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# Brown trout in Japan – introduction history, distribution and genetic structure

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**Abstract –** Brown trout *Salmo trutta* L. lives mainly in European rivers and is also bred in hatcheries for fishery purposes. Since the end of 19th century it has been introduced in all other continents. For the present survey most of the known self-sustaining brown trout river populations in Japan have been sampled and analyzed through sequences of the entire mitochondrial DNA control region and twelve microsatellites. In Japan, brown trout are genetically not homogeneous, probably as a consequence of several introductions, one in the Azusa river and at least one other in the remaining territory. The Chuzenji hatchery houses a genetically very distinct strain, probably due to intense manipulation in isolated scientific experimentations over 30 years. Finally, most populations showed high genetic diversity (Mamachi, Kane and Odori streams, Lake Chuzenji) with the exception of the Azusa river samples. This molecular analysis clearly demonstrates a European north Atlantic origin arrived in two distinct ways.

**Keywords:** brown trout / distant stocking / Japan / microsatellites / control region / trout management

**Résumé – La truite commune au Japon – Histoire de son introduction, sa distribution et sa structure génétique.** La truite commune, *Salmo trutta* L., est essentiellement une espèce vivant dans les rivières européenne. Elle est aussi élevée dans des pisciculture pour le repeuplement. Elle a été introduite dans tous les continents depuis la fin du XIX$^{e}$ siècle. Au Japon, la plupart des populations pérennes de truites ont été échantillonnées pour la présente étude et analysées au niveau de toute la région de contrôle de l'ADN mitochondrial et de douze microsatellites. Dans ce pays, les truites introduites ne sont pas génétiquement homogènes, probablement la conséquence de plusieurs introductions, une dans la rivière Azusa et au moins une dans le territoire restant. La pisciculture Chuzenji abrite une souche génétiquement très différente, ce qui est probablement dû à des manipulations intenses lors d'expériences scientifiques effectuées en isolement depuis une trentaine d'années. Finalement, la plupart des populations ont montré une grande diversité génétique (rivières Mamachi, Kane et Odori, lac Chuzenji) à l'exception des échantillons de la rivière Azusa. Cette étude moléculaire a clairement montré l'origine européenne nord-atlantique de ces populations introduites par deux voies distinctes.

**Mots clés :** truite commune / repeuplements distants / Japon / région de contrôle / gestion de la truite

## 1 Introduction

Brown trout, *Salmo trutta* Linnaeus, 1758, is one of the most man-handled fish species in the world (Laikre *et al.*, 1999). Its natural range covers Europe, Western Asia and North Africa (Behnke, 1986; Elliott, 1994). According to the control region (CR) marker of mtDNA, five main geographic lineages have been described: Atlantic (AT), Mediterranean (ME), Adriatic (AD), marmoratus (MA) and Danubian (DA) (Bernatchez *et al.*, 1992; Bernatchez, 2001). Several secondary lineages, placed at the basis of the main ones, have also been described, *e.g.* DU for Duero, TI for Tigris, the Balkan cluster, the Dades lineage in Morocco, NA for North Africa (respectively: Suarez *et al.*, 2001; Bardakci *et al.*, 2006; Snoj *et al.*, 2009, 2011; Tougard *et al.*, 2018).

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From 1748 onwards, brown trout, generally of the AT lineage has been domesticated in the Eastern Atlantic slopes for stocking in Westphalia, Germany (Leitritz and Lewis, 1980). Then, in the middle of the nineteenth century, it was massively produced in hatcheries in Alsace, France and Baden Wurttemberg, Germany for aquaculture and domestic forms were introduced into the wild as eggs in boxes, fry or sub-adults (Bohling *et al.*, 2016). European hatchery strains are composed of two lineages: (i) a more or less common one composed of admixed Atlantic original populations sampled in several north European countries and used currently as a commercial product for stocking (ComATL = common global Atlantic-based strain, according to Bohling *et al.*, 2016) and (ii) a very heterogeneous set of strains originating from local rivers and devoted to local stocking (LocATL = strains derived from local Atlantic watersheds, LocMED = strains derived from local Mediterranean watersheds *sensu* Bohling *et al.*, 2016). ComATL strains are mainly tagged by four D-loop haplotypes: haplotypes 1, 2, 3 and 4 (Cortey and García-Marín, 2000) that are widespread in Western Europe, from southern France to Norway, and northern Russia (Appendix 1). These haplotypes were then named At1a to d (Duftner *et al.*, 2003) or AT-s1 to 4 (Cortey and García-Marín, 2002; Cortey *et al.*, 2004) or Atcs1 to 4 (Cortey *et al.*, 2009). This last denomination is used in this study.

Brown trout is well known for its adaptive abilities to various ecological conditions, provided that there is plenty of running, clear, oxygenated, fresh water not exceeding 20 °C. These conditions are commonly met in mountain streams all over the world, which has resulted in brown trout transfers being successful outside the species natural range, including the southern hemisphere, where salmonids are not native (Bailey, 1966, MacCrimmon and Marsall, 1968; Elliott, 1989). Therefore, when introduced worldwide, brown trout frequently constitute naturalized (self-sustaining) populations (MacCrimmon and Marshall, 1968; MacCrimmon *et al.*, 1970). It has been reported that for self-sustainability, non-native brown trout require hydrologically stable streams with small snowmelt floods, low summer water temperatures and spawning ground availability (MacCrimmon and Marsall, 1968; Kawai *et al.*, 2013). The capacity of non-native brown trout to settle self-sustainable populations has been largely exploited to provide sport fishing for this species outside its native range.

On the basis of ecological impacts (Townsend, 1996; Jonsson and Jonsson, 2011; Budy *et al.*, 2013), brown trout are now considered one of the most pervasive and successful invaders often negatively affecting fishes and ecosystems, not only through predation and competition (Fausch and White, 1981; Ortiz-Sandoval *et al.*, 2017) but also by acting as a vector of exotic parasites (for details on brown trout invasive abilities, see Budy and Gaeta, 2018).

At the world scale, ancient introductions were probably made with wild eggs or fry. The first brown trout translocation outside the species natural range goes back to the mid-19th century, when it was introduced into Tasmania, Australia and New Zealand (Jones and Closs, 2018). At about the turn of the century it was also transferred to South Africa (Weyl *et al.*, 2018), USA and Patagonia (Budy and Gaeta 2018; Casalinuovo *et al.*, 2018). After that, brown trout spread across all the continents – even into Antarctica to the Kerguelen Islands (Labonne *et al.*, 2013). For details about brown trout translocations and distribution outside Europe, see Elliott, 1989 and Section 5 in Lobón-Cerviá and Sanz (2018).

Several studies have been made on introduced self-sustaining brown trout, including their reproduction, population density, growth, invasion dynamics etc., mainly in the Kerguelen Islands (Davaine and Beall, 1992; Labonne *et al.*, 2013; Jarry *et al.*, 2018). However, little has been done on genetic characterization of the translocated self-sustaining populations, which would reveal their origin, genetic structure, population genetic dynamics, and adaptive capacity as inferred from basic population parameters such as genetic diversity, genetic drift, gene flow etc.

In Japan, records on brown trout import are very rare and few data on the first brown trout introduction exist. Introductions were performed exclusively in the Islands of Honshu and Hokkaido. The population of Lake Chuzenji (Tochigi Prefecture, Honshu) is presumed to be the oldest one, supposedly originating in the early 1900's (Maruyama *et al.*, 1987) or even earlier, around the end of the 19th century (in 1892 according to Elliott, 1989). According to Kawanabe and Mizuno (1989) it arrived via trout hatcheries in the USA, due to the erroneous presence of brown trout eggs among those of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) or brook trout (*Salvelinus fontinalis* Mitchill, 1814).

