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Authors


Murphy, Terence M.
Ben-Yehuda, Nahum
Taylor, R.E.
et al.

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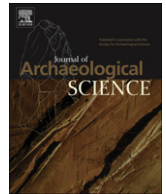
► We analyzed DNA from rope and fabric from the “Christmas Cave” in the Qidron Valley. ► Ribulose biphosphate carboxylase gene DNA was primarily from *Linum usitatissimum* L. ► Samples also had variable amounts of hemp (*Cannabis sativa* L.) DNA. ► ¹⁴C dating confirmed that samples represented both the Roman and Chalcolithic periods in Israel.

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Hemp in ancient rope and fabric from the Christmas Cave in Israel: talmudic background and DNA sequence identification

Terence M. Murphy^{a,*}, Nahum Ben-Yehuda^b, R.E. Taylor^{c,d,e}, John R. Southon^f

^a Department of Plant Biology, University of California, One Shields Avenue, Davis, CA 95616, USA

^b Department of Jewish History, CText ATI, Bar Ilan University, Ramat Gan 52900, Israel

^c Department of Anthropology, University of California, Riverside, CA 92521, USA

^d Cotsen Institute of Archaeology, University of California, Los Angeles, CA 90095, USA

^e Keck Accelerator Mass Spectrometry Laboratory, University of California, Irvine, CA 92697, USA

^f Department of Earth System Science, University of California, Keck Accelerator Mass Spectrometry Laboratory, Irvine, CA 92697, USA

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ABSTRACT

The “Christmas Cave”, a cave in the Qidron Valley near the Dead Sea and Qumran, has yielded a complex collection of plant-derived rope and fabric artifacts. Using polymerase chain reaction (PCR) to amplify DNA of the samples, we estimated the sizes and determined restriction patterns and base sequences of chloroplast genes, primarily *rbcl* (gene for the large subunit of ribulose biphosphate carboxylase). DNA was successfully extracted from all samples, but was limited to sizes of approximately 200–300 base pairs. As expected, the DNA extracted from the samples was identified as coming primarily from flax (*Linum usitatissimum* L.), but two samples had a significant fraction, and all samples had at least a trace, of hemp (*Cannabis sativa* L.) DNA. Artifacts from the Christmas Cave were thought to date from Roman times, but it was thought possible that some could be much older. Accelerator mass spectrometry (AMS)-based ¹⁴C dating confirmed that the samples contained representatives from both the Roman and Chalcolithic periods. This paper provides a synthesis of DNA, isotope, and literary analysis to illuminate textile history at the Christmas Cave site.

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1. Introduction

DNA analysis can identify the biological source of archaeological artifacts. This is true for many plant-based artifacts. Plant cells contain plastids, such as chloroplasts in leaves—often many copies—and plastids contain DNA sequences that are useful for identification. There is a great deal of information available concerning the base sequences of plastid genes in different plants, much of it gathered for use in determining evolutionary relationships. This information can be applied to objects like textiles and baskets.

At first glance, it may be surprising that DNA persists in manufactured objects, and some processes—e.g., mordanting—do break

down DNA. However, even present-day rope is made with natural fibers that receive a minimum of treatment, and the rope contains fragments of tissue with intact organelles (Dunbar and Murphy, 2009). We expect that treatments of fibers in the past were less stringent and the products from which they were made more likely to retain plastids and nuclei.

It may be even more surprising that DNA persists in ancient objects, since we can expect the rigors of time, with accompanying hydration, desiccation, and temperature extremes, to break down biological molecules. In fact, that does occur (Smith et al., 2003). But DNA may show a degree of resistance under certain conditions. Indeed its structure may have evolved in part to increase its stability (Lazcano et al., 1988). There have been many reports of ancient DNA isolated from, for example, mammoths preserved in glaciers (Gilbert et al., 2007), human mummies (e.g., Caramelli et al., 2008), wood (Liepelt et al., 2006), and rope (Mukherjee

* Corresponding author. Tel.: +1 530 752 2413; fax: +1 530 752 5410.

E-mail address: tmurphy@ucdavis.edu (T.M. Murphy).

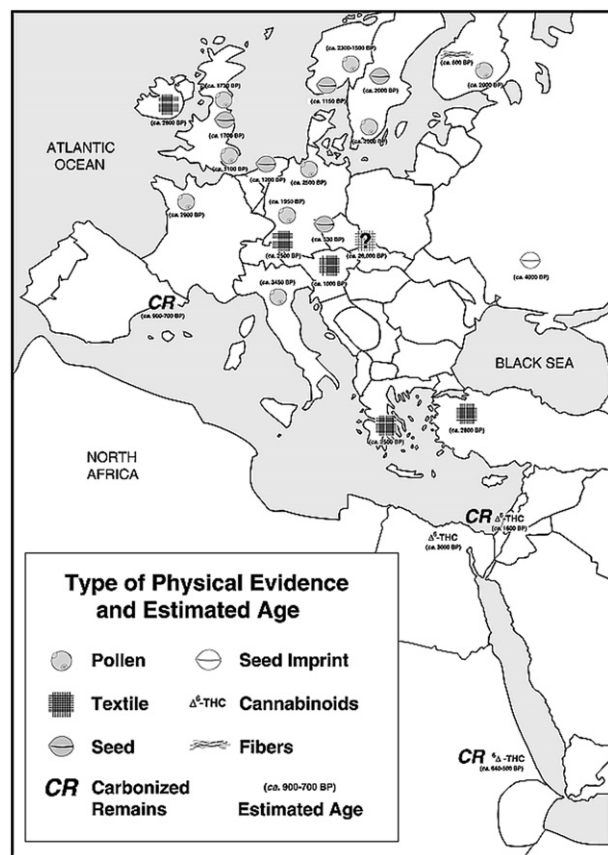


Fig. 1. Evidence of ancient uses of hemp in Europe and Asia Minor (Fleming and Clarke, 1998).

et al., 2008). The degradation of DNA results in a gradual reduction in its length as the polymeric strands become fragmented, but even relatively short fragments retain useful information in their base sequences.

With those considerations in mind, we resolved to identify DNA in fibers of samples of rope and cloth that have been found in an archeological site near Qumran and the Dead Sea. Microscopic observations have identified various samples of cloth from caves above Qumran as flax, cotton, and wool (Walton Rogers, 2003 and personal communication). Muller et al. (2004, 2006, 2007), using X-ray micro-diffraction, identified flax and cotton in Qumran samples. But there have been indications of the early use of hemp in East Asia (Mukherjee et al., 2008) and in Europe and Asia Minor (Fig. 1). Our objective was to learn whether the DNA of materials from an archeological site near the Dead Sea could confirm the presence of hemp or other fibers. We expected the use of DNA sequence information to confirm the identity of the major component (as flax), but also to indicate whether fibers from hemp or another plant species form a detectable fraction of one or more samples. As will be shown, our data do indicate that flax-linen dominates in every sample tested and that there is a small amount of hemp DNA in most samples.

