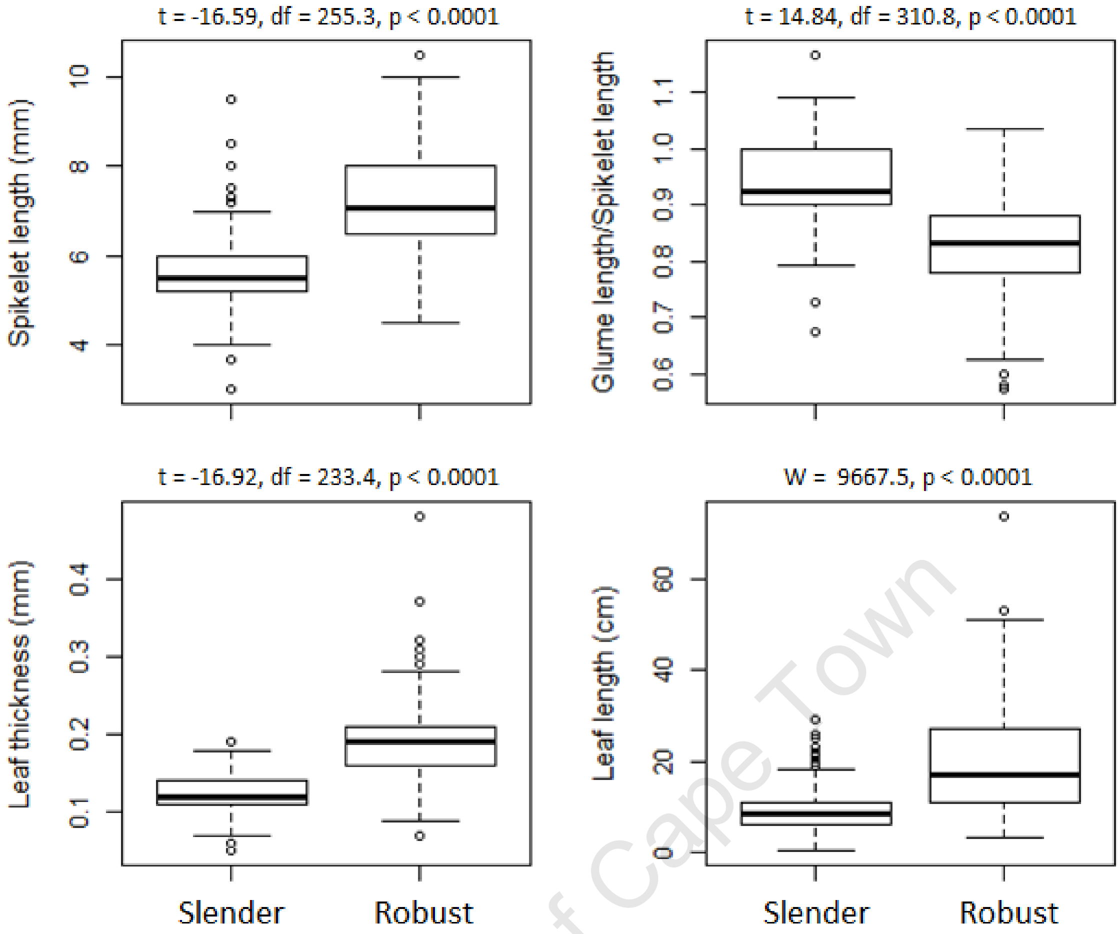

Figure 6: Boxplots comparing variation in morphological traits between forms identified in the LDA, with associated results of Welch's t-test or Wilcoxon's rank-sum test

Table 7: Contingency tables showing the assortment of the discrete traits by form (as defined by the LDA)

		Form	
Trait		**Slender**	**Robust**
Base angle			
	Spreading	194	35
	Upright	71	134
Leaf sinuations			
	Present	228	37
	Absent	62	143

Isolating mechanisms

Ploidy level

Table 8: Range and mean of meiotic chromosome counts for each form (localities as in Table 3)

			Chromosome count	
Form	**Voucher no.**	**Locality**	**Range**	**Mean**
Slender				
	SM0003	Skilpad	9 – 13	12
	SM0010	Farm	12 – 15	14
	SM0012	Kamieskroon	11 – 16	14
Robust				
	SM0001	Skilpad	26 – 33	30
	SM0007	Farm	20 – 24	22
	SM0005	Wildeperdehoek	22 – 26	24

Flowering time

Table 9: Summary of the ANCOVA results for collection date in relation to climate PC 1 and form

