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Ancient DNA typing indicates that the "new" glume wheat of early Eurasian agriculture is a cultivated member of the *Triticum timopheevii group*

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Abstract

We used polymerase chain reactions specific for the wheat B and G genomes with nine accessions of the "new" glume wheat (NGW), a type of cultivated wheat that was present across western Asia and Europe during the Neolithic and Bronze Ages but which apparently died out before the end of the 1st millennium BC. DNA sequences from the G genome were detected in two NGW accessions, the first comprising grain from the mid 7th millennium BC at Çatalhöyük in Turkey, and the second made up of chaff from the later 5th millennium BC site of Miechowice 4 in Poland. The Miechowice chaff also yielded a B genome sequence, which we ascribe to an admixture of emmer wheat chaff recorded in the sample from which the NGW material was extracted. Our results therefore provide evidence that NGW is a member of the *Triticum timopheevii* group of wheats. *Triticum timopheevii subsp. timopheevii* can therefore no longer be looked upon as a minor crop, restricted to western Georgia, but instead must be viewed as a significant component of prehistoric Eurasian agriculture, with implications for our understanding of the origins of agriculture in southwest Asia.

Keywords: Ancient DNA, Archaeobotany, New Glume Wheat, Polymerase Chain Reaction, Prehistoric Agriculture, *Triticum timopheevii*

1. Introduction

The "new" glume wheat (NGW) is a type of cultivated wheat found on archaeological sites but, unlike other wheat types found archaeologically, it is no longer cultivated. NGW was initially distinguished from other archaeologically attested glume wheat crops (einkorn, emmer and spelt) on the basis of its chaff in samples of charred remains from Neolithic and Bronze Age sites in northern

Greece (Jones et al., 2000). The same wheat type was found at Bronze Age Feudvar (Serbia) in the 1990s, where it was initially described as unripe, failed, or sieving waste of emmer wheat (Borojevic, 1991; Kroll, 2016), and NGW chaff is of the same morphological type as that identified as emmer 'machaoid type' by de Moulins (1997) at Neolithic Cafer Höyük (Turkey). The earliest recognition of this unusual type of wheat chaff may have been at Neolithic sites in Germany, where Knörzer (1974; 1980) observed a type of chaff (which shows similarities with NGW – Jones, et al. 2000) that could not be confidently identified to species. Criteria for the identification of NGW grains were established at Bronze Age Stillfried (Austria) by Kohler-Schneider (2003), and additional criteria for the identification of chaff, on the basis of near complete ears at Bronze Age Lucone D (northern Italy), were described by Perego (2017). NGW has been referred to in some publications as 'striate emmeroid' (Fuller, 2012) or Sanduri (Kroll, 2016), the latter reflecting the Georgian name (zanduri) given to a mixture of wheat species grown in Georgia in recent times (Menabde, 1948; Dorofeev, 1969; Jones et al., 2000; Mosulishvili et al., 2017). Since its initial discovery, NGW has been reported at archaeological sites across western Asia and Europe (Kenéz et al., 2014, Toulemonde et al., 2015), where it was a significant component of the crop repertoire during the Neolithic and Bronze Age, perhaps cultivated (in some instances) as a mixed crop with einkorn (Jones et al., 2000; Kohler-Schneider, 2003; Perego, 2017), and it has recently been identified as a separate pure crop in its own right at Neolithic Çatalhöyük (Turkey) (Bogaard et al., 2013, 2017). Its cultivation declined after the Bronze Age, and finds are relatively rare in the Iron Age, after which it apparently disappears from the archaeological record (Kenéz et al., 2014).

Despite the proliferation of descriptive terms for 'NGW', there is a general consensus among archaeobotanists working across Europe and Western Asia that all these synonyms refer to a distinct morphological type represented in the archaeological record. NGW has distinctive morphological features for both grain and chaff (spikelet bases) that distinguish it from the other cultivated glume wheats found at Neolithic and Bronze Age sites in Europe and western Asia: einkorn (*Triticum monococcum* L subsp. *monococcum*), emmer (*Triticum turgidum* L. subsp. *dicoccum* [Schrank] Thell.) and spelt (*Triticum aestivum* L. subsp. *spelta* (L.) Thell.). Of these glume wheats, the closest match is with emmer, both morphologically and, where this can be calculated, in the number of grains (two) per spikelet (Jones et al, 2000; Kohler-Schneider, 2003), though the chaff also shares some morphological traits with einkorn (de Moulin, 1997; Jones et al., 2000). In addition, both grains and chaff of NGW have morphological characteristics that are not found in any of the other three glume wheat species, and these characteristics are shared by all archaeological identifications of NGW (and its synonyms). This is significant as it means that a taxonomic identification of one archaeological accession of NGW can confidently be applied to all other archaeological finds of the same morphological type.

On the basis of comparison with 181 modern specimens of spikelet bases (both charred and uncharred) of twelve wild and cultivated glume wheat types (Supplementary Table 1), including *Triticum aestivum* L. subsp. *macha* (Dekapr. & Menabde) Mackey (from which the term 'machaoid' derives) and numerous accessions of *T. turgidum* subsp. *diccoccum* (to which NGW was initially assigned), the closest morphological match for NGW was found to be *Triticum timopheevii* (Zhuk.)

