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Horse flies (Diptera: Tabanidae) of three West African countries: A faunistic update, barcoding analysis and trypanosome occurrence

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ABSTRACT

Horse flies (Diptera: Tabanidae) are of medical and veterinary importance since they transmit a range of pathogens. The horse fly fauna of tropical Africa is still poorly known, and in some geographical areas has not been studied for decades. This study summarizes the results of tabanid collections performed in three West African countries where only sparse data were previously available, the Central African Republic (CAR), Gabon and Liberia. Of 1093 collected specimens, 28 morphospecies and 26 genospecies belonging to six genera were identified, including the first findings of eleven morphospecies in the countries where horse flies were collected: *Philoliche* (*Subpangonia*) *gravoti* Surcouf, 1908 and *Tabanus ianthinus* Surcouf, 1907 are new records for Liberia; *Ancala fasciata* f. *mixta* (Surcouf, 1914), *Tabanus fraternus* Macquart, 1846, and *T. triquetrornatus* Carter, 1915 for CAR; *Chrysops longicornis* Macquart, 1838, *Haematopota albihirta* Karsch, 1887, *H. bowdeni* Oldroyd, 1952, and *H. brucei* Austen, 1908 for Gabon; and *Tabanus secedens* f. *regnaulti* Surcouf, 1912 and *T. thoracinus* Palisot de Beauvois, 1807 for Gabon and Liberia. Species identification of all 28 morphospecies based on morphological features was further supplemented by barcoding of cytochrome oxidase I (COI). Based on the COI sequences of 115 specimens representing 74 haplotypes, a phylogenetic tree was constructed to illustrate the relationships among the tabanid species found and to demonstrate their intra- and interspecific divergences. Our study enriches the current number of barcoded tabanids with another 22 genospecies. Based on the analysis of molecular data we question the taxonomic relevance of the morphological forms *Ancala fasciata* f. *mixta* and *Tabanus secedens* f. *regnaulti*. A parasitological survey based on nested PCR of 18S rRNA revealed a high (˜25%) prevalence of *Trypanosoma theileri* in the studied horse flies, accompanied by two species of monoxenous trypanosomatids, *Crithidia mellificae* and *Blastocrithidia* sp.

1. Introduction

Horse flies (tabanids), haematophagous insects of the family Tabanidae (Diptera), are nuisance pests for people, wildlife and livestock because of their painful and irritating bite, persistent biting behavior, and blood ingestion. In addition, they act as biological or mechanical vectors in the transmission of several causative agents of medical and veterinary importance, including helminths, protozoa, bacteria and viruses. However, tabanids have received only little attention compared to other hematophagous Diptera (Baldacchino et al., 2014).

The family Tabanidae, with more than 4400 known species in 159 genera worldwide (Pape et al., 2011), is represented by approximately 800 species of 35 genera in the Afrotropical region (Chainey, 2017). Adults are stout-bodied flies with a striking appearance, bold stripes and large brilliant colored eyes, all providing useful taxonomic characteristics. However, the appearance of these traits can vary based on the age and preservation method. At present, tabanid identification is predominantly based on morphological characters, which while economical and not reliant on complicated equipment, does require experienced taxonomists. The generalized morphology of tabanids, the rarity of male specimens, the limitations of genital morphology for identifications, the use of variable characters like body and wing color, and in some cases the lack of reliable taxonomic characters in this family make species identification very difficult (Banerjee et al., 2015; Morita et al., 2016).

In light of the identification problems using traditional taxonomic tools, molecular-based approaches have started to be used to solve some

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of these problems. DNA barcoding has gained wide attention and plays an important role, not only for accurate species identification, but also for revealing genetic diversity and the presence of any biotypes/ haplotypes/ genotypes, with the power to resolve taxonomic ambiguities at the species level and within species complexes. The barcoding region of mitochondrial cytochrome oxidase subunit I (COI) has been shown to effectively discriminate species in a range of dipterans, including tabanids (e.g., Cywinska et al., 2010; Banerjee et al., 2015; Morita et al., 2016; Changbunjong et al., 2018; Mugasa et al., 2018; see also the BOLD database – http://www.boldsystems.org/). However, records of Sub-Saharan African tabanids are fragmentary and mostly cursory. COI sequences were obtained for several tabanid species in three East African countries (Uganda, Tanzania, and Kenya) by Mugasa et al. (2018), and in South Africa and Zambia by Taioe et al. (2017); however, no data are available for tabanids inhabiting West Africa.

