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In Search of the Lost Brown Trout (*Salmo trutta* Complex) Using DNA of Ancient and Modern Samples From the Southern Italian Peninsula

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ABSTRACT

The current diversity and distribution of species and populations have been shaped by the major climatic oscillations during the Quaternary. The brown trout (*Salmo trutta*) is a striking example of the strong effect of past climate changes on the evolutionary history of species, in fact, the alternation of glacial/interglacial cycles has led to continuous phenomena of expansion and contraction of its distribution and, consequently, to the evolution of different genetic lineages. This phenomenon was also directly observed by the genetic analysis of *S. trutta* bones recovered from the archaeological assemblage of a coastal cave named 'Grotta del Santuario Della Madonna' (Southern Italy). In this study, additional *S. trutta* remains were morphologically and genetically analysed, and the genetic results were compared with those of modern samples caught from streams close to the cave locality. The analysis of *S. trutta* bones confirms that changes in abundance, body size and genetic diversity observed in different stratigraphic sections were determined by environmental changes caused by the Younger Dryas-Holocene transition and by the ability of the species to perform anadromous migration, a behaviour that had disappeared at the end of the Pleistocene in the Mediterranean region. The comparison between ancient and modern samples highlights that extinctions and recolonizations due to past environmental changes affected also the current diversity and distribution of *S. trutta* in Southern Italian streams. Unfortunately, incorrect management policies related to sport fishing (i.e., stocking with alien brown trout strains) have been eroding millions of years of the trout evolution in just a few centuries.

1 | Introduction

Ocean sediments and ice cores have hidden valuable information about the Earth's history for millions of years. The oxygen isotopic composition of ice cores and of benthic/planktonic foraminifera remains in marine sediments have allowed the reconstruction of the Quaternary palaeoclimate, indicating that this period was characterized by the succession of several glacial/interglacial cycles (Dawson 2013). Glacial and interglacial periods were often punctuated by short events of rapid cooling (stadials)

or warming (interstadials) in the Northern Hemisphere during the last glacial-interglacial transition when, an initial warming phase (Bölling-Allerød interstadial, c. 14,700–12,800 cal yr BP) was followed by an abrupt restoration of cold conditions (Younger Dryas stadial, c. 12,800–11,600 cal yr BP) (Alley 2000; Dawson 2013; Hoek 2008; Rasmussen et al. 2006). The major climatic oscillations during the Pleistocene (from c. 2.58 million to 11,700 years ago) have influenced the geographical distribution of species, to the onset of adverse environmental conditions. Some of the species became extinct while others moved to find a

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suitable habitat and survived in refugial areas (e.g., Hewitt 1996, 2004; Stewart et al. 2010; Taberlet et al. 1998). The species that adapted to the cold of the Palaearctic region were usually confined in northern 'polar refugia' during warm interglacials, while temperate species used 'southern refugia' during glacial periods to escape the advance of the northern ice sheet (Stewart et al. 2010). When suitable environmental conditions were restored, species were able to re-expand their distribution range. Expansion/contraction phenomenon resulting from the alternation of glacial and interglacial periods, had important genetic consequences on species. Isolation in refugia for a long time prevented or limited contacts between individuals, bringing to the evolution of genetically differentiated and locally adapted populations and, in some cases, to speciation (Hewitt 1996, 2004).

Glacial–interglacial cycles during the Pleistocene had a strong impact on the evolutionary history of the brown trout of the *S. trutta* L. 1758 complex, partly explained by the analysis of the mitochondrial DNA control region (mtDNA CR) (Bernatchez 2001). The brown trout is a freshwater fish native to Europe, Western Asia and North Africa. It is currently considered as a 'species complex', as many differences in genetics/genomics (Hashemzadeh Segherloo et al. 2021; Splendiani et al. 2024), morphology (Delling et al. 2020) and life history traits (Klemetsen 2013) were recorded throughout the distribution range. The mtDNA CR analysis led initially to the description of five major lineages with a broad distribution. The Adriatic lineage (AD) is distributed in the Mediterranean area, predominantly in eastern tributaries, with populations in the eastern Iberian Peninsula, Corsica, Italian and Balkan basins, and the Rhone tributary. The marmoratus lineage (MA) is restricted primarily to northern Adriatic rivers in the Italian and Balkan peninsulas. The Mediterranean lineage (ME) is found in the western Mediterranean basin (Iberian Peninsula, France, Italian Peninsula and Corsica). The Danubian lineage (DA) is present in the Ponto-Caspian basin, while the Atlantic lineage (AT) is distributed across the European Atlantic and North African basins (Cortey et al. 2004; Oliver 2014; Pustovrh et al. 2011; Splendiani, Berrebi, et al. 2020; Splendiani et al. 2024). Within the AT lineage, it has been possible to distinguish between the Northern AT lineage (N-AT) and the Southern AT lineage (S-AT), the latter distributed in the Southern Atlantic Iberian Peninsula, North Africa and Sicily (Cortey et al. 2004; Snoj et al. 2011; Tougaard et al. 2018).

Isolation in glacial refugia followed by post-glacial recolonisation has been invoked to explain the present-day distribution of genetic lineages in several Palaearctic temperate fish species, such as the European perch (*Perca fluviatilis*; Nesbø et al. 1999), grayling (*Thymallus thymallus*; Koskinen et al. 2000) and European chub (*Squalius cephalus*; Seifertová et al. 2012). Specifically for *S. trutta*, individuals survived being confined in Southern ice-free refugia (e.g., Iberian, Italian and Balkan peninsulas) during glacial periods while they moved northward to re-expand their distribution range during interglacial periods. The dispersal was aided by the ability of *S. trutta* to perform anadromous migrations that allowed the sea dispersion and the colonization of new rivers (Cortey et al. 2009; D'Aurelio et al. 2025), a process promoted by straying behaviour. Despite natal homing is the predominant strategy in sea trout, some individuals are able to move between adjacent coastal catchments

and enter non-natal rivers to spawn, as shown by genetic, tagging and otolith microchemistry studies performed in northern Europe (Källo et al. 2022, 2024). In addition, populations living in the southernmost part of the distribution range may have survived warming phases by using the headstream river sections at high altitudes as refugia (Cortey et al. 2009). The isolation phases encouraged genetic differentiation while subsequent range expansion brought these differentiated lineages back into contact, thus shaping the current genetic diversity and distribution of *S. trutta* lineages. In addition to natural causes, the distribution of *S. trutta* has been manipulated by anthropogenic activities over time. For example, trout belonging to the N-AT lineage have been spread worldwide for farming and stocking purposes (Kottelat and Freyhof 2007). These introductions have eroded native genetic diversity due to introgression, and in some cases, have led to the complete replacement of local populations (Caputo et al. 2004; Splendiani et al. 2024).

Several life history strategies have been described for *S. trutta* including individuals who spend their entire lives in rivers (river-residency) or lakes (lake-residency) and individuals migrating between two distinct freshwater habitats (potamodromy) or from freshwater to the sea (anadromy) (Dodson et al. 2013; Ferguson et al. 2019). Residential and migratory forms of *S. trutta* can coexist in the same population (facultative migration) and the choice to migrate or not depends on a complex combination of genetic and environmental factors (Dodson et al. 2013; Ferguson et al. 2019). The anadromous behaviour is currently observed only in individuals of *S. trutta* inhabiting the Atlantic area, from Iceland to the northern Iberian peninsula, and those inhabiting the Black Sea and Caspian Sea (Elliott 1994); while in the Mediterranean area the anadromy probably disappeared during the Pleistocene–Holocene transition when the Mediterranean Sea became unsuitable for the salmonids' survival due to the increase in temperature and salinity (Bianco 1990; Durante 1978; Tortonese 1970). Sporadic captures of anadromous individuals in the Mediterranean basin, recognizable by the silver colouration assumed during the smoltification process, are attributable to the migration of Atlantic individuals with a farm origin that have retained their migratory behaviour or in which the anadromy was reactivated due to unfavourable environmental factors (Berrebi et al. 2022; Snoj et al. 2002; Splendiani, Fioravanti, et al. 2020).