2. Background

Samples described in this paper came from the “Christmas Cave” (herein abbreviated “CC”). The CC is located in the Judean Desert on the west bank of the Dead Sea, in the Qidron Valley, 1 km south of Qumran – (ICS coordinates 189887/121095)(Fig. 2). Its name commemorates the day on which it was discovered by John Allegro

in 1960 (Allegro, 1965, pp. 6–15). In 2007, the cave was surveyed again by Roi Porat and Hanan Eshel (Porat et al., 2007). They confirmed that the finds have no connection to the Qumran Caves (see also Shamir and Sukenik, 2010). This cave served as a refuge, beginning in the Chalcolithic Period and afterwards at the end of the Great Revolt in 73 CE and again in the Bar Kokhba Revolt in 135 CE. (Porat et al., 2009) Among the archaeological finds from this cave are wool and linen textiles from various periods. This assortment of textiles, in contrast to those found in Qumran – which are exclusively linen - is similar to those found in Masada (Belis, 2003, p. 211, 219, Sheffer and Granger-Taylor, 1994). In the opinion of Dr. Orit Shamir of the Israeli Antiquities Authority, comparison to the textiles found in The Cave of Letters (Granger-Taylor, 2006; Yadin, 1963) is more exact, those being generally coarser than the Masada textiles (personal correspondence).

Because this site is not considered part of the Qumran complex of caves, Humpert and Gunneweg’s (2003) inclusion of these textiles in the Qumran group of findings is confusing. The introduction (p. XIX) under the subheading “textiles” does not mention the CC at all, notwithstanding its textile finds being included in the subsequent chapters. The CC finds all appear numbered as category “QCC” (=“Qumran Christmas Cave”), and some have been given a parallel “QUM” number (Belis, 2003, p. 221; Müller et al., 2003, p. 277). In Humpert and Gunneweg (2003) Walton Rogers reports her analysis of some of these fibers, referring to them simply as coming from “a site in the Dead Sea region”, which is correct under any circumstances.

These cordage and textile samples were stored since their discovery at the Rockefeller Museum in Jerusalem, examined at École Biblique et Archéologique Française de Jérusalem (EBAF), and only recently relocated to the Israel Antiquities Authority. The articles had been bundled in batches from the various loci within the CC. Due to the difficulty of dating these samples, either by physical observation or by genetic testing, we chose to confirm the ages of selected samples through ^{14}C dating (see below).

It is generally accepted, based on both literary sources and archaeological finds, that the primary fibers in use in the 1st–4th centuries CE in the Land of Israel were sheep wool, goat and camel hair, and flax-linen¹. Additional fibers, such as hemp, cotton and silk are mentioned, albeit infrequently, in contemporary rabbinical literary sources, but have not been previously corroborated by archaeological finds of the period (Baginski, 2001; Shamir, 2001; Crowfoot and Crowfoot, 1961). This research project focuses on the genetic identification of vegetable fibers constituting cordage and textiles found in the CC. It has always been assumed that the fiber used in articles of this type is flax, which is well known as being widespread in the Land of Israel in this period, and is mentioned numerous times in the contemporary rabbinical literature (Mishnah; Toseftah and the Jerusalem Talmud)². As stated in the introduction, this assumption has been validated by optical microscopy (Walton Rogers, 2003) and X-ray diffraction (Müller et al., 2004, 2006, 2007). Nevertheless, it is difficult to distinguish cellulosic bast fibers such as flax and hemp by these means

¹ See: ASTM D 6798-02 *Standard Terminology Relating to Flax and Linen*. 2003. West Conshohoken, PA. “Flax” refers to the plant and its fiber. “Linen” refers to the products produced from spinning onwards in the production process. Both the Hebrew and Aramaic languages do not discern between these two designations, often causing ambiguity.

² The Mishnah, Toseftah and Jerusalem Talmud are all works redacted in the Land of Israel. Documentation of material culture appearing in all of them should be relevant to our research. The Mishnah (and probably Toseftah) represents 225 CE *terminus ante quem* and probably long before 70 CE *terminus post quem*. The Jerusalem Talmud, which is a work expounding on the Mishnah, is 350 CE *terminus ante quem*.

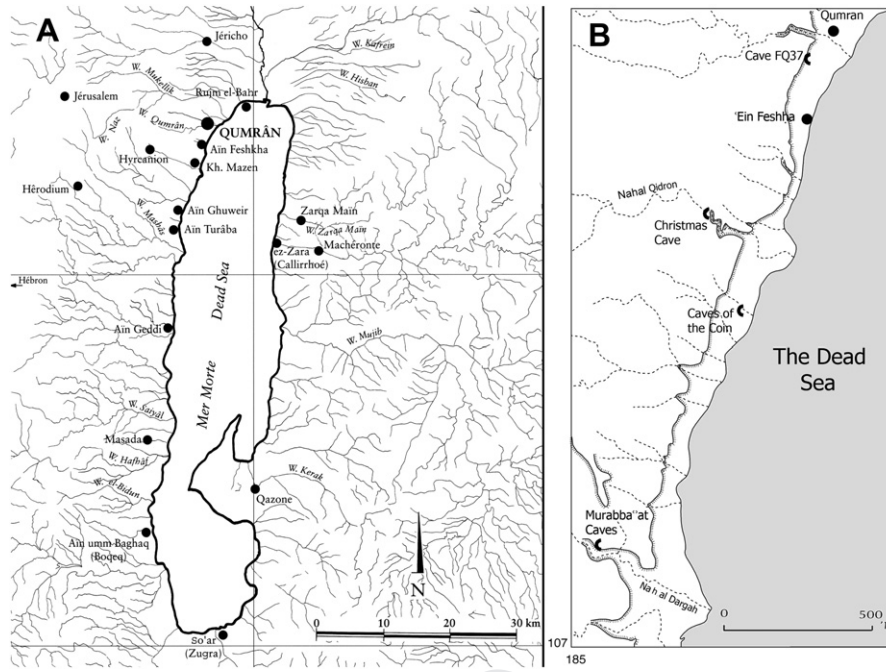


Fig. 2. A, Map of the Dead Sea with principal sites from the 1st century CE (Humpert and Chambon, 2003). B, Map of the refuge caves in the Qumran area. (Porat et al., 2009).

