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Molecular detection of *Rickettsia spp., Borrelia spp., Bartonella spp.* and *Yersinia pestis* in ectoparasites of endemic and domestic animals in southwest Madagascar

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ABSTRACT

Little is known about the presence of vector-borne bacteria in southwest Madagascar. Anthropogenic alteration of natural habitats represents an important driver for the emergence of new diseases. Especially the involvement of livestock and the involuntary maintaining of invasive synanthropic animals (particularly rats) facilitate disease transmission from wildlife to humans and associated animals and vice versa. The dissemination or acquisition of ectoparasites is most likely in regions where human/wildlife contact is increasing. Little is known about the presence of vector-borne bacteria in southwest Madagascar. In 2016 and 2017, ectoparasites were collected from various introduced (cattle and goats, cats, dogs and chicken, rats and mice) and native animal species (mouse lemurs [*Microcebus griseorufus*], Grandidier's mongooses [*Galidictis grandidieri*], bastard big-footed mice [*Macrotarsomys bastardi*], greater hedgehog tenrecs [*Setifer setosus*] and lesser hedgehog tenrecs [*Echinops telfairi*]) in the northern portion of Tsimanampetsotsa National Park and the adjacent littoral region. Thirteen species of blood-feeding ectoparasites (235 individuals of ticks [5 species], 414 lice [4 spp.] and 389 fleas [4 spp.]) were investigated for the presence and identity of rickettsiae, borreliae, bartonellae and *Yersinia pestis* using PCR techniques. *Rickettsia* spp. were detected in every single ectoparasite species (*Amblyomma variegatum, A. chabaudi, Rhipicephalus microplus, Haemaphysalis simplex, Argas echinops, Ctenocephalides felis, Echidnophaga gallinacea, Pulex irritans, Xenopsylla cheopis, Haematopinus quadripertusus, Linognathus africanus, L. vituli, Lemurpediculus verruculosus*). Lice and ticks were found harboring rickettsiae identified as *Rickettsia africae*, while *Rickettsia felis*-like bacteria were associated with fleas. *Borrelia* spp. were detected in 5% of *H. simplex* and 1% of *R. microplus* ticks. *Bartonella* spp. were detected in 40% of *H. quadripertusus* pools and in 5% of *L. verruculosus* pools. *Y. pestis* was detected in *X. cheopis* and *E. gallinacea* fleas collected from a rat. This study presents the detection of a broad spectrum of vector-borne bacteria including potential pathogens, and an unexpected finding of *Y. pestis* far off the known plague foci in Madagascar.

1. Introduction

Approximately 75% of human diseases originate from wildlife and/or domestic animals (Taylor et al., 2001), many of which require ectoparasites for transmission (The World Health Organization (WHO), 2017a). The drivers of emerging infectious diseases have been known for years and are debated intensively (Daszak et al., 2000; Githeko et al., 2000; Patz et al., 2000, 2003; Young et al., 2017). Several factors supporting disease emergence or zoonotic transmission pathways congregate in Madagascar: Importation of domestic animals and introduction of invasive species (Goodman, 1995; Stachurski et al., 2013), alteration of natural habitats through deforestation, replacement of forests with agricultural land, or habitat fragmentation (Harper et al., 2007; Brinkmann et al., 2014). In addition, climate change is expected

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to alter the distribution ranges of parasite species (Barrett et al., 2013). Biodiversity loss may weaken the dilution effect and hence may trigger increasing parasitism (Dobson et al., 2006; Civitello et al., 2015). Due to the rapid human encroachment of forest habitats the contact zones between synanthropic animals (i.e. livestock [Ratovonamana et al., 2013] as well as rats [Goodman, 1995; Ganzhorn, 2003]) and the endemic wildlife expand. The increased mutual contact can result in extended host spectra for ectoparasites or development of new reservoirs for pathogens in both ways.

The combination with a poor public health infrastructure causes a complex situation in Madagascar. The lack of human and animal health facilities, especially in rural areas, restricts the feasibility of proper diagnosis and treatment as well as the availability of epidemiological data on vector-borne infections (Barmania, 2015). Thus, there is a need for knowing the potential of ectoparasites to transmit diseases and how to prevent it (Bardsley and Thrusfield, 2014). Only a small number of studies about ectoparasitic fauna from recent years present data on harbored pathogens (e.g. Rakotonanahary et al., 2017; Qurollo et al., 2018). Since Madagascar seems to be susceptible to emerging vector-borne diseases to a high degree, there is a demand for investigations of this kind.

Here, we investigated several arthropod vectors (ticks, lice, fleas) from livestock (cattle and goats) and domestic animals (cats, dogs and chicken), peridomestic rodents (rats and mice) as well as endemic small mammals (mouse lemurs (*Microcebus griseorufus*), Grandidier's mongooses (*Galidictis grandidieri*), bastard big-footed mice (*Macrotarsomys bastardi*), greater (*Setifer setosus*) and lesser hedgehog tenrecs (*Echinops telfairi*) for the presence of rickettsiae, borreliae, bartonellae and *Y. pestis*. With the present data we provide a first overview of bacterial pathogens circulating in various groups of bloodsucking ectoparasites known for their vector potential, in order to estimate the relevance of vector-borne diseases in southwest Madagascar.

2. Methods

2.1. Study site

The study area is located in southwest Madagascar (Fig. 1) enclosing the coastal plain at the western edge of Tsimanampetsotsa National Park and parts of the Mahafaly plateau (~200 m a.s.l.) in the east of it. The region belongs to the spiny forest ecosystem characterized by dry and drought adapted vegetation (Fenn, 2003). Its climatic conditions are distinguished by irregular rainfall, often less than 400 mm averaged per year, and an annual mean temperature of 24 °C (Ratovonamana et al., 2011). The local population predominantly relies on pastoralism and agriculture. In the surroundings of the villages there are cultivated fields with hedges bordering on sparsely vegetated shrubland.