2. Material and methods

2.1. Study site

Tabanids were collected along with other hematophagous insects in three Sub-Saharan countries – the Central African Republic (CAR), Gabon and Liberia. The forested areas of Dzanga-Sangha Protected Areas (DSPA) in CAR include zones with various levels of protection and restricted human access, and are composed of secondary and, less frequently, primary forest (for more detail see Votýpka et al., 2015). Study sites are frequently visited by herds of forest buffalos (*Syncerus caffer nanus* (Boddaert, 1785)), bongos (*Tragelaphus eurycerus* (Ogilby, 1837)) and forest elephants (*Loxodonta cyclotis* (Matschie, 1900)); the surrounding primary forest is inhabited by several duiker antelopes (*Cephalophus* spp.), red river hogs (*Potamochoerus porcus* (Linnaeus, 1758)), giant forest hogs (*Hylochoerus meinertzhageni* Thomas, 1904), agile mangabeys (*Cercocebus agilis* A. Milne-Edwards, 1886), chimpanzees (*Pan troglodytes* (Blumenbach, 1775)) and western lowland gorillas (*Gorilla gorilla gorilla* (Savage & Wyman, 1847)). Sampling in Gabon occurred in the dense forest of the Loango National Park (for more detail see Červená et al., 2016) where similar host mammals like in CAR are available for blood feeding of tabanids: buffalos, bongos, elephants, duikers, both species of hogs, chimpanzees and gorillas. In Liberia, tabanids were collected (i) in clearings around the Viro camp located in primary dense forest near the border of Sapo NP (5°18′39.9″N, 8°46′53.1″W, 130 MASL) where hogs, duiker antelopes, fanged deer (*Hyemoschus aquaticus* (Ogilby, 1841)), chimpanzee and diana monkeys (*Cercopithecus diana* (Linnaeus, 1758)) are relatively abundant; (ii) in a gallery forest growing on the bank of the Sinoe River (5°19′36.2″ N, 8°48′42.5″W, 100 MASL) where, in addition to the above mentioned mammals, pygmy hippopotamus (*Hexaprotodon liberiensis* (Morton, 1844)) are available as a source of blood for tabanids; and (iii) in meadow-like openings in the vicinity of Jelay village (Jatia Town; 5°20′58.9″N, 8°48′44.8″W, 160 MASL) where local people keep goats, sheep, domestic pigs and dogs.

Our studied sites in Gabon and CAR were located on the edge of the vast Congo basins. The Congo Basin, located in Central Africa (also west equatorial Africa or the Congo), covers a total of 3.7 million square kilometers and contains some of the largest undisturbed tropical rainforests in the world. In the northeast, the Basin continues in the Guinean forests of West Africa, which are divided into the Upper and Lower Guinean forests. Both our collecting sites in Gabon and CAR are in the Lower Guinean forests, a biodiversity hotspot designated by Conservation International. Likewise, Liberia is in the Upper Guinean forests, known as the Guinean moist forests, which have been designated by the World Wide Fund for Nature (WWF) as one of the Global 200 critical regions for conservation.

Fig. 1. A tent-like Malaise trap with a black and dark-blue striped central wall localized in a clearing of the dense forest, the Loango National Park, Gabon.

2.2. Collection of insects

Tabanid flies were collected during September 2012 in the DSPA (CAR), May to July 2014 (Gabon) and September/October 2016 in Sapo National Park (Liberia). Tabanid flies were collected in range of habitats, including forest edges and forest clearings, river banks, and the margins and interiors of small villages, using tent-like Malaise traps with a black and dark-blue striped central wall and white roof that directs insects upwards to a cylinder containing a killing jar with 70% pure ethanol (Fig. 1). In Liberia, due to unexpected technical problems, local sugar cane liquor (with an approximate alcohol content of 40%) had to be used instead of 70% pure alcohol at one of the collection sites. Immediately after transfer to the laboratory, the collected insects were transferred from sugar cane alcohol to 70% pure alcohol. Subsequent DNA processing and analysis of tabanids and trypanosomes showed that this provisional solution had no significant effect on the obtained results. The captured insects were collected from the Malaise traps at least once a day. A small number of tabanids were obtained by sweep netting and hand collection. Ethanol-preserved specimens were stored at ambient temperature during the field work and later transported to the laboratory and stored in a refrigerator (+4 °C).

2.3. Species determination using morphological characters

In the laboratory, tabanids were sorted under a stereomicroscope according to species, sex, collection site and date of trapping. Species identification of dried specimens was done using various keys (Oldroyd, 1952, 1954, 1957) and compared with a reference collection (see Ježek et al., 2017). Selected pinned tabanid voucher specimens of all determined morphospecies were deposited in the National Museum in Prague.

2.4. DNA extraction, PCR amplification and sequencing

Several randomly selected specimens of all determined morphospecies were subjected to molecular barcoding. We attempt to analyze up to five specimens of each tabanid species (or its morphological forms) captured in particular collection sites (= countries). If a particular tabanid species was captured in more than one studied West African country, several individuals from each country were analyzed for intra-specific variability. Ethanol-preserved specimens were individually washed for 1 h in distilled water, and their genomic DNA was extracted from legs and/or thoracic muscle tissue using a High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. Isolated DNA was used as template for PCR amplification using standard barcoding primers LCO1490 and HCO2198 (Folmer et al., 1994) to amplify the cytochrome oxidase I (COI) gene. Amplification reactions were performed in a total volume of

25 µl with EmeraldAmp GT PCR master mix (Takara Bio) following the instructions stated in respective publications. Amplified products were visualized on 1% agarose gels, purified by QIAquick PCR Purification kits (Qiagen), and directly sequenced in one or both directions using the same amplification primer pairs (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit).

