CHAPTER 2

The taxonomic status of the eastern population of the Short-clawed Lark *Certhilauda chuana*

INTRODUCTION

Representatives of the genus *Certhilauda* include the Short-clawed Lark *C*. *chuana* and five species of Long-billed Larks namely, *Certhilauda curvirostris*, *C. brevirostris*, *C. semitorquata*, *C. subcoronata* and *C. benguelensis* (Ryan & Bloomer 1999). The genus constitutes the Long-billed Lark complex, a group of species characterised by their long bills, distinctive territorial call, spectacular aerial display flights and marked sexual size dimorphism. The Short-clawed Lark shares these characteristics with the five recognized Long-billed Lark species and is hence considered a sister species within the Long-billed Lark complex (Herremans *et al.* 1994; Ryan & Bloomer 1999).

The Short-clawed Lark is a highly localized species comprised of two geographically isolated populations in southern Africa (Barnes 2000). The species' western population occurs in south-eastern Botswana with scattered records from the North West, Northern Cape and the north-western Free State Provinces of South Africa (Herremans 1997). A smaller eastern population is largely restricted to the Polokwane Plateau of the Limpopo Province (Hockey *et al.* 2005). Due to its unobtrusive nature and highly localised distribution within its restricted range (Hunter 1991), the possible existence of the Short-clawed Lark's eastern population was first reported by Clancey (1966) and only later confirmed by Tarboton *et al.* (1987).

According to the Southern African Bird Atlas Project (SABAP) (Harrison *et al.* 1997), the western population occupies a maximum area of approximately 54000km^2 (Barnes 2000) and was recorded in 76 quarter degree grid squares (with 29 partly or completely falling within South Africa). The SABAP determined that the eastern population's area of occurrence covers approximately 10650km^2 and the species was recorded in 15 quarter degree grid squares within the Limpopo Province (Harrison *et al.* 1997). Since the distance between the closest occupied grid squares of the two populations is approximately 185 kilometres several authors have questioned the taxonomic affinities of the individuals belonging to the eastern and western populations (Herremans 1997; Barnes 2000; Birdlife International 2004). Engelbrecht (2005) also suggested that the taxonomic status of the eastern population be confirmed after a study of the eastern population revealed several differences in the breeding biology of the two populations, e.g. clutch size and number of breeding attempts. Furthermore, Herremans (1997) reported dialectal differences in the territorial calls of males from the western population over small geographical barriers and suggested that the species may have poor dispersal abilities. This is also reflected in the species' absence from areas of apparently suitable habitat within its area of occurrence (Hunter 1991). It is generally accepted that geographical isolation, coupled with poor dispersal abilities, is necessary to set the scene for independent evolutionary pathways, leading to speciation (McCullough 1996; Frankham *et al.* 2002). Should the eastern and western populations of the Short-clawed Lark prove to be distinct taxonomic entities, the eastern population is inadequately conserved and its conservation status will have to be re-assessed as a matter of urgency (Barnes & Tarboton 1998; Engelbrecht 2005).

Recent studies attempting to resolve taxonomic uncertainties have successfully incorporated data from a range of fields including genetics, morphology, plumage variation, vocalisations, biology and ecology. Ryan *et al.* (1998) used a number of the above criteria to recognize

Barlow's Lark *Calendulauda barlowi* as a distinct species in the Karoo Lark *C. albescens* complex of south-western Africa while Ryan & Bloomer (1999) described five sympatrically occurring species within the Long-billed Lark *Certhilauda* complex, previously regarded as subspecies. More recently, Barnes (2007) used phylogenetic analysis and morphometric data to recognize Beesley's Lark (*Chersomanes beesleyi*) as a distinct taxonomic entity from the Spike-heeled Lark *C. albofasciata*. From the foregoing it is evident that a combination of genetic, morphometric and call data has the potential to resolve taxonomic uncertainties in the *Alaudidae.* This was also the approach followed in the present study to confirm the taxonomic affinities of the two populations of the Short-clawed Lark.

MATERIALS AND METHODS

Morphometric Study

Sampling

Birds were captured over a five year period from 2002 to 2007 from various random locations within the geographical ranges of the two populations. A combination of mist nets and walk-in traps, baited with meal worms, was used to capture adult birds. A total of 47 birds were captured (13♂♂ and 3♀♀ from the western population and 24♂♂ and 7♀♀ from the eastern population. The Short-clawed Lark exhibits marked sexual size dimorphism and individuals can reliably be sexed on this basis (Engelbrecht 2005). Once all measurements were recorded and samples collected for genetic analysis, the birds were released where they were captured. In an effort to augment the sample sizes of the two populations, study skins were sourced from the collections of several South African museums. A total of six specimens were obtained from the National Flagship Institute Pretoria, Durban and McGregor museums (Table 2.1) and were included in the database for morphometric analysis.

Morphometrics

Only male birds were included in the morphometric comparative study by reason of a small sample size of female birds and the extent of sexual size dimorphism within the species (Table 2.1). A total of 37 captured male Short-clawed larks (*n* = 13 western population and *n* = 24 eastern population) were weighed to the nearest 1g using a Pesola 100g spring scale. Vernier calipers were used to measure head length, culmen length and tarsus length to the nearest 0.1mm, while wing and tail lengths were measured to the nearest 1mm as described by De Beer *et al.* (2001). As a result of damage to some of the study skins e.g. bills, some measurements were not recorded for certain individual skins. The Mann-Whitney U test was used to test for significant differences of the measured parameters between the two populations using the SPSS 13.0 software package (SPSS Inc. 1997). This non-parametric analogue test was selected as the dataset contained variables with only two values (Siegel & Castellan 1988).

Territorial song characterisation

Confirmation of the taxonomic status of the eastern and western populations of the species was supplemented by comparing territorial vocalisations of perched male Short-clawed Larks representing the two populations. A territorial call consists of a high-pitched, slow, drawn-out whistle comprised of 1 - 4 notes as described in Chapter 4. Calls were recorded in the field during the early breeding seasons of 2005 and 2006 using a portable digital voice recorder (Marantz Professional PMD670) and a uni-directional microphone (Sennheiser ME66). All recordings were made on windless days, from sunrise to mid-morning when territorial advertisement was at its peak. The recording distance never exceeded 35 metres. Analysis of the calls was performed using the Raven 1.2.1 sound analysis software package. Variation in territorial calls within and between the two populations was measured

by comparing the following variables for each note as well as for the total phrase in the case of double and triple-note calls:

1. The number of notes in a call,
2. DT - duration or delta time (ms),
3. LF - lowest frequency (kHz),
4. HF - highest frequency (kHz), and
5. DF - delta frequency, i.e. the frequency range (kHz) of a call.

Statistical analysis of vocalisations

The Kruskal–Wallis test is used when there is one attribute variable and one measurement variable, and the measurement variable does not meet the normality assumption of an anova. (Kruskal & Wallis 1952). This test was therefore adopted for indicating whether significant differences occur in various call variables recorded between individuals within the two populations. The Chi-square test was used to determine whether there is a significant association between the number of single- and triple-note phrases and the relevant population from which they were recorded. Mann-Whitney U non-parametric tests were employed to test for significant differences of double-note call variables measured between the two populations.

