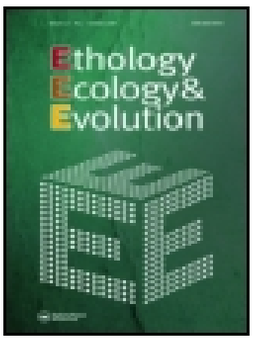

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Phylogeographic investigation of the bladder grasshopper *Bullacris unicolor* (Orthoptera Pneumoroidea) in South Africa

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There are several factors, such as genetic drift, gene flow and migration that affect the population genetic structure and phylogeographic distribution of genetic lineages within single species. Previous studies of the bladder grasshoppers, *Bullacris unicolor* of South Africa, showed divergence in mitochondrial CO1 (cytochrome *c* oxidase subunit 1) haplotype diversity and significant genetic structure. In this study, we revisit these findings adding more samples from different locations and using mitochondrial CO1 and Internal transcribed spacer (ITS) gene sequences. We tested the hypothesis that the western, northern and eastern distribution ranges of *B. unicolor* show different population genetic patterns, corresponding with isolation-by-distance. Mitochondrial CO1 and ITS data were collected for 99 individuals from 12 localities across the Western, Northern and Eastern sides of South Africa. Overall, significant variation in genetic structure was found across the localities as indicated by F_{ST} analyses. Haplotype and phylogeographical analyses suggested that restricted gene flow played a role in shaping current genetic patterns, although isolation-by-distance was not supported, as confirmed by Mantel tests. Phylogenetic trees of both genetic sequences revealed two major clades, with western and northern sides. Also, the major clades exhibit a few sub-clades within the localities, showing other factors shaping the genetic structure of *B. unicolor* include the geographical barriers, and most likely due to changes in habitat specificity and habitat fragmentation. Taken together, this study aims to contribute information on the population structure and genetic diversity of *B. unicolor* populations across South Africa.

KEY WORDS: CO1, genetic diversity, ITS, phylogeography, Pneumoroidea.

INTRODUCTION

The intraspecific variation in population genetic structure and phylogeographic distribution of genetic lineages are caused by various factors (Avise 2009), such as geographic barriers (Hirao et al. 2015; Ye et al. 2016), ecological differences (Katz

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et al. 2018; Matsubayashi & Yamaguchi 2022), and historical processes (Hewitt 2000; Campos et al. 2013). Geographic barriers play a significant role in promoting genetic differentiation by reducing gene flow within species (Zhang et al. 2006; Geist 2010; Bon et al. 2015). Conversely, greater gene flow and lower genetic structure between populations are caused by higher dispersal propensity (Chamberland et al. 2020). More genotypes and the possession of higher genetic diversity are also caused with the diverse environments with different selective pressure (Jin & Liu 2008). Genetic diversity within populations can be increased by habitat fragmentation (Vandergast et al. 2007). Furthermore, populations capacity to adapt to the novel environmental conditions can be associated with genetic variation (Futuyma & Peterson 1985; Robinson & Wilson 1996; Agashe 2009). Therefore, it is important to describe the genetic lineages that may reflect the adaptive potential of species (Moritz 1995; Godoy et al. 2004; Wares et al. 2005; Tahami et al. 2021).

The effect of climate changes is one of the major abiotic driving forces of genetic diversity and distribution of extant species (Gouws et al. 2010; Schnitzler et al. 2011; Pureswaran et al. 2018). These fluctuations in climate have resulted in extensive habitat fragmentation, altering distribution patterns of species, promoting isolation and finally leading to speciation (Hewitt 2003; Kobayashi et al. 2011). Although, comparative studies of the impact of biogeography and the evolutionary affinities among them are poorly understood (McDonald & Daniels 2012). Genetic studies can be used to define the evolutionary units to preserve the total diversity of populations or species (Ryder 1986; Flook & Rowell 1997; Flook et al. 2000; Green 2005).

Multiple molecular markers (e.g., internal transcribed spacer, microsatellite loci and mitochondrial DNA) have been used for phylogeographic studies (Garrick et al. 2015; Card et al. 2016; Martinez-Sañudo et al. 2019). Since lineage history of species is not always adequately represent by single locus datasets, studies combining multiple molecular markers in phylogeographic studies are increasing (Moritz 1995; Werhahn et al. 2020; Goyal & Sobti 2022). The use of CO1 continues to denote a significant factor of the phylogeographic toolbox (Avise et al. 1984; De Mandal et al. 2014; Tan et al. 2019), although recent studies have shown that CO1 may be more susceptible to introgression than many nuclear loci and it reduces the frequency of the introgressing haplotypes because of the lack of recombination (Kliman et al. 2000; Hey & Machado 2003; Ballard & Rand 2005).

