

Do dung beetles show interrelated evolutionary trends in wing morphology, flight biomechanics and habitat preference?

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Abstract

In flying organisms, wing shape and biomechanical properties are recognized as key traits related to dispersal, foraging behavior, sexual selection and habitat preferences. To determine if differences in dung beetle wing shape and flight biomechanics are consistent with habitat preferences in a phylogenetic context, we examined how wing morphology varied in a set of 18 Mozambique forest and grassland dung beetle (Scarabaeinae) species, representing nine genera and six tribes. Geometric morphometric measurements were taken of entire wings, as well as two additional shape characters comprising the RA4 and CuA to J regions of veins. Ordination (Principal Components Analysis and Canonical Variate Analysis) of landmark data revealed three different trends in wing shape related to expansion or contraction in external wing margins. These trends were consistent with published dung beetle phylogenies and a phylogenetic reconstruction of ancestral morphological changes using parsimony analysis of wing landmark configurations. Analysis of variance showed that the Procrustes distances between wing shapes were significantly correlated to species identity (~48% of variance), wing size (~27%), habitat (~11%) and two of the three, tested, biomechanical variables (wing loading, wing aspect ratio: ~1%). However, while a phylogenetic generalized least squares analysis confirmed a strongly significant phylogenetic signal for wing shape, it found no significant effect of any other variable. Therefore, wing shape evolution in dung beetles appears to have been phylogenetically constrained and habitat may constitute only a weak selective pressure for changes in wing shape.

INTRODUCTION

The evolution of wings represents a key adaptation that has contributed to the extraordinary ecological success of flying organisms (Kingsolver and Koehl 1994; Norberg 2006).

Evolutionary divergence in wing morphology may have contributed to this success since there is considerable variation in size, shape and function related to habitat preferences and foraging behavior (Norberg and Rayner 1987; Norberg 2006; DeVries et al. 2010; Chazot et al. 2016), mobility and migratory strategies (Sekar 2012; Flockhart et al. 2017), predation (Outomuro and Johansson 2015) and sexual selection (Outomuro et al. 2014). Since wing morphology, flight performance and ecological strategies are apparently interrelated (Norberg and Rayner 1987; Dudley 2002; Norberg 2006), the study of changes in these functional relationships over evolutionary time clearly requires an explicitly historical approach (Klingenberg and Ekau 1996; Monteiro and Nogueira, 2010). Along these lines, comparative methods based on phylogeny and morphometrics have been, recently, recognized as an important tool (Monteiro, 2013) for analyzing the potential role of natural selection and adaptation in driving phenotypic evolution among close relatives (Losos 2008).

In purely functional ecological terms, it has been shown that differences in wing morphology translate into differences in biomechanics, aerodynamics and flight performance (Dudley 2002). The most important biomechanical variables for aerodynamics include aspect ratio, wing loading and wing loading moment. As wing aspect ratio (WAR) affects the maneuverability and velocity of flight, low WAR values are related to increased maneuverability and slower flight (Dingle 1996). As wing loading (WL) is related to sustainability of flight, high WL values imply slow wing beat frequency and short flight duration (Dudley 2002). Finally, as wing loading moment (WLM) describes the relative energetic cost of beating the wing, low WLM values represent slow rate of wing beat and, presumably, reduced energetic cost (Viscor and Fuster 1987). It is likely

that such differences in flight performance conferred by wing shape would suit different ecological strategies, particularly since flight energetic costs could have a major influence on the behavioral ecology of a large number of flying organisms (Taylor and Merriam 1995; Dudley 2002; Hughes and Vogler 2004).

The relationships between wing shape, flight performance and ecological strategies have been the subject of much study in birds and bats (Norberg 1985; Rayner 1988, Gibb et al. 2006). While the aerodynamic properties conferred by biomechanical variables have also been used to compare wing morphologies in an evolutionary context (Norberg 1985; Rayner 1988; Warham 1977; Dudley 2002), there are relatively few studies in this regard, particularly in insects (Dudley 2002; Chazot et al. 2016). Nevertheless, the value of wing features in phylogenetic and taxonomic studies of insects has been recognized in flies (Houle et al., 2003; Krosch et al. 2013), dragonflies (Outomuro et al. 2013a,b), butterflies (Chazot et al. 2016), cantharid beetles (Su et al. 2015) and dung beetles (Philips et al. 2004; Bai et al. 2011; 2012). However, most studies focus on univariate rather than the multivariate descriptors that would better represent the phenotypic response to selective forces that have driven changes in wing shape.

To date, a cladistic approach to multivariate variation in wing shape of dung beetles has revealed evolutionary stability, suggesting that variation has been limited by functional constraints related to flight (Bai et al. 2011). Comparisons based on the whole wing and different wing regions (e.g. the R and M veins) also show different trends of variation within different tribes of Scarabaeinae, indicating that they experience different selective pressures (Bai et al. 2012). Thus, analyses of variation between whole wings and specific wing regions are essential for understanding the development and evolution of these complex morphological structures (Klingenberg et al. 2001) in relation to their environment.

Because of the patchy distribution and ephemeral nature of dung deposits, dung beetles have evolved specialized behaviors in order to locate and rapidly exploit available food resources (Hanski and Cambefort 1991) in habitats with different physical characteristics. Specialization to forest or grassland may demand different foraging strategies that would favor different wing shapes and flight behavior (Halfpeter and Mathews 1966; Philips et al. 2004; Tarasov and Génier 2015). Although foraging behavior of dung beetles seems relatively poorly documented, several observations have been made of search strategies for food (ALV Davis, pers. obs). Some large-bodied dung beetles (e.g. *Kheper lamarcki*) have been noted flying rapidly in a straight line at appreciable height above the soil surface, only descending in a spiral once the odor of food has been detected with at least one species (*Scarabaeus proboscideus*) known to land beside the dung. Other smaller bodied species have been observed to slowly search near the soil surface just above any herbaceous vegetation and land directly onto dung. Within forest, a number of species have been observed to alight on leaves (Gill 1991), which may assist in the location of nearby food. Such different strategies, particularly rapid flight and perching behaviour, would be expected to be influenced by presence and density of tall vegetation. In general, it is expected that large, narrow wings are associated with faster, sustained flight in open habitats compared to that of broad and short wings, which generate a lot of resistance and might be better suited to forested environments. However, to our knowledge, there are no studies of the putative relationships among wing shape, flight biomechanics and habitat preference in dung beetles.

In this study, we use a similar approach to that of Baylac et al. (2003) who illustrated the use of geometric morphometrics to explore how wing shape varies independently of body size and to estimate taxonomic differences in wing morphology within species complexes. Our geometric morphometric analysis uses 18 Mozambique dung beetle species to examine the phylogenetic and ecological trends in wing shape variation using a geometric morphometric analysis of the entire

wing and two wing regions (RA4, CuJ) that are assumed to be under different selective pressures (Bai et al. 2012). This includes an examination of the relationships between wing morphology, flight biomechanics and habitat preference of forest and grassland species.

