Mitochondrial DNA sequence divergence between “Kunimasu” *Oncorhynchus kawamurae* and “Himemasu” *O. nerka* in Lake Saiko, Yamanashi Prefecture, Japan, and their identification using multiplex haplotype-specific PCR

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Short running head: MHS-PCR identification of *Oncorhynchus kawamurae*

Short report

12 text pages

2 figures

Abstract In order to develop a simplified method for discriminating between *Oncorhynchus kawamurae* (Kunimasu) and sympatric *Oncorhynchus nerka* (Himemasu), sequence variations of mitochondrial DNA control region were examined on 71 specimens of Kunimasu and 103 of Himemasu. Sequence analyses for 530 bp revealed 3 variable sites, which defined three haplotypes in Kunimasu and one in Himemasu. Based on determined nucleotide sequences, species-specific primers were designed for the multiplex haplotype-specific polymerase chain reaction, which amplified ca. 900 and 1,040 bp fragments in Kunimasu and Himemasu, respectively. This simple rapid approach can be used to distinguish between the two species without the need to sacrifice specimens.

Key words: *Oncorhynchus kawamurae* · Lake Saiko · Molecular identification

Introduction

Oncorhynchus kawamurae Jordan and McGregor in Jordan and Hubbs 1925 (common name ‘‘Kunimasu’’), formerly restricted to Lake Tazawa, Akita Prefecture, the only known habitat of the species, had long been believed to have become extinct since the late 1930’s. Recently, however, Nakabo et al. (2011) discovered Kunimasu in Lake Saiko, Yamanashi Prefecture, one of the lakes to which eyed eggs of Kunimasu had been introduced from Lake Tazawa in 1935.

Stock assessment and intensive conservation efforts for Kunimasu are urgently needed, due to its present restricted distribution, i.e., Lake Saiko only. Although Kunimasu is known to have distinct morphological and ecological characteristics, having few pyloric caeca, and occurring and spawning on the deep bottom (Akitaken Suisanshikenjo 1915; Tanaka 1911; Okuyama 1939; Oshima 1941; Sugiyama 2000), both being unique within *Oncorhynchus*, comprehensive biological studies had at no time been conducted on Kunimasu in Lake Tazawa before its extinction. However, the discovery of Kunimasu in Lake Saiko has provided an unexpected opportunity to learn more of the morphological characteristics and life history of the species, especially with regard to spawning ecology and adaptations to its deeper, cooler water environment.

Kunimasu has been considered to have a close relationship with Kokanee, the land-locked sockeye salmon *Oncorhynchus nerka* (Japanese common name “Himemasu”) (Hosoya 2002; Eschmeyer 2012), sometimes having been treated as a subspecies or variation of the latter. Because Himemasu has been vigorously introduced to Lake Saiko over many years, both Kunimasu and Himemasu now occur sympatrically in the lake. However, the two species are clearly reproductively isolated (Nakabo et al. 2012; Muto et al. 2012), and are distinguishable by the numbers of pyloric caeca and gill rakers, in addition to spawning season and body coloration at such times (Jordan and Hubbs 1925; Akitaken Suisanshikenjo 1931; Oshima 1941; Hikita 1962; Sugiyama 2000). Notwithstanding, however, younger (immature)

and non-spawning adult fish show few differences in external morphology or coloration (Muto et al. 2012), resulting in difficulties in identification of the two species. Furthermore, it has been suggested that there is little difference in microhabitat or swimming depth between Kunimasu and Himemasu in Lake Saiko (Muto et al. 2012), unlike the situation in Lake Tazawa (Sugiyama 2000).

Detailed biological or ecological studies on Kunimasu in Lake Saiko would clearly be enhanced by a practical and reliable means of distinguishing between the former and Himemasu throughout all life history stages, from eggs to post-spawning carcasses. Morphological identification techniques, such as counting the numbers of pyloric caeca and gill rakers, are both time-consuming and labor intensive, apart from being inapplicable to eggs or juveniles. In addition, an identification method that does not sacrifice specimens is highly desirable, due to the apparently limited number of living individuals. Although microsatellite DNA analysis can discriminate precisely between Kunimasu and Himemasu without harming specimens, it is time-consuming and expensive, due to the need for genotyping at several loci (> 5), owing to the apparent absence of a diagnostic allele for Kunimasu (Muto et al. 2012). In addition, microsatellite DNA analysis usually requires an expensive automated fluorescent DNA sequencer, which makes general application of the method difficult.

