

RESEARCH PAPER

Phytochemical and genetic analyses of ancient cannabis from Central Asia

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Received 7 August 2008; Revised 24 September 2008; Accepted 25 September 2008

Abstract

The Yanghai Tombs near Turpan, Xinjiang-Uighur Autonomous Region, China have recently been excavated to reveal the 2700-year-old grave of a Caucasoid shaman whose accoutrements included a large cache of cannabis, superbly preserved by climatic and burial conditions. A multidisciplinary international team demonstrated through botanical examination, phytochemical investigation, and genetic deoxyribonucleic acid analysis by polymerase chain reaction that this material contained tetrahydrocannabinol, the psychoactive component of cannabis, its oxidative degradation product, cannabinol, other metabolites, and its synthetic enzyme, tetrahydrocannabinolic acid synthase, as well as a novel genetic variant with two single nucleotide polymorphisms. The cannabis was presumably employed by this culture as a medicinal or

psychoactive agent, or an aid to divination. To our knowledge, these investigations provide the oldest documentation of cannabis as a pharmacologically active agent, and contribute to the medical and archaeological record of this pre-Silk Road culture.

Key words: Archaeology, botany, cannabis, cannabinoids, archaeobotany, ethnopharmacology, genetics, medical history, phytochemistry.

Introduction

Uighur farmers cultivating the land at the base of the Huoyan Shan ('Flaming Mountains') in the Gobi Desert near Turpan, Xinjiang-Uighur Autonomous Region, China some 20 years ago uncovered a vast ancient cemetery (54 000 m²) that seemingly corresponds to the nearby Aidinghu, Alagou, and

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Subeixi excavations (Ma and Wang, 1994; Chen and Hiebert, 1995; Davis-Kimball, 1998; Kamberi, 1998; An, 2008) (see Supplementary Fig. S1 at *JXB* online) attributed to the *Gūshī* culture (later rendered Jūshi, or Cheshi) (Academia Turfanica, 2006). The first written reports concerning this clan, drafted about 2000 years BP (before present) in the Chinese historical record, *Hou Hanshu*, described nomadic light-haired blue-eyed Caucasians speaking an Indo-European language (probably a form of Tocharian, an extinct Indo-European tongue related to Celtic, Italic, and Anatolic (Ma and Sun, 1994). The *Gūshī* tended horses and grazing animals, farmed the land and were accomplished archers (Mallory and Mair, 2000). The site is centrally located in the Eurasian landmass (Fig. 1A, B), 2500 km from any ocean and located in the Ayding Lake basin, the second lowest spot on Earth after the Dead Sea (Fig. 1A, B). Formal excavations completed in 2003 revealed some 2500 tombs dating from 3200–2000 years BP (Xinjiang Institute of Cultural Relics and Archaeology, 2004). Other evidence from chipped stone tools and other items indicate a possible human presence in the area for some 10 000–40 000 years (Kamberi, 1998; Academia Turfanica, 2006). Due to a combination of deep graves (2 m

or more), an extremely arid climate (16 mm annual rainfall), and alkaline soil conditions (pH 8.6–9.1 (Pan, 1996), the remarkable preservation of the human remains resulted in the mummification of many bodies without a need for chemical methods. Numerous artefacts from the tombs included equestrian equipment and numerous Western Asian crops such as *Capparis spinosa* L. (capers) (Jiang et al., 2007), *Triticum* spp. (wheat), *Hordeum* spp. (naked barley), and *Vitis vinifera* L. (grapevines) (Jiang, 2008), often centuries before their first descriptions in Eastern China (Puett, 1998).

One tomb, M90 (GPS coordinates: 42° 48.395' N, 89° 38.958' E; elevation, 58 m) (see Supplementary Fig. S2A, B at *JXB* online), contained the skeletal remains of a male of high social status of an estimated age of 45 years, whose accoutrements included bridles, archery equipment, a *kon-gou* harp, and other materials supporting his identity as a shaman (see Supplementary Figs S3A, B, 4A–C at *JXB* online). His burial as a disarticulated skeleton, as opposed to a mummified body as more frequently was found, suggested that he probably died in the highlands of the *Tiān Shān* ('Heavenly Mountains,' or *Tāngri Tagh* in Uighur) (Fig. 1), and his bones were later interred at Yanghai, as



Fig. 1. Area maps. (A) Map of Turpan, Xinjiang, China and its location in Central Asia. (B) Map of Yanghai Tombs site and surrounding area (adapted from Xinjiang Institute of Cultural Relics and Archaeology, 2004).

nearby tombs contained large timbers of *Picea* (spruce) spp. that grow at 3000 m elevation. Modern Uighur pastoralists follow a similar annual migratory path to summer grazing lands some 60–80 km distant from the tombs. Near the head and foot of the shaman's bier lay a large leather basket and wooden bowl (see Supplementary Fig. S5A, B at *JXB* online) filled with 789 g of vegetative matter, initially thought to be *Coriandrum sativum* L. (coriander), but which, after meticulous botanical examination, proved to be *Cannabis sativa* L. (Jiang *et al.*, 2006). An initial radiocarbon date of 2500 years BP has subsequently been corrected to a calibrated figure of 2700 years BP based on additional analyses of equestrian gear and correlation to tree ring data (dendrochronology) in China. While an earlier publication (Jiang *et al.*, 2006) emphasized morphological features in identifying the cannabis, the current study used additional botanical, phytochemical, and genetic investigations to demonstrate that this cannabis was psychoactive and probably cultivated for medicinal or divinatory purposes. Great care was taken to prevent contamination of the sample throughout the analyses.

Materials and methods

Photomicrography methods

Upon courier delivery from China, a polythene bag containing 11 g of ancient cannabis was sterilized with ethanol, handled with

laboratory gloves in a laminar-flow hood, and transferred with a clean metal spatula (Fig. 2A). Two levels of light microscopy were used in this study. For the observations on the achenes (Fig. 2D), a low power Brunel MX3 microscope (Chippenham, Wiltshire, UK) was used and a $\times 3$ objective utilized in conjunction with an Olympus SP350 8 megapixel camera, stereo insert 30 mm lens tube, and Photonic PL2000 – double arm cold light source. Greater magnification was required for more detailed observations of trichomes (Fig. 2B, C): a high power stereo light microscope with a Trinocular Head for camera attachment (STE UK, Sittingbourne, Kent, UK) with an eye piece graticule for specimen size measurement fitted with $\times 4$, $\times 10$, and $\times 40$ objectives. The camera's $\times 3$ optical zoom capability provided additional magnification.