Subfossil remains of *S. trutta* are abundant in Italian archaeological sites dating from the Last Glacial Maximum (LGM) to the beginning of the Holocene, but they have a discontinuous distribution and are concentrated in few sites. Trout fishing, as an activity to complement mammal hunting, was recorded in the Eastern Alps at Riparo Dalmeri (Albertini and Tagliacozzo 2004) and, a large assemblage of trout remains were found in caves and shelters of the Fucino Basin, central Italy (Wilkens 1994; Russ 2010; Russ and Jones 2009). In central and southern Italy, especially along the Adriatic coast, the sea level rise in the early Holocene progressively hides a large part of the coastal settlements (Antonioli et al. 1996; Castagnino Berlinghieri et al. 2020; De Pippo and Pennetta 2004; Radić Rossi et al. 2020). Bone remains morphologically attributable to *S. trutta* were also found in the LGM levels of caves (e.g., Arene Candide and Balzi Rossi) located on cliffs overlooking the Ligurian Sea near Ventimiglia (Albertini et al. 2023) and in the

coastal cave of 'Grotta del Santuario Della Madonna' (Southern Italy, hereafter referred to as GSM) (Durante 1978). A high abundance of *S. trutta* remains in the Upper Palaeolithic layers of GSM was reported by Durante (1978), who describes these bones larger than those of trout caught from mountain streams of Central and Southern Italy, and more similar in size to anadromous trout from northern Europe. The combination of large bone sizes and the coastal location of many of these sites has led some authors to hypothesize the past presence of anadromous trout in the Mediterranean Sea. Salmonid remains, often associated with those of Atlantic gadids, were also found in archaeological sites located along the Spanish coast from Málaga to Catalonia (Juan-Muns 1982; Muñoz and Casadevall 1997; Morales-Muñiz and Roselló Izquierdo 2008), indicating that the Mediterranean area may have constituted in the past an important refuge for species restricted to colder waters (Kettle et al. 2011; Albertini et al. 2023).

The GSM is located south of the Praia a Mare village (Cosenza, Calabria, Italy) at the foot of the Vingiolo Mount (c. 50 m above sea level) (39°53'48" N, 15°47'04" E). The cave is 4 km away south of the mouth of the Noce River and about 500 m from the

Tyrrhenian Sea (Figure 1). This cave represents one of the most significant archaeological sites of Southern Italy, due to the exceptional sequence of deposits documenting the human occupation from the Upper Palaeolithic through the Middle Ages. Between 1959 and 1967, six different excavation campaigns were carried out by the 'Istituto Italiano di Paleontologia Umana' (IsIPU) (Bernabò Brea and Cavalier 2000; Cardini 1970). The excavation proceeded towards south-west along the rocky wall with a series of depth increases and several consecutive extensions by adding artificial sections with a thickness between 5 and 10 cm, and separating possible areas disturbed by percolation. The excavation unveiled a stratigraphic succession of about 12 m (Figure 2), divided into 11 layers labelled with alphabetic letters from A to M. Each layer was further subdivided into different sections numbered from 0 (top of the layer A) to 73 (bottom of the layer L). The base of the section 72 is at -7.30 m depth and the first evidence of human habitation was found there although human activity became more pronounced between sections 65 and 41, as demonstrated by a rich archaeological assemblage which includes lithic artifacts, charcoals and faunal remains (Bernabò Brea and Cavalier 2000; Cardini 1970). Besides fish remains, the faunal assemblage is largely dominated by wild

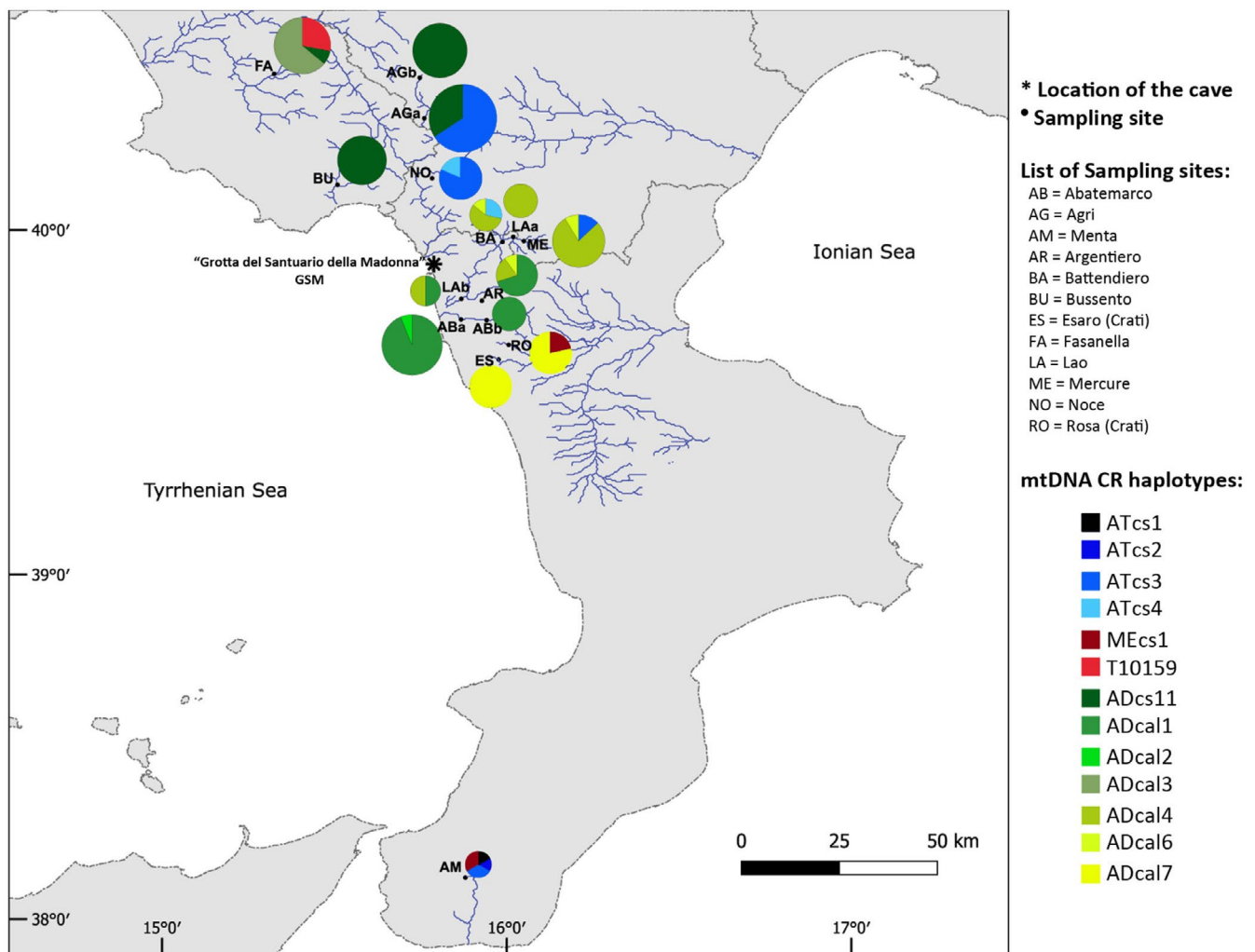


FIGURE 1 | Map of the study area showing the position of the cave 'Grotta del Santuario Della Madonna' (GSM) and sampling sites from which *Salmo trutta* modern individuals were caught. The geographic distribution and frequency of mtDNA CR haplotypes found in modern samples were visualised by pie charts.

