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Exploring the possibility of successive breeding of chickens in the Yayoi culture, Japan

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Abstract

Chickens are domestic animals whose main ancestor is the red junglefowl (*Gallus gallus*), and their natural habitat is Southeast Asia and Southern China. Domestic chickens were brought to the Japanese Archipelago by the middle of the Yayoi period (4th–3rd BCE). Research on chicken sexual dimorphism shows that Yayoi-era (10th BCE–3rd CE) birds were mostly male, hindering widespread breeding across Japan. In the 2011 research area at the Karakami site in Iki City, Nagasaki Prefecture, 18 Phasianidae bones were identified in a large ditch estimated to have been buried in the late Yayoi period. Although the tarsometatarsus and tibiotarsus were found to belong to chickens based on their morphological characteristics, it was impossible to determine whether the other bones, including the four juvenile bones, were derived from chickens. In our previous study, we established a method to distinguish between the bones of Japanese wild pheasants and chickens by using collagen peptide fingerprinting (ZooMS). In this study, we conducted ZooMS species identification of zooarchaeological bird remains and determined the sex of chicken remains in Karakami. We found six mature Phasianidae remains belonging to chickens, five of which were assumed to be males based on their sexual dimorphism. However, all five immature bird bones, including the three previously identified Phasianidae bones, were not derived from either chickens or Galliformes. These results do not support successful breeding of chickens at the Karakami site. Chickens were thought to have been brought to the Japanese Archipelago from the Korean Peninsula through Iki Island. Further studies must determine when chickens were introduced to Iki Island and whether they included both males and females during their earliest introduction to Iki Island and the Japanese Archipelago.

Keywords: Domestic chicken; Iki Island; Japanese Archipelago; Yayoi culture; ZooMS

Introduction

The chicken is a domestic animal whose main ancestor is the red junglefowl (*Gallus gallus*), native to the tropical forests of Southeast Asia and Southern China (Darwin 1868, Baker 1968, Fumihito et al. 1994, Wang et al. 2020). Although there are indications that archaeological records of domestic chickens date back to the early Holocene (West and Zhou 1988, Xiang et al. 2014), recent re-examination suggests that domestication probably did not occur until approximately 3,500 years ago (Eda 2016, Eda 2021, Peters et al. 2022). Among the Yayoi culture sites in the Japanese Archipelago, chicken or candidate chicken bones have been excavated from seven sites west of the Nobi Plain (Eda 2018). Because no reliable chicken bones were found at archaeological sites during the Jomon period (16,000–2,900 years ago), it is considered that domestic chickens were brought to the Japanese Archipelago during the Yayoi period (10th BCE–3rd CE) (Nishimoto 1993, Eda 2018). To date, the earliest evidence of domestic chickens was found at the Karako-Kagi site during the early middle Yayoi period (4th–3rd BCE) (Eda et al. 2023). Through research focusing on sexual dimorphism, we showed that most chickens in the Yayoi period were male and that breeding of these chickens may not have been possible in most regions of the Japanese Archipelago (Eda 2016, 2018). As an exception, immature chicken and hen bones were found at the Karako-Kagi site, Nara, suggesting successive breeding in the village (Eda et al. 2023, Eda et al. 2025). Because Karako-Kagi was one of the largest villages in the Yayoi culture (Fujita 2019), the generality of successive chicken breeding was not sufficiently clear, and further studies are required (Eda et al. 2023, Eda et al. 2025).

In the 2011 research area of the Karakami site in Iki City, Nagasaki Prefecture, 18 Phasianidae bones were identified in a large ditch estimated to have been buried during the late Yayoi period (1st–2nd CE) (Eda 2014, Tanaka and Matsumi 2014). Although the tarsometatarsus and tibiotarsus were found to belong to chickens based on their morphological characteristics, determining whether the other bones, including the four immature bones, were derived from chickens was left for future

research (Eda 2014). The Karakami site is located on Iki Island, between the Korean Peninsula and Kyushu Island, approximately 120 km from the Korean Peninsula and 20 km from Kyushu Island (Fig. 1). During the Yayoi period, Iki Island was a trading hub connecting the Korean Peninsula and Japanese Archipelago (Miyamoto 2013, Tanaka and Matsumi 2014). Several Yayoi chicken bones have been found at the Karakami and Harunotsuji sites (Kuroda 1959, Nayauchi and Matui 2009, Eda 2014), suggesting that chickens were brought to the Japanese Archipelago from the Korean Peninsula through Iki Island (Eda 2018). Considering the location of Iki Island, it is likely that successive breeding of chickens was conducted at the Karakami site, which was rarely performed in the Japanese Archipelago, except at the Karako-Kagi site during the early middle Yayoi period.

Zooarchaeological remains have been identified using matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (ToF) mass spectrometry (MS) of collagen peptides. This technique is referred to as zooarchaeology by mass spectrometry (ZooMS) (Buckley et al. 2010), and has been developed rapidly in recent years, primarily in Europe and North America (Hendy et al. 2018, Richter et al. 2022, Smith et al. 2024). The advantages of ZooMS over ancient DNA analysis include high success rate, low risk of contamination, small sample size required for analysis, and low cost (Buckley et al. 2010). However, the risk of contamination has been widely recognised, and methods to reduce this risk have been proposed (Hendy et al. 2018). Although fewer examples exist of ZooMS applied to birds than to other taxa (Codlin et al. 2022, Richter et al. 2022), we established a method for distinguishing between the bones of Japanese wild pheasants and those of chickens (Eda et al. 2020).

To obtain further insights into the use of chickens in Yayoi culture, particularly in terms of their successive breeding, we conducted ZooMS species identification of zooarchaeological Phasianidae and immature bird bones found at the Karakami site. Mature chicken bones were sexed based on sexual dimorphism to determine if they were from cocks or hens. In the ZooMS experiments,

we repeatedly observed peaks previously considered to be specific to chickens and wild Phasianidae from the Japanese Archipelago appearing simultaneously in the same samples. Therefore, liquid chromatography (LC)-MS/MS was used to determine the amino acid sequences and post-translational modifications of some samples to investigate the origin of these peaks. Based on these results, we discuss the identification of bird bones using ZooMS and the archaeological significance of chickens found at the Karakami site.