For the detection of trypanosomes, tissue homogenates of whole insect bodies were prepared from selected tabanid flies for all detected morphospecies and almost all localities (countries) of occurrence (Table 2). Due to the need to homogenize the whole insect body, tabanids from sites with only a few individuals collected were not included in the analysis and specimens were preserved for the museum collection. In the case of very numerous collections of the relevant species (e.g., *Tabanus ianthinus*), only part of the collected material was processed (Table 2). For parasite species identification, the trypanosome 18S rRNA genes were amplified using a newly designed specific nested PCR (Seward et al., 2017) with amplicon sizes reaching in the first and second round up to 2.2 kb and 2.1 kb, respectively. Positive samples on nested 18S rRNA PCR were subjected to subsequent direct sequencing.

2.5. Sequence alignment and analyses

To identify and compare the obtained sequences of tabanids (COI) as well as trypanosomatids (18S rRNA), they were blasted against the GenBank database using the BLASTn program and compared with the BOLD database (for tabanids only).

Using Geneious 11.0.5 software (Biomatters Ltd., Auckland, New Zealand), the tabanid COI sequences were corrected and aligned, nucleotide sequence divergences within and between species were calculated, and Neighbor-joining (NJ) analyses were performed with the Tamura-Nei genetic distance model and bootstrapping (1000 replicates). Maximum likelihood (ML) phylogenetic trees were performed with bootstrapping (1000 replicates) in MEGA 6.0.

3. Results

We collected a total of 1093 horse fly specimens from sites in Gabon (193 tabanid flies), CAR (172) and Liberia (728). Based on morphological classifications, 28 morphologically distinct species (morphospecies) were identified, of which the subfamily Pangoniinae was represented by one species of the genus *Philoliche*, the subfamily Chrysopsinae by three species of the genus *Chrysops*, and the subfamily Tabaninae by two species of the genus *Ancala*, one species of the genus *Euancala*, 13 species of the genus *Tabanus* and eight species of the genus *Haematopota* (see Table 1 and Fig. 2).

3.1. DNA barcoding

For genetic identification, an ˜700 bp fragment of the mitochondrial COI gene was successfully amplified from 115 specimens representing all 28 determined morphospecies (GenBank Acc. Nos. MK396261–MK396375; Supplementary Table 1). However, only a single sequence is available for some species due to the very limited number of collected specimens or due to the poor quality of the obtained sequence, even after repeated attempts.

No insertions, deletions, or stop codons were observed in the obtained COI sequences. A total of 74 haplotypes were identified, with the number of haplotypes varying from 1 to 6 within each morphospecies of horse flies. For most of the determined morphospecies, blasting against the GenBank and/or BOLD databases did not reveal any highly similar sequences, thus preventing associating the analyzed sequences with previously sequenced taxa. The only exceptions were for *Tabanus thoracinus*, *T. taeniola*, *Chrysops longicornis*, and *Ancala fasciata* (see Discussion); the available sequences of these species were included in the analysis.

3.2. Sequence divergence and phylogenetic analysis

The intraspecific divergence of horse flies varied from 0.0% to 6.5% (the average maximum intraspecific divergence was 2.1%). High intraspecific divergence was observed in three species: *Tabanus secendens* (0.0% to 6.5%), *Tabanus secendens* f. *regnaulti* (0.0% to 5.7%), and *Tabanus thoracinus* (0.0% to 6.2%); while intraspecific divergence of 0.0% to 3.5% was recorded in *Tabanus taeniola* and *Haematopota brucei*. The interspecific divergence of *Tabanus secendens* and *T. secendens* f. *regnaulti* showed a wider variation, ranging from 0.0%–6.5%, and the distinction between intra- and interspecific divergence for these two morphospecies was not sufficient.

Phylogenetic relationships among COI sequences of 28 morphologically distinct horse fly species were established using NJ (Fig. 3) and ML (similar results; not presented). The specimens of the same morphospecies were closely grouped together, except for *Tabanus secendens* and *T. secendens* f. *regnaulti*. For five species, *Ancala fasciata* (+ *A. fasciata* f. *mixta*), *Tabanus par*, *Tabanus taeniola*, *Tabanus thoracinus*, and *Haematopota brucei*, COI sequences of specimens originating from two different countries were available, while in the case of *Chrysops longicornis* and *Tabanus secendens* (+ *T. secendens* f. *regnaulti*), the specimens were collected and sequenced in all three studied countries (Table 1). The geographic origin of all above-mentioned tabanid species corresponds well with their intraspecific genetic differences (Fig. 3). The only exception is for *Tabanus secendens* and *T. secendens* f. *regnaulti*, with much more complicated patterns. Nominotypical *Tabanus secendens* was captured in all three studied countries, while *T. secendens* f. *regnaulti* was found in Gabon and Liberia only. On the phylogenetic tree, several distinct groups were formed (Fig. 3); however, their mutual position was unstable. The use of a haplotype network (Supplementary Fig. 1) did not help clearly distinguish these groups based on their morphology or geographic origin.

