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Original article

Molecular characterization of six *Hyalomma* species using mitochondrial markers

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ABSTRACT

Hyalomma species (Acari: Ixodidae) are vectors of several human and animal pathogens. However, due to their similar morphological properties, classification of related *Hyalomma* species is often challenging. Here we describe a combined approach for molecular characterization of six *Hyalomma* species: *H. aegyptium, H. dromedarii, H. excavatum, H. impeltatum, H. marginatum* and *H. turanicum*. This procedure was developed using a combination of PCR amplification of four molecular markers, followed by sequencing and species-specific restriction analysis. Segments from the following genes were used as markers: 12S rRNA, 16S rRNA, Cytochrome C oxidase subunit 1 (COX1), and Cytochrome B (CytB). Phylogenetic analysis based on the amplified sequences was consistent with the morphology-based classification. It revealed relative close proximity of *H. excavatum, H. marginatum* and *H. turanicum*, and close proximity of *H. aegyptium* and *H. dromedarii* to each other. *H. impeltatum* was examined using the COX1 and CytB markers, and in both cases was located on a separate clade from the other five species. Digestion of the amplified products using specific restriction enzymes enabled clear distinction between the six species. This report is the first to describe CytB marker sequences of the studied species, and the first to describe COX1 marker sequences of *H. aegyptium, H. excavatum, H. impeltatum* and *H. turanicum*. The information obtained in this study may therefore be useful for future combined morphological-molecular *Hyalomma* characterization.

1. Introduction

The ixodid tick genus *Hyalomma* contains 27 described species, which are present in Africa, Asia, the Middle East and parts of Europe (Guglielmone et al. (2010); Guglielmone et al., 2014). It is also considered to contain large intra-species morphological variation compared with other tick genera (Hoogstraal, 1956). In Israel, at least 8 *Hyalomma* species were documented on domestic and wild animals (Feldman-Muhsam, 1957). African *Hyalomma* species were thoroughly studied and described (Hoogstraal, 1956; Apanaskevich and Horak, 2005; Apanaskevich et al., 2008), but no recent reports were made on the abundance and classification of *Hyalomma* species in Israel. As mentioned above, a major challenge in performing a correct taxonomic classification of *Hyalomma* ticks is the morphological resemblance of related species, which can lead to misidentification (Guglielmone et al., 2014, Estrada-Peña et al., 2017). This is particularly important in studies aimed to assess the risk of tick-borne transmission of disease agents

such as *Theileria* species (Robinson, 1982; Samish and Pipano, 1983) and Crimean-Congo hemorrhagic fever virus (CCHFV, Hoogstraal, 1979). The development of molecular tools and the growing number of GenBank-annotated sequences of ticks, provide a complementing classification approach, based on genetic characteristics, rather than morphology alone (Estrada-Peña et al., 2017; Sanches et al., 2016). This approach was used for identification and classification of several tick groups in recent years, and allowed a re-definition of species dispersal, as well as comprehensive studies on tick-host preferences and population variations (Dinnis et al., 2014; de Lima et al., 2017; Kanduma et al., 2016). Mitochondrial genes are particularly useful as genetic markers, due to strict maternal inheritance, and, in some cases, a faster evolutionary change rate, compared with nuclear genes (Shao and Barker, 2007). Such markers were used by numerous research groups to classify and study tick populations, leading to the accumulation of data on tick mitochondrial markers and enabling improved classification of field samples (Dinnis et al., 2014; Kanduma et al., 2016). In previous studies,

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we used a combination of several mitochondrial markers and cuticular fatty acid profiling, to distinguish between related *Rhipicephalus* ticks (Erster et al., 2013b; Shimshoni et al., 2013). We thereby demonstrated that a clear distinction can be made between *R. turanicus* and *R. sanguineus* sensu lato, two very closely related species (Levin et al., 2012; Erster et al., 2013b). In this report, we apply the same combined approach, of PCR followed by restriction analysis, to distinguish between different *Hyalomma* species that are prevalent throughout the Middle East and have a veterinary and medical importance as ectoparasites and as potential disease vectors.

