Preplanned Studies

Molecular Identification and Genetic Characterization of Public Health Threatening Ticks — Chongming Island, China, 2021–2022

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Summary

What is already known about this topic?

Although ticks and tick-borne diseases are prevalent throughout China, there remains a knowledge gap regarding their biology and potential risk of distribution to human and animal populations on Chongming Island. The island, being China's third largest and a crucial component in the ecological preservation of the Yangtze Delta region, has yet to be comprehensively studied in this context.

What is added by this report?

In this study, employing molecular methodologies, a significant prevalence of *Haemaphysalis* (*H.*) *longicornis* and *H. flava* ticks — widely recognized for their high pathogenicity — is reported from Chongming Island. Additionally, the identification of two previously unreported species on the island, namely, *H. doenitzi* and *H. japonica*, expands our understanding of both the range and evolution of tick species.

What are the implications for public health practice?

The populations of humans and animals in nearly all 18 towns on Chongming Island are potentially at risk for transmission of tick-borne infectious agents. As a result, there is a pressing necessity for public health alerts, proactive tick surveillance, and effective screening of suspected clinical cases of tick-borne diseases within the Chongming population.

Ticks pose significant risks to human and animal health due to their capability of transmitting various pathogens, such as viruses, bacteria, and protozoans (*1*–*2*). To address this global health issue, the Chongming-based Center for One Health research was established on Chongming Island, China's third largest island and a crucial component in the ecological preservation of the Yangtze Delta region (*3*). The presence of ticks, including *Haemaphysalis* (*H.*) *longicornis* and *H. flava*, was previously confirmed in Dongping Forest Park and Dongtan Park on Chongming Island (*4*). Nonetheless, the potential risk ticks pose to human and animal populations on the island, along with their evolutionary impacts, remains unexplored.

In the course of 2021 to 2022, we conducted and identified ticks from Chongming Island utilizing 12S rRNA and co1 genes. We not only discovered an elevated prevalence of *H. longicornis* and *H. flava* on the island but also identified for the first time two additional tick species — *H. doenitzi* and *H. japonica*, both of which have been recognized for pathogenic properties and pose a threat to public health. Genetic diversity and neutrality tests suggested that the tick population was expanding or experiencing genetic hitchhiking. Consequently, there is an urgent need for sustained tick surveillance and targeted research on screening tick-borne pathogens and potential clinical cases to inform public health policies and actions.

In this study, ticks were collected from 18 towns and four protected regions on Chongming Island. Within each town, two to three sites were chosen for sample gathering, which was conducted bimonthly over two consecutive days between the hours of 10:00 AM and 4:00 PM from April 2021 to October 2022 (Figure 1). Each sample collection was performed by three collectors and lasted approximately 60±5 minutes.

Ticks were selected from two sources: those parasitizing domestic dogs and wild rabbits and free-living ticks collected from vegetation in parks and grasslands. The latter were sourced from areas in proximity to those parasitizing animals or from other external environments using a flag-dragging method.

After collection, ticks were classified as *Haemaphysalis* species based on their morphological characteristics as determined by microscopic examination. These characteristics include the shape of their prosthetic base, color, capitulum, conscutum, alloscutum, genital aperture, anal groove, anus, and arrangement on the posterior plate (*5*–*6*).

However, this study only distinguished between *H. flava* and *H. longicornis*, which were the most

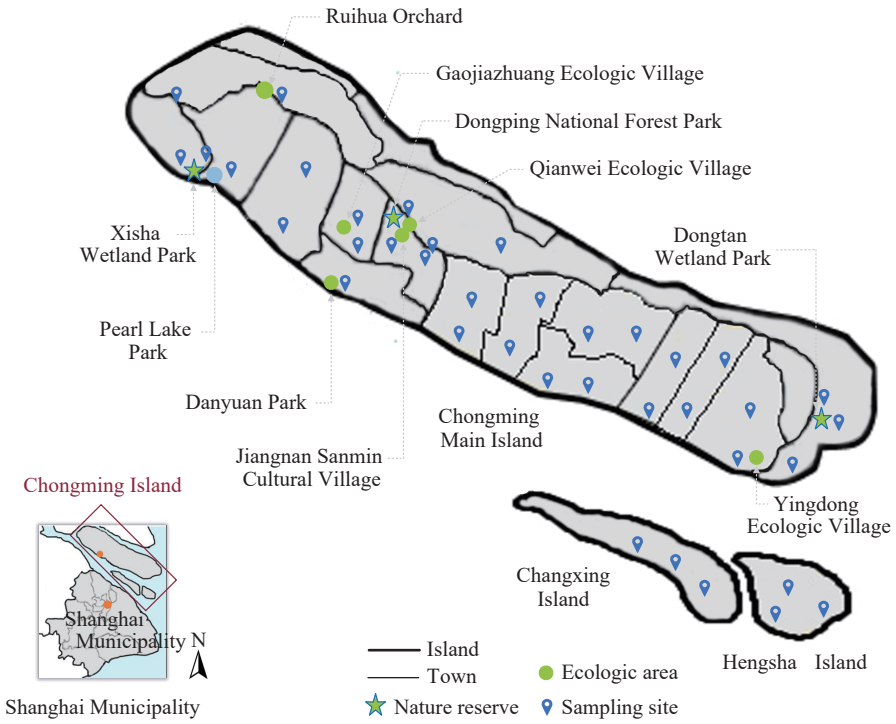


FIGURE 1. Locations on Chongming Island where ticks were collected, 2021–2022.

significantly represented species. *Haemaphysalis* species generally have eyeless, ciliated palisade, and rectangular basis capitula. *H. longicornis* is characterized by an abdomen of palpal segment 3 with a long conical spine and coxa II to IV internal spur, slightly larger and extending beyond the posterior (*5*). *H. flava* species possess thick and short abdominal spines on the palpal segment 3. Their coxa II to IV are short and triangular, and the scutum is marked with fine, shallow, evenly distributed engraved points (*6*). In females, the scutum is suborbicular, while in males, it is ovoid.