The Chuzenji brown trout hatchery (now the Nikko Station, National Research Institute of Fisheries Science) was established at the lake to propagate local material, which was afterwards mostly used for stocking in Japan. Later on, stocking with Chuzenji hatchery brown trout was discontinued and the fish were cultivated for experimental purposes without extra input from outside (personal communication from Nikko station scientists).

In Honshu, brown trout inhabit the upper part of the Azusa river in the remote Kamikouchi high mountain valley (Nagano Prefecture). This is one of the main trout habitats in this prefecture (*e.g.* Sakata, 1974; Kitano *et al.*, 2013). The prime source was eyed eggs from an American fish hatchery imported in the early 1930's and stocked by the Nagano prefectural government (Sakata, 1974).There is evidence that some Azusa trout were obtained directly from Europe in 1973 from Mepp Co. (a no longer operating French company; Maruyama *et al.*, 1987). Brown trout have also appeared in the lower part of the river (close to the city of Matsumoto), where they apparently spread via downstream migration or due to anthropogenic transfers (Yagyu *et al.*, 2016). Fishermen in Hokkaido introduced brown trout in the 1980s, which then appeared in numerous rivers in the southwestern part of the island.

Although there is plenty of indirect evidence pointing to self-sustainability of brown trout in Japan, some examples clearly show natural reproduction. Namely, the brown trout population from the Chitose river, a tributary to the Ishikari river in Hokkaido, has naturally propagated at least since 1984 when this species was first observed there (Urawa, 1989). In addition, some brown trout escaped into the Odori river (a tributary to the river Miya, central Honshu) from a small old hatchery in September 2004. Subsequently in 2008 and 2009, both juvenile and adult brown trout, including mature individuals, were observed and captured there (Ishizaki *et al.*, 2012).

In Japan, introduction of non-native salmonids (rainbow trout, brown trout and brook trout) seriously affected native species such as masu salmon (*Oncorhynchus masou* Brevoort, 1856), white-spotted charr (*Salvelinus leucomaenis* Pallas, 1814), Dolly Varden (*Salvelinus malma* Walbaum, 1792) and Sakhalin taimen (*Parahucho perryi* Brevoort, 1856) (Kitano *et al.*, 2013). Brown trout represent a major threat to the white-spotted charr, mainly through competition and genetic mixing. For example, earlier studies in Hokkaido populations have demonstrated replacement of white-spotted charr by brown trout (Takami *et al.*, 2002; Morita *et al.*, 2004; Hasegawa and Maekawa, 2009). Furthermore, brown trout that were released by local anglers into the Kane river (Fuji river system, Yamanashi Prefecture, Honshu) in 2004 began to hybridize with native white-spotted charr. This has become a problem of deep concern for conservation of the latter (Tanizawa *et al.*, 2016). Moreover, after an invasion that occurred above a collapsed dam in the Monbetsu stream in Hokkaido, brown trout replaced white-spotted charr through competition and possibly hybridization (Hasegawa, 2017). Brown trout have also reduced the number of species of fish fauna in other streams in Hokkaido (Shimoda 2012; Hasegawa *et al.*, 2017).

In response to its invasiveness in Hokkaido, brown trout stocking has been banned since 2003, while in Honshu, the Japanese government is now hurrying to prepare a law for brown trout management, mostly in terms of prohibiting stocking in natural streams.

The purpose of this article is twofold. Firstly in the introduction we have reviewed existing information on transfers of brown trout to Japan and the distribution of self-sustaining populations there. Then we have surveyed the evolution of the introduced populations by (i) testing present knowledge on the history of brown trout introduction in Japan through its genetic structure in the whole country, (ii) deducing the possible origins of self-sustaining populations in Japan by genetic comparison with Atlantic brown trout from Europe hatcheries and (iii) estimating the adaptive capacities of self-sustaining populations through genetic diversity.

The Japanese brown trout samples, which were collected between 2008 and 2017, were analyzed using mitochondrial DNA control region sequences and twelve microsatellite markers.

## 2 Materials and methods

### 2.1 Sampling

Specimens were caught between 2008 and 2017, mainly using electrofishing, but also nets and angling. Fin clips (adipose or ray fin) were taken and stored in 96% ethanol. Each sample was given a map number from 1 to 19 (Fig. 1). A description of the nineteen sampling localities, number of individuals analyzed (*N*) and the year of sampling are given in Table 1.

Sampling sites in the Mamachi and Monbetsu streams are described in detail, in Kitano *et al.* (2009) and Kawai *et al.* (2013) respectively (Tab. 1). Although both streams are tributaries to the Chitose river, they are physically isolated and are assumed to represent independent populations. Brown trout appeared in and invaded these streams from the 1980s onwards (Kawai *et al.*, 2013). As the Jigoku stream is the inlet of Lake Chuzenji, there is no migration barrier between them, so the Jigoku sample should represent the lake population. Brown trout have been reared in the Chuzenji hatchery for experimental purposes since 2000 without any input or output activities. In the Azusa river system specimens were collected in the upper part in the Kamikouchi valley (samples 7 to 11) and also downstream (samples 12 to 14) close to Matsumoto city (Fig. 1). They formed nine samples of 1 to 24 trout, due to capture difficulties in a low density situation. Between the upper (samples 7 to 11) and lower (samples 12 to 14) areas, there are at least four apparently insurmountable dams (Fig. 1), potentially preventing gene flow between the two. Statistical controls are necessary before the upper and lower samples are each considered as a true population. Brown trout from three French hatcheries (Lées Athas, Cauterets and Isère; Tab. 1) were included as reference material.

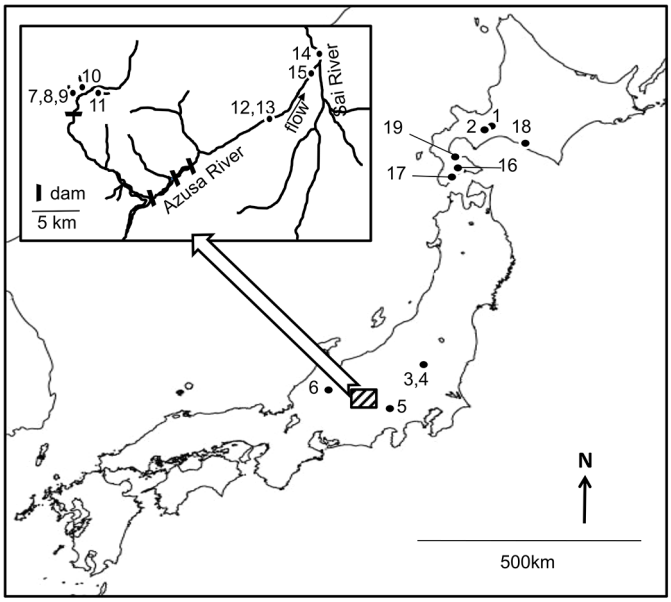


**Fig. 1.** Geographic position of the 19 Japanese samples analyzed.

### 2.2 DNA extraction, sequencing and alignment

DNA was isolated from fin tissue following the improved Chelex extraction procedure as described in Estoup *et al.* (1996).

The complete mitochondrial control region (mtDNA CR) was PCR-amplified using primers LRBT-25 and LRBT-1195 (Uiblein *et al.*, 2001), following the conditions in Marić *et al.* (2012). Both-directions sequencing was carried out on an ABI Prism 3130xl DNA sequencer (Applied Biosystems) according to the manufacturer's recommendations using the same primers. Sequences were aligned using the Clustal X computer program (Thompson *et al.*, 1997) implemented in MEGA version 6 (Tamura *et al.*, 2013). Haplotype nomenclature follows Cortey *et al.* (2009) for new haplotypes. The relationships among haplotypes detected in this study and reference haplotypes of the Atlantic lineage from Europe (Cortey *et al.*, 2009; Appendix 2) are presented as a 95% statistical parsimony network constructed using TCS 1.21 (Clement *et al.*, 2000).

