



**Does size matter for horny beetles? A geometric morphometric analysis of interspecific and intersexual size and shape variation in *Colophon haughtoni* Barnard, 1929, and *C. kawaii* Mizukami, 1997 (Coleoptera: Lucanidae).**

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1   **Title:** Does size matter for horny beetles? A geometric morphometric analysis of  
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4

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21 **Abstract**

22 *Colophon* is an understudied, rare and endangered stag beetle genus with all  
23 species endemic to isolated mountain peaks in South Africa's Western Cape.  
24 Geometric morphometrics was used to analyse intersexual and interspecific variation  
25 of size and shape in the mandibles, heads, pronota and elytra of two sympatric  
26 species: *C. haughtoni* and *C. kawaii*. All measured structures showed significant  
27 sexual dimorphism, which may result from male-male competition for females.  
28 Female mandibles were too small and featureless for analysis, but male *Colophon*  
29 beetles possess large, ornate mandibles for fighting. Males had significantly larger  
30 heads and pronota that demonstrated shape changes which may relate to resource  
31 diversion to the mandibles and their supporting structures. Females are  
32 indistinguishable across species, but males were accurately identified using  
33 mandibles, heads and pronota. Male *C. kawaii* were significantly larger than *C.*  
34 *haughtoni* for all structures. These results support the species status of *C. kawaii*,  
35 which is currently in doubt due to its hybridisation with *C. haughtoni*. We also  
36 demonstrate the value of geometric morphometrics as a tool which may aid  
37 *Colophon* conservation by providing biological and phylogenetic insights and  
38 enabling species identification.

39

40 **Keywords:** *Colophon* – Lucanidae – geometric morphometrics – morphology –  
41 species identification – sexual dimorphism.

42

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49

50 **Introduction**

51 *Colophon* Gray, 1832, is a genus of endangered montane stag beetles (subfamily  
52 Lucaninae Latreille, 1804 (Kim and Farrell 2015)) endemic to the Western Cape  
53 Province of South Africa (Fig. 1) (Geertsema and Owen 2007; Switala et al. 2015).  
54 The first species was described by Gray (1832) in 1832, but over 180 years later we  
55 still know very little about these understudied beetles

56 ***Colophon* biology**

57 The genus *Colophon* comprises 17 known species, each endemic to a particular  
58 mountain peak or range. All species are slow-moving and flightless, with adult  
59 activity spanning October to March (Barnard 1929; Endrödy-Younga 1986;  
60 Geertsema and Owen 2007). They inhabit montane fynbos habitats and are closely  
61 associated with *Restionaceae* bushes (Barnard 1929; Brinck 1956). Little else is  
62 known about the ecology, demography, physiology or behaviour of the genus  
63 (Geertsema and Owen 2007; Roets et al. 2013).

64 ***Colophon* conservation**

65 *Colophon* beetles are a research and conservation priority. Locally endemic species  
66 are especially vulnerable to extinction (Van Dyke 2008; Pizzo et al. 2011) and the  
67 restriction of *Colophon* species to the Western Cape mountain peaks makes them  
68 particularly vulnerable to global warming (Parmesan et al. 1999; Switala et al. 2015)  
69 Construction projects are damaging and reducing the already limited habitats of  
70 some populations (Geertsema and Owen 2007) and all *Colophon* species are  
71 threatened by illegal collection, with single specimens being sold for up to  
72 US\$15,000 on the black market (Beeton 1997; Melisch and Schütz 2000). *Colophon*

73 is an important flagship taxon for campaigns against illegal insect trade (New 2009)  
74 and all species are listed on CITES Appendix III (CITES 2015). The genus was  
75 added to the South African ToPS list in 2007 and species are also categorized from  
76 vulnerable to critically endangered on the IUCN Red List (IUCN 2015). However,  
77 these assessments need updating (IUCN 2015) and the effective management and  
78 conservation of *Colophon* is impaired by the paucity of knowledge on their natural  
79 history (New 2009; Roets et al. 2013).

80 **Morphology**

81 This study is the first attempt to statistically examine morphological variation in  
82 *Colophon* stag beetles. As morphology is determined by both genotype and  
83 phenotype it can provide insights into the phylogeny and ecology of *Colophon*  
84 beetles and the selective pressures driving their evolution (Losos and Miles 1994). A  
85 morphological study can also aid the development of a reliable and accurate  
86 identification method for this genus, which is fundamental to studying *Colophon*  
87 biology and ultimately to conserving the genus.

88 Traditionally, shape analysis relied on qualitative descriptions and linear  
89 measurements. These were often inadequate for describing the complexity of many  
90 organisms and the slight (but significant) variations between them. Modern  
91 geometric morphometrics provide a fast, cheap and accurate method for the  
92 detection, quantification and visualisation of subtle shape changes between  
93 organisms even at the intraspecific level (Bookstein 1997; Alibert et al. 2001; Adams  
94 et al. 2004; 2013).

