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ORIGINAL ARTICLE

Molecular Characterization of Hard Ticks by Cytochrome *c* Oxidase Subunit 1 Sequences

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Abstract: Although widely studied, the natural diversity of the hard tick is not well known. In this study, we collected 194 sequences from 67 species, covering 7 genera of hard tick. The 5' region of the mitochondrial cytochrome *c* oxidase subunit 1 region (586 bp) has been used to investigate intra- and inter-species variation and the phylogenetic tree of neighbor joining method has been used for assessment. As a result, by comparing the K2P-distance of intra- and interspecies, 30 samples (15.2%) shown that interspecies distance was larger than the minimum interspecfic distance. From the phylogenetic analysis, 86.8% (49) of the species were identified correctly at the genus level. On deeper analysis on these species suggested the possibility of presence cryptic species. Therefore, further work is required to delineate species boundaries and to develop a more complete understanding of hard tick diversity over larger scale.

Key words: *CO1*, hard tick, divergence, phylogenetic analyses

INTRODUCTION

Hard ticks are obligate ectoparasites, and seem to be second in importance only to mosquitoes as vectors of human and animal diseases [1]. Tick-borne diseases cause a huge loss to the livestock industry and increase the risk of disease such as Lyme disease, babesiosis, human granulocytic ehrlichiosis, forest encephalitis, spotted fever, anaplasmosis, and Crimean–Congo hemorrhagic fever [2-4]. All species are exclusively hematophagous in all feeding stages. Hard ticks are distributed worldwide with their hosts range from wild to domestic vertebrates except fishes.

Traditionally, classifications and phylogenetic inferences for Ixodidae were based on morphological, biological and ecological characteristics, often suggesting host specificity as the main factor [5,6]. However, methods for species determination are limited when taxa are morphologically very similar, specimens are damaged, and in frequent cases where immature stages are not described or are engorged [7].

Molecular systematics offered new possibilities to improve species recognition in hard ticks. ITS, 18S rDNA, 28S rDNA and other mitochondrial rDNA genes have been used to study these organisms and have played an important role in analyzing the classification and phylogenetics of hard ticks [8-10]. However, compared to the number of species of hard ticks, the extent of these studies are very limited [11].

Until recently, there has been little effort to standardize the methods for molecular identification of hard ticks, and no one gene has been formally selected as an admitted DNA marker to deal with problems of classification and phylogenetics in hard ticks. So, we chose the mitochondrial cytochrome *c* oxidase subunit 1 (*CO1*) gene fragment as a candidate molecular marker, and collected 194 samples (from 67 species of 7 genera) of hard ticks. Intra- and interspecies genetic divergences were assessed using the Kimura 2-parameter (K2P) distance model. Phylogenetic tree were performed to analyse their relationship at evolutionary level.

MATERIALS AND METHODS

Sample collection

Ticks used in this study were collected from field sites and different hosts in various regions of China (Table 1). After morphological identification, ticks were stored in 100% ethanol and conserved at 4˚C. Only male and unfed adult specimens were used.

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Table 1. Details of 36 samples collected from China in this study