METHODS

Study area and sampling

The Maputo Special Reserve in southern Mozambique (26°25'S, 32°45'E) supports a complex and exceptionally diverse mosaic of vegetation consisting of forest, savanna woodland, grassland, and wetland. Each habitat contains its own species assemblage and several of these vegetation types possess significant degrees of species-level endemism (Myre 1964). Dung beetles were sampled using pitfall traps (top diameter, 23 cm; depth, 17.5 cm) half filled with water containing a little detergent and baited with 250 ml of fresh domestic pig dung (following Jacobs et al. 2010). Sampling was conducted in three different habitats of the reserve during November 2006 (Table 1). The habitat preferences of the species were confirmed using previous studies conducted in the region (Davis et al. 2002; Jacobs et al. 2010). Survey and collecting procedures were conducted in line with the standards set by the ethics committees of the Universities of Pretoria (South Africa) and Eduardo Mondlane (Mozambique).

Data set

Selected species represented the overall phylogenetic and ecological diversity shown by the Mozambique assemblage. For phylogenetic diversity, we included two genera from each clade of dung beetles demonstrated by Mlambo et al. (2015) and all of the species recorded for those genera (i.e. groups that include the common ancestor and all of its descendants). In total, this represented 18 species belonging to 11 genera and six tribes. For ecological diversity, we

Table 1. Total number of sampled individuals for 18 dung beetle species in Maputo Special Reserve (Mozambique). In bold the habitat with more than 75% of the individuals captured.

Species	Habitat			Number of specimens measured
	Grassland	Sand Forest	Dune Forest	
<i>Catharsius harpagus</i> Harold	182	0	0	29
<i>Catharsius pandion</i> Harold	28	1208	474	29
<i>Catharsius tricornutus</i> Degeer	10	0	0	4
<i>Copris inhalatus</i> Quedenfeldt	124	10	0	21
<i>Copris puncticollis</i> Boheman	64	2	0	14
<i>Garreta caffer</i> (Fahraeus)	0	45	771	23
<i>Kheper lamarcki</i> (Macleay)	110	7	0	25
<i>Metacatharsius opacus</i> (Waterhouse)	314	4	0	21
<i>Neosisyphus fortuitus</i> Péringuey	8	0	0	4
<i>Neosisyphus mirabilis</i> (Arrow)	1	509	17	25
<i>Oniticellus planatus</i> Castelnau	2	0	0	2
<i>Onitis viridulus</i> Boheman	1	0	0	1
<i>Proagoderus aureiceps</i> (d'Orbigny)	2484	559	32	28
<i>Proagoderus chalcostolus</i> (d'Orbigny)	14	1	0	5
<i>Scarabaeus geminogalenus</i> Davis & Deschodt	10	0	3	8
<i>Scarabaeus goryi</i> (Castelnau)	27	114	1	23
<i>Sisyphus neobornemisszanus</i> Daniel, Davis & Scholtz	0	200	1100	23
<i>Sisyphus oralensis</i> Daniel, Davis & Scholtz	0	1648	158	18

included species showing variation in food relocation behaviors (telecoprid, paracoprid and endocoprid) and body sizes. In particular, we included species of genera (and their sister groups) that show extreme association with either unshaded grassland or shaded forest habitats, comprising hotter sand forest or cooler, coastal-fringing dune forest (Table 1) with their quite different structural characteristics that might influence foraging flight patterns of dung beetles. For each species, we used 30 specimens of the abundant taxa and all specimens of taxa recorded in low abundance. The final sample comprised only specimens with complete wings after discarding those damaged by handling during photography of wing shape. Digital photographs of the ventral side of the right wings were taken using a reflex camera (Canon D50) connected by a C tube to a Stereozoom microscope (SV11 ZEISS) in the morphometrics laboratory of the Instituto de Ecología, A. C. (Xalapa, Mexico).

Wing shape variation measurement

To measure wing shape variation, we obtained two-dimensional landmark configurations from images, using the program TpsDig 2.12 (Rohlf 2008). We recorded three configurations separately, one for the entire wing and two for different wing regions (Fig. 1) that play a significant role in flight performance and are also of biomechanical importance in dung beetles (Bai et al. 2012). Ventral views of the entire right wing (293 photographs) were recorded with a set of 19 landmarks (Fig. 1A). The area from vein RA4 to the top of the external margin of the hind wing was defined as region RA4 (273 Photographs). It was recorded using a collection of three landmarks and 15 semi-landmarks (Fig. 1B). It represents the bending zone, which is involved in structural support of the wing, maintenance of stability (Wootton 1992; Haas and Beutel 2001) and flexibility in terms of folding under the elytra (Fedorenko 2015). The external margin between veins CuA and J was defined as region CuJ (141 Photographs) and was recorded