Genetic Study

Sampling of genetic material

Six birds from each population were collected from random sites using the same techniques employed with the collection of birds for the morphometric study (Table 2.2). Two Rufous-naped Larks, *Mirafra africana* and three Spike-heeled Larks, *Chersomanes albofasciata*

were also captured to serve as outgroups for purposes of phylogenetic analysis. Blood was extracted by veni-puncture of the great wing vein, collected in capillary tubes and preserved in 95% ethanol. An inner secondary or a tail feather and five contour feathers were also plucked from each bird as backup to the blood samples. The feathers were stored in individual envelopes in dark, dry conditions at room temperature.

DNA extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from blood samples, using the DNeasy protocol for animal tissues and blood (Qiagen Inc.) according to the manufacturer's protocol. Polymerase Chain Reaction (PCR) (Saiki *et al.* 1988) of two mitochondrial protein coding genes was conducted. The two genes selected were; a partial Cytochrome *b* gene (Cyt *b*) and a near complete (972 bp out of 1119 bp) NADH dehydrogenase subunit 2 gene (ND2). PCR primers used for the Cyt *b* gene were L14990 and H15499 (Ryan & Bloomer 1999) and L5204 and H6312 for the ND2 gene (Cicero & Johnson 2001) (Table 2.3). The Cyt *b* fragment (530bp) amplified is situated at the 5' end of the gene. Both genes have been used successfully in the genetic analysis of *Alaudidae* in the past. Ryan *et al.* (1998) and Ryan & Bloomer (1999) used the Cytochrome *b* gene to resolve the evolutionary relationships among taxa within the Karoo and Long-billed Lark complexes while Guillaumet *et al.* (2005) used both Cyt *b* and ND2 genes to distinguish phylogenetic variation between two closely related *Galerida* larks of Morocco.

Cyt *b*

For amplification of Cyt *b* (524 bp) two microlitres of eluted DNA solution was added to a 48µl PCR cocktail containing 5µl 10x *Taq* buffer, 5µl 2.5mM magnesium chloride (Mg+ is the cofactor required by the enzyme to function), 10µl 2mM dNTPs, 0.25µl each of two 100µM primers (L14990 and H15499) and 0.3µl of 1.5 units of *Taq* polymerase Takara

Biochemicals (Takara Shuzo Co. Ltd.) and ddH_2O. Cycling parameters on a Hybaid Omn-e Thermal Cycler consisted of the following: an initial denaturation at 94ºC (3 min.) followed by 35 cycles of 94ºC (30 sec.), 48ºC annealing temperature (1 min.), 72ºC (1 min.). A final extension at 72ºC (10 min.) to complete elongation was performed before finally decreasing the temperature to 4ºC. Five microlitres of PCR products were electrophoresed at 150V for 10 minutes on a 1.5% agarose gel (0.7g agarose, 45ml 1x TAE buffer and 1 - 2µl Ethidium bromide) and visualized under ultraviolet light.

ND2

For amplification of the ND2 gene (969 bp), two microlitres of diluted DNA solution were added to a 48µl PCR cocktail containing the same concentrations as used in the Cyt *b* PCR mixture, except for 5µl 1mM dNTPs and 0.25µl of 1.25 units of *Taq* polymerase and primers L5204 and H6312. Cycling parameters started off with the same initial denaturation phase, at 94ºC (3 min.) followed by 50 cycles of 94ºC (30 sec.), 56ºC annealing temperature (1 min.), 72ºC (1 min.) and a final extension at 72ºC (10 min.). Once again five microlitres of PCR products were electrophoresed at 150V for 10 minutes on a 1.5% agarose gel (0.7g agarose, 45ml 1x TAE buffer and 1 - 2µl Ethidium bromide) and visualized under ultraviolet light. Due to the presence of many non-specific PCR products a touch down procedure was employed (Palumbi 1996). Cycling parameters for this procedure consisted of two cycles at 94ºC, 62ºC and 72ºC for 30 seconds each, followed by three more similar cycles, differing only in the annealing temperatures of 60ºC, 58ºC and 56ºC respectively. Finally 35 cycles were run at 94ºC, 62ºC annealing and 72ºC elongation for 30 seconds each. Since some non-specific PCR products were still present, the entire PCR product was added to a 0.9% agarose gel (0.5g agarose, 55ml 1x TAE buffer and 1 - 2µl ethidium bromide), electrophoresed at less than 150A with a size standard where after the required bands were excised from the gel.

Required PCR products were sent to the Pretoria laboratories of Inqaba Biotechnical Industries (Pty) Ltd. where they were purified and sequenced according to standard methods.

Sequencing

DNA sequences were generated in both directions according to standard protocols of Inqaba Biotechnical Industries (Pty) Ltd. In sequencing Cyt *b,* use was made of an additional internal primer L15245 nested within the amplified product which was designed by Inqaba Biotechnical Industries (Pty) Ltd. Primers L5216 and H6313 (Sorenson *et al*. 1999) were used in sequencing the ND2 gene (Table 2.3).

Phylogenetic Analysis

Sequences were verified and aligned using the Sequencher^{TM} software package (version 3.1.1, Gene Codes) and then imported into Clustal X (Thompson *et al*. 1997) for alignment. Adjustments were made by eye in Se-Al (version 1.0a1). A single Cyt *b* sequence from a Short-clawed Lark obtained from GENBANK (Accession number: AF033257) was included in the dataset. Sequences of two Rufous-naped Larks, *Mirafra africana* and three Spike-heeled Larks, *Chersomanes albofasciata* generated during the present study, served as outgroups (Table 2.2).

Phylogenetic analyses (using the parsimony algorithm) of both Cyt *b* and ND2 were carried out using PAUP* v4.0b10 (Swofford 1997). Parsimony trees were constructed for both genes. These were done, using an equally weighted analysis of each dataset, individually. All searches were heuristic, using the tree bisection and reconnection (TBR) branch swapping algorithm, with 1000 random taxon additions. Where multiple trees were obtained,

majority rule (50%) and strict consensus trees were constructed. Bootstrap analyses of 1000 replications with 10 random addition searches were performed to determine the nodal support. Additionally, a neighbor joining analysis was performed for the amplified ND2 genes in order to determine the sequence divergences among individuals, using uncorrected p-distances. The ND2 sequences of the ingroup were imported into MacClade 4.0 (Maddison & Maddison 2001) and translated to amino acids to verify the codon positions and resultant substitutions (synonymous or non-synonymous) of mutations.

Population genetic analysis to determine the haplotype diversity (i.e. how much variation occurred among the individual sequences) and the nucleotide diversity (i.e. extent of variety within all the sequences), thus, the diversity and differentiation among populations using ND2, was performed using DnaSP (Rozas & Rozas 1999). Additionally, DnaSP was used to test whether this part of the mitochondrial region evolved neutrally and will therefore be suitable for making population genetic inferences.

Lastly, phylogeographic analysis was carried out by means of a nested clade analysis using TCS (Clement *et al.* 2000) and GeoDis (Posada *et al.* 2000). A 95% parsimony haplotype network was estimated for ND2 in TCS according to the predictions derived from the coalescent theory, i.e.:

i. transitions are more likely to occur repeatedly than transversions,

ii. common haplotypes are more likely to be found at interior nodes of a cladogram with the rare haplotypes at the tips,

iii. haplotypes represented by a single individual (singletons) are more likely to connect to haplotypes from the same population than to haplotypes from different populations (Crandall & Templeton 1993). The estimated parsimony network was nested according to Templeton *et al*. (1987) in which haplotypes are treated as '0-step clades', groups of haplotypes

separated by a single mutation as '1-step clades' and groups of '1-step clades' separated by a single mutation as '2-step clades', etc. Geographic coordinates for the western population (n = 6) and the eastern population (n = 6) were obtained in the field by GPS. Nested clade analysis was performed using GeoDis which determines the clade distance (Dc) of a given group of haplotypes (which is a measure of the geographical range of the haplotypes within the clade) and the nested clade distance (Dn) of a given group of haplotypes (which measures how a haplotype group is distributed relative to all the haplotypes present in the nested clade).

