Original Research

Phylogenetic analysis and geographical distribution of *Theileria equi* and *Babesia caballi* sequences from horses residing in Spain.

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Abstract

The intraerythrocytic protozoans *Theileria equi* and *Babesia caballi* are the causative agents of equine piroplasmosis (EP), one of the most important equine tick-borne diseases due to its significant impact on global international horse trade. Although EP is known to be endemic in Spain, previous phylogenetic studies have only been conducted for limited geographical regions. Therefore, the objective of this study was to evaluate the genetic diversity and distribution of these parasite species nationwide. This was performed by amplification of the 18S small subunit (SSU) rRNA gene from 100 EP positive equine blood samples using a nested PCR protocol, and sequencing the obtained amplicons. Seventy-seven *T. equi* and six *B. caballi* isolates were successfully sequenced and phylogenetic analysis revealed that the *T. equi* isolates grouped into the previously described clades A (n = 21/77), D (n = 1/77) and E (n = 55/77), while *B. caballi* isolates were placed into clades A (n = 5/6) and B (n = 1/6). Isolates from *T. equi* clade D and *B. caballi* clade B have not previously been reported in Spain. A greater intra-clade diversity (97.3-98.3% identity) was observed between *T. equi* clade E isolates compared to those within clade A (99.7-100% identity). Additionally, a multivariable logistic regression model was used to analyse associations between the clade of *T. equi* infection and available epidemiological data. Horses residing in Spanish northern regions were statistically more likely to be infected with *T. equi* clade E (p=0.01). We conclude that while extensive sequence variation of equine piroplasms exists in Spanish infected horses, a requirement for increased equine movement controls between Spain and EP-endemic countries should be considered.

Keywords: Piroplasmosis; horses; nPCR; genotypes; Spain.

1. Introduction

Theileria equi and *Babesia caballi* are the primary pathogens of the intra-erythrocytic Apicomplexan parasitic disease, equine piroplasmosis (EP) (Wise et al., 2014). Natural transmission of EP occurs via specific ixodid tick vectors (Scoles and Ueti, 2015), of which *Ixodes*, *Haemaphysalis*, *Dermacentor* and *Rhipicephalus* species have been described in Spain (Nagore et al., 2004), although iatrogenic infection through the use of contaminated hypodermic needles or blood transfusions is also possible (Short et al., 2012).

EP is widespread worldwide and is of great economic importance to the Spanish equine industry (Camino et al., 2019b), not just due to the impact of acute clinical disease, but also because chronically infected horses often become asymptomatic carriers, a status that limits their movement to EP-free countries (Dewaal, 1992; Friedhoff et al., 1990). Both *T. equi* and *B. caballi* cause similar clinical signs (Wise et al., 2014), although a recent survey of Spanish EP cases indicated a greater severity of anaemia associated with *B. caballi* infection (Camino et al., 2019a).

Phylogenetic analysis based on 18S SSU rRNA gene sequences has revealed the presence of multiple genotypes that segregate in up to five gene clades (A, B, C, D and E) within *T. equi* (Qablan et al., 2013; Qablan et al., 2012), and three gene clades (A, B and C) within *B. caballi* (Qablan et al., 2013). Recent work has also indicated the presence of a new species, *Theileria haneyi*, within clade C of the current *T. equi* umbrella (Knowles et al., 2018), suggesting that the observed genetic diversity may represent more than intra-species clade multiplicity.

At present, there have been limited reports describing the genetic diversity of EP in Spain. Previously, Nagore et al. (2004) described the presence of two *T. equi* and two *B. caballi* genotypes in isolates from Spanish horses. One isolate from each parasite species showed a high degree of identity with previously described Spanish *T. equi* (Criado-Fornelio et al.,

2003b), and *B. caballi* (Allsopp et al., 1994) isolates. However, the other *T. equi* and *B. caballi* isolates demonstrated greater genetic diversity (96.8 and 97.4% identity respectively) which suggested the presence of novel genotypes (Criado-Fornelio et al., 2004). Therefore, the main goals of this study were (1) investigate the presence of *T. equi* and *B. caballi* clades currently in Spain, (2) describe the distribution of parasite clades within the country and (3) evaluate any potential epidemiological factors associated with detected species' clades.

2. Materials and methods

2.1 Sampling procedures

A total of 100 samples were selected from a larger set of 740 equine blood samples, submitted by private veterinarians from asymptomatic horses across Spain as part of a national EP prevalence survey (unpublished data). This sub-set of 100 samples was selected based on having strongly detectable parasite DNA (<35 quantification cycles (C_q)) when screened using a previously described multiplex real-time PCR using primers and TaqMan probes to specifically amplify the V4 hypervariable region of the 18S rRNA gene of *T. equi* and *B. caballi* (Camino et al., 2019a). Genomic DNA was extracted from whole blood samples using the QIAamp DNA mini kit (Qiagen, Spain) and stored at -40°C until analysis. A questionnaire to provide basic epidemiological data was completed by the submitting veterinarian from each sampled horse, including information regarding gender, age, breed, aptitude (purpose of use), geographical residence, tick presence at sampling and any history of de-worming or vaccination.

2.2 Nested PCR

All 100 DNA extractions were analysed by means of a *Theileria*/*Babesia* species catch-all nested PCR to amplifying a portion of the hypervariable V4 region of the 18S SSU rRNA gene.

Outer (BT1-F and BTH-1R) and inner (RLB-F2 and RLB-R2) primers were described in Criado-Fornelio et al. (2003a) and Oura et al. (2004) respectively, and the reaction conditions and PCR product visualization were conducted as described by Coultous et al. (2019a).

2.3 Purification and sequencing of PCR amplified products

PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Spain), before being send to Eurofins Genomics (Germany) for Sanger sequencing.

2.4 Phylogenetic analysis

Species identification of sequences obtained in this study was achieved using the basic local alignment search tool (BLAST) and comparison with sequences deposited in the non-redundant National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/). The MUSCLE function (Edgar, 2004), within the AliView alignment viewer and editor (Larsson, 2014), was used to compare the study sequences with those previously determined and deposited in GenBank for *T. equi* and *B. caballi*.

In order to assess the genetic diversity of both haemoparasites within the study samples, two species specific maximum likelihood phylogenetic trees were constructed using MEGA 7.0.26 software (Kumar et al., 2016). The 18S SSU rRNA gene sequences of *Theileria parva* (L02366) and *Theileria annulata* (KX375830) were included in the trees as outgroups. All sequences generated in this study were submitted to the NCBI GenBank database (accession numbers MN818861-MN818863 inclusive and MT563458-MT563531 inclusive for *T. equi* isolates; accession numbers MN818859, MN818860 and MT563454-MT563457 inclusive for *B. caballi*).