Zhuk. subsp. *timopheevi* (Jones et al., 2000). *Triticum timopheevii* subsp. *timopheevii* was cultivated in Georgia until at least the mid-twentieth century (Stoletova, 1924; Dekaprelevic and Menabde, 1932; Dekaprelevic, 1954; Menabde and Ertizian, 1960) and is thought to have been endemic to this region (Zohary et al., 2012; Mosulishvili et al., 2017) having been domesticated locally (and perhaps recently) from *Triticum timopheevii* (Zhuk.) Zhuk. subsp. *araraticum* Jakubz.. Despite this close match, the true taxonomic identity of NGW remains unknown. It may, as suggested by the morphological affiliation, result from an earlier domestication of *T. timopheevii* subsp. *araraticum* (the Georgian *T. timopheevii* subsp. *timopheevii* being either a relic of this early crop or the result of a later domestication) or it may be descended from wild emmer (*T. turgidum* L. subsp. *dicoccoides* [Korn. ex Asch. & Graebn.] Thell.), possibly by a separate domestication or alternatively by splitting of the initial domesticated lineage into distinct populations that diverged to give 'typical' emmer and NGW (Jones et al., 2000).

The taxonomic identity of NGW could be resolved by DNA typing and is essential for answering questions concerning its origins, such as whether this crop originated from a previously unknown domestication in southwest Asia or resulted from later genetic changes in an existing wheat crop (Jones et al., 2000; Toulemonde et al., 2015). There are unambiguous differences between the *T. timopheevii* and *T. turgidum* groups at the genetic level, the former possessing the G and A^t genomes, and the latter the B and A^u genomes (Zohary et al., 2012). The two types of A genome are very similar and difficult to distinguish by DNA sequencing, but the G and B genomes have distinctive genetic polymorphisms (Allaby et al., 1999; Sallares et al., 2004). These polymorphisms include variations in the G and B versions of the *Ppd-1* gene (Takenaka and Takenaka, 2012, 2013), coding for a protein involved in the photoperiod response, which we have used in design of a diagnostic set of polymerase chain reaction (PCR) tests that enable *T. timopheevii* and *T. turgidum* wheats to be unambiguously identified (Czajkowska et al., 2019).

Utilization of a PCR test for taxonomic identification of the NGW would require analysis of ancient DNA (aDNA), which is challenging with charred cereal specimens due to the extensive DNA breakdown that occurs during the heating process involved in preservation of these remains (Threadgold and Brown, 2003). It is clear that with many charred grains the degree of transformation to carbon is such that preserved aDNA cannot be detected by PCR (Brown et al., 1998; Brown, 1999; Oliveira et al., 2012; Fernandez et al., 2013; Lundstrom et al., 2018; Lempiäinen-Avci et al., 2020) or by next generation sequencing (Nistelberger et al., 2016). This does not, however, negate the conclusions of early work that some grains in some charred samples retain DNA fragments that are sufficiently intact to be amplified by highly specific and sensitive PCR tests (Brown, 1999), as shown by the numerous reports of aDNA sequences obtained from charred cereal samples (e.g. Allaby et al., 1994, 1997, 1999; Blatter et al., 2002; Schlumbaum et al., 1998; Fernandez et al., 2013; Bilgic et al., 2016; Tanaka et al., 2010, Castillo et al., 2016; Ciftci et al., 2019) as well as charred remains of other types of plant (e.g. grape: Manen et al., 2003; maize: Freitas et al., 2003; oak: Deguilloux et al., 2006; pea: Smýkal et al., 2014).

Two previous attempts to use aDNA typing to identify NGW have been reported, but in one of these papers the sequences obtained did not enable the B and G genomes to be distinguished

(Blatter et al., 2003), and in the second the PCRs were unsuccessful with the archaeological samples (Boscato et al., 2008). In addition, we previously reported G-specific sequences from two individual grains in an archaeobotanical sample from Assiros Toumba (Brown et al., 1998). Although NGW chaff had already been identified at this site the sample that was tested did not contain NGW chaff and was primarily composed of emmer mixed with smaller amounts of einkorn and spelt. We have therefore remained cautious about this G result.

In this paper we report use of the *Ppd-1* test with DNA extracts prepared from nine charred accessions of NGW and detection of G-specific signals with two of these.

2. Materials and methods

2.1 Ancient DNA regime

DNA extractions were prepared and PCRs set up in two physically-separated laboratories within the specialized ancient DNA research facility at the University of Manchester. DNA extractions were carried out in a Class II biological safety cabinet while PCRs were set up in a laminar flow PCR cabinet in a second, physically-isolated laboratory. These rooms had never previously been used to process grain or chaff of NGW or *T. timopheevii*. Each laboratory was supplied with ultra-filtered air under positive displacement. After each use, benches and equipment were decontaminated by overnight UV irradiation and by cleaning with 5% hypochlorite acid, 70% ethanol and DNA Away (Molecular Bioproducts). Small equipment, UV-stable plasticware and reagents were decontaminated by UV irradiation (254 nm, 120,000 mJ cm^{-2} for 2 $\times$ 15 min, with 180° rotation between the two exposures) before use. Sensitive plasticware was intensively wiped with ethanol and DNA away. Personnel wore a disposable forensic suit, face mask, double hair net, goggles, two layers of gloves and disposable shoe covers at all times. Each DNA extraction was accompanied by a blank (normal extraction but without botanical material) and every set of 11 PCRs was accompanied by three blanks (one extraction blank and two PCRs set up with water rather than DNA extract).

2.2 NGW accessions

The archaeobotanical material consisted of charred NGW grains and/or chaff fragments (spikelet bases) from five sites dating from the Neolithic to the Bronze Age (Tables 1–3). Charred grain from two of these sites has previously been reported to contain aDNA (Çatalhöyük – Bilgic et al., 2016; Assiros Toumba – Allaby et al., 1997). Two of the three accessions that were studied from Çatalhöyük were taken from samples made up entirely of NGW (Table 2), reducing the possibility of cross-contamination with non-NGW wheat debris. The wheat in a third sample, from Feudvar, was predominantly NGW. Other accessions came from mixed contexts where NGW was recovered from samples also containing other wheats such as einkorn and emmer.

