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An internal transcribed spacer 2 gene fragment, as a suitable phylogenetic marker in *Hyalomma* and *Rhipicephalus* (Acari: Rhipicephalinae) species

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Hyalomma and *Rhipicephalus* (Acari: Ixodidae: Rhipicephalinae) are the most important ectoparasites and biological vector of pathogen agents. The taxonomy of some *Hyalomma* and *Rhipicephalus* species is more debatable. The traditional taxonomy of ticks have been revolutionized using polymerase chain reaction. The specificity, efficiency and accuracy of PCR is highly dependent on the oligonucleotide primers. In this study, five primers designed for amplification of a fragment ITS2 so-called TAH-1, TAH-2, TRH (for *Hyalomma*) and TAH-3, TAH-4 (for *Rhipicephalus*). Successfully, 24 and 29 ITS2 sample was sequenced using these primers. As well as, an ITS2 phylogenetic tree were constructed using BEAST basis on the Bayesian Inference (BI) method. The most *Hyalomma* and *Rhipicephalus* species well differentiated using ITS2 fragment since the designed primer fail to amplify gene target in few cases.

Key words: PCR, Primer, ITS2, *Hyalomma*, *Rhipicephalus*, Phylogenetic relationship

INTRODUCTION

Hyalomma Koch, 1844 and *Rhipicephalus* Koch, 1844 (Acari: Ixodidae: Rhipicephalinae) are the most important ectoparasites and biological vector of pathogens (Sonenshine 1993). The taxonomy of some *Hyalomma* and *Rhipicephalus* species e.g., *R. sanguineus*, *H. excavatum* and *H. marginatum*is more debatable (Dantas-Torres *et al.* 2013; Hosseini-Chegeni *et al.* 2013). The traditional taxonomy of ticks (Acari: Ixodoidea) have been revolutionized over the past decades using molecular methods including polymerase chain reaction (Barker and Murrell 2008). The PCR performance is highly dependent on the oligonucleotide primers (Burpo 2001). Primers define the region of DNA template to be copied using hybridizeing to the target (Mcpherson and Moller 2006). These primers determine the specificity, efficiency and accuracy of polymerase chain reactions (Yuryev 2007). Internal transcribed spacer 2 (ITS2) locating in rDNA widely used for the identification of closly related species whitin suborder Acari (Navajas and Fenton 2000). The patial or complete ITS2 frequently used in a number of studies on hard ticks of the subfamilly Rhipicephalinae in order to identification and phylogentic relationship assessment (Zahler *et al.* 1997; Barker 1998; Murrell *et al.* 2001; Hlinka *et al.* 2002; Dergousoff and Chilton 2007; Lv *et al.* 2014). The present study was conduct to design a set of primer amplifying a fragment ITS2 gene in hard ticks of the genera *Hyalomma* and *Rhipicephalus* (Acari: Rhipicephalinae) for further works including molecular identification and phylogenetic relationship studies.

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MATERIAL AND METHODS

Primer design

In order to designing of primer, firstly the GenBank ITS2 sequences of representative *Hyalomma* (FJ416322, HQ123320, JQ737102, JQ737104, KC203370, KC203389, KC203394) and *Rhipicephalus* species (AF271283-4, DQ849268, JF758643, JQ412127, JQ625707-8, JQ737127, KC503269) were aligned using GeneDoc (version 2.7.000) (Nicholas *et al.* 1997). Second, the desired and conserved sites were selected using eye viewing, and the selected fragments were analyzed using online software Oligoanalyzer (version 3.1) (www.eu.idtdna.com/analyzer/applications/oligoanalyzer). The candidate primers were BLAST in GenBank. Finally, the Oligonucleotide primers were synthesized through the SinaClon Inc., Iran.

DNA isolation, PCR, gel electrophoresis and sequencing

Genomic DNA was extracted by using Phenol-Chloroform purification protocol based on Sambrook and Russell (2001). PCR was carried out at two thermocyclers MyGenie® (Bioneer, South Korea) and Bio-Rad® (U.S.). Temperature profiles for ITS2 gene (as touchdown in two round) as follows: first round; initial denaturation (95 °C, 5 min.), denaturation (94 °C, 1 min.), annealing (65-45 °C, 1 min.), extension (72 °C, 1.30 min.) then, repeating steps 2-4, 20 times, afterwards second round; the same cycle except annealing (45 °C, 1 min.) again repeating steps 2-4, 20 times and final extension (72 °C, 10 min.). During the annealing of first round temperature may be decrease 1 °C/cycle. The quantity and final concentration of PCR ingredients, respectively including: sterile water (14.8, 14.8 µl), PCR buffer, Bioflux®-10x (2.5, 1 mM), MgCl2, Bioflux®-50 mM (1, 2 mM), each forward and reverse primes-10 µM (1, 0.4 µM), dNTPs, Bioflux®-10 mM (0.5, 200 µM), Taq DNA polymerase enzyme, Bioflux®-5 U/µl (0.3, 1.5 U), gDNA template (4 µl, 50-100ng/µl). The PCR products were visualized by 1% agarose gel electrophoresis and the desired bands were purified using GeneJET Gel Extraction Kit®, then purified PCR products were submitted for sequencing to Sequetech®Company using ABI 3730XL DNA sequencer. Finally, all sequences were submitted in GenBank to assigning an accession number for each sequence.