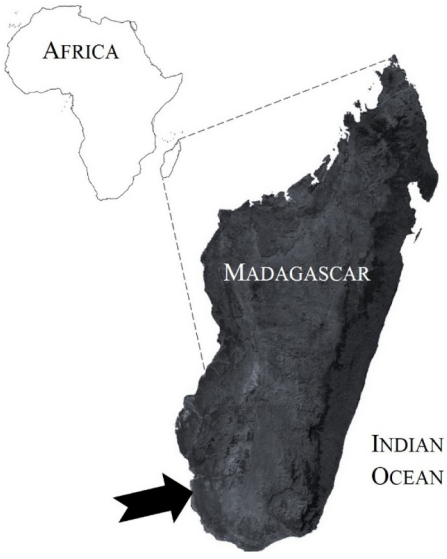

Fig. 1. Map of Madagascar. Madagascar is located in the Western Indian Ocean close to the coast of East Africa. The arrow points to the study site (E 43.6998, S 24.0786).

2.2. Arthropod collection

The arthropods processed in this study, were collected during a project investigating the ectoparasite burdens of mammalian hosts (Ehlers et al., 2019). In brief, ectoparasites were collected from livestock and domestic animals as well as trapped mice, rats and endemic small mammals in the dry season of 2016 and the rainy season of 2017. A smaller portion of the collection was included from sampling efforts conducted at the study site earlier (2013/2014). For a more detailed description of the trapping procedure and arthropod collection see Ehlers et al. (2019). All specimens were stored in 100% ethanol until further processing in the laboratory in Hamburg. The total collection consisted of 13 different ectoparasite species, comprising 1038 individuals (235 ticks, 389 fleas and 414 lice), found on 12 host species. An overview of the mammalian hosts portioned according to their ectoparasites and the results of the bacterial screening is provided in the Supplementary File 3.

2.3. Dissection of rat and mouse organs

Rats and mice were sacrificed by cervical dislocation and sprayed with ethanol to immobilize hosted fleas. Spleens and kidneys were dissected using disposable scalpel blades and stored in 100% ethanol. Personal protective equipment concluded FTP3 dust mask, goggles and disposable gloves.

2.4. DNA extraction

DNA of fleas and lice was extracted from pooled individuals (up to ten) resulting in 76 pools of fleas and 102 pools of lice. Ticks were processed individually. Arthropods were dried on filter paper, and transferred to 2.0 mL tubes containing two 3 mm steel beads. Ticks and the solid *Heamatopinus* lice were roughly cut into pieces using pointed scalpel blades (Bayha, Tuttlingen, Germany). The tubes were heated to 50 °C for 5 min to evaporate remaining ethanol. The samples were then homogenized in 180 µL PBS using the TissueLyzer LT (Qiagen, Hilden, Germany) for 4 min at 30 Hz. The next steps followed the insect protocol for the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) with minor modifications: The lysing temperature was raised to 65 °C and DNA was eluted with 100 µL buffer AE. A negative extraction control was processed along with each batch of arthropods (24 samples). Organ samples were treated according to the standard tissue protocol.

2.5. Pathogen DNA detection

Rickettsial DNA was detected by targeting the *ompB* gene with the primers 120-M59 and 120–807 (Roux and Raoult, 2000). Detection of *Borrelia* DNA was performed by combining an initial 16S rDNA screening qPCR (Parola et al., 2011) with conventional PCRs targeting *gyrB* (with three different primer pairs: gyrB-5′+1/gyrB-3′+1 (Schwan et al., 2005), gyrB-F/gyrB-R and gyrB-343/gyrB-1480, both designed for this study), *flaB* (with primers flaB-BOR1/flaB-BOR2 (Assous et al., 2006)), and a larger fragment portion of the 16S rDNA (Barbour et al., 1996). The qPCR reaction proceeded in a 20 µL volume containing 16 µL master mix (0.16 µL Platinum Taq polymerase, 4 mM $MgCl_2$, 0.2 mM each dNTP, 0.6 µM each primer, 0.2 µM probe and 0.04 µM BSA) and 4 µL template DNA. Flea samples were excluded from

Table 1
PCR based screening results of pathogen DNA isolated from individual ticks (PREV = prevalence; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis; n.d. = not done).

Tick species	*Rickettsia* sp. neg	pos	PREV	*Borrelia* sp. neg	pos	PREV	*Bartonella* sp. neg	pos	PREV
Amblyomma variegatum (*n* = 28)	0	28	100%	28	0	0%	28	0	0%
Amblyomma chabaudi (*n* = 2)	0	2	100%	2	0	0%		n.d.	
Argas echinops (*n* = 10)	9	1	10%	10	0	0%		n.d.	
Haemaphysalis simplex (*n* = 19)	17	2	11%	18	1	5%		n.d.	
Rhipicephalus microplus (*n* = 176)	148	28	16%	174	2	1%		n.d.	
total (*n* = 235)	174	61	26%	232	3	1%			

the *Borrelia* screening.

The presence of *Bartonella* DNA was verified by an initial qPCR targeting the 16S-23S ribosomal RNA intergenic spacer using primers ITS-Bart-F and ITS-Bart-R and the probe ITS-Bart-probe designed in our laboratory. The reaction mix consisted of 10 µL QuantiTect Probe PCR Master Mix, 0.4 µM each primer, 0.2 µM probe and 2µL template DNA in a final volume of 20 µL. Positive samples were confirmed by two consecutive conventional PCRs of the genes *gltA* (Norman et al., 1995) and *rpoB* (Renesto et al., 2001). Tick samples, except *Amblyomma variegatum*, were excluded from the *Bartonella* screening.

Flea DNA samples were additionally screened for *Y. pestis* by qPCR targeting the PCP1 gene using primers YpplaS1 and YpplaAs1 and probe YpplaP (Panning, unpublished). The PCR was performed using HotStarTaq *Plus* DNA polymerase in a 20 µL reaction volume with 0.12 µL of the enzyme, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each primer, 0.2 µM probe and 2 µL template DNA.

For the conventional PCR assays we used either Platinum Taq DNA polymerase or HotStarTaq DNA polymerase (for *Bartonella gltA* and *Borrelia gyrB*) in a 25 µL reaction volume composed of 0.15 µL polymerase, 1.5 mM $MgCl_2$ (2 mM for HotStarTaq) 0.2 mM each dNTP, 0.4 µM (0.6 µM) each primer and 2 µL template DNA. (Detailed PCR conditions and oligonucleotide sequences are listed in the Supplementary File 2).

