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ORIGINAL PAPER

Mitochondrial Gene Heterogeneity and Population Genetics of *Haemaphysalis longicornis* (Acari: Ixodidae) in China

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Abstract

Introduction *Haemaphysalis longicornis* is an important ectoparasite of domestic and wild animals that can transmit many pathogens including viruses, fungi, bacteria and protozoa.

Materials and methods In this study, we examined genetic variation and population genetics in three mitochondrial (mt) genes [*cox*1 (cytochrome *c* subunit 1), *rrn*L (large subunit ribosomal RNA) and *nad*5 (NADH dehydrogenase 5)] among four *H. longicornis* populations from China.

Results The sizes of the partial sequences of *cox*1, *rrn*L and *nad*5 were 776 bp, 409 bp, 510 bp, respectively. Among the obtained sequences, we identified 22 haplotypes for *cox*1, 2 haplotypes for *rrn*L and 17 haplotypes for *nad*5. Low gene flow and significant genetic differentiation (66.2%) were detected among *H. longicornis* populations. There was no rapid expansion event in the demographic history of four *H. longicornis* populations in China. In addition, phylogenetic analyses confirmed that all the *Haemaphysalis* isolates were *H. longicornis* which were segregated into two major clades.

Conclusion The mt DNA genes provide a potential novel genetic marker for molecular epidemiology of *H. longicornis* and assist in the control of tick and tick-borne diseases in humans and animals.

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

Zhong-Bo Li and Yi-Tian Fu contributed equally to this work and should be considered as co-first authors.

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Introduction

Ticks, as important ectoparasites, infect a broad range of animals and humans. They may also act as vectors of a range of pathogens (viruses, bacteria, fungi and protozoa) causing human and animal disease [3, 34, 36]. There are currently 896 species of ticks in the 3 families: the Ixodidae (hard ticks), the Argasidae (soft ticks), and the Nuttalliellidae. The Ixodidae is the most important family of veterinary and medical significance consisting of 702 species in 14 genera [15].

Haemaphysalis longicornis belongs to the Ixodidae and is distributed in Australia, New Zealand and eastern Asia [17]. This hard tick infests a variety of hosts (such as pigs, sheet, goat, cattle and dog), and causes lesions, dermatitis, weight loss, blood loss and even death [22]. More importantly, *H. longicornis* is a vector of many pathogens, such as *Anaplasma* spp., *Babesia ovata*, *Ehrlichia canis* and *Rickettsia conorii* [23, 30, 37, 38]. In China, *H. longicornis* is distributed in almost all the provinces or regions and is the dominant tick species in wild and domestic animals, causing major economic losses of livestock [31].

Mitochondrial DNA (mtDNA genes) sequences are preferred and reliable molecular markers for studying genetic diversity and phylogenetics in hard ticks [4, 18, 24, 25], given the advantages of mtDNA as a marker in the molecular research of species [14]. For example, mt *cox*1 (cytochrome *c* subunit 1) is a useful genetic marker for the identification and differentiation of ticks within the genus *Rhipicephalus* [26]. A recent study showed that mt genes can be used as standard genetic markers in discerning the genetic assemblages of *R. microplus* [27]. Furthermore, mt gene sequences are useful markers for studying genetic variation and phylogenetics of *H. flava* and *R. sanguineus* sensu lato tick populations [5, 25]. However, limited information is available about mt gene sequences of hard tick *H. longicornis* [6, 7]. Therefore, the objectives of the present study were to examine genetic variation and population genetics in three mt genes [*cox*1, *rrn*L (large subunit ribosomal RNA) and *nad*5 and *nad*5 (NADH dehydrogenase 5)] among *H. longicornis* isolates in China and to infer the phylogenetic relationships of *H. longicornis* with other *Haemaphysalis* ticks.

Materials and Methods

Parasites

All adult ticks of *H. longicornis* ($n=49$) were obtained from different hosts (goat: *Capra hircus*; hedgehog: *Erinaceus europaeus* and cattle: *Bos taurus*) and four provinces in China (Table 1). The goat, hedgehog and cattle were fed and tick specimens were collected by the veterinarians. The ticks were repeatedly washed in physiological saline, fixed in 70% (v/v) ethanol and stored at − 20 °C until use. These tick samples were preliminarily identified based on host preference and morphological characters [9].