Materials

1. Studied site

The Karakami site is a Yayoi period site located on Iki Island. Seven areas have been excavated since 1926 (Miyamoto 2013, Tanaka and Matsumi 2014). The Karakami site is located on a small hill, approximately 80 m above sea level, on the northwestern part of the island. A large ditch surrounding the top of the hill, a single burial jar tomb, the remains of a large circular pit dwelling, a pottery storage area where pottery and ritual vessels were discarded, an aboveground furnace for processing iron products, and a kiln for producing red ochre were confirmed. Besides the Yayoi pottery used at the time, numerous materials indicating exchanges with East Asian countries have been discovered in large ditches, such as tile-like pottery brought from mainland China and the Korean Peninsula and Korean-style plain pottery (Miyamoto 2013, Tanaka and Matsumi 2014). Large amounts of shells and animal bones have also been discovered; chicken bones were discovered in surveys conducted in 1952, 2008, and 2011 (Kuroda 1959, Nayauchi and Matui 2009, Eda 2014).

2. Studied samples

We analysed 11 bird bones found from the 2011 research area of the Karakami site. Eda (2014) reported six as mature Phasianidae, three as immature Phasianidae and two as immature

unidentified birds (Table 1, Fig. 2). Two mature Phasianidae bones (tarsometatarsus and tibiotarsus) were identified as chickens based on the criteria of Eda and Inoué (2011). These bones were excavated from three layers of covering soil in a large ditch and dated to the late Yayoi period based on the accompanying pottery (Tanaka and Matsumi 2014).

Methods

1. Chicken bone discrimination by mass spectrometry

Collagen peptides were isolated and digested, as described by Eda et al. (2020), in an ancient biomolecular laboratory at the Hokkaido University Museum. Briefly, the bone powder was demineralised with hydrochloric acid, the acid-insoluble pellet was gelatinised by heating in ammonium bicarbonate, and the gelatinised sample was digested using sequencing-grade trypsin. After digestion, the supernatant was acidified with trifluoroacetic acid and desalinated using C18 ZipTip.

1) MALDI ToF-MS analysis

The fractions of each collagen digest were analysed using an UltraflexTreme Mass Spectrometer (Bruker, Billerica, MA, USA) at the Central Institute of Isotope Science, Hokkaido University. The sample solution was spotted onto a target plate and mixed with α -cyano-4-hydroxycinnamic acid matrix solution. The obtained data were averaged using mMass 5.5.0 (Niedermeyer and Strohm 2012), and a peak list was created with a signal-to-noise (S/N) ratio of 6.0. Chicken and Japanese wild pheasant discrimination was conducted as described by Eda et al. (2020): chickens and red junglefowls had peaks of 1604.8 m/z (1+; GDPGPVGPVGPAGAFGPR) and 1620.8 m/z (1+; GDP*GPVGPVGPAGAFGPR, in which * shows post-transcript oxidation), whereas green (*Phasianus versicolor*) and copper pheasants (*Syrnaticus soemmerringii*) showed a peak of 1578.8 m/z (1+; GDPGPVGAVGPAGAFGPR). In addition, to confirm that the bones were

derived from Galliformes, including Phasianidae, the presence or absence of two peaks (2539.3 and 3192.5/3193.5 m/z) detected only in the Galliformes, including Phasianidae, were determined, as reported by Codlin et al. (2022). Peaks within $\pm 0.2 m/z$ were considered the same.

2) LC-MS/MS analysis

In the ToF-MS analysis, peaks (1578.8 and 1604.8 m/z) that were specific to chicken and Japanese wild pheasants, respectively, were commonly observed in all samples (see details in the Results section). To investigate the origin of these peaks, LC-MS/MS analysis of KI-01 and KM-02 was conducted in an open facility at Hokkaido University. Peptides were analysed using an Orbitrap Elite Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Scientific) in conjunction with a Thermo Easy-nLC (Thermo Scientific), as described by Komatsu et al. (2019), with the following modifications: a 110-min gradient was performed as 0% buffer B (0.1% formic acid/acetonitrile) to 5% B for 1 min, 5% B to 35% B for 80 min, 35% B to 90% B for 2 min, and finally 90% B for 27 min; a spray voltage of 2.0 kV and a transfer capillary temperature of 200 °C were used; an MS survey scan covered the m/z range of 300–1600; and MS/MS spectra were acquired by subjecting the top 15 ions to collision-induced dissociation. Raw data were processed using the PEAKS Studio 10.6 software (Bioinformatics Solutions, Canada). The search parameters were set as follows: trypsin (specific), oxidation of methionine and proline, and deamidation of asparagine and glutamine as variable modifications. Database: collagen type I (COL1) $\alpha 1$ sequences of 115 avian species, including five Phasianidae species [*G. gallus*, *Phasianus colchicus* (common pheasant), *Coturnix japonica* (Japanese quail), *Excalfactoria chinensis* (Blue-breasted quail) and *Tympanuchus pallidicinctus* (lesser Prairie Chicken)], and $\alpha 2$ sequences of 172 avian species, including nine Phasianidae species [*G. gallus*, *P. colchicus*, *C. japonica*, *E. chinensis*, *T. pallidicinctus*, *Lagopus muta* (rock ptarmigan), *L. leucura* (white-tailed ptarmigan), *Centrocercus urophasianus* (greater sage-

grouse), and *Meleagris gallopavo* (wild turkey)] (Supplementary Material 1). The nomenclature of the collagen peptide markers has been described by Brown et al. (2021). The results of LC-MS/MS analyses have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2024) partner repository with the dataset identifier PXD064367 and 10.6019/PXD064367.

2. Morphological sex identification

Sexing based on sexual dimorphism was performed on the newly identified chicken specimens using MS. The greatest length (GL) and width of the proximal and distal epiphyses (Did in the ulna and Bp and Bd in other elements) were measured according to von den Driesch (1976). At the proximal end of the tibiotarsus, the width between the medial edge of the medial articular surface and the lateral edge of the lateral articular surface (BP) was measured, as described by (Eda 2022). From the measured values, the log-difference scale was calculated according to Simpson's method using the following formula (Simpson 1941):

$$\text{Log difference scale} = \log_{10}X - \log_{10}Y$$

where X is the measured value of the analysed bone, and Y is the measured value of the reference specimen. The measurement value for the reference specimen was the average of the male and female mean values of captive red junglefowl, as shown in Eda (2022). For samples with multiple measurements, the log difference scale was calculated for each mensural position and the average was used as the log difference scale of the sample. Assuming that sexual size dimorphism in Yayoi chickens is similar to that in captive red junglefowls (Eda 2016), samples with positive log difference scales were identified as male, and samples with negative log difference scales were identified as female.

