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Classification of archaic rice grains excavated at the Mojaoshan site within the Liangzhu site complex reveals an *Indica* and *Japonica* chloroplast complex

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Abstract

To understand rice types that were utilized during postdomestication and in the modern age and the potential of genetic research in aged rice materials, archaeogenetic analysis was conducted for two populations of archaic rice grains from the Mojaoshan site during the Liangzhu Period in China (2940 to 2840 BC). Sequencing after the PCR amplification of three regions of the chloroplast genome and one region of the nuclear genome showed recovery rates that were comparable to those in previous studies except for one chloroplast genome region, suggesting that the materials used in this work were appropriate for recovering genetic information related to domestication traits by using advanced technology. Classification after sequencing in these regions proved the existence of *Japonica* and *Indica* chloroplasts in archaic grains from the west trench, which were subsequently classified into eight plastid groups (type I–VIII), and indicated that these rice grains derived from different maternal lineages were stored together in storage houses at the Mojaoshan site. Among these plastid groups, type V exhibited the same sequences as two modern *Indica* accessions that are utilized in basic studies and rice breeding. It was inferred that part of the chloroplast genome of archaic rice has been preserved in modern genetic resources in these two modern *Indica* accessions, and the results indicated that rice related to their maternal ancestor was present at the Mojaoshan site during the Liangzhu Period in China. The usefulness of archaeogenetic analysis can be demonstrated by our research data as well as previous studies, providing encouragement for the possibility that archaeogenetic analysis can be applied to older rice materials that were utilized in the rice-domesticated period.

Keywords: Agriculture, Archaeology, Archaic DNA, Diversity, Mojaoshan, Rice

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Introduction

Rice is believed to have been domesticated along the middle or lower Yangtze River, as suggested by archaeological studies (Fuller et al. 2007, 2009, 2010; Liu et al. 2007; Nakamura 2010; Deng et al. 2015; Zheng et al. 2016). Rice domestication has also been studied on the basis of genetic information, providing two hypotheses about Asian rice domestication: the single vs. multiple domestication hypotheses (Civán and Brown 2018; Choi and Purugganan 2018). The key to revealing how Asian rice was domesticated is to study archaic rice because rice domestication was carried out ca. 9000 BP (Zuo et al. 2017). Domestication studies with archaic rice have frequently been conducted by using morphometrical methods (Fuller et al. 2007; Liu et al. 2007). However, there is a problem in morphometrical research because size shrinkage is observed in plant remains, including those of rice (Fuller and Harvey 2006; Fuller 2007; Hopf 1955; Renfrew 1973; Smith 2014), which seems to lead to erroneous results if careful observations are not made. Grain size variation, which is commonly found in archaic rice, is thought to be an unsuitable criterion for domestication studies (Liu et al. 2007) or for the classification of archaic rice (Kumagai et al. 2016; Tanaka et al. 2016); thus, archaeogenetic studies are conducted on rice materials and provide

new insights into rice domestication and introduction (Castillo et al. 2016; Kumagai et al. 2016). On the basis of an archaeogenetic study on archaic rice, we can discuss the recovery of DNA from aged rice materials, the origins of cultivated rice, and the utilization of a portion of the genetic background in postdomestication activities, thus demonstrating the usefulness of archaeogenetic research in rice.

The Liangzhu site complex, which consists of several archaeological sites, is located within a plane area in the northwestern part of Hangzhou City, Zhejiang Province (30° 22' 36" N to 30° 26' 17" N, 119° 56' 41" E to 120° 03' 28" E), situated along the lower Yangtze River (Fig. 1). Among the sites in this complex, the Mojiaoshan site is enclosed within walls and thought to be the main city of the Liangzhu site complex. Many archaeological remains have been excavated, such as graves, irrigation systems, paddy fields, stone tools, and wooden tools, which are historically related to the cultures existing before and after the Liangzhu Period and to the culture carried along the middle Yangtze River and Yellow River. The Liangzhu site complex is thought to contain important remains for understanding the culture in the Liangzhu Period (2940–2840 cal. BC, or 5500–4300 year BP, estimated by Qin et al. 2015) and



Fig. 1 The excavated site of archaic rice in the Mojiaoshan site. White arrows indicate layers of archaic rice grains

the development of cities through the introduction of goods and culture from neighboring areas. Approximately 10 to 15 tons of archaic rice grains (estimated by Ningyuan Wang), together with burned wood, have been excavated from the west trench and south trench of the Mojiaoshan site. These rice grains exhibit high length and width variations, and it has been hypothesized that these rice grains were carried from other areas as tributes when the Mojiaoshan site was constructed, then mixed in storage houses along the west trench and south trench, accounting for the observed size variation and the likely different varieties present (Nakamura 2015). If the hypothesis can be supported by genetic data, these rice grains may provide genetic information about rice from neighboring areas and represent suitable materials for generating data on which rice types were utilized in post domestication activities in rice.