95 ***Colophon* morpho-taxonomy**

96 *Colophon* morphology was first investigated by Endrödy-Younga (1986), who used  
97 morphological characters to create a dichotomous key for the genus and  
98 hypothesize phylogenetic relationships between species. Switala et al. (2014) have  
99 recently confirmed these predictions using molecular techniques highlighting the  
100 value of morphology as a reliable and powerful taxonomic tool. The phylogenetic  
101 placement of one species, *C. kawaii*, remains unsolved (Switala et al. 2014). This  
102 species was undiscovered during Endrödy-Younga's study and is consequently not  
103 included in the identification key for the genus. Furthermore, Switala et al. analysed  
104 only two *kawaii* specimens, which either grouped as sister to *C. cameroni* Barnard,  
105 1929, or nested within *C. haughtoni*. The species status of *kawaii* is further  
106 confounded by molecular evidence for its hybridisation with *haughtoni*, with which it  
107 occurs sympatrically in the Hex River Mountains (Switala 2013; Switala et al. 2014).  
108 Geometric morphometrics have been successfully used to distinguish honeybee  
109 subspecies (*Apis mellifera* Linnaeus, 1761; Hymenoptera: Apidae) (Tofilski 2008)  
110 and to identify cryptic *Nebriola* ground beetle species (Coleoptera: Carabidae)  
111 (Roggero et al. 2013), and similar methods could shed light on *Colophon*  
112 systematics.

### 113 **Sexual dimorphism in *Colophon***

114 Male *Colophon* possess large mandibles which are highly diverse and species-  
115 specific in shape. By contrast, female mandibles are small, non-diagnostic structures  
116 (Endrödy-Younga 1986). Such sexual dimorphism may result from male-male  
117 competition for females; sex ratios in this genus appear to be male-biased for all  
118 species and male *Colophon* have been observed to attack each other using their  
119 mandibles (Geertsema and Owen 2007; T.E., pers. obs.). Male beetle weaponry is  
120 often positively allometric with body size (Petrie 1988; Kawano 1997; Kodric-Brown

121 et al. 2006) and larger weapons and/ or body sizes improve a males fighting success  
122 (Moczek & Emlen 2000; Moczek 2006). This favours sexual selection for increasingly  
123 large, weaponized males and is a likely driver of sexual dimorphism in many  
124 Coleopteran species including *Colophon* (Bonduriansky 2007; Painting and Holwell  
125 2013). Identifying and quantifying sexual dimorphism in *Colophon* is the first step  
126 towards a full understanding of the selective pressures driving their evolution (Emlen  
127 1997; Moczek 2006).

## 128 **Aims and hypotheses**

129 This study will focus on two sympatric species: *C. haughtoni* and *C. kawaii*. The  
130 research need for these species is highlighted by the IUCN Red List (IUCN 2015);  
131 the conservation status of *C. haughtoni* is endangered but requires updating, whilst  
132 *C. kawaii* is not even listed. Switala (2013) suggests that the latter species is  
133 critically endangered. Geometric morphometrics will be employed to quantify,  
134 visualise and test the statistical significance of interspecific and intraspecific size and  
135 shape variation in *Colophon* using four structures: the mandible, head, pronotum and  
136 elytron. Two hypotheses will be tested: (1) all of the above structures demonstrate  
137 sexual dimorphism in the species *C. haughtoni* and *C. kawaii*, and (2) all listed  
138 structures differ significantly in size and shape between male *C. haughtoni* and male  
139 *C. kawaii* beetles. Two additional aims of this study are (i) to provide support for the  
140 hypothesis that *C. kawaii* is a distinct species by testing the degree of morphological  
141 separation between male *C. haughtoni* and *C. kawaii* and (ii) evaluate the accuracy  
142 and value of geometric morphometrics as a tool for identifying *Colophon* species.

143

## 144 **Methods**

145 **Data collection**

146 Data were collected near the peak of Matroosberg Mountain by searching under  
147 shrubs for dead beetle fragments (04/Nov/2014- 10/Jan/2015; Location: S33°23'  
148 E19°40' 2231m; CapeNature Permit 0056-AAA007-00129). Sexes were  
149 distinguished using the mandibles, but females could not be identified to species  
150 level due to their extreme similarity and the consequent lack of any taxonomic key.  
151 Male *Colophon* beetles were identified using traditional qualitative characters based  
152 on Endrödy-Younga's (1986) dichotomous key. As previously mentioned this key  
153 does not include *C. kawaii*. However Endrödy-Younga describes how *C. haughtoni*  
154 can be distinguished from other *Colophon* species by the presence of "a flattened  
155 shiny surface" which connects the apex of the mandible and the dorsal process (see  
156 Fig. 1 for anatomical characters). This structure was found to be absent in *C. kawaii*  
157 (Figs. 1, 2, and so can be used as a basic, qualitative character to differentiate  
158 between these two species.

159 Fragmentary specimens were organised into the following groups: male *haughtoni*,  
160 male *kawaii*, females and "unidentified" *Colophon* individuals whose species and sex  
161 could not be determined. Complete specimens were dissected into the head,  
162 prothorax and abdomen. Pronota were separated from the prosterna and associated  
163 legs, and the elytra were separated from the abdomens and legs. This flattened out  
164 the pronota and elytra and avoided errors associated with placing 2D landmarks on a  
165 3D object (Cardini 2014). Segments were photographed by placing them on a stage  
166 with graph paper underneath. All segments were photographed dorsally and heads  
167 were also photographed ventrally to view the mandibles. A camera (Panasonic  
168 Lumix DMC-FT2 with a 28mm lens) was mounted above with the lens at a distance  
169 of 350mm from the stage. The camera was set at an angle parallel to the stage using

170 a spirit level. Fragments were placed in the centre of the image, which preliminary  
171 tests showed was free from distortion. Four datasets were created: mandible (n *C.*  
172 *haughtoni* = 39; n *C. kawaii* = 12); head (n *C. haughtoni* = 57; n *C. kawaii* = 13; n  
173 female= 19); pronotum (n *C. haughtoni* = 32; n *C. kawaii* = 11; n female = 26; n  
174 unidentified = 104) and elytron (n *C. haughtoni* = 20; n *C. kawaii* = 33; n female = 10;  
175 n unidentified = 113).

