The utilisation of CytB and COI barcodes for the identification of bloodmeals and *Culicoides* species (Diptera: Ceratopogonidae) reveals a variety of novel wildlife hosts in South Africa

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Highlights

- *Culicoides* midges are haematophagous vectors of economic and health importance.
- Hosts are attacked opportunistically and selection may depend on host availability.
- A variety of wildlife species are recorded as novel blood hosts.
- *Culicoides* are possibly transfer vectors of zoonosis.

Abstract

Biting midges in the genus *Culicoides* (Diptera; Ceratopogonidae) are vectors of pathogens that can cause diseases of major economic importance in humans and animals. Identifying host ranges of these biting midges might aid in understanding the complex epidemiology of such diseases, often involving reservoir hosts and multiple species. In this study, we aim to identify bloodmeal origin from engorged female *Culicoides* biting midges. All bloodfed females were opportunistically collected as part of an ongoing surveillance programme using Onderstepoort light traps in two provinces in South Africa. DNA of individuals was extracted and subjected to PCR targeting the *cytochrome B* (CytB) gene region of mammals and avians as well as *cytochrome oxidase I* (COI) for species identification. In total, 21 new reference barcodes were generated for *C. bedfordi, C imicola, C. leucosticus, C. magnus*, and *C. pycnostictus*. Seventy-four blood meals were identified, originating from 12 mammal and three avian species. COI sequence data performed well for species delimitation and 54 *Culicoides* specimens were identified with *C. imicola* the predominant species identified (41.8%). Generally, *Culicoides* species feed on a variety of hosts and host availability might be an important factor when selecting a host. *Culicoides* species thus appear to be opportunistic feeders rather than specialists. This implicates *Culicoides* as transfer vectors and demonstrates possible transmission routes of arboviruses and other pathogens from wildlife onwards to domestic animals and humans.

Keywords: Vector; Haematophagy; Arbovirus; Reservoir host; DNA barcode; Host preference; Zoonosis

1. Introduction

Various taxa of haematophagy flies (Diptera) are established vectors of pathogens that may cause medical and veterinary important diseases with substantial economic consequences (Braack et al., 2018; Gebresilassie et al., 2015; Riddin et al., 2019). *Culicoides* Latreille, 1809 (Ceratopogonidae) midges are perhaps best known for their role in perpetuating the transmission cycles of bluetongue virus (BTV) (Martinez-de la Puente et al., 2017; Steyn et al., 2016), African horse sickness virus (AHSV) (Guichard et al., 2014; Martinez-de la Puente et al., 2017) and equine encephalosis virus (EEV) (Barnard and Paweska, 1993; Snyman et al., 2021), however, these midges have been incriminated in transmission of viruses of the genera *Flavivirus, Alphavirus, Orthobunyavirus, Nairovirus, Phlebovirus* and *Orbivirus* (Carpenter et al., 2013; Martínez-de la Puente et al., 2015). They might therefore not only play an important role in the transmission of pathogens among humans, livestock, and wildlife, but between these taxa as well (Martinez-de la Puente et al., 2017). *Culicoides* is a diverse genus, comprising over 1 300 species, ranging between 1-3 mm in size with a near worldwide distribution, excluding only New Zealand, the Hawaiian Islands and the extreme Polar Regions (Borkent, 2017; Mellor et al., 2000). Of these, at least 120 species occur in southern Africa of which 105 have been recorded in South Africa with 10-20 species regularly recorded near livestock (Labuschagne, 2015; Meiswinkel et al., 2004).

Determining the feeding patterns and feeding ranges of *Culicoides* biting midges will be essential for determining host preference which in turn might aid in understanding the role of the midges in disease transmission (Bessell et al., 2014; England et al., 2020; Martinez-de la Puente et al., 2017; Mullens et al., 2004). Host choice is a critical determinant of the intensity at which pathogens are transmitted (Bobeva et al., 2015; Jupp and McIntosh, 1967). Due to the economic impact of viruses such as bluetongue virus (BTV) and African horse sickness (AHSV) (Grewar, 2016), both transmitted by *Culicoides* species, most studies have been focused on the agricultural sector and the surrounding matrix where domestic animals are the primary bloodmeal source (Nevill et al., 1988; Riddin et al., 2019; Venter et al., 2012). Wildlife species may, however, act as cycling hosts of arboviruses providing a constant source of pathogens that may spill over to livestock and/or humans (Barnard and Paweska, 1993; Haydon et al., 2002; Snyman, 2021). In the Afrotropical region, wildlife is thought to play a central role in pathogen dynamics, including viruses transmitted by *Culicoides* species, such as BTV, AHSV and equine encephalosis virus (EEV) (Barnard and Paweska, 1993; Barnard, 1997). Despite this, little is known about wildlife as potential blood hosts of *Culicoides* species and thus the possible spillover of pathogens from wildlife to domestic animals and/or humans. Several studies have contributed to elucidate aspects of host range utilisation of *Culicoides* in Europe (see review, Martínez-de la Puente et al., 2015), however, host range data in the Afrotropical region are still mainly restricted to livestock (Braverman and Phelps, 1981; Nevill et al., 1988; Nevill and Anderson, 1972; Riddin et al., 2019; Swanson and Turnbull, 2014).

