Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 11	Fasc. 2	213-224	2003

THE GENETIC ANALYSIS OF TROUT SKELETAL REMAINS: HOW SHOULD THE POTENTIAL OF ANCIENT FISH DNA BE UTILIZED?

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ABSTRACT. DNA can be retrieved from preserved biological remains; this provides scientists with an opportunity to directly measure molecular evolution over large periods of time. This means that the taxonomic affinity of extinct taxa can be ascertained by studying archival material found at archaeological sites. This paper describes a molecular approach which was used to identify the taxonomical position of a fish whose bones came from an archaeological site in Wolin in northern Poland. Ancient DNA was successfully extracted, and a fragment of about 350 bp from the mitochondrial control region was amplified with PCR and sequenced. The molecular analysis based on ancient and modern sequences of the mitochondrial control region proved that the archival bone was a component of a *Salmo trutta* skeleton. However, the mtDNA sequence examined was more similar to the haplotype of trout from the Adriatic Sea basin than to fish inhabiting the waters of the southern Baltic Sea coast.

Key words: ANCIENT DNA, ARCHAEOLOGICAL SITE, CONTROL REGION, MITOCHONDRIAL HAPLOTYPE, TROUT (*SALMO TRUTTA*)

INTRODUCTION

The development of molecular genetic techniques over the past two decades has made possible the study of processes of molecular evolution. It has become feasible to demonstrate at the molecular level even the smallest differences between the genomes studied. This, in turn, can resolve phylogenetic relationships among examined groups of organisms. Since the initial report by Higuchi et al. (1984), it has been known that molecular techniques not only allow scientists to study relationships among contemporary flora and fauna, but also among organisms that became extinct long time ago. Genetic information can be retrieved from the remains of ancient organisms; this offers great insight into the past and can be applied in solving many archaeozoological problems (Herrmann and Hummel 1993, Ciesielski 2001).

One common task of ancient DNA analysis is to determine the species origin of prehistoric material. The identification of fish remains from archaeological settlements can provide useful information not only about the hunting and management strategies of the inhabitants of particular archaeological sites, but also about the palaeoenvironment and the palaeodistribution of species (Mulkeen and O'Connor

1997). This approach has great potential to answer questions pertaining to fish palaeobiology and prehistoric human subsistence patterns.

Polymerase chain reaction (PCR) has made possible the study of trace amounts of DNA which sometimes survive in archaeological remains (Pääbo 1989). Repeated cycles of denaturation, annealing the primers to the target DNA and extending the segment between the primers by a DNA polymerase, result in the accumulation of a target DNA fragment that can be analyzed by conventional techniques. Recent studies have shown that PCR is a necessary tool for analyzing ancient DNA (Cooper and Wayne 1998).

The amount of preserved DNA is so small that only multi-copy sequences, such as mitochondrial genes, can be reliably amplified. As each vertebrate cell contains numerous mtDNA molecules, at least some undegraded mtDNAs often persist in samples of ancient tissues (Hagelberg et al. 1991). Moreover, a great number of mitochondrial sequences for a variety of fish species can be found in GenBank. Of the mitochondrial fragments, the control region (*CR*) is commonly studied in order to reveal genetic relationships among species and other taxa (Lee et al. 1995).

At present, ancient DNA research is focused mainly on mammalian remnants, especially those from humans (Cooper and Wayne 1998). The task of studying the ancient DNA preserved in old fish bones was recently initiated in the work of Butler and Bowers (1998). These authors were the first to demonstrate that it is possible to retrieve DNA from prehistoric fish bones. Subsequently, a few reports have confirmed that the retrieval and analysis of ancient DNA from fish skeletal remains is feasible (Ciesielski et al. 2002, Consuegra et al. 2002).

In the current study the aim was to identify the taxonomical position of fish from bones derived from an archaeological site. Based on morphometric criteria, this remain was identified as a fragment of the *os operculum* of a skeleton belonging to one of the salmonid species. One hypothesis is that it originated from the trout *Salmo trutta*; the other is that it came from the Atlantic salmon *Salmo salar*. The molecular approach based on the amplification of a mitochondrial DNA fragment was applied to determine which hypothesis is valid.