RESULTS

Morphometric results

The results of the morphometric comparison of males from the eastern and the western population is presented in Table 2.4. With the exception of a significant difference ($P < 0.05$) in the mean tarsus length between the eastern and western population, there were no other significant differences between populations for the morphometric parameters analysed (Table 2.4).

Territorial call analysis

Territorial calls were obtained from 13 males each in the eastern and western population and comprised 309 and 180 notes respectively. There were considerable structural differences within and between individuals with regard to the number and modulation of notes in territorial calls. A detailed analysis and description of the structure of territorial calls and individual variation between males from the eastern population is presented in Chapter

4. The data obtained for the analysis of territorial calls in Chapter 4 was used here to compare the territorial calls of males from the eastern and western population. This was done in an attempt to determine whether there is any variation in the selected territorial call parameters between the two populations.

The number of notes in each phrase ranged from 1 to 4 ($\bar{x}$ = 1.80, ± 0.69). The 4-note territorial call was only recorded twice for one individual from the eastern population and was therefore omitted from further statistical analyses. The number of single-, double- and triple-note phrases recorded for males from the eastern and western population and the relative contribution of each phrase to the total number of territorial phrases recorded, is presented in Table 2.5. Triple-note territorial calls are relatively uncommon in the western population and were recorded three times for one individual and once each for two other individuals from the western population. In this regard, the statistical analyses revealed a significant association between the number of single- and triple-note phrases and the population from which they were recorded (Chi-square = 14.14, d.f. = 2, $P < 0.05$). This may be ascribed to the relatively few triple-note recordings from the western population (Table 2.5). Due to the small sample size of triple-note calls for the western population, it was decided not to include triple-note data in the comparison of calls between the two populations. However, the results of the triple-note call variables analysed for the eastern population are presented in Chapter 4.

The descriptive statistics for single- and double-note variables analysed for individuals within the eastern and western population are presented in Table 2.6. Within the western population, significant differences were obtained between individuals for DF (Kruskal-Wallis test = 10.3, d.f. = 3, $P < 0.05$) of single-notes whereas the DT (Kruskal-Wallis test = 13.45, d.f. = 4, $P < 0.01$) of single-note calls was the only parameter that showed significant

differences between individuals within the eastern population. The Kruskal-Wallis test revealed statistically significant differences between the eastern and western population for DT (Kruskal-Wallis test = 19.70, d.f. = 1, $P < 0.0001$) , LF (Kruskal-Wallis test = 12.28, d.f. = 1, $P < 0.0001$) and HF (Kruskal-Wallis test = 17.71, d.f. = 1, $P < 0.0001$) of single-note territorial calls but not for the DF (Kruskal-Wallis test = 2.99, d.f. = 1, $P < 0.05$) of single notes (Table 2.6a).

The Mann-Whitney test showed significant differences between the eastern and western population for the following double-note territorial call variables analysed: LF2 (Mann-Whitney U test = 704.50, $P < 0.0001$), DF2 (Mann-Whitney U test = 1207.50, $P < 0.0001$), DT_{Tot} (Mann-Whitney U test = 667.50, $P < 0.0001$) and LF_{Tot} (Mann-Whitney U test = 1604.00, $P < 0.05$) but not for any of the other variables analysed (Table 2.6b). Within the western population, significant differences between individuals were observed for DT2 (Kruskal-Wallis test = 17.50, d.f. = 7, $P < 0.05$), DF2 (Kruskal-Wallis test = 14.64, d.f. = 7, $P < 0.05$) and DT_{Tot} (Kruskal-Wallis test = 16.88, d.f. = 7, $P < 0.05$). The Kruskal-Wallis tests showed significant differences ($P < 0.05$) between all individuals in the eastern population for the variables analysed.

Molecular genetic analyses

Sequence statistics

Cytochrome *b*

A total of 530 base pairs of the Cytochrome *b* gene were successfully amplified for all 12 individuals representing both populations of Short-clawed Larks as well as five individuals representing the outgroup species (Appendix 2.1). All the amplified sequences for the ingroup were identical. The sequences were also identical to the sequence obtained from

GENBANK and therefore this sequence was not included in further analyses. The complete dataset, including the outgroups, consisted of 430 constant characters, two variable characters that were parsimony uninformative and 98 parsimony informative characters.

ND2

A total of 972 base pairs of the ND2 gene were successfully amplified for all 12 individuals representing both populations of Short-clawed Larks as well as the outgroup individuals (Appendix 2.2). The dataset consists of 698 constant characters, 17 variable characters that were parsimony uninformative and 257 parsimony informative characters. Four haplotypes were found among the ND2 sequences of the Short-clawed Larks. Five of the individuals from the eastern population (1E, 2E, 3E, 5E and 6E) shared the same haplotype, with 4E having a mutation (transition) from CTG to CTA in position 183. This is a synonymous (Leucine) third codon position change. The individuals from the western population differed from those in the eastern population by the occurrence of a transversion (ACA to ACT) in position 24. This is also a synonymous (Threonine) third codon position change. Five of the individuals of the western population (1W, 2W, 3W, 4W and 6W) shared the same haplotype, with individual 5W having a transversion from CTA to ATA in position 481. This mutation represents a non-synonymous first codon position change that coded for amino acid Methionine instead of Leucine. Both these amino acids are large, non-polar compounds.

Sequence divergence

Pairwise estimates of nucleotide sequence divergence based on the ND2 gene resulted in very low values (Table 2.7). Among individuals of the eastern population sequence divergence values varied from 0.00% - 0.11% while among those of the western population

it ranged from 0.00% - 0.10%. The sequence divergence between individuals from the eastern and western populations varied between 0.10% - 0.31%.

Phylogenetic analyses

Cytochrome *b*

The parsimony analysis of the Cyt *b* sequences resulted in two most parsimonious trees with 106 steps (CI = 0.991, RI = 0.996). Since no variation was found in the sequences of all 12 individuals in the ingroup, the strict consensus tree resulted in a monophyletic clade consisting of all 12 individuals representing both populations (Fig. 2.1). Therefore no further analyses were performed using the Cyt *b* sequences.

ND2

The parsimony analysis of the ND2 sequences resulted in only a single topology with 416 steps (CI = 0.995, RI = 0.997). Both the bootstrap values (62% and 65% respectively) of the branches leading to the monophyletic grouping of individuals of the western population and separating individual 4E from the rest of the eastern population are too low to be considered with confidence (Fig. 2.2). Therefore, the individuals of both populations form a monophyletic group which is the sister group of the outgroup individuals (100%). A topology for only the ingroup individuals, rooted at the midpoint, had a high bootstrap value (100%) for the monophyletic origin of the western population individuals (Fig. 2.3). Due to the low levels of variation it was not deemed necessary to run tests for elaborate models of evolution.