176 **Geometric morphometrics**

177 Geometric morphometrics is the analysis of shape using geometric Cartesian  
178 coordinates instead of traditional qualitative variables or linear measurements  
179 (Zelditch et al. 2004). In geometric morphometrics the shape of organisms is  
180 described using a series of landmarks which are defined as discrete anatomical loci  
181 that are homologous across all specimens in the study (Zelditch et al. 2004). Shape  
182 information can be extracted from landmark coordinates and subjected to  
183 multivariate statistical analysis in order to answer a wide range of biological  
184 questions (Adams et al. 2004; 2013).

185 For each dataset in this study landmarks were digitized on photographs using  
186 tpsDig2 ver. 2.17 (Rohlf 2013; 2015; Fig. 2). Only the left side of structures were  
187 digitized to avoid bias caused by bilateral asymmetry (Marrone et al. 2014).  
188 Mandibles were analysed for males only as female mandibles were too small to  
189 reliably identify homologous landmarks. Procrustes superimposition was performed  
190 in MorphoJ ver. 1.06b (Klingenberg 2011) to scale, translate and rotate the landmark  
191 coordinates and produce Procrustes coordinates which contain information on shape  
192 only.

193 **Shape variation and visualisation**

194 A covariance matrix of the shape coordinates was generated in MorphoJ and a  
195 principal component analysis (PCA) performed on the matrix (preliminarily excluding  
196 unidentified beetles). Shape variation was visualised along each PC axis using thin-  
197 plate spline deformation grids produced in tpsRelw ver. 1.53 (Rohlf 2013; 2015).

198 **Size and allometry**

199 The size differences between groups (female, *C. haughtoni*, *C. kawai*) were tested  
200 for statistical significance using IBM SPSS Statistics 22. Each dataset was analysed  
201 separately. The natural log transformed centroid size (LnCS) was used as a  
202 measure of size for each individual. Centroid size is the square root of the summed  
203 squared distances between all landmarks and their centre of gravity or centroid  
204 (Bookstein 1997). A Shapiro-Wilk test showed that all LnCS distributions were  
205 normal ( $p>0.05$ ). Levene's test assessed the datasets for homogeneity of variances.  
206 The mandible and elytron datasets were then tested for differences between species  
207 and sexes using a one-way ANOVA followed by Tukey post-hoc tests to determine  
208 which groups differed significantly in LnCS. The head and pronotum datasets  
209 showed significant heterogeneity of variances ( $p<0.05$ ), so Welch's ANOVAs and  
210 Games-Howell post-hoc tests were applied instead.

211 A multivariate regression in tpsRegr ver. 1.41 (Rohlf 2013; 2015) tested for allometric  
212 effects by regressing partial warps (dependent variable) on LnCS (independent  
213 variable). Partial warps are shape variables calculated in tpsRegr from the raw  
214 landmark data. "Unidentified" beetles were again preliminarily excluded and each  
215 dataset was analysed separately. Deformation grids for the smallest, middle sized  
216 and largest individuals were constructed in tpsRegr to visualise the shape  
217 deformations associated with size changes.

218

219 **Group classification and differentiation**

220 The software PAST (Hammer et al. 2001) was used to test the significance of shape  
221 differences between male *C. haughtoni*, male *C. kawaii* and females (unidentified  
222 beetles were excluded). One-way non-parametric MANOVAs based on the  
223 Euclidean distance measure were performed on the PC scores for the mandible,  
224 head, pronotum and elytron datasets. Significance levels were calculated by  
225 permutation of beetle group membership with 9,999 permutations. Pairwise non-  
226 parametric MANOVAs between all pairs of groups were implemented as post-hoc  
227 tests.

228 Discriminant function analyses (DFA) using cross-validation methods were  
229 performed in IBM SPSS Statistics 22 to statistically test the ability of size and shape  
230 variables to predict which beetle group a specimen belonged to. Beetle category  
231 (male *C. haughtoni*, male *C. kawaii* and female) was used as the factor and LnCS  
232 and principal component (PC) scores were used as independent variables.  
233 Unidentified individuals were included and categorised as unknown. Procrustes  
234 Coordinates and LnCS were also used as independent variables but were less  
235 accurate and so were discarded (see also Meloro 2011 and Meloro et al. 2015).  
236 Stepwise methods were employed and a variable was entered into the model if the  
237 probability of its *F*-value was greater than 0.05 and removed if the probability value  
238 was lower than 0.10.

239 **Results**

240 **Shape variation and visualisation**

241 The mandible PCA shows two distinct clusters for *C. haughtoni* and *C. kawaii* when  
242 PC1 is plotted against PC2 (Fig. 3a). Together PC1 and PC2 explain 83.63% of total  
243 variance in the sample. *C. haughtoni* specimens are grouped at the extreme right of  
244 PC1, and are shown in the thin-plate spline to be more elongated with a longer  
245 ventral process (see Fig. 1 for anatomical traits). *C. kawaii* mandibles group at the  
246 extreme negative of PC1, being characterised by a squatter overall shape with a  
247 relatively short ventral process and a pronounced indent between the apex and  
248 dorsal process of the mandible.

