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SHORT COMMUNICATION

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No evidence for host plant associated genetic divergence in a population of *Bullacris unicolor* (Linnaeus, 1758) (Orthoptera: Pneumoridae)

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Host-associated genetic differentiation in grasshoppers has received limited attention, due to a lack of information on grasshopper-plant associations. The bladder grasshopper, *Bullacris unicolor* (Linnaeus, 1758) (Orthoptera: Pneumoridae), is a phytophagous species that can occur on at least six host plants within its geographic range. However, the relationship between host plant association and genetic variation of bladder grasshoppers has not been studied before. In light of this, the present study examined host plant-related genetic [mitochondrial cytochrome oxidase 1 (CO1) and the internal transcribed spacer (ITS) gene regions] and morphological (antennal length, body length, head width, abdomen width, femur length, tibia length and pronotum length) divergence within a population of *B. unicolor*. We used two plant species, belonging to different families, namely *Didelta spinosa* (L.fil.) Aiton (Asteraceae) and *Roepera morgsana* (L.) Beier & Thulin (Zygophyllaceae), to evaluate variation between individuals collected on these two sympatric host plants at a single locality in the Northern Cape, South Africa. The results demonstrated non-significant host related genetic variation with very low values of FST, indicating a low level of variation. The phylogram strongly indicated that there are no host-associated genetic differences in *B. unicolor* by displaying limited genomic clustering, whereas some differentiation was observed between the morphological measurements of males and females among host plants. Further studies using microsatellite molecular markers may help to discern population genetic structure. In addition, significant host-associated morphological divergence highlights the need to examine the mechanisms by which host utilization affects morphological features.

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Genetic and phenotypic differences between insects feeding on different species of host plants are well documented and consistent with theories of sympatric speciation (Diehl & Bush 1984; Fritz & Simms 1992; Via 2001; Drès & Mallet 2002; Hsu et al. 2018). Host plant associated ecological divergence among insect populations is often assessed in terms of traits, such as feeding preferences, mate choice, growth, survivorship, performance and fecundity (Funk et al. 2002). Bladder grasshoppers (Orthoptera: Pneumoridae) are a group of insects predominantly found in the coastal regions of southern Africa, and represent an ideal model system on which to study the interactions between insects and plants, and how this interaction may serve as a driver of diversification.

Pneumorids have a high degree of host plant specificity, with each species living and feeding on either one or a small number of host plant species. They rely heavily on cryptic camouflage to avoid predation, with individuals being extremely well matched in terms of colour pattern to their specific host plant. Preliminary investigations have shown significant geographic variation in the morphology of males within and between populations (Sathyan et al. 2017), and that these differences appear to be fixed rather than plastic. All *Bullacris* species are specialized to feed on a small number of host plant species and many of the host plants used by *Bullacris unicolor* (Linnaeus, 1758) occur sympatrically (personal observation). Adult females and nymphs of *B. unicolor* are unable to fly and are poor jumpers. They therefore remain on their host plant their entire lives, whereas adult males can fly and thus may disperse from their natal site. All individuals are cryptically camouflaged to match their host plant, and individuals feeding on different host species, even within the same area, display different colour variations.

Bullacris unicolor feeds selectively on host plant species from a number of unrelated plant genera throughout its geographic range, including *Didelta spinosa* (L.fil.) Aiton (Asteraceae), *Roepera morgsana* (L.) Beier & Thulin (Zygophyllaceae), *Salvia aurea* L. (Lamiaceae), *Tripteris oppositifolia* (Aiton) B. Nord. (Asteraceae), *Osteospermum moniliferum* subsp. *rotundatum* (DC.) J.C.Manning & Goldblatt (Asteraceae), and *Muraltia spinosa* (L.) F.Forest & J.C.Manning (Polygalaceae). In this study, *B. unicolor* was sampled from two plant species, *D. spinosa* and *R. morgsana*. We measured morphological and genetic variation between individuals from the two different host plants to determine if there was host-associated differentiation. Through this study, we aim to evaluate the potential for host-associated reproductive isolation in this species.

