

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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25

26 **Abstract**

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

44

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

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48

49 **Introduction**

50 *Oncorhynchus kawamurae* Jordan and McGregor *in* Jordan and Hubbs (1925) (common name  
51 “Kunimasu”) had been believed extinct in Lake Tazawa, the only known habitat of the species,  
52 since 1940, due to the introduction of acidic water to the Lake from the Tama River (Sugiyama  
53 2000). Although eyed-eggs of Kunimasu had been introduced to several other lakes, including  
54 Lake Saiko, Yamanashi Prefecture, before 1940, the lack of subsequent records of Kunimasu  
55 from these lakes had led to a belief that the species had become extinct. Accordingly, Nakabo et  
56 al’s. (2011) recent discovery of Kunimasu in Lake Saiko attracted a great deal of attention  
57 concerning the conservation of the species.

58 Introgressive hybridization, by which process genes are exchanged between distinct species,  
59 has often been observed between native and introduced salmonid species, including some  
60 lineages that have been isolated for more than a million years (Utter 2001), and is therefore  
61 regarded as a major threat to the persistence of native evolutionary lines. Although Nakabo et al.  
62 (2011) rejected the likelihood of extensive introgressive hybridization between Kunimasu and  
63 co-occurring kokanee (land-locked sockeye salmon, *Oncorhynchus nerka*; common name  
64 “Himemasu”) on the basis of clear morphological and genetic differences, the possibility of a  
65 low level of introgressive hybridization was not fully explored due to the small Kunimasu  
66 sample.

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

75 100,000 eyed eggs introduced from Lake Tazawa 70 years BP (Sugiyama 2000), the possibility  
76 of any recent bottleneck having occurred in the Kunimasu population, thereby reducing genetic  
77 diversity, was also investigated, using a suite of measures.

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

80

## 81 **Materials and Methods**

82 *Sampling.* A total of 115 specimens collected from Lake Saiko in 2010 and 2011 by gill net and  
83 angling, together with additional “beached” post-spawning (“Uki-yo”) specimens, were  
84 deposited in the Fish Collection of Kyoto University Museum (FAKU) and the Yamanashi  
85 Prefectural Fisheries Technology Center (YFTC) (Table S1). Each specimen was then  
86 provisionally identified for the genetic analysis on the basis of external morphology (body  
87 coloration) and collection date. On the basis of Nakabo et al’s (2011) observation that Kunimasu  
88 specimens collected during or just after spawning (in March and April) had a black body, 24 of  
89 the present specimens having a black body and collected from the presumed spawning ground  
90 in March and April (FAKU 98834–98847, YFTC 14, 15, 18–20, 22–25, 27) were provisionally  
91 identified as “Kunimasu”. The lack of dark dorsal spots agreed with previous reports on body  
92 coloration of Kunimasu (Jordan and Hubbs 1925; Hikita 1962). On the other hand, 21 male  
93 specimens collected in September (FAKU 99370–99383, 99385, 99387–99390, 99392, 99393),  
94 agreeing with the description of mature male Himemasu given by Jordan and Hubbs (1925)  
95 (non-black body with dark spots on dorsum), were provisionally identified as “Himemasu”. The  
96 remaining 58 specimens (FAKU 98737, 98738, 98742, 99167–99189, 99328, 99384, 99386,  
97 99391, 99394, 99396–99401, 99435–99445, 99452–99456, 99461–99463, YFTC 21, 26) plus  
98 12 black-bodied “Uki-yo” specimens collected in January and February (FAKU 98741, YFTC  
99 1–11), which may have included both Kunimasu and Himemasu, were left unidentified at this  
100 stage. Internal morphology, including numbers of gill rakers and pyloric caeca, was not used for

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

107 *Microsatellite genotyping.* Total genomic DNA was extracted using the DNeasy Tissue  
108 Kit (Qiagen, Germany) or the Wizard Genomic DNA Purification Kit (Promega, USA), both  
109 following the manufacturers’ protocols. Five microsatellite loci isolated from sockeye salmon  
110 (One102, One108, One110, One114, One115; Olsen et al. 2000) were amplified via the  
111 polymerase chain reaction (PCR) using fluorescent-labeled primers detected by an ABI-310  
112 genetic analyzer. PCR protocols followed Nakabo et al. (2011).

