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Molecular detection of *Anaplasma ovis* in small ruminants and ixodid ticks from Mongolia

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Abstract

Anaplasma ovis is a tick-borne obligate intracellular rickettsial bacterium that causes anaplasmosis in domestic and wild small ruminants. Sheep and goats, whose combined population is approximately 48.5-million in Mongolia, play a vital role in the country's economy. In this study, we conducted an epidemiological survey of *A. ovis* in sheep and goats from 19 of 21 provinces in Mongolia. Additionally, DNA samples extracted from unfed ticks collected in 11 Mongolian provinces were also screened for *A. ovis*. Of 1,179 and 871 blood DNA samples from sheep and goats, 813 (69.0%) and 621 (71.3%), respectively, were positive for *A. ovis* when screened by a PCR assay based on major surface protein 4 gene (*msp4*). On a per province basis, *A. ovis* infection rates ranged from 7.4%–93.3% and 13.3%–100% in sheep and goats, respectively. Subsequently, DNA samples prepared from 721 unfed ticks, including *Dermacentor nuttalli* (n=378), *Ixodes persulcatus* (n=95), *Haemaphysalis pospelovashtromae* (n=120), and *Hyalomma asiaticum* (n=128), were screened for *A. ovis* using the same PCR assay. Although nine *D. nuttalli* were *A. ovis*-positive, all other tick DNA samples were negative. In addition to reporting *A. ovis* in sheep and goats from all over Mongolia, this study identified *D. nuttalli* as a potential transmission vector of *A. ovis* in Mongolia. The present data highlight the importance of monitoring Mongolian sheep and goats for possible episodes of clinical anaplasmosis and controlling *D. nuttalli* throughout the country.

Keywords: *Anaplasma ovis*, epidemiology, goats, Mongolia, sheep, ticks

1. Introduction

Anaplasma ovis, a Gram-negative bacterium, belongs to genus *Anaplasma*, family Anaplasmataceae, and order Rickettsiales, and infects domestic and wild small ruminants [1, 2]. *Anaplasma ovis* is transmitted by ticks and infects host erythrocytes, where asexual reproduction occurs [3]. In sheep and goats, *A. ovis* infection might be characterised by mild-to-severe clinical disease [2, 4–6]. The disease development is often predisposed by co-infection with other pathogens and stress induced by various factors, such as hot climate and transportation [2, 4, 5]. The clinical signs of anaplasmosis caused by *A. ovis* in sheep and goats include fever, anaemia, jaundice, abortion, and production losses [7]. Therefore, control of *A. ovis* infection is vital for successful sheep and goat farming, and tick control is an integral part of any *A. ovis* control strategy, as tick control prevents *A. ovis* transmission from ticks to ruminants and vice versa.

Mongolia is an agricultural country, and the livestock industry plays a critical role in its national economy. However, growth of this industry has often been undermined by several factors, including infectious diseases [8]. Various species of tick-borne blood pathogens, including those of *Anaplasma*, have been reported in livestock in Mongolia [9–14]. The *Anaplasma* species reported in Mongolia include *A. marginale*, *A. phagocytophilum*, and *A. ovis* [13–15]; *A. ovis* infects sheep and goat populations [14, 16], of which there were 48.5 million animals in 2015 according to the national statistics census [17]. In addition, *A. ovis* has also been reported in cattle and reindeer in Mongolia [14, 18]. However, those studies were only conducted in a few Mongolian provinces, and a country-wide survey to determine *A. ovis* infection rates in

various Mongolian provinces has not yet been carried out. Additionally, potential tick species associated with *A. ovis* transmission are not known in Mongolia, although identification of tick vectors is very important for devising effective tick control measures to minimise *A. ovis* infection rates. In this study, we surveyed sheep and goats for *A. ovis* infection in 19 of 21 Mongolian provinces using a PCR assay. Additionally, we also screened DNA samples that were extracted from unfed ticks collected in 11 different provinces for *A. ovis* infection.

2. Materials and methods

2.1. DNA samples from sheep, goats, and ticks

Blood samples were collected from 1,179 sheep and 871 goats in 19 of 21 Mongolian provinces (Table 1) during 2013–2016. The sampling was not carried out in Darkhan-Uul and Orkhon provinces, formerly known as Darkhan and Erdenet cities, respectively, as the livestock farming is uncommon in these two urban areas. From each animal, approximately 2 ml of whole blood was collected from the jugular vein using a vacutainer tube that contained an anticoagulant (EDTA). All animals were apparently healthy during sampling. All blood samples were subjected to DNA extraction using phenol:chloroform:isoamyl alcohol (25:24:1, v/v) [19]. Moreover, a total of 601 questing adult ticks were collected in eight Mongolian provinces (Bayan-Ulgii, Dornod, Govi-Altai, Khovd, Selenge, Omnogovi, Ovorkhangai, and Tov) in 2012 and 2014–2016 using the flagging method. Based on morphology [20, 21], the questing ticks were identified as *Dermacentor nuttalli* (n=378)*, Ixodes persulcatus* (n=95)*,* and *Hyalomma asiaticum* (n=128). In addition, a total of 120 unfed adult *Haemaphysalis pospelovashtromae*, which was not detected among the questing ticks collected in the present study, were collected from the fur of both sheep and goats in four Mongolian provinces (Arkhangai, Bayankhongor, Bayan-Ulgii, and Zavkhan). The following morphological features were used to identify the tick species; 1) presence of spurs on the leg segments (*D. nuttalli*) or coxae (*Hae. pospelovashtromae*), 2) length of internal spur of coxae (*I. persulcatus* and *D. nuttalli*), 3) shapes of cervical groove, basis capituli, and lateral grooves on scutum (*Hya. asiaticum*), and 4) shape, length, and width of mouth parts (basis capituli, palp, and hypostome) and shapes of scutum and genital area

(*Hae*. *pospelovashtromae*). Subsequently, individual ticks were digested with a lysis buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA pH 7.5, 10 mM NaCl, 1% SDS, and 100 µg/ml Proteinase K) as previously described [22], and DNA samples were prepared using phenol:chloroform:isoamyl alcohol (25:24:1, v/v) [19]. All DNA samples were stored at –30ºC until further use. All animal procedures were approved by the Committee on the Ethics of Animal Experiments, Obihiro University of Agriculture and Veterinary Medicine (Approval number 28-45).

