REVEALING THE FACTORS THAT PROMOTE DIVERGENCE IN THE BLADDER GRASSHOPPER *BULLACRIS UNICOLOR* (ORTHOPTERA; PNEUMOROIDEA)

By

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DECLARATION

I, Rekha Sathyan, affirm that the work presented in this thesis is based on my own work. Neither the whole work nor any part of it has been submitted before for another degree in this or any other university, and that all the sources I have used have been indicated and acknowledged as a complete reference.

13 February 2019

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Signature Date

General Abstract

Variation in sympatric and allopatric populations is believed to be a precursor to eventual speciation. The dispersion of genes from one gene pool into another is prevented by various processes, including the founder effect, sexual selection, ecological differences and random genetic divergence. Examining patterns of intraspecific variation in phenotypic and genotypic traits may thus provide valuable insights into the processes that govern species origination.

Bladder grasshoppers (Orthoptera; Pneumoroidea) are an ideal model system to investigate patterns of geographic and ecological divergence due to their high host plant specificity, low dispersal and distinctive acoustic signals. This dissertation investigates intraspecific diversification in the bladder grasshopper *Bullacris unicolor* (Orthoptera: Pneumoroidea). Recent research on this species has shown significant intra- and inter-population variation in male advertisement calls and morphological characters. However, the exact cause of this variation has remained unclear. Furthermore, a previous study showed that the calls of *B. unicolor* from one particular population are highly differentiated from other populations, possibly due to the effects of anthropogenic noise. Here we aim to examine the drivers of diversity within *Bullacris unicolor* by embracing a multidisciplinary approach that encompasses the effects of environmental factors, genetics, anthropogenic noise and host plant associated divergence.

The first chapter examines genetic structuring among allopatric populations of *B. unicolor* and whether the observed variation in acoustic signals and morphological characters of *B. unicolor* is driven by environmental factors or genetic drift. Results revealed that there is

distinct genetic differentiation among populations. The population differences in acoustic signals were not significantly related to the variation in environmental factors or genetic factors, but morphological characters of males (body length) and females (head width and abdomen width) were significantly correlated with annual temperature. Moreover, environmental differences were significantly correlated with genetic distance, but not with latitude. Results show climate-induced geographic variation in genetic and morphological characters, suggesting that lineage diversification in general may be driven by climate mediated differences accompanied by genetic differentiation.

The second chapter investigated call parameter differences between noisy and quiet habitats of *B. unicolor*. We installed passive acoustic monitoring devices to record sound for three weeks in two different nature reserves, the Cape Flats Nature Reserve (CFNR) (high anthropogenic noise) and Tygerberg Nature Reserve (TNR) (low anthropogenic noise) in the Western Cape, South Africa. Our results suggest that call interval was positively correlated and call rate was negatively correlated with anthropogenic noise in CFNR. Peak frequency, call interval and call rate were affected by weather conditions; peak frequency increases and call rate decreases with wind speed. In addition, temperature was positively correlated and humidity was negatively correlated with both of these call parameters. We propose that both anthropogenic noise and weather conditions may constrain long-distance acoustic communication in *B. unicolor*.

The third chapter examined host related genetic and morphological divergence of *B. unicolor*. The genetic structure and morphological measurements of *B. unicolor* feeding on *Didelta spinosa* and *Roepera morgsana* at one locality (Springbok) was tested using mitochondrial

and nuclear markers. The results demonstrated that both genomes show non-significant host related variation with a very low value of F_{ST}, indicating a low level of variation. The phylogram strongly suggested that there are no host associated genetic differences in *B. unicolor* by displaying limited genomic clustering. On the other hand, significant differentiation was observed between the morphology of males and females on different host plants. Thus, further study is required to determine how these variation patterns associate with the host phylogeny, host phenology, nutritional values of host plants, host preferences and the development of polymorphic cryptic colouration in *B. unicolor*.

This is the first study to examine population genetics in pneumorids, and to integrate genetic and phenotypic variation with ecological factors, in order to better understand evolutionary processes in this unusual group of insects. In addition, acoustic monitoring is gaining popularity globally as an important conservation tool. Both the physiology and behaviour of many animal species are affected by noise pollution and it impacts on many ecological aspects of the lives of animals. In bladder grasshoppers, acoustic signals are used to exchange biological relevant information. The direct impact of noise on the behaviour of this species has not been studied before. It is also the first study of its kind in southern Africa to monitor acoustic soundscapes and explore the potential impacts of man-made noise on insect sound communication systems. The results of this thesis indicate that variation in the local environment, in combination with other ecological cues, has likely contributed to the diversification of morphological, acoustic and genetic characters within and among populations of *B. unicolor*. Furthermore, this thesis emphasizes that the effects of adaptation and drift are associated with differences on a regional scale. Our findings clearly suggest that gene flow in natural populations may mostly be environmentally structured.

I dedicate this to my family, friends and all people who dream.

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General introduction

Understanding how the variation within a population directs variation between populations and finally speciation is a fundamental goal of studies of ecology and evolution (Pfennig *et al*. 2010; Mullen & Shaw 2014). Multiple variants within and between populations are good model systems in speciation studies. Thus, to address allopatric speciation, geographic variation in acoustic, morphological, and genetic traits has been extensively studied (Amezquita *et al*. 2009; Hertach *et al*. 2015). Many studies (Lamichhaney *et al*. 2017; Campbell *et al*. 2018) have revealed various evolutionary forces that can contribute to divergence between populations, such as genetic drift, environmental variation, natural and sexual selection, and ecological differences. Recent empirical and theoretical studies suggest that, in allopatric models, geographic divergence in traits is promoted by both selective forces (natural and sexual selection) and non-selective forces (genetic drift) (Rundle & Nosil 2005). However, a single evolutionary force is not a mechanistic description for the evolution of new species. Instead, heterogeneity of divergence across different populations has been shaped by a combination of evolutionary forces. Concurrent operation of these factors in the same population causes intraspecific variation. Identifying these factors and the interactions among them may potentially enhance our understanding of the mechanisms that cause divergence of one species into two.

The process whereby populations or subsets of a single population become reproductively isolated through adaption to different ecological environments has been termed “ecological speciation” (Schluter 2000; 2001; Gilliam & McCracken 2007; Shieh *et al*. 2017). Ecological speciation requires that different populations encounter different environmental selective pressures, making it a distinct process from so-called mutation-order speciation that results

from the chance occurrence of different evolutionary solutions to similar selective pressures (Schluter 2009). In order for ecological speciation to progress to completion, ecologically divergent traits must be the same as or genetically linked to traits involved in reproductive isolation (Nosil & Rundle 2009); a requirement that is commonly fulfilled across taxonomically disparate groups (Funk *et al.* 2006). Indeed, mounting evidence for ecological speciation comes from a variety of widely spread taxonomic groups, including mimetic butterflies (reviewed in Jiggins 2008), *Anolis* lizards (Leal & Fleishman 2004), stick insects (Nosil 2004; 2007), *Mimulus* monkey flowers (Bradshaw & Schemske 2003), *Rhagoletis* flies (Bush, 1969; Feder 1998), sticklebacks (Rundle *et al*. 2000; Kozak *et al*. 2011) *Candidula* snails (Pfenninger *et al.* 2003), Darwin's finches (Grant 1999; Leon *et al*. 2010), killifish (Fuller *et al*. 2007), *Drosophila* flies (Matute *et al*. 2009), *Oryza* grasses (Zheng & Song 2011), and marine rockfish (Ingram 2011).

Variation in physical factors, such as altitude, temperature, rainfall, sunlight and resource quality and availability, may initiate diversity by causing a corresponding change in one or more of the morphological characteristics of an organism (Malhotra & Thorpe 1997; Calsbeek *et al*. 2006; Amiot *et al*. 2007; Capellini & Gosling 2007; Stillwell *et al*. 2007; Riesch *et al*. 2011). For example, salt marsh beetles that inhabit unstable salt marshes have longer wings than those inhabiting more stable marshes; an adaptation that permits enhanced dispersal ability in unpredictable environments (Dhuyvetter *et al*. 2004). Although there is a current scarcity of studies examining the role of biotic factors, such as competitors, predators and prey, in ecological divergence, they are also considered to be a significant driving force behind the process (Nosil 2004; Svanbäck & Bolnick 2007; Riesch & Deecke 2011).

It is not only morphological characteristics, but also communication systems that can vary according to the local environment (Nemeth *et al*. 2013; Lampe *et al*. 2014). A combination of geography and morphology is largely responsible for call variation (Pröhl *et al*. 2007). In animals that rely on signalling systems for mate choice cues, any alteration of the environment under which signalling occurs can critically influence both the nature and perception of signals. This divergence in mating signals and preferences resulting from ecological differences is referred to as sensory drive (Endler 1992, 1993), a process that can eventually lead to reproductive isolation and speciation (Boughman 2002), even in the absence of geographic barriers (Seehausen *et al*. 2008).

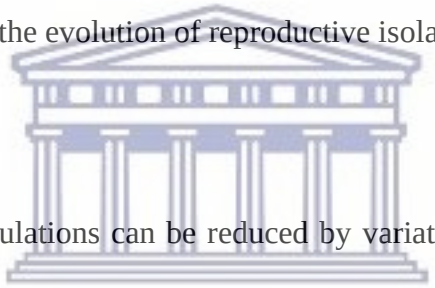
Speciation has traditionally been classified according to the degree of geographic isolation of diverging groups. Geographical variation among the populations of a species has long been identified as a likely precursor to allopatric speciation (Mayr 1954).This variation can arise for a number of reasons, including chance events, such as genetic drift and population bottlenecks. Isolated populations in different geographic areas are exposed to different adaptive pressures and chance events, and over time may diverge sufficiently to a point where they can no longer interbreed (Mayr 1963). Speciation in sympatry has always been much more contentious than speciation in allopatry (Coyne & Orr 2004). This is because it is much more difficult to achieve complete reproductive isolation in the absence of geographic barriers to gene flow. Sympatric speciation is most likely to occur via a combination of disruptive selection and direct selection on mating traits, provided the costs of assortative mating are weak or absent (Bolnick & Fitzpatrick 2007). Although strictly classifying speciation events according to the degree of geographic separation of putative species may be a less worthwhile endeavour than gaining a general understanding the processes that underlie

speciation events, be they in sympatry, parapatry or allopatry (Fitzpatrick *et al*. 2009), the terminology remains useful.

Ecological speciation is one such process that can occur under any geographic context. Ecologically driven sympatric speciation is gaining support from both empirical studies (Korol *et al*. 2006; Conde-Padin *et al*. 2007; Huber *et al*. 2007; Steinfartz *et al*. 2007) and theoretical models (Bürger & Schneider 2006; Bürger *et al*. 2006; Gavrilets & Vose 2007). Small-scale differences in ecological conditions or microhabitat within the same area may play a central role in the adaptive divergence of ecotypes within a population. For example, larvae of the fire salamander may inhabit either small streams or shallow ponds within the same forest. The salamanders have split into two genetically differentiated groups, each with its own adaptations to cope with the different ecological conditions (Steinfartz *et al*. 2007). One of the most convincing scenarios of how a geographically continuous population can split into two is through host shifts in plant-eating insects (Drès & Mallet 2002; Matsubayashi *et al*. 2010). If a subset of the population switches to a novel or previously unexploited food resource in order to reduce intraspecific competition, assortative mating can result in distinct host races (Bush 1994). If gene flow is sufficiently diminished, this may eventually result in complete reproductive isolation. Through this process of colonising and adapting to different ecological environments, insect host races may diverge with relative speed, in the order of dozens to hundreds of generations (Hendry *et al*. 2007).

Insect biotypes feeding on different host plants are most strongly associated with controversial theories of sympatric speciation (Via 2001). Many studies have been conducted on host races in phytophagous insects over the past decades and insect-plant relations have

been a major focus of study in ecology and evolution (Bernays & Chapman 1994). Polyphagous insects feeding on large number of plants from different families likely employ a number of different cues. Most phytophagous insects tend to specialize within their host plants and are associated with particular parts of plants. This is partly a matter of size, genes and mobility. Thus, the genetic variation of insects can certainly be affected by host plant association. Host plant-genetic diversity correlations have been reported in various species of insects (Dong *et al*. 2018). Furthermore, both mtDNA and nuclear DNA molecular markers have become useful markers in studies of phylogeography and population genetics of insects (Csilléry *et al*. 2010; Fontaine *et al*. 2012). Thus, assessing the genetic diversity of individuals from different host plant species by using multiple molecular markers can reveal the specific role of host use in the evolution of reproductive isolating barriers and speciation.



Indeed, gene flow among populations can be reduced by variation in ecological factors and adaptation to different habitats. Phenotypic divergence can also arise as a consequence of environmental effects on specific gene regulatory networks (Pfennig *et al* 2014). Divergent selection between environments is consistent in allopatric and sympatric populations (e.g. Mayr 1942, 1947). However, our understanding of the relationship between specific environmental conditions and divergence is not complete. For example, temperature and precipitation affect development and growth in biological organisms, and also play a role in ectotherms and homiotherms (Meiril *et al*. 2007). But, less attention has been paid as to how these specific environmental variations effect trait variations, notably the effects of temperature and rainfall, in determining the magnitude and direction of biological variation. Experiments that explore rainfall and temperature effects on specific behavioural traits causing reproductive isolation may be immensely useful to address these gaps.

Recent studies argue that genetic divergence also plays a dominant role in population divergence and speciation. Many studies have estimated the relationships, systematics and structure of populations by using several genetic markers and genetic information (Schneider *et al*. 2014; Alvarez *et al*. 2015), while patterns of genetic variation between populations and their relative role in divergence are still debated (Richards *et al*. 2009). Few studies have quantified the role of genetic drift in divergence of morphological and acoustic characters (For example, see Jiang *et al*. 2015; Duda & Costa 2015). Clarification of the relationship between patterns of genetic structure and trait designs is required to understand factors that can affect divergence between populations. Comparing acoustic and morphological divergence in conjunction with genetic differences helps to identify the role of genetic drift in geographical divergence. Combining a variety of different markers will be key to revealing the evolutionary changes that contribute to divergence within a species (Lozier *et al*. 2016).

More than ever before, acoustic habitats are being disturbed by changing environments as anthropogenic influences alter natural habitats. Soundscapes are no exception; with man-made noises (termed "anthrophony") infiltrating and impacting the acoustic space of animals (Pijanowski *et al*. 2011). Anthropogenic noise can mask acoustic cues of both land and under water animals (Merchant *et al*. 2015; Clark *et al*. 2009) and elicit changes in behaviour (Nowacek *et al*. 2007). Animals living in urban or noisy environments have been shown to significantly alter their signals and behaviour in response to increased noise levels. For example, male *Chorthippus biguttulus* grasshoppers that live adjacent to roads produce advertisement calls with a higher pitch than males away from roadsides (Lampe *et al* 2012). However, studies concerning the effects of anthropogenic noise on animals have concentrated almost exclusively on vertebrate species (but see Lampe *et al* 2012; Shieh *et al* 2012), despite there being significant potential consequences for invertebrates too (Morley *et al* 2014). More

studies that address the impacts of anthrophony on invertebrate species are therefore urgently needed.

Bladder grasshoppers (Orthoptera; Pneumoroidea) are a group of insects endemic to the coastal regions of southern Africa, and represent an ideal model system on which to study the roles of ecology and speciation. Bladder grasshoppers are most noteworthy for having evolved extreme specializations in the pursuit of long distance acoustic communication. This includes six pairs of abdominal hearing organs unique among the animal world (Dirsh 1965; van Staaden & Römer 1998), and a strongly inflated abdominal bladder that acts as an acoustic resonator, resulting in sound transmission distances of up to 1.9 km (van Staaden & Römer 1997). *Bullacris unicolor* is a fairly wide ranging pneumorid, with a geographic distribution extending along much of the coastal region of the Northern Cape and Western Cape Provinces of South Africa. This distribution spans two major vegetation biomes, the Fynbos and Succulent Karoo, with individuals in different regions of the distribution existing under dissimilar environmental conditions. Pneumorids have a high degree of host specificity, with each species living and feeding on either one or a small number of host plant species. They rely heavily on cryptic camouflage to avoid predation, with individuals being extremely well matched in terms of colour pattern to their specific host plant. Preliminary investigations have shown significant geographic variation in the acoustic and morphological components of males (Sathyan *et al*. 2017), and that these differences appear to be fixed rather than plastic. This variation in signal and body characteristics may be linked to various evolutionary forces.

Through a careful examination of the patterns of divergence within this ideal experimental system, at both intra- and inter-population level, the factors that contribute to speciation might be better understood. This study will examine 1) intraspecific genetic variation and how this correlates with acoustic, morphological and environmental differences 2) call character variation in response to anthropogenic noise and 3) host plant associated divergence of *B. unicolor*. This work will help to further our understanding of the ecological complexities that shape divergence and consequently promote speciation.

CHAPTER 1

GEOGRAPHICAL VARIATION IN *BULLACRIS UNICOLOR*: CONTRIBUTION OF ENVIRONMENTAL FACTORS AND GENETIC DRIFT

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Author's contributions: Rekha Sathyan and Vanessa Couldridge conceived and designed the study and carried out the fieldwork. Rekha Sathyan conducted the analysis with input from Vanessa Couldridge and Adriaan Engelbrecht.

Abstract

The patterns and causes of an animal's acoustic and morphological variation have been of long standing interest in evolutionary biology. However, little is known about the processes driving intraspecific acoustic and morphological variation. Here we used geographical variation in acoustic signals and morphological characters of the bladder grasshopper *Bullacris unicolor* to assess whether this divergence was driven by environmental factors and/or genetic drift. Our results revealed that acoustic and morphological characters varied significantly among populations. The population differences in acoustic signals of males were not significantly related to the variation in environmental factors or genetic factors, but morphological characters of males (body length) and females (head width and abdomen width) are significantly correlated with annual temperature. Moreover, environmental differences were significantly correlated with genetic distance, but not with latitude. Thus, climatic factors have likely contributed to the genetic divergence observed in *B. unicolor*. We

predict that the action of ecological selection and variation in habitat promotes divergence of individuals among geographically distributed populations.

Introduction

Determining the mechanisms that promote allopatric and sympatric models of speciation are central questions in evolutionary biology. The putative forces that promote divergence between and within populations are based on different macro- and micro-evolutionary processes. Interaction of different evolutionary forces leads to divergence in phenotypic traits. Studying these different forces will improve our understanding of the divergence in populations, niche differentiation and speciation (Lin *et al*. 2015). Potential evolutionary forces, such as the effects of climate, cause geographical variability in morphology and in acoustic characters, and may cause differences between populations (Dieckmann *et al*. 2004). These patterns of variation in the environment, such as habitat, geography, and climate, might lead to geographic speciation (Mayr 1954). But, it remains unclear whether there is a consistent association between phenotypic and genotypic variation and particular environmental variables (Medina & Francis 2012). Moreover, genetic drift represents another potential driver of acoustic and morphological divergence.

Decades of investigation of 'isolation by environment' (IBE) has revealed that patterns of morphological and acoustic differences increase with environmental differences (Ruegg *et al*. 2006; Wang & Wadburd 2014). In particular, elevation (Bradburd *et al*. 2013) and habitat type (Ramos & Peters 2017) have long been identified as possible mechanisms producing geographic variation. Janzen (1967) showed patterns of climatic variation across latitudes and the response of taxa to particular environmental gradients. He suggested body size within species increases with latitude (See Kozak & Wiens 2007). Since Janzen's hypothesis (Bergmann's rule) of climatic zonation was proposed, several studies and reviews highlighted the impact of latitudinal, climatic and geographic variation on particular traits of species. For

example, intraspecific morphological variation relative to latitude and climate has been observed in mammals (Meiri & Dayan 2003), birds (Campbell-Tennant *et al*. 2015), amphibians (Ashton 2002), and reptiles (Azócar *et al*. 2015). However, some studies of closely related taxa show negative relation of body size with latitude (Sota *et al*. 2000; Koyama *et al*. 2015). These processes address the important question of how patterns of geographic variation are generated.

Recent research on ecogeographical variation indicated that climatic variables, such as temperature, play an influential role in determining body size and dispersal among populations (Millien *et al*. 2006; Murphy *et al*. 2010). Other climatic variables, such as rainfall, may better correlate with latitude and influence body size variation (Ashton *et al*. 2000). Thus, body size variation among populations is expected to be higher with larger differences in environmental conditions (Ashton 2004). More studies are needed to see whether such variation is consistently linked with variation in particular environmental variables.

Not only morphological variation, but also mating signal divergence, leads to pre-mating isolation in animals, and it is often the first step towards divergence (Coyne & Orr 2004). It has been proposed that there is a complex interaction between an animal's acoustic signals and its morphology, ecology, and phylogenetic history (Luo *et al*. 2017). According to the acoustic adaptation hypothesis (AAH), divergence in calling songs are also often caused by different habitat features (Podos *et al*. 2004; Jang & Gerhardt 2006; Medina & Francis 2012). Examining the climatic effects on calls produced in variable environments may facilitate an understanding of how environmental variability is associated with acoustic variability in

intraspecific populations. Few studies have examined the relationship between precipitation and temperature and song traits, and these have suggested trait variation in songs may be shaped by environmental variability (Ruegg *et al*. 2006; Medina & Francis 2012).

It has been hypothesized that the physical properties of the environment promote signal divergence to minimize excess attenuation and distortion. Evolution of signals in response to local habitat features has been documented in insects (Schmidt & Balakrishnan 2015; Couldridge & van Staaden 2004; Zuk *et al*. 2001). The acoustic adaptation hypothesis predicts that structural differences in habitats influence the evolution of signals through their effects on signal transmission (Wilkins *et al*. 2013). Sexual selection via sensory drive may also explain intraspecific signal variation, as such variability promotes signal variation among populations inhabiting different environmental conditions. Inter-population variation via adaptation to different environmental conditions may be most common when species have large geographical ranges (Irwin *et al*. 2008).

The results of studies available on geographical call variation of insects support the 'genetic drift hypotheses' (Ortego *et al*. 2017). Species that exist in different geographic regions may show different patterns of genetic differentiation (Manier & Arnold 2006; Olsen *et al*. 2011). Even ecologically similar taxa can also show significantly different patterns of genetic differentiation (Van Buskirk 2012; Whiteley *et al*. 2014). Thus, a clear understanding of gene flow focused on a single species is of critical importance (Panhuis *et al*. 2001; Boughman 2002). There is growing literature to demonstrate that patterns of genetic variation within a population are caused by multiple factors such as mating patterns, random forces, distribution, or migration (Dyer 2015). This variation allows a population to survive in the

face of changing environmental circumstances. More molecular studies within species are needed for a better understanding of their evolution, genetic difference and population dynamics (Gonzalez-zerna *et al*. 2017)

Geographic variation in acoustic signals across a species' range has been demonstrated in a variety of animal taxa, including birds (Förschler & Kalko 2007; Koetz *et al*. 2007), mice (Campbell *et al*. 2010), bats (Sun *et al*. 2013), whales (Samarra *et al*. 2015), anurans (Castellano & Giacoma 2000; Pröhl *et al*. 2007; Rodríguez-Tejeda *et al*. 2014), fish (Phillips & Johnston 2008) and insects (Pinto-Juma *et al*. 2008). Likewise, morphological traits may also differ geographically (e.g. Castellano & Giacoma 2000; Pröhl *et al*. 2007; Huizenga *et al*. 2008; Cisneiros *et al*. 2012). However, studies examining the link between these phenotypic traits and genotypic variation have yielded mixed results, either supporting (Campbell *et al*. 2010; Velásquez *et al*. 2013; Warwick *et al*. 2015) or failing to support (Pröhl *et al*. 2006a, 2007b; Ohmer *et al*. 2009; Sun *et al*. 2013; Lee *et al*. 2016) a correlation between them. The absence of a correlation between genetic distance and morphological or behavioural distance points to the importance of either ecological or sexual selection on shaping phenotypic traits.

Understanding variation in abiotic factors may reveal various processes and patterns required for divergence. For example, changes in topography and climate can influence the evolution of many fundamental biological traits. Previous studies of speciation rarely quantify environmental factors that might cause allopatric and sympatric speciation due to the difficulty in obtaining detailed environmental data. Fortunately, nowadays GIS based data have been widely used to quantify spatial and temporal variation in temperature, rainfall and

topography (Review by Kozak *et al*. 2008). It has been particularly widely used in studies of phenotypic character evolution within and among species and the study of how new species originate (Ritchie *et al*. 2001; Salem 2003). Used in this way, GIS data combined with empirical data help to address intraspecific geographic variation and distinguish the effects of variation in the environment (e.g. changes in climate and topography).

Bullacris unicolor was used to study the role of environmental conditions in population divergence. Its distribution extends over parts of the Northern and Western Cape of South Africa, with varying environmental conditions. Consequently, the opportunity for differences between high latitude and low latitude populations should be large. However, it is not known whether this observed acoustic and morphological variation is linked to genetic variation.

In this chapter, I use both mtDNA and nDNA markers to examine genetic variation across multiple populations of *B. unicolor* to, firstly, determine whether allopatric populations show genetic divergence, and secondly, determine whether genetic variation is linked to acoustic and morphological variation. In addition, I examine whether inter-population differences may be linked to different environmental conditions, such as temperature and precipitation. By understanding the association between these key environmental factors (climate and latitude) and morphological, acoustic and genetic divergence, the roles of different factors in differentiation between populations may be revealed.