The observations on the seed were made on unmounted specimens. For these, small pieces of plant tissue were placed directly onto the low-power microscope plate. When using the high power microscope, samples were dry mounted on a glass slide. To achieve views where large proportions of the material were simultaneously in focus, flat samples specimens (as shown) gave the greatest success.

On the low power microscope the seed sample was illuminated with incident light, using a Photonic PL2000 – double arm 'cold light source' (Fig. 2D). Some samples, when placed on the high power microscope, were also illuminated using the cold light source. Others were illuminated from below. When viewing samples mounted beneath a cover slip, it is common to set up a microscope using the Köhler illumination method (Delly, 1988). This ensured that light from the condenser lens was focused correctly on the microscope slide. For uncovered specimens, the condenser height and aperture were adjusted while viewing the subject until optimum resolution was achieved. In all cases, the specimens were measured using a graticule within the eyepiece.

To enable photographs to be taken through the low power microscope, one eyepiece was replaced with a compatible 30 mm

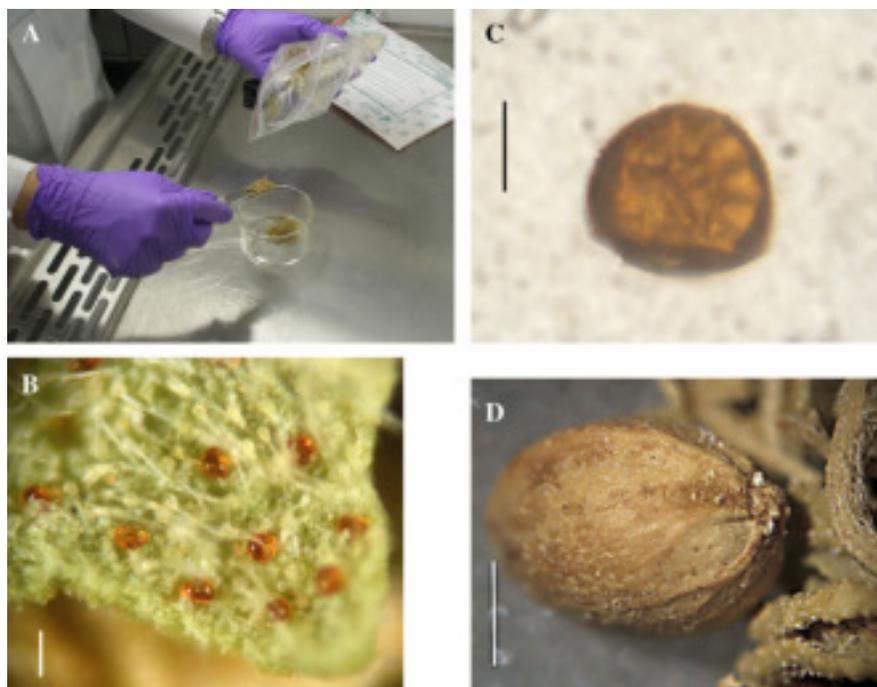


Fig. 2. Photomicrographs of ancient cannabis. (A) Photograph of the whole cannabis sample being transferred in laminar flow hood. (B) Photomicrograph of leaf fragment at low power displaying non-glandular and amber sessile glandular trichomes. Note retention of chlorophyll and green colour, scale bar=100 μ m. (C) Higher power photomicrograph of a single sessile glandular trichome. At least 4 of its 8 secretory cells are clearly visible on the right, and the scar of attachment to the stype cells in the centre, scale bar=25 μ m. (D) Low power photomicrograph of a cannabis achene ('seed') including the base with a non-concave scar of attachment visible, scale bar=1 mm.

lens tube to which single lens reflex or digital cameras would be attached. As in ordinary photography, the depth of field is considered to be the distance from the nearest object plain to the farthest object plain that is in focus. When objects are a long distance from the camera lens the depth of field is large. However, depth decreases as the image comes closer to the lens. When taking photomicrographs, depth of field is measured in microns (Delly, 1988). To maximize the chance of finding substantial areas of tissue simultaneously in focus within this narrow depth of field, multiple samples were laid as flat as possible onto glass slides. In all cases, photomicrographs were taken on a solid bench and the shutter activated remotely to reduce manually-induced camera-shake.

In no instance was any image modification technique used in these photographs.

Phytochemistry methods

Approximately 2 g of the dried plant material was extracted with 200 ml methanol:chloroform (9:1 v/v) by sonication at room temperature (21 °C), the standard extractive technique for this laboratory (GW Pharmaceuticals), a method that recruits >95% of phytocannabinoid content. The solvent layer was then transferred through a paper filter into a rotary evaporator flask. The flask was evaporated to dryness at 40 °C, under reduced pressure, prior to resuspension in 4 ml of methanol:dichloromethane (3:1 v/v). This sample was transferred to two autosampler vials to be analysed by GC-FID-MS and HPLC-UV. At all stages, the clean glassware was extracted with the same solvents to ensure that none of the observed peaks would be a result of contamination. GC-FID-MS analyses were performed on a HP6890 gas chromatograph, coupled to a 5975 inert mass spectrometer. The system was controlled with Agilent MSD chemstation D.03.00.611. The GC was fitted with a Zebron fused silica capillary column (30 m×0.32 mm inner diameter) coated with ZB-5 at a film thickness of 0.25 µm (Phenomenex). The oven temperature was programmed from 70 °C to 305 °C at a rate of 5 °C min⁻¹. The injector port and the transfer line were maintained at 275 °C and 300 °C, respectively. Helium was used as the carrier gas at a pressure of 55 kPa. The injection split ratio was 5:1. HPLC profiles were obtained using an Agilent 1100 series HPLC system controlled by Chemstation version A09.03 software. Cannabinoid profiles were generated using a C₁₈ (150×4.6 mm, 5 µm) analytical column fitted with a C₁₈ (10×4.6 mm, 5 µm) guard column. The mobile phase consisted of acetonitrile, 0.25% w/v acetic acid and methanol at a flow rate of 1.0 ml min⁻¹ and the column was kept at 35 °C. The UV profiles were recorded at 220 nm.