Phylogenetic analysis

Two ITS2 sequences of *Rhipicephalus sanguineus* (representative of two distinct group), one ITS2 sequence *R. bursa* and one ITS2 sequence of each *Hyalomma* species with a number of ITS2 sequences of GenBank database were selected for phylogenetic examination. Firstly, the sequence data were aligned by ClustalX® (version 2.0) (Larkin *et al.* 2007) as separate sheets. Afterwards, in order to constructing phylogenetic tree, the aligned sequences of ITS2 were analysed using BEAST® (version 1.8.1) (Drummond *et al.* 2012) based on the Bayesian Inference (BI) method. The genera *Rhipicephalus* and *Hyalomma* were choosed as outgroup for both *Hyalomma* and *Rhipicephalus* phylogenetic trees, respectively.

RESULTS

Primer design and sequencing

The designed primers for *Hyalomma* ITS2 gene including forward primers TAH-1: 5' GCG TGC TCG ATG GGA GAT G 3'; positions 118-136, TAH-2: 5' GAA AGC CTG CCT ACC G 3'; positions 222-237 both and a reverse primer TRH: 5' TCT TCG GGA CGG CGA CTG 3'; positions 809-826 all within ITS2 gene. As well as for *Rhipicephalus* ITS2 gene including forward primer TAH-3: 5' CTT CGT CTG TCT GAG GGT CGG 3'; positions 134-154 within 5.8S gene and reverse primer TAH-4: 5' GGA ACT GCG AAG CAC TTG G 3'; positions 699-717 within ITS2 gene. Successfully, 24 and 29 ITS2 sample was sequenced as unidirectional including GenBank