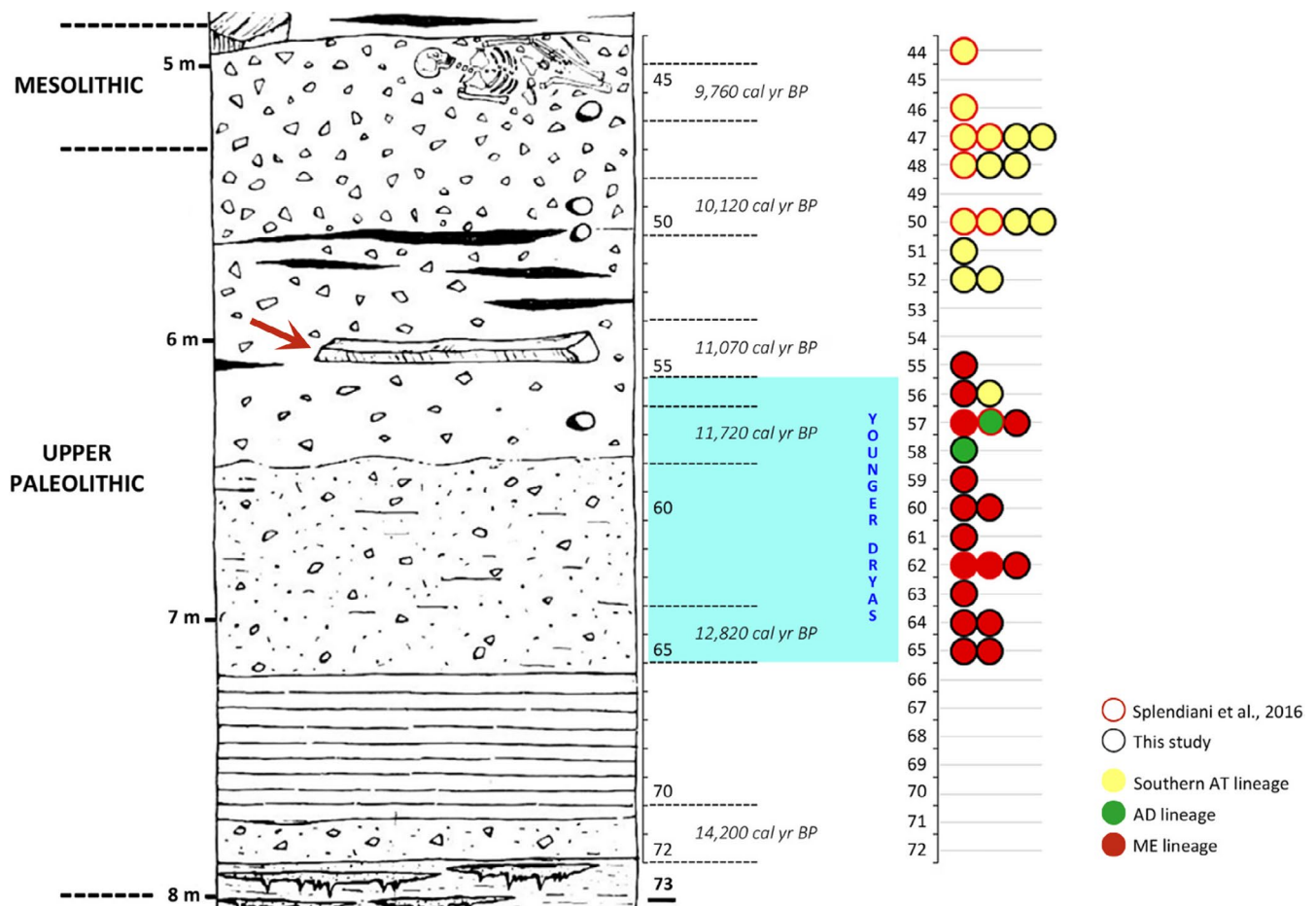


FIGURE 2 | Illustration of part of the stratigraphical sequence from 'Grotta del Santuario della Madonna' (modified from Cardini 1970) corresponding to Upper Palaeolithic and Mesolithic periods. The stratigraphical sequences were divided into sections (from 73 to 44) to which calibrated radiocarbon dating (from Splendiani, Fioravanti, et al. 2016) and the results of genetic analysis on ancient *Salmo trutta* bones were related. The Younger Dryas (c. 12,800–11,600 cal yr BP) is indicated approximately on the basis of the available radiocarbon dates. The red arrow indicates a large boulder probably fallen from the vault of the cave.

boar, red deer and roe deer, while carnivores, birds, turtles and molluscs are also represented (Durante and Settepassi 1972; Fiore, Gala, et al. 2004; Fiore, Pino Uría, and Tagliacozzo 2004; Gala and Tagliacozzo 2004; Pino Uría and Tagliacozzo 2008). Most of the *S. trutta* remains identified at the GSM site originate from the two deepest layers of the stratigraphic sequence (layers I and L). Level I (sections 42–47) is composed of sandy sediments with angular clasts, faunal remains and remains of fireplaces. Radiocarbon analysis has provided uncalibrated ^{14}C dates between 9070 ± 80 and 8735 ± 80 years BP (Alessio et al. 1966, 1967). Level L is composed of a first layer with more calcareous, sandy sediments with gravel and remains of fireplaces (sections 48–58), followed by sandy sediments with angular clasts (sections 59–65) and about 50 cm of finely stratified clay (sections 66–70) and sandy sediments with gravel at the base (sections 71–73) (Cardini 1970). Between sections 51 and 54 a large boulder had collapsed from the vault of the cave, probably as a consequence of ancient seismic activity, significantly reducing the excavation area (Robustelli and Lucà 2023). Layer L is characterized by abundant lithic industry and remains of mammals, birds, fish, marine and terrestrial molluscs (Tagliacozzo et al. 2016). Uncalibrated ^{14}C dates obtained from this layer range from $12,100 \pm 150$ to 9020 ± 125 years BP (Alessio et al. 1966, 1967). Radiocarbon dating measurements were later calibrated (Mussi

and Peresani 2011; Splendiani, Fioravanti, et al. 2016) suggesting that the first signs of human presence (section 72) date back to 14,200 cal yr BP while the following stratigraphic sections of interest for the present study (sections from 65 to 55) were deposited during the Pleistocene–Holocene transition (between Upper Palaeolithic and Mesolithic, c. 12,800–9000 cal yr BP) (Figure 2).

A preliminary ancient DNA (aDNA) study was carried out by Splendiani, Fioravanti, et al. (2016) on a small number of *S. trutta* bones from the GSM. Results from Splendiani, Fioravanti, et al. (2016) showed the presence of different genetic lineages inhabiting southern Italian rivers in a period between the Younger Dryas (c. 12,800–11,600 cal yr BP) and the early Holocene (c. 9000 cal yr BP). The study also allowed to identify, in addition to common lineages of the Mediterranean area (ME and AD), the presence of the S-AT lineage which is currently distributed only in the Iberian Peninsula, North Africa and Sicily. Sicily seems to be the only Italian region that now hosts trout belonging to this lineage (Fruciano et al. 2014; Sanz et al. 2023; Schöffmann et al. 2007; Snoj et al. 2011), while the Tyrrhenian area is characterized by trout belonging only to ME and AD lineages (Berrebi et al. 2019). However, the mtDNA CR diversity in the southern Italian peninsula was assessed by Berrebi et al. (2019) on a

limited number of samples from Calabrian rivers, making any conclusions about the possible presence of remnant S-AT trout in this area necessarily preliminary. In this study, fish remains excavated from GSM were morphologically re-analysed to accurately select those belonging to different *S. trutta* individuals. Genetic analyses were then performed on the selected specimens to validate, using a greater number of samples, the results previously obtained by Splendiani, Fioravanti, et al. (2016) and to assess the body size of the trout individuals. Furthermore, modern individuals from several streams surrounding the archaeological site were analysed to better investigate the presence of remnant S-AT trout populations in southern Italy and to fill a gap in the knowledge of the genetic diversity of the *S. trutta* complex in this region. The use of *S. trutta* as a model species in this work is due not only to the abundance of remains at the GSM archaeological site, but especially to its complex phylogeography, strong population structure, and sensitivity to environmental and anthropogenic changes, which make it particularly suitable for temporal genetic studies.

