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RESEARCH ARTICLE

Breeding sites, migration paths and phylogenetic relationships of mosquitoes in seven cities in northern and southern China

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ABSTRACT

Mosquito-borne diseases have wreaked havoc on human health, with consequences dramatically increasing in recent years. The incidence of mosquito-borne diseases is closely linked to the locations that are chosen for urban development. The aim of this study was to provide characteristics of mosquito breeding sites in northern and southern China and to document the most important arbovirus vectors found in the study area, the evidence generated here is critical for early prevention and control inter ventions. This research involved a random selection of various sites across four provinces, spanning both the northern and southern regions of China. The dwellings and accessible water storage containers in these sites were investigated to detect the presence of immature mosquitoes. Samples were then collected, mosquitoes were nurtured to adulthood, and the species that were present were identified. A total of 1 249 samples were collected during this survey of the mosquito breeding sites. A total of 80 samples were processed using the Chelex method to extract mosquito DNA from all the samples. The ITS2 gene fragment was then amplified by PCR and sequenced. A subsequent BLAST comparison allowed the identification of the mosquito species, and MEGA11 software was used for phylogenetic analysis. The results showed that there were four species of mosquitoes, including *Aedes albopictus*, *Culex quinquefasciatus*, *Lutzia fuscanus* and *Armigeres subalbatus*. The primary mosquito breeding grounds in the four provinces of China consisted of storm drains, discarded containers, garbage bins, and areas with standing water. Still-water environments, such as rice fields were the primary breeding locations in the southern cities. In contrast, in the northern regions, most breeding occurred at construction sites, and in similar water-prone areas. The most prevalent mosquitoes in the four provinces of China were of the genus *Aedes*, with a significant number originating from Fujian Province, China. This information sheds light on the migration patterns of mosquitoes and significantly enhances community-based protection measures and mobilization efforts.

Keywords: Mosquito; internal transcribed spacer 2; breeding site; migration path; phylogenetic relationship.

INTRODUCTION

Mosquitoes pose a substantial threat to public health worldwide, particularly in tropical and subtropical regions where the mosquitoes transmit many diseases that cause severe health risks (Giunti *et al*., 2023). According to the World Health Organization (WHO), vector-borne diseases account for more than 17% of all infectious diseases, with malaria alone claiming more than 400,000 lives each year. Dengue, yellow fever, chikungunya, and Zika virus have also caused widespread outbreaks in urban centers across the globe (Jones *et al*., 2020). Urbanization, global climate change, and rapid modernization in numerous low- and middle-income countries have created environments that are favorable for mosquito breeding and survival. As human populations expand and encroach upon natural habitats, mosquitoes are discovering new opportunities to adapt to urban conditions, leading to the spread of infectious diseases (Duval *et al*., 2023).

Climate change and human mobility have been central point of research, and these studies have highlighted their, impact on the diversity of mosquito populations. These studies underscore the significance of understanding how environmental and social factors interact to influence mosquito populations and the diseases the mosquitoes transmit (Lahondère & Bonizzoni, 2022). In the northeastern region of China, as well as the Qinghai-Tibet area, the high altitude and cold climate impose stringent conditions for the survival of mosquitoes. Among these, the *Aedes* genus is the most prominent. Conversely, in North China, Central China, South China, and Southwest China, where there are four distinct seasons, a greater diversity of mosquito species is observed. The primary mosquito genera in these regions are *Aedes*, *Culex*, and

Anopheles (Wang *et al*., 2022). The consistently high temperatures, abundant rainfall, and large population densities in Southeast Asian countries and southern Chinese regions have significantly elevated the risk of mosquito-borne disease outbreaks. These climatic and environmental conditions have created optimal breeding grounds for mosquitoes, subsequently increasing the probability of disease transmission (Chathuranga *et al*., 2023). Notably, rooftops, which are often neglected habitats, have emerged as significant breeding sites for *Aedes* mosquito species (Alarcón-Elbal *et al*., 2021). Additionally, environmental factors, such as waste tires contribute significantly to mosquito breeding, emphasizing the importance of proper waste management in disease prevention (Wilke *et al*., 2019).

The insights from these studies are paramount for developing effective prevention and control strategies. These strategies emphasize the need for comprehensive approaches that consider both the environmental and social determinants of health. Such approaches include modifying mosquito breeding sites, improving sanitation practices, implementing climate-smart urban planning, and enhancing public awareness and community engagement in mosquito control efforts.

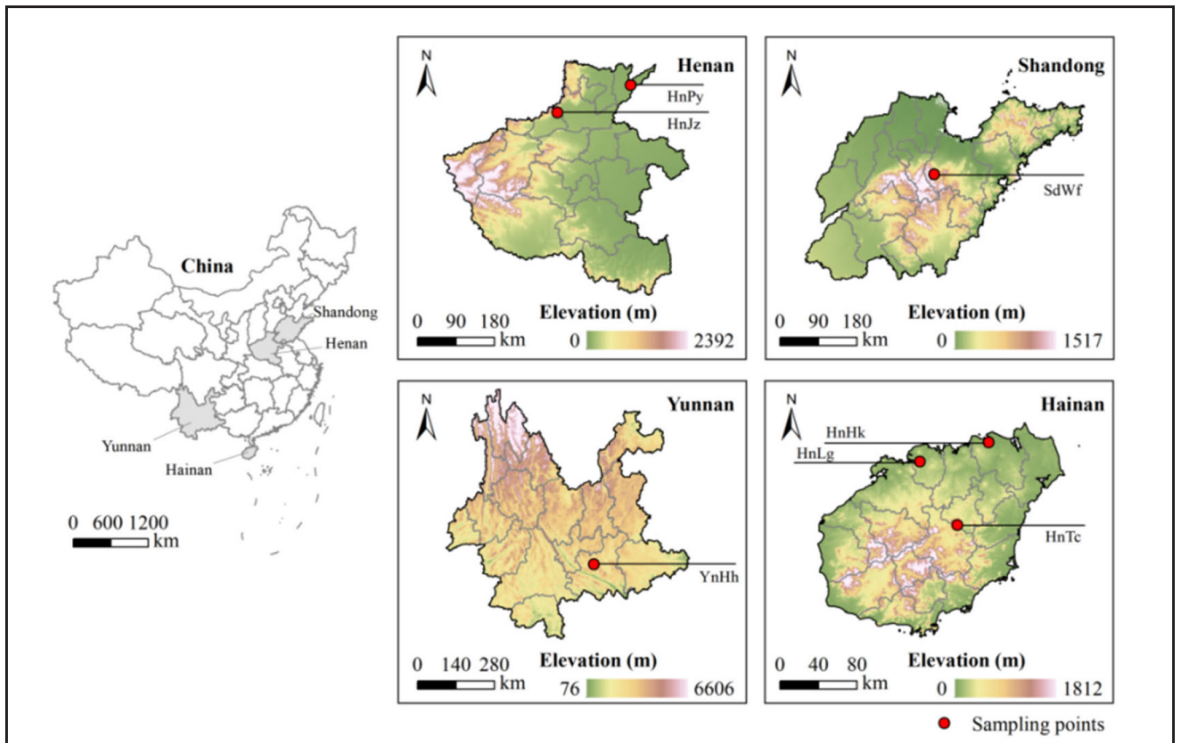
It is crucial to continue documenting and analyzing the characteristics of mosquito breeding sites across diverse regions of China and across the globe to shape the development of early prevention and control strategies. The objective of these studies is to identify the characteristics of mosquito breeding sites and dominant mosquito populations, eliminate mosquitoes at their source, predict types of mosquito-borne diseases, and initiate early prevention measures.

