



Title	A strategic sampling design revealed the local genetic structure of cold-water fluvial sculpin: a focus on groundwater-dependent water temperature heterogeneity
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1 **A strategic sampling design revealed the local genetic structure of cold-water fluvial**
2 **sculpin: a focus on groundwater-dependent water temperature heterogeneity**

3

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22

23 **Running title**

24 Sculpin genetic structure with water temperature

25

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28 **Abstract**

29 A key piece of information for ecosystem management is the relationship between the
30 environment and population genetic structure. However, it is difficult to clearly quantify the
31 effects of environmental factors on genetic differentiation because of spatial autocorrelation
32 and analytical problems. In this study, we focused on stream ecosystems and the
33 environmental heterogeneity caused by groundwater and constructed a sampling design in
34 which geographic distance and environmental differences are not correlated. Using
35 multiplexed ISSR genotyping by sequencing (MIG-seq) method, a fine-scale population
36 genetics study was conducted in fluvial sculpin *Cottus nozawae*, for which summer water
37 temperature is the determinant factor in distribution and survival. There was a clear genetic
38 structure in the watershed. Although a significant isolation-by-distance pattern was detected
39 in the watershed, there was no association between genetic differentiation and water
40 temperature. Instead, asymmetric gene flow from relatively low-temperature streams to high-
41 temperature streams was detected, indicating the importance of low-temperature streams and
42 continuous habitats. The groundwater-focused sampling strategy yielded insightful results
43 for conservation.

44

45 **Introduction**

46 How genetic structure is shaped across a landscape is an essential theme in evolutionary
47 biology, and this information provides an empirical basis for conservation biology by
48 elucidating habitat connectivity or predicting the effects of landscape change (Hohenlohe et
49 al. 2021). A pattern of ‘isolation by distance’ (IBD; Wright 1943), in which genetic
50 differentiation increases with geographic distance, is a very common pattern to explain
51 population structure (Meirmans 2012). In addition to space, another important component of
52 a landscape is the environment (Nosil et al. 2005; Thorpe et al. 2008). In the field of landscape
53 genetics, many studies have investigated the interactions between genetic differentiation and
54 landscape variables, and a couple of patterns have described the relationship between genetic
55 structure and environment. When gene flow between populations that inhabit different
56 environments is limited due to local adaptation or other factors, genetic differentiation
57 increases with environmental differences and a pattern of ‘isolation by environment’ (IBE;
58 Wang and Summers 2010) is generated. Another pattern that involves genetic structure and
59 the environment is ‘counter-gradient gene flow’ (Sexton et al. 2014). In this scenario, gene
60 exchange between different environments is high and forms a source-sink pattern in a
61 landscape. Because these interactions between genetic differentiation and landscape variables
62 are critically important for addressing classical evolutionary questions related to ecological
63 speciation or conservation, the environment is a factor that cannot be ignored when
64 describing the observed population structure (Orsini et al. 2013; Wang and Bradburd 2014).

65 However, it is very difficult to disentangle the relative strength of space (distance)
66 and environment in observed patterns of spatial genetic differentiation because
67 environmental differences are usually highly correlated with geographic distance (Sexton et
68 al. 2014; Wang and Bradburd 2014). To test spatial relationships, the Mantel test and partial
69 Mantel test are commonly used. However, the Mantel test family is strongly criticized for its
70 inflated Type I error rate, especially under conditions where the measured variables are
71 spatially correlated (Guillot and Rousset 2013; Harmon and Glor 2010; Meirmans 2012;

72 Raufaste and Francois 2001; Rousset 2002). Although there are several proposed alternative
73 methods to the Mantel test such as multiple regression on distance matrices (MRM; Lichstein
74 2007), Procrustes analysis, or redundancy analysis (RDA), and some of them perform much
75 better than the partial Mantel test, previous studies have suggested that all methods fail to
76 correctly model the relative importance of space and environment under certain patterns,
77 which cannot be known *a priori* (Diniz-Filho et al. 2013; Gilbert and Bennett 2010). It is
78 now recognized that no method has the ability to address the spatial-environmental
79 correlation and perform hypothesis testing simultaneously (Zeller et al. 2016). These studies
80 suggest the difficulty of separating spatial and environmental effects on genetic differences.
81 However, once a sampling design that decouples distance and environment is developed, we
82 will be able to clearly compare the relative strength of space and environment independent
83 of these controversies. In the field of community ecology, Gilbert and Lechowicz (2004) used
84 a sampling design that removed spatial autocorrelation of the environment sampled, and they
85 analyzed the species' spatial and environmental correlations to reject the neutral theory of
86 biodiversity. However, subsequent attempts to partition spatial and environmental
87 components have focused exclusively on statistical and analytical methodology (Gilbert and
88 Bennett 2010). Especially in landscape genetics, sampling design has not received much
89 attention (Meirmans 2015).

90 Here, we focused on stream ecosystems, which often exhibit heterogeneous
91 environments (Heino et al. 2013; Uno 2016). In stream ecosystems, water temperature is the
92 strongest factor affecting river organisms as well as flow regime (Olden and Naiman 2010),
93 and it is increasingly being an important variable under ongoing climate change. Water
94 temperature is determined by air temperature, riparian forests, and groundwater, among
95 others (Caissie 2006; Nakamura and Yamada 2005). In particular, the effect of groundwater
96 discharge on water temperature has been indicated to be very strong (Arscott et al. 2001).
97 However, the influence of groundwater discharge on the upper sections of streams has
98 received much less attention (Brown et al. 2007), and many ecological studies have used air

99 temperature instead of water temperature (e.g., Almodóvar et al. 2012; Middaugh et al. 2018).
100 Groundwater shows a very stable water temperature and flow regime; hence, groundwater
101 discharge greatly affects the stream environment (Poff et al. 2010). Given water temperature
102 in summer that is critical for cold-water organisms, streams with high groundwater display
103 lower values and these streams could be an important habitat for these species. Since the
104 amount of cold groundwater discharge vary locally within a watershed, groundwater creates
105 spatial heterogeneity of environments within the watershed. Thus, to reveal the ecological
106 role of water temperature, knowledge of the spatial heterogeneity caused by groundwater is
107 thought to be useful.

