A biogeographic, phylogenetic and taxonomic evaluation of South African orthopteran species (Orthoptera: Pneumoridae)

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Declaration

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Signed: October 2021

Abstract

The order Orthoptera contains a wide diversity of species and is the most speciose of the polyneopteran insect lineages. In South Africa, the orthopteran fauna is abundant and diverse, with approximately 88% of all Southern African species occurring in the region. One highly endemic and conservationally important group, the Pneumoridae (commonly known as bladder grasshoppers), is recognized as being an evolutionary distinct lineage, with all species occurring either fully or partially within South Africa. Unfortunately, the understanding of the true extent of diversity for this paleo-relictual group is hampered by inaccuracies in current taxonomic descriptions and the lack of adequate survey data. The last taxonomic revision was undertaken >50 years ago, during which several taxonomic discrepancies and uncertainties were introduced, particularly within the genus *Bullacris*. Specimens may be difficult to classify due to significant morphological overlap between species, geographic variation within species, and confusion arising from alternative male morphs being designated as separate species rather than conspecific within existing species. Furthermore, paleo-relictual insects are of great evolutionary and biogeographical interest by virtue of being survivors of highly derived lineages, which allows for insights into the paleo-history and phylogenetic diversity of a region. Thus, the aims of this research were, firstly, to investigate the biogeographic patterns of diversity for South African Orthoptera and to place the Pneumoridae family into this context, secondly, to construct a molecular phylogeny for the Pneumoridae and estimate divergence times by genetically analysing the relationships between Pneumoridae species, and lastly, to update taxonomic descriptions in the genus *Bullacris*. Biogeographic analyses were performed by making use of a national orthopteran database created from historical and current collecting records, and performing hierarchical cluster analyses to delimit zoogeographic regions and centres. A dated phylogeny was created by extracting DNA from fresh and museum specimens, and generating Bayesian Inference, Maximum Likelihood and BEAST phylogenetic trees for the Pneumoridae family. The updated revision of the genus *Bullacris* included morphological, acoustic and genetic comparisons. Results from the biogeographic analyses showed that there was a clear east (summer-rainfall) and west (winter-rainfall) primary biogeographic division for orthopteran species, which has also been previously noted for other insect taxa. Six zoogeographic centres were retrieved: the Central Nama-Karoo, the Cape Fynbos and the Succulent Karoo centre located in the west and the Savanna, the South-East Tropical and the Indeterminate Summer-Rainfall centre located in the east. Orthopteran species richness was found to be evenly distributed throughout the country; however, the Cape Fynbos centre has representatives for most orthopteran families. The dated phylogenetic analyses revealed that

bladder grasshoppers diverged from other orthopteran species during the early Cretaceous period, at an estimated 134.70 MYA. The first group to have diverged within the pneumorid family were the Forest species at approximately 116.91 MYA, followed by the Fynbos, the Succulent Karoo and then the Savanna species groups. It is suggested that bladder grasshoppers originated in South Africa and dispersed northward due to climatic changes. In addition, the phylogeny of the family showed that the species *Physemacris variolosa* integrated with members from the *Bullacris* genus. Lastly, the taxonomic revision of the *Bullacris* genus indicated that *B. discolor* and *B. serrata* were acoustically and morphologically similar, and had relatively low mitochondrial pairwise variation, and could thus possibly be represent sub-species. In addition, *B. membracioides* and *B. intermedia* were acoustically similar and also had low mitochondrial pairwise distances; however, statistical analyses showed significant morphological differences. Therefore, there was insufficient combined evidence to amalgamate species. Nevertheless, there are several environmental factors that could explain these variations, and it is therefore suggested that additional data is required to solve these taxonomic discrepancies. The results derived from this research have provided interesting insights into the evolutionary history of the Pneumoridae, the environments in which they occur, and share with other orthopteran species. Together with the newly discovered phylogenetic relationships and biogeographic studies, this information is useful data for conservation management strategies and expands our knowledge on the evolutionary histories of South Africa's entomofauna.

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Chapter 1: General Introduction

Orthopteran species are known to occur world-wide, with the exception of the polar regions and are found in a variety of habitats, ranging from deserts to rainforests (Kevan, 1982; Uvarov, 1966). The order Orthoptera has more than 26,000 extant species, making it the most diverse order amongst the polyneopteran insect lineages (Eades *et al*., 2014; Song *et al*., 2015). This order has been divided into two suborders, the Caelifera; which possesses short antennae and sound producing structures on the hind-legs, and the Ensifera; which possesses long antennae (longer than the body length), sings by means of organs on the wings and has tympanal organs (ears) located on the fore tibia (Picker *et al*., 2004). In South Africa, orthopterans are found across the entire region and comprise an estimated 323 genera and 809 species. The majority of endemic species are known to occur in the western arid parts of the country, whereas the more generalized species are known to be widespread Afrotropical forms (Bazelet, 2014; Picker *et al*., 2004).

The suborder Caelifera contains the infraorder Acrididea and has eight superfamily groups, of which Pneumoroidea (Thunberg, 1810) is one. Pneumoroidea, more commonly known as bladder grasshoppers, currently consists of nine genera and 17 described species (Dirsh, 1965). Bladder grasshoppers are primarily confined to southern Africa, with all species found along the coastal regions of South Africa and only four species extending variably northwards into East Africa, as far as Uganda (Dirsh, 1965). Bladder grasshoppers are primarily found in the following (mainly fire-free) biomes; the Succulent Karoo, Forest, Grassland, Savanna, Albany Thicket, Fynbos and Nama Karoo (Procheş, 2016). However, species distributional overlap between biomes on a finer scale, has been observed. Bladder grasshoppers are sexually dimorphic, with the primary males being macropterous and possessing an inflated balloon-like abdomen, which is used as a resonating chamber for producing advertisement calls; whereas the females are micropterous and do not possess the inflated abdomen (Figure 1.1). Males produce acoustic mating calls through abdominal-femoral stridulation (Figure 1.2), which are detectable by conspecifics up to a distance of 1.9 km in the species *B. membracioides* (van Staaden & Römer, 1997). Pneumorids are thought to be from a evolutionary distinct lineage due to the absence of classical ears in extant species (van Staaden & Römer, 1998) and having maintained their primitive nature of wing venation, which is observed in males (Ragge, 1963; Rehn, 1941; Smart, 1953).

FIGURE 1.1: Bladder grasshopper, *Bullacris membracioides*; showing the alternate male (left), female (centre) and primary male (right) (Image from Donelson *et al*., 2008).

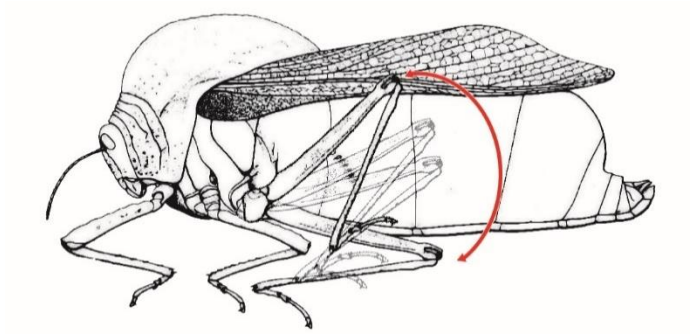

FIGURE 1.2: Stridulatory mechanism in bladder grasshopper, *Bullacris membracioides* (Image modified from Dirsh, 1965).

Taxonomic uncertainties in pneumorids

The most recent taxonomic revision of the Pneumoridae family was conducted in 1965, based on morphological characteristics (Dirsh, 1965), in which he proposed 17 species belonging to nine genera (six of which are monotypic). Prior to this revision, Dirsh (1963) discovered flightless non-inflated male forms, which he believed to be potentially neotenic forms and classified these individuals into separate genera, namely; *Parabullacris vansoni*, *Pneumoracris browni* and *Paraphysemacris spinosus*. However, he also stated that these individuals may have undergone parallel evolution to the existing *Bullacris unicolor*, *Peringueyacris namaqua* and *Physemacris variolosa* species, respectively. Later in 1979, the reprint of Skaife's *African Insect Life* (1953) made the first reference to two 'forms' of pneumorid males within one species, known as the 'primary' and 'alternate' males. Alexander & van Staaden (1989) subsequently reported that this phenomenon also occurs within *Bullacris membracioides*. The primary males develop their inflated abdomen during the final moult, at which stage the alternate males do not; however, the distinction between primary and alternate males are recognizable as early as the second or third instar (Alexander & van Staaden, 1989). Alternate males, like their female counterparts, possess micropterous wings that are hidden

underneath the pronotum. They make use of a "sneaking" tactic to exploit the acoustic mate location system between primary males and females in order to locate females for mating (Donelson & van Staaden, 2005). It was not until recent molecular analyses generated by Gordon (2017) and Laubscher (2021), that alternate males were verified as being a secondary male form of the same species; therefore, rendering the three uninflated taxa proposed by Dirsh (1963, 1965) invalid.

The taxonomy has yet to be formally corrected and updated. This would reduce the actual number of known species in the family Pneumoridae to 14 and the number of genera to six. In addition, due to morphological similarities between some species, as well as observed geographic variation in colour pattern and body size within species, existing species descriptions are currently inadequate. This is particularly problematic within the genus *Bullacris*. For example, colour pattern is the only known characteristic used to distinguish between *Bullacris discolor* and *B. serrata* (Dirsh, 1965), despite the described colour patterns not being ubiquitous for all individuals. Thus, there is an urgent need for taxonomic revision that encompasses acoustic variation and molecular data, as well as updated morphological descriptions.

Phylogenetic history of the Pneumoridae

Early orthopteran taxonomic studies relied predominantly on morphological characteristics to distinguish species, although acoustic characteristics were also later incorporated. These methodologies were used in other orthopteran taxonomic studies, such as Hebard (1913), Alexander (1957), Weissman and Rentz (1980) and Ragge and Reynolds (1988), to name a few. In 1995, a seminal study by Flook *et al*. published the first complete orthopteran mitochondrial genome. In addition, an extensive review by Chapco (1997) provided an overview of how molecular data can be used in the understanding of orthopteran evolution. Subsequent to this, many studies focusing on orthopteran phylogenies have been published (see references in Song, 2010).

Genetic analyses of the Pneumoridae family have not previously been attempted, although a preliminary phylogenetic investigation for the *Bullacris* genus has been conducted (Gordon, 2017). Thus, phylogenetic relationships within the family are currently uncertain. Nevertheless, members from the family have been included in a number of broad orthopteran phylogenetic studies (Dong *et al*., 2015; Flook & Rowell, 1997; Flook *et al*., 1999; Gordon *et al*., 2017; Laubscher *et al*., 2021; Song *et al*., 2014, 2015, 2018, 2020; Rutschamann, 2015; Sathyan *et al*., 2017). Furthermore, Song *et al*. (2015) produced a dated phylogenetic analysis

with the use of several fossil calibrations, to estimate divergence times for major orthopteran lineages. The species *Physemacris variolosa* was used as a representative for the family Pneumoridae and it was discovered that bladder grasshoppers may have originated during the early Cretaceous period at an estimated 116.97 million years ago (MYA). This time estimate, corroborates that pneumorids represent a phylogenetically distinct lineage of grasshoppers and further investigation of species within the family would provide greater insights into their evolutionary relationships and histories.

Biogeography of Orthoptera

The study of biogeography provides interesting insights into the evolutionary relationships and distributional patterns of species. The objective is to provide explanations for the geographical distributions of species that are based on past and present ecological and evolutionary processes (Sanmartín, 2012). Early techniques that were used to discover these processes were based on intuitive and expert knowledge, which was confined to the historical zoogeographical patterns of species. However, repeatable approaches were made possible with the use of geographical information systems (GIS), which has allowed for the integration of multiple disciplines such as biology, geography and ecology (Ricklefs & Jenkins, 2011; Sanmartín, 2012). In South Africa, biogeographical studies have been conducted on several different taxa such as insects, plants, reptiles, amphibians and birds (Born *et al*., 2007; Carcasson, 1964; Colville, 2009; Colville *et al*., 2014; Crowe, 1990; de Klerk *et al*., 2002; Endrödy-Younga, 1978; Herrel *et al*., 2011; Poynton, 1964; Tolley *et al*., 2006). However, a biogeographic analysis for South African orthopteran species has never been attempted. Exploring the species richness of the diverse South African orthopteran fauna would further improve our knowledge and understanding of patterns of insect species richness and areas of endemism; both essential for conservation strategies and management.

Research rationale

This research aims to improve the taxonomic, phylogenetic and biogeographic knowledge of a evolutionary distinct and near-endemic family of grasshoppers. It is clear that the taxonomy and species distributions of bladder grasshoppers are poorly understood, therefore this research is of particular importance. Insects are recognised as an understudied faunal group in South Africa (e.g., Melin & Colville, 2019) and a faunal group showing drastic global declines in diversity (see references in Cardoso *et al*., 2020). By understanding the identity and distribution of relictual insect taxa, it would help our understanding of human impacts on climatically sensitive ecosystems and taxa; and to better understand the impacts of global climate change on South Africa's biota. Relictual taxa confined to specific habitats have been shown to be sensitive indicators for climate change, and are considered of high conservation importance owing to their deep phylogenetic history (Hampe & Jump, 2011; Harrison & Noss, 2017). This study will be based on broad-scale patterns of orthopteran diversity from historical collecting records, and on field surveys and molecular investigations of pneumorid species and specimens from targeted populations spanning across several of South Africa's provinces and biomes.

Brief chapter overview

Chapter 1: General Introduction and Research rationale

A general introduction and research rationale stating the taxonomic uncertainties and phylogenetic histories of the family Pneumoridae, in addition to describing the current status of biogeographic studies of orthopteran species found in South Africa.

Chapter 2: Delimiting zoogeographic centres for South African Orthoptera

Biogeographic patterns of diversity for all major South African orthopteran families were identified and described by geographically depicting their distributions based on museum and personal collections. A hierarchical cluster analysis was generated based on the shared habitat preferences of species, to delimit zoogeographical centres and compare these regions to plant-based biomes and phytogeographic regions. In addition, the family Pneumoridae was placed into context and highlighted due to being a paleo-relictual lineage.

Chapter 3: Genetic and evolutionary insights into bladder grasshoppers (Orthoptera: Pneumoridae)

The taxonomic and evolutionary relationships among Pneumoridae species were investigated and time of divergence estimated. This first ever molecular phylogeny for the family was constructed from targeted collections of all 14 putative South African bladder grasshopper species. Evolutionary relationships among Pneumoridae species were phylogenetically illustrated by making use of mitochondrial COI and nuclear ITS and 18S gene regions and species divergence times estimated using BEAST analysis.

Chapter 4: An investigation of the genus *Bullacris* (Orthoptera: Pneumoridae)

The taxonomic descriptions of species belonging to the genus *Bullacris* were updated by including additional specimens and morphological variants, and incorporating additional methods for a more comprehensive comparison. *Bullacris* species were compared and differentiated by making use of morphological, acoustic and genetic data.

Chapter 5: General conclusions and summary

Overall conclusions and summaries presented for each chapter, together with suggestions for future research.

References

Alexander, A. J. & van Staaden, M. J. (1989). *Alternative sexual tactics in male bladder grasshoppers (Orthoptera, Pneumoridae)*. In Bruton, M. N. (Ed.). Alternative Life-History Styles of Animals, (pp. 261-277). Dordrecht, Netherlands: Kluwer Academic Publishers.

Alexander, R. D. (1957). The Taxonomy of the Field Crickets of the Eastern United States (Orthoptera: Gryllidae: *Acheta*). *Annals of the Entomological Society of America*, *50*(6): 584-602.

Bazelet, C. S. (2014). Checklist of South African Orthopteran; 2014 [cited 2019]. Database: Animal checklist [Internet]. Retrieved from: http://biodiversityadvisor.sanbi.org/research-and-modelling/checklists-and-encyclopaedia-of-life/south-african-animal-checklist/

Born, J., Linder, H. P. & Desmet, P. (2007). The Greater Cape Floristic Region. *Journal of Biogeography*, *34*: 147-162.

Carcasson, R. H. (1964). A preliminary survey of the zoogeography of the African butterflies. *East African Wildlife Journal*, *2*: 122–57.

Cardoso, P., Barton, P. S., Birkhofer, K., Chichorro, F., Deacon, C., Fartmann, T., …. & Samways, M. J. (2020). Scientists' warning to humanity on insect extinctions. *Biological Conservation, 242*, 108426. https://doi.org/10.1016/j.biocon.2020.108426

Chapco, W. (1997). *Molecular evolutionary genetics in orthopteroid insects.* In S. K. Gangwere, M. C. Muralirangan, M. Muralirangan (Eds.). The Bionomics of Grasshoppers, Katydids and their kin, (pp. 337-354). Oxon, United Kingdom: CAB International.

Colville, J. F. (2009). Understanding the evolutionary radiation of the mega-diverse Monkey Beetle fauna (Scarabaeidae: Hopliini) of South Africa. *Frontiers of Biogeography, 1*: 56-60.

Colville, J. F., Potts, A. J., Bradshaw, P. L., Measey, G. J., Snijman, D., Picker, M. D., Procheş, S., Bowie, R. C. K. & Manning, J. C. (2014). *Floristic and faunal Cape biochoria: do they exist?* In N. Allsopp, J. F. Colville, G. A. Verboom (Eds.), Fynbos: Ecology, Evolution and Conservation of a Megadiverse Region, (pp. 73-92). Oxford: University Press United Kingdom.

Crowe, T. M. (1990). *A quantitative analysis of patterns of distribution, species richness and endemism in southern African vertebrates*. In G. Peters and R. Hutterer (Eds.). Vertebrates in the tropics, (pp. 145–59). Bonn: Museum Alexander Koenig.

de Klerk, H. M., Crowe, T. M., Fjeldsa, J. & Burgess, N. D. (2002). Biogeographical patterns of endemic terrestrial Afrotropical birds. *Diversity and Distributions*, *8*: 147–62.

Dirsh, V. M. (1963). Three new genera and species of the family Pneumoridae (Orthoptera: Acridiodea). *Eos, Madr*. *39*: 177-184.

Dirsh, V. M. (1965). Revision of the family Pneumoridae (Orthoptera: Acridoidea). *Bulletin of the British Museum* (*Natural History*) *Entomology*, *15*: 325-96.

Donelson, N. C. & van Staaden, M. J. (2005) Alternate tactics in male bladder grasshoppers *Bullacris membracioides* (Orthoptera : Pneumoridae). *Behaviour*, *142*: 761–778.

Dong, L., Shi, J., Zhang, X., Zhang, Y., Li, X. & Yin, H. (2015). Molecular phylogenetic analysis of Acridoidea (Orthoptera: Caelifera) based on mitochondrial cytochrome oxidase subunit sequences. *Zootaxa*, *4018*(3): 411. doi:10.11646/zootaxa.4018.3.5

Eades, D. C., Otte, D., Cigliano, M. M. & Braun, H. (2014). Orthoptera Species File, version 5.0/5.0. [cited 8 January 2014]. Retrieved from: http://Orthoptera.SpeciesFile.org

Endrödy-Younga, S. (1978). *Coleoptera*. In M. J. A. Werger (Ed.), Biogeography and ecology of southern Africa (Vol. 31, no. 2, pp. 797-820). The Hague: Dr W. Junk by Publishers.

Flook, P. K. & Rowell, C. H. F. (1997). The effectiveness of mitochondrial rRNA gene sequences for the reconstructions of the phylogney of an insect order (Orthoptera). *Molecular Phylogentics and Evolution*, *8*: 177-192.

Flook, P. K., Klee, S. & Rowell, C. H. F. (1999). Combined Molecular Phylogenetic Analysis of the Orthoptera (Arthropoda, Insecta) and Implications for Their Higher Systematics. *Systematic Biology*, *48*(2): 233-253.

Flook, P. K., Rowell, C. H. F. & Gellissen, G. (1995). The sequence, organization, and evolution of the *Locusta migratoria* mitochondrial genome. *Journal of Molecular Evolution*, *41*: 928-941.

Gordon, M. L. (2017). *An acoustic, genetic and morphological review of the genus Bullacris (Orthoptera; Pneumoridae).* (Master's dissertation, University of the Western Cape, Cape Town). Retrieved from http://etd.uwc.ac.za/handle/11394/6119

Hampe, A. & Jump, A. S. (2011). Climate Relicts: Past, Present, Future. *Annual Review of Ecology, Evolution and Systematics*, *42*: 313–333. doi:10.1146/annurev-ecolsys-102710-145015

Harrison, S. & Noss, R. (2017). Endemism hotspots are linked to stable climatic refugia. *Annals of Botany*, *119*: 207–214. doi:10.1093/aob/mcw248

Hebard, M. (1913). A revision of the species of the genus *Nemobius* (Orthoptera: Gryllidae) found in North America North of the Isthmus of Panama. *Proceedings of the Academy of Natural Sciences of Philadelphia*, *65*(2): 394-492.

Herrel, A., Measey, G. J., Vanhooydonck, B. & Tolley, K. A. (2011). Functional consequences of morphological differentiation between populations of the Cape Dwarf Chameleon (*Bradypodion pumilum*). *Biological Journal of the Linnean Society*, *104*: 692-700.

Kevan, D. K. M. (1982). *Orthoptera*. In S. P. Parker (Ed.). Synopsis and Classification of Living Organisms, (pp. 352-379). New York: McGraw–Hill.

Laubscher, M. (2021). Genetic and morphological comparisons within the orthopteran family Pneumoridae. (Master's dissertation, University of the Western Cape, Cape Town). Retrieved from http://etd.uwc.ac.za/handle/11394/7949

Melin, A. & Colville, J. F. (2019). A review of 250 years of South African bee taxonomy and exploration (Hymenoptera: Apoidea: Anthophila). *Transactions of the Royal Society of South Africa*, *74*: 86–96. https://doi.org/10.1080/0035919X.2019.1572670

Picker, M., Griffiths, C. & Weaving, A. (2004). *Orthoptera: crickets, katydids, grasshoppers & locusts*. In J. Hromnik (Ed.). Field guide to insects of South Africa, (pp. 74-107). Cape Town: Struik Publishers.

Poynton, J. C. (1964). Amphibia of southern Africa: a faunal study. *Annals of the Natal Museum*, *17*: 1-334.

Procheş, Ş. (2016). Fire-free environments across southern Africa's biomes: distribution and refugial value. *North-Western Journal of Zoology*, *12*(2): 361-366.

Ragge, D. R. & Reynolds, W. J. (1988). The songs and taxonomy of the grasshoppers of the *Chorthippus biguttulus* group in the Iberian Peninsula (Orthoptera: Acrididae), *Journal of Natural History*, *22*(4): 897-929. doi: 10.1080/00222938800770611

Ragge, D. R. (1963). The nymphal wing-pad tracheation and adult axillary sclerites of the Pneumoridae (Orthoptera: Acridoidea). *Annals and Magazine of Natural History*, *13*(6): 185-191.

Rehn, J. A. G. (1941). On new and previously known species of Pneumoridae (Orthoptera: Acridoidea). *Transactions from the American entomological Society*, *67*: 137-159.

Ricklefs, R. E. & Jenkins, D. G. (2011). Biogeography and Ecology: towards the integration of two disciplines. *Philosophical Transactions of the Royal Society B, 366*: 2438-2448.

Rutschmann, S. (2015). Evolutionary processes in mayflies (Ephemeroptera): genomics approaches to the study of ancient origins and recent diversification. (Doctoral dissertation, Freie Universität Berlin, Berlin). Retrieved from https://refubium.fu-

berlin.de/bitstream/handle/fub188/5404/Dissertation_Rutschmann_online.pdf?sequence=1&isAllowed=y

Sanmartín, I. (2012). Historical Biogeography: Evolution in Time and Space. *Evolution: Education and Outreach, 5*: 555-568.

Sathyan, R., Engelbrecht, A. & Couldridge, V. C. K. (2017). Morphological, acoustic and genetic divergence in the bladder grasshopper *Bullacris unicolor*. *Ethology, Ecology and Evolution*, *29* (6): 552-573.

Skaife, S. H. (1953). *African Insect Life.* In Longmans, Green & Co. (Eds.). (pp. 388). London.

Skaife, S. H. (1979). *African Insect Life.* (Rev. ed.). In Ledger, J. & Bannister, A. (Revs.). (pp. 279). Cape Town: Struik.

Smart, J. (1953). On the wing-venation of *Physemacris variolosa* (Linn.) (Insecta: Pneumoridae). *Proceedings of the Zoological Society of London*, *123*(1): 199-202.

Song, H. (2010). Grasshopper Systematics: Past, Presence and Future. *Journal of Orthoptera Research*, *19*(1): 57-68.

Song, H., Amédégnato, C., Cigliano, M. M., Desutter-Grandcolas, L., Heads, S. W., Huang, Y., Otte, D. & Whiting, M. F. (2015). 300 million years of diversification: elucidating the patterns of orthopteran evolution on comprehensive taxon and gene sampling. *Cladistics*, *31*: 621-651. doi: 10.1111/cla.12116

Song, H., Béthoux, O., Shin, S. *et al.* (2020). Phylogenomic analysis sheds light on the evolutionary pathways towards acoustic communication in Orthoptera. *Nature Communications*, *11*: 4939. doi.org/10.1038/s41467-020-18739-4.

Song, H., Mariño-Pérez, R., Woller, D. A. & Cigliano, M. M. (2018). Evolution, Diversification and Biogeography Grasshoppers (Orthoptera: Acrididae). *Insect Systematics and Diversity*, *2*(4): 1-25.

Song, H., Moulton, M. J., Whiting, M. F. (2014). Rampant Nuclear Insertion of mtDNA across Diverse Lineages within Orthoptera (Insecta). *PLoS ONE 9*(10): e110508. doi:10.1371/journal.pone.0110508

Thunberg, C. P. (1810). Nya Arter af Pneumorae slägtet beskrifna. *Kungliga Svenska vetenskapsakakademiens handlingar, 31*: 54-65.

Tolley, K. A., Burger, M., Turner, A. A. & Matthee, C. A. (2006). Biogeographic patterns and phylogeography of dwarf chameleons (*Bradypodion*) in an African biodiversity hotspot. *Molecular Ecology*, *15*: 781-793.

Uvarov, B. P. (1966). Grasshopper and Locusts, V01.1. Cambridge: Cambridge University Press, 481 pp.

van Staaden, M. J. & Römer, H. (1997). Sexual signalling in bladder grasshoppers: tactical design for maximizing calling range. *Journal of Experimental Biology*, *200*: 2597–2608.

van Staaden, M. J. & Römer, H. (1998). Evolutionary transition from stretch to hearing organs in ancient grasshoppers. *Nature*, *394*: 773-776.

Weissman, D. B. & Rentz, D. C. F. (1980). Cytological, Morphological, and Crepitational characteristics of the Trimerotropine (Aerochoreuttes, Circotettix, and Trimerotropis) grasshoppers (Orthoptera; Oedipodinae). *Transactions of the American Entomological Society*, *106*(2): 253-272.

Chapter 2: Delimiting zoogeographic centres for South African Orthoptera

Abstract

Biogeography attempts to find explanations for the distributions of species, based on their past histories and present environmental conditions. Historically, biogeographic studies were modelled on intuitive and expert knowledge, whereas recent studies have advanced with the aid of computational power and repeatable methods. In South Africa, biogeographical studies on insects are greatly lacking. Two detailed southern African maps, produced over 50 years ago and based solely on expert knowledge, showed the broad zoogeographical zones for butterflies and beetles. However, very little is known about the zoogeographic patterns for other insect groups in the region. Orthopterans within this region are rich (> 800 species) and diverse, with a high level of endemism, making them an ideal group to investigate zoogeographic patterns. The aim of this study was to identify and describe the zoogeographical patterns of South Africa's orthopteran species, based on the distributions and levels of diversity of all major families. This was conducted by using point locality data and constructing a hierarchical cluster analysis based on the shared presence of species to delimit zoogeographical centres. In addition, delimited centres were compared to plant-based biomes and phytogeographic regions. Results showed that orthopteran species richness was evenly distributed across the region and clustered into six biogeographical centres. There was a primary split, separating species into a western winter-rainfall and an eastern summer-rainfall group. The west contained three centres: the Central Nama-Karoo, the Succulent-Karoo and the Cape Fynbos centre and the east included the Savanna, the Indeterminate Summer-Rainfall and the South-East Tropical centre. The eastern region was less diverse and distinctive than the western region. Strong consensus was seen between orthopterans and the Greater Cape Floristic Region, and between orthopterans and the Cape zoogeographic region for butterflies, suggesting that this region is representative of a biochorion. Additionally, the Cape Fynbos centre had the greatest numbers of orthopteran families, highlighting the importance of this area for orthopteran diversification. Finally, although several zoogeographic centres were found for orthopterans, there was indisputable evidence of under-sampling in South Africa. Further surveying of the South African orthopteran fauna may reveal additional centres and help resolve poorly retrieved centres, particularly in the eastern summer-rainfall region.

Introduction

Biogeographical studies can provide possible explanations for species' geographical distributions based on their evolutionary histories, present ecological processes and future predicted dispersals (Sanmartín, 2012). Earlier studies of biogeography did not incorporate the ecology of species' distributional patterns and confined their approaches to the historical distibution organisms. The major disadvantage of this approach is that the authors made use of their intuitive and often subjective knowledge to model species distributions, making it a non-repeatable approach (Linder, 2001). Nevertheless, over the intervening years, biogeography has evolved in its emergent understanding of historical processes (e.g., continental drift) and much attention has been devoted to the integration of disciplines, such as biology, geography and ecology (Ricklefs & Jenkins, 2011; Sanmartín, 2012). In addition, the modelling of species' spatial distribution patterns has greatly advanced with the emergence of geographic information systems (GIS) and remote sensing technologies, which have been used in the advancement of ecological processes (Sanmartín, 2012).

It has been suggested that when searching for shared historical and evolutionary patterns across taxa, 'units of area' is a useful standardized method for mapping and delineating species distributions (Hausdorf, 2002). This allows for the clustering of predefined operational geographic units (OGU) that are based on the shared presence-absence of species (Crovello, 1981). Historically, distributions of South African species were surveyed according to quarter degree square (QDS) levels of resolution (Edwards & Leistner, 1971). However, more recently, the use of GPS coordinates (locality point data) has become more effective and is widely used in a number of biogeographic studies. Therefore, with the use of point locality data from collection records together with QDS grid cells, studies have been able to delineate biogeographic divisions based on a modern quantitative approach (e.g., Bradshaw *et al*., 2015; Colville, 2009; Colville *et al*., 2014; Gonzalez-Orozco *et al*., 2013; Lenormand *et al*., 2019; Linder *et al*., 2012; Rodrigues *et al*., 2015; Seymour *et al*., 2001). This shift towards a more quantitative approach within biogeographic studies, with the use of numerical methods and increased computational power, now permits the analysis of larger datasets and more objectively defines biogeographic patterns (Bradshaw *et al*., 2015; Kreft & Jetz, 2010).

In South Africa, biogeographical studies have been conducted on butterflies (Carcasson, 1964), beetles (Endrödy-Younga, 1978; Colville, 2009), reptiles (Herrel *et al*., 2011; Tolley *et al*., 2006), plants (Born *et al*., 2007; Bradshaw *et al*., 2015; Colville *et al*., 2014; Jürgens, 1997; Linder & Mann, 1998), frogs (Crowe, 1990; Poynton, 1964) and birds

(Crowe, 1990; de Klerk *et al*., 2002). These can be broken into older studies that made use of expert knowledge (Carcasson, 1964; Crowe, 1990; Endrödy-Younga, 1978; Poynton, 1964) and recent ones using a more quantitative approach (Born *et al*., 2007; Bradshaw *et al*., 2015; Colville, 2009; Colville *et al*., 2014; de Klerk *et al*., 2002; Herrel *et al*., 2011; Tolley *et al*., 2006). Generating biogeographic regions or centres of plants and animals is important, as it allows for the discovery of links between taxa and their environments, in which the aforementioned studies have shown clear patterns of biogeographic structure. Some studies have identified some spatial congruence between plants and animals, such as strong arid links between areas of the Nama and Succulent Karoo for butterflies (Carcasson, 1964) and birds (de Klerk *et al*., 2002). Whereas, several different animal groups have shown strong connections between the Cape and Succulent Karoo areas (Endrödy-Younga, 1978; Vernon, 1999). Furthermore, studies by Tolley *et al*., (2006) and Herrel *et al*., (2011) discovered a correlation between the distribution of chameleons and the vegetation types in which they occur. Therefore, the delineating of species distributional regions assists in identifying areas for conservation importance and subsequently aids in conservation management strategies (Kreft & Jetz, 2010; Olivero *et al*., 2012).

In southern Africa, biogeographical studies on insects are greatly lacking (Colville *et al*., 2014). Nevertheless, there are two well detailed maps showing broad zoogeographical zones for butterflies (Carcasson, 1964) and beetles (Endrödy-Young, 1978). These studies were based on a mostly subjective approach and expert knowledge of the authors. Globally, there have been a number of studies that have discovered symbiotic relationships between insect and plant species (Basset *et al*., 2012; Crutsinger *et al*., 2006; Haddad *et al*., 2001; Johnson *et al*., 2006; Koricheva *et al*., 2000; Leal *et al*., 2016; Lewinsohn *et al*., 2006; Novotny & Basset, 2005; Siemann, 1998; Siemann *et al*., 1998; Zhang *et al*., 2016), although others have not (Hawkins and Porter, 2003). Within southern African biomes, there have only been a limited number of studies that have compared plant species and the structures of insect communities (Gess & Gess, 1993; Procheş & Cowling, 2006; Wright & Samways, 1998). The discovery of links between insect distribution patterns and phytogeographic regions or plant biomes would be of significant interest (Procheş & Cowling, 2007) from detailed mechanisms (host-plant and insect selection) to evolutionary interactions (Bernays, 1992). Recognizing these links in relation to insect diversity and identifying whether insects coevolve with plants, or if plant distribution patterns structures insect distributions, would be vital in understanding the driving force of herbivorous insect distributions.

South Africa has a rich and diverse orthopteran fauna, with approximately 323 genera and 809 species (Bazelet, 2014). There are approximately 366 genera native to southern Africa, of which an estimated 88% occur in South Africa (Eades *et al*., 2013; Lomer *et al*., 2001), making this a good taxonomic group for regional biogeographical studies. Orthopteran species occur globally in a wide variety of habitats, apart from the polar regions, and are the most diverse of the polyneopteran insect lineages (Eades *et al*., 2014; Grimaldi & Engel, 2005; Song *et al*., 2015). With the ability to inhabit various environments, orthopterans are known to be associated with certain plant species, by either being host plant specific (e.g., *Eremidium*; Armstrong & Brand, 2012; Brijlal *et al*., 2020) or agricultural pests (e.g., *Locustana*; Bam *et al*., 2020; Khambule, 2010; Todd, *et al*., 2002). In addition, some orthopterans also have the ability to camouflage well with their surroundings to avoid predation. For example, members from the families Pamphagidae, Lathiceridae and Lentulidae, are known to camouflage well with the substrates on which they occur (Brown, 1962; Scholtz *et al*., 2021).

Orthopterans also serve as bioindicators for ecological and conservation processes in a variety of environments, both globally and locally (Bazelet & Samways, 2011; Matenaar *et al*., 2015; Sauberer *et al*., 2004; Steck *et al*., 2007). However, insects are generally underrepresented in the planning and assessments of conservation strategies, largely due to the lack of available data (Cowling *et al*., 2004) as well as the poor taxonomic state of many groups (Melin & Colville, 2019). Recent studies have also observed that there has been a massive global decline in insects (Cardosao *et al*., 2020; Hallmann *et al*., 2017; Lister & Garcia, 2018). The results of an orthopteran biogeographic study for South Africa should therefore further improve our knowledge in identifying areas of richness and endemism for conservation importance. Furthermore, there is a high level of endemism of orthopteran species in South Africa, with the family Pneumoridae being an example of this, since all species occur within the region (Chapter 1 & 3; Dirsh, 1965; Matenaar *et al*., 2018; Naskrecki & Bazelet, 2009). Bladder grasshoppers are considered to be paleo-relictual taxa and may therefore show insightful historical biogeographic patterns regarding the evolution of South Africa's insect fauna.

A biogeographic study for South African Orthoptera has never been undertaken to date, therefore the aim of this chapter is to identify and describe the zoogeographical patterns of South African orthopteran species based on their distribution and diversity. This will be achieved by making use of a South African orthopteran database and conducting a hierarchical cluster analysis based on the shared presence of species to delimit zoogeographical centres. In addition, the study aims to test whether orthopteran species match or show spatial consensus

with plant-based biomes and phytogeographic regions, and to compare this information with what is known for butterflies (Carcasson, 1964).

Materials and Methods

Compilation of orthopteran distribution dataset

Point locality georeferenced data for orthopteran species records from South Africa were acquired from the following institutes; National Museum of Bloemfontein, Iziko South African Museum, Albany Museum, Durban Natural Science Museum, Ditsong National Museum of Natural History, the Karoo BioGaps Project (South African National Biodiversity Institute, SANBI), Stellenbosch University and the University of the Western Cape (Table 2.1). Locality points for species were plotted in QGIS 3.10.1 (QGIS Development Team, 2019) and checked to see if the locality information matched the GPS coordinates. Points that did not match the locality information and that fell outside of South African borders, were removed. In total, 19 of the 20 South African orthopteran families, 700 species and 4774 unique locality records were used for GIS mapping.

TABLE 2.1: A list of institutions from which data records were received and used in this study.

No.	Institution	Contact	Date Accessed	Source
1	National Museum of Bloemfontein	B. Muller	July 2018	*Unpublished*
2	Iziko South African Museum	A. Mayekiso	July 2018	*Unpublished*
3	Albany Museum	T. Bellingan	May 2018	*Unpublished*
4	Durban Natural Science Museum	N. Govender	October 2018	*Unpublished*
5	Ditsong National Museum of Natural History	T. Perregil	September 2018	*Unpublished*
6	Karoo BioGaps Project	C. Bazelet	August 2018	*Published online*
7	Stellenbosch University	C. Bazelet	June 2018	*Unpublished*
8	The University of the Western Cape	V. Couldridge	October 2018	*Unpublished*

Operational Geographic Unit (OGU)

Operational geographical units were obtained for further biogeographical analysis (Bradshaw *et al*., 2015; Moline & Linder, 2006). Orthopteran point distribution maps were created using QGIS 3.10.1 (QGIS Development Team, 2019) and overlaid onto a QDS vector file (Edwards & Leistner, 1971) of South Africa. The distributional point dataset was merged with the QDS cells and a list of species by QDS was generated. Similarly, to Bradshaw *et al*. (2015), all QDS cells that contained less than five records were removed.

Statistical and biogeographical analyses

The approach of Colville *et al*. (2014) and the detailed methodological steps given in Bradshaw *et al*. (2015) were followed in this study. A brief synopsis of these steps is given here. Firstly, all analyses were calculated using the statistical program R within the R-studio environment (R Development Core Team, 2012). A presence-absence matrix of species by OGU was created and then used to generate a dissimilarity matrix using Kulczinski's second (K2) similarity equation within the similarity index matrix package 'simba' (Juransinski, 2012). This matrix displays each row as representing a grid cell and each column a species. The Kulczinski's equation was used as it has been frequently utilised in several previous biogeographic studies (Born *et al*., 2007; Moline & Linder, 2006; Shi, 1993). Once the similarity matrix was generated, it was then used to run the cluster analysis with unweighted pair group method with arithmetic means (UPGMA; Sokal & Michener, 1958). This analysis groups grid cells using the hierarchical clustering function 'hclust' found in the 'stats' package within the R statistical environment (R Core Team, 2020).

A branch ranking technique derived from the Strahler stream order assignment (Borchert & Slade, 1981; Strahler, 1957) known as the branch order cut-off method (BOC; Bradshaw *et al*., 2015) was used to generate a dendrogram with branch order numbers. Each of these numbers were then assigned to all branches within the dendrogram using the 'phytools' package in R (Revell, 2012). The dendrogram was converted into a phylogram for better visualization of the branches using the 'ape' package (Paradis & Schliep, 2019). The hierarchical biogeographical units (clustered QDS grid cells) were identified based on species similarities, which resulted in determining the relationships between biogeographical areas. Branch orders were then plotted in QGIS 3.10.1 and the most realistic number (level) of centres were selected.

These clustered areas were carefully examined in QGIS and disjunct grid cells (cells of the same ranking and colour that were not part of the larger clustered group) were removed after further investigation, which entailed several steps. Firstly, if the disjunct cell contained < 3 records, it was removed, because fewer species can bias the results and overall conclusions. However, if disjunct cells had > 3 records, the species within the cell were inspected and based on known information regarding the species' biology and distribution, they were either retained or removed. An example of this is if species are known to only occur within the Fynbos biome, but they displayed in the Gauteng region, the QDS cell was removed. Species within their respective known habitats and regions were investigated post analyses, using platforms such

as the IUCN Red List of Threatened Species (www.iucnredlist.org) and Orthoptera Species File (http://orthoptera.speciesfile.org), to ensure that species were designated correctly. These disjunct occurrences may be the result of misidentifications of species or erroneous locality information. Once the final biological centres were identified, attribute tables for each of these centres were exported and descriptive statistics calculated. These calculations included the number of records and species found in each centre, as well as the number of families identified in each cluster.

Production of biogeographic maps

The final map representing the biogeographical centres was created and used as a template for creating additional maps. A species richness map was created with a colour gradient to indicate the abundance of species found within each QDS cell. This species richness map, as well as the resulting biogeographical centres, were then overlaid with South African biomes (Mucina & Rutherford, 2006). Finally, the biogeographic centres were additionally overlaid with Carcasson's (1964) southern African faunistic butterfly divisions for regional comparisons.

Results

Biogeographic centres

Results from the BOC technique showed a primary split at branch order cut-off level 6, producing a clear east and west zone (Figure 2.1). Roughly, these zones indicate differences in orthopteran species between the winter (west) and summer (east) rainfall regions. A total of six centres at level 5 were retrieved (Figure 2.2 & Table 2.2), with three of the six centres falling within each of the two primary zones. Within the western winter-rainfall zone, three distinct centres could be observed; the Cape Fynbos, the Central Nama-Karoo, and the Succulent-Karoo centre. The Succulent-Karoo and the Cape Fynbos centres, together align strongly with the Greater Cape Floristic Region (GCFR) and are comprised of fairly similar orthopteran families. The eastern summer-rainfall zone had less distinctive centres, showing an Indeterminate Summer-Rainfall, South-East Tropical and Savanna centre.

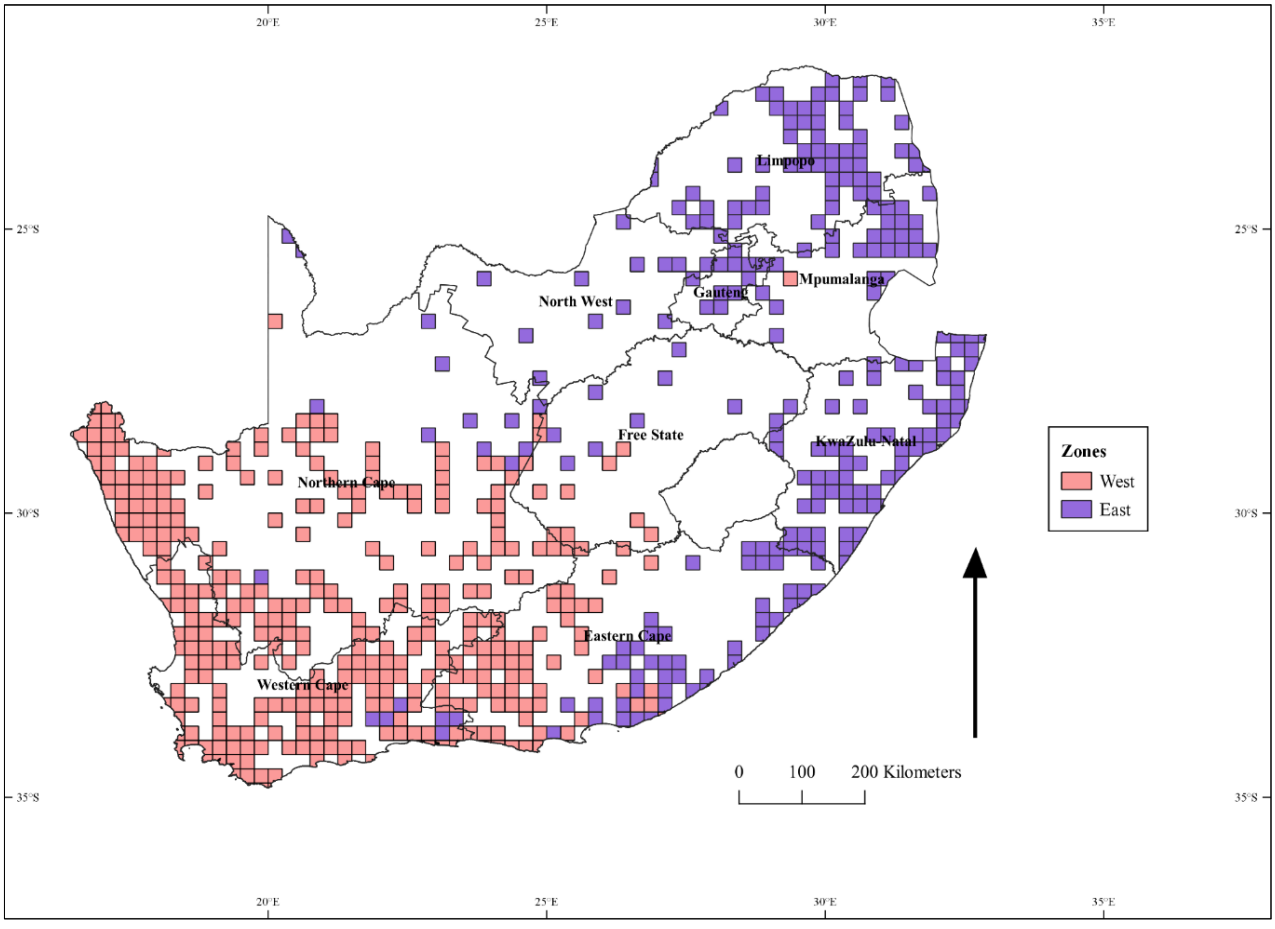

FIGURE 2.1: The primary split for South African orthopteran species, showing clear western (predominantly winter-rainfall) and eastern (predominantly summer-rainfall) biogeographical zones.

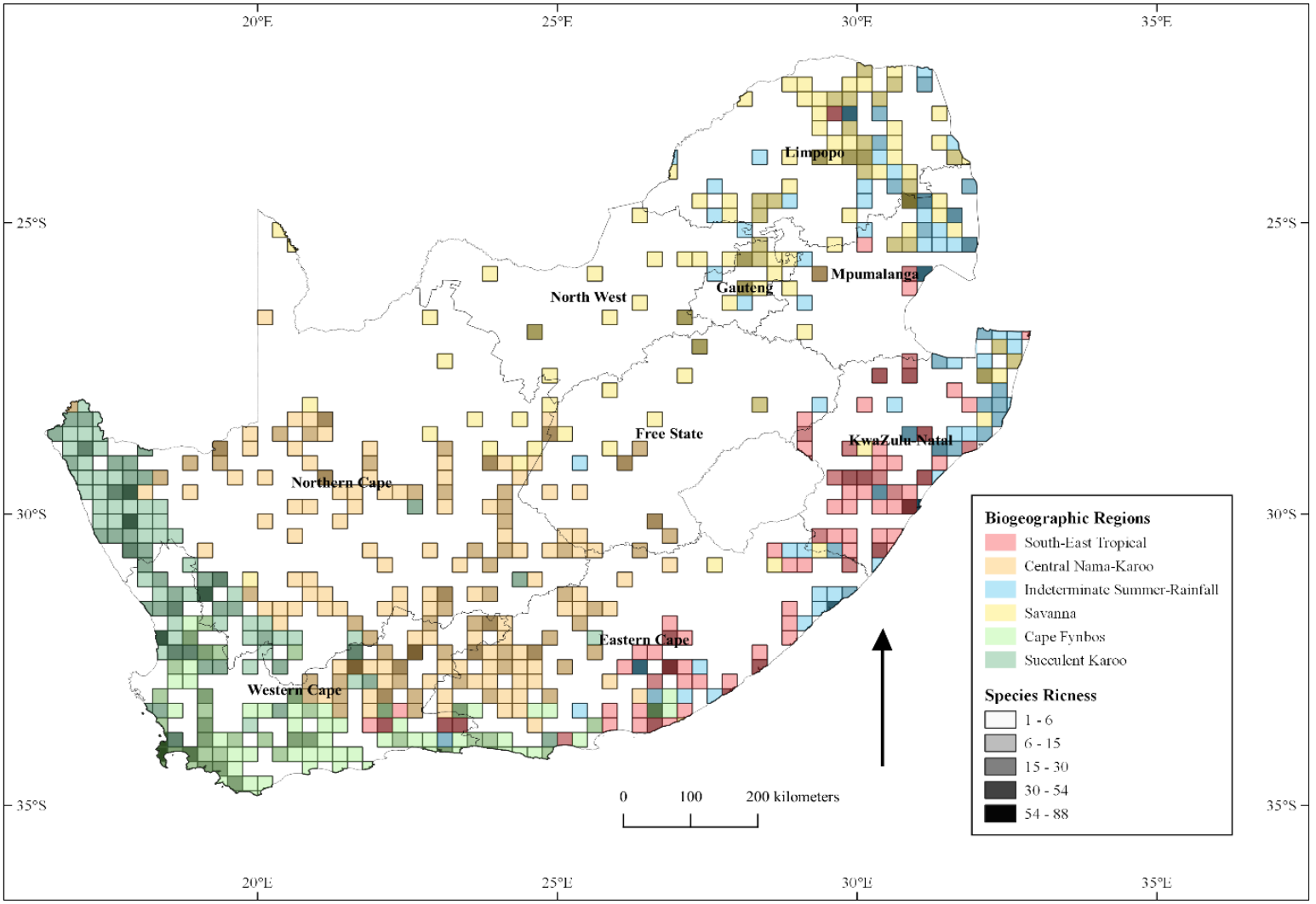

FIGURE 2.2: Species richness and clustered quarter degree square (QDS) cells showing the six identified centres (in colour) and species richness (darker shades) of South African orthopteran species.

The species richness map shows orthopteran species appear to be evenly distributed across South Africa's biomes (Figure 2.3) with grid cells displaying very high species richness (>50 species) seen scattered across all main biomes. The western, predominately winter-rainfall zone contained a slightly larger number of orthopteran families in comparison to the eastern summer-rainfall region (Table 2.2). The centre containing the highest family richness was the Cape Fynbos, with the Tettigoniidae and Acrididae having the largest number of species found within this centre. Generally, these two families were the most frequently represented families, whereas Rhaphidophoridae and Schizodactylidae were the least. The Pamphagidae were most abundant within the Central Nama-Karoo and the Succulent-Karoo centres, and Pneumoridae were largely found in the Cape Fynbos centre. Lentulidae were most commonly found in the Central Nama-Karoo and the Cape Fynbos centres.