249 PC1 and PC2 explain 77.89% of variance in the head PCA (Fig. 3b). This plot  
250 produces a total separation between male and female beetles along PC1, which  
251 mainly describes the relative length of the head. The thin-plate splines show females  
252 to have a protruding “forehead” or frons whilst males have a larger pre-orbital area at  
253 the front corner of the head (represented by the top left landmark in Fig. 3b).  
254 Although heterospecific males show substantial overlap in shape space, *kawaii*  
255 specimens are positioned more towards the positive end of the PC2 axis which  
256 describes a shorter, broader head shape than for *haughtoni* males.

257 Pronotum shape also produces a good separation of sexes (Fig. 3c). PC1 and PC2  
258 explain 80.77% of the total variance in this structure and display differences between  
259 sexes and heterospecific males respectively. Females are distributed at the positive  
260 end of PC1 and their pronota are slightly squatter and have shorter, narrower  
261 anterior regions compared to males. *C. kawaii* males have more protrusive shoulders  
262 than females and *haughtoni* males.

263 PC1 and PC2 explain 86.56% of variation in the elytra (Fig. 3d). This plot does not  
264 show any separation between groups but males generally have lower PC1 scores

265 than females. Thin-plate splines show that PC1 describes the relative elytron length  
266 and that male beetles have relatively shorter elytra compared to females. Changes  
267 on PC2 relates to a relative enlargement (negative scores) or shortening (positive  
268 scores) of the anterior edge of the elytra (Fig. 3d).

269 **Size and allometry**

270 Females consistently have the smallest LnCS for each body structure followed by  
271 male *C. haughtoni* then male *C. kawaii* (Figs. 4a-d). ANOVAs and post-hoc tests  
272 showed highly significant differences between all groups for the LnCS of every body  
273 structure (Table 1; all post-hoc tests:  $p \leq 0.001$ ). Regression analyses showed a  
274 significant correlation between size and shape for all body structures indicating  
275 strong allometry in all structures (Table 1). Allometry was visualised using  
276 deformation grids which depicted the shapes of the smallest and largest individuals  
277 and their deformation from the mean shape (Figs. 4a-d).

278 Allometry explains 15.8% of variation observed in the mandible dataset (Table 1)  
279 and deformation grids indicate a strong shape deformation from small to large  
280 mandible sizes (Fig. 4a). The smallest individual, represented by a *C. haughtoni*  
281 specimen, shows a contraction of the dorsal process compared to the mean shape  
282 for the sample. The largest specimen, a *C. kawaii*, shows an expansion of this region  
283 compared to the mean and a more pronounced indent between the dorsal process  
284 and the mandibular apex. The ventral process and apex are relatively shorter in the  
285 largest beetle.

286 Allometry accounts for 41.96% of the total shape variation in *Colophon* heads (Table  
287 1). Smaller individuals are characterized by more elongated, convex heads, as  
288 represented by the two landmarks on the right side of the deformation grids in Fig.

289 4b. Larger specimens have comparatively shorter heads, smaller eyes and larger  
290 genal and pre-orbital regions.

291 Allometry explains 42.68% of pronotal shape variation (Table 1). Deformation grids  
292 demonstrate that the smallest pronota are wide at the base but become increasingly  
293 narrower and shorter towards anterior portions (Fig. 4c). Larger pronota show the  
294 opposite trend of having enlarged anterior regions and contracted posterior regions.

295 Allometry explains 20.2% of variation observed in the elytron dataset. The smallest  
296 elytron has a smaller and more posteriorly placed scutellum compared to the mean,  
297 whilst the largest elytron depicts a larger, longer scutellum which extends further  
298 anteriorly compared to the mean (represented by the bottom right landmarks in Fig.  
299 4d).

300 **Group differentiation and classification**

301 Non-parametric MANOVAs showed that morphometric differences between male *C.*  
302 *haughtoni*, male *C. kawaii* and female *Colophon* were significant for all measured  
303 structures ( $p \leq 0.02$ ; Table 2). DFAs indicated that size and shape data from the  
304 mandibles, heads and pronota could be used to accurately predict the sex and  
305 species status of *C. haughtoni* and *C. kawaii* (Table 3). Data from the elytra could be  
306 used to accurately classify male *C. kawaii* and females, but not male *C. haughtoni*  
307 (Table 3).

308 The DFA for the mandible dataset selected LnCS, PC1, PC2, PC3 and PC4 (in order  
309 of decreasing loading - see Table 3a) to discriminate beetle groups. One significant  
310 discriminant function (DF) was extracted to distinguish between groups (Wilks  $\lambda =$

311 0.091,  $\chi^2(5)= 111.62$ ,  $p< 0.0001$ ). Following cross-validation, the percentage of  
312 correct classifications was 100% for both *C. haughtoni* and *C. kawaii*.

313 Two significant functions were derived for the head DFA (DF1: 93.6% variance,  
314 Wilks  $\lambda= 0.041$ ,  $\chi^2(10)= 267.951$ ,  $p< 0.0001$ ; DF2: 6.4% variance, Wilks  $\lambda=0.544$ ,  
315  $\chi^2(4)= 51.196$ ,  $p< 0.0001$ ). PC's 1, 2, 4, 5 and LnCS maximally differentiated  
316 between groups (Table 3b). All females, 82.5% of *C. haughtoni* and 84.6% of *C.*  
317 *kawaii* specimens were correctly classified following cross-validation.

318 The Pronotum DFA produced two significant DFs (DF1: 91.9% variance, Wilks  
319  $\lambda=0.108$ ,  $\chi^2(8)= 143.711$ ,  $p< 0.0001$ ; DF2: 8.1% variance, Wilks  $\lambda=0.682$ ,  $\chi^2(3)=$   
320 24.733,  $p< 0.0001$ ) loaded on LnCS, PC1, PC2 and PC8 (Table 3C). The percentage  
321 of correct classifications were high for all cross-validated groups (females: 96.2%; *C.*  
322 *haughtoni*: 90.6%; *C. kawaii*: 72.7%).

