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Title: A possible Middle Pleistocene Denisovan from the Annamite Chain of northern Laos

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Abstract: The Pleistocene presence of the genus *Homo* in continental Southeast Asia is primarily evidenced by a sparse stone tool record and rare human remains. Here we report the first Middle Pleistocene hominin specimen from Laos, with the discovery of a molar from the Tam Ngu Hao 2 (Cobra Cave) limestone cave in the Annamite Mountains. The age of the fossil-bearing breccia ranges between 164-131 kyr, based on the Bayesian modelling of luminescence dating of the sedimentary matrix from which it was recovered, U-series dating of an overlying flowstone, and U-series–ESR dating of associated faunal teeth. Analyses of the internal structure of the molar in tandem with palaeoproteomic analyses of the enamel indicate that the tooth derives from a young, likely female, *Homo* individual. The close morphological affinities with the Xiahe specimen from China indicate that they belong to the same taxon and that Tam Ngu Hao 2 represents, most likely, a Denisovan.

MAIN TEXT

82 **Introduction:** From the Early to Late Pleistocene, the presence of *Homo erectus* is well
83 documented in Asia, notably in China and Indonesia [1–3]. However, the taxonomic attribution of
84 most Asian late Middle Pleistocene *Homo* specimens remains a matter of contention [4-7]. The
85 recent description and analysis of the Harbin cranium from China has reignited this debate by
86 suggesting its attribution to a new species named *Homo longi* [8], but this taxonomic attribution of
87 this specimens remains highly debated. In fact, the Harbin cranium shows close morphological
88 similarities with other late Middle to early Late Pleistocene Asian *Homo* specimens from Dali,
89 Xujiayao, Xuchang and Hualongdong, whose taxonomy remains unclear [4,9,10]. These fossils are
90 considered to belong to a different taxon than *H. erectus* and are often grouped under the generic
91 label 'archaic humans' [9,10]. Due to the combination of features they exhibit, including
92 Neanderthal-like traits, it has been suggested that they belong to an Asian sister taxon of
93 Neanderthals, the Denisovans, even if this attribution to the latter group remains under debate
94 [5.11.12]. The small number of fossils currently securely attributed to this group (Denisova 2, a
95 lower left molar; Denisova 3, a distal manual phalanx; Denisova 4, an upper left M3; Denisova
96 8, an upper molar; and the Xiahe mandible) [13–16] prohibits a clear morphological picture of the
97 overall Denisovan morphology. Their geographic distribution also remains debated. Modern
98 Papuans, Aboriginal Australians, Oceanic/Melanesian, Philippine Ayta groups and, to a much
99 lesser extent, mainland Southeast Asian populations, retain a Denisovan genetic legacy [14,17,18,19].
100 Combined paleoproteomic and morphometric analyses recently suggested that the Middle
101 Pleistocene Xiahe mandible from Baishiya Karst Cave belonged to a Denisovan, extending the
102 known range of this group onto the Tibetan Plateau [15]. However, there is still no fossil evidence
103 explaining the Denisovans genetic imprint on modern southeast Asian populations and—due to
104 the paucity of the Middle Pleistocene fossil record—it is still unknown whether one or more
105 human lineages (co)existed in continental southern Asia. We present here the first unambiguous
106 Middle Pleistocene *Homo* specimen from mainland southeast Asia and discuss its taxonomic
107 attribution and implications for human evolution in the region.

108 In December 2018, a hominin permanent lower molar was recovered from a breccia block at
109 Tam Ngu Hao 2 (Cobra Cave), Huà Pan province, Laos (20°12'41.5"N, 103°24'32.2"E, altitude
110 1,116 m; Fig. 1, Fig. S1). The tower karst in which the cave was formed is positioned on the
111 south-eastern side of P'ou Loi Mountain with an entrance located 34 m above the alluvial plain
112 (Fig. 1A, Fig. S1). The site was discovered during a survey of the area around Tam Pà Ling,

113 where early *Homo sapiens* fossils have previously been recovered [20-22]. The tooth (TNH2-1) is a

114 mandibular left permanent molar crown germ (Fig. 2A-F; Fig. S2), and the absence of occlusal

115 and interproximal wear combined with the incipient root formation suggests that the tooth was

116 unerupted at the time of the individual's death. The morphology of the tooth is compatible with

117 an attribution to either a first or a second lower molar (Supplementary Material). In either case,

118 considering the early maturational stage of the root, this tooth belonged to a juvenile individual

119 corresponding to an age ranging from 3.5 to 8.5 years following modern developmental

120 standards [23].