2. Materials and methods

2.1. Tick collection and classification

Tick specimens were obtained either by collecting them from hosts in the field, or from laboratory collections, as described below. *Hyalomma aegyptium* was collected from tortoises, which are considered to be its predominant host (Hoogstraal, 1956). The species was identified according to accepted morphological characteristics (Apanaskevich, 2003a; D. Apanaskevich, personal communication). *Hyalomma dromedarii* was collected from camels, its known predominant host (Hoogstraal, 1956) and was similarly identified by published classification properties (Apanaskevich, 2002; Apanaskevich et al., 2008). *Hyalomma excavatum* and *H. marginatum* were collected from cattle and livestock. Both species were identified according to published classification characteristics (Hoogstraal, 1956; Apanaskevich (2003b); Apanaskevich and Horak, 2005, 2008). *Hyalomma impeltatum* and *H. turanicum* were retrieved from tick collections in the Kimron Institute (KVI) and the entomology laboratory of the Ministry of Health, Israel. Both species were identified according to accepted morphological characteristics (Apanaskevich and Horak, 2008, 2009). Tick collection from wild animals was performed by the Israel Natural Parks Authority (NPA) veterinarian as part of routine wildlife inspection, with special permission granted by the Israel NPA General Director. Collection from domesticated animals was performed during routine veterinary inspection of the Israeli Veterinary Services. Ticks were either kept alive in ventilated plastic cups, or were preserved in ethanol 70%, until morphological identification and subsequent DNA extraction were performed.

2.2. DNA extraction, PCR and cloning

DNA was extracted from ticks that were either collected directly from the host animal, or retrieved from collections in the KVI and in the Entomology laboratory of the Israeli Ministry of health. Extraction was performed using the MasterPure extraction kit (Epicentre,Illumina) according to the manufacturer's instructions. Primers were designed to amplify partially conserved fragments from the four genes that were used as markers, e.g. 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), cytochrome oxidase subunit 1 (COX1), and cytochrome B (CytB). Primers for 12S were based on the T1B and T2A primers (Beati and Keirans, 2001).Primers for *Hyalomma* COX1 were designed specifically for this study (Supplementary Table S1). The other primer pairs were designed based on multiple alignments of GenBank-annotated tick sequences as described before (Erster et al., 2013a,b). Conserved sequences between different genera were used as template. Primers were tested and PCR protocol was optimized to obtain the specific product. The primer sequences are listed in Supplementary Table S1. The PCR mix was as follows: 10 μl 2XDreamTaq™ (https://www.thermofisher.com/), 50–150 ng DNA, 1 μl of each primer (0.25 μM final), except for CytB 5′p primer, from which 2 μl were added (0.50 μM final), and H$_2$O to a final volume of 20 μl. PCR program was as described by Beati and Keirans (2001): 94 °C 2 min, 5x[94 °C 15 s 51 °C 30 s 72 °C 50 s] 25x [94 °C 15 s 53 °C 30 s 72 °C 50 s] 72 °C 5 min 4 °C hold.

PCR products were purified using GeneJet gel extraction kit (www.

Table 1
Intra-species Identity following multiple alignments of the PCR products studied in this work. Alignment was performed using the MUSCLE algorithm (Edgar, 2004).

Marker	Species	Positive/Total PCR[a]	Identity (%)
12S	*H.*[b] *aegyptium*	5/6	99.8
12S	*H. dromedarii*	6/6	99.3
12S	*H. excavatum*	9/9	99.5
12S	*H. impeltatum*	0/5	
12S	*H. marginatum*	4/5	99.8
12S	*H. turanicum*	0/4	
16S	*H. aegyptium*	6/6	99.7
16S	*H. dromedarii*	4/6	99.8
16S	*H. excavatum*	3/9	99.0
16S	*H. impeltatum*	0/5	
16S	*H. marginatum*	2/5	100
16S	*H. turanicum*	3/4	99.4
COX1	*H. aegyptium*	4/6	99.3
COX1	*H. dromedarii*	4/6	99.6
COX1	*H. excavatum*	7/9	99.8
COX1	*H. impeltatum*	4/5	99.8
COX1	*H. marginatum*	4/5	99.8
COX1	*H. turanicum*	3/4	99.6
CytB	*H. aegyptium*	4/6	99.8
CytB	*H. dromedarii*	5/6	99.8
CytB	*H. excavatum*	5/9	99.6
CytB	*H. impeltatum*	5/5	99.7
CytB	*H. marginatum*	5/5	92.0
CytB	*H. turanicum*	4/4	100

[a] Positive/ Total PCR title refers to the number of samples that enabled successful amplification of the marker for sequencing and digestion analysis, out of the total number of samples that were examined by PCR.

