Ticks and Tick-borne Diseases 11 (2020) 101382

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

# Molecular discrimination of *Hyalomma* tick species serving as reservoirs and vectors for Crimean-Congo hemorrhagic fever virus in sub-Saharan Africa

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ARTICLE INFO

*Keywords:*
CCHFV
Hyalomma
MALDI-TOF MS
DNA barcoding

ABSTRACT

The species identification of tick vectors of Crimean-Congo hemorrhagic fever virus (CCHFV), especially *Hyalomma* (*H.*) species, is a prerequisite to understand the eco-epidemiology of this disease and to reveal vector and virus reservoir species. However, the morphologic species discrimination can be difficult for damaged or blood-fed ticks and in case of species intercrosses. Therefore, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and restriction fragment length polymorphism (RFLP) analysis to distinguish the most common *Hyalomma* species from sub-Saharan Africa (*H. truncatum*, *H. rufipes* and *H. dromedarii*). Within the last years, MALDI-TOF MS analysis based on tick leg proteins has been shown to be a reliable method to distinguish several tick species. For this purpose, a reference spectral library of several European, American and African tick species was established. In this study, six different *Hyalomma* species were tested, all of which were all clearly distinguishable by mass spectrometric analyses. Moreover, MALDI TOF- MS was able to confirm morphologic findings where sequencing provided ambiguous results. In addition, a polymerase chain reaction (PCR) based on the CO1 gene amplification of ticks has been developed for the unequivocal species identification by amplicon sequencing and specific restriction endonuclease cleavage pattern analysis. RFLP proved to be a feasible auxiliary discrimination tool for selected *Hyalomma* species when access to sequencing methods is not available, as for instance during field studies.

## 1. Introduction

CCHFV (Crimean-Congo hemorrhagic fever virus) is one of the most widespread arthropod-borne viruses present in western parts of China, southern Asia, the Middle East, southwestern and southeastern Europe as well as throughout most parts of the African continent (Bente et al., 2013). CCHFV is primarily transmitted by ticks of the genus *Hyalomma*. Other possible transmission pathways are contacts to infectious blood, body fluids, or tissue. *Hyalomma* ticks serve as vectors as well as main reservoir (Gargili et al., 2017), as infected vertebrate hosts develop only a short viremias (Spengler et al., 2016). Massive tick infestations of livestock not only cause declining meat and milk yields in ruminants (Jonsson et al., 1998) and therefore huge financial losses, but also bear a health risk for livestock holders due to possible infections with zoonotic pathogens like CCHFV or *Rickettsia*. CCHFV can cause severe hemorrhagic fever in humans with case fatality rates of 5–30 % or higher (Bente et al., 2013). In contrast to humans, other susceptible livestock species, such as cattle, sheep and goats as well as wildlife do not show any clinical symptoms. Currently, the key molecular factors allowing certain tick species to receive, maintain and transmit CCHFV are unknown. It is also unclear whether all *Hyalomma* species are indeed vectors for CCHFV. The geographical distribution of CCHFV is nearly congruent with the occurrence of *Hyalomma* ticks and to date, there has not been a case of autochthonous infection in non-endemic *Hyalomma* areas. However, molecular detection of CCHFV in field samples of collected ticks alone can only indicate the presence of CCHFV in this region, but does not prove vector competence. Therefore, one of the most important conclusions of the review of Gargili et al. (Gargili et al., 2017) is the need for accurate tick species identification of CCHF virus-positive ticks to avoid any wrong conclusions on CCHFV circulation in the environment.

Currently, there are 27 different *Hyalomma* species described (Horak et al., 2002; Sands et al., 2017). However, more detailed references or literature are often only available for the most widespread species (such as *H. marginatum*, *H. rufipes*, *H. truncatum* or *H. dromedarii*), while the characterization of less prominent species is rather

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https://doi.org/10.1016/j.ttbdis.2020.101382
Received 8 October 2019; Received in revised form 10 January 2020; Accepted 23 January 2020
Available online 24 January 2020

sketchy. For some of them, it is not even clear whether they are independent species at all. Therefore, this study aimed to provide tools for the identification of *Hyalomma* species. As a first approach, morphologic and molecular methods were used to establish an unequivocal differentiation procedure for most common *Hyalomma* species with samples collected in Cameroon and Mauritania.

One major focus of molecular species identification was matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Species determination by MALDI-TOF MS has become more and more important during the last decade and has been proven to be a reliable tool for the identification of several arthropod species (Feltens et al., 2010; Kaufmann et al., 2012; Hoppenheit et al., 2013; Dvorak et al., 2014; Schaffner et al., 2014). For ticks, MALDI-TOF MS is a useful alternative method for species identification using the whole specimen (Karger et al., 2012) or only different body parts, such as legs (Yssouf et al., 2013a) or hemolymph (Yssouf et al., 2015). It has already been shown that different tick genera (including *Hyalomma*) can be discriminated on the basis of their leg proteins by MALDI-TOF MS (Kumsa et al., 2016; Diarra et al., 2017; Boucheikhchoukh et al., 2018). In previous studies up to three different *Hyalomma* species have been distinguished by mass spectrometric analysis (Yssouf et al., 2013a; Kumsa et al., 2016; Rothen et al., 2016; Diarra et al., 2017; Boucheikhchoukh et al., 2018). Therefore, one specific aim of this study was to provide a broader basis for MS-based discrimination of six *Hyalomma* species. Legs were preferred to whole animal samples in order to minimize the degree of potential contamination with host blood and thus ensure applicability to field-sampled, possibly engorged tick specimens.

