Multiple colonizations of Lake Biwa by *Sarcocheilichthys* fishes and their population history

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Abstract Rapid adaptation and speciation have not been well documented for organisms in Lake Biwa, Japan, the oldest ancient lake in East Asia. To examine these processes, we focused on the divergence of *Sarcocheilichthys* gudgeons and conducted phylogeographic and population genetic analyses using mitochondrial DNA sequences and microsatellite data. No remarkable genetic differentiation was found between two species, *S. biwaensis* and *S. variegatus microoculus*, both endemic to Lake Biwa, whereas this species group, including *S. v. variegatus*, was genetically divided into three major regional groups: the Lake Biwa, Ise Bay, and western groups. Divergent mtDNA haplotypes were included within the Lake Biwa and western groups, strongly suggesting secondary contacts among allopatrically isolated populations. Dating for mtDNA phylogeny using a geological constraint suggested the colonization of Lake Biwa by multiple *Sarcocheilichthys* lineages that diverged from each other in the early–middle Pleistocene. Coalescent-based population analyses indicated that the local populations colonized the rocky bottom habitat in Lake Biwa from other habitats after the Last Glacial Maximum, likely reflecting past environmental changes in the lake, including the disappearance of rocky areas during the glacial climate. Divergent morphological adaptation in *Sarcocheilichthys* associated with substrate type may have rapidly proceeded along with environmental changes.

Keywords Ancient lake · Central Japan · Gobioninae · Historical demography · Phylogeography · Trophic polymorphism

Introduction

Organisms that colonized novel environments often show ecological divergence in resource use, accompanied by morphological and physiological adaptations (Schluter 2000; Losos 2010). Such adaptation phenomena are important to understanding the roles of natural selection in species diversification. Physically isolated environments such as oceanic islands and lakes provide robust geographical systems to study adaptive evolution and speciation (Schluter 2000; Losos and Ricklefs 2009). For example, animal assemblages in ancient lakes such as the African Great Lakes and Lake Baikal in Siberia have been studied as typical cases of adaptive radiation, which involve long geological history, diversified environmental settings, and isolation from neighboring habitats to various extents (Martens 1997; Cristescu et al. 2010). On the other hand, rapid adaptive evolution and speciation, such as following the colonization of post-glacial lakes, are also important processes to elucidate the role of divergent natural selection in species diversification (e.g., Schluter 1998; Hudson et al. 2011). Such colonization–adaptation processes as those observed in post-glacial lakes may have repeatedly occurred along with environmental changes during historical radiation in ancient lakes. To understand the patterns of colonization–adaptation processes over a wide time scale and the formation of resultant endemic assemblages, phylogenetic and phylogeographic information of constituent species must be accumulated and compared.

Lake Biwa is an ancient lake in East Asia, located on central Honshu Island, Japan (35°20′N, 136°10′E, 84.4 m above sea level). It is the largest lake in Japan in terms of surface area (670.3 km^2) and volume (27.5 km^3) and is also the oldest lake in East Asia, dating to 4 million years ago (Mya) (Yokoyama 1984; Kawabe 1994). Similar to other ancient lakes, Lake Biwa harbors many animal and plant species (>1,000), including about 70 fish species/subspecies (Nishino 2003; Nishino and Hamabata 2005). Not surprisingly, the lake has been recognized as a focal research area for biodiversity and speciation of freshwater

organisms in Japan (Kawanabe 1978, 1996; Rossiter 2000). However, the number (~60) and proportion (<10%) of endemic species/subspecies are not very large when compared to levels of endemism in other ancient lakes throughout the world (e.g., >600 species in Lake Tanganyika; ~1,000 species in Lake Baikal; Martens 1997). Sixteen endemic/semi-endemic fish species (~24% of total) belonging to 12 genera of six families occur in Lake Biwa; this phylogenetic diversity also characterizes the fish assemblage in this lake (Watanabe, in press).

The organisms endemic to Lake Biwa generally exhibit distinct adaptations to the particular environments of the lake (e.g., the extensive pelagic area, deep zone, or rocky substrate), which started to be formed in the mid-Pleistocene (~0.4 Mya; "the present Lake Biwa"; Yokoyama 1984; Kawabe 1989, 1994; Meyers et al. 1993). The endemic organisms are often divided into two categories: species that evolved in the lake and relic species (Kawanabe 1978, 1996). Among these, the former are presumed to have differentiated from their ancestors through ecological adaptation after the formation of the present lake environments (e.g., Tomoda 1978; Takahashi 1989). However, recent molecular phylogenetic studies have revealed that several endemic cyprinids and a goby, that have typically been considered adapted to the lake, were derived from their closest relatives much earlier (in the Pliocene–early Pleistocene) than the formation of the present environments (Harada et al. 2002; Takada et al. 2010; Kakioka et al. 2013; Tabata and Watanabe 2013). In these cases, the ecological and morphological adaptations are inferred to have occurred much more recently than the divergence of the endemic lineages. In contrast, some endemic forms occur that exhibit adaptive ecological/morphological divergences without distinct genetic differentiation from their relatives living in or around the lake. Representatives of such species are the gudgeons of the genus *Sarcocheilichthys* (Cyprinidae: Cypriniformes), which exhibit trophic and body color polymorphisms (Komiya et al. 2011).

Sarcocheilichthys variegatus microoculus and *Sarcocheilichthys biwaensis* are endemic to Lake Biwa and are presumed to have evolved from the ancestral riverine species *S.*

variegatus variegatus (distributed widely in western Japan except Lake Biwa) via divergent adaptation to various local environments of the lake (Hosoya 1982). Trophic polymorphisms in their head and mouth shape clearly correspond to variation in the bottom environment (pebbly vs. rocky). Although not enough data are available, some genetic variation possibly contributes to their morphological variation (Nakamura 1969; Komiya et al., unpublished data). However, genetic analysis using presumably neutral markers (microsatellites) has not revealed any significant differentiation among local populations within or between the species in Lake Biwa (Komiya et al. 2011). Then, this case may represent an example of rapid adaptation and speciation in Lake Biwa, which have not yet been documented for organisms in this lake.

To estimate the spatiotemporal pattern of adaptive divergence of the *Sarcocheilichthys* fishes in Lake Biwa, their history of colonization and demography in Lake Biwa must be characterized. For this purpose, we conducted phylogeographic and population demographic analyses of population samples collected from the entire distribution of *Sarcocheilichthys* species in Japan using mitochondrial DNA and microsatellite markers. Our results suggest multiple colonization events of Lake Biwa by these species as well as their recent settlement and adaptation to a novel environment. On the basis of the estimated history of this group, the origins of the endemic species and their adaptation in Lake Biwa are discussed.

Materials and methods

Specimen collection

Fish samples of *Sarcocheilichthys variegatus variegatus*, *S. v. microoculus*, and *S. biwaensis* were collected from 28 localities throughout the entire range of these species in Japan (Fig. 1;

126 Table 1). Before selecting the local samples outside the Lake Biwa basin, we excluded
127 locations that included specimens obviously identified as *S. v. microoculus* by morphological
128 characteristics (Hosoya 1982), as this endemic Lake Biwa subspecies has been introduced
129 into several regions contaminated with fishery stocking of Ayu, *Plecoglossus altivelis*, from
130 Lake Biwa (e.g., Kurita et al. 2012; Watanabe 2012).
131 The localities were geographically grouped into five regions: Lake Biwa, Ise Bay, Kinki,
132 Chugoku, and Kyushu (Table 1). Almost all specimens were from wild populations, collected
133 by gill-net and dip-net, or purchased from local fishermen, with the exception of one local
134 sample of *S. biwaensis* (locality code #14; part), which was obtained from a captive
135 population that has been kept in the Lake Biwa Museum, Shiga, Japan, since 1995 or later
136 (Table 1). The bottom environments of the sampling sites in Lake Biwa were categorized into
137 rocky or pebbly (Fig. 1; Nishino 1991). Specimens from Lake Biwa include those used by
138 Komiya et al. (2011).
139
140 MtDNA sequencing and analyses
141
142 A total of 344 specimens from 26 localities, including 249 specimens from 15 sites in/around
143 Lake Biwa, were used for the mtDNA analysis (Table 1). Total genomic DNA was isolated
144 from fin clips preserved in 100 % ethanol using a Genomic DNA Purification Kit (Promega,
145 Madison, WI, USA). Polymerase chain reaction (PCR) amplification was performed using the
146 primer pair L14724 (5′-TGA CTT GAA RAA CCA YCG YYG-3′) (Palumbi et al. 1991) and
147 H15915 (5′-ACC TCC GAT CTY CGG ATT ACA AGA C-3′) (Aoyama et al. 2000) to
148 obtain the nucleotide sequences of the 3′-half of the mitochondrial cytochrome *b* gene (cyt*b*;
149 620 base pairs, bp). The PCR conditions and sequencing method were the same as those
150 described by Komiya et al. (2011). The obtained sequences were deposited in the DNA Data
151 Bank of Japan (DDBJ), GenBank, and European Nucleotide Archive (EMBL) (accession

152 numbers AB601449–601470, AB780978–780997). The haplotype frequencies of each
153 population were deposited in the Genetic Diversity and Distribution Map (GEDIMAP)
154 freshwater fish database (http://gedimap.zool.kyoto-u.ac.jp; Watanabe et al. 2010a) with
155 population IDs P1279–1290, P1292–1296, and P1691–1705.

156 Genetic diversity indices, i.e., the number of haplotypes, haplotype diversity, and
157 nucleotide diversity, were calculated for each region group using ARLEQUIN v3.5 (Excoffier
158 and Lischer 2010). Haplotype richness, an analogue to allelic richness, was also calculated
159 using the rarefaction method (Kalinowski 2004). Genetic differentiation among local samples
160 was assessed by pairwise F_{ST}, calculated for samples with ≥8 specimens using ARLEQUIN.
161 The significance level (0.05) for multiple comparisons was corrected using the sequential
162 Bonferroni method (Holm 1979; Rice 1989). A statistical parsimony network was constructed
163 using TCS v1.2.1 (Clement et al. 2000) at a 90 % confidence limit for the mtDNA haplotypes
164 obtained.