Genus	Species	Time	Locality	Source
Hyalomma	*Hya. dromedarii*	Sep. 2010	Gansu	Camel
	Hya.anatolicum anatolicum	Unknown	Gansu	Unknown
	Hya. detritum	Unknown	Inner Mongolia	Unknown
	Hya. asiaticum asiaticum	Unknown	Inner Mongolia	Ground
	Hya. asiaticum asiaticum	Jun. 2010	Xinjiang	Cattle
	Hya. asiaticum	Jun. 2011	Gansu	Camel
	Hya. rufipes	Jul. 2010	Gansu	Goat
Dermacentor	*D. silvarum*	Apr. 2010	Gansu	Sheep
	D. silvarum	Apr. 2010	Gansu	Goat
	D. silvarum	Apr. 2010	Gansu	Sheep
	D. silvarum	May. 2011	Gansu	Sheep
	D. silvarum	May. 2011	Gansu	Sheep
	D. everestianus	May. 2011	Xizang	Sheep
	D. niveus	Jun. 2011	Xizang	Sheep
Rhipicephalus	*R. microplus*	Jun. 2011	Gansu	Cattle
	R. microplus	Jun. 2010	Guizhou	Cattle
	R. sanguinens	May. 2010	Guangxi	Dog
	R.haemaphysaloides haemaphysaloides	Jun. 2011	Sichuan	Goat
	R. turanicus	May. 2010	Xinjiang	Sheep
Haemaphysalis	*H. longicornis*	May. 2011	Anhui	Goat
	H. longicornis	Sep. 2010	Henan	Sheep
	H. longicornis	Unknown	Gansu	Sheep
	H. longicornis	May. 2010	Hubei	Sheep
	H. longicornis	Jun. 2011	Gansu	Sheep
	H. longicornis	May. 2010	Zhejiang	Sheep
	H. qinghaiensis	Apr. 2010	Gansu	Sheep
	H. qinghaiensis	May. 2010	Gansu	Sheep
	H. qinghaiensis	May. 2011	Gansu	Sheep
	H. qinghaiensis	Jun. 2011	Qinghai	Ground
	H. qinghaiensis	Jun. 2011	Qinghai	Sheep
	H. qinghaiensis	May. 2008	Gansu	Ground
	H. flava	Sep. 2010	Henan	Sheep
Ixodes	*I. persulcatus*	Jun. 2011	Xinjiang	Sheep

DNA extraction, PCR amplification, and sequencing of *CO1*

DNA was extracted from the ticks using a tissue kit (Qiagen AG, Basel,Switzerland) according to the manufacturer's instructions. Each sample was cut with sterile scissors within a 1.5 ml microtube. After digestion with proteinase K (20 mg/ml), the samples were applied to the columns for DNA absorption and washing. Finally, the DNA was eluted in 100 ml of eluting buffer provided in the kit and stored at -20˚C. The primers used for PCR were LCO1490 (5′-GGTCAACAAATCATAAAGATA- TTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [12]. The 5′ region of *CO1* was amplified using the following thermal cycling program: 94˚C for 5 min, 35 cycles at 94˚C for 1 min, 53˚C for 1 min, and 72˚C for 1 min, followed by a final extension at 72˚C for 8 min. PCR products were purified using a PCR purification kit (Takara, Shiga, Japan). Sequencing reactions were resolved on automated DNA sequencer.

Data from GenBank

Some *CO1* sequences from the hard ticks were downloaded from GenBank. Sequences < 500 bp in length, with ambiguous bases (more than 15 'Ns'), or those belonging to unnamed species (sequences with 'sp.' in the species name) were removed from the analysis. In addition, we checked all the sequences using BLAST analysis (*E*-value < 0.001) to make sure that there were no host sequences in our data. The selected sequences were used to construct analysis datasets.

Sequence analysis

The *CO1* sequences were translated into amino acids with the program MEGA 4.0 in order to exclude sequencing errors and to avoid the inclusion of pseudogene sequences in the datasets. The sequences were aligned using ClustalW [13], and genetic distances were computed using MEGA 4.0 according to the K2P distance model. The maximal/mean/minimum intra- and interspecies distances were used to represent species

level divergence. Meanwhile, the maximal/mean/minimum intra-and intergenus distances were calculated to evaluate the genus level variation. Then a neighbor joining (NJ) tree was constructed and the genetic K2P distance was calculated within species and genera using MEGA 4.0. Evaluation of statistical confidence was based on 1,000 non-parametric bootstrap replicates. One soft tick isolate was used as the outgroup of phylogenetic tree.

RESULTS

Data acquisition

We collected 194 samples (36 from this study, 158 from GenBank) from 67 species and 7 genera of hard ticks (Table 1 and Supplementary Table S1). The mitochondrial *CO1* region of all samples collected in China was successfully amplified using PCR. The length of the PCR product was 707 bp. As some sequences of the *CO1* gene obtained from GenBank were shorter than 707 bp, all sequences were aligned with a consensus length of 586 bp, and no insertions, deletions, or stop codons were observed in any sequence. The sequences acquired in this study have been deposited in the GenBank database with accession numbers JQ737066-JQ737128.

