An acoustic, genetic and morphological review of the genus *Bullacris* (Orthoptera; Pneumoridae)

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Abstract

Bladder grasshoppers (Orthoptera: Pneumoridae) are nocturnal African herbivores that are endemic to the coastal regions of southern Africa. They rely heavily on sound communication for mate location and have a unique body structure, with an inflated abdomen seen only in males that aids in sound production. They have a continuous distribution that extends along the coast of South Africa from Namibia, into the eastern regions of Mozambique and beyond, as far as Uganda. *Bullacris* is the largest genus within the Pneumoridae family. Members of this genus are mostly found within South Africa and inhabit different vegetation biomes, namely the Succulent-Karoo, Fynbos and Savannah biomes. There are currently seven described species within the genus, based solely on morphological differences. However, these morphological differences are not well defined for all species pairs, leading to some degree of uncertainty in species delineation.

There have been no previous genetic studies looking at taxonomic relationships within the group. Furthermore, despite sound communication being an integral part of their biology and behaviour, male advertisement calls have not previously been compared across species to examine the extent of acoustic divergence between sister species. Therefore, this thesis aims to examine relationships within the *Bullacris* genus, based on morphological, acoustic and genetic differences.

A distribution map was created for each species by using the co-ordinates recorded upon collection, as well as museum collection data. Morphological measurements were obtained of nine linear measurements for male and female specimens. Acoustic analyses were conducted by recording male advertisement calls, with the exception of the species *B. boschimana*, and measuring temporal and frequency properties. Statistical analyses were then performed in which the morphological and acoustic data were compared and significant relationships reviewed.

Genetic analyses were performed on the mitochondrial (COI) and nuclear (ITS) gene regions of each species and phylogenetic relationships investigated. A mantel test was performed to correlate species pairwise differences in acoustic variables, with pairwise differences in morphology, as well as genetic pairwise distances.

It was found that by comparing acoustic and morphological characteristics, each *Bullacris* species was unique and could be classified as individual species, however, genetic analyses showed otherwise. It illustrated that *B. intermedia* does not form its own clade (as the rest of the species) and suggests that it may form part of *B. unicolor* instead. In addition, there were no significant correlations between the three datasets, thus proposing that morphology has no major influence on acoustic differences between species, nor does the genetic distance between species correlate with the differences in acoustics and morphology.

The results of this study have shown that species should not be distinguished based solely on one feature such as morphology or acoustics, but rather as a combination of attributes. Genetic analyses have slightly altered the classification of Dirsh (1965) by 'grouping' *B. unicolor* and *B. intermedia* thus suggesting *B. intermedia* as a junior synonym species for *B. unicolor*. However, it is possible that genetic analysis of *B. intermedia* was not sufficient to claim it being part of the *B. unicolor* lineage due to relatively small sample sizes for this species

Keywords: Pneumoridae, *Bullacris,* genetics, morphology, acoustics.

Declaration

I declare that this thesis, entitled **An acoustic, genetic and morphological review of the genus *Bullacris* (Orthoptera; Pneumoridae)**, is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Full name: Mikhaila Leigh Gordon **Date:** 02/ 02/ 2017

Signed:

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Chapter 1

General introduction

1.1. Background

Bladder grasshoppers (Orthoptera; Pneumoridae) encompass an ancient family of Acridid grasshoppers (Dirsh, 1965; Flook and Rowell, 1997a). Between the years 1775 and 1810, Thunberg originally labelled a number of *Bullacris* species as *Pneumora*, but this was later changed by Roberts in 1941. Little is currently known about this group, but molecular data indicates that the family is relatively ancient, possibly dating back to the Jurassic era (Flook and Rowell, 1997b). However, more recent taxonomic studies by Flook *et al*., 2000, Song, 2010 and Song *et al*., 2015 have looked at the phylogeny of families within the order Orthoptera. Their findings grouped bladder grasshoppers into the superfamily Pneumoroidae due to having distinctive characteristics.

The most recent taxonomic review of the family Pneumoridae was compiled by Dirsh in 1965, in which he described nine genera and 18 species, based on morphological characteristics, unique within the family. However, in his descriptions, Dirsh (1965) commented on the similarities in morphology of some of these species and noted the possibility that some of them may represent geographical races rather than distinct species.

The genus *Bullacris* has a continuous distribution that extends along the coast of South Africa, from the border of Namibia into the eastern regions of Mozambique; however there is a record of a single *Bullacris membracioides* specimen collected in Malawi (Dirsh, 1965). Each of these species can be found in various environments ranging from Albany Thicket to Succulent-Karoo biomes (Figure 1.1). This therefore indicates climatic, vegetation and habitat differences among species.

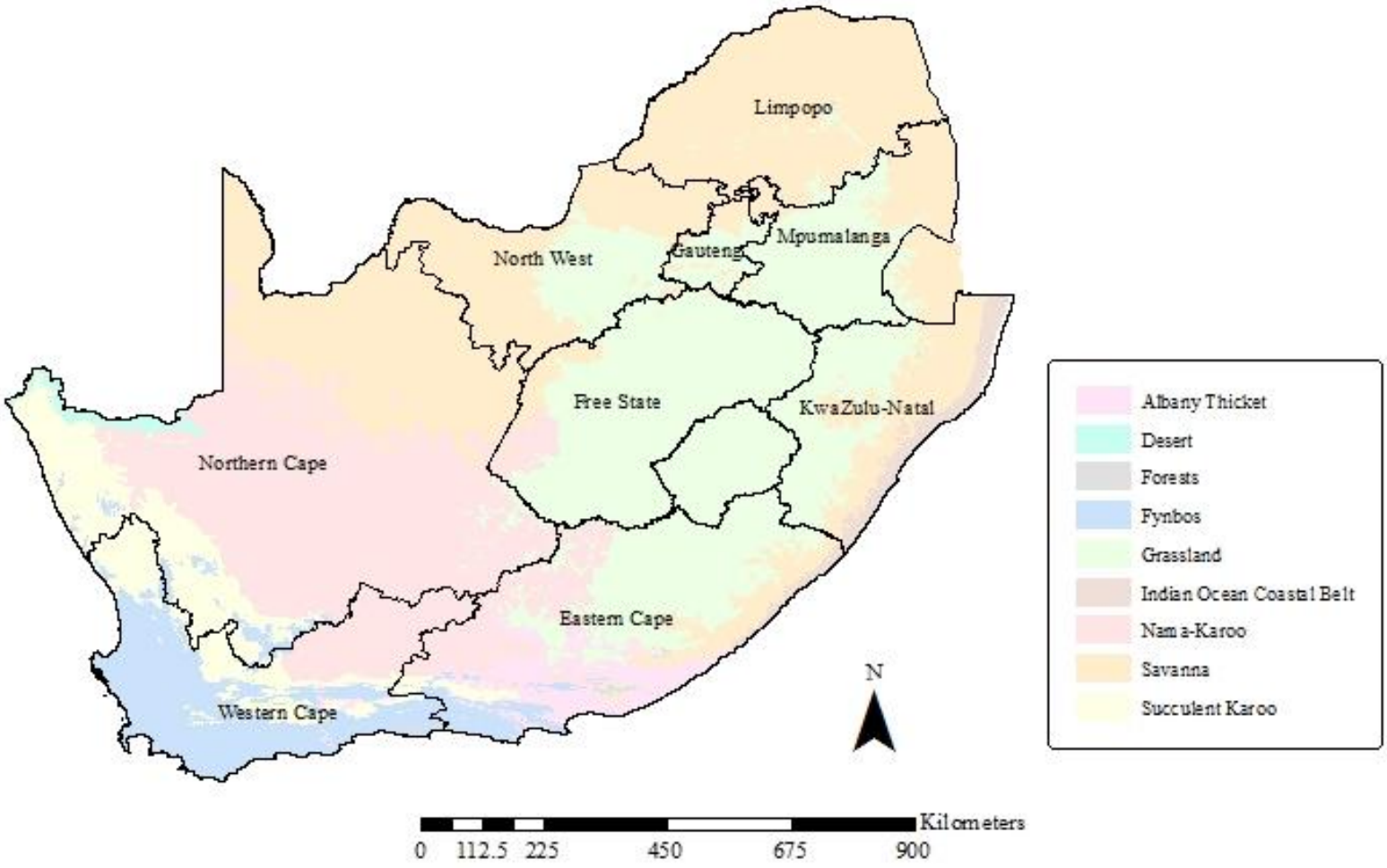

Figure 1.1: Biomes of South Africa (2006), from BGIS website created using ArcGIS 10.1.

1.2. Morphology

Studying the morphology of grasshoppers contributes to the understanding of how they function and survive. It is the description of their physical form that provides information on how certain structures are specialized and adapted. An advantage of morphological studies, is the ability to resolve phylogenetic relationships of fossil taxa and their relationships to living taxa (e.g. Maddison, 1996; Hillis and Wiens, 2000; Jenner, 2004). In addition, morphological traits are useful for making initial classifications of a species as well as possibly provide some information regarding its habitat. Even though morphological descriptions may be useful for the initial classification of species, it is not sufficient information to show that taxa may be reproductively isolated. An example of phase polyphenism can be seen in a study conducted by Uvarov (1921), in which two locust species were initially classified as separate taxa due to being in different metamorphism phases. Therefore, since taxonomists no longer rely solely on morphological differences, there is a need to revise this genus, not only morphologically but acoustically and genetically as well.

Speciation is the development of a new species when populations from a particular species genetically diverge into a reproductively isolated group (Lawrence, 2008). It is the promotion of genetic isolation that involves the accumulation of genetic differences that influences and alters the phenotypic appearance. An example of this is found in a study by Stange and Ronacher (2012), in which four geographically different populations of the *Chorthippus biguttulus* grasshopper were morphologically investigated. Their results showed that the body size of males and females differed between populations.

Bladder grasshoppers are sexually dimorphic, with males having an inflated abdomen and the ability to fly, which develops at the final moult. Females lack the inflated abdomen and are

micropterous and therefore have a strong host plant association, some even to a single species (van Staaden, pers. com.). An alternate form to the dominant inflated form was discovered in three pneumorid species by Donelson and van Staaden (2005), in which the males lack the air-filled abdomen and are incapable of flight. It was discovered that these males make use of the eavesdrop tactic on the duets of inflated males and females to exploit the acoustic mate location system (Donelson and van Staaden, 2005); whereas the winged males are responsible for mate localization by reciprocal duet.

1.3. Acoustics

Acoustic communication is a fundamental part of the biology and behaviour of many insects (Boake, 2002) and has been shaped by evolutionary processes (Bradbury and Vehrenkamp, 1998). It is a means of mate location by recognition of signals, the ability to localize the sound source and significant information (i.e. the identity of the signaller) (Gerhardt and Huber, 2002). The information given off by an individual is unique and a crucial component to the nature of species in an attempt to avoid hybridization. The basis on which females choose between males of the same species for reproductive purposes is determined by the information given off within signals that indicates the quality of the individual (Brown *et al*., 1996).

Sound communication is an integral part of the biology of pneumorid grasshoppers. The adult males have an inflated abdomen which functions as a resonating chamber and they are thus able to produce a very loud advertisement call (>98 dB Sound Pressure Level at 1 m), which can be heard at distances of up to 2 km by conspecific's (van Staaden and Römer, 1998). Further studies into the acoustic communication system in bladder grasshoppers have indicated that several selective pressures may influence the evolution of signals and signalling behaviour in

pneumorids (van Staaden and Römer 1997; 1998); van Staaden *et al*. 2003; Couldridge and van Staaden 2004; 2006). This includes the effects of habitat characteristics and weather conditions that may influence the transmission of signals, the alternative mating tactics of male morphs, as well as female mating preferences for signal characteristics, that may create sexual competition amongst males.

There has been significant progress with regards to animal communication research, specifically the general understanding of motor and sensory systems, evolution and speciation. Studies that have examined the relationship between the sound transmissions and physical properties of animal vocalizations within a variety of environments have revealed that individuals have morphologically and behaviourally adapted, in order to maximize the distance at which their signals may be transmitted (Marten and Marler, 1977; Bennet-Clark, 1998; Cocroft and Rodriguez, 2005).

It has been observed that long distance signallers are at a disadvantage when sound gradually becomes more degraded the further it propagates, which is directly influenced by environmental factors (Couldridge and van Staaden, 2004; Salaberria, 2010). There are many selective pressures that influence the optimal sound transmissions of signals, such as the physical habitat, weather conditions, urbanisation and noise pollution and signal masking (Endler, 1992; Wiley 2013; Morley *et al*., 2013). Therefore, factors such as absorption, refraction, reflection and the diffraction of sound waves into soil surfaces, surrounding vegetation and the atmosphere, are responsible for the degradation of signals (Wiley, 1978). These factors alter the amplitude, temporal structures and frequency content of acoustic signals (Forrest, 1994), therefore environmental conditions determine the distance at which signallers are able to communicate with conspecifics (Lang, 2000).

1.4. COI and ITS analysis

Phylogeography is used to describe the phylogenetic analysis of organismal data in the context of geographic distributions of organisms (Avise, 2000). The analysis of mitochondrial DNA in the early 1990's (Avise, 1989) at the species level resulted in the development of phylogeography, and was promoted as the molecular marker of choice due to the lack of recombination and presumed neutrality (Avise, 2000). The random effects of genetic drift causes changes in allele frequencies of DNA sequences, which are seen as novel mutations remain within populations and therefore results in a pattern of inherited sequences that can be used to deduce the genealogy of populations (Trewick and Morris, 2008). The amount of genetic differences or the distance between DNA sequences, suggests the length of time an existing barrier may have emerged, therefore the more genetically diverse species are from each other, the older the event that caused the split (Relethford, 2001; Brown, 2002; Trewick and Morris, 2008).

Mitochondrial and nuclear markers differ in their mode of inheritance and in the rate of evolution (Ballard and Whitlock, 2004). Mitochondrial DNA (mtDNA) has an accelerated rate of sequence evolution compared to nuclear DNA, which makes it perfectly suited for intra-specific variation and phylogeographical investigations (Brown *et al*., 1979; Brown *et al*., 1982). This genome does not recombine and is maternally inherited, therefore eliminating the effect of male dispersal abilities, which could homogenize genetic structures (Lansman *et al*., 1983; Avise, 1989; Avise, 1994). It has therefore been used extensively to understand the spatial distribution of genetic lineages within species and to identify the historical factor with the highest effect on the lineages spatial patterns (Avise, 1994).

Although there are many advantages to using mtDNA, it also has its limitations within population comparisons. When only making use of the mtDNA, to reveal the history of a species, only the maternal history is described (Zhang and Hewitt, 2003). In addition, mtDNA has shown to have pseudo-genes, which could lead to inaccurate evolutionary inference (Hlaing *et al*., 2009). Therefore, the use of nuclear DNA markers in conjunction with mtDNA is necessary. Combining these two data sets can reveal critical aspects of genealogical affinities. Unfortunately, nuclear DNA has a slow evolutionary rate in comparison to mtDNA, therefore receiving less information regarding taxa which have undergone recent divergence events (Zhang and Hewitt, 2003).

The COI (cytochrome oxidase I) mitochondrial gene used in this study has been widely applied to species level studies of insects (Caterino *et al*., 2000). This gene has proved useful in demonstrating close genealogical histories of morphologically and ecologically distinct taxa and revealing cryptic taxa to explore the spatial partitioning of the phylogenetic structure of species (phylogeography) (Funk *et al*., 1995; Szymura *et al*., 1996; Trewick, 2000). The nuclear gene used in this study is ITS (internal transcribed spacer) which has been classified as the universal DNA barcode marker for fungi (Schoch *et al*., 2012; Mahmoud and Zaher, 2015). However, this gene region is also popular for phylogenetic analyses at lower taxonomic levels in many taxa, including insects (Loxdale and Lushai, 1998; Wörheide *et al*., 2004).

Since *Bullacris* species are found in a number of different environments, it is highly likely that there is genetic variation, not only between species but also within (see Sathyan, *et al*., 2016). Currently, there is no published information regarding the genetic variation between *Bullacris* species, thus a phylogenetic study will be done to determine the genetic variation between species within the genus *Bullacris*. This will be done by examining mitochondrial (COI) and nuclear (ITS) genes.

1.5. Aims

There is currently very little published information regarding the genus *Bullacris* and its' species, although the acoustic communication system is reasonably well understood (van Staaden and Römer, 1997; 1998; van Staaden and Rieser, 2003; Couldridge and van Staaden, 2004; 2006). The existing and most current taxonomic information by Dirsh (1965), which is based on morphological comparisons, contains some uncertainties and additionally lacks the molecular approach and thus further research is required. Therefore this thesis aims to provide a taxonomic revision of the genus *Bullacris*, using contemporary methods combined with phylogentic reconstruction. This will be done by observing morphological, acoustic signal, genetic, as well as geographic, variation.

Chapter 2

Macro-evolutionary comparisons in morphological and acoustic adaptations between *Bullacris* species

Abstract

In 1965, the bladder grasshopper genus *Bullacris* was taxonomically reviewed in by Dirsh in which he described each of the seven known species based solely on morphological similarities and differences. Bladder grasshoppers are sexually dimorphic, with males having an inflated abdomen and the ability of flight, whereas the females lack the inflated abdomen and are micropterous. Communication between males and females are done by reciprocal duet-ting and this is responsible for mate location. Despite acoustics being a vital part of bladder grasshopper biology and reproduction success, the male advertisement calls of *Bullacris* males have not yet been compared across species. Therefore it is the aim of this chapter to compare and distinguish between the acoustic calls of *Bullacris* males as well as to differentiate between morphological characteristics between both male and female individuals from each of the known species.

Distribution maps show that species are mainly found along the coast of South Africa, occupying different habitat types. Morphological results have shown that each species is morphologically distinct, with the possible exception of females of *B. dicolor* and *B. serrata* which clustered together, which is in accordance with Dirsh (1965). *Bullacris membracioides* is found to have the largest male and female individuals, whereas *B. unicolor* has the smallest male and female individuals. According to Spearman's correlations, there is no significant relationship between total body length and carrier frequencies of males, however, there is a strong relationship between abdomen width and carrier frequency. Results for acoustic call properties

also indicate that each species is unique in terms of the sound signal produced. However, DFA analyses indicate that *B. unicolor*, *B. serrata*, and *B. discolor* share some similarities in acoustic signals, whereas *B. obliqua* has a much more distinct call from the rest.

In conclusion, results have shown that each species differs both morphologically and acoustically, thus broadly supporting the current classification by Dirsh (1965), however, there is some overlap between characteristics.

Keywords: Morphology, Acoustic signals, *Bullacris*, Distribution

2.1. Introduction

2.1.1 Background

Commonly known as the bladder grasshoppers, members of the genus *Bullacris* (Orthoptera: Pneumoridae) are almost exclusively endemic to South Africa, with a small number of specimen records from Malawi, Mozambique and Namibia. The first comprehensive taxonomic monograph of the family Pneumoridae was compiled by Dirsh (1965), in which species were described within the *Bullacris* genus; however, Dirsh's classifications were exclusively based on the differences between morphological characteristics. The key to each species which he described can be seen in Figure 2.1.

KEY TO SPECIES

MALES

1 (2) Antenna slightly club-like widened at apical part (Text-fig. 10). Supra-anal plate comparatively short and widely angular (Text-fig. 8) ***unicolor*** (Linnaeus)
2 (1) Antenna filiform. Supra-anal plate comparatively long and narrow, angular.
3 (6) Pronotum in profile highly arcuate (Text-figs. 11, 13).
4 (5) Pronotum in profile regularly arcuate (Text-fig. 11). Third episternum with brown patch. Size smaller (44–49 mm.) . . ***intermedia*** (Péringuey)
5 (4) Arc of pronotum, in profile, lower in prozona (Text-fig. 13). Third episternum without brown patch. Size larger (47–59 mm.) (Text-fig. 13) ***membracioides*** (Walker)
6 (3) Pronotum in profile low arcuate.
7 (10) Pronotum in profile regularly arcuate (Text-figs. 15, 17). Third abdominal tergite with 9–10 stridulatory ridges. Size larger (44–58 mm.).
8 (9) Pronotum without callosities. Veinlets of reticulation of elytra of the same colour as membrane. Sides of abdomen with ocellate pattern (Text-fig. 15) ***discolor*** (Thunberg)
9 (8) Pronotum with whitish callosities. Veinlets of reticulation of elytra darkened. Sides of abdomen with ocellate and marble pattern (Text-fig. 17) ***serrata*** (Thunberg)
10 (7) Arc of pronotum in profile lower in prozona. Third abdominal tergite with 13 stridulatory ridges. Size smaller (41–46 mm.) (Text-fig. 19) ***obliqua*** (Thunberg)

FEMALES

1 (4) Arc of pronotum in profile comparatively high (Text-figs. 11, 13).
2 (3) Smaller size (42 mm.) (Text-fig. 11) . . . . ***intermedia*** (Péringuey)
3 (2) Larger size (48–55 mm.) (Text-fig. 13) . . ***membracioides*** (Walker)
4 (1) Arc of pronotum in profile comparatively low.
5 (6) Pronotum narrow, slender (Text-fig. 10) . . . ***unicolor*** (Linnaeus)
6 (5) Pronotum comparatively wide, robust.
7 (8) Pronotum without dorsal callosities or with only traces of them (Text-fig. 15) ***discolor*** (Thunberg)
8 (7) Pronotum with dorsal callosities forming oblique whitish stripes.
9 (10) Dorsum of pronotum with convex sides and comparatively low obtuse median carina (Text-fig. 17) . . . . . ***serrata*** (Thunberg)
10 (9) Dorsum of pronotum with slightly concave sides and sharp median carina.
11 (12) General coloration greenish ; sides of abdomen with four rows of small whitish, oblique spots (Text-fig. 19) . . . . . ***obliqua*** (Thunberg)
12 (11) General coloration pale brownish ; sides of abdomen with two rows of large, whitish spots of irregular form (Text-fig. 21) . ***boschimana*** (Péringuey)

Figure 2.1: Key to *Bullacris* species according to Dirsh (1965).

The very wide distribution of *Bullacris* along the entire coast line of South Africa encompasses several different vegetation biomes. These species are known to occur in the Succulent-Karoo, Savanna and Fynbos biomes (van Staaden and Couldridge, 2004); however, looking at Figure 1.1 (see Chapter 1), the Grassland, Desert, Albany Thicket and Indian Ocean Coastal Belt biomes all occur along the coast and may additionally host members of this genus. Eco-geographical factors (latitude, altitude, temperature, *etc.*) have been shown to cause morphological gradations that are, in part, a response by the organism to the pressures exerted by its environment (Roff. 1986; Bidau *et al*., 2012). Varying conditions can have significant effects on the morphology of individuals (Chown and Gaston, 1999; Ashton, 2004; Lomolino *et al*., 2006). This would therefore suggest a distinct variation in morphological characteristics between species.

2.1.2 Morphology and Acoustics

Male bladder grasshoppers possess wings, whereas the females are micropterous (reduced wings) and have limited dispersal ability; therefore the males are responsible for mate location (Couldridge pers. com.). The males initiate this by using their inflated balloon-like abdominal bladder that acts as a resonating chamber, together with the stridulation of their hind-legs against their abdomen, to generate a very loud call (>98 dB SPL at 1 m). In *Bullacris membracioides* this call has a transmission distance of approximately 1.98 km at night (van Staaden and Romer, 1998). Based on the delectability and attractiveness of the call, the female will respond, which therefore allows the vagile male the opportunity to locate the female for mating (Couldridge and van Staaden, 2004).

Since bladder grasshoppers make use of long range acoustic communication, it is not unusual that physical properties of the environment may exert selection pressures on these calls (Couldridge and van Staaden, 2004). Therefore, based on the environment in which a species occurs, signals may differ in order to minimise excess attenuation and distortion (Ey and Fischer, 2009; Jain and Balakrishnan, 2011; Wilkins *et al*., 2012). A study by Couldridge and van Staaden (2004) tested the levels of attenuation and distortion of bladder grasshopper signals over certain distances in four distinct biomes, namely; the Succulent-Karoo, Fynbos, Savanna and Forest. They discovered that the species that occupied the Fynbos and Forest biomes had low levels of signal attenuation over distances in all environments (Couldridge and van Staaden, 2004). Therefore, it is possible that each species would have differences between call characteristics due to selective pressures resulting from differences in habitat acoustics.