2.5 Statistical analyses

Potential associations between horse epidemiological data and phylogenetic groups were evaluated by means of a multivariable logistic regression model using the software Small STATA version 12.0, with consideration of the odds ratios (ORs) and their corresponding 95% confidence intervals (CIs). To construct the model, epidemiological data were considered as individual variables and classified in several categories: sex (male/female), age (1-7/8-15/16-26 years), breed (Iberian/cross-breed/Central-European), aptitude (breeding/sport), geographical residence (north/south of Spain), presence of ticks on the horse (yes/no), deworming (previous treatment/never treated) and vaccination programme application (previous vaccination/no vaccination). The initial model contained all variables showing $p < 0.25$ in the univariable screening analysis along with confounders (sex, breed, age and aptitude). The final model was constructed by retaining the variables with $p < 0.05$ or where their removal resulted in a significant change in the effect of other variables, indicating confounding. Collinearity between variables was checked and excluded. Biologically plausible first-order interactions were determined between significant and confounders variables. Finally, the area under the receiver operating characteristic (ROC) curve and the Hosmer-Lemeshow goodness-of-fit test were calculated to evaluate fitting of the proposed model (Hosmer, 2000).

3. Results

3.1 EP detection by nested PCR

Nested PCR product was successfully generated and EP specific amplicons from 83 of the 100 samples sequenced. Only a single parasite species was detected in each sample.

3.2 Phylogenetic analysis of *T. equi* 18S rRNA gene

In total, 77 samples produced sequences identified as *T. equi*, and these were partitioned into three different clades (A, D and E). A representative sequence for each distinct *T. equi* clade detected in this study (accession numbers MN818861, MN818862 and MN818863) was selected to demonstrate this phylogenetic positioning (Figure 1). The sequences that clustered within clade A (27.3%, n = 21/77) shared 99.7% to 100% identity to each other (representative sequence MN818862). These sequences showed high identity (99.5-100%) to those from previous studies in Spain (AY150062.2-AY150064.2) (Criado-Fornelio et al., 2003b), and grouped with sequences derived from samples isolated in South Africa, Brazil, Jordan and USA. Sequences clustering in clade E (71.4%, n = 55/77) demonstrated 97.3-98.3% identity to each other (representative sequence MN818861), and showed high identity (99.5-100%) with previous sequences from Spanish isolates (AY534882.1 and DQ287951.1) (Nagore et al., 2004; Criado et al., 2006), as well as isolates from China, Korea, Mongolia, Saudi Arabia and Switzerland. Notably, only one sequence obtained clustered in clade D (MN818863). This clade has not been previously reported in Spanish samples, and grouped with isolates obtained from Turkey, Palestine, Israel and Sudan. An alignment of all *T. equi* isolates sequenced in this study is available in Appendix A.

3.3 Phylogenetic analysis of *B. caballi* 18S rRNA gene

Six *B. caballi* isolates were successfully sequenced in total, and these were distributed across two of the three previously described *B. caballi* clades, with the positioning demonstrated with a representative sample from each clade (Figure 2). Five isolates (representative sequence MN818860) clustered within clade A, showing 100% identity to each other and a previously reported *B. caballi* isolate from Spain (AY309955.1) (Criado-Fornelio et al., 2004), and displayed high identity (99.7%) to another sequence previously reported for a Spanish isolate (AY534883.1) (Nagore et al., 2004). This clade also contains isolates in China, Mongolia, South Africa and Italy. Only one isolate (MN818859) was clustered within a second clade,

clade B, and showed high identity (97.9-100%) to sequences of isolates from Israel, South Africa and Italy, but not with any previously reported Spanish *B. caballi* isolates. An alignment of all *B. caballi* isolates sequenced in this study is available in Appendix B.

3.4 Geographic distribution of the isolates and epidemiological data analyses

The samples in this study were collected from 12 of Spain's 17 autonomous communities; *Theileria equi* was detected in all the autonomous communities studied, but *B. caballi* was only detected in four communities (Table 1).

Figure 3 shows the geographic distribution of the detected parasite species and their representative clade types. The *T. equi* isolates grouped in clade E were present in all the sampled communities, however clade A parasites appear to be more restricted to central and southern communities. The singular *T. equi* isolate belonging to clade D was identified in Asturias, in the north of the country. Within the *B. caballi* isolates, all clade A sequences were only found in northern communities, whereas the single clade B isolate was detected in the far south in Andalusia.

Multivariable logistic regression analysis was performed to assess the statistical significance between *T. equi* clade and available epidemiological data. Since a clade D genotype was only represented by a single isolate, only clades A and E were considered in the analysis. The Hosmer–Lemeshow goodness-of-fit test indicated that the multivariable logistic regression analysis fitted the data adequately ($p = 0.38$). The area under the ROC curve was 0.86 (95% CI 0.77 to 0.96), indicating that the model had good overall predictive power. Location of residence was the only variable significantly associated with the distribution of clade types ($p = 0.01$). Thus, *T. equi* clade E was 5.6 times more likely to be detected in horses located in the north of Spain (OR = 5.6, 95% CI 1.50-21.22). Furthermore, a positive association, although not statistically significant, was identified between sport horses and clade E type infection (p

= 0.07; OR = 3.70, 95% 0.89-15.41). No statistical association was found between clade group and other variables tested: sex, age, breed, aptitude, presence of ticks on the horse, deworming and vaccination programme application.

The low number of *B. caballi* isolates obtained prevented meaningful statistical analyses of the sequences derived from these samples.

4. Discussion

This study set out to investigate the diversity and distribution of parasites that cause equine piroplasmosis in Spain. The large proportion of *T. equi* isolates compared to *B. caballi* isolates that were detected in the current study confirmed *T. equi* as the predominant EP parasite in Spain. This matches findings from other EP-endemic European countries such as Italy (Manna et al., 2018), France (Guidi et al., 2015) and Portugal (Ribeiro et al., 2013), as well as the situation seen in non-endemic European countries such as the UK (Coultous et al., 2019a). Although *T. equi* was overwhelmingly predominant in this study, it should be noted that for *B. caballi* natural recovery is possible, compared to the life-long asymptomatic carriers seen with *T. equi* infection, where parasitemia peaks may recrudesce in times of stress or immunocompromise (Bruning, 1996). Therefore, *B. caballi* infections are more difficult to detect in asymptomatic horses, so the real occurrence in Spain may be underrepresented in this study.

Although equi merozoite antigen (EMA-1), rhoptry associated protein (RAP-1), β-tubulin and mitochondrial cytochrome B (cytB) genes have been targeted by PCR to diagnose EP (Battsetseg et al., 2002; Montes Cortes et al., 2019; Nicolaiewsky et al., 2001), the 18S SSU rRNA gene is the most appropriate target for piroplasm identification and genetic variation analysis, primarily due to a level of sequence conservation that allows meaningful phylogenetic comparisons, and the occurrence of multiple copies within the genome (Allsopp and Allsopp,

2006; Hunfeld et al., 2008). Early studies of EP phylogeny based on this gene initially identified only two genotypes for both equine piroplasms (Nagore et al., 2004). However, further studies rapidly established the current systems of three clades for *B. caballi* (A-C), and five clades for *T. equi* (A-E) (Bhoora et al., 2009; Qablan et al., 2013; Salim et al., 2010), although recent authors have suggested combining *T. equi* clades B and E to give a four clade system (Hall et al., 2013; Alanazi et al., 2014; Coultous et al., 2019b). The present report indicates high heterogeneity for both hemoparasites across Spain, with the identification of isolates from three *T. equi* clades (A, D and E) and two *B. caballi* clades (A and B). This genetic variability among the *T. equi* and *B. caballi* 18S SSU rRNA sequences has been observed in previous studies performed in European (Criado-Fornelio et al., 2004; Manna et al., 2018) and non-European countries (Bhoora et al., 2009; Campos et al., 2019).