2.2. PCR detection of A. ovis *in sheep, goats, and ticks*

All DNA samples from sheep, goats, and ticks were screened for *A*. *ovis* infection using a previously described major surface protein gene (*msp4*)-based PCR assay [23]. Briefly, a 25-µl reaction mixture was prepared that contained 1.0 µl of template DNA, 1× PCR buffer (10× DreamTaq Buffer, Thermo Fisher Scientific, Vilnius, Lithuania), 200 µM of each dNTPs (Thermo Fisher Scientific), 0.4 µM of each forward (MSP45, 5′-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC-3′) and reverse (MSP43, 5′-CCGGATCCTTAGCTGAACAGGAATCTTGC-3′) primers, 0.25 µl of 5 U/µl *Taq* DNA polymerase (DreamTaq DNA Polymerase, Thermo Fisher Scientific), and 16.75 µl of ultra-pure water. Blood DNA sample from a sheep in Mongolia with *A*. *ovis* infection confirmed by microscopy as well as by PCR and sequencing [23] was used as a positive control (unpublished data), while a PCR reaction mixture that contained water instead of DNA was used as a negative control. The reaction mixture was then subjected to pre-denaturation at 95ºC for 5 min, and then to 40 cycles of denaturation at 95ºC for 45 s, annealing at 59ºC for 45 s, and extension at 72ºC for 1 min. Final elongation at 72ºC for 5 min was followed by agarose gel

127 electrophoresis, ethidium bromide staining, and visualisation under UV illumination.
128 Detection of a band at approximately 870-bp was considered positive.
129
130 ***2.3. Cloning and sequencing***
131 PCR products with the expected sizes were gel-extracted using NucleoSpin®
132 Gel and PCR Clean-up kit (MACHEREY-NAGGmbH & Co. KG, Düren, Germany).
133 The extracted DNA was ligated to a PCR 2.1 plasmid vector (TOPO, Invitrogen,
134 Carlsbad, CA, USA), and the inserts were sequenced using ABI PRISM 3100 genetic
135 analyzer (Applied Biosystems, Branchburg, NJ, USA).
136
137 ***2.4. Sequencing and phylogenetic analyses***
138 The newly generated *msp4* sequences were initially analysed by basic local
139 alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their origin.
140 The sequences were then trimmed at the both ends to obtain full-length (852-bp) *msp4*
141 sequences. The identity scores shared among the *msp4* sequences were calculated using
142 MatGAT version 2.01 [24]. The newly obtained Mongolian sequences and those
143 obtained from GenBank were used to construct a maximum likelihood phylogeny based
144 on Kimura 2-parameter substitution model [25] using MEGA version 6.0 [26]. The
145 evolutionary rate differences among sites were modelled using a discrete gamma
146 distribution (+G).
147
148 ***2.5. Statistical analyses***
149 The confidence intervals for the *A. ovis*-positive rates were calculated using
150 OpenEpi (http://www.openepi.com/Proportion/Proportion.htm) based on Wilson score

151 interval [27]. The *P* values to assess the statistically significant variations among the
152 rates were calculated using an "N-1" chi-squared test
153 (https://www.medcalc.org/calc/comparison_of_proportions.php) [28, 29]. *P* values <
154 0.05 were considered to indicate significant variation.
155
156

157 **3. Results and discussion**

158

159 Anaplasmosis caused by *A*. *ovis* in sheep and goats is widespread in Asian,
160 European, Mediterranean, and North and South American countries [2, 30]. This
161 infection may result in clinical disease that leads to severe economic losses [6]. Recent
162 studies detected *A*. *ovis* in sheep and goats from a few Mongolian provinces [14, 31].
163 However, the country-wide epidemiology of *A*. *ovis* and tick species that transmit this
164 pathogen were not known in Mongolia. Therefore, the aim of this study was to
165 determine the *A*. *ovis* infection rates in sheep and goats across Mongolia, and to identify
166 the potential tick vectors of *A*. *ovis* in this country.

167 *Anaplasma ovis* was detected by the *msp4* PCR assay from both sheep and goats
168 in all surveyed provinces (Table 1). The overall *A*. *ovis*-positive rates were comparable
169 between sheep and goats analysed in this study. Of 1,179 sheep and 871 goat DNA
170 samples, 813 (69.0%) and 621 (71.3%) were positive for *A*. *ovis* infection. The positive
171 rates were comparable to those determined in several other endemic countries, including
172 Portugal, Sudan, and Iraq [6]. On a per province basis, the positive rates in sheep and
173 goats ranged from 7.4%–93.3% and 13.3%–100%, respectively (Table 1). However,
174 the positive rates were less than 40% in small ruminants (sheep and goats) in only three
175 provinces (Dornod, Dornogovi, and Khentii), whereas the positive rates in the rest of
176 the surveyed provinces were greater than 40% (Fig. 1). In particular, *A*. *ovis* infection
177 was observed in more than 80% of small ruminants from Arkhangai, Bayankhongor,
178 Bulgan, Govisumber, Khovd, and Ovorkhangai (Fig. 1).

179 In general, animal age is a known risk factor for infections caused by tick-borne
180 pathogens [32–34]. Therefore, we investigated *A*. *ovis*-positive rates in two different age

groups. As the records on the age for 236 sheep and 143 goats were not available, 943 sheep and 728 goats were analysed for *A*. *ovis* infection in 1–3-year-old and >3-year-old age groups. However, the positive rates were not different between these age groups in sheep (64.4% and 67.4%, respectively) and goats (80.8% and 72.3%, respectively), which indicates that age is not a risk factor for *A*. *ovis* infection in Mongolia (Table 2). Although the reason why the positive rates were comparable between age groups is not very clear, the fact that the Mongolian livestock animals, including small ruminants, are extensively managed throughout their life might explain this observation [35]. Therefore, differences in the density and activity of tick vectors that transmit *A*. *ovis* might be a reason for the differential *A*. *ovis*-infection rates observed among Mongolian provinces.