Results

1. Chicken bone discrimination by mass spectrometry

1) MALDI ToF-MS analysis

Peaks of 1578.8 and 1604.8 m/z were observed in all samples, whereas the peak at 1620.8 m/z was found in six mature Phasianidae bones (KM-01 to KM-06) (Table 2; Fig. 3). The relative peak intensities of 1578.8 and 1604.8 m/z varied greatly among samples. In the bones of the six mature Phasianidae, the peak intensities of 1578.8 m/z ranged 0.7–3.6% and those of 1604.8 m/z ranged 48.2–100.0%. In contrast, in bones from immature birds (KI-01 to KI-05), the peak intensities of 1578.8 m/z ranged 0.5–62.9% and those of 1604.8 m/z ranged 2.0–14.0%. The peak intensities at 1620.8 m/z , which were found only in the mature bird bones, ranged 8.9–48.8%. In addition, all six mature bones showed 2539.3 and 3192.5/3193.5 m/z , whereas these two peaks were not observed in samples of any of the immature bones.

2) LC-MS/MS analysis

The amino acid sequence of 1577.8 m/z (uncharged mass) of KI-01 and 1603.8/1619.8 m/z (uncharged mass) of KM-02 was determined as GDPGPAGVVGPAGAFGPR and GDPGPVGPVGPAGAFGPR, respectively (Figs. 4a and b). Both sequences corresponded to COL1a2 889–906. The latter is identical to the sequence of *G. gallus*, whereas the former is identical to that of crested ibis (*Nipponia nippon*), California condor (*Gymnogyps californianus*), brown mesite (*Mesitornis unicolor*), rhinoceros hornbill (*Buceros rhinoceros*), and red-backed fairywren (*Malurus melanocephalus*) but different from those of *Phasianus colchicus* and *Meleagris gallopavo* (GDPGPVGAVGPAGAFGPR). The sequence of 1603.8 m/z (uncharged mass) detected in KI-01 was determined to be GEIGPAGN**VGPTGPAGPR (** shows post-transcript deamidation) (Fig. 4c). This sequence corresponded to COL1a2 175–192 in all Phasianidae birds and most of the birds in the database and differed from the sequence of COL1a2 889–906. The same sequence was confirmed in

KM-02. The amino acid sequence of the peak at 1577.8 m/z (uncharged mass) detected in KM-02 was determined to be GLPGESGAVGPAGPIGSR (Fig. 4d). This sequence is identical to COL1a2 502–519 in all Phasianidae species except *Meleagris gallopavo* and some of the birds in the database and could not be considered COL1a2 889–906. This amino acid sequence was not detected in KI-01. The highest coverage (%) of COL1a1 was obtained in *Gallus gallus* for KM-02 (43%) and *Grus americana* for KI-01 (49%), whereas ones of COL1a2 was observed in *Gallus gallus* for KM-02 (48%) and *Nipponia nippon* for KI-01 (48%)(10.6019/PXD064367).

2. Morphological sex identification

The log difference scale was calculated for the three specimens newly identified as chicken, and all values were positive, indicating that they were male (Table 1).

Discussion

1. Chicken bone discrimination by mass spectrometry

Because of the MALDI ToF-MS analysis, the peaks at 1578.8 and 1604.8 m/z were observed in all samples, although their intensity varied. Eda et al. (2020) revealed that both peaks were derived from residues 978–995 of COL1a2 [COL1a2 889–906 according to the collagen naming criteria by Brown et al. (2021)]. It was argued that as the former was found in Japanese wild pheasants (green and copper pheasants) and the latter in chickens, these peaks were useful for distinguishing chicken bones from medium-sized Phasianidae bones excavated from archaeological sites in the Japanese Archipelago. Eda et al. (2023) used this method to identify the immature bones of chickens found at the Karako-Kagi site. In Eda et al. (2020) and (2023), peaks at 1578.8 and 1604.8 m/z were not detected in the same sample. We therefore considered whether the detection of peaks specific to both

chicken and Japanese wild pheasants in the current study could be due to contamination, differences in sample storage conditions, or differences in the settings of the equipment and software used.

Despite several re-experiments, peaks at 1578.8 and 1604.8 m/z were commonly detected, making it unlikely that contamination was the cause. Compared to the samples from the Hiroshima Castle sites from the Edo period (1603–1868 CE) targeted by Eda et al. (2020), these samples were >1,500 years old, but approximately 300 years younger than the samples from the Karako-Kagi site. Visually, the samples did not appear to have considerably worse preservation conditions than those obtained from the Karako-Kagi site. For the analytical equipment, Eda et al. (2020) used Ultraflex III, whereas Eda et al. (2023) used UltraflexTreme. In addition, Eda et al. (2023) used Flex analysis, which was set to select the top 100 peaks as the analysis software, whereas this study analysed the raw data using mMass and selected all peaks with an S/N ratio of 6.0 or higher. Using UltraflexTreme, which has higher detection sensitivity, and mMass, which picks up minor peaks, it is possible that peaks that were not detected in previous studies were found.

This assumption was supported by the LC-MS/MS analysis results. The 1603.8 m/z (uncharged mass) peak detected in KI-01 differed from the chicken COL1a2 889-906 sequence and was a single deamidation in the amino acid sequence corresponding to pheasant and chicken COL1a2 175–192. Similarly, the amino acid sequence of the 1577.8 m/z (uncharged mass) peak detected in KM-02 was not the COL1a2 889-906 sequence but was equivalent to COL1a2 502-519. These results also support the fact that the two MALDI peaks of 1578.8 and 1604.8 m/z were repeatedly detected in all samples, which was not because of contamination.