[b] *H.* = *Hyalomma*.

thermofisher.com/order/catalog/product/K0691) and were either sequenced directly or, when the quality of the direct sequencing was insufficient, were cloned into a carrier plasmid. Cloning into pGEM-T Easy (Promega, Madison, WI, USA) by T/A ligation was performed according to the manufacturer's instructions. Ligation products were transformed into JM109 competent cells (www.promega.com/), and the plasmid was recovered using QIAPrep kit (QIAGEN, Hilden, Germany).

2.3. Phylogenetic analysis and restriction analysis design

Sequences that were analyzed by enzymatic restriction were amplified by PCR and purified as described above. Purified products were digested according to the following protocol: 200–500 ng DNA, 2 μl x10 reaction buffer, 1 unit of the corresponding restriction enzyme, H$_2$O to a final volume of 20 μl. Restriction products were analyzed by agarose gel electrophoresis and stained with ethidium bromide or GelRed (https://www.olerup.com). *EcoRI, DraI, HpaI, MunI, SacI, EcoRV, BfuI,* and *SmiI* restriction enzymes were from Thermo Fisher (https://www.thermofisher.com/). *BsaI* and *Bsu36I* enzymes were from NEB (New England Biolabs). Analysis of sequencing results, alignments and identification of restriction sites following sequencing of the studied samples was performed with the Geneious package (version 8, www.geneious.com). The multiple alignment files were converted into Mega format, and used to construct the phylogenetic dendrograms using Mega X (Kumar et al., 2018).

3. Results

A total of 35 individual ticks were examined using both morphological characteristics, as detailed in Section 2.1, and molecular markers restriction analysis followed by sequencing.

The molecular analysis was performed on six species, following an initial morphological classification: *Hyalomma aegyptium* (6 total samples), *H. dromedarii* (6 total samples), *H. excavatum* (9 total samples), *H.*
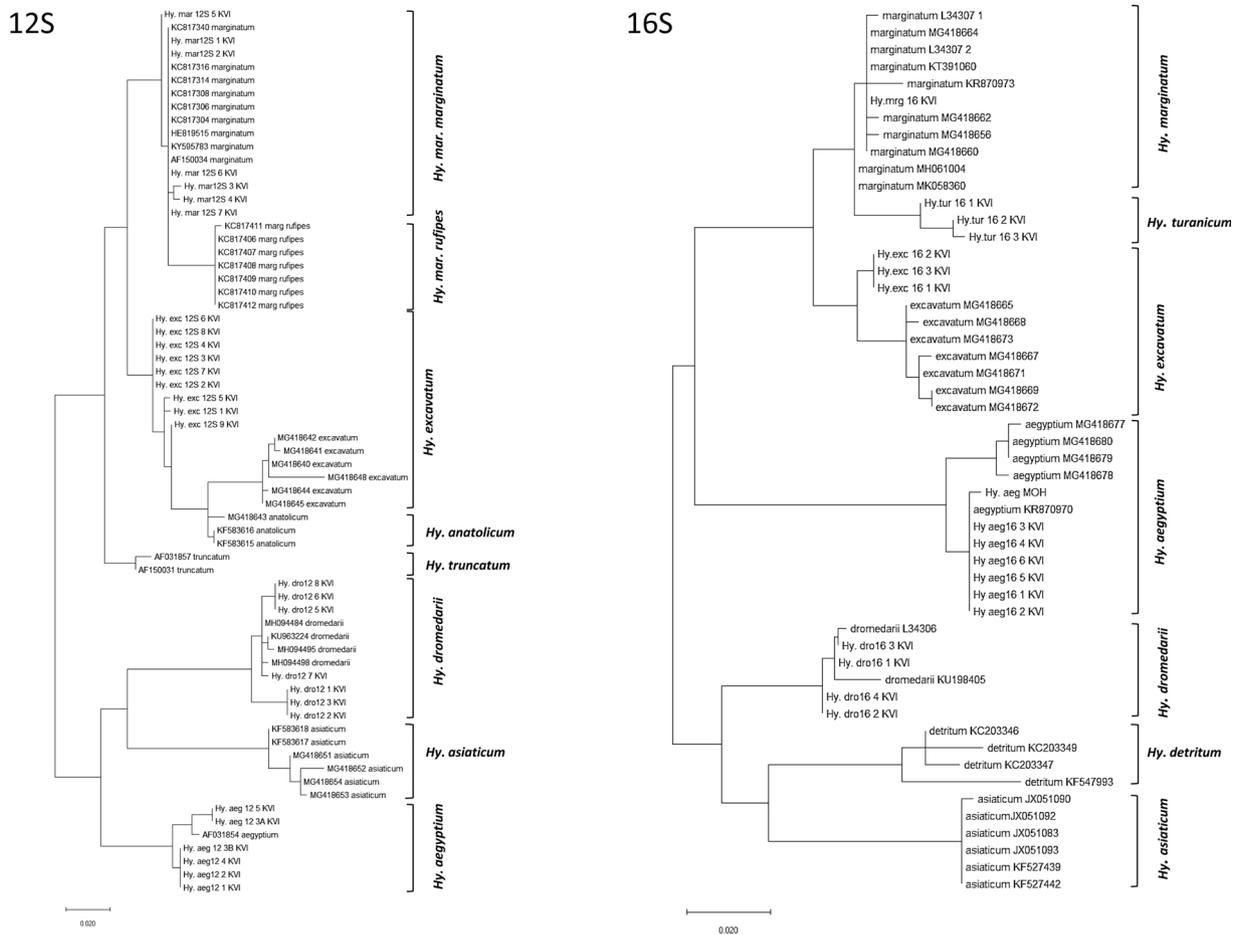
Fig. 1. Phylogenetic analysis of 12S and 16S markers. GenBank-annotated sequences and the sequences of the PCR products obtained in this study were used to construct phylogenetic dendrograms using the Maximum likelihood method in the MEGA X software (Kumar et al., 2018). The brackets and the adjacent labels denote each taxonomic group. The scale bar in each panel represents the genetic distance. Hy - *Hyalomma*.

