Genetic analyses of a Southern African millipede (*Bicoxidens* Attems 1928)

By

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Submitted in fulfilment of the academic requirements for the degree of

Master of Science

School of Life Sciences,

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Durban

July 2014

Authentication

As the candidate's supervisor I have agreed to the submission of this thesis.

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Declaration

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Acknowledgements

Abstract

Bicoxidens Attems, 1928 is an afrotropical millipede in the family Spirostreptidae which constitutes nine species. Based on the analysis of the male copulatory organs known as gonopods, *Bicoxidens* is monophyletic. However, gonopod morphology is central to millipede taxonomy, examination of gonopod morphology alone may not elucidate hidden genetic diversity thereby underestimating species richness in *Bicoxidens*. Analysis of genetic data as an additional source of taxonomic data has been noted to reveal genetic variation and flagged hidden species within millipedes. Hence there is a need to revise the morphological species definitions and phylogeny of *Bicoxidens* using both mitochondrial and nuclear DNA sequences. Furthermore, low vagility and habitat discontinuity may limit gene flow among *Bicoxidens* populations resulting differentiation and high genetic variation.

In this regard, the sequences of the mitochondrial *cytochrome oxidase* 1 (COI) and 16S rRNA genes were used to delimit species, unravel hidden species and test the monophyly of *Bicoxidens*. In addition, the phylogeography and population genetic structure of *B. flavicollis* was investigated based on COI and 16S rRNA sequences. Genetic distances were computed using COI. Phylogenetic inferences were done based on Maximum likelihood and Bayesian inference analyses of the COI and 16S rRNA sequences individually and with the combined data set (COI+16S rRNA). The phylogeography and population genetic structure of *B. flavicollis* was inferred based on genetic diversity indices, population genetic differentiation estimate PhiPT, haplotype network analyses and maximum likelihood analyses of both COI and 16S rRNA. Bayesian population structure analyses was done using the COI data set only. Furthermore, Mantel's isolation by distance among *B. flavicollis* specimens was tested.

Interspecific genetic distances (> 0.075) based on the Juke-Cantor model supported the morphological species definitions in *Bicoxidens*. However, high intraspecific sequence differences in *B. flavicollis* (> 0.14), *B. friendi* (0.084) and *B. brincki* (> 0.188) suggest the presence of cryptic species. The possible presence of cryptic species was supported by the phylogenic analyses which recovered a paraphyletic *Bicoxidens* phylogram with *B. flavicollis*, *B. brincki* and *B. friendi* exhibiting divergent lineages. *Bicoxidens flavicollis* populations exhibited high genetic diversity and strong population differentiation which suggests restricted gene flow. Haplotype network and Maximum likelihood analyses revealed limited sharing of haplotypes and cryptic species in

populations at Mazowe and Nyanga, respectively. *Bicoxidens flavicollis* populations were grouped in two four genetic clusters. Isolation by distance was insignificant among *B. flavicollis* specimens. The results support the utility of both COI and 16S rRNA in species delimitation in *Bicoxidens.* There is a growing body of evidence which also suggests presence of hidden species in *B. flavicollis, B brincki* and *B friendi.* Furthermore effort to uncover more hidden species should be made to elucidate the species richness before conservation strategies can be implemented.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Invertebrates dominate the earth's biodiversity constituting 80 % of all described species (Cardoso *et al.*, 2011). Despite their abundance in almost all biomes, there is a paucity in scientific literature on invertebrates. Millipedes are a diverse arthropod group which belongs to the class Diplopoda. Only 15 % of the estimated 80 000 millipede species have been described across six continents (Bond & Sierwald, 2002; Golovatch & Kime, 2009). Millipede distribution spans both hemispheres with only the northern hemisphere, in particular, North America and Europe, boasting of well documented diversity (Sierwald & Bond, 2007). In the millipede rich southern Africa 522 species grouped into 72 genera have been described (Hamer & Slotow, 2000). Such a statistic emphasizes how southern hemisphere millipedes have received little attention.

According to Lavelle *et al.* (2006) macro-invertebrate detritivores play a role in ecosystem processes. Millipedes accelerate decomposition by mechanical fragmentation as they ingest dead plant material and excrete faecal pellets (Hopkin & Read, 1992; Cárcamo *et al.*, 2000; Suzuki *et al.*, 2013). The faecal pellets which offer increased surface area become hotspots for microbial colonization and activity (Hättenschwiler & Gasser, 2005; Snyder & Hendrix, 2008). Furthermore, temperate and tropical forest-dwelling millipedes have been reported to enhance nutrient availability by consuming an estimated 36 % (Cárcamo *et al.*, 2000) and 39 % (Dangerfield & Milner, 1996) of the annual leaf litter, respectively. Snyder & Hendrix (2008) also highlighted the potential utility of millipedes in ecosystem rehabilitation as ecosystem health indicators and restoration tools. Unfortunately, incomplete distribution and taxonomic data will continue to hamper the application of tropical millipedes in such programs thereby further understating their potential role in ecosystems.

1.2 Millipede taxonomy

Traditionally, species delineation and elucidation of evolutionary relationships among taxa rely on morphological characters (Pires & Marinoni, 2010). For each taxon a set of taxonomically informative characters is identified. Sexually selected structures such as genitalia are widely recognized as reliable diagnostic traits in organisms such as arthropods (Tanabe *et al.*, 2001; Bond

et al., 2003; Padial *et al.*, 2010). Genital structures are believed to evolve rapidly and divergently due to sexual selection (Eberhard, 1985; Hosken & Stockley, 2004; Wheeler, 2008; Wojcieszek & Simmons, 2013). The rapid evolution results in species-specific variations in genital morphology which allows recognition of species. In millipedes, the male sperm transfer appendages, referred to as gonopods, are the traditional source of morphological taxonomic data (Hopkin & Read, 1992; Sierwald & Bond, 2007; Golovatch & Kime, 2009; Wojcieszek & Simmons, 2012). Gonopods are modified legs on the seventh body ring in the adult phase (Hopkin & Read, 1992). Gonopods are characterized by accessory structures called processes which are variable in structure/number thus enable recognition of taxa. Based on the variability in gonopod structures over 12 000 species in 16 orders of millipedes have been described (Brewer *et al.*, 2012).

However, there is a growing body of evidence which suggests that male genital divergence may proceed slowly and speciation may occur without a change in gonopod morphology (Bond *et al.*, 2003; Adams *et al.*, 2009; Wojcieszek & Simmons, 2012). Millipedes are short range endemics with low vagility hence prone to geographical isolation (Tanabe *et al.*, 2001; Enghoff & Seberg, 2006; Sierwald & Bond, 2007). Isolation may lead to restricted gene flow, genetic divergence and possibly speciation. If there is lack of conspicuous differences in male genitalia between recently divergent species, morphological cryptic species will have evolved. Thus, species delimitation based solely on gonopod morphology may not reveal cryptic taxa resulting in genetically distinct species being lumped (Bond & Sierwald, 2002; Jacob *et al.*, 2004; Adams *et al.*, 2009; Mwabvu *et al.*, 2013). Furthermore, species identifications of developmental stages is not possible because juveniles do not have gonopods and there are no taxonomically informative characters in millipede juvenile stages.

Accurate species delimitation is crucial for evolutionary studies (Claridge *et al.*, 1997) because species is the basic unit when examining relationships. Hence, incorrect species delimitation may result in incorrect phylogenetic conclusions within a taxon, this also occurs where morphological characters are not clearly defined. As such, phylogenetic relationships solely based on morphological traits may not recognize cryptic species and result in incorrect conclusions (Pires & Marinoni, 2010). This has implications on the accuracy of taxonomic conclusions and subsequent conservation decisions.

1.3 DNA in millipede taxonomy and phylogenetics

Although the utility of morphological characters is beyond question, DNA sequences also contain characters that are useful in species diagnosis and at inferring relationships among taxa (Hajibabaei *et al.*, 2007; Padial *et al.*, 2010; Goldstein & DeSalle, 2011). DNA sequences are obtained from amplifying segments of the mitochondrial and nuclear genomes, referred to as molecular markers. The variations in nucleotide sequences are used to make inferences on which clade an individual belongs to and relationships among taxa. Mitochondrial and nuclear markers offer taxonomically useful characters in terms of variability in the form of base substitutions within the nucleotide sequence (Danforth *et al.*, 2005; Galtier *et al.*, 2009). Thus, DNA markers present huge potential to millipede taxonomy.

Species specific DNA sequences provide more taxonomic data in cases where morphology is ambiguous (Avise, 2004). One of the often highlighted advantages of DNA sequences is the ability to identify morphologically cryptic species (Blaxter, 2004; Vogler & Monaghan, 2007). Taxonomy based solely on DNA sequence data has been widely debated (Wheeler, 2008) with several authors advocating for an integrative approach to taxonomy in which both morphological and molecular data are utilized (see Will *et al.*, 2005; Padial *et al.*, 2010; Pires & Marinoni, 2010; Schlick–Steiner *et al.*, 2010). Hence, DNA sequences are rapidly being applied to provide genetic data to supplement morphological data. Genetic data are proving successful in detecting cryptic diversity (e.g. Schlick–Steiner *et al.*, 2006; Ekrem *et al.*, 2010; Mitter *et al.*, 2011; Hamada *et al.*, 2010; Renaud *et al.*, 2012). There seems to be a shift towards combining morphological and molecular data in species recognition and phylogenetic relationship analysis. Schlick–Steiner *et al.* (2006) demonstrated the value of integrating molecular sequence data with morphological data for species recognition and phylogenetic analyses. According to Pires & Marinoni (2010) integrated approach provides better support for species boundaries and phylogenetic hypothesis.

Until recently millipede taxonomy relied solely on morphological data. Cryptic species have been flagged in genera such as *Anadenobolus* Karsch 1881 (Bond & Sierwald, 2002) and *Bicoxidens* Attems 1928 (Mwabvu *et al.*, 2013) based on mitochondrial DNA sequence data. Furthermore, Enghoff *et al.* (2011) highlighted that DNA sequence data may be a promising additional source of data for elucidating millipede evolutionary history and genetic diversity. Ordinal, familial,

generic and species relationships within millipedes have also been assessed using DNA sequence data (see Bond & Sierwald, 2002; Regier *et al.*, 2005; Marek & Bond, 2006; Wesener *et al.*, 2010; Pitz & Sierwald, 2010; Pimvichai *et al.*, 2014)

Millipedes are prone to geographic isolation due to low dispersal capabilities, hence they are suitable candidates for phylogeographic studies. Hence there is increased inbreeding which eventually leads to serial erosion of heterozygosity and an increase in homozygosity thus in breeding depression. Historical processes that have led to the present millipede distributions have been studied (Walker *et al.*, 2009; Loria *et al.*, 2011; Nistelberger *et al.*, 2014). However, genetic data on southern African millipedes are scare despite the former's potential utility in millipede phylogenetics and phylogeography.

1.4 Genus *Bicoxidens*

According to Sierwald & Bond (2007) 1 894 (16%) of the 12 000 described millipede species are grouped into four families (Spirostreptidae, Harpagophoridae, Odontopygidae and Julomorphidae) in the order Spirostreptida. In the family Spirostreptidae, 52 genera have been described in Africa (Hamer & Slotow, 2000). *Bicoxidens* is endemic to Southern Africa particularly areas south of the Zambesi and Kunene rivers and it is a member of the family Spirostreptidae (Mwabvu *et al.*, 2007). The genus consists of nine described species (*B. flavicollis* Attems 1928, *B. nigerrimus* Attems 1928, *B. nyathi* Mwabvu 2007, *B. gokwensis* Mwabvu 2007, *B. aridis* Mwabvu 2009, *B. grandis* Lawrence 1965, *B. matopoensis* Mwabvu 2007, *B. brincki* Schubart 1966, *B. friendi* Mwabvu 2000). Mwabvu *et al* (2007) reported that the species vary in size from 75 −170 mm in length and 6.6–10.6 mm in diameter. The body color ranges from shades of black, brown through to orange-yellow (Mwabvu *et al.*, 2007). According to Mwabvu *et al.* (2007) millipedes in the genus generally inhabit diverse habitats including savannah woodlands that are dominated by *Brachystegia* sp. or *Acacia* sp., riverine vegetation and mountain forests. *Bicoxidens* species are widely distributed, occupying the area south of the Zambezi River with Zimbabwe as the possible the center of origin (Mwabvu *et al.*, 2007). Mwabvu *et al.* (2007) further reported that distribution patterns within the genus may be influence by rainfall patterns and habitat preference in terms of vegetation type.

Based on gonopod morphology, *Bicoxidens* is monophyletic (Mwabvu *et al.*, 2007). However, morphological species definitions and the monophyly of *Bicoxidens* have not been tested using

genetic data. Furthermore, levels of interspecific and intraspecific genetic variation are unknown. However, high intraspecific genetic variation based on mtDNA revealed potential cryptic species in two *B. flavicollis* populations (Mwabvu *et al.*, 2013). Against this background such evidence warrants a molecular taxonomic revision of the genus.

1.5 Conclusion

Despite their ubiquity, millipede diversity is poorly described scientifically and millipedes are understudied, factors which may understate their ecological role. The lack of knowledge is compounded by the paucity of accurate taxonomic and phylogeographic data. As a result poor understanding of millipedes' evolutionary history may hinder their inclusion in conservation policies (Avise, 2008; Faith, 2008). The integration of morphology and DNA sequence data aims to improve the accuracy of species diagnoses since species are the basic unit in biodiversity. Correct species identification would result in accurate millipede species richness estimations. Morphology-based species definitions can also be validated based on genetic variations. Furthermore, genetic structure and divergence patterns in geographically fragment population may also be studied.

1.6 Aim of study

The aim of the study was to carry out a genetic analysis of *Bicoxidens* based on mitochondrial and nuclear DNA. This would help validate species definitions in the genus. Furthermore, levels of interspecific and intraspecific genetic variation and cryptic diversity could be identified. Millipede studies are biased towards temperate regions although tropical regions contain greater diversity. In addition, genetic data on tropical millipedes are scarce. Hence, the results of this study will improve our understanding of millipede diversity in southern Africa. In southern Africa few genetic studies have focused on Madagascan millipedes (Wesener *et al.*, 2010; Wesener *et al.*, 2011). The present study would be the first intensive molecular investigation in the family Spirostreptidae which focuses on the whole genus. Conservation efforts tend to prioritize evolutionary hotspots, thereby preserving future biodiversity (Faith, 2008; Avise, 2008). Genetic analysis of the afrotropical endemic *Bicoxidens* could further elucidate genetic diversity within the genus and possibly identify distinct gene pools. Molecular marker selection is important in genetic studies, hence the study will also evaluate the utility of mitochondrial DNA and nuclear markers in species discrimination and phylogenetic analysis of *Bicoxidens* species.

Objectives of study

1. To review the DNA markers in the small field of millipede molecular systematics.

2. To determine the phylogenetic relationships and within population and across population genetic variation among *Bicoxidens* species.

3. To determine the phylogeography and population genetics of *B. flavicollis* populations.

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CHAPTER 2

A REVIEW OF MITOCHONDRIAL AND NUCLEAR DNA MARKERS IN MILLIPEDE TAXONOMY.

