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# Molecular characterization of rare anadromous Rhône River brown trout

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**Abstract** – The brown trout form the *Salmo trutta* complex, a diversified assemblage of salmonids. Its native area mainly covers Europe. It can develop three ecological forms or ecotypes, depending on its migratory behaviours: resident, anadromous (going to sea) and lacustrine (going to lakes). The sea trout is the anadromous ecotype, born up river, living at sea where it reaches salmon size, and returning to the river of its birth for spawning. Like other anadromous fish species, this natural ecotype is protected in France. While its distribution along the Atlantic coasts is known, the sea trout is considered absent in the Mediterranean basin. However, some isolated individuals have been observed in the Rhône River and some other rivers from southern France. In order to understand the genetic position of these large specimens swimming upstream in Mediterranean rivers, and despite the degraded DNA due to bad tissue preservation, eight samples of these trout, mainly caught by anglers, were successfully genotyped at seven microsatellite loci and three sequenced at the mitochondrial Control Region. All specimens tested belong to the Atlantic lineage and are probably stocked domestic trout. This study provides preliminary elements for the conservation status of this ecotype in the Mediterranean basin.

**Keywords:** Degraded DNA / *Salmo trutta* / microsatellites / Control Region / Rhône River / stocking

## 1 Introduction

The brown trout *Salmo trutta* complex is, in its general acceptance, a diversified assemblage of salmonids whose native area covers Europe, Western Asia and North Africa. It has been studied for the last two centuries using multiple methods but reaching no clear agreement about its taxonomic organization (Guinand *et al.*, 2021; Tougard 2022). These taxonomic studies (1758–2007) were compiled by Kottelat and Freyhof (2007), resulting in the proposal of 28 species. After subsequent publications, there are more than 50 species (Delling, 2010; Turan *et al.*, 2009, 2011, 2012, 2014a, 2014b, 2017, 2020, 2021, 2022; Doadrio *et al.*, 2015; Segherloo *et al.*, 2021). The reasons of this taxonomic disorder and “inflation” (Isaac *et al.*, 2004; Zachos *et al.*, 2013; Guinand *et al.*, 2021) are first the diversity of ecological and geographic morphs within this group, the consideration of evolutionary lineages as

distinct species and the use of multiple species concepts (see Kottelat, 1997; Guinand *et al.*, 2021).

Brown trout populations frequently show distinct sedentary and anadromous behaviors in sympatry in north western Atlantic rivers of their native area (Behnke 1972; Dorefeyeva *et al.*, 1981; Charles *et al.*, 2005). Similarly, an *allacustrine formis* is observed, migrating to lakes. The migratory form differs from the resident ecotype by the life cycle, their morphological (especially the large body size), demographic and ecological characteristics (Frost and Brown, 1967; Campbell, 1977; Baglinière *et al.*, 2001). Some taxa from several European locations were first described as species according to their migration behavior, starting with Linnaeus (1758), who described two resident species (*S. trutta* Linnaeus, 1758 and *S. fario* Linnaeus, 1758 according to the color patterns and the mandible length), one anadromous (*S. eriox* Linnaeus, 1758) and one lacustrine species (*S. lacustris* Linnaeus, 1758). These forms were therefore considered distinct species until recently (e.g. Mills, 1971; Elliott, 1994) despite the early opposite opinion of Berg (1948), who considered these taxa to be the same species: *S. trutta*.

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Many ichthyologists later recognized them as subspecies: *S. trutta fario*, *S. trutta trutta* and *S. trutta lacustris* (e.g., Whitehead *et al.*, 1984; Lelek, 1987). However, several studies demonstrated that they are in fact ecotypes of the same population, thus of the same species. Indeed, genetic analyses revealed no difference between anadromous and resident trout of the same river (Fleming, 1983; Skaala and Naevdal, 1989), so they belong to the same population in a given watershed. Moreover, a population of one ecotype can produce progeny developing the other form (Skrochowska, 1969; Ombredane *et al.*, 1996; Dębowski *et al.*, 1999). This has been especially obvious in Kerguelen Islands where domestic sedentary Atlantic trout were introduced in troutless rivers, and anadromous forms appeared (Guyomard *et al.*, 1984; Lecomte *et al.*, 2013). It has also been demonstrated that distinct ecotypes in a given river do not develop heritable behavioral differentiation (Charles *et al.*, 2005; Ferguson *et al.*, 2017, 2019) and that these ecotypes are flexible with a continuum of life cycles, from purely resident to anadromous, with intermediate tactics along a continuum in time (age to reproduce) and space (distance of migration within the watershed up to the sea) (Cucherousset *et al.*, 2005).

The most consensual genetic structure description for the whole brown trout complex is based on mitochondrial DNA (mtDNA) sequences (Bernatchez *et al.*, 1992) and especially that of the Dloop, in the Control Region (CR). This marker discriminates seven main clusters: Danubian (DA), Atlantic (AT), Duero, (DU), Dadès, North Adriatic (MA; the marble trout), Adriatic (AD), Mediterranean (ME) and the North African (NA) lineages (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Weiss *et al.*, 2000; Bernatchez, 2001; Cortey and Garcia-Marin, 2002; Cortey *et al.*, 2004, 2009; Snoj *et al.*, 2011; Tougaard *et al.*, 2018; Sanz, 2018). The first molecular phylogenies grouped all the western Mediterranean lineages (MA+AD+ME), however they disagreed on the next closer lineage (DA in Bernatchez *et al.*, 1992 and in Cortey *et al.*, 2004; AT in Bernatchez, 2001).

The Rhône River is a French river drainage, flowing southward to the Mediterranean and known to harbor mainly the ME lineage (Bernatchez *et al.*, 1992; Bernatchez, 2001; Reynaud *et al.*, 2011). It is composed of dozens of large sub basins, mainly characterized by Mediterranean flow regimes (overflow in spring and autumn, low waters in summer and winter) and of mountainous flow regime (overflow in late spring when snow melts). Trout populations living there are highly diversified (Berrebi and Cherbonnel, 2009; Berrebi and Schikorski, 2016). The Mediterranean trout is deeply sedentary: some studies determined its range of movement to be less than 50 km (Berrebi and Shao, 2009).

With the successive dam buildings near the Rhône delta (Fig. 1) at Donzère-Mondragon (1952) and Vallabrègues (1970), several anadromous species such as the river lamprey *Lampetra fluviatilis* (Linnaeus, 1758) or the European sturgeon *Acipenser sturio* Linnaeus, 1758 have disappeared upstream (Keith *et al.*, 1992; Chassaing *et al.*, 2016). Anadromous trout are observed, but very rarely, in the Rhône drainage (Audouin and Maurin, 1958; Le Gurun *et al.*, 2012). A few specimens are sometimes caught near the Rhône delta (Camargue wetland) and in other coastal Mediterranean catchments, like the Var