Although *A*. *ovis* has been detected in several tick species, the vectorial capacity of these ticks is unknown, as most of these studies analysed ticks that were collected from the animal body [36–38]. The confirmed tick vectors of *A*. *ovis* include *Rhipicephalus bursa* and *Dermacentor andersoni* [2], both of which were not reported in Mongolia. In addition, a previous study found that *A*. *ovis* can be transmitted intrastadially (i.e, acquisition and transmission of infection by the same tick in the same stage when moves from one host to other without molting) by *D*. *nuttalli*, *Hya*. *asiaticum*, and *Rhipicephalus pumilio*, while transsatadial persistence was not observed [39]. Among these tick species, *D*. *nuttalli* and *Hya*. *asiaticum* are endemic in Mongolia, but their involvement in *A*. *ovis* transmission is unknown. Therefore, to identify potential tick vectors of *A*. *ovis* in Mongolia, we collected 721 unfed ticks, including 601 questing ticks (*D*. *nuttalli*, *I*. *persulcatus*, and *Hya*. *asiaticum*) collected from pastures in eight provinces and 120 *Hae*. *pospelovashtromae* that were attached to the

fur of sheep and goats in four provinces (Table 3). Among the questing ticks collected, *D. nuttalli* was detected in Bayan-Ulgii, Dornod, Govi-Altai, Khovd, and Tov, whereas *I. persulcatus* was only detected in Selenge. Alternatively, *Hya. asiaticum* was only collected in Omnogovi and Ovorkhangai Provinces (Table 3). The differences in the geography of sampling locations within provinces may explain why only a single tick species was collected in a given province, as the tick distribution in Mongolia varies among steppe, forest, and Gobi areas of each province [40].

When the DNA samples extracted from all 721 ticks were subjected to the *msp4* PCR assay, only nine *D. nuttalli* DNA samples (2.4%) were positive, which indicates that this tick species is a potential *A. ovis* vector in Mongolia. *Anaplasma ovis*-positive *D. nuttalli* ticks were detected in Bayan-Ulgii (2.9%), Govi-Altai (2.1%), and Khovd (2.9%) (Table 3). The positive rates in *D. nuttalli* were, however, very low as compared to those determined in sheep and goats. The present study analyzed only unfed adult ticks, and therefore *A. ovis* acquired by the nymphal stage might have been lost when they emerged as adults due to lack of transstadial transmission in *D. nuttalli* [39]. This could explain the low *A. ovis* positive rates in *D. nuttalli* compared with the small ruminants in Mongolia. In this study, we found that *A. ovis* infection rates in sheep and goats were relatively higher in western and central regions compared with the rest of Mongolia, except Sukhbaatar, which is an eastern province (Fig. 1). *Dermacentor nuttalli* abundance in western and central regions might explain the high prevalence of *A. ovis* in these regions, as the high-altitude forest and steppe areas favour colonisation of this tick species [40]. However, the questing ticks collected in Selenge, Omnogovi, and Ovorkhangai did not contain *D. nuttalli*. The differences in the geography of

228 sampling sites within provinces, as discussed elsewhere in this article, may explain why
229 *D*. *nuttalli* was not detected in these provinces [40].

230 To confirm the PCR findings, 10, 9, and 1 PCR amplicons with high band
231 intensity from sheep (2 from Khovd, 5 from Govisumber, and 3 from Bayan-Ulgii),
232 goats (3 from Khovd, 4 from Govisumber, and 2 from Bayan-Ulgii), and a *D*. *nuttalli*
233 tick (from Bayan-Ulgii), respectively, were cloned and sequenced. The resultant *A*. *ovis*
234 *msp4* sequences from sheep (GenBank accession numbers: LC412073–LC412082),
235 goats (LC412083–LC412091), and a *D*. *nuttalli* tick (LC412092) shared 99.6%–100%
236 identity. These sequences also shared 99.5%–100% identity scores with known *A*. *ovis*
237 *msp4* sequences from Mongolia (LC141078), China (KJ782397), Italy (AY702923),
238 Turkey (KY283958), Sudan (KU497710), Spain (HQ014384), Cyprus (FJ460454), the
239 USA (DQ674249), and Hungary (EF190512). In the phylogeny, the Mongolian *A*. *ovis*
240 *msp4* sequences clustered together with previously reported sequences from Mongolia
241 and with those reported from other endemic countries, which confirmed the PCR
242 findings of this study (Fig. 2).

243 In conclusion, this study demonstrated that *A*. *ovis* infects sheep and goats
244 throughout Mongolia, and that *D*. *nuttalli* is a potential vector in this country. Therefore,
245 *D*. *nuttalli* control is vital for minimising *A*. *ovis* prevalence in Mongolia.
246