Conventional PCRs were run with a Peqlab Primus 96 advanced® thermal cycler (PEQLAB Biotechnologie GMBH, Erlangen, Germany), for qPCR the LightCycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany) was used. A positive and a negative control were included in each run.

2.6. Calculation of prevalence and minimal infection rate (MIR)

The prevalence (percentage of infected individuals) was calculated by dividing the number of infected individuals by the total number of tested specimens. The MIR was calculated by dividing the number of positive pools by the total number of individuals from all pools per species.

2.7. Sequence analysis

The success of the conventional PCR amplification was verified by electrophoresis of the PCR products on 1% agarose gels in Tris-acetate-EDTA buffer (TAE) and staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, USA) added to the liquid gel. Positive samples were purified according to the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent to Microsynth Seqlab (Göttingen, Germany) for sequencing.

DNA sequences were checked for quality and ambiguous signals were trimmed using BioEdit Sequence Alignment Editor (version 7.2.5; Hall, 1999). Basic Local Alignment Search Tool (BLAST) was used for comparison to sequences deposited in the NCBI GenBank (Zhang et al., 2000). Sequences of PCR products and those obtained from GenBank were aligned by using ClustalW (Thompson et al., 1994) implemented in BioEdit. The MEGA 7 software (version 7.0.20; Kumar et al., 2016) was used for phylogenetic analysis by applying the neighbor-joining method (Saitou and Nei, 1987) in a complete deletion procedure, performing 1.000 bootstrap replications (Felsenstein, 1985). The evolutionary distances of the trees were computed using the Kimura 2-parameter method (Kimura, 1980).

3. Results

3.1. Rickettsia screening

OmpB rickettsial DNA was detected in all of the 13 ectoparasite species. In ticks, there was an overall prevalence of 26% (61/235) led by *Amblyomma* ticks (*A. variegatum* and *A. chabaudi*), which revealed a prevalence of 100% (30/30), followed by 16% *Rickettsia* prevalence in *Rhipicephalus microplus* (28/176), 11% in *Haemaphysalis simplex* (2/17) and 10% in *Argas echinops* (1/10; Table 1). Regarding fleas, 36 of 76 pools proved to contain rickettsial DNA (MIR 9%). In detail, the partial *ompB* gene was amplified from 5/6 *Ctenocephalides felis* pools (MIR 16%), from 22/49 *Echidnophaga gallinacea* pools (MIR 8%), from 1/4 *Pulex irritans* pools and from 8/17 *Xenopsylla cheopis* pools (MIR 14%; Table 2). *OmpB* amplification of lice pools revealed similar infection rates to fleas (overall MIR 10%; 43/102 positive pools). 12/35 *Haematopinus quadripertusus* (MIR 7%), 16/37 *Lemurpediculus verruculosus* (MIR 17%), 10/22 *Linognathus africanus* (MIR 11%) and 5/8 *Linognathus vituli* pools (MIR 8%) were successfully amplified (Table 3).

Analyses of sequenced partial *ompB* PCR products confirmed most of these sequences (16/20) matching to *Rickettsia africae* (Fig. 2). The sequences shared 99.9% (1/783 mismatch; GenBank accession numbers MN304852, MN304853, MN304858, MN304859, MN304861, and

Table 2
PCR based screening results of pathogen DNA isolated from pooled fleas (MIR = minimal infection rate; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis).

Flea species	*Rickettsia sp.* neg	pos	MIR	*Bartonella sp.* neg	pos	MIR	*Yersinia sp.* neg	pos	MIR
Ctenocephalides felis (*n* = 31)	1	5	16%	6	0	0%	6	0	0%
Echidnophaga gallinacea (*n* = 289)	27	22	8%	49	0	0%	46	3	1%
Pulex irritans (*n* = 10)	3	1	10%	4	0	0%	4	0	0%
Xenopsylla cheopis (*n* = 59)	9	8	14%	17	0	0%	16	1	2%
total (*n* = 389)	40	36	9%	76	0	0%	72	4	1%

Table 3
PCR based screening results of pathogen DNA isolated from pooled lice (MIR = minimal infection rate; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis).

Louse species	*Rickettsia sp.*			*Borrelia sp.*			*Bartonella sp.*		
	neg	pos	MIR	neg	pos	MIR	neg	pos	MIR
Haematopinus quadripertusus (*n* = 167)	23	12	7%	35	0	0%	21	14	8%
Lemurpediculus verruculosus (*n* = 96)	21	16	17%	37	0	0%	35	2	2%
Linognathus africanus (*n* = 87)	12	10	11%	22	0	0%	22	0	0%
Linognathus vituli (*n* = 64)	3	5	8%	8	0	0%	8	0	0%
total (*n* = 414)	59	43	10%	102	0	0%	86	16	4%

MN304862; detailed BLAST results are listed in the Supplementary File 1) to 100% sequence identity with *R. africae* strain ESF-5 (CP001612). The aberration, a C→T transition (a change from leucine to proline) at position 182, was seen in each one of the louse-derived sequences (MN304863, MN304864, MN304865 and MN304866), by all flea sequences except *P. irritans* (MN304848, MN304849, MN304850, MN304851 and MN304856) and by the tick *A. chabaudi* (MN304860). The PCR product of the remaining four sequences revealed a 6 bp insertion at position 848 (of the ORF) like *Rickettsia felis* and were clustered accordingly in the phylogenetic tree (Fig. 2). They all originated from flea samples. The detected DNA of a single *C. felis* pool showed 100% homology (789/789 bp; MN304847) compared to those of *R. felis* (CP000053). The other three sequences (MN304854, MN304855 and MN304857) showed only 96.2% homology to *R. felis*, but were closer related (98.4%) to another recently discovered candidate *Rickettsia sp*. called Candidatus *Rickettsia senegalensis* (KU499848). The flea species providing this deviating sequence were *E. gallinacea, X. cheopis* and *P. irritans*.