3.3. Prevalence of trypanosomes and their species identification

The detection of trypanosomes by the nested PCR approach was performed on DNA samples isolated individually from a randomly selected subset of 321 tabanid flies. Altogether, 79 specimens (24.6%) were infected by trypanosome parasites (Table 2). Based on 18S rRNA sequencing, only one trypanosome species, *Trypanosoma theileri*, was detected. Out of 28 tested tabanid species, 15 were found to be infected by this trypanosome (Table 2). However, for the remaining 13 trypanosome-negative species, in eight species only a single specimen was tested for the presence of parasites (Table 2). The overall prevalence of trypanosomes in the collected tabanids significantly differed in the three studied geographical regions, with an almost 50% prevalence in horse flies collected in CAR, 12.6% in Gabon and only 4.7% in Liberia (Table 2).

In addition to *Trypanosoma theileri*, two species of monoxenous trypanosomatids were detected by sequencing of 18S rRNA in two tabanid females collected in CAR. In monoxenous trypanosomatids, typing units (TUs) are used as proxies of species, similar to molecular operational taxonomic units (MOTUs) (Maslov et al., 2013). In *Haematopota griseicoxa* (voucher No. HF60), TU***246*** representing more likely *Crithidia mellificae* was found; the obtained sequence (MK395375) differs from the previously published sequences KM980183 and KM980182 in only one and four nucleotides, respectively. In *Chrysops silacea* (voucher No. HF49), the detected TU***247*** represents an unnamed species of the genus *Blastocrithidia*; the obtained sequence (MK395374) differs in only one nucleotide from the sequence KX138601 originating from the true bug *Lygus* sp.

4. Discussion

To improve our knowledge on Tabanidae in West Africa and on the occurrence of trypanosomes in these vectors, a baseline survey for the

Table 1
Summarized information about the identified tabanid morphospecies, their abundance in Gabon, CAR and Liberia (**in bold**). The number of specimens belonging to distinct cytochrome I (COI) haplotypes is presented in brackets: e.g., (2 + 1 / 2) means 2 specimens with haplotype "a" + 1 specimen with (closely related) haplotype "b" and 2 specimens with (distinct) haplotype "c" etc.

Morphospecies	Gabon	CAR	Liberia
Philoliche gravoti Surcouf, 1908			**20** (3 + 1 + 1)
Chrysops dimidiatus Wulp, 1885		**2** (1)	
Chrysops longicornis Macquart, 1838	**15** (2 + 2)	**23** (5)	**2** (1)
Chrysops silaceus Austen, 1907		**35** (1 + 1+1)	
Ancala fasciata (Fabricius, 1775)	**12** (2 + 1)		
Ancala fasciata f. *mixta* (Surcouf, 1914)		**2** (1)	
Euancala irrorata (Surcouf, 1909)	**1** (1)		
Tabanus argenteus Surcouf, 1907			**6** (1)
Tabanus boueti Surcouf, 1907			**43** (1 + 1)
Tabanus fraternus Macquart, 1846		**56** (6)	
Tabanus ianthinus Surcouf, 1907			**142** (3 + 1 + 1)
Tabanus obscurehirtus Ricardo, 1908		**14** (1 + 1+1)	
Tabanus par Walker, 1854	**22** (3 + 1 + 1+1 + 1+1)	**8** (3 + 1)	
Tabanus rufipes Palisot de Beauvois, 1806			**420** (3 + 1)
Tabanus secedens Walker, 1854	**24** (5 + 1)	**4** (1 + 1+1 / 1)	**70** (1 + 1 / 2)
Tabanus secedens f. *regnaulti* Surcouf, 1912	**17** (2 + 2 / 1)		**9** (2 + 2)
Tabanus taeniola Palisot de Beauvois, 1807	**40** (3 + 1 + 1)	**10** (2)	
Tabanus thoracinus Palisot de Beauvois, 1807	**23** (3 + 1 + 1+1)		**5** (1 + 1)
Tabanus triquetrornatus Carter, 1915		**2** (1)	
Tabanus variabilis Loew, 1858		**4** (1)	
Haematopota albihirta Karsch, 1887	**3** (2)		
Haematopota bowdeni Oldroyd, 1952	**2** (1)		
Haematopota brucei Austen, 1908	**3** (1 + 1)	**2** (1)	
Haematopota ciliatipes Bequaert, 1930		**4** (1 + 1)	
Haematopota furians Edwards, 1916			**7** (2)
Haematopota griseicoxa Oldroyd, 1952		**6** (1 + 1+1)	
Haematopota guineensis Bigot, 1891			**2** (1)
Haematopota torquens Gerstaecker, 1871			**2** (1)
Total	**193**	**172**	**728**

Table 2
Summarized information about the prevalence of *Trypanosoma theileri* detected by nested 18S rRNA PCR in tabanids collected in Gabon, CAR and Liberia: number of examined / number of positive specimens (prevalence in %).