In KM-02, the amino acid sequences of COL1a2 889-906 and COL1a2 175–192 were detected as a peak at 1603.8 m/z (uncharged mass). The amino acid sequences of COL1a2 175–192 were identical in chickens, as well as *Phasianus colchicus*, *Tympanuchus pallidicinctus*, *Lagopus muta*, *L. leucura*, *Centrocercus urophasianus*, *Coturnix japonica*, and *Meleagris gallopavo* and

several other non-Phasianidae birds. Codlin et al. (2022) also reported the same sequences in the Odontophoridae (*Colinus virginianus*), Laridae (*Leucophaeus attricilla*), Corvidae (*Corvus corax*), and Anatidae. In MALDI-ToF-MS analysis of collagen peptides from degenerated archaeological bones, the 1604.8 m/z peak can be widely detected in various bird species, including Phasianidae, due to deamidation.

The peak at 1620.8 m/z , which is considered a useful MALDI marker for chickens, was found in six mature Phasianidae bones (KM-01 to KM-06). Among these, two bones (KM-04 and KM-06) were identified as chicken rather than Japanese wild pheasants, because of the line-shaped posterior ligament of the tibiofibular joint for the former and the absence of medial planter crest for the latter (Eda 2014). In addition, according to Hsu and Eda (2022), the ulna (KM-01) could also be identified as chicken, as the distal end of the biceps ulnaris tubercle was distal to the distal end of the radial articular surface and was located in the extension of the intermuscular line. Furthermore, the six mature Phasianidae samples contained peaks at 2539.3 and 3192.5/3193.5 m/z , which were only found in Galliformes among the 23 samples analysed by Codlin et al. (2022), supporting the origin of these bones in the Galliformes. Morphological identification and identification using MALDI markers were consistent, thus confirming the validity of this method. The peak at 1604.8 m/z was also detected when the collagen peptide COL1a2 502–519 of birds, including Phasianidae, was deamidated; therefore, it is considered that 1620.8 m/z is a better indicator for identifying chickens in materials excavated from archaeological sites.

2. Immature bird bone identification by mass spectrometry

In five immature bones, including three identified as Phasianidae, peaks at 1578.8 and 1604.8 m/z were detected by MALDI-ToF-MS, but the peak at 1620.8 m/z was not detected. LC-MS/MS analysis of KI-01 showed a peak at 1577.8 m/z (uncharged mass) was GDPGPAGVVGPAGAFGPR,

which does not correspond to COL1a2 889-906 of Japanese indigenous pheasants (*Phasianus colchicus* and *Syrnaticus soemmerringii*) but corresponds to ones of *Nipponia nippon* (Pelecaniformes, Threskiornithidae), *Gymnogyps californianus* (Accipitriformes, Cathartidae), *Mesitornis unicolor* (Gruiformes, Mesitornithidae), *Buceros rhinoceros* (Bucerotiformes, Bucerotidae), and *Malurus melanocephalus* (Passeriformes, Maluridae). The sequence seemingly occurs sporadically in avian COL1a2 889-906, indicating that it is not a good stand-alone identification marker for specific taxa. As revealed by LC-MS/MS analysis of KI-01, the ToF-MS peak at 1604.8 *m/z* may be detected in various taxa of birds when the COL1a2 502-519 is deamidated. Furthermore, none of the three peaks detected in Galliformes by Codlin et al. (2022) were observed in the bones of the five immature birds. These results suggest that none of the five immature bird bones belong to either chicken or Galliformes. The morphological identification results of Eda (2014), who identified three immature bones as Phasianidae, were considered incorrect. This indicates that the identification of immature bird bones based on morphological characteristics should be performed with caution.

Compared to the MALDI key peaks provided by Codlin et al. (2022), KI-01, KI-02, KI-03, and KI-05 showed peaks at 3106.4/3107.4 and 3122.4/3123.4 *m/z*, which were only found in the Podicipedidae, including *Podilymbus podiceps*. Although the amino acid sequence of these peaks which were identical sequence with/without post-transcriptional modification(s) was not confirmed in the LC-MS/MS analysis of KI-01, the amino acid sequence (GPPGASGPPGPPGFQGVPGEPGPGQTGPHGPR; COL1a2 10-42) was also identified in six species, including *Nipponia nippon*, in the database. So far, *Nipponia nippon* is only species which shares identical COL1a2 10-42 and COL1a2 889-906 sequences with KI-01. The highest coverage (48 %) of COL1a2 was observed in *Nipponia nippon* for KI-01 and ten immature Threskiornithidae bones were reported in the same assemblage (Eda 2014). These facts suggest that KI-01 and others

were from Threskiornithidae although the COL1a2 database we compared against was still limited. In contrast, KI-04 showed all four MALDI peaks (2136.0, 2525.3, 3111.4, and 3127.4 m/z), which were only observed in *Corvus corax*. It is likely that KI-04 was from passerines, including Corvidae. It is expected that the establishment of key MALDI peaks for bird bone identification using ZooMS will enable further narrowing of the taxonomic groups.

3. Chickens used at the Karakami site and their archaeological implications

Using ZooMS, two chicken identifications were confirmed, and four specimens previously identified only to Phasianidae were identified as chicken. Furthermore, sexing indicated that all specimens were male, and no female bones were identified. In contrast, analysis of immature bones showed no chicken bones. Previous investigations at the Karakami site revealed three chicken bones, including two tarsometatarsi with spurs, which were assumed to originate from males (Nayauchi and Matui 2009, Eda 2014, 2016). This study confirmed that the chickens used at the Karakami site were predominantly male, supporting the hypothesis of Eda (2016) that chickens are rarely raised for successive generations in Yayoi cultural villages, including Karakami.

Chickens are considered to have been brought to the Japanese Archipelago from mainland China through the Korean Peninsula (Nishimoto 1993, Eda 2018). Iki Island, where the Karakami site was located, was described as “Iki-koku” in the ancient Chinese article of “Records of the Three Kingdoms vol. 30, Treatise on the Wuhuan, Xianbei, and Dongyi,” written between 280–297 BCE, was a trading hub connecting the Korean Peninsula and the Japanese Archipelago (Miyamoto 2013, Tanaka and Matsumi 2014). The oldest chicken bones found in the Japanese Archipelago date from the 4th – 3rd century BCE and were discovered at the Karako-Kagi site (Eda et al. 2023). The chickens were likely introduced to Iki Island at approximately this time, or even earlier. Further studies must

determine when chickens were introduced to Iki Island and whether they included both males and females during their earliest introduction to Iki Island and the Japanese Archipelago.

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Data availability statement

The data that support the findings of this study are available in the text, Supplementary Materials, or ProteomeXchange Consortium with the dataset identifiers PXD064367 and 10.6019/PXD064367.

