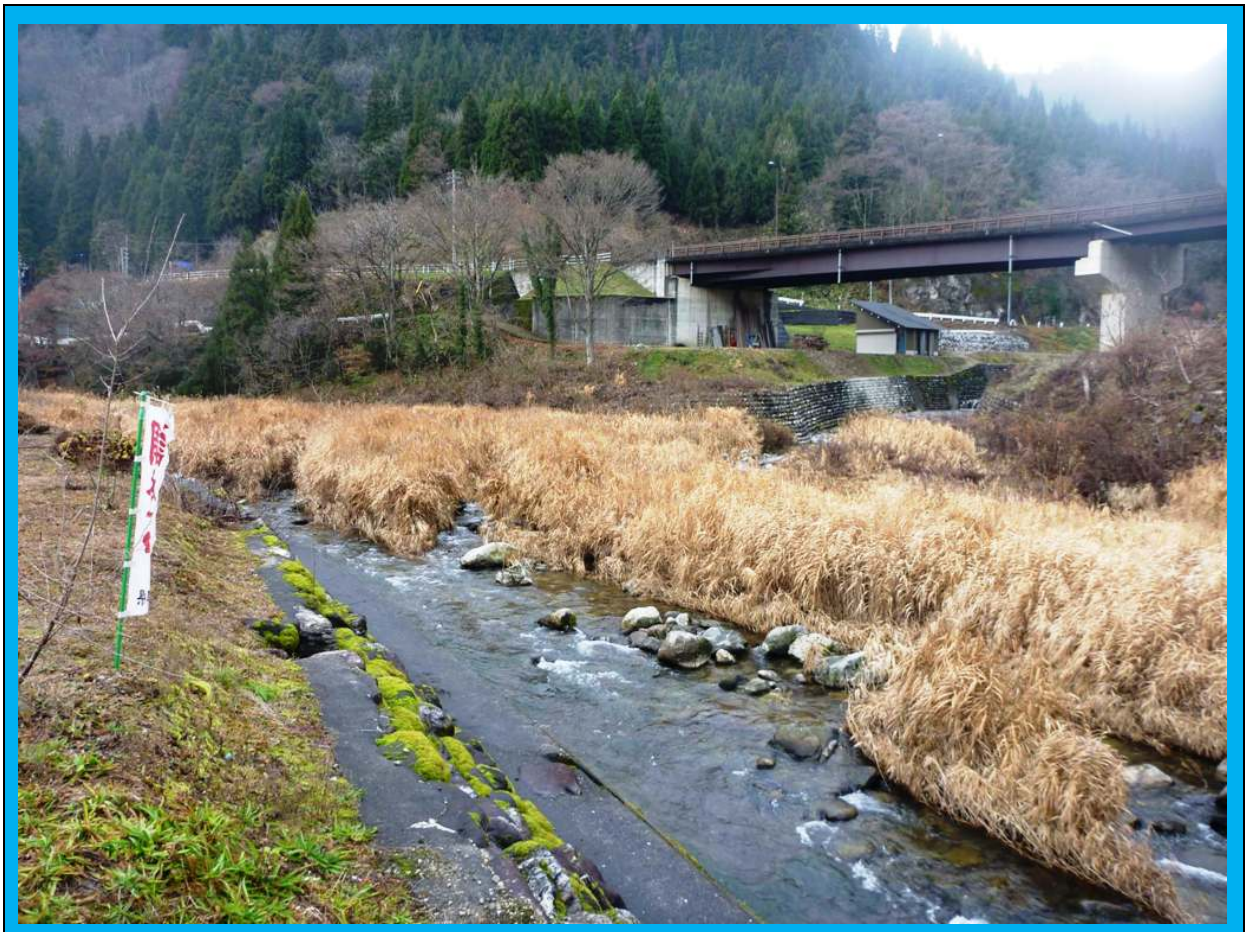
Genetic diversity of brown trout introduced in Japan

Report **JPNTROUT step1**
April 2017



Odori stream © K. Hasegawa

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1. Introduction

Brown trout (*Salmo trutta*) is one of the most handled fish in the world, together with some other salmonids and species of cichlids, silurids and cyprinids. The manipulation consists mainly in the domestication, generally for meat production, then for introduction of domestic forms in the wild, as eggs in boxes, fry or sub-adults. When introduced in the natural range of the species, complex genetic exchanges between the domestic and the wild part of the species occur, generally provoking negative consequences (Brockmark et al. 2010; Wollebaek et al. 2012; Hoxmeier and Dieterman 2013). This negative effect on natural populations has been described in other fish (Miller et al. 2004; Utter 2004; Harvey et al. 2015) and also on mammals (Feulner et al. 2013). When successfully introduced outside the natural range of the species, new self-sustaining populations are created, sometimes for the benefit of local managers (Dieterman and Hoxmeier 2011; Snook and Dieterman 2015), sometimes behaving as invasive forms with ecological consequences (Jonsson and Jonsson 2011; Budy et al. 2013; Splendiani et al. 2016).

Brown trout natural range covers Europe, Western Asia and North Africa (Figure 1). According to the Dloop marker of the mtDNA, five main geographic lineages have been described: AT for Atlantic, ME for Mediterranean, AD for Adriatic, MA for marble trout (north of Adriatic Sea) and DA for Danube (Bernatchez et al. 1992). Several secondary lineages, placed at the origin of the main ones, have also been described (e.g. DU for Duero, TI for Tigris, NA for North Africa…).

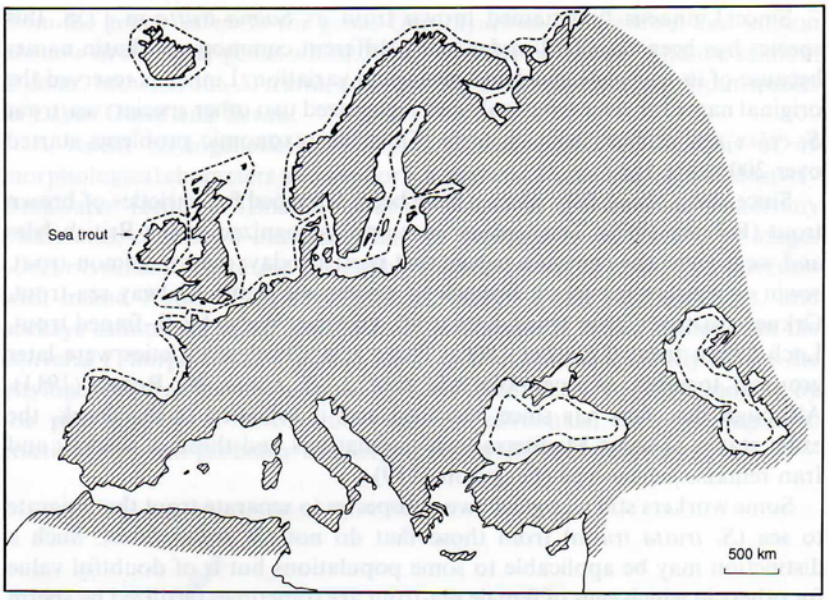

Figure 1: *Brown trout natural distribution (Elliot 1989)*

Brown trout is now introduced in all continents, in numerous locations. Figure 2 give an idea, dating from 1989, of the huge distribution of brown trout outside Europe. Nothing is known on its adaptation and on the characteristics of the self-sustaining populations: only some negative ecological effects have been investigated.