Materials and methods

Specimen collection

Bullacris unicolor was collected from five field sites located in the Western and Northern Cape provinces of South Africa – Springbok, Kamieskroon, Groenriviersmond, Cederberg, and Melkbosstrand. Altitudinal distribution of these localities ranges from 56m (Groenriversmond) to 954m (Springbok), and they are characterized by very different environmental and climatic conditions (Fig. 1.1, Table 1.1). The vegetation biome of Springbok, Kamieskroon, and Groenriviersmond is Succulent Karoo, while Cederberg and Melkbosstrand are part of the Fynbos biome (Cowling *et al*. 1997; Mucina & Rutherford 2006). The Succulent Karoo is more sparsely vegetated (semi-desert) and receives less precipitation than Fynbos. Due to the seasonal occurrence of bladder grasshoppers during the spring and early summer, sampling was done to coincide with peak times of emergence (September to November). A total of 98 grasshoppers (66 males and 32 females) were individually captured by hand between 2008 and 2014. As far as possible, adults or final instar nymphs were collected in order to prevent controlled laboratory conditions from influencing factors such as body size (61 adults and 37 nymphs). There was no significant difference in the distribution of adults and nymphs among the five locations ($\chi 2\ 4 = 6.182$; $P = 0.186$). Alternate males were excluded from the analysis due to their low occurrence ($n = 4$) and were only found at Springbok.

Animals collected in the field were transported to the University of the Western Cape. Nymphs were raised to adulthood in the laboratory under identical rearing conditions. Adult females and nymphs were housed in small groups (between one and three individuals) in

opaque plastic storage bins with a wire mesh top to allow for airflow and light. To prevent fighting, adult males were kept separate from each other. Grasshoppers were maintained under natural lighting conditions in a temperature controlled room maintained at approximately 22ºC to 25ºC. Individuals were fed *ad libitum* either on the host plant species on which they were found or, if this was unavailable, on lettuce leaves. Grasshoppers were examined daily to replenish their water and food, and to determine their moulting.

Environmental variables

Environmental variables from each unique location were obtained from an online free climate data set, WordClim - Global Climate Data (http://www.worldclim.org/bioclim). To provide a quantitative description of climatic conditions, we considered the following variables: (1) Annual temperature, (2) Annual rainfall, (3) Bio 2 = mean diurnal range, mean of monthly temperature max-min, (4) Bio 4 = temperature seasonality and (5) Bio 15 = precipitation seasonality (Table. 1.1). Other climatic variables were excluded from the analysis because of high correlation ($r > 0.9$), finally leaving five climatic variables and one topographical variable (latitude), as latitude influences the rate of warming (Deutsch *et al*. 2008) and it is a key factor in determining a region's climate. The six variables were chosen as the representatives of principal component analysis, and these variables represent the variation of temperature and rainfall and the effects of their interactions.

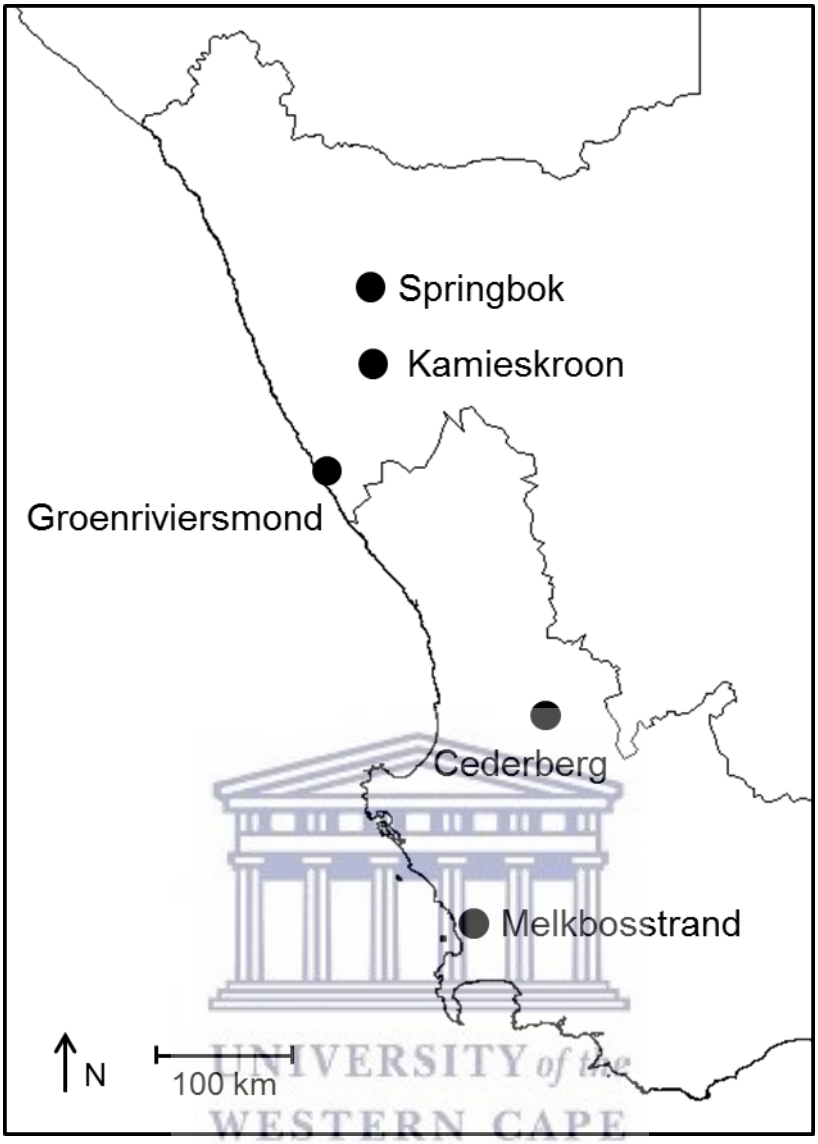


Figure 1.1 Map of South Africa showing the five locations where *Bullacris unicolor* was sampled.

Table 1.1 List of collection locations with basic climatic data

Population	Latitude	Longitude	Annual precipitation (mm)	Annual temperature range (max-min)	Mean diural range mean of monthly temp. (max-min)	Temperature seasonality (STD*100)	Precipitation seasonality (CV)
Cederberg	-32.6	19.01	327	18.27	15.46	471.50	68.57
Groenriversmond	-30.85	17.60	154	17.65	13.12	283.24	78.90
Kamieskroon	-30.2	17.93	221	15.63	13.93	392.14	63.81
Melkbosstrand	-33.73	18.50	621	16.55	11.04	316.92	62.21
Springbok	-29.66	17.88	182	17.34	13.41	423.32	63.85

Analysis of genetic differentiation

Genomic DNA was extracted from the hind legs of ethanol preserved specimens using standard phenol chloroform extraction procedures. For mtDNA, Polymerase chain reactions (PCRs) were conducted using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG - 3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA – 3') (Folmer *et al*. 1994) to amplify a 708-bp fragment cytochrome oxidase I (COI) mitochondrial gene of 43 individuals. We amplified nuclear genetic material (ITS) from 42 individuals. For nDNA, ITS forward (5'- AGA GGA AGT AAA AGT CGT AAC AAG G-3') and ITS reverse (5'- CCT TAG TAA TAT GCT TAA ATT CAG G-3') were used to amplify and sequence a 750 bp nuclear DNA segment. The sequence amplification and microsatellite genotyping for both mtDNA and nDNA were performed following the same protocols. We quantified genetic differentiation between populations using dnaSP ver 5.10 (Rozas 2009) by calculating F_{ST} separately for concatenated mtDNA and nDNA sequences.

Each PCR reaction contained 14.9µL of distilled water, 2.5 µl of 25mM $MgCl_2$, 2.5 µL of 10xMg^{2+} -free buffer, 0.5 µL of a 10mM dNTP solution and 0.5 µL (10mM) of the respective primer pairs, 0.1 µL of *Taq* polymerase and 2−5 µL of template DNA. PCR followed standard protocols with the following temperature cycle: 94ºC for 4 min, 94ºC for 30s, 48ºC for 45s and 72ºC for 35s. The last three steps were repeated for 40 cycles followed by a final extension of 15 min at 72ºC. Five microliters of amplified product was electrophoresed on 1 % of agarose gel, stained with ethidium bromide and observed under a UV transilluminator to confirm whether the amplification was successful. An automated sequencer (ABI 3100, applied Biosystems) was used to run cycle sequencing products. Sequences were aligned

using BIOEDIT sequence alignment editor, version 7.2.5. All the sequences were compared and blasted with other sequences of grasshoppers on GENBANK to authenticate the sequences and the lack of stop codons was checked using EMBROSS/Transec (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to determine whether a pseudo-gene was amplified. The sequences of the closely related *Bullacris membracioides* were used as outgroups.

To characterize the mode of nucleotide substitution, J MODELTEST version 3.06 was used (Posada & Crandall 1998). The best fit maximum-likelihood (ML) score was chosen using the Akaike information criterion (AIC) (Dabirra *et al.* 2012). Afterwards, as implemented in PAUP*4 version beta 10 (Swofford 2002), the phylogenetic relationships were inferred using maximum parsimony (MP) and neighbour joining tree (NJ). Genetic distance among populations was calculated by model averaged parameter values. Further analysis was done using Bayesian inference, Mr Bayes version 3.2 (Ronquist & Huelsenbeck 2003). Ten Monte Carlo Markov chains were run for five million generations of which 10% were burn in. The runs were stopped by the value of standard deviation of split frequencies below 0.01.

We conducted population genetic tests using DNAsp version 4.50 (Libardo & Rozas 2009). For each sampled population, we estimated haplotype (h) and nucleotide (π) diversities. We used Tajima's D test (Tajima 1989) and Fu's F_s test (FU 1997) to test the population changes and gene selection. Mismatch distribution was used to reveal the demographic history of the population. To understand the number of base substitutions per site between sequences we computed pairwise distance analysis (this was done in PAUP). An intra-specific phylogeny was constructed with the UPGMA using Splits tree version 4.13.1 (Huson & Bryant 2006). The evolutionary distance was calculated with the Maximum Composite Likelihood method,

and node support was evaluated with 1000 bootstrap replicates (Felsenstein 1985). Tests for the relationship between geographic distance and genetic distance were determined in Alleles in Space (AIS) version 1.0 (Miller 2005).

Geographical variation in morphological and acoustic characters

Previously collected data on morphological and acoustic variation of the same individuals from the same five populations was used in statistical analyses. Refer to Sathyan *et al*. 2017 for details on this data.

Statistical analysis

We conducted Principal Component Analysis (PCA) to test the correlation matrices of the six environmental factors. The relative contributions of each variable to principal components were determined from the matrix of factor-variable correlations. We ran PCAs on the climatic variables to represent the pattern of climate and geographical differences among locations. Axes with the Eigen value larger than 1 were retained. Principal Component Analyses were performed using SPSS version 23.

To test for correlations between acoustic and morphological variables with environmental factors and genetic variation, we first calculated pairwise differences of population mean values for each climatic, geographical, acoustic, morphological and genetic variable respectively. We also calculated combined Euclidean distances with latitude, annual rainfall, annual temperature, bio2, bio4 and bio15 to represent overall environmental variation between sampling localities. We then used pairwise Mantel tests to test the correlation

between acoustic and morphological distance matrices with climatic, geographical and genetic distance matrices, respectively. Mantel tests were used and implemented in the program R version 3.3.1.

We then used Pearson correlation analysis to test the relationships between individual climate variables (annual rainfall, temperature, bio 2, bio 4 and bio 15) and geography (latitude) with the two most strongly differentiated variables of male morphology, female morphology and the acoustic characters of males. The variables that show the highest difference in morphology and acoustic characters were derived from the discriminant function analysis of our previous study (see Sathyan *et al*. 2017). Pearson correlation was implemented in SPSS ver. 23.

Results

Variation in environmental factors

Principal component analysis showed that the first and second component explained together 76.1 percent of climatic variation. The first component (PC1) explained 50.9 percent of total climatic variation, and annual rainfall and latitude had high loadings on this component. The second component (PC2) explained 25.2 percent of climatic variation and temperature variables had high loadings on this component (Table 1.2). We plotted environmental variables as PC1, PC2, and PC3 (Fig. 1.2). Variables displayed on the left and right sides of Figure 1.2 formed distinct groups. Rainfall and latitude clustered together. Other climatic variables (temperature, bio2, bio4, and bio15) on the right side of the figure form a distinct group. In the full dataset of the PCA, all six variables differed significantly among locations (ANOVA $p < 0.05$). Thus, these six variables have been used to test phenotypic and genotypic variation. Eigen values were retained for all environmental variables and compared to the PC axes (Table 1.2). Overall climatic variation (pairwise distance) between sampling localities is shown in Table 1.3.

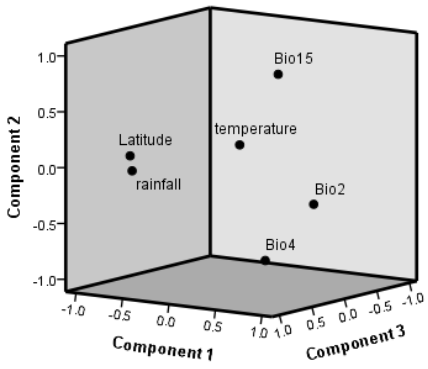


Figure 1.2 Component plot in rotated space of six environmental variables based on five locations.

Table 1.2 Loadings of environmental variables on principal component (PC) axes, with their eigenvalues (SD^2)

Variable	PC1	PC2	PC3
Latitude	0.961	0.121	0.218
Annual rainfall	0.963	-0.170	-0.200
Annual temperature	0.133	0.784	0.403
Mean monthly maximum-minimum temperature	-0.006	0.915	-0.131
Temperature seasonality	0.025	0.661	-0.748
Precipitation seasonality	-0.066	0.139	0.975
Eigenvalue (SD^2)	3.059	1.513	1.190

Table 1.3 Euclidean distances between sampled locations derived from environmental factors.

	Springbok	Kamieskroon	Groenriversmond	Cederberg
Kamieskroon	49.97			
Groenriversmond	143.65	128.771		
Cederberg	152.941	132.57	255.904	
Melkbosstrand	451.755	407.054	468.531	332.261

Genetic differentiation

After trimming short end sections of the COI and ITS sequences, 654 bp and 750bp were obtained from 43 and 42 specimens. There were 99 segregating sites of mtDNA and 13 sites of nDNA defined 30 and 12 haplotypes respectively. The number of observed haplotypes within populations from mtDNA ranged from: Cederberg-5, Groenriversmond-2,

Kamieskroon-7, Melkbosstrand-7, and Springbok-9. From nDNA, the number of haplotypes within populations was: Cederberg-5, Groenriversmond-1, Kamieskroon-1, Melkbosstrand-4, and Springbok-1. The average nucleotide diversity observed among all populations was 0.048 for mtDNA and 0.003 for nDNA. Both concatenated mtDNA and nDNA data showed significant genetic variation (mtDNA: F_{ST} – 0.637; nDNA: F_{ST} – 0.775). Pairwise population F_{ST} values based on mtDNA ranged from 0.467 to 0.962 and the pairwise population F_{ST} values based on nDNA ranged from 0.000 to 1.000 (Table 1.6, Fig. 1.3 and 1.4).

The haplotype diversity for the combined dataset of mtDNA was high (h = 0.96) with a nucleotide diversity of p = 0.048 (average nucleotide differences k = 32.021). As five main lineages were retrieved, we calculated genetic indices for each lineage. Springbok showed a high haplotype diversity (h = 1.00) and high nucleotide diversity (π = 0.040) compared to other locations. Conversely, Groenriversmond showed low haplotype diversity (h = 0.285) and very low nucleotide diversity (p = 0.0008). We applied the McDonald-Kreitman test to Springbok versus all other populations (P = not significant). We calculated Tajimas's D for the complete data set (DT = 0.383, P > 0.100) and for all individual populations. Melkbosstrand and Cederberg sequences both showed a significant deviation from zero. In nDNA, haplotype diversity of combined dataset was 0.75 and nucleotide diversity was 0.00 (average nucleotide difference k = 2.67). Springbok, Kamieskroon and Groenriversmond showed no haplotype and nucleotide diversity (p = 0.0000) and Cederberg and Melkbosstrand showed very low haplotype and nucleotide diversity (Table 1.4).

Haplotype diversity of mtDNA among the five populations varied from 0.28 (Groenriversmond) to 1.00 (Springbok), and nucleotide diversity varied from 0.000

(Groenriversmond) to 0.001 (Melkbosstrand). Haplotype diversity of nDNA among populations varied from 0.000 (Groenriversmond, Kamieskroon and Springbok) to 0.791 (Cederberg) and 0.558 (Melkbosstrand). Nucleotide diversity varied from 0.000 (Groenriversmond, Kamieskroon and Springbok) to 0.002 and 0.001 (Cederberg and Melkbostrand) (Table 1.8). Statistically significant differences in haplotype frequencies were observed among all populations in mtDNA ($X2 = 172.00$, $P < 0.001$) and nDNA ($X2 = 218.81$, $p < 0.001$).

A phylogenetic tree reconstructed from mtDNA sequencing data indicates five major haplotype lineages (Fig. 1.3). Both Cederberg (L2) and Groenriviersmond (L4) are clearly separated into distinct lineages, and do not share haplotypes with any other location. Melkbosstrand is divided into two lineages (L3 and L5). All of the individuals comprising lineage 5 were collected from the same area, which is separated from other collecting localities in the same Melkbosstrand region by urban development. The remaining lineage (L1) comprises a mixture of individuals from Kamieskroon and Springbok. On the other hand, nDNA data indicates four lineages (Fig. 1.4). Melkbosstrand (L4) and Cederberg (L2) separated into two distinct lineages. Cederberg shared a haplotype with L1 and Melkbosstrand with L3.

The genetic analysis revealed strong genetic structuring between locations. From the results of the mtDNA pairwise distance analysis, sequence divergence among individuals within the same population was generally small, with the exception of grasshoppers from Springbok, which showed more genetic variation (4.10%) compared to other populations There was no difference in the results when data was reanalysed excluding individuals from this

population. On the other hand, sequence divergence within Groenriviersmond was extremely low (0.09%). In nDNA, Cederberg (0.26%) and Melkbosstrand (0.17%) showed more genetic variation compared to other three populations, which is 0% (Table 1.5).

Table 1.4 Genetic diversity and demographic parametres for 654bp of cytochrome oxidase 1 (CO1) mtDNA and 750bp nuclear DNA (nDNA) of *Bullacris unicolor*. N - sampled size, Nh - haplotype number, S - number of polymorphic sites, h - haplotype diversity, π - nucleotide diversity, Tajima's D, Fu's Fs. *p <0.05, **p <0.01

Population	***N***	**Nh**	***S***	**h**	π	**D**	$\mathbf{F_S}$
mtDNA							
Cederberg	8	5	52	0.7857	0.0198	-1.894**	-2.279*
Groenriversmond	7	2	2	0.2857	0.0008	-1.2370	-1.3740
Kamieskroon	10	7	30	0.8666	0.0121	-1.2090	-1.5120
Melkbosstrand	10	7	30	0.9111	0.0243	2.412**	1.915*
Springbok	9	9	71	1.0000	0.0405	0.0740	-0.2470
nDNA							
Cederberg	14	5	6	0.7912	0.0024	-0.0771	-0.1230
Groenriversmond	14	1	0	0.0000	0.0000	0.0000	0.0000
Kamieskroon	20	1	0	0.0000	0.0000	0.0000	0.0000
Melkbosstrand	22	4	7	0.5584	0.0018	-0.8482	0.9440
Springbok	14	1	0	0.0000	0.0000	0.0000	0.0000

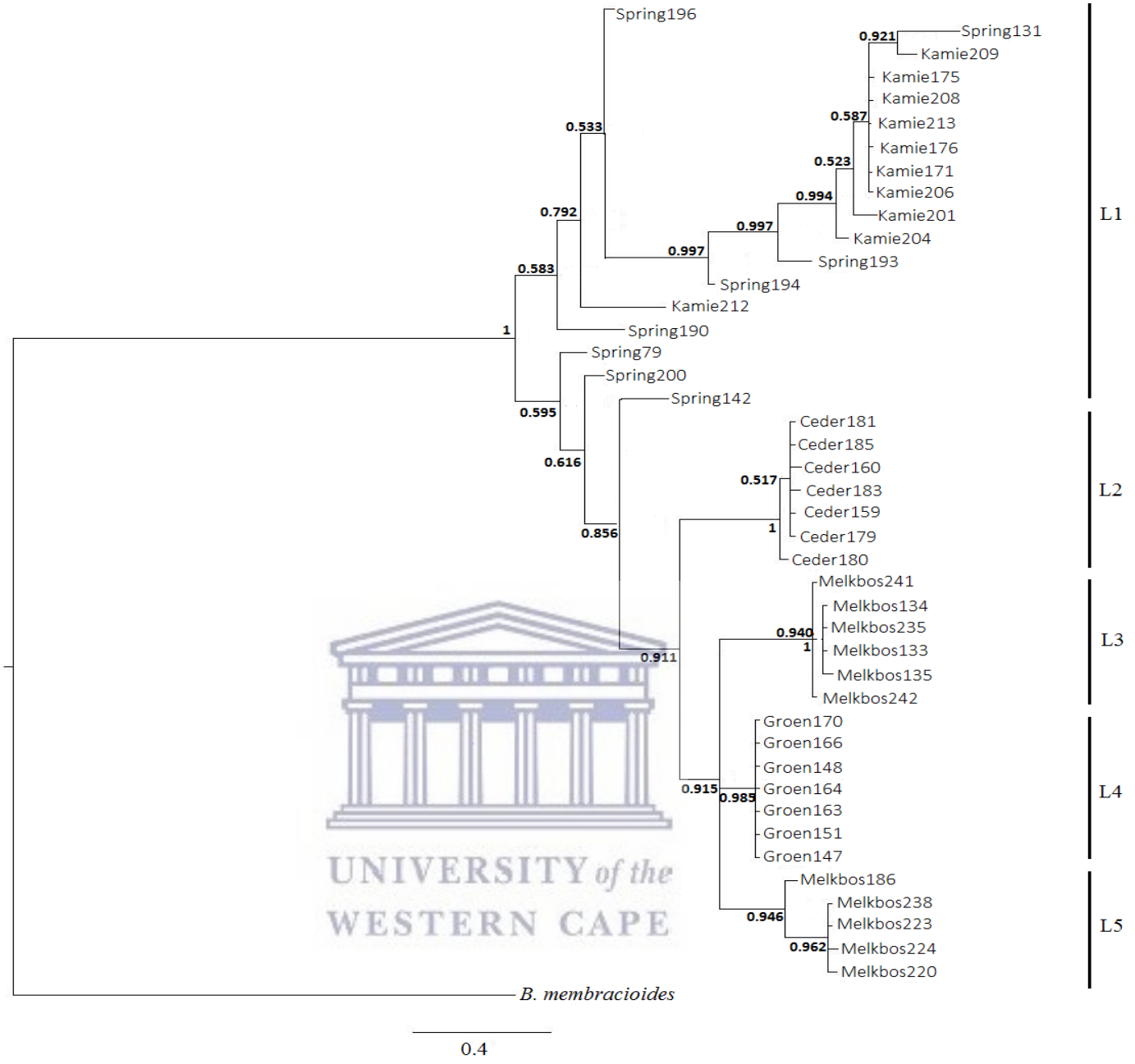


Fig 1.3 Linearized phylogenetic tree of *Bullacris unicolor* using CO1 genes (N = 43) based on 29 haplotypes, and rooted by *Bullacris membracioides*. All posterior probabilities of statistically well supported lineages that are greater and less than 0.95% are shown along the nodes. The five major lineages L1 to L5 that were retrieved are indicated by the vertical lines.

