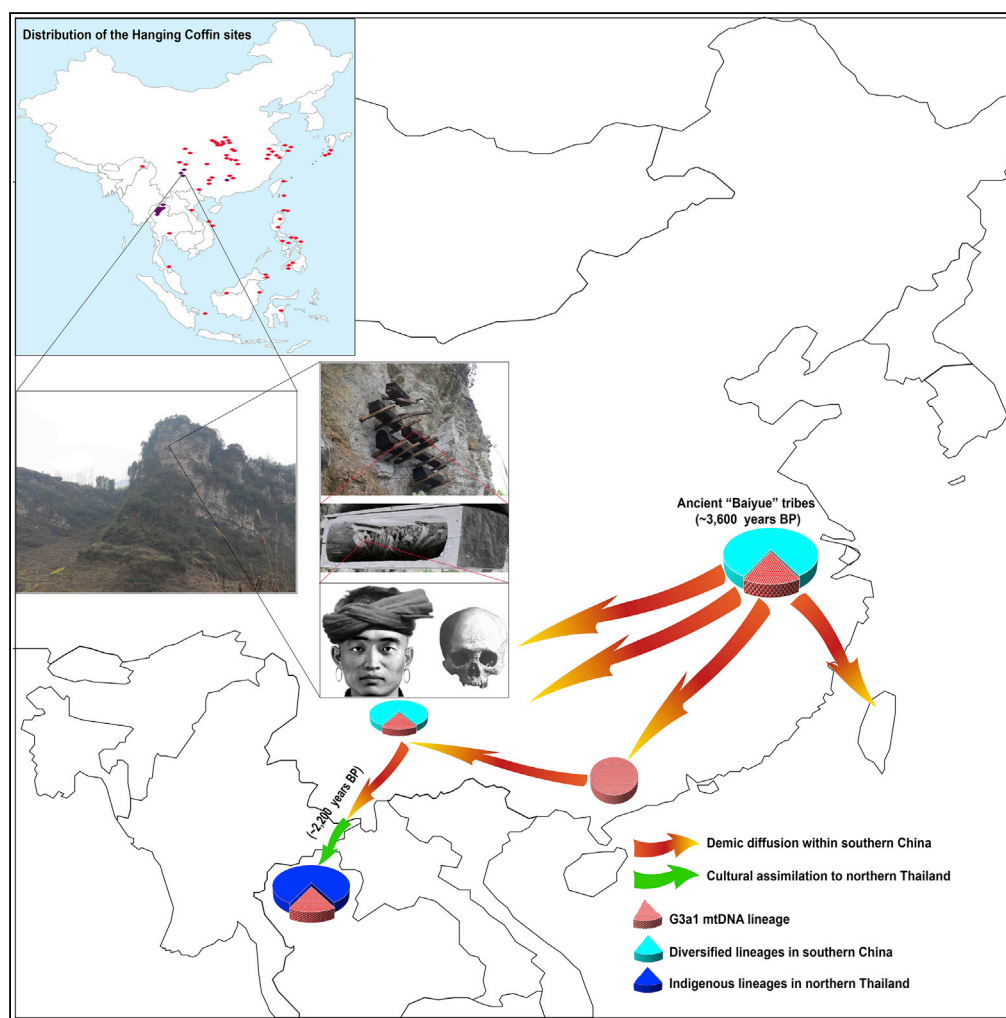


## Article

# A Matrilineal Genetic Perspective of Hanging Coffin Custom in Southern China and Northern Thailand



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## HIGHLIGHTS

The historical Hanging Coffin populations share partial genetic affinity

The mtDNA diversity of the Hanging Coffin people in southern China is high

The aDNA data are consistent with a single origin of the Hanging Coffin custom

Both cultural assimilation and demic diffusion occurred during the spread of the custom

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## Article

# A Matrilineal Genetic Perspective of Hanging Coffin Custom in Southern China and Northern Thailand

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## SUMMARY

**Hanging Coffin is a unique and ancient burial custom that has been practiced in southern China, Southeast Asia, and near Oceania regions for more than 3,000 years. Here, we conducted mitochondrial whole-genome analyses of 41 human remains sampled from 13 Hanging Coffin sites in southern China and northern Thailand, which were dated between ~2,500 and 660 years before present. We found that there were genetic connections between the Hanging Coffin people living in different geographic regions. Notably, the matrilineal genetic diversity of the Hanging Coffin people from southern China is much higher than those from northern Thailand, consistent with the hypothesized single origin of the Hanging Coffin custom in southern China about 3,600 years ago, followed by its dispersal in southern China through demic diffusion, whereas the major dispersal pattern in Southeast Asia is cultural assimilation in the past 2,000 years.**

## INTRODUCTION

Hanging Coffin (also known as *Log Coffin* in Thailand) is an ancient burial custom (Chen, 1996a), which has been practiced for ~3,000 years over wide geographic regions covering southern China, mainland Southeast Asia (MSEA), island Southeast Asia (ISEA), and near Oceania regions (Chen, 1996b). The earliest site was radiocarbon dated as  $3,620 \pm 30$  years before present (YBP), and it was proposed that the Hanging Coffin site located in Mount Wuyi of the coastal region in southeastern China was the origin area of the Hanging Coffin custom, followed by a massive population and cultural dispersal to other regions of southern China and further to the broad Southeast Asia (Chen, 1996c). Based on the radiocarbon dating series, distribution characteristics, and cultural connection pattern, it was hypothesized that the Hanging Coffin custom had an “East to West” and a “North to South” dispersion in southern China and Southeast Asia (Chen, 1996c), although the Log Coffin in northern Thailand has different shapes and sizes, carved elements, and wood materials, which was considered as a cultural adaptation to the local environment (Shoocongdej, 2018). However, the origin of the Hanging Coffin custom and its affiliated populations are still controversial. Craniological studies indicated the involvement of Daic (Tai-Kadai) people practicing the Hanging Coffin custom (Hu and Xiao, 1999a, 1999b; Ji et al., 2005; Lin and Mei, 1982), whereas the evidence of sacrificial objects showed that the Mon-Khmer (MK) (Shoocongdej, 2018) and Hmong-Mien (HM) (Lin, 1981) people might play an important role, and even Han people were likely involved (Sichuan Provincial Museum Gcc, 1980; Lin, 1982).

To test these long-standing debates, we performed mitochondrial whole genome analysis of 41 individual human remains (31 samples were sequenced in this study) from 13 Hanging Coffin sites in southern China and northern Thailand, to delineate the general population histories, present-day population affiliation, and dispersal pattern of the Hanging Coffin custom.

## RESULTS

## Authenticity of Ancient DNAs for the Hanging Coffin Samples

We collected 31 individual human remains from 3, 1, and 9 Hanging Coffin archaeological sites located in Yunnan and Guangxi provinces of China and northern Thailand, respectively. According to the published culture relics, the Yunnan sites were dated as  $1,246 \pm 65$  to  $660 \pm 30$  YBP (uncalibrated)

