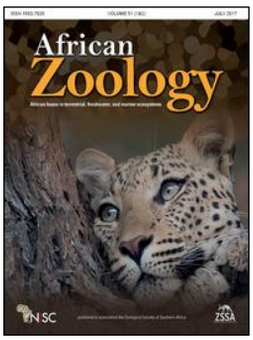

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Short Communication

Evidence of multiple divergent mitochondrial lineages within the southern African diplopod genus *Bicoxidens* Attems, 1928 (Spirostreptida)

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Two recent studies have suggested that divergent mitochondrial lineages may be present within spirostreptid genera such as *Bicoxidens* Attems, 1928. *Bicoxidens*, similar to many other endemic soil invertebrates, exhibits low dispersal capabilities and strict microclimate habitat preferences, attributes that often lead to geographic isolation. Given that prolonged geographic isolation often lays the foundation for population genetic differentiation, genetic divergence and possibly speciation, there was good reason to suspect that *Bicoxidens* may consist of several distinct lineages. On this basis, the mitochondrial cytochrome *c* oxidase subunit 1 (COI) was used to reconstruct the phylogeny of *Bicoxidens* and reveal divergent lineages within the genus. Maximum likelihood and Bayesian inference analyses recovered a paraphyletic *Bicoxidens* phylogram with divergent lineages present in three species – *B. friendi*, *B. flavicollis* and *B. brincki* – suggesting high genetic diversity within the genus. Bayesian genetic cluster analyses suggested the presence of multiple distinct mitochondrial lineages within the genus with four identified in *B. flavicollis* alone. It was therefore concluded that the divergent lineages observed among *Bicoxidens* populations may suggest the presence of hidden species.

Keywords: afrotropical, endemic, genitalia, hidden species, mitochondrial lineages, phylogeny

Macro-invertebrate detritivores such as millipedes play an important role in ecosystem processes (Lavelle et al. 2006). In terrestrial ecosystems millipedes contribute to vegetative litter breakdown, thereby accelerating decomposition and nutrient cycling in the soil (Hopkin and Read 1992; Suzuki et al. 2013). Estimates suggest that tropical and temperate forest-dwelling millipedes are responsible for the breakdown of 39% and 36% of the annual leaf litter, respectively (Dangerfield and Milner 1996; Cárcamo et al. 2000). Snyder and Hendrix (2008) also highlighted the potential utility of millipedes in ecological restoration programs as ecosystem health indicators. However, such studies, which aim to elucidate the role of millipedes in ecosystems, rely on accurate species richness estimates and distribution data.

Millipede species richness is estimated at 80 000 species worldwide, but to date only 15% of the estimated species richness is likely to have been described across six continents (Sierwald and Bond 2007). Millipede species descriptions have relied predominantly on the identification of differences in the male sperm-transfer genital structures known as gonopods (Hopkin and Read 1992; Sierwald and Bond 2007). Numerous studies have found that millipede genital structures evolve rapidly and divergently resulting in species specific variation, which allow species recognition (Hosken and Stockley 2004; Eberhard 2010). However, there is a more recent body of evidence which demonstrates that male genital divergence may proceed at a slower rate than genetic divergence and speciation may occur without any change in gonopod morphology (Bond et al. 2003; Adams et al. 2009; Wojcieszek and Simmons 2012). If there is a lack of conspicuous differences in male genital structures between recently divergent species, then morphologically cryptic species will have evolved. Hence, such a divergence and resultant increase in evolutionary diversity may not be detected based on gonopod morphology alone (Bond and Sierwald 2002; Jacob et al. 2004; Adams et al. 2009; Mwabvu et al. 2013).

This has implications for the inclusion of millipedes in conservation research and planning given that conservation planning requires accurate species richness estimates and distribution data. In addition, conservation prioritisation now focuses on preserving genetic diversity to ensure species survival by maximising the evolutionary potential. Hence, there is a need to elucidate levels and distribution of genetic diversity particularly in overlooked taxa such as millipedes. In this light, genetic data based on mitochondrial and nuclear DNA markers have been used to reveal cryptic species diversity and understand the spatial distribution of the genetic diversity in millipede phylogenetic and phylogeographic studies (Bond and Sierwald 2002; Walker et al. 2009; Mwabvu et al. 2013; Pimvichai et al. 2013; Nistelberger et al. 2014).