(Bergfjord and Holst, 2010) all the more so when they are extremely old, having suffered the damaging and deteriorating effects of time. Furthermore, optical and X-ray techniques may miss minor components of mixed fibers.

Hemp (*cannabis*) is mentioned in the Talmudic literature as an existing, marginal, textile product in the Land of Israel, similar, albeit inferior, to flax. Both flax-hemp blend and wool-camel's hair blend are mentioned in Mishnah Tractate Kil'ayim 9:1 and a parallel in Tractate Nega'im 11:2. The indication is that the two fibers blended together are quite similar. Hemp as a similar, albeit invalid, substitute for flax for the sacerdotal vestments is mentioned in Toseftah Tractate Menahot 9:17. In modern literature, mention has been made of the adulteration of flax with hemp as a possible means of deceit, as flax is more expensive (See Oakley, 1928, pp.167–9). But blending flax with hemp may be practiced to obtain rope that is both soft and strong (Weindling, 1947, p. 286). These two practices again indicate the similarity between hemp and flax.

Although linen cordage (rope or twine)³ is mentioned in rabbinical literature (Jerusalem Talmud Tractate Sukkah 1:52:2 and Tractate 'Erubin 5:22:4), it is possible that hemp was also used in these products and as well in other coarse textiles (Barber, 1991, p. 15; McKenna, 2004, pp. 4–5; Weindling, 1947, p. 286).

As opposed to the fabric and nets found in the Land of Israel, which have typically been the focus of extensive research, there is less information on the cordage finds (although see Shamir, <http://www.atnfriends.com>). Where were the ropes and textiles manufactured, and where did the raw materials come from? This being a cave of refuge, it is highly unlikely that the materials were manufactured *in situ*. A probable candidate is Beth Shean, which is

mentioned in the Diocletian Edict (26:23–28:30) by merit of its superior linen products, referred to there by its Roman name “Scythopolis”. Beth Shean, approximately 80 km north of the Dead Sea, is frequently mentioned in Talmudic sources (E.g. Jerusalem Talmud tractate Qiddushin 2:62:3, Midrash Beresheit Rabbah 19 and 20, Midrash Tan'umah Tazria' 7) as the famous center in this period for flax growing and manufacture of fine linen garments. Flax is mentioned in the Biblical period (c.1200 BCE), in nearby Jericho (Joshua 2, 6) and Crowfoot (1960, 1965, 1982) cites evidence of earlier finds there, too. In adjacent Hesban (Platt, 2009, pp.163–196. Mitchel, 1992 pp. 3–38. Personal correspondence with Paul Ray, Associate Curator, Horn Archaeological Museum at Andrew University), 'Ein Gedi (Shamir, 2007 “The Loomweights” and 2007 “The Textiles”) and Masada (Sheffer and Granger-Taylor, 1994), textile manufacturing implements from the relevant time periods have been unearthed, some of them possibly related to rope manufacture. Another possible source is Jerusalem, approximately 40 km away.

Talmudic literature mentions rope-making in several contexts, one of which is the halakhic injunction against using (even) a derelict synagogue (which is particularly suitable, being a long building) as a rope walk (Mishnah Tractate Megillah 3:3) due to the site's holiness. Others include rope making done by two halakhically impure individuals (Mishnah Tractate Zavim 3:2) and the injunction against making rope in a city of refuge (Toseftah Tractate Makot 3:10) (See also: Herzberg, 1924, pp. 140–146.).

Hemp in an agricultural context is mentioned only once in the Mishnah (Tractate Kil'ayim 2:5). According to Felix (1967, pp. 220–222) the text is corrupt⁴ and should be read “caraway”. Amar (2000, p. 336, and personal correspondence) believes that the printed text can be accepted as is, but in any case hemp was definitely a very marginal crop. The Mishnah and Jerusalem Talmud

³ See Denton and Daniels: “Rope” is an article of cordage more than approximately 4 mm in diameter”, and “twine” is twisted cordage less than 4 mm in diameter. The Talmudic term for rope is “hevel”, and for twine is “meshiha”. Our examples are border line in their diameters. Toseftah Tractate 'Erubin 2, 2 mentions rope of 8 cm in diameter, for measuring the *t'hum*. In Tractate Zavim 1, 11 a rope 50 m long is mentioned.

⁴ The printed text reads “knbs”, while variants including the Jerusalem Talmud read “krbs”. A perennial plant is necessary for the Mishnaic textual context. This designation fits caraway (“krbs”), not cannabis.



Fig. 3. Photographs of selected rope (928, 931, 795, 796) and fabric (786, 955, 019) samples used in this analysis.

(Tractate Kil'ayim 9:7) mentions that garments made of hemp (possibly blended or combined with sheep's wool) were imported from "overseas" perhaps from the Aegean and Asia Minor regions (Fig. 1) (Fleming and Clark, 1998; Herzberg, 1924), through the ports of Caesaria and Tyre and their respective vicinities. Perhaps fibers and/or yarn for weaving and rope manufacture were also imported, not locally grown. Pliny the Elder mentions hemp in a textile context (Book 19, ch. 8, and ch. 56.9). It is particularly suitable for rope and hunting nets. Two localities of production are mentioned: Alabanda and Mylasa, both in Asia Minor (Fig. 1).

In order to position these ropes' usage in their contemporary and local context, the following are the various uses for rope mentioned in Talmudic literature, any or all of which may very well have existed at CC.

Mishnah:

- Used as a guideline for harvesting straight rows of grain (Pe'ah 4:5)
- To support the branches of a grapevine (Kil'ayim 6:9)
- A leash for an animal (Shabbat 5:3 passim)
- A handle for a basket (Shabbat 8:2 passim)
- A sailor's knot (Shabbat 15:1)
- A cameleer's knot (ibid.) Tied to a bucket for drawing water from a cistern (Shabbat 15:2 passim)
- To secure a burden to an animal's back (Shabbat 24:1)
- To measure the "tehum", the distance permitted to walk on the Sabbath ('Eruvin 1:9)
- Rope bed or stool (Pesahim 4:9 passim)
- To measure a parcel of land (Bava Batrah 7:2–3)
- To bind an animal's legs (Parah 3:9)

Toseftah:

- To secure a person who is going to immerse in water - a "lifeline" (Shabbat 1:18)
- To bind bundles of branches (Betzah 3:10)
- Used for shade, placed on the roof of a booth (Sukkah 1:4)
- To climb up to the roof (Makot 2:6)
- To check the water level in a cistern - a "dipstick" (Makhshirin 9:6)

Additional maritime uses of rope are detailed in Sperber (1986). Shipping in the adjacent Dead Sea was well developed in the Roman period (Porat – personal correspondence). Porat et al. (2009) notes that entry and mobility in the CC require the use of rope, as in rappelling, or abseiling.