165 A Bayesian approach was used to estimate the phylogenetic tree and the divergence
166 times of lineages using the GTR+I model, selected by the Bayesian information criterion
167 (BIC) in jModeltest v2.1.1 (Darriba et al. 2012), and the Yule (speciation) tree prior using
168 BEAST v1.7.4 (Drummond and Rambaut 2007). Referring to the gobionine phylogeny
169 proposed by Tang et al. (2011), the following four outgroup sequences were added to the
170 analysis: *Sarcocheilichthys kiangsiensis* (AY952984; Yang et al. 2006), *S. nigripinnis*
171 (JN003319; Tang et al. 2011), *S. soldatovi* (JN003337; Tang et al. 2011), and *S. czerskii*
172 (EF193433; Zhang et al. 2008). We adopted the random local clock model, which assumes
173 one or more independent rates on different branches (Drummond and Suchard 2010). To
174 estimate the time of the most recent common ancestors (tMRCA), a geological constraint, i.e.,
175 the uplift of the Suzuka Mountains in central Honshu Island 1.0–1.5 Mya (Yokoyama 1988;
176 Kawabe 1994), was applied for the relevant node age. The constraint was specified as a
177 lognormal prior distribution, ranging from approximately 1.1 to 1.5 Mya in the 95% range

178 with mean = 1.3 Mya, log(SD) = 0.1, and offset = 0. All other model parameters used default
179 priors. For each Markov-chain Monte Carlo (MCMC) analysis, we performed two
180 independent runs of 50 million generations. We sampled every 1000th generation and
181 removed 10 % of the initial samples as burn-in. The convergence of the chains to the
182 stationary distribution and large effective sample size (ESS; >200) were confirmed using
183 TRACER v1.5 (Rambaut and Drummond 2009). The consensus tree was calculated using
184 LogCombiner v1.6.2 and TreeAnnotator v1.6.2 in the BEAST package, and the tree was
185 visualized using FigTree v1.3.1 (Rambaut 2009).
186 To estimate the demographic history of *Sarcocheilichthys* fishes in Lake Biwa, we
187 applied a Bayesian skyline plot (BSP) analysis (Drummond et al. 2005) for the mtDNA data,
188 implemented in BEAST. All Lake Biwa specimen data were combined (n = 242), but distinct
189 haplotype groups were analyzed separately or in combination. We performed two independent
190 runs with an MCMC chain length of 50 or 500 million generations, and their convergence to
191 the stationary distribution and large ESS (>200) were confirmed using TRACER. We
192 sampled every 1000th generation and removed 10% of the initial samples as burn-in. The
193 substitution model was selected using the BIC in jModeltest. The time to expansion was
194 estimated using the mutation rate obtained in the Bayesian phylogenetic analysis similar to
195 the above analysis but using only ingroup haplotypes [normal prior distribution, mean =
196 0.0122/million years (Myr), log (SD) = 0.0032, covering 0.0070–0.0175/Myr in the 95%
197 range; see "Results"]. The BSP results with the stepwise (constant) model were summarized
198 using Tracer.
199 Demographic parameters for pooled and respective local samples were also estimated
200 based on mismatch distributions of pairwise differences (Rogers and Harpending 1992). The
201 fitness of the observed data to a model of either sudden population expansion (Rogers and
202 Harpending 1992) or spatial expansion (Schneider and Excoffier 1999) was tested using
203 ARLEQUIN. We conducted neutrality tests by calculating Tajima's *D* (Tajima 1989) and

204 Fu's F_S (Fu 1997) to explore the demographic change, using ARLEQUIN. The significance
205 for all the estimates was tested by 10,000 permutations.
206
207 Microsatellite analyses
208
209 A total of 254 specimens from 15 localities, including 153 *S. v. microoculus* and 28 *S.*
210 *biwaensis* specimens from 10 sites in/around Lake Biwa and 73 *S. v. variegatus* from 5 sites
211 in western Japan, were used for the microsatellite analysis (Table 1). A total of 14
212 microsatellite loci isolated from *S. v. microoculus* (Fujita et al. 2008) were analyzed,
213 including 13 dinucleotide repeats (Svm03, Svm10, Svm32, Svm34, Svm46, Svm48, Svm49,
214 Svm50, Svm53, Svm56, Svm72, Svm82, and Svm166) and one trinucleotide repeat (Svm51).
215 PCR conditions for each locus were described by Fujita et al. (2008). PCR products were
216 sized on an automated DNA sequencer (ABI Prism GA310; Applied Biosystems, Foster City,
217 CA, USA) using GeneScan v3.1 and ROX400HD as the size standard (Applied Biosystems).
218 The mean observed (H_O) and expected (H_E) heterozygosities of the microsatellite data
219 and their deviation from Hardy–Weinberg equilibrium were analyzed using ARLEQUIN
220 [Electronic supplementary material (ESM) Table S1]. The pairwise F_{ST} was calculated to test
221 the differentiation among local samples using ARLEQUIN. A Bayesian clustering approach,
222 implemented in STRUCTURE v2.3.4 (Pritchard et al. 2000), was used to estimate the
223 population structure. Ten replicates for each predefined K = 1–16 were obtained with runs of
224 100,000 burn-in and 1,000,000 MCMC iterations, applying the admixture model with
225 correlated alleles. To determine the most likely value of K, the method of Evanno et al. (2005)
226 was implemented in the program STRUCTURE HARVESTER (Earl and vonHoldt 2012),
227 which determines the second-order rate of change in the distribution of L(K).
228 The migration and demographic histories of the *Sarcocheilichthys* fishes within Lake
229 Biwa were estimated using Migrate-n v3.3.2 (Beerli and Felsenstein 1999, 2001). To estimate

230 pairwise migration rates among 12 site/species samples and the tMRCA of each sample, we
231 employed a Brownian motion approximation of the stepwise mutation model. The mutation
232 rates were treated as variable among loci, and pairwise geographic distances among sites (as
233 coastal distances) were given for the analysis (ESM Table S2). Two independent runs
234 (1,000,000 burn-in steps followed by 5,000,000 steps sampled every 100 steps) were
235 conducted.
236
237
238 **Results**
239
240 Population structure and divergence time
241
242 In total, 42 mtDNA haplotypes were detected from 344 specimens of the Japanese
243 *Sarcocheilichthys* fishes, and of these, 24 haplotypes were obtained from Lake Biwa (Table 2;
244 Fig. 2). The haplotype tree revealed five major clades (A–E) (Fig. 3). Three of these (A, B,
245 and C) were found in Lake Biwa, resulting in moderately high genetic diversity (Table 2; Figs.
246 2, 3). Clade A was dominant, with 71 % of individuals (89 % in Lake Biwa) possessing
247 haplotypes in this clade. Clade A haplotypes were shared by *S. variegatus* and *S. biwaensis*
248 and were represented by a star-like network with haplotype A01 as the central and dominant
249 (73%) haplotype (Fig. 2). A subclade of clade A, consisting of A15 and A16, and a tip
250 haplotype, A18, were detected exclusively from the Chugoku region. Clade B haplotypes
251 were obtained from the Lake Biwa, Kinki, and Chugoku regions, but showed some regional
252 distribution patterns. The most interior haplotype of clade C (C03) was exclusively found in
253 Kyushu, but the tip haplotypes were shared by specimens from the Lake Biwa, Kinki, and
254 Chugoku regions. Clade D was endemic to the Yura River system (Kinki), and clade E was
255 the earliest divergent group and was endemic to the Ise Bay area (Figs. 2, 3).

256 The divergence times among the five major mtDNA clades (A–E) were estimated to be
257 0.62–1.26 Myr [lower and upper limits of 95 % highest probability density (HPD), 0.29–1.51
258 Myr; Table 3; Fig. 3]. The three clades occurring in Lake Biwa (A, B, and C) were suggested
259 to have differentiated from each other (tMRCA, 0.75–0.81 Myr, 95 % HPD, 0.38–1.29 Myr)
260 before the formation of the present Lake Biwa environments (~0.4 Mya).
261 In terms of F_{ST}, almost all sample pairs from different regions showed significant
262 differentiation for both mtDNA and microsatellites, but almost no differentiation was found
263 between *S. v. microoculus* and *S. biwaensis* (all 15 comparisons in mtDNA and 18 of 20
264 comparisons in microsatellites; ESM Table S3). In the microsatellite data, the assignment
265 analysis supported the division into three population groups (the maximum ΔK = 19.03 at K =
266 3), basically corresponding to the regional samples from the Lake Biwa, Ise Bay, and western
267 populations including the Kinki, Chugoku, and Kyushu samples (Fig. 4). Again, *S. v.*
268 *microoculus* and *S. biwaensis* were clustered into the single "Lake Biwa" population. Some of
269 the Yura River specimens were assigned to the Lake Biwa population, and this sample
270 included three loci exhibiting deviation from Hardy–Weinberg equilibrium (ESM Table S1);
271 they may be attributable to artificial introductions.
272
273 Population demography and migration in Lake Biwa
274
275 The mutation rate of the mtDNA cyt*b* segment was estimated at 0.0122/Myr/lineage (95 %
276 HPD, 0.0069–0.0180) by the Bayesian phylogenetic analysis using only the Japanese
277 *Sarcocheilichthys* data. Using this mutation rate and its approximate credibility range (see
278 "Materials and methods"), the demographic history of the Lake Biwa population was
279 estimated using the BSP analyses for all mtDNA haplotypes and respective haplotype clades
280 (Fig. 5). The BSP results indicated that the recent population expansion started 0.05 Mya or
281 later (i.e., during the last glacial period). This expansion pattern was obvious in the analyses

282 using all haplotypes and only the clade A haplotypes. This was also supported by neutrality
283 tests using Tajima's D and Fu's F_S (all $P < 0.05$), and partially supported by mismatch
284 distribution analysis (departure from the sudden expansion model or spatial expansion model,
285 $P > 0.05$; ESM Table S4).
286 The historical migration rates estimated by microsatellite data showed asymmetric
287 patterns among sites (Fig. 6a). The tendency of unidirectional immigration to rocky bottom
288 areas (Loc. #7 and 14) was remarkable (Fig. 6b). The tMRCAs of the rocky local populations
289 (average, 0.67/mutation/generation), with the exception of *S. v. microoculus* in Loc. #13, were
290 clearly shorter (0.1–0.4 times, 0.26 ± 0.13 SD times on average) than those of the other
291 populations (2.61 ± 0.06/mutation/generation; Fig. 6c).
292
293
294 **Discussion**
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296 Population structure of the *Sarcocheilichthys* fishes and multiple colonizations of Lake Biwa
297
298 Our microsatellite data revealed the regional population structure of the Japanese
299 *Sarcocheilichthys* fishes, and these results were also supported by mtDNA data. In particular,
300 the mtDNA tree showed that the fishes are differentiated into two major regional groups that
301 are divided by the Suzuka Mountains. These findings suggest that these two groups have been
302 isolated since the mountain system was formed 1.0–1.5 Mya (Yokoyama 1988; Kawabe
303 1994), and the western group expanding from Lake Biwa to Kyushu Island experienced gene
304 flow within this time scale. Considering that the western group includes two species (*S.*
305 *biwaensis* and *S. variegatus*) with two subspecies (*S. v. variegatus* and *S. v. microoculus*)
306 (Hosoya 1982, 2001), the unique population group in the Ise Bay area, east of the Suzuka
307 Mountains, could be treated as a unique taxonomic and conservation unit. Detailed

308 morphological/taxonomic reexaminations will be necessary.

309 In the area west of the Suzuka Mountains, except for Kyushu, largely diverged mtDNA
310 haplotypes were detected within regional population groups or even within local samples,
311 which were represented as single genetic groups by clustering analysis with microsatellite
312 data. Some exceptional specimens in the Yura River sample fully or partially exhibited
313 genetic characteristics of the Lake Biwa specimens in both microsatellite and mtDNA data.
314 This pattern would be attributable to an artificial introduction because Lake Biwa fishes
315 including *S. v. microoculus* are well known to have been introduced into several regions,
316 admixed with fishery stocking of Ayu from Lake Biwa (e.g., Kurita et al. 2012; Watanabe
317 2012). In the other localities, however, the occurrence of diverged mtDNA suggests historical
318 secondary contacts among differentiated populations through multiple dispersal events by, for
319 example, the connections of river systems (Avise 2000).

320 In samples from Lake Biwa, divergent mtDNA haplotypes from three distinct clades
321 were detected, each exhibiting moderate genetic diversity. Even taking the large credibility
322 intervals into consideration, it is highly probable that the divergence among the three clades
323 (A, B, and C; 95 % HPD, 0.38–1.29 Myr), at least that of C from the others (0.46–1.29 Myr),
324 preceded the formation of the present environments of the northern Lake Biwa basin, which
325 harbors a large, deep pelagic zone and rocky shore/bottom (~0.4 Mya). Considering the
326 heterogeneous distribution patterns of the mtDNA clades together, these results strongly
327 suggest multiple colonization events of Lake Biwa by the *Sarcocheilichthys* fishes, following
328 allopatric population differentiation.

329 Common or closely related haplotypes occurring in both Lake Biwa and the
330 Kinki–Chugoku regions around the Seto Inland Sea suggest some historical gene flow among
331 these regions. Such gene flow would be possible, at least partially, during marine regression
332 periods when river systems in these regions would have interconnected owing to desiccation
333 of the Seto Inland Sea (31 m water depth on average at present; Yonekura et al. 2001).

