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

Misa Osada and Yohey Terai*

Institutions

[1]*SOKENDAI (The Graduate University for Advanced Studies), Department of Evolutionary Studies of Biosystems, Shonan Village, Hayama, Kanagawa 240-0193, Japan.*

*Corresponding author:

Yohey Terai: Email: terai_yohei@soken.ac.jp

Abstract

Many marine fish species migrate to rivers, but little is known about whether these species switch their vision when inhabiting rivers or adapt their vision to the environment of both rivers and the sea. Grass puffer (*Takifugu niphobles*) is a marine fish species frequently migrating to rivers. In this study, we investigated grass puffers from riverine and marine populations and analyzed the gene expression in their eyes. The phylogeny and population genetics of riverine and marine grass puffers indicated that riverine and marine grass puffers are from the same population. Gene expression levels by high-throughput RNA sequencing indicated no differences in the expression patterns of vision-related genes in marine and riverine grass puffers. This result indicates that the adaption of their visual system to both marine and riverine environments rather than switching the expression of vision-related genes. Additionally, riverine grass puffers increase the expression levels of heat shock proteins and related genes. These genes showed higher expression in riverine grass puffer than in other marine and river pufferfish species, suggesting that the grass puffer individuals adapt to the environmental difference when they migrate to the river by increasing the expression levels of heat shock protein and related genes.

33 **Introduction**

34 The environments in the rivers and the sea differ in many aspects, including nutrient,
35 salinity, light intensity, and wavelength of light. For example, in freshwater with low
36 transparency, water with micro-particles scatters short-wavelength light, and the light in
37 turbid water is dominated by long wavelengths [1-3]. In contrast, long-wavelength light is
38 absorbed by water, and short-wavelength light is predominant in the clear ocean [4]. Since
39 fish species inhabit this heterogeneous light environment, they have adapted their vision
40 to the different light environments [2,3,5,6]. Accordingly, fish vision is known as a prime
41 example of adaptive evolution.

42 Vertebrate visual pigments consist of light-absorbing chromophores and the protein
43 component, opsin [7]. Spectral sensitivity is determined by the type of chromophore [with
44 11-*cis* retinal (A1-) or 11-*cis* 3-dehydroretinal (A2-derived retinal)] and by interactions
45 between chromophores and amino acid residues of opsin [8]. The replacement of A1- with
46 A2-derived retinal in the visual pigments shifts the absorption to a longer wavelength [9,10].

47 The enzyme CYP27c1 is known to be involved in the switch from A1 to A2, and the
48 expression level of this gene correlates with the amount of A2 [11,12]. Fish species use A1
49 and A2 retinal, with freshwater fish mainly using A2 and marine fish using A1 retinal [13].
50 A1 and A2 retinal are known to be differentially used at different life cycle stages of the
51 life cycle. Salmon use A1 retinal when living in the sea, and switch to A2 when they
52 migrate up the river to spawn [14]. In lamprey, A1 and A2 are differentially used for
53 juveniles and upstream migrating adult lamprey [11]. Marine fish species migrating to the
54 river use both A1 and A2 retinal [13]. This usage is a characteristic of both riverine and

marine environments. However, 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 in the rivers and the sea.

Grass puffer (*Takifugu niphobles*) is a marine fish species frequently migrating to rivers. Grass puffers are abundant in rivers and the sea during the same season. Fish with ancestry in marine fish and colonized freshwater have been reported to have adapted to freshwater by duplicating specific genes [15,16]. However, the grass pufferfish would not be a colonizing fish because they cannot live in freshwater for long periods [17]. Grass puffers possess both A1 and A2 retinal [13], therefore they seem to migrate to rivers for short periods. Hence, it is unclear how they adapt to different river environments compared to the sea and whether the populations in the rivers and the sea are the same. In this study, we analyzed the phylogenetic and population genetics of riverine and marine populations of grass puffer. Also, we 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 riverine and marine grass puffers to show how grass puffers inhabit the river and marine environments.