This study aims to contribute to our knowledge of blood hosts used by *Culicoides* species by analysing bloodmeals from engorged female midges collected in or near wildlife conservancies in South Africa, in order to implicate *Culicoides* species as potential transfer vectors of zoonotic arboviruses.

2. Material and methods

2.1. Study sites and *Culicoides* sampling

Engorged female *Culicoides* midges were opportunistically sampled from collections made for an ongoing arbovirus surveillance programme from four localities in South Africa, two sites in Limpopo Province (hereafter Limpopo) and five sites in Mpumalanga Province (hereafter Mpumalanga) (Fig. 1). Established *Culicoides* trapping procedure using 220 V Onderstepoort light traps were employed per site (Venter et al., 2009). Sampling took place for at least two days per month for the duration of 2015-2017 using one trap per site, running from approximately 17h00 to 08h00, hanging less than 2 m from ground level. The traps were hung as close as possible, never exceeding a few meters, from the boundary fence of the accommodation areas within Marekele National Park (MNP), Lapalala Wilderness (LW), Hans Hoheisen Wildlife Research Station (HHWRS) and Manyeleti Game Reserve, with the latter two representing Kruger National Park (KNP). Three additional sites in the rural community of Mnisi, northeastern Bushbuck Ridge, were selected due to their proximity to wildlife and the reliance of the community on livestock farming.

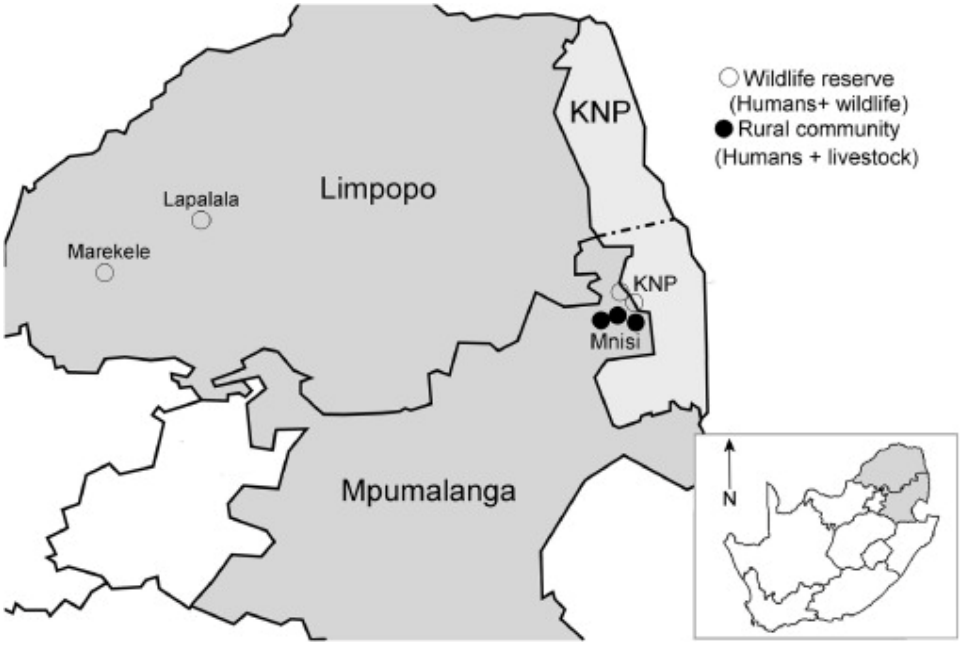

Fig. 1. Sampling sites in wildlife conservancies and rural communities adjacent to the conservancies monitored two days per month for 2015-2017. Mnisi comprised three pooled sites (Hluvukani, Ludlow and Hlalakahle), Kruger National Park comprised two pooled sites (HHWRS and Manyeleti game reserve). Open circles: Wildlife areas; Black circles: Rural farming communities. Abbreviations: KNP: Kruger National Park; HHWRS: Hans Hoheisen Wildlife Research Station; Lalapala: Lapalala Wilderness; Marekele: Marekele National Park.

Culicoides midges were separated from other insects using a light microscope. Midges were divided into pools of 100 to 500 individuals as part of the virus surveillance programme. Freshly blood-fed female midges were separated from the rest of the midges and stored in individually labelled Eppendorf tubes at -80 °C until molecular processing. The engorged females were not morphologically identified since the programme was not reliant on species identification. Subsamples (10% of pooled sample comprising 500 specimens) from a

few randomly selected pools from each locality were morphologically identified (Labuschagne (2015) to species level to establish a baseline of species composition at the surveillance sites (Supplementary metadata A).

2.2. DNA extraction and processing

The DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendation with minor changes, was used for DNA extraction. In short, stored bloodfed females were thawed to room temperature and placed into a new 1.5 ml Eppendorf tube with 180 µl lyses buffer (buffer ATL) and 20µl proteinase K. Midges were then homogenized using the Qiagen TissueLyzer™ and glass beads for 3 minutes at 300 rpm. The suspensions were incubated over night at 56 °C (Lassen et al., 2011). Buffer AL was added, and samples were incubated again for 10 min. DNeasy Mini Spin columns were then used to wash (x2) and elute the DNA to 200 µl with Buffer AE.