113 *Statistical analysis - population structure.* The population structure was explored using a  
114 Bayesian-based clustering method implemented in the program STRUCTURE 2.3 (Pritchard et  
115 al. 2000), wherein individuals are clustered on the basis of genotype into  $K$  randomly  
116 interbreeding groups by minimizing departures from Hardy-Weinberg equilibrium and linkage  
117 disequilibrium within the groups. Ten replicates for each pre-defined  $K = 1$  between 10 were  
118 obtained with runs of 100,000 burn-in and 200,000 Markov Chain Monte Carlo (MCMC)  
119 iterations, applying the admixture model with correlated alleles. Specimens were pooled into  
120 two sampling locations (Lakes Saiko and Akan) for use in the LOCPRIOR model (Hubisz et al.  
121 2009), which uses the sampling locations of specimens to assist in clustering. To determine the  
122 most likely value of  $K$ , the Evanno et al. (2005) method implemented in the program  
123 STRUCTURE HARVESTER (Earl 2009), which determines the second-order rate of change in  
124 the distribution of  $L(K)$ , was used. CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007) was  
125 used to summarize parameters across 10 replicate runs and DISTRUCT ver.1.1 (Rosenberg  
126 2004), to produce the corresponding graphical output. Each specimen was assigned to one or

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

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

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

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

153 within populations and increased heterozygosity relative to that expected under the  
154 mutation-drift equilibrium (heterozygosity excess) were tested in BOTTLENECK version  
155 1.2.02 (Cornuet and Luikart 1996), assuming an infinite alleles model of mutation (IAM).  
156 BOTTLENECK employs the Wilcoxon signed-rank test to detect heterozygosity excess. The  
157 statistic  $M$ , which measures allelic “vacancies” within the allelic range, was calculated  
158 according to Garza and Williamson (2001). Differences in expected heterozygosity, allelic  
159 richness and  $M$  values among populations were tested using Kruskal-Wallis test in R language  
160 version 2.11.1. (R developmental core team 2010). Where appropriate, all statistical tests were  
161 conducted at family-wise significance level of  $\alpha = 0.05$ , using Holm’s sequential Bonferroni  
162 adjustment (Holm 1979).

163

## 164 **Results**

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

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

179 “genetically-assigned Himemasu” are hereafter referred to simply as Kunimasu and Himemasu,  
180 respectively. Of the 70 unidentified specimens, 38 were assigned to Kunimasu and the  
181 remainder to Himemasu (Fig. 2). All but two specimens (FAKU 97737 and 97767) displayed  $q_i$   
182  $> 0.90$ , indicating a high probability of the former being purebred individuals (Vähä and  
183 Primmer 2006). Although the other two specimens were determined as possible hybrids, they  
184 were assigned to Kunimasu on the basis of their dominant Kunimasu genome, their  $q_i$  being  
185 0.87 and 0.73. The result of the NEWHYBRIDS analysis was consistent with that of the  
186 STRUCTURE analysis (not shown). Using a  $Q$  value of 0.50 as a threshold for assigning  
187 specimens to a specific genetic class, no specimens were assigned to any of the hybrid classes  
188 (F1, F2 and two backcrosses). Even when a threshold value of  $> 0.50$  for the sum of the  $Q$   
189 values of all hybrid classes was applied, no hybrid specimens were found.

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

193 **Genetic diversity and bottleneck effects.** Within-population genetic summary statistics  
194 for Kunimasu and the two Himemasu populations are presented in Table 1. Average expected  
195 heterozygosity and allelic richness were higher in Kunimasu than in Himemasu, both being  
196 significantly different among populations (Kruskal-Wallis test: expected heterozygosity  $\chi^2 = 9.5$ ,  
197  $P = 0.009$ , allelic richness  $\chi^2 = 9.4$ ,  $P = 0.009$ ). No mode shift in allele frequency distributions  
198 were detected (Fig. S1). Kunimasu had an excess of heterozygosity relative to that expected at  
199 mutation-drift equilibrium (Table 1), but this effect was not statistically significant ( $P = 0.031$ ,  
200 initial  $\alpha = 0.017$ ). The  $M$  values were all above the conservative significance threshold of  $M =$   
201 0.68 (Garza and Williamson 2001) and did not differ significantly among populations  
202 (Kruskal-Wallis test,  $\chi^2 = 0.67$ ,  $P = 0.715$ ).