2 | Materials and Methods

2.1 | Morphological Identification of Fish Remains

Fish remains excavated from GSM are currently stored at the 'Museo delle Civiltà' (MUCIV, Rome, Italy) and were recovered manually during the ISIPU excavation campaigns. The sediments were initially dry-sieved using a 1 cm mesh and subsequently water-sieved with a 2 mm mesh (Fiore, Gala, et al. 2004; Fiore, Pino Uría, and Tagliacozzo 2004). Remains recovered from sections 39 to 72 were examined and taxonomically assessed to recognise those belonging to *S. trutta*. The number of remains (NR) and the minimum number of individuals (MNI) were calculated, the latter using the method proposed by Bökönyi (1970), and trout vertebrae were classified following criteria and terminology proposed by Morales-Muñiz (1984). Among trout bones, 97 right dentaries were selected for successive analyses and were considered representative of different individuals as they all preserved the symphyseal region. In this way, the selection of bone samples that belong to the same individual can be avoided. All dentaries were weighed and measured with a calliper following the indications for the measurement provided by Morales-Muñiz and Rosenlund (1979). In the absence of specific regression tables, dentary dimensions were compared with a collection of skeletal elements from modern *S. trutta* individuals of known length to allow an approximate estimation of the archaeological specimens' total length (TL) and an assignment to wide size classes (c. 10 cm of TL) was performed. Taxonomic and osteometric analyses were carried out by comparing the selected trout bones with those of current *S. trutta* skeletons from the Bioarchaeology Service of the MUCIV and anadromous specimens from Asturias stored at the Zoological Archaeology Laboratory of the Universidad Autónoma de Madrid.

2.2 | Genetic Analysis of Ancient Samples

Among the 97 right dentaries chosen as suitable for DNA analysis (TP1–TP97), 32 representative samples of stratigraphic sections between 47 and 65 were used for DNA extraction (Table S1,

Figure S1). The bone surface was decontaminated as proposed in Speller et al. (2005), subsequently, the bone samples were crushed and used for DNA extraction following a modified silica-spin column method (Yang et al. 2004, 2008). In brief, c. 200 mg of bone powder was poured in 4 mL of digestion buffer containing 0.5 M EDTA (pH 8.0), 0.5% SDS and 0.5 mg/mL proteinase K and incubated in a shaking water bath at 55°C overnight. The undigested material was separated by centrifugation, and the supernatant was recovered and concentrated up to c. 125 µL using Amicon Ultra-15 centrifugal filter units (30 kDa MWCO; Millipore). The DNA was purified from the recovered solution using the QIAquick PCR Purification Kit (QIAGEN) and eluted in 100 µL of sterile ddH₂O.

A 633 bp region of the mtDNA, including 85 bp of the tRNA Pro gene and 548 bp of the mtDNA CR, was amplified by polymerase chain reaction (PCR) using a set of eight specific primer pairs designed and tested by Splendiani, Fioravanti, et al. (2016). All PCR amplifications were performed in a 25 µL reaction solution containing 2.5 µL of 10× PCR Buffer (Invitrogen), 0.75 µL of 50 mM magnesium chloride (Invitrogen), 0.2 µL of 10 mM dNTP mix (Invitrogen), 4.8 µL of 5 µM F + R primer solution, 0.2 µL of Platinum Taq DNA Polymerase (Invitrogen), 3 µL of DNA template and 13.55 µL of sterile ddH₂O. Thermal cycling conditions were set to have an initial denaturation step at 94°C for 7 min, followed by 60 cycles of denaturation at 94°C for 20 s, annealing at 53.9°C for 30 s, an extension at 72°C for 40 s and a final extension step at 72°C for 7 min. All amplicons were run on a 2% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotium) and visualised using a UV transilluminator. PCR products were purified using ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems) and Sanger sequenced in both directions on an ABI3730xl DNA Analyzer (Applied Biosystems) at the BMR Genomics (Padova, Italy).

Sequences obtained from ancient samples were compared using Nucleotide BLAST (Altschul et al. 1990) with all the sequences available in GenBank to detect any match. In addition, a multiple sequence alignment was performed using Clustal W (Larkin et al. 2007) and edited using BioEdit 7.2.5 (Hall 1999). The alignment includes mtDNA CR haplotypes representative of the major *S. trutta* lineages described so far for the Mediterranean area (ATcs1, ATcs2, ATcs3, ATcs4, ATcs25, ATcs33, Mecs1, ADcs1, ADcs11) and sequences previously obtained from 11 ancient samples from the same stratigraphical succession (Splendiani, Fioravanti, et al. 2016). To evaluate variation in haplotype composition over time, proposed by Splendiani, Fioravanti, et al. (2016), haplotypes were related to the stratigraphical sections of origin of each sample analysed and, when possible, to the calibrated radiocarbon dating (Alessio et al. 1966, 1967; Splendiani, Fioravanti, et al. 2016).

To minimize the risk of contamination by exogenous DNA of ancient samples, all pre-PCR steps (decontamination, DNA extraction, PCR reaction preparation) were carried out in a dedicated laboratory located in a different building from the post-PCR area and from the laboratory in which modern samples were analysed (Knapp et al. 2012; Poinar and Cooper 2000). Access to the aDNA laboratory was allowed only to authorized staff equipped with full-body disposable suits, specific shoes, protective face masks and gloves. Movements of the staff from

this laboratory to the post-PCR area should have always been unidirectional (Knapp et al. 2012; Poinar and Cooper 2000). To eliminate potential exogenous DNA in the aDNA laboratory, all surfaces were regularly cleaned with 10% bleach, while sensitive materials and instruments were cleaned with DNA AWAY Surface Decontaminant (Thermo Scientific) and/or UV irradiated for at least 30 min. In addition, contamination controls were always included during DNA extraction and PCR reaction preparation steps to detect any possible contamination (Knapp et al. 2012; Poinar and Cooper 2000).

2.3 | Genetic Analysis of Modern Samples

A total of 275 *S. trutta* individuals were sampled between 2014 and 2023 from 14 sites selected from different streams that crossed the same geographical area or regions closed to the GSM locality (Figure 1). Fish were caught by electrofishing and a small piece of anal fin was collected and stored in 96% ethanol until genetic analyses were performed. Total DNA was extracted using a MagCore HF 16 automated nucleic acid extractor (RBC Bioscience) with MagCore Genomic DNA Tissue Kit (Cartridge Code 401) according to the manufacturer's instructions and the amount and purity of DNA extracted was evaluated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

The mtDNA CR of all modern samples was screened using a PCR-RFLP (restriction fragment length polymorphism) procedure followed by a SSCP (single-strand conformation polymorphism) analysis. All PCR amplifications were performed using Rib28a (Schöffmann et al. 2007) and HN20 (Bernatchez and Danzmann 1993) primers in a 25 µL reaction solution containing 5 µL of 5× PrimeSTAR GXL DNA Polymerase (Takara), 2 µL of 10 mM dNTP mix (Invitrogen), 2 µL of 5 µM F + R primer solution, 0.8 µL of PrimeSTAR GXL DNA Polymerase (Takara), 3 µL of DNA template (40 ng/µL) and 12.2 µL of sterile ddH₂O. Thermal cycling conditions include an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 60 s and a final extension step at 72°C for 7 min. After amplification, PCR products were digested overnight at 37°C using the AluI restriction enzyme (Thermo Scientific), a step followed by SSCP analysis performed according to Righi et al. (2020). To attribute a complete mtDNA CR sequence to each modern sample analysed, a minimum of two amplicons for each SSCP pattern observed were sent to BMR genomics (Padova, Italy) for ExoSAP-IT (Applied Biosystems) purification and Sanger sequencing on an ABI3730xl DNA Analyzer (Applied Biosystems).

Sequences obtained from modern samples were compared using Nucleotide BLAST (Altschul et al. 1990) with all the sequences available in GenBank and aligned using Clustal W (Larkin et al. 2007) with 73 mtDNA CR sequences downloaded from GenBank and representative of the main *S. trutta* lineages and with sequences from ancient samples separately. Alignments were edited using BioEdit 7.2.5 (Hall 1999) and the genetic relationships among modern haplotypes were shown on a maximum parsimony haplotype network constructed on TCS v1.23 (Clement et al. 2000) and edited using tcsBU (Múrias Dos Santos et al. 2016). A phylogenetic tree was also constructed using

MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) implemented in Geneious Prime 2022.1.1 (<https://www.geneious.com>) follow the HKI + G + I model. The best nucleotide substitution model was selected according to the Bayesian information criterion (BIC) using the program jModeltest 2.1.3 (Darriba et al. 2012). The mtDNA CR of the Atlantic salmon (*Salmo salar*) was used as an outgroup during the phylogenetic tree construction.