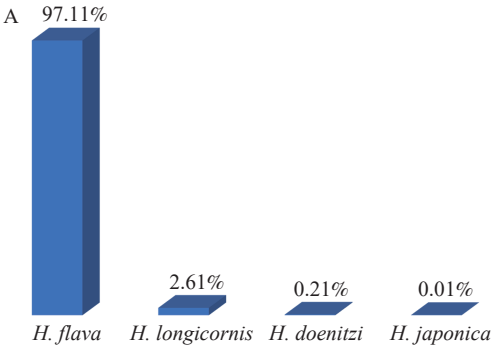
Microscopic examinations of ticks are depicted in Supplementary Figure S1 (available in https://weekly.chinacdc.cn/). Following identification, ticks were each individually preserved at −20 °C in 1.5 mL microcentrifuge tubes until further molecular analysis could be conducted.

Molecular identification and characterization were completed using polymerase chain reaction (PCR) amplification and sequencing of the produced amplicons. Genomic DNAs from a collection of 1,417 ticks were subjected to PCR amplification targeting 12S rRNA and col genes and were subsequently sequenced (*7*) with primer pairs T1B and T2A and CO1-F and CO1-R (Supplementary Table S1, Supplementary Figure S2, available in https://weekly.chinacdc.cn/). Amplification targeting the 12S rRNA locus proved more successful than that targeting the col locus, with success rates of 1,320/1,417 (93.15%) and 1,085/1,417 (76.57%), respectively. This disparity might be attributed to the limitations encountered in obtaining good-quality sequence reads from the col amplicons.

Analysis of 12S rRNA and co1 amplicons sequenced and verified in GenBank revealed that they predominantly belonged to the *Haemaphysalis* species, with *H. flava* constituting the majority at 97.11% (1,376/1,417), followed by *H. longicornis* at 2.61% (37/1,417), *H. doenitzi* at 0.21% (3/1,417), and *H. japonica* at 0.07% (1/1,417) (Figure 2A). When an intraspecific identity comparison based on the 12S rRNA gene was conducted, it was found that the ticks exhibited a high degree of identity with homologs from China and globally, with the exception of *H. japonica*, which presented an 83.48% degree of homology (Figure 2B).

Phylogenetic relationships were ascertained using both the neighbor-joining (NJ) and maximum likelihood (ML) methods. These relationships were established based on the correlation with publicly accessible homologous and orthologous sequences of the 12S rRNA and col genetic loci (Supplementary Table S2, available in https://weekly.chinacdc.cn/). From this examination, a dendrogram for 12S rRNA

A



B

Species	Marker	Confirmation/ total amplicons	Identity (%)
H. flava	12S rRNA	1,280/1,376	96.17
H. longicornis	12S rRNA	36/37	100
H. doenitzi	12S rRNA	3/3	99.11
H. japonica	12S rRNA	1/1	83.48

FIGURE 2. Molecular identification of ticks from Chongming Island (2021–2022) based on the 12S rRNA gene. (A) Distribution of species derived from Chongming Island. (B) Species confirmed through comprehensive sequence analysis, coupled with intraspecies validation, utilizing multiple alignments of the 12S rRNA gene amplicons.
Abbreviation: *H.=Haemaphysalis*.

suggested the existence of four main groups. These included *H. japonica* and *H. concinna* in the second cluster, *H. doenitzi* and *H. cornigera* in the third, and *H. longicornis* and *H. hystricis* in the fourth. Conversely, the first cluster distinctly comprised *H. flava* (Figure 3A). A comparison of the topological structure between the col and 12S rRNA phylogenetic trees yielded similar results (Figure 3B). Phylogenetic relationships derived from the ML method are available in Supplementary Figure S3 (available in https://weekly.chinacdc.cn/).

Genetic divergences were assessed at various taxonomic levels among tick species, utilizing Kimura's 2-parameter (K2P) distances based on the 12S rRNA locus (Supplementary Table S2). The highest within-species K2P distance was exhibited by *H. japonica* (0.0946), while the intraspecific distances for both *H. cornigera* and *H. hystricis* were zero. The zero intraspecific distance in these cases infers identical genetic sequences among populations within each species, offering potential insights into population history and overall biodiversity origins. The maximum interspecific K2P distance was identified between *H. hystricis* and *H. japonica* (0.1960). Conversely, the smallest interspecific distance of 0.0723 was observed between *H. cornigera* and *H. doenitzi*, indicating a potential genetic similarity between these species, as also depicted in the phylogenetic tree. An intermediate genetic distance was found between *H. hystricis* and *H. longicornis* (0.0900), comparable to the distance observed between *H. flava* and *H. japonica* (0.0903), both of which are in alignment with the phylogenetic topology.

