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1 **Genomic analysis of 6,000-year-old cultivated grain illuminates the**  
2 **domestication history of barley**

3

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43

44

45

46 **The cereal grass barley was domesticated about 10,000 years ago in the Fertile**  
47 **Crescent and became a founder crop of Neolithic agriculture<sup>1</sup>. Here, we report**  
48 **genome sequences of five 6,000-year-old barley grains excavated at a cave in the**  
49 **Judean Desert close to the Dead Sea. Comparison to whole exome sequence data**  
50 **from a diversity panel of present-day barley accessions revealed the close affinity**  
51 **of ancient samples to extant landraces from the Southern Levant and Egypt,**  
52 **consistent with a proposed origin of domesticated barley in the Upper Jordan**  
53 **Valley. Our findings suggest that barley landraces grown in present-day Israel in**  
54 **the past six millennia have not experienced a major lineage turnover although**  
55 **there is evidence for gene flow between cultivated and sympatric wild**  
56 **populations. We show the utility of ancient genomes from desiccated**  
57 **archaeobotanical remains in informing research into the origin, early**  
58 **domestication and subsequent migration of crop species.**

59

60 Genetic analyses of ancient DNA can greatly inform research into the origin, initial  
61 domestication and subsequent dispersal of crops and livestock as evidenced by studies  
62 involving ancient DNA samples and genomic datasets of present-day populations of  
63 cattle<sup>2</sup>, swine<sup>3</sup>, dogs<sup>4</sup> and maize<sup>5</sup>. Wheat and barley, founder crops of agriculture in  
64 the ancient Near East and Europe, were domesticated in the Fertile Crescent, where  
65 their wild relatives still thrive today<sup>1,6</sup>. Our current knowledge of their domestication  
66 is largely derived from morphological analysis of archaeobotanical remains<sup>1</sup> and the  
67 population genetic analysis of present-day samples<sup>7,8</sup>. Although domesticated wheat  
68 and barley appear in the archaeological record by 10,000 calendar years before  
69 present (cal BP)<sup>1</sup>, the oldest verified DNA sequences to date were retrieved from  
70 archaeobotanical specimens originating from Bronze Age China<sup>9</sup> and Ancient  
71 Egypt<sup>10</sup>. Claims about a small number of prehistoric wheat DNA molecules retrieved  
72 from Mesolithic paleosol<sup>11</sup> have remained contentious<sup>12,13</sup>. There have been no studies  
73 where large quantities of ancient DNA sequences have been retrieved that could  
74 underpin the comparison of modern and ancient samples of Old World cereals at a  
75 genome-wide scale.

76

77 Here, we report genome sequences of five 6000-year old barley grains excavated at  
78 Yoram Cave in the Judean Desert, Israel. Yoram Cave is part of a complex of three  
79 difficult-to-access caves, located in the south-eastern cliff of the Masada Horst facing

80 the Dead Sea. High-resolution excavation (Online Methods, Fig. 1a, Supplementary  
81 Figures 1, 2) revealed a single undisturbed anthropogenic layer of Chalcolithic origin  
82 (ca. 6,200-5,800 cal BP). The rich plant assemblage of more than 100 taxa was well  
83 preserved (Figure 1b), prompting us to attempt the retrieval of DNA sequences. We  
84 selected ancient barley grains (Figure 1c, Supplementary Figure 3) for DNA  
85 extraction because of barley's central role in ancient and modern agriculture and its  
86 remarkable adaptive features that make it a model plant in domestication genomics.

87

88 DNA extractions were performed from ten bisected grains and spikelet remains,  
89 whose other halves were subjected to direct radiocarbon dating, confirming the  
90 Chalcolithic origin of the specimens (Table 1). Illumina sequencing of libraries  
91 yielded between 7.3 and 21.5 million paired-end reads (Supplementary Table 1).  
92 Based on the fraction of reads that could be aligned to the barley reference genome,  
93 we estimated the content of endogenous DNA to range from 0.4 to 96.4 %. Sequence  
94 reads of eight samples showed fragment sizes and damage patterns characteristic of  
95 ancient DNA (Supplementary Table 1, Supplementary Figures 4, 5, 6), which  
96 demonstrates the authenticity of the samples<sup>14,15</sup>. Deamination-derived mismatches (C  
97 -> T, G -> A) occurred towards the ends of reads with frequencies between 1.9 and  
98 21.8 %. We only used five samples with on average more than 12 %  
99 misincorporations<sup>15</sup> at the first base of sequence fragments for further experiments.  
100 Once the authenticity of these samples had been established, we treated five DNA  
101 extracts containing a large fraction of endogenous DNA with uracil-DNA-glycosylase  
102 (UDG) to reduce nucleotide misincorporations caused by ancient DNA damage by  
103 removing deaminated cytosines<sup>16</sup>. Deep Illumina sequencing of the UDG-treated  
104 libraries yielded between 82.5 million and 5.1 billion reads (Table 1).

105

106 We compared the ancient barley genome sequences to present-day accessions on the  
107 basis of whole-exome capture<sup>17</sup> sequence data from 267 entries from *ex situ*  
108 collections representing extant populations of wild (*Hordeum vulgare* ssp.  
109 *spontaneum*) and domesticated (*H. vulgare* ssp. *vulgare*) barley from across the range  
110 of the species<sup>18</sup> (Figure 2a). This dataset<sup>18</sup> comprised 1,688,807 single nucleotide  
111 polymorphisms (SNPs) (Table 1).

112

113 Principal component analysis<sup>19</sup> has revealed fundamental patterns of population  
114 structure across the present-day accessions<sup>18</sup>. The first principal component (PC1)  
115 clearly differentiated wild and domesticated barleys, while PC2 represented the  
116 variation in the wild barleys (Figure 2b). Least-square projection<sup>20</sup> of the ancient  
117 samples onto the PCA axes defined by the extant samples revealed the close affinity  
118 of ancient barley with present-day domesticated barley. The deep coverage of sample  
119 JK3014 allowed us to ascertain the allelic status of the domestication genes *Non-*  
120 *brittle rachis 1 (Btr1)* and *Non-brittle rachis 2 (Btr2)*. In domesticated barley, one of  
121 *Btr1* and *Btr2* carry mutations that abolish the disarticulation of the spike at  
122 maturity<sup>21</sup>. JK3014 had a wild-type *Btr2* haplotype, but carried the previously  
123 described 1-bp deletion in the coding sequence of *Btr1* (Supplementary Figure 7),  
124 consistent with a high frequency of this mutation in barleys of the Southern Levant<sup>21</sup>.  
125 In agreement with the archaeobotanical classification of the ancient barley spike  
126 remains as being of the two-rowed type (Figure 2c), the extant accessions closest to  
127 the ancient samples were two-rowed domesticated barleys from the Southern Levant  
128 and Egypt (Figure 2b, d). A putative two-rowed phenotype of sample JK3014 can also  
129 be inferred from the allelic status of the *Six-rowed spike 1* gene<sup>22</sup> (Supplementary  
130 Figure 7).

131

132 Rare genetic variants can provide insights into the spatial structure of populations<sup>23,24</sup>.  
133 In inbreeding plants such as barley, isolation by distance<sup>25</sup> is common since gene flow  
134 across larger geographic distances is limited. We identified rare variants with minor  
135 allele counts of up to five across the ancient and extant barleys and determined the  
136 number of rare alleles shared between pairs of sequenced samples. Transitions were  
137 excluded from the analysis because deaminated cytosines cannot be repaired by UDG  
138 treatment if they are methylated<sup>16</sup> and thus can give rise to genotyping errors. The  
139 extant landraces that shared at least 30 rare alleles with the ancient samples were two-  
140 rowed accessions from Syria, Jordan, Egypt and Israel as well as six-rowed  
141 accessions from North Africa (Supplementary Table 2). Eight wild barleys from Israel  
142 also shared  $\geq 30$  rare alleles with the ancient samples (Supplementary Table 2).

143

144 We measured relatedness of ancient samples to each of the wild barley accessions  
145 based on the level of identity by state (IBS) calculated across all SNPs. The  
146 genetically closest wild accessions originated from a sampling site located in the

147 Upper Jordan Valley. We then calculated the geographic distance between Yoram  
148 Cave and each wild accession, but no significant correlation between IBS and  
149 geographic distance was found ( $R = -0.17$ ,  $P = 0.108$ ). However, when splitting the  
150 data into geographically proximal ( $< 250$  km) and distant ( $> 250$  km) samples,  
151 significant correlations between geographic distance and the relatedness score were  
152 detected for both subsets of the tested wild barley samples (proximal:  $R = 0.74$ ,  $P = <$   
153  $0.001$ ; distant:  $R = -0.34$ ,  $P = 0.006$ ) (Figure 3a). The same analysis was conducted  
154 between wild barleys and extant landraces from the Fertile Crescent (Figure 3b, c)  
155 pinpointing the Upper Jordan Valley as a peak for genetic similarity with  
156 domesticated barley. Conducting this analysis using only data from the deeply  
157 sequenced JK3014 yielded similar results (Supplementary Figure 8). The comparison  
158 of modern cultivars and landraces from outside of the Fertile Crescent (Europe, North  
159 Asia) to our wild barley panel accessions pinpointed accessions from the Upper  
160 Jordan Valley as the most closely related (Supplementary Table 3). The Israel-Jordan  
161 area was proposed earlier as one (though not the only) center of origin of  
162 domesticated barley<sup>8,26</sup>. This hypothesis is supported by two archeological sites, Tel  
163 Aswad and Ohalo II, with the earliest traces of barley cultivation<sup>27,28</sup>, which are  
164 within 80 km of the extant wild barley accessions in our panel that are genetically  
165 closest to the ancient samples.

