Oncorhynchus kawamurae "Kunimasu", a deepwater trout, discovered in Lake Saiko, 70 years after extinction in original habitat, Lake Tazawa, Japan

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Running head: Discovery of *O. kawamurae* in Lake Saiko

Abstract *Oncorhynchus kawamurae* (Osteichthyes: Salmonidae) (common name “Kunimasu”), a species endemic to Lake Tazawa, Akita Prefecture, Japan, was believed to have become extinct since 1940. However, nine specimens were discovered in March and April 2010, in Lake Saiko, Yamanashi Prefecture, one of the lakes to which eyed eggs of the species were introduced in 1935. These were identified with *O. kawamurae* in having 47–62 pyloric caeca, 37–43 gill-rakers, black-colored body, and spawning on 30–40 m depth at early spring, which are unique within *Oncorhynchus*. Furthermore, the distinctiveness of Kunimasu from sympatric kokanee (*O. nerka*) there was supported by microsatellite DNA data.

Keywords *Oncorhynchus kawamurae* · Deepwater trout · Supposed extinction · Discovery · Morphological and molecular data

Introduction

Oncorhynchus kawamurae Jordan and McGregor *in* Jordan and Hubbs (1925) (common name "Kunimasu") had been believed extinct from Lake Tazawa since 1940, at which time water from the Tama River was introduced to the Lake as part of a scheme for increased hydroelectric power generation before World War II in Japan (Sugiyama 2000; Ministry of Environment 2003). However, because of the acidic nature of the river, almost all of the fishes and small crustaceans disappeared from the lake. At that time, Kunimasu had been reported to occur and spawn on the deep bottom between 15 and 300 m (usually 150 and 180 m) depth (Tanaka 1911; Akitaken Suisanshikenjo 1915; Okuyama 1939; Oshima 1941; Sugiyama 2000), which was unique within *Oncorhynchus*. Although Kunimasu has been treated as a subspecies or variation of kokanee [land-locked sockeye salmon, *Oncorhynchus nerka* (Walbaum 1792); common name "Himemasu"] (Hosoya 2002; Eschmeyer 2010) because of the characteristic of spawning in a lake, none of the populations of kokanee (genetically distinct from sockeye) in North America (Wood 1995; Taylor et al. 1996; Wood and Foote 1996) occur and spawn in such deep water (Croot and Margolis 1991; Wydoski and Whitney 2003). Kunimasu had also been reported as spawning throughout the year (Akitaken Suisanshikenjo 1931; Okuyama 1939; Oshima 1941), which is a unique feature within *Oncorhynchus*.

Before 1940 and the demise of Kunimasu from Lake Tazawa, eyed eggs of the species had been introduced from Lake Tazawa to some other lakes, viz. 600,000 to some lakes (names not recorded) in Nagano, Yamanashi and Toyama Prefectures in 1930, 100,000 each to Lakes Saiko and Motosu, Yamanashi Prefecture in 1935 and 200,000 to Lake Biwa, Shiga Prefecture in 1935 (Sugiyama 2000). Notwithstanding, the lack of Kunimasu individuals recorded subsequently from these lakes has led to a belief in the extinction of the species. Only 17 specimens from the original habitat, including the holotype and paratypes, are known to exist,

being held in museum collections in the United States and Japan; one of these specimens, deposited in Akita Prefectural Museum, has been designated as a Cultural Property by the Agency for Cultural Affairs, Japan (http://www.bunka.go.jp/bsys/).

However, Kunimasu was recently discovered in Lake Saiko, Yamanashi Prefecture. Nine specimens, identified as Kunimasu, were collected by bottom gill net in 2010, most probably being descendents of eggs introduced in 1935. This is the first report of the present-day occurrence of this species, morphological and molecular data showing that it differs specifically from a kokanee population in Japan.

Materials and methods

The nine specimens collected from 30–40 m depth of Lake Saiko in March and April 2010 were deposited in the Kyoto University Museum, Kyoto University, Kyoto [FAKU 97736, 97737, 97742–97745, 97767–97769; 180.5–233.0 mm in standard length (SL)]. Measuring and counting methods are as follows: SL, linear distance from most anterior point of snout or upper lip with mouth closed to caudal-fin base (posterior end of hypurals, roughly where fold formed by bending caudal fin); gill-rakers (aligned in two rows on inner side of gill arches) counted on outer row on first gill arch (Hubbs and Lagler 1974; Nakabo 2002).

Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Germany) following the manufacturer's protocols, from muscle tissue preserved in 99% ethanol. Five microsatellite loci isolated from sockeye salmon (One102, One108, One110, One114, One115; Olsen et al. 2000) were amplified via the polymerase chain reaction (PCR) using fluorescent-labeled primers, detected by an ABI-310 genetic analyzer. PCR protocols are as follows: 92°C (5 min), cycles of 92°C (30 s)–T_a (annealing temperature) (30 s)–72°C (30 s), and

72°C (30 min). T_a used for One102, One108, One110, One114, and One115 was 56, 57, 58, 56, and 57°C, respectively, and the number of the cycles for them was 30, 30, 27, 33, and 30, respectively. Levels of genetic differentiation between populations were evaluated by pairwise F_{ST}, calculated using Arlequin ver. 3.5 (Excoffier et al. 2005). Comparative specimens of Himemasu were collected from Lake Saiko in March and April 2010 [20 specimens, 70.8–173.3 mm SL, introduced mainly from Lake Towada or Lake Shikotsu, but their original source was Lake Akan (Tokui 1964); FAKU 97747–97766] and from Lake Akan in May 2010 [30 specimens, 198.0–226.1 mm SL, indigenous, but partly reintroduced from some lakes of Japan (Tokui 1964); FAKU 97921–97950].

Results and discussion

The body color of Kunimasu was uniformly black when alive (Akitaken Suisanshikenjo 1907; Oshima 1940), becoming dark blue or almost black in spirits (Tanaka 1911; Jordan and Hubbs 1925). Okuyama (1939) noted that fresh specimens of the species changed from partly black to whitish when wrapped in cloth. The coloration of the recently-collected specimens agreed with these descriptions. FAKU 97767 was uniformly black when alive (Fig. 1a), fading somewhat after death. Although life color is unknown for the other specimens, they became grayish on the head behind the eye, the body being black behind the gill covers (males) or black on the snout, and pale on the head and body (females) (Fig. 1b) after cold storage or freezing. Kunimasu differed from other species of *Oncorhynchus* in having fewer pyloric caeca (49–59) and more gill-rakers (31–42) (Koshida 1910; Akitaken Suisanshikenjo 1915; Jordan and Hubbs 1925; Hikita 1962). The nine specimens from Lake Saiko conformed with these figures in having 47–62 pyloric caeca and 37–43 gill-rakers [Electronic supplementary material (ESM) Table S1,

Fig. S1].

The present nine specimens were apparently captured while spawning or just after spawning, two males being spent with broken pelvic and caudal (lower lobe) fins (FAKU 97737, 97768), two males spent with a broken caudal (lower lobe) fin (FAKU 97744, 97767) (Fig. 1a), two males spent with a slightly damaged caudal fin (FAKU 97736, 97769), two females spent with a broken caudal (upper and lower lobes) fin (FAKU 97743, 97745) (Fig.1b) and a single female with some matured eggs in the abdominal cavity (fins undamaged) (FAKU 97742). The collection period agreed closely with the established spawning season of Kunimasu, primarily from December through March (Akitaken Suisanshikenjo 1907, 1931; Okuyama 1939; Sugiyama 2000), the collection site being in 30–40 m depth, similar to the 40–50 m depth reported for Lake Tazawa (Akitaken Suisanshikenjo 1931). The matured eggs of FAKU 97742 were yellow and the flesh of the nine specimens pinkish-white, covered by a thick, mucous-layered skin (Fig. 1c), as previously reported (Akitaken Suisanshikenjo 1907; Okuyama 1939). From these information on morphological and ecological traits, we conclude that the present nine specimens of Lake Saiko are descended from Kunimasu of Lake Tazawa.

Kunimasu, previously treated as a subspecies of *O. nerka* (*O. nerka kawamurae*) (Hosoya 2002) or as a junior synonym of that species (Eschmeyer 2010), has a lake type life history (spawning and growing in lakes). Within the genus *Oncorhynchus*, such type of life history is seen only in kokanee populations of *O. nerka* (Himemasu) (Croot and Marcolis 1991; Behnke 1992; Wydoski and Whitney 2003). Although Kunimasu is similar to Himemasu, they differ in the former having fewer pyloric caeca (Hikita 1962).

