

Population genetic analysis of *Ixodes* tick species and *Toxoplasma gondii* in Tunisia and their relationships to global populations

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Introduction/Background

The *Ixodes ricinus* tick complex, consisting of 14 species, is widely distributed (Keirans and Needham, 1999; Xu et al., 2003). From this complex, only *I. ricinus* and *I. inopinatus*, have been reported in Tunisia (Estrada-Peña et al., 2014). Because of the high morphological similarities, these two species were previously confounded (Noureddine et al., 2011). In Africa, *I. ricinus* has been reported only in some humid and mountainous regions in Tunisia, Algeria and Morocco (Bouattour, 2001; Walker et al., 2003). Hoogstraal et al., (1964) recorded few numbers of *I. ricinus* in Egypt that were most likely introduced from Southern Europe by migratory birds. *I. ricinus* is a three-host tick species. The Immature stages are collected from small mammals, lizards (*Pseudodromus algirus*) or on ferns (*Pteridium aquilinum*), while adults feed mainly on cattle and sheep. In Tunisia, the larvae and nymphs were dragged from ferns and collected from lizards between April and August (Bouattour, 2001). Adults are active between September and May (Bouattour, 2001).

There are differences in the activity dynamics of *I. ricinus* populations found in North African countries which are probably due to differences in microclimate (temperature, humidity and rainfall) and hosts' availability (Estrada-Peña et al., 2013). The survey proposed here aims to

investigate the activity dynamics of *I ricinus*, to identify the abiotic factors influencing its distribution and to perform a phylogenetic analysis of populations of the species found in Ain Draham forest.

Toxoplasmosis is common in sheep in Tunisia (Gharbi et al., 2013; Khayeche et al., 2013) causing reproductive complications (Blewett et al., 1982) such as embryonic death followed by absorption or expulsion of small embryos if the infection takes place early during pregnancy. A later infection can cause abortion and birth of congenitally infected lambs (Buxton and Rodger, 2007). In males, there seems to be a relation between toxoplasmosis and disturbance of the reproductive parameters. In rams, Lopes et al. (2009) showed that *T. gondii*-infected rams produced smaller ejaculate volumes than healthy animals. In short and as a result of its simultaneous effects in both females and males, toxoplasmosis can be a major impediment to achieving optimal reproductive efficiency in sheep.

After estimating the serological and molecular prevalence in semen of natural infection by *T. gondii* in rams from different regions in Tunisia and with different reproductive history, the aim of this study was to characterize this parasite genetically and through phylogenetic analysis assess the relationship between *T. gondii* amplicons *vis-à-vis* the global haplotypes archived in the NCBI database.

Methods

Study area and samples

Two parasitic species were targeted in this study, the *Ixodes ricinus* ticks and *Toxoplasma gondii*. Samples of *Ixodes* were collected from five sites in Ain Draham region in Jendouba district North-west Tunisia.

