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Fish 'n chips: ZooMS peptide mass fingerprinting in a 96 well plate format to identify fish bone fragments

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ABSTRACT

Fish are a large, highly diverse, and anthropologically important group of vertebrates. However, fish bones are underrepresented in the archaeological literature because they are less stable than those of other taxa and identification of bone to species is often difficult or impossible. We explore a new identification system, ZooMS (Zooarchaeology by Mass Spectrometry), which is based upon protein barcoding. As proteins can be cleaved enzymatically and analyzed by mass spectrometry in a repeatable way, protein barcoding is used widely in microbiological contexts for quick and inexpensive protein identification; mass spectra reflect the differences in protein sequence and can therefore be reproducibly linked to a particular protein or protein fragment. ZooMS uses peptide fingerprinting of bone collagen as a method for rapid identification of archaeological bone. This has involved the identification of masses related to peptides of known sequence. For mammals, sufficient sequence information is available for this approach but for groups, such the teleost fish, species are highly diverse and there are few available collagen sequences. Here we report a preliminary investigation into the identification of fish species by peptide mass fingerprinting that does not require sequence information. Collagen mass spectra are used to identify eight species of bony fishes (Osteichthyes) from four orders: Clupeiformes, Salmoniformes, Gadiformes, and Perciformes. The method is applied to both modern and archaeological fish remains and offers the capacity to identify traditionally unidentifiable fish fragments, thus increasing the Number of Identified Specimens (NISP) and providing invaluable information in specialized contexts.

1. Introduction

Fish are a highly diverse and ubiquitous group, accounting for more than half of the 48,000 species of extant vertebrates. Fish occupy nearly all major aquatic habitats, from ice-covered lakes and polar oceans to tropical swamps and all the environments within these extremes (Helfman et al., 1997). They have been important in human development: from food to a source of economic power (Wheeler and Jones, 1989). Archaeologists have been interested in the species distribution of fish from assemblages because they can provide insight into fishing strategies (Van Neer, 1986; O'Connor, 2000; Casteel, 1976; Barrett et al., 1999), economic and trade patterns (Barrett, 1997; Barrett et al., 2008, 1999 Grant, 1988), diet (Galik and Kunst, 2004), resource availability, social relationships (O'Day, 2004; Speller et al., 2005; Van Neer and Ervynck, 2004; Pigiere et al., 2004), species ranges, diversity, and mobility (Speller et al., 2005; O'Connell and Tunnicliffe, 2001; Zohar et al., 2008; Koksvik and Steinnes, 2005), and past climate change (Robinson et al., 2009; Chen, 2000).

Fish bones are small and not as highly mineralized as mammal bones and, as a consequence, are typically underrepresented in the record despite their importance in archaeology (Olson and Walther, 2007; Nicholson, 1996). Even when fish bones are recorded, species identification is often difficult or impossible. A blind study revealed inconsistencies in fish identification between five zooarchaeological experts due to a lack of adequate reference collections (Gobalet, 2001). This is not surprising as the high diversity of fish often requires extenstive reference collections (Betts et al., 2011) and the most abundant elements, such as ribs, branchiostegal rays and fin bones are often indistinguishable between species

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(Wheeler and Jones, 1989; Lepiksaar, 1983; O'Connor, 2000). Moreover, taphonomic and diagenetic changes remove distinguishing morphological features (Jones, 1986; Wheeler and Jones, 1989; Zohar et al., 2008). Depending upon the element and the amount of taphonomic and diagenetic changes, bones may be identifiable to different taxonomic levels: order, family, genus, or species. As a result, much of the recovered fish bone and scale material from archaeological sites remains unidentifiable and many identifiable specimens are only classified to family or genus, not species.

In order to address the problem of fish bone identification, researchers have been exploring molecular methods with DNA based methods given most attention. This approach is being used increasingly in modern fisheries (Teletchea, 2009), and this information may be used by archaeologists to devise assays for archaeological remains. Discrimination between closely related species such as salmon and trout (Yang et al., 2004; Speller et al., 2005) and European and Atlantic sturgeon (Desse-Berset, 2009) has been possible using ancient DNA. Although costs are falling rapidly, the methods are currently still expensive, DNA survival is variable with some studies reporting low success rates (Hlinka et al., 2002), and universal primer sites for fish can be difficult to find (Jordan et al., 2010).

We describe an alternative method that uses peptide mass fingerprinting (PMF), a method used widely for quick and cost effective protein identification (Gonnet et al., 2003) based upon the pattern of mass to charge (m/z) ratios (Steen and Mann, 2004). PMF has already been used for species identification of modern fish muscle tissue. Mazzeo et al. (2008) were able to successfully classify 25 different commercial fish, including nine species in the order Gadiformes, using soluble proteins extracted from muscle. However, such data can be difficult to interpret as a consequence of changing solubility arising from decay. Carrera et al. (2007), therefore, used species-specific peptides from purified protein (nucleoside diphosphate kinase b) for discrimination between groups within the family Merlucciidae. We propose ZooMS (Zooarchaeology by Mass Spectrometry) as a means of rapid identification of archaeological remains using PMF of resistant extracellular matrix proteins (Buckley et al., 2009). As in the approach adopted by Carrera et al. (2007), a single target protein is analyzed for increased consistency, in our case the most common protein in bone, Type I collagen. Collagen is an ideal material for the identification of archaeological fish bones due to its stability over archaeological timescales and variability between species.