For over a decade, there have been no reports of mosquito breeding sites in northern or southern China. To gain a deeper understanding of the specific characteristics of these breeding habitats across seven sites in both regions, this study collected samples from residential and public locations in Henan, Shandong, Yunnan, and Hainan provinces of China. This comprehensive approach is aimed at identifying the unique features of these habitats, which is vital for developing targeted and effective prevention and control strategies.

MATERIALS AND METHODS

Sample collection

Straw was used to trap mosquito larvae and pupae (Li *et al*., 2022) in various watery locations such as water holes, tire tracks, water tanks, drainage ditches, abandoned tanks, water tanks, plastic rubber, tree holes and other dead corners, across seven distinct sites in China. These sites were categorized into northern and southern regions, and their respective longitudes and latitudes were recorded (Figure 1). Three of these locations were located in the north, namely



Sampling Locations	Codes	Coordinates
Puyang City, Henan Province, China	HnPy	35°44′26″ N, 115°23′14″ E
Jiaozuo City, Henan Province, China	HnJz	35°19′11″ N, 113°24′14″ E
Weifang City, Shandong Province, China	SdWf	36°29′17″ N, 118°27′16″ E
Honghe Prefecture, Yunnan Province, China	YnHh	23°38′22″ N, 102°48′29″ E
Haikou City, Hainan Province, China	HnHk	19°59′35″ N, 110°19′11″ E
Lingao County, Hainan Province, China	HnLg	19°50′30″ N, 109°38′22″ E
Tunchang County, Hainan Province, China	HnTc	19°12′72″ N, 109°58′34″ E

Figure 1. The distribution map of the mosquito sampling sites in the four provinces of northern and southern China. The locations, codes, latitudes and longitudes of the sampling sites in each province.

Puyang City, Jiaozuo City, and Weifang City, while the remaining four were located in the south, including Haikou City, Tunchang County, Lingao County, and Honghe Prefecture. Once captured, the mosquitoes at these sites were transported to the laboratory for initial classification, after which they underwent hatching and maturation processes (Ali *et al*., 2022).

Morphological classification

Newly emerged adult mosquitoes were observed using a Motic digital microscope, and Motic Images Plus 3.0 software from Motic China Group Co. Ltd was used to preliminarily classify the mosquitoes according to their morphological differences (Rahola *et al*., 2022).

Genomic DNA extraction and ITS2 gene amplification

Genomic DNA was extracted from 80 randomly selected mosquitoes. Each mosquito sample was washed with sterile ddH_2O several times, and genomic DNA was extracted from one leg of each mosquito using the Chelex method (Turan *et al*., 2015). The ITS gene was amplified using the forward primer 5.8S (5'-ATCACTCGGCTCGTGGATCG-3') and the reverse primer 28S (5'-ATGCTTAAATTTAGGGGGTAGTC-3') (Weeraratne *et al*., 2018) in a PCR assay, resulting in the amplification of target gene fragments of approximately 500 base pair (bp). PCRs were performed in a 50-µL reaction volume composed of 1 µL of genomic DNA, 1 µL each of forward primer and reverse primer (10 µM), 22 µL of ddH_2O, and 25 µL of Taq plus Master Mix (Biosharp, Hefei, China). The PCR procedure was conducted under the following conditions: an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 45°C for 20 seconds, and extension at 72°C for 50 seconds. A final extension step of 72°C for 5 minutes was then performed. The PCR products were electrophoresed on 1.5% agarose gel and were visualized using a gel imaging analysis system, and the specific DNA was excised for gel extraction using an Agarose Gel DNA Recovery Kit. The purified DNA fragments were inserted into the pMD 19T cloning vector (TaKaRa, Osaka, Japan), cloned, transformed into competent *Escherichia coli* DH5 alpha cells, which were subsequently sent to lGE Biotechnology Co., Ltd. (Guangzhou, China), for Sanger sequencing.

Sequence alignment and phylogenetic analysis

The obtained sequences were deposited at the National Center for Biotechnology Information (NCBI) and aligned using the Basic Local Alignment Search Tool (BLAST) nucleotide algorithm in the GenBank database. The sequence alignments were analyzed using sequence demarcation tool (SDT) version 1.2 software (Muhire *et al*., 2014) to generate a pairwise identity matrix. Haplotype and nucleotide diversity were inferred using DNAsp version 5.0 software (Librado & Rozas, 2009), and the haplotype network was mapped using network version 10.2.0.0 software (Bandelt *et al*., 1999). The nucleotide substitution saturation was analyzed using DAMBE version 7.3.11 software (Xia, 2018) to verify the effectiveness of the ITS2 gene as a DNA barcoding tool for mosquito species identification. Multiple sequence alignments were generated using MEGA version 11 software (Tamura *et al*., 2021), and a phylogenetic tree was constructed using the neighbor-joining method with the Kimura 2-parameter (K2P) model. The confidence value of each node in the system tree was tested using 1000 bootstraps.

RESULTS

Characteristics of mosquito breeding sites

During the sampling process, a significant number of mosquitoes were observed in residential areas, leisure areas, and water-holding containers with diverse materials. The breeding habitats of the mosquitoes in the northern and southern regions were predominantly puddles, tire tracks, water tanks, drains, plastic rubber, and tree holes. The breeding habitats of mosquitoes were notably different between the northern and southern regions. In the southern cities, stagnant water environments served as the primary breeding grounds, with household water buckets being the most frequent examples, followed closely by food waste boxes in commercial areas. Conversely, mosquito breeding sites in the northern regions predominantly included construction sites and water-retaining materials such as discarded tires. Furthermore, the number of breeding mosquitoes varied depending on the type of vegetation, with the highest populations being observed in humid environments. *Aedes* mosquito species were primarily distributed in tropical and subtropical regions, where the optimal temperature ranged from 35°C to 40°C and the relative humidity spanned from 35% to 45%. These mosquitoes were more inclined to breed in unvegetated, slow-moving, and sunny habitats, such as household waste buckets. On the other hand, *Culex* mosquito species were mainly found in temperate zones, with an optimal temperature of 25°C to 30°C and a relative humidity of 60% to 80%. They exhibited a greater propensity to breed in their natural habitats, characterized by slow-moving water and shaded areas.

Mosquito species identification

A total of 1 249 mosquito larvae and pupae were sampled during the study. In northern China, 640 mosquito samples were collected, including 433 from Puyang City, 79 from Jiaozuo City, and 128 from Weifang City. In southern China, a total of 609 samples were collected, 331 samples were collected in Haikou City, 108 samples were collected in Lingao County, 47 samples were collected in Tunchang County, and 123 samples were collected in Honghe Prefecture. A total of 80 newly emerged adult mosquitoes were randomly selected for molecular identification.

After sequencing, a total of 76 mosquito ITS2 gene sequences were obtained, with four samples being unsuccessfully sequenced. The sample sequences that were derived from Puyang City and Jiaozuo City in Henan Province of China were designated Hn01Py to Hn17Py and Hn18Jz to Hn28Jz, respectively. Those from Weifang City in Shandong Province of China were designated Sd01Wf to Sd13Wf. The sequences from Tunchang County in Haikou City and Lingao County in Hainan Province of China were designated Hn01Tc to Hn03Tc, Hn04Hk to Hn24Hk, and Hn25Lg to Hn27Lg. The samples from Honghe Prefecture in Yunnan Province of China were designated Yn01Hh to Yn08Hh. The sequence lengths ranged from 390bp to 586bp.