The majority of *T. equi* sequences were derived from clade E, showing a relatively higher genetic diversity (97.3-98.3% identity) between sequences compared to those isolates placed in clade A (99.7-100% identity). Both these clades have been previously described in Spain (Criado et al., 2006; Criado-Fornelio et al., 2003a; Nagore et al., 2004). As genetic diversity will have occurred over time due to recombination or SNPs within the genome (Mans et al., 2011), the greater sequence diversity of clade E samples, combined with their greater geographical distribution, may suggest that parasites within this clade could be related to the original EP strain that became endemic in Spain. *Theileria equi* clade E has been notably linked to Asian countries (Alanazi et al., 2014; Munkhjargal et al., 2013; Tian et al., 2013), but has also previously been described in Europe (Liu et al., 2016). A different situation is observed in the Americas, where a predominance of clade A isolates, and absence of clade E isolates, has been reported in studies carried out in North America (Hall et al., 2013) and Brazil (Peckle et al., 2018). An absence of clade E isolates has also been reported in southern and western Africa (Bhoora et al., 2009; Coultous et al., 2019b) and the Middle-East (Ketter-Ratzon et al., 2017).

Interestingly, Criado-Fornelio et al. (2003a) previously reported PCR detection of a *T. equi* clade A isolate from an asymptomatic Spanish dog (AY150064.2), suggesting a reduced host specificity of the piroplasm. However, this finding has been directly criticized by Uilenberg et al. (2018), arguing that a current trend of over-reliance on highly sensitive PCR detection methods may simply be detecting the transient presence of the parasite in a dead-end host; after all evidence of parasite detection is not evidence of host competence, but may indicate a degree of host persistence.

The present study is the first to report a *T. equi* clade D isolate from Spain. This clade was first reported in horses in Sudan (Salim et al., 2010), and later described in Tunisia (Ros-Garcia et al., 2013), Israel (Ketter-Ratzon et al., 2017) and Turkey (Ozubek and Aktas, 2018). The known movement of horses between African countries and Spain via Morocco has been cited as a potential route for EP strain introduction (Salim et al., 2010), and may explain the route of incursion for the isolate detected in this study.

Previous Spanish studies have only reported *B. caballi* isolates from the currently defined clade A (Criado-Fornelio et al., 2004; Nagore et al., 2004). Our results have revealed the existence of two *B. caballi* clades: clade A as the predominant genotype, and a single previously unreported clade B isolate. The novel presence of this clade may indicate increased incursion of *B. caballi* in the Spanish horse population, as suggested by our previous work (Camino et al., 2019b). Most likley, incursion occurs as a result of the introduction of asymptomatic carriers, as freedom from piroplasmosis is not a requirement when moving horses within the European Union (EU Directive 2009/156/CE).

Although only a single piroplasmid species was identified in each sample in our study, the detection of co-infected animals was not a study goal, and their existence cannot be discounted with our methods. Equally, the presence of mixed clade infections within single samples, or

indeed the presence of undetected genotypes, cannot be ruled out. Such investigation would require additional cloning of the generated amplicons, or further molecular screening, for example using species or clade-specific probes (Coultous et al., 2019b).

Widespread distribution of *T. equi* clade E throughout Spain was demonstrated, a result that agrees with the findings of previous work (Criado et al., 2006). In contrast, a more restricted geographic distribution of *T. equi* clade A was observed with these parasites detected in just four communities (Andalusia, Castile and Leon, Extremadura and Madrid) in central and southern Spain. This geographic division was shown to be statistically significant with horses residing in Spanish northern regions more likely to be infected with *T. equi* clade E genotypes ($p=0.01$). Clade A also had greatest occurrence in the community of Madrid, home to the country's capital, which has a higher risk of infectious disease transmission due to the frequent movement of horses for trade and equestrian competitions (Camino et al., 2018). Due to the high trafficking of horses in this region, it is possible that clade A was first introduced through this region, and subsequently spread to neighboring regions as a result of the continued national movement of horses. However, further investigation of the isolates with additional markers and epidemiological modeling would be required to fully investigate this hypothesis.

To evaluate whether any of the *T. equi* clades detected present a particular risk of disease to horses in Spain we tested for association of clade type with a number of risk factors. A previous study by Manna et al. (2018) reported a statistical correlation between horse clinical status (symptomatic and asymptomatic) and the phylogenetic clades, suggesting that clade A is more associated with horses showing clinical signs. In our study, no statistically significant correlations were obtained relating to biological parameters of the horses (sex, age or breed), or the management associated factors of vaccination, deworming and tick presence. However, our results did show that infections associated with *T. equi* clade E are more likely to be found in horses residing in northern regions. This might be related to the species of tick vectors

293 endemic to these regions, as speculated by previously (Hall et al., 2013). However, no studies
294 have been carried out to date recording the distribution of tick vectors in Spain, or the possible
295 affinity of different *T. equi* competent tick species for certain parasite genotypes. Additionally,
296 the model suggested a trend (p=0.07) for clade E to be more associated with sport horses than
297 breeding animals, a result which could be explained by the frequent trips of sport horses to
298 national competitions and the widespread distribution of clade E parasites throughout the
299 country.

300 **5. Conclusions**

301 This is the first study to assess the genetic diversity of equine piroplasmid isolates circulating
302 within the national horse population of Spain. Our results indicate the presence of *T. equi* and
303 *B. caballi* clades previously described in other limited Spanish studies, as well as identifying
304 new circulating *T. equi* and *B. caballi* genotypes. These newly identified genotypes are likely
305 a consequence of the currently uncontrolled equine movement between EP-endemic countries
306 and Spain. Following multivariable logistic regression analysis, *T. equi* clade E was
307 significantly more associated with Spanish northern regions, with clade A having a more
308 restricted geographic distribution in the south of the country. Further large-scale
309 epidemiological studies of EP in Spain are recommended to investigate risk factors association
310 with the different parasite genotypes and whether the horse population is at risk from incursion
311 of parasites from clades that are not regionally common. Additionally, a national survey of tick
312 vector distribution would be beneficial for identification of any association between parasite
313 genotype and tick species. The currently identified genetic diversity in both *T. equi* and *B.*
314 *caballi* will help to further inform both clinicians, horse owners and government officials in
315 the selection of appropriate diagnostic tools for the detection of infected carrier horses, helping
316 to reduce the future risk of introducing new genotypes into parasite free or endemic regions of
317 Spain.

Acknowledgments

We are grateful to all the veterinary surgeons and stud farm owners that participated in this study.

Funding

Eliazar Camino was supported by the Grants for Predoctoral Researchers of the Complutense University of Madrid (CT27/16). Robert Coultous at the University of Glasgow is currently supported by a Horserace Betting Levy Board (HBLB) research fellowship (VET/EPDF/2019-1).

Animal welfare

This study was exempt from undergoing the Ethical Committee of the Veterinary Faculty, Universidad Complutense, Madrid. However, all the owners from the horses included in the study received an information sheet with the details of the study and signed an informed consent for the collection and use of the blood sample.

Conflict of interest statement

The authors declare no conflict of interest in the subject, matter or materials discussed in this manuscript.

Appendices

Appendix A. Alignment of all *T. equi* isolate sequences.

Appendix B. Alignment of all *B. caballi* isolate sequences.

