Adaptation of the eyes of grass puffer (*Takifugu niphobles*) to the riverine and marine environments

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Abstract

Since fish inhabit heterogeneous light environments, fish vision is known as a prime example of adaptive evolution. Many species of marine fish migrate to rivers, but little is known about whether these species switch their vision when inhabiting rivers or whether they are adapted to the environment of both rivers and the sea. Grass puffer (*Takifugu niphobles*) is a marine fish species that frequently migrate to the rivers. In this study, we investigate grass puffers from both riverine and marine populations and analyzed the gene expression in the eyes. Phylogeny and population genetics of riverine and marine grass puffers indicated that riverine and marine grass puffers are from the same population. High-throughput RNA sequencing was used to compare expression levels of two marines and one riverine pufferfish species, as well as riverine and marine glass puffers. The results suggest that the grass puffers are estimated to be adapting their visual system to two environments, the river and the sea, rather than switching their visual system by changing the expression of vision-related genes. Four of the six genes with higher expression in riverine populations than in marine populations of grass puffer were heat shock proteins and related genes. The expression levels of these genes were higher in riverine grass puffer than in other marine and river pufferfish species, suggesting that the grass puffer individuals are adapting to the environmental difference that they face when they migrate to the river by increasing the expression levels of heat shock protein and related genes.

Introduction

The environments in the rivers and the sea differ in many aspects, including salinity, light intensity, and wavelength of light. For example, in freshwater with low transparency, water with micro-particles scatters short wavelength light, and the light in turbid water is dominated by long wavelengths (Maan, et al. 2006; Seehausen, et al. 2008; Terai, et al. 2017). In contrast, long-wavelength light is absorbed by water in the clear ocean, and short-wavelength light is predominant (Kirk 1994). Since fish species inhabit this heterogeneous light environment, they have adapted their vision to the light environment (Terai, et al. 2006; Seehausen, et al. 2008; Nagai, et al. 2011; Terai, et al. 2017). Accordingly, fish vision is known as a prime example of adaptive evolution.

Vertebrate visual pigments consist of light-absorbing chromophores and the protein component, opsin (Shichida 1999). Spectral sensitivity is determined by the type of chromophore [with 11-*cis* retinal (A1-) or 11-*cis* 3-dehydroretinal (A2-derived retinal)] and by interactions between chromophores and amino acid residues of opsin (Yokoyama 2000). The replacement of A1- with A2-derived retinal in the visual pigments shifts the absorption to a longer wavelength (Harosi 1994; Parry and Bowmaker 2000).

The enzyme CYP27c1 is known to be involved in the switch from A1 to A2, and the expression level of this gene correlates with the amount of A2 (Enright, et al. 2015; Morshedian, et al. 2017). Fish species use A1 retinal and A2 retinal, with freshwater fish mainly using A2 and marine fish using A1 retinal (Toyama, et al. 2008). At different stages of the life cycle, A1 and A2 retinal are known to be differentially used. Salmon use A1 retinal when living in the sea, and switch to A2 when they migrate up the river to

spawn (Beatty 1966). In lamprey, A1 and A2 are differentially used for juveniles and upstream migrating adult lamprey (Morshedian, et al. 2017). Interestingly, marine fish species migrating to the river use both A1 and A2 retinal (Toyama, et al. 2008). This represents a characteristic of both riverine and marine environments, but little is known about whether these species mainly use A2 retinal when they live in rivers or whether they have both A1 and A2 retinal in both rivers and the sea. Furthermore, little is known about whether these species express different levels of opsin genes between the rivers and the sea.

Grass puffer (*Takifugu niphobles*) is a marine fish species that frequently migrate to the rivers. Grass puffers are abundant both in rivers and in the sea during the same season. Grass puffer possesses both A1 and A2 retinal (Toyama, et al. 2008). In this study, we analyzed the phylogeny and population genetics of riverine and marine populations of grass puffer, and also analyzed gene expression levels in the eye of grass puffer from both riverine and marine populations. In addition, the gene expression levels in the eyes of marine and riverine pufferfish species were compared with those of two populations of grass puffer to show how grass puffer can inhabit two environments, one in the river and the other in the sea.

Result

No genetic differentiation between the riverine and marine populations of *T. niphobles*

80 Riverine grass puffers were collected 1.3 km upstream from the mouth of the river (Fig.
81 S1, Table S1). This is an environment where the water becomes brackish when the tide
82 rises and freshwater when the tide recedes. Based on our observations, the grass puffers
83 are not seen in the rivers during midwinter (mid-January through the end of February),
84 but many individuals were observed in the rivers from March through December. Many
85 grass puffers were also found in rivers that became freshwater at low tide. Marine grass
86 puffers were collected on a beach 10 km from the river grass puffer collection site. Since
87 this beach is more than 3 km from the nearest river, we expected less direct migration
88 with the river grass puffers to the marine population.

89 As a first step, we investigated the genetic relationships between the river and marine
90 populations of the grass puffer. Single nucleotide polymorphisms (SNPs) were extracted
91 from short reads of grass puffer from the riverine and marine populations (see Methods),
92 mapped to coding regions of a reference genome (*Takifugu rubripes*), and the mean of
93 Fst was calculated for a total of 172,214 SNPs. The mean Fst value was almost 0 (-
94 0.00026635), indicating no genetic differentiation between the riverine and marine
95 populations.

