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Unexpectedly high diversity of trypanosomes in small sub-Saharan mammals

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ABSTRACT

The extremely species-rich genus Trypanosoma has recently been divided into 16 subgenera, most of which show fairly high host specificity, including the subgenus *Herpetosoma* parasitizing mainly rodents. Although most Herpetosoma spp. are highly host-specific, the best-known representative, Trypanosoma lewisi, has a cosmopolitan distribution and low host specificity. The present study investigates the general diversity of small mammal trypanosomes in East and Central Africa and the penetration of invasive T. lewisi into communities of native rodents. An extensive study of blood and tissue samples from Afrotropical micromammals (1528 rodents, 135 shrews, and five sengis belonging to 37 genera and 133 species) captured in the Central African Republic, Ethiopia, Kenya, Malawi, Mozambique, Tanzania, and Zambia revealed 187 (11.2%) trypanosome-positive individuals. The prevalence of trypanosomes in host genera ranged from 2.1% in Aethomys to 37.1% in Lemniscomys. The only previously known trypanosome detected in our dataset was T. lewisi, newly found in Ethiopia, Kenya, and Tanzania in a wide range of native rodent hosts. Besides T. lewisi, 18S rRNA sequencing revealed 48 additional unique Herpetosoma genotypes representing at least 15 putative new species, which doubles the known sequence-based diversity of this subgenus, and approaches the true species richness in the study area. The other two genotypes represent two new species belonging to the subgenera Ornithotrypanum and Squamatrypanum. The trypanosomes of white-toothed shrews (Crocidura spp.) form a new phylogroup of Herpetosoma, unrelated to flagellates previously detected in insectivores. With 13 documented species, Ethiopia was the richest region for trypanosome diversity, which corresponds to the very diverse environments and generally high biodiversity of this country. We conclude that besides T. lewisi, the subgenus Herpetosoma is highly host-specific (e.g., species parasitizing the rodent genera Acomys and Gerbilliscus). Furthermore, several newly detected trypanosome species are specific to their endemic hosts, such as brush-furred mice (Lophuromys), dormice (Graphiurus), and white-toothed shrews (Crocidura).

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1. Introduction

Trypanosomes are both serious and biologically interesting parasites that have attracted considerable interest since their discovery in frogs almost 180 years ago (Gruby, 1843). Through extensive study, they have been found in all groups of vertebrates,

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being transmitted by a wide range of blood-feeding invertebrates (Gibson, 2017). The number of known *Trypanosoma* spp. is steadily growing, and this expanding diversity has recently been accommodated into 16 subgenera, frequently with distinct host specificity (Kostygov et al., 2021). Indeed, the specificity varies widely, from extremely low in *Trypanosoma cruzi* (subgenus *Schizotrypanum*), known from humans and more than 100 mammalian species (Jansen et al., 2017), to members of the subgenus *Herpetosoma* constituting trypanosomes parasitizing rodents, which are typically confined to a single mammalian host (Hoare, 1972; Molyneux, 1976; Maraghi and Molyneux, 1989; Santos-Gomes et al., 1993;

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Noyes et al., 2002). Rodent trypanosomes are considered highly host-specific, as only very few cross-infections under natural conditions were documented, while transmissions under laboratory conditions between closely related host species were likely facilitated by stress and suppressed immunity (Hoare, 1972; Noyes et al., 2002; Sato et al., 2007).

Only a single Herpetosoma sp., namely Trypanosoma lewisi, stands out in this respect. Its cosmopolitan distribution in rodents has been linked to historical human-mediated dispersal of its invasive host, rats of the genus Rattus, from Asia. Consequently, T. lewisi has been reported in more than 100 rodent species worldwide including Africa, where several surveys documented it in Nigeria, Niger, Mali (Dobigny et al., 2011; Schwan et al., 2016; Tatard et al., 2017), Uganda (Salzer et al., 2016), Egypt (Alsarraf et al., 2016; Dahesh and Mikhail, 2016), and Mozambique (Ortiz et al., 2018). To date, molecular data support Acomys iohannis, Arvicanthis niloticus. Graphiurus murinus. Hvbomvs lunaris. Lemniscomvs striatus, Lophuromys aquilus complex, Mastomys erythroleucus, Mastomys natalensis, Mus triton, Praomys daltoni, and Praomys jacksoni as its hosts and/or reservoirs (Dobigny et al., 2011; Salzer et al., 2016; Ortiz et al., 2018). Although T. lewisi is considered largely non-pathogenic for most of its rodent hosts, after its introduction by synanthropic rats to Christmas Island, this parasite very likely drove two native endemic rat species to extinction, representing the only known case of a trypanosomatid-mediated extinction of a vertebrate host (Wyatt et al., 2008). Trypanosoma lewisi is also recognised as potentially pathogenic to humans (Hoare, 1972; Molyneux, 1976) and several human infections have been reported from Gambia, Thailand, India, and China (Howie et al., 2006; Liu and Liu, 1990; Truc et al., 2013; Lun et al., 2015).