108 As a practical study, we investigated the genetic structure of the cold-water fluvial
109 sculpin *Cottus nozawae* in the upstream section of the Sorachi River, Hokkaido, Japan. Cold-
110 water fish are very vulnerable to climate change, and it is worth studying the relationships
111 between population structure and water temperature. Together with *C. nozawae*, *Salvelinus*
112 *malma*, *Salvelinus leucomaenis*, *Barbatula oreas*, and *Parahucho perryi* inhabit this
113 watershed. However, studies on *S. malma* and *S. leucomaenis* did not display strong genetic
114 structure within this watershed due to their active migration (Koizumi 2011; Nakajima et al.
115 2020). Due to the lower mobility of adult *C. nozawae* than these species (Goto 1998;
116 Okumura and Goto 1996), we predicted that *C. nozawae* should display clear genetic
117 differentiation within the watershed. In addition, the summer water temperature has been
118 shown to be the dominant factor determining the distribution and survival of this species
119 (Yagami and Goto 2000), and a relationship in which low water temperature in summer
120 increases the density of this species has also been confirmed in our study area (Suzuki et al.
121 2021). Therefore, summer water temperature can be explicitly used as an important variable
122 for *C. nozawae*. Hypothesis-driven studies that focus on a given variable have advantages
123 over exploratory studies in designing sampling strategies (Richardson et al. 2016). Since
124 ecologically important variables affecting survival and population density could cause local
125 adaptation between different environments (Kawecki and Ebert 2004), we predicted that the

126 differences in summer water temperature cause local adaptations and lead to the IBE pattern
127 within the watershed.

128 The aims of this study are (i) to minimize the correlation of geographic distance
129 and water temperature differences via a groundwater-focused sampling strategy, (ii) to
130 investigate the local genetic structure of *C. nozawae* and its determinant factors, and (iii) to
131 discuss the relationship between water temperature and the population structure of cold-water
132 fish. Although recent studies have frequently used adaptative genetic markers to detect local
133 adaptation, barriers to gene flow imposed by selection and local adaptation between
134 populations can be detected with neutral markers (Sexton et al. 2014). Given that the use of
135 adaptive markers is still challenging, the evaluation of current genetic differentiation and
136 connectivity based on neutral genetic markers is still informative and can assist in allocating
137 conservation units to preserve local genetic variation (Tsuda et al. 2015). To assess the genetic
138 structure, we used putatively neutral genome-wide SNPs on the inter simple sequence repeat
139 (ISSR) region and analyzed genetic differentiation and gene flow patterns.

140

141

142 **Materials and Methods**

143 *Study sites and sampling*

144 This study was conducted in the upper section of the Sorachi River, Hokkaido, Japan (Fig. 1;
145 Table 1). To conduct sampling across a heterogeneous landscape, we used an approach based
146 on watershed geology, which is known to cause significant variations in water temperature
147 through groundwater discharge (Caissie 2006; Nagasaka and Sugiyama 2010; Tague et al.
148 2007). In the Sorachi River, many small spring-fed inputs are found in the Quaternary
149 volcanic region (Koizumi and Maekawa 2004; Watz et al. 2019). Thus, the sampling sites
150 were selected so that tributaries with volcanic watersheds and nonvolcanic watersheds were
151 spatially intermingled. We also chose sites interspersed throughout the watershed such that
152 large spatial gaps among populations were eliminated as much as possible. Watershed

153 geology was assessed based on the Seamless Digital Geological Map of Japan V2 from the
154 National Institute of Advanced Industrial Science and Technology. There is a large dam
155 (Kanayama Dam) in the watershed. While the area upstream of Kanayama Dam represents
156 one of the most continuous river habitats in Japan and there are no barriers between
157 populations, the downstream region is slightly more influenced by anthropogenic impacts on
158 streams. For example, one population (Pop13) is located upstream of a check dam, and
159 another population (Pop19) is located in a tributary with a downstream portion that runs
160 through an agricultural landscape.

161 From 20 sites in the Sorachi River, 531 individuals of *C. nozawae* were caught for
162 genetic analysis. The fish were caught by electrofishing (model 12-B Backpack Electrofisher;
163 Smith-Root Inc.) or with hand nets. Small pieces of fin tissue were clipped, placed in 99.5%
164 ethanol, and stored at -20°C in the laboratory until DNA extraction. Total DNA was
165 extracted using QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Inc.) with a combination
166 of Genomic DNA Extraction Column (FAVORGEN Inc.).

167

168 *Water temperature*

169 At each sampling site, the summer water temperature was recorded hourly using data loggers
170 (Onset Computer Corp. or Gemini Data Loggers Ltd., depending on site). Water temperatures
171 were measured during the summer of 2020, and also during the summer of 2019 at some
172 locations. To align the data sampling periods, data from 16 July 2020 to 31 August 2020 were
173 extracted, and the maximum water temperature in this period was identified. At one site
174 (Pop10) where we failed to record water temperatures in some mid-summer periods, the
175 maximum temperature in 2019 was used as an alternative. We used the maximum water
176 temperature which significantly affects the populations of cold-water fish as a representative
177 of summer water temperature (Yagami and Goto 2000). The correlation of water temperature
178 differences with waterway geographic distance (hereafter referred to as geographic distance)
179 was examined with the Mantel test (9999 permutations) using the package ECODIST 2.0.3

180 (Goslee and Urban 2007) in R 3.6.0 (R Core Team 2019). Then, a Mantel correlogram
181 showing the spatial correlation of water temperature differences across multiple ranges of
182 geographic distance was constructed. The Mantel correlogram in 10 equal distance classes
183 was assessed with 9999 permutations using the package VEGAN 2.5.6 (Oksanen et al. 2019)
184 in R.