Morphospecies	Gabon	CAR	Liberia
Philoliche gravoti			11/0
Chrysops dimidiatus		1/0	
Chrysops longicornis	9/1 (11%)	17/16 (94%)	1/0
Chrysops silaceus		26/3 (12%)	
Ancala fasciata	10/0		
Ancala fasciata* f. *mixta		1/1 (100%)	
Euancala irrorata	1/0		
Tabanus argenteus			1/0
Tabanus boueti			9/0
Tabanus fraternus		50/22 (44%)	
Tabanus ianthinus			14/2 (14%)
Tabanus obscurehirtus		7/5 (71%)	
Tabanus par	20/0	5/3 (60%)	
Tabanus rufipes			9/1 (11%)
Tabanus secedens	21/1 (5%)		9/0
Tabanus secedens* f. *regnaulti	12/1 (8%)		2/0
Tabanus taeniola	34/8 (24%)	7/6 (86%)	
Tabanus thoracinus	19/5 (26%)		2/0
Tabanus triquetrornatus		1/0	
Tabanus variabilis		1/0	
Haematopota albihirta	2/1 (50%)		
Haematopota bowdeni	1/0		
Haematopota brucei	2/0	1/0	
Haematopota ciliatipes		2/1 (50%)	
Haematopota furians			4/0
Haematopota griseicoxa		3/2 (67%)	
Haematopota guineensis			1/0
Haematopota torquens			1/0
Total	**135/17 (12.6%)**	**122/60 (49.2%)**	**64/3 (4.7%)**

identification and description of horse flies and their parasites in the Central African Republic, Gabon and Liberia was conducted. 28 tabanid species belonging to six genera of three subfamilies were morphologically identified, including the first records of eleven tabanid species in these three countries. A parasitological survey focused on detecting trypanosomatids revealed *Trypanosoma theileri* infections in one third of the tested horse flies, and trypanosomes were detected in 15 out of 28 tabanid species tested.

We generated DNA barcode data of COI for 115 specimens from the 28 species of tabanid flies. The COI sequence clustering was congruent with the prevailing morphological identifications, and there were no sequences shared between any two taxa or between populations of the same species originating from different countries. Overall, this is the first study demonstrating the use of standardized COI barcodes for the identification of horse fly species in West Africa. This method may be applied in the future for proper tabanid identification in African countries and for the detection of potential cryptic species.

Despite their ubiquity, the taxonomy of the Tabanidae remains difficult, and the group remains poorly understood phylogenetically (Morita et al., 2016). They have received only little attention, and there is just limited molecular data available. New barcode data could help in the quick and precise species identification of African tabanids. Out of 28 tabanid species identified within this study, COI sequences of only four species (*Tabanus thoracinus*, *T. taeniola*, *T. par*, and *Ancala fasciata*) had been analyzed in the previous studies (Taioe et al., 2017; Mugasa et al., 2018). Unfortunately, sequences are publicly available only for the study made by Mugasa et al. (2018) in East Africa countries, with one sequence of *A. fasciata* from Nigeria (KM243527) and a morphologically undetermined specimen of the genus *Chrysops* from Kenya (BOLD:ABZ8804).

Our sequence analysis revealed a fairly good match with the geographic origin of tabanid flies in well-sampled taxa. Different haplotypes of *Tabanus taeniola* form three relatively well-separated clades according to their geographic origin: Kenya + Tanzania, Gabon and

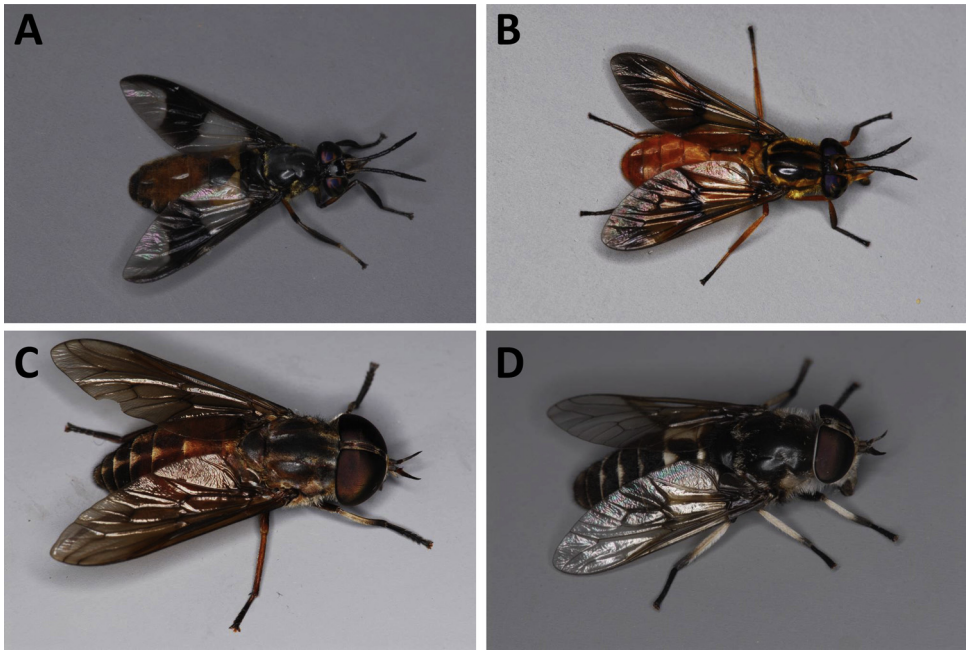

Fig. 2. Selected horse fly species of the genera *Chrysops* and *Tabanus* collected in forest clearings in Dzanga-Sangha Protected Areas, the Central African Republic: A, *Chrysops longicornis* Macquart, 1838; B, *Chrysops silaceus* Austen, 1907; C, *Tabanus secedens* Walker, 1854; D, *Tabanus variabilis* Loew, 1858.