BLAST alignments were conducted to verify the species identities of the mosquito samples collected from various sampling sites (Table 1). These mosquitoes were identified as belonging to the genera *Culex*, *Aedes* and *Armigeres*. The obtained sequences displayed a high degree of similarity to the sequences already present in the GenBank database, with all similarity percentages exceeding 94.92%. The sequence distribution included 61 *Aedes* mosquito species, accounting for 80.26% of the total; 14 *Culex* mosquito species, accounting for 18.42%; and 1 *Armigeres* mosquito species, accounting for 1.32%.

Table 1. BLAST alignment of mosquito samples

Number	Species	Per. Ident (%)	Accession No.	GC content (%)	Length (bp)	Country
Hn01Py	*A. albopictus*	97.88	KU497619	55.71	517	CHN
Hn02Py	*A. albopictus*	98.88	MW281907	54.35	552	BR
Hn03Py	*A. albopictus*	97.83	MN062759	54.89	552	CHN
Hn04Py	*A. albopictus*	98.25	KU497619	53.87	581	CHN
Hn05Py	*A. albopictus*	98.42	KU497619	54.22	581	CHN
Hn06Py	*A. albopictus*	97.91	KU497620	53.42	584	CHN
Hn07Py	*A. albopictus*	98.07	KU497619	53.53	581	CHN
Hn08Py	*A. albopictus*	97.90	KU497619	53.97	580	CHN
Hn09Py	*A. albopictus*	98.77	KU497619	53.87	581	CHN
Hn10Py	*A. albopictus*	97.05	L22060	53.23	573	USA
Hn11Py	*A. albopictus*	98.42	KU497619	54.39	581	CHN
Hn12Py	*A. albopictus*	97.90	KU497619	53.61	582	CHN
Hn13Py	*A. albopictus*	98.25	KU497619	54.14	580	CHN
Hn14Py	*A. albopictus*	97.83	KU497619	54.53	552	CHN
Hn15Py	*A. albopictus*	99.10	KU497619	54.78	555	CHN
Hn16Py	*A. albopictus*	98.25	KX495928	53.76	545	VN
Hn17Py	*A. albopictus*	97.23	L22060	53.14	574	USA
Hn18Jz	*C. quinquefasciatus*	97.90	U22122	53.14	574	USA
Hn19Jz	*A. albopictus*	97.48	KU497619	57.95	447	CHN
Hn20Jz	*A. albopictus*	97.79	AB231675	54.47	481	JPN
Hn21Jz	*A. albopictus*	97.63	KU497619	54.08	551	CHN
Hn22Jz	*A. albopictus*	98.25	KU497619	54.47	582	CHN
Hn23Jz	*C. quinquefasciatus*	97.50	GU562872	52.13	516	USA
Hn24Jz	*A. albopictus*	99.13	KU497619	54.36	585	CHN
Hn25Jz	*A. albopictus*	97.90	KU497619	53.87	581	CHN
Hn26Jz	*C. quinquefasciatus*	99.38	KX866008	53.39	487	AUS
Hn27Jz	*A. albopictus*	96.88	L22060	53.41	573	USA
Hn28Jz	*A. albopictus*	98.08	KU497619	54.20	583	CHN
Sd01Wf	*A. albopictus*	97.65	KU497619	55.25	552	CHN
Sd02Wf	*A. albopictus*	97.57	L22060	53.14	572	USA
Sd03Wf	*A. albopictus*	96.09	AB231675	53.60	569	JPN
Sd04Wf	*A. albopictus*	99.56	MN062759	53.87	581	IL
Sd05Wf	*A. albopictus*	97.84	KU497619	51.63	554	CHN
Sd06Wf	*A. albopictus*	98.60	L22060	53.16	570	USA
Sd07Wf	*A. albopictus*	97.64	KU497618	52.52	556	CHN
Sd08Wf	*A. albopictus*	97.68	AB231675	53.55	577	JPN
Sd09Wf	*A. albopictus*	98.78	KU497619	54.70	585	CHN
Sd10Wf	*A. albopictus*	96.73	KU497618	52.10	547	CHN
Sd11Wf	*A. albopictus*	94.92	L22060	52.18	550	USA
Sd12Wf	*A. albopictus*	96.72	L22060	52.86	575	USA
Sd13Wf	*A. albopictus*	99.64	AB231675	53.67	572	JPN
Hn01Tc	*L. fuscanus*	95.86	MF288840	54.12	510	CHN
Hn02Tc	*A. albopictus*	98.43	KU497619	54.36	585	CHN
Hn03Tc	*A. albopictus*	97.55	KU497619	53.36	581	CHN
Hn04Ml	*A. albopictus*	98.78	KU497618	54.60	586	CHN
Hn05Ml	*C. quinquefasciatus*	98.65	U22128	52.31	518	USA
Hn06Ml	*C. quinquefasciatus*	99.80	U33026	52.85	509	USA
Hn07Ml	*C. quinquefasciatus*	99.41	DQ341106	52.45	511	ZA
Hn08Ml	*A. albopictus*	97.22	L22060	53.32	572	USA
Hn09Ml	*A. albopictus*	99.47	KU497619	54.37	583	CHN
HnM10l	*A. albopictus*	99.12	KU497619	53.87	581	CHN
Hn11Xy	*C. quinquefasciatus*	99.40	KX866008	53.40	515	AUS
Hn12Xy	*C. quinquefasciatus*	98.84	GU562872	53.17	519	USA
Hn13Xy	*C. quinquefasciatus*	99.41	U22131	52.74	512	USA
Hn14Xy	*C. quinquefasciatus*	99.23	FJ416044	53.85	390	JPN
Hn15Xy	*C. quinquefasciatus*	99.22	GU562872	53.01	515	USA
Hn16Xy	*A. albopictus*	98.95	KU497619	54.53	585	CHN
Hn17Xy	*A. albopictus*	96.91	KU497618	53.61	554	CHN
Hn18Hk	*A. albopictus*	98.78	KU497619	54.26	586	CHN
Hn19Hk	*A. subalbatus*	99.10	KU497621	49.34	456	CHN
Hn20Hk	*C. quinquefasciatus*	98.65	U22132	53.00	517	USA
Hn21Hk	*A. albopictus*	98.43	KU497619	54.26	586	CHN
Hn22Hk	*A. albopictus*	97.30	KU497619	54.43	553	CHN
Hn23Hk	*A. albopictus*	97.65	KU497619	54.18	550	CHN
Hn24Hk	*L. fuscanus*	95.64	MF288840	54.31	510	CHN
Hn25Lg	*A. albopictus*	98.25	L22060	53.51	570	USA
Hn26Lg	*A. albopictus*	98.74	KU497619	54.42	555	CHN
Hn27Lg	*A. albopictus*	97.47	KU497619	55.25	552	CHN
Yn01Hh	*A. albopictus*	98.78	KU497619	54.60	586	CHN
Yn02Hh	*A. albopictus*	98.04	AB231675	53.54	579	JPN
Yn03Hh	*A. albopictus*	98.60	KU497619	54.43	586	CHN
Yn04Hh	*A. albopictus*	98.78	KU497619	54.43	586	CHN
Yn05Hh	*A. albopictus*	98.07	KU497619	53.87	581	CHN
Yn06Hh	*A. albopictus*	98.60	KU497617	53.62	580	CHN
Yn07Hh	*A. albopictus*	98.21	AB231675	54.17	576	JPN
Yn08Hh	*A. albopictus*	98.21	AB231675	53.91	575	JPN

Sequence features

The molecular identification of 76 sequences revealed that the average A+T and G+C contents were 46.23% and 53.77%. The alignments revealed comparisons between multiple mosquito sequences (Figure 2). The counts for constant registration sites, mutation sites, reduced information sites, and monomorphic sites were 67, 453, 431, and 22, respectively. Mutation sites represented 86.95% of the total.

