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1 Restricted gene flow between “Kunimasu” (*Oncorhynchus kawamurae*) and “Himemasu” (*O.*
2 *nerka*) in Lake Saiko, Yamanashi Prefecture, Japan, inferred from microsatellite analysis

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20 Short running head: Restricted gene flow of *Oncorhynchus kawamurae*

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Abstract

Introgressive hybridization between Kunimasu (*Oncorhynchus kawamurae*) and Himemasu (*O. nerka*) was investigated in a large number of specimens, in a search for basic data relevant to conservation needs of the former. A Bayesian-based clustering method using five microsatellite DNA loci separated 144 specimens from Lake Saiko, Yamanashi Prefecture, into two genetically distinct groups, corresponding to Kunimasu and Himemasu. Application of a threshold of individual proportion of membership (q_i) of 0.90, so as to separate hybrids from purebreds, resulted in only two specimens with $q_i < 0.90$ (therefore identified as hybrids), the remaining specimens all having $q_i > 0.90$ (indicating high purebred probability). The dominant Kunimasu genome in both of the hybrid specimens indicated that they were not F1 progeny, the overall results implying that any introgressive hybridization between Kunimasu and Himemasu has been highly restricted. A second Bayesian clustering analysis demonstrated the absence of hybrids with either a Kunimasu or Himemasu ancestor within two generations. An estimate of the contemporary genetic diversity of Kunimasu showed that species to have retained a relatively high genetic diversity compared to Himemasu and other kokanee populations. Both the limited introgressive hybridization with Himemasu and high genetic diversity indicated that the present Kunimasu population in Lake Saiko should not be considered to be seriously endangered, at least on the basis of its genetic characteristics.

Keywords *Oncorhynchus kawamurae* · *Oncorhynchus nerka* · Introgressive hybridization · Genetic diversity · Microsatellite DNA

Introduction

Oncorhynchus kawamurae Jordan and McGregor in Jordan and Hubbs (1925) (common name “Kunimasu”) had been believed extinct in Lake Tazawa, the only known habitat of the species, since 1940, due to the introduction of acidic water to the Lake from the Tama River (Sugiyama 2000). Although eyed-eggs of Kunimasu had been introduced to several other lakes, including Lake Saiko, Yamanashi Prefecture, before 1940, the lack of subsequent records of Kunimasu from these lakes had led to a belief that the species had become extinct. Accordingly, Nakabo et al.’s. (2011) recent discovery of Kunimasu in Lake Saiko attracted a great deal of attention concerning the conservation of the species.

Introgressive hybridization, by which process genes are exchanged between distinct species, has often been observed between native and introduced salmonid species, including some lineages that have been isolated for more than a million years (Utter 2001), and is therefore regarded as a major threat to the persistence of native evolutionary lines. Although Nakabo et al. (2011) rejected the likelihood of extensive introgressive hybridization between Kunimasu and co-occurring kokanee (land-locked sockeye salmon, *Oncorhynchus nerka*; common name “Himemasu”) on the basis of clear morphological and genetic differences, the possibility of a low level of introgressive hybridization was not fully explored due to the small Kunimasu sample.

Because the identification of potential risks for a population under threat is essential for appropriate conservation measures, the presence of introgressive hybridization between Kunimasu and Himemasu was assessed, based on variations in five microsatellite loci. A large number of specimens collected from Lake Saiko throughout 2010–2011, including some of equivocal body coloration, overcame the problem of insufficient sample. Contemporary genetic diversity of Kunimasu was also determined for comparison with Himemasu populations from several other Japanese lakes, in addition to some North American kokanee populations. Because the extant population of Kunimasu in Lake Saiko is most likely to have resulted from the ca.

100,000 eyed eggs introduced from Lake Tazawa 70 years BP (Sugiyama 2000), the possibility of any recent bottleneck having occurred in the Kunimasu population, thereby reducing genetic diversity, was also investigated, using a suite of measures.

The relationships of Kunimasu and *O. nerka* are also briefly discussed on the basis of genetic divergence between them.

Materials and Methods

Sampling. A total of 115 specimens collected from Lake Saiko in 2010 and 2011 by gill net and angling, together with additional “beached” post-spawning (“Uki-yo”) specimens, were deposited in the Fish Collection of Kyoto University Museum (FAKU) and the Yamanashi Prefectural Fisheries Technology Center (YFTC) (Table S1). Each specimen was then provisionally identified for the genetic analysis on the basis of external morphology (body coloration) and collection date. On the basis of Nakabo et al’s (2011) observation that Kunimasu specimens collected during or just after spawning (in March and April) had a black body, 24 of the present specimens having a black body and collected from the presumed spawning ground in March and April (FAKU 98834–98847, YFTC 14, 15, 18–20, 22–25, 27) were provisionally identified as “Kunimasu”. The lack of dark dorsal spots agreed with previous reports on body coloration of Kunimasu (Jordan and Hubbs 1925; Hikita 1962). On the other hand, 21 male specimens collected in September (FAKU 99370–99383, 99385, 99387–99390, 99392, 99393), agreeing with the description of mature male Himemasu given by Jordan and Hubbs (1925) (non-black body with dark spots on dorsum), were provisionally identified as “Himemasu”. The remaining 58 specimens (FAKU 98737, 98738, 98742, 99167–99189, 99328, 99384, 99386, 99391, 99394, 99396–99401, 99435–99445, 99452–99456, 99461–99463, YFTC 21, 26) plus 12 black-bodied “Uki-yo” specimens collected in January and February (FAKU 98741, YFTC 1–11), which may have included both Kunimasu and Himemasu, were left unidentified at this stage. Internal morphology, including numbers of gill rakers and pyloric caeca, was not used for