References

Alanazi, A.D., Said, A.E., Morin-Adeline, V., Alyousif, M.S., Slapeta, J., 2014. Quantitative PCR detection of *Theileria equi* using laboratory workflows to detect asymptomatic persistently infected horses. Vet Parasitol 206, 138-145. https://doi.org/10.1016/j.vetpar.2014.09.019

Allsopp, M.T., Cavalier-Smith, T., De Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108 (Pt 2), 147-152. https://doi.org/10.1017/S0031182000068232

Allsopp, M.T.E.P., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences 1081, 509-517. https://doi.org/10.1196/annals.1373.076

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Vet Parasitol 107, 351-357. https://doi.org/10.1016/S0304-4017(02)00131-0

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Vet Parasitol 159, 112-120. https://doi.org/10.1016/j.vetpar.2008.10.004

Bruning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. Br Vet J 152, 139-151. https://doi.org/10.1016/S0007-1935(96)80070-4

Camino, E., de la Cruz, M.L., Dominguez, L., Carvajal, K.A., Fores, P., de Juan, L., Cruz-Lopez, F., 2018. Epidemiological situation of the exposure to agents causing equine piroplasmosis in Spanish purebred horses in Spain: seroprevalence and associated risk factors. J Equine Vet Sci 67, 81-86. https://doi.org/10.1016/j.jevs.2018.03.012

Camino, E., Dorrego, A., Carvajal, K.A., Buendia-Andres, A., de Juan, L., Dominguez, L., Cruz-Lopez, F., 2019a. Serological, molecular and hematological diagnosis in horses with clinical suspicion of equine piroplasmosis: pooling strengths. Vet Parasitol 275, 108928. https://doi.org/10.1016/j.vetpar.2019.108928

Camino, E., Pozo, P., Dorrego, A., Carvajal, K.A., Buendia, A., Gonzalez, S., de Juan, L., Dominguez, L., Cruz-Lopez, F., 2019b. Importance of equine piroplasmosis antibody presence in Spanish horses prior to export. Ticks Tick Borne Dis, 101329. https://doi.org/10.1016/j.ttbdis.2019.101329

Campos, J.B.V., Andre, M.R., Goncalves, L.R., Freschi, C.R., Santos, F.M., de Oliveira, C.E., Piranda, E.M., de Andrade, G.B., Macedo, G.C., Machado, R.Z., Herrera, H.M., 2019. Assessment of

equine piroplasmids in the Nhecolandia sub-region of Brazilian Pantanal wetland using serological, parasitological, molecular, and hematological approaches. Ticks Tick Borne Dis 10, 714-721. https://doi.org/10.1016/j.ttbdis.2019.03.002

Coultous, R.M., Phipps, P., Dalley, C., Lewis, J., Hammond, T.A., Shiels, B.R., Weir, W., Sutton, D.G.M., 2019a. Equine piroplasmosis status in the UK: an assessment of laboratory diagnostic submissions and techniques. Vet Rec 184, 95. http://dx.doi.org/10.1136/vr.104855

Coultous, R. M., McDonald, M., Raftery, A. G., Shiels, B. R., Sutton, D. G. M., Weir, W. 2019b. Analysis of *Theileria equi* diversity in The Gambia using a novel genotyping method. Transbound Emerg Dis, 53, 385. https://doi.org/10.1111/tbed.13454

Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R., Irwin, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Vet Parasitol 142, 238-247. https://doi.org/10.1016/j.vetpar.2006.07.004

Criado-Fornelio, A., Gonzalez-del-Rio, M.A., Buling-Sarana, A., Barba-Carretero, J.C., 2004. The "expanding universe" of piroplasms. Vet Parasitol 119, 337-345. https://doi.org/10.1016/j.vetpar.2003.11.015

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003a. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part I. Epizootiological aspects. Vet Parasitol 113, 189-201. https://doi.org/10.1016/S0304-4017(03)00078-5

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A., Barba-Carretero, J.C., 2003b. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Vet Parasitol 114, 173-194. https://doi.org/10.1016/S0304-4017(03)00141-9

DeWaal, D.T., 1992. Equine Piroplasmosis - a Review. Brit Vet J 148, 6-14. https://doi.org/10.1016/0007-1935(92)90061-5

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 32(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Rev Sci Tech 9, 1187-1194. http://dx.doi.org/10.20506/rst.9.4.535

Guidi, E., Pradier, S., Lebert, I., Leblond, A. (2015). Piroplasmosis in an endemic area: analysis of the risk factors and their implications in the control of Theileriosis and Babesiosis in horses. Parasit Res, 114(1), 71–83. https://doi.org/10.1007/s00436-014-4161-9

Hall, C.M., Busch, J.D., Scoles, G.A., Palma-Cagle, K.A., Ueti, M.W., Kappmeyer, L.S., Wagner, D.M., 2013. Genetic characterization of *Theileria equi* infecting horses in North America: evidence for a limited source of U.S. introductions. Parasit Vectors 6, 35. https://doi.org/10.1186/1756-3305-6-35

Hosmer, D.W., Lemeshow, S., 2000. Applied Logistic Regression. Second Ed. Wiley Interscience Press, New York, USA, pp.143-188.

Hunfeld, K.P., Hildebrandt, A., Gray, J.S., 2008. Babesiosis: recent insights into an ancient disease. Int J Parasitol 38, 1219-1237. https://doi.org/10.1016/j.ijpara.2008.03.001

Ketter-Ratzon, D., Tirosh-Levy, S., Nachum-Biala, Y., Saar, T., Qura'n, L., Zivotofsky, D., Abdeen, Z., Baneth, G., Steinman, A., 2017. Characterization of *Theileria equi* genotypes in horses in Israel, the Palestinian Authority and Jordan. Ticks Tick Borne Dis 8, 499-505.