Ancient DNA analyses have been conducted in plant remains (Pääbo et al. 2004; Palmer et al. 2012) but are difficult because of DNA degradation caused by aging, such as sequence fragmentation and sequence substitution (Hofreiter et al. 2001; Gugerli et al. 2005). This DNA degradation has been verified by next-generation sequencing in three species, rice, maize and grape (Nistelberger et al. 2016). In the case of archaic rice, developed molecular markers have enabled DNA sequences to be recovered at low rates. Proto-Indica domestication was suggested by a study conducted by Castillo et al. (2016) using chloroplast genome markers developed for inter- and intragenic regions (*Orf100*, *petN-trnC* (I-32) and *rpl14-rp116* (PS-ID)) following previous studies (Nakamura et al. 1997; Takahashi et al. 2008; Tang et al. 2004). In this study, additional molecular markers were reconstructed to analyze the shattering genes *qSH1* and *sh4* and an intergenic region of rice chromosome number 6 (DJ6) following previous studies (Konishi et al. 2006; Li et al. 2006; Hanamori et al. 2011). *sh4* is related to the key domestication trait of nonshattering (Li et al. 2006; Zhang et al. 2009), and *qSH1* is found in a small subset of temperate *Japonica* accessions (Konishi et al. 2006). The DJ6 region is used to dominantly classify tropical and temperate *Japonica* (Hanamori et al. 2011; Tanaka et al. 2016). Among these six markers, I-32 and PS-ID and one nuclear genome marker, DJ6, were applied for the recovery of DNA from Japanese archaic rice grains dated to 2000 years BP, which enabled these archaic rice grains to be classified into two rice types corresponding to modern rice varieties (Tanaka et al. 2016). Kumagai et al. (2016) developed two single-nucleotide polymorphic markers (SNP markers) for the chloroplast genome after the whole-genome sequencing of 216 modern rice samples; these markers were located at positions 14,169 bp and 56,524 bp of the chloroplast genome in *O. rufipogon* (AP006728.1). These two SNP markers allowed the classification of not only two chloroplast genotypes corresponding to those of *Japonica* and *Indica* but

also archaic rice grains from Korea and Japan, among which the oldest grains were dated to 2800 years BP. These studies have encouraged the analysis of older rice using molecular markers. In addition, through the analysis of archaic rice grains at the Mojiaoshan site, we can demonstrate the possibility that genetic information can be recovered from rice materials that were utilized at the time of domestication.

In this study, archaic rice grains collected from the west trench and south trench of the Mojiaoshan site were classified by using molecular markers for the chloroplast genome and the nuclear genome. Based on the obtained DNA recovery rates and classification along with modern rice accessions, we discuss the following topics: 1) the possibility of recovering genetic information from more aged rice materials, 2) whether our data can support the hypothesis put forth by Nakamura (2015) regarding the possibility that rice grains were carried from other areas to the Mojiaoshan site, and 3) whether rice genotypes identified at the Mojiaoshan site can be found in modern rice accessions, to consider the coverage of diversity in modern breeding materials.

Materials and method

Plant materials

This study was conducted using two archaic charred rice populations from the Mojiaoshan site and 33 modern rice accessions (Fig. 2, Table 1). The two archaic rice populations were excavated from different trenches located in the western and southern areas of the Mojiaoshan site (Fig. 2). The archaic rice grains from the west trench were dated to a time range of 5500 year BP to 4300 year BP, corresponding to the Liangzhu Period, at Beijing University (Qin et al. 2015). These populations, which were collected by water flotation on a 2.0-mm mesh screen and preserved as dry specimens, were provided by the Zhejiang Provincial Institute of Cultural Relics and Archaeology, China (see an example of archaic grains from the west trench in Fig. 2b). Unhusked rice is thought to have been buried in the two trenches of the Mojiaoshan site based on the results of archaeological research communicated by Ningyuan Wang, who is one of the coauthors. Thus, the seed coat of the archaic rice could be identified (Fig. 2a), and the examined archaic rice samples are expected to have contained embryos.

All modern rice accessions included in our analyses were landraces and were selected to represent wide morphological and genetic diversity after Oka (Table 1; 1953, 1958). Among the modern rice accessions, 14 and 19 were *Japonica* and *Indica* accessions, respectively, from five countries, including China. Among the *Japonica* accessions, seven lowland landraces of tropical *Japonica*, 4

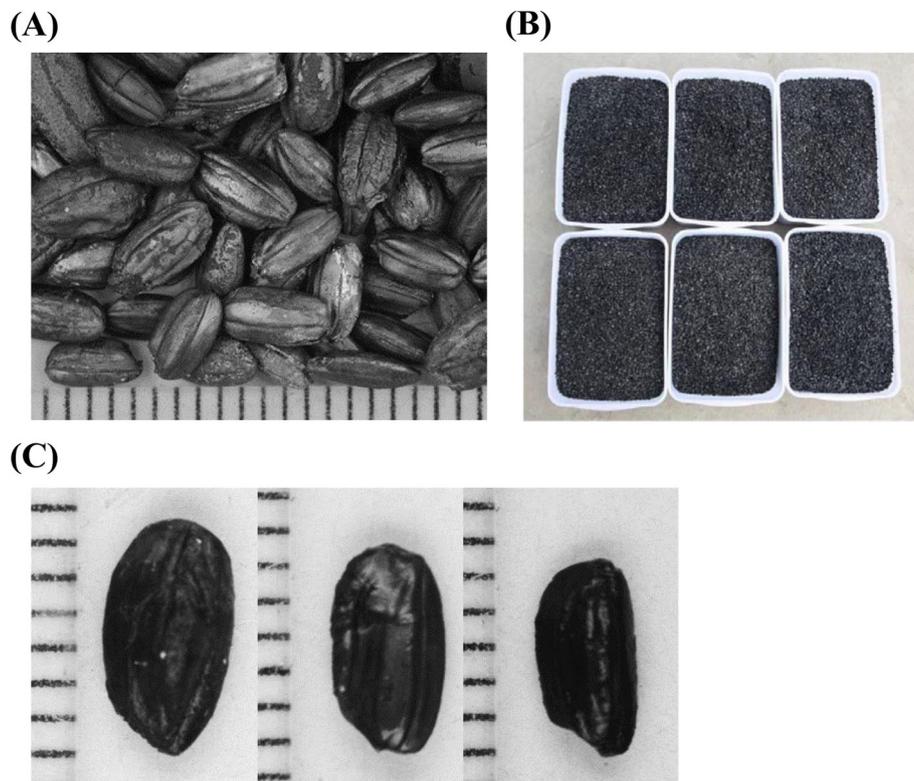


Fig. 2 Archaic rice examined in this study. **a** Rice grains excavated from the west trench at the Mojiaoshan site. **b** Collected rice grains from the west trench at the Mojiaoshan site. These grains were collected from soil blocks by water flotation. **c** Variation in rice grain excavated from northwestern trench-2 within the Liangzhu site complex

