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Genetic variation in mitochondrial genes of the tick *Haemaphysalis flava* collected from wild hedgehogs in China

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Abstract The tick *Haemaphysalis flava* (Acari: Ixodidae) is an important ectoparasite, which causes direct damage to their hosts and also acts as a vector of various infectious disease agents in China. Despite its significance, the epidemiology, genetics and biology of *H. flava* has not been studied in detail. In the present study, the genetic variation in three mitochondrial (mt) DNA regions, namely cytochrome *c* oxidase subunit 1 (*cox*1) and NADH dehydrogenase subunit 1 and 4 (*nad*1 and *nad*4), was examined in *H. flava* ticks collected from wild hedgehogs in China. A portion of *cox*1 (p*cox*1), *nad*1 (p*nad*1) and *nad*4 (p*nad*4) genes were PCR amplified from individual *H. flava* ticks and the amplicons were sequenced. The length of the sequences of p*cox*1, p*nad*1 and p*nad*4 were 849, 285 and 626 bp, respectively. The intra-specific sequence variation within *H. flava* was 0–0.4% for p*cox*1, 0–0.4% for p*nad*1 and 0–0.3% for p*nad*4. However, the inter-specific variation was significantly higher, 12.5–14.3%, 13.6–24.8% and 14.8–19% for p*cox*1, p*nad*1 and p*nad*4, respectively. Phylogenetic analysis based on Maximum likelihood (ML) method using the combined target mt gene sequences confirmed that all isolates of *Haemaphysalis* were *H. flava*. The molecular approach employed in this study provides a tool for further elucidating the molecular diversity of *H. flava* in China and elsewhere in Asia.

Keywords *Haemaphysalis flava* · Genetic variation · Mitochondrial DNA · Phylogenetic analysis

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Introduction

Ticks, as obligate hematophagous ectoparasites, play a significant role in the transmission of various pathogens, such as viruses, protozoa, fungi, bacteria and helminths, affecting humans and animals health worldwide (Silaghi et al. 2016; Benelli et al. 2016). The tick *Haemaphysalis flava* is widely prevalent in many countries and regions and feeds on blood from many wild and domestic animals (Andoh et al. 2013; Lu et al. 2013). This tick transmits various disease agents (*Francisella tularensis*, *Rickettsia* and *Ehrlichia*) and causes significant vector-borne diseases (encephalitis, lyme borreliosis and spotted fever), posing a huge threat to the health of both humans and animals (Xu et al. 2016).

Mitochondrial (mt) DNA sequences have been proven useful and reliable genetic markers due to their maternal inheritance, fast rate of evolutionary change and relatively conserved genome structures than nuclear ribosomal genome (Blouin 2002). mtDNA sequences can provide valuable markers for investigating population genetic structures, systematics and phylogenetics of ticks. For example, mt 16S ribosomal RNA gene sequences have been shown as useful genetic markers for the identification and differentiation of *Dermacentor nuttalli* (Kulakova et al. 2014). mt *cox*1 and 12S ribosomal RNA gene sequences are useful genetic markers for studying intra-specific variation of *Rhipicephalus appendiculatus* (Kanduma et al. 2016). mt *cox*1 gene sequences are new and useful markers for studying the population genetic variations in *Ixodes holocyclus* (Song et al. 2011). mt 16S ribosomal RNA and *cox*1 gene sequences are useful markers for studying of the genetic polymorphism of *I. persulcatus* and *I. pavlovskyi* tick populations (Livanova et al. 2015). Although genetic variation in a number of ticks have been studied, there is a paucity of information on sequence variation among populations of *H. flava* of socio-economic significance.

The objectives of the present study were to examine genetic variation in three mtDNA genes, namely cytochrome *c* oxidase subunits 1 (*cox*1) and NADH dehydrogenase subunits 1 and 4 (*nad*1 and *nad*4), among *H. flava* isolates from wild hedgehogs in China. Based on the combined sequences of these three mtDNA regions, phylogenetic relationships of *H. flava* with other two *Haemaphysalis* species were also re-constructed.