For a long time, the identification of trypanosomes was based solely on morphology (Hoare, 1967, 1972; Kaufer et al., 2017). However, due to the low number of reliable features and their variability, the morphology-based species concept was surpassed by species-specific molecular characters. Therefore, the current determination of trypanosomes, regardless of whether they are found in vertebrate hosts or invertebrate and insect vectors, is based on conserved gene sequences, such as the 18S rRNA and glyceraldehyde 3-phosphate dehydrogenase genes (Hamilton et al., 2007; Gibson, 2017; Hamilton and Stevens, 2017; Kostygov et al., 2021).

Approximately 50 nominal species classified into the subgenus *Herpetosoma* were named based on their morphology and presumed restriction to the host species from which they were isolated (Noyes et al., 2002; Dybing et al., 2016). *Herpetosoma* spp. were detected in the blood of a range of unrelated mammals such as rodents, lagomorphs, insectivores, sengis (elephant shrews), bats, and primates (Hoare, 1972; Marinkelle, 1977; Dybing et al., 2016). However, due to the almost identical morphology of the bloodstream stages, microscopic identification alone probably underestimates the true diversity (Maia da Silva et al., 2010; Ortiz et al., 2018). Indeed, a wider application of the sequencebased determination would likely document extensive diversity (Linardi and Botelho, 2002; Noyes et al., 2002; Sato et al., 2005, 2007; Maia da Silva et al., 2010).

So far, 18S rRNAs are available for 14 named *Herpetosoma* spp. that parasitize rodents, lagomorphs, and insectivores, although there is no clear distinction between intra- and interspecific genetic variability (Stevens et al., 1999; Maia da Silva et al., 2010; Ortiz et al., 2018; Egan et al., 2020). Previous phylogenetic studies divided this subgenus (also termed the *T. lewisi*-like clade) into several well-supported groups (Mafie et al., 2019; Egan et al., 2020) (Figs. 1 and 2).

The most important subclade currently encompasses *T. lewisi* together with four closely related species (*T. lewisi* subclade 1a) and *T. grosi* (subclade 1b), although its taxonomic composition remains ambiguous. Indeed, *T. musculi* of mouse (*Mus musculus*)

and T. blanchardi of dormouse (Eliomys guercinus) have identical ~2 kb-long 18S rRNA genes, whereas T. lewisi of rat, T. musculi of mouse, T. niviventerae of white-bellied rat, and T. rabinowitschae of hamster differ from each other by just a single nucleotide substitution, yet they constitute genuine species as confirmed by failed cross-infections (Mafie et al., 2019). At the same time, two to eight substitutions in this gene distinguish a range of isolates of closely related T. grosi (Sato et al., 2005; Guan et al., 2011). If the same rules applied to subclade 1a were applied to subclade 1b, the latter would have to be subdivided into several species, a motion that was repeatedly proposed (Noyes et al., 2002; Karbowiak and Wita, 2004; Sato et al., 2005). The less controversial subclade 2 brings together parasites of Cricetidae and Lagomorpha (subclade 2a) and Sciuridae (subclade 2b) (Grewal, 1957; Hamilton et al., 2005; Merino-Espinosa et al., 2016), while the recently established subclade 3 is formed by trypanosomes of insectivores (Mafie et al., 2019). In addition to these formally described trypanosomes, two isolates (B08-471 and BR042) without available morphological information, yet phylogenetically clearly distinct from the abovementioned Herpetosoma spp., have been found in a flea (Ceratophyllus sciurorum) and a rat (Rattus rattus), respectively (Votýpka et al., 2013; Egan et al., 2020).

Considering the vast diversity of Afrotropical rodents and insectivores, it is of interest to have a good understanding of the diversity, distribution, and host specificity of their trypanosomes. To reduce the dramatic disproportion between the known diversity of small mammals and their parasites in the Afrotropical biogeographic realm, we investigated trypanosomes from a wide range of sub-Saharan rodents and insectivores. The obtained 18S rRNA sequences provide insight into the biology and diversity of trypanosomes, especially those belonging to the subgenus *Herpetosoma*, and significantly extend the list of their rodent hosts. Due to the large-scale invasion of Asian rats into Africa, we have monitored the extent of penetration of invasive zoonotic *T. lewisi* into communities of native African small mammals.

2. Materials and methods

2.1. Sampling

Small mammals (rodents, shrews, and sengis) were trapped in Sherman live traps (subsequently sacrificed by cervical dislocation) and snap traps baited with a mixture of peanut butter, maize flour, and dried fish. All captured animals were dissected and tissue pieces (spleen in most samples, but also heart, lung, kidney, muscle, and tail) were stored in 96% ethanol until DNA extraction. Each specimen was assigned to a genus based on its external characteristics. The GPS coordinates of each locality were recorded. For more details on particular specimens, localities, and collectors, see Supplementary Table S1.