Collagen is composed of three chains, each containing over 1000 amino acid residues, wound into a triple helix. While most vertebrates have two genes (COL1A1 and COL1A2) that code for the α1 and α2 chains, many fish have a third gene COL1A3 that codes for a genetically distinct chain that replaces one of the α1 chains in the triple helix and others are homotrimeric. Collagen is the most abundant protein in the animal proteome, comprising 95% of the proteins in modern bone. Collagen also apparently becomes selectively enriched during burial (Schmidt-Schultz and Schultz, 2004) with the loss of other proteins such as osteocalcin (Buckley et al., 2008a). As a consequence of the ease of purification, collagen is routinely extracted for both radiocarbon and stable isotope analysis (Pollard et al., 2007) and can survive with almost no damage for at least 600,000 years (Holmes et al., 2005).

Collagen barcoding of archaeological bone (ZooMS) has been used to identify several mammal species (Buckley et al., 2008b, 2009) including discrimination between sheep and goats (Buckley et al., 2010). For mammals, sufficient sequences are available to allow identification by comparison with the masses expected for known peptides. This is not the case with fish bones for which we have very little sequence information. Therefore, we describe a variant of the ZooMS technique that uses statistical approaches to make comparisons based entirely upon peptide masses.

The method is applied to both modern and archaeological fish bone fragments from European herring, *Clupea harengus* (Clupeiformes); salmon, *Salmo salar*, and trout, *Salmo trutta* (Salmoniformes); cod, *Gadus morhua*, whiting, *Merlangius merlangius*, haddock, *Merlanogrammus aeglefinus*, and hake, *Merluccius merluccius* (Gadiformes); bass, *Dicentrarchus labrax*, lesser sand eel, *Ammodytes tobianus*, and mackerel, *Scomber scombrus* (Perciformes). In addition archaeological specimens from thronback ray, *Raja clavata* (Rajiformes) and plaice, *Pleuronectes platessa* (Pleuronectiformes) were also examined. The modern selection represents a small group of commonly landed species, from a wide taxonomic range, but containing two clusters of closely related species, which are often difficult to discriminate (the gadiforms and the salmoniformes). The sand eel was selected as an abundant member from the base of the food chain which may be confused with juvenile elements of other species. The archaeological specimens represent the same species as the modern selection (cod, haddock, herring, and bass), related species (whiting and mackerel), and distantly related species (ray and plaice). In addition, archaeological samples also represent bones identified only to family (Gadidae) and morphologically unidentifiable bones.

Table 1
Table of fish species used in the experiment.

Order name	Family name	Species name	Common name	BioTex	Boiled	Baked	Raw	Archaeo
Rajiformes	Rajidae	*Raja clavata*	ray					1
Clupeiformes	Clupeidae	*Clupea harengus*[a]	herring		4	2	2	2
Salmoniformes	Salmonidae	*Salmo salar*[a]	salmon		2			
Salmoniformes	Salmonidae	*Salmo trutta*[a]	trout		2			
Gadiformes	Gadidae	*Gadus morhua*[a]	cod	3	4	2	2	8
Gadiformes	Gadidae	*Merlangius merlangus*	whiting					1
Gadiformes	Gadidae	*Melanogrammus aegelfinus*[a]	haddock		2	2	2	3
Gadiformes	Gadidae	unidentifiable						2
Gadiformes	Merlucciidae	*Merluccius merluccius*[a]	hake		2	2	2	
Perciformes	Moronidae	*Dicentrarchus labrax*[a]	bass		4	2	2	2
Perciformes	Ammodytidae	*Ammodytes tobianus*[a]	sand eel		2[b]	2		
Perciformes	Scombridae	*Scomber scombrus*	mackerel					1
Pleuronectiformes	Pleuronectidae	*Pleuronectes platessa*	plaice					2
unidentifiable								4

Modern fish were processed by BioTex, boiling, baking, or removing bones from unprocessed fish. Archaeological bones were morphologically identified. Some were only identifiable to broad category or not at all. All species were used in the test set.

[a] Indicates species which had samples in the training set.

[b] The sand eel were boiled whole.

2. Materials and methods

2.1. Sample selection and preparation

Fish were frozen and then defrosted prior to preparation. In all, 47 modern fish were prepared: Clupeiformes: 8 herring (*C. harengus*); Salmoniformes: 2 salmon (*S. salar*), 2 trout (*S. trutta*); Gadiformes: 11 cod (*G. morhua*), 6 haddock (*M. aeglefinus*), 6 hake (*M. merluccius*); Perciformes: 8 bass (*D. labrax*), 4 lesser sand eel (*A. tobianus*). Modern bones were processed with several different methods in order to see the effect of different reference collection preparation methods and possible anthropological treatment of the bones on the collagen spectra. Bones were processed with BioTex which is used for preparation of reference collections, boiled for a short time (under 30 min), boiled for a long time (2 h), baked, or soaked in cool water to remove tissue. Table 1 summarizes the number of each species prepared by boiling, baking, soaking, or digestion with Biotex. For the 21 fish prepared by boiling, the head and axial skeleton including dorsal and ventral fins were boiled for 5–20 min depending upon the size of the fish. One cod was boiled for 2 h. The 12 fish prepared by baking were wrapped in aluminum foil and baked whole at 180° C on a baking sheet for 5–40 min depending upon size. Three cod heads and axial skeletons were prepared by digestion with two cups of BioTex in an incubator for seven days at 37° C and then washed in cold water. For the 10 raw fish, bones and scales were removed and then soaked in cool water for 30 min to soften tissue. In all cases, bones were brushed with a soft toothbrush under a cool stream of water to remove remaining tissue and scales were rinsed gently in cool water. After processing, 67 bone and 5 scale samples were chosen for collagen analysis from the 47 modern fish specimens. In addition, 26 archaeological specimens from Great Yarmouth were chosen and morphologically identified (Table S1).