Competing interest

The authors declare no conflict of interests.

Captions

Table 1 Bird bone samples from the Karakami site. No. in Fig. 2, description, layer, element, morphological identification (ID) in Eda (2014), ZooMS ID, log difference scale, and sex ID are shown.

Table 2. MALDI markers evaluated in this study. Relative intensities (Rel. Int.) and signal/noise (S/N) ratios are shown.

Fig. 1. Location of Iki Island and the Karakami site.

Fig. 2. Bird bones from Karakami site.

1–6. immature bird, 7–11. mature chicken. Note that 7–11 were identified in chickens based on mass spectrometry analysis, whereas 9 and 11 were also reported in chickens by Eda (2014) based on morphological characteristics.

Fig. 3. Time-of-flight mass spectrometry profiles of two samples from the Karakami site (a: KM-02, b: KI-01). KM-02 was identified as chicken, whereas KI-01 was identified as non-chicken.

Fig. 4. Liquid chromatography mass spectrometry (MS)/MS profiles of four amino acid sequences from the Karakami site (a: 1577.8 m/z of KI-01, b: 1619.8 m/z of KM-02, c: 1603.8 m/z of KI-01, d: 1577.8 m/z of KM-02).

Baker, C. M. 1968. Molecular genetics of avian proteins. IX. Interspecific and intraspecific variation of egg white proteins of the genus *Gallus*. *Genetics* **58**:211-226.

Brown, S., K. Douka, M. J. Collins, and K. K. Richter. 2021. On the standardization of ZooMS nomenclature. *Journal of Proteomics* **235**:104041.

Codlin, M. C., K. Douka, and K. K. Richter. 2022. An application of zooms to identify archaeological avian fauna from Teotihuacan, Mexico. *Journal of Archaeological Science* **148**:105692.

Darwin, C. R. 1868. *The variation of animals and plants under domestication*. John Murray, London.

Eda, M. 2014. Karakami Iseki 2011 Nendo oyobi 2013 Nendo Shutsudono Chourui Itai nitsuite (Bird remains found at the Karakami site during the 2011 and 2013 excavations). Pages 181-189 in S. Tanaka, and Y. Matsumi, editor. *Amenotanagao-Jinja Iseki Karakami Iseki Niji* (Amenotanagao-Jinja Site and Karakami Site Second). Iki City Board of Education.

Eda, M. 2016. Chickens in the Yayoi period: in the context of osteological microevolution under domestication. *Japanese Journal of Zooarchaeology* **33**:49-61.

Eda, M. 2018. Yayoijidi no Niwatori, Saiko (Reconsideration of chickens in Yayoi Period). *Archaeology Quarterly* **144**:43-46.

Eda, M. 2021. Origin of the domestic chicken from modern biological and zooarchaeological approaches. *Animal Frontiers* **11**:52-61.

Eda, M. 2022. The osteological microevolution of red junglefowl and domestic fowl under the domestication process. *Quaternary International* **626–627**:14-21.

Eda, M., H. Izumi, and S. Fujita. 2025. Karako-Kagi Iseki Shutudo Kijika Shiryouno Shitsuryoubunseki niyoru Doutei (Identification of Phasianidae bones from the Karako-Kagi site by mass spectrometry). *Annual Research Report of Archaeology in Tawaramoto Town* **29**:195-203.

Eda, M., H. Izumi, M. Yoneda, and S. Fujita. 2023. The earliest evidence of domestic chickens in the Japanese Archipelago. *Frontiers in Earth Science* **11**.

Eda, M., M. Morimoto, T. Mizuta, and T. Inoué. 2020. ZooMS for birds: Discrimination of Japanese archaeological chickens and indigenous pheasants using collagen peptide fingerprinting. *Journal of Archaeological Science: Reports* **34**:102635.

Fujita, S. 2019. Yamato Ouken Tanjo no Ishizue to natta Mura, Karako-Kagi Iseki (A village that laid the foundation for the birth of the Yamato sovereignty, Karako-Kagi site). Shinsen Sha, Tokyo.

Fumihito, A., T. Miyake, S. Sumi, M. Takada, S. Ohno, and N. Kondo. 1994. One subspecies of the red junglefowl (*Gallus gallus gallus*) suffices as the matriarchic ancestor of all domestic breeds. *Proceedings of the National Academy of Sciences of the United States of America* **91**:12505-12509.

Hendy, J., F. Welker, B. Demarchi, C. Speller, C. Warinner, and M. J. Collins. 2018. A guide to ancient protein studies. *Nature Ecology & Evolution* **2**:791-799.

Hsu, K.-h., and M. Eda. 2022. Morphological identification criteria for Phasianidae (chicken and indigenous fowls in Japan) bones and their application to investigate zooarchaeological remains from the Dejima Oranda Shōkan site. *Zoo-archaeology* **39**.

Komatsu, Y., Y. Wada, H. Izumi, T. Shimizu, Y. Takeda, T. Hira, and H. Hara. 2019. Casein materials show different digestion patterns using an in vitro gastrointestinal model and different release of glucagon-like peptide-1 by enteroendocrine GLUTag cells. *Food Chemistry* **277**:423-431.

Kuroda, N. 1959. On some bones from Iki I. and Yamaguchi Prefecture. *Bulletin of the Society of Japan* **21**:67-74.