A total of 38 individuals of *B. unicolor* were collected from *D. spinosa* and *R. morgsana* (Figure 1) around the town of Springbok (29°39′S, 17°53′E) in the Northern Cape, South Africa. The host plant species was recorded during sampling. Among the sampled specimens, 24 were collected from *D. spinosa* and 14 from *R. morgsana*. We collected a mixture of adults and sub-adults (final instar, n = 16) to permit accurate host association, as adult males may move around once they begin calling (typically about a week after their final moult). Nymphs were reared to adulthood in the laboratory, and fed either their original host plant (if available), or otherwise on lettuce leaves. As all nymphs used were in the final instar stage and thus within days of their final moult into

Figure 1. Two alternative host plants of *B. unicolor* (top) *Didelta spinosa* and (bottom) *Roepera morgsana*

adulthood, we assumed this had no significant impact on growth and adult body sizes. To maximize the coverage of genetic variation, we collected individuals from an approximately 25 km^2 area. Specimens were preserved in 95% ethanol at 4 °C for long-term storage until DNA extraction.

A series of seven linear measurements (mm), which included antennal length, body length, head width, abdomen width, femur length, tibia length and pronotum length were recorded for each ethanol preserved specimen following Donelson (2007) and Sathyan et al. (2017). We used multivariate analysis of variance (MANOVA) to compare the variation in male and female morphological characters within the population. The MANOVA compared each length variable against host plants, for both males and females. Mantel tests were used to test the relationship between morphological variation and genetic variation.

For each sample, we removed the hind legs and extracted genomic DNA using a MN Mechery Nagel tissue kit. The mitochondrial cytochrome oxidase 1 (CO1) and the internal transcribed spacer (ITS) gene regions were selected for this study. To amplify the partial sequence of mitochondrial gene COI (650bp), the forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) were used (Folmer et al. 1994). For ITS (750bp), the ITS forward primer DgITS-F (5′- AGAGGAAGTAAAAGTCGTAACAAGG-3′) and ITS reverse primer DgITS-R (5′- CCTTAGTAATATGCTTAAATTCAGG-3′) were used (Roy et al. 2009).

PCR amplifications for both COI and ITS were carried out in a final volume of 25 µl Ampliqon Taq DNA Polymerase Master Mix RED (Odense M, Denmark) – 12.5 µl Ampliqon Taq DNA Polymerase, 1.25 µl of the respective primer pairs and 10 µl ddH2O reactions, with 22.5 µl master mix and 2.5 µl of template DNA. For COI, the PCR cycling profile comprised an initial heating period of 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. A final extension step of 72 °C for 5 min was also added. For ITS, an initial heating period of 5 min at 94 °C was followed by 30 cycles of 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 1 min. A final extension step of 72 °C for 10 min and a final hold at 15 °C was used. To confirm whether amplification was successful, 2 µl of the amplified products were electrophoresed on 1.5% agarose gel (1x Tris borate-EDTA), stained with ethidium bromide, in a TAE buffer system and visualized under a UV transilluminator. Successfully amplified products were sequenced using an automated sequencer (ABI 3100, Applied Biosystems®) (Folmer et al. 1994).

The DNA sequences were edited manually and then aligned using Bioedit sequence alignment editor, version 7.2.5 (Hall 2005). All haplotype/allele sequences were deposited in GENBANK under the accession numbers OK428651–OK428679 for the ITS and OK570351–OK570373 for the COI. The COI and ITS sequences were compared and blasted against other sequences of grasshoppers on GENBANK to authenticate the sequences. The closely related *Bullacris membracioides* was used as an outgroup.