Table 1 Details of the NGW material analysed.

Table 2 Wheat identifications in analysed samples.

Table 3 Site context information.

[Tables at end of manuscript]

2.3 DNA extraction and analysis

Each accession, comprising 5 grains or 25 chaff fragments, was wrapped in UV-treated aluminium foil and ground into a fine powder that was placed in a microcentrifuge tube. DNA was extracted using materials provided in the NucleoSpin totalRNA FFPE kit and NTC buffer (Macherey-Nagel), using the following in-house procedure developed after extensive testing of alternative pipelines (Czajkowska and Brown, in preparation). An aliquot of 340 µl Lysis Buffer MLF + 30 µl Liquid Proteinase K was added to each accession, which was then vortexed, incubated at 56°C for 16 hours and centrifuged for 10 min at 16000 × *g*. The supernatant was transferred to a fresh microcentrifuge tube, 30µl Precipitation Buffer MKA added, and vortexed to distribute the precipitate homogeneously before placing at 4°C for 10 min and centrifuging for 10 min at 16000 × *g*. The supernatant was again transferred to a fresh microcentrifuge tube and 1 ml Binding Buffer MX added. After 1 min at room temperature, the mixture was loaded in 650 µl aliquots into a NucleoSpin RNA column which was placed in a collection tube and centrifuged for 30 s at 16000 × *g* after each addition. The eluate was discarded and the bound material washed by adding 600 µl Binding Buffer NTC and centrifuging at 30 s at 16000 × *g*, followed by 700 µl Wash Buffer MW2 and centrifugation for 30 s, and finally a further 700 µl Wash Buffer MW2 and centrifugation for 1 min. The eluates were discarded and the DNA eluted by adding 50 µl water and centrifuging for 1 min. Individual extractions from the same grain accession were pooled by rebinding on a column prior to PCR, to give three preparations for the Feudvar material (from 2 × 35 and 1 × 30 grains) and single preparations for each of the other eight accessions.

Two PCRs specific for the *Ppd-B1* gene giving products of 84 and 100 bp, and two specific for *Ppd-G1* with products of 61 and 69 bp (Czajkowska et al., 2019), were carried out in a LightCycler 480 (Roche) in 50 µl reaction volumes comprising 10 µl DNA extract, 1x SensiFAST SYBR No-ROX PCR master mix (Bioline), 100 nM forward primer, 100 nM reverse primer and PCR grade water. Cycling parameters were: 95°C for 5 min; followed by 35 cycles of 20 s at 95°C, 20 s at the annealing temperature (see Results), 20 s at 72°C; followed by a final extension at 72°C for 10 min. Product formation was assayed by melt curve analysis using the SYBR Green I/HRM Dye detection format (465 nm excitation, 510 nm emission), with melting data obtained by heating the products to 95°C for 5 s, cooling to 55°C for 30 s and then heating to 99°C with five data acquisitions per °C. Melting peaks were identified by plotting $-\left(\frac{dF}{dT}\right)$, where *F* is fluorescence level and *T* is temperature, against temperature.

Amplicons were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and cloned using the TOPO TA Cloning Kit for Subcloning, with One Shot TOP10 chemically competent *E. coli* cells (Invitrogen). Inserted DNA was reamplified from recombinant colonies with M13 forward and reverse primers, using the conditions described above with an annealing temperature of 55°C and omitting the final extension at 72°C. The PCR products were electrophoresed in a 2% agarose gel and those with insert sizes of c.50–150 bp purified as above and sequenced with the BigDye Terminator v3.1 kit chemistry (Applied Biosystems), using a protocol designed to prevent early signal

loss with difficult templates, in a reaction of 20.05 µl comprising 9 µl PCR product, 1 × BigDye sequencing buffer, 0.125 × BigDye v3.1 reaction mix, 0.0625 × dGTP BigDye v3.0 reaction mix, 4 pmoles primer, 0.95 M betaine (Sigma), 5% (v/v) dimethyl sulfoxide (Sigma) and UltraPure DNase/RNase-free distilled water. Cycling parameters were: 2 min at 96°C; 35 cycles of 40 s at 96°C, 15 s at 50°C, 4 min at 60°C; with products held at 4°C before purification (Beckman Coulter Agencourt CleanSEQ kit) and reading of paired-end sequences by capillary electrophoresis in a 3730 DNA Analyser (Applied Biosystems). BLAST (Altschul et al., 1990) was used to compare sequences with the GenBank database (Benson et al., 2006)

3. Results

PCRs were initially carried out using the annealing temperatures that had previously been optimized for the primer pairs with modern DNA (PCR1, 67°C; PCR2, 66°C; PCR3, 64°C; PCR4, 67°C; Czajkowska et al., 2019), but these amplifications gave no products that were detectable by melt curve analysis. A second of set of PCRs was therefore carried out with a lower annealing temperature of 59°C, in order to reduce the stringency of primer binding and hence improve the possibility of obtaining amplification if one or more of the nucleotides in the priming sites had become abasic due to DNA degradation (Brown and Brown, 2011). PCR1, targeting the B genome, still gave no products with any accession, but PCR2 (B genome) gave products with seven accessions, PCR3 (G genome) with nine accessions, and PCR4 (G genome) with all eleven accessions (Table 4).