marginatum (5 total samples), *H. impeltatum* (5 total samples) and *H. turanicum* (4 total samples).

3.1. Amplification of mitochondrial markers from Hyalomma species

Amplification of the following gene segments was performed: 12S, 16S, COX1 and CytB. Due to variable sample condition, in spite of repeating attempts, amplification of the 12S marker from *H. impeltatum* and *H. turanicum* samples was unsuccessful. Likewise, amplification of the 16S marker from *H. impeltatum* could not be accomplished. For some samples, certain markers were amplified successfully, but others were not. For example, the 12S marker was successfully amplified from 9 different *H. excavatum* samples, but the other three markers were successfully amplified only from three to seven samples (Table 1). A total number of 24 sequences were obtained for the 12S marker, 18 sequences for the 16S marker, 26 sequences for the COX1 marker and 28 sequences for the CytB marker (Table 1). The sequence lengths of the amplified segments were as follows: 385 bp for 12S, 425 bp for 16S, 763 bp for COX1 and 677 bp for CytB. For the 16S marker, this study was the first to sequence the *H. turanicum* species. For the COX1 marker, sequences from *H. aegyptium, H. excavatum*, *H. impeltatum* and *H. turanicum* were obtained for the first time. Finally, except for the CytB sequence of *Hyalomma asiaticum* (accession MF101817), all sequences obtained in this study were the first *Hyalomma* reported CytB sequences. Amplified marker sequences were deposited in the GenBank™ and the following accession numbers were assigned to them: KT391021 - KT391063, MG832792-MG832819, and KT989614-KT989638.

