Evaluating the morphological and molecular challenges in identifying the afrotropical *Atylotus* species (Diptera: Tabanidae)

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Highlights

•Afrotropical A*tylotus* horse flies are often misidentified morphologically.
•COI struggles to distinguish between aftrotropical A*tylotus* species.
•Horse flies transmit disease and must be correctly identified.

Abstract

The Afrotropical fly genus, *Atylotus* has previously shown little differentiation into species groups using the barcode gene COI. This study analysed all available *Atylotus* COI sequences from GenBank and BOLD to determine if COI is suitable for delimiting species of this genus. Morphological assessments of the different Afrotropical species were done to determine if these species have been accurately identified in recent publications. The results show that COI does not separate the species of this genus into species clades and these species are often misidentified in the literature. This is of concern as species of this genus are known vectors of pathogens and misidentifications have serious implications for management practices. Additional genes need to be used in future molecular studies to differentiate species.

Key words: Horse fly; Species identification; Disease vector; COI barcode

1. Introduction

There are approximately 35 genera and 800 species of Tabanidae in the Afrotropical region that are found in forest, savanna, montane, coastal and desert biomes (Chainey, 2017). Tabanidae in Africa have been largely neglected in the past 50 years with the number of studies slowly increasing in recent years (Desquesnes and Dia, 2003, 2004; Ježek et al., 2019; Keita et al., 2020; Kouam and Kamgno, 2016; Lydie et al., 2017; Morita, 2008; Mugasa et al., 2018; Sasaki, 2005; Snyman et al., 2020; Taioe et al., 2017; Votýpka et al., 2015, 2019). Tabanids are known vectors of pathogens including viruses, bacteria, protozoans and filarial nematodes (Baldacchino et al., 2014). This makes them of medical/veterinary and by extension, economic importance.

Atylotus (Tabaninae:Tabanini) are no different and several species have been implicated in pathogen transmission in the Afrotropics, including several recently published reports reaffirming its vectoral status. While investigating the role of tabanids in the transmission cycle of trypanasomosis, Mulandane et al. (2020) reported that *A. agrestis* Wiedemann, 1828 had the highest infection rate amongst the tabanid species in Mozambique. Desquesnes & Dia (2003, 2004) implicated *A. agrestis* and *A. fuscipes* Ricardo, 1908 as effective mechaniucal

vectors of *T. vivax* amongst cattle in Burkina Faso. Keita et al. (2020) detected, not only trypanosomes in *A. fuscipes* specimens from Senegal, but several species of *Rickettsia, Leishmania donovani* and the microfilarian, *Setaria digitata*. Taioe et al. (2017) in turn reported high infection rates of *Babesia bigemina* in both *A. agrestis* and *A. diurnus* Walker, 1850. Accurate identification of species is thus vital due to the downstream effects it may have in disease ecology or epidemiology.

Tabanidae are notoriously difficult to identify morphologically and this may be one of the reasons this group has been neglected, despite their vectoral status. The most complete keys and description for African Tabanidae are the comprehensive works of Oldroyd from the 1950′s (Oldroyd, 1957, 1954, 1952). Due to the age of these publications, they do not contain any colour photos and the line drawings are often difficult to interpret. The descriptions can also often be confusing and vague. Without having access to identified specimens, such as those in museum collections to compare them to, it is very easy to misidentify these species.

Recent studies in Africa on Tabanidae have produced several phylogenetic trees based on the COI gene (Keita et al., 2020; Mugasa et al., 2018; Taioe et al., 2017). These trees do not show any resolution between the species of the genus *Atylotus*. The five most commonly reported species of *Atylotus* from the Afrotropics are *A. agrestis, A. albipalpus* Walker, 1850*, A. diurnus, A. fuscipes,* and *A. nigromaculatus* Ricardo, 1900. *Atylotus fuscipes* and *A. albipalpus* were synonymised in 1976 (Moucha, 1976) but this synonymy seems to have been largely ignored as both names are widely used (Keita et al., 2020; Mugasa et al., 2018; Taioe et al., 2017). There is clearly a problem in the morphological identification of species of the *Atylotus* genus.

This study aimed to: (1) Evaluate the utility of COI as a tool for *Atylotus* species delimitation; (2) Review the COI markers as available from GenBank and BOLD libraries; (3) Elucidate the morphological species boundaries of *Atylotus* species occurring in the Afrotropical region and (4) Contribute four new COI barcodes and two AATS sequences from specimens confidently identified as *A. agrestis* collected in South Africa.

2. Material and methods

2.1. Morphological treatment

All the *Atylotus* specimens from the KwaZulu-Natal Museum (NMSA) in Pietermaritzburg, the National Museum in Bloemfontein (BMSA) and the Durban Natural Science Museum (DMSA) in Durban, South Africa were examined along with new material collected from the Lower Sabie area of the Kruger National Park, South Africa, with the use of Horizontal traps and Ngu traps baited with octenol during the 2018/2019 field season (supplementary material - Specimen Metadata). Photographs of the type specimens of *A. albipalpus, A. diurnus, A. fuscipes,* and *A. nigromaculatus* were obtained from the natural history museum (NHMUK) in London. Photographs of a non-type specimen of *A. agrestis* from the KwaZulu-Natal Museum in Pietermaritzburg (NMSA) were also taken. The museum specimens were compared to photographs of the type specimens and the published photos in several recent publications (Keita et al., 2020; Mugasa et al., 2018; Taioe et al., 2017). The taxonomic history of each species follows the catalogue of Chainey and Oldroyd (1980) accompanies the high-resolution photos graphs of each species (Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5). A table comparing the abdominal patterns of these species was also produced from the NHMUK photographs and Oldroyd's descriptions (Oldroyd, 1954).