MATERIAL AND METHODS

A number of the bone remains were collected from an archaeological site on Wolin Island in northern Poland. The archival bone (*os operculum*, Fig. 1) originated from the seventeenth habitation level which represents alluvial environments and

Fig. 1. *Os operculum* investigated in this work, total weight 0.90 g, scale bar = 10 mm.

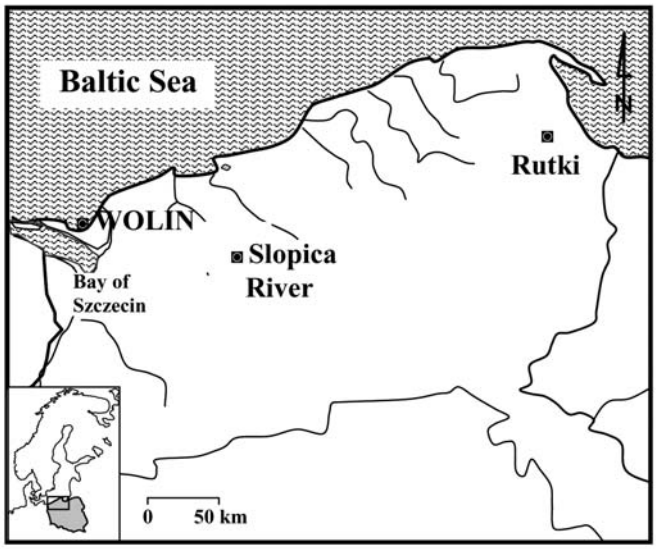

Fig. 2. Sampling locations of archival and modern samples for DNA analysis.

dates to the mid tenth century. Two contemporary *Salmo trutta* samples (Rut 19, 20) were obtained from the broodstock at the Rutki hatchery (Inland Fisheries Institute in Olsztyn), and one (Slup 32) was caught in the Slopica River (Fig. 2).

DNA EXTRACTION

The DNA extraction method was performed following the protocol of Ciesielski et al. (2002). The ancient remnant was soaked in a sodium hypochlorite solution to

decontaminate the surface and then rinsed several times in ethanol. Fine sand paper was used to powder the bone, and approximately 0.3 g of this bone powder was added to 0.5 M EDTA (pH 8.0) in order to decalcify the sample. After 90 h of incubation with EDTA, the decalcified bone powder was transferred into a new tube, and 500 μl of lysis buffer (100mM Tris-HCl; 10mM EDTA; pH 8.0, 0.5 mg of proteinase K, and 0.25 mg of dithiothreitol) was added. Then the sample was incubated at 55°C for 3 h. The aqueous phase was extracted twice with an equal volume of phenol, and once with a chloroform-isoamyl alcohol solution (24:1). The DNA of the sample was concentrated using Microcon - 50 microconcentrators (Millipore Corporation, Massachusetts, USA). Two consecutive rounds of concentration gave a final volume of 50 μl, and the retante was stored at - 20°C.

The modern DNA was extracted using a DNA - direct kit (Wizard® Genomic Purification Kit, Promega, Wisconsin, USA).

PCR AMPLIFICATION

The amplification of the DNA extracted from the archival bone was performed using primers L: 5′- CCACTACTCCCAAAGCT - 3′ (Bernatchez et al. 1992) and CRCH: 5′ - GGAAACATGTGTGAGCCA -3′ (Ciesielski 2001). The DNA from the modern samples was amplified using primers L and H2: 5′- CGTTGGTCGGTTCTT - 3′ (Bernatchez and Danzmann 1993). Both fragments correspond to the left domain of the mitochondrial control region.

Double stranded PCR amplification was performed in 50 μl reaction volumes containing 2 units of *Taq* DNA polymerase (Promega, Wisconsin, USA), 5 μl reaction buffer (500 mM KCl, pH 8.5; Triton X-100), 20 pmol of each primer (MWG-BIOTECH, Ebersberg, Germany), 2.5 mM $MgCl_2$, 500 of μM dATP, dCTP, dGTP, and dTTP, and 2 μl of DNA template. The DNA was amplified using a Perkin Elmer 9600 thermal cycler (PE-Applied Biosystems, California, USA) beginning with preliminary denaturation at 95°C for 5 min. The amplification cycle consisted of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s, for a total of 30 cycles, ending with a final elongation step at 72° C for 3 min. The initial PCR products of the archival sample were reamplified using the same procedure.