https://doi.org/10.1016/j.ttbdis.2017.02.010
Knowles, D.P., Kappmeyer, L.S., Haney, D., Herndon, D.R., Fry, L.M., Munro, J.B., Sears, K., Ueti, M.W., Wise, L.N., Silva, M., Schneider, D.A., Grause, J., White, S.N., Tretina, K., Bishop, R.P., Odongo, D.O., Pelzel-McCluskey, A.M., Scoles, G.A., Mealey, R.H., Silva, J.C., 2018. Discovery of a novel species, *Theileria haneyi n.* sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. Int J Parasitol 48, 679-690.
https://doi.org/10.1016/j.ijpara.2018.03.010
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33, 1870-1874.
https://doi.org/10.1093/molbev/msw054
Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics, 30(22), 3276–3278.
https://doi.org/10.1093/bioinformatics/btu531
Liu, Q., Meli, M. L., Zhang, Y., Meili, T., Stirn, M., Riond, B., Weibelb, B., Hofmann-Lehmann, R., 2016. Sequence heterogeneity in the 18S rRNA gene in *Theileria equi* from horses presented in Switzerland. Vet Parasitol 221, 24–29.
http://doi.org/10.1016/j.vetpar.2016.03.003
Manna, G., Cersini, A., Nardini, R., Bartolome Del Pino, L.E., Antognetti, V., Zini, M., Conti, R., Lorenzetti, R., Veneziano, V., Autorino, G.L., Scicluna, M.T., 2018. Genetic diversity of *Theileria equi* and *Babesia caballi* infecting horses of Central-Southern Italy and preliminary results of its correlation with clinical and serological status. Ticks Tick Borne Dis 9, 1212-1220.
https://doi.org/10.1016/j.ttbdis.2018.05.005
Mans, B.J., Pienaar, R., Latif, A.A., Potgieter, F.T., 2011. Diversity in the 18S SSU rRNA V4 hyper-variable region of *Theileria* spp. in Cape buffalo (*Syncerus caffer*) and cattle from southern Africa. Parasitology 138, 766-779.
https://doi.org/10.1017/S0031182011000187
Montes Cortes, M.G., Fernandez-Garcia, J.L., Habela Martinez-Estellez, M.A., 2019. A multinested PCR for detection of the equine piroplasmids *Babesia caballi* and *Theileria equi*. Ticks Tick Borne Dis 10, 305-313.
https://doi.org/10.1016/j.ttbdis.2018.11.008
Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M.A., Yokoyama, N., Igarashi, I., 2013. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 16, 178-185.
https://doi.org/10.1016/j.meegid.2013.02.005
Nagore, D., Garcia-Sanmartin, J., Garcia-Perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: epidemiological survey and phylogenetic analysis. Vet Parasitol 123, 41-54.
https://doi.org/10.1016/j.vetpar.2004.04.010
Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cunha, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Vet Parasitol 101, 9-21.
https://doi.org/10.1016/S0304-4017(01)00471-X
Oura, C.A., Bishop, R.P., Wampande, E.M., Lubega, G.W., Tait, A., 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. Int J Parasitol 34, 603-613.
https://doi.org/10.1016/j.ijpara.2003.12.012
Ozubek, S., Aktas, M., 2018. Genetic diversity and prevalence of piroplasm species in equids from Turkey. Comp Immunol Microbiol Infect Dis 59, 47-51.

https://doi.org/10.1016/j.cimid.2018.08.005
Peckle, M., Pires, M.S., Silva, C.B.D., Costa, R.L.D., Vitari, G.L.V., Senra, M.V.X., Dias, R.J.P., Santos, H.A., Massard, C.L., 2018. Molecular characterization of *Theileria equi* in horses from the state of Rio de Janeiro, Brazil. Ticks Tick Borne Dis 9, 349-353. https://doi.org/10.1016/j.ttbdis.2017.11.011
Qablan, M.A., Obornik, M., Petrzelkova, K.J., Sloboda, M., Shudiefat, M.F., Horin, P., Lukes, J., Modry, D., 2013. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. Parasitology 140, 1096-1103. https://doi.org/10.1017/S0031182013000486
Qablan, M.A., Sloboda, M., Jirku, M., Obornik, M., Dwairi, S., Amr, Z.S., Horin, P., Lukes, J., Modry, D., 2012. Quest for the piroplasms in camels: identification of *Theileria equi* and *Babesia caballi* in Jordanian dromedaries by PCR. Vet Parasitol 186, 456-460. https://doi.org/10.1016/j.vetpar.2011.11.070
Ribeiro, A. J., Cardoso, L., Maia, J. M., Coutinho, T., & Cotovio, M. (2013). Prevalence of *Theileria equi*, *Babesia caballi*, and *Anaplasma phagocytophilum* in horses from the north of Portugal. Parasit Res, 112(7), 2611–2617. https://doi.org/10.1007/s00436-013-3429-9
Ros-Garcia, A., M'Ghirbi, Y., Hurtado, A., Bouattour, A., 2013. Prevalence and genetic diversity of piroplasm species in horses and ticks from Tunisia. Infect Genet Evol 17, 33-37. https://doi.org/10.1016/j.meegid.2013.03.038
Salim, B., Bakheit, M.A., Kamau, J., Nakamura, I., Sugimoto, C., 2010. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene within *Theileria equi* from horses in Sudan. Parasitol Res 106, 493-498. https://doi.org/10.1007/s00436-009-1691-7
Scoles, G. A., & Ueti, M. W. (2015). Vector ecology of equine piroplasmosis. Annu Rev Entomol, 60(1), 561–580. https://doi.org/10.1146/annurev-ento-010814-021110
Short, M. A., Clark, C. K., Harvey, J. W., Wenzlow, N., Hawkins, I. K., Allred, D. R., et al. (2012). Outbreak of equine piroplasmosis in Florida. J Am Vet Med Assoc, 240(5), 588–595. https://doi.org/10.2460/javma.240.5.588
Tian, Z.C., Liu, G.Y., Yin, H., Luo, J.X., Guan, G.Q., Luo, J., Xie, J.R., Shen, H., Tian, M.Y., Zheng, J.F., Yuan, X.S., Wang, F.F., 2013. RPS8--a new informative DNA marker for phylogeny of *Babesia* and *Theileria* parasites in China. PloS one 8, e79860. https://doi.org/10.1371/journal.pone.0079860
Uilenberg, G., Gray, J., Kahl, O. 2018. Research on Piroplasmorida and other tick-borne agents: Are we going the right way? Ticks Tick-Borne Dis, 9(4), 860–863. https://doi.org/10.1016/j.ttbdis.2018.03.005
Wise, L.N., Pelzel-McCluskey, A.M., Mealey, R.H., Knowles, D.P., 2014. Equine piroplasmosis. Vet Clin North Am Equine Pract 30, 677-693. https://doi.org/10.1016/j.cveq.2014.08.008