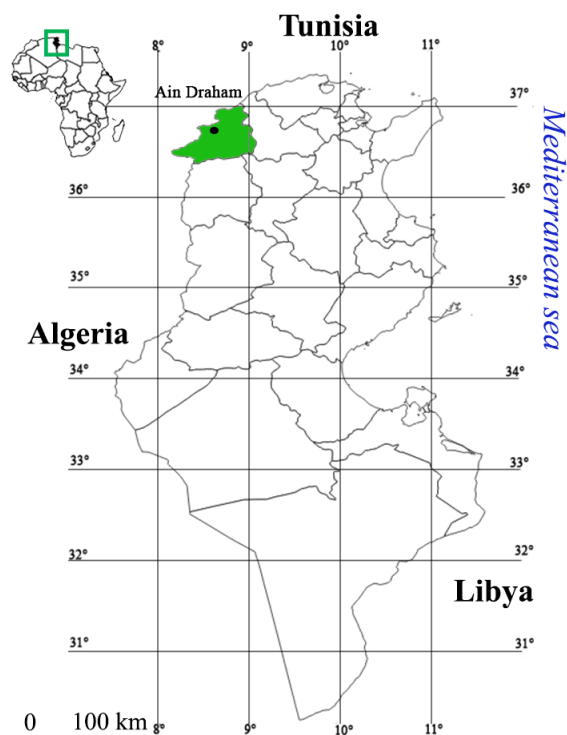


Figure 1: Location of Ain Draham forest (sites for tick collection) in Tunisia

The tick samples were collected monthly between September 2016 and August 2017 using the dragging technique (Tack et al., 2011). In total, 130 ticks were sampled during the study period. The tick samples were identified based on morphological characteristics following Walker et al., (2003). All the samples were stored at -20°C until analysis. The parameters of humidity and temperature were recorded using a thermo-hygrometer data logger, while slope and GPS coordinates were recorded with GPS calculator.

For *T. gondii*, semen samples were collected from rams of different ages and breeds in four locations *viz* Bou Salem (district of Jendouba), Mohamedia (district of Ben Arous), Saouaf (district of Zaghouan) and Oueslatia (district of Kairouan) of Tunisia (Figure 2). Semen samples were collected using an artificial vagina in the presence of a female in oestrus. Sperm samples were kept in sterile tubes, transported immediately to the National School of Veterinary Medicine of Sidi Thabet (Tunisia) and stored at -20 °C prior to DNA extraction. Semen samples were collected between April and September 2015; this corresponds to the peak of mating activity in sheep flocks in Tunisia.

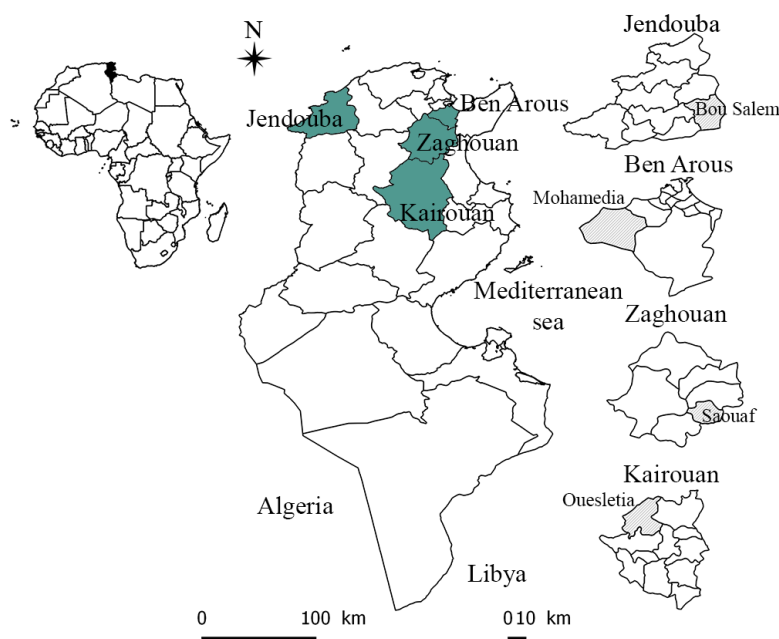


Figure 2: Districts in Tunisia where the semen samples were collected.

DNA extraction, PCR and sequencing

Genomic DNA was extracted from all the tick samples using the Wizard® Genomic DNA Extraction Kit (Promega, Madison, USA) according to the manufacturer's instructions. PCR reactions were performed in 30 µl reaction volumes made up of 1X PCR buffer, 2.25 mM MgCl₂, 0.8 mM dNTP, 0.5 µM of each primer, and one unit *Taq* polymerase. The target was to amplify the mitochondrial DNA gene 16S using the following primer pairs (16S-F 5'TGCTGTGGTATTTTGACTATAC3'; 16S-R 5'CCGGTCTGAACTCAGATCAAGT3') which amplified a fragment of 444 base pairs. The PCR reactions involved the following cycling profiles; an initial denaturation at 95°C for five minutes, followed by 35 amplification cycles made up of first denaturation step at 95°C for 30 secs, annealing at 59.4 °C for 30 secs and elongation at 72 °C for 1 minute. A final elongation step at 72 °C for 15 minutes completed the PCR reactions. A total of 20 PCR products were selected and sequenced by a private service provider.