166

167 Although self-fertilization is predominant in barley<sup>29</sup>, wild barley is fully interfertile  
168 with the domesticated crop and evidence for hybridization between the two has been  
169 reported<sup>7,30</sup>. To ascertain whether the genetic similarity between ancient and extant  
170 landraces is the outcome of shared ancestry or the result of later hybridization  
171 between local wild barley and domesticated forms, we performed a model-based  
172 assignment of present-day and ancient samples to two ancestral groups corresponding  
173 to wild and domesticated barleys using ADMIXTURE<sup>31</sup> considering only transversion  
174 variants. The analysis with two ancestral populations confirmed the strong  
175 differentiation between wild and domesticated accessions observed in PCA, with a  
176 perfect correlation between the domestication status and the assignment to ancestral  
177 populations. Thus, all ancient samples were assigned to the domesticated group  
178 (Figure 4a). However, wild ancestry coefficients of two ancient samples were 4.2 and  
179 8.7 %. Present-day landraces from the Levant (Israel, Jordan, Lebanon and Syria) also  
180 showed an elevated fraction of wild ancestry (6.8 %). By contrast, the average wild



181 ancestry for European landraces was only 0.14 %. We also performed ADMIXTURE  
182 runs with the number of ancestral population (K) set to five because this K value had  
183 the lowest cross validation error (Supplementary Figure 9). In this analysis,  
184 domesticated barley was separated into three and wild barley into two clusters  
185 (Supplementary Figure 10). The deeply sequenced ancient sample JK3014 had 7.6 %  
186 ancestry from a cluster predominantly composed of wild accessions, while the  
187 average ancestry fraction in this cluster was only 1.7 % for domesticated barley.  
188 These observations suggest gene flow between wild and cultivated barley in regions  
189 of sympatry.

190

191 We used D-statistics<sup>32</sup> to corroborate the hypothesis of archaic admixture between  
192 wild and domesticated barley populations in the Levant. We considered five  
193 categories: ancient barley, extant wild barley from the Levant (Israel, Syria, Jordan,  
194 Lebanon), extant landraces from the Levant, extant European landraces and outgroup  
195 *Hordeum pubiflorum*<sup>33</sup>. We calculated D for each ancient sample separately  
196 (Supplementary Table 4) and focus here on the results for the single deeply sequenced  
197 sample JK3014 (Figure 4b). D(extant Levantine landraces, extant European landraces,  
198 JK3014, outgroup) was significantly positive, confirming the close affinity of the  
199 ancient sample with the present-day Levantine landraces. The comparisons D(extant  
200 Levantine landraces, extant European landraces, Levantine wild barley, outgroup) and  
201 D(JK3014, extant European landraces, Levantine wild barley, outgroup) were also  
202 significantly positive, indicating admixture between wild and domesticated barleys  
203 from Israel after the lineage leading to Levantine landraces split from the progenitors  
204 of European landraces. The ancient sample did not show a closer affinity to extant  
205 Levantine wild barley than present-day landraces from this region, as D(JK3014,  
206 extant Levantine landraces, Levantine wild barley, outgroup) was not significantly  
207 different from zero. These findings indicate that the genomes of both ancient and  
208 present-day cultivated barley from the Levant show traces of archaic gene flow from  
209 sympatric wild accessions after the split between Levantine and European landraces,  
210 supporting the notion of hypothetical hybridization events between domesticated  
211 barley and sympatric wild stands<sup>7,34</sup>. As a consequence of this demographic scenario,  
212 the homogenization of the allele frequencies in sympatric wild and domesticated  
213 barley through bi-directional gene flow may complicate inferences about the origin(s)  
214 and domestication history<sup>34</sup> at the fine genomic scale, while key domestication genes

215 (such as the *btr* genes) are resistant to wild introgression due to a strong selection  
216 against shattering spikes. Despite hybridization events between wild and domesticated  
217 barleys in the last six millennia, the overall picture is that the genomes of extant  
218 Levantine landraces have remained remarkably similar to how they were 6000 years  
219 ago. This is despite climate change<sup>35</sup> and anthropogenic transformations of local flora  
220 and fauna, including changes in agricultural practices<sup>36</sup>, which might have favored the  
221 introduction of landraces from other regions that were better adapted to the changing  
222 agricultural environment. Whereas we found no indications of major lineage  
223 turnovers in the barley crop in the Southern Levant (as has, for example, been  
224 observed in Near Eastern pig populations<sup>37,38</sup>), the eventful history of this region  
225 makes it likely that the farmers that grew cereals there several millennia ago were not  
226 the ancestors of those that tended the present-day landraces<sup>39</sup>. One can speculate that  
227 conquerors and immigrants did not bring their crop seeds from their old homelands,  
228 but favored locally adapted landraces.

229

230 Expanding on previous studies that reported the PCR amplification and sequencing of  
231 single genes from ancient wheat and barley samples<sup>10,40</sup>, our results show that very  
232 ancient desiccated plant remains preserved under hot and arid conditions contain  
233 sufficient amounts of endogenous DNA to underpin genome-wide population genetic  
234 analyses in the context of diversity panels of extant individuals. Our analysis  
235 demonstrates the value of archaeogenomics in supporting contemporary genetic-based  
236 phylogeographic studies in exploring crop origins, and shows that 6,000 years BP  
237 domesticated barley appeared remarkably similar to proximate extant landraces,  
238 indicating that the major domestication events had occurred by that time.

239

240 **URLs**

241 Novosort, <http://www.novocraft.com>

242 R package mapdata, <https://cran.r-project.org/web/packages/mapdata/index.html>

243

244 **Accession Codes**

245 Raw read files of the ancient samples can be retrieved from the European Nucleotide  
246 Archive (ENA) under project ID PRJEB12197. The SNP genotype matrix is available  
247 under DOI 10.5447/IPK/2016/6. Passport information for the extant barley panel can  
248 be retrieved from DOI 10.5447/IPK/2016/3. DOIs were registered with e!DAL<sup>41</sup>.

249

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260

261 **Authors' contributions**

262 EW, TF, NS, and JK conceived the study. EW, TF, NS, JK, VJS and MM designed  
263 experiments. NM, UD, MD, SR and EW performed excavations and archaeobotanical  
264 analyses. VJS, AH and ER performed the ancient DNA experiments. MM, SH, AK,  
265 MS, SHV and REG analyzed data. JR, MM, IKD, BK, GJM, NS and RW provided  
266 exome capture data. MM, VJS, AH, SR, TF, JK, EW and NS wrote the manuscript  
267 with input from all co-authors. All authors read and approved the manuscript.

268

269 **Competing interests**

270 The authors declare no competing financial interests.

271

272 **References for the main text**

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378 **Figure legends for the main text**

379

380 **Figure 1: Ancient plant remains excavated at Yoram Cave.** Plan of the Southern  
381 Chamber of Yoram Cave showing the excavation grid and sub-units **(a)**. Photograph  
382 of Locus 3 in Square A2 during excavation – note the excellent dry preservation of  
383 rope, reeds, seeds and pellets **(b)**. Photograph of a well-preserved, desiccated barley  
384 grain found at Yoram Cave **(c)**.

385

386 **Figure 2: Ancient barley samples are closely related to present-day landraces**  
387 **from the Levant.** Ancient barley sequences were compared to exome sequence data  
388 of a present-day diversity panel. **(a)** The collection sites of landraces (black circles)  
389 and wild barleys (blue circles) are shown. Masada is marked with a red circle. **(b)**  
390 Principal component analysis showing ancient samples projected on the present-day  
391 diversity panel. The inset magnifies the PCA space around the ancient samples. ISR,  
392 SYR, LBN, EGY represent closely related landraces from Israel, Syria, Lebanon and  
393 Egypt, respectively. The proportion of variance explained by each principal  
394 component is indicated in parentheses. **(c)** Well-preserved rachis of two-rowed  
395 domesticated barley from Yoram Cave **(d)**. Spike of an individual of a present day  
396 two-rowed landrace barley (accession HOR8658) that is among the barleys most  
397 closely related to the ancient DNA sample from Yoram Cave.

398

399 **Figure 3: Relationship between genetic similarity and geographic distance.**  
400 Scatter plot of genetic similarity and geographic distance between 91 extant wild  
401 barley accessions sampled range-wide including the Fertile Crescent and **(a)**  
402 archaeological samples found at Yoram Cave, **(b)** two-row cultivated landrace from  
403 Israel, and **(c)** two-row cultivated landrace from Egypt. The geographic position  
404 attributed to each sample is: A (31.3141 N, 35.353 E), B (31.7156 N, 35.1871 E), C  
405 (31.193 N 29.904 E). Correlation coefficients and P-values for the geographically  
406 proximate and distant subsets are indicated in blue and red, respectively.

407

408 **Figure 4: Gene flow between wild and domesticated barleys in the Levant.**

409 **(a)** Wild ancestry coefficients of landraces from different geographic regions and  
410 ancient barleys as determined by ADMIXTURE. The wild ancestry proportion is  
411 shown in black for extant samples and in red for ancient samples. **(b)** D statistics

412 for different quadruples of barley populations ( $P_1$ ,  $P_2$ ,  $P_3$ ,  $O$ ). Positive D values  
413 indicate that  $P_1$  shares more derived allele with  $P_3$  than  $P_2$  does. Black bars  
414 indicate  $\pm 3$  standard errors (SE), gray bars  $\pm 1$  SE. Population names are  
415 abbreviated as follows: LevDom (Levantine landraces), LevWild (Levantine wild  
416 barley), Euro (European landraces),  $O$  (*H. pubiflorum*, outgroup). JK3014 is a  
417 deeply sequenced ancient sample.