identification purposes, due to these characters not being available in some specimens. Examples of Kunimasu (9) and Himemasu (20) (FAKU97747–97766) from Lake Saiko, examined by Nakabo et al. (2011), plus 30 “pure” Himemasu specimens from Lake Akan (FAKU97921–97950), for which no documented record of Kunimasu stocking exists, were also examined. Muscle tissues were obtained from all but three specimens and preserved in 99.5% ethanol. For the latter three, either a liver tissue sample or a fin clip was used.

Microsatellite genotyping. Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Germany) or the Wizard Genomic DNA Purification Kit (Promega, USA), both following the manufacturers’ protocols. 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 followed Nakabo et al. (2011).

Statistical analysis - population structure. The population structure was explored using a Bayesian-based clustering method implemented in the program STRUCTURE 2.3 (Pritchard et al. 2000), wherein individuals are clustered on the basis of genotype into K randomly interbreeding groups by minimizing departures from Hardy-Weinberg equilibrium and linkage disequilibrium within the groups. Ten replicates for each pre-defined $K = 1$ between 10 were obtained with runs of 100,000 burn-in and 200,000 Markov Chain Monte Carlo (MCMC) iterations, applying the admixture model with correlated alleles. Specimens were pooled into two sampling locations (Lakes Saiko and Akan) for use in the LOCPRIOR model (Hubisz et al. 2009), which uses the sampling locations of specimens to assist in clustering. To determine the most likely value of K , the Evanno et al. (2005) method implemented in the program STRUCTURE HARVESTER (Earl 2009), which determines the second-order rate of change in the distribution of $L(K)$, was used. CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007) was used to summarize parameters across 10 replicate runs and DISTRUCT ver.1.1 (Rosenberg 2004), to produce the corresponding graphical output. Each specimen was assigned to one or

more inferred groups on the basis of that individual's proportion of membership or proportion of the genome of individuals originating from those groups (q_i). As proposed by Vähä and Primmer (2006), specimens were assigned to one group if $q_i > 0.90$ (purebred individuals) or jointly to two or more groups if the proportion of membership to each was $q_i < 0.90$ (hybrid individuals), that value having been widely adopted in other studies seeking to detect hybrids (e.g., Burgarella et al. 2009; Sanz et al. 2009).

A second Bayesian method, NEWHYBRIDS 1.0 (Anderson and Thompson 2002), which aims specifically at detecting hybrids between species, was also applied. In NEWHYBRIDS, each individual's genotype frequency class (genetic class) is inferred, thus providing a posterior probability (Q) for reflecting the level of certainty of an individual belonging to a certain genetic class (for example, F1, backcross, purebred.) Six genetic classes were estimated, viz. pure Kunimasu, pure Himemasu, F1, F2, backcross of F1 with pure Kunimasu and that with pure Himemasu, with a burn-in of 50,000 iterations followed by a sampling period of 50,000 iterations, assuming no prior allele frequencies and a uniform prior distribution for diversity at each locus. The z option was used for Himemasu from Lake Akan to specify in advance that they were of known origin and belonged to the "pure Himemasu" class, thereby allowing their use in estimating allele frequencies among Himemasu.

Levels of genetic differentiation between populations were evaluated by pairwise F_{ST} , calculated using ARLEQUIN ver. 3.5 (Excoffier et al. 2005).

Statistical analysis - genetic diversity and bottleneck effects. The observed and expected heterozygosity, linkage disequilibrium between all combinations of locus pairs within populations and deviations from Hardy-Weinberg equilibrium were calculated using ARLEQUIN ver. 3.5 (Excoffier et al. 2005). Allelic diversity was assessed as allelic richness (calculated in FSTAT ver. 2.9.3.2 (Goudet 2002)), being a measure of the number of alleles per population corrected for sample size. Three measures were used to determine whether or not the Kunimasu population had experienced a recent bottleneck. Mode shifts in allele frequencies

within populations and increased heterozygosity relative to that expected under the mutation-drift equilibrium (heterozygosity excess) were tested in BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996), assuming an infinite alleles model of mutation (IAM). BOTTLENECK employs the Wilcoxon signed-rank test to detect heterozygosity excess. The statistic M , which measures allelic “vacancies” within the allelic range, was calculated according to Garza and Williamson (2001). Differences in expected heterozygosity, allelic richness and M values among populations were tested using Kruskal-Wallis test in R language version 2.11.1. (R developmental core team 2010). Where appropriate, all statistical tests were conducted at family-wise significance level of $\alpha = 0.05$, using Holm’s sequential Bonferroni adjustment (Holm 1979).