DNA Extraction, Genotyping and DNA Sequencing

Total genomic DNA was isolated from individual ticks using sodium dodecyl sulfate/proteinase K treatment, followed by spin column purification (Wizard® SV Genomic DNA Purification System, Promega, Madison, Wisconsin, USA). The primer sets (Table 2) were designed based on well-conserved mt sequences of *H. longicornis* (KY172954) and *H. flava* (NC_005292). PCR reactions (25 µL) were performed in 3.0 µL of $MgCl_2$ (25 mM), 0.25 µL of each primer (50 pmol/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, Germany). The cycling conditions were: 95 °C for 5 min (initial denaturation), followed by 40 cycles of 95 °C for 30 s (denaturation), 55 °C for 1 min (annealing), 72 °C for 2 min (extension) and then 72 °C for 5 min (final extension). Negative control (without DNA template) was included in each amplification run. PCR products were visualized by electrophoresis in 1% (w/v) agarose gel to validate amplification efficiency. PCR products were sent to Sangon Company (Shanghai, China) for sequencing from both directions.

Sequence Analysis and Reconstruction of Phylogenetic Relationships

The sequences were aligned with the available mt gene sequences (such as *H. longicornis* and *H. flava*) using the software MAFFT 7.122 [20]. Genetic diversity values, including polymorphic sites, A + T contents, haplotype number, haplotype diversity, average number of nucleotide differences, Tajima's *D*, Fu's *Fs* tests, mismatch distribution and nucleotide diversity of each gene were calculated using DnaSP v5.0 [28]. Genetic differentiation within and among four different populations was estimated using analysis of molecular variance (AMOVA) implemented in Arlequin v3.5 [10]. Gene flow (*N*m) among four populations was calculated as follows: (1-*F*st)/2*F*st using DnaSP v5.0 for 1000 permutations.

Phylogenetic trees inferred from the combined three mt gene datasets were constructed using the maximum likelihood (ML) method. In addition, *H. formosensis* (NC_020334), *H. parva* (NC_020335) and *H. flava* (NC_005292) were also included in the present study, with *Ixodes pavlovsky*i (NC_023831) as the outgroup. Phylogenetic analyses were performed using PhyML 3.0 [16]. The best fitting model with its parameter (GTR + I+G) of these sequence datasets was determined using JModeltest [32] based on the Akaike information criterion (AIC). ML analyses were checked on the basis of 100 bootstrap replicates (Br). Phylograms were drawn using FigTree v.1.31 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and Discussion

The mt *cox*1, *rrn*L and *nad*5 gene regions were amplified and sequenced individually from 49 *H. longicornis* samples that obtained sequences which were deposited in GenBank database (Table 1). The lengths of the mt sequences of *cox*1, *rrn*L and *nad*5 were 776 bp, 409 bp and 510 bp, respectively. The 49 mt sequences were closely related to *H. longicornis* mt sequences, and three mt gene regions (*cox*1, *rrn*L and *nad*5) had more than 98.0% identity to previously published mt sequences for *H. longicornis* from Australia and China (GenBank accession nos. AF132820, KU986720 and FN394350, respectively).

Table 1 The GenBank accession numbers for the three mtDNA sequences of 49 isolates *Haemaphysalis longicornis* from different hosts and geographical origins in China