TABLE 2.2: The number of species records and the total number of species and families found in each centre, for South African orthopteran species.

No.	Family Names	Cape Fynbos	Succulent-Karoo	Central Nama-Karoo	Savanna	Indeterminate Summer-Rainfall	South-East Tropical
1	Acrididae	219	188	398	77	152	247
2	Anostostomatidae	37	3	7	13	15	16
3	Euschmidtiidae	7	5	2	11	3	
4	Gryllacrididae	15	2		1	2	2
5	Gryllidae	17	5	14	9	16	10
6	Gryllotalpidae	8	1	1	1	1	1
7	Lentulidae	103	48	106	36	23	71
8	Lithidiidae	1	38	20	1		
9	Pamphagidae	87	229	362	72	26	20
10	Pamphagodidae				12		
11	Pneumoridae	119	96	9	4	25	73
12	Pyrgomorphidae	70	37	85	101	62	89
13	Rhaphidophoridae	1					
14	Schizodactylidae		3	2			
15	Stenopelmatidae	8	2		1	5	3
16	Tetrigidae	3			2	6	7
17	Tettigoniidae	220	131	108	210	317	85
18	Thericleidae	19	10	17	56	13	7
19	Tridactylidae	3	3			1	
No. of families per centre		**17**	**16**	**13**	**16**	**15**	**13**
No. of species per centre		**268**	**218**	**244**	**211**	**263**	**266**
No. of records per centre		**937**	**801**	**1131**	**607**	**667**	**631**

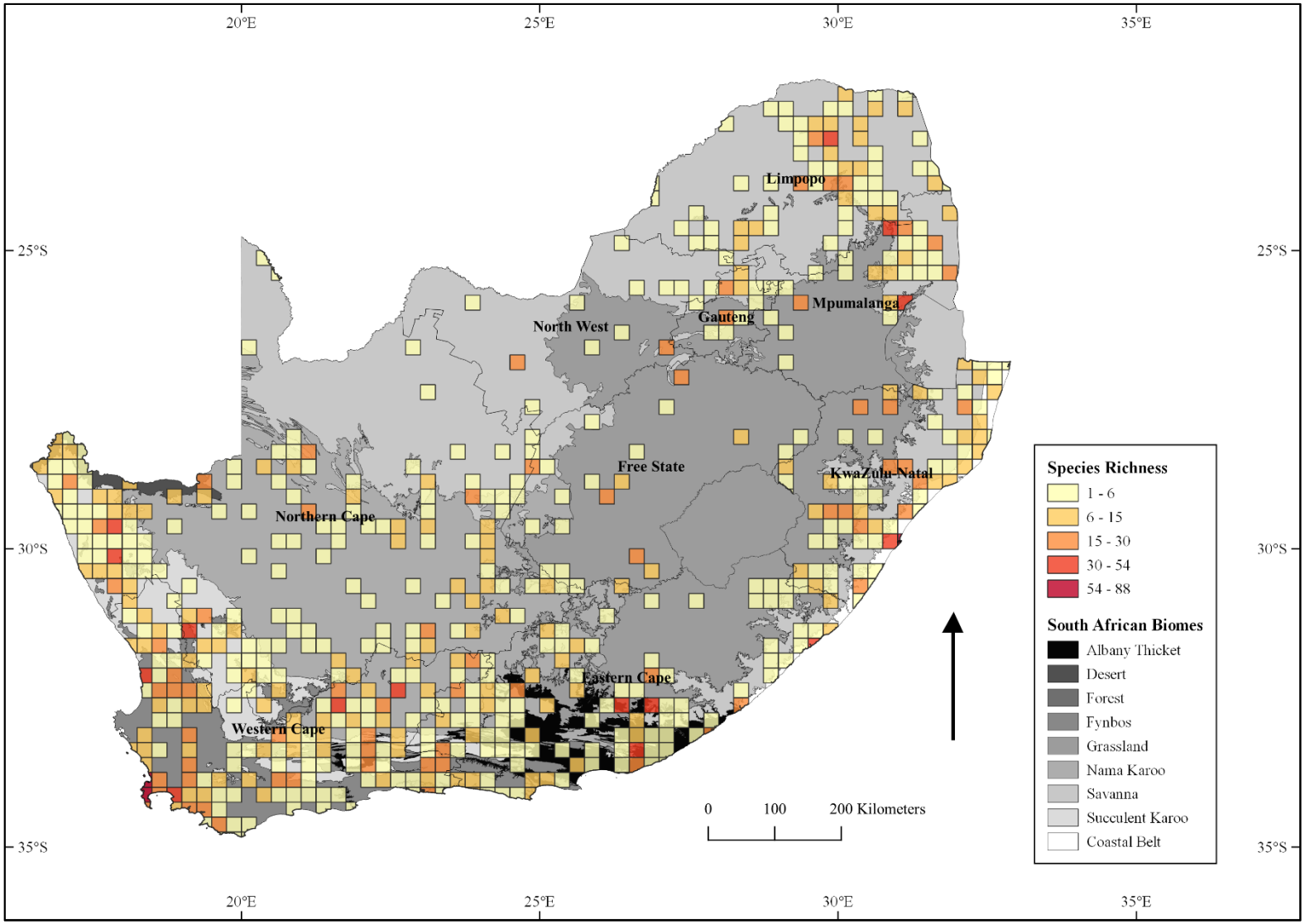

FIGURE 2.3: Distributional map of orthopteran species showing species richness based on the number of species found in each quarter degree square (QDS) overlaid with South African biomes (Mucina & Rutherford, 2006).

Spatial congruence with biomes and Carcasson's butterfly divisions

In general, there was a broad spatial overlap between centres and vegetation biomes (Figures 2.4 & 2.5). More specifically, the Cape Fynbos and Succulent-Karoo centres matched well with the GCFR and are known to be linked through shared species. Species distributions within the Central Nama-Karoo centre also broadly related to the Nama Karoo vegetation type. However, the South-East Tropical Rainfall, the Indeterminate Summer-Rainfall and the Savanna centres are spread across several different biomes and extend into the Cape Fynbos and the Central Nama-Karoo centres.

In Figure 2.6, the identified centres from this study were overlaid with faunistic butterfly divisions retrieved from Carcasson (1964). The Cape Fynbos centre largely coincided with Carcasson's Cape butterfly division. The Central Nama-Karoo orthopteran centre also showed good consensus with Carcasson's Karoo division. Orthopterans from the Succulent-Karoo centre overlap with two of Carcasson's faunistic divisions; the Namib and the Karoo divisions. The Savanna centre largely forms part of Carcasson's Kalahari division, but also has species present within his Cape Grasslands, Eastern Karoo, Coastal and South African Highlands Forest divisions. The majority of the South-East Tropical centre fell within Carcasson's Coastal and Eastern divisions; however, there were some outliers. The Indeterminate Summer-Rainfall centre fell across several of Carcasson's butterfly divisions, whereas Carcasson's Cape division was comprised of the Nama Karoo and the GCFR.

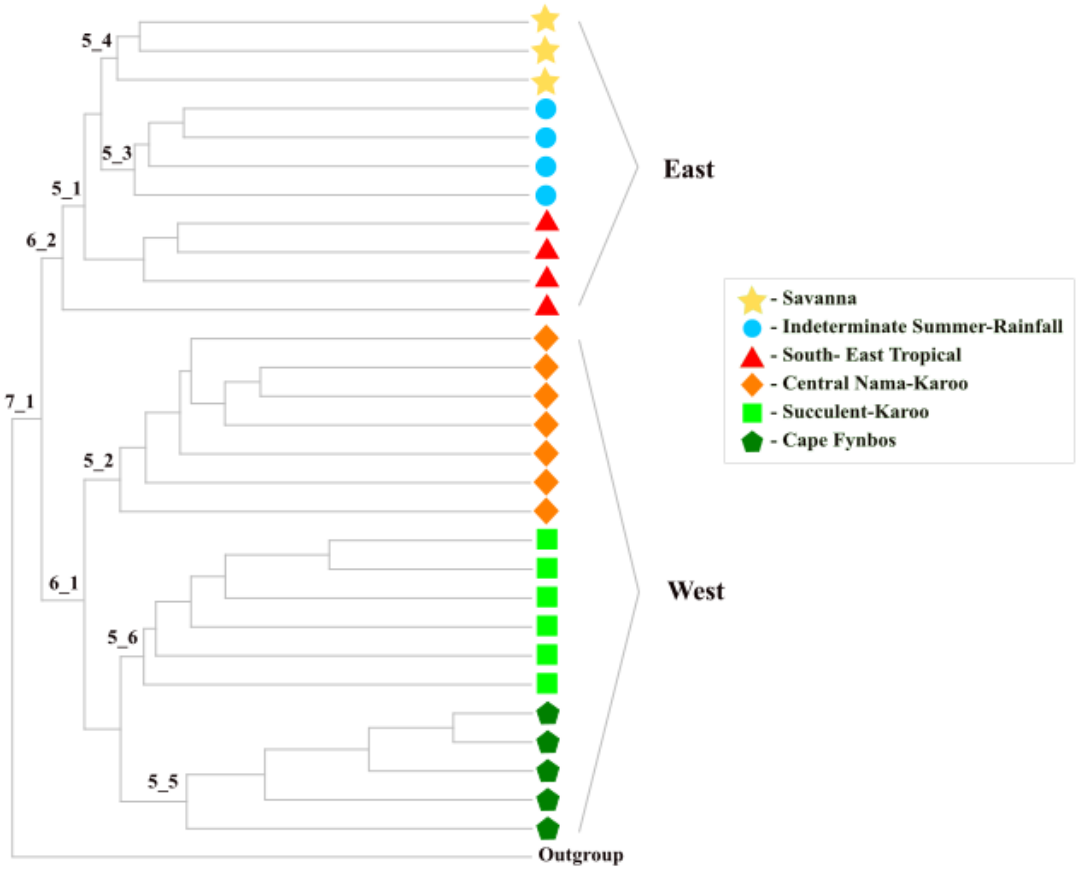

FIGURE 2.4: Phylogram showing the relationships between centres of quarter degree square (QDS) grid cells based on similarity of occurrence for South African orthopteran species (levels 7_1 to 5_6).

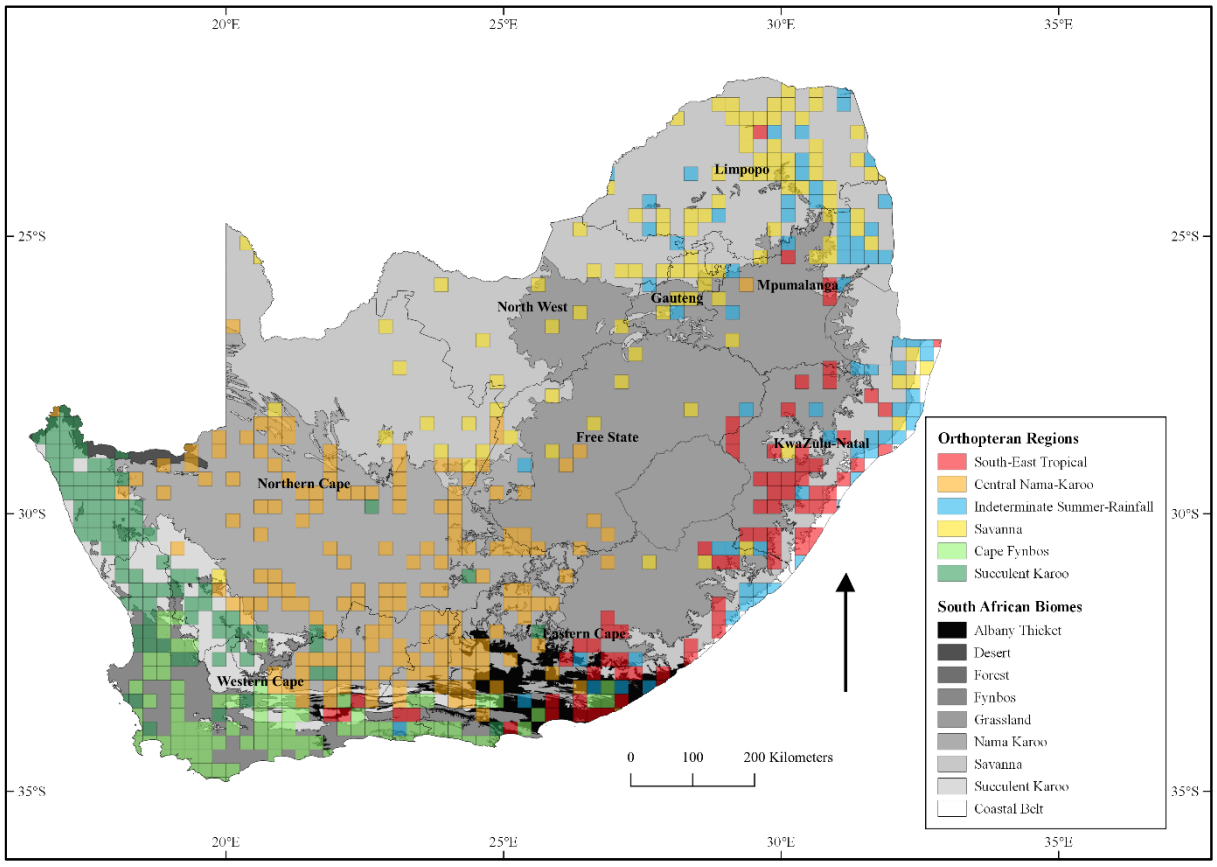

FIGURE 2.5: Clustered quarter degree square (QDS) centres of South African orthopteran species overlaid onto South African biomes (Mucina & Rutherford, 2006).

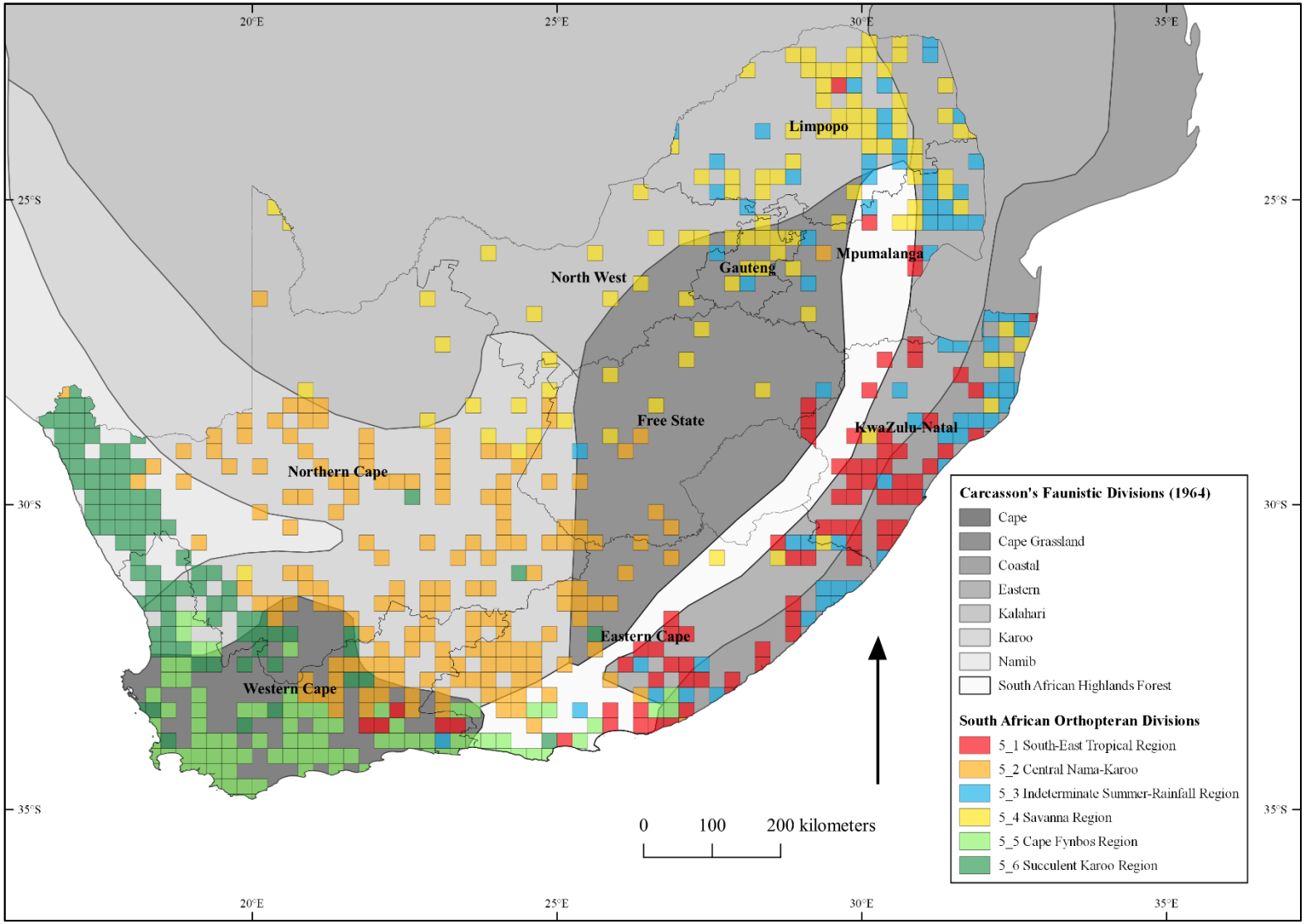

FIGURE 2.6: South African orthopteran centres from this study, overlaid with a map of the faunistic divisions from Carcasson's (1964) biogeographical analysis of African butterflies.

Discussion

Biogeographic patterns of diversity

Two broad biogeographic regions were seen, with a clear primary break between the western winter-rainfall and the eastern summer-rainfall zones (Figure 2.1). This is in broad congruence with Carcasson's (1964) butterflies, Colville's (2009) monkey beetles, Colville *et al*.'s (2014) plants and butterflies, Crowe's (1990) reptiles and Endrödy-Younga's (1978) beetles. Each zone contained three biogeographic centres, in which the western zone showed distinct centres, whereas the eastern zone had less defined centres. Orthopteran species richness was generally evenly distributed across South Africa, with similar counts of families and species represented in each of the identified biogeographic zones and centres (Table 2.2). Orthopteran diversity was however, lower in the eastern zone in comparison to the western zone, even though well-collected areas such as those close to major towns showed comparable grid square diversity between the two zones (Figure 2.3).

The eastern zone contained three summer-rainfall centres: the Savanna, the South-East Tropical and the Indeterminate Summer-Rainfall centres. The Savanna centre had several disjunct cells present within the Grassland biome, as well as within the tropical and summer-rainfall regions found along the east coast (Figure 2.5). The South-East Tropical centre extended into the Cape Fynbos, a distributional pattern that has previously been observed for birds and beetles (Colville *et al*., 2014; Endrödy-Younga, 1978). The Savanna centre had the most familial diversity within the eastern zone; however, the South-East Tropical centre had the highest species count. Two families Rhaphidophoridae and Schizodactylidae were not found in any of the summer-rainfall regions and are restricted to the western winter-rainfall regions of South Africa. In contrast, the family Pamphagodidae was not present in the western regions but rather confined to the eastern summer-rainfall region. The Indeterminate Summer-Rainfall centre was concentrated in the north-eastern and south-eastern regions of South Africa and extended along the coast into the winter-rainfall Cape (Figure 2.5). This geographically large, but less distinctive region is most likely the result of data deficiencies and an increase in sampling efforts could potentially provide better biogeographic resolution. Alternatively, this region may share several faunal links with tropical and subtropical areas further north and therefore remain unresolved (Carcasson, 1964).

The western zone of the primary break also had three distinct centres: the Cape Fynbos, the Succulent-Karoo, and the Central Nama-Karoo centre. The distinctiveness of the centres

retrieved is potentially the result of having higher orthopteran endemism, which has also been identified for other insect groups in this zone, such as monkey beetles (Colville *et al*., 2014), lacewings (Sole *et al*., 2013), heelwalkers (Klass *et al*., 2003), cockroaches (Picker *et al*., 2012), weevils (Colville *et al*., 2014) and bladder grasshoppers (Chapter 3). This zone also showed high levels of orthopteran species and familial richness, both within the Cape Fynbos and Succulent-Karoo centres, which corresponds well for what is known for several other insect groups (Colville *et al*., 2014). The Central Nama-Karoo centre had the highest number of orthopteran records; however, this is attributed to the increase in knowledge of the Karoo region, through a recent national survey project (the Karoo BioGaps Project conducted by SANBI in 2016). The Cape Fynbos region is known for being particularly rich in orthopteran species (Naskrecki & Bazelet, 2009), many being endemic and flightless (Matenaar *et al*., 2014). The Cape Fynbos and the Succulent-Karoo centres are interesting in that they represent an area of faunal transition or overlap. Additionally, results identified the Cape Fynbos centre as the area with the most familial taxa present and the highest species diversity (Table 2.2) providing further evidence that this area is of importance for insect diversification (Chapter 3).

There are several interesting Orthoptera in this region, such as the quantity and high level of diversity of paleo-relictual Pneumoridae (Chapter 3; Dirsh, 1965). A dated phylogeny indicated that the forest species are the first to have diverged, and split from the non-forest species at approximately ± 116.91 MYA (Chapter 3). Therefore, it is suggested that these species may have been more widely distributed at one stage. A similar distribution pattern was identified for forest butterflies and it was suggested that this was the result of forest vegetation being forced to break up and retreat to higher levels (mountainous areas or dispersal further north into Africa) during the hot and dry interpluvial stages (Carcasson, 1964). Furthermore, the grasshopper family Lentulidae is highly concentrated in certain areas of the Cape Fynbos region, and appears to be a Fynbos endemic clade (Matenaar, *et al*., 2018). In contrast, speciose families such as Acrididae and Tettigoniidae appear widespread throughout the country (Picker *et al*., 2004; Song *et al*., 2018). Overall, there are both similarities and differences between the defined centres from this study and that of Carcasson's (1964) butterflies and Colville *et al*. (2014) plants.

Biogeographic consensus with other animals and plants

This study highlighted several significant findings for the biogeographic patterns of orthopteran species, as well as supporting the validity of a GCFR biochorion (Colville *et al*.,

2014). In the western zone, the Cape Fynbos and the Succulent-Karoo centres matched well with the 'Cape Sub-region' of Carcasson's (1964) butterflies, as well as the winter-mesic and aseasonal-mesic subregions of Colville *et al*. (2014) for plants. Similar to the Cape Fynbos centre presented here, these sub-regions extended as far as East London, in which Carcasson (1964) retrieved a strong afrotemperate forest element for butterflies. There was also strong consensus for the Cape Fynbos centre of orthopterans and the Cape Floristic Region (*sensu* Manning & Goldblatt, 2012). This suggests that orthopterans and plants may share a common biogeographical history, and or that orthopterans have evolved specializations towards Fynbos host plants. The Central Nama-Karoo centre seems to be more related to that of the 'Karoo Sub-region' from Carcasson's (1964) map, in addition to the phytogeographical region for arid plants of Jürgens (1997). However, it is possible that if a larger dataset was used, there would likely be a split into a western winter-rainfall group and an eastern summer-rainfall group.

In the eastern zone, the South-East Tropical centre extended into the Cape Fynbos centre, which was also seen for beetles (Endrödy-Younga, 1978). These extensions could be explained through the presence of Cape relictual taxa that had been previously found within the temperate coastal forests and Drakensberg Mountains, causing these radiations to be a younger and more adaptive group (Endrödy-Younga, 1978). The Indeterminate Summer-Rainfall region roughly matches the 'South African Highland Forest' distribution pattern for beetles (Endrödy-Younga, 1978) and the 'Coastal and Eastern' pattern for butterflies (Carcasson, 1964). Carcasson (1964) suggested that the eastern South African butterfly region extends into the northern African tropical and subtropical areas, because of shared faunal links further north into Africa; this is most likely also true for Orthoptera (Otte & Armstrong, 2017). The Savanna centre encompasses both Savanna and Grassland biomes and has potentially separated from the drier grassland areas around the Kalahari. A study on arthropod assemblages (Botha *et al*., 2016) discovered that there are high levels of similarity in parasitoids and pollinators between the Savanna and Grassland biomes, in which the ability of flight was ascribed. Nevertheless, with better sampling efforts, these centres may become more defined and additional centres identified.

Conclusion

Biogeographic patterns are important for conservation because they identify centres of endemism and areas of evolutionary interest. From this study, it is important to note that the Cape Fynbos and the Succulent Karoo biomes are important regions for orthopteran species, because of their familial richness. In addition, there was good spatial congruence found between orthopteran centres and phytogeographic centres within the GCFR. This would suggest that the GCFR may be a biochorion or an area of distinct biogeographic patterns for a range of taxonomic groups. It must be stressed that due to the large number of unidentified species and extremely broad locality information, the final dataset was reduced significantly. In addition, there was clear evidence of under-sampling. A large portion of the South African region has not been surveyed for orthopteran species, which is evident by the number of grid cells (69%) that did not contain any records. As such, even though distributional datasets collated from natural history museums are useful for biogeographical studies, there are recognized limitations when using such data (Graham, *et al*., 2004). Future biogeographic studies for orthopteran species would benefit from using a larger dataset; however, this would require extensive sampling throughout the South Africa region, which in turn will potentially provide additional biogeographic insights and align less distinctive biogeographic patterns observed in this study's summer-rainfall zone.

References

Armstrong, A. J. & Brand, R. F. (2012). Invertebrates on isolated peaks in the uKhahlamba-Drakensberg Park World Heritage Site, South Africa. *Koedoe: African Protected Area Conservation and Science*, *54*(1): #1082, 10 pages. Retrieved from http://dx.doi.org/10.4102/koedoe.v54i1.1082

Bam, A., Addison, P. & Conlong, D. (2020). Acridid ecology in the sugarcane agro-ecosystem in the Zululand region of KwaZulu-Natal, South Africa. *Journal of Orthoptera Research, 29*: 9.

Basset, Y., Cizek, L., Cuénoud, P., Didham, R., *et al*. (2012). Arthropod diversity in a tropical forest. *Science, 338*: 1481–1484.

Bazelet, C. S. & Samways, M. J. (2011). Identifying grasshopper bioindicators for habitat quality assessment of ecological networks. *Ecological Indicators, 11*(5): 1259-1269.

Bazelet, C. S. (2014). Checklist of South African Orthopteran. Database: Animal checklist (Internet). Retrieved from: http://biodiversityadvisor.sanbi.org/research-and-modelling/checklists-and-encyclopaedia-of-life/south-african-animal-checklist/

Bernays, E. A. (1992). Interactions of insects and plants. *Scientific Progress* (Oxford), *76*: 247-271.

Borchert, R. & Slade, N. A. (1981). Bifurcation ratios and the adaptive geometry of trees. *Botanical Gazette, 142*: 394–401.

Born, J., Linder, H. P. & Desmet, P. (2007). The Greater Cape Floristic Region. *Journal of Biogeography, 34*: 147-162.

Botha, M., Siebert, S. J. & van den Berg, J. (2016). Do arthropod assemblages fit the grassland and savanna biomes of South Africa? *South African Journal of Science*, *112*(9/10): #2015-0424, 10 pages. http://dx.doi.org/10.17159/sajs.2016/20150424

Bradshaw, P. L., Colville, J. F. & Linder, H. P. (2015). Optimising regionalisation techniques: Identifying centres of endemism in the extraordinarily endemic-rich Cape Floristic Region. *PLoS ONE*, *10*(7): e0132538. doi:10.1371/journal.pone.0132538

Brijlal, R., Rajak, A., Armstrong, A. J. (2021). Aspects of the life history and ecology of two wingless grasshoppers, *Eremidium armstrongi* and *Eremidium browni* (Lentulidae), at the Doreen Clark Nature Reserve, KwaZulu-Natal, South Africa. *Journal of Orthoptera Research, 30*(1): 73-80.

Brown, H. D. (1962). New and Interesting Grasshoppers from Southern Africa – 2 (Orthoptera: Acridoidea). *Journal of Entomological Society of Southern Africa, 25*(1): 1-19.

Carcasson, R. H. (1964). A preliminary survey of the zoogeography of the African butterflies. *East African Wildlife Journal*, *2*: 122–57.

Cardoso, P., Barton, P. S., Birkhofer, K., Chichorro, F., Deacon, C., Fartmann, T., …. Samways, M. J. (2020). Scientists' warning to humanity on insect extinctions. *Biological Conservation, 242*, 108426. https://doi.org/10.1016/j.biocon.2020.108426

Colville, J. F. (2009). Understanding the evolutionary radiation of the mega-diverse Monkey Beetle fauna (Scarabaeidae: Hopliini) of South Africa. *Frontiers of Biogeography, 1*: 56-60.

Colville, J. F., Potts, A. J., Bradshaw, P. L., Measey, G. J., Snijman, D., Picker, M. D., Procheş, S., Bowie, R. C. K. & Manning, J. C. (2014). Floristic and faunal Cape biochoria: do they exist? In N. Allsopp, J. F. Colville, G. A. Verboom (Eds.), *Fynbos: Ecology, Evolution and Conservation of a Megadiverse Region*, (pp. 73-92). Oxford: University Press United Kingdom.

Cowling, R. M., Knight, A. T., Faith, D. P., Ferrier, S., Lombard, A. D., Rouget, M., Maze, K. & Desmet, P. (2004). Nature conservation requires more than a passion for species. *Conservation Biology*, *18*: 1674-1676.

Crovello, T. J. (1981). Quantitative biogeography: an overview. *Taxon, 30*: 563-575.

Crowe, T. M. (1990). A quantitative analysis of patterns of distribution, species richness and endemism in southern African vertebrates. In G. Peters and R. Hutterer (Eds.) *Vertebrates in the tropics*, (pp. 145–59). Bonn: Museum Alexander Koenig.

Crutsinger, G. M., Collins, M. D., Fordyce, J. A., Gompert, Z., Nice, C. C. & Sanders, N. J. (2006). Plant Genotypic Diversity Predicts Community Structure and Governs an Ecosystem Process. *Science, 313*: 966-968.

de Klerk, H. M., Crowe, T. M., Fjeldsa, J. & Burgess, N. D. (2002). Biogeographical patterns of endemic terrestrial Afrotropical birds. *Diversity and Distributions*, *8*: 147–62.

Dirsh, V. M. (1965). Revision of the family Pneumoridae (Orthoptera: Acridoidea). *Bulletin of the British Museum* (*Natural History*) *Entomology*, *15*: 325-96.

Eades, D. C., Otte, D., Cigliano, M. M. & Braun, H. (2013). Orthoptera Species File, version 5.0/5.0. Retrieved from: http://Orthoptera.SpeciesFile.org

Eades, D. C., Otte, D., Cigliano, M. M. & Braun, H. (2014). Orthoptera Species File, version 5.0/5.0. Retrieved from: http://Orthoptera.SpeciesFile.org

Edwards, D. & Leistner, A. O. (1971). A degree reference system for citing biological records in southern Africa. *Mitteilungen der Botanischen Staatssammlung*, München, *10*: 501-509.

Endrödy-Younga, S. (1978). Coleoptera. Monographiae *Biologicae*, 797–821. doi:10.1007/978-94-009-9951-0_26

Gess, F. W. & Gess, S. K. (1993). Effects of increasing land utilization on species representation and diversity of aculeate wasps and bees in the semi-arid areas of southern Africa. In J. LaSalle & I. D. Gauld (Eds.). *Hymenoptera and Biodiversity*, (pp. 83-113). United Kingdom: Wallingford.

Gonzalez-Orozco, C. E., Laffan, S. W., Knerr, N. & Miller, J. T. (2013). A biogeographical regionalization of Australian Acacia species. *Journal of Biogeography*, *40*: 2156–2166. doi: 10.1111/jbi.12153

Graham, C. H., Ferrier, S., Huettman, F., Moritz, C. & Petersen, A. T. (2004). New developments in museum-based informatics and applications in biodiversity analysis. *Trends in Ecology and Evolution*, *19*: 497-603.

Grimaldi, D. & Engel, M. S. (2005). Evolution of the Insects. Cambridge University Press, New York.

Haddad, N. M., Tilman, D., Haarstad, J., Ritchie, M. & Knops, J. M. H. (2001). Contrasting effects of plant richness and composition on insect communities: a field experiment. *The American Naturalist*, *158*(1): 17-35.

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D. & de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, *12*(10): e0185809. https://doi.org/10.1371/journal.pone.0185809

Hausdorf, B. (2002). Units in biogeography. *Systematic Biology*, *51*, 648–52.

Hawkins, B. A. & Porter, E. E. (2003). Does herbivore diversity depend on plant diversity? The case of California butterflies. *The American Naturalist*, *161*(1): 40–49. doi:10.1086/345479

Herrel, A., Measey, G. J., Vanhooydonck, B. & Tolley, K. A. (2011). Functional consequences of morphological differentiation between populations of the Cape Dwarf Chameleon (*Bradypodion pumilum*). *Biological Journal of the Linnean Society of London*, *104*: 692-700.

IUCN Red List of Threatened Species. (c1964). [Internet]. International Union for Conservation of Nature. Retrieved from: www.iucnredlist.org

Johnson, M. T. J., Lajeunesse, M. J. & Agrawal, A. A. (2006). Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. *Ecology Letters*, *9*: 24-34.

Jurasinski, G. (2012). 'simba': A collection of functions for similarity analysis of vegetation data. (R package, version 0.3-5). Retrieved from: https://CRAN.R-project.org/package=simba

Jürgens, N. (1997). Floristic biodiversity and history of African arid regions. *Biodiversity and Conservation, 6*: 495-514.

Kambule, I. N. (2010). Investigation of egg development in the brown locust, *Locustana pardalina* (Walk.) (Orthoptera: Acrididae) (Master's dissertation, University of Witwatersrand, Johannesburg, South Africa). Retrieved from https://wiredspace.wits.ac.za/bitstream/handle/10539/8276/MSC%20THESIS%202009%20EGG%20DEVELOPMENTcorr%20final.pdf?sequence=1&isAllowed=y

Klass, K. D., Picker, M. D., Damgaard, J., van Noort, S. & Tojo, K. (2003). The taxonomy, genitalic morphology, and phylogenetic relationships of southern African Mantophasmatodea (Insecta). *Entomologische Abhandlungen, 61*: 3–67.

Koricheva, J., Mulder, C. P. H., Schmid, B., Joshi, J. & Huss-Danell, K. (2000). Numerical responses of different trophic groups of invertebrates to manipulations of plant diversity in grasslands. *Oecologia*, *125*: 271-282.

Kreft, H. & Jetz, W. (2010). A framework for delineating biogeographical regions based on species distributions. *Journal of Biogeography*, *37*: 2029–2053.

Leal, C. R. O., Silva, J. O., Sousa-Souto, L. & Nerves, F. S. (2016). Vegetation structure determines insect herbivore diversity in seasonally dry tropical forests. *Journal of Insect Conservation, 20*: 979-988.

Lenormand, M., Papuga, G., Argagnon, O., Soubeyrand, M., de Barros, G., Alleume, S. & Luque, S. (2019). Biogeographical network analysis of plant species distribution in the Mediterranean region. *Ecology and Evolution*, *9*: 237-250.

Lewinsohn, T. M., Prado, P., Jordano, P., Bascompte, J. & Olesen, J. (2006). Structure in plant-animal interaction assemblages. *Oikos*, *113*(1): 174-184. doi: 10.1111/j.0030-1299.2006.14583.x

Linder, H. P. & Mann, D. M. (1998). The phylogeny and biogeography of *Thamnochortus* (Restionaceae). *Botanical Journal of the Linnean Society, 128*: 319-357.

Linder, H. P., de Klerk, H. M., Born, J., Burgess, N. D., Fjeldså, J. & Rahbek, C. (2012). The partitioning of Africa: statistically defined biogeographical regions in sub-Saharan Africa. *Journal of Biogeography, 39*(7): 1189-1205.

Linder, P. (2001). On areas of endemism, with an example from the African Restionaceae. *Systematic biology, 50*: 892-912.

Lister, B. C. & Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proceedings of the National Academy of Science of the United States of America*, *115*(44): E10397-E10406. Retrieved from: www.pnas.org/cgi/doi/10.1073/pnas.172247711

Lomer, C. J., Bateman, R. P., Johnson, D. L., Langewald, J. & Thomas, M. (2001). Biological control of locusts and grasshoppers. *Annual Review of Entomology*, *46*: 667-702.

Manning, J. C. & Goldblatt, P. (Eds.). (2012). *Plants of the Greater Cape Floristic Region I: The core Cape flora*. Pretoria: South African National Biodiversity Institute. (Strelitzia 29).

Matenaar, D., Bazelet, C. S. & Hochkirch, A. (2015). Simple tools for the evaluation of protected areas for the conservation of grasshoppers. *Biological Conservation*, *192*: 192–199. doi.org/10.1016/j.biocon.2015.09.023

Matenaar, D., Broder, L., Bazelet, C. S. & Hochkirch, A. (2014). Persisting in a windy habitat: population ecology and behavioral adaptations of two endemic grasshopper species in the Cape region (South Africa). *Journal of Insect Conservation*, *18*: 447-456.

Matenaar, D., Fingerle, M., Heym, E., Wirtz, S. & Hochkirch, A. (2018). Phylogeography of the endemic grasshopper genus *Betiscoides* (Lentulidae) in the South African Cape Floristic Region. *Molecular Phylogenetics and Evolution*, *118*: 318-329.

Melin, A. & Colville, J. F. (2019). A review of 250 years of South African bee taxonomy and exploration (Hymenoptera: Apoidea: Anthophila). *Transactions of the Royal Society of South Africa*, *74*: 86–96. https://doi.org/10.1080/0035919X.2019.1572670

Moline, P. M. & Linder, H. P. (2006). Input data analytical methods and biogeography of elegia (Restionaceae). *Journal of Biogeography*, *33*: 47-62.

Mucina, L. & Rutherford, M. C. (Eds.). (2006). The vegetation of South Africa, Lesotho and Swaziland. Pretoria: South African National Biodiversity Institute. (Strelitzia, vol. 19).

Naskrecki, P. & Bazelet, C. S. (2009). A species radiation among South African flightless spring katydids (Orthoptera: Tettigoniidae: Phaneropterinae: Brinckiella Chopard). *Zootaxa*, *2056*: 46-62.

Novotny, V. & Basset, Y. (2005). Host specificity of insect herbivores in tropical forests. *Proceedings of the Royal Society of London*. *Series B, Biological Sciences*, *272*: 1083–1090.

Olivero, J., Marquez, A. L. & Real, R. (2012). Integrating fuzzy logic and statistics to improve the reliable delimitation of biogeographic regions and transition zones. *Systematic Biology*, *62*: 1–21.

Otte, D. & Armstrong, A. (2017). Additional *Eremidium* species from Eastern Cape Province and KwaZulu-Natal Province in South Africa and a new genus, *Zulutettix* (Acridoidea: Lentulidae). *Transactions of the American Entomological Society*, *143*(2): 305-433. doi: 10.3157/061.143.0211

Paradis, E. & Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, *35*: 526-528.

Picker, M., Griffiths, C. & Weaving, A. (2004). Orthoptera: crickets, katydids, grasshoppers & locusts. In J. Hromnik (Ed.), *Field guide to insects of South Africa*, (pp. 74-107). Cape Town: Struik Publishers.

Poynton, J. C. (1964). Amphibia of southern Africa: a faunal study. *Annals of the Natal Museum*, *17*: 1-334.

Procheş, Ş. & Cowling, R. M. (2006). Insect diversity in Cape fynbos and neighbouring South African vegetation. *Global Ecology and Biogeography*, *15*: 445-451.

Procheş, Ş. & Cowling, R. M. (2007). Do insect distributions fit our biomes? *South African Journal of Science, 103*: 258-261.

QGIS Development Team (2019). Quantum Geographic Information System Software. Version 3. Retrieved from: https://qgis.org/en/site/

R Core Team. (2020). R: A language and environment for statistical computing. R Foundation for statistical computing. Vienna, Austria. Retrieved from: https://www.R-project.org/

R Development Core Team. (2012). R: A language and environment for statistical computing. Version 2.15.0. R Foundation for Statistical Computing Vienna Austria.

Revell, L. J. (2012). Phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, *3*: 217–23.

Ricklefs, R. E. & Jenkins, D. G. (2011). Biogeography and Ecology: towards the integration of two disciplines. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *366*: 2438-2448.

Rodrigues, P., Figueira, R., Vaz Pinto, P., Araujo, M. B. & Beja, P. (2015). A biogeographical regionalisation of Angolan mammals. *Mammal Review, 45*: 103-116.

Sanmartín, I. (2012). Historical biogeography: evolution in time and space. *Evolution: Education and Outreach, 5*: 555-568.

Sauberer, N., Zulka, K. P., Abensperg-Traun, M., Berg, H. M., Bieringer, G., Milasowszky, N., Moser, D., Plutzar, C., Pollheimer, M., Storch, C., Trostl, R., Zechmeister, H. & Grabherr, G. (2004). Surrogate taxa for biodiversity in agricultural landscapes of eastern Austria. *Biological Conservation, 117*:181-190. doi: 10.1016/S0006-3207(03)00291-X

Scholtz, C., Scholtz, J. & de Klerk, H. (2021). *Pollinators, Predators & Parasites: The ecological role of insects in southern Africa*. In M. Mössmer (Ed.), Cape Town: Struik Nature.

Seymour, C. L., de Klerk, H. M., Channing, A. & Crowe, T. M. (2001). The biogeography of the Anura of sub-equatorial Africa and the prioritisation of areas for their conservation. *Biodiversity and Conservation*, *10*: 2045-2076.

Shi, G. R. (1993). A comparative study of 39 binary similarity coefficients. *Memoirs of the Association of Australasian Palaeontologists*, *15*: 329–41.

Siemann, E. (1998). Experimental tests of effects of plant productivity and diversity on grassland arthropod diversity. *Ecology*, *79*: 2057-2070.

Siemann, E., Tilman, D., Haarstad, J. & Ritchie, M. (1998). Experimental Tests of the Dependence of Arthropod Diversity on Plant Diversity. *The American Naturalist*, *152*(5): 738-750.

Sokal, R. R. & Michener, C. D. (1958). A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin*, *38*: 1409–38.

Sole, C. L., Scholtz, C. H., Ball, J. B. & Mansell, M. W. (2013). Phylogeny and biogeography of southern African spoon-winged lacewings (Neuroptera: Nemopteridae: Nemopterinae). *Molecular phylogenetics and evolution*, *66*: 360-368.

Song, H., Amédégnato, C., Cigliano, M. M., Desutter-Grandcolas, L., Heads, S. W., Huang, Y., Otte, D. & Whiting, M. F. (2015). 300 million years of diversification: elucidating the patterns of orthopteran evolution on comprehensive taxon and gene sampling. *Cladistics*, *31*: 621-651. doi:10.1111/cla.12116

Song, H., Mariño-Pérez, R., Woller, D. A. & Cigliano, M. M. (2018). Evolution, diversification and biogeography grasshoppers (Orthoptera: Acrididae). *Insect Systematics and Diversity*, *2*(4): 1-25.

Steck, C. E., Burgi, M., Coch, T. & Duelli, P. (2007). Hotspots and richness pattern of grasshopper species in cultural landscapes. *Biodiversity and Conservation*, *16*: 2075-2086. doi:10.1007/s10531-006-9089-7

Strahler, A. N. (1957). Quantitative analysis of watershed geomorphology. *Transactions, American Geophysical Union*, *38*: 913–20.

Todd, M. C., Washington, R., Cheke, R. A. and Kniveton, D. (2002). Brown locust outbreaks and climate variability in southern Africa. *Journal of Applied Ecology*, *39*(1): 31–42.

Tolley, K. A., Burger, M., Turner, A. A. & Matthee, C. A. (2006). Biogeographic patterns and phylogeography of dwarf chameleons (*Bradypodion*) in an African biodiversity hotspot. *Molecular Ecology*, *15*: 781-793.

Vernon, C. J. (1999). Biogeography, endemism, and diversity of animals in the karoo. In W. R. J. Dean and S. J. Milton (Eds.). *The karoo: ecological pattern and processes*, (pp. 57-8). Cambridge: Cambridge University Press, Cambridge.

Wright, M. G. & Samways, M. J. (1998). Insect species richness tracking plant species richness in a diverse flora: gall-insects in the Cape Floristic Region, South Africa. *Oecologia*, *115*: 427-433.

Zhang, K., Lin, S., Ji, Y., Yang, C., Wang, X., Yang, C., Wang, H., Jiang, H., Harrison, R. D., & Yu, D. W. (2016). Plant diversity accurately predicts insect diversity in two tropical landscapes. *Molecular Ecology*, *25*(17): 4407-4419.

Chapter 3: Genetic and evolutionary insights into bladder grasshoppers (Orthoptera: Pneumoridae)

Abstract

The orthopteran family Pneumoridae is a paleo-relictual insect group endemic to the African continent, with all 14 species found along the coastal regions of southern Africa. The majority of these species are found within the Greater Cape Floristic Region, with only three species extending into East Africa, as far north as Uganda. Pneumorids are thought to be a evolutionary distinct family due to the lineage retaining relatively simple hearing organs, as well as possessing the primitive form of wing venation. A phylogenetic study and estimated time of divergence for this family has not been previously attempted and thus the intrafamilial evolutionary patterns are unknown. In this chapter, 10 of the 14 Pneumoridae species were barcoded using the mitochondrial gene region COI, in conjunction with the two nuclear gene regions, ITS and 18S, in order to investigate the taxonomic and evolutionary relationships among species. In addition, a BEAST analysis was conducted to determine the time at which species originated and diverged. Results showed that bladder grasshoppers diverged from other orthopteran species during the Cretaceous period at an estimated 134.70 MYA. The first split within the family was between the forest and non-forest species at approximately 116.91 MYA. The subsequent clades to have diverged from the forest clade were predominantly the Succulent Karoo species, then the Fynbos species and finally the Savanna species, which were the most recently diverged species. It is suggested that bladder grasshoppers may have originated in South Africa and then dispersed northwards into Africa. In addition, phylogenetic analyses identified taxonomic discrepancies, with the genus *Physemacris* falling within the *Bullacris* clade and *B. unicolor* falling outside of the main *Bullacris* clade, and *B. membracioides* and *B. intermedia* being monophyletic, with only a 5% mitochondrial divergence. More intensive sampling to include additional species and populations is required to further resolve the evolutionary relationships within Pneumoridae and deal with taxonomic inconsistencies.

Introduction

The family Pneumoridae (Thunberg, 1810), more commonly known as bladder grasshoppers (Figure 3.1), is endemic to Africa, with almost all species restricted to the southernmost region of the continent (Dirsh, 1965). The family consists of six genera and 14 known species that are mainly found along the coastal areas of southern Africa, with 10 species found within the Greater Cape Floristic Region (Born *et al*., 2007; Snijman, 2013) (Figure 3.2). Apart from a handful of records of a single genus *Physophorina* (containing two species), occurring in Uganda, Tanzania, Malawi and Mozambique, as well as a single record each for *Pneumora inanis* and *Bullacris membracioides* from Tanzania and Malawi respectively, all other species are believed to be endemic to South Africa (Dirsh, 1965).

Bladder grasshoppers are sexually dimorphic with males possessing an inflated abdomen, whereas females do not. The males also have macropterous flight wings, while the females have micropterous or brachypterous wings and are unable to fly. In males, the unique balloon-like abdomen acts as a resonating chamber for sound production and *Bullacris membracioides* is known to produce an acoustic call that can be detectable by conspecifics up to distances of 1.9 km (van Staaden & Römer, 1997). Pneumorids are thought to be an evolutionary distinct family due to the absence within lineages of species with classical ears, and that extant species have retained this ancient form of hearing, which represents the transitional stage of evolution in insect ears (van Staaden and Römer, 1998). Furthermore, these species have maintained the primitive pattern of wing venation, which has been observed in the primary males (Ragge, 1963; Rehn, 1941; Smart, 1953).

In 1999 the first modern molecular phylogeny for the order of Orthoptera was produced, making use of two mitochondrial and one nuclear DNA marker, based on 31 taxa that represented all major lineages (Flook *et al*., 1999). This study concluded that the superfamily Pneumoroidea consists of one family, as opposed to the three families conceived by Dirsh (1975). More recent studies have made use of the complete mitochondrial genome, as well as four nuclear loci for 36 of the 40 families, representing all 15 currently recognized super-families of Orthoptera (Song *et al*., 2015). Together with the use of fossil calibrations, divergence times were estimated for the major orthopteran lineages, and Pneumoroidea, represented by a single representative of *Physemacris variolosa* (Linnaeus, 1758), was discovered to have originated within the Mesozoic era, at an estimated 116.97 million years ago (MYA) (Song *et al*., 2015). However, a phylogenetic study and estimation of species

divergence within the family has not been previously attempted and thus the intrafamilial evolutionary patterns are currently unknown.

All species of Pneumoridae are found in South Africa, either entirely or partially, with the highest radiation of Pneumoridae species found within the Greater Cape Floristic Region (hereafter referred to as the Greater Cape). This region consists of two globally biodiverse plant hotspots and is known to have one of the highest levels of plant species richness and endemism in the world (Kreft & Jetz, 2007; Goldblatt & Manning, 2002; Snijman, 2013). The plants of this region show divergences dating back to approximately 64 - 71 MYA (Linder, 2003), and very recent and rapid divergences occurring within the past 3.8 - 8.7 MYA (Klak *et al*., 2004; Verboom, *et al*., 2009). The divergences of species and high levels of endemism are thought to be the direct result of long-term climate stability (Colville *et al*., 2020); the current winter-rainfall and summer arid climatic characteristics of the Greater Cape, which are thought to have initiated around the end of the Miocene (Diekmann *et al*., 2003; Kottek, *et al*., 2006; Linder, 2003; Zachos, *et al*., 2001). In addition, the presence of remarkably predictable winter-rainfall, when interrupted by occasional drought, provides opportunities for plant diversification as a result of fragmentation and generation turnover (Snijman, 2013).