Results

Genetic divergence and introgressive hybridization. All five loci used in this study were polymorphic, the total number of alleles per locus ranging from 11 (One102) to 17 (One110) and allelic richness ranging from 8.9 (One102) to 12.3 (One115). Significant linkage disequilibrium was observed between one pair of loci (One102 and One115) in Himemasu from Lake Akan among a total of 30 tests (initial $\alpha = 0.0017$). There were no significant departures from Hardy-Weinberg equilibrium after sequential Bonferroni adjustment (initial $\alpha = 0.0033$). Allele frequencies for the five loci are presented in Table S2.

In the STRUCTURE analysis, the most likely number of clusters was shown to be $K = 2$, according to the Evanno et al. (2005) method (Fig. 1). Each specimen was then assigned to one or other of the clusters based on q_i . All 33 specimens of provisionally-identified “Kunimasu” were assigned to one cluster, whereas all 41 specimens of “Himemasu” from Lake Saiko were to the other, together with 30 specimens of indigenous Himemasu from Lake Akan (Fig. 2). Therefore, we regarded the former cluster as “genetically-assigned Kunimasu” and the latter as “genetically-assigned Himemasu”. Note that “genetically-assigned Kunimasu” and

“genetically-assigned Himemasu” are hereafter referred to simply as Kunimasu and Himemasu, respectively. Of the 70 unidentified specimens, 38 were assigned to Kunimasu and the remainder to Himemasu (Fig. 2). All but two specimens (FAKU 97737 and 97767) displayed $q_i > 0.90$, indicating a high probability of the former being purebred individuals (Vähä and Primmer 2006). Although the other two specimens were determined as possible hybrids, they were assigned to Kunimasu on the basis of their dominant Kunimasu genome, their q_i being 0.87 and 0.73. The result of the NEWHYBRIDS analysis was consistent with that of the STRUCTURE analysis (not shown). Using a Q value of 0.50 as a threshold for assigning specimens to a specific genetic class, no specimens were assigned to any of the hybrid classes (F1, F2 and two backcrosses). Even when a threshold value of > 0.50 for the sum of the Q values of all hybrid classes was applied, no hybrid specimens were found.

Moderate but highly significant genetic differentiations (F_{ST}) were observed between Kunimasu and the two populations (Lakes Saiko and Akan) of Himemasu, such differentiation not being apparent between the latter (Table 2).

Genetic diversity and bottleneck effects. Within-population genetic summary statistics for Kunimasu and the two Himemasu populations are presented in Table 1. Average expected heterozygosity and allelic richness were higher in Kunimasu than in Himemasu, both being significantly different among populations (Kruskal-Wallis test: expected heterozygosity $\chi^2 = 9.5$, $P = 0.009$, allelic richness $\chi^2 = 9.4$, $P = 0.009$). No mode shift in allele frequency distributions were detected (Fig. S1). Kunimasu had an excess of heterozygosity relative to that expected at mutation-drift equilibrium (Table 1), but this effect was not statistically significant ($P = 0.031$, initial $\alpha = 0.017$). The M values were all above the conservative significance threshold of $M = 0.68$ (Garza and Williamson 2001) and did not differ significantly among populations (Kruskal-Wallis test, $\chi^2 = 0.67$, $P = 0.715$).

Discussion

Genetic divergence and introgressive hybridization. The introduction of non-indigenous species and habitat modification can result in introgressive hybridization between otherwise allopatric species, which may lead to the extinction of participating species through the loss of distinct gene pools and local adaptive features, and breakdown of co-adapted gene complexes (Allendorf and Waples 1995; Rhymer and Simberloff 1996; Seehausen 2006). Previous studies examining introgressive hybridization in salmonid fishes following secondary contact have described various findings. In Crane Prairie Reservoir in the Northwestern US, Matala et al. (2008) observed genetic distinctiveness and highly restricted, if any, gene flow between wild red band trout (*Oncorhynchus mykiss gairdneri*) and hatchery rainbow trout (*Oncorhynchus mykiss irideus*), despite extensive stocking of the latter. In Lake Biwa, Japan, Kuwahara et al. (2012) recently reported a moderate level of introgression of the *Oncorhynchus masou* subsp. (Biwamasu) [*Oncorhynchus* sp. sensu Nakabo (2009)] genome into lake-run *Oncorhynchus masou ishikawae* (Amago), the latter being absent from Lake Biwa before stocking was initiated in 1970. Bettles et al. (2005) reported ongoing introgressive hybridization between cutthroat trout (*Oncorhynchus clarki clarki*) and rainbow trout (*O. mykiss irideus*) in 13 streams on Vancouver Island, Canada, the degree of which varied among streams from F1 dominance to the formation of a hybrid swarm.