Result

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

77 We collected riverine grass puffers 1.3 km upstream from the mouth of Tagoe River,
78 Kanagawa, Japan (Fig. S1, Table S1). Based on our observations, the grass puffers are
79 not seen in the rivers during midwinter (mid-January through the end of February). In
80 contrast, we visually observed many grass puffer individuals in the rivers from March
81 through December, even at low tide. We collected Marine grass puffers on a beach 10 km
82 from the mouth of Tagoe River. Since this beach is more than 3 km from the nearest river,
83 we expected less direct migration of the river grass puffers. We measured salinity and
84 light environments at the Tagoe River and Tateishi Park beach to verify the differences
85 between the river and the sea environments (Fig. S2).

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

93 *T. rubripes* and *T. poecilonotus* are marine species in the same genus as grass puffer.
94 SNPs were extracted from individuals of these species collected from the sea (Fig. S1,
95 Table S1) using the same method as grass puffer individuals (see Methods). The SNPs
96 were combined with the grass puffer SNPs, and a phylogenetic tree was constructed by
97 the ML method using *C. rivulata* and *T. nigroviridis* as out-group species with a total of
98 116,941sites. Phylogenetic trees showed that grass puffer formed a monophyletic group

99 with no population differentiation (Fig. 1). We then performed a population genetic
100 analysis with 275,155 sites. Using the same data set, a principal component analysis
101 (PCA) showed similar results, with no differentiation between the riverine and marine
102 populations of grass puffer (Fig. 2A). Furthermore, ADMIXTURE analysis using the
103 same data set showed that the grass puffers formed either a single cluster (K=2, 3) or
104 multiple clusters (K=4-6), but the riverine and marine populations did not differentiate
105 (Fig. 2B). These results indicate that riverine and marine grass puffers are from the same
106 population. Grass puffer is known to spawn simultaneously in the surf on the beach in
107 June, and subpopulations are presumed to mix at this time, which may prevent genetic
108 differentiation.
109

110 **Expression differences in the eyes between the riverine and marine populations of *T.***
111 ***niphobles***

112 We performed RNA sequencing to compare gene expression levels in the eyes of grass
113 puffers from the riverine and marine populations. Total RNAs were extracted from eyes,
114 and 10-15 Gb sequences were determined from four individuals, each of grass puffers
115 from the riverine and marine populations. These high-throughput sequences were mapped
116 to a reference genome sequence of *T. rubripes* to compare gene expression levels between
117 the riverine and marine populations of grass puffers. As a result, we found 25
118 differentially expressed genes with statistical significance (Fig. 3, Table S2). Among the
119 25 genes, six genes were highly expressed in the riverine population, and 19 genes were
120 highly expressed in the marine population (Fig. 3, Table S2). In this study, we focused

121 on six of the 25 genes highly expressed in the riverine population to reveal how marine
122 grass puffer individuals can inhabit a freshwater environment. Three of these were heat
123 shock protein genes (Hsp 9, 47, 90) [18,19]. unc45b has also been reported to be part of a
124 chaperone system that interacts with HSP90 [20].

125 Next, we examined whether the expression levels of genes related to vision varied
126 between the riverine and marine populations. The photosensitivity of visual pigments is
127 tuned by the amino acid sequence of opsins and the type of chromophores [8]. Therefore,
128 we first searched for the opsin genes in the genome of *T. rubripes* and CYP27c1 gene for
129 switching chromophores. The color opsins, blue-sensitive (*SWS2)*, green-sensitive (*RH2)*,
130 and long-wavelength sensitive (*LWS)* opsin genes were annotated in the genome of *T.*
131 *rubripes*. In addition, the scotopic opsin, *RH1* gene, and *CYP27c1* were present in the
132 genome. Comparisons of gene expression levels of the four opsin genes and *CYP27c1*
133 between the river and marine populations of grass puffer showed no difference with
134 statistical significance (Fig. 4). In addition, the amino acid sequences of four opsins were
135 identical between the two populations.