The Greater Cape is also recognized as a zoogeographical region for insects (Chapter 2). It is known as a centre of radiation for several insect groups and harbours many paleo-relictual insects of ancient Gondwanan lineages (see Colville *et al*., 2014). Available insect phylogenetic data suggests that the Fynbos component of the Greater Cape has many basal lineages. For example, some of the earliest diversifying bee lineages are found in the winter-rainfall regions of the Greater Cape (Kuhlmann, 2009). There also appears to be a pattern of radiations out of the Fynbos into neighbouring biomes, such as the Succulent Karoo of the Greater Cape, or further northwards (Born, *et al*., 2007; Naskrecki & Bazelet, 2009; Predel *et al*., 2012; Procheş & Cowling, 2006; Sole *et al*., 2013; Switala *et al*., 2014; Verboom *et al*., 2003). In addition, there is prominent phylogeographic structure of invertebrates, indicating isolated populations within the Greater Cape since the Early Pliocene. For example, in flies (de Jager & Ellies, 2013) and velvet worms (McDonald & Daniels, 2012), as well as since the Pleistocene, as seen in beetles (Pitzalis & Bologna, 2010) and cicadas (Price *et al*., 2007). Other animal taxa, such as reptiles, also have radiations dating back to the Middle to Late Miocene; e.g., the southern Rock agama (Matthee & Flemming, 2002), the southern African gecko (Bauer & Lamb, 2005), the Cape fossorial skink (Daniels *et al*., 2009) and southern African chameleons (Tolley *et al*., 2008). Furthermore, amphibians and birds found within the Greater Cape have also been observed to have ancient lineages (Tolley, *et al*., 2014).

Understanding the radiations of the bladder grasshoppers within South Africa and particularly within the Greater Cape, would provide important insights into the evolution of the insect fauna in this region, particularly for a faunal group of such ancient lineage. In addition, there are several inaccuracies and misidentifications that are recognized in pneumorid taxonomy (Chapter 1). These attributes therefore make bladder grasshoppers suitable for a detailed phylogenetic assessment that would provide a deeper understanding into the evolutionary processes and species divergence events within the family. In this study, members from the Pneumoridae family will be barcoded using the mitochondrial *cytochrome c oxidase* subunit I (COI), as well as two additional nuclear gene regions (ITS and 18S) in order to investigate the taxonomic and evolutionary relationships between species. Analysis is also conducted to determine the time at which these species originated and diverged, and to place this in the context of landscape evolution of the Greater Cape.

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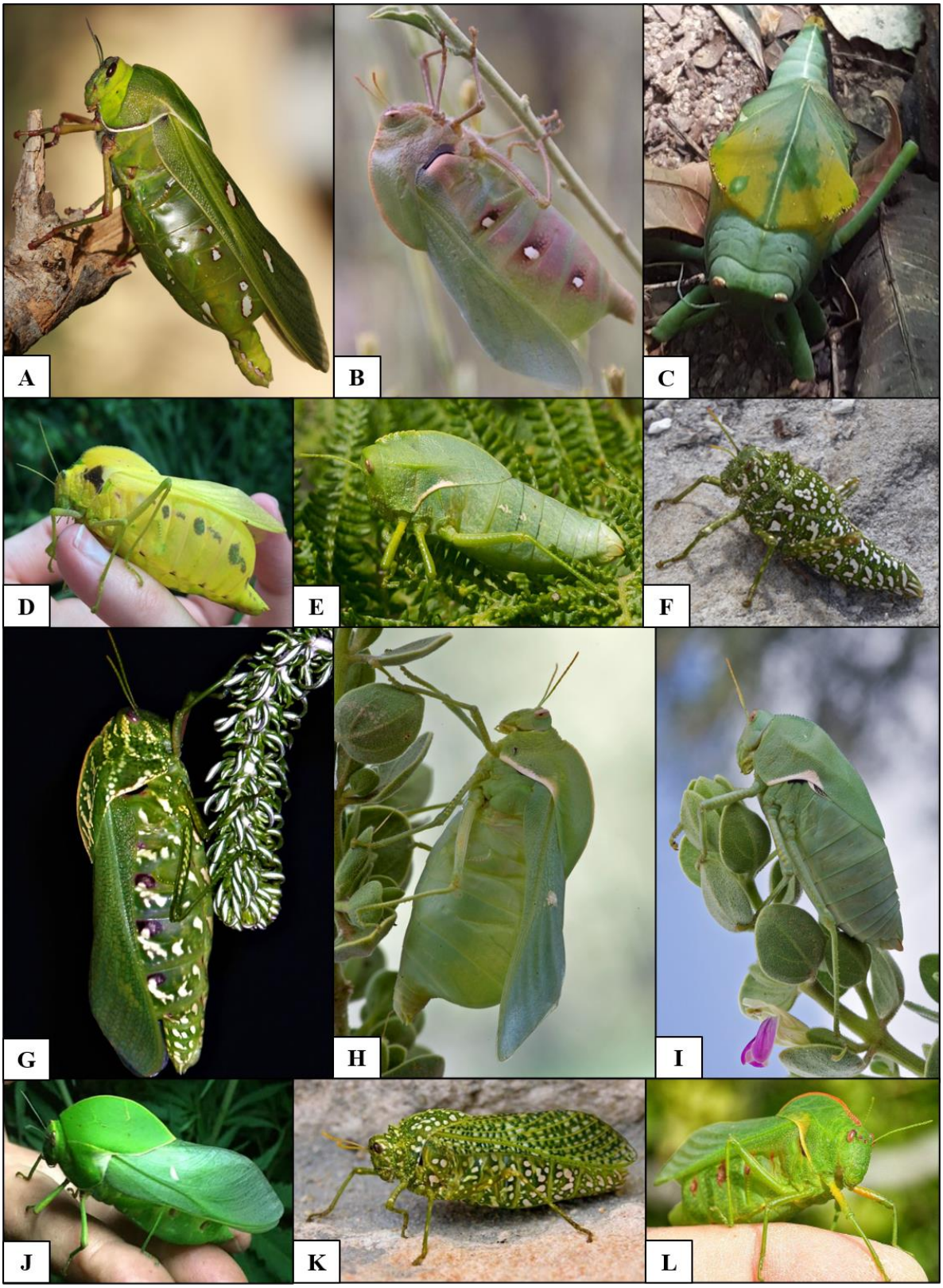

FIGURE 3.1*:* Representatives of some Pneumoridae species; (A): *Pneumora inanis* male (Image by: Noel Potgieter), (B): *Bullacris unicolor* male (Image by: Frank Gaude), (C): *Physophorina miranda* female (Image by: Madelein Zaayman), (D): *Bullacris membracioides* male (Image by: Suncana Bradley), (E): *Bullacris discolor* female (Image by: Chris Whitehouse), (F): *Physemacris variolosa* female (Image by: Corinne Merry), (G): *Bullacris serrata* male (Image by: Vanessa Couldridge), (H): *Bullacris boschimana* male (Image by: Piotr Naskrecki), (I): *Bullacris boschimana* female (Image by: Piotr Naskrecki), (J): *Physophorina miranda* male (Image by: Rowan Forbes), (K): *Physemacris variolosa* male (Image by: Chris Whitehouse), (L): *Bullacris discolor* male (Image by: Darryl Lampert).

Materials and Methods

Taxon sampling and distribution

A species distribution map of all pneumorid species for South Africa was created using the digitized and georeferenced localities from personally collected specimens as well as museum collections and online databases, namely; the Iziko South African Museum, Ditsong National Museum of Natural Sciences, British Museum, Rhodes University, National Museum of Bloemfontein, Durban Natural Science Museum, National Collection of Insects- ARC, Stellenbosch University, and iSpot Nature. Distribution maps were created in QGIS 3.10.1 (QGIS Development Team, 2019), together with an underlaying representation of the South African biomes (Mucina and Rutherford, 2006).

Specimens for genetic analysis were collected in the field during the austral spring and summer seasons, in the Northern, Western, and Eastern Cape, and KwaZulu-Natal provinces of South Africa. Two specimens were also collected from Tanzania. For outgroups, two species of Pamphagidae, *Hoplolopha* (Stål, 1876) and *Lamarckiana* (Kirby, 1910) from South Africa were collected.

Ten of the 14 valid species belonging to the family Pneumoridae were used in this study (Table 3.1). Genetic samples of the remaining four species could not be obtained, which is due to both the rarity of some species and the difficulty in locating highly cryptic individuals in the field, as well as the severe degradation of the genetic material of museum specimens. However, all but one of the six genera are represented. The only genus not included is the monotypic genus *Prostalia*, which has not been collected for several decades and is known only from a handful of museum specimens.

TABLE 3.1: A list of all known pneumorid species and those from which sequences were successfully obtained and used in this study, or not obtained (shaded in grey).

No.	Species name	No. of samples	Localities	Biome	Province/ Country
1	*Bullacris boschimana*	0	-	-	-
2	*Bullacris unicolor*	6	Kamieskroon	Succulent Karoo	Northern Cape
			Cape Point, Cape Town	Fynbos	Western Cape
			Belville	Fynbos	Western Cape
			West Coast National Park	Fynbos	Western Cape
3	*Bullacris obliqua*	6	Groenriviersmond	Succulent Karoo	Northern Cape
			West Coast National Park	Fynbos	Western Cape
			Lambert's Bay	Fynbos	Western Cape
4	*Bullacris discolor*	4	Port Elizabeth	Albany Thicket	Eastern Cape
			Cape Town	Fynbos	Western Cape
			Jonkersberg Mountain	Fynbos	Western Cape
			Outeniqua Nature Reserve	Fynbos	Western Cape
5	*Bullacris serrata*	3	Grahamstown	Fynbos	Eastern Cape
6	*Bullacris intermedia*	3	The Haven	Grassland	Eastern Cape
			Silaka Nature Reserve	Grassland	Eastern Cape
7	*Bullacris membracioides*	4	Durban	Indian Ocean Coastal Belt	KwaZulu-Natal
			Inchanga		KwaZulu-Natal
8	*Peringueyacris namaqua*	5	Spektakel Pass	Succulent Karoo	Northern Cape
			Kamieskroon		Northern Cape
9	*Physemacris variolosa*	7	St Francis Bay	Fynbos	Eastern Cape
			Simonskloof, Montagu	Fynbos	Western Cape
			Wilderness National Park	Fynbos	Western Cape
			Cape Town	Fynbos	Western Cape
			Betty's Bay	Fynbos	Western Cape
10	*Physemacris papillosa*	0	-	-	-
11	*Pneumora inanis*	2	Grahamstown	Forest	Eastern Cape
			Bulwer	Forest	KwaZulu-Natal
12	*Prostalia granulata*	0	-	-	-
13	*Physophorina miranda*	0	-	-	-
14	*Physophorina livingstoni*	2	Matemanga	Forest	Tanzania

DNA extraction, amplification and sequencing

One mitochondrial and two nuclear DNA markers were used in this study. These included the mitochondrial marker *cytochrome c oxidase* subunit I (COI: LCO1490 and HCO2198; Folmer *et al*., 1994), and nuclear markers; inter-transcribed spacer (ITS: ITS-F and ITS-R; Roy *et al*., 2008) and 18S (18-F and 18S-R; Song *et al*., 2015). Internal primers for each of the primers was personally created, using Geneious v 7.1.3 (Kearse *et al*., 2012). A detailed representation of the primers is listed in Table 3.2.

TABLE 3.2: A list of primers that were used in this study. Internal primers (e.g., ITSa-F) were created using Geneious v7.1.3.

Primer Name	Primer Sequence
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198	5'-TAAACTTCAGGGTGAGGGTGACCAAAAAATCA-3'
ITS-F	5'-AGAGGAAGTAAAAGTCGTAACAAGG-3'
ITS-R	5'-CCTTAGTAATATGCTTAAATTCAGG-3'
18S-F	5'-TGCTTGTCTCAAAGATTAAGC-3'
18S-R	5'-GCATCACAGACCTGTTATTGC-3'
COIa-F	5'- WCCATTAATRATTGGAGCACCA3'
COIb-R	5'-RATDGGGTCACCYCCTCCTGC-3'
ITSa-F	5'-ACCGACTGCATATCCGAACG-3'
ITSb-R	5'-CTGCGTTCTTCATCGACCCA-3'
18Sa-F	5'-GATCGCACGGTCTCTGTACC-3'
18Sb-R	5'-CCTCGACACTCGGTGAAGAG-3'

Genomic DNA was isolated from ethanol preserved whole specimens. A salt extraction protocol (Aljanabi & Martinez, 1997) was used on the hind-leg of recent specimens, whereas phenol-chloroform extractions were used on degraded specimens. Gene fragments were amplified in 25 µL reaction volumes containing 60-100 ng/ µL genomic DNA, 10 µL double distilled H_2O, 1.25 µL of each forward and reverse primer, and 12.5 µL of 2G Robust HotStart ReadyMix (KM5701- KAPA Biosystems) enzyme. The polymerase chain reaction (PCR) profile for the 18S gene had an initial denaturation step at 94 °C for 2 min, a 35-cycle amplification (94 °C for 30 secs, 52.6 °C for 30 secs, and 72 °C for 1 min 45 secs) with a final extension step of 72 °C for 3 min and the final hold at 15 °C. The PCR protocol 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 the final hold at 15 °C. Lastly, 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 secs, 49 °C for 45 secs, 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. Successfully amplified samples were sent to Macrogen Inc. (Amsterdam, Netherlands) for sequencing.

Phylogenetic analyses

Sequences were aligned in Geneious v. 7.1.3 (Kearse *et al*., 2012) and screened for base ambiguities and stop codons. A total of 44 sequences (including the outgroup species) were amplified for 18S (501 bp), 42 were sequenced for COI (578 bp), and 38 for ITS (492 bp). Mitochondrial sequences were translated into amino acids using Geneious v. 7.1.3 translation options, with the genetic code set to invertebrate mitochondrial and Frame 1 was selected. Pairwise distance analysis was calculated in MEGA v. 7 (Kumar, *et al*., 2016) for the COI mitochondrial gene, by making use of the Kimaru 2-parameter model to estimate the rate of sequence divergence. A nucleotide substitution model was selected, that included transitions and transversions. In addition, uniform rates were applied among sites, with a pairwise deletion treatment for gaps / missing data.

Phylogenetic trees were constructed in both Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks (RAxML-VI-HPC, Stamatakis, 2006; Mr Bayes, Ronquist *et al*., 2012) for the mitochondrial and nuclear genome combined dataset. Both analyses were run using the desktop interface. For BI, four Markov chains were run in parallel, with each chain starting from random trees, running for 10 million generations, sampling every 1 000 generations. The analyses were run according to the substitution models and partitioning scheme inferred by jModelTest v2.1.7 (Diarriba *et al*., 2012); 18S: JC; COI: TIM2+G and ITS F81+G. A total of 25% was discarded as burn-in, and a 50% majority rule consensus tree was generated. Analyses were terminated when standard deviation of split frequencies fell below 0.1. Phylogenetic trees were edited using FigTree v. 1.4.4 (Rambaut, 2018) where posterior probabilities (PP) values for the BI tree were added and bootstrap values added for the ML tree. Only support values greater than 0.95 were retained for PP, whereas support values greater than 75% were retained for ML (Felsenstein, 1985).

Divergence time estimates

To estimate timing and rates of divergence amongst the species found within the superfamily Pneumoroidea, a divergence time estimate analysis was performed using BEAST v2.4.6 (Drummond *et al*., 2012). For this analysis, 44 individuals were used, representing a total of 10 species within the family and two alternate orthopteran species were used as outgroups. Fossil evidence or a reliable geological event to calibrate the molecular clock for Pneumoridae is not available; therefore, a secondary calibration point for the family was used, similar to a study by Mariño-Pérez & Song (2019). This calibration point was determined by Song *et al*. (2015), in which nine fossils calibration points from other orthopteran families were used. According to Song *et al*. (2015) the families Pneumoridae and Trignopterygidae were estimated to diverge at approximately 116.97 MYA.

An xml file was created in BEAUti (Drummond *et al*., 2012), specifying monophyly constraints, molecular clock models and priors. Each gene was assigned their respective site model (18S: JC69; COI: GTR and ITS: HKY), which was determined using JModelTest v2.1.7 (Darriba *et al*., 2012). The relaxed clock log normal model was used for the clock model, the Yule model with uniform distribution as a tree prior, and a normal distribution as a distribution prior for calibration points. The normal distribution model was selected due to the uncertainty of date estimates while making use of secondary calibration points. Two distinct analyses were generated to assess convergence across independent runs. Each analysis was run for 50 million generations, sampling every 5000 generations. Results were then inspected using Tracer v.1.6. (Rambaut & Drummond, 2007), with 25% of each run discarded as burn-in and the three best trees were combined using LogCombiner v. 2.3.0 (Rambaut & Drummond, 2014). TreeAnnotator v. 2.3.0 (Rambaut & Drummond, 2014) was used to summarize a maximum clade credibility tree, which was then visualized in FigTree v. 1.4.4 (Rambaut, 2018).

Results

All species are found along the coastline of South Africa, with documented localities up to a maximum of approximately 300 km inland (Figure 3.2). Several species can be seen to have a large distribution and are found across multiple biomes (Figure 3.2 & Table 3.3). However, for several species this overlap is seen near the intersection of biomes. Older location records are not very accurate and may account for these outlying distribution records. Table 3.3 provides a list of Pneumoridae species and the South African biomes in which they are most commonly found. The Fynbos and Thicket biomes can be seen to host the majority of the pneumorids, whereas a single species is found in the arid Nama Karoo (*B. boschimana*).

TABLE 3.3: A list of bladder grasshopper species and their respective biomes within South Africa [x indicates the presence of a species in a biome].

Species	**Biomes of South Africa**						
	Forest	**Fynbos**	**Grassland**	**Nama Karoo**	**Savanna**	**Succulent Karoo**	**Albany Thicket**
B. boschimana				x		x	
B. unicolor		x				x	
B. obliqua		x				x	
B. discolor		x					x
B. serrata		x					x
B. intermedia					x		x
B. membracioides					x		x
Pe. namaqua						x	
Ph. variolosa		x					x
Ph. papillosa		x					
Pn. inanis	x						x
Pr. granulata			x		x		
Phy. miranda	x						
Phy. livingstoni	x						
Total no. of species	**3**	**6**	**1**	**1**	**3**	**4**	**6**

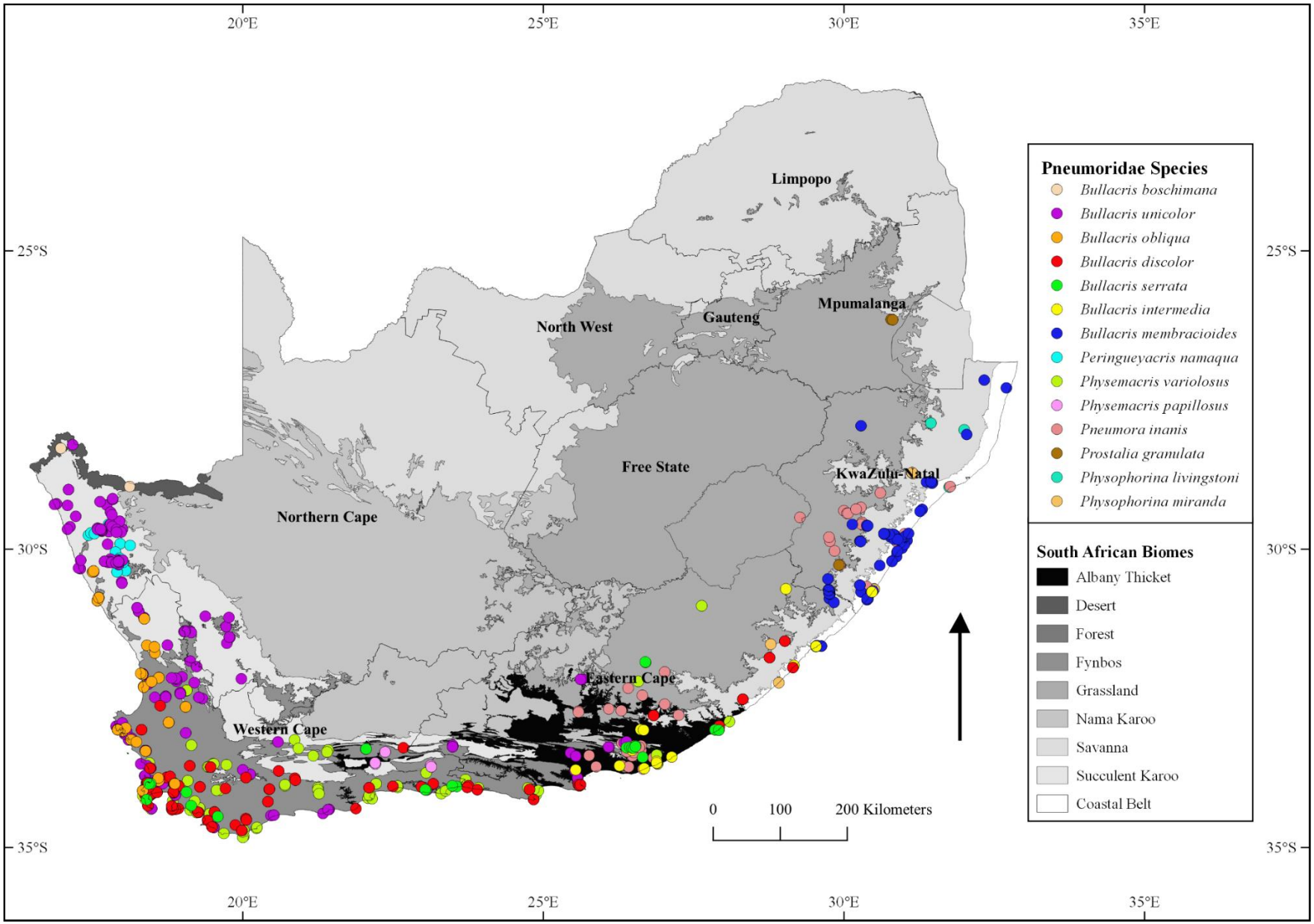

FIGURE 3.2: A distribution map of Pneumoridae species (colour dots) found in South African biomes (grey shaded areas), based on personal and museum specimen records (Mucina & Rutherford, 2006).

Phylogenetic analyses

Ten of the 14 species listed (Table 3.1) provided suitable sequences for phylogenetic analyses. A concatenated dataset amounting to 1571 bp was analysed from one mitochondrial (COI) and two nuclear (ITS and 18S) markers. Species sequence divergence for the COI gene region showed a mean of 15.48% for interspecific variation (Table 3.4). The species with the highest amount of intraspecific diversity is *B. unicolor* (6.64%), whereas the least amount of intraspecific variation is observed within *B. serrata* and *Phy. livingstoni* (0%). The species pair that has the greatest amount of interspecific genetic diversity is *B. obliqua* and *Phy. livingstoni* (25.85%), whereas the least amount of divergence is observed between *B. intermedia* and *B. membracioides* (5.01%).

Phylogenetic methods, BI and ML produced different topologies for total evidence datasets (Figures 3.3 & 3.4), as well as the topology resulting from the divergence time analyses (Figure 3.5). Support values are represented at the nodes with the following conditions; BI ≥ 95 pp and ML ≥ 70% bootstrap for strong support, and BI ≤ 95 pp and ML ≤ 70% is considered as weak support. The monophyly of Pneumoridae was well supported, with 1.00 Bayesian posterior probabilities and 100% Maximum likelihood bootstrap, relative to the *Hoplolopha* sp. and *Lamarckiana* sp. outgroup species. The clade composed of *Phy. livingstoni* and *Pn. inanis* had strong BI and ML support (BI = 1 and ML = 89), and was the first to diverge from other bladder grasshopper species. Within the second major clade, there is a monophyletic clade consisting of *B. unicolor* and *Pe. namaqua*; however, there was only BI support for this split (BI = 1) as ML shows weaker support (ML = 57). For the *B. discolor*, *B. intermedia*, *B. membracioides* and *B. serrata* clade, there is strong support (ML = 98 and BI = 1). There is weak ML support for the *Ph. variolosa* split (ML = 38) and BI analyses show a polytomy for this split, and thus further investigation is required. In addition, *B. intermedia* and *B. membracioides* also form a monophyletic group, with strong supporting values for BI (BI = 1) and ML (ML = 99).

TABLE 3.4: Mitochondrial COI pairwise distances for species within the Pneumoridae family, calculated in MEGA v. 7. and making use of the Kimura 2-parameter model.

	B. discolor	***B. intermedia***	***B. membracioides***	***B. obliqua***	***B. serrata***	***B. unicolor***	***Pn. inanis***	***Phy. livingstoni***	***Pe. namaqua***	***Ph. variolosa***
B. discolor	**4.641 ± 0.021**									
B. intermedia	11.668 ± 0.017	**2.422 ± 0.009**								
B. membracioides	11.444 ± 0.011	5.013 ± 0.011	**2.953 ± 0.027**							
B. obliqua	14.814 ± 0.012	16.078 ± 0.012	16.064 ± 0.008	**4.279 ± 0.028**						
B. serrata	9.476 ± 0.005	9.462 ± 0.015	10.192 ± 0.006	15.852 ± 0.006	**0.000 ± 0.000**					
B. unicolor	16.842 ± 0.020	18.239 ± 0.020	17.429 ± 0.012	15.258 ± 0.016	16.007 ± 0.009	**6.643 ± 0.033**				
Pn. inanis	22.722 ± 0.017	23.330 ± 0.012	22.535 ± 0.008	22.635 ± 0.013	23.322 ± 0.010	21.832 ± 0.007	**3.025 ± 0.021**			
Phy. livingstoni	22.768 ± 0.027	24.256 ± 0.026	24.238 ± 0.026	25.848 ± 0.024	23.836 ± 0.033	22.727 ± 0.033	19.253 ± 0.009	**0.000 ± 0.000**		
Pe. namaqua	16.761 ± 0.015	19.902 ± 0.028	18.581 ± 0.011	17.750 ± 0.020	18.981 ± 0.048	11.904 ± 0.016	23.017 ± 0.007	25.760 ± 0.010	**0.448 ± 0.006**	
Ph. variolosa	13.104 ± 0.015	12.382 ± 0.035	11.729± 0.029	12.513 ± 0.028	11.858 ± 0.022	11.665 ± 0.042	19.538 ± 0.025	24.109 ± 0.010	11.775 ± 0.027	**0.980 ± 0.013**

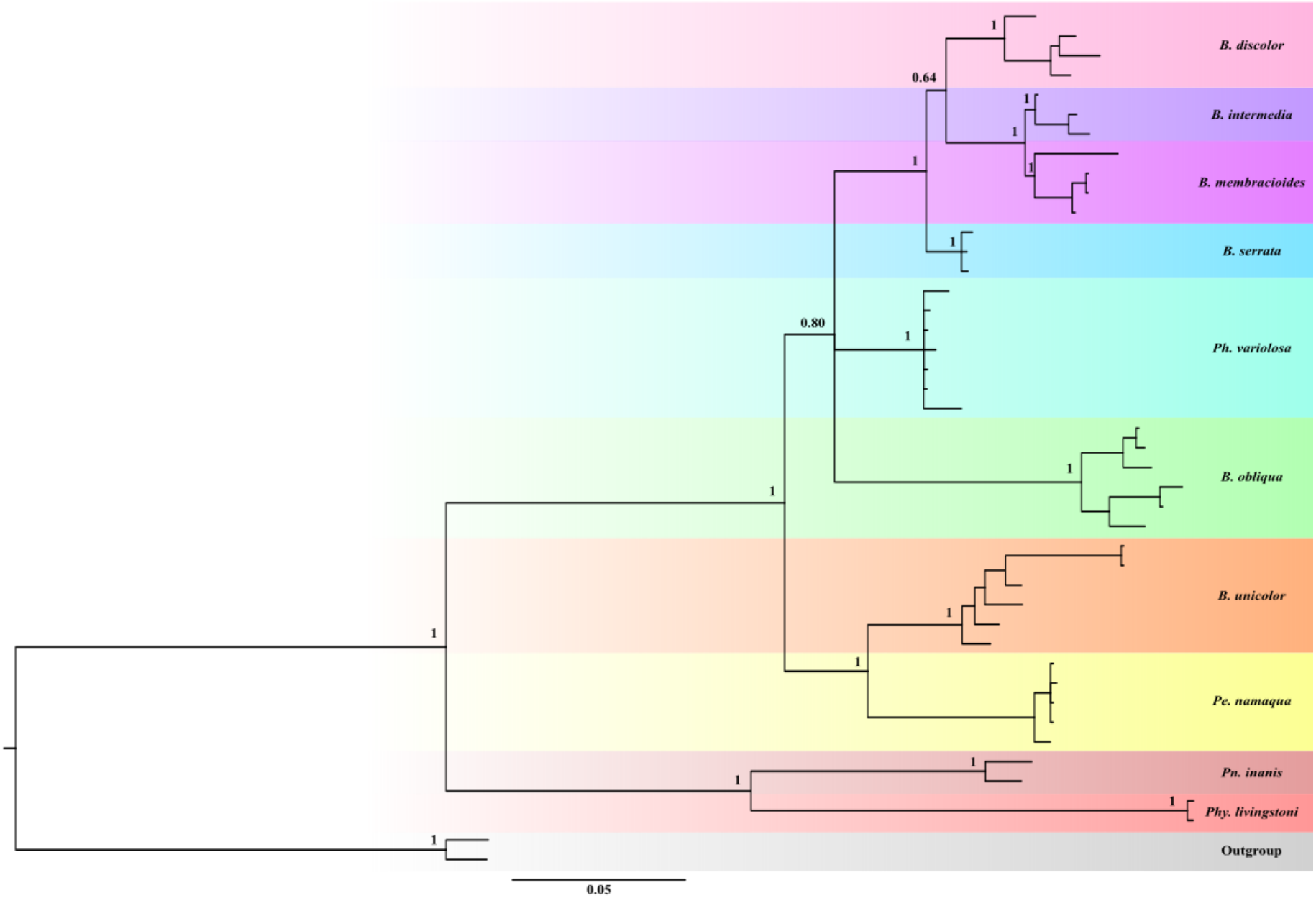

FIGURE 3.3: Bayesian Inference (BI) topology for total DNA dataset evidence (18S, COI, ITS) for species belonging to the family Pneumoridae. Support values for BI posterior probability (≥ 0.95 pp) are presented at the nodes. Values that are greater than 0.95 are considered as strong support, and values that are less than 0.95, are considered as weak support.

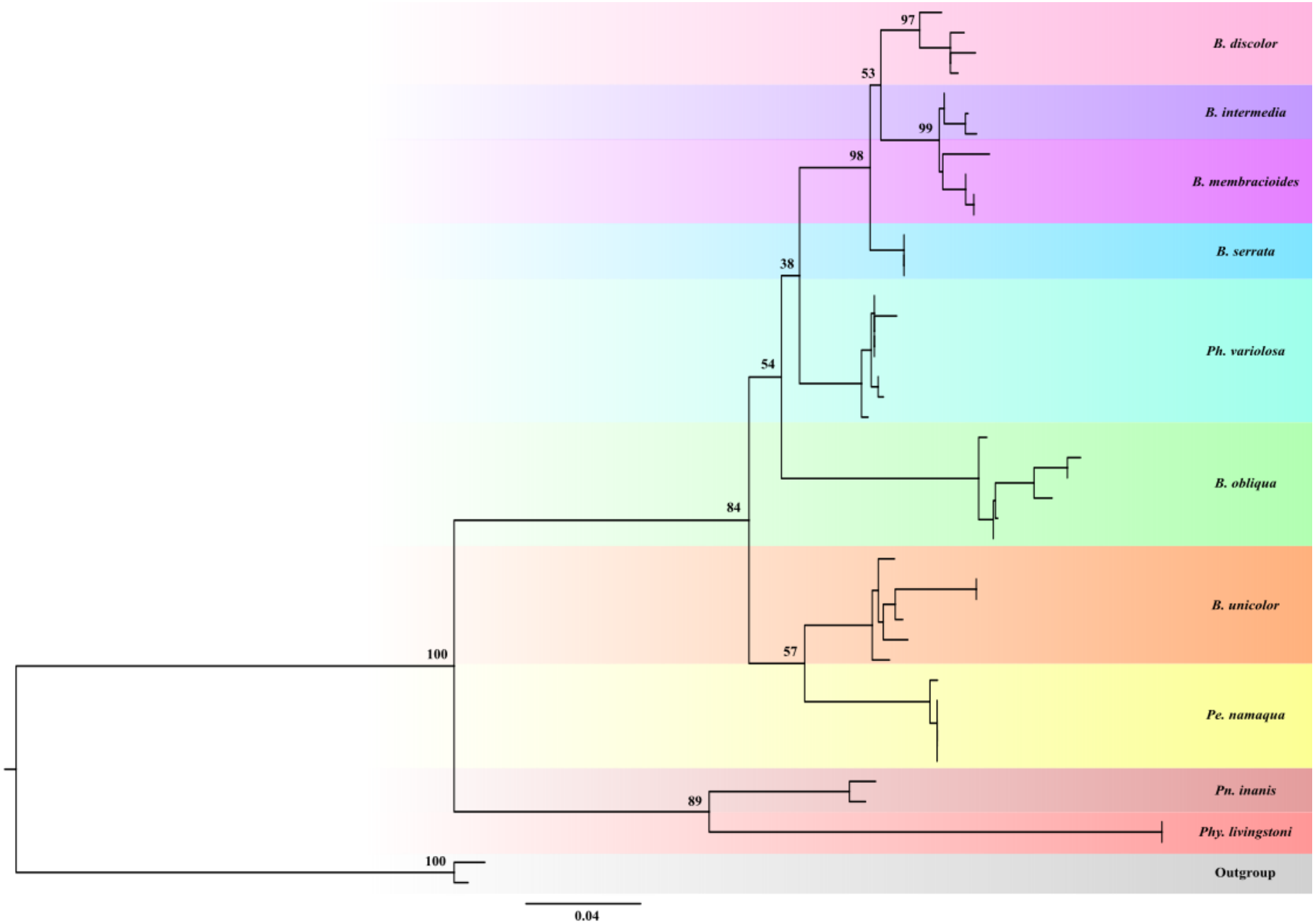

FIGURE 3.4: Maximum Likelihood (ML) topology for total DNA dataset evidence (18S, COI, ITS) for species belonging to the family Pneumoridae. Support values for ML bootstrap (≥ 70%) are indicated at the nodes. Values that are greater than 70%, are considered as strong support, and values that are less than 70%, are considered as weak support.

Divergence time estimates

Making use of a secondary calibration point of 116.97 MYA (Song *et al*., 2015), estimated divergence times are presented in Figure 3.5, with the blue bars indicating the 95% high posterior density (HDP) interval of each divergence. These estimates suggest that Pneumoridae diverged from other grasshopper families during the early Cretaceous period, at approximately 134.70 MYA (95% HPD: 115.61 – 165.76). The first split between species within the family was approximately 116.91 MYA (95% HPD: 114.98 – 118.90), in which the two species inhabiting forest type vegetation, *Pn. inanis* and *Phy. livingstoni*, are found at the base of the node. The second major split is between *Bullacris*, *Physemacris* and *Peringueyacris* during the late Cretaceous period (80.74 MYA, 95% HPD: 60.92 – 100.95), in which the Succulent Karoo inhabiting species split from the Fynbos and Savanna species groups. In addition, the genus *Physemacris* falls within the *Bullacris* species clades and *Phy. variolosa* is observed to have diverged from *B. obliqua* during the Paleogene period (66.15 MYA, 95% HDP: 47.28 – 85.02). Fynbos inhabiting species are seen to split from the later evolving Savanna species during the mid-Paleogene period (38.06 MYA, 95% HDP: 21.33 – 47.13). The most recent divergence between species took place between the two Savanna species, *B. intermedia* and *B. membracioides* during the Miocene (18.64 MYA, 95% HDP: 9.80 – 27.99), indicating the most recent speciation split.

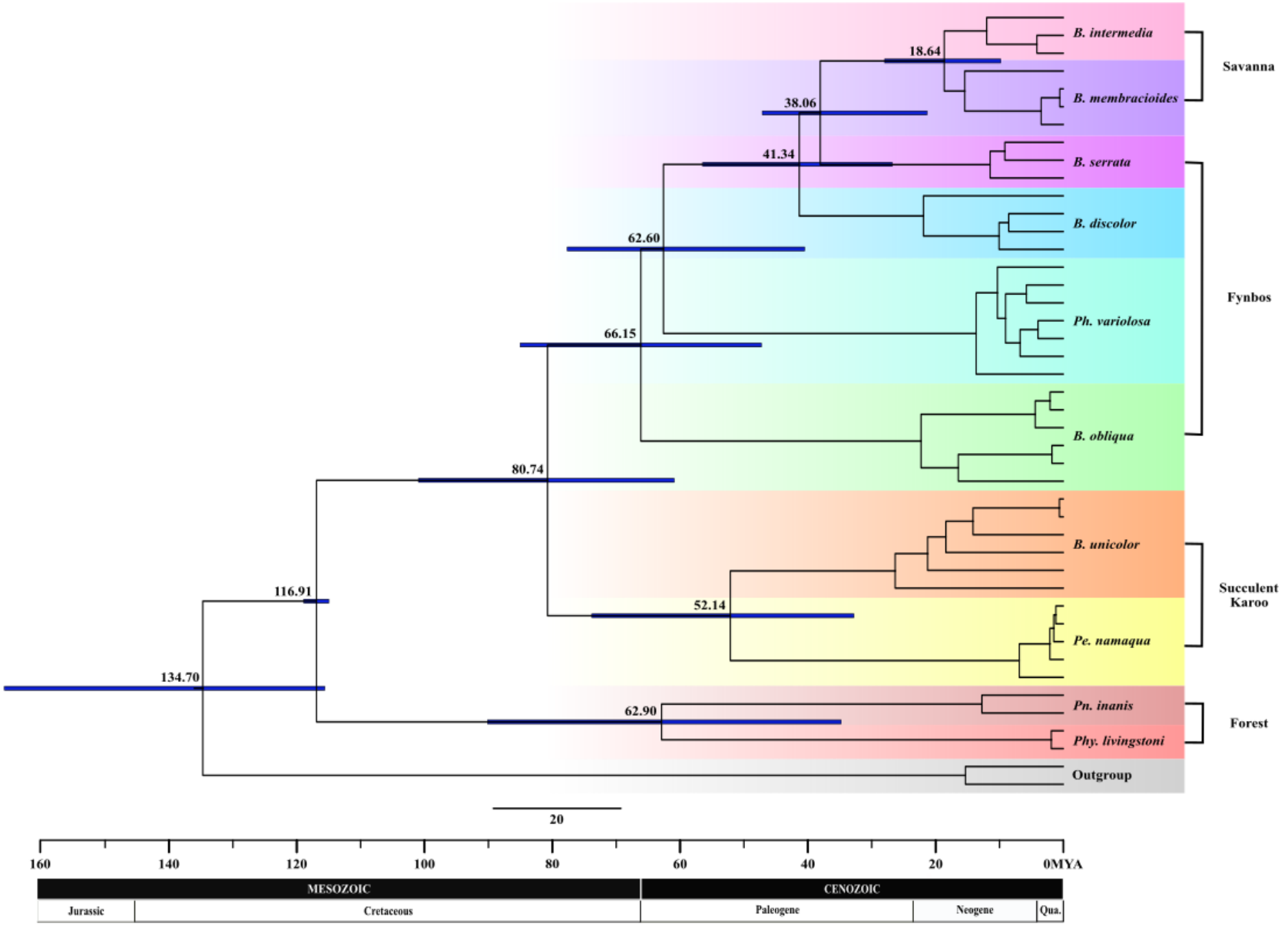

FIGURE 3.5: Dated phylogeny of the family Pneumoridae based on BEAST analysis. Estimated divergence times in millions of years (MYA) are represented at the nodes, which fall within the Cenozoic and Mesozoic Eras. The blue bars represent credible intervals and the 95% node age. Species group according to the biome in which they are primarily found.

Discussion

An intrafamilial phylogenetic study was conducted to assess the evolutionary history and interspecies relationships of bladder grasshoppers. This study is the first phylogenetic analysis of the family Pneumoridae. Ten of the 14 bladder grasshopper species (five of the six genera) were sequenced and showed divergence times from the early Cretaceous (134.70 MYA, 95% HDP 115.61 – 165.76; Pneumoridae family split from other orthopteran families) through to the most recent species split during the early Miocene (18.64 MYA, 95% HDP: 9.80 – 27.99). The phylogenetic results provided some important insights into understanding the taxonomic relationships between species, as well as the role of habitat divergence in the evolution of species groups.

Phylogenetic analyses

The results also reveal that the current taxonomic classifications, which are based on morphology, do not reflect evolutionary history. The genera *Bullacris* and *Physemacris* are morphologically distinguished based largely on the shape of the prozona of the pronotum. In *Physemacris* species, the prozona has two or three large teeth, whereas in *Bullacris* the prozona is more regular. However, phylogenetic analyses from this study showed that *Physemacris*, which consists of two species (*Ph. variolosa* and *Ph. papillosa*) falls within the *Bullacris* species clade (Figures 3.1, 3.3 – 3.5). Only *Ph. variolosa* was included here, as *Ph. papillosa* specimens could not be obtained for genetic analysis. This species is extremely rare in the field and known from very few historical specimens, all of which were collected within a narrow geographic area. While the phylogenetic position of *Ph. papillosa* remains uncertain, it is suggested that *Ph. variolosa* should be renamed and incorporated into the *Bullacris* genus. Similarly, the position of *Pe. namaqua* suggests that it should also be incorporated into the *Bullacris* genus, as it clusters with *B. unicolor* as part of the larger predominantly *Bullacris* clade. Alternatively, *B. unicolor* could be reclassified as it falls outside of the main *Bullacris* group and is more strongly aligned to *Pe. namaqua* than to other *Bullacris* species.

In addition, *B. membracioides* and *B. intermedia* form a strong BI and ML monophyletic clade (Figures 3.3 & 3.4) with only a 5% mitochondrial pairwise variation (Table 3.4). This suggests that these species may either be a single species or are currently undergoing a speciation event, since they are the most recent pair to diverge. Considering that these species were differentiated based on a very minor morphological differences, (i.e., slight differences

in body size, small variations in the profile of the pronotum and slight colour variations; Dirsh, 1965), a more detailed taxonomic assessment is required (see Chapter 4). Results showed that *B. unicolor* had the highest intraspecific genetic diversity. However, this may be because individuals used in this study were representatives taken from across a wide distribution range for the species. This result is not unexpected for the species, as a study by Sathyan *et al*. (2017) discovered that populations of *B. unicolor* may be undergoing genetic isolation. Conversely, for the two species with the lowest intraspecific variation, *B. serrata* and *Phy. livingstoni*, individuals were collected from the same area.

Divergence time estimates

Results of the BEAST analyses (Figure 3.5) indicated that bladder grasshoppers emerged during the early Cretaceous period, which is known to be the most significant geological period for insect evolution (Grimaldi & Engel, 2005). This time estimate also coincides with the breakup of Gondwana (Ali & Atchnison, 2008; Rabinowitz *et al*., 1983; Reeves *et al*., 2002) and as a result had a drastic effect on global climate (Grimaldi & Engel, 2005), particularly within the southern hemisphere (Bradshaw & Cowling, 2014). Thereafter, the first divergence within the family was between the two forest genera (*Pneumora* and *Physophorina*) and the non-forest clade, at an estimated 116.91 MYA (95% HDP: 114.98 – 118.90). This divergence coincides with the time estimate of when the southern regions of Africa were dominated by temperate and sub-tropical forests (Axelrod & Raven, 1978; Greenway, 1970; Patridge & Maud, 2000).

The subsequent divergence occurred between the predominantly Succulent Karoo (*B. unicolor* and *Pe. namaqua*) and Fynbos species (*B. serrata*, *B. discolor*, *Ph. variolosa* and *B. obliqua*), which together make up the Greater Cape region. The Fynbos biome is known to contain older endemic lineages of plant species than that of the Succulent Karoo biome (Verboom *et al*., 2009) and there are several other taxa that are thought to have radiated from the Fynbos biome to the Succulent Karoo (Manning & Goldblatt, 2012; Naskrecki & Bazelet, 2009; Predel *et al*., 2012; Verboom *et al*., 2003). However, this is not the case for bladder grasshoppers, since the Fynbos and Succulent Karoo species split at approximately 80.74 MYA (95% HDP: 32.80 – 73.81), suggesting that the Fynbos clade is as old as the Succulent Karoo clade. Furthermore, bladder grasshoppers had several divergences during the Cenozoic era (*ca*. 66 MYA), a time at which the global climate altered drastically, becoming more temperate (Bender, 2013) and grasses becoming dominant (Strömberg, 2011). Moreover, a number of

taxa are also known to have diverged more frequently during this time, which is thought to be related to the geological stability of the Greater Cape (Cowling *et al*., 2009; Linder, 2005).

The most recent split within the family is between *B. membracioides* and *B. intermedia* at an estimated 18.64 MYA (95% HDP: 9.80 – 27.99). Both of these species primarily occur within the Savanna biome of South Africa, although *B. intermedia* is primarily found in the Eastern Cape and *B. membracioides* in KwaZulu-Natal. The 95% confidence interval for this split encompasses the assumed time (8 - 10 MYA: Diekmann *et al*., 2003; Linder, 2003; Siesser, 1978; 1980) for the formation of the cold Benguela current off the coast of south-western Africa, resulting in a climatic shift towards cooler and drier conditions (Cowling *et al*., 2009; Goldblatt & Manning, 2000; Linder, 2003). During the Miocene, large climatic fluctuations occurred and this may have promoted the diversification (Daniel *et al*., 2020a; Linder *et al*., 2003; Tolley *et al*., 2008). This resulted in species adapting to new environmental conditions (deMenocal, 2004; Potts 1996), as well as stimulating speciation within the Greater Cape (Verboom *et al*., 2003). In the middle Miocene, African grasslands and savannas became more extensive (Jacobs, 2004; Jacobs *et al*., 1999) and with the current fire regime suggested to have been established at *ca.* 15 MYA as a result of global cooling, led to the expansion of open habitats.

Pneumorid evolution

By combining dated phylogenies with ecological and geographical information, it is possible to explore the evolutionary histories of a region's diversity. Pneumorids, being a paleo-relictual insect group, with most found within the Greater Cape, are therefore of great evolutionary and biogeographical interest. Being survivors of ancient Gondwanan lineages, this study provides insights into the paleo-history of southern Africa and the Greater Cape, assisting in identifying patterns of insect diversification.

According to the divergence time estimates from this study, the forest species (*Pneumora* and *Physophorina*) are the first taxa to have diverged and have the largest distribution range, extending from South Africa to Uganda. This coincides with the East African distribution of forested vegetation during the Cretaceous period (Axelrod and Raven, 1978; Greenway, 1970; Patridge & Maud, 2000). Several hypotheses, including refugial and geographical uplift models, have been suggested to explain the distribution patterns and speciation mechanisms of forested regions in eastern Africa (Hemp *et al*., 2014). In addition, the vicariant fragmentation of species is thought to be the result of various geographical events,

which includes continental drift (Gamble *et al*., 2008), changes in sea-level (Vandergast *et al*., 2007) or climatic changes (Knowles, 2001). Therefore, it is possible that during a period when southern Africa was characterized by warm and humid climatic conditions, which supported extensive forested regions (Dingle *et al*., 1983; Patridge & Maud, 2000), this provided favourable conditions for promoting the evolution of bladder grasshoppers. However, when conditions were not favourable as a result of climate change, it has been suggested that the coastal regions of the Cape Floristic Region probably functioned as a refugia during this time (Matenaar *et al*., 2016). Alternatively, orographic changes caused by the upliftment of the Cape Fold belt, in addition to the repeated oceanic regression, may have allowed taxa to distribute into the coastal and lowland habitats of Africa (Hemp *et al*., 2020; Matenaar *et al*., 2016). Nevertheless, it is suggested that bladder grasshoppers originated and started to diversify within South Africa before radiating northwards along the eastern regions of Africa. This trend has been suggested for other taxa (Daniel *et al*., 2020b; Tolley *et al*., 2011), including the endemic African flightless grasshopper family Lentulidae (Hemp *et al*., 2020; Matenaar *et al*., 2015), as well as for the Colophon stag beetles (Switala *et al*., 2014).

In addition, during the Cenozoic era, bladder grasshoppers diverged on several occasions (Figure 3.5). Species within the Fynbos clade were the first to separate during this time (*ca*. 66.15 MYA), which coincides with the time estimates for the Fynbos floral clades in a study conducted by Verboom *et al*. (2009). The leading hypothesis for diversification of species within the Cape during this time is the response to climate change and stability during the Cenozoic (Barraclough, 2006; Cowling *et al*., 2009; Linder, 2005; Switala *et al*., 2014; Tolley *et al*., 2006; Verboom *et al*., 2009). Furthermore, it has been observed that bladder grasshoppers are associated with certain plant species that are common and widely distributed throughout South Africa and more specifically along the coast (e.g., *B. unicolor*: *Didelta spinosa*, *Mulraltia spinosa*, *Osteospermum moniliferum* and *Tripteris oppositifolia*; *B. discolor*: *Senecio halimifolius* and *Metalasia muricata*; *B. membracioides*: *Berkheya* sp.; *Pe. namaqua*: *Pentzia incana*; *Ph. variolosa*: *Elytropappus rhinocerotis* and *Metalasia muricata*). Thus, with the radiation of flora within the Greater Cape it is possible that pneumorids may have evolved and co-diversified with their respective host plant species. The Greater Cape has been extensively investigated for its angiosperm diversity and diversification, but little is known about the diversification of its insect fauna, although it is assumed that co-diversification with the rich flora has occurred (Proches *et al*., 2009; Colville *et al*., 2014).

Conclusion

A shortcoming of this study was the inability to sequence genetic material from all of the 14 Pneumoridae species, as well as not having a wider representation of populations within each species (Table 3.1). Due to the difficulty in locating some species, this study was only able to use a 71% representation of species. In addition, not all sequences were generated for each gene region, due to older specimens having degraded DNA, thus making it very difficult to sequence. It is suggested that to generate a more complete and detailed phylogenetic analysis for the Pneumoridae, additional material for all species is required, including further dense sampling across the family's geographic range from southern and eastern Africa. Nevertheless, most genera were included in the study (with the exception of *Prostalia*) and so the inclusion of additional species would most likely not alter the conclusions of this study.

With regards to the genetic dating of species, this study made use of a secondary calibration point retrieved from Song *et al*. (2015), in which available orthopteran fossil evidence was used to determine the placement of Pneumoridae within the order. However, if fossil evidence for the pneumorid family is found, it would provide for more accurate support towards dating calibrations and divergence times. In addition, since *Ph. variolosa* was found nested within the *Bullacris* genus, it would be beneficial to include the only other species from this genus, *Ph. papillosa*, to determine the taxonomic relationships between these two species and the phylogenetic placement and validity of the genus *Physemacris*. Based on these results, *B. intermedia* and *B. membracioides* had a low mitochondrial DNA variation of 5% (Table 3.4) and it is suggested that they should be considered as a single species. However, further taxonomic investigation of the *Bullacris* genus is essential (see Chapter 4).