2.2. Collagen extraction

Small fish bone or scale samples were placed into a low protein binding polystyrene 96 well plate (Nunc). Several method variations were tested for collagen extraction. The first method (modified from Buckley et al. (2009)) was acid digestion where the bone matrix is demineralized with acid to extract the proteins. Acid digestion samples were incubated with 0.6 M hydrogen chloride for 1 h at room temperature. The bone chips were washed three times with distilled water and then incubated for 3 h at 65° C in 100 μl of 50 mM ammonium bicarbonate. The supernatant was transferred to a new plate, incubated with 1 μg trypsin overnight at 37° C, acidified to 0.1% trifluoroacetic acid (TFA), and purified on a 10 μl Millipore C-18 resin ZipTip (conditioning and eluting solutions 50% acetonitrile and 0.1% TFA, lower hydrophobicity buffer 0.1% TFA). The sample was transferred completely five times through the tip and eluted in 10 μl of solution (25 μl for large tips).

The second method does not use acid, but only uses warm ammonium bicarbonate to remove the protein from the bone matrix. An advantage over acid extraction is that in this method the bone samples are not damaged. Instead of the acid incubation, samples were incubated twice with 100 μl of 100 mM ammonium bicarbonate at 65° C for 1 h. The first supernatant was discarded, and following re-incubation, the second supernatant was transferred to a new plate, incubated with trypsin, acidified, and ZipTip eluted identically to the acid digestion. In addition, two other variables were tested in a small number of samples. Samples from the original plate were digested testing different trypsin amounts (1 or 2 μg) and different volume ZipTips (10 μl and 100 μl). In total, 155 digestions were run on the bone and scale fragments.

For mass spectrometry, 1–1.5 μl of digestion extract was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid) on a MALDI target plate and run along with calibration standards in reflectron mode using a calibrated Bruker Daltonics Ultraflex III TOF/TOF analyzer. With replication and re-spotting, a total of 330 individual spectra were collected (Table S2).

2.3. Spectra pre-processing and method of classification

Many spectra showed baseline shifts as a result of noisy matrix peaks from 500 to 800 *m*/*z* and few larger peptides. As this would affect normalization, necessary to account for differences in total ion counts, the spectra were first differentiated and the absolute values of the changes in intensity used to provide normalization constants. That is, the spectra were normalized so that the total integral of these absolute values was the same for each spectra. After normalization the spectra were binned to allow integrated peaks rather than individual *m*/*z* values to be used as variables. This was achieved using the method of Davis et al. (2007), in which the bin ends are determined from the peaks in a reference spectrum. The median value over all samples for each *m*/*z* value was calculated and the resulting spectrum smoothed before the minima were located and used to provide the bins. Fig. 1 shows the bins obtained.

Spectra were visually inspected and those of very poor quality, low signal to noise ratio and no discrete peaks, were discarded. Approximately two-thirds of the remaining modern spectra (the replicate analysis of 28 specimens) formed the training set, used to identify discriminatory *m*/*z* values. In total, the training set consisted of 66 spectra from eight fish species (10 herring, 7 salmon, 3 trout, 17 cod, 7 haddock, 7 hake, 11 bass, and 4 sand eel spectra). The test set consisted of spectra from the archaeological samples, eleven modern specimens not in the training set, and due to low sample size, spectra from two specimens (one trout and one salmon) that were used during training (although from different digestions). The final test set consisted of 164 spectra (see Supplementary Material).

Partial least squares linear discriminant analysis (PLS-LDA) was performed on the binned training data to identify discriminatory variables. For each of the eight species, a PLS-LDA model was used to

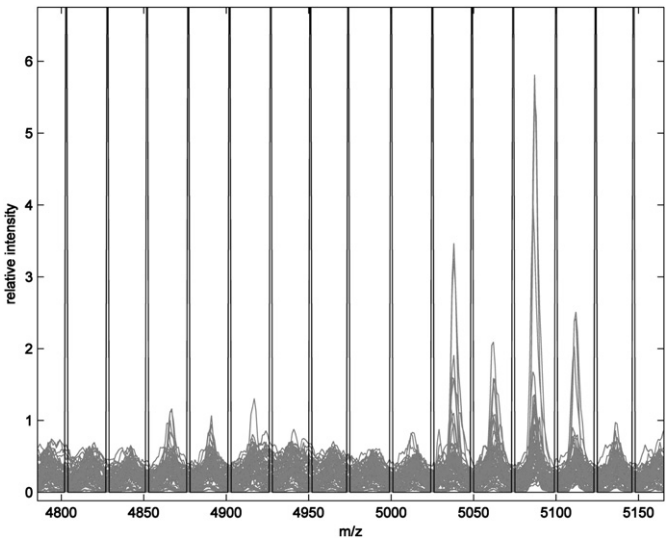

Fig. 1. A close-up showing the bins obtained using a smoothed reference spectrum. The black lines indicate the bin ends overlaid on spectra from the training set, shown in gray.

identify consistent differences between that species and the rest of the data (i.e. eight separate PLS-LDA models were calculated). In each case the highest variable importance in projection (VIP) scores were used to identify possible discriminatory markers for the species in question. Fig. 2 shows that some biomarkers are characteristic to particular species in the training set whereas others are shared by related species. In all, 89 potential biomarkers were identified.