3.2. Borrelia screening

The *Borrelia* qPCR generated many false positive results (78/235

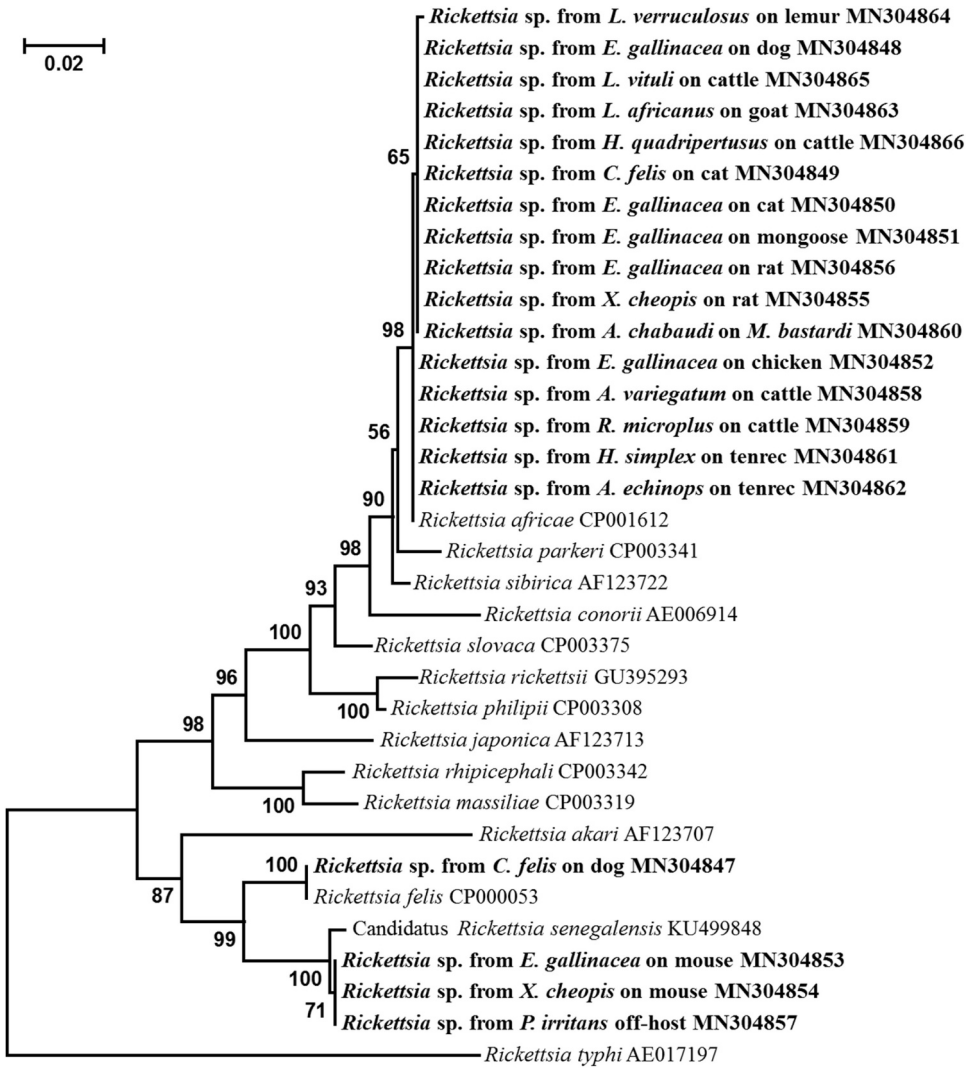

Fig. 2. Evolutionary relationship of *Rickettsia* spp. based on *ompB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.52448091 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 777 positions in the final dataset.

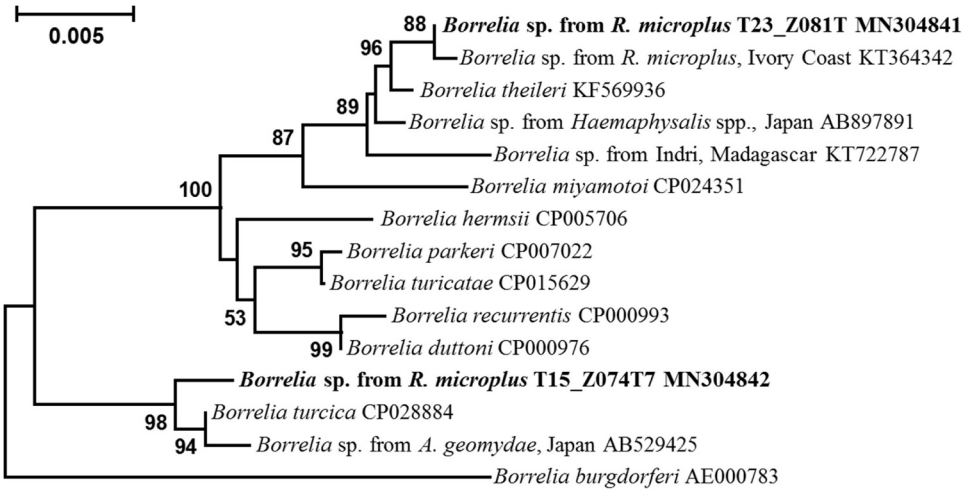

Fig. 3. Evolutionary relationship of *Borrelia* spp. based on 16S rDNA alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.08247409 is shown. The bootstrap values >50 are shown next to the branches. The tree is drawn to scale. There were a total of 1083 positions in the final dataset.

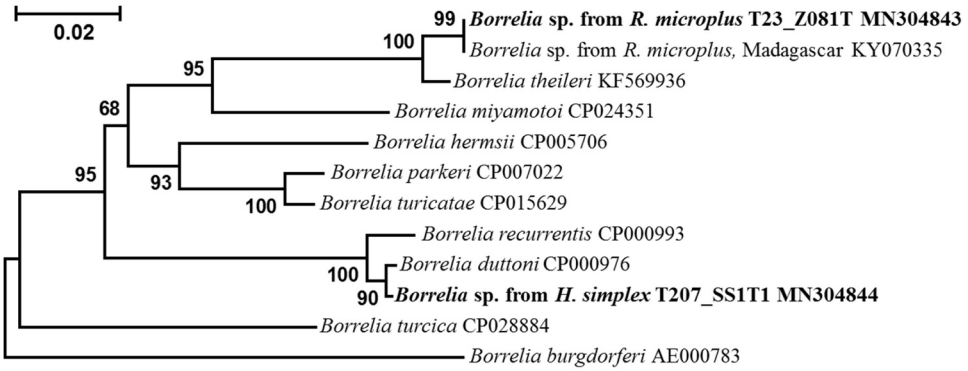

Fig. 4. Evolutionary relationship of *Borrelia* spp. based on *flaB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.41705634 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 704 positions in the final dataset.

