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# Current and Historical Genetic Variability of Native Brown Trout Populations in a Southern Alpine Ecosystem: Implications for Future Management

**Caterina M. Antognazza [1,*], Anja Palandačić [2,3], Giovanni B. Delmastro [4], Giuseppe Crosa [1] and Serena Zaccara [1]**

[1] Department of Theoretical and Applied Sciences, University of Insubria, Via H. Dunant 3, 21100 Varese, Italy; giuseppe.crosa@uninsubria.it (G.C.); serena.zaccara@uninsubria.it (S.Z.)
[2] Natural History Museum Vienna 1, Zoological Department, Fischsammlung, Burgring 7, 1010 Vienna, Austria; anja.palandacic@nhm-wien.ac.at
[3] Department of Biology, Biotechnical Faculty, University of Ljubljana, 1516 Ljubljana, Slovenia
[4] Laboratorio di Ittiologia e Biologia Acque, Museo Civico di Storia Naturale, Via San Francesco di Sales 188, 10022 Carmagnola, Italy; gbdelmastro@gmail.com
* Correspondence: caterina.antognazza@uninsubria.it; Tel.: +39-0332-421422

**Abstract:** The highly polymorphic taxon European brown trout (genus *Salmo*) has high phenotypic plasticity, displaying a complex pattern of morphological and life-history variation, contributing to taxonomic confusion. Three main mitochondrial lineages (Adriatic, Mediterranean, and *marmoratus*) developed during the Pleistocene climatic events in the southern Alpine ecosystem. Here, the natural distribution of native brown trout *S. trutta* is controversial, complicated by introductions of the Atlantic strain. By investigating museum vouchers, this study aimed to retrace the historical presence of brown trout in the southern Alpine ecosystem before the beginning of mass introductions, which occurred since the middle of the 19th century. By examining the combination of historical and current genetic variability, this study aims to depict the actual impact of introductions of the introduced strain, increasing knowledge and informing conservation strategies and future management plans. The molecular approaches selected were: (i) sequencing of the mitochondrial control region and (ii) genotyping of the nuclear gene *LDH-C1**. Vouchers dated the presence of the native Adriatic strain since 1821, while current genetic variability showed the widespread signature of introgression, a consequence of several decades of introductions. Focused plans to preserve local lineages are urgently needed, including short-term solution to avoid complete pauperization of this ecosystem.

**Keywords:** *Salmo trutta* complex; museum vouchers; mtDNA; conservation genetics

**Key Contribution:** The study of museum specimens and material from recent field collections showed the degree to which historical introductions changed the native character of extant brown trout populations in the southern Alpine region. The results indicate that while there is considerable evidence of introgression of introduced lineages into the native background, native stocks have persisted, a finding which is of great importance for conservation management.

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## 1. Introduction

Aquatic ecosystems, especially freshwater ones, are among those most altered by human activities. In addition to hydraulic, morphological, and chemical alterations, alterations of fish driven by anthropic introductions as well as processes linked to climate change add to the problem of loss of nature characteristics [1]. In Europe, human activities have drastically changed terrestrial and aquatic (marine and freshwater) ecosystems over the last 150 years [2]. Habitat destruction, fragmentation, and overexploitation of resources together with climate change and biological invasions are drastically altering ecosystems,

abruptly reducing biodiversity [3]. Further, in the 19th century, massive, worldwide fish translocations took place, with no regard to the possible detrimental effects of introductions of alien species on native biodiversity [3]. There are not many reliable accounts of how many freshwater fish species were originally distributed before massive stocking started. Therefore, it is challenging to accurately determine the consequences that decades of stocking had on native populations.

The highly polymorphic taxon European brown trout (*Salmo trutta* complex) is represented by up to 20 endemic species in the Mediterranean region [4]. Brown trout has high phenotypic plasticity and displays a complex pattern of morphological and life-history variation within the same geographical area, contributing to taxonomic confusion, thus hindering understanding of the evolutionary history of the species complex [5]. Investigation of phylogenetic relationships between morphologically and geographically remote populations of the *Salmo trutta* complex across Europe allowed for the description of five main mitochondrial lineages, developed during Pleistocene climatic events in glacial refugia (Adriatic, Mediterranean, *marmoratus*, Danubian, and Atlantic), described by [5] and confirmed in many publications [6–9]. In the Italian peninsula, the native brown trout belongs to only three lineages, Adriatic, Mediterranean, and *marmoratus* [9–12]. However, the natural distribution of native brown trout in the southern Alpine ecosystem is still controversial [12–16], complicated by uncontrolled stocking with introduced hatchery-reared Atlantic *S. trutta* (L., 1758) [8,16], since the middle of the 19th century (1859) [17]. Thus, Atlantic *S. trutta* is currently considered as one of the most invasive species [18], whilst it is imperiled in much of its native range [19]. This massive stocking was driven for food and fisheries reasons throughout Europe and in the specific case of southern Alpine River ecosystems (i.e., the Po River basin). As a consequence, its introduction led to a high level of farm-derived gene introgression, which enhanced hybridization, thus diminishing the ability of morphological discrimination of native and introduced populations [8,20].

Invaluable information can be yielded by comparing contemporary and past genetic diversity, before the natural distribution of native fishes was altered because of human activity [21]. A way to recognize a historical alteration of the gene pools that occurred over the last 150 years can be to investigate the historical and biological memory preserved in museums. In the middle-to-late 19th century, the construction of most natural-history collections (NHCs) began; hence, freshwater ichthyological specimens can be highly useful for retracing the initial geographic distribution of brown trout lineages [12,22]. Enhanced with advances in molecular techniques, museum specimens have been useful for evaluating the impact of stocking of commercially important fishes [23–25], and to offer insight into their phylogeny [26], population genetics [27] and biogeographic studies [28]. The same approach can be used in the brown trout species complex, which would allow recognition of brown trout lineages populating the respective basins before 1900, when major hydro-morphological alterations and introductions of fish species were carried out. The demonstration of historical natural existence of specific lineages in dedicated geographic areas would have important implications for managing freshwater ecosystems.