In addition to MALDI-TOF MS, a second molecular species discrimination method was used based on the PCR amplification of specific marker genes. The mitochondrial gene cytochrome *c* oxidase I (CO1) has been shown to be a valuable target gene for DNA barcoding and is used for molecular identification of a great variety of eukaryotic and prokaryotic species (Hebert et al., 2003). Sequencing of certain marker gene amplicons, like CO1 for molecular species identification, represents the gold standard, although this method cannot guarantee a one hundred percent correct result either. Interpretation of the sequence data may be difficult, especially if high homologies to more than one single species occur or if databases contain incorrectly annotated sequences (Zhang and Zhang, 2014). Therefore, MALDI-TOF MS was included in this study as an alternative molecular method for the identification of selected *Hyalomma* species. However, during field studies in developing countries, access to such gene analysis methods or MALDI-TOF MS is generally problematic due to a lack of infrastructure. Another alternative approach of tick species identification is restriction fragment length polymorphism (RFLP), which enables the distinction of specific gene sequences within a short time and without access to costly laboratory equipment. For this purpose, we developed a "*Hyalomma*-specific" primer pair for the CO1 gene amplification containing suitable restriction enzyme cleavage sites for species discrimination.

## 2. Material and methods

### 2.1. Ticks

Field samples as well as laboratory-reared tick colonies used as reference material are summarized in Table 1. All ticks were stored in 70 % ethanol at −20 °C for at least two weeks prior to analysis. Field samples which had been collected from ruminants and camelids were classified as "blood-fed / engorged" while ticks from laboratory colonies were considered to be "non-engorged". Only adult ticks were used. The morphologic species identification of *Hyalomma* spp. was performed according to keys of Apanaskevich et al. (Apanaskevich and Horak, 2008a, 2008b; Apanaskevich et al., 2008, 2010). Ninety-two ticks belonging to 14 different species from five genera, obtained from field collections or from established laboratory colonies, were used for MALDI-TOF MS analysis (Table 1 and Supplementary Table 1). *H. rufipes*, *H. truncatum*, *H. nitidum* and *Amblyomma (A.) variegatum* were field- collected in Cameroon in 2015. *H. dromedarii* originated from field collections in Mauritania in 2018. *H. scupense* and *H. marginatum* ticks were field-collected in Croatia (kindly provided by Dr. Relja Beck, Croatian Veterinary Institute, Zagreb). Moreover, *H. marginatum* tick samples were also obtained from a laboratory colony established by Hans Dautel (Insect Services, Berlin, Germany). *A. hebraeum*, *A. americanum*, *Ixodes* (*I.) hexagonus*, *I. ricinus*, *Rhipicephalus* (*R.) appendiculatus*, *R. sanguineus sensu lato (s. l.)* and *Dermacentor* (*D.) reticulatus* ticks were obtained from laboratory colonies (kindly provided by MSD Animal Health Innovation GmbH, Schwabenheim, Germany) in order to compare the *Hyalomma* spp. to a broad spectrum of different tick genera to obtain representative data sets. The main focus of this study was the molecular differentiation of *Hyalomma* species, other samples served as outgroups and for validation purposes (Table 1). For the establishment

**Table 1**
Tick species and their origins used for reference database of MALDI-TOF MS and restriction digest analysis.

| Tick species | Origin | Sex | MALDI-TOF MS | RFLP | | | |
|---|---|---|---|---|---|---|---|
| | | | applied | n | pos. PCR *[a] | RFLP*[b] | applied |
| *Hyalomma rufipes* | CMR | f,m | ✓ | 47 | 46 | 33 | ✓ |
| *Hyalomma truncatum* | CMR | f,m | ✓ | 31 | 30 | 29 | ✓ |
| *Hyalomma marginatum* | CRO | f,m | ✓ | 9 | 9 | 7 | ✓ |
| | LC | f,m | | | | | |
| *Hyalomma scupense* | CRO | f | ✓ | 3 | 2 | 2 | ✓ |
| *Hyalomma dromedarii* | MRT | f,m | ✓ | 5 | 5 | 5 | ✓ |
| *Hyalomma nitidum* | CMR | f,m | ✓ | 6 | 6 | 4 | ✓ |
| *Hyalomma* sp. *[c] | CMR | f,m | – | 21 | 19 | – | ✓ |
| *Amblyomma variegatum* | CMR | f,m | ✓ | – | – | – | – |
| *Amblyomma hebraeum* | LC | f,m | ✓ | – | – | – | – |
| *Amblyomma americanum* | LC | f,m | ✓ | – | – | – | – |
| *Rhipicephalus sanguineus s. l.* | LC | f,m | ✓ | – | – | – | – |
| *Rhipicephalus appendiculatus* | LC | f,m | ✓ | – | – | – | – |
| *Ixodes ricinus* | LC | f | ✓ | – | – | – | – |
| *Ixodes hexagonus* | LC | f,m | ✓ | – | – | – | – |
| *Dermacentor reticulatus* | LC | f,m | ✓ | – | – | – | – |

LC = laboratory colony strain; CMR = Cameroon; MRT = Mauritania; CRO = Croatia; f = female; m = male.
*a = Samples which showed specific bands in 1 % agarose gel.
*b = Samples which were digested by restriction enzymes.
*c = spoiled ticks used from a pool consisting of *H. truncatum* and *H. rufipes*.

and adaptation of *Hyalomma*-specific CO1 PCR, only ticks from the genus *Hyalomma* were used (Table 1).

### 2.2. Sample preparation for MALDI-TOF MS

The sample preparation protocol was based on published protocols (Diarra et al., 2017; Nebbak et al., 2017). After morphologic assessment under the stereomicroscope (Nikon SMZ 745 T), ticks were rinsed with purified water followed by 70 % ethanol to remove coarse impurities and minimize cross-contamination of the samples. Four legs of the ticks were cut off with a scalpel and transferred into an Eppendorf Safe-Lock Tube (1.5 ml). For each tick sample the scalpel was cleansed carefully with purified water and 70 % ethanol and dried before use. The remaining idiosoma and gnathosoma were used for DNA or RNA analysis. Tick legs were mixed (shaken by hand) with 20 µl of 70 % formic acid and a spatula of glass powder (Sigma-Aldrich, G4649) and the mixture was sonicated for 10 min in a water bath (Branson Digital Sonifier 450D) at 4 °C. After sonication, the samples were shaken for 10 min at 300 rpm in an Eppendorf Thermomixer at room temperature and 20 µl of 50 % acetonitrile were added. Before loading the samples on a MALDI target plate, they were centrifuged for 4 min at 14,000 g. After drying, the spot was overlaid with 2 µl of a α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (HCCA saturated in 50 % ACN/2.5 % trifluoroacetic acid). Every sample was spotted sixfold on a MALDI steel target and from every spot four spectra were acquired for the generation of main spectra projections (MSP) as suggested by the manufacturer of Biotyper and ClinProTools software (Bruker Daltonics, Bremen, Germany). Spectra of low quality were excluded after visual inspection, leaving a minimum of 20 single spectra per sample for the calculation of a MSP.