All fieldwork complied with legal regulations in the African countries concerned and the sampling was carried out according to local legislation. DNA samples analysed in this study were previously collected during several expeditions over two decades in the framework of various research projects in seven sub-Saharan countries, namely the Central African Republic, Ethiopia, Kenya, Malawi, Mozambique, Tanzania, and Zambia (Supplementary Fig. S1). The samples utilised in the study have been lawfully acquired and were collected prior to The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from Their Utilisation to the Convention on Biological Diversity being in effect. All animal tissues used in this study were previously published as part of an inquiry into the biogeography of micromammals in Africa (Bryja et al., 2014, 2019; Aghová et al., 2017, 2019; Hánová et al., 2021). Animal collections



Fig. 1. An 18S rRNA-based maximum likelihood phylogenetic reconstruction of the subgenus *Herpetosoma* comprised of all available (nearly full-length) sequences and all 49 detected genotypes clustered into 16 phylogroups designated here as colour-coded proxy (phylo)species AFxx. Hitherto known and described (sub)clades within the genus *Herpetosoma* are indicated by blue numerals 1a,b, 2a,b, and 3 (for more information, see Section 4.1); for details of the *Trypanosoma lewisi* subclade (1a), see Fig. 2. A phylogenetic position of two additional species, AFO1 and AFO2, members of the subgenera *Ornithotrypanum* and *Squmatrypanum*, respectively, is presented with the selected closely related sequences. Country ISO (International Organisation of Standardisation) alpha-3 codes and vertebrate host genera are indicated for all taxa/genotypes. Asterisks mark branches with maximal statistical support (bootstrap values for maximum likelihood >90, Bayesian posterior probabilities >0.95); double-crossed branches are 50% of the original length; the scale bar denotes the number of substitutions per site.

– T. grosi SESUJI (RUS, Apodemus)



Fig. 2. An 18S rRNA-based maximum likelihood phylogenetic reconstruction of the *Trypanosoma lewisi* subclade (see Fig. 1; subclade 1a). Country ISO alpha-3 codes and host genera are indicated for all taxa/genotypes; double-crossed branches are 30% of the original length; the scale bar denotes the number of substitutions per site.

and handling adhered to the wildlife research regulations of the respective institutions and countries, including the Ethiopian Wildlife Conservation Authority (Ethiopia), the Sokoine University of Agriculture in Morogoro (Tanzania), the Kenyan Forest Service and the Kenyan Wildlife Service (Kenya), the Zambian Wildlife Authority (Zambia), the National Directorate for Protected Areas (Mozambique), the National Research Council and Forestry Department (Malawi), the Ministre de l'Education Nationale, de l'Alphabetisation, de l'Enseignement Superieur et de la Recherche, the World Wildlife Fund and Primate Habituation Programme (the Central African Republic).

2.2. DNA extraction, amplification, sequencing, phylogeny, and estimation of species richness

DNA from 96% ethanol-preserved tissue samples was extracted using a DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions. The species of mammalian hosts were established by DNA barcoding. We amplified and sequenced the mitochondrial gene for cytochrome b (CYTB) using a protocol described elsewhere (Bryja et al., 2014). The obtained sequences were compared with datasets from recent taxonomic, phylogenetic, and phylogeographic studies on particular mammalian genera, or with unpublished data available at the Institute of Vertebrate Biology, Czech Academy of Sciences.

To amplify the trypanosome 18S rRNA gene, approximately 10 ng of previously extracted DNA was subjected to the trypanosomatid-specific nested PCR protocol as described by Seward et al. (2017) and the PCR products were directly sequenced. Sequences were checked using Geneious software (version 10.0.6, https://www.geneious.com) and an alignment for phylogenetic analysis was generated by MAFFT v.7. All related sequences available in GenBank of the (nearly) full-size 18S rRNA gene were used. The final dataset contained 2146 molecular characters and 86 sequences representing formally described species or molecular operational taxonomic units (MOTUs), which constitute proxy (geno)species, both represented in some cases by several genotypes. Phylogenetic reconstructions were conducted using maximum likelihood (ML; PhyML v.3.0.1) and Bayesian inference (BI; MrBayes v.3.2.2) with model optimisation in ModelTest v.3.06. A general time-reversible substitution model with a mixed model for among site rate variation (GTR + Γ + I) was chosen as the best fitting model of sequence evolution. Bootstrap analyses involved heuristic searches with 1000 replicates (ML). BI analysis was run for five million generations with covarion and sampling every 100 generations. All other parameters were left in their default states. The distribution of detected species was visualised in QGIS (https://www.qgis.org/en/site/).

Estimation of trypanosome (*Herpetosoma*) species richness (observed plus undetected) was based on an asymptotic approach which compares the estimated asymptotes (with non-parametric estimators) of species accumulation curves (for more detail see Chao and Chiu, 2016) using PAST (Paleontological Statistics) v. 4.09 (https://www.nhm.uio.no/english/research/infrastruc-ture/past/).

