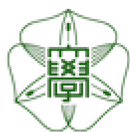

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1 **Screening of tick-borne pathogens in argasid ticks in Zambia: expansion of the geographic**
2 **distribution of *Rickettsia lusitaniae* and *Rickettsia hoogstraalii* and detection of putative novel**
3 ***Anaplasma* species**

4

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31

32 Abbreviations: ancestral group (AG), transitional group (TRG), typhus group (TG), spotted fever group

33 (SFG), citrate synthase (*gltA*), outer membrane protein A (*ompA*), outer membrane protein B (*ompB*), 17-

34 kDa common antigen (*htrA*), surface cell antigen-4 (*sca4*), and 16S ribosomal RNA (16S rRNA), heat-

35 shock protein (*groEL*).

36 **Abstract**

37 Ticks (Ixodidae and Argasidae) are important arthropod vectors of various pathogens that
38 cause human and animal infectious diseases. Many previously published studies on tick-borne
39 pathogens focused on those transmitted by ixodid ticks. Although there are increasing reports of viral
40 pathogens associated with argasid ticks, information on bacterial pathogens they transmit is scarce.
41 The aim of this molecular study was to detect and characterize *Rickettsia* and *Anaplasmataceae* in
42 three different argasid tick species, *Ornithodoros faini, Ornithodoros moubata,* and *Argas walkerae*
43 collected in Zambia. *Rickettsia hoogstraalii* and *Rickettsia lusitaniae* were detected in 77 % (77/100)
44 of *Ar. walkerae* and 10 % (5/50) of *O. faini*, respectively. All *O. moubata* pool samples (n = 124) were
45 negative for rickettsial infections. *Anaplasmataceae* were detected in 63 % (63/100) of *Ar. walkerae*
46 and in 82.2 % (102/124) of *O. moubata* pools, but not in *O. faini*. Phylogenetic analysis based on the
47 concatenated sequences of 16S rRNA and *groEL* genes revealed that *Anaplasma* spp. detected in the
48 present study were distinct from previously validated *Anaplasma* species, indicating that the current
49 knowledge on the diversity and vector range of *Anaplasma* spp. is incomplete. Our findings highlight
50 new geographical records of *R. lusitaniae* and *R. hoogstraalii* and confirm that the wide geographic
51 distribution of these species includes the African continent. The data presented here increase our
52 knowledge on argasid tick-borne bacteria and contribute toward understanding their epidemiology.

53 **Keywords**: Argasid tick; *Anaplasma*; *Rickettsia lusitaniae*; *Rickettsia hoogstraalii*; Zambia

54 **Introduction**

55 Ticks are recognized as the second most important disease vectors after mosquitoes.
56 Worldwide, more than 950 species of ticks are recorded, belonging three families: Ixodidae, Argasidae,
57 and Nuttalliellidae (Guglielmone et al., 2014; Dantas-Torres 2018). Ticks harbor and transmit a wide
58 variety of viruses, bacteria, and protozoa, some of which cause diseases in humans and animals. Recent
59 studies have expanded our knowledge on the diversity of tick-associated microorganisms and a
60 number of novel microorganisms have been reported (Yu et al., 2011; Guo et al., 2018; Matsuno et al.,
61 2018; Torii et al., 2019).

62 Argasid ticks differ morphologically from ixodid ticks in that they have an oval body shape
63 and the head and mouthparts are hidden underneath the body. They are associated with all classes of
64 terrestrial volant and non-volant vertebrates (Hoogstraal, 1985). Their phylogeny and taxonomic
65 classification remain unresolved. Different systematic approaches have resulted in several species
66 being assigned to different genera (Guglielmone et al., 2010) and new species continue to be
67 discovered (Muñoz-Leal et al., 2020).

68 Rickettsiae (family *Rickettsiaceae*, order Rickettsiales) are obligate intracellular Gram-
69 negative bacteria that are divided into four major groups phylogenetically: ancestral group (AG),
70 transitional group (TRG), typhus group (TG), and spotted fever group (SFG). AG and SFG rickettsiae
71 are predominantly maintained and transmitted by ticks, while TRG and TG rickettsiae are mostly

72 transmitted by other arthropods such as mites, lice, and fleas. Some of the TRG and SFG rickettsiae
73 have been reported from argasid ticks (Gillespie et al., 2007). For example, *Rickettsia hoogstraalii*,
74 *Rickettsia lusitaniae*, and "*Candidatus* Rickettsia wissemanii" were reported in *Ornithodoros capensis*,
75 *Ornithodoros erraticus*, and *Ornithodoros hasei*, respectively (Duh et al., 2010; Milhano et al., 2014;
76 Tahir et al., 2016). However, the pathogenicity, host range, and distribution of *Rickettsia* spp.
77 associated with argasid ticks are not fully understood.

78 The genera *Anaplasma, Aegyptianella,* and *Ehrlichia* belong to the family
79 *Anaplasmataceae*, which are obligate intracellular bacteria. These bacteria include pathogenic
80 species and are transmitted to humans and animals by ticks worldwide. Many of the epidemiological
81 studies on *Anaplasmataceae* were carried out on vertebrate hosts and ixodid ticks in many regions
82 including Africa. For example, *Anaplasma marginale* was detected from cattle and buffalo in
83 Southern Africa, Tunisia, Senegal, Madagascar, Morocco, and Kenya (Ait Hamou et al., 2012; Adjou
84 Moumouni et al., 2015; Belkahia et al., 2015; Eygelaar et al., 2015; Pothmann et al., 2016; Sisson et
85 al., 2017; Dahmani, et al., 2019); *Anaplasma platys* and *Ehrlichia canis* were detected from dogs in
86 Zambia (Qiu et al., 2018; Vlahakis et al., 2018); and *Anaplasma phagocytophilum* and *Ehrlichia* spp.
87 were detected from ixodid ticks in Ghana and South Africa (Mtshali et al., 2017; Adenyo et al., 2020).
88 However, few studies have explored the presence of *Anaplasmataceae* associated with argasid ticks
89 in African continent. *Aegyptianella pullorum*, a small bacterium that infects and replicates only in