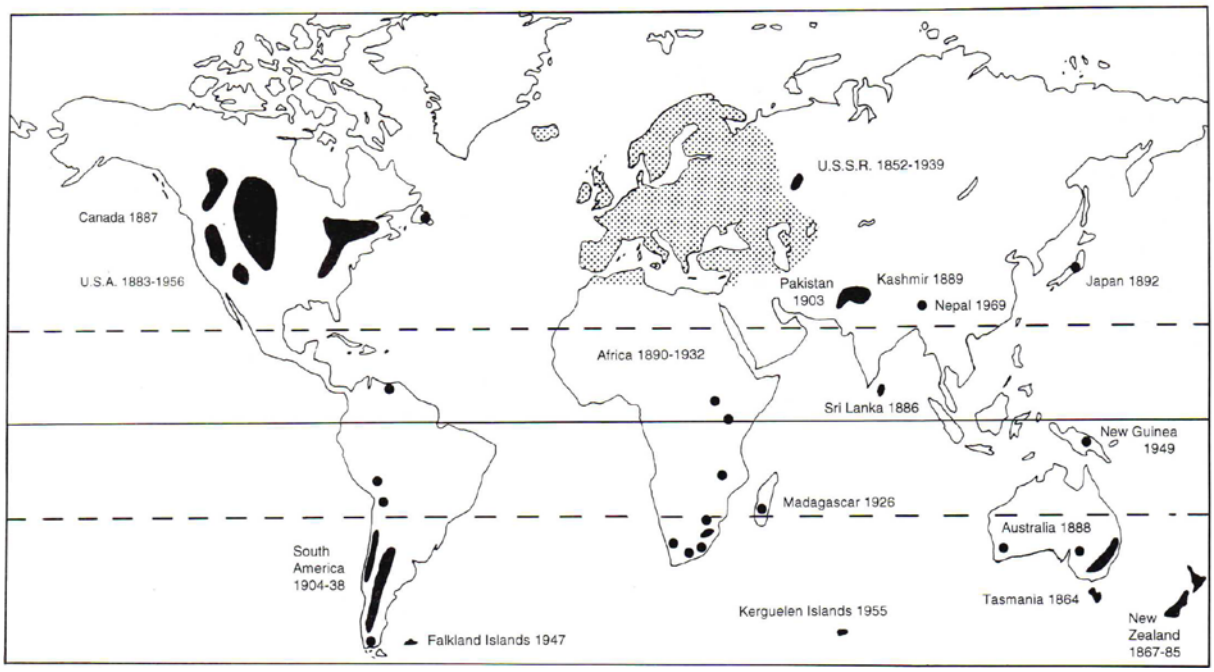

Figure 2: *Brown trout introduced distribution with some expected dates of arrival (Elliot 1989)*

In Japan, the first introduction of brown trout is due to its erroneous presence among eggs of rainbow trout or brook trout from USA before 1900 (1892 according to Elliot 1989; 1877 according to Maruyama et al. 1987). So, there is no official record on brown trout introduction. However, by investigating on the field and in literature, more information could be gathered:

The population of Lake Chuzenji in Nikko city, Tochigi prefecture (about 100km north from Tokyo) may be the oldest brown trout population in Japan (there is no official record, but it was maybe introduced around 1900's), and other brown trout in Japan are thought to be introduced via Lake Chuzenji.

In Feburary 1973, eyed eggs were imported from Mepp Co., a French private company, and stocked to rivers in Nagano Prefecture (Maruyama et al. 1987). It was not possible to find this company nowadays.

In Chitose River in western Hokkaido, anglers released brown trout in 1980's, and first found evidence of reproduction in 1984 (Urawa 1989). But, there is no record of the first introduction in this river.

In Odori stream, brown trout have escaped from a fish hatchery in September 2004 due to the damage of a typhoon. It was a very old and small hatchery (see photo in Appendix 1). Probably as a consequence, in 2008 and 2009, juvenile and adult brown trout, including mature individuals, were observed and captured (Ishizaki et al. 2012).

In Hokkaido, northern part of Japan, salmonid species are major species in freshwater fish fauna. Thus, nonnative brown trout (also nonnative rainbow trout) became problematic firstly in Japan (since late 1990's). The biggest problem is that nonnative brown trout replaced native white-spotted charr (*Salvelinus leucomaenis*) through competition and maybe hybridization

(Hasegawa 2017). On the other hand, their impact on another major native salmonid, the masu salmon (*Oncorhynchus masou*), seems to be not so serious due to habitat niche segregation (Hasegawa and Maekawa 2006; Hasegawa et al. 2012). Globally, the impacts of brown trout on local fauna are not studied enough. Hasegawa et al. 2017 paper is the first study which evaluated the impact on local fish fauna in Japanese streams. Due to these backgrounds, stocking brown trout has been prohibited since 2003 in Hokkaido.

On the other hand, the climate of Honshu, the biggest island and the main part of Japan, is warmer than that of Hokkaido. In Honshu, salmonid species are not so popular fish, and their main habitat is limited in upstream rivers. Thus, nonnative salmonids like brown and rainbow trout had not been recognized as "a danger", and there is no law to restrict keeping them in hatcheries and also stocking into natural streams. However, new populations of nonnative brown trout has been found in many regions in Honshu since 2000's, Japanese government is now in a hurry to make a law for brown trout management, mostly prohibiting stocking into natural streams.

In order to understand the capacity of brown trout to establish and reproduce out of its natural range, an informal international research consortium was established in 2016 with the objective to:

- describe the genetic diversity of European hatcheries stocks (project DOM-E) in order to detect the strains at the origin of worldwide introductions;
- describe the world self-sustaining introduced population diversity and adaptation (project WTROUT).

