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Millennia-Long Co-Existence of Two Major European Whitefish (*Coregonus* spp.) Lineages in Switzerland Inferred from Ancient Mitochondrial DNA

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Abstract: Archaeological fish remains are an important source for reconstructing past aquatic ecosystems and ancient fishing strategies using aDNA techniques. Here, we focus on archaeological samples of European whitefish (*Coregonus* spp.) from Switzerland covering different time periods. *Coregonus* bones and scales are commonly found in archaeological assemblages, but these elements lack species specific features and thus inhibit morphological species identification. Even today, fish taxonomy is confusing and numerous species and ecotypes are recognized, and even more probably existed in the past. By targeting short fragments of the mitochondrial d-loop in 48 morphologically identified *Coregonus* scales and vertebrae from 10 archaeological sites in Switzerland, endogenous d-loop sequences were found in 24 samples from one Neolithic, two Roman, and four Medieval sites. Two major mtDNA clades, C and N, known from contemporary European whitefish populations were detected, suggesting co-occurrence for at least 5000 years. In the future, NGS technologies may be used to explore *Coregonus* or other fish species and ecotype diversity in the past to elucidate the human impact on lacustrine/limnic environments.

Keywords: *Coregonus*; ancient DNA; mitochondrial DNA; archaeology; Neolithic; Roman; Medieval; Switzerland

1. Introduction

Whitefish (*Coregonus* spp.) are widespread across central and northern European lakes [1–3]. They display high phenotypic diversity with several ecologically, morphologically, and genetically distinct species co-occurring within single lakes, which is thought to be the result of recent adaptive radiation following postglacial colonization [4–8].

In Swiss sub-Alpine lakes, up to six whitefish species coexist [9,10]. They are genetically distinct, vary in body size, growth rate, gill-raker numbers, food preferences, and spawning behaviour, and occupy littoral, pelagic, and benthic habitats [7,10–13]. In Europe, two diverse mitochondrial (mtDNA) d-loop clades were detected in whitefish (*C. lavaretus* complex), which coexist across Swiss lakes and are shared among species: one so-called ‘northern lineage’ (N clade) with a predominant distribution range from north-west Russia to Denmark and one ‘central European lineage’ (C clade) with a higher frequency from Denmark to the European Alps [12,14]. The co-occurrence of both lineages suggests that whitefish populations originating from at least two different refugia colonized the Swiss lakes after the last glacial maximum (LGM), giving rise to new whitefish species [12].

However, human impact, such as eutrophication, overfishing, regulations, barrages, or sewage plants, led to a drastic decrease in diversity and the loss of species, e.g., lacustrine and river-spawning coregonids including those supposedly living exclusively in rivers [10,11,15,16]. Today, the European whitefish is one of the major fish groups in Switzerland declared endangered, and thus all species are protected by the 1982 Bern Convention on the Conservation of European Wildlife and Natural Habitats. Biological research into the processes that maintain and generate biodiversity may help to understand and prevent such extinctions [10]. Yet, archaeology, archaeozoology, and ancient DNA (aDNA) analyses of whitefish remains offer a direct view on the life history of a species during past cultural periods in relation to the human impact and thus permit a long-term perspective on biological conservation efforts [17].

In Switzerland, the archaeological remains of whitefish are frequently found in different time periods from the Neolithic onwards [18–25]. They are well preserved both in waterlogged and dry conditions. In the Neolithic, whitefish, along with other fish remains, document the regular exploitation and on-site consumption of fish depending on seasonal availability. These finds, along with dendrochronological dated lake shore settlements, provide rich and detailed chronological insights into prehistoric fishing techniques, e.g., harpoons for pike fishing or gillnets for cyprinids in shallow water, while the fishing of whitefish in open water required watercraft and more refined catching techniques [18,19].

During the Roman era, whitefish was on the menu in military camps, as well as in wealthy households across Switzerland [20,21]. As lakes are the usual habitat of whitefish [22], the finding of their remains at Roman sites close to the Rhine River and its tributaries [21] suggest that migratory populations spawning in the Rhine existed in the past, some of which may have survived until recently [16].