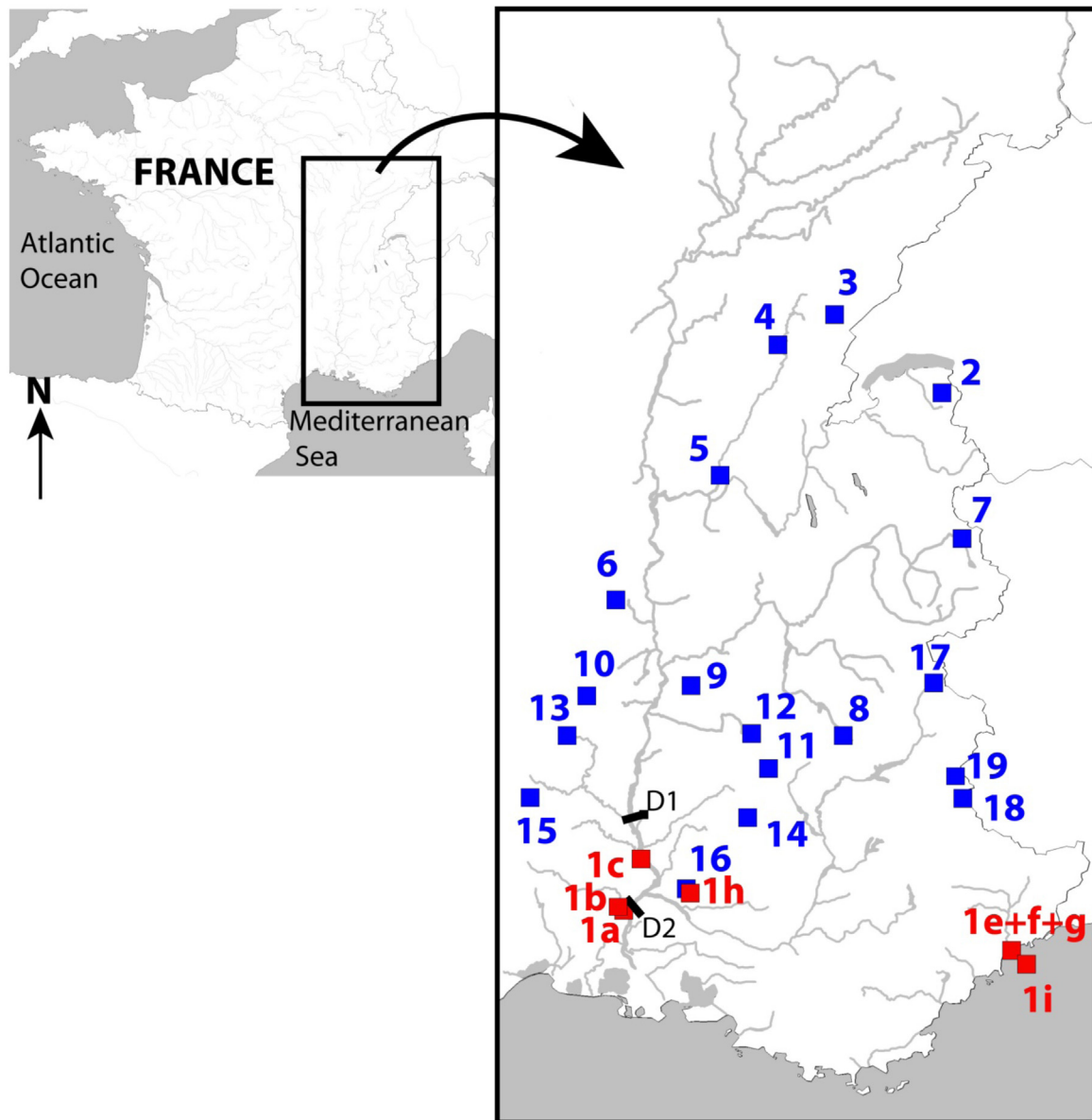
River, or captured by trawl nets at sea (Didry, 1953; Audouin and Maurin, 1958; Spillmann, 1961; local fishermen personal communications). Iglésias *et al.* (2021) recorded a similar capture of trout at sea, but this is probably a resident specimen washed out to the sea (recognizable by its general punctuation including the dorsal fin and indented caudal fin). Didry (1953) affirmed that Mediterranean trout can easily migrate from a coastal drainage to another one through the sea but provided no supporting data. On the other hands Spillmann (1961) claimed that restocking in headwaters of these coastal Mediterranean drainages from Danish eggs explained the presence of sea trout in this area. As anadromous species are the most threatened taxa (UICN Comité Français *et al.*, 2019), essentially due to the presence of dams preventing their migrations, degrading local environmental conditions and fragmentation of the hydrographic networks (Merg *et al.*, 2020), all diadromous fish species are attentively monitored by riverine managers. Thus, as a diadromous species, sea trout management is driven by the French code of the environment, establishing rules for its fishing (i.e. open fishing season, minimum catch size, catch quota per year and fisherman; art. R436-44 of the 5 August 2005).

The aim of this study is to assign sea trout caught in French Mediterranean rivers using molecular data to their closest genetic lineage, in order to clarify the mysterious identity of these large-bodied trout going up the Rhône River and often blocked downstream of the dams. Two hypotheses will be tested: (i) the sea trout are genetically similar to some natural Mediterranean populations of the Rhône basin (if Mediterranean trout can spontaneously develop an anadromous strategy) or (ii) they are similar to hatchery stocks and come from restocking with Atlantic domestic trout. This determination will provide key information to protect this Mediterranean population of sea trout.

## 2 Materials and methods

### 2.1 Sampling rare specimens

Sea trout are reported from time to time along the main Rhône River bed, in some of its tributaries like the Sorgues, Veuze and Gardon Rivers, in the delta formed by its estuary, the Camargue wetland, and at sea, in trawl nets, off the coast of this Mediterranean zone. They have also been observed in another Mediterranean river of southern France: the Var River. The rarity of this category of trout is such that it cannot reliably be captured using scientific methods (e.g., programmed electric fishing). In our study, we considered 14 trout specimens caught by anglers or in rare occurrences by electrofishing between 2004 and 2007, in the Rhône and Var rivers. Among them, as an example, the 1b specimen, featured in Figure 2, was fished with a spinning spoon (i.e. artificial metallic bait) in the Gardon River, a tributary of the Rhône River. The *Migrateurs-Rhône-Méditerranée* association (MRM), a nongovernmental organization for the protection of the migratory fish of the Rhône River, has for several years collected and conserved some organs (eggs, digestive tracts, fin-clips or scales) from these sporadic captures. These tissues were conserved in more or less concentrated ethanol, in small or big jars, without guarantee as to the good preservation of the tissues or their DNA.



**Fig. 1.** Geographic localization of the samples. Red letters = sea trout individuals (Tab. 2), blue numbers = comparative samples (Tab. 1). Trout 1d is missing because of unknown origin. D1 and D2 = large downstream dams (Donzère-Mondragon and Vallabregues dams, respectively). Hatcheries are not represented.

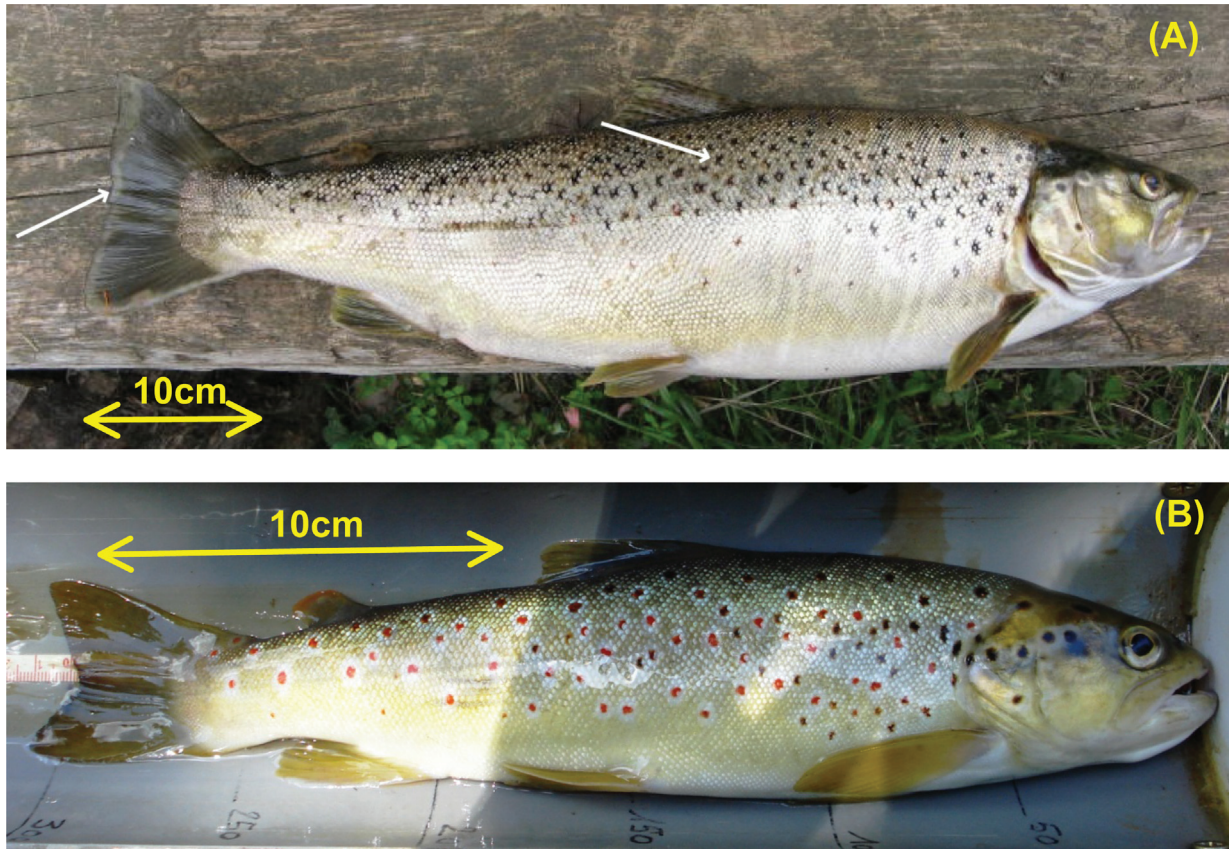
Specimens were identified morphologically according to several external criteria. Sea trout is differentiated from the resident trout by (i) a larger size in total length reaching easily 50 cm (*vs.* not exceeding 35 cm), (ii) a silver coat (*vs.* greenish to brownish skin coloration), even if in large rivers this typical coloration pattern tends to disappear in favor of being brownish, (iii) the presence of star-shaped black spots (*vs.* round red and black spots as well as 4 or 5 blackish vertical bands on the flanks) and (iv) the straight margin of caudal fin (*vs.* lightly forked) (Fig.2: Spillmann, 1961; Baglinière *et al.*, 2000; Jonsson and Jonsson, 2007). As a last parameter for identification, the stations of capture of all sea trout (with the exception of specimen 1h) were outside the distribution of resident trout, strictly distributed in salmonid-favorable rivers whose mapping is well known in France.