2.1 Introduction

The process of identifying, naming and classifying organisms based on a hierarchal system is known as taxonomy (Wheeler, 2008). Evolutionary relationships among organisms including line of descent can be inferred through a phylogeny. According to Godfray & Knapp (2004) phylogeny is one of the three components of taxonomy, therefore it will be treated as such in this review. The advent of molecular markers, which are short DNA sequence segments obtained from the mitochondrial or nuclear genome has revolutionized taxonomy (Dayrat, 2005; Schlick–Steiner *et al.*, 2010; Padial *et al.*, 2010). DNA sequences are an additional source of taxonomic data to the traditional morphology especially when morphology becomes ambiguous (Blaxter, 2004; Hebert & Gregory, 2005). Cryptic taxa which are morphologically indistinguishable have been detected upon analysis of DNA sequence data (Hebert *et al.*, 2004; Witt *et al.*, 2006; Schlick–Steiner *et al.*, 2006; Pfenninger *et al.*, 2007; Renaud *et al.*, 2012; Hernández–Triana *et al.*, 2012). Furthermore, levels of genetic diversity and the historical events which led to the spatial distribution of the genetic variation with taxa have also been studied using genetic data (Derkarabetian *et al.*, 2011; Cooper *et al.*, 2011; Kodandaramaiah *et al.*, 2012).

Millipedes exhibit several characteristics which warrant the inclusion of DNA in their taxonomy. For example millipedes are isolation prone and have low dispersal ability hence long periods of isolation would result in genetic divergence (Sota & Tanabe, 2010; Nistelberger *et al.*, 2014). Genetic divergence and variation may increase due to discontinuity in millipede habitats which would restrict migration and immigration thereby limiting gene flow. Thus, diversification without morphological change may result giving rise to cryptic species which may not be detected through morphological analysis alone (Bond & Sierwald, 2002; Bond *et al.*, 2003; Wojcieszek & Simmons, 2012).

In general, millipedes are under-studied, in the past decade molecular data have been used to augment morphology in millipede taxonomy. However, the apparent need for millipede genetic data lies in contrast to the paucity in millipede genetic data especially on African millipedes. Given the need for DNA in millipede taxonomy there is merit in attempting to establish the current status

in relation to other well studied groups. This review aims to be an appraisal of the inclusion of mitochondrial and nuclear DNA sequence markers in millipede taxonomy including phylogenetics and phylogeographic studies. This review attempts to explore the influences which underlie the trends in the use of mitochondrial and nuclear DNA. There are lessons to be gleaned from over a decade of integrating DNA sequence data in millipede studies. This may hopefully be a foundation for a framework of an integrated taxonomy approach tailored for millipedes.

2.2 Literature survey

The ISI web of Knowledge search tool was used to survey for published literature wherein molecular markers were used to i) recognize species and ii) construction of evolutionary relationships within millipedes iii) understand population genetic structure and phylogeography. The literature survey was conducted for journal articles published over a 20 year period. Millipede studies that used mitochondrial and nuclear DNA sequences for species recognition, phylogenetics, phylogeography and population genetics were selected.

2.3 Results and Discussion

A total of 21 references were recovered based on the search criteria (Table 1). All taxonomic levels within class Diplopoda have been studied using 12 DNA sequence markers, with most studies biased towards generic and species levels (Fig. 1). Mitochondrial DNA (mtDNA) markers were more frequently used in comparison to nuclear DNA (nDNA) and COI was the most popular marker among mtDNA markers (Fig. 1). According to Galtier *et al.* (2009) mtDNA is easy to amplify, characterized by low recombination and highly variable due to a rapid mutation rate making it the genome of choice. Typically animal mtDNA encodes for 37 genes comprising 13 protein coding, two ribosomal RNAs and 22 for tRNAs (Boore, 1999; Lavrov *et al.* 2002; Woo, 2007). Protein coding mitochondrial genes have the highest evolution rates among mitochondrial genes which allow for separation of cryptic species (Hajibabaei *et al.*, 2007; Hebert *et al.*, 2003). In millipedes, cryptic species are more likely present in lower taxonomic categories such as genus and species which are often defined by structural variations of the gonopods. There may be a decoupling of gonopod and genetic divergence (Bond *et al.*, 2003; Huber *et al.*, 2005; Derkarabetian *et al.*, 2011), an event which often complicate species differentiation.

Millipede species in *Cylindroiulus* Verhoeff 1894 (Seeber *et al.*, 2010), *Aphistogoniulus* Silvestri 1897 (Wesener *et al.*, 2011), *Brachybe* Wood 1864 (Brewer *et al.*, 2012)*, Thyropygus* Pocock 1894 (Pimvichai *et al.*, 2014) were differentiated based on the standard barcoding gene COI. Sota & Tanabe (2010) reported intraspecific genetic divergences among *Parafontaria tonominea*

Verhoeff 1936 species complex based on COI-COII gene region. Cyt-b gene seems to be a well-established species level marker in both vertebrates and invertebrates (Farias *et al.*, 2001)**.**

Studies on sequence divergences in *Brachybe* suggested that cyt-b may be useful at species delineation in millipedes (Brewer *et al.*, 2012). It would be interesting to evaluate cyt-b in deep level millipede evolutionary relationships given that it characteristically has both slower and rapid evolving regions (Farias *et al.*, 2001). Even though COI has been applied at higher taxonomic categories such as class and family (Wesener *et al.*, 2010) it may have been purely to separate species.

Mitochondrial ribosomal genes 12S rRNA and 16S rRNA are highly conserved with less variability as opposed to their protein coding counterparts (Boore, 1999; Lavrov *et al.* 2002; Woo *et al*. 2007). The low sequence variability suggests that 12S rRNA and16S rRNA are unsuitable markers at species or population level. The 12S rRNA and 16S rRNA genes do not evolve fast enough to accumulate adequate substitutions required to discriminate recently diverged taxa (Lane *et al.*, 1985; Janda & Abbot, 2007). Hence, 12S rRNA would be useful at targeting higher categories such as phyla or subphyla, likewise 16S rRNA utility would be restricted to family and genus level. However, for some taxa intraspecific and intrageneric level relationships have been resolved based on 16S rRNA (Schulze, 2006; Jolly *et al.*, 2006). Furthermore, variability in 16S rRNA sequences has revealed cryptic species in *Anadenobolus excisus* Karsch 1881 (Bond & Sierwald, 2002) and *B. flavicollis* Attems, 1928 (Mwabvu *et al.*, 2013). Mitochondrial tRNA-valine is the only one of the 22 tRNA that has taxonomic value in millipedes (see Marek & Bond, 2006).

Measures of genetic diversity and speciation mechanisms within a particular species can be inferred through population genetic and phylogeographic studies (Avise, 2000; 2009). Biodiversity and conservation also require the ascertainment of patterns and processes which influence the spatial distribution of a particular species (Avise, 2008). Assessment of COI gene revealed vicariance in *Atelomastix bamfordi* Verhoeff 1924 (Nistelberger *et al.*, 2014) while Loria *et al.* (2011) reported limited gene flow in the cave millipede *Tetracion* Hoffman 1956. In addition, Walker *et al.* (2009) found evidence, based on 12S rRNA and tRNA-valine genes, that habitat suitability is directly linked to high levels of genetic diversity.

Table 1. References wherein DNA markers were used for species recognition and determining evolutionary relationships in millipedes.

Source	Taxonomic level	DNA sequence marker (s)
Nistelberger *et al.* (2014)	species	COI
		16S rRNA
Pimvichai *et al.* (2014)	genus	COI
		16S rRNA
Enghoff *et al.* (2013)	family	16S rRNA
		28S rRNA
Mwabvu *et al.* (2013)	species	16S rRNA
Brewer *et al.* (2012)	genus	COI
		Cyt b
		Glutamyl and prolyl-tRNA synthetase
Wojcieszek and Simmons (2012)	species	COI
Frederiksen *et al.* (2012)	species	COI
		16S rRNA
		28S rRNA
Wesener (2012)	family	COI
Enghoff *et al.* (2011)	family	16S rRNA
		28S rRNA
Spelda *et al.* (2011)	species	COI
Wesener *et al.* (2011)	genus	COI
		16S rRNA
		18S rRNA
Loria *et al.* (2011)	genus	COI
Sota and Tanabe (2010)	species	COI-COII region
		EF-1a
Seeber *et al.* (2010)	genus	COI
Pitz and Sierwald (2010)	order	18S rRNA
		28S rRNA
Wesener *et al.* (2010)	order	COI
		16S rRNA
		18S RNA
Walker *et al.* (2009)	genus	12S rRNA
		tRNA valine
Cong *et al.* (2009)	class	18S rRNA
		28S rRNA
Marek and Bond (2006)	genus	16S rRNA
		12S rRNA
		tRNA valine
Regier *et al.* (2005)	order	EFI-a
		EF-2
		RNA polymerase 3
Bond and Sierwald (2002)	species	16S rRNA
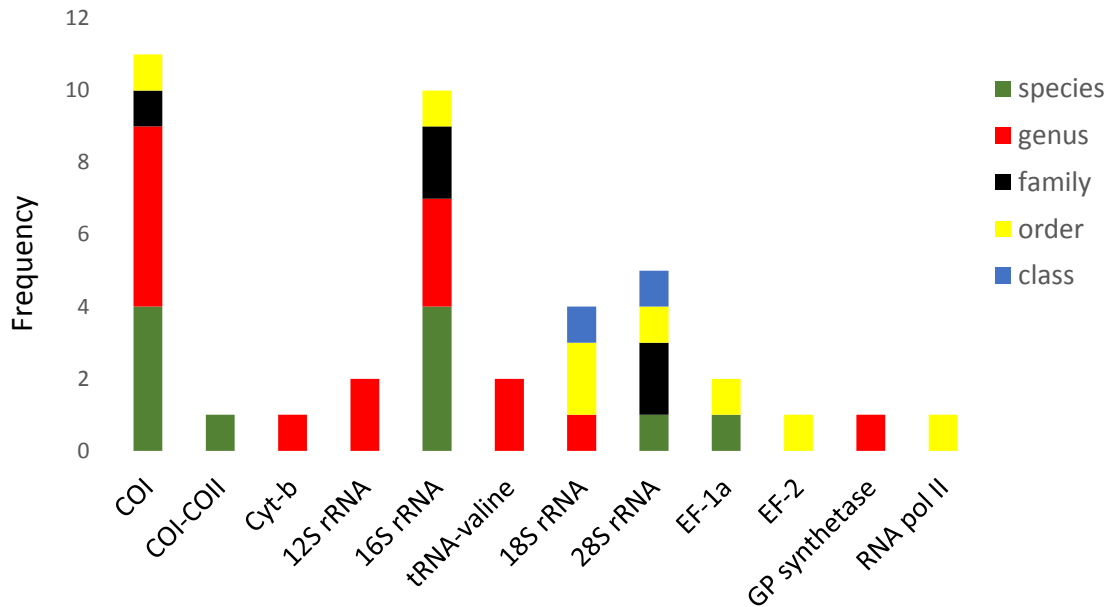
Figure 1 Frequency of DNA sequence marker use at the different taxonomic levels in millipedes.

Nuclear DNA consists of short tandem repeat units with three ribosomal RNAs encoding genes (18S rRNA, 5.8S rRNA and 28S rRNA) separated by non-coding spacers (introns) (Hwang & Kim, 1999). Generally nDNA encoding genes are more conserved relative to the mitochondrial genes, hence, are more suited for studying higher taxonomic levels. As such, ordinal (Wesener *et al.*, 2010; Pitz & Sierwald, 2010) and familial (Enghoff *et al.*, 2011, 2013) millipede taxonomic studies have been done basing on 18S rRNA and 28S rRNA. In contrast both the 18S rRNA and 28S rRNA have been used at low taxonomic levels. Both larger 28S rRNA and smaller 18S rRNA are characterized by a combination of highly conserved and rapidly evolving segments (Hwang & Kim, 1999). Therefore, variable segments of the 18S rRNA and 28S rRNA highlighted by Raupach *et al.* (2010) may account for their utility at genus and species level in millipedes. However, the variable sites may distort resolution at higher levels. For example, Wesener *et al.* (2010) reported good resolution at family level using the 18S rRNA after excluding the hypervariable regions from the analysis.

Protein coding nuclear genes (exons) are also a source of nDNA useful in taxonomy. This class of genes has a slower evolution rate in comparison to mitochondrial genes (Danforth *et al.*, 2005; Wild & Maddison, 2008). According to Danforth *et al.* (2005) substitution rates vary within and among the genes making them suited for both higher and lower level taxonomic studies. Four nuclear protein coding genes; elongation factor 1 alpha (EF-1α), elongation factor 2 (EF-2), RNA

polymerase II (RNA pol II) and glutamyl and prolyl-tRNA synthetase have been used in millipede taxonomy (Table 1). EF-2 and RNA pol II have been used to test millipede ordinal relationships while EF-1α and glutamyl and prolyl-tRNA synthetase have been employed in studying relationships of recently diverged taxa (Table 1).

The popularity of mtDNA over nDNA in millipede taxonomic studies may be attributed to the former's properties highlighted by Galtier *et al.* (2009). However, wide use could just be due to the fact that most studies are focusing on lower level taxonomic categories (see Table 1; Fig.1). Hence the preference for mtDNA would be inevitable in such a scenario.

Since the incorporation of DNA sequences data in taxonomy one of the mute points has been whether to use one or more genes for the same question. It is often argued that adding more taxa rather than genes improves the resultant phylogeny (Zwickl & Hillis, 2002; Heath *et al.*, 2008). An ideal DNA marker that can resolve taxonomic issues at all taxonomic levels may not exist (Curole & Kocher, 1999). Jenner (2004) suggested that a single gene based molecular phylogeny increases in likelihood when the same phylogeny is supported by a non-linked marker. Besides enhanced resolution, sequence data from different genomes also reduces gene specific deficiencies (Foster & Hickey, 1999). Brewer *et al.* (2012) reported concordance in the general topology of mitochondrial and nuclear gene trees. Results based on EF-1α and COI-COII both supported isolation by distance hypothesis in *Parafontaria tonominea* species complex (Sota & Tanabe, 2010).

Furthermore, sampling sequence data from both genomes presents an opportunity to concatenate (combined analysis of datasets) the datasets there by maximizing on the advantages of each gene (Capella–Gutierrez *et al.*, 2014). Studies such as Cong *et al.* (2009), Wesener *et al.* (2011) and Enghoff *et al.* (2011) have reported millipede phylogenies based on combined sequence data from either nuclear ribosomal genes or nuclear protein coding genes in addition to mitochondrial genes. Genetic analysis by Pimvichai *et al.* (2014) is an example where a combined data set yielded a tree similar in topology to the one obtained from the separate analysis of data sets. Although concatenated result are useful care should be taken in selecting appropriate marker combinations (Capella–Gutierrez *et al.*, 2014).

Generally, the integration of DNA with traditional morphology based taxonomy is widely accepted. For example, Loria *et al.* (2011) reported that COI differentiated between two species of the North American troglobiotic millipede *Tetracion* as did morphology. Marek & Bond (2006)

described a genus in their exemplar approach of a combined data analysis which included both morphological and molecular data. Pitz & Sierwald (2010) presented phylogenetic trees of relationships in the order Spirobolida based on both morphological and molecular data.

2.4 Conclusion

Given that 15 % of the estimated diversity of millipedes has been described (Sierwald & Bond, 2007; Golovatch & Kime, 2009) the contribution of DNA sequence markers to the relatively understudied millipedes cannot be understated. Based on their biology and ecology it is ideal to study the genetic variability of millipedes. Since morphology is too inclusive (Bond & Sierwald, 2002), molecular taxonomic revisions should become standard targeting all known millipede species including poorly studied African millipedes. Millipedes are microhabitat specialists and the habitats are discontinuous thereby limiting gene flow. Therefore, understanding historical processes or events underlying the millipede distribution may improve our understanding of millipede evolutionary relationships. Against such a background more attention should also be focused on understanding the phylogeography and population genetic structure of millipedes.

Mitochondria are the genome of choice in millipede evolutionary studies although evolutionary relationships may be distorted by introgression and incomplete lineage sorting (McGuire *et al.*, 2007; Petit & Excoffier, 2009). Therefore, as suggested by Enghoff *et al.* (2011) millipede species diagnosis and phylogenetics would benefit from the inclusion of a single copy nuclear gene. Such nuclear genes, although difficult to amplify, would complement mitochondrial genes in population genetics and phylogeographic studies. In addition, to developing and evaluating more protein coding genes, focus can also include the internal transcribed spacers (ITSs) of the nDNA.