**Table 1.** Description of the nineteen sampling stations and three reference hatcheries, number of individuals analyzed (*N*) and the date of sampling. E = flowing to eastern Japan: Pacific Ocean; W = to the west: Sea of Japan. All the sampling stations are in Honshu Island unless indicated as "Hokk." = Hokkaido Island.

| Map | Station | N | date | Medium | Drainage | Year of first introduction or observation | Reference |
|---|---|---|---|---|---|---|---|
| 1 | Mamachi stream (trib. of Chitose river) | 25 | 2012 | River | Ishikari (W, Hokk) | | |
| 2 | Monbetsu stream (trib. of Chitose river) | 25 | 2012 | River | Ishikari (W, Hokk)) | 1984 in the main river | Urawa (1989) |
| 3 | Jigoku stream (flows to Lake Chuzenji) | 22 | 2017 | Lake | Tone (E) | Early 1900's | Maruyama *et al.* (1987) |
| 4 | Chuzenji hatchery | 25 | 2016 | Hatchery | Honsu Japan | Early 1900's | Fukuda (1999)[1] |
| 5 | Kane stream | 25 | 2016 | River | Fuji (E) | 2012 | Tanizawa *et al.* (2016) |
| 6 | Odori stream | 24 | 2016 | River | Jinzu (W) | 2004 | Ishizaki *et al.* (2012) |
| Upper Azusa (in Kamikouchi valley) | | | | | | | |
| 7 | station 1 | 24 | 2013 | River | Shinano (W) | | |
| 8 | station 2 | 18 | 2013 | River | Shinano (W) | | |
| 9 | station 3 | 19 | 2013 | River | Shinano (W) | | |
| 10 | Zenrokusawa stream (trib. of Azusa) | 1 | 2013 | River | Shinano (W) | Early 1930's[2] | Sakata (1974) |
| 11 | Shimizusawa stream (trib. of Azusa) | 3 | 2013 | River | Shinano (W) | | |
| Lower Azusa (close to Matsumoto city) | | | | | | | |
| 12 | Azusa (adults) | 7 | 2008 | River | Shinano (W) | | |
| 13 | Azusa (YOY) | 13 | 2008 | River | Shinano (W) | | |
| 14 | Azusa (Sai) (close to Toyoshina) | 9 | 2016 | River | Shinano (W) | Early 1990's | Yagyu *et al.* (2016) |
| 15 | Azusa (close to Shimauchi) | 21 | 2016 | River | Shinano (W) | | |
| 16 | Hekirichi stream | 25 | 2013 | River | Hekirichi (E; Hokk.) | 1990 | Morita *et al.* (2004) |
| 17 | Shiriuchi stream | 25 | 2013 | River | Shiriuchi (E; Hokk.) | | |
| 18 | Shizunai stream | 25 | 2013 | River | Shizunai (E; Hokk.) | | |
| 19 | Torizaki stream | 25 | 2013 | River | Torizaki (E) | | |
| 20 | Lées Athas hatchery | 20 | 2014 | Hatchery | French Dept. 64 | | Bohling *et al.* (2016) |
| 21 | Cauterets hatchery (2014 strain raised in Babeau hatchery) | 28 | 2014 | Hatchery | French Dept. 65 & 34 | | Bohling *et al.* (2016) |
| 22 | Isère hatchery | 30 | 2008 | Hatchery | French Dept. 38 | | Bohling *et al.* (2016) |

1: Fukuda (1999): fishermen started cultivation of brown trout in the Chuzenji area in 1924.
2: In addition, eyed eggs were supposed to be imported from Mepp Co. (a defunct French company) in 1973 (Maruyama *et al.*, 1987).

### 2.3 Microsatellite genotyping and statistical analyses

Twelve microsatellite loci (Mst543, MST85, Omm1105, Omy21Dias, Oneμ9, Sfo1, Ssa197, SsoSL311, SsoSL438, SsoSL417, Str591 and StrBS131; Tab. 2) were amplified in three multiplex PCR (Tab. 2). PCR amplifications were carried out using the Qiagen multiplex PCR kit (Qiagen) and a set of forward primers with various concentrations (Tab. 2 and references herein). Amplifications were conducted in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems), according to the supplier's instructions (Qiagen multiplex PCR kit) with an initial denaturation step at 95 °C for 15 min; followed by 35 cycles of denaturation at 94 °C (30 s), annealing (59 °C, 90 s) and extension (72 °C, 59 s); with a final extension step at 59 °C for 30 min. Amplicons were separated on a capillary ABIPRISM 3130xl sequencer using GeneScan500Rox dye as the standard size. Fragment lengths were assessed using a GeneMapper v4.1 software system (Life Technologies™).

In order to draw the overall genetic structure of the analyzed samples in a unique diagram, Factorial Correspondence Analysis (FCA; Benzécri, 1973), implemented in GENETIX 4.04 (Belkhir *et al.*, 2004), was first performed.

Assignment tests, using the Bayesian STRUCTURE 2.1 program (Pritchard *et al.*, 2000), were used to subdivide the whole sample-set into K subgroups characterized by the best genetic equilibrium in terms of best panmixia and lower linkage. The admixture ancestry model and correlated allele frequencies options were chosen. A burn-in of 100,000 iterations followed by 200,000 additional Markov Chain Monte Carlo iterations was run for all tests except from step four of the hierarchical analysis (see below) for which they were 50,000/100,000 respectively. For each K value, five runs were repeated in order to check the stability of the assignment.

**Table 2.** Twelve microsatellite loci characteristics. The second column indicates the three multiplexes developed.

| Locus | multiplex | Primers concentration | Reference |
|---|---|---|---|
| MST543 | C | 0.15 µM | Presa *et al.* (1994) |
| MST85 | B | 0.15 µM | Presa and Guyomard (1996) |
| Omm1105 | A | 0.8 µM | Rexroad *et al.* (2002) |
| OMY21DIAS | B | 0.1 µM | Holm and Bendixen (2000) |
| Oneµ9 | C | 0.2 µM | Scribner *et al.* (1996) |
| Sfo1 | A | 0.1 µM | Angers *et al.* (1995) |
| Ssa197 | A | 0.2 µM | O'Reilly *et al.* (1996) |
| SsoSL311 | C | 0.6 µM | Slettan *et al.* (1995) |
| SSOSL417 | A | 0.1 µM | Slettan *et al.* (1995) |
| SSOSL438 | C | 0.1 µM | Slettan *et al.* (1996) |
| STR591 | B | 0.2 µM | Presa and Guyomard (1996) |
| STRBS131 | C | 0.4 µM | Charles *et al.* (2005) |

Estimation of the best K value (number of biological subgroups in the entire sample) was approached using the "Delta K method" of Evanno *et al.* (2005) through STRUCTURE HARVESTER (Earl and von Holdt, 2012).

Two methods were used to explore the assignment deeply:

The entire sampling was first analyzed as a whole from $K=1$ to $K=10$. While using the method of Evanno *et al.* (2005), all runs that made sense were represented in a tree describing all subdivisions. This precaution was taken because, as explained by Gilbert *et al.* (2012), "*selecting the optimal K can be quite a subjective procedure and is best inferred when the biology and history of the organism are taken into account*". Therefore, levels of K higher than that suggested by the Delta K method were also explored.

The hierarchical STRUCTURE assignment analysis was then performed (Pritchard *et al.*, 2000; Vähä *et al.*, 2007; Marić *et al.*, 2017). After the first analysis and determination of the most probable K using the method of Evanno *et al.* (2005), which set the first hierarchical level, each subgroup of the first level was analyzed separately, allowing for more precise clustering of individuals without eliminating admixed individuals. This hierarchical method was applied until no further substructure was observed.

Genetic diversity is also an important parameter for introduced populations, because it can provide information on the evolutionary potential of the strain used and on the number of individuals introduced. In addition, it can be the mark of small population size or of bottlenecks. This was estimated through three parameters using GENETIX software, *i.e.* observed heterozygosity (Ho), the non-biased estimated heterozygosity (Hnb), which is the calculated proportion of heterozygote genotypes according to the allele frequencies pondered by the sample size (Nei, 1978) and the mean number of alleles per locus (A).