Figure legends

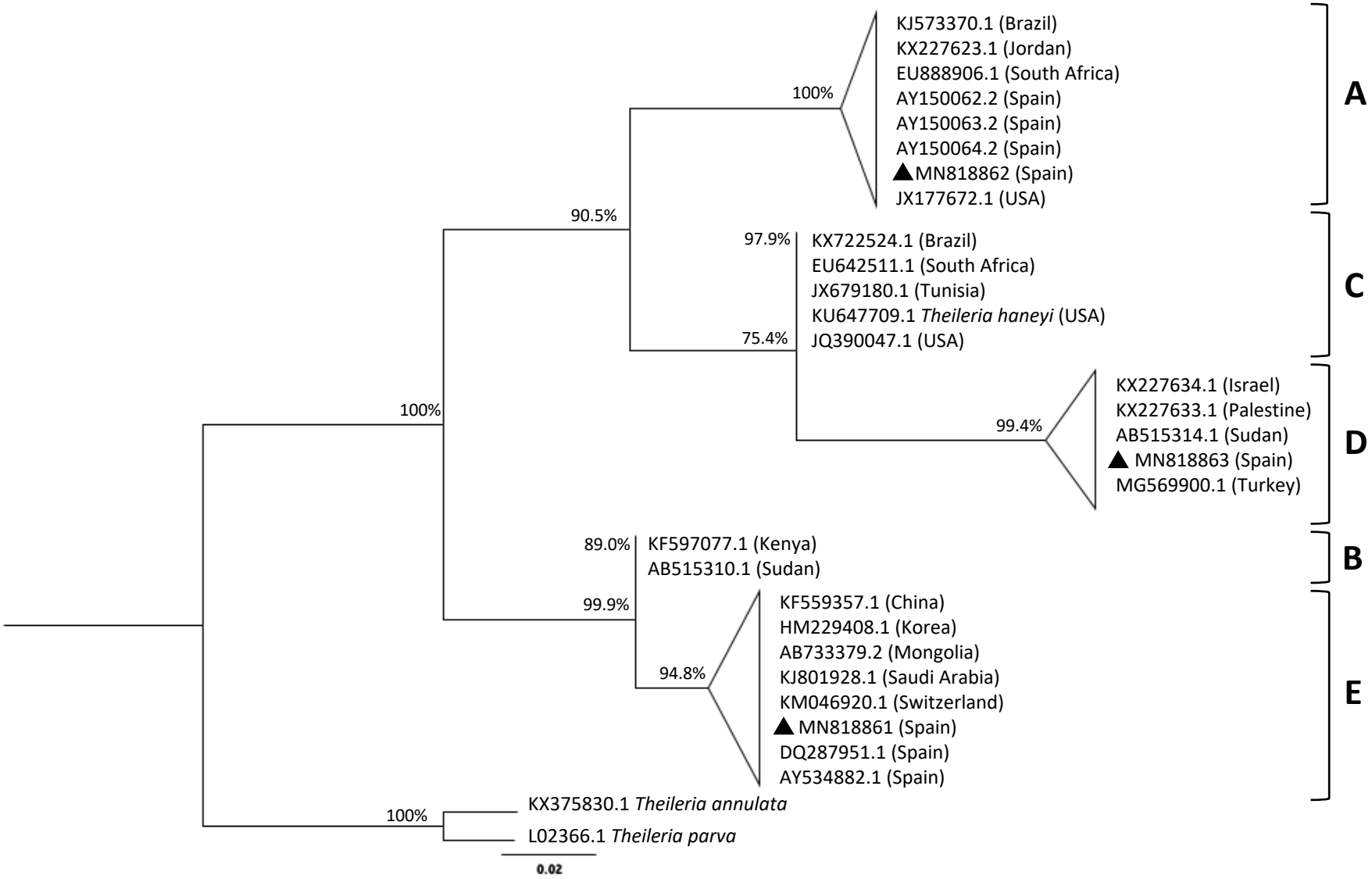
Figure 1. A maximum likelihood tree of *Theileria equi* isolates based on sequences derived from the amplified 18S SSU rRNA V4 hypervariable region; *Theileria equi* sequences reported previously are denoted by GenBank accession number and country of isolation. Bold triangles refer to sequences obtained from the present study. Numbers above the branches correspond to bootstrap values (1000 replications). Previously described clades (A-E) are highlighted.

Figure 2. A maximum likelihood tree of *Babesia caballi* isolates based on sequences derived from the amplified 18S SSU rRNA V4 hypervariable region; *Babesia caballi* sequences reported previously are denoted by GenBank accession number and country of isolation. Bold triangles refer to sequences obtained in the present study. Numbers above the branches correspond to bootstrap values (1000 replications). Previously described clades (A-C) are highlighted.

Figure 3. The geographic distribution of *Theileria equi* and *Babesia caballi* clade types within the sampled communities across Spain. The abbreviations of the autonomous communities are expanded in Table 1.

Table 1. A breakdown by community of the total number of samples analysed, and the parasite clades detected.

Communities	Number of samples collected	*T. equi*-clade	*B. caballi*-clade
Andalusia	22	A, E	B
Asturias	17	D, E	A
Basque Country	4	E	-
Canary Islands	1	E	-
Cantabria	12	E	A
Castile and Leon	16	A, E	A
Catalonia	2	E	-
Extremadura	5	A, E	-
Galicia	4	E	-
Madrid	9	A, E	-
Navarre	6	E	-
Valencia	2	E	-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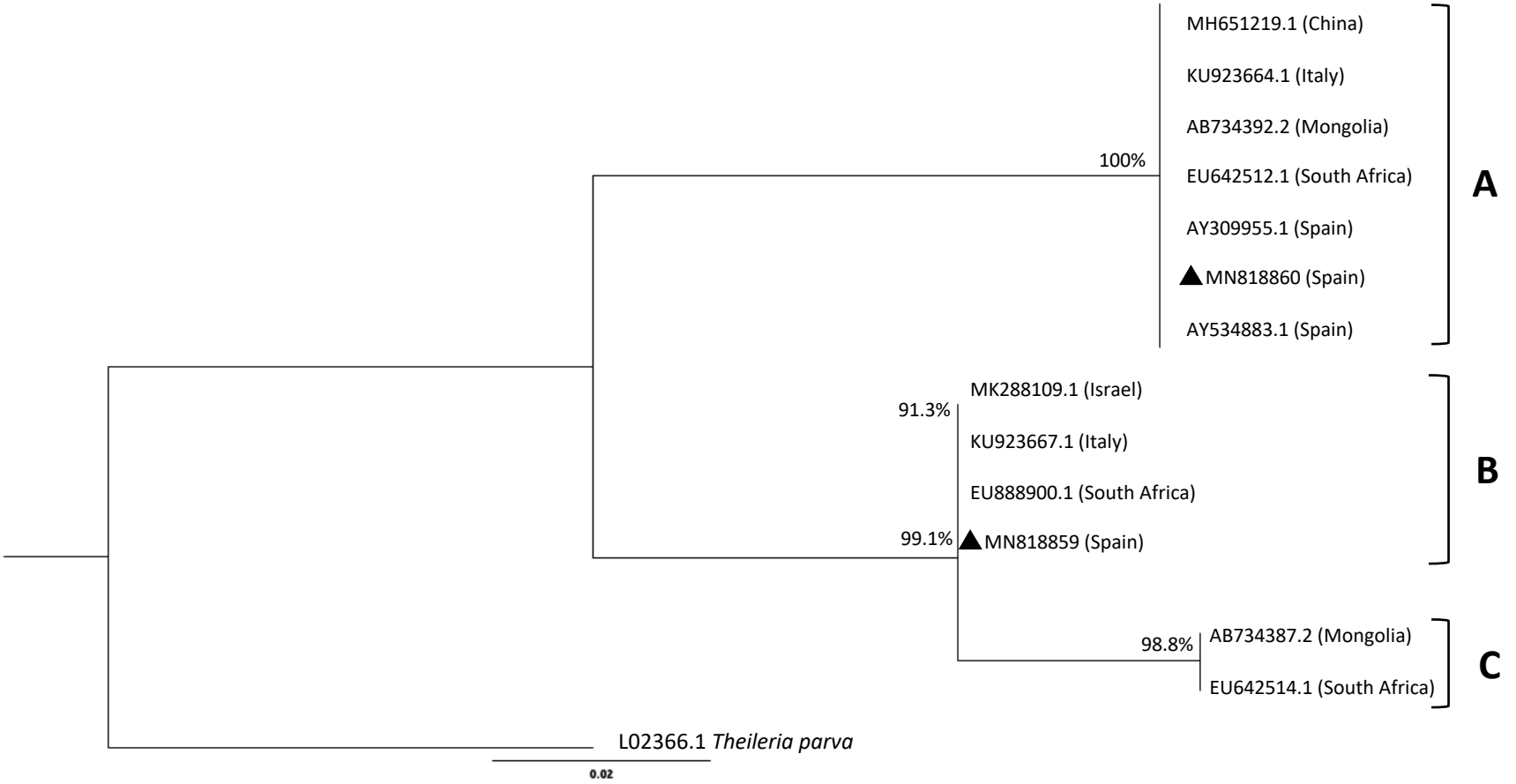
KX227623.1 (Jordan)
EU888906.1 (South Africa)
AY150062.2 (Spain)
AY150063.2 (Spain)
AY150064.2 (Spain)
▲MN818862 (Spain)
JX177672.1 (USA)
100%
90.5%
97.9%
KX722524.1 (Brazil)
EU642511.1 (South Africa)
JX679180.1 (Tunisia)
KU647709.1 Theileria haneyi (USA)
75.4%
JQ390047.1 (USA)
100%
99.4%
KX227634.1 (Israel)
KX227633.1 (Palestine)
AB515314.1 (Sudan)
▲ MN818863 (Spain)
MG569900.1 (Turkey)
89.0%
KF597077.1 (Kenya)
AB515310.1 (Sudan)
99.9%
KF559357.1 (China)
HM229408.1 (Korea)
AB733379.2 (Mongolia)
KJ801928.1 (Saudi Arabia)
KM046920.1 (Switzerland)
94.8%
▲ MN818861 (Spain)
DQ287951.1 (Spain)
AY534882.1 (Spain)
100%
KX375830.1 Theileria annulata
L02366.1 Theileria parva
0.02
A
C
D
B
E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KU923664.1 (Italy)
AB734392.2 (Mongolia)
100%
EU642512.1 (South Africa)
A
AY309955.1 (Spain)
MN818860 (Spain)
AY534883.1 (Spain)
MK288109.1 (Israel)
91.3%
KU923667.1 (Italy)
B
EU888900.1 (South Africa)
99.1%
MN818859 (Spain)
98.8%
AB734387.2 (Mongolia)
C
EU642514.1 (South Africa)
L02366.1 Theileria parva
0.02