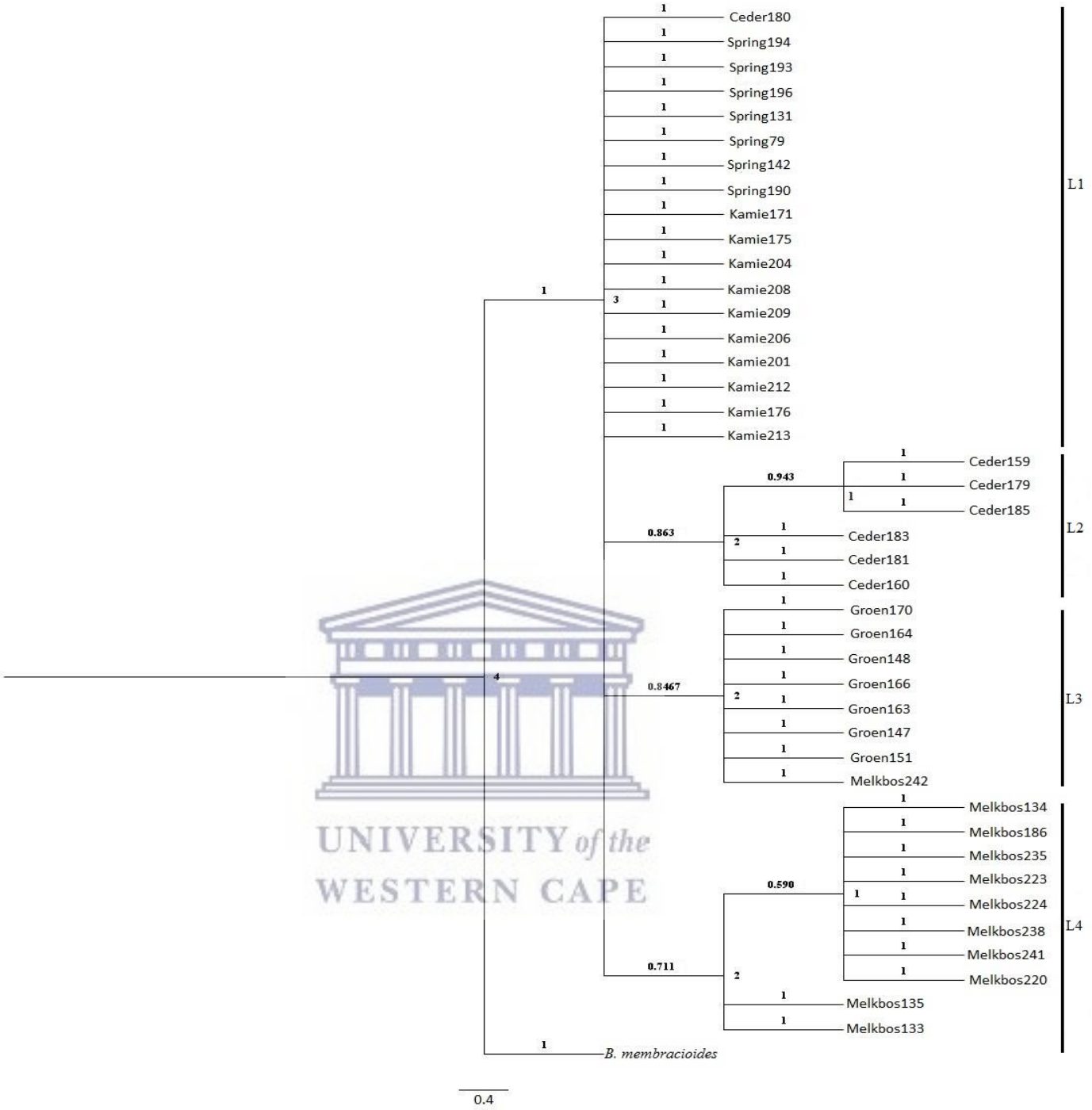


Fig 1.4 Linearized phylogenetic tree of *Bullacris unicolor* using ITS genes (N = 42) based on 9 haplotypes, and rooted by *Bullacris membracioides*. Posterior probabilities of statistically well supported lineages are shown along the nodes. The four major lineages L1 to L4 that were retrieved are indicated by the vertical lines.

Table 1.5 Population pairwise genetic differentiation (mean ± SD) within (bold) and between sampled populations of *B. unicolor*.

mtDNA	Springbok	Kamieskroon	Groenriversmond	Cederberg	Melkbosstrand
Springbok	**4.10 ± 0.02**				
Kamieskroon	4.95 ± 0.02	**1.22 ± 0.02**			
Groenriversmond	4.65 ± 0.01	6.80 ± 0.01	**0.09 ± 0.00**		
Cederberg	5.23 ± 0.02	7.15 ± 0.01	3.75 ± 0.01	**1.99 ± 0.04**	
Melkbosstrand	5.66 ± 0.01	7.96 ± 0.01	2.97 ± 0.02	5.07 ± 0.01	**2.37 ± 0.02**
nDNA					
Springbok	**0.00 ± 0.00**				
Kamieskroon	0.00 ± 0.00	**0.00 ± 0.00**			
Groenriversmond	0.13 ± 6.57	0.13 ± 1.31	**0.00 ± 0.00**		
Cederberg	0.24 ± 0.00	0.24 ± 0.00	0.37 ± 0.00	**0.26 ± 0.00**	
Melkbosstrand	0.56 ± 0.00	0.56 ± 0.00	0.67 ± 0.00	0.26 ± 0.02	**0.17 ± 0.00**

Table 1.6 Estimates of genetic differentiation (F_{ST}) derived from both mtDNA and nDNA.

	Melkbosstrand	Cederberg	Springbok	Kamieskroon
mtDNA				
Cederberg	0.7238			
Springbok	0.4747	0.6177		
Kamieskroon	0.7762	0.9021	0.4805	
Groenriversmond	0.5855	0.9650	0.6239	0.9047
nDNA				
Cederberg	0.7419			
Springbok	0.8415	0.5029		
Kamieskroon	0.8415	0.5029	0.0000	
Groenriversmond	0.8661	0.6769	1.0000	1.0000

Table 1.7 Haplotype diversity and nucleotide diversity of five populations of *Bullacris unicolor*.

Population	N	Haplotype diversity	Nucleotide diversity
mtDNA			
Cederberg	8	0.785	0.019
Groenriversmond	7	0.285	0.000
Kamieskroon	10	0.866	0.012
Melkbosstrand	10	0.733	0.024
Springbok	8	1.000	0.040
nDNA			
Cederberg	7	0.791	0.002
Groenriversmond	7	0.000	0.000
Kamieskroon	10	0.000	0.000
Melkbosstrand	11	0.558	0.001
Springbok	7	0.000	0.000

The relationship of acoustic and morphological divergence with environmental factors and genetics

Results of the Mantel tests are shown in Table 1.8. A significant correlation was found between climatic and genetic distance. Female and male morphology were significantly correlated with genetic distance (nDNA and mtDNA). Both mtDNA and nDNA are correlated with climatic distance across all populations. There was no significant correlation between acoustic or morphological distance and geographical distance. Male and female morphological characters were correlated with climatic variables in a similar way among populations.

Looking more specifically at individual variables, Pearson correlation showed a negative significant correlation between head and abdomen width of females and body length of males with annual temperature (Table 1.9). Also, the analysis showed significant negative correlation with annual temperature in both male and female morphology. In males and females, smaller size was predominantly correlated with higher temperature. We found that temperature partially explained morphological variation in both males and females of *B. unicolor*, but this was not the case for latitude. Overall, male and female body parts were most strongly influenced by temperature.

Table 1.8 Mantel correlations between climate, geography and genetic distance (mtDNA and nDNA) and acoustic and morphological characters (males and females) of *Bullacris unicolor* across populations. The significant p-values are shown in bold.

Variables		R	p
Mantel tests			
Climate	Female morphology	-0.404	**0.044**
	Male morphology	-0.235	**0.031**
	Acoustic	-0.077	0.599
	mtDNA	0.342	**0.030**
	nDNA	0.696	**0.002**
Geography	Female morphology	-0.142	0.727
	Male morphology	0.092	0.374
	Acoustic	-0.011	0.480
	mtDNA	-0.110	0.683
	nDNA	0.071	0.362
mtDNA	Female morphology	0.728	**0.032**
	Male morphology	0.938	**0.025**
	Acoustic	0.208	0.324
nDNA	Female morphology	0.677	**0.006**
	Male morphology	0.224	**0.040**
	Acoustic	-0.196	0.759

Table 1.9 The relationship coefficients between environmental factors and acoustic, morphological (males and females) and genetics (mtDNA and nDNA). Abbreviations are: AR-annual rainfall, AT- annual temperature, Bio 2- mean of monthly temperature max-min, Bio4- temperature seasonality and Bio15- precipitation seasonality.

	Environmental factors					
	AR	AT	Bio 2	Bio 4	Bio 15	Latitude
Acoustic						
Length of entire call	–0.762	0.144	0.185	–0.424	0.738	–0.616
Length of final syllable	–0.145	–0.365	–0.570	–0.737	0.191	–0.634
Male morphology						
Head width	0.462	–0.572	–0.717	–0.363	–0.633	–0.423
Body length	0.142	–0.834*	–0.634	–0.640	–0.288	–0.628
Female morphology						
Head width	0.419	–0.913*	–0.578	–0.156	–0.800	–0.343
Abdomen width	0.133	–0.905*	–0.238	0.101	–0.784	–0.45
Genetic diversity						
mtDNA	0.319*	0.524*	0.399*	0.345*	0.345*	–0.24
nDNA	0.738*	0.167	0.772*	0.379*	0.154	0.521

Note *indicating significant relationship

The relationship between environmental factors and genetics

Mantel tests revealed both mtDNA and nDNA are significantly positively correlated with the climatic variables (Table 1.8). The univariate correlations also showed significant positive relationships with most of the climatic variables and genetic characters of both mtDNA and nDNA genes (Table 1.9).

Discussion

In a previous study, we found that acoustic and morphological characters of *Bullacris unicolor* varied significantly across populations (R Sathyan unpublished master's thesis). The results of the genetic analysis conducted here confirm that these populations are also genetically differentiated. Studies have indicated that climatic factors are one of the primary sources that promote the evolution of acoustic and morphological characters in different taxa, such as birds (Maluleke *et al*. 2017), bats (Xie *et al*. 2017), and frogs (Yasumiba *et al*. 2016). Here, morphology was not correlated with latitude, suggesting that variation in characters was not the result of associated variation in body size as proposed by Bergmann's rule. The results provided evidence that genetic drift was responsible for the variation of morphological characters, as both male and female morphology were associated with genetic distance. Although, genetic drift is not a key driving factor. The association between climatic and genetic distance indicated that there is support for genetic divergence based on climatic variables. This suggests that genetic differences have exerted an influence on regional differences in morphology.

Variation in temperature and humidity in different locations will lead to intra-specific variation in acoustic features of insects (Walker 1962; Gerhard & Huber 2002). However, in this study, a non-significant correlation was found between climatic and acoustic variables, indicating that climate was not responsible for the divergence of acoustic characters. However, it is known that atmospheric attenuation in signals is the result of complex interactions between temperature, humidity, and the frequency of the sound being propagated (Luo *et al*. 2014). For example, the calls of *B. unicolor* differ in their signal attenuation and fidelity when broadcast at different times of the night (Couldridge & Gordon 2015), although

there was no clear pattern of association between prevailing climatic variables and calling time. This suggests that regional differences in calls are not simply due to climatic differences.

Ecological factors may also help to drive acoustic divergence, for example, sound transmission is more difficult with high background noise and growth of vegetation (Barbour & Billings 1988). Birds sing for longer and at lower frequencies in order to compensate for background noise generated by rainfall and higher density vegetation (Ruegg *et al*. 2006; Nemeth *et al*. 2013; Lampe *et al*. 2014). I suggest adaptation to local habitat may influence geographic variation in call characters in *B. unicolor*. Future analyses combining ecological data with background noise (abiotic and biotic) and vegetation may provide insight into the relative significance of the direct and indirect effects of ecological factors on spectral and temporal components of *B. unicolor* calls (see also chapter 2).

Our data did not support the relationship between genetic drift and acoustic divergence. Here, the population differences in acoustic characters were not correlated with either mtDNA or nDNA based genetic distance. Moreover, both genetic markers showed a significant differentiation among populations. The mtDNA genetic data points to the fragmented nature of the Melkbosstrand population, with individuals forming two distinct clusters. These two genetic clusters within the Melkbosstrand population cannot be attributed to geographic distances between individual samples, as the geographic distances separating sampled individuals is greater within the L3 cluster than the average distance between individuals from L3 and L5. It is more likely that this clustering arrangement has resulted from isolation

due to habitat fragmentation. However, these individuals did not show any significant morphological or acoustic differentiation, and so were still treated as a single population.

Morphological variation is only partly influenced by variation in climate, although climate (apart from temperature) was not a strong correlate of morphology (But see also Donelson 2007). Landscape features may be a barrier to gene flow and were congruent with observed patterns of morphology. Morphology of individuals from Springbok and Kamieskroon showed some overlap. Genetic analysis (mtDNA and nDNA) also showed overlap of individuals between these two locations. In general, individuals occupying nearby populations show less variation, indicating similar climatic niches and similar phenotypes, and also showing a close association between ecological attributes and their morphology (Zurano *et al*. 2017).

The genetic structure of *B. unicolor* was high along its distribution range. Five distinct mitochondrial phylogenetic clades could be identified, and these coincided with collecting locality, indicating that our sampled populations have undergone genetic differentiation. Genetic data further indicated that Springbok has a higher number of haplotypes, polymorphic sites, and haplotype and nucleotide diversity in comparison with the other sampled populations. The significant positive values of both Tajima's D and Fu's Fs from Melkbosstrand are indicative of a possible recent population bottleneck or decreasing population. This may be a consequence of the loss and fragmentation of habitat due to urban expansion in this area. Conversely, significant negative values in the Cederberg population suggest recent expansion in this population. Tajima's values for all other populations were non-significant, indicating that there have been no recent demographic changes (Table 1.4).

However, lower nucleotide diversity within Groenriversmond is suggestive of a possible recent population expansion in this area as well.

The isolation by environment patterns we obtained from Pearson correlation analysis indicated that annual temperature is significantly negatively correlated with female and male morphology. However, annual rainfall showed a non-significant positive relationship between these three morphological characters. These discrepancies in the influence of climatic factors on morphology are the result of complex interactions between annual rainfall and temperature. Runemark *et al*. (2017) suggested temperature seasonality best explains population divergence in beak size of the Italian sparrow. Similarly, our findings also suggest that abdomen width differences in *B. unicolor* females result from a plastic response to local temperature variation. The significant positive correlation of genetic and morphological characters indicates that some morphological differences are independent of climatic factors.

Genetic differentiation in local populations is mainly influenced by natural selection, resulting in adaptation to local environmental conditions (Slatkin 1987). A review by Sexton *et al*. (2014) indicated the strongest patterns of genetic divergence by Isolation by Environment (IBE) are in invertebrates. They showed a high percentage of studies found evidence for IBE. The average F_{ST} was higher in studies that focussed on IBE only than in the studies which found IBD. We found that the F_{ST} value of mtDNA for Cederberg and Groenriversmond was higher than other populations. For nDNA, Springbok and Kamieskroon showed higher values compared to other populations. The reasons for a difference between Groenriversmond and three other populations might be the effects of distance and variation in climatic factors. The annual rainfall of 154 mm in Groenriversmond

compared to the annual rainfall of Cederberg, Springbok and Kamieskroon of 327, 182 and 221 mm respectively, suggests climatic factors affect different organisms in different regions. At the same time, the F_{ST} value of Kamieskroon and Cederberg was also high despite low environmental variation and distance. This pattern of divergence of organisms with similar spatial scales is unclear. The causes of disparity among organisms on smaller geographic scales need to be investigated to complete this scenario.

Here we sampled individuals from a relatively wide distribution in South Africa, which stretches from south to north over a distance of 560km. Non-significant relationships were observed between latitude and temperature ($r = 0.59$, $P = 0.21$) and latitude and rainfall ($r = 0.36$, $P = 0.47$). So the effects of latitude on genetic variation are not an equivalence relation to the effects of rainfall and temperature, and the effects of genetic diversity may be considered as the effects of IBE rather than IBD. Therefore, temperature and rainfall are the determinant factors influencing the genetic structure of *B. unicolor*. Isolation by environment may be a result of strong natural selection on allozymes and influence the structure of genes. Studies are needed that can reveal the contributions of natural selection and environmental structuring to patterns of gene flow (Leal *et al*. 2017). Local environmental conditions may influence the pattern of natural selection and support different gene flow scenarios and finally lead to higher genetic diversity.

We have investigated the importance of environmental factors and genetic drift acting on morphological and acoustic characters of *B. unicolor* by relating divergence of neutral markers and climatic factors with population divergence of characters. We found evidence for genetic drift and the role of climatic factors. These conclusions were further supported by

our morphological analysis where we found that a climatic variable (temperature) affected morphological characters in this species. The approach using environmental factors and genetic drift suggests that our interpretation about adaptive causes behind population divergence in this species is strong. The results support the role of environmental factors and genetic drift affecting population divergence. However, our results do not reject a role for frequency-dependent selection. Instead, morphology is partially influenced by divergent selection caused by local environmental differences, and genetic drift, although the relative balance between these different forces is likely to differ between populations.

In summary, our study supports climate-induced geographic variation in genetic and morphological characters, suggesting that lineage diversification in general may be driven by climate mediated differences accompanied by genetic differentiation. Change in climate can modify the environmental stimuli, activating polyphenisms, and some epigenetic marks, thus modifying separate phenotype proportions within populations. Our study also proposes that climate variation in conjunction with other ecological discontinuities leads to phenotypic plasticity and local adaptation allow lineages to diverge. In general, we suggest that the interaction between local ecology and female preference for male traits needs to be considered to further understand population divergence in this species of bladder grasshopper.

CHAPTER 2

THE EFFECT OF ANTHROPOGENIC NOISE AND WEATHER CONDITIONS ON MALE CALLS OF *BULLACRIS UNICOLOR*

Abstract

Acoustic communication in animals mainly relies upon specific contexts and environments for effective signal transmission. Increasing anthropogenic noise pollution and different weather conditions can disrupt acoustic communication in animals. In this study, we investigated call parameter differences between noisy and quiet environments in the bladder grasshopper *Bullacris unicolor*. We installed passive acoustic monitoring devices to record sound for three weeks in two different nature reserves in close proximity to each other, the Cape Flats Nature Reserve (CFNR) (high anthropogenic noise) and Tygerberg Nature Reserve (TNR) (low anthropogenic noise) in the Western Cape, South Africa. Weather conditions, including wind speed, temperature and humidity, were also recorded. We quantified the emission of calls of *B. unicolor* through visual inspection of the recordings. The CFNR presented significantly higher anthropogenic noise levels than the TNR. Surprisingly, the quantitative comparison of calls of *B. unicolor* between the two locations showed more calling activity in the noisy location than in the location with less anthropogenic noise. Call interval was positively and call rate was negatively correlated with anthropogenic noise in CFNR. Peak frequency and call rate were affected by weather conditions; peak frequency increased and call rate decreased with increased wind speed. On the other hand, temperature was positively correlated with both of these call parameters. Our results indicate that both anthropogenic noise and weather conditions influence signalling in *B. unicolor* and may thus constrain long-distance acoustic communication in this species.

Given that anthropogenic noise and weather conditions may be triggering grasshoppers to adjust their call parameters to avoid masking of their signals; our results highlight both the complexity of call evolution and the need to consider multiple causes when exploring this issue.

Introduction

Increases in anthropogenic noise as a result of urban development and transportation networks affect acoustic communication in animal species in their natural habitats (Slabbekoorn & Peet 2003; Ey and Fischer 2009; Halfwerk *et al*. 2011; Parks *et al*. 2011; Rosa & Koper 2018). Anthropogenic noises are very different compared to the sounds emitted from biotic and abiotic sources (Hildebrand 2009), and have the potential to degrade and mask acoustic signals, and thus, affect intraspecific acoustic communication of many different species (Barber *et al*. 2009; Halfwerk *et al*. 2011). Rapidly burgeoning research has identified the impacts of anthropogenic noise on biological community disruption. For example, grasshoppers from roadside habitats produce significantly higher frequency songs compared to conspecifics from quiet habitats (Lampe *et al*. 2012). Furthermore, conspecific frogs from noisy and quiet habitats were found to call at different pitches (Parris *et al*. 2009). Birds inhabiting urban areas also shift their songs to higher frequencies compared to conspecifics from rural habitats (Dowling *et al*. 2012). To date, however, studies have mostly focused on the effect of noise on amphibians and birds (Parris *et al*. 2009; Cunnington & Fahrig 2010). While anthropogenic noise has been shown to have a significant effect on invertebrates (Morley *et al*. 2014), surprisingly, and despite their central role in food webs and fulfilling ecosystem services, little research has been conducted on the effects of anthropogenic noise on invertebrates.

Insects produce sounds or vibrations for a variety of reasons: for example, aggression, mate location, attraction and courtship, predator avoidance, detection of parasite host species and social communication (Owings & Morton 1998; Morley *et al*. 2014). Increased urbanization gives rise to noisy environments that can have detrimental effects on insect communication

through acoustic masking of signals. For instance, female response rate to male courtship songs in *Drosophila montana* has decreased in the presence of background noise (Sammara *et al*. 2009). Einhaupl *et al*. (2011) found that females were more attracted by the courtship songs of male grasshoppers, *Chorthippus biguttulus*, which were more robust against broad band white noise. This process of masking interference of acoustic signals could negatively affect insect populations by reducing reproductive success, decreasing the chances of mating, or increasing predation risk (Kaiser & Hammers 2009; Vargas-Salinas & Amézquita 2013). Hence, the effectiveness of insect communication and ultimately breeding success depends on the recognition of signals against background noise. Thus, interference of road traffic noise has developed as a field of interest and concern in many taxa, including insects (Morley *et al*. 2014).

Animals that communicate acoustically may counteract masking interference by means of evolutionary adaptations or behavioural adjustments (Brumm & Slabbekoom 2005). Populations may change communication traits in the long term, and senders may modify call intensity, call rate, call duration and call frequency in the short term (see Vargas-Salinas & Amézquita 2013). Anthropogenic noise can inhibit calling in insects leading to a significant increase of acoustic features, i.e. insects shift the energy distribution of calling songs to higher frequencies in the presence of higher noise levels (Shieh *et al*. 2012). Studies on anurans have demonstrated that frogs tend to call at times when ambient noise is lower, and stop calling when it increases, whereas some species call more frequently under noisy conditions (Sun & Narins 2005). Furthermore, frogs exposed to traffic noise alter the pitch of their calls, the length of calling periods or the timing of their signals (Parris *et al*. 2009; Kaiser *et al*. 2011). Overall, the evidence suggests that different strategies are being used to

avoid masking of acoustic communication signals across different vertebrate and invertebrate taxa.

Both natural abiotic and biotic sound sources, such as wind, rain and the choruses of other animals, can also make the environment noisy. Invertebrates evolve different mechanisms to cope with this noise to survive and reproduce. For example, the cricket *Paroecanthus podagrosus* modifies its auditory tuning to conspecific songs in noisy rainforests with high levels of acoustic competition (Schmidt *et al*. 2011). Similarly, the Australian bush cricket (*Sciarasaga quadrata*) tunes in to lower frequencies of singing conspecific males to filter out noise generated by heterospecifics (Römer & Bailey 1998). In addition, environmental variables, which include temperature, wind speed and moisture, provide meaningful indicators for habitat selection and other activities (Chen *et al*. 1999). Moreover, multiple studies have demonstrated the influence of the environment on population dynamics and patterns of acoustic signal differences in amphibians (Combes *et al*. 2018; Goutte *et al*. 2018; Velásquez *et al*. 2018). However, the various mechanisms through which climate effects invertebrate population dynamics are unknown and remain to be measured.

In bladder grasshoppers, acoustic communication plays a major role in mate location (van Staaden & Römer 1997, 1998, van Staaden *et al*. 2003, Couldridge & van Staaden 2004, 2006). To attract females, males produce a loud advertisement call by rubbing a line of ridges on their hind-femur against a crescent-shaped ridge on the side of the inflated abdominal bladder, functioning as an acoustic resonator. A female responds to male calls with short, low frequency acoustic signals and this response depends upon the attractiveness of call characteristics (Couldridge & van Staaden 2006). Males orient and fly to receptive females to

mate with them. The advertisement calls of males possess species specificity, with variation in both temporal and structural properties both between and within species (Couldridge & van Staaden 2004; Sathyan *et al*. 2017). A previous study found some association between habitat and signal characteristics in bladder grasshoppers (Couldridge & van Staaden 2004). Hence, the effect of weather conditions and noise on bladder grasshoppers that rely on acoustic communication need to be better studied to understand their impact on acoustic behaviour and ecology.

Few studies have examined the effects of traffic noise on the call composition and behaviour of invertebrates (Reviewed by Morley *et al*. 2014). Most of the studies concluded modification of call frequency in invertebrates is a mechanism for avoiding masking. This study describes the call characteristics of *Bullacris unicolor* at two nearby sites that differ in their levels of road noise, and evaluates the effects of road noise on the temporal and spectral characteristics of the calls of this species. In addition, abiotic factors, such as wind speed, temperature, phase of the moon, etc. may also impact population distribution, species abundance and diversity (Drent & Daan 1980) and these climatic variables were thus also considered in the study. This is the first African study documenting the effects of anthropogenic noise and weather conditions on insect call parameters. Successful signal transmission is essential for males to convey information and for mate choice, therefore we expect male grasshoppers from high background noise levels to produce greater low-frequency signals compared to grasshoppers from quieter conditions, as high-frequency signals are expected to suffer greater acoustic interference from traffic noise (Parris & Schneider 2008; Lampe *et al*. 2012). We investigated the potential adaptations of *B. unicolor* to changing environments by measuring changes in acoustic features in response to anthropogenic noise and weather conditions in the field.