334 Regional freshwater fish fauna (Tsubokawa 1988; Watanabe 2012) and phylogeographic
335 patterns of some other fish species also support such riverine connections; i.e., the
336 populations around Seto Inland Sea share close mtDNA haplotypes, for example, in Medaka
337 *Oryzias latipes*, the cyprinid *Biwia zezera* and *Gnathopogon elongatus*, respectively
338 (Takehana et al. 2003; Watanabe et al. 2010b; Kakioka et al. 2013). The observed genetic
339 diversity pattern in the *Sarcocheilichthys* fishes reflects such a geographical history associated
340 with the formation processes of freshwater fauna in western Japan. Lake Biwa would have
341 served as a pool accepting colonizers at multiple time scales and preserving their genetic
342 diversity, and also as a source for dispersal to neighboring areas.
343
344 Colonization and demographic history in Lake Biwa and the origin of adaptation
345
346 The *Sarcocheilichthys* fishes in Lake Biwa exhibit remarkable, continuous variation in head
347 shape from short to long (Nakamura 1969; Hosoya 1982; Tomoda 1991). As in other fishes
348 (Smith and Skúlason 1996), the head shape divergence is associated with diverse habitats and
349 feeding habits in this group and is thus considered a trophic polymorphism (Komiya et al.
350 2011, unpublished data). *Sarcocheilichthys variegatus microoculus* includes short,
351 intermediate, and long head types, which occur in sandy, pebbly, and rocky bottom habitats,
352 respectively. *Sarcocheilichthys biwaensis* is the long head extreme form inhabiting rocky
353 bottom areas (Hosoya 1982; Tomoda 1991; Komiya et al. 2011). The longer headed
354 individuals from rocky areas are equipped with a narrow head, elongated mouth, large jaw,
355 and large, anteriorly positioned eyes. In addition, they have a deep and laterally compressed
356 body. All of these traits are considered to be advantageous for handling attached and/or
357 cryptic prey in crevices in the rocky bottom or floating in open water (Komiya et al. 2011;
358 unpublished data). Because the rocky bottom habitat is unique within the species' range and is
359 restricted even in Lake Biwa, the long head type of *S. v. microoculus* and *S. biwaensis* was

360 very likely derived from ancestral shorter head forms.

361 The head shape divergence could be caused by phenotypic plasticity, genetic
362 differences, or a combination of both. Despite the remarkable morphological differences,
363 there was no distinct genetic differentiation between the short and long head samples or even
364 between *S. v. microoculus* and *S. biwaensis* in presumably neutral genetic markers (Komiya et
365 al. 2011; present study). However, Nakamura's (1969) brief description and our unpublished
366 result of experimental breeding suggest that the head shape variation in this group is
367 genetically controlled to some extent. These facts imply a short history of divergence and/or
368 partial gene flow among these forms and species.

369 The more detailed, coalescent-based analysis revealed intriguing patterns of migration
370 and establishment of the local *Sarcocheilichthys* populations within Lake Biwa. The detected
371 unidirectional immigration patterns to the rocky bottom areas were consistent with the
372 hypothesis of the recent establishment of local populations in the rocky habitat. Their shorter
373 estimated tMRCAs also support this idea. Geological and paleoclimatic studies have indicated
374 that the water level of the lake was lowered (about –30 m) and the rocky bottom environment
375 almost disappeared during the last glacial period, which was mainly associated with low
376 precipitation (Uemura and Yokoyama 1983; Ishida et al. 1984). The absolute ages of the
377 tMRCAs are difficult to estimate because of the uncertainty associated with the mutation rates
378 of our microsatellite markers. However, if the population expansion of the entire Lake Biwa
379 population occurred about 0.05 Mya or later, as estimated by the mtDNA BSP analysis, the
380 rocky habitat populations, with tMRCAs one-fourth shorter than the others, may have
381 established later than 0.02 Mya, i.e., after the Last Glacial Maximum (LGM; ~0.02 Mya).
382 This pattern implies that the rocky *S. v. microoculus* and *S. biwaensis* populations proceeded
383 to colonize and establish in re-emerged rocky environments as temperatures rose and
384 precipitation increased after the LGM. The shortest tMRCA for the *S. biwaensis* population in
385 Loc. #14, however, could be a result of a founder effect and/or bottleneck in this captive

population. On the other hand, the larger tMRCA of the rocky *S. v. microoculus* around Okishima Island (Loc. #13) may be because the rocky environment around the island appears to have exceptionally remained even during the last glacial period owing to the position and geology of the island (Uemura and Yokoyama 1983; Ishida et al. 1984).

The morphological characteristics of the rocky populations are inferred to be strongly linked to the rocky bottom environments as mentioned above. Thus, such traits are supposed to have adaptively evolved along with colonization of the rocky areas, probably after the LGM. This also suggests the rapid speciation of *S. biwaensis* from an ancestral *S. v. microoculus* form. These patterns support the hypothesis that divergent adaptive evolution has been recurrently occurring even in the fish assemblage in Lake Biwa in the absence of explosive adaptive radiation. To prove their rapid evolution and speciation, the genetic contribution to the adaptive traits and the intensity of reproductive isolation between the two species must be examined in detail.

In contrast with the cases of several Lake Biwa endemics whose origins are inferred to precede the formation of the present Lake Biwa environments (~0.4 Mya; e.g., Kakioka et al. 2013; Tabata and Watanabe 2013), the probable rapid adaptation/speciation inferred for the *Sarcocheilichthys* fishes is a rare known case in Lake Biwa and represents an ideal candidate for the study of divergent adaptation in Lake Biwa. Their multiple colonization history over several hundred thousand years implies an accumulation of genetic variation in their genome for adapting to the various environmental conditions that appeared in the Paleo-Lake Biwa. Such standing variation may have played an important role in the rapid adaptation of this group. Recently developed genomic methods (see, e.g., Gilad et al. 2009; Schoville et al. 2012) will be applied to further elucidate the genetic basis and evolutionary dynamics of the flexible adaptation of these fishes, including their trophic polymorphisms.

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References

Aoyama J, Watanabe S, Ishikawa S, Nishida M, Tsukamoto K (2000) Are morphological characters distinctive enough to discriminate between two species of freshwater eels, *Anguilla celebesensis* and *A. interioris*? Ichthyol Res 47:157–161

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge

Beerli P, Felsenstein J (1999) Maximum-likelihood estimation of migration rates and effective population numbers in two populations using a coalescent approach. Genetics 152:763–773

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. Proc Nat

438 Acad Sci USA 98:4563–4568
439 Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene
440 genealogies. Mol Ecol 9:1657–1659
441 Cristescu ME, Adamowicz SJ, Vaillant JJ, Haffner DG (2010) Ancient lakes revisited: from
442 the ecology to the genetics of speciation. Mol Ecol 19:4837–4851
443 Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new
444 heuristics and parallel computing. Nat Methods 9:772
445 Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees.
446 BMC Evol Biol 7:214
447 Drummond AJ, Suchard MA (2010) Bayesian random local clocks, or one rate to rule them
448 all. BMC Biol 8:114
449 Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of
450 past population dynamics from molecular sequences. Mol Biol Evol 22:1185–1192
451 Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for
452 visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet
453 Resour 4:359–361
454 Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using
455 the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620
456 Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform
457 population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567
458 Fu YX (1997) Statistical tests of neutrality of mutations against population growth,
459 hitchhiking, and background selection. Genetics 147:915–925
460 Fujita S, Komiya T, Takeshima H, Watanabe K, Nishida M (2008) Isolation and
461 characterization of 15 microsatellite loci for the Japanese gudgeon *Sarcocheilichthys*
462 *variegatus*. Mol Ecol Resour 8:1335–1337
463 Gilad Y, Pritchard JK, Thornton K (2009) Characterizing natural variation using

464 next-generation sequencing technologies. Trends Genet 25:463–471
465 Harada S, Jeon SR, Kinoshita I, Tanaka M, Nishida M (2002) Phylogenetic relationships of
466 four species of floating gobies (*Gymnogobius*) as inferred from partial mitochondrial
467 cytochrome *b* gene sequences. Ichthyol Res 49:324–332
468 Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Statist
469 6:65–70
470 Hosoya K (1982) Classification of the cyprinid genus *Sarcocheilichthys* from Japan, with
471 description of a new species. Jpn J Ichthyol 29:127–138
472 Hosoya K (2001) Higai, genus *Sarcocheilichthys*. In: Kawanabe H, Mizuno N, Hosoya K
473 (eds) Freshwater fishes of Japan. Yama-Kei Publishers, Tokyo, pp 310–313 (in Japanese)
474 Hudson AG, Vonlanthen P, Seehausen O (2011) Rapid parallel adaptive radiations from a
475 single hybridogenic ancestral population. Proc R Soc B:278:58–66
476 Ishida S., Kawada K, Miyamura M (1984) Geology of the Hikone-seibu district. Chiiki
477 chishitsu kenkyu hokoku, scale 1:50,000 Kyoto (11), no 17, Chishitsu Chosajo (in
478 Japanese with English summary)
479 Kakioka R, Kokita T, Tabata R, Mori S, Watanabe K (2013) The origins of limnetic forms
480 and cryptic divergence in *Gnathopogon* fishes (Cyprinidae) in Japan. Environ Biol Fish
481 96:631–644
482 Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical
483 sampling designs. Conserv Genet 5:539–543
484 Kawabe T (1989) Stratigraphy of the lower part of the Kobiwako group around the Ueno
485 Basin, Kinki District, Japan. J Geosci, Osaka City Univ 32:39–52
486 Kawabe T (1994) Chapter 1. Biwako no Oitachi (formation of Lake Biwa). In: Research
487 Group for Natural History of Lake Biwa (ed) Biwako no Shizenshi (The natural history
488 of Lake Biwa). Yasaka Shobo, Tokyo, pp 24–72 (in Japanese)
489 Kawanabe H (1978) Some biological problems. Verh Internat Ver Limnol 20:2674–2677

Kawanabe H (1996) Asian great lakes, especially Lake Biwa. Environ Biol Fish 47:219–234

Komiya T, Fujita S, Watanabe K (2011) A novel resource polymorphism in fish, driven by differential bottom environments: an example from an ancient lake in Japan. PLoS One 6:e17430

Kurita Y, Nakajima J, Inui R (2012) Host mussels of the introduced Lake Biwa endemic, *Sarcocheilichthys variegatus microoculus* (Cyprinidae), in northern Kyushu Island, Japan. Jpn J Ichthyol 59:95–97 (in Japanese)

Losos JB (2010) Adaptive radiation, ecological opportunity, and evolutionary determinism. Am Nat 175:623–639

Losos JB, Ricklefs RE (2009) Adaptation and diversification on islands. Nature 457:830–836

Martens K (1997) Speciation in ancient lakes. Trends Ecol Evol 12:177–182

Meyers PA, Takemura K, Horie S (1993) Reinterpretation of late Quaternary sediment chronology of Lake Biwa, Japan, from correlation with marine glacial–interglacial cycles. Quatern Res 39:154–162

Nakamura M (1969) Cyprinid fishes of Japan: studies on the life history of cyprinid fishes of Japan. Research Institute for Natural Resources, Tokyo (in Japanese).

