Investigation of *Leishmania* infection and blood sources analysis in *Phlebotomus chinensis* (Diptera: Psychodidae) along extension region of the Loess Plateau, China

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Abstract

Background

Visceral leishmaniasis (VL) was one of the most important parasitic diseases in China, caused by *Leishmania* protozoans and transmitted by sandflies. Recently VL cases have reappeared in China, including the extension region of the Loess Plateau. The purpose of this study was to detect the infection rate of Leishmania and analyze the blood sources of the sandflies vectors to guide the development of prevention and control measures.

Methods

Sandflies were collected by light traps from rural areas in Shanxian, Henan, China in 2015, as well as in Wuxiang and Yangquan, Shanxi, China in 2017. The blood sources of sandflies were analyzed by PCR detecting the host-specific mitochondrial cytochrome *b* (mtDNA *Cyt b*) gene fragments. *Leishmania* infection in sandflies was detected by amplifying and sequencing ribosomal DNA internal transcribed spacer 1 (*ITS1*). The *Leishmania* specific antibodies in the sera of local dogs were detected by ELISA.

Results

Blood sources showed diversity in the extension region of the Loess Plateau, including human, chicken, dog, cattle, pig and goat. Multiple blood sources within a sandfly individual were observed in samples from Yangquan (17/118, 14.4%) and Wuxiang (12/108, 11.1%). *Leishmania* DNA was detected in sandflies collected from Yangquan, of which 8.5% pooled and 1.9% individual samples were positive. The *ITS1* sequences were conserved with the *Leishmania donovani* complex. The positive rate of *Leishmania* specific antibodies in dogs was 5.97%.

Conclusion

This study detected the blood sources and *Leishmania* parasites infection of sandflies by molecular methods in the extension region of Loess Plateau, China. A high epidemic risk of leishmaniasis is currently indicated by the results as the infection of *Leishmania* in sandflies, the extensive blood sources of sandflies including humans, and positive antibody of *Leishmania* in local dog sera. Given the recent increase of VL cases, asymptomatic patients, dogs and other potential infected animals should be screened and treated. Furthermore, the density of sandflies needs to be controlled and personal protection should be strengthened.

1 Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a sandfly borne disease caused by *Leishmania* protozoans. VL was one of the most important parasitic diseases in China [1–4]. At present, VL is mainly endemic in western China, and focal and sporadic cases occurred in Xinjiang, Inner Mongolia, Gansu, Sichuan, Shaanxi, and Shanxi [2, 3]. A total of 3,337 cases were reported from 2004 to 2012, of which

97.03% were distributed in Xinjiang, Gansu and Sichuan [5]. In 2018, 180 cases of VL were reported in 78 counties of 11 provinces in China, mainly distributed in Gansu, Shanxi and Shaanxi Province, and the epidemic area has expanded [6].

There are three epidemic types of VL described in China, the anthroponotic type, the zoonotic mountain type and the zoonotic desert type [7]. The zoonotic desert type VL was dominated in Xinjiang and Inner Mongolia, China. The extension region of Loess Plateau in Henan, Shaanxi and Shanxi Provinces has been a representative of the zoonotic mountain type VL endemic region. The characteristics of the zoonotic mountain type VL in China include: the pathogen was mainly *Leishmania infantum,* the vector was *Phlebotomus chinensis,* and the zoonotic hosts were dog (*Canis familiaris)* as well as raccoon dog (*Nyctereutes procyonoides)* [8–11]. VL was eliminated in most endemic areas after stringent implementation of control programs by the government in the 1950s [8]. No VL case was reported in Henan Province from 1983 to 2013 [4]. However, local VL cases began to reappear after that [12, 13]. In 2018, 38 VL cases were reported in Shanxi Province, mainly in Yangquan City (15 cases) and Pingding County (8 cases); 3 and 27 cases reported in Henan and Shaanxi Provinces[6].

Blood meal identification is important evidence for the determination of the host preferences of hematophagous arthropods. Detection of *Leishmania* infection in sandflies and animal hosts could provide critical information to estimate the vector competence and assess the epidemic risk of VL in the endemic areas [14, 15]. However, there was no report on *Leishmania* infection and blood source animals of sandflies in the extension region of Loess Plateau.