This study performed genetic analyses of brown trout museum vouchers and current populations in a southern Alpine ecosystem, to increase the useful knowledge for informing conservation strategies. The molecular approaches, largely adopted in the literature, were: (i) sequencing of the mitochondrial control region and (ii) genotyping of the nuclear gene *LDH-C1**, in order to achieve taxonomic attribution and estimation of level of current introgression, respectively. The nuclear *LDH-C1** gene discriminates European hatchery stocks (fixed for the *90 allele) from native populations, characterized by the *100 allele [29].

## 2. Materials and Methods

### *2.1. Museum Vouchers: Collection Strategy, Laboratory Procedures, and Data Analyses of Voucher Samples*

Vouchers of *Salmo* sp. were selected for genetic analysis combining two criteria: (i) collection period from 1820 to 1900, and (ii) geographic location. The purpose was to complete characterization of the historical and geographical southern Alpine scenario that occurred in pre-stocking times (1859) [17] and during the initial management period, not included in [12]. The museum material analyzed in this study are housed in four NHCs: the Naturhistorisches Museum of Vienna, Austria (NMW), the Museum of Natural History of Milan, Italy (MSNM Pi), the Museum of Natural History "Kosmos" of Pavia, Italy (MSNPV), and the Civic Museum of Natural Sciences of Brescia, Italy (MCSNB) (Table 1). Specifically, there were five specimens from Torbole (confluence with Lake Garda), six from a fish farm operating nearby Lake Garda and two from Rovereto (Adige River). Six specimens were collected in three Alpine tributaries of the Po River (River Ticino, River Caffaro, and River Serio; Table 1; Figure 1). The oldest specimen is from 1821 in Torbole (Table 1).

**Table 1.** Information about museum *Salmo* sp. specimens retrieved for this study (*cf* Figure 1; Supplementary Table S1). Museum housing specimens, collector, catalogue number (Cat_Num), locality, year, and identification code (ID) used for genetic analysis of each specimen are detailed. In addition, amplification method (primer pairsL15998-PRO and HDL-C1 [31], three pieces: when three smaller overlapping fragments 200—250 bp were amplified (*cf* Supplementary Table S2), are indicated.

| Museum | Collector | Cat_Num | River Drainage | Locality | Year | ID | Method |
|---|---|---|---|---|---|---|---|
| Vienna | unknown | NMW_96796 | Lake Garda | Torbole | 1821 | SZ01 * | [31] |
| | Steindachner | NMW_65951 | Lake Garda | Torbole | 1884 | SZ02 | Three pieces |
| | Steindachner | NMW_65952 | Lake Garda | Torbole | 1884 | SZ03 | [31] |
| | Steindachner | NMW_65954 | Lake Garda | Torbole | 1884 | SZ04 | Three pieces |
| | Sturany | NMW_65415 | Lake Garda | Fish farm | 1892 | SZ05 | [31] |
| | Sturany | NMW_66083_1 | Lake Garda | Fish farm | 1892 | SZ06 | [31] |
| | Sturany | NMW_66083_2 | Lake Garda | Fish farm | 1892 | SZ07 | [31] |
| | Sturany | NMW_66489 | Lake Garda | Fish farm | 1892 | SZ08 | [31] |
| | Sturany | NMW_66490_1 | Lake Garda | Fish farm | 1892 | SZ09 | [31] |
| | Sturany | NMW_66490_2 | Lake Garda | Fish farm | 1892 | SZ10 | [31] |
| | unknown | NMW_67946 | Adige | Rovereto | End 1800 | SZ11 | [31] |
| | unknown | NMW_90252 | Adige | Rovereto | End 1800 | SZ12 | [31] |
| | Steindachner | NMW_59654 | Lake Garda | Torbole | 1884 | SZ13 | [31] |
| Brescia | unknown | MCSNB 57 | Caffaro | - | Beginning 1900 | SZ14 | Failed |
| | unknown | MCSNB 13 | Caffaro | - | Beginning 1900 | SZ15 | [31] |
| Pavia | unknown | MSNPV CP0433—Ex 435/593 | Serio | Bergamo | End 1800 | SZ16 | Failed |
| | unknown | MSNPV CP0435—Ex 438/604 | Ticino | Pavia | 1877 | SZ17 | Failed |
| Milano | unknown | MSNM Pi 3139_B | Serio | - | 1858 | SZ18 | [31] |
| | unknown | MSNM Pi 3139_A | Serio | - | 1858 | SZ19 | [31] |

* Amplified and sequenced twice.

Laboratory procedures were performed in a DNA clean room with sterilized and UV-irradiated utensils. DNA was extracted from branchial arch tissue from the right side of the specimens. The tissue was air-dried to remove residual ethanol, and DNA extracted with the QIAamp® DNA Blood and Tissue Kit (Qiagen Inc., Hilden, Germany) using Mini Elute columns and following the manufacturer's protocol. All extractions included extraction controls to ensure that there was no contamination of the buffers. After the extraction, DNA concentration was measured using the Qbit High Sensitivity Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the fragmentation of the DNA was measured using the Tapestation High Sensitivity Kit (Agilent, Santa Clara, CA, USA). The results are available in Supplementary Table S1.