### 2.3. MALDI-TOF MS conditions and construction of a main spectrum library

Spectra were recorded with an Ultraflextreme instrument (Bruker Daltonics, Bremen, Germany, Flexcontrol 3.0). Reference spectra, or main spectra projections (MSP) as they are denominated in the Biotyper software, were constructed with MALDI Biotyper software (version 2, Bruker) from 20 to 24 single spectra with 500 laser pulses each in the m/z range of 2,000–15,000. The mass spectrometer was externally calibrated with Bacterial Test Standard Bruker, #255,343 in the m/z range between 3,637.8 and 16,592.3. The calibrant was placed on a central spot of the target and eight surrounding spots were measured. Spectra were evaluated and edited with FlexAnalysis software (version 3.0, Bruker). A library of 92 MSP was constructed with Biotyper software which was then used for the database queries. For the construction of MSP in the m/z range between 2,000–15,000, the following parameters were applied: spectra were smoothed using the Savitzky-Golay algorithm, peak search was carried out by spectra differentiation, the maximum mass error of each single spectrum was set to 2000 ppm, the desired mass error for the main spectrum to 200 ppm. The minimum peak frequency was 50 %. Peak intensities were normalized to the most intense peak and a minimum intensity of 0.1 % of the most intense peak was required. The peak number was limited to 70 per spectrum.

### 2.4. Statistical evaluation of mass spectra

Results of the MALDI Biotyper query were exported and transferred to the statistical programming language R (version 3.5.1., (R Development Core Team, 2011)). Score values were arranged as a distance matrix for the construction of a Sammon map by two-dimensional scaling (Sammon, 1969). Statistical models were calculated for pairs of tick species with ClinProTools software (version 2.2, Bruker) using all four available algorithms, the genetic algorithm (GA), support vector machine (SVM), supervised neural network (SNN), and the quick classifier (QC). The suggested standard parameters were applied for GA (maximum of 10 peaks, maximum of 3 generations, k-nearest neighbor (KNN) classification with 1 neighbor), SVM (automatic detection of 1–25 peaks, KNN with 3 neighbors), SNN (automatic peak detection 1–25 peaks), and QC (automatic peak detection 1–25 peaks, p-value T-test/ANOVA). The cross validation was performed using the random mode with 50 % leave out and 10 iterations.

### 2.5. Development of a Cytochrome c oxidase subunit 1 (CO1) PCR assay for the molecular discrimination of Hyalomma spp.

Primers were designed to target the CO1 gene sequence of different *Hyalomma* species. Tick species were selected that were expected to occur in Cameroon and Mauritania. Finally, *H. truncatum*, *H. dromedarii*, *H. rufipes, H. impeltatum* and, in addition as the most important representative species in Europe, *H. marginatum* were chosen. This decision was made based on published studies about the local distribution of *Hyalomma* ticks (Apanaskevich and Horak, 2008a, 2008b; Apanaskevich et al., 2008; Apanaskevich and Horak, 2009). Considering differences between CO1 sequences also within a single species (due to genetic diversity, hybridization (Rees et al., 2003) or incorrect morphologic identification) available in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) database, a CO1 alignment was generated for each chosen *Hyalomma* species and the most similar sequence to the consensus sequence was chosen and used for the alignment comparing CO1 genes of different selected *Hyalomma* species. Based on this alignment, primers were designed to amplify a conserved but for each species-specific section. Although the focus was primarily on ticks of the genus *Hyalomma*, a PCR product of the same size was additionally amplified in *A. hebraeum* and *A. variegatum*. For this purpose the following primers were selected: For_Hya_COI-237 5´-TGGTAATGATCAAATTTATAATGTA-3´ and Rev_Hya_COI-1267 5´- CGATCTGTTAATAATATAGTAAT-3´. The predicted size of the amplified fragment is 495 bp. Remaining parts of tick samples after preparation of the legs for MALDI-TOF MS were homogenized together with 300 µl of lysis-buffer Buffer AVL, Qiagen and one 5 mm steel bead in 2 ml Eppendorf Safe-Lock tubes in a Qiagen Tissuelyser II 3 min/30 Hz. The homogenate was clarified by centrifugation for 10 min at 8000 rpm and DNA/RNA extraction was performed with the Viral RNA mini Kit Qiagen as suggested by the manufacturer. For PCR, 2 µl of template DNA and 10 pmol of each primer were used along with DreamTaq DNA Polymerase Kit Thermo Fisher Scientific in a total reaction volume of 20 µl. The PCR reaction was performed with a BioRad C1000 Thermal Cycler Bio-Rad Laboratories under the following conditions: 95 °C for 3 min (initial denaturation), 95 °C for 30 s (denaturation), 50 °C for 30 s (annealing), 72 °C for 40 s (extension) and 72 °C for 15 min (final extension). The amplified PCR products were analyzed by agarose gel (1 %) electrophoresis with a runtime of one hour at 120 V.