96 *T. rubripes* and *T. synderi*, the same genus as grass puffer, are marine species. SNPs
97 were extracted from individuals of these species collected from the sea (Fig. S1, Table
98 S1) in the same method as grass puffer individuals (see Methods). The SNPs were
99 combined with the grass puffer SNPs and a phylogenetic tree was constructed using the
100 ML method with a total of 266,954 sites. A phylogenetic tree showed that grass puffer
101 formed a monophyletic group, but the riverine and marine populations were not

102 differentiated (Fig. 1). Using the same data set, a principal component analysis (PCA)
103 showed similar results, with no differentiation between the riverine and marine
104 populations of grass puffer (Fig. 2A). Furthermore, ADMIXTURE analysis using the
105 same data set showed that the grass puffers formed either a single cluster (K=2, 3) or
106 multiple clusters (K=4-6), but the riverine and marine populations did not differentiate
107 (Fig. 2B). These results indicate that riverine and marine grass puffers are from the same
108 population. Grass puffer is known to spawn simultaneously in the surf on the beach in
109 June, and subpopulations are presumed to mix at this time, which may prevent genetic
110 differentiation.
111
112 **Expression differences in the eyes between the riverine and marine populations of *T.***
113 ***niphobles***
114 RNA sequencing was performed to compare gene expression levels in the eyes of grass
115 puffer from the riverine and marine populations. Total RNAs were extracted and 10-15
116 Gb sequences were determined from four individuals each of grass puffer from the river
117 and marine populations. These high-throughput sequences were mapped to a reference
118 genome sequence of *T. rubripes* to compare gene expression levels between the riverine
119 and marine populations of grass puffer. As a result, we found 25 differentially expressed
120 genes with statistical significance (Fig. 3, Table S2). Among the 25 genes, 6 genes were
121 highly expressed in the riverine population and 19 genes were highly expressed in the
122 marine population (Fig. 3, Table S2). In this study, we focused on six of the 25 genes that
123 are highly expressed in the riverine population to reveal how marine grass puffer

124 individuals can inhabit a freshwater environment. Three of these six genes were heat
125 shock protein genes (Hsp 9, 47, 90) (Sørensen, et al. 2003; Abumourad 2009). unc45b
126 has also been reported to be part of a chaperone system that interacts with Hsp90 (Hansen,
127 et al. 2014).

128 Next, we examined whether the expression levels of genes related to vision varied
129 between the riverine and marine populations. The photosensitivity of visual pigments is
130 tuned by the amino acid sequence of opsins and the type of chromophores (Yokoyama
131 2000). Therefore, we first searched for the opsin genes and the CYP27c1 gene for
132 switching chromophores in the genome of *T. rubripes*. The green-sensitive opsin gene,
133 *RH2*, and the long-wavelength sensitive opsin gene, *LWS*, were present as color vision
134 opsins in the annotation of the genome of *T. rubripes*. In addition, the *RH1* gene for
135 scotopic vision and *CYP27c1* were also present in the annotation. Many fish species have
136 the *SWS* gene, a blue-sensitive opsin, in their genome and are sensitive to blue light.
137 Therefore, we searched for the *SWS* gene in the genome sequence of *T. rubripes* using
138 the cichlid *SWS* sequence as a query but did not find any sequences with high similarity.
139 We also searched for the *SWS* gene in the assembled RNA sequences of the grass puffer
140 but did not find any homologous sequence. Based on these results, the grass puffer has
141 an opsin repertoire of *RH2*, *LWS*, and *RH1*. Comparisons of gene expression levels of the
142 three opsin genes and *CYP27c1* between the river and marine populations of grass puffer
143 showed no difference in gene expression levels with statistical significance (Fig. 4C, 4D,
144 4F).
145

146 **Comparisons of gene expression between species in Tetraodontidae**

147 The opsin repertoire was examined for *T. rubripes* and *T. synderi*, as well as
148 *Dichotomyctere nigroviridis*, which is a Tetraodontidae species inhabiting rivers and
149 ponds in Southeast Asia (Rainboth 1996). We also searched for the *SWS*, *RH2*, *LWS*, and
150 *RH1* genes in the assembled RNA sequences of *T. rubripes*, *T. synderi,* and *D.*
151 *nigroviridis*, and found the homologs of these genes, while we did not find any
152 homologous sequence of the *SWS* gene. Based on these results, we infer that the genus
153 *Takifugu*, including grass puffer, and *D. nigroviridis* have an opsin repertoire of *RH2*,
154 *LWS*, and *RH1* and use a dichromatic color vision system.