	Df	Deviance	Resid. Df	Resid. Dev	F value	Pr (>F)
NULL			304	515986		
Climate PC 1	1	41750	303	474236	27.2963	3.27E-07
Form	1	13400	302	460835	8.7612	0.003322
Climate PC 1:Form	1	449	301	460386	0.2935	0.588373

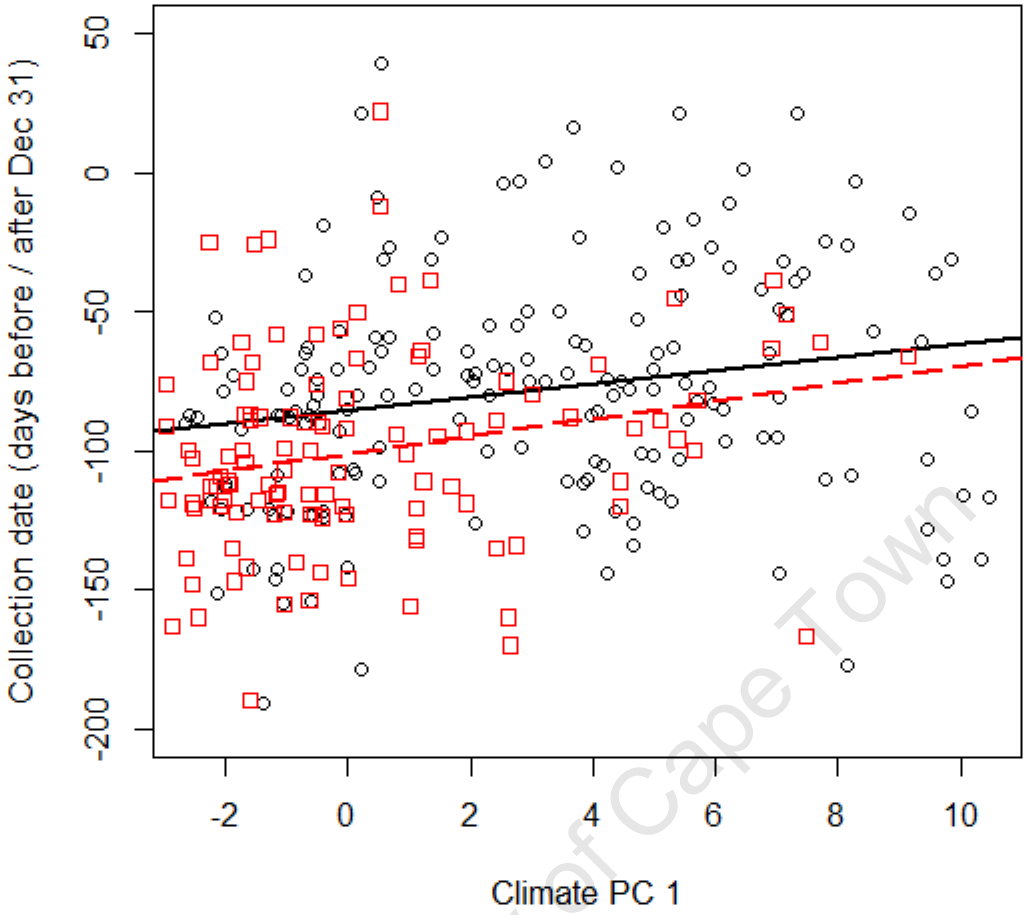

Figure 7: Collection dates in relation to climate PC 1 and form, with linear regression lines (robust forms = red squares, dashed red trend line; slender forms = black circles, solid black trend line).

Table 10: Summary of the GLM results for anthesis proportions

	Df	Deviance	Resid. Df	Resid. Dev	Pr(> χ^2)
NULL			27	101.068	
Form	1	17.187	26	83.881	0.00047
Site	1	32.935	25	50.945	<0.0001
Form:Site	1	13.68	24	37.265	0.00181