Bicoxidens Attems, 1928 is endemic to the savanna woodlands south of the Zambezi River with most known localities in Zimbabwe (Hamer et al. 2006; Mwabvu et al. 2007). The genus consists of nine described species: *B. aridis* Mwabvu, 2009, *B. brincki* Schubart, 1966, *B. flavicollis* Attems, 1928, *B. friendi* Mwabvu, 2000, *B. gokwensis* Mwabvu, 2007, *B. grandis* Lawrence, 1965, *B. matopoensis* Mwabvu, 2007, *B. nigerrimus* Attems, 1928 and *B. nyathi* Mwabvu, 2007 (Attems 1928; Lawrence 1965; Schubart 1966; Mwabvu 2000; Mwabvu et al. 2009). Based on gonopod morphology, *Bicoxidens* is monophyletic and consists of distinct species groups (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. Despite the caveat of using few samples collected from two populations, a recent molecular study reported high intraspecific genetic variation based on mitochondrial DNA in *B. flavicollis* populations (Mwabvu et al. 2013). Furthermore, an additional study that sought to recover a molecular phylogeny for seven genera within the Spirostreptidae reported that *B. flavicollis* samples occurred in two distinct clades (Mwabvu et al. 2015). Against this background, we hypothesised that there may be multiple divergent lineages within *Bicoxidens*, which suggests the presence of hidden species and this is possible given that reliance on gonopod morphology alone may fail to account for cryptic species.

Hence, the aim of the present study was to test the monophyly of *Bicoxidens* using a molecular marker and potentially reveal any divergent lineages within the genus. In order to identify divergent evolutionary lineages within the previously defined species, a molecular phylogeny was constructed based on mitochondrial DNA and the congruency between current morphological species definitions was tested. *Bicoxidens flavicollis*, the most ubiquitous species, was selected as the surrogate species to elucidate current genetic structuring of populations and spatial distribution of the genetic diversity within the genus.

Forty-eight *Bicoxidens* specimens (from eight species collected from localities in Zimbabwe and parts of western Mozambique) were utilised in this study. Fresh male specimens were collected by hand and preserved in 100% ethanol to preserve the integrity of the genetic material. Only males were collected because females do not have taxonomically useful characters that enable species identification. Sampling for most of the species was hindered by anthropogenic modifications to their known localities, some of which have become residential areas and agricultural farms. Thus, 15 specimens were loaned from the KwaZulu-Natal Museum, Pietermaritzburg (South Africa) and the Natural History Museum, Bulawayo (Zimbabwe). All of the species used were identified based on gonopod morphology by Tarombera Mwabvu. *Doratogonus* Attems, 1914 (Spirostreptidae) was selected as one outgroup taxon. Additional taxa, namely *Pachyiulus varius* (Fabricius, 1781) (Julidae) and *Tetracion tennesseensis* Causey, 1959 (Abacionidae), which are distantly related to the family Spirostreptidae, were also used to root the phylogenetic tree. Selection of outgroup taxa was also based on the availability of mitochondrial cytochrome *c* oxidase subunit 1 (CO1) sequence data in the GenBank database.

Total genomic DNA was extracted from 10 legs removed from the midbody rings of each specimen using the ZR Genomic DNA™ Tissue Miniprep kit in accordance with the manufacturer's standard protocol (Zymo Research, Irvine, CA, USA). The mitochondrial CO1 gene was amplified by PCR using the primers LCO1490 and HCO2198 (Folmer et al. 1994). The CO1 fragment was amplified using the following thermal profile: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1.5 min, and final extension at 72 °C for 10 min. The amplification reactions were performed in 25 μl reaction volumes containing 12.5 μl Econotaq® (Lucigen), 7.82 μl PCR H_2O, 1 μl bovine serum albumin, 0.84 μl (10 mM) of each primer and 2 μl DNA template. Amplification products (5 μl) were electrophoresed in 1% agarose gels to verify successful amplification of the targeted regions. Successful PCR products were sequenced through Sanger sequencing at Inqaba Biotech Industries (Pretoria, South Africa) using an Applied Biosystems 3500XL sequencer.

Sequencing of the CO1 gene was successful for all 48 specimens and nucleotide sequences were deposited in the NCBI GenBank (see Table 1). Consensus sequence alignments were carried out using BioEdit 7 (Hall 1999). Sequences for the outgroup taxa *Doratogonus* sp. (AY288738.1), *Pachyiulus* varius (JN619384.1) and *Tetracion tennesseensis* (JN656611.1) were downloaded from the NCBI GenBank database.

Based on MrModeltest 2.3 (Nylander 2004), the best-fit model of substitution appropriate for the CO1 alignment was the general time reversible with a gamma distribution (GTR+G) model. Phylogenetic analysis was conducted using both maximum likelihood and Bayesian methods. The maximum likelihood tree was estimated using GARLI 0.951 (Zwickl 2006) to conduct a heuristic search with a starting tree obtained via a neighbour-joining, branch-swapping algorithm and resampling with a 1 000 replicates. MrBayes 3.1 (Ronquist and Huelsenbeck 2003) was used for the Bayesian Inference wherein four Markov Chain Monte Carlo (MCMC) chains (three heated and one 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 1.6 (Rambaut and Drummond 2013).