155 The expression levels of three opsin genes, *CYP27c1*, and genes with higher expression
156 levels in the riverine population than in the sea population of the grass puffer were
157 compared with that of *T. rubripes*, *T. synderi*, and *D. nigroviridis*, and the grass puffer
158 populations. The relative expression of *RH*2 in marine *T. rubripes* and *T. synderi*
159 exceeded 50% of all opsins, and the expression of *LWS* was also relatively higher than in
160 the grass puffer (Fig. 4A, 4B). Therefore, these two marine species primarily express
161 color vision opsin genes. In contrast, the relative expression of *RH1* in riverine *D.*
162 *nigroviridis* exceeded 95%, indicating that a scotopic vision opsin gene was
163 predominantly expressed in this species (Fig. 4E). *RH1* was predominantly expressed in
164 the grass puffer, but less than in *D. nigroviridis*, with 67% and 84% in the riverine and
165 marine populations, respectively (Figs. 4C, 4D). *RH2* and *LWS* were highly expressed in
166 grass puffer than in *D. nigroviridis*. *CYP27c1* was expressed at lower levels in the marine
167 species *T. rubripes* and *T. synderi* than in the grass puffer, and at a higher level in *D.*

168 *nigroviridis* compared to grass puffer (Fig. 4F). Among the six genes with higher
169 expression levels in the riverine population than in the marine population of the grass
170 puffer, we focused on four genes with higher expression (TPM > 5). These four genes
171 expressed higher levels in the river population of the grass puffer than in the other species
172 (Fig. 5).
173
174 **Discussion**
175 When marine fish migrate to a river, they face a riverine environment that differs from
176 that of the sea. Therefore, we expected that marine fish that migrate to the river may
177 respond to a different environment. This study investigated mRNA expression in the eyes
178 of the grass puffer, which inhabit both the river and the sea. As for vision, the repertoire
179 of opsin genes and their expression levels, were comparable between the riverine and
180 marine populations of the grass puffer. The expression levels of *CYP27c1*, representing
181 the amount of A2 retinal, were also comparable. Thus, grass puffers are presumed to have
182 the same usage of vision in both marine and riverine environments. In contrast, *RH1* was
183 predominantly expressed in the river species *D. nigroviridis*, while cone opsins were
184 predominantly expressed in the marine species *T. rubripes* and *T. synderi*. Comparing the
185 expression of the opsin genes of all species used in this study, the grass puffer showed an
186 expression pattern intermediate between that of marine and river species. The expression
187 of *CYP27c1* was also intermediate between marine and riverine species. Based on these
188 results, the grass puffers were inferred to be using a visual system that functions in both
189 the river and the sea. In other words, the grass puffers are estimated to be adapting their

190 visual system to two environments, the river and the sea, rather than switching their visual
191 system by changing the expression of vision-related genes depending on the ambient
192 environments.

193 Unlike the vision-related genes, the expression levels of the six genes were
194 significantly upregulated in the riverine population compared to the marine population of
195 grass puffer. Focusing on the four genes with the higher expression among these six genes,
196 the expression levels were upregulated in the riverine grass puffer population compared
197 to the marine *T. rubripes* and *T. synderi*. Interestingly, the expression levels of these four
198 genes were higher in the riverine grass puffer population than in the riverine *D.*
199 *nigroviridis*. These four genes were heat shock proteins (Hsp), Hsp 90, Hsp47, Hsp9, and
200 unc45b. Hsp functions in protein folding, maintenance of structural integrity, and proper
201 regulation for a wide range of proteins (Sørensen, et al. 2003). Hsp90 has been reported
202 to play a role in lens formation in the eye (Hooven, et al. 2004). In addition, unc45b is
203 known to interact with Hsp90 and is involved in lens formation (Hansen, et al. 2014).
204 Hsp47 is a molecular chaperone specific to collagen and plays an important role during
205 ocular development, especially in corneal morphogenesis (TANAKA, et al. 1996).
206 Considering that these three chaperone-related genes are involved in lens and cornea
207 formation, these three genes may be involved in normal eye formation and maintenance
208 in freshwater environments, with lower salt concentrations than in seawater. *D.*
209 *nigroviridis* also inhabits the river, but the expression levels of these three genes were not
210 upregulated. This species is adapted to the river, thus the riverine environment is probably
211 not a major stressor for them. Hsp9 has been proposed to be involved in the immune

212 response against pathogenic bacteria (Abumourad 2009). The grass puffer individuals
213 that have migrated to the river likely face a different type of pathogenic bacteria than
214 those in the sea and have upregulated expression of Hsp9.

215 Taken together, we hypothesize that the grass puffer individuals are adapting to the
216 environmental difference (low salinity and pathogenic bacteria) that they face when they
217 migrate to the river by increasing the expression levels of heat shock protein and related
218 genes.

219

220 **Conclusion**

221 In this study, we investigated how the grass puffer inhabits two different environments,
222 the river and the sea, based on gene expression in the eyes. Opsin and *CYP27c1* genes
223 were expected to be adapted to two environments, riverine and marine, by making their
224 expression patterns intermediate between those of riverine and marine species. On the
225 other hand, the high expression of Hsp genes in the riverine grass puffer population
226 suggests that they are adapted to the river environment through the expression of heat
227 shock proteins. Future studies of other marine fish migrating to rivers will reveal whether
228 these two adaptive strategies to different environments are shared by other fish species.

229

230 **Methods**

231 **Sampling**

232 Grass puffers were collected from Tagoe river and Tateishi park (Fig. S1) by fishing. *T.*
233 *rubripes* and *T. synderi* individuals were collected from Tokyo bay (Fig. S1) by fishing.