The present results indicated highly restricted introgressive hybridization between Kunimasu and Himemasu, despite the large scale introduction of the latter into both Lakes Tazawa and Saiko. According to the STRUCTURE analysis based on five microsatellite loci, all but two (FAKU 97737 and 97767) of a total of 144 specimens were assigned to Kunimasu and Himemasu clusters with high probability. Although the remaining two specimens were suggested as being hybrids, the Kunimasu genome was dominant in both of them, indicating that they were not F1 progeny. Furthermore, the NEWHYBRIDS and STRUCTURE analysis results were consistent, indicating an absence of hybrids having either a Kunimasu or Himemasu ancestor within two generations (F1, F2, backcross of F1 with pure Kunimasu and

those with pure Himemasu) in the specimens sampled, leading to our conclusion that introgressive hybridization between Kunimasu and Himemasu has been rare, resulting in the persistence of distinct gene pools of the two species in Lake Saiko. Notwithstanding, some traces of past introgressive hybridization were identified, although it is not clear whether this occurred (pre-1940) in Lake Tazawa or subsequently in Lake Saiko.

Vähä and Primmer's (2006) simulation study using STRUCTURE proposed the use of an arbitrary threshold of $q_i = 0.90$ for separating hybrids from purebreds, despite showing at that time that such a value tended to overestimate the proportion of hybrids. Application of a more stringent threshold ($q_i = 0.80$) in the present study did not affect the result significantly, one specimen still being assigned to hybrids. The STRUCTURE analysis also tends to overestimate the proportion of hybrids when the number of loci used is small (Vähä and Primmer 2006), but this effect should have been compensated for by the inclusion of reference population allele frequency information (Himemasu from Lake Akan) and the relatively large genetic divergence between Kunimasu and Himemasu (Vähä and Primmer 2006). Nevertheless, further analysis with an increased number of loci may further clarify the relationship between the two species.

Spatial and temporal segregation of spawning have both been reported as primary factors underlying the divergence of sympatric species or populations of the genus *Oncorhynchus* (e.g., Taylor et al. 1997; Fillatre et al. 2003; Lin et al. 2008; Pavey et al. 2010). Although the ecology and life history of Kunimasu remain largely unknown, Nakabo (2011) and Nakabo et al. (2011) suggested that Kunimasu in Lake Tazawa had become adapted to a particularly low water temperature spawning ground, occurring in deeper water than for any other species of *Oncorhynchus*. Nakabo et al. (2011) also noted that their Kunimasu specimens had been collected while or just after spawning in March and April, in accordance with the putative main spawning season of Kunimasu in Lake Tazawa (February) (Nakabo 2011), whereas Himemasu generally spawn in autumn, as described by, for example, Tokui (1959, 1961). Apparently, both spatial and temporal segregation of spawning of Kunimasu and Himemasu in Lake Saiko has

led to their reproductive isolation.

Genetic differentiation (F_{ST}) between Kunimasu and Himemasu (Table 2) was comparable to that previously reported among populations of *O. nerka*, inferred from microsatellite DNA variations. Genetic differentiation between Kunimasu and Himemasu was greater than that observed among Japanese Himemasu stocks (pairwise $F_{ST} = -0.008$ – 0.032 ; Yamamoto et al. 2011), but less than that between sockeye salmon from the Abira River, Hokkaido [derived from Himemasu in Lake Shikotsu, Hokkaido (Urawa et al. 1999)] and other sockeye salmon populations from various localities around the Pacific rim (pairwise $F_{ST} = 0.199$ – 0.310 ; Beacham et al. 2006), suggesting that Kunimasu represents one of a number of populations of kokanee. Nevertheless, Kunimasu have evolved unique morphological and ecological characteristics within *O. nerka*, presumably because of the unique environment of Lake Tazawa, being on the periphery of the range of *O. nerka*, such uniqueness contributing to the reproductive isolation between Kunimasu and Himemasu (see above).

Kunimasu has been reported by several authors as having a uniformly black body in life (Akitaken Suisanshikenjo 1907; Oshima 1940; Nakabo et al. 2011), lacking dark dorsal spots (Jordan and Hubbs 1925; Hikita 1962). In contrast, Oshima (1941) described Kunimasu as having small indistinct dark spots on the dorsum (on the basis of one male specimen). In the present study, all specimens of provisionally-identified “Kunimasu”, which had a black body without dark dorsal spots, were subsequently genetically assigned to the Kunimasu cluster, according to the STRUCTURE analysis. On the other hand, the genetic assignment of the 70 previously unidentified specimens yielded mixed results, 38 and 32 specimens being assigned to the Kunimasu and Himemasu clusters, respectively. The former included 10 specimens with a black body with dark dorsal spots (FAKU 99442, 99444, 99452, 99454, 99562, 99463, YFTC 6, 8, 9, 11), 19 with a black body without dark spots (FAKU 98737, 98738, 98741, 98742, 99396–99400, 99443, 99453, 99461, YFTC 1–5, 7, 10), seven with a non-black body with dark spots (FAKU 99187, 99189, 99386, 99435, 99437, 99439, YFTC 26) and two with a non-black

body without dark spots (FAKU99182, 99328). Such variations in body coloration, previously unreported, need to be considered in relation to life history phase. A detailed morphological description of Kunimasu will be considered in a forthcoming publication.