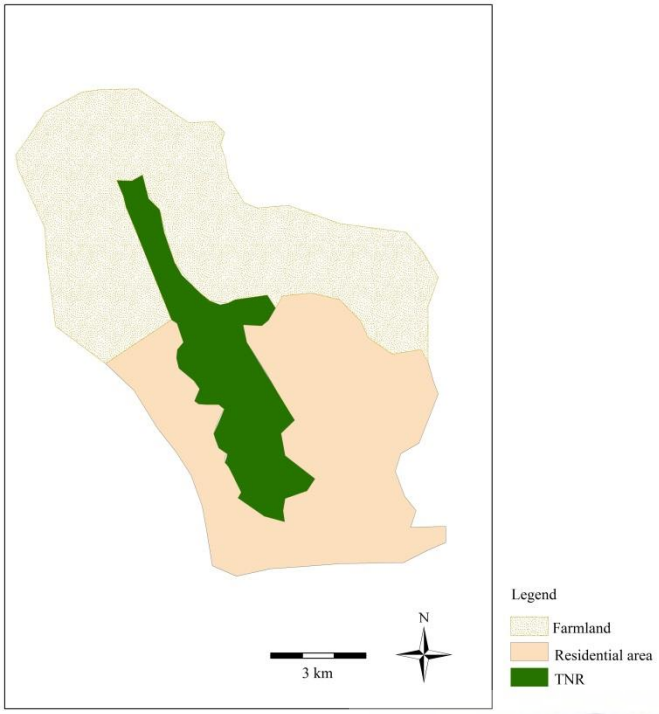
Materials and methods

Study area

Two nature reserves that differ in their levels of background noise were selected as study sites. These sites were chosen due to their proximity to each other and the confirmed presence of the study species at both locations. The two reserves were the 30 ha Cape Flats Nature Reserve (CFNR) (33.9333°S, 18.6277°E), situated adjacent to a major road and a railway line, and the 388 ha Tygerberg Nature Reserve (TNR) (33.8775° S, 18.6041° E), a reference site with less noise. Both nature reserves are located in Bellville, in the northern suburbs of Cape Town. This study was conducted during October and November 2016, at the beginning of summer, coinciding with the peak calling season of *B. unicolor* in this area.

The predominant vegetation in the CFNR is the endangered Cape Flats Dune Strandveld, and in Tygerberg Nature Reserve is the Swartland Shale Renosterveld; both are endemic to the Western Cape. Various species of birds, reptiles, frogs, insects and mammals are found in both reserves. Another bladder grasshopper species, *Physemacris variolosus*, is also present in both nature reserves. Despite their close proximity, the climate differs somewhat between the reserves, as TNR stands on a series of hills (elevation 237 m) with 360 degree views over Cape Town. Most of the TNR is covered by natural vegetation, and it is surrounded by agricultural fields and some residential properties, while a large part of CFNR (elevation 80 m) is surrounded by industrial areas, as well as a university campus and residential houses. Commuter trains and road traffic also produce noise pollution during the day and night in this location (Fig. 2.1).

(A)



(B)

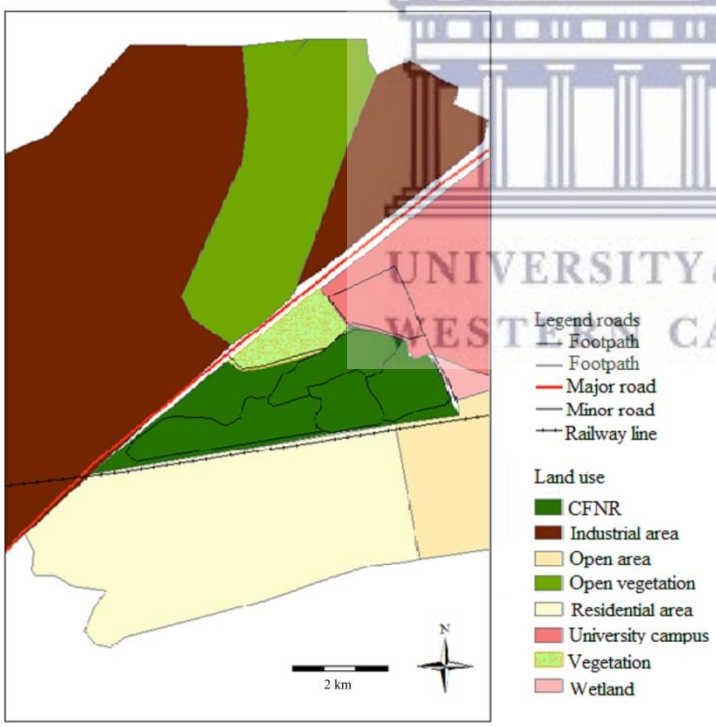


Figure 2.1 Land cover map of (A) TNR (Tygerberg Nature Reserve) and (B) CFNR (Cape Flats Nature Reserve).

Data collection and analysis

Passive acoustic recorders (SM3 Wildlife Acoustics), equipped with multidirectional acoustic microphones (SM3-A1) were installed at the two reserves. The recorders were programmed to record from sunset to sunrise, over three consecutive weeks at both sites, at a 16 kHz sampling rate (90 dB signal to noise ratio). Each recorder was placed approximately 2m above the ground, leaving the two lateral microphones free from any interference. Recorders were moved to new positions (20m apart) each week to monitor different male calls and to prevent double sampling. Humidity, temperature and wind speed were continuously logged every 5 minutes by a weather meter (Kestrel 4000).

The level of anthropogenic noise was calculated based on 30 min sampling intervals between 19:00 and 05:00 hours. To quantify the disturbance from the road, we counted all instances of noise generated by passing vehicles for each 30 min period. Counts were done by visual and audio identification of broadband patterns in spectrograms generated in Raven Pro 1.5 (Cornell Lab of Ornithology, Ithaca, NY).

The advertisement call of *B. unicolor* is a short and high intensity call produced by males (Fig. 2.2). We used four call parameters to characterize and compare *B. unicolor* calling behaviour between locations: call duration (length of call from beginning to end of the call, s) peak frequency (frequency at which the call is of greatest intensity, kHz), inter-call interval (duration between two calls from the same individual, s) and call rate (number of calls per 10 minute period). Call rates of grasshoppers were calculated based on manual, individual call detections per 30 min intervals. Call duration, call interval and peak frequency were extracted

by the measurement of individual calls. Sound spectrograms and power spectrums were generated using Raven Pro (version 1.5).

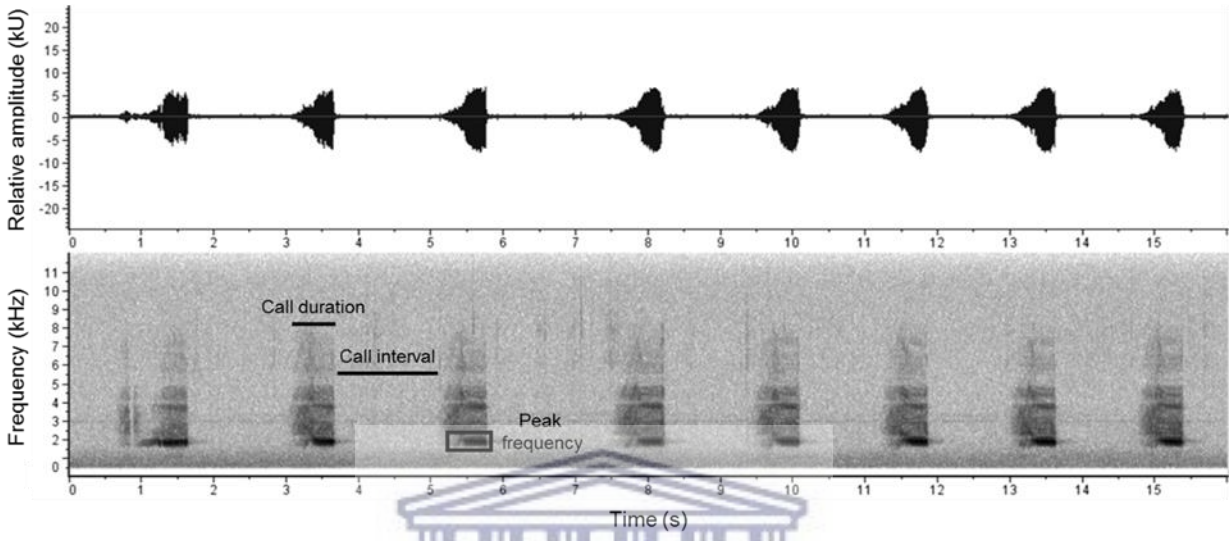


Figure 2.2 Example of advertisement calls of *Bullacris unicolor* recorded in the field with their respective oscillogram (above) and spectrogram (below).

Statistical analysis

We used ANOVAs to determine whether anthropogenic noise, weather conditions and call parameters differed significantly between the two sites (CFNR and TNR). We calculated anthropogenic noise, weather conditions and call parameters as dependent variables to test whether these differed between the two locations. Time and date were included as a fixed covariate and factor respectively. We used 2-tailed Pearson correlation analyses to detect the magnitude of a potential effect of anthropogenic noise and weather conditions on call parameters. All statistical analyses were performed using SPSS 25.0.

Results

Difference in anthropogenic noise, call parameters and weather conditions

Anthropogenic noise level in the CFNR was significantly greater than in the TNR (Fig. 2.3, Table 2.1). Out of the four measured acoustic variables, peak frequency, call interval and call rate were significantly different between the two nature reserves (Fig. 2.4, Table 2.1). Wind speed, temperature and humidity were also significantly different between the two study areas (Table 2.1).

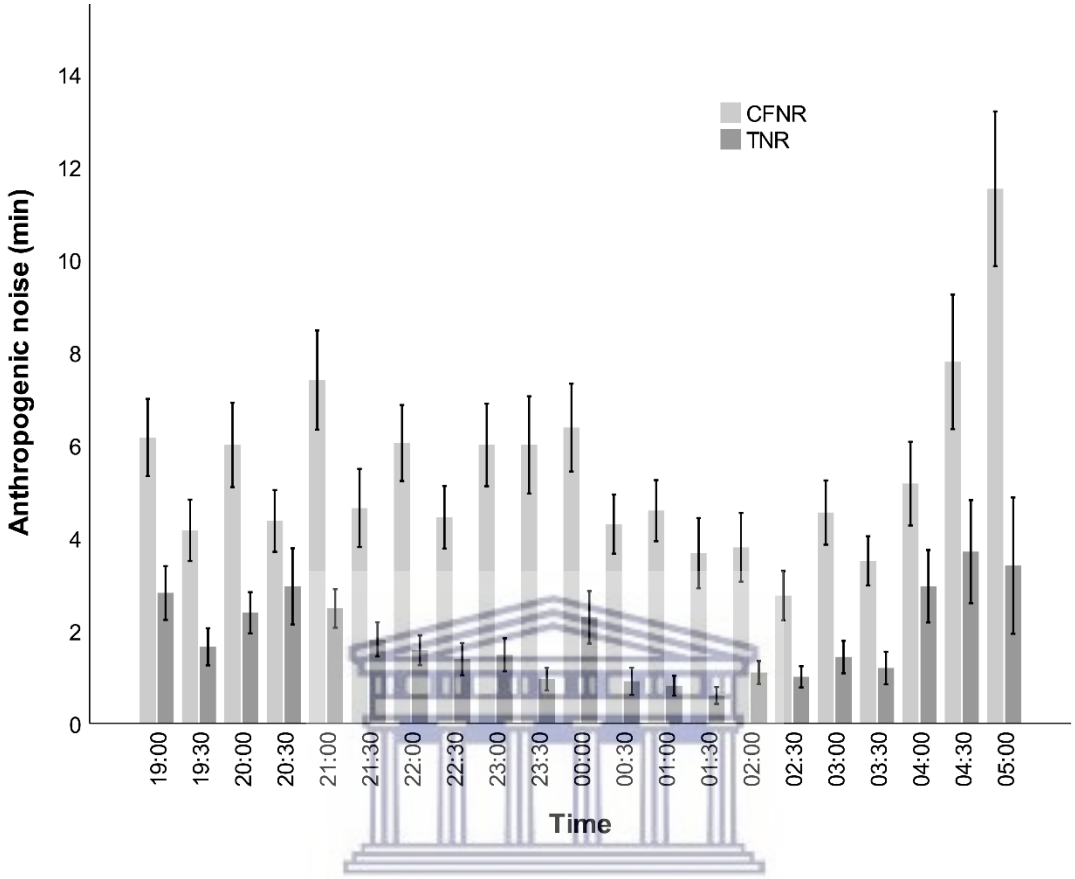


Figure 2.3 Anthropogenic noise levels in the CFNR and TNR measured over a period of three weeks in October and November 2016. Data is presented as averages for each 30 minute interval during the night (mean ± SD).

Table 2.1 Summary of ANOVA results for differences between call parameters of *Bullacris unicolor*, weather conditions and anthropogenic noise between CFNR and TNR.

	CFNR Mean ± SD	TNR Mean ± SD	*N*	Mean	SD	SE	Mean square	*F*	df	*p*
Call parameters										
Call duration (sec)	0.8 ± 0.2	0.8 ± 0.2	89	0.82	0.27	0.03	0.01	0.19	87	0.66
Call interval (sec)	11.4 ± 7.4	7.3 ± 5.7	89	8.58	6.67	0.71	132.02	3.03	87	0.04*
Peak frequency (kHz)	1656.2 ± 184.3	1749.2 ± 92.5	89	1702.24	152.79	16.20	192496.22	8.99	87	0.00*
Call rate (calls/min)	9.4 ± 29.7	6.6 ± 26.2	976	9.33	28.36	0.91	5705.70	7.14	1	0.00*
Weather										
Temperature (°C)	13.0 ± 1.9	14.8 ± 5.6	690.00	13.73	3.21	0.12	2000.06	270.75	1	0.00*
Wind speed (m/sec)	0.3 ± 0.6	0.9 ± 0.7	690.00	0.87	0.84	0.03	76.32	126.39	1	0.00*
Humidity (%)	95.1 ± 8.8	66.0 ± 21.4	691.00	86.03	14.18	0.54	13973.16	77.20	1	0.00*
Anthropogenic noise (min)	6.9 ± 3.6	2.4 ± 2.0	975.00	4.42	5.55	0.18	5155.16	201.88	1	0.00*

* $p < 0.05$

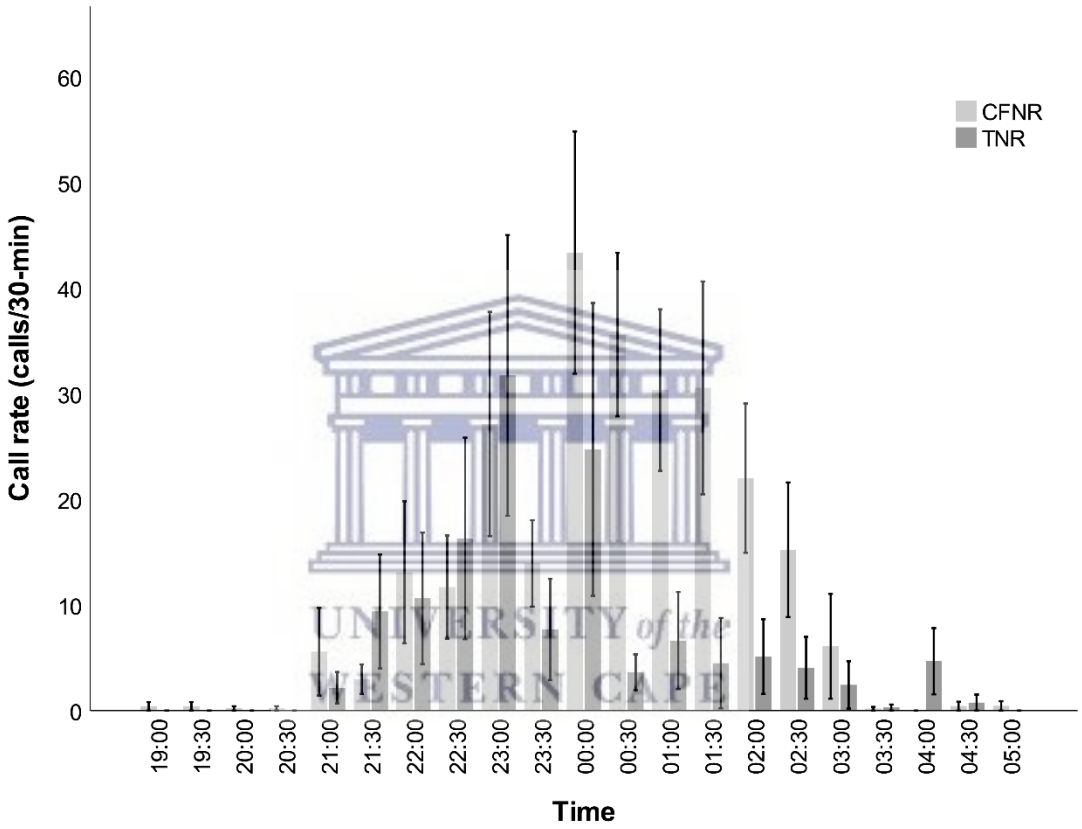


Figure 2.4 Call rates of *B. unicolor* measured over 30 minute intervals. Values are means ± SD.

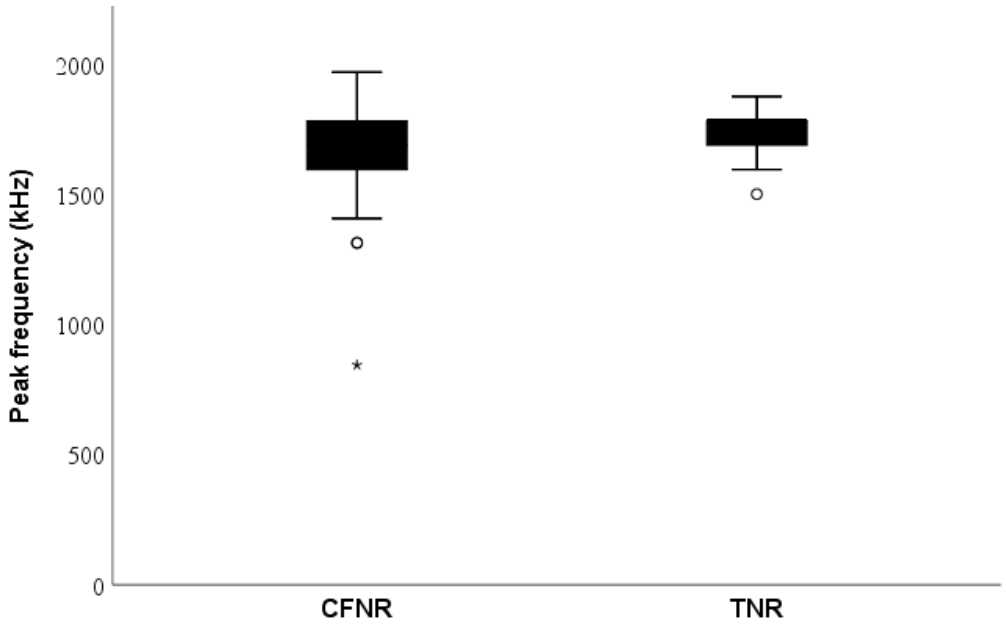


Figure 2.5 Peak frequency of *B. unicolor* calls from the CFNR and TNR.

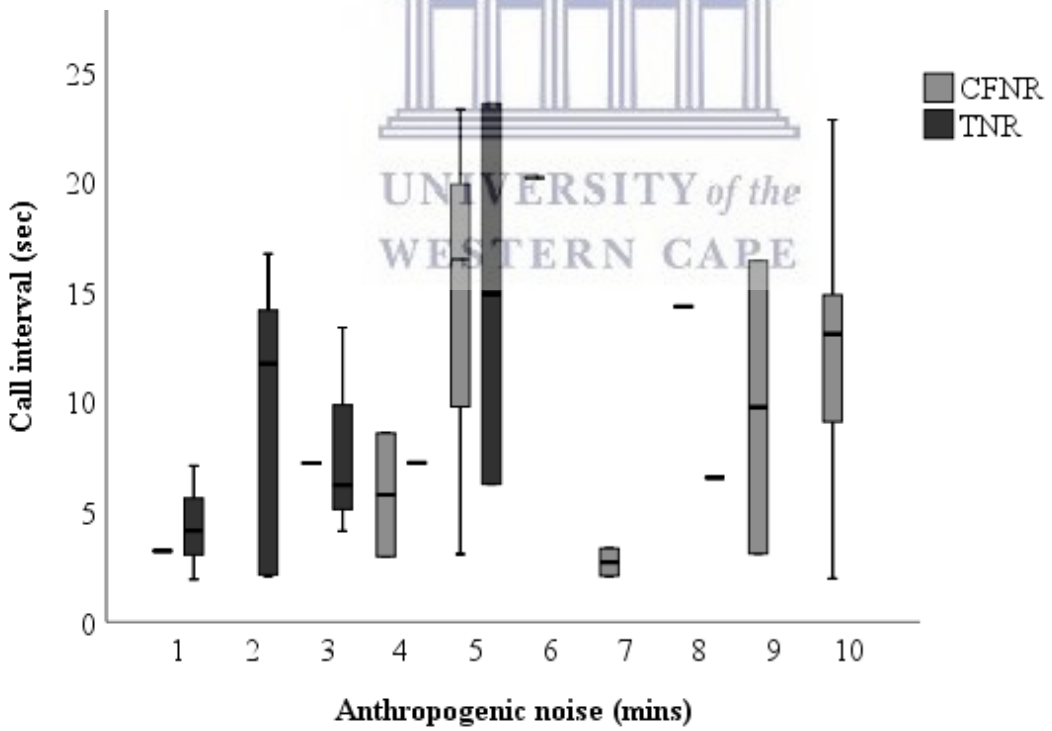


Figure 2.6 Relationship between call interval and anthropogenic noise from CFNR and TNR.

Effects of anthropogenic noise and weather conditions on call parameters

The calling activity of *Bullacris unicolor* started between 21h00–21h30 and ended between 04h00–04h30 in both study areas (Fig. 2.4). The call rate, defined as the number of calls per time period, was significantly correlated with the anthropogenic noise level at the CFNR, but not at the TNR (Table 2.2). However, males from the CFNR, with high levels of anthropogenic noise, called significantly more (higher call rate) than males from the TNR. Call duration did not differ significantly between the two study areas, and was not correlated with any of the measured variables. There was a positive correlation between anthropogenic noise and call interval (interval between two calls) at the CFNR (Fig. 2.6). CFNR males have a higher call interval than males in the TNR, this difference was marginally non-significant (Tables 2.1). Furthermore, males from the TNR, with low anthropogenic noise, called at significantly higher peak frequencies (Fig. 2.5). There was no interaction between anthropogenic noise and the peak frequency of calls.

There were significant relationships between acoustic variables and weather conditions. Peak frequency increased with wind speed and temperature, and decreased with humidity. The number of calls (call rate) decreased with wind speed and humidity, and increased with temperature (Table 2.2). Call interval was negatively correlated with wind speed at the CFNR, but otherwise did not vary significantly with weather conditions.

Table 2.2 The relationship between anthropogenic noise and weather conditions and the call parameters of *B. unicolor* at two locations.

	Call parameters	Anthropogenic noise (mins/30 mins interval)	Wind speed (m/sec)	Temperature (°C)	Humidity (%)
CFNR	Call duration (s)	0.01	-0.25	-0.24	-0.25
	Call interval (s)	0.34**	-0.33*	0.00	-0.27
	Peak frequency (kHz)	-0.33	0.07	0.43**	-0.03
	Call rate (calls/min)	-0.11**	-0.07	0.15**	-0.11*
TNR	Call duration (s)	0.06	0.22	0.10	-0.22
	Call interval (s)	0.31	0.19	-0.07	0.09
	Peak frequency (kHz)	-0.27	0.46**	0.17	-0.35*
	Call rate (calls/min)	0.02	-0.12*	0.22**	-0.25**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Discussion

Our results showed acoustic adaptations of *B. unicolor* by (1) increasing call interval and decreasing call rate in response to increasing levels of anthropogenic noise, and (2) shifting to a higher frequency or reducing the call rate to avoid the potential negative impacts of adverse climate conditions. To deal with anthropogenic noise, many animal taxa use higher frequencies to avoid signal degradation and masking. Studies on mammals (Duarte *et al*. 2018), birds (Narango & Rodewald 2018) and anurans (Kruger and Du Preez 2016; Goutte 2018) have indicated frequency adjustments to cope with noise. However, this result differs from the findings of previous studies that invertebrates produce higher frequency calls in noisy habitats (Lampe *et al*. 2012). We found that male grasshoppers from the CFNR (noisy area) produced signals with lower frequency compared to males from the TNR (quieter area) (also see Caorsi *et al*. 2017). Our results showing significant changes in call interval and call rate during high anthropogenic noise levels. Male *B. unicolor* from the noisier site (CFNR) also had a significantly longer call interval than males from the quieter site (TNR). Significant difference in call rate between the two sites is dependent on the number of males active at the site, so even if individual males are spacing their calls more widely, there may still be more individuals active.

On the other hand, the interval between calls was positively correlated with traffic noise level suggesting that the grasshopper population we studied dealt with the potential masking effect of traffic noise by not calling or producing a lower number of calls in high background traffic noise rather than by modifying their call frequency. This appropriate timing of calling may help grasshoppers to reduce the detrimental effects of masking signals by anthropogenic

noise (Sun & Narins 2005). The adjustment of calling activities to avoid interference from noise sources has already been established in various animal taxa (Parks *et al*. 2007; Sousa-Lima & Clark 2008; Slabbekoorn & Ripmeester 2008). Our results supported this expectation by showing significant changes in call interval during high anthropogenic noise.