3 | Results

3.1 | Morphological Analysis

A total of 8311 fish remains were identified between sections 39 and 72, making them one of the most abundant faunal components at the GSM archaeological site (Table S2). Excluding unidentifiable bones from the overall fish sample (4915 out of 8311 fish remains), the analysis of the remaining 3396 bones has allowed the identification of 2479 bones (73%) belonging to *S. trutta*, 752 bones (22.1%) belonging to *Anguilla anguilla*, and 165 bones (4.9%) belonging to other fish species (Table S3). The Holocene levels contain few fish remains, even considering the larger area excavated for these periods (ca 15.30 m²), with remains of eel and various marine species. Fish remains were in an excellent state of preservation. In fact, lepidotrichia and vertebrae were identified still in anatomical connection, probably aided by the formation of calcareous concretions due to the abundant dripping of water, and the bone surfaces were also quite well preserved. A large number of cranial and post-cranial bones belonging to *S. trutta* were found in all the stratigraphical column between the sections 40 and 65 (Table S4) with two main apparent abundance peaks, one in correspondence with section 50 and the other with section 61 (Figure 3). However, the decrease in fish remains was probably more gradual, as these two peaks could be an artefact due to the collapse of a boulder from the cave vault, with the consequent alteration of sections 53 and 54. The presence of the large boulder is shown in Figure 2, exactly at the transition between sections 51 and 54. Two hundred and ninety-six trout dental fragments (including right symphyseal portions) were referable to at least 111 individuals. Eel remains became abundant starting from section 56, with two apparent abundance peaks in sections 55 and 52, and bone remains of other fish species were instead present with low and constant abundances in all the sections examined (Figure 3).

The total length estimation for *S. trutta* individuals performed on the dentaries highlights that individuals between 27 and 52 cm in length were discovered in most of the sections. Individuals smaller than 27 cm were found exclusively in section 61 and the largest individuals (> 52 cm in length) were present almost exclusively in sections between 58 and 65, whereas all size classes were represented only in section 61 (Figure 3). In addition, sections with the greatest abundance of *S. trutta* remains and with the largest estimated size were those included in the stratigraphical sections from 55 to 65 (Figure 3), which, considering the calibrated radiocarbon dates, were deposited during the Younger Dryas stadial (Figure 2).

In sections 44–47, trout bones still represented more than half of the remains (274 NR) and belonging also to large specimens, reaching or exceeding 57 cm of TL (at least two quadrates, one

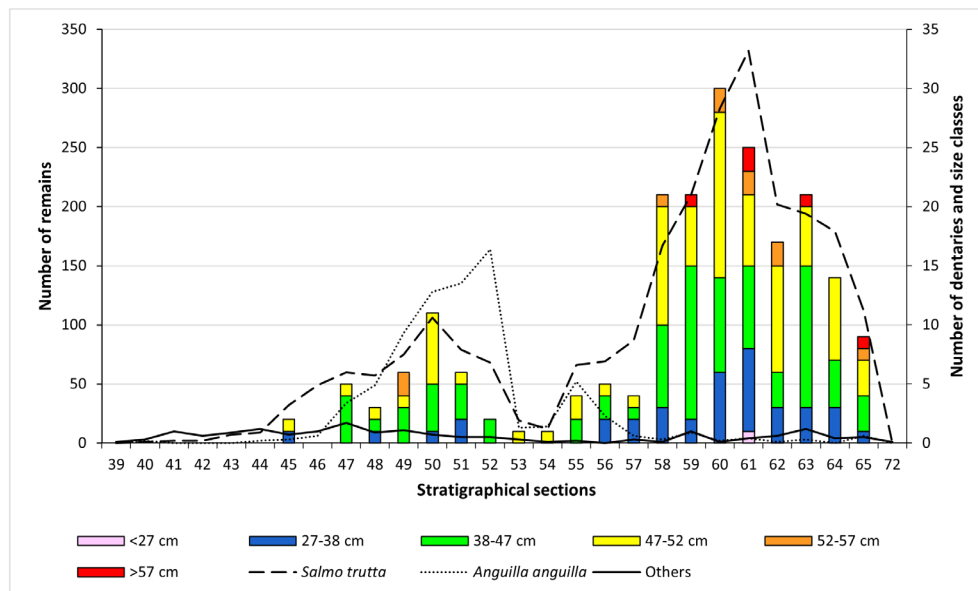


FIGURE 3 | Graph showing the relationship between the abundance of fish remains (line chart), number of dentaries and estimated size classes (bar chart) and stratigraphical sections.

articular and one palatine) and 21 NR with a TL between 50 and 57 cm (Figure 3). The section 48 is characterized by the progressive increase in *S. trutta* remains, which exceed 90% of fish remains between sections 57 and 65, with a maximum of 99% in section 60. Between sections 52 and 65, different skeletal elements (some dentals, but also two palatines and two glossohyals) belonging to individuals with a TL > 57 cm were identified (Table S4; Figure 3). The relative scarcity of vertebral elements, only 4.7% of those expected based on the MNI calculation (Table S4), is evident when observing the skeletal components especially in sections 48–65 of the stratigraphic column.

3.2 | Ancient DNA

For ancient samples, the set of primers designed by Splendiani, Fioravanti, et al. (2016) allowed us to obtain a 448bp discontinuous sequence of the mtDNA CR. The amplification with all the primer pairs failed for eight samples out of the 32 analysed (TP34, TP82, TP64, TP13, TP42, TP39, TP73, TP80) while the complete sequence of interest was successfully obtained for eight samples (TP11, TP29, TP33, TP58, TP52, TP61, TP71, TP10). A 435bp in length sequence was obtained for five samples (TP15, TP31, TP18, TP63, TP12) and shorter sequences, between 92 and 406bp in length, were obtained for the 11 remaining samples (TP44, TP41, TP47, TP59, TP26, TP60, TP17, TP24, TP05, TP40, TP50) (Table S5). The alignment of the complete region of interest with some of the mtDNA CR haplotypes belonging to the major *S. trutta* lineages highlights the presence of 10 diagnostic sites (Table S6). All diagnostic sites are recognizable in both 448 and 435 bp in length sequences, while for shorter sequences from two to nine diagnostic sites were identified. However, for all the ancient samples analysed, the haplotypes were determined by comparing the diagnostic sites obtained with those of modern haplotypes and with those of samples coming from other stratigraphic sections (Table S6). Ten samples were classified

as belonging to the ATcs33 haplotype (TP11, TP29, TP33, TP58, TP52, TP44, TP41, TP47, TP59, TP15), 13 samples were identified as MEcs1 haplotype (TP26, TP60, TP17, TP24, TP61, TP71, TP05, TP10, TP18, TP48, TP63, TP12) and one sample was classified as ADcs11 haplotype (TP31). This latter came from the stratigraphical section 58, all the samples classified as belonging to the MEcs1 haplotype came from sections between 55 and 65, and samples belonging to the ATcs33 haplotype come from the sections between 47 and 52, except the TP15 sample which came from the section 58. Including data from Splendiani, Fioravanti, et al. (2016), samples belonging to the ATcs33 haplotype were distributed mainly among stratigraphic sections between 44 and 56 while samples identified as MEcs1 and ADcs11 haplotypes were mainly found in sections between 55 and 65, which were deposited during the Younger Dryas (Figure 2). We also found that the oldest sample (layer 65) from Splendiani, Fioravanti, et al. (2016), described as belonging to the Southern AT lineage, was misidentified and actually came from the section 47 (dated to c. 10,000 calyr BP) leading us to identify a single change in haplotype composition (from ME-AD to AT) exclusively at the end of the Younger Dryas.

