Phylogeography and morphometric variation of the spike-heeled lark *Chersomanes albofasciata* complex

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Abstract

The Spike-heeled Lark *Chersomanes albofasciata* is a peculiar, short-tailed lark confined to southern, central and East Africa. Typical of resident larks, it exhibits marked geographic variation in plumage colour which has historically resulted in large numbers of subspecies being described. This variation was examined by sequencing 630 base pairs of the cytochrome *b* gene in 39 individuals and morphometric measurements of 320 birds. Nine of the 14 southern African *C. albofasciata* subspecies defined by Clancey in 1980 were sampled for assessing genetic variation, as well as one population from northern Namibia (*C. a. boweni*) and the isolated *C. [a.] beesleyi* population in Tanzania. Within South Africa, 16 maternal lineages were identified. The haplotype network revealed three geographically distinct haplogroups: Namaqualand, Karoo and Eastern grasslands, which did not follow past or existing subspecies ranges. Our data thus suggest incongruence between genetic variation and phenotype in South Africa. The boundaries and relationship among the phylogeographic haplogroups and morphometric measurement were similar to the closely related long-billed lark *Certhilauda curvirostris* complex suggesting similar processes during their evolutionary history. The existing subspecies designations are clearly not supported by the genetic data presented here and thus more research into this complex is required. Further research should include sampling of Spike-heeled Larks in underrepresented areas of the species' range and the use of additional genetic markers to improve our understanding of the evolutionary history of this complex. *C. [a.] beesleyi* was sister to all haplotypes sampled and smaller in size than all subspecies measured. While the status of the contentious *C. [a.] beesleyi* has still not been resolved, we have added morphometric data supporting its isolation from other subspecies.

Its limited and isolated range warrants increased conservation efforts and we propose its recognition as an evolutionarily significant unit.

Keywords: cytochrome *b*; intraspecific variation; Alaudidae

Introduction

The Spike-heeled Lark *Chersomanes albofasciata* is a distinctive lineage within the Alaudidae, characterised by its short, white-tipped tail, erect stance, and occurrence in small family groups, with at least some pairs aided at the nest by group members (Dean 2005). It is sedentary throughout its range (Dean and Hockey 1989; Keith et al. 1992), and occurs in a wide range of open habitats, from sea-level to over 2000 m above sea-level in grassland, semi-desert and desert edge. It avoids areas with trees (Dean 2005), and thus most of its range is confined to the area south of the central African woodlands. Spike-heeled Larks are widely distributed across southern Africa, with populations ranging north into south-central Angola (Dean et al. 1997; Dean 2000; 2005, Figure 1A). Farther north, there are two small, known isolated populations: one recently discovered population in Katanga Province, SE Democratic Republic of Congo (DRC), currently placed with the Angolan *C. a. obscurata* (Louette and Hasson 2017), and *C. [a.] beesleyi* from the Asogati Plain in the rain shadow of Mt Kilimanjaro in northern Tanzania (Benson and Forbes-Watson 1966). The latter taxon has a highly restricted range and very small population (Zimmerman et al. 1996; Lanham 1997; Figure 1A) and is treated as a separate species by some authorities (e.g. Gill and Donsker 2019).

Given their sedentary nature, broad habitat tolerance and large geographic range, Spike-heeled Larks exhibit considerable phenotypic variation linked to differences in rainfall, vegetation structure and soil colour (Meinertzhagen 1951; Macdonald 1952; de Juana et al. 2004; Dean 2005; Donald et al. 2017). This morphological and plumage variation has resulted in the description of numerous subspecies over the years, with 21 different subspecies names in existence (Roberts 1940; Meinertzhagen 1951; Macdonald 1952; Winterbottom 1958; Rudebeck et al. 1970; Clancey 1980; Dean and Keith 1992; Ryan 2004a; Dean 2005; Ryan 2017; Gill and Donsker 2019). Clancey (1980) recognised the most subspecies concurrently with 16 demarcated on the range map. Most modern texts recognise nine subspecies (Dean and Keith 1992; de Juana et al. 2004; Dean 2005; Ryan 2017; Gill and Donsker 2019). Dean (2005) suggested that some of these nine may not be valid and also redefined several subspecies boundaries compared to Clancey (1980; Figure 1B). These subspecies