Sample codes	Geographical origin	Host	Year	Sex	Season	GenBank accession number		
						*Cox*1	*Rrn*L	*Nad*5
HLHN1	Hunan (Changsha)	Goat	2017	Female	Spring	MF666880	MF666970	MF666969
HLHN2	Hunan (Changsha)	Goat	2017	Male	Spring	MF666881	MF666971	MF666968
HLHN3	Hunan (Changsha)	Goat	2017	Female	Spring	MF666882	MF666972	MF666967
HLHN4	Hunan (Changsha)	Goat	2017	Female	Spring	MF666883	MF666973	MF666966
HLHN5	Hunan (Changsha)	Goat	2017	Male	Spring	MF666884	MF666974	MF666965
HLHN6	Hunan (Changsha)	Goat	2017	Male	Spring	MF666885	MF666975	MF666964
HLHN7	Hunan (Changsha)	Goat	2017	Male	Spring	MF666886	MF666976	MF666963
HLHN8	Hunan (Changsha)	Goat	2017	Male	Spring	MF666887	MF666977	MF666962
HLHN9	Hunan (Changsha)	Goat	2017	Male	Spring	MF666888	MF666978	MF666961
HLHN10	Hunan (Changsha)	Goat	2017	Female	Spring	MF666889	MF666979	MF666960
HLHN11	Hunan (Changsha)	Goat	2017	Female	Spring	MF666890	MF666980	MF666959
HLHN12	Hunan (Changsha)	Goat	2017	Female	Spring	MF666891	MF666981	MF666958
HLHN13	Hunan (Changsha)	Goat	2017	Female	Spring	MF666892	MF666982	MF666957
HLHN14	Hunan (Changsha)	Goat	2017	Female	Spring	MF666893	MF666983	MF666956
HLHN15	Hunan (Changsha)	Goat	2017	Female	Spring	MF666894	MF666984	MF666955
HLSD16	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666895	MF666985	MF666954
HLSD17	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666896	MF666986	MF666953
HLSD18	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666897	MF666987	MF666952
HLSD19	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666898	MF666988	MF666951
HLSD20	Shangdong (Taian)	Hedgehog	2014	Female	Summer	MF666899	MF666989	MF666950
HLSD21	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666900	MF666990	MF666949
HLSD22	Shangdong (Taian)	Hedgehog	2014	Female	Summer	MF666901	MF666991	MF666948
HLSD23	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666902	MF666992	MF666947
HLSD24	Shangdong (Taian)	Hedgehog	2014	Female	Summer	MF666903	MF666993	MF666946
HLSD25	Shangdong (Taian)	Hedgehog	2014	Female	Summer	MF666904	MF666994	MF666945
HLSD26	Shangdong (Taian)	Hedgehog	2014	Female	Summer	MF666905	MF666995	MF666944
HLSD27	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666906	MF666996	MF666943
HLSD28	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666907	MF666997	MF666942
HLSD29	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666908	MF666998	MF666941
HLSD30	Shangdong (Taian)	Hedgehog	2014	Male	Summer	MF666909	MF666999	MF666940
HLHeN31	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666910	MF667000	MF666939
HLHeN32	Henan (Xinyang)	Hedgehog	2014	Female	Autumn	MF666911	MF667001	MF666938
HLHeN33	Henan (Xinyang)	Hedgehog	2015	Female	Autumn	MF666912	MF667002	MF666937
HLHeN34	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666913	MF667003	MF666936
HLHeN35	Henan (Xinyang)	Hedgehog	2013	Male	Autumn	MF666914	MF667004	MF666935
HLHeN36	Henan (Xinyang)	Hedgehog	2013	Male	Autumn	MF666915	MF667005	MF666934
HLHeN37	Henan (Xinyang)	Hedgehog	2013	Male	Autumn	MF666916	MF667006	MF666933
HLHeN38	Henan (Xinyang)	Hedgehog	2013	Male	Autumn	MF666917	MF667007	MF666932
HLHeN39	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666918	MF667008	MF666931
HLHeN40	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666919	MF667009	MF666930
HLHeN41	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666920	MF667010	MF666929
HLHeN42	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666921	MF667011	MF666928
HLHeN43	Henan (Xinyang)	Hedgehog	2013	Male	Autumn	MF666922	MF667012	MF666927
HLHeN44	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666923	MF667013	MF666926
HLHeN45	Henan (Xinyang)	Hedgehog	2013	Female	Autumn	MF666924	MF667014	MF666925
HLYN46	Yunnan (Chuxiong)	Cattle	2013	Male	Autumn	MH024516	MH024508	MH024512
HLYN47	Yunnan (Chuxiong)	Cattle	2013	Male	Autumn	MH024517	MH024509	MH024513

Table 1 (continued)

Sample codes	Geographical origin	Host	Year	Sex	Season	GenBank accession number		
						*Cox*1	*Rrn*L	*Nad*5
HLYN48	Yunnan (Chuxiong)	Cattle	2013	Female	Autumn	MH024518	MH024510	MH024514
HLYN49	Yunnan (Chuxiong)	Cattle	2013	Female	Autumn	MH024519	MH024511	MH024515

Table 2 Sequences of primers used to amplify a portion of the *cox*1, *rrn*L and *nad*5 of *Haemaphysalis longicornis* in the present study

Name of primer	Sequence (5′ to 3′)
For p*cox*1	
*cox*1F	GGAACAATATATTTAATTTTTGG
*cox*1R	ATCTATCCCTACTGTAAATATATG
For p*rrnL*	
16SF	CTGCTCAATGATTTTTTAAATTGCTGTGG
16SR	CCGGTCTGAACTCAGATCAAGT
For p*nad*5	
*nad*5F	TCTAAAATTAAATCCTTTGAAT
*nad*5R	AAGAGCCCAAATTCCATTTTC

The A + T content of these sequences was 67.0–67.7% for *cox*1, 77.8–78.0% for *rrn*L and 73.2–74.6% for *nad*5, respectively. The intra-specific sequence variation within *H. longicornis* was 0–2.8% for *cox*1, 0–2.9% for *rrn*L and 0–6.7% for *nad*5, however, the inter-specific sequence differences among other members of the genus *Haemaphysalis* were 13.8–15.3% for *cox*1, 14.7–15.7% for *rrn*L and 13.3–18.1% for *nad*5. Similarly, sequence diversity has also been detected in *H. flava* [25], *H. qinghaiensis* [29] and *H. punctate* [6] by analysis of mt gene sequences. These studies have clearly indicated that mt gene sequences provide reliable genetic markers for identification and differentiation of *Haemaphysalis* species.