This study has provided new insights into the evolution of the Pneumoridae and insect diversification in the Greater Cape. However, more detailed phylogenetic information is required to fully explore these patterns. By understanding the evolution and distribution of relictual insect taxa such as pneumorids, it may be possible to improve human impacts on climatically sensitive ecosystems. Bladder grasshoppers are confined to climatically-vulnerable "refugial" habitats and they are therefore considered as useful indicators for climate change (Hampe & Jump, 2011; Harrison *et al*., 2017). They are also considered of high conservation importance owing to their deep phylogenetic history and the rarity of several species within the group.

References

Ali, J. R. & Atchnison, J. C. (2008). Gondwana to Asia: plate tectonics, paleogeography and the biological connectivity of the Indian sub-continent from the Middle Jurassic through latest Eocene (166–35 Ma). *Earth-Science Reviews*, *88*: 145–166. doi:10.1016/j.earscirev.2008.01.007

Aljanabi, S. M. & Martinez, I. M. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acid Research*, *25*: 22.

Axelrod, D. I. & Raven, P. H. (1978). Late cretaceous and tertiary vegetation history of Africa. In M. J. A. Werger, (Ed.), *Biogeography and Ecology of Southern Africa*, (pp. 77–130). The Hague: W. Junk.

Barraclough, T. G. (2006). What can phylogenetics tell us about speciation in the Cape? *Diversity and Distributions*, *12*(1): 21-26.

Bauer, A. M. & Lamb, T. (2005). Phylogenetic relationships of southern African geckos in the *Pachydactylus* group (Squamata: Gekkonidae). *African Journal of Herpetology*, *54*: 105–29.

Bender, M. L. (2013). *Paleoclimate*. New Jersey: Princeton University Press. (Princeton Primers in Climate; no. 8).

Born, J., Linder, H. P. & Desmet, P. (2007). The Greater Cape Floristic Region. *Journal of Biogeography*, *34*:147–162.

Bradshaw, P. L. & Cowling, R. M. (2014). Landscapes, rock types, and climate of the Greater Cape Floristic Region. In N. Allsopp, J. F. Colville, G. A. Verboom (Eds.), *Fynbos: Ecology, Evolution, and Conservation of a Megadiverse Region*, (pp. 26- 46). United Kingdom: Oxford University Press.

Colville, J. F., Beale, C. M., Forest, F., Altwegg, R., Huntley, B. & Cowling, R. M. (2020). Plant richness, turnover, and evolutionary diversity track gradients of stability and ecological opportunity in a megadiversity centre. *Proceedings of the National Academy of Sciences*, *117*(33): 20027–20037.

Colville, J. F., Potts, A. J., Bradshaw, P. L., Measey, G. J., Snijman, D., Picker, M. D., Procheş, S., Bowie, R. C. K. & Manning, J. C. (2014). Floristic and faunal Cape biochoria: do they exist? In N. Allsopp, J. F. Colville, G. A. Verboom (Eds.), *Fynbos: Ecology, Evolution and Conservation of a Megadiverse Region*, (pp. 73-92). United Kingdom: Oxford University Press.

Cowling, R. M., Procheş, Ş. & Partridge, T. C. (2009). Explaining the uniqueness of the Cape flora: incorporating geomorphic evolution as a factor for explaining its diversification. *Molecular Phylogenetics and Evolution, 51*: 64–74.

Daniel, G. M., Davis, A. L. V., Sole, C. L. & Scholtz, C. H. (2020a). Evolutionary history and eco-climatic diversification in southern African dung beetle *Sisyphus*. *Journal of Biogeography, 47*(12): 2698-2713.

Daniel, G. M., Sole, C. L., Davis, A. L. V., Strümpher, W. P. & Scholtz, C. H. (2020b). Systematics of the dung beetle tribe Sisyphini Mulsant (Scarabaeidae: Scarabaeinae) inferred from a molecular phylogeny and biogeography of southern African species. *Systematic Entomology, 45*(1): 73-84.

Daniels, S. R., Heideman, N. J. & Hendricks, M. G. (2009). Examination of evolutionary relationships in the Cape fossorial skink species complex (Acontinae: *Acontias meleagris meleagris*) reveals the presence of five cryptic lineages. *Zoologica Scripta, 38*: 449–63.

de Jager, M. L. & Ellies, A. G. (2013). The influence of pollinator phylogeography and mate preference on floral divergence in a sexually deceptive daisy. *Evolution, 67*(6): 1706-1714.

deMenocal, P. B. (2004). African climate change and faunal evolution during the Pliocene-Pleistocene. *Earth and Planetary Science Letters, 220*: 3–24.

Diarriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods, 9*(8): 772-772.

Diekmann, B., Falker, M. & Kuhn, G. (2003). Environmental history of the south-eastern South Atlantic since the Middle Miocene: evidence from the sedimentological records of ODP Sites 1088 and 1092. *Sedimentology, 50*: 511–29.

Dingle, R. V., Siesser, W. G. & Newton, A. R. (1983). *Mesozoic and Tertiary geology of southern Africa*. Rotterdam: Salem, N. H., USA: A. A. Balkema; Distributed in the U.S.A. & Canada by M.B.S.

Dirsh, V. M. (1965). Revision of the family Pneumoridae (Orthoptera: Acridoidea). *Bulletin of the British Museum* (*Natural History*) *Entomology, 15*:325–396.

Dirsh, V. M. (1975). Classification of the Acridomorphoid Insects. Farringdon E. W. Classey Ltd., 8: 171 p.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. (2012). Bayesian phylogenetics with beauty and the beast v. 1.7. *Molecular Biology and Evolution, 29*: 1969-1973.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Naturalist, 125*(1): 1–15.

Flook, P. K., Klee, S. & Rowell, C. H. F. (1999). Combined molecular phylogenetic analysis of the Orthoptera (Arthropoda, Insecta) and implications for their higher systematics. *Systematic Biology*, *48*(2): 233-253.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*:294-297.

Gamble, T., Bauer, A. M., Greenbaum, E. & Jackman, T. R. (2008). Evidence for Gondwanan vicariance in an ancient clade of gecko lizards. *Journal of Biogeography, 35*(1): 88-104.

Goldblatt, P. & Manning, J. C. (2002). Plant Diversity of the Cape Region of Southern Africa. *Annals of the Missouri Botanical Garden*, *89*: 281-302.

Goldblatt, P. & Manning, J.C. (Eds.). (2000). *Cape plants. A Conspectus of the Cape Flora of South Africa.* Cape Town and Missouri: National Botanical Institute and Missouri Botanical Gardens. (Strelitzia 9; pp. 1-743).

Greenway, P. J. (1970). A classification of the vegetation of East Africa. *Kirkia*, *9*:1-68.

Grimaldi, D. & Engel, M. S. (Eds.). (2005). *Evolution of the insects*. New York: Cambridge University Press.

Hampe, A. & Jump, A. S. (2011). Climate Relicts: Past, Present, Future. *Annual Review of Ecology, Evolution and Systematics*, *42*: 313–333. doi:10.1146/annurev-ecolsys-102710-145015

Harrison, S. & Noss, R. (2017). Endemism hotspots are linked to stable climatic refugia. *Annals of Botany*, *119*: 207–214. doi:10.1093/aob/mcw248

Hemp, C., Kehl, S., Schultz, O., Wägele, J. W. & Hemp, A. (2014). Climatic fluctuations and orogenesis as motors for speciation in East Africa: case study on *Parepistaurus*, Karsch, 1896 (Orthoptera). *Systematic Entomology*, *40*(1): 17-34.

Hemp, C., Scherer, C., Brandl, R. & Pinkert, S. (2020). The origin of the endemic African grasshopper family Lentulidae (Orthoptera: Acridoidea) and its climate-induced diversification. *Journal of Biogeography, 47*(8): 1805-1815.

Jacobs, B. F. (2004). Palaeobotanical studies from tropical Africa: relevance to the evolution of forest, woodland and savannah biomes. *Philosophical Transactions of the Royal Society, 359*: 1573-1583. doi: 10.1098/rstb.2004.1533

Jacobs, B. F., Kingston, J. D. & Jacobs, L. L. (1999). The origin of grass-dominated ecosystems. *Annals of the Missouri Botanical Garden*, *86*(2): 590-643.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. &

Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12): 1647-1649.

Kirby, W. F. (1910). A Synonymic Catalogue of Orthoptera. *Bulletin of the British Museum* (*Natural History*) *of London*, *3*(2): 343.

Klak, C., Reeves, G. & Hedderson, T. (2004). Unmatched tempo of evolution in Southern Africa semi-desert ice plants. *Nature*, *427*(6969): 63–65.

Knowles, L. L. (2001). Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology*, *10*(3): 691–701.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. & Rubel, F. (2006). World map of the Koppen-Geiger climate classification updated. *Meteorologische Zeitschrift*, *15*: 259–63.

Kreft, H. & Jetz, W. (2007). Global patterns and determinants of vascular plant diversity. *Proceedings of the National Academy of Sciences of the United States of America*, *104*: 5925–5930.

Kuhlmann, M. (2009). Patterns of diversity, endemism and distribution of bees (Insecta: Hymenoptera: Anthophila) in southern Africa. *South African Journal of Botany*, *75*: 726-738.

Kumar, S., Strecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7): 1870-4.

Linder, H. P. (2003). The radiation of the Cape flora, southern Africa. *Biological Review*, *78*: 597–638.

Linder, H. P., Eldenäs, P. & Briggs, B. G. (2003). Contrasting patterns of radiation in African and Australian Restionaceae. *Evolution*, *57*(12): 2688-2702.

Linder, P. H. (2005). Evolution of diversity: the Cape Flora. *TRENDS in Plant Science*, *10*(11): 536-541.

Linnaeus, C. (1758). Systema Naturae per Regna tria naturae (10th ed.) 1:427.

Manning, J. C. & Goldblatt, P. (Eds.). (2012). *Plants of the Greater Cape Floristic Region 1: The Core Cape flora*. Pretoria: South African National Biodiversity Institute. (Strelitzia; vol. 29).

Mariño-Pérez, R., & Song, H. (2019). On the origin of the New World Pyrgomorphidae (Insecta: Orthoptera). *Molecular Phylogenetics and Evolution*, *139*: 106537.

Matenaar, D., Bazelet, C. S. & Hochkirch, A. (2015). Simple tools for the evaluation of protected areas for the conservation of grasshoppers. *Biological Conservation, 192*: 192–199. doi.org/10.1016/j.biocon.2015.09.023

Matenaar, D., Bröder, L. & Hochkirch, A. (2016). A preliminary phylogeny of the South African Lentulidae. *Hereditas, 153*:1.

Matthee, C. A. & Flemming, A. F. (2002). Population fragmentation in the southern rock agama, *Agama atra*: more evidence for vicariance in Southern Africa. *Molecular Ecology, 11*: 465–71.

McDonald, D. E. & Daniels, S. R. (2012). Phylogeography of the Cape velvet worm (Onychophora: *Peripatopsis capensis*) reveals the impact of Pliocene—Pleistocene climatic oscillations on Afromontane Forest in the Western Cape, South Africa. *Journal of Evolutionary Biology, 25*: 824–35.

Mucina, L. & Rutherford, M. C. (Eds.). (2006). The vegetation of South Africa, Lesotho and Swaziland. Pretoria: South African National Biodiversity Institute. (Strelitzia, vol. 19).

Naskrecki, P. & Bazelet, C. S. (2009). A species radiation among South African flightless spring katydids (Orthoptera: Tettigoniidae: Phaneropterinae: *Brinckiella*, Chopard). *Zootaxa, 2056*: 46-62.

Partridge, T. C. & Maud, R. R. (2000). Macro-scale geomorphic evolution of southern Africa. In T. C. Partridge, & R. R. Maud, (Eds.), *The Cenozoic of Southern Africa*, (pp. 3-18). New York: Oxford University Press.

Pitzalis, M. & Bologna, M. A. (2010). Time of diversification in the Cape fauna endemisms, inferred by phylogenetic studies of the genus *Iselma* (Coleoptera: Meloidae: Eleticinae). *Systematic Entomology, 35*: 739–752.

Potts, R. (1996). Evolution and Climate Variability. *Science, 273*: 922-923.

Predel, R., Neupert, S., Huetteroth, W., Kahnt, J., Waidelich, D. & Roth, S. (2012). Peptidomics-based phylogeny and biogeography of Mantophasmatodea (Hexapoda). *Systematic Biology, 61*(4): 609–629.

Price, B. W., Barker, N. P. & Villet, M. H. (2007). Patterns and processes underlying evolutionary significant units in the *Platypleura stridula* L. species complex (Hemiptera: Cicadidae) in the Cape Floristic Region, South Africa. *Molecular Ecology, 16*: 2574–88.

Procheş, Ş. & Cowling, R. (2006). Insect diversity in Cape fynbos and neighbouring South African vegetation. *Global Ecology and Biogeography, 15*: 445-451.

Procheş, Ş. Forest, F., Veldtman, R. Chown, S. L., Cowling, R. M., Johnson S. D., Richardson, D. M., & Savolainen, V. (2009). Dissecting the plant-insect diversity relationship in the Cape. *Molecular Phylogenetics and Evolution, 51*: 94–99.

QGIS Development Team (2019). Quantum Geographic Information System Software. Version 3. Retrieved from: https://qgis.org/en/site/

Rabinowitz, P. D., Coffin, M. F. & Falvey, D. (1983). The separation of Madagascar and Africa. *Science, 220*: 67–69. doi:10.1126/science.220.4592.67

Ragge, D. R. (1963). The nymphal wing-pad tracheation and adult axillary sclerites of the Pneumoridae (Orthoptera: Acridoidea). *Annals and Magazine of Natural History*, *13*(6): 185-191.

Rambaut, A. & Drummond A. (2007). Tracer v. 1.6. computer program. Retrieved from: http://tree.bio.ed.ac.uk/software/

Rambaut, A. & Drummond, A. J. (2014). TreeAnnotator v. 2.3.0. Part of the BEAST package. Retrieved from: http:// beast2.org/

Rambaut, A. (2018). FigTree Version 1.4.4. Retrieved from: http://tree.bio.ed.ac.uk/software/figtree/

Reeves, C. V., Sahu, B. K. & de Wit, M. (2002). A re-examination of the paleo-position of Africa's eastern neighbours in Gondwana. *Journal of African Earth Science*, *34*: 101–108. doi:10.1016/S0899-5362(02)00011-8

Rehn, J. A. G. (1941). On new and previously known species of Pneumoridae (Orthoptera: Acridoidea). *Transactions from the American Entomological Society*, *67*: 137-159.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*:539–42.

Roy, L., Dowling, A. P. G., Chauve, C. M. & Buronfosse, T. (2008). Delimiting species boundaries within Dermanyssus Duges, 1834 (Acari: Dermanyssidae) using a total evidence approach. *Molecular Phylogenetics and Evolution*, *50*: 446–470.

Sathyan, R., Engelbrecht, A. & Couldridge, V. C. K. (2017). Morphological, acoustic and genetic divergence in the bladder grasshopper *Bullacris unicolor*. *Ethology, Ecology and Evolution*, *29* (6): 552-573.

Siesser, W. G. (1978). Aridification of the Namib desert: evidence from oceanic cores. In E. M. van Zinderen Bakker, (Ed.), *Antarctic Glacial History and World Palaeoenvironments*, (pp. 105-113). Rotterdam: Balkema.

Siesser, W. G. (1980). Late Miocene origin of the Benguela upwelling system off northern Namibia. *Science*, *208*: 283–285.

Smart, J. (1953). On the wing-venation of *Physemacris variolosa* (Linn.) (Insecta: Pneumoridae). *Proceedings of the Zoological Society of London*, *123*(1): 199-202.

Snijman, D. A. (2013). *Plants of the Greater Cape Floristic Region. 2: The extra Cape flora*. Pretoria: South African National Biodiversity Institute. (Strelitzia; vol. 30).

Sole, C. L., Scholtz, C. H., Ball, J. B. & Mansell, M. W. (2013). Phylogeny and biogeography of southern African spoon-winged lacewings (Neuroptera: Nemopteridae: Nemopterinae). *Molecular phylogenetics and evolution*, *66*: 360-368.

Song, H., Amédégnato, C., Cigliano, M. M., Desutter-Grandcolas, L., Heads, S. W., Otte D. & Whiting M. F. (2015). 300 million years of diversification: elucidating the patterns of orthoptera evolution based on comprehensive taxon and gene sampling. *Cladistics, 31*, 621-651. doi:10.1111/cla.12116

Stål, C. (1876). *Öfversigt af Kongl. Vetenskaps-akademiens forhandlingar*, *33*(3): 36.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*: 2688–2690.

Strömberg, C. A. E. (2011). Evolution of Grasses and Grassland Ecosystems. *Annual Review of Earth and Planetary Sciences*, *39*: 517-544.

Switala, A. K., Sole, C. L. & Scholtz, C. H. (2014). Phylogeny, historical biogeography and divergence time estimates of the genus Colophon Gray (Coleoptera: Lucanidae). *Invertebrate Systematics*, *28*: 326-336.

Thunberg, C. P. (1810). Nya Arter af Pneumorae slägtet beskrifna. *Kungliga Svenska Vetenskapsakademiens Handlingar*, *31*: 54-65.

Tolley, K. A., Bowie, R. C. K., Measey, J., Price, B. W. & Forest, F. (2014). The shifting landscape of genes since the Pliocene: terrestrial phylogeography in the Greater Cape Floristic Region. *Fynbos: Ecology, Evolution, and Conservation of a Megadiverse Region*, Chapter 7: 142-163.

Tolley, K. A., Burger, M., Turner, A. A. & Matthee, C. A. (2006). Biogeographic patterns and phylogeography of dwarf chameleons (*Bradypodion*) in an African biodiversity hotspot. *Molecular Ecology*, *15*: 781–93.

Tolley, K. A., Chase, B. M. & Forest, F. (2008). Speciation and radiations track climate transitions since the Miocene Climatic Optimum: a case study of southern African chameleons. *Journal of Biogeography*, *35*: 1402–14.

Tolley, K. A., Tilbury, C. R., Measey, G. J., Menegon, M., Branch, W. R., & Matthee, C. A. (2011). Ancient forest fragmentation or recent radiation? Testing refugial speciation models in chameleons within an African biodiversity hotspot. *Journal of Biogeography*, *38*(9): 1748-1760.

van Staaden, M. J. & Römer, H. (1997). Sexual signalling in bladder grasshoppers: tactical design for maximizing calling range. *Journal of Experimental Biology*, *200*: 2597–2608.

van Staaden, M. J. & Römer, H. (1998). Evolutionary transition from stretch to hearing organs in ancient grasshoppers. *Nature*, *394*:773–776.

Vandergast, A. G., Bohonak, A. J., Weissman, D. B. & Fisher, R. N. (2007). Understanding the genetic effects of recent habitat fragmentation in the context of evolutionary history: phylogeography and landscape genetics of southern California endemic Jerusalem cricket (Orthoptera: Stenopelmatidae: *Stenopelmatus*). *Molecular Ecology*, *16*(5): 977-992. doi: 10.1111/j.1365-294x.2006.03216.x

Verboom, G. A., Archibald, J. K., Bakker, F. T., Bellstedt, D. U., Conrad, F., Drey-Er, L. L., Forest, F., Galley, C., Goldblatt, P., Henning, J. F., Mummenhoff, K., Linder, H. P., Muasya, M., Oberlander, K. C., Savolainen, V., Snijman, D. A., Van Der Niet, T. & Nowell, T. L. (2009). Origin and diversification of the Greater Cape flora: ancient species repository, hot-bed of recent radiation, or both? *Molecular Phylogenetics and Evolution*, *51*: 44–53.

Verboom, G. A., Linder, H. P. & Stock, W. D. (2003). Phylogenetics of the grass genus Ehrharta: evidence for radiation in the summer-arid zone of the South African Cape. *Evolution*, *57*: 1008–1021.

Zachos, J., Pagani, M., Sloan, L., Thomas, E. & Billups, K. (2001). Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science*, *292*: 686–93.

Chapter 4: An investigation of the genus *Bullacris* (Orthoptera: Pneumoridae)

Abstract

The genus *Bullacris* in the family Pneumoridae was most recently revised by Dirsh in 1965 based on morphological comparisons between species. However, since that time, new information about the genus and the family has come to light, necessitating a revision of the genus. In addition, the species *B. boschimana* was originally described based on a single female specimen. Here, the male of the species is presented and described for the first time. The aim of this study was to update the current species descriptions by including additional specimens and incorporating additional methods for a more comprehensive comparison. Analyses consisted of morphometric measurements from high-quality images of type specimens, existing South African museum specimens, as well as personally collected specimens. Acoustic signals are also presented and compared between species. In addition, phylogenetic analyses were conducted on the barcoding mitochondrial gene COI and two nuclear genes, namely ITS and 18S. Results show that according to morphological, acoustic and genetic data, *B. discolor* and *B. serrata* as well as *B. intermedia* and *B. membracioides* share notable similarities. *Bullacris discolor* and *B. serrata* share similar phenotypic traits, in which *B. discolor* can either appear uniform in colour or have a speckled variation that is very similar in appearance to *B. serrata*. *Bullacris intermedia* and *B. membracioides* have a 5% mitochondrial DNA pairwise distance, suggesting that they may have not be fully diverged; however, morphological analysis shows that these species are morphologically distinguishable. It is suggested that these species may have undergone spatial separation at one point; however, further investigation is required. Additional sampling across a wider geographic range is essential to clarify the relationships between *B. discolor* and *B. serrata*, as well as between *B. intermedia* and *B. membracioides*.

Introduction

In the past, species were generally classified according to morphological variation. However, morphological features are not always reliable, due to factors such as phenotypic plasticity, geographic variations within widely distributed species and the presence of cryptic species, which leads to potential taxonomic inaccuracies and misidentifications (Bickford *et al*., 2007; Peralta-Rincon *et al*., 2017; Sathyan *et al*., 2017; Song *et al*., 2017). An example of this is observed within the family Pneumoridae, in which members within the family were originally described solely on morphological differences (Dirsh, 1965). However, in recent years, personal observations of species found within the *Bullacris* genus made by Dr Couldridge, have revealed a fair amount of colour variation within some species (e.g., *Bullacris unicolor*, Figure 4.1). In addition, Dirsh (1963) discovered a flightless non-inflated male form, which he believed to be a neotenic form and classified these individuals into separate genera, namely; *Parabullacris vansoni*, *Paraphysemacris spinosus* and *Pneumoracris browni*. It was not until Alexander & van Staaden (1989) and later, Donelson & van Staaden (2005), that two 'forms' of pneumorid males within the species *B. membracioides* were reported. These forms are known as the 'primary' and 'alternate' males, in which the alternate males do not develop the inflated abdomen during the final moult; however, the initial distinction is recognizable as early as the second or third instar (Alexander & van Staaden, 1989). Alternate males (Figures 4.2 & 4.3), have subsequently been observed in additional *Bullacris* species, namely; *B. unicolor*, *B. discolor* and *B. obliqua* but the taxonomy was never formally updated.

In 2003, genetic barcoding was suggested as a streamlined method for identifying and grouping species together according to genetic similarities (Hebert *et al*., 2003). However, species-level identifications made using DNA barcoding have also been thoroughly debated (Čandek & Kuntner, 2015; Hubert & Hanner, 2015; Krishnamurthy & Francis, 2012; Lipscomb *et al*., 2003; Pires & Marinoni, 2010; Rubinoff, 2006; Scotland *et al*., 2003; Tautz *et al*., 2002, 2003; Trewick, 2008; Will & Rubinoff, 2004; Will, *et al*., 2005). It has been argued that DNA sequencing is often limited when species pairs have recent origins (new sister species will share alleles as a result of ongoing gene flow) (Tautz *et al*., 2003) and therefore still results in taxonomic inaccuracies. It is further suggested that taxonomic methods should not only rely on DNA barcodes for the identification of species, but rather use it as a basis, whilst maintaining the importance of morphological variation (Tautz *et al*., 2003).

The *Bullacris* genus currently consists of seven described species. One of the species, *B. boschimana* was described from a single female specimen collected in 1911 and no other

specimens have since been collected. However, recent and careful examinations of museum collections have uncovered five male specimens of *B. boschimana*, hitherto undetected and incorrectly labelled, necessitating that the male of the species be described. Furthermore, preliminary genetic analyses for the genus *Bullacris* based on the barcoding *cytochrome oxidase* I (COI) and internal transcribed spacer (ITS) markers suggested that members morphologically classified as *B. serrata* and *B. discolor*, as well as *B. membracioides* and *B. intermedia*, had very little genetic variation (Gordon, 2017), warranting further investigation. According to Dirsh (1965), which is the most recent previous revision for the family, *B. discolor* and *B. serrata* were differentiated based on minor morphological differences. *Bullacris discolor* and *B. serrata* differ predominantly in colour pattern, with *B. serrata* having white spots or stripes on the pronotum and the abdomen, which are thought to be absent in *B. discolor* (Figure 4.4). Furthermore, the main distinguishing features between *B. intermedia* and *B. membracioides* are body size, with *B. membracioides* being slightly larger, and small differences in the shape of the arc of the pronotum, this being less regular in *B. membracioides*. In addition, the alternate form of males first reported by Alexander & van Staaden (1989) in *B. membracioides* and subsequently observed in additional species, have not been formally included into their respective species descriptions. Thus, there is a need for a taxonomic re-evaluation of the genus.

The intent of this study is to re-examine and update the previous descriptions of each species within the *Bullacris* genus and try to and resolve some of the taxonomic confusion. This will be done by re-evaluating morphological characteristics of specimens, with the aid of high-quality photographs, incorporating and presenting acoustic signal data as well as evaluating three gene regions to establish genetic relationships within the genus.

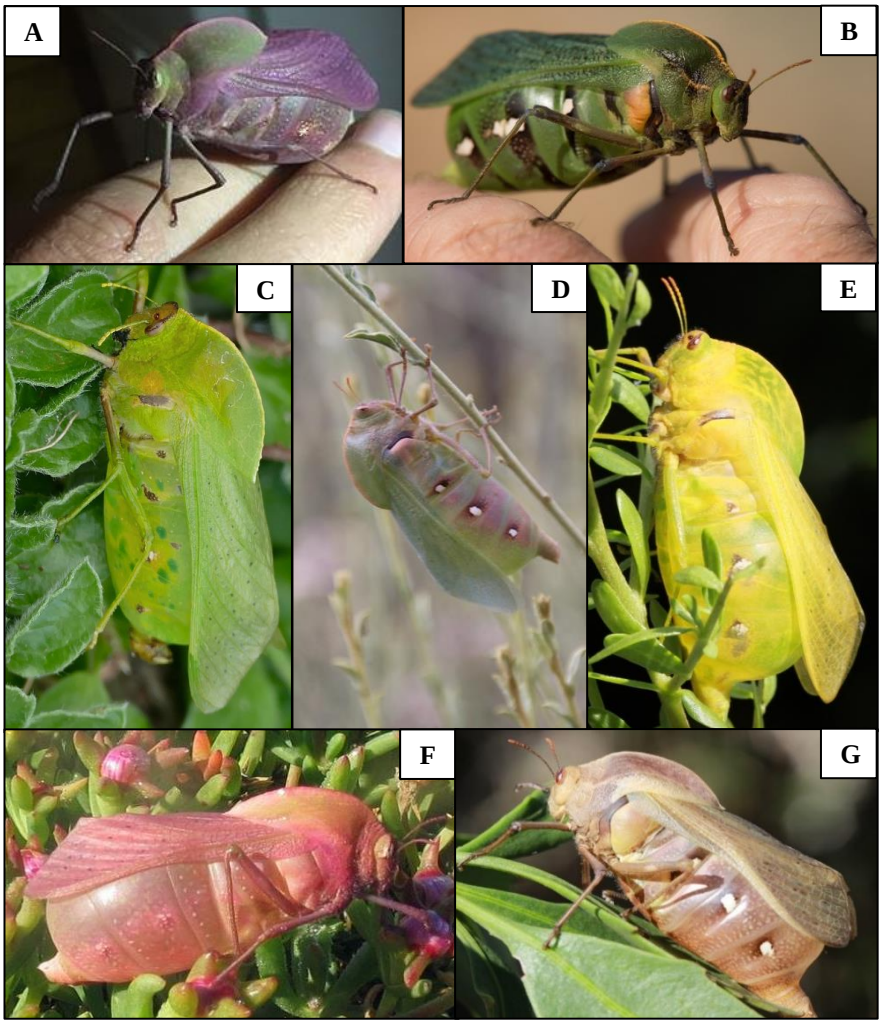

FIGURE 4.1: Colour variation among *Bullacris unicolor* males. Images by: (A) Mollie Brown, (B) Etwin Aslander, (C) Bernard Dupont, (D) Frank Gaude, (E) Vanessa Couldridge, (F) Tony Rebelo, (G) Vanessa Couldridge.

FIGURE 4.2: *Bullacris obliqua* alternate male (left) and female (right) (Image by: Vanessa Couldridge).

FIGURE 4.3: *Bullacris unicolor* alternate male and female (Image by: Vanessa Couldridge).

FIGURE 4.4: Potential phenotypic morphs; (A): *Bullacris serrata* (Image by: Vanessa Couldridge) and (B): *Bullacris discolor* (Image by: Gertrude Panzenberger Smith).

Material and methods

Sampling and locality information

Specimens were collected during the spring and summer seasons along the coastal provinces of South Africa, which includes the Northern Cape, Western Cape, Eastern Cape and KwaZulu-Natal (Table 4.1). Individuals used in this study were from a combination of earlier personal collections and freshly collected specimens. Rarer species such as *B. boschimana*, *B. serrata* and *B. intermedia* were targeted for field collections as they are poorly represented in archival collections; however, fresh samples for *B. boschimana* and *B. intermedia* were not located. Therefore, due to the degradation of *B. boschimana* DNA in museum specimens, which could not be successfully sequenced, this species was excluded from genetic analyses. In addition, to make up for uneven numbers across species groups, museum specimens were used in the morphological comparisons.

TABLE 4.1: The sum of *Bullacris* specimens collected in the field or examined from pinned museum specimens, and the South African province in which they occur.

Species		Field	Museum	Total	Northern Cape	Western Cape	Eastern Cape	KwaZulu-Natal
B. boschimana	male	-	5	**6**	x			
	female	-	1					
B. unicolor	male	9	18	**48**	x	x	x	
	female	15	6					
B. obliqua	male	15	2	**21**	x	x		
	female	1	3					
B. discolor	male	9	13	**54**		x	x	
	female	30	2					
B. intermedia	male	5	6	**12**			x	x
	female	-	1					
B. serrata	male	5	10	**22**		x	x	
	female	-	7					
B. membracioides	male	5	18	**32**			x	x
	female	8	1					

Locality information for species distributions can be seen in Figure 4.5, where species are primarily found along the coastline of South Africa. However, two species have distributions that extend into neighbouring countries, *B. boschimana* has been found in southern Namibia, and a single (unconfirmed) record of *B. membracioides* from Malawi was reported by Dirsh (1965). In the Northern Cape, *B. boschimana*, *B. unicolor* and *B. obliqua* can be found. The Western Cape province houses *B. unicolor*, *B. obliqua*, *B discolor* and *B. serrata*.

Bullacris unicolor, *B. discolor*, *B. serrata*, *B. intermedia* and *B. membracioides* occupy the Eastern Cape province and *B. intermedia* and *B. membracioides* are found in the Kwa-Zulu Natal region. A distribution map was created using a combination of personal, museum and online data records, which aided in the sampling of species (Figures 4.5 & 4.6).

Freshly collected specimens were preserved in 95% ethanol for DNA extraction. Pinned museum specimens were examined from archival collections from the Iziko South African Museum in Cape Town; the ARC Plant Protection Research Institute in Pretoria; the Albany Museum in Grahamstown; Ditsong National Museum of Natural History in Pretoria and the Durban Natural Science Museum in Durban. In addition, type specimens were also examined for each species, either physically or via high resolution photographs for specimens housed in overseas collections that were not available for loan.

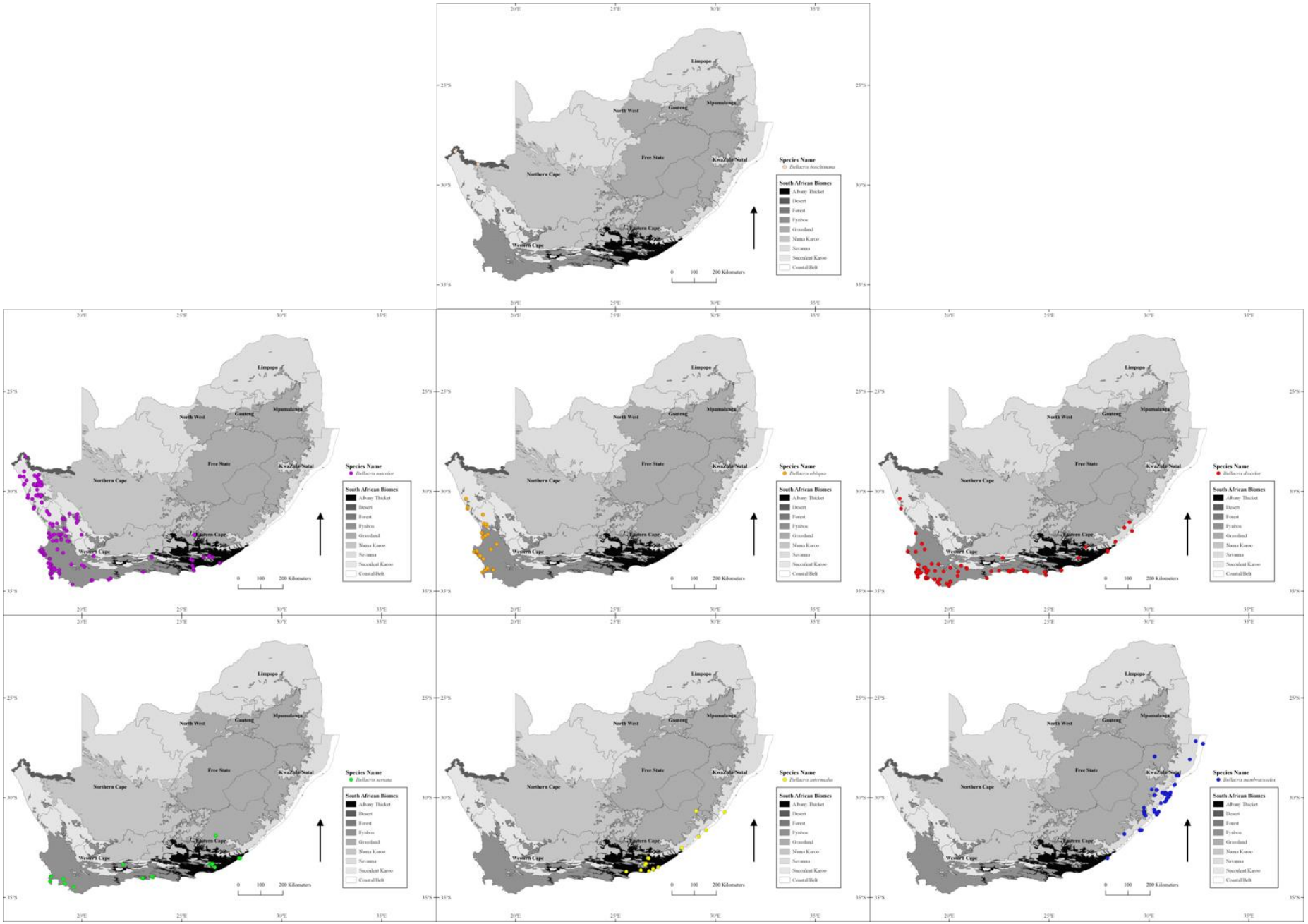

FIGURE 4.5: Distribution maps of *Bullacris* species across the different biomes of South Africa (Mucina & Rutherford, 2006).

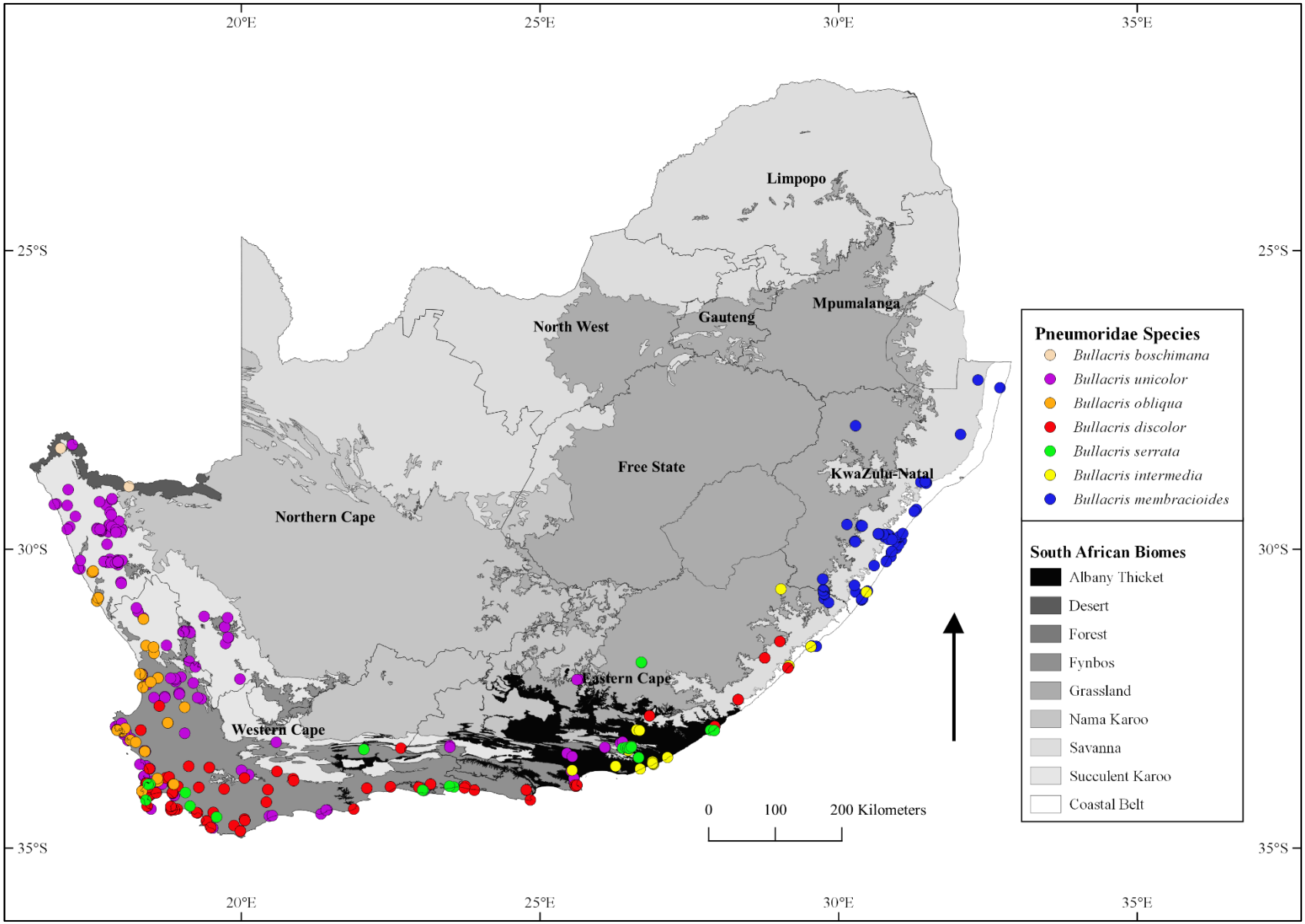

FIGURE 4.6: Distribution map of *Bullacris* species from personal, museum and online location data, with South Africa and its biomes (Mucina & Rutherford, 2006).

Taxonomy

Five male bladder grasshopper specimens housed at the ARC Plant Protection Research Institute (n = 2) and at the Ditsong National Museum of Natural History (n = 3), both in Pretoria, South Africa were examined and based on morphological features and collecting locality were determined to be the male counterpart of the already classified female *B. boschimana* specimen. These specimens were all collected in Rosh Pinah, Namibia, approximately 200 km from the locality for the type specimen. These males are very clearly morphologically distinct from the males of all other known pneumorid species. In addition, male and female individuals of *B. boschimana* were observed together and photographed in the Richtersveld, South Africa in 2009 (P. Naskrecki, *pers. comm.*), further confirming their identity. These males are described here for the first time. Furthermore, alternate males are described for *B. unicolor*, *B. discolor*, *B. obliqua* and *B. membracioides*. It is important to note that the alternate male of *B. unicolor* was originally described by Dirsh (1963) as *Parabullacris vansoni*, which now falls away.

Morphology

A series of morphometric measurements for male and female *Bullacris* specimens were taken using digital callipers, calibrated to the nearest 0.001 mm. All morphological characteristics were measured on the right side to ensure standardization. Following Donelson & van Staaden (2005), nine linear measurements (mm) were acquired, which included; PA= the pronotum arc, PL= pronotum length and PH= pronotum height; the AL= antennae length; HW= head width; AW= abdomen width; HL= hind femur length; TL= tibia length and TB= total body length (Figure 4.7).

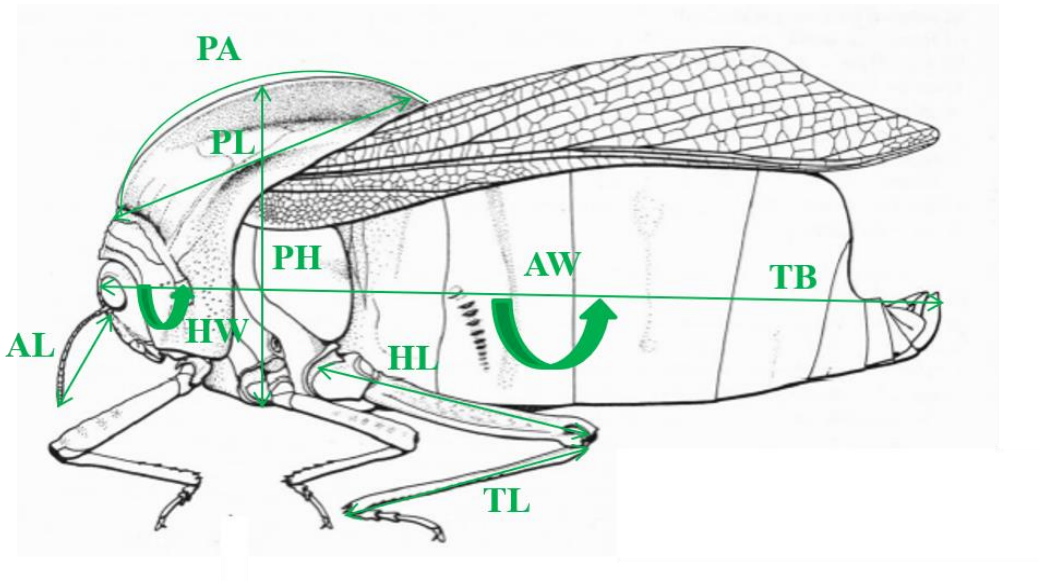

FIGURE 4.7: Male *Bullacris membracioides* (Dirsh, 1965), showing the nine linear anatomical measurements (males and females). Abbreviations: PA: pronotum arc; PL: pronotum length; PH: pronotum height; AL: antennae length; HW: head width; AW: abdomen width; HL: hind femur length; TL: tibia length and TB: total body length.

The starting point for measuring the abdomen width began directly between the two stridulatory ridges for the males and between the second and the third abdominal segments for females. The hind femur and tibia length for both males and females started from the base of the trochanter to the distal tibial articulation. Total body length was measured from the most anterior point of the head to the end of the abdomen; head width was taken directly behind the compound eyes and, antennae length was obtained from the antennal socket to the tip of the flagellum. Pronotum height was measured from the bottom of the thorax to the highest point of the pronotum. The arc was measured by placing a cotton string along the curve of the pronotum and then measuring the string, and the length of the pronotum was linearly measured from the base of the ridge to its pointed end. Photographs of type specimens from Uppsala,

Sweden were measured using the digital imaging software, ImageJ (Schindelin *et al*., 2015; https://imagej.net/).

Digital images of morphological characteristics from freshly collected and museum specimens were taken using a Leica Z16 APO Camera system at the Entomology Department at the Iziko South African Museum in Cape Town (Table 4.2). Multiple images were captured at different focal lengths, using a 1 x magnifier and a montage of the multiple images was created using the Las v 4.7.1 application suite.

TABLE 4.2: A list of museum codes for specimen images presented in this study, with their respective locality information.

Species	Sex	Museum and ID	Location	Province
***B. boschimana* (Type)**	Female	Iziko - SAM-ORT-A004325	Henkries	Northern Cape
	Male	ARC - National Insect Collection	Richtersveld	Northern Cape
B. unicolor	Female	Iziko - SAM-ORT-A004379	Kamieskroon	Northern Cape
	Male	Iziko - SAM-ORT-A004367	De Hoop Nature Reserve	Western Cape
	Alternate male	University of the Western Cape	Springbok	Western Cape
B. obliqua	Female	Iziko - SAM-ORT-A004360	Salt River	Western Cape
	Male	Iziko - SAM-ORT-A004358	Wallekraal	Northern Cape
	Alternate male	Albany Museum	Darling	Western Cape
B. discolor	Female	Iziko - SAM-ORT-A004327	Kirstenbosch	Western Cape
	Male	Iziko - SAM-ORT-A004330	Rosmead Junction	Western Cape
	Alternate male	University of the Western Cape	Cape Town	Western Cape
***B. intermedia* (Type)**	Female	Iziko - SAM-ORT-A004343	Kentani	Eastern Cape
	Male	Iziko - SAM-ORT-A004344	Kowie River	Eastern Cape
B. serrata	Female	Iziko - SAM-ORT-A004362	Cape Town	Western Cape
	Male	Iziko - SAM-ORT-A004361	Stanford	Western Cape
B. membracioides	Female	Iziko - SAM-ORT-A004351	Krantzkloof	KwaZulu-Natal
	Male	Iziko - SAM-ORT-A004347	Durban	KwaZulu-Natal
	Alternate male	University of the Western Cape	Inchanga	KwaZulu-Natal

Acoustics

Acoustic calls for each species were obtained from a library of previously recorded calls. These sounds had been recorded either in the field or in a controlled laboratory environment (Table 4.3) using a Marantz PMD-670 digital recorder and a Sennheiser K6/Me-66 microphone. Majority of the calls used in this analysis were recorded in a controlled environment; however, a small percentage of calls that could not be obtained in a laboratory and field recordings were used. Although field recordings may be of a lesser quality, the minority of calls should not drastically affect the overall conclusions. The calls were analysed using RavenPro 1.5 software (Center for Conservation Bioacoustics, 2014). Temporal and frequency properties of each call were measured for a maximum of 10 calls per individual for each species (fewer than 10 calls were available for some individuals). Each call was first filtered by removing background noise frequencies that were below 500 Hz. The following properties were then measured; the frequency of the first harmonic and of the introductory syllables, the carrier frequency, the total length of the call, the length of the introductory syllables, inter-syllable pause and the length of the final syllable (Figure 4.8).

TABLE 4.3: A list of recorded *Bullacris* male acoustic calls and their corresponding localities.

Species	Location	No. of individuals	No. of calls
B. boschimana	Richtersveld	1	5
B. unicolor	Cederberg	5	50
	Springbok	5	50
	Kamieskroon	5	50
	Bellville	2	20
	Darling	5	52
	Goegap Nature Reserve	3	35
	Groenriviersmond	7	71
	Spektakel Pass	8	85
B. obliqua	Oudtshoorn	2	16
	Groenriviersmond	5	50
	West Coast National Park	4	36
B. discolor	Betty's Bay	5	50
	Hangklip	5	46
	Ashton	6	58
B. intermedia	Port St Johns	3	32
B. serrata	Grahamstown	2	24
B. membracioides	Inchanga	10	104
Totals		**83**	**834**

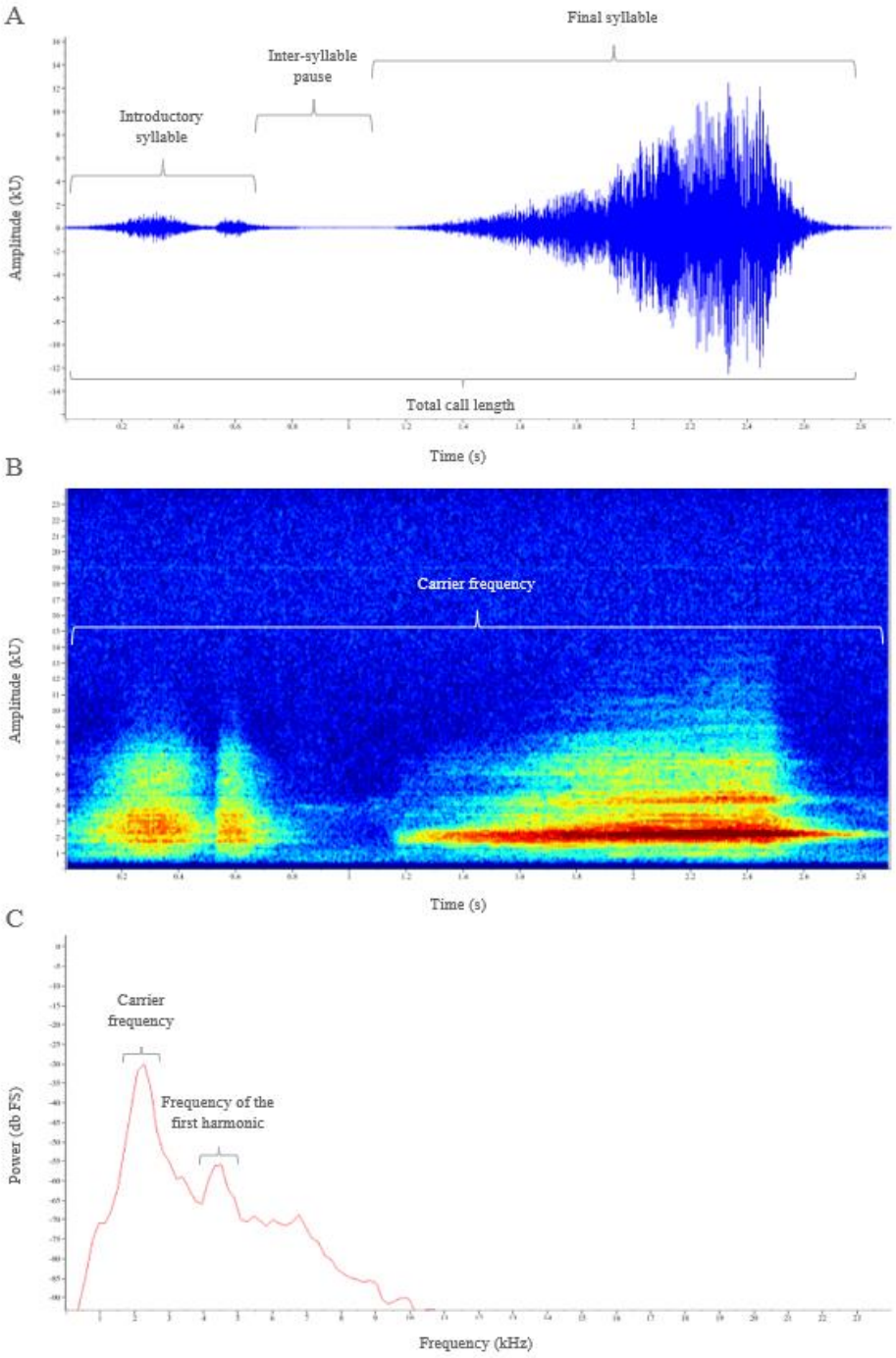

FIGURE 4.8: Waveform (A), spectrogram (B) and spectrum (C) for a *Bullacris unicolor* advertisement call, showing the measurements taken.