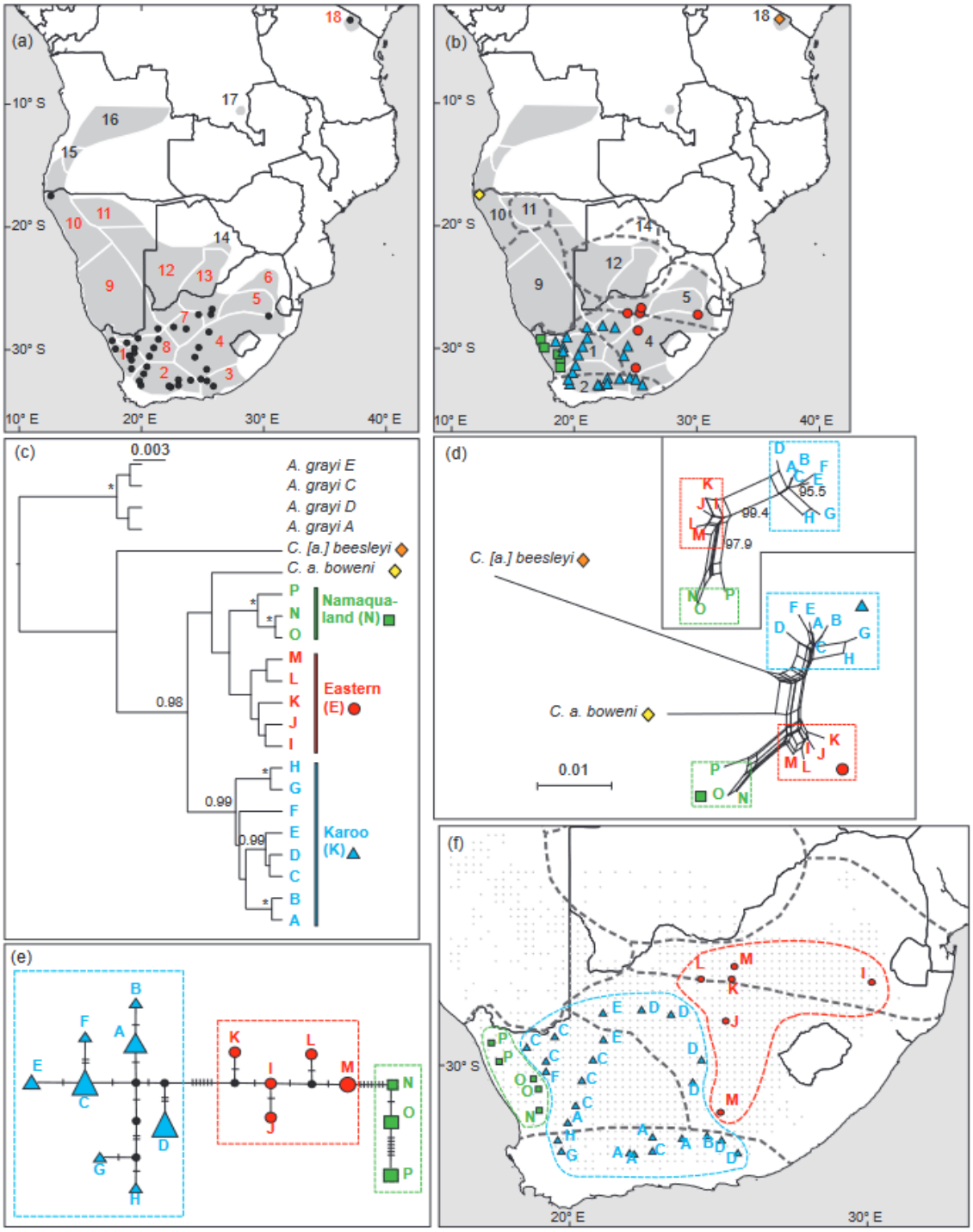

Figure 1. Spike-heeled Lark distribution, sampling and genetic clustering. (A) Spike-heeled Lark subspecies designation *sensu* Clancey 1980 with two additions from Democratic Republic of Congo (17) and Tanzania (18). *Clancey map upper limit 10 °S. Numbers refer to subspecies designations as follows: 1. *C. a. garrula*; 2. *C. a. macdonaldi*; 3. *C. a. latimerae*; 4. *C. a. albofasciata* 5. *C. a. alticola*; 6. *C. a. subpallida*; 7. *C. a. baddeleyi*; 8. *C. a. bushmanensis*; 9. *C. a. arenaria*; 10. *C. a. boweni*; 11. *C.*

a. erikssoni; 12. *C. a. kalahariae*; 13. *C. a. bathoeni*; 14. *C. a. barlowi*; 15. *C. a. longispina*; 16. *C. a. obscurata*; 17. putative *C. a. obscurata*; 18. *C. [a.] beesleyi*. Bold numbers indicate subspecies with morphometric data. Genetic sampling indicated with ●. (B) Spike-heeled subspecies designation in Roberts VII (Dean 2005), compared to Clancey (1980), showing locations of samples in the haplotype analysis indicated with ■ for the Eastern, ▲ for the Karoo, ● for the Namaqualand haplogroups, ♦ for *C. a. boweni* (10) and ♦ for *C. [a.] beesleyi* (18); colours and symbols used throughout the Figure. (C) The phylogenetic relationship within the spike-heeled lark complex inferred using the Yule model and Bayesian Inference (BI) rooted on *Ammomanopsis grayi*. Posterior probabilities above 0.90 are shown above nodes; * indicates support of 1.00. Letters refer to haplotypes defined in Supplementary Table S5. (D) NeighborNet networks for Spike-heeled Larks generated in SplitsTree. Proposed genetic groups are indicated. Inset shows support levels between the South African groups. (E) A haplotype network for Spike-heeled Larks (35 step maximum) constructed with TCS. The size of each symbol is proportional to the frequency of the haplotype. Each connecting line represents a single mutational change and the small closed circles represent unsampled or extinct haplotypes. Boxes represent the geographical haplogroups that are represented on the map (panel F). (F) Haplotype distribution compared to Roberts VII subspecies designations with proposed genetic haplogroups. Grey dots represent Southern African Bird Atlas Project 2 data (the recorded range of the species within the region).

designations do not include the controversial *C. [a.] beesleyi* found in Tanzania. Initially categorised as a subspecies of *C. albofasciata* after its discovery in 1966 (Benson and Forbes-Watson 1966), it was split as *C. beesleyi* by Ryan (2004b), a decision reflected in the IOC list (Gill and Donsker 2019). Concerns regarding this split were raised by Donald and Collar (2011) and BirdLife International (2020) still categorises it as a subspecies, *C. a. beesleyi,* as it does not meet their criteria for full species status. Handbook of Birds of the World Alive (HBWAlive) follows the BirdLife International list and also lists *C. a. beesleyi* as a subspecies (Ryan 2017).

A molecular phylogeny of the Alaudidae showed the Spike-heeled Lark to be nested in a well-supported archaeo-endemic southern African radiation along with Gray's Lark *Ammomanopsis grayi*, Short-clawed Lark *Certhilauda chuana* and the long-billed lark *Certhilauda curvirostris* (*sensu lato*) complex (Barnes 2007; Alström et al. 2013). Alström et al. (2013) only included two Spike-heeled Lark samples in their analyses: *C. a. boweni* (found in northern Namibia) and *C. [a.] beesleyi,* adding support for the splitting of *C. beesleyi* as a separate species.