The pattern of changes in peptide mass reflects the underlying evolution of the collagen molecule, the relatively slow rate of which means it is unlikely that there will be a single unique biomarker for each species. Therefore, in the absence of sequences for most peptides (Buckley et al., 2009), we attempted to discriminate between species using combinations of all the discriminatory markers. A pattern for each fish species was obtained as a series of 89 ones and zeros, where a value of one indicates a peak consistently recorded for that species in the training set spectra. For classification, a score for each fish species was obtained by multiplying the relevant binned intensities by these templates. Thus, the score for a particular species represents the sum of the intensities recorded for *m*/*z* values chosen as representative of that species. As the number of markers chosen varied between species, each score was divided by the number of markers for that species. To avoid large peaks dominating the analysis, the variables were first standardized (i.e. each binned value was divided by the median value for that bin) taken over all spectra in the training set. The scores obtained for each of the eight species were re-scaled so that they summed to unity to provide a probability of correct identification. Although this assumes that one of the eight species used during training is correct, similar scores for all classes could provide an indication of an entirely different species. Normalization of the spectra is not necessary for individual classifications as it is the relative intensities within a spectrum that determines the species. However, normalization allows scores for replicates to be combined and comparisons made between the scores obtained for different spectra. As replicate analyses were available for both training set and test set specimens, the scores were combined to identify a species for each set of replicates, as well as, for individual spectra. Different measures of confidence in the classification were also assessed, including the ratio of and difference between the probabilities obtained for the two highest scoring species (Figure S1).

3. Results

3.1. Fish preparation, type of element, and extraction method

The spectra obtained for cod prepared by BioTex were significantly different to those obtained for raw, boiled, baked, and archaeological cod, possibly because the enzymes in BioTex

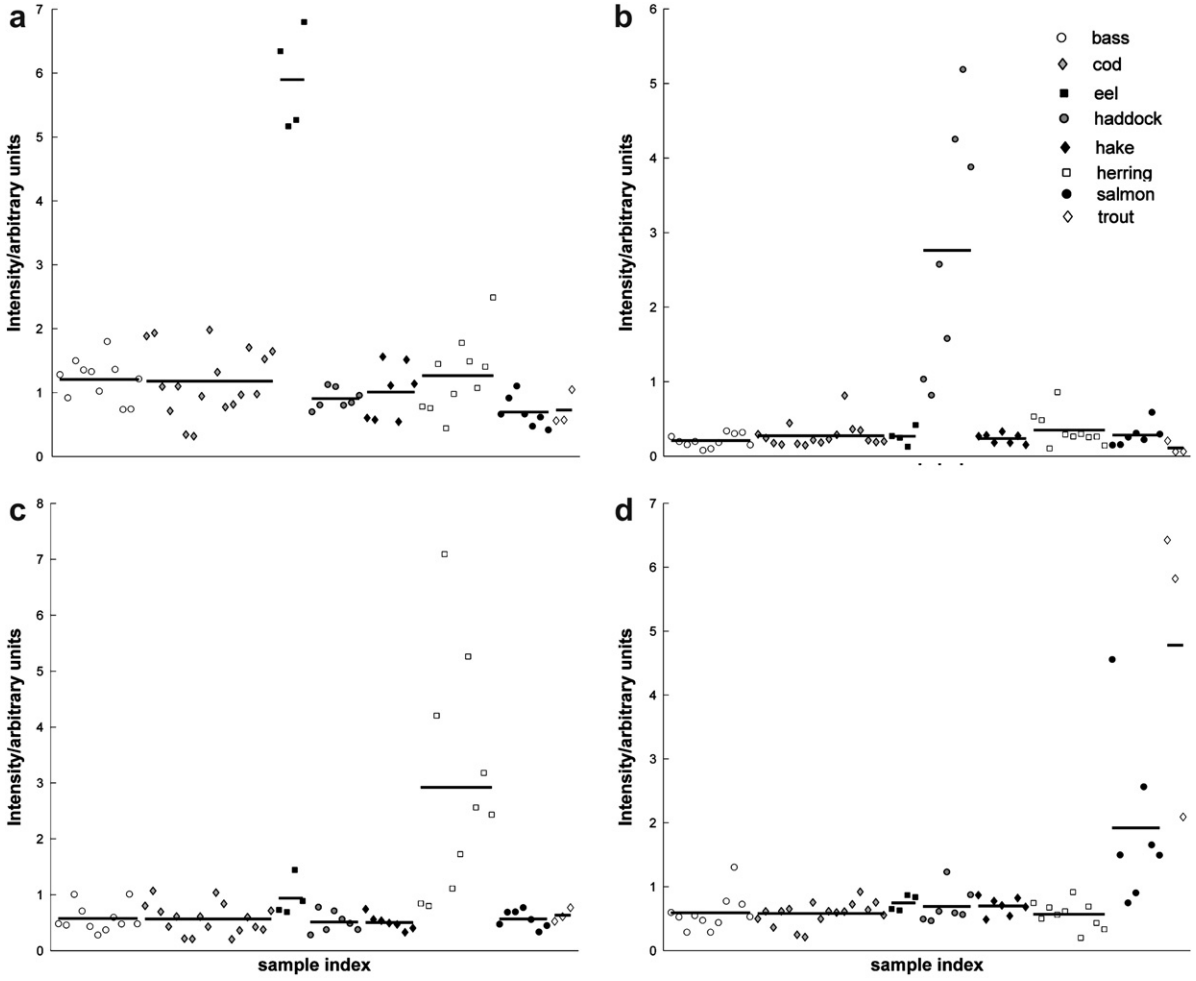

Fig. 2. The relative intensities for some *m*/*z* bins identified as biomarkers. The values for the *m*/*z* bins shown in (a), (b) and (c) appear to be characteristic of sand eel, haddock and herring respectively whereas that shown in (d) does not distinguish between the closely related species of salmon and trout. In each case, bass, haddock and salmon are represented by circles colored white, gray and black respectively. Diamonds represent cod, hake and trout, colored gray, black and white respectively. Sand eel and herring are represented by squares colored black and white respectively.