234 *D. nigroviridis* individuals were obtained from a tropical fish dealer. The information on
235 each species used in this study is listed in Table S1. The guidelines for experimental
236 animal management of SOKENDAI were followed throughout the study. The
237 Institutional Animal Care and Use Committee of SOKENDAI approved the animal
238 protocols and procedures.
239

240 **RNA sequencing and comparison of expression levels**

241 Total RNAs were extracted from the eyes of *T. rubripes*, *T. synder*i, *D. nigroviridis*, and
242 grass puffer using TRIzol RNA Isolation Reagent (Thermo Fisher Scientific, Waltham,
243 MA, USA) according to the manufacturer's instructions. RNA libraries were constructed
244 using the NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Ultra
245 RNA Library Prep Kit for Illumina (New England Bio Labs, Ipswich, MA) following the
246 manufacturer's instructions. Short cDNA sequences (paired-end 150 bp) were determined
247 from the libraries using the Illumina HiseqX platform (RNA-seq). RNA-seq reads from
248 each of three of four individuals of *T. rubripes*, *T. synder*i, and grass puffer (10-15 Gbp)
249 were trimmed to remove adaptor sequences and mapped to the reference genome of *T.*
250 *rubripes*. Reads showing similarity (90%) with 90% read lengths were mapped to the
251 reference genome and expression levels were calculated using CLC Genomics
252 Workbench ver. 21 (https://www.qiagenbioinformatics.com/). TPM (Transcript Per
253 Million) was used for normalized expression values. The average TPM of the four
254 individuals of the grass puffer was compared. Differential expression was assessed using
255 the TMM (trimmed mean of M values) method (Robinson and Oshlack 2010)

256 implemented in the CLC genomics workbench using default parameters. False Discovery
257 Rate (FDR) correction was applied to the calculated p-values and differentially expressed
258 genes with corrected p-value < 0.05 were considered significant. For *D. nigroviridis*, the
259 short reads were assembled by the CLC genomics workbench using "Auto" parameters,
260 then short reads were mapped to the assembled contigs. TPM was used for normalized
261 expression values. The TPM values of *D. nigroviridis* were normalized with that of the
262 grass puffer using the TPM values of the GAPDH gene. The average TPM values of
263 Hsp90, Hsp47, Hsp9, and unc45b were compared using a t-test with Bonferroni
264 correction.
265
266 **Phylogenetic, principal component, and ADMIXTURE analyses**
267 The mapping data of eight grass puffers as well as two outgroup *Takifugu* species (three
268 individuals each) were exported in bam file format and sorted and indexed using samtools
269 (Li, et al. 2009). The duplicated reads in bam files were marked by the MarkDuplicates
270 algorithm in GATK v4.2 (https://gatk.broadinstitute.org/hc/en-us). We performed
271 genotype calling using the HaplotypeCaller algorithm in GATK v4.2. Genotypes of all
272 individuals were output as gvcf format (-ERC GVCF option) and filtered by the
273 VariantFiltration algorithm in GATK v4.2 with default parameters. All gvcf files were
274 combined into a single gvcf format file by the CombineGVCFs algorithm in GATK v4.2.
275 The combined file was genotyped by the GenotypeGVCFs algorithm in GATK v4.2. We
276 removed all indels, singleton, and doubleton sites to eliminate PCR and sequencing errors,
277 and extracted bi-allelic sites with coverage equal to or more than five in all individuals
278 and with GQ values equal to or more than ten in all individuals.
279 The variants in a vcf file were converted to PHYLIP format. 10 kb sequences from the
280 5' end of the PHYLIP format file were extracted and a model for the Maximum Likelihood

281 method was selected using MEGA ver. X (Kumar, et al. 2018). A phylogenetic tree was
282 constructed using the Maximum Likelihood (ML) method using PhyML ver. 3.2
283 (Guindon, et al. 2010) with a model selection option "-m GTR" and with 100 bootstrap
284 replications (Fig. 1: 266,954 sites).

285 Using the same data set, we performed a principal component analysis (PCA) using
286 PLINK ver. 1.9 (Purcell, et al. 2007) with an option "--indep-pairwise 50 10 0.1" to
287 explore the genetic relationship among all individuals (Figure 2A).

288 ADMIXTURE ver. 1.3 (Alexander and Lange 2011) was run on the same dataset
289 (Figure 2B) assuming 2 to 6 clusters (K=2-6).

290

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365

366 **Figure legends**

367 Fig. 1 Phylogenetic relationships between *Takifugu* species.

368 Maximum likelihood tree based on 266,954 SNPs. Node labels indicate bootstrap

369 replicates.

370

371 Fig. 2 Genetic relationships between *Takifugu* species.

372 Principal Components Analysis (PC1 versus PC2) based on 266,954 SNPs (A). Colored

373 circles correspond to the names of species or populationss in the panel. (B) ADMIXTURE

374 results based on the same SNP data with (A) for K = 2-6.

375

376 Fig. 3 A volcano plot for the gene expression differences between the riverine and marine

377 grass puffer populations

378 Each blue dot represents the gene expressed in the eye. The log2 fold changes of average

379 TPM values of the marine grass puffer population compared to those of the riverine grass

380 puffer population and the -log10 p-values resulting from a t-test comparing average TPM

381 values between the riverine and marine grass puffer populations for each gene are plotted

382 on the x- and y-axis, respectively.

383

384 Fig. 4 Expression of opsin and CYP27c1 genes

385 The relative expression of opsin genes in (A) *T. rubripes*, (B) *T. synderi*, (C) riverine

386 population of grass puffer, (D) marine population of grass puffer, and (E) *D. nigroviridis*.

387 (F) the expression of the CYP27c1 gene.