We sequenced part of the mitochondrial DNA (mtDNA) cytochrome *b* gene to assess the relationships among taxa within the Spike-heeled Lark within South Africa. Mitochondrial DNA is still useful for assessing phylogeographic patterns (Hung et al. 2016). We assessed geographical variation in morphology in relation to the genetically-defined and traditionally assigned taxa with a focus on South Africa. We also present additional morphometric data for *C. [a.] beesleyi*.

Materials and methods

Sampling for genetic testing

Fresh tissue (liver, heart and pectoral muscle) or blood samples were taken at 37 localities from 39 individuals in the Spike-heeled Lark range between 1997 and 2002. Because Clancey (1980) provided maps for taxon boundaries, this was the most useful treatment for subspecific designation at the time of sampling. Ten of Clancey's (1980) 14 southern African subspecies (but only five of the nine subspecies retained in Roberts VII (Dean 2005); Figure 1A, B) were sampled as well as the Tanzanian population of *C. [a.] beesleyi*. Gray's Lark was identified as a suitable outgroup based on an overall phylogeny of the Alaudidae (Barnes 2007; Alström et al. 2013). Seven Gray's Lark samples from six localities were collected. All sample names, sample sources, including voucher specimen and GenBank details, subspecific designation, and collection localities are listed in Supplementary Table S1. Tissue was stored in a 20% dimethylsulphoxide (DMSO) and saturated salt (NaCl) solution (Amos and Hoelzel 1991), whereas blood samples were stored in 0.1 M Tris-HCL, 0.04 M EDTA·Na_2, 1.0 M NaCl, 0.5% SDS buffer. Samples were refrigerated as soon as possible after collection.

DNA extraction, amplification and sequencing

Samples of 0.01–0.02 g ground tissue or 15–20 µl of blood were digested in 500 µl amniocyte buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 1 M EDTA, pH 8.0, 0.5% SDS) and total genomic DNA extracted using standard techniques of proteinase K digestion (0.5 mg, Roche Diagnostics, Johannesburg) at 55 °C for 12–24 hours. RNA digestion (0.1 mg RNAse A, Roche Diagnostics) followed at 37 °C for 1 hour. DNA was then extracted three times with phenol and once with a 24:1 solution of chloroform:isoamyl alcohol solution and total DNA precipitated overnight at -20 °C with 0.1 volumes 3 M sodium acetate and 2 volumes 96% ethanol. The DNA pellets were collected in a microcentrifuge at 13 000 rpm for 30 min. This was followed by a 70% ethanol wash and collection of the pellet by centrifugation at 13 000 rpm for 30 min. The pellet was resuspended in 50 µl Sabax® water (Adcock Ingram, Johannesburg) preheated to 37 °C and then stored at -20 °C.

A 630 base pair (bp) fragment of the cytochrome *b* gene, corresponding to positions 15029–15659 in the chicken genome (Desjardins and Morais 1990), was amplified in a PCR using primers L14990/L14841 (Kocher et al. 1989) and H15696 (Edwards et al. 1991). This fragment was chosen for assessing the variation within the spike-heeled lark complex over the more traditional COI barcode fragment (Hebert et al. 2004; Ratnasingham and Hebert 2007) as this fragment has proven useful as a first step in exploring variation within the lark family (Alström et al. 2013). Amplifications were performed in 50 µl volumes using 1 x reaction buffer, 2.0 mM $MgCl_2$, 2 mM dNTPs, 50 pmol of each primer and 1.5 units of Super-therm® *Taq* DNA polymerase (Southern Cross Biotechnology, Cape Town). The PCR conditions were: initial denaturation of 2 min at 94 °C, 35 cycles of denaturation (94 °C, 30 s), primer annealing (50-52 °C, 30 s) and polymerase extension (72 °C, 45 s) and final extension of 5 min at 72 °C in a GeneAmp® PCR System 9700 (Applied Biosystems, Johannesburg). Negative controls were included in all PCRs. The quality and quantity of PCR products was checked on 1.0% agarose (Promega, Johannesburg) gels, stained with ethidium bromide, before purification. Products showing specific amplification were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim, Johannesburg) and the concentration quantified using a fluorometer.

Both heavy and light strands were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase (Applied Biosystems). Approximately 30-90 ng of template, 3.2 pmol of the relevant primer and 4 µl of the BigDye ready reaction kit was made up to 10 µl with Sabax® water and cycled in a Geneamp PCR System 9700 (Applied Biosystems). Cycle sequence products were precipitated using a modified NaAc precipitation method (Applied Biosystems). Products were run on ABI Prism 377 or ABI 3100 DNA sequencers (Applied Biosystems).

Phylogenetic and genealogical analyses

Contigs of cytochrome *b* heavy and light strands were aligned in Sequence Navigator v.1.1 (Applied Biosystems). Consensus sequences were then aligned with the full cytochrome *b* sequence of the chicken (*Gallus gallus*; GenBank Ref. L08376) and translated into amino acid sequences in CLC Sequence Viewer 7.8 (www.qiagenbioinformatics.com/products/clc-sequence-viewer/) using the vertebrate mitochondrial translation table to confirm mtDNA authenticity due to the potential of avian nuclear mtDNA segments (numts) (Sorenson and Quinn 1998). Removal of identical sequences reduced the number of ingroup individuals from

34 to 18 and the number of outgroup individuals from seven to four for a total of 22 sequences. Transition and transversion substitutions were plotted in DAMBE7 against Kimura's two parameter distances to determine the substitution saturation (Xia 2013; Xia 2018).

Model selection using the Maximum Likelihood (ML) method (24 models assessed) was performed in MEGA X v10.0.5 (Nei and Kumar 2000; Kumar et al. 2018). Both Bayesian Information Criterion (BIC) and Akaike's Information Criterion corrected for small numbers (AICc) identified the Hasegawa-Kishino-Yano with Gamma (HKY+G) as best fit model. Pairwise distances, phylogenetic trees – estimated by Maximum Parsimony (MP), Maximum Likelihood (ML) and by Bayesian Inference (BI) – a haplotype network and a phylogenetic network were all constructed to identify haplogroups within the spike-heeled lark complex. We have used the term haplogroup rather than clade throughout to avoid confusion between different methods and terminologies.