In order to genetically characterize the putative sea trout of our sampling, other reference samples, already genotyped by the same team (grey literature), were added for comparison. These reference samples, belong to the *Institut des Sciences de l'Evolution* (Montpellier University, France) tissues collection and include numerous resident populations living in tributaries all along the Rhône River basin as well as commercial domestic strains bred in several French hatcheries for stocking (details in Tab. 1).

## 2.2 DNA extraction, mitochondrial DNA and microsatellites amplifications

Total DNA was isolated from fin tissue preserved in 96% ethanol following the protocol of Medrano *et al.* (1990) for





**Fig. 2.** (A) Sea trout 1b (52 cm TL for 1745 g) fished with spinning spoon in the Gardon River in 2007. White arrows highlight morphological characters allowing the identification as sea trout: straight margin of caudal fin and the presence of star-shaped black spots. (B) Resident Mediterranean trout (28 cm TL, 460 g) electro-fished in the Sorgue River, left tributary of the Rhône River. This relatively large river trout shows a typical forked tail, black and red spots on the flanks and the dorsal fin, a yellow-brown skin color.

mtDNA sequencing and using Chelex 100 according to Walsh *et al.* (1991) method.

The complete CR (*ca.* 1100bp) was amplified following the protocol in Marić *et al.* (2012) using primers LRBT-25 (5'-AGA GCG CCG GTG TTG TAA TC-3') and LRBT-1195 (5'-GCT AGC GGG ACT TTC TAG GGT C-3'; Uiblein *et al.*, 2001). Purified PCR products were Sanger sequenced in one direction using primer LRBT-1195 (3'-end) and BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.).

Primers of the seven microsatellite markers used in this study were obtained from the literature (Oneμ9, Ss0SL-311, Omy21DIAS, Mst543, Sfo1, Ssas1197 and OMM1105, see Tab. 2 for details). Microsatellite repeated sequences are dinucleotide except Ssa197 which is a tetranucleotide microsatellite (O'Reilly *et al.*, 1996). For each marker, one of the 5' ends of the two primers was end-labeled with a fluorescent dye. Polymerase chain reactions (PCR) were performed using the Qiagen multiplex PCR kit in a final volume of 10 μl, containing 3 μl of genomic DNA diluted at 10 ng/μl, 5 μl of Qiagen PCR Master Mix, 1 μl of Qiagen Q-solution, and 1 μl of primer mix at 2 μM each (Eurofins MWG Operon).

Amplifications and genotyping were carried out in a Gene Amp PCR System 2700 thermal cycler (Applied Biosystems), according to the supplier's instructions (Qiagen multiplex PCR kit): initial denaturation step (95 °C, 15 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C for all loci, 90 s), and extension (72 °C, 60 s), with a final extension step (60 °C, 30 min). Amplified PCR fragments were then diluted and separated on an ABIPRISM 3130/xl/sequencer (Applied Biosystems) with Gene Scan 500 Rox dye size standards. Allele sizes were determined using the Gene Mapper v4.1 software system (Applied Biosystems, Life Technologies). A genotype matrix was then constructed and used as a basis for all the following statistical analyses.

### 2.3 Data processing

MtDNA partial CR sequences were aligned with haplotypes previously identified in the literature using the Muscle package (Edgar, 2004) in Mega X (Kumar *et al.*, 2018). CR sequences of *S. trutta* haplotypes available in the literature (Cortey and García-Marín, 2002; Duftner *et al.*, 2003; Cortey *et al.*, 2004, 2009; Meraner *et al.*, 2007; Snoj *et al.*, 2011) as

**Table 1.** Composition of the 27 samples considered in the present study.

Sample n°	Stations	Sub-basin (Rhône tributaries)	Sample sizes	Year of sampling
	Rivers			
st01	Mediterranean sea trout	Rhône, Var and sea	9	2004–2007
st02	Dranse d'Abondance	Drance	22	2008
st03	Saine	Ain	29	2008
st04	Merlue	Ain	30	2008
st05	Ain	Ain	19	2001
st06	Riotet	Cance	29	2009
st07	Isère	Isère	30	2010
st08	Drac	Isère	20	2012
st09	Véore	Véore	30	2009
st10	Dorne	Eyrieux	20	2006
st11	Drôme	Drôme	30	2009
st12	Bez	Drôme	24	1997
st13	Ardèche	Ardèche	20	2006
st14	Ouvèze	Ouvèze	24	1997
st15	Cèze	Cèze	20	2011
st16	Sorgue	Ouvèze	20	2010
st17	Durance	Durance	20	2010
st18	Lake Lauzanier	Durance	15	2014
st19	Ubayette	Durance	23	2000
	Hatcheries			
st20	Pas-de-Calais hatchery	–	29	2008
st21	Seine-Maritime hatchery	–	30	2008
st22	Oise hatchery	–	20	2010
st23	Ain hatchery	–	30	2008
st24	Isère hatchery	–	30	2008
st25	Hautes-Pyrénées hatchery	–	28	2014
st26	Haute-Garonne hatchery	–	29	2011
st27	Pyrénées-Atlantiques hatchery	–	20	2014

**Table 2.** Characteristics of the seven microsatellite loci. The second column indicates the gathering of each locus to one of the three multiplexes developed.

Locus	Multiplex	Primers concentration	References
MST543	C	0.15 µM	Presa <i>et al.</i> , 1994
Omm1105	A	0.8 µM	Rexroad <i>et al.</i> , 2002
OMY21DIAS	B	0.1 µM	Holm and Bendixen 2000
Oneµ9	C	0.2 µM	Scribner <i>et al.</i> , 1996
Sfo1	A	0.1 µM	Angers <i>et al.</i> , 1995
Ssa197	A	0.2 µM	O'Reilly <i>et al.</i> , 1996
SsoSL311	C	0.6 µM	Slettan <i>et al.</i> , 1995

well as those of *S. ohridanus* and *S. salar* used as outgroups are reported in [Appendix A](#) with their GenBank accession numbers.