Genetic divergence and gap

Using the K2P model, sample divergences at various taxonomic levels are shown in Tables 2 and 3. As expected, the genetic divergence increased with higher taxonomic ranking: 0.001±0.001 to 0.016±0.003 at intraspecies level, 0.002±0.001 to 0.248±0.023 at interspecies level, 0.005±0.002 to 0.175±0.011 at intragenus level, and 0.186±0.012 to 0.243±0.016 at intergenus level. The *Bothriocroton* showed the lowest mean intraspecies divergence (0.005±0.002), while *Rhipicephalus* showed the highest mean intraspecies divergence (0.062±0.039) (Fig. 1). The largest ratio between the average intra- and interspecies divergence was in the *Ixodes* with a 7.5-fold difference, and the lowest ratio was in the *Dermacentor* with a 2.4-fold difference. As shown in Fig. 1, there was not a distinct gap between the distribution of the intra- and interspecies divergence. The overlapping regions were mainly distributed in the *R. turanicus, Hya. dromedarii, D. marginatus, D. silvarum*, and *A. testudinarium*.

Phylogenetic tree

The NJ tree of the overall analysis is shown in Fig. 2. The phylogenetic relationship at the genus level was well resolved

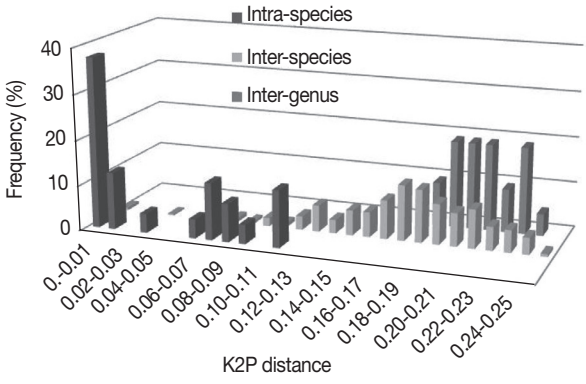

Fig. 1. Frequency distribution of genetic K2P-distances in a 586 bp segment of the *CO1* gene in Ixodidae at species and genus level.

Table 2. Measures of inter- and intra-species divergences for *CO1* sampled in 7 genera of Ixodidae

	Intra-species distance			Inter-species distance		
	Minimum	Mean	Maximum	Minimum	Mean	Maximum
Hyalomma	0.004±0.002	0.039±0.046	0.110±0.010	0.035±0.006	0.113±0.027	0.155±0.017
Dermacentor	0.003±0.001	0.050±0.042	0.084±0.008	0.002±0.001	0.122±0.058	0.179±0.016
Haemaphysalis	0.008±0.002	0.033±0.042	0.016±0.003	0.150±0.016	0.175±0.021	0.191±0.019
Bothriocroton	0.005±0.002	0.005±0.002	0.005±0.002	0.000±0.000	0.000±0.000	0.000±0.000
Rhipicephalus	0.014±0.004	0.062±0.039	0.116±0.015	0.051±0.010	0.156±0.028	0.207±0.020
Amblyomma	0.002±0.002	0.057±0.077	0.112±0.010	0.147±0.016	0.177±0.028	0.206±0.018
Ixodes	0.001±0.001	0.026±0.043	0.077±0.010	0.094±0.017	0.196±0.030	0.248±0.023

Table 3. Measures of inter- and intragenus divergences for *CO1* sampled in family Ixodidae

	Intra-genus distance			Inter-genus distance		
	Minimum	Mean	Maximum	Minimum	Mean	Maximum
Ixodidae	0.005±0.002	0.118±0.056	0.175±0.011	0.186±0.012	0.211±0.017	0.243±0.016