Materials and methods

Parasites and DNA extraction

All adult ticks of *H. flava* (n = 20) were obtained from wild hedgehogs in Henan and Hunan provinces of China (Table 1). These samples were fixed in 70% (v/v) ethanol and stored at −20 °C until use. Total genomic DNA was extracted from individual samples using sodium SDS/proteinase K treatment, followed by spin column purification (TIANamp Genomic DNA Purification System, TIANGEN) and eluted into 50 μL H_2O according to the manufacturer's recommendations.

Enzymatic amplification and sequencing

The primer sets (Table 2) for amplifying mt *cox*1, *nad*1 and *nad*4 were designed based on well-conserved mt sequences of *H. flava* (NC_005292) (Shao et al. 2004). PCR reactions (25 μL) were performed in 3.0 μL of $MgCl_2$ (25 mM), 0.25 μL of each primer (50 pmol/

Table 1 Geographical origins in China of *Haemaphysalis flava* samples used in the present study, as well as their GenBank accession numbers for sequences of partial mitochondrial cytochrome *c* oxidase subunit 1 gene (p*cox*1), and NADH dehydrogenase subunits 1 and 4 genes (p*nad*1 and p*nad*4)

Sample codes	Geographical origin	GenBank accession number		
		*cox*1	*nad*1	*nad*4
HF1	Henan (Xinyang)	KY003181	KY065299	KY065319
HF2	Henan (Xinyang)	KY021801	KY065300	KY065320
HF3	Henan (Xinyang)	KY021802	KY065301	KY065321
HF4	Henan (Xinyang)	KY021803	KY065302	KY065322
HF5	Henan (Xinyang)	KY021804	KY065303	KY065323
HF6	Henan (Xinyang)	KY021805	KY065304	KY065324
HF7	Henan (Xinyang)	KY021806	KY065305	KY065325
HF8	Henan (Xinyang)	KY021807	KY065306	KY065326
HF9	Henan (Xinyang)	KY021808	KY065307	KY065327
HF10	Henan (Xinyang)	KY021809	KY065308	KY065328
HF11	Hunan (Changde)	KY021810	KY065309	KY065329
HF12	Hunan (Changde)	KY021811	KY065310	KY065330
HF13	Hunan (Changde)	KY021812	KY065311	KY065331
HF14	Hunan (Changde)	KY021813	KY065312	KY065332
HF15	Hunan (Changde)	KY021814	KY065313	KY065333
HF16	Hunan (Changde)	KY021815	KY065314	KY065334
HF17	Hunan (Changde)	KY021816	KY065315	KY065335
HF18	Hunan (Changde)	KY021817	KY065316	KY065336
HF19	Hunan (Changde)	KY021818	KY065317	KY065337
HF20	Hunan (Changde)	KY021819	KY065318	KY065338

Table 2 Sequences of primers used to amplify a portion of the cytochrome *c* oxidase subunit 1 gene (p*cox*1), NADH dehydrogenase subunit 1 and 4 genes (p*nad*1 and p*nad*4) from *Haemaphysalis flava*

Name of primer	Sequence (5′–3′)
For p*cox*1	
cox1 F	GGAACAATATATTTAATTTTTGG
cox1 R	ATCTATCCCTACTGTAAATATATG
For p*nad*1	
nad1 F	AAGCCCATCCTCTTCACA
nad1 R	TTGGGATATTGTCACATTCG
For p*nad*4	
nad4 F	CATAATACCCAGCCTTCTCC
nad4 R	ATGACTCCCAAAGGCTCA

μL), 2.5 μL 10 × rTaq buffer (100 mM Tris–HCl and 500 mM KCl), 2 μL of dNTP Mixture (2.5 mM each), 0.25 μL of r*Taq* (5 U/μL) DNA polymerase (TaKaRa Biotechnology, Dalian, China) and 2 μL of DNA sample in a thermocycler (Biometra, Göttingen, German). The cycling conditions were: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 s (denaturation), 53–56 °C for 30 s (annealing), 72 °C for 1 min

(extension) and then 72 °C for 5 min (final extension). Negative control (without DNA template) was included in each amplification run. Each amplicon (5 μL) was examined by 1% (w/v) agarose gel electrophoresis to validate amplification efficiency. PCR products were sent to BGI-Shenzhen (Shenzhen, China) for sequencing from both directions.