When sampling in a given country or region allow it, special studies are developed in order to understand local origin of brown trout and its adaptation. The different special studies will feed the WTROUT project.

All these projects are developed with the objective to be finally published.

The present step report aims at analyzing and interpreting Japanese trout samples genotypes (project JPNTROUT).

2. Sampling

The sampling was done by the Japanese part of the consortium. The characteristics of all analyzed trouts are given Table 1. Their geographic location is represented in Figure 3. Each river was given a map number between 1 and 7 and each sample a working number from 1 to 18.

Map n°	Calculs n°	Stations	River basin	Elevation (m)	Sampling date	Tissue N	Genotypes N	Donator	Reports	ISEM samples n°	ISEM individuals n°
1	1	Mamachi stream	Ishikari (W)	12	September 2012	27	25	K. HASAGAWA	JPNTROUT+WTROUT	F514	T30427-T30451
2	2	Monbetsu stream	Ishikari (W)	51	September 2012	28	25	K. HASAGAWA	JPNTROUT+WTROUT	F515	T30454-T30478
3	3	Jigoku (Lake Chuzenji)	Tone (E)	1269	January 2017	23	22	K. HASAGAWA	JPNTROUT+WTROUT	F528	T30714-T30736
4	4	Chuzenji hatchery	P (E)	1269	December 2016	30	25	K. HASAGAWA	JPNTROUT+WTROUT	F516	T30482-T30506
5	5	Kane stream	Fuji (E)	658	Nov. & Dec 2016	43	25	K. HASAGAWA	JPNTROUT+WTROUT	F517	T30512-T30536
6	6	Odori stream	Jinzu (W)	428	November 2016	40	24	K. HASAGAWA	JPNTROUT+WTROUT	F518	T30555-T30579
7A	7	Azusa (Kamikouchi) without name 1	Shinano (W)	593	June 2013	24	24	K. HASAGAWA	JPNTROUT+WTROUT	F519	T30595-T30618
7A	8	Azusa (Kamikouchi) without name 2	Shinano (W)		June 2013	18	18	K. HASAGAWA	JPNTROUT+WTROUT	F520	T30619-T30636
7A	9	Azusa (Kamikouchi) without name 3	Shinano (W)		September 2013	19	19	K. HASAGAWA	JPNTROUT+WTROUT	F521	T30637-T30655
7A	10	Azusa (Kamikouchi) Zenrokusawa stream	Shinano (W)		November 2013	2	1	K. HASAGAWA	JPNTROUT+WTROUT	F522	T30656
7A	11	Azusa (Kamikouchi) Shimizusawa stream	Shinano (W)		November 2013	4	3	K. HASAGAWA	JPNTROUT+WTROUT	F523	T30659-T30661
7B	12	Azusa (Matsumoto city)	Shinano (W)		May 2008	7	7	K. HASAGAWA	JPNTROUT+WTROUT	F524	T30662-T30668
7B	13	Azusa (Matsumoto city) YOY	Shinano (W)		May to Nov. 2008	13	13	K. HASAGAWA	JPNTROUT+WTROUT	F525	T30669-T30681
7B	14	Azusa (Matsumoto) Toyoshina	Shinano (W)		November 2016	11	9	K. HASAGAWA	JPNTROUT+WTROUT	F526	T30682-T30692
7B	15	Azusa (Matsumoto) Shimauchi	Shinano (W)		November 2016	21	21	K. HASAGAWA	JPNTROUT+WTROUT	F527	T30693-T30713
-	16	Lées Athas hatchery	hatchery	-	2014	20	20	P. BERREBI	TFP1	L443	T26033-T26052
-	17	Cauterets hatchery	hatchery	-	2014	28	28	P. BERREBI	MAE1	L556	T28112-T28140
-	18	Isère hatchery	hatchery	-	2008	30	30	P. BERREBI	GSALM2	L266	T16926-T16955

Table 1: *Characteristics of the Japanese samples considered. For comparison, a French commercial strain has been added (last line)*

To complete information:

- Two samples were stocked in the freezing chamber in the Hokkaido National Fisheries Research Institute: the brown trout sampled in 2012 from Mamachi stream (locations described in Kitano et al. 2009) and Monbetsu stream (see Kawai et al. 2013 for the location). Both streams are tributaries of Chitose River, but these streams are separated by a big dam, so the samples probably belong to different populations. Brown trout have invaded these streams from 1980's.

- Figure 3 gives the geographic location of each sampled region.

- Azusa River sampling was divided into two sub-samples (7A & B). At least, there are four unpassable dams between 7A and 7B zones (Figure 4). Some samples from Azusa River are pieces of adipose fin, because these were sampled few years ago.