323 Although beetle groups could not be separated by plotting elytron PC1 values  
324 against PC2 values, two significant discriminant functions were selected for in the  
325 DFA (DF1: 95.4% variance, Wilks  $\lambda=0.264$ ,  $\chi^2(6)= 78.683$ ,  $p< 0.0001$ ; DF2: 4.6%  
326 variance, Wilks  $\lambda=0.896$ ,  $\chi^2(2)= 6.458$ ,  $p< 0.0001$ ). LnCS, PC1 and PC2 were the  
327 variables which most separated the groups (Table 3d). Females (85%) and *C. kawaii*  
328 (80%) showed high percentages of correct classifications, whilst *C. haughtoni* was  
329 correctly classified only 66.7% of the time after cross-validation.

330 **Discussion**

331 This study has made the first exploration of *Colophon* morphology using geometric  
332 morphometrics. *C. kawaii* is found at only one location where it occurs sympatrically  
333 with *C. haughtoni* (Switala 2013), but there is currently no standard method for

334 discriminating between these species. We identified a structure (a flattened shiny  
335 surface connecting the mandible apex with the dorsal process (Fig. 1)) which is  
336 present in *C. haughtoni* but absent in *C. kawaii* and so can be used as a basic  
337 qualitative character for distinguishing these species. However, traditional qualitative  
338 morphometric characters cannot be subjected to statistical analysis or demonstrate  
339 the significance of morphological variations in the genus (Bookstein 1997; Alibert et  
340 al. 2001; Adams et al. 2004; 2013). Modern geometric morphometric methods  
341 provide a new, analytical perspective which advance the taxonomy of this genus,  
342 enable the visualisation of previously undetected shape changes and allow us to  
343 answer a range of biological questions regarding *C. haughtoni* and *C. kawaii*.

344 We found that sexual dimorphism is significant in the heads, pronota and elytra of  
345 these species (Hypothesis 1). The morphology of the mandibles, heads, pronota and  
346 elytra also differs significantly between male *C. haughtoni* and male *C. kawaii*  
347 (Hypothesis 2). This result fulfils our first aim by giving support to the hypothesis that  
348 *C. kawaii* is a distinct species. We were able to accurately distinguish sexes and  
349 males of different species, suggesting geometric morphometrics are a powerful tool  
350 for the classification of *C. haughtoni* and *C. kawaii* (Aim (ii)).

351 **1. Sexual dimorphism**

352 Some studies suggest natural selection, such as divergence in feeding niches, leads  
353 to shape divergence between sexes (Temeles and Roberts 1993; Temeles et al.  
354 2000). However, unambiguous examples of ecologically driven sexual dimorphism  
355 are rare and most studies suggest sexual selection is the primary cause (Shine  
356 1989). Observations of sexual dimorphism in this study will therefore only be  
357 discussed in relation to sexual selection.

358 Sexual dimorphism is evident in the mandibles of *Colophon* but geometric  
359 morphometrics also revealed significant size and shape differences in the head,  
360 pronotum and elytron that were not previously apparent (Table 2). Deformation grids  
361 allowed the visualisation of subtle shape changes which may have important  
362 biological functions. Regression showed that shape variation due to allometry was  
363 significant for all structures (Table 1) and females had consistently smaller LnCS  
364 values than males (Figs. 4a-d). This may be due to sexual selection for increased  
365 overall size in males, which is associated with positive allometric mandible growth  
366 and increases their success in male-male competition (Petrie 1988; Kawano 1997;  
367 Kodric-Brown et al, 2006).

368 **1.1. Head**

369 Female heads were significantly smaller than in males but were relatively longer with  
370 larger eye diameters and a more protrusive frons. Males had a more concave frons,  
371 a larger pre-orbital area and larger genae (Fig. 3a). Sexual dimorphism in *Colophon*  
372 heads may be related to mandibular form and function. Goyens et al. (2014) found  
373 that longer mandibles and stronger bite forces in male *Cyclommatus metallifer* stag  
374 beetles (Boisduval, 1835; Coleoptera: Lucanidae) are compensated for by longer  
375 input levers and larger closer muscles. These structures in turn require larger heads  
376 with broader anterior portions compared to females. A similar mechanism could  
377 explain the large size and pre-orbital region of male *Colophon* heads.

378 Male *Colophon* also had smaller eye diameters and wider genae than females (Fig.  
379 3a). Okada and Miyatake (2009) observed similar trends in large-mandibled  
380 *Gnatocerus cornutus* Fabricius, 1798, beetles (Coleoptera: Tenebrionidae)  
381 compared to small-mandibled males. They suggest that larger genae may develop in

382 compensation for enlarged mandibles or may be used for display in male-male  
383 competition (Okada and Miyatake 2009). Emlen (2001) demonstrated that excessive  
384 horn growth in *Onthophagus* Latreille, 1802, dung beetles can divert resources from  
385 nearby structures such as eyes and antennae and a similar trade-off could underlie  
386 the relatively smaller eyes of male *Colophon* beetles compared to females.

387 Male-male competition in *Colophon* beetles may produce sexual selection for  
388 increasing mandible size and power in males leading to corresponding sexual  
389 dimorphism in head morphometry.

