ACOUSTIC SIGNALLING IN BLADDER GRASSHOPPERS (ORTHOPTERA: PNEUMORIDAE)

by

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Abstract

Sound plays a vital communicative role in many Orthopteran insect species. Acoustic signals are often used during courtship behaviour and mate location. The Pneumoridae, commonly referred to as bladder grasshoppers, are a family of insects native to southern and eastern Africa. They are highly adapted for long-distance sound communication, with the males emitting very loud advertisement calls that are typically distinctive to each species. However, relatively few previous studies have examined sound communication within the entire family, and little is known about interspecific variation in signals and how this relates to evolutionary history. The aim of this study was to compare acoustic signal characteristics, as well as the morphology of the sound-producing structures, across multiple species in order to better understand morphological constraints on sound production. Both morphological and acoustic features were then correlated with previously published genetic distances to determine whether acoustic signals reflect phylogenetic relationships. The results showed significant differences between the acoustic characteristics of species and between the morphological properties of the sound-producing structures. Furthermore, there was a significant relationship between morphological and acoustic characteristics, indicating that species with a similar morphology of the sound producing structure produce calls that are more similar. Finally, genetic distances were positively correlated with morphological distances, but not with acoustic distances. This suggests that phylogenetic history somewhat constrains acoustic signalling in this family with regards to morphology, but genetic relatedness cannot fully account for the extent of acoustic variation between species.

Declaration

I declare that this thesis, entitled "***Acoustic signalling in bladder grasshoppers (Orthoptera; Pneumoridae)",*** is my own work and 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: _____

Signature: **Date:**

(ELECTRONIC SIGNATURE)

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CHAPTER 1: INTRODUCTION

1.1 Acoustic signalling

Many animals use sound as a communicative tool to transmit important biological information to members of their own or other species. These sounds perform a variety of crucial functions, such as attracting potential mates, defending territories, alerting conspecifics to predator presence, defence against predators, maintaining social cohesion, discovering prey, and helping with orientation (Goutte *et al*., 2013; Goutte *et al*., 2018).

Sound communication is relatively rare in insects, occurring in only six orders, including the Orthoptera (Greenfield, 2016; Song *et al.*, 2020), the focus of this thesis. The Orthoptera are a large and diverse order of insects, including grasshoppers, crickets and katydids, that have become highly specialised for sound communication, and are the largest group of sound producing animals (Song *et al.*, 2020). In Orthopterans, like all sound producing insects, sounds are primarily used for mate attraction and location, and are usually highly species specific (Alexander, 1962), although they may also function in predator avoidance or territoriality (Greenfield, 1997; Robinson and Hall, 2002; Larrosa *et al.*, 2010).

In order to attract potential mating partners, males typically produce an advertisement call that is detected and recognised by female receivers (Heinrich *et al*., 2012; García *et al*., 2014). Sound signals used in courtship behaviour may contain several kinds of information about an individual, such as size, sex, species, and physical condition (Boake, 2002; Gerhardt and Huber, 2002). Thus, sound signals may be reliable indicators of male quality, and hence mating suitability. For example, females of the tree frog *Agalychnis moreletii* preferred males

that called more frequently and produced calls that were longer in duration and had shorter inter-call intervals, which is indicative of a higher energy investment (Briggs, 2010).

There are several factors that may influence variation in acoustic signals, both within and between species. These include sexual selection, natural selection, stochastic processes and pleiotropic effects (Wilkins *et al*., 2013). Acoustic variation may occur at different levels, ranging from the intra-individual level to the inter-specific level. Due to their prominent role in mate attraction, sexual selection is hypothesised to be one of the major driving forces behind the evolution of acoustic signals (Hall and Robinson, 2021). Although it has received comparatively less attention, natural selection is also expected to influence acoustic signal evolution. For example, habitat differences have been shown to alter the properties of sound signals in order to optimise sound transmission in a particular environment (Slabberkoorn and Smith, 2002).

Interspecific interactions may drive signal divergence between closely related species, particularly those which are recently diverged (Kyogoku and Wheatcroft, 2020). This divergence in acoustic signals may help to avoid signal masking (Gröning and Hochkirch, 2008), wasted effort courting heterospecifics (Friberg *et al.*, 2013), or inbreeding (Servedio and Noor, 2003). Reproductive character displacement leads to otherwise similar species having unusually divergent calls and results from co-existing species modifying their calls in order to reduce the fitness costs associated with reproductive interference from heterospecifics (Kyogoku and Wheatcroft, 2020). Reproductive character displacement has been documented in a wide variety of acoustically signalling species, including frogs (Höbel and Gerhardt, 2003; Malone *et al.* 2014), birds (Kirschel *et al.*, 2009), cicadas (Marshall and

Cooley, 2000), crickets (Jang and Gerhardt, 2006; Jang *et al.*, 2009) and katydids (Cole 2016).

1.2 Sound production mechanisms

Insects use a variety of different mechanisms to produce sound signals, such as tymbals in cicadas, wing vibrations (buzzing) in mosquitoes and midges, striking a part of the body against the substrate in some beetles, and expelling air in caterpillars (Bennet-Clark, 1998). However, the most common form of sound production in insects is via stridulation, whereby two specialised body parts, known as the file and the scraper, are rubbed against each other. For example, the Pamphagidae produce a song by rubbing together the ventral edge of the metanotum and the basalar sclerite (Bennet-Clark, 1998; López *et al*., 2007).

Orthopteran insects produce a wide variety of sounds. These range in frequency from audible to well into the ultrasonic range (up to 100 kHz), and in duration from less than a millisecond up to several minutes (Robinson and Hall, 2002). Amplitude also varies widely, with the bladder grasshopper *Bullacris membracioides* having one of the loudest documented calls at 98 dB SPL at 1 m (van Staaden and Römer, 1997). The size and shape of the sound producing structures, as well as the speed of stridulation, may all affect the characteristics of the sound produced (Bennet-Clark 1998). Male size also affects call amplitude and carrier frequency, with larger males generally producing louder calls with a lower carrier frequency (Robinson and Hall, 2002).

Different groups of Orthoptera may have distinct sound production mechanisms (Riede 1987). In the suborder Ensifera (crickets and katydids), the tegmina are used to produce sound, whereby the stridulatory apparatus typically consists of a file on one wing and a plectrum on the other (tegmino-tegminal stridulation), although there may be some exceptions to this (Song *et al.*, 2020). This apparatus can be completed by a resonator known as either the harp or the flute present on both forewings in crickets (Schubnel *et al*., 2021). However, in the suborder Caelifera (grasshoppers), sound production is less common, but also more varied (Greenfield 1997; Song *et al.*, 2020). Grasshoppers are also unique among animals in that they make use of two sound organs at the same time, on the left and right hand sides of the body (Robinson and Hall, 2002). Grasshopper songs are most commonly produced by rubbing a stridulatory file on each hind femur against each forewing (Hall and Robinson, 2021). However, different groups of grasshoppers make use of various different body parts for stridulation. For example, the Pneumoridae use abdomino-femoral stridulation and the Pamphagidae use Krauss's organ-femoral stridulation (Massa, 2012; Song *et al.*, 2020). Sound production mechanisms sometimes vary even among closely related species. For example, in grasshoppers of the *Stenobothrus eurasius* group, *Stenobothrus eurasius* generates sounds by leg stridulation whereas *Stenobothrus eurasius hyalosuperficies* produces sound via wing clapping (Tarasova *et al*., 2021). Some grasshoppers may also produce sounds during flight, known as crepitation, by clapping their hind wings together (Kuga and Kasuya, 2021). This is thought to be an antipredator mechanism. Although many grasshoppers communicate via acoustic signals, some grasshopper families do not produce sounds and conclude pairing and mating without the production of sound (Otte, 1970; Riede, 1987). For example, certain diurnal grasshoppers communicate by visual displays and are completely silent (Riede, 1987; Heinrich *et al*., 2012).

1.3 Relationship between acoustics and morphology

Acoustic signal differences among organisms are often linked to morphological differences, as morphological features often underpin, and thus influence, differences in sound production (Marten and Marler, 1977; Bennet-Clark, 1998; Cocroft and Rodriguez, 2005; Rivera-Correa *et al*., 2022). This variation may occur both within and between species. For example, body size is typically negatively correlated with call frequency (pitch) and positively correlated with call amplitude, in both insects and other animals (Brown *et al*., 1996; Bennet-Clark, 1998; Briggs 2010). For example, a recent large-scale study on parrots found that species with larger body sizes produced songs that were longer and had a lower frequency and a wider frequency bandwidth (Marcolin *et al*., 2022). This highlights the influential role of body size in the evolution of intraspecific differences in acoustic signals. Indeed, body size is arguably the best predictor of acoustic signal differences between species (Marcolin *et al*., 2022).

In Orthoptera, acoustic characteristics have been shown to correlate with the morphology of the sound producing structures (e.g. Stange and Ronacher, 2012). For example, studies on katydids have revealed that song parameters across multiple species are most strongly predicted by aspects of the structures that generate sound, rather than by absolute body size (Chivers *et al*., 2017; Montealegre-Z *et al.*, 2017). At the intraspecific level, a study on sagebrush crickets, *Cyphoderris strepitans*, which use their tegmina to produce sound, found that differences in wing morphology between males are linked to differences in song structure in this species (Ower *et al*., 2017). However, in contrast, another study on four field cricket species revealed that wing shape and male songs co-evolve and can be linked across species, but do not co-vary within species (Blankers *et al*., 2018). However, relatively few

studies have examined the morphological variation that underlies male acoustic signal variation and the relationship between morphology and behaviour remains poorly understood.