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CHAPTER 3

GENETIC VARIATION, CRYPTIC DIVERSITY AND MOLECULAR PHYLOGENY OF *Bicoxidens* species Attems 1928 (DIPLOPODA, SPIROSTREPTIDA, SPIROSTREPTIDAE)

ABSTRACT

Bicoxidens Attems 1928 is an afrotropical millipede in the family Spirostreptidae which constitutes nine species. Based on the analysis of the male copulatory organs known as gonopods, *Bicoxidens* is monophyletic. Although gonopod morphology is central to millipede taxonomy, examination of gonopod morphology alone may not uncover hidden genetic diversity thereby underestimating species richness in *Bicoxidens*. Analysis of genetic data as an additional source of taxonomic data has revealed the genetic variation and flagged hidden species within millipedes. Hence, there was a need to revise the morphological species definitions and phylogeny of *Bicoxidens* based on DNA sequences. In this regard, the sequences of the mitochondrial *cytochrome c oxidase* subunit 1 (COI) and 16S rRNA genes were used to delimit species, uncover hidden species and test the monophyly of *Bicoxidens*. Genetic distances (Jukes-Cantor model) were computed based on COI only. Phylogenetic inferences were done based on Maximum likelihood and Bayesian inference analyses of the COI and 16S rRNA sequences individually and the combined data set (COI+16S rRNA). Interspecific genetic distances (> 0.075) based on the Juke-Cantor model supported the morphological species definition in *Bicoxidens*. However, high intraspecific sequence differences in *B. flavicollis* (> 0.14), *B. friendi* (0.084) and *B. brincki* (> 0.188) suggested the presence of cryptic species. The possible presence of cryptic species was supported by the phylogenetic analyses which recovered a paraphyletic *Bicoxidens* phylogram with *B. flavicollis*, *B. brincki* and *B. friendi* exhibiting divergent lineages. *Bicoxidens flavicollis* populations exhibited high genetic diversity and strong population differentiation which suggests restricted gene flow. The results support the utility of both COI and 16S rRNA in species delimitation in *Bicoxidens*. The high genetic divergence also suggests the presence of hidden species in *B. flavicollis, B brincki* and *B friendi*. The findings further highlight the important role of DNA sequences in uncovering cryptic diversity in millipedes.

3.1 Introduction

Millipedes (Class Diplopoda) are important macro-decomposers in terrestrial ecosystems (Hopkin & Read, 1992; Cárcamo *et al.*, 2000; Suzuki *et al.*, 2013). About 7 000 (Shear, 2011) to 12 000 species have been described in the Americas, Asia Australia, Europe and Africa (Sierwald & Bond, 2007; Golovatch & Kime, 2009; Shelley & Golavatch, 2011). Southern Africa accounts for less than 7 % (Hamer & Slotow, 2000; Sierwald & Bond, 2007) of the currently described species despite being cited as rich in millipede fauna. According to Hamer *et al.* (2006) tropical savannah woodlands south of the Zambezi river are dominated by members of the order Spirostreptida mainly the families Odontopygidae, Harpagophoridae and Spirostreptidae.

Bicoxidens Attems 1928 (Spirostreptidae) is endemic to savannah woodlands (Hamer *et al.*, 2006) south of the Zambezi river with Zimbabwe as the possible the center of radiation (Mwabvu *et al.*, 2007). Millipedes exhibit high endemism due to low dispersal capacity coupled with strict habitat preference (Hamer *et al.*, 2006; Enghoff & Seberg, 2006). According to Mwabvu *et al.* (2007) *Bicoxidens* species distribution is influenced by rainfall patterns and vegetation type. The nine recognized *Bicoxidens* species (*B. flavicollis* Attems 1928, *B. nigerrimus* Attems 1928, *B. nyathi* Mwabvu 2007, *B. gokwensis* Mwabvu 2007, *B. aridis* Mwabvu 2009, *B. grandis* Lawrence 1965, *B. matopoensis* Mwabvu 2007, *B. brincki* Schubart 1966, *B. friendi* Mwabvu, 2000) are restricted to habitats such as savannah woodlands that are dominated by *Brachystegia* sp. or *Acacia* sp. and miombo woodlands which receive high annual rainfall (Mwabvu *et al.*, 2007). All these factors including patchiness of habitats such as miombo woodlands (see Nyamapfene, 1991) may have contributed to speciation in this genus through geographical isolation and genetic divergence.

Millipede taxonomy relies on the identification of species specific differences in the male sperm transfer organs known as gonopods (Hopkin & Read, 1992; Sierwald & Bond, 2007). Gonopod morphology remains central to the taxonomy of spirostreptids including *Bicoxiden*s (Mwabvu *et al.*, 2007; Mwabvu *et al.*, 2013). *Bicoxidens* is characterized by an L-shaped gonopod teleopodite (see Fig. 1) which has no femoral process or torsion of the femur but has one or two lobes just before the bend after the femur which distinguishes the genus from other spirostreptid genera (Mwabvu *et al.*, 2007). Species definitions within the genus are based on the consistent variations in the telocoxite structural components including apical lobes and processes (Mwabvu *et al.*, 2007). A gonopod morphology based revision of *Bicoxidens* recovered a monophyletic phylogeny

with distinct species groups (see Mwabvu *et al*., 2007). However, the position of *B aridis* in this phylogeny is unknown as it was not part of the ingroup then.
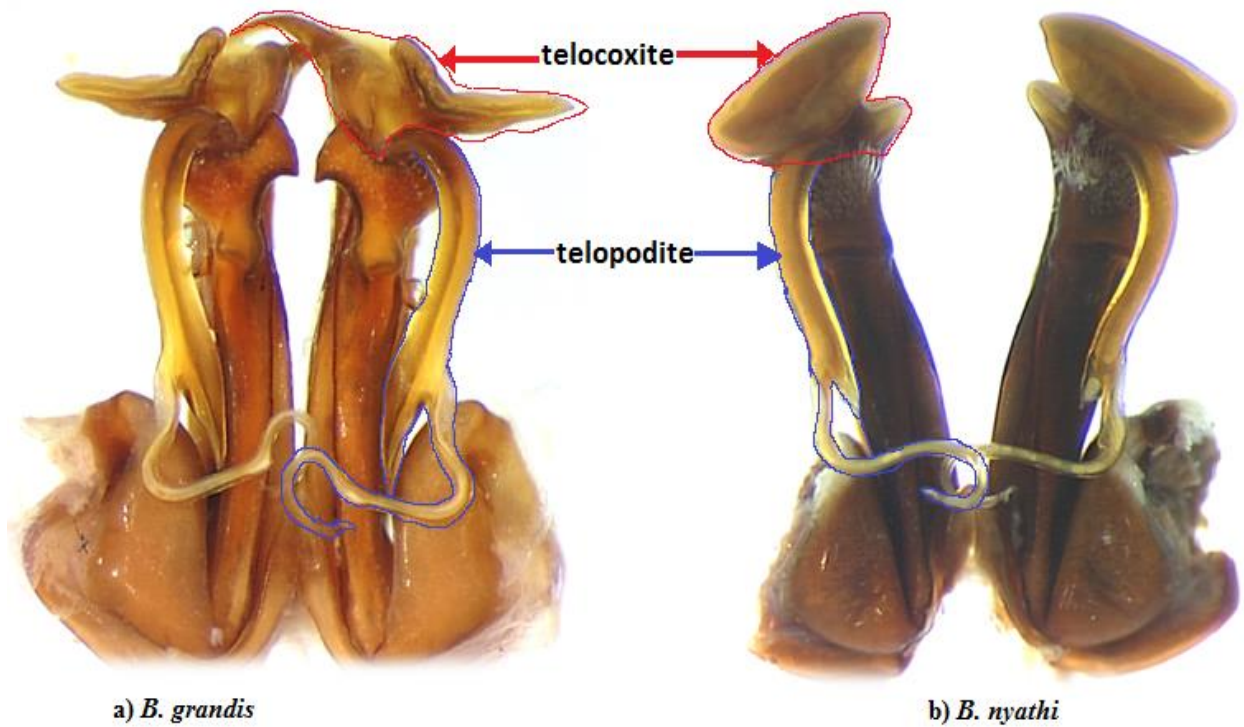
Figure 2. Oral view of gonopods of *B. grandis* and *B. nyathi* showing the characteristic L-shaped telopodite and the structural differences in the telocoxite between the two species (Images extracted from Mwabvu *et al.* 2007)

Although the utility of gonopods in millipede evolutionary studies is unquestionable, gonopods however fail to account for hidden species whose gonopods are morphologically similar (Bond & Sierwald, 2002; Mwabvu *et al.*, 2013). In light of this, genetic data based on mitochondrial and nuclear DNA markers have been used as additional source of data in millipede taxonomy, phylogenetics and phylogeographic studies (Bond & Sierwald, 2002; Marek & Bond, 2006; Walker *et al.*, 2009; Wesener *et al.*, 2011; Spelda *et al.*, 2011; Brewer *et al.*, 2012; Wesener, 2012; Frederiksen *et al.*, 2012; Pimvichai *et al.*, 2014; Nistelberger *et al.*, 2014). Congruency between genetic data from DNA markers such as COI and morphological data has provided support for the observed species boundaries and phylogenies in troglobiotic millipedes

(Loria *et al.*, 2011). However, no genus in the family Spirostreptidae has been evaluated based on molecular data.

The present molecular study is the first of an afrotropical spirostreptid millipede genus. Although a taxonomic revision based on gonopod morphology of *Bicoxidens* recovered distinct species, the present study seeks to test the congruency between interspecific genetic variations and the current species definitions. The utility of mitochondrial and nuclear DNA in species discrimination in this group would also be tested. Furthermore, the genetic structure of an isolation prone taxon such as *Bicoxidens* can be examined to elucidate the historical vicariance based on the patchiness of the preferred microhabitat. .

3.2 Materials and methods
3. 2.1 Taxon sampling

Bicoxidens specimens were collected from their known localities (Table 2). Fresh male specimens were collected by hand and preserved in 100 % ethanol to preserve the integrity of genetic material. Efforts were made to collect at least five samples per locality in order to get a representative sample but fewer specimens were sampled at some locations. Sampling for most of the species was hindered by anthropogenic modification to their known geographic ranges. Henceforth, some specimens were borrowed from the KwaZulu–Natal Museum, Pietermaritzburg (South Africa) and the Natural History Museum, Bulawayo (Zimbabwe) where single specimens of each species were available. A total of 48 *Bicoxidens* specimens comprising eight species were used in this study. Out of the nine species only *Bicoxidens gokwensis* was unavailable for use in this present study. All the species used in this study were identified by Tarombera Mwabvu.

3.2.2 DNA extraction

DNA extraction protocols, phenol chloroform isoamyl-alcohol extraction method (PCI) and ethanol precipitation were attempted, however, the Zymogen Genomic DNA™-Tissue MiniPrep commercial DNA extraction kit gave the best DNA yield. Total genomic DNA was extracted from 10 legs removed from the mid-body rings from each specimen using Zymogen Genomic DNA™-Tissue MiniPrep (Zymogen Research) according to the manufacturer's standard protocol.

The integrity and quantity of the extracted DNA was assessed through electrophoresis on 1 % TBE (tris-boris-EDTA) gel stained with 100 μl of ethidium bromide. DNA samples (5 μl) stained with

1 µl Loading dye and 2 µl of DNA ladder (l kb) were loaded into the wells and run at 100 volts for one hour in running buffer. The resultant gels were visualized using Chemidoc UV trans-illuminator (Bio-Rad) and single compact bands of ~1kb in size indicated good quality and quantity DNA yield.

3.2.3 DNA amplification and sequencing

Fragments of the mitochondrial (COI and 16S rRNA) and nuclear genome (Internal transcribed spacer region and Elongation factor–1α) were targeted for amplification using polymerase chain reaction (PCR). Unless mentioned all PCRs were performed in 25 µl reaction volumes contained 12.5 µl Econotaq, 7.82 µl PCR H_2O, 1 µl Bovine Serum Albumin (BSA), 0.84 µl of each primer and 2 µl of DNA template. A negative control was included in every PCR to check for contamination during the preparation of reaction volumes.Mitochondrial COI was amplified using universal primers by Folmer *et al.* (1994); LCO1490 and HCO2198 (see Table 3).The 16S rRNA gene fragment was amplified using primer sets by Kessing *et al.* (1989). 16Sar and 16Sbr and 16Sarl and 16Sbrh (see Table 3).

Only Mitochondrial 16S rRNA primers Rhino 16SJB and Rhino 16SNC (Bond & Sierwald, 2002) (Table 3) were used in a reaction mixture made up of 5 µl of 10 x PCR buffer, 4 µl of magnesium chloride at 25 mM, 4 µl of each primer at 6 µM, 0.5 µl of deoxyribonucleic triphosphates (dNTPs) (40 mM), 0.2 µl of 5 Units/µl *Taq* Polymerase (Supertherm), 9 µl of DNA template, made up a total volume of 25 µl with PCR H_2O.Amplification of the internal transcribed spacer region (ITS) and nuclear protein coding gene Elongation factor 1 alpha (EF1α) was carried out using primers from Presa *et al.* (2002) and Sota and Tanabe (2010), respectively (see Table 3).

After amplification, 5 µl of each PCR was electrophoresed in 1 % agarose gels stained with ethidium bromide (100 µl) to verify amplification of targeted region based on size. DNA ladder (2 µl) was also included in one of the wells as a size reference and the gels were run at 100 volts for one hour in running buffer before being viewed using Chemidoc UV trans–illuminator (Bio–Rad). PCR products were sequenced at Inqaba Biotechnical Industries, South Africa, using the forward primers from the initial PCR primer sets in Table.

Table 2 Locality and number of specimen for *Bicoxidens* species**.**

Locality	Species	Number of specimens	Latitude	Longitude
Chitombo	*B. flavicollis*	4	18º 28' 9.28''S	30 º 49'27.64''E
Chihota	*B. flavicollis*	6	17 º 50' 0.00''S	31 º 02'0.00''E
Mzinga	*B. flavicollis*	5	18 º 25' 0.00''S	32 º 58'0.00''E
Mazowe	*B. flavicollis*	6	17 º 28' 0.00''S	30 º 59'0.00''E
Marange	*B. flavicollis*	1	19 º 10'0.00''S	32 º 18'0.00''E
Nyanga	*B. flavicollis*	3	18º21'0.00''S	32º74'0.00''E
Muterere	*B. flavicollis*	3	18 º25'0.00''S	32 º 57'0.00''E
Chegutu	*B. flavicollis*	1	18° 8' 24.00"S	30° 9' 0.00"E
Dwala ranch	*B. matopoensis*	1	2129B2	
Maleme camp	*B. nigerrimus*	1	20 º 33'0.00''S	28 º 30'0.00''E
Chipise	*B. grandis*	1	2230B2	
Maleme camp	*B. matopoensis*	1	20 º 33'0.00''S	28 º 30'0.00''E
Lumene falls	*B. flavicollis*	1	20 º 34'0.00''S	28 º 25'0.00''E
Pande mine	*B. brincki*	1	22 º 18'0.00''S	30 º 16'0.00''E
Hovi crossing	*B. flavicollis*	1	20 º 37'0.00''S	28 º 30'0.00''E
Mtawatawa	*B. friendi*	8	17 º 09'0.00''S	31 º 59'0.00''E

Table 3 Primers and PCR thermal profiles used to amplify the CO1, 16S, ITS2 and EFI **α** regions for *Bicoxidens* species.