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248

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255 **References**

256 1. K.L. Kuttler, *Anaplasma* infections in wild and domestic ruminants: a review, J.
257 Wildl. Dis. 20 (1984) 12–20.
258 2. K.T. Friedhoff, Tick-borne diseases of sheep and goats caused by *Babesia*,
259 *Theileria* or *Anaplasma* spp., Parassitologia 39 (1997) 99–109.
260 3. A. Rymaszewska, S. Grenda, Bacteria of the genus *Anaplasma* – characteristics of
261 *Anaplasma* and their vectors: a review, Vet. Med. Czech 53 (2008) 573–584.
262 4. S.M. Khayyat, A.A. Gilder, Ovine piroplasmoses in Iraq, Trans. R. Soc. Trop. Med.
263 Hyg. 41 (1947) 119–120.
264 5. R. Manickam, Epidemiological and clinical observations of acute anaplasmosis in
265 sheep, Indian J. Vet. Med. 7 (1987) 159–160.
266 6. S. Renneker, J. Abdo, D.E. Salih, T. Karagenc, H. Bilgic, A. Torina, A.G. Oliva, J.
267 Campos, B. Kullmann, J. Ahmed, U. Seitzer, Can *Anaplasma ovis* in small
268 ruminants be neglected any longer?, Transbound. Emerg. Dis. 60 Suppl 2 (2013)
269 105–112.
270 7. T. Tibbitts, W. Goff, W. Foreyt, D. Stiller, Susceptibility of two Rocky mountain
271 bighorn sheep to experimental infection with *Anaplasma ovis*, J. Wildl. Dis. 28
272 (1992) 125–129.
273 8. World Bank, Synthesis Report, Vol. I, Livestock Sector Study, Mongolia (2009).
274 9. K. Altangerel, B. Battsetseg, B. Battur, T. Sivakumar, E. Batmagnai, G. Javkhlan,
275 B. Tuvshintulga, I. Igarashi, K. Matsumoto, H. Inokuma, N. Yokoyama, The first
276 survey of *Theileria orientalis* infection in Mongolian cattle, Vet. Parasitol. 182
277 (2011) 343–348.
278 10. K. Altangerel, T. Sivakumar, B. Battsetseg, B. Battur, A. Ueno, I. Igarashi, N.

279 Yokoyama, Phylogenetic relationships of Mongolian *Babesia bovis* isolates based
280 on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes, Vet.
281 Parasitol. 184 (2012) 309–316.
282 11. T. Sivakumar, K. Altangerel, B. Battsetseg, B. Battur, M. Aboulaila, T.
283 Munkhjargal, T. Yoshinari, N. Yokoyama, I. Igarashi, Genetic detection of *Babesia*
284 *bigemina* from Mongolian cattle using apical membrane antigen-1 gene-based PCR
285 assay, Vet. Parasitol. 187 (2012) 17–22.
286 12. T. Yoshinari, T. Sivakumar, M. Asada, B. Battsetseg, X. Huang, D.T. Lan, T.
287 Inpankaew, A.P. Ybañez, A. Alhassan, O.M. Thekisoe, A.C. De Macedo, H.
288 Inokuma, I. Igarashi, N. Yokoyama, A PCR based survey of *Babesia ovata* in cattle
289 from various Asian, African and South American countries, J. Vet. Med. Sci. 75
290 (2013) 211–214.
291 13. A.P. Ybañez, T. Sivakumar, B. Battsetseg, B. Battur, K. Altangerel, K. Matsumoto,
292 N. Yokoyama, H. Inokuma, Specific molecular detection and characterization of
293 *Anaplasma marginale* in Mongolian cattle, J. Vet. Med. Sci. 75 (2013) 399–406.
294 14. N. Ochirkhuu, S. Konnai, R. Odbileg, S. Murata, K. Ohashi, Molecular
295 epidemiological survey and genetic characterization of *Anaplasma* species in
296 Mongolian livestock, Vector Borne Zoonotic Dis. 17 (2017) 539–549.
297 15. C. Karnath, A. Obiegala, S. Speck, S. Essbauer, H. Derschum, H. Scholz, D. Kiefer,
298 D. Tserennorov, O. Dashdavaa, N. Tsogbadrakh, B. Jigjav, M. Pfeffer, Detection of
299 *Babesia venatorum*, *Anaplasma phagocytophilum* and *Candidatus Neoehrlichia*
300 *mikurensis* in *Ixodes persulcatus* ticks from Mongolia, Ticks Tick Borne Dis. 7
301 (2016) 357–360.
302 16. P. Sophia, G. Battsetseg, H. Kass Philip, E. Foley Janet, Detection and

303 Epidemiology of Tick-Borne Pathogens in Free-Ranging Livestock in Mongolia, J.
304 Clin. Exp. Pathol. S3 (2012) 006.
305 17. Ministry of Food and Agriculture of Mongolia, National statistical census (2015)
306 18. J.C. Haigh, V. Gerwing, J. Erdenebaatar, J.E. Hill, A novel clinical syndrome and
307 detection of *Anaplasma ovis* in Mongolian reindeer (*Rangifer tarandus*), J. Wildl.
308 Dis. 44 (2008) 569–577.
309 19. J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular cloning: A laboratory manual.
310 Cold Spring Harbor Laboratory Press, NY, USA (1989).
311 20. D.R. Arthur, Ticks: A Monograph of the Ixodoidea. Part V. On the Genera
312 *Dermacentor*, *Anocentor*, *Cosmiomma*, *Boophilus* and *Margaropus*, Cambridge
313 Univ. Press, Cambridge, UK, (1960).
314 21. N.A. Filippova, Ixodid tick of the subfamily ixodinae, Fauna SSSR, Arachnoidea,
315 Lzd. Nauka Leningrad (in Russian) 4 (1977) 316–330.
316 22. B. Tuvshintulga, T. Sivakumar, B. Battsetseg, S. Narantsatsaral, B. Enkhtaivan, B.
317 Battur, K. Hayashida, K. Okubo, T. Ishizaki, N. Inoue, I. Igarashi, N. Yokoyama,
318 The PCR detection and phylogenetic characterization of *Babesia microti* in
319 questing ticks in Mongolia, Parasitol. Int. 64 (2005) 527–532.
320 23. J. de la Fuente, M.W. Atkinson, V. Naranjo, I.G. Fernandez de Mera, A.J. Mangold,
321 K.A. Keating, K.M. Kocan, Sequence analysis of the msp4 gene of *Anaplasma ovis*
322 strains, Vet. Microbiol. 119 (2007) 375–381.
323 24. J.J. Campanella, L. Bitincka, J. Smalley, MatGAT: an application that generates
324 similarity/identity matrices using protein or DNA sequences, BMC Bioinformatics
325 4 (2003) 29.
326 25. M. Kimura, A simple method for estimating evolutionary rate of base substitutions

through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120.