90 avian red blood cells and transmitted by argasid tick, was reported in Nigeria (Leeflang and
91 Ilemobade 1977). Another *Aegyptianella* sp. was described from wild helmeted guineafowls in South
92 Africa (Earlé et al., 1991). Recently, *Anaplasma* spp. were detected from *Argas persicus* in Algeria
93 (Lafri et al., 2017). The present study was designed to detect and characterize *Rickettsia* and
94 *Anaplasmataceae* in three species of argasid ticks collected in Zambia.
95
96 **Materials and Methods**
97 *Sample collection and DNA extraction*
98 *Ornithodoros faini* ticks were recovered from bat guano in Leopards Hill cave (15.44 S,
99 28.51 E), Lusaka, Zambia in April 2016. DNA was extracted from 50 individual ticks using DNAzol
100 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions, and used for screening
101 of *Borrelia* spp. in a previous study (Qiu et al., 2019). *Argas walkerae* ticks were obtained from a
102 chicken coop in Isoka (10.14 S, 32.64 E), Zambia in November 2017. DNA was extracted from 100
103 individual ticks using DNAzol (Invitrogen). A total of 639 *Ornithodoros moubata* ticks were collected
104 from African warthog burrows in Mosi-oa-Tunya National Park (17.85 S, 25.79 E), Livingstone,
105 Zambia in April 2019. DNA was extracted from a total of 124 pools (each pool consisting of three to
106 five ticks) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), and used for screening
107 of African swine fever virus (ASFV) in a previous study (Chambaro et al., 2020). This sampling

108 activity was authorized by the Department of National Parks and Wildlife, Ministry of Tourism and
109 Arts of the Republic of Zambia.

110 *Conventional polymerase chain reaction (PCR)*

111 The DNA samples were initially subjected to screening for *Rickettsia* spp. using a citrate
112 synthase gene (*gltA*) PCR method. PCR was conducted using the primers gltA8211Fc and gltA8211Rc
113 and Ex-Taq HS (Takara, Shiga, Japan) with the following conditions: 1 min denaturation step at 98 °C
114 followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension step
115 at 72 °C for 5 min (Gaowa et al., 2013). For a negative control, UltraPure™ distilled water (Invitrogen)
116 was added instead of template DNA. The resulting PCR products were electrophoresed on a 1.2 %
117 agarose gel stained with Gel-Red (Biotium, Hayward, CA, USA), and visualized with a UV trans-
118 illuminator. Further characterization of *Rickettsia* spp. was conducted based on five additional genes:
119 outer membrane protein A (*ompA*), outer membrane protein B (*ompB*), 17-kDa common antigen (*htrA*),
120 surface cell antigen-4 (*sca4*), and 16S ribosomal RNA (16S rRNA) (Regnery et al., 1991; Roux and
121 Raoult 2000; Sekeyova et al., 2001; Labruna et al., 2004; Anstead and Chilton 2013). Table 1 shows
122 the primer sets used for each assay. PCR conditions were as described above except for the annealing
123 temperature (48 °C for *ompA* and *ompB* PCRs, 52 °C for 16S rRNA and *htrA* PCRs, and 50 °C for
124 *sca4* PCR).

125 To screen for *Anaplasmataceae*, a 345 bp fragment of the 16S rRNA gene of the family

126 *Anaplasmataceae* was PCR-amplified using the primers EHR16SD and EHR16SR (Parola et al.,
127 2000). Ten positive samples were randomly selected and used for further characterization with
128 additional PCRs: a PCR targeting the heat-shock protein (*groEL*) gene, a nested-PCR amplifying
129 almost the full length (approximately 1,300 bp) of the 16S rRNA gene, and a semi-nested PCR
130 targeting the *gltA* gene of *Anaplasma* spp. (Sumner et al., 1997; Liz et al., 2000; Kawahara et al., 2006;
131 Loftis et al., 2006; Gofton et al., 2016). All PCR primer sets are shown in Table 1. Tick DNA samples,
132 which were positive for *Rickettsia* and *Anaplama* in the previous studies (Thu et al., 2019; Adenyo et
133 al., 2020), were included as a positive control for each PCR assay.

134 *Sequence and phylogenetic analysis*

135 Sanger sequencing was performed using BigDye Terminator version 3.1 chemistry (Applied
136 Biosystems, Foster City, CA, USA). Sequencing products were run on an ABI Prism 3130xl genetic
137 analyzer according to the manufacturer's instructions. The sequence data were assembled using ATGC
138 software version 6.0.4 (GENETYX, Tokyo, Japan). The DDBJ/EMBL/GenBank accession numbers
139 for the sequences obtained are as follows: *Rickettsia gltA* (LC558315 to LC558316), *ompA* (LC558316
140 to LC558317), *ompB* (LC558318), *htrA* (LC558319 to LC558320), and 16S rRNA gene (LC558311
141 to LC558312); *Anaplasma* 16S rRNA gene (LC558313 to LC558314), and *groEL* (LC558321 to
142 LC558323). Phylogenetic trees were constructed by using MEGA 6.6 software (Tamura et al., 2013)
143 with maximum-likelihood and/or neighbor-joining methods.

144

145 **Results**

146 *Detection and characterization of* Rickettsia *spp.*

147 Five out of 50 *O. faini* samples produced positive rickettsial *gltA* PCR results (Table 2).
148 Subsequent analysis revealed that the sequences of all five amplicons were identical. The rickettsial
149 *gltA* sequence amplified from the *O. faini* samples showed 100 % (537/537 bp) identity with that of a
150 *Rickettsia lusitaniae* isolate Turkmenia/1948 (MK761227). Positive *gltA* PCR results with identical
151 sequences were obtained for 77 out of 100 *Ar. walkerae* samples. The *gltA* sequence amplified from
152 the *Ar. walkerae* samples showed 99.8 % (536/537 bp) identity with that of a *R. hoogstraalii* isolate,
153 TR-Rg685 (MK929389). None of the *O. moubata* pools was PCR-positive for the *gltA* gene.
154 Randomly selected positive samples (2 from *O. faini* and 10 from *Ar. walkerae*) were used for further
155 characterization by additional PCR and sequence analyses. The PCRs targeting the *htrA* and 16S rRNA
156 genes gave amplicons in all selected samples and the sequence of these genes were identical within
157 each tick species. The *ompA* and *ompB* PCRs yielded amplicons only from the *O. faini* samples and
158 only one sequence type was obtained for each gene. None of the selected samples produced positive
159 *sca4* PCR amplicons. The sequence similarities with the closest *Rickettsia* species are listed in Table
160 3. Phylogenetic trees based on the sequences of the *gltA*, *ompA*, *ompB*, *htrA,* and 16S rRNA gene
161 fragments are shown in Figure 1.