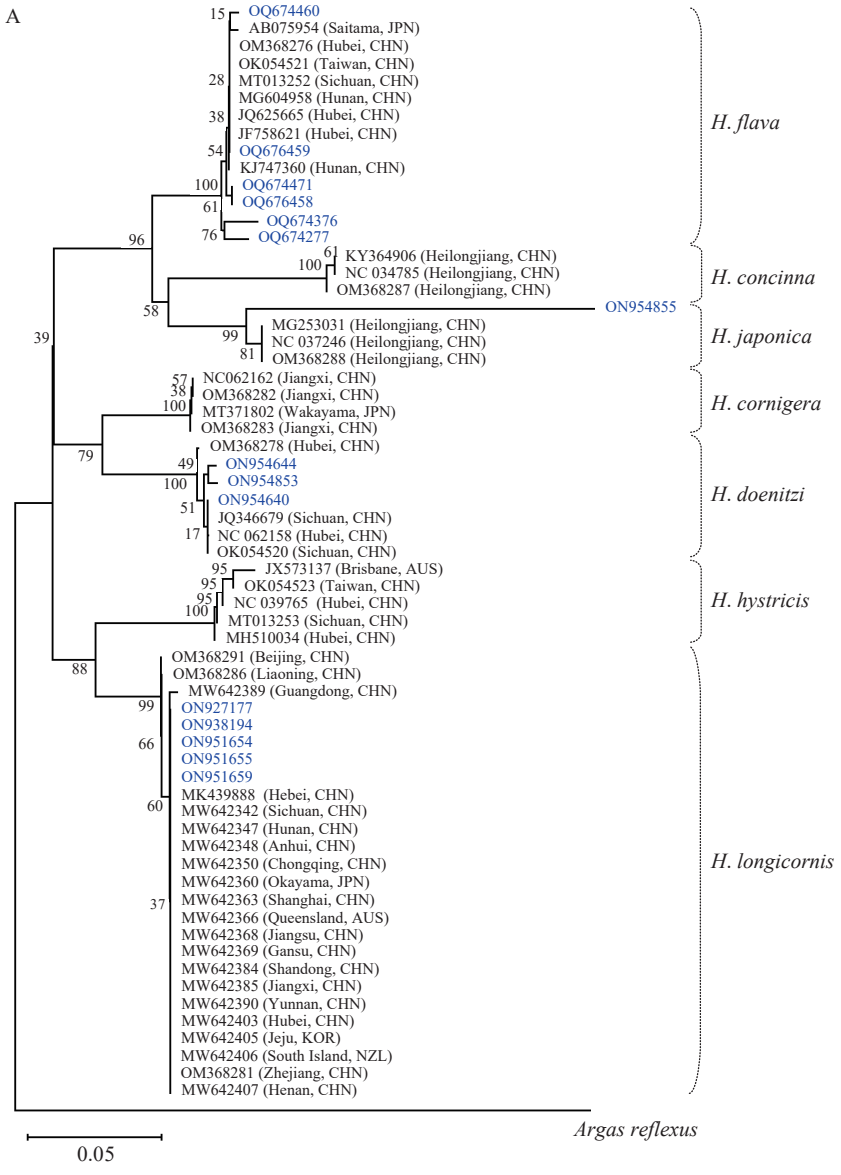
The mapping of K2P genetic distances revealed that *H. japonica* and *H. longicornis* maintained notable genetic differentiation compared to other tick species on Chongming Island. In particular, *H. japonica* presented the highest intraspecific and interspecific K2P distances. Its intraspecific distance, notably, exceeded the lowest interspecific distance (between *H. doenitzi* and *H. cornigera*) by 0.0946 (Figure 4A). Collectively, distinct interspecific boundaries were evident in more than 96% of the 12S rRNA fragments examined. We conducted genetic diversity indices and neutrality tests for these tick species using the 12S rRNA locus as a reference (Figure 4B). Among the species examined, the greatest number of polymorphic loci was found in *H. japonica* (n=56), while *H. flava* reported the lowest number (n=1). In terms of nucleic acid diversity (Pi), *H. japonica* was the most diverse (0.08333), while *H. doenitzi* exhibited the highest haplotype diversity (0.714). Neutral tests signified consistent negative values for Fu and Li's D and Tajima's D for the four species, yet they reported positive values for *H. concinna*. These findings suggest that there has been a recent expansion in global populations of *H.* longicornis, *H. doenitzi*, *H.* flava, and *H. japonica* ticks, including those based in Chongming.

DISCUSSION

The current study identified four tick species on Chongming Island, notably *H. flava* (97.11%), *H. longicornis* (2.61%), *H. doenitzi* (0.21%), and *H. japonica* (0.07%). This contrasts with a previous study that indicated *Rhipicephalus sanguineus* and *H. longicornis* as the main tick species in Shanghai (*8*). However, our findings indicate that *H. flava* and *H. longicornis* represent the majority of ticks on Chongming Island, corroborating recent research conducted in Dongping Forest Park and Dongtan Park

on the island (*4*). Notably, our research newly reports the presence of *H. doenitzi* and *H. japonica* from Dongtan Wetland Park and Dongping Forest Park, respectively, a fact not previously documented on the island. *H. doenitzi*, known as an avian tick, infests birds; thus, its presence on Chongming Island suggests potential for pathogen spillover. The low prevalence rates of *H. doenitzi* (3/1,417) and *H. japonica* (1/1,417) could imply their random occurrence and indicate that the likelihood of their detection in previous studies was relatively low. Furthermore, we posit that *H. japonica*, often referred to as "the northern tick", may have been introduced to the island via migratory birds breeding annually or increasingly common northern-to-southern trading transportation (*3*).

H. flava, a tick species, holds significant importance in public health, medical, and veterinary arenas due to its potential to cause lesions, blood loss, weight loss, and, in some cases, death. This species acts as a vector for various pathogens, including *Borrelia burgdorferi* (*9*–*10*), severe fever with thrombocytopenia syndrome virus (*11*), and tick-borne encephalitis virus (*12*). An epidemiological investigation on ticks and associated pathogens in pet dogs revealed that *H. longicornis* (*9*) was the predominant tick species across 1,140 counties in eastern and northeastern China, exposing over 40% of the population. Notably, a high incidence of *H. flava* and *H. longicornis* in 18 towns across Chongming, China, suggests a significant public health threat due to potential transmission risks of tick-borne infectious agents. *H. japonica* and *H. doenitzi* are