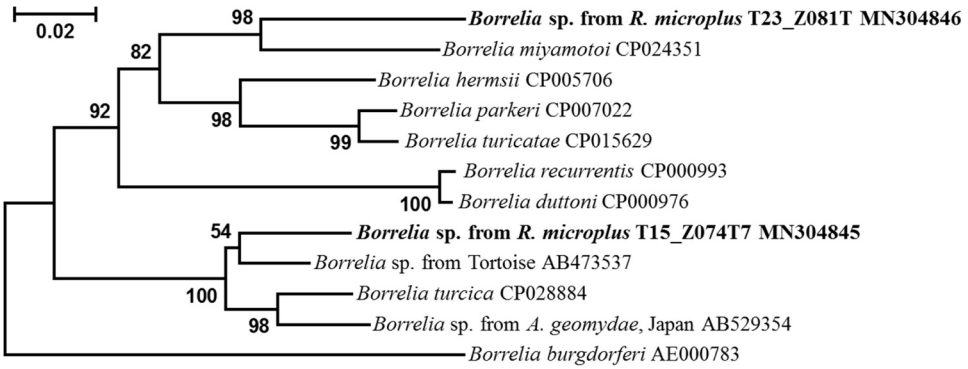

Fig. 5. Evolutionary relationship of *Borrelia* spp. based on *gyrB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.61223084 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 594 positions in the final dataset.

tick and 52/110 louse samples were positive), and only three tick samples could be confirmed by at least one of the three consecutive conventional PCRs. Ct-values of false positive ranged between 40 and 27.6 but the three positive samples showed clearer exponentially ascending fluorescence curves in the qPCR with ct-values of 23.13, 27.22 and 32.71, respectively. *Borrelia* DNA was detected in 1/19 (5%) *H. simplex* and in 2/174 (1%) *R. microplus* ticks (table 1). Sequence analysis of a 1084 bp portion of 16S DNA showed 99.9% similarity of the first *R. microplus* sample (MN304841; Fig. 3) with an uncultured *Borrelia* sp. from Ivory Coast, also detected in *R. microplus* (KT364342) and 99.7% identity to *Borrelia theileri* (KF569936). A *Borrelia* sp. from Madagascar detected in the blood of the lemur *Indri indri* equaled to 99.2% (KT722787). The *flaB* sequence (MN304843) analysis confirmed the close relationship to *B. theileri* (98.7%, 9/713 mismatches), and additionally revealed 100% identity to another Madagascan *Borrelia* sequence deposited in GenBank (KY070335; Fig. 4). The closest match for the *gyrB* (MN304846) sequence was *Borrelia miyamotoi* with 91% (CP024351; Fig. 5). There was no fitting *gyrB* sequence available for *B. theileri*. The second *Borrelia* sp. from *R. microplus* clustered together with *Borrelia turcica* (CP028884) and other tortoise associated but uncultured *Borrelia* spp. (see Fig. 3 and Fig. 5). In the *gyrB* sequence alignment this sample (MN304845) showed the highest similarity to a *Borrelia* sp. detected in tissue from a tortoise in Japan (AB473537; 95.8%), followed by 94.4% identity to *B. turcica* and 93.8% identity to a *Borrelia* sp. hosted by tortoise tick *Amblyomma geomydae* (AB529356; Fig. 5). The corresponding values for the 16S sequence analysis (MN304842) were 99.6% (*B. turcica*) and 99.4% (*Borrelia* sp., *A. geomydae*), respectively (Fig. 3). We were not able to get a *flaB* amplificate for this sample. Yet, the *flaB* amplification was the only successful for the *Haemaphysalis* DNA sample. Here, we obtained 97.3% identity of the 722 bp sequence (MN304844) to *Borrelia duttoni* (CP000976) and 97% identity to *Borrelia recurrentis* (CP000993; Fig. 4).

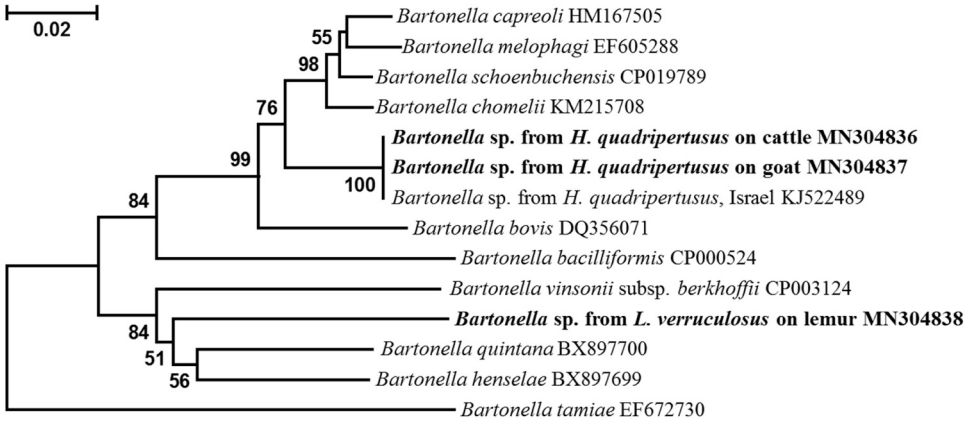

Fig. 6. Evolutionary relationship of *Bartonella* spp. based on *rpoB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.55666004 is shown. The bootstrap values >50 are shown next to the branches. The tree is drawn to scale. There were a total of 839 positions in the final dataset.