For the semen samples, DNA was also extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. The B1 gene of *T. gondii* was amplified by PCR using two primers: forward B22 (5'-AACGGGCGAGTAGCACCTGAGGAGA-3') and reverse B23 (5'-TGGGTCTACGTCGATGGCATGACAAC-3').

The PCR reaction cocktails consisted of 10X PCR buffer, 2.5 mM MgCl₂, 200 µM of each dNTP, 0.6 mg BSA, 10 pmol of primers, 1U *Taq* polymerase, 6 µl of sample DNA and distilled water to a total volume of 25 µl. The PCR reaction profile was composed of 2 preliminary steps of 2 min at 50 °C and 6 min at 95 °C, respectively. These steps were followed by 40 cycles of 30 secs at 94 °C, 30 secs at 57 °C and 1 min at 72 °C adding 1 sec/cycle. A final elongation step of 7 min at 72 °C was performed. A total of 30 *T. gondii* amplicons were randomly chosen and sequenced through a private service provider.

Prior to sequencing, all the PCR products were purified with the ExoASP-IT[®] PCR Product Cleanup Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Sequencing reactions were performed with the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIORAD) using the ABI BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's protocol. The sequenced fragments were visualised using an ABI 3730xl DNA Analyser (Applied Biosystems). The same primers used for PCR were used for sequencing (16F and 16S R for ticks and B22 and B23 for *T. gondii* samples).

Data analysis

All chromatograms for both *Ixodes ricinus* and *T. gondii* were visualised and edited manually to correct base calling errors using ChromasPro software (version 1.7.4). Multiple sequence alignments will be performed using MEGA6 software and the sequences will be compared with the GenBank database with BLAST. A phylogenetic tree will be established for the analyses of the relationship between *Ixodes* isolates/ *T. gondii* in Tunisia and those from the world available in NCBI database.

To further assess the genetic groups present in the dataset while providing support for the phylogenetic analysis, a median joining network will be constructed using the Network software version 4.6. The default values in the program will be employed in the analysis. To put the *Ixodes* ticks and *T. gondii* found in Tunisia in the context of their global cohorts, haplotypes representing the two species will be downloaded from the NCBI's GeneBank database and incorporated in the phylogenetic and median joining network analysis.

Results

Ixodes ricinus

The BLAST search comparison between twenty 16S rDNA sequences showed a difference between 11 haplotypes (98-99% homology) which were submitted to GenBank.

I. ricinus K2 (MH375820) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaagcgtgattaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K9 (MH375816) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K14 (MH375824) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K16 (MH375823) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K1 (MH375826) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K8 (MH375817) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K51 (MH375818) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K47 (MH375819) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K18 (MH375822) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K12 (MH375825) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K19 (MH375821) 1 tttgactatcgaaggtattgaataaagattttaaatgagtgctaaaggaatgattaaaacattaaaagctttcttaattgataatgaatttaatt-tttttagtcgaaaagcaaaaataaaaattag
I. ricinus K2 (MH375820) 129 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K9 (MH375816) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K14 (MH375824) 131 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K16 (MH375823) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K1 (MH375826) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K8 (MH375817) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K51 (MH375818) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K47 (MH375819) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K18 (MH375822) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K12 (MH375825) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K19 (MH375821) 130 ggacagaagaagccctatgaattttttttaaaattttataaaaactttttataaaaatttaatttaattgggggtgagtggaagaaagataaatatc-tttttttt-aaagaaaattcggttcogtttttagogatt
I. ricinus K2 (MH375820) 258 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K9 (MH375816) 259 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K14 (MH375824) 260 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K16 (MH375823) 260 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K1 (MH375826) 258 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K8 (MH375817) 258 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K51 (MH375818) 258 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K47 (MH375819) 258 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K18 (MH375822) 259 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K12 (MH375825) 259 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K19 (MH375821) 259 aaatgaaaaaataactctagggataaacagcgttaataattttggatagtcctatagatagataaaatagtttggaactcgtatgtggattaggattcttttttggggaagagttaaaaaaa-gaagtttgg
I. ricinus K2 (MH375820) 387 caacttataatttccctactgatctg
I. ricinus K9 (MH375816) 388 caacttataatttccctactgatctg
I. ricinus K14 (MH375824) 389 caacttataatttccctactgatctg
I. ricinus K16 (MH375823) 389 caacttataatttccctactgatctg
I. ricinus K1 (MH375826) 387 caacttataatttccctactgatctg
I. ricinus K8 (MH375817) 387 caacttataatttccctactgatctg
I. ricinus K51 (MH375818) 387 caacttataatttccctactgatctg
I. ricinus K47 (MH375819) 387 caacttataatttccctactgatctg
I. ricinus K18 (MH375822) 388 caacttataatttccctactgatctg
I. ricinus K12 (MH375825) 389 caacttataatttccctactgatctg
I. ricinus K19 (MH375821) 388 caacttataatttccctactgatctg