Pairwise distances were calculated in MEGA X with the Maximum Composite Likelihood (MCL) model (Tamura et al. 2004; Kumar et al. 2018). Rate variation among sites was modelled with both an uncorrected gamma distribution (shape parameter of 1), to allow for comparison with other studies, as well as a corrected gamma distribution (shape parameter of 0.0573 as calculated in the MEGA X HKY+G model).

A MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar 2000) with search level 1, implemented in MEGA X. Initial trees were obtained by random addition of sequences (with 10 replicates). One thousand bootstrap replicates were performed and a 50% majority rule consensus tree inferred (Felsenstein 1985).

A ML tree was obtained based on the HKY model (Hasegawa et al. 1985) using MEGA X. Initial trees for the heuristic searches were obtained with the "Neighbour-Joining" and "BioNJ" algorithms applied to a matrix of pairwise distances estimated using the MCL approach and then selecting the topology with the superior log likelihood values. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The fast "Subtree-Pruning-Regrafting" heuristic method (SPR level 3) was applied for tree inference. One thousand bootstrap replicates were performed and a 50% majority rule consensus tree inferred (Felsenstein 1985).

The BI tree was computed within the BEAST2 v2.5.1 framework (Bouckaert et al. 2014) using a Yule model prior and a relaxed log normal clock (Drummond et al. 2006) with a substitution rate of 2.1% ± 0.68 as calculated in Weir and Schluter (2008) as the average cytochrome *b* substitution rate in a wide range of avian species. Trees were sampled every 1000th generation over 10 million generations. Tracer v1.7.1 (Rambaut et al. 2018) was used to determine that valid estimates of posterior distribution of the parameters were obtained. The first 10% of trees were discarded as burn-in and the maximum credibility tree was calculated in TreeAnnotator v2.5.1 and visualised with FigTree v1.4.3.

The underlying assumptions of MP and model-based methods are often violated when analysing intraspecific datasets (reviewed by Posada and Crandall 2001), thus network approaches were also used to explore phylogeographic structure among populations of the Spike-heeled Lark. A NeighborNet network was generated using SplitsTree v 4.15.1 with and without *C. a. boweni* and *C. [a.] beesleyi* to visualise the conflicting signals or alternative phylogenetic histories which were not represented in the phylogenetic trees. The HKY85 model was applied to distance calculations with equal base frequencies and equal rates of site variation (Bryant and Moulton 2004; Huson and Bryant 2006).

The TCS v1.01 statistical parsimony network software (Clement et al. 2000) was used to construct a haplotype network with a 95% confidence limit for 630 base pairs of 35 samples in the spike-heeled lark complex in South Africa.

Morphometrics

Measurements were taken from 275 museum skins and 45 freshly collected and live birds from 13 of Clancey's (1980) southern African Spike-heeled Lark subspecies as well as *C. [a.] beesleyi* (Figure 1A). No measurements were obtained from *barlowi* (Botswana) or for the Angolan subspecies *obscurata* and *longispina*. All measurements were made by KNB in the field and from skins in the British Museum of Natural History, Kenyan National Museum, Ditsong Museum of Natural History of South Africa (formerly Northern Flagship Institution) and Iziko South African Museum. A wing rule was used to measure flattened wing chord and tail length to the nearest 1 mm. Other measurements were made with digital callipers to the nearest 0.1 mm: head length, culmen lengths (both from the anterior edge of the skull to the bill tip – C1 – and from the anterior edge of the nares to the bill tip – C2), bill depth and tarsus length. Fresh specimens and live birds were weighed to the nearest 0.5 g on a 100-g Pesola spring balance. The sex of specimens was determined by gonad inspection. Fourteen

additional individuals of *C. [a.] beesleyi* were measured by Lanham (1997) and included in some of our analyses as outlined below.

Given possible discrepancies in weights and measurements between museum skins, fresh specimens and live birds (Winker 1998), differences in univariate measures among these groups were tested using ANOVA. Due to strong sexual dimorphism, data for males and females were analysed separately. We tested differences both within traditionally described subspecies and among pooled data for all taxa. Only one measure, culmen length, C1, was significantly longer in museum skins than in live birds (average 18%, range 13–30% among subspecies). For each subspecies, the appropriate correction factor was applied to freshly collected birds. The pooled correction factor was applied to live *C. [a.] beesleyi* because there were too few museum skins to calculate a reliable correction factor.

R v3.4.2 (R Core Team 2018) in RStudio (RStudio Team 2016) was used for all remaining analyses. Data were standardised using the *scale* command in R prior to principle component analysis (PCA). Visual inspection for outliers was performed and analysis with and without outliers was explored (see results for details). Collinearity was assessed using Pearson's correlation coefficients (*corrplot;* Wei and Simko 2017) to determine which, if any, of the measurements could be combined in linear regression modelling.

As all of the measurements were closely correlated (see results) we could not combine them in one model. Further analyses were done on the raw data and not the standardised data as we were not combining different measurements. Boxplots of the seven univariate morphometric measures between traditionally defined subspecies (Clancey 1980) were generated for each measurement (*ggplot2;* Wickham 2016). Linear regression of each morphometric measurement (response variable) with subspecies based on Clancey (1980) and sex as covariates was performed to determine which, if any, of the measurements could be used to distinguish *C. [a.] beesleyi* from other sampled subspecies. The *C. [a.] beesleyi* measurements by Lanham (n = 14) were included as a separate "subspecies" grouping in these analyses where available (wing chord, tail, and tarsus). An interaction between subspecies and sex was also included and retained where significant (see Supplementary Tables S2-S4 for details). Simulation-based model diagnostics created readily interpretable scaled (quantile) residuals for the fitted linear mixed models (*DHARMa*; Hartig 2020). Differences in estimated marginal means (EMMs) of PCA components of the subspecies were compared graphically (*emmeans*; Lenth 2020). Overlapping arrows indicate no "significant"

difference between groups after Tukey adjustment ($\alpha < 0.05$). Modelling was performed with and without five identified outliers using transformations as required to ensure the best fit of the models.