185

186 *MIG-seq library preparation and sequencing*

187 To obtain neutral genome-wide SNP data, we used multiplexed ISSR genotyping by
188 sequencing (MIG-seq; Suyama and Matsuki 2015), a technique in which loci between two
189 microsatellite regions are amplified by PCR and next-generation sequencing. MIG-seq is one
190 of the reduced representation sequencing methods along with restriction site-associated DNA
191 sequencing (RAD-seq), but the number of available informative loci (MIG-loci) detected by
192 MIG-seq is less than that of RAD-seq, and most of the MIG-loci are putatively neutral.
193 Although MIG-seq is not suitable for outlier analysis or gene identification, acquired loci are
194 sufficient for population genetic analysis. A MIG-seq library was prepared following the
195 protocol outlined in Suyama and Matsuki (2015), except for the minor modifications outlined
196 below. The first PCR was conducted using eight ISSR primer sets with tail sequences at an
197 annealing temperature of 38 °C. The second PCR was conducted using primer pairs including
198 tail sequences, adapter sequences for Illumina sequencing, and five-base (forward) and nine-
199 base (reverse) barcode sequences to identify each individual sample. The conditions for the
200 second PCR were as follows: 12 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C
201 for 15 s, and extension at 68 °C for 1 min. The second PCR products of all 531 samples were
202 mixed, and then fragments in the size range of 400–800 bp in the purified library were
203 isolated. After library quantification, the products were sequenced on the Illumina MiSeq
204 platform using MiSeq Reagent Kit v3 (150 cycles). Both ends of the fragments and the
205 indexes were read by paired-end and index sequencing: 80, 80, 9, and 5 bases of sequences
206 were determined as read 1, read 2, index-1, and index-2, respectively. The first 17 bases (SSR

207 and anchor regions) in both reads were skipped using the *DarkCycle* option of the MiSeq
208 system. Read 1 and 2 cannot overlap within each fragment, and the size range of the library
209 was 400–800 bp. Thus, following to Suyama and Matsuki (2015), we treated reads 1 and 2
210 as independent reads. The final data output to the next step included sequences of 80 bases
211 from both ends of 400–800 bp forward-reverse amplicons of various ISSR regions.

212

213 *SNP detection*

214 Read quality filtering was performed using Trimmomatic 0.39 (Bolger et al. 2014).
215 Extremely short reads containing adapter sequence were filtered out. Then, to remove low
216 quality reads, the reads were scanned with a 4-base wide sliding window, and reads with an
217 average quality per base below 15 were removed. After quality filtering, a total of 39,956,740
218 reads were obtained. SNP selection was performed using STACKS 2.41 (Catchen et al. 2013).
219 First, reads were grouped to each locus using the *ustacks*, *cstacks*, *sstacks*, *tsu2bam*, and
220 *gstacks* commands with the following parameters recommended by Paris et al. (2017):
221 minimum depth option creating a stack (m) = 3, maximum distance between stacks (M) = 2,
222 maximum mismatches between loci when building the catalog (n) = 2, and number of
223 mismatches allowed to align secondary reads (N) = 4. After this process, the dataset of
224 assembled loci (*stacks dataset*) was obtained. For most analyses (except for gene flow
225 analysis), loci present at a rate of more than 80% among all samples were extracted using the
226 *populations* command. Several options were included with this command: the minimum
227 minor allele count over the entire dataset was set to three to exclude potentially artificial loci
228 found in only one individual, sites showing excess heterozygosity (>0.6) were removed to
229 filter potential heterozygotes resulting from artificial loci built from several paralogous
230 genome regions, and the output was limited to one SNP per locus to exclude linkage
231 disequilibrium among SNPs. After filtering, 489 SNPs were obtained.

232

233 *Genetic diversity and differentiation*

234 For each population, the expected heterozygosity (H_E) and fixation index (F_{IS}) were
235 calculated using the *populations* command in STACKS. Significant deviations from Hardy–
236 Weinberg equilibrium (HWE), as indicated by F_{IS} deviating from zero, were tested by 1000
237 randomizations using FSTAT 2.9.4 (Goudet 1995). Genetic differentiation among
238 populations was evaluated by calculating global/pairwise F_{ST} values (Weir and Cockerham
239 1984) using GenAlEx 6.5 (Peakall and Smouse 2012). To understand the spatial trend of the
240 genetic structure, a Mantel correlogram displaying correlations between F_{ST} and geographic
241 distance for each of the 10 distance classes was constructed with 9999 permutations using
242 the package VEGAN in R.

243

244 *Population structure*

245 The population structure was examined using STRUCTURE 2.3.4 (Pritchard et al. 2000),
246 which implements a Bayesian clustering method using multi-locus allele frequency data. The
247 STRUCTURE settings were the admixture and allele frequency correlated model with
248 previous sampling location information (LOCPRIOR; Hubisz et al. 2009). The algorithm was
249 run 20 times for each K from 1 to 14 with a burn-in of 20,000 followed by 30,000 Markov
250 chain Monte Carlo (MCMC) replicates. The program CLUMPAK (Kopelman et al. 2015)
251 was used to compile the results of the STRUCTURE analysis for each K. STRUCTURE
252 HARVESTER (Earl and vonHoldt 2012) was employed to calculate the probability of the
253 data for each K ($\text{LnP}(D)$; Pritchard et al. 2000), the corresponding standard deviation, and
254 Evanno’s delta K (Evanno et al. 2005).

255

256 *Association of genetic variation with geographic distance and water temperature*

257 To evaluate the effects of space and water temperature on genetic differentiation, the
258 independent correlations of F_{ST} with water temperature differences and geographic distance
259 were calculated by Mantel tests with 9999 permutations. To identify confounding effects
260 between space and water temperature if present, multiple regression on distance matrices

261 (MRM; Lichstein 2007) was used, with “water temperature differences + geographic distance”
262 as the explanatory variable. Mantel tests and MRM were performed with the package
263 ECODIST in R. These analyses were conducted at three scales: (a) using all 20 populations
264 (waterway distance 0.3-70.6 km), (b) using upstream 12 populations (Pop1-12; waterway
265 distance 0.3-24.1 km), and (c) using structured 9 populations (Pop4-12; populations assigned
266 as one large cluster in STRUCTURE). We used scale (b) to account for the mobility of *Cottus*
267 and to exclude the effects of a long spatial gap and a dam, and scale (c) was used to prevent
268 biases when including sites displaying high genetic divergence (‘outliers’; Koizumi et al.
269 2006).

270 Some studies have considered it inappropriate to apply the Mantel test to the raw
271 vector data and to take the difference to create a matrix (Legendre and Legendre 2012). Hence,
272 we also used distance-based RDA, which is a constrained ordination approach that can use
273 environmental variables as vector data and has higher power than the Mantel test (Harmon
274 and Glor 2010; Legendre et al. 2015). We used genetic variation as the response variable and
275 water temperature and spatial variables derived from geographic distance as explanatory
276 variables. For genetic variation, a principal coordinate analysis (PCoA) of the pairwise F_{ST}
277 matrix was conducted, and all axes were used as response variables. Spatial predictors were
278 generated as a set of distance-based Moran’s eigenvector maps (MEMs; Griffith and Peres-
279 Neto 2006), vectors that capture broad- to small-scale spatial structures. MEMs were
280 generated from the geographic distance matrix using the package ADESPATIAL 0.3.8 (Dray
281 et al. 2020) in R. To identify meaningful MEM predictors, forward selection (999
282 permutations) was used for generated MEM dataset. In each RDA with the explanatory
283 variables of water temperature and geographic MEM variables, adjusted R-square values
284 (R_{adj}^2), which penalize the increase in explanatory power due to an increase in the number of
285 explanatory variables, were calculated. RDAs were performed on three scales, as in the
286 Mantel tests and MRM analyses. RDAs including forward selections were performed with
287 the package VEGAN in R.