Introduced to Lake Saiko from other Japanese lakes since 1912 (Tokui 1964), Himemasu also breed in a creek to the hatchery at lakeside in autumn (Y. Miura, personal communication). But, Kunimasu here spawned in the bottom of the lake in March as stated above. Our analysis using five microsatellite loci revealed significant differentiation between Himemasu and

Kunimasu from Lake Saiko, such differentiation not being apparent between Himemasu from Lake Saiko and Himemasu from Lake Akan, one of the original habitats of Himemasu in Japan (Tokui 1959a) (Table 1, ESM Tables S2, S3). There was no specimen having intermediate genetic characteristics between Kunimasu and Himemasu in Lake Saiko from Bayesian model-based clustering analysis (ESM Fig. S2), although a certain possibility of introgression could not be excluded. Accordingly, Kunimasu in Lake Saiko has been completely, or at least largely, isolated reproductively from co-occurring Himemasu, and, following Mayr's biological species concept (Mayr 1942, 1969), should not be treated as a subspecies or the same species as Himemasu. As discussed in Nakabo (2011), Kunimasu should be treated as a distinct species, *Oncorhynchus kawamurae*, as originally described by Jordan and McGregor *in* Jordan and Hubbs (1925).

Almost all biological characteristics of Kunimasu have remained unknown. Photographs known to date (Tanaka 1911; Akitaken Suisanshikenjo 1915; Okuyama 1939; Oshima 1940; Hikita 1962) were all taken of matured individuals and the present specimens had all completed (or were in the process of) spawning. Coloration of immature stages remains unknown. Early reports of Kunimasu spawning throughout the year (Akitaken Suisanshikenjo 1931; Okuyama 1939; Oshima 1941) need to be considered in relation to body color. Judging from the number of gill-rakers (highest in *Oncorhynchus*; Hikita 1962), the species probably eats mesopelagic or benthopelagic zooplankton, similar to kokanee (Himemasu). Early reports of Kunimasu captures include those from 150 m depth in November 1910 and 37.5–120 m depth (usually 90 m depth) from February 1913 to March 1914, by bottom gill net (Akitaken Suisanshikenjo 1915). Another local fisherman reported capturing Kunimasu from Lake Tazawa prior to 1940 in 150–180 m depth (Sugiyama 2000), the water temperature (below 100 m depth) being 3.8–3.9°C throughout the year (Akitaken Suisanshikenjo 1930). Kunimasu occurred in 30–40 m depth in Lake Saiko in March and April 2010, the water temperature below 30 m depth being ca. 5°C from June through

December, and 3.9°C in March (Haga et al. 1996; Ohama et al. 1996). On the other hand, Himemasu occurs in temperature zones of 10–13°C from early summer to late autumn in Lake Towada (near Lake Tazawa) (Tokui 1959b). Accordingly, Kunimasu appears to have adapted to cooler temperature zones than Himemasu, suggesting that present-day Kunimasu habitat may represent a Pleistocene glacial age relict.

The discovery of Kunimasu in Lake Saiko has provided an unexpected opportunity to learn more of the life history of this species and its evolutionary relationships. In particular, we have been given a second chance to conserve and maintain this species, and an opportunity to learn from mistakes of the past. Appropriately, confirmation of a living population of Kunimasu has been achieved in 2010, the United Nations International Year of Biodiversity.

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Table 1 Pairwise F_{ST} values between populations (*below diagonal*) and associated *P*-values (*above diagonal*) inferred from five microsatellite loci

	Ok-LS	On-LS	On-LA
Ok-LS		0.0000	0.0000
On-LS	0.1206		0. 6340
On-LA	0.1330	–0.0025	

Ok-LS Kunimasu, *Oncorhynchus kawamurae*, *n* = 9; *On-LS* Himemasu, *O. nerka*, from Lake Saiko, *n* = 20; *On-LA* Himemasu from Lake Akan, *n* = 30

Fig. 1 *Oncorhynchus kawamurae* from Lake Saiko, Yamanashi Prefecture, Japan. **a** FAKU 97767, male, 180.5 mm SL, living, just after capture, collected on 4 April 2010 (photo by H. Ito). **b** FAKU 97743, female, 203.1 mm SL, one day following preservation on ice, collected on 19 March 2010 (photo by T. Nakabo). **c** FAKU 97742, female with matured eggs one day following preservation on ice, collected on 19 March 2010 (photo by T. Nakabo)