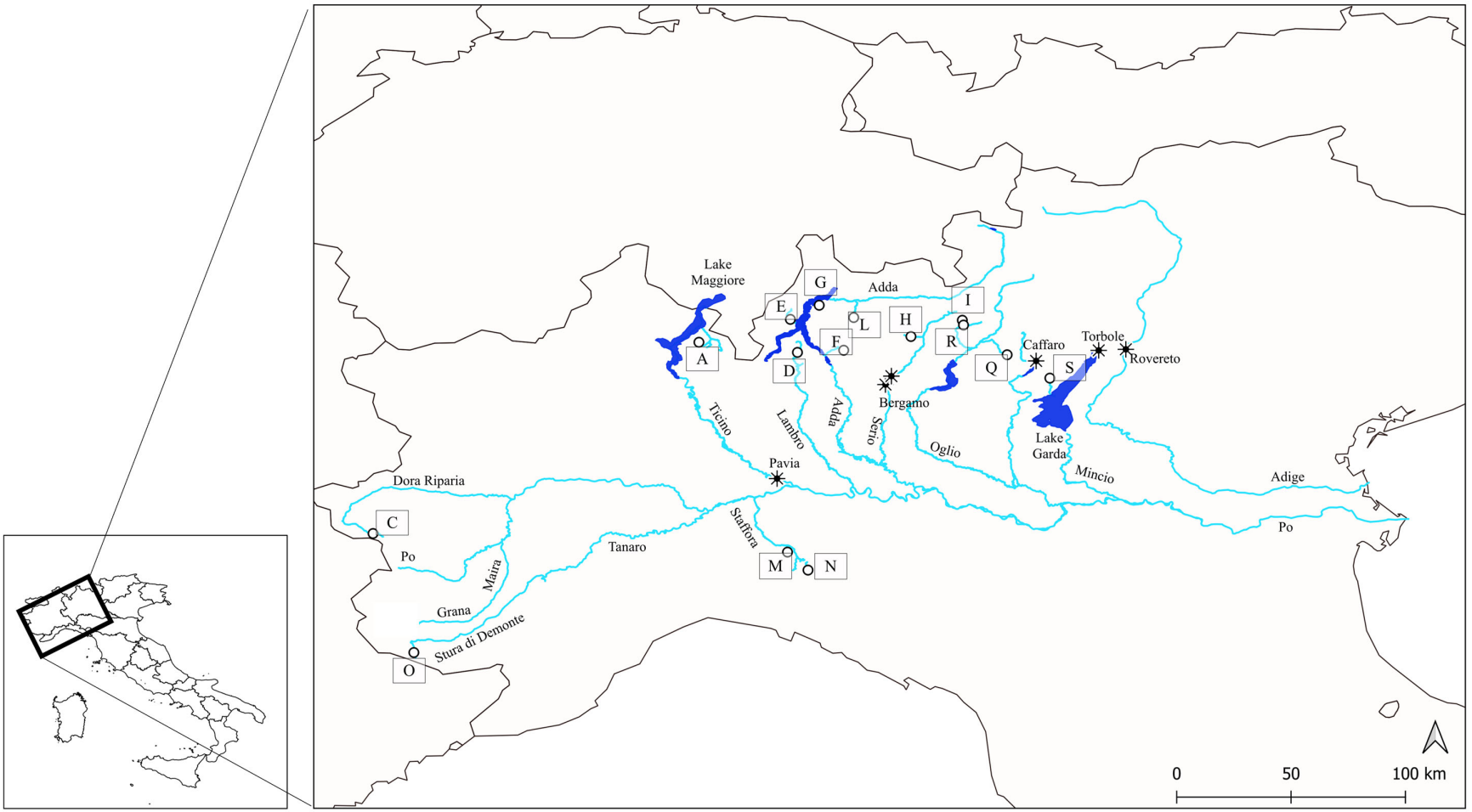


**Figure 1.** Geographic origins of *Salmo* sp. samples analyzed. Museum voucher origins are indicated with a star shape symbol (*cf* Table 1); locations of specimens collected during an electrofishing campaign in spring 2022 are indicated with a white dot and ID letter (*cf* Supplementary Table S3). Populations C and O were collected from south-western Alpine rivers, where the native evolutionary lineages have been recently attributed [30]. Asterisk refers to known locations of museum vouchers.

The partial mtDNA control region (CR) was amplified using the primer pairs L15998-PRO and HDL-C1 [31], which were designed to amplify an approximately 450 bp fragment of the CR. This area of the mtDNA was selected because it has several single nucleotide polymorphism (SNP) diagnostics for the main *S. trutta* lineages that were first identified by [6] and later verified by numerous investigations [7,12,32,33]. Due to DNA fragmentation, in some samples the complete fragment did not amplify. Thus, new primers were designed to amplify three smaller, overlapping fragments (called F1–F3), which added up to the complete, 450 bp fragment. Details are given in Supplementary Table S2 and Supplementary Figure S1. All PCRs (polymerase chain reactions) included a negative extraction control as well as a negative PCR control.

The PCR reaction volume was 20 µL, with 10 µL Mastermix (Qiagen Multiplex PCR Kit, Qiagen Inc., Hilden, Germany) with 0.5 µL of each primer (10 µM), with varying amount of water and DNA according to the measured concentration (from 2 to 7 µL per reaction). The same PCR protocol as the one for fresh samples was used for all fragments together with an increased number of cycles, 45. PCR products were purified with the Qiagen PCR purification kit and sent for sequencing in both directions by Mycrosynth (Balgach, Switzerland) using PCR primers.

After sequencing, the fragments were aligned with MEGA 5.0 [34] and combined into a single sequence. Combined sequences were added to the dataset. For additional quality control, the oldest sample dated from 1821 was amplified and sequenced twice.

The taxonomic attribution of museum vouchers was then completed by aligning new sequences and by comparing them to four sequences of the *S. trutta* complex, in agreement with [12]: Atlantic lineage (ATcs-1, GenBank: AF273086), Adriatic lineage (Adcs-1, GenBank: AY836330), Mediterranean lineage (MEcs-1, GenBank: AY836350), and *marmoratus* lineage (MA2c, GenBank: JQ582461) (Table 2). Positions of diagnostic sites referred to a sequence of *S. trutta* complete mitochondrial genome (GenBank accession number: AM10409) included in the alignment [12]. Newly discovered haplotypes were named while the existing nomenclature was adopted for sequences that matched with haplotypes previously described in the literature.