Indeed, rope (both laid and braided) was certainly used in antiquity for a multitude of purposes. Many rope finds have been retrieved from CC and other sites in the Land of Israel. The vast majority of these ropes are made from date-palm fiber; additional ropes are of goat and camel hair; and the distinct minority is of bast fiber, which has always been assumed to be flax. Up until now, no archaeological textile finds in the Land of Israel have been identified as hemp, but up until now DNA technology has not been employed.

3. Methods

3.1. Samples

Samples of cordage and textile, part of the National Treasures Collection of the Israel Antiquities Authority, were identified by curator Dr. Orit Shamir as coming from the "Christmas Cave" collection. Cordage samples were numbered I.A.A. 582928 (herein abbreviated 928), 582931 (931), 585795 (795), 585796 (796), and 637538 (538). Textile samples were numbered 582812 (812), 582955 (955), 583019 (019), 585440 (440), 585786 (786), 577061 (061), and 577289 (289). Two samples of modern rope were included: one stated to be of linen from Japan, and one of flax from the United Kingdom. A positive control sample of flax, *Linum usitatissimum* L., was obtained from the garden of the Plant Sciences Department, University of California, Davis, California, USA. Positive control samples of *Cannabis sativa* L. were obtained from the police department of Bakersfield, California, USA.

3.2. Extraction of DNA

Samples of cordage and textile, ranging in weight from 0.04 g to <0.01 g, were each ground to dust in ceramic mortars and pestles or (less effectively) in disposable microfuge tubes with plastic pestles; 0.7 ml of buffer containing 1% cetyltrimethyl ammonium bromide, 0.7 M NaCl, 10.5 mM ethylenedinitrilotetraacetic acid, and 55 mM Tris buffer, pH 8 was added, and the slurry was ground to homogeneity. The mixture was heated at 65 °C for 10 min or more and then cooled; 4 µl of RNAase containing 107 units were added; and the solution was incubated at 37 °C for 20 min. The mixture was extracted with 350 µl of chloroform, and DNA in the aqueous layer was precipitated with an equal volume of isopropanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and dissolved in water. The DNA was further purified by adsorption on and elution from glass filters (Qiagen or Promega). Samples of fresh

L. usitatissimum L. and dried *C. sativa* L. were treated similarly after having been ground in disposable microfuge tubes with plastic pestles.

3.3. DNA amplification

Identification of the plant species contributing fibers to the cordage and textile samples began with the polymerase chain reaction (PCR)-amplification of the ribulose biphosphate carboxylase-oxygenase large subunit (*rbcl*) gene (GenBank references: *L. usitatissimum*, FJ169596; *C. sativa*, AF500344). This gene is located in chloroplast DNA. Thus, it is specific for photosynthetic organisms and its use avoids confusion from the presence of animal or fungal DNAs. Initial attempts at amplifying the gene used primers recommended by Dunbar and Murphy (2009), which produce a DNA fragment of approximately 771 base pairs. These attempts were unsuccessful, generating DNA that was identified as coming from a *Salvia* species, a laboratory contaminant. We recognized that amplifying a large sequence would select against ancient DNA templates, which tend to be fragmented. We then tested other combinations of primers giving smaller fragments (Fig. 4 below) and adopted two primers that generated a 184 bp DNA fragment: *rbcl*F2, TGTTACTCCATTGTGGGTAATG, and *rbcl*R3a, TTCGGTTTAATAGTACAGCCCAAT. The DNA segment defined by these primers included an *Eco*R1 restriction site in the *Salvia* gene, but not the *Linum* or *Cannabis* genes. Treating the templates with *Eco*R1 blocked amplification of the contaminant. A separate set of samples extracted under conditions that avoided the contaminant confirmed the results obtained with the first set.

Two other sets of primers, representing two other sections of the chloroplast genome, were tested. One was specific for *trnL*, the leucine transfer RNA gene (GenBank references: *L. usitatissimum*, FJ160887; *C. sativa*, AY958396). Primers (*trnL*F1: AGCTGTCTAA-CAAATGGAGTTG; *trnL*R1: GGACTCTATCTTTGTTCTCGTCC) were chosen to give amplified fragments of 311 base pairs from *Linum*, 180 base pairs from *Cannabis*, and 283 base pairs from any

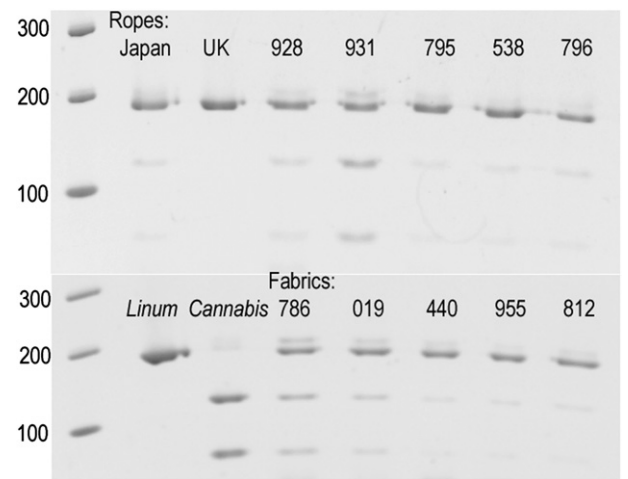


Fig. 5. DNA analysis of rope and fabric samples. Following PCR amplification using *rbcl* primers, DNA was subjected to cleavage by *Bam*H1 restriction enzyme. As shown in the lower frame, control DNA from *C. sativa* was completely cleaved, whereas DNA from *L. usitatissimum* was not. DNAs from modern linen (Japan) and flax (UK) ropes were not cleaved. Rope 931 and fabrics 786 and 019 showed significant cleavage. Numbers at the left show the size of DNA fragments in base pairs.

contaminating *Salvia*. A second set of primers was specific for *matK*, which codes for the maturase enzyme for lysine tRNA (GenBank references: *L. usitatissimum*, FJ160842; *C. sativa*, AF345317). Primers (*matK*F1, AAAAGGTTGGGGTCGGAATT; *matK*R1, CCTGCCCTTTA-TATAATCTTTG; *matK*R2, TCATTATAATTGACCAGATCG) were chosen to give amplified fragments of 109 base pairs from *Linum* and 162 base pair from *Cannabis*. No product was expected from *Salvia*, since *Salvia* lacked a complementary base sequence, at least in that region of the genome.