3.2. Phylogenetic analysis

In order to establish whether sequence information of the amplified markers could be used for classification, phylogenetic dendrograms were generated, in which the sample-derived sequences are designated according to their morphological characteristics. The sequences obtained from the studied samples, as well as GenBank™-annotated sequences, were analyzed using BLAST, followed by construction of Maximum Likelihood dendrograms, for each marker. The 12S analysis shows clear clustering of each species, with the *H. rufipes* group diverging from the *H. marginatum* clade, and the *H. anatolicum* group diverging from the *H. excavatum* clade (Fig. 1). The *H. excavatum* clade was divided into two branches, one consisting of the KVI-sequenced ones, and the other consisting of samples originated from Turkey (Hekimoglu and Ozer, 2017). The intra-species variability within the samples analyzed in this work ranged from 99.3%–99.8% identity (Table 1), and the overall identity within the entire 12S alignment was 93.0%. The amplified 16S region was somewhat less conserved, with 90.8% identity among different species, including GenBank-annotated samples. The intra-species identity was much higher, ranging between 99% and 100% (Table 1). The COX1 amplified marker identity was the same as that of the 16S (90.6%, Fig. 2), with intra-species identity of 99.3%–99.8% (Table 1). When this study as conducted, only a single *Hyalomma* Cytochrome *B* (CytB) sequences were available from Gen-Bank (accession MF101817), hence, the phylogenetic analysis of this marker was based almost entirely on samples obtained in this study, leading to clear distribution of each species on a separate clade in the dendrogram (Fig. 2). The identity among CytB marker from different
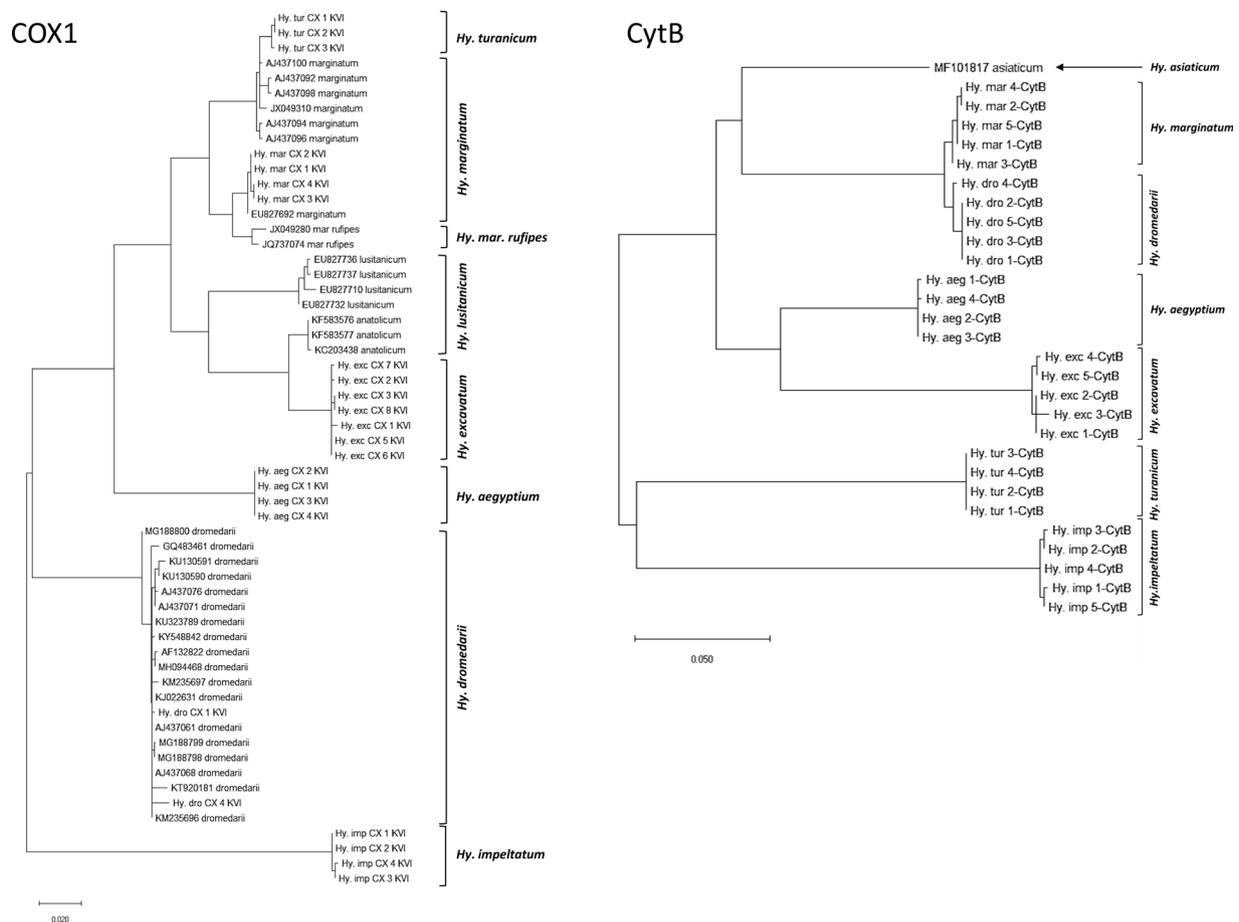
Fig. 2. Phylogenetic analysis of COX1 and CytB markers. The analyses for the COX1 and CytB markers were performed as described in the legend of Fig. 2. The scale bar in each panel represents the genetic distance. Hy – *Hyalomma*.

species was 83.7%, significantly lower than the other markers tested. The intra-species variability ranged between 92% and 100% (Table 1). The single available GenBank-annotated *Hyalomma* CytB sequence, *H. asiaticum* (accession MF101817), was branched from the *H. marginatum* – *H. dromedarii* clade (Fig. 2).