Statistical analyses

Statistical analyses were calculated using IBM SPSS Statistics 21. Multivariate Analysis of Variance (MANOVA) was used to test for differences between species in morphological measurements for males and females, as well as male acoustic characteristics. Pillai's Trace was used as a multivariate statistic for the MANOVA, since Box's test indicated that the assumption of equality for covariance matrices were not met. Statistical analyses were not performed on the morphological measurements of alternate males due to low sample sizes, as alternate males are relatively rare within populations.

Discriminate Function Analysis (DFA) was also conducted and associated canonical centroid plots generated to examine how well species cluster based on morphological and acoustic characteristics.

Genetics

A total of 23 *Bullacris* individuals were used for genetic analyses and two *Physemacris variolosa* individuals used as the outgroup (Table 4.4). Each individual had a hind-leg removed and washed for 60 min in double distilled water and then air dried in a vacuum centrifuge for 45 minutes. The dried tissue was placed into an Eppendorf tube, crushed and DNA extracted using salt extraction protocol for fresh samples or by using KAPA Express DNA Extraction Kit (KK 7151) for the more degraded samples.

A total of two nuclear and one mitochondrial gene marker were used in this study. Nuclear genes were 18S ribosomal RNA (18Sa and 18Sb; Song, 2015) and inter-transcribed spacer (ITS) (ITS-F and ITS-R; Roy *et al*., 2008). The mitochondrial gene is a universal primer, *cytochrome c oxidase* subunit I (LCO1490 and HCO2198; Folmer *et al*., 1994).

Gene fragments were amplified in 25 µL volumes, containing a master mix comprised of 10 µL distilled 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 18S polymerase chain reaction (PCR) profile had an initial denaturation step of 94 °C for 2 min, a 35-cycle amplification of 94 °C for 30 s, 52.6 °C for 30 s, and 72 °C for 1 min 45 s, followed by a final extension step of 72 °C for 3 min with a final hold of 15 °C.

For the COI gene, the PCR profile 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 continued for 5 min at 72 °C and final hold at 15 °C. In addition, 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 s, 49 °C for 45 s, and 72 °C for 1 min), followed by a final extension of 72 °C for 10 min and a final hold of 15 °C.

To confirm the successful DNA amplification, electrophoresis was carried out using 1 x TBE buffer on a 1% gel and successful samples were sequenced at Macrogen Inc. (Amsterdam, Netherlands).

TABLE 4.4: List of species with locality records for each individual used for genetic sequencing. Gene regions successfully sequenced are highlighted in grey.

Species name	Locality record	Gene regions		
		COI	ITS	18S
B. discolor	Cape Town			
	Port Elizabeth			
	Grootbos Nature Reserve			
	St Francis Bay			
B. intermedia	The Haven			
	The Haven			
	Silaka Nature Reserve			
B. membracioides	Inchanga			
	Inchanga			
	Port St Johns			
B. obliqua	Groenriviersmond			
	West Coast National Park			
	Groen Rivier Nature Reserve			
	Lamberts Bay			
	Groenriviersmond			
	West Coast National Park			
B. serrata	Grahamstown			
	Grahamstown	-		
	Highlands	-		
B. unicolor	UWC Nature Reserve, Bellville			
	West Coast National Park			
	Cape Point			
	Groen Rivier Nature Reserve			
P. variolosa (Outgroup)	Betty's Bay			
	St Francis Bay			

Phylogenetic analyses

Sequenced samples were aligned using Geneious v. 7.1.3 (Kearse *et al*., 2012). A total of 25 individuals were sequenced for 18S (490 bp), and ITS (497 bp), and only 23 individuals for COI (529 bp). Mitochondrial sequences were translated into amino acids to confirm functionality. Phylogenetic analyses were conducted using RaxML-VI-HPC (Stamatakis, 2006) for Maximum Likelihood (ML) and MrBayes v. 3.2 (Ronquist *et al*., 2012) for Bayesian Inference (BI). A phylogenetic tree was produced using the combined genomic dataset and analyses were run through the CIPRES Science Gateway v. 3.3 (Miller *et al*., 2010). Calculating BI, four Markov chains were run, with each chain beginning at random trees for 10 million generations and a tree was sampled at every 1000 generations. Analyses were run according to the substitute models inferred from JModelTest; COI (TIM2+G), ITS (F81+G) and 18S (JC). A total of 10% was discarded as burn-in, and a 50% majority rule consensus tree was generated. Analyses were terminated when standard deviation of split frequencies fell below 0.1.

FigTree v 1.4.1 (Rambaut, 2014) was used to edit the respective trees, in which the BI posterior probabilities (PP) and ML bootstrap support values (Felsenstein, 1985) were added. Support values for both bootstrap (≥ 70%) and PP (≥ 0.95 pp) were indicated using half black circles at the nodes (BI on the left and ML on the right). Further editing was done using InkScape 0.92.4 (https://inkscape.org/en/). Genetic pairwise distances were calculated for the mitochondrial COI dataset, using MEGA v. 7 (Kumar, *et al*., 2016), in which a table was generated and genetic distances calculated (averages and standard deviations) for within and between species groups, in Microsoft Excel.

Results

FIGURE 4.9: *Bullacris boschimana* male, collected in Rosh Pinah, Namibia (1974).

Taxonomy

Order: Orthoptera

Superfamily: Pneumoroidea

Family: Pneumoridae

Genus: *Bullacris* Roberts, 1941

Bullacris boschimana Péringuey, 1916

Type locality: South West Africa: Namibia, Rosh Pinah

Type: Male

Examined materials: South West Africa: Namibia, Rosh Pinah, 11.1970, ♂♂♂ (coll. J. Botha). All three pinned specimens are housed at Ditsong National Museum of Natural History in Pretoria, South Africa (Museum ID numbers: TMOR-105, TMOR-154 and TMOR-155). 04.06.1974, ♂ (coll. A. P. H. Maritz); 07.1974, ♂ (coll. J. Botha). These two pinned specimens are stored at the ARC Plant Protection Research Institute in Pretoria, South Africa (Figures 4.9 & 4.16).

Male: Large in size (mean body length = 49.41 mm). The integument of the head and thorax is rugulose and granulose. Antennae are filiform with 18-20 segments. The abdomen is smooth and robust. Relatively small granulose head, with a slightly convex face. The fastigial furrow is faint and the pronotum profile is moderately arcuate, with slight depressions between the metazona and prozona areas. The pronotum is divided by the longitudinal median carina with transverse wrinkled furrows (sulci). The third abdominal tergum possesses 9-10 stridulatory ridges. Weak hind-legs, with the tibia slightly tuberculate, together with a long and narrow femur. The supra-anal plate is narrow and angular, with the sub-genital plate being short and angular with an incised apex. Elytra and wings exceed the end of the abdomen. General colouration is a uniform green. The median carina of the pronotum is slightly yellow, and the posterior margin of the lateral lobe is whitish. There is a white spot on each transparent tegmina. The colouration in Figure 4.9 is the result of the specimen aging.

Morphological analyses

According to the morphological measurements recorded in Table 4.5, the average smallest males and females were represented by *B. unicolor* (Figures 4.18 & 4.19), having an average total body length of 40.62 mm for males and 39.63 mm for females, whereas the average largest male and female individuals were recorded for *B. membracioides* (Figures 4.31 & 4.32) with a total body length of 52.70 mm and 54.76 mm, respectively.

The head size of males varies among species, with *B. unicolor* (Figure 4.18 F) having the smallest average head width (4.89 mm); and *B. obliqua* (Figure 4.21 E) and *B. boschimana* (Figure 4.16 D) having a slightly larger head size (5.33 mm and 5.41 mm respectively); whereas *B. discolor* (Figure 4.24 E), *B. serrata* (Figure 4.27 D) and *B. membracioides* (Figure 4.31 B) have the largest head sizes (6.32 mm; 6.80 mm and 6.78 mm). Females have relatively large head sizes compared to males, ranging from 5.63 mm for *B. unicolor* (Figure 4.19 C) to 9.35 mm for *B. intermedia* (Figure 4.30 B).

The pronotum arc, height and length varies quite significantly between species and between males and females. *Bullacris unicolor* (Figure 4.18) males have the lowest averages for length (17.58 mm) and height (15.21 mm), and *B. obliqua* (Figure 4.21) the lowest arc length (19.43 mm), whereas *B. membracioides* (Figures 4.31 & 4.32) males and females have the largest averages for all pronotum measurements. Females with the smallest pronotum arc are *B. unicolor* (Figure 4.19) individuals (23.66 mm); however, the lowest value for pronotum height was in *B. boschimana* (Figure 4.17) at 12.91 mm and for pronotum length was *B. obliqua* at 21.75 mm (Figure 4.22).

Bullacris unicolor (Figures 4.18 & 4.19) have the lowest averages recorded for the remaining morphological measurements, making this species the smallest *Bullacris* species overall within the genus. The species with the largest abdomen width for males is *B. boschimana* at 17.93 mm (Figure 4.16) and for females, *B. serrata* (Figure 4.28) has the largest abdomen width (12.71 mm). The smallest averages for tibia length, total body size and antennae length calculated for males and females, belong to *B. unicolor* (Figures 4.18 & 4.19), whereas the largest averages belong to *B. membracioides* (Figures 4.31 & 4.32). *Bullacris intermedia* females (Figure 4.30) have the largest hind femur (21.05 mm) and *B. unicolor* (Figure 4.19) the smallest (13.11 mm), whereas *B. membracioides* males (Figure 4.31) have the largest hind femur length (18.06 mm) and *B. unicolor* the smallest (13.63 mm).

TABLE 4.5: Morphological measurements (mm) for *Bullacris* species, both male and female, showing the mean and standard deviation. The species with the highest and lowest average values for each variable are highlighted in bold.

Species	No.	sex	pronotum			abdomen (width)	hind femur (length)	tibia (length)	total body (length)	head (width)	antennae (length)
			length	arc	height						
Bullacris boschimana	5	Male	21.22 ± 0.48	23.32 ± 0.96	16.22 ± 2.18	**17.93 ± 0.94**	16.46 ± 0.93	17.27 ± 0.68	49.41 ± 1.24	5.41 ± 0.18	7.60 ±0.30
	1	Female	24.28	25.49	**12.91**	12.37	19.06	17.35	54.05	6.66	7.21
Bullacris unicolor	27	Male	**17.58 ± 1.43**	20.75 ± 1.84	**15.21 ± 1.14**	**13.99 ± 1.10**	**13.63 ± 0.66**	**12.09 ± 1.00**	**40.62 ± 2.33**	**4.89 ± 0.35**	**6.98 ± 0.45**
	21	Female	21.83 ± 2.20	**23.66 ± 2.46**	13.42 ± 1.61	**8.74 ± 0.91**	**13.11 ± 0.93**	**11.81 ± 0.78**	**39.63 ± 3.70**	**5.63 ± 0.44**	**6.17 ± 0.58**
Bullacris obliqua	17	Male	**17.58 ± 1.31**	**19.43 ± 1.43**	15.50 ± 1.20	14.06 ± 1.34	14.98 ± 0.84	13.51 ± 0.77	42.81 ± 1.93	5.33 ± 0.31	9.25 ± 0.63
	4	Female	**21.75 ± 1.63**	23.96 ± 1.73	14.40 ± 0.70	10.20 ± 1.26	14.72 ± 1.55	13.70 ± 2.46	41.17 ± 6.10	6.85 ± 1.08	7.41 ± 0.57
Bullacris discolor	22	Male	19.58 ± 1.84	21.90 ± 2.31	17.04 ± 1.34	14.45 ± 1.71	15.71 ± 1.61	13.97 ± 1.68	46.51 ± 4.16	6.32 ± 0.77	10.96 ± 0.97
	32	Female	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
Bullacris intermedia	11	Male	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
	1	Female	27.25	28.86	17.75	11.22	**21.05**	18.97	42.36	**9.35**	10.53
Bullacris serrata	15	Male	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
	7	Female	25.46 ± 2.50	27.91 ± 2.69	17.35 ± 1.62	**12.71 ± 1.36**	17.19 ± 1.54	16.21 ± 1.17	44.60 ± 5.49	8.75 ± 0.84	10.37 ± 1.24
Bullacris membracioides	23	Male	**23.13 ± 2.40**	**25.20 ± 2.93**	**20.10 ± 1.14**	16.98 ± 3.51	**18.06 ± 0.66**	**16.96 ± 0.73**	**52.70 ± 3.04**	6.78 ± 0.37	**11.26 ± 1.58**
	9	Female	**33.22 ± 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**

MANOVA results for male morphological measurements

Multivariate analysis indicates there are variations in morphological characteristics between *Bullacris* males (Pillai's Trace = 2.576; $F_{54, 660} = 9.194$; $p < 0.001$) (Table 4.6). The pronotum length of *B. membracioides* differs significantly to all other species ($p < 0.001$), with the exception of *B. boschimana* and *B. intermedia* ($p > 0.05$). There are similarities in the length of the pronotum arc between *B. discolor* and *B. serrata* ($p > 0.05$), however both are significantly different to *B. boschimana*, *B. intermedia* and *B. membracioides*. With regards to the pronotum height, *B. membracioides* and *B. intermedia* share similarities, but are significantly different to the remaining species ($p < 0.001$).

The width of the abdomen is similar between *B. boschimana*, *B. serrata*, *B. intermedia* and *B. membracioides* ($p > 0.05$), whereas the remaining species are significantly different to each other, with the exception of *B. obliqua* and *B. discolor* that share similarities with *B. intermedia*. The hind femur of *B. unicolor* is significantly smaller than all other species ($p < 0.001$); whereas *B. discolor* and *B. intermedia* are significantly different to *B. membracioides*, *B. serrata* and *B. unicolor* ($p < 0.001$). *Bullacris intermedia*, *B. obliqua* and *B. discolor* have no significant differences in tibia length amongst each other, which is also true for *B. boschimana*, *B. serrata* and *B. membracioides* ($p > 0.05$). However, *B. unicolor* is significantly different to all species ($p < 0.001$) for tibia length.

There are also similarities in body size between *B. serrata*, *B. membracioides* and *B. boschimana* ($p > 0.05$), whereas *B. unicolor* has significant differences to all species with the exception of *B. obliqua* ($p > 0.05$). *Bullacris discolor* has significant differences in head width to other species ($p < 0.001$); however, *B. boschimana*, *B. obliqua* and *B. intermedia* share similarities, as do *B. membracioides* and *B. serrata* ($p > 0.05$). Antennae length varies between species, however *B. membracioides*, *B. serrata* and *B. discolor* share similarities, as do *B. unicolor*, *B. boschimana* and *B. intermedia* ($p > 0.05$). In addition, the antennae length for *B. obliqua* is significantly different to all species ($p < 0.05$).

TABLE 4.6: Multivariate comparisons table for male morphology between *Bullacris* species, showing mean differences and standard error for pairwise differences. Significant differences are highlighted in bold.

Pronotum (length)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	**3.640 ± 0.812**						
B. obliqua	**3.642 ± 0.849**	0.002 ± 0.516					
B. discolor	1.644 ± 0.826	**-1.996 ± 0.479**	**-1.998 ± 0.539**				
B. serrata	0.836 ± 0.861	**-2.804 ± 0.537**	**-2.806 ± 0.591**	-0.808 ± 0.558			
B. intermedia	-0.272 ± 0.900	**-3.911 ± 0.597**	**-3.914 ± 0.645**	**-1.916 ± 0.616**	-1.108 ± 0.662		
B. membracioides	-1.907 ± 0.823	**-5.547 ± 0.473**	**-5.549 ± 0.533**	**-3.551 ± 0.497**	**-2.743 ± 0.554**	-1.636 ± 0.611	

Pronotum (arc)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	2.564 ± 1.007						
B. obliqua	**3.887 ± 1.053**	1.323 ± 0.641					
B. discolor	1.421 ± 1.025	-1.143 ± 0.594	**-2.466 ± 0.668**				
B. serrata	1.069 ± 1.069	-1.496 ± 0.666	**-2.818 ± 0.733**	-0.353 ± 0.693			
B. intermedia	**-3.467 ± 1.116**	**-6.031 ± 0.740**	**-7.353 ± 0.801**	**-4.888 ± 0.764**	**-4.535 ± 0.821**		
B. membracioides	-1.879 ± 1.021	**-4.443 ± 0.587**	**-5.765 ± 0.662**	**-3.300 ± 0.617**	**-2.947 ± 0.687**	1.588 ± 0.759	

Pronotum (height)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	1.016 ± 0.585						
B. obliqua	0.722 ± 0.611	-0.294 ± 0.372					
B. discolor	-0.811 ± 0.595	**-1.827 ± 0.345**	**-1.533 ± 0.388**				
B. serrata	-0.867 ± 0.620	**-1.883 ± 0.387**	**-1.588 ± 0.426**	-0.055 ± 0.402			
B. intermedia	**-3.301 ± 0.648**	**-4.316 ± 0.430**	**-4.022 ± 0.465**	**-2.489 ± 0.444**	**-2.434 ± 0.477**		
B. membracioides	**-3.877 ± 0.593**	**-4.893 ± 0.341**	**-4.599 ± 0.384**	**-3.065 ± 0.358**	**-3.010 ± 0.399**	-0.576 ± 0.440	

Abdomen (width)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	**3.940 ± 0.937**						
B. obliqua	**3.870 ± 0.979**	-0.072 ± 0.596					
B. discolor	**3.480 ± 0.953**	-0.455 ± 0.553	-0.383 ± 0.621				
B. serrata	1.787 ± 0.994	**-2.150 ± 0.602**	**-2.080 ± 0.682**	-1.697 ± 0.644			
B. intermedia	1.857 ± 1.038	**-2.080 ± 0.688**	-2.010 ± 0.745	-1.627 ± 0.711	0.070 ± 0.764		
B. membracioides	0.957 ± 0.950	**-2.980 ± 0.546**	**-2.910 ± 0.616**	**-2.530 ± 0.574**	-0.831 ± 0.639	-0.901 ± 0.706	

Hind femur (length)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	**2.830 ± 0.462**						
B. obliqua	**1.480 ± 0.483**	**-1.350 ± 0.294**					
B. discolor	0.752 ± 0.470	**-2.080 ± 0.273**	-0.726 ± 0.306				
B. serrata	-0.710 ± 0.490	**-3.540 ± 0.306**	**-2.190 ± 0.336**	**-1.460 ± 0.318**			
B. intermedia	0.801 ± 0.512	**-2.030 ± 0.339**	-0.677 ± 0.367	0.049 ± 0.350	**1.510 ± 0.377**		
B. membracioides	**-1.600 ± 0.468**	**-4.430 ± 0.269**	**-3.080 ± 0.303**	**-2.350 ± 0.283**	-0.891 ± 0.315	**-2.400 ± 0.348**	

Tibia (length)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	**5.180 ± 0.501**						
B. obliqua	**3.760 ± 0.524**	**-1.430 ± 0.319**					
B. discolor	**3.300 ± 0.510**	**-1.880 ± 0.296**	-0.451 ± 0.332				
B. serrata	1.017 ± 0.531	**-4.170 ± 0.331**	**-2.740 ± 0.365**	**-2.290 ± 0.345**			
B. intermedia	**2.720 ± 0.555**	**-2.470 ± 0.368**	-1.040 ± 0.398	-0.589 ± 0.380	**1.700 ± 0.409**		
B. membracioides	0.313 ± 0.508	**-4.870 ± 0.292**	**-3.440 ± 0.329**	**-2.990 ± 0.307**	-0.704 ± 0.342	**-2.400 ± 0.377**	

Total body (length)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	**8.790 ± 1.398**						
B. obliqua	**6.600 ± 1.461**	-2.192 ± 0.889					
B. discolor	2.900 ± 1.422	**-5.890 ± 0.825**	**-3.700 ± 0.927**				
B. serrata	-2.561 ± 1.483	**-11.350 ± 0.925**	**-9.160 ± 1.017**	**-5.460 ± 0.961**			
B. intermedia	4.146 ± 1.549	**-4.640 ± 1.027**	-2.452 ± 1.111	1.247 ± 1.060	**6.710 ± 1.140**		
B. membracioides	-3.286 ± 1.417	**-12.080 ± 0.815**	**-9.880 ± 0.918**	**-6.190 ± 0.856**	-0.724 ± 0.953	**-7.430 ± 1.053**	

Head (width)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	0.523 ± 0.229						
B. obliqua	0.087 ± 0.239	-0.437 ± 0.146					
B. discolor	**-0.910 ± 0.233**	**-1.430 ± 0.135**	**-0.990 ± 0.152**				
B. serrata	**-1.390 ± 0.243**	**-1.910 ± 0.152**	**-1.480 ± 0.167**	**-0.480 ± 0.158**			
B. intermedia	-0.111 ± 0.254	**-0.630 ± 0.168**	-0.197 ± 0.182	**0.800 ± 0.174**	**1.280 ± 0.187**		
B. membracioides	**-1.370 ± 0.232**	**-1.890 ± 0.134**	**-1.450 ± 0.151**	**-0.460 ± 0.140**	0.025 ± 0.156	**-1.260 ± 0.173**	

Antennae (length)

	B. boschimana	*B. unicolor*	*B. obliqua*	*B. discolor*	*B. serrata*	*B. intermedia*	*B. membracioides*
B. boschimana							
B. unicolor	0.619 ± 0.508						
B. obliqua	**-1.650 ± 0.531**	**-2.270 ± 0.323**					
B. discolor	**-3.360 ± 0.517**	**-3.980 ± 0.300**	**-1.710 ± 0.337**				
B. serrata	**-2.910 ± 0.539**	**-3.530 ± 0.336**	**-1.260 ± 0.370**	0.447 ± 0.349			
B. intermedia	-0.364 ± 0.563	-0.983 ± 0.373	**1.290 ± 0.404**	**3.000 ± 0.385**	**2.550 ± 0.414**		
B. membracioides	**-3.660 ± 0.515**	**-4.280 ± 0.296**	**-2.010 ± 0.334**	-0.298 ± 0.311	-0.745 ± 0.346	**-3.300 ± 0.383**	

DFA results for male morphological measurements

Eigenvalues for male morphology shows that 56.7% of the variation was explained by Discriminant Function 1 and 26.5% of the variation by Discriminant Function 2 (Table 4.7). DF 1 has a positive correlation with antennae length and head width and a negative correlation with abdomen width, whereas DF 2 has a positive correlation with tibia length and pronotum height and a negative correlation with head width (Table 4.8). The DFA for male morphology indicates that *Bullacris* males are morphologically different ($p < 0.05$). The majority of species had overlapping clusters for the canonical centroid plot (Figure 4.10). *Bullacris intermedia* overlaps with *B. boschimana,* and *B. discolor* overlaps with both *B. serrata* and *B. obliqua*. In addition, *B. serrata* and *B. discolor* overlap slightly with *B. membracioides*.

TABLE 4.7: Eigenvalues for *Bullacris* male morphological measurements. The percentage of variation for Function 1 and 2 are highlighted in bold.

Eigenvalues				
Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	6.543[a]	**56.7**	56.7	0.931
2	3.054[a]	**26.5**	83.2	0.868
3	1.478[a]	12.8	96.0	0.772
4	0.364[a]	3.2	99.2	0.517
5	0.062[a]	0.5	99.7	0.242
6	0.034[a]	0.3	100.0	0.182

a. First 6 canonical discriminant functions were used in the analysis.

TABLE 4.8: Standardized canonical discriminant function coefficients for *Bullacris* male morphological measurements. The strongest correlation values are highlighted in bold.

Standardized Canonical Discriminant Function Coefficients						
	Function					
	1	2	3	4	5	6
Pronotum (length)	0.019	0.277	0.000	0.182	0.065	0.999
Pronotum (arc)	-0.168	0.299	0.287	0.285	0.607	-0.418
Pronotum (height)	0.016	**0.505**	0.860	-0.222	-0.432	-0.149
Abdomen (width)	**-0.445**	0.406	-0.206	0.236	0.148	-0.038
Hind femur (length)	-0.113	0.000	-0.510	-1.246	-0.671	-0.294
Tibia (length)	0.220	**0.655**	-0.516	-0.093	0.676	-0.329
Total body (length)	0.269	-0.264	-0.385	0.748	-0.606	0.494
Head (width)	**0.596**	**-0.619**	0.476	0.799	0.135	-0.388
Antennae (length)	**0.757**	-0.311	0.223	-0.342	0.468	0.169

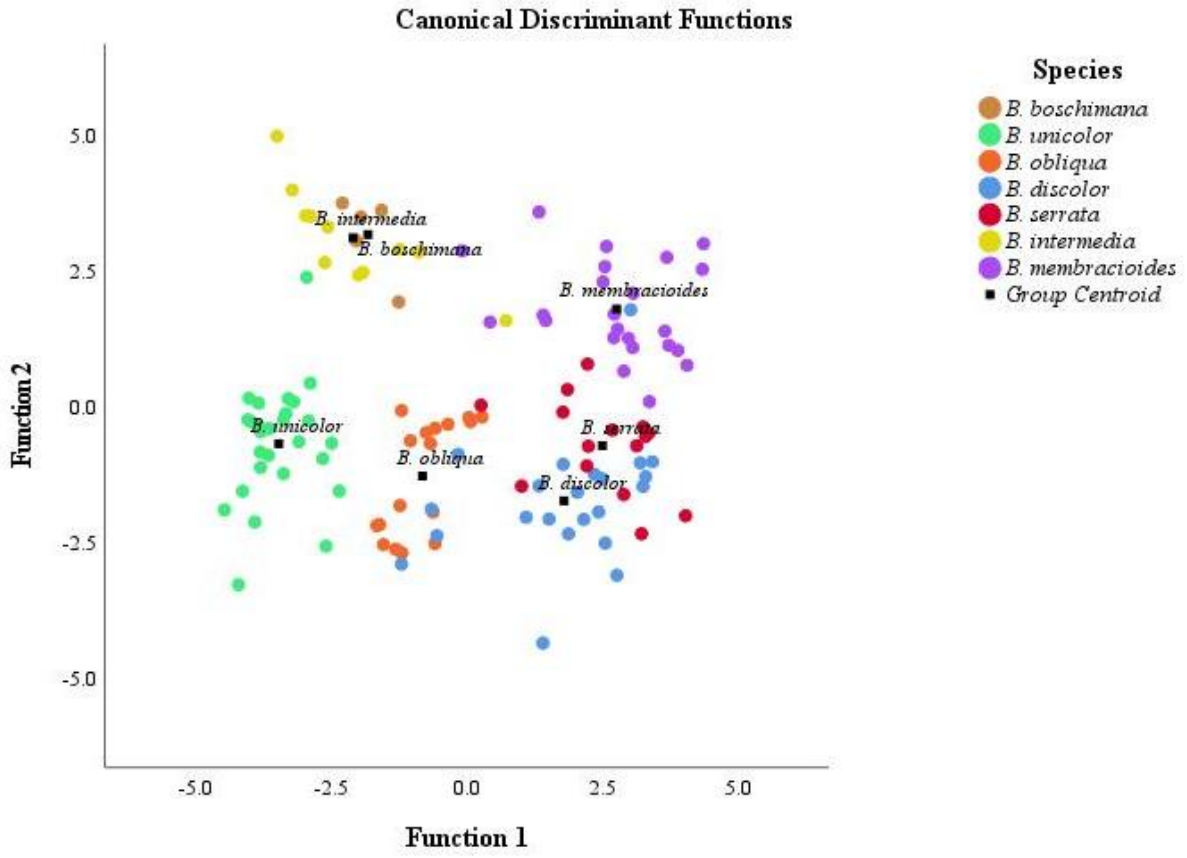

FIGURE 4.10: Discriminant function analysis (DFA) showing the canonical centroid plot for *Bullacris* male morphological measurements.

MANOVA results for female morphological measurements

Results from multivariate analysis for female morphological characters shows that there are significant differences amongst species (Pillai's Trace = 2.295; $F_{36, 252} = 9.424$; $p < 0.001$) (Table 4.9). *Bullacris intermedia* and *B. boschimana* were excluded from the analysis due to low sample sizes.

The pronotum length of *B. membracioides* has significant differences to all species ($p < 0.001$). *Bullacris unicolor* and *B. discolor* also have significant differences to the remaining species, with the exception of *B. obliqua* and *B. serrata* ($p > 0.05$), respectively. The pronotum height and arc of *B. unicolor* varies significantly to other species, with the exception of *B. obliqua* ($p > 0.05$). However, the pronotum arc of *B. obliqua* is significantly different to *B. discolor* and *B. membracioides*, whereas the pronotum height of *B. discolor* and *B. membracioides* are significantly different to each other and to both *B. unicolor* and *B. obliqua* ($p < 0.001$).

Bullacris unicolor and *B. obliqua* are significantly different to all species for abdomen width ($p < 0.001$), whereas *B. discolor*, *B. serrata* and *B. membracioides* differ significantly to *B. unicolor* and *B. obliqua*. All species differ significantly for both hind femur and tibia length, with the exception of *B. serrata* and *B. discolor*, which share similarities for hind femur and tibia length ($p > 0.05$). Body size for *B. discolor* and *B. membracioides* are significantly different to all species ($p < 0.001$), whereas *B. unicolor* and *B. serrata* share similarities with *B. obliqua* ($p > 0.05$). *Bullacris unicolor* and *B. obliqua* both vary significantly in head width from the remaining species ($p < 0.001$), whereas there are similarities between *B. discolor*, *B. serrata* and *B. membracioides*. Antennae length for *B. serrata* is significantly different for all species; however, *B. unicolor* and *B. obliqua* share similarities, as do *B. membracioides* and *B. discolor*.

TABLE 4.9: Multivariate comparison table for female morphology between *Bullacris* species, showing mean differences and standard error for pairwise differences (excluding *Bullacris boschimana* and *Bullacris intermedia*). Significant differences are highlighted in bold.

Pronotum (length)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	0.079 ± 1.226				
B. discolor	**-4.592 ± 0.631**	**-4.672 ± 1.191**			
B. serrata	**-3.625 ± 0.980**	-3.705 ± 1.408	0.967 ± 0.937		
B. membracioides	**-11.391 ± 0.895**	**-11.471 ± 1.350**	**-6.799 ± 0.848**	**-7.766 ± 1.312**	

Pronotum (arc)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	-0.303 ± 1.580				
B. discolor	**-4.902 ± 0.813**	**-4.599 ± 1.536**			
B. serrata	**-4.253 ± 1.264**	-3.950 ± 1.815	0.649 ± 1.208		
B. membracioides	**-5.577 ± 1.154**	**-5.273 ± 1.7402**	-0.675 ± 1.093	-1.323 ± 1.459	

Pronotum (height)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	-0.971 ± 1.234				
B. discolor	**-3.792 ± 0.635**	-2.821 ± 1.200			
B. serrata	**-3.926 ± 0.987**	-2.955 ± 1.418	-0.134 ± 0.944		
B. membracioides	**-7.034 ± 0.901**	**-6.063 ± 1.359**	**-3.242 ± 0.854**	-3.108 ± 1.140	

Abdomen (width)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	**-1.457 ± 0.510**				
B. discolor	**-3.671 ± 0.262**	**-2.215 ± 0.495**			
B. serrata	**-3.973 ± 0.408**	**-2.517 ± 0.586**	-0.302 ± 0.390		
B. membracioides	**-3.494 ± 0.372**	**-2.037 ± 0.5613**	0.178 ± 0.352	0.480 ± 0.471	

Hind femur (length)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	**-1.607 ± 0.534**				
B. discolor	**-4.348 ± 0.275**	**-2.742 ± 0.519**			
B. serrata	**-4.075 ± 0.427**	**-2.468 ± 0.613**	0.274 ± 0.408		
B. membracioides	**-7.451 ± 0.390**	**-5.845 ± 0.588**	**-3.103 ± 0.369**	**-3.377 ± 0.493**	

Tibia (length)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	**-1.886 ± 0.511**				
B. discolor	**-4.222 ± 0.263**	**-2.336 ± 0.496**			
B. serrata	**-4.393 ± 0.409**	**-2.507 ± 0.587**	-0.171 ± 0.391		
B. membracioides	**-7.561 ± 0.373**	**-5.674 ± 0.562**	**-3.339 ± 0.353**	**-3.167 ± 0.472**	

Total body (length)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	-1.537 ± 2.062				
B. discolor	**-9.756 ± 1.061**	**-8.219 ± 2.004**			
B. serrata	**-4.969 ± 1.650**	-3.432 ± 2.369	**4.787 ± 1.577**		
B. membracioides	**-15.132 ± 1.506**	**-13.595 ± 2.271**	**-5.376 ± 1.426**	**-10.163 ± 1.905**	

Head (width)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	**-1.222 ± 0.292**				
B. discolor	**-3.194 ± 0.151**	**-1.972 ± 0.284**			
B. serrata	**-3.122 ± 0.234**	**-1.900 ± 0.336**	0.072 ± 0.224		
B. membracioides	**-3.075 ± 0.214**	**-1.853 ± 0.322**	0.119 ± 0.202	0.047 ± 0.270	

Antennae (length)

	B. unicolor	*B. obliqua*	*B. discolor*	*B. serrata*	*B. membracioides*
B. unicolor					
B. obliqua	0.614 ± 0.920				
B. discolor	**-5.538 ± 0.474**	**-6.151 ± 0.895**			
B. serrata	**-2.717 ± 0.736**	**-3.331 ± 1.057**	**2.821 ± 0.704**		
B. membracioides	**-6.728 ± 0.672**	**-7.342 ± 1.014**	-1.190 ± 0.636	**-4.011 ± 0.850**	

DFA results for female morphological measurements

Eigenvalues from the DFA indicate that 60.2% of the morphological variation is explained by Function 1, whereas 34.8% is explained by Function 2 (Table 4.10). Discriminant Function 1 has a positive correlation with head width and abdomen width, and a negative correlation with the arc of the pronotum (Table 4.11). In addition, DF 2 has a positive correlation with pronotum length and tibia length and a negative correlation with head width. The DFA for female morphological measurements shows that species are morphologically different ($p < 0.05$). *Bullacris discolor* and *B. serrata* have overlapping clusters on the centroid plot (Figure 4.11), indicating that these species are morphologically similar. In addition, the *B. obliqua* cluster overlaps somewhat with *B. unicolor*, *B. discolor* and *B. serrata*, whereas *B. membracioides* clusters strongly on its own.

TABLE 4.10: Eigenvalues for *Bullacris* female morphological measurements. The percentage of variation for Function 1 and 2 are highlighted in bold.

Eigenvalues				
Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	10.827[a]	**60.2**	60.2	0.957
2	6.251[a]	**34.8**	94.9	0.928
3	0.846[a]	4.7	99.6	0.677
4	0.063[a]	0.4	100.0	0.244

a. First 4 canonical discriminant functions were used in the analysis.

TABLE 4.11: Standardized canonical discriminant function coefficients for *Bullacris* female morphological measurements. The strongest correlation values highlighted in bold.

Standardized Canonical Discriminant Function Coefficients				
	Function			
	1	2	3	4
Pronotum (length)	-0.256	**0.933**	-0.124	0.350
Pronotum arc	**-0.261**	0.135	0.161	0.651
Pronotum (height)	-0.118	-0.014	-0.057	0.533
Abdomen (width)	**0.268**	-0.137	-0.528	0.413
Hind femur (length)	0.234	0.462	0.093	-0.247
Tibia (length)	0.051	**0.860**	-0.652	-0.044
Total body (length)	-0.247	-0.036	0.766	-0.948
Head (width)	**1.039**	**-1.373**	0.030	-0.373
Antennae (length)	0.122	-0.108	0.895	0.457

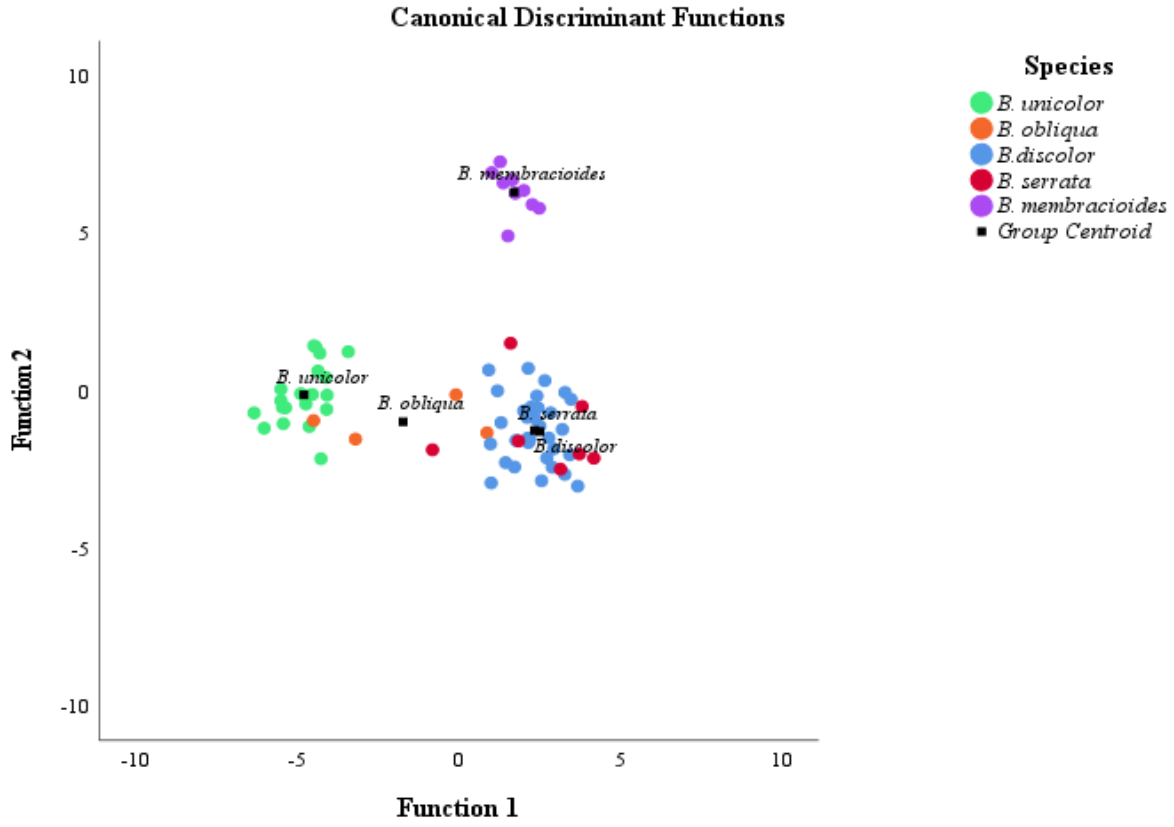

FIGURE 4.11: Discriminant function analysis (DFA) showing the canonical centroid plot for *Bullacris* female morphological measurements.

Revised descriptions and species images

Morphological characters of *Bullacris* species have suitably been described by Dirsh in 1965 and species can be distinguished morphologically based on the updated taxonomic key (Figure 4.12). However, distinguishing morphological features are briefly described here. *Bullacris unicolor* has slightly club-like antennae (Figure 4.13), widening at the apical part (A) in comparison to the filiform antennae (B) found in the remaining species. *Bullacris unicolor* males also have a well-rounded abdomen whereas *B. obliqua*, *B. discolor* and *B. serrata* have a more elongated abdomen.

Pneumorids have distinctive elytra and wing characteristics that distinguish them from other superfamilies within the Caelifera suborder (Dirsh 1965). All *Bullacris* species possess wings, with primary males being macropterous with fully developed elytra and wings (Figure 4.14). Females have micropterous elytra (Figure 4.15), which are partially or wholly hidden under the pronotum. Male and female wings within the genus *Bullacris* are alike; however, a detailed description of the male and female wings (Figures 4.14 & 4.15) can be found in the article 'Revision of the family Pneumoridae (Orthoptera: Acridoidea)' by Dirsh (1965). Alternate males have vestigial elytra, with wings completely hidden under the pronotum (Figures 4.20; 4.23; 4.26 & 4.33). *Bullacris boschimana* males (Figure 4.16 G) have one white spot on each fore wing, which easily distinguishes them from all other *Bullacris* species

The pronotum of *Bullacris* males varies in shape, length, height and pattern. *Bullacris discolor* (Figure 4.24 C) and *B. serrata* (Figure 4.27 B) males have a red line that runs down the middle of the pronotum, however *B. serrata* has speckled pronotum. This characteristic (markings on the pronotum) is also occasionally seen in *B. discolor*, as opposed to the more frequent uniform green. *Bullacris obliqua* (Figure 4.21 C) often has oblique white stripes on the pronotum; however, many specimens lack this feature and are uniform green with no markings. The pronotum profile for *Bullacris intermedia* (Figure 4.29 E) males is regularly arcuate and the third episternum has a brown patch, whereas the morphologically similar *B. membracioides* (Figure 4.31 A) has a highly arcuate profile, but lower at the prozona, without a brown patch on the third episternum. The remaining species have a relatively low arc of the pronotum.

The *Bullacris* females also show variation of the pronotum in respect to size, pattern and shape. Several species have white markings covering the pronotum, which can be seen here in *B. boschimana* (Figure 4.17 E), *B. obliqua* (Figure 4.22 D) and *B. serrata* (Figure 4.28 E); however, this is not a distinguishing feature, since uniform colouration has also been

observed in these species. The pronotum profile is generally high in *B. intermedia* (Figure 4.30 A) and *B. membracioides* (Figure 4.32 C), whereas the pronotum for *B. unicolor* (Figure 4.19 B) and *B. boschimana* (Figure 4.17 C) is narrow and slender with a relatively low profile. *Bullacris discolor* (Figure 4.25 E) has a wide and robust pronotum, without dorsal callosities. The dorsum of *B. serrata* (Figure 4.28 C) has convex sides with a low obtuse median carina, and *B. obliqua* (Figure 4.22 B) has slightly concave sides with a sharp median carina.

The blotched white and brown markings on the sides of the abdomen of *Bullacris* males are highly variable and appear to not be species specific. Nevertheless, the specimens that have been observed in this study shows that *B. boschimana* males (Figure 4.16) have no markings on the abdomen, whereas *B. serrata* males (Figure 4.27) have a speckled abdomen in addition to circular white markings. The remaining species are more variable and may either have three or four circular markings or lack these completely. The females of *B. unicolor* (Figure 4.19) and *B. membracioides* (Figure 4.32) may also sometimes have three or four markings along the abdomen. The remaining species have many different markings covering the abdomen, which can be observed in the figures below (Figures 16 – 33).

The stridulatory mechanism, which is only found in males, consists of sclerotized ridges found on the abdomen. The number and arrangement of these ridges is somewhat unique to each species and can potentially be used as a taxonomic characteristic. When investigating the stridulatory ridges between *Bullacris* males, *B. boschimana* (Figure 4.16 E) has 8-9 ridges, *B. membracioides* (Figure 4.31 E) has 9 ridges, *B. discolor* (Figure 4.24 F) and *B. intermedia* (Figure 4.29 F) have between 9 and 10 ridges. *Bullacris serrata* (Figure 4.27 E) has 10 ridges, whereas *B. obliqua* (Figure 4.21 F) has 13 ridges. The most variable number of ridges is seen in *B. unicolor* (Figure 4.18 G), which has between 11 and 13 stridulatory ridges.

The alternate males lack the inflated abdomen and are much smaller than primary males. By examining the handful of elusive alternate males, *B. unicolor* is typically dark brown in colour, with three white circular markings along the sides of the abdomen (Figure 4.20), which are absent in the remaining species. *Bullacris obliqua* alternate males have two forms of colouration; the first being green-brown with white markings covering the pronotum and the abdomen, and the second green-yellow with three pink lines on the pronotum and pink appendages (Figure 4.23). Furthermore, *B. discolor* (Figure 4.26), as well as *B. membracioides* (the largest of the alternate males; Figure 4.33) have also been observed to possess the red line along the pronotum and on the extremities, in addition to the overall uniform light green colour.

KEY TO SPECIES
MALES

1. (2) Antennae slightly club-like widened at apical part (Figure 4.13 A). Supra-anal plate comparatively short and widely angular (Figure 4.18). ***unicolor*** (Linnaeus)
2. (1) Antennae filiform (Figure 4.13 B). Supra-anal plate comparatively long and narrow, angular.
3. (6) Pronotum in profile highly accurate.
4. (5) Pronotum in profile regularly accurate. Third episternum with brown patch and third abdominal tergite with 9 – 10 stridulatory ridges. Size smaller (42 – 52 mm) (Figure 4.29) ***intermedia*** (Péringuey)
5. (4) Arc of pronotum, in profile, lower in prozona. Third episternum without brown patch and third abdominal tergite with 9 stridulatory ridges. Size larger (45 – 58 mm) (Figure 4.31). ***membracioides*** (Walker)
6. (3) Pronotum in profile low arcuate. Elytra each with white spot. Third abdominal tergite with 8 stridulatory ridges (Figure 4.16). ***boschimana*** (Péringuey)
7. (10) Pronotum in profile regularly arcuate. Third abdominal tergite with 9-10 stridulatory ridges.
8. (9) Pronotum without callosities. Veinlets of reticulation of elytra is the same colour as membrane. Sides of abdomen with ocellate pattern or uniform (Figure 4.24). ***discolor*** (Thunberg)
9. (8) Pronotum with whitish callosities. Veinlets of reticulation of elytra darkened. Sides of abdomen with ocellate and marble pattern (Figure 4.27) ***serrata*** (Thunberg)
10. (8) Arc of pronotum in profile lower in prozona. Third abdominal tergite with 13 stridulatory ridges. Size smaller (41 – 46 mm) (Figure 4.21). ***obliqua*** (Thunberg)

KEY TO SPECIES
FEMALES

1. (4) Arc of pronotum in profile comparatively high.
2. (3) Smaller size (42 mm) (Figure 4.30). ***intermedia*** (Péringuey)
3. (2) Larger size (51 – 60 mm) (Figure 4.32). ***membracioides*** (Walker)
4. (1) Arc of pronotum in profile comparatively low.
5. (6) Pronotum narrow, slender (Figure 4.19). ***unicolor*** (Linnaeus)
6. (5) Pronotum comparatively wide, robust.
7. (8) Pronotum without dorsal callosities or with only traces of them (Figure 4.25) ***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 (Figure 4.28). ***serrata*** (Thunberg)
10. (9) Dorsum of pronotum with slightly concave sides and sharp median carina.
11. (12) General colouration greenish. Sides of abdomen with four rows of small whitish, oblique spots (Figure 4.22). ***obliqua*** (Thunberg)
12. (11) General colouration pale brownish or green. Side of abdomen with two rows of large whitish spots of irregular form or uniform in colour (Figure 4.17). ***boschimana*** (Péringuey)

FIGURE 4.12: Key to *Bullacris* male and female species, modified from Dirsh (1965).

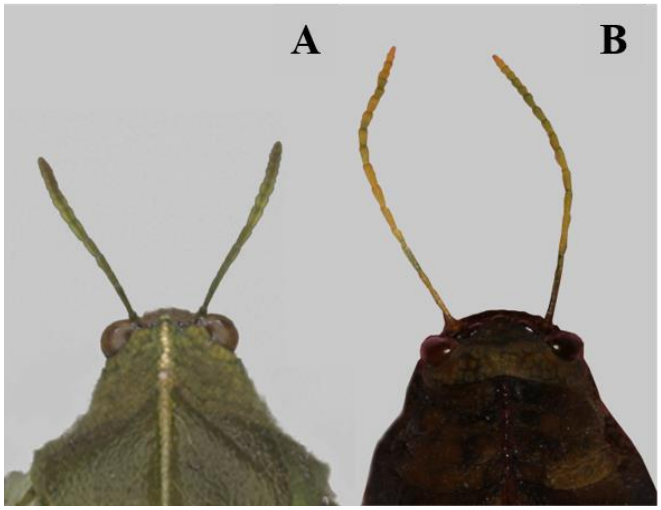

FIGURE 4.13: Antennae comparison, *Bullacris unicolor* have a club-like shape (A), whereas all other *Bullacris* species have a filiform shape (B).

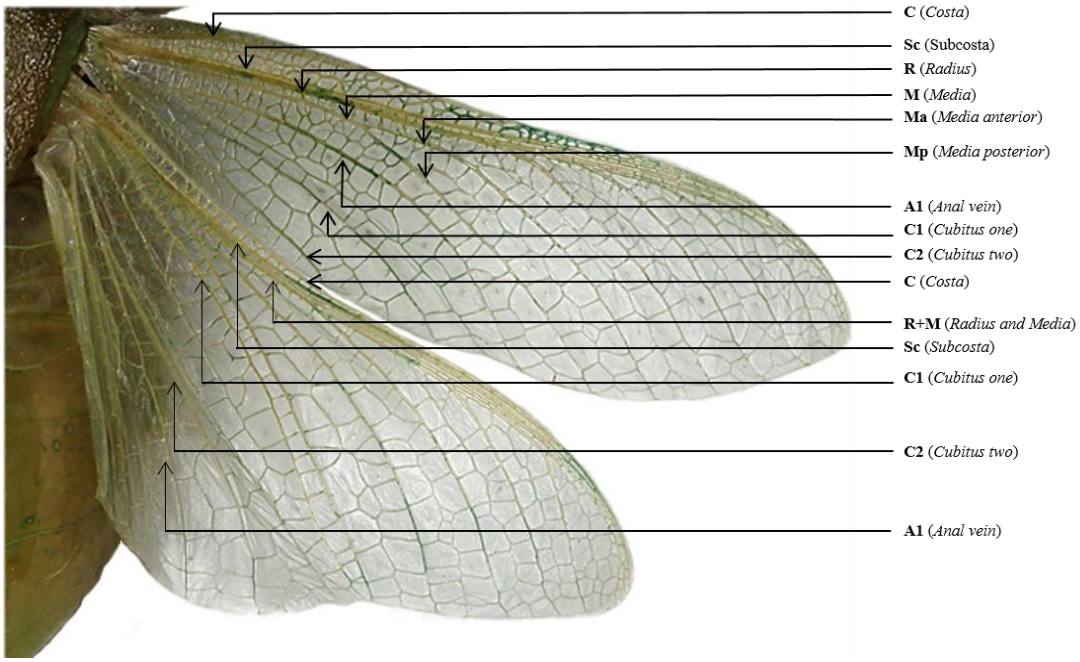

FIGURE 4.14: Wing venation of a *Bullacris discolor* primary male.