Population genetic variability and population subdivision

The haplotype and nucleotide diversity of both the eastern and the western populations was relatively low. The haplotype diversity of both populations was 0.33333 and the nucleotype

diversity of both populations was 0.00034. The relative measure of population structure, population divergence or the inbreeding coefficient of a subpopulation with respect to the total population (F_{st}) (Wright 1951) calculated in DnaSP was relatively high (F_{st} = 0.75) with the number of migrants per generation, very low (N_m = 0.08). Both the calculated Fu and Li's D* and F* statistics (Fu & Li 1993) to test the neutrality of the ND2 sequenced region were statistically non-significant (P > 0.10) which is consistent with neutral evolution.

Phylogeographic analysis

The 95% parsimony cladogram estimated with TCS yielded a network that formed unambiguous connections among four haplotypes (two each from both the eastern and western populations) (Fig. 2.3). The size of each oval is scaled to the number of individuals possessing that haplotype, with the inferred ancestral haplotype A, (according to coalescence theory) represented by a square. The polymorphisms in the ingroup never exceeded one mutation with a single substitution between the singleton C (representing individual 1E) and the rest of the eastern population individuals (haplotype A), one substitution between the singleton D (representing individual 5W) and the rest of the western population individuals (haplotype B) and one substitution between haplotype A and B. In the subsequent nested clade analysis using Geodis (Fig. 2.4), none of the clades were significantly different (P = 1.00) from random expectations and therefore no phylogeographic inferences could be made.

DISCUSSION

The present study has resolved the long-standing uncertainty that has surrounded the taxonomic status of the isolated eastern population of the Short-clawed Lark. The results indicate that the eastern and western populations of the species are virtually

indistinguishable based on the analyses of morphometric, call and molecular data. Although the sample sizes used in the present study were small, particularly those employed for molecular data, similar sample sizes have been used to resolve taxonomic uncertainties in the *Alaudidae* (Ryan & Bloomer 1997; Ryan & Bloomer 1999). The small sample size used in the present study is due in part to the Short-clawed Lark's listing as a species of conservation concern and the difficulty in capturing live birds.

The study revealed minor morphometric differences between the two populations, with only the length of the tarsus proving to be statistically significantly longer in males from the western population. Similarly, a quantitative and qualitative comparison of the territorial calls of males from the eastern and western populations of the Short-clawed Lark showed significant differences in their territorial calls for only some of the variables analysed. However, the variation in certain call parameters among individuals within a population showed just as much variation as between the two populations, e.g. the parameters analysed for double-note territorial calls of eastern population males showed significant differences among individuals for all 12 of the parameters analysed. Ryan & Bloomer (1999) found the same pattern in individual variation in the call structure of species in the Long-billed Lark complex. Herremans (1997) reported differences in the calls of males from the western population over small geographic distances and ascribed these "regional dialects" as a consequence of the species' poor dispersal abilities. The song characterisation results of the present study and that of Ryan & Bloomer (1999) suggest that although the structure of territorial calls are similar in the Short-clawed Lark and Long-billed Lark complex, the extent of individual variation in territorial calls complicates the recognition of dialects within populations. Thus, although there are minor differences in the territorial calls of males from the two populations it does not warrant the recognition of two separate taxa.

The results of the molecular analyses of the two populations support the abovementioned findings. A total of 530 base pairs of the Cytochrome *b* gene were amplified for individuals representing both Short-clawed Lark populations. The results obtained from the amplified Cyt *b* sequences showed the populations to be 100% identical. The Cyt *b* gene has been used successfully in other similar phylogenetic studies involving species in the *Alaudidae*. In a study aimed at clarifying the lineation of species within the Long-billed Lark complex, Ryan & Bloomer (1999) described five distinct species on account of Cyt *b* sequence divergences ranging between 2 and 9%. Correspondingly, Ryan *et al.* (1998) described Barlow's Lark *Calendulauda barlowi* as a distinct species within the Karoo Lark complex with pairwise sequence divergences of 1.9 - 4.9%. In order to corroborate the Cyt *b* results obtained in the present study it was decided to use a second mitochondrial gene which represents a faster rate of evolution (Johnson & Sorenson 1998). Hence the ND2 gene was used, from which a total of 972 base pairs were successfully amplified. Sequence divergence between the eastern and western populations ranged between 0.10 - 0.31%. This was also significantly lower than the results obtained in other similar studies on members of *Alaudidae*. Drovetski (2005) reported sequence divergences of 0.72 – 0.79% between three clades representing the Streaked Horned Lark *Eremophila alpestris strigata* from across the US Pacific Coast states. Furthermore, both the eastern and western individuals exhibited only four haplotypes of the partial ND2 gene investigated with a single mutational step separating each of the haplotypes (Fig. 2.4). Consequently, the level of divergence derived from the ND2 data in the current study would thus too be insufficient to recognize the eastern population of the Short-clawed Lark as a different taxonomic entity.

The use of subspecies as a formal taxonomic category has been challenged by some quarters, but it remains a useful label for regional variation (Ryan *et al.* 1998). The results of the current morphological, song characterisation and molecular study suggests that there is

insufficient consistent regional variation between the two populations to recognize the two populations as subspecies.

The similarity of the Cyt *b* sequences between all individuals from both populations and the high fixation index (F_{st} = 0.75) and low rate of migration (N_m = 0.08 migrants per generation) calculated for ND2 sequences suggests that there is practically no movement between the eastern and western populations. The results also indicate that the two populations are genetically depauperate (although based on only two gene regions), which raises some concern with regards to the effects of inbreeding. The risk of possible extinction is high in small populations that have a small geographic range and show low genetic diversity (Allendorf 1983; Spellerberg 1996). The two isolated populations of the Short-clawed Lark are thus extremely vulnerable to environmental stochasticity of any sort as the consequences of genetic drift and inbreeding leads to decreased heterozygosity and subsequent poor adaptability. Contrary to the aforementioned, Gray (1996) suggested that a lack of genetic diversity does not necessary constitute a threat to a species survival as there are examples of successful species which are genetically depauperate. In some cases where low genetic diversity is believed to be caused by a population bottleneck, population numbers have recovered rapidly from very low numbers e.g. Fallow Deer *Cervus dama* (Linnaeus, 1758) and Northern Elephant Seal *Mirounga angustirostris* (Gill, 1866) (Gray 1996). However, this should be viewed in relation to the impacting demographic factors. The low genetic diversity shown in the Short-clawed Lark suggests a bottleneck event in the species' evolutionary past. This is aggravated by the species' apparent limited dispersal abilities (Herremans 1997) which restricts gene flow between sub-populations and which may contribute to inbreeding. From an evolutionary point of view, it is imperative that as much as possible of the present genetic variation in the two populations be conserved to enable the species to respond to future stochastic environmental effects.

The reason for the lack of divergence between the two populations can be twofold. Firstly, a geographical corridor of suitable habitat linking the two populations may exist, although several factors suggest this to be an unlikely scenario. No Short-clawed Larks were recorded within the 180km "breach" zone during the Southern African Bird Atlas Project (see Herremans 1997) or during surveys conducted as part of the present study (Chapter 3). The environment in the "breach" zone is characterized by habitat that the species generally avoids, e.g. mountainous areas, dense woodland and/or a sandy substrate in certain areas. Furthermore, population genetic analysis shows a high inbreeding coefficient within the populations and a particularly low migration rate between the populations. This is supported by the apparently poor dispersal abilities of the Short-clawed Lark (Herremans 1997). As with many other habitat specific larks, e.g. the Karoo Lark *Calendulauda albescens* (Ryan *et al*. 1998), the Short-clawed Lark also appears to be reluctant to cross unsuitable habitat.