Genetic methods

DNA was extracted from pulverized dried leaves, from two seeds probably belonging to *Cannabis* spp., and from three seeds probably from other unidentified species. The DNeasy Plant Mini Kit (Qiagen) was used, according to the Qiagen protocol, but with some modification to increase the final DNA amount and to avoid external and artificial contamination. For this reason, pre-PCR and post-PCR operations were physically separated and carried out in different environments. Ancient DNA extraction and other pre-PCR works were performed under a UV-filtered ventilation system and a positive pressure airflow. Filtered pipette tips and sterile tubes and plastics were always used; gloves, masks, and laboratory coats were always worn. The quality of DNA obtained was estimated by A₂₆₀/A₂₈₀ absorbance ratio. In order to obtain the highest possible fidelity during PCR synthesis, PCR reactions were performed using the Pwo Master ready-to-use proofreading master mix (Roche Applied Science) according to their protocol. The primers designed to test DNA integrity and suitability for PCR analysis and species identification were from the ITS region of nuclear ribosomal DNA (Blattner, 1999), and from a non-coding region of chloroplast DNA

(Taberlet *et al.*, 1991). The reaction mixtures were subjected firstly to an initial heat denaturation at 94 °C for 3 min; then, they were subjected to 35 cycles of heat denaturation at 94 °C for 30 s, 1 min of primer annealing at 55 °C for the ITS region, and 50 °C for cpDNA, and DNA extension at 72 °C for 40 s. Finally, the samples were maintained at 72 °C for 5 min for the final extension. PCR reactions were performed in an MJ Research PTC-100 thermal cycler (MJ Research, USA). The amplification products were separated by electrophoresis in a 1.5% agarose gel. The bands were excised and purified with the MinElute Gel Extraction Kit (Qiagen). PCR-purified products were quantified and directly forward- and reverse-sequenced, using the GenomeLab™ Dye Terminator Cycle Sequencing with a Quick Start Kit on a CEQ8000 Genetic analyser (Beckman Coulter). Primer sequences were identified and removed manually, and database searches were performed with the BLASTN algorithm (Altschul *et al.*, 1990). The sequences results proved that the pulverized dried tissue was from *Cannabis sativa* L., despite our observation in the mixed sample of some small seeds of different species, removed before the DNA extraction; no differences were observed between the sequences obtained and those deposited at the NCBI gene-bank (for THCA- and CBDA-synthases, GeneBank accession numbers E55108/GI 18529739 and E33091/GI 18623981). By contrast, no amplification was obtained from DNA extracted from seeds of both cannabis and the other, unidentified species. The allelic status at a single locus, *B*, known to be the major gene determining the CBD/THC ratio in cannabis (de Meijer *et al.*, 2003), was investigated in the ancient material. The primer pairs described (de Meijer *et al.*, 2003) are not sufficiently associated with the chemotype (Pacífico *et al.*, 2006), and the sequence-based primers described therein (Pacífico *et al.*, 2006) failed to yield any amplification, probably due to the limited integrity of DNA from ancient cannabis tissues, which did not sustain the amplification of a 1100 Da DNA fragment. Therefore, three different primer pairs (Fw150×Rev328, Fw166×Rev318, and Fw154×Rev318) were used. These primers were designed on two conserved small regions of a zone varying between the known sequences of THC and CBD alleles. When tested on fresh cannabis tissues, these primers were demonstrated to be able to amplify both alleles (PCR and sequences data not shown). Using different primer pair combinations, the risk of a no-match or a mismatch because of possible mutations in the 3' end of primer region was overcome. The primer sequences are listed in Supplementary Fig. S8 at *JXB* online. All reaction mixtures were subjected first to heat denaturation at 94 °C for 3 min and then to 35 cycles consisting of heat denaturation at 94 °C for 15 s, primer annealing at 54 °C for 30 s, and DNA extension at 72 °C for 1 min. Finally, the samples were maintained at 72 °C for 5 min for the final extension of DNA. PCR products were separated by electrophoresis in a 1.5% agarose gel. The bands were excised and purified with MinElute Gel Extraction Kit (Qiagen). PCR-purified products were quantified and directly sequenced in forward and reverse, using the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit on a CEQ8000 Genetic analyser (Beckman Coulter).

Results

Microscopic botanical analysis

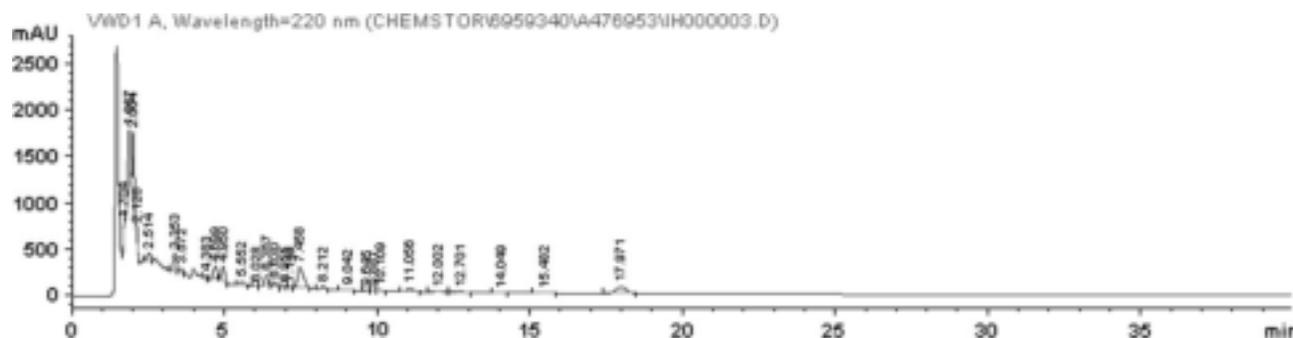
Gross examination of the 11 g sample of cannabis provided by the Chinese Academy of Sciences revealed loose dry vegetative material (Fig. 2A). The impression that the vegetative material had been lightly pounded was supported by examination of the wooden bowl, whose internal surface was worn smooth, apparently from use as a mortar (see Supplementary Fig. S5B at *JXB* online). The cannabis