lowland landraces of temperate *Japonica* and three upland landraces of temperate *Japonica* were examined. Seeds of these accessions were provided by the National Institute of Genetics (NIG) of Japan and the Genebank of the Genetic Resources Center (GRC) at NARO, Japan. The rice types listed in Table 2 refer to the Oryzabase classification (<http://www.shigen.nig.ac.jp/rice/oryzabase/>) of NBRP for accessions from NIG and to the database of the Genebank Project of NARO (https://www.gene.affrc.go.jp/databases-plant_search.php) for accessions from GRC.

Archaeogenetic methodology

To prevent contamination by modern rice DNA, the extraction and analysis of aDNA were performed in the Ancient DNA laboratory within the Institute of Food Crops at the Jiangsu Academy of Agricultural Sciences, China, according to the following procedure:

1. The archaic rice grains were not manipulated in an ancient DNA laboratory where modern rice had previously been analysed.
2. The ancient DNA laboratory was closed off to other researchers during the period of all ancient DNA experiments.
3. Extraction was carried out in a sterile flow hood chamber that had previously been bleached, sealed, and UV irradiated for over 1 day.
4. The ethanol, bleach, and filters used for DNA purification and sealing were UV-irradiated for over 1 day after introduction to the sterile flow hood chamber.
5. Other disposable materials, such as stainless-steel beads, tubes, filter tips, gloves, ultrapure water and all liquids employed for DNA extraction except for ethanol, were electron ray irradiated by the supplier and were UV irradiated for over 1 day after introduction to the sterile flow hood chamber. All materials were only opened inside the flow hood chamber after the procedure indicated above.
6. Tweezers and pipets were autoclaved and were UV irradiated for over 1 day after introduction to the sterile flow hood chamber.
7. Lab coats were also UV irradiated for over 1 day.
8. PCR amplification of ancient DNA was performed with a thermal cycler in which modern rice DNA had not previously been used to be amplified.

Table 1 DNA genotype of modern rice accessions examined in this study

Variety name/ number ^a	Accession	Country	Group/ Rice type/ Species	Cultivation type	Seed ^b source	Chloroplast genome ^c						Nuclear genome ^c			
						<i>rps16</i> intron1	<i>Orf100</i> In/del ^d		<i>trnI</i>	<i>petN-trnC</i>	<i>qSH1</i>	<i>sh4</i>	DJ6 In/del ^d		
							F1-R1	F1-R2					F1-R1	F1-R2	
Gendjah ratji	/ T0624	Indonesia	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Paid kasalle	/ T0642	Indonesia	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Sinanpablo	/ T0220	Philippines	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Malagkit sinaguig	/ T0221	Philippines	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Macan menita	/ T0224	Philippines	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Hsu-chou-san-pei	/ T0718	China	<i>Japonica</i> / Tropical	Lowland	1	152	100	162	A	75	G	T	315	102	
Koddi	/ T0757	China	<i>Japonica</i> / Tropical	Lowland	1	159	NA	93	A	75	G	T	98	NA	
Ta-pei-mang	/ T0708	China	<i>Japonica</i> / Temperate	Lowland	1	152	100	162	A	75	G	T	98	NA	
Tsao-siao-pei-tao	/ T0709	China	<i>Japonica</i> / Temperate	Lowland	1	152	100	162	A	75	G	T	98	NA	
Bohai	/ T0848	China	<i>Japonica</i> / Temperate	Lowland	1	152	100	162	A	75	G	T	315	102	
Kinoshita-mochi	/ T0563	Japan	<i>Japonica</i> / Temperate	Lowland	1	152	100	162	A	75	G	T	98	NA	
Akamochi	/ JP4424	Japan	<i>Japonica</i> / Temperate	Upland	2	152	100	162	A	75	G	T	315	102	
Abaremochi	/ JP5085	Japan	<i>Japonica</i> / Temperate	Upland	2	152	100	162	A	75	G	T	315	102	
Akayakan	/ JP4663	Japan	<i>Japonica</i> / Temperate	Upland	2	152	100	162	A	75	G	T	315	102	
P.T.B.10	/ T0414	India	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
A.D.T. 4	/ T0415	India	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
A.D.T. 14	/ T0417	India	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
M.T.U. 9	/ T0420	India	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
P.T.B. 8	/ T0421	India	<i>Indica</i>	Lowland	1	152	100	162	G	107	G	T	315	102	
Mugi of bogra	/ T0440	India	<i>Indica</i>	Lowland	1	152	NA	93	G	107	G	T	315	102	
Pduhai perumal	/ T0435	Sri Lanka	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
70 ^a som cau	/ T0001	Vietnam	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
He-nan-tsao	/ T0706	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Mao-tü-tas	/ T0710	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Chiang-si-wan	/ T0715	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Chin sen	/ T0719	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Kun-mih-tsieh-huan	/ T0724	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Ming-hou-tsiu-tsao	/ T0729	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Siao-chung-kuh	/ T0761	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
U-kuh-tsing-you	/ T0101	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Peh-kuh	/ T0108	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Tuan-Kuang-hwa-lo	/ T0130	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	
Patpaku	/ T0868	China	<i>Indica</i>	Lowland	1	159	NA	93	G	107	G	T	315	102	

^aAccession numbers refer to varieties registered in the National Institute of Genetics (NIG), Japan