Molecular identification of the bloodmeals from individual bloodfed *Culicoides* females were determined using Cytochrome B (CytB) mammal and avian specific primer sets producing fragments of 727 base pairs (bp) and 518 bp respectively (Cicero and Johnson, 2001; Ngo and Kramer, 2009) (Table 1). All extractions were subjected to both mammal and avian protocols in separate reactions. *Culicoides* individuals that produced positive bloodmeal host data were identified to species level using universal primers amplifying a 710 base pair (bp) fragment of the subunit I of the cytochrome oxidase (COI) (Folmer et al., 1994) (Table 1). PCR reactions consisted of 20 µM of each primer, 10 mM dNTPs and 10 µl DNA with Phusion® High Fidelity DNA Polymerase (ThermoFisher Scientific™) to produce a total volume of 50 µl. CytB reactions were subjected to 95 °C for 10 min followed by 36 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s with a final extension of 72 °C for 7 min using a Termocycler (Applied Biosystems™). PCR conditions for COI were similar to the CytB assay except for the annealing temperature of 48-50 °C. A re-amplification PCR was performed on samples yielding faint gel electrophoreses bands in 2% Agarose stained with ethidium bromide (120 V, 40 min), or for inadequate sequencing results. Re-amplification PCR conditions were kept the same, but the primer concentration was reduced to 10 µM per primer. PCR amplification success rate was calculated by the number of samples that yielded sequence data divided by the total number of samples subjected to PCR.

Table 1. Primer names, sequences and references for *Culicoides* bloodmeal analysis and species identification.

Gene fragment and primer	**Primer sequence (5'-3')**	**Reference**
Cytochrome B		
Mammal (F)	CGAAGCTTGATATGAAAAACCATCGTTG	Ngo and Kramer (2009)
Mammal (R)	TGTAGTTRTCWGGGTCHCCTA	
Avian (F)	GACTGTGACAAAATCCCNTTCCA	Cicero and Johnson (2001)
Avian (R)	GGTCTTCATCTYHGGYTTACAAGAC	
Cytochrome oxidase I		
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HC02198	TAAACTTCAGGGTGACCAAAAAATCA	

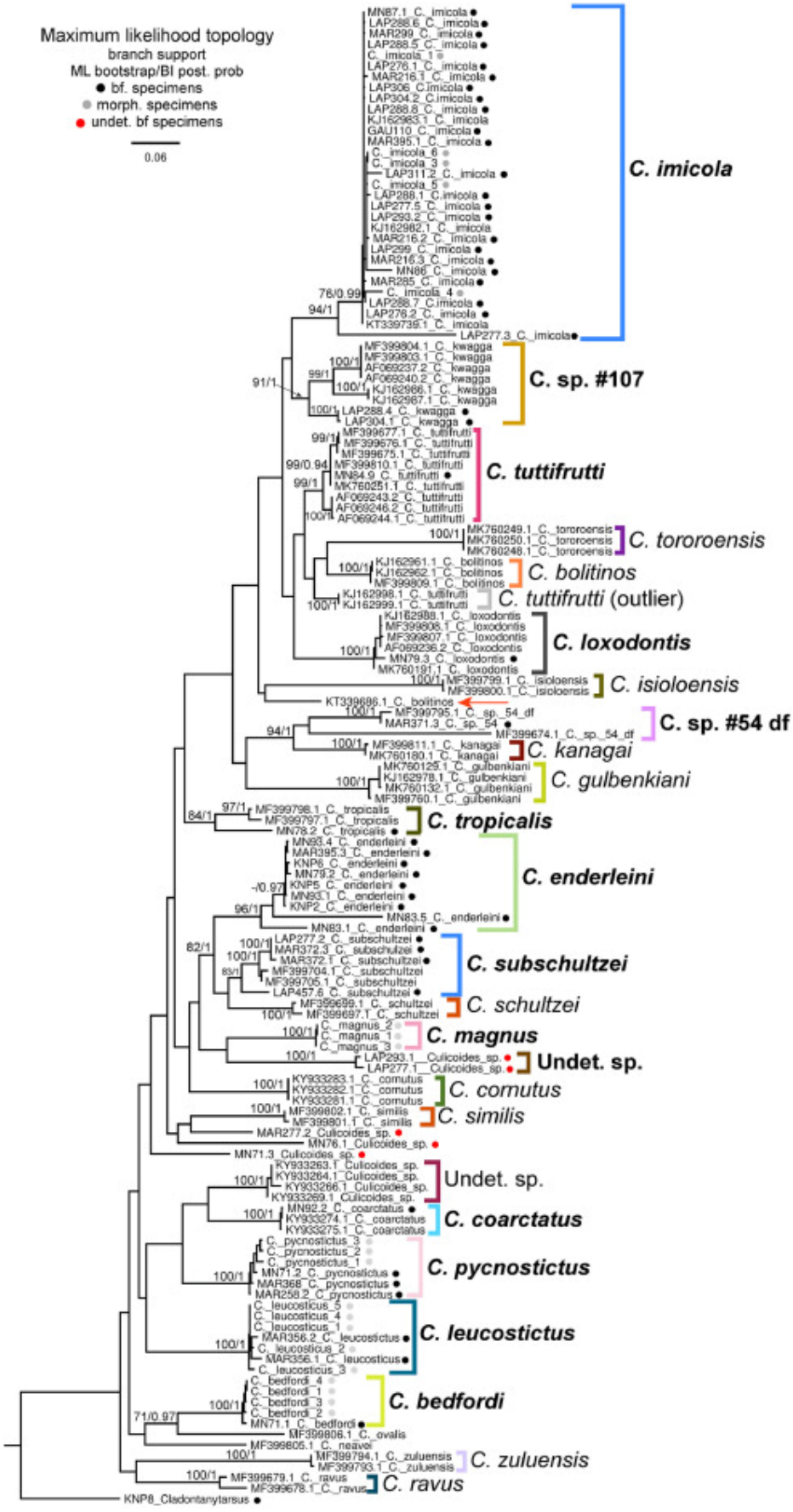
Maximum likelihood topology
branch support
ML bootstrap/BI post. prob
bf. specimens
morph. specimens
undet. bf specimens
0.06
C. imicola
C. sp. #107
C. tuttifrutti
C. tororoensis
C. bolitinos
C. tuttifrutti (outlier)
C. loxodontis
C. isioloensis
C. sp. #54 df
C. kanagai
C. gulbenkiani
C. tropicalis
C. enderleini
C. subschultzei
C. schultzei
C. magnus
Undet. sp.
C. cornutus
C. similis
Undet. sp.
C. coarctatus
C. pycnostictus
C. leucostictus
C. bedfordi
C. ovalis
C. zuluensis
C. ravus
KNP8_Cladontanytarsus