Genetic diversity and population bottleneck. Small isolated or recently bottlenecked populations are most likely to suffer from an increased chance of extinction or reduced adaptive potential due to reduced genetic diversity, as well as from other deterministic and/or stochastic effects (Frankham et al. 1999, 2002). Although the extant Kunimasu population was established recently from a single founding event involving ca. 100,000 eggs, the genetic diversity of Kunimasu, measured by expected heterozygosity and allelic richness, was no less than that of Himemasu from Lakes Saiko and Akan (this study), or from other Japanese (Yamamoto et al. 2011) and North American kokanee populations (Young et al. 2004). In addition, the Kunimasu population did not show any evidence of having been subjected to a bottleneck, according to three measures used herein to determine the likelihood of such, i.e., a mode shift in allele frequency distributions, heterozygosity excess and a reduced value for M . A mode shift of allele frequency distributions in a bottlenecked population occurs such that the proportion of alleles at an intermediate frequency exceeds the proportion of rare alleles, because rare alleles are lost rapidly as the result of small population size (Luikart et al. 1998). The observed heterozygosity in bottlenecked population would be increased relative to the heterozygosity expected under mutation-drift equilibrium estimated from the observed number of alleles, as allelic diversity is reduced faster than heterozygosity during a bottleneck. The statistic M , which measures allelic “vacancies” within the allelic range, can be expected to be reduced in bottlenecked populations as the latter have more “vacancies” than non-bottlenecked populations. However, each of these genetic tests failed to detect any signs of a bottleneck in the Kunimasu population.

In conclusion, the present Kunimasu population in Lake Saiko is not considered to be seriously endangered, at least by introgressive hybridization and reduced genetic diversity.

However, the rate of introgressive hybridization may differ in different situations. In fact, two Kunimasu-like specimens collected in Lake Motosu in December 2010, to which ca. 100,000 eyed eggs of Kunimasu were introduced in 1935 (Sugiyama 2000), have both been shown to be hybrids of Kunimasu and Himemasu, the Himemasu genome being dominant, following a preliminary genetic analysis by STRUCTURE. Environmental changes in Lake Saiko have the potential to intensify introgressive hybridization between Kunimasu and Himemasu, as evident already for several sympatric species pairs (Seehausen 2006), some of which have resulted in breakdown into a hybrid swarm. Clearly, therefore, future conservation efforts for Kunimasu in Lake Saiko should include periodic monitoring of the genetic properties of the species.

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Table 1 Sample size (n), number of alleles, allelic richness (A), expected and observed heterozygosity (H_E , H_O), expected heterozygosity under mutation-drift equilibrium (H_{Eq}) and M values (M) in five microsatellite loci of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*

	Ok-LS	On-LS	On-LA
<i>One102</i>			
n	71	73	30
No. of alleles	11	5	5
A	10.005	4.559	4.967
H_E	0.853	0.522	0.486
H_O	0.859	0.562	0.433
H_{Eq}	0.736	0.482	0.547
M	0.917	0.833	0.833
<i>One108</i>			
n	71	73	30
No. of alleles	11	10	8
A	10.175	8.588	7.932
H_E	0.891	0.768	0.742
H_O	0.789	0.726	0.633
H_{Eq}	0.734	0.707	0.711
M	0.786	0.667	0.533
<i>One110</i>			
n	71	73	30
No. of alleles	11	11	10
A	9.805	7.837	9.766

	H_E	0.882	0.618	0.584
	H_O	0.859	0.603	0.533
	H_{Eq}	0.735	0.733	0.773
	M	0.524	0.786	0.625
<i>One114</i>				
	n	71	72	29
	No. of alleles	11	9	8
	A	10.257	8.615	8.000
	H_E	0.885	0.819	0.825
	H_O	0.831	0.792	0.655
	H_{Eq}	0.734	0.675	0.713
	M	0.917	1.000	0.889
<i>One115</i>				
	n	71	73	30
	No. of alleles	12	11	9
	A	10.672	9.792	8.899
	H_E	0.884	0.621	0.567
	H_O	0.845	0.630	0.567
	H_{Eq}	0.757	0.735	0.743
	M	0.800	0.917	0.900
	Mean H_E	0.879	0.670	0.641
	Mean number of alleles	11.2	9.2	8.0
	Mean A	10.183	7.878	7.913
	Mean M	0.789	0.840	0.756
	Heterozygosity excess (P -value)	0.031	1.000	0.219

