Molecular survey of *Babesia*, *Theileria*, *Trypanosoma*, and *Anaplasma* infections in camels (*Camelus dromedaries*) in Egypt

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Abstract

The one-humped camel (*Camelus dromedarius*) or dromedary is an economically important domestic animal. However, infectious diseases, including those caused by vector-borne hemopathogens, frequently compromise the health and production of camels. In this study, we examined infections caused by *Babesia*, *Theileria*, *Trypanosoma*, and *Anaplasma* species in camels in Egypt. We analyzed blood DNA samples from 148 camels reared in six Egyptian governorates (Giza, Asyut, Sohag, Qena, Luxor, and the Red Sea) using pathogen-specific Polymerase Chain Reaction (PCR) assays. Our results indicated that 29 (19.6%), 22 (14.9%), 1 (0.7%), 2 (1.4%), 1 (0.7%), 2 (1.4%), and 28 (18.9%) of the surveyed animals were infected with *Babesia bovis*, *B. bigemina*, *Babesia* sp. Mymensingh, *Theileria* sp. Yokoyama, *Theileria equi*, *Trypanosoma evansi*, and *Anaplasma marginale*, respectively. We found that a total of 68 (45.9%) animals were infected with at least one of the detected hemopathogens. Sequencing analyses of PCR amplicons confirmed our diagnostic results. This study is the first to report *Theileria* sp. Yokoyama and *Babesia* sp. Mymensingh in Egypt. This is also the first report of infection with these two species in one-humped camel. In conclusion, this study found that camels in Egypt are infected with several vector-borne hemopathogens, including novel parasite species.

Keywords: *Anaplasma*, *Babesia*, Camel, Egypt, *Theileria*, *Trypanosoma*

The one-humped camel *Camelus dromedarius* or dromedary is a domestic animal belonging to the Camelidae family and is widely distributed in the arid and semi-arid regions of Africa, Arabia, and western Asia extending up to India [1]. Camels significantly contribute to Egypt's local economy and culture. They produce milk, meat, and wool [1]. Additionally, camels serve as a mode of transportation, particularly in the desert; therefore, they are an important element of nomadic life. Camel rearing is primarily practiced for recreation and entertainment purposes in tourist areas such as the Luxor and Red Sea governorates [2]. Camels in Egypt are maintained by different management strategies depending on the topography. In the countryside, smallholders occasionally rear camels together with other livestock or in solitary farms, and they are also reared in desert rangelands, such as the Sinai Peninsula, northwest coastal zone, and the coastline along the Red Sea [3].

Infections caused by vector-borne hemopathogens are highly prevalent among camels. They are known to be infected with various species of *Babesia* (*Babesia bovis* (*B. bovis*), *B. bigemina*, and *B. caballi*), *Theileria* (*Theileria annulata*, *T. mutans*, *T. ovis*, and *T. equi*), *Trypanosoma* (*Trypanosoma evansi*), *Anaplasma* (*Anaplasma marginale*, *A. centrale*, *A. platys*, and *A. phagocytophilum*), *Ehrlichia* (*Ehrlichia canis*), and *Hepatozoon* (*Hepatozoon canis*) [4–9]. Several tick species, including *Hyalomma dromedarii* (*H. dromedarii*), *H. rufipes*, *H. truncatum*, *H. anatolicum excavatum*, *H. marginatum rufipes*, and *H. impeltatum*, frequently infest the camels in Egypt [5,10]. Some of these tick species are known to transmit several hemopathogens, such as species of *Babesia* (*B. caballi* by *H. marginatum* and *H. truncatum*), *Theileria* (*T. annulata*, *T. lestoquardi*, and *T. ovis* by *Hyalomma* species), and *Anaplasma* (*A. marginale* by *H. rufipes*) [11–13]. Therefore, infections with these hemopathogens are widespread in Egyptian camels, causing clinical diseases, such as theileriosis, trypanosomiasis, and anaplasmosis [5,14–17]. Previous investigations conducted using microscopy and Polymerase Chain Reaction (PCR) assays

reported that camels in Egypt were infected with *B. bovis*, *B. bigemina*, *T. annulata*, *T. evansi,* *A. marginale*, *A. centrale*, and *A. phagocutophilim* [5,18–20]. However, several hemoparasites, including *T. equi* and *Babesia* sp. Mymensingh, were not studied in Egyptian camels. Therefore, in this study, we conducted a comprehensive survey of *Babesia*, *Theileria*, *Trypanosoma*, and *Anaplasma* species in camels reared in Egypt.