Partial CR diagnostic sites for each lineage were identified with the Quiddich package ([Kühn and Haase, 2019](#)) for R ([R Core Team, 2013](#)). Lineage affiliation using the entire reference sequences (1125 bp) was made with a phylogenetic tree by Bayesian inference (MrBayes 3.2, [Ronquist \*et al.\*, 2012](#)), with the HKY+I+G model selected by JModelTest 2.1.1 ([Darriba \*et al.\*, 2012](#)) according to Bayesian criteria.

Bayesian analysis was performed launching two runs with 5 million generations and sampling every 100 generations. The subsequent tree files were summarized and 10% of trees eliminated as burn' in after checking for convergence. The analysis was reiterated twice cutting our alignment to our partial CR sequences lengths (532 bp and 325 bp) using respectively HKY+G and HKY+I models.

For microsatellites, standard parameters were calculated using the Genetix 4.05 software ([Belkhir \*et al.\*, 2004](#)): the

expected heterozygosity ( $H_e$ ), corrected for sample sizes (Nei, 1978), the observed heterozygosity ( $H_o$ ) and the mean number of alleles by locus ( $A$ ). While the sea trout sample st01 is far from constituting a functional population (sampled over several years, in several locations, during migration stage), it is interesting to compare its global diversity with the diversity of the other river and hatchery samples. In order to estimate the sea trout genetic diversity, only  $H_o$  and  $A$  were calculated because these parameters do not require a Hardy-Weinberg equilibrium. For this, the Genetix software was used for the 26 comparative samples calculations. The allelic richness of a population,  $A_r$ , is the expected number of distinct alleles in a sample and can provide strict comparisons if the sample sizes are equalized to the smaller one (rarefaction method, Kalinowski, 2004). The HP-Rare 1.0 software (Kalinowski, 2005) was used, reducing all samples to eight individuals.

A general picture of the trout genetic structure was first researched through multidimensional analyses on microsatellites data. Here, Factorial Correspondence Analyses (FCA: Benzécri, 1973) were carried out as implemented in Genetix, allowing the overall structure of the sampling to be explored. The clusters (or clouds) observed in the diagrams correspond to nuclear genetic homogeneous lineages. The mathematical method is detailed in She *et al.* (1987).

In order to detect differentiated subgroups, assignment tests using the Bayesian Structure 2.1 program (Pritchard *et al.*, 2000), subdivided the whole sample into  $K$  subgroups ( $K$  is the number of biological subgroups tested within the entire sample), characterized by their best genetic equilibrium in terms of best panmixia and lower linkage disequilibrium. The admixture ancestry model and correlated allele frequencies option were chosen. A burn-in of 50,000 iterations followed by 100,000 additional Markov Chain Monte Carlo iterations were applied on 5 runs for each  $K$  value. Here  $K$  has been tested between 1 and 8. The DeltaK method (Evanno *et al.*, 2005) was applied through Structure Harvester (Earl and von Holdt, 2012) in order to suggest the best  $K$  value in terms of likelihood. The objective of the test is to link the sea trout to one of the lineages included in the sampling: the domestic Atlantic lineage or one of the natural Mediterranean lineages present in the detailed sampling of the tributaries of the Rhône River.

### 3 Results

#### 3.1 Degraded DNA amplification

Among the 14 sea trout tissues, nine provided amplifiable DNA: one sample of eggs and digestive tract, two samples of fin-clip and six samples of scales (Tab. 3). One trout (specimen 1c) was not used because of too much missing data.

The mtDNA CR marker was amplified on only three out of the nine specimens (sea trout 1f, 1g and 1i). All sequences were short, between 270 and 489 bp, and at the 3' end of the CR. All three DNA samples came from scales (Tab. 3).

Concerning microsatellites, we obtained complete genotypes from only five out of the nine specimens. For the following analyses on microsatellite data, only eight specimens were considered, showing at most two missing locus genotypes (Tab. 3).

#### 3.2 MtDNA affiliation

Three short CR sequences (270 and 489 bp) were obtained and aligned with other reference sequences of the main lineages of *S. trutta*. The phylogenetic tree obtained by Bayesian inference (Fig. 3) follows mainly the one of Snoj *et al.* (2011) with an irresolution grouping AT, DU, DA and Dadès lineages, and a second one clustering AD, MA and ME lineages but distinguishing well this last one (Fig. 3). Our three partial sequences are placed in the AT+DU clade, and do not share diagnostic sites with the ME lineage (T in positions 878 and 961 vs. C and A in position 901 vs. G. See Appendix B). The two specimens 1g and 1i are clustered with the haplotype ATcs4 sharing the A in positions 908 and 937. The specimen 1f differs from the ATcs1, ATcs3, ATcs14, ATcs15, ATcs25, ATcs45, ATM2 haplotypes by only the T in position 548 (vs. C). The specimens 1f and 1h seem to correspond to unknown AT haplotypes. The three new sequences were deposited in GenBank with the accession numbers OP719777 to OP719779. The two phylogenetic trees on shorter sequences (532 bp and 325 bp) gave the same results (Appendix C).

#### 3.3 Microsatellite genotyping information

Observed heterozygosity (Tab. 4) was 0.74 for the sea trout assemblage, 0.60 for the river populations ( $0.41 < H_o < 0.68$ ) and 0.73 for the hatchery strains ( $0.55 < H_o < 0.82$ ). The calculation of the  $A$  parameter gave 6.6 for the sea trout, 7.8 ( $5.4 < A < 10.9$ ) for the river populations and 8.3 ( $5.5 < A < 12.14$ ) for the hatchery ones. The eight sea trout samples are as diverse as hatchery samples and about 20% more diverse than the resident Mediterranean lineage.

In order to overcome the differences in samples sizes, rarefaction calculations allowed the homogenization of all samples to eight individuals. The expected number of alleles in the sea trout sample was 4.82; it was 4.36 in average for hatcheries and 3.91 in average for river samples. Among rivers, only one sample was more diverse than the sea trout sample: the population 8 of the Drac River flowing to the Isère River, an upstream left tributary of the Rhône River. Also, two hatcheries were shown more diverse than sea trout.