### *2.2. Current Trout Population: Sampling Strategy, Tissue Collection, Laboratory and Data Analyses*

Sampling sites for extant populations were selected according to the following criteria: (i) lack of previous molecular data, prevalently in central southern Alpine rivers; (ii) absence of official introduction records of the *S. trutta* Atlantic lineage; and (iii) in high Alpine brooks, preferentially occupied by brown trout which should not overlap with the ecological zone of marble trout (*Salmo marmoratus*, Cuvier 1829), a distinct species that occurs from the Po (only its northern tributaries) to the Soca and Rizana drainages in Italy and Slovenia [10]. In addition, two populations were collected from south-western Alpine rivers, where the native evolutionary lineages have been recently attributed [30] (Figure 1).

Fifteen populations of brown trout (for a total of 224 fish) were sampled from 13 Alpine and two Apennine tributaries of the Po River (Supplementary Table S3; Figure 1) between May and June 2022 using backpack electrofishing. Specimens were randomly selected, and following their capture, they were anesthetized in water and eugenol and measured (fork length, nearest mm); tissue samples (pelvic fin clips) were taken from each individual. After tissue collection, each fish was returned in situ. The lack of diagnostic morphological characters among lineages impeded any classification of lineages in the field.

Tissue samples were stored in 95% ethanol and subsequently total DNA was extracted in laboratory using a salting-out method [30]. The partial mtDNA control region (CR) was amplified using primer pair L15998-PRO and HDL-C1 [31]. PCR reactions were prepared in a total volume of 10 µL, with 2 µL genomic DNA, 5 µL Mastermix (Qiagen Multiplex PCR Kit), and 0.25 µL of each primer with the following profile: initial denaturation at 95 °C/15 min; 95 °C/60 s, 52 °C/60 s, and 72 °C/60 s (40 cycles); followed by a final extension step at 72 °C/10 min. Negative controls were included. PCR products were

purified using the Exo-SAP (Euroclone, Pero, Italy) kit. Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Waltham, MA, USA) on an ABI 310 DNA analyzer, in the forward direction.

The *LDH-C1** nuclear locus was amplified using Ldhxon3F and Ldhxon4R primers [29,35] in a total volume of 10 µL, with 2 µL genomic DNA, 5 µL Mastermix (Qiagen Multiplex PCR Kit), and 0.25 µL of each primer. The PCR profile was obtained by initial denaturation at 95 °C/15 min; 95 °C/60 s, 60 °C/60 s, 72 °C/60 s (35 cycles); followed by a final extension step at 72 °C/10 min. PCR products were then digested with BslI restriction enzyme (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions. The fragments produced were ascertained through a fragment analysis procedure using a PCR amplification labelled Ldhxon4R primer with 6Fam fluorescent dye [36]. Labelled amplicons were separated through capillary electrophoresis in an ABI 3130xl genetic analyzer (Thermo Fisher Scientific) and alleles *90 and *100 [35] were scored in PeakScanner 1.0 V (Applied Biosystems) using GeneScan 500 ROX size standard (Thermo Fisher Scientific). For all reactions, negative controls were included.

CR mtDNA sequences were manually aligned using BioEdit ver. 5.0.9 [37] and then grouped into haplotypes using DnaSP ver. 5.10 [38]. Novel haplotypes generated in the present study were deposited in GenBank (Accession Number: OQ676372; OQ676375; OQ676376, detailed in Table 3). In the multiple alignment, 48 available sequences of *Salmo trutta* complex from the main mitochondrial lineages were incorporated (Supplementary Table S4). Phylogenetic analyses were performed using the HKY + I + G model of DNA sequence evolution [39] as estimated with J-ModelTest 3.7 software [40]. Confidence values for the respective nodes were estimated by 1000 bootstrap replicates. The analyses were computed using PAUP 4.0b [41], Neighbor-Joining (NJ), GARLI v1.0 [42], and Maximum Likelihood (ML) criteria, respectively. Bayesian analysis was performed using MrBayes v3.2 software [43] with a Markov Chain Monte Carlo algorithm (MCMC): four simultaneous and independent Markov chains from random trees were started and run for 1,000,000 generations, with the first 25,000 generations (2500 trees) discarded as the burn-in. Phylogenetic trees were visualized using FigTree v1.4.3 software [44] and rooted using *Salmo salar* (Supplementary Table S4). To analyze the genealogical relationship within the dataset, a Minimum Spanning Network (MSN) was built on the same multiple alignment through a statistical parsimony criterion as available in TCS v1.3 software [45].

**Table 2.** Information about museum specimens successfully analyzed and mtDNA CR diagnostic positions, numbered referring to the complete *Salmo trutta trutta* mtDNA genome (GenBank number: AM910409.1), detected after the alignment with four *Salmo trutta* complex sequences following [12] (Supplementary Table S3).