121 To best document THN2-1, morphological description and comparative analyses were

122 performed. We also developed a specific sampling protocol that allowed us to sample for

123 palaeoproteomic and future isotopic analyses while preserving the whole occlusal surface

124 morphology of the crown. Sampling for these destructive analyses took place after microCT

125 analyses of the entire tooth, ensuring full morphological data were saved. No additional sampling

126 for ancient DNA analyses was performed at this stage given the old age of the specimen and the

127 tropical conditions under which the sediment and fossils were deposited. The invasive sampling

128 strategy to collect dental tissues for molecular analyses only focused on the distal part of the

129 inferior aspect of the crown, keeping the mesial portion of the crown intact.

130

131

132 **Results**

133 **Context and Dating**

134 The geological setting, stratigraphy and micromorphology of the sediment sequence were

135 analysed to obtain a comprehensive, multi-scalar assessment of the depositional context and

136 taphonomic history of the fossils recovered from the cave (Supplementary Material). The

137 partially eroded sediments that infill the studied entrance passage comprise a lower and an upper

138 facies representing two phases of sediment accumulation separated by an erosional surface and

139 an unknown period of time (Fig. 1B). The lower facies (Lithological Unit 1, LU1) is weakly

140 cemented and forms an arenitic silty clay deposit that is devoid of fossils (Fig. 1E). The upper,

141 fossiliferous facies (Lithological Unit 2, LU2) is well cemented and coarse grained, containing

142 intrakarstic angular limestone clasts and extrakarstic rounded pebbles, forming a very hard

143 breccia/conglomerate layer from which skeletal elements—and in particular, teeth—were

144 recovered in high frequencies (Fig. 1D). The change in lithology between the two facies most

145   likely reflects a reconfiguration of the karstic hydrological system as would be associated with a
146   major flood, eroding space in LU1 onto which the sediments of LU2 were unconformably
147   overlain. The sediments of LU2 are laterally contiguous and densely packed throughout the
148   exposure excavated for this study, precluding major reworking of material and confirming the
149   stratigraphic context of the fossils contained within, including the hominin tooth (see detailed
150   observations described in Supplementary Material). The upper facies (LU2) is draped with two
151   carbonate flowstones, indicating a final change in hydrology and the passage of surface water out
152   of the cave and the precipitation of laminar speleothem (Fig. 1C).

153

154   Three bovid teeth (TNH2-10/CC10, TNH2-11/CC11, TNH2-12/CC12) recovered from the upper
155   fossil-bearing breccia (LU2) were directly dated using coupled uranium series and electron spin
156   resonance (US-ESR), providing a weighted mean age estimate of 151+/-37 thousand years ago
157   (kyr) (2-sigma) (Fig. 1B; Tables S1, S2) and an age range of 188-117 kyr. Two large blocks of
158   breccia (LCC1 and LCC2) from LU2 (upper) and one block of the silty clay unit (LCC3) from
159   LU1 (lower) were removed for luminescence dating (Fig. 1B). These samples produced coeval
160   age estimates of 143 ± 24 kyr (LCC1) and 133 ± 19 kyr (LCC2) for the deposition of the LU2
161   breccia and 248 ± 31 kyr (LCC3) for the underlying LU1 silty clay deposit (Table S3). These
162   ages are in stratigraphic agreement with the age of the overlying flowstone (CCF1), which was
163   precipitated earlier than 104 ± 27 kyr based on the weighted mean of U-series age estimates on
164   four separate sub-samples of flowstone carbonate (Table S4). Bayesian modelling was performed
165   on all independent age estimates to determine an overall geochronological framework for the site
166   and tooth (Supplementary Material and Fig. S3). The fossiliferous breccia including the tooth
167   was deposited between 164-131 kyr (at 68% confidence limit).