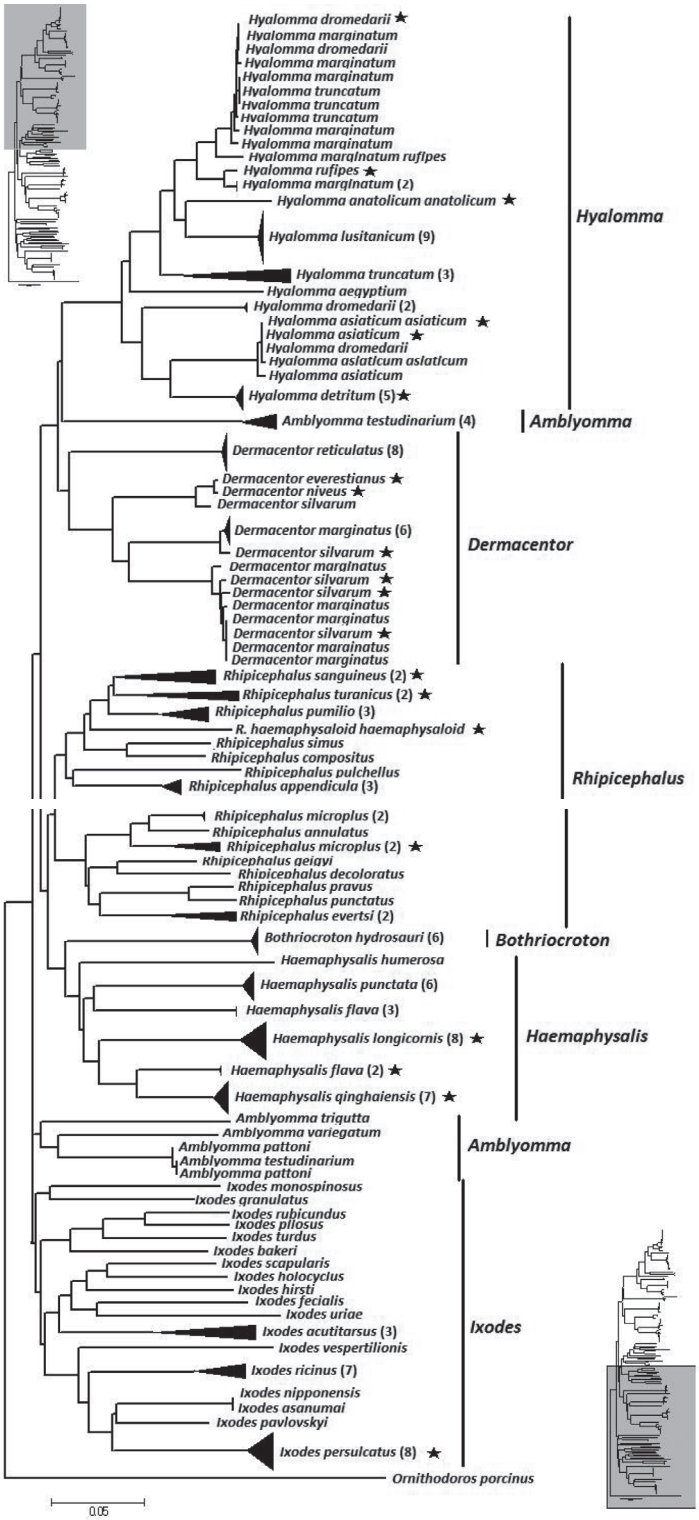

Fig. 2. Neighbor-joining tree of 194 isolates from the family Ixodidae and related species. The tree is constructed with 586 bp of *CO1*. Bracketed numbers represent the number of isolates sequenced for each species. Asterisk represent samples collected from China in this study.

with the exception of *Amblyomma*. From the tree, *Hyalomma*, *Dermacentor*, *Amblyomma*, and *Rhipicephalus* formed 1 clade. *Bothriocroton* and *Haemaphysalis* formed another clade. *Ixodes* as distinct difference at morphous to other hard ticks, formed a third clade. However, at a species level, 9 species (13.4%) did not form a monophyletic group. They were *Hya. dromedarii*, *Hya. marginatum*, *Hya. asiaticum asiaticum*, *D. marginatus*, *D. silvarum*, *A. testudinarium*, *R. microplus*, and *H. flava*.

DISCUSSION

In this study, the mean sequence divergence in hard ticks (0.197 ± 0.063) is higher than that observed in other organisms [14-16]. Such high values of genetic distance reflect possible biological diversity within the Ixodidae. Such as the distance between *Amblyomma testudinarium* (HM193893) and *A. testudniarium* (HM193895) was 0.112 ± 0.010, and they were in different clades of the phylogenetic tree. However, *Rhipicephalus microplus* and *Dermacentor marginatus* also gave similar results. The reason may be geographic variation or comprise cryptic species [17]. Additionally, the distance between the species *Dermacentor everestianus* (JQ737079) and *D. niveus* (JQ737080) was only 0.004 ± 0.002, and also formed into 1 clade. Therefore, these analyses might indicate hybridization or a misidentification among these species.