This study aimed to develop a simple, rapid, cost-saving molecular method for discriminating between Kunimasu and Himemasu in Lake Saiko. We applied multiplex haplotype-specific (MHS) PCR to mitochondrial (mt) DNA for these species, such being a single step method based on haplotype-specific variations in DNA sequences (e.g., Rocha-Olivares 1998; Durand et al. 2010; Yamanoue et al. 2010). On the premise that species-specific DNA sequence variations exist, this method was expected to be labor, cost and time-saving, and applicable even to small juveniles, yet yielding reliable, sensitive and highly reproducible identifications (reviewed in Teletchea 2009), all the while without the need to harm specimens.

Materials and methods

Specimens. A total of 144 specimens, including 71 Kunimasu and 73 Himemasu, collected from Lake Saiko in 2010 and 2011 by gill net and angling, or the collection of carcasses ("Uki-yo") washed ashore after spawning, plus 30 specimens of indigenous Himemasu from Lake Akan (but see Kaeriyama 1993), in which Himemasu is not sympatric with Kunimasu, were utilized in the study. The same specimens were previously used for microsatellite DNA analyses by Muto et al. (2012). The specimens were unambiguously assigned to either Kunimasu or Himemasu, using Bayesian clustering, and deposited in the Kyoto University Museum (FAKU) or the Yamanashi Prefectural Fisheries Technology Center (YFTC).

DNA extraction, PCR amplification and sequencing. Total genomic DNA was extracted from white muscle preserved in 99 % ethanol using the DNeasy Tissue Kit (Qiagen, Germany) or the Wizard Genomic DNA Purification Kit (Promega, USA), both following the manufacturers' protocols. The control region of mtDNA was amplified by PCR, using the forward primer tRNAthr-2 5'-TCT TGT AAT CCG GAA GTC GGA-3' and reverse primer tRNAphe-2 5'-AAC AGC TTC AGT GTT ATG CT-3' (Sato et al. 2001). PCR was conducted using the Takara EX *Taq* polymerase kit (Takara Bio Inc., Shiga, Japan) under the following temperature profile: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were purified with ExoSAP-IT (USB. Co., OH, USA) and directly sequenced using the BigDye 1.1 Terminator sequencing kit (Applied Biosytems Inc., CA, USA) following the manufacturer's protocol, with the primer tRNAthr-3 5'-GGT TAA AAC CCT CCC TAG TG-3' (Sato et al. 2001). Alignments were checked by eye with the help of DNASIS software (Hitachi Software Engineering Co.,

111 Kanagawa, Japan).

112 *Primer design for MHS-PCR.* On the basis of sequence variations among Kunimasu
113 and Himemasu, species-specific forward primers were designed to anneal to sequences with a
114 diagnostic nucleotide for each species on the 3'-end, taking intra-specific sequence variations
115 into account, and to amplify different-sized PCR products. To enhance the specificity of
116 primer annealing, the third nucleotide from the 3'-end of each primer was designed to be
117 mismatched, as noted in Sommer et al. (1992) and Watanabe and Umetsu (2007). The
118 sequences of the designed primers were as follows: Ok-spL (Kunimasu specific primer) 5'-ATT
119 ATC AAC ATA CRG TGG TGTC-3'), On-spL (Himemasu specific primer) 5'-TAA ACT ACC
120 CTC TGA CGG CTAC-3'). Via MHS-PCR using these species-specific forward primers and
121 the common reverse primer tRNAphe-2 (see above), a species-specific fragment of ca. 900 base
122 pairs (bp) in Kunimasu and ca. 1040 bp in Himemasu were predicted to be amplified. For
123 internal positive control of the PCR conditions, a pair of primers expected to commonly anneal
124 to either of the two species, L-M-12S; 5'-ACA AAC TGG GAT TAG ATA CCC CAC TAT
125 GCC-3' and H-A-16S; 5'-GTT TTT GGT AAA CAC GCG AGG CTT ATG TTT GCC GAG-3'
126 (Matsui et al. 2012), which are complements of the primers of Kawaguchi et al. (2001),
127 producing a ca. 1,500 bp amplicon, were also included in MHS-PCR. Thus, for either of the
128 two species, two different-sized fragments (a species-specific amplicon and internal positive
129 control) could be expected to be amplified.