168

169   **Fauna**
170   The Tam Ngu Hao 2 faunal assemblage comprises 186 identified dentognathic specimens (NISP)
171   dominated by isolated teeth of large mammals, including several megaherbivores (Table S5).
172   Their analyses reveal typical taphonomic pathways of assemblages from karstic systems in terms
173   of representation of specimens and types of damage. Due to the energy associated with the
174   deposition of (LU2), only teeth of large mammals are present in the assemblage, and we note the
175   absence of small and light teeth of any microvertebrates. Moreover, most teeth are gnawed by
176   porcupines, known to be a major accumulator agent in the region [24]. Therefore, the poor

177   preservation of specimens as shown in Fig. S15, precludes identification to the species level for

178   most of the recorded taxa. The fauna bears close affinities to those known from the late Middle

179   Pleistocene of southern China and northern Indochina and, to a lesser extent, Java, which is

180   consistent with the sedimentary chronology of the site. It can be assigned to the "*Stegodon-*

181   *Ailuropoda* faunal complex" [25–28]. We note the absence of Neogene taxa that persist in the Early

182   Pleistocene and that of two key-species, *Pachycrocuta brevirostris* and *Gigantopithecus blacki*,

183   which are good indicators of pre-300 kyr faunas in the region [29–31]. The archaic *Stegodon*

184   persisted in Asia most likely until the end of the Late Pleistocene [32]. We recovered herbivores

185   including *Tapirus*, *Stegodon*, and Rhinocerotidae, animals that were adapted to canopied

186   woodlands in the area. We also found animals such as the *Bos* species, small-sized Caprinae and

187   large-sized Cervidae (possibly *Rusa unicolor*), which are all known to exhibit a great variability

188   in their preferred habitats, from closed and intermediate forests to open grassland [33], and feeding

189   behaviour.

190

191   **Ancient proteins analyses**

192   The enamel from the TNH2-1 tooth specimen was analysed using nanoLC-MS/MS and the

193   recently developed approach for ancient enamel proteomes [29]. The TNH2-1 proteome is

194   composed of a common set of enamel-specific proteins, all of which have previously been

195   observed in Pleistocene enamel proteomes [34-36] (Table S6). The enamel proteome has elevated

196   levels of diagenetic protein modifications (Fig. S4A-D, Table S7) and preserves serine (S)

197   phosphorylation within the S-x-E motif previously observed in ancient dental enamel [34,35] (Fig.

198   S4E). Based on proteome composition and modification, as well as the absence of peptides

199   matching to any of these proteins in our extraction and mass spectrometry blanks, we consider

200   our proteomic data as indicative of endogenous proteins deriving from the sampled enamel.

201

202   Unfortunately, no high-confidence peptides overlapped diagnostic amino acid positions with

203   sequence differences between *H. sapiens*, Denisovans, or Neanderthals, making further

204   taxonomic assignment based on palaeoproteomics impossible. This is in line with previous

205   research, which indicated that closely related hominin populations can be distinguished based on

206   dentine and bone proteomes, while enamel proteomes are less informative in the context of close

207   phylogenetic proximity [35]. Nevertheless, by comparing the sequences recovered from the TNH2-

208   1 enamel proteome with that of extant hominids for which protein sequences are available, we

209   find that the specimen belongs to a member of the genus *Homo* (Table S8).

210   The absence of peptides specific to male-diagnostic amelogenin Y (AMELY) suggests that either

211   the sampled molar was from a female individual or that AMELY-specific peptides were not

212   observed due to degradation beyond the limit of detection of the instrument.