Table 4 Results of PCRs carried out at an annealing temperature of 59°C. [Table at end of manuscript]

The PCR products were cloned and clones of reasonable sizes (50–150 bp) were sequenced (Table 5). Cloning was successful with each of the seven PCR2 products, yielding 36 clones (five or six clones per product). Two of these 36 clones, both from the Miechowice accession, gave sequences identical to the *Ppd-B1* target (Supplementary Fig. 1A); the other 34 clones gave sequences that either had no significant similarity to entries in the GenBank database, or gave hits to bacterial or other non-plant sequences. Three of the nine products amplified by PCR3, directed at the G genome, were not sequenced due to significant amplicon size discrepancies. The other six products gave a total of 14 clones, nine of which (all five from Çatalhöyük accession 12015, and all four from the Miechowice accession) gave authentic *Ppd-G1* sequences (Supplementary Fig. 1B); the other four sequences either had no significant hits in the database or similarities with non-plant sequences. With PCR4, again directed at the G genome, seven products gave 18 clones and the other four products were not sequenced. Seventeen of the clones gave sequences that had no significant hits in the database or were similar to non-plant sequences, and one, from the Miechowice accession, yielded an authentic *Ppd-G1* sequence (Supplementary Fig. 1C).

Table 5 Sequences obtained from the cloned PCR products. [Table at end of manuscript]

4. Discussion and conclusions

4.1 Amplification of DNA from charred NGW accessions

Since the initial report of aDNA in charred cereal grains (Allaby et al., 1994), there has been extensive debate regarding the extent to which DNA is preserved in this type of material. Early conclusions that aDNA is present in, at best, only a few grains from an archaeobotanical sample (Allaby et al., 1997; Brown, 1999) have been confirmed by several papers reporting negative results (Brown et al., 1998; Brown, 1999; Oliveira et al., 2012; Fernandez et al., 2013; Nistelberger et al., 2016; Lundstrom et al., 2018; Lempiäinen-Avci et al., 2020). Recently, the ability of DNA to withstand the heating conditions needed to produce charred archaeobotanical material has been questioned (Nistelberger et al., 2016; Lundstrom et al., 2018), but this view is arguably over-pessimistic, requiring that the numerous positive reports of aDNA in charred wheat, barley and rice remains (Allaby et al., 1994, 1997, 1999; Blatter et al., 2002; Schlumbaum et al., 1998; Fernandez et al., 2013; Bilgic et al., 2016; Tanaka et al., 2010, Castillo et al., 2016; Ciftci et al., 2019) be dismissed as illusory. Threadgold and Brown (2003) showed that there is a critical threshold of c.200°C above which the DNA in modern cereal grains degrades relatively rapidly, but that at temperatures below this threshold DNA can be detected by fluorimetry and PCR even after several hours of heating. Other studies have shown that material resembling archaeobotanical samples can be obtained after heating modern grain for 2–3 h at 220–240°C (Fraser et al., 2013; Charles et al., 2015). Taken together, these studies suggest that there may be a small window of heating parameters that can, in some cases, give rise to charred archaeobotanical material in which some intact DNA is preserved. It is therefore reasonable to attempt to use aDNA typing in the taxonomic identification of NGW.

We used two strategies to maximize our chances of success. First, prior to commencing the project we performed an extensive reappraisal of the methods used previously to study aDNA in charred grain. From trials with a combination of modern grain and non-NGW archaeobotanical samples, we devised procedures that are optimized for retrieval of short double- and single-stranded DNA fragments from material has undergone the type of heat damage occurring during charring (Czajkowska and Brown, in preparation).

Second, where possible, we selected archaeobotanical samples that appeared most likely to contain aDNA. Our initial focus was on two sites, Çatalhöyük and Assiros Toumba, where we and others have previously obtained positive aDNA detections with non-NGW varieties of charred wheat (Brown et al., 1997; Bilgic et al., 2016). The NGW material from these two sites has very good or excellent morphological preservation, and most grains have attached embryos. Additionally, two of the Çatalhöyük samples comprised only NGW, which meant that the aDNA analysis would not be complicated by possible contamination with debris from non-NGW wheat remains. We subsequently extended the study to include sites from which there has been no previous aDNA work. These were Feudvar and Stillfried, where again there is very good or excellent archaeobotanical preservation, and Miechowice 4, where the degree of preservation was less optimal.

We used PCRs specific for the B and G genomes of wheat and obtained evidence for the presence of the G genome in two accessions, from Çatalhöyük and Miechowice 4. The grain from Çatalhöyük gave consistent results for the G genome with PCR3, all clones of this PCR product

corresponding with the reference G sequence. With PCR4, this accession did not give an authentic G genome PCR product, and instead yielded amplicons of varying sizes whose sequences gave only poor matches with sequences in the GenBank database. The chaff from Miechowice 4 again gave consistent G results with PCR3 but also yielded an authentic G sequence with PCR4. The Miechowice chaff also gave two clones with B genome sequences after amplification with PCR2.

In order to obtain amplicons from the NGW accessions it was necessary to reduce the annealing temperatures for each of the four PCRs to 59°C, which is 5–8°C lower than the optimal annealing temperatures for these PCRs with modern DNA samples (PCR1, 67°C; PCR2, 66°C; PCR3, 64°C; PCR4, 67°C; Czajkowska et al., 2019). The rationale was that by lowering the annealing temperature, priming could occur at sites where one or possibly two of the nucleotides in the annealing sequence had become damaged by loss of their nucleotide base(s), as is anticipated for aDNA templates (Brown and Brown, 2011). One consequence of reducing the stringency of the PCR in this way is that non-target sequences can also be amplified, as was observed with the majority of accessions with PCRs 2, 3 and 4, these accessions giving amplicons of various 'incorrect' lengths whose sequences gave only poor matches with sequences in the GenBank databases. These amplicons probably derive from a mixture of uncharacterized microbial contaminants from reagents and the burial environment and, possibly, highly degraded wheat DNA (Fernandez et al., 2012; Nistelberger et al., 2016).