Table S1 Meristic characters of Kunimasu *Oncorhynchus kawamurae* from Lake Saiko

	FAKU97736	FAKU97737	FAKU97742	FAKU97743	FAKU97744	FAKU97745	FAKU97767	FAKU97768	FAKU97769
Sex	Male	Male	Female	Female	Male	Female	Male	Male	Male
Total length (mm)	234.0	238.4	275.0	227.0	225.0	–	214.1	226.0	252.2
Standard length (mm)	200.0	200.1	233.0	203.1	195.2	181.0	180.5	181.4	209.0
Gill rakers	40	38	37	38	40	41	40	43	39
Pyloric caeca	62	55	62	51	58	48	55	54	47

Table S2 Sample size (*n*), number of alleles (*A*), expected and observed heterozygosity (H_E, H_O) in five microsatellite loci of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*

	Ok-LS	On-LS	On-LA
One102			
n	9	20	30
A	6	4	5
H_E	0.824	0.558	0.486
H_O	0.889	0.650	0.433
One108			
n	9	20	30
A	7	6	8
H_E	0.869	0.765	0.742
H_O	0.889	0.800	0.633
One110			
n	9	20	30
A	9	5	10
H_E	0.895	0.524	0.584
H_O	0.889	0.600	0.533
One114			
n	9	20	29
A	6	8	8
H_E	0.791	0.799	0.825
H_O	0.667	0.800	0.655
One115			
n	9	20	30
A	8	7	9
H_E	0.856	0.592	0.567
H_O	0.667	0.600	0.567

Ok-LS Kunimasu, *Oncorhynchus kawamurae*; *On-LS* Himemasu, *O. nerka*, from Lake Saiko; *On-LA* Himemasu from Lake Akan

Significant deviation from Hardy–Weinberg equilibrium was not observed ($P \geq 0.05$)

Table S3 Allele frequencies for five microsatellite loci of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*

	Ok–LS	On–LS	On–LA
One102			
n	9	20	30
226	0.111	–	–
230	0.222	0.300	0.150
234	–	0.075	0.067
238	0.278	0.600	0.700
242	0.056	–	0.067
246	0.278	–	–
250	–	0.025	0.017
254	0.056	–	–
One108			
n	9	20	30
183	0.056	–	–
187	0.167	–	–
191	0.056	–	–
195	0.111	0.225	0.100
199	–	0.150	0.217
203	–	0.100	0.150
207	0.278	0.400	0.433
211	0.167	0.100	0.033
215	0.167	–	0.033
219	–	–	–
223	–	–	–
227	–	0.025	0.017
231	–	–	–
235	–	–	–
239	–	–	–
243	–	–	–
247	–	–	–
251	–	–	0.017
One110			
n	9	20	30
207	0.056	–	–
211	–	–	–
215	0.056	–	–
219	–	–	–
223	–	–	–
227	–	–	–
231	0.111	0.650	0.600
235	–	0.025	0.017
239	–	–	–
243	–	–	–
247	–	–	–
251	0.056	–	0.017
255	0.278	–	–
259	0.111	0.050	0.017
263	–	–	0.017
267	0.056	0.025	0.017
271	–	–	0.033
275	0.111	–	0.017
279	–	0.250	0.250

283	–	–	–
287	0.167	–	–
291	–	–	0.017
One114			
n	9	20	29
227	–	0.100	0.052
231	–	0.075	0.190
235	–	0.025	0.052
239	0.111	–	–
243	–	0.325	0.259
247	0.333	0.275	0.259
251	0.056	0.150	0.103
255	–	0.025	0.034
259	–	0.025	0.052
263	0.111	–	–
267	0.333	–	–
271	–	–	–
275	–	–	–
279	–	–	–
283	0.056	–	–
One115			
n	9	20	30
181	0.056	–	–
185	–	–	–
189	–	–	–
193	0.056	–	0.017
197	0.111	–	0.067
201	0.056	0.125	0.017
205	–	0.050	0.033
209	0.167	0.025	–
213	0.167	0.050	0.083
217	0.333	0.625	0.650
221	–	0.100	0.067
225	–	–	0.017
229	–	0.025	0.050
233	0.056	–	–