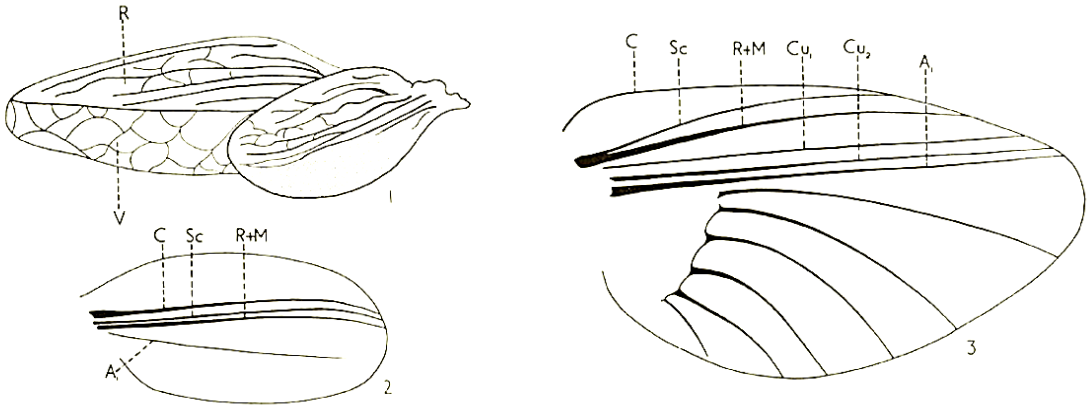

FIGURE 4.15: Wing venation of a *Bullacris discolor* female (reproduced from Dirsh, 1965).

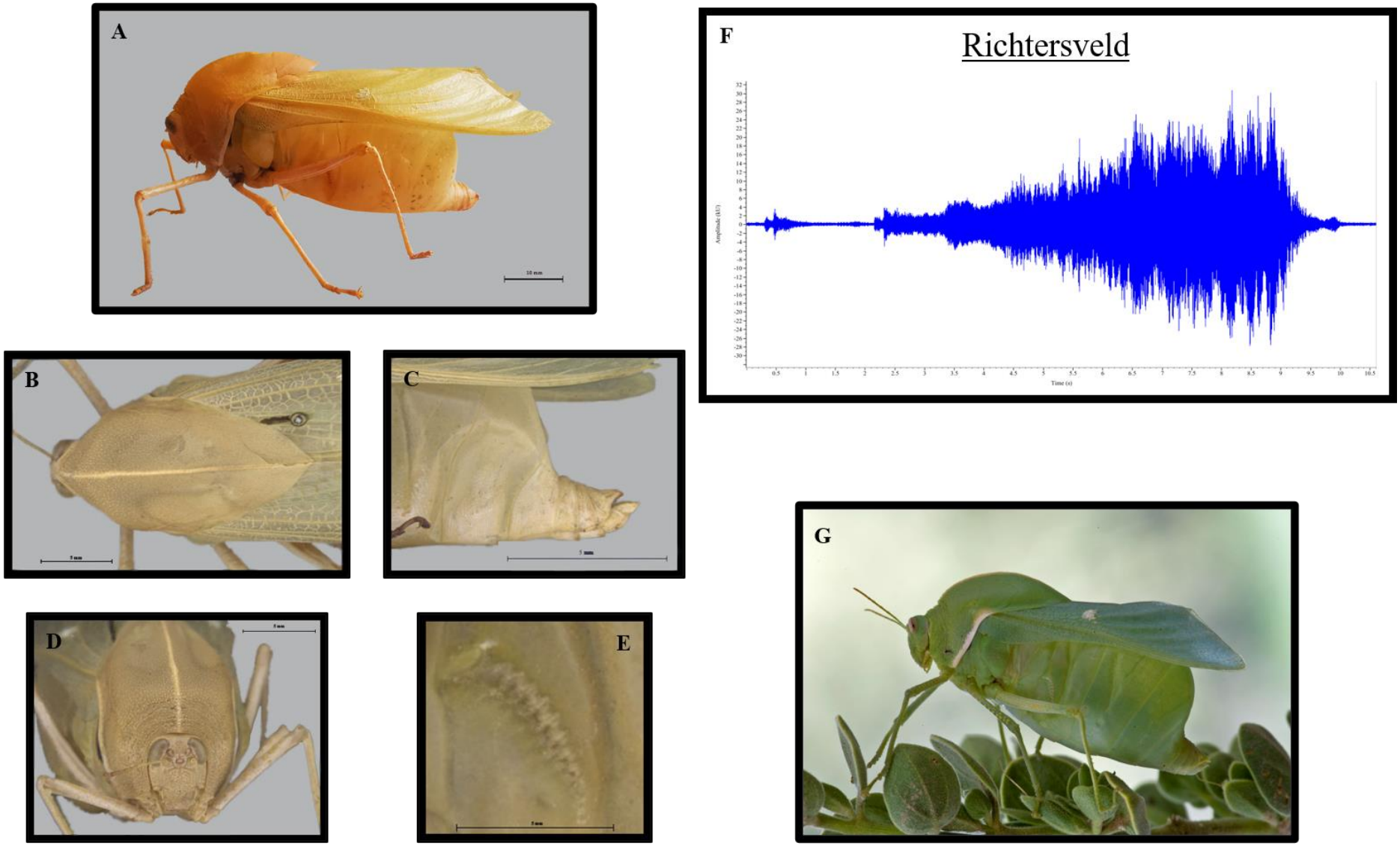

FIGURE 4.16: *Bullacris boschimana* male showing; (A): full image of specimen, (B): dorsal view of the pronotum, (C): end of abdomen side view, (D): front view of head, (E): stridulatory ridges, (F): acoustic call from the Richtersveld and (G): a live image of the species (Image and sound recording by: Piotr Naskrecki).

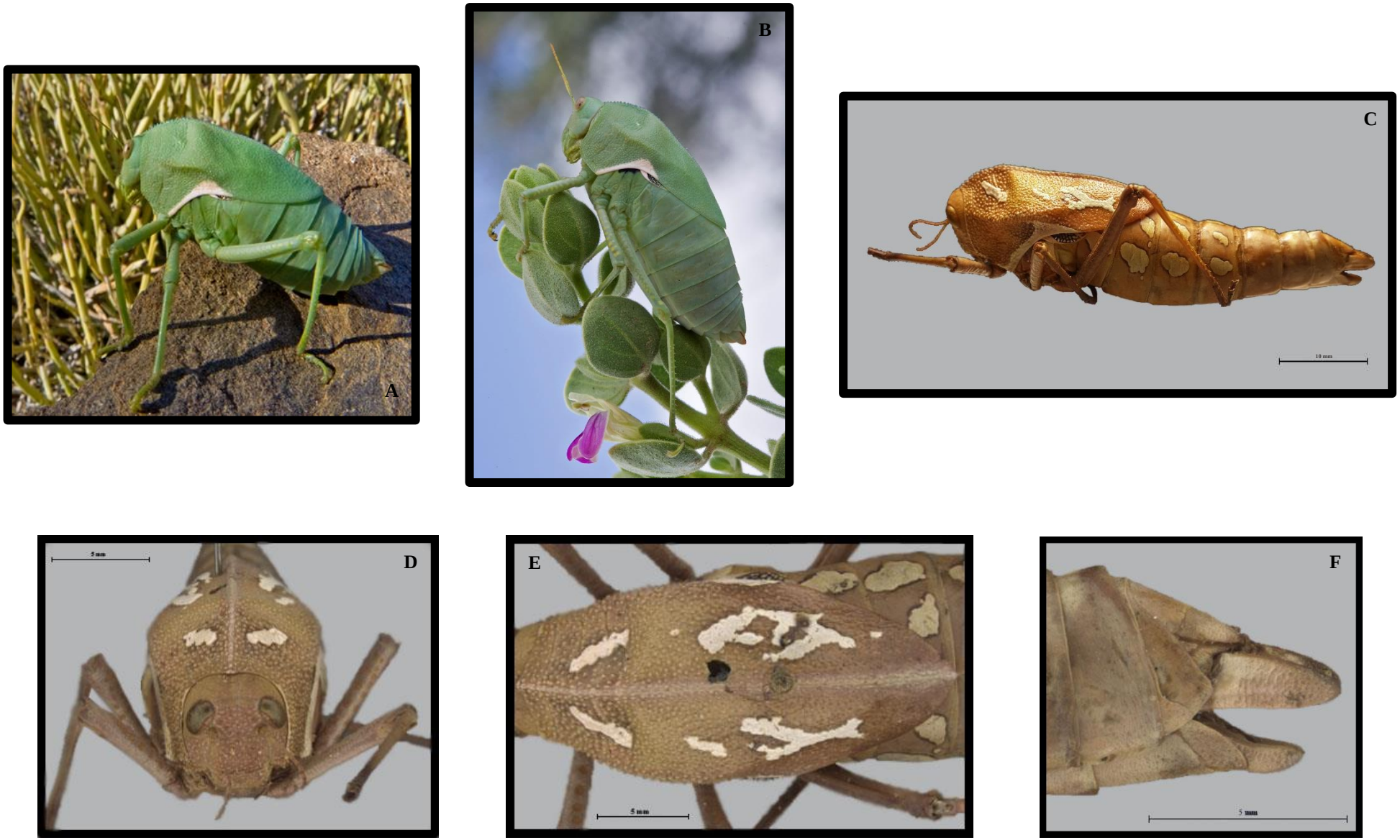

FIGURE 4.17: *Bullacris boschimana* female showing; (A & B): live images of the species (Images by: Piotr Naskrecki), (C): full image of type specimen, (D): front view of head, (E): dorsal view of the pronotum and (F): end of abdomen side view.

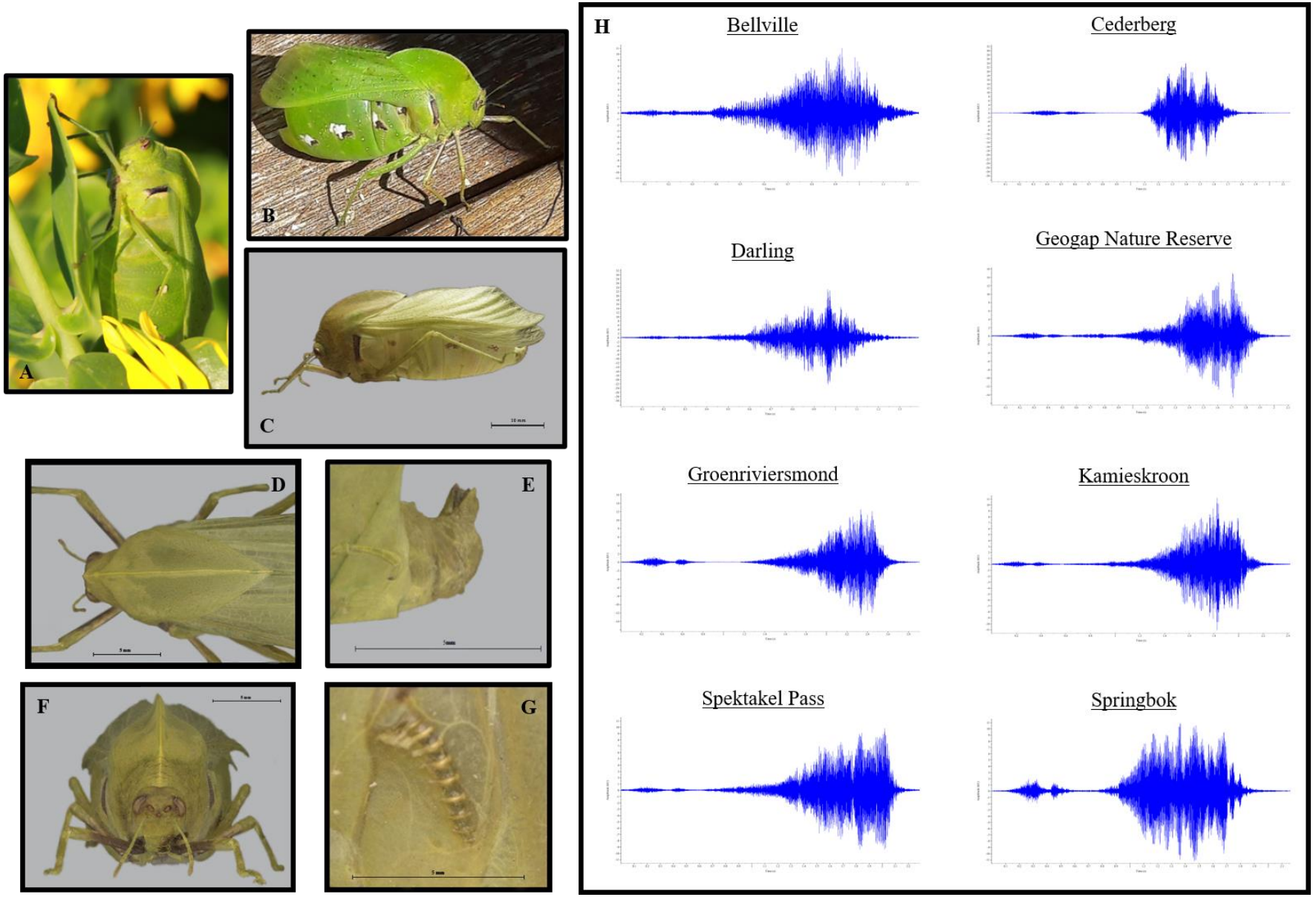

FIGURE 4.18: *Bullacris unicolor* male showing; (A & B): live images of the species (Images by: Vanessa Couldridge & Mike Cawood), (C): full image of specimen, (D): dorsal view of the pronotum, (E): end of abdomen side view, (F): front view of head, (G): stridulatory ridges and (H): acoustic calls from several locations.

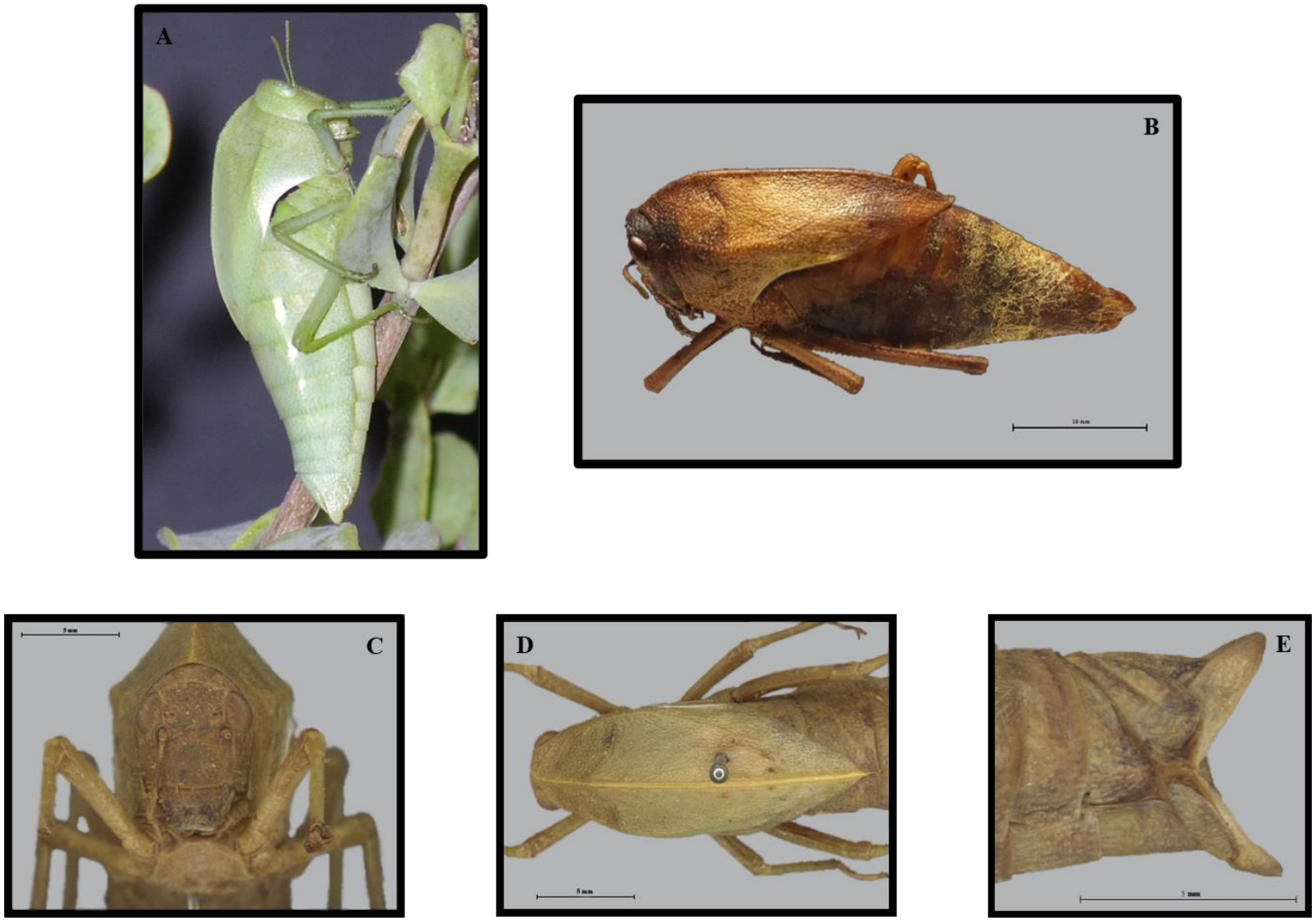

FIGURE 4.19: *Bullacris unicolor* female showing; (A): a live image of the species (Image by: Vanessa Couldridge), (B): full image of specimen, (C): front view of head, (D): dorsal view of the pronotum and (E): end of abdomen side view.

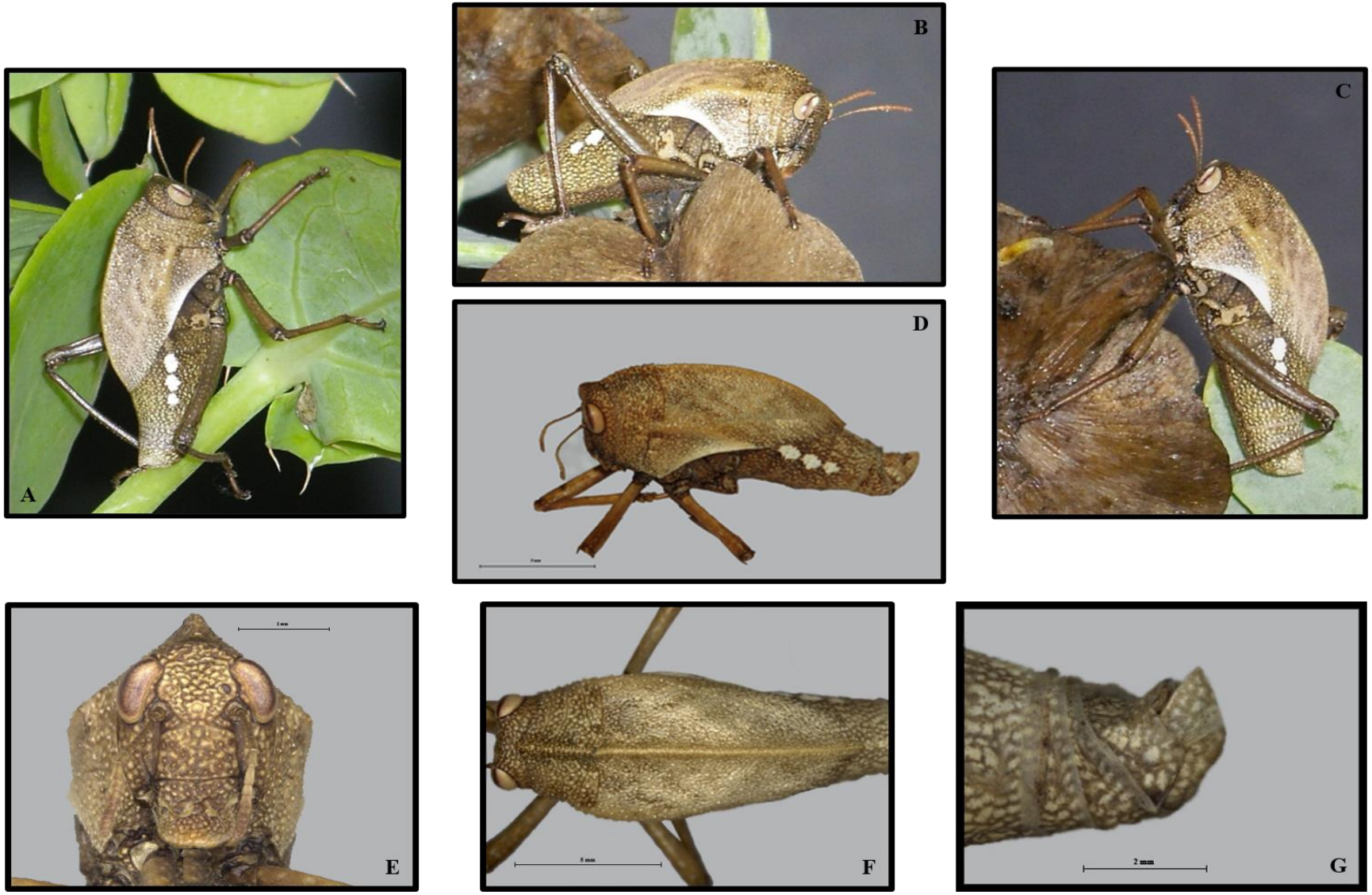

FIGURE 4.20: *Bullacris unicolor* alternate male showing; (A, B & C): live images of the species (Images by: Vanessa Couldridge), (D): full image of specimen, (E): front view of head, (F): dorsal view of the pronotum and (G): end of abdomen side view.

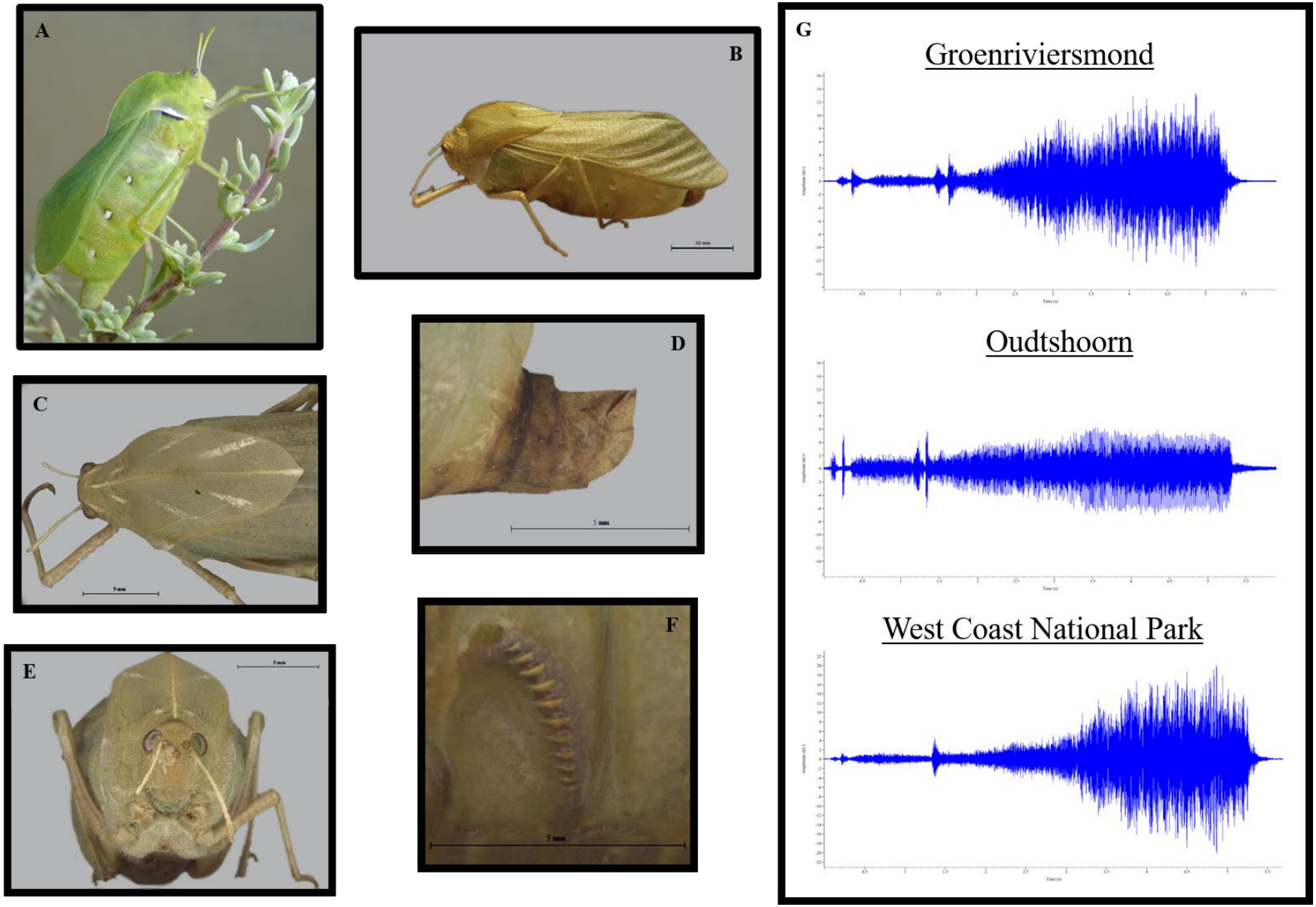

FIGURE 4.21: *Bullacris obliqua* male showing; (A): live image of the species (Image by: Vanessa Couldridge), (B): full image of specimen, (C): dorsal view of the pronotum, (D): end of abdomen side view, (E): front view of head, (F): stridulatory ridges and (G): acoustic calls from several locations.

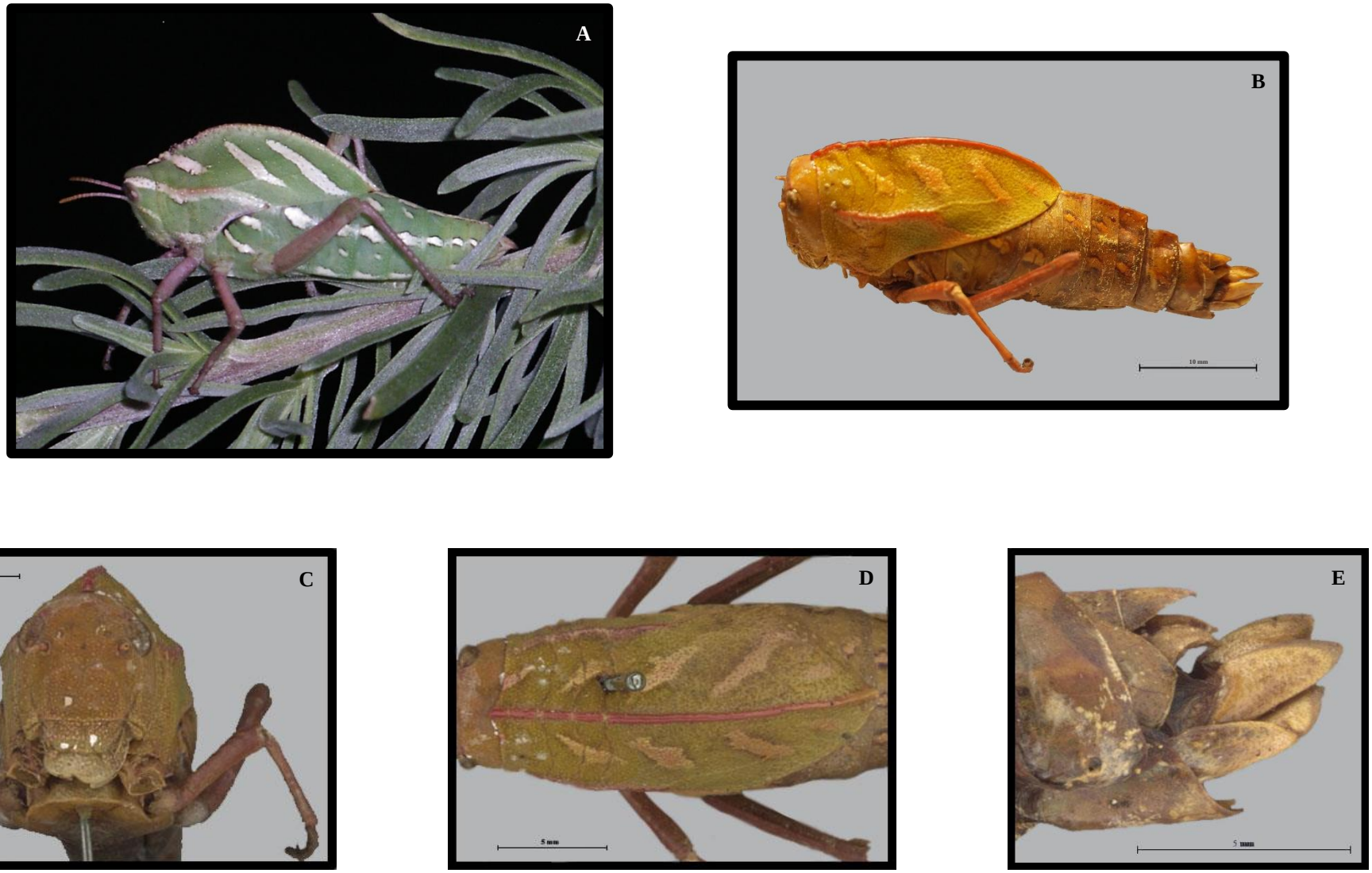

FIGURE 4.22: *Bullacris obliqua* female showing; (A): live image of the species (Image by: Vanessa Couldridge), (B): full image of specimen, (C): front view of head, (D): dorsal view of the pronotum and (E): end of abdomen side view.

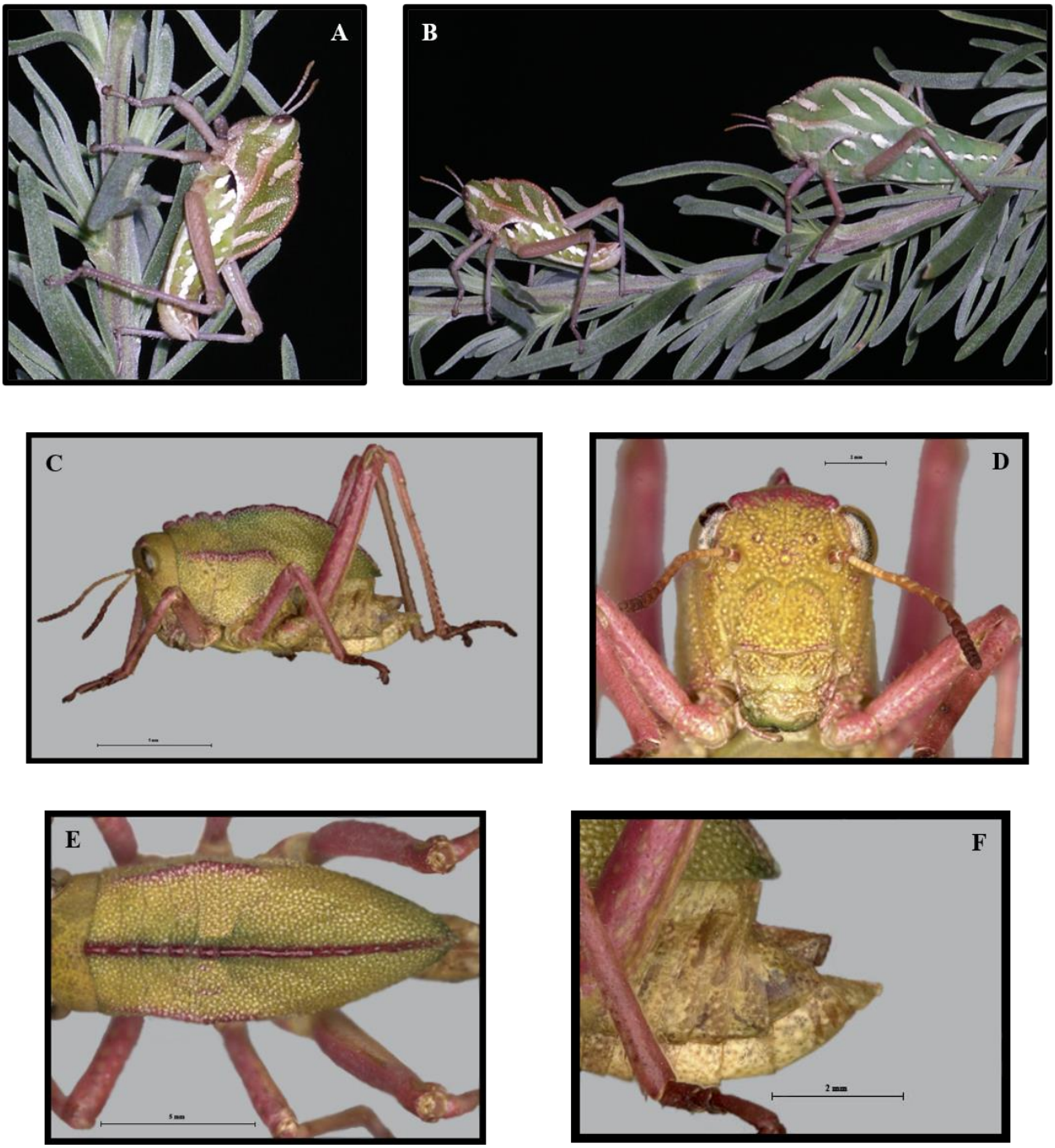

FIGURE 4.23: *Bullacris obliqua* alternate male showing; (A & B): live images of the species (Images by: Vanessa Couldridge), (C): full image of specimen, (D): front view of head, (E): dorsal view of the pronotum and (F): end of abdomen side view.

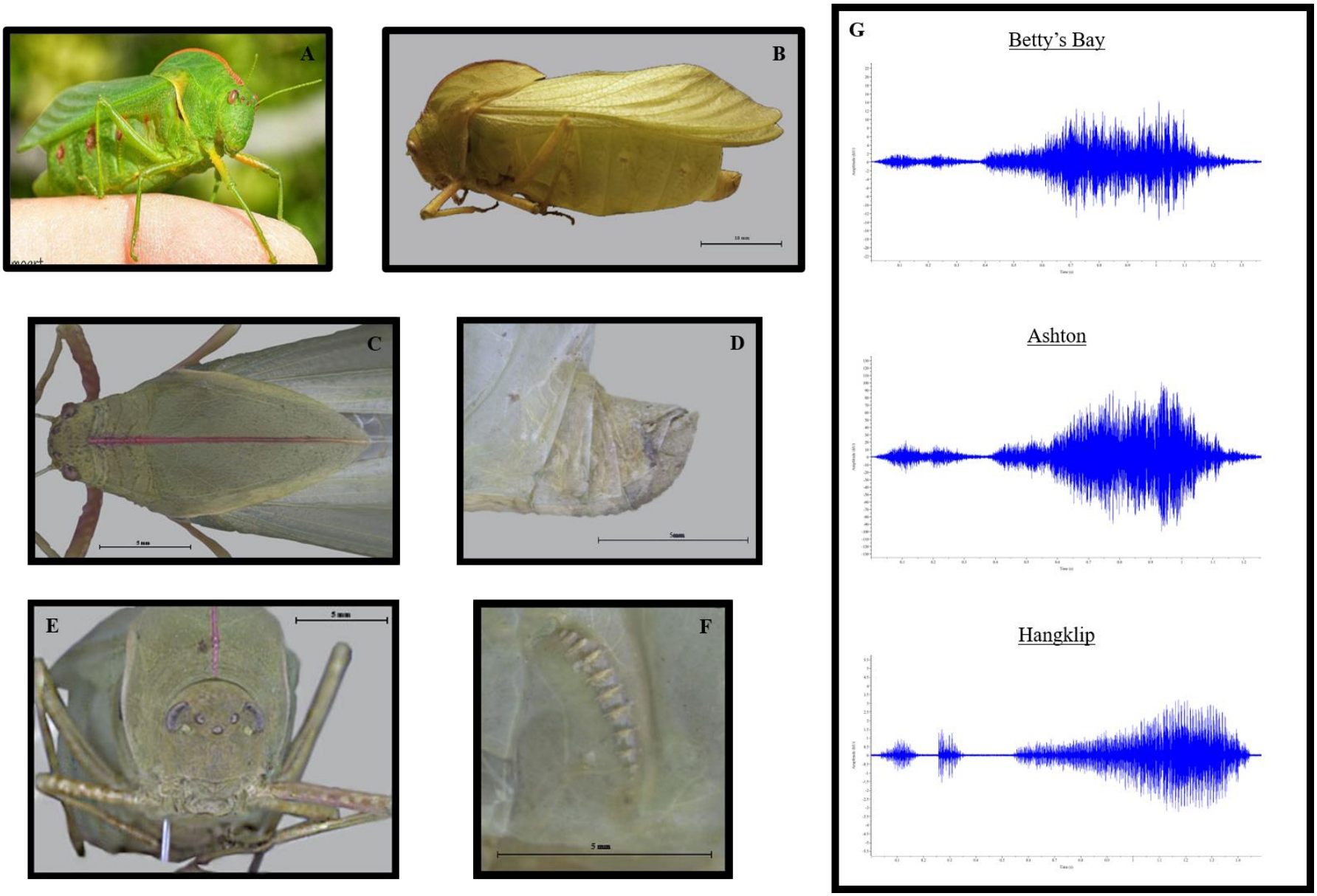

FIGURE 4.24: *Bullacris discolor* male showing; (A): live image of the species (Image by: Darryl Lampert), (B): full image of specimen, (C): dorsal view of the pronotum, (D): end of abdomen side view, (E): front view of head, (F): stridulatory ridges and (G): acoustic calls from several locations.

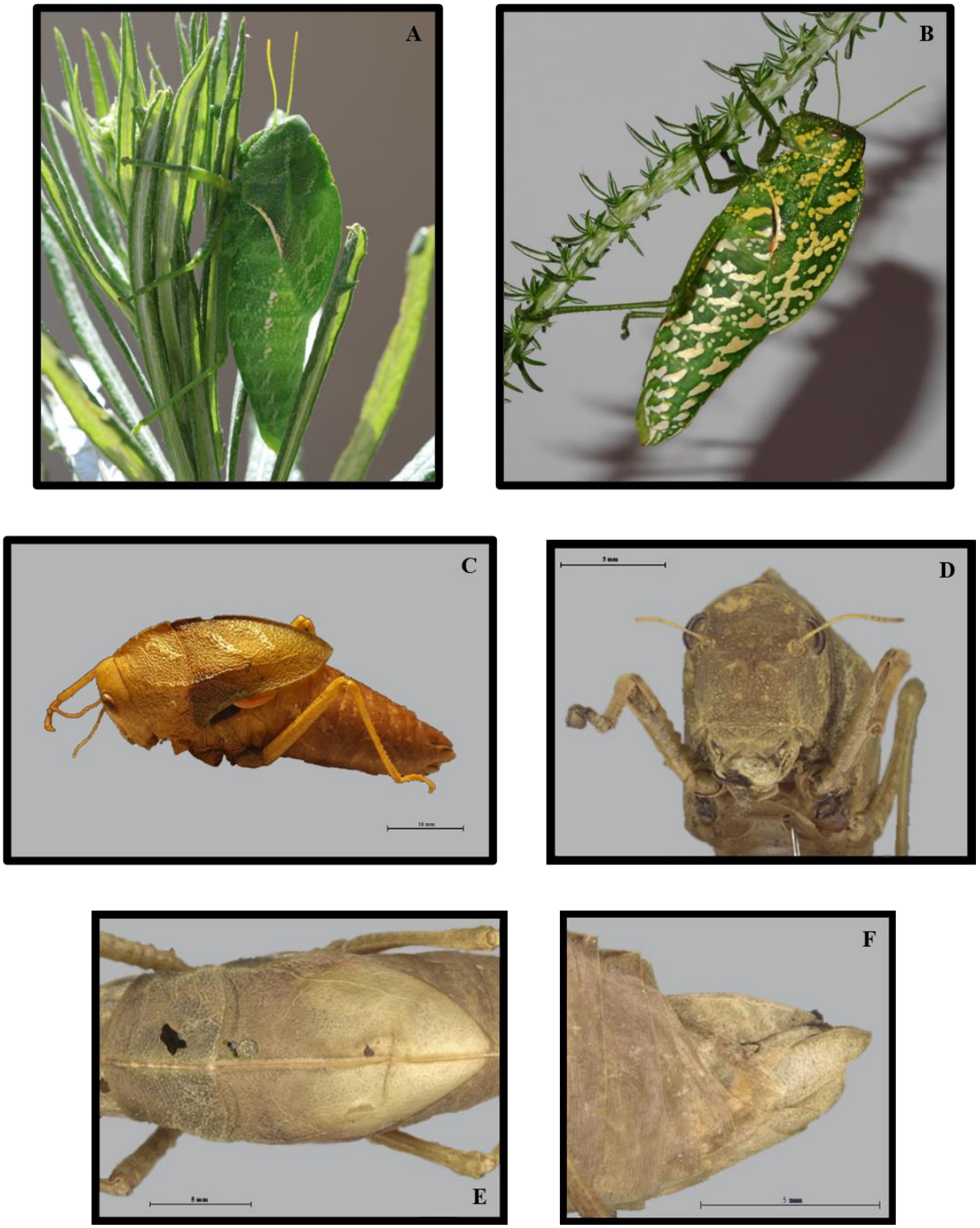

FIGURE 4.25: *Bullacris discolor* female showing; (A & B): live images of the species (Images by: Vanessa Couldridge), (C): full image of specimen, (D): front view of head, (E): dorsal view of the pronotum and (F): end of abdomen side view.

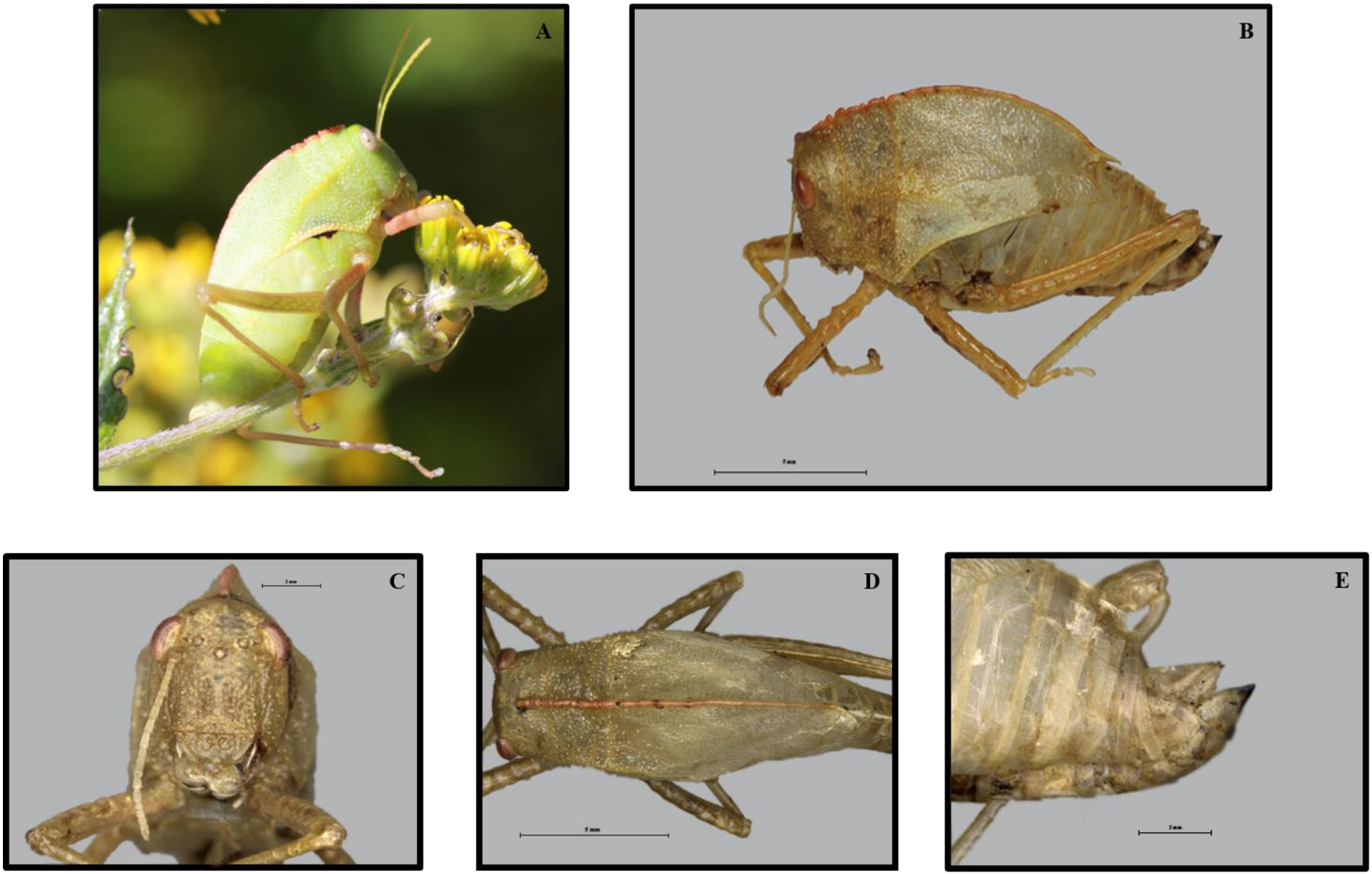

FIGURE 4.26: *Bullacris discolor* alternate male showing; (A): live image of the species (Image by: Vanessa Couldridge), (B): full image of specimen, (C): front view of head, (D): dorsal view of the pronotum and (E): end of abdomen side view.

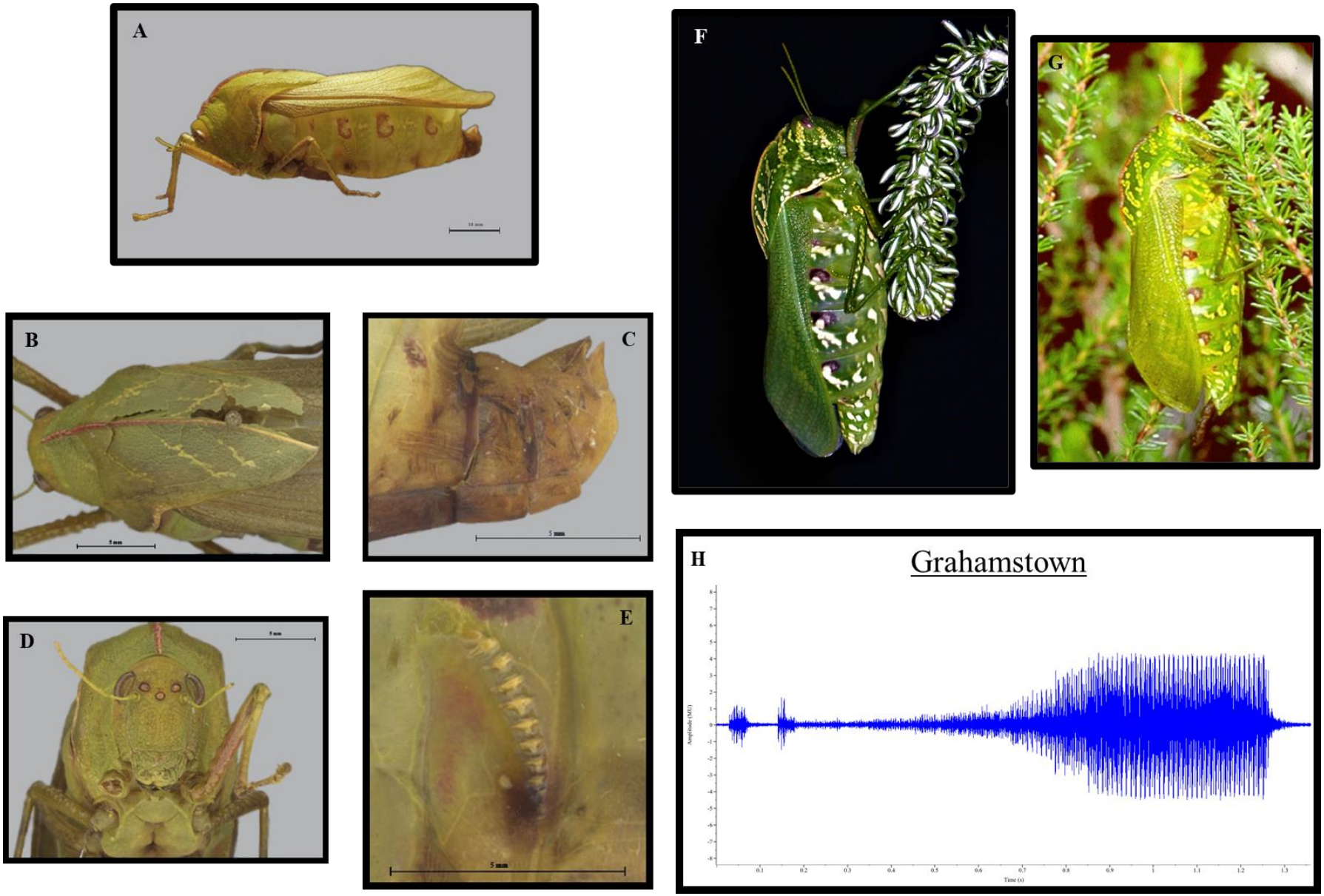

FIGURE 4.27: *Bullacris serrata* male showing; (A): full image of specimen, (B): dorsal view of the pronotum, (C): end of abdomen side view, (D): front view of head, (E): stridulatory ridges, (F & G): live images of the species (Images by: Vanessa Couldridge and Alan Weaving) and (H): an acoustic call from Grahamstown.