### 2.6. Genetic typing by restriction fragment length polymorphism (RFLP)

Sequences of the amplicon of the *Hyalomma*-specific PCR described above were used to identify suitable restriction enzymes sites for genetic typing by RFLP. All samples were sequenced by Sanger sequencing (Eurofins scientific). Using Geneious (Version 11.1.5) software, different combinations of restriction enzymes were tested to achieve a large interspecies variation of the expected fragment pattern in a virtual gel. Combining restrictions enzymes AluI and HinfI revealed a highly diverse fragment pattern for all selected species. The FastDigest Kit (Thermo Fisher Scientific) was used for the restriction digest according to the manufacturer's instruction. The master mix used for one single reaction was 2 µl of Green Buffer (Thermo Fisher Scientific), 1 µl each of AluI and HinfI (New England Biolabs GmbH), 2 µl of DNA template and 12 µl $H_20$. The mixed samples were incubated for 10 min at 37 °C followed by an inactivation step of 10 min at 80 °C. 15 µl of digested DNA master mix were then loaded on a polyacrylamide gel composed of a 12

% resolving gel and a 4 % stacking gel cast according to the Mini-PROTEAN® (Bio-Rad Laboratories) instruction manual, but omitting the addition of SDS (sodium dodecyl sulfate). Fragments were separated at 60 V for 230 min in a Mini-PROTEAN® Tetra System electrophoresis chamber and stained with ethidium bromide for 10 min.

## 3. Results

### 3.1. Tick species identification by MALDI-TOF MS analysis

For the evaluation of MALDI-TOF mass spectra, a reference library containing 92 samples was established using the Biotyper software. The sample pool partly overlapped with the RFLP approach (Table 1), although it was not completely identical. All data sets were queried against the library. The identity scores of the top-ranking hits indicated the correct species for 91/92 samples showing that the Biotyper library was useful for MALDI-TOF MS based identification of the included species. The highest scores calculated for a diverging species were also monitored and added to Supplementary Table 1 as column 'High score (incorrect)' together with the respective species (column 'High-scoring diverging species'). For at least some of the samples belonging to *H. marginatum*, *H. truncatum*, *H. nitidum*, and *H. rufipes*, the differences between the highest scores achieved with representatives of the same and of a differing species in the reference spectra library were only small, indicating that identification based on MALDI Biotyper scores may be problematic for these species. A score-based distance matrix was calculated and the distances between the individual samples were visualized after two-dimensional scaling (Fig. 1). While some species showed well separated clusters like the *Amblyomma* species, representatives of *H. rufipes* and *H. truncatum* appeared more as an unresolved continuum indicating only slight differences of the spectral features used for the calculation of the MALDI Biotyper scores. Note that the *R. sanguineus s. l.* samples only seem to overlap with the *Hyalomma* samples for restrictions in the two-dimensional representation of the distances. For a more detailed view, an interactive three-dimensional version of the Sammon plot is provided as Supplementary Fig. 1. As shown in Fig. 2, numerous identification scores ≥ 1.8 were observed also for diverging *Hyalomma* species and thus ranged above the minimum identification score suggested in previous studies (Yssouf et al., 2013b; Nebbak et al., 2017). It should be noted that for some *H. nitidum*, *H. rufipes* and *H. truncatum* identification score values above 2.0 were also observed in representatives of a different species. To test whether spectra from these species were distinct enough for a reliable species discrimination using a different statistical approach, spectra of the respective *Hyalomma* species were transferred to ClinProTools software and MS based statistical models were calculated for every pair of these species. The models were then validated using a leave-50 %-out cross validation strategy. As shown in Supplementary Table 2 one of the available algorithms offered in ClinProTools (Genetic algorithm) resulted in high cross validation rates between 100 and 94 %, indicating that MS-based separation of the respective *Hyalomma* species was indeed possible. The cross-validation rates obtained with the other available algorithms ranged between 87 % and 100 % and are presented in detail in Supplementary Table 2.

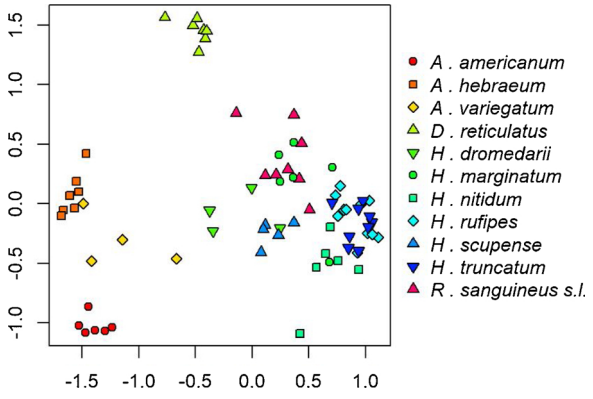


**Fig. 1.** Distances of the spectra from *Amblyomma* species, *Hyalomma* species and two more distinct tick species (*D. reticulatus* and *R. sanguineus s. l.*) based on MALDI Biotyper scores are presented as Sammon plot after two-dimensional scaling.

### 3.2. Hyalomma spp.-specific Cytochrome c oxidase subunit 1 (CO1) PCR assay

A total number of 122 ticks were tested using a newly developed CO1-specific primer pair (Table 1). For 117 CO1 amplicons specific bands of the expected size of 495 bp were observed in the 1 % agarose gel (Fig. 3). In five cases (*H. truncatum*, 1x female; *H. rufipes*, 1x male; *H. scupense*, 1x female; not further determined *Hyalomma* spp., 2 x sex unknown) no specific bands were detected. Table 2 provides an overview of 33 sequenced CO1 amplicons of chosen ticks for an additional verification of the morphologic determination. Sequences were analyzed and compared by using the Blast tool (NCBI). The *H. marginatum* sample MN244329 was identified as *H. turanicum* with 99 % query coverage and 100 % sequence identity. It is important to note that the second most similar alignment was *H. marginatum* with 100 % query coverage and 99.76 % identity. It has already been described that the very close relationship between these two species might lead to genetic misidentification (Apanaskevich, 2002; Zhang and Zhang, 2014; Sands et al., 2017). One sequenced specimen of *H. rufipes* (MN244339) was identified as *H. marginatum*, despite of given morphologic characteristics of *H. rufipes*. Interestingly, all six *H. nitidum* CO1 amplicons were clearly assigned to *H. truncatum* by sequencing.