Mitochondrial Cytochrome oxidase 1

Primer	Direction	Sequence	PCR thermal profile
LCO 1490	F	5'GGTCAACAAATCATAAAGATATTGG3'	initial denaturation 95 ºC for 5 minutes, followed by 40 cycles of 94 ºC for
HCO2198	R	5'TAAACTTCAGGGTGACCAAAAAATCA3'	60 seconds, 40 ºC for 60 s and 72ºC for 90 s and final extension at 72 ºC for 10 min

Mitochondrial 16S rDNA

Primer	Direction	Sequence	PCR thermal profile
16Sar	F	5'CGCCTGTTTTTCAAAAACAT3'	initial denaturation temperature of 94 °C for 2 min,
16Sbr	R	5'CCGGTTTGAACTCAGATCATGT3'	followed by 36 cycles of 94 °C for 30 seconds, 55 °C for 30 s and 72 °C for 2 min, and then a final 72 °C for 5 min.
16Sarl	F	5'TGCCTGTTTATCAAAAACAT–3'	initial denaturation temperature of 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s,
16Sbrh	R	5'CCGGTCTGAACTCAAATCATGT3'	59.3 °C for 30 s and 72 °C for 2 min, and then a final 72 °C for 5 min.
Rhino 16SJB	F	CCA TGT ATT TGA TAA ACA GGC A	95ºC initial denaturation for 5 minutes followed by 25 cycle of 94 ºC denaturation for 30 s,
Rhino 16SNC	R	GTG GGG GTA TTG GAA AAT GTT C	annealing at 52 ºC to 55 ºC for 30 s, a 1 min extension at 72 ºC,final 10 min extension at 72 ºC

Internal Transcribed Spacer 2

Primer	Direction	Sequence	PCR thermal profile
5.8sr	F	5'CTACGCCTGTCTGAGTGTC'	initial denaturation 94 °C for 2 min,
28s	R	5'ATATGCTTAAATTCAGCGGG3'	followed by 36 cycles of 94 °C for 20 s, 48.5– 52 ºC for 20 s and 72 °C for 45 s, a final 72 °C extension for 5 min.

Elongation Factor 1 alpha (EF1α

Primer	Direction	Sequence	PCR thermal profile
DiploEF1aF	F	5'GCCTGG GTT TTG GATAAA CTTAAG GC3	initial denaturation 94 °C for 2 min,
DiploEF1aR3	R	5'CCT CCA ATC TTG TAA ACG TC3'	followed by 36 cycles of 94 °C for 20 s, 48.5– 52 ºC for 20 s and 72 °C for 45 s, a final 72 °C extension for 5 mi

3.2.4 Analysis

Sequences were manually scanned for ambiguous nucleotide reads and edited in BioEdit (Hall, 1998). Consensus sequence alignments were constructed in BioEdit using the Clustal W alignment accessory option. Sequences for outgroup taxa *Doratogonus* sp. AY288738.1 (COI), AY288715.1 (16S rRNA), *Pachyiulus varius* (Accession number JN619384.1) and *Tetracion tennesseensis* (Accession number JN656611.1) were downloaded from NCBI Genebank database.

The levels of saturation in each data set were assessed using DAMBE (Data Analysis in Molecular Biology and Evolution) version 5 (Xia & Xie, 2001) prior to phylogenetic analyses. Saturation tests evaluate phylogenetic utility of a data set because high substitution levels erode the phylogenetic signal. The index of substitution saturation (Iss) was calculated for the data set, where Iss values lower than both the critical indices of substitution saturation symmetrical (Iss_c) suggests little saturation within the data set. Analysis was carried out for COI and 16S rRNA individually and as a concatenated data set of both genes.

Genetic distances and phylogenies were inferred using Neighbor joining (NJ) trees Bayesian inference (BI) and maximum likelihood (ML) trees. Models of nucleotide substitution that best fit each data set were selected using MrModeltest 2.3 (Nylander, 2004). Based on the Akaike Information Criterion (AIC), the General Time Reversible with a Gamma distribution (GTR+G) model seemed appropriate for the COI data set while the General Time Reversible (GTR) model was selected for the 16S rRNA data set. For the combined data set analysis two partitions were created and each partition was assigned the appropriate nucleotide substitution model.

Genetic distance matrices were computed in MEGA 6 (Kumar *et al.*, 2008). The NJ trees were estimated in PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 (Swofford, 2003) with nodal support for the trees being estimated by resampling with a 1000 replicates. The maximum likelihood tree was estimated using GARLI (Zwickl, 2006) with commands including a heuristic search with starting tree(s) obtained via neighbor–joining, branch–swapping algorithm and resampling with a 1000 replicates. MrBayes version (Ronquist & Huelsenbeck, 2003) was used for the Bayesian Inference wherein four Markov chains (three heated and 1 cold) were run. Two parallel runs were conducted each for 10 million generations with a 25 % burnin. The burnin period was determined by analysis of optimal parameters and likelihood files generated by the sump

command in the MrBayes command block using Tracer v1.4.1 (Rambaut and Drummond 2007). Trees generated before the plot had plateaued were discarded as burn in.

3.3 Results

Fresh specimens yielded high quality DNA templates for subsequent PCR as opposed to older museum specimens. The museum specimens were preserved in formalin-containing preservation solutions which affected the recovery of DNA from these specimens (Linville *et al.* 2004). Sequence alignments were obtained for both mitochondrial COI and 16S rRNA. EF1–α did not amplify despite repeated attempts. The nuclear ITS 2 region amplified for fresh specimens only, however, the sequences obtained could not be aligned due to failed sequence reads.

According to the saturation test, the COI, 16S rRNA and combined (COI+ 16S rRNA) data set had a useful phylogenetic signal. The data sets yielded an average Iss value that was significantly lower than the Iss_c assuming both symmetrical and asymmetrical topologies (Table 4).

Table 4 Saturation test for COI, 16S rRNA and COI+16S rRNA

	Iss	Iss_c	P
COI	0.166	0.708	<0.001
16S rRNA	0.198	0.682	<0.001
COI+16S rRNA	0.185	0.722	<0.001

The base composition of the 535 base pair COI alignment were as follows: A= 0.27618, C= 0.22969, G= 0.15188, T= 0.34224 with 255 variable sites and 188 parsimony informative sites. The chi squared homogeneity test demonstrated significantly homogenous nucleotide frequencies among taxa (X^2= 16.716005, df =141, p =1.00). The base pair composition of the 16S rRNA data set (443bp) were as follows: A=0.3294, C=0.0995, G= 0.2036, with 373 variable and 151 parsimony informative sites. The homogeneity test indicated significant homogenous nucleotide frequencies among taxa (X^2= 12.63 df= 102, p= 1.00). The concatenated data set (COI+16S rRNA) had A, C, G, T nucleotide frequencies of 0.3009, 0.1728, 0.1754 and 0.3511, respectively. The homogeneity test among taxa was significant (X^2= 10.45, df= 102, p= 1). The concatenated alignment consisted of 978 base pairs with 562 variable and 297 parsimony informative sites.

Sequence divergence analysis was carried out for the COI gene only because sequences for the gene were available from all study specimens. Genetic distances based on the Jukes–Cantor model in MEGA 5 (Kumar *et al.*, 2008) based on the COI alignment revealed genetic variation range of 0.0096 – 0.227 with a mean of 0.1340 among *Bicoxidens* species (see Table 5). Mean intraspecific genetic divergence could only be estimated for *B. flavicollis, B. friendi* and *B. brincki*. Intraspecific genetic divergence in *B. flavicollis* ranged from 0 to 0.280 with a mean of 0.107 (Table 7). *Bicoxidens flavicollis* specimens from Mazowe (BflvD1–6) were separated from other conspecific members by distances between 0.140 and 0.220. Furthermore, the intra-population genetic distances within the Mazowe population were also very high with two groupings apparent. The group consisting BflvD1, BflvD2 and BflvD3, and another consisting of BflvD4, BflvD5 and BflvD1 had genetic distances ranging from 0.140–0.160 (Table 7). The intraspecific genetic divergence exhibited by *B. flavicollis*–Nyanga1 (BflvF1) (0.217–0.282) was higher than the average 0.134 between species. *Bicoxidens brincki* (BbriP) from Pande, although morphologically similar, also showed high divergence (0.2369) (Table 6) from other specimens of *B. brincki* and the mean was higher than the species mean (0.134) (Table 5).

Table 5. Comparison of genetic distances (below diagonal) between *Bicoxidens* species under the Jukes-Cantor model based on COI. Mean interspecific variation was 0.134

	B. flav	*B. fri*	*B. bri*	*B. nig*	*B. gra*	*B. mat*	*B. nyathi*
B. flavicollis							
B. friendi	0.127						
B. brincki	0.157	0.126					
B. nigerrimus	0.136	0.096	0.109				
B. grandis	0.131	0.104	0.108	0.075			
B. matopoensis	0.147	0.104	0.136	0.100	0.101		
B. nyathi	0.194	0.145	0.196	0.159	0.166	0.171	
B. aridis	0.167	0.160	0.165	0.166	0.158	0.171	0.227

B. flav= *B. flavicollis*; B. fri= *B. friendi;*   B. bri=*B. brincki;*   B.nig=*B. nigerrimus*; B. gra= *B. grandis*; B. mat=*B. matopoensis.*

Phylogenetic tree support based on both bootstrap support and posterior probabilities was delimited as follows: 0.65/64 < weak supported, 0.66/65< moderate support >0.89/89 and 0.90/90> strong support. For the 16S rRNA and combined data sets sequences were only obtained for *B. flavicollis*, *B friendi*, *B. brincki* and *B. grandis* only.

The COI tree, 16S rRNA tree and combined dataset (COI+16S rRNA) tree exhibited a degree of congruence with some major clades and sub-clades recurring in all trees with moderate to strong support. The 16S rRNA tree and the combined data set tree were similar in topology.

Table 6. Comparison of intraspecific genetic distances within *B. friendi* and *B. brincki* under the Jukes-Cantor model based on *COI*. Mean intraspecific divergence for each species is in bold.

	*B.friendi*1	*B. friendi*2	*B. friendi*3	*B. friendi*4	*B.friendi*5	*B brincki*P	*B. brinki*B
*B. friendi*1	**0.0349**						
*B. friendi*2	0.0134						
*B. friendi*3	0.0232	0.0232					
*B .friendi*4	0.0333	0.0212	0.0153				
*B. friendi*5	0.0375	0.0272	0.0212	0.0252			
*B. friendi*6	0.0840	0.0724	0.0611	0.0611	0.0459		
*B. brincki*P	0.2174	0.2174	0.2206	0.2238	0.2174	**0.1881**	
*B. brincki*B	0.1029	0.0956	0.0884	0.0884	0.0791	0.2303	
*B.brincki*D	0.1204	0.1230	0.1255	0.1281	0.1360	0.2369	0.0980

Numbers next to the species name are replicates, letters next to the species name are locations. P=Pande mine, B=Beitbridge and D=Doddieburn Ranch.

All phylogenetic trees recovered a paraphyletic *Bicoxidens* clade with morphologically congeneric members basally splitting from the genus (Figs 3 & 4). The *Bicoxidens* clade (Figs 3 & 4) recovered was paraphyletic but it shares consistencies with the monophyletic *Bicoxidens* clade based on gonopod morphology by Mwabvu *et al.* (2007).

Table 7. Comparison of intraspecific genetic distances within *B. flavicollis* under the Jukes-Cantor model based on COI. Mean intraspecific divergence is in bold.

	BflvA1	BflvB1	BflvC1	BflvD1	BflvD2	BflvD3	BflvD4	BflvD5	BflvD6	BflvE	BflvF1	BflvF2	BflvF3	BflvG
BflvA1	**0.1071**													
BflvB1	0.05													
BflvC1	0.05	0.05												
BflvD1	0.15	0.15	0.15											
BflvD2	0.15	0.15	0.15	0.01										
BflvD3	0.14	0.14	0.14	0.01	0.01									
BflvD4	0.17	0.18	0.16	0.16	0.16	0.16								
BflvD5	0.17	0.18	0.16	0.16	0.16	0.16	0.00							
BflvD6	0.17	0.17	0.15	0.15	0.15	0.14	0.03	0.03						
BflvE	0.05	0.05	0.04	0.15	0.14	0.14	0.16	0.16	0.17					
BflvF1	0.24	0.23	0.23	0.22	0.21	0.21	0.22	0.22	0.21	0.22				
BflvF2	0.10	0.10	0.10	0.21	0.21	0.20	0.20	0.20	0.20	0.10	0.28			
BflvF3	0.07	0.06	0.06	0.17	0.16	0.16	0.17	0.17	0.18	0.06	0.23	0.05		
BflvG	0.05	0.05	0.00	0.15	0.15	0.14	0.16	0.16	0.15	0.04	0.23	0.10	0.07	
BflvJ	0.08	0.06	0.08	0.14	0.14	0.13	0.15	0.15	0.15	0.08	0.22	0.12	0.08	0.08
BflavK	0.14	0.13	0.15	0.04	0.04	0.05	0.17	0.17	0.16	0.14	0.22	0.19	0.14	0.15

Bflv= *B. flavicollis*. Letters next to the acronym represent locality, where A=Chitombo, B=Chihota, C=Mzinga, D=Mazowe, E=Marange, F=Nyanga, G=Muterere, J=Chegutu and K=Maguge. The numbers next to the letters are replicates.