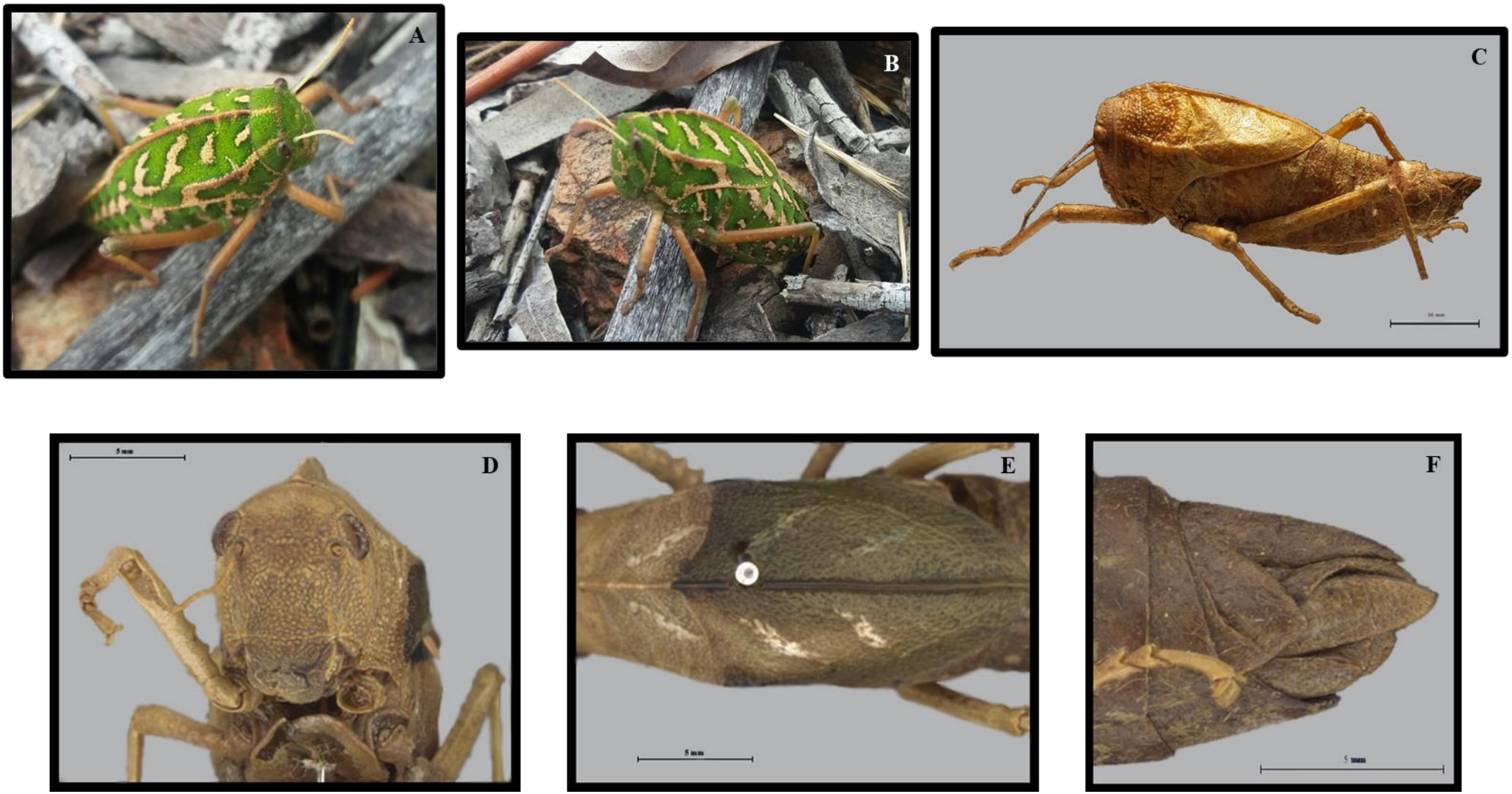

FIGURE 4.28: *Bullacris serrata* female showing; (A & B): live images of the species (Images by: Fiona Hellman), (C): full image of specimen, (D): front view of head, (E): dorsal view of the pronotum and (F): end of abdomen side view.

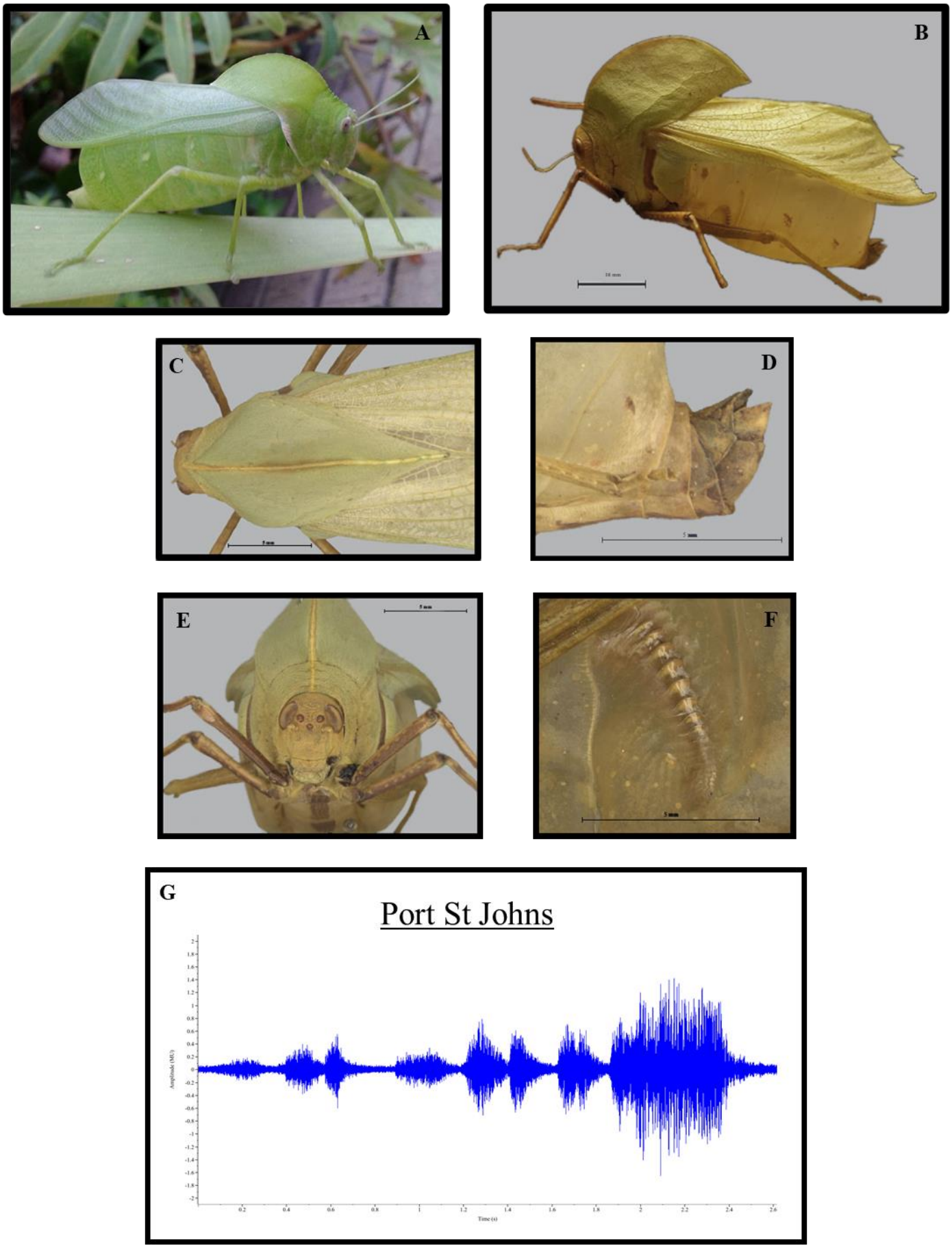

FIGURE 4.29: *Bullacris intermedia* male showing; (A): live image of the species (Image by: Lizzi Tarr), (B): full image of specimen, (B): dorsal view of the pronotum, (C): end of abdomen side view, (D): front view of head, (E): stridulatory ridges and (F): an acoustic call from Port St Johns.

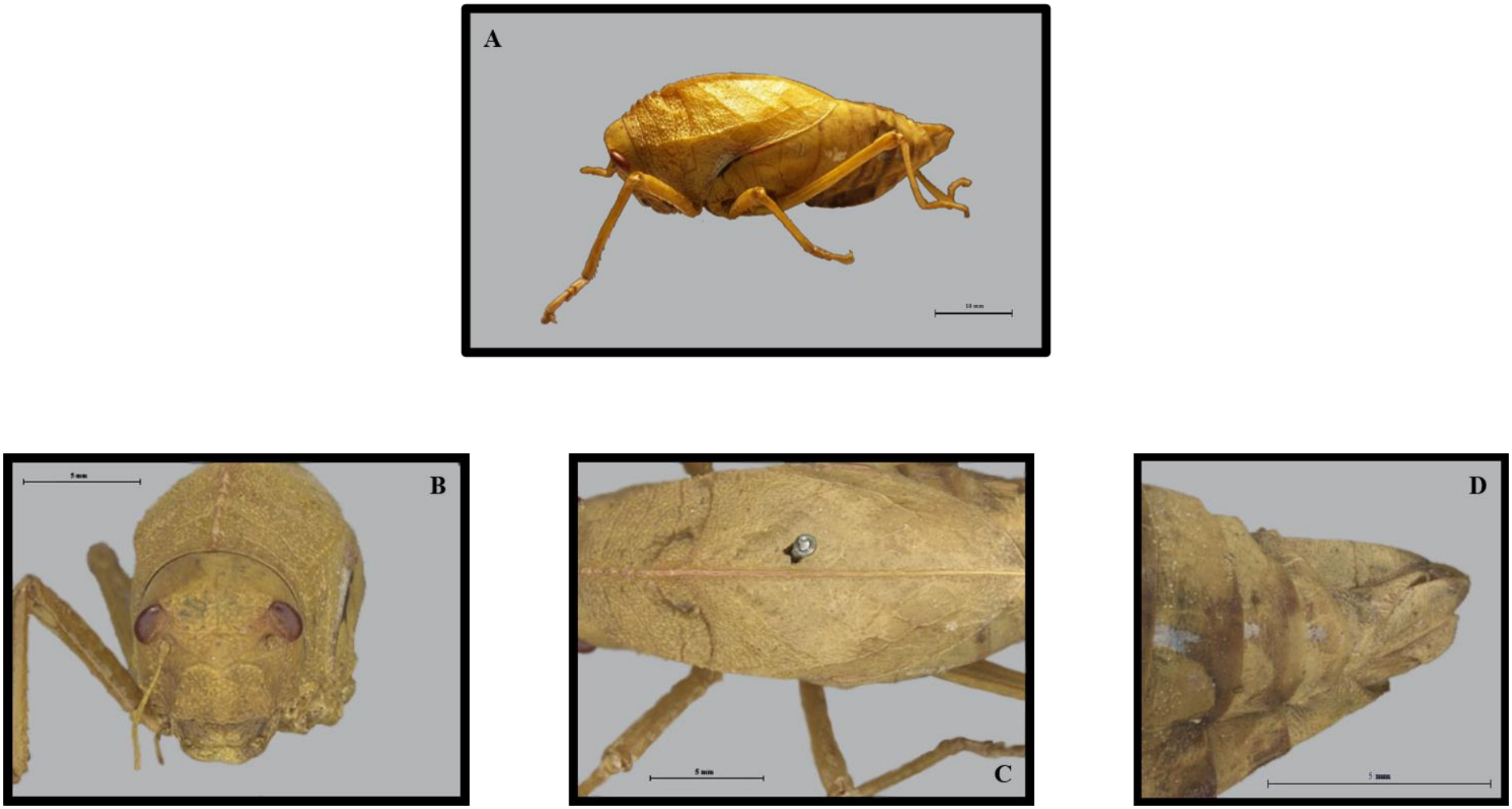

FIGURE 4.30: *Bullacris intermedia* female showing; (A): full image of specimen, (B): front view of head, (C): dorsal view of the pronotum and (D): end of abdomen side view.

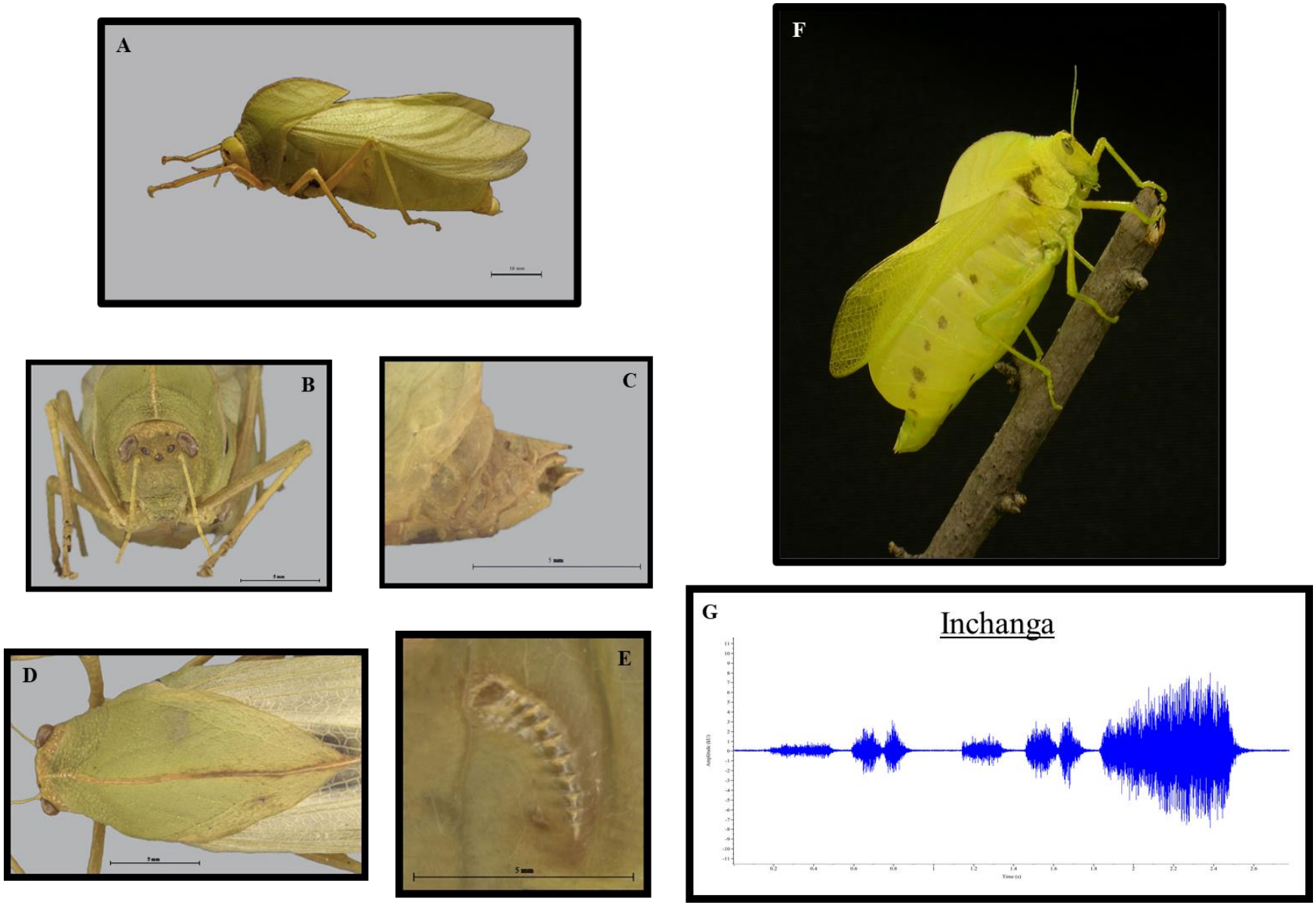

FIGURE 4.31: *Bullacris membracioides* male showing; (A): full image of specimen, (B): front view of head, (C): end of abdomen side view, (D): dorsal view of the pronotum, (E): stridulatory ridges, (F): live image of species (Image by: Vanessa Couldridge) and (G): an acoustic call from Inchanga.

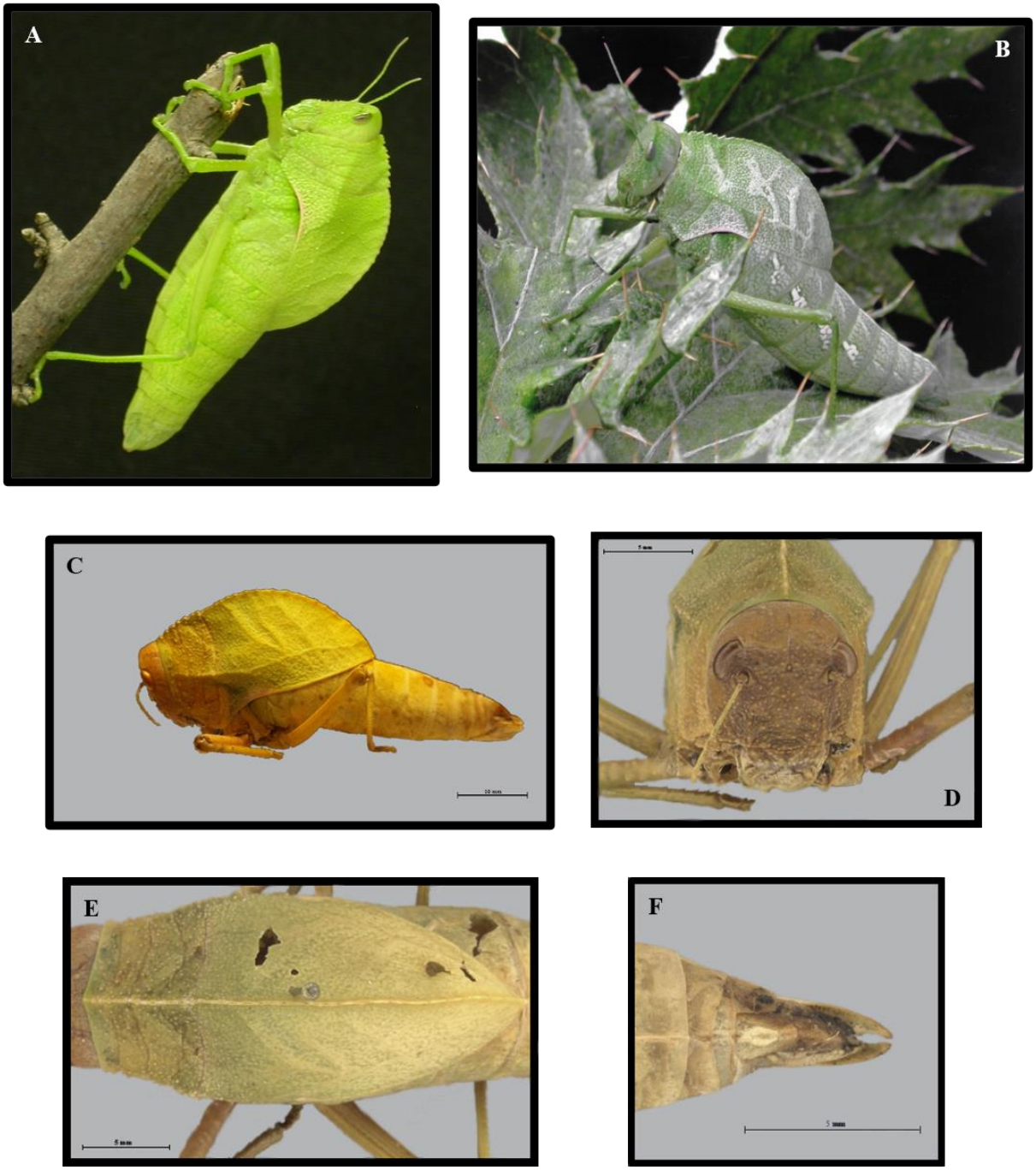

FIGURE 4.32: *Bullacris membracioides* female showing; (A & B): live images of species (Images by: Vanessa Couldridge), (C): full image of specimen, (D): front view of head, (E): dorsal view of the pronotum and (F): end of abdomen side view.

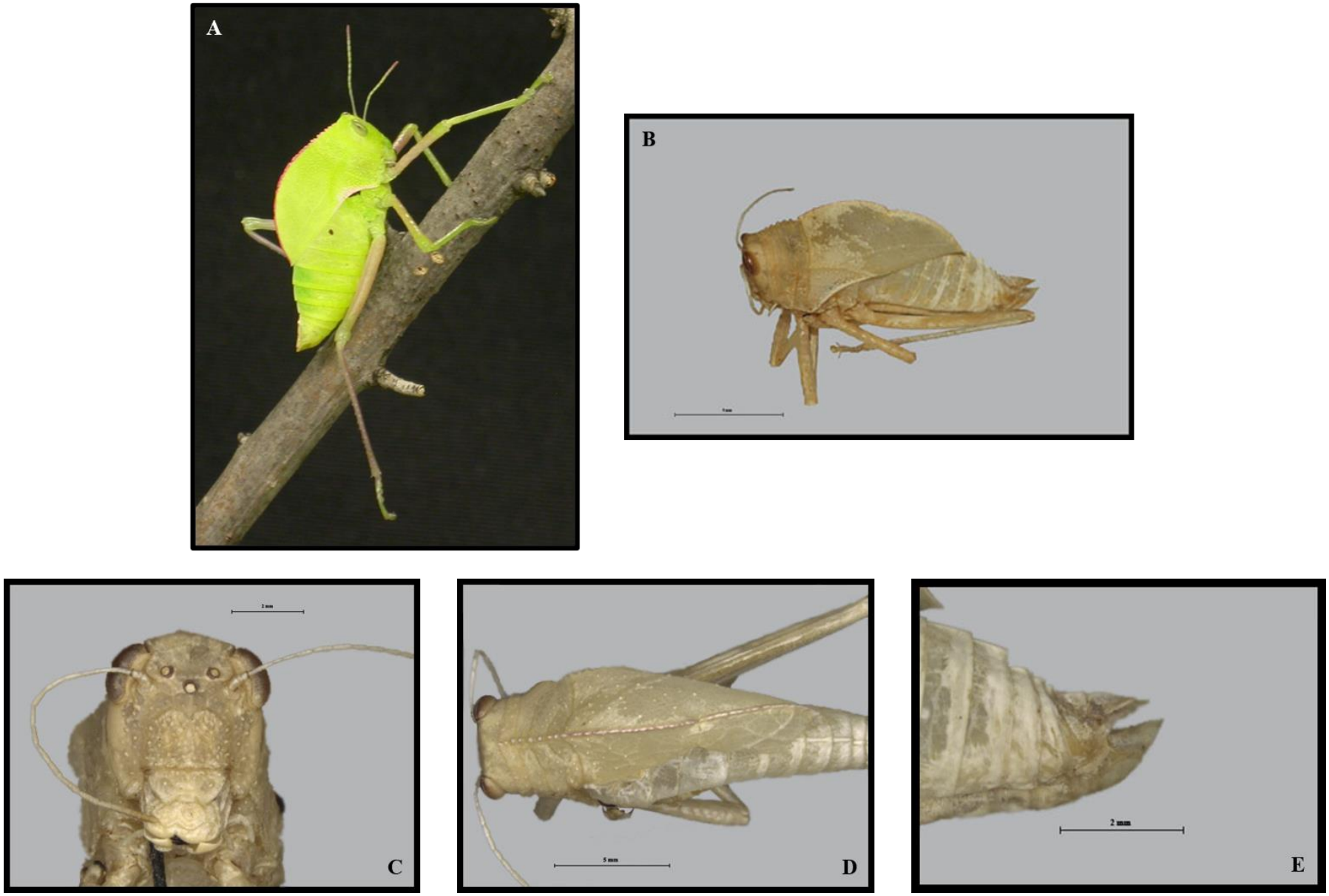

FIGURE 4.33: *Bullacris membracioides* alternate male showing; (A): live image of the species (Image by: Vanessa Couldridge), (B): full image of specimen, (C): front view of head, (D): dorsal view of the pronotum and (E): end of abdomen side view.

Acoustic analyses

A total of 834 male acoustic calls were analysed, with temporal and frequency properties calculated (Figures 4.34 & 4.35). Mating calls are quite distinctive between most species as well as between some populations (Figures 4.36 – 4.41). However, calls between populations are still recognisable as belonging to the same species. Averages of temporal and frequency call properties for all *Bullacris* males are presented in Table 4.12. *Bullacris boschimana* has the longest call with an average of 9.565 s, whereas *B. serrata* has the shortest call of 1.328 s (Figures 4.36 & 4.41). The call with the lowest carrier frequency is *B. boschimana* at 1550.4 Hz, whereas *B. discolor* has the highest carrier frequency at 2347.9 Hz and also has the shortest introductory syllables (Figures 4.36 & 4.39).

Bullacris obliqua, *B. intermedia* and *B. serrata* have no inter-syllable pauses, while *B. boschimana* has the longest pause (1.03 s). In addition, there is some variation in inter-syllable pauses within species. For example, *B. discolor* recorded in Betty's Bay does not have inter-syllable pauses; however, recordings from Hangklip and Ashton have clear pauses between the introductory and final syllables (Figure 4.39). This is also true for *B. unicolor* populations, in that clear inter-syllable pauses are present in some locations, but not others, and individuals from southern locations often drop the introductory syllables altogether, producing only the final syllable (Figures 4.36 – 4.38).

Bullacris unicolor, *B. discolor* and *B. serrata* (Figures 4.36 – 4.38; 4.39 & 4.41) have two introductory syllables, whereas *B. membracioides* and *B. intermedia* have six, but sometimes seven (Figure 4.41). *Bullacris discolor* and *B. serrata* have very similar call characteristics, as do *B. membracioides* and *B. intermedia*.

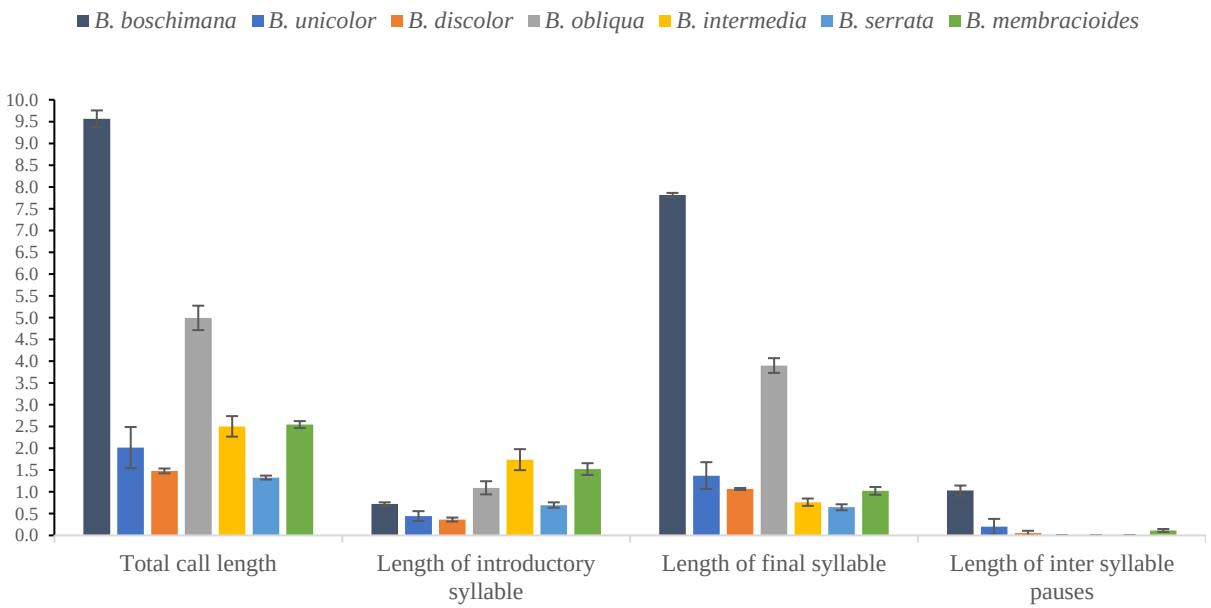

FIGURE 4.34: Bar graph showing the mean and standard deviation (error bars) for the total call length, introductory syllable length, final syllable length and the inter-syllable pause for *Bullacris* male calls.

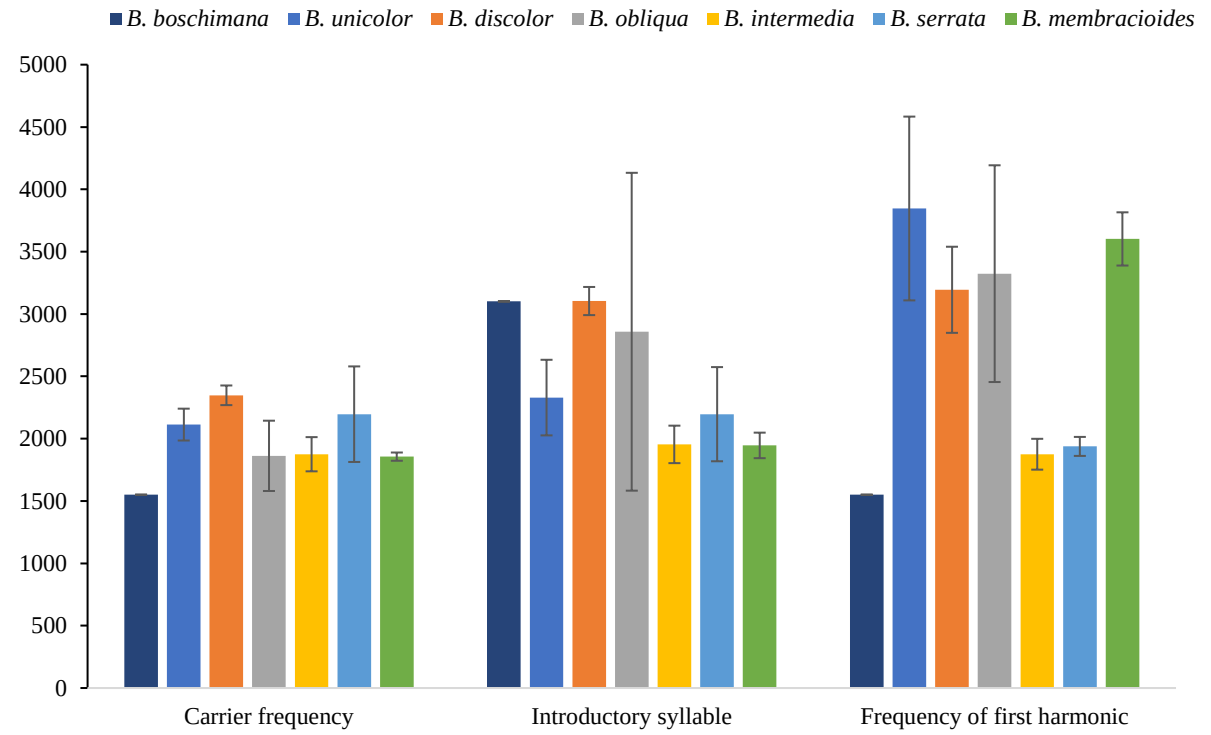

FIGURE 4.35: Bar graph showing the mean and standard deviation (error bars) for peak frequencies of the total call and, introductory syllables and the frequency of the first harmonic for *Bullacris* male calls**.**

TABLE 4.12: Temporal properties of *Bullacris* male advertisement calls, showing the highest and lowest values in bold.

Species	Location	Number of calls	total call length	length of introductory syllables	length of final syllable	inter syllable pause	carrier frequency	introductory syllables	frequency of 1st harmonic
B. boschimana	Richtersveld	5	**(9.565 ± 0.1909)**	(0.720 ± 0.0397)	**(7.814 ± 0.0481)**	**(1.03 ± 0.1155)**	**(1550.4 ± 0)**	(3100.8 ± 0)	**(1550.4 ± 0)**
B. unicolor	Bellville	20	1.550	0.273	1.222	0.055	1875.0	2643.8	3009.4
	Cederberg	50	1.809	0.497	0.906	0.407	2107.5	2013.8	2947.9
	Darling	52	1.350	0.280	1.070	0.000	2210.3	2318.8	4323.3
	Goegap Nature Reserve	35	2.028	0.459	1.570	0.000	2223.2	2512.5	4516.1
	Groenriviersmond	71	2.597	0.574	1.586	0.437	2226.2	2466.5	4476.2
	Spektakel Pass	85	2.700	0.490	1.851	0.359	1972.1	1846.3	2931.6
	Kamieskroon	50	2.206	0.550	1.485	0.166	2171.3	2148.8	4331.3
	Springbok	50	1.888	0.433	1.296	0.158	2118.8	2685.0	4233.8
		413	(2.016 ± 0.472)	(0.444 ± 0.113)	(1.373 ± 0.308)	(0.198 ± 0.181)	(2113.0 ± 127.8)	(2329.4 ± 303.2)	**(3846.2 ± 737.0)**
B. obliqua	Groenriviersmond	50	4.905	1.137	3.768	0	1687.5	2902.5	3003.8
	Oudtshoorn	16	5.306	1.216	4.090	0	1711.9	1561.2	2659.3
	West Coast National Park	36	4.767	0.925	3.842	0	2187.5	4109.4	4307.3
		102	(4.993 ± 0.280)	(1.093 ± 0.151)	(3.900 ± 0.169)	**0**	(1862.3 ± 281.9)	(2857.7 ± 1274.7)	(3323.5 ± 869.3)
B. discolor	Ashton	58	1.542	0.404	1.091	0.047	2269.4	3109.9	2844.8
	Betty's Bay	50	1.436	0.374	1.062	0.000	2347.5	3213.8	3202.5
	Hangklip	46	1.466	0.314	1.045	0.108	2426.7	2988.4	3535.2
		154	(1.481 ± 0.055)	**(0.364 ± 0.046)**	(1.066 ± 0.023)	(0.052 ± 0.054)	**(2347.9 ± 78.7)**	**(3104.0 ± 112.8)**	(3194.2 ± 345.3)
B. intermedia	Port St Johns	32	(2.503 ± 0.236)	**(1.739 ± 0.241)**	(0.763 ± 0.0840)	**0**	(1875.2 ± 137.1)	(1954.0 ± 150.3)	(1875.2 ± 123.7)
B. serrata	Grahamstown	24	**(1.328 ± 0.045)**	(0.697 ± 0.062)	**(0.646 ± 0.069)**	**0**	(2052.8 ± 383.2)	(2196.4 ± 377.6)	(1938.0 ± 76.2)
B. membracioides	Inchanga	104	(2.545 ± 0.079)	(1.523 ± 0.135)	(1.022 ± 0.089)	(0.114 ± 0.033)	(1855.9 ± 33.0)	**(1945.9 ± 102.4)**	(3602.1 ± 213.5)

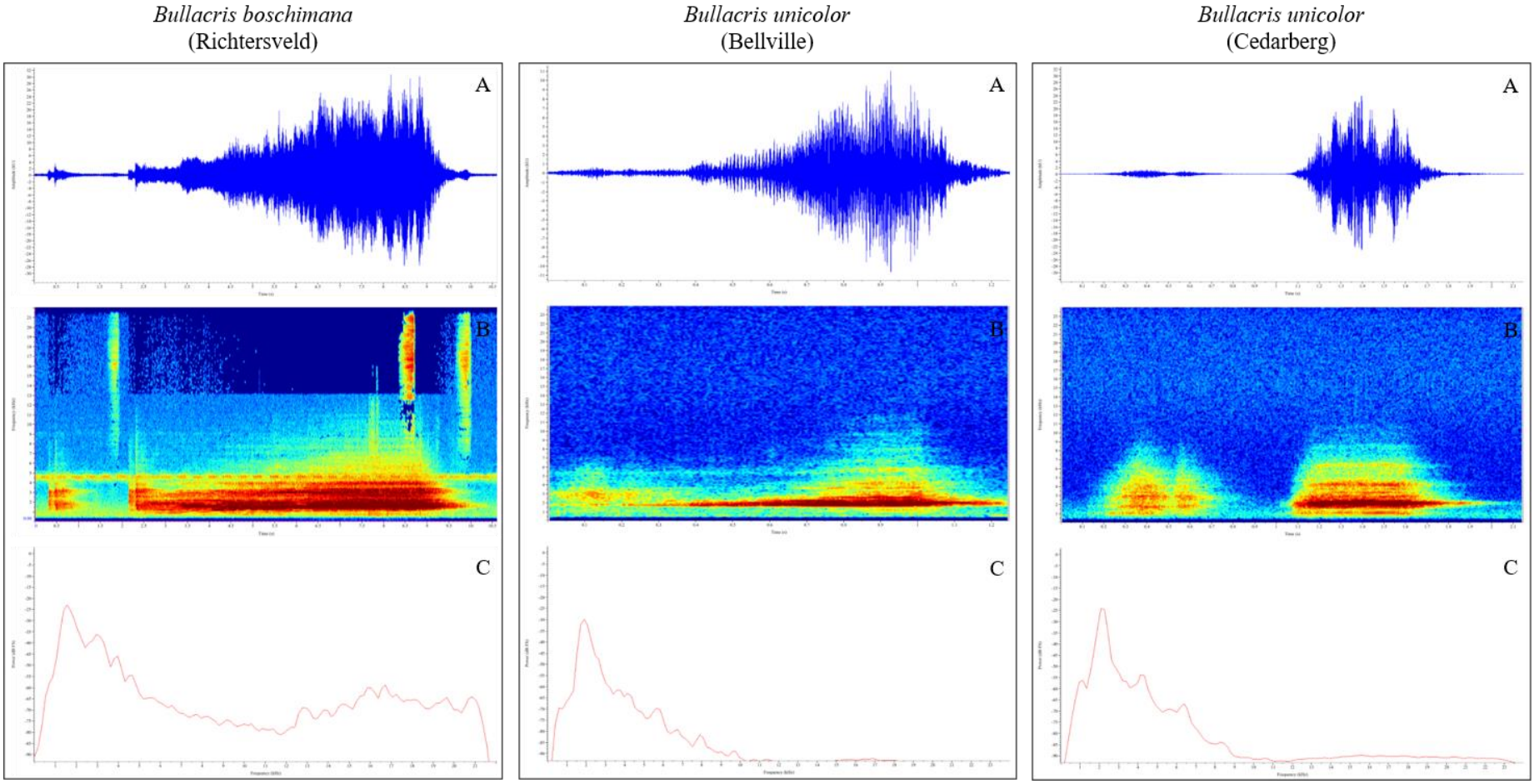

FIGURE 4.36: Acoustic calls for *Bullacris boschimana* (Richtersveld) and *Bullacris unicolor* (Bellville and Cederberg) showing the waveform (A), spectrogram (B) and spectrum (C).

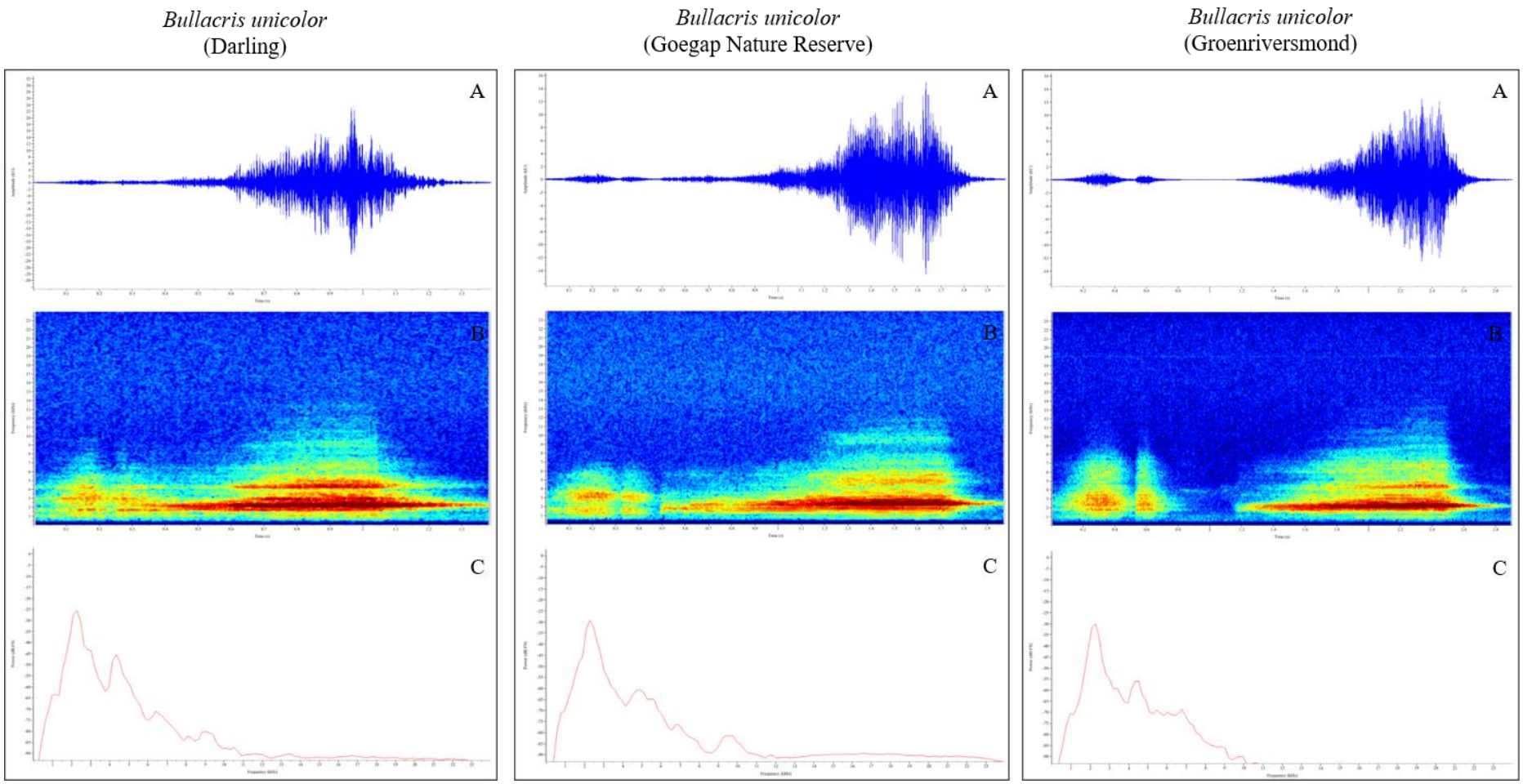

FIGURE 4.37: Acoustic calls for *Bullacris unicolor* (Darling, Geogap Nature Reserve and Groenriviersmond) showing the waveform (A), spectrogram (B) and spectrum (C).

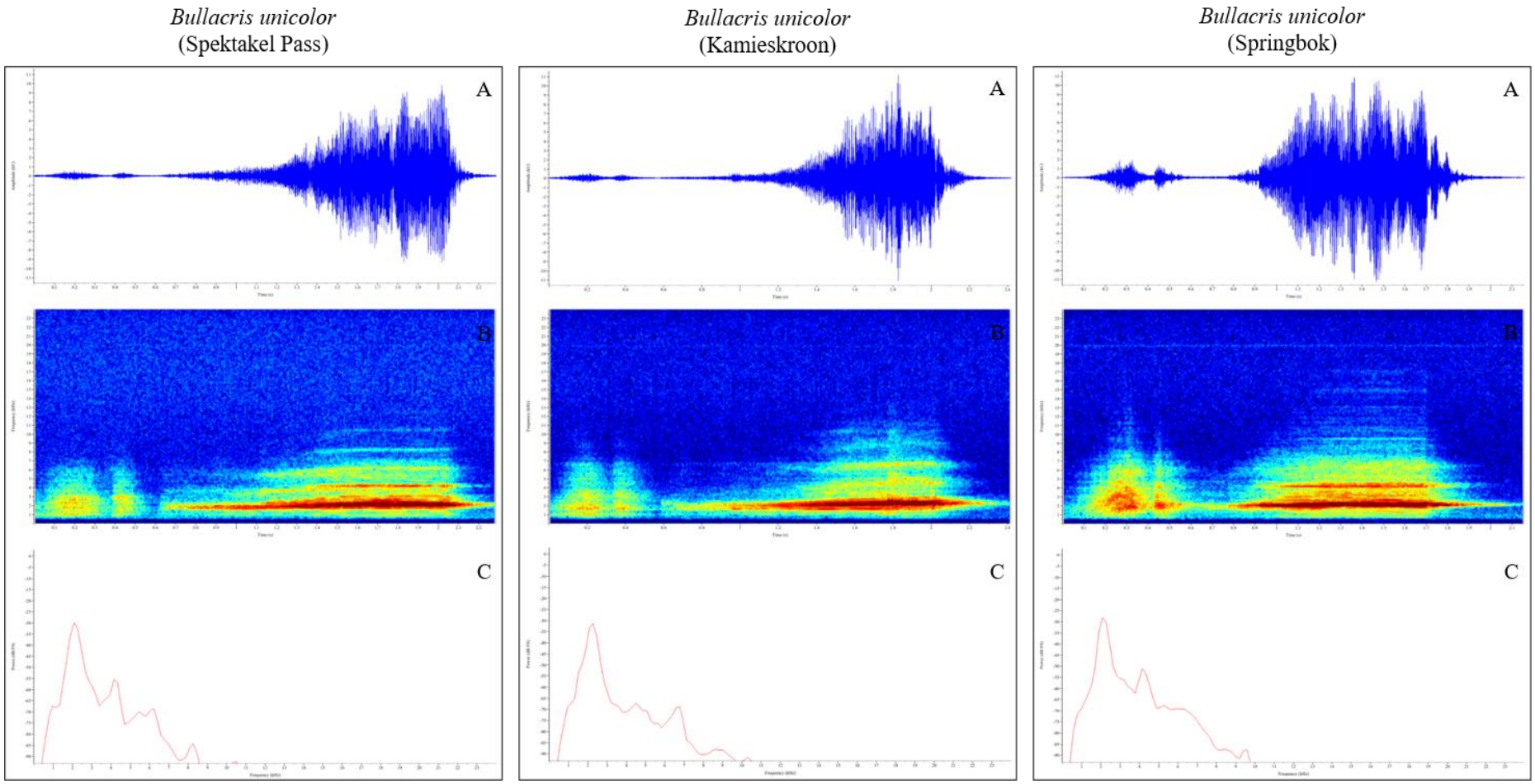

FIGURE 4.38: Acoustic calls for *Bullacris unicolor* (Spektakel Pass, Kamieskroon and Springbok) showing the waveform (A), spectrogram (B) and spectrum (C).

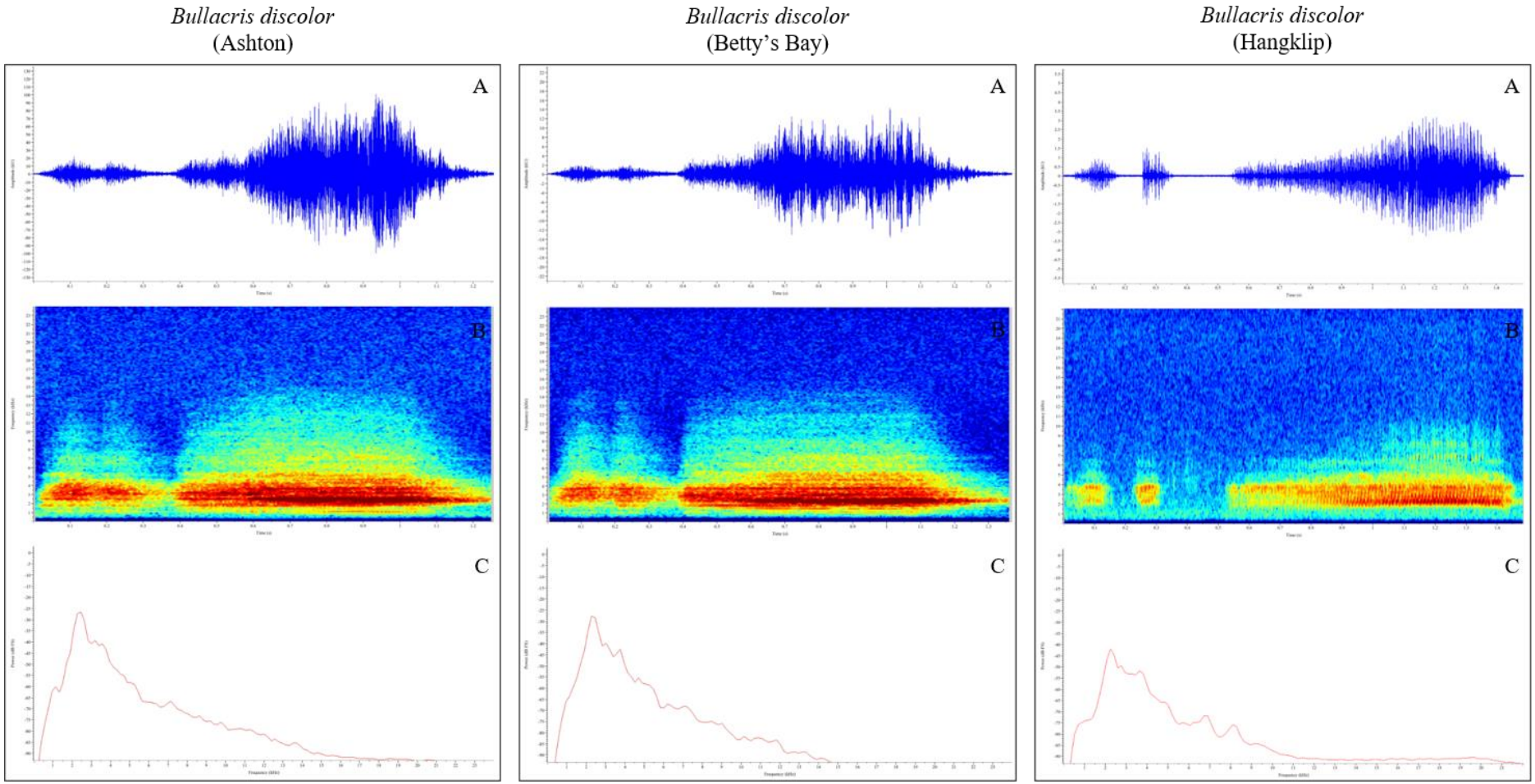

FIGURE 4.39: Acoustic calls for *Bullacris discolor* (Ashton, Betty's Bay and Hangklip) showing the waveform (A), spectrogram (B) and spectrum (C).