- Miyamoto, K.** 2013. Karakami Site, Iki Island, Japan: 1977 and 2011 Excavations at the fifth to the seventh areas of Karakami site. Division of Archaeology, Faculty of Humanities, Kyushu University, Fukuoka.
- Nayauchi, T., and A. Matui.** 2009. Zooarchaeological remains found at the Karakami site during the 2007 and 2008 excavations. Pages 131-142 in K. Miyamoto, editor. Karakami Site, Iki Island, Japan: Excavation at the first area of Karakami site. Division of Archaeology, Faculty of Humanities, Kyushu University, Fukuoka.
- Niedermeyer, T. H. J., and M. Strohal.** 2012. mMass as a Software Tool for the Annotation of Cyclic Peptide Tandem Mass Spectra. *PLoS One* **7**:e44913.
- Nishimoto, T.** 1993. Fowls in the Yayoi. *Japanese Journal of Zooarchaeology* **1**:45-48.
- Perez-Riverol, Y., C. Bandla, Deepti J. Kundu, S. Kamatchinathan, J. Bai, S. Hewapathirana, Nithu S. John, A. Prakash, M. Walzer, S. Wang, and Juan A. Vizcaíno.** 2024. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Research* **53**:D543-D553.
- Peters, J., O. Lebrasseur, E. K. Irving-Pease, P. D. Paxinos, J. Best, R. Smallman, C. Callou, A. Gardeisen, S. Trixl, L. Frantz, N. Sykes, D. Q. Fuller, and G. Larson.** 2022. The biocultural origins and dispersal of domestic chickens. *Proceedings of the National Academy of Sciences* **119**:e2121978119.
- Richter, K. K., M. C. Codlin, M. Seabrook, and C. Warinner.** 2022. A primer for ZooMS applications in archaeology. *Proceedings of the National Academy of Sciences* **119**:e2109323119.
- Simpson, G. G.** 1941. Large Pleistocene felines of North America. *American Museum Novitates* **1136**:1-27.
- Smith, G., K. Ruebens, V. Sinet-Mathiot, and F. Welker.** 2024. Special Issue: Integrating ZooMS and Zooarchaeology: Methodological Challenges and Interpretive Potentials Towards a Deeper Integration of ZooMS and Zooarchaeology at Paleolithic Sites: Current Challenges and Future Directions. *PaleoAnthropology* **2024**:186-211.
- Tanaka, S., and Y. Matsumi.** 2014. Amenotanagao-Jinja Iseki and Karakami Iseki Niji (Amenotanagao-Jinja Site and Karakami Site Second). Iki City Board of Education.
- von den Driesch, A.** 1976. A guide to the measurements of animal bones from archaeological sites. *Peabody Museum Bulletin* **1**:1-136.
- Wang, M.-S., M. Thakur, M.-S. Peng, Y. Jiang, L. A. F. Frantz, M. Li, J.-J. Zhang, S. Wang, J. Peters, N. O. Otecko, C. Suwannapoom, X. Guo, Z.-Q. Zheng, A. Esmailizadeh, N. Y. Hirimuthugoda, H. Ashari, S. Suladari, M. S. A. Zein, S. Kusza, S. Sohrabi, H. Kharrati-Koopae, Q.-K. Shen, L. Zeng, M.-M. Yang, Y.-J. Wu, X.-Y. Yang, X.-M. Lu, X.-Z. Jia, Q.-H. Nie, S. J. Lamont, E. Lasagna, S. Ceccobelli, H. G. T. N. Gunwardana, T. M. Senasige, S.-H. Feng, J.-F. Si, H. Zhang, J.-Q. Jin, M.-L. Li, Y.-H. Liu, H.-M. Chen, C. Ma, S.-S. Dai, A. K. F. H. Bhuiyan, M. S. Khan, G. L. L. P. Silva, T.-T. Le, O. A. Mwai, M. N. M. Ibrahim, M. Supple, B. Shapiro, O. Hanotte, G. Zhang, G. Larson, J.-L. Han, D.-D. Wu, and Y.-P. Zhang.** 2020. 863 genomes reveal the origin and domestication of chicken. *Cell Research* **30**:693-701.
- West, B., and B. X. Zhou.** 1988. Did chickens go north? New evidence for domestication. *Journal of Archaeological Science* **15**:515-533.
- Xiang, H., J. Gao, B. Yu, H. Zhou, D. Cai, Y. Zhang, X. Chen, X. Wang, M. Hofreiter, and X. Zhao.** 2014. Early Holocene chicken domestication in northern China. *Proceedings of the National Academy of Sciences* **111**:17564-17569.

Table 1 Bird bone samples from the Karakami site. No. in Fig. 2, description, layer, element, morphological identification (ID) in Eda (2014), ZooMS ID, log difference scale, and sex identification results are shown.