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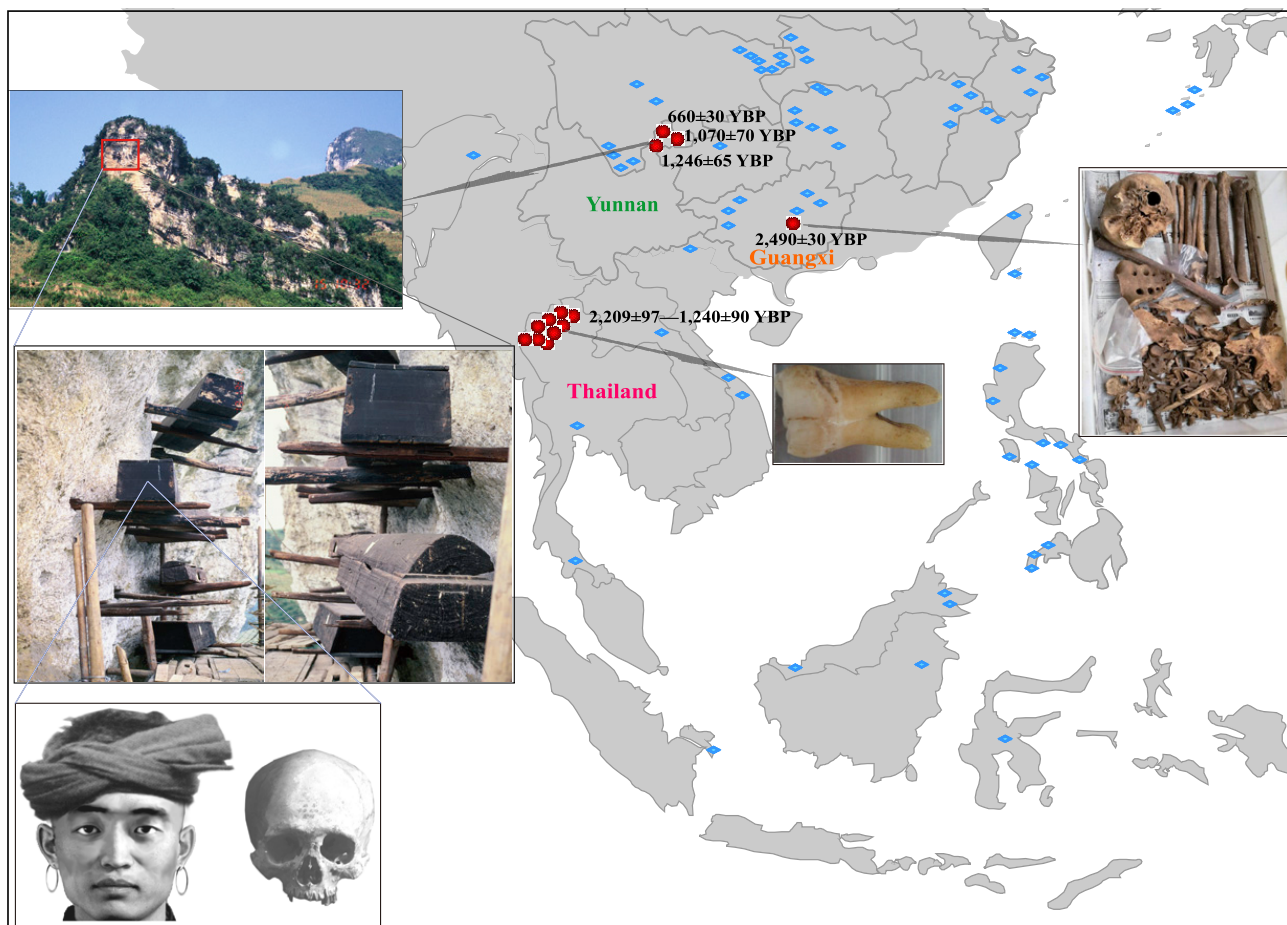
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Continued





**Figure 1. Geographic Distribution and Panorama View of the Unearthed Hanging Coffin Sites**

The red labels indicate the sampling sites with uncalibrated dating. The blue labels indicate the reported Hanging Coffin sites, and very few of them contained human remains. We conducted a medicolegal cranial reconstruction using an almost complete skull from the Washi Hanging Coffin site (the bottom left panel). The two panels on the right show the unearthed human remains from the Hanging Coffin sites. The distribution map of the reported Hanging Coffin sites was redrawn based on the map in the book entitled “Cliff-Burial in China” (Chen, 1996). See also in Figure S3.

(radiocarbon-dated in Peking University, China) (Ji et al., 2005) and the northern Thailand sites were dated as  $2,209 \pm 97$  to  $1,240 \pm 90$  YBP (uncalibrated) (Figure 1) (McCull et al., 2018; Shoocongdej, 2018). For the Guangxi site, we performed radiocarbon dating using wood chips sampled from the coffins (Beta Analytic testing laboratory, USA), and the uncalibrated date is  $2,490 \pm 30$  YBP (Figure S3C) and the calibrated date is 2,730–2,460 YBP.

We extracted ancient DNAs (aDNAs) from these human remains using the published method (Allentoft et al., 2015; Dabney et al., 2013; Rohland and Hofreiter, 2007). With the use of the human mtDNA probes (Enk et al., 2014), we enriched the aDNA extracts for the mitochondrial genome (see details in Supplemental Information). We then sequenced the constructed aDNA libraries using the Illumina HiSeq X Ten platform. We first assessed the authenticity of the obtained mtDNA genome data by evaluating several key indexes, including terminal damage rate (Figure S1A), average fragmental length, mapping rate, as well as mtDNA contamination rate. All samples showed damage patterns typical of aDNA with minimal amounts of contamination (Table S1). The sequencing depth is high ( $>10\times$ ) for most of the samples, from which we obtained complete mtDNA genome sequences ( $>95\%$ ), and only three samples had relatively low coverages (66.55%–88.88%) and were excluded from the following analyses. In addition, we included the published mtDNA genome data of 10 Log Coffin samples from the Long Long Rak archaeological site, northern Thailand (McCull et al., 2018).

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### Maternal Affinity of the Hanging Coffin Samples Revealed by the mtDNA Haplotype

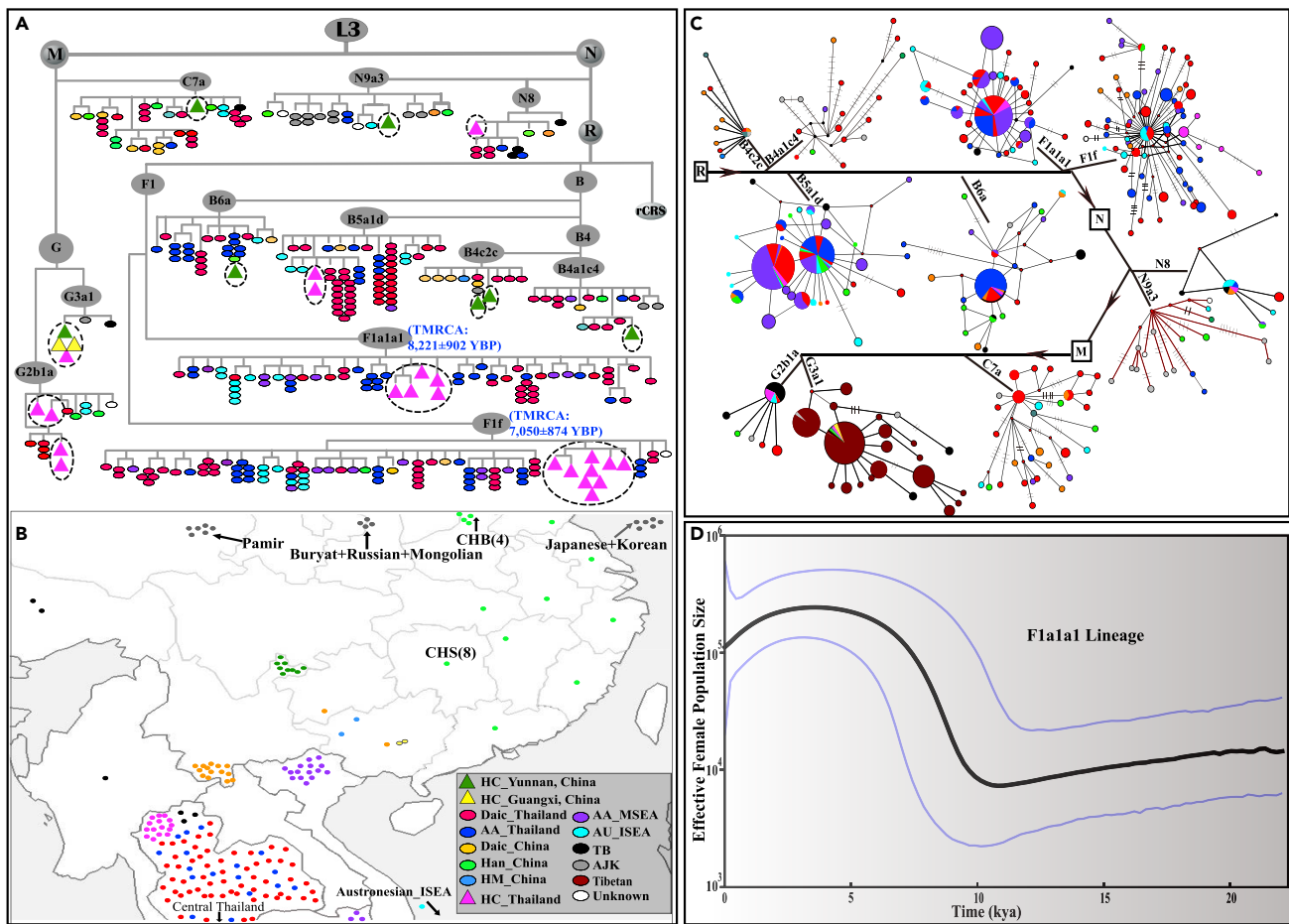
Considering the same burial custom, similar relic culture, and close geographic locations, we grouped the Hanging Coffin samples into two populations from Yunnan and Thailand, respectively. In view of mtDNA haplotype, the Hanging Coffin samples from Yunnan were highly diversified, and among the nine individuals, we detected eight different mtDNA lineages, including B4c2c, B4a1c4, B6a, C7a, F3a1, G3a1, N9a3, and R9. In contrast, the haplotype diversity of the samples from northern Thailand is relatively low, and only 7 lineages (B5a1d, F1a1a1, F1f, F1c1a2, G2b1a, G3a1, and N8) were detected in 28 individuals. The two Guangxi samples both belong to the G3a1 lineage, which is also present in both Yunnan and northern Thailand.