4.2 Authenticity of the results

Non-authentic 'detections' of aDNA from charred cereal accessions can arise in three ways: (1) from contamination of extracts with modern DNA, in particular amplicons from previous PCR experiments; (2) from contamination of the ancient material with modern plant material (e.g. pollen from wild or cultivated plants) or debris from other charred material from the archaeobotanical sample; (3) by misinterpretation of sequences. We do not believe that any of these issues are responsible for the G genome detections that we obtained with the Çatalhöyük and Miechowice material.

We carried out the aDNA procedures under a strict technical regime involving physically-separated laboratories, decontamination of work areas, personnel, equipment and reagents, etc. (Section 2.1), which we believe is robust and prevents contamination with modern DNA. In particular, *T. timopheevii* plant material or DNA extracts had never previously been handled in the aDNA laboratories, so any modern contamination must have been brought into those laboratories when the charred grain extracts were being prepared or when PCRs were set up. However, no work with *T. timopheevii* plant material or DNA extracts was performed in any laboratory in the University of Manchester during the month prior to preparation of the charred grain extracts, and all nine charred accessions were processed before the first PCRs were performed with those extracts. The technical regime is the same as that used by us in other projects, for example in genotyping of *Mycobacterium tuberculosis* and *Mycobacterium leprae* DNA in human bones (e.g. Bouwman et al., 2012; Kerudin et al., 2019), where contamination with PCR amplicons would be evident when next-generation sequencing reads are mapped to the reference sequences. We have never observed such contamination. Additionally, during the preliminary method development we found no evidence that any of the reagents were contaminated with wheat DNA. The need to reduce the annealing

temperatures in order to obtain amplicons with the ancient extracts also provides evidence that the wheat sequences that we obtained were derived from aDNA, rather than from modern contaminating DNA, as the latter would not be expected to display chemical damage and therefore would yield amplicons at the higher annealing temperatures. At those higher temperatures, which consistently give positive PCR results with modern wheat extracts (Czajkowska et al., 2019), no amplicons were obtained with any charred accession-PCR combination.

Possible contamination of the archaeobotanical samples prior to their use in this project is also unlikely, at least as an explanation of the G genome detections that we report. None of the sites from which we obtained material, nor the institutes in which the samples have been curated, are located in the wild range of *T. timopheevii* or in areas where this species is cultivated. Contamination with *T. timopheevii* pollen or other modern explants is therefore very unlikely. Neither could contamination with debris from non-NGW grain within the archaeobotanical samples give rise to the G detections, as the samples as a whole did not contain other grains that could conceivably contain a G genome (Table 2). Note, however, that this type of contamination could account for the B genome detection obtained with the material from Miechowice 4, as the sample from which NGW spikelet bases were extracted was an external rubbish pit, and the sample as a whole also contained emmer wheat. Additionally, surface concretions on the chaff from this site may have introduced extraneous material from the rest of the sample, causing contamination with material containing B genome aDNA.

The third possibility, misinterpretation of sequences, can also be discounted. Although the use of lowered annealing temperatures allowed amplification of non-target microbial loci, the sequences from Çatalhöyük and Miechowice 4 that we identify as *Ppd-G1* have identity with the reference sequences and significant difference to the closest non-target sequences present in the databases.

A second approach to authentication of an aDNA result is to ask how reasonable it is for the material being studied to contain preserved DNA, taking into account factors such as the preservation status of the material and the environmental conditions at the site from which it was recovered (Gilbert et al., 2005). Çatalhöyük is located in semi-arid steppe with low rainfall (300–350 mm p.a.) and a monthly average temperature range of 0–22°C (Table 3), conditions that are generally considered to be compatible with aDNA preservation (Kistler et al., 2017). Ancient DNA has previously been reported in both charred wheat remains (Bilgic et al., 2016) and human bones (Chyleński et al., 2019) from Çatalhöyük, confirming that the environmental conditions allow DNA survival. The NGW grains that we studied were recovered from a storage deposit c.2 m below the modern surface of the occupation mound and the sample (unlike that from Miechowice 4) was made up entirely of NGW with no macroscopically detectable admixture of any other type of wheat. The NGW grains displayed excellent morphological preservation (Fig. 1A). Overall, the environmental and preservational information for the Çatalhöyük material is consistent with the results that we obtained (Supplementary Table 2).

Fig. 1. Examples of the material giving positive results for the G genome. (A) Charred NGW grain from Çatalhöyük, Building 131, showing excellent preservation and lack of distortion. (B) Charred NGW spikelet bases from Miechowice 4, pit 30, showing surface abrasion and concretions.

The Miechowice results are also consistent with the context and preservation of the material (Supplementary Table 2). The chaff from Miechowice 4 was in a relatively poorly preserved state due to post-depositional abrasion of the surface, rather than damage and distortion incurred during charring (Fig. 1B). This form of damage is unlikely to affect the preservation of aDNA but it inevitably reduces the confidence with which the material can be identified on the basis of morphological criteria. This increases the possibility of accidental inclusion of chaff from another wheat species.