All morphometric measurements were plotted against rough latitude for each subspecies and linear regression performed as above. Measurements from Lanham (1997) were not included in the analysis, but were plotted along with measurements for one individual of unknown sex measured in DRC (87 mm wing chord; 40.5 mm tail; 31mm tarsus; latitude -10.5; Louette and Hasson 2017).

Modelling of morphometric measurements grouped by genetic haplogroups in South Africa (n = 225) with sex as a covariate, was also performed using the same approach as above.

Results

Forty-seven partial cytochrome *b* sequences (630 bp) were uploaded to GenBank, with accession numbers MG706025–71 (details in Supplementary Table S1). While sequences for *C. a. boweni* (AY165165) and *C. [a.] beesleyi* (KF060440) were available (and based on some of the same samples used here), our sequences included an additional nine basepairs at the 3' end.

Sequence variation

The HKY+G model was identified as the best fit of the data with estimated base frequencies (A = 25.0%, C = 35.7%, G = 15.8%, T = 23.4%) and a Gamma distribution (with shape parameter $\alpha = 0.0573$). There was no evidence of numts.

Spike-heeled Larks from South Africa included 16 haplotypes (Supplementary Table S5) differing by 0.002–3.41% with 33 (5.2%) variable sites: 27 (84.4%) at the third position of codons, and only two at first and three at second position sites. There were very few transversions between Spike-heeled Lark samples (Supplementary Figure S1). Both transitions and transversions increased linearly with distance and transitions consistently outnumbered transversions, suggesting a lack of substitution saturation (Supplementary Figure S1).

Pairwise distances between Spike-heeled Larks and Gray's Larks (uncorrected average: 21.2%, range 19.0-22.9%) were consistent with inter-generic comparisons elsewhere in the

Table 1. Percentage genetic distances between members of the spike-heeled lark complex based on 630 bp of cytochrome *b* gene. Analyses were conducted using the Maximum Composite Likelihood model corrected with a gamma distribution value above the diagonal (shape parameter = 0.057 as calculated in MEGA X) (Tamura et al. 2004). Uncorrected values are displayed below the diagonal. Proposed haplogroups are indicated with boxes.

		C. albofasciata A	*C. albofasciata B*	*C. albofasciata C*	*C. albofasciata D*	*C. albofasciata E*	*C. albofasciata F*	*C. albofasciata G*	*C. albofasciata H*	*C. albofasciata I*	*C. albofasciata J*	*C. albofasciata K*	*C. albofasciata L*	*C. albofasciata M*	*C. albofasciata N*	*C. albofasciata O*	*C. albofasciata P*	*C. a. boweni*	*C. [a.] beesleyi*
C. albofasciata A	**Karoo**	-	0.16	0.34	0.72	0.52	0.72	0.93	0.94	1.87	2.15	2.42	2.45	2.43	4.15	4.54	3.75	5.94	19.9
C. albofasciata B		0.16	-	0.52	0.92	0.72	0.92	1.15	1.15	2.13	2.43	2.71	2.75	2.73	4.54	4.95	4.12	6.46	21.2
C. albofasciata C		0.32	0.48	-	0.71	0.16	0.34	0.92	0.93	1.85	2.13	2.40	2.43	2.42	4.12	4.51	3.72	5.90	19.8
C. albofasciata D		0.64	0.81	0.64	-	0.92	1.14	1.37	1.38	2.42	2.73	1.87	3.07	3.05	4.95	5.38	3.75	5.94	17.4
C. albofasciata E		0.48	0.64	0.16	0.81	-	0.52	1.15	1.15	2.13	2.43	2.71	2.75	2.73	4.54	4.95	4.12	6.46	19.8
C. albofasciata F		0.64	0.81	0.32	0.97	0.48	-	1.37	1.38	2.42	2.73	3.02	3.07	3.05	4.95	5.38	4.51	7.01	19.9
C. albofasciata G		0.81	0.97	0.81	1.14	0.97	1.14	-	0.34	2.73	3.07	3.38	3.42	3.40	5.41	5.86	4.95	5.41	22.6
C. albofasciata H		0.81	0.97	0.81	1.14	0.97	1.14	0.32	-	2.13	2.43	2.71	2.75	2.73	4.54	4.95	4.12	4.47	19.8
C. albofasciata I		1.48	1.65	1.48	1.82	1.65	1.82	1.99	1.65	-	0.16	0.33	0.34	0.34	1.87	2.13	2.12	3.69	23.9
C. albofasciata J		1.64	1.82	1.65	1.99	1.82	1.99	2.16	1.82	0.16	-	0.52	0.53	0.52	2.15	2.43	2.42	4.12	25.4
C. albofasciata K		1.82	1.99	1.82	1.48	1.99	2.17	2.34	1.99	0.32	0.48	-	0.72	0.71	2.42	2.71	2.12	3.69	21.1
C. albofasciata L		1.81	1.99	1.82	2.16	1.99	2.16	2.33	1.99	0.32	0.48	0.64	-	0.34	1.87	2.13	2.12	4.57	23.9
C. albofasciata M		1.82	1.99	1.82	2.16	1.99	2.16	2.34	1.99	0.32	0.48	0.64	0.32	-	1.36	1.60	2.10	3.72	21.0
C. albofasciata N		2.69	2.87	2.69	3.05	2.87	3.05	3.23	2.87	1.48	1.64	1.82	1.48	1.14	-	0.16	1.35	6.02	25.4
C. albofasciata O		2.87	3.05	2.87	3.23	3.05	3.23	3.41	3.05	1.65	1.82	1.99	1.65	1.31	0.16	-	1.13	5.52	26.9
C. albofasciata P		2.51	2.69	2.52	2.51	2.69	2.87	3.05	2.69	1.65	1.82	1.65	1.65	1.65	1.14	0.97	-	6.50	26.8
C. a. boweni		3.27	3.45	3.27	3.27	3.45	3.64	3.09	2.72	2.36	2.54	2.36	2.71	2.36	3.26	3.07	3.45	-	32.6
C. [a.] beesleyi		5.86	6.07	5.87	5.45	5.87	5.86	6.29	5.87	6.51	6.72	6.08	6.51	6.09	6.72	6.94	6.95	7.00	-

Eastern

Namaq.