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Figure 2. Distribution of *Bicoxidens* species sampled
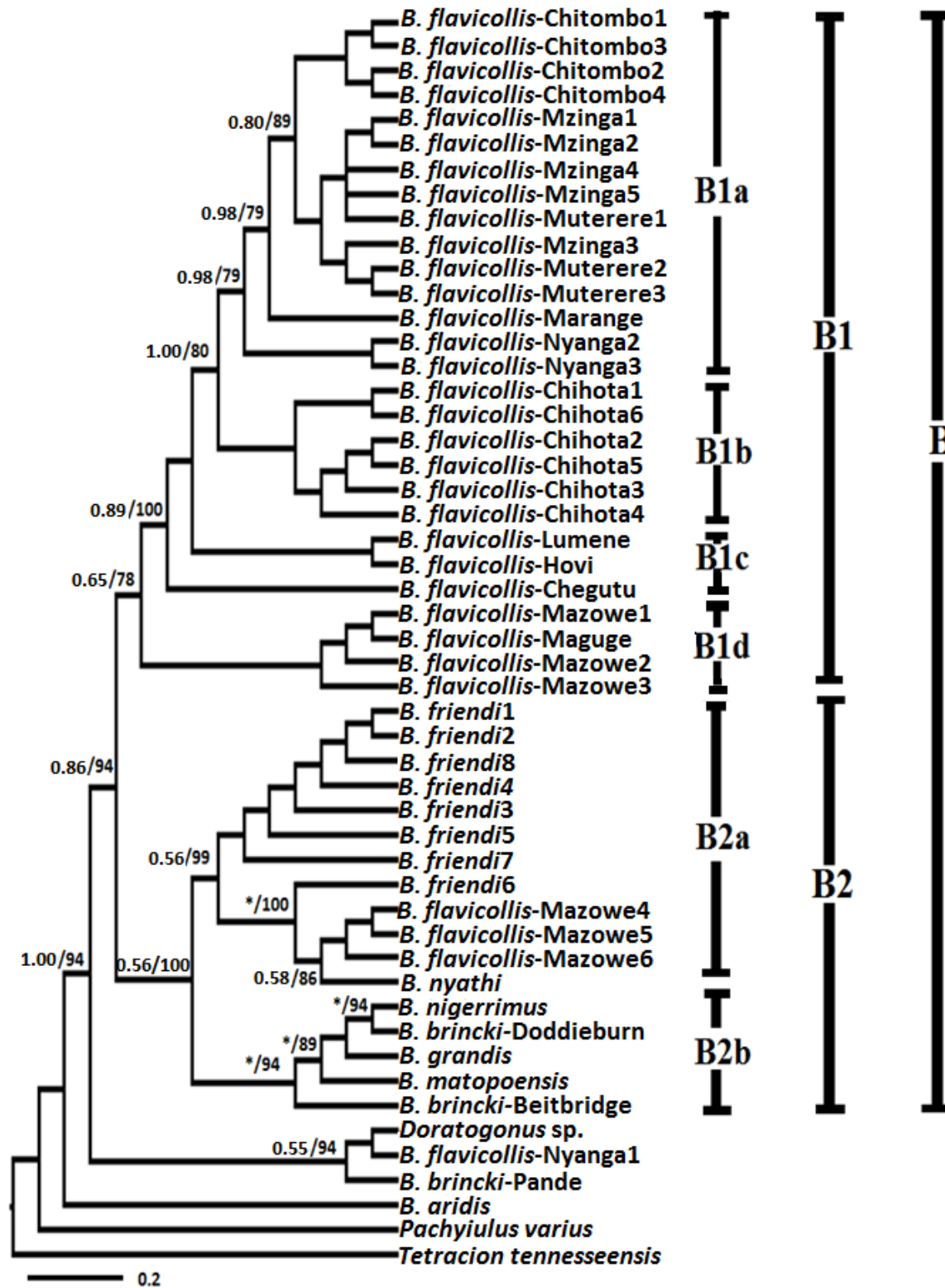
Figure 3. Phylogenetic relationships within *Bicoxidens* based on the ML analysis of CO1. Nodal support values are indicated, as Bayesian inference (BI) posterior probabilities/ML bootstrap support. Vertical bars denote major clades and subclades. * indicates BI posterior probabilities that are less than 0.5. Branch labels include the species, locality and replicate number.
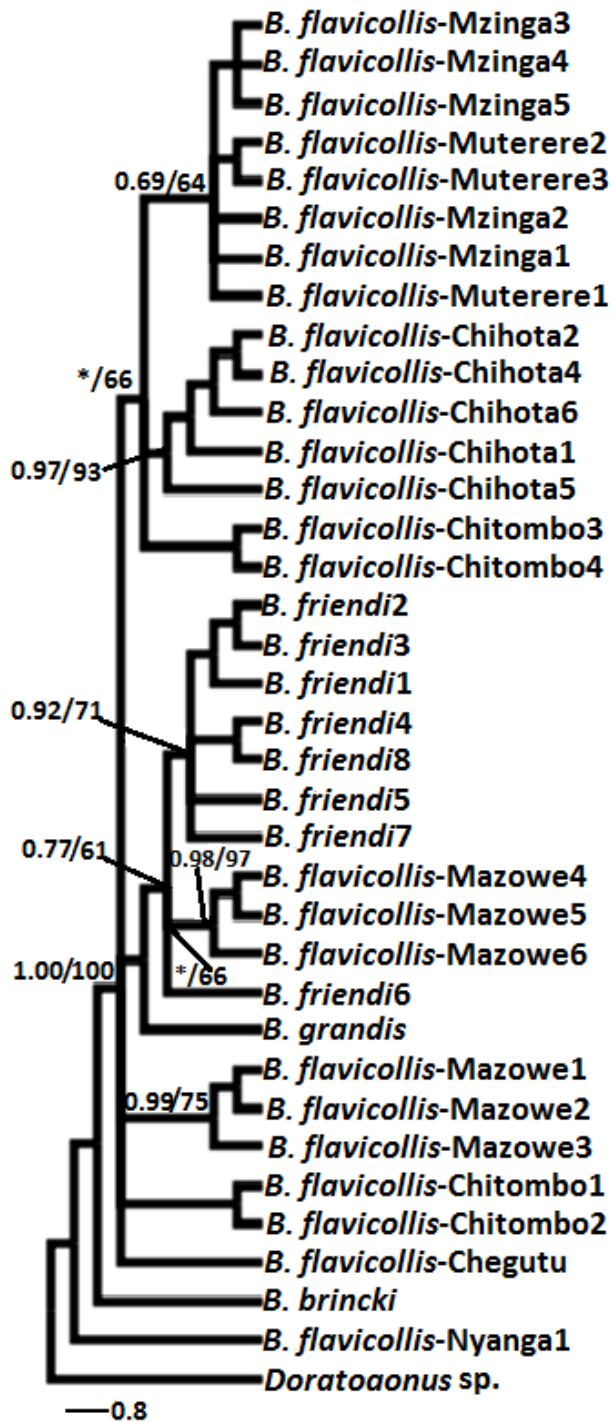
Figure 4. Phylogenetic relationships within *Bicoxidens* based on the ML analysis of combined data set (COI+16S rRNA). Nodal support values are indicated, as Bayesian inference (BI) posterior probabilities/ML bootstrap support. Vertical bars denote major clades and subclades. * indicates BI posterior probabilities that are less than 0.5.

Based on the COI tree, two sister clades were evident, one with *B. flavicollis* (clade B1) and clade B2 comprising *B. nigerrimus, B. nyathi, B aridis, B. grandis, B matopoensis, B. brincki, B. friendi* with three *B. flavicollis*-Mazowe members (Fig 3). Furthermore, a split in clade B2

further separates into clade B2a and clade B2b in the COI tree (Fig. 3) which are similar to the species groups identified by Mwabvu *et al.* (2007) except for the swap in position between *B. nyathi* and *B. grandis*. In clade B2b, the large body-sized *B. brincki*-Beitbridge is a sister taxon to *B. nigerrimus, B. grandis, B. matopoensis* and *B. brincki*-Doddieburn. The clade between *B. nigerrimus* and *B. brincki*-Doddieburn is strongly supported and is consistent with their morphology-based relationship. *Bicoxidens matopoensis* still retains its basal position in relation to the *B. nigerrimus* and *B. brincki*-Doddieburn clade (Fig. 3). Despite constituting only four species, the combined tree (Fig. 4) concurred with the *B. friendi–B. grandis* clade in Mwabvu *et al.* (2007).

Both COI tree (Fig. 3) and combined data set tree (Fig. 4) retrieved paraphyletic groupings of *B. flavicollis*. The *B. flavicollis* clade (Clade B1) recovered by the COI gene had moderate support with members from Mazowe (1, 2 & 3) and Mozambique occupying the basal position (Fig. 3). Clade B1 also includes a sub-clade with *B. flavicollis*–Chegutu, a population in Central Zimbabwe, at the basal node followed by three distinct clades nested within the clade according to geographic location as south-western (B1c), northern (B1b) and eastern (B1a) clades (see Figs. 2 & 3). In both the COI tree (Fig. 3) and combined gene tree (Fig 4), *B. flavicollis* members from Chihota and Chitombo formed clades according to sampling site and proximity in the case of Muterere–Mzinga clade (see Fig. 2).Three species, *B. friendi*, *B. flavicollis* and *B. brincki* which had more than one replicate, exhibited paraphyly. The northern *B. flavicollis*–Mazowe population occurred in two distinct clades in all trees with the population exhibiting high genetic distances (CO1: 0.14–0.22; Table 7) from other *B flavicollis* populations (Figs. 3 & 4). The sub–clade of *B. flavicollis*–Mazowe (4; 5 & 6) and *B friendi* 6 (Fig. 3; Clade B2a) further demonstrates the paraphyly of *B. flavicollis* and *B friendi*. The *B. flavicollis*-Mazowe (4; 5 & 6) and *B. friendi* 6 clade is also moderately supported and strongly supported in the combined tree (Fig. 4).

The positions of *B. flavicollis*-Nyanga 1, *B. brincki*-Pande and the recently described *B. aridis* in all trees were inconsistent. *Bicoxidens flavicollis*-Nyanga 1 and *B. brincki*-Pande both formed a strongly supported clade with the outgroup *Doratogonus* (Fig. 3). High COI genetic distances for both *B. flavicollis*-Nyanga 1 and *B. brincki*-Pande lend support to their grouping (Table 6 & 7). In

the combined data tree, *B. flavicollis*-Nyanga 1 and *B. brincki*-Pande appear to be outside the *Bicoxidens* clade (Fig. 4).

3.4 Discussion

Advances in DNA molecular technology have provided the means to examine species definitions and phylogenetic relationships within taxa using DNA sequence data. However, besides the ongoing debate on reliance on DNA sequence data in systematics, there are challenges in acquiring the good quality DNA from preserved specimens. According to Linville *et al*. 2004 specimens preserved in formalin containing solutions generally yield low quality DNA which in turn presents a challenge in obtain good quality DNA sequence data. The present study sampled from museum collection stored in formalin as fresh specimens could not be obtained for six species (*B. nigerrimus*, *B. nyathi*, *B aridis*, *B. grandis*, *B. matopoensis* and *B. brincki*) out of the eight species studied. Preserved museum specimens are valuable and are not always readily available for destructive DNA extraction methods. Single specimens of each of the six species were obtained from the museums. This effectively impacted on the sample size of the study as five replicates could be obtained for fresh specimens only. Millipedes are short range endemics with low dispersal capabilities and some are restricted site endemism (Harvey, 2002; Hamer & Slotow, 2002). Most *Bicoxidens* species have been collected once and the collection has been done from a single site. Some of these sites have undergone anthropogenic modification. Despite rigorous sampling efforts these species were not found. It may be hypothesized that populations may have been fragmented and disappeared as numbers dwindled to unviable states leading to extirpation therefore hampering population recovery.

Mitochondrial COI was amplified and sequenced for both museum and fresh specimens while the nuclear rDNA (ITS) amplified for fresh specimens only and the resultant sequences where of low quality based on the ABI file chromatogram peaks therefore not useful. The success in obtaining mtDNA sequence data may be attributed to the universal primers, ease of amplification and high copy numbers. A few ITS sequences that were obtained could not be used due to high heterogeneity which may not be uncommon in millipedes (Nistelberger *et al.*, 2014). Appropriate primers are also critical when amplifying a gene fragment. Nuclear EF1–α failed to amplify despite the use of diplopod specific primers which further highlights the difficulty in amplifying nuclear protein coding genes. Mitochondrial 16S rRNA also did not amplify using primers designed for the Rhinocricidae millipedes (Bond & Sierwald, 2002). This is a bit unusual considering that the

same primers were employed successfully in studying phylogenetic relationships of some genera in the family Spirostreptidae (Mwabvu *et al.*, 2013). Inconsistencies of this magnitude may make it difficult to exploit DNA sequence data in millipede taxonomic and evolutionary studies. In addition, unclear specimen preservation methods coupled with millipede defensive secretions could have negatively impacted on the quality of DNA extracted from museum specimens and the inherent amplification success.

Phylogenetic trees based on COI, 16S rRNA and the combined data set had relative similarities in their general topologies. Mitochondrial COI has a higher evolutionary rate compared to the 16S rRNA gene which may account for the phylogenetic incongruence between the former and latter trees (Lavrov *et al.* 2002; Woo *et al*. 2007;Galtier *et al.*, 2009). The COI tree was well resolved and with few polytomous clades in comparison to the 16S rRNA tree suggesting that COI is a better suited marker for this genus. However, the difference in the sizes of the data sets may have induced some of the observed differences between the COI and 16S rRNA trees. Studies by Pollock *et al.* (2002) and Hedtke *et al.* (2006) suggest that the number of taxa considered for analysis may impact the tree topology. All sequences were analyzed for COI while not all sequences were available for the 16S rRNA. Ultimately the incongruence exhibited by combined data may have also been influenced by the fore mentioned possibilities.

Despite minor inconsistencies among themselves, the gene trees recovered a paraphyletic *Bicoxidens* phylogram. Out of all three phylogenetic trees, the COI tree showed a high degree of concordance with the gonopod based consensus tree. Based on the COI gene, three distinct species groups as in the gonopod based consensus tree were also apparent in the molecular analysis albeit minor positional differences in some clades. All trees generally recovered *B. flavicollis* as a distinct clade while *B. nigerrimus, B. nyathi, B. aridis, B. grandis, B. matopoensis, B. brincki* and *B. friendi* formed the other clade. This congruency between the morphological and molecular trees is supported by the high degree of overlap in gonopod structure similarities and differences. In *Bicoxidens* the gonopod apical telocoxite varies in shape and structure between species (Mwabvu *et al.*, 2007). *Bicoxidens nigerrimus, B. nyathi, B. grandis, B matopoensis, B. brincki*–Doddieburn and *B. friendi* characteristically share a similar distal telocoxite with lateral processes and lack median processes, traits which they are likely to have inherited from a common ancestor. It also interesting to note that *B. friendi* is the only species in this clade occurring in northern Zimbabwe while the rest occur in the south to south–western parts (see Figs. 2 & 3). Since the clade includes

larger species, *B. brincki* and *B. grandis,* it may be assumed that the common ancestor may have been large-bodied and therefore more mobile. The basal position occupied by *B. brincki*–Beitbridge in the sub-clade which includes *B. nigerrimus, B. grandis, B matopoensis* and *B. brincki*-Doddieburn also suggests dispersal northwards from a southern locality by a large bodied ancestor (Figs 2 & 3)

The strongly supported clade of *B. brincki*-Doddieburn and *B. nigerrimus* is a constant feature in both morphological and molecular phylogenetic trees. This may also be expected because *B. brincki*-Doddieburn and *B. nigerrimus* share a resemblance in folding of the telocoxite along with *B. matopoensis* (Mwabvu *et al*. 2007) which may explain the position of *B. matopoensis* in relation to this clade. Furthermore, localities of *B. nigerrimus* and *B. brincki*-Doddieburn and *B. matopoensis* are in the south-western pocket of Zimbabwe, a factor which may influence their relationship. However, not all species relationships in Clade B2 seem to be influenced much by geographic proximity (Fig. 3). For example, Clade B2a consist of *B. friendi* and *B. flavicollis*-Mazowe (4, 5, & 6) collected from northern Zimbabwe and *B. nyathi* collected from south central Zimbabwe (see Figs 2 & 3). Under a speciation by distance model, *B. nyathi* would be expected to be closely related to species in clade B2b, *B. matopoensis* and *B. nigerrimus,* due to their close proximity (Figs 2 & 3). Estimating the divergence times of each species and correlating the divergence times to historical events may shed more light on the speciation mechanisms of *Bicoxidens* species.

The position of the recently described *B. aridis* was not considered in the morphological consensus phylogeny of *Bicoxidens*. Based on the molecular phylogram (see Fig 3), *B. aridis* is outside the general *Bicoxidens* clade. Congeners have a characteristic L-shaped telopodite which among other characteristics separates *Bicoxidens* from other spirostreptid genera (Mwabvu *et al.*, 2007). In addition to the unique clockwise coil, the post knee telopodite in *B. aridis* lacks the L-shape. This difference in the shape of the telopodite coupled with other characteristics described in Mwabvu *et al.* (2009) lends support to the mitochondrial COI data and raise questions about the position of *B aridis*. However, *B. aridis* could be demonstrating divergence, as a result of adaptations to different local conditions in the Zambezi valley.

High levels of genetic divergences coupled with paraphyly were observed in *B. flavicollis*, *B. brincki* and *B. friendi,* despite homogeneity in gonopod morphology. *Bicoxidens flavicollis* is the most widely distributed species in the genus (Mwabvu *et al.*, 2007). According to Mwabvu *et al*.

(2007) populations of the species vary in color depending on the geographic location. Generally the *B. flavicollis* populations were clustered according to the geographic ranges (see Figs 2 & 3). Most species were collected from the north to the eastern region of Zimbabwe where rainfall is more reliable than the southern parts. Given that *B. flavicollis* is relatively small and less mobile, the transition in climatic conditions and vegetation type from the north and east to south may have restricted gene flow encouraging isolation and greater genetic divergence between populations.