The study area included several locations (villages and small towns) in six main governorates of central, eastern, and southern Egypt (Fig. 1). In total, 148 camels were investigated between June 2016 and October 2019. Blood samples were collected from all 148 camels and placed in EDTA-containing tubes. All animals were apparently healthy during sampling. From each sample, 100 µl of blood was spotted on a circular filter paper (Whatman™, Fisher Scientific, UK). Blood spots were allowed to completely dry and then stored at room temperature until used for DNA extraction. The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (approval number: 20–30) approved all animal protocols.

Genomic DNA was extracted from a filter paper punch containing dried blood (approximately 50 µl). Briefly, a single punch from each filter paper was immersed in 300 µl of phosphate-buffered saline at 4°C for 36 h. The mixture was vortexed and incubated at 70°C for 3 h with frequent agitation after adding 150 µl of RLT lysis buffer (QIAGEN, Hilden, Germany) and 6 µl of 20 mg/ml proteinase K (QIAGEN). The liquid portion was transferred into a new tube and then subjected to DNA extraction using phenol–chloroform–isoamyl alcohol, as described previously [21]. The DNA pellet was dissolved in 50 µl of elution buffer (QIAGEN) after ethanol precipitation and then stored at −30°C until further use.

The existence of PCR-amplifiable DNA was confirmed in all camel DNA samples using a previously reported PCR assay that can amplify a 208-bp fragment of the camel

mitochondrial genome's cytochrome b gene (Table 1) [22]. Subsequently, all the DNA samples were screened using previously reported pathogen-specific PCR assays. Nested PCR assays targeting rhoptry-associated protein 1 (*rap-1*), apical membrane antigen 1 (*ama-1*), *rap-1* (*bc48*), merozoite antigen 2 (*ema-2*), and major surface protein 5 (*msp-5*) genes were used to detect *B. bovis*, *B. bigemina*, *B. caballi*, *T. equi*, and *A. marginale*, respectively [23–25]. However, single-step PCR assays based on *ama-1*, merozoite-piroplasm surface antigen (*tams-1*), major piroplasm surface protein (*mpsp*), and internal transcribed spacer 1 (*its-1*) genes were used to detect *Babesia* sp. Mymensingh, *T. annulata*, *T. orientalis*, and *Trypanosoma* species, respectively [23,26,27]. Moreover, the samples were also screened using a primer set targeting *T. evansi* Rode Trypanozoon antigen type (RoTat) 1.2 Variable Surface Glycoprotein (VSG) gene [28]. The PCR primers used in this study are listed in Table 1.

Each single-step PCR assay was conducted in a 10-µl reaction mixture containing 1 µl of genomic DNA, 1× PCR buffer (10× PCR buffer, Applied Biosystems, Branchburg, NJ, USA), 200 µM of each dNTP (Applied Biosystems), 0.5 µM of each forward and reverse primer, 0.1 µl of 5 U/µl Taq polymerase (Applied Biosystems), and 5.9 µl of distilled water. Conversely, we conducted the first and second round of nested PCR assays using a 10-µl PCR mixture containing 5.0 µl of ready master mix (KAPA2G Robust HotStart ReadyMix PCR Kit, KAPA Biosystems, Wilmington, MA, USA), 3.8 µl of distilled water, 0.1 µl of 50 mM from each primer (forward and reverse), and 1 µl of DNA template. The cycling conditions were described in previous reports [23–27]. The PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide, and then visualized under UV light. The detection of bands with sizes equivalent to those obtained with positive controls indicated positive results for the tested hemopathogens.