For the Bayesian phylogenetic analysis and maximum likelihood (ML) analysis, the best-fit nucleotide substitution model was chosen in MODELTEST version 3.06 (Posada & Crandall 1998). Bayesian analyses were performed by means of the best-fit model using Mr Bayes version 3.2 (Ronquist & Huelsenbeck 2003). We ran four chains with 5 × 107 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 mtDNA and ITS analysed separately. Genetic parameters for grasshoppers on each host plant species, including the number of haplotypes (Nh), haplotype diversity (Hd), nucleotide diversity (π) and the number of segregating sites, were analysed. Pairwise genetic divergence estimates (FST values) were calculated and tested for significance to assess the relative degree of divergence between individuals from the two host plants. Isolation by distance among sampled individuals was obtained by examining the correlations between matrices of pairwise genetic distance (FST) and morphological distance using PAUP version 4.0 (Swofford 2002). Mantel tests were used to test the strength of the relationship between the morphology and genetics using the *ppcor* package (Kim 2015) in R 4.1.0 (R Core Team, 2021).

We carried out a neighbour joining analysis in PAUP* 4.0 (Swofford 2002), with rooted phylogenetic trees constructed using the congeneric *B. membracioides* as an outgroup. Splits tree version 4.13.1 (Huson & Bryant 2006) was used to display haplotype sequence variation and the genetic distances of individuals collected from the two host plant species.

The results demonstrated non-significant host related genetic variation with very low values of FST, indicating a low level of variation. The phylogram strongly indicated that there were no host-associated genetic differences in *B. unicolor*, with limited genomic clustering evident. In contrast, some differentiation was observed between the morphological measurements of males and females for each of the host plant species. Monte Carlo nonparametric tests indicated non-significant differences from normality for all body measurements. MANOVA results revealed differences in the morphological characters of both males (Wilks' Lambda = 0.328, $F = 1.172$, $P < 0.005$) and females (Wilks' Lambda = 0.075, $F = 3.529$, $P < 0.005$) among individuals

feeding on alternative host plants within the population. Males differed in pronotum length and females differed in head width, femur length and tibia length (Table 1). In all cases, measurements for grasshoppers feeding on *D. spinosa* were slightly larger.

The absence of clustering sequences, non-significant *P*-values and negative or low FST values indicated that host plants do not support phylogenetic tracking in *B. unicolor* (Figures 2 and 3). Moreover, the substitution rates of ITS between the two host plants showed low values (0.3%). These results suggest that the individuals from different host plants are not genetically isolated. Pairwise distances of CO1 and ITS showed similar levels of genetic variation within *B. unicolor* found on *D. spinosa* (3.64% and 0.38%) and *R. morgsana* (2.56% and 0.39%) and between host plants (3.00% and 0.37%) (Table 2). These results suggest that the level of host-associated intrapopulation genetic variation was extremely low and little sequence divergence was evident both within *D. spinosa* and *R. morgsana*. Mean mitochondrial intrapopulation divergence values were higher than nuclear DNA as predicted, because of the faster mutation rate in the mitochondrial genome (Simon et al. 1994).

Morphological distance was not significantly correlated with genetic distance, suggesting that morphological differences between individuals within the population were not predicted by genetic distance. Mantel tests revealed that both male and female morphological distance was not correlated with either the CO1 or ITS gene [males (CO1: −0.103, $P = 0.690$ and ITS: $r = 0.077$, $P = 0.312$), and females (CO1: −0.030, $P = 0.544$ and ITS: 0.089, $P = 0.326$)].

Despite the lack of genetic divergence related to host plant use, there was evidence of morphological variation, with individuals feeding on *D. spinosa* being comparatively larger in size. This finding may not be surprising given the evidence of host-associated growth rate and the effects of host plant quality on fecundity of herbivorous insects (reviewed in Bush & Butlin 2001; Awmack & Leather 2002). The components of host plant quality, such as chemical compounds and nutritional content directly affect herbivorous insects' fecundity, and reproductive strategies. The success of predators and parasitoids may also be affected by host plant characteristics (Matsubayashi et al. 2010). Thus, the observed relationship between host plants and morphology may be the product of multiple mechanisms, such