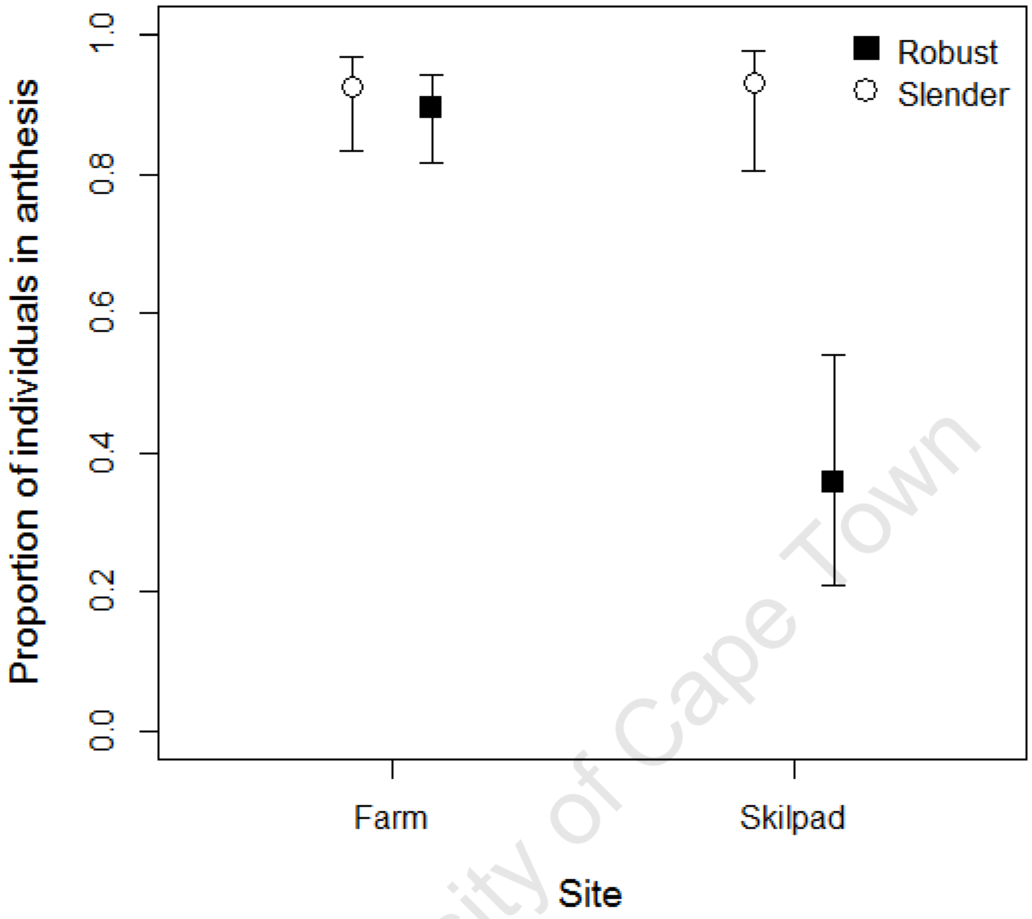

Figure 8: Predicted mean (±95% CI) proportions of individuals in anthesis per transect for each form at the two field sites (as in Table 3), based on the GLM