Ok-LS Kunimasu, *Oncorhynchus kawamurae*; *On-LS* Himemasu, *O. nerka*, from Lake Saiko; *On-LA* Himemasu from Lake Akan

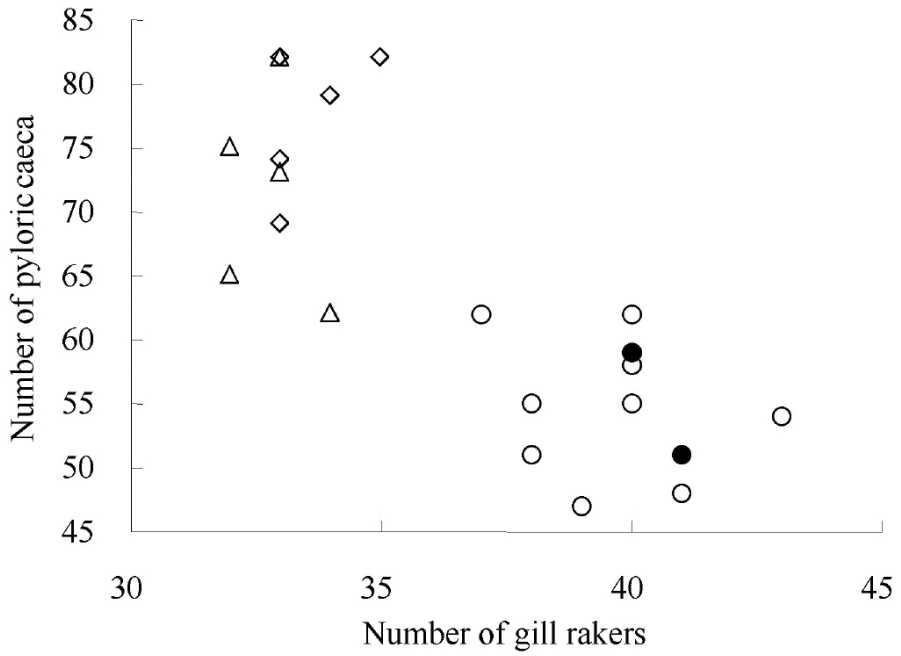

Fig. S1 Relationship between the number of gill rakers and pyloric caeca in Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*. *Open circles* Kunimasu from Lake Saiko, $n = 9$; *Solid circles* Kunimasu from Lake Tazawa [from original description of Jordan and McGregor *in* Jordan and Hubbs (1925)], $n = 2$; *triangles* Himemasu from Lake Saiko, $n = 5$; *diamonds* Himemasu from Lake Akan, $n = 5$

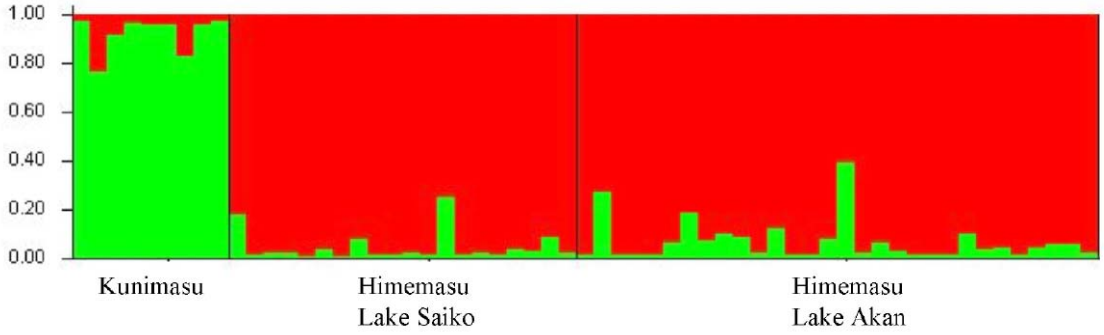

Fig. S2 Model-based clustering analysis on Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka* computed by Structure version 2.3 with $K = 2$ (Pritchard et al. 2000). *Vertical bars* represent individuals, and colors correspond to proportional membership of that individual. The LOCPRIOR model (Hubisz et al. 2009) was used. Burn-in was 10,000 iterations, followed by 10,000 iterations

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