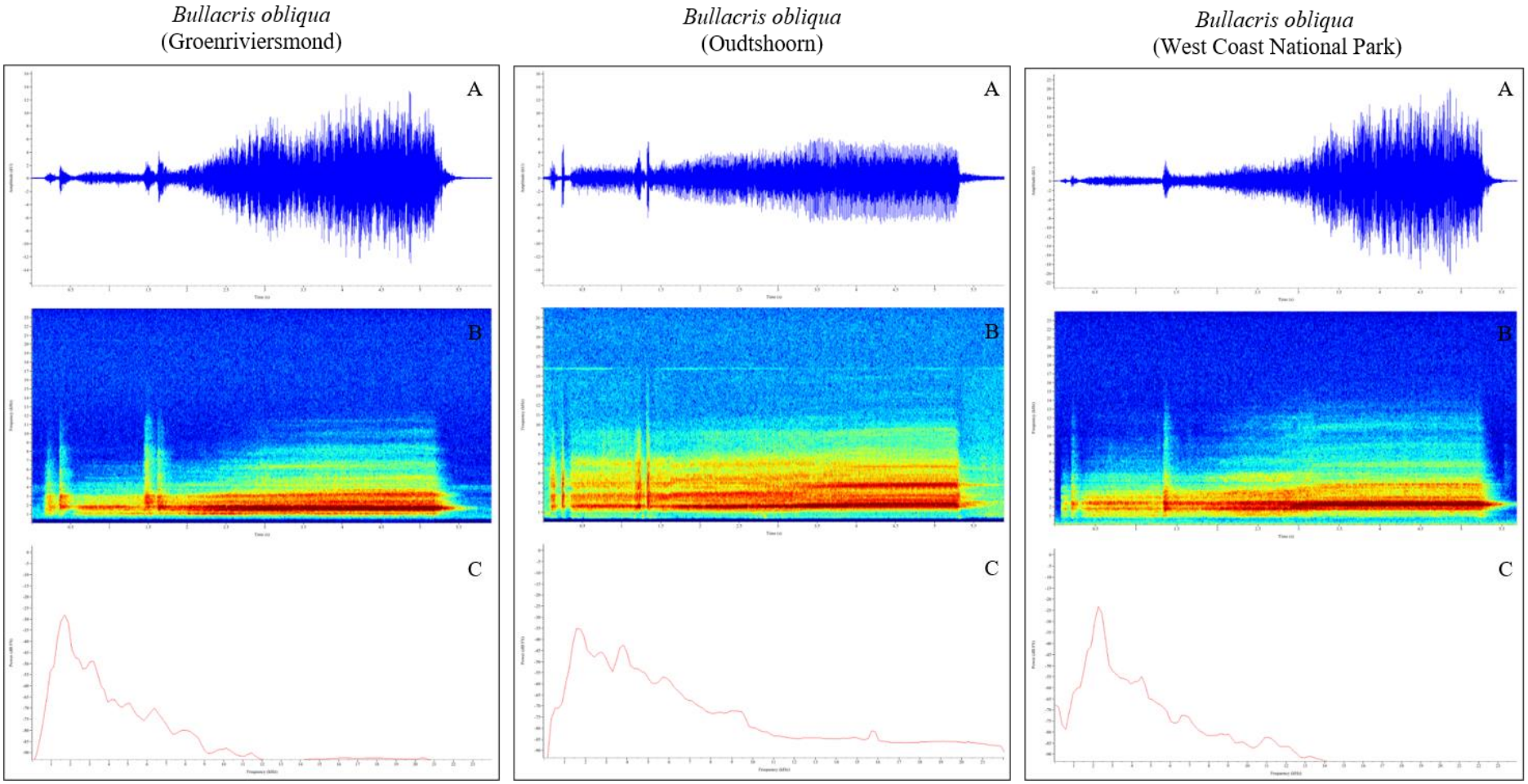

FIGURE 4.40: Acoustic calls for *Bullacris obliqua* (Groenriviersmond, Oudtshoorn and West Coast National Park) showing the waveform (A), spectrogram (B) and spectrum (C).

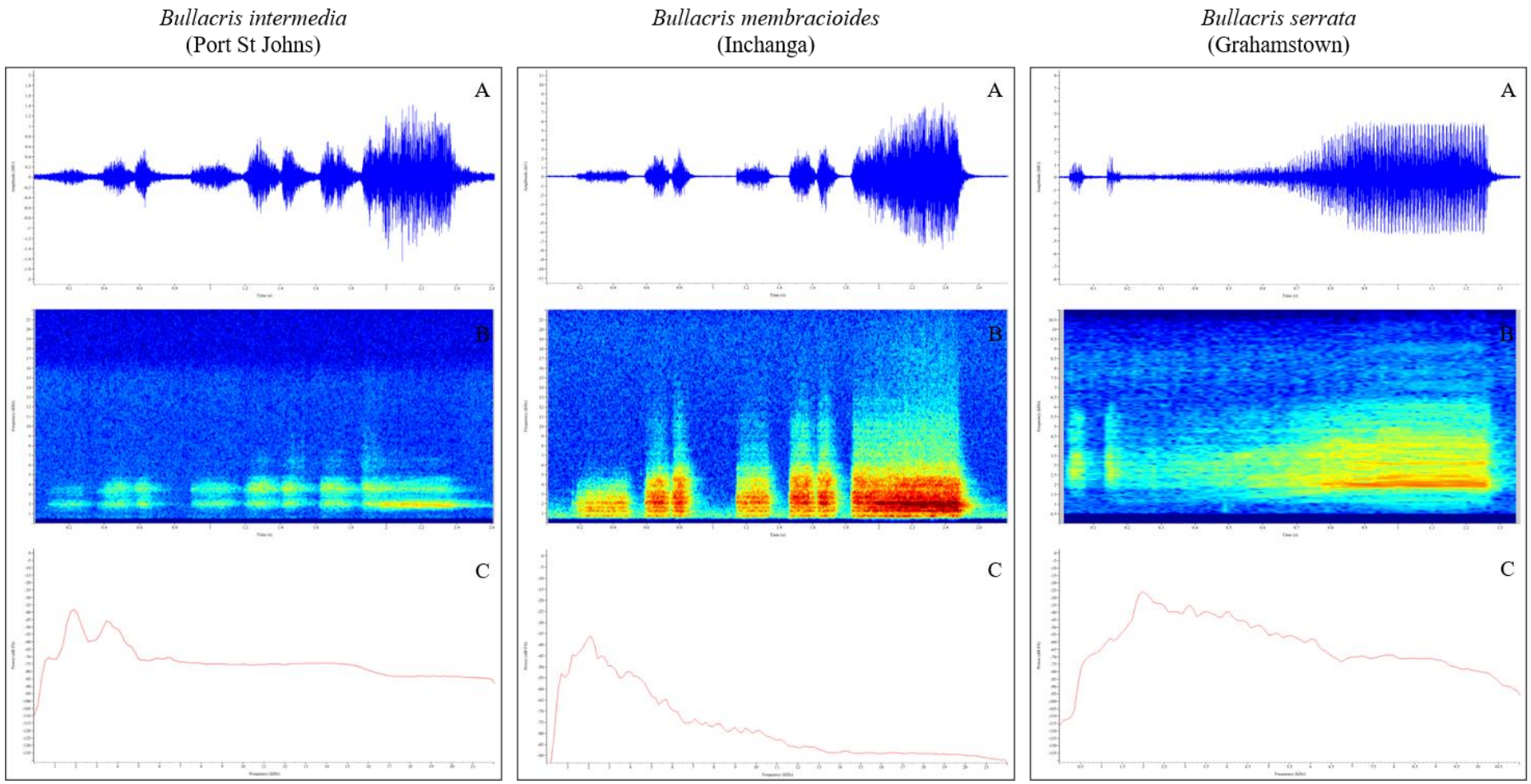

FIGURE 4.41: Acoustic calls for *Bullacris intermedia* (Port St Johns), *Bullacris membracioides* (Inchanga) and *Bullacris serrata* (Grahamstown) showing the waveform (A), spectrogram (B) and spectrum (C).

MANOVA for male acoustic variables

Multivariate analysis (Table 4.13), indicates that there is an overall significant difference in male acoustic call characteristics among species (Pillai's Trace = 2.943; $F_{42, 4962} = 113.758$; $p < 0.001$). Multiple comparisons show that call length differs significantly for *B. boschimana*, *B. unicolor* and *B. obliqua* ($p < 0.001$), whereas *B. discolor* and *B. serrata* share similarities ($p > 0.05$), as do *B. membracioides* and *B. intermedia* ($p > 0.05$). The length of the introductory syllables shows differences between all species with the exception of *B. boschimana*, which is similar to *B. serrata* ($p > 0.05$). There are similarities within the length of the final call between *B. discolor* and *B. membracioides* ($p > 0.05$), and between *B. serrata* and *B. intermedia* ($p > 0.05$). Significant differences are also observed between species for the inter-syllable pauses, such as *B. boschimana*, *B. unicolor* and *B. membracioides*; however, the remaining species share similarities ($p > 0.05$).

The carrier frequency of the acoustic calls indicates that *B. boschimana* and *B. discolor* are significantly different to all species ($p < 0.001$); however, there are similarities between *B. membracioides*, *B. intermedia* and *B. obliqua* ($p > 0.05$), as well as between *B. unicolor* and *B. serrata* ($p > 0.05$). There is not much significant variation in the frequencies of the introductory syllables between species; however, *B. boschimana* differs from *B. membracioides* and *B. intermedia*, as well as *B. serrata* from *B. discolor* and *B. obliqua* ($p < 0.001$). *Bullacris unicolor* is the only species to significantly differ in the frequency of the first harmonic ($p < 0.001$); whereas *B. boschimana*, *B. intermedia* and *B. serrata* share similarities ($p > 0.05$). In addition, *B. obliqua* has similar frequencies to *B. discolor* ($p > 0.05$) and *B. membracioides* ($p > 0.05$).

TABLE 4.13: Multiple comparisons for *Bullacris* male acoustic calls between species, showing mean differences and standard error. Significant differences are highlighted in bold.

Total call (length)

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**8.080 ± 0.220**						
B. unicolor	**7.429 ± 0.218**	**-0.651 ± 0.041**					
B. obliqua	**4.645 ± 0.222**	**-3.434 ± 0.055**	**-2.784 ± 0.048**				
B. membracioides	**7.058 ± 0.222**	**-1.021 ± 0.055**	**-0.371 ± 0.048**	**2.413 ± 0.061**			
B. serrata	**8.237 ± 0.235**	0.157 ± 0.095	**0.808 ± 0.091**	**3.592 ± 0.099**	**1.179 ± 0.098**		
B. intermedia	**7.062 ± 0.229**	**-1.018 ± 0.081**	**-0.367 ± 0.077**	**2.416 ± 0.085**	0.004 ± 0.085	**-1.175 ± 0.115**	

Introductory syllables (length)

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**0.353 ± 0.077**						
B. unicolor	**0.254 ± 0.077**	**-0.099 ± 0.014**					
B. obliqua	**-0.354 ± 0.078**	**-0.707 ± 0.019**	**-0.608 ± 0.017**				
B. membracioides	**-0.738 ± 0.078**	**-1.091 ± 0.019**	**-0.992 ± 0.017**	**-0.384 ± 0.021**			
B. serrata	0.023 ± 0.082	**-0.330 ± 0.033**	**-0.231 ± 0.032**	**0.377 ± 0.035**	**0.761 ± 0.035**		
B. intermedia	**-1.019 ± 0.080**	**-1.372 ± 0.029**	**-1.273 ± 0.027**	**-0.665 ± 0.030**	**-0.281 ± 0.030**	**-1.042 ± 0.040**	

Final syllable (length)

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**6.746 ± 0.146**						
B. unicolor	**6.386 ± 0.145**	**-0.360 ± 0.027**					
B. obliqua	**3.969 ± 0.147**	**-2.777 ± 0.037**	**-2.417 ± 0.032**				
B. membracioides	**6.766 ± 0.147**	0.020 ± 0.037	**0.379 ± 0.032**	**2.800 ± 0.040**			
B. serrata	**7.168 ± 0.156**	**0.422 ± 0.063**	**0.781 ± 0.061**	**3.199 ± 0.065**	**0.402 ± 0.065**		
B. intermedia	**7.051 ± 0.152**	**0.304 ± 0.054**	**0.664 ± 0.051**	**3.081 ± 0.057**	**0.285 ± 0.056**	-0.117 ± 0.076	

Inter-syllable pause (length)

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**0.980 ± 0.079**						
B. unicolor	**0.789 ± 0.078**	**-0.191 ± 0.015**					
B. obliqua	**1.030 ± 0.079**	0.050 ± 0.020	**0.241 ± 0.017**				
B. membracioides	**0.906 ± 0.079**	**-0.074 ± 0.020**	**0.116 ± 0.017**	**-0.124 ± 0.022**			
B. serrata	**1.030 ± 0.084**	0.050 ± 0.034	**0.241 ± 0.033**	0.000 ± 0.035	**0.124 ± 0.035**		
B. intermedia	**1.030 ± 0.082**	0.050 ± 0.029	**0.241 ± 0.027**	0.000 ± 0.030	**0.124 ± 0.030**	0.000 ± 0.041	

Carrier frequency

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**-791.34 ± 85.43**						
B. unicolor	**-569.90 ± 84.75**	**221.44 ± 15.93**					
B. obliqua	**-317.40 ± 85.98**	**473.94 ± 21.53**	**252.50 ± 19.66**				
B. membracioides	**-297.09 ± 85.95**	**494.26 ± 21.41**	**272.81 ± 18.51**	20.31 ± 23.51			
B. serrata	**-502.42 ± 91.10**	**288.93 ± 37.02**	67.49 ± 35.42	**-185.02 ± 38.27**	**-205.33 ± 38.20**		
B. intermedia	**-324.84 ± 89.03**	**466.51 ± 31.59**	**245.06 ± 29.70**	-7.44 ± 33.04	-27.75 ± 32.96	**177.58 ± 44.71**	

Introductory syllable frequency

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	-6.54 ± 301.16						
B. unicolor	836.66 ± 298.77	**843.20 ± 56.17**					
B. obliqua	-17.25 ± 303.10	-10.71 ± 75.91	**-853.91 ± 65.77**				
B. membracioides	**1102.85 ± 302.99**	**1109.38 ± 75.48**	**266.19 ± 65.26**	**1120.10 ± 82.87**			
B. serrata	904.40 ± 321.15	**910.94 ± 130.50**	67.74 ± 124.87	**921.66 ± 134.91**	-198.44 ± 134.66		
B. intermedia	**1146.83 ± 313.86**	**1153.36 ± 111.35**	**310.16 ± 104.70**	**1164.08 ± 116.49**	43.98 ± 116.20	242.42 ± 157.60	

Frequency of the first harmonic

	B. boschimana	*B. discolor*	*B. unicolor*	*B. obliqua*	*B. membracioides*	*B. serrata*	*B. intermedia*
B. boschimana							
B. discolor	**-1616.77 ± 340.10**						
B. unicolor	**-2288.80 ± 337.39**	**-672.03 ± 63.43**					
B. obliqua	**-1859.40 ± 342.28**	-242.63 ± 85.73	**429.40 ± 74.27**				
B. membracioides	**-1944.84 ± 342.16**	**-328.07 ± 85.23**	**343.96 ± 73.69**	-85.44 ± 93.58			
B. serrata	-387.58 ± 362.67	**1229.19 ± 147.37**	**1901.22 ± 141.01**	**1471.82 ± 152.35**	**1557.26 ± 152.07**		
B. intermedia	-324.83 ± 354.43	**1291.93 ± 125.75**	**1963.97 ± 118.23**	**1534.56 ± 131.55**	**1620.00 ± 131.23**	62.741 ± 177.97	

DFA results for acoustic calls

The DFA for male acoustic calls indicates that there are significant differences between calls of the *Bullacris* species ($p < 0.05$). The eigenvalues indicated that 51.1% of the variation was contributed by Discriminant Function 1 and 39.6% of the variation contributed by Discriminant Function 2 (Table 4.14). Discriminant Function 1 has a strong positive correlation with the length of the final syllable, and a negative correlation with the length of the entire call (Table 4.15). Furthermore, DF 2 has a positive correlation with the length of the introductory and final syllables and a negative correlation with the total length of the call. The canonical centroid plot shows that *B. unicolor* and *B. discolor* have overlapping clusters, as do *B. intermedia* and *B. membracioides* (Figure 4.42). *Bullacris boschimana* and *B. obliqua* have unique calls and do not overlap with any other species.

TABLE 4.14: Eigenvalues for *Bullacris* male acoustic calls. The percentage of variation for Function 1 and 2 are highlighted in bold.

Eigenvalues				
Function	**Eigenvalue**	**% of Variance**	**Cumulative %**	**Canonical Correlation**
1	13.584[a]	**51.1**	51.1	0.965
2	10.532[a]	**39.6**	90.8	0.956
3	1.904[a]	7.2	97.9	0.810
4	0.323[a]	1.2	99.1	0.494
5	0.165[a]	0.6	99.8	0.376
6	0.061[a]	0.2	100.0	0.240

a. First 6 canonical discriminant functions were used in the analysis.

TABLE 4.15: Standardized canonical discriminant function coefficients for *Bullacris* male acoustic calls. The strongest correlation values are highlighted in bold.

Standardized Canonical Discriminant Function Coefficients						
	Function					
	1	**2**	**3**	**4**	**5**	**6**
Total call (length)	**-0.394**	**-2.763**	-0.390	-5.484	-5.011	2.485
Introductory syllable (length)	0.045	**1.968**	0.089	1.781	1.683	-0.537
Final syllable (length)	**1.307**	**1.716**	0.236	3.657	3.352	-1.688
Inter-syllable pauses (length)	-0.093	0.525	0.618	1.426	2.284	-0.370
Carrier frequency	-0.119	-0.171	-0.699	-0.016	0.061	0.479
Introductory syllable frequency	0.270	0.043	-0.468	0.195	0.274	0.443
Frequency of the first harmonic	-0.018	-0.043	1.049	0.413	-0.418	0.275

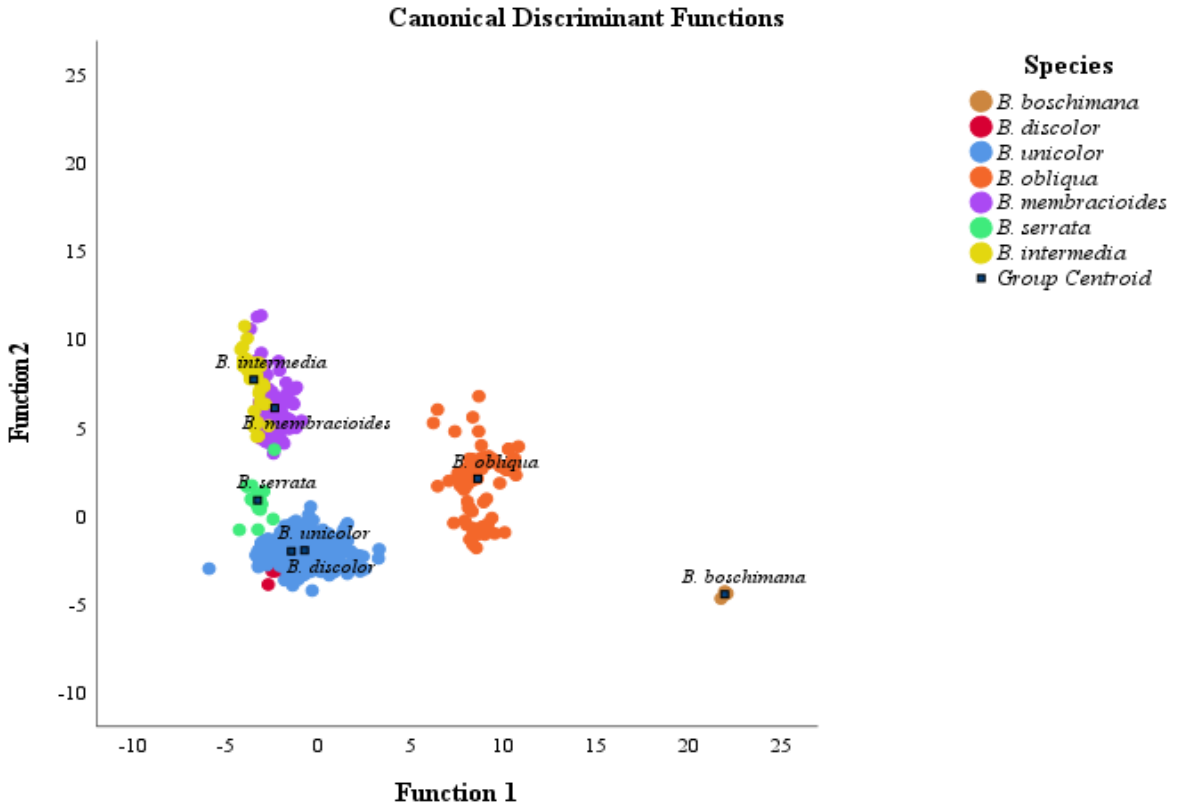

FIGURE 4.42: Discriminant function analysis (DFA) showing the canonical centroid plot for *Bullacris* male acoustic characteristics.

Molecular analyses

For the 25 individuals used (including the outgroup), a total of 1516 base pairs (bp) from one mitochondrial and two nuclear markers were used for analyses. The nuclear gene 18S was used to amplify 490 base pairs (bp), the ITS gene had 497 bp amplified and COI had 529 bp amplified. For degraded samples, internal primers for COI (COI a & b), ITS (ITS a & b) and 18S (18S a & b) genes were created using Geneious v. 7.1.3 (Kearse *et al*., 2014) (Table 4.16).

TABLE 4.16: A list of primers used in this study. Internal primers (e.g., COIa-F), were created using Geneious v. 7.1.3.

Primer Name	**Primer Sequence**
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198	5'-TAAACTTCAGGGTGAGGG TGACCAAAAAATCA-3'
ITS-F	5'-AGAGGAAGTAAAAGTCGTAACAAGG-3'
ITS-R	5'-CCTTAGTAATATGCTTAAATTCAGG-3'
18S-F	5'-TGCTTGTCTCAAAGATTAAGC-3'
18S-R	5'-GCATCACAGACCTGTTATTGC-3'
COIa-F	5'- WCCATTAATRATTGGAGCACCA3'
COIb-R	5'-RATDGGGTCACCYCCTCCTGC-3'
ITSa-F	5'-ACCGACTGCATATCCGAACG-3'
ITSb-R	5'-CTGCGTTCTTCATCGACCCA-3'
18Sa-F	5'-GATCGCACGGTCTCTGTACC-3'
18Sb-R	5'-CCTCGACACTCGGTGAAGAG-3'

The two phylogenetic methods, Baysian Inference (BI) and Maximum Likelihood (ML) produced identical phylogenies (Figure 4.43). Support values greater than 0.95 for posterior probability (pp) and greater than 75% for boostrap (BS) values were considered as strong support, whereas lesser values were considered as not supported.

The outgroup *Physemacris variolosa* is paraphyletic to *Bullacris* and was well supported (1.00 pp and 100% BS). Monophyletic clades can be seen for *B. obliqua* and *B. unicolor*, as well as for *B. membracioides* and *B. intermedia* for both the BI and ML anlyses. In addition, there is a lack of support between *B. intermedia*, *B. membracioides* and *B. discolor* species groups for both analyses (≤ 95 pp; 70% BS); however, the remaining species had strong BI and ML support.

Genetic pairwise distances for the mitochondrial COI dataset (Table 4.17) indicates that there is relatively little intraspecific variation (< 5%). However, interspecific variation is quite high for most species' pairs, with the exception of *B. intermedia* and *B. membracioides* (4.75%) and *B. serrata* and *B. discolor* (7.13%). The highest variation is between *B. intermedia* and *B. unicolor* (18.52%), whereas the lowest interspecific variation is between *B. intermedia* and *B. membracioides*.

TABLE 4.17: Pairwise genetic differences COI dataset (mean ± SD) within (bold) and between sampled species of *Bullacris*.

	N	*B. unicolor*	*B. discolor*	*B. membracioides*	*B. obliqua*	*B. serrata*	*B. intermedia*
B. unicolor	4	**4.158 ± 0.010**					
B. discolor	3	17.261 ± 0.021	**4.776 ± 0.025**				
B. membracioides	3	17.662 ± 0.008	11.371 ± 0.010	**0.515 ± 0.004**			
B. obliqua	6	15.187 ± 0.018	14.894 ± 0.011	16.012 ± 0.008	**4.301 ± 0.028**		
B. serrata	2	16.279 ± 0.010	7.126 ± 0.031	11.596 ± 0.009	15.531 ± 0.010	**0.000 ± 0.000**	
B. intermedia	3	18.522 ± 0.020	11.756 ± 0.017	4.750 ± 0.011	16.118 ± 0.012	11.048 ± 0.020	**2.397 ± 0.009**

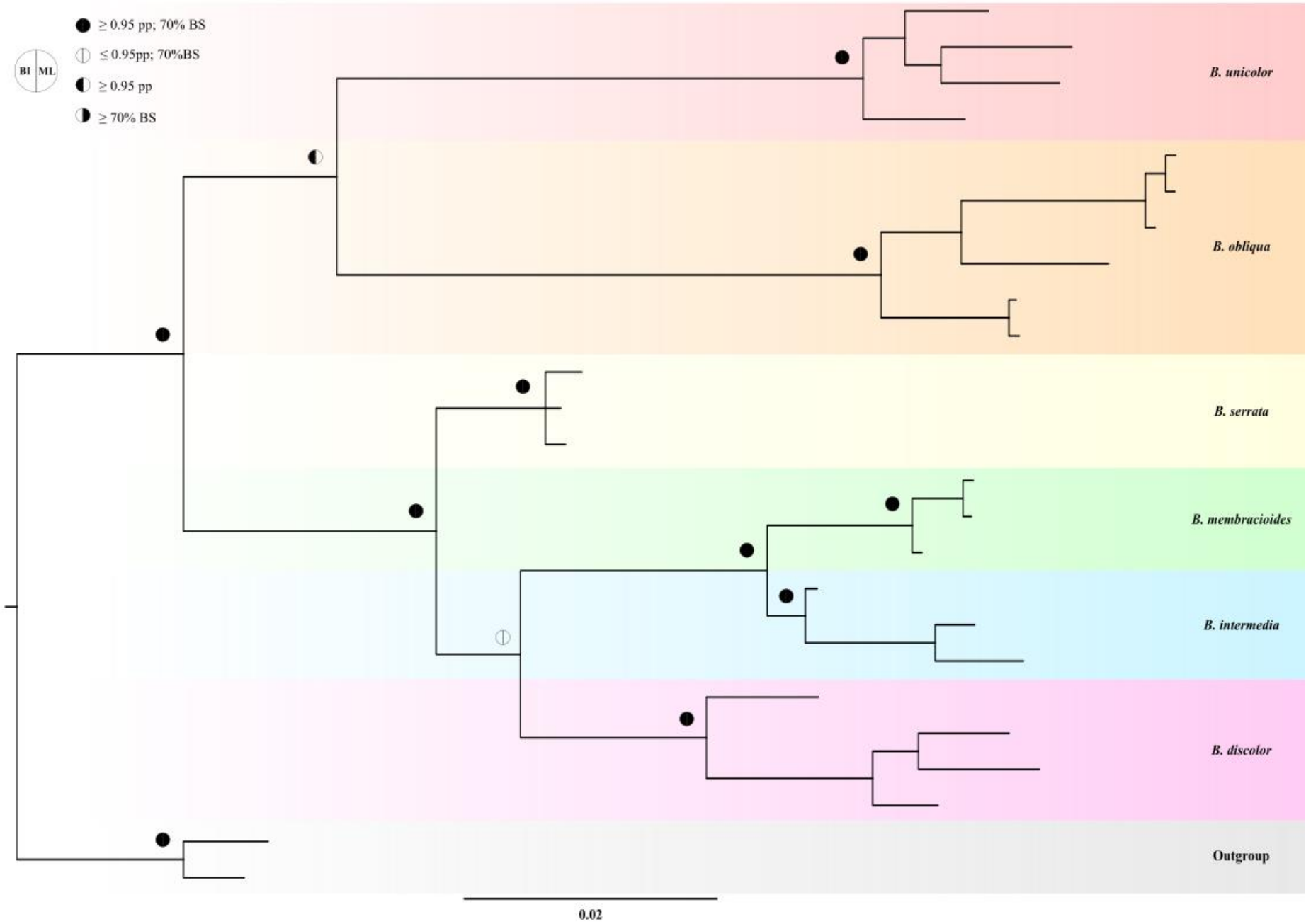

FIGURE 4.43: Bayesian Inference topology for the total evidence of DNA data (CO1, ITS, 18S) for *Bullacris*. Support values for both bootstrap (≥ 70%) and posterior probability (≥ 0.95 pp) are indicated by half black circles (BI on the left and ML on the right) at the nodes.

Discussion

Taxonomy is the foundation for classifiying organisms. There are various ways in which species can be described and clustered together, however each aspect feeds into the other and becomes more accurately and strongly supported when used in conjuction. This study used morphology, acoustics, as well as genetics to reexamine the taxonomy of *Bullacris* species. Results indicated that *B. discolor* and *B. serrata*, as well as *B. intermedia* and *B. membracioides* share a substantial amount of similarities, and that these two pairs of species thus warrant a more extensive investigation.

Bullacris discolor and *B. serrata* are morphologically, acoustically and genetically quite similar, and also have overlapping distributions (Figures 4.10 - 4.11; 4.42 & Table 4.17). It is now understood that *B. discolor* has a highly variable appearance, ranging from uniform light green to a strongly speckled dark green and white pattern. The speckled appearance of *B. serrata* was the initial distinguishing characteristic that separated the two species; however, with the aid of genetic analyses, it is reiterated that taxonomic classifications cannot solely rely on morphological variations. Nevertheless, this phenotypic variation can be explained by colour polymorphism and more specifically homochromy. Homochromy is a frequently occurring function in grasshoppers, whereby individuals match their body colouration with the background patterns of their local habitat (Booth, 1990; Castner & Nickle, 1995; Dearn, 1990; Peralta-Rincon *et al*., 2017; Rowell, 1971). Bladder grasshoppers are known to be host plant specific (Couldridge & van Staaden, 2004) and it is highly probable that the uniform versus speckled appearance is the result of homochromy. Another example of colour polymorphism was observed within the *B. unicolor* group, in which geographically different populations have extensive colour variations (Figure 4.1). This is not unusual for grasshoppers since studies have indicated that there is a significant degree of intraspecific variability, including phenotypic colour variations (Dearn, 1990; Nabours *et al*., 1933; Peralta-Rincon *et al*., 2017; Richards & Waloff, 1954; Rowell, 1971; Rubtzov, 1935).

Bullacris intermedia and *B. membracioides* were genetically and acoustically similar (Figure 4.42 & Table 4.17), however, surprisingly, morphological analysis showed very little overlap (Figure 4.11). Dirsh (1965) stated that these species only differed in body size and the profile of the pronotum, which may have been sufficient to combine these species, since there are several orthopteran studies showing that body size variation between populations is possible, as a result of developmental and or evolutionary factors (Berven & Gill, 1983; Bidau

et al., 2012; Masaki, 1967; Peters, 1983). However, the analysis from this study indicates that these species differ more extensively in morphology than may be expected from geographic size variation and, therefore, a more detailed investigation between *B. intermedia* and *B. membracioides* is required.

Acoustic signals are key features to a number of insects groups as they represent important behaviours, such as sexual selection (Hirtenlehner & Römer, 2014), species recognition (Wilkins *et al*., 2013) and predator defence (Kowalski, *et al*., 2014). For bladder grasshoppers, the use of acoustic signals is for mate location and thus each species typically has very distinct calls (Figures 4.36 – 4.41). However, when comparing the advertisement calls between *B. discolor* and *B. serrata*, as well as between *B. intermedia* and *B. membracioides*, there are only slight variations. Such slight variations are more commonly observed between geographically separated populations and has been observed between *B. unicolor* populations. Acoustic calls for *B. serrata* were recorded from a single population and therefore, additional recordings would be beneficial. It is important to note that the quality of the acoustic call presented for *B. intermedia* is poor, due to the recording taken from field observations (Figure 4.41). Furthermore, environmental pressures and conditions are known to influence acoustic signal properties (Endler, 1992; Forrest, 1994; Morton, 1975), therefore, wide-ranging species such as *B. discolor* and *B. unicolor*, that inhabit more than one vegetation biome, are likely to have slight acoustic variations between populations. An example of this can be understood from a study conducted by Couldridge and Gordon (2015), in which two populations of *B. unicolor* (Northern and Western Cape) were found to call at different times of the night.

Populations that exist across a wide geographic range may also be genetically different, which can be caused by several factors, such as random selection, environmental forces or migration (Dyer, 2015; Manier & Arnold, 2006; Whitney *et al*., 2014). A study by Sathyan *et al*. (2017), discovered that there was a significant correlation between genetic distances and slight morphological variation between *B. unicolor* populations. However, results derived from this study proves to be insufficient to conclude that species pairs *B. discolor* and *B. serrata*, and *B. intermedia* and *B. membracioides*, as widely distributed populations. Therefore, these species should retain their current taxonomic names.

Conclusion

There are several factors that may influence and contribute to variations in acoustic and morphological characteristics (Whitman & Agrawal, 2009). Factors such as environmental conditions and phenotypic plasticity have shown to siginficantly impact acoustic signals, morphological variations and developmental differences (Bidau *et al*., 2012; Donelson & van Staaden, 2005; Forrest, 1994; Gross, 1996; Peralta-Rincon *et al*., 2017). Therefore, when attempting to accurately describe and classify single species, a combination of taxonomic methods are required.

Based on the results of this study, there was insufficient combined evidence to amalgamate species and it is suggested that additional data is required to fully untangle the relationships between *B. serrata* and *B. discolor*, and between *B. intermedia* and *B. membracioides*. Although both pairs of sister species showed considerable overlap, they also differed notably in certain respects. *Bullacris serrata* and *B. discolor* showed greater genetic varaition that what may be expected for typical intraspecific geographic variation, despite morphological and acoustic similarities. Likewise, *B. intermedia* and *B. membracioides* showed significant morphological variation, despite genetic and acoustic similarities.

Future studies should include a genetic evaluation of *B. boschimana* as well as denser sampling of species and acoustic data across their respective distribution ranges. In addition, the locating and formal taxonomic descriptions of alternate males for the remaining three *Bullacris* species would be beneficial towards a more comprehensive evaluation of the *Bullacris* genus. Furthermore, based on the results derived in Chapter 3, individuals from the genera *Physemacris* and *Peringueyacris* formed part of the *Bullacris* clade; however, these individuals were not included in this taxonomic analysis, due to having insufficient genetic data for *Bullacris boschimana* and *Physemacris papillosa*, as well as retaining the initial *Bullacris* classifications made by Dirsh (1965).

The need for further research on *Bullacris* is crucial due to the fact that there is currently very little published information on these unique and endemic species, which are considered to be of high conservation importance.

References

Alexander, A. J. & van Staaden, M. J. (1989). *Alternative sexual tactics in male bladder grasshoppers (Orthoptera, Pneumoridae)*. In Bruton, M. N. (Ed.). Alternative Life-History Styles of Animals, (pp. 261-277). Dordrecht, Netherlands: Kluwer Academic Publishers.

Berven, K. A. & Gill, D. E. (1983). Interpreting geographic variation in life history traits. *American Zoologist, 23*: 85-97.

Bickford, D., Lohman, D. J., Sodhi, N., S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K. & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution, 22*(3): 148-55.

Bidau, C. J., Miño, C. I., Castillo, E. R. & Martí, D. A. (2012). Effects of abiotic factors on the geographic distribution of body size variation and chromosomal polymorphism in two neotropical grasshopper species (*Dichroplus*: Melanoplinae: Acrididae). *Psyche: A Journal of Entomology*, *2012*: 1-11.

Booth, C. L. (1990). Evolutionary significance of ontogenetic colour change in animals. *Biological Journal of the Linnean Society*, *40*(2):125–63.

Čandek, K. & Kuntner, M. (2015). DNA barcoding gap: reliable species indentification over morphological and geographical scales. *Molecular Ecology Resources, 15*(2): 268-277.

Castner, J. L. & Nickle, D. A. (1995). Observations on the behavior and biology of leaf-mimicking katydids (Orthoptera: Tettigoniidae: Pseudophyllinae: Pterochrozini). *Journal of Orthoptera Research, 4*:93. doi:10.2307/3503463

Center for Conservation Bioacoustics. (2014). Raven Pro: Interactive Sound Analysis Software (Version 1.5). Ithaca, NY: The Cornell Lab of Ornithology. Retrieved from: http://ravensoundsoftware.com/

Couldridge, V. C. K. & Gordon, M. L. (2015). Diel variation in signalling and signal transmission in the bladder grasshopper, *Bullacris unicolor* (Orthoptera; Pneumoridae). *Journal of Behaviour*, *152* (12-13): 1701-1718.

Couldridge, V. C. K. & van Staaden, M. J. (2004) Habitat-dependent transmission of male advertisement calls in bladder grasshoppers (Orthoptera; Pneumoridae). *The Journal of experimental biology*, *207*: 2777-2786. doi: 10.1242/jeb.01092

Dearn, J. M. (1990). Color pattern polymorphism. In R. F. Chapman & A. Joern (Eds.). *Biology of grasshoppers*, (pp. 517-549). New York: John Wiley & Sons.

Dirsh, V. M. (1963). Three new genera and species of the family Pneumoridae (Orthoptera: Acridiodea). *Eos, Madr*, *39*: 177-184.

Dirsh, V. M. (1965). Revision of the family Pneumoridae (Orthoptera: Acridoidea). *Bulletin of the British Museum* (*Natural History*) *Entomology*, *52*: 1950–1977.

Donelson, N. C. & van Staaden, M. J. (2005) Alternate tactics in male bladder grasshoppers *Bullacris membracioides* (Orthoptera : Pneumoridae). *Behaviour*, *142*: 761–778.

Dyer, R. J. (2015). Is there such a thing as landscape genetics? *Molecular Ecology*, *24*(14): 3518-3528.

Endler, J. A. (1992). Signals, signal conditions and the direction of evolution. *The American Naturalist, 139*: 125-153.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using bootstrap. *Journal of Evolution, 39*: 783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial *cytochrome c oxidase* subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5): 294-299.

Forrest, T. G. (1994). Propagation and Environmental Effects on Acoustic Signals. *American Zoologist*, *34*: 644–654.

Gordon, M. L. (2017). *An acoustic, genetic and morphological review of the genus Bullacris (Orthoptera; Pneumoridae).* (Master's dissertation, University of the Western Cape, Cape Town). Retrieved from http://etd.uwc.ac.za/handle/11394/6119

Gross, M. R. (1996). Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology and Evolution, 11*: 92-98.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B, Biological Science*, *270* (1512): 313-321.

Hirtenlehner, S. & Römer, H. (2014). Selective phonotaxis of female crickets under natural outdoor conditions. *Journal of comparative physiology A*, *200*: 239–250. doi: 10.1007/s00359-014-0881-7

Hubert, N. & Hanner, R. (2015). DNA Barcoding, species delineation and taxonomy: a historical perspective. *DNA Barcodes*, *3*: 44-58.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software

platform for the organization and analysis of sequence data. *Bioinformatics*, *28*:1647–1649.

Kowalski, K. N., Lakes-Harlan, R., Lehmann, G. U. C. & Strauss, J. (2014) Acoustic defence in an insect: characteristics of defensive stridulation and differences between the sexes in the tettigoniid *Poecilimon ornatus* (Schmidt 1850). *Zoology*, *117*: 329–336. doi: 10.1016/j.zool.2014.04.007

Krishnamurthy, P. K. & Francis, R. A. (2012). A critical review on the utility of DNA barcoding in biodiversity conservation. *Biodiversity and Conservation*, *21*: 1901-1919.

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA 7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7):1870-1874.

Lipscomb, D., Platnick, N. & Wheeler, Q. (2003). The intellectual content of taxonomy: a comment on DNA taxonomy. *Trend in Ecology & Evolution*, *18*(2): 64–66.

Manier, M. K. & Arnold, S. J. (2006). Ecological correlates of population genetic structure: a comparative approach using a vertebrate metacommunity. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *273*: 3001-3009. doi:10.1098/rspb.2006.3678

Masaki, S. (1967). Geographic variation and climate adaptation in a field cricket (Orthoptera: Gryllidae). *Evolution*, *21*(4): 725-741.

Miller, M. A., Pfeiffer, W. & Schwartz T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments workshop* (*GCE*). Conducted by San Diego Supercomputer Center. New Orleans, pp. 1-8. doi: 10.1109/GCE.2010.5676129

Morton, E. S. (1975). Ecological sources of selection on avian sounds. *The American Naturalist*, *109*(965): 17-34.

Mucina, L. & Rutherford, M. C. (Eds.). (2006). The vegetation of South Africa, Lesotho and Swaziland. Pretoria: South African National Biodiversity Institute. (Strelitzia, vol. 19).

Nabours, R. K., Larson, I. & Hartwig, N. (1933). Inheritance of colorpatterns in the grouse locust *Acrydium arenosum* Burmeister (Tettigidae). *Genetics*, *18*: 159–171.

Peralta-Rincon, J. R., Escudero, G., Edelaar, P. (2017). Phenotypic plasticity in color without molt in adult grasshoppers of the genus *Sphingonotus* (Acrididae: Oedipodinae). *Journal of Orthoptera Research*, *26*(1): 21-27.

Peters, R. H. (1983). *The Ecological Implications of Body Size*. (Cambridge studies in Ecology). Cambridge: Cambridge University Press. doi:10.1017/CBO9780511608551

Pires, A. C. & Marinoni, L. (2010). DNA barcoding and traditional taxonomy unified through Intergrative Taxonomy: a view that challenges the debate questioning both methodologies. *Biota Neotropica, 10*(2): 339-346.

Rambaut, A. (2014). FigTree. Version 1.4.1. Retrieved from: http://tree.bio.ed.ac.uk/software/figtree/

Richards, O. W. & Waloff, N. (1954). *Studies on the biology and population dynamics of British grasshoppers.* London: Anti-locust Research Centre (Anti-locust bulletin; vol. 17).

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu L., Suchard, M. A. & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*: 539-42.

Rowell, C. H. F. (1971). The variable coloration of the acridoid grasshoppers. *Advances in Insect Physiology, 8*: 145-98.

Roy, L., Dowling, A. P. G., Chauve, C. M. & Buronfosse, T. (2008). Delimiting species boundaries within *Dermanyssus* Dugès, 1834 (Acari: Dermanyssidae) using a total evidence approach. *Molecular Phylogenetics and Evolution, 50*: 446-470.

Rubinoff, D. (2006). DNA Barcoding Evolves into the Familiar. *Society for Conservation Biology, 20*(5): 1548-1549.

Rubtzov, I. A. (1935). Phase variation in non-swarming grasshoppers. *Bulletin of Entomological Research, 26*(4): 499-520.

Sathyan, R., Engelbrecht, A. D. & Couldridge, V. C. K. (2017). Morphological, acoustic and genetic divergence in the bladder grasshopper *Bullacris unicolor*. *Ethology, Ecology and Evolution*, *29*(6): 552-573.

Schindelin, J., Rueden, C. T. & Hiner, M. C. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development, 82*(7-8): 518-529. https://doi.org/10.1002/mrd.22489

Scotland, R. W., Olmstead, R. G. & Bennett, J. R., (2003). Phylogeny reconstruction: The role of morphology. *Systematic Biology, 52*(4): 539–548.

Song, H., Amédégnato, C., Cigliano, M. M., Desutter-Grandcolas, L., Heads, S. W., Huang, Y., Otte, D. & Whiting, M. F. (2015). 300 million years of diversification: elucidating the patterns of orthopteran evolution based on comprehensive taxon and gene sampling. *Cladistics, 31*: 621-651.

Song, H., Foquet, B., Mariño-Pérez, R. & Woller, D. A. (2017). Phylogeny of locusts and grasshoppers reveals complex evolution of density-dependent phenotypic plasticity. *Scientific Reports*, *7*: 6606. https://doi.org/10.1038/s41598-017-07105-y

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*: 2688-2690.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. & Vogler, A. P. (2002). DNA points the way ahead in taxonomy. *Nature*, *418*(368): 479.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. & Vogler, A. P. (2003). A plea for DNA taxonomy. *Trends in Ecology & Evolution*, *18*(2): 71-74.

Trewick, S. A. (2008). DNA Barcoding is not enough: mismatch of taxonomy and genealogy in New Zealand grasshoppers (Orthoptera: Acrididae). *Cladistics*, *24*: 240-254.

Whitman, D. W. & Agrawal, A. A. (2009). What is phenotypic plasticity and why is it important? In Whitman, D. W. and Ananthakrishman, T. N. (Eds.). *Phenotypic Plasticity of Insects*, (pp. 1-63). Washington: Science Publishers.

Whitney, J. E., Gido, K. B. & Propst, D. (2014). Factors associated with the success of native and nonnative species in an unfragmented arid-land riverscape. *Canadian Journal of Fisheries and Aquatic Sciences*, *71*(8):1134-1145. doi: 10.1139/cjfas-2014-0153

Wilkins, M. R., Seddon, N. & Safran, R. J. (2013) Evolutionary divergence in acoustic signals: causes and consequences. *Trends in Ecology & Evolution*, *28*: 156–166. doi: 10.1016/j.tree.2012.10.002

Will, K. W. & Rubinoff, D. (2004). Myth of the Molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*, *20*: 47-55.

Will, K. W., Mishler, B. D. & Wheeler, Q. D. (2005). The Perils of DNA Barcoding and the Need for Integrative taxonomy. *Systematic Biology*, *54*(5): 844-851.

Chapter 5: General conclusion and summary

The findings presented in this thesis have provided new insights into the evolutionary history of bladder grasshoppers and the environments in which they occur. Bladder grasshoppers are largely endemic to South Africa, with most species found within two plant global biodiversity hotspots, the Cape Floristic Region and the Succulent Karoo. Biogeographic analyses provided further insights into the shared habitats of bladder grasshoppers and other orthopteran species and the zoogeographic regions in which the majority of pneumorid species occur. A dated species-level phylogeny was undertaken, the first genetic analyses for the family, signifying the time at which bladder grasshopper lineages diverged, as well as highlighting taxonomic discrepancies. Furthermore, by updating the taxonomic methods for classifying species found within the genus *Bullacris*, results indicated that making use of multiple criteria is essential for delineating species boundaries.

In Chapter 2, biogeographic analyses suggested that South African orthopteran species richness is evenly distributed throughout the region. There was, however, a clear primary biogeographic break between the eastern summer-rainfall and the western winter-rainfall species. This east-west break has been previously reported for butterfly, beetle, plant and reptile taxa, providing further evidence that South Africa is made up of two bioregions broadly aligned along rainfall seasonality. Finer-scale biogeographic patterns were also seen from the analyses of the orthopteran dataset, with six secondary centres were discovered, of which three were found in the east and three in the west. The centres found in the west were the Succulent Karoo, the Cape Fynbos and the Central Nama-Karoo, and the centres found in the east were the South-East Tropical, the Savanna and the Indeterminate Summer-Rainfall region. The western centres were more distinctive and taxonomically diverse than the eastern centres. The majority of orthopteran families were seen in the Cape Fynbos and the Succulent Karoo centres, validating the boundaries of a Greater Cape. In this Greater Cape, Pneumoridae species were also concentrated, thus providing additional support for the biological diversity of this region, including the presence of a paleo-relictual element. Furthermore, results from this study support the Greater Cape as a biochorion of high biogeographic interest.

Analyses from Chapter 3 suggested that pneumorids diverged from other orthopteran species during the early Cretaceous period at approximately 134.70 MYA. It is suggested that bladder grasshoppers originated within South Africa, and dispersed in a northward direction, along the eastern regions of Africa. The first divergence within the family occurred between the forest (*Physophorina* and *Pneumora*) and the non-forest taxa (*Bullacris*, *Physemacris* and

Peringueyacris) at an estimated 116.91 MYA. The species to have subsequently diverged were the predominantly Succulent Karoo (*B. unicolor* and *Pe. namaqua*), Fynbos (*B. discolor, B. serrata, B. obliqua* and *Ph. variolosa*), and Savanna (*B. membracioides* and *B. intermedia*) species groups, respectively. Phylogenetic analyses also indicated that *Physemacris variolosa* nested within species from the *Bullacris* genus, and thus the current genus names do not reflect evolutionary history. This may suggest that the genus *Physemacris* should be absorbed into *Bullacris.* However, only one of the two *Physemacris* species (*Ph. variolosa*) was used in this study and therefore *Ph. papillosa* needs to be genetically investigated to provide a more definitive taxonomic placement and status of *Physemacris*. The monotypic genus *Peringueyacris* clustered together with *B. unicolor*, both being found in semi-arid environments, providing further evidence that the genus *Bullacris* is not a monophyletic group. A genetic evaluation of *B. boschimana*, which is the only pneumorid species to occur in a desert environment and the only species to have a distribution that extends into Namibia, would help to shed even more light on the role of habitat shifts in pneumorid evolution.

In Chapter 4, a taxonomic revision of species belonging to the genus *Bullacris* used a combination of morphological, acoustic and genetic analyses to differentiate between the seven known species. All species were included in the study, although genetic data for *B. boschimana* was not available, as a consequence of not being able to extract DNA from the material available. Evidence showed that *B. discolor* and *B. serrata* were similar morphologically and acoustically, with only minor variations that could be explained by environmental factors. Genetically, representatives from these two species had low pairwise distances for the mitochondrial COI gene marker, but still larger than variations typically seen within a species and thus overall, there was insufficient basis to combine these species. The same rationalizations were seen for *B. membracioides* and *B. intermedia*. While these two species are genetically very similar, within the range of a single species, and are also difficult to distinguish acoustically, morphological data indicated significant divergence. The alternate males found in four of the *Bullacris* species are unarguably a secondary male form, although relatively rarely encountered in the field (~5% of individuals collected from host plants) and thus, although it is assumed that they also occur in the remaining three species, they have yet to be documented. It is therefore suggested that all seven species currently found within the genus *Bullacris* retain their specific status at this time.

Directions for future research

Future research should include denser sampling and increased survey data for South African orthopteran biogeographic analyses, as a large portion of South Africa remains unsurveyed. This information will improve our knowledge of species distributions across the region and aid in refining biogeographic regions of species and therefore provide useful information for conservation strategies and management. The investigation into the distribution of other paleo-endemic grasshoppers, such as species from the superfamilies Tetrigoidea and Grylloidea, would further our understanding of anthropogenic and climatic impacts on sensitive insect species and ecosystems. In addition, increased phylogenetic data, in combination with biogeographic studies, would help us better understand the evolutionary history of Orthoptera in South Africa. Data for additional paleo-relictual species such as individuals from the genus *Lithidiopsis*, would add significant value to our current evolutionary knowledge about South Africa's entomofauna.

In the context of Pneumoridae research, phylogenetic analysis would benefit from incorporating all remaining species from the family for future genetic evaluations, such as *B. boschimana*, for a more complete and robust analysis of the *Bullacris* genus in order to resolve species-level relationships. Similarly, a denser sampling across species distributions together with the use of additional genes regions (e.g., 12S, 16S, 28S, COII) would substantiate the current findings presented in this study. The use of additional fossil evidence from the family or sister taxa would undoubtingly also strengthen divergence time estimates that are currently based on a single calibration point. Morphological results would be more robust if additional female specimens from multiple populations for *B. intermedia* and *B. membracioides* were used for comparative measures.

Pneumoridae are phylogentically distinct and unusual group of grasshoppers that are regarded as being of high conservation importance and are considered particularly sensitive to, and indicators of global climate change. Half of all species have been evaluated as either threatened or near-threatened. Thus, resolving taxonomic uncertainties and identifying areas of diversity should be considered a priority.