288

289 *Contemporary gene flow*

290 To infer gene flow patterns, BA3SNP (Mussmann et al. 2019), a reconstructed version of
291 BayesAss (Wilson and Rannala 2003) that estimates dual-direction pairwise migration rates
292 (here migration is successful dispersal that contributes to gene flow) over a few generations,
293 was used for the 20 populations. To improve the convergence of BayesAss, another SNP
294 filtering criterion that increases the variance among populations was used. From the *stacks*
295 *dataset*, loci present at a rate of more than 40% among at least two populations were extracted
296 with a minimum minor allele count of three. As in the other analyses, sites showing excess
297 heterozygosity (>0.6) were removed, and the output was limited to one SNP per locus. Then,
298 1291 SNPs were obtained. We set 20,000,000 MCMC iterations, including a burn-in period
299 of 10,000,000. Following the proposal of Meirmans (2014), we performed 10 independent
300 runs with different seed values and chose the run with the lowest Bayesian deviance to
301 overcome poor MCMC sampling. The inferred migration rates with 95% credible intervals
302 that did not include zero were regarded as significant. We then defined the *net immigration*
303 *rate* into a given population A, from another population B, as the [estimated value of B->A
304 minus that of A->B]. Then, the *mean net immigration rate* for each population (the mean net
305 immigration rate for a given population from all of its paired net immigration rate values
306 between all other populations) was calculated to measure the degree to which a population is
307 a donor or a recipient of migrants (Hänfling and Weetman 2006; Sexton et al. 2016). To
308 investigate whether water temperature affects the source-sink structure independent of
309 upstream-downstream dispersal, we constructed a linear model with a response variable of
310 the mean net immigration rate and explanatory variables as maximum water temperature and
311 elevation. The Pearson's correlation between water temperature and elevation was -0.39; thus,
312 no multicollinearity was considered. The independent effects of water temperature and
313 elevation were assessed by the partial regression coefficient (β) and standardized partial
314 regression coefficient (std β). Likelihood ratio tests were used to determine the significance

315 of explanatory variables. This analysis was conducted using the package LMTEST (Zeileis
316 and Hothorn 2002) in R.

317

318

319 **Results**

320 *Heterogeneity of water temperature*

321 Summer water temperatures varied within the watershed, and the maximum water
322 temperature ranged from 12.5 to 23.5 °C. The correlation between geographic distance and
323 water temperature difference was low (Mantel $r = 0.08$, $p = 0.45$). In the Mantel correlogram
324 for water temperature, r values were not significant in all distance classes (Fig. 2A),
325 indicating little spatial autocorrelation.

326

327 *Genetic diversity, divergence, and population structure*

328 F_{IS} did not deviate significantly from zero except in one population (Pop20), suggesting that
329 HWE could be assumed in most populations. The H_E was similar across the watershed, but
330 one population located in the upstream section of a check dam (Pop13) displayed a slightly
331 lower value (Table 1). Across the 20 populations, the F_{ST} value was 0.038, indicating weak
332 population differentiation, and pairwise F_{ST} values ranged from 0.001 to 0.127 (Table S1). In
333 a Mantel correlogram, r values were significantly positive in up to the second distance classes
334 (approximately 15 km) and reached zero at a distance of 27 km (Fig. 2B).

335 In the STRUCTURE analysis, the probability of the data (LnP(D)) increased
336 progressively with each K , and ΔK was highest at $K = 4$ (Fig. S1). Distinct clusters
337 corresponding to the geographic structure were detected up to $K = 6$ (Fig. 1). For $K = 2$, the
338 genomes of the individuals in Pop13 were grouped into a single unique cluster. For $K = 4$,
339 Pop1-3 and Pop16-19 were grouped as additional clusters. Pop14,15,20 was inferred to be
340 an admixed cluster when $K = 4$ and a distinct unique cluster when $K = 6$. Pop3 also formed
341 a unique cluster at $K = 6$. Pop4-12 was assigned to almost one cluster at even large K .

342

343 *Effects of space and water temperature on genetic differentiation*

344 Mantel tests captured significant correlations between pairwise F_{ST} and waterway geographic
345 distance (Mantel $r = 0.27$, $p < 0.05$; Fig. 3), indicating a significant effect of IBD. Conversely,
346 no correlation between F_{ST} and water temperature differences was detected (Mantel $r = -0.10$,
347 $p = 0.56$). The MRM with these two matrices as an explanatory variable did not show a
348 significant relationship, and the R^2 value was not very different from that of geographic
349 distance alone. The same pattern was obtained at different spatial scales (Table 2). The
350 forward selection of the MEM variables identified six significant predictors and the RDA
351 with selective MEMs was significant with an R_{adj}^2 value of 0.61 (Fig. S2). Even in RDA,
352 water temperature did not explain the genetic variation at all.

353

354 *Gene flow*

355 A total of 11 significant migration rates were detected (Tables 3 and S2). All of them were
356 migration from relatively low-temperature sites (mean 14.3 °C [SD 2.90]) to high-
357 temperature sites (mean 20.0 °C [SD 1.84]). Most of them were upstream to downstream
358 direction, but they also included migration from downstream to upstream direction (Pop4 to
359 5 and Pop12 to 11). The linear model indicated that water temperature had a significant effect
360 on the mean net immigration rates (std $\beta = 0.542$; $p < 0.05$) but elevation did not (std $\beta = -$
361 0.077 ; $p = 0.70$; Table 4). Populations with lower water temperatures displayed lower mean
362 net immigration rates, indicating that they are the source populations (Fig. 4).