4.3 Implications of the results

Our results provide evidence that NGW contains a G genome and is therefore a member of the *T. timopheevii* group, which comprises both the wild and domesticated G genome tetraploid wheats. This discovery has implications beyond the taxonomic identification of NGW at these two sites. First, since archaeobotanists are agreed that the various synonyms assigned to NGW all refer to the same morphological wheat type (Kenéz et al., 2014, Toulemonde et al., 2015), the aDNA confirmation of the G genome can be extrapolated to all other synonymous finds. There is also no doubt that NGW was a cultivated wheat throughout most, if not all, of its range. It has been identified (often in quantity) at agricultural settlements across Europe and western Asia, extending well beyond the geographic range of wild *T. timopheevii* subsp. *araraticum*. It has been found in storage contexts from at least the mid 7th millennium to the 1st millennium BC (as at Çatalhöyük, Assiros, Stillfied and possibly Feudvar), where it was stored alongside, or as a mixture with, other cultivated wheats, notably einkorn (the other main component, along with *T. timopheevii*, of the cultivated zanduri crop grown until recently in Georgia – Menabde, 1948; Mosulishvili et al., 2017). Its separate storage at Çatalhöyük, and its predominance in samples at other sites (such as Feudvar) confirm that it is not simply a contaminant of other wheat crops.

Secondly, the identification of NGW as a cultivated member of the *T. timopheevii* group is important for the evaluation of competing hypotheses concerning the origins of agriculture in southwest Asia. While it is generally agreed that agriculture in this region arose in the so-called Fertile

Crescent (an arc stretching from the Levant, through southeast Turkey to Mesopotamia), it is a matter of debate whether the species that make up the southwest Asian package of Neolithic grain crops were domesticated once only in a well-defined 'core area' within this arc (Lev-Yadun et al., 2000; Abbo et al., 2010; 2013) or whether they were domesticated in multiple locations over a wider geographic area (Willcox 2002, 2005; Fuller et al., 2011; 2012). One of the criteria suggested for evaluating the likelihood of these alternative hypotheses is crop species diversity (Zohary, 1999; Fuller et al., 2011; 2012; Abbo et al., 2013). Zohary (1999), for example, argued that, when there are multiple wild species from the same genus in a region, only one of which becomes domesticated, this suggests single rather than multiple domestication events. In this context, he draws particular attention to the 'sibling' wild species *T. turgidum* subsp. *dicoccoides* (wild emmer) and *T. timopheevii* subsp. *araraticum*, which occur sympatrically in the northern and eastern part of the Fertile Crescent, but of which only emmer was thought to have been domesticated. It has been suggested, however, that NGW may result from the domestication of *T. timopheevii* subsp. *araraticum,* alongside wild emmer, during the emergence of agriculture in southwest Asia (Jones et al., 2000). For this reason, NGW is listed (under the term 'striate emmeroid') as one of nine potentially 'lost' or 'failed' founder crop species, additional to the eight conventional founder crops domesticated in southwest Asia (Fuller et al., 2012, Table 2). Fuller et al. argue that it is unlikely that so many species were brought into cultivation in the same restricted core area, though the status of some of these additional founder crops has been questioned (Abbo et al., 2013).

NGW has been identified in the Fertile Crescent, at the Pre-pottery Neolithic B (PPNB) site of Cafer Höyük (SE Turkey), where it was apparently the dominant wheat crop (referred to as *T. turgidum* subsp. *dicoccum* (machaoid type), from the early PPNB (late 9th millennium BC – de Moulins, 1997) onwards. More recently, it has been identified at a slightly earlier date further west (in Central Turkey) from early PPNB (c.8400 cal. BC) and later levels at Aşıklı Höyük (Ergun, 2018; Quade et al., 2018), where it was a secondary cultivar alongside the predominant emmer crop. It has also been suggested, based on the presence of both tough (domesticated type) and brittle (wild type) rachis abscission scars (following criteria used by Tanno and Willcox, 2012), that the glume wheats (including NGW) at Aşıklı Höyük were still in the process of domestication. Grains and chaff at other PPNB sites that have previously been identified as domesticated emmer (*T. turgidum* subsp. *dicoccum*) – especially those from sites excavated before criteria for the identification of NGW were published – would also benefit from re-examination to determine whether some of these too are NGW*,* potentially expanding its early geographic range, and so further broadening the possible locations for its domestication.

Ancient DNA confirmation of NGW as *T. timopheevii* (rather than a genetic variant of cultivated emmer) therefore provides concrete evidence that *T. timopheevii* was domesticated alongside emmer in southwest Asia. This both undermines the 'sibling species' argument for a single domestication event and provides confirmation that the number of founder crop species was indeed greater than the eight conventionally accepted Neolithic domesticated crops, adding weight to the argument for more numerous, and potentially dispersed, instances of domestication. This is also in keeping with recent results, based on modern DNA research, suggesting that southwest Asian cereal

crops were initially cultivated as widespread 'metapopulations', rather than emanating from a core area of domestication (Civáň et al., 2013; Poets et al. 2015; Allaby, 2015; Pankin et al., 2018; Oliveira et al. 2020). The subsequent wide distribution of *T. timopheevii* at sites in western Asia and Europe dating from the Early Neolithic to the Iron Age (9th to 1st millennium BC) indicates that, although it may be described as a lost crop (depending on its relationship to the *T. timopheevii* of western Georgia), it was certainly not a failed crop, having persisted over a broad geographic area for at least seven thousand years. *Triticum timopheevii* can therefore no longer be looked upon as an endemic crop species restricted to western Georgia (Mosulishvili et al., 2017) or a "local episode in wheat-crop evolution" (Zohary et al., 2012). Instead this species must be viewed as a significant component of prehistoric Eurasian agriculture, with implications for our understanding of the origins of agriculture in southwest Asia.