To relate these Hanging Coffin samples to current human populations, we collected the mtDNA genome data of 280 Asia-Pacific populations (28,618 sequences) from the previous publications (Table S2). We first constructed a phylogenetic tree by integrating our data with the published 433 complete mtDNA genome sequences (Brandao et al., 2016; Cavadas et al., 2015; Delfin et al., 2014; Genomes Project et al., 2015; Jiang et al., 2014; Jinam et al., 2012; Ko et al., 2014; Kong et al., 2003; Kutanan et al., 2017, 2018; Lippold et al., 2014; Liu et al., 2012; Ma et al., 2016; Macaulay et al., 2005; Peng et al., 2018; Tanaka et al., 2004; Zhang et al., 2008, 2013) (Figures 2A, 2B, and S2). The results show that the Hanging Coffin individuals are closely related with several present-day populations in southern China and MSEA, including the Daic populations from southern China (such as the Dai, Shui, and Zhuang ethnic groups) and northern Thailand (the Tai ethnic group), the MK populations from northern Thailand, as well as the HM populations from southern China (such as Yao in Guangxi) and northern Vietnam. In particular, we observed several cases of identical mtDNA mutation motifs between the Hanging Coffin samples and the current populations. For example, in the B6a lineage, the Yunnan Hanging Coffin sample (WXWS2) shares the same mutation motif with six present-day MK individuals from northern Thailand and one Han individual from southern China. In the B4c2c lineage, the Yunnan Hanging Coffin sample (DSG-4) shares the same mutation motif with one Thai individual from northern Thailand and one Dai individual from southwestern China. In addition, in the C7a lineage, the mtDNA haplotype of the Hanging Coffin sample from Yunnan (WXWS-1) is ancestral to many present-day individuals including Thai, MK, and Karen populations from northern Thailand, consistent with the proposed “North to South” dispersal route (Chen, 1996b) (Figures 2A, 2B, and S2). Importantly, the Hanging Coffin populations from southern China (Yunnan and Guangxi) and northern Thailand share the G3a1 lineage, an indication of a close genetic affinity, suggesting that the custom dispersed to different regions in a relatively short period of time. The genetic affinity of the Hanging Coffin samples is further supported by network analysis with more global populations using the hypervariable segment-I and the mitochondrial genome sequence data (Figure 2C).

Although there is genetic affinity among the Hanging Coffin populations from different geographic regions, we saw a clear divergence between the Yunnan and the northern Thailand populations as only one haplotype (G3a1) out of the 14 detected haplotypes is shared between them. For the northern Thailand population, according to the Bayes Skyline Plot simulation, the prevalent mtDNA lineages (B5a1d, F1a1a1, and F1f) all show a rapid population expansion starting about 7,500 YBP (~8,200 YBP for the F1a1a1 lineage and ~7,000 YBP for the F1f lineage) (Figures 2A, 2C and 2D). This date not only precedes the known massive migration of Thai people from southern China into Thailand between the 8th and 10th centuries (He, 2015; Pittayaporn and Pittayawat, 2014) but also is earlier than the earliest radiocarbon date (~2,200 YBP) of the Log Coffin in northern Thailand. Given that these mtDNA lineages are also prevalent in the present-day MK populations from northern Thailand, we speculate that these mtDNA lineages likely originated locally. In other words, the Log Coffin human remains from northern Thailand were probably due to cultural assimilation rather than demic diffusion of the Hanging Coffin custom.

### Maternal Affinity of the Hanging Coffin Samples with Current Asia-Pacific Populations

We analyzed the relationships among the Hanging Coffin populations and the present-day Asia-Pacific populations. First, we constructed a PCA (principal-component analysis) map of 280 populations based on their mtDNA haplotype frequencies (Table S2). The Guangxi Hanging Coffin site has only two samples and was therefore excluded. The two Hanging Coffin populations cluster closely with each other, and they both belong to the “Southern Population” group (Figure S1B). Further analysis including only those “Southern Populations” indicates that the Hanging Coffin populations from Yunnan and northern Thailand cluster together with one Thai, two MK, and one Tibeto-Burman (Karen people) populations from northern Thailand and one MK, one Austronesian, and one HM populations from Vietnam (Figure 3A). The PCA



**Figure 2. The mtDNA Genome Diversity of the Hanging Coffin Populations**

(A) The schematic phylogeographic tree of the Hanging Coffin related mtDNA lineages, which was constructed based on the complete mtDNA sequences in Figure S2. The Hanging Coffin samples are those triangles highlighted with broken circles.

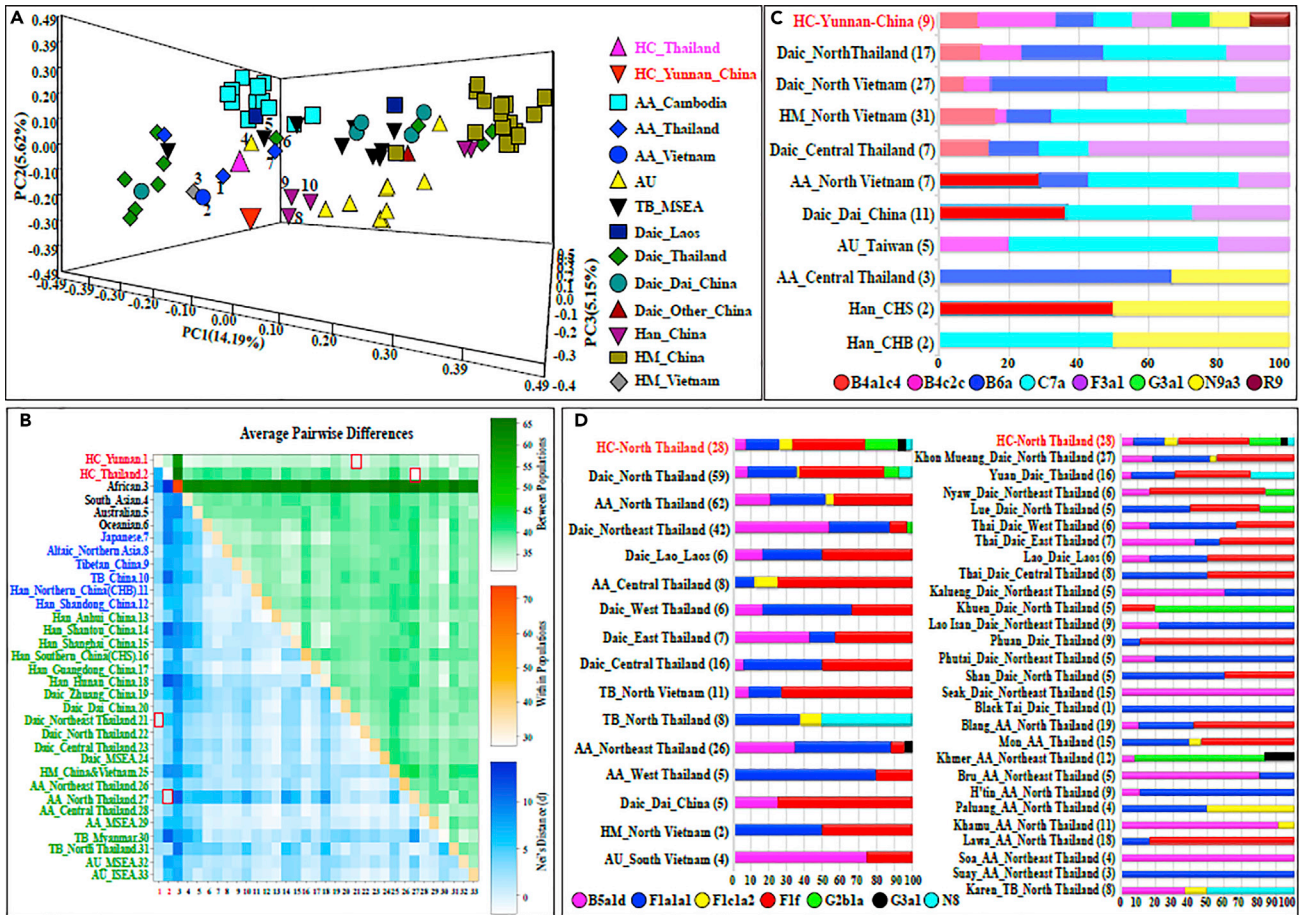
(B) The geographic distribution of the included present-day populations.