213

214   **External and internal structural analyses of the tooth**

215   Externally, the TNH2-1 crown displays a coarse wrinkling pattern that is found in Pleistocene

216   *Homo* (*H. erectus* s.l., European and Asian Middle Pleistocene *Homo* and Neanderthals), but is

217   rare in modern *H. sapiens*. The mid-trigonid crest is well developed as commonly recorded in

218   European Middle Pleistocene *Homo* and Neanderthals, while it is generally absent or less

219   frequent in *H. erectus* s.l. and fossil and extant *H. sapiens* [37]. Below the external surface, the

220   enamel-dentine junction (EDJ) of the tooth shows the dentine horns of the five main cusps and of

221   a tuberculum intermedium and a low but uninterrupted mid-trigonid crest (Fig. 2A-H, Fig. S2;

222   Supplementary Material). The latter feature is generally found in Neanderthals (80-100%

223   depending on the molar position) [38-40] but is less frequent in *H. erectus* s.l. and *H. sapiens* [41–47]

224   (Fig. S5). In addition, the EDJ of TNH2-1 shows an internally-positioned metaconid reminiscent

225   of Neanderthal molars [40] and a low crown topography similar to that of *H. erectus* [41–47]. These

226   features, as well as a slight buccal shelf present on the EDJ of TNH2-1, are all expressed on the

227   EDJ of the Denisovan molars from Baishiya Karst Cave (Xiahe, Gansu, China) (Fig. S5) [15].

228   TNH2-1 dentine differs from the much higher and proportionally more mesiodistally compressed

229   EDJ of Neanderthals and *H. sapiens* [39,40], as well as from the shorter dentine horns and more

230   densely wrinkled occlusal basin of *H. erectus* s.l. [41–47] (Fig. S5).

231

232   In terms of absolute dimensions, only Asian Middle Pleistocene *Homo* have larger tooth crowns

233   than TNH2-1 (Tables S9, S10). TNH2-1 crown metrics are within the ranges of variation for *H.*

234   *erectus* s.l., *H. antecessor*, Asian Middle Pleistocene *Homo* and Neanderthals, but they

235   statistically differ from the smaller crowns of European Middle Pleistocene *Homo* and from

236   Pleistocene and Holocene *H. sapiens* (Fig. 2I-J; Tables S10, S11). With respect to tooth crown

237   tissue proportions, TNH2-1 has a high percentage of crown dentine (Vcdp/Vc: 55.37%) with

238   moderately thick enamel as shown by absolute and relative enamel thickness values (3D AET:

239   1.18 mm; 3D RET: 17.00; Table S12). These crown tissue proportions match to those of the

240 nearly unworn M2 of the Xiahe mandible [15] (Vcdp/Vc: 54.62%; 3D AET: 1.47 mm; 3D RET:
241 18.97) and the upper molar of Denisova 4 (3D RET: 15.27; B. Viola, pers. comm.), but within
242 the ranges of variation of all comparative fossil and extant human groups (Fig. S6A-C; Tables
243 S12, S13). Three-dimensional maps of topographic enamel thickness distribution show that
244 TNH2-1 has the thickest enamel at the top of the hypoconid and hypoconulid cusps and in the
245 distobuccal quarter of the crown (Fig. S6D). In comparison, all other samples tend to have the
246 thickest enamel distributed on all buccal cusps and more spread on the buccal aspect of the
247 crown, even if variable between groups and between molar positions. The M2 of the Xiahe
248 specimen shows thicker enamel spread along the buccal crown aspect but its distribution pattern
249 is partly obliterated by occlusal wear.