A total of 32 sequences of the CO1 fragment belonging to *B. flavicollis* specimens grouped into 11 populations were used as surrogates to assess the genetic structuring of *Bicoxidens* populations. Bayesian clustering analysis was performed using Bayesian Analysis of Population Structure (BAPS) 5 (Corander et al. 2008) to evaluate the genetic structure among *B. flavicollis* populations. Mixture and admixture analysis of the samples was estimated using BAPS 5 based on five MCMC runs of 5×10^5 iterations with the first 10% discarded as burnin. The number of populations (K) was estimated from the posterior probability distribution of the data [ln $p(D)$].

Table 1: *Bicoxidens* cytochrome *c* oxidase subunit 1 (COI) sequences deposited in the GenBank database

	Laboratory code	Locality	COI
B. flavicollis Attems, 1928			
B. flavicollis-Chitombo 1	A21	Chitombo, Zimbabwe	KM982490
B. flavicollis-Chitombo 2	A22	Chitombo, Zimbabwe	KM982491
B. flavicollis-Chitombo 3	A23	Chitombo, Zimbabwe	KM982492
B. flavicollis-Chitombo 4	A24	Chitombo, Zimbabwe	KM982493
B. flavicollis-Chihota 1	B21	Chihota, Zimbabwe	KM982494
B. flavicollis-Chihota 2	B22	Chihota, Zimbabwe	KM982495
B. flavicollis-Chihota 3	B23	Chihota, Zimbabwe	KM982496
B. flavicollis-Chihota 4	B24	Chihota, Zimbabwe	KM982497
B. flavicollis-Chihota 5	B25	Chihota, Zimbabwe	KM982498
B. flavicollis-Chihota 6	B26	Chihota, Zimbabwe	KM982499
B. flavicollis-Mzinga 1	C21	Mzinga, Zimbabwe.	KM982500
B. flavicollis-Mzinga 2	C22	Mzinga, Zimbabwe.	KM982501
B. flavicollis-Mzinga 3	C23	Mzinga, Zimbabwe.	KM982502
B. flavicollis-Mzinga 4	C24	Mzinga, Zimbabwe.	KM982503
B. flavicollis-Mzinga 5	C25	Mzinga, Zimbabwe.	KM982504
B. flavicollis-Mazowe 1	F21	Mazowe, Zimbabwe	KM982513
B. flavicollis-Mazowe 2	F22	Mazowe, Zimbabwe	KM982514
B. flavicollis-Mazowe 3	F23	Mazowe, Zimbabwe	KM982515
B. flavicollis-Mazowe 4	F24	Mazowe, Zimbabwe	KM982516
B. flavicollis-Mazowe 5	F25	Mazowe, Zimbabwe	KM982517
B. flavicollis-Mazowe 6	F26	Mazowe, Zimbabwe	KM982518
B. flavicollis-Marange	G21	Marange, Zimbabwe	KM982519
B. flavicollis-Nyanga 1	H21	Nyanga, Zimbabwe	KM982520
B. flavicollis-Nyanga 2	H22	Nyanga, Zimbabwe	KM982521
B. flavicollis-Nyanga 3	H23	Nyanga, Zimbabwe	KM982522
B. flavicollis-Muterere 1	I21	Muterere, Zimbabwe	KM982523
B. flavicollis-Muterere 2	I22	Muterere, Zimbabwe	KM982524
B. flavicollis-Muterere 3	I23	Muterere, Zimbabwe	KM982525
B. flavicollis-Chegutu	J21	Chegutu, Zimbabwe	KM982526
B. flavicollis-Lumene	K21	Lumene Falls, Zimabwe	KM982527
B. flavicollis-Hovi	P21	Hovi Crossing, Zimabwe	KM982532
B. flavicollis-Maguge	U21	Maguge, Mozambique	KM982537
B. friendi Mwabvu, 2000			
*B. friendi*1	D11	Mtawatawa, Zimbabwe	KM982505
B. friendi 2	D12	Mtawatawa, Zimbabwe	KM982506
B. friendi 3	D13	Mtawatawa, Zimbabwe	KM982507
B. friendi 4	D14	Mtawatawa, Zimbabwe	KM982508
B. friendi 5	D15	Mtawatawa, Zimbabwe	KM982509
B. friendi 6	D16	Mtawatawa, Zimbabwe	KM982510
B. friendi 7	E11	Maramba curves, Zimbabwe	KM982511
B. friendi 8	E12	Maramba curves, Zimbabwe	KM982512
B. brincki Schubart, 1966			
B. brincki-Pande	L31	Pande Mine, Zimbabwe	KM982528
B. brincki-Beitbridge	Q31	Beitbridge, Zimbabwe	KM982533
B. brincki-Doddieburn	R31	Doddieburn Ranch, Zimbabwe	KM982534
B. matopoensis Mwabvu, 2007	O61	Dwala Ranch, Zimabwe	KM982531
B. nigerrimus Attems, 1928	M51	Maleme Rest Camp, Zimbabwe	KM982529
B. grandis Lawrence, 1965	N41	Chipise school, Zimabwe	KM982530
B. nyathi Mwabvu, 2007	S71	Mudziwuri River, Zimbabwe	KM982535
B. aridis Mwabvu, 2009	T91	Mushumbi Pools, Zimbabwe	KM982536