Nishino M (1991) Regional division of the lake shore based on benthic animals. In: Lake Biwa Environmental Research Institute (ed) Project Report (No 90-A05): Landscape-ecological division of the Lake Biwa shore. Lake Biwa Environmental Research Institute, Otsu, pp 47–63 (in Japanese)

Nishino M (2003) Biwako no Koyushu wo Meguru Mondai. Koyushu list no Ichibu Shusei nit suite (The problem of endemics in Lake Biwa). Oumia 76:3–4 (in Japanese)

Nishino M, Hamabata E (2005) Naiko karano Message [The message from “Naiko (lagoon around Lake Biwa)”]. Sun-rise Press, Shiga (in Japanese)

Palumbi S, Martin A, Romano S, McMillian WO, Stice, et al. (1991) The simple fool’s guide to PCR. Hawaii University Press, Honolulu

516 Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using
517 multilocus genotype data. Genetics 155:945–959
518 Rambaut A (2009) FigTree Version 1.3.1. http://tree.bio. ed.ac.uk/software/figtree/
519 Rambaut A, Drummond AJ (2009) Tracer version 1.5. http://
520 tree.bio.ed.ac.uk/software/tracer/
521 Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225
522 Rogers AR, Harpending H (1992) Population growth makes waxes in the distribution of
523 pairwise genetic differences. Mol Biol Evol 9:552–569
524 Rossiter A (2000) Lake Biwa as a topical ancient Lake. Adv Ecol Res 31:571–598
525 Schluter D (1998) Ecological speciation in postglacial fishes. In: Grant PR (ed) Evolution on
526 islands. Oxford University Press, Oxford, pp 163–180
527 Schluter D (2000) Ecology of adaptive radiation. Oxford University Press, Oxford
528 Schneider S, Excoffier L (1999) Estimation of demographic parameters from the distribution
529 of pairwise differences when the mutation rates vary among sites: application to human
530 mitochondrial DNA. Genetics 152:1079–1089
531 Schoville SD, Bonin A, François O, Lobreaux S, Melodelima C, Manel S (2012) Adaptive
532 genetic variation on the landscape: methods and cases. Ann Rev Ecol Evol Syst 43:23–43
533 Smith TB, Skúlason S (1996) Evolutionary significance of resource polymorphisms in fishes,
534 amphibians, and birds. Annu Rev Ecol Syst 27:111–133
535 Tabata R, Watanabe K (2013) Hidden mitochondrial DNA divergence in the Lake Biwa
536 endemic goby *Gymnogobius isaza*: implications for its evolutionary history. Environ Biol
537 Fish 96:701–702
538 Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA
539 polymorphism. Genetics 123:585–595
540 Takada M, Tachihara K, Kon T, Yamamoto G, Iguchi K, Miya M, Nishida M (2010)
541 Biogeography and evolution of the *Carassius auratus*-complex in East Asia. BMC

Evolutionary Biology 10:7

Takahashi S (1988) A review of the origins of endemic species in Lake Biwa with special reference to the goby fish, *Chaenogobius isaza*. J Paleolimnol 1:279–292

Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2003) Geographic variation and diversity of the cytochrome *b* gene in Japanese wild populations of medaka, *Oryzias latipes*. Zool Sci 20:1279–1291

Tang KL, Agnew MK, Chen WJ, Vincent Hirt M, Raley ME, Sado T, Schneider LM, Yang L, Bart HL, He S, Liu H, Miya M, Saitoh K, Simons AM, Wood RM, Mayden RL (2011) Phylogeny of the gudgeons (Teleostei: Cyprinidae: Gobioninae). Mol Phylogenet Evol 61:103–124

Tomoda Y (1978) Biwako to Namazu (Lake Biwa and catfish). Chobunsha, Tokyo (in Japanese)

Tomoda Y (1991) Fish of Lake Biwa: endemic fish of Lake Biwa and fish remains collected from Paleo Lake Biwa (Kobiwako Group). In: the Editorial Committee of Natural History of Shiga Prefecture (ed) Natural history of Shiga Prefecture: a report of comprehensive academic study. Foundation of Nature Conservation of Shiga Prefecture, Otsu, pp 1399–1457 (in Japanese with English abstract)

Tsubokawa K (1988) Zoogeographical consideration on the freshwater fish fauna of the Okayama Region, Japan. Bull Kurashiki Mus Nat Hist 3:1–30 (in Japanese with English abstract)

Uemura Y, Yokoyama T (1983) Biwako no shizen—chikei to chisou · chishitsu (Nature of Lake Biwa—topography, stratigraphy, and geology). Bewako-henshu-iinkai (ed) Biwako sono shizen to shakai (Lake Biwa, its nature and society). Sun Bright Publ, Otsu, pp 39–52 (in Japanese)

Watanabe K (2012) Faunal structure of Japanese freshwater fishes and its artificial disturbance. Environ Biol Fish 94:533–547

Watanabe (in press) Origin and diversification of freshwater fishes in Lake Biwa. In: Okuda N (ed) Biodiversity in aquatic systems and environments: in Lake Biwa. Springer, Tokyo

Watanabe K, Kano Y, Takahashi T, Mukai T, Kakioka R, Tominaga K (2010a) GEDIMAP: a database of genetic diversity for Japanese freshwater fishes. Ichthyol Res 57:107–109

Watanabe K, Kawase S, Mukai T, Kakioka R, Miyazaki J-I, Hosoya K (2010b) Population divergence of *Biwia zezera* (Cyprinidae: Gobioninae) and the discovery of a cryptic species, based on mitochondrial and nuclear DNA sequence analyses. Zool Sci 27:647–655

Yang JQ, He SP, Freyhof J, Witte K, Liu HZ (2006) The phylogenetic relationships of the Gobioninae (Teleostei: Cyprinidae) inferred from mitochondrial cytochrome *b* gene sequences. Hydrobiologia 553:255–266

Yokoyama T (1984) Stratigraphy of the quaternary system around Lake Biwa and geohistory of the ancient Lake Biwa. In: Horie S (ed) Lake Biwa, monographiae biologicae (volume 54). Dr W Junk Publishers, Dordrecht, pp 43–128

Yokoyama T (1988) Seinan Nihon no Shizenshi (Natural history of southwestern Japan). Sanwa-shobo, Kyoto (in Japanese)

Yonekura N, Kaizuka S, Nogami M, Chinzai K (2001) Regional geomorphology of the Japanese Islands, vol 1 Introduction to Japanese geomorphology. University of Tokyo Press, Tokyo (in Japanese)

Zhang L, Tang QY, Liu HZ (2008) Phylogeny and speciation of the eastern Asian cyprinid genus *Sarcocheilichthys*. J Fish Biol 72:1122–1137

590 **Figure legends**

591

592 **Fig. 1** Sampling localities of *Sarcocheilichthys* fishes. *Numbers* correspond to those in Table
593 1. *Triangles*, *S. biwaensis*; *circles*, *S. variegatus microoculus*; *squares*, *S. v. variegatus*. *
594 Rocky bottom area, ** including both rocky and pebbly areas, and others pebbly areas

595

596 **Fig. 2** Statistical parsimony network for mtDNA cyt*b* haplotypes of the Japanese
597 *Sarcocheilichthys* fishes. The *areas of the circles* containing a haplotype number are
598 proportional to haplotype frequency (but shown as a half for A01). Haplotypes are separated
599 by colors that correspond to species and localities, as depicted in the pie graph. *Sb*, *S.*
600 *biwaensis*; *Svm*, *S. variegatus microoculus*; *Svv*, *S. variegatus variegatus.* The dashed line
601 indicates an alternative connection (loop). The *closed small circles* indicate unobserved
602 hypothetical haplotypes.

603

604 **Fig. 3** Bayesian phylogenetic tree of *Sarcocheilichthys* fishes based on partial mtDNA
605 cytochrome *b* sequences with the GTR + I model. The tree was dated using the random local
606 clock model with a node age constraint [*Cs* a lognormal prior distribution with mean = 1.3
607 Mya, log(SD) = 0.1, and offset = 0]. *Numbers at nodes* indicate Bayesian posterior
608 probabilities (values <80 % not shown). *Horizontal bars at nodes* show credibility intervals as
609 95% HPD. Species and locality where the haplotype was detected are shown as *filled squares*
610 on the right side of the tree. Abbreviations: *Sb*, *S. biwaensis*; *Svm*, *S. variegatus microoculus*;
611 *Svv*, *S. variegatus variegatus*; *IB*, Ise Bay; *KK*, Kinki; *CG*, Chugoku; *KS*, Kyushu. *Haplotype*
612 *codes* are the same as those in Fig. 2. *Node numbers shown in a circle* correspond to those in
613 Table 3

614

615 **Fig. 4** Bayesian assignment analysis of *Sarcocheilichthys* samples based on microsatellite

616 data using STRUCTURE (Pritchard et al. 2000). **a** Δ*K* following Evanno et al. (2005) as a
617 function of the number of assumed populations (*K* = 1–17). **b** Results of the assignment for *K*
618 = 3. *Each vertical bar* represents an individual partitioned into the three clusters defined by
619 STRUCTURE. Abbreviations: *Sb*, *S. biwaensis*; *Svm*, *S. variegatus microoculus*; *Svv*, *S.*
620 *variegatus variegatus*; *IB*, Ise Bay; *KK*, Kinki; *CG*, Chugoku; *KS*, Kyushu. *Sample codes*
621 correspond to those in Fig. 1 and Table 1
622
623 **Fig. 5** Bayesian skyline plots for *Sarcocheilichthys* fishes in Lake Biwa for all haplotypes
624 (*All*) and for each clade of haplotypes (*A*–*C*). The *central bold line* represents the mean value
625 for the relative effective female population size, and the *shaded area* denotes the 95 % HPD
626
627 **Fig. 6** The historical migration patterns and tMRCAs for local populations of
628 *Sarcocheilichthys* fishes in Lake Biwa estimated using Migrate-n (Beerli and Felsenstein
629 1999, 2001). *Sample codes* correspond to those in Fig. 1 and Table 1. *7b* and *14b* are *S.*
630 *biwaensis* samples; others are *S. variegatus microoculus*. * Rocky bottom area, ** including
631 both rocky and pebbly areas, and others pebbly areas. **a** Pairwise migration rate matrix [*M*, as
632 immigration rate/(mutation rate × km)]. Darker *squares* indicate higher migration rates. **b**
633 Major migration patterns among local populations. Only migration links to neighboring sites
634 are indicated. *Closed triangles*, *S. biwaensis*; *closed circles*, *S. v. microoculus*. **c** Mean
635 tMRCAs for local populations as scaled in mutation/generation. *Horizontal lines* indicate
636 standard deviation
637

Table 1 Localities and sample size of *Sarcocheilichthys* fishes analyzed

Locality code	Locality (bottom type)	Species	mtDNA	Microsatellite	Remarks[a]	GEDIMAP ID
Lake Biwa						
1	Off Kitakomatsu, Otsu, Shiga (P)	Svm	–	15	P8	
2	Yotsu River Mouth, Yotsugawa, Adogawa, Shiga (P)	Svm	10	–		P1691
3	Ado R. Mouth, Kitafunaki, Adogawa, Shiga (P)	Svm	21	16	P9	P1284
		Sb	1	–		P1294
4	Off Imazu, Shiga (P)	Svm	10	–		P1692
5	Off Momose, Chinai, Makino, Shiga (P)	Svm	21	16	P10	P1286
		Sb	2	–		P1293
6	Off Oura, Nishiazai, Shiga (P)	Svm	25	–	R2	P1281
7	Around off Onoe, Kohoku, Shiga (P&R)	Svm	26	32	P1, R1	P1279, 1280
		Sb	17	20	BN	P1295, 1296
8	Off Minamiham, Nagahama, Shiga (P)	Svm	12	–	P2	P1289
9	Tributary of Ane R., Bessho, Kohoku, Shiga (P)	Svm	12	–		P1693
10	Around Takeshima Island, Off Hikone, Shiga (P)	Svm	25	22	P3	P1287
11	Channel, Ryohama, Hikone, Shiga (P)	Svm	10	–		P1694
12	Off Notogawa, Notogawa, Shiga (P)	Svm	9	–	P4	P1283
13	Around Okishima Is., Off Oumihachiman, Shiga (R)	Svm	–	9	R3	
14	Off Mizugahama, Oumihachiman, Shiga (R)	Svm	11	16	R5	P1290
		Sb	7	8 [b]	BE	P1292
15	Off Chuzu, Yasu, Shiga (P)	Svm	10	9	P5	P1285
16	Off Moriyama, Shiga (P)	Svm	10	8	P6	P1282
17	Off Shimosakamoto, Otsu, Shiga (P)	Svm	10	10	P7	P1288
Ise Bay area						
18	Hijie R., Ibi R. s., Tado, Kuwana, Mie	Svv	3	–		P1695
19	Harai R., Kushida R. s., Matsusaka, Mie	Svv	19	15		P1696
Kinki						
20	Kizu R., Yodo R. s., Kyotanabe, Kyoto	Svv	4	–		P1697
21	Yura R., Ayabe, Kyoto	Svv	20	20		P1698
22	Muko R., Sasayama, Hyogo	Svv	10	11		P1699
23	Maruyama R., Toyooka, Hyogo	Svv	2	–		P1700
Chugoku						
24	Takahashi R., Kurashiki, Okayama	Svv	4	–		P1701
25	Ashida R., Fukuyama, Hiroshima	Svv	14	19		P1702
26	Hii R., Unnan, Shimane	Svv	3	–		P1703
Kyushu						
27	Naka R., Fukuoka, Fukuoka	Svv	8	8		P1704
28	Futatsu R., Yanagawa, Fukuoka	Svv	8	–		P1705