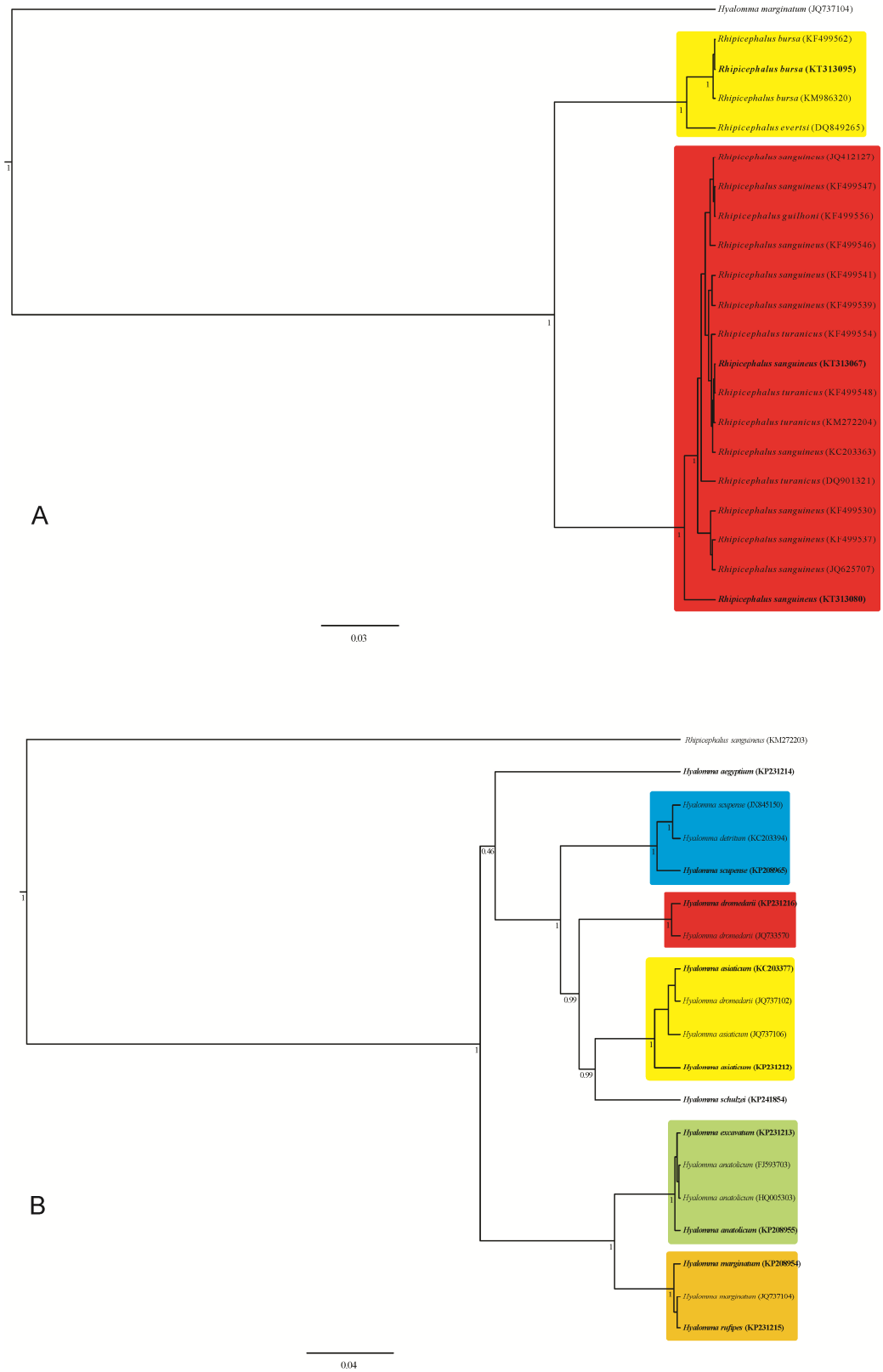

FIGURE 1- The phylogenetic relationship reconstructed using BEAST package based on Bayesian Inference (BI) indicating of *Rhipicephalus* (A) and *Hyalomma* ITS2 sequences of GenBank and sequences of the present study. Taxa of the present study are bold and GenBank accession number of each taxa are given in the parenthesis. Nodes indicated with credibility values.

accession numbers KP208952-59, KP208960-61, KP208962-66, KP208967-68, KP231211-16 and KP241854 for *Hyalomma* species as well as, KT313067-78, KT313079-88, KT313089-91, KT313093, KT313095-96 and KT313102 for *Rhipicephalus* species.

Phylogenetic analysis

The phylogenetic trees counsructed using Bayesian Inference (BI) method shown in Figure 1. The *Rhipicephalus* phylogenetic tree were considered as two main clades *R. bursa* and *R. sanguineus* (with intraspecific genetic distance) according to low intra (1%) and high (12%) genetic distance. As well as, according to these criteria, also the morphological differences of aong distinct species, seven clades *Hyalomma aegyptium*, *H. anatolicum*, *H. asiaticum*, *H. dromedarii*, *H. marginatum*, *H. schulzei* and *H. scupense* were created in *Hyalomma* phylogenetic tree. No intraspecific genetic distance were seen within species except 1% for *H. asiaticum*.

DISCUSSION

The ITS2 were amplified using cloning in a number of studies (Zahler *et al.* 1997; Rees *et al.* 2003; Chitimia *et al.* 2009; Ganjali *et al.* 2011; Tian *et al.* 2011), however, in the present study ITS2 fragments were achieved just using the PCR no need to time consuming cloning procedures. The designed primer of the present study can be used in the further studies as mixture (as Multiplex PCR with modification) to identifying morphologically unknown *Hyalomma* and *Rhipicephalus* specimens only based on a portion of body e.g., legs. Second internal transcribed spacer (ITS2) widely used as a molecular marker in phylogeny and evolutionary analysis of ticks as well as in order to molecular identification and determining taxonomical status of closely related tick taxa (McLain *et al.* 1995; Rich *et al.* 1997; Zahler *et al.* 1997; Barker 1998; Fukunaga *et al.* 2000 ; Murrell *et al.* 2001; Hlinka *et al.* 2002; Labruna *et al.* 2002; Rees *et al.* 2003; Dergousoff and Chilton 2007; Chitimia *et al.* 2009; Moshaverinia *et al.* 2009; Song *et al.* 2011; Lv *et al.* 2014). ITS2 is one of the most useful DNA fragment for phylogenetic analysis of the history of life (Hillis and Dixon 1991). In the present study, most *Hyalomma* and *Rhipicephalus* species well differentiated using ITS2, however, closely related morphological taxa *H. marginatum* (KP208954) and *H. rufipes* (KP231215) (4% genetic distance), *H. excavatum* (KP231213) and *H. anatolicum* (KP208955) (no genetic distance) as well as two *R. sanguineus* different phylogenetic clade (KT313067 and KT313080) not separated in the species level (2% genetic distance). Similar to the results of the present study, Barker (1998) declared a genetic distance equal to 3.3% among two *Rhipicephalus* species confirming some *Rhipicephalus* species could not be determined using ITS2. An acceptable amount of nucleotide base pair (bp) circa 596 bp and 655 bp plus gaps were used to analyzing phylogenetic relationship of *Hyalomma* and *Rhipicephalus* species, respectively.

Primer as a key factor in PCR improveing specificity, efficiency and accuracy of PCR amplification. Annealing temprature of mixed primers (forward and reverse) in a reaction play a major role in primer design. In the present study two pair primers TAH1-2, TRH and TAH3-4 could amplify target ITS2 gene in most specimens, successfully. In the failed PCR cases caution should be taken whereas DNA extraction or genetic variation in desired locus may affect a successful PCR. The most *Hyalomma* except *H. rufipes* and *H. excavatum* could be identified using ITS2. As well as, two deatale species *R. sanguineus* and *R. turanicus* are a distinct species based on low interspecific variation of ITS2.

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