2.1.3 Aims

It was therefore the aim of this study to compare morphological and acoustic characteristics between the known *Bullacris* species as well as to test for a correlation between morphological and acoustic variation. It is hypothesized that each species will be morphologically as well as acoustically distinct. A series of linear measurements (mm) were acquired from male and female specimens of each species, for morphological comparisons. In addition, sound recordings were obtained of male advertisement calls for each species and these were compared in terms of their temporal and frequency properties.

2.2. Materials and Methods

2.2.1. Geographical Distribution

Location data for each of the seven species of *Bullacris* was obtained from a database created using a combination of the GPS co-ordinates recorded upon collection of specimens in the field, as well as museum data. Information was used from five museums within South Africa with the largest collections of pneumorids. This included Iziko Museum, Albany Museum, Ditsong Museum, the ARC National Insect Collection, and the Durban Natural Science Museum. Additional records were also used from the university collections at Stellenbosch University and Rhodes University. Distribution maps were created using Arc GIS 10.3.1. Individual maps were created for each species, which included an overlay of the vegetation biomes of South Africa, as well as a combined map for all species, showing overlaps in distributions.

2.2.2 Morphology

Recent bladder grasshopper samples (2013- 2015) were collected as adults in the field in the Northern Cape, Western Cape and KwaZulu-Natal Provinces of South Africa (Table 2.1). Where there was insufficient material that could not be collected in the field, it was supplemented with museum specimens from archival collections, from the Iziko Museum in Cape Town; the ARC National Collection of Insects in Pretoria, and the Albany Museum in Grahamstown. Difficulties in locating certain species in the field due to species being very well camouflaged as well as scarce resulted in sample sizes being uneven across species (Table 2.1). It should be noted that only one female of *Bullacris boschimana* (type specimen) has ever been collected, and there are no male specimens available. This individual is not a misidentification as

it has unique characteristics and therefore is included in this study for completeness. However, this species was not used in any statistical analyses.

Table 2.1: Sample sizes and location information for specimens used in the morphological analyses.

Species	Locations	Sex	Collection Type	Count	Total
B. discolor	Betty's Bay, Ashton, Cape Town, East London, Bellville, Namaqualand	Male	Field	15	20
			Museum	5	
		Female	Field	30	32
			Museum	2	
B. unicolor	Kamieskroon, Springbok, Groenriviersmond, Melkbosstrand, Spektakel Pass, Citrusdal, Overberg, Namaquland, Garies, Daring, Cape Town, Langebaan	Male	Field	15	25
			Museum	10	
		Female	Field	15	20
			Museum	5	
B. intermedia	Grahamstown, Addo, Kowie, Transkei	Male	Field	3	13
			Museum	10	
		Female	Field	0	2
			Museum	2	
B. membracioides	Inchanga, Durban, Eshowe, Umkomaas	Male	Field	5	17
			Museum	12	
		Female	Field	8	9
			Museum	1	
B. obliqua	Groenriviersmond, Darling, West Coast National Park, Saldhana Bay, Namaqualand, Cape Town	Male	Field	15	17
			Museum	2	
		Female	Field	0	3
			Museum	3	
B. serrata	Grahamstown, Swartberg Pass, Cape Town	Male	Field	0	15
			Museum	15	
		Female	Field	0	6
			Museum	6	
B. boschimana	Henkries	Male	Field	0	0
			Museum	0	
		Female	Field	0	1
			Museum	1	

Once the animals were anesthetized, the specimens were preserved in 90% ethanol for later DNA extraction. A series of morphometric measurements for male and female specimens were obtained using a digital caliper, adjusted to the nearest 0.001 mm in a laboratory after its demise. All body parts were carefully measured on the right side, in order to standardize results. Following Donelson and van Staaden (2005), a series of nine linear measurements (mm) were acquired from each female and male specimen, which included the abdomen width (point directly between the two stridulatory ridges and female abdomen width was measured from point directly between the second and third abdominal segments), the length of the hind femur (from the base of the trochanter to the distal tibial articulation) as well as the tibia, total body length (the most anterior point of the head to the end of the abdomen), head width (from directly behind the compound eyes anterior to the head), antennae length (from the antennal socket to the tip of the flagellum) as well as the pronotum length, arc and height (Figure 2.2).

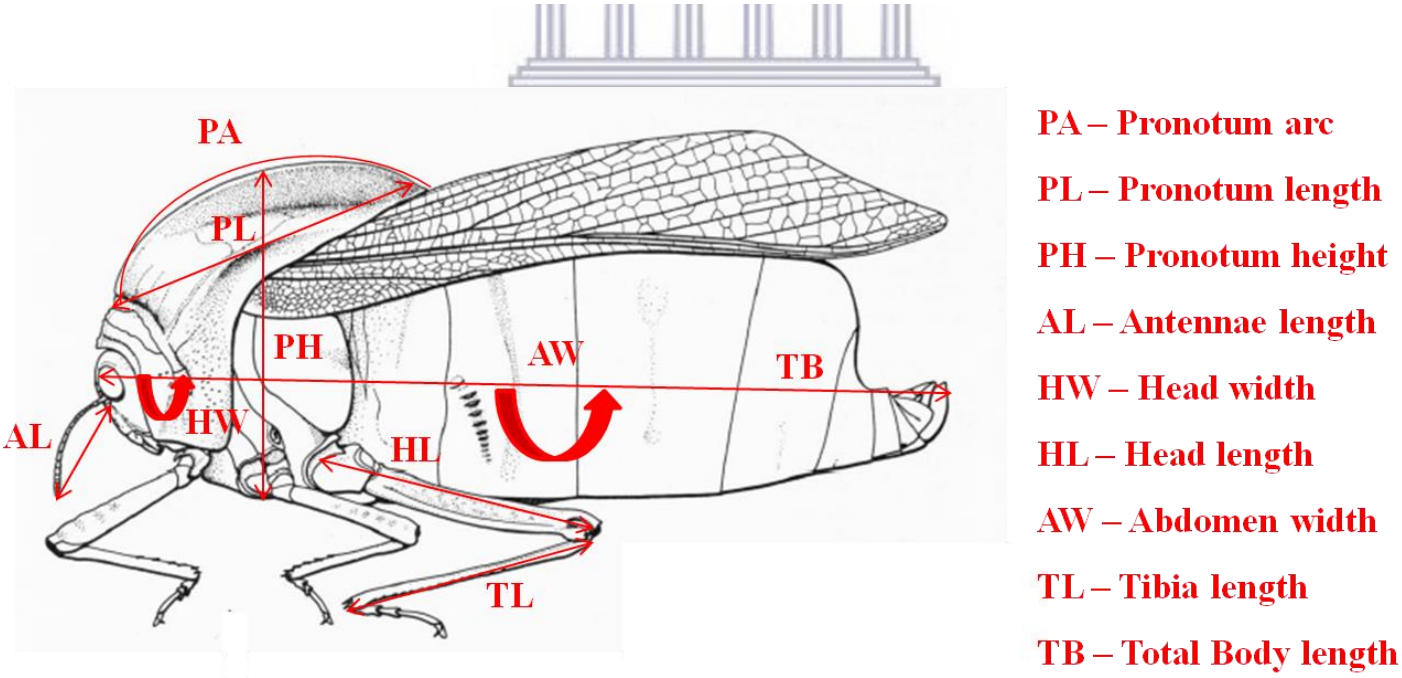

Figure 2.2: Diagram of a male *B. membracioides* (Dirsh, 1965), showing the nine linear anatomical measurements taken from females and males. For paired structures, the right hand sides were used for each measurement.

2.2.3. Acoustic Calls

Male advertisement calls were recorded for each species, excluding *B. boschimana*. These calls were either recorded in the field, in which temperature was not recorded, or in a controlled laboratory environment using a Marantz PMD-670 digital recorder and a Sennheiser K6/Me-66 microphone. The microphone was positioned at a distance of approximately 1 m in front of the calling male. Analyses of these calls were made using Raven Pro 1.5 software (Cornell Bioacoustics Research Program) where temporal and frequency properties were measured. As with the morphological analyses, sample sizes were uneven across species due to certain species being difficult to locate in the field (Table 2.2). The majority of the calls were recorded in the lab from specific individuals, however it is not certain whether field recordings were from single or multiple individuals. Field recordings were less reliable due to an excess of background noise. All measurements were done after filtering background noise to remove frequencies below 500 Hz (Figure 2.3). For each call, seven characteristics were measured; namely, the total call length, the length of introductory syllables, the length of the final syllable, inter-syllable pauses, the carrier frequency of the call, the frequency of the introductory syllables and the frequency of the first harmonic (Figure 2.4).

Table 2.2: The number of male advertisement calls that were used to measure temporal and frequency properties, taken from various locations.

Species	Loaction	No. of calls
B. discolor	Betty's Bay	50
	Hangklip	50
	Ashton	58
B. unicolor	Cederberg	50
	Springbok	50
	Kamieskroon	50
	Bellville	20
B. obliqua	West Coast National Park	50
	Oudtshoorn	10
	Citrusdal	17
	Groenriviersmond	9
B. membracioides	Inchanga	104
B. serrata	Grahamstown	24
B. intermedia	Port St John's	32

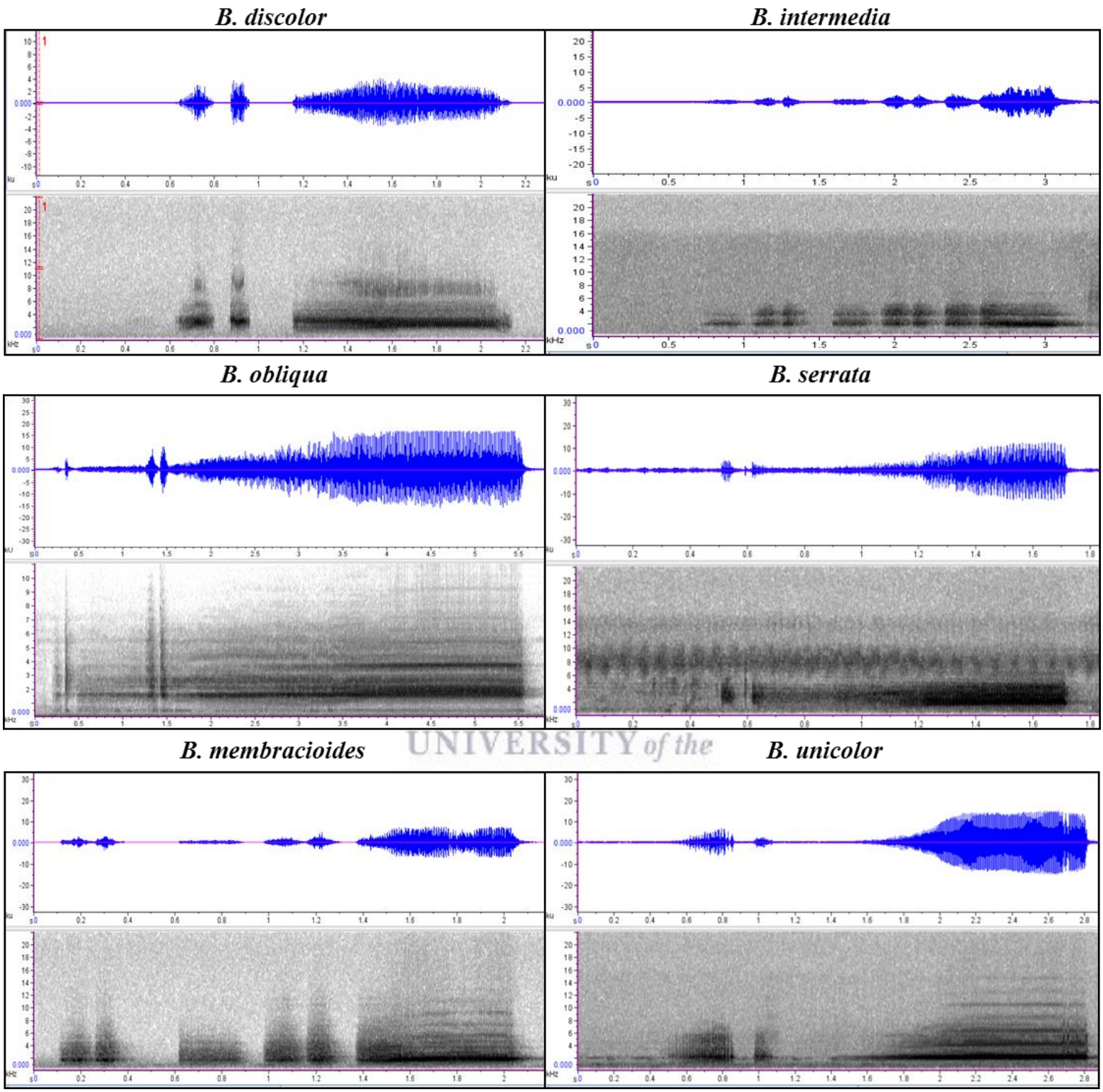

Figure 2.3: Waveform (above) and spectogram (below) showing the differences between advertisement calls of *Bullacris* male species.

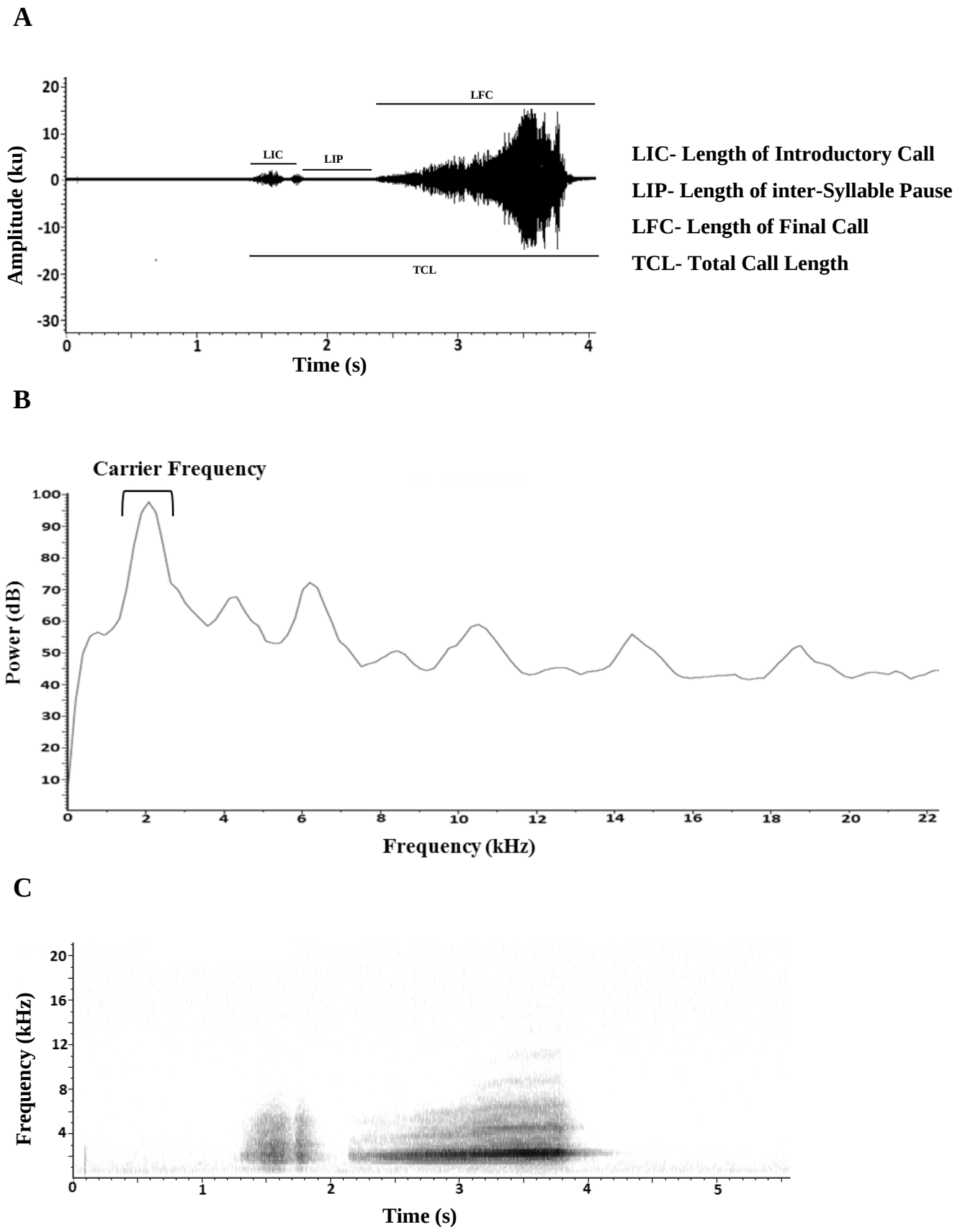

Figure 2.4: Waveform (A), sonogram (B), and spectrogram (C) exemplars of the male advertisement call of *B. unicolor* as well as the properties of the call that was measured.

2.2.4. Statistical analyses

All statistical analyses were carried out using IBM SPSS Statistics 21, where Multivariate Analyses of Variance (MANOVA) and Discriminate Function Analyses (DFA) were used to evaluate differences between call characteristics as well as morphological measurements between each species. MANOVA was used to morphologically compare the six species by using the measurements taken as dependent variables, to find any significant differences among species, with a significant level of less than 0.05. In addition, Pillai's Trace was used as a multivariate statistic for the MANOVA, since Box's test indicated that the assumption of equality of covariance matrices could not be met. Spearman's correlation was also performed between the average abdomen width of males as well as their total body length and corresponding carrier frequencies, to test if there is any relationship.

For acoustics, a similar MANOVA was conducted where the measured acoustic characteristics were also compared across species in order to find any significant differences. Canonical centroid plots illustrate the difference between canonical group means and how well species separate based on the measured variables. This was produced to show how species cluster based on morphological and acoustic characteristics. A Mantel test was performed using the Ade4 package in R 3.3.2, to correlate species pairwise Euclidean distances in acoustic variables with pairwise distances in morphology.

2.3. Results

2.3.1. Distribution

Looking at the distribution of *Bullacris* species in Figures 2.5 to 2.11, there is only one locality for *B. boschimana* (A) in the Northern Cape, on the border of Namibia and South Africa, *B. membracioides* (B) and *B. intermedia* (E) can be found along the east coast, located in the Eastern Cape and Kwa-Zulu Natal. The Eastern and Western Cape is occupied with *B. discolor* (C) as well as *B. serrata* (G). *Bullacris obliqua* (D) can be found in the Northern and Western Cape, whereas *B. unicolor* (F) occupies at least three provinces, the Northern, Western and Eastern Cape.

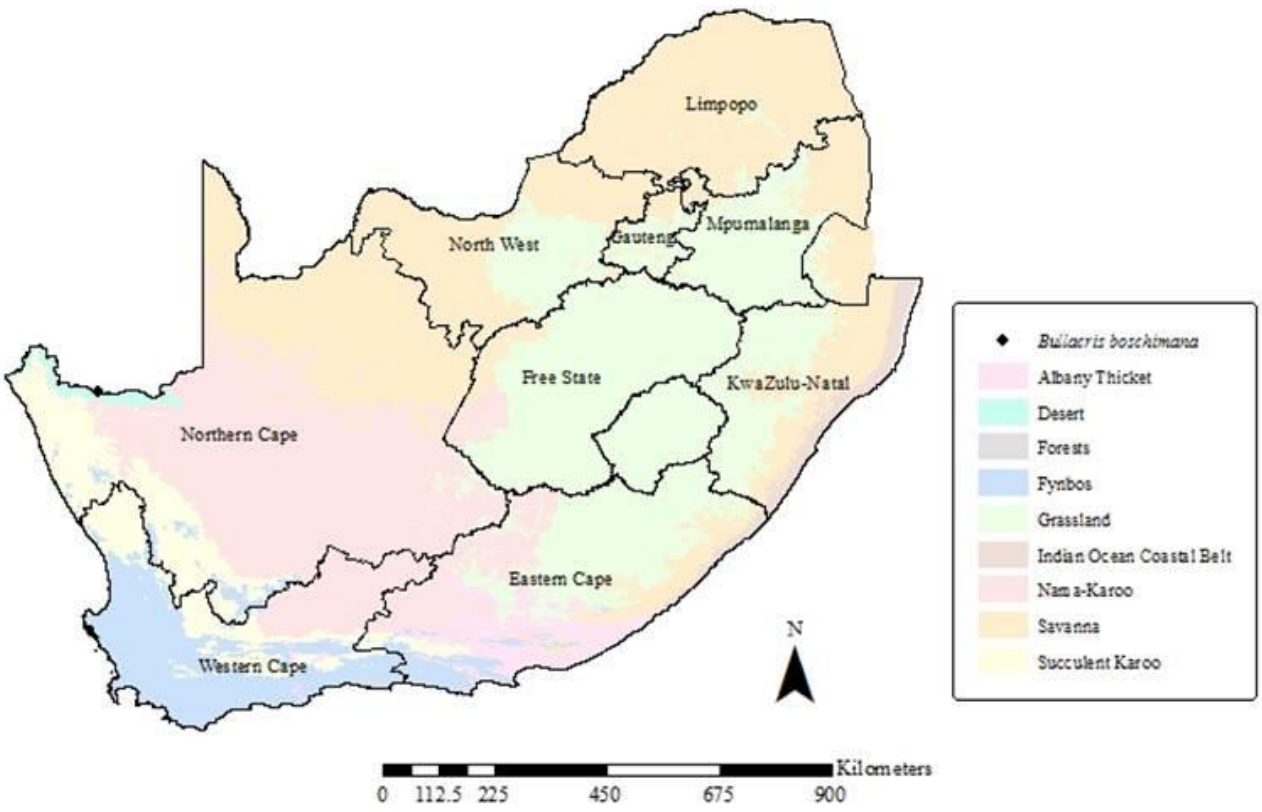

Figure 2.5: Distribution maps showing the recorded location of *B. boschimana* (A).

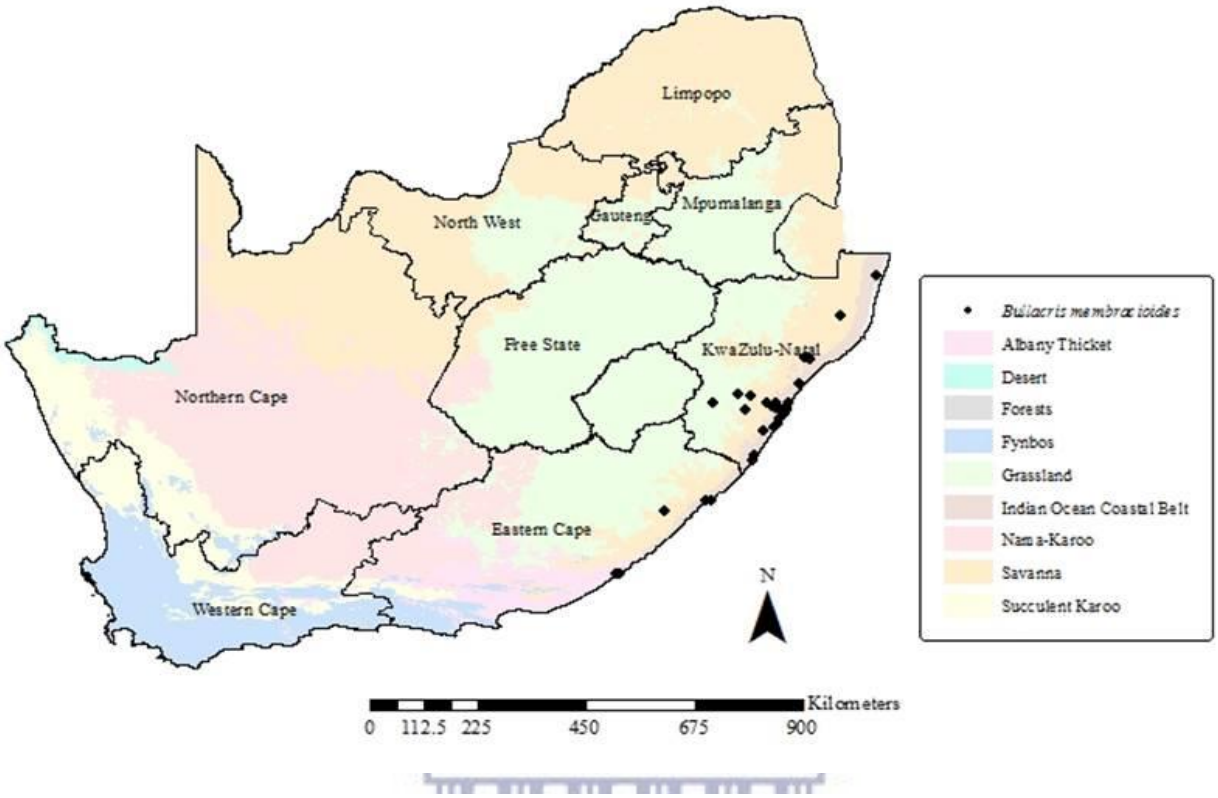

Figure 2.6: Distribution map showing the recorded locations of *B. membracioides* (B).