250

251 The EDJ shape of TNH2-1 was quantitatively compared with those of Pleistocene and Holocene
252 human groups using geometric morphometrics (Supplementary materials). Landmark-based and
253 surface deformation-based approaches were used, with both methods similarly distinguishing
254 between *H. erectus* s.l., European Middle Pleistocene *Homo* and Neanderthals and *H. sapiens*
255 using canonical variate and a between-group principal component analyses (Fig. 3, Fig. S7).
256 Along CV2 and bgPC1, the higher EDJ and more externally set dentine horns of Neanderthals
257 and *H. sapiens* are discriminated from the lower and more centrally positioned dentine horns of
258 *H. erectus* molars. The CV1 and bgPC2 axes separate Neanderthals from modern humans, with
259 the former having more internally placed mesial dentine horns and a more developed
260 hypoconulid than the latter. TNH2-1 falls outside the ranges of all other groups. It has an
261 intermediate EDJ shape between the low crown of *H. erectus* (but exceeding the variation of the
262 latter group along CV1 and bgPC2) and the cusp position of Neanderthal molars (even if outside
263 their range of variation along CV2 and bgPC1). TNH2-1's closest morphological affinity lies
264 with the Denisovan specimen Xiahe, which also displays Neanderthal-like features (Fig. 3, Fig.
265 S7).

266

267 **Discussion**

268 Reconstructing dispersals and ultimately evolutionary trajectories of *Homo* in Asia depends on a
269 currently poor fossil record. The Asian late Middle Pleistocene fossil record is mostly limited to
270 the eastern part of the continent [4,8–10,15,48]. Any additional human remains from this time period
271 documenting the evolution of *Homo* in southern Asia might thus help confirm previous

272   hypotheses or reveal new lineages. Proteomic analysis of the TNH2-1 molar indicates that it
273   belongs to a female individual of the genus *Homo*. Morphometric analyses of the external and
274   internal crown structural organisation allow us to reject a number of hypotheses regarding
275   species assignment. TNH2-1 has large crown dimensions and a complex occlusal surface that
276   differentiates it from the smaller and morphologically simpler teeth of *H. floresiensis* [49], *H.*
277   *luzonensis* [50] and *H. sapiens*. The EDJ shape shows a mixture of Neanderthal-like and *H.*
278   *erectus*-like features, closely resembling the M1 morphology of the Denisovan specimen from
279   Xiahe (Fig. 2, Fig. S5). The similarities between TNH2-1 and *H. erectus* are mostly related to the
280   proportionally lower crown, although *H. erectus* molars display even lower molar crowns and a
281   narrower occlusal basin (Fig. 2, Fig. S5). The Lao fossil shows clear Neanderthal-like features
282   such as a well-developed mid-trigonid crest and internally-positioned mesial dentine horns, but
283   differs with its much lower EDJ topography and occlusal basin shape.

284   The differences from Neanderthals that we observe do not preclude TNH2-1 from belonging to
285   this taxon and would make it the south-eastern-most Neanderthal fossil ever discovered.
286   However, considering the morphological particularities of TNH2-1 in unison, as well as the
287   high-degree of morphodimensional similarities with the molars of the Denisovan specimen from
288   Xiahe, the most parsimonious hypothesis is that TNH2-1 belongs to this sister group of
289   Neanderthals. If TNH2-1 indeed belongs to a Denisovan, this occurrence, along with the recent
290   discovery of a Denisovan mandible from the Tibetan Plateau, a high-altitude, hypoxic
291   environment [15], would suggest that this Pleistocene Asian population possessed a high degree of
292   plasticity to adapt to very diverse environments [51]. Available Denisovan dental remains indicate
293   a mixture of traits consistent with the current paleogenetic evidence that Denisovans and
294   Neanderthals are sister taxa [13,14,51–53] and are therefore expected to share some craniodental
295   features [15,54]. This is further supported by recent analyses that identified possible Denisovan
296   skeletal characteristics based on unidirectional methylation changes including traits that have
297   been linked to Chinese fossils such as Xujiayao and Xuchang [9,54]. Denisovans are notable for
298   their large dentition, with some Neanderthal-like crown features [15,48,54], as well as distinctive
299   cusp and root morphology [14–16]. In the absence of molecular analyses, looking for these
300   combined features in the Asian human fossil record, including in fossils like the Penghu 1
301   mandible from the Taiwan Strait [55], may help identify more Denisovan specimens (Fig. S8).