The PCR product was separated by electrophoresis in 1.5% agarose gel, using 1x TBE buffer (0.5M EDTA, pH 8.0). Ethidium bromide (0.1 mg ml^{-1}) was added to the gel and the PCR product was visualized with UV light. As a molecular weight

marker, ΦX 174 DNA digested with *Hinf* I was used. Before sequencing the PCR product was purified of oligonucleotides, primers, and dimers using Microcon-50 spin columns (Millipore Corporation, Massachusetts, USA).

DNA SEQUENCING

Sequencing was performed using a Perkin Elmer ABI 373 automated DNA sequencer and the DyeDeoxy Cycling Sequencing reaction (PE-Applied Biosystems, California, USA) at the Institute of Biochemistry and Biophysics in Warsaw, Poland. The nucleotide sequences were deposited in GenBank under accession numbers: AY 236220, AY 236221, AY 236222, and AF 363686.

SEQUENCE ANALYSIS

Correlations among the ancient and modern mtDNA sequences as a result of their shared phylogenetic history were inferred by Bayesian analysis using the Markov chain Monte Carlo (MCMC) method (Huelsenbeck et al. 2000, Huelsenbeck and Bollback 2001). In this case, the question of phylogenetic relationships of a species group is addressed on all possible trees (τ_i), weighted by the probability that each tree is correct. It can be supposed, for example, that each possible phylogenetic tree is either consistent with (c) or inconsistent with (n) an evolutionary hypothesis, X. Then, the overall probability that the hypothesis is correct will be the sum of the posterior probabilities of trees consistent with the hypothesis, $f(\tau_c \mid X)$. The sum of the posterior probabilities of all trees, i.e. $f(\tau_c \mid X)$ and $f(\tau_n \mid X)$, will be 1.

In the current study, the question concerned the taxonomical position of the salmonid fish whose bone remain was examined. One hypothesis was that studied remains were of the trout *S. trutta*, whereas the other considered it to be of the Atlantic salmon *S. salar*. The probability that the *S. trutta* hypothesis is correct was the sum of the posterior probabilities of a reconstruction that places the archival sample into one clade with contemporary *S. trutta*. Consequently, in the calculation of the overall probability of the *salar* hypothesis, only those trees were considered which contained the clade of the ancient sample and *S. salar*. The phylogeny was based on mtDNA sequences sampled from the archival specimen, contemporary trout *S. trutta* (North Atlantic haplotype; Suarez et al. 2001) and salmon *S. salar* (Shedlock et al. 1992). The trees were rooted using rainbow trout *Oncorhynchus mykiss* as the outgroup. The hypotheses were examined by approximating the posterior probabilities of the trees for *CR* fragments using the MrBAYES program (Huelsenbeck and Ronquist 2001). For the analysis, it was

assumed a priori that all trees had equal probabilities. The HKY85 (Hasegawa et al. 1985) model of DNA substitution was assumed with Γ rate variation (Yang 1994). Both models allow for different rates of transition, transversion, different stationary nucleotide frequencies, and among site rate variation. Four Markov chains were run simultaneously for 50 000 generations, sampling the chains every 10 generations. The first 2 000 generations of the chains were discarded.

The second part of the phylogenetic survey, which included modern mitochondrial haplotypes originated from Poland (AY 236220, AY 236221, AY 236222) and other parts of Europe (AF 253558 - North Atlantic, AF 253545 - Atlantic, AF 253551 - Adriatic; Suarez et al. 2001), was performed in the same way.

RESULTS

The DNA extraction method applied yielded a sufficient amount of good quality DNA. The polymerase chain reaction on the extract from the bone remains was successful and produced a piece of ancient mtDNA approximately 350 bp in length. A 275 bp fragment located in the middle part of this sequence was used in further phylogenetic analysis.