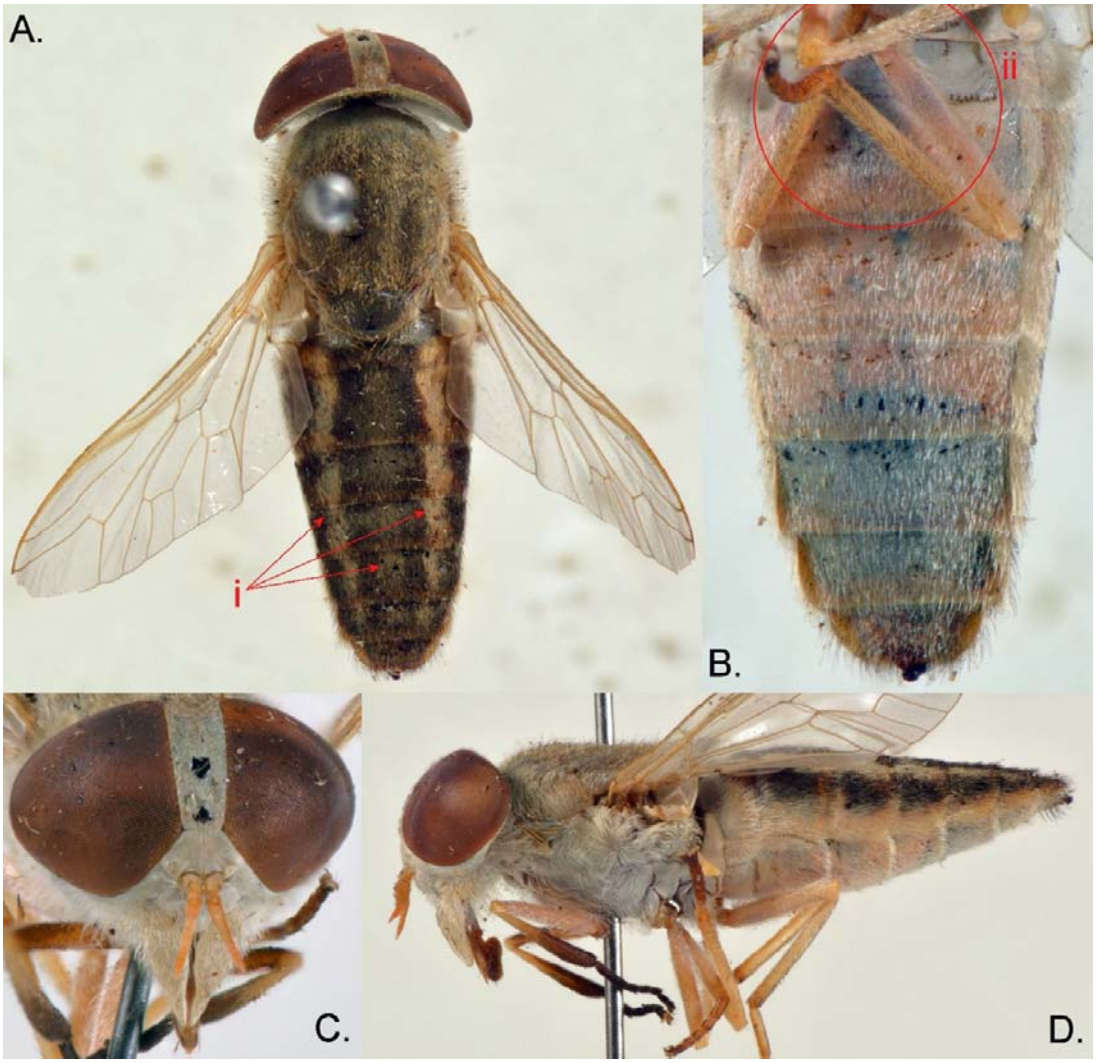

Fig. 1. *Atylotus agrestis* NSMA - DIP049514. A. dorsal view; B. ventral view of abdomen; C. facial view; D. lateral view. i. arrows indicating two distinct sublateral lines and indistinct/faint median line; ii. Presence of a black/dark basomedial mark/spot on the abdomen in ventral view.

2.2. Sequencing and analysis

DNA was extracted from legs of the specimens using the Chelex® protocol obtained from Walsh et al. (1991) with modifications. Amplification of COI was done as described by Mulandane et al. (2020) and amplification of the Alanyl-tRNA synthetase (AATS) gene was conducted with the use of primers detailed in Table 1 using standard methods.

Table 1. Primer information for this study, including: target region, primer name, length, sequence, expected product size and reference.

Target Region	Primer	Length	Sequence (5′–3′)	Expected product size	References
COI	LCO1490	25-mer	GGT CAA CAA ATC ATA AAG ATA TTG G	708bp	Folmer et al. (1994)
	HC02198	26-mer	TAA ACT TCA GGG TGA CCA AAA AAT CA		
AATS1	M13A1×40F	40-mer	TGT AAA ACG ACG GCC AGT GNA TGA AYC ART TYA ARC CAN T	587bp	Morita *et al*. (2016)
	M13rA1×244R	38-mer	CAG GAA ACA GCT ATG ACC ATN CCR CAR TCN ATR TGY TT		

All available COI sequences of *Atylotus* species were downloaded from GenBank and BOLD (Table S1). Sequences were aligned using the online version of MAFFT with default parameters (http://mafft.cbrc.jp/alignment/server/). The aligned matrix was manually viewed, edited and truncated using Mega X (Kumar et al., 2018). Bayesian inference (BI) analysis was performed in MrBayes 3.2 (Huelsenbeck and Ronquist, 2001) using the best-fitting nucleotide substitution mode (GTR+*G*) from jModelTest (Posada, 2008). Four MCMC chains searched for 10 million generations sampling every 1 000th tree. The first 15% of trees were discarded as burn-in. After analysis, the effective sample size (ESS) was inspected in Tracer 1.6 where values of > 200 were considered adequate. A maximum likelihood (ML) analysis was done using RAxML 8 (Stamatakis, 2014) invoking the same model. Bootstraps were calculated invoking the autoMRE bootstopping function. Finally, a data-display network (DDN) was generated from uncorrected p-distances using all characters in SplitsTree4 (Huson and Bryant, 2006). *Tabanus longirostris* was used as an outgroup.

3. Results

3.1. Morphological results

Five distinct abdominal patterns are observed and can be used to separate *Atylotus agrestis, A. albipalpus, A. diurnus, A. fuscipes* and *A. nigromaculatus* from the Afrotropical region **(Table 2).**

Table 2. Patterns on the abdominal dorsum of five Afrotropical *Atylotus* species adapted from Oldroyd (1954).

Species	Diagnosis
A. agrestis	Two orange/reddish yellow sub-lateral longitudinal stripes with a medial stripe of yellow hairs on a black background (Fig. 1A);
A. albipalpus	Two ashy grey sub-lateral stripes on a dark brown background with no orange (Fig. 2A)
A. diurnus	Three longitudinal stripes formed from golden hairs (Fig. 3A)
*A. fuscipes**	Two broad orange sub-lateral stripes that end on the 5th tergite, a medial black stripe with no yellow hairs (Fig. 4A)
A. nigromaculatus	Three well-defined, parallel-sided, yellowish grey longitudinal stripes, equal in width (Fig. 5A)

∗*Atylotus fuscipes* is currently regarded as a synonym of *A. albipalpus*.

The photo comparisons show that there are five distinct species of *Atylotus* in the Afrotropical region (Figs. 1- 5). The published photos of *A. diurnus* (Mugasa et al., 2018: Fig. 3I, pg. 127; Taioe et al., 2017: Fig. 2C, pg. 6) and *A. nigromaculatus* (Mugasa et al., 2018: Fig. 3H, pg. 127; Taioe et al., 2017: Fig. 2E, pg. 6) do not match the photos of the type specimens obtained from NHMUK (Fig. 3& 5). The *A. albipalpus* type specimen is in poor shape which limits comparisons (Fig. 2). The published photos of *A. agrestis* and *A. fuscipes* (Taioe et al., 2017: Fig. 2B, 2D, pg. 6) are similar to the specimens from the NHMUK (Figs 1& 4). The photo of *A. fuscipes* (Keita et al., 2020: Fig. 1d, pg4) is more similar to the type photos of *A. albipalpus* (Fig. 2) than *A. fuscipes* (Fig. 4) but due to the poor condition of the *A. albipalpus* type, this is difficult to confirm.