In order to explore the reasons for the VL recurrence in the area, and provide a scientific basis for the development of prevention and control measures, we collected sandflies from Shanxian in Henan Province, Yangquan and Wuxiang in Shanxi Province, which are located in the extension region of the Loess Plateau, China. *Leishmania* infection and blood sources were detected, and the *Leishmania* specific antibodies were determined in the sera of local dogs.

2 Materials And Methods

2.1 Ethics statement

This study was carried out in strict accordance with the National Natural Science Foundation of China ethical guidelines for biomedical research involving living animals and human subjects.

2.2 Sandfly collection and species identification

The sandfly samples were collected at three sites located in the extension region of the Loess Plateau China (Table 1; Fig. 1): Xizhang Village (111.22°E/34.62°N, 985m) in Shanxian County (SX), Henan Province in July 2015, and Hedi Village (113.56°E/38.00°N, 895m) in Yangquan City (YQ) and Moyu Village (113.09°E/36.79°N, 1050m) in Wuxiang County (WX), Shanxi Province, China in June 2017.

Light traps (MYFS-HJY−1, Houji Dianzi, Dongguan China) were used to catch sandflies. With the consent of the owners, light traps were set up in utility room, cave dwelling, courtyard and chicken farm from 5:30 pm to 8:30 am, and collected manually in the evening by mouth aspirators. The captured sandflies were sorted and counted by male and female, respectively.

The fresh female sandfly adults were randomly dissected and the pharyngeal armature and spermatheca were observed under microscope. The species were identified according to the morphology [16]. The rest of the specimens were preserved in RNAfixer (Aidlab Biotechnologies, China) and brought back to the laboratory. Blood source analysis was conducted on those female specimens with visible blood residues, whereas all female sandflies were used to detect *Leishmania* infection.

2.3 The ecological niches of the sandflies

The ecological niches of the sandflies in the extension region of the Loess Plateau China were described in our published article[17]. In brief, the collection sites are located in hilly lands with altitude ranges from 895m to 1050m, with similar geographical features and typical northern temperate climate. The buildings are cave dwellings or brick houses with tile roof. There are a variety of domesticated animals in the villages, including chickens, goats, pigs, cattle and goats. Most animals were kept in caves or semi-closed livestock circles adjacent to the houses, and some animals are kept open in the courtyard..

2.4 DNA extraction and molecular identification of species

Genomic DNA of sandfly samples was extracted using DNAzol (Life Technologies, USA) following the manufacturer's instructions. The fragment of the mitochondrial cytochrome *b* (mtDNA *cyt b)* genewas amplified according to the method reported by Esseghir [18]. The primers were forward CB1 (5'-TAT GTA CTA CCA TGA GGA CAA ATA TC−3') and reverse CB3-R3A (5'-GCT AAT TAC TCC TCC TAA CTT ATT−3'). The positive PCR products were sequenced using four-color fluorescently labeled dideoxy termination method in Boshang Biotech Co., Ltd. (Shanghai, China). The sequences were Blast aligned in GenBank on the NCBI website to determine the sandfly species.

2.5 Blood sources identification

The female sandflies with visible blood residues were used for blood sources analysis, including 31 pooled and 216 individual samples. There were 12 pools from SX, 9 pools from YQ and 10 pools from WX. Every pooled sample contained 10 individuals. The individual samples include 118 from YQ and 108 from WX. The mtDNA *cyt b* fragments of different animals and human were amplified by PCR [19–22]. According to the main animal species in the collection site, PCR assay was developed with primers specific to human, chicken, goat, pig, cattle and dog. The information of primer sets was listed in Table 2. The PCR reaction was carried out in 25μl containing 1.5μl DNA template, 0.2μmol/L primers and 12.5μl

2×PCR mix reagents (Aidlab Biotechnologies, China). The PCR running parameters were starting at 94 °C for 2 min; continuing with 35 cycles of 94°C for 15s, 51°C for 30s, and 72°C for 1min; and a final extension with 72°C for 8min. The PCR products were electrophoresed on a 1.5% agarose gel to determine the size and were sequenced to confirm.

2.6 *Leishmania* spp. detection in sandflies

Leishmania infection was identified in all female sandflies. The ribosomal DNA internal transcribed spacer 1 (*ITS 1)* fragment of *Leishmania* was amplified using the following primers: forward LITSR 5' -CTG GAT CAT TTT CCG ATG–3', reverse L5.8S 5' -TGA TAC CAC TCG CAC TT–3' [23]. The PCR reaction was performed in 25μl contained 1.5μl DNA template, 0.2μmol/L primers and 12.5μl 2×PCR mix reagents. The PCR temperature profile was as follows: starting at 94°C for 2min; continuing with 35 cycles of 94°C for 30s, 52°C for 30s, and 72°C for 30s; and a final extension with 72°C for 8min. A positive control containing *Leishmania donovani* DNA (provided by National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention) and a negative control without DNA template were utilized.