contaminated the samples. The bones boiled for 2 h yielded no collagen, whereas boiling times of less than 30 min yielded equivalent intensities to baked and raw bones. Other preparation methods and element choice did not show any consistent difference in over all spectra. Variation in the pattern of peaks recorded between species was observed. Principal Components Analysis (PCA) shows greater separation for some species than others. The scores plot of the first two principal components (Fig. 3) shows herring to be most distinct among the species in the training set. The similarity between trout and salmon; and cod, haddock, and hake is also obvious. Separation within these two groups and between other species is, however, possible using more dimensions. There are also differences between archaeological and modern samples of the same species with fewer peaks recorded for archaeological samples (Fig. 4). Not surprisingly, the samples towards the extremities of the PCA plot are those for which more species biomarkers were recorded.

Fewer high quality spectra (high signal to noise ratio and discrete peaks) were obtained from fish bones than from mammal bones treated in a similar fashion. In fact, 54% (178/330) of the spectra were considered to be poor quality (low signal to noise ratios or few discrete peaks) and 100 of these were excluded from further analysis (spectra with both low signal to noise ratios and few discrete peaks). Only 7% (24/330) of the spectra were of good quality, in contrast to around 80% obtained for archaeological and modern mammal specimens. The other 39% (128/330) of the spectra were of mediocre quality. We found no correlation in the quality of spectra with location on the plate, sample size or color, fish species, element, age of bone, or trypsin concentration. There was no consistent difference between the two methods: partial acid digestion plus heat or warm ammonium bicarbonate. Replicates of the same sample also resulted in variable quality spectra. It seems unlikely that this was a direct result of the extraction method and may indicate inconsistencies in peptide production, purification, or co-crystallization with the matrix.

3.2. Classification results

Classification of the 66 spectra in the training set shows internal consistency with only three misclassifications: one within Order (haddock classified as hake; Gadiformes), the other two misclassifying as unrelated species (one hake classified as eel and one cod classified as herring). Probability scores were considered to assess the possibility of providing a measure of confidence in the classification. The misclassified cod specimen was classed as herring with a probability of 0.138, where the second highest probability was 0.137 (hake). This very small difference in scores does suggest that the classification is ambiguous. The probabilities associated with the other two misclassified specimens were also relatively low at 0.153 and 0.219 where 0.125 would indicate that all species were equally likely. In both cases the correct species was given the second highest probability (0.144 and 0.139 respectively).

Various measures of confidence involving comparison of probability scores were considered. The simplest of these appear to be the most useful with little difference between two simple measures: the highest score divided by the second highest score (so that a value close to 1.0 indicates a low confidence level), and the difference between these two scores (so that a value close to zero indicates a low confidence level). With either of these measures, the misclassified cod specimen is assigned with the lowest confidence. However, although the other two misclassifications are given relatively low confidence levels by both measures, there are some correctly classified specimens in the training set identified with lower confidence. In fact, if the highest level of confidence with a misclassification were used as the threshold for correct identification of species, the classification rate for the training set

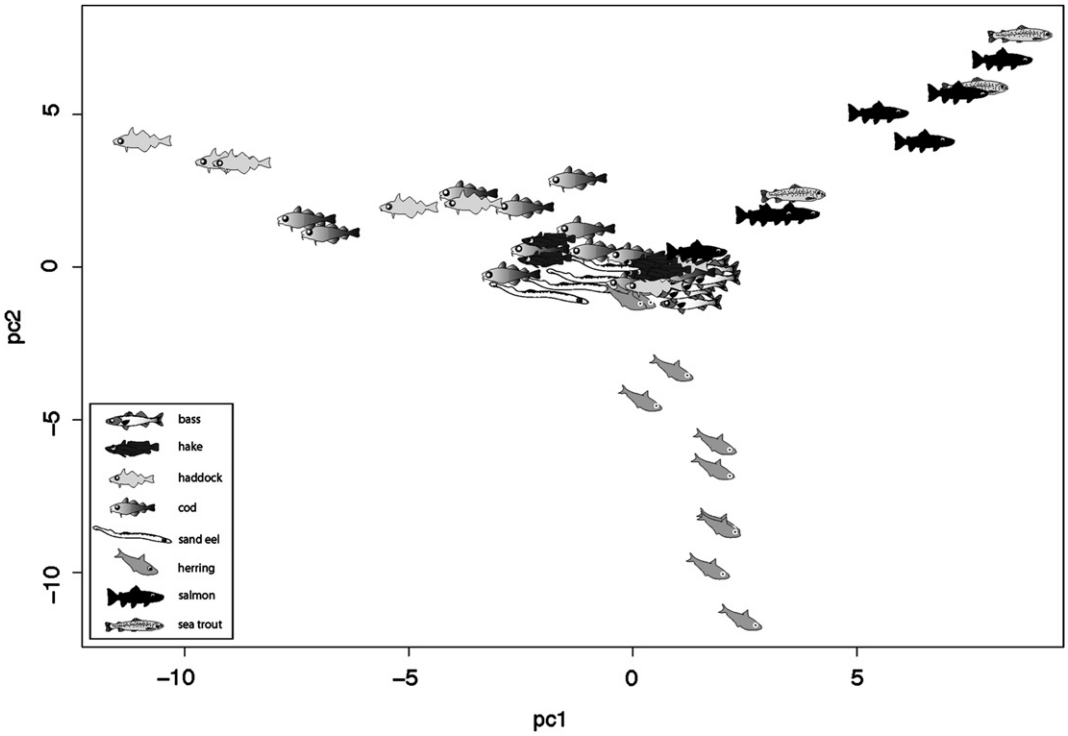

Fig. 3. Scores plot for the first two principal components obtained for the training set. Values for the 89 *m*/*z* bins identified as potential biomarkers were used as input variables. The plot shows clustering of haddock, cod and hake as well as trout and salmon with specimens for which fewer biomarkers were recorded lying in the center of the plot. Further separation can be seen using further dimensions.