Many studies have indicated that mt sequences are unique genetic markers to indicate geographical movements and population genetic structure of parasites [11–13]. In the present study, 22 polymorphic sites, 11 haplotypes, Hd = 0.696 and Pi = 0.00917 were determined in all sequences of p*cox*1. 11 polymorphic sites, 3 haplotypes, Hd = 0.041 and Pi = 0.0011 were determined in all sequences of p*rrn*L. 34 polymorphic sites, 17 haplotypes, Hd = 0.849 and Pi = 0.01296 were determined in all sequences of p*nad*5 (Table 3). A moderate level of haplotype diversity (except for Yunnan) was maintained in the *H. longicornis* populations, but their nucleotide diversity was relatively low due to the richness of single-nucleotide substitutions. Similar results also were reported for *I. ricinus* in Baltic countries [33]. The low level of nucleotide diversity was found across all four *H. longicornis* populations, revealing a relative lack of genetic variation across the *H. longicornis*, regardless of geographical origin and hosts. The combined three mt gene sequences of *H. longicornis* gave a negative Tajima's *D* value of −0.2 ($P > 0.05$) and Fu's *Fs* tests value of −2.105 ($P > 0.05$). The positive values from Tajima's D test signify that *H. longicornis* might not have experienced population expansion in the past. The *H. longicornis* sequences had a negative Tajima's *D* value of −0.2 and Fu's *Fs* tests value of −2.105, but the result was not statistically significant ($P > 0.05$). The genetic differentiation (66.2%) was mainly observed among populations, while the remaining 33.8% was observed between individuals within populations. These results indicated that there was higher genetic differentiation among populations across the four *H. longicornis* populations examined here. The AMOVA analysis has also confirmed that there was significant genetic differentiation across the four *H. longicornis* populations in the China mismatch distribution analysis of the combined three gene datasets which revealed the presence of a multi-peak not shown and a low rate of gene flow value ($Nm = 0.18$). The low levels of gene flow indicated less gene flow among the *H. longicornis* populations from the four provinces over

Table 3 Genetic diversity indices of *cox*1, *rrn*L and *nad*5 genes in *Haemaphysalis longicornis* from four populations in China

Populations	*Cox*1					*Rrn*L					*Nad*5				
	N	*S*	*H*	*Hd*	*Pi*	*N*	*S*	*H*	*Hd*	*Pi*	*N*	*S*	*H*	*Hd*	*Pi*
Hunan	15	2	3	0.257	0.00049	15	12	3	0.59	0.00484	15	16	8	0.867	0.01016
Shangdong	15	6	6	0.648	0.00155	15	1	2	0.476	0.00116	15	1	2	0.248	0.00049
Henan	15	4	5	0.476	0.0012	15	1	2	0.476	0.00116	15	28	9	0.905	0.01669
Yunnan	4	0	1	0	0	4	0	1	0	0	4	0	1	0	0
Total	49	22	22	0.696	0.00917	49	12	3	0.041	0.0011	49	34	17	0.849	0.01296

N number of sequence used, *S* number of polymorphic, *H* number of haplotypes, *Hd* haplotype diversity, *Pi* nucleotide diversity