^bSeeds were provided by three source institutes indicated by the following numbers: 1 = National Institute of Genetics (NIG), Japan, 2 = Genebank, Genetic Resources Center, NARO, Japan

^cElectrophoresis and sequencing were used to detect an expected DNA fragment in regions *rps16* intron1, *Orf100*, *trnI*, *petN-trnC*, *qSH1*, *sh4* and DJ6 and the size of each DNA fragment was used as the genotype name. "F1-R1" or "F1-R2" are primer set in *Orf100* and DJ6. NA = Not amplification by specific primer set

^dGenotype data, except for "P.T.B. 8" in *orf100* region, are referred to the study of Castillo et al. (2016)

Table 2 Primer information used in this study and sequence polymorphism detected by using their primers

Genome	Gene name ^a	Application	Forward (F), Reverse (R) Primer (5' to 3') ^b	Position (bp) ^a	Product size (bp) ^{a,c}	Polymorphism	
						Type	Sequence ^d
Chloroplast	<i>rps16</i> intron1:	Ancient rice	F2 CCTTATCCGGTCCAATTCTA	4946-4966	152	In/del	+ 7 bp
	OsC01	Modern rice	R2 GGGTATGTTGCTACTCTTTTGAA	5097-5075			
	<i>Orf100</i>	Ancient rice	F1 TGGATTTGAAAGTCAATTTT	8500-8520		In/del	-69 bp
		Modern rice	R1 CCTTTCCCACTCGCTCTCTA	8599-8579	100		
			R2 TCCATGATTCTATTTCCAAG	8661-8641	162		
	<i>trnL</i> :	Ancient rice	F TCGATTCTCTCCGTTAACTTT	14,116-14,137	152	SNP	A/G
	14,169	Modern rice	R GGAAGAAGTCTTTGGTAATGG	14,267-14,247			
	<i>petN-trnC</i> :	Ancient rice	F2 ATCAGTTCAAAGAATTTACTC	17,758-17,778	75	In/del	+ 32 bp
l-32	Modern rice	R2 TATTATACTTAATGCTCCCC	17,832-17,812				
Nuclear	chr. 1 <i>qSH1</i> promoter	Ancient rice	F ATGGTATTGATGTACTGGA	38,217,614-38,217,634	71	SNP	G/T
			R CATCTCGTCCAAGATCCTTA	38,217,684-38,217,664			
	chr. 4 <i>sh4</i> exon1	Ancient rice	F AGACGTCATCCTCATCACC	34,631,533-34,631,552	142	SNP	G/T
		Modern rice	R TAGTTCTCCACCCACTTCCAC	34,631,431-34,631,411			
	chr. 6 Non-coding region: DJ6	Ancient rice	F1 TGACCGTCTGTAGCAGTG	9,043,039-9,043,058		In/del	+ 217 bp
		Modern rice	R1 CCAGTTTAATGTTTTTCATTGCC	9,043,136-9,043,113	98		
			R2 GATTTCCGTTTCCGTGCC	-	NA		

^aGene name, position and product size were referred to chloroplast genome (accession No. NC_001320) and nuclear genome of chromosome no.1, 4 and 6 (accession No. NC_008394, NC_008397, NC_008399) in *Japonica* 'Nipponbare'

^bThe specific forward primer was F1 for specific reverse primers, R1 and R2, in *Orf100* region and DJ6 region. Y = C or T

^c"NA" indicated PCR amplification was not successful with specific forward and reverse primer set

^dNumerical number indicates insertion or deletion length corresponding with *Japonica* 'Nipponbare' sequence. SNP sequence shows about wild type or mutant type by "wild-type sequence/mutant-type sequence" in a cell

To check for DNA contamination, a sample without seed remains was used as a negative control for DNA extraction. In addition, aDNA was amplified in the aDNA laboratory of the Faculty of Agriculture and Life Science at Hirosaki University to establish that the aDNA results were authentic.

DNA extraction

A total of 120 grains from the west trench and 50 grains from the south trench with an unbroken shape were selected (Fig. 2a, c) for DNA analysis. Ancient DNA was extracted using the procedure described in our previous study (Mutou et al. 2014; Tanaka et al. 2016). For DNA extraction from modern rice accessions, one seed from each accession was sown on filter paper and grown at 30 °C under a 16 h light-8 h dark cycle at a light intensity of 46.5 $\mu\text{Ms}^{-1}\text{m}^{-2}$. Ten-day-old seedlings were individually ground in liquid nitrogen, and total DNA was extracted using the procedure of Murray and Thompson (1980) with minor modifications in different rooms from the above ancient DNA laboratory.

Primer design and utilization

To classify the archaic grains, sequences corresponding to an insertion or deletion (in/del) of 7 bp in the intron of *rps16* (assigned as OsC01, Okoshi et al. 2016), an in/del of

69 bp in *Orf100* (Takahashi et al. 2008), a SNP in a pseudo *trnL* sequence (14,169; Kumagai et al. 2016), and an in/del of 32 bp in the intergenic region between *petN* and *trnC* (l-32) in the rice chloroplast genome were obtained (Tang et al. 2004) (Table 2). Three nuclear markers included SNPs in each of the promoter regions of *qSH1* (Konishi et al. 2006), exon 1 of *sh4* (Li et al. 2006), and one set of in/dels of 4 bp and 221 bp in the intergenic region of rice chromosome number 6 (assigned as the DJ6 region, Hanamori et al. 2011). Among these seven markers, the primer sets targeting the intron of *rps16* and pseudo *trnL* were reconstructed using Primer 3 (Untergasser et al. 2012) from rice chloroplast genome sequences NC_001320 (*Japonica*) and JN861109 (*Indica*), and the expected products are reasonably short, with a predicted length of 152 bp in *Japonica* 'Nipponbare', which is within the range that is expected to be preserved in ancient DNA based on previous studies (Palmer et al. 2009, 2012). The remaining primer sets for *Orf100*, *petN-trnC*, *qSH1*, *sh4* and DJ6 were developed in a previous study (Castillo et al. 2016). All primer sets were used to analyze both modern DNA and ancient DNA.