388

389 Fig. 5 Expression of heat shock protein and related genes

390 The expression of (A) Hsp 90, (B) Hsp47, (C) Hsp 9, and (D) unc45b in *T. rubripes*, *T.*

391 *synderi*, riverine and marine populations of grass puffer, and *D. nigroviridis*. Asterisks

392 indicate statistical significance ($p < 0.05$ after Bonferroni correction) of differences in

393 expression levels between riverine grass puffer and *T. rubripes* and *T. synderi*.

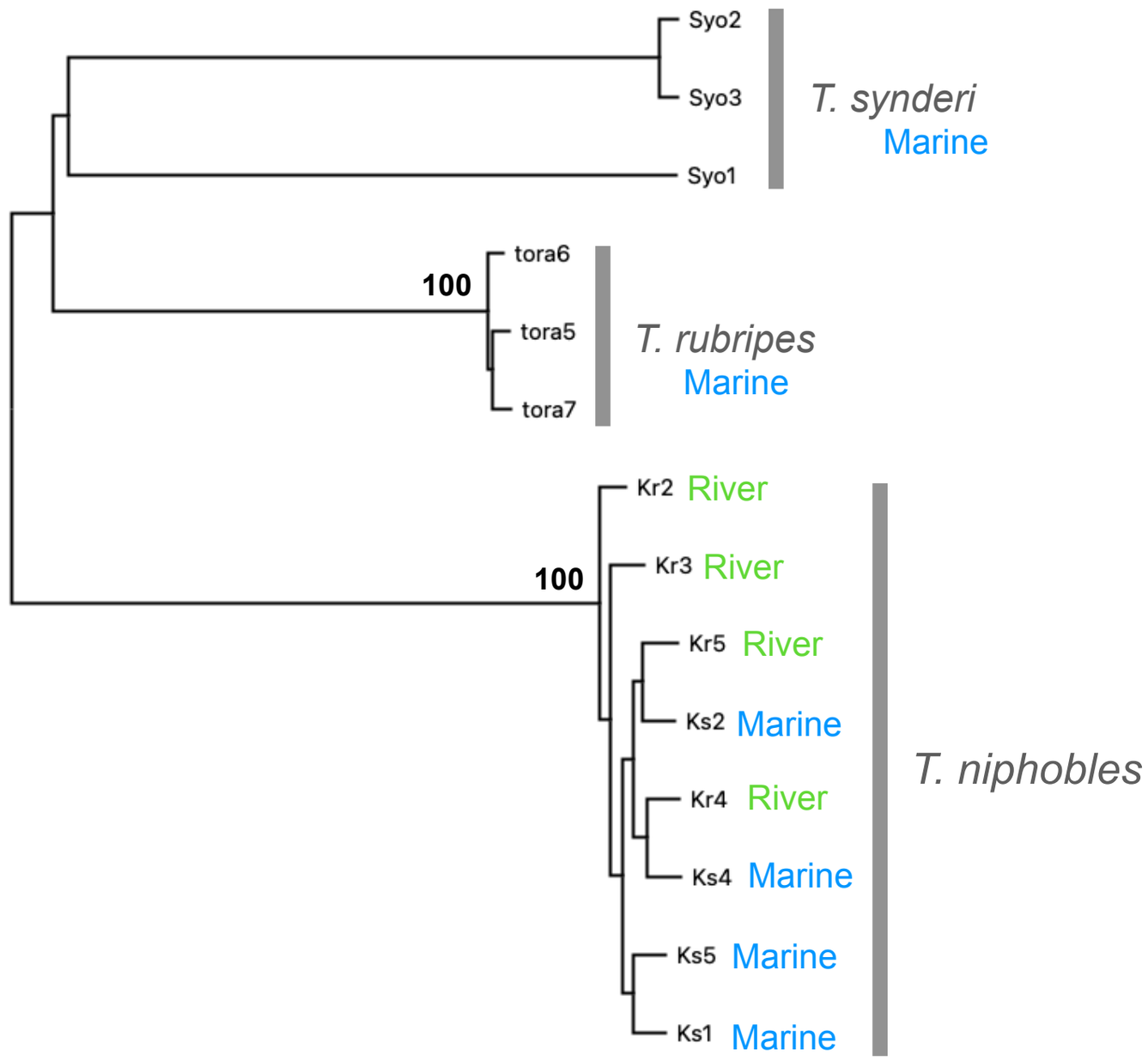

Fig. 1 Phylogenetic relationships between *Takifugu* species.
Maximum likelihood tree based on 266,954 SNPs. Node labels indicate bootstrap replicates.