AS
CA
BC
GA
NA
CT
CL
MA
NORTH
SOUTH
EX
VA
AN
CN

Theileria equi (piechart)

- Clade A
- Clade D
- Clade E

Babesia caballi (hatching)

- Clade A
- Clade B

Appendix A – Alignment of all *T. equi* isolate sequences

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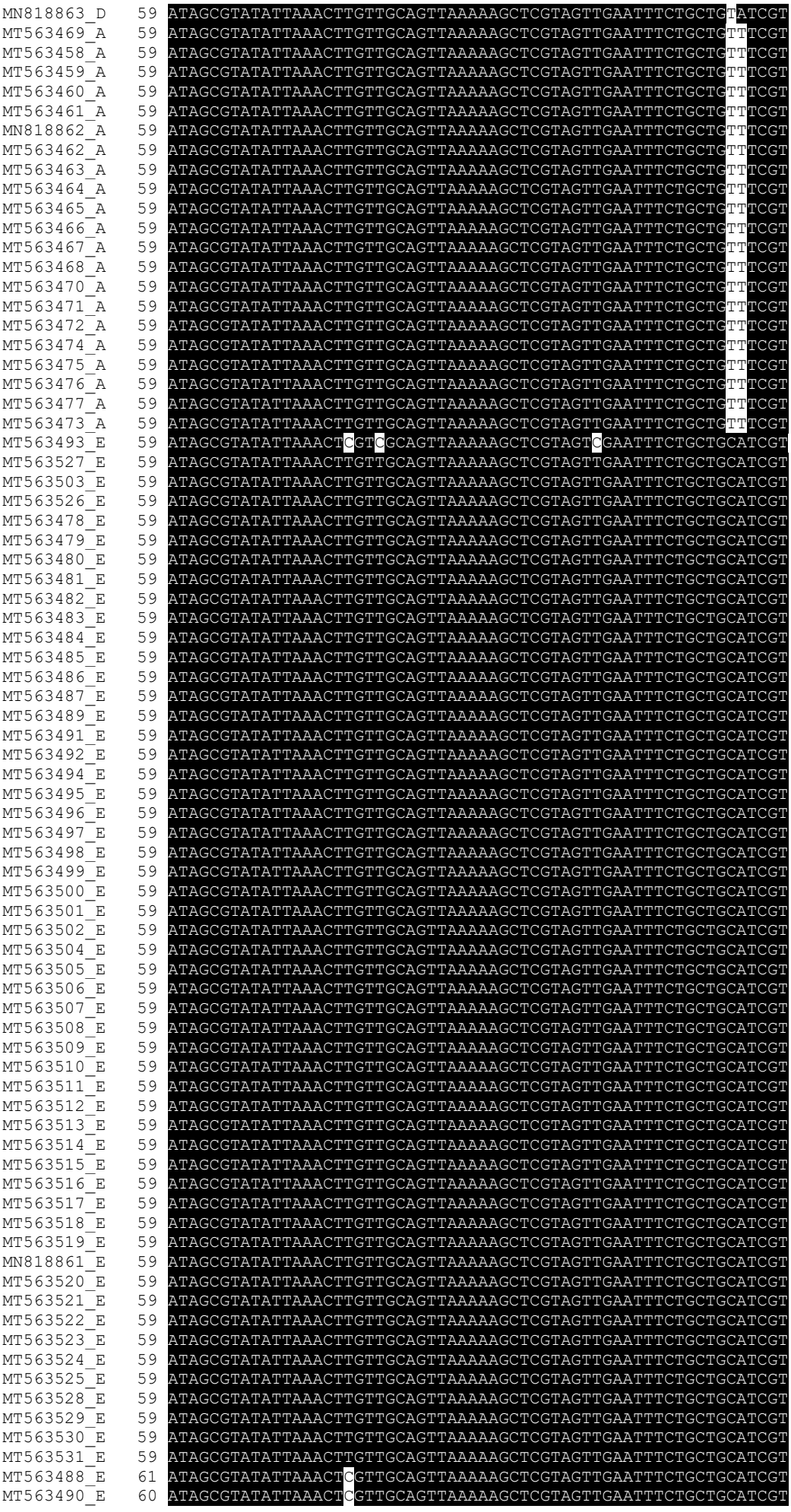
Appendix A – Alignment of all *T. equi* isolate sequences