Furthermore, upon blasting the 76 sample sequences in GenBank, 20 sequences were retrieved. A comparison of these sequences revealed that the average contents of A+T and G+C were 46.60% and 53.40%, respectively. Within these sequences, the counts for constant anchor sites, mutation sites, reduced information sites, and monomorphic sites were 159, 330, 247, and 83, respectively, with mutation sites representing 66.13% of the total.

Taken together, the 76 sample sequences and the 20 sequences obtained from BLAST totaled 96 sequences. Within these 96 sequences, the average content of A+T was 51.69%, while that of C+G was 48.31%. Additionally, the numbers of constant anchor sites, mutation sites, reduced information sites, and monomorphic sites were 51, 451, 430, and 21, respectively, with mutation sites accounting for 89.84% of the total.

The pairwise sequence alignment (Figure 3a) effectively revealed the clustering patterns of 76 sequences, reflecting a strong alignment with the unique attributes of the sample collection points. Notably, the lower portion of the figure shows significant disparities among the sample sequences that were collected from various urban areas within Henan Province of China. Although there are certain variations in sequence consistency within Puyang City, these differences are relatively minor compared to those observed across different provinces, which aligns agree well with the actual

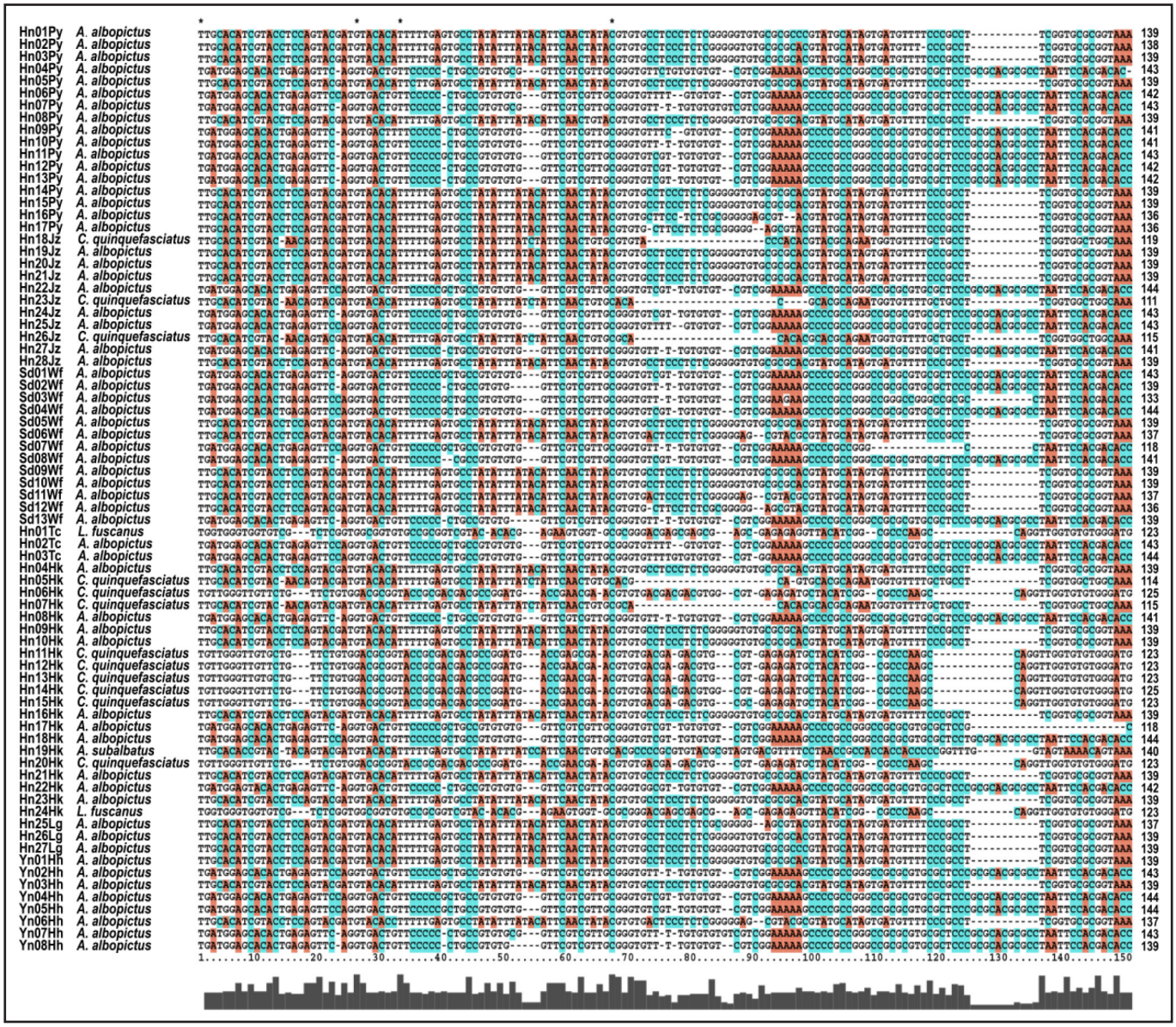


Figure 2. Seventy-six sequences were used for multiple sequence alignment via ClustalW in MEGA.