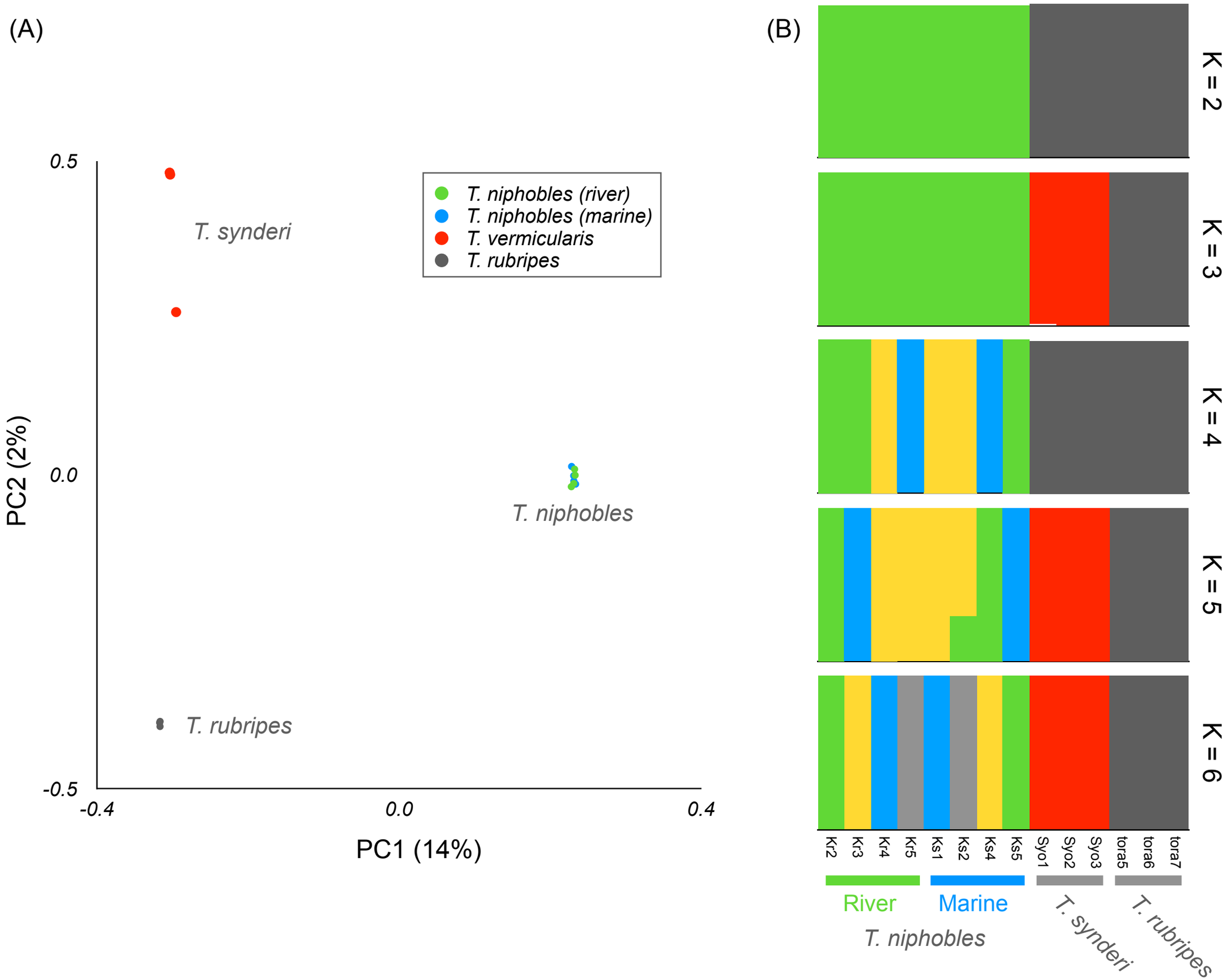

Fig. 2 Genetic relationships between *Takifugu* species.
Principal Components Analysis (PC1 versus PC2) based on 266,954 SNPs (A). Colored circles correspond to the names of species or populationss in the panel. (B) ADMIXTURE results based on the same SNP data with (A) for K = 2-6.