On the other hand, morphological characteristics may not necessarily correlate with acoustic characteristics. For example, a study on tree crickets revealed that the relationship between song frequency and male body size has become decoupled and males are able to adjust the frequency of their songs independently of body size (Mhatre *et al.*, 2012). Similarly, populations of the same species may exhibit variation in acoustic characteristics in the absence of morphological variation (Hernández-Herrera and Pérez-Mendoza, 2021). Cryptic species may likewise differ acoustically in the absence of morphological variation (Tan *et al.*, 2020).

1.4 Relationships between acoustics and genetics

The relationship between acoustic signal characteristics and genetic characteristics is not fully understood, especially in insects. There is contradictory evidence as to whether acoustic cues correlate with genetic variation. Chen *et al*. (2021) found that genetic distance in thirteen species of Tettigoniidae was positively correlated with acoustic distance, which suggests consistency of evolutionary speed between genetics and acoustic cues in these species. Similar relationships have been uncovered in birds (Irwin *et al.* 2008) and primates (Thinh *et al*., 2011; Meyer *et al*., 2012). Thus, there is at least some evidence of a link between genetic and acoustic distances.

On the other hand, acoustic variation does not always correspond to genetic variation. For example, Rivera-Correa *et al*. (2022) found significant differences in call characteristics among geographically separated populations of the frog *Pristimantis jaguensis*, but no genetic structuring of populations, with individuals from the same population showing greater genetic variation than individuals from different populations.

1.5 Relationship between morphology and genetics

The study of morphological and genetic variation can provide important information for recreating evolutionary histories (Tihelka *et al*., 2021). Morphological differences may reflect genetic variation between taxa and may be used as classification tools, to guide conservation efforts, or as a starting point for genetic investigations. For example, a large-scale study on frogs revealed a general agreement between genetic and morphological characteristics (Rivera-Correa *et al*., 2017). However, morphological differences may not always correspond with genetic differences. A study of the grasshopper *Mioscirtus wagneri* found that intraspecific morphological variation was significantly associated with microsatellites, but not with mtDNA markers (Ortego *et al.*, 2012). Furthermore, Ortego *et al.* (2012) found that genetically differentiated populations sometimes displayed morphological convergence.

1.6 Bladder grasshoppers

The superfamily Pneumoroidea is composed of a single family, the Pneumoridae, with 14 known species (Dirsh, 1965; Laubscher, 2021). However, some of these species are rarely

encountered in the field and at least two species have not been sighted for many years. All species are cryptically camouflaged. The Pneumoridae are largely endemic to southern Africa and found mainly in the coastal regions, with the majority of species endemic to South Africa. However, at least two species are found in eastern Africa and one species in southern Namibia. Different bladder grasshopper species inhabit different vegetation biomes throughout this distribution, ranging from desert to forest.

Bladder grasshoppers produce sound primarily for mate location, but also occasionally as an anti-predator or possible spacing mechanism (van Staaden *et al.*, 2004). Adult males emit extremely loud calls at night to advertise themselves to females. Male bladder grasshoppers have a large inflated abdomen, which develops at the final moult and serves to amplify their call (van Staaden and Römer, 1998). Males produce their song through stridulation, by rubbing a row of rasps on the hind femur against a row of ridges on the side of the abdomen. These ridges vary in number and arrangement between males of different species, and may be used in species identification. The male call is not only loud, but also relatively low in frequency for their body size. This leads to unusually long sound transmission distances for an insect (van Staaden and Römer, 1997). Alternative males that adopt a sneaker strategy and lack the sound producing structures of primary males have been documented in several species (Donelson and van Staaden, 2005; Laubscher, 2021). However, these males are relatively rare in natural populations.

Receptive females respond to the calls of males with a much softer call, since they lack the inflated abdomen, and the male then tracks the location of the female through reciprocal duetting (van Staaden and Römer 1997). The call of the female is not species specific. Depending on the species, the sound of the females is produced by either rubbing strong wing

veins with teeth against teeth on the posterior femur or against the abdomen, while also raising the pronotum at a high angle (van Staaden and Römer 1997).

Male calls vary greatly between bladder grasshopper species (Couldridge and van Staaden, 2004) and are generally highly species specific. They may also vary geographically within a species, as a result of sexual selection or ecological differences (Couldridge and Gordon, 2015; Sathyan *et al*., 2017; Sathyan and Couldridge, 2021).

1.7 Aims of the study

The first aim of this study was to examine and compare species of bladder grasshopper in terms of acoustic and morphological characteristics, and to determine whether this variation can be related to morphological differences in the sound producing structures. There is currently little known regarding the interspecific variation and the relationships between morphological and acoustic characteristics in bladder grasshopper species across the family. Because the abdomen of male bladder grasshoppers not only contributes to overall body size, but is also an integral part of the sound producing mechanism, variation in the shape and size of the abdominal bladder might be expected to heavily influence the characteristics of the sound that is produced. For example, a larger bladder may result in louder calls that also have a lower fundamental frequency. In addition, the number and arrangement of elements in the file and scraper mechanism might also be expected to influence sound production. In this way, morphology may constrain acoustic signal characteristics.

Recent phylogenetic work has revealed the broad evolutionary relationships within the Pneumoridae family (Gordon, 2022). Thus, the second aim of the study was to correlate interspecific genetic distances obtained from Gordon (2022) with both acoustic and morphological differences to determine to what extent the observed differences may be related to genetic variation between species.

CHAPTER 2: MATERIALS AND METHODS

2.1 Species included in the study

Of the 14 known species of bladder grasshopper, a total of 12 species were included in this study, of which acoustic data was available for 10 species, morphological data for 12 species and genetic data for 10 species (Table 2.1). These species occupy a range of different habitat types (Table 2.1).

Table 2.1. Bladder grasshopper species included in the study and the vegetation biomes in which each species is found.

Species	Acoustic data*	Morph. data	Genetic data	Vegetation biome(s)
Bullacris boschimana	Yes	Yes	-	Succulent Karoo; Desert
Bullacris discolor	Yes	Yes	Yes	Fynbos
Bullacris intermedia	Yes	Yes	Yes	Savanna; Grassland
Bullacris membracioides	Yes	Yes	Yes	Savanna; Indian ocean coastal belt
Bullacris obliqua	Yes	Yes	Yes	Fynbos; Succulent Karoo
Bullacris serrata	Yes	Yes	Yes	Fynbos
Bullacris unicolor	Yes	Yes	Yes	Fynbos; Succulent Karoo
Physemacris variolosa	Yes	Yes	Yes	Fynbos
Peringueyacris namaqua	-	Yes	Yes	Succulent Karoo
Pneumora inanis	Yes	Yes	Yes	Forest
Physophorina livingstoni	-	Yes	Yes	Forest
Physophorina miranda	-	Yes	-	Forest

* This excludes one additional species for which the identity could not be verified.

2.2 Call recording and analysis

To analyse the acoustic differences between species of bladder grasshopper, we used sound recordings of the calls of 91 individuals from ten different species. The individuals were collected or recorded from various locations throughout South Africa, within the Western Cape, Eastern Cape, Northern Cape and KwaZulu-Natal Provinces (Table 2.2). All collections and recordings took place during the austral spring and summer due to the seasonal nature of the study animals.

The calls used in the study were previously recorded sounds that had been collected over several years. The majority of these calls were laboratory recordings of known individuals and were recorded using a Sennheiser ME66/K6 shotgun microphone connected to a Marantz PMD670 portable digital recorder, conducted within a temperature range of 20 °C to 25 °C. The sounds were recorded at a sampling rate of 48 kHz and saved in 16-bit .wav format. However, calls for two species (*B. boschimana* and 'unknown') were field recordings, obtained from other sources. For laboratory recordings, sounds were recorded at night at a distance of approximately 2 metres from spontaneously calling males, which were housed in individual enclosures with plastic mesh lids for sound transparency.

Table 2.2. Locations and number of males used for the acoustic analysis. Numbers in brackets after each location indicate the number of males recorded from that location.

Species	Sample size	Location
Bullacris boschimana	1	Richtersveld (1)
Bullacris discolor	15	Ashton (1), Bettys Bay (1) Hangklip (1), Cape Town (12)
Bullacris intermedia	2	Port St Johns (2)
Bullacris membracioides	8	Inchanga (8)
Bullacris obliqua	8	Groenriviersmond (3), Oudtshoorn (1) West Coast National Park (3), Citrusdal (1)
Bullacris serrata	3	Grahamstown (3)
Bullacris unicolor	36	Bellville (2), Citrusdal (6), Darling (1) Groenriviersmond (1), Kamieskroon (15) Springbok (11)
Physemacris variolosa	13	Bettys Bay (1), Citrusdal (3), Montagu (1) Rooi Els (1), Stanford (4), Overberg (1) Pringle Bay (2)
Pneumora inanis	3	Grahamstown (3)
Unknown species*	2	Eswatini (Swaziland) (2)

* The identity of this species could not be confirmed. The calls were recorded in the field and the specimens were not collected. However, the call is very different from all other known pneumorid calls.