Phylogenetic tree support based on bootstrap analysis was interpreted as follows: <64 weak support; 65–89 moderate support; >90 strong support. Based on CO1 data *Bicoxidens* was paraphyletic. Three nominal species, *B. brincki*, *B. flavicollis* and *B. friendi*, also exhibited paraphyly with each of the species consisting of at least two divergent mitochondrial lineages (Figure 1). All 32 *B. flavicollis* specimens analysed occurred in four distinct and well-supported clades (clades B1, B2, B4 and C; Figure 1). Furthermore, *B. flavicollis* specimens collected from Mazowe, which is in the north of Zimbabwe, were split into two clades, B2 and B4 (Figure 1). Clade B4, which consisted of half of the *B. flavicollis*-Mazowe samples and *B friendi* 6, demonstrated the presence of separate lineages within *B. flavicollis* and *B friendi*. The positions of *B. flavicollis*-Nyanga 1 and *B. brincki*-Pande were inconsistent with the positions of conspecifics. *Bicoxidens flavicollis*-Nyanga 1 and *B. brincki*-Pande both formed a

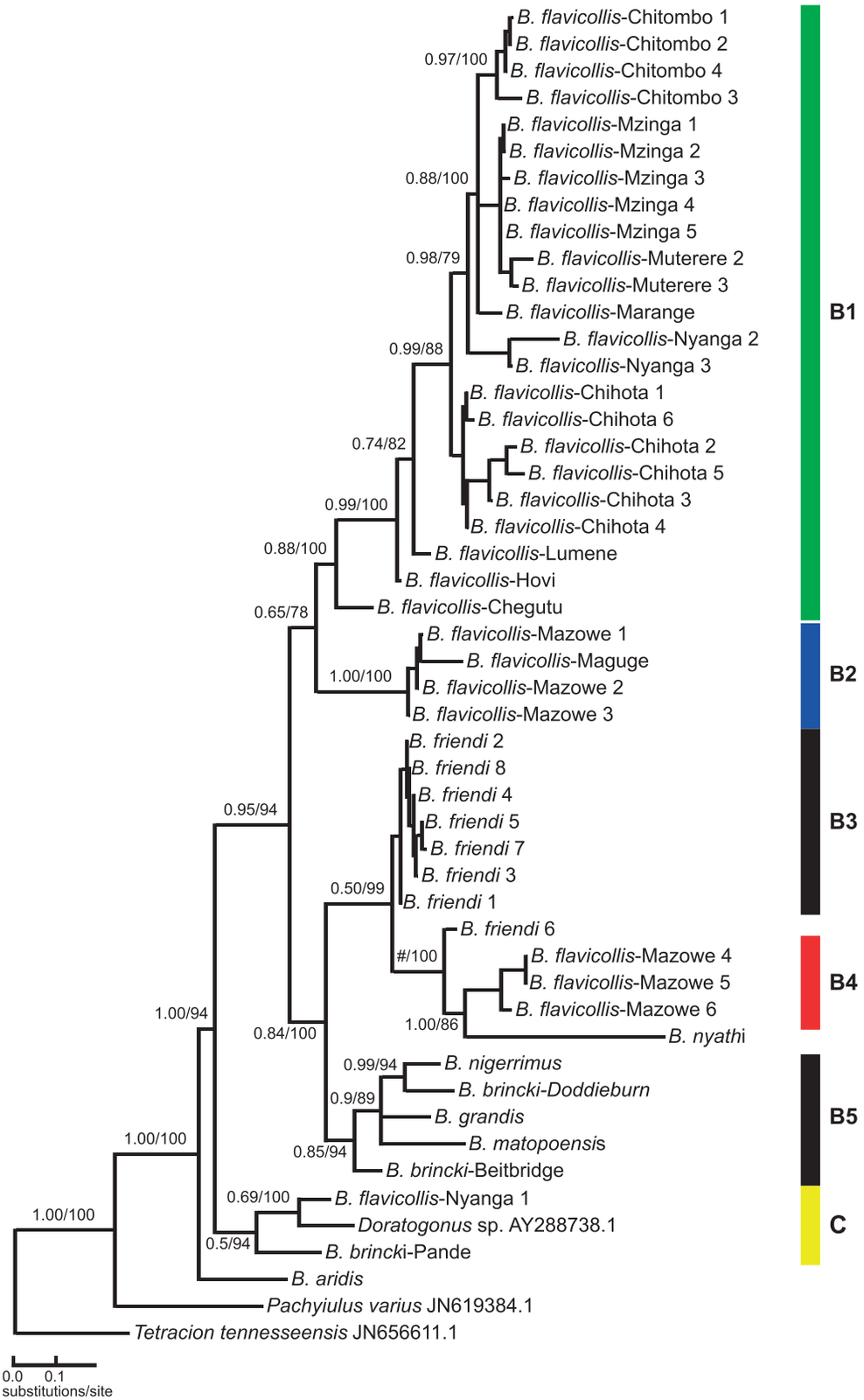

Figure 1: Phylogenetic relationships within *Bicoxidens* from Zimbabwe based on maximum likelihood (ML) analysis of the COI gene. Nodal support values indicated are the Bayesian inference posterior probability/ML bootstrap support. Coloured bars denote major clades and subclades. # indicates posterior probabilities that were less than 0.5. Branch labels include species name, locality and replicate number