418 **Table 1: Summary of ancient barley samples used for genetic analyses.**

419

Sample name	Radiocarbon age <sup>1</sup>	Number of raw reads	Percentage of mapped reads	Percentage of unique reads	Average read depth <sup>2</sup>	Number of called SNPs
<b>JK2281</b>	5290 ± 27	256.1 M	31.4%	26.0%	0.54	162,110
<b>JK3009</b>	5034 ± 36	89.2 M	61.7%	67.3%	0.46	133,365
<b>JK3010</b>	5032 ± 37	94.7 M	62.3%	57.9%	0.96	278,505
<b>JK3013</b>	5227 ± 37	82.5 M	49.4%	39.8%	0.19	18,949
<b>JK3014</b>	4988 ± 36	5,131.2 M	86.4%	28.3%	20	1,283,396

420

421 <sup>1</sup>Uncalibrated radiocarbon years before the present, see for Supplementary Table 1 for calibrated dates.

422 <sup>2</sup>Average read depth in target regions of the exome capture assay for JK2281-JK3013. The mode of the coverage distribution is given for

423 JK3014.

424 **Online Methods**

425

426 **Archaeology**

427

428 Yoram Cave

429 Yoram Cave is archaeologically significant as one of the rare cave sites with a  
430 single layer of human occupation according to current radiocarbon dating  
431 findings. Unlike most other Judean Desert caves, there are no findings from the  
432 later Roman and Byzantine Periods. In addition, it is one of the rare cave sites  
433 that has not suffered from modern looting or hyena burrowing. It is the only  
434 Chalcolithic cave site in the Judean Desert that has been excavated by high-  
435 resolution sampling methods. The cave's plant assemblage has been preserved  
436 by drying, supporting use in possible DNA-based studies.

437 Location and description of the cave:

438 Yoram Cave is part of a cave-complex ("Masada caves – South"), with  
439 three caves located in the south-eastern cliff of Masada Horst (Supplementary  
440 Figure 2), facing the Dead Sea. Access to the cave complex is relatively difficult as  
441 it requires walking along narrow goat paths on a sharp incline rock-fall. The  
442 Yoram Cave entrance is on an almost vertical cliff, some 4m above a goat trail at  
443 its base (Fig. 1B).

444 Water sources are scarce. Some small rock depressions, holding flood  
445 waters for a few months are found about 150 meters southwest of the  
446 cave complex. The nearest permanent springs are in Tze'elim canyon, some 5 km  
447 walking distance northward.

448 The cave entrance is 2.9 m wide with a fieldstone wall stretching along the  
449 entrance. The interior has two rooms (Figure 1a and Supplementary Figure 1).  
450 The northern room is approximately 7 m long and between 3.5 and 5 m wide,  
451 and contains large boulders. The southern room consists of three areas: an  
452 entrance (A), a short corridor (B), and a small inner cubicle (C). The latter room's  
453 maximum length is 6.5 m, and its width is between 2 and 2.5 m. (Figure 1a,  
454 Supplementary Figures 1, 2). The heights of these 3 areas range between 0.2 and  
455 1.8 m. Most human activities and related plant remains were found in the  
456 southern room.

457 Stratigraphy

458 Excavations in the southern room revealed three phases (from top to bottom):

459 1. A biogenic layer, mostly the result of nesting activities of large birds of prey  
460 (possibly Bearded Vulture, *Gypaetus barbatus*, or members of the eagle family),  
461 including large and small twigs, bones and droppings;

462 2. An anthropogenic layer, representing the Chalcolithic period of human  
463 activity (Fig. S2); and

464 3. A pre-floor Chalcolithic layer.

465 Scant evidence of modern human activity was discovered on top of layer 1. An  
466 initial round of radiocarbon dating validated the excavators' field observation of  
467 a Chalcolithic origin of the anthropogenic layer (ca. 6,200-5,800 cal. BP). The  
468 biogenic layer was dated to the LB/IAI period.

469 Reeds (possibly *Typha/Phragmites* spp. see Schick et al.<sup>42</sup>) were found  
470 abundantly among the anthropogenic layer plant remains (Figure 1b). The  
471 appearance of reeds alongside various rope segments (plants still to be  
472 identified) and a small mat section in one of the excavated samples hint that  
473 simple mats probably covered the cave floor. Such mats indicate preparations for  
474 prolonged stay in the cave, rather than a chance occupation. The human-built  
475 wall in the entrance is further evidence for the prudent use of the cave.

476

477 Excavation and sampling

478 Excavation was conducted by a high-resolution excavation method, with the  
479 excavated space being divided into sub-units (Figure 1a). These were  
480 meticulously sampled, with each sample going through a sorting procedure,  
481 using 1 mm and 100 µm mesh sieves. During sifting, various categories of finds  
482 were separated (e.g. archaeological artifacts, macro- and microecofacts –  
483 archaeozoological and archaeobotanical remains), which were packed  
484 separately. Additional separation was undertaken on plant material, with 1 liter  
485 of sediment out of each excavated bucket from the "anthropogenic" loci (#1, 3, 4,  
486 5, 7, 9) was kept for archaeobotanical analysis in the laboratory. Larger samples  
487 were also retained when plant remains were visible to the naked eye during  
488 excavation.

489 Mapping of Yoram Cave (Fig 1a, Supplementary Fig. 1) was performed by the  
490 excavation team headed by Uri Davidovich and Nimrod Marom in 2007 using  
491 standard cave mapping equipment, including a Leica Disto D3 laser inclinometer  
492 and a Silva Ranger 3 prismatic compass; the grade of mapping was 5C. Field  
493 maps were later graphically edited using Limelight software.

494

#### 495 **DNA extraction and library preparation**

496

497 A panel of 13 samples was initially selected for this study consisting of eight  
498 barley grains, two barley ear fragments, two wheat emmer grains and one  
499 emmer ear fragment. All subsequent sampling procedures, DNA extractions and  
500 library preparations were carried out in clean room facilities dedicated to  
501 ancient DNA research at Tuebingen University. During the sampling process, all  
502 samples weighing more than 15 mg were divided into two parts, of which one  
503 part was used for subsequent DNA extraction and the other one sent for  
504 radiocarbon dating at Curt-Engelhorn-Zentrum Archaeometrie gGmbH  
505 Mannheim (Germany). The DNA extractions were conducted on 5 to 30 mg of  
506 plant material using the PTB extraction protocol detailed by Kistler<sup>43</sup> with the  
507 following modifications: all samples were extracted twice (E1 and E2). After a  
508 first incubation for two hours at 37°C the plant remains were pelleted, the  
509 supernatants were taken off and stored at 4°C over night. Plant pellets were  
510 resuspended in extraction buffer a second time and incubated over night at 37°C.  
511 All extracts were purified simultaneously on the next day.

512 For library preparation, a well established protocol by Meyer and Kircher<sup>44</sup> was  
513 used to convert a 20 µl aliquot of each DNA extract into double-stranded Illumina  
514 libraries. Adaptor ligation to the fragments was quantified using a quantitative  
515 PCR with the primers IS7 and IS8<sup>44</sup>, the reagents of the DyNAmo Flash SYBR  
516 Green qPCR Kit (Biozym) and the Lightcycler 96 (Roche). Then, double indexed  
517 libraries were created by adding sample specific barcodes to both library  
518 adapters via amplification<sup>45</sup> followed by another quantification assay using the  
519 primers IS5 and IS6<sup>44</sup> to estimate the efficiency of the indexing PCR. All  
520 extraction and library blanks were treated accordingly. These libraries were  
521 used subsequently for initial shotgun sequencing.

522 For genome-wide shot-gun sequencing and enrichment additional libraries for  
523 the extracts JK2281E1, JK2281E2, JK3009E1, JK3010E1, JK3013E1 and  
524 JK3014E1 (Table 1, Supplementary Table 1) were prepared from 50 µl aliquots  
525 of all DNA extracts following the methods described above<sup>44,45</sup> with one  
526 modification: all extracts and blanks were treated with uracil-DNA glycosylase  
527 (UDG) and endonuclease VIII during the library preparation to avoid potential  
528 sequencing artifacts that are caused by the characteristic ancient DNA damage  
529 pattern due to the deamination of cytosine to uracil over time<sup>16</sup>.

530 For all indexed libraries, a second amplification was carried out in 100 µl  
531 reactions using 5µl library template, 4 units AccuPrime Taq DNA Polymerase  
532 High Fidelity (Invitrogen), 1 unit 10X AccuPrime buffer (containing dNTPs) and  
533 0.3 µM IS5 and IS6 primers<sup>44</sup>. The following thermal profile was performed: 2-  
534 min initial denaturation at 94°C, followed by 4 to 17 cycles consisting of 30-sec  
535 denaturation at 94°C, a 30-sec annealing at 60°C and a 2-min elongation at 68°C  
536 with a final 5-min elongation at 68°C. After amplification the products were  
537 purified using MinElute spin columns (Qiagen) according to the manufacturer's  
538 protocol and quantified using an Agilent Bioanalyzer DNA 1000 Chip.