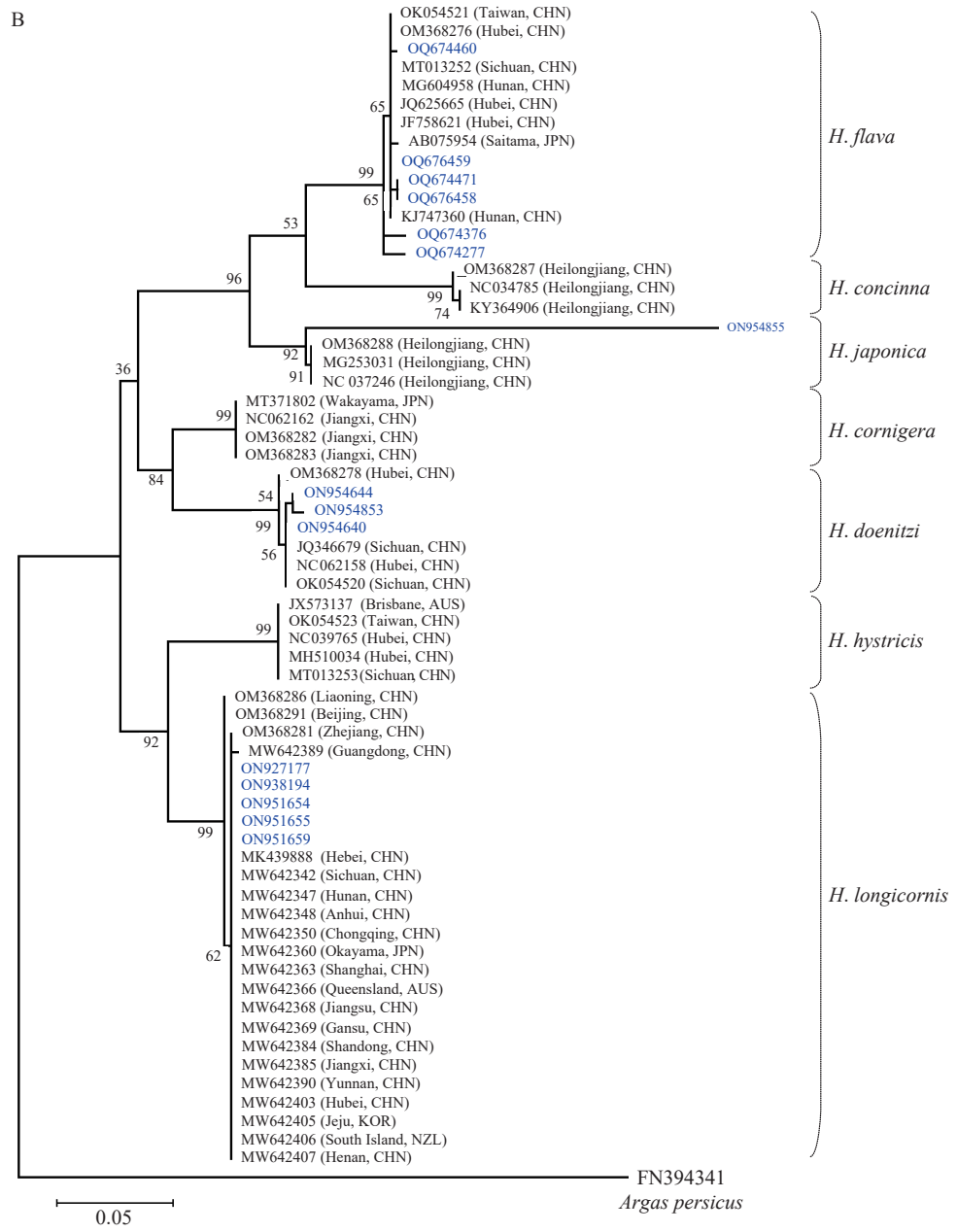
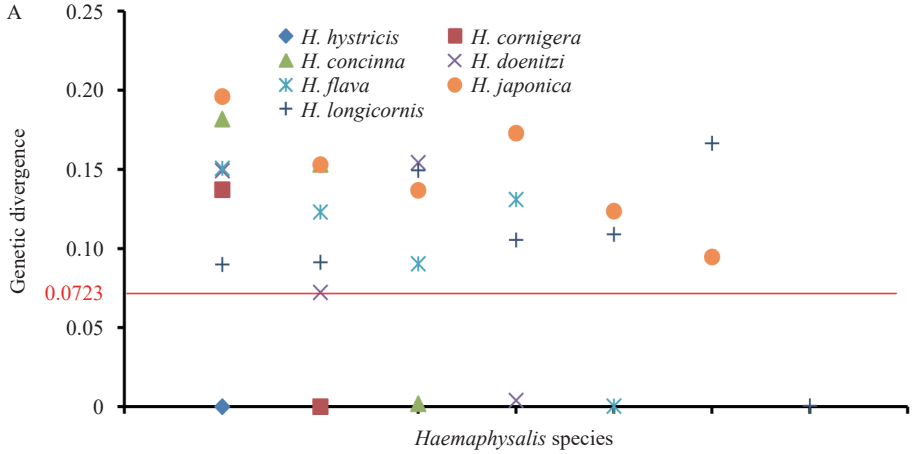


FIGURE 3. Comparison of genetic and geographical relationships between *Haemaphysalis* species from Chongming Island, 2021–2022, and those on record in GenBank. (A) Neighbor-joining tree based on 12S rRNA sequences. (B) Neighbor-joining tree based on col sequences.
Note: Percentage values on the tree branches denote bootstrapping values from 1,000 replicates. The identification process for each *Haemaphysalis* species sequence involved its accession number and geographical location (country). The gene sequences relevant to the species identified in this study are highlighted in blue. For the 12S rRNA and col phylogenetic trees, *Argas reflexus* and *Argas persicus* were used as outgroups, respectively. The scale bar signifies nucleotide substitutions per site.
Abbreviation: *H.=Haemaphysalis*.

prevalent across several regions in China, including Fujian Province, Yunnan Province, Gansu Province, Hebei Province, and the Inner Mongolia Autonomous Region and Taiwan, China (*9*), and are known to transmit a range of pathogens. These cause infections such as Lyme borreliosis (*10*) and babesiosis in humans (*13*). Despite their low prevalence on Chongming Island, these species also pose a public health threat.