3. Results

We examined tissue samples from 1,668 small mammals (rodents, shrews, and sengis) belonging to 133 species and 37 genera (Supplementary Table S1–S3) that were captured during field research in the Central African Republic, Ethiopia, Kenya, Malawi, Mozambique, Tanzania, and Zambia. The sampling sites and the number of captured animals are summarised in Supplementary Fig. S1 and Supplementary Table S4, respectively. Overall, 187 individuals (11.2%) were infected with trypanosomes based on nested PCR. In the individuals for whom sex was detected and recorded (Supplementary Table S1), no significant differences (Chi² = 0.525; P = 0.47) in infection prevalence were observed between females and males, which were infected in 7.2% and 8.2% of cases, respectively.

Infection rates varied significantly between the studied countries; while sample sets from Kenya and Ethiopia contained a large fraction of positive individuals (22.9% and 18.3%, respectively), significantly lower infection rates were encountered in Tanzania (5.8%), Mozambique (4.8%), Zambia (4.7%), the Central African Republic (2.3%), and Malawi (1.1%) (Supplementary Table S4). Of the 37 mammalian genera examined, trypanosomes were detected in 19; however, within the host genera, of which at least a dozen individuals were captured and screened (Supplementary Table S3), the proportion was significantly higher (16/18; 89%) (Supplementary Fig. S2). The overall prevalence within the investigated mammalian genera ranged from 2.1% (*Aethomys*) to 32.1% (*Rattus*), 36.7% (*Acomys*), and 37.1% (*Lemniscomys*) (Supplementary Table S3). The obtained 18S rRNA gene sequences represent 51 unique genotypes (Supplementary Table S5) that cluster, based on their phylogenetic analysis, into at least 18 phylogroups (designated as AFxx) exemplifying different trypanosome species, with nine of them represented by more than one genotype (Figs. 1 and 2; Supplementary Table S6). While the subgenera Ornithotrypanum (AF01), which infects mainly birds, and Squamatrypanum (AF02), parasitizing reptiles and mammals, are represented by a single new species (with a single genotype) each, the vast majority of detected genotypes (49) belongs to the subgenus Herpetosoma. With a single exception (see below), all detected phylogroups can be considered as new species of this subgenus.

The picture of the phylogroup AF05 (the *T. lewisi* subclade; 1a) is quite complicated (Figs. 1, 2, and Supplementary Fig. S7; Supplementary Table S6). Found in five countries (Central African Republic. Ethiopia, Kenva, Tanzania, and Zambia), this subclade is represented in our dataset by 10 different genotypes (AF05a-i) derived from 19 rodent species belonging to the following 12 genera: Praomys (represented by 13 individuals in two species), Lemniscomys (11/1), Mus (10/3), Rattus (9/1), Stenocephalemys (7/2), Aethomys (3/1), Acomys (3/3), Mastomys (3/2), Dendromus (1/1), Desmomys (1/1), Grammomys (1/1), and Lophuromys (1/1). Although all of these genotypes cluster within the genetically polymorphic T. lewisi subclade, only a single genotype (AF05b), detected in the invasive R. rattus and eight native rodent species belonging to seven genera (Aethomys chrysophilus, Dendromus mystacalis, Desmomys harringtoni, Grammomys macmillani, Mastomys erythroleucus, Mus mahomet, Mus triton, and Stenocephalemys albipes) and originating from three African regions (Ethiopia, Kenya, and Tanzania), is 100% identical to the *T. lewisi* sequences available in GenBank and therefore can be unambiguously associated with this species. The assignment of the remaining nine genotypes of the phylogroup AF05 to T. lewisi or their consideration as separate species is problematic and cannot be unambiguously established.



Fig. 3. A *Herpetosoma* spp. richness accumulation curve generated for all tested rodent species captured in the seven studied sub-Saharan countries; number of observed (detected) species and three non-parametric species richness estimators (Chao 2, first- and second-order jackknife) are included in the inset panel.

In Herpetosoma spp. (49 genotypes belonging to at least 16 putative trypanosome species), we observed a positive correlation between the number of examined (as well as infected) individuals per mammalian genus (in host genera with an average or high prevalence) and the number of trypanosome genotypes (Supplementary Fig. S3). In rodents, based on accumulation curves, we slowly reached the anticipated Herpetosoma richness, estimated to be between 20 and 22 species (Fig. 3), while an increase in genotype number, which is estimated to be between 67 and 76, can still be expected (Supplementary Fig. S4). Furthermore, there were significant differences in the number of trypanosome species detected in individual countries. In Ethiopia, 13 parasite species were found, while in Tanzania, with the second highest number of micromammals tested, only five species were detected (Supplementary Table S7). Furthermore, their frequency varies significantly. For example, in Ethiopia, the three most dominant trypanosome species (AF05, AF06, and AF09) represent 73% of all positive cases. while the remaining 27% were assigned to 10 species (Fig. 4). Such an uneven distribution is strongly associated with the abundance of mammalian host species and their relative presence in captures.