Additionally, we found weather conditions correlate with the calls of grasshoppers. Our results showed that peak frequency and call rate tended to be affected by wind speed, temperature and humidity. Males increased the peak frequency of calls with increasing wind speed and temperature. However, higher humidity reduced the frequency of calls. The emission of loud calls in the middle of the night when humidity is lower could make sound transmission more efficient (Campbell *et al*. 2010; Snell-Rood 2012). In our study system, male *B. unicolor* in TNR call at a higher frequency than males from CFNR, where traffic noise is significantly higher. This difference in peak frequency and call rate with temperature and wind speed was observed in both study locations. In fact, in TNR we observed a very low calling activity throughout the night from around 12:00 to 03:00 hours, but higher activity from 21:30 to 23:30 compared to CFNR (Fig. 2.4). But, our result indicated no relationship between anthropogenic noise and call rates of males from TNR. This result supports predictions derived from the AAH by correlating wind speed and temperature with call parameters. We predict that more pronounced variation in peak frequency modulations of grasshoppers from TNR is linked to wind speed. Therefore a potential explanation for variation in the call parameters is that males in the habitat with high wind speed increase the frequency of the call even if the anthropogenic noise is low. On the other hand, we found that the relationships between call rate and wind speed were negative, indicating that in areas with high wind speed, grasshoppers might conserve energy by reducing the number of calls, as the production of advertisement calls is one of the most energetically expensive activities

and cost of calling is substantial, as reported in insects, amphibians and birds (Ryan 2001; Ophir *et al*. 2010).

The role of environmental constraints on acoustic signals of animals has been widely investigated. Here, TNR males call at a higher frequency, where wind speed and temperature are significantly higher, although call rate correlates negatively with wind speed. This suggests *B. unicolor* males adjust their calls under variable wind and temperature conditions. There are many examples of associations between climate variation and frequency in birds and bats (Price 2008; Sun *et al*. 2013). Population variation in social spacing due to different resource distributions selects for song transmission over different distances (McIntyre 1995; Ries *et al*. 2004; Laurance *et al*. 2007). Signals with high peak frequency modulation attenuate faster and lower frequency signals attenuate less with distance (Bradbury & Vehrencamp 1998). Hence, climate can influence sound scape patterns of individuals from different landscape structure and habitat (Hillerband 2004). Here, the spectral properties of grasshopper calls were found to be potentially adaptive and weather conditions appeared to be a constraint impacting their evolution. Thus, climatic conditions should favour variation in lower frequency signals, a hypothesis that could be tested with comparison of different populations and spectral properties of advertisement songs across climatically distinct sites.

Recently, considerable evidence has emerged showing that landscape structure can influence the interaction of species at multiple spatial and temporal scales (Laurance *et al*. 2007; Narango & Rodewald 2018). For instance, density of vegetation between urban and rural landscapes might influence the higher frequencies in different animal species (for e.g. Derryberry 2018; Helversen *et al*. 2015). Here, habitat quality may also be correlated with

acoustic features, although CFNR and TNR have similar habitats. Furthermore, climate, and land forms influence the timing of specific life-history events (Ahola *et al*. 2004; Pijanowski *et al*. 2011; Combes *et al*. 2018). Considering that divergence in weather conditions affects communication signals in this species of grasshopper, further investigation of the interplay between vegetation, noise and other forms of grasshopper behaviour is needed to elucidate the signal quality in both sites studied.

The biotic soundscape of both study sites was shaped by animal communities that were mainly composed of birds, mammals and insects. In both sites, calls of *Physemacris variolosus* (co-existing bladder grasshopper) were present, while in TNR the call rate of this species was higher (Average: CFNR - 0.14 and TNR - 3.12 calls/mins). *Physemacris variolosus* is a silver spotted bladder grasshopper with an average peak frequency of around 3.14 kHz (see Couldridge & Bazelet 2018). These two species of grasshoppers (heterospecific) occur in sympatry, creating competition in acoustic space (Parris *et al*. 2009). In bladder grasshoppers, females generally prefer conspecific calls and discriminate against heterospecifics (Couldridge & van Staaden 2006). Also, intraspecific competition may play a significant role in influencing frequency change. Therefore, peak frequency variation of *B. unicolor* calls between locations may be due to inter and intraspecific competition.

Our results suggest that *B. unicolor* reduce calling effort during moments of high anthropogenic noise level, and therefore, avoid potential masking of signals in noisy environments. Gurule-Small & Tinghitella (2018) tested if pre-reproductive exposure to noise improves adult performance in noisy environments in field crickets (*Teleogryllus oceanicus*). They reared female crickets in one of three noisy environments: masking traffic noise, non-

masking and silence. They found female crickets reared in masking noise took 80% longer than females reared in silence to locate a simulated singing male who was less than 1 m away. Hence, we predict that males that emit their calls by avoiding the sudden episodes of anthropogenic noise should achieve higher reproductive success. Further playback experiments should be conducted to investigate the potential effects of signal masking on mating behaviour in *B. unicolor*.

Studies on the impact of noise on insect call parameters have so far yielded inconsistent results, with some studies verifying an increase in the peak frequency of calls in noisy environments; however others indicated a decrease or no change in peak frequency. For example, in the cicada *Cryptotympana takasagona*, males increased the dominant frequency of calls in response to traffic noise (Shieh *et al*. 2012). They were able to increase the resonant frequency of their calls by decreasing the volume of the abdominal cavity. In this study, we cannot rule out the possibility that factors other than noise and climate are associated with the observed differences (e.g. morphology). It is proposed that small-sized males produce higher frequency calls with smaller abdominal cavities and that large-sized male reduce the volume of the abdominal cavity to produce higher frequency calls in birds (Patricelli & Blickley 2006). Other possible explanations for the observed variation in calls with traffic noise are that males from noisier sites are smaller than the males from quieter sites. Although we did not study the effects on body size with increasing traffic noise, it is possible that further study may reveal differences. Bladder grasshoppers have stridulatory files on the abdomen that are rubbed by scrapers on the legs to produce calls (van Staaden & Römer 1997). We consider that these would create a difference in the peak frequency of calls in TNR. Thus, like other taxa of insects, whether grasshoppers could in fact reduce the

volume of the abdominal cavity to shift their songs to higher frequencies requires further investigation.

Grasshoppers might also be adjusting spectral and temporal characteristics of acoustic signals to reduce competition pressure or predation risk. Noise from traffic has been demonstrated to alter predator-prey relationships (Barber *et al*. 2010). Habitat features, such as vegetation height and background sound level, interferes with the ability of an animal to detect predators (Verdolin 2006). Few studies have documented the increase of vigilance behaviour under high noise levels (Rabin *et al*. 2006; Quinn *et al*. 2006). Here, individuals from CFNR call later in the night compared to TNR individuals. This could be the effect of the relationship between predator and prey. The major predators in the study areas are a variety of bird species and bats. It is probable that the nocturnal predators rely heavily on acoustic cues to find their prey. This highlights the potential complexity of the relationship between noise exposure and predation risk. Another possible scenario is the pattern of biotic and abiotic noise effects on the diel variation in signalling. In addition, males adjust their call frequencies to reduce overlap with other competing males. Studies incorporating predator-prey relationships, diel variation and signal adjustment to reduce overlap in different parts of its distribution would be valuable.

Spectral and temporal parameters of calls are very important in localization and mate selection (Forrest 1994). But, the development of masking mechanisms of acoustic signals does not ensure the success of mating in many species. We observed that modifications of temporal properties of calls in response to anthropogenic noise might be related to reduce masking effects. Our study is based on long-term exposure to traffic noise, and on individuals from their own habitat. Therefore, we only assessed long term effects caused by noise and

cannot exclude the chances of additional changes in call parameters, which might occur in a short-term exposure. To assess this hypothesis, field or laboratory experiments are required to measure the call parameters of individuals before and after exposure to noise. It is known that in anurans, females decrease in their ability to decode a message from male calls in the presence of acoustic noise (Goutte *et al*. 2013). Therefore, whether changes in call parameters increase the chances of mating in a noisy environment is yet to be identified. In addition, variable habitat quality imposed by high to low traffic noise with increasing distance from the road may also cause a spatial effect (Caorsi *et al*. 2017).

Changes in call parameters with anthropogenic noise not only effect calling activity but also indirectly affect the animal's life function (Kaiser *et al*. 2015). Studies have shown that animals select quiet areas to perform their daily activities (Duarte *et al*. 2011) and noise can disrupt these. Anthropogenic noise can also cause physiological stress (Kight & Swaddle 2011), and alter population distribution (Bejder *et al*. 2006), species abundance (Bayne *et al*. 2008) and diversity (Proppe *et al*. 2013). Many endemic species of flora and fauna are experiencing negative effects derived from urban development and road noise.

This study adds to the growing literature concerning the effects of anthropogenic noise on acoustic signals. Since different species are differently affected by anthropogenic noise, detailed studies and a clear understanding of the behaviour of individual organisms is required to know the ecological and evolutionary consequences of increasing anthropogenic noise and changing signalling environments.

CHAPTER 3

HOST PLANT ASSOCIATED GENETIC DIFFERENTIATION IN A SYMPATRIC POPULATION OF *BULLACRIS UNICOLOR*

Abstract

The importance and prevalence of variation within populations has been debated for decades. Host adaptation has been cited as a major reason for sympatric divergence, resulting in the evolution of biotypes and cryptic species. Studies of herbivorous insects have played a key role in understanding how host associated differences can facilitate genetic variation. There has been only limited study of host-associated genetic differentiation in grasshoppers, due to the lack of intimate grasshopper-plant associations. The bladder grasshopper, *Bullacris unicolor* (Orthoptera: Pneumoroidea), is a highly phytophagous species. The relationship between host plant association and genetic variation of bladder grasshoppers has not been studied before, despite their observed dependence on a relatively small number of host plant species. In light of this, the present study was conducted to examine host plant related genetic and morphological divergence within a population of *Bullacris unicolor*. This species has been previously documented to occur on at least six host plants within its entire geographic range. Here we used two plant species, belonging to different families, *Didelta spinosa* (Asteraceae) and *Roepera morgsana* (Zygophyllaceae), to evaluate variation between individuals collected on these two sympatric host plants at a single locality in the Northern Cape, South Africa. The results demonstrated non-significant host related genetic variation with very low values of F_{ST}, indicating a low level of variation. The phylogram strongly suggested that there are no host associated genetic differences in *B. unicolor* by displaying limited genomic clustering, whereas strong differentiation was observed between the

morphological measurements of males and females among host plants. In addition, significant host-associated morphological divergence highlighted the need to further examine the mechanisms by which host utilization affects morphological features.

Introduction

Elucidating the factors that promote sympatric speciation or divergence-with-gene-flow has been the subject of renewed interest among evolutionary ecologists during the past decades (e.g. Orr & Smith 1998; Funk *et al*. 2002; Coyne & Orr 2004; Doellman *et al*. 2018). Divergent selection pressures experienced by members of the same population under different conditions, such as ecological selection and genetic incompatibilities, can facilitate the evolution of reproductive isolation (Barton *et al*. 2009; Smadja & Butlin 2011). Several studies in this field have shed light on particular scenarios favouring divergence in the face of gene flow. The studies of ecologically driven selection and genetic architecture on reinforcement or sympatric speciation has intensified in the past few years, leading to major empirical advances (Dieckmann *et al*. 2004; Gavrilets 2004; Maan & Seehausen 2011; Smadja & Butlin 2011).

Speciation is more or less a continuous process in which populations become genetically segregated, but the exact cause or route of speciation remains unclear (Turelli *et al*. 2001). It is important to discern the pattern of phenotypic and genetic differentiation, and the processes responsible for forming and shaping clines to understand the evolution of reproductive barriers without spatial separation (Bolnick & Fitzpatrick 2007). Implications from this endeavour may be strengthened by experiments testing how the individual's biotype differences within a population cause reproductive isolation. Genetic and phenotypic differences between insects feeding on different species of host plants are well documented and also strongly supportive of theories of sympatric speciation (Diehl & Bush 1984; Fritz & Simms 1992; Via 2001; Drès & Mallet 2002; Hsu *et al*. 2018). Many studies have described host-associated speciation in phytophagous insects (Matsubayashi *et al*. 2010; Knolhoff &

Heckel 2014; Antwi *et al*. 2015). Therefore, studies of herbivorous insects and their isolation due to differences in plant preferences have played a central role in our understanding of the evolution of ecological specialization (Diehl & Bush 1984). Host plant associated ecological divergence among populations is often assessed in terms of traits such as preference to feed and mate, growth, survivorship, performance and fecundity in association with the different host plants (Funk *et al*. 2002). But phylogenetic tracking between phytophagous insects and their host plants has rarely been tested (reviewed in de Vienne *et al*. 2013; Suchan & Alvarez 2015) and there is a lack of support for phylogenetic tracking in insect–plant relationships.

Genetic difference is not only the result of natural selection acting on the individuals within a species (Jaenike 1981). According to Thorpe's (1930) definition of biological races, individuals of a species occurring in the same locality can be isolated into groups to some extent by food preferences, suggesting biotypes' isolation due to differences in plant preferences. Most of the studies of insect-plant interactions reviewed monophagous insects on single plant species or closely related species (Bernays 1998; de Vienne *et al* 2013; Suchan & Alvarez 2015). It is difficult to distinguish phylogenetic tracking between herbivores and their host plants in such a narrow host range. For example, highly similar host plants (sister taxon) will not generate disruptive selection required for speciation and an insect is unlikely to colonize host plants with little similarity to the original host (Nyman 2010). Thus, the insect-plant interaction hypothesis posits that the maximum possibility of insect speciation occurs when alternate hosts are of a different resource space, such as chemical compounds, phenology, nutritional content, etc. (Nyman 2010; Heard 2012). Comprehensive quantifications of host plant associated ecological divergence within populations that utilize different plants are rare. Closely related plants often share similar morphological, physiological and phenological characteristics due to phylogenetic

conservatism (Liu *et al*. 2015). Therefore, phylogenetic comparisons between individuals from highly different host plants (different family) may represent the relative effect of plant variation that potentially affects herbivore populations.

Untangling population structure, genetic diversity and adaptation processes are important for developing effective procedures for speciation in insects. DNA markers such as mtDNA and nuclear DNA have been used as popular genetic markers for the research of insect genetics (Meng *et al*. 2018). Compared to nuclear DNA, some of the unique characteristics of mtDNA are a high rate of nucleotide substitution, maternal inheritance and little recombination (Avise 2000; Behura 2006). Generally, mtDNA is assumed to evolve under neutral selection; therefore the use of only mtDNA for phylogeographic and population genetic studies is questionable. It is necessary to utilise both mtDNA and nuclear DNA molecular markers together to produce a comprehensive picture of intraspecific diversity (Fontaine *et al*. 2014).

Most insect populations do not experience disruptive selection associated with host shifts. However, some insects are truly host specific with restricted host ranges (Thompson 1994; Mopper & Strauss 1998). Host plant specializations in grasshoppers appear to be an exception and are thought to promote ecological diversification and reproductive isolation (Sword & Dopman 1999, Sword *et al*. 2005). However, such studies have been quite rare in grasshoppers (Sword & Dopman 1999). Allopatric speciation in the polyphagous bladder grasshopper *Bullacris unicolor* has been studied by Sathyan *et al*. (2017). *B. unicolor* feeds selectively on host plant species from a number of unrelated plant genera, including *Didelta spinosa* (family-Asteraceae), *Roepera morgsana* (Zygophyllaceae), *Salvia africana-lutea* (Lamiaceae), *Tripteris oppositifolia* (Asteraceae) *Osteospermum moniliferum* (Asteraceae),

and *Muraltia spinosa* (Polygalaceae). The wide range of host plants for the *B. unicolor* species complex provides a unique opportunity to test host associated genetic divergence of individuals within a population.

How interactions with host plants may have shaped the evolution of *B. unicolor* has not been studied before. All *Bullacris* species are specialized to feed on a small number of host plant species and many of the host plants used by *B. unicolor* occur sympatrically (personal observation). The degree of morphological differentiation of *B. unicolor* varies among males and females (R Sathyan unpublished Master's thesis). Males produce loud calls and females reply to the male calls by duetting (Donelson & van Staaden 2005). Adult females and nymphs of *B. unicolor* are unable to fly and are poor jumpers and thus remain on their host plant, whereas adult males can fly and thus move further from their natal site than do females. All individuals are cryptically camouflaged to match their host plant, and individuals feeding on different host species, even within the same area, adopt different colour variations.

The specific prediction of insect-plant interaction was derived by matching the phylogeny of *B. unicolor* with their host plants. The genetic diversity of *B. unicolor* was investigated using COI and ITS genes. *B. unicolor* was sampled from two plant species, *Didelta spinosa* and *Roepera morgsana*, belonging to the families Asteraceae and Zygophyllaceae respectively. Additionally, morphological variation was expected to occur, caused by the host plant phenology and nutrition. We measured morphological variation between individuals from the two different host plants to determine if there was host-associated morphological differentiation. Through this study, we aim to improve the understanding of host associated reproductive isolation in this species. In this present study, COI and nuclear DNA were used

for molecular diversity analysis of *B. unicolor* collected on two different host plants from the same geographic area. Phylogenetic tracking of individuals may uncover host related divergence between individuals found on these two host plants.

Materials and methods

Specimen collection

A total of 35 *B. unicolor* individuals were collected from two host plant species, *D. spinosa* and *R. morgsana* (Fig. 3.1) in and around the town of Springbok in the Northern Cape, South Africa. Host plant species were recorded upon sampling. Among the sampled specimens, 22 were collected from *D. spinosa* and 13 from *R. morgsana*. We collected a mixture of adults as well as sub-adults (final instar, n = 16) to permit accurate host association, as adult males may move around. Nymphs were reared to adulthood in the laboratory, and fed either their original host plant (if available) or otherwise on lettuce leaves. As all nymphs used were final instar and thus within days of their final moult into adulthood, this is unlikely to have significantly impacted on growth and adult body sizes. To maximize the coverage of genetic variation, we collected individuals from an approximately 25 km area. After morphological measurement, all specimens were preserved in 95% ethanol at 4°C for long term storage until DNA extraction.

(A)

(B)

Figure 3.1 Two different host plants of *B. unicolor* (A) *Didelta spinosa* and (B) *Roepera morgsana* (Photo credit: VCK Couldridge).

Determining morphological variation

A series of seven linear measurements (mm) were made for each specimen following Donelson (2007) and Sathyan *et al*. (2017). These measurements were used to establish whether morphology differed according to host plant as well as whether it was correlated with genetic characters.

We used multivariate analysis of variance (MANOVA) to determine the variation in male and female morphological characters within the population. The MANOVA compared each length variable against host plants, for both males and females. This analysis shows how each male and female morphological variable is responding to the two different host plants. Mantel tests were used to test the relationship between morphological variables and genetics.

DNA extraction and PCR amplification

For each sample, we removed the hind legs and extracted genomic DNA using a DNeasy animal tissue kit (Qiagen Inc., Valencia, CA, USA). The collected materials will be deposited in a museum. We collected gene sequences from the mitochondrial cytochrome oxidase 1 (CO1) gene and internal transcribed spacer (ITS). To amplify the partial sequence of mitochondrial gene COI (650bp), we used the PCR forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994) and for ITS (750bp), the PCR ITS forward primer DgITS-F (5'- AGAGGAAGTAAAAGTCGTAACAAGG-3')

and ITS reverse primer DgITS-R (5'- CCTTAGTAATATGCTTAAATTCAGG-3') were used.

PCR amplifications for both COI and ITS were carried out in a final volume of 25 µl reactions with 23 µl Taq PCR MasterMix, 1 × PCR buffer, 0.5–1 µl of each primer, 2 µl of $MgCl_2$, 4 µl of dNTPs, 19 µl ddH_2O and 2 µl template DNA. For CO1, the PCR cycling profile comprised an initial heating period of 94 °C for 3 min followed by35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. A final extension step of 72 °C for 5 min was also added. For ITS, an initial heating period of 94 °C for 5 min was followed by 30 cycles of 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 1 min. A final extension step of 72 °C for 10 min and a final hold 15 °C was done. To confirm whether amplification was successful, 2µl of the amplified product was electrophoresed on 1.5% agarose gel (1x Tris borate-EDTA), stained with ethidium bromide, and observed under a UV transilluminator. PCR products were then purified using a QIAquick PCR purification kit and an automated sequencer (ABI 3100, applied Biosystems) was used to run cycle sequencing products (Folmer *et al*. 1994; Sathyan *et al*. 2017).

Molecular phylogenetic analysis

The DNA sequences were edited manually and aligned using Bioedit sequence alignment editor, version 7.2.5. Unique sequences were obtained after manual correction and assembly. The CO1 and ITS sequences were compared and blasted with other sequences of grasshoppers on GENBANK to authenticate the sequences. The closely related *Bullacris membracioides* was used as an outgroup.

For the Bayesian phylogenetic analysis and maximum likelihood (ML), the best fit nucleotide substitution model was chosen in MODELTEST version 3.06 (Posada & Crandall 1998). Bayesian analyses were performed under the best-fit model using Mr Bayes version 3.2 (Ronquist & Huelsenbeck 2003). We ran four chains with $5 \times 10_7$ generations in the Markov Chain Monte Carlo (MCMC) process. When the average standard deviation of the split frequencies was zero, convergence of the MCMC process was established. The first 25% of the MCMC samples were discarded as burn-in.

Genetic diversity was calculated in DnaSP version 5.10 (Librado and Rozasv 2009) with mtDNA and ITS analysed separately. Genetic parameters for each individual, grouped by host plant, including number of haplotypes (Nh), haplotype diversity (Hd) and nucleotide diversity (π), and the number of segregating sites, were analysed with concatenated mtDNA and ITS. Pairwise genetic divergence estimates (F_{ST} values) were calculated and tested for significance to assess the relative degree of divergence between individuals from the two host plants. Isolation by distance among sampled individuals was obtained by examining the correlations between matrices of pairwise genetic distance (F_{ST}) and morphological distance using PAUP version 4.0 (Swofford 2002). Mantel tests were used to test the strength of the relationship between the morphology and genetics in R software.

We conducted a neighbour joining analysis in PAUP 4.0 (Swofford 2002) and rooted phylogenetic trees were constructed using the congeneric *B. membracioides* as an outgroup. The resulting trees were visualized using Splits tree version 4.13.1 (Huson & Bryant 2006) based on haplotype sequence variation and the genetic distances of individuals from two host plant species.

Results

Morphological variation of males and females

Mean values and standard deviations for morphological measurements of males and females are given in Table 3.1. Monte Carlo nonparametric tests indicated non-significant differences from normality for all body measurements. MANOVA results revealed differences in the morphological characters of both males (Wilks' Lambda = 0.328, F = 1.172, P < 0.005) and females (Wilks' Lambda = 0.075, F = 3.529, P < 0.005) among individuals feeding on alternative host plants within the population. Males differed in pronotum length (Table 3.1A), and females differed in head width, femur length and tibia length (Table 3.1B).

Table 3.1 Morphological measurments (mm), given as mean ± standard deviation for (A) males (n=13) and (B) females (n=14) of *Bullacris unicolor* from two host plants, and multivariate analysis of variance (MANOVA) results of the variation in the morphological characters. Morphological measurments: AL = antennae length; HW = head width; BL = body length; AW = abdomen width; FL = femur length; TL = tibia length; and PL = pronotum length.

(A)	Mean ± SD					
Morphological characters	*Didelta spinosa* N-7	*Roepera morgsana* N-6	df	Mean square	F	Sig.
AL	6.08 ± 1.24	6.46 ± 0.96	1	0.46	0.36	0.55
HW	4.58 ± 0.22	4.61 ± 0.29	1	0.00	0.02	0.87
BL	39.09 ± 1.78	37.84 ± 1.91	1	5.02	1.47	0.25
AW	10.95 ± 0.98	10.87 ± 0.66	1	0.01	0.02	0.88
FL	11.16 ± 0.43	10.93 ± 0.57	1	0.17	0.69	0.42
TL	11.73 ± 0.78	11.22 ± 0.59	1	0.84	0.70	0.21
PL	17.78 ± 0.25	16.55 ± 0.91	1	4.87	11.66	0.01*
(B)						
	N-8	N-6				
AL	5.73 ± 0.64	5.59 ± 0.55	1	0.65	0.17	0.68
HW	5.72 ± 0.35	5.24 ± 0.15	1	0.76	9.31	0.02*
BL	39.59 ± 3.71	37.31 ± 3.16	1	17.71	1.44	0.25
AW	8.73 ± 0.86	8.65 ± 0.54	1	0.02	0.36	0.85
FL	10.99 ± 0.65	9.72 ± 0.50	1	5.52	15.53	0.00*
TL	11.75 ± 0.64	10.54 ± 0.69	1	5.08	11.45	0.02*
PL	22.31 ± 1.10	21.32 ± 0.81	1	3.36	3.38	0.24

* Significant P < 0.05

Molecular phylogenetic analysis

After trimming short end sections, a sequence of the 654 bp mitochondrial COI and 700 bp ITS DNA gene were obtained from 23 specimens within the population. The aligned COI fragments contain a total of 23 mitochondrial haplotypes and 90 polymorphic sites. Overall nucleotide diversity was 0.030 (ranging from 0.03 in *D. spinosa* to 0.02 in *R. morgsana*) and haplotype diversity was 1.000. There was no significant variation in base composition between sequences of samples from different host plants ($X^2 = 23.00$, df = 22, p = 0.40).

The aligned ITS fragments contain a total of 15 nuclear haplotypes and 17 polymorphic sites. Overall nucleotide diversity was 0.003 (0.00 for both host plants) and haplotype diversity was 0.928 (ranging from 0.92 in *D. spinosa* to 0.82 in *R. morgsana*). There was no significant variation in base composition between sequences of samples from host plants ($X^2 = 35.82$, df = 14, p = 0.30). The non-significant negative values of CO1 (Tajima's D: –0.92201and Fu's F_S:–9.450) and ITS (Tajima's D: –0.74918 and Fu's F_S: –4.545) is indicative of no recent demographic divergence and an excess of rare haplotypes over what would be expected under neutrality.