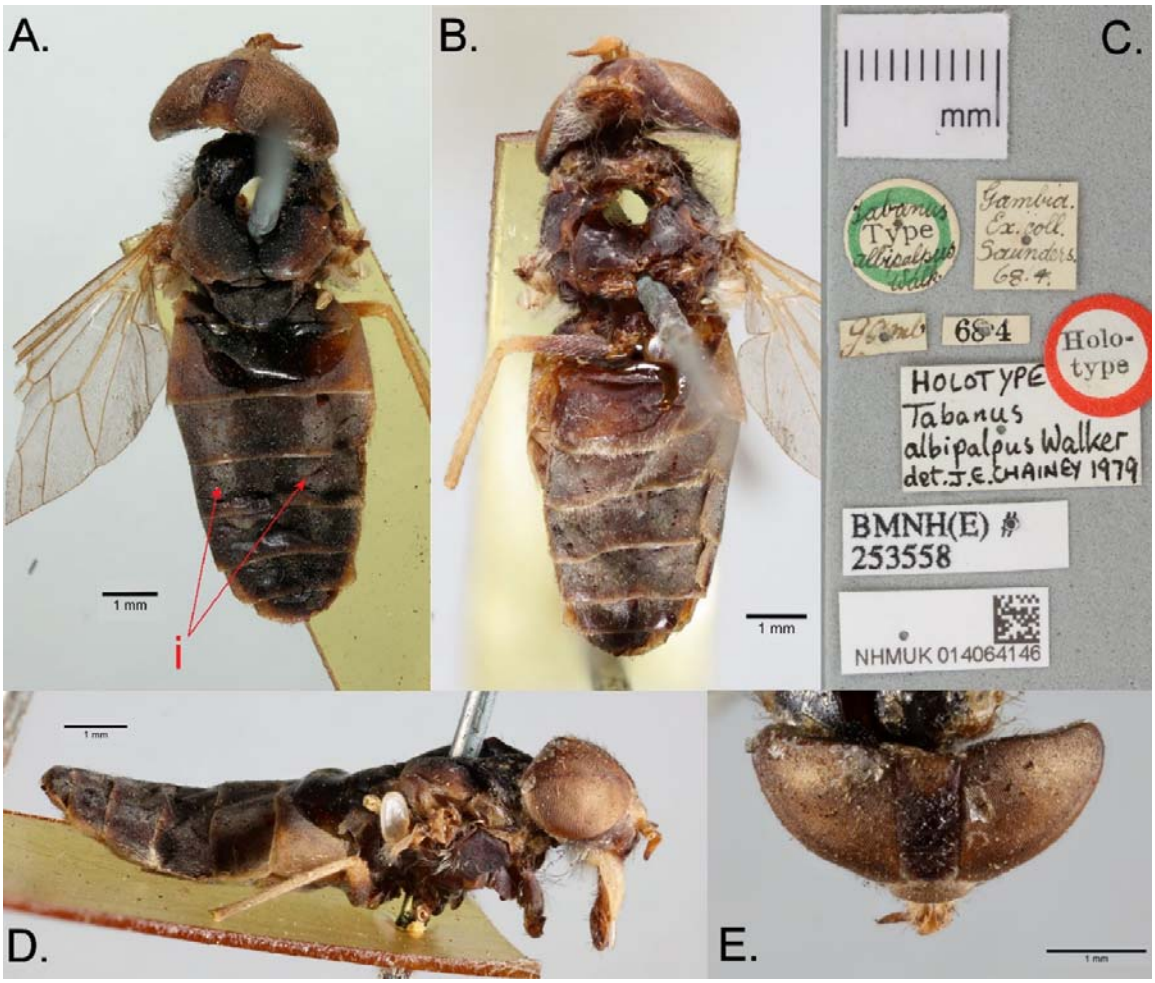

Fig. 2. *Atylotus albipalpus* NHMUK 014,064,146 holotype. A. dorsal view; B. ventral view; C. pin labels; D. lateral view; E. dorsal view, head. i. arrows indicating two distinct sublateral lines, median line absent. All scale bars represent 1 mm.

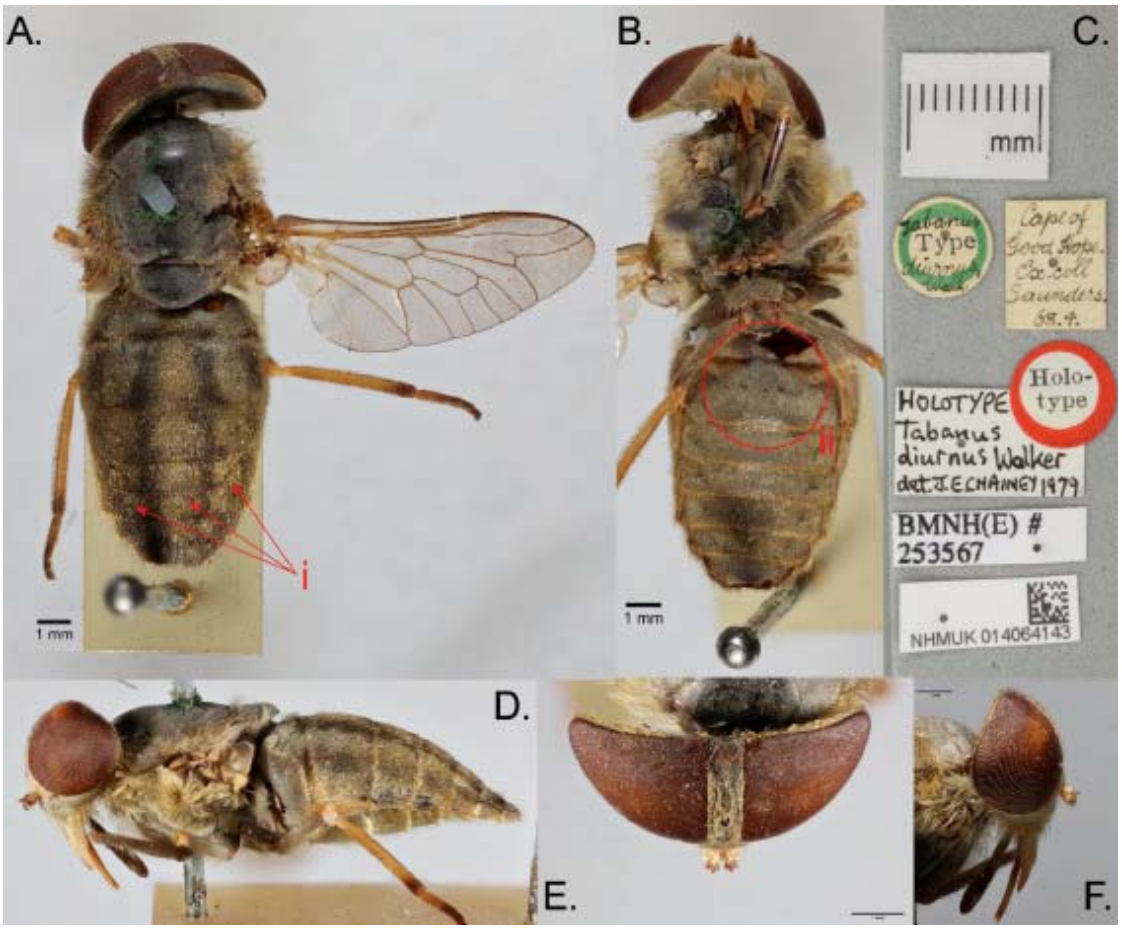

Fig. 3. *Atylotus diurnus* NHMUK 014,064,143 holotype. A. dorsal view; B. ventral view; C. pin labels; D. lateral view; E. dorsal view, head; F. lateral view, head. i. arrows indicating two distinct sublateral lines and one distinct median line; ii. Black/dark basomedial mark absent. All scale bars represent 1 mm.