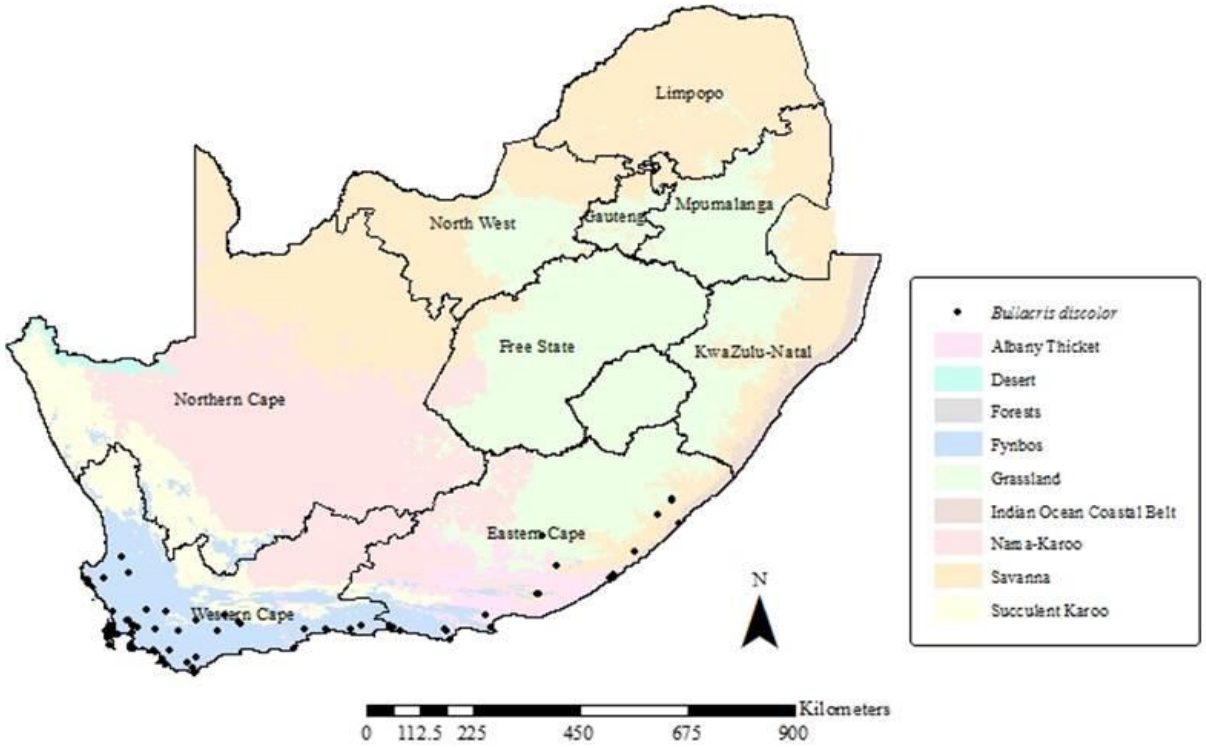

Figure 2.7: Distribution map showing the recorded locations of *B. discolor* (C).

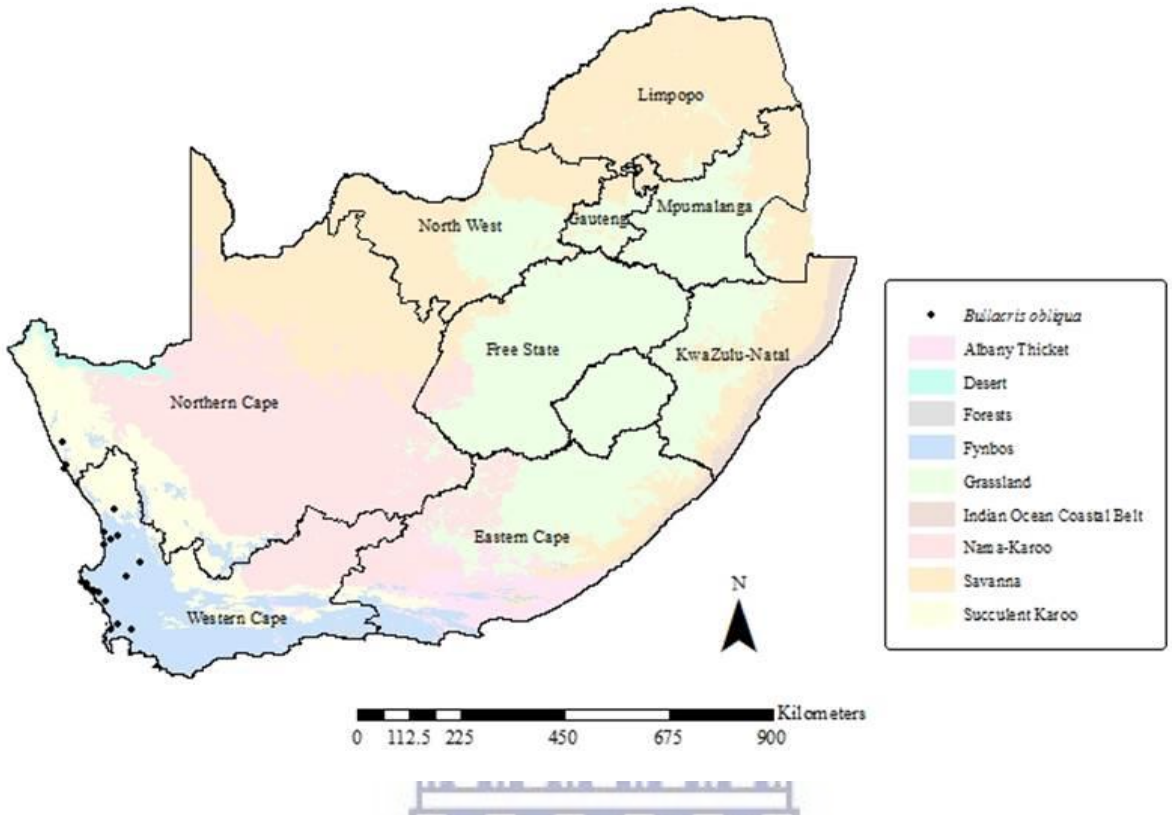

Figure 2.8: Distribution map showing the recorded locations of *B. obliqua* (D).

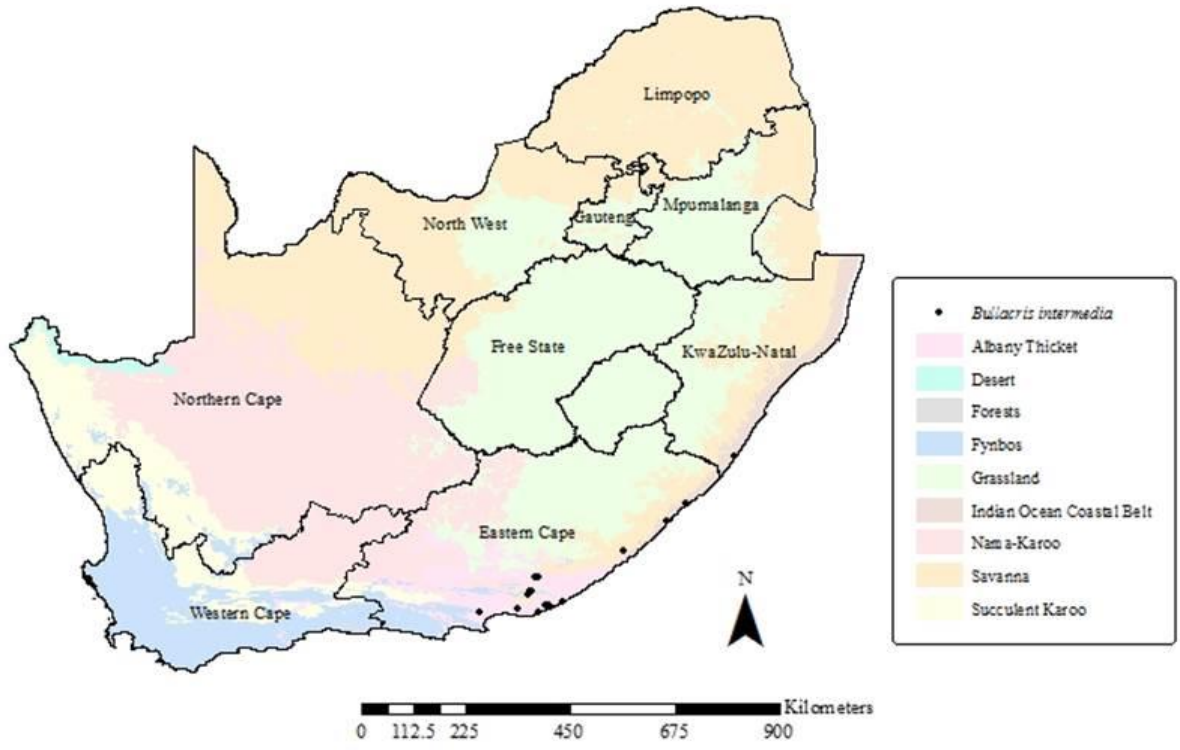

Figure 2.9: Distribution map showing the recorded locations of *B. intermedia* (E).

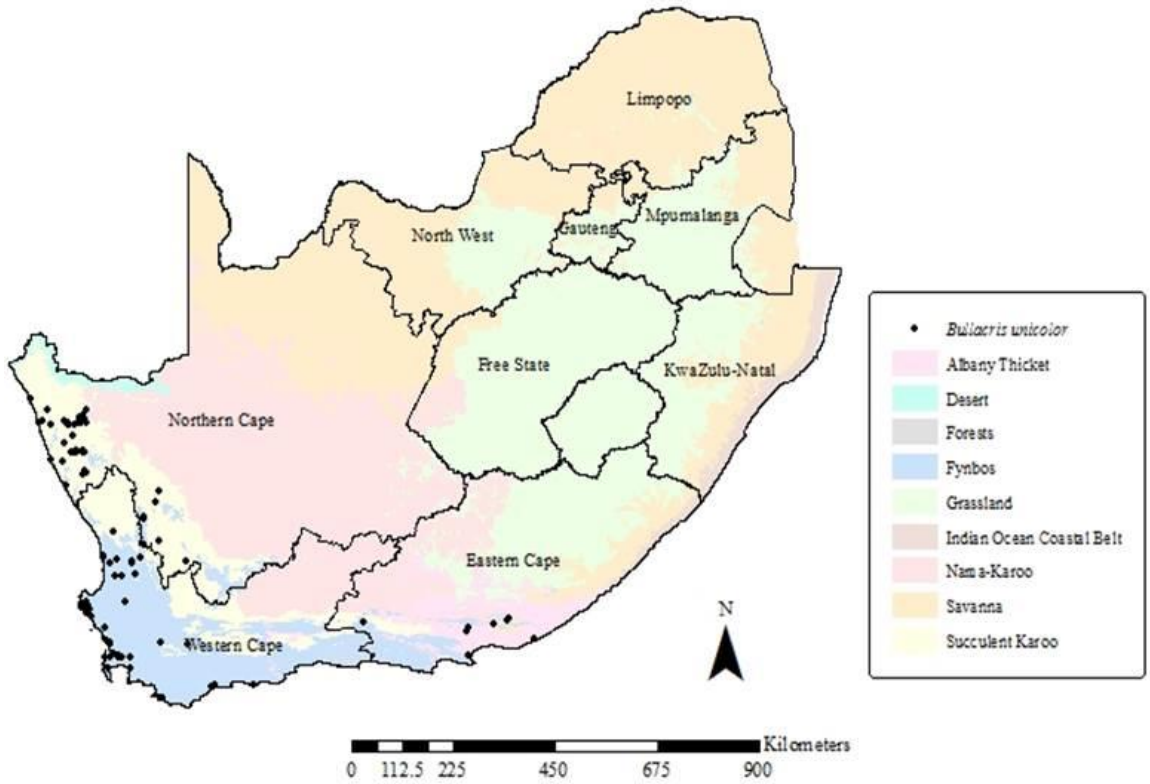

Figure 2.10: Distribution map showing the recorded locations of *B. unicolor* (F).

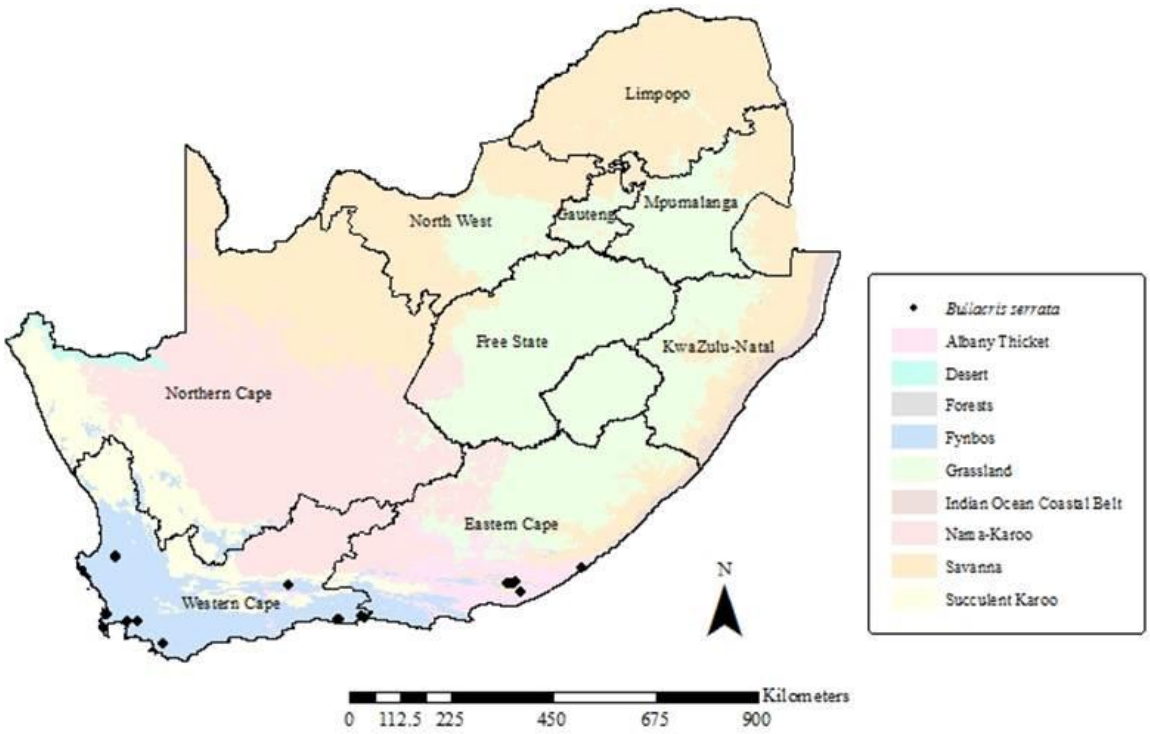

Figure 2.11: Distribution map showing the recorded locations of *B. serrata* (G).

The single specimen locality for *B. boschimana* (A) occurs within a desert type biome. *Bullacris unicolor* (F) has a south-east distribution, and occupies the Fynbos, Succulent-Karoo and Albany Thicket type biomes, which is also true for *B. serrata* (G); however it has a distribution that extends from the south-west to the south-east regions. *Bullacris obliqua* (D) only occupies the Fynbos and Succulent-Karoo biomes, but along the west coast. *Bullacris membracioides* (B) occupies the south eastern regions of and inhabits the Indian Ocean Coastal Belt, Savanna, Grassland as well as the Albany Thicket vegetation types. *B. intermedia* (E) has a relatively small distribution in the south-west, where it inhabits Albany Thicket, Savanna as well as the Indian Ocean Coastal Belt. Lastly, *B. discolor* (C) has a large distribution range from south-west to south-east and can be found within Fynbos, Succulent-Karoo, Albany Thicket, the Indian Ocean Coastal Belt, Savanna and also Grassland type biomes. A collective distribution map was created in order to see the overlap in species distribution (Figure 2.12).

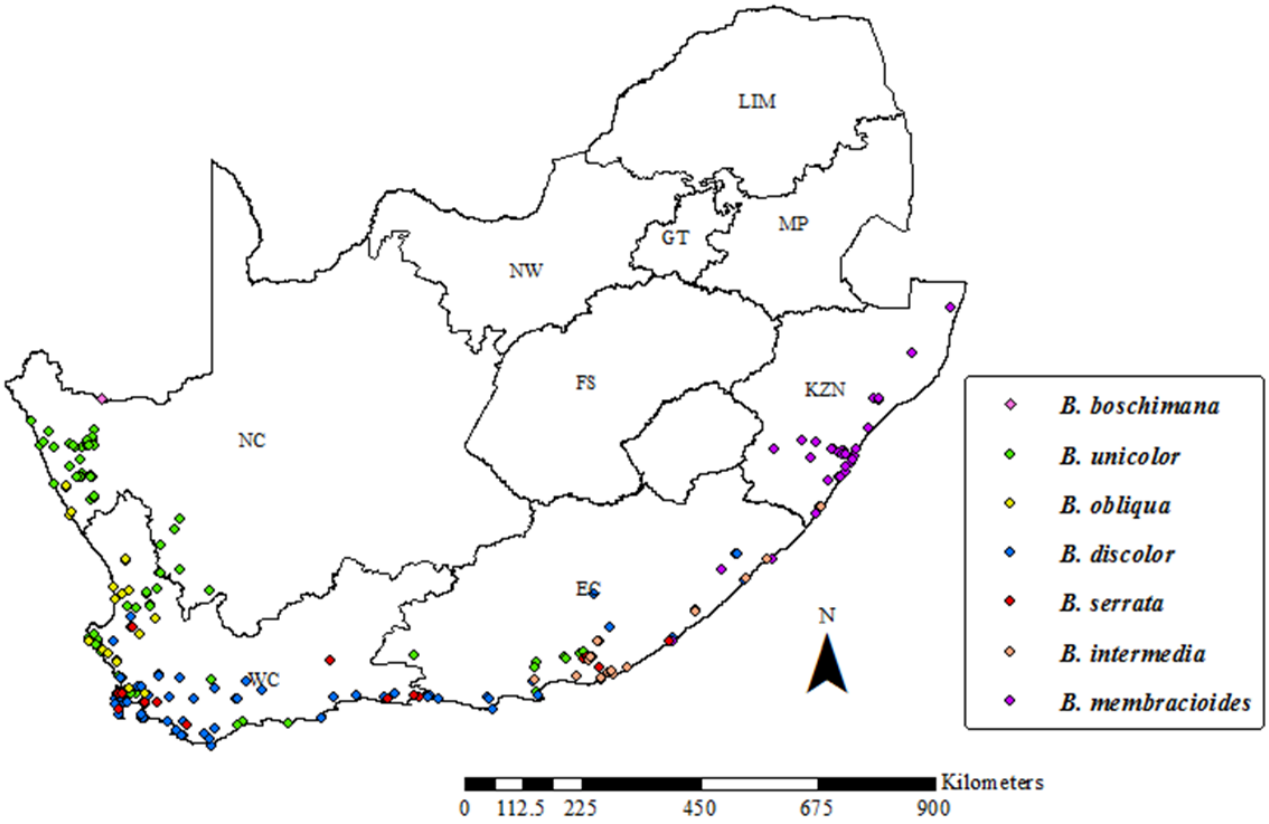

Figure 2.12: A collective distribution map of *Bullacris* species.

2.3.2. Morphological measurements

Visual observations of gross morphology among the *Bullacris* species (Figure 2.13 and Figure 2.14) show that there are differences in wing spans and lengths as well as in pronotum heights and lengths. *Bullacris unicolor* is also visibly smaller than the rest of the species, whereas *B. membracioides* is the largest. In addition, *B. unicolor* has more of a rounded abdomen, whereas the rest of the species have a more oval shaped abdomen. The species also vary in their colouration. *Bullacris* unicolor, *B. discolor* and *B. serrata* have three or four ocellated markings along the side of the abdomen, while *B.* boschimana, *B. serrata* and *B. obliqua* have more extensive whitish markings covering the body, which may be a result of blending or camouflaging to their specific host plants. The markings on *B. serrata* females are typically more spot-like and differ to those of *B. obliqua* females which are more elongated. In addition, *B. serrata* males have white spots on the pronotum, which is only seen in this species.

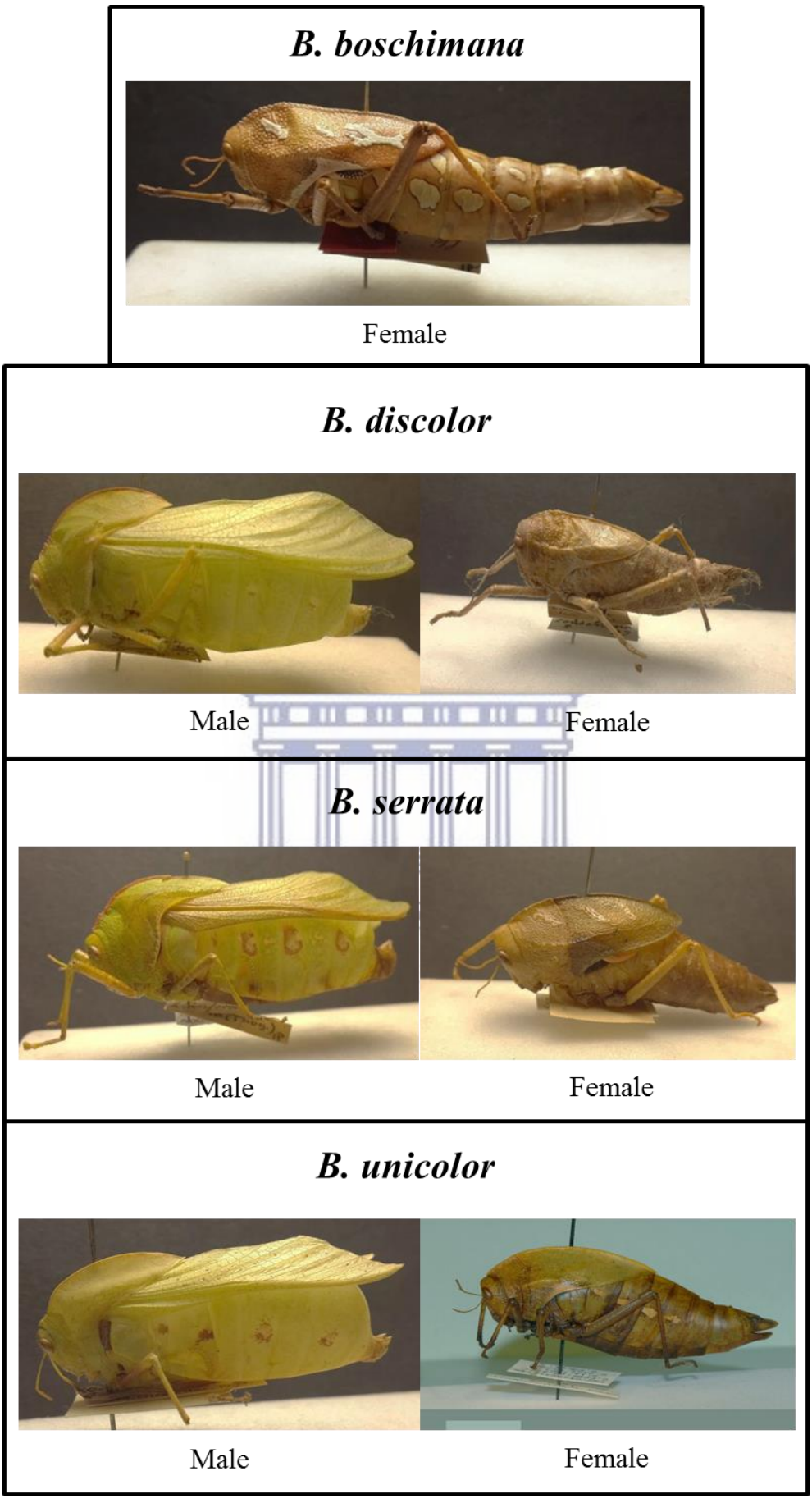

Figure 2.13: Image comparisons of *B. boschimana* (excluding male), *B. discolor*, *B. serrata* and *B. unicolor* male and female specimens.