539 All libraries for initial and genome-wide shotgun sequencing were then diluted  
540 to 10nM and pooled in equimolar amounts. Initial shotgun sequencing of  
541 libraries was undertaken with an Illumina HiSeq 2500 platform, using a paired-  
542 end dual index run with 2\*100+7+7 cycles and the manufacturer's protocols for  
543 multiplex sequencing (TruSeq PE Cluster Kit v3-cBot-HS). Genome-wide shotgun  
544 sequencing of the UDG treated libraries was performed on an Illumina NextSeq  
545 500 platform with 2 x 150+8+8 cycles using the NextSeq High Output reagent kit  
546 v1 and the manufacturer's protocol for multiplex sequencing.

547 The UDG-treated libraries from the extracts JK2281E1, JK2281E2 (Table 1,  
548 Supplementary Table 1) were treated separately: after the second amplification  
549 the libraries were enriched using a sequence capture assay for the barley  
550 exome<sup>17</sup> as described by Himmelbach *et al.*<sup>46</sup> with one modification: the  
551 concentration of the DNA fragments recovered from the capture was determined  
552 by quantitative PCR using the primers IS5 and IS6<sup>44</sup>, the SYBR Green PCR Master  
553 Mix (Qiagen, Hilden) and the 7900 HT Fast Real-Time PCR system (Applied  
554 Biosystems).

555 After a dilution to 10 nM the sequencing was carried out on the Illumina HiSeq  
556 2500 platform as described by Mascher *et al.*<sup>17</sup> with a paired-end single index  
557 run using 101+6+100 cycles and the manufacturer's protocols for multiplex  
558 sequencing (TruSeq PE Cluster Kit v3-cBot-HS).

559 Four additional UDG-treated libraries of the JK3014E1 were produced for deeper  
560 sequencing as detailed previously, diluted to 10 nM and pooled in equimolar  
561 amounts together with the already sequenced JK3014E1 UDG-library. The  
562 sequencing of the pools was conducted on a HiSeq4000 platform with 2 x  
563 75+8+8 cycles using the HiSeq 3000/4000 PE Cluster Kit, HiSeq 3000/4000 SBS  
564 Kit and the manufacturer's protocol for multiplex sequencing.

565 Raw sequence reads have been uploaded to EMBL ENA short read archive  
566 (accession: PRJEB12197).

567

#### 568 **Processing and mapping of sequence reads**

569

570 Overlapping paired-end reads were merged using scripts provided by Kircher<sup>47</sup>  
571 for samples JK2279 – JK2284 or with leeHom<sup>48</sup> (using the parameter “—  
572 ancientdna”) for the other samples. Length distribution of the merged reads was  
573 calculated using AWK and the Unix tools “sort” and “uniq” as described in  
574 Supplement S4 of Gallego Llorente *et al.*<sup>49</sup>. Merged reads were aligned to (i) the  
575 whole-genome shotgun assembly of barley cv. Morex<sup>50</sup> and (ii) the chloroplast  
576 genome assembly of cv. Morex<sup>51</sup> with BWA-MEM version 0.7.12<sup>52</sup> using default  
577 parameters. Conversion to BAM format and calculation of mapping statistics  
578 were performed with SAMtools<sup>53</sup>. Sorting of BAM files and duplicate removal  
579 was done with novosort (Novocraft Technologies Sdn Bhd, Malaysia). Nucleotide  
580 misincorporation profiles were generated with mapDamage version 2.0<sup>54</sup> for the  
581 nuclear and the chloroplast genome. Genotypes of five UDG-treated ancient  
582 samples at 1,688,807 known SNP positions<sup>18</sup> were called using single-sample  
583 variant calling with SAMtools (version 0.1.19, commands samtools mpileup and  
584 bcftools view)<sup>55</sup> using only reads with mapping quality above Q30 and  
585 considering bases with quality above Q20. SNP positions were retained if their  
586 quality score was at least 30 and they were covered by at least two reads. In the  
587 case of the deeply sequenced sample JK3014, we also required the read depth to

588 be not larger than 30 (= x 3 the mode of the coverage distribution in exome  
589 capture target regions). Heterozygous calls were set to missing. In the deeply  
590 sequenced ancient DNA sample JK3014, 0.7 % of variants were called  
591 heterozygous (compared to 1.7 % in the extant samples). In an inbreeding crop,  
592 the divergence between parental haplotypes of an individual is very low.  
593 Contamination with DNA of extant barley would thus become evident in an  
594 elevated fraction of heterozygous calls. The absence of such a pattern lends  
595 further support to the authenticity of the ancient samples. Coverage statistics  
596 were calculated with SAMtools<sup>53</sup> and BEDTools<sup>56</sup>.

597

### 598 **Population genetic analysis**

599

600 Principal component analysis (PCA) was performed with EIGENSOFT 6.0.1<sup>19</sup> for  
601 five ancient barley samples and 228 extant barley *ex situ* accessions with clear  
602 domestication status and well-described geographic origins<sup>18</sup>. Least-square  
603 projection as implemented in the smartPCA program of EIGENSOFT was used to  
604 project the ancient samples onto the PCA axes defined by the extant samples.

605 To investigate the relatedness between the Yoram Cave samples (and extant  
606 landraces) and wild barley accessions representing the entire natural  
607 distribution range, the corresponding data was extracted from the filtered SNP  
608 table. Relatedness between the archeological samples and each wild accession  
609 was measured by the level of IBS calculated across all SNPs using the SNPRelate  
610 package<sup>57</sup> in R. For each wild accession, relatedness to the five archeological  
611 samples was averaged using the geometrical mean to obtain one relatedness  
612 score (RS). We then calculated the geographic distance (GD) between the Yoram  
613 Cave location (latitude: 31.314 N, longitude: 35.353 E) and the sampling position  
614 of each wild accession based on its coordinates, converting the distances  
615 between coordinate positions to kilometres using the rough conversion metrics  
616 of 1 degree = 111 km. To capture the change in the correlation coefficient sign  
617 observed between geographic distance and genetic relatedness, the data were  
618 split into geographically proximate and distant categories using the most related  
619 wild accession coordinates rounded up to the nearest 50 km as a break point.

620 Model-based ancestry estimation was performed with ADMIXTURE (ref. 31). For  
621 each K from 1 to 10, twenty replicate ADMIXTURE runs were performed on the  
622 genotype matrix of 233 samples (228 geo-referenced extant accessions of known  
623 domestication status<sup>18</sup> plus five ancient samples) using only transversion  
624 variants with a present genotype call for at least one ancient samples. Before  
625 running ADMIXTURE, linkage disequilibrium pruning was done with the R  
626 package SNPRelate<sup>57</sup> using the function snpgdsLDpruning() with the parameters  
627 “ld.threshold=0.4, slide.max.bp=100000, slide.max.n=50”. Replicate ADMIXTURE  
628 runs were combined with CLUMPP<sup>58</sup>.

629 D statistics were calculated using ADMIXtools<sup>59</sup> after the SNP matrix had been  
630 converted to the EIGENSOFT format with the SNPRelate<sup>57</sup> function  
631 snpgdsGDS2Eigen(). The barley relative *Hordeum pubiflorum* was used as an  
632 outgroup. We used exome sequencing reads of *H. pubiflorum* published by  
633 Mascher et al. (ref. 17) to call genotypes at variant positions with SAMtools.  
634 Read alignments at the *Btr1/2* and *Vrs1* loci were manually inspected with  
635 SAMtools “tview”.