The genetic analysis does not reveal strong genetic structuring between host plants. Results of pairwise distance analysis showed sequence divergence among individuals within the population using two different host plants was generally low. COI substitution rates of individuals from *D. spinosa* and *R. morgsana* showed 3.54% and 2.53% respectively, and between these two host plants showed 3.00%. On the other hand, ITS substitution rates of *D.*

spinosa and *R. morgsana* were 0.36% and 0.39% respectively, and between host plants was 0.377% (Table 3.2).

Analysis of gene flow and genetic differentiation revealed a non-significant variation among the individuals from different host plants. The overall analysis supported the hypothesis of no effect of host plants on genetic structure, and rather indicated significant sub-structuring within the population. Sub-structuring within the location was clearly attributable to genetic variation between the individuals, but not affiliated to host plants, there should be another cause for this sub structuring. There was no significant genetic structuring by host plant use and there was no evidence of significant sub-structuring between insects feeding on the different plants at the same site.

Pairwise comparisons of F_{ST} values between host plants further supported the results reported above. F_{ST} values were lowest for COI (– 0.010) and ITS (0.043) genes in comparison between host plants. The genetic distance was drawn into the phylogenetic tree. The tree does not show a clear split between the two host plants (Fig. 3.3 and 3.4). The topology of the phylogenetic tree based on mtDNA data does not show identical phylogenetic relationships among samples from different plants (Table 3.2 and 3.3, Fig. 3.3). Mitochondrial haplotypes were not clustered according to the host plants, although the data produces three major groups. ITS data also produces a similar population tree (Table 3.2 and 3.3, Fig. 3.3), with a posterior probability of one. But, the topology forms three different clusters with seven samples. Overall low molecular variance occurred within groups associated with host plants.

Table 3.2 Pairwise genetic distance of mtDNA and ITS (mean ± standard deviation) within (bold) and between individuals of *B. unicolor* from two host plants.

		Didelta spinosa	*Roepera morgsana*
CO1	*Didelta spinosa*	**3.54 ± 0.02**	
	Roepera morgsana	3.00 ± 0.02	**2.53 ± 0.01**
ITS	*Didelta spinosa*	**0.36 ± 0.00**	
	Roepera morgsana	0.377 ± 0.00	**0.39 ± 0.00**

Table 3.3 Genetic diversity and demographic parameters for 654 bp of cytochrome oxidase I (COI) mtDNA and 760 bp of ITS of *Bullacris unicolor*. N = sample size; Nh = haplotype number; S = number of polymorphic sites; Hd = haplotype diversity; π = nucleotide diversity.

	Host plant	N	Nh	S	Hd	**π**
CO1	*Didelta spinosa*	13	13	78	1.00	0.03
	Roepera morgsana	10	10	56	1.00	0.02
ITS	*Didelta spinosa*	26	11	9	0.92	0.00
	Roepera morgsana	20	6	12	0.82	0.00

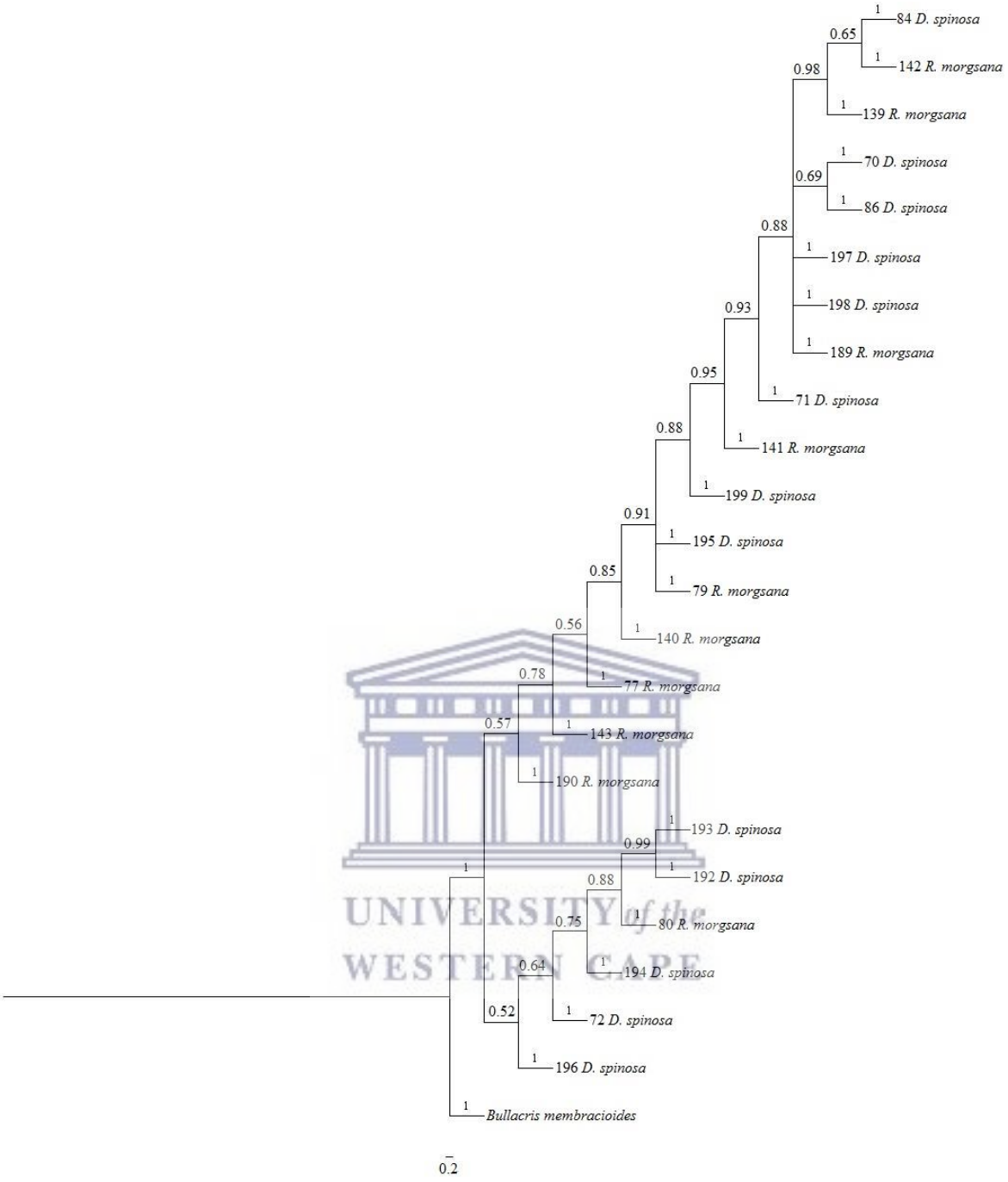


Figure 3.2 Linearised phylogenetic tree of *Bullacris unicolor* from two host plants using the cytochrome oxidase I (COI) gene (n = 23), based on 23 haplotypes and rooted by *Bullacris membracioides*. Posterior probabilities are included above branches.

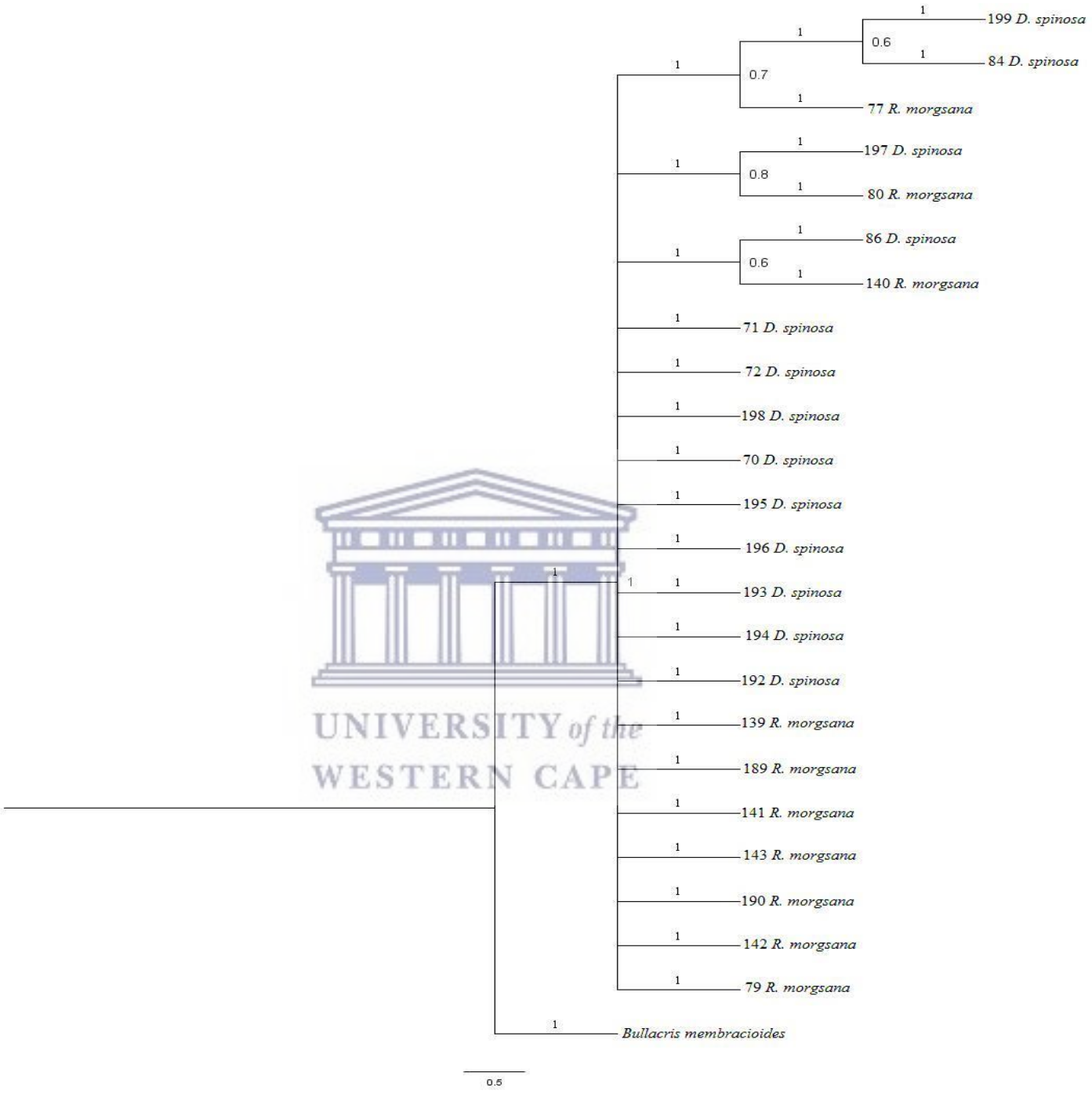


Figure 3.3 Linearised phylogenetic tree of *Bullacris unicolor* from two host plants using the internal transcribed spacer (ITS) gene (n = 46) based on 15 haplotypes and rooted by *Bullacris membracioides*. Posterior probabilities are included above branches.

Relationships among morphology and genetics

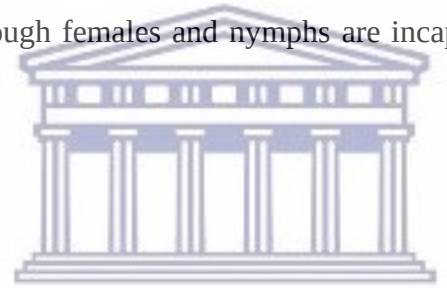
Morphological distance was not significantly correlated with genetic distance, suggesting that morphological differences between individuals within a population were not predicted by genetic distance. Mantel tests revealed that both male and female morphological distance was not correlated with either the CO1 or ITS gene; males (CO1: –0.103, $p = 0.690$ and ITS: $r = 0.077$, $p = 0.312$, and females (CO1: –0.030, $p = 0.544$ and ITS: 0.089, $p = 0.326$).

Discussion

In the present study, the genetic structure of *B. unicolor* collected from two different host plants, *Didelta spinosa* and *Roepera morgsana,* at one locality (Springbok) was analyzed. The absence of clustering sequences, non-significant *P* values and negative or low F_{ST} values indicate that host plants do not support phylogenetic tracking in *B. unicolor*. Moreover, the substitution rates of ITS between the two host plants showed low values (0.3%). These results suggest that the individuals from different host plants are not genetically isolated. Our results showed that pairwise distances of CO1 and ITS showed genetic variation within *B. unicolor* found on *D. spinosa* (3.54% and 0.36%) and *R. morgsana* (2.53% and 0.39%) and between host plants (3.00% and 0.37%). Results obtained suggested that the level of host associated intrapopulation genetic variation was extremely low and little sequence divergence was evident within *D. spinosa* and *R. morgsana*. F_{ST} values were very low for both sequences, which indicates a low level of phylogenetic tracking within the population. Mean mitochondrial intrapopulation divergence values were higher than nuclear DNA as predicted, because of the faster mutation rate in the mitochondrial genome (Simon *et al*.1994). These results indicate that host shifting may have been the product of evolutionary history of *B. unicolor*. Furthermore, the relationship between host plants and morphology indicates that the plant-insect interactions may have been the product of multiple mechanisms (Hsu *et al.* 2018).

The genetic structure of *B. unicolor* for both CO1 and ITS showed three different clades, but these did not coincide with host plant use, indicating that our sampled individuals have undergone partial genetic differentiation. ITS genetic data further indicated a smaller number of haplotypes and segregating sites in comparison with the mtDNA. The non-significant

negative values of CO1 and ITS is indicative of no recent dempographic divergence and an excess of rare haplotypes over what would be expected under neutrality (de Jong *et al*. 2011). Following this test, the hypothesis of neutral evolution was significantly rejected for both host plants. We predict that the clustering may be a consequence of habitat fragmentation or heterogeneity. This low level of genetic variation supports the hypothesis that host fidelity is not perfect in *B. unicolor*, indicating the occurrence of migration between hosts. Reproductive isolation in a sympatric population can only evolve if there is a premating selection against migrants or post mating selection against hybrid progeny (Liou & Price 1994). Adult males may also have migrated to mate with females found on different hosts. Adult males of *B. unicolor* are capable of flight, enabling them to easily travel between neigbouring host plants, although females and nymphs are incapable of flight and are weak jumpers.



Despite the lack of genetic divergence related to host plant use, there was evidence of morphological variation, with individuals feeding on *D. spinosa* being comparatively larger in size. This finding may not be surprising given the evidence of host associated growth rate and the effects of host plant quality on fecundity of herbivorous insects (reviewed in Bush & Butlin 2001; Awmack & Leather 2002). The components of host plant quality, such as chemical compounds and nutritional content directly affect herbivorous insects' fecundity, reproductive strategies, etc. Performance of predators and parasitoids may also be affected by host plant quality. Insect divergence is frequently associated with their uitilizastion of the contents of resource space from each host plant (Wood & Keese 1990; Bruce 2015). Host shifting is traditionally expected between closely related plant species, as they share similarities in chemical compounds (Janz & Nylin 1997). Given the lack of support for phylogenetic tracking, this is unlikely to be the case between *B. unicolor* and their host plants

because these plants do not share any physical similarity with each other and belong to different families. However, results suggest that males and females are significantly bigger when feeding on *D. spinosa* compared to *R. morgsana*, indicating that plant quality might be responsible for the observed morphological differences.

As observed in previous studies, host specificity in insects influences reproductive isolation when migration occurs between host plants due to premating or postmating selection against migrants, thus favoring host adaptation through the evolution of biotypes (Wang & Qiao 2009; Foottit *et al*. 2010, Rebijith *et al*. 2013). However, the present study showed no evidence of host-associated genetic differentiation in *B. unicolor* based on mitochondrial and nuclear molecular markers. Overall, the data suggest that there is gene flow among individuals using different hosts. The absence of evident genetic differentiation could be accounted for by a high behavioral plasticity that is not dependent on genetic variability. Meanwhile, to resolve the deep host relationships of *B. unicolor*, further studies should be undertaken to discern the exact systematic status by adding microsatellite markers. Host-associated preferences are thought to be a complex phenomenon involving the interaction of many genes (Komazaki & Toda 2008).

Sufficent gene flow within a population can prevent the process of sympatric speciation, and leave little population structure over big areas. This is usually observed in flying insect species that can migrate (for e.g. Brower & Boyce 1991; Estoup *et al*. 1995; Freeland *et al*. 2003). In contrast, *B. unicolor* does not migrate, and allopatric population divergence has been already found in this species (Sathyan *et al*. 2017). However, sympatric speciation is a progressive process, with complete reproductive isolation depending on the intensity of

divergent selection, and the degree of host preference and assortative mating (Johnson *et al*. 1996). We assume that these grasshoppers might shift between host plants to overcome strong host selection and to maintain sufficient gene flow (Wood 1993; Wood *et al*. 1999).

The combination of low nucleotide diversity and high haplotype diversity in our data can be a signature of rapid demographic expansion (Avise 2000). Pair-wise differences between sequences within populations were developed to test selective neutrality of mutations (Ramos-Onsins & Rozas 2002). Here, we chose Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) to detect population expansion and these differ in their approach. Tajima's D test is based on the allele frequency distribution of segregating nucleotide sites. A negative value indicates a bias towards rare alleles and being a signature of recent population expansion. While Fu's F_S test is based on the distribution of alleles or haplotypes. In the present study, Tajima's D test and Fu's Fs shows non-significant negative values for both COI and ITS sequences within populations. The negative values resulting from both tests indicate that there is an excess of rare mutations in this population, which can indicate recent expansion in the population. Alternatively, these values can result from balancing selection on a nearby locus. Studies demonstrating direct or indirect selection on the mitochondrial genome and nuclear DNA markers in natural populations could give a more complete perspective on the neutral sympatric population structure (Silva-Brandão *et al*. 2018).

Despite the prevalence of polyphagy among grasshoppers (Chapman & Sword 1997), few previous studies have demonstrated host plant associated ecological and genetic divergence in grasshoppers (Sword *et al*. 2005). They indicated evolutionary consequences of host plant associated divergence are consistent across phytophagous insect groups. Although detailed

examinations of local host use patterns are rare in grasshoppers, such studies will elucidate occurrences of local adaptation and resource associated divergence (Sword & Dopman 1999; Traxler & Joern 1999). Particularly, the current study aimed the relative contribution of host plant associated genetic drift. However, limitations on sample sizes were encountered and this compromised the number of individuals that could be analysed. Therfore additional investigations of host associated sympatric and allopatric population divergence of morphology and genetics need to be conducted in this species.

In conclusion, this study provides information on host associated genetic diversity within a population of *B. unicolor*. Grasshoppers on different host plants do not show genetic differentiation. Interestingly, the sympatric population shows morphological variation associated with host plants. These findings suggest that differentiation in morphology within the population may occur as an environmental response to host plant use. This study provides a much needed framework for the investigation of adaptive host associated variation at the phenotypic or molecular level in additional populations of *B. unicolor*. Further studies combining additional microsatellite molecular markers should be undertaken to discern population genetic structure at a finer scale.

General conclusion

The present work explores the factors that influence divergence in the bladder grasshopper *Bullacris unicolor*. This is the first population genetic study to be conducted on a bladder grasshopper species, and provides insights into genetic structuring and divergence within the species and how this links to previously observed morphological and acoustic variation, as well as different environmental conditions, such as temperature and precipitation. Results support climate-induced geographic variation in genetic and morphological characters, suggesting that lineage diversification in general may be driven by climate mediated differences accompanied by genetic differentiation. However, divergence in calls is not associated with environmental factors, which indicates that other factors may also help to drive acoustic divergence. In particular, mate choice may help to drive geographic variation in call characters in *B. unicolor* and this needs to be examined in future studies.

The analysis of the effects of anthropogenic noise and weather conditions on signalling in *B. unicolor* indicates that these may trigger grasshoppers to adjust their call parameters to avoid masking of their signals. Several factors may potentially be involved in the origin and maintenance of call differentiation. A positive and negative correlation between call interval and call rate with anthropogenic noise, and the observed effects of weather conditions on peak frequency and call rate in both locations indicates that both anthropogenic noise and weather conditions affect the properties of the call of *B. unicolor*. Moreover, we predict that the evolution of acoustic signals can be shaped by several selective pressures, such as sexual selection, physiology, phylogeny, predation, parasitism, competition, vegetation structure and the environment. Thus, our results highlight both the complexity of call evolution and the need to consider multiple causes when exploring this issue.

There are no host-associated genetic differences in *B. unicolor* within a single population. However, host-associated morphological divergence highlighted the need to target the effect of host utilization, considering its potential importance as a key trait for promoting diversification. Future research efforts will be needed to empirically assess the factors shaping the observed variation in morphology and colour associated with host plants. Further studies that combine additional microsatellite molecular markers should be undertaken to discern the population genetic structure on a finer scale. Moreover, assessing host-associated divergence between allopatric populations is critical to elucidate instances of host shifting and resource associated divergence. Many additional aspects of the ecology of *B. unicolor*, such as predator community, female choice, male competition and related spatial and temporal variation in the call, will have to be clarified to define a clearer evolutionary scenario of allopatric and sympatric variation in this invertebrate.

To conclude, this is the first study to examine population genetics and ecological influences in the evolution of this unusual group of insects, bladder grasshoppers. The results of this study further support prior research which has suggested that environmental variation and local habitat variation are imperative to population divergence. Our data suggest that variation in the local environment, in combination with other ecological cues, has likely contributed to the diversification of morphological, acoustic and genetic characters within and among populations of *B. unicolor*. Thus, our findings clearly suggests that gene flow in natural populations may mostly be environmentally structured, although several issues remain to be addressed, such as 1) the connection between mechanisms and patterns of gene flow 2) the association between gene flow patterns and local adaptation 3) the long-term impacts of different patterns of gene flow on the evolution of plasticity and 4) finally, the effect of climate change on all of the above patterns remains to be evaluated.

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Appendix

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Morphological, acoustic and genetic divergence in the bladder grasshopper Bullacris unicolor

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Although the processes that promote biodiversity remain poorly understood, geographic variation resulting from selection and/or neutral processes is thought to be a precursor to allopatric speciation. An examination of intraspecific divergence in multiple traits and their co- variation is an essential part of understanding the origin of new species. Here we investigate patterns of geographic variation in acoustic, morphological and genetic characters of allopatric populations of the bladder grass-hopper Bullacris unicolor. We found significant geographic variation in both temporal and frequency components of male advertisement calls, as well as in morphological variables of males and females. However, acoustic characters were much more strongly differentiated between populations than were morphological characters, with no correlation between acoustic and morphological traits. Furthermore, analysis of the mitochondrial DNA marker cytochrome c oxidase I indicated strong genetic structuring, pointing towards genetic isolation among populations in the absence of isolation by distance. Furthermore, we found genetic distance was significantly cor-related with morphological differentiation, but not with acoustic differentiation. The high levels of variation in male advertisement calls between populations, as well as the lack of association between acoustic and genetic distance, suggests that diver-gence in acoustic traits cannot be attributed to genetic features, and is more likely due to alternative selective pressures, such as mate choice or the ecological environment. Our results further suggest that morphological and acoustic features are uncorrelated at the intraspecific level and appear to be evolving under separate selective pressures.

KEY WORDS: advertisement call, COI, morphology, population divergence, Pneumoridae.

INTRODUCTION

Geographic variation among allopatric populations is believed to be a precursor to eventual speciation (Mayr 1954). Examining patterns of geographic variation in phenotypic and genotypic traits may thus provide valuable insights into the processes

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that govern species origination. The dispersion of alleles from one gene pool into another is prevented by various processes, including the founder effect, sexual selection, ecological differences and random genetic divergence (Rabosky 2016).

Across the geographic range of a species, populations may experience differential selective pressures. Species with large geographic ranges that span multiple habitat types may be particularly susceptible to divergence through adaptation to different ecological environments (Schluter 2001). Ecological agents of divergent selection may include either the physical environment or other interacting organisms (Rundle & Nosil 2005). Both morphological (Calsbeek et al. 2006; Amiot et al. 2007; Capellini & Gosling 2007; Stillwell et al. 2007; Riesch et al. 2011) and acoustic characteristics (Nicholls & Goldizen 2006; Ruegg et al. 2006) have been shown to vary in accordance with local ecological conditions.

Alongside natural selection, sexual selection has also been identified as one of the most important factors in the generation of biological diversity (Boake 2002; Coyne & Orr 2004). The advertisement calls of males are subject to sexual selection in many species, including insects (Hoffman et al. 2006; Balakrishnan 2016) and may exhibit minor inter-population differences (Zuk et al. 2001; Gerhardt & Huber 2002; Philips & Johnston 2008). Divergence in the acoustic characters of males among populations as a result of sexual selection may have important consequences for speciation (Pröhl et al. 2007). Recent studies have indicated that the evolution of sexual signalling is associated with diversification and accelerated rates of speciation (Mendelson & Shaw 2005; Grace & Shaw 2012).

Species that exist across different geographic regions may show corresponding patterns of genetic differentiation (Manier & Arnold 2006; Olsen et al. 2011). These patterns of genetic variation between populations may be caused by multiple factors, including random forces, selection or migration (Olsen et al. 2011; Whiteley et al. 2014; Dyer 2015). However, even phylogenetically similar taxa can show significantly differ-ent patterns of spatial genetic variation (Van Buskirk 2012; Whiteley et al. 2014). Thus, a clear understanding of gene flow focused on a single species is of critical importance (Panhuis et al. 2001; Boughman 2002).