162 According to the guideline for the identification of *Rickettsia* species (Fournier et al., 2003;
163 Fournier and Raoult, 2009), *Rickettsia* spp. detected from *O. faini* and *Ar. walkerae* were identified as
164 *R. lusitaniae* and *R. hoogstraalii,* respectively. Phylogenetic analysis also supported this classification
165 (Figure 1). In the phylogenetic trees based on *gltA*, *htrA*, and 16S rRNA gene fragments rickettsial
166 sequences from *Ar. walkerae* clustered together with those from *R. hoogstraalii*. The rickettsial
167 sequences from *O. faini* formed a cluster with those from *R. lusitaniae* in the phylogenetic trees based
168 on *gltA*, *htrA*, and *ompA* genes. The *ompB* sequence of the same region and the sequence of the 16S
169 rRNA gene of *R. lucitania* were not available in public database. Thus, the sequences of *ompB* and
170 16S rRNA genes from *O. faini* were not clustered with any other validated rickettsial species in the
171 phylogenetic trees based on these genes.

172 *Detection and characterization of* Anaplasma *spp.*

173 The screening of *Anaplasmataceae* was carried out using the PCR targeting 345 bp of 16S
174 rRNA gene. The positive results of that PCR were obtained from 63 out of 100 *Ar. walkerae* samples
175 (Table 2). In addition, 50 out of 100 *Ar. walkerae* ticks were infected with both *Rickettsia* sp. and
176 *Anaplasma* sp. Subsequent sequence analyses revealed that the sequences of all 63 *Ar. walkerae*
177 amplicons were identical. The partial sequence of 16S rRNA gene from *Ar. walkerae* amplicons
178 showed 99.0 % (302/305 bp) identity with that of an *An. phagocytophilum* strain Hubei E4
179 (KF569909) detected in a goat in China. Ten randomly selected positive samples were used for further

180 characterization by amplifying the *groEL*, 16S rRNA, and *gltA* genes of *Anaplasmataceae*. Partial
181 sequences of the *groEL* gene from these 10 samples were identical and showed 77.8 % (941/1,209 bp)
182 identity with *Anaplasma centrale* (AF414866) in *Rhipicephalus simus* tick reported from South Africa.
183 The almost full-length 16S rRNA gene sequences obtained from 10 nested PCR amplicons were
184 identical and showed 96.7 % (1,283/1,326 bp) identity with *An. marginale* from cattle in Uganda
185 (KU686792). None of these 10 *Ar. walkerae* samples produced amplicons in the *gltA* semi-nested PCR.
186 The positive results of the PCR targeting 345 bp of *Anaplasmataceae* 16S rRNA gene were
187 obtained from 102 out of 124 *O. moubata* DNA pools. Subsequent sequence analyses revealed that
188 the sequences from these 102 *O. moubata* pools were identical. The sequence of the amplicons showed
189 99.3 % (303/305 bp) identity with that of *Anaplasma* sp. (MN317255) detected from cattle in Senegal.
190 Ten randomly selected pool samples were used for further characterization as described above. The
191 partial sequences of the *groEL* gene from the 10 samples revealed two variants with 10 nucleotide
192 differences within 1,281 bp. The sequences of both variants showed 79.8 % (1,024/1,283 bp) identity
193 with that of *Anaplasma ovis* strain Haibei (CP015994) from a sheep in Haibei, China. The almost full
194 length 16S rRNA gene sequences from the 10 pools were identical and showed 96.1 % (1,279/1,330
195 bp) identity with *An. phagocytophilum* (MK814406) from a dog in South Africa. None of these 10 *O.*
196 *moubata* pool samples produced amplicons in the *gltA* semi-nested PCR. All the *O. faini* samples
197 showed negative results in the *Anaplasmataceae* PCR.

198 Phylogenetic trees were constructed based on the concatenated sequences of 16S rRNA and

199 *groEL* genes (Figure 2). The maximum likelihood and neighbor-joining methods produced

200 phylogenetic trees with consistent topologies. The *Anaplasma* spp. sequences detected in *O. moubata*

201 clustered together, and formed a sister clade with that of "*Candidatus* Anaplasma sphenisci", which

202 was detected from an African penguin in South Africa (Vanstreels et al., 2018), while the *Anaplasma*

203 sp. sequences from *Ar. walkerae* formed a monophyletic clade distinct from other validated *Anaplasma*

204 species (Figure 2).

205

206 **Discussion**

207 We investigated *Rickettsia* spp. and *Anaplasmataceae* in three different argasid ticks, *O.*

208 *faini*, *O. moubata*, and *Ar. walkerae*, collected in Zambia. We identified two rickettsial species, namely

209 *R. lusitaniae and R. hoogstraalii*, and two putative novel *Anaplasma* species that have very low

210 sequence identities with known *Anaplasma* spp. This study was the first investigation of *Rickettsia*

211 spp. and *Anaplasmataceae* in argasid ticks in Zambia.

212 Most of the previous epidemiological studies on tick-borne *Rickettsia* and *Anaplasmataceae*

213 examined ixodid ticks. Only a few attempts have been made to investigate these pathogens in argasid

214 ticks. For examples, a *Rickettsia felis*-like organism was found in *O. capensis* in the United States

215 (Reeves et al., 2006). Previously uncharacterized *Rickettsia* spp. were detected from *O. erraticus*,

216 *Ornithodoros rupestris*, and *O. capensis* in Algeria (Lafri et al., 2015). *Rickettsia japonica*, a causative
217 agent of Japanese spotted fever, was isolated from the bat-associated tick, *Argas dewae*, in Australia
218 (Izzard et al., 2018). More recently, "*Candidatus* R. wissemanii" was detected in *Ornithodoros hasei*
219 from Argentina (Colombo et al., 2020) and two novel *Rickettsia* spp. were found in argasid ticks in
220 northern Africa (Buysse and Duron, 2020). These findings indicate that the diversity of
221 microorganisms in argasid ticks has not yet been fully explored.