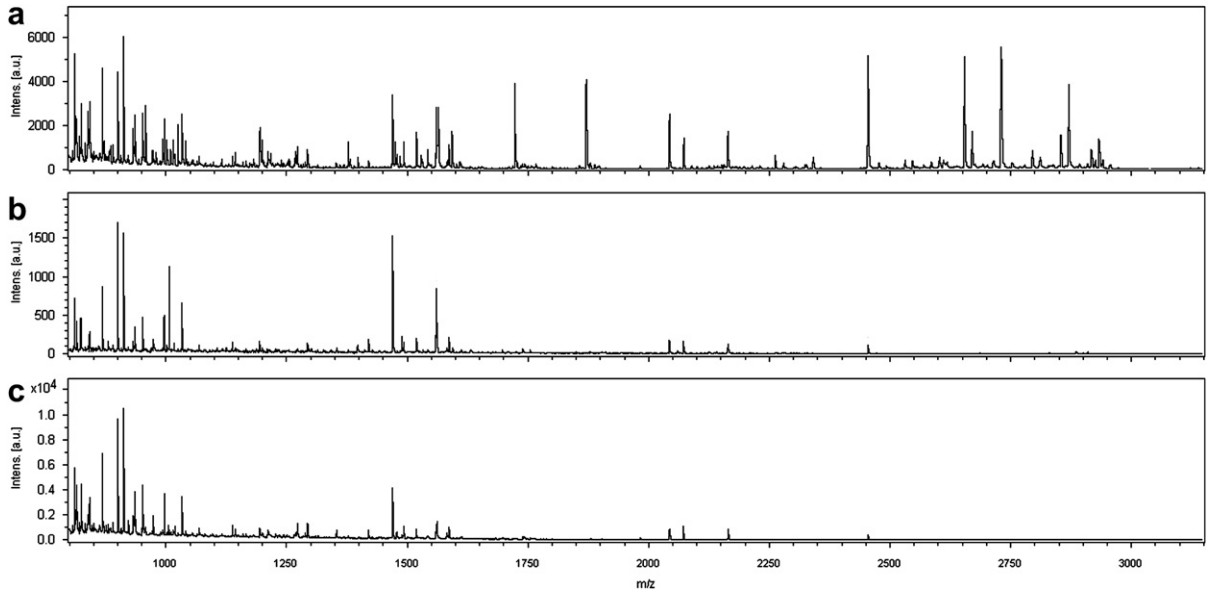

Fig. 4. The spectrum for a modern haddock specimen is shown in (a) with that of an archaeological specimen identified by morphology as haddock in (b) that of a previously unidentified archaeological specimen, classified by our method as haddock in (c).

would be reduced from 95% to 68% (with both measures). As there is little difference between the two measures of confidence here we will consider only the difference between the two highest probability scores and refer to this as the confidence measure.

The generalization of the classification was tested using the further 164 spectra (between 2 and 8 replicates each of 39 specimens) in the test set. They were considered in three separate categories: 28 specimens (modern or archaeological) with secure morphological identifications from any of the eight species in the training set; 5 specimens (archaeological) with secure morphological identifications from species other than those in the training set; and due to difficulty in identification of some ancient fish bones, 6 specimens from bones which are morphologically unidentifiable. The probability scores for each set of replicates were combined to provide a score for the specimen. Of the 28 specimens known to be of a species used in training (bass, cod, eel, haddock, hake, herring, salmon or trout), all bar three (89%) were correctly classified (Table 2). Fig. 4 shows the spectrum obtained for one of the unknown specimens that was classified confidently as haddock together with that for an archaeological specimen identified as haddock by morphology and a modern haddock specimen. The three incorrect classifications consisted of two haddocks classed as hake, (both Gadiformes (Teletchea et al., 2006)), and a trout classed as a herring.

A limitation of the comparative approach is the small size of the training set. This can be seen when we ran samples not in the training set. Of the 5 specimens of other known species, the ray and whiting were both classified as herring, the mackerel as bass and one plaice specimen was classed as eel and the other as trout; however, all had low confidence scores (Table 2).

4. Discussion

There are clear differences between modern fish species that allow peptide fingerprints to be classified. Furthermore, the archaeological samples have similar spectra to modern fish of the same species. In all, 89 biomarkers were identified which together were capable of distinguishing between the eight different species in this study. This represents approximately 20 peptide masses per species; although a number of biomarkers were shared by closely related species such as trout and salmon, or haddock and hake.

Table 2
Table of identifications for the test set.