Our study revealed a significant overlap between the intraspecific and interspecific K2P distances in *H.*

A



B

Species	*n*	S	Pi	h	Hd	Fu and Li's D	Tajima's D
H. flava	14	1	0.000,44	2	0.143	−1.397,49	−1.155,24
H. longicornis	26	2	0.000,66	3	0.218	−0.689,07	−1.223,94
H. doenitzi	7	4	0.003,98	4	0.714	−0.789,27	−0.876,42
H. japonica	4	56	0.083,33	2	0.500	−0.868,31	−0.868,31
H. cornigera	4	0	0	1	0	0	NA
H. hystricis	4	0	0	1	0	0	NA
H. concinna	3	1	0.001,97	2	0.667	1.632,99	1.632,99

FIGURE 4. Analysis of Kimura's two-parameter distance, genetic diversity, and neutrality tests for *Haemaphysalis* ticks collected from Chongming Island, 2021–2022. (A) Plot of the K2P distance of the *Haemaphysalis* species using NJ-K2P distances. (B) Genetic diversity indices and neutrality tests based on the 12S rRNA gene in the *Haemaphysalis* species.
Note: The red solid line in panel A represents the minimum interspecific distance between *H. cornigera* and *H. doenitzi*.
Abbreviation: *n*=the number of species; S=the number of polymorphism loci; Pi=nucleic acid diversity; h=haplotype number; Hd=haplotype diversity; *H.*=*Haemaphysalis*.

japonica. However, clear boundaries for the barcoding gap (ranging from 0.0040 to 0.0723) were observed in other species under observation. The overlap in *H. japonica* might be attributable to a high count of polymorphic loci (*n*=56) and considerable diversity (Pi=0.0833) in its 12S rRNA. Furthermore, the 12S rRNA topology indicates that the *H. japonica* species clusters separately from the other three species, implying that 12S rRNA may not serve as an effective biomarker for its identification. All observed tick species exhibited negative neutrality tests, as evidenced by Fu and Li's D and Tajima's D values, which might suggest that the four identified tick species in Chongming may have undergone population expansion or global genetic hitchhiking (*14*). This could enhance the adaptability of the ticks to environmental variations, extend their distribution, and potentially harbor a greater number of pathogens, escalating their capacity to cross-transfer diseases and intensifying their public health risks.

This study revealed a high prevalence of *Haemaphysalis* tick distribution on Chongming Island. These findings suggest potential transmission risks of tick-borne infectious agents to both the human and animal populations on the island. Consequently, it is crucial to urgently issue public health warnings, implement active tick surveillance, and efficiently screen suspected tick-borne disease cases within the Chongming population. To address intricate ecological and health challenges, we recommend future research into the epidemiological distribution of tick-borne pathogens on Chongming Island. Such research could expedite the successful application of the One Health approach to public health threats in China.

Conflicts of interest: Xiao-nong Zhou is an editorial board member of the journal China CDC Weekly. He was not involved in the peer-review or handling of the manuscript. The authors have no other competing interests to disclose.

Acknowledgements: The authors express their gratitude to the staff at the Shanghai Chongming Centre for Disease Control and Prevention for their assistance in facilitating the collection and identification of ticks.

Supporting information: The manuscript and accompanying supplementary file contain all pertinent data. The sequences of both the 12S rRNA and col genes, central to the phylogenetic analysis, have been

lodged with the National Center for Biotechnology Information (NCBI). The GenBank accession numbers for these sequences are as follows: for 12S RNA genes — *H. flava* [OQ674277, OQ674376, OQ674460, OQ676458, OQ676459, OQ674471]; *H. longicornis* [ON927177, ON938194, ON951654, ON951655, and ON951659]; *H. doenitzi* [ON954640, ON954644, and ON954853]; *H. japonica* [ON954855]. For col genes — *H. flava* [ON954774, ON954780, ON959178, and ON959193-ON959195]; *H. longicornis* [ON954776].

Funding: Supported by the financial backing of the Science and Technology Innovation Project Fund from the School of Global Health, Shanghai Jiao Tong University School of Medicine (SGHKJCX2021-05, SGHKJCX2021-04) and the International Joint Laboratory on Tropical Diseases Control in Greater Mekong Subregion (21410750200).

doi: 10.46234/ccdcw2023.156

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Submitted: June 01, 2023; Accepted: September 12, 2023

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SUPPLEMENTARY MATERIAL

A

H. flava (female, abdomen)

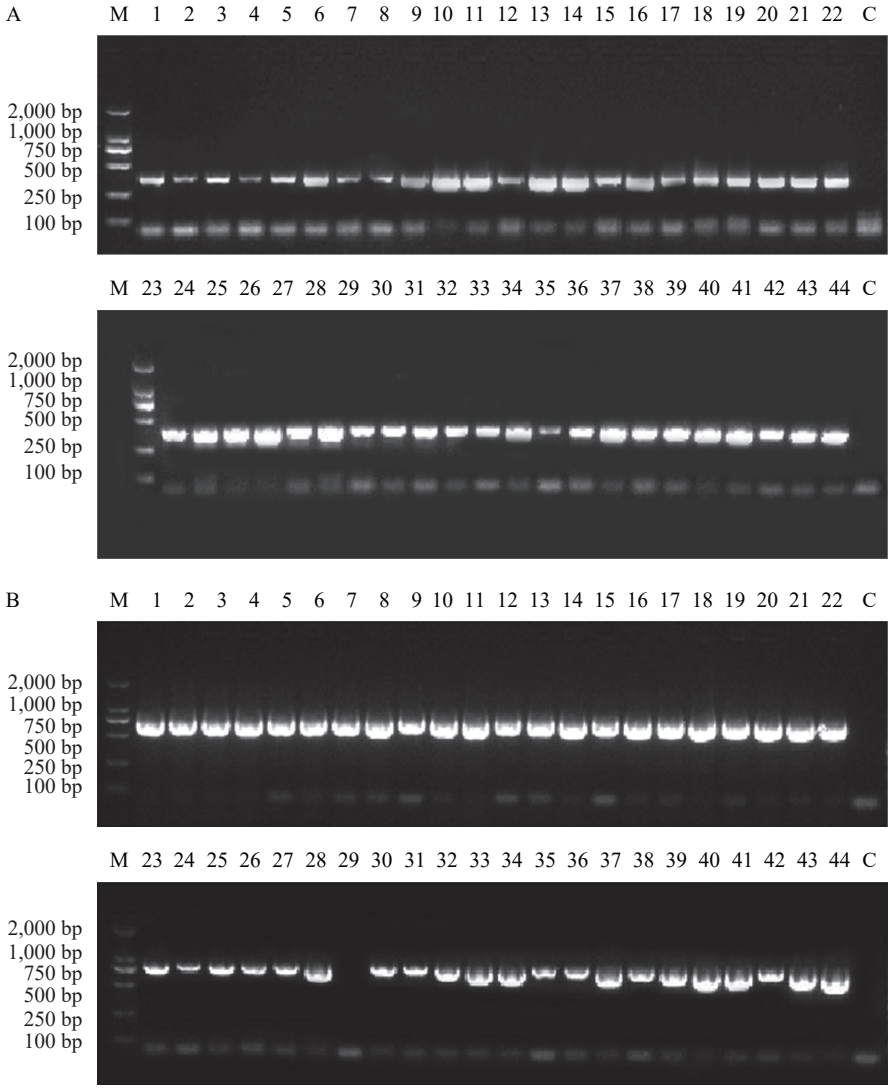
H. longicornis (female, dorsum)