The *CO1* gene appears to be an informative molecular marker on several taxonomic scales, but particularly at the species level [18]. Our analysis shows a general increase in the molecular divergence of *CO1* with taxonomic rank. The diversity within species is especially high, with a maximum of 0.116 ± 0.015. It makes *CO1* suitable for investigating intraspecies variation. DNA barcoding assumes that the genetic distances between species are greater than within species. In that way, clusters of similar sequences represent species, clearly separated from other clusters (species) [19]. However, there also 30 samples where the maximum interspecies distance was larger than the minimum interspecfic distance. This means that the gap might be absent in these samples because of insufficient variation between them [20,21]. From the NJ phylogenetic tree, nine of the 67 species (13.4%) examined in this study (*Hya. dromedarii*, *Hya. marginatum*, *Hya. asiaticum asiaticum*, *Hya. truncatum*, *D. marginatus*, *D. silvarum*, *A. testudinarium*, *R. microplus*, and *H. flava*.) did not form a monophyletic group. *Hya. asiaticum asiaticum* and *Hya. dromedarii* shared similar morphologic characters from capitulum, scutum, Haller's organ, peritrematal plate, the first caruncle, coax and spur of feet of adults and larval stages. Ecologically, these 2 species also share the same desert intertidal area. They are 2 different species proved by previous studies [22-24]. However, they formed one clade in this study. This phenomenon was also found for other hard ticks. For example, *Hya. dromedarii*, *Hya. marginatum* and *Hya. truncatum* formed a complex clade. These results agreed with some studies using mt 12S rDNA, 16S rDNA or ITS, in which *Hyalomma* spp. shown high divergence distance and low bootstrap value [25,26]. As many results indicated that there is a high diversity in hard ticks [27,28].

This study provides that using the *CO1* gene is a potential tool for species identification in Ixodidae. However, it is inadequate to use a single mitochondrial gene (*CO1*) for DNA taxonomy. Therefore, an integrative approach is needed to combine nuclear and mitochondrial genes, morphological characters, and ecological information into further studies of hard ticks.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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Supplementary Table S1. The taxa and GenBank accession of 194 hard ticks used in this study

Taxon	Locality	GeneBank accessions
Amblyomma limbatum	Australian	FJ584434
Amblyomma limbatum	Australian	FJ584430
Amblyomma limbatum	Australian	FJ584435
Amblyomma limbatum	Australian	FJ584433
Amblyomma limbatum	Australian	FJ584429
Amblyomma pattoni	China	HM193875
Amblyomma pattoni	China	HM193876
Amblyomma testudinarium	China	HM193895
Amblyomma testudinarium	China	HM193893
Amblyomma testudinarium	China	HM193894
Amblyomma testudinarium	China	HM193892
Amblyomma trigutta	Japan	AB113317
Amblyomma variegatum	Senegal	GU062743
Bothriocroton hydrosauri	Australian	FJ584426
Bothriocroton hydrosauri	Australian	FJ584424
Bothriocroton hydrosauri	Australian	FJ584422
Bothriocroton hydrosauri	Australian	FJ584427
Bothriocroton hydrosauri	Australian	FJ584425
Bothriocroton hydrosauri	Australian	FJ584423
Dermacentor albipictus	Canada	GU968842
Dermacentor everestianus	China	JQ737079
Dermacentor marginatus	Romania	FN394327
Dermacentor marginatus	Romania	FN394331
Dermacentor marginatus	Romania	FN394332
Dermacentor marginatus	Romania	FN394330
Dermacentor marginatus	Romania	FN394328
Dermacentor marginatus	China	HM193891
Dermacentor marginatus	China	HM193889
Dermacentor marginatus	China	HM193887
Dermacentor marginatus	China	HM193890
Dermacentor niveus	China	JQ737080
Dermacentor reticulatus	China	HM193885
Dermacentor reticulatus	China	HM193883
Dermacentor reticulatus	China	HM193881
Dermacentor reticulatus	China	HM193879
Dermacentor reticulatus	China	HM193886
Dermacentor reticulatus	China	HM193884
Dermacentor reticulatus	China	HM193882
Dermacentor silvarum	China	JQ737075
Dermacentor silvarum	China	JQ737076
Dermacentor silvarum	China	JQ737077
Dermacentor silvarum	China	JQ737078
Dermacentor silvarum	China	JQ737081
Dermacentor steini	China	HM193861
Haemaphysalis concinna	China	EU670047
Haemaphysalis flava	China	JQ737097
Haemaphysalis flava	China	HM193864
Haemaphysalis flava	China	HM193865
Haemaphysalis flava	Japan	AB075954
Haemaphysalis flava	China	JF758632
Haemaphysalis humerosa	Australian	AF132819
Haemaphysalis longicornis	China	JQ737087