222 This is the first report of *R. lusitaniae* in Africa. This species was first found in *Ornithodoros*
223 *erraticus* collected from a pigpen in Portugal (Milhano et al., 2014) and subsequently reported from
224 *Ornithodoros yumatensis* collected in a cave in Mexico (Sánchez-Montes et al., 2016). A recent study
225 on bat-associated ticks, including *Ar. vespertilionis*, confirmed a new geographical distribution of *R.*
226 *lusitaniae* in China (Hornok et al., 2019). Thus, *R. lusitaniae* have been detected in four different
227 continents, namely Europe, America, Asia, and Africa. *O. erraticus* is a common argasid tick of pigs
228 in Europe, while *O. yumatensis*, *O. faini*, and *Argas vespertilionis* are well known as bat-associated
229 ticks in America, Africa, and Eurasia, respectively. Although the role of vertebrate hosts (pigs and
230 bats) in the maintenance cycle of *R. lusitaniae* is unknown, their wide habitat range could be linked to
231 the wide geographic distribution of this rickettsial species. It is notable that a case of human borreliosis
232 transmitted by *O. faini* recently occurred in Zambia (Qiu, et al., 2019). Therefore, further studies are
233 needed to investigate the transmission potential of *R. lusitaniae* via the bite of *O. faini* and its

234 pathogenicity to humans and animals.

235 This study provides the first direct evidence of *R. hoogstraalii* in *Ar. walkerae,* which is
236 known as a poultry tick, in Zambia. *Rickettsia hoogstraalii* is a member of SFG rickettsiae with
237 unknown pathogenicity and is closely related to *R. felis*, an emerging pathogen known to be
238 transmitted by arthropods including fleas and possibly mosquitoes (Dieme et al., 2015; Moonga et al.,
239 2019). Although *R. hoogstraalii* was originally reported from an ixodid tick *Haemaphysalis sulcata*
240 in Croatia (Duh et al., 2006), its presence in argasid ticks, namely *O. capensis* in Croatia and *Ar.*
241 *persicus* in Ethiopia, has also been demonstrated (Duh et al., 2010; Pader et al., 2012). Moreover, *R.*
242 *hoogstraalii* has been reported from a number of geographically diverse countries including Japan, the
243 western Indian Ocean islands, the United States, Cyprus, Spain, Turkey, and Italy (Kawabata et al.,
244 2006; Mattila et al., 2007; Márquez, 2008; Chochlakis et al., 2012; Dietrich et al., 2014; Orkun et al.,
245 2014; Chisu et al., 2017). Since *H. sulcata, O. capensis,* and *Ar. persicus* are seabird-associated ticks,
246 migratory birds may have contributed to the wide distribution of *R. hoogstraalii*. Although there is no
247 record of seabirds in Zambia, which is a land locked country in Southern African region, some
248 Eurasian birds such as white storks, annually migrate to Southern Africa for overwintering (Olsen et
249 al., 2006). Tick-borne pathogens such as SFG rickettsiae and *Borrelia burgdorferi* sensu lato are
250 dispersed among European countries by migratory birds and their carrying pathogen-infected ticks
251 (Comstedt et al., 2006; Elfving et al., 2010). Furthermore, it has been proposed that birds could serve

252 as potential reservoirs of tick-borne pathogens including *Rickettsia* (Hornok et al., 2014). Thus, there
253 is a possibility that migratory birds or their attached ticks have introduced *R. hoogstraalii* to the
254 Zambian fowl or *Ar. walkerae*. To evaluate this hypothesis, further investigations of *R. hoogstraalii* in
255 migratory and domestic birds as well as their ectoparasites including ticks are required.

256 The infection rate of *R. hoogstraalii* in *Ar. walkerae* was 77 % in the present study. A high
257 infection rate of this rickettsial species was also reported in a previous study that tested *O. capensis*
258 collected in the western Indian Ocean islands for rickettsial infections (Dietrich et al., 2014). Some
259 *Rickettsia* spp. can be transmitted through transovarial and transstadial routes in ticks (Moore et al.,
260 2018) and this is the most probable explanation of the high prevalence of *R. hoogstraalii*. In general,
261 symbiotic rickettsia tend to have a high prevalence among host arthropod populations, and provide a
262 positive effect on the host fitness (Chiel et al., 2009). The benefit of symbiotic rickettsia has been
263 reported from several ixodid ticks, namely *Amblyomma americanum*, *Dermacentor variabilis*, and
264 *Ixodes scapularis*, where an increased larval motility was observed under laboratory conditions
265 (Kagemann and Clay, 2013). Moreover, the genome of *Rickettsia monacensis*, a symbiont of *Ixodes*
266 *pacificus* (Hunter et al., 2015), contains the complete folate biosynthesis pathway and the
267 dihydrofolate reductase activity of the rickettsial gene was confirmed *in vitro* (Bodnar et al., 2018),
268 suggesting its beneficial role for host ticks. Further studies regarding the effect of *R. hoogstraalii* on
269 the fitness of *Ar. walkerae* may also help to elucidate the role of rickettsial symbionts in argasid ticks.