The PCR amplicons were purified from the agarose gel using a commercial kit (QIAquick Gel Extraction Kit, QIAGEN), ligated into a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), transformed into TOP 10 *E. coli* cells (Invitrogen), and then plated onto Luria–Bertani (LB) agar plates (Invitrogen). Clones were identified and cultured in LB broth. The plasmids were extracted using the QIAprep Spin Miniprep Kit (QIAGEN) and then sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resultant sequences were analyzed using the basic local alignment search tool (https://blast.ncbi.nlm.nih. gov/blast.cgi) to verify their origins. In addition, sequences from the pathogens, which were detected for the first time in Egypt, together with those retrieved from GenBank were aligned using MAFFT online software and used to construct maximum likelihood phylogenetic trees with MEGA X software.

The positive infection rates were analyzed using the OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm) to calculate the 95% confidence intervals based on the Wilson score interval. The *P* values were calculated to determine the statistical significance of differences in positive rates using a Chi-Square test (https://www.medcalc.org/calc/comparison_of_proportions.php). Differences in positive rates were considered statistically significant when the *P* values were <0.05.

We found that the surveyed animals were infected with seven of the nine tested pathogens, except for *B. caballi* and *T. orientalis*. Sixty-five (43.9%) of the 148 animals screened were infected with at least one hemopathogen. *B. bovis* was the most common (positive rate: 29/148, 19.6%), followed by *A. marginale* (28/148, 18.9%) and *B. bigemina* (22/148, 14.9%) (Table 2). *T. annulata* was detected in two camels (1.4%), whereas *Babesia* sp. Mymensingh and *T. equi* were each detected in one camel only (0.7%). Based on the size of PCR amplicons (480 bp), two animals (1.4%) were considered positive for *T. evansi* [27]. The PCR targeting RoTat1.2 VSG gene further confirmed that these two animals were

150 infected with *T. evansi*. *B. bovis* and *A. marginale* were detected in all six governorates
151 surveyed, whereas *B. bigemina* was detected in camels from all governorates, except for
152 Luxor (Table 2). The remaining species were detected only in one (*Babesia* sp. Mymensingh
153 and *T. equi* in Giza) or two governorates (*Theileria annulata* in Giza and Sohag and *T. evansi*
154 in the Giza and Red Sea governorates). Although differences were observed among the
155 positive rates of hemopathogens, a fair comparison was not possible due to small sample size.

156 *T. equi*, which has been previously reported in camels in other countries [7,29], had
157 not been demonstrated in Egyptian camels. Therefore, this is the first study to report *T. equi*
158 infection in camels in this country. This is not an entirely unexpected finding as a previous
159 study had reported that *T. equi* infection is widespread in horses and donkeys in Egypt [30].

160 The detection of the recently identified *Babesia* sp. Mymensingh is one of the key
161 findings of this study [26]. *Babesia* sp. Mymensingh, which causes severe clinical babesiosis
162 in cattle, is a large-type of *Babesia*, but differs from *B. bigemina* morphologically. This
163 species has also been detected in other livestock animals, including buffalo, sheep, and goats,
164 in several Asian, African, and American countries [31]. However, the *Babesia* sp.
165 Mymensingh had never been surveyed in camels or Egypt. Therefore, our study is the first to
166 report *Babesia* sp. Mymensingh infection in camels and Egypt. Furthermore, our findings
167 indicate that infection with *Babesia* sp. Mymensingh might be common among cattle
168 populations in this country.