DNA analysis

aDNA was amplified using the first PCR product as a template. The same primer set was used in the first and second rounds of PCR. PCR amplification was carried

out three times for all ancient DNA extracts. In addition, a negative control amplification was carried out to assess possible contamination. The amplification of two regions (the intron of *rps16* and pseudo *trnI*) was performed in a 20 μ L mixture including 2.0 μ L aDNA, 1 \times ExTaq[™] buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 0.25 U ExTaq[™] polymerase (TAKARA, Japan), 0.1 mM dNTPs, 2.0 mM MgCl₂ and each primer at 0.25 μ M by using a Mastercycler Ep Gradient system (Eppendorf, Germany). Initial denaturing was performed at 95 °C for 3 min, followed by 35 PCR cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 3 min. DNA amplification of the remaining five regions (*Orf100*, *petN-trnC*, *qSH1*, *sh4* and DJ6) was performed using the same procedure applied by Castillo et al. (2016). Electrophoresis and sequencing for all markers were performed using the same procedures employed by Castillo et al. (2016).

Modern DNA analyses were performed for seven regions, including the intron of *rps16*, pseudo *trnI*, *qSH1*, and *sh4*. The genotyping data for *Orf100*, *petN-trnC* and DJ6 in 32 of the modern rice accessions (with the exception of accession 'P.T.B. 8', for which *Orf100* genotyping was performed in this study) were generated in a previous study (Castillo et al. 2016) and are referenced in the data analysis. The PCR amplification and electrophoresis procedures were basically the same as for the aDNA

analysis, except that during PCR amplification, the modern DNA was amplified using the DNA extraction liquid as the template. The first PCR products were used for electrophoresis and sequencing for all markers.

Based on the sequences of the obtained PCR amplicons, genotyping was performed as follows: archaic grains and modern rice accessions were classified as insertion or deletion types for each of three regions of the chloroplast genome (intron 1 of *rps16*, *Orf100* and *petN-trnC*) and according to the SNPs in the sequences of pseudo *trnI*, *qSH1* and *sh4* (Table 2). PCR amplification with the F1 and R2 primer set targeting *Orf100* generated either a 93 bp fragment or a 162 bp fragment, which included a 69 bp insertion. PCR amplification with primers F1 and R1 generated null and 100 bp fragments, including part of the 69 bp insertion because the R1 primer was constructed within the 69 bp insertion (Castillo et al. 2016). In the DJ6 region, an 98 bp or 315 bp fragment was detected by using the F1 and R1 primer set. Among these fragments, the 315 bp fragment shared a 4 bp deletion and 221 bp insertion with the 98 bp fragment (Castillo et al. 2016). The archaic grains were classified according to the 98 bp sequence type or the 98 bp sequence type that was part of the 315 bp fragment as follows: the 98 bp sequence type could be amplified by using primer set F1 and R1, but the 315 bp fragment was not successfully amplified by the primer set because of DNA degradation; or the 98 bp fragment could be detected

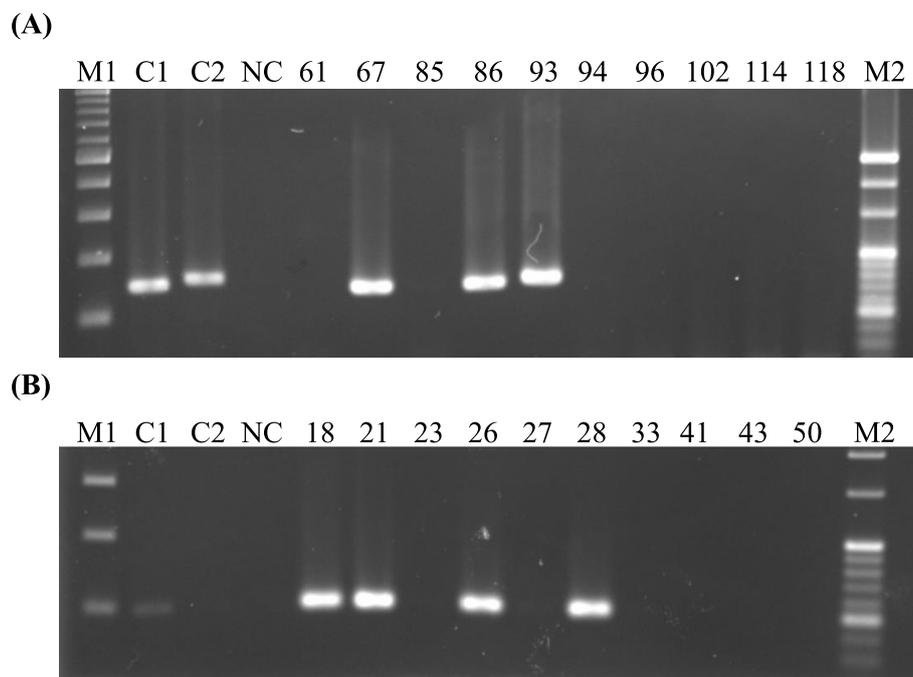


Fig. 3 An example of the electrophoresis of PCR products amplified from rice aDNA samples from the west trench at the Mojaoshan site. **a** Amplicons obtained by using the specific primer targeting *rps16* intron 1. **b** Amplicons obtained by using the specific primer targeting *Orf100* F1 and R1. M1 = 100 bp DNA ladder (Takara, Japan), C1 = *Oryza sativa Japonica* 'Ta-pei-mang', C2 = *Oryza sativa Indica* "P.T.B.10", 61–118 and 18–50 = Archaic rice grains, M2 = 20 bp DNA ladder (Takara, Japan)