2.7 Detection of anti-*Leishmania* antibody

The sera of 67 dogs (*Canis familiaris)* from YQ were provided by Yangquan Center for Disease Control and Prevention. The dogs were (0.5–14 years old). The owners of the dogs had been informed in advance and consented to the blood collection. The sera were separated by centrifugation at 3,000 rpm for 10 minutes after blood collection in the field. Then the sera were refrigerated and transported to the laboratory for testing. The *Leishmania* specific antibodies were detected using the commercialized Dogs *Leishmania* Ab ELISA kit (Fusheng Industrial Co., Ltd. Shanghai, China) in accordance with the manufacturer's instructions.

3 Results

3.1 Sandfly species identification

Thirty species were identified by morphology as *Ph. chinensis.* All other samples were identified as *Ph. chinensis* by molecular marker,which mtDNA *cyt b* gene sequence conserved with the HM747267 sequences in GenBank database. The *Ph. chinensis* is the absolutely dominant sandfly species inthe extension region of the Loess Plateau China.

3.2 Blood sources identification

The blood sources of the fed sandflies include human, pig, chicken, goat, dog and cattle (Table 3). In SX, chicken blood was positive in all 12 pooled samples (100%), followed by cattle (66.7%), human (33.3%),

dog (33.3%) and pig (16.7%), whereas goat was negative in all samples. In YQ, chicken and human were the most common blood sources. Chicken blood was identified in 88.9% of pooled samples and 59.3% of individuals, and human blood was positive in 66.7% pooled samples and 26.3% individuals. However, goat and human were the most common blood sources in WX. Goat blood was found in all pooled samples (100%) and 34.3% individuals; human blood was detected in 70.0% pooled samples and 64.8% individuals. Chicken blood was positive in 10.0% pooled samples and 21.3% individuals. Dog blood was positive in 10.0% pooled samples and 0.9% individuals.

Multiple blood sources within a sandfly were found in 54 individuals. Among them, 17 individual samples collected from YQ (14.4%), all of which were blood mixture of human and chicken. The other 37 individual sanflies were collected from WX (34.3%), including chicken+human (n = 10), goat+human (n = 24), chicken+goat (n = 1), chicken+goat+human (n = 1) and dog+goat+human (n = 1).

In general, the sandfly has a wide range of blood sources, and multiple blood sources means the sandflies take a variety of blood meal from different hosts in a short period.

3.3 *Leishmania* infection in sandflies

Out of the 59 pooled samples (10 individuals in a pooled sample) and the 108 individuals from YQ, *Leishmania* DNA was detected in five pooled samples and two individual specimens. The minimum infection rate of *Leishmania* in the sandfly population of YQ was 1.00% (7/698). All sequence of *Leishmania* amplicons were conserved with *L. donovani* complex (*L. donovani/L. infantum)* (MH200624) [24]. Of the two positive individuals, one had human blood meal while the other had chicken blood meal. No sample of SX and WX populations was positive in *Leishmania* DNA detection.

3.4 Detectionof *Leishmania* specific antibodies in dog sera

Out of the 67 dog sera samples, four were identified as positive (5.97%) in the detection of *Leishmania* specific antibodies using Dogs *Leishmania* IgG ELISA. The four positive dogs had no obvious pathological manifestations such as hair removal, desquamation, mental wilting, etc.

3.5 Comprehensive results of Yangquan

Comprehensive results were obtained in the samples collected from Yangquan Shanxi Province, China, which were summarized as below: Total of 3,599 sandflies were collected, *Ph. chinensis* was absolutely dominant species [23]. Chicken and human were the most common blood sources. *Leishmania* DNA was detected in five pooled samples and two individual specimens, which sequences were conserved with *L. donovani* complex (*L. donovani/ L. infantum)*.. The positive rate of dogs was 5.97% (4/67) in the detection of *Leishmania* specific antibodies.