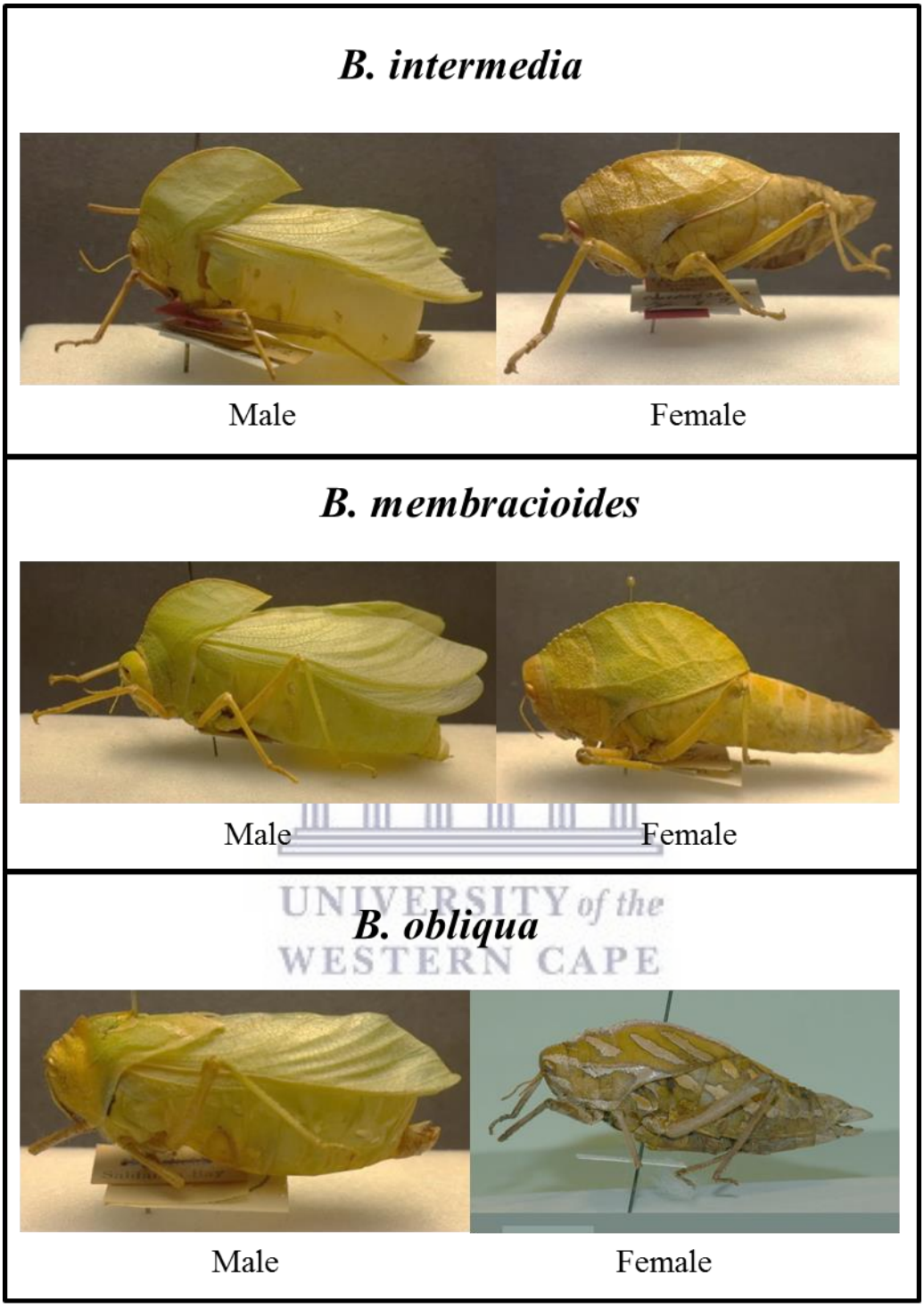

Figure 2.14: Image comparisons of *B. intermedia*, *B. membracioides* and *B. obliqua* male and female specimens.

According to the measured morphological results in Table 2.3 and the bar graphs in Figures 2.15 and 2.16, *B. membracioides* has the largest females and males in comparison to the rest of the species. Males have an average total body length of 52.63 mm and an average abdomen of width of 16.70 mm; whereas females have an average total body length of 54.76 mm, however, *B. serrata* males have the largest abdomen width, 13.04 mm. The smallest species, for both males and females is *B. unicolor*, with an average total body length of 40.57 mm and 39.74 mm respectively, as well as an abdomen width of 13.96 mm and 8.75 mm, however, the *B. boschimana* specimen is the second largest female, with a body length of 54.05 mm.

Table 2.3: Means (± standard error) for anatomical measurements of *Bullacris* males.

Species	Pronotum			abdomen (width)	hind femur (length)	tibia (length)	total body (length)	head (width)	antennae (length)
	length	arc	height						
B. discolor	19.36 ± 4.59	21.72 ± 5.23	16.06 ± 2.95	**13.18 ± 2.69**	15.80 ± 3.17	14.25 ± 2.87	44.33 ± 8.53	6.50 ± 1.70	10.35 ± 2.39
B.unicolor	**17.42 ± 1.27**	20.68 ± 1.66	**14.97 ± 0.85**	13.96 ± 1.13	**13.33 ± 0.69**	**11.98 ± 0.63**	**40.57 ± 2.19**	**4.83 ± 0.35**	**7.02 ± 0.55**
B. membracioides	**23.73 ± 2.65**	26.07 ± 2.87	**20.25 ± 1.27**	**16.70 ± 4.04**	**18.00 ± 0.60**	**16.93 ± 0.79**	**52.63 ± 3.27**	6.74 ± 0.38	**11.16 ± 1.84**
B. obilqua	17.65 ± 1.35	**19.65 ± 1.57**	15.46 ± 1.19	14.12 ± 1.33	15.11 ± 0.87	13.61 ± 0.87	43.09 ± 2.08	5.31 ± 0.32	9.26 ± 0.63
B. serrata	20.39 ± 1.46	22.25 ± 1.59	17.09 ± 1.06	16.14 ± 0.99	17.17 ± 0.47	16.25 ± 0.72	51.97 ± 2.52	**6.80 ± 0.35**	10.51 ± 1.48
B. intermedia	21.49 ± 0.70	**26.78 ± 1.49**	19.52 ± 0.73	16.07 ± 0.92	15.66 ± 0.90	14.55 ± 0.63	45.26± 2.64	5.52 ± 0.57	7.96 ± 0.77
B. boschimana	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*Minimum and maximum values highlighted in bold.

Table 2.4: Means (± standard error) for anatomical measurements of *Bullacris* females.

Species	Pronotum			abdomen (width)	hind femur (length)	tibia (length)	total body (length)	head (width)	antennae (length)
	length	arc	height						
B. discolor	26.42 ± 2.40	28.56 ± 3.18	17.22 ± 2.96	12.41 ± 0.92	17.46 ± 0.84	16.04 ± 0.76	49.38 ± 3.35	8.82 ± 0.46	11.71 ± 0.61
B.unicolor	**21.80 ± 2.25**	**23.69 ± 2.51**	13.36 ± 1.62	**8.75 ± 0.94**	**13.16 ± 0.93**	**11.83 ± 0.80**	**39.74 ± 3.76**	**5.62 ± 0.44**	**6.18 ± 0.60**
B. membracioides	**33.23 ± 1.67**	**29.23 ± 3.25**	**20.46 ± 0.93**	12.23 ± 0.30	20.56 ± 0.77	**19.37 ± 0.62**	**54.76 ± 2.68**	8.71 ± 0.42	**12.90 ± 2.04**
B. obilqua	22.47 ± 0.96	24.60 ± 1.42	14.64 ± 0.63	10.56 ± 1.26	15.95 ± 0.23	15.28 ± 0.92	44.44 ± 3.66	7.41 ± 0.69	7.63 ± 0.36
B. serrata	26.00 ± 2.24	28.61 ± 2.14	17.61 ± 1.61	**13.04 ± 1.15**	17.68 ± 0.90	16.58 ± 0.70	45.90 ± 4.69	8.98 ± 0.65	10.37 ± 1.24
B. intermedia	27.12 ± 0.19	28.71 ± 0.22	17.6 ± 0.21	11.27 ± 0.07	**21.02 ± 0.04**	18.91 ± 0.09	48.02 ± 0.18	**9.28 ±0.10**	10.43 ± 0.15
B. boschimana	24.28	25.49	**12.91**	12.37	19.06	17.35	54.05	6.66	7.21

*Minimum and maximum values highlighted in bold.

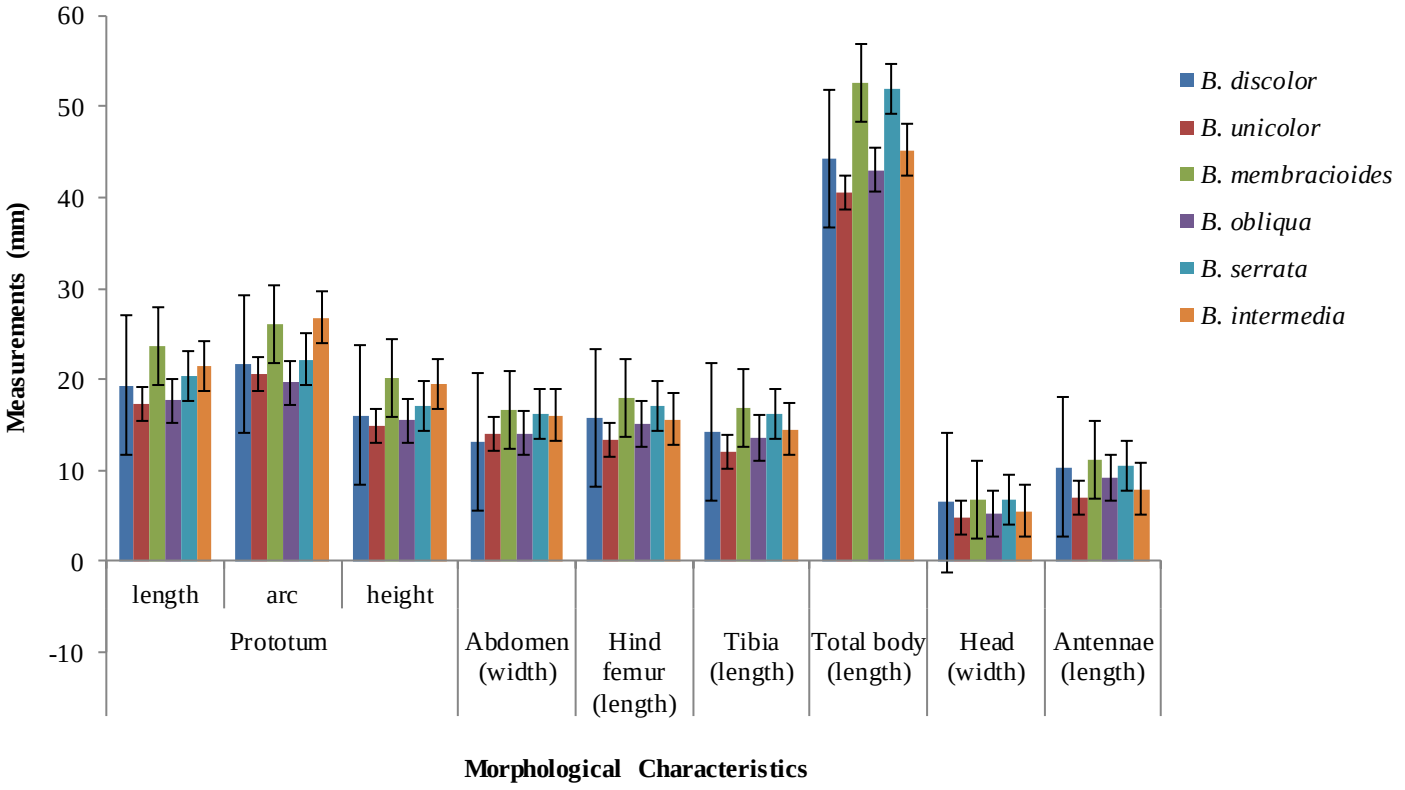

Figure 2.15: Graph showing mean lengths (± standard error) for nine anatomical characteristics for male morphology, excluding *B. boschimana*.

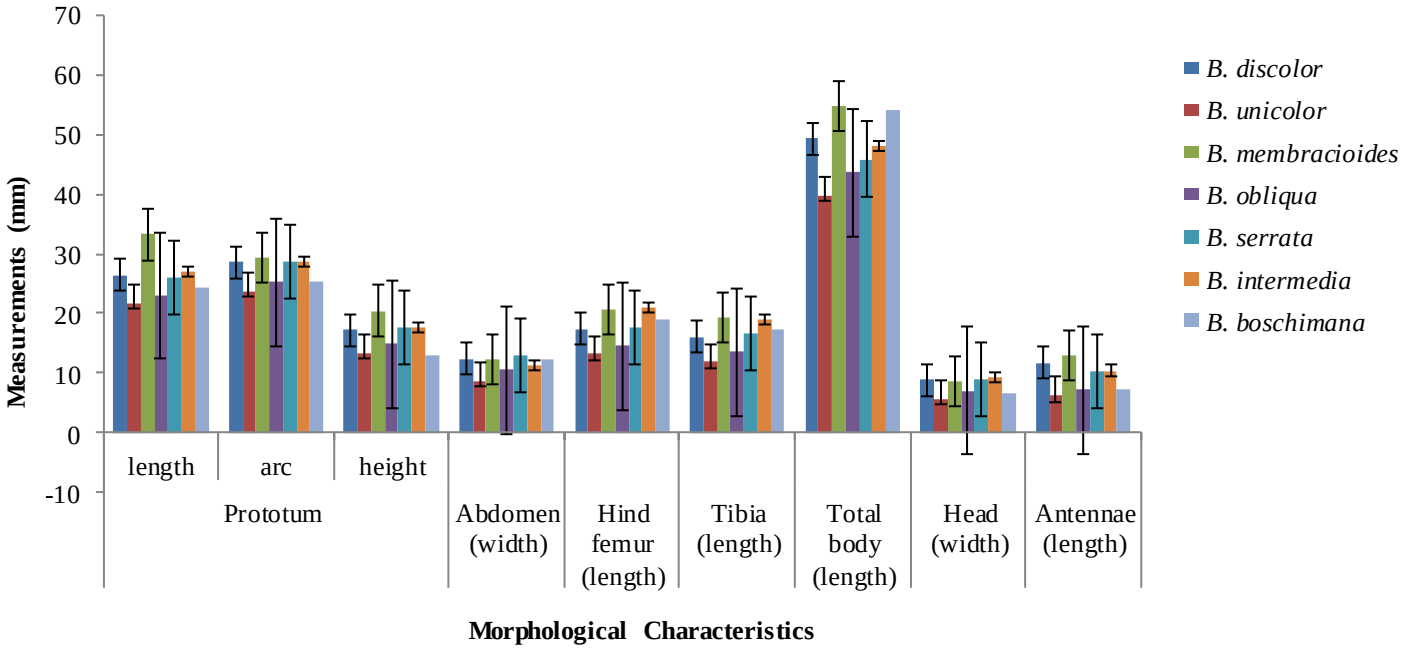

Figure 2.16: Graph showing mean lengths (± standard error) for nine anatomical characteristics for female morphology.

2.3.3. MANOVA for results for male morphology

Multivariate analyses in Table 2.5 (A), shows that males have significant differences among their morphological characteristics (Pillai's Trace = 2.272; $F_{45, 475} = 8.792$; $p < 0.001$). Pronotum length for *B. membracioides* is significantly different to all other species ($p < 0.05$), and the pronotum arc is also different to all others, with the exception of *B. intermedia* ($p = 0.949$). *Bullacris discolor*, *B. intermedia*, *B. serrata* and *B. membracioides* show significant differences to *B. obliqua* ($p < 0.05$), with the exception of *B. intermedia* ($p = 0.732$), for pronotum height. Abdomen width varies among species, with *B. discolor* being significantly different to *B. membracioides* ($p = 0.001$) and *B. serrata* ($p = 0.034$); as well as *B. unicolor* to *B. membracioides*, *B. intermedia* and *B. serrata* ($p < 0.05$).

There are no significant differences in the length of the hind femur between *B. intermedia* and both *B. discolor* ($p = 0.954$) and *B. obliqua* ($p = 0.311$); otherwise all pairwise comparisons are significant. In Table 2.5 (B), tibia length differs significantly between all pairs except between *B. serrata* and *B. membracioides* ($p = 0.093$) and between *B. discolor* and *B. intermedia* ($p = 0.985$). Similarly, body length differs between all species pairs except between *B. intermedia* and both *B. discolor* ($p = 0.969$) and *B. obliqua* ($p = 0.190$) and between *B. membracioides* and *B. serrata* ($p = 0.971$).

Head width does not differ between *B. obliqua* and *B. intermedia* and between *B. membracioides* ($p = 0.838$) and *B. serrata* ($p = 0.998$), but is otherwise significantly different between all species pairs. Antenna length shows similarities only between some species pairs including, *B. serrata* and both *B. membracioides* ($p = 0.673$) and *B. obliqua* ($p = 0.055$); *B. intermedia* with both *B. unicolor* ($p = 0.287$) and *B. obliqua* ($p = 0.081$); and lastly between *B. discolor* and *B. serrata* ($p = 1.000$).

Table 2.5 (A): Multiple comparisons table between species, showing mean differences and standard error for pairwise differences for male morphology. Significant differences are highlighted in bold.

Pronotum (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	(-1.166 ± 0.495)	**N/A**				
B. intermedia	**(2.911 ± 0.619)**	**(4.077 ± 0.597)**	**N/A**			
B. membracioides	**(5.149 ± 0.544)**	**(6.316 ± 0.519)**	**(2.239 ± 0.639)**	**N/A**		
B. obliqua	(-0.929 ± 0.531)	(0.237 ± 0.519)	**(-3.840 ± 0.639)**	**(-6.078 ± 0.567)**	**N/A**	
B. serrata	**(1.803 ± 0.022)**	**(2.969 ± 0.539)**	(-1.108 ± 0.655)	**(-3.346 ± 0.585)**	(2.732 ± 0.585)	**N/A**

Pronotum (arc)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	(-0.278 ± 0.622)	**N/A**				
B. intermedia	**(5.827 ± 0.779)**	**(6.105 ± 0.751)**	**N/A**			
B. membracioides	**(5.114 ± 0.684)**	**(5.392 ± 0.652)**	(-0.712 ± 0.803)	**N/A**		
B. obliqua	(-1.309 ± 0.684)	(-1.031 ± 0.652)	**(-7.135 ± 0.803)**	**(-6.423 ± 0.712)**	**N/A**	
B. serrata	(1.291 ± 0.709)	(1.569 ± 0.678)	**(-4.535 ± 0.824)**	**(-3.823 ± 0.735)**	**(2.600 ± 0.735)**	**N/A**

Pronotum (height)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-1.744 ± 0.335)**	**N/A**				
B. intermedia	**(2.811 ± 0.420)**	**(4.555 ± 0.404)**	**N/A**			
B. membracioides	**(3.534 ± 0.369)**	**(5.278 ± 0.351)**	(0.723 ± 0.432)	**N/A**		
B. obliqua	**(-1.251 ± 0.369)**	(0.494 ± 0.351)	**(-4.061 ± 0.432)**	**(-4.784 ± 0.383)**	**N/A**	
B. serrata	(0.377 ± 0.382)	**(2.122 ± 0.365)**	**(-2.434 ± 0.444)**	**(-3.157 ± 0.396)**	**(1.627 ± 0.396)**	**N/A**

Abdomen (width)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	(-0.228 ± 0.564)	**N/A**				
B. intermedia	(1.889 ± 0.705)	**(2.116 ± 0.680)**	**N/A**			
B. membracioides	**(2.515 ± 0.620)**	**(2.743 ± 0.591)**	(0.627 ± 0.727)	**N/A**		
B. obliqua	(-0.066 ± 0.620)	(0.162 ± 0.591)	(-1.955 ± 0.727)	**(-2.581 ± 0.644)**	**N/A**	
B. serrata	**(1.959 ± 0.642)**	**(2.186 ± 0.614)**	(0.070 ± 0.747)	(-0.557 ± 0.665)	**(2.025 ± 0.665)**	**N/A**

Hind Femur (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-2.552 ± 0.205)**	**N/A**				
B. intermedia	(-0.221 ± 0.257)	**(2.331 ± 0.247)**	**N/A**			
B. membracioides	**(2.117 ± 0.225)**	**(4.670 ± 0.215)**	**(2.339 ± 0.264)**	**N/A**		
B. obliqua	**(-0.769 ± 0.225)**	**(1.783 ± 0.215)**	(-0.548 ± 0.264)	**(-2.887 ± 0.234)**	**N/A**	
B. serrata	**(1.290 ± 0.233)**	**(3.842 ± 0.223)**	**(1.511 ± 0.271)**	**(-0.828 ± 0.242)**	**(2.059 ± 0.242)**	**N/A**

Table 2.5 (B): Multiple comparisons table between species, showing mean differences and standard error for pairwise differences for male morphology. Significant differences are highlighted in bold.