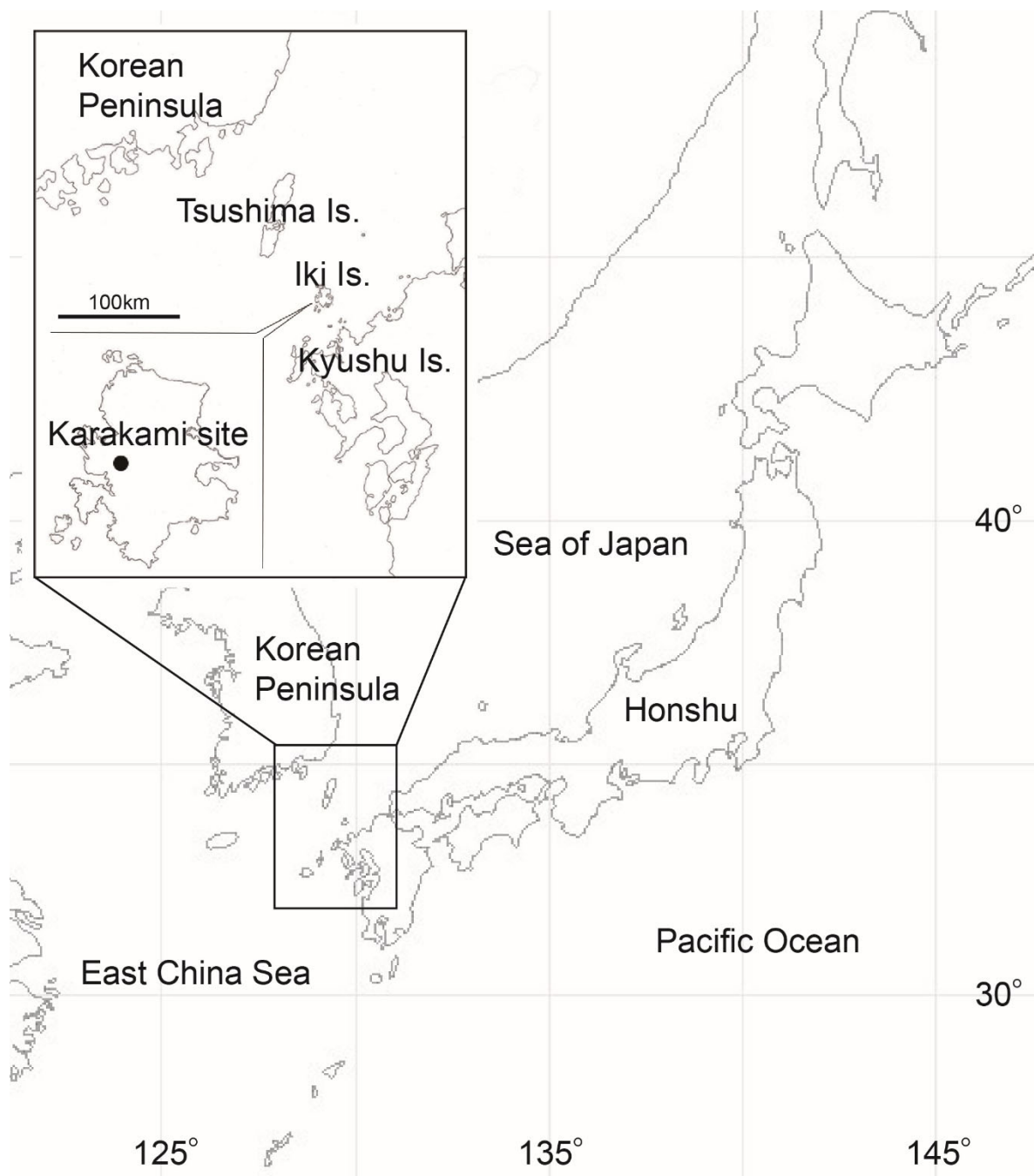
ID	No. in Fig. 2		Layer	Element	Morphological ID in		Log difference scale	Sex ID
		Description			Eda (2014)	ZooMS ID		
KI-01	1	IK023/98004	Shell and bone layer	Femur L	Phasianidae/immature	<i>Not</i> Phasianidae	Not determined	Not determined
KI-02	2	IK023/98003	Middle layer	Tibiotarsus R	Phasianidae/immature	<i>Not</i> Phasianidae	Not determined	Not determined
KI-03	3	1012	Lower layer	Tarsometatarsus L	Phasianidae/immature	<i>Not</i> Phasianidae	Not determined	Not determined
KI-04	4	856	7th trench	Humerus R	Aves/immature	<i>Not</i> Phasianidae	Not determined	Not determined
KI-05	5	1254	Shell and bone layer	Humerus R	Aves/immature	<i>Not</i> Phasianidae	Not determined	Not determined
KM-01	6	IK023/98002	Lower layer	Ulna L	Phasianidae	Chicken	0.1057	Male
KM-02	7	IK023/98006	Shell and bone layer	Femur L	Phasianidae	Chicken	0.0859	Male
KM-03	8	1148	Shell and bone layer	Femur R	Phasianidae	Chicken	Not determined	Not determined
KM-04	9	IK023/98007	Middle layer	Tibiotarsus L	Chicken	Chicken	0.0782	Male
KM-05	10	201	Middle layer	Tibiotarsus R	Phasianidae	Chicken	0.0525	Male
KM-06	11	IK023/98005	Middle layer	Tarsometatarsus L	Chicken	Chicken	0.0851	Male

Table 2 MALDI markers evaluated in this study. The relative intensities (Rel. Int.) and signal/noise (S/N) ratios are shown.

ID	1578.8 <i>m/z</i>		1604.8 <i>m/z</i>		1620.8 <i>m/z</i>		2539.3 <i>m/z</i>		3192.5 <i>m/z</i>		3193.5 <i>m/z</i>	
	Rel. Int. (%)	S/N ratio	Rel. Int. (%)	S/N ratio	Rel. Int. (%)	S/N ratio	Rel. Int. (%)	S/N ratio	Rel. Int. (%)	S/N ratio	Rel. Int. (%)	S/N ratio
KI-01	62.9	769.3	7.1	85.2	Not observed		Not observed		Not observed		Not observed	
KI-02	50.0	770.6	14.0	213.6	Not observed		Not observed		Not observed		Not observed	
KI-03	43.1	761.6	4.0	70.5	Not observed		Not observed		Not observed		Not observed	
KI-04	0.5	9.1	11.3	202.0	Not observed		Not observed		Not observed		Not observed	
KI-05	35.9	613.0	2.0	33.6	Not observed		Not observed		Not observed		Not observed	
KM-01	0.7	11.0	48.2	720.8	48.8	723.3	31.1	644.9	0.3	8.2	1.8	52.3
KM-02	1.3	17.0	59.6	776.7	37.9	490.6	17.1	353.9	Not observed		0.7	23.7
KM-03	0.8	14.5	58.5	1075.3	44.1	803.1	32.1	774.8	0.3	10.2	1.8	64.2
KM-04	3.6	51.6	94.4	1329.6	8.9	125.0	30.0	622.5	0.2	6.8	1.1	35.7
KM-05	1.9	24.7	100.0	1304.7	17.6	227.7	40.5	610.2	0.3	6.8	1.7	35.8
KM-06	0.9	10.4	53.1	603.4	40.1	450.3	20.3	323.7	0.3	8.3	2.0	50.1

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461 Fig. 1 Location of Iki Island and the Karakami site.

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Fig. 2 Bird bones from Karakami site.

1–5. immature bird, 6–11. mature chicken. Note that 6–11 were identified in chickens based on mass spectrometry analysis, whereas 9 and 11 were also reported in chickens by Eda (2014), based on morphological characteristics.