Examination of the dendrograms generated by the four analyses showed that for 12S, 16S and COX1 markers, the *H. excavatum*, *H. marginatum* and *H. turanicum* (when present) sequences clustered together on the same main clade, while *H. aegyptium* and *H. dromedarii* were clustered on a separate main clade (Figs. 1,2). The *H. impeltatum* group in the COX1 dendrogram was located on a separate clade from all other groups (Fig. 2). The CytB analysis gave a somewhat different partition, where the *H. marginatum* samples were clustered together with *H. dromedarii*, and the *H. excavatum* group was clustered with *H. aegyptium*. In this analysis the sequences were located on a separate clade, closer to the *H. impeltatum* group, which was located, again, on a separate main branch (Fig. 2). In all four analyses, all the samples that were identified morphologically as one species, clustered together on the same branch. These data therefore demonstrate that for the examined samples, there is good agreement between the species-specific morphology-based classification and the partition derived from the genetic markers that were used in this work.

3.3. Species differentiation by restriction analysis

In order to combine morphological classification with the advantages of molecular analysis, a combined Restriction analysis was applied to the studied samples. Restriction patterns that enable discrimination between the examined species were determined and the

restriction maps of each amplified marker were compared between the six species. Species-specific restriction enzymes were selected and were then tested with the purified PCR products (Fig. 3). For the 12S amplified fragment, two differential digestions were performed: only the *H. aegyptium* marker was digested by *EcoRI,* at position 190, and the *H. dromedarii* marker was uniquely digested twice, at positions 111 and 173 with *DraI* (Fig. 3A). Sequence analysis of the amplified 16S region showed that it did not contain any specific restriction sites that would enable clear distinction between the examined species (data not shown). The COX1 marker was more informative, containing four species-specific restriction sites, as follows: *BsaI* for *H. dromedarii* at position 631, *DraI* for *H. excavatum* at position 376, *SacI* for *H. impeltatum* at position 214 and *HpaI* for *H. marginatum* at position 551 (Fig. 3B, C). The CytB amplified sequence contained six species-specific restriction sites for the following markers: *BfuI* and *BsaI* for *H. aegyptium* at positions 392 and 172, respectively, *MunI* for *H. dromedarii* at position 192, *SmiI* for *H. impeltatum* at position 140, *EcoRV* for *H. marginatum* at position 136, and *Bsu36I* for *H. turanicum* at position 335 (Fig. 3C–F).