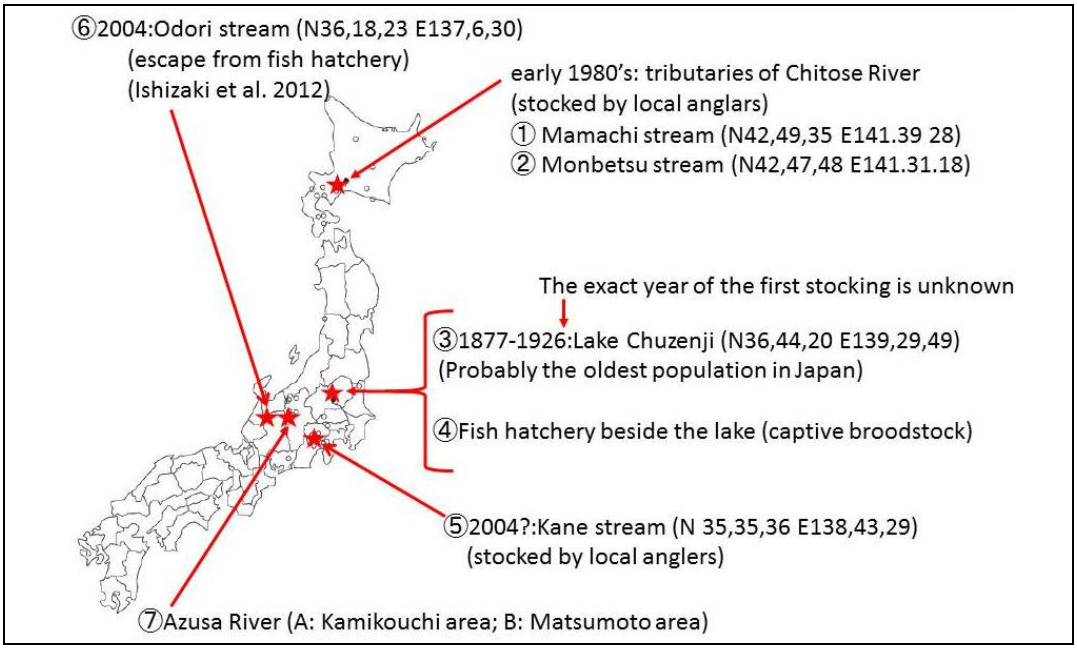

Figure 3: *Synthetic map of the seven samples or groups of samples*

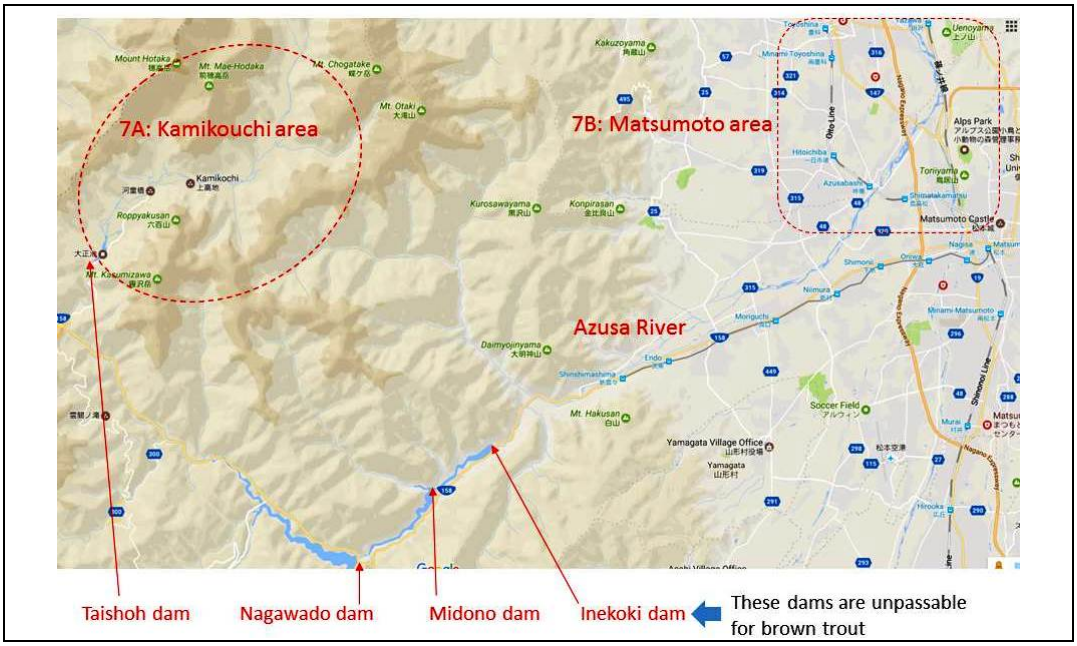

Figure 4: *Details of the Azusa River sampling. Kamikouchi (7A) area is composed of 5 subsamples, Matsumoto (7B) of 4 (see Table 1)*

The samples arrived in Montpellier the 23 January and 3 February 2017. Genotyping took place between the 3rd and 14th of April 2017.

3. Molecular methods

Fin clips were cleared of alcohol in the Labofarm-Genindexe private laboratory. A very small piece of fin was treated with Chelex method for DNA extraction. The improved Chelex extraction procedure is based on the method of Estoup et al. (1996). A set of 12 nuclear microsatellite loci were selected for analysis according to their polymorphism (*Mst543, MST85, Omm1105, Omy21Dias, Oneμ9, Sfo1, Ssa197, SsoSL311, SsoSL438, SsoSL417, Str591* and *StrBS131*; Berrebi *et al.*, 2013). The characteristics of the markers are developed in Table 2. The twelve loci were amplified within tree multiplex PCR (Table 2). PCR amplifications were carried out using the Qiagen multiplex PCR kit (Qiagen) in a 10 µL volume containing 3 µL of genomic DNA diluted at 10ng/µL, 5 µL of Qiagen PCR Master Mix, 1 µL of Qiagen Q-solution, 1 µL of primer mix of variable concentration (Table 2) with forward primers labelled at 5' end using different fluorescent dyes (FAM, HEX, or NED). Amplifications were conducted in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems), according to the supplier's instructions (Qiagen multiplex PCR kit): 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 during 30 min. Amplified PCR fragments were then diluted and separated on a capillary ABIPRISM 3130*xl* sequencer (Applied Biosystems) with the use of GeneScan500Rox dye as standards size. Fragment lengths were assessed using GeneMapper v4.1 software system (Life Technologies TM).

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

Table 2: *Twelve microsatellite loci characteristics*

4. Statistical methods

4.1. Multidimensional analysis

I order to draw the overall genetic structure of the involved samples in a unique diagram, a Factorial Correspondence Analysis (FCA, Benzécri, 1973), implemented within the GENETIX 4.04 program (Belkhir et al., 2004), is first performed. This method is well adapted to genotype data and mathematical details are friendly given in She et al. (1987). The matrix necessary to feed the program is constituted of the trout individuals as lines and the alleles as

columns. In the cells, the alleles are counted as 0 if absent, 1 if heterozygote and 2 if homozygote. Each trout was positioned in a hyperspace according to its 24 alleles. Correlations are the frequency, for two alleles, to be found in the same individual. Clusters (clouds) detected on the diagram correspond to homogeneous genetic lineages, gathering individuals according to their multilocus genotype and independently of their geographic origin.

4.2. Assignment tests

Assignment tests, using the Bayesian STRUCTURE 2.1 program (Pritchard et al., 2000), subdivided the whole sample into K = 2 to 15 subgroups characterized by the best genetic equilibrium in terms of panmixia and lower linkage. The admixture ancestry model and correlated allele frequencies option were chosen. A burn-in of 100,000 iterations followed by 200,000 additional Markov Chain Monte Carlo iterations were run. For each K value, 10 runs were repeated in order to check the stability of the assignment.

The 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 & von Holdt, 2012). This automatic estimation was completed by the "higher K which makes sense" method (Berrebi et al., 2013). 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". So, levels of K higher than the more significant suggested by the Delta K method have been explored.