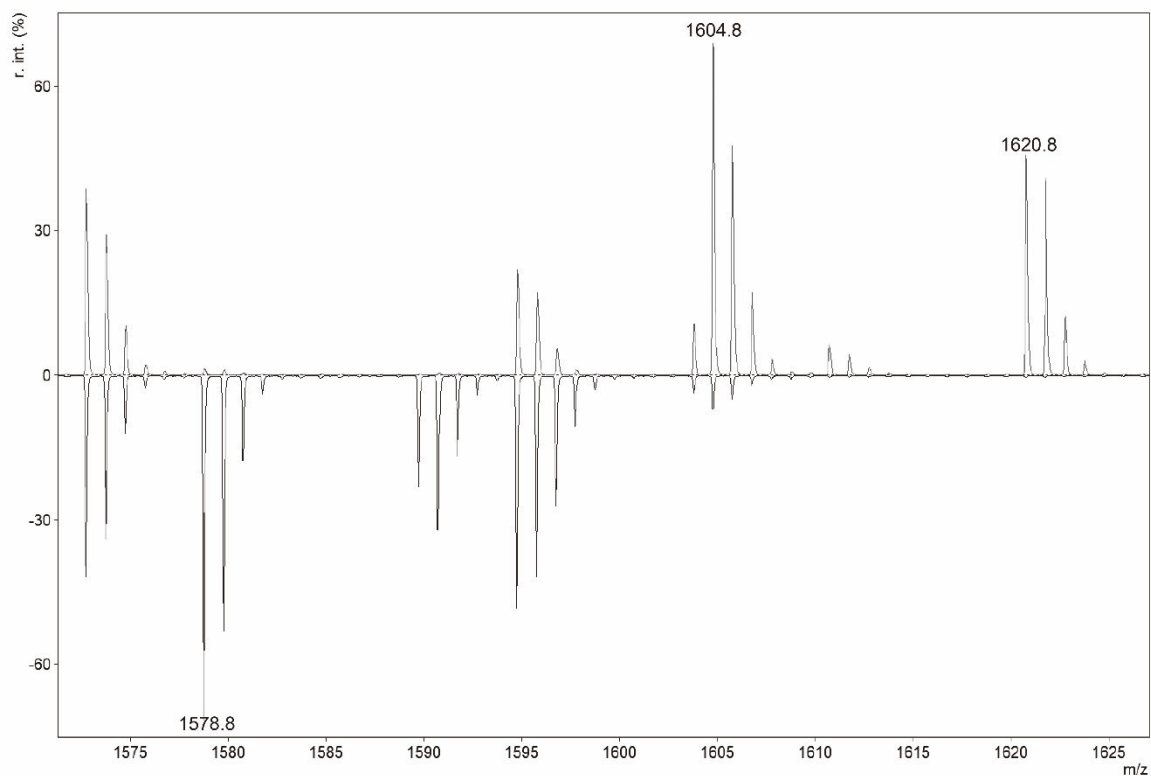


Fig. 3 Time-of-flight mass spectrometry profiles of two samples from the Karakami site (a: KM-02, b: KI-01). KM-02 was identified as chicken, whereas KI-01 was identified as non-chicken .

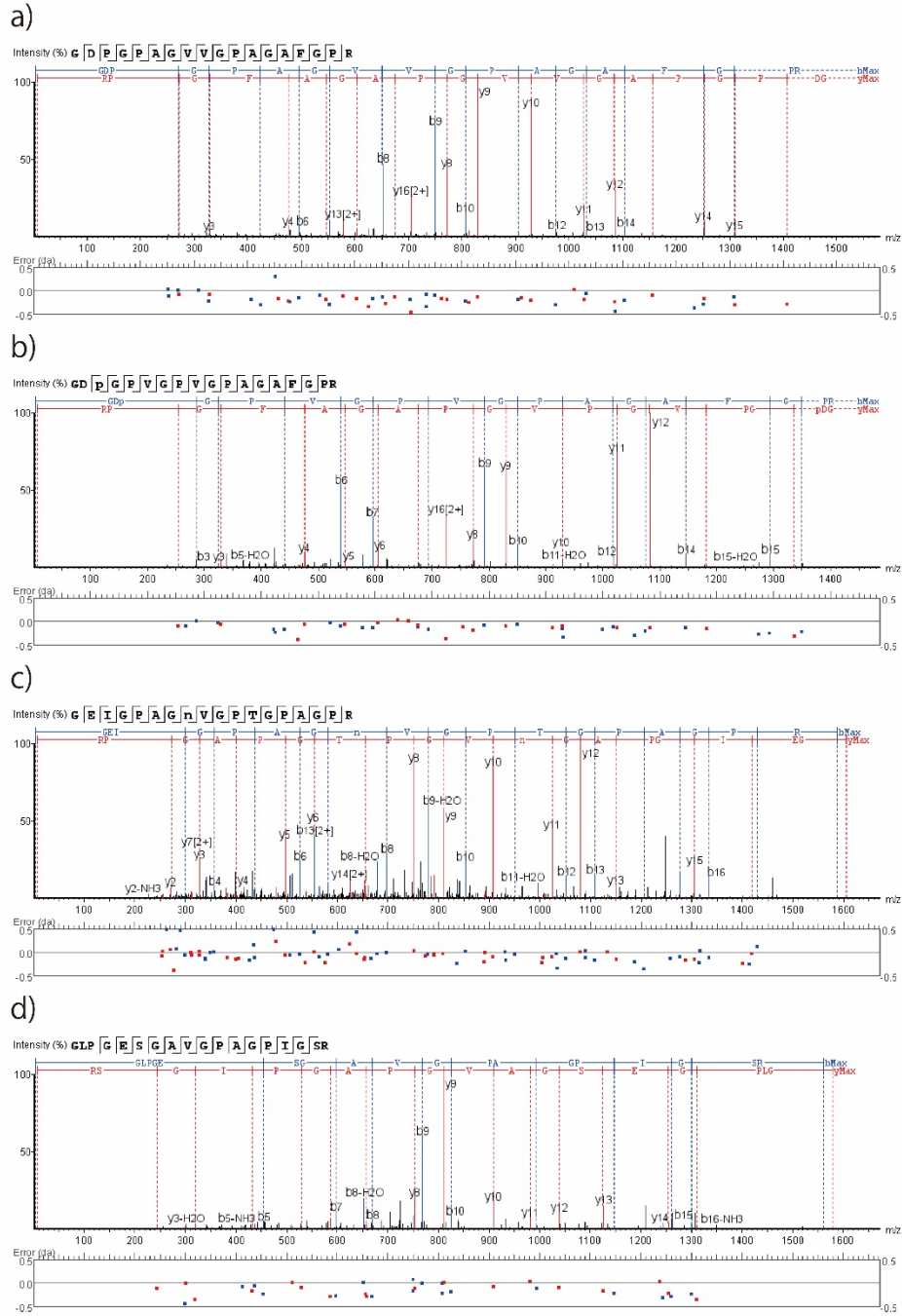


Fig. 4. Liquid chromatography-tandem mass spectrometry profiles of four amino acid sequences from the Karakami site (a: 1577.8 m/z of KI-01, b: 1619.8 m/z of KM-02, c: 1603.8 m/z of KI-01, d: 1577.8 m/z of KM-02).