26. K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
27. E.B. Wilson, Probable inference, the law of succession, and statistical inference, J. Am. Stat. Assoc. 22 (1927) 209–212.
28. I. Campbell, Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations, Stat. Med. 26 (2007) 3661–3675.
29. J.T. Richardson, The analysis of 2 × 2 contingency tables—yet again, Stat. Med. 30 (2011) 890.
30. V. Rar, I. Golovljova, *Anaplasma*, *Ehrlichia*, and "*Candidatus Neoehrlichia*" bacteria: pathogenicity, biodiversity, and molecular genetic characteristics, a review, Infect. Genet. Evol. 11 (2011) 1842–1861.
31. M.J. Lagerkvist, Cross-sectional study of *Anaplasma* spp. in goats and sheep in Mongolia: A comparison between species in relationship to pasture conditions, Examensarbete (2017) 67.
32. F. Iqbal, M. Fatima, S. Shahnawaz, M. Naeem, R. Shaikh, M. Ali, A. Shaikh, M. Aktas, M. Ali, A study on the determination of risk factors associated with babesiosis and prevalence of *Babesia* sp., by PCR amplification, in small ruminants from Southern Punjab (Pakistan), Parasite 18 (2011) 229–234.
33. N. Abdela, N. Ibrahim, F. Begna, Prevalence, risk factors and vectors identification of bovine anaplasmosis and babesiosis in and around Jimma town, Southwestern Ethiopia, Acta. Trop. 177 (2018) 9–18.
34. D.A. Salih, A.M. El Hussein, M.N. Kyule, K.H. Zessin, J.S. Ahmed, U. Seitzer,

Determination of potential risk factors associated with *Theileria annulata* and *Theileria parva* infections of cattle in the Sudan, Parasitol. Res. 101 (2007) 1285–1288.

35. B. Battsetseg, T. Sivakumar, K. Naranbaatar, S. Narantsatsral, P. Myagmarsuren, B. Enkhtaivan, B. Davaasuren, D. Mizushima, G. Weerasooriya, I. Igarashi, B. Battur, N. Yokoyama, Serosurvey of *Babesia bovis* and *Babesia bigemina* in cattle in Mongolia, Vet. Parasitol. Reg. Stud. Rep. 13 (2018) 85–91.
36. A. Saghafi Pour, A. Sofizadeh, B. Farzinnia, S. Chinikar, N. Jesri, Z. Telmadarreyi, Molecular detection of *Anaplasma ovis* in ticks in Qom County, Qom Province, J. Zoonoses 1 (2014) 54–59.
37. A. Jafarbekloo, H. Bakhshi, F. Faghihi, Z. Telmadarraiy, A. Khazeni, M.A. Oshaghi, M.R. Ramzgouyan, M.M. Sedaghat, Molecular detection of *Anaplasma* and *Ehrlichia* infection in ticks in borderline of Iran-Afghanistan, J. Biomed. Sci. Eng. 7 (2014) 919–926.
38. A. Matysiak, P. Dudko, K. Dudek, M. Dudek, A. Junkuszew, P. Tryjanowski, The occurrence of pathogens in *Rhipicephalus microplus* ticks from cattle in Madagascar, Veterinární Medicína 61 (2016) 516–523.
39. L. Wenshun, L. Wenxiang, Z. Qicai, Y. Feng, D. Huifang, Y. Hong, Ovine anaplasmosis in Northwest China, Trop. Anim. Health Prod. 29(1997) S18-S18.
40. D. Mu, Pasture ticks in Mongolia and preventive measures, Doctoral dissertation, Berlin (1986).

373 **Figure legends**

374

375 **Fig. 1.** Epidemiological mapping of *A*. *ovis* in Mongolia. Epidemiological maps were
376 prepared to illustrate the differential *A*. *ovis* infection rates in small ruminants (both
377 sheep and goats) from 19 Mongolian provinces. The geographical variations of the *A*.
378 *ovis*-positive rates among small ruminants are indicated by different background colours.
379 The red, blue, and green circles indicate geographical locations at which sheep, goats,
380 and both sheep and goats were sampled, respectively.

381

382 **Fig. 2.** Phylogenetic tree of *A*. *ovis msp4*. A maximum-likelihood phylogeny was
383 constructed using the *A*. *ovis msp4* sequences obtained in this study and those retrieved
384 from GenBank. Two *A*. *marginale msp4* sequences were used as outgroup sequences.
385 The sequences generated in this study are highlighted in boldface. Note that the newly
386 determined Mongolian gene sequences clustered together with known *A*. *ovis* sequences
387 from different countries.

Table 1. PCR detection of *A. ovis* in sheep and goats from 19 Mongolian provinces