The coastline of southern Africa presents an ideal opportunity to study biogeographic processes due to the high degree of habitat fragmentation and climatic modification (Rutherford & Westfall 1986; Rutherford 1997; Tyson & Partridge 2000; Hewitt 2004). Within the north and western coast, the biomes are comprised of fynbos and succulent karoo. Fynbos is a natural shrub found in the Western Cape province of South Africa and the succulent Karoo biome is found along the west coast of the Northern Cape province and the northern parts of the Western Cape province. The Eastern Cape coastal regions are also mostly comprised of fynbos (Mucina & Rutherford 2006). The relative role of geographic isolation and its impact on evolutionary mechanisms on fynbos and succulent karoo biomes are largely unexplored in the absence of phylogeographic studies on invertebrates (McDonald & Daniels 2012).

Bladder grasshoppers belong to the family Pneumoridae (Linnaeus 1758; Thunberg 1810). The unique characteristics of the family include their long-range acoustic signalling with strongly inflated abdomen and a hearing distance of almost 2 km (van Staaden & Römer 1997, 1998). The males use abdominal bladder as a resonating chamber, together with the stridulation of their hind-legs against their

abdominal ridges for acoustical communication. Host plant specificity is strong in pneumorid species and they are confined to their food plants. Their body colouration perfectly camouflages to their host plant (see also Couldridge & Gordon 2015; Sathyan et al. 2017, 2022; Sathyan & Couldridge 2021).

The genus *Bullacris* is endemic to southern Africa (Roberts 1941) and presently includes seven species; among them *Bullacris unicolor* is a species with a relatively wide distribution along the west coast of South Africa (Gordon 2017, an unpublished Master's Thesis). It also extends along the southern coast of South Africa into the Eastern Cape (Dirsch 1965). It can be effectively employed to reconstruct biogeographic patterning of different vegetations, such as fynbos and succulent karoo areas. This ecological hotspot includes Cape floral kingdom, one of the most species-rich areas on earth with a richly complex environment, phenological specialization and plant diversity (McDonald et al. 1996; Linder 2003). Thus, chances for geographic variation is high in *B. unicolor* making it ideal for studying species diversification processes. Their dependence on specific habitat and restricted ability of dispersal make them suitable to reconstruct the affinities of different biomes. At present, the molecular phylogeny of *B. unicolor* is poorly known (see also Sathyan et al. 2017).

An analysis of CO1 genetic information on the populations of *B. unicolor* from different distributions are studied by Sathyan et al. (2017). The relationship of the genus was analysed based on acoustical, morphological and genetic evidence. They demonstrated that *B. unicolor* comprises different clades corresponding to distinct biogeographical regions in the Western and Northern Cape. They further suggested a link between nearby locations based on the close phylogenetic relationships, however, wider sampling of the species can improve biogeographic inferences. Presently, the population genetic structure of *B. unicolor* is poorly known.

We investigated comprehensive information on this species molecular phylogeny and genetics and discussed the processes leading to the diversification of species. Previously, a genetic study of *B. unicolor* revealed genetic variation of CO1 genes between five populations of the western and northern cape of South Africa (Sathyan et al. 2017). In the current research, we added more samples and had the opportunity to include two gene sequences from 11 populations from the western, northern, and eastern capes of South Africa. The level of genetic diversity and divergence of *B. unicolor* from locations covering the distribution is assessed using genetic samples using mitochondrial cytochrome C oxidase subunit 1 (CO1) and Internal transcribed spacer (ITS) gene sequences.

We aimed to establish a knowledge base of the genetic diversity level and phylogeographic patterns amongst the geographic populations of *B. unicolor*, in order to determine the phylogeographic structure and the factors that influence it. This type of study, by the use of different molecular markers are important to analyse gene flow or isolation-by-distance. The result can facilitate a better understanding of the evolutionary dynamics in insect studies and its influence on the insect ecology and genetics.

In this study, specifically, we use population genetic analysis to evaluate phylogenetic diversity and structuring in this endemic species. We hypothesize that, due to limited dispersal capacity and strict habitat requirements, there will be little gene flow between neighbouring populations, leading to the development of genetic structuring among the different populations. The evolutionary frameworks can assess the distribution of intraspecific genetic diversity. A separate study of genetic variation in invertebrates adapting to heterogeneous environments will reveal the speciation

processes. This study of intra-specific genetic diversity and population structure illustrates how the change in intra-specific diversity alters species response to environments and hence the consequences of the ecosystem.

MATERIALS AND METHODS

Sample collection

In total, 99 individuals were collected from 11 isolated populations of *B. unicolor* (Fig. 1, Table 1) from the Western, Northern and Eastern Cape provinces of South Africa. Specimens were collected by hand and preserved in 95% ethanol for molecular studies.