136

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

138 The opsin repertoire was examined for *T. rubripes* and *T. poecilonotus*, as well as
139 *Tetraodon nigroviridis*, which is a Tetraodontidae species inhabiting rivers and ponds in
140 Southeast Asia [21]. We also searched for the *SWS2*, *RH2*, *LWS*, and *RH1* genes in the
141 assembled RNA sequences of *T. rubripes*, *T. poecilonotus,* and *T. nigroviridis*, and found
142 the homologs of these genes. Therefore, we infer that the genus *Takifugu*, including grass

143 puffer, and *T. nigroviridis* have an opsin repertoire of *SWS2*, *RH2*, *LWS*, and *RH1* and
144 use a trichromatic color vision.

145 We focused on four opsin genes, CYP27c1, and genes with higher expression levels in
146 the riverine population than in the marine population of the grass puffer and compared
147 the expression levels with those of *T. rubripes*, *T. poecilonotus*, and *T. nigroviridis*. The
148 relative expression of *RH*2 in marine *T. rubripes* and *T. poecilonotus* exceeded 30% of
149 total opsin expressions, and the expression of *SWS2* and *LWS* was also relatively higher
150 than that of the grass puffer (Fig. 4A, 4B). Therefore, these two marine species primarily
151 express color opsin genes. In contrast, the relative expression of *RH1* in riverine *T.*
152 *nigroviridis* exceeded 95% of total opsin expressions, indicating that a scotopic opsin
153 gene was predominantly expressed in this species (Fig. 4E). In the grass puffer, *RH1* was
154 predominantly expressed, but less than in *T. nigroviridis*, with 67% and 84% in the
155 riverine and marine populations, respectively (Figs. 4C-4E). *SWS2*, *RH2,* and *LWS* were
156 highly expressed in grass puffer than in *T. nigroviridis*. *CYP27c1* was expressed at lower
157 levels in the marine species *T. rubripes* and *T. poecilonotus* than in the grass puffer and
158 higher levels in *T. nigroviridis* compared to the grass puffer (Fig. 4F). Among the six
159 genes with higher expression levels in the riverine population than in the marine
160 population of the grass puffer, we focused on four highly expressed genes with (TPM >
161 5). These four genes expressed higher levels in the river population of the grass puffer
162 than in the other species (Fig. 5).

163

164 **Discussion**

165 When marine fish migrate to a river, they face a riverine environment that differs from
166 the sea. Therefore, we expected that marine fish that migrate to the river might respond
167 to a different environment, and this study investigated mRNA expression in the eyes of
168 the grass puffer, which inhabits both the river and the sea. As for vision, the repertoire of
169 opsin genes and their expression levels were comparable between the riverine and marine
170 populations of the grass puffer. The expression levels of *CYP27c1*, representing the
171 amount of A2 retinal, were also comparable. Thus, grass puffers are presumed to have
172 the same vision usage in both marine and riverine environments. In contrast, *RH1* was
173 predominantly expressed in the river species *T. nigroviridis*, while cone opsins were
174 predominantly expressed in the marine species *T. rubripes* and *T. poecilonotus*. In the
175 expression of the opsin genes of all species used in this study, the grass puffer showed an
176 expression pattern intermediate between that of marine and river species. The expression
177 of *CYP27c1* was also intermediate between marine and riverine species. According to
178 these results, we inferred that the usage of the visual system in the grass puffers is the
179 same in both the river and the sea. In other words, the grass puffers adapt their visual
180 system to two environments, the river and the sea, rather than switching their visual
181 system by changing the expression of vision-related genes depending on the ambient
182 environments.