PCRs were conducted in 20 μ L of solution containing 12.1 μ L of water, 4 μ L of 5 \times Green Go Taq Buffer (Promega Corporation,

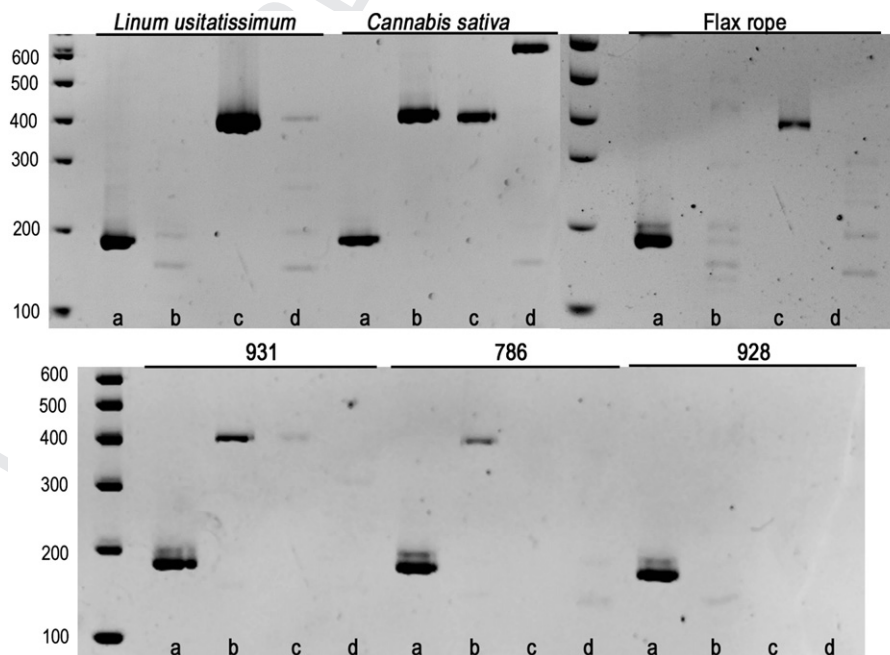


Fig. 4. Selection of *rbcl* primers for the analysis of ancient samples. Extracts of modern flax (*L. usitatissimum*) and hemp (*C. sativa*) plants were compared to those of flax rope and three ancient samples, using primers designed to give amplified DNA of 184 (a), 390 (b), 378 (c), or 584 (d) base pairs. Under the PCR conditions used, modern flax gave products only with primers a and c; modern hemp gave products with all four primer pairs. Archeological samples gave products with primers a and b. Numbers at the left show the size of DNA fragments in base pairs.

Hemp CGCTCTACGTCTGGAGAGATTGGAATCCCTACTTCTTATACT
Flax AGCCCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >955+F2 AGCCCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >955+R3a AGCCCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >786+F2 AG CCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >786+R3a AGCCCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >795+F2 AG CCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >795+R3a AGCCCTACGTCTGGAGGATTGCGAATCCCTCCTGCTTATACT
 >931+F2 MGCYCTACGTCTGGAGGATTGMGAATCCCTMCTGCTTATACT
 >931+R3a MGCYCTACGTCTGGARGATTGMGAATCCCTCCTKCT ATACT
 M=A/C Y=C/T R=A/G M=A/C M=A/C K=G/T

Hemp AAAACTTTCCAAGGACCGCCTCATGGGATCCAAGTTGAGAGAG
Flax AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGTTGAGAGAG
 >955+F2 AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGTTGAGAGAG
 >955+R3a AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGT GAGAGAG
 >786+F2 AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGTTGAGAGAG
 >786+R3a AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGT GAGAGAG
 >795+F2 AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGTTGAGAGAG
 >795+R3a AAAACTTTCCAAGGCCCGCCTCACGGGATTCAAGT GAGAGAG
 >931+F2 AAAACTTTCCAAGGYCCGCTCAYGGGATYCAAGTTGAGAGAG
 >931+R3a AAAACTTTCCAAGGYCCGCTCAYGGGATYCAAGT GAGAGAG
 Y=C/T Y=C/T Y=C/T

Fig. 6. Base sequences of PCR-amplified *rbcl* DNA from representative samples of rope and fabric, with corresponding flax and hemp sequences for comparison. Sequences 795 and 931 are from rope samples; sequences 786 and 955 are from fabric samples. All samples were amplified using forward (*rbclF2*) and reverse (*rbclR3a*) primers. The sequencing procedure was performed with each primer; the results from both primers are shown. Base differences in the hemp and flax DNA are shown in boldface black and the corresponding superpositions in sample 931 are indicated by code letters (M,Y,R,K).

Madison, WI, USA), 1.6 μ L dNTPs (2.5 mM of each dNTP), 0.125 μ L *Taq* DNA Polymerase (Go *Taq*, 5 μ / μ L, Promega), 0.6 μ L of each primer (forward and reverse, 20 μ M), and 1 μ L of template DNA. Normal PCR conditions were 96 °C for 1 min; 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min; 72 °C for 5 min; 4 °C hold. For study of amplification kinetics (Fig. 9), samples were removed after the 72 °C step of the 24th, 28th, 32nd, 36th, 40th, and 44th cycles.

3.4. Analysis of PCR products

The products of PCR were identified in two ways. Restriction analysis and gel electrophoresis were used to distinguish *Linum* from *Cannabis* products. The sequence of the *Cannabis* DNA fragment contained a BamH1 restriction site, which was missing in the *Linum* fragment. PCR solutions, 8 μ L, were treated with 1 μ L of

BamH1 (Promega) and 1 μ L of appropriate buffer at 37 °C for at least 1 h. The mixtures were then subjected to acrylamide gel electrophoresis for 2 h at 110 V on 10-cm long gels. The separating gel contained 10% acrylamide (37.5:1 acrylamide:bis-acrylamide, Bio-rad) and Tris-HCl, pH 8.8; the stacking gel contained 4% acrylamide and Tris-HCl, pH 6.8. Electrode buffer contained 0.004 M Tris and 0.192 M glycine. The gels were stained with ethidium bromide and images of their fluorescence recorded. Quantification of fluorescence (Fig. 9) was performed using an Alpha Innotech FluorChem 8900 imager (Cell Biosciences Corp., Santa Clara, CA, USA).