Geographic variation in acoustic signals across a species' range has been demon-strated in a variety of animal taxa, including birds (Förschler & Kalko 2007; Koetz et al. 2007), mice (Campbell et al. 2010), bats (Sun et al. 2013), whales (Samarra et al. 2015), anurans (Castellano & Giacoma 2000; Pröhl et al. 2007; Rodríguez-Tejeda et al. 2014), fish (Philips & Johnston 2008) and insects (Pinto-Juma et al. 2008). Likewise, morpho-logical traits may also differ geographically (e.g. Castellano & Giacoma 2000; Pröhl et al. 2007; Huizenga et al. 2008; Cisneiros et al. 2012). However, studies examining the link between these phenotypic traits and genotypic variation have yielded mixed results, either supporting (Campbell et al. 2010; Velásquez et al. 2013; Warwick et al. 2015) or failing to support (Pröhl et al. 2006, 2007; Ohmer et al. 2009; Sun et al. 2013; Lee et al. 2016) a correlation between them. The absence of a correlation between genetic dis-tance and morphological or behavioural distance points to the importance of either ecological or sexual selection in shaping phenotypic traits, rather than of isolation through drift.

The integration of different sources of intraspecific variation provides valuable insights into the causes of that variation and, ultimately, speciation (Noguerales et al. 2016). Genetic differentiation within a species may be caused by a combination of deterministic and stochastic processes. Neutral genetic markers may be used not only to infer phylogenetic relationships among populations, but also to assess the relative

contributions of natural selection and genetic drift to phenotypic divergence, by com-paring the relationship between genotypic versus phenotypic variation (Leinonen et al. 2006). If phenotypic variation follows genetic variation of neutral markers, then it is likely that the observed phenotypic variation is a consequence of genetic drift. Alternatively, if phenotypic variation does not follow neutral genetic variation, then selective pressures are likely to be at play (Lehtonen et al. 2009). These could be either stabilising or disruptive selective pressures, depending on whether phenotypic variation is less than or greater than neutral genetic variation, respectively (Lehtonen et al. 2009).
Bladder grasshoppers (Orthoptera Pneumoroidea) are a group of endemic south-ern African insects specialised for long-range acoustic communication (van Staaden & Römer 1997). The distinctive feature of this family is the strongly inflated, balloon-like abdominal bladder of males that acts as an acoustic resonator and includes six pairs of abdominal hearing organs, unique in the animal world (van Staaden & Römer 1998). Males generate a loud signal at night by rubbing their hind legs and abdomen together, creating a transmission distance of 1.5–1.9 km (van Staaden & Römer 1997). Females respond acoustically to male advertisement calls based on their detectability and attrac-tiveness, thereby allowing flighted males to locate them (Couldridge & van Staaden 2006). Alternate male morphs (sneaker males) are present in low numbers, and exploit the acoustic signalling system between primary males and females to their own benefit (Donelson & van Staaden 2005). While the male call varies greatly between species, the softer female signal is relatively simple in structure and is not species-specific (van Staaden et al. 2004). Any alteration to the male call would thus likely have implications for species integrity.

Apart from selective pressures, gene flow in bladder grasshoppers is thought to be inhibited by two additional factors, namely low vagility and high host-plant fidelity. Bladder grasshoppers lack the saltorial legs typical of most grasshoppers, instead rely-ing on crypsis to avoid predation (Alexander & van Staaden 1989). Together with the inability of females to fly, this is expected to impede migration between neighbouring populations. Furthermore, bladder grasshoppers are strongly dependent on their host plants. Each species lives and feeds on either one or a small number of host plant species. These factors may create small isolated populations and a high potential for genetic drift and adaptive divergence (Antwi et al. 2015).

Bullacris unicolor is a fairly wide-ranging species, with a geographic distribution extending along the coastal areas of the western region of South Africa. This distribu-tion spans two vegetation biomes: the sparsely vegetated, semi-arid Succulent Karoo biome, dominated by low- growing succulent shrubs, and the Fynbos biome, which is extremely rich in plant diversity and dominated by fine-leaved shrubs. Both areas are characterised by winter rainfall, between 20 and 290 mm per year, and extreme summer aridity (Guo et al. 2016).

Due to its wide geographic distribution, limited dispersal abilities, and reliance on acoustic signals for mate recognition, Bullacris unicolor is an ideal model species with which to study patterns of intraspecific divergence. The aims of this study were to assess the degree of morphological and acoustic variation among populations of B. unicolor, and to correlate this phenotypic variation with genetic variation based on mitochondrial DNA markers. Knowledge of the patterns of intraspecific differentiation and the relationships between phenotypic and genotypic traits will contribute to a deeper understanding of the evolutionary processes that have led to this differentiation. We hypothesise that populations will be differentiated due to a combination of geo- graphic distance and differing selective pressures in different environments, and that as

phenotypic variation is likely to be influenced by divergent selective pressures, it will be uncorrelated with neutral genetic variation.

MATERIALS AND METHODS

Specimen collection

Bullacris unicolor was collected from five field sites located in the Western and Northern Cape provinces of South Africa: Springbok, Kamieskroon, Groenriviersmond, Cederberg and Melkbosstrand (Fig. 1). The vegetation biome of Springbok, Kamieskroon and Groenriviersmond is Succulent Karoo, while Cederberg and Melkbosstrand are part of the Fynbos biome (Cowling et al. 1997; Mucina & Rutherford 2006). The Succulent Karoo is more sparsely vegetated (semi-desert) and receives less precipitation than Fynbos. Due to the seasonal occurrence of bladder grasshoppers during the spring and early summer, sampling was done to coincide with peak times

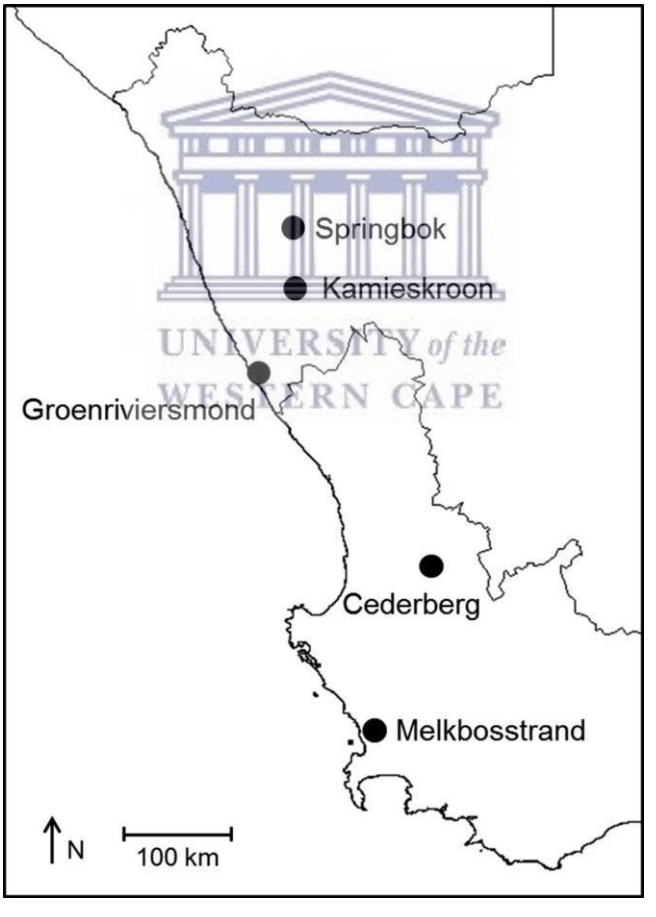

Fig. 1. — Map of the west coast of South Africa showing the five locations where Bullacris unicolor was sampled.

of emergence (September to November). A total of 98 grasshoppers (64 males and 34 females) were individually captured by hand between 2008 and 2014. As far as possible, adults or final instar nymphs were collected in order to prevent controlled laboratory conditions from influencing factors such as body size (61 adults and 37 nymphs). There was no significant difference in the distribution of adults and nymphs among the five locations ($\chi^2 = 6.182$; $P = 0.186$). Alternate males were excluded from the analysis due to their low occurrence (n = 4), and were only found at Springbok.

Animals collected in the field were transported to the University of the Western Cape. Nymphs were raised to adulthood in the laboratory under identical rearing conditions. Adult females and nymphs were housed in small groups (between one and three individuals) in opaque plastic storage bins with a wire mesh top to allow for airflow and light. To prevent fighting, adult males were kept separate from each other. Grasshoppers were maintained under natural lighting conditions in a temperature-controlled room maintained at approximately 25 °C. Individuals were fed ad libitum either on the host plant species on which they were found or, if this was unavailable, on lettuce leaves. Grasshoppers were examined daily to replenish their water and food, and to determine their moulting.

Sound recording and analysis

To record male advertisement calls, individual adult males were placed in a clear plastic container (230 mm diameter × 240 mm high) fitted with a plastic mesh cover on top. Spontaneously calling males were recorded using a Marantz PMD-670 digital recorder (48 kHz sampling frequency; 16-bit resolution per sample) and a Sennheiser K6/ME-66 microphone. The tip of the microphone was positioned at a distance of approximately 1 m in front of the calling male. Songs of 44 males were recorded. These sound files were downloaded onto a computer and analysed using Raven Pro 1.3 software (Cornell Bioacoustics Research Programme).
Bullacris unicolor produces a relatively short advertisement call (< 3 sec) with only two short introductory syllables preceding the final long resonant syllable, and a carrier frequency of around 2 kHz (Fig. 2). The song recordings were characterised in terms of their temporal structure and frequency spectra. All the measurements were done after filtering background noise to remove frequencies below 500 Hz. Ten calls per male were analysed. For each call we measured seven characteristics: length of the introductory syllables, inter- syllable pause, length of the final syllable, entire call length, peak frequency of the introductory syllables, peak frequency of the final syllable, and the rise time (time taken to reach maximum amplitude) of the final syllable (Fig. 2).

Morphology

Morphometric measurements of both male and female grasshoppers were made to the nearest 0.01 mm using digital callipers. Measurements were made on the right side of each specimen in order to standardise results. Following Donelson and van Staaden (2005) a series of seven linear measurements (mm) were obtained, which included antennal length, body length, head width, abdomen width, femur length, tibia length and pronotum length. Antennal length (AL) was measured from the base of the antenna to its tip. Body length (BL) was measured from the most anterior point of the head to the end of the abdomen. Head width (HW) was measured immediately behind the compound eyes. Male abdomen width (AW) was measured from the point directly between the two stridulatory ridges, and female abdomen width was measured from the point directly between the second and third abdominal segments. Hind femur length (FL) was measured from the point of articulation with the trochanter to the point of articulation with the tibia. Hind tibia length (TL) was measured from the point of articulation with the femur to the point of articulation with the tarsus. Pronotum length (PL) was measured linearly from the base of the ridge where it meets the head to its pointed end.

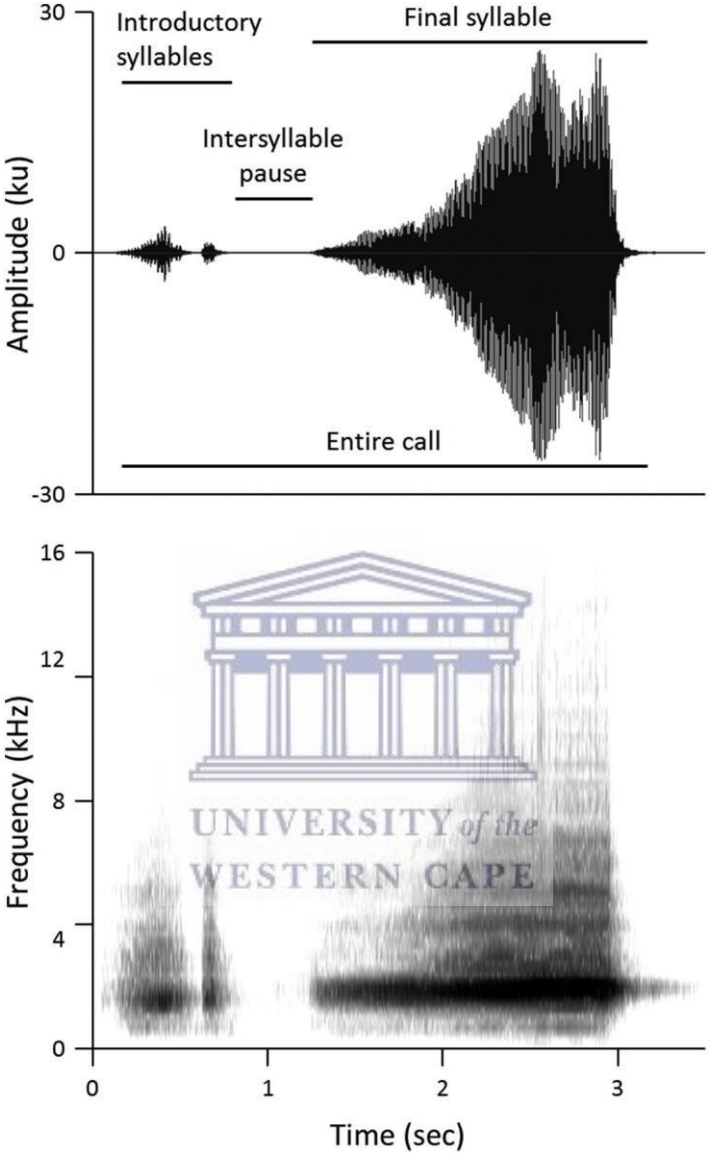

Fig. 2. — Oscillogram (top) and sonogram (bottom) of the male advertisement call of Bullacris unicolor.

Genetic analysis

Genomic DNA was extracted from the hind legs of ethanol-preserved specimens using standard phenol chloroform extraction procedures. Polymerase chain reactions (PCRs) were con-ducted using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) to amplify a 708-bp fragment cyto-chrome oxidase I (COI) mitochondrial gene. Each PCR reaction contained 14.9 µL of distilled

water, 2.5 µL of 25 mM $MgCl_2$, 2.5 µL of 10x Mg^{2+}-free buffer, 0.5 µL of a 10 mM dNTP solution and 0.5 µL (10 mM) of the respective primer pairs, 0.1 µL of Taq polymerase and 2−5 µL of template DNA.

PCR followed standard protocols with the following temperature cycle: 94 °C for 4 min, 94 °C for 30 sec, 48 °C for 45 sec and 72 °C for 35 sec. The last three steps were repeated for 40 cycles, followed by a final extension of 15 min at 72 °C. Five microlitres of amplified product was electro-phoresed on 1% of agarose gel, stained with ethidium bromide and observed under a UV transillu-minator to confirm whether the amplification was successful. An automated sequencer (ABI 3100, applied Biosystems) was used to run cycle sequencing products. Sequences were aligned using BIOEDIT sequence alignment editor, version 7.2.5. All the sequences were compared and blasted with other sequences of grasshoppers on GENBANK to authenticate the sequences. To further evaluate the functionality of the COI sequences they were checked on EMBROSS/Transec (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html). The GENBANK sequences of the more distantly related Melanoplus bivittatus and closely related Bullacris membracioides were used as outgroups.

To characterise the mode of nucleotide substitution, J MODELTEST version 3.06 was used (Posada & Crandall 1998). The best-fit maximum-likelihood (ML) score was chosen using the Akaike information criterion (AIC) (Darriba et al. 2012). Further analysis was done using Bayesian inference, with Mr Bayes version 3.2 (Ronquist & Huelsenbeck 2003). Ten Monte Carlo Markov chains were run for five million generations of which 10% were burn-in. The runs were stopped by the value of standard deviation of split frequencies below 0.01.
We conducted population genetic tests using DNAsp version 4.50 (Librado & Rozas 2009). For each sampled population, we estimated haplotype (h) and nucleotide (π) diversities. We used Tajima's D test (Tajima 1989) and Fu's F_S test (Fu 1997) to test the population changes and gene selection. Mismatch distribution was used to reveal the demographic history of the population. To understand the number of base substitutions per site between sequences we computed pairwise distance analysis in PAUP*4 version beta 10 (Swofford 2002). An intra-specific phylogeny was constructed with the unweighted pair group method with arithmetic mean (UPGMA) using Splits tree version 4.13.1 (Huson & Bryant 2006). Tests for the relationship between geographic distance and genetic distance were determined in Alleles in Space (AIS) version 1.0 (Miller 2005).

Statistical analysis of acoustic and morphological characters

All statistical tests were performed with IBM SPSS version 22, and graphics generated with JMP 12. To assess the variation in both morphological and acoustic characters between different groups we performed multivariate analysis of variance (MANOVA), and canonical discriminant analysis.

We tested for differences among individuals according to geographic location. Separate MANOVAs were used to analyse morphological and acoustic data. For the acoustic data, measure-ments from 10 calls per male were first averaged and the mean values used in further statistical analyses. We used the seven acoustic variables as dependent variables and location as the inde-pendent variable. Similarly, for the MANOVAs on morphological data, the seven morphological measurements were dependent variables and location was the independent variable. Males and females are sexually dimorphic and morphological data from males and females was therefore analysed separately. Where the assumption of equality of covariance matrices (tested using Box's test) could not be met for the MANOVA, we used Pillai's criterion as our multivariate statistic; otherwise Wilks' lambda was used. Following each MANOVA, univariate ANOVAs with Tukey multiple comparison tests were used to determine which individual characteristics differed among locations.

To examine patterns of separation between grasshoppers from different areas in more detail, we performed discriminant function analysis (DFA) using the same data as the MANOVAs. This technique predicts group membership based on combinations of the original variables, and iden-tifies which of these variables are the best predictors of the defined groups. Standardised coeffi-cients of the first discriminant functions thus estimate the degree of morphological and acoustic

divergence, with higher values indicating greater divergence of the variable. Canonical centroid plots were created to visually represent how the populations differed from each other.

Relationships among genetic, morphological and acoustic distances

To test for relationships between genetic, morphological and acoustic data, we conducted Mantel and partial Mantel tests, controlling for geographic distance, with 10,000 permutations using the Vegan package in R version 3.0.2. Acoustic and morphological distance matrices were created from Euclidean distances of averaged population traits, and the genetic distance matrix from F_{ST} values. Geographic distances were calculated from global positioning system (GPS) coordinates taken at each location.

RESULTS

Morphological variation of males and females

Mean values and standard deviations for morphological characters of males and females are shown in Table 1A–B. MANOVA results revealed significant differences in the morphological characters of both males (Wilks' Lambda = 0.271, $F_{28,164} = 2.555$, $P < 0.001$) and females (Pillai's Trace = 1.498, $F_{28,92} = 1.968$, $P = 0.009$) among popula-tions. Males differed in head width, body length and pronotum length (Table 1A), and females differed in head width, abdomen width and pronotum length (Table 1B). In addition, the femur length of females was only marginally non-significant between populations ($P = 0.082$).

From the DFA conducted on males, DF1 explained 75.4% of total variance and DF2 explained 15.1% (Fig. 3a). Body length and abdomen width showed the highest correlation with DF1, whereas antennae and body length showed the highest correla-tion with DF2. Males from Cederberg form a distinct cluster, whereas there is some degree of overlap between males from the remaining four areas (Fig. 3a). For females, DF1 explained 66.7% and DF2 22.6% of the total variance (Fig. 3b). Head width and tibia length show the highest correlation with DF1, and tibia and pronotum length show the highest correlation with DF2. Females from Melkbosstrand form a more distinct cluster than females from other areas, while there is some overlap between Kamieskroon and Springbok and between Cederberg and Groenriviersmond (Fig. 3b).

Acoustic variation of males

Visual inspection of calls among populations revealed the absence of an inter syllable pause in the calls of individuals from Melkbosstrand, whereas individuals from the other populations show more similar song types (Fig. 4). Mean values and standard deviations for acoustic characters of males are shown in Table 2. MANOVA results showed that acoustic characters of males from the five areas differed significantly (Pillai's Trace = 2.203, $F_{28,144} = 6.308$, $P < 0.001$), with all except one of the measured characteristics (peak frequency of the introductory syllables) differing among locations.

The DFA on the acoustic characters of male calls revealed that DF1 explained 78.0% of the total variance and DF2 explained 12.3% (Fig. 5). Length of the entire call and length of the final syllable were the two variables most strongly correlated with the

Table 1.

Morphological measurements (mm), expressed as mean and standard deviation, of Bullacris unicolor from five locations, and analysis of variance (ANOVA) results of the variation in morphological characters of (A) males and (B) females. Abbreviations: AL = antennae length; HW = head width; BL = body length; AW = abdomen width; FL = femur length; TL = tibia length; and PL = pronotum length. Superscript letters indicate significant pairwise differences, with different letters indicating a significant difference ($P < 0.05$; Tukey multiple comparison tests).

(A)	Springbok	Kamieskroon	Groenriviersmond	Cederberg	Melkbosstrand		
Variable	(n = 17)	(n = 18)	(n = 8)	(n = 6)	(n = 11)	$F_{4.51}$	P
AL	6.18± 1.03	6.00± 0.73	5.75± 0.95	6.15± 0.59	6.51± 1.02	0.849	0.501
HW	4.57± 0.25^{ab}	4.47± 0.21^{ab}	4.33± 0.16^{ab}	4.22± 0.15^{a}	4.70± 0.44^{b}	3.971	0.007*
BL	38.38± 1.79^{a}	39.61± 2.04^{a}	37.74± 1.75^{ab}	35.05± 1.28^{b}	39.84± 2.23^{a}	7.776	< 0.001*
AW	10.90± 0.77	11.49± 0.82	10.72± 0.71	11.20± 0.52	11.13± 0.71	1.912	0.123
FL	11.02± 0.48	10.88± 0.82	10.75± 0.61	10.35± 0.25	11.18± 0.57	1.579	0.194
TL	11.44± 0.73	11.24± 0.63	10.99± 0.34	11.05± 0.59	11.57± 0.62	1.297	0.284
PL	17.19± 0.85^{a}	17.33± 1.10^{ac}	16.68± 0.60^{ab}	15.91± 0.47^{b}	18.23± 0.60^{c}	7.263	< 0.001*
(B)	Springbok	Kamieskroon	Groenriviersmond	Cederberg	Melkbosstrand		
Variable	(n = 12)	(n = 5)	(n = 6)	(n = 6)	(n = 5)	$F_{4.26}$	P
AL	5.67± 0.59	5.91± 0.58	5.17± 0.74	5.27± 0.47	5.74± 1.68	1.080	0.387
HW	5.51± 0.36^{a}	5.84± 0.51^{a}	4.92± 0.29^{b}	4.85± 0.19^{b}	5.80± 0.19^{a}	9.065	< 0.001*
BL	38.61± 3.55	38.29± 2.02	35.43± 1.86	39.01± 4.68	39.15± 0.70	1.163	0.350
AW	8.70± 0.71^{a}	9.35± 0.31^{a}	7.41± 0.59^{b}	7.59± 1.43^{ab}	8.67± 0.26^{ab}	4.625	0.006*
FL	10.45± 0.86	11.11± 0.12	9.96± 0.72	10.25± 0.27	11.41± 0.75	2.336	0.082
TL	11.23± 0.89	11.50± 0.26	10.69± 0.68	10.90± 0.39	11.21± 1.40	0.829	0.519
PL	21.8± 1.08^{a}	21.89± 1.92^{abc}	20.20± 0.69^{b}	20.73± 0.99^{ab}	24.50± 1.26^{c}	7.049	0.001*

* Significant, $P \leq 0.007$ (after Bonferroni corrections for multiple tests).