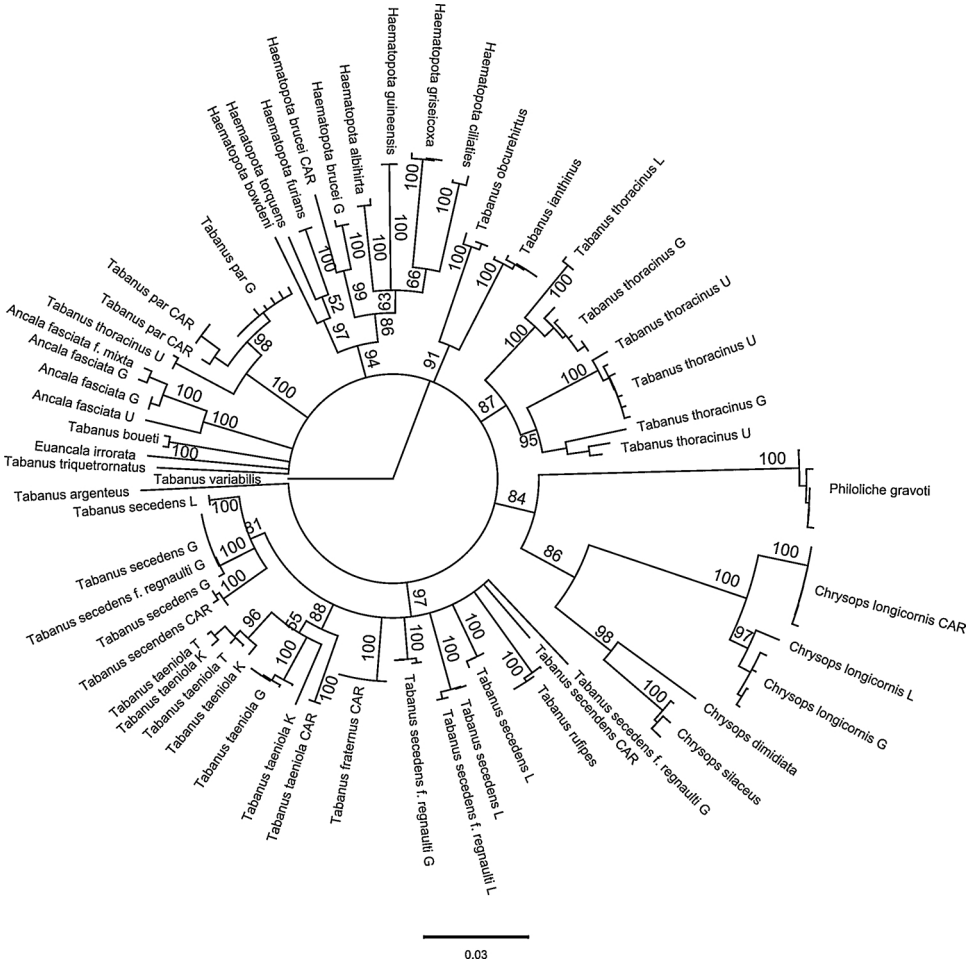

Fig. 3. Phylogram based on data from the mitochondrial COI gene as recovered by Neighbor-joining (NJ) analysis. Branch lengths are relative to the number of changes on that branch. If the species has been recorded in different geographical areas, the abbreviation of the country is given: L (Liberia), G (Gabon), CAR (the Central African Republic), K (Kenya), T (Tanzania), and U (Uganda).

CAR; however, one haplotype from Kenya remains outside this otherwise logical phylogenetic pattern (Fig. 3). Similarly, several haplotypes of *Tabanus thoracinus* from Uganda form two related clades, and another two distinct clades are formed by haplotypes originating from Gabon and Liberia (Fig. 3). This aesthetic geographic pattern is disturbed by one haplotype from Gabon, clustering among haplotypes from Uganda. This finding may either demonstrate the wide genetic plasticity of *T. thoracinus*, or it may be evidence of the existence of morphologically indistinguishable sibling genospecies inhabiting the same area (at least in Gabon).

Forma (abbreviation f. = form), similar to *varietas* (var., v.), is more likely a morphological term; however, it has also relevant taxonomic value. *Forma* is below the species level and is considered an infrasubspecific category (with the exception of names published before 1961, in which case forma is considered subspecific; see ICZN 1999: Art. 45.6). *Forma* is the most common infrasubspecific category among those introduced in the past, but it has been used inconsistently and sometimes even interchangeably. Our sequencing data do not reveal any significant variability between nominotypical *Ancala fasciata* (from Gabon) and *Ancala fasciata* f. *mixta* (from CAR); the sequences differ in only two nucleotides. We conclude that these two morphotypes should only be considered as color variations without any taxonomic value. This view is further supported by a comparison of our *A. fasciata / A. fasciata* f. *mixta* sequences with sequences of *A. fasciata* from Uganda (KX946497–KX946505) and Nigeria (KM243527). While Ugandan samples are represented by two haplotypes closely related to our samples (the closest haplotype differs just in 4–8 nt from our sequences), specimen named as *A. fasciata* from Niger differs in 18–22 characters (nt). Such considerable sequential differences suggest the possibility that while *Ancala* specimens captured in Gabon, CAR and Uganda represent the same species, *Ancala fasciata*, specimen from Nigeria represents another (sibling) species.