Raven Pro 1.5 software (Cornell Bioacoustics Research Program) was used to analyse the calls according to a range of parameters. Bladder grasshopper calls typically consist of a number of short and relatively soft introductory syllables followed by a longer and louder

final syllable (Figure 2.1). Calling males repeat the entire call every few seconds during a calling bout (Figure 2.1). For each call, we measured nine characteristics of the call, which included both temporal and frequency components. These were: 1) the length of the introductory syllable(s) (sec), 2) the length of the final syllable (sec), 3) total call length (sec), 4) the number of introductory syllables, 5) the number of final syllables, 6) delta frequency (the difference between the minimum and maximum frequency) (Hz), 7) peak frequency (the frequency where the highest amplitude value was observed) (Hz), 8) interquartile range (IQR) bandwidth (the difference between the first and third quartile frequencies) (Hz), and 9) 90% bandwidth (Hz).

Five calls from each individual male were measured. These values were first averaged, and the mean values for each individual were then used in further statistical analyses. Thus, sound characteristics were measured from a total of 455 calls.

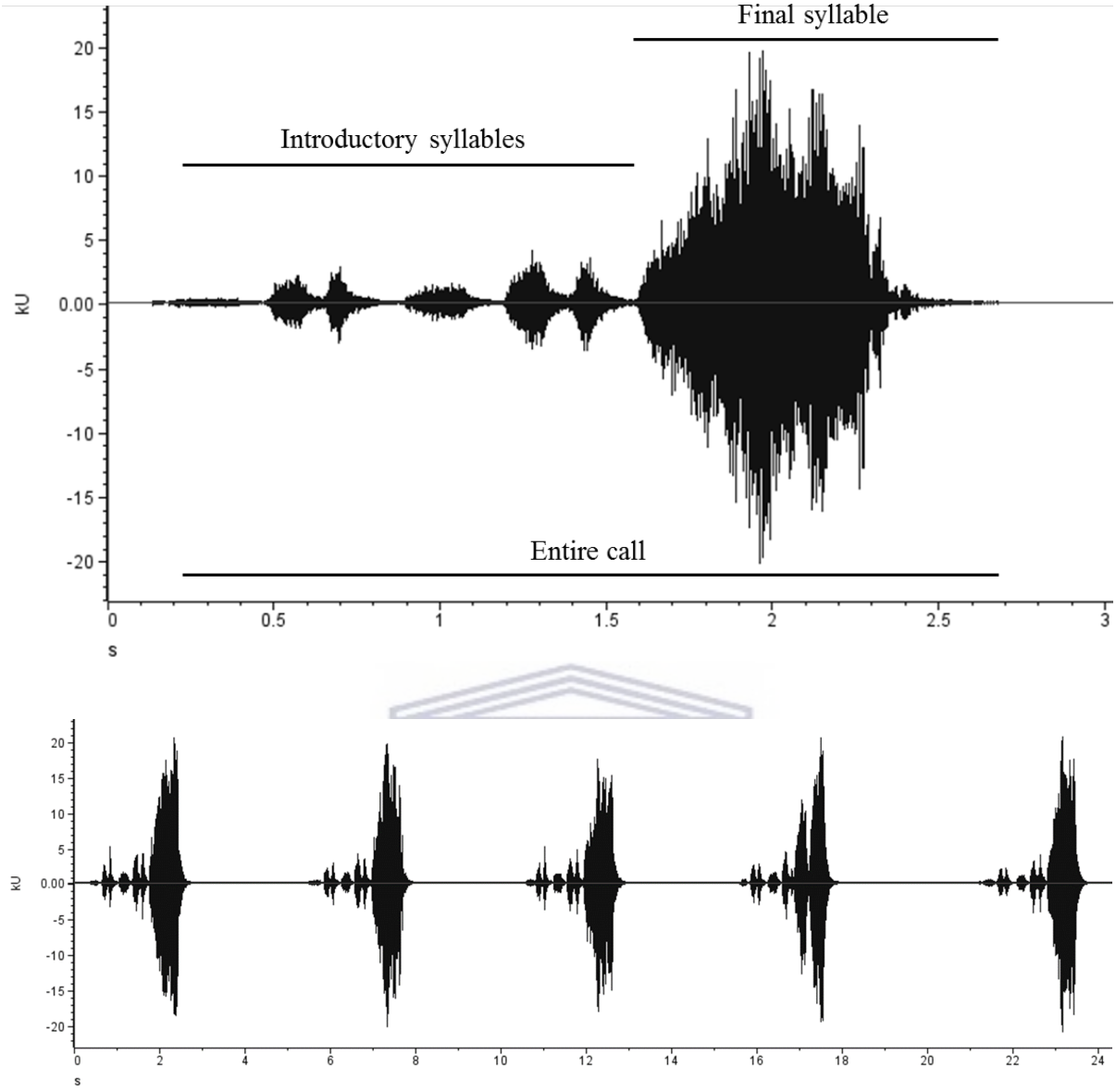


Figure 2.1. Waveform of the male advertisement call of *Bullacris membracioides* showing the structure of the call (top). Calling bout consisting of five calls (bottom).

2.3 Morphological measurements and analysis

A total of 136 male bladder grasshoppers belonging to twelve species were used in the morphological analysis (Table 2.3). The specimens had been previously collected between 1993 and 2018 from the field. They were preserved in ethanol and housed at the University of the Western Cape (n = 100). Supplementary material from pinned collections from the National Insect Collection in Pretoria (n = 7), the Ditsong Museum in Pretoria (n = 3) and the Iziko Natural History Museum in Cape Town (n = 26) were also analysed to increase sample sizes for less common species.

A total of seven morphological measurements were taken. These measurements were: 1) total body length (the most anterior point of the head to the end of the abdomen) (mm), 2) abdomen height (measured at the third abdominal segment) (mm), 3) abdomen width (measured at the widest part of the abdomen) (mm), 4) abdomen length (measured from the thorax to the end of the body) (mm), 5) number of abdominal ridges, 6) hind femur length (mm), and 7) number of leg ridges (Figure 2.2). All measurements were done with the aid of a digital calliper, and were done on the right-hand side of the body for paired structures. The number of abdominal and leg ridges were counted using a dissecting microscope.

Table 2.3. Locations and sample sizes of males used in the morphological analyses. Numbers in brackets indicate the number of males sampled from that location.

Species	Sample size	Locations
Bullacris boschimana	5	Rosh Pinah, Namibia (5)
Bullacris discolor	20	Cape Town (16), Paarl (1), George (1), Port Elizabeth (1), St Francis Bay (1)
Bullacris intermedia	5	Bashee (2), Kowie River (2), Port St Johns (1)
Bullacris membracioides	15	Durban (3), Eshowe (2), Hillcrest (1), Inchanga (7), Port St Johns (1), Umkomaas (1)
Bullacris obliqua	15	Groenriviersmond (8), Saldanha Bay (1), Wallekraal (1), West Coast National Park (5)
Bullacris serrata	11	Grahamstown (5), Stanford (1), Swartberg Pass (5)
Bullacris unicolor	30	Bellville (4), Citrusdal (1), Darling (1), Dwarskersbos (6), Groenriviersmond (6), Jakkalsfontein (1), Kamieskroon (3), Melkbosstrand (5), Springbok (2), Vanrhynsdorp (1)
Physemacris variolosa	22	Bettys Bay (8), Citrusdal (2), Hawston (1), Karakul River (1), Paleisheuwel (1), Rooi Els (1), Simonskloof (3), Stanford (3), Somerset West (1), Uniondale (1)
Peringueyacris namaqua	2	Spektakel (1), Springbok (1)
Pneumora inanis	5	East London (2), Grahamstown (3)
Physophorina livingstoni	2	Tanzania (2)
Physophorina miranda	4	Eshowe (1), Nkandhla (1), Port St Johns (2)

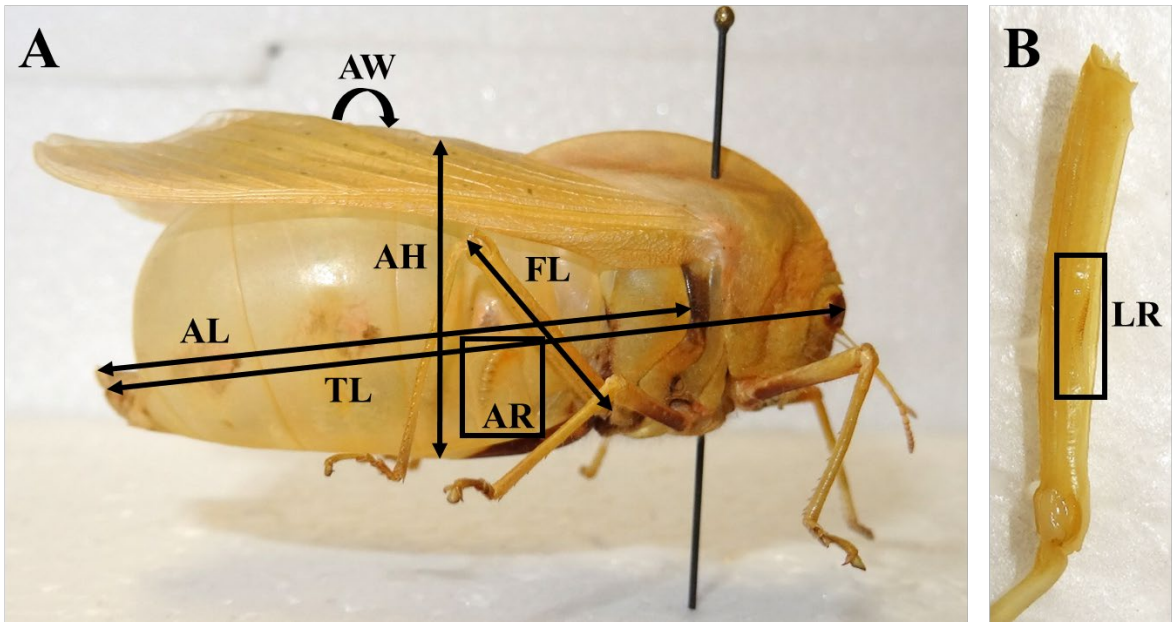


Figure 2.2. Photograph of a male bladder grasshopper (*Bullacris unicolor*) showing the morphological measurements (A) and close up of the inner surface of the hind femur showing the scraper (B). AH = abdomen height; AL = abdomen length; AR = number of abdominal ridges; AW = abdomen width; FL = femur length; LR = number leg ridges; TL = total body length.