Allopatric populations are expected to be genetically distant due to the low vagility of millipedes (Bond & Sierwald, 2002; Sota & Tanabe, 2010; Wojcieszek & Simmons, 2012) and this is likely to be reflected in molecular phylogenetic trees. However, the *B flavicollis*-Mazowe (1, 2 & 3) and *B flavicollis*-Maguge clade which occurred in all trees is puzzling. Members of the Maguge population would be expected to be genetically closer to geographically proximal eastern *B. flavicollis* population than Mazowe, which is approximately 300 km away. Such evidence implies that geographic distance is not necessarily positively proportional to genetic divergence among *Bicoxidens* populations. This warrants testing population structure and isolation by distance among *Bicoxidens* populations.

The occurrence of the *B. flavicollis*-Mazowe population in two distinct clades in all trees despite similarity in gonopods suggests cryptic diversity within this species. The high intra-population genetic variation may be explained by low vagility, strict habitat preference and high levels of site endemism which may have caused such genetic divergence due to isolation (Hamer *et al.*, 2006; Moir *et al.*, 2009). Such intra-population differences suggest that *B. flavicollis* is a species complex. Despite the caveat of using few samples high genetic divergences observed by Mwabvu *et al.* (2013) between two populations of *B. flavicollis* support this notion. Millipedes tend to exhibit variations in characteristics according to geographic location (Tanabe *et al.*, 2001). Findings by (Mwabvu *et al.*, 2013) suggest that there may be a correlation between the color morphs and genetic divergence in *B. flavicollis.*

The position of *B. brincki*-Pande, *B. flavicollis*-Nyanga1 and *B. friendi*6 in the molecular trees was unexpected and inconsistent with morphological definitions. Although unexpected this may be due to the presence of potential cryptic species within *Bicoxidens* rather than incorrect identification. The decoupling of genetic diversification and change in gonopod morphology has been observed in opiliones (Derkarabetian *et al.*, 2011), spiders (Huber *et al.*, 2005) and millipedes (Bond &

Sierwald, 2002; Bond *et al.*, 2003). Although gonopods are central to species delineation, examination of gonopods alone may not reveal hidden species (Jacob *et al.*, 2004).

3.5 Conclusion

This is the first molecular study of phylogenetic relationships within an afrotropical millipede genus. Despite the initial aim of utilizing both mitochondrial and nuclear genes, mitochondrial COI and 16S rRNA were analyzed. Based on both the COI gene and 16S rRNA gene, *Bicoxidens* was paraphyletic. However, the utility of COI was emphasized by the congruency in the topology of the morphological tree and the COI gene tree. The presence of cryptic species in *Bicoxidens* is supported by the high intraspecific genetic divergences and the presence of divergent lineages for *B. flavicollis, B. friendi* and *B. brincki.* Given such evidence population genetics for each species should be studied to better understand the levels of cryptic diversity. Paraphyly was observed despite similarity in gonopod morphology. Genitalia of cryptic species flagged in this study may be evaluated using morphometric landmark analyses. Morphometric landmark analyses may reveal taxonomically useful but subtle differences or variation in morphology which may have been overlooked by qualitative analyses.

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CHAPTER 4: PHYLOGEOGRAPHY AND POPULATION GENETICS OF *Bicoxidens flavicollis* Attems 1928.

ABSTRACT

Bicoxidens flavicollis Attems, 1928 is a small bodied and color polymorphic southern African millipede. However, despite strict microhabitat specialization and low vagility, *B. flavicollis* is widely distributed across geographic range characterised by heterogeneity in vegetation type and climate. Since vicariance and dispersal are known instigators of distribution in taxa, this present study sought to ascribe the observed distribution of *B. flavicollis* populations to either vicariance or dispersal by assessing the phylogeography and population structure within the species. DNA sequences from the mitochondrial *cytochrome oxidase* 1 and 16S rRNA genes were analysed in order to infer levels of genetic diversity, population differentiation and structure. Genetic diversity indices, revealed high genetic diversity within *B. flavicollis*. Strong population differentiation suggested limited gene flow thus inbreeding among *B. flavicollis* populations. In addition, population paraphyly was observed in two populations at Mazowe and Nyanga, based on the phylogenetic relationships. Bayesian population structure grouped the populations into four genetic clusters with no admixture. Mantel's test for isolation by distance was insignificant suggesting no correlation between genetic distance and spatial geographic distance. Based on the results, historical vicariance is the more likely mechanism by which *B. flavicollis* has achieved such a wide distribution. Furthermore populations at Mazowe may be hidden species within *B. flavicollis*.

4.1 Introduction

Millipedes are strict microhabitat specialists with low dispersal ability (Hopkin & Read, 1992; Hamer & Slotow, 2000). Millipede habitat specialization could lead to allopatric populations because preferred habitats maybe separated by unfavourable habitats or other barriers, therefore, the habitats occur in discrete patches. Low dispersal ability and habitat patchiness are likely to limit migrations and connectivity between populations causing population subdivisions. As a result, gene flow between the sub-divided millipede populations is restricted leading to genetic isolation and divergence due to genetic drift or local adaptation (Hamer & Slotow, 2000). Therefore, high genetic variation is expected among populations that display such vicariance and dispersal.

DNA sequence data have been used to infer the extent of genetic variation within populations of low dispersal taxa and flag genetically distinct populations. Cooper *et al.* (2011) observed high mitochondrial and nuclear DNA genetic divergence among geographically isolated populations of the trapdoor spider, *Maggridgea* Cambridge 1875. Sub-populations formed due to discontinuous habitats usually exhibit high genetic diversification as exemplified by populations of the cave spider, *Nesticus barri* Gertsch 1984 (Snowman *et al.*, 2010). High genetic variation between populations has also been observed in millipedes. Investigations into the spatial distribution of genetic variation and gene flow patterns in millipedes suggested a strong relationship between genetic and geographic distance between populations of the millipede *Narceus* Rafinesque 1820 (Walker *et al.*, 2009). In contrast, spatial isolation with no connectivity in *Atelomastix bamfordi* Edward & Harvey 2010 revealed strong genetic differentiation between populations (Nistelberger *et al.*, 2014).

Bicoxidens flavicollis Attems 1928 (Spirostreptida, Spirostreptidae) is the most ubiquitous species in the genus (Mwabvu *et al.* 2007). The known distribution of *B. flavicollis* populations includes northern, south-western, eastern and central parts of Zimbabwe and western Mozambique (Mwabvu *et al.*, 2007). Despite the ubiquity in a geographical range with heterogeneous vegetation type and rainfall patterns, *B. flavicollis* populations are confined to fragmented moist savannah woodlands (Mwabvu *et al.*, 2007). According to Mwabvu *et al.* (2007) *B. flavicollis* populations exhibit colour polymorphism ranging from black, brown to yellow. Although widely distributed, *B. flavicollis* is among the small-bodied members of the genus (Mwabvu *et al.*, 2013) which would probably reduce its vagility. A fundamental question would be how a small-bodied micro-endemic

species is more widely distributed than larger and presumably more mobile species. Genetic divergence levels reported by (Mwabvu *et al.*, 2013) suggest that *B. flavicollis* may be a species complex. Hidden species may have been undetected due to discordance between morphological change and genetic divergence preceding a speciation event (Bond & Sierwald, 2002; Mwabvu *et al.*, 2013).

Even though high levels of cryptic diversity may underlie the wide distribution, there is little knowledge about the mechanisms which influenced the spatial distribution and genetic diversity in *B. flavicollis*. *Bicoxidens flavicollis* may be a series of metapopulations where exchange of genetic material occurs through migration or dispersal. Hence, from a phylogeographic analysis of *B. flavicollis*, a strong correlation between genetic distance and geographic distance would be expected among the populations. Alternatively, the distribution of *B. flavicollis* populations may have resulted from a once ubiquitous ancestral population which fragmented and genetically diverged in isolation over long periods (see Nistelberger *et al*., 2014). In this scenario, proximal populations may not necessarily be more genetically related. Population fragmentation may have occurred due to shrinking of forests due to climate change (see Walker *et al*., 2009). Understanding genetic structure and the processes which underlie the observed population genetic structure in a particular group are necessary and often give context to the latter's phylogenetic relationships.

This is the first phylogeographic and population genetics study of an afrotropical millipede. The present study aims to assess the phylogeographic and population structure across *B. flavicollis* populations given the potential complexity of the species. The objectives were to determine; the levels of genetic differentiation and cryptic diversity in *B. flavicollis,* genetic structure within and among *B. flavicollis* populations and infer levels of gene flow among the populations. Furthermore, the utility of both mitochondrial COI and 16S rRNA at population level studies was evaluated.

4.2 Materials and methods
4.2.1Taxon sampling
A total of 32 individuals of *B. flavicollis* grouped into 11 populations were collected from their known localities. Fresh male specimens were collected by hand and preserved in 100 % ethanol to preserve the integrity of genetic material (see Post *et al* 1993). Efforts were made to collect at least five samples per locality in order to get a representative sample but fewer specimens were sampled

at some locations. Sampling for most of the species was hindered by anthropogenic modification to their

known geographic ranges. Henceforth, some specimens were borrowed from the KwaZulu–Natal Museum, Pietermaritzburg (South Africa) and the Natural History Museum, Bulawayo (Zimbabwe) where single specimens of each species were available. All the specimens used in this study were identified by Tarombera Mwabvu

Table 8 Locality and number of specimen for *Bicoxidens flavicollis* populations**.**

Locality	Species	Number of specimens	Latitude	Longitude
Chitombo	*B. flavicollis*	4	18º 28' 9.28"S	30 º 49'27.64"E
Chihota	*B. flavicollis*	6	17 º 50' 0.00"S	31 º 02'0.00"E
Mzinga	*B. flavicollis*	5	18 º 25' 0.00"S	32 º 58'0.00"E
Mazowe	*B. flavicollis*	6	17 º 28' 0.00"S	30 º 59'0.00"E
Marange	*B. flavicollis*	1	19 º 10'0.00"S	32 º 18'0.00"E
Nyanga	*B. flavicollis*	3	18º21'0.00"S	32º74'0.00"E
Muterere	*B. flavicollis*	3	18 º25'0.00"S	32 º 57'0.00"E
Chegutu	*B. flavicollis*	1	18° 8' 24.00"S	30° 9' 0.00"E
Maleme camp	*B. nigerrimus*	1	20 º 33'0.00"S	28 º 30'0.00"E
Lumene falls	*B. flavicollis*	1	20 º 34'0.00"S	28 º 25'0.00"E
Hovi crossing	*B. flavicollis*	1	20 º 37'0.00"S	28 º 30'0.00"E

4.2.2 DNA extraction

DNA extraction protocols, phenol chloroform isoamyl–alcohol extraction method (PCI) and ethanol precipitation were attempted, however, the commercial kit gave the best DNA yield. Total genomic DNA was extracted from 10 legs removed from the mid-body rings from each specimen

using Zymogen Genomic DNA™–Tissue MiniPrep (Zymogen Research) according to the manufacturer's standard protocol.

The integrity and quantity of the extracted DNA was assessed through electrophoresis on 1 % TBE (tris–boris–EDTA) gel stained with 100 µl of ethidium bromide. DNA samples (5 µl) stained with 1 µl Loading dye and DNA ladder (2 µl) were loaded into the wells and run at 100 volts for one hour in running buffer. The resultant gels were visualized using Chemidoc UV trans–illuminator (Bio–Rad) and single compact bands of ~1kb in size indicated good quality and quantity DNA yield.

4.2.3 DNA amplification and sequencing

Fragments of the mitochondrial (CO1 and 16S rRNA) and nuclear genome (Internal transcribed spacer region and Elongation factor–1α) were targeted for amplification using polymerase chain reaction (PCR). Unless mentioned all PCRs were performed in 25 µl reaction volumes contained 12.5 µl Econotaq, 7.82 µl PCR H_2O, 1 µl Bovine Serum Albumin (BSA), 0.84 µl of each primer and 2 µl of DNA template. A negative control was included in every PCR to check for contamination during the preparation of reaction volumes.

Mitochondrial CO1 was amplified using universal primers by Folmer *et al.* (1994); LCO1490 and HCO2198 (Table 9). The 16S rRNA gene fragment was amplified using primer sets by Kessing *et al.* (1989), 16Sar and 16Sbr and 16Sarl and 16Sbr (see Table 9) Only mitochondrial 16S rRNA primers Rhino 16SJB and Rhino 16SNC (Bond & Sierwald, 2002) (Table 9)were used in a reaction mixture made up of 5 µl of 10 x PCR buffer, 4 µl of magnesium chloride at 25 mM, 4 µl of each primer in 6 µM, 0.5 µl of deoxyribonucleic triphosphates (dNTPs) (40 mM), 0.2 µl of 5 Units/µl Taq Polymerase (Supertherm), 9 µl of DNA template, made up a total volume of 25 µl with PCR H_2O. Amplification of the internal transcribed spacer region (ITS) and nuclear protein coding gene EF1α was done using the following primers from Presa *et al.* (2002) and Sota and Tanabe (2010) respectively (Table 9). After amplification, 5 µl of each PCR was electrophoresed in 1 % agarose gels stained with ethidium bromide (100 µl) to verify amplification of targeted region based on size. DNA ladder (1kb) was also included in one of the wells as a size reference and the gels were ran at 100 volts for one hour in running buffer before being viewed using Chemidoc UV trans–illuminator (Bio–Rad). PCR products were sequenced Inqaba at Biotechnical Industries, South Africa, using the forward primers from the initial PCR primer sets.

Table 9 Primers and PCR thermal profiles used to amplify the CO1, 16S, ITS2 and EFI **α** regions for *Bicoxidens flavicollis* populations.