Sequences analysis and reconstruction of phylogenetic relationships

Sequences of the three mt genes were separately aligned using the software Clustal X 1.83 (Thompson et al. 1997). The level of sequence differences (D) among *H. flava* isolates were calculated by pairwise comparisons using the formula $D = 1-(M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton et al. 1995). The haplotypes, nucleotide diversity (Pi) and haplotype diversity (Hd) of each gene were determined using the DnaSP 5.0 program (Librado and Rozas 2009).

The combined sequences of p*cox*1, p*nad*1 and p*nad*4 of all tick samples in this study were used for phylogenetic analyses. Maximum likelihood (ML) was used for phylogenetic re-constructions. ML analyses were performed using PhyML 3.0 (Guindon et al. 2010), and the GTR + I model with its parameter for the concatenated dataset was determined for the ML analysis using JModeltest (Posada 2008) based on the Akaike information criterion (AIC). Bootstrap support (BS) for ML trees was calculated using 100 bootstrap replicates. To study the phylogenetic relationships with other *Haemaphysalis* species, *H. formosensis* (NC_020334) (Burger et al. 2013), *H. parva* (NC_020335) (Burger et al. 2013) and *H. flava* (NC_005292) (Shao et al. 2004) were considered into the present study, with *Bothriocroton concolor* (NC_017756) as the outgroup. Phylograms were drawn using the Tree View program version 1.65 (Page 1996).

Results and discussion

Genetic variation is widespread in tick populations, and the accurate analysis of genetic variation in ticks has important implications for studying epidemiology, population genetics and biology of ticks. Advances in molecular genetics and bioinformatic methods are providing unique opportunities to explore the biology of ticks. mtDNA markers are receiving more attention for this purpose than nuclear DNA (e.g., internal transcribed spacer) due to high mutation rate (Blouin 2002). However, given the different evolutionary rates occurring in different gene regions of tick mtDNA and the high degree of conservation of the *cox*1 gene (Guo et al. 2016). Therefore, in the present study, three mt markers (*cox*1, *nad*1 and *nad*4) were combined to further explore the genetic variation of the tick *H. flava*.

Amplicons of p*cox*1, p*nad*1 and p*nad*4 (approximately 900, 330 and 670 bp, respectively) were amplified individually and subjected to agarose gel electrophoresis. For each mtDNA region, in no case was product amplified from no DNA sample or host DNA control (not shown). The sequences of p*cox*1, p*nad*1 and p*nad*4 were 849, 285 and 626 bp in size, respectively. There were no difference in the lengths of any of the three p*cox*1, p*nad*1 and p*nad*4 sequences among *H. flava* from different geographic origins. These sequences have been deposited in the GenBank database (Table 1). The A + T contents of the sequences were 60.6–62.6% (p*cox*1), 56.3–60.6% (p*nad*1) and 67–73.6% (p*nad*4),

respectively. Five haplotypes, nucleotide diversity (Pi = 0.00092) and haplotype diversity (Hd = 0.653) were determined among p*cox*1 sequences. Two haplotypes, nucleotide diversity (Pi = 0.00118) and haplotype diversity (Hd = 0.337) were determined among p*nad*1 sequences. Three haplotypes, nucleotide diversity (Pi = 0.00046) and haplotype diversity (Hd = 0.279) were determined among p*nad*4 sequences. The intra-specific sequence variations among different populations of *H. flava* isolates were 0–0.4% for p*cox*1, 0–0.4% for p*nad*1 and 0–0.3% for p*nad*4, while the inter-specific sequence differences among members of the *Haemaphysalis* were significantly higher, being 2.5–14.3%, 13.6–24.8% and 14.8–19% for p*cox*1, p*nad*1 and p*nad*4. These studies have clearly indicated that mt *cox*1, *nad*1 and *nad*4 gene sequences provide reliable genetic markers for specific identification and differentiation of *H. flava*. These results were consistent with that of previous studies (Chitimia et al. 2010; Mangold et al. 1998).