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

440   *On-LA* Himemasu from Lake Akan

441

442

443

Table 2 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	0.1342		0.5387
On-LA	0.1416	-0.0005	

Ok-LS Kunimasu, *Oncorhynchus kawamurae*, $n = 71$; *On-LS* Himemasu, *O. nerka*, from Lake Saiko, $n = 73$; *On-LA* Himemasu from Lake Akan, $n = 30$

454 **Figure legends**

455 **Fig. 1** Average delta K over 10 replicate runs of STRUCTURE for a given number of clusters
456 (K) for the specimens examined

457 **Fig. 2** Model-based clustering analysis of Kunimasu *Oncorhynchus kawamurae* and two
458 populations of Himemasu *O. nerka* computed by STRUCTURE version 2.3 with $K = 2$. Vertical
459 bars represent individuals and probability of assignment to each cluster (black; Kunimasu,
460 white; Himemasu). Arrows indicate putative hybrids. Provisional identification of each
461 specimen shown at bottom.

Fig. 1 Average delta K over 10 replicate runs of STRUCTURE for a given number of clusters (K) for the specimens examined

Fig. 2 Model-based clustering analysis of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka* computed by STRUCTURE version 2.3 with $K = 2$. Vertical bars represent individuals and its probability of assignment to each cluster (black; Kunimasu, white; Himemasu). Arrows indicate putative hybrids. Provisional identification of each specimen shown at bottom.

Fig. S1 Allele frequency distribution of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*

Table S1 Sampling locality, date, method, body color, sex, provisional identification, genetic assignment and catalogue number of each specimen examined

Locality	Date	Method	Body color	sex	Provisional identification	Genetic assignment	Catalogue number
Lake Saiko	20100306	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU97736
Lake Saiko	20100306	Gill net	b	Male	"Kunimasu"	Kunimasu*	FAKU97737
Lake Saiko	20100319	Gill net	b	Female	"Kunimasu"	Kunimasu	FAKU97742
Lake Saiko	20100319	Gill net	b	Female	"Kunimasu"	Kunimasu	FAKU97743
Lake Saiko	20100319	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU97744
Lake Saiko	20100319	Gill net	b	Female	"Kunimasu"	Kunimasu	FAKU97745
Lake Saiko	20100404	Gill net	c	Unknown	"Himemasu"	Himemasu	FAKU97747
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97748
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97749
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97750
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97751
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97752
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97753
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97754
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97755
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97756
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97757
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97758
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97759
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97760
Lake Saiko	20100404	Gill net	d	Unknown	"Himemasu"	Himemasu	FAKU97761
Lake Saiko	20100404	Gill net	d	Male	"Himemasu"	Himemasu	FAKU97762
Lake Saiko	20100404	Gill net	c	Male	"Himemasu"	Himemasu	FAKU97763
Lake Saiko	20100404	Gill net	d	Female	"Himemasu"	Himemasu	FAKU97764
Lake Saiko	20100403	Angling	d	Unknown	"Himemasu"	Himemasu	FAKU97765
Lake Saiko	20100404	Gill net	c	Male	"Himemasu"	Himemasu	FAKU97766
Lake Saiko	20100404	Gill net	b	Male	"Kunimasu"	Kunimasu*	FAKU97767
Lake Saiko	20100306	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU97768
Lake Saiko	20100306	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU97769
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97921
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97922
Lake Akan	20100511	Gill net	c	Female	Pure Himemasu	Himemasu	FAKU97923
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97924
Lake Akan	20100511	Gill net	c	Female	Pure Himemasu	Himemasu	FAKU97925
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97926
Lake Akan	20100511	Gill net	c	Female	Pure Himemasu	Himemasu	FAKU97927
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97928
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97929
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97930
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97931
Lake Akan	20100511	Gill net	c	Female	Pure Himemasu	Himemasu	FAKU97932
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97933
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97934
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97935
Lake Akan	20100511	Gill net	c	Female	Pure Himemasu	Himemasu	FAKU97936
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97937
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97938
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97939
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97940
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97941
Lake Akan	20100511	Gill net	d	Female	Pure Himemasu	Himemasu	FAKU97942
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97943
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97944
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97945
Lake Akan	20100511	Gill net	d	Male	Pure Himemasu	Himemasu	FAKU97946
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97947
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97948
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97949
Lake Akan	20100511	Gill net	c	Male	Pure Himemasu	Himemasu	FAKU97950
Lake Saiko	20101219	Angling	b	Female	"Unidentified"	Kunimasu	FAKU98737
Lake Saiko	20101219	Angling	b	Male	"Unidentified"	Kunimasu	FAKU98738
Lake Saiko	20110106	Beached	b	Male	"Unidentified"	Kunimasu	FAKU98741
Lake Saiko	20101229	Angling	b	Female	"Unidentified"	Kunimasu	FAKU98742
Lake Saiko	20110302	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98834
Lake Saiko	20110302	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98835
Lake Saiko	20110302	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98836

Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98837
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98838
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98839
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98840
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98841
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98842
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98843
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98844
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98845
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98846
Lake Saiko	20110303	Gill net	b	Male	"Kunimasu"	Kunimasu	FAKU98847
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99167
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99168
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99169
Lake Saiko	20110620	Angling	d	Unknown	"Unidentified"	Himemasu	FAKU99170
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99171
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99172
Lake Saiko	20110620	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99173
Lake Saiko	20110621	Angling	d	Unknown	"Unidentified"	Himemasu	FAKU99174
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99175
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99176
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99177
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99178
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99179
Lake Saiko	20110622	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99180
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99181
Lake Saiko	20110623	Angling	d	Unknown	"Unidentified"	Kunimasu	FAKU99182
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99183
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99184
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99185
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99186
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Kunimasu	FAKU99187
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99188
Lake Saiko	20110623	Angling	c	Unknown	"Unidentified"	Kunimasu	FAKU99189
Lake Saiko	20110426	Angling	d	Female	"Unidentified"	Kunimasu	FAKU99328
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99370
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99371
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99372
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99373
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99374
Lake Saiko	20110908	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99375
Lake Saiko	20110910	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99376
Lake Saiko	20110907	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99377
Lake Saiko	20110907	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99378
Lake Saiko	20110907	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99379
Lake Saiko	20110910	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99380
Lake Saiko	20110910	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99381
Lake Saiko	20110910	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99382
Lake Saiko	20110910	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99383
Lake Saiko	20110910	Gill net	d	Male	"Unidentified"	Himemasu	FAKU99384
Lake Saiko	20110911	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99385
Lake Saiko	20110911	Gill net	c	Female	"Unidentified"	Kunimasu	FAKU99386
Lake Saiko	20110911	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99387
Lake Saiko	20110911	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99388
Lake Saiko	20110911	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99389
Lake Saiko	20110911	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99390
Lake Saiko	20110907	Gill net	d	Male	"Unidentified"	Himemasu	FAKU99391
Lake Saiko	20110907	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99392
Lake Saiko	20110907	Gill net	c	Male	"Himemasu"	Himemasu	FAKU99393
Lake Saiko	20110907	Gill net	c	Female	"Unidentified"	Himemasu	FAKU99394
Lake Saiko	20111001	Angling	b	Female	"Unidentified"	Kunimasu	FAKU99396
Lake Saiko	20111001	Angling	b	Male	"Unidentified"	Kunimasu	FAKU99397
Lake Saiko	20111001	Angling	b	Female	"Unidentified"	Kunimasu	FAKU99398
Lake Saiko	20111001	Angling	b	Female	"Unidentified"	Kunimasu	FAKU99399
Lake Saiko	20111001	Angling	b	Male	"Unidentified"	Kunimasu	FAKU99400
Lake Saiko	20111001	Angling	a	Male	"Unidentified"	Himemasu	FAKU99401
Lake Saiko	20111002	Angling	c	Male	"Unidentified"	Kunimasu	FAKU99435
Lake Saiko	20111002	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99436
Lake Saiko	20111002	Angling	c	Female	"Unidentified"	Kunimasu	FAKU99437
Lake Saiko	20111002	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99438
Lake Saiko	20111002	Angling	c	Male	"Unidentified"	Kunimasu	FAKU99439