Bottom type for Lake Biwa; *P* pebbly, *R* rocky

GEDIMAP: http://gedimap.zool.kyoto-u.ac.jp

Svm, *S. variegatus microoculus*; *Sb*, *S. biwaensis*; *Svv*, *S. variegatus variegatus*

[a] Data fully or partially from Komiya et al. (2011) and sample codes in the reference

[b] Specimens from the captive population kept in Lake Biwa Museum, except two specimens for microsatellite analysis

Table 2 Genetic diversity of the regional population groups of the Japanese *Sarcocheilichthys* fishes

Region	Number of local samples	Number of total specimens	Total number of haplotypes	Proportion of each clade (number of haplotypes)					Haplotype richness at *n* = 16 (95% CI)	Haplotype diversty (± SE)	Nucleotide diversiy × 100 (± SE)
				A	B	C	D	E			
S. variegatus microoculus											
Lake Biwa	15	222	22	0.89 (16)	0.05 (3)	0.06 (3)	–	–	4.67 (2–7)	0.4513 ± 0.0425	0.3220 ± 0.2018
S. biwaensis											
Lake Biwa	4	27	6	0.93 (4)	0.07 (2)	–	–	–	4.63 (3–6)	0.5442 ± 0.1058	0.2270 ± 0.1598
S. v. variegatus											
Ise Bay area	2	22	4	–	–	–	–	1.00 (4)	3.16 (2–4)	0.2597 ± 0.1202	0.1159 ± 0.1006
Kinki	4	36	11	0.19 (1)	0.36 (6)	0.08 (1)	0.36 (3)	–	8.43 (6–10)	0.9000 ± 0.0236	0.9240 ± 0.5035
Chugoku	3	21	7	0.57 (3)	0.33 (3)	0.10 (1)	–	–	6.47 (5–7)	0.8476 ± 0.0440	0.9892 ± 0.5471
Kyushu	2	16	1	–	–	1.00 (1)	–	–	1.00 (1–1)	0	0

Regions correspond to those in Table 1

Table 3 Estimated divergence times of the major clades of the Japanese *Sarcocheilichthys* fishes

Node		tMRCA (mean ± SE)	95% HPD
1	Clade A	0.457 ± 0.009	0.208–0.793
2	Clade B	0.429 ± 0.007	0.181–0.746
3	Clade C	0.473 ± 0.007	0.165–0.839
4	Clade D	0.178 ± 0.003	0.027–0.375
5	Clade E	0.286 ± 0.003	0.075–0.504
6	Clades A + D	0.616 ± 0.012	0.286–1.083
7	Clades A + D + B	0.750 ± 0.013	0.384–1.239
8	Clades A–D	0.811 ± 0.013	0.455–1.292
Cs	Clades A–E (all)	1.262 ± 0.000	1.017–1.510

Node codes correspond to those in Fig. 3

Cs the node given the constraint of 1.1–1.5 Mya (see "Materials and methods")

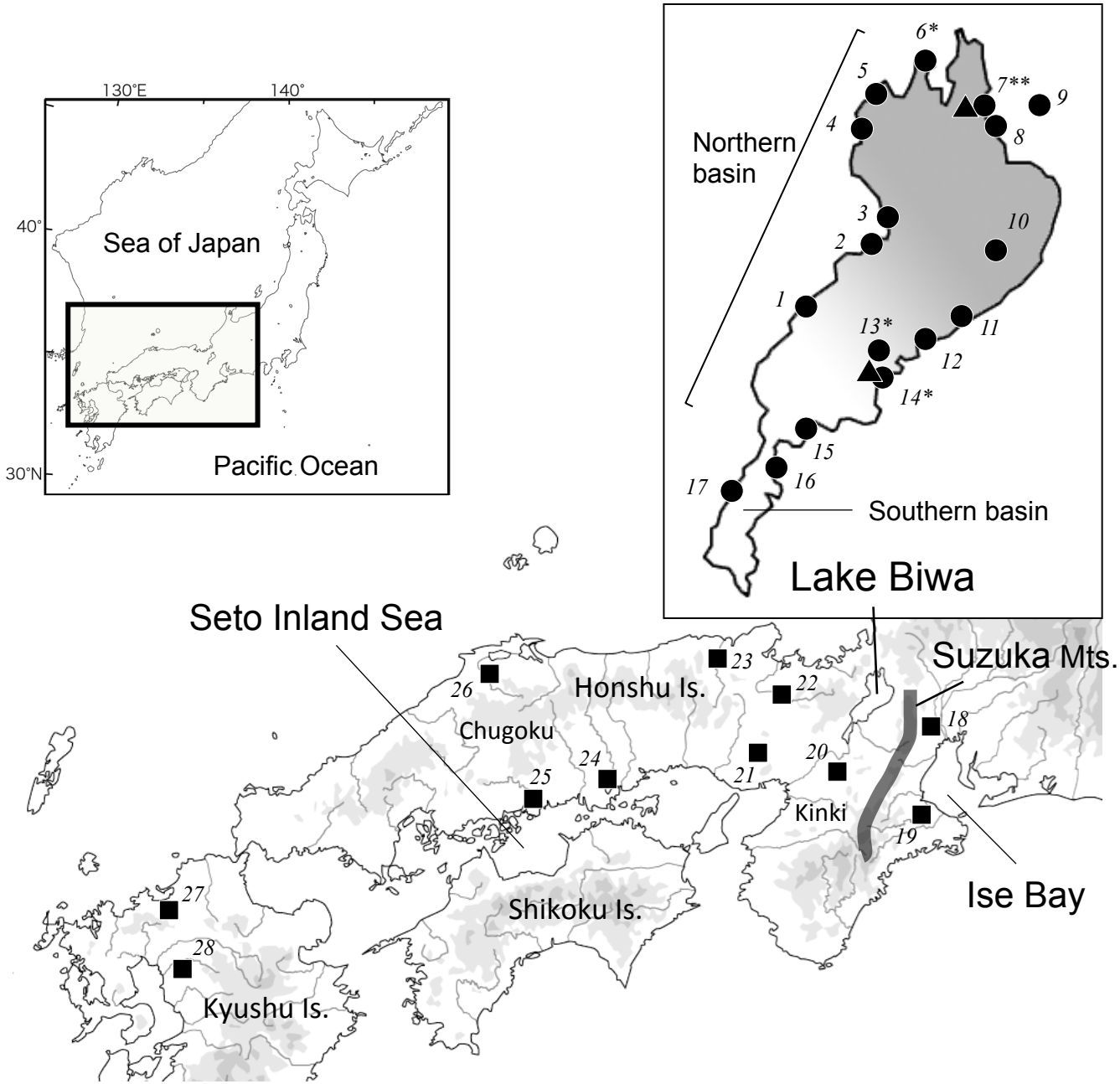
130°E
140°
40°
30°N
Sea of Japan
Pacific Ocean
Northern basin
Southern basin
Lake Biwa
Seto Inland Sea
Suzuka Mts.
Honshu Is.
Chugoku
Kinki
Shikoku Is.
Kyushu Is.
Ise Bay
1
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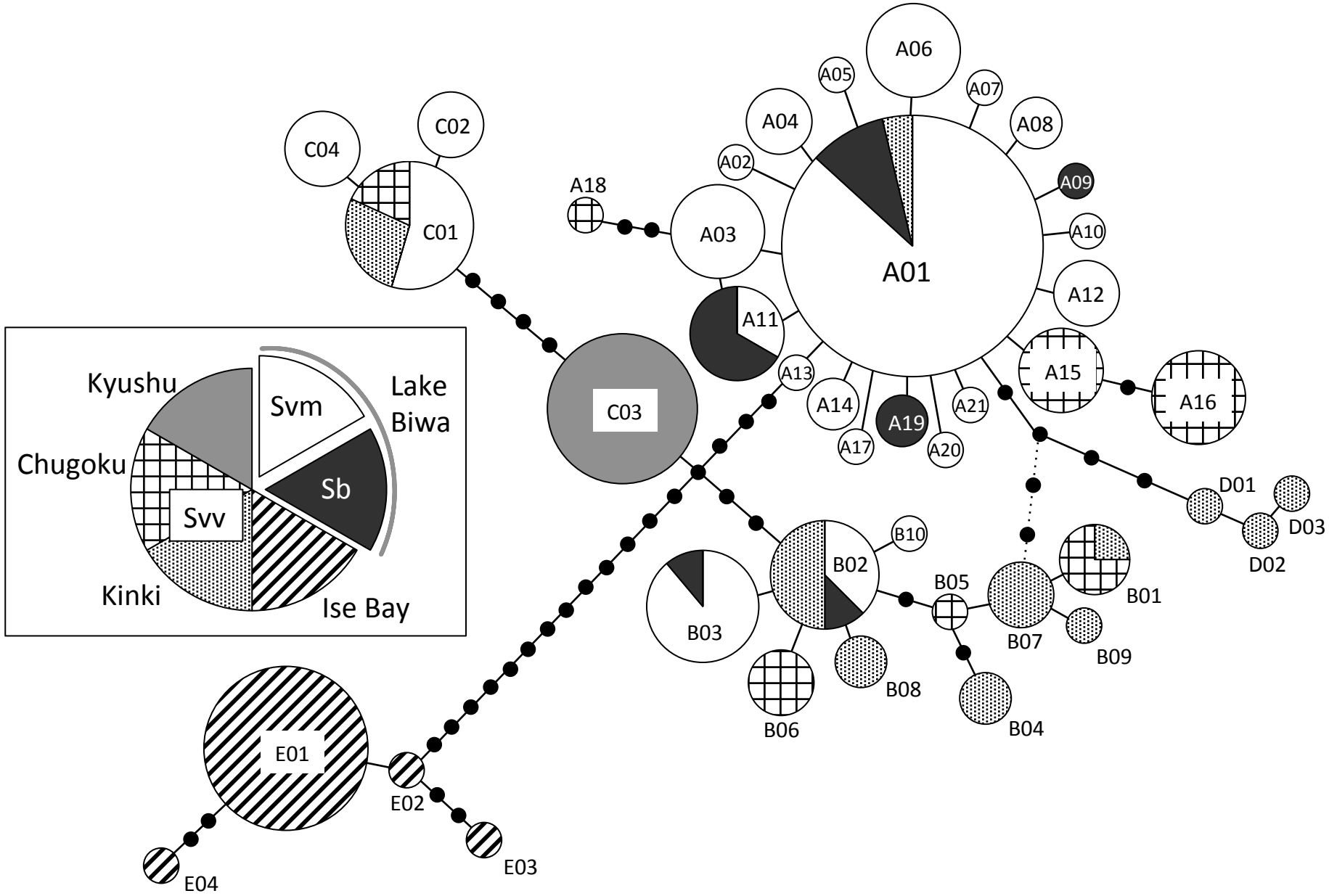
Fig. 1