363

364

365 **Discussion**

366 *Genetic differentiation*

367 In riverscapes, lower genetic diversity upstream than downstream has been recognized as a
368 typical pattern (Blanchet et al. 2020; Thomaz et al. 2016). However, in this study, genetic

369 diversity was similar regardless of longitudinal position (Table 1). The relative suitability of
370 the upstream environment for the sculpin and the source-sink gene flow that is not only in
371 the upstream-downstream direction (Table 4) may have suppressed the decrease in genetic
372 diversity of upstream populations.

373 Among the entire sampling sites, a low level of genetic differentiation was
374 observed ($F_{ST} = 0.038$). A study of *C. nozawae* in Tohoku district displayed high genetic
375 differentiation within one watershed (Ito et al. 2018), and previous studies investigating other
376 fluvial *Cottus* species within 30-50 km² scale have often reported high F_{ST} values (Hänfling
377 and Weetman 2006; Junker et al. 2012; Ruppert et al. 2017; but see Peacock et al. 2016). In
378 the present study, the degree of genetic differentiation was somewhat lower than those
379 observed in these previous studies. Other than gene flow, the large effective population size
380 and consequent low genetic drift from the common ancestor may result in low genetic
381 differentiation. Nevertheless, high-resolution genome-wide SNP analysis allowed genetic
382 differentiation within the watershed to be clearly detected.

383 Distinct genetic structure along the geographic structure was inferred within the
384 watershed (Fig. 1). There was a significant correlation between genetic differentiation and
385 geographic distance, but summer water temperature had no effect on the strength of genetic
386 differentiation (Fig. 3). Thus, the possibility that differences in water temperature lead to
387 genetic differentiation via local adaptation is low.

388 Only a handful of studies have investigated the associations between the
389 environment and the genetic structure of sculpin. Ruppert et al. (2017) analyzed the
390 relationship between genetic and elevation differences and displayed a pattern of IBE along
391 elevation. Another study demonstrated genetic divergence between different habitat types
392 (Dennenmoser et al. 2014). However, most studies have been unable to eliminate the
393 interaction of environment and geographic distance. It is not clear whether populations in
394 these different regions and species actually have IBE patterns, but the IBE of sculpin may
395 not be very common in wild populations.

396 There may be some factors related to genetic differentiation that are not clear from
397 this study (residuals). For example, the factors promoting the genetic divergence between
398 Pop1-3 and Pop4-12 detected in STRUCTURE analysis are still unknown. Since we assume
399 the IBE pattern as a hypothesis, we used only an environmental variable of each site, but the
400 factor of genetic differentiation may also lie on the pathway between populations (e.g.,
401 streambed slope). To identify those factors contributing to the residuals of genetic
402 differentiation, analyses using environmental data across the entire watershed (e.g., White et
403 al. 2020) and denser sampling are necessary.

404

405 *Gene flow*

406 Another important insight into population structure was obtained via gene flow analysis.
407 Almost all significant migration was detected in population pairs within approximately 15
408 km, the range where the spatial correlation of genetic variation was significant in the Mantel
409 correlogram (Fig. 2B). Most of the 11 cases of significant migration were downstream
410 direction (Tables 3 and S2), which is consistent with several previous studies about fluvial
411 sculpins that have revealed the source-sink structure from upstream to downstream sites
412 (Hänfling and Weetman 2006; Junker et al. 2012). However, the present study also detected
413 migrations toward tributaries at higher elevations, from lower-temperature tributaries to
414 higher-temperature tributaries. Of course, we should be cautious about the accuracy of
415 BayesAss estimates (Meirmans 2014). However, the estimated pattern displayed a clear trend,
416 and it could be concluded that suitable habitats with low summer water temperatures behave
417 as “source” habitats in the watershed. The effect of summer water temperature on the mean
418 net immigration rate was larger than that of elevation (Table 4) and populations with lower
419 temperatures had a greater degree of individual supply into other populations (Fig. 4).

420 Although BayesAss estimates migration rates over a short period, the estimates
421 should reflect the gene flow pattern in the studied ecosystem. In this study, significant
422 migration was estimated between populations with different summer water temperatures at

423 close locations (Fig. 1; Table 3). This finding implies that gene flow in the direction of across
424 dissimilar environments is high. This type of gene flow is referred to as ‘counter-gradient
425 gene flow’ (Sexton et al. 2014). The IBE pattern is detected when gene flow among similar
426 environments is high; therefore, the counter-gradient gene flow is the opposite pattern to IBE
427 and suppresses IBE. The ecological processes driving this gene flow pattern could not be
428 identified in this study, but it may at least be related to differences in population density
429 documented by Suzuki et al. (2021) in the same watershed as the present study (i.e., high
430 density in low-temperature streams).

431 Goto (1998) used mark-recapture methods and reported that most mature
432 individuals stay at the same site for more than one year, indicating almost no migration. While
433 the mark-recapture method addresses only adult movement, genetic approaches also reflect
434 movement during early life stages that is not easily captured by mark-recapture approaches
435 (Lamphere and Blum 2012). If passive gene flow (i.e., the transportation of juveniles or eggs)
436 is the driving force of gene flow, some high migration rates obtained in our study do not
437 conflict with the results of mark-recapture methods. Furthermore, some previous studies
438 using genetic methods on *Cottus* reported similar migration rates to those obtained in the
439 present study and discrepancies between genetic and mark-recapture estimates (Junker et al.
440 2012; Lamphere and Blum 2012), suggesting that the detected degree of gene flow is
441 reasonable. Still, it remains to be seen whether the estimated degree of gene flow is constantly
442 observed. For instance, flooding is a factor that increases fish dispersal (Blondel et al. 2021;
443 Natsumeda 2003), and it should be noted that we cannot rule out the possibility that recent
444 large disturbances (e.g., relative large typhoon in 2016) may have temporarily promoted
445 dispersal. To make the findings on gene flow more general, more research under different
446 conditions and other evidence such as differences in turnover rates are needed.

447

448 *Implications for future management*

449 Because sculpins are not commercially or recreationally important fish, they are impacted

450 relatively little by human translocations (Lucek et al. 2018). Thus, sculpin genes have a
451 footprint of a long-term past history, and the diversity of each watershed should be protected.
452 Within the watershed, while each tributary is characterized by a unique genetic composition,
453 loose connectivity via gene flow between tributaries is maintained. Indeed, fragmented
454 populations such as Pop13 show lower genetic diversity. In particular, populations in streams
455 with less suitable environments may be maintained by immigration from low-temperature
456 streams that are more suitable habitats. Therefore, under ongoing climate change, (i) the
457 preservation of low-temperature streams and (ii) the prevention of making unpassable
458 barriers between source and sink populations would be the key measures for conserving
459 sculpin populations. Considering that cold-water fish other than benthic fish have been well
460 studied and revealed to show high mobility between tributaries, these measures will be
461 effective for overall cold-water fish conservation. Groundwater and its associated factors
462 such as watershed geology are useful for identifying low-water-temperature streams and
463 conserving possible “source” populations.