Tibia (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-2.396 ± 0.217)**	**N/A**				
B. intermedia	(0.181 ± 0.272)	**(2.577 ± 0.262)**	**N/A**			
B. membracioides	**(2.561 ± 0.239)**	**(4.957 ± 0.228)**	**(2.380 ± 0.280)**	**N/A**		
B. obliqua	**(-0.765 ± 0.239)**	**(1.630 ± 0.228)**	**(-0.946 ± 0.280)**	**(-3.357 ± 0.248)**	**N/A**	
B. serrata	**(1.879 ± 0.247)**	**(4.275 ± 0.237)**	**(1.698 ± 0.288)**	(-0.682 ± 0.257)	**(2.644 ± 0.257)**	**N/A**

Total Body (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-5.400 ± 0.723)**	**N/A**				
B. intermedia	(-0.710 ± 0.905)	**(4.689 ± 0.872)**	**N/A**			
B. membracioides	**(6.658 ± 0.795)**	**(12.058 ± 0.757)**	**(7.369 ± 0.932)**	**N/A**		
B. obliqua	**(-2.888 ± 0.795)**	**(2.511 ± 0.757)**	(-2.178 ± 0.932)	**(-9.547 ± 0.826)**	**N/A**	
B. serrata	**(5.997 ± 0.823)**	**(11.397 ± 0.787)**	**(6.708 ± 0.957)**	(-0.661 ± 0.854)	**(8.886 ± 0.854)**	**N/A**

Head (width)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-1.331 ± 0.135)**	**N/A**				
B. intermedia	**(-0.633 ± 0.169)**	**(0.698 ± 0.163)**	**N/A**			
B. membracioides	**(0.581 ± 0.148)**	**(1.912 ± 0.141)**	**(1.214 ± 0.174)**	**N/A**		
B. obliqua	**(-0.841 ± 0.148)**	**(0.490 ± 0.141)**	(-0.208 ± 0.174)	**(-1.422 ± 0.154)**	**N/A**	
B. serrata	**(0.647 ± 0.154)**	**(1.979 ± 0.147)**	**(1.281 ± 0.179)**	(0.066 ± 0.159)	**(1.489 ± 0.159)**	**N/A**

Antenna (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-3.524 ± 0.370)**	**N/A**				
B. intermedia	**(-2.580 ± 0.463)**	(0.945 ± 0.446)	**N/A**			
B. membracioides	(0.621 ± 0.407)	**(4.145 ± 0.388)**	**(3.201 ± 0.477)**	**N/A**		
B. obliqua	**(-1.284 ± 0.407)**	**(2.241 ± 0.388)**	(1.296 ± 0.477)	**(-1.905 ± 0.423)**	**N/A**	
B. serrata	(-0.029 ± 0.421)	**(3.496 ± 0.403)**	**(2.551 ± 0.489)**	(-0.650 ± 0.437)	(1.255 ± 0.437)	**N/A**

2.3.4. MANOVA for results for female morphology

Multivariate analyses in Table 2.6 (A), shows that females have significant differences among their morphological characteristics (Pillai's Trace = 2.610; $F_{45, 305} = 7.400$; $p < 0.001$). *Bullacris membracioides* shows significant differences in the length of the pronotum ($p < 0.05$) to all other species. The length of the pronotum arc of *B. intermedia* and *B. obliqua* shows no significant differences to the other species ($p > 0.05$). Significant differences in pronotum arc can be seen between *B. unicolor* and *B. discolor* as well as *B. serrata* ($p < 0.05$); whereas *B. membracioides* differs to *B. obliqua* and *B. obliqua* differs to *B. discolor* ($p < 0.05$). Abdomen width does not differ greatly between species, with exceptions to *B. discolor* and *B. unicolor* as well as *B. obliqua*; *B. unicolor* to *B. intermedia*, *B. membracioides* and *B. serrata* and lastly *B. obliqua* and *B. serrata* ($p < 0.05$).

The length of the hind femur has a number of significant differences between species, however, *B. discolor* shows similarities with *B. obliqua* (p =0.101) and *B. serrata* ($p = 0.993$); *B. intermedia* and *B. membracioides* ($p = 0.983$); as well as *B. obliqua* and *B. serrata* ($p = 0.095$). Similarities in tibia length (Table 2.6 (B)), are only shown between *B. discolor* and *B. obliqua* ($p = 0.577$) as well as *B. serrata* ($p = 0.592$); *B. intermedia* and *B. membracioides* ($p = 0.967$); *B. obliqua* and *B. serrata* ($p = 0.193$).

Total body length varies significantly between *B. discolor* and *B. unicolor* as well as *B. membracioides*; between *B. unicolor* and *B. intermedia*; *B. membracioides* and *B. serrata*; as well as between *B. membracioides* and *B. obliqua* ($p < 0.05$). The head width of *B. unicolor* species is the only species to show significant differences to the rest of the species ($p < 0.05$) and antenna length shows similarities between *B. discolor* and *B. intermedia* ($p = 0.451$); between *B. unicolor* and *B. obliqua* ($p = 0.497$) and lastly between *B. intermedia* and *B. serrata* ($p = 1.000$).

Table 2.6 (A): Multiple comparisons table between species, showing mean differences and standard error pairwise differences female morphology. Significant differences are highlighted in bold.

Pronotum (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-4.625 ± 0.635)**	**N/A**				
B. intermedia	(0.691 ± 1.624)	**(5.316± 1.652)**	**N/A**			
B. membracioides	**(6.799 ± 0.840)**	**(11.424 ± 0.894)**	**(6.108 ± 1.741)**	**N/A**		
B. obliqua	(-3.429 ± 1.624)	(1.196 ± 1.652)	(-4.120 ± 2.227)	**(-10.228± 1.741)**	**N/A**	
B. serrata	(-0.422 ± 0.991)	**(4.075 ± 2.237)**	(-1.113 ± 1.819)	**(-7.222 ± 1.174)**	(3.006 ± 1.819)	**N/A**

Pronotum (arc)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-4.868 ± 0.823)**	**N/A**				
B. intermedia	(0.146 ± 2.104)	(5.015 ± 2.141)	**N/A**			
B. membracioides	(0.675 ± 1.089)	**(5.543 ± 1.159)**	(0.528 ± 2.257)	**N/A**		
B. obliqua	(-3.269 ± 2.104)	(1.599 ± 2.141)	(-3.415 ± 2.887)	(-3.943 ± 2.257)	**N/A**	
B. serrata	(0.046 ± 1.284)	**(4.915± 1.344)**	(-0.100 ± 2.357)	(-0.628 ± 1.521)	(3.315 ± 2.357)	**N/A**

Pronotum (height)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-3.860 ± 0.654)**	**N/A**				
B. intermedia	(0.384 ± 1.673)	(4.244 ± 1.702)	**N/A**			
B. membracioides	**(3.242 ± 0.866)**	**(7.102 ± 0.921)**	(2.858 ± 1.794)	**N/A**		
B. obliqua	(-2.416 ± 1.673)	(1.444 ± 1.702)	(-2.800 ± 2.2947)	**(-5.658 ± 1.794)**	**N/A**	
B. serrata	(0.396 ± 1.021)	**(4.256 ± 1.068)**	(0.012 ± 1.874)	(-2.846 ± 1.209)	(2.812 ± 1.874)	**N/A**

Abdomen (width)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-3.662 ± 0.256)**	**N/A**				
B. intermedia	(-1.142 ± 0.661)	**(2.520 ± 0.671)**	**N/A**			
B. membracioides	(-0.178± 0.341)	**(3.484 ± 0.363)**	(0.964 ± 0.708)	**N/A**		
B. obliqua	**(-1.962 ± 0.667)**	(1.700 ± 0.671)	(-0.820 ± 0.905)	(-1.784 ± 0.708)	**N/A**	
B. serrata	(0.626 ±0.403)	**(4.288 ± 0.421)**	(1.768 ± 0.739)	(0.804 ± 0.477)	**(2.588 ± 0.739)**	**N/A**

Hind Femur (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-4.303 ± 0.242)**	**N/A**				
B. intermedia	**(3.506 ± 0.620)**	**(7.864 ± 0.631)**	**N/A**			
B. membracioides	**(3.103 ± 0.321)**	**(7.406 ± 0.341)**	(-0.458 ± 0.665)	**N/A**		
B. obliqua	(-1.639 ± 0.620)	**(2.664 ± 0.631)**	**(-5.200 ± 0.850)**	**(-4.742 ± 0.665)**	**N/A**	
B. serrata	(0.216 ± 0.378)	**(4.519 ± 0.396)**	**(-3.345 ± 0.694)**	**(-2.887 ± 0.448)**	(1.855 ± 0.694)	**N/A**

Table 2.6 (B): Multiple comparisons table between species, showing mean differences and standard error pairwise differences female morphology. Significant differences are highlighted in bold.

Tibia (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-4.204 ± 0.215)**	**N/A**				
B. intermedia	**(2.869 ± 0.550)**	**(7.073 ± 0.560)**	**N/A**			
B. membracioides	**(3.339 ± 0.285)**	**(7.542 ± 0.303)**	(0.469± 0.590)	**N/A**		
B. obliqua	(0.901 ± 0.550)	**(3.303 ± 0.560)**	**(-3.770 ± 0.755)**	**(-4.239 ± 0.590)**	**N/A**	
B. serrata	(-0.542 ± 0.336)	**(4.746 ± 0.351)**	**(-2.327 ± 0.616)**	**(-2.796 ± 0.398)**	(1.443 ± 0.616)	**N/A**

Total Body (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-9.641 ± 1.004)**	**N/A**				
B. intermedia	(-1.364 ± 2.569)	**(8.277 ± 2.613)**	**N/A**			
B. membracioides	**(5.376 ± 1.330)**	**(15.017 ± 1.414)**	(6.740 ± 2.755)	**N/A**		
B. obliqua	(-4.289 ± 2.569)	(5.352 ± 2.613)	(-2.925 ± 3.524)	**(-9.665 ± 2.755)**	**N/A**	
B. serrata	(-3.488 ± 1.568)	**(6.153 ± 1.640)**	(-2.123 ± 2.877)	**(-8.863 ± 1.857)**	(0.802 ± 2.877)	**N/A**

Head (width)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-3.204 ± 0.136)**	**N/A**				
B. intermedia	(0.456 ± 0.348)	**(3.660 ± 0.354)**	**N/A**			
B. membracioides	(-0.119 ± 0.180)	**(3.086 ± 0.191)**	(-0.574 ± 0.373)	**N/A**		
B. obliqua	**(-1.349 ± 0.348)**	**(1.855 ± 0.354)**	**(-1.805 ± 0.477)**	**(-1.273 ± 0.373)**	**N/A**	
B. serrata	(0.154 ± 0.212)	**(3.358 ± 0.222)**	(-0.302 ± 0.389)	(0.273 ± 0.251)	**(1.503 ± 0.389)**	**N/A**

Antennae (length)						
	B. discolor	***B. unicolor***	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***
B. discolor	**N/A**					
B. unicolor	**(-5.527 ± 0.273)**	**N/A**				
B. intermedia	(-1.281 ± 0.698)	**(4.246 ± 0.710)**	**N/A**			
B. membracioides	**(1.190 ± 0.361)**	**(6.718 ± 0.384)**	**(2.472 ± 0.748)**	**N/A**		
B. obliqua	**(-4.276 ± 0.698)**	(1.251 ± 0.710)	**(-2.995 ± 0.957)**	**(-5.467 ± 0.748)**	**N/A**	
B. serrata	**(-1.340 ± 0.426)**	**(4.187 ± 0.446)**	(-0.583 ± 0.782)	**(-2.530 ± 0.505)**	**(2.937 ± 0.782)**	**N/A**

The DFA for male morphology shows that that the species are morphologically different ($p < 0.05$); however there is still a small amount of overlap, which suggests some similarities (Figure 2.17). Morphologically, *B. obliqua*, *B. discolor* and *B. serrata* are very closely related in phenotypic appearance. According to eigenvalues, (Table 2.7), 62.88% of the variation is explained by Function 1 and 30.54% variation by Function 2. DF1 has a positive correlation with leg length (hind femur and tibia length) and a negative correlation with abdomen width and DF2 has a positive correlation with pronotum height and a negative correlation with antennae length (Table 2.8).

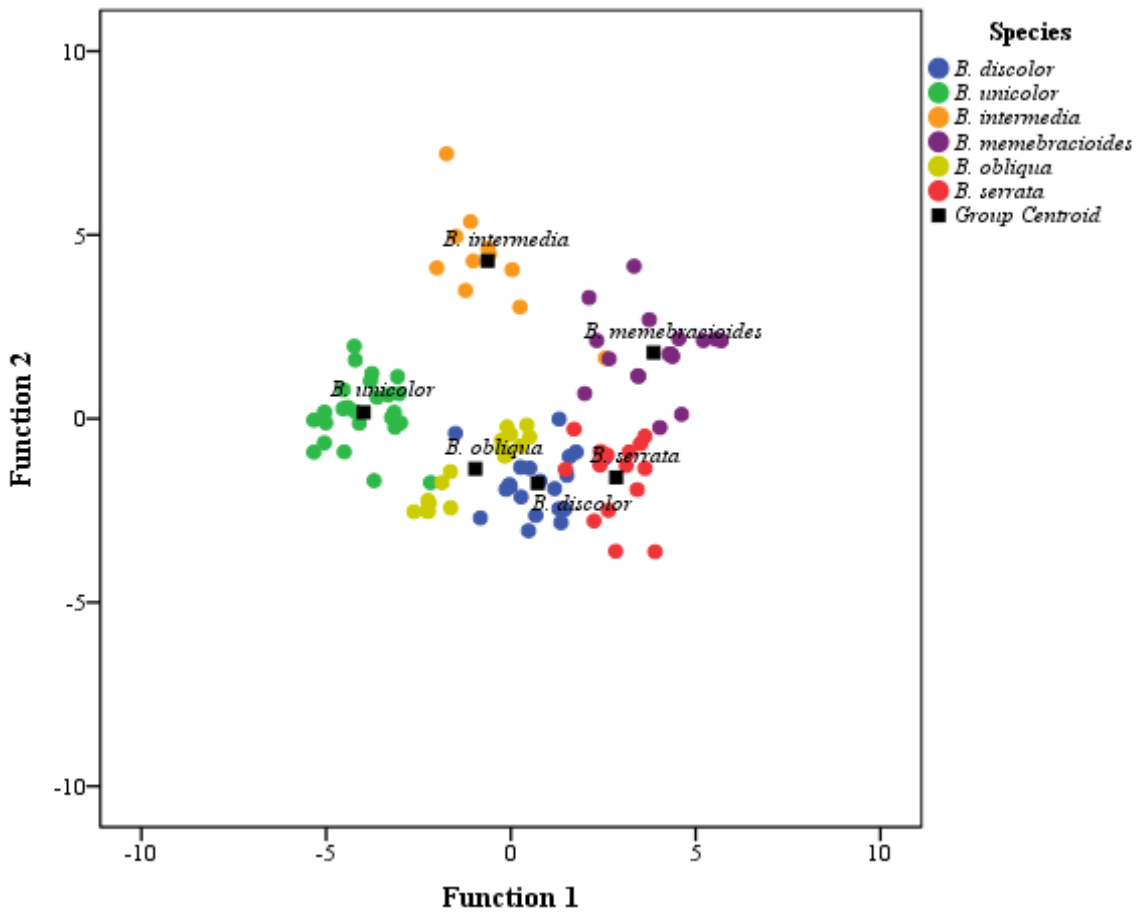

Figure 2.17: Canonical centroid plot of the discriminant function analysis (DFA) for male morphology.

Table 2.7: Table showing eigenvalues for male morphology. The percentage of variation for Function 1 and 2 are highlighted in bold.

Eigenvalues				
Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	8.106[a]	**62.88**	62.88	0.94
2	3.938[a]	**30.54**	93.42	0.89
3	0.626[a]	4.86	98.27	0.62
4	0.133[a]	1.03	99.31	0.34
5	0.089[a]	0.69	100.00	0.29
a. First 5 canonical discriminant functions were used in the analysis.				

Table 2.8: Standardized canonical discriminant function coefficients for male morphology, with positive and negative correlation values in bold.

Standardized Canonical Discriminant Function Coefficients					
	Function				
	1	**2**	**3**	**4**	**5**
Prototum length	0.045	0.290	0.137	-0.050	0.838
Prototum arc	-0.141	0.424	0.120	0.102	-0.196
Prototum height	0.056	**0.800**	-0.474	0.335	-0.154
Abdomen width	**-0.198**	0.358	0.429	-0.244	-0.380
Hind femur length	**0.427**	-0.412	-0.720	-0.335	0.125
Tibia length	0.427	0.453	0.218	-0.435	-0.735
Total body length	0.073	-0.326	0.747	0.039	0.720
Head width	0.182	-0.462	0.389	0.792	-0.439
Antenna length	0.321	**-0.469**	-0.561	0.078	0.372

The DFA for female morphology shows that the species are morphologically different ($p < 0.05$). *B. discolor* and *B. serrata* are the only two species that show any overlap in clustering on the centroid plot, indicating morphological similarities between these two species (Figure 2.18). According to the eigenvalues (Table 2.9), 78.51% of the variation is explained by Function 1and 17.73% variation by Function 2. DF1 has a positive correlation with head width and a negative correlation with pronotum length and DF2 has a positive correlation with pronotum length and a negative correlation with head width (Table 2.10).

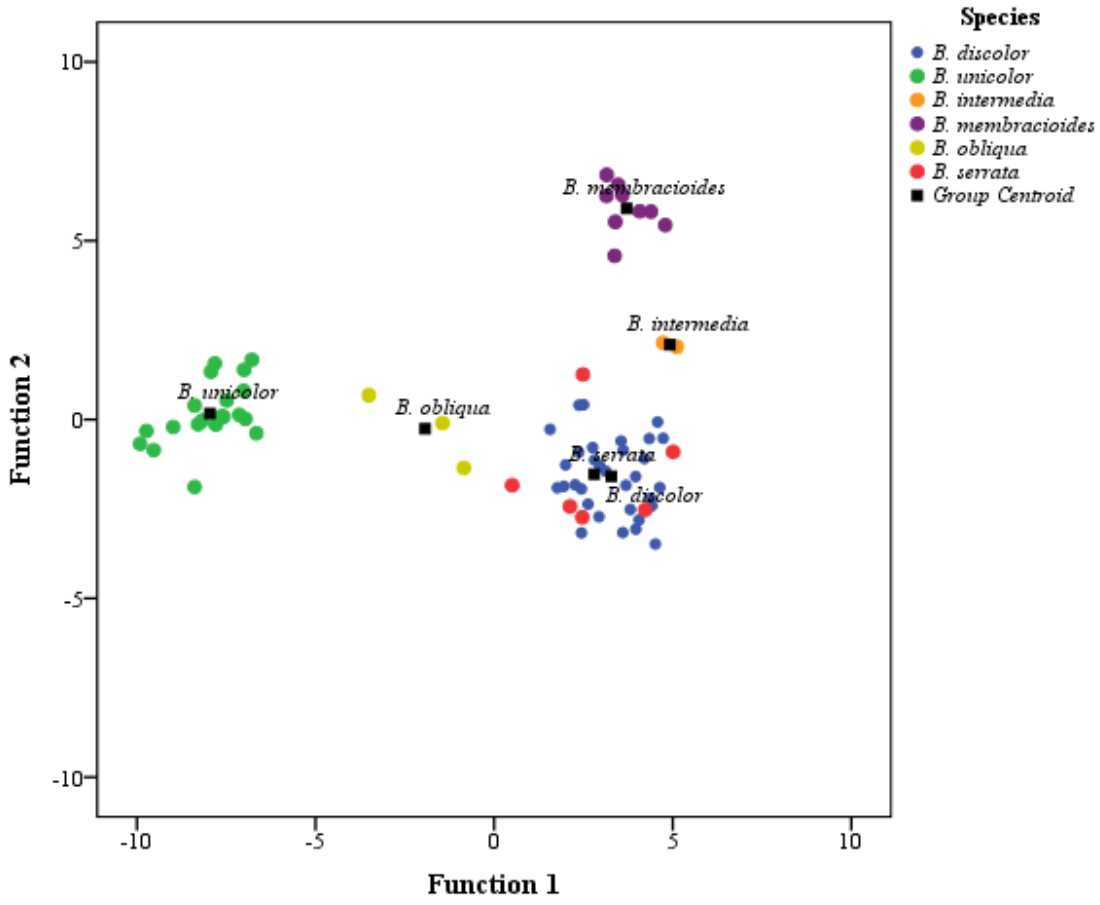

Figure 2.18: Canonical centroid plot of the discriminant function analysis (DFA) for female morphology.

Table 2.9: Table showing the eigenvalues for female morphology. The percentage of variation for Function 1 and 2 are highlighted in bold.

Eigenvalues				
Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	28.034[a]	**78.51**	78.51	0.983
2	6.330[a]	**17.73**	96.24	0.929
3	0.994[a]	2.78	99.02	0.706
4	0.283[a]	0.79	99.82	0.470
5	0.066[a]	0.18	100.00	0.248

a. First 5 canonical discriminant functions were used in the analysis.

Table 2.10: Standardized canonical discriminant function coefficients for female morphology, with positive and negative correlation values in bold.

Standardized Canonical Discriminant Function Coefficients					
	Function				
	1	**2**	**3**	**4**	**5**
Prototum length	**-0.673**	**0.886**	0.116	0.579	0.178
Prototum arc	0.045	0.143	0.374	0.033	0.581
Prototum height	-0.091	-0.026	0.169	0.365	0.263
Abdomen width	-0.019	-0.143	-0.217	0.934	-0.121
Hind femur length	0.335	0.381	-0.059	-0.392	0.650
Tibia length	0.138	0.707	-0.612	0.191	-0.585
Total body length	0.078	-0.001	0.662	-0.706	-0.694
Head width	**0.898**	**-1.268**	-0.363	-0.327	-0.026
Antenna length	0.694	-0.093	0.776	-0.127	0.110

2.3.5. Acoustic Calls

According to the average values for acoustic call properties (Table 2.11), *B. serrata* has the shortest call length (1.336 s) and *B. obliqua* has the longest call length (4.836 s). Inter-syllable pauses are not present in *B. serrata* and *B. obliqua* calls; however, *B. unicolor* has the longest pause (0.196 s) and *B. discolor* has the shortest pause (0.052 s). *Bullacris membracioides* has the longest introductory call, whereas *B. serrata* has the shortest. The species with the longest final call is *B. obliqua* and the shortest call is *B. intermedia*.

Looking at the graphs below (Figure 2.19 and 2.20), *B. discolor* has the highest carrier frequency (2347.866 kHz), whereas *B. membracioides* has the lowest frequency (1855.925 kHz). *B. obliqua* has the highest introductory syllable frequency (3505.938 kHz), whereas *B. membracioides* has the lowest frequency (1945.873 kHz). *B. obliqua* has the highest frequency of the first harmonic (3655.521 kHz), whereas *B. intermedia* have the lowest frequency (2584.000 kHz).

Table 2.11: Mean and standard error values for temporal and frequency call properties for six species. Minimum and maximum values are highlighted in bold.

Species	Total call length	Length of introduction	Length of final	Inter-syllable pauses	Carrier frequency	Introductory syllables	Frequency of first harmonic
B. discolor	(1.481 ± 0.055)	(0.364 ± 0.046)	(1.066 ± 0.023)	(0.052 ± 0.054)	**(2347.866 ± 78.653)**	(3104.030 ± 112.77)	(3194.170 ± 345.253)
B. unicolor	(1.863 ± 0.270)	(0.438 ± 0.120)	(1.227 ± 0.241)	**(0.196 ± 0.149)**	(2068.125 ± 131.713)	(2372.813 ± 341.564)	(3630.573 ± 754.250)
B. obliqua	**(4.836 ± 0.098)**	(1.031 ± 0.150)	**(3.805 ±0.052)**	**(0.000 ± 0.000)**	(1937.500 ± 353.553)	**(3505.938 ± 853.389)**	**(3655.521 ± 921.743)**
B. membracioides	(2.545 ± 0.079)	**(1.523 ± 0.135)**	(1.022 ± 0.089)	(0.114 ± 0.033)	**(1855.925 ± 33.039)**	**(1945.873 ± 102.438)**	(3602.113 ± 213.545)
B. serrata	**(1.336 ± 0.045)**	**(0.116 ± 0.062)**	(1.220 ± 0.069)	**(0.000 ± 0.000)**	(1955.210 ± 383.232)	(2859.600 ± 377.580)	(3083.550 ± 76.213)
B. intermedia	(2.352 ± 0.236)	(1.509 ± 0.241)	**(0.843 ± 0.084)**	(0.084 ± 0.000)	(2067.233 ± 137.118)	(2354.333 ± 150.324)	**(2584.000 ± 123.732)**

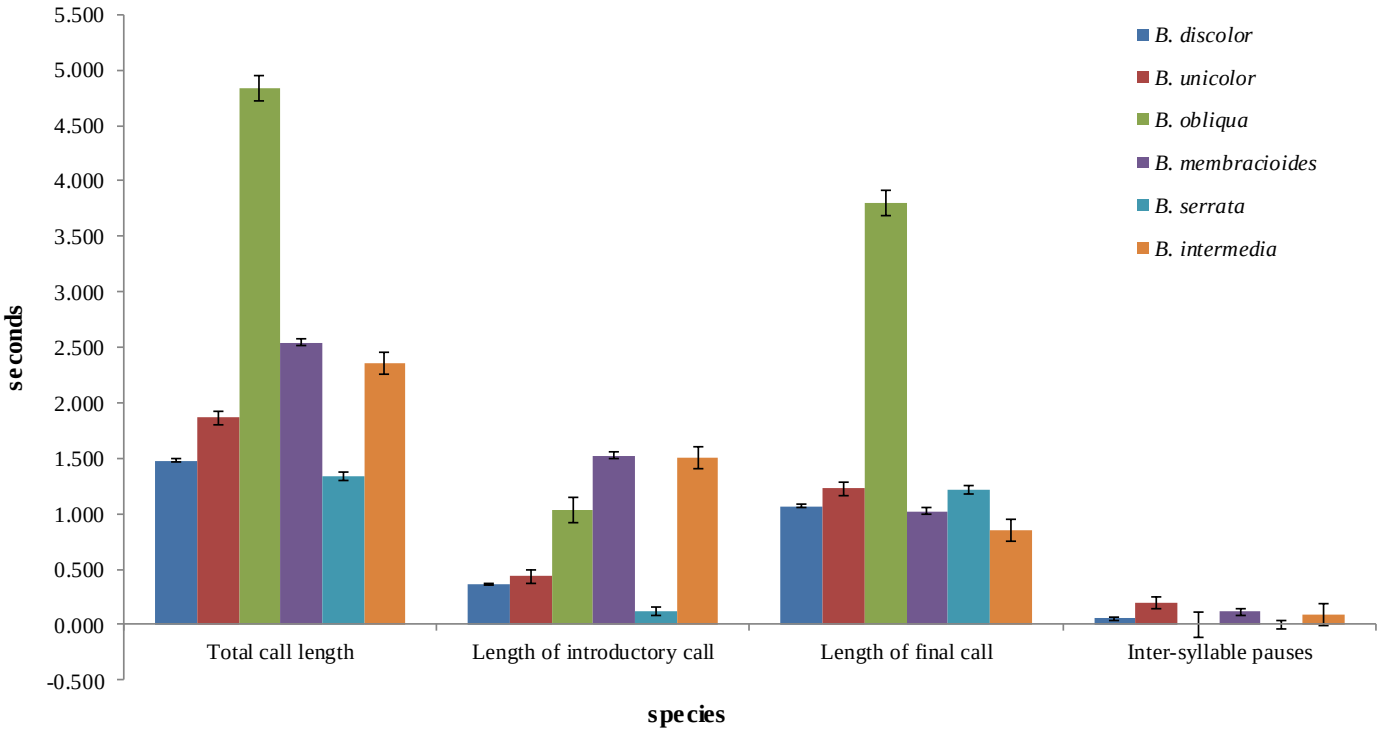

Figure 2.19: Mean lengths (± standard error) of temporal properties for male acoustic calls.