636 The map in Figure 2a was generated with the R package ‘mapdata.’  
637

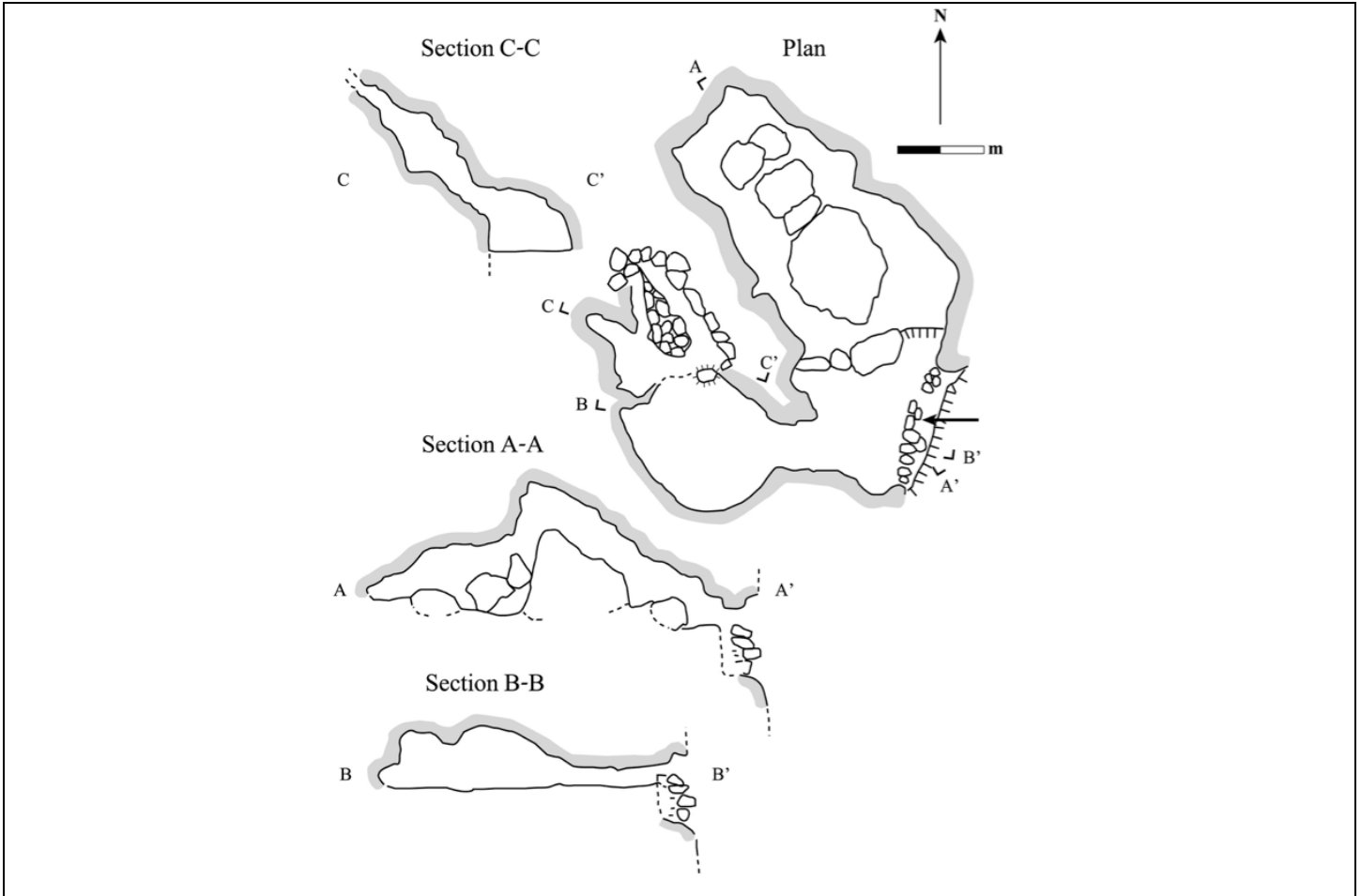


638 **Additional references**

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- 684
- 685



**Supplementary Figure 1**

Plan of Yoram Cave – top plan and sections.

Arrows indicates the entrance to the cave and the human-made wall across it. Note the boulders in the northern room and the relatively horizontal surface in the southern room. Gray shaded borders indicate that the edge of the cave is cut into rock.



**Supplementary Figure 2**

Photographs from the excavation.

Masada Southern Cave complex, with three caves located in the south-eastern cliff of the Masada Horst **(a)**. The dotted red line marks the easiest trail giving access to the caves. The entrance of Yoram Cave, facing south-east, in an almost vertical cliff, some 4 meters above the trail leading to the cave **(b)**. The south room during excavation of locus 3 **(c)**.

**JK3009**



**JK3010**



**JK3011**



**JK3012**



**JK3013**

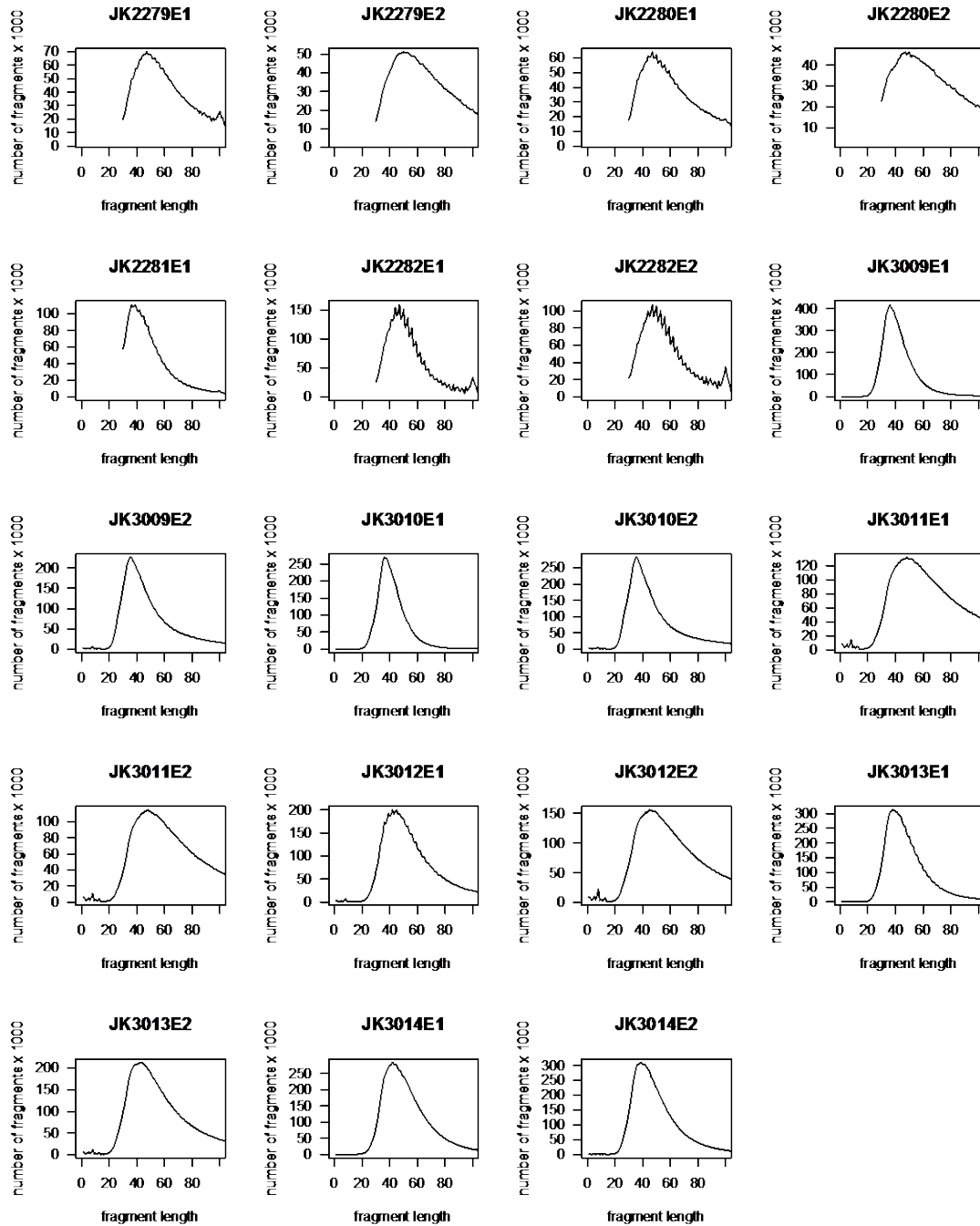


**JK3014**



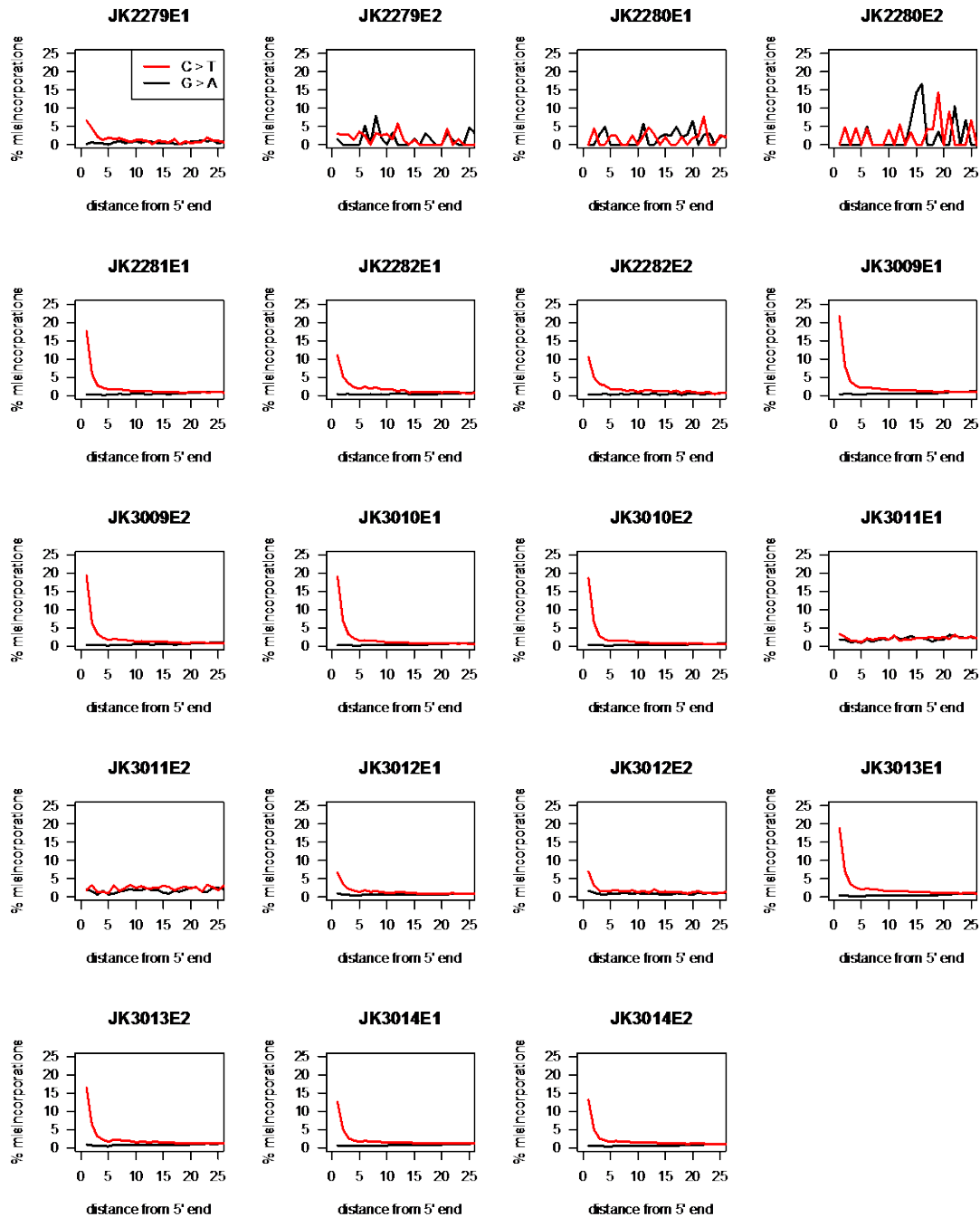
**Supplementary Figure 3**

Photographs of ancient barley grains used for DNA extraction.