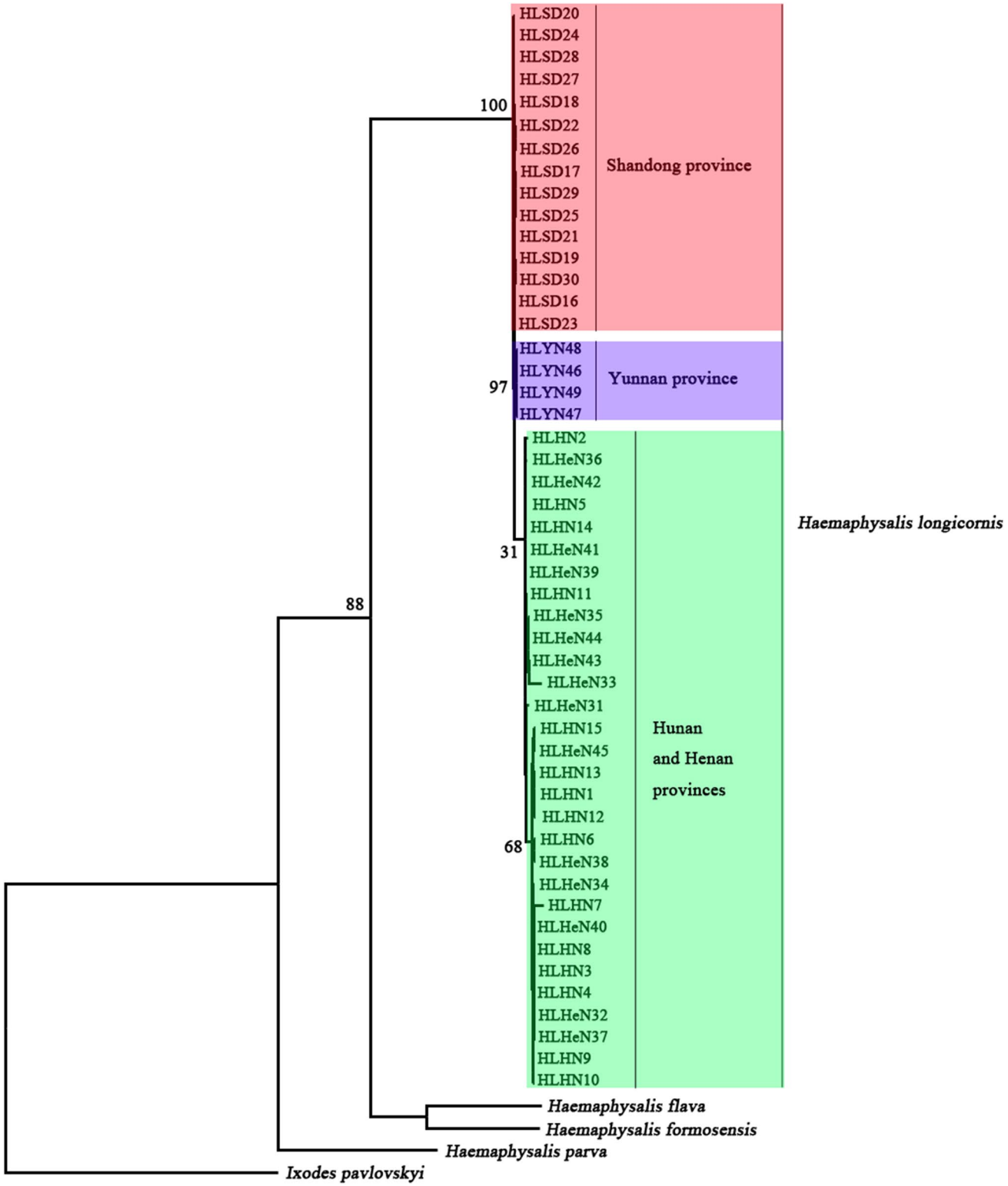

Fig. 1 Phylogenetic relationship among *Haemaphysalis longicornis* isolates in China with other *Haemaphysalis* species inferred by maximum likelihood analyses using the combined dataset (*cox*1 + *rrn*L + *nad*5), with *Ixodes pavlovsk*yi (NC_023831) as outgroup. Bootstrapping frequency (Br) values were indicated at nodes

time. Some studies have observed that *R. appendiculatus* went through a demographic expansion in Kenya [2, 21, 35]. However, our finding suggests that there was no rapid expansion event in the demographic history of all four *H. longicornis* populations.

mtDNA genes are useful molecular markers for phylogenetic studies of many ectoparasites, including ticks [1, 8, 19, 21]. In the present study, all the *H. longicornis* isolates were grouped together, indicating that all studied isolates represent the species *H. longicornis* (Fig. 1). The *H. longicornis* forms a monophyletic group with high statistical support (Br = 100), and all the *H. longicornis* isolates were segregated into two major clades (Fig. 1). Isolates from Shandong and Yunnan provinces clustered together in one clade with high statistical support (Br = 97) (Fig. 1). However, isolates from Hunan and Henan provinces clustered together in another clade without reflecting geographical origin, with weak statistical support (Br = 31) (Fig. 1). Our results suggest that *H. longicornis* may exist in multiple genotypes or distinct lineages. A previous study also supports the division of *I. scapularis* into several distinct lineages based on mt *cox*1 and 16S genes, and nuclear genes (serpin2, ixoderin B and lysozyme) [35].

In conclusion, our study made the first attempt to characterize genetic variation of *H. longicornis* isolated from different hosts and four provinces in China, by comparing and analyzing mt *cox*1, *rrn*L and *nad*5 genes. These datasets of *H. longicornis* provide a potential novel genetic marker for molecular epidemiology of *H. longicornis* in animals.

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Compliance with Ethical Standards

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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