One of the most important aspects of the detected trypanosome genotypes and species (phylogroups) concerns their host specificity. When analysing the trypanosome species richness in mammalian host genera (Figs. 5 and 6; Table 1, Supplementary Table S3, S6), the highest diversity, that is 16 genotypes representing 10 species, was found in spiny mice (Acomys spp.); however, additional findings of Herpetosoma spp. (4-7; Supplementary Fig. S5) and genotypes (9–16; Supplementary Fig. S6) can still be expected. The second highest genotypic diversity was detected in gerbils (Gerbilliscus spp.), but their 11 genotypes clustering into two closely related phylogroups (AF12 and AF13) more likely represent only two genetically highly variable Herpetosoma spp. (Fig. 1).

A trypanosome species detected in a large number of rodents implies rather low host specificity (Table 1 and Supplementary Table S3), but a closer look reveals a more complex picture. By far the lowest host specificity, and thus the widest host range. was detected for the phylogroup AF05 (the *T. lewisi* subclade). which was found in five out of seven studied sub-Saharan countries in 19 rodent species belonging to 12 genera (Figs. 2, 5, and Supplementary Fig. S7; Table 1 and Supplementary Table S6).

However even in this case it should be noted that only a single genotype AF05b (100% T. lewisi) shows a truly wide host spectrum (eight rodent genera), while the other genotypes of the phylogroup AF05 are much more host-specific, infecting maximally-two rodent genera, with one of them being dominant (Supplementary Table S6). Moreover, for the other four trypanosome species with more than a dozen positive individuals (AF06, AF09, AF12/AF13), only one dominant mammalian host genus was identified, while the infections in other host genera were rare (Figs. 1, 5; Table 1 and Supplementary Table S9), indicating a rather high host specificity.

4. Discussion

Our sample set comprises 1,640 individuals of native micromammals (represented by 132 species) and 28 individuals of the invasive black rat (Rattus rattus). The revealed 11.2% trypanosome prevalence correlates well with other studies (Hoare, 1972; Molyneux, 1976; Karbowiak et al., 2009; Noyes et al., 2002). The same applies to equal infection rates in males and females, reflecting the presumed transmission of the subgenus Herpetosoma through fleas, which do not markedly distinguish between sexes (Kiffner et al., 2013), although in some cases males were more infested than females (Krasnov et al., 2011; Kiffner et al., 2014).

Considerable variety in the prevalence of trypanosomes within the studied geographic area (Supplementary Table S4) merits attention, as they cannot be explained by substantial differences in the relative and/or absolute abundances of the hosts. However, we assume that the true infection rates are higher because the low prevalence is typical for sample sets in areas where sampling has taken place a long time ago (e.g., Malawi and Zambia sampled before 2010). The handling and processing of samples frequently occurred under substandard conditions, and it is likely that these circumstances influenced the artificial rate of false- negative samples.

Although some mammalian host species and genera appear to be trypanosome-free (Supplementary Tables S2 and S3), in most of these cases only a very limited number of individuals were screened (Supplementary Fig. S2). Within our dataset, the only exceptions are the genera Arvicanthis and Heliophobus, represented by five and one species, respectively, with over a dozen negative



Ethiopia

Fig. 4. Proportion of 13 trypanosome spp. detected in Ethiopia (the suffix "x" indicates that the species consist of more than one genotype).



Fig. 5. Host specificity of four *Trypanosoma* phylogroups/species where more than a dozen infected individuals were recorded. While AF05 (*Trypanosoma lewisi* subclade, 1a) shows very low host specificity (typical for *T. lewisi*), for the other three trypanosome species (AF06, AF09, and combined closely related AF12 + AF13) only one host genus (blue sector) is predominant, which indicates rather high host specificity. (The suffix "x" indicates that the species consist of more than one genotype.).

individuals per genus (more precisely 20 and 16, respectively). Hence, we propose that as long as a sufficient number of individuals has been examined, trypanosomes would be found in (almost) every rodent species.

The genera Acomys and Lophuromys (both of the murid subfamily Deomyinae) showed the highest prevalence of trypanosomes (36.7 and 18.9%, respectively), while at the other end of the spectrum the genera Aethomys and Arvicanthis (both of the tribe Arvicanthini) had an infection rate of 2.1% and 0%, respectively. These differences are robust, as each of these genera, except grass rat (Arvicanthis spp.) with only 20 individuals, was represented by over 100 examined specimens captured in different countries and biotopes (Supplementary Tables S1-S3). The high positivity of spiny mice (genus Acomys) may be a consequence of their life in colonies with facilitated transmission of fleas, the main vectors of rodent trypanosomes. The influence of colonial lifestyle on increased infection rates was documented for Trypanosoma nabiasi parasitizing rabbits (Grewal, 1957). A similar explanation based on facilitated transmission between individuals could also be applied to the second most infected group of rodents (brush-furred mice; genus Lophuromys), which often occur at high densities. On the contrary, the geographical distribution and type of habitat do not seem to significantly influence the overall trypanosome prevalence in these rodents, as brush-furred mice inhabit moist areas and grass edges of forests, while spiny mice occupy relatively arid savannahs and bushland.