| | | | | **CR mtDNA Variable Positions** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Source** | **Year** | **Haplotype** | **15,757** | **15,777** | **15,790** | **15,810** | **15,842** | **15,860** | **15,864** | **15,926** | **15,927** | **16,011** | **16,053** | **16,054** | **16,067** |
| AF273086 | | | ATcs-1 | C | - | C | G | T | A | C | G | C | G | C | T | T |
| AY836330 | | | ADcs-1 | . | . | . | . | . | . | . | C | . | . | T | C | C |
| AY836350 | | | MEcs-1 | . | . | . | A | . | C | . | . | . | . | T | C | C |
| JQ582461 | | | MA2c | . | A | . | . | C | . | . | A | T | . | T | C | C |
| SZ01 | Torbole | 1821 | ADcs-1 | . | . | . | . | . | . | . | C | . | . | T | C | C |
| SZ02 | Torbole | 1884 | Adriatic ± | . | . | . | . | . | . | . | / | / | / | T | C | C |
| SZ03 | Torbole | 1884 | AD_SZ3 * | . | . | . | . | . | . | . | C | . | A | T | C | C |
| SZ04 | Torbole | 1884 | Adriatic ± | . | . | . | . | . | . | . | C | . | . | T | C | C |
| SZ05 | Fish farm | 1892 | ATcs-1 | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SZ06 | Fish farm | 1892 | ATcs-1 | . | . | . | . | . | . | . | . | . | . | . | . | . |
| SZ07 | Fish farm | 1892 | ADcs-1 | . | . | . | . | . | . | . | C | . | . | T | C | C |
| SZ08 | Fish farm | 1892 | Ma2c | . | A | . | . | C | . | . | A | T | . | T | C | C |
| SZ09 | Fish farm | 1892 | Ma2c | . | A | . | . | C | . | . | A | T | . | T | C | C |
| SZ10 | Fish farm | 1892 | ADcs-1 | . | | . | . | . | . | . | C | . | . | T | C | C |
| SZ11 | Rovereto | - | Ma2c | . | A | . | . | C | . | . | A | T | . | T | C | C |
| SZ12 | Rovereto | - | MA_SZ12 * | T | A | T | . | C | . | T | A | T | . | T | C | C |
| SZ13 | Torbole | 1884 | Ma2c | . | A | . | . | C | . | . | A | T | . | T | C | C |
| SZ15 | Caffaro | 1900 | AD/MA ± | / | / | / | / | / | / | / | / | / | . | T | C | C |
| SZ18 | Serio | 1858 | ADcs-1 | . | . | . | . | . | . | . | C | . | . | T | C | C |
| SZ19 | Serio | 1858 | AD/MA± | / | / | / | / | / | / | / | / | . | . | T | C | C |

/ Undetected diagnostic position due to incomplete CR mtDNA sequence available. * Unique haplotype (GenBank accession number: OQ676373-OQ676374). Full stop indicates nucleotide identity. ± Main lineage attributed through synapomorphic site. - Gap.

**Table 3.** Haplotype distribution based on CR mtDNA sequences (408 bp), according to the major trout evolutionary lineages (Atlantic, AT, Adriatic, AD, *marmoratus*, MA, Danubian, DA and Mediterranean, ME (Bernatchez et al., 1992)), and population distribution of LDH-C1* polymorphism results: homozygous for Mediterranean lineage (*100/*100, MED), homozygous for Atlantic lineage (*90/*90, ATL), and heterozygous (*100/*90, Hyb). Population (ID), stream, basin, and number of samples (N) are indicated.

| | | | | **Haplotype Distribution** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Lineages** | | **AT** | **AD** | | | | | **Ma** | | **DA** | **ME** | | | | |
| | | | | **ATcs1** | **AD-Thyrr1** | **ADK1** | **ADcs1** | | **Ma2C** | **Ma2A** | | **Da1f** | | **Mecs1** | **Genotype** | | |
| **ID** | **Stream** | **Basin** | **N** | **Hap1** | **Hap2** | **Hap4** | **Hap11** | **Hap9 *** | **Hap3** | **Hap5** | **Hap7 *** | **Hap6** | **Hap8 *** | **Hap10** | **ATL** | **Hyb** | **MED** |
| A | Marianna ± | Ticino | 20 | 17 | 1 | | | | 1 | | | | | | 8 | 10 | 2 |
| C | Ripa ¥ | Dora Riparia | 10 | | | | | | | | | | | 10 | - | 7 | 3 |
| D | Valle Rezzago | Lambro | 20 | 17 | | 3 | | | | | | | | | 3 | 17 | - |
| E | Senagra | Adda | 20 | 18 | | | | | | 2 | | | | | 9 | 11 | - |
| F | Caldone | Adda | 20 | 19 | | | | | | | | 1 | | | 10 | 10 | - |
| G | Valle Merla | Adda | 20 | 15 | | | | | | 3 | 2 | | | | 4 | 16 | - |
| H | Acqualina | Adda | 20 | 18 | 1 | | | | | | | | 1 | | 5 | 14 | 1 |
| I | Vò | Oglio | 10 | 10 | | | | | | | | | | | 9 | 1 | - |
| L | Valle della Pietra | Adda | 20 | 19 | | | | | | | | | 1 | | 7 | 13 | - |
| M | Lella | Staffora | 20 | 20 | | | | | | | | | | | 9 | 11 | - |
| N | Avagnone | Trebbia | 10 | 7 | 1 | | | 1 | | | | | 1 | | 1 | 9 | - |
| O | Rio Freddo ¥ | Tanaro | 10 | | | | 10 | | | | | | | | 2 | 5 | 3 |
| Q | Grigna | Oglio | 7 | 7 | | | | | | | | | | | 6 | 1 | - |
| R | Allione | Oglio | 12 | 11 | | | | | | 1 | | | | | 10 | 2 | - |
| S | Valle di Vesta | Mincio | 5 | 5 | | | | | | | | | | | 4 | 1 | - |
| | | | 224 | 183 | 3 | 3 | 10 | 1 | 1 | 6 | 2 | 1 | 3 | 10 | 87 | 128 | 9 |

* Unique haplotype (GenBank Accession Number: OQ676372; OQ676376; OQ676375), ± one sample failed amplification of CR mtDNA, and ¥ populations collected from southwestern Alpine rivers, where the native evolutionary lineages have been recently attributed [30].