The Middle Ages was a period of increasing fish consumption [23,24], including whitefish. An example for the transportation of whitefish over 100 km from Lake Lucerne to the city of Basel is known from a written source dated to the 12th century AD [25]. However, the consumption of fresh fish from sources at long distances may have been limited to wealthy people, e.g., members of the clergy and upper classes and the demands of most people were likely satisfied by local fisheries [23,24].

Archaeological sites for later periods are rare in Switzerland. Therefore, no data are included from periods between the ca. 16th century AD and modern times. Today, whitefish are very important economically for professional fishers, who supply local restaurants around the lakes that flourish during times of eutrophication. However, the recent re-oligotrophication of the lakes may reduce whitefish catches, causing economic loss to the fishers [15].

With the routine wet-sieving of archaeological sediments in Switzerland, using small mesh sizes, small fish remains such as cranial bones, vertebrae, and scales, provide evidence for a much more important role of fish as a food source throughout history than previously believed [18,21,23]. However, the species identification of *Coregonus* based on the morphological criteria of skeletal elements is not possible as it lacks diagnostic features, and in the case of cranial bones, scales, or vertebrae most commonly preserved in the archaeological record, identification is only possible on the genus level, preventing the assessment of species diversity in the past. Therefore, the application of DNA-based methods to distinguish modern *Coregonus* species may help to overcome this limitation provided that DNA has survived in the archaeological remains.

Ancient DNA studies have been successfully applied to a variety of fish from marine and freshwater environments of cold and temperate regions, but rarely from tropical regions [26–31], using maternally inherited mitochondrial DNA markers. These papers support the potential of aDNA to address past diversity, historic trade routes, and economies, to reconstruct expansions and colonization routes of past wild fish populations, and to detect climatic effects on fish species. Nuclear markers have been targeted in a few publications to study historic genetic fish diversity [29,30,32]. The application of SNP typing, NGS, and DNA capture-enrichment methods to ancient fish, however, awaits the development of genomic tools for population studies that are, unfortunately, still not readily available for most modern fish groups [33]. Nuclear markers (SNPs) and NGS are currently being developed and adopted for extant *Coregonus* species [1,34], and they will be available for species determinations in ancient *Coregonus* remains in the near future.

Here, we show that small *Coregonus* remains are a source of aDNA by the PCR amplification of mtDNA d-loop fragments in 48 individual elements from two waterlogged and eight dryland archaeological sites in Switzerland. The presence of two main maternal lineages, C and N, was found from the Neolithic onwards. This study provides a basis for further genetic research using archaeological *Coregonus* and other fish remains to reconstruct fishing practices, subsistence, trade, economy, or fish speciation and diversity in the context of the past and present anthropogenic impact.

2. Materials and Methods

2.1. Sampling

Fish remains are recovered from 4, 1, and 0.35 mm mesh size sieves after the wet-sieving of sediment samples together with archaeobotanical and other small faunal remains. An archaoezoological analysis of *Coregonus* samples was performed at Integrative Prehistory and Archaeological Science (IPAS), University of Basel, Switzerland. *Coregonus* vertebrae and scales were obtained and morphologically determined following standard procedures at the IPAS [23,24,35]. The specimens were stored at IPAS for up to 20 years in a dark, dry place at room temperature. From this collection, 40 morphologically well preserved *Coregonus* remains (34 vertebrae, six scales) were selected for aDNA analysis. Additionally, eight *Coregonus* elements (four vertebrae, four scales) from the Neolithic lake-shore settlement Arbon Bleiche 3 were “freshly” re-sampled from wet sediments that had been cold-stored for about 20 years. These waterlogged preserved remains were dried at room temperature for one week, after which DNA was extracted (Tables 1 and S1).

2.2. Archaeological Sites

Fish remains were recovered from two Neolithic waterlogged sites, and three Roman, one Early Medieval, and four Medieval dryland sites in Switzerland. Neolithic sites were dated by dendrochronology, and all others were dated by a typo-chronological analysis of the artefacts (coins and pottery) in the same layers (Figure 1, Tables 1 and S1).