DNA extraction, PCR amplification and sequencing

For each sample, we removed the hind legs and extracted genomic DNA using a DNeasy animal tissue kit (Qiagen Inc., Valencia, CA, USA). We collected gene sequences from the mitochondrial cytochrome oxidase 1 (CO1) gene and internal transcribed spacer (ITS). To amplify the partial sequence of mitochondrial gene CO1 (650 bp), we used the PCR forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) and for ITS (750 bp), the PCR ITS forward primer DgITS-F (5"- AGAGGAAGTAAAAGTCGTAACAAGG-3") and ITS reverse primer DgITS-R (5"- CCTTAGTAATATGCTTAAATTCAGG-3") were used (Roy et al. 2008).

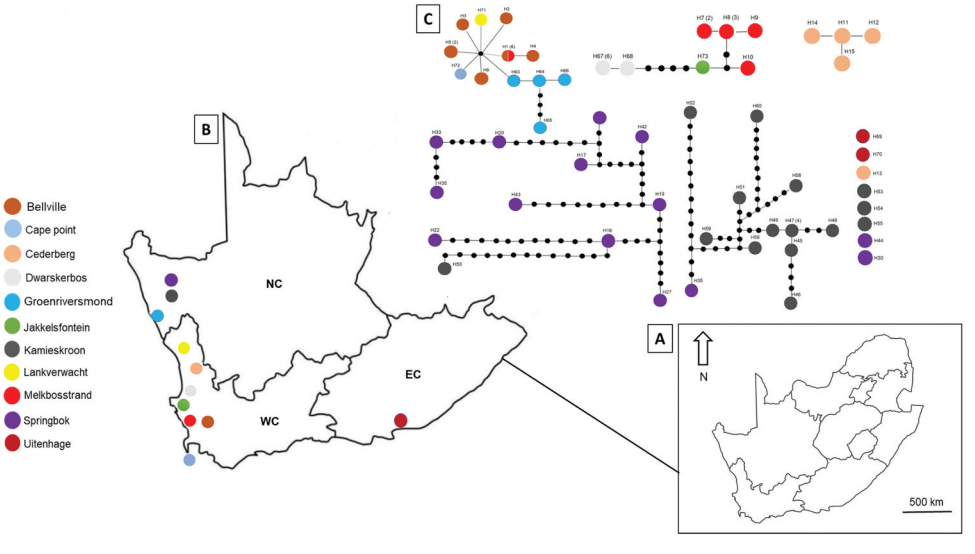

Fig. 1. — (A) and (B), map of the Western, Northern and Eastern Cape (WC, NC and EC) provinces of South Africa (SA) showing the localities where *Bullacris unicolor* was sampled. The different colours represent the 12 population groups. (C), Haplotype network of CO1 sequences from *B. unicolor* specimens from 12 populations. Each line represents a single mutational step, irrespective of length. Individual numbers of the respective haplotypes and their frequency (in the brackets) are shown.

Table 1.

Sampled localities, geographic coordinates and the number of *B. unicolor* specimens sequenced for CO1 and ITS.

				Bullacris unicolor (n)	
Province	Locality	Latitude	Longitude	CO1	ITS
Western cape	Bellville	– 33.89	18.63	9	8
	Cederberg	– 32.58	19.01	8	7
	Cape point	– 34.25	18.42	1	1
	Dwarskerbos	– 32.65	18.30	7	7
	Jakkelsfontein	– 33.41	18.26	1	1
	Melkbosstrand	– 33.71	18.45	10	9
	Lankverwacht	– 31.49	18.91	1	1
Northern Cape	Kamieskroon	– 30.21	17.92	20	20
	Springbok	– 29.66	17.89	29	28
	Groenriversmond	– 30.85	17.60	11	11
Eastern Cape	Uitenhage	– 33.76	25.41	2	2

Amplification and sequencing of the mitochondrial cytochrome oxidase subunit 1 (CO1) was performed using the techniques outlined in Sathyan et al. (2017). PCR amplifications for ITS were carried out in a final volume of 25 µL Ampliqon Taq DNA Polymerase Master Mix RED (Odense M, Denmark), 22.5 µL master mix and 2.5 µL of template DNA. For ITS, an initial heating period of 94 °C for 5 min was followed by 30 cycles of 94 °C for 30 sec, 49 °C for 30 sec, and 72 °C for 1 min. A final extension step of 72 °C for 10 min and a final hold 15 °C was done. To confirm whether amplification was successful, 2 µL of the amplified product was electrophoresed on 1.5% agarose gel (1× Tris borate-EDTA), stained with ethidium bromide, and observed under a UV transilluminator. PCR products were then purified using a QIAquick PCR purification kit and an automated sequencer (ABI 3100, applied Biosystems) was used to run cycle sequencing products (Folmer et al. 1994).