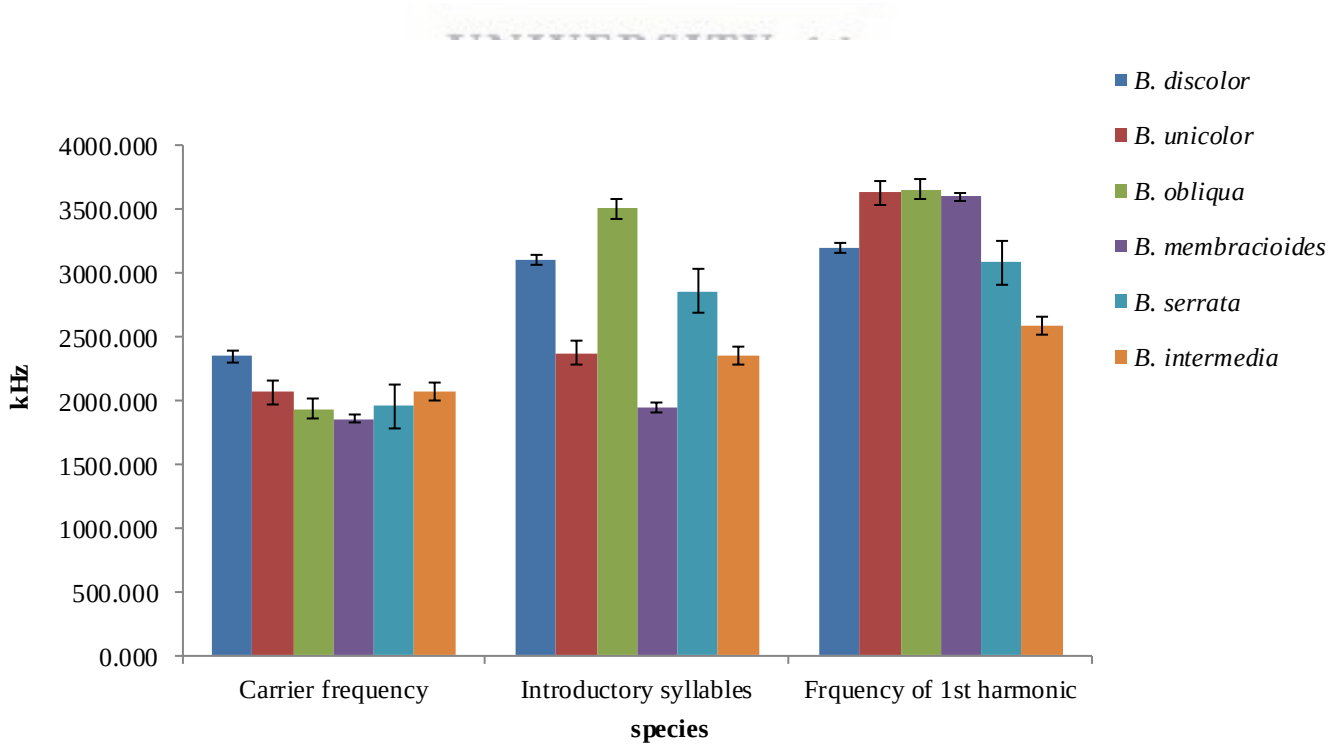

Figure 2.20: Mean (± standard error) of frequency properties for male acoustic calls.

2.3.6. MANOVA results for acoustic calls

Multivariate analyses in Table 2.12, shows that males have significant differences among their acoustic call characteristics (Pillai's Trace = 2.857; $F_{35, 2755}$ = 104.943 $p < 0.001$) for the six *Bullacris* species. Multiple comparisons shows that total call length differs between all species, aside from *B. membracioides* and *B. intermedia* (p = 1.000). The length of the introductory syllables shows significant differences across all species ($p < 0.05$). The length of the final syllable differs between all species, except for *B. serrata* and *B. intermedia* (p = 0.266). Carrier frequency is also significantly different between all species, except for *B. unicolor and B. serrata* (p = 0.312); *B. obliqua* and *B. membracioides* (p = 0.391) as well as *B. intermedia* (p =0.990); and *B. membracioides* and *B. intermedia* (p = 0.966).

The frequencies of the introductory syllables differs between all species, with the exception of *B. unicolor* and *B. serrata* (p = 0.983); *B. membracioides* and *B. serrata* (p = 0.631) as well as *B. intermedia* (p = 0.999); and *B. serrata* and *B. intermedia* (p = 0.586). The frequency of the first harmonic is similar between *B. discolor* and *B. membracioides* (p = 1.000) as well as *B. obliqua* (p = 0.996); between *B. obliqua* and *B. membracioides* (p = 0.994), and lastly between *B. intermedia* and *B. serrata* (p = 0.999). There are significant differences in inter-syllable pauses between most species, with an exception of *B. discolor* and *B. membracioides* (p = 0.581); *B. obliqua* and both *B. serrata* and *B. intermedia* (p = 1.000).

Table 2.12: Multiple comparisons between subjects (MANOVA) for male acoustic calls (mean differences and standard error). Significant differences presented in bold.

Total call length

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(-0.481 ± 0.027)**	**N/A**				
B. obliqua	**(-3.361 ± 0.031)**	**(-2.880 ± 0.032)**	**N/A**			
B. membracioides	**(-1.020 ± 0.030)**	**(-0.539 ± 0.030)**	**(2.340 ± 0.034)**	**N/A**		
B. serrata	**(0.159 ± 0.052)**	**(0.640 ± 0.052)**	**(3.520 ± 0.055)**	**(1.179 ± 0.054)**	**N/A**	
B. intermedia	**(-1.017± 0.044)**	**(-0.535 ± 0.045)**	**(2.344 ± 0.048)**	(0.004 ± 0.046)	**(-1.175 ± 0.063)**	**N/A**

Length of introductory call

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(-0.108 ± 0.018)**	**N/A**				
B. obliqua	**(-0.663 ± 0.021)**	**(-0.555 ± 0.021)**	**N/A**			
B. membracioides	**(-1.073 ± 0.020)**	**(-0.965 ± 0.020)**	**(-0.410 ± 0.023)**	**N/A**		
B. serrata	**(-0.312 ± 0.036)**	**(-0.204± 0.036)**	**(0.351 ± 0.037)**	**(0.761 ± 0.0367)**	**N/A**	
B. intermedia	**(-1.355 ± 0.030)**	**(-1.246 ± 0.030)**	**(-0.692 ± 0.033)**	**(-0.281 ± 0.032)**	**(-1.042 ± 0.043)**	**N/A**

Length of final call

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(-0.090 ± 0.023)**	**N/A**				
B. obliqua	**(-2.660 ± 0.028)**	**(-2.570 ± 0.028)**	**N/A**			
B. membracioides	**(0.090 ± 0.026)**	**(0.181 ± 0.026)**	**(2.751 ± 0.030)**	**N/A**		
B. serrata	**(0.493 ± 0.045)**	**(0.583 ± 0.045)**	**(3.153 ± 0.048)**	**(0.402 ± 0.047)**	**N/A**	
B. intermedia	**(0.373 ± 0.039)**	**(0.465 ± 0.039)**	**(3.036 ± 0.041)**	**(0.285 ± 0.040)**	(0.117 ± 0.055)	**N/A**

Carrier frequency

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(135.131 ± 20.036)**	**N/A**				
B. obliqua	**(370.829 ± 23.572)**	**(235.698 ± 23.845)**	**N/A**			
B. membracioides	**(420.144 ± 22.205)**	**(285.013 ± 22.495)**	(49.315 ± 25.695)	**N/A**		
B. serrata	**(214.815 ± 38.590)**	(79.683 ± 38.757)	**(-156.014 ± 40.698)**	**(-205.329 ± 39.922)**	**N/A**	
B. intermedia	**(392.394 ± 32.897)**	**(257.263 ± 33.093)**	(21.565 ± 35.346)	(-27.750 ± 34.450)	**(177.580 ± 46.721)**	**N/A**

Frequency of introductory syllables

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(659.271 ± 64.223)**	**N/A**				
B. obliqua	**(-465.933 ± 75.556)**	**(-1125.203 ± 76.432)**	**N/A**			
B. membracioides	**(943.817 ± 71.177)**	**(284.546 ± 72.105)**	**(1409.750 ± 82.361)**	**N/A**		
B. serrata	**(745.375 ± 123.696)**	(86.104 ± 124.233)	**(1211.308 ± 130.453)**	(-198.442 ± 127.966)	**N/A**	
B. intermedia	**(987.796 ± 105.447)**	**(328.526 ± 106.076)**	**(1453.729 ± 113.298)**	(43.980 ± 110.425)	(242.422 ± 149.761)	**N/A**

Frequency of first harmonic

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(-330.808 ± 76.096)**	**N/A**				
B. obliqua	(-43.977 ± 89.525)	**(286.831 ± 90.563)**	**N/A**			
B. membracioides	(10.207 ± 84.336)	**(341.015 ± 85.436)**	(54.184 ± 97.859)	**N/A**		
B. serrata	**(1567.467 ± 146.565)**	**(1898.275 ± 147.201)**	**(1611.444 ± 154.572)**	**(1557.260 ± 151.625)**	**N/A**	
B. intermedia	**(1630.208 ± 124.943)**	**(1961.016 ± 125.688)**	**(1674.184 ± 134.245)**	**(1620.000 ± 130.841)**	(62.741 ± 177.449)	**N/A**

Length of inter-syllable pauses

	B. discolor	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. discolor	**N/A**					
B. unicolor	**(-0.140 ± 0.011)**	**N/A**				
B. obliqua	**(0.103 ± 0.013)**	**(0.243 ± 0.014)**	**N/A**			
B. membracioides	(-0.021 ± 0.021)	**(0.119 ± 0.013)**	**(-0.124 ± 0.015)**	**N/A**		
B. serrata	**(0.103 ± 0.022)**	**(0.243 ± 0.022)**	(0.000 ± 0.023)	**(-0.124 ± 0.022)**	**N/A**	
B. intermedia	**(0.103 ± 0.019)**	**(0.243 ± 0.019)**	(0.000 ± 0.020)	**(-0.124 ± 0.020)**	(0.000 ± 0.027)	**N/A**

2.3.7. DFA results for acoustic calls

According to the eigenvalues, 76.8% of the variation was contributed by Discriminant Function 1 and 17.7% of the variation contributed by Discriminant Function 2 (Table 2.13). DF1 has a positive correlation with total call length and a negative correlation with the length of the introductory syllables. DF 2 has a positive correlation with the length of introductory syllable and a negative correlation with the length of the final syllable (Table 2.14). The calls are mostly separated on the basis of temporal, rather than frequency components of the call.

Table 2.13: Table showing eigenvalues for male advertisement calls. The percentage of variation for Function 1 and 2 are highlighted in bold.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	37.972[a]	**76.8**	76.8	0.987
2	8.767[a]	**17.7**	94.5	0.947
3	2.354[a]	4.8	99.3	0.838
4	0.32[a]	0.6	99.9	0.493
5	0.042[a]	0.1	100	0.202

a. First 5 canonical discriminant functions were used in the analysis.

Table 2.14: Standardized canonical discriminant functions coefficients for all male *Bullacris* calls, with positive and negative correlation values highlighted in bold.

Standardized Canonical Discriminant Function Coefficients					
	Function				
	1	**2**	**3**	**4**	**5**
Total length of call	**1.096**	0.181	-1.676	-1	0.392
Length of introductory call	**-0.784**	**0.852**	1.06	0.841	0.055
Length of final call	0.345	-0.24	1.461	0.811	-0.323
Carrier frequency	-0.179	-0.148	0.335	0.069	0.801
Introductory syllable	0.093	0.022	0.357	0.378	0.341
First harmonic	-0.149	0.028	-0.898	0.584	-0.154
Inter-syllable pauses	0.282	**-0.175**	-0.228	0.397	0.272

The centroid plot for acoustic calls (Figure 2.21) *B. membracioides* and *B. intermedia* differ from the other species in having longer introductory syllables. *Bullacris unicolor*, *B. serrata* and *B. discolor* is grouped together, thus having similar acoustic signals. *Bullacris obliqua* is most widely separated from all other species, having a much longer total call length and shorter introductory syllable (Figure 2.19).

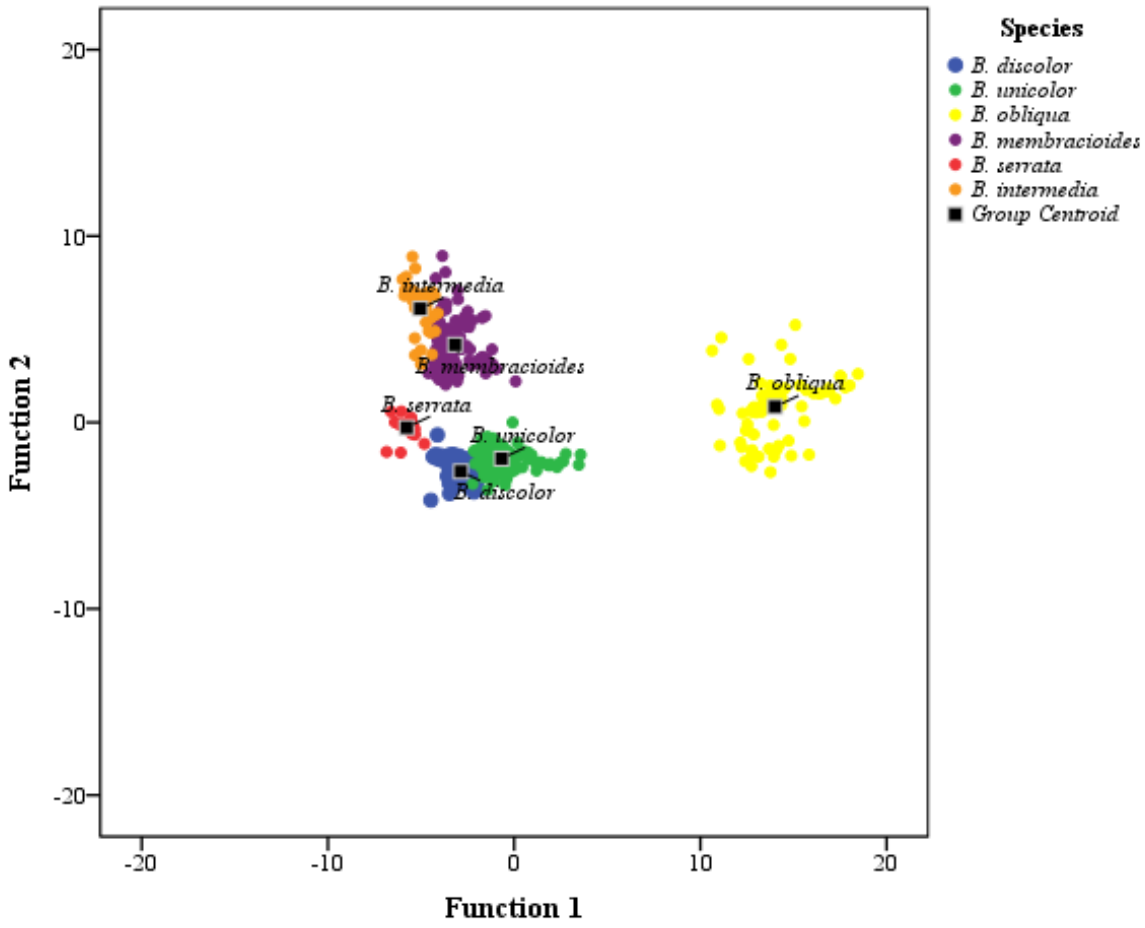

Figure 2.21: Canonical centroid plot of the discriminant function analysis (DFA) for male acoustic calls.

The scatter plot (Figure 2.22) shows the relationship between the average abdomen width of the species and their average carrier frequency (r = -0.829; p = 0.042). This indicates that the smaller the abdomen width gets, the higher the carrier frequency becomes and vice versa. However, this relationship does not appear to hold true for *B. serrata*, since it has an abdomen width of 16.14 mm and a carrier frequency of 2052.82 kHz.

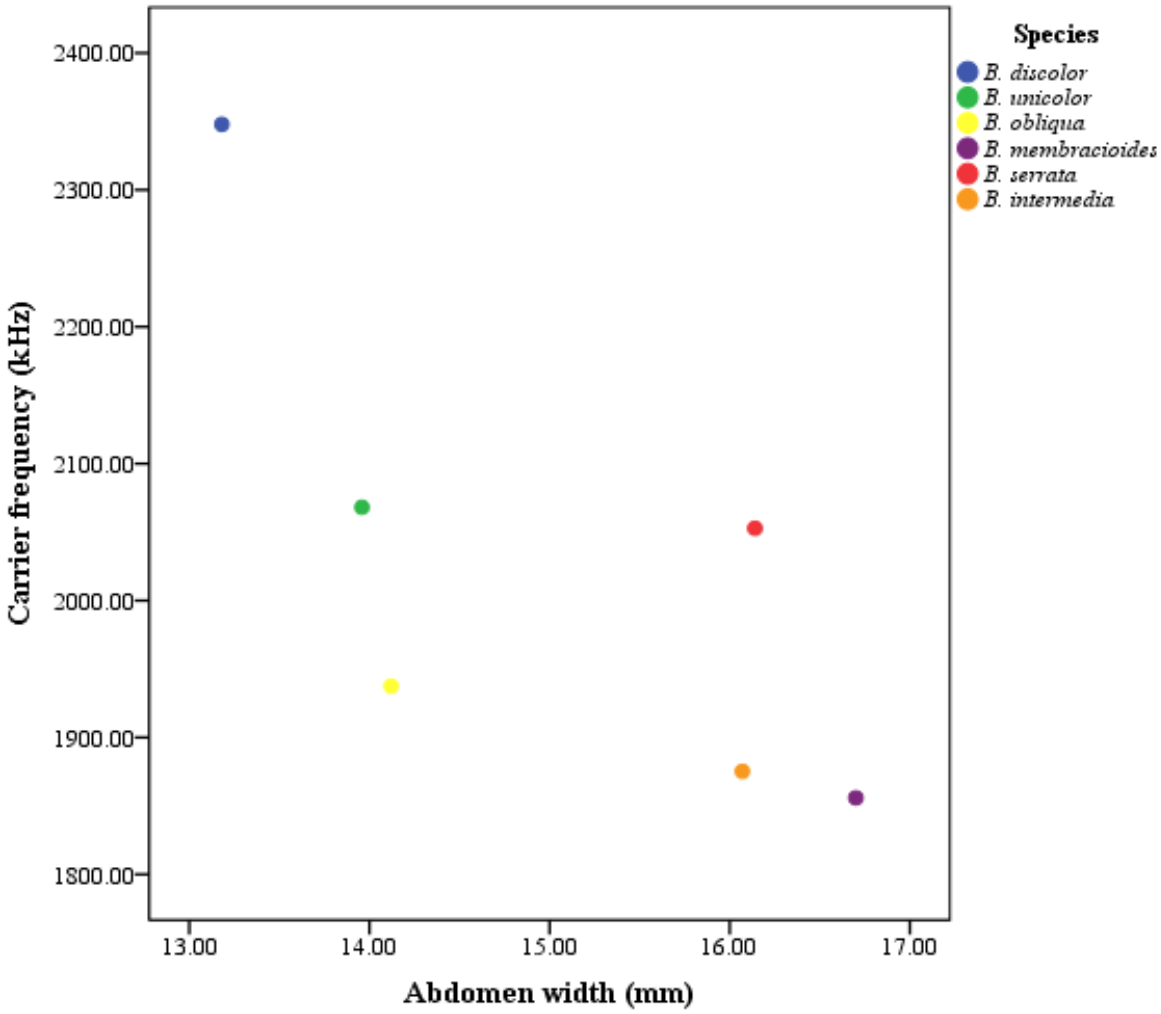

Figure 2.22: Scatter plot showing the relationship between the average abdomen width of each species and their average carrier frequency.

According to Spearman's correlation results, between abdomen width and carrier frequency, the correlation coefficient is r = -0.829 which indicates a strong relationship. Since it is a negative value, this indicates that if one variable increases (abdomen width); the second variable will decrease (carrier frequency). The significant (2-tailed) value is p = 0.042, therefore there is a statistical significance between abdomen width and carrier frequency. However, for Spearman's correlation results between the total body length and carrier frequency, there is no significant relationship between total body length and carrier frequency (r = -0.314; p = 0.544).

2.3.8. Mantel test

Mantel test results for the correlation of pairwise distances between acoustic and morphological variables, based on 9999 replicates, produced an R-value = 0.236 and no significant relationships was found (P = 0.179), thus illustrating that morphology does not have a significant relationship with the advertisement call properties of male bladder grasshoppers across species.

2.4. Discussion

2.4.1 Distribution

The distribution pattern Dirsh (1965) constructed is very similar to the collective distribution maps that were created for this study (Figure 2.12), with an exception of the inland locations for *B. unicolor*. The distribution of each *Bullacris* species (Figure 2.5 – 2.11) can be seen occupying only certain areas of coastal regions in South Africa. For example, *B. unicolor* only occurs in the north-western parts, whereas *B. membracioides* only occurs in the south-eastern regions. Since they occupy specific areas which are exposed to specific climatic variations, this may therefore result in differences in morphological characteristics. However, species with overlapping distributions such as, *B. unicolor, B. obliqua* and *B. discolor* (which are the three smallest species) and *B. serrata*, *B. intermedia* and *B. membracioides* may share morphological similarities. Climate variation can also be one of the reasons why their reproductive seasons differ, with seasonality peaking earlier in the west and later in the east of South Africa. For example, *B. unicolor* reproduces earlier (start of summer) and *B. membracioides* reproduces later in the season (towards the end of summer) (Couldridge and Gordon, 2015).