### 3.3. Restriction fragment length polymorphism (RFLP) as a decision support for species determination of Hyalomma ticks

DNA from 80 individual tick specimen, representing six different *Hyalomma* species, were digested by restriction enzymes (Table 1). An overview of the obtained different types of electrophoretic patterns is depicted in Fig. 3. Four out of five *Hyalomma* species tested were clearly distinguishable. Restriction fragment patterns of *H. truncatum* and *H. nitidum* were completely identical and RFLP did not allow a differentiation between these two species. However, only slight deviations within a species were observed overall. Two distinct but not substantially different digestion patterns were identified in 29 tested *H. truncatum* specimens (Fig. 4a). For reasons of clarity, these variants are referred to as Type 1 (e.g. HT Z0802 in Fig. 4a) and 2 (e.g. HT Z0801 in Fig. 4a) in the following text. With one exception, all of the 33 *H. rufipes* samples showed a uniform gel electrophoresis pattern (Fig. 4b). HR Z0794 (Fig. 4b) was not only totally different from all other *H. rufipes* samples, but was also completely identical to the seven *H. marginatum* samples tested (Fig. 4c). Five *H. dromedarii* ticks (Fig. 4d) were tested and four of them showed identical patterns (with four evenly arranged bands). Sample HD Z0774 exhibited a variation of the first band at the level of the 200 bp marker. The six *H. nitidum* specimens showed the same gel pattern as *H. truncatum* (Fig. 4e) and also differentiated into Type 1 and 2 patterns. Two *H. scupense* samples were tested (Fig. 3) and were identical.

### 3.4. C01 gene sequencing of specimens with unclear RFLP results

For CO1 sequencing, PCR products were generated by the primer pair described in the method section. To clarify the different RFLP patterns of analyzed *H. truncatum* samples, three samples of pattern Type 1 (HT Z0801, HT Z0808, HT Z0809) and Type 2 (HT Z0802, HT Z0803, HT Z0806) were sequenced. All six sequenced CO1 amplicons, regardless of Type 1 or 2, were clearly classified as *H. truncatum* with an

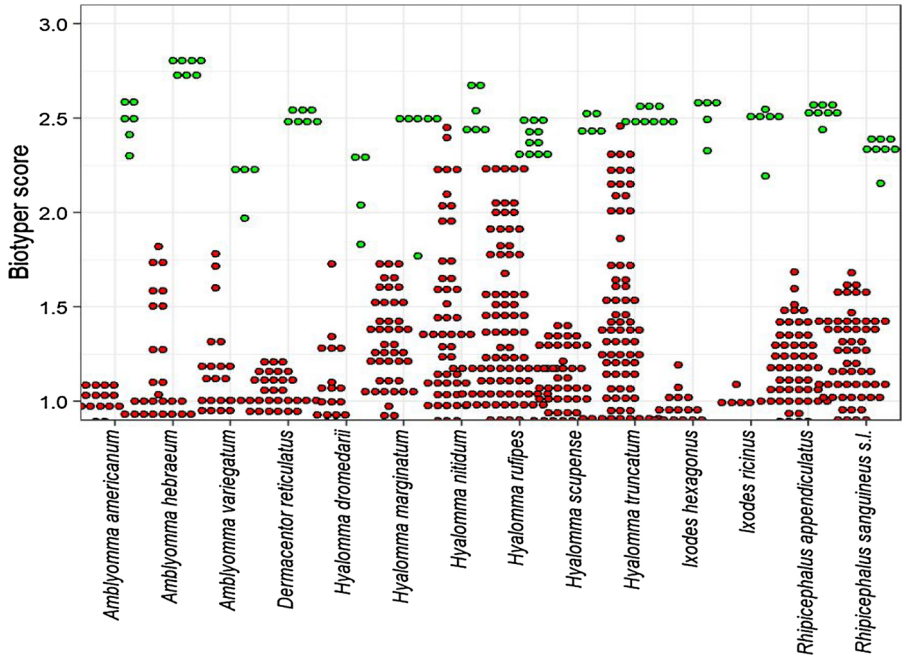


**Fig. 2.** MALDI Biotyper identification scores. The scores obtained for a representative of the same species as given on the x-axis are in green, scores with differing species are in red.

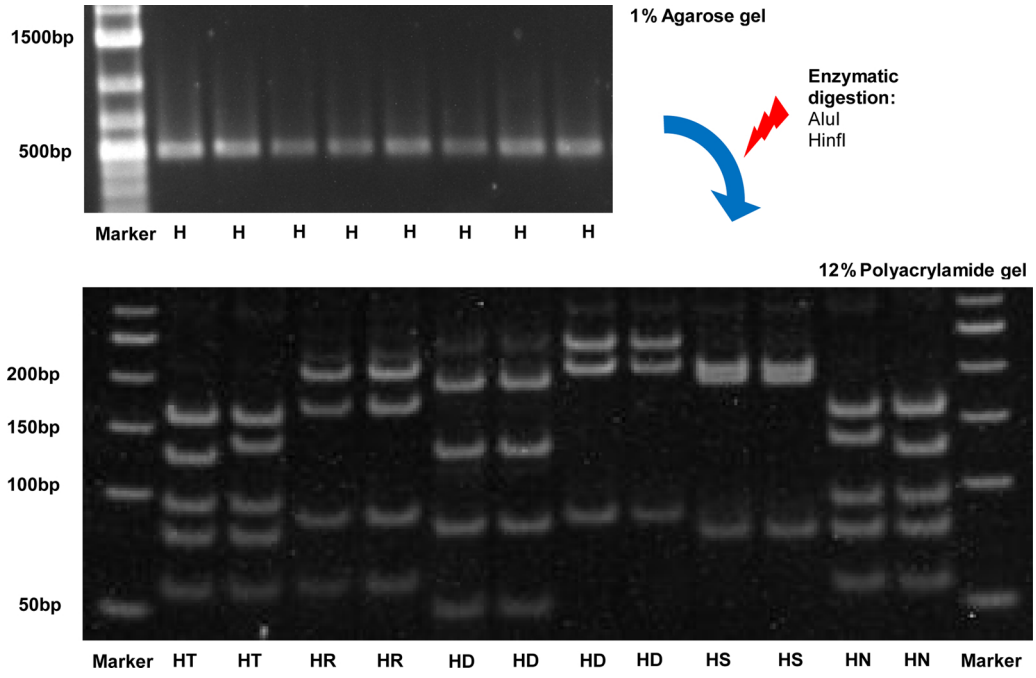


**Fig. 3.** Restriction enzyme digest patterns of different *Hyalomma* species CO1 amplicons before and after enzymatic digestion. H: *Hyalomma* sp.; HT: *H. truncatum*; HR: *H. rufipes*; HD: *H.dromedarii*; HM: *H. marginatum*; HS: *H. scupense*; HN: *H. nitidum.*