A second more plausible explanation for the lack of divergence between the two populations may be as a result of a relatively recent separation as is indicated by a high F_{st} value. Moreover, the results showed that the inferred ancestral haplotype of ND2 is part of the eastern population (Fig. 2.4), suggesting that the Short-clawed Lark's distribution range was initially restricted to the Polokwane Plateau, but expanded when suitable habitat became available to the west. The Short-clawed Lark's close association with "subsistence agricultural man" (Herremans & Herremans 1992) and the habitat changes associated with this may have contributed to a range expansion of the species in the last few centuries followed by separation into geographically isolated populations. One of the contributing factors to this vegetation change may have been the great iron-age civilizations and mega-complexes of the Rolong, Bahurutshe, Kwena and Kgatla clans in what is today south-eastern and eastern Botswana and the North West Province of South Africa. Towards the

north and east, other large iron-age communities included the Ndebele and the Pedi civilizations. These civilizations numbered hundreds of thousands of people who possessed as many cattle (Bulpin 1965; Mitchell 2002). In his diaries, explorer/scientist Dr. Andrew Smith described vast concentrations of cattle and heavily grazed “open” landscapes during the early to mid 1800’s in what is today the Vryburg and Mafikeng areas of the North West Province (Kirby 1939). It is likely that such large “rural” human populations contributed to the creation of open habitats through deforestation (for fuel and charcoal) and overgrazing by their vast cattle herds (F. Roodt, pers. comm.), thus creating suitable habitat for the Short-clawed Lark. A network of trade routes linked these iron-age communities. One of the major routes linked the port of Sofala in Mozambique via Mapungubwe, the Polokwane Plateau and the southern parts of the Waterberg Mountain range to the metal workers at Rooiberg near modern day Thabazimbi (South Africa) in the south-west (Changuion & Bergh 1998). It is well known that many large tribal villages were established along this route (Changuion & Bergh 1998). It is believed that after the collapse of these civilizations, the fragmentation of tribes during the Mfecane and the Rinder Pest epidemic of 1896 (Mack 1970), a gradual change in vegetational structure occurred whereby woodland habitats replaced grasslands (Liebenberg 1998; Kepe & Scoones 1999). Grasslands and open savannas only remained in populated rural areas and at higher altitudes and elevated plateaus, e.g. the Polokwane Plateau, where climatic factors such as frost would have restricted the growth of woody vegetation (F. Roodt, pers. comm.). In this regard, Carton (2003) suggested that prior to the 1896 Rinder Pest (Cattle Plague) epidemic which caused the decimation of millions of large ungulates (Liebenberg 1998) in southern and eastern Africa, large tracts of the northern parts of southern Africa consisted of open grassland. Thus, it is postulated that habitat change by iron-age communities created a corridor for dispersal of individuals from the ancestral population on the Polokwane Plateau (according to the coalescence theory) to the south-west. A combination of the collapse of these civilizations and the Rinder Pest

epidemic indirectly resulted in a change in the vegetation structure from a grassland or open savanna to woodland. This, in turn, rendered the habitat unsuitable for the Short-clawed Lark which resulted in isolation of the eastern and western population.

In conclusion it can safely be assumed that the present study has confirmed that the eastern population of the Short-clawed Lark is not a distinct taxonomic entity, thus resolving a long-standing uncertainty. Despite this, it has also been established that the two populations are geographically isolated and appears to have a remarkably low genetic diversity. There are genetic differences in the ND2 gene between the two populations which suggest that the two populations may be on separate evolutionary pathways that may lead to speciation.

Table 2.1. Details of Short-clawed Lark specimens used for morphometric measurements from the western (W) and eastern (E) populations. Polokwane Nature Reserve = PNR (eastern population) and Botsalano Nature Reserve = BNR (western population).

Live specimens

Number & Population	Collection date	Collection locality	Co-ordinates	SAFRING number
E1	31/10/2002	PNR	S 23 56 076, E 29 27 457	BD94073
E2	16/11/2002	PNR	S 23 56 002, E 29 27 320	BD94078
E3	31/10/2002	PNR	S 23 58 129, E 29 31 173	BD96247
E4	16/10/2003	PNR	S 23 58 458, E 29 28 273	BE16404
E5	24/10/2003	PNR	S 23 58 131, E 29 28 472	BE16409
E6	29/10/2003	PNR	S 23 56 952, E 29 27 124	BE16414
E7	29/10/2003	PNR	S 23 58 107, E 29 31 878	BE16415
E8	06/05/2004	PNR	S 23 58 088, E 29 28 056	BE16452
E9	09/06/2004	PNR	S 23 58 279, E 29 31 108	BE16426
E10	09/06/2004	PNR	S 23 56 101, E 29 27 637	BB02965
E11	09/06/2004	PNR	S 23 58 347, E 29 31 928	BB02967
E12	18/05/2005	PNR	S 23 58 467, E 29 31 060	BE16470
E13	18/05/2005	PNR	S 23 58 445, E 29 30 539	BE16471
E14	24/05/2005	PNR	S 23 58 074, E 29 31 017	BE16474
E15	24/05/2005	PNR	S 23 58 062, E 29 31 006	BE16406
E16	22/06/2005	PNR	S 23 58 258, E 29 28 288	BE16476
E17	05/09/2006	PNR	S 23 58 202, E 29 28 105	BE16493
E18	15/09/2006	PNR	S 23 58 000, E 29 28 380	BE16496
E19	15/09/2006	PNR	S 23 58 121, E 29 27 917	BE16497
E20	22/09/2006	PNR	S 23 58 050, E 29 30 894	BE16498
E21	22/09/2006	PNR	S 23 58 733, E 29 31 154	BE17405
E22	22/09/2006	PNR	S 23 58 544, E 29 30 803	BE17407
E23	13/10/2006	PNR	S 23 58 472, E 29 30 473	BE17409
E24	09/01/2007	PNR	S 23 58 733, E 29 31 476	BE17433
W1	18/09/2004	Ga-Pitsane (west of BNR)	S 25 34 900, E 25 41 504	-
W2	19/09/2004	Ga-Pitsane (west of BNR)	S 25 30 489, E 25 43 334	-
W3	19/09/2004	Ga-Pitsane (west of BNR)	S 25 33 157, E 25 41 058	-
W4	19/09/2004	Ga-Pitsane (west of BNR)	S 25 33 068, E 25 43 321	BE16451
W5	11/02/2005	BNR	S 25 33 347, E 25 43 590	BE16461
W6	12/02/2005	BNR	S 25 33 885, E 25 43 300	BE16462
W7	12/02/2005	BNR	S 25 33 885, E 25 43 300	BE16463
W8	07/12/2006	BNR	S 25 32 268, E 25 41 122	BE17424
W9	07/12/2006	BNR	S 25 33 481, E 25 41 383	BE17425
W10	08/12/2006	BNR	S 25 32 737, E 25 38 487	BE17428
W11	08/12/2006	Ga-Pitsane (west of BNR)	S 25 34 330, E 25 43 165	BE17429
W12	09/12/2006	Ga-Pitsane (west of BNR)	S 25 32 244, E 25 42 968	BE17431
W13	09/12/2006	BNR	S 25 32 034, E 25 41 212	BE17432

Study skins

Number & Population	Museum	Original Collection site	Museum Number
W14	NFI Pretoria	Schweizer-Reneke (RSA)	39081
W15	NFI Pretoria	Wolmaransstad (RSA)	7340
W16	NFI Pretoria	Wolmaransstad (RSA)	7326
W17	Durban	Marapeng (Botswana)	30309
W18	Durban	Morobana (Botswana)	30308
W19	McGregor	Kanye (Botswana)	B780

Table 2.2. Sampling localities for genetic analyses. Short-clawed Larks *Certhilauda chuana* from the eastern population (*n* = 6) (1E – 6E) and from the western population (n = 6) (1W – 6W). Outgroup birds consisted of Rufous-naped Lark *Mirafra africana* (*n* = 2) (1R & 2R) and Spike-heeled Lark *Chersomanes albofasciata* (*n* = 3) (1S – 3S).