270 Although members of the genus *Anaplasma* are very common in ixodid ticks, very few
271 research efforts have been made to test for their presence in argasid ticks. For example, *Anaplasma*
272 spp. have been reported in an argasid tick, *Ar. persicus*, in Algeria (Lafri et al., 2017). Vanstreels et al.
273 (2018) detected "*Candidatus* An. sphenisci" in the African penguin and suspected *O. capensis* as a
274 potential vector for this bacterium, since this argasid tick is a common blood sucking ectoparasite of
275 the African penguin. In this study, we detected two putative novel *Anaplasma* species from *Ar.*
276 *walkerae* and *O. moubata*. Phylogenetic analyses of the concatenated sequences of 16S rRNA and
277 *groEL* genes suggested that *Anaplasma* spp. from *O. moubata* were closely related to "*Candidatus* An.
278 sphenisci", while *Anaplasma* sp. from *Ar. walkerae* formed a separate clade. Lack of sequence data
279 from species closely related to the *Anaplasma* detected in this study suggests that the diversity and
280 vector range of *Anaplasma* spp. may have been underestimated. Further studies on the *Anaplasma* spp.
281 in argasid ticks will provide valuable insights into the evolution and epidemiology of this group of
282 bacteria.

283 The detection rate of *Anaplasma* sp. in *Ar. walkerae* was as high as 63 %, which is
284 comparable to a previous study where *Anaplasma* sp. was detected in 62 % of *Ar. persicus* in Algeria
285 (Lafri et al., 2017). Generally, a tick acquires *Anaplasma* through blood feeding on an infected host.
286 The transovarial transmission of *Anaplasma* in ticks has not been demonstrated but transstadial
287 transmission is known to occur (Vimonish et al., 2020). Argasid ticks have multiple nymphal stages

288 and each stage requires blood feeding. This could provide increased opportunity for argasid ticks to

289 acquire *Anaplasma* from host blood. Further investigations on *Anaplasma* in chickens are required to

290 understand the causes of the high infection rate of *Anaplasma* in *Ar. walkerae*.

291 In our previous studies, the same (pool) samples of *O. faini* and *O. moubata* were tested for

292 *Borrelia* and ASFV infections, respectively (Qiu et al., 2019; Chambaro et al., 2020). Of note, ASFV

293 was detected from 47.6% (59/124) of *O. moubata* pool samples (Chambaro et al., 2020). Considering

294 that *Anaplasma* sp. was detected from 82.3% (102/124) of the pools, co-infection of *Anaplasma* sp.

295 and ASFV in *O. moubata* may be occurring. Further investigation examining individual *O. moubata*

296 ticks is needed to evaluate the co-infection rate in ticks.

297 In conclusion, we detected *R. lusitaniae* for the first time in Africa and provide the first

298 evidence of *R. hoogstraalii* in *Ar. walkerae*. In addition, two putative novel *Anaplasma* species were

299 detected from *Ar. walkerae* and *O. moubata*. Since this study examined the argasid ticks from a limited

300 source, further studies employing ticks from various geographic locations and chicken coops are

301 necessary to give a clear picture on the distribution of these pathogens in Zambia and neighboring

302 countries. Nonetheless, the findings expand our knowledge on tick-borne bacteria in argasid ticks and

303 contribute towards understanding of their epidemiology.

304

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316 **Authors' contributions**

317 YQ: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing -
318 Original Draft, Funding acquisition; RN: Conceptualization, Methodology, Writing - Review &
319 Editing, Funding acquisition; MS: Resources; MK: Resources, Investigation; HC: Resources,
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323 approved the final version of the manuscript.

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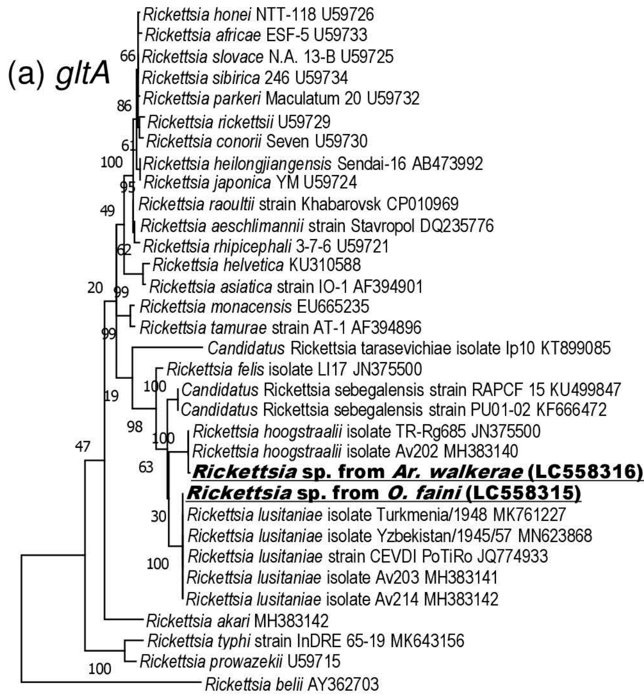
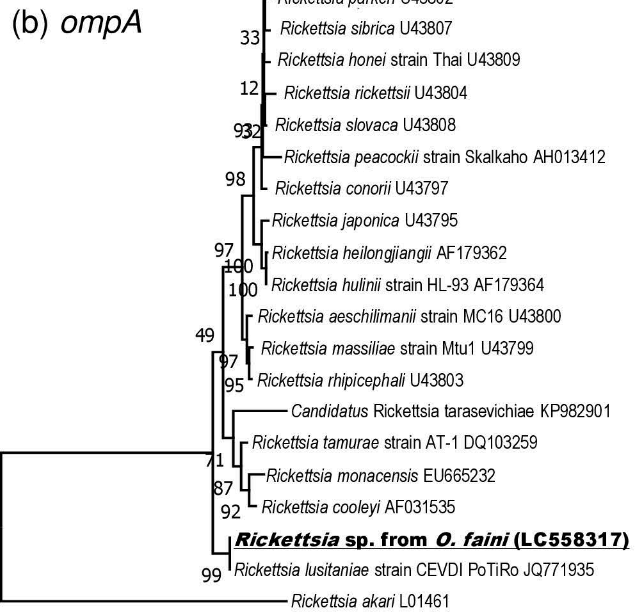
592