strongly supported clade (Clade C) with the outgroup taxon *Doratogonus* sp. (Figure 1).

Bayesian analysis revealed four distinct mitochondrial lineages among the 11 *Bicoxidens flavicollis* populations based on CO1 data (Figure 2). The first genetic lineage consisted of half of the population at Mazowe (red in Figure 2) and the second lineage constituted the second half of the population at Mazowe together with the population at Maguge (blue in Figure 2). One-third of the Nyanga population formed the third cluster, whereas the remainder of the populations at Chihota, Chitombo, Mzinga, Muterere, Hovi, Lumene, Chegutu and Marange formed the largest lineage (green in Figure 2). Members of the populations at Mazowe as well as Nyanga were split between two genetic clusters (Figure 2). There was no evidence of admixture, suggesting that none of the individuals in this data set have mixed ancestry.

Previous studies have only reported the presence of high genetic divergence among two populations of *B. flavicollis* (Mwabvu et al. 2013, 2015). This study demonstrates that the presence of divergent mitochondrial lineages is not unique to *B. flavicollis* alone but is also apparent in *B. brincki* and *B. friendi*. For taxa such as millipedes, the presence of divergent mitochondrial lineages despite the absence of discrete variation in gonopod morphology often suggests the presence of hidden diversity (Bond and Sierwald 2002; Bond et al. 2003). Furthermore, the present study indicates the existence of four distinct mitochondrial lineages within *B. flavicollis* populations, which is previously unreported. The presence of multiple mitochondrial lineages within *Bicoxidens* may be misconstrued as an overestimation resulting from our reliance on data from the mitochondrial CO1 region, whose advantages and disadvantages are documented in Silva and Willows-Munro (2016). However, we argue that cryptic lineage diversity is a common phenomenon in soil-dwelling endemics with low dispersal abilities, such as millipedes, and this is supported by studies on ants (Norman et al. 2016), springtails (Porco et al. 2012; Zhang et al. 2014), earthworms (Novo et al. 2010; Shekhovtsov et al. 2016) and centipedes (Wesener et al. 2016). Hence, we attribute our findings to the fact that *Bicoxidens* follows a diversification pattern similar to other soil-dwelling invertebrates and this can either be further validated or dispelled by data from a nuclear region.

Divergent genetic lineages are more likely expected among short-range endemic populations. Short-range endemic populations within other millipede taxa have been characterised by low vagility, microhabitat specialisation and an inherent tendency to be isolated geographically, influencing genetic divergence (Hamer et al. 2006; Moir et al. 2009). Furthermore, persistence of multiple isolated refugia has been reported to be the driving force behind high