Geographic range

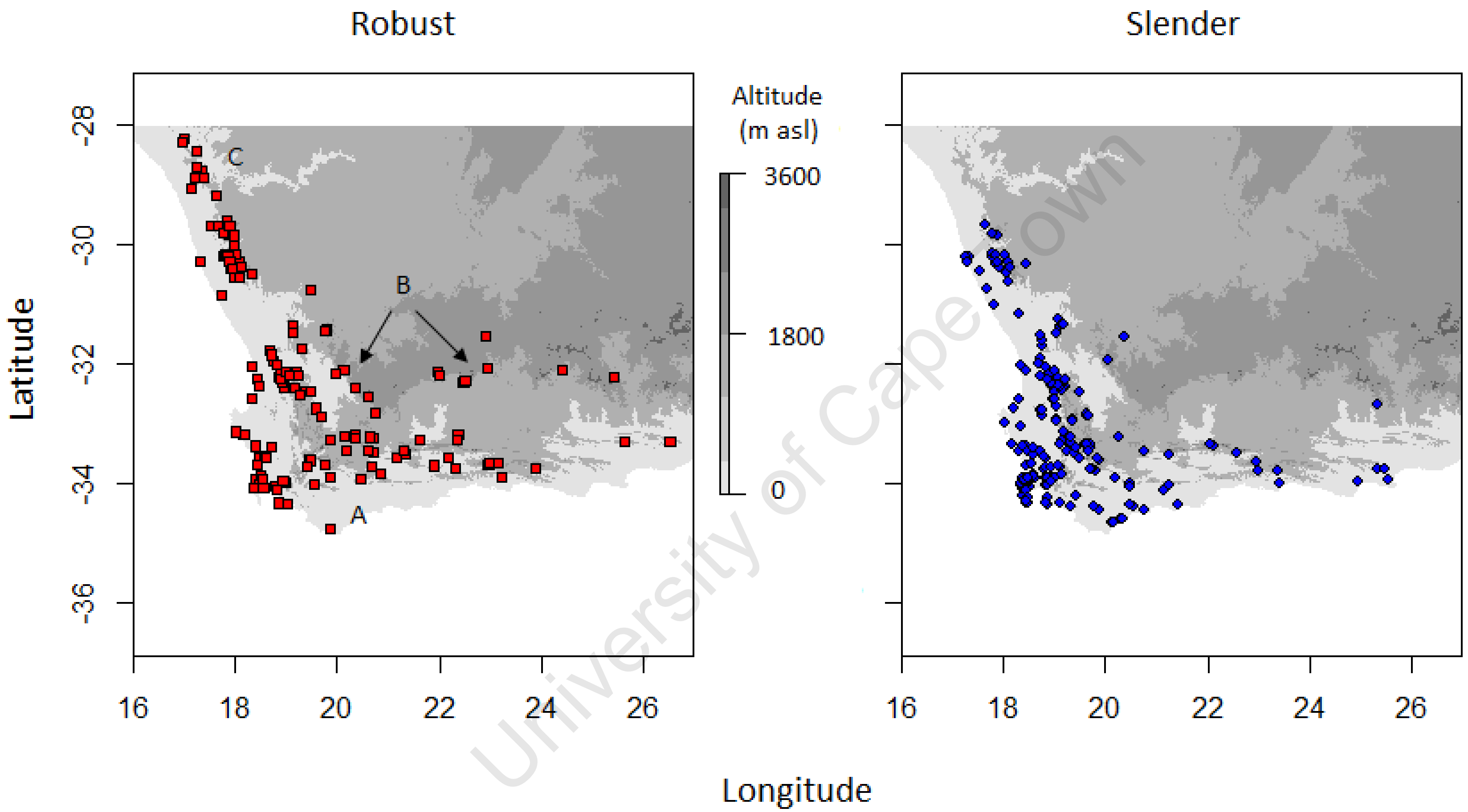

Figure 9: Geographic ranges of the different forms. Points represent specimen localities from BOL, NBG and SAM. Labels A, B and C referred to in text

Climatic niche

Table 11: Summary of bioclimatic variable PCA scores (ordered by absolute magnitude per variable type) for the GCFR. Variables used in the univariate analysis (Figure 12) are in bold type

Bioclimatic variable	**Variable type**	**Description**	**PC 1**	**PC 2**
	Temperature			
BIO2		Mean Diurnal Range (Mean of monthly (max temp - min temp))	-0.301	-0.148
BIO5		**Max Temperature of Warmest Month**	-0.291	0.071
BIO7		Temperature Annual Range (BIO5-BIO6)	-0.290	-0.194
BIO4		**Temperature Seasonality (standard deviation *100)**	-0.266	-0.211
BIO10		Mean Temperature of Warmest Quarter	-0.217	0.203
BIO6		Min Temperature of Coldest Month	0.152	0.375
BIO1		Annual Mean Temperature	-0.092	0.343
BIO8		Mean Temperature of Wettest Quarter	-0.064	-0.107
BIO11		Mean Temperature of Coldest Quarter	0.063	0.387
BIO3		Isothermality (BIO2/BIO7) (* 100)	0.054	0.212
BIO9		Mean Temperature of Driest Quarter	-0.018	0.322
	Precipitation			
BIO12		**Annual Precipitation**	0.322	-0.090
BIO17		**Precipitation of Driest Quarter**	0.298	-0.133
BIO14		Precipitation of Driest Month	0.297	-0.132
BIO16		Precipitation of Wettest Quarter	0.282	-0.048
BIO13		Precipitation of Wettest Month	0.275	-0.065
BIO19		Precipitation of Coldest Quarter	0.261	0.069
BIO18		Precipitation of Warmest Quarter	0.230	-0.217
BIO15		Precipitation Seasonality (Coefficient of Variation)	-0.112	0.195
	Altitude		-0.100	-0.377