593 **Figure legends**

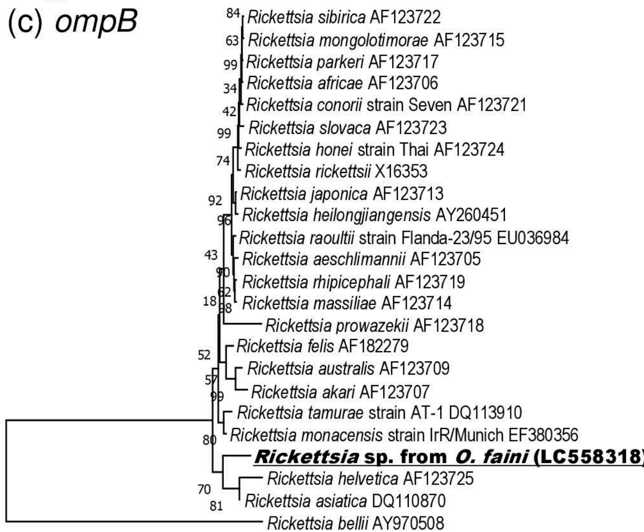
594 **Figure 1. Phylogenetic trees of *Rickettsia* spp. based on the sequences of 5 different genes. (**a) *gltA*
595 (537 bp), (b) *ompA* (493 bp), (c) *ompB* (780 bp), (d) *htrA* (465 bp), and (e) 16s rRNA (1,245 bp). The
596 trees were constructed using the maximum-likelihood method with the Kimura 2-parameter model.
597 All bootstrap support values from 1,000 replicates are shown at the interior branch nodes. The
598 sequences obtained in this study are shown in bold and underlined.
599
600 **Figure 2. Phylogenetic trees of *Anaplasma* spp. based on the concatenated sequences of 16S**
601 **rRNA and *groEL* genes.** The phylogenetic trees were constructed using maximum-likelihood (a) and
602 neighbor-joining (b) methods. Bootstrap support values from 1,000 replicates are shown at the interior
603 branch nodes. The sequences obtained in this study are shown in bold and underlined.



(a) gltA
Rickettsia honei NTT-118 U59726
Rickettsia africae ESF-5 U59733
Rickettsia slovace N.A. 13-B U59725
Rickettsia sibirica 246 U59734
Rickettsia parkeri Maculatum 20 U59732
Rickettsia rickettsii U59729
Rickettsia conorii Seven U59730
Rickettsia heilongjiangensis Sendai-16 AB473992
Rickettsia japonica YM U59724
Rickettsia raoultii strain Khabarovsk CP010969
Rickettsia aeschlimannii strain Stavropol DQ235776
Rickettsia rhipicephali 3-7-6 U59721
Rickettsia helvetica KU310588
Rickettsia asiatica strain IO-1 AF394901
Rickettsia monacensis EU665235
Rickettsia tamurae strain AT-1 AF394896
Candidatus Rickettsia tarasevichiae isolate Ip10 KT899085
Rickettsia felis isolate LI17 JN375500
Candidatus Rickettsia sebegalensis strain RAPCF 15 KU499847
Candidatus Rickettsia sebegalensis strain PU01-02 KF666472
Rickettsia hoogstraalii isolate TR-Rg685 JN375500
Rickettsia hoogstraalii isolate Av202 MH383140
Rickettsia sp. from Ar. walkerae (LC558316)
Rickettsia sp. from O. faini (LC558315)
Rickettsia lusitaniae isolate Turkmenia/1948 MK761227
Rickettsia lusitaniae isolate Yzbekistan/1945/57 MN623868
Rickettsia lusitaniae strain CEVDI PoTiRo JQ774933
Rickettsia lusitaniae isolate Av203 MH383141
Rickettsia lusitaniae isolate Av214 MH383142
Rickettsia akari MH383142
Rickettsia typhi strain InDRE 65-19 MK643156
Rickettsia prowazekii U59715
Rickettsia belii AY362703
0.05



(b) ompA
Rickettsia africae U43790
Rickettsia parkeri U43802
Rickettsia sibrica U43807
Rickettsia honei strain Thai U43809
Rickettsia rickettsii U43804
Rickettsia slovaca U43808
Rickettsia peacockii strain Skalkaho AH013412
Rickettsia conorii U43797
Rickettsia japonica U43795
Rickettsia heilongjiangii AF179362
Rickettsia hulinii strain HL-93 AF179364
Rickettsia aeschilimanii strain MC16 U43800
Rickettsia massiliae strain Mtu1 U43799
Rickettsia rhipicephali U43803
Candidatus Rickettsia tarasevichiae KP982901
Rickettsia tamurae strain AT-1 DQ103259
Rickettsia monacensis EU665232
Rickettsia cooleyi AF031535
Rickettsia sp. from O. faini (LC558317)
Rickettsia lusitaniae strain CEVDI PoTiRo JQ771935
Rickettsia akari L01461
0.1



(c) ompB
Rickettsia sibirica AF123722
Rickettsia mongolotimorae AF123715
Rickettsia parkeri AF123717
Rickettsia africae AF123706
Rickettsia conorii strain Seven AF123721
Rickettsia slovaca AF123723
Rickettsia honei strain Thai AF123724
Rickettsia rickettsii X16353
Rickettsia japonica AF123713
Rickettsia heilongjiangensis AY260451
Rickettsia raoultii strain Flanda-23/95 EU036984
Rickettsia aeschlimannii AF123705
Rickettsia rhipicephali AF123719
Rickettsia massiliae AF123714
Rickettsia prowazekii AF123718
Rickettsia felis AF182279
Rickettsia australis AF123709
Rickettsia akari AF123707
Rickettsia tamurae strain AT-1 DQ113910
Rickettsia monacensis strain IrR/Munich EF380356
Rickettsia sp. from O. faini (LC558318)
Rickettsia helvetica AF123725
Rickettsia asiatica DQ110870
Rickettsia bellii AY970508
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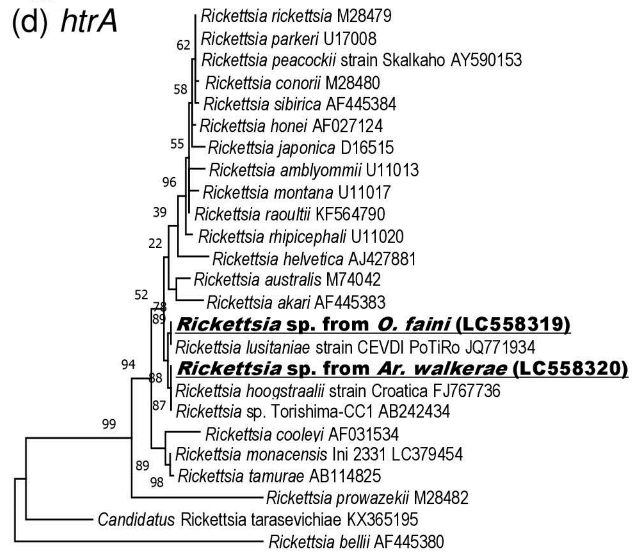