4. Discussion

Correct classification of related tick species is important for taxonomic research purpose, as well as for identification of vectors of a wide variety of pathogens (Guglielmone et al. (2010); Jongejan and Uilenberg, 2004). In this study, we used four mitochondrial gene markers to classify morphologically-related *Hyalomma* ticks. The use of molecular markers and mitochondrial markers in particular, has become an increasingly useful tool for tick identification (Burger et al.,
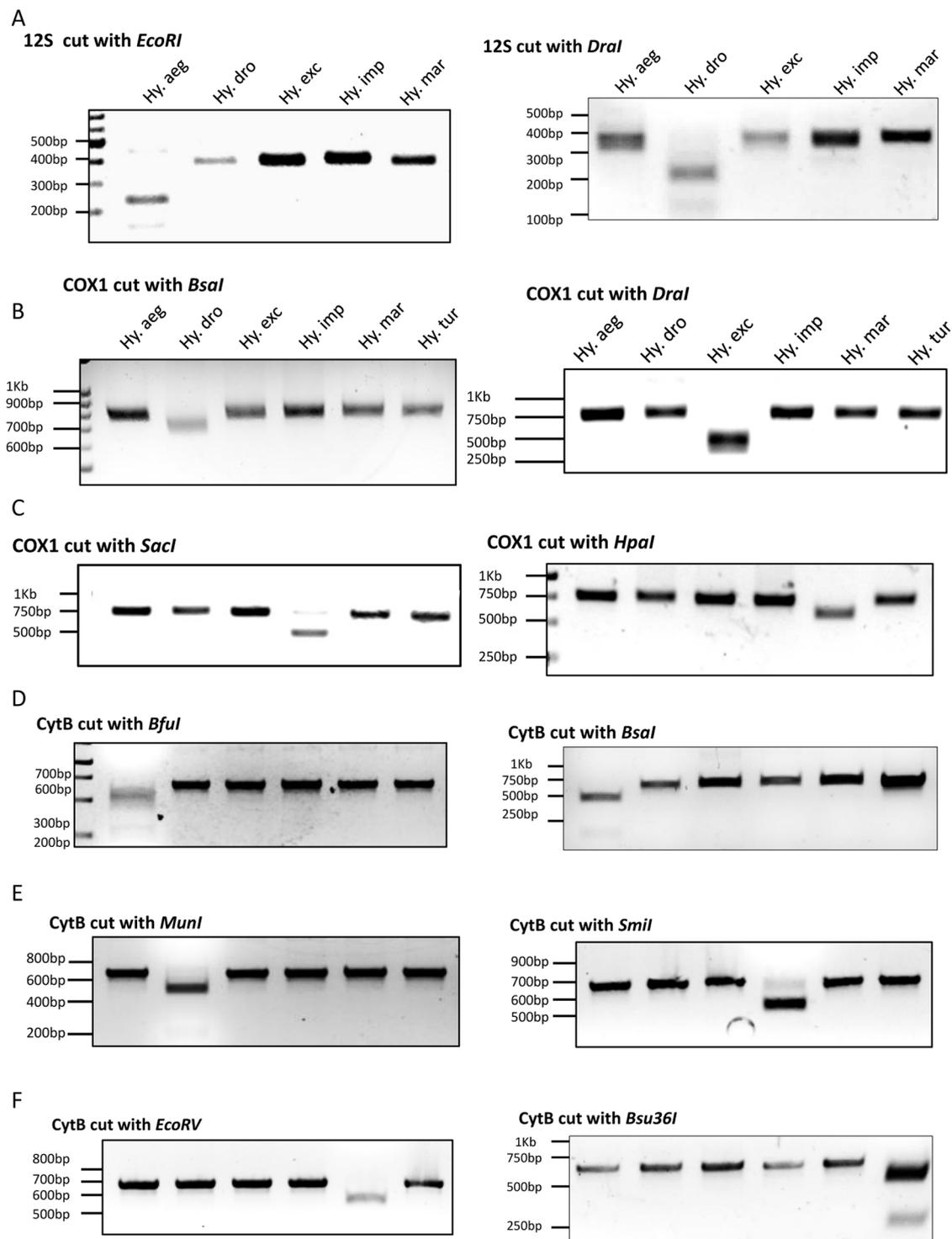
Fig. 3. Digestion analysis of the amplified markers. The PCR products of the four markers were purified and subjected to restriction analysis, as follows. (A) Digestion of the 12S marker PCR products with *EcoRI* (left) and *DraI* (right). (B) Digestion of the COX1 marker PCR products with *BsaI* (left) and *DraI* (right). (C) Digestion of the COX1 marker PCR products with *SacI* (left) and *HpaI* (right). (D) Digestion of the CytB marker PCR products with *BfuI* (left) and *BsaI* (right). (E) Digestion of the CytB marker PCR products with *MunI* (left) and *SmiI* (right). (F) Digestion of the CytB marker PCR products with *EcoRV* (left) and *Bsu36I* (right). Samples were separated on 1.2% agarose gel and stained with GelRed™ or Ethidium bromide.

2012; Zhang and Zhang, 2014). Combinations of nuclear and mitochondrial markers were used by us and by other groups, to identify and classify ixodid ticks of several genera: *Amblyomma* (Burger et al., 2012; Erster et al.,2015; de Lima et al., 2017), *Ixodes* (Poucher et al., 1999; Erster et al.,2013a) and *Rhipicephalus* (Erster et al., 2013b; Sanches et al., 2016). The entire mitochondrial genome of *Rhipicephalus, Dermacentor* and *Amblyomma* ticks, and recently that of *H.*

asiaticum, has been sequenced (Guo et al., 2016; McCooke et al., 2015; Williams-Newkirk et al., 2015; de Lima et al., 2017; Liu et al., 2018). However, in spite of their medical and veterinary importance, a considerably smaller number of studies was dedicated to the molecular classification of *Hyalomma* ticks (Abdullah et al., 2016; Hekimoglu and Ozer, 2017; Liu et al., 2018). *Hyalomma* ticks transmit the causative agents of several veterinary and zoonotic diseases, such as *Theileria*