Panmixia was determined using the Fis parameter. For this, the estimator F of Weir and Cockerham (1984) was calculated, together with its significance after 5,000 permutations of alleles within each sample, using GENETIX software. This test was applied sample by sample, then to the upper and lower groups of Azusa river sampling sites in order to collapse them into only two populations.

Differentiation between samples (populations) was evaluated using the Fst parameter (Weir and Cockerham, 1984) using GENETIX software. In addition, 5000 permutations of individuals among samples allowed the significance of the differentiations to be calculated.

Sequential Bonferroni correction (Rice, 1989) was performed for repeated tests.

When calculating Fst and Fis values, the upper and lower samples of the Azusa river system were joined in two independent, upper and lower assemblages. Fis value was calculated also for the entire Azusa sample-set. Genetic diversity was estimated for each sample and also for the upper and lower assemblages, respectively.

## 3 Results

### 3.1 Mitochondrial diversity

About 1100 bp, representing the complete CR mtDNA, were resolved with sequence analysis in 92 individuals (six to eight per wild population and altogether sixteen from hatcheries; Tab. 3).

After alignment, the CR sequences of Japanese samples collapsed into six haplotypes. They corresponded to previously described haplotypes ATcs1 to 4 and A17 (aka At1f). We also detected one previously undescribed haplotype that we named ATcs53 (GenBank Accession No MK330940).

The geographic distribution of the haplotypes observed in Japan is given in Table 3. The most common haplotypes were ATcs3 and ATcs4 (26% each) followed by Atcs2 (25%), ATcs1 and ATcs53 (10% each) and A17 (3%). The number of haplotypes varied from one to four per sample being the highest in Jigoku stream (Lake Chuzenji) and the lowest in the Chuzenji hatchery, the Kane stream and Azusa river (Shimauchi). Haplotypes ATcs3 and 4 were observed in the Cauterets hatchery, but only haplotype ATcs4 was found in the Isère hatchery.

Data on the distribution of haplotypes ATcs 1 to 4 outside Japan are collected in Appendix 1. When the haplotypes found in Japan were plotted onto the maximum parsimony network (Fig. 2) consisting of various haplotypes of the Atlantic brown

**Table 3.** Control region haplotype distribution. Numbers in parentheses indicate the number of haplotypes in a sample. For Map numbers, see Figure 1.

| Map | Station | | Haplotype frequencies | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | N | ATcs1 | ATcs2 | ATcs3 | ATcs4 | ATcs53 | A17 |
| 1 | Mamachi stream | 7 (2) | | | 3 | 4 | | |
| 2 | Monbetsu stream | 8 (2) | | 1 | 7 | | | |
| 3 | Jigoku (Lake Chuzenji) | 8 (4) | | 1 | 5 | 1 | 1 | |
| 4 | Chuzenji hatchery | 7 (1) | | | | | 7 | |
| 5 | Kane stream | 7 (1) | | | | 7 | | |
| 6 | Odori stream | 7 (2) | | | 5 | 2 | | |
| 7 | Azusa (Kamikouchi) station 1 | 7 (2) | | 6 | | | 1 | |
| 15 | Azusa (Matsumoto) Shimauchi | 6 (1) | | 6 | | | | |
| 16 | Hekirichi stream | 7 (3) | | 4 | 1 | 2 | | |
| 17 | Shiriuchi stream | 7 (2) | 4 | | | | | 3 |
| 18 | Shizunai stream | 6 (2) | 5 | | 1 | | | |
| 19 | Torizaki stream | 6 (2) | | 5 | | 1 | | |
| 21 | Cauterets hatchery | 5 (2) | | | 2 | 3 | | |
| 22 | Isère hatchery | 4 (1) | | | | 4 | | |
| | Σ | 92 | 9 | 23 | 24 | 24 | 9 | 3 |

trout (Appendix 2), they fell into clades 3-1 and 3-2 (*sensu* Cortey *et al.*, 2009).

### 3.2 Multidimensional analysis

In the multidimensional FCA graph, in which both samples from Japan and specimens from three hatcheries in France were included (Fig. 3A), three distinct clouds emerged. The cloud encircled by a black ellipse comprises individuals from French hatcheries along with most of the individuals from the Japanese rivers, while the cloud encircled by red includes samples from the Azusa river and that encircled by green represent the Chuzenji hatchery.

In the second step (Fig. 3B) the individuals from the Chuzenji hatchery and the Azusa samples were removed in order to obtain clearer resolution of the remaining samples (black circled in Fig. 3A). Thus, individuals from French hatcheries clustered on the left side of the graph, while Japanese samples grouped on the right with a possible cline of the Kane sample to the top and the Shiriuchi sample to the bottom.

### 3.3 Assignment structure

Classical assignment and the Delta K test recognized $K=2$ as the most probable, followed by $K=5$ and $K=7$. The complete analysis, synthesized as a tree in Figure 4, described the steps from $K=2$ to $K=7$. The "Structure tree" recognized Azusa river brown trout as the most differentiated group followed by the samples from French hatcheries and the Chuzenji hatchery.

Hierarchical assignment analysis is presented in Figure 5. Azusa river was the first cluster excluded (cluster 2), which, in further steps, collapsed into clusters 2.1 and 2.2 corresponding to the upper and the lower sampling areas. Stemming from cluster 1, three genetically homogenous sub-clusters emerged: French hatcheries (1.3), Kane stream and Chuzenji hatchery (1.2) and the remaining samples (1.1) constituting a rather heterogeneous, weakly structured genetic group (Steps 3 to 6).

### 3.4 Population parameters

Very low levels of genetic diversity were observed in the Chuzenji hatchery (Hnb = 0.39, $A=2.25$) and in most Azusa river samples ($0.44 < \text{Hnb} < 0.50$, $2.83 < A < 4.5$; Tab. 4). High values were obtained for the Jigoku stream with Hnb = 0.71 and $A=6.33$. Note that most other samples showed Hnb values over 0.6, which is also considered as high (Bohling *et al.*, 2016). The Hnb descriptors were generally in congruence with the level of genetic diversity of the populations described as measured by an average number of alleles per locus ($A$).

The Fis parameter, measuring deviation from the Hardy-Weinberg (H-W) expectation (Wright, 1951), indicated only a few panmixia deviations. Table 4 shows that five samples were significantly in H-W disequilibrium, but this was lost in three after Bonferroni correction and one was too small. As a result, only samples 15 and 17 were significantly out of panmixia at $p < 0.05$, demonstrating that globally, brown trout populations in Japan are in panmixia. The Fis value of the upper and the lower Azusa assemblage was estimated with high statistical support not to be different from zero, while for the entire Azusa sample-set, Fis reached 0.029 and the probability to differ from zero was 92.5%.