4.3. Population parameters and equilibria

Panmixia was estimated through Fis parameter. For this, the estimator f of Weir & Cockerham (1984) was calculated, together with its significance after 5,000 permutations of alleles within each sample, performed with the GENETIX software.

Differentiation between samples is estimated using the Fst parameter through the estimator of Weir & Cockerham (1984). 5,000 permutations of individuals among samples allow estimating the significance of the differentiations.

Another potentially important statistical calculation is the LD. This estimation can indicate a recent introduction of foreign lineage (Walhund effect: Sinnock, 1975). It can also indicate differential behavior between markers kinds. The Black & Krafsur (1985) method is implemented in GENETIX software, followed by an estimation of the significance with 5,000 permutations.

Sequential Bonferroni corrections (Rice, 1989) have been performed for repeated tests.

5. Results

5.1. Multidimensional picture

This method places each trout in a diagram according to all its genetic components. Because based on the Khi-2 distances, it favors the exceptions with respect to the Gaussian distribution of the variables.

Two aspects of the same analysis are presented: Figure 5 positions the individuals while Figure 6 positions the barycentres of the samples. What are the results?

- Figure 5: the overall structure is divided into 3 subgroups:

i) nearly all Japanese samples, from North and South of the country and French hatcheries (black ellipse) suggesting that Japan stocking was made from a unique origin, close to the French commercial hatcheries;

ii) the Lake Chuzenji hatchery suggesting that this strain has no impact on the present Japanese brown trout populations;

iii) the Azusa River samples, suggesting the history of this river's stocking is totally different from that of the remaining sampled rivers.

- Figure 6 shows 4 clusters: not only the Lake Chuzenji hatchery, but also the French hatcheries strains are distinct of the main Japanese river populations. Azusa River form a well distinct group, slightly divided into Matsumoto and Kamikouchi sub groups.

All these suggestions will be tested by assignment analysis.

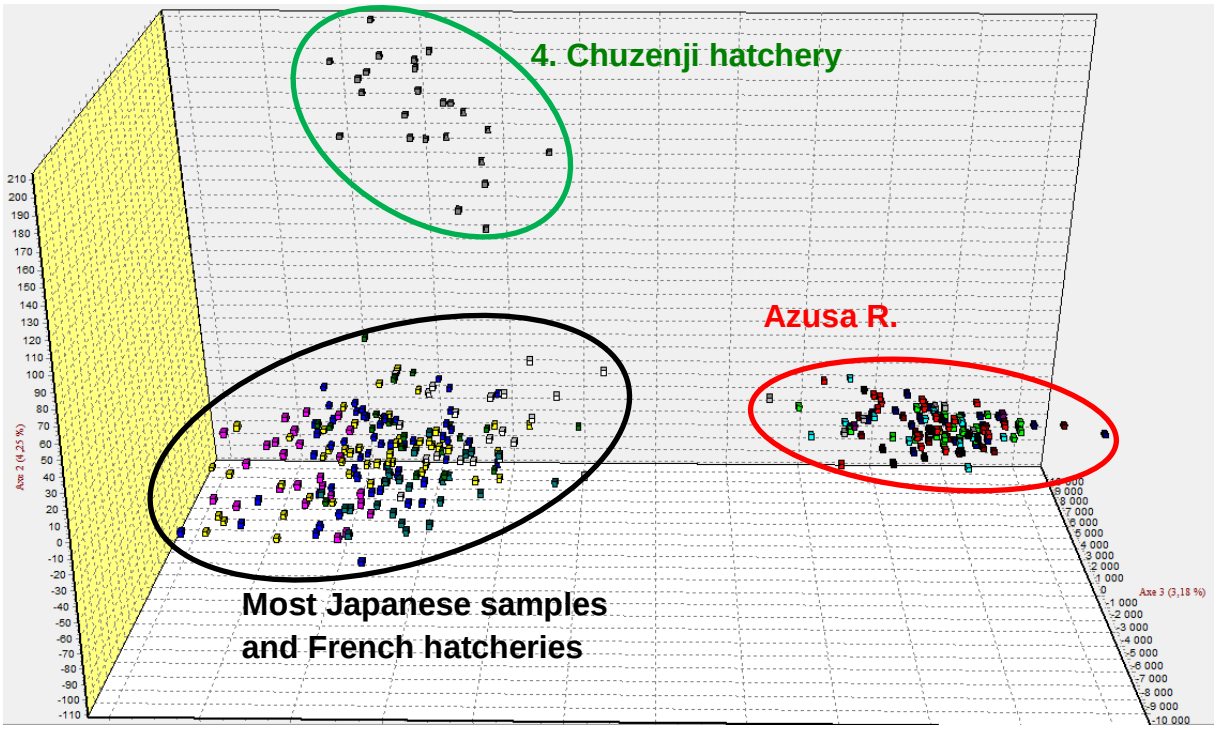

Figure 5: FCA of the whole sampling showing 3 main subgroups.