(Samish and Pipano, 1983*)*, *Rickettsia* and *Ehrlichia* species (Pereira et al., 2018), CCHF Virus and other viruses (Hoogstraal, 1979; Lutomiah et al., 2014). Therefore, correct identification of potential *Hyalomma* vectors is of particular importance, necessitating adequate tools to meet that need. As mentioned above, during the preparation of this report, a single *Hyalomma* mitochondrial genome was published (Liu et al., 2018). We therefore sought to generate a dataset of local *Hyalomma* markers, to improve the currently available tools for classification of *Hyalomma* ticks. In the absence of a comprehensive genetic dataset, Liu et al. (2018) highlight the importance of using a large amount of data to adequately resolve phylogenetic controversies. Due to the limited genetic information from *Hyalomma* species, it was not possible to conclude why the phylogenetic relationships that were reflected in the 12S, 16S and COX1 dendrograms are different from the pattern that emerges from the CytB analysis. One possible explanation is a different mutation rate in the different genes that led to a variable proximity of each marker between the different species; e.g., for the 12S, 16S and COX1 markers, *H. marginatum* is most closely related to *H. excavatum*, whereas for the CytB marker, *H. marginatum* is most closely related to *H. dromedarii*. However, since only a single GenBank-annotated Cytochrome B sequence was available during the preparation of this work (Liu et al., 2018), very limited conclusions could be drawn from the data. Additional CytB sequences, from other geographic regions are needed to determine whether this is indeed the case. The *H. impeltatum* samples did not yield any PCR results with the 16S test, and their PCR-generated sequences were located on a separate clade in both the COX1 and CytB dendrograms. This may indicate a possible divergence from the other species studied in this work, at least with respect to mitochondrial genes evolution. Again, since there is very limited information on relevant markers from this species, it was not possible to clearly establish its genetic relationship with the other species studied in this work, let alone other *Hyalomma* species. In this respect, it is important to note that further improvement of PCR procedure for some of the markers is expected to result in more and better marker sequences than what was accomplished in this study. In addition to the lack of comprehensive data, deposition of misidentified sequences can further complicate molecular classification, as was recently described (Zhang and Zhang, 2014; Estrada-Peña et al., 2017). Deposition of additional marker sequences in the GenBank is therefore of crucial importance for improvement of correct *Hyalomma* classification.

In order to provide additional classification tools to complement the morphological identification, we developed restriction fragment length polymorphism (RFLP) tests, based on the four markers studied. A similar analysis was described for *Ixodes* species, but required PCR standards and was therefore less applicable for a wide use (Poucher et al., 1999). We previously applied this approach to distinguish between four *Rhipicephalus* species (Erster et al., 2013b). In the present study, we demonstrate that related *Hyalomma* ticks from the same geographic region can be rapidly classified without the need to sequence each sample, once the marker sequences of these species is determined. Although the 12S and 16S markers are very frequently used for species identification, we found that the COX1 and CytB markers were more informative for the restriction analysis and provided a complete tool set for differentiation between the six species studied. It should be noted, however, that the efficiency of the PCR varied between different species, leading to unsuccessful amplification of some of the markers in some of the species. This problem was previously described in a similar study that characterized *Hyalomma* ticks in Turkey (Hekimoglu and Ozer, 2017). Nevertheless, the COX1 and CytB markers combination enabled a complete restriction-based differentiation between all six species studied. Importantly, this approach was tested here on six local species. It should be used with caution when attempting to classify other species, whose restriction maps are not yet established. Moreover, when applying it to related tick populations in other geographic regions, it should be adjusted and validated for each region, to accommodate specific genetic variations. Taking these limitations into considerations, generation of a genus-wide dataset of molecular markers, should serve as a useful tool to improve classification of related ticks and complement established morphological keys.

In conclusion, the relative intra- and inter-species identity, and the relationships between the species were determined for each marker, based on their phylogenetic analysis. The sequence information was then used to devise a rapid procedure that distinguishes between these species without the need to sequence newly obtained PCR products. Due to the challenging situation of *Hyalomma* morphological classification, this molecular approach may help to improve accurate classification of *Hyalomma* samples, whose marker sequences are established. The genetic analyses described herein may further advance the understanding of relationship between populations of related *Hyalomma* species that share the same geographic region.

Conflict of interests

Acknowledgements

Appendix A. Supplementary data

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