(d) htrA
Rickettsia rickettsia M28479
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Rickettsia peacockii strain Skalkaho AY590153
Rickettsia conorii M28480
Rickettsia sibirica AF445384
Rickettsia honei AF027124
Rickettsia japonica D16515
Rickettsia amblyommii U11013
Rickettsia montana U11017
Rickettsia raoultii KF564790
Rickettsia rhipicephali U11020
Rickettsia helvetica AJ427881
Rickettsia australis M74042
Rickettsia akari AF445383
Rickettsia sp. from O. faini (LC558319)
Rickettsia lusitaniae strain CEVDI PoTiRo JQ771934
Rickettsia sp. from Ar. walkerae (LC558320)
Rickettsia hoogstraalii strain Croatica FJ767736
Rickettsia sp. Torishima-CC1 AB242434
Rickettsia cooleyi AF031534
Rickettsia monacensis Ini 2331 LC379454
Rickettsia tamurae AB114825
Rickettsia prowazekii M28482
Candidatus Rickettsia tarasevichiae KX365195
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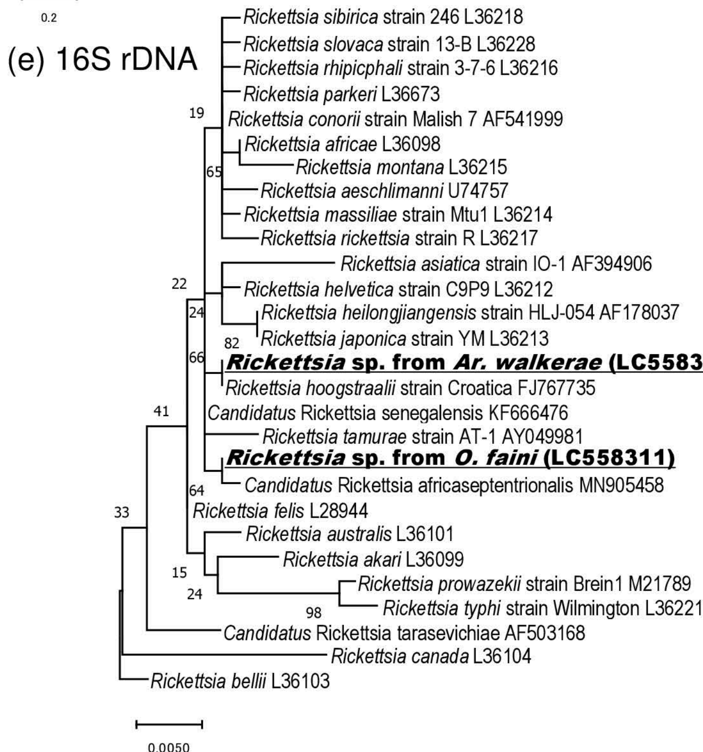