Mitochondrial Cytochrome oxidase 1

Primer	Direction	Sequence	PCR thermal profile
LCO 1490	F	5'GGTCAACAAATCATAAAGATATTGG3'	initial denaturation 95 ºC for 5 minutes, followed by 40 cycles of 94 ºC for 60 seconds, 40 ºC for 60 s and 72ºC for 90 s and final extension at 72 ºC for 10 min
HCO2198	R	5'TAAACTTCAGGGTGACCAAAAAATCA3'	

Mitochondrial 16S rDNA

Primer	Direction	Sequence	PCR thermal profile
16Sar	F	5'CGCCTGTTTTTCAAAAACAT3'	initial denaturation temperature of 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 seconds, 55 °C for 30 s and 72 °C for 2 min, and then a final 72 °C for 5 min.
16Sbr	R	5'CCGGTTTGAACTCAGATCATGT3'	
16Sarl	F	5'TGCCTGTTTATCAAAAACAT–3'	initial denaturation temperature of 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, 59.3 °C for 30 s and 72 °C for 2 min, and then a final 72 °C for 5 min.
16Sbrh	R	5'CCGGTCTGAACTCAAATCATGT3'	
Rhino 16SJB	F	CCA TGT ATT TGA TAA ACA GGC A	95ºC initial denaturation for 5 minutes followed by 25 cycle of 94 ºC denaturation for 30 s, annealing at 52 ºC to 55 ºC for 30 s, a 1 min extension at 72 ºC, final 10 min extension at 72 ºC
Rhino 16SNC	R	GTG GGG GTA TTG GAA AAT GTT C	

Internal Transcribed Spacer 2

Primer	Direction	Sequence	PCR thermal profile
5.8sr	F	5'CTACGCCTGTCTGAGTGTC3'	initial denaturation 94 °C for 2 min, followed by 36 cycles of 94 °C for 20 s, 48.5– 52 ºC for 20 s and 72 ºC for 45 s, a final 72 °C extension for 5 min.
28s	R	5'ATATGCTTAAATTCAGCGGG3'	

Elongation Factor 1 alpha (EF1α

Primer	Direction	Sequence	PCR thermal profile
DiploEF1aF	F	5'GCCTGG GTT TTG GATAAA CTTAAG GC3	initial denaturation 94 °C for 2 min, followed by 36 cycles of 94 °C for 20 s, 48.5– 52 ºC for 20 s and 72 ºC for 45 s, a final 72 °C extension for 5 min
DiploEF1aR3	R	5'CCT CCA ATC TTG TAA ACG TC3'	

4.2.4 Analysis

Sequences were aligned and edited using Bioedit (Hall, 1998). Levels of genetic variation among *B. flavicollis* populations were assessed by calculating haplotype number, nucleotide diversity and haplotype diversity for each population in DNASP 5 (Librado & Rozas, 2009). Neutrality assumptions were tested using Tajima's (1989) *D* statistic within *B. flavicollis* populations. Population differentiation among populations with at least two individuals was assessed based on pairwise PhiPT ($\mathbf{\Phi_{PT}}$) values which were tested over 10 000 random permutations in GenAlEx 6.5 (Peakall & Smouse, 2012). Population differentiation based on PhiPT values was delimited as follows $\mathbf{\Phi_{PT}} > 0.25$ (strong differentiation), $0.15 < \mathbf{\Phi_{PT}} < 0.25$ (moderate differentiation) and $0.05 > \mathbf{\Phi_{PT}}$ (negligible differentiation) (Wright, 1978). Analysis of Molecular Variance (AMOVA) was performed in GenAlEx version 6.5 (Peakall & Smouse, 2012) in order to partition genetic variation among *B. flavicollis* populations, and the significance with 10 000 permutations. Isolation by distance pattern was assessed using Mantel's (1967) tests over 10 000 permutations. Genetic distance and geographic distances between populations generated in GenAlEx (Peakall & Smouse, 2012) were used for this correlation. The relationship among the haplotypes was assessed by plotting a haplotype network in R using the Pegas package (Paradis, 2010). The maximum likelihood tree was estimated using GARLI (Zwickl, 2006) with commands including a heuristic search with starting tree(s) obtained via neighbor–joining, branch–swapping algorithm and resampling with a 1000 replicates. For the phylogenetic analysis *Doratogonus*, *B. brincki, B. friendi* and *B. grandis* were included as out-groups. Bayesian clustering analysis was performed in BAPS 6 (Corander *et al.*, 2008) to evaluate the genetic structure among *B. flavicollis* populations. Mixture and admixture analysis of the sample was estimated using 5 Markov chain Monte-Carlo (MCMC) runs of 5×10^5 iterations with the first 10 % discarded as burnin. The number of populations (K) was estimated using posterior probability of the data [LnP (D)].

4.3 Results

4.3.1 Genetic diversity

The mitochondrial (mtDNA) gene *cytochrome oxidase 1* gene was successfully sequenced for 32 individuals of *B. flavicollis* while 25 individuals were successfully sequenced for the 16S rRNA gene. Analysis of the 535 base pairs of the COI yielded 31 haplotypes from 32 individuals. A total of 19 haplotypes were inferred from the 25 sequences in the 443 bp 16S rRNA alignment. Six populations (Chitombo, Chihota, Mzinga, Mazowe, Muterere and Nyanga) were represented by at

least three individuals while the majority of the populations had one representative for the COI gene. For the 16S rRNA five populations consisted of more than three individuals (Chitombo, Chihota, Mzinga, Mazowe, and Muterere).

Genetic analysis of the COI revealed consistent measures of genetic diversity with high haplotype (h=1.00) diversity and low nucleotide diversity ($\pi_n < 0.5$) across all populations (Table 10). *Bicoxidens flavicollis* populations from Chitombo and Nyanga had among the highest nucleotide diversities of 0.1842 and 0.13725, respectively, against an overall average of 0.0772. Tajima's *D* for all six populations was not significantly different from 0 suggesting neutral variation.

Table 10. mtDNA *cytochrome oxidase* 1 diversity measures for *B. flavicollis* populations with more than three individuals

Population	*n*	*Nh*	*h*	π_n	Tajima's *D*
Chitombo	4	4	1.00	0.1842	-0.02994
Chihota	6	6	1.00	0.03185	0.29356
Mzinga	5	5	1.00	0.00606	-0.80734
Mazowe	6	6	1.00	0.08188	1.60398
Nyanga	3	3	1.00	0.13725	N/A
Muterere	3	3	1.00	0.02198	N/A

n=number of individuals, *Nh* = number of haplotypes, *h*=haplotype diversity, nucleotide diversity=π_n

Table 11. 16S rRNA diversity measures for *B. flavicollis* populations with more than three individuals

Population	*n*	*Nh*	*h*	π_n	Tajima's *D*
Chitombo	4	4	1.00	0.36070	-0.86401
Chihota	5	3	0.7	0.00448	-1.12397
Mzinga	5	3	0.70	0.06007	1.81061
Mazowe	6	4	0.80	0.05149	0.54394
Muterere	3	3	1.00	0.20896	N/A

n=number of individuals, *Nh* = number of haplotypes, *h*=haplotype diversity, nucleotide diversity=π_n

Haplotype diversity (Nei, 1987) estimates for the 16S rRNA data set all approached 1 (although less than 0.95 diversity is considered relatively moderate diversity) while the nucleotide diversity (Nei and Li, 1979).estimates were all less than 0.5 (Table 11). Furthermore, the null hypothesis that the 16S rRNA sequences are under selective neutrality was not rejected for all populations.

4.3.2 Genetic differentiation and population structure

Analysis of molecular variance (AMOVA) revealed evidence of population genetic differentiation. Based on COI, 58 % of the genetic variation occurs within the populations and 42 % among populations (Fig. 5). Overall Φ_{PT} was high among populations, at 0.418 ($P < 0.05$). Pairwise Φ_{PT} comparisons ranged from 0.121 to 0.765 (Table 12). All pairwise comparisons (Table 13) based on COI were significant except for the comparisons between Muterere and Mzinga and Muterere and Nyanga. High Φ_{PT} values (> 0.25) suggested strong population differentiation among the *B. flavicollis* populations (Table 13). However, moderate pairwise Φ_{PT} values (< 0.25) were observed between Nyanga and three populations Chitombo, Mzinga and Mazowe (Table 13).

Analysis of the 16S rRNA data set revealed that the genetic variation among *B. flavicollis* populations was partitioned as follows, 73 % within and 27 % among populations (Fig. 5). Strong differentiation was also apparent among the *Bicoxidens flavicollis* populations (Table 12) based on the 16S rRNA gene. All pairwise Φ_{PT} values were significant at the 5 % level ($p < 0.05$). Populations at Chitombo shared negligible to moderate Φ_{PT} values with populations at Chihota, Mzinga, Mazowe and Muterere (Table 13). Furthermore, the population genetic differentiation between Mzinga and Muterere was moderate (Table 13).
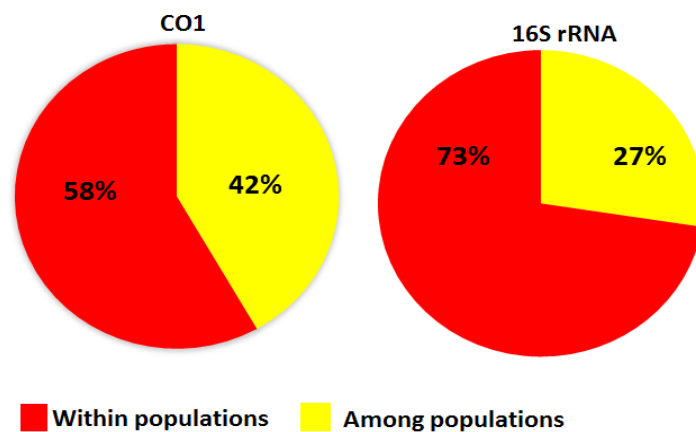

Figure 5. Partitioning of genetic variation within and among samples of *B. flavicollis* populations from Zimbabwe, Southern Africa.

Table 12. Estimation of differentiation among Φ_{PT} among *B. flavicollis* populations

	Φ_{PT}	p
CO1	**0.418**	**0.001**
16S rRNA	**0.274**	**0.007**

Table 13. Pairwise Φ_{PT} values among populations of *B. flavicollis* based on the mtDNA COI gene (below diagonal and bold) and 16S rRNA gene (above diagonal). All values were significant at the 5% level.* 16S rRNA sequences from Nyanga were unavailable.

	Chitombo	**Chihota**	**Mzinga**	**Mazowe**	**Nyanga**	**Muterere**
Chitombo	---	0.128	0.205	0.155	*	0.00
Chihota	**0.428**	---	0.509	0.481	*	0.337
Mzinga	**0.694**	**0.671**	---	0.469	*	0.235
Mazowe	**0.464**	**0.527**	**0.539**	---	*	0.282
Nyanga	**0.204**	**0.366**	**0.230**	**0.241**	---	*
Muterere	**0.765**	**0.654**	**-**	**0.456**	**0.121**	---

Table 14 Nei's (1972) pairwise population nucleotide divergences matrix based on CO1 gene (below diagonal and bold) and 16S rRNA gene (above diagonal). * 16S rRNA sequences from Nyanga were unavailable.

	Chitombo	**Chihota**	**Mzinga**	**Mazowe**	**Nyanga**	**Muterere**
Chitombo	---	0.066	0.225	0.085	*	0.114
Chihota	**0.135**	---	0.182	0.044	*	0.090
Mzinga	**0.125**	**0.157**	---	0.190	*	0.212
Mazowe	**0.351**	**0.363**	**0.358**	---	*	0.101
Nyanga	**0.215**	**0.226**	**0.231**	**0.350**	---	*
Muterere	**0.135**	**0.150**	**0.020**	**0.325**	**0.237**	---

Mazowe had the highest Nei's (1972) genetic distances between *B. flavicollis* populations ranging from 0.325 to 0.363 (COI) (Table 14). The highest nucleotide divergence was recorded between two northern populations at Mazowe and Chihota (41 km apart). The eastern region Nyanga population was also highly divergent based on COI particularly from other eastern region populations at Chitombo, Mzinga, and Muterere along with the northern populations Chihota and Mazowe (Table 14). The highest divergences based on the 16S rRNA gene were between Chitombo and Mzinga, Mazowe and Mzinga and, Muterere and Mzinga (Table 14).

Haplotypes based on the COI gene generally formed clusters according to spatial proximity and no haplotype sharing was evident except for Mzinga and Muterere (Fig. 6). The relationship among the haplotypes based on the haplotype network were mirrored by the phylogenetic tree (see Fig. 6). *Bicoxidens flavicollis* populations from the eastern region Chitombo, Marange, Mzinga, Muterere and Nyanga formed a cluster although Nyanga was paraphyletic. However, northern populations Chegutu, Chihota and Mazowe were not monophyletic despite the close proximity. Proximal populations at Muterere and Mzinga share haplotypes between them. The position of Chihota haplotypes in both the haplotype network and phylogenetic tree suggest a closed relationship with the eastern population rather the northern population (Fig. 6). South-western populations at Lumene and Hovi formed a clade which seemed to be the link between the northern region populations (Fig. 6). Mazowe showed paraphyly at population level occurring in two clades and forming a clade with a geographically distant Maguge haplotype.

Based on the 16S rRNA gene, Mazowe and Chegutu shared haplotypes while the rest were unique haplotypes (Fig. 7). Eastern populations at Mzinga, Chitombo and Muterere formed a clade although the clade included a northern population from Chihota. Mazowe haplotypes maintained their paraphyly along with their close relationship with the Chegutu haplotype (Fig. 7). A distant haplotype highlighted the paraphyly of Muterere based on the 16S rRNA gene.

Isolation by distance analysis of COI and the 16S rRNA also reflected a weak correlation between genetic and geographic distance. The Pearson correlation slope (R^2) which is a measure of the strength of the relationship between pairwise genetic distances and pairwise straight line distances was weak at 0.0554 and 0.0003 for COI and the16S rRNA, respectively.

Bayesian analysis revealed four distinct genetic clusters (k=4) among 11 populations based on COI data (Fig 8). The first cluster consisted of the half of the Mazowe population (red) and the second

constituted the second half of the Mazowe population and the Maguge population. A third of the Nyanga population formed the third cluster while the rest formed the largest cluster (green). There was no evidence of admixture suggesting that none of the individuals in this dataset have mixed ancestry.
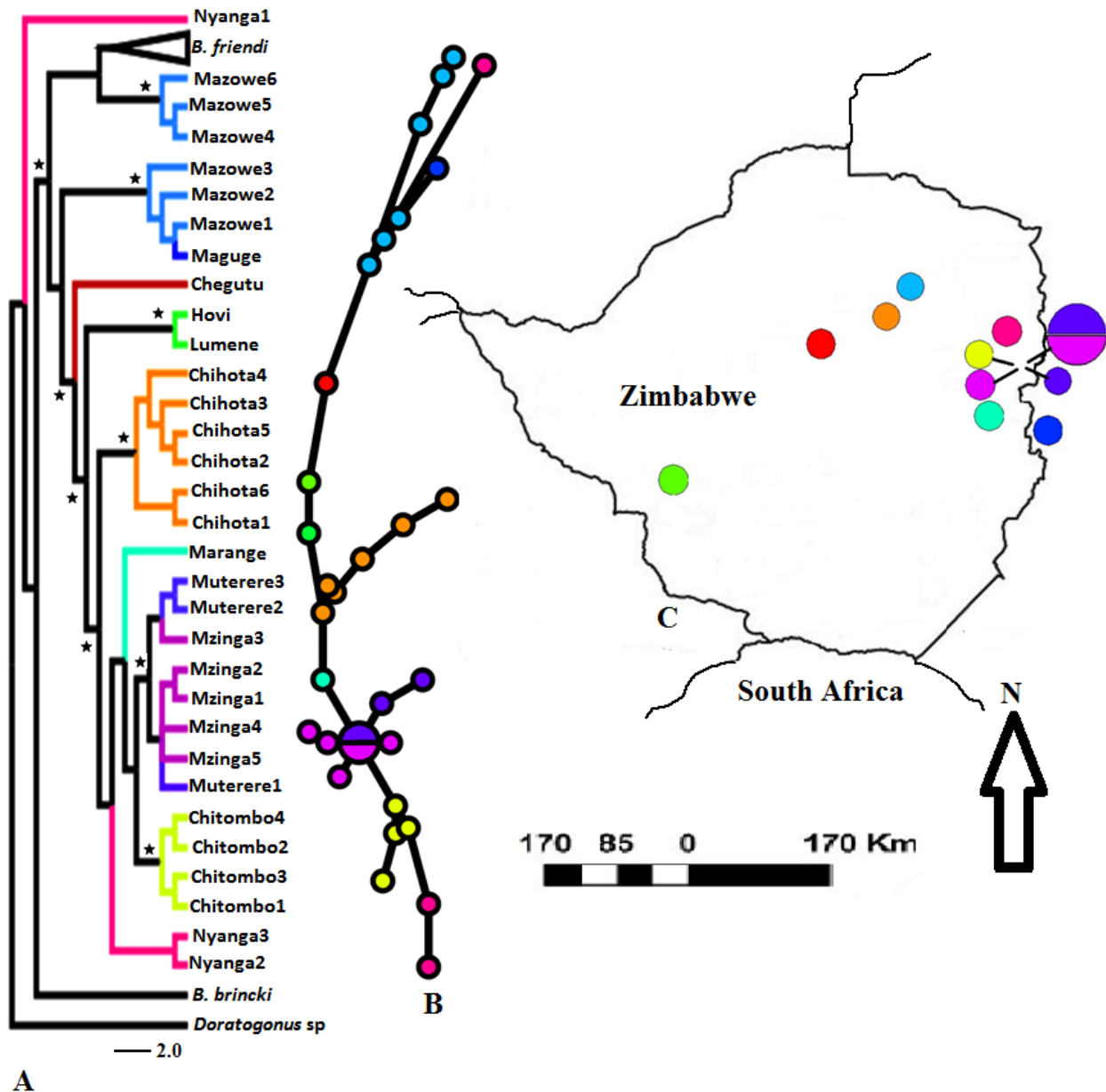