Inter-population differentiation based on pairwise Fst values is shown in Table 5. The nine Azusa samples were expected to constitute two populations isolated by impassable dams, so they were computed as two samples. The highest values were observed in comparison with the Chuzenji hatchery sample ($0.31 < \text{Fst} < 0.44$). The Azusa upper and

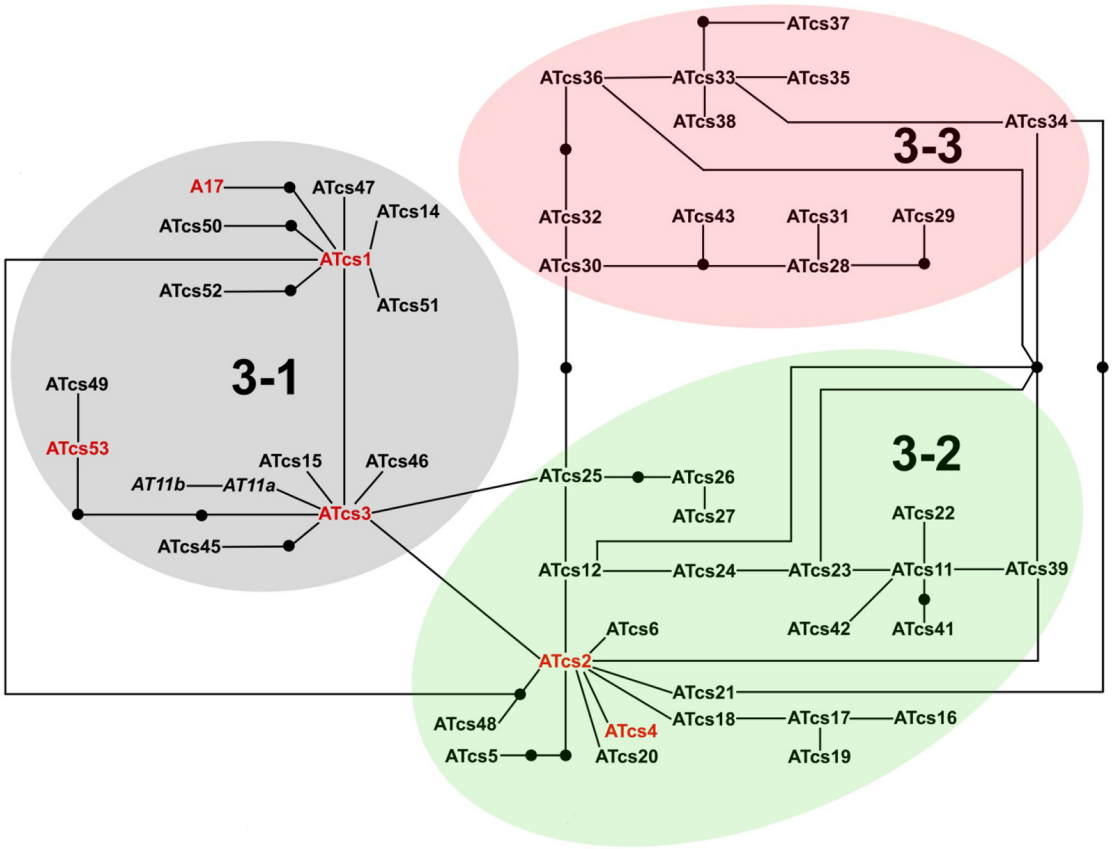


**Fig. 2.** Haplotype network relating the haplotypes of the Atlantic clade found in brown trout in Japan (in red) with previously published data (Appendix 2). Lines, regardless of length, represent single mutational events and link the haplotypes; black dots represent a missing or theoretical haplotype. 3-1, 3-2 and 3-3 designate the clades as in Cortey *et al.* (2009) and are included in grey, pink and green envelopes.

lower assemblages were the next most differentiated units ($0.22 < Fst < 0.33$ excluding comparison with the Chuzenji hatchery sample). Genetic differentiation between the upper and lower assemblage was relatively low ($Fst = 0.05$) but highly significant. Within the upper and within the lower Azusa assemblages, there was genetic identity (non-significant Fst) between the samples (data not shown).

## 4 Discussion

### 4.1 Origin of Japanese brown trout

The distribution of most of the haplotypes found in Japan is highly dispersed throughout Europe. In addition, the haplogroup ATcs1 to 4, which is characteristic of European domestic strains, also appears in most natural Atlantic populations along the whole Atlantic side of Europe from Norway to Spain, including Iceland, and also in Central and Eastern Europe up to Russia (Appendix 1). This is the main reason why, in most cases, it was not possible to recognize the origin of Japanese populations, despite some differences in the distribution of the four haplotypes. The presence of rare haplotype A17 was not supportive either. This haplotype was previously observed in the river Libĕchovka in the Elbe river system, Czech Republic, with the status of wild origin (Kohout *et al.*, 2012), and in the Eisack/Isarco river, South Tyrol (named as At1f; Meraner *et al.*, 2007). However, it cannot be ascertained that this haplotype is endemic to the Elbe river system nor are there any reports indicating brown trout transportation from the Czech Republic to Asia. Moreover, Meraner *et al.*, (2007) suggested that this haplotype was also distributed from hatchery strains. The appearance of haplotype ATcs53, which has not been found previously, requires a more detailed explanation. We observed it only in the Chuzenji hatchery, in the Jigoku stream apparently reflecting the population of Lake Chuzenji, and in the upper part of the Azusa river. The introduction of brown trout into the lake goes back to the beginning of the 20th century (Maruyama *et al.*, 1987). Nowadays, this haplotype is not part of commercial domestic lines, but it is possible that it might have been in the past and that it was later lost due to founder effects and/or random genetic drift. The first introductions of brown trout into the U. S., from where this species was assumed to be transferred to Lake Chuzenji (see Introduction), started from Germany in 1883 and 1884, but over the next few years, brown trout eggs arrived in U.S. hatcheries from Scotland and England as well (Behnke and Williams, 2007). Haplotype ATcs53 differs from haplotype ATcs49 by a single base mutation. It was detected in the Coquet river, British Isles (Cortey *et al.*, 2009) and also does not belong to the contemporary hatchery haplogroup.