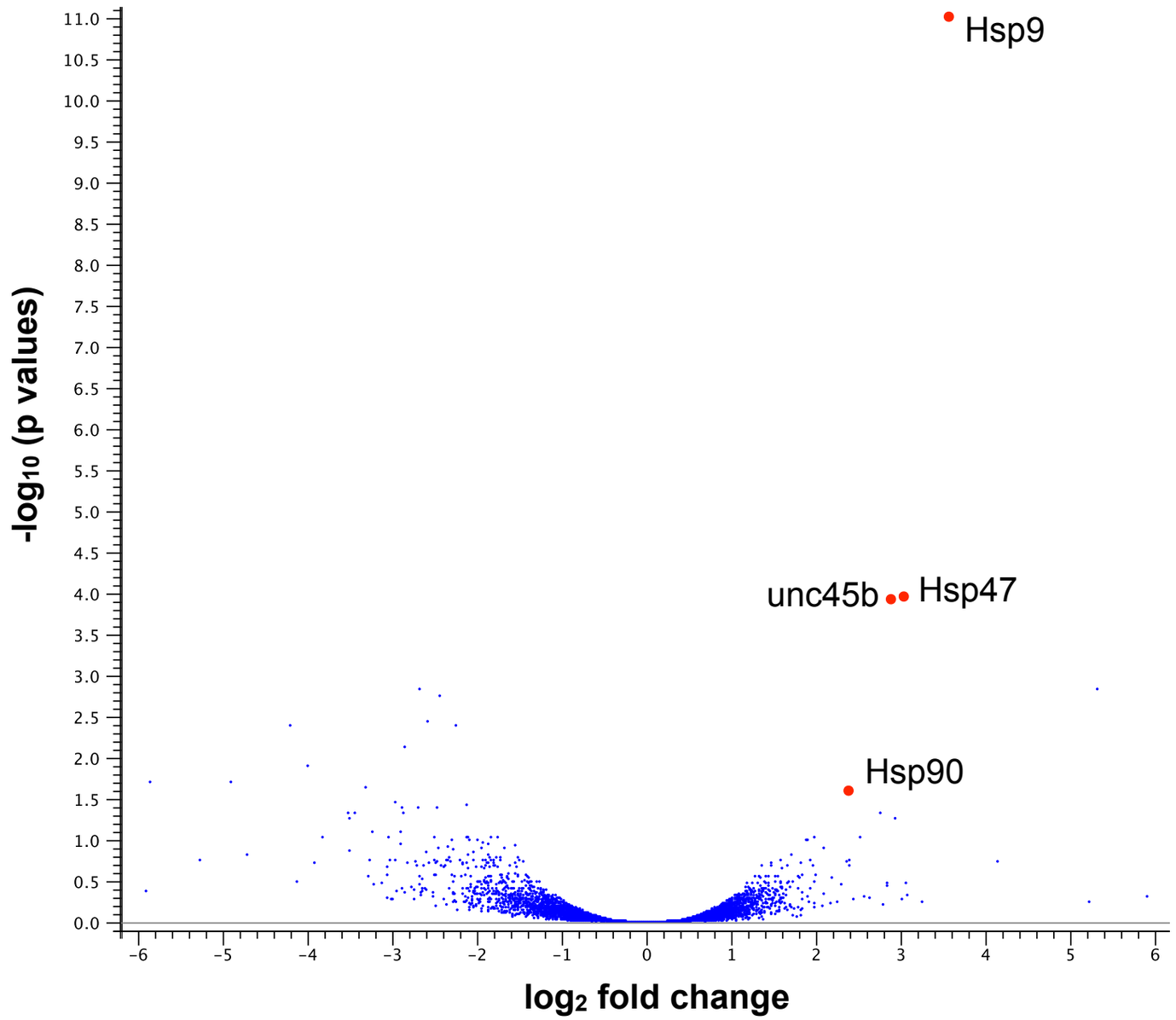

Fig. 3 A volcano plot for the gene expression differences between the riverine and marine grass puffer populations
Each blue dot represents the gene expressed in the eye. The log2 fold changes of average TPM values of the marine grass puffer population compared to those of the riverine grass puffer population and the -log10 p-values resulting from a t-test comparing average TPM values between the riverine and marine grass puffer populations for each gene are plotted on the x- and y-axis, respectively.