retained a surprisingly green colour in its leafy parts and displayed visible glandular trichomes (Fig. 2B), the phytochemical factory of the plant and site of manufacture of cannabinoids and terpenoids (Potter, 2004; McPartland and Russo, 2001; Kim and Mahlberg, 2003). However, the ancient sample lacked the typical cannabis odour. Microscopic examination confirmed the presence of intact sessile trichomes with an amber tint (Fig. 2B), while higher resolution documented the retention of visible secretory cells within the trichomes (Fig. 2C). Achenes ('seeds') averaged 2.2–3.6 mm in length (Jiang *et al.*, 2006), were light in colour with some striations, but demonstrated rough, non-concave fruit attachment (Fig. 2D), all traits of domestication (Schlumbaum *et al.*, 2008) associated with cultivated cannabis strains (Vavilov, 1926). In contrast, achenes of wild strains are typically smaller and darker with concave attachment zones that favour shattering and easy spread (Vavilov, 1926). Germination was attempted with 100 achenes in compost, but no emergence was observed after 21 d.

Phytochemical analysis

Phytochemical and genetic teams were initially blinded to one another's results. The extraction of 2 g of plant

material produced 67.9 mg of solids after the removal of solvents. Using high performance liquid chromatography (HPLC), the largest cannabinoid peak was cannabitol (CBN) at 7.4 min, but concentration levels were very low, averaging 0.007% w/w. CBN is an oxidative breakdown product of THC, generated non-enzymatically, with increasing age (Brenneisen, 2007). There were also peaks corresponding to expected retention times for cannabidiol (CBD) at 4.9 min and cannabichromene (CBC) at 12 min (Fig. 3). Both are phytocannabinoids resulting from alternative enzymatic pathways than that yielding THC (de Meijer *et al.*, 2003). There were very few peaks in the first 20 min of the gas chromatogram where mono- and sesquiterpenes elute (Fig. 4). This lack of terpenoid volatiles supports the physical observation that the plant material lacked the herbal smell traditionally associated with cannabis (McPartland and Russo, 2001). Shown in Fig. 5A–C, (and in Supplementary Fig. S7A, B at *JXB* online) are breakdowns of sub-regions of the gas chromatogram. The major peaks in the 13–30.5 min region are free fatty acids (see Supplementary Fig. S7A at *JXB* online). The largest peak identified as palmitic acid was the most abundant in the sample. Methyl and propyl cannabinoids eluted in the 27–30 min region and the