3.3. Bartonella screening

Neither *A. variegatum* nor any flea species DNA sample was screened positive by the *Bartonella* ITS qPCR. For louse pools we received 24 positive reactions, yet 11 of them had a ct-value above 40, three of which were confirmed by *gltA* or *rpoB* conventional PCR. 14/35 positive *H. quadripertusus* pools (MIR 8%) and 2/37 (MIR 2%) positive *L. verruculosus* pools were found finally (Table 3). Two different strains of *Bartonella* sp. were detected, one from *H. quadripertusus* and the other from *L. verruculosus*. Both *H. quadripertusus* associated PCR products (*rpoB*: MN304836; *gltA*: MN304840) showed 100% similarity to an uncultured *Bartonella* sp. from Israel (KJ522489; KJ522487), which was also recovered from *H. quadripertusus* (Fig. 6). The species most related to this phylum was represented by *Bartonella chomelii* sharing 96.2% identical bases of *rpoB* sequence (KM215708) and 96.1% identity of *gltA* sequence (KM215692). The *rpoB* amplification of the *L. verruculosus* pools (MN304838) revealed 90.6% sequence identity with *Bartonella henselae* (BX897699) and 90.1% identity to a GenBank entry of *Bartonella quintana* (BX897700). The phylogenetic tree based on *gltA* sequences is not shown.

3.4. Y. pestis screening

The presence of *Y. pestis* DNA was proved in 4/76 flea samples (MIR 2%), comprising three pools of *E. gallinacea* and one pool of *X. cheopis*, by specific qPCR (Table 2). All four pools contained fleas collected from the same black rat (*R. rattus*) individual. Spleen and kidney DNA samples from this rat were also screened positive by qPCR. *Y. pestis* DNA was further detected in spleen samples of two other hosts, one mouse and one rat. However, ct-values where quite high (fleas: 38.84–45; kidney: 40.82; spleens: 34.93/39.58 (rats) and 40.59 (mouse)).

3.5. Co-infection

Among all investigated ectoparasite individuals or pools, seven were co-infected by rickettsiae and bartonellae (six *H. quadripertusus* and one *L. verruculosus*).

4. Discussion

Current changes in land use and the transformation of habitats bear the threat of yet unknown transmissions of pathogens between native and introduced animal species as well as between animals and humans. These transmissions can have fatal effects on all hosts affected (e.g., Ostfeld et al., 2005; Köndgen et al., 2017). The data presented here add analyses of possible bacterial pathogens to previous studies on the occurrence of arthropod ectoparasites on various animal species of southwestern Madagascar (Ehlers et al., 2016, 2019). While rickettsial DNA was detected in all species of arthropod hosts, findings of borreliae, bartonellae and *Y. pestis* were limited to only two ectoparasite species each. In the following paragraphs we review and discuss the addressed pathogens separated by genus.

4.1. Rickettsiae

The screening results presented in this study revealed a remarkably broad spectrum of ectoparasite species containing rickettsial DNA. Rickettsiae are Gram-negative obligate intracellular pathogens which reveal a remarkably wide range of arthropod hosts including blood-sucking ectoparasites (Weinert et al., 2009). Recent phylogenetic analyses subdivide this genus into four main groups, the ancestral, transitional, typhus (TG), and spotted fever group (SFG; Gillespie et al., 2008). SFG rickettsiae are predominantly transmitted by ticks and cause mild to severe forms of spotted fever (Fournier et al., 2009). The TG rickettsiae, *Rickettsia typhi* and *Rickettsia prowazekii*, are the agents of endemic (murine) and epidemic typhus, respectively. Whereas *R. prowazekii* is transmitted by the body louse (*Pediculus humanus corporis*; Parola et al., 2006), *R. typhi* is transmitted by fleas, usually *X. cheopis*, but also by other flea species and arthropod vectors (Azad, 1990). *Rickettsia felis,* a further flea-borne rickettsial pathogen, belongs to the transitional group and is transmitted mainly by the cat flea *C. felis*. As for *R. felis* the pathogenic potential of the other so called *R. felis*-like organisms (RFLOs) is still unclear and new species are described constantly (Jiang et al., 2013; Lopez-Velez et al., 2015; Mediannikov et al., 2015).

Each of the 13 species of ticks, fleas or lice was tested positive for rickettsial DNA (Tables 1-3). Compared to this broad diversity of host species, the diversity of *Rickettsia* spp. obtained from the phylogenetic analyses appeared relatively narrow. The majority of the DNA sequences could be assigned to *R. africae* (Fig. 2), resembling 100% of the *ompB* sequence or holding one mismatch, respectively. Except *P. irritans* (n = 10), all ectoparasite species provided *R. africae* DNA. *Rickettsia africae* is widely distributed on the African continent and has been detected in at least 13 hard tick species (Parola et al., 2013; Halajian et al., 2018) and one bird flea species (Sekeyová et al., 2012), but, to our knowledge, there are no further reports of detection of *R. africae* in soft ticks, louse or flea species so far. High prevalence of *R. africae* seems to be common for *A. variegatum* ticks. Previous studies in African countries found prevalences of *R. africae* beyond 50% in Nigeria and Uganda (Lorusso et al., 2013), the Union of the Comoros (Yssouf et al., 2014), Cameroon (Vanegas et al., 2018) and Côte d'Ivoire (Ehounoud et al., 2016). However, the situation in Madagascar seems to be special since this is the third study reporting 100% of a cohort of *Amblyomma* ticks being infected by *R. africae* (Keller et al., 2016; Ehlers et al., 2016). Due to transovarial and transstadial (vertical) transmission of *R. africae* in *A. variegatum* (Socolovschi et al., 2009), this phenomenon is consequential. Nevertheless, human infections by SFG rickettsiae are only anecdotally documented in Madagascar

(Fournier et al., 2009). Lower prevalence, as discovered in Mozambique (5.6%; Matsimbe et al., 2017) or Nigeria (Ogo et al., 2012), for instance, seems to be an exception. Throughout the literature involving rickettsial burdens of *Rhipicephalus* spp. from cattle, prevalences are significantly lower, ranging from 0 to 10% (Ogo et al., 2012; Yssouf et al., 2014; Ehounoud et al., 2016; Vanegas et al., 2018). Again, our results from Madagascar depict another situation heading with 16% (28/176) of *R. microplus* containing *R. africae* DNA.