Based on the almost full-size 18S rRNA sequences, the majority of detected trypanosome species show a clear phylogenetic affinity with the already described *Herpetosoma* spp. Our finding of 49 *Herpetosoma* genotypes representing at least 16 different species (15 of them being novel), more than doubles the species diversity of this subgenus (Figs. 1 and 2).

4.1. Diversity of Herpetosoma in African micromammals

Besides 10 genotypes belonging to the *T. lewisi* subclade (see below), we have detected a further 15 phylogroups (putative new species), which more than double the currently known and molecularly verified diversity of the subgenus *Herpetosoma*. Host identity constituted the main criterium for the descriptions of new *Herpetosoma* spp., even when minimal or no morphological variation was observed (Hoare, 1972), and our data are compatible with Hoare's concept of high host specificity. In rare cases, when certain trypanosome species were encountered in several rodent genera (e.g., AF06, AF09, AF12, and AF13), a single host genus clearly predominated, implying its importance for parasite maintenance (Fig. 5).



Fig. 6. Geographical distribution of 10 *Herpetosoma* phylogroups/species detected in spiny mice (*Acomys* spp.) in the seven studied sub-Saharan countries. Localities with multiple *Herpetosoma* spp. are indicated by arrows. Black dots show localities with only negative *Acomys* samples.

Estimation of true species richness (observed plus undetected) is statistically difficult, especially for highly diverse assemblages with many rare species (Chao and Chiu, 2016) and strongly depends on sampling effort, sample completeness, and in case of parasites, on host specificity and sensitivity of detection methods. Based on richness accumulation curves, we assume that we detected more than half of the rodent *Herpetosoma* spp. which inhabited the geographical area studied. Although the dataset of 1640 examined native micromammals is respectable, considering the presumed high host specificity of *Herpetosoma* and the vast diversity of rodents, as well as the still geographically limited surveys, we believe that the findings of several other trypanosome species can be expected, especially in rodent species and genera (as well as in insectivores), which are represented in our dataset by only a few individuals.

Some large rodent genera host several trypanosome species. Spiny mice (*Acomys*) are parasitised by at least 10 *Herpetosoma* spp. (up to six co-occurring in the same locality), with six of them (mainly AF06, but also AF10, AF11, AF15, AF16, and AF18) primarily confined to this rodent genus (Fig. 6). The strict host specificity of two closely related *Herpetosoma* spp. is well documented due to frequent infections encountered in gerbils (*Gerbilliscus* spp.), with AF12 confined to the Somali-Masai region, while AF13 has a much broader distribution throughout the Somali-Masai and Zambezian savannahs (Supplementary Fig. S8). Due to their endemic hosts, other *Herpetosoma* spp. are restricted to the Ethiopian Highlands, which is the case for AF09 and AF26 from brush-furred mice (*Lophuromys*) and dormice (*Graphiurus*), respectively (Supplementary Fig. S9). The uniqueness of Ethiopia in terms of species diversity is further reflected by being home to at least 10 additional *Herpetosoma* spp. detected in our dataset (Fig. 4; Supplementary Table S7), while Tanzania, which was equally heavily sampled, was second with just five trypanosome species. Therefore, the generally very high biodiversity of rodents in various ecosystems of Ethiopia (Bryja et al., 2019), which is considered one of the world's top biodiversity hotspots, also provides fertile ground for the speciation of their blood parasites.

Out of the 135 examined insectivores, six (4.4%) hosted a new, putatively shrew-specific trypanosome species (AF08, three genotypes), restricted to Ethiopian Highlands due to its endemic hosts, Glass's (*Crocidura glassi*) and Beletta (*C. yaldeni*) white-toothed shrews. Interestingly, this phylogroup is unrelated to trypanosomes recently described from insectivores (Mafie et al., 2019), namely *T. talpae, T. anourosoricis*, and *T. sapaensis*, which form a separate subclade (Fig. 1), indicating several independent acquisitions of trypanosomes by this mammalian group.

The well-supported basal branch AF22 of the now significantly expanded *Herpetosoma* clade is represented by flagellates of target rats (*Stochomys*) and wood mice (*Hylomyscus*) captured in the understudied Congo basin. This phylogroup differs considerably from other members of the subgenus and therefore constitutes an attractive taxon that may differ significantly from other *Herpetosoma* spp. from both the ecological and biological perspectives. It is even plausible that AF22 does not belong to the monophyletic subgenus *Herpetosoma* and represents a new subgenus.