4 Discussion

VL cases along the extension region of Loess Plateau in China belong to zoonotic mountain type, which patients were mostly children under 10 years of age [2, 3]. High *Leishmania* spp. infection rate was detected in local dogs, so the number of human VL cases can be reduced by the elimination and suppression of local dogs [11, 25, 26]. The dog was the principal reservoir host in this epidemic area. The primary vector of the zoonotic mountainous type VL was *Ph. chinensis,* a zoophilic species that also feeds on human. Recently, VL cases in extension region of the Loess Plateau, China were recurrence after the eradication of the disease for 20 years, which posed a challenge for exploration and control.

Sandflies have a broad range of hosts as blood sources [19, 20, 27–29]. They take blood mostly from mammals [20, 30–33] and cold-blooded animals [21]. In Jiuzhaigou Sichuan, China, swine was the dominant blood source of sandflies, followed by chickens and dogs [22]. However, there has been no report concerning the blood sources of sandflies in extension region of the Loess Plateau, China. In this study, chickens and humans were the most common blood sources of sandflies in Yangquan, Wuxiang Shanxi and Shanxian Henan from China, while dogs, goats, cattle and pigs were blood sources of sandflies as well. The proportion of blood sources was different with the locations of collection and the environment. As a good and sufficient blood source in the region, chickens may contribute to the sustainability of a large sandfly population, which was similar to what has been documented in other investigations [34, 35]. Although chickens attract sandflies in Brazil and in this study, the role of chickens in the epidemiology of the sandfly-borne diseases has not been defined yet [35].

There are also many reports on the detection of *Leishmania* spp. in animals by PCR assay, such as in bovines (5%), buffaloes (4%) and goats (16%) [36], even in desert lizards [37]. In another case, goats were believed to constitute a reservoir host of *L. donovani* in Nepal [7]. Therefore, other reservoir animal hosts of *Leishmania* should be further investigated. In Yangquan Shanxi, China, *Leishmania* was found in sandflies andthe specific antibody was also positive in local dogs. However, there was no dog blood in the sandfly samples, probably because the collection sites were far away from the dogs' haunts in Yangquan. In Shanxian and Wuxiang, where the ecologic niches of sandflies were similar to that in Yangquan, dog was one of the blood sources animals. Consequently, we believed that dogs were still the blood source of sandflies in Yangquan, which frequently moving around sandflies' habitats. Multiple blood sources were found in individual fed specimens, suggesting a complex feeding behavior, which was critical for sandflies to transmit zoonotic diseases to humans.

In China, vector sandflies with *Leishmania* infection have been reported in Sichuan and Shaanxi Province [16, 38]. The natural infection rate of a new haplotype of *L. donovani* was 1.98% in some villages in Sichuan Province [39], consistent with the results of sandfly infection rate in this study. To the best of our knowledge, this study was the first report of sandfliesinfected with *Leishmania* spp. in the extension region of the Loess Plateau, China. Monitoring natural *Leishmania* infection in sandflies would provide critical information to estimate the vector competence and assess the local epidemic risk of VL situation. Of the seven positive samples, the amplified sequences were all conserved with *L. donovani* complex (*L.*

donovani/L. infantum).. Multiple lines of evidence suggested that there were heterogeneous *Leishmania* strains in China. These strains were distinct from but phylogenetically related to *L. donovani/L. infantum* complex [24, 26, 37, 39, 40]. Our results would be a complement for the heterogeneity information of *Leishmania* in China.

Dogs were confirmed as the reservoir host of *Leishmania* spp. in mountainous type of zoonotic VL in the extension region of Loess Plateau, China [2, 3]. It has been reported that the positive rate of *Leishmania* spp. in dogs was above 50% in Jiuzhaigou Sichuan, 41.9% in Heishui Sichuan, and 77.21% in Wenxian Gansu, China [11, 25, 41]. In Shanxi Province, there was only one investigation in 1959, in which the *Leishmania* positive rate in dogs was 0.01% [16]. In this study, the positive rate of serum antibodies in dogs from Yangquan Shanxi was 5.97%, and there was no disease manifestation in these dogs. Among the seven positive sandfly samples tested, at least three contained human blood. Based on the preceding investigation, local asymptomatic patients and dogs should the important sources of VL infection, and the screening and treatment of the disease need to be strengthened.