Table 1. Morphological measurements (mean ± standard deviation) (mm) for (A) males ($n = 20$) and (B) females (n = 18) of *Bullacris unicolor* from two host plants, and multivariate analysis of variance (MANOVA) results of the variation in the morphological characters

Morph. charact.	*Didelta spinosa* (Mean ± SD)	*Roepera morgsana* (Mean ± SD)	df	Mean square	*F*	*P*
(A)	N-14	N-6				
AL	5.85 ± 1.02	6.46 ±0.96	1	1.55	1.51	0.23
HW	4.70 ± 0.38	4.61 ±0.29	1	0.03	0.26	0.61
BL	39.12 ±2.02	37.84 ± 1.91	1	6.86	1.72	0.20
AW	11.21 ± 0.99	10.87 ± 0.66	1	0.46	0.55	0.46
FL	10.95 ±0.93	10.93 ±0.57	1	0.00	0.02	0.96
TL	11.62 ±0.93	11.22 ±0.59	1	0.70	0.96	0.33
PL	17.88 ±0.98	16.55 ±0.91	1	7.34	7.86	0.01**
(B)	N-10	N-8				
AL	5.70 ±0.57	5.59 ±0.55	1	0.00	0.00	0.95
HW	5.74 ± 0.39	5.32 ±0.23	1	0.80	7.09	0.02*
BL	39.17 ±3.46	37.90 ± 3.20	1	18.42	1.63	0.25
AW	8.84 ±0.80	8.68 ±0.58	1	0.11	0.22	0.85
FL	11.03 ±0.58	9.93 ±0.62	1	5.37	14.98	<0.001*
TL	11.70 ±0.59	10.63 ±0.69	1	4.19	10.18	0.02**
PL	21.28 ± 0.69	21.33 ±0.69		3.99	3.38	0.85

Morphological measurements: AL = antennae length; HW = head width; BL = body length; AW = abdomen width; FL = femur length; TL = tibia length; and PL = pronotum length
*significant $P < 0.05$

Table 2. Pairwise genetic distance of CO1 and ITS (mean ± standard deviation) within (bold) and between individuals of *B. unicolor* from two host plants

		Didelta spinosa	*Roepera morgsana*
CO1	*Didelta spinosa*	**3.64 ± 0.02**	
	Roepera morgsana	3.00 ± 0.02	**2.56 ± 0.01**
ITS	*Didelta spinosa*	**0.38 ± 0.00**	
	Roepera morgsana	0.37 ± 0.00	**0.39 ± 0.00**