(C and D) (C) The network of mtDNA lineages based on hypervariable segment-1 and complete mtDNA sequences. The short lines on the branches indicate mutation steps, and those branches without short lines have only one mutation step. (D) The Bayesian skyline plot showing historic changes of female effective population size of the F1a1a1 lineage in samples from Southeast Asia. HC, Hanging Coffin; AA, Austro-Asiatic; HM, Hmong-Mien; AU, Austronesian; TB, Tibeto-Burman; AJK, Altaic + Japanese + Korean.

See also in Figure S2.

pattern is further supported by the calculated average number of pairwise differences (measured by  $\Phi_{ST}$ ) of the 31 representative Asia-Pacific populations and the two Hanging Coffin populations (Figure 3B).

The comparison of mtDNA lineage component frequency spectrum confirmed the close relationship of the southern populations with the Hanging Coffin populations (Figures 3C and 3D). The Hanging Coffin populations show the closest affinity with present-day Daic populations in southern China and northern Thailand, and some of the other surrounding populations in Southeastern Asia also share maternal lineages with the Hanging Coffin populations, an implication of gene flow among populations belonging to different language families. Of the eight lineages that appeared in the Hanging Coffin population from Yunnan, four are present in two present-day Thai populations from northern Thailand and Vietnam (Figure 3C). In contrast, the present-day Thai population from northern Thailand harbors six of the seven Hanging Coffin lineages in Thailand, a clear indication of genetic affinity with the historic Log Coffin population (Figure 3D, left panel). Furthermore, within the present-day Thai populations from northern Thailand, we found two Thai sub-branches (Khon Mueang and Thai Yuan) that showed the closest affinity



**Figure 3. Genetic Affinity of the Hanging Coffin Populations with Current Asia-Pacific Populations**

(A) The PCA plot indicates a relatively close relationship of the Hanging Coffin populations with the “Southern” populations. The present-day populations showing close relationship to the two Hanging Coffin population are labeled with numbers, including 1, MK\_Central Thailand; 2, AA\_North Vietnam; 3, HM\_North Vietnam; 4, AU\_South Vietnam; 5, TB\_North Thailand; 6, Thai\_North; 7, AA\_Northeast Thailand; 8, Han\_CHS\_China; 9, Han\_Shanghai\_China; and 10, Han\_CHB\_China.

(B) The average pairwise differences are calculated based on the  $\Phi_{ST}$  index using the Arlequin (version 3.5.0) software, which confirms the genetic affinity of the Hanging Coffin populations with the “Southern” populations as shown in the PCA map. The red boxes indicate those populations showing the smallest genetic distance to the two Hanging Coffin populations in the heatmap.

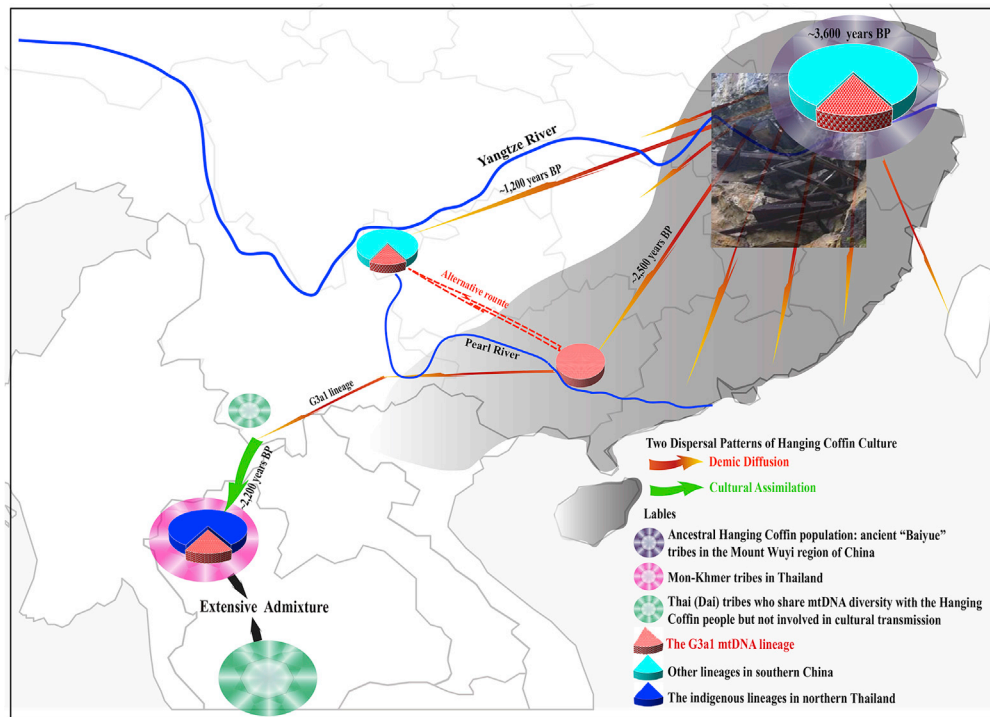
(C) The spectrum of mtDNA lineages of the Yunnan Hanging Coffin population when compared with those of the present-day “Southern” populations.

(D) The spectrum of mtDNA lineages of the Hanging Coffin population from northern Thailand when compared with those of the present-day “Southern” populations. The present-day “Southern” populations are grouped in large (left panel) and small (right panel) geographic regions. The number in the bracket refers to the sample size of each population. HC, Hanging Coffin; AA, Austro-Asiatic; HM, Hmong-Mien; AU, Austronesian; TB, Tibeto-Burman. See also in Figure S1.

to the Log Coffin population by harboring four of the seven lineages with similar lineage frequency spectrum (Figure 3D, right panel).

## DISCUSSION

The Hanging Coffin burial custom is a unique cultural relic once widely distributed in the Yangtze River and the Pearl River basins of southern China, Southeast Asia, and near Oceania regions. This custom had vanished in mainland China and MSEA a couple of hundred years ago, but is still practiced in some remote ISEA regions, e.g., the Yami ethnic group in Lanyu region of Taiwan, China, and the Toraja people in Sulawesi of Indonesia (Chen, 1996d). In regard to the chronological order of the Hanging Coffin archaeological sites, the proposed route of dispersal was called “the more east, the more ancient, the more west, the more recent,” and the archaeological sites in southern China are in general older than those in Southeast Asia, consistent with an “East to West and North to South” dispersal pattern (Chen, 1996c).