A01
A02
A03
A04
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A07
A08
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A10
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A16
A17
A18
A19
A20
A21
B01
B02
B03
B04
B05
B06
B07
B08
B09
B10
C01
C02
C03
C04
D01
D02
D03
E01
E02
E03
E04
Kyushu
Svm
Lake Biwa
Chugoku
Sb
Svv
Kinki
Ise Bay

Fig. 2

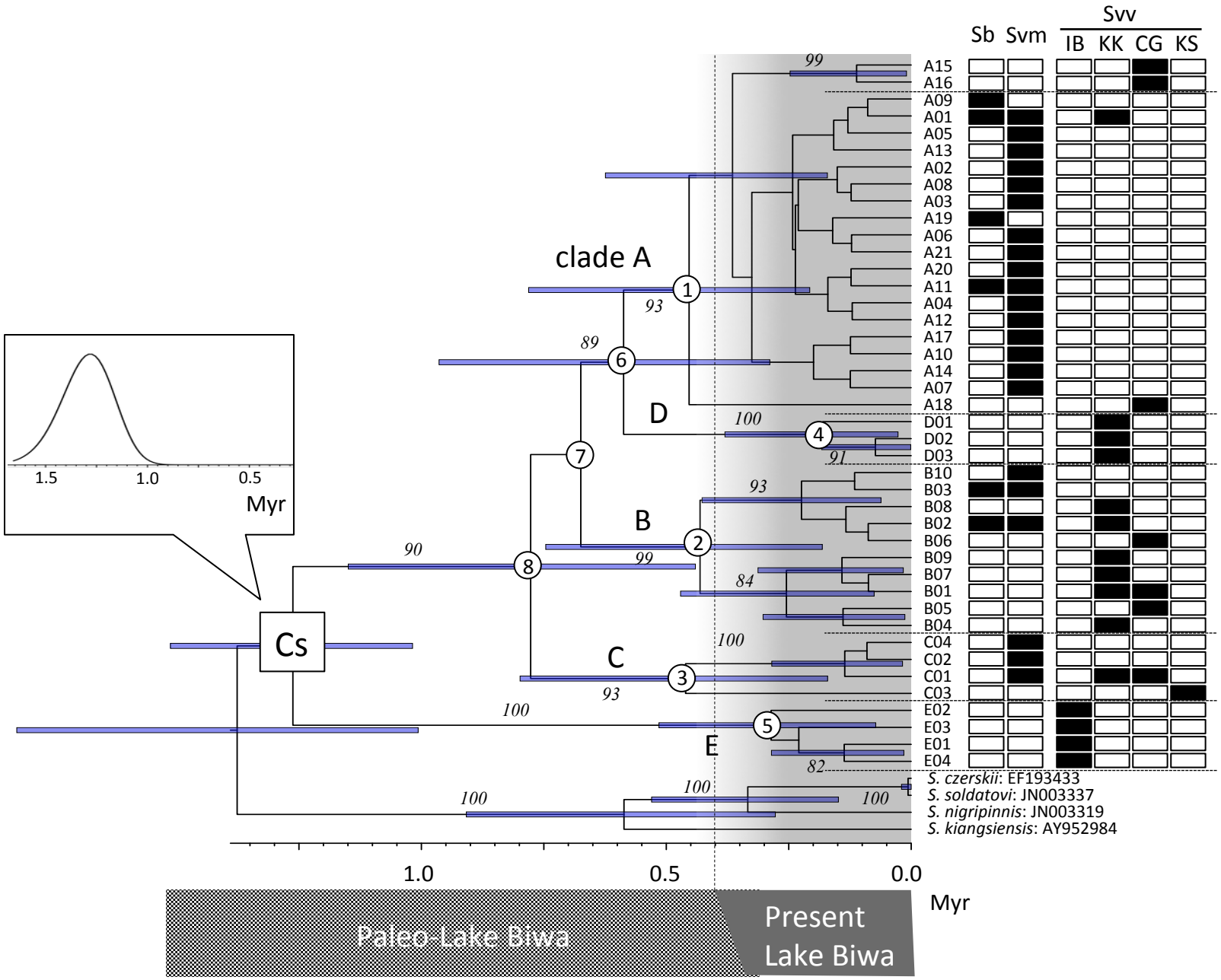
Sb
Svm
Svv
IB
KK
CG
KS
clade A
D
B
C
E
Cs
1.5
1.0
0.5
Myr
1.0
0.5
0.0
Myr
Paleo-Lake Biwa
Present
Lake Biwa
A15
A16
A09
A01
A05
A13
A02
A08
A03
A19
A06
A21
A20
A11
A04
A12
A17
A10
A14
A07
A18
D01
D02
D03
B10
B03
B08
B02
B06
B09
B07
B01
B05
B04
C04
C02
C01
C03
E02
E03
E01
E04
S. czerskii: EF193433
S. soldatovi: JN003337
S. nigripinnis: JN003319
S. kiangsiensis: AY952984

Fig. 3

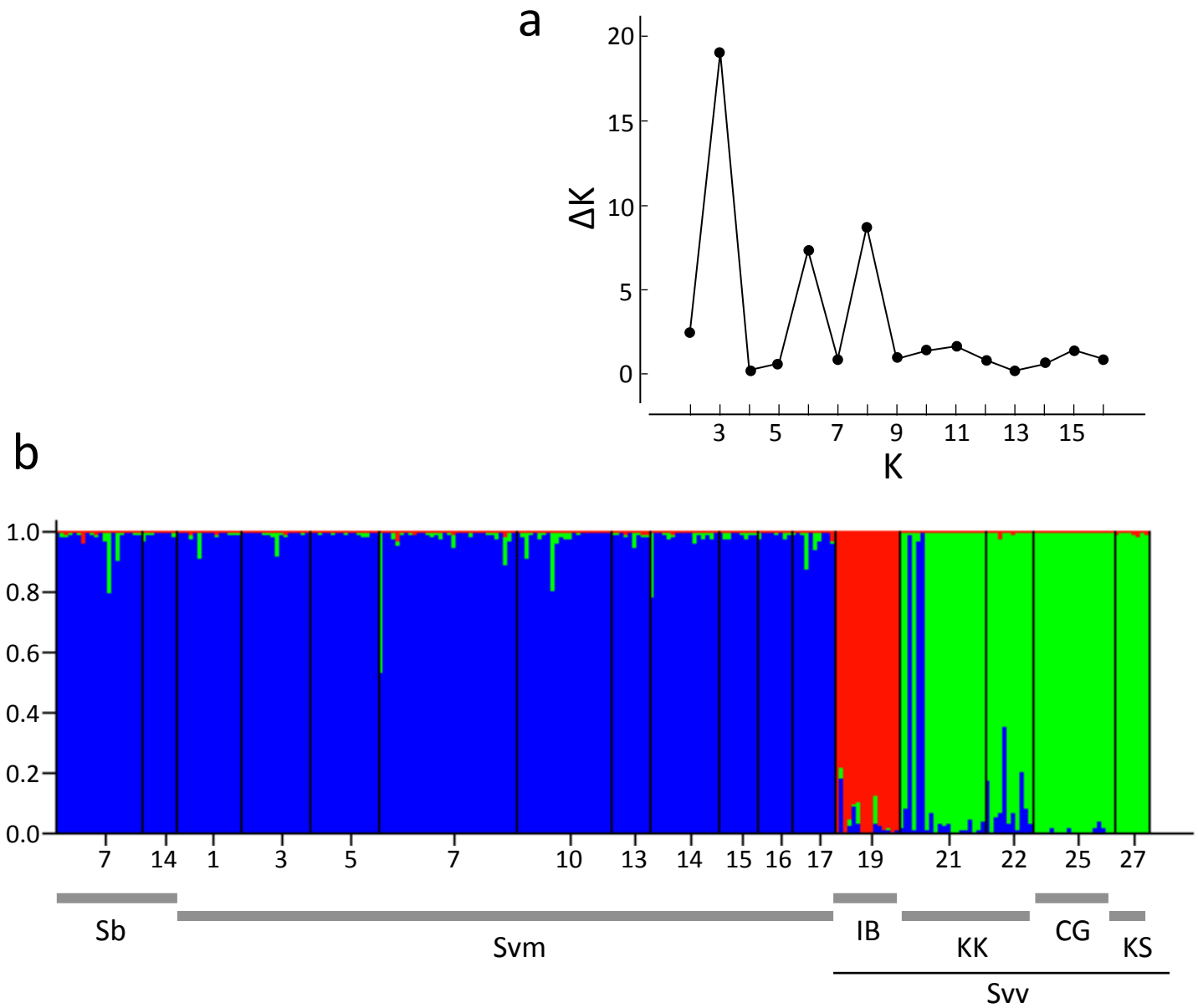
a
ΔK
20
15
10
5
0
3
5
7
9
11
13
15
K
b
1.0
0.8
0.6
0.4
0.2
0.0
7
14
1
3
5
7
10
13
14
15
16
17
19
21
22
25
27
Sb
Svm
IB
KK
CG
KS
Svv

Fig. 4

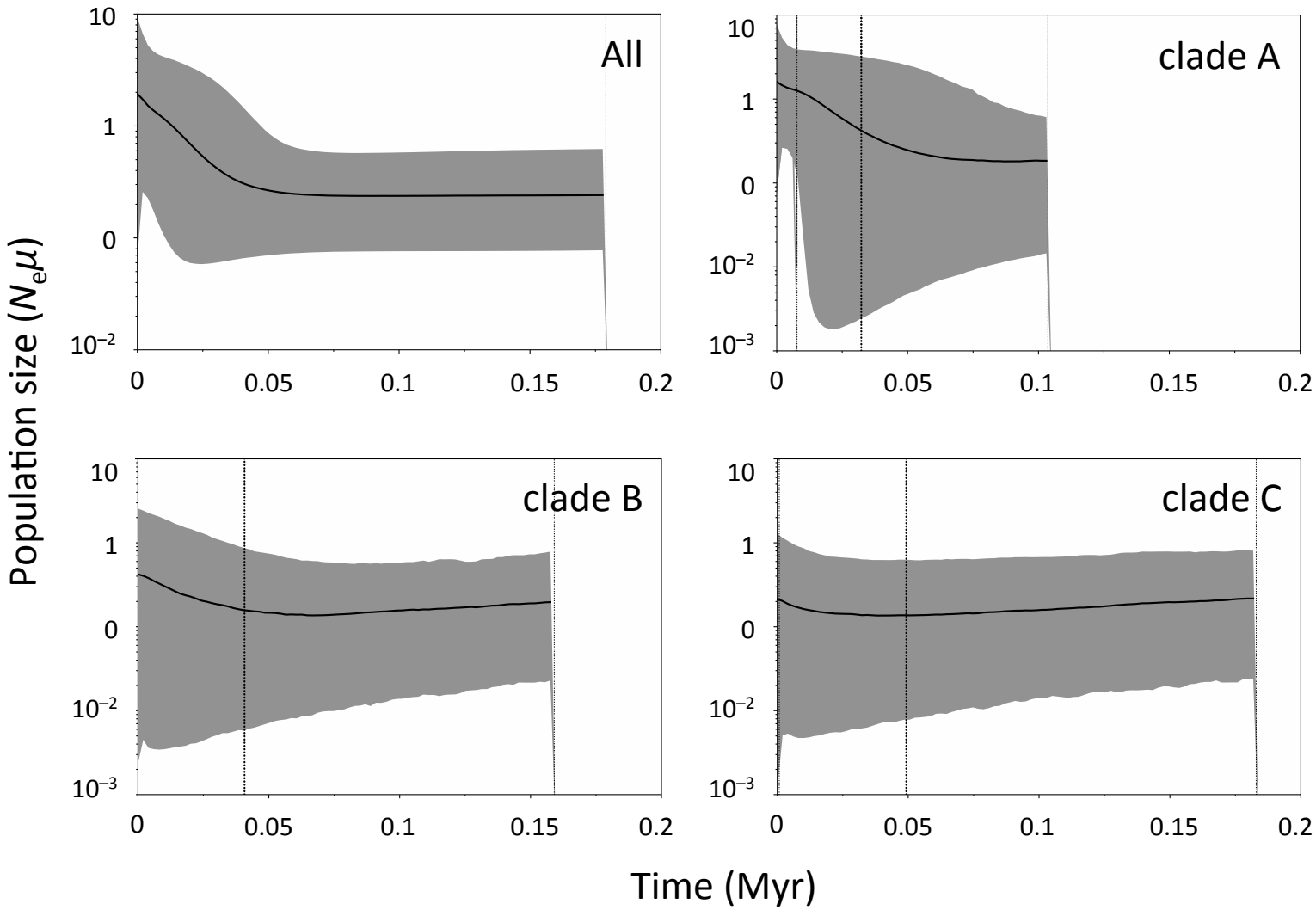
All
clade A
clade B
clade C
Population size ($N_e\mu$)
Time (Myr)
10
1
0
10^{-2}
10^{-3}
0
0.05
0.1
0.15
0.2