Base sequence analysis was used to confirm the identity of the reaction products. Products were purified by separation on 1.5% agarose gels; the bands were excised, and the DNA isolated using glass filters (Promega). The concentrations of nucleic acid in the samples were measured by their fluorescence when combined with

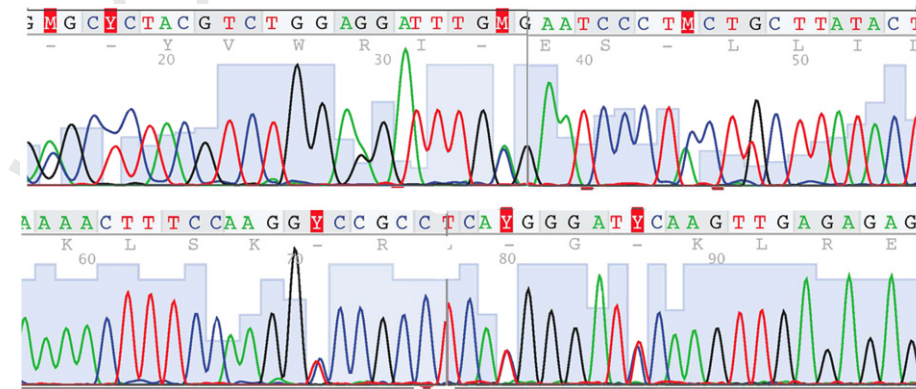


Fig. 7. Chromatograms showing superpositions in the DNA base sequence of samples 931 (top) and 786 (bottom). Bases marked M indicate superpositions of A (green) and C (blue); bases marked Y indicate superpositions of T (red) and C (blue). These chromatograms show partial sequences using primer *rbclF2*; the chromatograms produced by primer *rbclR3a* showed additional superpositions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

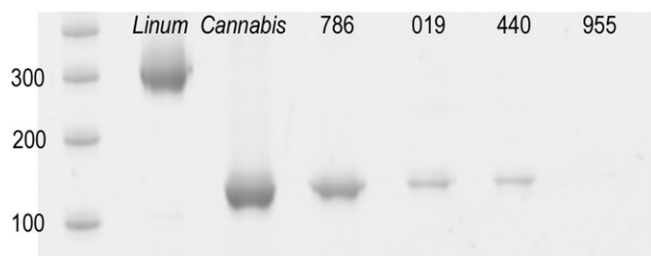


Fig. 8. DNA analysis of fabric samples using *trnL* primers. Numbers at the left show the size of DNA fragments in base pairs. "Linum" and "Cannabis" were modern control samples. Under the conditions of this experiment, only hemp and no flax DNA was amplified from archeological samples.

ethidium bromide, and the base sequences of samples were determined by the UC Davis Gene Sequencing Facility, using both forward and reverse primers.

3.5. AMS-based ^{14}C analyses

Radiocarbon measurements were performed at the Keck Carbon Cycle Accelerator Mass Spectrometry Laboratory, University of California, Irvine (Southon et al., 2004). Samples were chemically pretreated by successive heating in 1 N HCl (to remove carbonates and acid-soluble humates), 1 N NaOH (to remove base-soluble humates), 1 N HCl (to neutralize NaOH), and ddH₂O (to remove HCl) before combustion.

4. Results

The study focused on five samples of cordage and five of textile, examples of which are shown in Fig. 3.

4.1. PCR

As noted in the Methods section, the choice of primers was a critical part of the study. Following the discovery that no *rbcl* DNA product 771 base pairs long could be amplified from the archeological DNA templates, we tested various *rbcl* primer combinations to find a successful set (Fig. 4). Using template DNA from modern hemp, amplified DNA was obtained with all four primer pairs tested. However, templates from flax plants and from modern flax rope only gave product using primer pairs *rbclF2/rbclR3a* and *rbclF2/rbclR4a*. Three template DNAs extracted from archeological samples gave product corresponding to the smallest (184 bp) band obtained with flax (and hemp). Two archeological DNAs gave a product with primer pairs *rbclF1/rbclR3a* that likely represented hemp. For subsequent tests, we concentrated on *rbclF2/rbclR3a*, which gave the smallest product and thus was least likely to discriminate against the gene from a minor species.

The *rbcl* PCR products obtained using the template DNA extracted from all the archeological cordage and textile samples contained strong bands of approximately 184 base pairs (Fig. 5). To distinguish between flax and hemp templates, we noted that the band produced using authentic *C. sativa* DNA template was cut over 90% by *BamH1*, yielding fragments of 115 and 69 base pairs. The PCR product of the modern UK rope did not show a detectable amount of cutting and thus was entirely flax. Interestingly, the PCR product of the modern Japanese rope showed a small amount of cutting, indicating the presence of some *C. sativa* DNA. Most of the archeological samples showed only faint bands at 115 and 69 base pairs after *BamH1* treatment, indicating that, like the Japanese rope, they contained little *C. sativa* DNA. Rope sample 931 and textile sample 786 were the most notable exceptions, with sample 931