Many studies have demonstrated that mtDNA sequences are valuable genetic markers for phylogenetic studies of different groups of parasites, including ticks (Song et al. 2011; Liu et al. 2013; Burger et al. 2014). In the present study, phylogenetic analyses of the combined sequences of p*cox*1, p*nad*1 and p*nad*4 among 20 individual *H. flava* isolates from China, 3 other ticks including *B. concolor* as outgroup, using ML is shown in Fig. 1. In this tree, the *H. flava* form monophyletic group with high statistical support (BS = 100), and all the *H. flava* isolates were more closely related to *H. formosensis* than to *H. parva*.

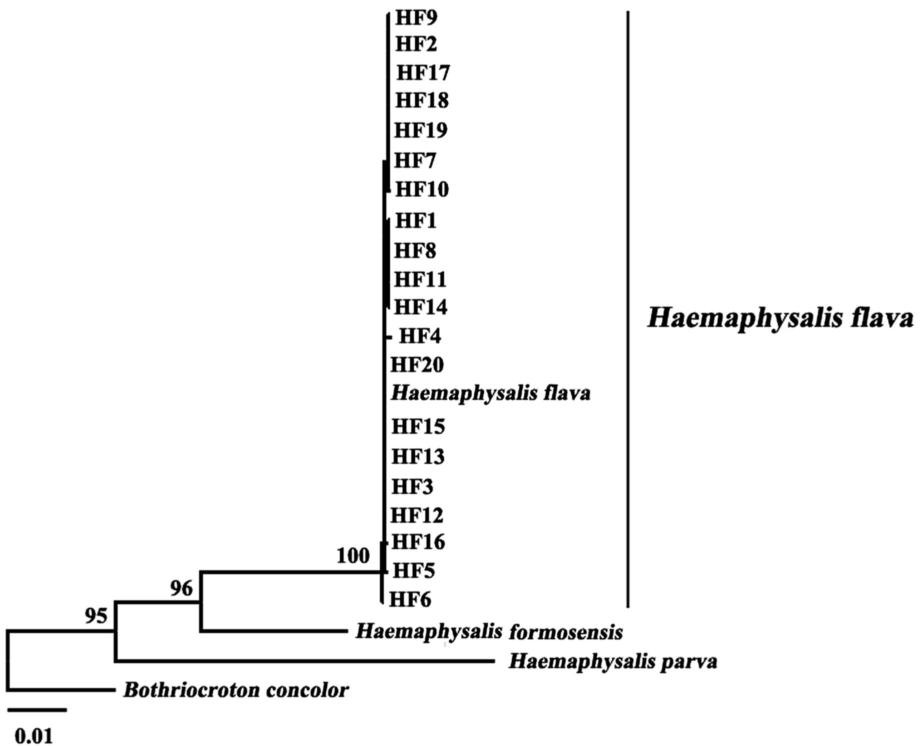

Fig. 1 Phylogenetic relationship among *Haemaphysalis flava* isolates in China with other *Haemaphysalis* species inferred by maximum likelihood analyses using the combined dataset (*cox*1 + *nad*1 + *nad*4), with *Bothriocroton concolor* (NC_017756) as outgroup

Our results were consistent with those of previous studies (Burger et al. 2013; Williams-Newkirk et al. 2015).

Our study presents the first attempt to characterize genetic variation in mt *cox*1, *nad*1 and *nad*4 genes of *H. flava* isolates from wild hedgehogs in China, but we believe it is still necessary to carry out more experimental research. Future studies could (1) examine population structure using larger number of samples from different hosts and geographical locations, (2) employ other more variable molecular markers, (3) detailed morphological re-description of these ticks *H. flava.*

In conclusion, sequence variations among *H. flava* isolates from different geographical localities in China were revealed by sequence analyses of mt *cox*1, *nad*1 and *nad*4 genes. The molecular approach employed provides a powerful tool for elucidating the epidemiology, genetics and biology of *H. flava* in China and elsewhere in Asia.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The performance of this study was strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and our protocol was reviewed and approved by the Research Ethics Committee of Hunan Agricultural University.

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