Based on the comparison of our sequences with the available databases (GenBank and BOLD), we obtained one more sequence from an undetermined specimen of the genus *Chrysops*, which was captured around Mombasa, Kenya. Phylogenetic analysis demonstrates a close relationship with our sequences of *Ch. longicornis* (Fig. 3). The specimen was not identified, but according to the picture provided in the BOLD database (BIN ID: BOLD: ABZ8804), the tabanid fly morphologically roughly corresponds to *Ch. longicornis*.

In most cases, the genetic variability (expressed as various haplotypes) of tabanid species captured in different countries (Gabon, CAR, and Liberia) correlates with their geographical origin (Fig. 3). Similar findings have also been demonstrated in previous studies (Taioe et al. 2017; Mugasa et al., 2018). The observed genetic differences can be explained as intra-specific (inter-population) variance. The only exception is the *Tabanus secendens* complex (Fig. 3 and Supp. Fig. 1). Nominotypical *Tabanus secendens* and *Tabanus secendens* f. *regnaulti* show considerable genetic heterogeneity that do not correspond even to their morphology or geographic origin. Studied populations more likely represent even the highly variable species or closely related sibling species under the species complex. Our results indicate the presence of at least five group (possible cryptic species) within this complex (Fig. 3 and Supp. Fig. 1); however do not support f. *regnaulti* as a true subspecies or any other taxonomically relevant unit (see below).

Besides its typical form, *Tabanus secedens* Walker, 1854 has the morphological variation *Tabanus secedens* f. *regnaulti* Surcouf, 1912, which is habitually chocolate brown, i.e., much more deeply dark compared to the typical form. Oldroyd (1954) revised the species *Tabanus secedens* and accepted that *regnaulti* is likely a true species. The differences between typical specimens and their variation are: (I) *secedens* – about 19–22 mm; thoracic stripes yellow; abdomen reddish, especially towards the base; median stripe narrow and faint, but not broken into indistinct triangles; lateral stripes faint and broken; (II) *regnaulti* – same size, or a little smaller; much darker in color, chocolate brown, with whitish thoracic stripes and three rows of abdominal triangles that are much more prominent than in *secedens*. However, the later works of Moucha (1976) and Chainey and Oldroyd (1980) recognized f. *regnaulti* and nine other forms as only synonyms of *secedens*.

Our analysis does not provide a clear answer to the question about the existence of f. *regnauti* as an actual species. Several detected clades, with deep intraspecific genetic divergence compared to the interspecific divergence, support the presence of several discrete genetic entities that do not correspond either with the morphology (typical *secedens* vs. f. *regnaulti*) or geographical origin (Ghana vs. CAR vs. Liberia). Unfortunately, it is not possible to unambiguously determine which clades are closer to each another and how many cryptic genospecies are present in our material. Morphospecies *secendens* (the typical form) from Ghana and Liberia are genetically distinct, although morphologically identical. At the same time, f. *regnaulti* is genetically different from the typical form *secendens* cohabitating the studied localities in both countries, Ghana and Liberia; however, similar differences exist between populations of forma *regnaulti* originating from both studied geographical areas. Thus, our analysis does not support f. *regnaulti* as being a true subspecies or any other taxonomically relevant unit. The most plausible explanation is that the species *Tabanus secedens* is highly morphologically and genetically variable, and geographically distant populations differ considerably.

Our analysis using COI sequence divergence demonstrated that some species, e.g. *Tabanus fraternus*, are genetically very uniform in the studied sites; on the other hand, barcoding data revealed that some species, e.g. *Tabanus par* or *Tabanus thoracinus*, are represented by several co-occurring haplotypes. However, because our dataset is still very limited and the number of individuals surveyed within each population was rather low, additional molecular data coupled with morphological characters will be useful for a more comprehensive picture.