169 PCR amplicons were cloned and sequenced to validate the screening results. Two
170 PCR amplicons were sequenced for *B. bigemina*, *T. annulata*, and *A. marginale*, whereas
171 single amplicons were sequenced for *B. bovis*, *Babesia* sp. Mymensingh, *T. equi*, and *T.*
172 *evansi*. The newly determined sequences of *B. bovis rap-1* (GenBank accession no.
173 LC653106), *B. bigemina ama-1* (LC653107 and LC653108), *Babesia* sp. Mymensingh *ama-*
174 *1*(LC653109), *T. equi ema-2* (LC653110), *T. evansi its-1* (LC653115), and *A. marginale*

175 *msp-5* (LC653113 and LC653114) shared high identity scores with the GenBank sequences
176 from the following pathogens: *B. bovis* (AB917246; 99.7%), *B. bigemina* (LC438499; 98.6
177 and 99.1%), *Babesia* sp. Mymensingh (LC506545; 99.5%), *T. equi* (KM264373; 98.6%), *T.*
178 *evansi* (MN121259; 99.0%), and *A. marginale* (MK834272; 99.0% and 98.5%), respectively.
179 The *B. bovis* and *T. evansi* sequences also shared 99.3% and 98.1% identity scores with
180 previously reported sequences (MF737083 and MF737081, respectively) in Egypt. As the
181 present study is the first to report *Babesia* sp. Mymensingh in camels as well as in Egypt, a
182 maximum phylogenetic tree was constructed based on Kimura 2-parameter substitution
183 model. The *Babesia* sp. Mymensingh *ama-1* sequence generated in this study clustered
184 together with previously determined sequences and formed a distinct clade, confirming our
185 finding (Fig. 2A).

186 Interestingly, the sequences of two amplicons obtained from *T. annulata*-specific
187 PCR assay (LC653111 and LC653112) shared higher identity scores (99.5% and 99.6%) with
188 a sequence (LC467555) from recently identified *Theileria* sp. Yokoyama than those with a
189 sequence (AF214831) from *T. annulata* (93.1% and 93.0%). The newly generated sequences
190 also shared low identity scores (87.6 – 91.2%) with previously reported *T. annulata*
191 sequences (AB917275 – AB917302) in Egypt. In phylogeny constructed based on Jukes-
192 Cantor substitution model, the Egyptian sequences determined in the present study clustered
193 together with the *Theileria* sp. Yokoyama sequences from Sri Lanka and formed a sister
194 clade to the common ancestor of *T. annulata* and *T. lestoquardi* sequences (Fig. 2B). These
195 findings indicated that the animals detected positive for *T. annulata* were indeed infected
196 with *Theileria* sp. Yokoyama, which is a recently identified, novel *Theileria* species closely
197 related to *T. annulata* [32]. The parasite species was reported only in cattle in Sri Lanka;
198 therefore, this is the first report of infection of *Theileria* sp. Yokoyama outside Sri Lanka as
199 well as in a non-cattle host. Previous studies conducted in Egypt reported that some camels

200 that tested positive for pan-*Theileria* species PCR were negative for *T. annulata* [5,14].
201 *Theileria* sp. Yokoyama may have infected such *Theileria*-positive but *T. annulata*-negative
202 camels according to our findings.

203 The positive rates of the some of hemopathogens, in particular *T. evansi*, were low
204 compared to those reported previously [15,16]. This might be due to the differences in the
205 DNA extraction methods and sensitivities of PCR assays employed [33]. However, a fair
206 comparison of positive rates is not possible because of the small sample size. Therefore,
207 large-scale surveys with statistically calculated sample size are essential to investigate the
208 prevalence of hemopathogens in camels. The lack of microscopic data is also a limitation of
209 our study. Especially, future studies should focus on microscopic detection of *Babesia* sp.
210 Mymensingh and *Theileria* sp. Yokoyama in camels. Moreover, identification of
211 transmission vectors of these two parasite species might shed an additional light on their
212 epidemiology. Several risk factors, such as age and management practices, affect the
213 infections with hemopathogens. However, analysis of risk factors for individual pathogens
214 detected in our study was not possible due to the low positive rates for some of the pathogens
215 and the fact that the samples were collected over a long period of time from small numbers of
216 animals reared in a large geographical area.