464 Local conservation measures can also be proposed. If the distance at which the
465 spatial coefficient becomes nonsignificant is considered to represent the appropriate
466 management unit size (Diniz-Filho and De Campos Telles 2002), stream networks should not
467 be subdivided into stretches of less than 15 km to maintain genetic resources. However, even
468 if a large continuous habitat is maintained, then sections consisting solely of high-
469 temperature streams will lead to local population declines due to the absence of individual
470 supply sources. Thus, care must be taken whenever rivers are fragmented. In the Sorachi
471 River, many *C. nozawae* inhabit high density (Suzuki et al. 2021), and they are not considered
472 to be in danger of immediate extinction. However, maintaining the abundance of general
473 species is very important to avoid ecosystem breakdown (Baker et al. 2019). In some regions
474 in Japan (Tohoku district), *C. nozawae* populations are in danger of local extinction (Ministry
475 of the Environment Government of Japan 2020). Therefore, regardless of how large the
476 population is, maintaining continuous stream networks and the connectivity to low-

477 temperature sections in the networks is needed for future population persistence.

478

479 *Implications for landscape/riverscape genetics*

480 Decoupling distance and environment in a locality is a strong strategy for rigorously
481 evaluating competing hypotheses (Gilbert and Lechowicz 2004). Through this approach, we
482 revealed a pattern that has not been proposed for the examined genus. Freshwater populations
483 have been identified as potentially fruitful targets for the application of a landscape genetic
484 approach to delineate population structure (Kanno et al. 2011), and we focused on
485 groundwater to reveal the association of water temperature with the genetic structure of cold-
486 water fish populations.

487 In this study, the possibility of a source-sink structure caused by counter-gradient
488 gene flow was suggested instead of IBE. Many source-sink structures from core populations
489 to edge (peripheral) populations have been studied. However, local gene flow patterns are
490 also important for discussing population structure in changing environments. The IBE pattern
491 is easily detected when spatial-environmental correlation is present, but it is important to
492 scrutinize whether it truly exists. Although the local source-sink pattern provides notable
493 insights for conservation and evolution (Iles et al. 2018), a previous meta-analysis showed
494 that counter-gradient gene flows are much less frequently reported than IBE (Sexton et al.
495 2014). Counter-gradient gene flow is inherently exclusive to IBE and may tend to be
496 neglected. In population genetic studies, the results often differ from the hypotheses (Myers
497 et al. 2019). As detecting the relationship between the environment and genetic differentiation
498 is still challenging, we hope that the role of the environment in genetic divergence will be
499 better understood in many studies with the help of sampling strategies.

500

501

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510

511 **Conflict of Interest**

512 The authors declare no conflict of interest.

513

514 **Data Archiving**

515 Raw MIG-seq reads were deposited in the DDBJ Sequence Read Archive under accession
516 number DRA011249. The other data are available on Figshare
517 (<https://doi.org/10.6084/m9.figshare.13383245>).

518

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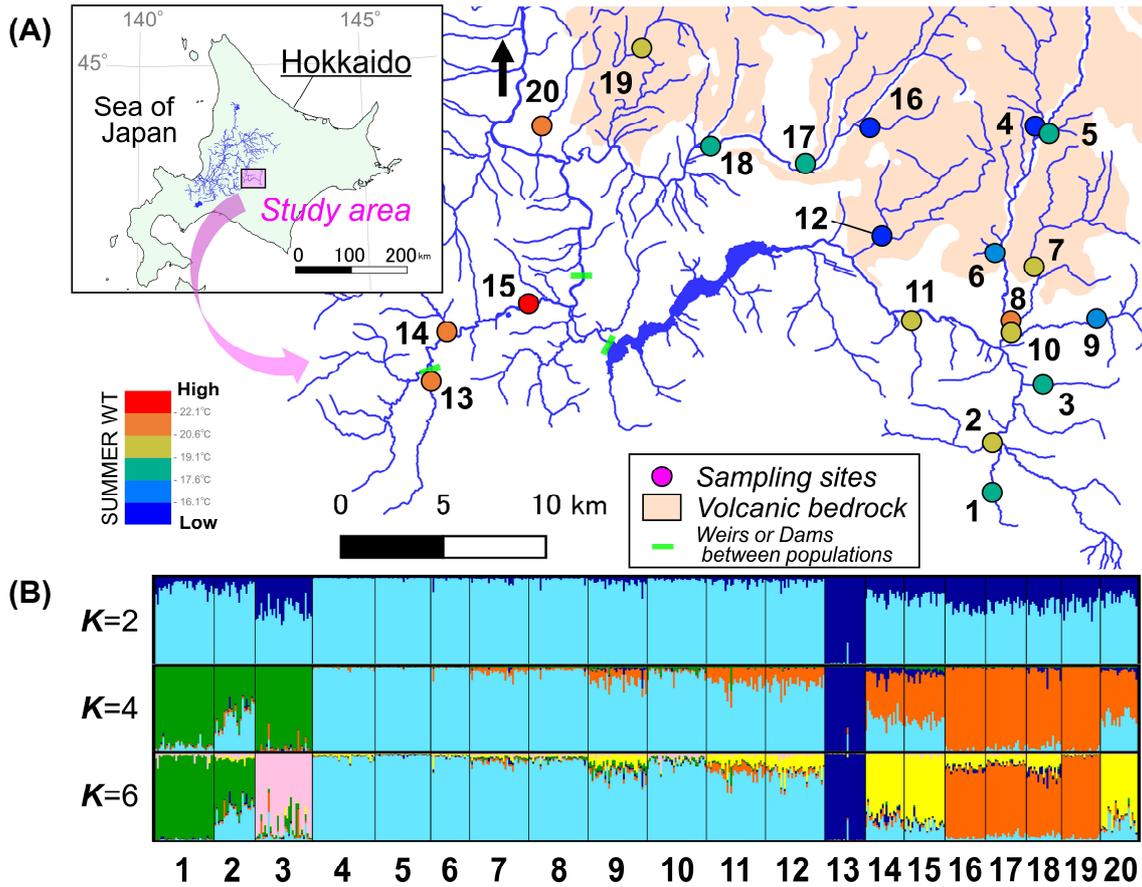
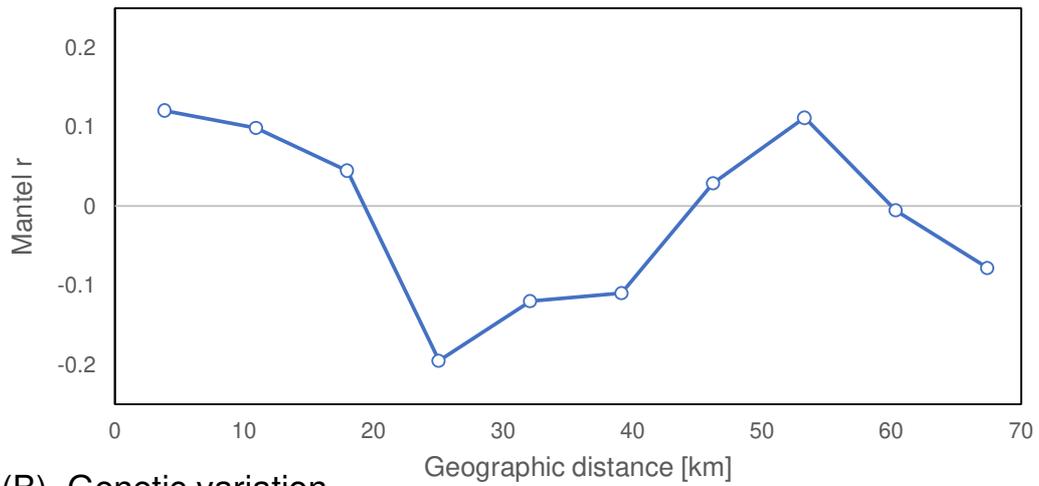