130 *MHS-PCR amplification.* MHS-PCR was performed on some representative
131 specimens selected so as to involve all haplotypes of the two species found in this study, using
132 five primers [two species-specific forward primers (Ok-spL and On-spL), a reverse primer
133 common to the two species (tRNAphe-2), and a pair of primers for internal positive control
134 (L-M-12S and H-A-16S)]. The PCR reaction was conducted using the Takara *Taq* Hot Start
135 Version polymerase kit (Takara Bio Inc., Shiga, Japan), with a reaction mixture containing 1.0
136 µl of 10X provided buffer, 1.0 µl of provided dNTPs mixture, 1.0 µl each of five primers, 0.06

137 µl of Takara *Taq* Hot Start Version polymerase and 0.40 µl of extracted total genomic DNA as
138 template, with the total volume brought up to 10 µl with sterilized water. The PCR
139 temperature profile was as follows: initial denaturation at 94 °C for 5 min, followed by 35
140 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 15 s and extension at 72 °C for 1
141 min, with a final extension at 72 °C for 7 min. PCR products, together with a DNA size
142 standard, were electrophoresed on a 1.5 % agarose gel in TAE buffer and visualized with
143 ethidium bromide stain.
144
145
146 **Results**
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148 Nucleotide sequences were obtained for 530 bp containing the part of the mitochondrial proline
149 transfer RNA gene and the 5' end of the control region for both Kunimasu and Himemasu.
150 Three variable sites were found among 174 specimens, with two transitions, one transversion
151 and no indels, thereby yielding three and one haplotypes from Kunimasu and Himemasu,
152 respectively. No haplotypes were shared between the two species. The names of haplotypes
153 and their frequency in Kunimasu were as follows: K-01 (85.9 %), K-10 (11.3 %) and K-29
154 (2.8 %). A representative haplotype of Kunimasu (K-01) was deposited in the DNA Data
155 Bank of Japan (DDBJ), with the accession number AB774473. The uncorrected pairwise
156 sequence divergence between species was 0.4–0.6 %. Two diagnostic nucleotide sites were
157 found, making it possible to design species-specific primers for each species and employ the
158 MHS-PCR method for discriminating between the two species. Inter- and intraspecific
159 variations of aligned sequences at the annealing sites of the designed species-specific primers
160 are shown in Fig. 1, and the MHS-PCR results for each of three specimens each of Kunimasu
161 and Himemasu in Fig. 2. As predicted, each Kunimasu (lanes 1–3 in Fig. 2) and Himemasu
162 individual (lanes 4–6 in Fig. 2) showed a species-specific fragment of ca. 900 and ca. 1,040 bp,

163 respectively, plus a common fragment of ca. 1,500 bp.

164

165

166 **Discussion**

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168 Kunimasu has been considered to have a close affinity with Himemasu (*Oncorhynchus nerka*,
169 otherwise referred to as kokanee or anadromous sockeye salmon) (Sugiyama 2000), although
170 their exact evolutionary relationship remained unclear, because only 17 formalin-fixed
171 specimens of Kunimasu from the species' original habitat, Lake Tazawa (including the holotype,
172 paratype and nontype specimens), were known prior to its rediscovery in Lake Saiko. In
173 addition, an attempt to extract DNA from formalin-fixed Kunimasu specimens had resulted in
174 failure (Sugiyama 2000). Recently, Muto et al. (2012) suggested that Kunimasu represented one
175 of a number of populations of kokanee, based on comparisons of genetic distances obtained
176 from microsatellite DNA data from Kunimasu, Himemasu and populatons of kokanee or
177 sockeye salmon from the Pacific rim. Mitochondrial DNA sequence information in the present
178 study indicated that the relationship of Kunimasu with Himemasu was the closest amongst
179 species of *Oncorhynchus*, the uncorrected pairwise sequence divergence between the former
180 being only 0.4–0.6 %, whereas those between Kunimasu and other *Oncorhynchus* species were
181 > 5 % (calculated from DDBJ/EMBL/GenBank sequence data: Accession nos. AB039892;
182 *Oncorhynchus keta*, NC010959; *O. gorbuscha*, NC009263; *O. kisutch*, NC002980; *O.*
183 *tshawytscha*, DQ864465; *O. masou*, NC001717; *O. mykiss*).
184 The result of MHS-PCR indicated that Kunimasu from Lake Saiko could be clearly
185 distinguished from sympatric Himemasu, by differences in the sizes of PCR products. In this
186 method, *Oncorhynchus masou*, another *Oncorhynchus* species inhabiting in Lake Saiko, yielded
187 neither of the two species-specific fragments but only the internal positive control fragment (the
188 result not shown). This method is advantageous in time or cost than other molecular