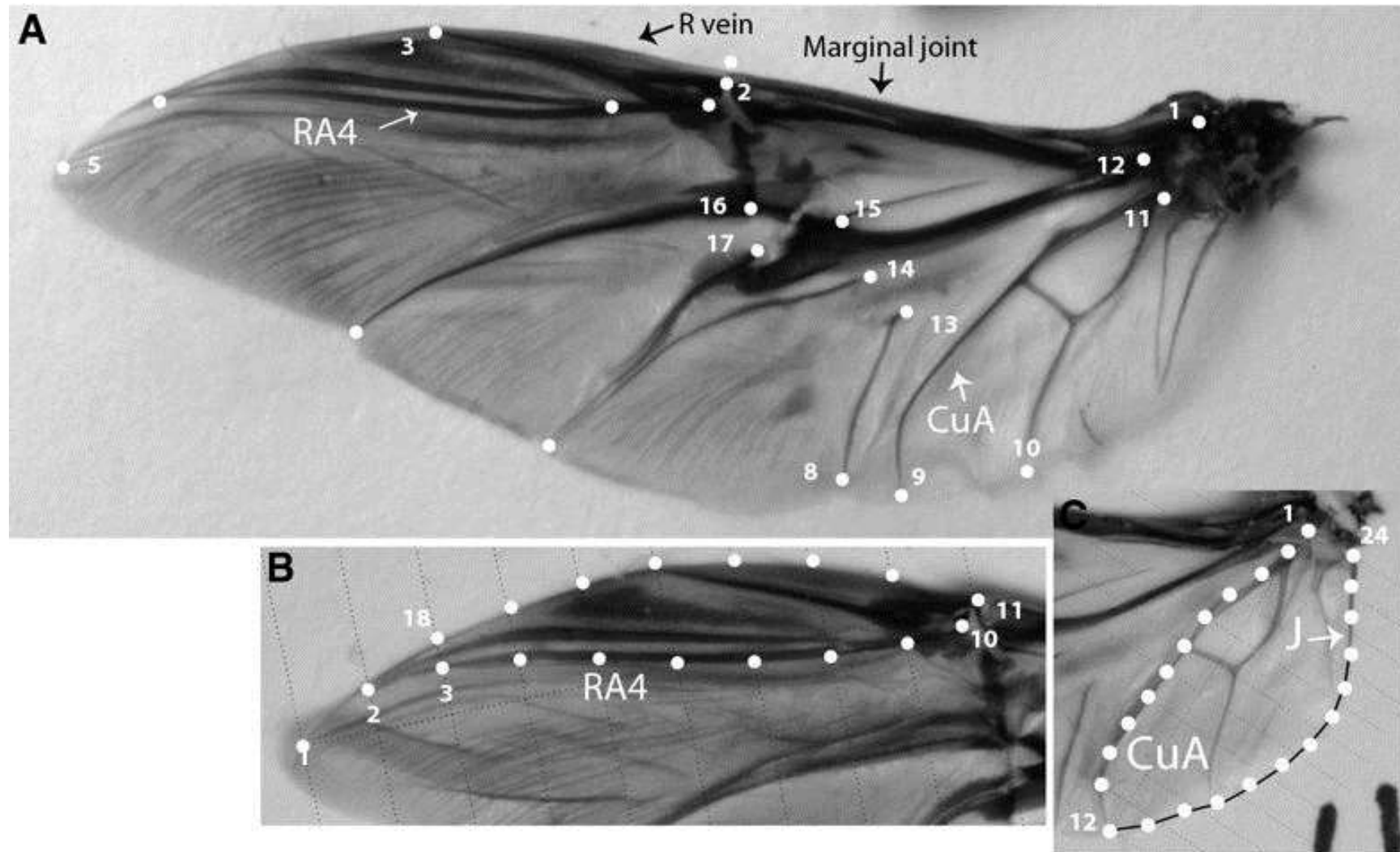


Fig. 1. A-C. Three configurations of the sets of points used to record ventral wing shape characters in Scarabaeinae species: A. Whole wing with 19 landmarks; B. Region RA4 (region of vein RA4) with 18 points, three landmarks (1,9,10) and fifteen semi-landmarks (2-8,12-18); C. Region CuJ (region of veins CuA and J) with 24 points, two landmarks (1,12) and 22 semi-landmarks (2-11,13-24). Other relevant variable regions are indicated.

using two landmarks and 22 semi-landmarks (Fig. 1C). This region represents the elongation of the apical membrane independently of the folding pattern of the wing (Fedorenko 2015). Semi-landmarks were recorded with guides drawn from the program Make Fan 8 from the IMP series (Sheets 2014). These data were uploaded as a Tps file (see Supplementary Material 1, 2 and 3).

Wing shape variation analysis

An analysis protocol was implemented in order to study wing shape variation using routines in the Geomorph library, version 3.0.5 (Adams et al. 2017) of the R (R Development Core Team 2016) and IMP software packages (Sheets 2014). Following digitization, differences in landmark configurations due to the size, position and orientation of specimens were removed by optimally superimposing the configurations using a generalized Procrustes analysis (GPA) (Rohlf and Slice 1990). Size was estimated as centroid size prior to the GPA superimposition. Semi-landmarks were aligned using a method that minimized Procrustes distances between the corresponding curve in the consensus configuration and each specimen in the sample (Zelditch et al. 2004).

We conducted a partial least squares (PLS) regression to evaluate covariance between aligned morphological matrices for the whole wing and two wing regions in the program PLS, version 8 (Sheets 2014). Correlations were highly significant according to a permutation test with 1000 replicates ($P < 0.01$) (whole wing vs. region RA4: $r = 0.52$; whole wing vs. region CuJ: $r = 0.60$; region RA4 vs. region CuJ: $r = 0.64$). Considering the high and significant covariance values between the entire wing and the two wing regions, the subsequent statistical and comparative methods were applied to the entire wing as a wing shape variable (see below).

We used Principal Components Analysis (PCA), run in PCAgen 8 (Sheets 2014), to explore (1) the dispersion of landmark configurations in multivariate space; (2) to detect major variation in the shape of entire wings and wing regions among all 18 species; and (3) to determine if coding

of species data points for habitat associations was consistent with wing shape variation. Patterns of similarities were calculated from the variance-covariance matrix of the shape variables. The variance explained by each principal component describes the difference in wing shape relative to the consensus shape in the morphospace (Zelditch et al. 2004).

To evaluate the differences among the 17 species (*Oniticellus planatus* was excluded since it was represented by a single specimen and, thus, there was no variance), we also used Canonical Variate Analysis (CVA) in the program CVAgen 8 (Sheets 2014). In the CVA, we selected the first 10 principal components obtained from the exploratory analyses to reduce the effect of the low number of specimens in some groups. We obtained deformations with respect to the consensus mean configuration shown by groups in directions of major variation along the first two canonical axes. The significance of differences among groups on canonical axes was calculated using Bartlett's test for differences in the value of Wilk's lambda. For details of the procedure, see Zelditch et al. (2004).

Wing shape evolution

Using wing shape characters, we determined phylogenetic relationships among species using spatial parsimony in the program TNT (Goloboff et al. 2008). This analysis was performed following the protocol of Catalano et al. (2010) using the default parameters for superimposing configurations on the tree. We then assessed congruence between the parsimony phylogeny for species, where branch lengths were equal, and a molecular phylogeny for genera (Mlambo et al. 2015) where branch lengths were based on numbers of differences between molecular sequences.

We used the function *plotGMPhyloMorphoSpace* in R to determine if a plot of the average landmark configurations for species in PCA morphospace (dimensions 1 and 2) (see above) was congruent with phylogenetic trees. These trees comprised the consensus molecular phylogeny

(Mlambo et al. 2015) for assigning generic position and the parsimony phylogeny for assigning species position. This descriptive approach, using a combination of proportional branch length data for genera and equal branch lengths for species, was unavoidable as there is no molecular phylogeny that includes all of the species selected for the present study.

On the phylogeny derived from each of the three wing shape characters, we also obtained ancestral configurations at each node and identified the nodes that represented major morphological changes. The amounts of change were estimated as Procrustes distances and were represented on the tree as vector lines from estimated ancestral to descendant configurations. Reconstructed configurations in TNT were saved in a Tps file and the pairwise distance matrix between wing shapes in ancestors and descendants was calculated using the program Coordgen 8 (Sheets 2014).

Flight biomechanics and phylogeny

To evaluate the relationship between wing shape and the biomechanics of flight, we calculated three variables related to flight performance: Wing aspect ratio (WAR), Wing loading (WL) and wing loading moment (WLM) (Viscor and Fuster 1987; Dudley 2002). The biomechanical variables were calculated using the expressions shown in Table 2. Total wing area (Wa) and wingspan (Ws - longest distance from the axillary region to the tip) were derived from images of the right wing using Image *J* software (Rasband 1997).

In addition, we plotted the position of each species in a three-dimensional space with regards to the average values for the three biomechanical variables. We then mapped the phylogenetic trees onto this plot using the library Phytools (Revell 2012) in R. In this biomechanical morphospace we, again, used the molecular phylogeny for genera (branch lengths based on differences between

Table 2. Expressions and measurements used to quantify the biomechanical variables of flight.

Biomechanical variables	Expressions	Measurements used
Wing aspect ratio (WAR)	$WAR = W_s^2 / Wa$	W_s = wing span (m)
Wing loading (WL)	$WL = B_w / Wa$	Wa = wing area (m ²)
Wing loading moment (WLM)	$WLM = B_w / W_s^2$	B_w = body weight (kg)

sequences) (Mlambo et al. 2015) and the parsimony phylogeny for species (equal branch lengths).