**Figure 3A**

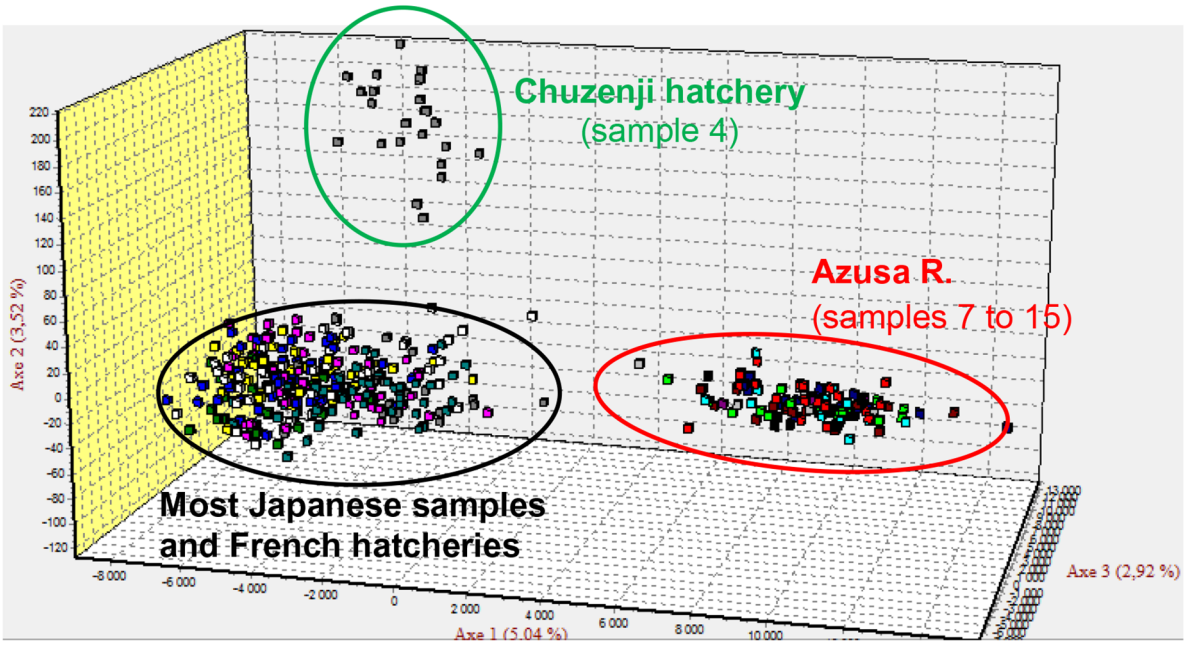


**Figure 3B**

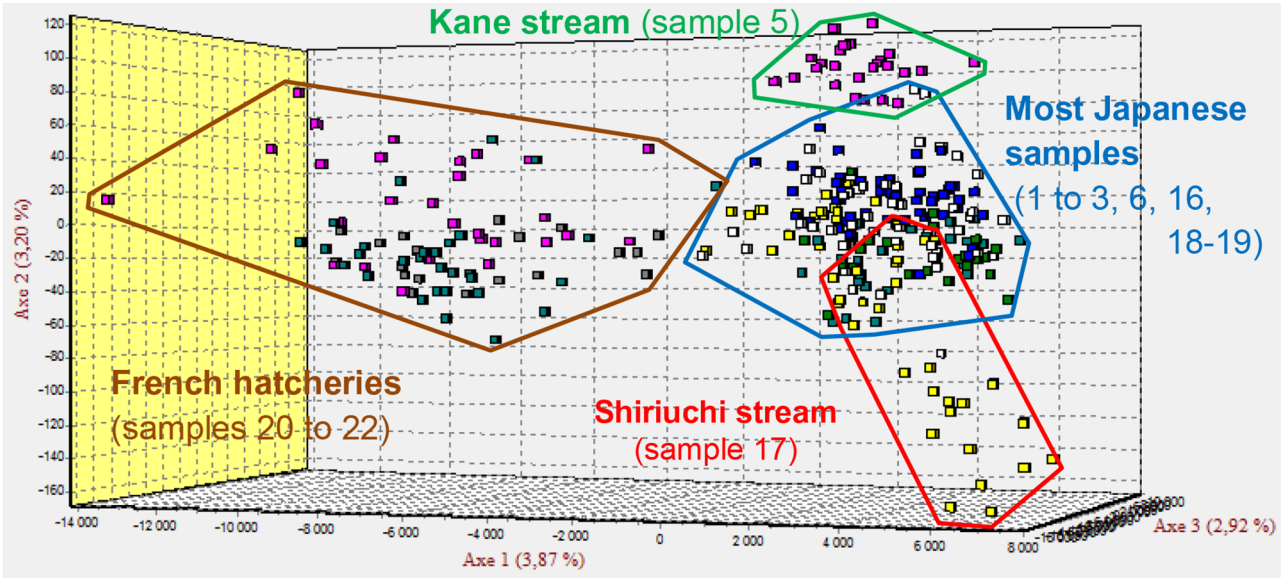


**Fig. 3.** Graphical presentation of the results inferred from multidimensional factorial correspondence analysis. Clouds detected on the diagram correspond to genetically similar individuals constituting lineages. A: the entire sample-set from Japan and specimens from three hatcheries in France; B: only the samples encircled with black in A.

Thus, these pieces of evidence suggest that haplotype ATcs53 found in Lake Chuzenji could have an origin in the British Isles. The lake population had served as a source for brown trout cultivation in Chuzenji hatchery (Fukuda, 1999), which explains how this haplotype came there. However, because the hatchery population has been maintained without extra input from outside since 2000 (*e.g.* from the lake), haplotype ATcs53 could have become fixed, due to random genetic drift and founder effects. Given that the material from the hatchery before 2000 was used to supply the remaining Japanese rivers, it is possible that this haplotype was also transferred into the Azusa river. Alternatively, it is also possible that it came there directly in eggs imported from America in the early 1930's when the upper Azusa river was supposedly first stocked (Sakata, 1974). This latter assumption is supported by inter-population differentiation tests (FCA, STRUCTURE and F indexes) all suggesting genetic distinction of the Azusa sample from all the other Japanese samples and the possibility of an

| K=2 | K=3 | K=4 | K=5 | K=6 | K=7 | |
|---|---|---|---|---|---|---|
| | | | | **3** + 17 | 17 | Shiriuchi stream |
| | | | 1 + 3 + 6 + 16 + **17** + 19 | | 1+**3**+**18** | various locations |
| | | | | 1 + **2** + 6 + 16 + 19 | | |
| | | 1 to 3 + 5-6 + 16 + 18-19 | | | 2 + 6 + 16 + 19 | various locations |
| | 1 to 6 + 16 to 19 | | 2 + 5 + 18 | 5 + 18 | 5 | Kane stream |
| 1 to 6 + 16 to 22 | | 4 + 17 | 4 | 4 | 4 | Chuzenji hatchery |
| | 20 to 22 | 20 to 22 | 20 to 22 | 20 to 22 | 20 to 22 | French hatcheries |
| 7 to 15 | 7 to 15 | 7 to 15 | 7 to 15 | 7 to 15 | 7 to 15 | Azusa samples |

**Fig. 4.** Successive assignment analyses, performed with STRUCTURE 2.3.4 software, from $K=2$ to $K=7$ represented as a tree. Numbers represent samples as in the first column of Table 1. Numbers in bold correspond to samples that "jumped" from one branch to another (= statistical artifacts).

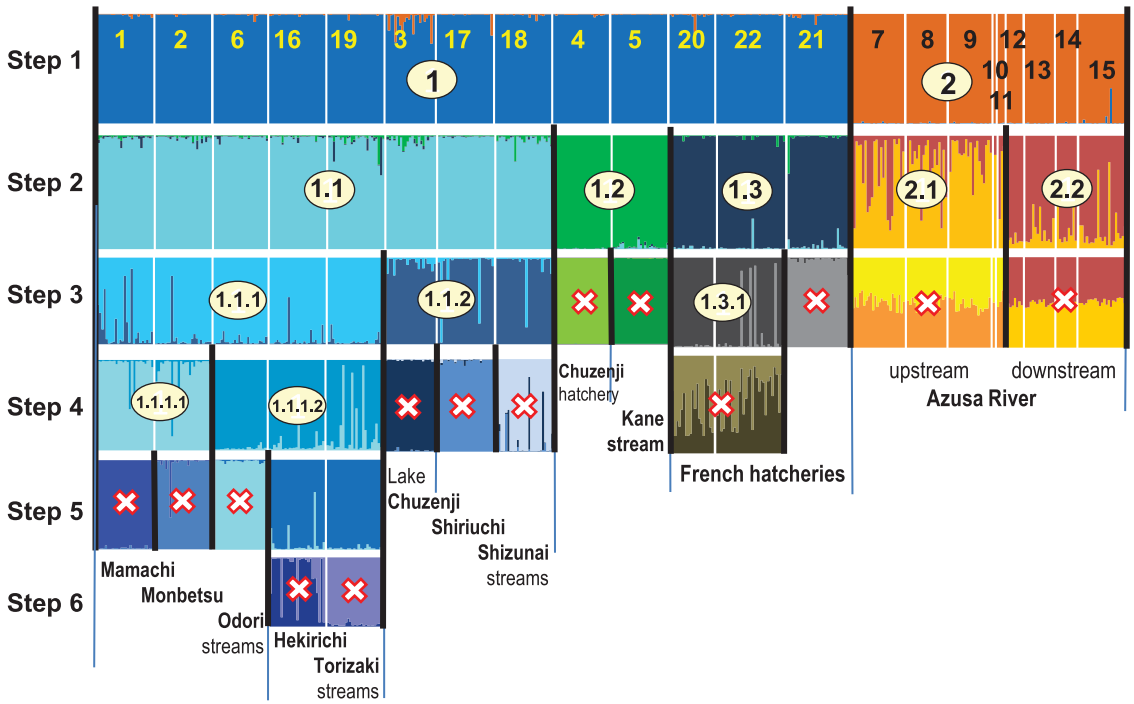


**Fig. 5.** Hierarchical assignment analysis. At each step and each cluster, STRUCTURE assignment is processed until absence of structure is reached (red crosses), *i.e.* multiple samples without subgroups (below 1.3.1, 2.1 and 2.2 where assignment cuts each individual and not between individuals) or a single sample with low diversity (all other cases).

independent import source of brown trout in the Azusa river system.