## 3. Results

### *3.1. Taxonomic Attribution of Museum Vouchers*

Of the nineteen museum specimens, the DNA extraction failed for three. Among the remaining samples, eleven were successfully amplified using the method adopted by [31] and five samples using the three pieces method (Table 1). Twelve sequences yielded a 328 bp CR mtDNA, whilst for four sequences (SZ02, SZ04, SZ15 and SZ19) only partial fragments of the CR region were obtained. Thirteen synapomorphic sites were used to classify sequences into the major S. trutta mitochondrial lineages (Table 2). Ten sequences overlapped with three haplotypes already known from the literature (ATcs-1, Adcs-1, Ma2c; Table 2), and two are described here for the first time: one belonging to the Adriatic lineage, AD_SZ3, and one to the marmoratus lineage, MA_SZ12 (Table 2). The attribution of AD_SZ3 was based on the state of the character observed at positions 15926, 16053, 16054, and 16067 (Table 2) and MA_SZ12 due to the characters at positions 15777, 15842, 15926, and 15927 (Table 2). Regarding the attribution of the partial sequences, sequence SZ02 had all the characters of the Adriatic lineage, except at sites 15926, 15927, and 16011 (Table 2). Partial sequence SZ04 was ascribed to the Adriatic lineage due to the nucleotide at sites 15926, 16053, 16054, and 16067 (Table 2), as only an intermediate portion of the sequence, where no synapomorphic sites are present, was missing. Sequences SZ15 and SZ19 had available only the last six synapomorphic sites, which could be characteristic of either the Adriatic or the marmoratus lineage (Table 2).

Overall, five vouchers belonged to the marmoratus lineage, of which two were collected in a fish farm in the late 1800s, two in the Adige River, but date of collection was unknown, and one at Torbole in 1884 (Table 2). The two vouchers attributed to the Atlantic lineage (haplotype ATcs-1) were found only at the fish farm in the late 1800s. Finally, nine vouchers bore the Adriatic lineage, of which four overlapped with haplotype Adcs-1, found at Torbole in 1821, in the Serio River in 1858, and in the fish farm (Table 2). Four vouchers ascribed to the Adriatic lineage were collected in the Serio River in 1858, Caffaro River (beginning in 1900), and at Torbole in 1884 (Table 2; Figure 1).

### *3.2. Current Trout Populations*

Amplification and sequencing of the CR mitochondria (408 bp in length) was successfully performed for all but one specimen (pop. A, Marianna stream). In the multiple alignment, 12 variable sites, 9 of which were parsimony informative, defined 11 haplotypes, of which 3 are original to this study (Hap7, Hap8, and Hap9) (Table 3).

In order to attribute new haplotypes to different lineages, a phylogenetic tree and a Minimum Spanning Network were constructed. The tree topology was concordant between the reconstruction methods; the Atlantic, Danubian, and Mediterranean lineages formed a statistically supported cluster, while the Adriatic and marmoratus lineages did not show statistical support (Figure 2a).

The minimum spanning network shows the complex structure of brown trout lineages (Figure 2b).

Hap1 was the most geographically widespread haplotype ($n$ = 183) (Figure 2b; Table 3), overlapping within the ATcs1 Atlantic cluster, the haplotype associated with a hatchery origin [46]. It was observed in all populations with the exceptions of the population Ripa, C (Dora Riparia basin) and population Rio Freddo, O (Tanaro basin) (Table 3; Figure 3). The least represented haplotype Hap6 overlapped with the Da1f haplotype [47] clustering in the DA lineage; it was found in only one individual in population Caldone, F (Adda basin). Among 9 sequences, three marmoratus lineage haplotypes were found, of which one original (Hap7, hereafter named Ma1A), was restricted to one population (Valle Merla, G, Adda basin), whilst Hap3 and Hap5 have been previously described in the Po River basin (Ma2C and Ma2A, respectively) and retrieved from four populations in the Ticino (Marianna, A), Adda (Senagra, E and Valle Merla, G) and Oglio basins (Allione, R) (Table 3; Figure 3). Among 13 sequences belonging to the Mediterranean lineage, two haplotypes were observed. An original haplotype Hap8 (hereafter named MeA1) was retrieved from

three specimens from Adda (Acqualina, H, and Valle della Pietra, L) and Trebbia (Avagnone, N) basins. Ten sequences of Hap10 were only found in the population Ripa, C (Dora Riparia basin); this haplotype coincided with Mecs1 previously described in this area [30] (Table 3, Figure 3). The Adriatic lineage was found in 14 sequences defined in four haplotypes: Hap11 was observed in 10 specimens collected in the Tanaro basin (Rio Freddo, O) and overlapped with haplotype Adcs-1 (Table S4), whilst Hap4, previously described as ADK1, was detected in 3 specimens from the Lambro basin (Valle di Rezzago, D), and Hap2, previously described as AD-Thyrr1 (Table S4), was recorded in 3 specimens from the Ticino (Marianna, A), Adda (Acqualina, H), and Trebbia basins (Avagnone, N). A newly described haplotype, Hap9 (hereafter named AD-A1) was found in the Trebbia basin (Avagnone N) (Table 3; Figure 3).