189 identification methods such as PCR restriction fragment length polymorphism (PCR-RFLP)
190 (e.g., Larmuseau et al. 2008) or microsatellite DNA analysis (e.g., Madsen et al. 2009). The
191 method is clearly applicable to field research, including ecological, stock management and
192 enhancement, and conservation studies, without any need for further appraisal. It is important
193 to note, however, that the method is based upon interspecific differences in mitochondrial
194 (mt)DNA, which is a maternally inherited non-recombinant. Accordingly, identifications
195 using mtDNA may not always be appropriate if introgressive hybridization between species is
196 likely. In Lake Saiko, no common haplotype was found between Kunimasu and Himemasu,
197 genetically identified from biparentally-inherited microsatellite DNA analyses, in this study.
198 This suggests that any effects of introgression would be very minor, if present at all, in Lake
199 Saiko. Notwithstanding, Muto et al. (2012), reported two specimens, identified as hybrids of
200 Kunimasu and Himemasu, the Himemasu genome being dominant, as preliminary results from
201 Lake Motosu, Yamanashi Prefecture, to which eyed eggs of Kunimasu had also been introduced
202 in 1935 (Sugiyama 2000). We observed that one of these specimens had a haplotype of
203 Kunimasu and the other had a haplotype of Himemasu (data not shown). Furthermore,
204 Yamamoto et al. (2011) and Kogura et al. (2011) reported some mtDNA sequence variation in
205 Himemasu, although we found no polymorphism within Himemasu specimens from Lake Saiko
206 and Lake Akan. Caution must therefore be exercised when applying MHS-PCR identification
207 techniques for Kunimasu to specimens from lakes other than Lake Saiko.
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292