203

204 **Discussion**



205 **Genetic divergence and introgressive hybridization.** The introduction of non-indigenous  
206 species and habitat modification can result in introgressive hybridization between otherwise  
207 allopatric species, which may lead to the extinction of participating species through the loss of  
208 distinct gene pools and local adaptive features, and breakdown of co-adapted gene complexes  
209 (Allendorf and Waples 1995; Rhymer and Simberloff 1996; Seehausen 2006). Previous studies  
210 examining introgressive hybridization in salmonid fishes following secondary contact have  
211 described various findings. In Crane Prairie Reservoir in the Northwestern US, Matala et al.  
212 (2008) observed genetic distinctiveness and highly restricted, if any, gene flow between wild  
213 red band trout (*Oncorhynchus mykiss gairdneri*) and hatchery rainbow trout (*Oncorhynchus*  
214 *mykiss irideus*), despite extensive stocking of the latter. In Lake Biwa, Japan, Kuwahara et al.  
215 (2012) recently reported a moderate level of introgression of the *Oncorhynchus masou* subsp.  
216 (Biwamasu) [*Oncorhynchus* sp. sensu Nakabo (2009)] genome into lake-run *Oncorhynchus*  
217 *masou ishikawae* (Amago), the latter being absent from Lake Biwa before stocking was  
218 initiated in 1970. Bettles et al. (2005) reported ongoing introgressive hybridization between  
219 cutthroat trout (*Oncorhynchus clarki clarki*) and rainbow trout (*O. mykiss irideus*) in 13  
220 streams on Vancouver Island, Canada, the degree of which varied among streams from F1  
221 dominance to the formation of a hybrid swarm.

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

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

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

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

257 led to their reproductive isolation.

258 Genetic differentiation ( $F_{ST}$ ) between Kunimasu and Himemasu (Table 2) was comparable  
259 to that previously reported among populations of *O. nerka*, inferred from microsatellite DNA  
260 variations. Genetic differentiation between Kunimasu and Himemasu was greater than that  
261 observed among Japanese Himemasu stocks (pairwise  $F_{ST} = -0.008$ – $0.032$ ; Yamamoto et al.  
262 2011), but less than that between sockeye salmon from the Abira River, Hokkaido [derived from  
263 Himemasu in Lake Shikotsu, Hokkaido (Urawa et al. 1999)] and other sockeye salmon  
264 populations from various localities around the Pacific rim (pairwise  $F_{ST} = 0.199$ – $0.310$ ;  
265 Beacham et al. 2006), suggesting that Kunimasu represents one of a number of populations of  
266 kokanee. Nevertheless, Kunimasu have evolved unique morphological and ecological  
267 characteristics within *O. nerka*, presumably because of the unique environment of Lake Tazawa,  
268 being on the periphery of the range of *O. nerka*, such uniqueness contributing to the  
269 reproductive isolation between Kunimasu and Himemasu (see above).

270 Kunimasu has been reported by several authors as having a uniformly black body in life  
271 (Akitaken Suisanshikenjo 1907; Oshima 1940; Nakabo et al. 2011), lacking dark dorsal spots  
272 (Jordan and Hubbs 1925; Hikita 1962). In contrast, Oshima (1941) described Kunimasu as  
273 having small indistinct dark spots on the dorsum (on the basis of one male specimen). In the  
274 present study, all specimens of provisionally-identified “Kunimasu”, which had a black body  
275 without dark dorsal spots, were subsequently genetically assigned to the Kunimasu cluster,  
276 according to the STRUCTURE analysis. On the other hand, the genetic assignment of the 70  
277 previously unidentified specimens yielded mixed results, 38 and 32 specimens being assigned to  
278 the Kunimasu and Himemasu clusters, respectively. The former included 10 specimens with a  
279 black body with dark dorsal spots (FAKU 99442, 99444, 99452, 99454, 99562, 99463, YFTC 6,  
280 8, 9, 11), 19 with a black body without dark spots (FAKU 98737, 98738, 98741, 98742,  
281 99396–99400, 99443, 99453, 99461, YFTC 1–5, 7, 10), seven with a non-black body with dark  
282 spots (FAKU 99187, 99189, 99386, 99435, 99437, 99439, YFTC 26) and two with a non-black