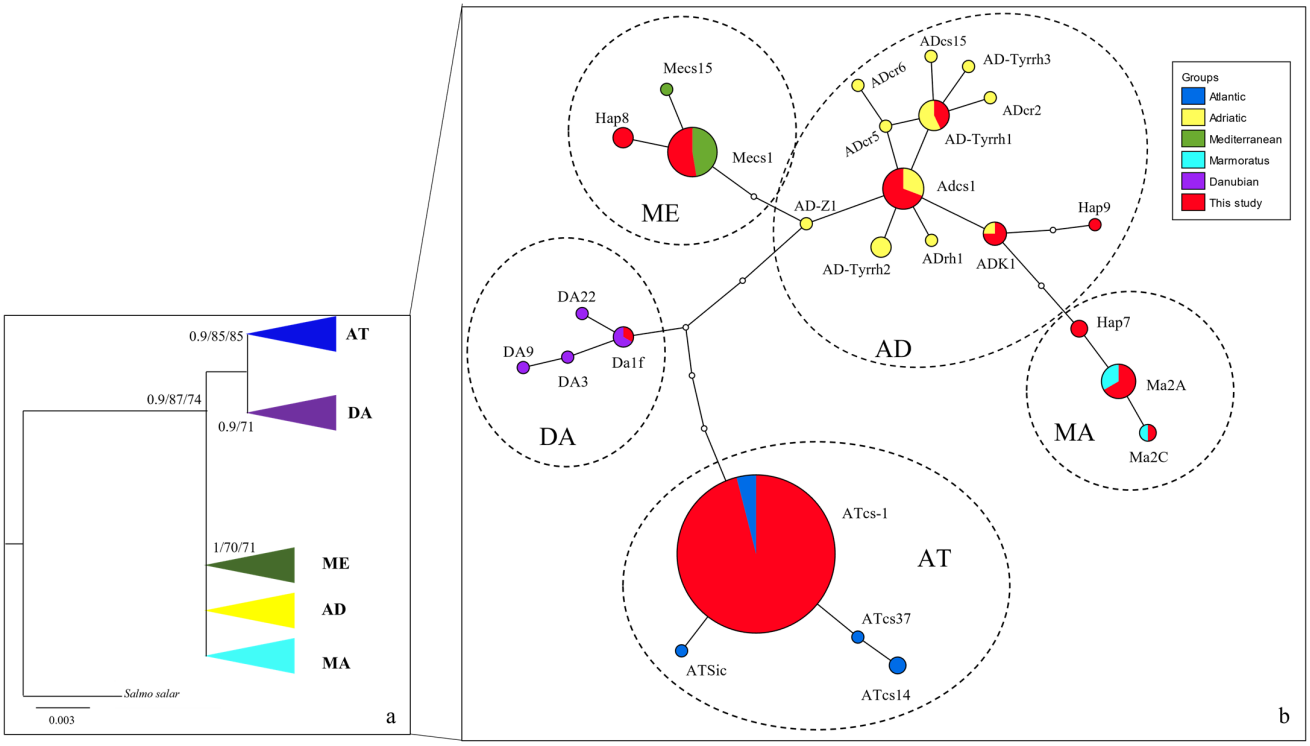


**Figure 2.** (**a**) Bayesian (BI) tree for *S. trutta* sp. based on the DNA sequences of the mtDNA control region (408 bp in length). Only significant node supports of the three optima criteria adopted (≥70%) are displayed. Trees were rooted using *S. salar* (*cf* Supplementary Table S3), and (**b**) Minimum Spanning Network of *S. trutta* haplotypes based on mtDNA control region (408 bp in length) and GenBank sequences (*cf* Supplementary Table S4). Each circle represents one haplotype, and the sizes of circles are proportional to the number of individuals sharing the same haplotype. White small circles represent missing haplotypes (mutational steps). Sequences from this study are highlighted in red.

Genotyping of the LDH-C1* locus was successful for all 224 samples and revealed that most fish, 55%, were hybrids (*90/100 alleles) at the marker locus, although that frequency might underestimate the degree of introgression across the genome in a population with advanced-generation hybrids (Table 3; Figure 3). The homozygous *90/90 genotype (41%) was observed in all populations, except in the Dora Riparia basin (Ripa, C). Finally, a small frequency, 4%, of homozygous *100/100 alleles were restricted to four populations (Table 3; Figure 3).