183 Unlike the vision-related genes, the expression levels of the six genes were
184 significantly upregulated in the riverine grass puffer population compared to the marine
185 population. In the four highly expressed genes among these six genes, the expression
186 levels were upregulated in the riverine grass puffer population compared to the marine *T.*

187 *rubripes* and *T. poecilonotus*. Interestingly, the expression levels of these four genes were
188 higher in the riverine grass puffer population than in the riverine *T. nigroviridis*. These
189 four genes were heat shock proteins (Hsp), Hsp 90, Hsp47, Hsp9, and unc45b. Hsp
190 functions in protein folding, maintenance of structural integrity, and proper regulation for
191 a wide range of proteins [18]. Hsp90 has been reported to play a role in lens formation in
192 the eye [22]. In addition, unc45b is known to interact with Hsp90 and is involved in lens
193 formation [20]. Hsp47 is a molecular chaperone specific to collagen and plays an essential
194 role during ocular development, especially in corneal morphogenesis [23]. Considering that
195 these three chaperone-related genes function in lens and cornea formation, these genes
196 may be involved in normal eye formation and maintenance in freshwater environments
197 with lower salt concentrations than seawater in grass puffer. *T. nigroviridis* also inhabits
198 the river, but the expression levels of these three genes were not upregulated. This species
199 adapts to the river, thus the riverine environment is probably not a major stressor for them.
200 Hsp9 has been proposed to be involved in the immune response against pathogenic
201 bacteria [19]. The grass puffer individuals that have migrated to the river likely face a
202 different type of pathogenic bacteria than those in the sea and have upregulated
203 expression of Hsp9.
204 Taken together, we hypothesize that the grass puffer individuals adapt to the
205 environmental difference (low salinity and pathogenic bacteria) that they face when they
206 migrate to the river by increasing the expression levels of heat shock protein and related
207 genes.
208

Conclusion

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

Methods

Measurement of salinity and light environments

We measured salinity at the Tagoe River and Tateishi Park (Fig. S1). In the Tagoe River, we collected water from a depth of 50 cm every hour from low to high tide at the point where we collected the grass puffers and measured salinity using a hydrometer (SpectrumBrands Japan, Kanagawa Japan) (Fig. S2A). In Tateishi Park, we measured salinity only once since salinity does not change with tides. We measured the wavelength of light in the horizontal direction at a depth of 50 cm with an Ocean Optics spectrophotometer (Ocean Photonics, Tokyo Japan) at the sites where we collected the grass puffers in the Tagoe River and Tateishi Park (Figs. S1 and S2 B).

Sampling

231 Grass puffers were collected from Tagoe river and Tateishi park (Fig. S1) by fishing. *T.*
232 *rubripes* and *T. poecilonotus* individuals were collected from Tokyo bay (Fig. S1) by
233 fishing. *C. rivulata* was collected from Tateishi park (Fig. S1) by fishing. *T. nigroviridis*
234 individuals were obtained from tropical fish dealer. The information of each species used
235 in this study are listed in Table S1. The guidelines for experimental animal management
236 of SOKENDAI were followed throughout the study. The Institutional Animal Care and
237 Use Committee of SOKENDAI approved the animal protocols and procedures.
238
239 **RNA sequencing and comparison of expression levels**
240 Total RNAs were extracted from eyes of *C. rivulata, T. rubripes*, *T. poecilonotus*, *T.*
241 *nigroviridis*, and grass puffer using TRIzol RNA Isolation Reagent (Thermo Fisher
242 Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA
243 libraries were constructed using the NEBNext Poly(A) mRNA Magnetic Isolation
244 Module and the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Bio
245 Labs, Ipswich, MA) following the manufacturer's instructions. Short cDNA sequences
246 (paired-end 150 bp) were determined from the libraries using the Illumina HiseqX
247 platform (RNA-seq). RNA-seq reads from each of three or four individuals of *T. rubripes*,
248 *T. poecilonotus*, and grass puffer (10-15 Gbp) were trimmed to remove adaptor sequences
249 and mapped to the reference genome of *T. rubripes*. Reads showing similarity (90%) with
250 90% read lengths were mapped to the reference genome and expression levels were
251 calculated using CLC Genomics Workbench ver. 21
252 (https://www.qiagenbioinformatics.com/). TPM (Transcript Per Million) was used for