Data analysis

The DNA sequences were edited manually and aligned using Bioedit sequence alignment editor, version 7.2.5 (Hall 1999). Ambiguities and missing data were excluded by trimming the end sections of the sequences. Unique sequences were obtained after manual correction and assembly. The CO1 and ITS sequences were compared and blasted with other sequences of grasshoppers on GENBANK to authenticate the sequences. EMBROSS/Transec (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) were used to check and further evaluate the functionality of the CO1 and ITS sequences. The closely related *Bullacris membracioides* was used as an outgroup.

For the Bayesian phylogenetic analysis and maximum likelihood (ML), the best fit nucleotide substitution model was chosen in MODELTEST version 3.06 (Posada & Crandall 1998).

Bayesian analyses were performed under the best-fit model using Mr Bayes version 3.2 (Ronquist & Huelsenbeck 2003). We ran four chains with 5×10^7 generations in the Markov Chain Monte Carlo (MCMC) process. When the average standard deviation of the split frequencies was zero, convergence of the MCMC process was established. The first 25% of the MCMC samples were discarded as burn-in.

Genetic diversity was calculated in DnaSP version 5.10 (Librado & Rozas 2009) with CO1 and ITS analysed separately. Genetic parameters for each individual, including number of haplotypes (Nh), haplotype diversity (Hd) and nucleotide diversity (π), and the number of segregating sites, were analysed with concatenated CO1 and ITS. We used Tajima's *D* test (Tajima 1989a, 1989b) and Fu's *F*s test (Fu 1997) to test the population changes and gene selection. Pairwise genetic divergence estimates (F_{ST} values) were calculated and tested for significance to assess the relative degree of divergence between individuals from different populations using PAUP version 4.0 (Swofford 2002). Mantel tests were used to determine the strength of the relationship between genetic distance and geographic distance using the isolation-by-distance (IBD) in Alleles In Space (AIS) version 1.0 (Miller 2005). Geographic coordinates were measured from global positioning system (GPS) coordinates taken at each location. A data matrix was then formed using CO1 genetic distances and the corresponding linear geographical distance.

We analysed the population size expansion using mismatch distribution. The histograms of mismatch frequency were plotted in DNAsp to determine whether the number of pairwise differences within *B. unicolor* reflected a rapid population expansion model or a constant population history model (Harpending 1994). Ragged and erratic curves of mismatch distribution reflect the stationary populations over long periods of time, whereas a smooth bell-shaped curve reflect growth and expansion in populations (Harpending 1994; Ray et al. 2003). The raggedness r and R2 statistics were used to calculate the mismatch distribution.

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

A probability cut-off of 95% haplotype parsimony network was reconstructed using the TCS 1.21 software (Clement et al. 2000) and the geographical relationships among haplotypes were depicted for the CO1 data only. Based on the frequency, network location and geography the ambiguous connections (loops) were resolved (Crandall & Templeton 1993; Crandall 1994).

RESULTS

Genetic diversity of B. unicolor

After trimming the short end sections of the CO1 and ITS sequences, 654 bp were obtained from 99 specimens for CO1 and 762 bp were obtained from 95 specimens for ITS. The aligned CO1 fragments were comprised of 73 mitochondrial haplotypes, 238 substitutions and 191 polymorphic sites. The ITS fragments were comprised of 13 haplotypes, 26 substitutions and 26 polymorphic sites. The average number of pairwise differences for CO1 and ITS were 32.528 and 2.659% respectively. Overall nucleotide diversity and haplotype diversity for CO1 was 0.049 and 0.985 and for ITS was 0.003 and 0.779 respectively. There was significant variation in base composition between sequences (CO1: C2 = 594.000, df = 432, $P < 0.01$; ITS: C2 = 380.000, df = 48, $P < 0.01$).

Genetic structure

A phylogenetic tree reconstructed from DNA sequencing data of CO1 indicates eight haplotype clades (Fig. S1 in Supplemental Data). The Western Cape populations are separated into four distinct clades (Clades 1, 2, 4 and 5). Clade 1 shared haplotypes from Melkbosstrand, Jakkelsfontein and Dwarskerbos. Clade 2 shared haplotypes from Bellville and Melkbosstrand. The Northern Cape population, Groenriversmond (Clade 3) and the Western Cape population, Cederberg (Clade 5) are clearly separated, and do not share haplotypes with any other population. The two individuals from Lankverwacht and Cape point are separated in Clade 4. Similarly, two individuals from the Eastern Cape, Uitenhage comprised Clade 6. The remaining Clades 7 and 8 comprises a mixture of individuals from Kamieskroon and Springbok. A strong genetic structuring between locations was observed in the genetic analysis.