CRediT author statement

Beata Czajkowska: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, review and editing, Project administration. **Amy Bogaard:** Resources, Writing – review and editing. **Michael Charles:** Conceptualization, Resources, Writing – review and editing. **Glynis Jones:** Conceptualization, Resources, Writing – original draft, review and editing, Funding acquisition. **Marianne Kohler-Schneider:** Resources. **Aldona Mueller-Bieniek:** Resources. **Terence Brown:** Conceptualization, Writing – original draft, review and editing, Supervision, Project administration, Funding acquisition.

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647

Table 1

Details of the NGW material analysed.

Accession	Site	Sample number	Context/Unit number	Date	Material	Reference
1	Feudvar, Serbia		W3063	Bronze Age (1600–1500 cal. BC)	100 grains	*Kroll* (2016)
2	Çatalhöyük, Turkey	10830	20703	Neolithic (6640–6220 cal. BC)	30 grains	Bogaard et al. (2017)
3	Çatalhöyük, Turkey	11995	22656	Neolithic (6640–6220 cal. BC)	30 grains	Bogaard et al. (2017)
4	Çatalhöyük, Turkey	12015	22637	Neolithic (6640–6220 cal. BC)	25 grains	Bogaard et al. (2017)
5	Assiros Toumba, Greece	4355	38014	Late Bronze Age (c. 1360 cal. BC)	30 grains	Jones et al. (2000)
6	Stillfried, Austria	11392	pit 643	Late Bronze Age (1031–819 cal. BC)	30 grains	Kohler-Schneider (2003)
7	Assiros Toumba, Greece	4355	38014	Late Bronze Age (c. 1360 cal. BC)	25 chaff fragments	Jones et al. (2000)
8	Stillfried, Austria	11392	pit 643	Late Bronze Age (1031–819 cal. BC)	25 chaff fragments	Kohler-Schneider (2003)
9	Miechowice 4, Poland		pit 30	Middle Neolithic (4400–4000 cal. BC)	25 chaff fragments	Bieniek (2002, 2007)

651 **Table 2**

652 Wheat identifications in analysed samples.

Site		**Çatalhöyük**	**Çatalhöyük**	**Çatalhöyük**	**Miechowice 4**	**Feudvar**	**Assiros Toumba**	**Stillfried**
Sample number		10830	11995	12015			4355	11392
Unit number		20703	22656	22637		W3063	38014	
Context		Building 122	Building 131	Building 131	Pit 30		Room 24	Pit 643
Context type		Storage deposit	Storage deposit	Storage deposit	Outdoor refuse pit	Pit	Storage area	Storage pit
Wheat identifications								
T. monococcum subsp. *monococcum*	Grains	32	0	0	12	1411	136	141
T. monococcum subsp. *monococcum*	Glume bases	2	0	0	58	2378	131	12
T. monococcum subsp. *monococcum* or *T. turgidum subsp dicoccum*	Grains	/	/	/	/	/	4	/
T. turgidum subsp dicoccum	Grains	66	0	0	25	224	/	44
T. turgidum subsp dicoccum	Glume bases	125	0	0	86	4	7	2
T. turgidum subsp dicoccum or NGW	Grains	/	/	/	/	/	310	3
T. turgidum subsp dicoccum or NGW	Glume bases	/	/	/	/	/	28	9
NGW	Grains	701	905	3304	/	85 957	/	176
NGW	Glume bases	1226	772	1392	721	77 508	200	63
probable NGW	Grains	/	/	/	2	/	/	/
probable NGW	Glume bases	/	/	/	52	/	/	/
T. turgidum subsp dicoccum or *T. aestivum* subsp. *spelta*	Grains	0	0	0	0	0	73	/
T. aestivum subsp. *spelta*	Grains	0	0	0	0	0	57	69
T. aestivum subsp. *spelta*	Glume bases	0	0	0	0	0	70	13
Triticum (glume wheat)	Grains	1138	0	0	0	/	/	62
Triticum (glume wheat)	Glume bases	0	0	0	2960	/	40	18
T. aestivum subsp. *spelta* or *aestivum*	Grains	/	/	/	/	/	/	10
T. aestivum subsp. *aestivum*	Grains	0	0	0	0	7	0	3
T. aestivum subsp. *aestivum*	Rachis internodes	0	0	0	1	2	0	0
Triticum sp.	Grains	/	/	/	17	/	/	8
%NGW		58.6%	100%	100%	19.5%	97.6%	27.9%	38.5%
Degree of distortion		Very little	Very little	Very little	Very little	Very little	Very little	Very little
State of preservation		Excellent	Excellent	Excellent	Poor	Excellent	Very good	Very good
Confidence of identification		Very confident	Very confident	Very confident	Fairly confident	Very confident	Very confident	Very confident
Grain embryos present?		Mostly present	Mostly present	Mostly present	–	Often present	Mostly present	Often missing

653 'Glume bases' includes whole spikelet bases (pairs of glume bases), each counted as two glume bases.

654 / = category not used.

Table 3

Site context information.

Site	Çatalhöyük	Miechowice 4	Feudvar	Assiros Toumba	Stillfried
Location	Central Anatolian plateau	Kuyavia, North European Plain	Loess plateau, Titel	Langadas Basin	Loess plateau, E. Austria
Coordinates	37°40′00″N; 32°49′41″E	52°37′30″N; 18°49′08″E	45°17′15″N; 20°13′48″E	40°48′10″N; 23°01′13″E	48°24′49″N; 16°50′14″E
Height above sea level	1000 m	90m	120 m	150 m	199 m
Environment	semi-arid steppe	temperate	temperate	temperate	temperate
Köppen-Geiger classification	BSk	Cfb	Csb	Csa	Cfb
Mean monthly temperature range	0–22°C	-4–19°C	0–22°C	4–25°C	–2–19°C
Average monthly precipitation range	300–350 mm	526 mm	635 mm	458 mm	559 mm
Site type	Tell/mound	Flat	Tell/mound	Tell/mound	Hillfort
Depth below surface[a]	c.2 m	–	1.6 m	c.4 m	2.7–2.8 m

[a] For tells/mounds, depth below surface of tell or mound.