253 normalized expression values. The average TPM of the four individuals of the grass
254 puffer were compared. Differential expression was assessed using the TMM (trimmed
255 mean of M values) method [24] implemented in the CLC genomics workbench using default
256 parameters. False Discovery Rate (FDR) correction was applied to the calculated p-values
257 and differentially expressed genes with corrected p-value < 0.05 were considered
258 significant. For *T. nigroviridis*, the short reads were assembled by CLC genomics
259 workbench using "Auto" parameters, then short reads were mapped to the assembled
260 contigs. TPM was used for normalized expression values. The TPM values of *T.*
261 *nigroviridis* were normalized with that of the grass puffer using TPM values of *GAPDH*
262 gene. The average TPM values of *Hsp90, Hsp47, Hsp9*, and *unc45b* were compared using
263 a t-test with Bonferroni correction.
264
265 **Phylogenetic, principal component, and ADMIXTURE analyses**
266 The mapping data of eight grass puffers as well as two outgroup *Takifugu* species (three
267 individuals each) were exported in bam file format and sorted and indexed using samtools
268 [25]. The duplicated reads in bam files were marked by the MarkDuplicates algorithm in
269 GATK v4.2 (https://gatk.broadinstitute.org/hc/en-us). We performed genotype calling
270 using the HaplotypeCaller algorithm in GATK v4.2. Genotypes of all individuals were
271 output as gvcf format (-ERC GVCF option). All gvcf files were combined into a single
272 gvcf format file by the CombineGVCFs algorithm in GATK v4.2 and filtered by the
273 VariantFiltration algorithm in GATK v4.2 with default parameters. The combined file was
274 genotyped by the GenotypeGVCFs algorithm in GATK v4.2. We removed all indels,
275 singleton, and doubleton sites to eliminate PCR and sequencing errors, and extracted bi-
276 allelic sites with coverage equal to or more than five in all individuals and with GQ values
277 equal to or more than twenty in all individuals.

The variants in a vcf file were converted to PHYLIP format. 10 kb sequences from the 5' end of the PHYLIP format file were extracted and a model for Maximum Likelihood method was selected using MEGA ver. X [26]. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method using PhyML ver. 3.2 [27] with a model selection option "-m GTR" and with 100 bootstrap replications (Fig. 1: 116,941 sites).

We selected the Takifugu species from a gvcf file and filtered with the same condition described above, and performed a principal component analysis (PCA) using PLINK ver. 1.9 [28] with an option "--indep-pairwise 50 10 0.1" to explore the genetic relationship among all individuals (Figure 2A: 275155 sites).

ADMIXTURE ver. 1.3 [29] was run on the same dataset (Figure 2B: 275,155 sites) assuming 2 to 6 clusters (K=2-6).

References

1 Maan, M. E., Hofker, K. D., van Alphen, J. J. & Seehausen, O. Sensory drive in cichlid speciation. *The American Naturalist* **167**, 947-954 (2006).

2 Seehausen, O. *et al.* Speciation through sensory drive in cichlid fish. *Nature* **455**, 620-626, doi:10.1038/nature07285 (2008).

3 Terai, Y. *et al.* Visual adaptation in Lake Victoria cichlid fishes: depth-related variation of color and scotopic opsins in species from sand/mud bottoms. *BMC Evol. Biol.* **17**, 200, doi:10.1186/s12862-017-1040-x (2017).

4 Kirk, J. T. *Light and photosynthesis in aquatic ecosystems.* (Cambridge university press, 1994).

5 Terai, Y. *et al.* Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. *PLoS Biol.* **4**, e433, doi:10.1371/journal.pbio.0040433 (2006).

6 Nagai, H. *et al.* Reverse evolution in RH1 for adaptation of cichlids to water depth in Lake Tanganyika. *Mol. Biol. Evol.* **28**, 1769-1776, doi:10.1093/molbev/msq344 (2011).

7 Shichida, Y. in *The Retinal Basis of Vision* (ed J. Toyoda) (Elsevier Science, 1999).

8 Yokoyama, S. Molecular evolution of vertebrate visual pigments. *Prog Retin Eye Res* **19**, 385-419 (2000).

9 Harosi, F. I. An analysis of two spectral properties of vertebrate visual pigments. *Vision research* **34**, 1359-1367 (1994).