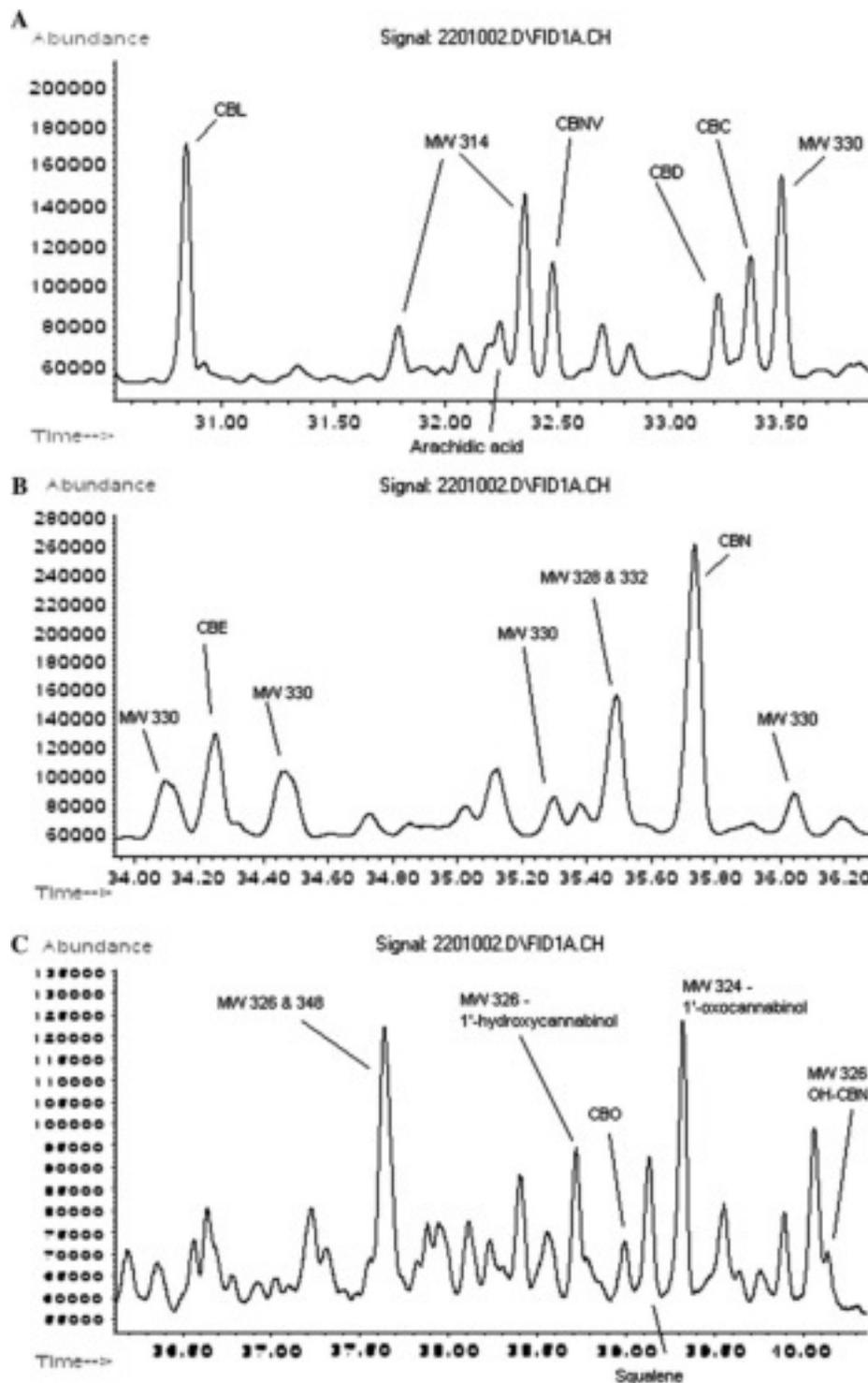


Fig. 5. Gas chromatography of ancient cannabis subsections. (A) GC of the 30–34 min region demonstrates several phytocannabinoids: cannabidiol (CBD), cannabichromene (CBC), cannabicyclol (CBL), and cannabinavarin (CBNV). (B) GC of the 34–36.3 min region displays the highest peak, cannabiol (CBN), the direct non-enzymatic oxidative metabolite of THC, with possible cannabielsoin (CBE) at 34.2 min. (C) GC of the 36.3–40.5 min region displays cannabitril (CBO) a THC degradant, and CBN variants (see text).

peaks marked as 286 Da and 302 Da all had MS spectra consistent with propyl cannabinoids. There were two phthalate peaks at approximately 23.5 min (believed to have originated from the polythene bags in which the

samples were supplied). A number of phytocannabinoids were identified in the 30–34 min region (Fig. 5A) including cannabidiol (CBD), cannabichromene (CBC), cannabicyclol (CBL, a heat-generated artefact of CBC

(Brenneisen, 2007), and cannabinavarin (CBNV, a propyl analogue of CBN). In the 34–36.3 min region (Fig. 5B), apart from cannabinol (CBN), the largest individual phytocannabinoid component, there were at least four peaks of 330 Da with cannabielsoin (CBE, an artefact derived from CBD (Brenneisen, 2007) a likely identification of the peak at 34.2 min. In the 36.3–40.5 min region (Fig. 5C), the known THC degradant cannabitol (CBO) (Brenneisen, 2007) was seen, as well as a series of peaks with spectral similarities to CBN, three of which are tentatively identified by the NIST database as either hydroxyl- or oxo-CBN. The last region (42–50 min; see Supplementary Fig. S7B at *JXB* online), contained phytosterols and triterpene alcohols with beta-sitosterol the most abundant compound.

Mass spectra (MS) of selected phytocannabinoids corresponding to the above are displayed (Fig. 6). Values are all in agreement with those in NIST and GW Pharmaceutical databases.

There was a very small peak detected at the correct retention time in the sample for THC, but the spectra could not confirm its identity.

Genetic analysis

Because of the unique degree of preservation of the cannabis, a genetic analysis was undertaken. The two ancient DNA sequences determined were labelled *China F* and *China F(h)*. Alignment of these paleo-sequences (excluding the primers' region, in red in Fig. 7A) with the presently available databases demonstrated:

(i) *China F* (Fig. 7A) is identical 134/134 nucleotide agreement to other deposited sequences: AB212841, AB212839, AB212836, AB212833, AB212830, all belonging to tetrahydrocannabinolic acid synthases (THCA-synthases), a species-specific genetic region (Schlumbaum *et al.*, 2008) from *Cannabis sativa* L.

(ii) *China F(h)* (Fig. 7A) is a new variant, not previously present in the genetic databases (submitted to NCBI, GenBank accession number EU839988), showing a maximum identity of 132/134 nucleotides with the above-mentioned sequences and with *China F*.

Utilizing BLASTX, i.e. performing searches through amino acid translation, it was again shown that the *China F(h)* amino acid sequence is not registered in the database, and this is an obvious but necessary confirmation of the originality of this variant of the THCA synthase allele. These results also prove that both sequences encode for THCA synthase, the biosynthetic enzyme for THCA that decarboxylates via heat or ageing to yield psychoactive THC (Russo, 2007). Direct comparison of the two ancient sequences, identified the nature of the small differences observed: the samples have two 'mutations' (highlighted

in yellow in Fig. 7A), which can be considered transversions: from guanine to cytosine, and from cytosine to adenine. The first of these two nucleotide substitutions is synonymous, i.e. it does not change the amino acid sequence, while the second one is a non-synonymous substitution, leading to a serine-threonine exchange (highlighted in light blue in Fig. 7B) in the encoded amino acid sequence; these two amino acids, however, have similar physico-chemical properties. No CBDA synthase, the biosynthetic enzyme for CBD (de Meijer *et al.*, 2003), was identified in the sample.

Discussion

The results presented collectively point to the most probable conclusion which is that the *Gūshī* culture cultivated cannabis for pharmaceutical, psychoactive or divinatory purposes. In examining the botanical evidence from this 'old and cold' site with its unique degree of preservation, the cannabis consisted of a processed (pounded) sample whose seed size, colour, and morphology, at least according to principles of Vavilov (Vavilov, 1926), suggest that it was cultivated rather than merely gathered from wild plants. The considerable amount of cannabis present (789 g) without any large stalks or branches would logically imply a pooled collection rather than one from a single plant. Importantly, no obvious male cannabis plant parts (e.g. staminate flowers, not infrequently observed in Indian herbal cannabis, or *bhāng* (Russo, 2007) were evident, implying their exclusion or possible removal by human intervention, as these are pharmacologically less psychoactive.

The HPLC, GC, and MS analyses confirm the identity of the supplied plant sample as *Cannabis sativa* L. The predominance of CBN indicates that the original plants contained Δ^9 -tetrahydrocannabinol (THC) as the major phytocannabinoid constituent. The presence of CBO and numerous CBN-related substance peaks further supports this view. CBD and CBC, together with their known thermo-oxidative degradation products CBE and CBL (Brenneisen, 2007), are present, but the GC analysis would appear to indicate that, in both cases, CBC and CBL are represented in greater quantities, as expected in a high-THC cannabis strain wherein CBD is only a minor component. In addition, there is a peak for CBNV which confirms that the plant also contained Δ^9 -tetrahydrocannabinavarin (THCV), a propyl phytocannabinoid. All of these observations are consistent with strains of cannabis with a high THC content and in an alternative taxonomy suggests it should be assigned to *Cannabis indica* Lamarck (Hillig and Mahlberg, 2004).