Fig. 2. Maximum likelihood phylogeny generated from COI sequence data including branch support from ML analysis (bs) and Bayesian inference (pp). Black dots indicate bloodfed midges successfully identified, red dots indicate bloodfed midges not successfully identified, grey dots indicate new reference COI barcodes from morphologically identified specimens (Accession numbers MN329588- MN329662). Bootstrap support greater than 75 and posterior probabilities greater than 0.95 are displayed on the branches. Arrow points to a reference specimen possibly with an erroneous identity.

Amplicons of the correct sizes *i.e.* 727 bp for mammals, 518 bp for avian and 710 bp for COI were excised using a ultraviolet (UV) case and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, California, USA) according to the manufacturer's instructions with an extra centrifuge step after the second wash and addition of heated (37 °C) elution buffer. Two µl of purified product was viewed on a 2% gel to measure the quantity of the product. Purified products were then sent to Inqaba Biotec™ (Pretoria, South Africa) for Sanger sequencing of both the forward and reverse strands.

PCRs were validated and optimized using known mammal (cattle, lion, buffalo, sable, goat, wild dog, vervet monkey, nyala, and human), avian (ostrich, cape vulture, and white back vulture) obtained through the arbovirus syndromic surveillance program in the Zoonotic arbo and respiratory virus program (ZARV), Department Medical Virology, University of Pretoria and *Culicoides* species as controls (*Culicoides imicola, C. bedfordi, C. leucosticus, C. magnus, C. pycnostictus,* Fig. 2, indicated by filled grey circles). All COI sequences are available on GenBank (Accession numbers MN329588- MN329662).

2.3. Phylogenetic analysis

All sequences were viewed and assembled using CLC Main workbench version 8.0.1 (https://www.qiagenbioinformatics.com). All assembled sequences were compared to the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). Values greater than 97% were considered significant and identification reported on (Murugan et al., 2016; Slama et al., 2015).

Assembled COI sequences were added to relevant sequences from Afrotropical *Culicoides* species downloaded from GenBank (Figure 2, supplementary data) and aligned using the online version of MAFFT (https://mafft.cbrc.jp/alignment/server/) with default parameters. The aligned matrices were viewed, edited and truncated in MEGA 7 (Kumar et al., 2016).

Splitstree 4 was used to generate a data-display network (DDN) using all characters and uncorrected p-distances (Huson and Bryant, 2006) with bootstrap support calculated from 1 000 replicates. A maximum likelihood (ML) analysis on the same aligned matrix was performed in RAxML version 8 using a GTR+G model with the autoMRE function invoked for the calculation of bootstrap support (Stamatakis, 2014). A Bayesian approach for phylogenetic inference was conducted in MrBayes version 3 (Ronquist and Huelsenbeck, 2003). Model TIM+I+G, as estimated by jModeltest 2 (Darriba et al., 2012) using AI criteria via the CIPRES gateway (www.phylo.org) was employed in the Bayesian analysis. Four simultaneous cold Monte-Carlo Markov Chains searched for five million generations, with each 1 000th tree sampled. The first 10% of the sampled trees were discarded as burn-in for the calculation of the posterior probabilities (pp). Effective sample

size was calculated and viewed using Tracer version 1.6 with ESS values greater than 200 considered to be adequate (Rambaut et al., 2014).