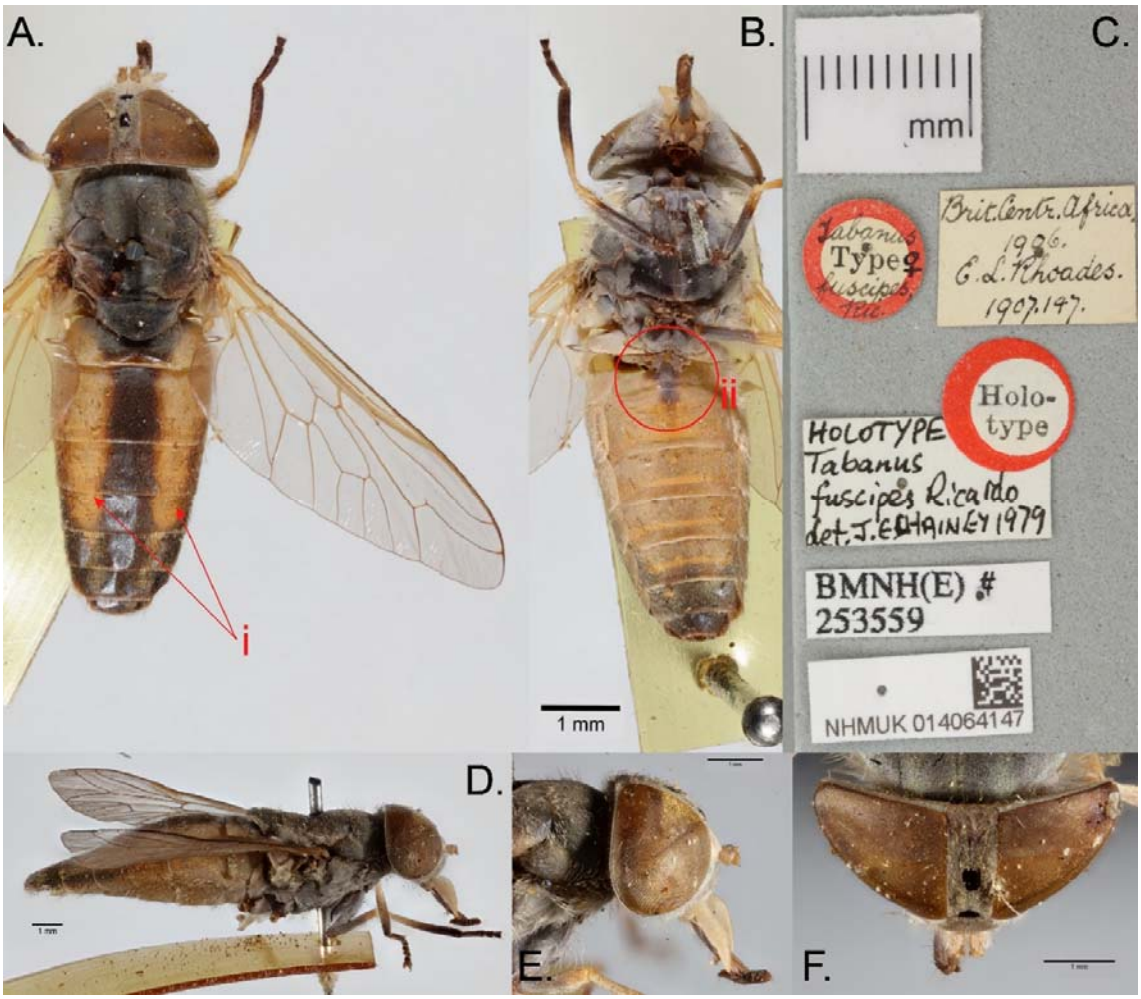

Fig. 4. *Atylotus fuscipes* NHMUK 014,064,147 holotype. A. dorsal view; B. ventral view; C. pin labels; D. lateral view; E. lateral view, head; F. dorsal view, head. i. arrows indicating two distinct sublateral lines, median line absent; ii. Small but distinct black/dark basomedial mark. All scale bars represent 1 mm.

Taxonomic Treatment:

3.1.1Atylotus agrestis Wiedemann, 1828: 557 (*Tabanus*). Type locality: Egypt (Fig. 1)

albicans Macquart, 1834: 204 (*Tabanus*). Senegal.

ditoeniatus Macquart, 1838: 130 (126) (*Tabanus*). Mauritius & Reunion.

bipunctatus Wulp, 1885:75 (*Tabanus*). Ghana & South Africa.

soudanensis Cazalbou in Laveran, 1904: 352. *Nomen nudum,*

lacustris Ghidini, 1938: 343 (*Tabanus*). Ethiopia. [Junior homonym]

ghidini Pechuman, 1949: 84 (*Tabanus*). Replacement name for *lacustris* Ghidini.

ditaeniatus. Incorrect subsequent spelling of *ditoeniatus*.

sudanicus. Incorrect subsequent *spelling of soudanensis.*

3.1.2Atylotus albipalpus Walker, 1850: 44 (*Tabanus*). Type locality: The Gambia (Fig. 2.)

fuscipes Ricardo, 1908:332 (*Tabanus*). Malawi (as 'British Central Africa').

oculipilus Carter, 1915:175 (*Tabanus*, as var. of *fuscipes*). South Africa.

3.1.3Atylotus diurnus Walker, 1850: 43 (*Tabanus*). Type locality: South Africa (Fig. 3).

3.1.4Atylotus fuscipes Ricardo, 1908: 332 (*Tabanus*), regarded as a synonym of *Tabanus albipalpus* Walker (=*Atylotus albipalpus*). Type locality: Malawi (as 'British Central Africa') (Fig. 4).

3.1.5Atylotus nigromaculatus Ricardo, 1900:165. Type locality: South Africa.

ditoeniatus, Bequaert 1930: 917. Liberia [not to be confused with *ditoeniatus* Macquart which is a synonym of *Tabanus agrestis* Wiedemann (=*Atylotus agrestis*)] (Fig. 5)

3.2. Sequencing results and phylogenetic analysis

A total of four COI and two AATS sequences were generated and uploaded to GenBank (Table 3). The final COI matrix comprised 79 ingroup taxa, one outgroup taxon and a length of 657 characters.