Figure 3: Clustal-W alignment of 16S rDNA amplicons from 11 *Ixodes ricinus* haplotypes in Ain Draham region (Jendouba district, Northwest Tunisia)

The amplicons showed 95-100% homology with sequences from Algeria, Morocco, France, Germany, Netherlands, Portugal and North America.

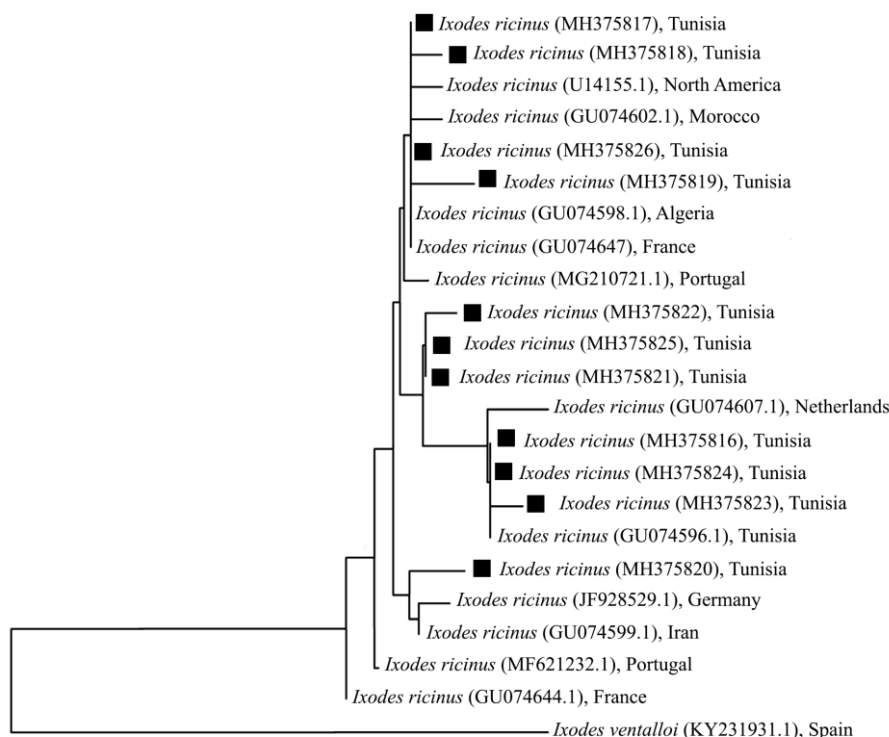


Figure 4: Phylogenetic tree showing the relationships between Tunisian isolates identified in the present study (indicated by black squares) and those deposited in GenBank from different countries based on 16S rDNA gene of *Ixodes ricinus* collected from Northwest Tunisia.

The tree was constructed using the neighbour-joining method of Saitou and Nei (1987) and distance were estimated using the Tamura-Nei model (Tamura and Nei, 1993).

The *I. ricinus* amplicons in Aïn Draham forest collected from vegetation and cow are relatively diverse and showed a genetic variability comparing to those from other Mediterranean regions. The Tunisian and the other North African isolates have a close relationship and belong to the same group. This divergence could be explained by the interaction of ticks with their environment and hosts. In fact, the variation among microclimates and the geographical discontinuity could affect the genetic structure and drives the geographic distribution of *I. ricinus* population (Jore et al., 2014). The presence of the Mediterranean Sea could be a natural barrier leading to the isolation of North African tick groups from those present in Europe. Furthermore, the differences in the activity dynamics and the interaction with different host communities could contribute to this divergence. For example, in Europe *I. ricinus* parasitizes mainly sheep but in Tunisia it was found mainly in cattle (Bouattour, 2001; Nouredine et al., 2011).