Code	Date	Locality name	Coordinates
1E		Polokwane Nature Reserve	S 23 58 303, E 29 17 355
2E		Rondepan Farm (Polokwane)	S 23 46 812, E 29 23 505
3E		Polokwane Nature Reserve	S 23 58 467, E 29 31 060
4E		Polokwane Nature Reserve	S 23 58 445, E 29 30 539
5E		Polokwane Nature Reserve	S 23 58 074, E 29 31 017
6E		Polokwane Nature Reserve	S 23 58 062, E 29 31 006
1W		Botsalano Nature Reserve	S 25 32 268, E 25 41 122
2W		Botsalano Nature Reserve	S 25 33 481, E 25 41 383
3W		Botsalano Nature Reserve	S 25 32 737, E 25 38 487
4W		Ga-Pitsane	S 25 34 330, E 25 43 165
5W		Ga-Pitsane	S 25 32 244, E 25 42 968
6W		Botsalano Nature Reserve	S 25 32 034, E 25 41 212
3R		Botsalano/Ramatlabama	S 25 36 547, E 25 41 951
2R		Palmietfontein Farm (Polokwane)	S 23 45 571, E 29 25 025
1S		Botsalano Nature Reserve	S 25 34 389, E 25 42 938
2S		Polokwane Nature Reserve	S 23 98 444, E 29 51 102
3S		Botsalano Nature Reserve	S 25 33 532, E 25 41 917

Table 2.3. Sequences and locations of primers used for amplification and sequencing of Cytochrome *b* and ND2 genes.

Primer (a)	Sequence (b)	Source
Cyt *b*		
L14990	5'-CCATCCAACATCTCAGCATGATGAAA-3'	Ryan & Bloomer (1999) from Kocher *et al*. (1989)
H15499	5'-GGTTGTTTGAGCCTGATTC-3'	Ryan & Bloomer (1999) from Avise *et al.* (1994)
L15245 nested	5'-GATCCCTACTAGGCATCTG-3'	Customised - Inqaba Biotechnical Industries (Pty) Ltd.
ND2		
L5204	5'-TAACTAAGCTATCGGGCCCAT-3'	Inqaba Biotechnical Industries (Pty) Ltd. modified from Cicero & Johnson (2001)
H6312	5'-CTTATTTAAGGCTTTGAAGGCC-3'	Cicero & Johnson (2001)
L5216	5'-GCCCATACCCCRAgAATG-3'	Sorenson *et al.* (1999)
H6313	5'-CTCTTATTTAAGGCTTTGAAGGC-3'	Sorenson *et al*. (1999)

(a) Letters refer to light (L) and heavy (H) strands. Numbers correspond to locations on the chicken (*Gallus*) sequence (Desjarins & Morais 1990) of the 3' end of the primer sequence.

(b) All primers are listed in the 5' to 3' direction

Table 2.4. Results of morphometric comparisons between male Short-clawed Larks from the eastern and western populations (Mann-Whitney U test).

Parameter	Population	*n*	Mean	SD	*P*
Mass (g)	East	24	37.13	2.68	0.10
	West	13	38.58	2.09	
Wing length (mm)	East	24	103.29	3.27	0.28
	West	19	104.26	2.35	
Head length (mm)	East	23	43.93	1.05	0.70
	West	17	43.77	1.61	
Culmen length (mm)	East	23	21.56	1.18	0.21
	West	17	22	0.88	
Tarsus length (mm)	East	23	29.41	1.09	0.02
	West	18	30.31	1.27	
Tail length (mm)	East	23	76.54	5.03	0.99
	West	18	76.52	4.09	

Table 2.5. The number of single-, double- and triple-note territorial call phrases and their relative contributions recorded for Short-clawed Lark males from the eastern and western population.

Number of notes		West	East	Total
Single	*n*	47	49	96
	% for both populations	49%	51%	100%
	% within populations	42.3%	30.1%	35.0%
Double	*n*	59	82	141
	% for both populations	41.8%	58.2%	100%
	% within populations	53.2%	50.3%	51.5%
Triple	*n*	5	32	37
	% for both populations	13.5%	86.5%	100%
	% within populations	4.5%	19.6%	13.5%

Table 2.6. Single and double-note territorial call variables analysed for Short-clawed Lark males from the eastern and western population of the species. * Statistically significant ($P < 0.05$) differences between populations.

a) Single-note territorial call variables - DT = delta time (ms), LF1 = Lowest Frequency (kHz), HF1 = Highest Frequency (kHz), DF1 = Delta frequency (kHz).

b) Double-note call variables - DT1, DT2 and DT_{Tot} = delta time of the first and second note and total phrase respectively (ms), LF1, LF2 and LF_{Tot} = Lowest Frequency of the first and second note and total phrase respectively (kHz), HF1, HF2 and HF_{Tot} = Highest Frequency of the first and second note and total phrase respectively (kHz), DF1, DF2 and DF_{Tot} = Delta frequency of the first and second note and total phrase respectively (kHz).

a) Single-note territorial call								
	East (*n* = 47)				**West (*n* = 49)**			
Variable	**Mean**	**SD**	**Median**	**Range**	**Mean**	**SD**	**Median**	**Range**
*DT**	0.78	0.16	0.78	0.14 - 1.13	0.91	0.11	0.89	0.64 - 1.26
*LF**	4.77	1.19	4.83	1.95 - 7.08	4.08	0.85	3.76	3.22 - 6.68
*HF**	5.94	1.11	6.14	3.86 - 7.80	4.92	1.02	4.54	3.83 - 7.63
DF	1.17	0.76	0.95	0.34 - 3.54	0.85	0.30	0.81	0.37 - 1.53
b) Double-note territorial call								
	East (*n* = 82)				**West (*n* = 59)**			
Variable	**Mean**	**SD**	**Median**	**Range**	**Mean**	**SD**	**Median**	**Range**
DT1	0.45	0.28	0.50	0.06 - 0.91	0.43	0.25	0.31	0.09 – 0.89
DT2	0.92	0.37	0.76	0.15 – 1.64	0.77	0.13	0.77	0.44 – 1.06
DT_{Tot}*	0.89	0.42	0.95	0.12 – 2.34	1.38	0.18	1.33	1.12 – 1.81
LF1	5.23	1.16	5.61	3050.80 – 7817.80	5.61	0.80	5.64	4135.60 – 7186.40
*LF2**	4.07	0.84	3.93	3050.80 – 6440.70	5.29	0.73	5.46	3762.70 – 6440.70
LF_{Tot}*	4.55	1.07	4.10	3084.70 – 6881.40	5.10	0.78	5.32	3762.00 – 6440.70
HF1	6.48	0.88	6.27	4678.00 – 8466.10	6.64	0.74	6.81	5016.90 – 7762.70
HF2	6.18	1.08	6.37	4508.50 – 7694.90	6.19	0.87	6.36	4359.00 – 7491.50
HF_{Tot}	6.79	0.88	6.98	4203.40 – 8466.10	6.86	0.46	6.92	5846.20 – 7762.70
DF1	1.25	0.54	1.13	419.50 – 2915.30	1.03	0.22	1.05	474.60 – 1435.90
*DF2**	2.11	1.21	2.07	305.10 – 4339.00	0.91	0.23	0.92	508.50 – 1423.70
DF_{Tot}	2.24	1.13	2.07	474.60 – 4843.20	1.77	0.53	1.49	1152.50 – 2931.30

Table 2.7. Pairwise estimates of ND2 nucleotide sequence divergence between Short-clawed Larks from the Western population (1W – 6W) and the Eastern population (1E – 6E) with outgroups, Rufous-naped Lark (1R & 2R) and Spike-heeled Lark (1S – 3S). Sequence differences are depicted in percentage.