Five major clades were identified from the ITS data (Fig. S2 in Supplemental Data). All populations were observed to be separate from each of the five major clades. Clade 1 comprised samples from the Western and Eastern Cape. Two individuals from Uitenhage were positioned in Clade 1 despite being from the Eastern Cape. Only one individual from Melkbosstrand (Western Cape) clustered in Clade 2 together with the Groenriversmond samples (Northern Cape). Clades 3 and 4 were comprised of individuals from Springbok and Cederberg respectively. Clade 5 included all Springbok and Kamieskroon samples, but one individual from Clade 5 came from Cederberg and this location is nearest to the Northern Cape.

Pairwise distance analysis of CO1 (Table 2) revealed sequence divergence among individuals within the same population was generally small, with the exception of grasshoppers from Springbok and Kamieskroon (Clade 8), which showed high genetic variation (3.42%) compared to other populations. On the other hand, individuals within Groenriversmond showed low levels of sequence divergence (0.26%). The nucleotide diversity (π) of Clade 8 is 0.034 while haplotype diversity (h) for this clade was 0.988 (Table 3). Clades 4, 6,7 and 8 recorded the highest π and h. A high h and low π indicate moderate genetic diversity in the studied populations of *B. unicolor*. Pairwise distance analysis of ITS revealed sequence divergence within Clade 1, that was all locations from the Western Cape and two samples from the Eastern Cape, showed the highest genetic variation (0.24%). Individuals from Clade 5 showed the second highest percentage of variation (0.06%). Clade 1 showed the highest π and h, with all populations from Western Cape and two individuals from Eastern Cape. Although, Cederberg, despite being from The Western Cape, was separated as Clade 4 with the second highest haplotype diversity.

Haplotype analysis

Statistical parsimony haplotype network was done only for CO1, not ITS. A total of 73 haplotypes were derived from the 99 individuals sequenced with 65 of them being unique haplotypes and four being shared haplotypes (Fig. 1). The shared haplotypes of Western Cape and Northern Cape populations are Hap1 (Bellville, Melkbosstrand), Hap5 (Bellville), Hap7 (Melkbosstrand), Hap8 (Melkbosstrand), Hap11 (Cederberg), Hap47 (Kamieskroon), Hap62 (Groenriversmond), Hap67 (Dwaserkbos). All shared haplotypes were within location except for Hap1. The population from Clade 8 (Springbok and Kamieskroon) recorded the highest total number

Table 2.

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

CO1	Clade 1 Melkbosstrand (WC) Dwarskerbos (WC) Jakkelsfontein (WC)	Clade 2 Melkbosstrand (WC) Bellville	Clade 3 Groenriversmond (NC)	Clade 4 Lankverwacht (WC) Cape point (WC)	Clade 5 Cederberg (WC)	Clade 6 Uitenhage (EC)	Clade 7 Springbok (NC) Kamieskroon (NC)	Clade 8 Springbok (NC) Kamieskroon (NC)
Clade 1	**0.76 ± 0.01**							
Clade 2	3.99 ± 0.00	**0.66 ± 0.00**						
Clade 3	2.72 ± 0.00	3.24 ± 0.00	**0.26 ± 0.00**					
Clade 4	2.18 ± 0.00	3.55 ± 0.00	2.18 ± 0.00	**2.59 ± 0.00**				
Clade 5	3.68 ± 0.01	5.16 ± 0.01	3.68 ± 0.01	4.28 ± 0.01	**1.72 ± 0.02**			
Clade 6	5.54 ± 0.00	6.11 ± 0.00	5.54 ± 0.00	5.27 ± 0.00	6.68 ± 0.01	**2.44 ± 0.02**		
Clade 7	4.12 ± 0.01	5.24 ± 0.01	4.12 ± 0.01	4.17 ± 0.01	4.75 ± 0.01	5.77 ± 0.01	**2.13 ± 0.02**	
Clade 8	6.23 ± 0.01	6.98 ± 0.01	6.23 ± 0.01	6.39 ± 0.01	6.41 ± 0.01	6.92 ± 0.01	5.69 ± 0.02	**3.42 ± 0.01**

ITS	Clade 1 Melkbosstrand Bellville Dwarskerbos Cape point Uitenhage Jakkelsfontein Lankverwacht	Clade 2 Groenriversmond Melkbosstrand	Clade 3 Springbok	Clade 4 Cederberg	Clade 5 Springbok Kamieskroon Cederberg
Clade 1	**0.24 ± 0.00**				
Clade 2	0.66 ± 0.00	**0.00 ± 0.00**			
Clade 3	0.67 ± 0.00	0.26 ± 0.00	**0.00 ± 0.00**		
Clade 4	0.69 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	**0.04 ± 0.00**	
Clade 5	0.57 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.18 ± 0.00	**0.06 ± 0.00**

Table 3.