The other rickettsiae found in our study derived from fleas and were 100% homologous to *R. felis* (one pool of *C. felis*.) or clustered together with Candidatus *R. senegalensis* (Fig. 2), which was first discovered in cat fleas (Mediannikov et al., 2015). Due to our findings we can now add three flea species (*E. gallinacea, X. cheopis* and *P. irritans*) to the list of potential hosts for this RFLO. *Rickettsia felis* has been associated with 12 flea species in continental Africa (Brown and Macaluso, 2016). Its principal vector, *C. felis*, was demonstrated to be capable of vertical transmission of *R. felis* (Wedincamp and Foil, 2002) and high prevalence of this bacterium (up to 95%) in central Africa seems to be common (Mediannikov et al., 2012; Leulmi et al., 2014). In Madagascar, *R. felis* had been detected in *X. cheopis* (2%) and in *P. irritans* fleas (26%; Rakotonanahary et al., 2017). The same study reports *R. typhi* infection of *X. cheopis*; and there are also reports of murine typhus in travelers acquired in Madagascar (Walter et al., 2012). Yet, we did not find *R. typhi* in our study.

4.2. Borreliae

Compared to *Rickettsia* spp., borreliae seem to be less prevalent in Madagascar. The genus *Borrelia* phylogenetically splits up into three main groups: Lyme disease (LD) borreliae, relapsing fever (RF) borreliae, and reptile-associated (REP) borreliae (Takano et al., 2010; Franke et al., 2013). LD is the most relevant tick-borne disease from the northern hemisphere, while in Sub-Saharan Africa pathogenic borreliae are represented by the RF group of the genus. An infection with *B. recurrentis*, transmitted to humans by *Pediculus humanus corporis*, is called epidemic or louse-borne relapsing fever (LBRF). In South and East Africa and Madagascar tick-borne relapsing fever (TBRF) is caused by *B. duttoni* transmitted by soft ticks of the *Ornithodorus moubata* complex. Yet, there are many more *Borrelia* spp., each transmitted by its specific soft tick vector worldwide, while some RF species such as *Borrelia lonestari, B. theileri* and *B. miyamotoi* are transmitted by hard ticks (Lee et al., 2014a; Talagrand-Reboul et al., 2018). Endemic RF has not been diagnosed in Madagascar since the early 1950s, probably due to the decline of the *O. moubata* s.l. vector population (Colas-Belcour et al., 1952; Rodhain and Fontenille, 1989). Epidemic RF has never been diagnosed in Madagascar, but seems to be emerging regularly in continental Africa (Elbir et al., 2013). Borrelioses of domestic and wildlife animals are comparably neglected in Madagascar despite the presence of their potential vectors. For example, the cattle tick *R. microplus* known to host *B. theileri* (Smith et al., 1978), a species causing bovine borreliosis, is well established over the whole country (Stachurski et al., 2013). *Borrelia* spp. was also detected in the blood of three lemur species (*Lepilemur mustelinus, Propithecus diadema* and *Indri indri*; Larsen et al., 2016; Qurollo et al., 2018).

In the course of our *Borrelia* screening, only three tick individuals were found to carry borreliae (Table 1). These were two out of 176 *R. microplus* (1%). Interestingly, one of them clustered together with the tortoise associated portion of the genus as confirmed by the 16S rDNA and *gyrB* alignment (Figs. 3 and 5). The other *Borrelia* DNA sample obtained from *R. microplus* was similar to *R. theileri* 16S rDNA (99.7%) and also showed 99.2% identity to borreliae detected in lemur blood (Larsen et al., 2016), indicating a high distribution of this pathogen. Hagen et al. (2018) detected the same borreliae in close proximity to our study site. The prevalence reported here is congruent to our results and these of other studies, which found *B. theileri* infecting less than 1% of *R. microplus* and 0.5% of *Rhipicephalus geigyi* ticks, respectively (Yparraguirre et al., 2007; McCoy et al., 2014). While *Haemaphysalis* spp. hosting borreliae are well known from east Asia (Lee et al., 2014b; Furuno et al., 2017; Khoo et al., 2017), our finding of *Borrelia* DNA in a *Haemaphysalis* tick is apparently a rarity regarding Africa. This sample showed 97.3% identity to *B. duttoni* (Fig. 4), the causative agent of endemic RF in human. This could be a hint of the *Haemaphysalis* tick and perhaps its tenrec host maintaining the pathogen in absence of its main vector *O. moubata*.

4.3. Bartonellae

There is a very limited number of studies about the presence of *Bartonella* sp. in Madagascar, reporting the detection of *Bartonella* DNA in fruit bats and their ectoparasites (bat flies and fleas; Brook et al., 2015), in rats, and *Synopsyllus fonquerniei* fleas (Brook et al., 2017), in one *Haemaphysalis lemuris* tick (Lado et al., 2018) and in both head (Eremeeva et al., 2019) and body lice (Sangaré et al., 2014). Bartonellae are Gram-negative facultative intracellular bacteria whose vectors are known distinctively for only a few species: *Bartonella bacilliformis* (the agent of Oroya fever) is transmitted by sandflies of the genus *Lutzomyia* (Battistini, 1931), *B. henselae* (causative agent of cat scratch disease) is transmitted by *C. felis*, and *B. quintana* (the trench fever pathogen) by *P. humanus* (Billeter et al., 2008).

We found *Bartonella* infection of two louse species: The cosmopolitan cattle louse, *H. quadripertusus*, and *L. verruculosus* (table 3), endemic in Madagascar and specialized in mouse lemurs (Ehlers et al., 2019). A potentially new *Bartonella* variant, closely related to other bartonellae infecting ruminants, was detected in 14/35 *H. quadripertusus* pools. As indicated by 100% identity of the amplified *rpoB* sequence (Fig. 6), the same strain was detected earlier in Israel, also in *H. quadripertusus* (Gutiérrez et al., 2014). The *rpoB* sequence derived from *Bartonella* DNA isolated from 2/70 *L. verruculosus* pools interestingly revealed closest relationship to the two pathogenic *Bartonella* spp., *B. henselae* and *B. quintana*. Yet, there is only similarity of about 90% to both species, indicating that this *Bartonella* sp. might be a novel strain as well.