KK #	Species	Comp ID	Score	Confidence	# Spectra
KK44	bass	✓	0.490	0.400	4
KK7	bass	✓	0.316	0.202	4
KK29	haddock	hake	0.346	0.194	4
KKA26	bass	✓	0.255	0.126	4
KKA25	bass	✓	0.241	0.120	6
KKA24	herring	✓	0.257	0.119	4
KK33	herring	✓	0.243	0.117	4
KKA21	cod	✓	0.240	0.110	4
KK24	cod	✓	0.246	0.109	4
KK21	haddock	✓	0.261	0.098	4
KKA01	cod	✓	0.228	0.093	4
KK53	salmon	✓	0.235	0.061	2
KKA14	?	haddock	0.184	0.060	6
KK28	hake	✓	0.185	0.058	4
KKA07	haddock	✓	0.190	0.045	6
KKA08	haddock	✓	0.181	0.045	4
KKA02	cod	✓	0.175	0.043	6
KK13	hake	✓	0.181	0.042	4
KKA16	?	cod	0.170	0.035	4
KK16	cod	✓	0.152	0.026	2
KKA06	haddock	hake	0.165	0.025	2
KKA23	herring	✓	0.165	0.025	6
KKA22	cod	✓	0.165	0.022	4
KKA13	mackerel	bass	0.182	0.013	8
KKA09	plaice	eel	0.142	0.013	4
KKA18	?	bass	0.156	0.013	4
KKA03	cod	✓	0.166	0.012	6
KKA17	?	bass	0.144	0.011	6
KKA04	cod	✓	0.154	0.010	4
KKA19	?	bass	0.147	0.009	4
KKA20	cod	✓	0.137	0.006	4
KKA05	cod	✓	0.137	0.006	4
KKA12	ray	herring	0.137	0.003	4
KKA11	whiting	herring	0.170	0.002	4
KKA10	plaice	trout	0.138	0.002	4
KK50	trout	herring	0.138	0.002	2
KK40	eel	✓	0.132	0.002	4
KKA15	?	bass	0.132	<0.000	4
KK35	herring	✓	0.146	<0.000	2

KK – modern, KKA – archaeological, ? – unable to be morphologically identified, ✓– correct identifications. Line – boundary between reliable and unreliable confidence scores. 25 fish were correctly identified. All of the species not in the database were unreliable. Two of the morphologically unidentifiable fragments have high confidence scores and the identifications are consistent with archaeological information.

Fig. 2 shows the relative intensities recorded for the training set for some of the unique and shared biomarkers. Analysis of replicate cod spectra (multiple replicates from two modern and two archaeological specimens) revealed analytical differences; half of the replicate spectra from each specimen were poor quality with few peaks none of which were specific for cod. The modern spectra had replicates containing between one and eight of the seventeen biomarkers for cod, while the archaeological samples only contained one or two biomarkers. The classification of the archaeological specimen with only one biomarker recorded in one spectrum was unreliable based upon the confidence score. The other archaeological sample was determined confidently with just two biomarkers present in two out of six spectra. This level of confidence may fall as the library expands and these two biomarkers are shared with a larger number of species. However, the result shows that even when only a small number of biomarkers are detected, accurate identification is possible if the target library of species is small (e.g. from a restricted source).

Analysis of different processing methods shows that reference collections processed by BioTex cannot be used for reference spectra. The proteases and surfactants in BioTex which break down the tissue most likely interfere with ZooMS. Fish bones processed under cold water, by boiling, or by baking all produce the same biomarker patterns. Fish bones recovered from archaeological sites have often been processed by humans by baking or boiling and these tests show that long term boiling (2 h) destroys the collagen enough to prevent identification. However, shorter boiling (up to 30 min) or baking (180° C for up to 45 min and an internal temperature near the bones of up to 67° C) does not affect ZooMS. These results support the view that mineralized collagen can tolerate extreme temperatures, in excess of 140° C prior to melting (Kronick and Cooke, 1996; Covington et al., 2008).

The classification algorithm will assign any input to one of the species used in training set, even if the sample is from a species not included in the training set. Therefore, some indication that a classification is unreliable is required. Table 2 shows that there is a pattern between high confidence measures and identification success. If classification is considered unreliable when the confidence measure is less than 0.02, then all five classifications for species not in the training data set would be highlighted as being unreliable. Although this would flag 19 of the 25 correct classifications as reliable and one incorrect classification as unreliable, six correct and two incorrect classifications would be flagged erroneously. It seems likely that with a larger training set a confidence measure could be developed to identify false positives, but further analysis with more data will be necessary.

Of the unidentifiable bone fragments, one specimen was identified as cod, one as haddock, and four as bass. Using the confidence measure with a cut-off of 0.02, the four bass are identified as unreliable. In fact, two of the bass specimens were morphologically identified as belonging to the family Gadidae and another was possibly a bird from its morphology. Since many species of fish found at Great Yarmouth are not included in the training set, including several species from the family Gadidae, it is unsurprising that four of the six classifications are unreliable (Rogerson, 1976). The two specimens identified as cod and haddock have higher confidence measures and would be considered reliable classifications on this basis. The similarity between the spectrum from one of the specimens identified as cod and that from an archaeological specimen identified morphologically as cod can be seen in Fig. 5. Since both cod and haddock are found in high numbers at Great Yarmouth, the classification of the fragments is consistent with the archaeological evidence.

Herring, salmon, trout, cod, haddock, hake, bass, and lesser sand eel were consistently identified by unique mass fragments from modern ($n = 43$) and archaeological ($n = 26$) bone. These identifications were highly concurrent with traditional morphological identification for modern bone (11/13) and for archaeological bones that could be morphologically identified (14/15). Additionally, the method assigned species to morphologically unidentifiable bones (2/5) with high confidence levels, whereas spectra from fish of species other than those in the training set were given low probabilities for all classes and could therefore be recognized as distinct.