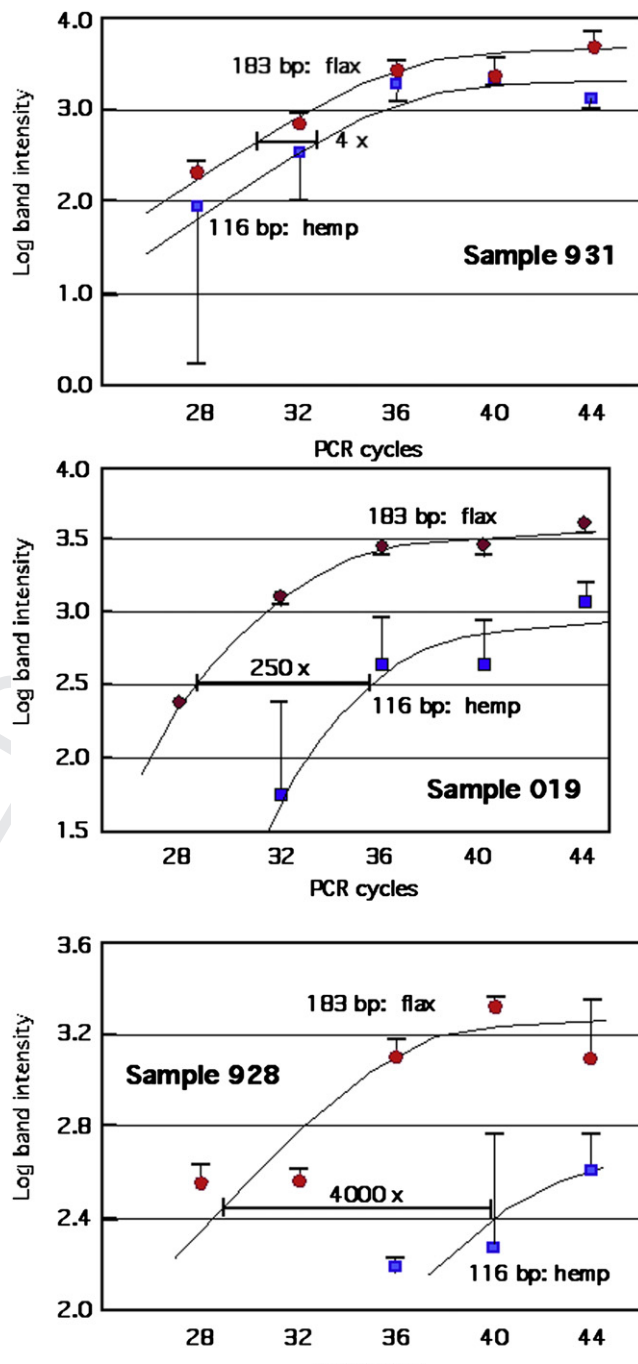


Fig. 9. Analysis of the relative amounts of flax and hemp DNA templates in three archaeological samples: 931 (rope), 019 (fabric), 928 (rope). See Table 1 for sample ages. Estimates shown were calculated by the amplification factor (2^n , where n = number of cycles) needed to give equal intensity of flax and hemp bands.

showing (in the experiment presented) 44% cutting and sample 786 showing 39% cutting. However, repetitions of the PCR reaction and restriction digestion, particularly of sample 786, did not consistently show the smaller bands produced by *BamH1*.

The base sequences of DNA from the archeological samples confirmed their identity as primarily *L. usitatissimum* Fig. 6. Within the 184-base pair amplified DNA, there was a stretch of 86 base pairs in which accurate sequence determinations could be obtained from both primers. Within that region were nine sites at which the sequences of the *Linum* and *Cannabis* genes differed. The

Table 1
Radiocarbon ages of representative samples of rope and fabric.

Laboratory number	Accession number	Material	$\delta^{13}\text{C}$ (‰)	^{14}C Age (yrs BP)	Calibrated age (cal BCE/CE) ^a
UCIAMS-79816	585440	Fabric (flax)	-24.3	1870 ± 15	CE 80–215 ^b
UCIAMS-79814	582928	Rope (flax)	-	1950 ± 15	CE 5–85
UCIAMS-79813	582931	Rope (flax, hemp)	-25.3	1900 ± 15	CE 65–130
UCIAMS-79817	583019	Fabric (flax, hemp)	-24.3	4760 ± 15	BCE 3635–3520 ^b
UCIAMS-79815	585786	Fabric (flax, hemp)	-	4770 ± 15	BCE 3635–3625 (0.12) BCE 3605–3525 (0.88)

^a Calibration of the ^{14}C age for each measurement utilized CALIB 6.0 program protocols employing the Intcal09 data set (Reimer et al., 2009). Single interval 2σ range calibration values are expressed for intercepts representing ≥ 0.95 of the relative area under the probability distribution. In cases of multiple intercepts, the 2σ ranges with relative areas under probability distribution of ≥ 0.05 are noted in parenthesis for intercept separations of >20 yrs. Age ranges are rounded to nearest 5 yr increments.

^b Multiple intercepts with probability distribution separations of <15 yrs.

sequence of rope sample 931 showed superpositions of two bases at all nine sites (from at least one reading direction)(Figs. 6 and 7), confirming that this sample contained a significant amount of *Cannabis* DNA. Chromatograms of the other samples, including textile sample 786, were not interpreted by the computer as having a significant amount of *Cannabis* DNA, but small peaks corresponding to *Cannabis* bases could be seen in the chromatogram for sample 786 (Fig. 7).

A few other base-sequence superpositions, e.g. K (=G/T) or a present/deleted base (=T/-), occurred near the ends of the 86-base pair stretch, but probably represented sequencing errors rather than the inclusion of a variant *Linum* or another species, since in each case the superpositions were found with only one primer. However, one of the superpositions in sample 931 indicated C/T (confirmed in three independent determinations), whereas the sequences of *Linum* and *Cannabis* at that position were C and A,

Flax	TGTTTACTTCCATTGTGGGTAATGTGTTTGGGTTTAAAGCCC
Hemp	TGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAGGCC
Date palm	TGTTTACTTCCATTGTGGGTAATGTATTTGGTTCAAAGCCC
Cotton	TGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAGGCC
Abaca	TGTTTACTTCCATTGTGGGTAATGTATTTGGTTCAAAGCCT
Ramie	TGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAGGCC
Flax	TGCGAGCCCTACGTCTGGAGGATTTGCGAATCCCTCCTGCTT
Hemp	TGCGCGCTCTACGTCTGGAAGATTTGAGAATCCCTACTTCTT
Date palm	TACGAGCTCTACGTCTGGAGGATCTGCGAATCCCACTTCTT
Cotton	TGCGCGCTCTACGTCTAGAGGATCTGCGAGTCCCTACTGCTT
Abaca	TACGAGCTCTACGTCTGGAGGATCTGCGAATCCCACTTCTT
Ramie	TGCGCGCTCTACGTCTGGAGGATTTGCGAATCCCTCCTGCTT
Flax	ATACTAAAACCTTTCCAAGGCCCGCCTCACGGGATTCAAGTTG
Hemp	ATACTAAAACCTTTCCAAGGACCGCCTCATGGGATCCAAGTTG
Date palm	ATTCCAAACCTTTCCAAGGCCCGCCTCATGGGATCCAAGTTG
Cotton	ATATTAACCTTTCCAAGGCCCGCCTCATGGGATCCAAGTTG
Abaca	ATTCCAAACCTTTCCAAGGCCCGCCTCACGGCATTCAAGTTG
Ramie	ACACTAAAACCTTTCCAAGGCCCGCCTCATGGGATCCAAGTTG
Flax	AGAGAGATAAATTGAACAAGTACGGTCGCCCCCTATTGGGCT
Hemp	AGAGAGATAAATTGAACAAGTATGGTCGCCCACTATTGGGAT
Date palm	AAAGAGATAAAGTTGAACAAGTATGGTCGGCCTCTATTGGGAT
Cotton	AAAGAGATAAATTGAACAAGTATGGTCGCCCCCTATTAGGAT
Abaca	AAAGAGATAAAGTTGAACAAGTATGGTCGTCGCCCTATTGGGAT
Ramie	AGAGAGATAAATTGAACAAGTATGGCCGGCCCTATTGGGAT
Flax	GTACTATTAAACCGAA
Hemp	GTACTATTAAACCTAA
Date palm	GTACTATTAAACCAA
Cotton	GTACTATTAAACCTAA
Abaca	GCACTATTAAACCAA
Ramie	GTACTATTAAACCTAA