390 **1. 2. Pronotum**

391 Visual examination suggested pronotal morphology was homogenous across sexes  
392 but our analyses revealed otherwise. Size was a prominent distinguishing factor  
393 between the sexes and females had significantly smaller pronota than males of both  
394 species (Table 3, Fig. 4c). Hlavac (1969) and Okada and Miyatake (2009) suggest  
395 that larger prothoraces in male beetles may contain a greater muscle mass and  
396 energy store and so may evolve to compensate for their large mandibles and the  
397 forces produced during combat (Tomkins et al. 2005). This may also explain the  
398 expansion of male *Colophon* pronota towards the head region, where most support  
399 is needed (Fig. 3c).

400 Deformation grids also revealed that female pronota are generally squatter than in  
401 males and although they are smaller anteriorly they are wider towards the abdomen  
402 (Fig. 3c). In contrast to male beetles, females may divert their resources posteriorly  
403 to improve their reproductive success. Preziosi et al. (1996) and Adams and Funk  
404 (1997) hypothesize that larger abdomens increase the egg carrying capacity and  
405 consequently the fecundity of female insects. Fairn et al. (2007) found that the

406 pronota of female *Dineutus nigrior* Roberts, 1895, beetles (Coleoptera: Gyrinidae)  
407 were shorter compared to males and suggested this was in compensation for longer  
408 elytra and increased egg storage. Very few studies have explored pronotal sexual  
409 dimorphism in beetles but the distinctive pronotal morphology of male and female  
410 *Colophon* suggests that this structure may be influenced by sexual selection and so  
411 may give insight into the reproductive biology of *C. haughtoni* and *C. kawaii*.

412 **1.3. Elytron**

413 Although elytral shape was significantly different between all beetle groups (Table 2),  
414 considerable overlap between sexes was observed in the elytron PCA plots (Fig.  
415 3d). This suggests that sexes are morphologically more similar in their elytra  
416 compared to other structures. Size was the most discriminating factor between  
417 males and females (Table 3D), and allometric effects were primarily associated with  
418 an increase in scutellum size from small females to large males. Females tended  
419 towards a relatively longer elytron than males (Fig. 3a) and as previously discussed  
420 this may result from selection for increased egg carrying capacity (Preziosi et al.  
421 1996; Adams and Funk 1997). This hypothesis has been used to explain relatively  
422 wider abdomens observed in female Chilean Magnificent Beetles (*Ceroglossus*  
423 *chilensis* Eschscholtz, 1829) (Benitez et al. 2011) and the longer abdomens in  
424 female *Neochlamisus bebbianae* Brown, 1943, leaf beetles (Adams and Funk 1997).  
425 The observed dimorphism in *Colophon* may equally be caused by selection for  
426 shorter elytra in males. Kawano (1997) found that elytral size was negatively  
427 allometric to body length in Lucanid males and Okada and Miyatake (2009) observed  
428 that increased horn growth in male *G. cornutus* beetles was correlated with reduced  
429 elytral length. This suggests that resources are diverted anteriorly to structures more  
430 important for competition in male beetles (Okada and Miyatake 2009). Our results

431 indicate gender specific trends in elytral morphology but the detection and  
432 visualisation of shape changes was probably hindered by the lack of homologous  
433 landmarks on this structure. Further studies should be conducted using semi-  
434 landmark methods (Zelditch et al. 2004; Van Bocxlaer and Schultheiß 2010).

435 Most studies focus on sexual dimorphism in beetle weaponry and little information is  
436 available on other body structures. However, gender-specific trends in head,  
437 pronotal and elytral shape may provide additional insight into the sexual selective  
438 pressures underlying morphological diversification.

439 **2. Interspecific variation**

440 Interspecific morphological variation was not explored in female *Colophon* as they  
441 could not be identified to species level and it remains for genetic methods or more  
442 detailed anatomical analyses to separate females according to species. This  
443 highlights their extreme morphological homogeneity, as is typical for most  
444 Coleopteran species (Kawano 2006; Switala 2013). Male *C. haughtoni* and male *C.*  
445 *kawaii* demonstrated significant morphological differences in the mandibles, heads,  
446 pronota and elytra (Table 2). and these species could be accurately classified using  
447 these structures . Size (LnCS) significantly affected shape variation in all structures  
448 (Figs. 4a-d) and made a large contribution to species separation in both PCAs and  
449 DFAs (Tables 1 and 3). *C. kawaii* were significantly larger than *C. haughtoni* for all  
450 structures. The results of this study showed species-specific morphological variation  
451 in *C. haughtoni* and *C. kawaii*, giving support to the hypothesis that these are distinct  
452 species that are able to hybridise.

453 **2.1. Mandible**

454 Coleopteran weapons are impressive not only for their size but also their diversity  
455 and male *Colophon* are no exception to this. *C. haughtoni* were shown to have a  
456 longer ventral process than *C. kawaii* and less distinct mandibular horns due to a  
457 flattened surface connecting the mandible apex to the dorsal process (Figs. 1, 3a).  
458 Although all male beetle weapons are used in male-male competition it is not yet  
459 clear why these organs are so diverse, given their common function (Emlen et al.  
460 2005). Palmer (1978), Eberhard (1981) and Siva-Jothy (1987) suggest that the  
461 variation in Coleopteran weapon morphology mirrors species-specific differences in  
462 how they are utilised in combat; specialized knobs and spikes may provide leverage  
463 and friction specific to particular fighting tactics (Emlen et al. 2005). Male *C.*  
464 *haughtoni* have been observed to use their mandibles to attack an opponent's gula  
465 (throat) or leg (T.E., pers. obs.). However further observations and statistical  
466 analyses for both species are necessary to confirm whether the divergent mandible  
467 morphology of *C. haughtoni* and *C. kawaii* is related to differing combat behaviour.