Wing shape, flight biomechanics and habitat preference

Using the function *procD.lm* in R, a single ANOVA model was developed to determine the extent to which wing shape variation can be explained by co-variation with the three biomechanical variables, wing size and habitat preference in 16 species (*Onitis* and *Oniticellus* were excluded due to insufficient sample size, $n \leq 2$). This model included the effect of the interaction between biomechanical variables and wing size (allometry expressed as centroid size). The variance was described using the sums of squares of the coordinates, Procrustes distances between each individual configuration and the consensus shape for each factor level. Function, *procD.lm*, estimates the probability of the variation compared to a null model by resampling with 999 permutations (Adams and Otálora-Castillo 2013). We also used the function *advanced.procD.lm* to test an additional complete linear model that included the effect of the interaction between habitat and biomechanical variables as well as that between species, wing size and habitat. The script for these analyses in R is available in Supplementary Material 4.

Since species identity explained the greatest proportion of the variance, we evaluated the degree of phylogenetic signal in shape data, relative to that expected under a Brownian motion model of evolution. For this test, we used the multivariate version of the K-statistic with the function *physignal* (Adams 2014a), which represents a mathematical generalization of the Kappa statistic (Blomberg et al. 2003).

We also used the function, *procD.pgls*, to conduct a phylogenetic generalized least square (PGLS) test (Adams 2014b) on the phylogenetic variance in the residuals. This test evaluated the

effect of the centroid size, habitat preference and biomechanical variables on the specific wing shape average. We estimated the probability of this variation compared to a null model by resampling the residuals using 1000 permutations.

Finally, we used the package PDAP (Midford et al. 2005) of the program Mesquite 1.1 (Maddison and Maddison 2006) to run a phylogenetically corrected regression based on the molecular phylogeny of Mlambo et al. (2015). This analysis used a Felsenstein independent contrasts test (Felsenstein 1985) to compare differences in the average shapes of the entire wings between genera as described by PC1, wing size and biomechanical variables.

RESULTS

Wing shape variation

Ordination biplots generated from the principal components (PCA) (Fig. 2A) and canonical variate (CVA) analyses of entire wing shape (Fig. 3) show similar separations between species. Dispersion in the ordinal space defined by both PCA and CVA (dimensions 1 and 2) suggest three different groups of species. *Garreta caffer* and *Onitis viridulus* were those most distant from the consensus shape. *Sisyphus*, *Neosisyphus*, *Proagoderus* and *Oniticellus* species formed a second agglomeration, and the remaining species formed the third group. Superimposition of phylogenetic trees onto the mean PCA coordinates for each species shows that the variation in wing shape is consistent with phyletic relationships between sister groups and species of the same genus (Fig. 2B).

In the CVA, major deformations of the group means from the consensus shape for entire wings were in the same three directions as those suggested by PCA with 14 significant discriminant axes (Wilk's $\lambda < 0.74$, $\text{Chi}^2 > 63.97$, $P < 0.001$). Group 1 (*Garreta-Onitis*) changed along canonical axis 2, with the largest deformations in the region of the radius anterior

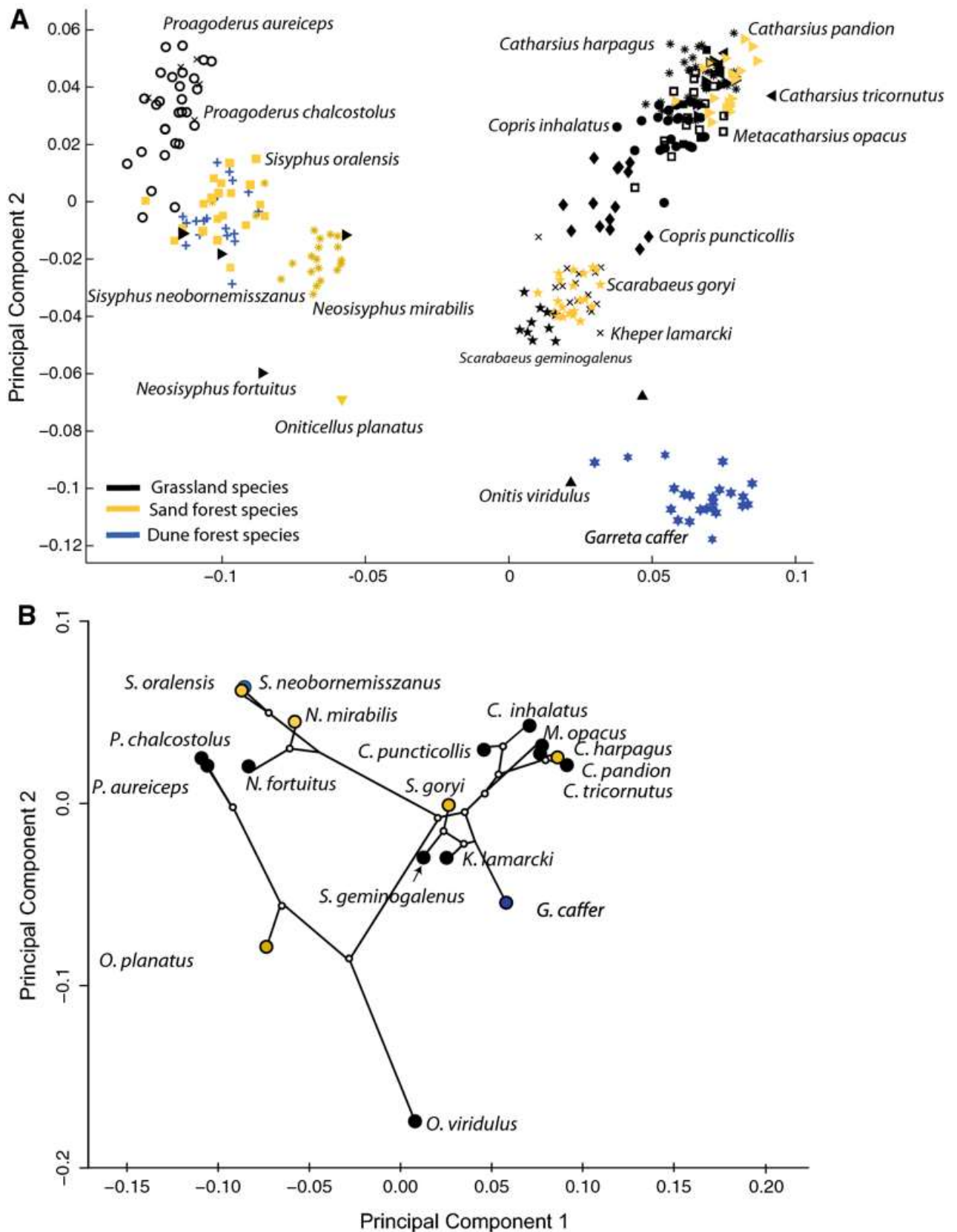


Fig. 2. A-B. Ordination plot for Principal Components 1 and 2 representing differences in morphology among the whole wings of 18 Scarabaeinae species based on shape vectors. A. Data points for individuals of each species. B. Molecular phylogenetic tree superimposed onto mean data points for each genera where branch length does not reflect phyletic distance.