2.4 Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 27. We tested differences in acoustic characteristics using a Multivariate Analysis of Variance (MANOVA) and Discriminant Function Analysis (DFA). The nine different acoustic variables were used as dependent variables, and the species as the independent variable. Similarly, a MANOVA and DFA were used to test for differences in morphological characteristics between species. The

seven morphological features were used as dependent variables, and the species as the independent variable.

A Mantel test correlating morphological and acoustic measurements was conducted for all bladder grasshopper species that had both morphological and acoustic data available. The correlation was based on the Euclidean distance matrices of species averages. The analysis was conducted using the ade4 Package (Thioulouse *et al*., 2018) in R statistical software (R Core Team, 2022). Morphological and acoustic distances were also separately correlated with genetic distance, using mitochondrial COI pairwise distance values taken from Gordon (2022). Genetic distances were available for ten pneumorid species; these included all of the known species examined here, with the exception of *B. boschimana* and *Phy. miranda*.

CHAPTER 3: RESULTS

3.1 Acoustic comparison between species

The calls of the different species differ substantially in length, carrier frequency and number of elements. Some species, such as *Physemacris variolosa, B. unicolor, B. discolor* and *B. serrata* have relatively short and simple songs, while *Pneumora inanis*, the unknown species, *B. membracioides, B. boschimana, B. obliqua* and *B. intermedia* have longer and more complex songs. Waveforms and spectrograms of the different species of bladder grasshoppers are shown in Figure 3.1.

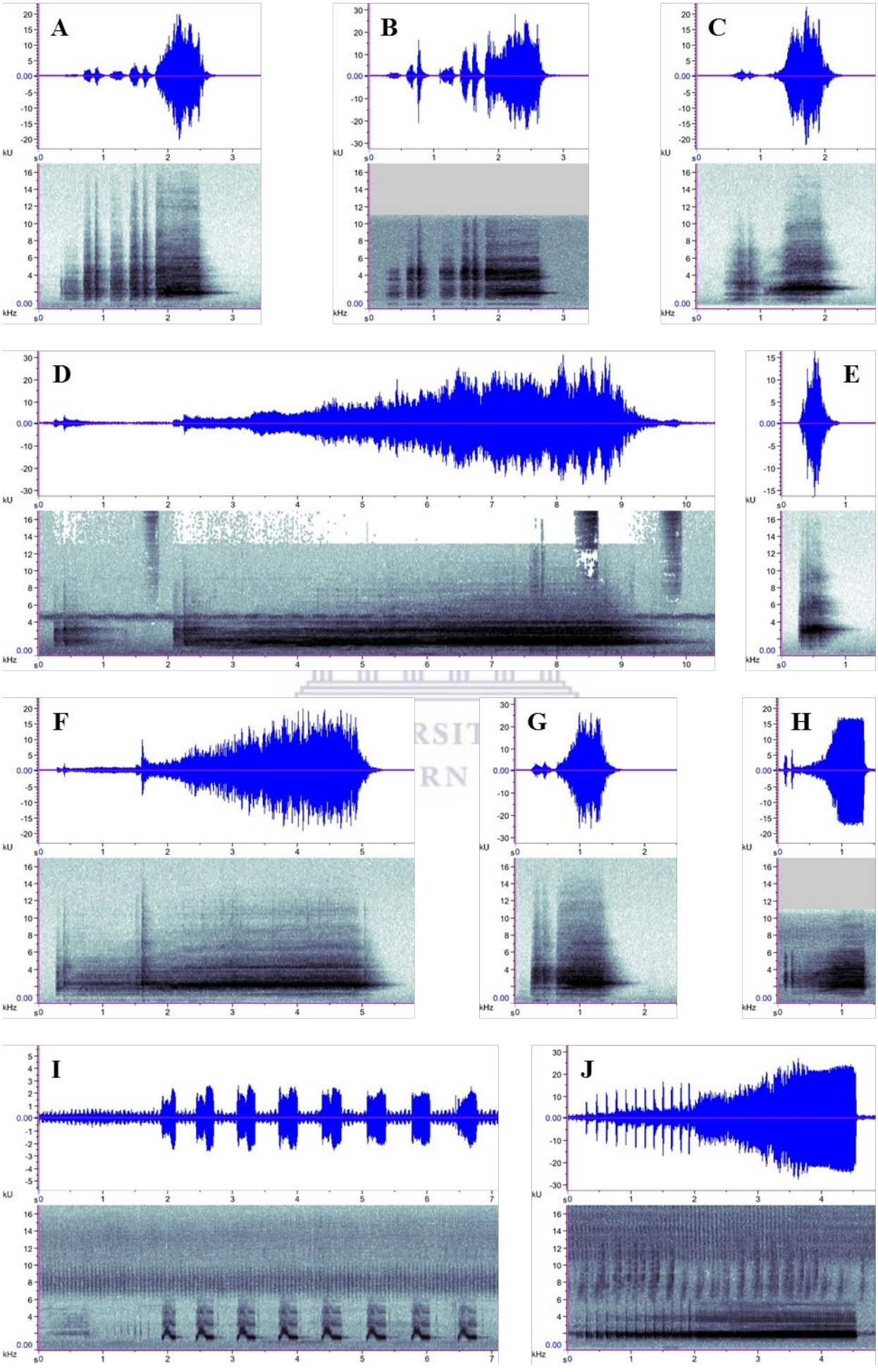


Figure 3.1: Waveforms (above) and spectrograms (below) of the calls of the ten species of bladder grasshopper. A = *B. membracioides*; B = *B. intermedia*; C = *B. unicolor*; D = *B. boschimana*; E = *Ph. variolosa*; F = *B. obliqua*; G = *B. discolor*; H = *B. serrata*; I = *Pn. inanis*; J = unidentified species.

3.2 Acoustic variation between species

Mean values and standard deviations of acoustic variables for each species are shown in Table 3.1. *Bullacris boschimana* has the longest call length and length of the final syllable, whereas *P. variolosa* has the shortest call length (Table 3.1). The 'unknown' species has the highest number of introductory syllables. *Bullacris serrata, B. discolor, B. unicolor* and *P. variolosa* all have the lowest number of introductory syllables. The repetition of a final syllable occurs only in *Pneumora inanis*. On the other hand, all other species have a single final syllable.

Physemacris variolosa has the shortest introductory syllables and *Pneumora inanis* has the shortest final syllable. *Pneumora inanis* also has the highest 90% bandwidth, whereas the lowest is for the Unknown species. The 'unknown' species and *B. discolor* showed the lowest and highest IQR bandwidth respectively. The peak (carrier) frequency was lowest for *Pn. inanis* and highest for *Ph. variolosa*. The 'unknown' species has the longest introductory syllables, while *B. serrata* showed the shortest introductory syllable length. *Bullacris discolor* and *B. obliqua* showed the highest and lowest delta frequency respectively.

Table 3.1. Mean values (± standard deviation) of acoustic characteristics of males. The sample size is indicated in brackets after the name of each species.

Characteristic	*B. boschimana* (1)	*B. discolor* (15)	*B. intermedia* (2)	*B. membracioides* (8)	*B. obliqua* (8)	*B. serrata* (3)	*B. unicolor* (36)	*Ph. variolosa* (13)	*Pn. inanis* (3)	Unknown (2)
Length of introductory syllables (sec)	2.115	0.355 ± 0.089	1.668 ± 0.301	1.255 ± 0.079	1.651 ± 0.544	0.203 ± 0.030	0.872 ± 0.272	0.291 ± 0.094	1.484 ± 0.255	2.282 ± 0.218
Length of final syllable (sec)	7.506	0.926 ± 0.093	0.753 ± 0.101	1.023 ± 0.169	3.167 ± 0.548	1.127 ± 0.051	1.222 ± 0.301	0.399 ± 0.144	0.317 ± 0.039	2.232 ± 0.433
Total call length (sec)	9.621	1.287 ± 0.142	2.422 ± 0.199	2.304 ± 0.219	4.791 ± 0.760	1.327 ± 0.029	2.160 ± 0.508	0.800 ± 0.432	7.983 ± 1.855	4.505 ± 0.215
Number of introductory syllables	5.000	2.000 ± 0	6.400 ± 0.849	5.675 ± 0.465	5.000 ± 0	2.000 ± 0	1.944 ± 0.232	2.000 ± 0	6.856 ± 0.769	16.500 ± 2.121
Number of final syllables	1.000	1.000 ± 0	1.000 ± 0	1.000 ± 0	1.000 ± 0	1.000 ± 0	1.000 ± 0	1.000 ± 0	9.159 ± 1.362	1.000 ± 0
Peak frequency (Hz)	1,507.350	2,357.950 ± 214.024	1,843.258 ± 48.702	1,813.924 ± 102.017	1,947.911 ± 261.404	1,830.627 ± 126.898	2,140.654 ± 122.996	3,008.929 ± 301.201	1,504.886 ± 43.278	1,817.410 ± 109.587
Delta frequency (Hz)	1,360.100	2,363.867 ± 534.915	1,667.694 ± 74.990	1,207.540 ± 341.994	769.777 ± 209.334	1,335.818 ± 431.229	805.402 ± 130.059	1,531.073 ± 358.612	1,847.802 ± 1118.530	1,616.500 ± 52.750
IQR bandwidth (Hz)	258.400	671.913 ± 220.439	447.889 ± 194.894	625.438 ± 871.119	324.661 ± 218.135	618.374 ± 230.979	302.083 ± 181.056	253.973 ± 107.398	566.549 ± 737.552	172.300 ± 0
90% bandwidth (Hz)	1,550.400	1,731.248 ± 236.425	2,489.245 ± 450.688	1,423.339 ± 1255.634	1,018.121 ± 888.525	1,984.995 ± 772.417	587.500 ± 211.183	764.117 ± 309.120	2,585.508 ± 3189.850	430.700 ± 0

The MANOVA results revealed that there were significant differences in sound characteristics between bladder grasshopper species (Pillai's Trace = 4.964, $F_{81;702} = 10.660$, $p < 001$). Significant differences were found between species for each of the nine individual acoustic variables (Table 3.2).