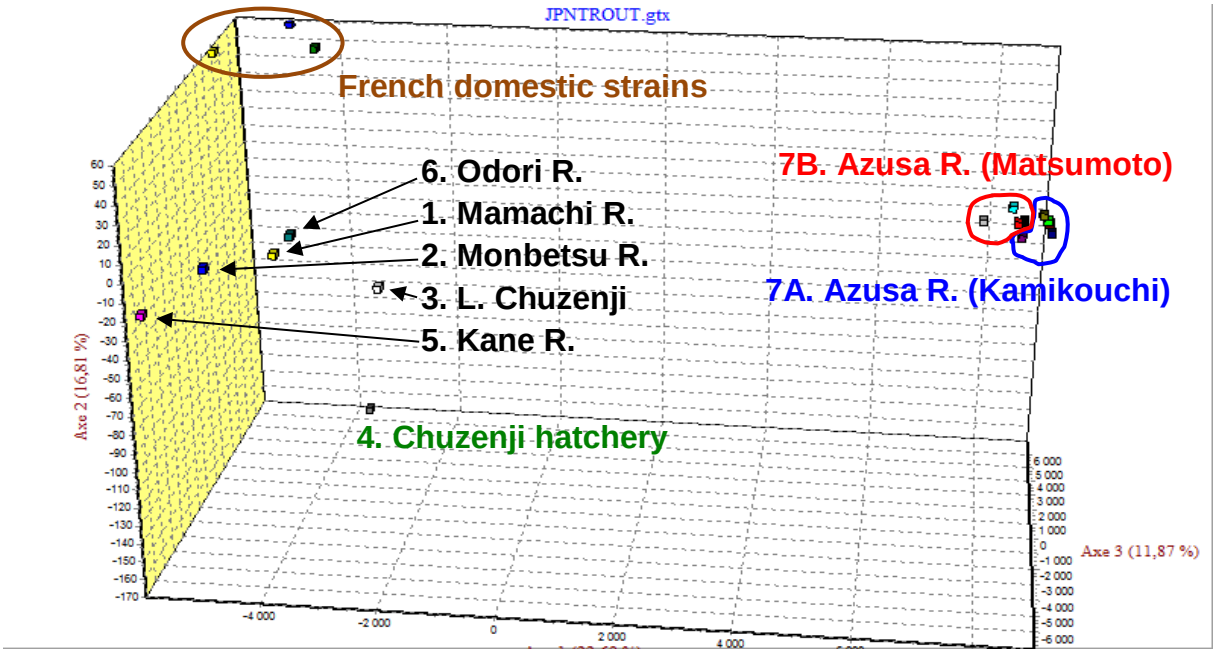

Figure 6: *Same FCA presenting the barycenters (centers of gravity) of each sample.*

5.1. Assignment structure

This method consists of dividing the global sample (Japanese and French) into K sub units by trial-and-error method. At each step, the STRUCTURE software give a random partition, this partition is checked according to equilibrium populations parameters (panmixia and linkage). By learning (artificial intelligence), each tentative improve the equilibrium parameters (here, 100,000 iterations are not recorded, and then the 200,000 following iterations try to reach the best equilibrium according the imposed K value).

The decision help method (Evanno et al 2005 method) suggest that the partition into 2 (K=2), 4, 8 then 13 are the more significant (decreasing order). They are represented in Figure 8 in orange then in yellow.

Figure 7 represent the K=4 and K=8 partitions. Table 3 gives the assignment percentages and Figure 8 restitutes all the partitions obtained successively. For K=4, the 4 clusters of Figure 6 are reproduced. K=8 show that Mamachi and Monbetsu streams are very similar (they belong to the Ishikari basin), that Azusa River is very homogeneous and that the French hatcheries strains show two slightly differentiated groups.

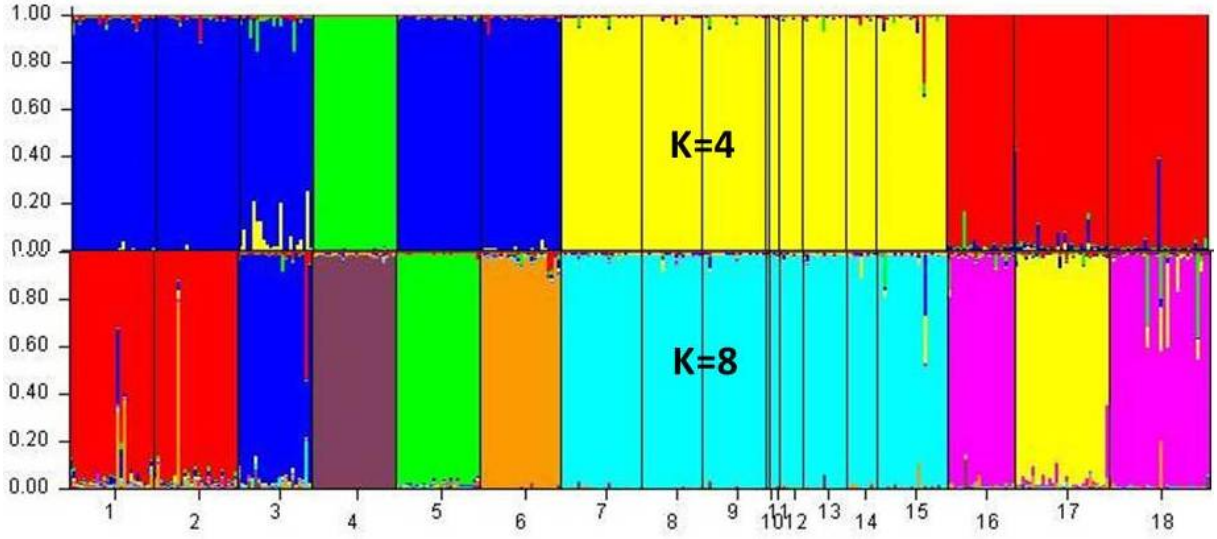

Figure 7: *STRUCTURE output for K=4 and K=8. Each color, dispatched at random by the software, represents a genetic sub unit. The numbers at the bottom correspond to the "Calcul n°" column of Table 1.*

Calculs n°	Stations	Genotypes N	dominant	Chuzenji hatchery	Azusa	French domestic	Ishikari	Lake Chuzenji	Chuzenji hatchery	Kane	Odori	Azusa	64 and 38 French hatcheries	65 French hatchery
1	Mamachi stream	25	0,97	0,01	0,01	0,01	0,90	0,03	0,01	0,01	0,04	0,01	0,01	0,01
2	Monbetsu stream	25	0,98	0,01	0,00	0,01	0,91	0,01	0,01	0,01	0,05	0,00	0,01	0,01
3	Jigoku (Lake Chuzenji)	22	0,91	0,03	0,06	0,01	0,04	0,90	0,01	0,01	0,01	0,02	0,01	0,01
4	Chuzenji hatchery	25	0,00	0,99	0,00	0,00	0,00	0,00	0,98	0,00	0,00	0,00	0,00	0,00
5	Kane stream	25	0,99	0,01	0,00	0,01	0,01	0,00	0,01	0,97	0,01	0,00	0,00	0,00
6	Odori stream	24	0,98	0,00	0,01	0,01	0,01	0,01	0,01	0,01	0,95	0,00	0,01	0,00
7	Azusa (Kamikouchi) without name 1	24	0,00	0,01	0,99	0,00	0,00	0,00	0,01	0,00	0,00	0,98	0,00	0,00
8	Azusa (Kamikouchi) without name 2	18	0,00	0,00	0,99	0,00	0,00	0,00	0,00	0,00	0,00	0,98	0,00	0,01
9	Azusa (Kamikouchi) without name 3	19	0,00	0,01	0,99	0,00	0,00	0,01	0,00	0,00	0,00	0,98	0,00	0,00
10	Azusa (Kamikouchi) Zenrokusawa stream	1	0,00	0,00	0,99	0,00	0,00	0,01	0,00	0,00	0,00	0,97	0,00	0,00
11	Azusa (Kamikouchi) Shimizusawa stream	3	0,00	0,00	0,99	0,00	0,00	0,00	0,00	0,00	0,00	0,98	0,00	0,00
12	Azusa (Matsumoto city)	7	0,00	0,00	0,99	0,00	0,00	0,01	0,00	0,00	0,00	0,97	0,00	0,00
13	Azusa (Matsumoto city) YOY	13	0,00	0,01	0,99	0,00	0,00	0,01	0,01	0,00	0,00	0,97	0,00	0,00
14	Azusa (Matsumoto) Toyoshina	9	0,00	0,00	0,99	0,01	0,00	0,01	0,00	0,00	0,01	0,96	0,00	0,01
15	Azusa (Matsumoto) Shimauchi	21	0,01	0,01	0,97	0,02	0,00	0,02	0,01	0,01	0,01	0,94	0,00	0,01
16	Lées Athas hatchery	20	0,01	0,01	0,00	0,97	0,01	0,01	0,01	0,01	0,01	0,00	0,94	0,01
17	Cauterets hatchery	28	0,04	0,01	0,01	0,95	0,01	0,01	0,00	0,01	0,01	0,01	0,03	0,93
18	Isère hatchery	30	0,02	0,01	0,00	0,97	0,01	0,01	0,01	0,03	0,01	0,00	0,91	0,03