While chromatography elution times may vary with temperature, column type, and other factors, confirmation was evident with corroboratory mass spectra values that

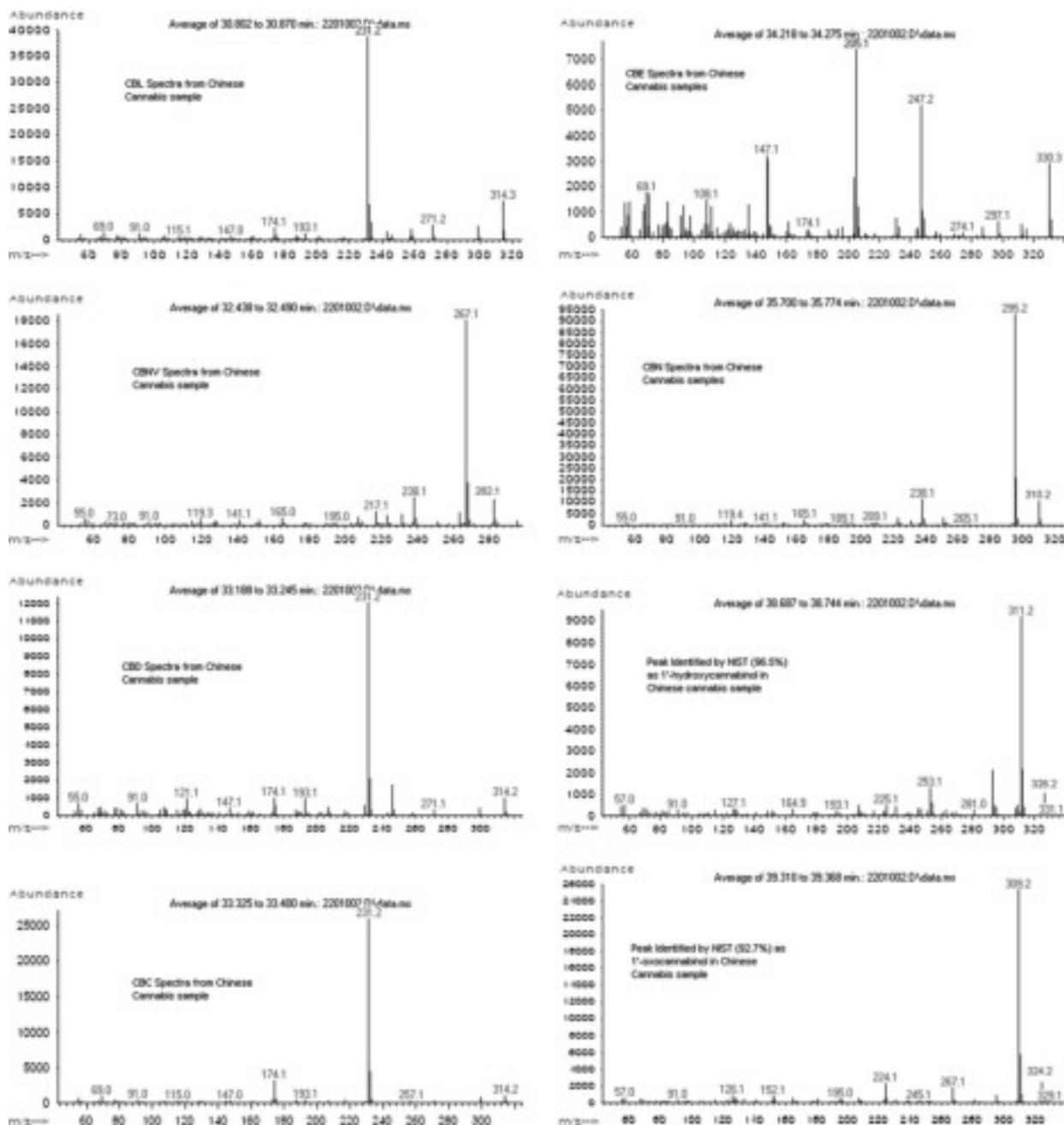


Fig. 6. Mass spectra of ancient cannabis. Subsections demonstrate the phytocannabinoids cannabiniol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabiolivarin (CBNV), cannabichromene (CBC), cannabielsoin (CBE), 1'-oxcannabinol, and 1'-hydroxycannabinol.

were identical to those seen daily in assays performed on fresh cannabis extracts in this laboratory.

The presence of so many recognized cannabinoid degradants is consistent with very old cannabis samples. The very low concentration levels measured in the HPLC analysis may indicate that the sample provided contained significantly more leaf and twig material than flower material, rather than being evidence in itself that the sample was of low potency originally. This plant material is therefore conclusively cannabis derived from a population

of plants within which THC was the dominant cannabinoid. By contrast, a sample taken from a mix of wild-type *Cannabis sativa* would customarily harbour a more equal mixture of THC and CBD (de Meijer *et al.*, 2003). It would appear, therefore, that humans selected the material from plants on the basis of their higher than average THC content. To elaborate, a chemotaxonomy of cannabis previously outlined indicates three types (Small and Beckstead, 1973): chemotype I (drug) strains with high-THC:CBD ratios, chemotype II low-THC, higher-CBD