Table 3 DNA genotype of archaic grains and modern rice accessions by specific primer set in the chloroplast genome

Site name/ Rice type	No. of grains/ ^a accessions	<i>rps16</i> intron1 ^b			<i>Orf100</i> F1–R1 ^b		<i>Orf100</i> F1–R2 ^b			<i>trnI</i> ^b			<i>petN–trnC</i> ^b		
		152	159	NA	100	NA	93	162	NA	A	G	NA	75	107	NA
West trench	120	4	1	115	5	115	0	8	120	11	2	107	0	0	120
South trench	50	0	0	50	0	50	0	0	50	0	0	50	0	0	50
<i>Japonica</i> / Tropical/ Lowland	7	6	1	0	6	1	1	6	0	7	0	0	7	0	0
<i>Japonica</i> / Temperate/ Lowland	4	4	0	0	4	0	0	4	0	4	0	0	4	0	0
<i>Japonica</i> / Temperate/ Upland	3	3	0	0	3	0	0	3	0	3	0	0	3	0	0
<i>Indica</i>	19	2	17	0	1	18	18	1	0	0	19	0	0	19	0

^aNumerical number indicates number of accessions in modern rice accessions

^bElectrophoresis and sequencing were used to detect an expected DNA fragment in regions *rps16* intron1, *Orf100*, *trnI* and *petN–trnC* and the size of each DNA fragment was used as the genotype name. “F1–R1” or “F1–R2” are primer set in *Orf100*. NA = Not amplification by specific primer set

by primers F1 and R2, which was developed based on the 221 bp insertion in the 315 bp fragment.

Results

On the basis of PCR amplification, compared with modern rice accessions, the expected DNA fragments were detected in the three regions of the chloroplast genome (intron 1 of *rps16*, *Orf100* and pseudo *trnI*) and the DJ6 region in archaic rice grains from the west trench but only in the DJ6 region in rice grains from the south trench (Fig. 3ab; Supplementary data 1 and 2). Among the 120 archaic grains from the west trench, one grain shared all three regions of the chloroplast genome, seven grains shared two of the three regions, and 12 grains shared one of the three regions. Thus, at least one of the three regions in the chloroplast genome was recovered from 20 grains (16.7%).

The expected fragment in intron 1 of *rps16* was observed in five grains among the 120 grains (4.2%) from the west trench (Table 3), and sequencing revealed that four and one grains exhibited the 152 bp and 159 bp target fragments, which were mainly found in modern *Japonica* and *Indica*, respectively. On the other hand, the number of expected fragments was found in 5 grains for the *Orf100* F1–R1 region and 8 grains for the *Orf100* F1–R2 region, among which the expected fragments were

generated from the amplification of both regions in only one grain (Supplementary data 1). Thus, the recovery of the expected fragment in *Orf100* was achieved in 12 grains (10.0%), and a comparable number, of 13 grains, for pseudo *trnI*. In the case of the PCR amplification of DJ6, the expected fragment of 98 bp was observed in one archaic grain from the west trench (0.8%) and two grains (4.0%) from the south trench by using the F1 and R1 primer set, which shared a sequence that was mainly found in temperate *Japonica* (Table 4). In addition, by using primer set F1 and R2, a 98 bp fragment including a portion of the 315 fragment was found in five grains from the south trench (10%), whose sequence was mainly shared by tropical *Japonica*, upland rice and *Indica*.

On the basis of the combined genotypes from the three regions of intron 1 of *rps16*, *Orf100* and pseudo *trnI*, we were able to classify the modern rice accessions into five genotypes and the archaic rice grains from the west trench into eight genotypes (type I to VIII) (Table 5). According to this classification, the dominant genotype was found in each modern *Japonica* and *Indica* accession. One *Japonica* accession, ‘Koddy’, from China was classified as a genotype different from the other 13 *Japonica* accessions for two of the regions, intron 1 of *rps16* and *Orf100* (Table 1). Two *Indica* accessions from India, “P.T.B. 8” and “Mugi of bogra”, were classified as

Table 4 DNA genotype of archaic grains and modern rice accessions by specific primer set in the nuclear genome

Site name/ Rice type	No. of grains/ ^a accessions	<i>qSH1</i> ^b			<i>sh4</i> ^b			DJ6 F1–R1 ^b			DJ6 F1–R2 ^b	
		G	T	NA	T	G	NA	98	315	NA	102	NA
West trench	120	0	0	120	0	0	120	1	0	119	0	120
South trench	50	0	0	50	0	0	50	2	0	48	5	45
<i>Japonica</i> / Tropical/ Lowland	7	7	0	0	0	7	0	1	6	0	6	1
<i>Japonica</i> / Temperate/ Lowland	4	4	0	0	0	4	0	3	1	0	1	3
<i>Japonica</i> / Temperate/ Upland	3	3	0	0	0	3	0	0	3	0	3	0
<i>Indica</i>	19	19	0	0	0	19	0	0	19	0	19	0

^aNumerical number indicates number of accessions in modern rice accessions

^bElectrophoresis and sequencing were used to detect an expected DNA fragment in regions *qSH1*, *sh4* and DJ6 and the size of each DNA fragment was used as the genotype name. “F1–R1” or “F1–R2” are primer set in DJ6. NA = Not amplification by specific primer set