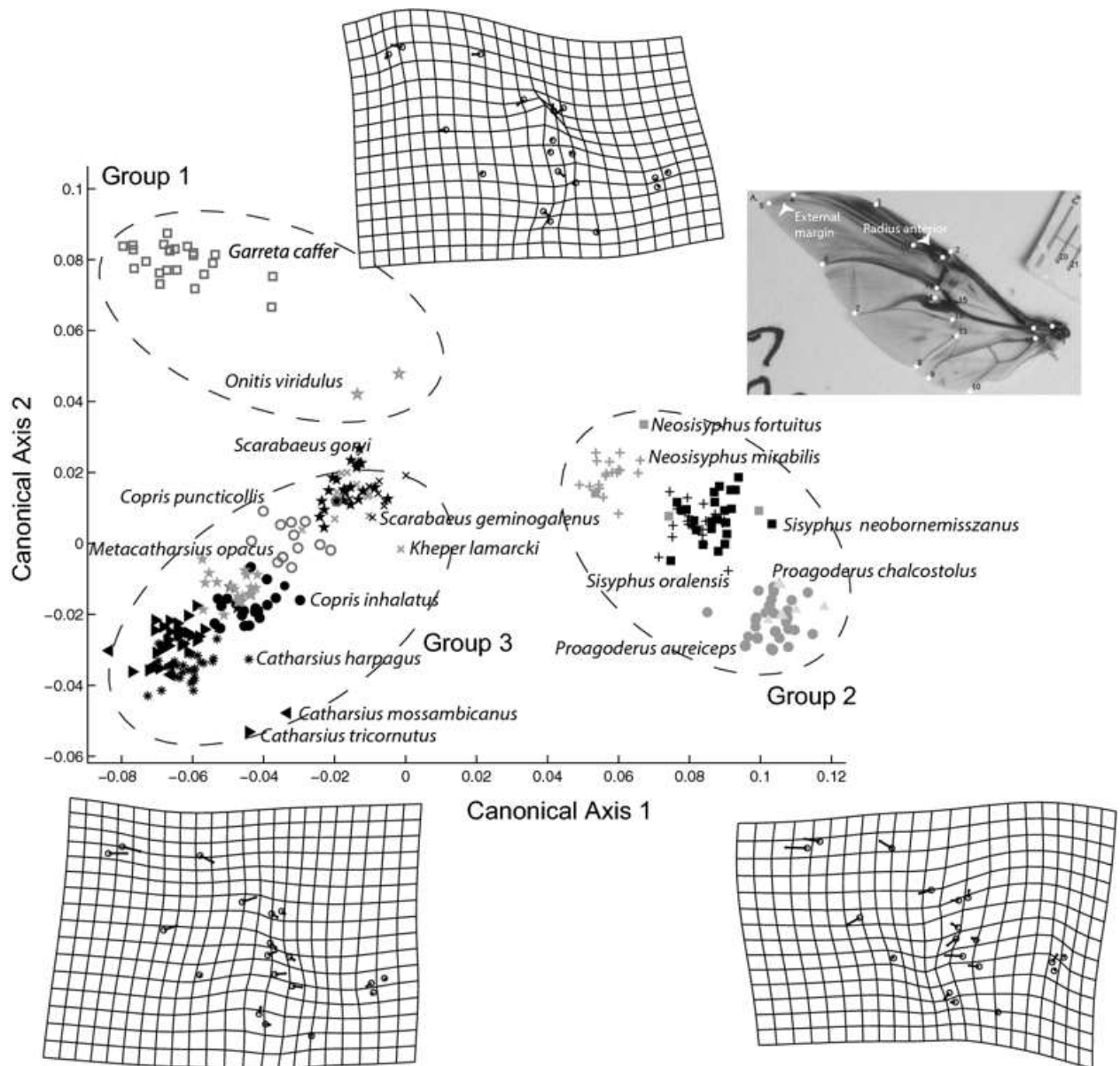


Fig. 3. Ordination plot for canonical axes 1 and 2 representing differences in morphology between the whole wings of 17 Scarabaeinae species based on shape vectors. Groups of species changing in the same direction are indicated in dotted circles. Deformation grids represent variation in shape among species along axis 1 in Group 1 (*Garreta*, *Onitis*) and variation in consensus shape along axis 2 in Group 2 and Group 3.

(landmarks 18-19) and the external margin of the wing (landmarks 3-6). Group 2 (*Sisyphus*, *Neosisyphus*, *Proagoderus* and *Oniticellus* species) changed along canonical axis 1 due to the expansion of the external wing margin (landmarks 3-5), while the remaining species in Group 3 changed as a result of contraction in this wing margin, although they represented the lowest degree of overall deformation (Fig. 3). A total of 92% of cases were correctly reassigned to the original groups.

Ordination of shape data for regions RA4 and CuJ yielded results similar to those found for the entire wing, but the three species groups were differentiated to a lesser degree (Fig S1). There were 12 significant discriminant axes for wing region RA4 (Wilk's $\lambda < 0.65$, $\text{Chi}^2 > 106.12$, $P < 0.02$) (Fig S1A) and 11 for region CuJ (Wilk's $\lambda < 0.08$, $\text{Chi}^2 > 273.15$, $P < 0.001$) (Fig. S1B). Major deformations were shown in the wing shape of *Scarabaeus* species and *Garreta caffer* (Figs S1C, S1D), which all belong to the same phylogenetic clade.

Wing shape evolution

In general, the phylogenetic reconstruction of wing shape characters was congruent with the molecular phylogeny of Mlambo et al. (2015) (Fig. 4). We recovered the *Catharsius*, *Metacatharsius* and *Copris* clade as well as clades comprising species from the tribes Scarabaeini (*Kheper*, *Scarabaeus*) and the Sisyphini (*Sisyphus*, *Neosisyphus*). Phylogenetic reconstructions of the ancestral shapes of entire wings indicated large changes in vectors and Procrustes distances at nodes 27, 31 and 32 (Fig. 4). However, the wing shapes of descendants following nodes 23, 28 and 30, were similar to those of the ancestors with little change in vectors and mostly low Procrustes distances (Fig. 4). Estimated vectors for the RA4 region indicated the largest changes in shape with larger Procrustes distances at nodes 30, 31, 32 (Fig. S2). Vectors in the CuJ region indicated few changes as well as low Procrustes distances (Fig. S2).