The Azusa river system was sampled at two sub-regions known as Kamikouchi (upstream) and Matsumoto (downstream) areas that are isolated from each other by four dams (Taishoh, Nagawado, Midono and Inekoki). While we do not know if construction of the dams occurred before or after trout introduction and are not sure if these dams are insurmountable to trout, our assignment and Fst tests clearly pointed out genetic interregional differentiation of the divided populations. Moreover, H-W disequilibrium was detected, if the entire Azusa sample-set was considered as one population, suggesting the presence of genetic substructures. Nevertheless, when calculated for the upper and the lower Azusa assemblage separately, both were found in panmixia. The coherence observed above and below the dams indicates gene flow between the samples within each area and limited or no transfer of genetic variation between the upstream and downstream

**Table 4.** The population parameters describe the samples' genetic diversity and their panmictic equilibrium. Panmixia is globally respected with some exceptions: * = significant at $p < 0.95$ (no longer significant after Bonferroni correction when between parentheses); na = not analyzed because the sample size was too small; ns = not significant (respects the panmixia). Azusa river trout were analyzed sample by sample, and then grouped into upstream and downstream assemblages (see text).

| Map | Station | N | Hnb | Ho | A | Fis | signif. |
|---|---|---|---|---|---|---|---|
| 1 | Mamachi stream | 25 | 0.6607 | 0.6563 | 5.5833 | 0.00696 | ns |
| 2 | Monbetsu stream | 25 | 0.5889 | 0.5817 | 4.7500 | 0.01261 | ns |
| 3 | Jizoku (Lake Chuzenji) | 22 | 0.7086 | 0.6854 | 6.3333 | 0.03340 | ns |
| 4 | Chuzenji hatchery | 25 | 0.3905 | 0.4267 | 2.2500 | -0.09480 | ns |
| 5 | Kane stream | 25 | 0.6541 | 0.6800 | 4.0000 | -0.04037 | ns |
| 6 | Odori stream | 24 | 0.6127 | 0.5859 | 4.6667 | 0.04464 | ns |
| 7 | Azusa (Kamikouchi) without name 1 | 24 | 0.4779 | 0.4618 | 3.7500 | 0.03441 | ns |
| 8 | Azusa (Kamikouchi) without name 2 | 18 | 0.4643 | 0.4679 | 3.8333 | -0.00792 | ns |
| 9 | Azusa (Kamikouchi) without name 3 | 19 | 0.4354 | 0.4474 | 3.0833 | -0.02828 | (*) |
| 10 | Azusa (Kamikouchi) Zenrokusawa stream | 1 | na | na | na | na | na |
| 11 | Azusa (Kamikouchi) Shimizusawa stream | 3 | na | na | na | na | na |
| 7 to 11 | Upper Azusa R. | 65 | 0.4700 | 0.4655 | 4.2500 | 0.00962 | ns |
| 12 | Azusa (Matsumoto city) | 7 | 0.4644 | 0.4802 | 2.8333 | -0.03596 | ns |
| 13 | Azusa (Matsumoto city) YOY | 13 | 0.4951 | 0.5385 | 3.7500 | -0.09150 | ns |
| 14 | Azusa (Matsumoto) Toyoshina | 9 | 0.4670 | 0.4213 | 3.5000 | 0.10297 | ns |
| 15 | Azusa (Matsumoto) Shimauchi | 21 | 0.4509 | 0.3974 | 4.5000 | 0.12127 | * |
| 12 to 15 | Lower Azusa R. | 50 | 0.4848 | 0.4879 | 4.2500 | -0.00652 | ns |
| 16 | Hekirichi stream | 25 | 0.6837 | 0.7129 | 6.1667 | -0.04368 | (*) |
| 17 | Shiriuchi stream | 25 | 0.6349 | 0.5667 | 4.0000 | 0.10946 | * |
| 18 | Shizunai stream | 25 | 0.5967 | 0.6133 | 4.9167 | -0.02853 | (*) |
| 19 | Torizaki stream | 25 | 0.6828 | 0.6567 | 6.3333 | 0.03902 | ns |
| 20 | Lées Athas hatchery | 20 | 0.6355 | 0.5500 | 4.9167 | 0.13755 | ns |
| 21 | Cauterets hatchery | 28 | 0.7765 | 0.7629 | 8.0833 | 0.01782 | ns |
| 22 | Isère hatchery | 30 | 0.6705 | 0.6694 | 6.0833 | 0.00164 | ns |

**Table 5.** Fst estimation between sample pairs, considering Azusa samples as separate, upper and lower assemblages. All comparisons are highly significantly different from zero ($p < 0.0002$, ***). Colored cells designate low values below 0.1 (red, orange, yellow and beige show increasing values). The framed cell shows the significant Fst between the upper and lower Azusa river.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 to 11 | 12 to 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mamachi** stream | 1 | 0 | 0,100 | 0,085 | 0,321 | 0,173 | 0,149 | 0,243 | 0,239 | 0,102 | 0,152 | 0,178 | 0,099 | 0,125 | 0,109 | 0,110 |
| **Monbetsu** stream | 2 | | 0 | 0,128 | 0,373 | 0,171 | 0,137 | 0,267 | 0,268 | 0,080 | 0,165 | 0,160 | 0,094 | 0,154 | 0,144 | 0,137 |
| **Jigoku** (Lake Chuzenji) | 3 | | | 0 | 0,294 | 0,168 | 0,156 | 0,224 | 0,218 | 0,114 | 0,129 | 0,156 | 0,124 | 0,141 | 0,091 | 0,138 |
| **Chuzenji** hatchery | 4 | | | | 0 | 0,340 | 0,380 | 0,434 | 0,439 | 0,356 | 0,353 | 0,380 | 0,334 | 0,388 | 0,309 | 0,360 |
| **Kane** stream | 5 | | | | | 0 | 0,200 | 0,331 | 0,324 | 0,155 | 0,215 | 0,178 | 0,138 | 0,206 | 0,129 | 0,177 |
| **Odori** stream | 6 | | | | | | 0 | 0,278 | 0,279 | 0,079 | 0,171 | 0,211 | 0,085 | 0,161 | 0,150 | 0,157 |
| upper **Azusa** r. | 7 to 11 | | | | | | | 0 | 0,051 | 0,260 | 0,305 | 0,314 | 0,276 | 0,250 | 0,240 | 0,245 |
| lower **Azusa** r. | 12 to 15 | | | | | | | | 0 | 0,255 | 0,309 | 0,326 | 0,280 | 0,233 | 0,221 | 0,229 |
| **Hekirichi** stream | 16 | | | | | | | | | 0 | 0,127 | 0,149 | 0,054 | 0,129 | 0,116 | 0,110 |
| **Shiriuchi** stream | 17 | | | | | | | | | | 0 | 0,201 | 0,141 | 0,185 | 0,137 | 0,184 |
| **Shizunai** stream | 18 | | | | | | | | | | | 0 | 0,156 | 0,188 | 0,175 | 0,180 |
| **Torizaki** stream | 19 | | | | | | | | | | | | 0 | 0,127 | 0,111 | 0,105 |
| **Lees Athas** hatchery | 20 | | | | | | | | | | | | | 0 | 0,104 | 0,033 |
| **Cauterets** hatchery | 21 | | | | | | | | | | | | | | 0 | 0,089 |
| **Isère** hatchery | 22 | | | | | | | | | | | | | | | 0 |

samples. This suggests that each can be considered a single population.

The Chitose river was also examined at two stations (Mamachi and Montbetsu streams: samples 1 and 2). The Fst value between them is significant (Tab. 5), while they were not assigned to different lineages before K=7 in the one run assignment analysis (Fig. 4) nor before step 5 in the hierarchical assignment analysis (Fig. 5). All these results indicate slight genetic differences and the two stations, physically isolated by damming, cannot be considered as hosting the same population.