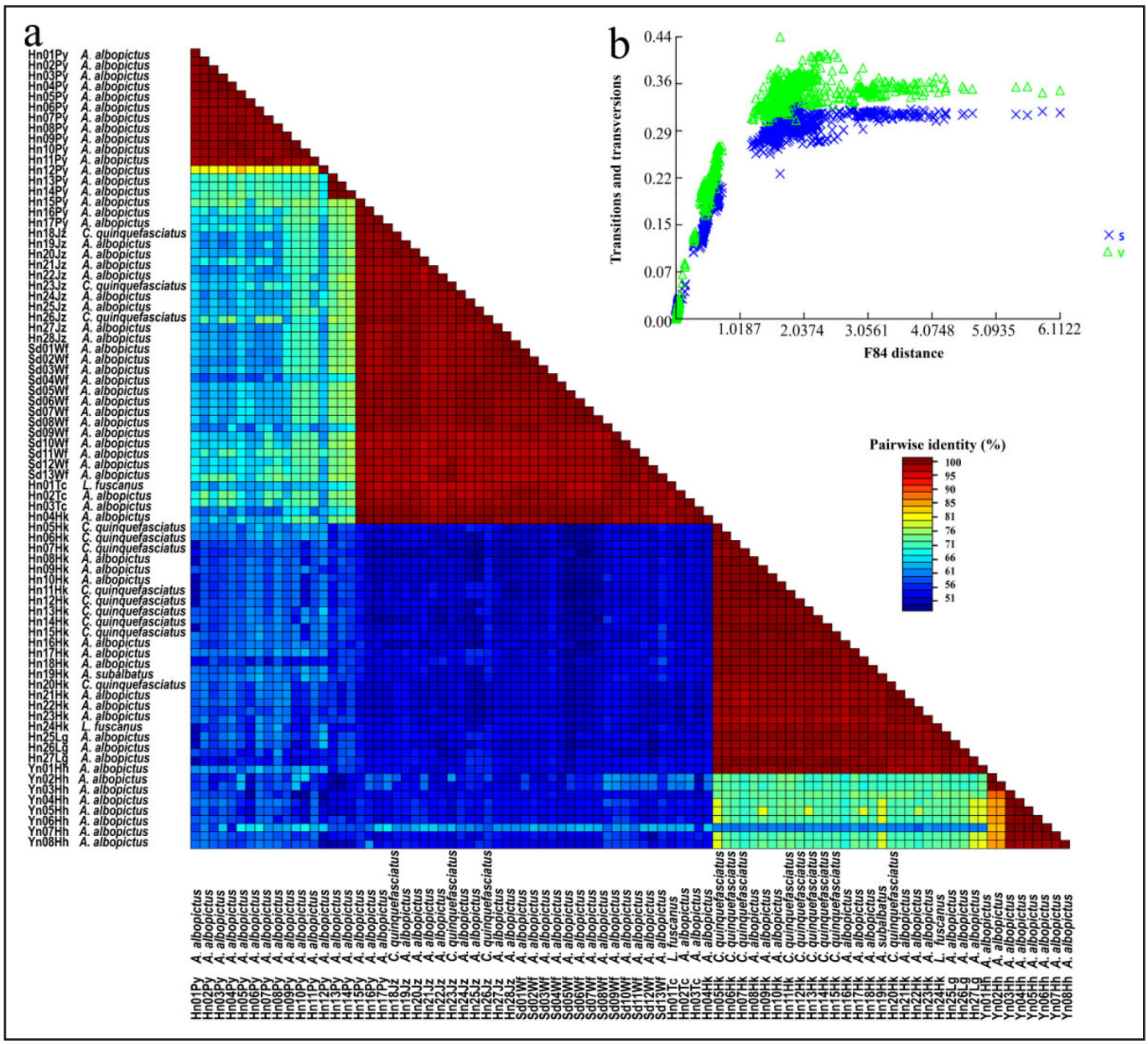


Figure 3. (a) Color-coded pairwise identity matrix generated from 76 mosquito genomes. Each colored cell represents a percentage identity score between two sequences (one indicated horizontally to the left and the other vertically at the bottom). A colored key indicates the correspondence between the pairwise identities and the colors displayed in the matrix. (b) Analysis of the ITS2 gene replacement saturation in mosquitoes by DAMBE.

observations. Furthermore, from a north south perspective, Henan and Shandong Provinces of China, both of which are located in the north, exhibit narrower color contrasts in their respective maps than do the southern provinces, indicating a certain level of sequence consistency.

Haplotype polymorphisms

The haplotype polymorphism analysis of the ITS2 gene sequence revealed the existence of 39 distinct haplotypes across all sequences (Figure 4). There were 26 haplotypes of *Aedes albopictus*, 10 haplotypes of *Culex quinquefasciatus*, 2 haplotypes of *Lutzia fuscanus*, and 1 haplotype of *Armigeres subalbatus*. Each colored circle represents an individual haplotype, with the size of the circle indicating the number of samples belonging to that haplotype. Lines connecting two circles indicate a relationship between these haplotypes. Additionally, red dots indicate haplotypes that were theoretically inferred by the software system but lacked actual sample data for confirmation. Hap1 encompassed 11 sequences, with five originating from Henan Province of China, two from Shandong Province of China, three from Hainan Province of China, and one from Yunnan Province of China. Hap8 comprised six sequences distributed as follows: one from Henan Province of China, three from Shandong Province of China, one from Hainan Province of China, and one from Yunnan Province of China. Furthermore, Hap9 contained 10 sequences, including three from Henan Province of China, one from Shandong Province of China, four from Hainan Province of China, and two from Yunnan Province of China. The fact that these three haplotypes were found in samples from different provinces suggests migration patterns and genetic exchanges among mosquitoes across the four provinces. The existence of multiple haplotypes indicated genetic diversity within the mosquito population. Notably, the overall haplotype distribution did not exhibit a single star pattern, indicating the absence of a dominant population.

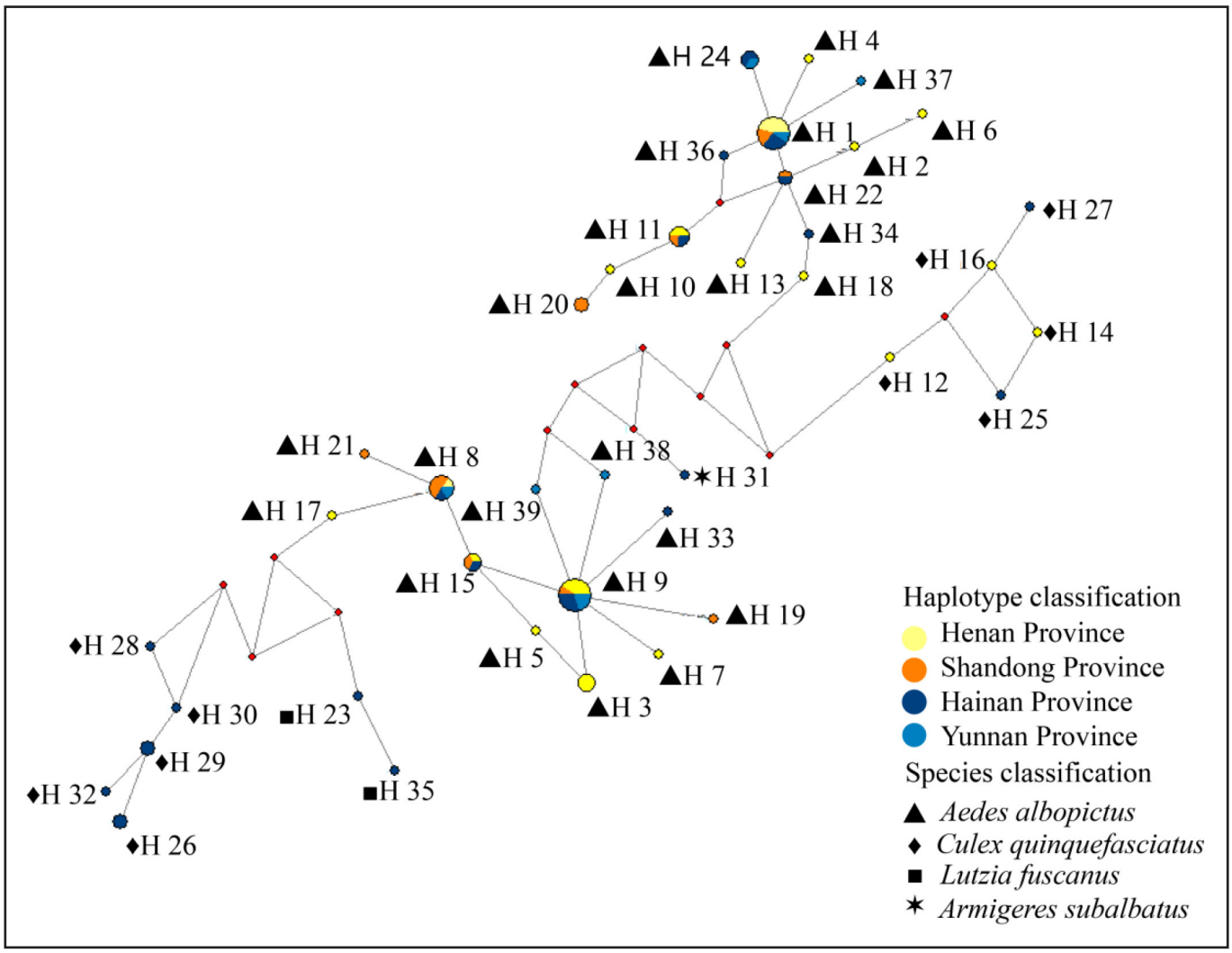


Figure 4. Haplotype network diagram of 76 mosquito gene sequences. Samples were collected in Henan, Shandong, Hainan and Yunnan provinces of China. Dashes on the lines indicate the number of mutations. The color of each circle represents the geographic location of the mosquito collection site. Different symbols before haplotype indicate different species.