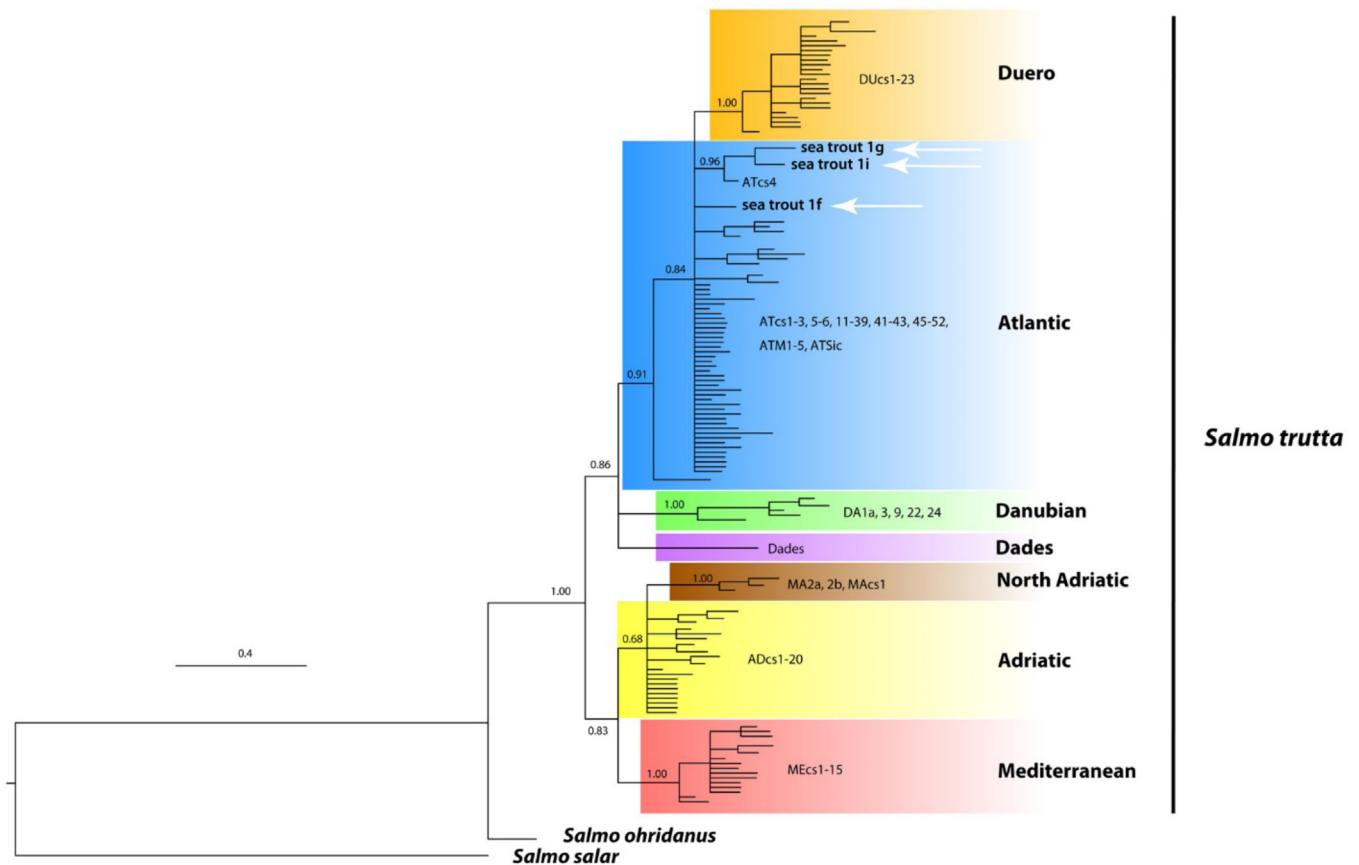
The numerous reference samples used (18 river samples from the Rhône watershed and 8 hatchery strains from France) allowed the multidimensional construction of a background map (Fig. 4), clustering Atlantic domestic strains at the left of the diagram and wild Mediterranean populations at its right. All eight sea trout are grouped in the domestic Atlantic zone of the hyperspace.

The DeltaK method (Evanno *et al.*, 2005) suggested a partition into two groups ( $K=2$ , obviously Atlantic and Mediterranean lineages). The separation between wild Mediterranean populations and domestic Atlantic strains permitted to clearly place all the sea trout in the domestic lineage (Fig. 5). Exploration of higher partitions (here up to  $K=5$ ) only allowed the distinction of several Mediterranean sub-lineages. For the Atlantic lineage, for  $K=5$ , it remained a single unit and grouped sea trout with domestic Atlantic strains (st20 to st27) in Figure 5.



**Table 3.** Genotypes and mtDNA sequences lengths (bp) of the sea trout samples with details on tissues used for DNA extraction (G = eggs, DT = digestive tract, F = fin, S = scales), locality, date of capture and total length. na = no amplification. Trout 1c, with 4 missing loci, has been removed.

N	Tissue	Locality	Year	Size (mm)	Ssa197	Oneμ9	SsoSL-311	Omy21DIAS	Sfo1	MST 543	Omm1105	mtDNA
1a	ω DT	Difffluence Grand/Petit Rhône R.	2004	545	131135	203203	134150	122122	118132	126146	274294	na
1b	F	Gardon R. (at Comps)	2007	520	123127	201211	128148	100102	106118	124146	262262	na
1d	S	?	?	—	131135	205205	134150	120122	118132	126146	274294	na
1e	S	Var R. (Napoléon 3 threshold)	2005	190	123127	205211	142152	126126	114134	122122	242294	na
1f	S	Var R. (Napoléon 3 threshold)	2005	200	123135	203205	128150	108114	110118	126146	258294	489bp
1g	S	Var R. (Napoléon 3 threshold)	2005	205	127135	205205	130156	114122	0	122122	238258	490bp
1h	S	Sorgues de Valleron R.	2004	380	123127	205205	130142	106114	116138	124146	262274	na
1i	S	Mediterranean (4 miles off Nice)	2005	—	127127	0	130130	120122	130130	0	242266	270bp

**Fig. 3.** Phylogenetic tree by Bayesian inference with the CR marker (1125 bp) on 124 sequences of *Salmo trutta* haplotypes. Colored blocks represent *S. trutta* lineages. White arrows designate the three Mediterranean sea trout 1f, 1g and 1i. Posterior probability values are indicated above the nodes.



**Table 4.** Genetic diversity of the analyzed samples measured with: Expected heterozygosity (He, unbiased parameter of Nei 1978), Observed heterozygosity (Ho), mean number of alleles by locus (A) and Allele richness (Ar after rarefaction calculation). For the sea trout sample, He cannot be calculated because, composed of several independent captures, it does not represent a population. For each parameter, high values are indicated in grey.

	Rivers	He	Ho	Ar
st01	Mediterranean sea trout	–	0.741	4.82
st02	Dranse d'Abondance	0.663	0.610	3.85
st03	Saine	0.746	0.616	4.08
st04	Merlu	0.783	0.619	4.33
st05	Ain	0.789	0.677	4.62
st06	Riotet	0.636	0.591	3.6
st07	Isère	0.718	0.611	4.27
st08	Drac	0.809	0.589	4.88
st09	Véore	0.725	0.633	4
st10	Dorne	0.644	0.586	3.24
st11	Drôme	0.625	0.571	3.36
st12	Bez	0.761	0.618	4.31
st13	Ardèche	0.673	0.657	3.78
st14	Ouvèze	0.560	0.560	3.09
st15	Cèze	0.781	0.690	4.71
st16	Sorgue	0.613	0.610	3.48
st17	Durance	0.766	0.671	4.39
st18	Lake Lauzanier	0.561	0.411	3.11
st19	Ubayette	0.619	0.516	3.22
<b>Hatcheries</b>				
st20	Pas-de-Calais hatchery	0.805	0.739	4.93
st21	Seine-Maritime hatchery	0.798	0.743	4.4
st22	Oise hatchery	0.690	0.641	3.6
st23	Ain hatchery	0.818	0.810	4.62
st24	Isère hatchery	0.736	0.700	3.99
st25	Hautes-Pyrénées hatchery	0.814	0.796	4.69
st26	Haute-Garonne hatchery	0.851	0.822	5.19
st27	Pyrénées-Atlantiques hatchery	0.672	0.557	3.5