Although we have systematically detected the blood sources, *Leishmania* infection and the antibody of *Leishmania* in dog sera in this study, there were still some limitations in this study. First, the primers for PCR detection of blood sources were designed based on the observation of the environment of the collection sites, but some blood sources animals may not be observed, so they may be missed. Moreover, the pooled samples were used for blood sources and *Leishmania* infection detection, making the positive rates not accurate. In addition, the investigation of potential reservoir hosts was insufficient in the study. In the future, we will collect more animal blood samples to detect *Leishmania* infection and antibodies, so as to provide more accurate guidance on the prevention and control of VL in the extension region of Loess Plateau in China.

5 Conclusions

This study detected the blood sources and *Leishmania* parasites infection of sandflies by molecular methods in the extension region of Loess Plateau, China. A high epidemic risk of leishmaniasis is currently indicated by the comprehensive information as below: high density of *Ph. chinensis* vector, the infection of *Leishmania* in sandflies, the extensive blood sources of sandflies including humans and dogs, and positive antibody of *Leishmania* in local dog sera. Given the recent increase of VL cases, asymptomatic patients, dogs and other potential infected animals should be screened and treated. Furthermore, the density of sandflies needs to be controlled and personal protection should be strengthened.

List Of Abbreviations

DNA, deoxyribonucleic acid;

VL, visceral leishmaniasis;

PCR, Polymerase Chain Reaction;

ELISA, Enzyme Linked Immunosorbent Assay;

cyt b, mitochondrial cytochrome b;

ITS1, internal transcribed spacer 1;

YQ, Yangquan;

WX, Wuxiang;

SX, Shanxian.

Declarations

Ethics approval and consent to participate

This study was carried out in strict accordance with the National Natural Science Foundation of China ethical guidelines for biomedical research involving living animals and human subjects. The owners of the dogs had been informed in advance and consented to the blood collection.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed to the collection of sandflies. YM and HP conceived and designed the experiments. HYC and YM identified specimens by morphological characters. HMC and HYC performed PCR assay. HP did ELISA test. FT, HP and YM did data analysis and wrote manuscript.

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Tables

Table 1. The information on sample collection of sandflies in the extension of the Loess Plateau, China

Collection site		Date	Longitude	Latitude	Altitude (m)
Xizhang Village, Shanxian County, Henan Province, China (SX)	Chicken sheds, cave dwelling and courtyard	July 2015	111.22°E,	34.62°N	985
Hedi Village, Yangquan City, Shanxi Province, China (YQ)	Chicken farm, utility room and courtyard	June 2017	113.56°E	38.00°N	895
Moyu Village, Wuxiang County, Shanxi Province, China (WX)	Livestock sheds	June 2017	113.09°E	36.79°N	1 050

Table 2. PCR primers for mtDNA-*cytb* amplification of blood sources of sandflies.

Species	Primers	Sequence (5' to 3'□	Amplicon length (bp)
Human (*Homo sapiens*)	HF	GGC TTA CTT CTC TTC ATT CTC TCC T	334
	UR	GGT TGT CCT CCA ATT CAT GTT A	
Chicken (Gallus gallus)	ChF	CAT ACT CCC TCA CTC CCC CA	802
	ChR	CCC CTC AGG CTC ACT CTA CT	
Goat (*Capra hircus*)	GoatF	AAT CAT CCG ATA CAT ACA CG	506
	GoatR	ATA TAG TTG TCT GGG TCT CC	
Pig (*Sus domesticus*)	PF	CCT CGC AGC CGT ACA TCT C	453
	UR	GGT TGT CCT CCA ATT CAT GTT A	
Cattle (*Bos taurus*)	CF	CAT CGG CAC AAA TTT AGT CG	561
	UR	GGT TGT CCT CCA ATT CAT GTT A	
Dog (*Canis familiaris*)	DF	GGA ATT GTA CTA TTA TTC GCA ACC AT	680
	UR	GGT TGT CCT CCA ATT CAT GTT A	

Table 3. Blood sources detection results of the fed sandflies in the extension region of the Loess Plateau, China.

Collection site	Samples (n)	Blood sources (Number of positive samples (%))					
		Human	Pig	Cattle	Dog	Chicken	Goat
SX	Pooled samples (12)	4 (33.3%)	2 (16.7%)	8 (66.7%)	4 (33.3%)	12 (100%)	0 (0.0%)
YQ	Pooled samples (9)	6 (66.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (88.9%)	0 (0.0%)
	Individual samples (118)	31 (26.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	70 (59.3%)	1 (0.8%)
WX	Pooled samples (10)	7 (70.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)	1 (10.0%)	10 (100%)
	Individual samples (108)	70 (64.8%)	0 (0.0%)	0 (0.0%)	1 (0.9%)	23 (21.3%)	37 (34.3%)

SX, Shanxian; YQ, Yangquan; WX, Wuxiang.