The remaining Japanese populations sampled (*i.e.* without Azusa) seem to stem from a single or several introductions based on genetically very similar material (Figs. 3–5; Tab. 5), which, taking into account the assumed history of brown trout stocking, is very likely for brown trout from Lake Chuzenji. An exception was the Chuzenji hatchery sample, which differed considerably from the Jigoku sample in fixation index (Fst = 0.29) on the basis of microsatellites, although sharing its only haplotype with the Jigoku (lake) sample. This differentiation can be explained by the fact that the hatchery population has been kept in captivity for more than 30 years without input of any fish from other populations. Its genetic diversity was found to be the lowest in the entire sample-set, even lower than for the fragmented Azusa river samples. This is probably a consequence of a small effective population size (small number of genitors) and selection pressure due to the hatchery environment accompanied by founder effects and random genetic drift. Altogether, this could have specifically shaped the distinct genetic profile of the Chuzenji hatchery population and impoverished its genetic diversity. Interestingly, the pairwise Fst-values between the hatchery sample and each of the remaining Japanese samples is nevertheless much higher ($0.30 < Fst < 0.44$) than between the hatchery and lake samples (Fst = 0.29), which implies that the hatchery population originating from the lake has differentiated due to its long-lasting separation.

According to the records on brown trout imports to Japan, a French source was assumed to be involved. Using FCA, genetic proximity of most Japanese populations with individuals from French hatcheries was implied at first sight (Fig. 3A). This was supported with the very low Fst value 0.09 between the Lake Chuzenji and Cauterets hatchery samples suggesting a French origin of part of current Japanese populations as possible. However, more in-depth multidimensional analysis (Fig. 3B) revealed that the individuals from French hatcheries are actually rather separated from all the Japanese samples, suggesting that these Japanese populations are not of French origin, even though the Nagano Prefecture (mid part of Honshu island) is known to have been stocked with a brown trout strain shipped over by a French private company (Maruyama *et al.*, 1987). Moreover, the Azusa river and Chuzenji hatchery samples appeared to be genetically very distinct from modern hatchery individuals, and so, as argued above, their source should probably be connected with early introduction from the USA. However, it should not be neglected that the genetic profile of microsatellites characterizing trout in modern French hatcheries could differ from that of fish imported from France, whose origin is not known. For more precise identification of the origin of Japanese trout, additional out-groups from the Atlantic basin should be included.

### 4.2 Adaptation of introduced populations

One of the known difficulties of adaptation of introduced species is their lack of genetic diversity. An introduced species is often characterized by a small founder population inducing strong founder effect and genetic drift (Sakai *et al.*, 2001; Allendorf and Lundquist, 2003; Yonekura *et al.*, 2007). The consequences can be inbreeding depression, poor ability to adapt and low success in a new environment (Reed and Frankham, 2003; Spielman *et al.*, 2004).

In Japan, most introduced brown trout populations in rivers exhibited genetic diversity similar to that in domestic French hatcheries, which are considered as highly polymorphic (Hnb around 0.65, Bohling *et al.*, 2016). The main exception is Azusa river samples, which always showed Hnb $< 0.5$, suggesting distinct origins and/or methods of management. The high genetic diversity of most Japanese brown trout populations results from the introduction of highly polymorphic strains and the suitability of Japanese rivers as a habitat for brown trout, since no founder effect was detectable except in the Azusa basin. Maintenance of wide diversity and genetic similarity all around Japan, except for the Azusa river, may also be due to a relatively recent common introduction and/or the presence of large populations able to avoid genetic drift.

## 5 Conclusions

Several useful observations can be inferred from this study on self-sustaining brown trout populations in Japan:

- The introduced brown trout are genetically not homogeneous, which is probably a consequence of several introductions, at least two.
- In most cases, there is no real geographic logic in the clustering of samples, which is expected for introduced populations depending mostly on human decisions; the exception is Azusa brown trout, which are differentiated from other populations and probably represent an independent import from the USA in the 1930's.
- The Chuzenji hatchery (sample 4) houses a genetically very distinct strain. This is likely due to intense manipulation under isolated experimental conditions over 30 years.
- Most populations showed high genetic diversity (Mamachi, Kane and Odori streams, Lake Chuzenji) and so favorable capacities for adaptation, with the exception of the Azusa river samples, where a certain lack of genetic polymorphism was detected.
- While of limited efficiency, the genetic comparison between some European domestic samples and the Japanese ones suggested that brown trout in Japan stem from hatchery raised strains originating from north Atlantic European Rivers.

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## Appendix 1

Distribution of haplotypes ATcs1, 2, 3 and 4.

| Haplotype | Accession number | Distribution – country/drainages |
|---|---|---|
| ATcs1 | AF273086 | [1]Denmark (Skals), Norway (Bjornes Lake, Sima), Spain (hatchery stocks), [2]Spain (Garona), France (Gulf of Biscay), Iceland (Skorradalsvatn), British Isles (Coquet, Wear, Lune and Melvin), [3]Czech (Elbe and Oder), Slovakia (Vistula) |
| ATcs2 | AF273087 | [1]Denmark (Skals and Karup), Norway (Guddal and Sima), Spain (hatchery stocks), [2]France (Gulf of Biscay), [2]British Isles (Coquet, Stour, Rother, Fowey, Teifi, Conwy, Loch Romoch), [2]Russia (Nilima and Vorobiev), [3]Czech (Elbe and Oder), Slovakia (Vistula) |
| ATcs3 | AF274574 | [1]Denmark (Skals), Norway (Bjornes Lake, Guddal and Sima), Spain (hatchery stocks), [2]Spain (Garona), France (Gulf of Biscay), British Isles (Coquet, Wear, Rother, Teifi, Conwy, Melvin), [3]Czech (Elbe and Oder), Slovakia (Vistula) |
| ATcs4 | AF274575 | [1]Denmark (Skals and Karup), Norway (Bjornes Lake, Guddal and Sima), Spain (hatchery stocks), [2]France (Gulf of Biscay), British Isles (Lune) |

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## Appendix 2

List of mtDNA CR haplotypes used for genealogical analysis, with their GenBank accession numbers. The six haplotypes detected in Japanese populations are underlined.

| Haplotype | Accnb | Haplotype | Accnb |
|---|---|---|---|
| ATcs1 | AF273086 | ATcs28 | EF530490 |
| ATcs2 | AF273087 | ATcs29 | EF530491 |
| ATcs3 | AF274574 | ATcs30 | EF530492 |
| ATcs4 | AF274575 | ATcs31 | EF530493 |
| ATcs5 | AF274576 | ATcs32 | EF530494 |
| ATcs6 | AF274577 | ATcs33 | EF530495 |
| A17 = At1f | HQ848368 / DQ841193 | ATcs34 | EF530496 |
| ATcs11 | AY836327 | ATcs35 | EF530497 |
| At11a | AY185578 | ATcs36 | EF530498 |
| At11b | AY185579 | ATcs37 | EF530499 |
| ATcs12 | AY836328 | ATcs38 | EF530500 |
| ATcs14 | EF530476 | ATcs39 | EF530501 |
| ATcs15 | EF530477 | ATcs41 | EF530502 |
| ATcs16 | EF530478 | ATcs42 | EF530503 |
| ATcs17 | EF530479 | ATcs43 | EF530504 |
| ATcs18 | EF530480 | ATcs45 | EF530505 |
| ATcs19 | EF530481 | ATcs46 | EF530506 |
| ATcs20 | EF530482 | ATcs47 | EF530507 |
| ATcs21 | EF530483 | ATcs48 | EF530508 |
| ATcs22 | EF530484 | ATcs49 | EF530509 |
| ATcs23 | EF530485 | ATcs50 | EF530510 |
| ATcs24 | EF530486 | ATcs51 | EF530511 |
| ATcs25 | EF530487 | ATcs52 | EF530512 |
| ATcs26 | EF530488 | ATcs53 | MK330940 |
| ATcs27 | EF530489 | | |