Fig. 5

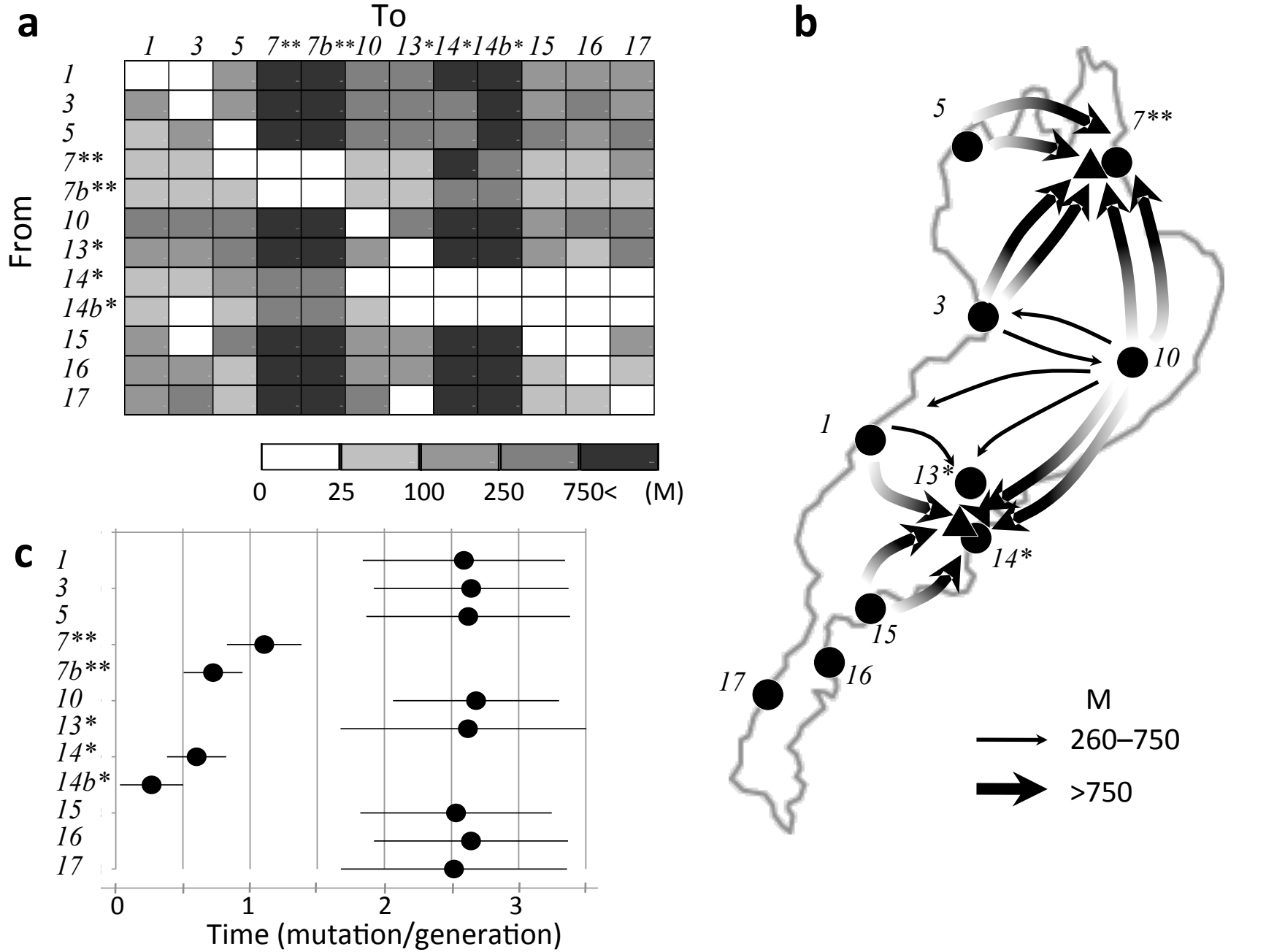
a
To
1 3 5 7** 7b** 10 13* 14* 14b* 15 16 17
From
1
3
5
7**
7b**
10
13*
14*
14b*
15
16
17
0 25 100 250 750< (M)
b
5
7**
3
10
1
13*
14*
15
16
17
M
260–750
>750
c
1
3
5
7**
7b**
10
13*
14*
14b*
15
16
17
0 1 2 3
Time (mutation/generation)

Fig. 6

ESM Table S1 Average expected heterozygosity (± SD) and number of loci that showed the deviation from Hardy–Weinberg equilibrium

Locality code	Locality	Species	Number of specimens	Average expected heterozygosity	H-W-E deviation[a]
Lake Biwa					
1	Off Kitakomatsu, Otsu, Shiga	Svm	15	0.7378 ± 0.3843	0
3	Ado R. Mouth, Kitafunaki, Adogawa, Shiga	Svm	16	0.7098 ± 0.3700	0
5	Off Momose, Chinai, Makino, Shiga	Svm	16	0.7216 ± 0.3756	0
7	Off Onoe, Kohoku, Shiga	Svm	32	0.7316 ± 0.3747	0
		Sb	20	0.7462 ± 0.3852	0
10	Around Takeshima Island, Off Hikone, Shiga	Svm	22	0.7265 ± 0.3749	0
13	Around Okishima Is., Off Oumihachiman, Shiga	Svm	9	0.7208 ± 0.3846	0
14	Off Mizugahama, Oumihachiman, Shiga	Svm	16	0.7111 ± 0.3705	0
		Sb	8	0.6839 ± 0.3689	0
15	Off Chuzu, Yasu, Shiga	Svm	9	0.7330 ± 0.3907	0
16	Off Moriyama, Shiga	Svm	8	0.7226 ± 0.3884	0
17	Off Shimosakamoto, Otsu, Shiga	Svm	10	0.7090 ± 0.3766	0
Ise Bay area					
19	Harai R., Kushida R. s., Matsusaka, Mie	Svv	15	0.3956 ± 0.2171	1
Kinki					
21	Yura R., Ayabe, Kyoto	Svv	20	0.7522 ± 0.3881	3
22	Muko R., Sasayama, Hyogo	Svv	11	0.7443 ± 0.3922	0
Chugoku					
25	Ashida R., Fukuyama, Hiroshima	Svv	19	0.7833 ± 0.4037	0
Kyushu					
27	Naka R., Fukuoka, Fukuoka	Svv	8	0.4893 ± 0.2709	0

Svm, *S. variegatus microoculus*; *Sb*, *S. biwaensis*; *Svv*, *S. variegatus variegatus*

[a] Number of loci that showed significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$ after sequencial Bonferroni correction by each sample, $n = 14$ loci)

ESM Table S2 Matrix of geographical distances as shorter coastal distances (km) for "geofile" in Migrate-n analysis

		Samples											
Samples		1	3	5	7	7b	10	13	14	14b	15	16	17
1	Off Kitakomatsu, Otsu, Shiga	-											
3	Ado R. Mouth, Kitafunaki, Adogawa, Shiga	12.5	-										
5	Off Momose, Chinai, Makino, Shiga	31.9	19.4	-									
7	Off Onoe, Kohoku, Shiga	72.4	59.9	40.5	-								
7b	Off Onoe, Kohoku, Shiga	72.4	59.9	40.5	0.0	-							
10	Around Takeshima Island, Off Hikone, Shiga	63.7	76.2	74.7	34.2	34.2	-						
13	Around Okishima Is., Off Oumihachiman, Shiga	42.5	55.0	74.4	50.6	50.6	27.0	-					
14	Off Mizugahama, Oumihachiman, Shiga	39.6	52.1	71.5	47.7	47.7	24.1	2.9	-				
14b	Off Mizugahama, Oumihachiman, Shiga	39.6	52.1	71.5	47.7	47.7	24.1	2.9	0.0	-			
15	Off Chuzu, Yasu, Shiga	25.2	37.7	57.1	62.1	62.1	38.5	17.3	14.4	14.4	-		
16	Off Moriyama, Shiga	22.0	34.5	53.9	68.3	68.3	44.7	23.5	20.6	20.6	6.2	-	
17	Off Shimosakamoto, Otsu, Shiga	27.2	39.7	59.1	73.5	73.5	49.9	28.7	25.8	25.8	11.4	6.0	-

Samples 7b and 14b are of *Sarcocheilichthys biwaensis*; others *S. variegatus microoculus*

ESM Table S3 Pairwise F_{ST} for the *Sarcocheilichthys* samples with sample sizes ≥ 8 based on mtDNA and microsatellite data

(a) mtDNA

Samples	Samples 7b	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	19	21	22	25	27	28
S. biwaensis																						
7b Off Onoe	–																					
S. variegatus microoculus (Lake Biwa)																						
2 Yotsu River Mouth	-0.0654	–																				
3 Ado R. Mouth	0.0021	-0.0236	–																			
4 Off Imazu	-0.0715	-0.1029	-0.0277	–																		
5 Off Momose	0.0021	-0.0236	-0.0448	-0.0277	–																	
6 Off Oura	-0.0047	-0.0299	0.0139	-0.0386	0.0139	–																
7 Off Onoe	-0.0275	-0.0528	0.0033	-0.0605	0.0010	-0.0262	–															
8 Off Minamiham	-0.0204	-0.0520	-0.0569	-0.0449	-0.0569	-0.0107	-0.0163	–														
9 Tributary of Ane R.	0.0665	0.0527	0.0517	0.0553	0.0517	0.0723	0.0600	0.0428	–													
10 Around Takeshima Island	0.0466	0.0271	0.0436	0.0258	0.0436	0.1308	0.0927	0.0323	0.1333	–												
11 Channel, Ryohama	0.0124	0.0000	0.0013	0.0000	-0.0054	-0.0339	-0.0287	-0.0179	0.0784	0.1041	–											
12 Off Notogawa	-0.0017	-0.0235	-0.0433	-0.0241	-0.0433	0.0355	0.0031	-0.0632	0.0443	0.0011	-0.0143	–										
14 Off Mizugahama	0.0359	0.0254	0.0181	0.0272	0.0181	0.0113	0.0143	0.0108	0.1106	0.1198	0.0487	0.0465	–									
15 Off Chuzu	0.1531	0.1211	0.1186	0.1242	0.1186	0.2937	0.2347	0.0992	0.2474	-0.0374	0.2281	0.0423	0.2477	–								
16 Off Moriyama	-0.0633	-0.0870	-0.0277	-0.0938	-0.0277	-0.0297	-0.0539	-0.0449	0.0553	0.0301	0.0000	-0.0241	0.0272	0.1286	–							
17 Off Shimosakamoto	-0.0654	-0.0959	-0.0236	-0.1029	-0.0236	-0.0299	-0.0528	-0.0414	0.0527	0.0250	0.0000	-0.0235	0.0254	0.1211	-0.0870	–						
S. v. variegatus (Ise Bay)																						
19 Harai R.	0.9535	0.9702	0.9449	0.9738	0.9449	0.9748	0.9593	0.9613	0.9819	0.8651	0.9926	0.9470	0.9936	0.8972	0.9738	0.9702	–					
S. v. variegatus (Kinki)																						
21 Yura R.	0.4086	0.3776	0.4164	0.3811	0.4164	0.5109	0.4774	0.3860	0.4577	0.2795	0.4464	0.3397	0.4593	0.2402	0.3871	0.3776	0.8703	–				
22 Muko R.	0.5575	0.5560	0.5917	0.5669	0.5917	0.7147	0.6477	0.5815	0.6791	0.3078	0.6909	0.5074	0.7063	0.3014	0.5669	0.5560	0.9397	0.4073	–			
S. v. variegatus (Chugoku)																						
25 Ashida R.	0.2239	0.1934	0.2544	0.1966	0.2544	0.3324	0.2904	0.2187	0.2825	0.1742	0.2622	0.1779	0.2816	0.1918	0.1966	0.1934	0.8948	0.3613	0.3755	–		
S. v. variegatus (Kyushu)																						
27 Naka R.	0.8031	0.8519	0.7795	0.8681	0.7795	0.9015	0.8441	0.8207	0.9162	0.5036	0.9638	0.7502	0.9690	0.4933	0.8681	0.8519	1.0000	0.5472	0.6657	0.6255	–	
28 Futatsu R.	0.8031	0.8519	0.7795	0.8681	0.7795	0.9015	0.8441	0.8207	0.9162	0.5036	0.9638	0.7502	0.9690	0.4933	0.8681	0.8519	1.0000	0.5472	0.6657	0.6255	0.0000	–