Figures

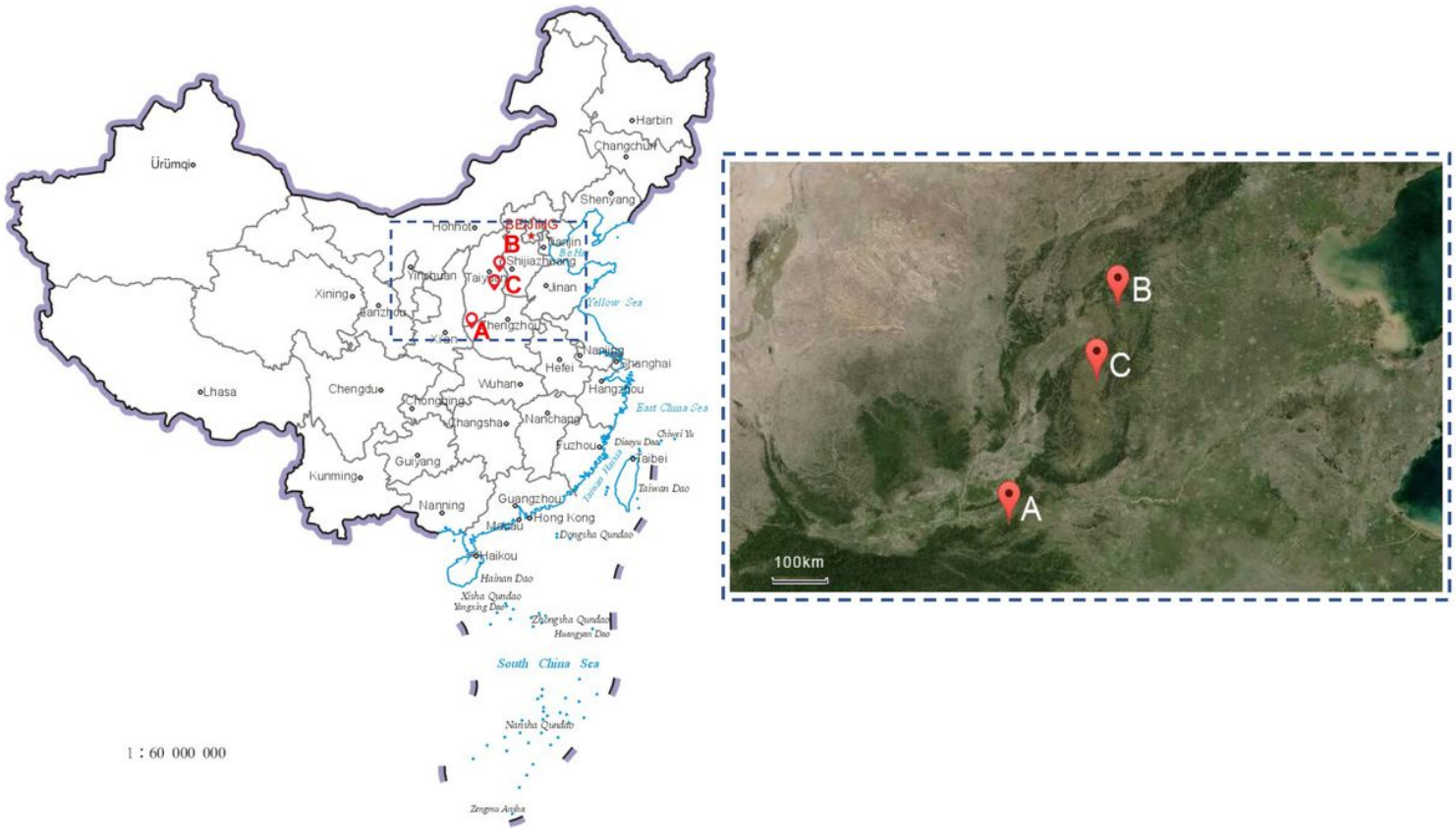

Figure 1

Schematic diagram of the collection location. A, Xizhang Village, Shanxian County, Henan Province, China; B, Hedi Village, Yangquan City, Shanxi Province, China; C, Moyu Village, Wuxiang County, Shanxi Province, China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.