Ticks are under growing suspicion of being capable to transmit *Bartonella* spp., at least the genera *Dermacentor* and *Ixodes* (Angelakis et al., 2010a, b). In Taiwan, *R. microplus* exhibited a *Bartonella* prevalence of 15.7% (Tsai et al., 2011a). However, all ticks subjected to our *Bartonella* screening were tested negative (Table 1).

The same applies to fleas which seem to be commonly infected by bartonellae elsewhere (Sackal et al., 2008; Tsai et al., 2010; Fernandez-Gonzalez et al., 2016), but not at our study site (Table 2). Negative results are also presented from the Democratic Republic of the Congo, where 123 fleas were screened for bartonellae (Leulmi et al., 2014). These findings are coherent to the situation in Sub-Saharan Africa, where the occurrence of bartonellae is rare, or related studies have not been conducted (see reviews by Billeter et al., 2008; Tsai et al., 2011b).

4.4. Y. pestis

Plague, caused by the Gram-negative bacterium *Y. pestis*, is undeniably the most notorious disease in Madagascar. The WHO (Bertherat, 2016) reported 3248 human cases from 2010 to 2015 worldwide, including 584 deaths. Madagascar is by far the most affected country with 2604 reported cases (476 deaths) attributed to pneumonic and bubonic plague and sepsis. From August to November 2017 Madagascar was faced with a plague epidemic of unusual size. The WHO (2017b) reported a total of 2417 confirmed, probable and suspected cases of plague, with the majority (1854) classified as pneumonic plague during this outbreak.

In Madagascar the persistence of plague is strongly connected to the highly invasive character of the black rat, which not only spreads in cities but also adapts well to rural areas and natural habitats (Duplantier and Rakotondravony, 1999). Not only *X. cheopis* is involved

in the epidemic cycles in Madagaskar, but also the endemic flea *S. fonquerniei* (Duplantier and Duchemin, 2003). The contribution of *S. fonquerniei* is required to maintain sylvatic transmission cycles, because *X. cheopis*, the principal vector for humans, predominantly occurs in human housings (Duplantier and Duchemin, 2003). In 1998, plague re-emerged in the district of Ikongo between 250 m and 540 m of altitude (Duplantier et al., 2005), considerably below the postulated limit of Madagascar's plague focus of 800 m (not including Mahajanga; Brygoo, 1966). After this outbreak four individuals of endemic small mammals were found to be seropositive for IgG anti-*Y. pestis* (Duplantier et al., 2001). The effect of involvement of a new reservoir host was demonstrated by the introduced *Suncus murinus* facilitating several outbreaks in the coastal city of Mahajanga in the 1990s (Duplantier and Duchemin, 2003).

Here we present the molecular detection of *Y. pestis* DNA in two flea species, *E. gallinacea* and *X. cheopis* (Table 2), and in the rat they were collected from, in a coastal region of Madagascar. As described above, the plague foci normally lie above 800 m, but under certain circumstances, this boundary could fall. However, the mentioned rat was trapped in a village, and samples from a forest site were all negative. Additionally, no endemic fleas have been found in the region.

Echidnophaga gallinacea was proven to become infected with *Y. pestis* in a laboratory study (Weyer, 1942). Yet, for its life history this flea species seems unsuitable for serving as a vector, because once it has bitten the host it stays attached, and it was observed that it would not leave even when the host died (Suter, 1964). In contrast to *E. gallinacea* the vector potential of *X. cheopis* is verified, but indicated by the low ct-value of the qPCR (>38) found in our study, the bacterial burden inside the fleas may have been insufficient for transmission.

5. Conclusion

In conclusion, we identified a broad range of vector-borne bacteria present in southwest Madagascar. In that particular rural region of southwest Madagascar, where humans live in close contact to livestock and rats, we found pathogenic bacteria circulating in ectoparasites which are also known to feed on humans (ticks and fleas). We illustrated a large distribution of rickettsiae among the ectoparasite fauna of the region. Borreliae and bartonellae were both found to be restricted to only two ectoparasite species each, which likely do not have the potential for interspecific transmission due to host specificity. The finding of *Y. pestis* DNA in fleas of a rat in a geographical region far off the known plague foci of Madagascar was unexpected.

Our results do not prove that the detected bacteria actually live and reproduce in or are transmitted by the examined arthropods. This limitation is due to the fact that our study was based on the mere detection of bacterial DNA. We also cannot explain the high distribution of *R. africae* that stands in contrast to the distinct host specificity of most infected arthropods. However, this indicates that these pathogens have crossed species boundaries in the past.

Future studies should address the epidemiological relevance of the vector-borne pathogens detected in our study. Seroprevalence studies for past infections as well as pathogen detection in acutely infected hosts, should be performed at the study site in order to demonstrate the resulting disease burdens in the local human population, livestock and the endemic fauna.

Ethical approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. The field work was approved by the ethics committee of the Institute of Zoology of Hamburg University before the initiation of this study and authorized by the Ministère de L'Environnement, de l'Ecologie, de la Mer et des Forêts. (Research permits: N°136/16/- and N°002/ 17/MEEF/SG/ DGF/DSAP/SCB.Re; export permit: N°345-17/MEEF/SG/DGF/ DREEFAAND/SFR). This article does not contain any studies with human participants performed by any of the authors.

CRediT authorship contribution statement

Julian Ehlers: Writing - original draft, Conceptualization, Investigation, Methodology. **Andreas Krüger:** Conceptualization, Resources, Writing - review & editing, Supervision. **Solofomalala Jacques Rakotondranary:** Project administration, Methodology. **Rakotomalala Yedidya Ratovonamana:** Project administration, Resources. **Sven Poppert:** Conceptualization. **Jörg Ulrich Ganzhorn:** Conceptualization, Writing - review & editing, Supervision. **Dennis Tappe:** Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

None.

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Supplementary materials

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