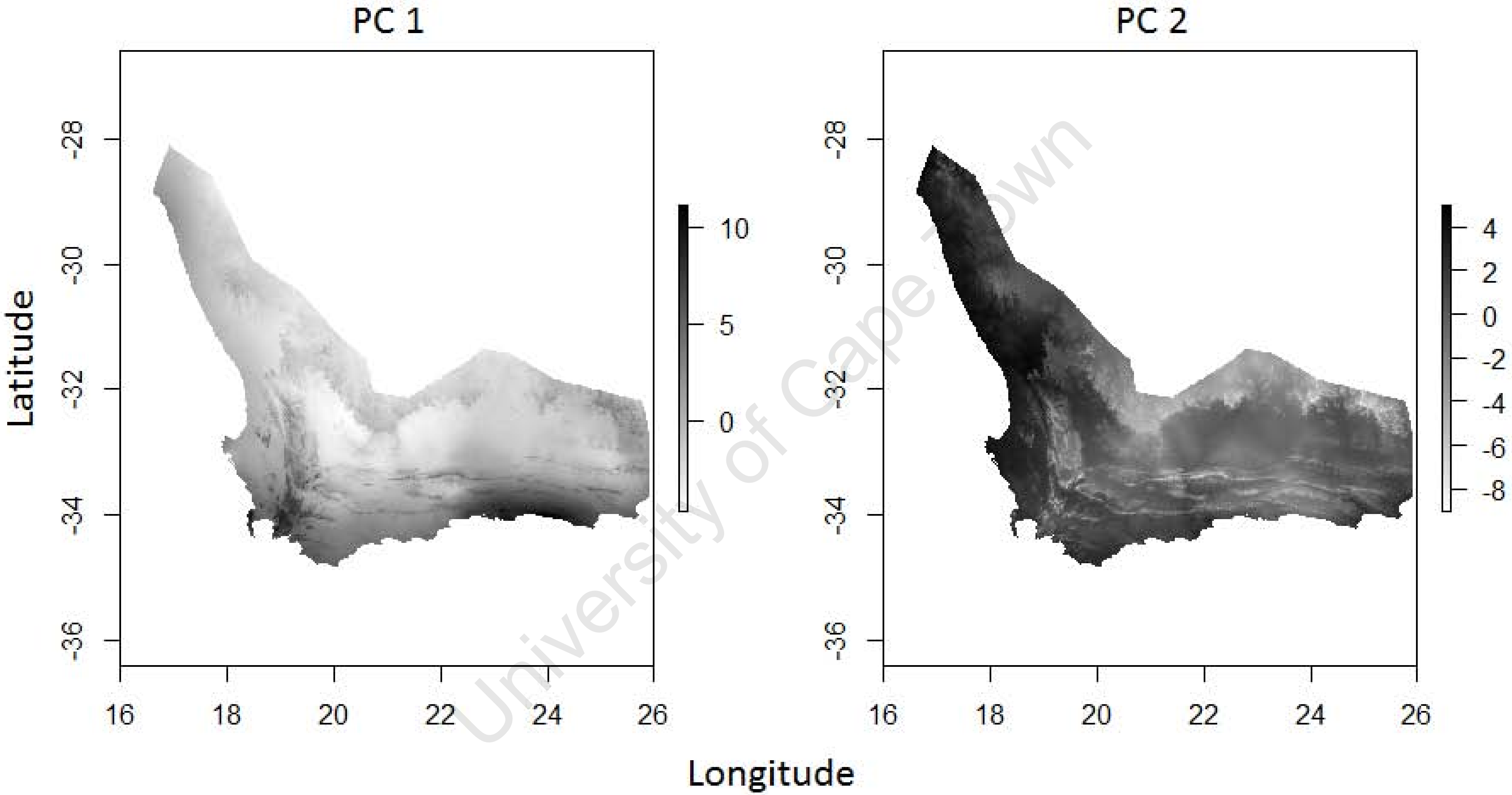

Figure 10: Maps showing geographic variation in PC1 and PC2 scores for the PCA on bioclimatic variables plus altitude