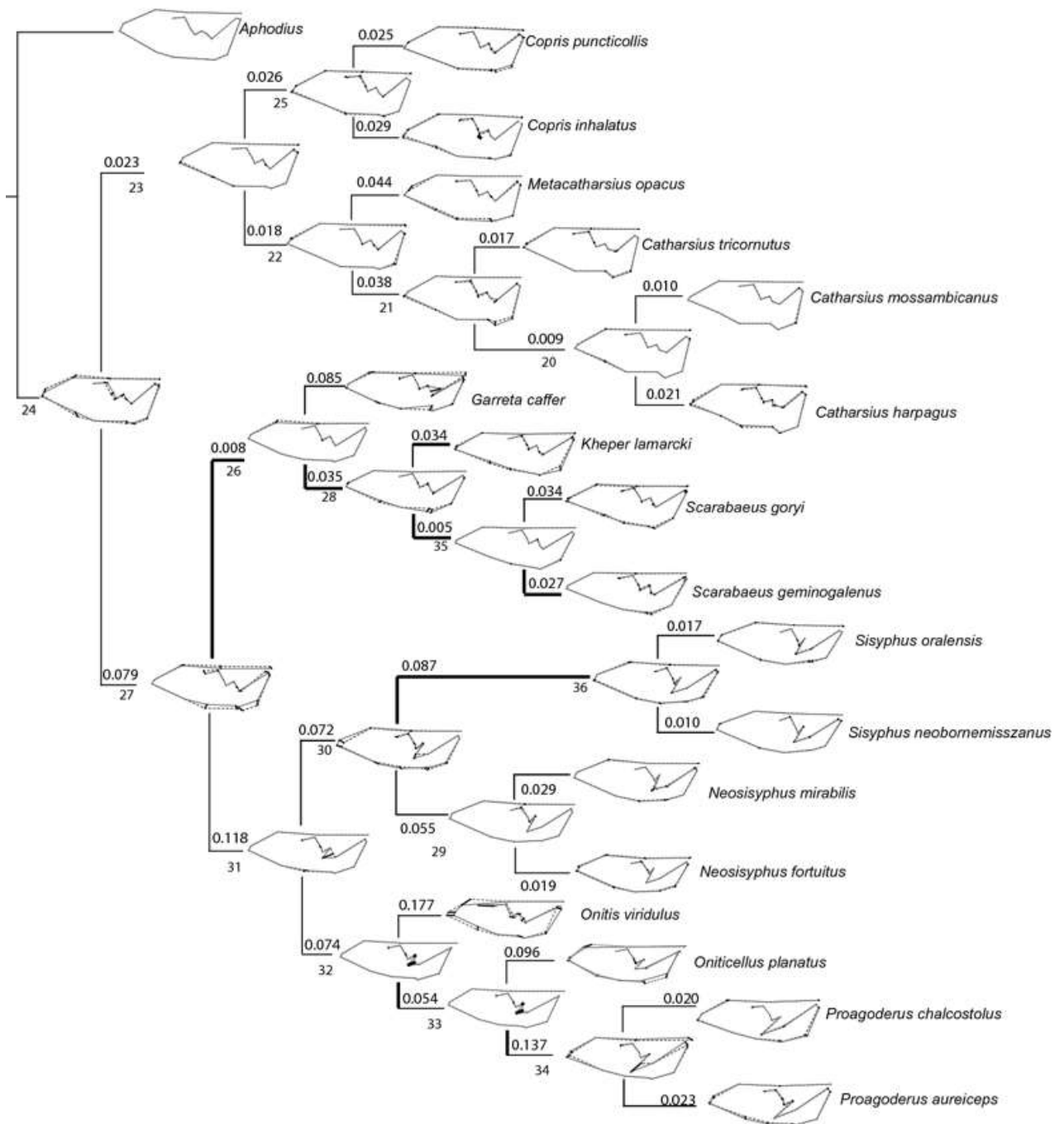


Fig. 4. Observed and reconstructed ancestral configurations of whole wings for 18 Scarabaeinae species mapped onto the TNT morphological phylogeny with reference numbers for nodes below branches and Procrustes distances between configurations on adjacent nodes above branches. Vectors marked with black lines represent changes with respect to the ancestral configurations. Configurations in descendants are shown at the ends of branches.

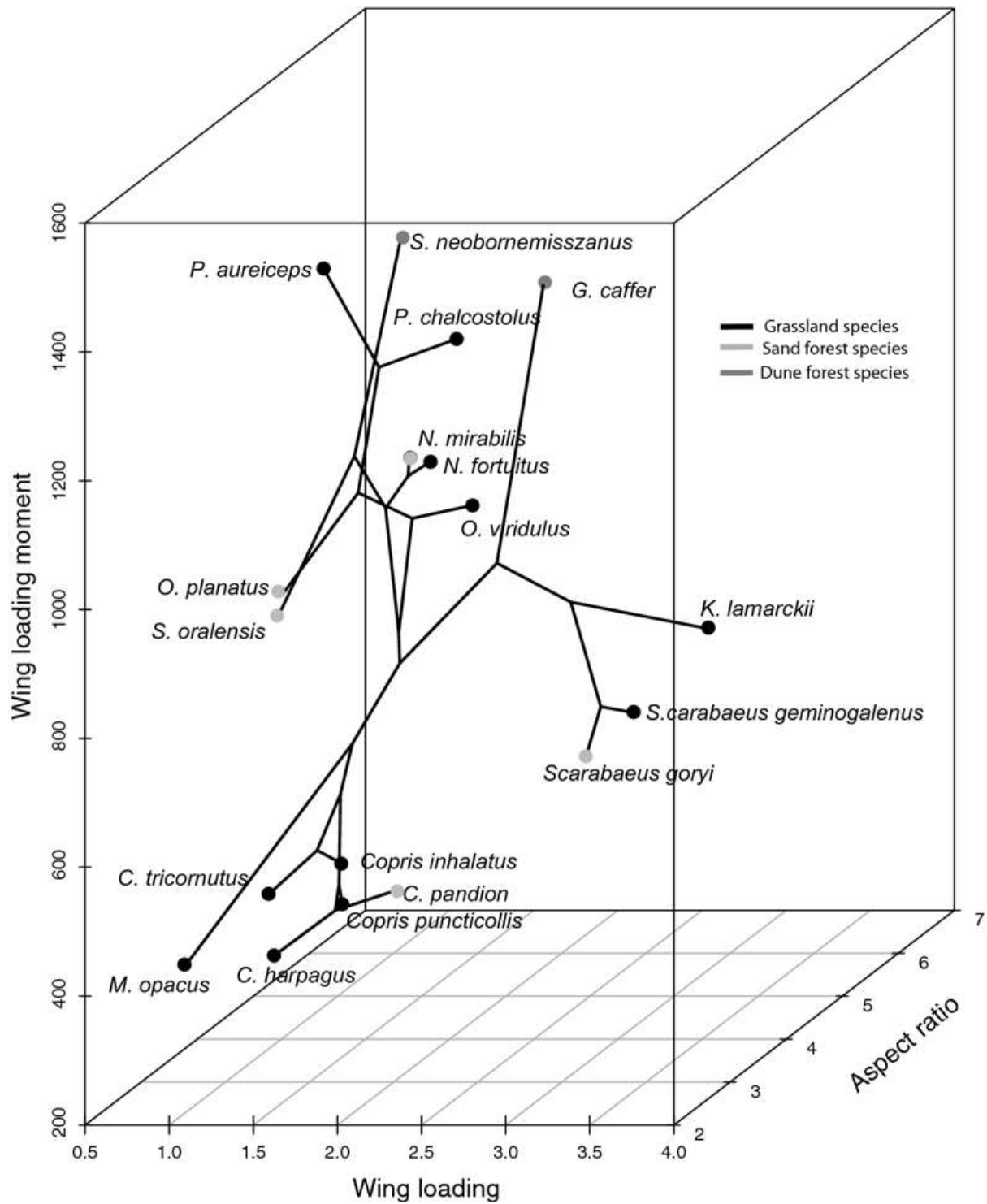


Fig 5. Three-dimensional biomechanical space with phylogeny superimposed. Each axis corresponds to a biomechanical variable that was tested. Color labels illustrate the habitat preference recorded for each species.