(b) Microsatellite

Samples	Samples 7b	14b	1	3	5	7	10	13	14	15	16	17	19	21	22	25	27
S. biwaensis																	
7b Off Onoe, Kohoku, Shiga	–																
14b Off Mizugahama, Oumihachiman, Shiga	0.0246	–															
S. variegatus microoculus (Lake Biwa)																	
1 Off Kitakomatsu, Otsu, Shiga	-0.0030	0.0333	–														
3 Ado R. Mouth, Kitafunaki, Adogawa, Shiga	0.0087	0.0584	0.0152	–													
5 Off Momose, Chinai, Makino, Shiga	-0.0055	0.0342	0.0049	-0.0018	–												
7 Off Onoe, Kohoku, Shiga	-0.0024	0.0312	0.0057	0.0077	-0.0034	–											
10 Around Takeshima Island, Off Hikone, Shiga	-0.0014	0.0447	0.0095	0.0067	-0.0036	0.0004	–										
13 Around Okishima Is., Off Oumihachiman, Shiga	-0.0009	0.0219	-0.0031	0.0191	0.0077	0.0054	0.0130	–									
14 Off Mizugahama, Oumihachiman, Shiga	-0.0025	0.0303	0.0051	0.0109	-0.0040	0.0030	0.0031	-0.0025	–								
15 Off Chuzu, Yasu, Shiga	-0.0041	0.0218	-0.0039	0.0141	-0.0013	-0.0037	-0.0013	-0.0139	-0.0075	–							
16 Off Moriyama, Shiga	0.0197	0.0581	0.0139	0.0315	0.0147	0.0200	0.0203	0.0232	0.0161	0.0146	–						
17 Off Shimosakamoto, Otsu, Shiga	0.0125	0.0501	0.0183	0.0224	0.0129	0.0056	0.0140	0.0036	0.0116	0.0121	0.0116	–					
S. v. variegatus (Ise Bay)																	
19 Harai R., Kushida R. s., Matsusaka, Mie	0.3767	0.4437	0.3952	0.4125	0.4023	0.3709	0.3947	0.4034	0.4069	0.4263	0.4323	0.4179	–				
S. v. variegatus (Kinki)																	
21 Yura R., Ayabe, Kyoto	0.0624	0.1018	0.0672	0.0796	0.0623	0.0697	0.0611	0.0804	0.0669	0.0610	0.0684	0.0915	0.3917	–			
22 Muko R., Sasayama, Hyogo	0.0708	0.0776	0.0808	0.0808	0.0740	0.0713	0.0734	0.0732	0.0735	0.0633	0.0991	0.1039	0.4063	0.1044	–		
S. v. variegatus (Chugoku)																	
25 Ashida R., Fukuyama, Hiroshima	0.1029	0.1089	0.0965	0.1052	0.1075	0.1054	0.1009	0.1068	0.1017	0.0863	0.1090	0.1151	0.3697	0.1053	0.0814	–	
S. v. variegatus (Kyushu)																	
27 Naka R., Fukuoka, Fukuoka	0.2976	0.3475	0.3146	0.3248	0.3124	0.2955	0.3091	0.3238	0.3207	0.3271	0.3312	0.3318	0.5099	0.3039	0.3101	0.2930	–

Sample codes correspond to those in Fig. 1 and Table 1

Samples 7b and 14b are of *Sarcocheilichthys biwaensis*; others *S. variegatus microoculus*

The value indicated in red was significant after the sequential Bonferroni correction (α = 0.05)

ESM Table S4 Results of mismatch distribution analysis and neutrality tests by Tajima's *D* and Fu's F_S for pooled and respective samples of *Sarcocheilichthys* species

						Sudden expansion model				Spatial expansion model							
Region	Code	Population/clade	Bottom type	Species	*n*	τ; 95 % C.I.	ϑ_0	ϑ_1	*P* (SSD)	τ; 95 % C.I.	ϑ	*M*	*P* (SSD)	Tajima's *D*	*P*	Fu's F_S	*P*
Lake Biwa																	
	2–12, 14–17	Lake Biwa (all)	P&R	Svm	222	0.039 0.002–0.543	0.000	inf.	<0.001	8.540 0.001–63.356	0.582	0.239	0.649	-1.543	0.031	-9.231	0.008
	3, 5, 7, 14	Lake Biwa (all)	P&R	Sb	27	0.703 0.201–1.367	0.000	inf.	0.113	0.692 0.247–1.532	0.011	inf.	0.046	-1.484	0.054	-0.789	0.323
		Clade A	P&R	Svm	195	3.000 0.000–3.500	0.000	0.460	0.431	0.374 0.206–0.601	0.001	inf.	0.025	-2.168	<0.001	-23.360	<0.001
		Clade B	P&R	Svm	12	0.768 0.000–1.885	0.000	inf.	0.781	0.751 0.082–2.324	0.016	inf.	0.573	-0.047	0.442	-0.137	0.338
		Clade C	P&R	Svm	13	1.074 0.000–2.236	0.000	inf.	0.153	1.073 0.324–2.353	0.001	inf.	0.092	0.879	0.819	0.436	0.556
	2	Yotsu R. mouth, Adogawa	P	Svm	10	3.000 0.457–3.807	0.000	0.499	0.230	7.549 0.000–98.222	0.366	0.267	0.470	-1.873	0.007	1.453	0.794
	3	Ado R. mouth, Kitafunaki	P	Svm	21	3.000 0.410–3.000	0.000	0.272	0.104	10.743 0.000–170.500	0.140	0.252	0.356	-1.510	0.052	1.735	0.831
	4	Off Imazu	P	Svm	10	3.000 0.430–3.000	0.000	0.133	0.034	7.712 0.000–170.625	0.001	0.277	0.308	-1.839	0.010	2.985	0.911
	5	Off Momose	P	Svm	21	3.250 0.000–46.250	0.330	0.630	0.259	9.490 0.000–109.286	0.532	0.217	0.429	-1.510	0.051	0.645	0.670
	6	Off Oura	P	Svm	25	3.000 0.344–3.574	0.000	0.397	0.392	7.708 0.000–85.000	0.366	0.067	0.431	-2.160	0.002	-1.431	0.118
	7	Around off Onoe	P&R	Svm	26	3.250 0.121–4.746	0.330	0.623	0.411	7.148 0.000–89.775	0.548	0.153	0.519	-1.693	0.028	-1.152	0.211
				Sb	17	0.594 0.000–2.393	0.000	188.438	0.768	7.021 0.000–183.75	0.740	0.274	0.495	-1.195	0.116	0.114	0.553
	8	Off Minamihama	P	Svm	12	0.500 0.000–0.093	0.000	inf.	0.193	9.460 0.000–112.424	0.647	0.179	0.464	-2.067	0.003	0.717	0.656
	9	Ane R., Kohoku	P	Svm	12	1.117 0.000–2.289	0.000	inf.	0.140	1.114 0.373–2.454	0.003	inf.	0.080	-0.379	0.367	-0.895	0.137
	10	Takeshima Is.	P	Svm	25	9.529 0.096–95.529	0.000	2.644	0.177	8.338 0.437–19.617	0.680	1.173	0.720	0.130	0.601	0.961	0.701
	11	Hikone	P	Svm	10	0.490 0.000–1.367	0.000	inf.	0.457	0.475 0.000–2.323	0.014	inf.	0.350	-1.401	0.034	-1.164	0.037
	12	Off Notogawa	P	Svm	9	1.217 0.000–2.643	0.000	inf.	0.180	10.411 0.000–116.660	2.132	0.222	0.120	-1.723	0.027	0.087	0.505
	14	Off Mizugahama	R	Svm	11	0.436 0.000–1.232	0.000	inf.	0.444	0.432 0.000–48.125	0.004	inf.	0.273	-0.100	0.331	0.356	0.366
				Sb	7	0.898 0.000–22.750	0.000	inf.	0.284	0.898 0.000–101.190	0.001	inf.	0.223	1.342	0.972	0.856	0.604
	15	Off Chuzu	P	Svm	10	10.203 0.314–99.203	0.002	11.437	0.084	8.573 0.455–36.388	1.185	1.944	0.469	0.479	0.719	1.806	0.816
	16	Off Moriyama	P	Svm	10	3.000 0.457–3.801	0.000	0.504	0.244	6.548 0.000–100.111	0.361	0.272	0.479	-1.839	0.008	1.160	0.767
	17	Off Shimosakamoto	P	Svm	10	3.000 0.457–3.836	0.000	0.499	0.248	7.550 0.000–103.889	0.366	0.267	0.480	-1.873	0.006	1.453	0.800
Ise Bay																	
	19	Harai R., Mie		Svv	19	–				–				0.000		–	
Kinki	21	Yura R., Kyoto		Svv	20	10.516 0.000–16.887	0.000	6.231	0.514	6.916 0.397–22.933	2.773	1.197	0.691	0.048	0.570	2.573	0.877
	22	Muko R., Hyogo		Svv	10	4.770 0.410–8.551	0.000	6.417	0.492	2.986 0.577–8.247	1.335	4.248	0.494	-0.250	0.416	0.322	0.559
Chugoku																	
	25	Ashida R., Hiroshima		Svv	14	9.562 0.000–98.562	0.000	9.727	0.053	9.100 4.099–16.662	0.001	3.723	0.073	0.637	0.770	2.527	0.887
Kyushu																	
	27	Naka R., Fukuoka		Svv	8	–				–				0.000		–	
	28	Futatsu R. Fukuoka		Svv	8	–				–				0.000		–	

Population codes corespond to those in Table 1 and Fig. 1

Sb, *S. biwaensis*; *Svm*, *S. variegatus microoculus*; *Svv*, *S. variegatus variegatus*

τ time parameter with 95% confidence interval, ϑ_0 pre-expansion population size, ϑ_1 post-expansion population size, ϑ population size, *M* number of migrants exchanged with other demes, *inf.* infinite, – no result obtained

P(SSD) probability for sum of squred deviation by a permutation test

P values smaller than 0.05 are indicated in red