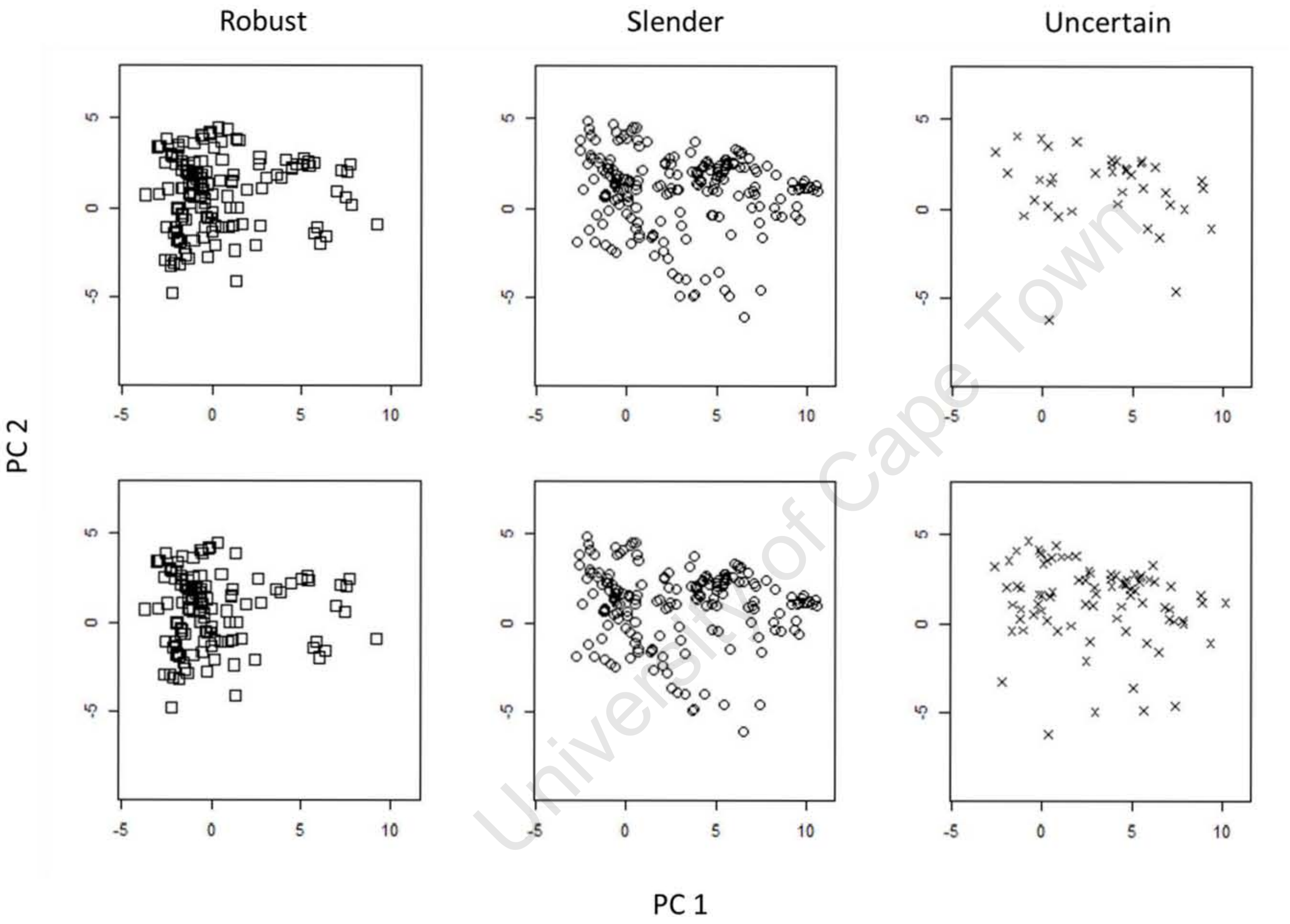

Figure 11: Principal component scores for robust and slender forms and uncertain specimens for bioclimatic variables plus altitude. Individuals identified with 90% (top) and 99% (bottom) confidence by the LDA