Table 3: *Frequencies of each lineage detected by assignment in each sample for K=4 then K=8 (the colors of the headings correspond to that of the Figure 7). Orange cells show dominant lineage of each sample. A frequency of 0.05 and below is considered as not significant. The numbers 38, 64 and 65 for the French hatchery samples correspond to administrative departments where are settled the fish farms.*

Figure 8: *Report in a tree of the successive assignments until K=9. In orange the more significant structure, in yellow the following structures.*

When we increase the K value over K=9, something changes at K=11: the very homogeneous Azusa River populations split into Kamikouchi and Matsumoto sub groups. The two French hatcheries strains from 38 and 64 department do not split at K=15.

5.3. Population parameters

A way to estimate the "health" of the population is measuring its genetic diversity. It is considered that more a population is polymorphic, more it can overcome modifications (anthropization, global warming…). There is no "normal" value for polymorphism, but it is known that European Atlantic domestic strains are highly polymorphic, which can be used to estimate the Japanese populations polymorphism.

The best descriptor of genetic diversity is the Hnb parameter (non-biased heterozygosity, because pondered according to the sample size). Other parameters as Ho (observed heterozygosity) and A (mean number of alleles by locus) can help the estimated also (Table 4).

Clearly Mamachi, Kane and Odori rivers together with Lake Chuzenji are inhabited by highly polymorphic brown trout population, similar to European domestic strains. The Chuzenji hatchery raises a stock that has a weak polymorphism.

Another interesting parameter is the Fst: a measure of the differentiation between samples, 2 by 2. Small samples (under 10) are not considered. Most samples are differentiated from most others. Exceptions are observed between Azusa River samples (green cells in Table 5).

Calculs n°	Stations	Genotypes N	Hnb	Ho	A	Fis	signif.
1	Mamachi stream	25	0.661	0.656	5.6	0.007	ns
2	Monbetsu stream	25	0.589	0.582	4.7	0.013	ns
3	Jigoku (Lake Chuzenji)	22	0.709	0.685	6.3	0.033	ns
4	Chuzenji hatchery	25	0.390	0.427	2.2	-0.095	*
5	Kane stream	25	0.654	0.680	4,0	-0.040	ns
6	Odori stream	24	0.613	0.586	4.7	0.045	ns
7	Azusa (Kamikouchi) without name 1	24	0.478	0.462	3.7	0.034	ns
8	Azusa (Kamikouchi) without name 2	18	0.464	0.468	3.8	-0.0082	ns
9	Azusa (Kamikouchi) without name 3	19	0.435	0.447	3.1	-0.028	ns
10	Azusa (Kamikouchi) Zenrokusawa stream	1	0.727	0.727	1.7	0.000	-
11	Azusa (Kamikouchi) Shimizusawa stream	3	0.583	0.528	2.7	0.116	-
12	Azusa (Matsumoto city)	7	0.464	0.480	2.8	-0.036	-
13	Azusa (Matsumoto city) YOY	13	0.495	0.538	3.7	-0.091	ns
14	Azusa (Matsumoto) Toyoshina	9	0.467	0.421	3.5	0.103	-
15	Azusa (Matsumoto) Shimauchi	21	0.451	0.397	4.5	0.121	**
16	Lées Athas hatchery	20	0.635	0.550	4.9	0.138	***
17	Cauterets hatchery	28	0.776	0.763	8.1	0.018	ns
18	Isère hatchery	30	0.670	0.669	6.1	0.002	ns

Table 4: *Main population parameters of genetic diversity (green headings) and of panmixia (blue headings). Not that very small samples are not considered. Orange value cells designate the highly polymorphic estimations, the yellow one the polymorphic ones. Grey cells highlight the very low polymorphism. *, ** and *** are three levels of departure from zero significance.*

Calculs n°	Stations	N	1	2	3	4	5	6	7	8	9	13	14	15	16	17	18
1	Mamachi stream	25	0	0.09989	0.08475	0.32144	0.17348	0.14896	0.23127	0.23112	0.22696	0.20226	0.21311	0.24995	0.12509	0.10898	0.10968
2	Monbetsu stream	25		0	0.12814	0.37267	0.17068	0.137	0.25314	0.25943	0.26549	0.25713	0.24595	0.26831	0.15357	0.14421	0.13684
3	Jigoku (Lake Chuzenji)	22			0	0.29428	0.16783	0.15614	0.20421	0.21095	0.21061	0.18141	0.19703	0.22121	0.14075	0.09069	0.13807
4	Chuzenji hatchery	25				0	0.34014	0.37959	0.43465	0.46381	0.46499	0.45178	0.47464	0.46457	0.38772	0.30898	0.36011
5	Kane stream	25					0	0.2	0.30922	0.31008	0.32645	0.29689	0.29993	0.31712	0.20588	0.12902	0.17672
6	Odori stream	24						0	0.26328	0.26083	0.27815	0.2708	0.24547	0.27636	0.16102	0.15027	0.1565
7	Azusa (Kamikouchi) without name 1	24							0	0.00367	0.01197	0.06618	0.03696	0.04895	0.23583	0.21507	0.2314
8	Azusa (Kamikouchi) without name 2	18								0	0.02578	0.07546	0.03001	0.04677	0.24203	0.21719	0.22449
9	Azusa (Kamikouchi) without name 3	19									0	0.06089	0.06753	0.0796	0.23664	0.22052	0.23106
13	Azusa (Matsumoto city) YOY	13										0	0.02904	0.04058	0.19691	0.17681	0.19928
14	Azusa (Matsumoto) Toyoshina	9											0	-0.00154	0.21634	0.19448	0.20432
15	Azusa (Matsumoto) Shimauchi	21												0	0.23997	0.21907	0.22908
16	Lées Athas hatchery	20													0	0.10422	0.03347
17	Cauterets hatchery	28														0	0.08941
18	Isère hatchery	30															0