Table 3.2. MANOVA results of the acoustic characteristics of males.

Dependent variable	Sum of squares	Mean square	d.f.	*F*	*P*
Length of introductory syllables	22.729	2.525	9	38.319	<0.001*
Length of final syllable	84.041	9.338	9	120.605	<0.001*
Total call length	202.087	22.454	9	107.075	<0.001*
Number of introductory syllables	577.518	64.169	9	543.140	<0.001*
Number of final syllables	109.618	12.180	9	939.351	<0.001*
Delta frequency	29443470.61	3271496.734	9	32.469	<0.001*
Peak frequency	13216680.60	1468520.067	9	41.718	<0.001*
IQR bandwidth	2794435.382	310492.820	9	2.862	0.006*
90% bandwidth	36510608.51	4056734.278	9	9.020	<0.001*

*Significant, $P < 0.05$

The Discriminant Function Analysis (DFA) for the male acoustic characteristics shows that species separate out according to their acoustic differences, although there is some overlap between the call characteristics of certain species (Figure 3.1). *Bullacris boschimana*, *B. intermedia*, *B. membracioides* and *B. obliqua* grouped together, indicating similarities in the

signal characteristic of these four species. Similarly, *B. discolor*, *B. serrata*, *B. unicolor* and *Ph. variolosa* also clustered together. The calls of *Pn. inanis* as well as the 'unknown' species were very distinctive and did not overlap with any other species (Figure 3.2).

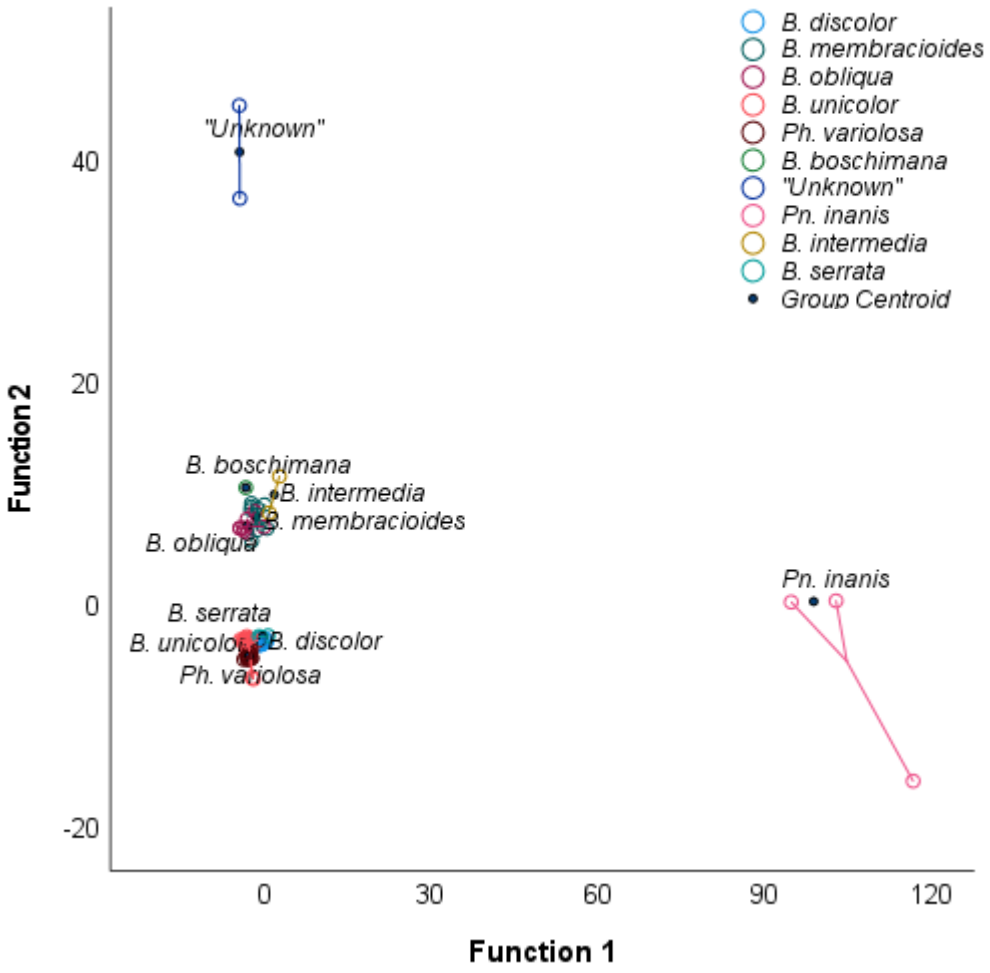


Figure 3.2. Canonical centroid plot of the discriminant function analysis (DFA) for the acoustic characteristics of male calls.

The first two functions of the DFA together explained 94.3% of the variation in the data. Discriminant Functions 1 and 2 explained 74.2% and 20.1% of variation respectively (Table 3.3). Discriminant Function 1 has a strong positive correlation with the number of final

syllables and with the 90% bandwidth (Table 3.4), and *Pn. inanis* is thus separated from all of the other species along this axis as it has a repeated final syllable and a larger bandwidth (Figure 3.1). Discriminant Function 2 has a strong positive correlation with the number of introductory syllables as well as the length of the final syllable (Table 3.4). The 'unknown' species separates from the remaining species mostly due to having many introductory syllables, but also a relatively long final syllable (Figure 3.2). Species belonging to the genus *Bullacris*, as well as *Ph. variolosa*, form two clusters along this axis. *Bullacris boschimana*, *B. intermedia*, *B. membracioides* and *B. obliqua* group together as they have longer final syllables and more introductory syllables than the cluster consisting of *B. discolor*, *B. serrata*, *B. unicolor* and *Ph. variolosa.*

Table 3.3. Eigenvalues of the first five canonical discriminant functions for male acoustic signals. The percentage of variation for Functions 1 and 2 are highlighted in bold.

	Eigenvalues	Variance %	Cumulative %	Canonical correlation
1	257.794	**74.2**	74.2	0.998
2	69.814	**20.1**	94.3	0.993
3	12.708	3.7	97.9	0.963
4	4.008	1.2	99.1	0.895
5	2.480	0.7	99.8	0.844

Table 3.4. Standardised Canonical Discriminant Function Coefficients of acoustic characteristics for bladder grasshoppers calls.

	Function				
	1	2	3	4	5
Length of introductory syllables	0.366	0.164	0.471	-0.368	-0.075
Length of final syllable	-0.004	0.380	1.299	0.585	0.003
Total call length	-0.287	-0.307	-0.645	-0.171	0.127
Number of introductory syllables	-0.070	1.023	-0.237	0.075	0.097
Number of final syllables	1.486	-0.119	0.211	0.063	0.070
Delta frequency	0.225	0.038	-0.282	0.600	-0.630
Peak frequency	-0.203	-0.162	-0.083	0.551	0.802
IQR bandwidth	-0.611	0.149	-0.118	0.279	-0.150
90% bandwidth	1.378	0.033	0.083	-0.193	-0.156

3.3 Morphological variation between species

The morphological differences between pneumorid species are illustrated in Table 3.5. *Peringueyacris namaqua* was the smallest species and *Phy. livingstonii* was the largest species examined. Although other bladder grasshopper species showed comparable mean values, *B. discolor* had the fewest abdomen ridges. *Pn. namaqua* and *Phy. livingstonii*, on the other hand, had a much higher number of abdomen ridges than all other species, followed by *Pn. inanis* and *Phy. miranda*, which also had a relatively high number of abdomen ridges. *Physophorina miranda*, *Phy. livingstonii* and *Pn. inanis* were further distinguishable from the other species by the presence of two distinct types of ridges in the stridulatory file that varied in size. Across species, the number of leg ridges was comparable and did not differ greatly from one species to another. This feature was also difficult to measure from pinned museum specimens due to the fixed position of the leg against the abdomen which obscured the ridges. The number of leg ridges was thus removed from further statistical analysis.

Table 3.5. Mean values (± standard deviation) of morphological characteristics of bladder grasshopper males. Numbers of specimens used are indicated in brackets after the name of each species.