Alaudidae. Within the spike-heeled lark complex, three South African haplogroups were identified: Karoo, Eastern and Namaqualand. Within haplogroup pairwise distances were as follows: (1) Karoo: corrected average 0.84%, range 0.16-1.3%, (2) Eastern: corrected average 0.45%, range 0.16-0.72% and (3) Namaqualand: corrected average 0.88%, range 0.16-1.4%. Between the haplogroups, pairwise distances were smallest between the geographically isolated Eastern and Namaqualand: corrected average 2.1%, range 1.4-2.7%. Both were more distantly related to the Karoo: Karoo – Eastern corrected average 2.6%, range 1.9-3.4%; Karoo – Namaqualand corrected average 4.6%, range 3.7-5.9% (Table 1). The sample of *C. a. boweni* from Namibia differed from South African birds by 3.7-7.0% (corrected average 5.3%) and was closest to the Eastern haplogroup.

Phylogenetic analyses

Relationships within the Spike-heeled Larks differed among phylogenetic analyses, mainly due to branch-swapping among the many closely related South African Spike-heeled Lark haplotypes reducing support (Figure 1C, Supplementary Figure S2). However, in all analyses haplotypes A–H split from haplotypes I-P, supporting the distinction and monophyly of a Karoo haplogroup. The analyses also showed consistent support for the Namaqualand haplogroup (haplotypes N–P clustered together with statistical support), while the support for an Eastern haplogroup (haplotypes I – M) was lacking. *C. a. boweni's* position was unclear (Figure 1C, Supplementary Figure S2); its placement relative to the South African haplotypes was not statistically supported in any of the analyses*. C. [a.] beesleyi* was consistently sister to all other haplotypes, although not always with strong support.

Network analyses

Despite reticulation, the NeighborNet network confirmed the distinction of *C. [a.] beesleyi* (long branch and 100% support) and *C. a. boweni* (long branch and 100% support) from the South African haplogroups (Figure 1D). Analysis of the South African haplotypes in isolation supported a split between the Karoo and Eastern groups (99.4%) and a split between Eastern and Namaqualand groups (97.9%; Figure 1D inset).

Geographical structuring was strong, despite the relatively small sample size (Figure 1E, 2F). Haplotypes differed by 1–33 steps and supported the three proposed haplogroups among South African birds (Figure 1E). The Karoo haplogroup comprised eight haplotypes: A, C and D were widely distributed, with 5–7 individuals each, whereas haplotypes G and H were confined to the Tankwa Karoo, differing from A–D by at least five mutational steps (Figure 1E,

2F). The Karoo haplogroup differed from the Eastern haplogroup by a minimum of eight mutational steps, and from the Namaqualand haplogroup by 19 steps. Surprisingly, the Eastern and Namaqualand haplogroups were more closely related to each other (differing by only seven mutational steps) than the geographically intermediate Karoo haplogroup (Figure 1E, 1F). There was also significant variation within the Namaqualand haplogroup, with haplotype O differing from P by six mutational steps. There was little correspondence between existing subspecies (according to Clancey 1980 or Dean 2005) and the genetic groups identified here (Figure 1F). With a relaxed connection limit of 90% *C. a. boweni* was 14 steps from haplotypes K, I and M in the Eastern haplogroup.

Morphological comparisons

Of the 320 birds examined, 180 were male, 137 were female and three were unconfirmed and removed from further analysis. Five of the females (spread across five different subspecies) clustered as males in PCA analysis (Supplementary Figure S3). These birds may have been mis-sexed (sexing was based on museum labels). As we had no way of verifying the sexes, they were discarded from further analyses after confirming that no major trends were altered in the modelling (data not shown).

Males averaged larger than females in all measurements. Dimorphism was greatest in culmen measurements (15.1%–43.6%) and least in tarsus length (5.1%–12.5%). *C. [a.] beesleyi* was the smallest taxon in almost all linear measures (Figure 2, Supplementary Figure S4). PCA analysis separated them from the other subspecies with the first two components explaining 83.5% of the variation for both sexes (Figure 2A, 2B).

Individual morphometric measures were highly correlated with one another (Figure 2C). Sexual dimorphism was significant for all measurements (Supplementary Tables S2-S4), however interactions between subspecies and sex were almost entirely absent (*C. a. baddleyi* was the only subspecies with a consistent interaction) and thus interactions were not included in the models. Tail length separated *C. [a.] beesleyi* from all other subspecies based on EMMs with Tukey-adjusted *P*-values, (Figure 2D, 2G). Culmen 2 length separated *C. [a.] beesleyi* from most other subspecies (Figure 2E, 2H). *C. [a.] beesleyi* wing chord lengths were smaller than all subspecies other than the northern Namibian *C. a. boweni* and *C. a. erikssoni* (Figure 2F, 2I). There was no separation of *C. [a.] beesleyi* from other subspecies based on other morphometric measurements (Supplementary Table S2, Figure S4).