4.2. Trypanosoma lewisi in African rodents and the quest for true T. lewisi

Within the 10 detected genotypes (AF05a-j) forming the *T. lewisi* subclade (Fig. 2), only a single one (AF05b) is 100% identical to the *T. lewisi* sequences available in GenBank (e.g., AB242273, AJ009156, and AJ223566), and can thus be unambiguously assigned to this species. Among the eight native rodent species captured in Ethiopia, Kenya, and Tanzania, the following six were detected as hosts of *T. lewisi* for the first time: *Aethomys chrysophilus, Dendromys mystacalis, D. harringtoni, Grammomys macmillani, Mus mahomet*, and Stenocephalemys albipes.

Several studies revealed a particularly high prevalence of *T. lewisi* in *Mastomys natalensis* (Maia da Silva et al., 2010; Ortiz et al., 2018; Egan et al., 2020), incriminating this very abundant, widespread, and synanthropic African rodent as the main reservoir of *T. lewisi* in domestic and peridomestic habitats. However, in our survey, only one *M. erythroleucus* out of 211 specimens of *Mastomys* spp. (143 *M. natalensis*, 53 *M. erythroleucus*, 14 *M. awashensis*, and one *M. kollmannspergeri*) was infected by *T. lewisi* (AF05b), with two individuals of *M. natalensis* being infected by the closely related genotype AF05i, and an additional seven individuals carrying other species of the subgenera *Herpetosoma* and *Squamatrypanum*. Therefore, our results do not support the key role of the genus *Mastomys* in the maintenance of *T. lewisi* in the studied geographical areas.

While AF05b corresponds to *T. lewisi* sensu stricto, the assignment of the other nine AF05 genotypes is less straightforward and demonstrates the limits of trypanosome taxonomy based solely on the 18S rRNA gene. The minute nucleotide differences (one to six substitutions) do not allow us to determine which of these newly detected genotypes merely extend the genetic variability of *T. lewisi* (e.g. AF05h) and which are genuine species (e.g. AF05j). Hence, our assumption that AF05h represents the true *T. lewisi* is based on a single nucleotide substitution distinguishing it from the available *T. lewisi* sequences and, more importantly, on its occurrence in black rat (*R. rattus*). Our findings also question the robustness of the previously published subdivision of *T. lewisi* into European and Asian isolates on one side and Brazilian isolates on

Table 1

Overview of all detected trypanosome phylogroups/species (the suffix "x" indicates that more than one genotype was detected) belonging to the genera *Herpetosoma*, *Ornithotrypanum* (*Orn.*), and *Squamatrypanum* (*Sq.*) and their mammalian host genera captured in seven sub-Saharan countries.

	Orn.	Sq.	Herpetosoma														\sum indiv.	\sum <i>Tryp.</i> species		
Host species	AF01	AF02	AF05x	AF06x	AF08x	AF09x	AF10	AF11x	AF12x	AF13x	AF15	AF16	AF18	AF20	AF21x	AF22	AF23	AF26x		
Acomys			3	36		1	2	4			6	1	3	1	1				58	10
Aethomys			3																3	1
Dendromus			1																1	1
Desmomys			1																1	1
Gerbilliscus									13	7									20	2
Grammomys			1																1	1
Graphiurus																		3	3	1
Hylomyscus																1			1	1
Lemniscomys	1		11						1										13	3
Lophuromys			1		1	23					1								26	4
Mastomys		1	3	3											3				10	4
Mus			10	1													1		12	3
Ochromyscus ^b															1				1	1
Praomys			13																13	1
Rattus			9																9	1
Saccostomus										2									2	1
Stenocephalemys			7			1													8	2
Stochomys																1			1	1
Crocidura ^c					6	1													7	2
No. individulas	1	1	63	40	7	26	2	4	14	9	7	1	3	1	5	2	1	3	190 ^a	
No. host genera	1	1	12	3	2	4	1	1	2	2	2	1	1	1	3	2	1	1		

indiv., individuals; Tryp., Trypanosoma.

^a Mixed infections were detected in one Lophuromys brunneus (AF05c + AF09a) and in two Gerbilliscus vicinus (AF12d + AF13d).

^b Formerly Myomyscus (see Nicolas et al., 2021; https://doi.org/10.1016/j.ympev.2021.107263).

^c Insectivores.

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the other side, which is based on a single nucleotide difference (Egan et al., 2020).