302   The alternative hypothesis that TNH2-1 belongs to a group of Neanderthals that made an

303   incursion into southeast Asia (see for example discussions on fossils that may demonstrate this

304   dispersal from Maba and Dali)[56, 57] cannot be outright rejected.

305

306   The tooth from Tam Ngu Hao 2 Cave in Laos thus provides direct evidence of a Denisovan or

307   Neanderthal female individual with associated fauna in mainland Southeast Asia by 164-131 kyr.

308   This discovery further attests that this region was a hotspot of diversity for the genus *Homo* (Fig.

309   S8), with the presence of at least five late Middle to Late Pleistocene species: *H. erectus* [58],

310   Denisovans/Neanderthals, *H. floresiensis* [49], *H. luzonensis* [50] and *H. sapiens* [20–22].

311

312

313   **Data and material availability:** All mass spectrometry proteomics data have been deposited in
314   the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE
315   partner repository with the dataset identifier PXD018721.

316

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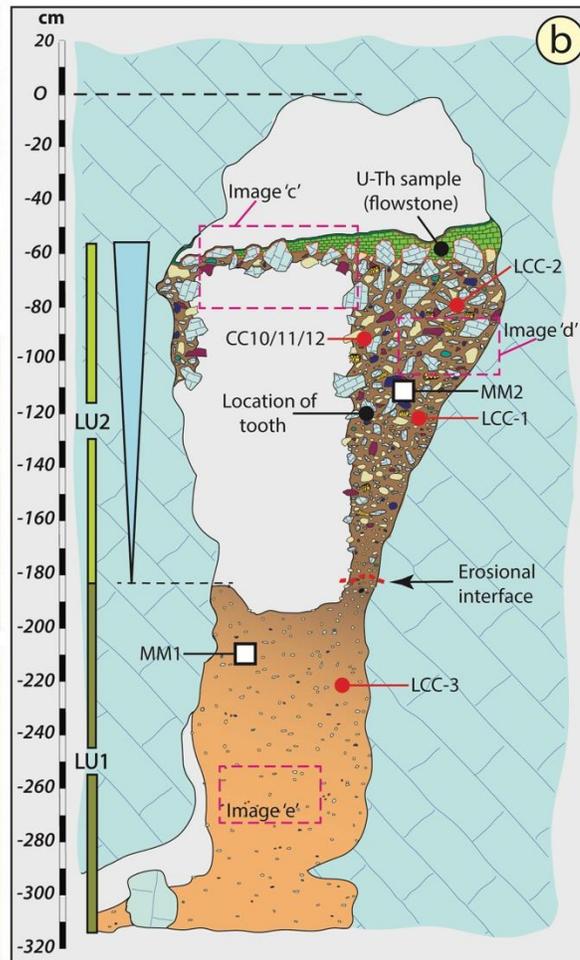445   years ago. *Nature* **577**, 381-385 (2020).