3. Results

The mean number of *Culicoides* specimens caught per trap per trapping night was highest in Mnisi ($(\bar{x})_n$ = 836), followed by KNP ($(\bar{x})_n$ = 580), Marekele($(\bar{x})_n$ = 567) and lowest in Lapalala ($(\bar{x})_n$ = 285). A total of 142 bloodfed females were sampled, with the most originating from KNP (n = 49) with similar numbers from Lapalala (n = 33), Marekele (n = 30) and Mnisi (n = 30) (Supplementary Table SM 2). A total of 35 *Culicoides* species were identified during the course of this study across all sites using COI sequencing and morphological subsamples. Thirty-one of the 35 species were present in the Mnisi area, followed by 22 species recorded in KNP, 14 in Marekele and lastly 10 species recorded in LW. Nineteen species of the 35 species were only present in the morphological subsamples, seven species were only present as bloodfed individuals and not present in the morphological subsamples and finally, nine species were present as both bloodfed individuals and in the morphological subsamples (Table 2, black circles). It should be noted that while C. sp. #54 (d/f) and *C. subschultzei* were present in both molecular and morphological samples, the were not simultaneously present at the same locality.

Table 2. Species per site either morphologically identified from subsamples (filled black circle) or using molecular inference from COI data (open circles).

Culicoides species	**KNP**	**Mnisi**	**MNP**	**LW**
C. albopunctatus	●	●		
C. bedfordi		○		
C. bolitinos	●	●	●	●
C. coarctatus		○		
C. enderleini	●○	●○	●○	●
C. eriodendroni	●	●		
C. exspectator	●	●	●	
C. imicola	●○	●○	●○	●○
C. kanagai		●		`
C. leucosticus	●	●	●○	●
C. loxodontis	●○	●		
C. neavei		●		
C. nevilli		●		
C. nigeriae	●	●		
C. nigripennis grp	●	●		
C. nivosus	●	●	●	
C. olyslageri	●	●		
C. pretoriensis	●	●		●
C. punctithorax	●	●		
C. pycnosticus	●	●○	●○	●
C. ravus	●	●	●	
C. schultzei		●	●	
C. similis	●	●	●	

Culicoides species	**KNP**	**Mnisi**	**MNP**	**LW**
C. sp. #50			●	●
C. sp. #54 (d/f)	●	●	○	
C. sp. # 61	●	●		
C. sp. #107				○
C. subschultzei	●	●	○	○
C. tropicalis	●	●○		
C. tuttifrutti		●○		
C. walkeri	●	●		
Undet sp.[1]				○
Undet sp.[2]			○	
Undet sp.[3]		○		
Undet sp.[4]		○		
Chironomidae sp.[5]				
Total ●	22	27	11	7
Total ○	3[5]	9	7	4
Grand total	22	31	14	10

KNP: Kruger National Park; MNP: Marekele National Park; LW: Lapalala Wilderness;

Undetermined specimens: LAP277.1[1], LAP293.1[1]: MAR277.2[2]; MN76.1[3]; MN71.3[4]; Not counted[5]

The KNP samples had the lowest PCR amplification success rate, with only 24.5% and 18.4% of bloodfed specimens yielding CytB and COI sequences respectively. Marekele samples had higher success rates with 53.3% and 50% of samples yielding successful CytB and COI sequences respectively. Lapalala in turn had 63.6% and 60.6% CytB and COI success, respectively. Mnisi samples had a high success rate for CytB amplification (83.3%), but very low success when COI was targeted (33.3%) (Supp. Table SM2).

Identification of vertebrate hosts via CytB BLAST revealed five, four, six and five hosts used by *Culicoides* species in KNP, Mnisi, Marekele and Lapalala respectively for a total of 15 different hosts across all sites. The hosts comprised of 12 different mammal species, of which cattle were the most prominent (31.3%) followed by human and wildebeest (both 11.3%) and three different avian species (*Turdus libonyana* (Kurrichane thrush), *Cisticola* spp (Warblers), *Tockus leucomelas* (Southern yellow-billed hornbill)). In collections where more than one female of a species was tested, host blood meal differed by up to four different hosts (Table 3).

Table 3. Bloodmeal hosts identified from CytB sequences, including site and relevant *Culicoides* species identified from COI sequences.