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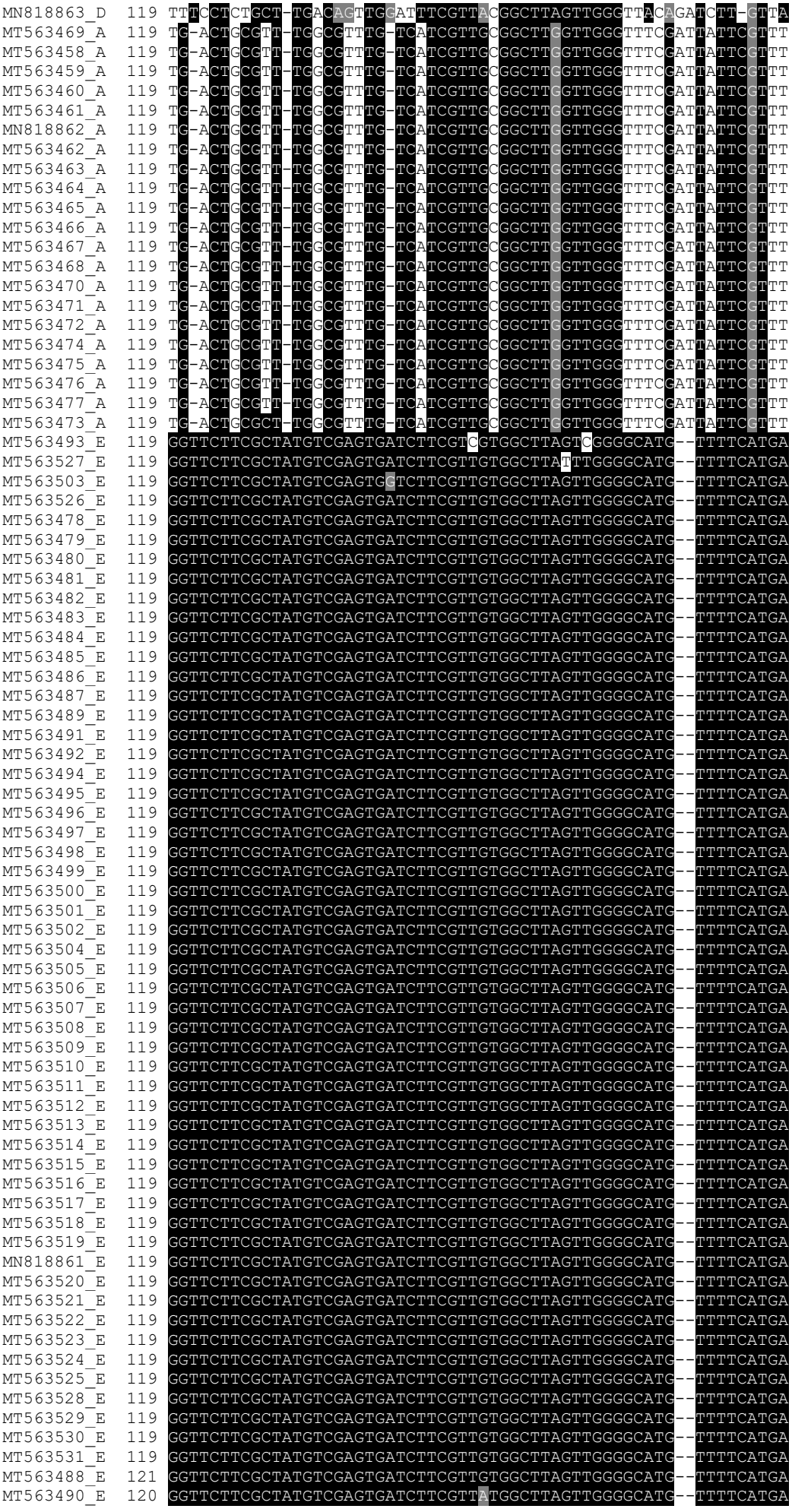
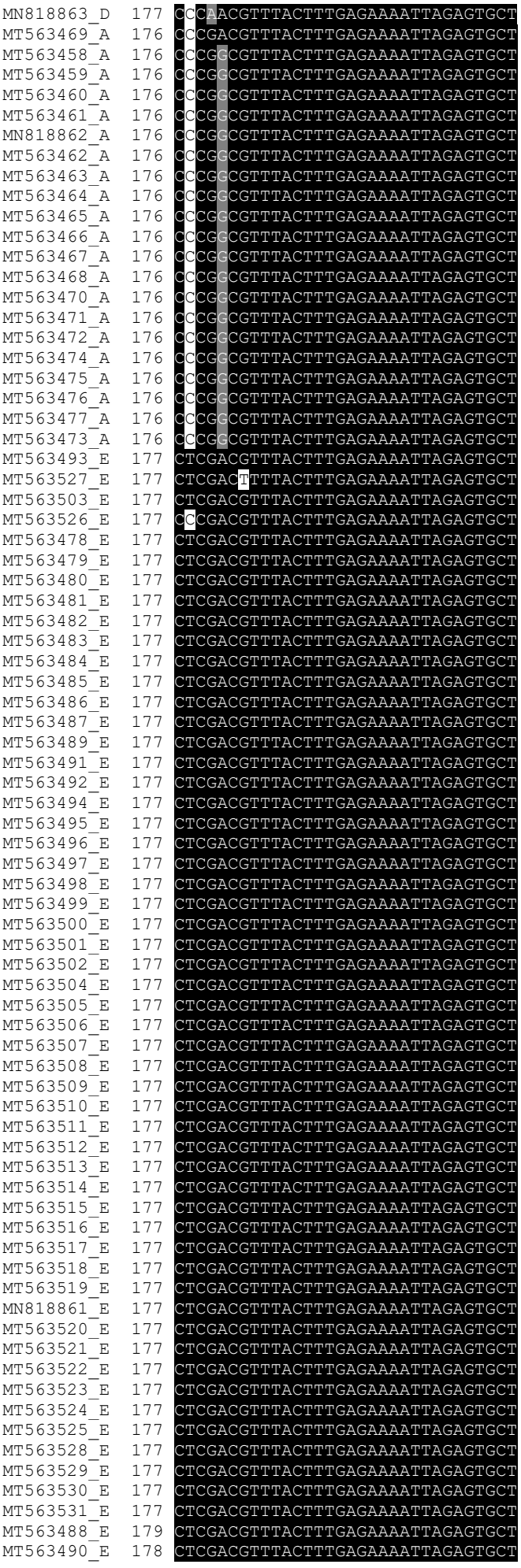


Appendix A – Alignment of all *T. equi* isolate sequences



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MT563456_A    61 CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAG
MT563457_A    61 CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAG
MN818860_A    61 CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAG

MN818859_B   121 TTGAATTTCTGCGTTGCGTTGTTCTTGCTTTTTGCTTGATTTTCGCTTCGCTTTTTGTTT
MT563454_A   121 TTGAACTTTTGCGTTGTCCTTTTCCTGCTTTGTGCGGGTTATTGACTTCGCTTTTTCTTT
MT563455_A   121 TTGAACTTTTGCGTTGTCCTTTTCCTGCTTTGTGCGGGTTATTGACTTCGCTTTTTCTTT
MT563456_A   121 TTGAACTTTTGCGTTGTCCTTTTCCTGCTTTGTGCGGGTTATTGACTTCGCTTTTTCTTT
MT563457_A   121 TTGAACTTTTGCGTTGTCCTTTTCCTGCTTTGTGCGGGTTATTGACTTCGCTTTTTCTTT
MN818860_A   121 TTGAACTTTTGCGTTGTCCTTTTCCTGCTTTGTGCGGGTTATTGACTTCGCTTTTTCTTT

MN818859_B   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA
MT563454_A   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA
MT563455_A   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA
MT563456_A   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA
MT563457_A   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA
MN818860_A   181 TTACTTTGAGAAAATTAGAGTGTTTATCGCAGACTTTTGTCTTGAATACTTCAGCATGGA

MN818859_B   241 ATAACATAGTAGGACCTTGGTTCTATTTTGTTGG-TTTGGGACCTTGGTAATGG
MT563454_A   241 ATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTGGAACCTTGGTAATGG
MT563455_A   241 ATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTGGAACCTTGGTAATGG
MT563456_A   241 ATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTGGAACCTTGGTAATGG
MT563457_A   241 ATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTGGAACCTTGGTAATGG
MN818860_A   241 ATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTGGAACCTTGGTAATGG
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