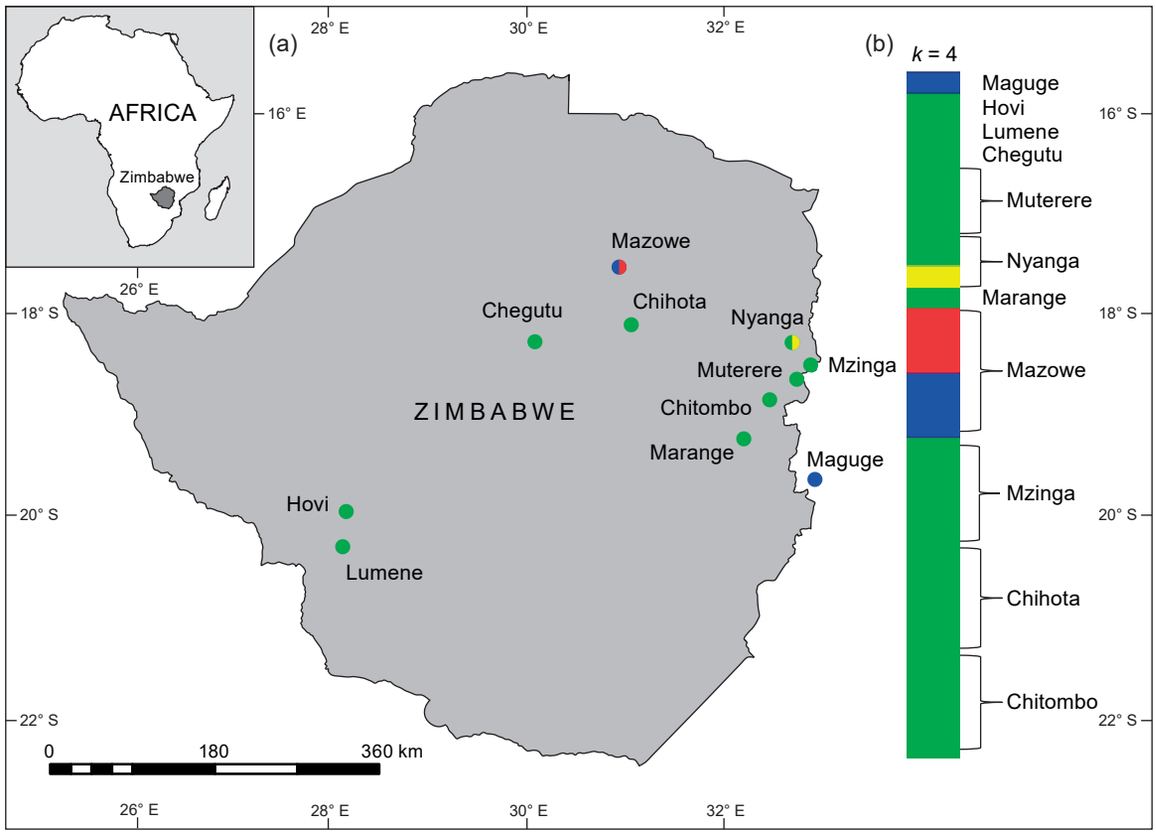

Figure 2: Assignment of *Bicoxidens flavicollis* individuals to genetic clusters and the geographic distribution of the genetic clusters across sampling sites. (a) Spatial distribution of the genetic clusters across sampling sites. (b) Bayesian genetic clustering among *B. flavicollis* individuals ($k = 4$) where each colour represents a genetic cluster

genetic diversification within several millipede taxa, which include *Aphistogoniulus* (Wesener et al. 2011), *Atelomastix bamfordi* (Nistelberger et al. 2014), *Pogonosternum* (Decker 2016b) and *Somethus* (Decker 2016a).

Bicoxidens members generally have a strict preference for humid microclimate habitats (Mwabvu et al. 2007). Changes in climate during the Pleistocene era across Africa (Nicholson and Flohn 1980) may have led to the constriction of the forests and woodlands into isolated patches, thereby fragmenting *Bicoxidens* populations. Subsequently, the isolated populations may have diverged genetically due to genetic drift and possible local adaptation leading to allopatric speciation.

Divergent mitochondrial lineages in *Bicoxidens* species were also apparent in populations at the same locality. *Bicoxidens flavicollis* populations at Mazowe and Nyanga and a *B. friendi* population at Mtawatawa exhibited high genetic divergence at population level despite similarity in gonopod morphology. For soil-dwelling invertebrates, divergent mitochondrial lineages that occur in sympatry can either be an indication of the presence of cryptic species (Shekhovtsov et al. 2016) or a highly polymorphic species (Giska et al. 2015). In millipedes, Bond and Sierwald (2002) uncovered cryptic species after observing divergent lineages within the *Anadenobolus excicus* Karsch, 1881 species complex that occurred in sympatry.

Furthermore, evidence from a study on earthworms (Jones et al. 2016) suggests that the establishment of polymorphisms and the evolution of reproductive isolation through mechanical or behavioural barriers to reproduction may lead to such genetic divergence within *Bicoxidens* populations. Therefore, sympatric divergent lineages observed within *B. flavicollis* populations at Mazowe and Nyanga can be attributed to secondary contact after divergence in allopatry, an explanation that is supported by Sechi (2013). Reproductive isolation barriers may have developed while the *Bicoxidens* populations were separated allowing two or more divergent genetic lineages to persist in sympatry. However, further investigations are required to demonstrate reproductive isolation and establish the exact mechanisms by which the divergent lineages observed in *Bicoxidens* species have become established and continue to persist. Such studies would also need to use nuclear data and include greater population-level sampling.

The present study is one of the few molecular phylogenetic studies that have focused on an entire millipede genus from southern Africa. The results suggest a high prevalence of divergent mitochondrial lineages within *Bicoxidens*. Furthermore, there is high local genetic diversity as well as substantial geographic structuring of the genetic diversity in *Bicoxidens* species. Both allopatric and sympatric speciation mechanisms may be at the core of the cryptic species diversity and speciation within *Bicoxidens*. Based on the present study, future molecular taxonomic revisions of other Spirostreptidae genera are warranted. The results of the study highlight the need for conservation management strategies to focus on preserving the unique genetic diversity in short-range endemics. Failure to preserve such genetic diversity may have a negative impact on the ability of the taxa to adapt to fluctuating environmental conditions, increasing their risk of extinction.