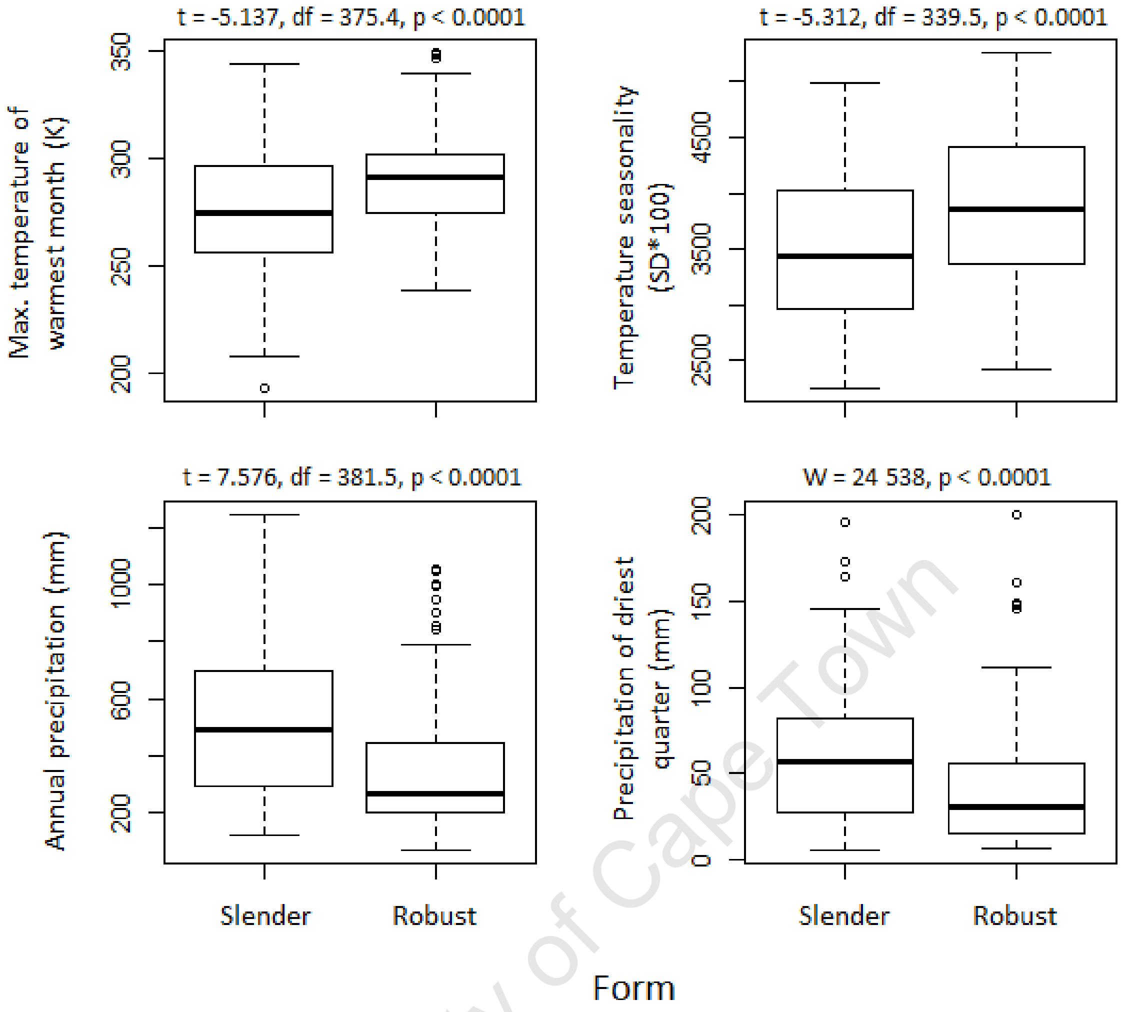

Figure 12: Boxplots of differences in key bioclimatic variables for slender and robust forms (identified with 90% confidence in the LDA). Results of Welch's t-test or Wilcoxon's rank-sum test are shown above each plot

Palatability

Table 12: ANOVA table showing importance of site and form in explaining distance from the edge of shrubs

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Site	1	110.72	110.72	63.735	<0.0001
Form	1	66.54	66.54	38.304	<0.0001
Residuals	352	611.47	1.74		