2.3. Neolithic Period

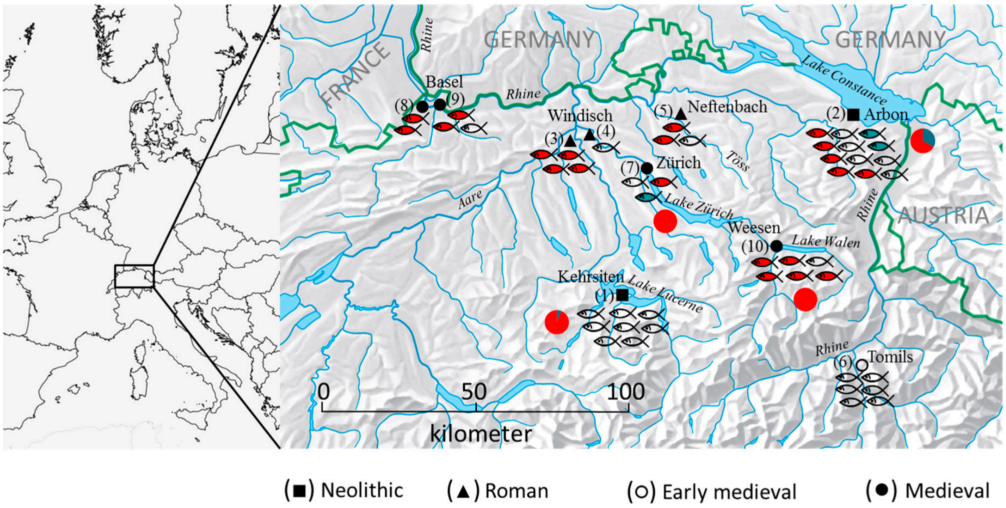
2.3.1. Stansstad-Kehrsiten

The lakeshore settlement Stansstad-Kehrsiten is located at Lake Lucerne, in the Canton of Nidwalden and dendro-dates point to occupation between ca. 3500–3400 BC. The settlement was established on a lake beach and is a key site of the Neolithic transitional time period between the Cortaillod and Pfyn cultures [19]. Underwater excavations took place between 2003 and 2011. The animal remains at the site are 75% fish, 20% mammals, and 5% amphibians after wet-sieving. The final assessment of the site is ongoing.

Table 1. Specifications of archaeological sites from which whitefish remains were taken for this study.

Archaeological Site	City/Canton	Dating	Code Figure 1	Elements	Site Type	Depositional Context	Reference
Stansstad-Kehrsiten	Kehrsiten/NW	3500–3400 BC	1	4 scales 4 vertebrae	Lake-shore settlement	Layer samples	[19]
Arbon Bleiche 3	Arbon/TG	3384–3370 BC	2	4 vertebrae 4 scales [1] 4 vertebrae [1]	Lake-shore settlement	Cultural layers	[36]
Breite	Windisch/AG	1st century AD	3	2 scales 2 vertebrae	dryland	Barrel pits, pits	[21,37]
Römerblick	Windisch/AG	1st century AD	4	1 vertebra	dryland	Kitchen floor of a peristyle house	[21,37]
Neftenbach	Neftenbach/ZH	3th/4th century AD	5	3 vertebrae	dryland	Cesspits	[38,39]
Tomils	Tomils/GR	7th century AD	6	6 vertebrae	dryland	Floor insulation structure	[40–42]
Fraumünsterstrasse	Zürich/ZH	1010–1160 AD	7	3 vertebrae	dryland	Occupation layer, filling of a fireplace	[43]
Bäumleingasse 14	Basel/BS	13th century AD	8	2 vertebrae	dryland	Cesspits	[44]
Weesen Rosengärten	Weesen/SG	14th century AD	10	6 vertebrae	dryland	Waste trench	[45,46]
Museum der Kulturen, Im Schürhof	Basel/BS	15th/16th century AD	9	3 vertebrae	dryland	Cesspits	[45,47]

[1] "Freshly" re-sampled from cold stored sediments as described in Materials and Methods.