## 4 Discussion

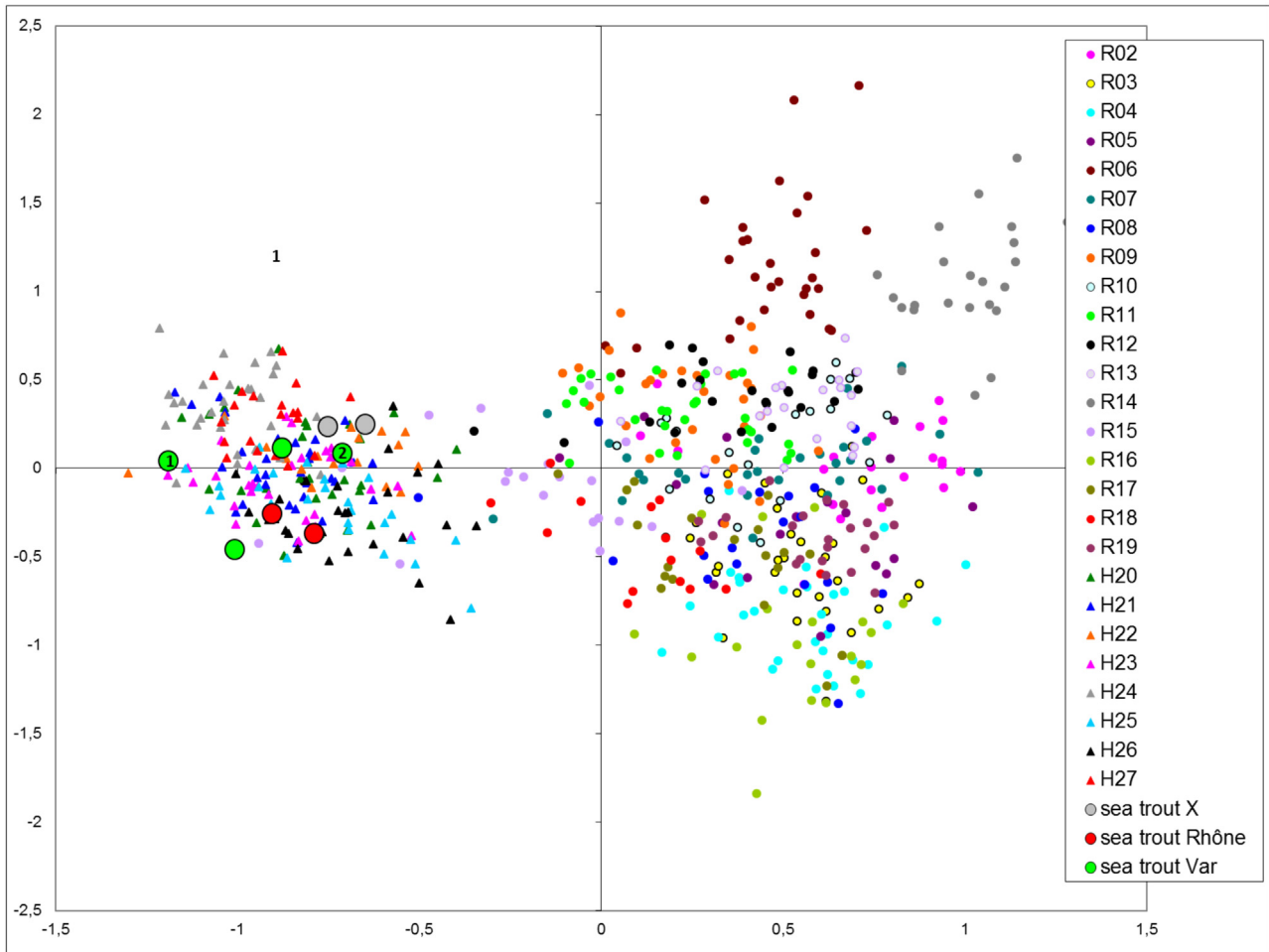
The present study addresses a category of trout that is hard – if not impossible – to sample using classical sampling approaches, making inferences complicated. Yet, the distinction between resident and anadromous forms is essential for conservation. The method used and the confidence that can be given to this determination condition the strength of the conclusions. The first element for determination was the overall morphology of the trout, as reported by the fishermen who brought the specimens to the MRM association: generally large, silvery skin with only a few cross-shaped spots, end of the tail straight (Spillmann, 1961; Baglinière *et al.*, 2000; Jonsson and Jonsson, 2007). This morphology is only reported, not observed and measured by researchers. However, the MRM association has worked on diadromous fish species since nearly 30 years with collaboration networks all over the country with the National Federation of Angling in France (FNPF) and the French Biodiversity Agency (OFB) as well as several research labs. So, it has good knowledge in fish identification and is therefore a trustful source. The other important element is location. French rivers are classified as first category (environments favorable to salmonids, generally upstream of the rivers) and as second category (downstream of the rivers, water temperature in summer incompatible with the life of salmonids, cyprinids

dominant). This is particularly true for the Rhône drainage (Changeux, 1995). All trout analyzed, except specimen 1 h, were caught in second category rivers, or even at sea. These characteristics as well as the molecular results obtained all point toward anadromous trout. In addition, the behavior observed by anglers (large trout “waiting” downstream from major dams) corresponds to anadromy.

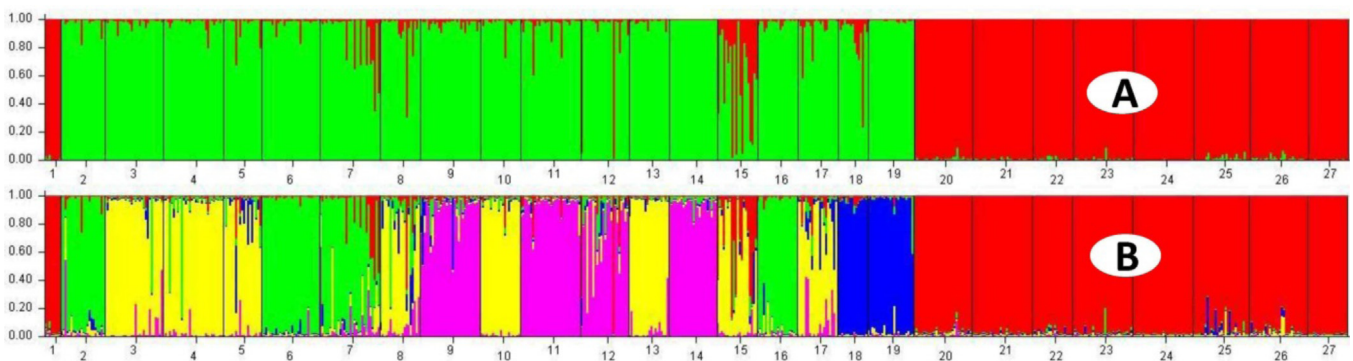
### 4.1 Degraded DNA analysis

Among the 14 sea trout tissues, nine provided amplifiable DNA, three gave partial mtDNA CR sequences and eight gave microsatellites data, five completely genotyped at seven loci.

The incomplete molecular data is a sign that the DNA was probably not well preserved, coherent with an apparent state of degradation of several samples. It is known that dried tissues give better results than other materials stored in ethanol in uncontrolled conditions (Rowe *et al.*, 2011). Dried DNA on scales can be preserved over 60 years for molecular studies (*e.g.*, Nielsen *et al.*, 1997, 1999) giving some perspectives of research including historical data (Nielsen and Hansen 2008; Levin *et al.*, 2018). However, in the samples of the present study, DNA is degraded probably due to low ethanol concentration. Obtaining microsatellite data appears to be more efficient than obtaining short mtDNA sequences in this case.



**Fig. 4.** The FCA (axis 1 horizontal and 2 vertical) presents a very clear classification of the genotypes of the 27 analyzed samples into two clusters: domestic Atlantic at the left and wild Mediterranean at the right. Legend at the right uses the numbers from the first column of [Table 1](#): R samples with small circles = river sample; H with small triangles = hatchery sample; large circles represent sea trout (st01), with Rhône River in red, Var River in green; unknown origin (X = Rhône or Var River) in grey. In circles are given the number of missing locus genotypes among 7.



**Fig. 5.** Different histograms produced by the STRUCTURE software after assignment analysis of the 649 sampled specimens (individual 1c has been removed). A = Assignment of the whole sampling to two expected subgroups ( $K=2$ ). B = STRUCTURE histogram when  $K=5$  showing the wild Mediterranean populations diversity (domestic Atlantic strains, in red, stay homogeneous).

## 4.2 Nature and origin of the Rhône River anadromous trout

Mitochondrial data on the three sequenced specimens affiliates them clearly, despite the short lengths of sequences, to the Atlantic lineage and not to the Mediterranean cluster.

Among the nine genotyped sea trout, two had more than one missing locus: individual 1i (2 missing locus genotypes) but also the individual 1d with 4 missing loci. This last individual has been discarded from the last analyses. The eight remaining sea trout were assigned to the Atlantic domestic trout lineage, according to microsatellites multidimensional (Fig. 4) and assignment (Fig. 5) analyses.

The genetic composition of the Rhône River anadromous trout contrasts with that of the resident populations sampled in tributaries all around the Rhône watershed that are mostly composed of wild Mediterranean trout (among the 425 trout belonging to the 18 Rhône River samples, 7 are stocked individuals and 30 are hybrids between Atlantic and resident trout, according to microsatellite genotypes).