The main question of the study concerned the taxonomical position of the fish whose bone remains were examined. Therefore, Bayesian analysis of the *CR* fragment was performed to find its most probable phylogeny. The tree (*O. mykiss*, *S. salar*,(Archival, *S. trutta* North Atlantic)) had the highest possible posterior probability $f(\tau \mid X) = 1.00$. The maximum credibility value (1.00) for the clade containing the two sequences indicated that they were highly similar to each other. The nucleotide difference between the archival sample sequence and that of *S. salar* was 16 substitutions.

The next step in the investigation was to resolve phylogenetic relationships among the archival and modern specimens of brown trout. For this purpose, mitochondrial *CR* fragments from modern samples of trout were sequenced, which yielded three haplotypes - Rut 19, Rut 20, and Slup 32. Among all the sequences of the *CR* fragment, nine variable positions were detected (Fig. 3). The most divergent sequence was Rut 20. The archival nucleotide sequence differed from the contemporary trout sequences Rut 20, Rut 19 and Slup 32 by five, two and three substitutions, respectively.

Figure 4 shows the tree relating the studied mitochondrial haplotypes. The archival sample was in a group with the Adriatic haplotype. The two other clusters

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        5          15         25         35         45         55
North Atlantic      TTTCA-GCTA TGTACAATAA CAATTGTTGT ACNATGCTAA CCCAATGTTA TACTACATCT
Atlantic            .....A.... .......... .......... .......... .......... ..........
Adriatic            .....-.... .......... .......... .......... .......... ..........
Archival            .....-.... .......... .......... .......... .......... ..........
Rut 19              .....-.... .......... .......... .......... .......... ..........
Slup 32             .....-.... .......... .......... .......... .......... ..........
Rut 20              .....-.... .......... .......... .......... .......... ..........

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        65         75         85         95        105        115
North Atlantic      ATGTATAATA TTACATATTA TGTATTTACC CATATATATA ATATAGCATG TGAGTAGTAC
Atlantic            .......... .......... .......... .......... .......... ..........
Adriatic            .......... .......... .......... .......... .......... ..........
Archival            .......... .......... .......... .......... .......... ..........
Rut 19              .......... .......... .......... .......... .......... ..........
Slup 32             .......... .......... .......... .......... .......... ..........
Rut 20              .......... .......... .......... .......... .......... ..........

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       125        135        145        155        165        175
North Atlantic      ATCATATGTA TTATCAACAT TAATGAATTT AACCCCTCAT ACATCAGCAC TAACTCAAGG
Atlantic            .......... .......... ..G....... .......... .......... ..........
Adriatic            .......... .......... ..G....... .......... .......... ..........
Archival            .......... .......... ..G....... .......... .......... ..........
Rut 19              .......... .......... ..G....... .......... .......... ..........
Slup 32             .......... .......... ..G....... .......... .......... ..........
Rut 20              .......... .......... ..G....... .......... .......... ..........

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       185        195        205        215        225        235
North Atlantic      TTTACATAAA GCAAAACACG TGATAATAAC CAACTAAGTT GTCTTA-ACC CGATTA-ATT
Atlantic            .......... .......... .......... .......... ......-... ......-...
Adriatic            .......... .......... .......... .......... ......-... ......-...
Archival            .......... .......... .......... .......... ......-... ......-...
Rut 19              .......... .......... .......... .......... ......-... ......-...
Slup 32             .......... .......... .......... .......... ......-... ......-...
Rut 20              .......... .......... .......... .......... ......C... A.....G...

                    ....|....| ....|....| ....|....| ....|....|
                       245        255        265        275
North Atlantic      GTTATATCAA TAAAACTCCA GCTAACACGG GCT-CCGTC.
Atlantic            .......... .......... .......... ...-......
Adriatic            .......... .......... .......... ...-......
Archival            .......... .......... .......... ...-......
Rut 19              .......... ..C....... .......... .A.-......
Slup 32             .......... ..C....... .......... .A.-.G....
Rut 20              .......... ..C....... .......... .A.G.G....
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Fig. 3. Comparison between the aligned sequences of mitochondrial control region fragment obtained from archival and modern samples. Identity is indicated with dots, gaps in the sequence are shown by dashes. Sequence described as North Atlantic, Atlantic, and Adriatic were published by Suarez et al. (2001).