(**a**) (**b**)

Figure 1. (**a**) Switzerland within Europe and (**b**) showing an enlarged part of Switzerland with the locations of cities and archaeological sites (numbers in parenthesis) providing the samples for this study. Main lakes and rivers are in italics. Number of fish symbols corresponds to number of archaeological samples tested per site. Pie charts represent extant samples from Lake Lucerne ($n = 49$), Lake Zürich ($n = 20$), Lake Walen ($n = 23$), and Lake Constance ($n = 42$) (as listed in Table S2). Red coloured fish symbols and pie charts denote C lineage; blue coloured fish symbols and pie charts denote N lineage; empty fish symbols denote no aDNA detected. Number of fish symbols corresponds to number of archaeological samples tested per site. (1) Stansstad-Kehrsiten (3500–3400 BC); (2) Arbon Bleiche 3 (3384–3370 BC); (3) Windisch Breite (1st century AD); (4) Windisch Römerblick (1st century AD); (5) Neftenbach (3th/4th century AD); (6) Tomils (7th century AD); (7) Zürich Fraumünsterstrasse (1010–1160 AD); (8) Basel Bäumleingasse 14 (13th century AD); (9) Basel Museum der Kulturen, Im Schürhof (15th/16th century AD); (10) Weesen Rosengärten (14th century AD). (Map (**b**) © swisstopo).

2.3.2. Arbon-Bleiche 3

Arbon Bleiche 3 is a single-phase settlement of the Horgen culture on Lake Constance, in the Canton of Thurgau and has been dated dendrochronologically to between 3384 and 3370 BC [36]. Excavations started in 1993, and the final evaluation of the site took place from 1997 to 2002. After wet-sieving, a high proportion of fish remains were recovered: 79% fish, 20% amphibians, and <1% birds and mammals, which underlines the importance of fish as a protein source in these Neolithic cultures.

Both sites belong to the UNESCO World heritage site "Prehistoric Pile dwelling around the Alps" (http://sites.palafittes.org/home). Thanks to well-preserved timber, layers of these sites have been dated dendrochronologically, and uncarbonised organic material is mostly well preserved under waterlogged conditions, providing an excellent opportunity for archaeobiological studies of early agricultural societies [48–50].

2.4. Roman Era

The sites Römerblick and Breite at Windisch, in the Canton of Aargau, belong to a large Roman legionary camp excavated in several, still ongoing, campaigns lasting more than 100 years. Both are dated typologically to the 1st century AD, and samples are preserved in dry conditions. Excellently preserved samples were obtained from the floor of a high-ranking centurion's kitchen

(Windisch Römerblick) and from three pits (Windisch Breite), and the latter were filled with 45% fish from all faunal remains [21,37].

The site Neftenbach, in the Canton of Zürich, is a Roman villa rustica dating to the 3th/4th century AD [38,39]. Samples are from a cesspit of construction 25.

2.5. Medieval Period

Samples are from a floor insulation of the annex constructions G and F of an early Christian church (7th century AD; Sogn Murezi) at Tomils, in the Canton of Grisons [40–42]. The constructions were physically connected with each other and with the church. The samples had been moved from their primary and unknown archaeological context to their final destination within the annex constructions. Faunal samples were retrieved during a student field course of IPAS in 2005. Excavations were carried out between 1994 and 2011.

Zürich Fraumünsterstrasse, in the Canton of Zürich (1010–1160 AD) was a living quarter close to the lake of Zürich and the abbey of Fraumünster. The samples were taken from the first occupation layers with an unclear context and from the filling of a fire pit [43].

The fish remains from Basel Bäumleingasse 14, in the Canton of Basel-Stadt (13th century AD) [44] were from the upper layer of a cesspit outside the Medieval building.

Further samples were taken from cesspit 2 from the excavation at Basel Museum der Kulturen, Im Schürhof, in the Canton of Basel-Stadt (15th/16th century AD) [45,47]. An assessment of the site is ongoing.

Fish remains were retrieved from waste ditches (German Ehgraben) built between houses for the disposal of faeces and other waste at Weesen Rosengärten, in the Canton of St. Gallen (14th century AD) [45,46]. A final assessment of the site is ongoing.