All sea trout analyzed showed microsatellite genotypes and mitochondrial sequences of Atlantic origin. Because the Atlantic trout is not natural in the Rhône basin or in any Mediterranean river, it is most likely that these eight sea trout come from restocking. These results are similar to other molecular studies realized on sea trout from the Adriatic Sea; sea trout from this area were also assigned to stocked Atlantic strains from hatcheries (Snoj *et al.*, 2002; Splendiani *et al.*, 2020).

While not constituting a functional population, the assembled sea trout samples showed a high level of diversity, as generally observed in the commercial strains found everywhere in France and in other countries (Bohling *et al.*, 2016; Berrebi *et al.*, 2019). This provides another line of evidence for a domestic origin of sea trout. In hatcheries, this high diversity derives from the stock constitution more than thirty years ago by crossing several north European natural reproducers (Bohling *et al.*, 2016; Berrebi *et al.*, 2019).

As a first result, the Rhône sea trout is likely originating from stocking using the national (and international) commercial Atlantic strain, far from what can be seen in natural populations of the Rhône watershed. Because of its North European origins, the Atlantic domestic strain seems to produce migratory individuals first swimming to sea, crossing obstacles thanks to their small size at the smolt stage, then stopped by dams when returning for reproduction, due to the large size of the adults. According to Jorgensen and Berg (1991), Atlantic domestic trout are known to produce post-stocking downstream movements. Domestic stocked populations have showed such migratory behavior in other circumstances, as when introduced in Kerguelen Islands' troutless streams (Davaine and Beall, 1997; Lecomte *et al.*, 2013). Similarly, after introduction in Newfoundland, Canada, of brown trout fertilized eggs shipped in 1883 from Stirling, Scotland and from Germany (Westley and Fleming, 2011), brown trout developed an invasive behavior. The dispersal of these trout was facilitated principally by anadromy along the north and south coasts of the Avalon Peninsula, expressing both anadromous and resident ecotypes (O'Toole *et al.*, 2021).

## 4.3 Ecology of Rhône River anadromous vs resident trout

Neither Risso (1810, 1826) nor Crespon (1844) mentioned the sea trout in their inventories of southern France fishes. According to fossil data, this ecotype was probably absent from the Mediterranean catchments since the last ice age events between the Holocene and Pleistocene (Durante, 1978; Hamilton *et al.*, 1989; Bouza *et al.*, 1999; Splendiani *et al.*, 2016). As an ancestral character (since also present in Atlantic salmon *Salmo salar*), anadromy should have disappeared in modern Mediterranean trout. Several explanations have been suggested. (i) This absence could be attributed to some physicochemical properties of the sea water, such as surface salinity around 38‰ and surface temperature peaks over 25 °C, which correspond to upper critical values for the trout (Tortonesi, 1970). (ii) Possibly, the anadromous behavior has been counter-selected in Mediterranean trout lineage since the Pleistocene and Holocene (Splendiani *et al.*, 2016). (iii) Another possibility could be that the migratory capacity of Mediterranean trout is silent during hot interglacial periods but triggered by glacial ecological conditions, a hypothesis invoked to explain the Mediterranean trout invasion of Corsica during or after the last glaciations (Gauthier and Berrebi, 2007). (iv) The lower Rhône River is characterized by disproportionately large floods after particular Cevenol-type storm events, with discharges reaching 1720 m<sup>3</sup> s<sup>-1</sup> at Beaucaire, corresponding to the discharge of the Loire and Seine rivers added together (Pardé, 1925). These conditions are clearly inadequate for the establishment of the sea trout in the Mediterranean catchments (Snoj *et al.*, 2002; Splendiani *et al.*, 2020).

Anadromous trout reappeared in the Mediterranean (at least along French Mediterranean coasts and in north Adriatic Sea) due to Atlantic strains introductions. One of the first trout restocking in the Rhône River took place in 1851 from an Alsatian fish farm breeding a strain coming from the Rhine catchment (Vivier, 1956). After 1950, because of the construction of important dams few kilometers upstream to the delta (Donzère-Mondragon and Vallabrègues dams), several anadromous fish species disappeared upstream. For sea trout, nowadays, according to local anglers, this big form is in drastic regression, but growing stocking reduction is a part of the cause.

## 4.4 Consequences for the conservation and the management

Our results clarified the status of Mediterranean sea trout as an allochthonous lineage of *S. trutta*, which confirms and supports previous findings in the Adriatic Sea (Snoj *et al.*, 2002; Splendiani *et al.*, 2020). The justification of protective measures for an introduced taxon may be questioned, like for the European bitterling *Rhodeus amarus* (Linnaeus, 1758). This species, considered as threatened in most of Europe, have been evidenced as recently invasive in west and central Europe, following carp culture, and threatening freshwater mussels (Van Damme *et al.*, 2007). The rare (and regressing) presence of the sea trout in the French Mediterranean basin has no incidence on the management of the anadromous species already established (Lebel *et al.*, 2007). However, autochthonous resident Rhône trout populations, called *Salmo rhodanensis* Fowler, 1974 by some authors

(e.g., Kottelat and Freyhof, 2007), belonging mainly to the Mediterranean lineage (*sensu* Bernatchez *et al.*, 1992), are threatened by human activities. Threat comes especially from hydropower reservoir building (Grimardias *et al.*, 2017) or non-native Atlantic brown trout stockings (Caudron and Champigneulle, 2011), leading to natural/domestic introgression (Poteaux *et al.*, 1999). Thus, there is no interest in protecting an allochthonous lineage which could also be a threat for the autochthonous one. Managers can then focus their efforts on the three remaining threatened diadromous species: the Mediterranean shad *Alosa agone* (Scopoli, 1786), the sea lamprey *Petromyzon marinus* Linnaeus, 1758 and the European eel *Anguilla Anguilla* (Linnaeus, 1758).

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**Appendix A: GenBank accession numbers of CR sequences used in this study**

Lineage	Haplotype	GenBank	Source
Atlantic	ATcs1	AF273086	Cortey & García-Marín, 2002
Atlantic	ATcs2	AF273087	Cortey & García-Marín, 2002
Atlantic	ATcs3	AF274574	Cortey & García-Marín, 2002
Atlantic	ATcs4	AF274575	Cortey & García-Marín, 2002
Atlantic	ATcs5	AF274576	Cortey & García-Marín, 2002
Atlantic	ATcs6	AF274577	Cortey & García-Marín, 2002
Atlantic	ATcs11	AY836327	Cortey <i>et al.</i> , 2004
Atlantic	ATcs12	AY836328	Cortey <i>et al.</i> , 2004
Atlantic	ATcs13	AY836329	Cortey <i>et al.</i> , 2004
Atlantic	ATcs14	EF530476	Cortey <i>et al.</i> , 2009
Atlantic	ATcs15	EF530477	Cortey <i>et al.</i> , 2009
Atlantic	ATcs16	EF530478	Cortey <i>et al.</i> , 2009
Atlantic	ATcs17	EF530479	Cortey <i>et al.</i> , 2009
Atlantic	ATcs18	EF530480	Cortey <i>et al.</i> , 2009
Atlantic	ATcs19	EF530481	Cortey <i>et al.</i> , 2009
Atlantic	ATcs20	EF530482	Cortey <i>et al.</i> , 2009
Atlantic	ATcs21	EF530483	Cortey <i>et al.</i> , 2009
Atlantic	ATcs22	EF530484	Cortey <i>et al.</i> , 2009
Atlantic	ATcs23	EF530485	Cortey <i>et al.</i> , 2009
Atlantic	ATcs24	EF530486	Cortey <i>et al.</i> , 2009
Atlantic	ATcs25	EF530487	Cortey <i>et al.</i> , 2009
Atlantic	ATcs26	EF530488	Cortey <i>et al.</i> , 2009
Atlantic	ATcs27	EF530489	Cortey <i>et al.</i> , 2009
Atlantic	ATcs28	EF530490	Cortey <i>et al.</i> , 2009
Atlantic	ATcs29	EF530491	Cortey <i>et al.</i> , 2009
Atlantic	ATcs30	EF530492	Cortey <i>et al.</i> , 2009
Atlantic	ATcs31	EF530493	Cortey <i>et al.</i> , 2009
Atlantic	ATcs32	EF530494	Cortey <i>et al.</i> , 2009
Atlantic	ATcs33	EF530495	Cortey <i>et al.</i> , 2009
Atlantic	ATcs34	EF530496	Cortey <i>et al.</i> , 2009
Atlantic	ATcs35	EF530497	Cortey <i>et al.</i> , 2009
Atlantic	ATcs36	EF530498	Cortey <i>et al.</i> , 2009
Atlantic	ATcs37	EF530499	Cortey <i>et al.</i> , 2009
Atlantic	ATcs38	EF530500	Cortey <i>et al.</i> , 2009
Atlantic	ATcs39	EF530501	Cortey <i>et al.</i> , 2009
Atlantic	ATcs41	EF530502	Cortey <i>et al.</i> , 2009
Atlantic	ATcs42	EF530503	Cortey <i>et al.</i> , 2009