Genetic diversity and demographic parameters for 654 bp of cytochrome oxidase 1, CO1 (F_{st} = 0.654) mtDNA and 762 bp of Internal transcribed spacer, ITS (F_{st} = 0.810) of *Bullacris unicolor*. N = sample size; Nh = haplotype number; S = number of polymorphic sites; h = haplotype diversity; π = nucleotide diversity; Tajima's *D*, Fu's *F*s. *P < 0.05, **P < 0.01.

Population	*N*	Nh	*S*	h	π	*D*	*F*s
CO1							
Clade 1	15	7	11	0.819	0.007	1.831	0.736
Clade 2	12	6	20	0.757	0.006	– 1.670	0.629
Clade 3	11	5	8	0.618	0.002	– 1.548	– 0.763
Clade 4	2	2	17	1.000	0.025	0.000	0.000
Clade 5	8	5	45	0.786	0.017	– 1.887**	2.692*
Clade 6	2	2	16	1.000	0.024	0.000	0.000
Clade 7	16	16	71	1.000	0.024	– 1.570	– 6.503*
Clade 8	33	30	94	0.988	0.034	– 0.783	– 8.984
ITS							
Clade 1	28	5	10	0.696	0.002	– 0.653	1.293
Clade 2	12	1	0	0.000	0.000	0.000	0.000
Clade 3	6	1	0	0.000	0.000	0.000	0.000
Clade 4	6	2	1	0.333	0.000	– 0.933	– 0.003
Clade 5	43	4	11	0.136	0.000	– 2.390**	– 4.647*

Location details of each clade is defined in Table 2.

of haplotypes (CO1–30), while Clades 4 (Lankverwacht and Cape Point) and 6 (Uitenhage) recorded the lowest number of haplotypes (2).

Population size changes

Population size changes and deviations from neutrality were investigated through different analyses. All of the CO1 analyses produced significant negative Fu's *F*s values (*F*s = – 19.749, $P \leq 0.05$), indicating population expansion. However, Tajima's D was not significant (Tajima's *D* = – 0.989, $P \geq 0.05$). Individually analysed populations showed that Clade 5 (Cederberg) and Clade 7 (Springbok and Kamieskroon) have significantly negative *F*s values, implying population expansion in the past, while the remainder of the populations produced no significant values (Table 3). F_{ST} values (0.654) of all populations were high, indicating substantial genetic structuring. A mismatch distribution analysis based on Dnasp performed on all of the CO1 haplotypes produced a smooth and bimodal curve (Fig. 2), revealing that the populations were undergoing expansion. The raggedness values for the CO1 data set was 0.002 and R2 statistic of 0.081 respectively. The low values of raggedness index did not reject a sudden expansion model.

For the ITS data sets for all of the populations, Tajima's *D* value was negative, but non-significant (Tajima's *D* = – 1.433, $P \geq 0.050$), implying deviations from neutrality, while Fu's *F*s (*F*s = 1.293, $P \geq 0.05$) value was not significant. F_{ST} values (0.810) of all populations were high. The Raggedness (0.068) and R2 statistic (0.056) values for all the alleles were low. Analysis of populations separately showed Fu's *F*s values were non-significant for all of the populations, except for Clade 5 (Springbok, Kamieskroon and Cederberg). Fu's *F*s and Tajima's *D* value calculated for all other clades were not statistically significant. Other than this, all of the ITS datasets produced a bimodal curve (Fig. 1), these populations were undergoing population expansion.

Relationships among geographic distance and genetics

The Mantel test for matrix correlation between CO1 genetic distance and linear geographic distance gave no significant correlation coefficient (Mantel test: r = – 0.431, $P = 0.954$) suggesting that *B. unicolor* does not fit an isolation-by-distance model.