	1E	**3E**	**5E**	**6E**	**6W**	**2E**	**4E**	**1W**	**5W**	**2W**	**3W**	**4W**	**2R**	**1R**	**1S**	**3S**	**2S**
1E																	
3E	0.00																
5E	0.00	0.00															
6E	0.00	0.00	0.00														
6W	0.11	0.11	0.10	0.10													
2E	0.00	0.00	0.00	0.00	0.11												
4E	0.11	0.11	0.10	0.10	0.21	0.10											
1W	0.11	0.11	0.10	0.10	0.00	0.11	0.21										
5W	0.21	0.21	0.21	0.21	0.10	0.21	0.31	0.10									
2W	0.11	0.11	0.10	0.10	0.00	0.11	0.21	0.00	0.10								
3W	0.11	0.11	0.10	0.10	0.00	0.11	0.21	0.00	0.10	0.00							
4W	0.11	0.11	0.10	0.10	0.00	0.11	0.21	0.00	0.10	0.00	0.00						
2R	17.84	17.75	18.03	18.03	18.09	17.89	17.92	18.01	18.19	18.25	18.09	18.09					
1R	17.94	17.86	18.13	18.13	18.19	18.00	18.02	18.11	18.29	18.35	18.19	18.19	0.21				
1S	16.96	16.98	17.08	17.08	17.24	16.74	16.97	17.26	17.34	17.20	17.24	17.24	19.54	19.33			
3S	17.18	17.19	17.28	17.28	17.44	16.95	17.18	17.46	17.55	17.50	17.44	17.44	20.05	19.84	1.14		
2S	17.07	17.09	17.18	17.18	17.34	16.84	17.08	17.36	17.44	17.40	17.34	17.34	19.95	19.74	1.03	0.10	

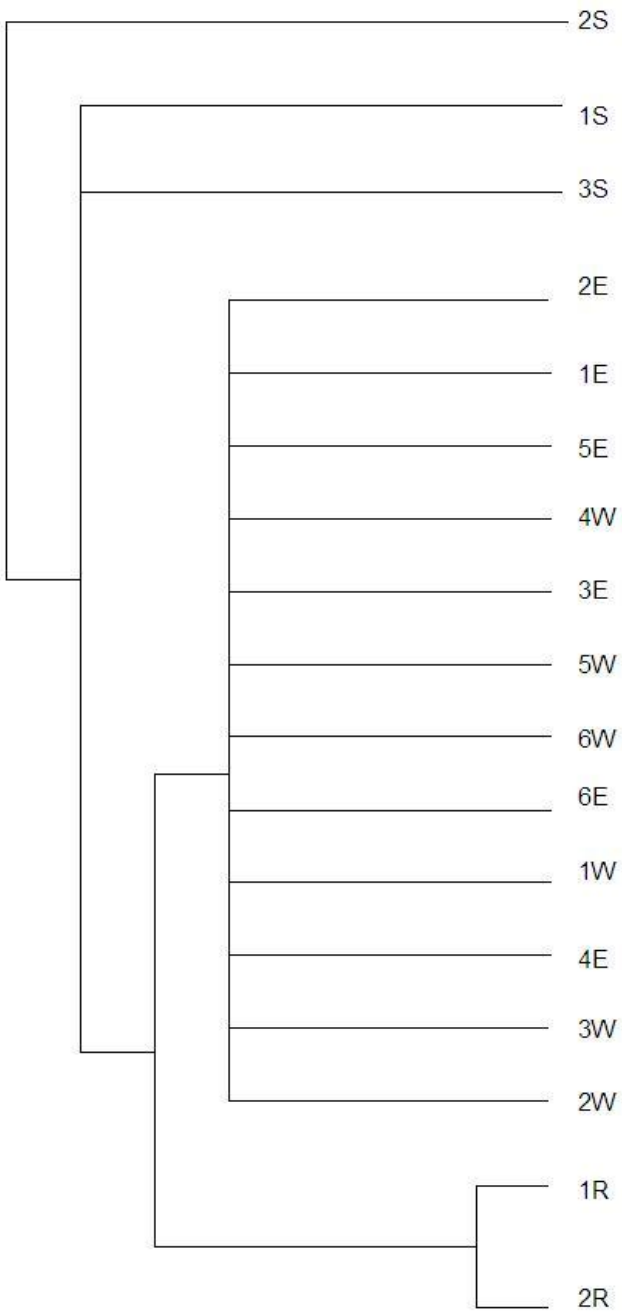

Fig. 2.1. A strict consensus tree of the most parsimonious trees of the partial Cytochrome *b* gene from individuals from the western (1W – 6W) and eastern (1E – 6E) populations of the Short-clawed Lark with outgroup individuals represented by the Rufous-naped Lark (1R & 2R) and the Spike-heeled Lark (1S – 3S).