Characteristic	*B. boschimana* (5)	*B. discolor* (20)	*B. intermedia* (5)	*B. membracioides* (15)	*B. obliqua* (15)	*B. serrata* (11)	*B. unicolor* (30)	*Ph. variolosa* (22)	*Pn. inanis* (5)	*Pe. namaqua* (2)	*Phy livingstonii* (2)	*Phy. miranda* (4)
Total body length (mm)	49.496 ± 1.169	46.017 ± 2.470	48.100 ± 3.325	52.201 ± 2.661	42.567 ± 2.598	51.874 ± 2.598	39.247 ± 3.130	41.897 ± 3.233	65.022 ± 4.005	24.220 ± 0.085	87.129 ± 1.579	58.740 ± 2.626
Length of abdomen (mm)	34.316 ± 1.249	33.858 ± 2.292	30.530 ± 2.909	37.149 ± 2.086	30.764 ± 2.519	35.640 ± 3.958	27.400 ± 2.850	29.251 ± 3.254	49.722 ± 11.793	17.400 ± 0.141	52.933 ± 1.534	42.870 ± 5.859
Height of abdomen (mm)	19.565 ± 0.869	17.131 ± 1.419	15.266 ± 2.516	17.613 ± 1.685	15.161 ± 2.025	16.349 ± 2.327	15.868 ± 2.028	12.202 ± 1.470	25.662 ± 11.916	11.830 ± 0.382	29.452 ± 2.017	19.618 ± 1.979
Width of abdomen (mm)	17.916 ± 0.992	14.634 ± 1.186	14.770 ± 2.157	16.701 ± 1.822	14.033 ± 1.558	13.742 ± 1.790	13.199 ± 1.341	11.978 ± 1.336	21.994 ± 1.459	9.800 ± 0.665	25.447 ± 0.834	19.493 ± 1.320
Length of hind femur (mm)	15.698 ± 0.669	17.157 ± 1.171	15.500 ± 1.238	17.364 ± 1.383	14.033 ± 1.558	17.472 ± 3.879	12.914 ± 1.904	13.714 ± 1.546	18.120 ± 1.353	11.875 ± 0.488	20.811 ± 1.793	16.270 ± 1.427
Leg ridges	22.000	25.150 ± 2.560	20.333 ± 1.528	24.222 ± 2.635	21.071 ± 2.702	23.000	22.965 ± 3.168	24.000 ± 2.360	23.970 ± 7.113	*	*	*
Abdomen ridges	10.25 ± 1.500	8.000 ± 0.000	9.200 ± 1.304	8.615 ± 0.768	11.467 ± 0.743	8.700 ± 0.823	11.233 ± 1.406	13.634 ± 1.677	20.000 ± 2.944	27.500 ± 0.707	27.000 ± 0.000	20.000 ± 0.000

* Leg ridges could not be counted

The MANOVA results revealed that there is a significant difference in morphology between species (Pillai's Trace = 3.001, $F_{66;678} = 10.279$, $p < 0.001$). Each of the measured variables differed significantly between species (Table 3.6).

Table 3.6. MANOVA results of the morphological characteristics of males.

Dependent variable	Sum of squares	Mean square	d.f.	*F*	*P*
Total body length	8,890.903	808.264	11	97.577	<0.001*
Abdomen length	4,278.844	388.986	11	29.247	<0.001*
Abdomen height	1,206.092	109.645	11	13.246	<0.001*
Abdomen width	863.937	78.540	11	37.007	<0.001*
Hind femur length	532.569	48.415	11	14.540	<0.001*
Number of abdomen ridges	2,103.471	191.225	11	151.064	<0.001*

*Significant, $P < 0.05$

The canonical centroid plot clearly distinguishes *Pe. namaqua, Phy. livingstonii*, *Phy. miranda*, and *Pn. inanis* from the rest of the species (Figure 3.3). In particular, *Phy. livingstonii* and *Pe. namaqua* are morphologically very distinct from all other species, while *Phy. miranda* and *Pn. inanis* were more similar to each other. Among the remaining species, *B. serrata*, *B. discolor*, *B. intermedia*, *B. membraciodes*, and *B. boschimania* formed a tight cluster, indicating morphological similarities between these five species. Similarly, *B. obliqua, B. unicolor* and *Ph. variolosa* also clustered tightly together, indicating that these three species share morphological similarity (Figure 3.3).

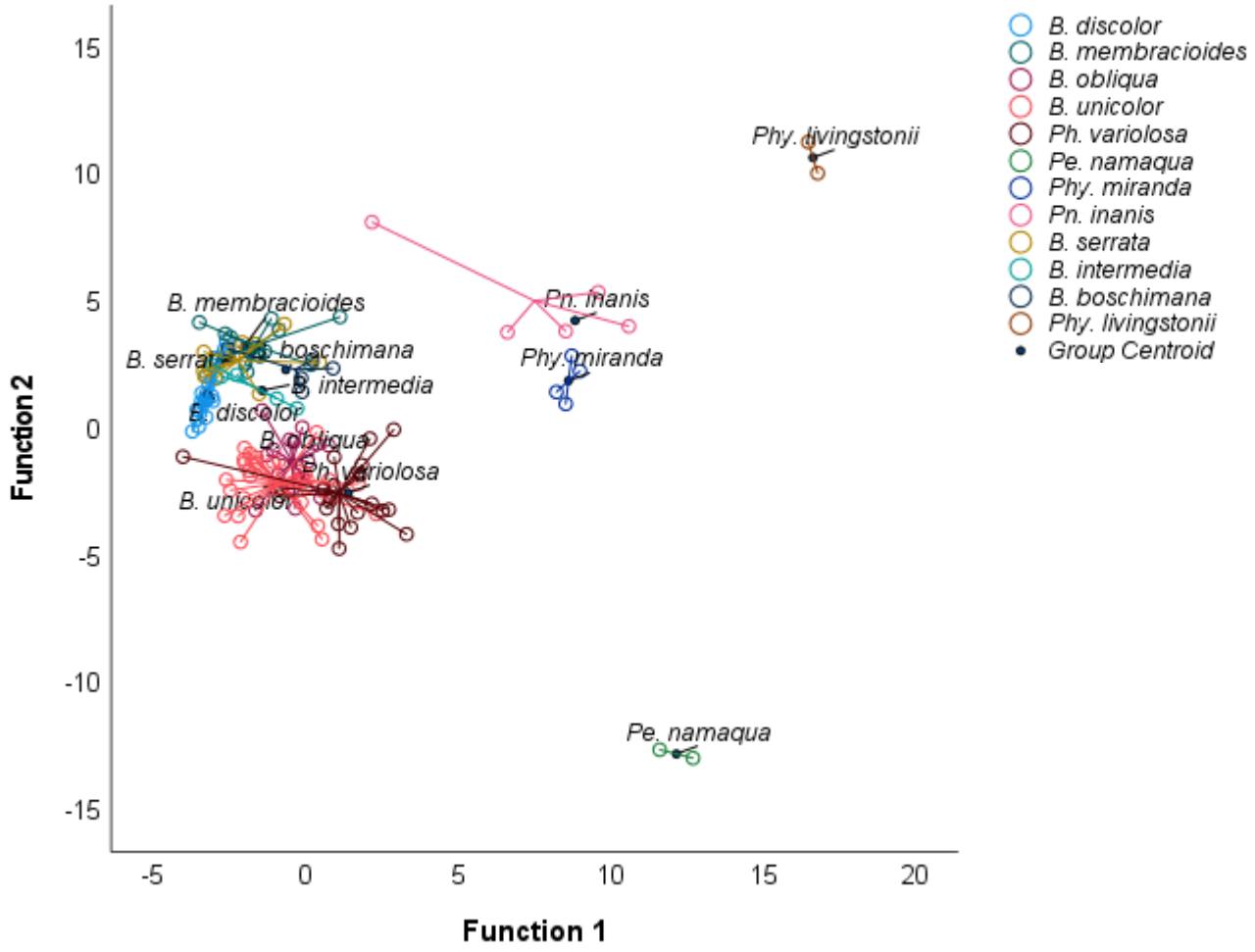


Figure 3.3. Canonical centroid plot of the Discriminant Functions Analysis (DFA) for the morphological characteristics of males.

Together DF1 and DF2 accounted for 93.3% of the variance in male morphology (Table 3.7). Discriminant Function 1 had a strong, positive correlation with the number of abdominal ridges, whereas DF2 was most strongly related to total body length (Table 3.8) and thus males separate out primarily on the basis of these two characteristics (Figure 3.3). Along the first axis, *Phy. livingstonii*, *Pe. nanaqua*, *Phy. miranda* and *Pn. inanis* separate out from the remaining species due to having a greater number of abdominal ridges. Along the second

axis, *Phy. livingstonii* and *Pe. namaqua* separate out from the remaining species due to having the largest and smallest body sizes respectively (Figure 3.3).

Table 3.7. Eigenvalues of the first six canonical discriminant functions for male morphological characteristics. The percentage of variation for Functions 1 and 2 are highlighted in bold.

	Eigenvalues	Variance %	Cumulative %	Canonical correlation
1	16.352	**56.7**	56.7	0.971
2	10.583	**36.7**	93.3	0.956
3	1.044	3.6	96.9	0.715
4	0.547	1.9	98.8	0.594
5	0.245	0.8	99.7	0.443
6	0.092	0.3	100.0	0.290

Table 3.8: Standardised Canonical Discriminant Function Coefficients for the morphological characteristics of male bladder grasshoppers.