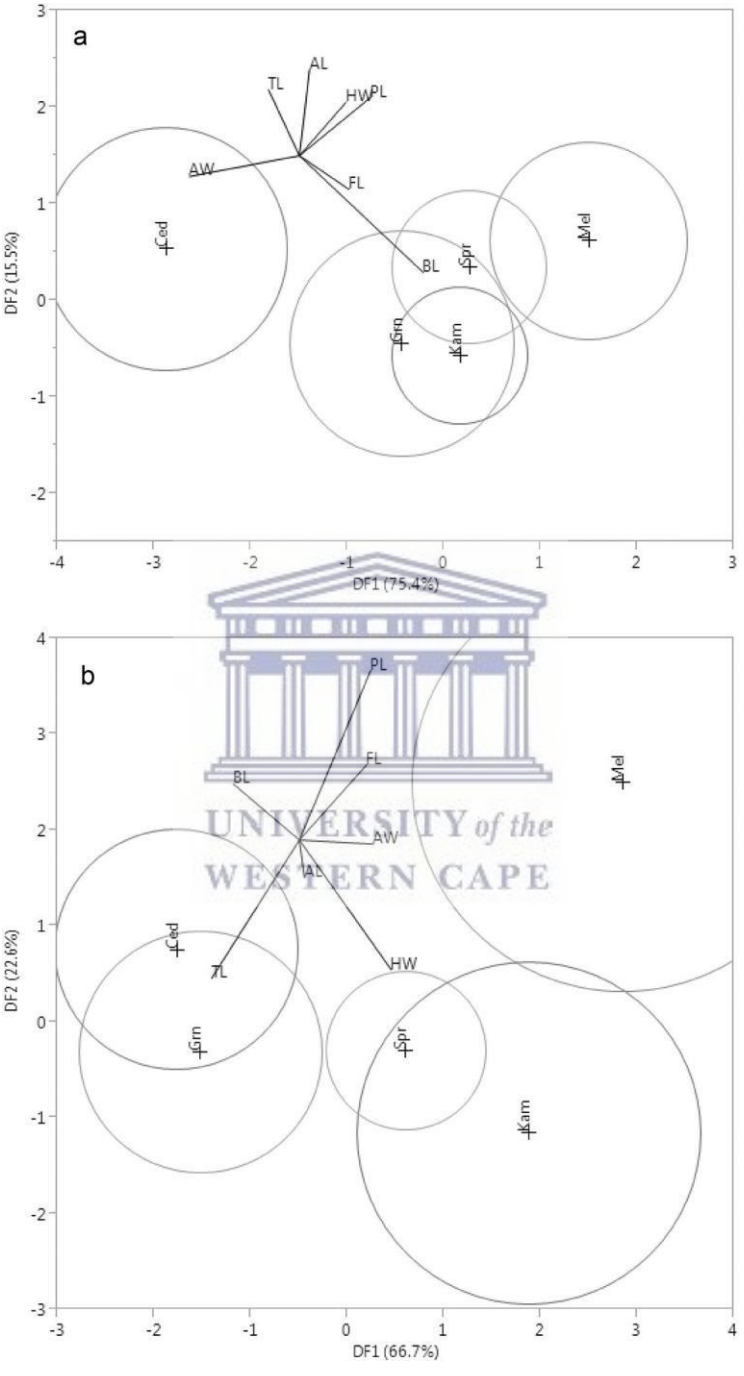

Fig. 3. — Canonical centroid plots of the discriminant function analysis for morphological data for (a) males and (b) females. Abbreviations: Ced = Cederberg; Grn = Groenriversmond; Kam = Kamieskroon; Mel = Melkbostrand; Spr = Springbok; AL = antennae length; HW = head width; BL = body length; AW = abdomen width; FL = femur length; TL
= tibia length; and PL = pronotum length. Circles indicate 95% confidence intervals for group means and radiating lines indicate the strength and direction of association of individual variables with discriminant functions.

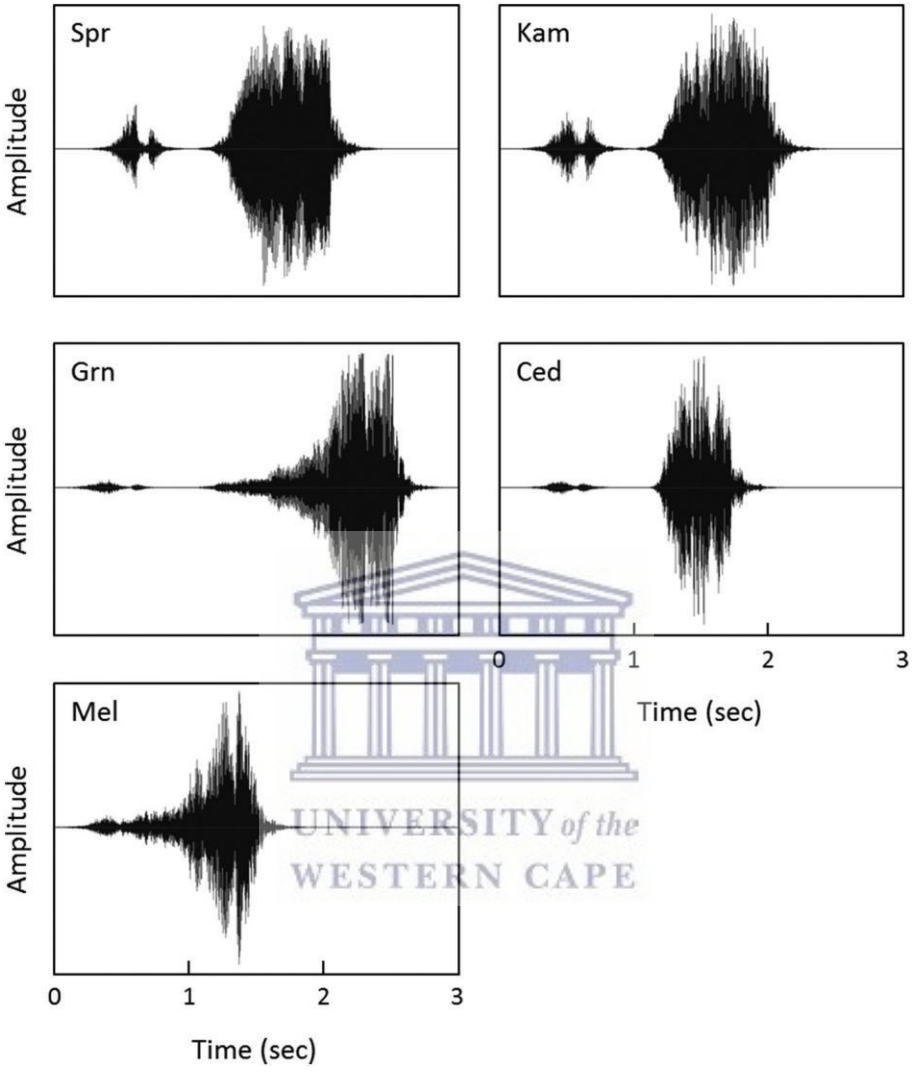

Fig. 4. — Oscillogram exemplars of Bullacris unicolor male calls from the five locations. Spr = Springbok; Kam = Kamieskroon; Grn = Groenriviersmond; Ced = Cederberg; Mel = Melkbosstrand.

first two discriminant functions. The only overlap observed was between male calls from Springbok and Kamieskroon; otherwise calls from the different areas formed very distinct clusters (Fig. 5).

Table 2.

Measurements of the acoustic characteristics of Bullacris unicolor males (n = 44) from five locations, expressed as mean and standard deviation, and results of analysis of variance (ANOVA) testing for differences in each characteristic according to location. Superscript letters indicate significant pairwise differences, with different letters indicating a significant difference (P < 0.05; Tukey multiple comparison tests).

Springbok		Kamieskroon	Groenriviersmond	Cederberg	Melkbosstrand	F 4.39	P
Length of intro. syllable (sec)	0.439 ± 0.101^{ab}	0.518 ± 0.080^{ab}	0.583 ± 0.048^{a}	0.407 ± 0.097^{b}	0.240 ± 0.224^{c}	12.815	< 0.001*
Intersyllable pause (sec)	0.177 ± 0.107^{a}	0.146 ± 0.068^{a}	0.508 ± 0.076^{b}	0.381 ± 0.083^{b}	0^{c}	33.041	< 0.001*
Length of final syllable (sec)	1.586 ± 0.289^{ac}	1.431 ± 0.251^{ac}	1.699 ± 0.152^{a}	0.881 ± 0.124^{b}	1.460 ± 0.528^{bc}	10.875	< 0.001*
Length of entire call (sec)	2.192 ± 0.432^{a}	2.098 ± 0.319^{ac}	2.791 ± 0.214^{b}	1.659 ± 0.271^{cd}	1.594 ± 0.742^{d}	11.385	< 0.001*
Peak freq. of intro. syllables (Hz)	2331.10 ± 573.51	2199.27 ± 349.87	2431.87 ± 222.01	1914.95 ± 324.02	1400.27 ± 309.21	1.412	0.248
Peak freq. of final syllable (Hz)	2102.67 ± 121.03^{ab}	2179.18 ± 122.58^{b}	2238.75 ± 109.81^{b}	2196.18 ± 75.23^{b}	1876.92 ± 378.01^{a}	4.013	0.008*
Rise time of final syllable (sec)	1.166 ± 0.327^{a}	0.831 ± 0.285^{b}	1.243 ± 0.139^{a}	0.430 ± 0.077^{c}	0.914 ± 0.201^{ab}	10.385	< 0.001*

* Significant, P ≤ 0.007 (after Bonferroni corrections for multiple tests).

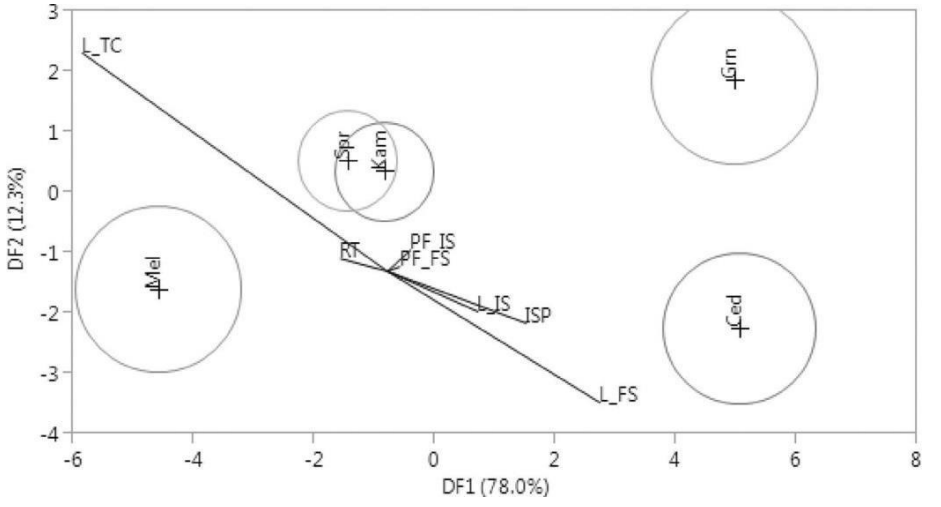

Fig. 5. — Canonical centroid plot of the discriminant function analysis showing the separation of male advertisement calls according to location. Abbreviations: Ced = Cederberg; Grn = Groenriversmond; Kam = Kamieskroon; Mel = Melkbostrand; Spr = Springbok; L_IS = length of the introductory syllables; ISP = inter-syllable pause; L_FS = length of the final syllable; L_TC = total call length; PF_IS = peak frequency of the introductory syllables; PF_FS = peak frequency of the final syllable; and RT = rise time of the final syllable. Circles indicate 95% confidence intervals for group means and radiating lines indicate the strength and direction of association of individual variables with discriminant functions.

Population genetic analysis

After trimming short end sections of the COI sequences, 654 bp were obtained from 44 specimens. The aligned COI fragments were comprised of 29 mitochondrial haplotypes, 126 substitutions and 113 polymorphic sites. The general time-reversible model with a proportion of invariable sites (GTR+I) was selected as the model of substitution using the AIS criteria. The mean number of pairwise difference was 1.958% (Table 3). Overall nucleotide diversity was 0.048 (ranging from 0.001 to 0.024) and haplotype diversity was 0.966 (ranging from 0.286 in Groenriversmond to 1.000 in Springbok) (Table 4). There was no significant variation in base composition between sequences (C_2 = 171.356, df = 112, P = 0.01).

A phylogenetic tree reconstructed from DNA sequencing data indicates five major haplotype lineages (Fig. 6). Both Cederberg (L2) and Groenriviersmond (L4) are clearly separated into distinct lineages, and do not share haplotypes with any other location. Melkbosstrand is divided into two lineages (L3 and L5). All of the individuals compris-ing lineage 5 were collected from the same area, which is separated from other collect-ing localities in the same Melkbosstrand region by urban development. The remaining lineage (L1) comprises a mixture of individuals from Kamieskroon and Springbok.

The genetic analysis revealed strong genetic structuring between locations. From the results of the pairwise distance analysis, sequence divergence among individuals within the same population was generally small, with the exception of grasshoppers from Springbok, which showed more genetic variation (4.10%) compared to other populations (Table 3). There was no difference in the results when data was reanalysed excluding individuals from this population. On the other hand, sequence divergence within Groenriviersmond was extremely low (0.09%) (Table 3).

Table 3.

Population pairwise genetic differentiation (mean ± standard deviation) within (bold) and between sampled populations of Bullacris unicolor.

	Springbok	Kamieskroon	Groenriviersmond	Cederberg	Melkbosstrand
Springbok	4.10 ± 0.02				
Kamieskroon	4.95 ± 0.02	1.22 ± 0.02			
Groenriviersmond	4.65 ± 0.01	6.80 ± 0.01	0.09 ± 0.00		
Cederberg	5.23 ± 0.02	7.15 ± 0.01	3.75 ± 0.01	1.99 ± 0.04	
Melkbosstrand	5.66 ± 0.01	7.96 ± 0.01	2.97 ± 0.02	5.07 ± 0.01	2.37 ± 0.02

Table 4.

Genetic diversity and demographic parameters for 654 bp of cytochrome oxidase I (COI) mtDNA of Bullacris unicolor. N = sampled size; Nh = haplotype number; S = number of polymorphic sites; h = haplotype diversity; π = nucleotide diversity; Tajima's D, Fu's F_S. *P < 0.05, **P < 0.01.

Population	N	Nh	S	h	π	D	F_S
Springbok	9	9	71	1.0000	0.0405	0.074	− 0.247
Kamieskroon	10	7	30	0.8666	0.0121	− 1.209	− 1.512
Groenriversmond	7	2	2	0.2857	0.0008	− 1.237	− 1.374
Cederberg	8	5	52	0.7857	0.0198	− 1.894**	− 2.279*
Melkbosstrand	10	7	30	0.9111	0.0243	2.412**	1.915*

The haplotype diversity for the combined data set was high (h = 0.96), with a nucleotide diversity of P = 0.048 (average nucleotide differences k = 32.021). As five main lineages were retrieved, we calculated genetic indices for each lineage (Table 4). Springbok showed a high haplotype diversity (h = 1.00) and high nucleotide diversity (π = 0.040) compared to other locations. Conversely, Groenriversmond showed low haplotype diversity (h = 0.285) and very low nucleotide diversity (P = 0.0008). We applied the McDonald-Kreitman test to Springbok versus all other populations (P = not significant). We calculated Tajimas's D for the complete data set (D_T = 0.383, P > 0.100) and for all individual populations. Melkbosstrand and Cederberg sequences both showed a significant deviation from zero (Table 4).

Relationships among genetics, acoustics and morphology

Genetic distance was not significantly correlated with geographic distance (Mantel test: r = – 0.336, P = 0.867), suggesting that genetic differences between populations were not predicted by geographic distance. Partial Mantel tests controlling for geographic distance revealed that genetic distance was significantly correlated with morphological distances in males (r = 0.938, P = 0.025) and females (r = 0.728, P = 0.032), but not with male acoustic distances (r = 0.257, P = 0.233). Male and female morphological distances were significantly correlated with each other (r = 0.836,

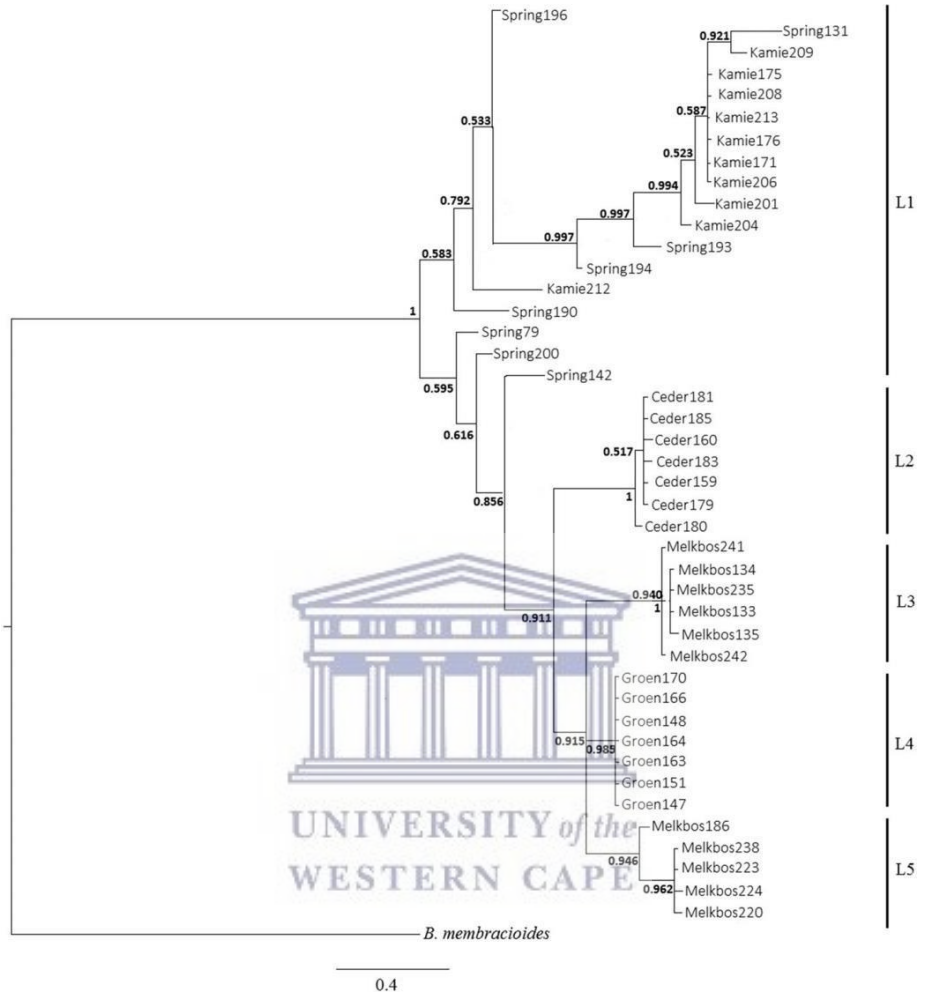

Fig. 6. — Linearised phylogenetic tree of Bullacris unicolor using cytochrome oxidase I (COI) genes (n = 43) based on 29 haplotypes, and rooted by Bullacris membracioides. Posterior probabilities of statistically well supported lineages which are > 0.95 are shown along the nodes. The five major lineages L1 to L5 that were retrieved are indicated by the vertical lines.

P = 0.008), while there was no relationship between male morphological and acoustic distances (r = 0.298, P = 0.283). Furthermore, geographic distance was not correlated with either acoustic distance (r = – 0.082, P = 0.672) or morphological distance (males: r = – 0.366, P = 0.942; females: r = 0.014, P = 0.475).

DISCUSSION

Our results reveal that Bullacris unicolor shows significant variation in morphol-ogy, male advertisement calls and genetic traits across its geographic range. However,

acoustic characters were much more strongly differentiated between populations than was morphology. In terms of morphology, males were found to differ significantly in body length, pronotum length and head width, whereas females differed significantly in pronotum length, head width and abdomen width. The reason why females were not found to differ in body length may be the fact that the abdomen of females can be extended and contracted, becoming distended with eggs, unlike the abdomen of males which is much more rigid and fixed in size (Dirsh 1965). Therefore, pronotum length may be a more reliable indicator of body size in females. This is supported by the observation that the body length and pronotum length measurements of males followed the same pattern of differentiation among populations, whereas female body length and pronotum length measurements showed little correlation with each other, and female pronotum lengths more closely mirrored the pattern of variation observed among male pronotum and body length measurements. Although females were found to differ significantly in abdomen width, egg development can also cause some degree of varia-tion in abdomen width, and so this result should be interpreted with caution.

The comparison of morphology across the spatial geographic range of the species was relatively complex and uncorrelated with geographic distance. Some morphologi-cal characters between populations that are close geographically show significant differences, whereas comparison of populations which are well separated in geographic space shows much less difference. For example, the body size of males from Melkbosstrand and Springbok showed slight overlap in discriminant function classifi-cation (Fig. 3a), despite the fact that these sampling locations are separated by a linear distance of ± 500 km. This may be because size differences did not follow a gradient from north to south, but rather that grasshoppers from areas with a similar temperature were more likely to be similar in size. Previous studies on other grasshopper species have shown that differences in morphology are linked to differences in local environ-mental conditions, particularly temperature (Gomez & Van Dyck 2012; Noguerales et al. 2016).

We found that the advertisement call of B. unicolor varies greatly on a geographic scale, both in temporal and in frequency components. Populations were very distinct in their call properties, showing very clear patterns of clustering (Fig. 5). Indeed, it was only Springbok and Kamieskroon that showed any overlap with each other. This over-lap of call characteristics between Springbok and Kamieskroon may be explained by migration (e.g. Attisano et al. 2013; Bell et al. 2013) promoting similarity among these two geographically adjacent populations. As the male call functions in mate attraction, these differences in the call may have implications for species recognition. Further research to investigate mating barriers among adjacent and distant population pairs will do much to shed light the extent of reproductive isolation of B. unicolor populations.

Interestingly, calls of males from Melkbosstrand completely lack pauses between the introductory and final syllables. Furthermore, males regularly omitted the intro-ductory syllables altogether, producing a call consisting of only the final syllable. When the introductory syllables were produced they were very short compared to those of other populations. This unusual adjustment of the call structure, together with the relatively low carrier frequency of the call, makes this population more acoustically distinctive than the other populations. This may be due to Melkbosstrand being the most southerly and geographically isolated of the sampled locations, or because of stronger selective pressures at this location. Melkbosstrand has a much greater degree of urbanisation compared to the other locations, and populations in urban

environments are typically more fragmented and also have to contend with higher levels of noise pollution. Previous studies have shown that anthropogenic noise significantly impacts animal sound communication systems in grasshoppers and other animals (McMullen et al. 2014). Further studies will test to what extent anthropogenic influ-ences are impacting sound communication in bladder grasshopper populations that exist in urban environments, and whether this could potentially lead to reproductive isolation. The genetic data points to the fragmented nature of the Melkbosstrand population, with individuals forming two distinct clusters (Fig. 6). These two genetic clusters within the Melkbosstrand population cannot be attributed to geographic dis-tances between individual samples, as the geographic distances separating sampled individuals is greater within the L3 cluster than the average distance between indivi-duals from L3 and L5. It is more likely that this clustering arrangement has resulted from isolation due to habitat fragmentation. However, these individuals did not show any significant morphological or acoustic differentiation, and so were still treated as a single population.

We found no significant correlation between acoustic and morphological dis-tances of males across the geographic range of the species, suggesting that differences in the physical characteristics of males have little influence on the sexual signalling system. However, there may be additional morphological characteristics that were not considered here which correlate more strongly with song production and thus acoustic variables. Alternatively, morphological and acoustic characters may be evolving semi-independently under different selective pressures. This is supported by the finding that males were much more strongly differentiated in acoustic than in morphological char-acteristics. Given the nature of the sexual signalling system in B. unicolor, it seems likely that the male call is assessed by females and that differences in the advertisement call can lead to differential male mating success. While no mate choice studies have been conducted in B. unicolor, a previous study of female preferences in the congeneric B. membracioides revealed that females do discriminate amongst males on the basis of their advertisement calls (Couldridge & van Staaden 2006). Further study of female preferences in B. unicolor will provide insights as to whether mate choice is contribut-ing to signal diversification.

The genetic structure of B. unicolor was high along its distribution range. Five distinct mitochondrial phylogenetic clades could be identified, and these coincided with collecting locality, indicating that our sampled populations have undergone genetic differentiation. Genetic data further indicated that Springbok has a higher number haplotypes, polymorphic sites, and haplotype and nucleotide diversity in comparison with the other sampled populations. The significant positive values of both Tajima's D and Fu's F_S from Melkbosstrand are indicative of a possible recent population bottleneck or decreasing population. This may be a consequence of the loss and fragmentation of habitat due to urban expansion in this area. Conversely, significant negative values in the Cederberg population suggest recent expansion in this population. Tajima's values for all other populations were non-significant, indicating that there have been no recent demo-graphic changes (Table 4). However, lower nucleotide diversity within Groenriversmond is suggestive of a possible recent population expansion in this area as well.

We found that genetic distances were correlated with morphological distances only, and not with acoustic distances. An association between genetic and acoustic traits has been reported in some taxa (Amézquita et al. 2009; Velásquez et al. 2013), but not in others (Ruegg et al. 2006; Ohmer et al. 2009; Rodríguez-Tejeda et al. 2014). These contradictory results reported in the literature may reflect the complex and intertwined nature of the evolutionary processes at play. An observed lack of

association with genetic markers is, however, not unexpected for traits important in mate recognition and mate choice, as these traits are generally under strong selective pressure. We also found that genetic distance was not significantly correlated with geographic distance. While geographically distant populations are generally expected to be more genetically isolated, this relationship may not hold true if other factors are also contributing to patterns of genetic differentiation (Pröhl et al. 2006). It should be borne in mind that our study made use of mitochondrial DNA (mtDNA) markers, which may yield different results to nuclear DNA markers due to their different modes of inheritance. A previous study on the grasshopper Mioscirtus wagneri found that morphological variation was significantly linked to microsatellite, but not to mtDNA, markers (Ortego et al. 2012). Different types of genetic markers may thus be more informative at different spatiotemporal scales, and future studies should make use of a wider range of genetic markers to gain further insights into patterns of genetic divergence. Furthermore, we made use of a relatively small number of populations in order to infer relationships, and future sampling from a larger number of locations would be needed to confirm these results.

Our findings suggest a high degree of morphological, acoustic and genetic diver-gence within B. unicolor. However, the relationships between morphology, acoustics, genetics and geography were found to be fairly complex, demonstrating the intricate nature of the forces behind allopatric divergence, and the importance of considering multiple traits when examining intraspecific variation. The lack of correlation between the degree of acoustic variation and both genetic and morphological variation suggests that differences in male advertisement calls have not arisen through neutral variation, but instead may be attributed to alternative selective pressures, such as female mating preferences or ecological conditions. A detailed examination of these alternative factors will do much to clarify the observed patterns of geographic variation in this species.

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DISCLOSURE STATEMENT

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