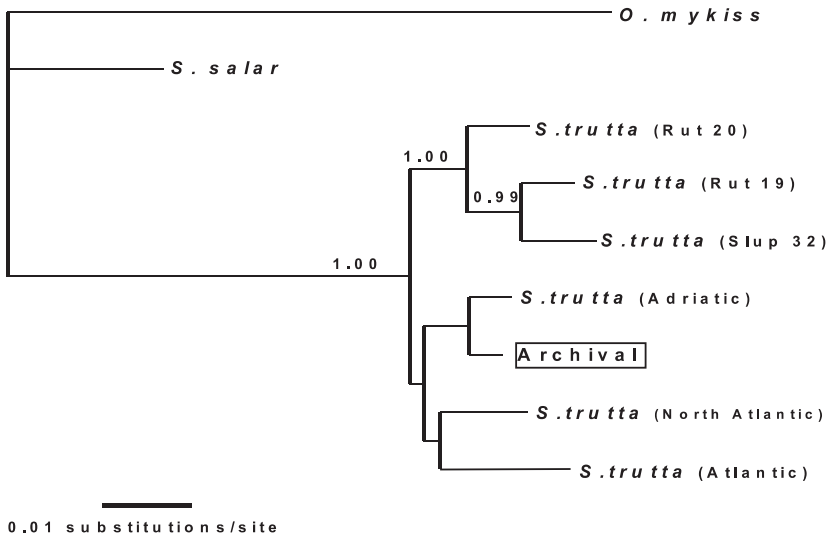

Fig. 4. Phylogram showing the relationships between the *CR* mtDNA sequences of archival and contemporary *S. trutta* and *S. salar*. The estimates of phylogeny are based on Bayesian criteria. Only clades with greater than 0.5 posterior probability are shown. The numbers at the interior nodes represent the posterior probability that the clade is correct. The posterior probabilities of clades were approximated with the MrBAYES program (Huelsenbeck and Ronquist 2001). The tree was rooted using rainbow trout (*Oncorhynchus mykiss*) as the outgroup.

included haplotypes of fish from Poland (the Rut 19, Rut 20, and Slup 32 haplotypes) and one joined the haplotypes of the Atlantic trout.

DISCUSSION

The main goal of this work was to present a molecular approach to retrieve genetic information from a fish remnant. The extraction, amplification, and sequencing processes were successfully performed, and allowed an ancient mitochondrial DNA sequence to be characterized. One of the disadvantages of ancient DNA research is DNA fragmentation which makes it impossible to amplify DNA segments longer than 200 bp (Pääbo 1989, Consuegra et al. 2002). The method used in this study yielded a DNA fragment 350 bp long, which, to the best of the authors' knowledge, is one of the longest pieces of ancient DNA ever retrieved from the bone of a vertebrate.

Another aim of the study was to ascertain the taxonomical position of the fish whose remains were analyzed. Bayesian analysis was performed on both archival mtDNA *CR* and modern *S. salar* and *S. trutta* species. In the tree relating the

haplotypes, the archival sample was included in a clade with contemporary *S. trutta*; this confirmed previous presumptions that remains were of a salmonid species.

Having confirmed the taxonomic status of the archival fish, the aim was to test how the sequence obtained from the ancient sample corresponded to sequences of trout currently inhabiting Polish waters. To make this analysis more readable, mitochondrial haplotypes characteristic of brown trout living in other parts of Europe were included. Surprisingly, phylogenetic analysis revealed that the fish formed one clade with those from the Adriatic Sea basin, whereas other Polish trout were in a separate clade.