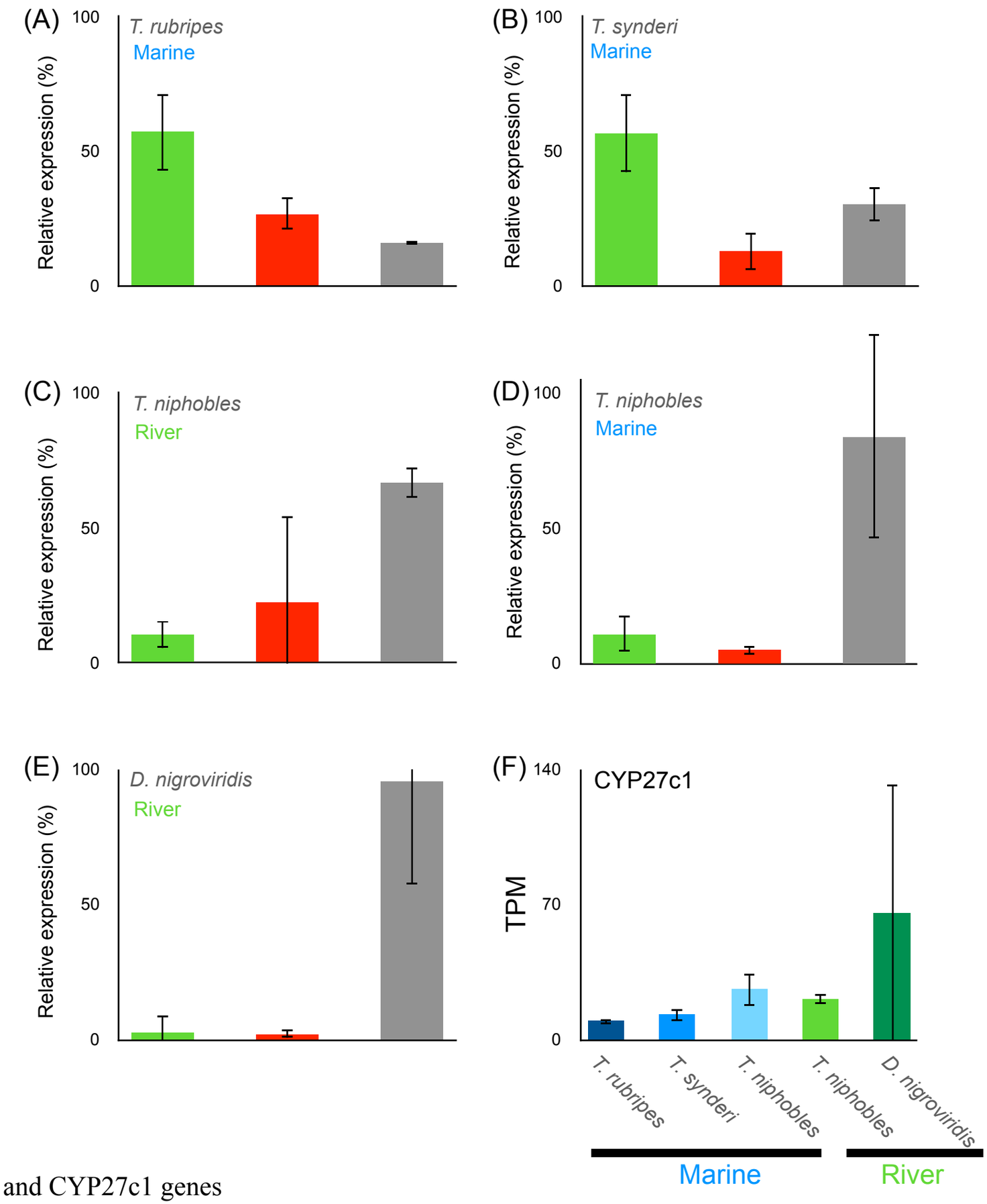

Fig. 4 Expression of opsin and CYP27c1 genes
The relative expression of opsin genes in (A) *T. rubripes*, (B) *T. synderi*, (C) riverine population of grass puffer, (D) marine population of grass puffer, and (E) *D. nigroviridis*. (F) the expression of CYP27c1 gene.

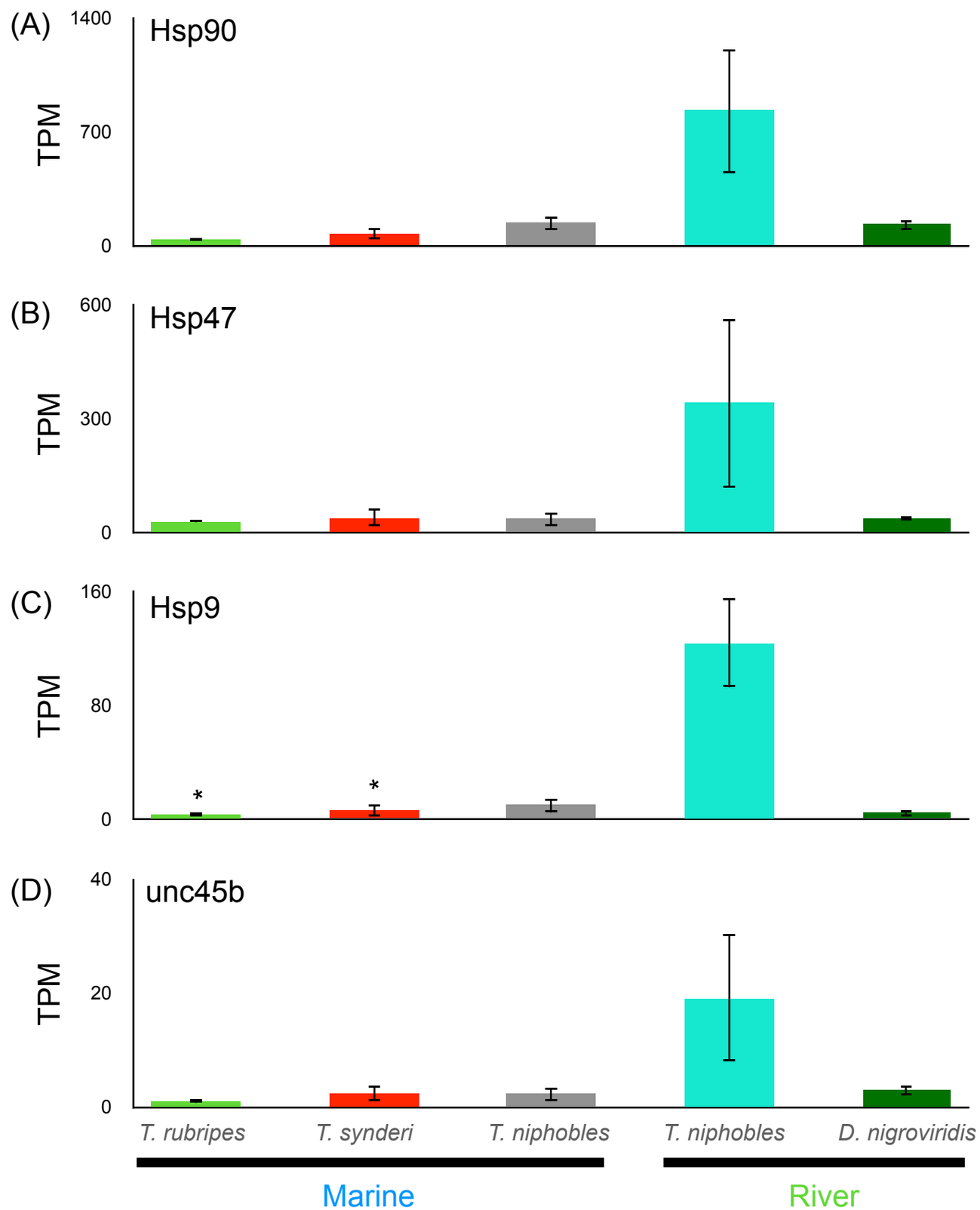

Fig. 5 Expression of heat shock protein and related genes
The expression of (A) Hsp 90, (B) Hsp47, (C) Hsp 9, and (D) unc45b in *T. rubripes*, *T. synderi*, riverine and marine populations of grass puffer, and *D. nigroviridis*. Asterisks indicate statistical significance ($p < 0.05$ after Bonferroni correction) of differences in expression levels between riverine grass puffer and *T. rubripes* and *T. synderi*.