B

H. longicornis (female, dorsum)

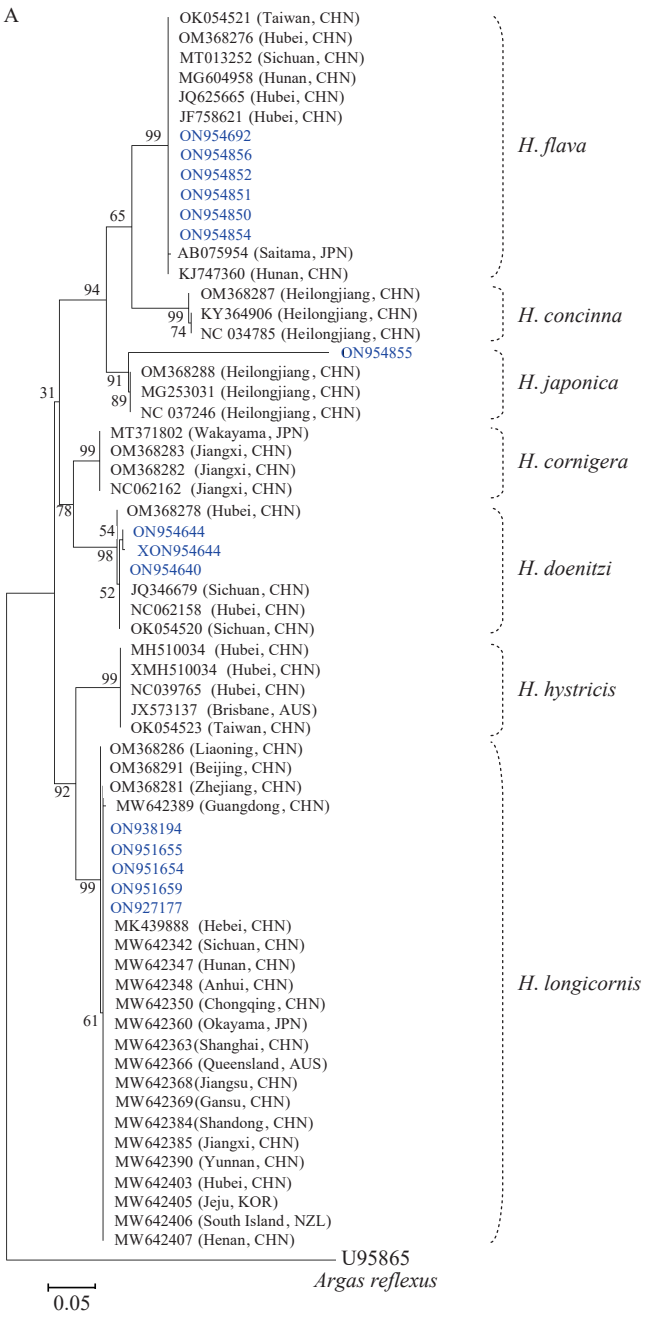
H. longicornis (female, abdomen)

SUPPLEMENTARY FIGURE S1. Identification of primary ticks (A) *H. flava* and (B) *H.* longicornis on Chongming Island, 2021–2022.
Abbreviation: *H.=Haemaphysalis.*



SUPPLEMENTARY FIGURE S2. Specific bands of amplified mitochondrial genes were identified through agarose gel electrophoresis. (A) Amplicons of the 12S rRNA gene at approximately 380 base pairs. (B) Amplicons of the co1 gene at approximately 650 base pairs.

Note: In panel A, “M” denotes DNA Marker (DL2000, Takara); “numbered” amplicons of the 12S rRNA gene; “C” denotes negative template control. In panel B, “M” denotes DNA Marker (DL2000, Takara); “numbered” denotes amplicons of co1 gene; “C” denotes negative template control.