Table 5: *Fst estimation between samples of 9 individuals at least. Green cells are not-significant differentiation (after Bonferroni correction), mainly between Azusa River populations.*

6. Interpretation and discussion

This is the first molecular analysis ever done on brown trout populations stemming from ancient introductions in Japan. For this, several basins have been sampled (Ishikari, Tone, Fuji, Jinzu and Shinano basins flowing in Hokkaido and Honshu parts of the country). Hatcheries are represented by a Japanese one near the Lake Chuzenji and three more from France, representing the main commercial present strain in Western Europe.

The overall picture given by the analyses is:

i) Japanese introduced brown trout are not homogeneous, probably a consequence of several introductions (Figure 5).

ii) There is no geographic logic in the samples clustering, opposing Azusa trout to the remaining populations. Popular literature (Sakata 1973) described that brown trout eggs which were imported from a fish hatchery in USA had been stocked in the Kamikouchi area

during 1925 to 1933. The stocking was conducted for leisure fishing by Fisheries Division of Nagano Prefecture. So, brown trout in Azusa River were introduced from the different origin. It may cause the different genetic characteristics from others.

iii) Azusa basin has been stocked by only one strain. The very slight differentiation between Kamikouchi and Matsumoto sub groups (appearing at K=11) can be due to simple isolation during a long time (Figure 7).

iv) The differentiation of Kane trout (at K=5), Odori trout (K=7) and Lake Chuzenji trout (K=8) are rather light and can be due to different strains introduced or to long time of isolation after a common introduction (Figure 8).

v) The Chuzenji hatchery releases are not found in any sampled population (Figure 8). The genetic diversity of the hatchery stock is very low, reducing the potential benefic effect of stocking with that strain (Table 3). The difference between Chuzenji hatchery (sample 4) and Lake Chuzenji (3) may be caused by genetic drift occurred in the hatchery and because this sampled hatchery is for scientific research and not used for stocking. The brown trout in the scientific fish hatchery has been bred for more than 30 years without input of any fish from other populations. These fish has not been used for stocking into the lake. Today, 50,000 fry are annually stocked in to the lake from another fish hatchery beside the lake, not sampled here, but they are different from the fish in the analyzed hatchery. Also, natural spawning is observed in the inlet of the lake, and these mature fish have been used few times for artificial fertilization in the fish hatchery. The scientific fish hatchery, analysed here, was never used for stocking.

vi) Several populations have high genetic diversity (Mamachi, Kane and Odori streams, Lake Chuzenji) while Azusa River numerous samples miss polymorphism (Table 4).

vii) Most pairs of samples are significantly divergent (Table 5). The only exceptions are within Azusa River samples. It is difficult to say if this differentiation is due to distinct introductions or to long time isolation. However the Azusa River was certainly stocked with a particular brown trout, genetically distinct from the Western Europe commercial strains.

6.1. Adaptation after introduction

Two parameters can be influent for brown trout adaptation in Japan: the climatic difference between Hokkaido and Honshu islands and the history of introduction bay man.

Concerning the climatic north-south cline, the results showed that there is a large homogeneity between samples located in the two islands and that the differences occur:

- between Azusa and other rivers (and lake)
- between Lake Chuzenji hatchery and river populations

So this climatic cline is not the key parameter.

Concerning the human factor, its role in the Azusa River / most Japanese rivers and lake / Japanese hatchery differentiation is obvious. This differentiation is due to different origins of the introduced trout.

The general evolution theory considers also that genetic diversity is the prerequisite for adaptation capacity of a population. As a reference, French hatchery strains show a diversity of $0.63<Hnb<0.78$, which is considered as highly polymorphic. The high polymorphism observed in Mamachi, Kane and Odori River populations and in Lake Chuzenji population indicate that introduction was made with enough individuals to maintain the adaptive capacities of the European domestic strains. However, the brown trout living in Azusa River showed clearly too low polymorphism ($0.43<Hnb<0.49$ for sampled with more than 10 trouts).

6.2. Origin of introduction

Only some recorded introductions are known. It is so difficult to suggest a precise origin of introduced strains:

- before 1900, brown trout was mistakenly introduced with rainbow trout from USA, but we do not know where they were introduced;
- around the year 1930, the Lake Chuzenji was stocked and then, from this first introduction, the country was stocked. This seems correspond to the "dominant" lineage (Table 3), however there is no explanation for the different stock present in the Lake Chuzenji hatchery;
- the 1973 importation of French brown trout is another hypothesis for the origin of the "dominant" lineage, mostly because this lineage is close to the 3 French hatcheries stocks (Figure 5);
- finally, the escaped hatchery trout along Odori river probably dispatched the "dominant" lineage from the hatchery to the river.

6.3. Conclusions and perspectives

This first tentative to understand the way brown trout have been introduced in Japan and the adaptive phenomenon following it gave us several interesting information. This introduction has been heterogeneous and most of it was done in a good way, i.e. which maintained most of the genetic diversity.

The interpretation of these introductions in terms of beneficial or detrimental depends of different points of view. For anglers, since the brown trout is bred in several hatcheries (at least the Lake Chuzenji hatchery analyzed here, but also numerous hatcheries all around the country), this species is welcome for a part of Japanese people. However, recent studies showing the negative influence of brown trout on native white-spotted charr *Salvelinus leucomaenis* (Hasegawa 2017) show that brown trout can also be considered as an invasive species. Its influence on the masu salmon (*Oncorhynchus masou*) is less negative (Hasegawa et al. 2012).

The present report is called "step 1". A second step should need more samples and a better exploration of the local literature in order to add new data to the knowledge of the arrival of the European brown trout in Japan.

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8. Appendices

Appendix 1: The old fish farming facilities

Fish hatchery along Odori stream

Tank for brown trout near the Lake Chuzenji

Appendix 2: Some brown trout morphotypes.

Mamachi stream morphotype

Monbetsu stream morphotype

Kane stream morphotype