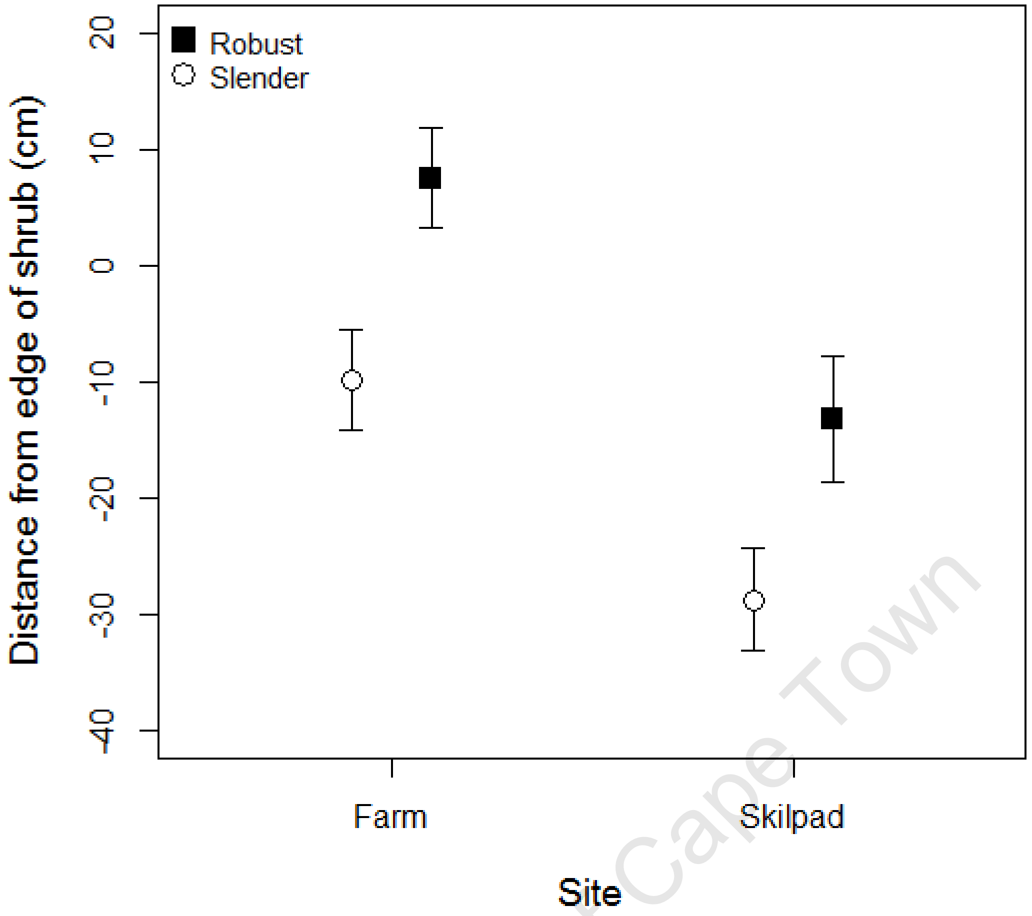

Figure 13: Predicted mean (±95% CI) distances from the edge of shrubs of both forms at the two sites where field surveys were conducted. The Farm site was presumed to be regularly grazed, whereas the Skilpad site was presumed to experience a low grazing intensity

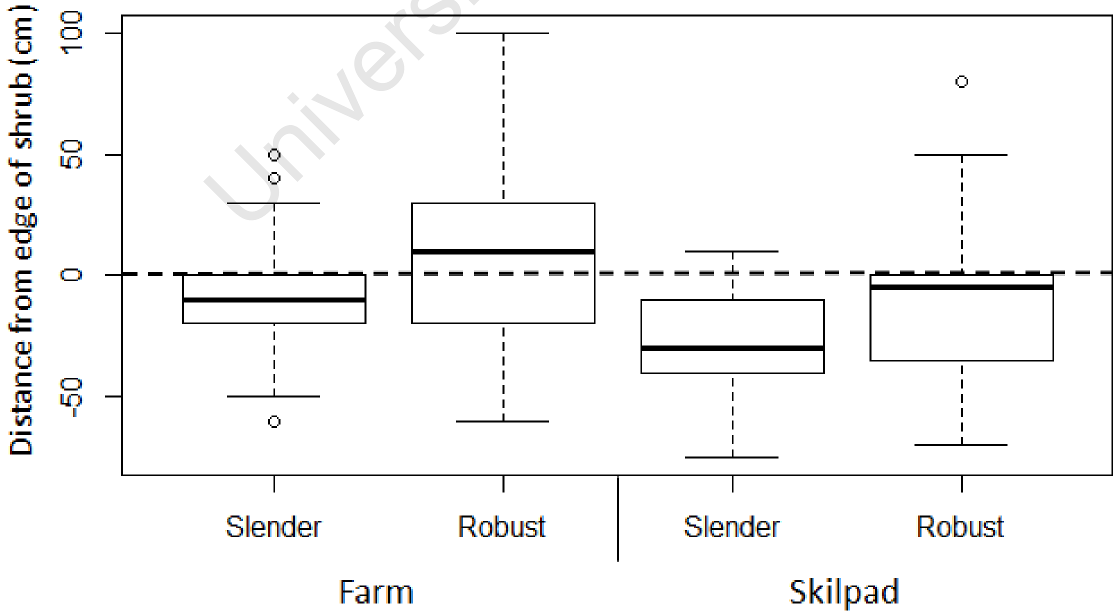

Figure 14: Boxplots showing variation in distance from the edge of shrub in relation to site and form. The dashed horizontal line indicates the edge of the shrub

Table 13: Contingency tables showing counts of individuals of each form located either outside or on the edge of shrubs, or within shrubs, at the two field sites

Site	Location	Form	
		Slender	**Robust**
Farm			
	Within	66	40
	Outside	38	94
Skilpad			
	Within	52	24
	Outside	17	24