The bone analyzed in the study was derived from an archaeological site on Wolin Island, which is located at the mouth of the Oder River. Wolin was one of the largest urban settlements along the southern coast of the Baltic Sea from the eighth to the twelfth centuries. Thanks to its geographical location, Wolin was also one of the largest trade centers in central Europe during this period. It is plausible that merchants arriving there from southern Europe could have transported for sale specimens of this valuable fish. This scenario might be supported by the fact that salmonid fish were caught very rarely by fishermen from this Baltic coast region during the Bronze Age (Makowiecki 2000). This could explain why the DNA of the studied trout bone is more similar to DNA of fish from the Adriatic Sea basin than to DNA of fish that were native to the Baltic Sea coast.

Genetic studies performed on brown trout strongly support the idea that this species is characterized by a complex genetic structure and large genetic differentiation, including subspecies, sympatric isolated populations, and ecological forms (e.g. Bernatchez et al. 1992, Bouza et al. 2001, Presa et al. 2002). This seems to be the result of species habitat fragmentation, homing behavior, and complex evolution during the Pleistocene (Bernatchez 2001). Taking into consideration the great genetic variation among *S. trutta* lineages, it is possible that the investigated fish could have been native to the southern coast of the Baltic Sea. Since current genetic data on trout inhabiting Polish water is scant, this question cannot be resolved yet.

CONCLUSIONS

Many new fields are beginning to utilize the potential of ancient DNA with the aim of identifying and analyzing ancient DNA recovered from archival fish bone. This paper suggests that it is possible to combine the three diverse scientific disci-

plines of ichthyology, archaeology, and molecular biology. It has been demonstrated here that fish remains obtained from an archaeological site can provide unique information about extinct individuals. This kind of information may be helpful in explaining the assignment of particular fish species. The methods presented could provide additional tools for the successful restitution of endangered fish species and in defining factors which influence biodiversity in natural fish populations. The molecular study of the archival fish collection can increase knowledge of prehistoric fisheries and explain the human factors that could have changed the composition of natural fish populations.

This paper was written in memory of Dr. Jaroslaw Filipiak, Professor of the Agriculture University of Szczecin, who provided us with the opportunity to begin research on ancient fish DNA.

ACKNOWLEDGEMENTS

We are grateful to Professor Z. Chelkowski (Agriculture University of Szczecin) for kindly providing us with archival bone and to Dr. J. Kozlowski (University of Warmia and Mazury in Olsztyn) for collecting modern samples. This research was supported by the Foundation for Polish Science under the ARCHEO Nr 5 scheme.

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STRESZCZENIE

MOŻLIWOŚCI WYKORZYSTANIA ARCHIWALNEGO DNA RYB

Odkrycia poczynione w ciągu ostatnich kilkunastu lat dowodzą, że techniki genetyki molekularnej można wykorzystać nie tylko do poznawania współczesnej flory i fauny, ale również tego świata ożywionego, który dawno przeminął. Dysponując szczątkami wymarłych roślin i zwierząt można odzyskać i analizować zawarte w nich DNA, co pozwala między innymi poznać ich stanowisko systematyczne oraz powiązania filogenetyczne ze współcześnie żyjącymi gatunkami.

W pracy przedstawiono podejście badawcze umożliwiające poznanie pozycji taksonomicznej ryby, której szczątki (rys. 1) odkryto na stanowisku archeologicznym zlokalizowanym na wyspie Wolin (rys. 2). Analizowano fragment szkieletu, który najprawdopodobniej należał do jednej z ryb łososiowatych. Zastosowane metody pozwoliły uzyskać sekwencję regionu kontrolujacego replikację i transkrypcję (*CR*) mitochondrialnego DNA (rys. 3). Przeprowadzona analiza filogenetyczna potwierdziła wcześniejsze przypuszczenia - badana kość rzeczywiście należała do ryby łososiowatej, dokładnie do troci, *S. trutta*.

Do drugiego etapu analizy filogenetycznej włączono haplotypy mtDNA troci obecnie zasiedlających wody Polski oraz sekwencje charakterystyczne dla ryb pochodzących z innych części Europy (Suarez et al. 2001). Niespodziewanie okazało się, iż ryba, której szczątki pochodziły z wyspy Wolin była bliższa genetycznie troci złowionej w zlewisku Morza Adriatyckiego niż rybom zasiedlającym wody północnej Polski (rys. 4).

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