(*Continued to the next page*)

Supplementary Table S1. Continued

Taxon	Locality	GeneBank accessions
Haemaphysalis longicornis	China	JQ737090
Haemaphysalis longicornis	China	JQ737091
Haemaphysalis longicornis	China	JQ737092
Haemaphysalis longicornis	China	JQ737093
Haemaphysalis longicornis	China	JQ737096
Haemaphysalis longicornis	Australian	AF132820
Haemaphysalis longicornis	China	EU670048
Haemaphysalis longicornis	China	JF758631
Haemaphysalis longicornis	China	JF758635
Haemaphysalis punctata	Romania	FN394335
Haemaphysalis punctata	Romania	FN394336
Haemaphysalis punctata	Romania	FN394337
Haemaphysalis punctata	Romania	FN394338
Haemaphysalis punctata	Romania	FN394339
Haemaphysalis punctata	Romania	FN394340
Haemaphysalis punctata	Romania	FN394340
Haemaphysalis qinghaiensis	China	JQ737088
Haemaphysalis qinghaiensis	China	JQ737089
Haemaphysalis qinghaiensis	China	JQ737094
Haemaphysalis qinghaiensis	China	JQ737095
Haemaphysalis qinghaiensis	China	JQ737098
Haemaphysalis qinghaiensis	China	JQ737099
Haemaphysalis qinghaiensis	China	JQ737100
Hyalomma aegyptium	Belgium	AF132821
Hyalomma anatolicum anatolicum	China	JQ737067
Hyalomma asiaticum	China	JQ737072
Hyalomma asiaticum	China	JQ737073
Hyalomma asiaticum asiaticum	China	JQ737070
Hyalomma asiaticum asiaticum	China	JQ737071
Hyalomma detritum	China	JQ737068
Hyalomma detritum	China	JQ737069
Hyalomma detritum	Unknow	EU827695
Hyalomma detritum	Unknow	EU827696
Hyalomma detritum	Unknow	EU827694
Hyalomma dromedarii	China	JQ737066
Hyalomma dromedarii	Egypt	AF132822
Hyalomma dromedarii	Ethiopia	AJ437082
Hyalomma dromedarii	Ethiopia	AJ437080
Hyalomma dromedarii	Ethiopia	AJ437062
Hyalomma lusitanicum	Unknow	EU827739
Hyalomma lusitanicum	Unknow	EU827737
Hyalomma lusitanicum	Unknow	EU827735
Hyalomma lusitanicum	Unknow	EU827697
Hyalomma lusitanicum	Unknow	EU827699
Hyalomma lusitanicum	Unknow	EU827701
Hyalomma lusitanicum	Unknow	EU827703
Hyalomma lusitanicum	Unknow	EU827705
Hyalomma lusitanicum	Unknow	EU827742
Hyalomma marginatum	Unknow	EU827693
Hyalomma marginatum	Unknow	EU827692
Hyalomma marginatum	Ethiopia	AJ437100
Hyalomma marginatum	Ethiopia	AJ437098

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Supplementary Table S1. Continued