The basis of taxonomic identification relies on the close observation of morphological details on specific elements in the fish skeleton (Wheeler and Jones, 1989). This work was pioneered in the eighteenth century and formed the basis for nineteenth and twentieth century ichthyology. This requires an extensive collection of fish skeletons and at least one highly trained ichthyologist. To identify fish remains with confidence it is necessary that the reference collection include every species of the family that occurs in the region today and also any species that may be locally extinct. Care and judgment are required to exclude damaged, diseased, and aberrant specimens from this process, so a working knowledge of the morphology of living populations is necessary. This requires considerable ichthyological and ecological knowledge. Having drawn up a list and acquired the relevant species, the next stage is careful observation of a number (at least 5) specimens of each species which cover the entire size range: immature, medium, and large specimens. Each specimen needs to be carefully defleshed, cleaned, and adequately crated, making it difficult for comprehensive collections to exist outside of museums of international standing (O'Connor, 2000). Long hours of careful macroscopic examination are then required to identify characteristics that are consistently uniquely present on each species.

The ability to process 96 bone fragments simultaneously and detect peptide fragments using MALDI MS means that ZooMS provides a high-throughput method for the identification of bone fragments. ZooMS offers two major benefits to the traditional taxonomic identification. First, while it still requires a complete reference collection, it only needs to be in one location. Any mass spectra obtained from a species can be placed in a global database and then used in all locations. In addition, only a few specimens from each species need to be used for the reference collection, and physical characteristics such as size and maturity level of the fish do not matter. Finally, since only a few bones are needed to create the reference spectra collection, they can be taken from processing waste so the cost can be minimal to create the collection. If the method can be successfully shown to be applied to scales, samples could be taken for a reference collection without killing any fish.

Second, the barcoding of collagen provides a repeatable identification technique for archaeological and modern bone fragments too small or too fragmented to be recognized by conventional zooarchaeological methods. From the results presented, it is probable that with additional optimization each sample would only need to be extracted once and run using MALDI in duplicate or triplicate in order to identify the sample to species. This would make the cost for ZooMS identification of one sample approximately £15 (assuming 96 samples were analyzed together); however, the method is still in development and costs could be expected to fall. We envisage ZooMS as an additional tool in support of traditional archaeozoological methods, rather than a replacement for them. It provides no information on cut marks, size, sex, and age; and only limited information on disease markers (diseases which involve a mass shift in collagen peptides could possibly be detected). However, it can aid traditional morphological analyses in cases where the bones are non-diagnostic, fragmented, or do not match any of the species in the local reference collection.

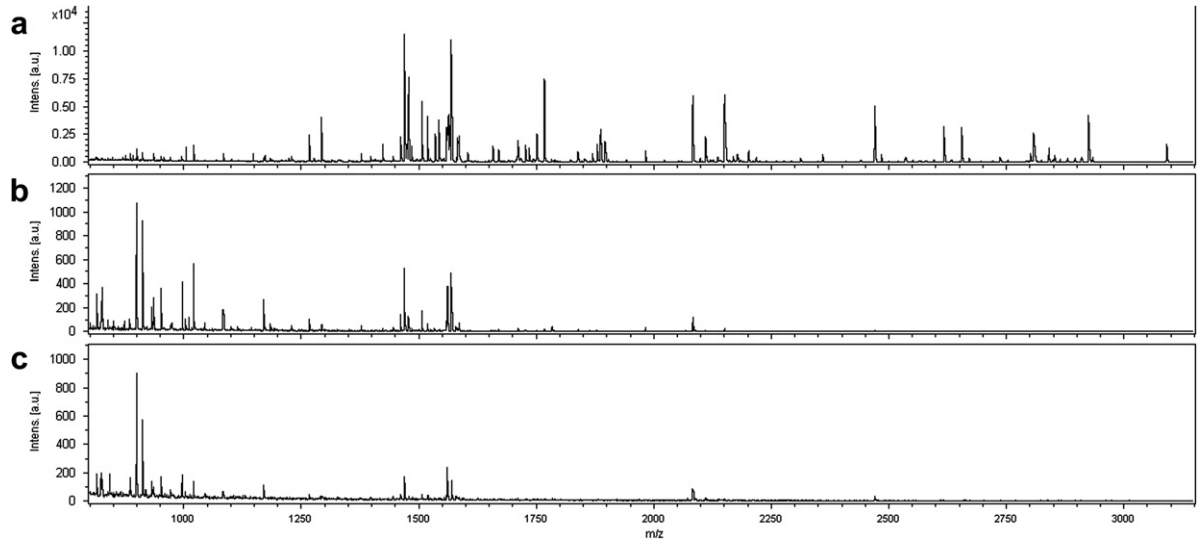

Fig. 5. The spectrum from a modern cod (a) with that of an archaeological specimen identified by morphology as cod in (b) and that obtained from a previously unidentified archaeological specimen, classified by our method as cod, is shown in (c).

One fundamental problem with the ZooMS approach at present is the number of species in the database, a problem analogous to having too few species in a skeletal reference collection (Gobalet, 2001). As more samples are processed, the database will be extended, improving the classification ability and permitting improved confidence levels. We envisage that ZooMS can be used to increase NISP number, partially mitigate differential preservation, and identify exotic species without the need for access to an extensive reference collection. These benefits could be particularly useful for those who work with small sample numbers, small fragments, or ground bone: forensics, food industry quality control, remains from sediment cores, specialized archaeological contexts including drains and food offering sites and site evaluation digs. Thus the ZooMS barcoding technique has great potential for species identification of fish bones, answering the fundamental archaeological question, "What is it?"

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Appendix. Supplementary data

Supplementary data associated with the article can be found in online version, at doi:10.1016/j.jas.2011.02.014.

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