Province	Year/Month	Sheep			Goat		
		No. Sample	No. Positive	% (CI[a])	No. Sample	No. Positive	%(CI)
Arkhangai	2013/7, 2014/7	119	102	85.7 (78.3-90.8)	37	33	89.2 (75.2-95.7)
Bayankhongor	2014/10, 2015/4	50	45	90 (78.6-95.6)	59	47	79.7 (67.7-87.9)
Bayan-Ulgii	2014/8, 2015/4	51	30	58.8 (45.1-71.2)	49	42	85.7 (73.3-92.9)
Bulgan	2013/7, 2014/7	160	136	85 (78.6-89.7)	120	113	94.2 (88.4-97.1)
Dornod	2013/4	18	4	22.2 (9-45.2)	14	5	35.7 (16.3-61.2)
Dornogovi	2014/9	39	6	15.4 (7.2-29.7)	72	17	23.6 (15.3-34.6)
Dundgovi	2013/4, 2014/9	58	28	48.3 (35.9-60.8)	68	23	33.8 (23.7-45.6)
Govi-Altai	2015/4	10	9	90 (59.5-98.2)	16	10	62.5 (38.6-81.5)
Govisumber	2016/6	68	57	83.8 (73.3-90.7)	57	49	86 (74.6-92.7)
Khentii	2013/4, 2015/5	27	2	7.4 (2-23.3)	15	2	13.3 (3.7-37.8)
Khovd	2014/7	49	33	67.3 (53.3-78.7)	50	50	100 (92.9-100)
Khovsgol	2014/7	112	77	68.8 (59.6-76.5)	46	42	91.3 (79.6-96.5)
Omnogovi	2014/9, 2015/4	33	18	54.5 (37.9-70.1)	33	18	54.5 (37.9-70.1)
Ovorkhangai	2014/10	30	28	93.3 (78.6-98.1)	20	18	90 (66.8-98.2)
Selenge	2013/7, 2014/7	110	81	73.6 (64.7-80.9)	68	50	73.5 (61.9-82.5)
Sukhbaatar	2013/4, 2016/5	35	23	65.7 (49.1-79.1)	28	19	67.9 (49.3-82)
Tov	2013/5, 2014/9	109	84	77.1 (68.3-83.9)	40	19	47.5 (32.9-62.5)
Uvs	2016/4	55	23	41.8 (29.7-54.9)	41	31	75.6 (60.6-86.1)
Zavkhan	2014/10	26	14	53.8 (35.4-71.2)	14	12	85.7 (60-95.9)
Total		1179	813	69 (66.2-71.5)	871	621	71.3 (68.2-74.2)

[a] 95% confidence interval

Table 2. *Anaplasma ovis*-positive rates in different age groups of sheep and goats from 19 Mongolian provinces

Province	Sheep							Goat						
	1-3 years			> 3 years			*P* value	1-3 years			> 3 years			*P* value
	No. sample	No. postive	% (CI[a])	No. sample	No. postive	% (CI)		No. sample	No. postive	% (CI)	No. sample	No. postive	% (CI)	
Arkhangai	42	36	85.7 (72.1-93.2)	37	29	78.4 (62.8-88.6)	0.3935	20	19	95.0 (76.3-99.1)	12	11	91.7 (64.6-98.5)	0.7055
Bayankhongor	19	18	94.7 (75.3-99.0)	31	25	80.6 (63.7-90.8)	0.1679	24	19	79.2 (59.5-90.7)	29	24	82.8 (65.4-92.4)	0.7415
Bayan-Ulgii	29	10	34.5 (19.9-52.6)	21	16	76.2 (54.9-89.3)	0.0039	24	22	91.7 (74.1-97.6)	20	19	95.0 (76.3-99.1)	0.6604
Bulgan	86	70	81.4 (71.9-88.2)	63	56	88.9 (78.8-94.5)	0.2133	54	53	98.1 (90.2-99.6)	55	53	96.4 (87.6-99.0)	0.5713
Dornod	14	3	21.4 (7.5-47.5)	4	1	25.0 (4.5-69.9)	0.882	10	5	50.0 (23.6-76.3)	4	0	0.0 (0.0-48.9)	
Dornogovi	15	3	20.0 (7.0-45.1)	24	3	12.5 (4.3-31.0)	0.533	4	1	25.0 (4.5-69.9)	42	14	33.3 (21.0-48.4)	0.7378
Dundgovi	22	14	63.6 (42.9-80.2)	23	10	43.5 (25.6-63.1)	0.1794	18	6	33.3 (16.2-56.2)	34	11	32.4 (19.1-49.1)	0.9422
Govi-Altai	3	3	100 (43.8-100)	7	6	85.7 (48.6-97.4)		6	4	66.7 (30.0-90.3)	10	6	60.0 (31.2-83.1)	0.7983
Govisumber	31	27	87.1 (71.1-94.8)	37	30	81.1 (65.8-90.5)	0.5075	38	33	86.8 (72.6-94.2)	19	16	84.2 (62.4-94.4)	0.7919
Khentii	20	1	5.0 (0.8-23.6)	7	1	14.3 (2.5-51.3)	0.4318	10	1	10.0 (1.7-40.4)	5	1	20.0 (3.6-62.4)	0.6038
Khovd	25	18	72.0 (52.4-85.7)	24	16	66.7 (46.7-82.0)	0.8002	20	20	100 (83.8-100)	30	30	100 (88.6-100)	
Khovsgol	23	11	47.8 (29.2-67.0)	34	16	47.1 (31.4-63.2)	0.9531	30	26	86.7(70.3-94.6)	15	15	100 (79.6-100)	
Omnogovi	8	6	75.0 (40.9-92.8)	16	8	50.0 (28.0-72.0)	0.2516	5	3	60.0 (23.0-88.2)	17	11	64.7 (41.3-82.6)	0.8512
Ovorkhangai	11	11	100 (74.1-100)	19	17	89.5 (68.6-97.0)		13	11	84.6 (57.7-95.6)	7	7	100 (64.5-100)	
Selenge	50	36	72.0 (58.3-82.5)	56	41	73.2 (60.4-83.0)	0.8905	33	25	75.8 (58.9-87.1)	35	25	71.4 (54.9-83.6)	0.6902
Sukhbaatar	7	1	14.3 (2.5-51.3)	13	9	69.2 (42.3-87.3)	0.0222	20	18	90.0 (69.9-97.2)	0	0	0	
Tov	13	8	61.5 (35.5-82.2)	28	25	89.3 (72.8-96.2)	0.0399	3	0	0	7	5	71.4 (35.9-91.7)	
Uvs	22	11	50.0 (30.7-69.2)	33	12	36.4 (22.1-53.3)	0.3173	18	16	88.9 (67.2-96.9)	23	15	65.2 (44.8-81.1)	0.0011
Zavkhan	12	4	33.3 (13.8-60.9)	14	10	71.4 (45.3-88.2)	0.0568	5	5	100 (56.5-100)	9	7	77.8 (45.2-93.6)	0.4215
Total	452	291	64.4 (59.9-68.7)	491	331	67.4 (63.1-71.4)	0.3317	355	287	80.8 (76.4-84.6)	373	270	72.3 (67.6-76.6)	0.5482