3.3 | Modern DNA

The PCR-SSCP screening allowed the successful identification of 14 patterns corresponding to distinct haplotypes (c. 996bp in length) in 252 *S. trutta* modern individuals. The comparison of these haplotypes with those previously published showed the presence of seven haplotypes already described belonging to the AT (ATcs1, ATcs2, ATcs3, ATcs4; GenBank accession numbers: AF273086, AF273087, AF274574, AF274575), ME (MEcs1, T10159; GenBank accession numbers: AY836350, LT617622) and AD lineages (ADcs11; GenBank accession number: AY836340). Moreover, seven new haplotypes belonging to the AD lineage (ADcal1, ADcal2, ADcal3, ADcal4, ADcal5, ADcal6, ADcal7) were also found. After trimming the 82bp repeats from four of

them (ADcal1, ADcal4, ADcal5, ADcal6), ADcal4 and ADcal5 haplotypes collapsed into the same sequence which will hereafter be referred to as ADcal4 (Figure 4, Figure S2). The haplotypes identified seem to have a precise geographical distribution (Figure 1). Specifically, haplotypes of the ME lineage were found at the northernmost and southernmost investigated sites, and the MEcs1 haplotype was observed in the Fasanella, Rosa and Menta rivers while the MEcal1 haplotype was detected only in the Fasanella River. The AD lineage followed a north to south distribution. The ADcal3 haplotype was detected only in the northernmost site in Fasanella samples followed by the ADcs11 haplotype in the Bussento and Agri Rivers. ADcal4 and ADcal6 haplotypes were spread in the Lao basin, principally in sampling sites further upstream while the ADcal1 haplotype was distributed in the Abatemarco River and in the most coastal sampling site of the Lao basin; ADcal2 was observed at very low frequencies only in the Abatemarco River. Finally, the ADcal7 haplotype was detected in the Rosa and Esaro Rivers, flowing into the Ionian Sea. Finally, considering the allochthonous AT lineage, the Noce River is totally characterized by the presence of AT lineage from fish farms (ATcs3 and ATcs4 haplotypes), indicating a high degree of anthropogenic influence on this watercourse. In the Agri and Menta, sampling sites were also highly represented

by AT lineage, while their reduced presence was observed in the Battendiero and Mercure rivers.

The haplotype network (Figure 4) and the phylogenetic tree (Figure S2) show the relationship among all the haplotypes highlighting that the T10159 haplotype was very close to the MEcs1 haplotype; in fact, they only differ by a single mutation step. New haplotypes belonging to the AD lineage are the most common in the study area; ADcal2 and ADcal3 are related to the ADcs11 haplotype and differ from each other by few mutational steps (Figure 4, Figure S2). The ADcal1, ADcal4 and ADcal6 haplotypes, instead, are characterized by the presence of the 82 bp repeats towards the 3'-end of the mtDNA CR, and they are strictly related with the AD-Tyrrh4 haplotype (Figure 4; Berrebi et al. 2019). The observed AT haplotypes have a domestic origin belonging to the Northern AT lineage. Finally, the comparison among ancient and modern dataset, considering only the 448 bp in common, indicates that the T10159 haplotype is identical to the MEcs1 haplotype (Table S6), and all new AD haplotypes are identical to the ADcs11 haplotype except the ADcal2 haplotype, which differs from the latter due to a mutation in the diagnostic site 448 (Table S6). Instead, the AT haplotypes (ATcs1, ATcs2,

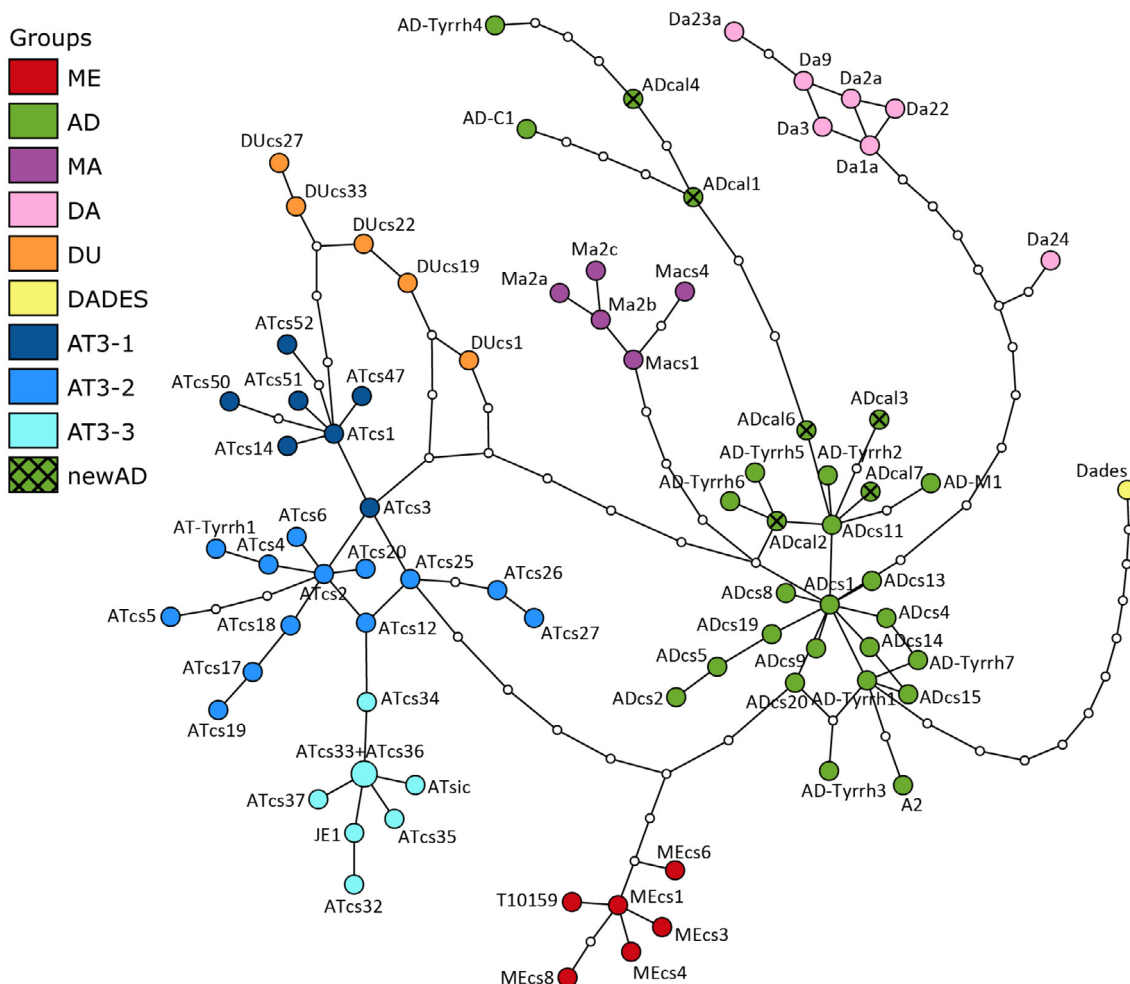


FIGURE 4 | Maximum Parsimony haplotype network showing the relationship between mtDNA CR haplotypes from modern *Salmo trutta* individuals analysed in this study and sequences obtained from the GenBank database. The sizes of circles are not proportional to observed haplotype frequencies and colours indicate the main evolutionary lineages described for *S. trutta*.

ATcs3, ATcs4) found in modern samples are different from the ATcs33 haplotype belonging to the Southern AT lineage, as shown comparing mutations in diagnostic sites 89, 123, 448 (Table S6).

4 | Discussion

4.1 | Preservation and Recovery of Trout Remains at GSM

The state of conservation of *S. trutta* bones analysed in the present study may explain the success of genetic analyses, only eight samples out of 32 selected did not allow the extraction of good quality DNA to allow the amplification of at least one mtDNA fragment out of eight. The amount of endogenous DNA that can be recovered from archaeological remains is influenced by the degree of bone preservation (Kendall et al. 2018). Natural processes of degradation of organic molecules are activated immediately after the death of an organism together with the decomposition due to microorganisms which lead to the degradation of DNA molecules in small fragments (Pääbo et al. 2004). Hydroxyapatite crystals, that make up the inorganic matrix of bone tissue, bind DNA molecules protecting them from degradation and promote the recovery of DNA suitable for genetic analysis. *S. trutta* bones from GSM were in an excellent state of preservation, as shown by the maintenance of anatomical connection between fins and some vertebrae, and by the recovery of many fragile skeletal elements such as lepidotrichia, spines and thin cranial bones. Although the taphonomic study is still ongoing, it is worth noting that the high presence of cranial elements in the stratigraphical sections under investigation may suggest on-site manipulation of carcasses (e.g., Russ and Jones 2009) and is also an indicator of the state of preservation of fish skeletons in archaeological sites. The salmonid skull is in fact characterized by the presence of very fragile bones with few well-ossified elements (except for the symphyseal portion of the dentary; Roselló Izquierdo and Morales-Muñiz 1990).