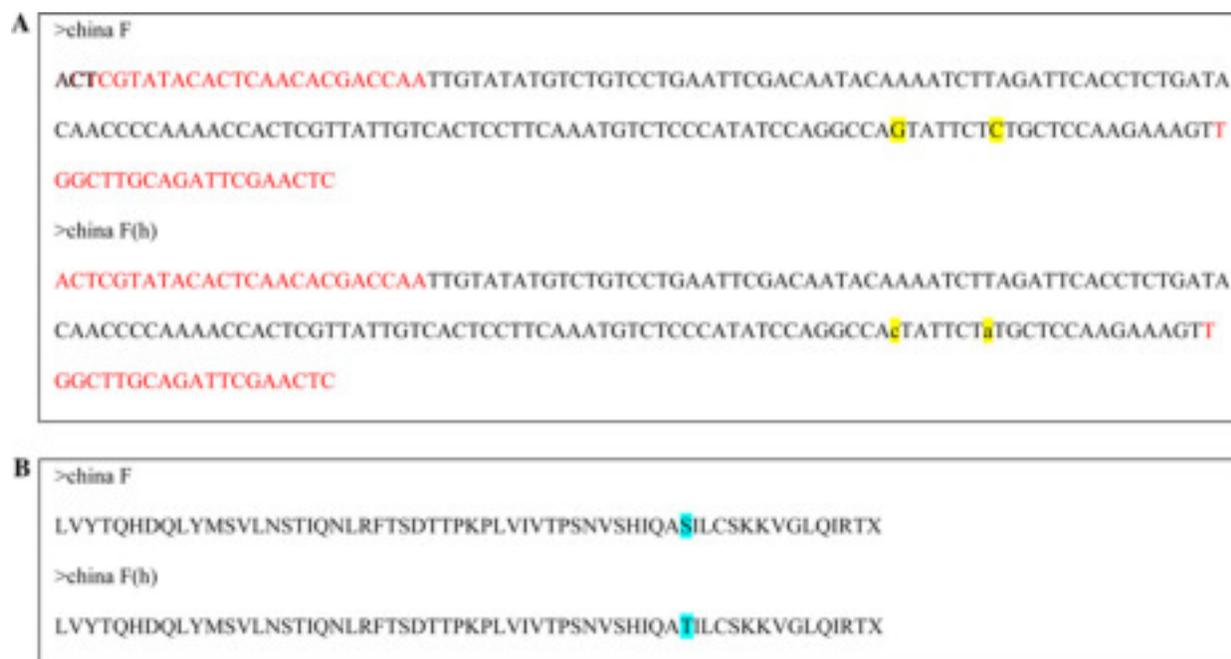


Fig. 7. DNA analysis of ancient cannabis. (A) Nucleotide sequences of the wild-type tetrahydrocannabinolic acid synthase, China F, and the mutant sequence, China F(h), with two single nucleotide polymorphisms highlighted in lower case yellow. (B) Amino acid translation of China F and China F(h), demonstrating divergence in a change from serine (wild-type) to threonine (mutant), highlighted in blue.

(fibre) strains, and chemotype II with more equal ratios. THC and CBD production are mediated by co-dominant alleles B_T and B_D , respectively (de Meijer *et al.*, 2003). By comparison, pooled samples from cannabis fields in Morocco and Afghanistan will normally produce 25% high-THC plants, 25% high-CBD plants, and 50% with lower, mixed titres, combining to yield roughly equivalent amounts of the two phytocannabinoids (Russo, 2007), a pattern not observed in our specimen.

Isotopic analysis of cellulose from this cannabis sample might conceivably be used in comparison with other samples in an attempt to establish its geographic origin.

While multi-purpose cannabis plants used simultaneously for food (seed), fibre (stalks), and pharmaceutical uses (flowering tops) have been recently reported from Darchula in far western Nepal (Clarke, 2007), more customarily, a given plant is best suited toward a single purpose. Of additional key importance is the absence of hemp artefacts from the Yanghai Tombs. The *Gūshī* fabricated clothing from wool (see Supplementary Fig. S6B at *JXB* online) and ropes from *Phragmites* (reed) spp. fibres (see Supplementary Fig. S6C at *JXB* online). Whereas hemp textiles have been collected from the Northern China Yangshao Culture from 6000–7000 years BP, their appearance in the west was not documented before 2000 years BP, for example, 1500 years BP in Kucha, 600 km west of Turpan (Mallory and Mair, 2000).

Previous phytochemical analyses of antique cannabis preparations have demonstrated THC remnant fingerprints

from 19th century cannabis preparations (Harvey, 1990) including a 140-year-old sample of Squire's Extract (Harvey, 1985). A study in 1992 reported the presence of Δ^8 -THC (previously termed Δ^6 -THC) from burned cannabis that was reportedly inhaled as an aide to childbirth in a Judean cave 1700 years BP (Zias *et al.*, 1993), supported by the finding of cannabinoid residues in an adjacent glass vessel (Zias, 1995). In the Mustang region of Nepal, mummified human remains of probable Mongolian ancestry have been dated 2200–2500 years BP in association with cannabis, probably transported from elsewhere (Knörzer, 2000; Alt *et al.*, 2003), but with insufficient detail to ascertain its use. Rudenko recovered cannabis seeds, censors, and hempen clothing in Pazyryk, Siberia from Scythian *kurgans* (burial mounds) from 2400–2500 years BP (Rudenko, 1970; Brooks, 1998), closely matching Herodotus' descriptions of funeral rites for that culture (Herodotus, 1998). Sarianidi also claimed cannabis use in the Bactria–Margiana Archaeological Complex (BMAC) (present day Turkmenistan) (Sarianidi, 1994, 1998), but this interpretation has been debated (see discussion in Russo, 2007).

Another independent genetic analysis of this material published subsequent to our analysis (Mukherjee *et al.*, 2008) confirmed the presence of THCA synthase, but not the single nucleotide polymorphisms. The authors posited a European–Siberian origin for the material.

Current genetic data also confirm that the plant material examined is *Cannabis sativa* L. according to ITS and cpDNA analysis. The results also support the hypothesis

of the existence of at least two THCA-synthase nucleotide sequences in the ancient plant material examined. One of these sequences perfectly matches the corresponding sequence of already-known THCA-synthases deposited in GenBank, both as gene and protein sequences; the second sequence is a novel one, with two single nucleotide polymorphisms (SNPs) encoding for a protein with presumably very similar characteristics. Whether these two sequences coexisted in a single cannabis plant or a strain heterozygous at the *B* locus, or belong to different plants, could not be concluded.