217 In conclusion, we found that Egyptian camels are infected with *Babesia*, *Theileria*,
218 *Trypanosoma*, and *Anaplasma*. This study is the first to report *Theileria* sp. Yokoyama and
219 *Babesia* sp. Mymensingh in one-humped camel and in Egypt.
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Acknowledgments

We appreciate Ms. Hiroko Yamamoto (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan) for her outstanding technical support. We also thank the owners of camels and field veterinarians in all surveyed governorates for their assistance in handling camels and sampling.

Funding: This study was supported by grants from the Japan Society for Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (JSPS KAKENHI Numbers 19KK0174 and 19K23704), from Open Partnership Joint Project of the JSPS Bilateral Joint Research Projects, and from the Ministry of Higher Education and Scientific Research, Egypt.

Declarations of interest: none

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Figure legend

Fig. 1. Geographical map showing the sampling sites. The camels were sampled from various localities in six governorates of Egypt.

Fig. 2. Phylogenetic analyses. Two phylogenetic trees were constructed using the *Babesia* sp. Mymensingh *ama-1* (panel A) and *Theileria* sp. Yokoyama *tyms1* (panel B) sequences generated in this study. The *Babesia* sp. Mymensingh and *Theileria* sp. Yokoyama sequences from Egyptian camels clustered together with previously reported sequences of these parasite species and formed distinct phylogenetic clades.

Table 1. List of PCR primers used in this study

Pathogen[a]	Target gene	PCR primers (5′-3′)		Size (bp)	Reference
		Forward	Reverse		
B. bovis	*rap-1*	CACGAGCAAGGAACTACC GAT GTTGA	CCAAGGACCTTCAACGTACGAGGTCA	360	[23]
		TCAACAACGTACTCTATATGGCTACC	CTACCGACCAGAACCTTCTTCACCAT	298	
B. bigemina	*ama-1*	GTATCAGCCGCCGACCTCCGTAAGT	GGCGTCAGACTCCAACGGGGAACCG	738	[23]
		TACTGTGACGAGGACGGATC	CCTCAAAAGCAGATTCGAGT	211	
B. caballi	*bc48*	ACGAATTCCCACAACAGCCGTGTT	ACGAATTCGTAAAGCGTGGCCATG	530	[24]
		GGGCGACGTGACTAAGACCTTATT	GTTCTCAATGTCAGTAGCATCCGC	430	
Babesia sp. Mymensingh	*ama-1*	TGGCGCCGACTTCCTGGAGCCCATCTCCAA	AGCTGGGGCCCTCCTTCGATGAACCGTCGG	371	[26]
T. annulata	*tams-1*	ATGCTGCAAATGAGGAT	GGACTGATGAGAAGACGATGAG	768	[23]
T. orientalis	*mpsp*	CTTTGCCTAGGATACTTCCT	ACGGCAAGTGGTGAGAACT	776	[23]
T. equi	*ema-2*	CGTTGTCACTCTCGGAGC	TGACCCAGGAATCACCAG	587	[24]
		CGTTGTCACTCTCGGAGCCAC	GAGCAGGGACAACGCAGACAG	221	
Trypanosoma species	*its-1*	CCGGAAGTTCACCGATATTG	TTGCTGCGTTCTTCAACGAA	250-700	[27]
T. evansi	*RoTat1.2 VSG*	CTGAAGAGGTTGGAAATGGAGAAG	GTTTCGGTGGTTCTGTTGTTGTTA	151	[28]
A. marginale	*msp-5*	GTGTTCCTGGGGTACTCCTATGTGAACAAG	AAGCATGTGACCGCTGACAAACTTAAACAG	547	[25]
		AAGCACATGTTGGTAATATTCGGCTTCTCA	AATTCTCGCATCAAAAGACTTGTGGTACTC	195	
Camel	*cytochrome b*	AGCCTTCTCTTCAGTCGCACAC	GCCCATGAAAGCTGTTGCT	208	[22]

[a] Nested PCR assays were used to detect *B. bovis*, *B. bigemina*, *B. caballi*, *T. equi*, and *A. marginale*.