468 **2.2. Head**

469 Compared to *C. haughtoni*, *C. kawaii* heads were bigger and broader. As previously  
470 discussed, changes in mandible size, form and function are shown to produce  
471 changes in the head morphology of male and female *C. metallifer* stag beetles  
472 (Goyens et al. 2014) and also between different ant species (Paul and Gronenberg  
473 1999). The observed divergence in *Colophon* head shape may similarly result from  
474 the larger, differentially shaped mandibles of *C. kawaii* compared to *C. haughtoni*.

475 **2.3. Pronotum**

476 The larger pronotum in *C. kawaii* may be due to the increase in overall body size but  
477 no explanation could be found for the more protrusive shoulders observed in this

478 species (Fig. 3c). Some studies find that morphological variations are correlated with  
479 ecological factors (Forsythe 1991; Barton et al. 2011). For example, mandible length  
480 is correlated with prey size in tiger beetles (Coleoptera: Carabidae) (Pearson and  
481 Mury 1979; Ganeshiah and Belavadi 1986) and many insect species show  
482 significant trends in body size with increasing altitude (Chown and Klok 2003). There  
483 is insufficient information on *Colophon* to make assumptions regarding the ecological  
484 drivers of their morphological diversification but quantifying this diversity is the first  
485 step in elucidating the selective pressures underlying their speciation.

486 **3. Accuracy of geometric morphometrics**

487 This study has confirmed both the power and the shortcomings of geometric  
488 morphometrics for *Colophon* identification. Intraspecific variation could not be  
489 analysed in females without *a priori* species identification but the DFA distinguished  
490 them from males with 85-100% accuracy for all structures. Males could be identified  
491 to species level with 100% accuracy based on their mandibles but also with high  
492 percentages of accuracy (72.7-90.6%) using just heads and pronota. The remote  
493 distribution and elusive nature of *Colophon* poses a barrier to scientific research  
494 (Switala et al. 2015) and often only a few incomplete fragments are the only  
495 evidence to be found in the field (Endrödy-Younga 1986; T.E., pers. obs.). Scientists  
496 are heavily reliant on mandible morphology for species and sex identification  
497 (Switala 2013) but due to *Colophon*'s relatively small size the head and mandibles  
498 are often missing (T.E., pers. obs.). Whilst genetic methods are most popular for  
499 insect species identification (Behura 2006) the provincial laws protecting *Colophon*  
500 beetles (CITES 2015) also hinder the acquisition of fresh specimens for genetic  
501 analysis. In addition, species including *C. haughtoni* and *kawaii* inhabit private land  
502 and approval from landowners to remove these endangered and valuable beetles

503 may be problematic (T.E., pers. obs.). To aid species identification in the field  
504 Switala (2013) attempted to determine diagnostic larval characters for *Colophon*.  
505 However as with many scarabaeoids, interspecific differences were too small for this  
506 purpose. Geometric morphometrics could provide a reliable, fast and cost effective  
507 technique for *Colophon* identification which could be particularly valuable when  
508 attempting to map species distributions or locate new populations.

509 **Conclusion**

510 Prior to this study interspecific and intersexual shape variations were observed but  
511 not yet quantified in *Colophon* mandibles and the morphological variation of the  
512 head, pronotum or elytra was not considered for the genus. Only *C. haughtoni* and  
513 *C. kawaii* were analysed in this study but we showed that geometric morphometrics  
514 can be a powerful technique for exploring interspecific and intersexual variation in  
515 any *Colophon* species. These methods allowed the detection and statistical analysis  
516 of subtle shape variations that were previously unknown and identified body  
517 structures other than the mandibles that could be used for species and sex  
518 discrimination. Sexual dimorphism is evident in the mandibles of all *Colophon*  
519 species but we also showed significant dimorphisms in the head, pronotum and  
520 elytra of *C. haughtoni* and *C. kawaii*. The morphological distinction of heterospecific  
521 male beetles gave support to the hypothesis that *kawaii* is a valid species.

522 Our results provided novel insights into the interspecific and intersexual shape  
523 diversity of *C. haughtoni* and *C. kawaii* and identified additional avenues for study.  
524 Further research on sexual dimorphism could give insight into the reproductive  
525 biology of the genus, which is currently unknown. Also, it is important to understand  
526 how sexual selection has shaped *Colophon* evolution (e.g. male weaponization and

527 hypertrophy) and whether interspecific differences in male beetles could reflect  
528 ecological adaptations such as altitudinal size variation. Finally, geometric  
529 morphometrics could help to elucidate *Colophon* phylogeny and create a taxonomic  
530 key for the entire genus.

531 Ultimately, geometric morphometrics could aid *Colophon* conservation by facilitating  
532 accurate species identification, thereby enabling taxon-specific, targeted  
533 conservation strategies. Thereafter geometric morphometrics could give insight into  
534 the reproductive biology, ecology and distribution of this rare and endangered group  
535 of beetles.

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716 **Tables:**

717 **Table 1.** Size variation and allometry in male *C. haughtoni*, male *C. kawaii* and female *Colophon*  
 718 (species unknown). ANOVAs tested for significant differences in the natural logarithm of centroid size  
 719 (LnCS) between beetle groups. Shape variables were regressed on LnCS to test for allometry in each  
 720 structure. % expl. var. is the variation in each dataset explained by size. Goodall's *F*-test analyses the  
 721 significance of the regression.