THC represents one of the possible phytocannabinoid end-products manufactured by cannabis plants; THC (or, in its native form, THCA) is synthesized by a well-characterized enzyme (THCA- or THC-synthase) from a precursor (CBG or CBGA) common to most chemotypes that represents the metabolic ‘switching point’, downstream of which the variability of the different chemotypes is concentrated. The agents of such variability found in cannabis germplasm are exclusively the different synthases, among which THC(A)-synthase is the only one responsible for making that specific cannabinoid, THC. Therefore, the presence of the allelic variant responsible for coding the THC(A)-synthase may well be considered to be diagnostic, or at least strongly suggestive of a THC-producing plant. The fossil cannabis plants found were therefore genetically equipped to produce THC. How much THC they actually produced, cannot of course be specified because they depend on a number of anatomical, environmental, and nutritional factors that remain unknown.

Numerous questions remain. Current data do not permit it to be ascertained how the cannabis from the tomb was administered. If used orally, perhaps it was combined in some fashion with *Capparis spinosa* L., as these plants were found together in a nearby but later tomb at Yanghai (Jiang *et al.*, 2007). That date for that tomb was initially reported as 2700 years BP via radiocarbon methods, and since corrected to 2200–2400 years BP with additional calibration employing tree ring data. If this cannabis were smoked or inhaled, no mechanism for so doing has been excavated in the area. The *Gūshī* could have sifted the cannabis through fabric after pounding, then fumigated it, much as described for the alleged cannabis candidate, the Sumerian A.ZAL.LA, administered medicinally for ‘hand of ghost’ (Thompson, 1923, 1949), since posited as nocturnal epilepsy (Russo, 2007; Wilson and Reynolds, 1990). While this culture could have arrived from the earlier BMAC region as ‘oasis hoppers’ (Barber, 1999), and certain cultural relationships are apparent to the Scythian culture with respect to cannabis use and equestrian prowess, those peoples were Iranian speakers (Mallory and Mair, 2000). In addition, *Gūshī* cultural affinities and burial practices much more closely resemble those of the presumed proto-Tocharian speaking, incense-burning (Kuzmina, 1998) Afanasievo peoples in the

Yenisei Valley to the north (Anthony, 1998, 2007; Mallory, 1998; Renfrew, 1998; Mallory and Mair, 2000), whose putative southward migration some authorities have attributed to ‘global cooling’ *c.* 4000 years BP (Hsü, 1998), and to their proto-Indo-European-speaking Yamnaya forebears further west, dating to 6000 years BP (Mallory, 1989; Anthony, 1998; Winter, 1998). Abundant mysteries remain as to the origins and customs of the *Gūshī*. Additional answers may accrue from future archaeological excavations or human genetic analyses that elucidate relationships with other ancient cultures and modern peoples of the region. The unique SNPs discovered in this ancient sample may yet be of critical importance in tracing the phylogeny and geographic spread of cannabis and the humans who used it.

The excellent preservation of the cannabis from this tomb allowed an unprecedented level of modern botanical investigation through biochemistry and genetics to conclude that the plant was cultivated for psychoactive purposes. While cultivation of hemp for fibre has been documented in Eastern China from a much earlier date (*vide supra* Mallory and Mair, 2000), the current findings represent the most compelling physical evidence to date for the use of cannabis for its medicinal or mystical attributes.

Supplementary data

Photographs and diagrams of the Yanghai Tombs site, Tomb M90 contents including fabric and ropes, and additional chromatographic and genetic analysis primer sequence information are presented in Supplementary Figs S1–S8, available online.

Fig. S1. Study site at the Yanghai tombs with Huoyan Shan mountain range in background (photo EBR).

Fig. S2. Diagrams of the Yanghai Tombs (adapted from Xinjiang Institute of Cultural Relics and Archaeology, 2004, with permission).

Fig. S3. The shaman’s tomb, M90 [previously published in Mandarin (Xinjiang Institute of Cultural Relics and Archaeology, 2004), used with permission].

Fig. S4. The shaman’s skull (photos EBR).

Fig. S5. Containers in which cannabis was stored in tomb [previously published in Mandarin (Xinjiang Institute of Cultural Relics and Archaeology, 2004) used with permission].

Fig. S6. Re-excavation of Tomb M90. This was undertaken to re-examine artefacts, measure GPS coordinates, and assess environment conditions (photos EBR).

Fig. S7. Chromatography subsections from phytochemical analysis.

Fig. S8. Primer sequences employed in the genetic analysis to amplify THC- and CBD-allele specific fragments and their sequences (5′ → 3′).

Acknowledgements

The authors are grateful to the Chinese Academy of Sciences and GW Pharmaceuticals for support of the project. Kim Laughton facilitated communication and logistics between the Chinese authorities and the British Home Office for exportation and importation of the ancient cannabis. Daniel Adams, Laura-Jane Everitt, and Helen Keogh performed phytochemical analytical preparation, supervised by Peter Gibson. Ying Li provided translation and logistical support to EBR during field work in Xinjiang. Gregory Gerdeman is thanked for his helpful review of the article, as are the anonymous reviewers for their suggestions. No competing financial interests were operative in this study.

Author contributions: EBR proposed and co-ordinated the current study, engaged in field work, and wrote the article drafts. HEJ performed the majority of the background research and was actively engaged in current investigations. AS performed the phytochemical analysis and wrote the pertinent methods and results sections. AC, FDB, and GM performed the genetic analysis and wrote the pertinent methods and results sections. DJP co-ordinated the handling of the ancient cannabis in the UK, performed the microphotography, and wrote the pertinent methods and results. EGL, XL, DKF, FH, YBZ, YFW, LCZ, and CJL were all engaged in earlier investigations in relation to this study. YXZ analysed the phytochemistry of the cannabis sample and SB and his colleagues analysed the phytochemistry and genetics of the cannabis sample independently. CSL conceived the concept of studying the archaeological cannabis samples by multidisciplinary methods, and organized, co-ordinated and supervised all aspects of the current study and its performance.

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