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

286 ***Genetic diversity and population bottleneck.*** Small isolated or recently bottlenecked  
287 populations are most likely to suffer from an increased chance of extinction or reduced adaptive  
288 potential due to reduced genetic diversity, as well as from other deterministic and/or stochastic  
289 effects (Frankham et al. 1999, 2002). Although the extant Kunimasu population was established  
290 recently from a single founding event involving ca. 100,000 eggs, the genetic diversity of  
291 Kunimasu, measured by expected heterozygosity and allelic richness, was no less than that of  
292 Himemasu from Lakes Saiko and Akan (this study), or from other Japanese (Yamamoto et al.  
293 2011) and North American kokanee populations (Young et al. 2004). In addition, the Kunimasu  
294 population did not show any evidence of having been subjected to a bottleneck, according to  
295 three measures used herein to determine the likelihood of such, i.e., a mode shift in allele  
296 frequency distributions, heterozygosity excess and a reduced value for  $M$ . A mode shift of  
297 allele frequency distributions in a bottlenecked population occurs such that the proportion of  
298 alleles at an intermediate frequency exceeds the proportion of rare alleles, because rare alleles  
299 are lost rapidly as the result of small population size (Luikart et al. 1998). The observed  
300 heterozygosity in bottlenecked population would be increased relative to the heterozygosity  
301 expected under mutation-drift equilibrium estimated from the observed number of alleles, as  
302 allelic diversity is reduced faster than heterozygosity during a bottleneck. The statistic  $M$ , which  
303 measures allelic “vacancies” within the allelic range, can be expected to be reduced in  
304 bottlenecked populations as the latter have more “vacancies” than non-bottlenecked populations.  
305 However, each of these genetic tests failed to detect any signs of a bottleneck in the Kunimasu  
306 population.

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

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

318

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433

434

435 **Table 1** Sample size ( $n$ ), number of alleles, allelic richness ( $A$ ), expected and observed  
 436 heterozygosity ( $H_E$ ,  $H_O$ ), expected heterozygosity under mutation-drift equilibrium ( $H_{Eq}$ ) and  $M$   
 437 values ( $M$ ) in five microsatellite loci of Kunimasu *Oncorhynchus kawamurae* and two  
 438 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

444 **Table 2** Pairwise  $F_{st}$  values between populations (below diagonal) and associated  $P$ -values  
 445 (above diagonal) inferred from five microsatellite loci

446

	Ok-LS	On-LS	On-LA
447 Ok-LS		0.0000	0.0000
448 On-LS	0.1342		0.5387
449 On-LA	0.1416	-0.0005	

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

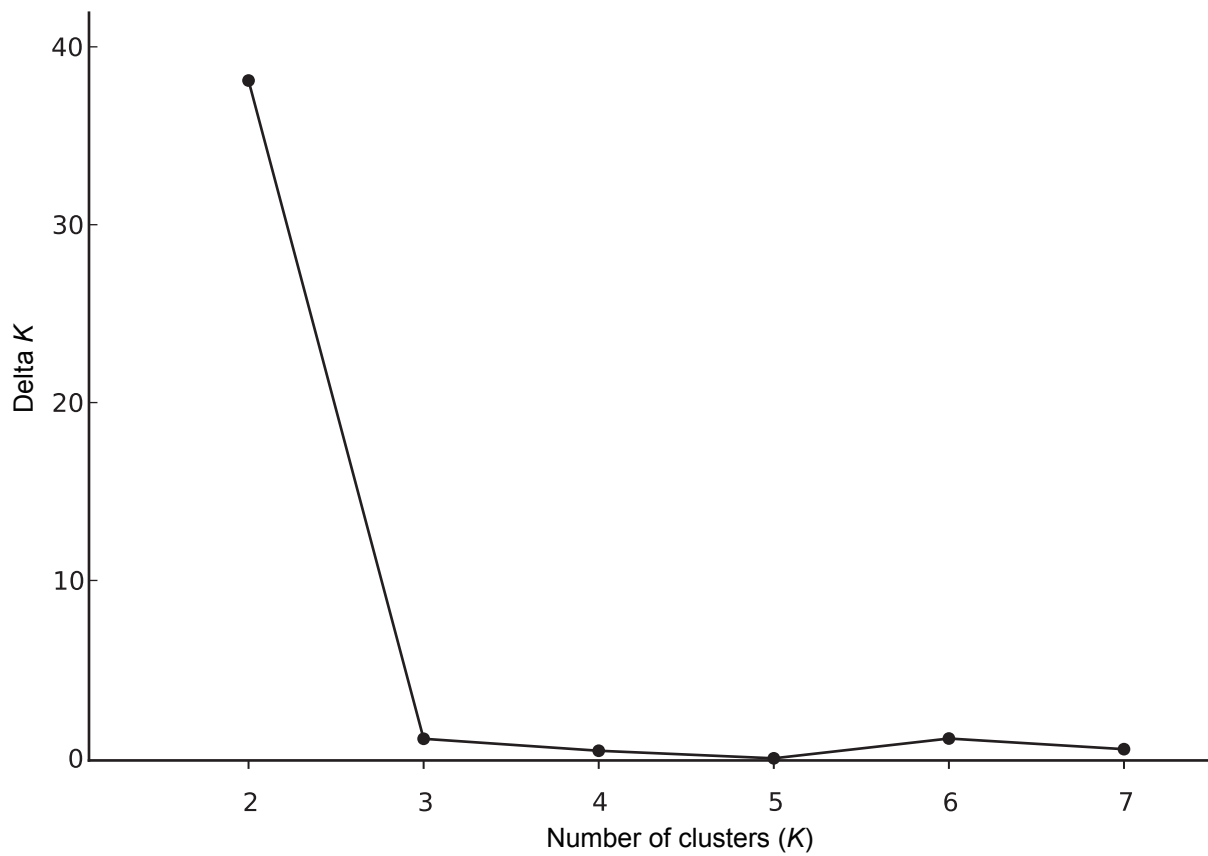
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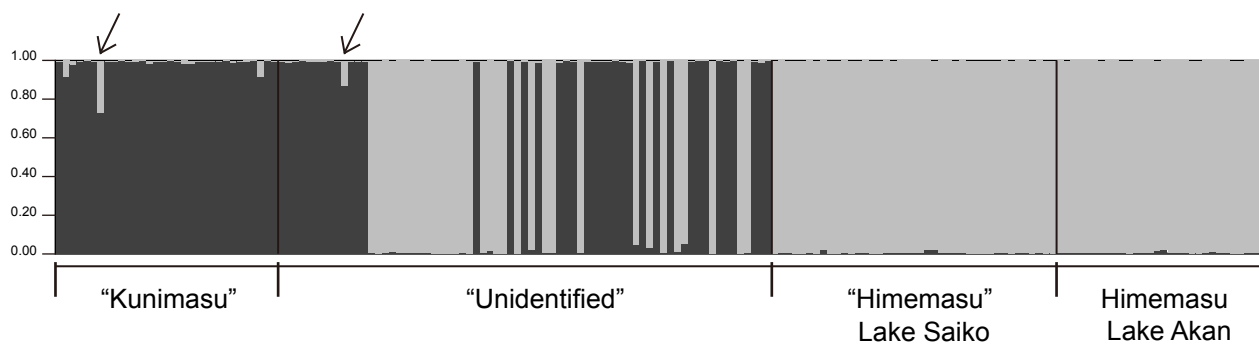
454 **Figure legends**