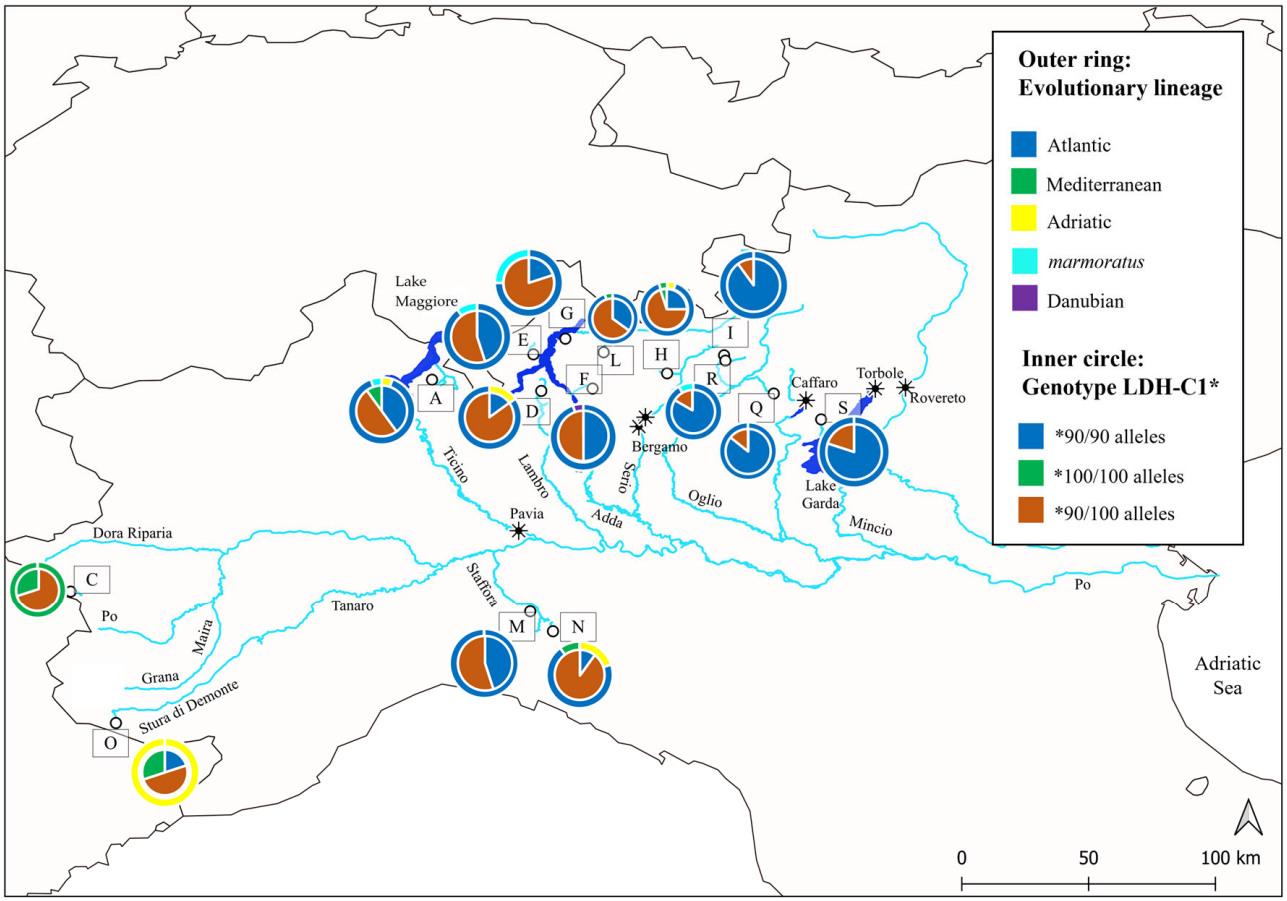


**Figure 3.** Distribution of mitochondrial lineages based on the mtDNA control region (408 bp in length) (outer ring) and *LDH-C1** alleles (inner circle) for brown trout complex populations (ID letter locations, *cf* Table 3).

## 4. Discussion

The current study shows the value of using museum vouchers to better understand the native distribution of within-species genetic variation, especially in areas (and of species) with known fish introductions. Despite the challenges involved in employing museum materials, such as degradation and fragmentation of the DNA [21,48,49], here sixteen vouchers were successfully analyzed and added valuable information of genetic diversity of the southern Alpine *S. trutta* complex before the extensive fish introductions [50]. Furthermore, the museum vouchers analyzed in this study highlighted the presence of haplotype ADcs-1, considered the most ancestral and widespread haplotype of the Adriatic lineage [6,32]. The ADcs-1 haplotype was detected in two specimens, the first from Torbole dated to the beginning of the 19th century (1821), and the second from the Serio River was collected in the mid-19th century (1858). According to these results, the Adriatic lineage populated the two streams before the beginning of massive stocking with the Atlantic strain [8], testifying for a natural presence of the Adriatic lineage in the central southern Alpine ecosystem (Figure 1). Moreover, the findings corroborate the hypothesis previously proposed by [8,12,30] in which local brown trout may have survived the Pleistocene glacial phases in a few ice-free refugia sites in the southern Alpine ecosystem. The finding of three original haplotypes (AD-A1, MeA1, and MaA1) belonging to the three southern Alpine native lineages (Adriatic, Mediterranean, and *marmoratus*) further supports the hypothesis. The lack of the introduced lineage (i.e., the Atlantic strain) recorded in voucher samples collected in the first half of the 19th century supports previous findings [12], which suggest that the impacts of significant *S. trutta* introductions were still sporadic at the end of the 19th century. Indeed, in this study, the Atlantic variant associated with a hatchery

origin [44] was found in only two museum vouchers collected from a fish farm in 1892. In a similar study, including museum specimen up to the end of the 19th century, no hatchery-specific Atlantic variants were detected [12].

In contrast, the impact of prolonged introduction of the introduced strain is nowadays widely present. Current trout populations showed only one Atlantic variant, ATcs-1 (Hap1) of hatchery origin [44], which was widespread; its genetic uniformity likewise is a sign of introduction from a single stock.

Further, introgression, resulting from contact between the introduced and local strains, has also been shown to be widespread in the southern Alpine area. This introgression is visible in the two populations sampled in the south-western Alpine rivers (Tanaro and Dora Riparia basins), of which native status has been already disentangled [30]. Indeed, the frequency of the hybrid genotype indicates a high level of introgression of introduced lineages into the native background. Thus, focused planning for conservation of the native *S. trutta* complex in the southern Alpine region is urgently needed. Nevertheless, the finding of the original haplotypes AD-A1, MeA1, and MaA1, together with five additional haplotypes, which belong to native southern Alpine *S. trutta* lineages [9–12], suggest their long presence in this area, which enabled them time to develop genetic variability. Therefore, their presence implies that the natural situation, despite the introgression and presence of hatchery variants, has not been destroyed. Hence, focused management plans and actions are necessary at once.

## 5. Conclusions

This study supports the focal role of both local as well as national museums in the conservation of the natural resources for both past and future research purposes. The combination of museum and recent material used in this study has offered a tool to understand the impact of management in the southern Alpine area occurring over the last 150 years. This application has also highlighted the direction that should be taken to promote conservation of local genetic variants. Indeed, museum vouchers add a temporal dimension to conservation genetic inferences, by providing information baseline levels of diversity, where modern data lack biogeographical information and may be compared with and used to guide conservation and management plans [51]. In the southern Alpine area, the results from the museum vouchers analyzed in this study provided historical evidence of the original occurrence of the Adriatic lineage, before the stocking with the introduced Atlantic strain began, supporting the hypothesis of the natural presence of the Adriatic strain [5,9,12,33,52]. Focused plans to preserve the local lineages are urgent in order to avoid the complete pauperization of the Alpine area, which long-term viability should be monitored considering the challenges of climate change upon regional ecosystems.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8080411/s1, Figure S1: schematic representation of the newly designed primer pairs to amplify three smaller overlapping fragments. Nucleotide positions refer to the fragment of CR mtDNA 450 bp length amplified with the primer pairs L15998-PRO and HDL-C1 [31]; Table S1: Description of museum vouchers. Table S2: Details of new primers designed to amplify three smaller overlapping fragments (F1, F2, F3), methods three pieces" (*cf* Figure S1). Nucleotide positions refer to the fragment of CR mtDNA 450 bp length amplified with the primer pairs L15998-PRO and HDL-C1 [31]. Table S3: sampled populations: genetic population identification (ID), stream, basin, sub-basin, geographic coordinates, and number of samples (N) are indicated (*cf* Figure 1). Table S4: Genbank references of *Salmo trutta* complex sequences used in this study for the phylogenetic analysis and parsimony network reconstruction. For each sequence evolutionary lineage based on the mitochondrial control region (D-Loop), name of the haplotype deposited in Genbank (GenBank), accession number at GenBank database (Acc. Num.), and source are indicated. References [53–64] are cited in the Supplementary Materials.

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