Flight biomechanics and phylogeny

The biomechanical variables represent different trends of variation among the 18 species (Table 3). Values for wing loading moment (WLM) were lowest and most similar in the *Catharsius*, *Metacatharsius* and *Copris* group. Much higher values were found for all other groups. In general, wing loading (WL) values were relatively low in all groups except for the species of *Garreta*, *Onitis*, *Scarabaeus* and *Kheper*, which showed intermediate to high values. The wing aspect ratio (WAR) values were less variable than those for the other biomechanical variables. The values for 17 of the species lay within narrow ranges (2.798-3.510 or 4.085-5.313), with that of a single species offset to a higher value (6.995). Congruence of biomechanical variation and phylogenies for genera (Mlambo et al. 2015) and species position (parsimony reconstructed tree) were greatest within the *Catharsius*, *Metacatharsius* and *Copris* clade (Fig. 5) where values of WLM, WL and WAR were all low. These are genera in which most of the species are associated with grasslands, except for *Catharsius pandion* (Table 1).

Wing shape, flight biomechanics and habitat preferences

Analyses of variance in wing shape showed that species identity accounted for close to 50% of the overall variation, although significant proportions of variation were also influenced by wing size, habitat preference and two biomechanical variables (WL, WAR) in rank order (Table 4). However, none of the interactions between wing size and biomechanical variables were significant (Table 4). The full model, including the interaction between habitat preference, centroid size and biomechanical variables, also had no effect on wing shape variation ($F = -3.45$, $P = 0.25$).

Table 3. Values for biomechanical variables and body weight for 18 Scarabaeinae species (see Table 2 for derivation of biomechanical values). Dry body weight (a reliable measure of body mass) for each individual was obtained using a digital balance (precision ± 0.0001). F = Forests, G = Grassland.

Tribe	Species	Wing Aspect Ratio	Wing Loading (kg/m ²)	Wing Loading Moment	Body Weight (g)
	GROUP 1		m ²)	Moment	
Gymnopleurini	<i>Garreta caffer</i> (F)	3.228	2.889	1423.216	0.354
i Onitini	<i>Onitis viridulus</i> (G)	4.385	2.049	1002.906	0.187
	GROUP 2				
Sisyphini	<i>Neosisyphus fortuitus</i> (G)	5.313	1.479	1008.714	0.057
	<i>Neosisyphus mirabilis</i> (F)	4.907	1.494	1041.871	0.060
	<i>Sisyphus neobornemisszanus</i> (F)	4.603	1.537	1394.410	0.039
	<i>Sisyphus oralensis</i> (F)	5.288	0.556	770.076	0.005
Oniticellini	<i>Oniticellus planatus</i> (G)	4.688	0.794	846.977	0.014
Onthophagini	<i>Proagoderus aureiceps</i> (G)	3.469	1.457	1431.727	0.042
	<i>Proagoderus chalcostolus</i> (G)	4.085	2.054	1281.190	0.124
	GROUP 3				
Coprini*	<i>Catharsius harpagus</i> (G)	2.798	1.385	409.987	0.542
	<i>Catharsius pandion</i> (F)	4.173	1.633	416.471	0.486
	<i>Catharsius tricornutus</i> (G)	4.123	1.348	401.316	0.354

	<i>Copris inhalatus</i> (G)	4.829	1.102	417.077	0.153
	<i>Copris puncticollis</i> (G)	4.951	0.619	362.148	0.035
	<i>Metacatharsius opacus</i> (G)	3.366	0.648	358.033	0.060
Scarabaeini	<i>Kheper lamarcki</i> (G)	3.510	3.776	871.384	1.941
	<i>Scarabaeus geminogalenus</i>	6.995	2.137	508.101	0.519
	(G) <i>Scarabaeus goryi</i> (F)	4.195	2.827	629.983	1.304

* Polyphyletic tribe (see* Tarasov and Dimitrov 2016)

Table 4. Results of the ANOVA model testing for the effect on whole wing shape (defined by Procrustes distances) of wing size (WS), species identity, habitat and biomechanical variables (WL, WAR, WLM) in 16 dung beetle species. Significant effects are in bold. WS = Wing size (centroid size as a descriptor, details in Methods), WL = Wing loading, WAR = Wing aspect ratio, WLM = Wing loading moment.

Effect	Df	SS	MS	Variance (%)	F	Pr (>F)
WS	1	0.847	0.847	27.41	538.91	0.001
Species	13	1.469	0.113	47.67	71.92	0.001
Habitat	2	0.339	0.169	11.027	108.14	0.001
WL	1	0.003	0.003	1.26	2.462	0.002
WAR	1	0.003	0.003	1.09	2.142	0.009
WLM	1	0.002	0.002	0.05	0.838	0.537
WL*WS	1	0.002	0.002	0.073	1.441	0.09
WAR*WS	1	0.001	0.001	0.040	0.796	0.556
WLM*WS	1	<0.001	<0.001	0.002	0.535	0.875
Residuals	263	0.413	0.001			
Total	285	3.083		88.62		

The phylogenetic generalized least square method reveals a significant phylogenetic signal in shape variation for the entire wing ($K = 1.03$, $P = 0.01$). However, the multivariate k-statistic did not detect any significant effects on the residuals for wing shape variance: wing centroid size ($r^2 = 0.08$, $F_{1,10} = 1.8$, $P = 0.98$), habitat selection ($r^2 = 0.21$, $F_{3,10} = 0.99$, $P = 0.99$), WL ($r^2 = 0.09$, $F_{1,10} = 0.24$, $P = 0.73$), WAR ($r^2 = 0.15$, $F_{1,10} = 2.17$, $P = 0.25$) or and WLM ($r^2 = 0.22$, $F_{1,10} = 3.13$, $P = 0.14$). This means that all of the reported differences in previous comparisons are mostly explained by shared ancestry, suggesting that wing shape variation is not explained by any variable other than a neutral model of evolution (Brownian motion model).

Using average values for genera, the phylogenetically corrected regressions do not support significant positive correlations between wing size and wing shape ($r = -0.27$, $P = 0.211$) or wing aspect ratio and wing loading moment ($r = -0.37$, $P = 0.125$). But, they do support a positive correlation between aspect ratio and wing loading ($r = 0.69$, $P = 0.009$) and a negative correlation between aspect ratio and wing loading moment ($r = -0.53$, $P = 0.004$).