Table 3. Sequences submitted to GenBank.

	Specimen code	COI	AATS	Locality
A. agrestis	DVTD_23	OL534387	OL536328	Kruger National Park, South Africa
A. agrestis	DVTD_104	OL534388	OL536329	Kruger National Park, South Africa
A. agrestis	DVTD_1140	OL534389		Kruger National Park, South Africa
A. agrestis	DVTD_673	OL534390		Kruger National Park, South Africa

Three general groups with varying support were recovered across the three analyses (support in brackets DDN bootstrap / ML bootstrap / BI posterior probabilities): an Afrotropical cluster (100/100/1), a Holarctic cluster (55/62/0.99) and an Oriental cluster (80/no grouping/no grouping). *Atylotus horvathi*, a specimen collected in South Korea, grouped with the Oriental species in the data-display network and fell sister to the Afrotropical + Holarctic groups (Fig. 6A, orange arrow).

The *Atylotus* species from the Afrotropical region showed no resolution into species clades apart from an *A. fuscipes* group that formed a separate clade with strong support, all originating from a single study in Senegal (Fig. 6A & B). The *A. agrestis* specimens from this study (red dots on tree) fell amongst the other Afrotropical species (Fig. 6B). There is a clade

of *A. agrestis* that groups with the Oriental species separate from the Afrotropical *A. agrestis* (O1 Fig. 6B).

The other species of *Atylotus* from the Holarctic and Oriental regions showed clear resolution into well-defined species clades (Fig. 6A & B). A previously unidentified sequence (KR441648) is here determined to belong to *Atylotus thoracius*.

4. Discussion

The use of DNA sequence analysis for species identification, or species barcoding, has been employed regularly in recent years especially for species that are difficult to differentiate morphologically, especially Diptera (Mulandane et al., 2020; Sonet et al., 2012; Williams and Villet, 2013). Although useful, barcodes have had varying success in Diptera, especially when utilising COI (Snyman et al., 2021; Dowton et al., 2014; Jordaens et al., 2013; Whitworth et al., 2007; Meier et al., 2006). In order to overcome this, molecular libraries need a variety of different barcodes generated from accurately identified species available for comparison and confirmation (Snyman et al., 2021; Meiklejohn et al., 2019; Beebe, 2018; Meier et al., 2006).

Importantly, COI performed well to distinguish between *Atylotus* species from regions outside of the Afrotropics. The inability of the same marker in distinguishing *Atylotus* species within the Afrotropics is odd and in all probability rather points to a misunderstanding of where the species boundaries are, resulting in misidentification of these species. This in turn result in a confusing DNA reference library that might not reflect the true species diversity of *Atylotus*, which in turn might lead to erroneous inferences when sequences from these libraries are used or analysed.

The tree in Fig. 6B shows a lack of resolution between the species of *Atylotus* using the COI sequences from GenBank and BOLD (Table S1). The sequences in many cases are identical despite being identified as different species (Mugasa et al., 2018; Taioe et al., 2017). This suggests three possibilities – (1) the specimens have been incorrectly identified, (2) COI is not able to differentiate Afrotropical species of this genus or (3) there is only one species of *Atylotus* in the Afrotropical region present in these DNA libraries. The most likely is that these specimens have been misidentified. The photos of these species in the different publications are incredibly similar looking (Mugasa et al., 2018; Taioe et al., 2017). They most closely resemble the type photo of *A. fuscipes* (Fig. 4). However, apart from the difficulty COI has in delimiting dipteran species, *Atylotus* specimens stored in 70% EtOH for long periods tend to resemble *A. fuscipes* due to the loss of setae and the degradation of abdominal colour patterns (Mulandane et al., 2020).

Atylotus agrestis was recorded by Oldroyd (1954) as occurring “from Senegal to Natal and westwards to Angola and lower Congo”. This is a very broad description encompassing most of Africa which is in agreement with the more recent study of Ježek et al. (2017) which lists 31 African countries. There is a cluster of *A. agrestis* from the orient from specimens that originated from Bangladesh and India (Fig. 6B). Oldroyd (1954) and Ježek et al. (2017) record the distribution of *A. agrestis* as extending into the Palaearctic and Oriental regions, however if they are the same species as the *A. agrestis* found in Africa, one would expect these sequences to cluster with the other *A. agrestis* in the tree. All the *A. agrestis* sequences from Genbank and BOLD are from southern Africa (Table S1). The photo of the specimen on BOLD for one of the sequences from Bangladesh (GMBCE2141–15) does not look like

photos of *A. agrestis* received from the NHMUK (not shown in this publication) or the photos of *A. agrestis* from the KwaZulu-Natal Museum or any specimens observed in this study (Supplementary Material: Fig. SM1). It is likely that this is another incorrectly identified record and should be investigated.

Atylotus nigromaculatus is recorded by Usher (1972) as being endemic to South Africa, but Oldroyd (1954) suggested it may also occur in Malawi. All the *A. nigromaculatus* records from the museums in South Africa are from South Africa and Lesotho (Snyman et al., 2020). Recently published records of *A. nigromaculatus* are from Kenya, Tanzania and South Africa (Mugasa et al., 2018; Taioe et al., 2017). The photographs in these publications of *A. nigromaculatus* do not resemble the type specimen, suggesting incorrect morphological interpretation and thus misidentification (Fig. 5). The description of the abdomen of *A. nigromaculatus* states "…with three well-defined, parallel-sided, yellowish grey longitudinals stripes, all equally clear, and about equal in breadth…" (Oldroyd, 1954: pg 123). None of the photos in these publications show three well-defined longitudinal stripes and the identifications are therefore considered doubtful. The geographic distribution of this species is likely still confined to the southern parts of the Afrotropics but warrants investigation.