2.6. Methods

All DNA extractions and pre-PCR steps were performed in dedicated aDNA facilities and strictly followed the accepted standards as established at IPAS and in aDNA research (see e.g., [51,52]).

2.6.1. DNA Extraction

Single *Coregonus* vertebrae and scales (samples Cor1 to Cor48) were ground in 360 µL buffer ATL (provided from DNeasy® Blood & Tissue Kit, Qiagen, Basel, Switzerland) using a mortar and pestle, and for every eight samples, two blank extracts were included in the preparation. All extraction steps followed the "User-Developed Protocol: Purification of total DNA from compact animal bone" for ≤100 mg bone using the DNeasy® Blood & Tissue Kit. Thereafter, extracts were further purified with Ultra-0.5 mL 30 kDa centrifugal filters (Amicon/Millipore, Zug, Switzerland) with buffer AE (provided from DNeasy® Blood & Tissue Kit) to a final volume of 100–150 µL per sample. Samples were stored at −20 °C.

2.6.2. Primer Design, PCR, and Sequencing

European whitefish populations including those from Switzerland belong to two divergent mitochondrial d-loop lineages: the Northern and the Central European clade [12,14]. Compared to the reference sequence NC002646 of the Central (C) European lineage, the Northern (N) European lineage typically shows one nucleotide insertion, 15,847_15,848insT or 15,846_15,847insT (SNP1), and three substitutions, 15,887C>T (SNP2), 16,498A>G (SNP3), and 16,726G>A (SNP4). The whole region spans about 992 bp and as aDNA is highly fragmented, five primer pairs were designed to target SNP sites by generating short non-contiguous d-loop fragments and allowing for high specificity to *Coregonus* (Table 2). The fragment lengths generated were less than 100 bp for most primer pairs, except for the combination CORb1F and COR1R, which produced a larger fragment of 135 bp. However, this primer pair was less successful and was therefore not further used in this study.

Table 2. Primer pairs used in this study.

Primer Pairs	Coordinates Reference Sequence NC002646	Nucleotide Sequence (5′–3′)	Annealing Temperature (°C)	Primer Length (bps)	Amplicon Length (bps)	Target Region Contains
CORb1F	15,794–15,818	TCAACATAAGTGATTTTAAGCCCTC	54–55	25	90–91	SNP1
CORb1R	15,864–15,883	AGAACGGTTCCGTTGGTGAT		20		
CORb1F	15,794–15,818	TCAACATAAGTGATTTTAAGCCCTC	54	25	134–135	SNP1 and SNP2
COR1R	15,908–15,927	GCCCGTGTTAGTTGGAGGTT		20		
CORc1F	15,853–15,874	AGACTCGGATAATCACCAACGG	54–55	22	80	SNP2
CORc1R	15,914–15,932	ACGGAGCCCGTGTTAGTTG		19		
CORa2F	16,447–16,466	TGTCAAACCCCAAAACCAGG	54–55	20	78	SNP3
CORa2R	16,502–16,524	TGTCGGTGCCAAAGTTTGTTAAT		23		
COR3F	16,690–16,709	TTGGCACCGACAACCCTATC	54–55	20	86	SNP4
COR3R	15–38	ACAGCTTCAGTGTTATGCTTTAGT		24		

PCR amplifications were performed in a Mastercycler pro S (Eppendorf, Allschwil, Switzerland) in 25 µL volumes with 5–8 µL DNA sample, 2 µM of each primer, and 400 µM dNTP Mix (Promega, Dübendorf, Switzerland), plus 1.5 U AmpliTaq Gold, 1×GeneAmp PCR Gold buffer (150 mM Tris-HCl, 500 mM KCL, pH 8.0) and 2 mM $MgCl_2$ (all from Applied Biosystems, Hombrechtikon, Switzerland). PCR started with a 12-min initial denaturing step, followed by 70 cycles of denaturing at 94 °C for 1 min, annealing at 54–55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplifications were performed in sets of six to eight samples per primer pair including at least one blank extract and one non-template control. None of the controls produced a product after PCR amplification.