Empty Cell	**Mpumalanga**		**Limpopo**		Empty Cell	Empty Cell
Bloodmeal host (CytB)	**Mnisi**	**KNP**	**MNP**	**LW**	**Total**	***Culicoides* species (COI)‡**
***Ceratotherium simum* (White Rhino)**	-	1	-	5	6	*C. imicola* (4/22); *C. loxodontis* (1/1); Undet sp.
***Bos taurus* (Domestic cattle)**	19	2	-	-	21	*C. coarctatus* (1/1); *C. enderleini* (2/9); *C. imicola* (1/22); *C. tropicalis* (1/1); *C. tuttifrutti* (1/1)
***Tragelaphus strepsiceros* (Greater Kudu)**	-	1	-	4	5	*C. imicola* (3/22); C. subschultzei (1/4)
***Aepyceros melampus* (Impala)**	-	-	3	-	3	*C. imicola* (1/22); Undet sp.
***Hippopotamus amphibious* (Hippo)**	-	3	4	-	7	*C. enderleini* (4/9); *C. imicola* (3/22)
***Syncerus caffer* (African Buffalo)**	-	3	-	1	4	*C. enderleini* (2/9); *C. imicola* (1/22); Undet sp.
***Loxodonta africana* (African Elephant)**	-	1	1	-	2	*C. enderleini* (1/9); Dasyops group sp. (1/1)
***Connochaetes taurinus* (Blue Wildebeest)**	-	-	3	5	8	*C. imicola* (7/22); *C. leucosticus* (1/2)
***Homo sapiens* (Human)**	-	-	2	6	8	*C. imicola* (2/22); C. sp. # 107 (2/2); *C. pycnostictus* (2/3); *C. subschultzei* (1/4)
***Equus burchellii chapmani* (Plains Zebra)**	1	-	3	-	4	*C. leucosticus* (1/2); *C. subschultzei* (2/4)
***Capra hircus* (Domestic goat)**	1	-	-	-	1	No COI amplification
***Steatomys* spp. (Fat mice)**	1	-	-	-	1	Undet sp.
***Turdus libonyana* (Kurrichane thrush)**	1	-	-	-	1	*C. bedfordi* (1/1)
***Cisticola* spp. (Warbler)**	2	-	-	-	2	*C. pycnostictus* (1/3); Undet sp.
***Tockus leucomelas* (Yellow-billed hornbill)**	-	1	-	-	1	Non-biting midge†
Totals	**25**	**12**	**16**	**21**	**74**	***Culicoides*: 11 spp., 6** Undet sp**. *Cladontanytarsus*: 1** Undet sp.

KNP: Kruger National Park; MNP: Marekele National Park; LW: Lapalala Wilderness; ‡ (positive for host/total positive); †Non biting midge, Chironomidae species. Undet.sp: Undetermined species.

A total of 54 COI sequences were generated from 142 bloodfed females (38% success rate). Successful identification could be inferred from either phylogenetic analysis or BLAST results for 48 of the specimens (filled black circles on Fig. 2). Five bloodfed females were molecularly determined to be *Culicoides*, but without reference sequences, no species level identification could be inferred (red dots on Fig. 2). The phylogenetic matrix used for identification inference via analysis consisted of 139 taxa and a maximum of 622 characters (Table SM3; Metadata B). Species clusters were generally well supported across all analysis. Subgenera and/or species groups were generally recovered as monophyletic but rarely with strong support. Six specimens comprising four species remains unidentified and one result, due to possible contamination, was identified as a Chironomidae species, a non-biting midge (filled red circles on Fig. 2). The BLAST results, bootstrap support from both data-display networks (DDN) and Maximum Likelihood (ML) analysis and posterior probabilities (pp) from the Bayesian inference (BI) are summarised in supplementary Table SM 4. Reference barcodes generated for *C. bedfordi* (four specimens), *C imicola* (six specimens), *C. leucosticus* (five specimens), *C. magnus* (three specimens), and *C. pycnostictus* (three specimens) all formed well-supported monophyletic clusters (indicated by filled grey circles on Fig. 2).

4. Discussion

Bloodmeal analysis of *Culicoides* species collected in wildlife and rural areas in north eastern parts of South Africa described here suggest that *Culicoides* species are either generalist feeders or perhaps specialists exhibiting opportunistic feeding behaviours. Seemingly, host availability also influences feeding patterns and/or host choice. *Culicoides imicola*, as an example, would readily feed on livestock and wildlife, depending on the collection habitat (Martinez-de la Puente et al., 2017). In mosquitoes, both host availability and habitat drives spatial distribution of species composition, much in agreement with the findings presented here (Burkett-Cadena et al., 2013).

The sites chosen in this study presents habitats with a wider variety of potential blood hosts than that of an intensive agricultural setting with several arboviruses circulating in these sampling localities. *Culicoides* midges has been implicated in the transmission of BTV in Mnisi (Steyn et al., 2015) and EEV in Lapalala Wilderness and Marekele National Park (Snyman et al., 2021) and the presence of EEV antibodies in Zebra from Kruger National Park is also of note (Barnard and Paweska, 1993). Several other arboviruses might also be circulating in these areas, not necessarily linked to *Culicoides*, but with zoonotic potential including Middelburg virus, Shuni virus and West Nile virus present in Lapalala Wilderness, Marekele National Park as well as in the Kruger National Park (Steyn et al., 2020b, 2020a, 2019). Information regarding the species of *Culicoides* present in these areas as well as the hosts utilised by the midges for obtaining blood is consequently of importance.

In the north-eastern corner of Bushbuckridge (Mnisi) where cattle are the primary agricultural livestock, over 60% of the bloodmeals were identified as cattle (*Bos taurus*) with various other species, including a rodent and birds commonly found around houses. This points to the role host availability might play in *Culicoides* feeding behaviour. Here, *C. imicola* was reaffirmed as a dominant species in South Africa (Meiswinkel, 1989; Venter and Meiswinkel, 1994). Surprisingly, humans were not identified as hosts among the *Culicoides*

from the Mnisi area, although humans live in close contact to the animals in this area. The small sample size may however have influenced the finding if cattle significantly outnumber humans as available hosts.