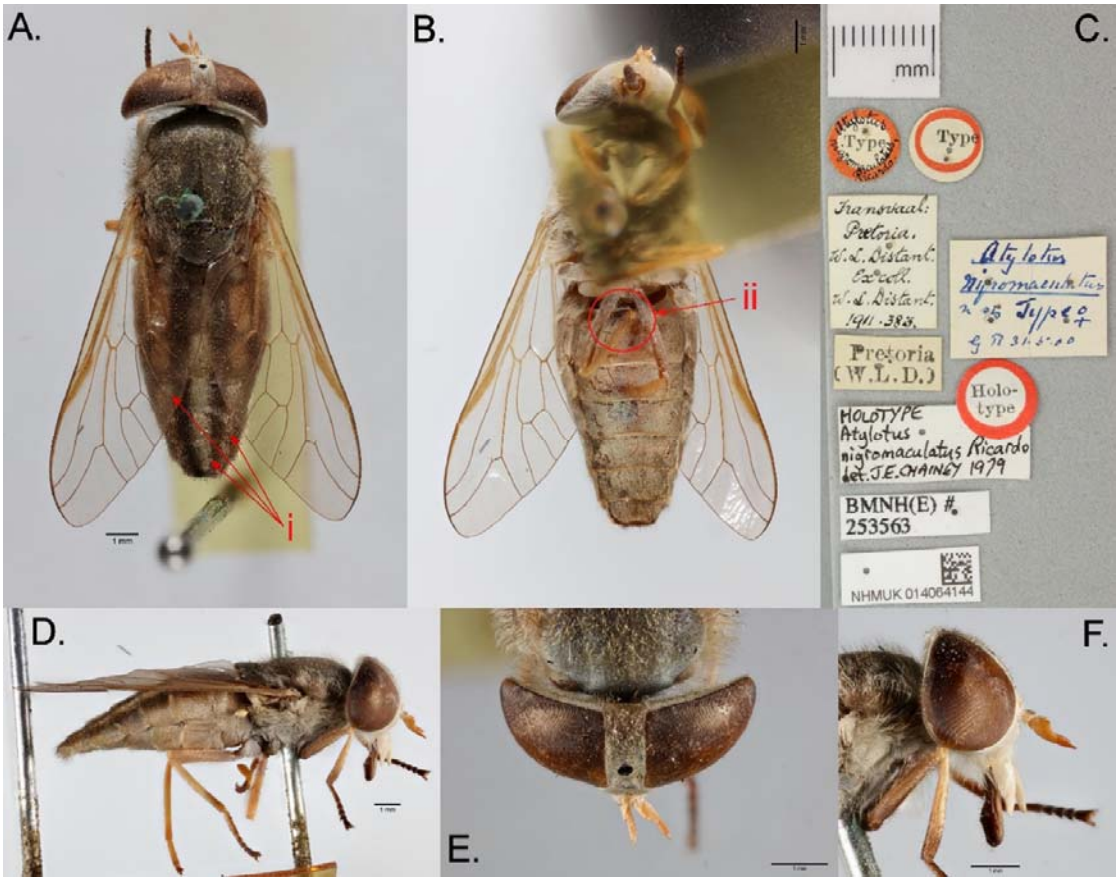

Fig. 5. *Atylotus nigromaculatus* NHMUK 014,064,144 holotype. A. dorsal view; B. ventral view; C. pin labels; D. lateral view; E. dorsal view, head; F. lateral view, head. i. arrows indicating two distinct sublateral lines and one distinct median line; ii. Small but distinct black/dark basomedial mark. All scale bars represents 1 mm.

Atylotus albipalpus is recorded as only occurring in west Africa with *A. fuscipes* being widely distributed across Africa having the same distribution as *A. agrestis* (Oldroyd, 1954). The

synonymy of *A. albipalpus* and *A. fuscipes* was published in both the World Synoptic Catalogue of Tabanidae (Moucha, 1976) and the Catalogue of Afrotropical Diptera (Chainey and Oldroyd, 1980), with no publication justifying the decision. These two species are however morphologically different looking (Fig. 2& 4) and it is difficult to understand why they were synonymised. Both names have continued to be used in recent publications suggesting the authors' rejection of this synonymy (Keita et al., 2020; Mugasa et al., 2018; Taioe et al., 2017; Desquesnes and Dia, 2003, 2004).

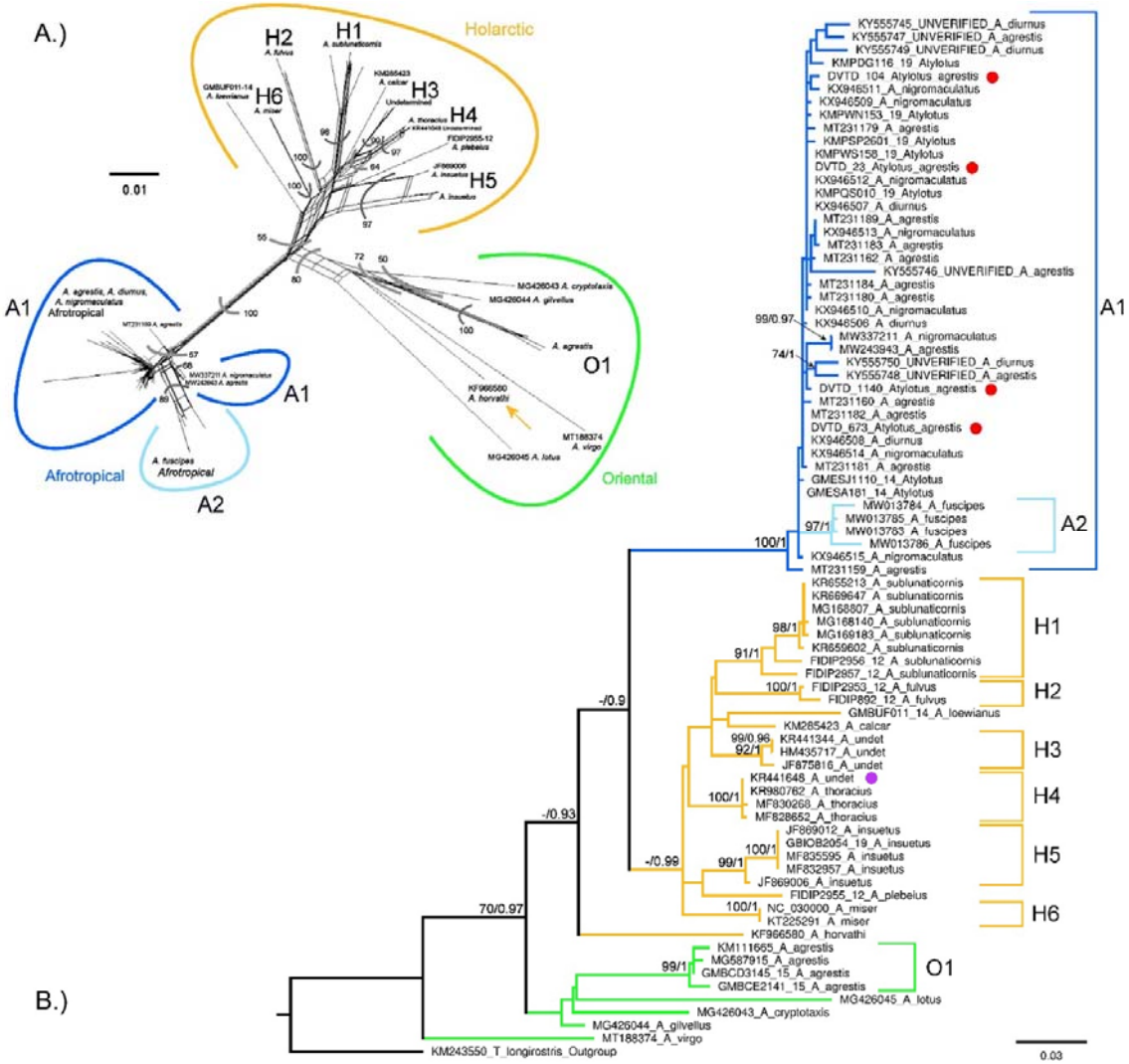

Fig. 6. (A) Data-display network generated from all characters using p-distances, bootstrap support calculated from 1000 replicates based on COI sequences. Orange arrow indicate a Holarctic species, *A. horvathi*, falling within the Oriental clade. (B). ML topology from RAxML analysis, GTR + G model based on the same COI data. Nodal support: ML bootstrap/BI posterior probabilities. Legend: A1: Undefined *Atylotus* cluster; A2: A. fuscipes; H1: *A sublunaticornis*; H2: *A. fulvus*; H3: Undetermined species; H4: *A. thoracinus*; H5: *A insuetus*; H6: *A. miser*; O1: Oriental *A. agrestis*. Red dots: COI sequences generated in this study. Purple dot: Taxon with new assigned identification.