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490 **Author Contributions:**
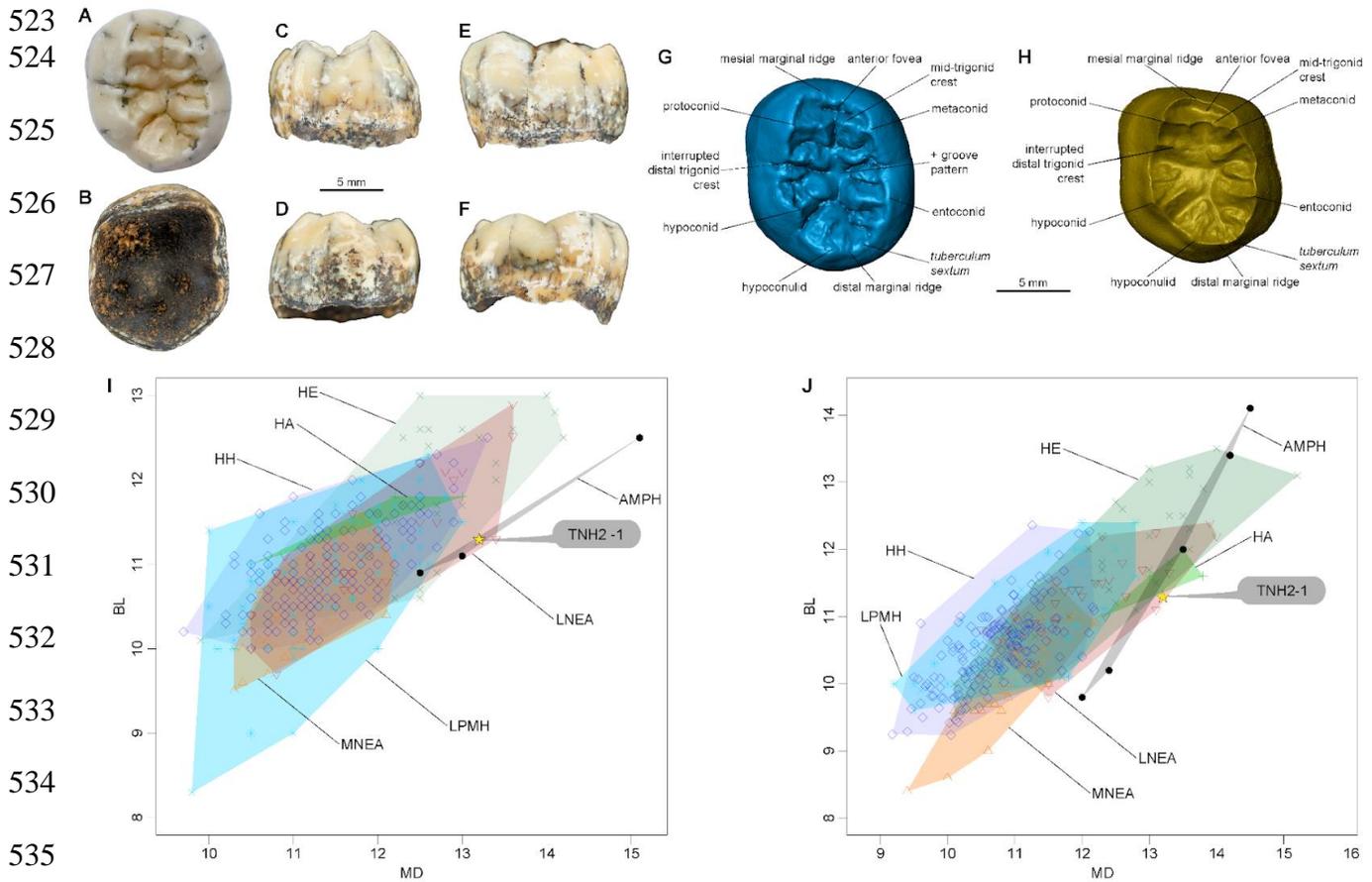491 Conceptualization: FD, LS, AMB, TS, VS, TL

492 Methodology: CZ, MMS, KEW, RJ, RMB

493   Formal analysis: CZ, MMS, AMB, POA, MWM, LP, XS, J-JH, PD, J-LP, QB, FW, PLR, JVO,

494   EC, HMC, KEW, RJ, J-XZ, RMB

495   Investigation: FD, CZ, LS, PD, J-LP, ES, SF, TD, QB, AMB, FW, PLR, JVO, EC, HMC, KEW,

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499   Supervision: FD, LS, AMB

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507   **Supplementary Materials**
508   Materials and Methods