2.4.1 Morphological differences

There are some discrepancies in morphological measurements between this study in comparison to that of Dirsh (1965). This is unlikely to be due to measurement error, since the measurements obtained for *B. boschimana* on the same individual matched those reported by Dirsh. The body length for *B. obliqua* females is smaller in this study (average of 44.44 mm) compared to the range reported by Dirsh of 45 – 51 mm. *Bullacris discolor* males from this study also differ with a previous average range of 44 – 58 mm and a current average of 44.33 mm. *Bullacris serrata* females had a range of 45-55 mm but here have an average body length of 45.90 mm. In contrast, the average body length of *B. intermedia* individuals had an initial range of 44-49 mm and now an average of 51.97 mm for males, and females with a previous average of 42 mm to a current average of 48.02 mm.

Morphologically, Dirsh (1965) believed that *B. intermedia* individuals are very similar to *B. membracioides* and also to a lesser extent *B. unicolor*. In addition, *B. serrata* are very close to *B. discolor*, however he mentions that the patterns on the pronotum as well as the abdomen are different. He also mentions that *B. obliqua* was very similar to *B. discolor* and *B. serrata*. Conversely, this study shows that *B. serrata*, *B. discolor* and *B. obliqua* males are morphologically alike (Figure 2.17), whereas *B. serrata* and *B. discolor* females are similar (Figure 2.18). Possible reasons for these similarities may be the result of similar types of habitats due to overlapping distributions, which suggests experiencing the same climate types as well as possible host plant similarities. Alternatively, these species may share similarities due to more recent speciation events.

The discrepancies in body sizes between the two studies could be due to the measurement of individuals from different sampling locations. A study by Bai *et al*. (2016) used geometric morphometric methods to show that 39 populations of *Trilophidia annulata* in China had varying fore and hind-wing lengths as well as body size among the geographical different populations. Smaller individuals that possessed shorter forewings were located in lower latitudinal and mountainous areas whereas the larger bodied individuals had larger forewings and were found in higher latitudinal areas. Previous studies have shown that bladder grasshoppers are known to exhibit variation in size within a species based on geographical differences (Sathyan, 2014; Sathyan *et al*., 2016). According to Couldridge (pers. com.), due to *B. discolor* and *B. serrata* being so acoustically similar and having overlapping distributions, it is possible that these two species could be geographic variants of the same species. However, due to the lack of genetic material, this theory cannot be tested as of yet.

The morphology of insects may be influenced by environmental conditions as well as ecological factors (Bernays, 1991). Latitude, altitude and resource availability have been shown to cause morphological adaptations, which is an organisms' response to the pressures exerted by its environment (Rhymer, 1992; Williams, 2001). However, some studies have shown that morphological gradients have not always been consistent with environmental factors (Ashton, 2004). When looking at the dispersion of bladder grasshoppers, there are two factors that restrict their distribution, namely host plant fidelity and vagility. This is due to the females, nymphs and alternate males possessing only rudimentary sub-pronotal wings and being non-volant. In addition, pneumorids demonstrate high host-plant specificity for plants that have very patchy distributions; and this may create isolated populations with a high potential for genetic drift (Römer *et al*., 2014).

The results of this study have shown that the *Bullacris* species described by Dirsh (1965) differ both morphologically and acoustically, thus supporting the current classification, although there is some overlap, especially in morphology, between species. However, genetic analyses together with morphological comparisons may be a more accurate way to verify species boundaries (Friedheim, 2016).

2.4.2. Acoustic variation

According to Bohn (1988), changes in the velocity and absorption of sound travelling through the air can be altered by environmental effects. The loss of amplitude from signals is the result of spreading, scattering and absorption of sound waves, which diminishes the range at which receivers detect the signals (Couldridge and van Staaden, 2004). Studies in birds (Brenowitz, 1983) and primates (Brown *et al*., 1995) have shown that calls broadcast in diverse environments show signs of different rates of excess attenuation and/or distortion.

The acoustic adaptation hypothesis (Morton, 1975; Hansen, 1979) was based on the observation that environmental factors greatly influence the evolution of long-range acoustic signals by enforcing selection pressures that modify the properties of sound signals in order to maximize their broadcast range, as well as the number of potential receivers (Endler, 1992; Forrest, 1994). An example of this can be seen in the divergence of songs in birds (Ruegg *et al*., 2006). The earlier mentioned habitat types vary extensively, from dense, humid areas to open, semi-desert, arid areas. Since sound properties can be influenced by their surroundings, and each species of *Bullacris* occurs is a different habitat, there should be variation between the characteristics of their acoustic signals.

This study shows that when visually comparing the acoustic signals of each species, it is very obvious to see variation. However, according to the DFA results (Figure 2.21), *B. membracioides* and *B. intermedia* have overlapping clusters which shows that they have longer introductory calls. Clusters for *B. serrata* and *B. discolor* also overlap; as well as *B. discolor* and *B. unicolor*, however, *B. obliqua* seems to cluster separately, which suggests that they hardly share any similarities with the rest of the species. Results show that this is due to *B. obliqua* having a shorter introductory call and a much longer total call length. Species such as *B. discolor* and *B. unicolor* have overlapping distributions (Figure 2.12) and share similarities between call characteristics which could possibly be a result of selective pressures on the calls due to transmitting in the same environment. However there are still slight differences in the calls, which may help to avoid hybridization. This is also true for *B. intermedia* and *B. membracioides.*

Communication plays a vital role in the social behavior of all animals and assists in attracting and courting of potential mates, maintaining territories and minimizing predation (Walker 1998). Each signal contains biologically significant information, i.e. the identity of the signaler (sex and species), size and physical condition (Gerhardt and Huber, 2002), however these signals are often degraded over distance and time within a natural habitat. Thus the production of a signal is only beneficial to the sender, if the signal is successfully transmitted over a certain distance. There are a number of factors that determine the effectiveness of signals over a certain distance. The environment in which the call is being transmitted, adds constraints on signal transmission and thus the detection thereof.

Closely related species often tend to have similarities between characteristics such as morphology, but are often divergent in sexually selected traits such as acoustic signals (Dominey, 1984; Ryan and Rand, 1993). Studies have shown that the signal structure and signaling behaviour between and within species may vary due to geographical variation and this then influences speciation (Gray and Cade, 2000; Filatova *et al*., 2012; Oh *et al*., 2012). Examples of this can be seen between geographically varied populations of *Chorthippus biguttulus* species (Stange and Ronacher, 2012), as well as between *Chorthippus parallelus* (Tregenza *et al*., 2000). Thus any change to an environment, in which signaling occurs can greatly influence and affect the nature and perception of a signal and therefore result in the divergence of mating signal preferences (Endler, 1992).

2.5. Conclusion

The result of this study has shown that each of the known *Bullacris* species varies both morphologically as well as acoustically. However, similarities between species may be the result of overlapping distributions, which implies similar habitat types and thus similar environmental conditions. Morphological differences are in accordance with Dirsh (1965); however some measurements were slightly different. In addition, morphological characteristics were found to not have any significant correlation with acoustic signals, indicating that morphology and acoustics may be under separate selective pressures. In future, a larger sample size of individual from a greater variety of geographic locations would benefit this study to better understand variation within and between species and specifically the collection of *B. boschimana* specimens. Furthermore, given the limitations of using morphology and acoustic signals, a genetic approach would be of great value to assist in distinguishing between species.

Chapter 3

A taxonomic and phylogenetic construction of the genus *Bullacris*

Abstract

The genus *Bullacris* (Orthoptera; Pneumoridae) has solely been differentiated based on morphological studies by Dirsh in 1965 and no phylogenetic studies have previously been done. This study focuses on the genetic structure among species within the genus *Bullacris*. However, due to insufficient sampling data, *B. boschimana* and *B. serrata* were excluded from this study. Analyses were performed by sequencing the mitochondrial (COI) and nuclear (ITS) gene regions from the hind-leg of each bladder grasshopper. Phylogenetic analyses were conducted using genetic pairwise distance tests, Bayesian inference and SplitsTree analyses. Results indicated that each species formed its own clade, with the exception of *B. intermedia*, which partially formed part of *B. unicolor*. This outcome is unexpected since *B. unicolor* and *B. intermedia* are morphologically distinct and have a limited overlap in distribution. Results also indicated a lack of correlation between genetic divergence and both morphological and acoustic divergence. Future studies should be performed with a larger sampling group for *B. intermedia* as well as include the two previously mentioned species that were unavailable.

Keywords: *Bullacris*, phylogenetics, COI, ITS

3.1. Introduction

3.1.1. Background

Bladder grasshoppers from the family Pneumoridae are a fairly small group, comprising of only nine genera, the largest of which is the genus *Bullacris*. However, to date no comprehensive genetic studies have been done on this group. Species were determined based solely on morphological comparisons by Dirsh (1965) and this was the last taxonomic review of the genus, in which seven distinctive species were recognized. However, of the seven species, only one *Bullacris boschimana* female (type specimen), which is considered to be a species on its own, was ever located.

Pneumoridae are predominantly confined to southern Africa and species from the genus *Bullacris* are known to occur in a number of different biomes, such as the Succulent-Karoo, Savanna and Fynbos biomes within the coastal areas of South Africa (Mucina and Rutherford, 2006), however, there is a single record for *B. membracioides* from Malawi (Dirsh, 1965). Environmental conditions within these regions differ extensively (see Couldridge and van Staaden, 2004), thus the development of phenotypic variation is immense. According to Donelson (2007), the gene flow in pneumorids is thought to be inhibited by two factors, such as host plant specificity and low vagility.

3.1.2. Genetic analyses

Over the past 250 years, taxonomic decisions were solely based on morphological structures of individual organisms (Herbert and Gregory, 2005). However, there are a limited number of common physical characters across major groups of organisms (Hillis, 1987). Therefore there is no one standard agreement on morphological definitions and thus species classification (Nazari *et al*., 2007), which causes there to be a barrier in the flow of knowledge. In recent years, there has been a developing understanding in molecular systematics to differentiate between species; due to each individual organism having a unique genome (Mallet, 1995; Herbert and Gregory, 2005) and similarities can be found between individuals from the same species. However, this method has its advantages and disadvantages.

The genomic approach to distinguish between taxa illustrates the diversity of DNA sequences used to identify organisms. Studies by Flook and Rowell (1997 a; 1997 b) examined the evolutionary history of the order Orthoptera, by reconstructing phylogenies from nucleotide sequences. They discovered that mitochondrial DNA (mtDNA) sequences were suitable for studying the phylogenies of insects and PCR primers permit the amplification of the gene being sequenced (Simon *et al*., 1994). However according to the phylogenetic population studies by Avise *et al*. (1979) and Lansman *et al*. (1981) mtDNA is the most rapidly evolving DNA. A single mtDNA genome is transmitted from each breeding female to its offspring and thus the genetic effective population size is proportional to the number of breeding females (Hebert *et al*., 2003). In contrast, nuclear genes, which are approximately 100 000 times larger, males and females contribute two full genomes to the gene pool and the effective size is therefore proportional to twice the total population size (Hebert *et al*., 2003).

The use of mtDNA, *cytochrome c oxidase subunit I* (COI) fragment has been effectively applied to intra- and interspecific studies of a wide range of invertebrate taxa (Lunt *et al*., 1996), including orthopterans (e.g., Funk *et al*., 1995; Szymura *et al*., 1996; Zhang and Hewitt, 1996; Trewick *et al*., 2000). The analysis of the COI (mtDNA) is maternally transmitted and is known to have a high evolutionary rate, and can therefore be used to determine the variation between closely related species (Giles *et al*., 1980; Jenuth *et al*., 1997). It has proved useful in both revealing cryptic taxa to explore the spatial partitioning of phylogenetic structure of species (phylogeography) and demonstrating close genealogical histories of morphologically or ecologically distinct taxa (e.g. Funk *et al*., 1995; Szymura *et al*., 1996; Trewick, 2000).

Conversely, the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) has also been widely used in molecular phylogenies and taxonomy studies, due to the high amplification of rRNA genes (Das and Deb, 2015). Recent statistics shows that the ITS-region is one of the most extensively sequenced molecular markers and it has one of the highest success rates for identification (Alvarez and Wendel, 2003). This region is comprised of 18S rRNA (an internally transcribed region – ITS1), the entire 5.8S rRNA sequence (an internally transcribed region – ITS2) and a partial 28S rRNA sequence.

The ITS barcoding region has been used successfully to disseminate taxonomic questions in an array of organisms such as algae (Bakker *et al*., 1995; An *et al*., 1999), plants (Jobes and Thien, 1997; Baldwin and Markos, 1998; Alvarez and Wendel, 2003), mites (Roy *et al*., 2009; Engelbrecht *et al*., 2014; Engelbrecht *et al*., 2016), oomycota (Cooke *et al*., 2000; Leclerc *et al*., 2000; Robideau,, 2011) and grasshoppers (Sword *et al*., 2007; Ullrich *et al*., 2009). It has also been useful to identify species at different geographical areas as well as within species and this could be due to having relatively low evolutionary pressure acting on "non-functional" sequences (e.g. Asteraceae; Baldwin *et al*., 1995).

3.1.3. Aims

This study focuses on the phylogenetics among species within the genus *Bullacris* and to determine to what extent the currently described species are genetically distinct, as well as to determine whether there is a correlation between genetic divergence and morphological and acoustic divergence. It is anticipated that each species described by Dirsh (1965), will be genetically distinct. Assessment of spatial genetic variation in such taxa may facilitate novel insights into genetically effective dispersal, social and mating behaviors, as well as evolutionary selection pressures on signaling systems (Zamudio *et al*., 2016).

3.2. Materials and Methods

3.2.1. Sampling

Specimens were collected in the field during the months from August 2013 to February 2015, along the coast line of South Africa, in the Northern Cape, Western Cape, Eastern Cape and KwaZulu-Natal (see Table 2.1, Chapter 2). Certain species were difficult to collect in the field and sampling numbers were supplemented with material from museum collections, however there was also limited museum specimens and thus sample sizes were uneven across species.

A distribution map was created using the GPS coordinates recorded upon collection together with museum locality data, in Arc GIS 10.3.1 (see Figure 2.12, Chapter 2). Furthermore, *Physemacris variolosus* was used as an outgroup, which was analyzed in previous studies and found on Genbank (accession number, GU 122585.1).

3.2.2. DNA extraction, PCR and DNA sequencing

The hind leg of specimens from each species (museum and fresh individuals) was removed, crushed and then placed into a drying block overnight at 37 °C to remove all excess moisture and ethanol. DNA was then extracted from the muscle tissue by using a KAPA Express DNA Extraction Kit (KK 7151) following the protocol of the manufacturer, KAPA Biosystems. The number of individuals analyzed can be seen in Table 3.1. Universal primers, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG -3') and HCO2198 (5'-TAAACTTCAGGGTGAGGG TGACCAAAAAATCA-3') (Folmer *et al*., 1994) of COI were

used to amplify 633 base pairs (bp) of the gene. The nuclear ITS gene, for 754 bp was amplified using primers described by Roy *et al*. (2008), forward (5'-AGAGGAAGTAAAAGTCGTAACAAGG-3') and reverse (5'CCTTAGTAATATGCTTAAATTCAGG-3').

Table 3.1: Total number of taxa genetically analyzed for mitochondrial (CO1) and nuclear (ITS) genes.

	B. unicolor	***B. discolor***	***B. membracioides***	***B. obliqua***	***B. intermedia***
COI gene	45	25	11	13	1
ITS gene	27	10	8	9	3

Each PCR reaction contained 22.5 µL of the PCR master mix, which comprised of 10 µL Millipore water, 1.25 µL of the respective primers, 12.5 µL of 2G Robust Hotstart ReadyMix (KM5701- KAPA Biosystems) enzyme, and 2.5 µL of template DNA. The PCR reaction for the COI gene, had an initial denaturation step at 95 °C for 1 min, a 10-cycle amplification (95 °C for 1 min, 43 °C for 1 min, and 72 °C for 1 min), followed by a 30-cycle amplification (93 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min). The final extension step was continued for 5 min at 72 °C and final hold at 15 °C.

The PCR protocol for the ITS gene, had an initial denaturation at 94 °C for 5 min, then a 30 cycle amplification (94 °C for 30 sec, 49 °C for 45 sec, and 72 °C for 1 min), followed by a final extension of 72 °C for 10 min and a final hold at 15 °C. To confirm the successful DNA amplification, electrophoresis was carried out using 1 x TBE buffer on a 1% agarose gel. Successful amplified samples were sent to the DNA sequencing facility at Stellenbosch University for PCR clean up and sequencing.

3.2.3. Sequencing alignment and phylogenetic reconstructions

Sequences were aligned manually using BioEdit Sequence Alignment Editor Version 7.2.5 (Hall, 1999). The COI data matrix consisted of 633 aligned base pairs and 95 different taxa, whereas ITS had a data matrix of 754 aligned base pairs and 57 different taxa. Mitochondrial sequences were translated into amino acids using EMBOSStranseq (www.ebi.ac.uk/Tool/st/emboss_transeq) to confirm functionality and thus exclude the possible presence of pseudogenes.

Phylogenetic analyses were conducted using Bayesian inference (BI) using MrBayes v. 3.2.5 (Ronquist *et al*., 2012), a genetic pairwise distance test was also calculated using PAUPup version 1.0 (Calendini and Martin, 2005) and a table was generated to measure the distances in genetic differentiation. Unique evolutionary lineages was investigated using SplitsTree version 4 (Huson, 1998), as it assists in visualizing the complexity of phylogenic data. These analyses were done for both COI and ITS genetic markers.

The sample frequency was set to 1000 with a convergence rate for the COI region being 4 million generations and the ITS region being 6 million generations. Analyses were terminated once the standard deviation of split frequencies fell below 0.1. The trees were edited using FigTree v. 1.4.1 (Rambaut, 2014) where posterior probabilities (PP) values for the Bayesian tree were added (above), together with bootstrap values for the maximum likelihood (ML) (below). Only support values greater than 0.95 were retained for PP, and the following scale was used: 0.50-0.94, weak; and 0.95-1.0, strong; whereas support values greater than 75% was retained for ML (Felsenstein, 1985).

3.2.4. Statistical analyses

A statistical mantel test was performed using the Ade4 package in R 3.32, to test whether genetic pairwise distances were correlated with both acoustic and morphological differences.

3.3. Results

3.3.1. Data characteristics

Sampling was done in the Northern, Western and Eastern Cape, however due to unsuccessful sampling; numbers were limited and then supplemented with museum specimens. However, there was great difficulty in the sequencing of pinned museum specimens due to individuals being timeworn. A total of 152 *Bullacris* individuals were successfully sequenced, of which, 95 specimens were sequenced for COI and 57 for ITS. Since *B. serrata* was not collected in the field, museum speciecimens were unsuccessfully sequenced and only three museum specimens of *B. intermedia* were successful. A JMODELTEST selected the GTR + G + I model as the best model of substitution for both gene fragments.

3.3.2. Pairwise distances

Genetic pairwise distances generated in PAUPup version 1.0 (Calendini and Martin, 2005) was summarized into Tables 3.2 and 3.3. Differentiation between the COI and ITS gene for *Bullacris* species can be seen between and within species.

There is a large amount of variation for the mitochondrial gene (COI) within species, which is to be expected due to it being maternally inherited and having a high evolutionary rate. *Bullacris obliqua* has a greater variation within species with a 3.11% difference, whereas *B. membracioides* has the least amount of variation, with a 0.05% difference (Table 3.2). Between species, *B. membracioides* and *B. intermedia* had the greatest variation, with an average of 16.60% difference and *B. unicolor* and *B. intermedia* had the least variation, with an average of 2.73% and 7.32% difference, respectively (Table 3.2).

Table 3.2: Pairwise genetic distance table showing the differentiation between the COI gene of *Bullacris* species (mean ± SD), both within (bold) and between sampled species.

	B. unicolor	***B. discolor***	***B. membracioides***	***B. obliqua***	***B. intermedia***
B. unicolor	**2.726 ± 0.014**				
B. discolor	15.806 ± 0.011	**1.881 ± 0.015**			
B. membracioides	16.483 ± 0.004	11.193 ± 0.004	**0.057 ± 0.001**		
B. obliqua	13.213 ± 0.005	14.917 ± 0.006	16.078 ± 0.004	**3.107 ± 0.021**	
B. intermedia	7.323 ± 0.005	16.291 ± 0.009	16.602 ± 0.000	14.716 ± 0.002	**n/a**

For ITS, *B. intermedia* has the greatest variation within species, with an average of 6.95% difference and *B. unicolor* has the least amount of variation, with a 0.45% difference. *Bullacris obliqua* and *B. intermedia* shows greater variation between species, with an 11.75% difference and *B. membracioides* and *B. discolor* has the least amount of variation, with a 3.35% difference (Table 3.3).

Table 3.3: Pairwise genetic distance table showing the differentiation between the ITS gene of *Bullacris* species (mean ± SD), both within (bold) and between sampled species.

	B. unicolor	*B. discolor*	*B. membracioides*	*B. obliqua*	*B. intermedia*
B. unicolor	**0.453 ± 0.043**				
B. discolor	6.332 ± 0.019	**1.343 ± 0.042**			
B. membracioides	6.763 ± 0.018	3.353 ± 0.028	**2.048 ± 0.043**		
B. obliqua	11.319 ± 0.028	10.034 ± 0.030	9.701 ± 0.029	**3.768 ± 0.036**	
B. intermedia	3.933 ± 0.013	6.589 ± 0.024	7.054 ± 0.023	11.746 ± 0.030	**6.951 ± 0.036**

3.3.3. Phylogenetic Tree

The Bayesian tree in Figure 3.1 for the COI region showed strong support (PP > 0.95) for *B. membracioides*, *B. discolor* and *B. obliqua* species. However, *B. unicolor* had a number of different clades within the species, which showed differences between populations based on the different geographical locations. These distinct evolutionary lineages can also be seen in Figure 3.2, the Bayesian tree for the ITS region, however, *B. intermedia* seems to fall within the *B. unicolor* clade, with PP values of 0.993 and 0.906.

The tree topologies retrieved for PP and ML were nearly identical with regards to the major clades. The monophyly of *B. unicolor* for COI was moderately well supported, with a 75% PP-value and a 58 ML-value. In addition, *B. discolor* and *B. obliqua* had strong support 100% (PP) and 100 (ML), *B. membracioides* and *B. intermedia* had a strong PP-values of 100% and 99% respectively, but low support values for ML (47 and 16).

With regards to the ITS Bayesian Tree, each monophyletic group was strongly supported for each species. *B. unicolor* had a PP-value of 83% and a ML-value of 1; *B. discolor* had a PP-value of 95% and a ML-value of 1; and lastly, both *B. membracioides* and *B. obliqua,* had a PP-value of 100% and a ML-value of 1.