**Figure 4. The Proposed Origin and Dispersal Pattern of the Hanging Coffin Custom**

The approximate range of distribution of the ancient “Baiyue” tribes is highlighted in gray. The two different dispersal patterns of the Hanging Coffin custom are indicated by two arrows with different colors. The major historic tribes involved in the Hanging Coffin custom are labeled with different symbols. The Yangtze River and the Pearl River are indicated with blue lines. The pie shows the rough mtDNA lineage component in different Hanging Coffin tribes.

The Hanging Coffin sites not only show continuity in terms of time and geographic regions but also have similar cultural connotation (choice of burial location, coffin shape, and funerary objects) (Chen, 1996e). In addition, the Hanging Coffin relics in southern China and Southeast Asia share many common cultural elements with the ancient “Baiyue” (means hundreds of “Yue” tribes, who were the ancestors of the present-day Daic speakers) tribe relics, such as the stepped adze (found in Guangxi, Guangdong, and Fujian of China, Borneo and Sulawesi of Indonesia and the Philippine archipelago), the shoulder stone artifacts (originated in the Pearl River Delta region and found in Guangxi and Hainan island of China, Vietnam, the Malay peninsula, and west to Assam of India and Bangladesh), and the geometric impression pottery (the most ancient unearthed cultural relics in the Hemudu and the Liangzhu sites of China, and found from southern mainland China to Taiwan, Indo-China, and the Kalumparp region of Sulawesi) (Chen, 1996f).

The human culture and language spread patterns around the world are mainly explained by two alternative models: the demic diffusion model, which involves massive human migration to other places, and the cultural assimilation model, which refers to major cultural transmission between populations and involves limited genetic exchange between them (Cavalli-Sforza et al., 1994). Our genetic data reveal that the Hanging Coffin populations from southern China and northern Thailand are genetically linked with a “North to South” decline of mtDNA haplotype diversity, consistent with the hypothesized migratory route of the Hanging Coffin custom based on cultural relics (Chen, 1996a). In addition, they both show a close genetic affinity to the present-day Daic populations living in these regions, congruent with the “North to South” dispersal route (Chen, 1996b). However, these two Hanging Coffin populations share very few mtDNA lineages (1 of 14), an indication of limited genetic exchange, which fits the model of cultural assimilation rather than demic diffusion during the historic spread of this custom to Southeast Asia. Combining our genetic results with the archaeological evidence, we speculate that the Hanging Coffin culture originated from the southeastern coastal region (likely in the Mount Wuyi region of China) ~ 3,600 YBP among the ancient “Baiyue” tribes, and this unique burial custom expanded to the wider southern China regions in

the following millennia through demic diffusion. Then the Hanging Coffin custom spread southward to Southeast Asia mostly by cultural assimilation (Figure 4). It should be noted that due to the limited human remain samples from restricted geographic regions in this study, we cannot rule out other possible scenarios in view of direction and route of custom spread. Future studies with more samples covering more relic sites are needed to test the proposed dispersal pattern of the Hanging Coffin custom.

### Limitations of the Study

In this study, we only explored the matrilineal genetic perspective due to the difficulty of acquiring genomic and Y-DNA data. Also, the sampled Hanging Coffin sites are limited.

### METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

### DATA AND CODE AVAILABILITY

GenBank accession numbers for the mtDNA genome sequences of the 31 Hanging Coffin samples are MN006845–MN006875.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101032>.

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### AUTHOR CONTRIBUTIONS

B.S., X.J., and R.S. designed the study; X.J., R.S., X.L., Y.G., S.H., H.L., and T.Y. collected the samples; X.Z., Y.Z., and J.H. conducted the experiment; H.S. and C.L. provided technical assistance in the experiments; X.Z., C.L., Y.Z., and J.H. analyzed the data; X.Z., C.L., S.C., S.H., R.S., X.J., and B.S. wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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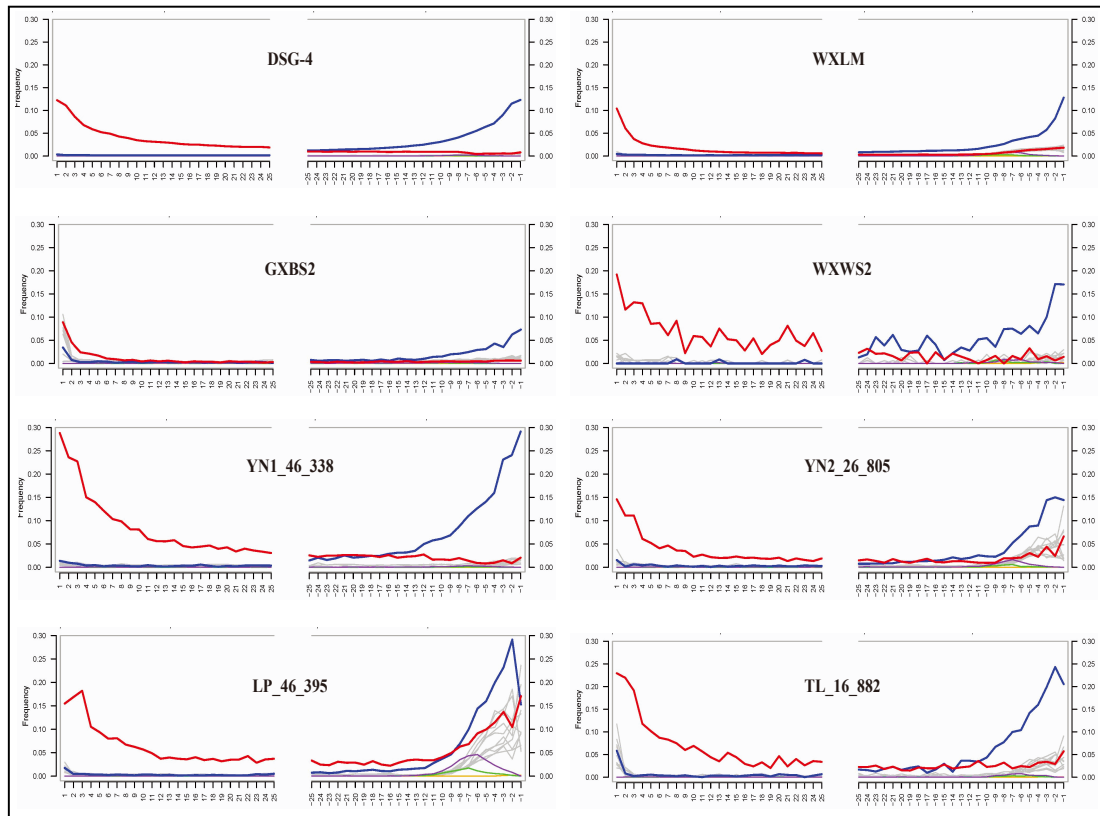
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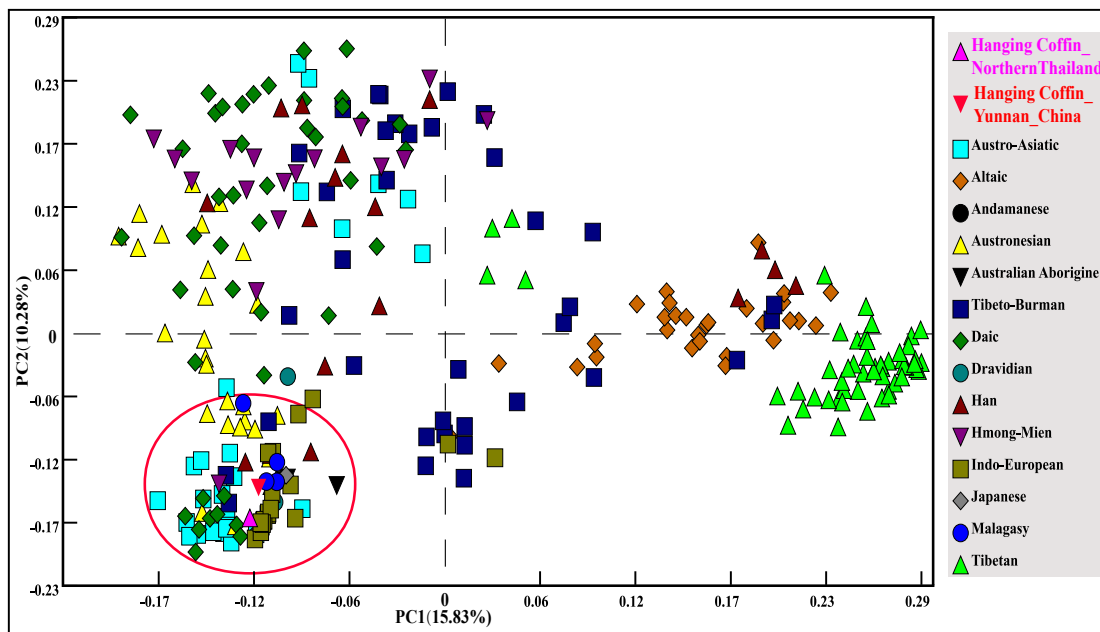
## **Supplemental Information**