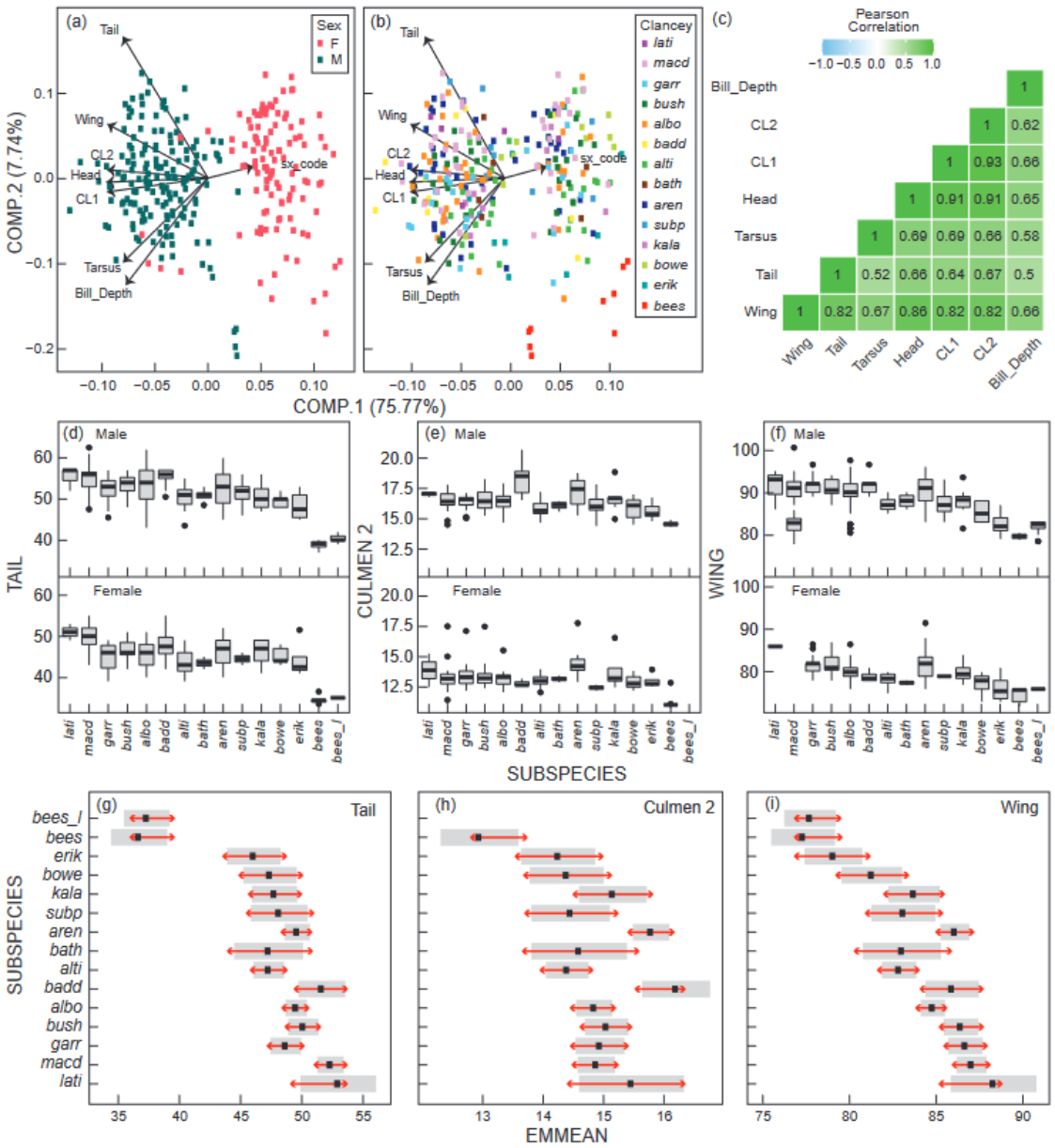

Figure 2. Morphological variation in the spike-heeled lark complex divided by subspecies according to Clancey (1980). (A) Principle component analysis (PCA) by sex. (B) PCA by subspecies. (C) Correlations among all measurements. Box plots of measurements of (D) Tail, (E) Culmen 2 and (F) Wing. Estimated Marginal Means (EMMs) based on linear regression analyses for (G) Tail, (H) Culmen 2 and (I) Wing. Non-overlapping arrows in the EMM plots indicates significant differences between groups after Tukey adjustment ($\alpha < 0.05$).

The overall trend was for a significant decrease in size towards the equator (Supplementary Figure S5, Supplementary Table S3). Head measurements for males followed this trend, however for females there was no decrease in size with latitude (Supplementary Table S2; Supplementary Figure S5C); wing measurements also had an interaction between sex and latitude (Supplementary Table S2). Tail lengths of *C [a.] beesleyi* specimens were all shorter than expected based on modelling (Supplementary Figure S5A). Lanham's (1997) measurements were more in line with the models for tail (Supplementary Figure S5A), wing chord (Supplementary Figure S5B) and tarsus (Supplementary Figure S5D). The unknown sex bird measured in DRC had an intermediate tail length (Supplementary Figure S5A), a long wing chord (Supplementary Figure S5B) and a long tarsus (Supplementary Figure S5D).

While the PCA analyses of the morphometric data failed to separate the three genetically-identified haplogroups in South Africa (Supplementary Figure S6A), there were significant differences in several measurements (Supplementary Table S4, Figure S6). The Eastern haplogroup birds were the consistently smaller than Karoo birds across all measures (Supplementary Figure S6). Due to small sample size in the Namaqualand haplogroup, their relative size was not as well defined, however they appear to be intermediate between the Eastern and Karoo haplogroups with some measurement overlapping with the Eastern haplogroup and some with the Karoo haplogroup (Supplementary Figure S6).