It is reasonable to assume that at least some AF05 genotypes actually represent separate species (e.g., AF05j differs from the typical T. lewisi AF05b genotype by four substitutions and from other AF05 genotypes by up to six substitutions). In any case, taxonomically relevant information on this most widely used marker should be weighed with caution, particularly in the case of the T. lewisi subclade, within which some verified species differ from each other by only a single nucleotide (Fig. 2). A detailed comparison of genetic variability within and between the 16 detected Herpetosoma phylogroups suggests that different mutation rates likely exist within individual clades, as was already noted for other trypanosomes (e.g. T. cruzi complex versus T. brucei complex) and the subfamily Leishmaniinae (Kaufer et al., 2017; Kostygov et al., 2021). While in the case of various genotypes clustering into the non-T. lewisi phylogroups we have good reason to believe that they represent a particular genetic variability within a single species (e.g. AF06, AF08, AF09, AF12, AF13, and AF21), this view can hardly be extended to the T. lewisi subclade (AF05). Therefore, we cannot apply the same scale and threshold to all detected phylogroups. It is plausible that within the *T. lewisi* subclade (Fig. 2), there is either a single species with several host-specific strains (and therefore T. blanchardi, T. musculi, T. niviventerae, and T. rabinowitschae are not valid species) or multiple very closely related species, the validity of which have to be confirmed by host specificity and crossinfections.

The geographical distribution of the *T. lewisi* subclade is largely consistent with the mid-elevations of the East African mountains, with two obvious exceptions (Supplementary Fig. S7): (i) the AF05e genotype was found only in two species of soft-furred mice (*Praomys*) living in forests at the source of the Zambezi River in Zambia, which biogeographically belong to the Congo basin (Bohoussou et al., 2015; Mizerovská et al., 2019); (ii) the AF05h genotype preferentially parasitizes the black rat (*R. rattus*) in two very distant geographical regions – low elevation regions in Ethiopia and the southern part of the Central African Republic. The data are consistent with the spreading of some *T. lewisi* genotypes (AF05b and AF05h) by non-native rodents, such as black rats.

4.3. Other trypanosome subgenera

Together with *Herpetosoma* spp., rodents are infected by a plethora of other trypanosomes. In the Neotropics, *T. (Schizotrypanum) cruzi, T. (Trypanozoon) brucei evansi,* and *T. (Squamatrypanum) lainsoni* have been found; furthermore, rodents also host *T. rangeli* and *T. conorhini* (both subgenus *Aneza*), as well as several undetermined trypanosomes morphologically classified into the subgenus *Megatrypanum* (Hoare, 1972; Pinto et al., 2006; Dobigny et al., 2011; Naiff and Barrett, 2013; Roque and Jansen, 2014; Salzer et al., 2016).

Although the majority of the detected genotypes belong to *Herpetosoma*, the two outliers encountered are affiliated with different subgenera (Fig. 1 and Supplementary Fig. S10). Genotype AF02 from Natal multimammate rat (*Mastomys natalensis*) captured in Zambia clusters within the subgenus *Squamatrypanum*, close to *T. varani* and other trypanosomes from African reptiles (Wenyon, 1908; Sato et al., 2009). This subgenus is a mixture of African and South American trypanosomes parasitizing vertebrates that range from lizards and snakes to rodents, marsupials, and bats (Viola et al., 2009; Rodrigues et al., 2019; Kostygov et al., 2021). The host promiscuity of some of these flagellates is particularly striking in some Neotropical *Squamatrypanum* species. *Trypanosoma lainsoni* was detected in Amazonian rodents, as well as in marsupials and bats (Naiff and Barrett, 2013; Rodrigues et al., 2019) and *T. cascavelli*, otherwise typical for reptiles (Ayala,

1970; Pessôa and De Biasi, 1972), was also detected in marsupials (Rodrigues et al., 2019).

Interestingly, species of the *T. varani* subclade were previously encountered in the blood of African rodents belonging to the genera *Mastomys* in Niger (Dobigny et al., 2011) and *Hylomyscus* and *Praomys* in Uganda (Salzer et al., 2016) but, regrettably, their sequences are not available in GenBank. The occurrence of *T. varani*-like trypanosomes in native rodents suggests that they are at least occasionally transmitted from prey to predators, such as pythons and monitor lizards, although this has not yet been experimentally proven. Whether trypanosomes from the *T. varani* subclade were originally parasites of reptiles or mammals remains an open question. In any case, our findings further support the recent establishment of the subgenus *Squamatrypanum* that accommodates trypanosomes of reptiles and mammals (Kostygov et al., 2021).

Finally, our finding of a single 18S rRNA sequence (AF01) in typical striped grass mouse (*Lemniscomys striatus*) from Tanzania is unexpected, as this trypanosome falls within the recently established subgenus *Ornithotrypanum*, formerly the *T. bennetti* clade (Kostygov et al., 2021). This cosmopolitan subgenus of avian trypanosomes is commonly present in various wild birds, and thus our isolated finding may be dismissed as possible contamination in the field. However, it should be noted that another avian-type trypanosome (PNW5) was previously detected in Dalton's praomys (*Praomys daltoni*) in Niger (Dobigny et al., 2011), again with publicly unavailable sequence. Therefore, at this point, rare infections of rodents with avian trypanosomes cannot be excluded.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2022.06.002.

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