### **A Matrilineal Genetic Perspective of Hanging Coffin Custom in Southern China and Northern Thailand**

**Xiaoming Zhang, Chunmei Li, Yanan Zhou, Jiahui Huang, Tengsong Yu, Xu Liu, Hong Shi, Hong Liu, Stephen Chia, Shenmin Huang, Yaozheng Guo, Rasmi Shoocongdej, Xueping Ji, and Bing Su**

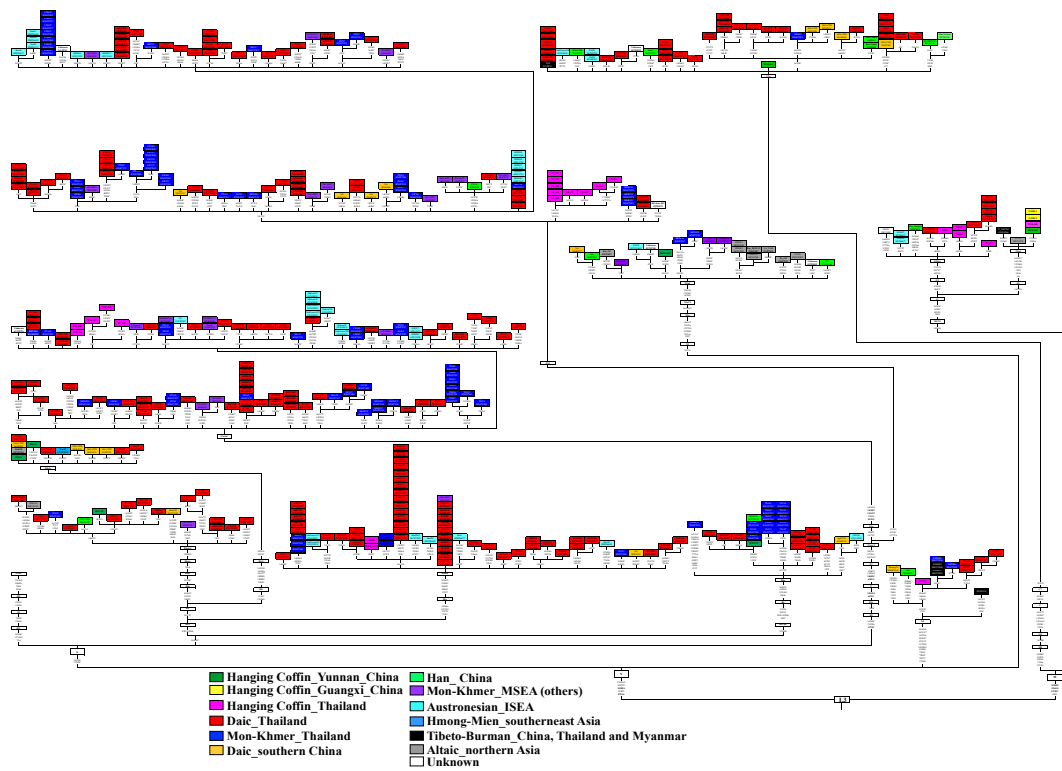


A

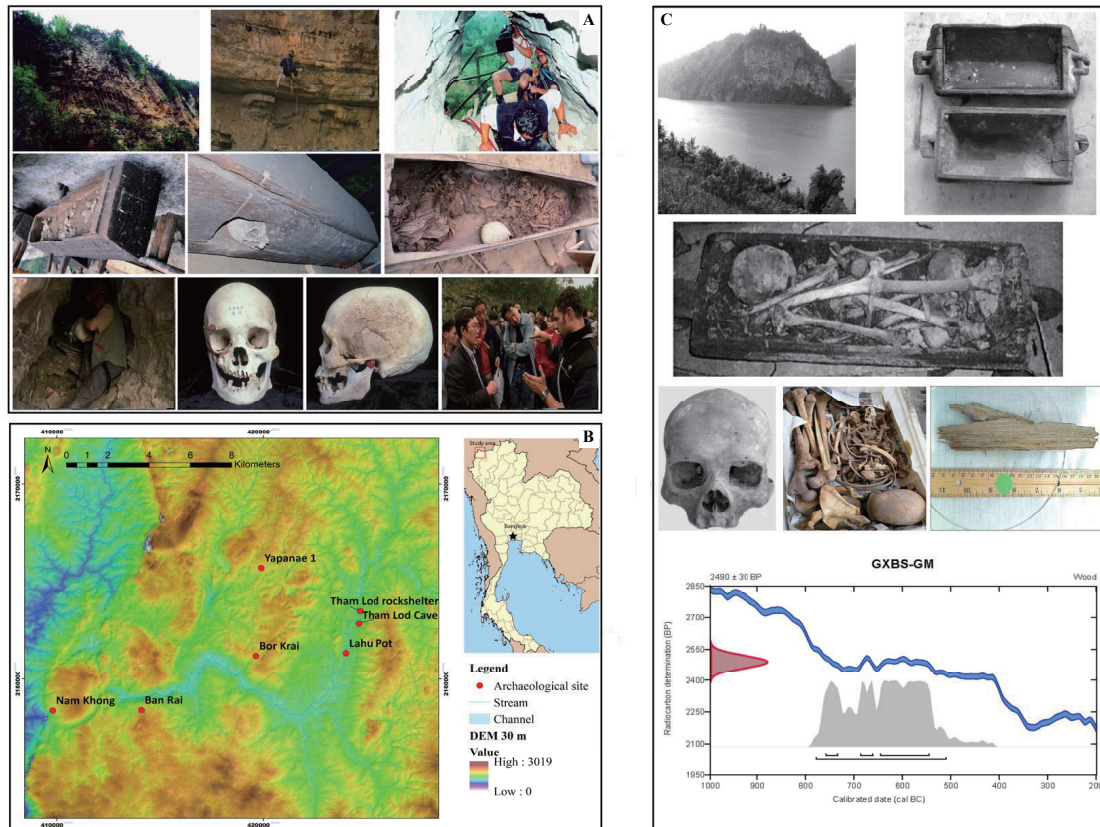


B

**Figure S1. aDNA quality assessment and population relationship.** Related to Figure 2, Figure 3 and Transparent Methods. **A:** The terminal damage rate (TDR) maps of eight representative aDNA samples calculated by MapDamage 2.07. **B:** The PCA plot showing the relationship between the Hanging Coffin populations and the present-day Asia-Pacific populations.



**Figure S2. The phylogenetic tree based on the 38 Hanging Coffin samples and 392 published mitochondrial whole genomes.** Related to Figure 2A and Transparent Methods. The populations are labeled with different colors based on their language families. The sample IDs are shown in the box, and the mutations used to define the mtDNA lineages are given along the branches. For transversions, the derived alleles are shown in lowercase. Back mutations are indicated with a ‘!’ suffix.



**Figure S3. The information of the Hanging Coffin relic sites.** Related to Figure 1 and Transparent Methods. A: The pictures showing excavation of the Hanging Coffin burial sites in Yunnan, China. B: The distribution map of the Log Coffin archaeological sites in Pang Mapha ensemble, northern Thailand. C: The overview of the 'Huacun Hanging Coffin site' from Guangxi province, China (the photos were provided by Mr. Yaozheng Guo). The radiocarbon dating in this study used the wood coffin fragments, and was undertaken by Beta Analytic testing laboratory in USA; the radiocarbon age was calibrated with BetaCal3.21 using HPD method INTCAL13.