Discussion

Three genetic divisions were apparent among the South African haplotypes of Spike-heeled Lark examined here: Karoo, Namaqualand and Eastern. This was far fewer than expected based on traditional subspecies designations (Clancey 1980; Keith et al. 1992; Dean 2005). While Dean (2005) had reduced the number of subspecies found in South Africa compared to earlier published works (Clancey 1980), his subspecies designations were not supported by the genetic data presented here. Specifically, the eastward expansion of the Namaqualand subspecies *C. a. garrula* (Dean 2005) was not supported, with the Namaqua haplogroup being largely confined to succulent Karoo west of the Great Escarpment range, as initially described by Winterbottom (1958) and maintained by Clancey (1980). There was similar variation within this Namaqualand haplogroup as there was between it and the Eastern haplogroup and between the Eastern and Karoo haplogroups, thus a further split may be warranted in a north-south direction. However, without further sampling within this region, we are not proposing this at this time. East of this range the situation was much less complex than suggested by the number of subspecies currently recognised, with only a Karoo and an Eastern haplogroup

apparent. These two haplogroups appeared to be split roughly along the west-to-east transition from the Nama-Karoo to the Grassland biome around 25 °E, extending north through the Savanna biome. This also roughly follows the west-to-east transition from “Hot and Cold Desert” to “Hot and Cold Semi-arid” Köppen climate types (Rubel and Kottek 2010). A similar geographic split exists between *Certhilauda curvirostris, C. semitorquata* and *C. subcoronata* in the long-billed lark complex (Ryan and Bloomer 1999).

The genetic distances between Spike-heeled Lark haplogroups also followed a similar pattern to that observed in the long-billed lark complex. The Grassland (*Certhilauda semitorquata*) and Namaqualand (*C. curvirostris*) forms of Long-billed Lark are more closely related to each other than to the intervening central Karoo form (*C. subcoronata*; Ryan and Bloomer 1999; Alström et al. 2013). This was mirrored in the spike-heeled lark complex where the Namaqualand haplogroup was more closely related to the geographically separated Eastern haplogroup compared to the intervening Karoo haplogroup. This pattern was particularly surprising in the Spike-heeled Lark given that both Namaqualand and Eastern populations occurred in close proximity to the adjacent Karoo population (within 15–25 km on either side), without any obvious barriers to gene flow. This was further substantiated by the network analyses with distinct clustering of the haplotypes into the three proposed haplogroups.

Morphometrics of the Spike-heeled Larks also mimicked that found in the long-billed lark complex. The more arid and geographically adjacent Karoo and Namaqualand haplogroups (*C. curvirostris* and *C. subcoronata* equivalents in the long-billed lark complex) were of similar size to each other, and larger on average than the Eastern haplogroup (*C. semitorquata* equivalent in the long-billed lark complex; Ryan and Bloomer 1999). This, combined with the genetic patterns, suggests similar processes shaping the evolutionary history of Spike-heeled Larks and the long-billed lark complex.

Additional sampling of birds in eastern South Africa, Botswana and southern Namibia is needed to further explore the evolutionary history of this complex within the region. The addition of nuclear genes and/or genome-wide nuclear DNA data such as SNPs would also be beneficial as, while there is some evidence that phylogeography can be successfully determined based solely on mtDNA (Hung et al. 2016), there have also been criticisms (McKay and Zink 2010; Toews et al. 2016). The SNP approach has been successful in resolving other cryptic lark families such as the *Calandrella* larks (Stervander et al. 2016).

Even without the addition of more geographic coverage and nuclear data, it is fairly clear that the current morphologically-based subspecies designations as defined in both Clancey (1980), and more recently, Roberts VII (Dean 2005), do not accurately represent the genetic differentiation within the Spike-heeled Lark. This was not surprising as Phillimore and Owens (2006) found that only 36 percent of global avian subspecies were phylogenetically distinct. Donald et al. (2017) explored the possible mechanisms of substrate-colour matching in larks and the implications this has for taxonomy. They speculated that the phenomenon of lark plumage matching substrate colour may be as simple as dust sticking to feathers or that birds may be able to alter the colour of their feathers during moulting to match their surrounding habitat (Donald et al. 2017). The seemingly temporary nature of plumage colour questions its use as a taxonomic tool (Donald et al. 2017) as exemplified here.

While we were not able to add much to previous assessments of *C. [a.] beesleyi* (Alström et al. 2013), we have expanded the genetic evidence to include more haplotypes from southern Africa (*C. [a.] beesleyi* was sister to all haplotypes included here) as well as morphometric data. Morphometrics supported that *C. [a.] beesleyi* was significantly smaller compared to its southern counterparts across several measurements. While smaller birds were expected towards the equator (James's Rule adapted from Bergman's Rule in Blackburn et al. 1999), there was some evidence that tail measurements of *C. [a.] beesleyi* were consistently smaller than predicted by latitude in both sexes. We did not have any measurements from *C. a. obscurata*. Donald and Collar (2011) found that tails of these birds overlapped in size with those of *C. [a.] beesleyi*, and the one measurement of a DRC individual (Louette and Hasson 2017) was also similar. Therefore *C. [a.] beesleyi* may not be significantly smaller than other subspecies found at similar latitudes. Hopefully these data will be useful for future studies of this complex.

The population of *C. [a.] beesleyi* appears to be declining with <1000 individuals estimated in the 1990s (Lanham 1997; N. Baker pers. comm.) and likely only a few hundred today (pers. obs. KNB). Combined with its highly restricted range (<10 km^2), genetic distinctness and smaller size, this population could be treated as an evolutionarily significant unit (Moritz 1994) for conservation purposes regardless of taxonomic status. There is an urgent need to assess the population status, ecology and behaviour of *C. [a.] beesleyi* to better understand its habitat requirements and to identify potential threats to its persistence.

In conclusion, the discrepancies between genotype and phenotype within the spike-heeled lark complex have been highlighted by this study. More sampling, both morphometric and genetic, of data depauperate populations is required. Future genetic testing should include SNP analysis or similar genome-wide approaches to augment the analysis presented here.

Acknowledgements

KNB thanks Neil and Liz Baker who introduced him to Beesley's Lark and provided hospitality and assistance in the field in Tanzania. Financial support was received from the South African National Research Foundation and the Universities of Cape Town and Pretoria. SMM was funded by a Clancey Trust Postdoctoral Fellowship. Thanks to Jessica van der Wal and Anina Coetzee for useful discussions on linear regression analyses. Thanks to several anonymous reviewers for helpful comments on earlier drafts of this manuscript.

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