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References

Adams DC, Berns CM, Kozak KH ,Wiens JJ. 2009. Are rates of species diversification correlated with rates of morphological evolution? *Proceedings of the Royal Society B: Biological Sciences* 276: 2729–2738.

Attems C. 1928. The Myriapoda of South Africa. *Annals of the South African Museum* 26: 1–431.

Bond JE, Beamer DA, Hedin MC, Sierwald P. 2003. Gradual evolution of male genitalia in a sibling species complex of millipedes (Diplopoda: Spirobolida: Rhinocricidae: *Anadenobolus). Invertebrate Systematics* 17: 711–717.

Bond JE, Sierwald P. 2002. Cryptic speciation in the *Anadenobolus excisus* millipede species complex on the Island of Jamaica. *Evolution* 56: 1123–1135.

Cárcamo H, Abe T, Prescott C, Holl F ,Chanway C. 2000. Influence of millipedes on litter decomposition, N mineralization, and microbial communities in a coastal forest in British Columbia, Canada. *Canadian Journal of Forest Research* 30: 817–826.

Corander J, Marttinen P, Sirén J, Tang J. 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics* 9: 539.

Dangerfield J, Milner A. 1996. Millipede fecal pellet production in selected natural and managed habitats of southern Africa: implications for litter dynamics. *Biotropica* 28: 113–120.

Decker P. 2016a. Integrative taxonomic revision of the polymorphic flat-millipede genera *Oncocladosoma* and *Somethus* in South Australia (Diplopoda: Polydesmida: Paradoxosomatidae). *Invertebrate Systematics* 30: 201–218.

Decker P. 2016b. Phylogenetic analysis of the Australian trans-Bass Strait millipede genus *Pogonosternum* (Carl, 1912) (Diplopoda, Polydesmida, Paradoxosomatidae) indicates multiple glacial refugia in southeastern Australia. *ZooKeys* 578: 15–31.

Eberhard WG. 2010. Evolution of genitalia: theories, evidence, and new directions. *Genetica* 138: 5–18.

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

Giska I, Sechi P, Babik W. 2015. Deeply divergent sympatric mitochondrial lineages of the earthworm *Lumbricus rubellus* are not reproductively isolated. *BMC Evolutionary Biology* 15: 217.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

Hamer M, Slotow R, Lovell S. 2006. The South African savanna millipede (Diplopoda) fauna: taxonomic diversity, endemism spatial and temporal effects on conservation assessments. *Norwegian Journal of Entomology* 53: 321–334.

Hopkin PS, Read JH. 1992. *Biology of millipedes*. New York: Oxford University Press.

Hosken DJ, Stockley P. 2004. Sexual selection and genital evolution. *Trends in Ecology and Evolution* 19: 87–93.

Jacob A, Gantenbein B, Braunwalder ME, Nentwig W, Kropf C. 2004. Complex male genitalia (hemispermatophores) are not diagnostic for cryptic species in the genus *Euscorpius* (Scorpiones: Euscorpiidae). *Organisms Diversity and Evolution* 4: 59–72.

Jones G, Wills A, Morgan A, Thomas R, Kille P, Novo M. 2016. The worm has turned: behavioural drivers of reproductive isolation between cryptic lineages. *Soil Biology and Biochemistry* 98: 11–17.

Lavelle P, Decaëns T, Aubert M, Barot S, Blouin M, Bureau F, Margerie P, Mora P, Rossi J-P. 2006. Soil invertebrates and ecosystem services. *European Journal of Soil Biology* 42: S3–S15.

Lawrence RF. 1965. New Spirostreptidae and Harpagophoridae (Diplopoda) from southern Africa. *Memórias do Instituto de investigaçao cientifica de Moçambique serie A* 7: 38–39.

Moir ML, Brennan KEC, Harvey MS. 2009. Diversity, endemism and species turnover of millipedes within the south–western Australian global biodiversity hotspot. *Journal of Biogeography* 36: 1958–1971.

Mwabvu T. 2000. Two new species of *Bicoxidens* Attems, 1928, from Zimbabwe (Diplopoda: Spirostreptida: Spirostreptidae). *African Zoology* 35: 115–119.

Mwabvu T, Hamer ML, Slotow RH. 2007. A taxonomic review of the southern African millipede genus, *Bicoxidens* Attems, 1928 (Diplopoda: Spirostreptida: Spirostreptidae), with the description of three new species and a tentative phylogeny. *Zootaxa* 1452: 1–23.

Mwabvu T, Hamer M, Slotow R. 2009. A new species of *Bicoxidens* Attems, 1928 (Diplopoda, Spirostreptida, Spirostreptidae) from northern Zimbabwe. *ZooKeys* 7: 75–81.