**Table S3 The  $^{14}\text{C}$  dates of the Log Coffin sites in northern Thailand.** Related to Figure 1 and Figure S3B

<b>NO.</b>	<b>Sites</b>	<b>Abbr</b>	<b>Date</b>	<b>Reference</b>
1	Bor Krai	BK	1,090±210 (OAP2162) to 2,230±230 (OAP2207)	Wannasri 2004
2	Ban Rai rockshelter	BR	1,520±210 (OAP2201) to 2,260±240 (OAP2197)	Wannasri 2004
3	Lahu Pot Coffin Cave	LP	1,540±120 (AMS384) to 2,080±60 (AMS385)	Grave, P. et al. 1994
4	Nam Khong Coffin Cave	NK	NA	NA
5	Tham Lod Cave	TL	1,240±90 (AMS387) to 1,450±110 (AMS388)	Grave, P. et al. 1994
6	Tham Lod rockshelter	TLR	NA	NA
7	Yappanae 1	YN1	NA	NA
8	Yappanae 2	YN2	1,200±200 (OAP2251) to 2,020±220 (OAP2242)	Wannasri 2004
9	Long Long Rak	LLR	1,761±25 to 1,870±30	McColl, et al. 2018

## **Transparent Methods**

### **Sampling of the Hanging Coffin Human Remains**

We collected a total of 31 samples from 13 Hanging Coffin archaeological sites in southern China (Yunnan and Guangxi provinces) and northern Thailand (Figure 1). The radiocarbon dating of these sites range from ~2,500 to 660 YBP (Table S1). The description of these archaeological sites can be found in the last section of supplemental information.

### **Radiocarbon Dating of the Baise Hanging Coffin archaeological site from Guangxi, China**

We sent the wood chips that was taken down from the Coffin (Figure S3C) to Beta Analytic testing laboratory, USA, for  $^{14}\text{C}$  dating analysis. All tests were performed in Beta Analytic located in Miami using 4 in-house NEC accelerator mass spectrometers (AMS) and 4 Thermo IRMSs under strict chain of custody and quality control using ISO/IEC 17025:2005 testing accreditation PJLA #59423 accreditation protocols. The test sample, and modern and blank controls were analyzed in the same chemistry lines by professional technicians using identical reagents and counting parameters. The "Conventional Radiocarbon Age" was calculated using the Libby half-life (5,568 years), corrected for total isotopic fraction and used for calendar calibration where applicable. The age was rounded to the nearest 10 years and was reported as radiocarbon years before present (BP), "present" = AD-1950. The modern reference standard was 95% the  $^{14}\text{C}$  signature of NIST SRM-4990C (oxalic acid). The calculated ages less than 30 BP on the Conventional Radiocarbon Age were rounded up to 30. The radiocarbon age was calibrated with BetaCal3.21 using HPD method INTCAL13 (Reimer, et al. 2013).

### **Ancient DNA (aDNA) Extraction, Library Preparation, mtDNA Capture and Sequencing**

We have built the aDNA clean room facility in the main building of Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). The aDNA clean

room is far from the ordinary molecular biology labs to prevent potential contaminations. The aDNA facility is equipped with positive air pressure, universal UV irradiation, super clean bench, separate rooms for pre-preparation of sample treatment, aDNA extraction, library build, PCR set up and PCR experiments. We performed the post-PCR procedures in the ordinary molecular biology lab.

DNA was extracted from teeth or phalanx. Firstly, we conducted 2 hours UV-irradiation on the samples, and removed a layer of surface using a sterile dentistry drill, then again irradiated with 1 hour UV-light on the samples. We drilled out ~80 mg of bone powder for every sample with the sterile dentistry drill, and only do 2 samples at one time (include following procedures until performing sequencing; samples from different archaeological sites were never handled together) to avoid potential individual cross-contamination. Using the 80 mg bone powder, we performed DNA extraction following the silica suspension protocol from an early report (Rohland and Hofreiter 2007), which was modified afterwards (Allentoft, et al. 2015) for customizing recovering of more shorter DNA fragments, that finally resulting a total of 100  $\mu$ l aliquots for each sample. In brief, the bone powder was digested over night with proteinase K in 0.5M EDTA plus 10% N-Laurylsarcosyl suspension, then the released DNA was absorbed in solution which includes PB buffer, 5M sodium acetate, 5M sodium chloride and SiO<sub>2</sub> suspension, and followed by three times of purification using 80% ethyl alcohol. Finally, after airing, the DNA was eluted with 100  $\mu$ l EB buffer.

Next, to perform preliminary aDNA preservation situation screening, using 20 $\mu$ l DNA aliquots of each sample, we built the double strand library (DSL) with no Uracil-DNA-Glycosylase (UDG) treatment under a single indexing with commercial kit (cat no: E7370) from New England Biolabs (Ipswich, MA) following the manufacturer's guidelines, as previously reported (Meyer and Kircher 2010) that includes end prep, adaptor ligation, purification, PCR amplification and size selection steps. PCRs were conducted in a final volume of 50  $\mu$ l using AmpliTaq Gold 360 DNA Polymerase (AmpliTaq Gold, Life Technologies Applied Biosystems) which is able to well amplify across uracils, preserve the DNA damage pattern that induced by deamination, which indicating of authentic aDNA (Krause, et al. 2010). We performed all the sequencing



(also the following captured library sequencing) on the Illumina HiSeq X Ten (PE-150) platform (<https://www.illumina.com.cn/systems/sequencing-platforms/hiseq-x.html>). The calculated appraise indexes of aDNA quality and preservation are shown in Table S1. Lastly, we rebuilt the DSLs with 3 hours UDG treatment using the remaining DNA extraction aliquots, which could largely remove uracil residues from DNA fragmental end to leave abasic sites, and cuts the DNA at the 5' and 3' sides of the abasic sites with enzyme endonuclease VIII (Endo VIII). For these libraries, we performed the mtDNA capture using myBaits® Mito-Target Capture Kits as previous report (Enk, et al. 2014). Briefly, we used the biotinylated RNA “baits” that are transcribed from the human genomic DNA to perform the capture in solution overnight at 65°C, then mixed in streptavidin-coated magnetic beads and sequestered the targets with a magnetic stand. The PCRs for both pre-capture and post-capture are performed using KAPA HiFi Hot start Polymerase (KAPA BIOSYSTEMS).

### **Process of Data Mapping, Alignment, Filtering and mtDNA Assembly**

We merged forward and reverse sequencing reads that overlapped by at least 11 bp into single sequences to recover full-length molecule sequences as described in previous reports (Reich, et al. 2010; Fu, et al. 2013), the base with the higher quality score was called in the overlapping sequence, and we used the reconstructed full-length molecule sequences for further analysis. Non-merged sequences and sequences < 35 bp were discarded. All the merged reads were mapped to the human reference genome hs37d5 ([ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2\\_reference\\_assembly\\_sequence](ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequence)) using BWA (Li and Durbin 2009) with parameters: `aln -l 16500 -n 0.01 -o 2 -t 10`, and the SAM/BAM-alignment files were processed and analyzed using Samtools (Li, et al. 2009) and Picard (<http://picard.sourceforge.net/>). We excluded the sequences with a mapping quality score < 25 from further analysis. We collapsed sequences with identical alignment start and end coordinates into single sequences by consensus calling as report (Reich, et al. 2010). For the BAM which mapped from captured data, we rescaled the mtDNA BAM files using mapDamage2.07 (Jonsson, et al. 2013) to reduce the impact by sequence damage. Table S1 summarized

the number of sequences retained for every samples after each step. After all these steps, 0.01% to 1.28% of reads were mapped to reference sequence that reflect endogenous rates (Table S1). For the mtDNA captured data, the final mtDNA BAM files were manually and visually inspected for all mutations against the revised Cambridge reference sequence (rCRS, NC\_012920.1), and the entire mtDNA consensus sequences fasta files were extracted using Integrative Genomics Viewer (IGV), to corroborate every mutation for each individual. The average length of the mtDNA molecules range from 55 to 99 bp (Table S1). The mitochondrial genome coverage as determined from unique sequences range from 1.04- to 110.45-fold, with an average coverage of 32.84 fold (Table S1).