PCR products were run on 3% agarose gel and bands of an expected size were cut and purified using a MinElute Gel Extraction Kit (Qiagen, Basel, Switzerland). Products were directly sequenced in both directions by Microsynth (Balgach, Switzerland) using Sanger technology with modified PCR primers (the same as used in amplification reactions) to which a non-specific 40-bp nucleotide tail (5′-AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA-3′) had been added to the 5′-end [53]. Three independent repeat amplifications from the same extracts were performed per sample and targeted to ensure the reproducibility of the sequencing and genotyping results. All ancient *Coregonus* sequences are available at GenBank accession numbers MF441228-MF441251.

Sequences were aligned using BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the *Coregonus lavaretus* mitochondrion sequence (NC002646) as a reference sequence. Lineage identifications were done by eye at the respective SNPs. Concatenated consensus ancient sequences (all four d-loop fragments combined into a single fragment of 140–141 bp, primer sites excluded, *n* = 24), along with published sequences (concatenated to 140–141 bp) of *Coregonus lavaretus* spp. from the European pre-Alpine/Alpine Region (Rhône Drainage, France, *n* = 12; Rhine Drainage, Switzerland, *n* = 270; Danube Drainage, South Germany, *n* = 57), Northern Europe (*n* = 34) [12] (see Table S2), were used to construct a median-joining network [54] with NETWORK 4.6.1.3 (http://www.fluxus-engineering.com/sharenet.htm).

3. Results and Discussion

PCR reactions were performed on 48 archaeological *Coregonus* remains using primers that target four small non-contiguous segments of *Coregonus* d-loop DNA. Amplifications were successful and sequences were reliably retrieved for all d-loop segments in 50% of elements (five scales, 19 vertebrae) from seven out of 10 sites, covering the whole time range tested, except for the Early Medieval period (Figure 1, Table S1). This success rate is in the order of magnitude observed for PCR-based approaches when targeting the mtDNA of archaeological fish [32,55] or other animal remains from central European climate zones [56]. Even from unusually small quantities of starting material (50% of specimen weighing <10 mg and the smallest weighing <2 mg), vertebrae and scales provided a source of aDNA (Table S1). Sequences generated from all segments were repeatedly identical within one individual and SNPs were consistently typed. All fragments were identical to published *Coregonus* d-loop sequences. Therefore, a morphological and d-loop-based determination of *Coregonus* spp. gave congruent results.

For 19 vertebrae and five scales from three sites, amplifications completely failed for all segments/primer combinations. This applied in the first instance to the Neolithic lake-dwelling settlements of Stansstad-Kehrsiten and Arbon Bleiche 3 when stored samples were processed (Table S1). This agrees with earlier observations that PCR-based aDNA amplification from animal remains from waterlogged Neolithic contexts in Switzerland failed [56], with few exceptions [52]. The performance of advanced DNA techniques (NGS) when using the same type of material is as yet unknown. Interestingly, when we used specimens "freshly" re-sampled from cold stored sediments from Arbon Bleiche 3, d-loop sequences were obtained for seven out of eight samples tested (Table S1). We observed similar behaviour at other Neolithic lake shore settlements (e.g., Zürich Opéra, data not shown). Therefore, material for DNA analyses from waterlogged Neolithic specimens in the Alpine foreland is preferably taken directly from the excavations or whole sediment blocks (not individuals) should be cold stored [57,58]. Quite unexpectedly, all six samples of the Early Medieval dryland site at Tomils (Figure 1, Table S1) also failed to yield any amplifiable aDNA, suggesting the existence of taphonomic particularities associated with this site (e.g., specimens were not found in situ and were used to construct a floor insulation structure).