In the present study, the average intra- and interspecific divergence of morphologically identified horse fly species were 2.1% (range 0.0%–6.5%) and 5.5% (range 3.5%–12.5%), respectively. A comparison of intra- and interspecific genetic divergence (barcoding gap) has been proposed as a method for species discrimination; however, genetic divergence is specific to different taxa and a universal cutoff is inappropriate. For Canadian, Indian, and Thai tabanids, average intra- and interspecific genetic divergence varies from 0.5 to 1.3% (range 0.0%–6.4%), and from 5.9 to 8.5% (range 0.0%–16.2%), respectively (Cywinska et al., 2010; Banerjee et al., 2015; Changbunjong et al., 2018). However, the calculated intra- and interspecific genetic divergence average is highly dependent on the analyzed dataset, number of sequenced specimens and number of studied populations within the whole geographical range of distribution, number of closely related species (species complexes and sibling species), etc. It is therefore necessary to consider each study individually and to accept the fact that a universal cutoff does not exist, even for lower taxonomical levels, e.g. families. We have to admit that our study was limited by the fact that captured localities within each country were very close to each other and it is therefore necessary to assume that the captured individuals belonged to one tabanid population. Any analysis of intraspecific variability is thus influenced by this considerable bias. In the case that a given species was captured in two (*Ancala fasciata* (+ *A. fasciata* f. *mixta*), *Tabanus par*, *T. taeniola*, *T. thoracinus*, and *Haematopota brucei*) or three (*Chrysops longicornis*, *Tabanus secendens* (+ *T. secedens* f. *regnaulti*)) countries, the compared populations were from very remote sites and we have no data on the variability of the populations in other countries. Analysis of a dataset of our newly obtained sequences from Gabon, CAR and Liberia with sequences added of already available in GenBank/BOLD from flies captured in Uganda, Tanzania and Kenya, increased the range of intraspecific divergence of some studied horse fly species: *A. fasciata* (from 0.0%–1.2% to 0.0%–1.6%), *Ch. longicornis* (from 0.0%–2.8% to 0.0%–2.9%), *T. taeniola* (from 0.0%–3.5% to 0.0%–4.8%), and *T. thoracinus* (from 0.0%–6.2% to 0.0%–8.5%).

One of the main advantages of DNA barcode data is the recognition of cryptic species among morphologically indistinguishable specimens,

and detection of cryptic species using DNA barcoding data has been reported in a wide array of taxa including tabanids. Cywinska et al. (2010) demonstrated intraspecific divergence in Canadian tabanids, supporting the presence of cryptic species in *Chrysops dawsoni* and *C. montanus*, and Banerjee et al. (2015) revealed the presence of cryptic species in Indian *C. dispar*. In the current study, we also observed several well-supported clades with high bootstrap values both in the NJ and ML analyses in the *Tabanus secendens* complex originating from our three distinct geographical areas.

Tabanids are neglected subjects of research but important vectors of disease agents. In the current study, the DNA of 321 tabanid flies were tested by nested PCR for the presence of trypanosomes; however, only one species, *Trypanosoma theileri*, was detected. *Trypanosoma theileri* was first identified in cattle in 1902 by Laveran and Bruce. The hosts are different bovine, ovine, and cervids in Africa, Asia, Europe and Americas and the principal vectors are considered tabanid flies (Hoare, 1972), although other vectors have also been suggested (Schoener et al., 2018; Calzolari et al., 2018). *T. theileri* belongs to a group of trypanosomes that are commonly considered nonpathogenic and may persist in hosts for many years without any evidence of clinical disease. The broad spectrum of tabanid species in which *T. theileri* has been detected is not conclusive evidence of the involvement of particular species in parasite transmission.

Differences in the prevalence of trypanosomes in tabanids are most likely related to the availability of reservoir vertebrate hosts (large ungulates) in the studied sites. In CAR, where the prevalence of trypanosomes reaches almost 50%, tabanids were captured in areas with a high density of forest buffaloes. In Gabon, with 12% prevalence, wild ungulates were less abundant; however, cattle herds were observed close to some sampling sites. In Liberia, where the prevalence of trypanosomes in tabanids reaches only 5%, sampling was carried out near villages where no cattle were kept. The trypanosomes detected in Liberia apparently originate from wild forest ungulates.

Our finding of monoxenous trypanosomatids in tabanids is not surprising. These flagellates are confined to a single insect host and represent the bulk of the known diversity of the family Trypanosomatidae (Maslov et al., 2013; Lukeš et al., 2018). Due to the scarcity of data on trypanosomatid infections in dipterans (e.g., Teixeira et al., 2011; Týč et al., 2013; Borghesan et al., 2018), only limited conclusions can be drawn for this group. However, to our surprise and in contrast to our previous experience (e.g., Týč et al., 2013; Votýpka et al., 2018, 2019), both trypanosomatids most likely represent already-described/named species. Tabanids (including otherwise blood sucking females) feed on various sugar sources, e.g. flowers, where can get infected by monoxenous trypanosomatids shed from the digestive tract of other (primary) insect hosts. We can hypothesize that both the detected trypanosomatids are not primarily parasites of horse flies but rather originated from other native hosts, e.g., bees in the case of *Crithidia mellificae* (Langridge and McGhee, 1967) and true bugs in the case of *Blastocrithidia* sp. (Záhonová et al., 2016).

5. Conclusion

The horse fly family Tabanidae is one of the economically most important groups of bloodsucking dipterans, and correct species identification is crucial for determining their role in disease transmission and for planning effective vector control. So far, COI barcodes for 468 out of more than 4400 described horse fly species are available in GenBank/BOLD databases; however, data from Sub-Saharan African tabanids are sparse. Generation of a DNA barcode library on different tabanid species with more exhaustive sampling will not only provide a platform for an easy and reliable identification system, but also help biologists to link genetic variability with taxonomy, biology and vector management. We hope that enrichment of the public databases with another 22 genospecies significantly increases the knowledge of African tabanid fauna and will be helpful in horse fly identification.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.105069.

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