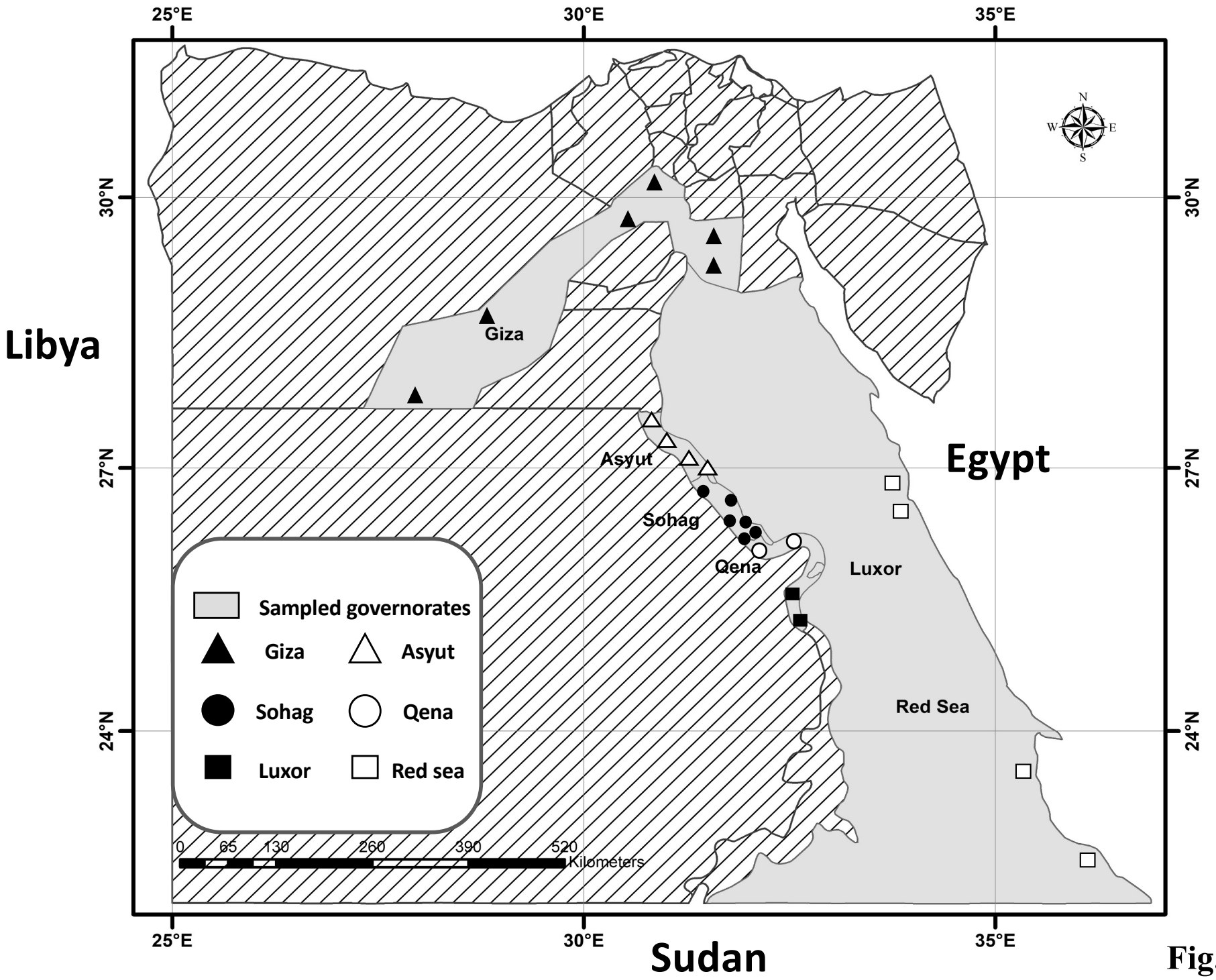
25°E
30°E
35°E
30°N
27°N
24°N
Libya
Egypt
Sudan
Giza
Asyut
Sohag
Qena
Luxor
Red Sea
N
W
E
S
Sampled governorates
Giza
Asyut
Sohag
Qena
Luxor
Red sea
0
65
130
260
390
520
Kilometers