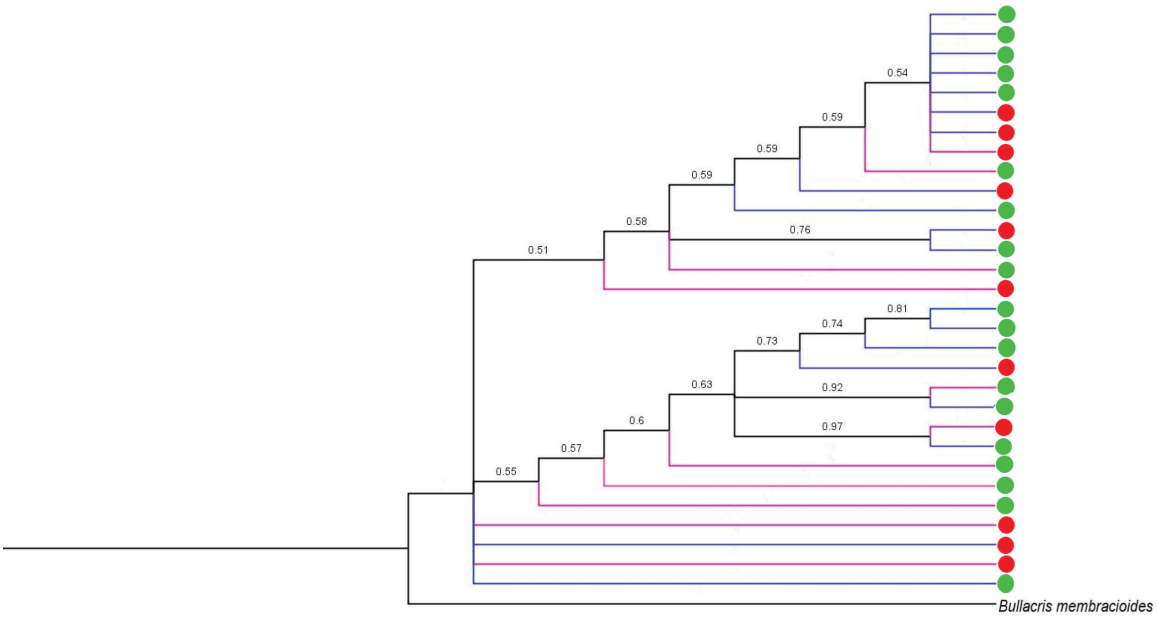

Figure 2. Linearised phylogenetic tree of *Bullacris unicolor* from two host plants using the cytochrome oxidase 1 (CO1) gene ($n = 30$), based on 30 haplotypes and rooted by *Bullacris membracioides*. Posterior probabilities are included above branches. Blue and pink lines represent males and females accordingly. The host plants *D. spinosa* and *R. morgsana* are represented by green and red dots respectively

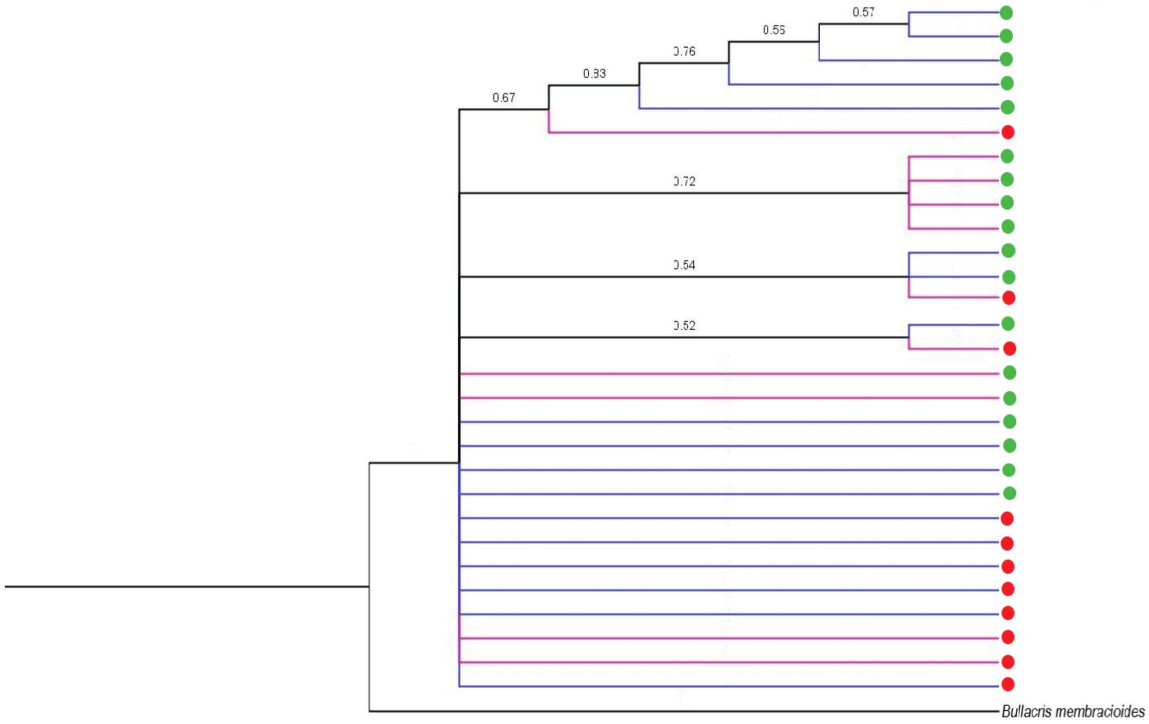

Figure 3. Linearised phylogenetic tree of *Bullacris unicolor* from two host plants using the internal transcribed spacer (ITS) gene (n = 58) based on 21 haplotypes and rooted by *Bullacris membracioides*. Posterior probabilities are included above branches. Blue and pink lines represent males and females accordingly. The host plants *D. spinosa* and *R. morgsana* are represented by green and red dots respectively.