	Function					
	1	2	3	4	5	6
Total body length	0.160	1.001	-0.082	-0.926	-0.084	-0.030
Abdomen length	-0.040	-0.361	-0.888	1.224	-0.484	0.407
Abdomen height	-0.196	0.228	1.016	-0.401	0.436	0.643
Abdomen width	0.288	0.258	0.555	0.527	-0.211	-0.552
Hind femur length	-0.019	0.158	-0.239	0.300	0.905	-0.221
Number of abdomen ridges	0.978	-0.374	-0.074	0.036	0.101	-0.005

3.4 Relationship between morphological, acoustic and genetic characteristics

The scatter plot (Figure 3.4) shows the relationship between morphological and acoustic features based on pairwise distances between species. There is a weak but significant positive correlation between morphology and acoustics ($r = 0.351$; $p = 0.039$), indicating that species with similar sound-producing structures have similar calls.

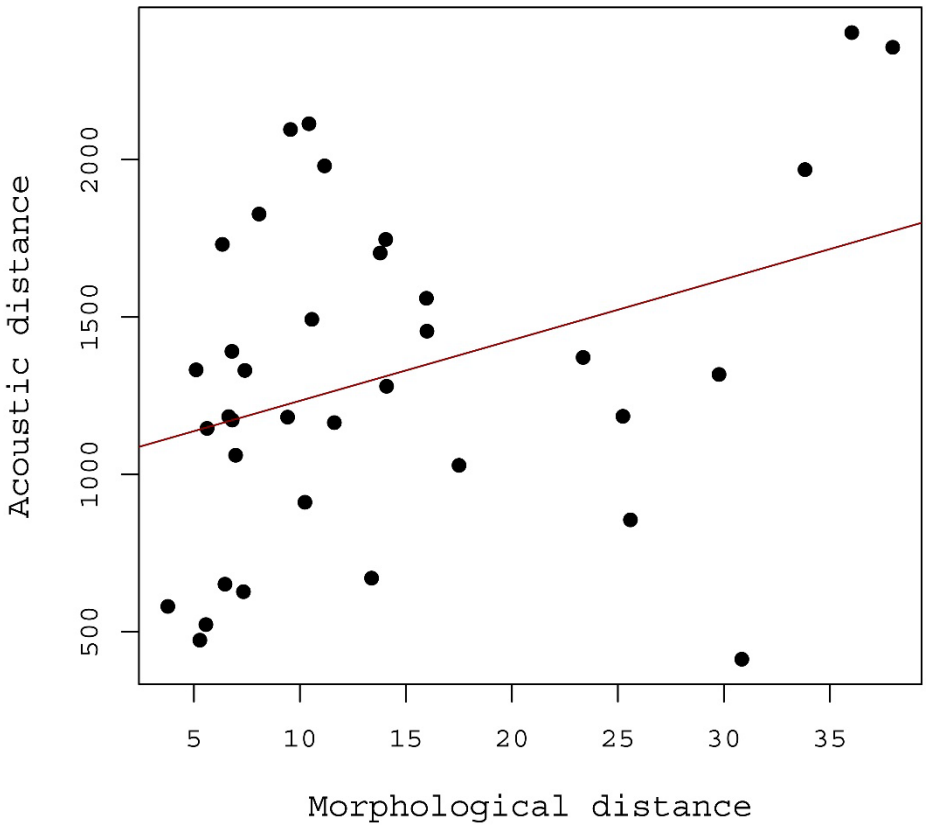


Figure 3.4. Scatter plot showing the relationship between morphological and acoustic characteristics of bladder grasshopper species.

There was a significant positive correlation between morphology and genetic distance ($r = 0.814$; $p < 0.001$), indicating that morphologically similar species are more closely related to

each other (Figure 3.5). However, there was a non-significant relationship between genetic and acoustic distances ($r = 0.191$; $p = 0.179$), suggesting that more closely related species do not have more similar calls (Figure 3.6).

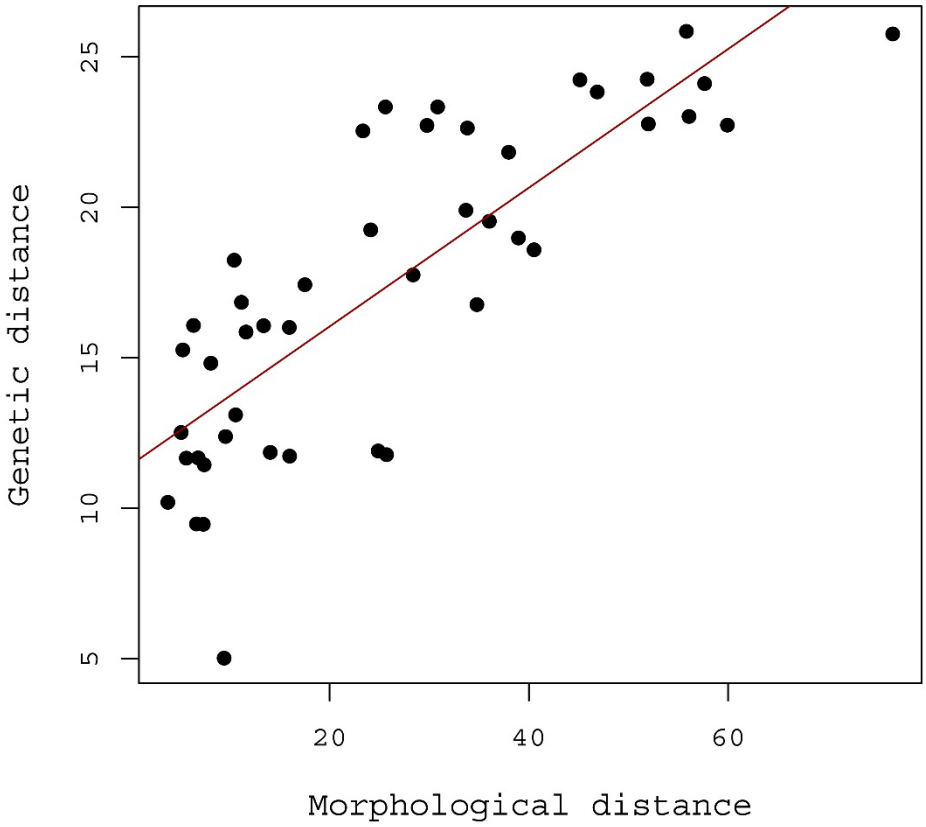


Figure 3.5. Scatter plot showing the relationship between morphological and genetic characteristics of bladder grasshopper species.

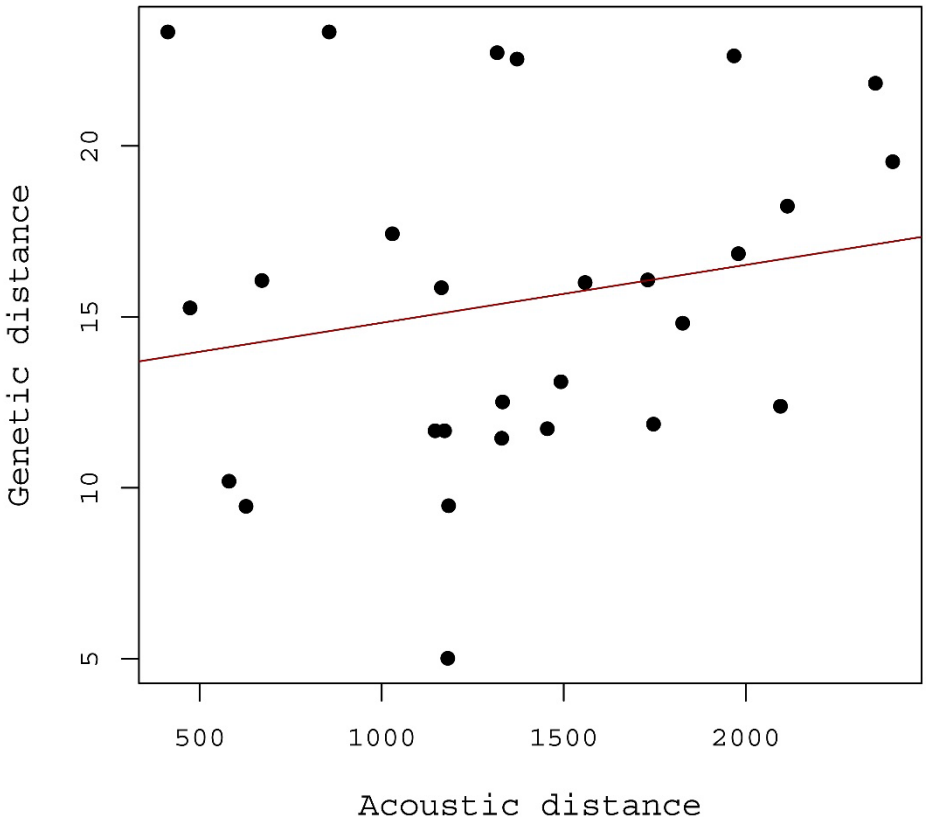


Figure 3.6. Scatter plot showing the relationship between genetic and acoustic characteristics of bladder grasshopper species.

CHAPTER 4: DISCUSSION

This is the first study to compare the advertisement call characteristics of bladder grasshopper species across the entire family. Results show that there are significant differences between species, with forest species, in particular, having very distinctive and more structurally complex calls. However, certain species were found to have more similar calls. Similarly, although there were significant differences in the morphological characters examined, certain species showed greater divergence than others.