Phylogenetic relationships

A graph showing the nucleotide substitution saturations (Figure 3b) was generated by plotting the uncorrected P distances along the horizontal axis against transitions and transversions along the vertical axis. By comparing the simple index of substitution saturation (Iss) with the critical Iss value (Iss.c) of the ITS2 genes, it was determined that Iss<Iss.c and P<0.001, indicating that base substitution was not saturated. This finding was considered suitable for both phylogenetic analysis and phylogenetic trees display (Figure 5). The sequences of Hn01Py~Hn03Py, Hn05Py, Hn08Py, Hn14Py~Hn17Py, Hn19Jz~Hn21Jz, Hn28Jz, Sd05Wf~Sd06Wf, Sd09Wf~Sd12Wf, Hn04Hk, Hn09Hk~Hn10Hk, Hn16Hk, Hn21Hk, HnHk23, Hn25Lg~Hn27Lg, Yn01Hh, Yn03Hh, and Yn06Hh, as well as those from GenBank (MN062759, AB231675, KX495928, MW281907, KU487619, KU497617, L22060, and KU497618) converged into a single clade, revealing that they were *Aedes albopictus*. These mosquitoes were collected from various locations, including Fujian, Palestine, Brazil, Vietnam, and Japan, with the closest genetic ties to mosquitoes from Fujian, China. On the other hand, sequences such as Hn18Jz, Hn23Jz, Hn26Jz, Hn05Hk, Hn06Hk, Hn07Hk, Hn11Hk~Hn15Hk, and Hn20Hk, as well as those from GenBank (DQ341106, FJ416044, U33026, KX866008, GU562872, U22122, U22128, U22131, and U22132) converged into a separate clade, indicating that they belonged to *Culex quinquefasciatus*. These mosquitoes were found in diverse locations such as Fujian, China, the United States, Australia, Bangladesh, and South Africa. Among these sequences, the most closely related sequence was U33026, which originated from the United States. Furthermore, sequences including Hn04Py, Hn06Py~Hn07Py, Hn09Py~Hn13Py, Hn22Jz, Hn24Jz~Hn25Jz, Hn27Jz, Sd01Wf~Sd04Wf, Sd07Wf~Sd08Wf, Sd13Wf, Hn02Tc~Hn03Tc, Hn08Hk, Hn17Hk, Hn18Hk, Hn22Hk, Yn02Hh, Yn04Hh~Yn05Hh, and Yn07Hh~Yn08Hh converged into another clade, all belonging to *Aedes albopictus*. Additionally, the Hn19Hk and KU497621 sequences clustered together, and these samples were identified as *Armigeres subalbatus*. Finally, the Hn01Tc and Hn24Hk sequences converged into a clade, revealing them to be *Lutzia fuscanus*.

Inference of Migration Paths

By integrating haplotype polymorphisms and phylogenetic relationships, it was speculated that Hn01Py, Hn03Py to Hn09Py, Hn11Py to Hn15Py, Hn19Jz, Hn21Jz, Hn22Jz, Hn24Jz, Hn25Jz, Hn28Jz, which were from Henan Province of China, Sd01Wf, Sd05Wf, Sd07Wf, Sd09Wf, Sd10Wf, which were from Shandong Province of China, Hn01Tc to HnTc03, Hn04Ml, Hn09Ml, Hn10Ml, Hn16Xy, Hn17Xy, Hn18Hk, Hn19Hk, Hn21Hk to Hn24Hk, Hn26Lg, Hn27Lg, which were from Hainan Province of China, Yn01Hh, Yn03Hh to Yn06Hh, which were from Yunnan Province of China, originating

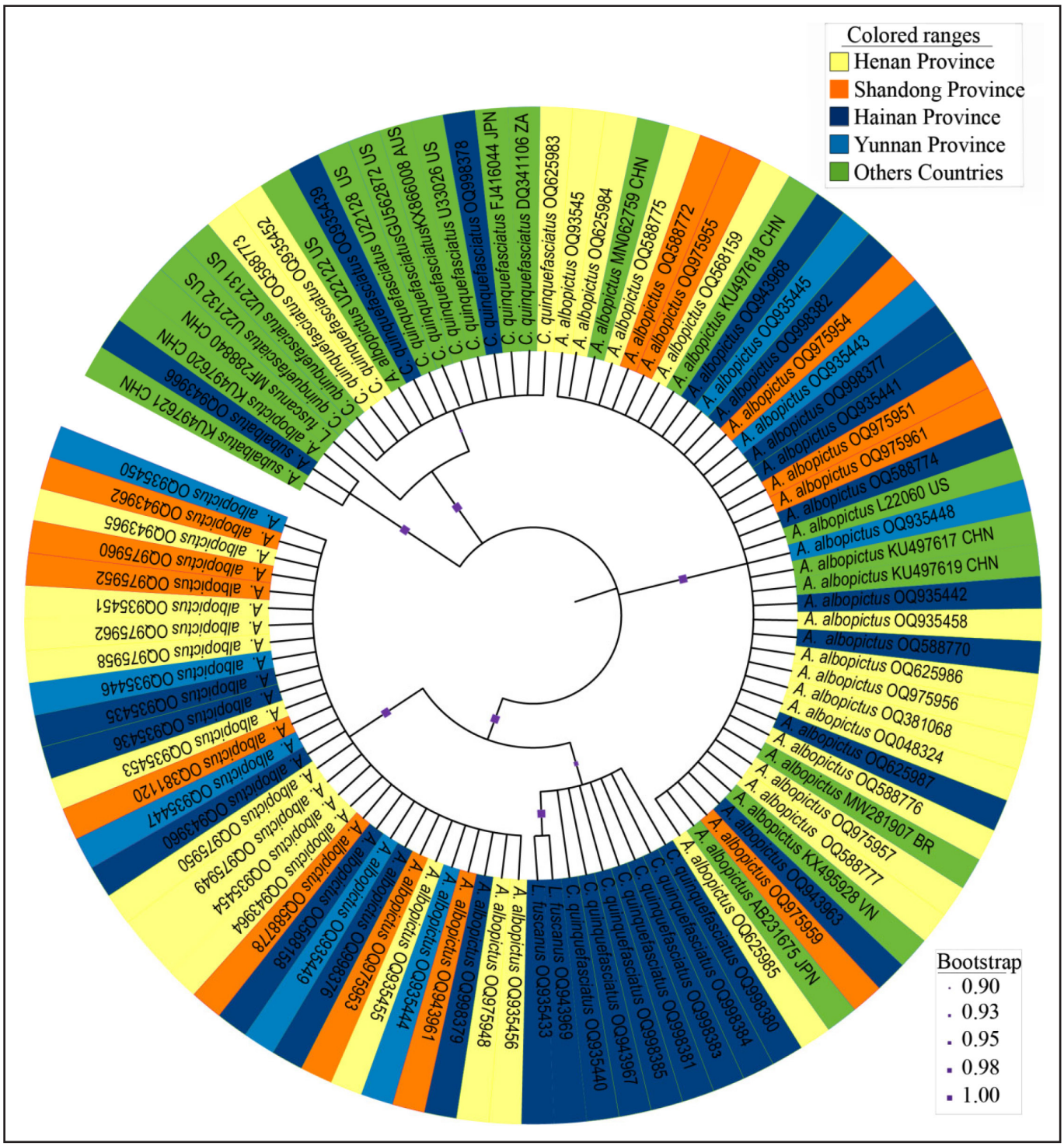


Figure 5. A phylogenetic tree were constructed based on 20 ITS2 sequences in GenBank and 76 raw sequences. Different colors represent different sampling points, and sequences obtained from GenBank are annotated with the origin at the tail of each branch.