[a] 95% confidence interval

Table 3. PCR detection of *A. ovis* in unfed ticks collected from 11 Mongolian provinces

Province	Year/Month	*Dermacentor nuttalli*[a]		*Ixodes persulcatus*[a]		*Hyalomma asiaticum*[a]		*Haemaphysalis pospelovashtromae*[b]	
		No. samples	No. positive (%)	No. samples	No. positive (%)	No. samples	No. positive (%)	No. samples	No. positive (%)
Arkhangai	2015/4	NC	-	NC	-	NC	-	6	0
Bayankhongor	2015/4, 2016/4	NC	-	NC	-	NC	-	78	0
Bayan-Ulgii	2012/4, 2015/4	136	4 (2.9)	ND	-	ND	-	32	0
Dornod	2016/5	20	0	ND	-	ND	-	NC	-
Govi-Altai	2015/5	48	1 (2.1)	ND	-	ND	-	NC	-
Khovd	2014/5	138	4 (2.9)	ND	-	ND	-	NC	-
Omnogovi	2016/6	ND	-	ND	-	91	0	NC	-
Ovorkhangai	2016/6	ND	-	ND	-	37	0	NC	-
Selenge	2014/6	ND	-	95	0	ND	-	NC	-
Tov	2015/4	36	0	ND	-	ND	-	NC	-
Zavkhan	2015/4	NC	-	NC	-	NC	-	4	0
Total		378	9 (2.4)	95		128		120	0

NC, not collected; ND, not detected.

[a] Questing *Dermacentor nuttalli*, *Ixodes persulcatus*, and *Hyalomma asiaticum* ticks were collected by the flagging method.

[b] Unfed *Haemaphysalis pospelovashtromae* ticks were collected from sheep and goat fur.

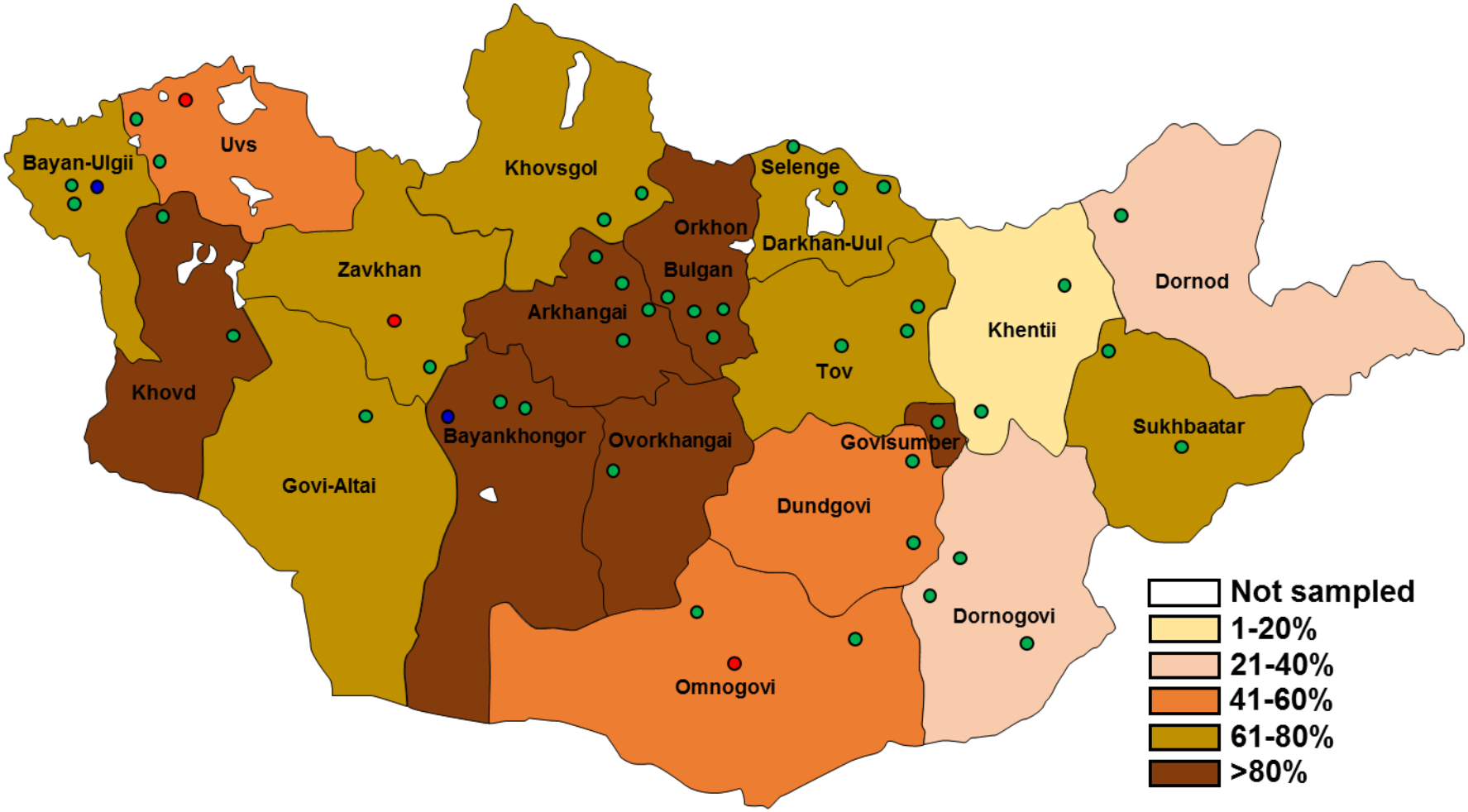
Bayan-Ulgii
Uvs
Khovsgol
Selenge
Orkhon
Darkhan-Uul
Zavkhan
Bulgan
Dornod
Arkhangai
Khentii
Tov
Khovd
Bayankhongor
Ovorkhangai
Govisumber
Sukhbaatar
Govi-Altai
Dundgovi
Dornogovi
Omnogovi
Not sampled
1-20%
21-40%
41-60%
61-80%
>80%