Atlantic	ATcs43	EF530504	Cortey <i>et al.</i> , 2009
Atlantic	ATcs45	EF530505	Cortey <i>et al.</i> , 2009
Atlantic	ATcs46	EF530506	Cortey <i>et al.</i> , 2009
Atlantic	ATcs47	EF530507	Cortey <i>et al.</i> , 2009
Atlantic	ATcs48	EF530508	Cortey <i>et al.</i> , 2009
Atlantic	ATcs49	EF530509	Cortey <i>et al.</i> , 2009
Atlantic	ATcs50	EF530510	Cortey <i>et al.</i> , 2009
Atlantic	ATcs51	EF530511	Cortey <i>et al.</i> , 2009
Atlantic	ATcs52	EF530512	Cortey <i>et al.</i> , 2009
Atlantic	ATM1	JF297978	Snoj <i>et al.</i> , 2011
Atlantic	ATM2	JF297979	Snoj <i>et al.</i> , 2011
Atlantic	ATM3	JF297980	Snoj <i>et al.</i> , 2011
Atlantic	ATM4	JF297975	Snoj <i>et al.</i> , 2011
Atlantic	ATM5	JF297977	Snoj <i>et al.</i> , 2011
Atlantic	ATSic	JF297974	Snoj <i>et al.</i> , 2011
<hr/>			
Mediterranean	MEcs1	AY836350	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs2	AY836351	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs3	AY836352	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs4	AY836353	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs5	AY836354	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs6	AY836355	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs7	AY836356	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs8	AY836357	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs9	AY836358	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs10	AY836359	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs11	AY836360	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs12	AY836361	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs13	AY836362	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs14	AY836363	Cortey <i>et al.</i> , 2004
Mediterranean	MEcs15	AY836364	Cortey <i>et al.</i> , 2004
<hr/>			
Adriatic	ADcs1	AY836330	Cortey <i>et al.</i> , 2004
Adriatic	ADcs2	AY836331	Cortey <i>et al.</i> , 2004
Adriatic	ADcs3	AY836332	Cortey <i>et al.</i> , 2004
Adriatic	ADcs4	AY836333	Cortey <i>et al.</i> , 2004
Adriatic	ADcs5	AY836334	Cortey <i>et al.</i> , 2004
Adriatic	ADcs6	AY836335	Cortey <i>et al.</i> , 2004
Adriatic	ADcs7	AY836336	Cortey <i>et al.</i> , 2004
Adriatic	ADcs8	AY836337	Cortey <i>et al.</i> , 2004
Adriatic	ADcs9	AY836338	Cortey <i>et al.</i> , 2004
Adriatic	ADcs10	AY836339	Cortey <i>et al.</i> , 2004
Adriatic	ADcs11	AY836340	Cortey <i>et al.</i> , 2004
Adriatic	ADcs12	AY836341	Cortey <i>et al.</i> , 2004
Adriatic	ADcs13	AY836342	Cortey <i>et al.</i> , 2004

Adriatic	ADcs14	AY836343	Cortey <i>et al.</i> , 2004
Adriatic	ADcs15	AY836344	Cortey <i>et al.</i> , 2004
Adriatic	ADcs16	AY836345	Cortey <i>et al.</i> , 2004
Adriatic	ADcs17	AY836346	Cortey <i>et al.</i> , 2004
Adriatic	ADcs18	AY836347	Cortey <i>et al.</i> , 2004
Adriatic	ADcs19	AY836348	Cortey <i>et al.</i> , 2004
Adriatic	ADcs20	AY836349	Cortey <i>et al.</i> , 2004
Dadès	Dades	JF297981	Snoj <i>et al.</i> , 2011
Danubian	Da1a	AY185568	Duftner <i>et al.</i> , 2003
Danubian	Da3	AY185571	Duftner <i>et al.</i> , 2003
Danubian	Da9	AY185572	Duftner <i>et al.</i> , 2003
Danubian	Da22	AY185573	Duftner <i>et al.</i> , 2003
Danubian	Da24	AY185576	Duftner <i>et al.</i> , 2003
Duero	DUcs1	EF530513	Cortey <i>et al.</i> , 2009
Duero	DUcs2	EF530514	Cortey <i>et al.</i> , 2009
Duero	DUcs3	EF530515	Cortey <i>et al.</i> , 2009
Duero	DUcs4	EF530516	Cortey <i>et al.</i> , 2009
Duero	DUcs5	EF530517	Cortey <i>et al.</i> , 2009
Duero	DUcs6	EF530518	Cortey <i>et al.</i> , 2009
Duero	DUcs7	EF530519	Cortey <i>et al.</i> , 2009
Duero	DUcs8	EF530520	Cortey <i>et al.</i> , 2009
Duero	DUcs9	EF530521	Cortey <i>et al.</i> , 2009
Duero	DUcs10	EF530522	Cortey <i>et al.</i> , 2009
Duero	DUcs11	EF530523	Cortey <i>et al.</i> , 2009
Duero	DUcs12	EF530524	Cortey <i>et al.</i> , 2009
Duero	DUcs13	EF530525	Cortey <i>et al.</i> , 2009
Duero	DUcs14	EF530526	Cortey <i>et al.</i> , 2009
Duero	DUcs15	EF530527	Cortey <i>et al.</i> , 2009
Duero	DUcs16	EF530528	Cortey <i>et al.</i> , 2009
Duero	DUcs17	EF530529	Cortey <i>et al.</i> , 2009
Duero	DUcs18	EF530530	Cortey <i>et al.</i> , 2009
Duero	DUcs19	EF530531	Cortey <i>et al.</i> , 2009
Duero	DUcs20	EF530532	Cortey <i>et al.</i> , 2009
Duero	DUcs21	EF530533	Cortey <i>et al.</i> , 2009
Duero	DUcs22	EF530534	Cortey <i>et al.</i> , 2009
Duero	DUcs23	EF530535	Cortey <i>et al.</i> , 2009
North Adriatic	MAcs1	AY836365	Cortey <i>et al.</i> , 2004
North Adriatic	MA2a	DQ841189	Meraner <i>et al.</i> , 2007
North Adriatic	MA2b	DQ841190	Meraner <i>et al.</i> , 2007
<i>Salmo ohridanus</i>		AY926564	Sušnik <i>et al.</i> , 2006
<i>Salmo salar</i>		AF133701	Arnason <i>et al.</i> , unpublished data

(DA), Duero (DU), Marbled (MA)) with the three partial sequences obtained from the sea trout specimens. GenBank accession numbers are listed in [Appendix A](#).

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[illegible]



**Appendix C.** Phylogenetic trees by Bayesian inference with the partial CR marker (532 bp (A) and 325 bp (B)) on respectively 123 and 124 sequences of *Salmo trutta*

haplotypes. Colored blocks represent *S. trutta* lineages. White arrows designate the three Mediterranean sea trout 1f, 1g and 1i. Posterior probability values are indicated above the nodes.