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.

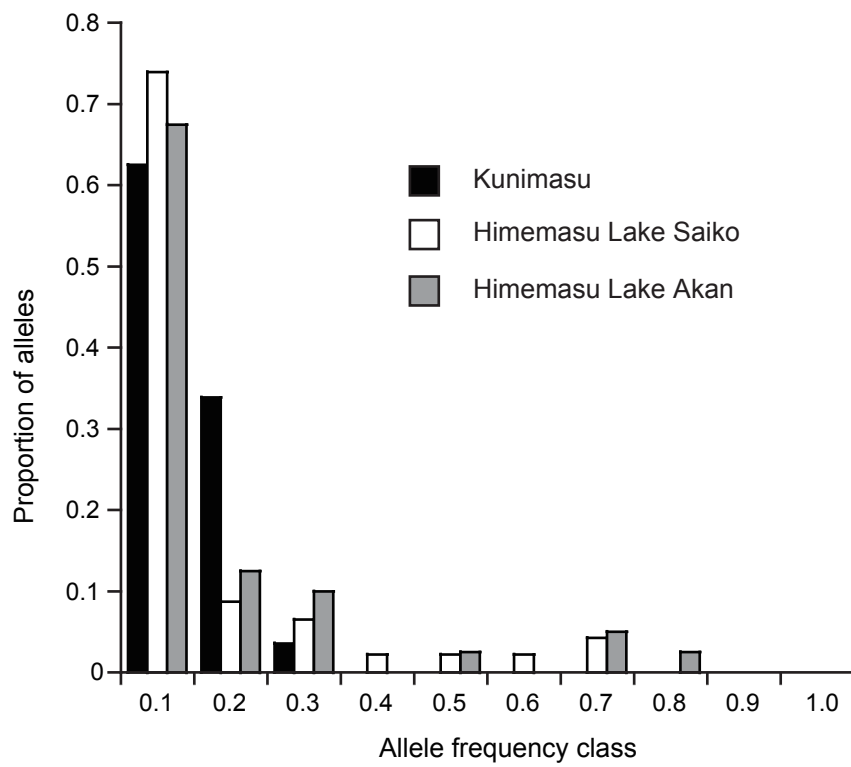


**Fig. 1** Average delta  $K$  over 10 replicate runs of STRUCTURE for a given number of clusters ( $K$ ) for the specimens examined



**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	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	–

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