from various localities in China, might have originated from Fujian, Guangdong Provinces, or Chongqing City of China. Furthermore, Hn10Py, Hn17Py, Hn17Jz, Hn23Jz, Hn27Jz, which were from Henan Province of China, Sd02Wf, Sd06Wf, Sd11Wf, Sd12Wf, which were from Shandong Province of China, Hn05Ml, Hn06Ml, Hn08Ml, Hn12Xy, Hn13Xy, Hn15Xy, Hn20Hk, and Hn25Lg, which were from Hainan Province of China, might have migrated from the United States. Hn16Py was postulated to have migrated via Vietnam, whereas Hn20Jz, which was from Henan Province of China, Sd03Wf, Sd08Wf, Sd13Wf, which were from Shandong Province of China, Hn14Xy, which was from Hainan Province of China, Yn02Hh, Yn07Hh, and Yn08Hh, which were from Yunnan Province of China, might have migrated from Japan. Additionally, Hn26Jz which was from Henan Province of China and Hn11Xy which was from Hainan Province of China might have migrated from Australia, Sd04Wf which was from Shandong Province of China from Israel, Hn07Ml which was from Hainan Province of China from South Africa, and Hn02Py which was from Yunnan Province of China from Brazil.

DISCUSSION

The survey revealed close relationships between mosquito breeding locations and multiple variables, including container type, the amount of water present, and adjacent vegetation. The *Aedes* mosquito species exhibited a notable preponderance in household buckets, constituting 34.21% of the identified breeding locations. Puddles and canals emerged as the subsequent preferred habitats, succeeded by bushes, takeaway boxes, and tourist attractions, which accounted for 13.16%, 13.16%, 11.84%, 6.58%, and 2.63% of their breeding sites, respectively. In contrast, *Culex* mosquito species demonstrated a higher concentration in sewers, representing 6.58% of the recorded breeding sites. This was trailed by ditches, bushes, takeaway boxes, puddles, and household buckets, which comprised 3.94%, 2.63%, 2.63%, 1.32%, and 1.32% of their breeding locales, respectively. Household buckets have emerged as the most common container type, potentially due to their ubiquity and frequent utilization. As primary water storage vessels, buckets are prone to water accumulation, which aligns with previous research conducted in Mꞌrida City (Baak-Baak *et al*., 2014). The research further revealed that different mosquito species exhibit specific preferences for their breeding habitats. *Aedes albopictus* preferred to breed in flowerpots and containers made of diverse materials, whereas *Culex* mosquito species favored sewers and pools. Additionally, *Aedes* mosquito species were more inclined to lay eggs in containers with higher water levels, similar to the findings from studies that were conducted in Zika virus-affected regions. The current study highlighted that the largest mosquito populations were present in moist environments, with a high abundance of *Aedes* specimens in sheltered herbaceous layers, echoing similar observations that were made in Oldenburg (Sauer *et al*., 2021).

Mosquitoes, as vector-borne arthropod insects, are ubiquitously in tropical, subtropical, and temperate regions. The experimental results indicate that, in recent years, the breeding habitats of mosquitoes have undergone significant transformations due to changes in climate, economic growth, and population migration patterns. These alterations have had profound impacts on the genetic structure and diversity of mosquito populations, leading to a surge in genetic diversity, haplotype polymorphism, and an increased frequency of gene exchange within these populations. The mosquito species that were found in the northern and southern regions of the study area exhibited strong genetic affinities with their counterparts in various countries, including the United States, Japan, Brazil, Australia, Vietnam, South Africa, Israel, and specific regions within China, such as Chongqing and Fujian. The phylogenetic analysis conducted by Samson *et al*. (2013) predicted that these genetic relationships are likely linked to mosquito migration and species invasion patterns. Among the mosquito species that were collected during the immature stage, *Aedes* emerged as the dominant species (Allgood & Yee, 2014). *Aedes* mosquito species are a crucial vector for dengue fever, and their densities are especially high in both the northern and southern regions. The emergence of imported dengue cases poses a significant threat to global dengue epidemic prevention efforts. *Aedes albopictus* has been demonstrated to be markedly adaptable to domestic environments and is classified as an ectophilic species, which aligns with the observations. This species appears to be a dominant competitor, and asymmetric competition may explain the scarcity of *Culex quinquefasciatus* in certain areas. The findings not only increase the understanding of mosquito biology and ecology but also have significant implications for disease prevention and control strategies. Given the close genetic ties among mosquito species across different regions, it is crucial to monitor and manage mosquito populations effectively to mitigate the risk of disease transmission.

Based on haplotype analysis, Hap12, Hap14, Hap16, Hap25, and Hap27 clustered together and were identified as *Culex* mosquito species. This clustering suggested the occurrence of genetic exchange between mosquitoes in Henan and Hainan provinces of China. Furthermore, both Hap23 and Hap35 are attributed to *Lutzia fuscanus*. While Hap23 could result from a gene mutation in either *Aedes* or *Culex*, a closer examination of phylogenetic relationships indicates that the mutation is more likely to have originated from in *Culex*. On the other hand, Hap31 belongs to the species *Armigeres*, and a comprehensive analysis of haplotype and phylogenetic suggested that it is more likely to have mutated from *Aedes*.

Mosquito-borne diseases are closely linked to the selection of urban development sites. Despite the significant burdens of dengue, chikungunya, and Zika, there are currently no vaccines available on the market. The treatment strategies for all three diseases primarily aim to alleviate symptoms, and the sole effective method to mitigate the burden of these diseases lies in prevention. This prevention heavily relies on vector control (Elsinga *et al*., 2017). The southern part of China, with its emphasis on tourism development and increased human flow, represents a potential factor for the proliferation of mosquito breeding grounds. Residential areas with numerous discarded plastic food and beverage containers are particularly susceptible to becoming breeding havens. Therefore, preventing littering in these areas, intensifying awareness campaigns, and fostering collaboration with local authorities to enhance monitoring are crucial (Yang *et al*., 2020). Given the distinct climatic conditions in the north and south, along with the unique characteristics of urban development, tailored control measures for breeding sites have been implemented based on local conditions. Effective water source management has proven to be a viable method for reducing the number of mosquito breeding sites. To guide future mosquito control strategies in urban environments, potential breeding sites in construction sites and communities have been reduced by eliminating water storage containers in living and working areas, thus limiting water sources. However, urbanization brings higher temperatures and drier conditions, which, in some areas, have led to reductions in mosquito populations but also suggested mosquito migration. This emphasizes the need for ongoing analyses of mosquito phylogeny and documentation of breeding sites.