293 **Figure legends**

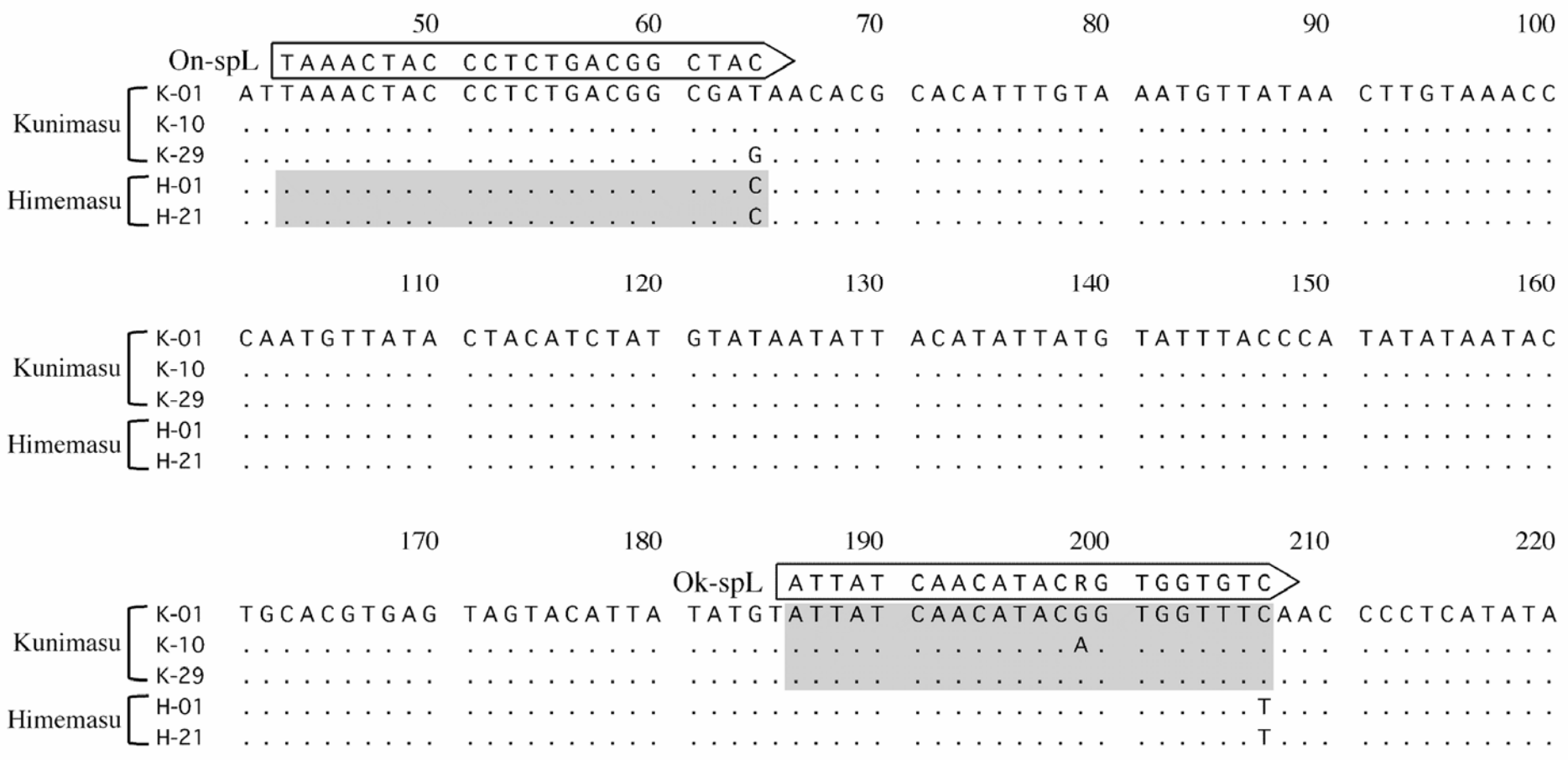
294

295 **Fig. 1** Partial sequences of mitochondrial DNA control region and proline transfer RNA gene of
296 Kunimasu *Oncorhynchus kawamurae* and Himemasu *O. nerka*. Annealing locations of
297 species-specific primers designed in this study indicated by arrows. *Dots* indicate nucleotides
298 identical to those of haplotype K-01. Nucleotide positions numbered from the 5'-end of
299 determined sequences. *Shaded areas* represent target sequences of the species-specific primers.
300 All haplotypes found in this study are presented

301

302 **Fig. 2** An ethidium bromide-stained 1.5 % agarose gel with TAE buffer after electrophoresis of
303 the MHS-PCR products. Lanes 1–3: Kunimasu *Oncorhynchus kawamurae* (lane1–3
304 correspond to K-01, K-10 and K-29 haplotypes, respectively), lanes 4–6: Himemasu *O. nerka*,
305 lane 7: 100-bp DNA ladder size marker (Takara Bio Inc., Shiga, Japan). *K*, *H* and *IC* represent
306 species-specific fragments for Kunimasu (ca. 900 bp), Himemasu (ca. 1,040 bp) and internal
307 positive control, respectively

308


50
60
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80
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100
On-spL
TAAACTAC CCTCTGACGG CTAC
Kunimasu
Himemasu
K-01
K-10
K-29
H-01
H-21
ATTAAACTAC CCTCTGACGG CGATAACACG CACATTTGTA AATGTTATAA CTTGTAAACC
G
C
C
110
120
130
140
150
160
CAATGTTATA CTACATCTAT GTATAATATT ACATATTATG TATTTACCCA TATATAATAC
170
180
190
200
210
220
Ok-spL
ATTAT CAACATACRG TGGTGTC
TGCACGTGAG TAGTACATTA TATGTATTAT CAACATACGG TGGTTTCAAC CCCTCATATA
A
T
T