Fig. 1

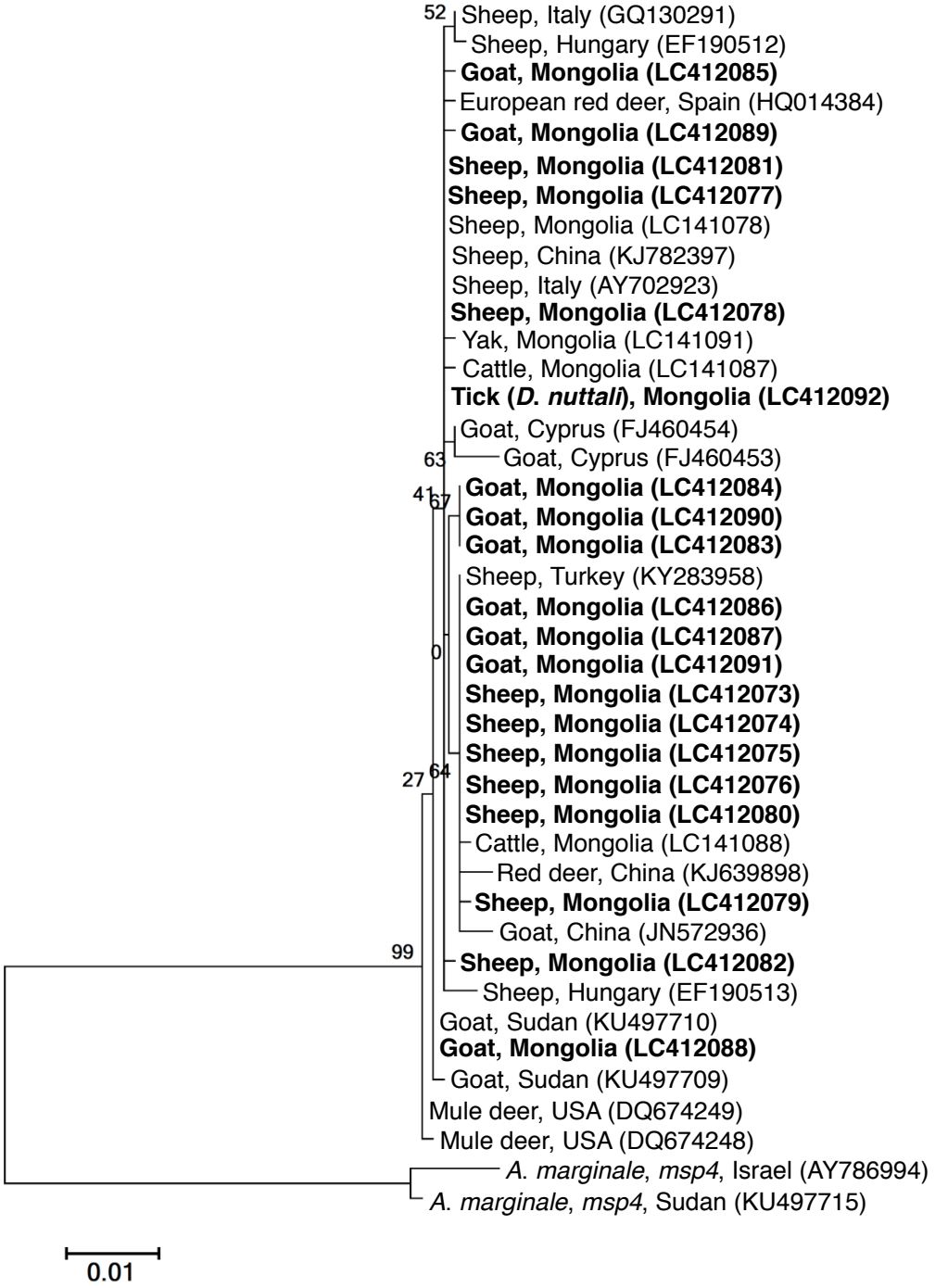
52
Sheep, Italy (GQ130291)
Sheep, Hungary (EF190512)
Goat, Mongolia (LC412085)
European red deer, Spain (HQ014384)
Goat, Mongolia (LC412089)
Sheep, Mongolia (LC412081)
Sheep, Mongolia (LC412077)
Sheep, Mongolia (LC141078)
Sheep, China (KJ782397)
Sheep, Italy (AY702923)
Sheep, Mongolia (LC412078)
Yak, Mongolia (LC141091)
Cattle, Mongolia (LC141087)
Tick (D. nuttali), Mongolia (LC412092)
Goat, Cyprus (FJ460454)
63
Goat, Cyprus (FJ460453)
41
67
Goat, Mongolia (LC412084)
Goat, Mongolia (LC412090)
Goat, Mongolia (LC412083)
Sheep, Turkey (KY283958)
Goat, Mongolia (LC412086)
Goat, Mongolia (LC412087)
0
Goat, Mongolia (LC412091)
Sheep, Mongolia (LC412073)
Sheep, Mongolia (LC412074)
Sheep, Mongolia (LC412075)
27
64
Sheep, Mongolia (LC412076)
Sheep, Mongolia (LC412080)
Cattle, Mongolia (LC141088)
Red deer, China (KJ639898)
Sheep, Mongolia (LC412079)
Goat, China (JN572936)
99
Sheep, Mongolia (LC412082)
Sheep, Hungary (EF190513)
Goat, Sudan (KU497710)
Goat, Mongolia (LC412088)
Goat, Sudan (KU497709)
Mule deer, USA (DQ674249)
Mule deer, USA (DQ674248)
A. marginale, msp4, Israel (AY786994)
A. marginale, msp4, Sudan (KU497715)
0.01

Fig. 2