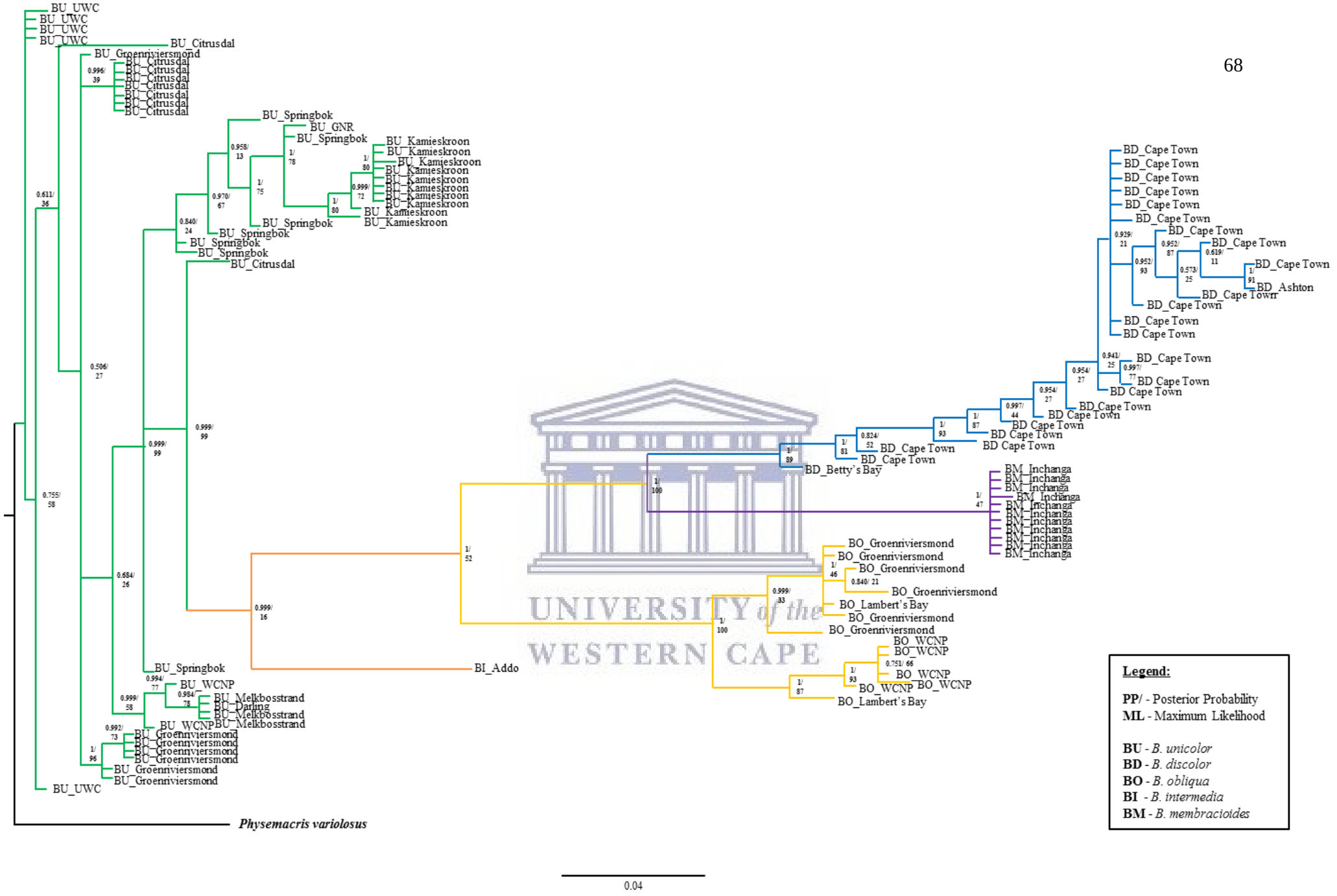

Figure 3.1: Phylogenetic tree from the Bayesian analyses of the COI region for *Bullacris* species, showing posterior probabilities (PP) values (above) and maximum likelihood (ML) values below.

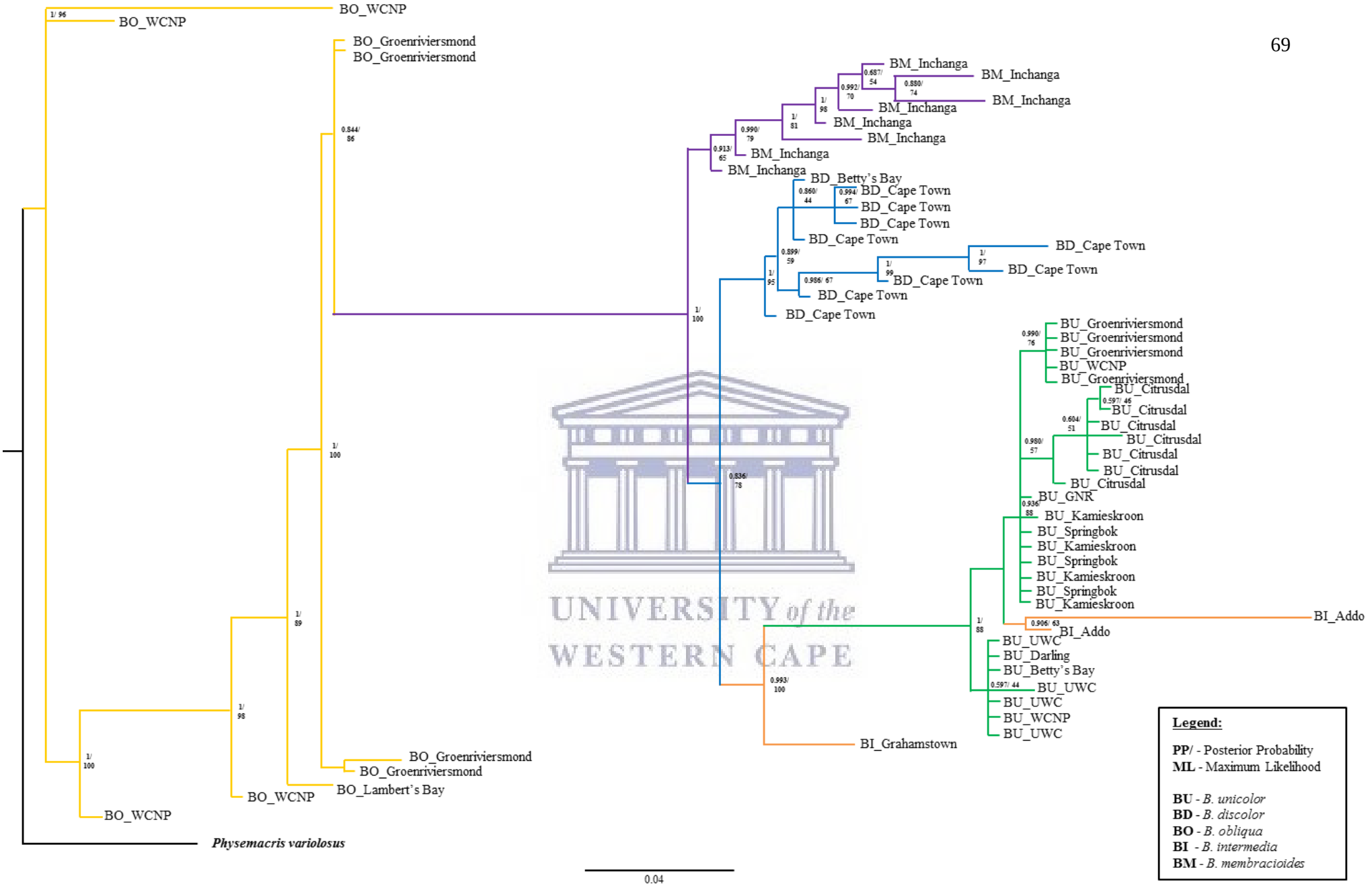

Figure 3.2: Phylogenetic tree from the Bayesian analyses of the ITS region for *Bullacris* species, showing posterior probabilities (PP) values (above) and maximum likelihood (ML) values below.

3.3.4. SplitsTree

SplitsTree computed an unrooted phylogenetic network for the COI (Figure 3.3) and ITS (Figure 3.4) region of *Bullacris* species. Both the COI and ITS trees clearly show that each species, with an exception of *B. intermedia* has unique evolutionary clades. *Bullacris membracioides* shows very little conflict within its cluster due to all specimens being collected in the same area. However, *B. obliqua*, *B. unicolor* and *B. discolor* shows greater distortion within their lineages, resulting from multiple sampling localities. *Bullacris intermedia* falls within the *B. unicolor* clade and are therefore cannot be classified as a distinct species.

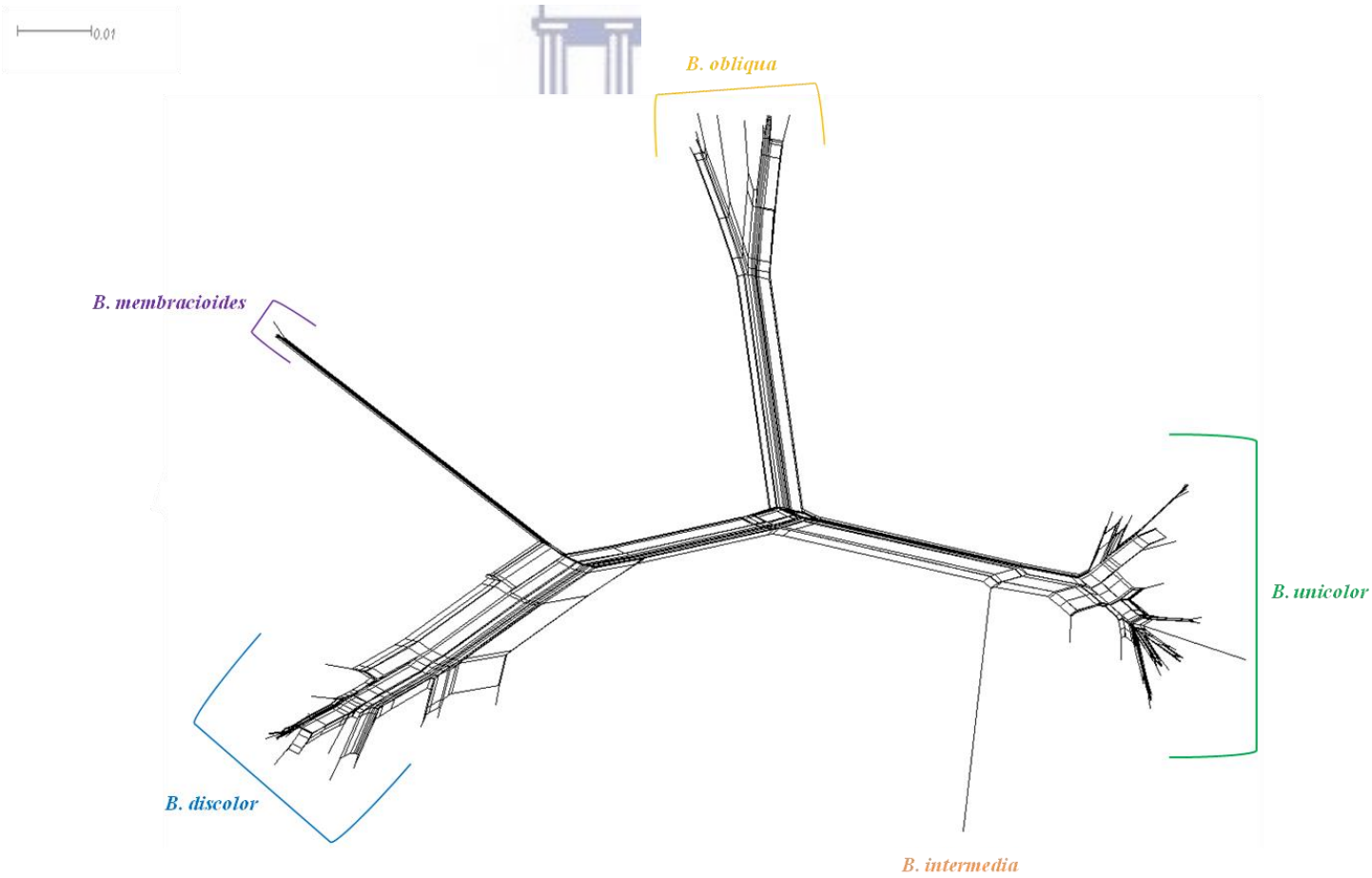

Figure 3.3: SplitsTree diagram showing the COI region for five *Bullacris* species.

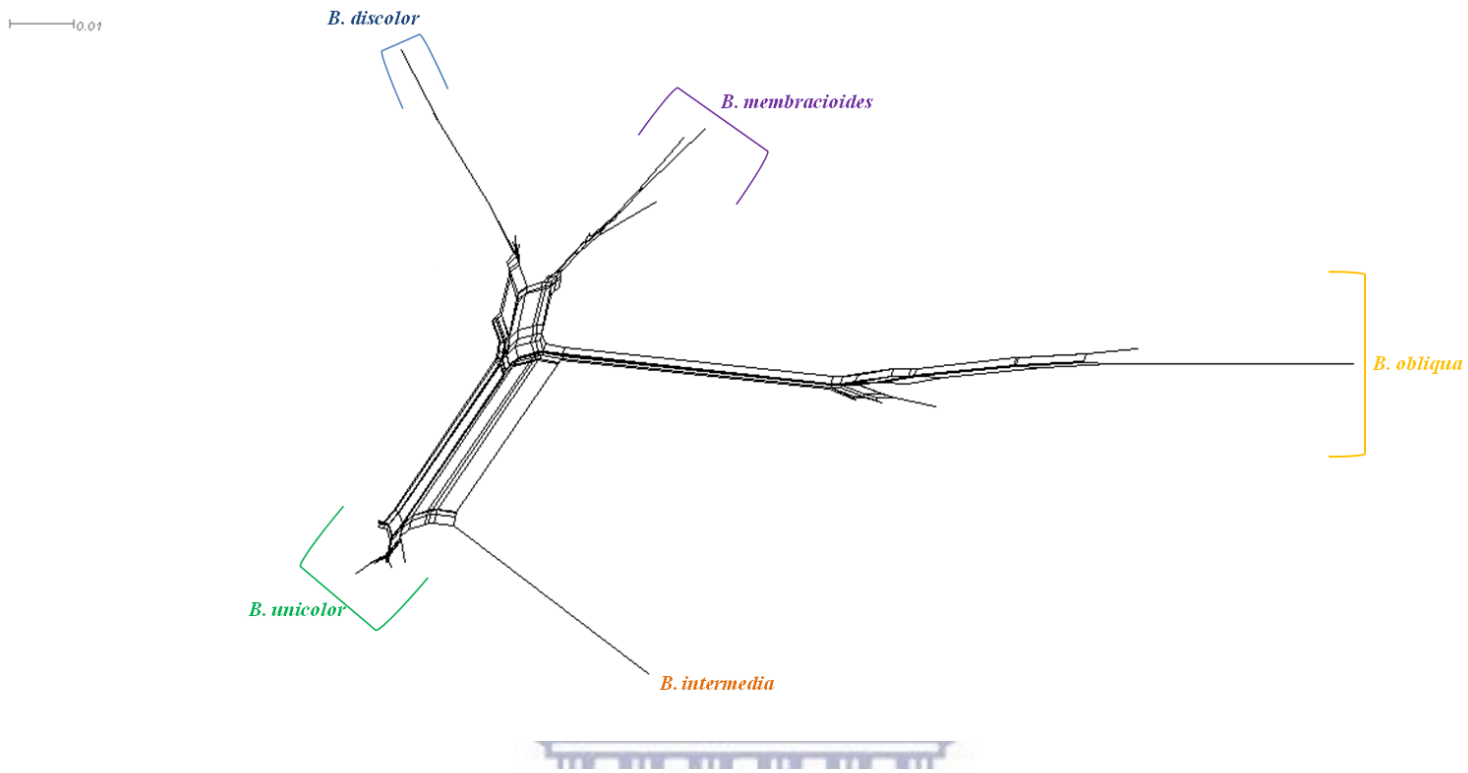

Figure 3.4: SplitsTree diagram showing the ITS region for five *Bullacris* species.

3.3.5. Statistical analyses

Mantel test results showed that genetic pairwise distances had no significant correlation with either morphological distances (R = 0.037; *P* = 0.426) or acoustic distances (R = 0.417; *P* = 0.137). In addition, partial mantel tests controlling for each of the three variables, also yielded non-significant results (*P* > 0.05).

3.4. Discussion

This study is the first attempt to reconstruct the phylogeny of the *Bullacris* genus. It has been suggested that a genetic approach together with morphological and acoustic analyses, would yield more accurate and definitive results in reconstructing phylogenetic relationships (Hillis, 1987). The results of this study were significant, in that each species (excluding *B. serrata* and *B. boschimana*) described by Dirsh (1965) were genetically distinct, with the exception of *B. intermedia*.

As previously mentioned, the use of the mitochondrial COI gene has assisted in the classifications of a number of taxa (Lunt *et al*., 1996). Since the mitochondrial genome is maternally inherited (Zhang and Hewitt, 2003), it is expected to have a higher evolutionary rate (Giles *et al*., 1980; Jenuth *et al*., 1997) and thus more genetic variation between species, due to each species having unique evolutionary sequences. Therefore, mtDNA is useful for distinguishing between taxa on a population level (Trewick, 2000). Conversely, the nuclear ITS gene has been used to classify taxa on a species level, due to having a high amplification rate of rRNA genes (Das and Deb, 2015).

Several types of analyses were generated based on the amplification of the COI and ITS gene markers of each *Bullacris* species. Pairwise genetic distances between species for the COI marker (Table 3.2) had a high average range from 11.2% to 16.6%. However, within species there was a relatively low divergence, with an average in variation ranging from 0.05% to 3.1%. This range excludes *B. intermedia* (within species) due to only one sequence being correctly amplified. The two species with the highest amount of variation of 16.6% was between *B. membracioides* and *B. intermedia* and the species that shared the least amount of variation, 7.3% was between *B. unicolor* and *B. intermedia*.

The variation between *Bullacris* species for the ITS marker (Table 3.3) had an average range of 3.4% to 11.7% and the variation within species had an average range of 0.45% to 6.9%. The highest variation was contributed by *B. intermedia* and *B. obliqua* (11.7%) as well as between *B. unicolor* and *B. obliqua* (11.3%), whereas the least amount of variation between species was found between *B. intermedia* and *B. unicolor* (3.9%). These results suggest that the genus *Bullacris* is composed of several independent species with the possibility of *B. unicolor* and *B. intermedia* being sister species.

Tree based analyses all show that each species was grouped according to geographical locations, and had relatively strong support values (PP < 1), except for *B. intermedia*, which had a strong support PP-value of 0.99, but a weak ML score of 16. The ITS phylogenetic tree (Figure 3.2) has similar results in that each species was geographically grouped, with very strong PP-values (PP < 1); however *B. intermedia* had a PP-value of 0.99 and fell within the *B. unicolor* clade. This same outcome can be seen in Figure 3.3 and 3.4 for the SpitsTree analyses of COI and ITS. SplitsTree analyses were used to demonstrate evolutionary changes on a finer scale and once more, each species forms its own clade with the exception of *B. intermedia*, which falls within the *B. unicolor* clade.

These results somewhat differs to that of the previous chapter (Chapter 2), in which morphological and acoustic differences had separated each species according to their unique evolutionary characteristics. This contrasting result may be due to the fact that morphological and acoustic characteristics are highly influenced by environmental conditions (Heinrich *et al*., 2012; Pitchers *et al*., 2014). Factors such as predation, competition, sexual selection, climatic conditions, habitat and host plant specificity may all contribute to the variations between acoustic and morphological characteristics between species (Whitman and Agrawal, 2009). Nevertheless, this technique could possibly prove to be more useful when evaluating species on a population

level that have different geographical locations. An example of this can be seen in Donelson (2007) and Sathyan *et al*., (2016), in which populations of *B. unicolor* which are geographically isolated, showed significant variations between morphological and acoustic characteristics.

This is not unexpected, since *B. unicolor* occurs within the Fynbos, Succulent-Karoo and Albany Thicket biomes, and thus experiences differences in climate as well as vegetation types (Figure 2.10). In addition, this then confirms the separation of individuals within clades for *B. unicolor*, *B. discolor* and *B. obliqua* as seen in Figure 3.1 and 3.2, which is based on differences in sampling localities. This is with the exception of *B. membracioides*, due to all individuals being sampled from one location. SplitsTrees diagrams (Figure 3.3 and 3.4) share similar results to that of the phylogenetic trees, since SplitsTrees shows unique evolutionary lineages for each individual, which correlates with the sampling locality.

Other possible reasons for *B. intermedia* having the least amount of genetic variation with *B. unicolor* may be due to convergent evolution. This is when both species may have similar traits that have evolved independently due to adapting to similar environments, and this is supported by both of these species having overlapping distributions (Figure 2.9). Alternatively, this variation may also be explained by divergent evolution, which occurs when two species from a common ancestor evolved such that they differ from one another (Lawrence, 2008). However, there is no relevant literature on this genus to motivate these possibilities. Nevertheless, sister species seems to be more relevant on the basis that *B. unicolor* and *B. intermedia* occur in climatically different regions. An example of this is seen in a study by Bidau *et al*. (2012), where the body size two neotropical grasshopper species, *Dichroplus pratensis* and *D. vittatus* have been influenced by environmental factors.

Furthermore, *B. intermedia* have a partially overlapping distribution with *B. unicolor* as well as *B. membracioides* (Figure 2.9). However, genetic variation between *B. intermedia* and *B. membracioides* is far greater (COI- 16.6% and ITS- 7.1%) than the variation between *B. intermedia* and *B. unicolor* (COI- 7.3% and ITS- 3.9%). A study by Bulgarella *et al*., (2015) found that populations of the Wellington tree weta, *Hemideina crassidens*, inhibit distinct localities and therefore experience different environmental conditions and climatic pressures. The results of their study showed that the females grew faster and to a larger size in areas with higher elevation, than individuals from a lower elevation. It is possible that observed size differences between *Bullacris* species could also be due to environmental variables rather than genetic differences.

There is a change of altitude in South Africa, with a gradual increase starting in the Western Cape, along the coast into the Eastern Cape and finally into KwaZulu-Natal, and these differences in elevation may influence growth. In addition, the Western Cape and KwaZulu-Natal provinces fall on either side of the Bedford gap, which implies differences in seasonal rainfall (Conradie, 2012). This is significant because rainfall and moisture availability are believed to trigger the hatching of pneumorids. The Western Cape is known to have winter rainfall, whereas KwaZulu-Natal has summer rainfall. Furthermore, species are known to thrive in areas that have summer rainfall, due to warmer temperatures together with rainfall promoting growth, whereas the Western Cape and the Northern Cape experience rainfall during the coldest months of the year when temperatures for growth are not optimal. Therefore, it is suggested that *B. membracioides* from KwaZulu-Natal experiences optimal climatic conditions for growth and reproduction, thus attaining larger body sizes. This major shift in climate may also promote genetic variation between species that inhabit summer versus winter rainfall areas.

The fitness of individuals is often correlated with body size, since body size influences a number of traits, such as dispersal ability, longevity, number and size of offspring, and competitiveness (Peters, 1983; Honek, 1999). Evolutionary and developmental explanations for this variation include the availability and quality of resources, population density and competition, climatic variations, predation, clinal variation in development rates and sexual selection (Peter 1983; Bervan and Gill, 1983). Therefore, with an increase in growth and developmental rates, comes an increase in reproduction rates and thus a greater rate in genetic diversity. Even though partial mantel tests in this study between genetic and both acoustic and morphological characteristics showed no significant relations, these results could possibly be due to the influence of multiple selective pressures acting on phenotypic characteristics, including both ecological selection and sexual section.

3.5. Conclusion

The results of this study have added more insights into the evolutionary adaptations and diversification of species from the genus *Bullacris*. This is the first molecular study that has been conducted on this genus. When the classification of taxa relies solely on one concept, such as morphology or acoustics, important information can be overlooked. It was expected that the results of this study would show each would be genetically distinct and in conjunction with the species concept. However, this study discovered that *B.* unicolor and *B. intermedia* shared closely related DNA sequences. This study also shows that even though an examination of the phenotypic component of species reveals insights into patterns of diversification, a genetic approach provides additional and sometimes contradictory evidence regarding interspecific variation.

In future, a larger sample size for *B. intermedia* should be incorporated in this study to verify the distinctiveness of the species. In addition, genetic analyses from the two species that have been excluded from this study, *B. boschimana* and *B. serrata* should be included to have a more robust and complete understanding of the genetic structure and differences between *Bullacris* species.

Chapter 4

General Conclusion

In conclusion, the results of this thesis have indicated that in order to comprehensively distinguish between species, there are a number of factors that need to be taken into consideration. Even though morphological differences are in accordance with Dirsh (1965), genetic analyses has shown that *B. intermedia* and *B. unicolor* have a low genetic variation which could possibly be the result of one species that had recently diverged from a common ancestor, or simply the effects of environmental and climatic conditions that have influenced the rate of genetic diversity between these species.

The variation in morphology between these two species appears to be due to variations in environmental differences as well as vegetation types, since geographically separated populations may adapt to local conditions. Acoustic variation between species gives the impression that it may be influenced by both mate choice and the environment and thus signals may vary not only on a species level but on a population level as well.

It is evident that species cannot be solely differentiated by one grouping factor such as morphology, but by acoustics and genetics as well. Likewise, the phylogenetic position of *B. serrata* also needs to be looked at in more detail in future studies. This species is morphologically and acoustically very similar to *B. discolor* and an examination of genetic material of *B. serrata* will help resolve the taxonomic status of these two species. Furthermore, the use of a second nuclear DNA marker such as 18S should be analysed in order to substantiate the level of divergence found in this study.

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