A
OK054521 (Taiwan, CHN)
OM368276 (Hubei, CHN)
MT013252 (Sichuan, CHN)
MG604958 (Hunan, CHN)
JQ625665 (Hubei, CHN)
JF758621 (Hubei, CHN)
ON954692
ON954856
ON954852
ON954851
ON954850
ON954854
AB075954 (Saitama, JPN)
KJ747360 (Hunan, CHN)
H. flava
OM368287 (Heilongjiang, CHN)
KY364906 (Heilongjiang, CHN)
NC 034785 (Heilongjiang, CHN)
H. concinna
ON954855
OM368288 (Heilongjiang, CHN)
MG253031 (Heilongjiang, CHN)
NC 037246 (Heilongjiang, CHN)
H. japonica
MT371802 (Wakayama, JPN)
OM368283 (Jiangxi, CHN)
OM368282 (Jiangxi, CHN)
NC062162 (Jiangxi, CHN)
H. cornigera
OM368278 (Hubei, CHN)
ON954644
XON954644
ON954640
JQ346679 (Sichuan, CHN)
NC062158 (Hubei, CHN)
OK054520 (Sichuan, CHN)
H. doenitzi
MH510034 (Hubei, CHN)
XMH510034 (Hubei, CHN)
NC039765 (Hubei, CHN)
JX573137 (Brisbane, AUS)
OK054523 (Taiwan, CHN)
H. hystricis
OM368286 (Liaoning, CHN)
OM368291 (Beijing, CHN)
OM368281 (Zhejiang, CHN)
MW642389 (Guangdong, CHN)
ON938194
ON951655
ON951654
ON951659
ON927177
MK439888 (Hebei, CHN)
MW642342 (Sichuan, CHN)
MW642347 (Hunan, CHN)
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MW642403 (Hubei, CHN)
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MW642406 (South Island, NZL)
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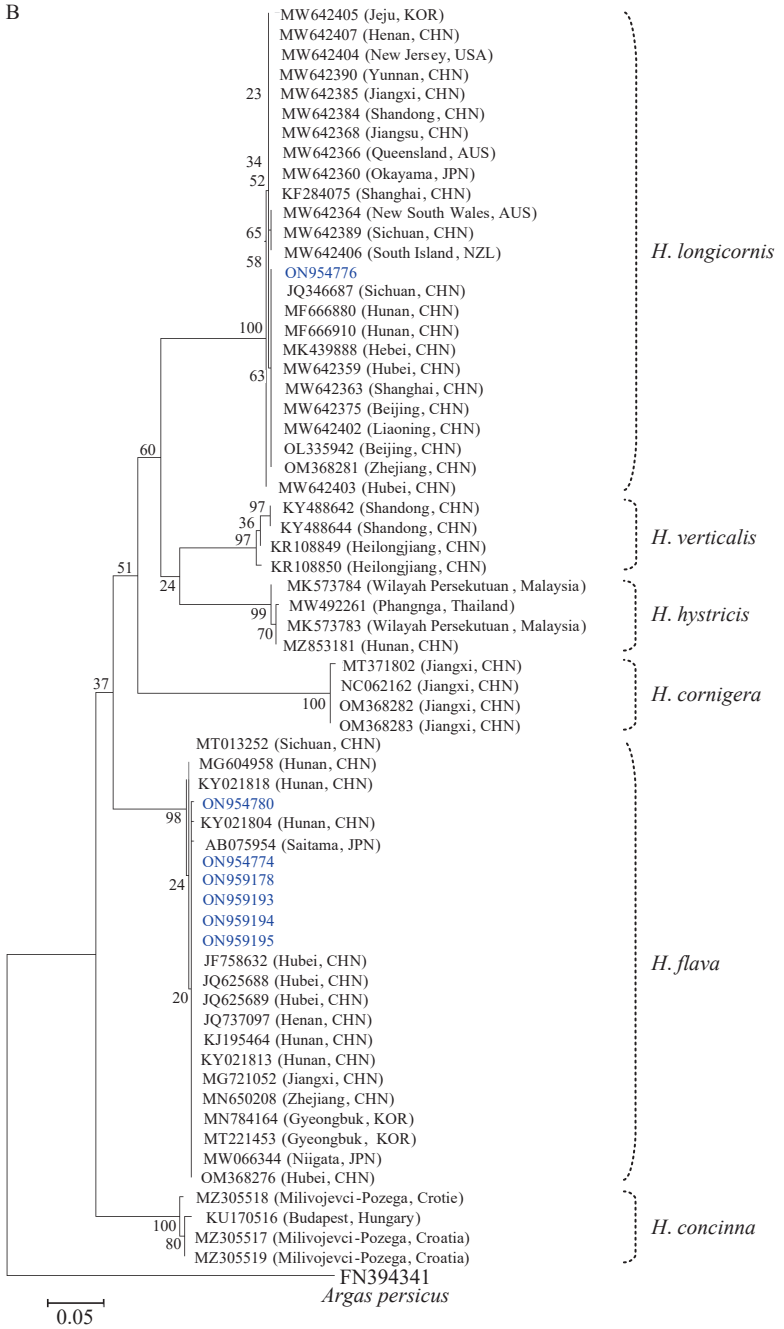
H. longicornis
U95865
Argas reflexus
0.05



SUPPLEMENTARY FIGURE S3. Comparison of the genetic and geographical relationships between *Haemaphysalis* species from Chongming Island, 2021–2022, and those deposited in GenBank. (A) Maximum likelihood tree based on 12S rRNA sequences. (B) Maximum likelihood tree based on co1 sequences.

Note: The relationships among *Haemaphysalis* species were discerned via analysis of partial 12S rRNA and col gene sequences. The percentages on the branches represent the bootstrap values from 1,000 replications. Each *Haemaphysalis* species sequence was identified based on its accession number and geographical location (country). Sequences representing the species identified herein are highlighted in blue. *Argas reflexus* and *Argas persicus* were utilized as outgroups for 12S rRNA and col phylogenetic trees, respectively. The scale bar symbolizes the rate of nucleotide substitutions per site.

Abbreviation: *H.=Haemaphysalis.*

SUPPLEMENTARY TABLE S1. Primer sequences employed for the PCR amplification of mitochondrial genes in ticks.

Amplification fragment	Primers	Primer sequences	Annealing temperature (°C)	Amplicon size (bp)
12S rRNA	T1B T2A	AAACTAGGATTAGATACCCT AATGAGAGCGACGGGCGATGT	51/ 53	380
co1	CO1-F CO1-R	GGAACAATATATTTAATTTTTGG ATCTATCCCTACTGTAAATATATG	55	650–820

Abbreviation: bp=base pairs.

SUPPLEMENTARY TABLE S2-1. Data on the mitochondrial gene sequences (12S rRNA) utilized for phylogenetic and genetic diversity analysis.