identity range of 99%–100%. The specific 495 bp segment amplified by our primer pair was identified in the *H. truncatum* reference sequences in GenBank. This sequence was aligned with the sequences produced from HT Z0801, HT Z0808 HT Z0809 (Type 1) and HT Z0802, HT Z0803 and HT Z0806 (Type 2). It was shown that an additional HinfI restriction site in samples of Type 1 in the interval between 328 and 332 bp is responsible for the slight gel pattern shift. This results in an additional approximately 10 bp digestion product, which cannot be detected in the RFLP due to its small size. Several sequences with the same deviating restriction sites as Type 1 and 2 do exist in the NCBI. *H. rufipes* sample HR Z0794 (MN244339) deviating in Fig. 4b was sequenced along with eight other *H. rufipes* samples as comparisons. All ticks were previously identified as *H. rufipes* due to clear morphologic characteristics. However, according to the gel pattern HR Z0794 seemed to represent *H. marginatum* (for comparison see Fig. 4c). Querying the GenBank revealed identification of HR Z0794 as *H. marginatum* with a sequence identity of 100 % including the same positions of restriction sites. All other specimens were confirmed unambiguously as *H. rufipes* (Table 2). In contrast to these results, all samples were determined as *H. rufipes* by MALDI-TOF MS analysis. The sequencing of the CO1 PCR product of HD Z0774 (MN244323) in Fig. 4d revealed *H. dromedarii* with an identity of 99 % (Table 2). Furthermore, an

**Table 2**
Overview of 33 Hyalomma ticks identified by CO1 gene sequencing.

| Species | GenBank accesion no. | MALDI TOF-MS reference | Origin | GenBank Identity | GenBank Identification |
|---|---|---|---|---|---|
| *H. dromedarii* | MN244321 | Z0775 | MRT | 100 % | *H. dromedarii* |
| | MN244322 | Z0776 | MRT | 99 % | *H. dromedarii* |
| | MN244323 | Z0774 | MRT | 99 % | *H. dromedarii* |
| | MN244324 | Z0779 | MRT | 99 % | *H. dromedarii* |
| *H. marginatum* | MN244325 | Z0853 | LC | 99 % | *H. marginatum* |
| | MN244326 | Z0855 | LC | 99 % | *H. marginatum* |
| | MN244327 | Z0856 | LC | 99 % | *H. marginatum* |
| | MN244328 | Z0870 | CRO | 100 % | *H. marginatum* |
| | MN244329 | – | CRO | 100 % | *H. turanicum* |
| | MN244330 | – | CRO | 100 % | *H. marginatum* |
| *H. nitidum* | MN244331 | Z0859 | CMR | 99 % | *H. truncatum* |
| | MN244332 | Z0860 | CMR | 99 % | *H. truncatum* |
| | MN244333 | Z0861 | CMR | 100 % | *H. truncatum* |
| | MN244334 | Z0862 | CMR | 99 % | *H. truncatum* |
| | MN244335 | Z0857 | CMR | 99 % | *H. truncatum* |
| | MN244336 | Z0858 | CMR | 99 % | *H. truncatum* |
| *H. rufipes* | MN244337 | Z0792 | CMR | 100 % | *H. rufipes* |
| | MN244338 | Z0793 | CMR | 100 % | *H. rufipes* |
| | MN244339 | Z0794 | CMR | 100 % | *H. marginatum* |
| | MN244340 | Z0795 | CMR | 99 % | *H. rufipes* |
| | MN244341 | Z0796 | CMR | 100 % | *H. rufipes* |
| | MN244342 | Z0797 | CMR | 100 % | *H. rufipes* |
| | MN244343 | Z0798 | CMR | 100 % | *H. rufipes* |
| | MN244344 | Z0799 | CMR | 100 % | *H. rufipes* |
| | MN244345 | Z0800 | CMR | 100 % | *H. rufipes* |
| *H. truncatum* | MN244348 | Z0801 | CMR | 100 % | *H. truncatum* |
| | MN244349 | Z0802 | CMR | 100 % | *H. truncatum* |
| | MN244350 | Z0803 | CMR | 99 % | *H. truncatum* |
| | MN244351 | Z0806 | CMR | 100 % | *H. truncatum* |
| | MN244352 | Z0808 | CMR | 100 % | *H. truncatum* |
| | MN244353 | Z0809 | CMR | 99 % | *H. truncatum* |
| *H. scupense* | MN244346 | Z0788 | CRO | 100 % | *H. scupense* |
| | MN244347 | Z0789 | CRO | 99 % | *H. scupense* |

MRT = Mauritania; CMR = Cameroon; CRO = Croatia; LC = laboratory Colony.

additional AluI restriction site between 121 and 124 bp was detected caused by a substitution from guanine to adenine leading to the fragmentation of the 180 bp band into two 85–90 bp segments (clearly recognizable by the prominent double band just below the 100 bp marker in Fig. 4d). Since at least two further sequences with the same additional restriction site exist in GenBank (KU130591 and GQ483461), it can be assumed that this was not an accidental mutation. In total, six individual specimens of *H. nitidum* were sequenced. With an identity range of 99–100 %, all of them matched with *H. truncatum* and were not distinguishable. In MALDI-TOF MS, however, a slight but measurable difference between the two species was observed.