Taxon	Locality	GeneBank accessions
Hyalomma marginatum	Ethiopia	AJ437096
Hyalomma marginatum	Ethiopia	AJ437094
Hyalomma marginatum	Ethiopia	AJ437097
Hyalomma marginatum rufipes	Ethiopia	AF132823
Hyalomma rufipes	China	JQ737074
Hyalomma truncatum	Ethiopia	AF132824
Hyalomma truncatum	Ethiopia	AJ437090
Hyalomma truncatum	Ethiopia	AJ437088
Hyalomma truncatum	Ethiopia	AJ437086
Hyalomma truncatum	Ethiopia	AJ437084
Hyalomma truncatum	Ethiopia	AJ437089
Ixodes acutitarsus	Japan	AB105166
Ixodes acutitarsus	China	HM193862
Ixodes acutitarsus	China	HM193896
Ixodes asanumai	Japan	AB231674
Ixodes bakeri	South African	GU437873
Ixodes cornuatus	Australia	FJ571511
Ixodes fecialis	Australia	FJ571509
Ixodes granulatus	Unknow	AB231673
Ixodes granulatus	China	JF758633
Ixodes hirsti	Australia	FJ571510
Ixodes holocyclus	Japan	AB075955
Ixodes lividus	United Kingdom	GU124743
Ixodes monospinosus	Japan	AB231672
Ixodes nipponensis	Japan	AB231671
Ixodes ovatus	Japan	AB231670
Ixodes pavlovskyi	Japan	AB231669
Ixodes persulcatus	China	HM193868
Ixodes persulcatus	China	HM193870
Ixodes persulcatus	China	HM193872
Ixodes persulcatus	China	HM193867
Ixodes persulcatus	China	HM193869
Ixodes persulcatus	China	HM193871
Ixodes persulcatus	China	JF758629
Ixodes persulcatus	Japan	AB073725
Ixodes philipi	Japan	AB231663
Ixodes philipi	Japan	AB231665
Ixodes philipi	Japan	AB231664
Ixodes philipi	Japan	AB231666
Ixodes pilosus	South African	GU437874
Ixodes ricinus	France	GU074940
Ixodes ricinus	France	GU074942
Ixodes ricinus	France	GU074944
Ixodes ricinus	France	GU074946
Ixodes ricinus	France	GU074948
Ixodes ricinus	France	GU074950
Ixodes ricinus	Romania	FN394342
Ixodes rubicundus	South African	GU437875
Ixodes scapularis	USA	GU074891
Ixodes turdus	Japan	AB231668
Ixodes uriae	Japan	AB087746
Ixodes vespertilionis	Japan	AB231667

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Supplementary Table S1. Continued

Taxon	Locality	GeneBank accessions
Ixodida persulcatus	China	JQ737101
Ixodiphagus hookeri	France	JQ315225
Rhipicephalus annulatus	Israel	AF132825
Rhipicephalus appendicula	Zimbabwe	AF132833
Rhipicephalus appendicula	Rwanda	DQ901363
Rhipicephalus appendicula	Rwanda	DQ901361
Rhipicephalus appendicula	Rwanda	DQ901359
Rhipicephalus appendicula	Rwanda	DQ901357
Rhipicephalus appendicula	Rwanda	DQ901362
Rhipicephalus appendicula	Rwanda	DQ901360
Rhipicephalus appendicula	Rwanda	DQ901356
Rhipicephalus appendicula	Rwanda	DQ901358
Rhipicephalus compositus	Zimbabwe	AF132834
Rhipicephalus decoloratus	Kenya	AF132826
Rhipicephalus evertsi	Kenya	AF132835
Rhipicephalus evertsi	Namibia	AF132836
Rhipicephalus geigyi	Unknow	AY008680
Rhipicephalus haemaphysaloid haemaphysaloid	China	JQ737085
Rhipicephalus maculatus	Australia	AY008681
Rhipicephalus microplus	Australia	AF132827
Rhipicephalus microplus	China	JQ737082
Rhipicephalus microplus	China	JQ737083
Rhipicephalus microplus	China	JF758636
Rhipicephalus microplus	China	JF758630
Rhipicephalus microplus	China	HM193863
Rhipicephalus pravus	Zimbabwe	AF132837
Rhipicephalus pulchellus	Australia	AY008682
Rhipicephalus pumilio	China	HM193877
Rhipicephalus pumilio	China	HM193878
Rhipicephalus pumilio	Australia	AY008684
Rhipicephalus punctatus	South Africa	AF132838
Rhipicephalus sanguinens	China	JQ737084
Rhipicephalus sanguineus	China	JF758634
Rhipicephalus sanguineus	China	HM193873
Rhipicephalus sanguineus	Egypt	AF132839
Rhipicephalus simus	Turkey	AF132840
Rhipicephalus turanicus	China	JQ737086
Rhipicephalus turanicus	South Africa	AF132841