Fig. 1

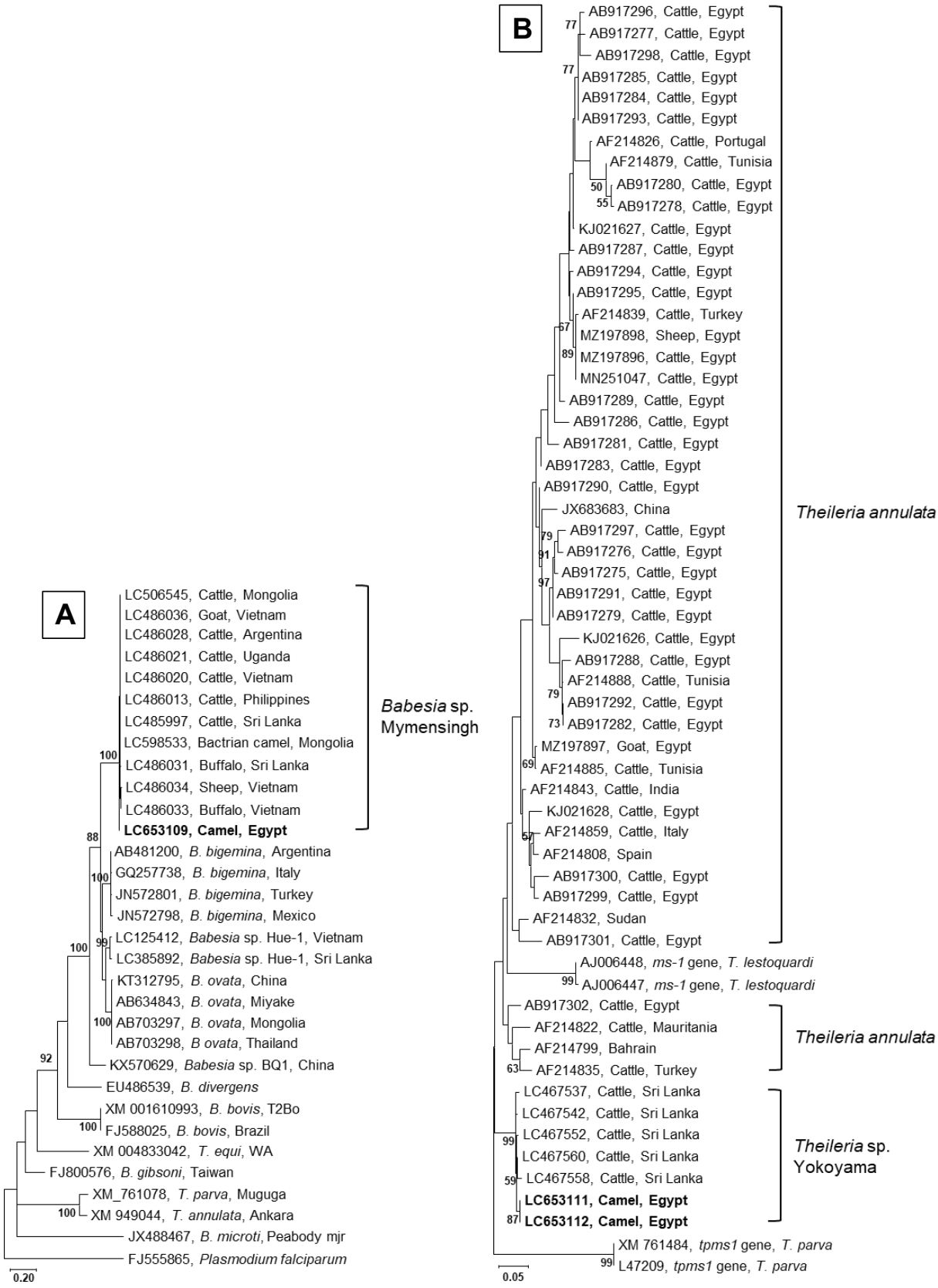
A
LC506545, Cattle, Mongolia
LC486036, Goat, Vietnam
LC486028, Cattle, Argentina
LC486021, Cattle, Uganda
LC486020, Cattle, Vietnam
LC486013, Cattle, Philippines
LC485997, Cattle, Sri Lanka
LC598533, Bactrian camel, Mongolia
LC486031, Buffalo, Sri Lanka
LC486034, Sheep, Vietnam
LC486033, Buffalo, Vietnam
LC653109, Camel, Egypt
Babesia sp. Mymensingh
AB481200, B. bigemina, Argentina
GQ257738, B. bigemina, Italy
JN572801, B. bigemina, Turkey
JN572798, B. bigemina, Mexico
LC125412, Babesia sp. Hue-1, Vietnam
LC385892, Babesia sp. Hue-1, Sri Lanka
KT312795, B. ovata, China
AB634843, B. ovata, Miyake
AB703297, B. ovata, Mongolia
AB703298, B ovata, Thailand
KX570629, Babesia sp. BQ1, China
EU486539, B. divergens
XM 001610993, B. bovis, T2Bo
FJ588025, B. bovis, Brazil
XM 004833042, T. equi, WA
FJ800576, B. gibsoni, Taiwan
XM_761078, T. parva, Muguga
XM 949044, T. annulata, Ankara
JX488467, B. microti, Peabody mjr
FJ555865, Plasmodium falciparum
0.20
B
AB917296, Cattle, Egypt
AB917277, Cattle, Egypt
AB917298, Cattle, Egypt
AB917285, Cattle, Egypt
AB917284, Cattle, Egypt
AB917293, Cattle, Egypt
AF214826, Cattle, Portugal
AF214879, Cattle, Tunisia
AB917280, Cattle, Egypt
AB917278, Cattle, Egypt
KJ021627, Cattle, Egypt