Table 5 Classification by sequence type in chloroplast genome regions

Rice type	No. of ^a grains/accessions	<i>trnI</i> ^b SNP	<i>rps16</i> ^b intron1 In/del	<i>Orf100</i> ^b In/del
Modern				
<i>Japonica</i>	13	A	–	Insertion
<i>Japonica</i>	1	A	Insertion	–
<i>Indica</i>	17	G	Insertion	–
<i>Indica</i>	1	G	–	–
<i>Indica</i>	1	G	–	Insertion
Archaic rice grains at West trench				
I	1	A	–	Insertion
II	2	A	UN	Insertion
III	1	A	Insertion	UN
IV	7	A	UN	UN
V	1	G	UN	Insertion
VI	1	G	UN	UN
VII	4	UN	UN	Insertion
VIII	3	UN	–	Insertion

^aNumerical number indicates number of accessions in modern *Japonica* and *Indica*

^bUN Sequence was not detected because of DNA fragmentation, –: Not insertion type

different genotypes at two of the three regions than 17 other *Indica* accessions. However, all modern *Japonica* rice accessions shared the “A” sequence for the SNP of pseudo *trnI*, whereas all *Indica* rice accessions exhibited a “G” in this SNP sequence. This sequence differentiation between the two rice subspecies is common, as shown in a previous study by Kumagai et al. (2016) using 216 modern rice samples. Among the archaic rice grains from the west trench, type I exhibited the same sequence as the modern *Japonica* genotype, which was dominant; type II may also have the same genotype as type I, but the sequence of the intron of *rps16* could not be recovered from two grains of type II. One grain showing unsuccessful amplification of the *Orf100* region was classified as type III, which presented the same sequence as a modern *Japonica* genotype found at a low frequency. In Type IV, an “A” SNP sequence was found within the pseudo *trnI* region, which was shared with the modern *Japonica* accessions examined, although the DNA sequences recovered for the region indicated that seven grains of type IV exhibited a chloroplast genome similar to that of *Japonica* rather than that of *Indica*. Types V and VI may share the *Indica* sequence, at least in the pseudo *trnI* region, because a “G” SNP sequence was found for this region in two grains of both types, which was a sequence found only in modern *Indica* accessions. Between these two types, type V presented a sequence insertion in the *Orf100* region, which was also

found in a minor *Indica* genotype among the modern rice accessions from India, as mentioned above. Therefore, it was inferred that the archaic rice grains consisted of at least two types: one exhibiting a chloroplast genome sequence corresponding to that of modern *Japonica* and the other to that of *Indica*, implying that these grains were derived from different maternal lineages.

Discussion

The recovery of archaic DNA from plant remains through PCR sequencing via the Sanger method with PCR amplification or even next-generation sequencing is often difficult (Palmer et al. 2012; Nistelberger et al. 2016). The different ages and states of preservation of plant remains may delay the development of useful methods for recovering archaic DNA. DNA survives under cool, dry, dark, anaerobic and slightly alkaline conditions (Bollongino et al. 2008; Schlumbaum et al. 2008), and the desiccation of plants is an excellent sample preservation method to allow the recovery of DNA (Palmer et al. 2009; Bunning et al. 2012). These favorable conditions are related not only to the conditions during aging but also the preservation conditions after the excavation of plant remains. Thus, without careful consideration, we cannot compare the recovery rates of archaic DNA from samples across different studies. Our charred rice grain samples were preserved under dry conditions after their excavation, which was similar to the preservation conditions employed in three previous studies (Castillo et al. 2016; Kumagai et al. 2016; Tanaka et al. 2016). However, the charring conditions for all of these remains, including our samples, are unknown; charring under artificial conditions has shown to be successful at temperatures of less than 200 °C with anaerobic conditions in wheat, barley and sunflower (Hopf 1955; Renfrew 1973; Smith 2014), but DNA degradation caused by artificial charring has been observed in wheat (Threadgold and Brown 2003). Our study did not demonstrate DNA recovery for the *petN-trnC* region from the two populations of archaic grains (Table 3), although two other studies have successfully recovered archaic DNA from the *petN-trnC* region, for which the recovery rates were 32.7% (69/211 grains) across samples in a study by Castillo et al. (2016) and 14.0% (35/250 grains) in a study by Tanaka et al. (2016). The difficulty of recovering archaic DNA may simply be related to sample aging as follows: the estimated ages of 5500 years BP to 4300 years BP for the samples in this study were older than those of the samples in these other two studies (2250 years BP to 1650 years BP in the study by Castillo et al. (2016) and 2400 years BP to 600 years BP in the study by Tanaka et al. (2016)). Moreover, no recovery of chloroplast DNA regions was observed in the archaic grains from the south trench, as shown in Table 3. DNA degradation, including that occurring during sample aging, is thought to lead to a

reduced recovery rate, as suggested in previous studies (Threadgold and Brown 2003). On the other hand, the recovery rates for the *Orf100* region were comparable between this study and a previous study (10.0% (12/120 grains) in this study and 7.1% (15/211 grains) in the study by Castillo et al. (2016)). Normal PCR-based sequencing with several primer sets may be effective for capturing a single target region in charred rice grains, as indicated by Kumagai et al. (2016), as opposed to using high-throughput sequencing, which has been reported to produce no informative sites and a low recovery rate (Nistelberger et al. 2016). Alternatively, a large number of archaic rice grains could be applied for DNA analysis to recover genetic information, as was the case in this study and the previous study by Castillo et al. (2016). However, there may be quantitative and qualitative limitations to the recovery of genetic information under either approach, especially in genetic regions related to domestication traits, such as the shattering genes *sh4* and *qSH4*, as observed in this study and previous studies (Castillo et al. 2016). If a sample with a large volume was applied for archaeogenetic analysis, a large amount of genotyping data could be recovered to reveal plant domestication, as reported for maize and bottle gourd by using the shotgun and next-generation sequencing (Kistler et al. 2014; Ramos-Madriral et al. 2016). Recently, a large number of SNPs were detected from archaic melon seeds with comparable dimensions to rice grains by using a medium-throughput genotyping platform (Sabato et al. 2019). Our materials appeared to contain targeted genetic regions based on the observation of DNA fragments after normal PCR amplification, and there is potential to recover more genetic information related to domestication traits from these samples via advanced technologies. These results indicated the possibility that archaic DNA may remain in aged rice grains older than our materials that were utilized in the rice domestication period.