Fig. 1. (A) Study area. The fill colors of the sampling site depend on the maximum water temperature. Site labels correspond to population IDs in the text and Table 1. (B) Population structure inferred by STRUCTURE. The number indicates the population IDs.

(A) Water temperature



(B) Genetic variation

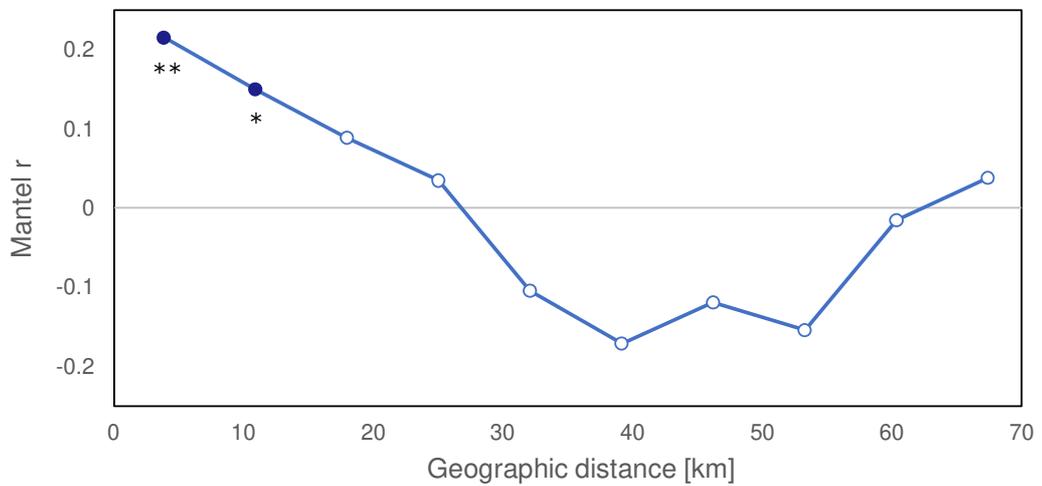


Fig. 2. Mantel correlograms showing spatial autocorrelation of (A) water temperature and (B) genetic data. Filled circles indicate significant correlations from a null model of spatial structure (* $p < 0.05$, ** $p < 0.01$).

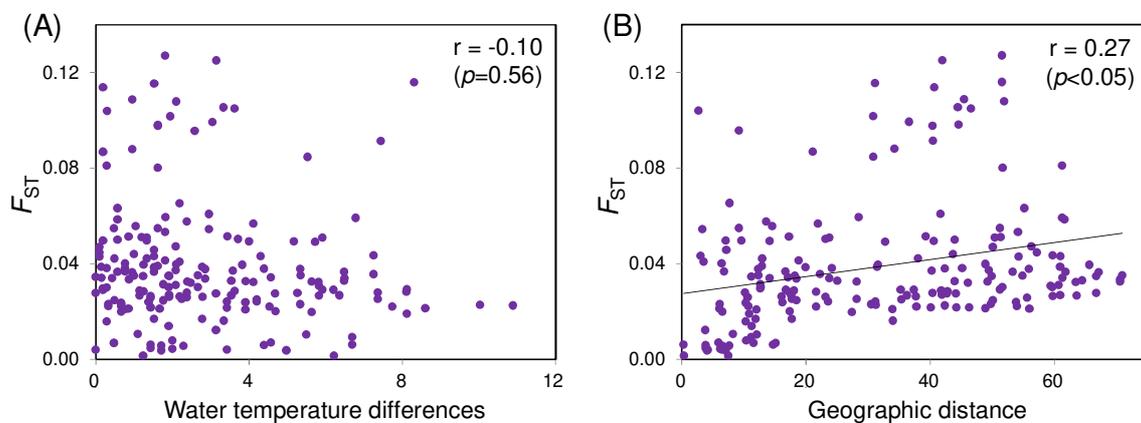


Fig. 3. Relationship of genetic differentiation with (A) water temperature and (B) geographic distance.