### **DNA Fragmental Terminal Damage Rate (TDR), mtDNA Sequence Consensus, Contamination Estimate and Sex Determination**

The percentage of C-to-T changes at the 5'-ends of the endogenous sequences from libraries that were non-UDG-treated was estimated using mapDamage2.07 (Jonsson, et al. 2013). Except for a few samples showing less than 10%, all the other samples possess more than 10% damage at the terminal (table S1) and therefore meet our criteria of aDNA authenticity for further processing. We checked the positions with a major allele frequency of > 80% and only extracted the mutations greater than 90%, which could largely exclude the mutations resulted from molecular damage. The final BAM files were used for estimating mtDNA contamination levels with Schmutzi (Renaud, et al. 2015). In total, only two samples show detectable contamination level (Table S1), suggesting that the DNAs largely derived from a single endogenic biological source.

As males have only one X and one Y chromosome, we expected the data coverage of X chromosome nucleotides is about half of the autosomal coverage, and substantial number of reads mapped to the Y chromosome (Skoglund, et al. 2013). However, females were expected have comparable X chromosome coverage to the autosomal coverage, and significant fewer reads mapped to the Y chromosome (largely due to certain regions of similarity between the X and Y chromosomes). Thus, The ratio of mapped reads to X and Y chromosomes could be used to infer sex (Skoglund, et al.

2013). We used this method to infer the sex of all samples, and found that almost all the samples are male from the Yunnan and Guangxi sites, and there were more males than females in the northern Thailand sites (Table S1).

### **Biogeographical Phylogeny and Population Cluster Analysis**

We used the online tools Haplogrep (Kloss-Brandstatter, et al. 2011) and MitoTool (Fan and Yao 2011) to perform Haplogroup assignment. To get a broad picture of Asia-pacific wide population relationships with the Hanging Coffin populations, we collected an extensive array of 280 published present-day Asia-pacific populations with 28,618 sequences (Table S2). To determine the phylogeographic positions of the Hanging Coffin samples, we employed the published 392 complete mtDNA genome sequences from previous studies (Kong, et al. 2003; Tanaka, et al. 2004; Macaulay, et al. 2005; Zhang, et al. 2008; Jinam, et al. 2012; Liu, et al. 2012; Delfin, et al. 2014; Jiang, et al. 2014; Ko, et al. 2014; Lippold, et al. 2014; Cavadas, et al. 2015; Genomes Project, et al. 2015; Brandao, et al. 2016; Ma, et al. 2016; Kutanan, et al. 2017; Kutanan, et al. 2018; Peng, et al. 2018; Zhang, et al. 2013) to construct the phylogenetic tree. We used the  $\rho \pm \sigma$  statistics to estimate the coalescence time to the most recent common ancestor (TMRCA) of the F1a1a1 and F1f lineages, and a strict molecular clock with a fixed rate of  $2.14 \times 10^{-8}$  substitutions/site/year was used (Rieux et al., 2014). The Bayesian Skyline Plot (BSP) (Drummond, et al. 2005) in BEAST (version 1.10.3) (Drummond, et al. 2012) with MCMC algorithms (Drummond, et al. 2002) were used to reconstruct the demographic history for the F1a1a1 lineage with 108 complete sequences. To establish the genetic relationships between the Hanging Coffin populations and the present-day populations, we performed PCA based on the frequency spectrum of mtDNA haplogroups according to the method developed by Richards et al (Richards, et al. 2002) in the MVSP3.13 software. To reveal the detailed structure of the Hanging Coffin population haplogroups, based on the HVS-I sequence data or complete sequences, we constructed reduced median networks using the programme NETWORK version 5.0.0.3 (Fluxus Engineering) (Bandelt, et al. 1999). Arlequin (version 3.5.0) (Excoffier et al., 2005) was used to calculate the average

number of pairwise differences based on the  $\Phi$ ST index using mtDNA genome sequences, including 31 major Asia-pacific populations covering the major language family groups and two Hanging Coffin population shown in Figure 3B. Considering the sample sizes of the Hanging Coffin human remains from Yunnan and Thailand are 9 and 22 samples, respectively, our strategy for the modern populations was randomly sampling 10 sequences for each population.

### **General Information of the Hanging Coffin Sites in the Present Study**

**The Hanging Coffin sites from Yunnan province of China:** The Hanging Coffin sites in Yunnan is restricted to Zhaotong city in northeastern Yunnan that along the Jinsha river basin (a tributary of Yangtze River), majorly distribute in Yongshan, Yanjin and Weixin county, near the provincial border between Sichuan and Yunnan (Liu and Sun, 1996). These Hanging Coffins sites (include from Matangba, Gong county in southern Sichuan) have been called the 'Bo people's Hanging Coffins' (Zhang, 1990), and the craniological studies indicate their affinity to Zhuang ethnic group, which is the largest present day Daic speaker and mostly living in Guangxi province (Hu and Xiao, 1999a; Hu and Xiao, 1999b; Ji et al. 2005). The earliest appearance of the 'Bo people' dates back to the 3rd century BC, and they established a 'Bo vassal kingdom' at around the border of Sichuan, Yunnan and Guizhou provinces; 'Bo people' disappeared from the literature after the end of the Ming dynasty, which accompanied by fade away of the hanging coffin burial practice (Ji et al. 2005). In September 2000, the China Exploration of Hong Kong, the Yunnan Institute of Geography, and the Yunnan Institute of Cultural Relics and Archaeology conducted joint research for the preservation of hanging coffins at the site of Washi, which is located 37 km from Weixin city (GPS: 27°49'27"N 104°46'49"E, altitude 615 m) (Figure S3A). A salvage excavation of three naturally destroyed coffins (WG1, WG2, WG3) unearthed a complete human skull and other postcranial bones of the same individual (Figure S3A), together with a few wooden, bamboo, or textile relics from WG3. <sup>14</sup>C dating of the coffin wood samples resulted in a date of 1,246 ± 65 YBP for WG3. Subsequently in April 2003, the Bang Productions Ltd. of Hong Kong, the Yunnan Institute of Cultural Relics and

Archaeology, and the Yunnan Institute of Geography conducted an investigation of the Yanjin and Weixin counties for the purpose of making a television program for the Discovery Channel (USA). The investigation team found a new hanging coffin site at the natural cliffs of Jiudongyan, Longma, and Jiucheng, 50 km from Weixin city (GPS: 28°01'56.1"N 105°00'16.2"E, altitude 538 m). A human skull with a mandible and a few postcranial bones were found in a cleft on the cliff, where the surrounding coffin wood was completely decayed by weathering. <sup>14</sup>C dating was carried out for intact wood samples taken from another coffin in the vicinity of the human skeleton, giving a date of 1,070 ± 60 YBP (Ji. et al. 2005). Dr. Xueping Ji, one of the designer of this study, played as scientific consultant to film this Discovery Channel Award-winning documentary--Mysterious Hanging Coffins of China (hosted by Joan Chen (Hong Kong adventure association) and published by Discovery Channel, NHK in 2004-English narration).

**The Hanging Coffin sites from northern Thailand:** The information is presented in Figure S3B.

**The Hanging Coffin site from Youjiang District of Baise city, Guangxi province of China (Figure S3C):** The 'Huacun Hanging Coffin site' located at the 500 meter northwest of Huacun village, Yangxu town, Youjiang district of Baise city, Guangxi province of China. This site found at June, 2006, locating at escarpment of north shore of Youjiang River, a tributary of the Pearl River. This site harbors two cave positions, which has 3 coffins and 3 human skeletons in total (cave NO.1 has one coffin and cave NO.2 has two, which coffin NO.2 has 2 human skeletons, but coffin NO.1 have been destroyed beyond recognition), and very few burial objects been found. It is reported that all three the human individuals performed tooth-digging custom, which is one of the cultural symbols for ancient "Baiyue" tribes (<http://www.gxmuseum.cn/a/science/31/2013/3331.html>).

#### **Data and Code Availability**

GenBank accession numbers for the mtDNA genome sequences of the 31 Hanging Coffin samples are MN006845-MN006875.

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