Mwabvu T, Lamb J, Slotow R, Hamer M, Barraclough D. 2013. Is millipede taxonomy based on gonopod morphology too inclusive? Observations on genetic variation and cryptic speciation in *Bicoxidens flavicollis* (Diplopoda: Spirostreptida: Spirostreptidae). *African Invertebrates* 54: 349–356.

Mwabvu T, Lamb J, Slotow R, Hamer M, Barraclough D. 2015. Do cytochrome *c* oxidase 1 gene sequences differentiate species of spirostreptid millipedes (Diplopoda: Spirostreptida: Spirostreptidae)? *African Invertebrates* 56: 651–661.

Nicholson SE, Flohn H. 1980. African environmental and climatic changes and the general atmospheric circulation in late Pleistocene and Holocene. *Climatic Change* 2: 313–348.

Nistelberger H, Byrne M, Coates D, Roberts JD. 2014. Strong phylogeographic structure in a millipede indicates Pleistocene vicariance between populations on banded iron formations in semi-arid Australia. *PLoS ONE* 9: e93038.

Norman V, Darras H, Tranter C, Aron S, Hughes WO. 2016. Cryptic lineages hybridize for worker production in the harvester ant *Messor barbarus. Biology Letters* 12: 20160542.

Novo M, Almodóvar A, Fernández R, Trigo D ,Cosín DJD. 2010. Cryptic speciation of hormogastrid earthworms revealed by mitochondrial and nuclear data. *Molecular Phylogenetics and Evolution* 56: 507–512.

Nylander J. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Pimvichai P, Enghoff H ,Panha S. 2013. Molecular phylogeny of the *Thyropygus allevatus* group of giant millipedes and some closely related groups. *Molecular Phylogenetics and Evolution* 71: 170–183.

Porco D, Potapov M, Bedos A, Busmachiu G, Weiner WM, Hamra-Kroua S, Deharveng L. 2012. Cryptic diversity in the ubiquist species *Parisotoma notabilis* (Collembola, Isotomidae): a long-used chimeric species? *PLoS ONE* 7: e46056.

Rambaut A, Drummond A. 2013. Tracer 1.6. University of Edinburgh, Edinburgh, UK.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Schubart VO. 1966. *Diplopoda III*: *Pselaphognatha*, *Opisthospermophora, Colobognatha*. Stockholm: Almqvist and Wilksell.

Sechi P. 2013. An evolutionary history of the peregrine epigeic earthworm *Lumbricus rubellus*. PhD thesis, Cardiff University, UK.

Shekhovtsov S, Berman D, Bazarova N, Bulakhova N, Porco D, Peltek S. 2016. Cryptic genetic lineages in *Eisenia nordenskioldi pallida* (Oligochaeta, Lumbricidae). *European Journal of Soil Biology* 75: 151–156.

Sierwald P, Bond JE. 2007. Current Status of the Myriapod Class Diplopoda (Millipedes): taxonomic diversity and phylogeny. *Annual Review of Entomology* 52: 401–420.

Silva JMD, Willows-Munro S. 2016. A review of over a decade of DNA barcoding in South Africa: a faunal perspective. *African Zoology* 51: 1–12.

Snyder BA, Hendrix PF. 2008. Current and potential roles of soil macroinvertebrates (earthworms, millipedes, and isopods) in ecological restoration. *Restoration Ecology* 16: 629–636.

Suzuki Y, Grayston SJ, Prescott CE. 2013. Effects of leaf litter consumption by millipedes *Harpaphe haydeniana* on subsequent decomposition depends on litter type. *Soil Biology and Biochemistry* 57: 116–123.

Walker MJ, Stockman AK, Marek PE, Bond JE. 2009. Pleistocene glacial refugia across the Appalachian Mountains and coastal plain in the millipede genus *Narceus*: evidence from population genetic, phylogeographic, and paleoclimatic data. *BMC Evolutionary Biology* 9: 25.

Wesener T, Raupach MJ, Decker P. 2011. Mountain refugia play a role in soil arthropod speciation on Madagascar: a case study of the endemic giant fire-millipede genus *Aphistogoniulus*. *PLoS ONE* 6: e28035.

Wesener T, Voigtländer K, Decker P, Oeyen JP, Spelda J. 2016. Barcoding of Central European *Cryptops centipedes* reveals large interspecific distances with ghost lineages and new species records from Germany and Austria (Chilopoda, Scolopendromorpha). *ZooKeys* 564: 21–46.

Wojcieszek JM, Simmons LW. 2012. Evidence for stabilizing selection and slow divergent evolution of male genitalia in a millipede (*Antichiropus variabilis*). *Evolution* 66: 1138–1153.

Zhang F, Yu D, Luo Y, Ho SY, Wang B, Zhu C. 2014. Cryptic diversity, diversification and vicariance in two species complexes of *Tomocerus* (Collembola, Tomoceridae) from China. *Zoologica Scripta* 43: 393–404.

Zwickl D. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD thesis, University of Texas, Austin, Texas, USA.

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