Figure 6. Maximum Likelihood tree and haplotype network showing distribution of COI genetic variation among *B. flavicollis* populations. **A**, Maximum Likelihood tree computed on COI sequences. Scale bar represents the expected mutation per site. ★ denotes node values are strong bootstrap support (<80). **B**, Haplotype network. **C**, Distribution of haplotypes across sampling site in Zimbabwe.
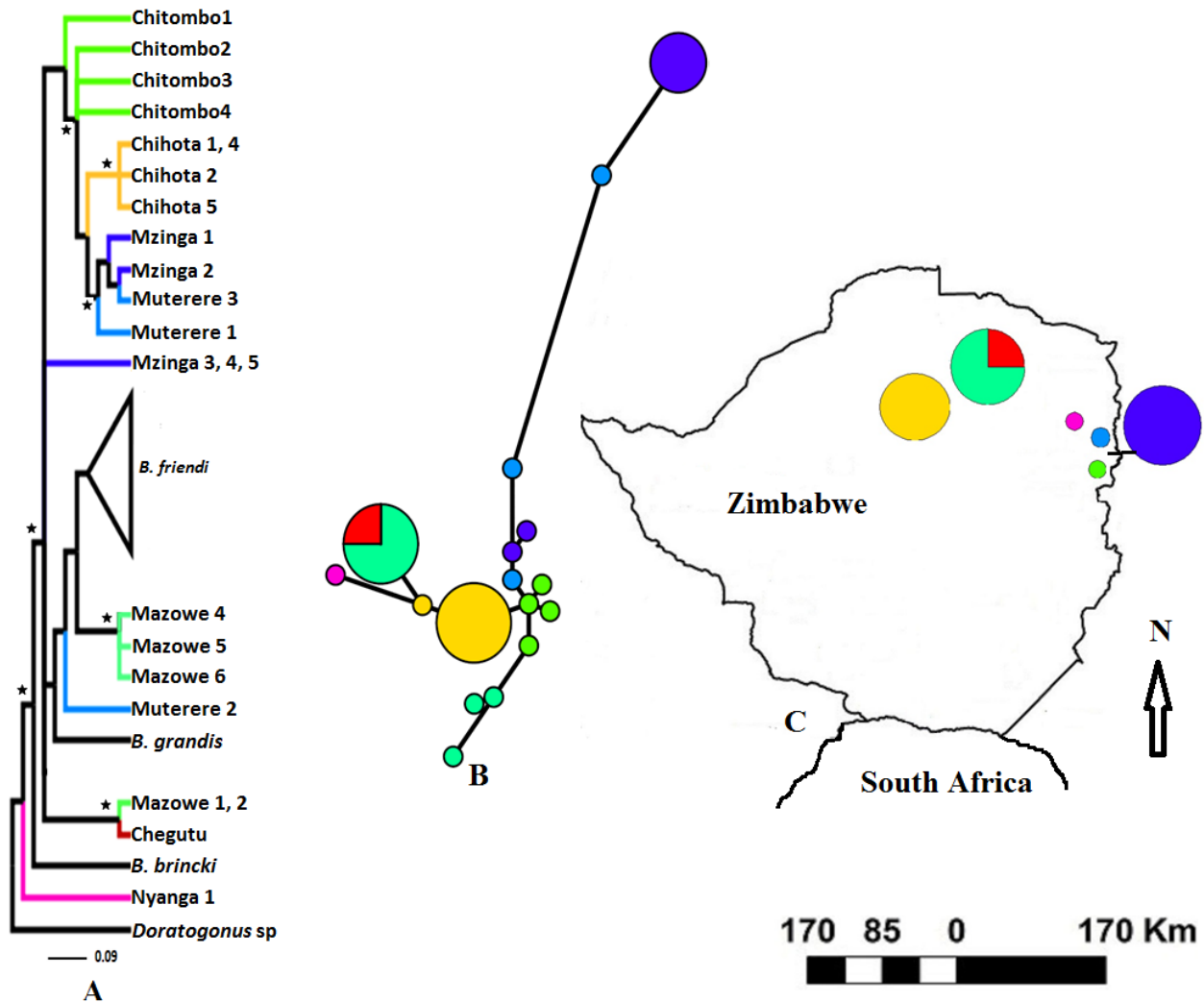
Figure 7. Maximum Likelihood tree and haplotype network showing distribution of 16S rRNA genetic variation among *B. flavicollis* populations. **A,** Maximum Likelihood tree computed on 16S rRNA sequences. Scale bar represents the expected mutation per site. ★ denotes node values are strong bootstrap support (>80). **B,** Haplotype network. **C,** Distribution of haplotypes across sampling site in Zimbabwe.

4.4 Discussion

MtDNA evolves rapidly enough to separate closely related taxa (Hebert *et al.*, 2004). In millipedes, COI and both ribosomal rRNAs (12S rRNA and 16S rRNA) seem to have the ability to elucidate population genetics and phylogeography (see Walker *et al*., 2009; Sota & Tanabe 2010; Loria *et al*., 2011; Nistelberger *et al.,* 2014). DNA of both COI and 16S rRNA demonstrated substantially high levels of intraspecific genetic divergence and strong population differentiation among *B. flavicollis* populations.
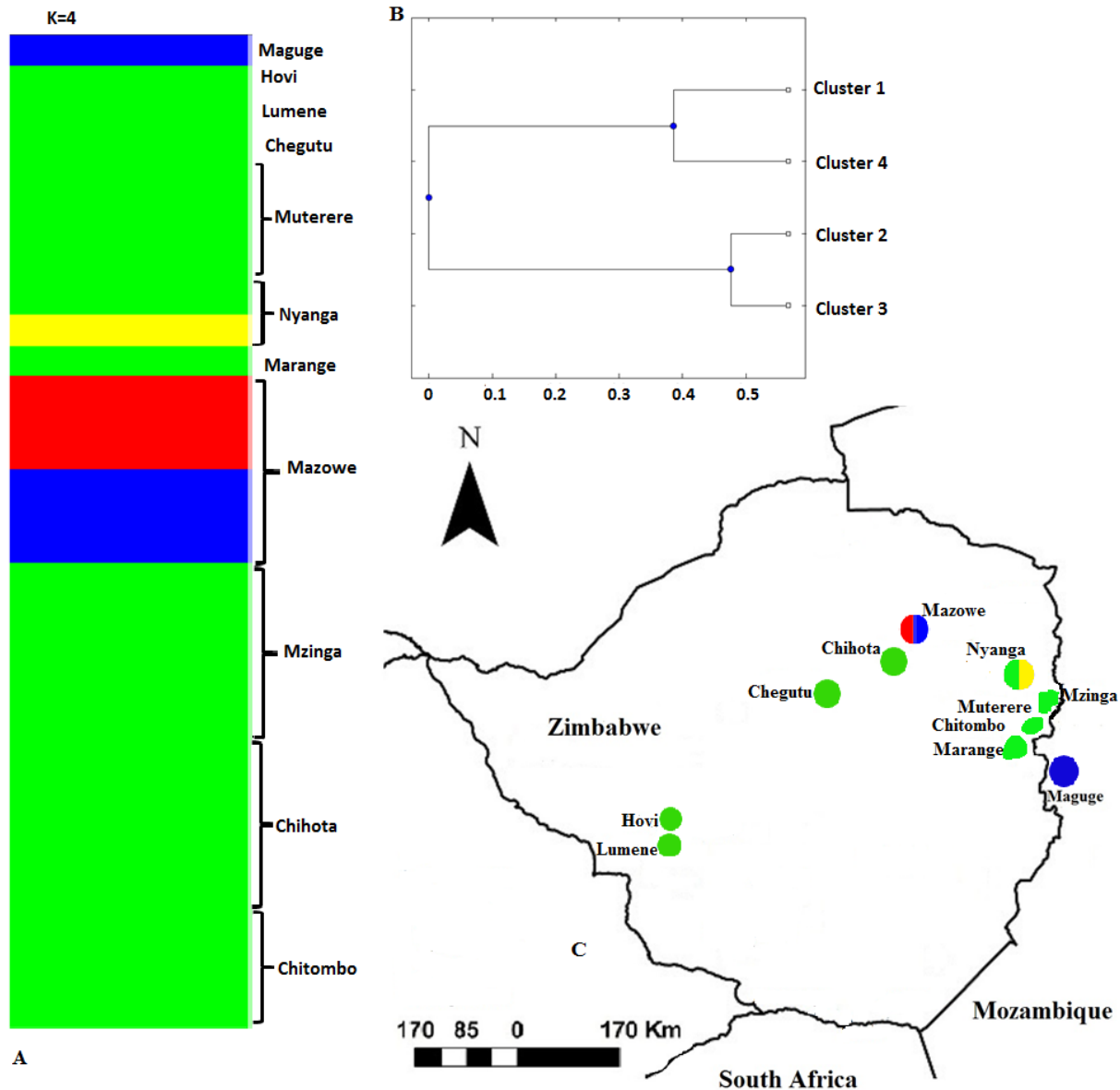
Figure 8. Assignment of *B. flavicollis* individuals to Bayesian genetic clusters (K=4) (**A and B)** and their distribution of the genetic clusters across sampling sites in Zimbabwe (**C**).

High haplotype diversity (> 0.70) based on both the COI and 16S rRNA gene (Table 11 & 12) also suggests that *B. flavicollis* populations consist of unique and unshared haplotypes therefore high genetic diversity. High Φ_{PT} values among populations may be attributed to barriers to gene flow such as limited dispersal propensity and habitat fragmentation which are driving population differentiation. Haplotype networks of *B. flavicollis* support the notion of restricted gene flow among populations. Low vagility and habitat discontinuity, either anthropogenic or naturally induced may be at the core of structuring the genetic variability observed in the small-bodied *B.*

flavicollis. Pleistocene changes in climate are hypothesized to have influenced genetic diversity in taxa such as millipedes by either modifying habitats and or inducing of suitable habitat discontinuity through habitat fragmentation (see Walker *et al*., 2009). Therefore *B. flavicollis* populations may have become isolated resulting genetic divergence due to genetic drift and local adaptation.

In this study populations were sampled from the northern, south-western and eastern regions of Zimbabwe, therefore, barriers to gene flow may differ according to region. The eastern region consists of mountain ranges dominated by pristine montane forests and miombo woodlands with a cool and humid climate (Whitlow, 1987). Such conditions provide suitable habitats for millipedes. However, population differentiation among eastern *B. flavicollis* populations despite spatial proximity may have been driven by changes in altitude (see Hodkinson, 2005). In contrast, isolation and differentiation among the northern populations in Mazowe and Chihota located in the crop farming region according to Nyamapfene (1991), may occur as a result of the agricultural activities which constrict woodlands into small isolated patches. Although differentiation among regions was not tested it may be assumed that a combination of differences in vegetation and soil type may be influencing differentiation at the regional level.

Bayesian analysis of the COI dataset grouped *B. flavicollis* populations into four genetic clusters. Unexpectedly, the Mazowe population as well as the Nyanga population were paraphyletic with each population split between two clusters. Furthermore, isolation by distance was insignificant among *B. flavicollis* populations. Evidently half of the Mazowe population shared closer genetic relationship with the Maguge population (over 400 km away) than with the other half of the Mazowe population. Also population pairwise genetic distances (COI) suggested that the northern Chihota population is more genetically related to Mzinga, an eastern population more than 215km away than Mazowe which is just 41 km away. Such population paraphyly and genetic distribution in the absence of isolation by distance can be ascribed to incomplete lineage sorting (Masta, 2000) and fragmentation of large historical population sizes (Nistelberger *et al.*, 2014). The lack of admixture may be driven by propensity of millipedes to become isolated with minimal overlap between populations.

Strong population differentiation and lack of connectivity between the populations supports historical vicariance rather than dispersal as a plausible account for the wide distribution of *B. flavicollis*. Similarly Nistelberger et *al.* (2014) observed evidence for Pleistocene induced vicariance in the spirostreptid millipede *A. bamfordi*. In addition to low vagility, climate changes, altitude and food availability are likely to have suppressed the dispersal of *B. flavicollis,* a small-bodied, strict habitat specialists and desiccation prone taxon. Although, tentative the findings of this present study warrant further investigations into understanding the observed genetic distribution in *B. flavicollis.* Unpredictable population densities in millipedes resulted in skewed sample sizes, however, the high differentiation highlighted in the present study cannot be ignored. Large population sizes would allow gene flow estimates to be tested within paraphyletic populations and regions where monophyly is expected. Molecular dating that estimates the divergence time of the *B. flavicollis* populations would further elucidate the role of historical climate and landscape changes. Further studies should include non-linked markers such as from the nuclear genome in addition to mitochondrial markers. Furthermore such studies which flag unique genetic entities provide valuable information for conservation of these ecologically important taxa.

4.5 References

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5.0 Overall Conclusion

Sequence markers may differ in their biological properties such as rate of evolution, phylogenetic resolution and ease of amplification (Galtier *et al.*, 2009). Despite differences in general properties mtDNA gene COI and 16S rRNA were both useful in differentiating species, elucidating phylogenetic relationships and revealing cryptic diversity within *Bicoxidens*. In addition, the congruency in the general topologies between COI and 16S rRNA further highlights the utility of multiple gene analysis in millipede taxonomy.

Based on evidence from Bond & Sierwald (2002), morphology based taxonomy may be limited in revealing hidden species and therefore needs to be augmented by DNA sequence data. Failure to account for hidden species may result in underestimation of species richness (Brewer *et al.*, 2012). In the present study mtDNA data demonstrated the presence of hidden species within *Bicoxidens.* The patterns of cryptic diversity observed in *Bicoxidens* specie*s* may be present in other millipede genera hence there is a need to reassess the taxonomy of other genera by integrating DNA sequence data into taxonomy. However, morphological taxonomic revision should precede the DNA sequence based taxonomic reassessment. Morphological taxonomic revisions allow for congruency tests between morphological and molecular species definitions, thereby revealing presence or absence of cryptic species in millipedes.

Millipede populations are likely to experience restricted gene flow due to their low vagility and high propensity to become isolated (see Tanabe *et al*., 2001; Enghoff & Seberg, 2006). Phylogeographic and population genetic structure analysis of *B. flavicollis* revealed strong population differentiation with unique haplotypes. The presence of unique haplotypes and cryptic diversity in *Bicoxidens* has implications on the inclusion of millipedes in conservation and management strategies. Habitat fragmentation and subsequent loss through anthropogenic modification and climate change may pose an increased threat to the genetic diversity within *Bicoxidens*. Therefore, any conservation management strategies should focus on preserving the unique genetic diversity in the genus.

More importantly, because soil invertebrates including millipedes are under studied, *Bicoxidens* species could become a surrogate taxa for other microhabitat specialist in conservation and

environmental assessments. Given that biologists are unable to survey all habitats the use of suitable surrogates is a logical approach in conservation. Based on their characteristics, millipedes (in this case *Bicoxiden*s) appear to be suitable candidates.

Future research could include:

1. Morphological and molecular taxonomic revision of other millipede genera to uncover hidden species and validate species definitions.
2. Increased taxon sampling covering a wider geographic range to further elucidate the distribution of the genetic diversity.
3. Developing taxon specific primers in order to improve the amplification of nuclear markers
4. Whole genome sequencing in order construct evolutionary relationships based on d the whole genome.
5. Niche based modelling to further understand the role of climate change, vegetation and soil type in species distribution.

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