The *A. fuscipes* from Senegal form a well-supported clade within the Afrotropical species clade (Fig. 6B). It should be noted that the remaining Afrotropical specimens are all from eastern and southern Africa. The support for the *A. fuscipes* clade comprising the only west African specimens (Senegal) might then be a result of incomplete sampling rather than species delimitation. Additionally, the habitus photo of this species in the Keita et al. (2020: Fig. 1d, pg. 4) publication also closely resembles the *A. albipalpus* type specimen from the NHMUK (Fig. 2) (Keita et al., 2020). As *A. albipalpus* is recorded as occurring in west Africa, in sympatry with *A. fuscipes*, the correct designation of a species name to the sequences are questionable (Oldroyd, 1954).

The studies of Desquesnes & Dia (2003, 2004) reaffirmed the difficulty differentiating between *A. albipalpus* and *A. fuscipes* in West Africa using the Oldroyd (1954) key. The authors however had no problem differentiating between *A. agrestis* and *A fuscipes* in the same region, suggesting the presence of at least two distinct sympatric species. If this is the case, the ability of COI to distinguish between species in the Afrotropics will perhaps prove to be inadequate. This highlights the need to diversify the markers uploaded to DNA libraries and rather than focusing soley on COI as a barcode.

Atylotus diurnus was described from one specimen with the locality label "Cape of Good Hope" (Oldroyd, 1954). There are no specimens of this species in any of the museums in South Africa (Snyman et al., 2020). In recent studies, *A. diurnus* is recorded from Kenya and Zambia (Mugasa et al., 2018; Taioe et al., 2017). The photo of the type specimen does not resemble any of the photos accompanying the new records from beyond the Cape floristic Region (Fig. 3) suggesting incorrect identifications. *Atylotus diurnus* is most likely a very rare species or an odd specimen belonging to another species. Investigating the validity of the species will be trying due to the vague locality descriptor, a very large area in South Africa. A concerted collection effort in the Western Cape Province of South Africa (the area represented by the "Cape of Good Hope") could potentially answer this question by producing new specimens.

5. Conclusion

There are clearly problems with the identification of Afrotropical species in the *Atylotus* genus. Due to the vectoral status of several species of *Atylotus*, accurate identification is crucial. Incorrectly identified species thus might result in major downstream consequences and/or contribute to our misunderstanding of complex interwoven epidemiological cycles. Further studies are warranted using fresh material that has been identified by expert entomologists and using several genes to determine how many species of *Atylotus* occur in the Afrotropical region. It is advised to rely less on COI as a sole barcode and barcoding type studies should perhaps aim at diversifying the markers uploaded/contributed to open access DNA libraries.

CRediT authorship contribution statement

Kirstin A. Williams: Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing. **Andeliza Smit:** Investigation. **Luis Neves:** Supervision, Resources. **Louwtjie P. Snyman:** Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Baldacchino, F., Desquesnes, M., Mihok, S., Foil, L.D., Duvallet, G., Jittapalapong, S., 2014. Tabanids: neglected subjects of research, but important vectors of disease agents! *Infect. Genet. Evol*. 28, 596–615. https://doi.org/10.1016/j.meegid.2014.03.029.

Beebe, N.W., 2018. DNA barcoding mosquitoes: advice for potential prospectors. *Parasitology* 145 (5), 622–633.

Carter, H.F., 1915. On some previously undescribed Tabanidae from Africa. *Ann. Trop. Med. Parasito*l. 9, 173–196. https://doi.org/10.1080/00034983.1915.11687677.

Chainey, J.E., 2017. Tabanidae (Horse flies, Deer flies and clegs), in: Kirk-Spriggs, A. and Sinclair, B.J. (Eds), *Manual of Afrotropical Diptera* vol 2. SANBI, Pretoria, pp. 893–913.

Chainey, J.E., Oldroyd, H., 1980. Family tabanidae, in: Crosskey, R.W. (Ed.), *Catalogue of the Diptera of the Afrotropical Region. British Museum* (Nat. History), London, pp. 275–306.

Desquesnes, M., Dia, M.L., 2003. Trypanosoma vivax: mechanical transmission in cattle by one of the most common African tabanids, *Atylotus agrestis*. *Exp. Parasitol*. 103, 35–43. https://doi.org/10.1016/S0014-4894(03)00067-5.

Desquesnes, M., Dia, M.L., 2004. Mechanical transmission of Trypanosoma vivax in cattle by the African tabanid *Atylotus fuscipes. Vet. Parasitol*. 119, 9–19. https://doi.org/10.1016/j.vetpar.2003.10.015.

Dowton, M., Meiklejohn, K., Cameron, S.L., Wallman, J., 2014. A preliminary framework for DNA barcoding, incorporating the multispecies coalescent. *Syst. Biol*. 63, 639–644. https://doi.org/10.1093/sysbio/syu028.

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. *Mol. Mar. Biol. Biotechnol*. 3, 294–299.

Ghidini, G., 1938. Ditteri ematofagi dell'Africa orientale Italiana, Genus Tabanus s.l. *Riv. di Biol. Colon. Roma* 1, 321–364.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755. https://doi.org/10.1093/bioinformatics/17.8.754.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23, 254–267.

Ježek, J., Oboňa, J., Wamiti, W., Njoroge, N., 2017. Upswing of collections of horse flies (Diptera, Tabanidae) held at the National Museums of Kenya. *Nairobi. Acta Mus. Siles. Sci. Natur.* 66, 53–63.

Ježek, J., Votýpka, J., Brzoňová, J., Oboňa, J., 2019. Horse flies (Diptera: tabanidae) collected in Central African republic, Gabon and Liberia with comments on their updated distribution. *Acta Musei Silesiae, Sci. Nat.* 68, 263–274. https://doi.org/10.2478/cszma-2019-0025.

Jordaens, K., Sonet, G., Richet, R., Dupont, E., Braet, Y., Desmyter, S., 2013. Identification of forensically important *Sarcophaga* species (Diptera: sarcophagidae) using the mitochondrial COI gene. *Int. J. Legal Med.* 127, 491–504. https://doi.org/10.1007/s00414-012-0767-6.