509   Supplementary Text

510   Figs. S1 to S15

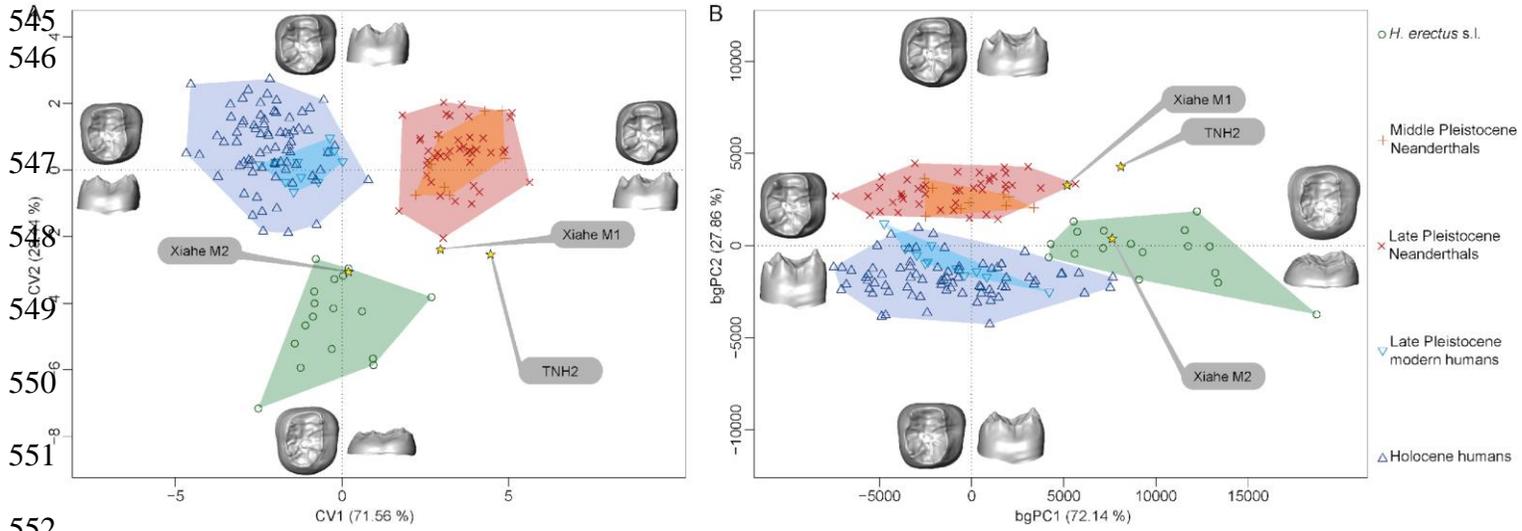511   Tables S1 to S17

512   Data File 1

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515 **Fig. 1. Geomorphological context and stratigraphy of TNH2.** A) aerial view of the site. The

516 red circle indicates the entrance of Tam Ngu Hao 2 cave; B) stratigraphy and sampling locations

517 of the infilling of the cave, showing Lithological Unit 1 and 2 (LU1 and LU2) with the erosional

518 interface between these layers indicated by a dashed red line; Micromorphological

519 (microstratigraphic) samples (MM1 and MM2) are also shown. Encircled numbers denote

520 approximate positions of photographs in C, D & E; C) view of the flowstone capping the upper

521 remaining part of LU2. D) detail of the arenitic breccia/conglomerate of LU2 ; E) detail of the

522 arenitic   silty   clay   of   LU1.

Fig. 2. Morphological and metrical features of the TNH2-1 specimen. A-F, pictures of TNH2 in occlusal (A), inferior (B), mesial (C), distal (D), buccal (E) and lingual (F) views. G-H, virtual renderings of the outer enamel surface (G) and enamel-dentine junction (H) in occlusal view showing the main morphological features. I-J, bivariate scatter plots of the mesiodistal and buccolingual crown dimensions of TNH2 compared with the M1s (I) and M2s (J) of *H. erectus* (HE), *H. antecessor* (HA), Middle Pleistocene Neanderthals (MNEA), Late Pleistocene Neanderthals (LNEA), Asian Middle Pleistocene *Homo* (AMPH), Late Pleistocene modern humans (LPMH) and Holocene humans (HH).

Fig. 3. Canonical variate analysis (A) and between-group principal component analysis (B) of the EDJ deformation-based shape comparison of TNH2-1, *H. erectus* s.l., the Denisovan specimen from Xiahe, Neanderthals and *H. sapiens*.