## 4. Discussion

CCHFV vector competence of various tick species is still enigmatic and necessitate urgent clarification. A conclusive statement whether a species is vector competent for a given pathogen requires very complex animal infection studies (Gargili et al., 2017) with multiple parameters to be considered (Gargili et al., 2013; Thangamani and Bente, 2014). Studies based on ticks which were collected from animals whilst feeding or from the environment have been published. However, as these may have incorporated blood from viremic animals without intrinsic virus amplification, such studies can only be indicative in terms of vector competence.

This study assessed alternative species determination methods such as RFLP pattern (plus facultative sequencing) and MALDI-TOF MS which are also suitable for large numbers of field samples. While morphologic species identification may be the fastest and most economical method, it requires well-trained tick experts in order to avoid incorrect species identification, as there is large variation of morphologic phenotypes within a single species. In most cases, hybridization (Rees et al., 2003) and different genetic clades (Cangi et al., 2013) cannot be detected by morphological determination either. DNA sequencing is the best way to perform phylogenetic analyses, but is less suitable for high throughput screening of samples.

As described in literature (Yssouf et al., 2013a; Kumsa et al., 2016; Rothen et al., 2016; Diarra et al., 2017; Boucheikhchoukh et al., 2018) MALDI-TOF MS analyses based on tick leg proteins can be a well suited screening method with a relatively easy sample preparation. All six *Hyalomma* species studied here were well separable by both MALDI Biotyper score-based analyses (Figs. 1 and 2) as well as by ClinProTools multivariate statistics (Supplementary Table 2) with only few misidentifications. An optimization of the statistical models with ClinProTools (Supplementary Table 2) or freely available statistical tools (Karger, 2016a; 2016b) seems feasible, however, this approach would require higher sample numbers coming from a wider range of geographical locations. It was proven for *H. rufipes* that there are at least two different spatially separated genetic clades (Cangi et al., 2013). Also, slight spectral differences within the same species may be caused by environmental influences, as it was shown for *I. ricinus* (Karger et al., 2019). *H. marginatum* was the only *Hyalomma* species of which field samples and specimens from laboratory colonies were available. While the ticks from laboratory colonies differed significantly from the other *Hyalomma* species, the field samples were more difficult to classify, i.e. could not always be clearly assigned to *H. marginatum*. Finally, only one *H. marginatum* field sample was included in the MALDI-TOF MS reference database (Z0870) and it produced comparatively low scores with the laboratory-reared ticks of the same species. However, it is so far unclear whether spectra of *H. marginatum* that were reared in the laboratory or collected in the field differ systematically. In contrast, field samples of *H. dromedarii* and *H. scupense* could be very well differentiated by MALDI-TOF MS (Fig. 2). *H. truncatum* and *H. nitidum* were more difficult to distinguish - as expected given their uncertain taxonomic classifications at present. While *H. truncatum* is widespread in Africa, *H. nitidum* is only found in humid areas of West and Central Africa (Tomassone et al., 2005). So far, these two species have been distinguished by morphologic features and there is only limited information about their genetic background. Presently, it is not known whether *H. nitidum* is an independent species or only a geographical variation of *H. truncatum* (Apanaskevich and Horak, 2008b).

In conclusion, MALDI-TOF MS performs well for tick identification. The per-sample costs for reagents are low, while the availability of costly mass spectrometers may limit its application. In addition, samples collected in the field always carry a certain risk of contamination such as dust, dirt and foreign protein, which may influence the quality of the spectra.

RFLP can serve as an alternative molecular method for the differentiation of species when there is no access to sequencing tools. As for MALDI-TOF MS, a high sample throughput is possible. Moreover, it may be more suitable for field studies (e.g. in Africa) than MALDI-TOF MS, as only simple laboratory equipment is sufficient. *H. rufipes* could be very well distinguished from the other species displaying a typical fragment pattern. However, sample HR Z0794 raised questions. It was determined as *H. rufipes* by morphologic and MALDI-TOF MS analyses, whereas the results of RFLP and sequencing indicated *H. marginatum*. There is at least one other sequence in the NCBI sequence data base (JX049279) in which a *H. rufipes* specimen appears to have a *H. marginatum* genotype. Since *H. marginatum* does not occur in sub-Saharan Africa (Apanaskevich and Horak, 2008a) and has not yet been described in Cameroon, hybridization seems unlikely. We included this species in the study as it is the main vector of CCHFV in Europe/Mediterranean basin (Bente et al., 2013). Sample Z0794 showed that MALDI-TOF MS, in combination with morphologic identification, can help to distinguish genetically questionable ticks. Although only five *H. dromedarii* were tested in the RFLP with one slightly deviating sample