**Supplementary Figure 4**

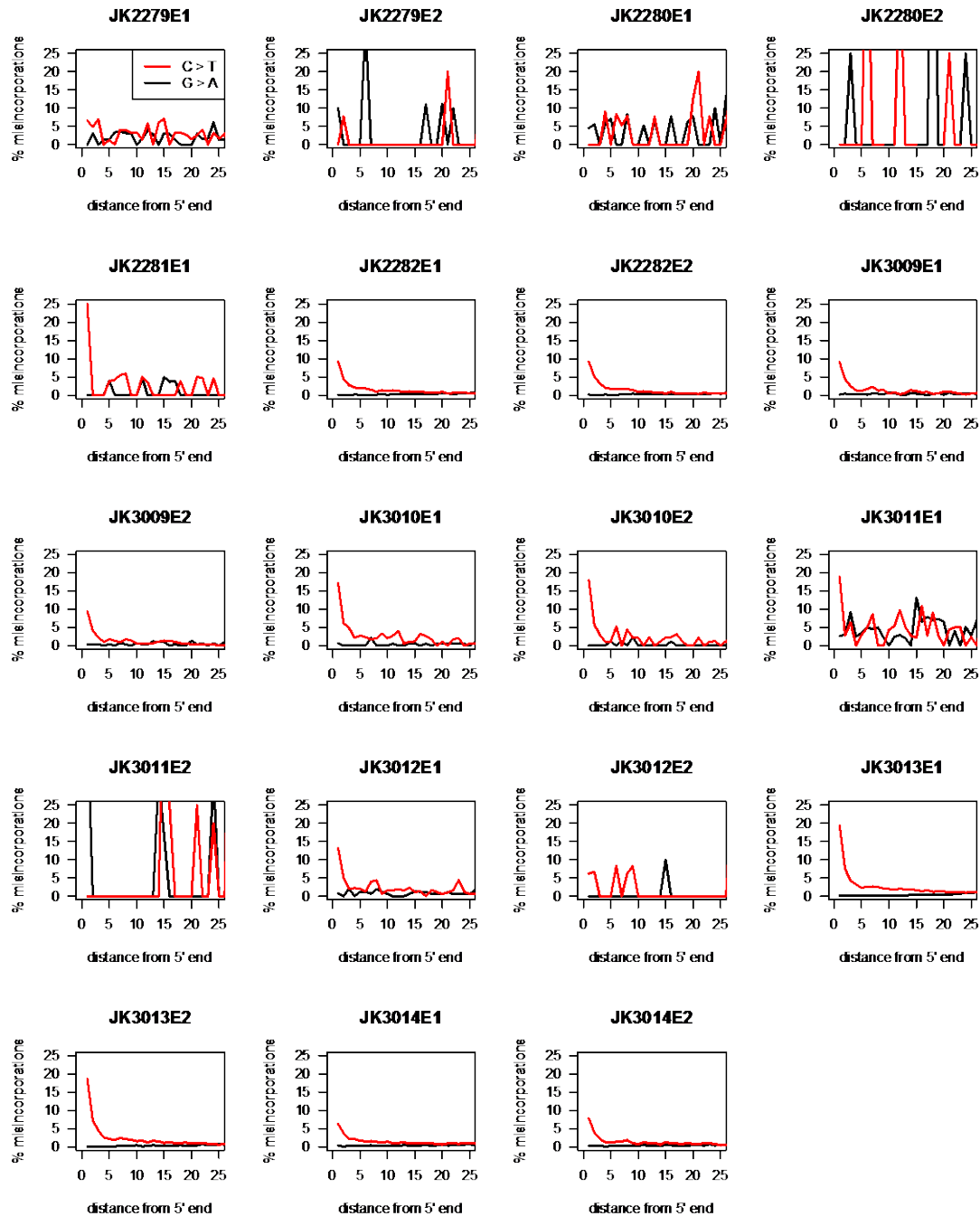
Length distribution of sequence fragments.



### Supplementary Figure 5

Nucleotide misincorporation profiles in reads mapped to the whole-genome shotgun assembly of barley cv. Morex.

The proportion of C > T misincorporations (red) is compared to the G > A baseline (black).

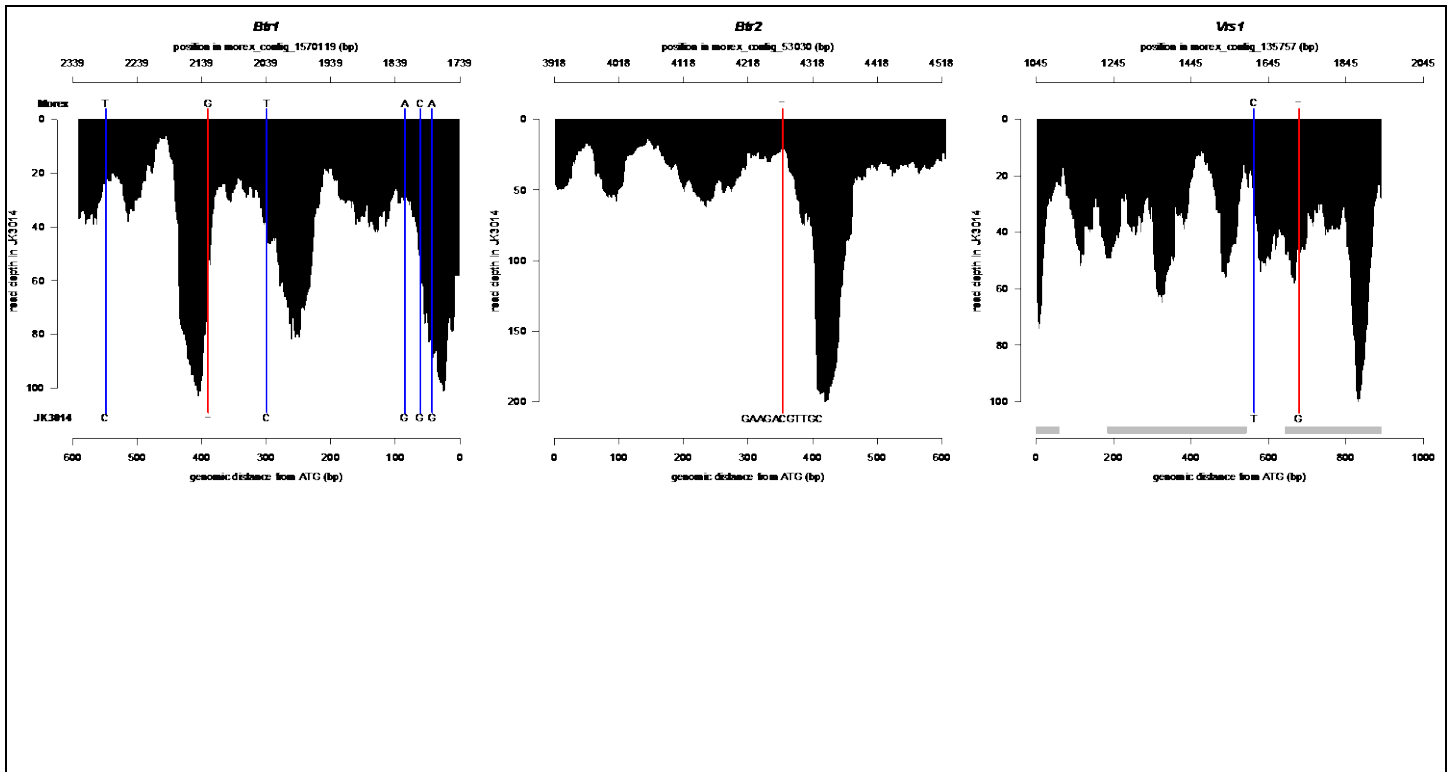


### Supplementary Figure 6

Nucleotide misincorporation profiles in reads mapped to the barley chloroplast genome assembly.

The proportion of C > T substitutions (red) is compared to the G > A baseline (black).