Keita, M.L., Medkour, H., Sambou, M., Dahmana, H., Mediannikov, O., 2020. Tabanids as possible pathogen vectors in Senegal (West Africa). *Paras. Vect.* 13, 1–15. https://doi.org/10.1186/s13071-020-04375-w.

Kouam, M.K., Kamgno, J., 2016. The African Chrysops. *Biol. Control Pest Vector Insects* 285–298.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Lydie, A.G., Lendzele, S.S., Desquesnes, M., Dia, M.L., 2017. An updated list of Tabanidae (Diptera: insecta) in Ivory Coast. *J. Biodivers. Syst*. 3, 69–79.

Macquart, J., 1838. Diptéres Exotiques Nouveaux Ou Peu connus. Vol. 1. [Part 1]. *Librairie Encylopedique de Roret, Paris*.

Macquart, J., 1834. *Histoire naturelle des insectes. Diptéres. Tome premier. Collection des suites a Buffon*.

Meiklejohn, K.A., Damaso, N., Robertson, J.M., 2019. Assessment of BOLD and GenBank – Their accuracy and reliability for the identification of biological materials. *PLoS ONE* 14, e0217084. https://doi.org/10.1371/journal.pone.0217084.

Meier, R., Shiyang, K., Vaidya, G., Ng, P.K., 2006. DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. *Syst. Biol.* 55 (5), 715–728.

Morita, S.I., 2008. A revision of the *Philoliche aethiopica* species complex (Diptera: tabanidae). *African Invertebr* 49, 129–158.

Moucha, J., 1976. Horse-flies (Diptera: tabanidae) of the World Synoptic Catalogue. *Acta Entomologica Musei Nationalis Pragae*. Supplement 7, 1–319.

Mugasa, C.M., Villinger, J., Gitau, J., Ndungu, N., Ciosi, M., Masiga, D., 2018. Morphological re-description and molecular identification of Tabanidae (Diptera) in East Africa. *Zookeys* 2018, 117–144. https://doi.org/10.3897/zookeys.769.21144.

Mulandane, F.C., Snyman, L.P., Brito, D.R.A., Bouyer, J., Fafetine, J., Van Den Abbeele, J., Oosthuizen, M., Delespaux, V., Neves, L., 2020. Evaluation of the relative roles of the Tabanidae and Glossinidae in the transmission of trypanosomosis in drug resistance hotspots in Mozambique. *Parasites and Vectors* 13, 1–17. https://doi.org/10.1186/s13071-020-04087-1.

Oldroyd, H., 1957. The Horseflies of the Ethiopian region. Vol. III. *Subfamiles Chrysopsinae, Scepsidinae and Pangoniinae, and a Revised Classification.*

Oldroyd, H., 1954. *The Horseflies of the Ethiopian Region. Vol. II. Tabanus and Related Genera*. British Museum of Natural History, London.

Oldroyd, H., 1952. *The Horseflies of the Ethiopian Region. I. Haematopota and Hippocentrum*. British Museum of Natural History, London.

Pechuman, L.L., 1949. Some Notes on Tabanidae (Diptera) and the Description of Two New *Chrysops. Can. Entomol*. 81, 77–84. https://doi.org/10.4039/Ent8177-4.

Posada, D., 2008. jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* 25, 1253–1256. https://doi.org/10.1093/molbev/msn083.

Ricardo, G., 1908. XLIII.—Descriptions of thirty new species of Tabani [sic] from Africa and Madagascar. *Ann. Mag. Nat. Hist*. 1, 268–278.

Sasaki, H., 2005. Tabanid Flies (Diptera: Tabanidae) of the Mahale Mountains National Park. *East Africa. J. Rakuno Gakuen Univ, Tanzania.*

Snyman, L.P., Neves, L., Lempereur, L., Bosman, A.C., 2020. Overview of the horseflies (Diptera: tabanidae) of South Africa: assessment of major collections for spatiotemporal analysis. *Austral Entomol* 59, 549–560. https://doi.org/10.1111/aen.12466.

Snyman, J., Snyman, L.P., Labuschagne, K., Venter, G.J., Venter, M., 2021. 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. *Acta Trop* 219, 105913.

Sonet, G., Jordaens, K., Braet, Y., Desmyter, S., 2012. Why is the molecular identification of the forensically important blowfly species *Lucilia caesar* and L. illustris (family Calliphoridae) so problematic? *Forensic Sci. Int.* 223, 153–159. https://doi.org/10.1016/j.forsciint.2012.08.020.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313.

https://doi.org/10.1093/bioinformatics/btu033.

Taioe, M.O., Motloang, M.Y., Namangala, B., Chota, A., Molefe, N.I., Musinguzi, S.P., Suganuma, K., Hayes, P., Tsilo, T.O.I.J., Chainey, J.E., Inoue, N., Thekisoe, O.M.M., 2017. Characterization of tabanid flies (Diptera: tabanidae) in South Africa and Zambia and detection of protozoan parasites they are harbouring. *Parasitology* 1–17. doi:10.1017/S0031182017000440.

Usher, P.J., 1972. A review of the South African horsefly fauna (Diptera: tabanidae). *Ann. Natal Museum* 21, 459–507.

Votýpka, J., Rádrová, J., Skalický, T., Jirků, M., Jirsová, D., Mihalca, A.D., D'Amico, G., Petrželková, K.J., Modrý, D., Lukeŝ, J., 2015. A tsetse and tabanid fly survey of African great apes habitats reveals the presence of a novel trypanosome lineage but the absence of *Trypanosoma brucei. Int. J. Parasitol.* 45, 741–748. https://doi.org/10.1016/j.ijpara.2015.06.005.

Votýpka, J., Brzoňováa, J., Ježekc, J., Modrý, D., 2019. Horse flies (Diptera: tabanidae) of three West African countries: a faunistic update, barcoding analysis and trypanosome occurrence. *Acta Trop.* 197 https://doi.org/10.1016/j.actatropica.2019.105069.

Walker, F., 1850. Diptera. [Part 1]. In: Saunders, W.W. (Ed.), I*nsecta Saundersiana: Or Characters of Undescribed Insects in the Collection of William Wilson Saunders [1850–1856]*. John Van Voorst, London, pp. 1–76.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10, 506–513.

Whitworth, T.L., Dawson, R.D., Magalon, H., Baudry, E., 2007. DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: calliphoridae). *Proc. R. Soc. B Biol. Sci.* 274, 1731–1739. https://doi.org/10.1098/rspb.2007.0062.

Wiedemann, C.R.W., 1828. *Aussereuropäische zweiflügelige Insekten. 1.* Schulzischen Buchhandlung, Hammburg. 608 pp.(xxxii+ 7 pls.).

Williams, K., Villet, M., 2013. Ancient and modern *hybridization between Lucilia sericata and L. cuprina (Diptera : calliphoridae). Eur*. J. Entomol. 110, 187–196.

Wulp [van der], F.M., 1885. Note XIV on exotic Diptera. *Notes from Leyden Mus*. 7, 57–87.