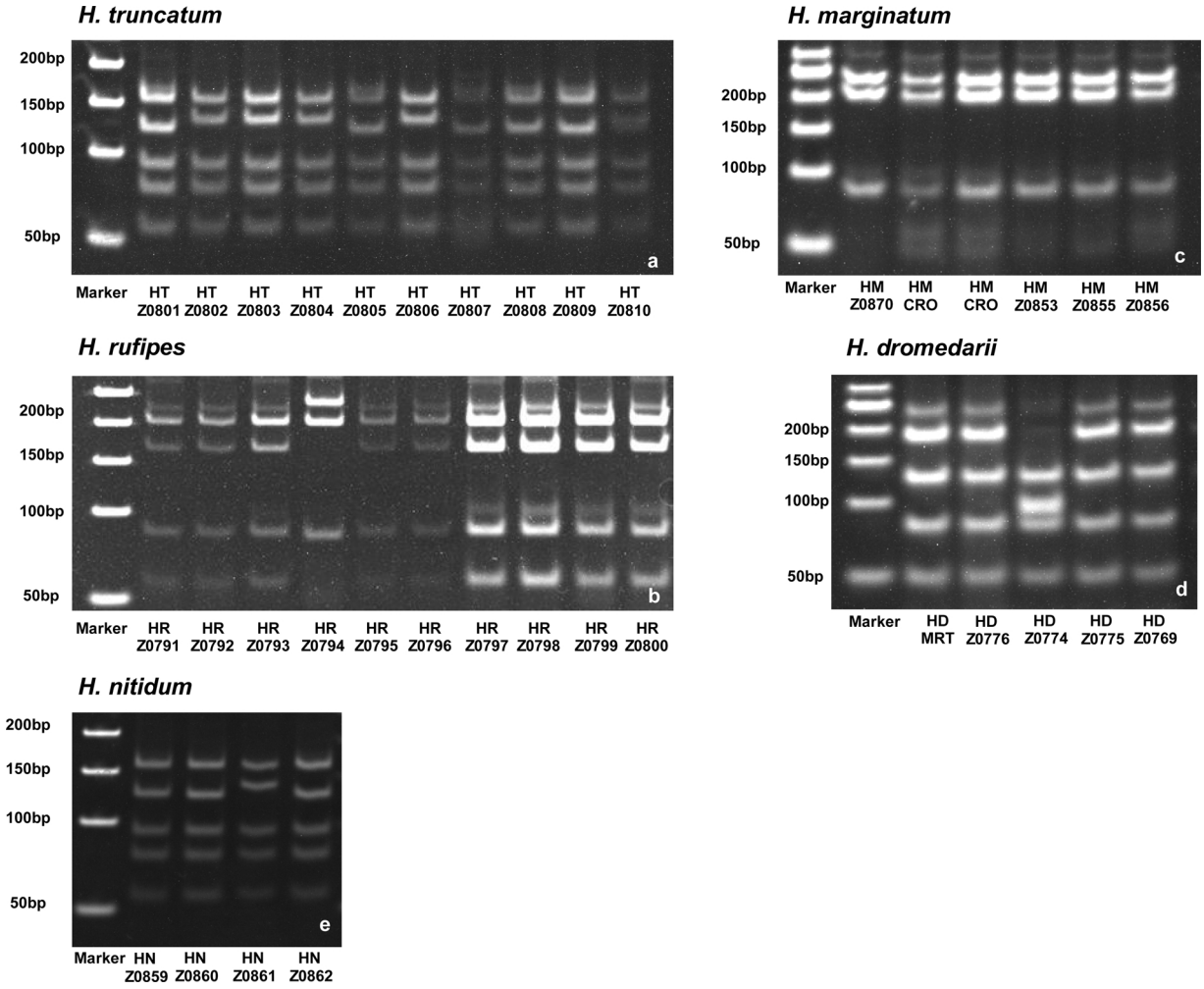


**Fig. 4.** Restriction enzyme digest patterns of *Hyalomma* species, which had been used for reference spectra in MALDI-TOF MS (12 % polyacrylamide gel). Despite *H. truncatum* und *H. nitidum* all species could be well distinguished by RFLP. HT: *Hyalomma truncatum*; HR: *H. rufipes*; HM: *H. marginatum*; HD: *H. dromedarii;* HN: *H. nitidum.*

(HD Z0774), the results seem to be reliable. *H. scupense* could be distinguished from other species by RFLP, but the primary focus of this work was on African *Hyalomma* ticks and only few specimens were available. The findings of *H. truncatum* and *H. nitidum* must be considered separately. Both species obviously cannot be distinguished genetically by the CO1 sequence. Neither RFLP nor sequence comparison using the Blast tool (NCBI) yielded conclusive results, while the MALDI-TOF MS analyses showed a noticeable difference between the species. None of our six *H. nitidum* sequences, and neither the three sequences (KU130613, KU130614 and KU130615) available in NCBI, differed distinctly from *H. truncatum*. However, the study of Sands et al. (2007), which compared almost all known *Hyalomma* species by using five different marker genes (CO1, 28 s, 16 s, ITS II and H3), was not able to resolve this complicated taxonomic situation between *H. truncatum* and *H. nitidum* either. More genetic investigations are required in order to provide a more meaningful statement about the relationship between these two species.

## 5. Conclusion

All three speciation approaches, morphological determination, MALDI-TOF MS and RFLP showed advantages and disadvantages, whereby no method as stand-alone can guarantee a 100 % correct result. Using MALDI-TOF, all *Hyalomma* species could be distinguished, including even the two phylogenetically questionable species *H. truncatum* and *H. nitidum*. It is a reliable screening method that allows a high sample throughput. RFLP can be a useful auxiliary tool for molecular identification of selected *Hyalomma* species, especially if there is no possibility of sequencing.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

## CRediT authorship contribution statement

**A. Schulz:** Conceptualization, Methodology, Investigation, Visualization, Data curation, Validation, Writing - original draft. **A. Karger:** Visualization, Methodology, Investigation, Software, Validation, Formal analysis, Writing - review & editing. **B. Bettin:** Visualization, Methodology, Investigation, Software, Validation. **A. Eisenbarth:** Writing - review & editing, Conceptualization. **M.A. Sas:** Writing - review & editing, Supervision. **C. Silaghi:** Writing - review & editing, Resources, Supervision. **M.H. Groschup:** Conceptualization, Resources, Funding acquisition, Project administration, Supervision, Writing - review & editing.

## Acknowledgement

We thank Dr. Relja Beck (Croatian Veterinary Institute, Zagreb) for providing the ticks samples from Croatia, Samuel Abah (Laboratoire National Vétérinaire du Cameroun, Ngaoundéré, Cameroon) for the ticks from Cameroon, Yahya Barry (Office National de Recherche et de Développement de l'Elevage, Nouakchott, Mauritania) for the ticks from Mauritania and IS Insect Services GmbH, Berlin (Germany) together with MSD Animal Health Innovation GmbH, Schwabenheim (Germany), for the laboratory-reared ticks.

Special thanks go to Dr. Lidia Chitimia-Dobler (Institut für Mikrobiologie der Bundeswehr, Munich, Germany), for help with morphological determination of *Hyalomma* ticks and to René Schöttner (FLI) for technical support. This study was co-funded by the German Federal Foreign Office (German Partnership Program for Biosecurity), by the Deutsche Forschungsgemeinschaft (grant number: GR 980/4-1) as well as by the European Union (LEAP-AGRI LEARN project (ID 222).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101382.

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