DISCUSSION

Wing shape variation and its evolution

Although the present sample is limited to 18 species represented by ~300 individuals, it nevertheless, represents the wide phylogenetic and ecological diversity of Scarabaeinae. The results identify three principal trends of wing shape variation comprising one group of small-bodied, dung beetle species (genera: *Sisyphus*, *Neosisyphus*, *Proagoderus*) and two groups of primarily larger-bodied species (genera: *Copris*, *Catharsius*, *Scarabaeus*, *Kheper*; genera: *Garreta*, *Onitis*). All statistical tests indicate a strongly significant effect of species relatedness on differences in wing shape, a pattern that was recovered using genera or species and, equal or asymmetric branch length on phylogenies. Depending on the analytical method, effects of wing

size, habitat association and biomechanical attributes are of lesser rank importance, or, not at all important. For this reason, in order to detect a possible relationship between wing shape and habitat, future studies should consider a larger sample size.

The results are consistent with those of Bai et al. (2011) using a sample of 81 species, who observed an effect of phylogenetic constraint in wing shape variation of scarabaeine dung beetles with no substantial changes in wing morphology over evolutionary time. However, contrary to the suggestion of Bai et al. (2012), high degrees of correlation between wing regions (veins RA4 and CuA to J) and the entire wing indicate that wing shape is changing as a unit of ontogenetic development in response to selective pressures, with the differences primarily explained by phylogenetic divergence. In general, the positions of aligned landmark configurations are quite conservative, with considerable overlap between species. This suggests that dung beetle wings have evolved very slowly, possibly limited by structural and functional constraints although these are difficult to identify in this study. These observations agree with those made for fruit flies of the genus *Drosophila* (Houle et al. 2003, Houle et al. 2017), in which wing shape is generally conserved across populations and species, providing an example of evolutionary stasis that is primarily the result of strong selective pressures on wing shape (Frankino et al. 2009). It is possible that an integrated combination of selective factors (e.g. functional ecological, developmental, genetic) have influenced variation in wing shape of dung beetles leading to relative evolutionary stasis as has been the case in other taxa (Beldade et al. 2002; Wagner 2011; Matamoros-Vidal et al. 2015).

Wing morphology and habitat association

Although an appreciable correlation between wing shape and vegetation type was demonstrated, this association disappeared upon statistical removal of the phylogenetic effect. Thus, although

differences in vegetation structure between unshaded grassland and shaded forest habitats could conceivably influence wing morphology in dung beetles, the present study does not convincingly support this hypothesis as the groups defined from wing shape variables show a mix of habitat associations. Similarly, Gibb et al. (2016) found no evidence that habitat or landscape characteristics have any influence on the wing morphology of ground-dwelling carabid beetles. They instead suggest that determinants of variation in morphology comprised phylogenetic relatedness, as in dung beetles, or a trade-off between dispersal ability and body size. Nevertheless, Chazot et al. (2016) provide evidence that microhabitat has driven wing shape evolution in *Morpho* butterflies with canopy and understory species showing different wing morphology and flight behavior. Furthermore, Dempster (1991) showed that morphological changes in butterfly species are possibly related to changing configurations of habitat at the landscape scale.

It might be predicted that flying organisms with large, narrow wings and fast, sustained flight would be better suited to open habitats whereas species with broad, short wings would be better suited to densely-vegetated woody habitats (Dudley 2002, Chazot et al. 2016). However, dung beetles showed an opposite trend to this prediction since wider wings, resulting from expansion of the external wing margin, showed greater frequency in species of the grassland than in those of the forest. A broad wing base has several advantages in that it is better adapted for take-off and allows a wider range of speed (Outomuro et al. 2013b) although maintaining flight is more difficult and energetically more expensive compared to a narrow wing base. Even so, broad-winged species could undertake shorter flights in order to mitigate the energetic costs. A narrow wing tip is, energetically, less costly (Outomuro et al. 2013b) and allows for more extensive flight activity whereas a long, narrow wing shape is well suited for fast, gliding flight patterns. However, these traits may not be so well suited to foraging activity by most dung beetle species,

irrespective of habitat. The traits may be driven more by the importance of foraging for dung considering the high competition for such quick-drying resources (Hanski 1991) that are found mainly on the soil surface. However, further research is required to support or disprove this hypothesis.

Flight biomechanics and foraging behavior

A surprisingly low level of correlation was found between biomechanical variables and wing morphology in the present study. However, this may be because, in addition to wing shape and wing size of insects, aerodynamic performance is highly dependent on wing deformation during flight (Shyy et al. 2016), a factor that has been little studied in dung beetles. Nevertheless, differences in flight biomechanics conferred by wing shape variation can result in different degrees of flight efficiency (Johansson et al. 2012), which may be important for foraging behavior in dung beetles.

Thus, estimated values for biomechanical variables may provide some insight into foraging behavior shown by the present subset of species. In >75% of the species, values for wing aspect ratio (WAR) varied between 3 and 5. Insects with similar WAR have short, wide wings that increase drag (Dudley 1990; Wootton 1992) and favor short distance flight (Gibb et al. 2006). High maneuverability and slower flight conferred by low WAR values would suit foraging for a scattered ephemeral food resource such as dung. This is consistent with observations of wing load values (WL) that were >0.55 kg / m² in all but one species. Such higher values suggest they are mostly slower dispersers dependent on a high spatial frequency of food, particularly, in comparison to other beetle species with WL values <0.6 kg / m² that are considered to be faster dispersers (Dudley 2002; Gibb et al. 2006). Measurements of wing loading moment (WLM) varied from lower to higher extremes in different taxa, largely related to body size, although

values were mostly similar in close relatives. Mostly large-bodied species in the Scarabaeini and Coprini tended to lower WLM implying a lower energy strategy with a lower rate of wing beat. By contrast, the higher WLM of smaller bodied species imply a higher energy strategy with a higher rate of wing beat. Such differences in WLM may make smaller-bodied species more competitive in the race to find food before larger-bodied species. This is consistent with the observation of Outomuro et al. (2013b) that the relationships among wing shape, speed, maneuverability and energetic costs seem to be taxon specific.

Conclusions

In a subset of dung beetles inhabiting three contrasting vegetation types, we demonstrated three different species groups in terms of wing shape, one with small body and wing size, two with mostly large body and wing size. Two statistical tests showed that closely related taxa have similar wing shapes that are apparently independent of variation in body and wing size. The same two statistical tests showed that wing shape variation was less strongly or not at all correlated to wing size, habitat and flight biomechanics variables. Therefore, the evolution of wing shape in dung beetles appears to have been phylogenetically constrained. This study suggests that research should be expanded to many different lineages of related species in order to better determine the ecological factors that have driven evolutionary variation in wing morphology.

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Data Availability

The following information is provided regarding data availability:

The raw data are available in Supplementary Material 1, 2, 3 and 5. The script for the morphometric and statistical analyses in R is provided in Supplementary Material 4. Additional results are provided in Figures 1S and 2S.

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