The high number of remains discovered in GSM confirms an intensive exploitation of fish resources during the first phase of occupation of the cave by humans (see also Durante 1978). The Upper Palaeolithic was characterized by an increase in fishing activities recorded in several archaeofauna assemblages as the human diet, based up to that moment principally on large mammals, was supplemented by hunting small mammals and exploiting marine and freshwater resources (Albertini and Tagliacozzo 2004; Blanco-Lapaz et al. 2023; Colonese et al. 2011; Dias et al. 2016; Russ 2010; Russ and Jones 2009; Wilkens 1994). Palaeolithic hunter-gatherers were opportunistic as they mainly exploited the most available resources near human shelters, freshwater fish when settled near rivers and marine resources when settled near the coast (Mannino et al. 2011; Turrero, Ardura, and García-Vázquez 2014; Turrero, García-Vázquez, and de Leaniz 2014). So, the geographical location of the cave (not far from both the Noce River and Tyrrhenian Sea) and the presence of remains belonging to migratory and brackish water species (as the European eel and the mullet, respectively) suggest that cavemen fished mainly in the lower part of the river and/or along the coast. Between the LGM and the beginning of the Holocene, salmonid fishing appears to have been

continuously practiced along the Atlantic coasts of the Iberian Peninsula (Álvarez Fernández and Fernández García 2012; Bicho and Esteves 2022; Blanco-Lapaz et al. 2023; Fuertes-Prieto et al. 2023), with evidence dating back to the Mousterian (Roselló-Izquierdo and Morales Muñiz 2005). Similar patterns are observed at various sites in southwestern France, including notable artistic representations dated to the Magdalenian (Cleyet-Merle 1991). Thanks to their anadromous behaviour, trout could feed in the sea, thus reaching particularly large sizes, which made their use as food by humans energetically advantageous. During the seasonal migration from the sea to rivers for reproduction, *S. trutta* individuals could be easily caught in shallow waters by hand or using rudimentary nets or harpoons (Russ 2016; Russ and Jones 2009; Turrero et al. 2012, 2013).

The morphological identification of fish bones from the faunal assemblage of GSM allows us to detect that most of them belong to *S. trutta* and, considering the radiocarbon dating, it is also evident that *S. trutta* remains are the most abundant in the assemblage during the cold phase of the Younger Dryas (12,800–11,600 calyr BP). An estimation of the size of *S. trutta* individuals was also carried out, and they were classified into different size classes. In general, most of the remains belonged to individuals between 27 and 52 cm in length; individuals larger than 52 cm were quite common while those smaller than 27 cm were rare. The estimated sizes for the trout exploited by the inhabitants of the Calabrian cave (mainly larger than 27 cm in length) are thus consistent with the anadromous lifestyle, as previously suggested also by Durante (1978). Anadromous populations of *S. trutta* are currently found from North Portugal to the White Sea, including Iceland, the Black Sea and the Caspian Sea, while the Mediterranean Sea hosts exclusively resident populations (Elliott 1994). Anadromy likely temporarily disappeared from this area at the end of the Last Glaciation, when increased water temperature and salinity made the Mediterranean Sea unsuitable for trout survival. Water temperature, dissolved oxygen and salinity are, in fact, limiting factors for growth and reproduction of *S. trutta* (Elliott and Elliott 2010; Jonsson 2023; Jonsson and Jonsson 2007, 2009). Resident trout are generally smaller than anadromous ones, as the growth rate at sea is higher than in freshwater. Anadromous trout, in fact, feed in a nutrient rich environment and, having a larger size, they can also exploit fish as prey, contrary to resident individuals which almost exclusively feed on zoobenthos and zooplankton (Jonsson and Jonsson 2007). The length at maturity in partially migratory populations was estimated between 15.2 and 23.5 cm in resident individuals, while anadromous individuals measure between 19.9 and 75.8 cm (Jonsson and Jonsson 2007, 2021). Subsequently, with the return of colder climatic conditions during the Younger Dryas, anadromous life histories may have reappeared in Mediterranean brown trout populations. The definitive loss of marine migratory behaviour in these populations likely occurred with the onset of the warmer climatic conditions of the early Holocene.

4.2 | Lineage Turnover and Body Size Variation Across Climatic Phases

The analysis of the mtDNA CR performed on 32 ancient samples confirms the preliminary results obtained by Splendiani,

Fioravanti, et al. (2016), analysing only few samples, indicating that the trout caught during different climate periods belong to distinct evolutionary lineages. During the cold period of the Younger Dryas, trout caught by the inhabitants of GSM belonged exclusively to the ME lineage and, during the following warming phase, only individuals of the S-AT lineage were caught. ME, AD and S-AT lineages seems to coexist during the transition among Younger Dryas and the beginning of the Holocene, when an overall decrease in size is observed, although some large individuals are still present (section 49 in stratigraphic column). Therefore, size variation could be related to change in environmental conditions rather than the effect of artificial selection due to fishing activity on a single population (e.g., Turrero, García-Vázquez, and de Leaniz 2014). The drastic thermal fluctuations during the Pleistocene–Holocene transition could explain such morphological change considering that bones belonging to larger individuals were mainly recovered in sections accumulated during the colder period of the Younger dryas while size and abundance tend to decrease at the beginning of the Holocene, while trout disappeared completely from the stratigraphic column starting from section 43. The Bergmann's rule describes the relationship between temperature and body size, stating that organisms living in cold areas are bigger than those living in warmer areas, however this ecological theory is not followed by all organisms (Ahti et al. 2020; Audzijonyte et al. 2020; Solokas et al. 2023). The analysis of *S. trutta* remains from Gran Dolina (Sierra de Atapuerca, Spain) did not highlight a statistical relationship between temperature and estimated body size of Pleistocene trout, demonstrating that body size of *S. trutta* do not follow the Bergmann's rule (Blanco-Lapaz et al. 2021). Changes in body size and genetics of *S. trutta* individuals from the Calabrian cave could therefore be directly linked to the transition from the cold period of the Younger Dryas to a warmer climate which characterized the beginning of the Holocene.

Multi-proxy studies demonstrate that short-term climatic events (e.g., Younger Dryas) recorded in the North Atlantic Ocean have also affected the Mediterranean palaeoclimate (Cacho et al. 2001; Dormoy et al. 2009). Marine pollen records of both western and eastern Mediterranean Sea detect cold and dry conditions during both the Oldest and Younger Dryas, while the Bölling-Allerod and the Holocene were characterized by temperate conditions. Furthermore, the Younger Dryas was affected by a reduction in winter precipitations while a 'Mediterranean' climate (hot dry summers and cool wet winters) was established during the Holocene (Dormoy et al. 2009). Sea surface temperature (SST) and sea surface salinity (SSS) of the southern Tyrrhenian Sea were reconstructed by the analysis of two sediment cores and compared with results from North Atlantic Ocean showing synchronous variations among these basins during the Last Glacial Maximum (Kallel et al. 1997). SSTs were similar to those recorded during the Bölling-Allerod and at the beginning of the Holocene while, during the cooling periods of the Oldest and Younger Dryas, SSTs values decreased according to North Atlantic conditions. Consequently, SSSs follow the temperature trend with warm periods characterized by higher salinity values while cold periods, Oldest Dryas and Younger Dryas, were characterized by a strong decrease in salinity (Kallel et al. 1997). The high-resolution integrated stratigraphy for the Late Quaternary described for the south-eastern Tyrrhenian Sea (Lirer et al. 2013) has brought us to confirm, based on differences

in the abundance and diversity of planktonic foraminiferal and nannoplankton in different stratigraphical layers, the influence of the Younger Dryas in this area. An increase of cool water foraminiferal species and a strong turnover in the abundance between calcareous nannofossils *Globigerina oceanica* and *Emiliana huxley* were recorded in the layer corresponding to the Younger Dryas event, indicating a rapid return to a cold climate. In addition, a high abundance of *Globigerina bulloides* and a rare occurrence of *Florisphaera profunda* were also observed during the Younger Dryas suggesting a period of high surface productivity (Lirer et al. 2013). During the Younger Dryas, environmental conditions suitable for anadromous migration (e.g., low temperature, low salinity, high productivity) characterize the Tyrrhenian area, and the greater availability of food at sea enabled the trout to reach larger sizes and to be fished easily in the period of return to the river for spawning. The re-establishment of a warm climate at the beginning of the Holocene led to temperature and salinity values of the Mediterranean similar to the current ones, causing the progressive loss of anadromy for native Mediterranean trout. In fact, the currently known cases of anadromous trout in this basin concern individuals of domestic origin that come from wild northern European populations where the anadromous tendency is still active (Splendiani, Fioravanti, et al. 2020). Climate conditions of the Younger Dryas have been linked to the increase in anadromous fish consumption by humans and animals also in other areas of the world. In North America, bone remains of anadromous salmonids are particularly abundant in archaeological excavations dating back to the end of the Pleistocene, suggesting that environmental conditions and the productivity of water basins were probably favourable to the establishment of spawning runs that made these species easily accessible for food consumption (Fedje et al. 2011; Halffman et al. 2015; Potter et al. 2023).