659 **Table 4**

660 Results of PCRs carried out at an annealing temperature of 59°C.

Accession	Site	PCR1 (B genome)	PCR2 (B genome)	PCR3 (G genome)	PCR4 (G genome)
1A[a]	Feudvar	No product	Positive	Positive	Positive
1B[a]	Feudvar	No product	Positive	Positive	Positive
1C[a]	Feudvar	No product	No product	No product	Positive
2	Çatalhöyük	No product	Positive	Positive	Positive
3	Çatalhöyük	No product	Positive	Weak positive	Weak positive
4	Çatalhöyük	No product	No product	Weak positive	Positive
5	Assiros Toumba	No product	No product	Positive	Weak positive
6	Stillfried	No product	Positive	Positive	Weak positive
7	Assiros Toumba	No product	No product	Positive	Positive
8	Stillfried	No product	Positive	No product	Positive
9	Miechowice 4	No product	Positive	Positive	Positive

661 [a] The Feudvar accession was divided into three subsamples of 35, 35 and 30 grains, respectively.

662

663 **Table 5**

664 Sequences obtained from the cloned PCR products.

Accession	Site	Clones[a]	Sequence length (bp)	Closest match in GenBank	Coverage (%)	Similarity (%)
PCR2 (B genome) expected sequence length 100 bp						
1A	Feudvar	1	41	*Jonesia denitrificans*	85	100
		2–5	103, 100, 119, 86	No significant matches	–	–
1B	Feudvar	1	45	*Hylemonella gracilis*	74	100
		2	45	*Crassostrea gigas*	71	100
		3	58	*Cottoperca gobio*, *Ipomoea triloba*	94	100
		4	68	*Anabas testudineus*, *Oryzias latipes*	85	96
		5	100	No significant match	–	–
2	Çatalhöyük	1–5	82, 49, 126, 39, 48	No significant matches	–	–
3	Çatalhöyük	1–5	76, 33, 39, 47, 48	No significant matches	–	–
6	Stillfried	1	23	*Ovis 22anadensis canadensis*	95	100
		2–5	85, 100, 39, 47	No significant matches	–	–
8	Stillfried	1	100	Uncultured gamma proteobacterium	65	98
		2	110	Uncultured gamma proteobacterium	51	100
		3	40	*Paraburkholderia caffeinilytica*	90	100
		4	48	*Neurospora crassa*	82	96
		5	46	No significant match	–	–
9	Miechowice 4	1	100	*Triticum turgidum*	100	100
		2	100	*Triticum turgidum*	100	100
		3	41	*Lysinibacillus* sp. B2A1	95	100
		4–6	40, 112, 47	No significant matches	–	–
PCR3 (G genome) expected sequence length 61 bp						
1A	Feudvar	1	47	No significant match	–	–
1B	Feudvar	–	–	–	–	–
2	Çatalhöyük	–	–	–	–	–
3	Çatalhöyük	–	–	–	–	–
4	Çatalhöyük	1	61	*Triticum timopheevii*	100	100
		2	61	*Triticum timopheevii*	100	100
		3	61	*Triticum timopheevii*	100	100
		4	61	*Triticum timopheevii*	100	100
		5	61	*Triticum timopheevii*	100	100
5	Assiros Toumba	1	74	No significant match	–	–
6	Stillfried	1	74	*Scophthalmus maximus*	100	100
		2	52	No significant match	–	–
7	Assiros Toumba	1	57	*Blumeria graminis*	100	100
9	Miechowice 4	1	61	*Triticum timopheevii*	100	100
		2	61	*Triticum timopheevii*	100	100
		3	61	*Triticum timopheevii*	100	100
		4	61	*Triticum timopheevii*	100	100

PCR4 (G genome) expected sequence length 69 bp

1A	Feudvar	1	69	*Microbacterium hominis*, *Rhizobium tropici*	68	100
1B	Feudvar	–	–	–	–	–
1C	Feudvar	1, 2	54, 54	No significant matches	–	–
2	Çatalhöyük	–	–	–	–	–
3	Çatalhöyük	1	68	*Rhizobiales* PAMC 29148, *Xenorhabdus poinarii*	75	100
		2	46	*Cottoperca gobio*	88	91
		3	46	No significant matches	–	–
4	Çatalhöyük	1	66	*Linum usitatissimum*	76	100
		2	62	*Oryzias latipes*	86	100
		3	64	*Microvirga* sp. 17 mud 1-3	87	95
		4	70	*Pangasianodon hypophthalmus*	68	100
		5	69	No significant match	–	–
5	Assiros Toumba	1	69	*Pongo abelii*	79	96
6	Stillfried	–	–	–	–	–
7	Assiros Toumba	1	78	No significant match	–	–
8	Stillfried	–	–	–	–	–
9	Miechowice 4	1	69	*Triticum timopheevii*	100	100
		2	69	*Canis lupus dingo*, *Methanohalophilus portucalensis*	65	100
		3	67	Cloning vector PBTH-mwtGFP	100	100
		4	69	*Mus musculus*	65	100
		5	69	*Miniopterus natalensis*	86	96

[a] Clones with inserts 50–150 bp are listed. Clones with inserts outside of this size range were considered to contain non-specific products.