Species	Geographic localities	GenBank accession No.
H. hystricis	Brisbane, Australia	JX573137
	Hubei, China	MH510034
	Sichuan, China	MT013253
	Hubei, China	NC_039765
	Taiwan, China	OK054523
H. cornigera	Wakayama, Japan	MT371802
	Guangzhou, China	NC062162
	Guangzhou, China	OM368282
	Guangzhou, China	OM368283
H. concinna	Heilongjiang, China	KY364906
	Heilongjiang, China	NC_034785
	Heilongjiang, China	OM368287
H. doenitzi	Sichuan, China	JQ346679
	Hubei, China	NC_062158
	Sichuan, China	OK054520
	Hubei, China	OM368278
	Shanghai, China	ON954640
	Shanghai, China	ON954644
	Shanghai, China	ON954853
H. flava	Saitama, Japan	AB075954
	Hubei, China	JF758621
	Hubei, China	JQ625665
	Hunan, China	KJ747360
	Hunan, China	MG604958
	Sichuan, China	MT013252
	Taiwan, China	OK054521
	Hubei, China	OM368276
	Shanghai, China	OQ676427
	Shanghai, China	OQ676437
	Shanghai, China	OQ674460
	Shanghai, China	OQ674471
	Shanghai, China	OQ676458
	Shanghai, China	OQ676459
H. japonica	Heilongjiang, China	MG253031
	Heilongjiang, China	NC_037246
	Heilongjiang, China	OM368288
	Shanghai, China	ON954855

Continued

Species	Geographic localities	GenBank accession No.
H. longicornis	Hebei, China	MK439888
	Sichuan, China	MW642342
	Hunan, China	MW642347
	Anhui, China	MW642348
	Chongqing, China	MW642350
	Okayama, Japan	MW642360
	Shanghai, China	MW642363
	Queensland, Australia	MW642366
	Jiangsu, China	MW642368
	Gansu, China	MW642369
	Shandong, China	MW642384
	Jiangxi, China	MW642385
H. longicornis	Guangdong, China	MW642389
	Yunnan, China	MW642390
	Hubei, China	MW642403
	Jeju, Republic of Korea	MW642405
	South Island, New Zealand	MW642406
	Henan, China	MW642407
	Zhejiang, China	OM368281
	Liaoning, China	OM368286
	Beijing, China	OM368291
Argas reflexus		U95865.1

Abbreviation: *H.=Haemaphysalis*.

SUPPLEMENTARY TABLE S2-2. Data on the mitochondrial gene sequences (co1) utilized for phylogenetic and genetic diversity analysis.

Species	Geographic localities	GenBank accession No.
H. hystricis	Wilayah Persekutuan, Malaysia	MK573783
	Wilayah Persekutuan, Malaysia	MK573784
	Phangnga, Thailand	MW492261
	Hunan, China	MZ853181
H. cornigera	Jiangxi, China	MT371802
	Jiangxi, China	NC_062162
	Jiangxi, China	OM368282
	Jiangxi, China	OM368283
H. concinna	Budapest, Hungary	KU170516
	Milivojevci-Pozega, Croatia	MZ305517
	Milivojevci-Pozega, Croatia	MZ305518
	Milivojevci-Pozega, Croatia	MZ305519
H. doenitzi	Hubei, China	JF758632
	Hubei, China	JQ625688
	Hubei, China	JQ625689
	Henan, China	JQ737097
	Hunan, China	KJ195464

Continued

Species	Geographic localities	GenBank accession No.
	Hunan, China	KY021804
	Hunan, China	KY021813
	Hunan, China	KY021818
	Hunan, China	MG604958
	Jiangxi, China	MG721052
	Zhejiang, China	MN650208
	Gyeongbuk, Republic of Korea	MN784164
	Sichuan, China	MT013252
	Gyeongbuk, Republic of Korea	MT221453
	Niigata, Japan	MW066344
	Hubei, China	OM368276
	Shanghai, China	KF284075
	Shanghai, China	ON954774
	Shanghai, China	ON954780
	Shanghai, China	ON959178
	Shanghai, China	ON959193
	Shanghai, China	ON959194
	Shanghai, China	ON959195
H. longicornis	Sichuan, China	JQ346687
	Hunan, China	MF666880
	Hunan, China	MF666910
	Hebei, China	MK439888
	Hubei, China	MW642359
	Okayama, Japan	MW642360
	Shanghai, China	MW642363
	New South Wales, Australia	MW642364
H. longicornis	Queensland, Australia	MW642366
	Jiangsu, China	MW642368
	Beijing, China	MW642375
	Shandong, China	MW642384
	Jiangxi, China	MW642385
	Sichuan, China	MW642389
	Yunnan, China	MW642390
	Dalian, China	MW642402
	Hubei, China	MW642403
	New Jersey, the United States	MW642404
	Jeju, Republic of Korea	MW642405
	South Island, New Zealand	MW642406
	Henan, China	MW642407
	Beijing, China	OL335942
	Zhejiang, China	OM368281
	Shanghai, China	ON927177
	Shanghai, China	ON938194

Continued

Species	Geographic localities	GenBank accession No.
H. longicornis	Shanghai, China	ON951654
(continued)	Shanghai, China	ON951655
	Shanghai, China	ON951659
	Shanghai, China	ON954776
H. verticalis	Heilongjiang, China	KR108849
	Heilongjiang, China	KR108850
	Shandong, China	KY488642
	Shandong, China	KY488644
Argas persicus	Southwestern Romania	FN394341

SUPPLEMENTARY TABLE S3. Average intraspecific and interspecific K2P distances predicated on the 12S rRNA gene in *Haemaphysalis* species.

Species	*N*	*H. hystricis*	*H. cornigera*	*H. concinna*	*H. doenitzi*	*H. flava*	*H. japonica*	*H. longicornis*
H. hystricis	5	0						
H. cornigera	4	0.1371328831	0					
H. concinna	3	0.1817426049	0.1532318083	0.0019841330				
H. doenitzi	4	0.1491843529	0.0723418465	0.1542762170	0.0039905820			
H. flava	14	0.1507102241	0.1230000555	0.0903495504	0.1309744074	0.0004275540		
H. japonica	4	0.196004581*	0.1529088927	0.1368672590	0.1728339139	0.1235672509	0.0945783130	
H. longicornis	26	0.0899835374	0.0913522722	0.1493494257	0.1054751596	0.1089099103	0.1665811766	0.0006666250

Note: Intraspecific distance data are shown in boldface for clarity. The underlined data indicate the highest intraspecific and lowest interspecific distances.
Abbreviation: *N*=number of sequences. *H.=Haemaphysalis.*
* the highest interspecific distance.