DISCUSSION

Our results show that *B. unicolor* has a high level of genetic structuring, similar to that observed within many other widespread invertebrate species in southern Africa (Daniels et al. 2001; Wishart & Hughes 2001; Gouws et al. 2004). The CO1 gene showed much higher levels of variation as expected compared to the ITS alleles, presumably due to the faster rate of substitution of the mitochondrial genome (Brown et al. 1979). The mismatch analyses of population size change through time indicate support for a population size expansion with CO1 and moderate support with ITS. The different results from the two different loci reflect the fact that CO1 responds more strongly to population size changes due to the smaller effective population size relative to nuclear genes. Furthermore, the recovery of demographic details can be

prevented by the low levels of variation in the ITS alleles. The negative Tajima's *D* statistic assessed from CO1 and ITS indicates an excess of rare variants relative to standard neutral expectations. One explanation for this observation is a departure from the neutral model due to a past population expansion or an ancient population bottleneck (Slatkin & Hudson 1991; Fay & Wu 1999; Wakeley & Aliacar 2001; Fischer et al. 2004). Longer sequences and comparison with other nuclear loci is required to gain a more complete picture of the processes affecting the genetic variation of these two genes.

The CO1 data revealed eight major clades are largely limited to a well-defined geographical region. The ITS data showed five major clades but there was an overlap in the geographical distribution of the clades, especially Western and Northern Cape. Some individuals also possessed alleles that were otherwise restricted to geographically distant locations, for example, Melkbos242 from Western Cape sharing the allele with individuals from Groenriversmond, Northern Cape and Ceder180 from the Western Cape sharing the alleles from with the individuals from Springbok and Kamieskroon, Northern Cape. The broad corresponding distributions of the eight and five major clades from the two loci reflect population divergences rather than random patterns due to lineage arrangement. The occurrence of admixed specimens

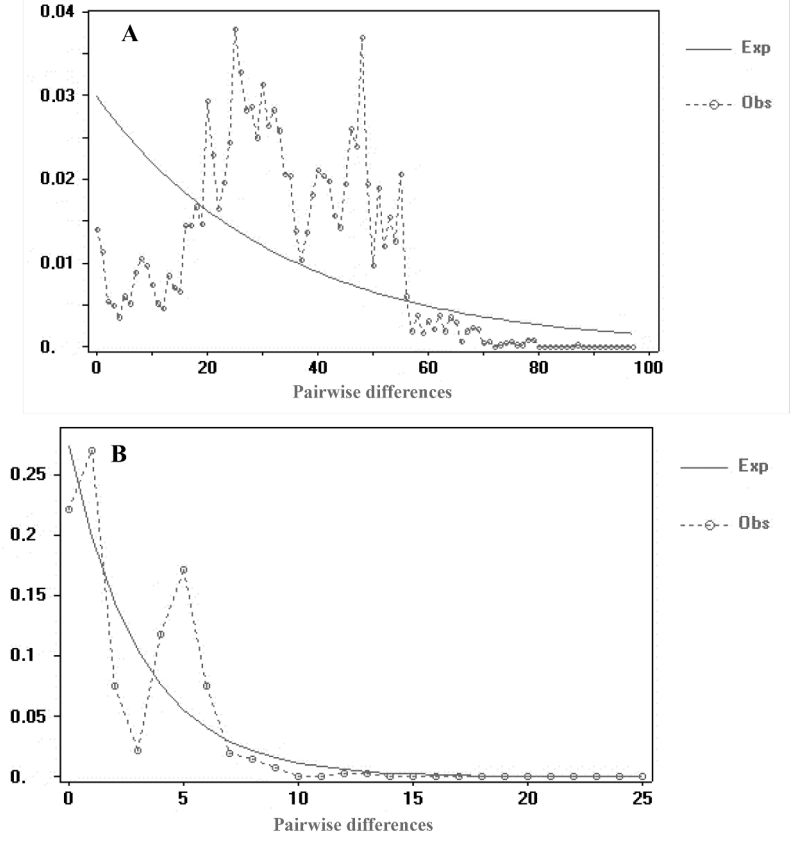

Fig. 2. — Mismatch distribution of all pairwise combinations. (A) CO1 haplotypes and (B) ITS alleles. The observed distribution is represented by a dotted line (Obs), and the expected frequencies by a continuous line (Exp).

and boundaries between the major clades divergent somewhat between the two loci specifies secondary contact and following gene flow has occurred.

Biogeographically, the Western Cape population (Clades 1, 2, 4 and 5) is separated by a phylogeographic barrier of approximately 60 km, the Cape Flats. The emergence of Cape Flats leads to the separation of Table Bay and False Bay (Schalke 1973). Several phylogeographic studies on invertebrates have identified the Cape Flats as a barrier to gene flow within the Western Cape region (Daniels et al. 2001; Wishart & Hughes 2003; Gouws et al. 2010). Biogeographically, within the Western Cape, mountains also form a barrier between populations (Visser et al. 2014). The CO1 Clades 1, 2, 4 and 5 are located in the south and north-western cape and is separated by the Boland, Olifants River and Hottentots Holland Mountains and the Hex River, Skurweberg Cederberg and Langeberg Mountains. The four clades therefore appear to have resulted from these physical barriers. The individuals from Bellville and Melkbosstrand populations are clustered and likely represent gene-flow event between geographically close localities. More specifically, the phylogenetic trees retrieved indicated that it has a closer genetic relationship, suggesting there is ongoing gene flow between these locations.