In the wildlife areas *Culicoides* midges fed on a variety of animals. In Kruger National Park, hippopotamus (*Hippopotamus amphibious*) and buffaloes (*Syncerus caffer*) were the major bloodmeal sources amongst the selected samples with *C. imicola* and *C. leucostictus* the predominate midge species identified. Limited data is available on the *Culicoides* species and abundance in Kruger National Park (Meiswinkel and Braack, 1994; Steyn et al., 2015). In Marakele National Park bloodmeal origins were mostly from hippopotamus, however, the host range for *Culicoides* from Marakele National Park included the widest range of animals. *Culicoides imicola* and *Culicoides schultzei* were identified as the most abundant species present in Marakele National Park reflecting previous species compositions recorded from Limpopo province (Venter et al., 1996). In Lapalala Wilderness, where humans were most frequently recorded as blood hosts, *C. imicola* was again the predominate species identified. The results are, however, probably influenced by light trap placement. In Marakele National Park, light traps were placed near a permanent water source housing hippopotamus and in Lapalala Wilderness, traps were placed close the rangers' houses. The biased trap placement was unfortunately unavoidable due to limited availability of electricity needed to operate the light traps.

Historically, sampling of bloodfed *Culicoides* was mostly restricted to agricultural transformed habitats also yielding biased host results (Braverman et al., 1971; Braverman and Phelps, 1981; Nevill et al., 1988; Walker and Boreham, 1976) (Supplementary metadata B). Here the wide variety of novel records of blood hosts are probably due to sampling in non-agricultural habitats presenting a wider variety of possible hosts available to *Culicoides* females. Even though inferring host preference from bloodfed females should be approached with caution, these results do indicate that wider host ranges are utilised by midges for bloodmeal than previously postulated. *Culicoides leucostictus*, for instance, previously hypothesised to be an avian specialist (Labuschagne, 2015), has since been recorded as feeding on livestock and wild mammals and are now reaffirmed here (Riddin et al., 2019). Conversely, *C. bedfordi* usually associated with mammal hosts, fed on a common house bird, the Kurrichane thrush (*Turdus libonyana*). *Culicoides enderleini*, in turn, seemingly targets only large mammals (hippopotamus, cattle and buffalo) but should receive more scientific scrutiny before concrete hypotheses are presented. It is however important to keep in mind that host ranges and host preferences are subequal and studies aiming to determining true host preference should ideally be laboratory based, using choice experiments, where variables can be controlled (Martínez-de la Puente et al., 2015; Takken and Verhulst, 2012). This study and others with overlapping aims, does however, highlight the potential of *Culicoides* species to be implicated as transfer vectors due to its generalist and/or opportunistic feeding behaviour.

This is especially significant given that over 10% of bloodfed females from a variety of species screened here fed on humans. While human handling might result in contamination of samples, all necessary care was taken to reduce such contamination. While this is not the first report implicating humans as a utilised blood source for *Culicoides*, several new sources are added to the list. *Culicoides* species can therefore duly be regarded as possible transfer

vectors between birds, wildlife, livestock and humans. Opportunistic feeding might be important when assessing disease patterns that might be connected to the movement and/or migratory patterns of reservoir hosts, another valuable avenue in need of scrutiny. Although generalists or opportunistic feeders may exhibit a decline in vectoral capacity due to the diverse range of hosts utilised, including a reduction of the impact on a target species, it will allow transmission of pathogens from a variety of potential reservoirs to livestock and/or humans (Santiago-Alarcon et al., 2013). Human feeding of midges indicates that arboviruses can be spread directly to humans without an animal precursor or midges acting as a bridge between animal and man.

While the identification of hosts and midges using molecular barcodes worked well, the amplification success was unexpectedly low. It should be noted that low success was reported in other studies as well. England *et al.* (2020), as an example, managed to generate sequences from only 46% of the collected bloodmeals using similar methodology. Several factors might have contributed to the low amplification success, but freeze-thawing was probably central. Samples were returned to the laboratory frozen from the field, then thawed to sort and pool specimens for virus screening. The bloodfed midges were removed at this stage and separately frozen for later processing. This additional freezing step might have contributed to DNA degradation. Perhaps transferring the specimens to EtOH might have improved the amplification rate. Additionally, especially with larger collections, sorting can take considerable time to complete, conducted under a light dissection microscope, with specimens being exposed to unfavourable conditions throughout the process.

A further complication in using engorged females to identify blood meal host, is that theoretically midges only take up 0.045 µl blood per feeding due to their small size (Venter et al., 2005). Therefore, unidentifiable blood meals could have been from midges not being fully engorged or partly digested blood. Additionally, midges were kept in a 5% Savlon® solution at 4 °C until processing, which could also result in DNA degradation. These are, however, standard sampling protocols (Barnard 1997b; Gordon et al., 2015), and a suggestion would be for future studies to focus on fully engorged females with visibly fresh blood including prompt processing of to facilitate bloodmeal analysis and thus molecular identification of midges and hosts.

The differences between the morphological subsamples and the bloodfed females can probably be due to incomplete sampling. The subsamples are not reflective of the true *Culicoides* richness of any of the sampling localities, but rather of a specific trapping event. Beyond adding useful occurrence records of *Culicoides* species to the literature, the morphological subsamples are indicative of a richer *Culicoides* fauna than what is represented by the bloodfed females. Bloodfed females are difficult to sample and generally comprise less than 1% of the total catch (Riddin et al., 2019). In hindsight, a more exhaustive list of morphologically identified specimens from the regions would have been useful, but by the time of analysis, the sample were already macerated.