(e) 16S rDNA
Rickettsia sibirica strain 246 L36218
Rickettsia slovaca strain 13-B L36228
Rickettsia rhipicphali strain 3-7-6 L36216
Rickettsia parkeri L36673
Rickettsia conorii strain Malish 7 AF541999
Rickettsia africae L36098
Rickettsia montana L36215
Rickettsia aeschlimanni U74757
Rickettsia massiliae strain Mtu1 L36214
Rickettsia rickettsia strain R L36217
Rickettsia asiatica strain IO-1 AF394906
Rickettsia helvetica strain C9P9 L36212
Rickettsia heilongjiangensis strain HLJ-054 AF178037
Rickettsia japonica strain YM L36213
Rickettsia sp. from Ar. walkerae (LC558312)
Rickettsia hoogstraalii strain Croatica FJ767735
Candidatus Rickettsia senegalensis KF666476
Rickettsia tamurae strain AT-1 AY049981
Rickettsia sp. from O. faini (LC558311)
Candidatus Rickettsia africaseptentrionalis MN905458
Rickettsia felis L28944
Rickettsia australis L36101
Rickettsia akari L36099
Rickettsia prowazekii strain Brein1 M21789
Rickettsia typhi strain Wilmington L36221
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Rickettsia canada L36104
Rickettsia bellii L36103
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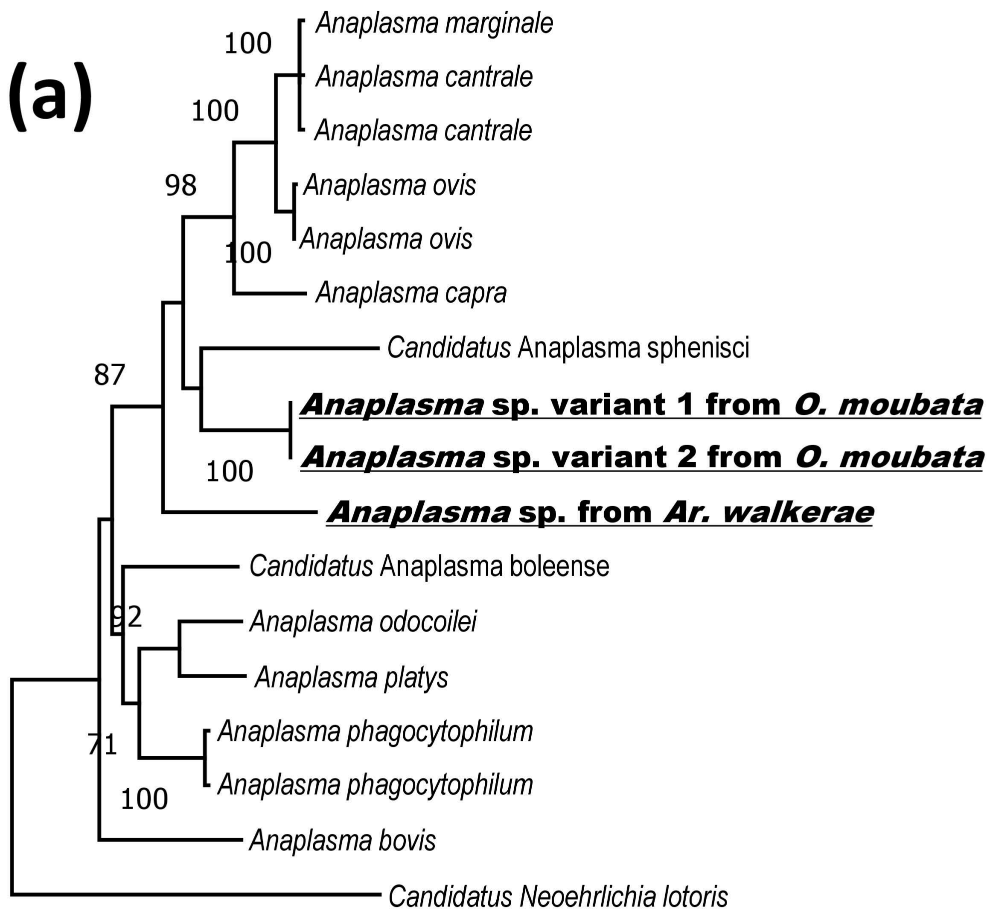
(a)
100
Anaplasma marginale
Anaplasma cantrale
100
Anaplasma cantrale
98
Anaplasma ovis
Anaplasma ovis
100
Anaplasma capra
Candidatus Anaplasma sphenisci
87
Anaplasma sp. variant 1 from O. moubata
Anaplasma sp. variant 2 from O. moubata
100
Anaplasma sp. from Ar. walkerae
Candidatus Anaplasma boleense
92
Anaplasma odocoilei
Anaplasma platys
Anaplasma phagocytophilum
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Anaplasma phagocytophilum
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Anaplasma bovis
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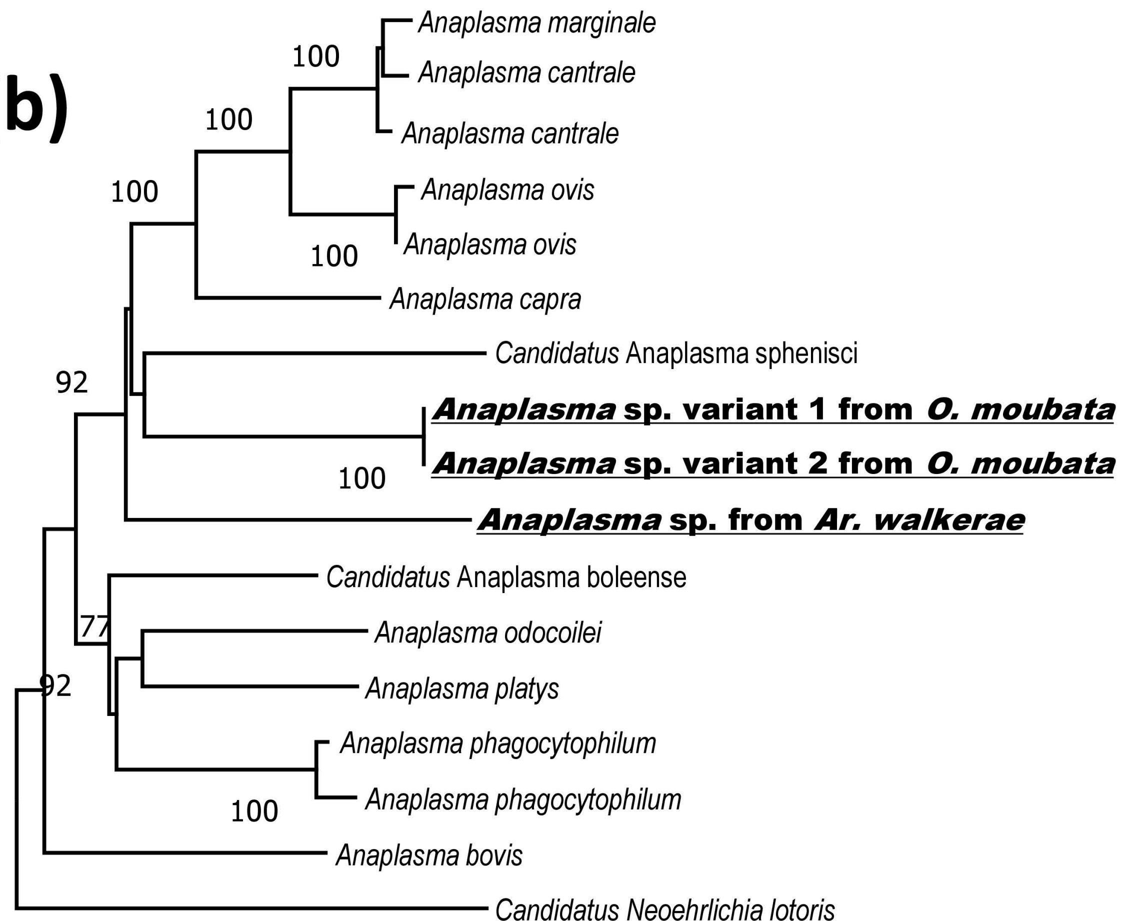
(b)
Anaplasma marginale
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Anaplasma cantrale
100
Anaplasma cantrale
100
Anaplasma ovis
Anaplasma ovis
100
Anaplasma capra
Candidatus Anaplasma sphenisci
92
Anaplasma sp. variant 1 from O. moubata
Anaplasma sp. variant 2 from O. moubata
100
Anaplasma sp. from Ar. walkerae
Candidatus Anaplasma boleense
77
Anaplasma odocoilei
92
Anaplasma platys
Anaplasma phagocytophilum
Anaplasma phagocytophilum
100
Anaplasma bovis
Candidatus Neoehrlichia lotoris
0.02