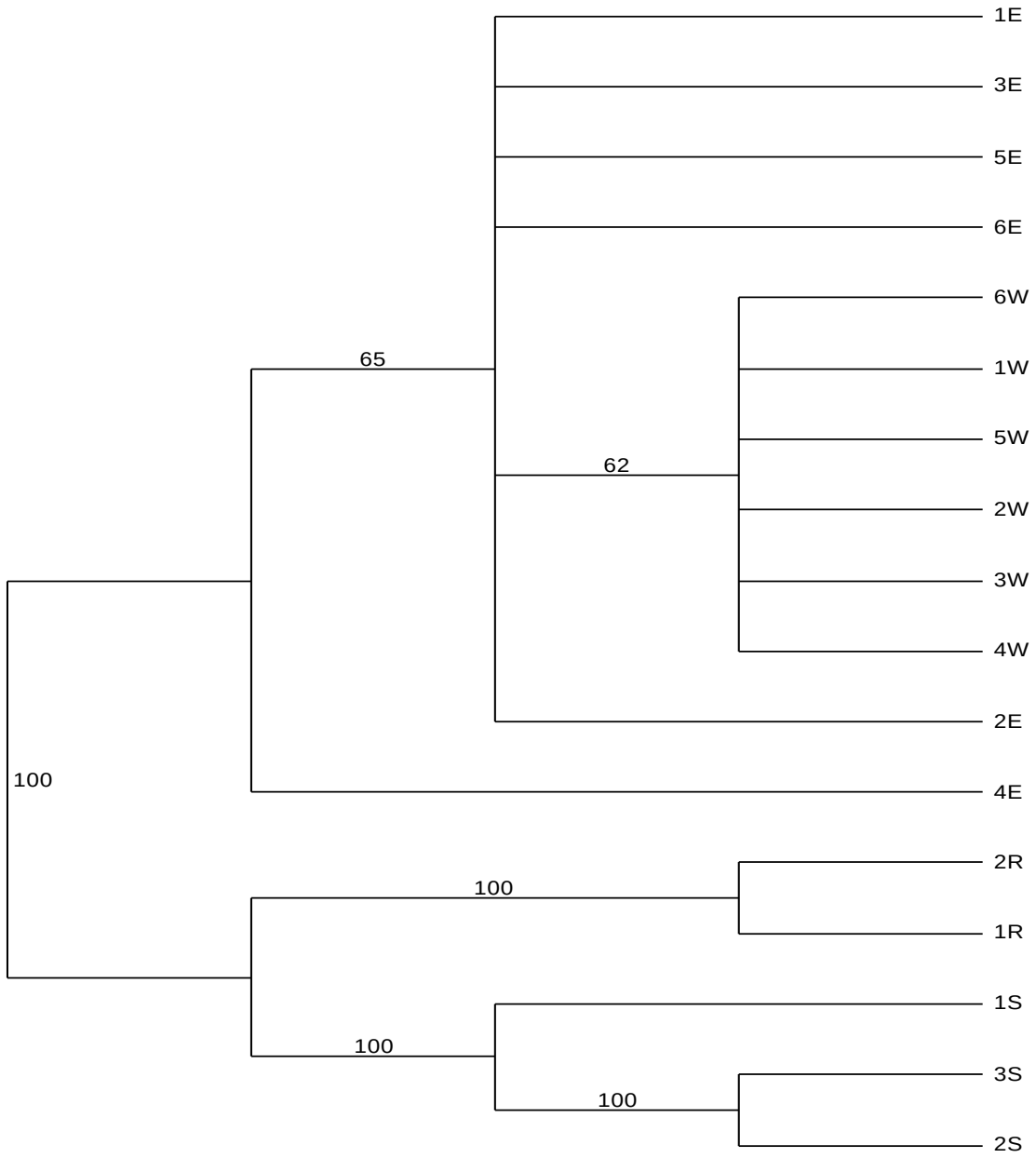

Fig. 2.2. Most parsimonious tree with bootstrap values for the ND2 dataset (CI = 0.995, RI = 0.997) from individuals from the western (1W – 6W) and eastern (1E – 6E) populations of the Short-clawed Lark with outgroup individuals represented by the Rufous-naped Lark (1R & 2R) and the Spike-heeled Lark (1S – 3S). Numbers in the branches indicate the bootstrap support values.

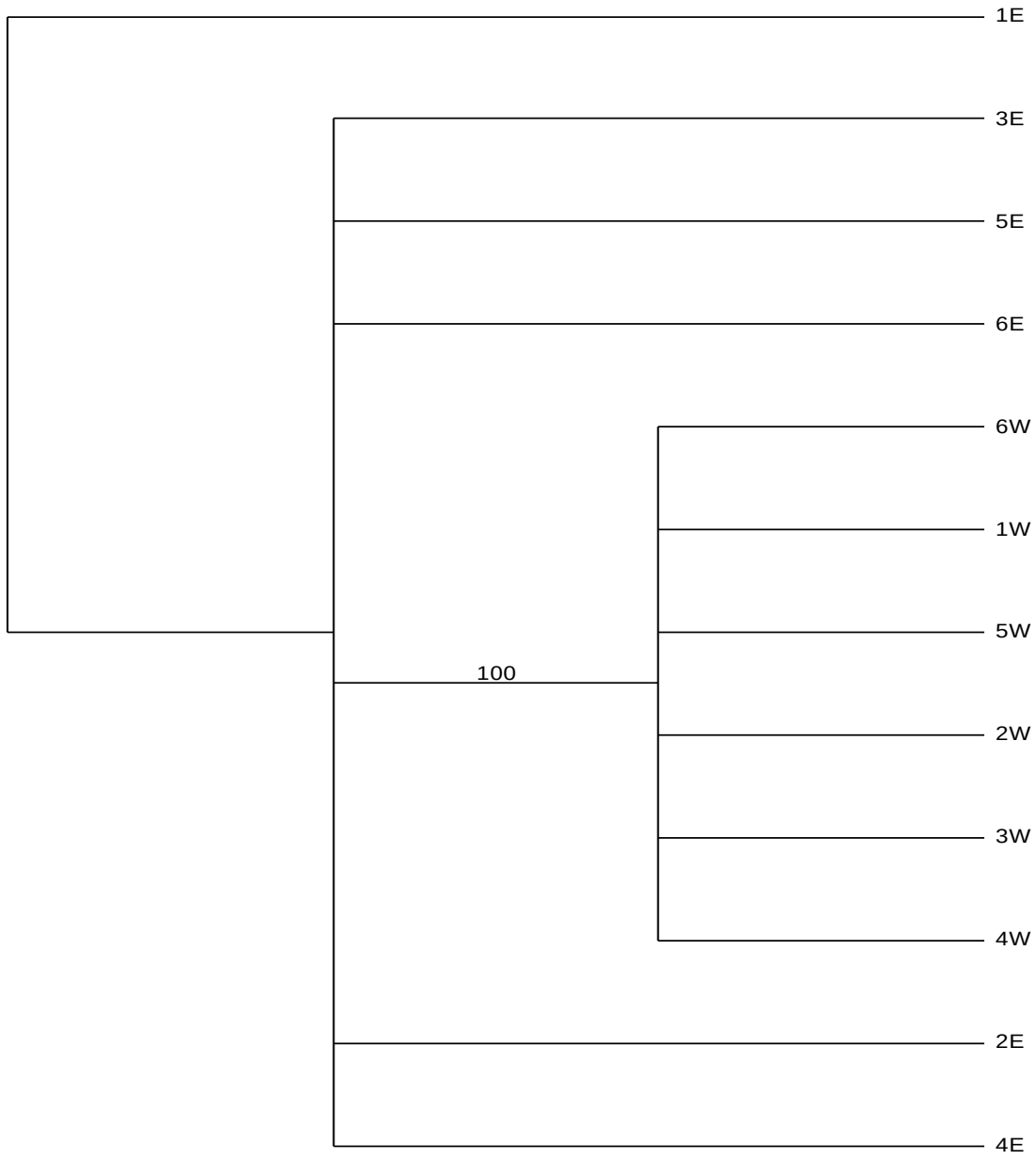

Fig. 2.3. The ND2 Midpoint-rooted bootstrap tree representing the in-group individuals from the western (1W – 6W) and eastern (1E – 6E) populations of the Short-clawed Lark.

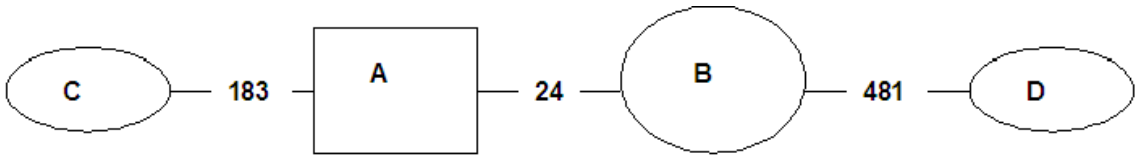

Fig. 2.4. TCS network of four haplotypes of individuals from the eastern and western Short-clawed Lark populations where C = the 4E singleton, D = the 5W singleton, A = the rest of the eastern population individuals and B = the rest of the western population individuals. The ancestral population is labelled A.