4.3 | Loss of Anadromous Behaviour in Mediterranean Trout

The results of genetic analysis have highlighted that only trout of the Mediterranean lineages (i.e., ME and AD, sensu Bernatchez 2001) were caught by humans during the Younger Dryas, probably because they were among the most abundant prey in that period in the lower part of the Noce river. The onset of colder climatic conditions may have promoted the activation of anadromous behaviour in ME and AD trout, bringing to the colonization of coastal river systems. At the end of the Younger Dryas, a change in haplotype composition of *S. trutta* populations was observed and the complete substitution of the ME and AD trout with those of the S-AT lineage was recorded. The transition from cool to warm conditions could have caused severe hydrological events (Forzoni et al. 2015), to which salmonids are particularly vulnerable (Pujolar et al. 2011). These events may have led to population declines or local extinctions of ME and AD trout, thereby providing anadromous S-AT trout with the opportunity to colonize newly available habitats (e.g., Ibrahim et al. 1996; Bernatchez 2001). The replacement of ME and AD lineages could also be explained by considering that, during this period of climatic transition, ME and AD trout may have reverted to a resident life history, whereas S-AT trout reacted to climatic change by shifting their range northwards through marine migration. This process likely ceased when rising salinity

and temperature rendered the Mediterranean unsuitable for anadromous salmonids (e.g., Kettle et al. 2008).

Individuals of the S-AT lineage seem to survive only in North Africa and Spain in the present day (Schöffmann et al. 2007; Snoj et al. 2011; Tougard et al. 2018), while in Italy, it exclusively lives in a few rivers of the Hyblean plateau, in eastern Sicily (Fruciano et al. 2014; Schöffmann et al. 2007). The possible existence of residual populations of the S-AT lineage was tentatively addressed by Berrebi et al. (2019), who analysed 13 individuals of *S. trutta* from Calabrian rivers. Unfortunately, this limited sample showed high levels of introgression with farmed AT strains, and the presence of native populations carrying exclusively AD haplotypes. Given the restricted number of samples examined, the findings were inconclusive regarding the persistence of S-AT lineages. Consequently, this limitation prompted us to expand the sampling effort in this geographic area to more accurately assess the potential presence of relict S-AT populations within the Italian Peninsula. However, the only AT haplotypes detected in our study are those belonging to the northern AT lineage, widespread in Italy as a consequence of intense stocking activities carried out in recreational fishing using trout from Northern Europe (Berrebi et al. 2019, 2021; Caputo et al. 2004; Splendiani et al. 2013; Splendiani, Ruggeri, et al. 2016). The analysis of modern individuals from the Noce River reveals the presence of only N-AT haplotypes of domestic origin (ATcs3 and ATcs4), while previous studies also found haplotypes of the AD lineage but at a low frequency in hybrid/introgressed individuals (Caricato et al. 2016). We can therefore hypothesize that during the progressive warming following the Younger Dryas, the presence of resident ME and AD trout in the upper courses of Southern Peninsular rivers may have prevented the establishment of the S-AT lineage. Considering that brown trout is an aggressive fish, forming territories and dominance hierarchies (Finstad et al. 2011), competitive exclusion under conditions of reduced productivity and climate change probably affects the colonization. Trout belonging to the S-AT lineage may have been able to persist only in a few rivers of the Hyblean Plateau in south-eastern Sicily (Schöffmann et al. 2007), likely due to the absence of competition and/or favourable hydrological conditions in Sicilian watercourses. The Hyblean plateau is characterized by carbonate substrates and aquifer systems that promote relatively stable flow regimes, conditions similar to those associated with the persistence of native genetic variability in *S. trutta* populations in Central Italy (Splendiani et al. 2013).

4.4 | Genetic Diversity and Evolutionary History of Southern Italian Trout

Among native haplotypes found in modern samples, six out of eight had never been observed in other areas, which is an expected result considering the high genetic diversity observed in brown trout populations of the Tyrrhenian region and that southern Italian trout have never been studied in depth so far (Berrebi et al. 2019). The ME lineage is not very common among sampled sites and is represented by only two haplotypes: haplotype MEcs1, T10159. The latter was detected also in one Corsican site (Tougard et al. 2018). In accordance with the first description of the genetic diversity of *S. trutta* in the Tyrrhenian area, the majority of haplotypes found in modern samples belong to the AD

lineage, being strictly related to the haplotype ADcs11 and with most of the AD haplotypes described in the Tyrrhenian area by Berrebi et al. (2019). The geographic distribution of haplotypes in the studied area and the evidence that connected watercourses host principally private haplotypes, indicates that a long history of the isolation of rivers has led to the differentiation of trout populations. The comparison of haplotypes found in ancient and modern samples suggests that ME and AD lineages have inhabited Southern Italy at least since the Pleistocene, although AD haplotypes were rare at the transition from Younger Dryas to Holocene they are currently more widespread. The different distribution of genetic lineages in space and time clearly suggests that the evolutionary history of brown trout in Southern Italy has been strongly influenced by local extinctions and recolonizations due to river captures (e.g., Bianco 1995) or by anadromous individuals coming from adjacent areas when sea conditions were favourable (e.g., Cortey et al. 2009).

5 | Concluding Remarks

In conclusion, our study depicts how the combined effects of system productivity, covarying with temperature conditions, and different trade-offs between energy efficiency and competitive strength are of potential importance as drivers of animal distribution in a changing climate (see Menge et al. 2008 for similar considerations). Furthermore, the intertwining of biotic and abiotic forces over geological time contributed to produce the complex genetic make-up observed in Mediterranean trout populations. Unfortunately, in an almost instantaneous lapse of time (about a century) compared to the slowness of evolution, the introduction of domestic stocks in Italian rivers has already heavily erased the biodiversity that has settled over millennia. If there is not a profound rethink soon in conservation practices, in the near future we will be able to get an idea of the original fish fauna by studying only ancient DNA from museum samples (Splendiani et al. 2017) or from archaeological remains (Splendiani, Fioravanti, et al. 2016 and this work).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The new mtDNA CR sequences obtained in this study are available in the GenBank database under accession numbers PZ293215–PZ293221.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Right dentaries selected among *S. trutta* bone remains from Grotta del Santuario della Madonna. Dentaries chosen for genetic analyses are circled in red (all details in Table S1). **Figure S2:** Bayesian phylogenetic tree built using mtDNA CR haplotypes from modern *S. trutta* samples analysed in this study and sequences obtained from GenBank database. The tree was rooted using the mtDNA CR sequence of *S. salar*. The name of each haplotypes was coloured based on the *S. trutta* lineage and haplotypes described in this study are in bold italics. **Table S1:** Information about the right dentaries selected among *S. trutta* bone remains. Dentaries chosen for genetic analyses are highlighted in grey. **Table S2:** Number of faunal remains identified in the Mesolithic and Upper Palaeolithic sections of Grotta del Santuario della Madonna. **Table S3:** Number of fish remains identified in the stratigraphical sections under investigation. **Table S4:** Information on skeletal elements of *S. trutta* identified in the Mesolithic and Palaeolithic sections of Grotta del Santuario della Madonna. **Table S5:** PCR amplification success rates per sample and primer pair used for genetic analysis. **Table S6:** Diagnostic sites identified after the alignment of mtDNA CR haplotypes obtained from modern and ancient *S. trutta* samples.