Toxoplasma gondii

The BLAST comparison of the partial sequences (114 base pairs) of the B1 gene revealed 100% homology between the five sequenced amplicons from Tunisia. The PCR amplification of the genomic DNA recovered from the 92 semen samples corresponded to *Toxoplasma gondii* thus confirming the infection of the rams' semen.

The amplicons showed a 100% sequence homology with recently reported sequences of *T. gondii* from Tunisia (GU249509.1), Brazil (JX218225.1), Saint Kitts and Nevis (KU599369.1) and Great Britain (KC928250.1) archived at the genebank. They shared 99% homology with sequences from Puerto Rico (KU599371.1) and 98% homology with four human isolates (GU139479.1, KU599432.1, KU599427.1 and KT310098.1). The amplicon also shared a 97% sequence homology with many others from Tunisia, Portugal, USA, Gabon, France, Ethiopia and Turkey. A homology of 96% was shared with an amplicon from French Guiana (KU599429.1) and 95% with samples from Tunisia, Austria, Turkey and China (GU139472.1, KU599330.1, KU599480.1, KU599377.1 and KU599387). A phylogenetic tree incorporating the *T. gondii* amplicon from the current study and sequences of other amplicons downloaded

from the genebank was constructed (Figure 3). It confirms that the five sequences form a single haplotype that has also been observed in chicken, cats, European pole rat and humans in Brazil, St Kits and Nevis, Great Britain and Tunisia, respectively.

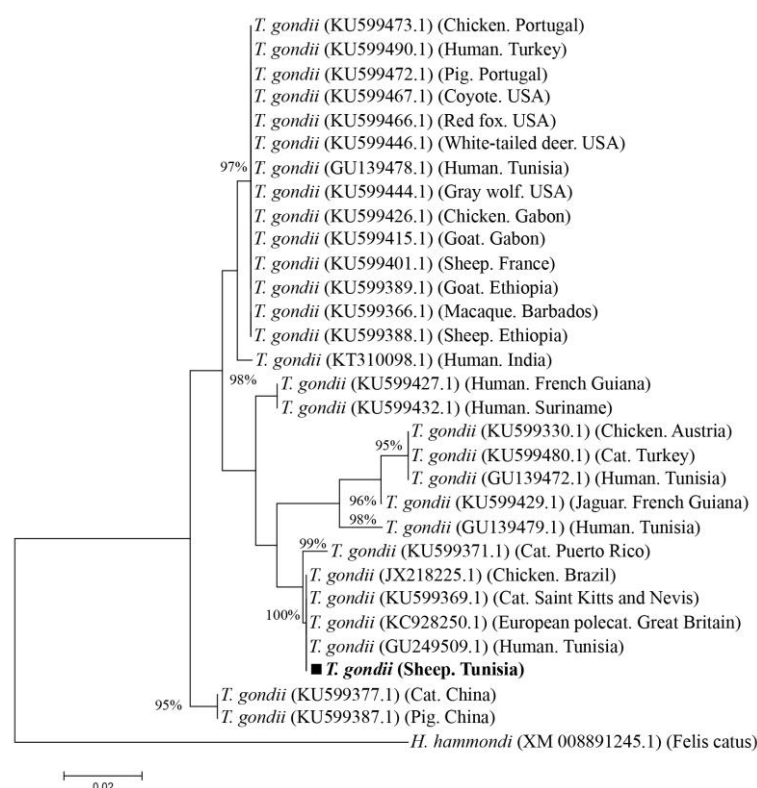


Figure 5: Phylogenetic tree of B1 gene for *Toxoplasma gondii* isolated from rams' semen and the main *T. gondii* species deposited in GenBank.

Output

The genetic analysis output of this work will be incorporated in at least two papers under preparation.

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