4.1 Morphology

In this study, species belonging to the genus *Bullacris*, as well as *Ph. variolosa*, displayed more similar morphological characteristics and clustered more closely together than species from the other genera examined (Figure 3.2). In particular, *B. obliqua, Ph. variolosa*, and *B. unicolor* were very similar in morphology. This is somewhat surprising as the calls of these three species are very distinct, particularly that of *B. obliqua*. These three species often coexist with each other and the observed acoustic divergence despite similarities in the sound producing structures suggests that the species may be under selective pressure to avoid reproductive interference from heterospecifics. Likewise, the species *B. serrata*, *B. discolor*, *B. intermedia*, *B. membraciodes* and *B. boschimana* also clustered quite strongly together despite there being some notable differences in the calls of these five species. Apart from the two pairs of sister species (*B. serrata* and *B. discolor*, and *B. intermedia* and *B. membraciodes*), there is little or no geographic overlap among these species that might promote acoustic divergence. *Bullacris serrata*, *B. discolor*, *B. intermedia* and *B.*

membraciodes have been shown to be the most recently diverged pneumorid species (Gordon, 2022) and this may explain why they display similar sound production morphologies. *Pneumora inanis*, *Phy. miranda* and *Phy. livingstonii* separated out from the remaining species, with *Pn. inanis* and *Phy. miranda* being more similar to each other than to *Phy. livingstonii*. These are the three largest species and also the only three species that have two distinct types of abdominal ridges. *Physophorina livingstonii* is the largest pneumorid species and also has a slightly more abdominal ridges than *Phy. miranda* and *Pn. inanis*, which are more similar to each other both in body size and in abdominal ridge number. *Peringueyacris namaqua* was also morphologically distinct from all other species, having the smallest body size and yet the highest number of abdominal ridges.

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). Thus, the morphology of bladder grasshoppers may be affected by environmental conditions. Previous studies have shown that bladder grasshoppers exhibit variation in size within a species based on geographical differences (Donelson, 2007; Sathyan *et al*., 2017; Sathyan and Couldridge, 2021). According to a study by Donelson (2007) the morphology of male bladder grasshoppers may be affected by climate, and males vary in body length due to differences in the geographical region from which they were collected. A further study, when comparing male morphology across the geospatial range of a species of bladder grasshopper, found that there are differences in morphological characteristics (Sathyan *et al*., 2017). Populations of the same species of bladder grasshopper collected from areas of similar temperature are likely to be similar in size (Sathyan *et al*., 2017).

Environmental factors, along with geographical distributions, may affect morphological characteristics in bladder grasshoppers. As the climate varies, so too may morphological characteristics. Bladder grasshoppers can be found in a variety of habitats, ranging from dense, forested, humid areas to open, semi-desert, and arid areas. *Peringueyacris namaqua*, the smallest species, occurs in Namaqualand, which is a semi-desert, with a total body length much smaller than all other species. On the other hand, *Pn. inanis*, *Phy. miranda* and *Phy. livingstonii* are the three largest pneumorid species, and inhabit forests. They also have the most complex calls. In a study on two species of neotropical grasshoppers, *Dichroplus pratensis and D. vittatus*, it was shown that body size is affected by different climates (Bidau *et al*. 2012).

4.2 Acoustics

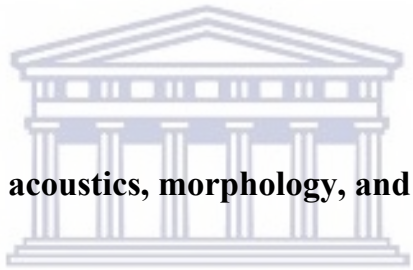
According to the DFA results (Figure 3.1), *B. boschimana*, *B. membracioides*, *B. intermedia* and *B. obiqua* have overlapping clusters, indicating similar acoustic features. This includes having longer calls with more syllables making up the call, as well as slightly lower carrier frequencies than other *Bullacris* species. The remaining *Bullacris* species (*B. serrata*, *B. discolor*, *B. unicolor*) as well as *Ph. variolosa* also showed overlap in signal characteristics. These four species have relatively simple songs, being fairly short in duration and they all have the same number of syllables making up the call (two introductory syllables and one final syllable). On the other hand, *Pneumora inanis* and the 'unknown' species do not show any overlap in signal features with any other species, indicating that the calls of both these species are highly distinctive. They both have calls that are far more complex than other

pneumorid species, containing a greater variety of elements making up the call. *Pneumora inanis* is the only species to repeat the final syllable and the only species to display frequency modulation of the call.

Environmental factors have a big impact on the formation of long-range acoustic signals because they impose selection pressures in different habitats, changing the properties of sound signals (Lang, 2000). It is possible that the acoustic differences between pneumorid species are due to selective pressures imposed by environmental conditions, and different habitats that influence the properties of acoustic signals. Bladder grasshopper species living in a different habitats may have different acoustic signal characteristics. Similar effects of geographical and environmental factors on sound signals have been found in other taxa. For example, birds in inhabiting similar environments have been shown to have similar sound signals (Ruegg *et al*., 2006).

The evolution of acoustic signals may be shaped by various selective pressures, such as sexual selection, predation, parasitism, competition, vegetation structure and ecology, and also constrained by factors such as physiology and phylogeny (Medina and Francis, 2012; Ruegg *et al*. 2006). Variation in mating signals may provide the first catalyst for speciation events (Boake, 2002). Sexual selection is considered to be one of the major drivers of acoustic signal variation (Hall and Robinson, 2021). Females of the bladder grasshopper *Bullacris membracioides* have been shown to preferentially respond to certain male signal characteristics, suggesting that female choice may be operating in this family and contributing to signal divergence (Couldridge and van Staaden, 2006).

Different bladder grasshopper species inhabit distinct regions of southern Africa where they occupy very dissimilar habitat types. For example, *B. unicolor* is predominantly found along the west coast of South Africa in the succulent Karoo biome, while *B. membracioides* is found along the east coast in the savanna biome. This diversity of habitats may explain some of the interspecific variation among the characteristics of their acoustic signals (Couldridge and van Staaden, 2004). In particular, *Pn. inanis* was found to have a unique call that differed substantially from that of all other species. This species inhabits forested areas, which are expected to have different sound transmission properties to more open habitats, which may favour more complex calls with lower carrier frequencies in order to enhance signal transmission (Padgham, 2004).



4.3 Relationships between acoustics, morphology, and genetics

The results of this study show that there is a positive relationship between morphological and acoustic characteristics in pneumorid species. This has also been documented in other taxa (e.g. Nevo and Capranica 1985; Castellano *et al*., 1999; Gingras *et al*. 2013). This relationship with morphology can thus explain, to some extent, the similarity or difference between the acoustic signals of these species.

The result of this study has shown a positive relationship between both morphological and acoustic distances (Figure 3.3), and between morphological and genetic distances (Figure 3.4). However, there was no significant relationship between acoustic and genetic distances (Figure 3.5). The lack of a correlation between male songs and genetic relatedness among species suggests that the differences in acoustic traits are not directly related to genetic

divergence, but are rather evolving due to selective pressures such as mate choice or ecological environment. These results are in contrast to previous studies which have shown a link between genetic and acoustic features (Amézquita *et al*. 2009; Velásquez *et al*. 2013). For example, a previous study that looked at *Tettigoniidae* species found that acoustic distances were positively correlated with genetic distances (Chen *et al*. 2021).

4.4 Conclusion

In conclusion, we investigated the morphological and acoustic aspects of bladder grasshopper species and their similarities and differences. We detected differences in both the acoustic calls and in the morphological characteristics of the sound producing structures among species. Acoustic variation may be impacted by mate selection and environmental conditions; therefore signals may vary at the species and population levels. The results of this study indicate that differences in the morphological features responsible for sound production affects the characteristics of sound signals, contributing to, and constraining, the diversity of acoustic characteristics between the studied species. However, this relationship, while significant, was not very strong, and thus acoustic characteristics are not necessarily strictly constrained by sound production morphology. Thus, species with similar sound producing structures may sometimes have divergent calls, and vice versa.

The results revealed that the morphology of the sound producing structures was significantly associated with genetic distance, but that acoustic distance was not. Similar results were found by Sathyan *et al*. (2017) when looking at intraspecific variation among populations of *B. unicolor*, where there was a significant relationship between morphology and genetics but

not between acoustics and genetics. Thus, interspecific patterns of divergence appear to mirror intraspecific patterns of divergence. However, morphology and acoustic distance were not correlated at the intraspecific level in *B. unicolor* (Sathyan *et al.*, 2017), which is in contrast to the results obtained here. However, here we looked specifically at the morphology of the sound producing structures, rather than overall morphology as in Sathyan *et al.* (2017). These results suggest that acoustic signals are under strong selective pressures.

Unfortunately sample sizes were small for some of the species examined here, and data were only available for a slightly different subset of species for each of the three variables examined (acoustic, morphology and genetics). Due to the rarity of certain species, it is difficult to obtain samples in the field for acoustic and genetic analyses, and even museum material is scant for some species. Future studies should ideally include all species, as well as more individuals of those species where sample sizes were low, for more robust analyses. In addition, the sound recordings used in this study were not all obtained using the same equipment or under the same environmental conditions, which may possibly have influenced some of the call parameters measured, and additional recordings taken under more controlled conditions are needed. However, due to the generally stereotypical nature of male advertisement calls and low intraspecific variation compared to interspecific variation, larger sample sizes are unlikely to change the overall conclusions of the study.

This study contributes to the understanding and knowledge of acoustic variation among bladder grasshoppers, and the relationship between male calling songs, sound producing morphology and phylogenetic relationships. This is important for understanding the evolution of sound signalling in this unusual group of insects.

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