	ANOVA			% expl. var.	Goodall's <i>F</i> -test		
	<i>F</i> -ratio	d.f.	<i>p</i> -value		<i>F</i> -ratio	d.f.	<i>p</i> -value
<b>Mandible</b>	30.92	1, 49	<0.0001	15.80%	9.25	6, 294	<0.0001
<b>Head</b>	241.9	2, 36.64	<0.0001	41.96%	63.07	8, 696	<0.0001
<b>Pronotum</b>	145.28	2, 27.14	<0.0001	42.68%	49.94	8, 536	<0.0001
<b>Elytron</b>	31.42	2, 60	<0.0001	20.21%	15.45	4, 244	<0.0001

722

723

724 **Table 2.** Results of non-parametric MANOVAs (9999 permutations) and pairwise comparisons  
 725 performed on principal component scores from the mandibles, heads, pronota and elytra of male  
 726 *Colophon haughtoni*, male *C. kawaii* and female *Colophon* (species unknown).

	MANOVA		Pairwise comparisons		
	<i>F</i>	<i>p</i>	groups	<i>F</i>	<i>p</i>
<b>Mandible</b>	65.17	0.001	<i>hau</i> x <i>kaw</i>	65.17	0.001
<b>Head</b>	70.09	0.0001	<i>hau</i> x <i>kaw</i>	16.32	0.0001
			<i>hau</i> x <i>fem</i>	110.3	0.0001
			<i>kaw</i> x <i>fem</i>	93.45	0.0001
<b>Pronotum</b>	38.33	0.0001	<i>hau</i> x <i>kaw</i>	9.392	0.0001
			<i>hau</i> x <i>fem</i>	53.83	0.0001
			<i>kaw</i> x <i>fem</i>	42.76	0.0001
<b>Elytron</b>	14.38	0.0001	<i>hau</i> x <i>kaw</i>	3.629	0.02
			<i>hau</i> x <i>fem</i>	19.22	0.0001
			<i>kaw</i> x <i>fem</i>	21.35	0.0001

727

728 **Table 3.** Canonical discriminant coefficients and loadings for the discriminant function analyses  
 729 applied to body structures of *Colophon* beetles. The shape (principal component (PC) scores) and  
 730 size (natural logarithm of centroid size (LnCS)) of body structures were used to distinguish between  
 731 male *C. haughtoni*, male *C. kawaii* and female *Colophon* (species unknown). The standardized  
 732 coefficient indicates the contribution of each size/shape variable to the discriminant function(s)  
 733 derived from the analysis. Loading represents the correlation between the discriminant function(s) and  
 734 an independent variable (beetle group).

	Standardized Coefficient		Loading	
	Function 1	Function 2	Function 1	Function 2
<b>(A) Mandible*</b>				
LnCS	-0.634		-0.251	
PC1	1.342		0.545	
PC2	-0.490		-0.073	
PC3	0.603		0.088	
PC4	-0.604		-0.034	
<b>(B) Head</b>				
PC1	1.133	0.125	0.785	-0.443
PC2	0.723	0.775	0.08	0.732
PC4	0.094	0.552	0.007	0.344
PC5	-0.307	-0.003	-0.021	-0.072
LnCS	-0.135	0.491	-0.336	0.607
<b>(C) Pronotum</b>				
LnCS	-0.494	0.164	-0.764	0.172
PC1	0.739	0.308	0.777	0.520
PC2	0.285	-0.588	0.152	-0.742
PC8	-0.137	0.586	-0.42	0.642
<b>(D) Elytra</b>				
LnCS	0.688	0.623	0.657	0.336
PC1	0.675	-0.814	0.593	-0.788
PC2	0.666	0.331	0.221	0.451

735 \*Only one Discriminant Function was selected for the mandible dataset

736

737 **Figure Legends:**

738 **Fig. 1.** Adult *Colophon* beetles. (A) *Colophon haughtoni*. (B) Ventral photograph of *C. haughtoni* head  
739 showing (1) gena, (2) mandible base, (3) ventral process, (4) dorsal process, and (5) apex of the  
740 mandible. (C) Ventral view of *C. kawaii* head. Scale bars represent 4mm (A) and 2mm (B-C).  
741 Photographs by H.J. de Klerk.

742 **Fig. 2.** Landmarks used for geometric morphometric analysis of *Colophon* specimens: (A) male *C.*  
743 *haughtoni* mandible; (B) male *C. kawaii* mandible; (C) male *C. haughtoni* head; (D) female head; (E)  
744 pronotum; (F) elytron. Scale bars represent 2mm.

745 **Fig. 3.** Plots of the first two principal component (PC) scores obtained from principal component  
746 analyses on the shapes of four structures in *Colophon* beetles; (A) mandible, (B) head, (C) pronotum  
747 and (D) elytron. The analysis was carried out on male *C. haughtoni*, male *C. kawaii* and female  
748 *Colophon* of unknown species. The values in brackets for each axis represent the percentage of  
749 shape variance explained by each PC. Deformation grids show the shape change from the consensus  
750 to the extreme positive and negative of each PC axis.

751 **Fig. 4.** Box plots and deformation grids showing size variations and shape deformations in *Colophon*  
752 beetles: female *Colophon*, male *C. haughtoni* and male *C. kawaii*. Size was measured as natural log  
753 transformed centroid size (LnCS). Deformation grids show the shape changes related to size from the  
754 smallest to the largest individuals. Values in parentheses are the magnification applied to improve  
755 visualisation of shape deformations.

756