Currently, there is a paucity of studies that focus on mosquito breeding sites in China, particularly in the northern and southern regions, where no comprehensive investigations have been conducted. Consequently, the examinations of mosquito breeding sites and their phylogenetic analysis in both regions hold significant value in medicine and public health. This study not only contributes to the identification and classification of mosquito species but also improves the understanding of the genetic variations, population structures, and biological evolution within these species.

Competing Interests

The co-authors declare that they have no conflict of interest.

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REFERENCE

Alarcón-Elbal, P.M., Rodríguez-Sosa, M.A., Ruiz-Matuk, C., Tapia, L., Arredondo, Abreu, C.A., Fernández, González, A.A., Rodríguez, Lauzurique, R.M. & Paulino-Ramírez, R. (2021). Breeding Sites of Synanthropic Mosquitoes in Zika-Affected Areas of the Dominican Republic. Journal of the American Mosquito Control Association **37**: 10-19. https://doi.org/10.2987/20-6953.1

Ali, S., Samake, J.N., Spear, J. & Carter, T.E. (2022). Morphological identification and genetic characterization of Anopheles stephensi in Somaliland. Parasites & Vectors **15**: 247. https://doi.org/10.1186/s13071-022-05339-y

Allgood, D.W. & Yee, D.A. (2014). Influence of resource levels, organic compounds and laboratory colonization on interspecific competition between the Asian tiger mosquito Aedes albopictus (Stegomyia albopicta) and the southern house mosquito Culex quinquefasciatus. *Medical and Veterinary Entomology* **28**: 273-286. https://doi.org/10.1111/mve.12047

Baak-Baak, C.M., Arana-Guardia, R., Cigarroa-Toledo, N., Puc-Tinal, M., Coba-Tún, C., Rivero-Osorno, V., Lavalle-Kantun, D., Loroño-Pino, M.A., Machain-Williams, C., Reyes-Solis, G.C. *et al*. (2014). Urban mosquito fauna in Mérida City, México: immatures collected from containers and storm-water drains/catch basins. *The Southwestern Entomologist* **39**: 291-306. https://doi.org/10.3958/059.039.0207

Bandelt, H.J., Forster, P. & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37-48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Chathuranga, W.G.D., Weeraratne, T.C., Abeysundara, S.P., Karunaratne, S.H.P.P. & de Silva, W.A.P.P. (2023). Breeding site selection and co-existing patterns of tropical mosquitoes. *Medical and Veterinary Entomology* **37**: 550-561. https://doi.org/10.1111/mve.12656

Duval, P., Antonelli, P., Aschan-Leygonie, C. & Valiente, Moro, C. (2023). Impact of Human Activities on Disease-Spreading Mosquitoes in Urban Areas. *Journal of Urban Health: Bulletin of The New York Academy of Medicine* **100**: 591-611. https://doi.org/10.1007/s11524-023-00732-z

Elsinga, J., Veen, H.T., Gerstenbluth, I., Burgerhof, J.G.M., Dijkstra, A., Grobusch, M.P., Tami, A. & Bailey, A. (2017). Community participation in mosquito breeding site control: an interdisciplinary mixed methods study in Curaçao. *Parasites & Vectors* **10**: 434. https://doi.org/10.1186/s13071-017-2371-6

Giunti, G., Becker, N. & Benelli, G. (2023). Invasive mosquito vectors in Europe: From bioecology to surveillance and management. *Acta Tropica* **239**: 106832. https://doi.org/10.1016/j.actatropica.2023.106832

Jones, R.T., Ant, T.H., Cameron, M.M. & Logan, J.G. (2020). Novel control strategies for mosquito-borne diseases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **376**: 20190802. https://doi.org/10.1098/rstb.2019.0802

Lahondère, C. & Bonizzoni, M. (2022). Thermal biology of invasive Aedes mosquitoes in the context of climate change. *Current Opinion in Insect Science* **51**: 100920. https://doi.org/10.1016/j.cois.2022.100920

Li, J., Tang, J., Wu, J., Yang, M., Zhang, M., Liang, C., Zhou, H., Zhu, G. & Cao, J. (2022). Surveillance of Aedes populations in Jiangsu Province in 2020. *Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi* **35**: 63-67. https://doi.org/10.16250/j.32.1374.2022107

Librado, P. & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Muhire, B.M., Varsani, A. & Martin, D.P. (2014). SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS One* **9**: e108277. https://doi.org/10.1371/journal.pone.0108277

Rahola, N., G nay, F., Öztürk, M., Alten, B., Aqeehal, H.A., Saadawi, W.K., Shaibi, T., Kavran, M., Petrić, D., Mitrović, J. *et al*. (2022). Assessment of expertise in morphological identification of mosquito species (Diptera, Culicidae) using photomicrographs. *Parasite* **29**: 45. https://doi.org/10.1051/parasite/2022045

Samson, D.M., Qualls, W.A., Roque, D., Naranjo, D.P., Alimi, T., Arheart, K.L., Müller, G.C., Beier, J.C. & Xue, R.D. (2013). Resting and energy reserves of Aedes albopictus collected in common landscaping vegetation in St. Augustine, Florida. *Journal of the American Mosquito Control Association* **29**: 231-236. https://doi.org/10.2987/13-6347R.1

Sauer, F.G., Grave, J., Lühken, R. & Kiel, E. (2021). Habitat and microclimate affect the resting site selection of mosquitoes. *Medical and Veterinary Entomology* **35**: 379-388. https://doi.org/10.1111/mve.12506

Tamura, K., Stecher, G. & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* **38**: 3022-3027. https://doi.org/10.1093/molbev/msab120

Turan, C., Nanni, I.M., Brunelli, A. & Collina, M. (2015). New rapid DNA extraction method with Chelex from Venturia inaequalis spores. *Journal of Microbiological Methods* **115**: 139-143. https://doi.org/10.1016/j.mimet.2015.06.005

Wang, T., Fan, Z.W., Ji, Y., Chen, J.J., Zhao, G.P., Zhang, W.H., Zhang, H.Y., Jiang, B.G., Xu, Q., Lv, C.L. *et al*. (2022). Mapping the Distributions of Mosquitoes and Mosquito-Borne Arboviruses in China. *Viruses* **14**: 691. https://doi.org/10.3390/v14040691

Weeraratne, T.C., Surendran, S.N., Parakrama, & Karunaratne, S.H.P. (2018). DNA barcoding of morphologically characterized mosquitoes belonging to the subfamily Culicinae from Sri Lanka. *Parasites & Vectors* **11**: 266. https://doi.org/10.1186/s13071-018-2810-z

Wilke, A.B.B., Caban-Martinez, A.J., Ajelli, M., Vasquez, C., Petrie, W. & Beier, J.C. (2019). Mosquito Adaptation to the Extreme Habitats of Urban Construction Sites. *Trends in Parasitology* **35**: 607-614. https://doi.org/10.1016/j.pt.2019.05.009

Xia, X. (2018). DAMBE7: New and Improved Tools for Data Analysis in Molecular Biology and Evolution. *Molecular Biology and Evolution* **35**: 1550-1552. https://doi.org/10.1093/molbev/msy073

Yang, P.L., Nelson-Hurwitz, D.C. & Katz, A.R. (2020). Evaluating Potential Mosquito Breeding Sites on a University Campus. *Hawai'i Journal of Health & Social Welfare* **79**: 347-352.