Genetic variation was high within grasshoppers from Springbok and Kamieskroon (Clade 8, CO1). High genetic variation and overlap of haplotypes suggest that dispersal among localities at the Northern Cape is occurring. However, Groenriversmond does not share any haplotypes with these two locations despite its proximity to Kamieskroon and an absence of any obvious barriers to gene flow. However, the ITS tree showed one specimen from Melkbosstrand nested within the Groenriversmond clade. Both of these localities occur along the coast but are separated by a distance of 500 km. Gene flow between these two locations suggesting the possibility of a suitable habitat may have connected these localities in the past.

Genetic information showed high values of haplotype diversity from all populations together. Conversely, low genetic variation within the population of *B. unicolor* occurs maybe due to the genetic founder effects related to small population size. In addition, geographical ranges may also play a major role in structuring genetic diversity in *B. unicolor*. The different haplotypes across the distribution of *B. unicolor* explains demographic increase in each population. The different genetic clusters, especially in the Western and Northern Cape, cannot be explained by isolation-by-distance alone. Thus, it seems that genetic variation is affected by environmental conditions and biogeographic differences of the local population (Hendry et al. 2007; Handley et al. 2011; Serrana et al. 2019b; Ward & Aukema 2019).

The separation between populations were observed in the network and confirmed by population differentiation test, indicating the limitation in gene flow between populations. The main drivers of genetic structure in phytophagous insects are geographical or reproductive barriers, dispersal capacity, host plant fidelity, and habitat fragmentation (Barrett & Kohn 1991; Bertheau et al. 2013; Lesieur et al. 2016; Martinez-Sañudo et al. 2020). The low dispersal ability of this flightless females could have favoured the lack of gene flow among its distribution ranges (Cavaletto et al. 2019). Male *B. unicolor* show a low dispersal ability and move only relatively short distances (Alexander & van Staaden 1989). The development of population structure is greatly influenced by factors that affect dispersal, such as historical variance and geographic distance coupled with differences in habitat discontinuity and dispersal ability (Serrana et al. 2019a; Wang et al. 2022).

The haplotype network revealed five major clusters, but the two specimens from Uitenhage, Eastern Cape clustered separately. In general, haplotypes from this region did not form a monophyletic group, but appeared to be randomly distributed across the haplotype network. The pairwise differences within this location shows 2.44% of variation between these two specimens. This is the second highest difference after the Northern Cape population. Despite the low number of samples from this location, they display high diversity. However, more specimens covering a larger sampling locality across the Eastern Cape distribution range are necessary in order to understand this novel lineage.

In *B. unicolor* the nucleotide diversity is low compared to the haplotype diversity for both CO1 and ITS data for all clades. This suggests that the population has shown fluctuations in the past, and generally points to population growth in the more recent past following population bottlenecks (Rogers & Harpending 1992; Avise 2000). The mismatch analysis of the CO1 data, where all the populations included in the analysis generated a bimodal profile, indicates population expansion (Xiao et al. 2011). Fu's *F*s (CO1 = – 19.749) is significantly negative, indicating the excess of rare haplotypes in an expanding population. Non-significant and negative Tajima's D imply that populations of *B. unicolor* did not diverge from neutrality. In population size changes, Fu's *F*s is more powerful and sensitive than Tajima's D (Ramos-Onsins & Rozas 2002). Again, the ITS region generated a bimodal curve indicating population expansion. Overall, population demographic analyses and mismatch analysis, in addition to the combination of high haplotype and low nucleotide diversity of *B. unicolor* populations for both the CO1 gene and the ITS regions, propose rapid demographic expansion from a small population in the past.

To summarize, we found high haplotype diversity but low nucleotide diversity among *B. unicolor* populations from all three provinces, suggesting population expansion of *B. unicolor*. The genetic clusters were observed in the phylogenetic tree and haplotype network, clear population structuring was also noticed between geographically different populations. The population structuring was noticed among geographically separate populations. Absence of haplotypes from several populations in each genetic cluster indicate high genetic divergence among the populations. The haplotype composition surveyed in the present study may provide a baseline for future comparisons to monitor the temporal variability of haplotype frequency and population structure. Further studies increasing the sampling size, mostly in areas where the samples are low (e.g., Eastern Cape), combined with study of other markers could help to acquire a better image of *B. unicolor* population structure. This research contributed valuable data for future big scale biogeographical and taxonomic studies of this species.

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