Fig. 1

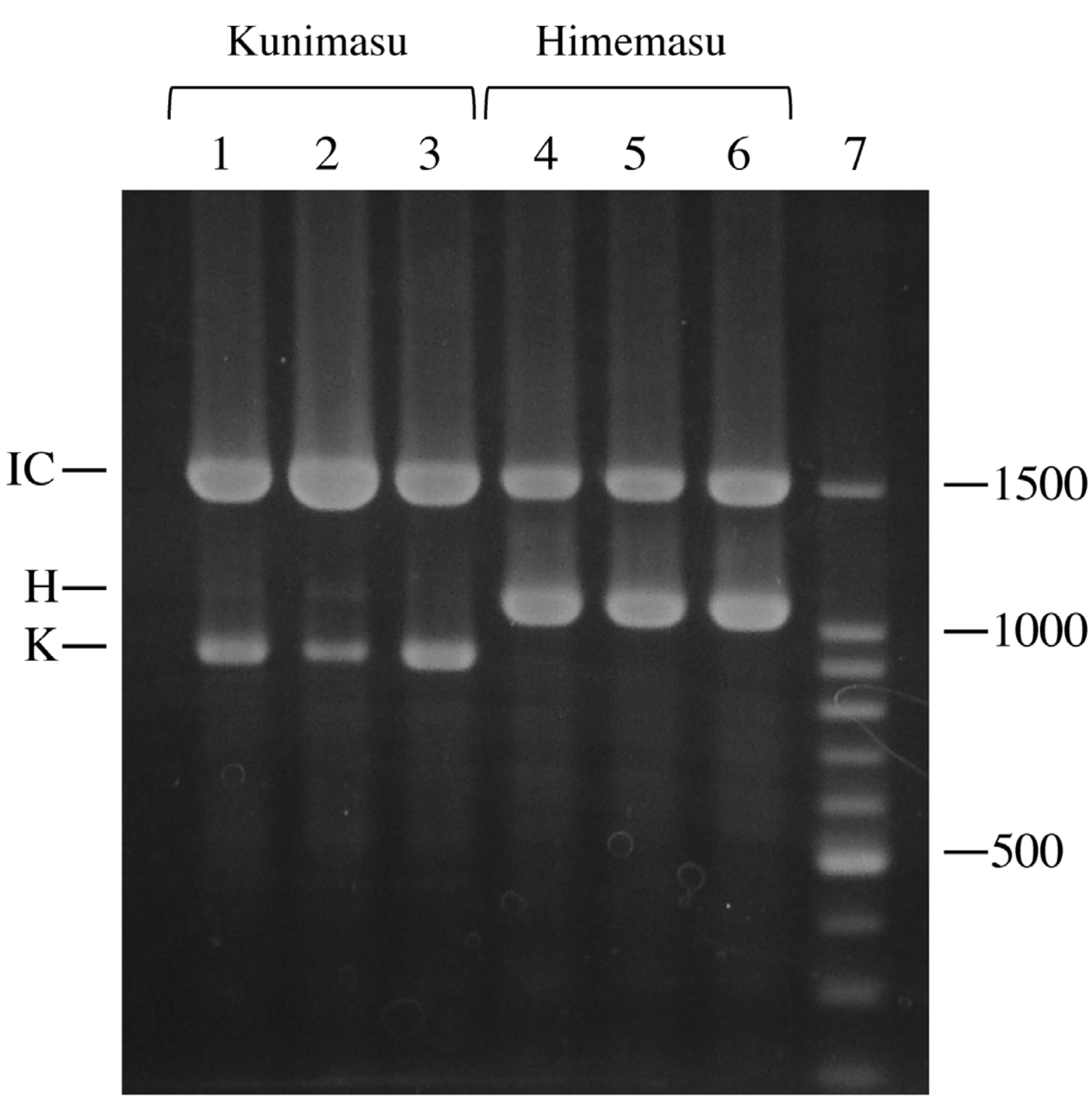

Fig. 2