AB917287, Cattle, Egypt
AB917294, Cattle, Egypt
AB917295, Cattle, Egypt
AF214839, Cattle, Turkey
MZ197898, Sheep, Egypt
MZ197896, Cattle, Egypt
MN251047, Cattle, Egypt
AB917289, Cattle, Egypt
AB917286, Cattle, Egypt
AB917281, Cattle, Egypt
AB917283, Cattle, Egypt
AB917290, Cattle, Egypt
JX683683, China
AB917297, Cattle, Egypt
AB917276, Cattle, Egypt
AB917275, Cattle, Egypt
AB917291, Cattle, Egypt
AB917279, Cattle, Egypt
KJ021626, Cattle, Egypt
AB917288, Cattle, Egypt
AF214888, Cattle, Tunisia
AB917292, Cattle, Egypt
AB917282, Cattle, Egypt
MZ197897, Goat, Egypt
AF214885, Cattle, Tunisia
AF214843, Cattle, India
KJ021628, Cattle, Egypt
AF214859, Cattle, Italy
AF214808, Spain
AB917300, Cattle, Egypt
AB917299, Cattle, Egypt
AF214832, Sudan
AB917301, Cattle, Egypt
Theileria annulata
AJ006448, ms-1 gene, T. lestoquardi
AJ006447, ms-1 gene, T. lestoquardi
AB917302, Cattle, Egypt
AF214822, Cattle, Mauritania
AF214799, Bahrain
AF214835, Cattle, Turkey
Theileria annulata
LC467537, Cattle, Sri Lanka
LC467542, Cattle, Sri Lanka
LC467552, Cattle, Sri Lanka
LC467560, Cattle, Sri Lanka
LC467558, Cattle, Sri Lanka
LC653111, Camel, Egypt
LC653112, Camel, Egypt
Theileria sp. Yokoyama
XM 761484, tpms1 gene, T. parva
L47209, tpms1 gene, T. parva
0.05

Fig. 2