Fig. 10. Comparison of base sequences of PCR-amplified *rbcl* DNA from six old-world fiber plants: flax (*L. usitatissimum* L.), hemp (*C. sativa* L.), date palm (*Phoenix dactylifera* L.), cotton (*Gossypium hirsutum* L.), abaca (*Musa textilis* Nêe), and ramie (*Boehmeria nivea* (L.) Gaud). Aqua- and yellow-colored segments represent the sequences binding to the forward and reverse primers, respectively. The unshaded central area represents the region in which our sequences of the DNA from archaeological samples were confirmed (see Fig. 6). Bold-face letters within the central area highlight differences from the flax sequence. The *Bam*H1 site in the hemp sequence is shown in blue and underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. In this position, the codons containing C, T, and A (GGC, GGT, GGA) all code for glycine, so this is a “silent” substitution. It is possible that the *rbcl* gene of the archeological *Cannabis* differed from the modern species used for identification.

In an attempt to confirm the data obtained from the *rbcl* analysis, we used the extracts of DNA as templates to amplify fragments of two other chloroplast genes, *trnL* and *matK*. Using the *trnL* primers, we obtained *Cannabis* fragments from rope sample 931 and most of the textile samples, especially 786 (Fig. 8). There was no indication of *Linum* template DNA in the ancient samples, although the modern control gave a good band. Using the *matK* primers, we could not obtain *Linum* or *Cannabis* fragments from any archeological DNA templates, although again the modern control templates worked well (data not shown). Assuming that the primer-complementary sequences of the *trnL* and *matK* target genes have not changed drastically over the last 2000 years, these results suggest that different regions of chloroplast DNA fragment at different rates.

Finally, we estimated the relative amounts of flax and hemp DNA using a semi-quantitative “kinetics” technique in which we compared the amount of *rbcl* PCR product as a function of the number of replication cycles. Although under appropriate conditions, the amplification of DNAs can give amounts of products that represent the relative amounts of different templates, many problems with the technique can confound the analysis. It is more accurate to compare the number of cycles that give equal band intensities upon staining, even given the uncertainty inherent in the relationship between staining intensity and DNA size. We applied this technique to three samples that appeared to show three different levels of hemp DNA, 931, 019, and 928. In fact, as shown in Fig. 9, the levels of hemp varied from ca one-fourth that of flax (sample 931) to 1/4000 (sample 928).

4.2. AMS-based ^{14}C dating

The ages of five samples, including two cordage and three textile samples, were determined by AMS-based ^{14}C measurements. Radiocarbon ages are expressed as conventional ^{14}C dates normalized to -25‰ $\delta^{13}\text{C}$ (Stuiver and Polach, 1977). The calibration of the ^{14}C age for each measurement utilized CALIB 6.0 program protocols employing the Intcal09 data set (Reimer et al., 2009). Calibrated values are expressed as a 2 sigma range. The data (Table 1) revealed that the samples represented two distinct periods. The two rope samples, 928 and 931, and textile sample 440 were produced in the first two centuries CE; two textile samples, 019 and 786, were much older, from the fourth millennium BCE.

5. Discussion

The PCR data confirmed the identities of the contents of all the cordage and textile samples as primarily flax-linen. The sequence data revealed the presence of hemp DNA, and by inference hemp fibers, but only in one rope sample, 931, and one textile sample, 786. The gel electrophoresis patterns, more sensitive to minor components, indicated the presence of hemp DNA in those samples and all the other samples, with the exception of the control *Linum* DNA and the DNA from the contemporary flax rope.

The lack of linearity inherent in PCR made it impossible to estimate the relative amounts of flax and hemp accurately from the initial analyses, which were performed with a fixed number of amplification cycles, but a modification of the PCR protocol (Fig. 9) revealed that the fraction of DNA from hemp varied widely, from 25% to 0.025% that of the amount of flax DNA. The ubiquity of the hemp DNA, particularly in the small amounts found in most of the archeological samples, forces us to consider the possibilities for its

origin. These include deliberate incorporation of hemp into flax rope and linen textiles *in situ* and the importation of hemp-containing rope and textiles from other places. Samples 931 and 786 most likely acquired their substantial amounts of hemp DNA in their fabrication. Samples that had much smaller amounts of hemp DNA might have acquired it as dust, through their storage over several centuries in contact with some (perhaps a small number) of hemp products. In the same way, samples might have acquired small amounts of hemp dust during their excavation and transport to museums, particularly if their bundles were bound with hemp rope or twine. Finally, we cannot ignore the possibility of contamination in the analytical laboratory, although controls did not indicate this. The lack of contemporary hemp bands in the archeological samples in Fig. 4 strongly suggests that the hemp DNA, whether incorporated deliberately or through contamination, was ancient.

What is the possibility that the results we interpret as representing hemp actually reflect another fiber? A comparison of the amplified segments of the *rbcl* genes from six old-world fiber plants, flax, hemp, date palm, cotton, banana palm, and ramie, shows that the base sequences of the central regions (Figs. 6 and 10, unshaded) differ among all six. The *BamHI* site in the hemp sequence occurs only in that species, so that the minor bands seen in Fig. 5 must represent hemp. The specific base superpositions shown in Fig. 7 occur only in the flax-hemp pairing. The lack of other minor bases indicates that, if present, the amounts of other fibers (or strictly speaking, their DNA) must be very small.

The ^{14}C dating of the samples confirms a suggestion by Dr. Orit Shamir (personal communication) that they represent two distinct periods of use, one prehistoric and one from the time of the Roman conquest. Previous ^{14}C dating of two wood samples from CC found even earlier dates, 3670 and 4830 BCE (Rasmussen et al., 2005), but the authors pointed out that the dates might have represented “cultural activity 6000 years ago or the use of old wood.” Samples from the “Cave of the Warrior,” dated to approximately the same times, ca. 3800 BCE and ca. 4400 BCE (Jull et al., 1998), included textiles and baskets, a clear indication of prehistoric culture. Our three dated samples from the Early Bronze period, though somewhat younger, confirm the suggestion of very old cultural activity and add the information that this activity included the use of hemp.

In conclusion, this work points out the value of PCR in determining the plant-fiber composition of textile and cordage, particularly when there are mixtures of fibers. Our multiple-disciplinary approach, combining the PCR data with literary-historical analysis and AMS-based ^{14}C dating of the textile samples, has enabled us to unearth this little known aspect of textile history – i.e. ancient hemp in the Land of Israel.

Uncited references

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