Lake Saiko	20111002	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99440
Lake Saiko	20111004	Angling	c	Male	"Unidentified"	Himemasu	FAKU99441
Lake Saiko	20111004	Angling	a	Male	"Unidentified"	Kunimasu	FAKU99442
Lake Saiko	20111016	Angling	b	Male	"Unidentified"	Kunimasu	FAKU99443
Lake Saiko	20111016	Angling	a	Male	"Unidentified"	Kunimasu	FAKU99444
Lake Saiko	20111016	Angling	a	Male	"Unidentified"	Himemasu	FAKU99445
Lake Saiko	20111020	Angling	a	Male	"Unidentified"	Kunimasu	FAKU99452
Lake Saiko	20111020	Angling	b	Female	"Unidentified"	Kunimasu	FAKU99453
Lake Saiko	20111020	Angling	a	Female	"Unidentified"	Kunimasu	FAKU99454
Lake Saiko	20111017	Angling	c	Unknown	"Unidentified"	Himemasu	FAKU99455
Lake Saiko	20111017	Angling	c	Female	"Unidentified"	Himemasu	FAKU99456
Lake Saiko	20111109	Angling	b	Male	"Unidentified"	Kunimasu	FAKU99461
Lake Saiko	20111109	Angling	a	Male	"Unidentified"	Kunimasu	FAKU99462
Lake Saiko	20111109	Angling	a	Male	"Unidentified"	Kunimasu	FAKU99463
Lake Saiko	20110114	Beached	b	Female	"Unidentified"	Kunimasu	YFTC1
Lake Saiko	20110115	Beached	b	Unknown	"Unidentified"	Kunimasu	YFTC2
Lake Saiko	21110119	Beached	b	Male	"Unidentified"	Kunimasu	YFTC3
Lake Saiko	20110130	Beached	b	Female	"Unidentified"	Kunimasu	YFTC4
Lake Saiko	20110216	Beached	b	Female	"Unidentified"	Kunimasu	YFTC5
Lake Saiko	20110218	Beached	a	Female	"Unidentified"	Kunimasu	YFTC6
Lake Saiko	20110218	Beached	b	Female	"Unidentified"	Kunimasu	YFTC7
Lake Saiko	20110218	Beached	a	Female	"Unidentified"	Kunimasu	YFTC8
Lake Saiko	20110218	Beached	a	Female	"Unidentified"	Kunimasu	YFTC9
Lake Saiko	20110218	Beached	b	Male	"Unidentified"	Kunimasu	YFTC10
Lake Saiko	20110224	Beached	a	Female	"Unidentified"	Kunimasu	YFTC11
Lake Saiko	20110302	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC14
Lake Saiko	20110302	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC15
Lake Saiko	20110324	Gill net	b	Female	"Kunimasu"	Kunimasu	YFTC18
Lake Saiko	20110325	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC19
Lake Saiko	20110325	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC20
Lake Saiko	20110325	Gill net	c	Female	"Unidentified"	Himemasu	YFTC21
Lake Saiko	20110325	Gill net	b	Female	"Kunimasu"	Kunimasu	YFTC22
Lake Saiko	20110325	Gill net	b	Female	"Kunimasu"	Kunimasu	YFTC23
Lake Saiko	20110325	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC24
Lake Saiko	20110325	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC25
Lake Saiko	20110325	Gill net	c	Male	"Unidentified"	Kunimasu	YFTC26
Lake Saiko	20110331	Gill net	b	Male	"Kunimasu"	Kunimasu	YFTC27

a black with dark spots, *b* black without dark spots, *c* not black with dark spots, *d* not black without dark spots

* Putative hybrids

Table S2 Allele frequencies for five microsatellite loci of Kunimasu *Oncorhynchus kawamurae* and two populations of Himemasu *O. nerka*

	Ok-LS	On-LS	On-LA
<i>One102</i>			
<i>n</i>	71	73	30
218	0.014	—	—
222	0.028	—	—
226	0.134	—	—
230	0.106	0.226	0.15
234	0.021	0.075	0.067
238	0.141	0.651	0.7
242	0.148	0.034	0.067
246	0.268	—	—
250	0.028	0.014	0.017
254	0.092	—	—
258	—	—	—
262	0.021	—	—
<i>One108</i>			
<i>n</i>	71	73	30
179	0.007	—	—
183	0.049	—	—
187	0.148	—	—
191	0.134	—	—
195	0.141	0.11	0.1
199	0.07	0.144	0.217
203	0.077	0.068	0.15
207	0.134	0.425	0.433
211	0.12	0.116	0.033
215	0.099	0.034	0.033
219	—	—	—
223	—	—	—
227	—	0.068	0.017
231	0.021	—	—
235	—	0.014	—
239	—	—	—
243	—	—	—
247	—	0.014	—
251	—	0.007	0.017
<i>One110</i>			
<i>n</i>	71	73	30
207	0.007	—	—
211	—	—	—

215	0.007	—	—
219	—	—	—
223	—	—	—
227	—	—	—
231	0.07	0.562	0.6
235	—	0.007	0.017
239	—	0.007	—
243	—	—	—
247	—	0.014	—
251	0.085	—	0.017
255	0.176	—	—
259	0.141	0.062	0.017
263	0.085	0.041	0.017
267	0.183	0.021	0.017
271	0.085	0.014	0.033
275	0.085	0.007	0.017
279	—	0.253	0.25
283	—	0.014	—
287	0.077	—	—
291	—	—	0.017
<i>One114</i>			
<i>n</i>	71	72	29
227	—	0.097	0.052
231	—	0.069	0.19
235	—	0.028	0.052
239	0.113	0.042	—
243	0.077	0.306	0.259
247	0.162	0.222	0.259
251	0.183	0.146	0.103
255	0.077	0.069	0.034
259	0.028	0.021	0.052
263	0.049	—	—
267	0.141	—	—
271	0.092	—	—
275	0.07	—	—
279	—	—	—
283	0.007	—	—
<i>One115</i>			
<i>n</i>	71	73	30
177	0.007	—	—
181	0.07	—	—
185	0.007	—	—
189	0.028	—	—

193	0.148	0.007	0.017
197	0.056	0.048	0.067
201	0.127	0.055	0.017
205	0.099	0.034	0.033
209	0.106	0.048	—
213	0.07	0.027	0.083
217	0.211	0.603	0.65
221	—	0.089	0.067
225	—	0.034	0.017
229	—	0.027	0.05
233	0.07	—	—
237	—	0.027	—

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