Cytochrome oxidase I proved as an effective molecular barcode for *Culicoides* identification on species level, despite previous reports questioning the capability of the marker for such purposes, especially used on taxa with high diversity including species complexes (Meier et al., 2006). Here, well-supported monophyletic species clusters were generally

recovered, and consistent and congruent results were obtained across all analysis methods. Additionally, the topology followed the higher classification of species groups and subgenera as summarised in Labuschagne (2015) and Bakhoum et al., (2018) quite well. Even though these nodes were not always statistically supported, the phylogenetic signal obtained from COI seemed to be adequate for a study of this nature.

Twenty-three specimens could confidently be identified as *C. imicol*a with one specimen (LAP277.3) falling outside of the species cluster. Collectively, however, the cluster was also a well-supported clade. The identification of these specimens as *C. imicola* was consistent across all methodologies and therefore viewed with confidence. Similarly, identification of sequences generated in this study could confidently be assigned to C. sp. #107 ("kwagga"), *C. tropicalis, C. enderleini, C. subshultzei, C. bedfordi, C. coarctatus, C. pycnostictus and C. leucostictus* as indicated by the black dots on Fig. 2.

Culicoides tuttifrutti formed two non-sister monophyletic clusters. The outlying cluster comprise of sequences generated from two specimens caught in KwaZulu-Natal, South Africa (KJ162998-999) and were recovered as sister to *C. bolitinos* + *C. tororoensis*. The reason for the grouping is unclear. The grouping is probably a result of incomplete sampling or incorrectly identified sequences in the molecular libraries but perhaps some genetic variance might be present in the species and may warrant investigation. A reference *C. bolitinos* sequence from GenBank (KT339686) did not group with the remaining *C. bolitinos* sequences from this study (red arrow in Fig. 2) and is probably indicative of initial erroneous identification and should be reconsidered as an effective reference sequence.

Sequence data generated from specimen MAR371-3 formed a well-supported group with MF399675, a specimens identified as C. sp. #54 dark form (d/f) of the Dasyops group, a well-known species morph yet to be described (Bakhoum et al., 2018; Labuschagne, 2015). Another reference sequence from the same study, also identified as C. sp. #54, however, distorts the support even though it is consistently recovered as the sister sequence (Bakhoum et al., 2018). The reference sequences were generated from specimens collected in Harare, Zimbabwe and Port Elizabeth, RSA and increased support might perhaps be achieved by including more geographically representative sampling.

Two specimens could confidently be separated as a distinct species (LAP277.1, LAP293.1), but lacks identity. Specimens MN71.3, MN76.1 and MAR277.2 remains unidentified and did not form clusters with reference sequences included in this analysis. A series of sequences (KY933263-69; PopSet: 1207851499) uploaded to GenBank formed a well-supported cluster but remain unidentified.

The sequence generated from a specimen collected in Kruger National Park, KNP8, was not identified as a species of *Culicoides*, but a Chironomidae, a non-biting midge associated with water due to the aquatic larval phase. Chironomids are not easily mistaken for *Culicoides*, especially given the bloodmeal sequence results that indicated that the midge fed on a bird. Since Chironomids are not haematophagous and morphologically very dissimilar, the result is quite puzzling. The traps are equipped with size-selection netting that should exclude larger insects such as chironomids. Even in the case of a chironomid entering the trap, bloodfed females were washed with distilled water before being macerated for extraction

and PCR processing and consistent results were obtained for all other specimens. Contamination seems unlikely but might be the most probable reason. An unlikely scenario is amplification of the haemolymph of a chironomid in the gut of the midge. Several *Culicoides* species have been recorded feeding on arthropods (Borkent, 2017). Nevertheless, identification results for bloodfed specimen KNP8 was not obtained and proved to be a good outgroup for phylogenetic analysis.

In addition to the host ranges utilised by *Culicoides* females, this study highlights the useful utility of molecular barcodes and the importance platforms such as NCBI/GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.barcodinglife.org/). Species identification using molecular methods is only achievable through collaborative efforts aiming to populate such open access sequence libraries with accurate high-quality sequence data in reference to all species and in the Afrotropics, much more sequence data is needed.

In conclusion, no clear pattern of hosts utilised as blood sources by *Culicoides* females can be established from this work or elsewhere (Supplementary metadata B). Currently, insufficient data or biased sampling procedure might be obscuring host selection patterns exhibited by *Culicoides* species and more studies are needed to elucidate the pattern, if there are any. Nevertheless, this study provides new insight into feeding hosts of several *Culicoides* species at the human/livestock/wildlife interface in South Africa including evidence of human feeding for *C. imicola*, C. sp. # 107, C*. pycnostictus* and *C. subschultzei*. This has implications for cross species transmission of arboviral infections at the interface including zoonotic infections.

CRediT authorship contribution statement

Jumari Snyman: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Louwrens P. Snyman:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Karien Labuschagne:** Investigation, Writing – review & editing. **Gert J. Venter:** Investigation, Writing – review & editing. **Marietjie Venter:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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