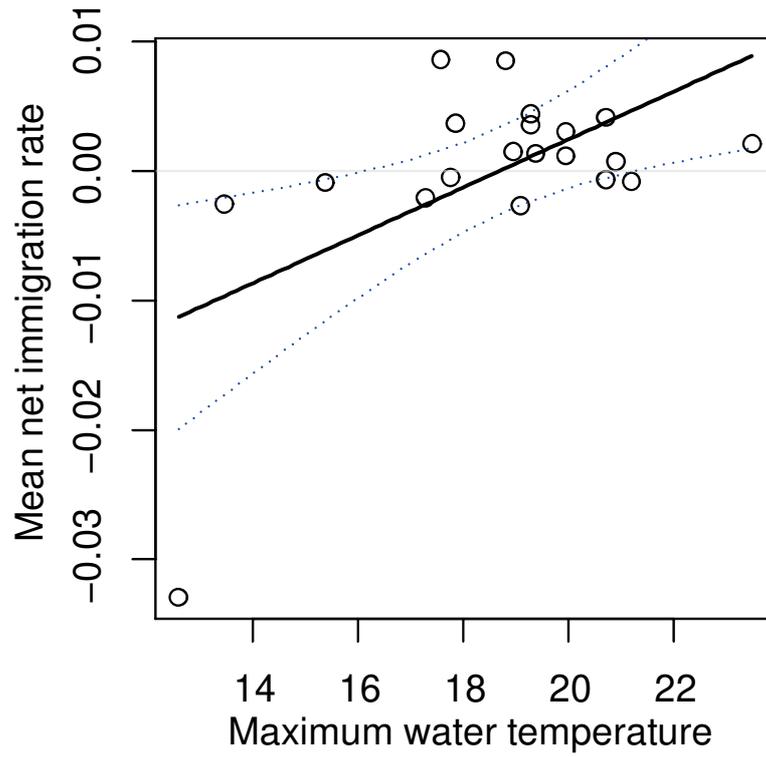


Fig. 4. Variation in the mean net immigration rate with maximum water temperature. Populations with negative net immigration (i.e., greater emigration) indicate relative source populations, whereas populations with positive values (i.e., greater immigration) indicate relative sink populations. The dashed lines indicate the 95% confidence interval.

Table 1. Details of the sampling sites and genetic diversity of each population.

Population ID	Localities	Longitude (E°)	Latitude (N°)	Elevation (m)	N	H_E	F_{IS}
1	Tomamu River	142.670	43.057	526	32	0.121	0.016
2	Kinnosawa River	142.672	43.085	466	22	0.113	0.005
3	Shikerebenaizawa River	142.699	43.107	451	31	0.112	0.006
4	Ehoroakanbetsu River	142.699	43.218	510	34	0.114	0.012
5	Ozawa River	142.703	43.218	511	30	0.113	0.004
6	A tributary of the Seesorapuchi River	142.678	43.163	445	21	0.111	0.009
7	Pankeyara River (up)	142.699	43.155	450	32	0.112	0.007
8	Pankeyara River (down)	142.680	43.133	414	32	0.114	0.001
9	Peiyurushiebe River (up)	142.739	43.136	498	32	0.108	0.007
10	Peiyurushiebe River (down)	142.680	43.129	413	32	0.113	0.014
11	Ecchudantai-no-sawa River	142.624	43.137	387	32	0.113	-0.001
12	Ikutora River	142.603	43.173	376	32	0.113	0.009
13	Tonashibetsu River (up)	142.335	43.118	378	22	0.085	0.003
14	Tonashibetsu River (mid)	142.346	43.135	340	21	0.106	0.007
15	Tonashibetsu River (down)	142.393	43.149	289	22	0.113	0.014
16	Nishitappu River (up)	142.593	43.224	375	22	0.115	0.003
17	Nishitappu River (mid)	142.558	43.207	325	22	0.117	0.000
18	Nishitappu River (down)	142.501	43.216	292	19	0.112	-0.001
19	Roseppu River	142.463	43.260	336	21	0.101	0.002
20	Onkosawa River	142.403	43.218	239	20	0.108	0.038*

N, number of individuals; H_E , expected heterozygosity; F_{IS} , fixation index (significant deviation from zero is denoted by asterisks; * $p < 0.05$ after Bonferroni correction).

Table 2. Summary of Mantel tests, MRM, and RDAs on three spatial scales for examining the effect of space and water temperature on genetic differentiation (F_{ST}).

Explanatory matrices	All 20 populations	Upstream 12 populations	Structured 9 populations
Mantel test (r^2)			
Water temperature	0.010 [†] ($p=0.56$)	0.023 [†] ($p=0.53$)	0.000 ($p=0.94$)
Geographic distance	0.072 ($p<0.05$)	0.074 ($p=0.24$)	0.298 ($p<0.05$)
MRM (R^2)			
Water temperature+ Geographic distance	0.087 ($p=0.13$)	0.116 ($p=0.32$)	0.304 ($p<0.05$)
RDA (R_{adj}^2)			
Water temperature	-0.017 ($p=0.51$)	-0.073 ($p=0.92$)	-0.083 ($p=0.69$)
Geographic distance (MEMs)	0.609 ($p<0.01$)	0.374 ($p<0.001$)	0.486 ($p<0.05$)

Mantel r values were squared to compare MRM R^2 values. R_{adj}^2 values can take negative values when the relation is very small. P -values were obtained by two-tailed tests. Upstream 12 populations, Pop1-12; Structured 9 populations, Pop4-12. [†]: original r values were negative.

Table 3. Significant migration estimated by BA3SNP (BayesAss). The estimated immigration rate and its 95% credible interval (95% CI) are shown.

Direction			Estimate	95% CI
Pop1	to	Pop2	0.033	0.003-0.063
Pop4	to	Pop2	0.040	0.007-0.072
Pop4	to	Pop5	0.201	0.156-0.246
Pop4	to	Pop6	0.155	0.105-0.205
Pop4	to	Pop7	0.084	0.045-0.123
Pop4	to	Pop10	0.064	0.029-0.098
Pop4	to	Pop11	0.038	0.010-0.066
Pop4	to	Pop15	0.032	0.003-0.060
Pop12	to	Pop11	0.026	0.002-0.050
Pop14	to	Pop15	0.045	0.010-0.080
Pop16	to	Pop17	0.071	0.031-0.112

Table 4. The linear model explaining the mean net immigration rate. *P*-values were obtained by likelihood ratio tests.

Variable	β (SE)	std β	χ^2	<i>p</i>
(Intercept)	-2.97*10 ⁻² (1.85*10 ⁻²)	--	--	--
elevation	-7.86*10 ⁻⁶ (2.21*10 ⁻⁵)	-0.077	0.15	0.70
water temperature	1.76*10 ⁻³ (6.97*10 ⁻⁴)	0.542	6.35	<0.05