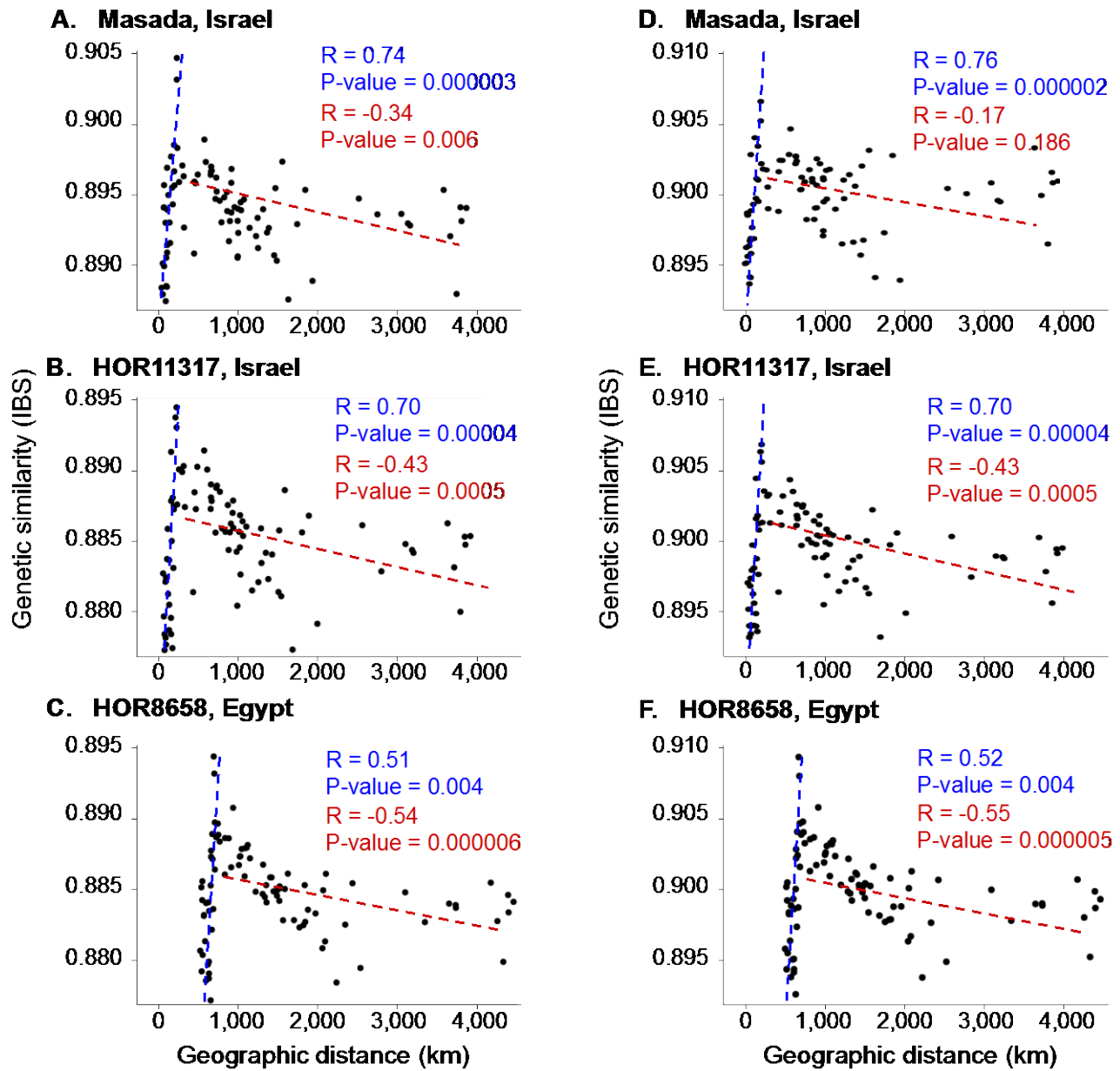




### Supplementary Figure 7

#### Haplotype of *Btr1*, *Btr2* and *Vrs1*

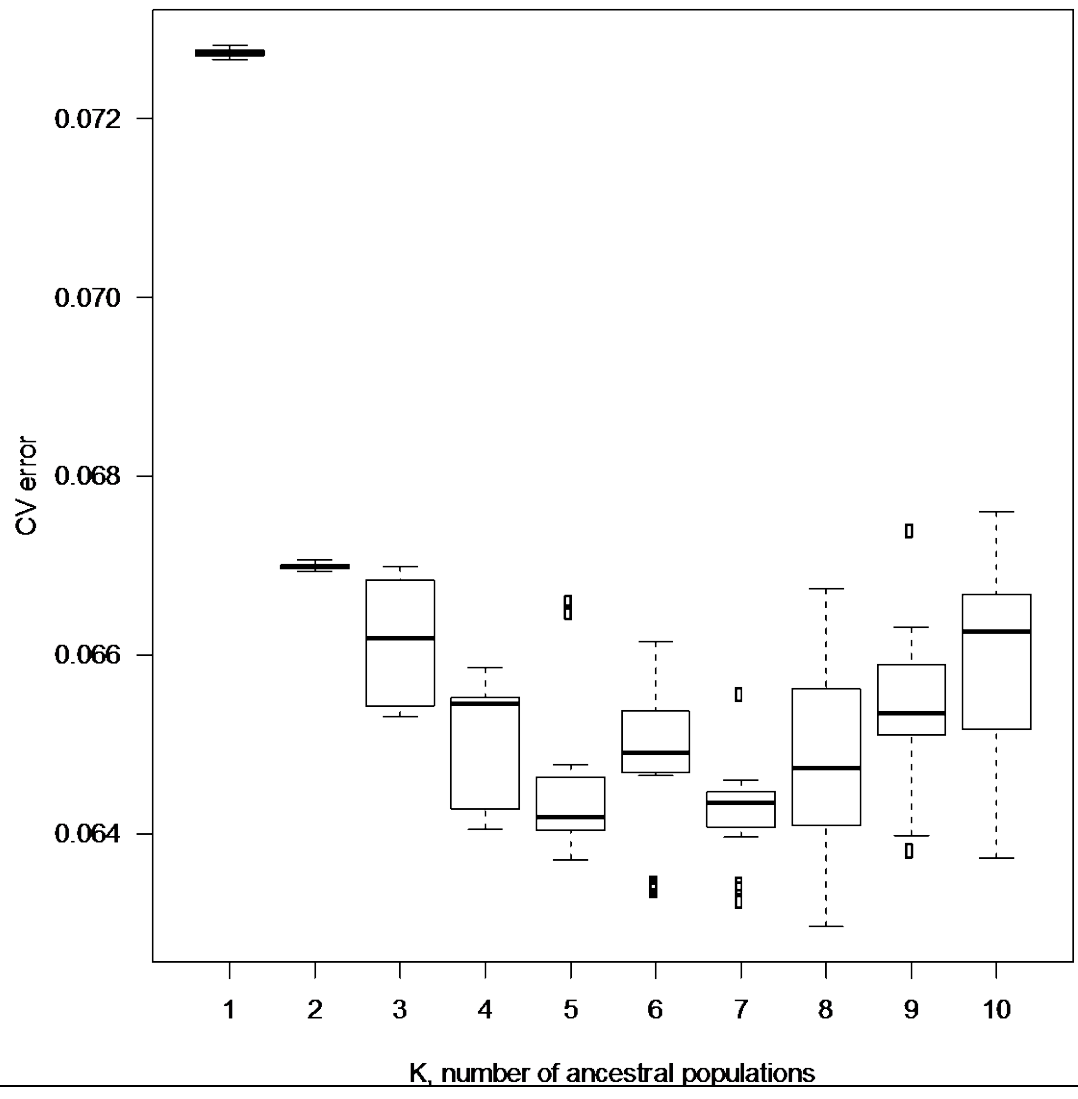
Contigs of the Morex WGS assembly harboring these genes were identified by BLAST searches. The read depth in the deeply sequenced sample JK3014 is shown in black. The position in the respective WGS contigs is indicated in the upper axis. The distance (in the genomic sequence) from the start codon of each gene is shown in the bottom axis. *Btr1* and *Btr2* are single-exon genes. Gray bars indicate the positions of the three exons of *Vrs1*. The positions of SNPs are highlighted by vertical lines. The Morex allele is shown above, and the JK3014 allele below the lines. SNPs with a previously reported functional effect are shown in red. JK3014 carries a loss-of-function allele of *Btr1*, while *Btr2* and *Vrs1* are wild-type alleles. The coding sequence of *Btr1* and *Btr2* is identical to the haplotype of cv. Haruna Nijo as reported by Komatsuda et al., 2015 (NCBI GenBank accession KR813337.1). The sequence of *Vrs1* matches the *Vrs1.b2* allele as designated by Komatsuda et al., 2007.