Sequences of ancient and modern concatenated d-loop segments from the whitefish radiation across Europe, including Switzerland, were compared to each other: Median-joining network analysis (Figure 2) showed that all ancient sequences fit within the haplotype diversity of the two major maternal lineages known from modern European whitefish (*C. lavaretus* complex), i.e., the C and N lineage [12]. Two ancient haplotypes (20 samples and one sample, respectively) affiliated to the C lineage and one haplotype (three samples) clustered with the N lineage (Figure 2). This is consistent with contemporary lineage distribution in the sub-Alpine region and, more specifically, within Switzerland (228 samples C lineage, 42 samples N lineage, see Table S2). In the future, SNP genotyping and NGS may be used to discern single *Coregonus* individuals, as well as species, properly and allow for more accurate quantitative estimates of diversity and population sizes, for e.g., from sediments [59].

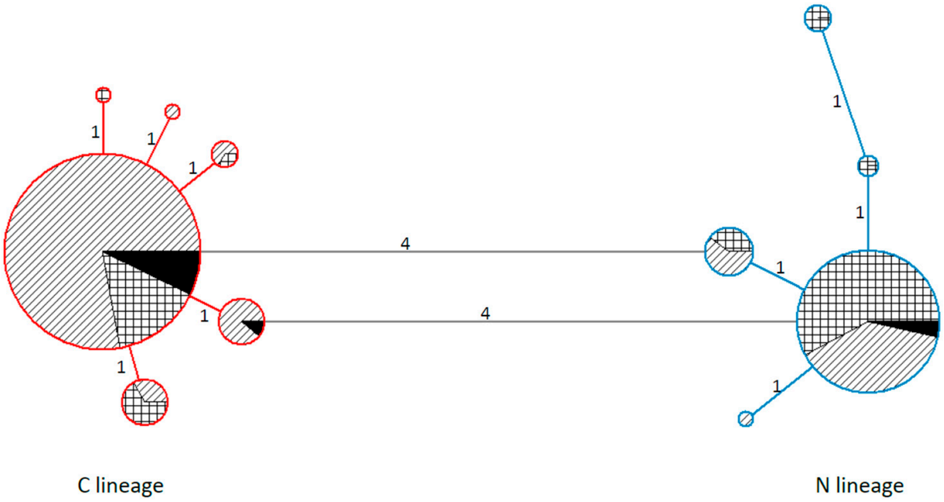


Figure 2. Median-joining network of concatenated *Coregonus* d-loop sequences (140–141 bp) displaying lineage distribution of archaeological samples from Switzerland (n = 24) (solid black) compared to published modern sequences from the European pre-Alpine/Alpine region including France (n =12), Switzerland (n = 270) and South-Germany (n = 57) (backward diagonal lines), and Northern Europe (n = 34) (cross lines). Size of nodes are proportional to haplotype frequencies. Numbers denote number of mutations between nodes.

The presence of both divergent mtDNA lineages C and N at the Neolithic site of Arbon Bleiche 3 (Lake Constance) and at Fraumünsterstrasse (Lake Zürich) from the 11th century AD (Figure 1) suggests co-existence for more than 5000 years in the sub-Alpine region. The Neolithic *Coregonus* from Lake Constance are probably among the ancestors of the recent adaptive radiation and both lineages are still present today at Lake Constance (28 samples C lineage, 14 samples N lineage (Figure 1, Table S2), and [12]. Furthermore, if, in the future, we will be able to identify the whitefish at Arbon Bleiche 3 as *C. wartmanni* ('Blaufelchen'), a contemporary pelagic whitefish species in Lake Constance [10], this would explain the archaeozoological evidence that during the Horgen culture people were fishing in open water using boats and sophisticated fishing techniques [18]. Given that in Lake Zürich only lineage C (20 samples) has been detected in modern whitefish (Figure 1, Table S2) and [10,12], it is possible that lineage N has gone extinct in this lake following recent or more ancient natural and/or anthropogenic impacts at some point after the 11th century AD. It is known that in recent times, whitefish types disappeared and re-appeared in the Swiss lakes [15].

In the future, SNP genotyping and NGS may be used to discern ancient whitefish species and allow for more accurate assessments of past diversity important not only for archaeological issues, but also for evaluating the history of freshwater ecosystems and consequences of species protection.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/3/34/s1, Table S1: Specifications of samples and d-loop PCR amplification success, Table S2: Compiled samples used in median-joining network analysis.

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