as selective predation, developmental plasticity, adaptive and non-adaptive processes (Augustyn et al. 2017; Hsu et al. 2018).

Insect divergence is frequently associated with host preferences, as the latter can directly result in assortative mating (Wood & Keese 1990; Bruce 2015). Host shifting is traditionally expected between closely related plant species, as they share similarities in chemical compounds (Janz & Nylin 1997). Given the lack of support for phylogenetic tracking, this is unlikely to be the case between *B. unicolor* and their host plants, as these plants do not share any physical similarities with each other and belong to different families.

The combination of low nucleotide diversity and high haplotype diversity in our data can be a signature of rapid demographic expansion (Avise 2000). Pairwise differences between sequences within populations were developed to test selective neutrality of mutations (Ramos-Onsins & Rozas 2002). Here, we chose Tajima's *D* (Tajima 1989) and Fu's Fs (Fu 1997) to detect population expansion as they differ in their approach. Tajima's *D* test is based on the allele frequency distribution of segregating nucleotide sites. In the present study, Tajima's *D* test and Fu's Fs show non-significant negative values for both COI and ITS sequences within the population. The negative values resulting from both tests indicate that there is an excess of rare mutations in this population, which can indicate recent expansion in the population (Table 3). Alternatively, these values can result from balancing selection on a nearby locus.

As in this study on *B. unicolor*, there is little evidence for host plant associated ecological and genetic divergence in grasshoppers (Chapman & Sword 1997; Sword et al. 2005). This is surprising given the evolutionary consequences of host plant associated divergence that are consistently noted across phytophagous insect groups. For example, evidence of host-associated genetic structure is consistent with processes of host-associated divergence in leaf mining fly species (Scheffer et al. 2021). In addition, divergence in host-acceptance behaviours on different host plants is a key aspect of evolutionary differentiation among closely-related taxa of phytophagous parasitic insects

Table 3. Genetic diversity and demographic parameters for 654 bp of cytochrome oxidase 1 (CO1) and 760 bp of ITS of *Bullacris unicolor*

	Host plant	*N*	*Nh*	*S*	*Hd*	∏
CO1	*Didelta spinosa*	19	19	116	1.00	0.04
	Roepera morgsana	11	11	62	1.00	0.03
ITS	*Didelta spinosa*	36	16	12	0.95	0.00
	Roepera morgsana	22	7	12	0.84	0.00

N = sample size; Nh = haplotype number; *S* = number of polymorphic sites; Hd = haplotype diversity; π = nucleotide diversity

(Bierbaum & Bush 1990). Detailed examinations of local host use patterns are rare in grasshoppers, but such studies would be valuable as they would elucidate occurrences of local adaptation and resource associated divergence in this group (Sword & Dopman 1999; Traxler & Joern 1999).

There was no evidence of host-associated genetic differences in *B. unicolor* within a single population. However, host-associated morphological divergence highlighted the need to target the effect of host utilization, considering its potential importance as a key trait for promoting diversification. However, limitations on sample sizes were encountered and this compromised the number of individuals that could be analysed. Future research efforts will be needed to assess the factors shaping the observed variation in morphology and colour associated with host plant use. Further studies that make use of additional microsatellite molecular markers should also be carried out to discern the population genetic structure on a finer scale. Moreover, assessing host-associated divergence between allopatric populations is critical to elucidate instances of host shifting and resource associated divergence.

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