**Supplementary Figure 8**

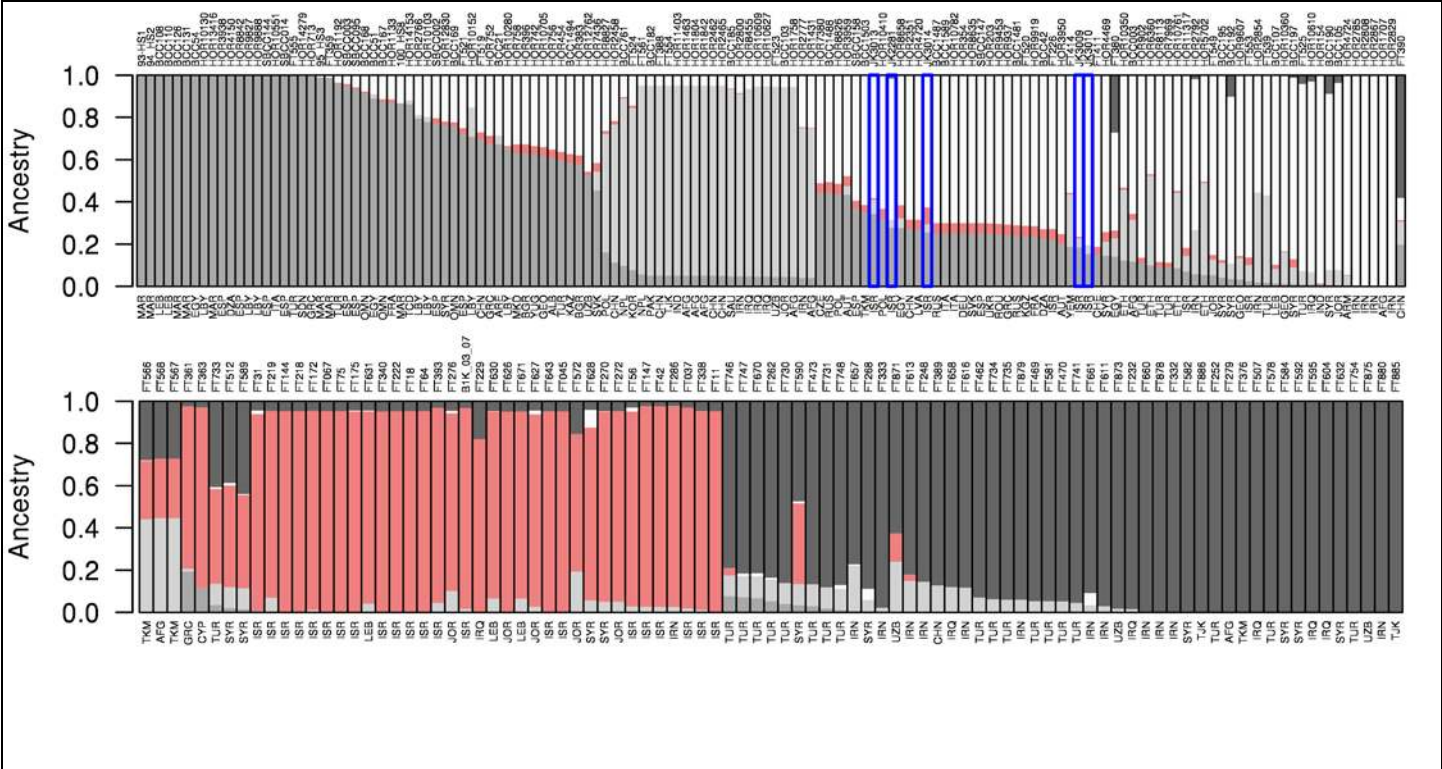
Relationship between genetic similarity and geographic distance

Scatter plot of genetic similarity and geographic distance between 91 extant wild barley accessions sampled across the Fertile Crescent and (A) archaeological sample JK3014 found at Yoram Cave and sequenced to higher depth using all SNPs, (B) two-row cultivated landrace from Israel, (C) a two-rowed cultivated landrace from Egypt, (D) the ancient sample JK3014 found at Yoram Cave and sequenced to higher depth excluding transition SNPs, (E) two-rowed cultivated landraces from Israel excluding transition SNPs, and (F) two-rowed cultivated landraces from Egypt excluding transition SNPs. The geographic position attributed to each sample is: A, D (31.3141 N, 35.353 E); B,E (31.7156 N, 35.1871 E); C,F (31.193 N 29.904 E). Correlation coefficients and P-values for the geographically proximate and distant subsets are indicated in blue and red, respectively.



**Supplementary Figure 9**

Cross-validation error of ADMIXTURE analysis. Box plots were calculated from 20 replicate runs for each K.



**Supplementary Figure 10**

ADMIXTURE analysis for K=5 for domesticated (top) and wild (bottom) samples.

Colors in both panels correspond to the same ancestral populations. Sample names and countries of origin are indicated above and below the plots, respectively. Ancient samples are highlighted by blue borders.

**Supplementary Table 1.** Sequencing statistics of ancient barley DNA without UDG treatment. Read statistics after UDG treatment are provided in Table 1.

Sample	C14 age <sup>1</sup>	Cal age <sup>2</sup>	Extraction	Raw reads	Merged reads	% mapped	% unique	% damaged
JK2279	5065 ± 27	3942-3802	1	8,208,520	3,548,936	31.5%	18.0%	6.6%
JK2279	5065 ± 27	3942-3802	2	7,809,034	3,240,281	2.8%	27.2%	3.0%
JK2280	NA	NA	1	7,410,426	3,192,474	18.6%	8.4%	4.4%
JK2280	NA	NA	2	8,537,472	3,150,021	4.7%	15.0%	4.8%
JK2281	5290 ± 27	4226-4047	1	7,257,940	3,210,243	56.9%	55.8%	17.7%
JK2282	5275 ± 27	4225-4006	1	9,601,504	4,513,912	95.9%	9.8%	11.1%
JK2282	5275 ± 27	4225-4006	2	8,401,768	3,772,270	75.7%	10.4%	10.5%
JK3009	5034 ± 36	3939-3775	1	18,769,156	8,976,322	67.5%	77.5%	21.8%
JK3009	5034 ± 36	3939-3775	2	15,308,126	7,037,725	33.2%	68.6%	19.5%
JK3010	5032 ± 37	3940-3773	1	11,237,658	5,427,102	71.6%	78.1%	19.2%
JK3010	5032 ± 37	3940-3773	2	17,609,772	8,071,918	37.8%	58.3%	18.8%
JK3011	5115 ± 37	3968-3812	1	19,100,026	8,175,478	0.7%	54.4%	3.4%
JK3011	5115 ± 37	3968-3812	2	15,648,476	6,838,637	0.4%	58.9%	1.9%
JK3012	4987 ± 37	3886-3707	1	17,736,280	7,985,422	42.6%	12.3%	6.6%
JK3012	4987 ± 37	3886-3707	2	20,000,020	8,788,418	2.8%	24.4%	7.0%
JK3013	5227 ± 37	4048-3976	1	19,482,948	9,219,005	60.7%	50.2%	18.9%
JK3013	5227 ± 37	4048-3976	2	21,479,860	9,742,769	11.7%	48.9%	16.5%
JK3014	4988 ± 36	3886-3708	1	20,069,866	9,681,200	87.8%	81.9%	12.5%
JK3014	4988 ± 36	3886-3708	2	20,901,314	9,939,663	73.3%	74.4%	13.1%

<sup>1</sup>Uncalibrated radiocarbon years before the present; <sup>2</sup>Calibrated age, calendar years before Christ

**Supplementary Table 2:** Rare allele sharing between ancient and modern samples. The table lists extant samples that share at least 30 rare alleles (minor allele count  $\leq 5$ ) with the ancient samples. Only transversion variants were considered for this analysis.

<b>sample</b>	<b>country of origin</b>	<b>domestication status</b>	<b>number of shared alleles</b>
HOR4469	Syria	landrace 2-row	89
HOR2766	Libya	landrace 6-row	63
FT549	Jordan	landrace 2-row	56
HOR8658	Egypt	landrace 2-row	56
FT75	Israel	spontaneum	45
HOR10280	Libya	landrace 2-row	42
BCC107	Lebanon	landrace 2-row	40
FT037	Israel	spontaneum	38
FT31	Israel	spontaneum	38
FT340	Israel	spontaneum	38
100_HS8	Morocco	landrace 6-row	37
HOR10103	Libya	landrace 6-row	36
FT218	Israel	spontaneum	35
HOR11317	Israel	landrace 2-row	35
HOR14153	Chad	landrace 6-row	35
FT276	Jordan	spontaneum	34
FT631	Lebanon	spontaneum	32
FT393	Israel	spontaneum	30

**Supplementary Table 4:** D statistics for different comparisons between wild and domesticated barleys from the Levant, European landraces and ancient samples. Standard errors and Z scores (D/SE) are given in columns SE and Z.

P1	P2	P3	Outgroup	D	all SNPs			transversions only		
					SE	Z	D	SE	Z	
Levantine landraces	European landraces	Levant wild	H. pubiflorum	0.037	0.004328	8.546	0.0342	0.005323	6.418	
Levantine landraces	European landraces	JK2281	H. pubiflorum	0.1076	0.019226	5.594	0.1007	0.021775	4.623	
Levantine landraces	European landraces	JK3009	H. pubiflorum	0.0898	0.015975	5.62	0.0499	0.01801	2.768	
Levantine landraces	European landraces	JK3010	H. pubiflorum	0.1671	0.016772	9.963	0.1534	0.018811	8.155	
Levantine landraces	European landraces	JK3013	H. pubiflorum	0.0955	0.027177	3.516	0.0986	0.037957	2.598	
Levantine landraces	European landraces	JK3014	H. pubiflorum	0.1864	0.015474	12.045	0.1854	0.016067	11.538	
JK2281	European landraces	Levant wild	H. pubiflorum	0.061	0.01632	3.737	0.0116	0.021853	0.531	
JK2281	Levantine landraces	Levant wild	H. pubiflorum	0.0265	0.01447	1.83	0.0018	0.020225	0.088	
JK3009	European landraces	Levant wild	H. pubiflorum	0.0562	0.0166	3.386	0.0662	0.023755	2.789	
JK3009	Levantine landraces	Levant wild	H. pubiflorum	0.019	0.014787	1.288	0.0617	0.022587	2.731	
JK3010	European landraces	Levant wild	H. pubiflorum	0.0366	0.010696	3.424	0.0227	0.015691	1.445	
JK3010	Levantine landraces	Levant wild	H. pubiflorum	0.0031	0.009611	0.325	-0.0068	0.014167	-0.48	
JK3013	European landraces	Levant wild	H. pubiflorum	0.0883	0.028889	3.058	0.1282	0.056598	2.266	
JK3013	Levantine landraces	Levant wild	H. pubiflorum	0.0809	0.029933	2.704	0.1068	0.055261	1.933	
JK3014	European landraces	Levant wild	H. pubiflorum	0.0413	0.007807	5.288	0.0385	0.010813	3.558	
JK3014	Levantine landraces	Levant wild	H. pubiflorum	0.0077	0.007189	1.066	0.0068	0.009839	0.696	