Classification by using sequences from three regions (intron 1 of *rps16*, *Orf100* and pseudo *trnI*) of the chloroplast genome revealed two genotypes corresponding to modern *Japonica* and *Indica* in the archaic grains from the west trench (Table 5). This result indicated that rice grains with *Japonica* and *Indica* chloroplast genotypes were stored together at the Mojiaoshan site during the Liangzhu Period (5500–4300 year BP) in China. Castillo et al. (2016) detected *Japonica* and *Indica* coexisting in India during the early Historic period (2250–2090 year BP) through archaic DNA analysis, although coexistence of the two rice varieties has not been found at the Metal Age sites in Thailand (1980–1950 year BP), leading to the conclusion that the Indian *Indica* subspecies arrived in Thailand at a later period in the first centuries AD. Unfortunately, no archaeological evidence reveals the relationship between South and Southeast Asia and China in the Liangzhu

Period. Thus, it would be completely speculative to suggest that the introduction of *Indica* was carried out in China prior to Thailand, but our research results suggested that rice grains were stored together in a storage house at the Mojiaoshan site, as inferred by Nakamura (2015). Thus, we observed two chloroplast genotypes derived from different maternal lineages in archaic grains from the west trench, even though different genotypes were found for the DJ6 region in archaic rice from the south trench (Tables 4 and 5).

The sequence analysis of three regions (intron 1 of *rps16*, *Orf100* and pseudo *trnI*) also revealed that each of the archaic grains of type III or type V exhibited the same sequence as all modern *Japonica* or modern *Indica* accessions, respectively (Table 5). To explore rice accessions showing the same genotype as the *Japonica* or *Indica* accessions in these three regions, we performed BLAST searches of these rice accessions in the sequence database of the National Center for Biotechnology Information, USA (<https://www.ncbi.nlm.nih.gov/>). No *Japonica* accessions with the same genotype as type III were found in the sequence database, whereas a modern *Indica* accession with the same genotype as type V exhibited the same chloroplast sequence as two other *Indica* accessions, ‘Shuhui498’ from China (nucleotide accession number: CP018170) and ‘Milyang23’ from Korea (KM103382). ‘Shuhui 498’, a restorer line from a three-hybrid system with heavy panicles, is used for superhigh-yield breeding programs; the heavy panicle trait, which is defined as more than 5 g of grain weight per panicle, is controlled by two key genes, *grain number 1a* (*Gn1a*) and *grain size 3* (*GS3*) (Wang et al. 2018). Based on the database of the China Rice Data Center (<http://www.ricedata.cn/variety/>), this line was inbred via cytoplasmic displacement, and the cytoplasmic donor accession is unknown. The whole-genome sequence of this line is available as a reference (Du et al. 2017). ‘Milyang23’, a high-yield rice variety derived from a three-way cross between *Indica* and *Japonica* (IR8//Yukara/TN1) that contributed to the “Korean green revolution”, has also been utilized in basic studies and introduced into breeding activities (Kim et al. 2014; Manigbas et al. 2019). It seems that the chloroplast genome corresponding to type V has been preserved in genetic resources and utilized for breeding and basic studies, indicating that a related maternal ancestor was present in the Mojiaoshan site during the Liangzhu Period in China.

Conclusion

In conclusion, an archaeogenetic analysis based on three regions of the chloroplast genome and one region of the nuclear genome revealed comparable recovery rates to previous studies, which suggested that our materials were

useful for recovering genetic information related to domestication traits by using advanced technology. Classification after the sequencing of these regions revealed eight plastid groups in archaic grains from the west trench and two genotypes for one nuclear genome region in archaic grains from the south trench, and the results indicated that these rice grains were stored together in a storage house at the Mojiaohsan site. Among the identified plastid groups, type V presented the same sequences as modern *Indica* accessions that are utilized in basic studies and rice breeding. These two modern *Indica* genetic resources were considered to exhibit a chloroplast genome similar to that of type V, which indicated that rice related to their maternal ancestor was present at the Mojiaohsan site during the Liangzhu Period in China. The usefulness of archaeogenetic analysis is demonstrated by our research data as well as previous studies, which provides encouragement that archaeogenetic analysis may be applied to older rice materials that were utilized in the rice domestication period.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s43014-020-00028-8>.

Additional file 1: Supplementary data 1. Genotyping after sequencing for the amplicons generated by using specific primer sets in rice grains from the west trench.

Additional file 2: Supplementary data 2. Genotyping after sequencing for the amplicons generated by using specific primer sets in rice grains from the south trench.

Abbreviations

PCR: Polymerase chain reaction; cal. BC: Calibrated BC; BP: Before present; SNP: Single-nucleotide polymorphism; NBRP: National Bio Resource Project, Japan; NARO: National Agricultural Food and Research Organization, Japan; aDNA: Ancient DNA; Sec: Seconds; Min: Minutes; BLAST: Basic Local Alignment Search Tool

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Authors' contributions

K.T., B.L., R.I., S. N., T.U. and C.W. designed the experiments; K.T. and C.Z. prepared the laboratory for the experiments and performed analyses; M.K., M.K., N. W. and M.C. prepared the archaic rice materials, M.K., N.K., S.K., R.I., H.T., N.W., S.N. and T.U. interpreted the data based on archaeological knowledge; K.T. and C.Z. wrote the manuscript, which was modified by C.W. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data referenced in this manuscript are available.

Competing interests

The authors declare that they have no competing interests.

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