10 Parry, J. W. & Bowmaker, J. K. Visual pigment reconstitution in intact goldfish retina using synthetic retinaldehyde isomers. *Vision Res.* **40**, 2241-2247 (2000).

11 Morshedian, A. *et al.* Cambrian origin of the CYP27C1-mediated vitamin A1-to-A2 switch, a key mechanism of vertebrate sensory plasticity. *Royal Society open science* **4**, 170362 (2017).

12 Enright, J. M. *et al.* Cyp27c1 red-shifts the spectral sensitivity of photoreceptors by converting vitamin A1 into A2. *Curr. Biol.* **25**, 3048-3057 (2015).

13 Toyama, M. *et al.* Presence of rhodopsin and porphyropsin in the eyes of 164 fishes, representing marine, diadromous, coastal and freshwater species—a qualitative and comparative study. *Photochem. Photobiol.* **84**, 996-1002 (2008).

14 Beatty, D. D. A study of the succession of visual pigments in Pacific salmon (Oncorhynchus). *Canadian journal of zoology* **44**, 429-455 (1966).

15 Ishikawa, A. *et al.* A key metabolic gene for recurrent freshwater colonization and radiation in fishes. *Science* **364**, 886-889 (2019).

16 Ishikawa, A., Yamanouchi, S., Iwasaki, W. & Kitano, J. Convergent copy number increase of genes associated with freshwater colonization in fishes. *Philosophical Transactions of the Royal Society B* **377**, 20200509 (2022).

17 Kato, A., Maeno, Y. & Hirose, S. Brief migration of the grass puffer, Takifugu niphobles, to fresh water from salt water. *Ichthyol. Res.* **57**, 298-304 (2010).

18 Sørensen, J. G., Kristensen, T. N. & Loeschcke, V. The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037 (2003).

19 Abumourad, I. Tilapia Heat Shock Protein: Molecular Cloning and

Characterization. *Science* **7**, 46-57 (2009).

20 Hansen, L. *et al.* The myosin chaperone UNC45B is involved in lens development and autosomal dominant juvenile cataract. *Europ. J. Hum. Genet.* **22**, 1290-1297 (2014).

21 Rainboth, W. J. *Fishes of the cambodian mekong*. (Food & Agriculture Org., 1996).

22 Hooven, T. A., Yamamoto, Y. & Jeffery, W. R. Blind cavefish and heat shock protein chaperones: a novel role for hsp90alpha in lens apoptosis. *Int. J. Dev. Biol.* **48**, 731-738 (2004).

23 TANAKA, Y. *et al.* Expression of 47 kDa heat shock protein (HSP47) during development of mouse cornea. *Experimental eye research* **63**, 383-393 (1996).

24 Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology* **11**, 1-9 (2010).

25 Li, H. *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

26 Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547 (2018).

27 Guindon, S. *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307-321 (2010).

28 Purcell, S. *et al.* PLINK: a toolset for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559-575 (2007).

29 Alexander, D. H. & Lange, K. Enhancements to the ADMIXTURE algorithm for individual ancestry estimation. *BMC Bioinformatics* **12**, 1-6 (2011).

Figure legends

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

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

371 replicates.

372

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

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

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

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

377

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

379 grass puffer populations

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

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

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

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

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

385

386 Fig. 4 Expression of opsin and CYP27c1 genes

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

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

389 (F) the expression of CYP27c1 gene.

390

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

392 The expression of (A) Hsp 90, (B) Hsp47, (C) Hsp 9, and (D) unc45b in *T. rubripes*, *T.*
393 *poecilonotus*, riverine and marine populations of grass puffer, and *T. nigroviridis*.
394 Asterisks indicate statistical significance ($p < 0.05$ after Bonferroni correction) of
395 differences in expression levels between riverine grass puffer and *T. rubripes* and *T.*
396 *poecilonotus*.

397

398 **Acknowledgement**

399 This work was supported by JST SPRING, Grant Number JPMJSP2104.

400

401 **Data availability statement**

402 The nucleotide sequences were deposited in the DDBJ Sequenced Read Archive under a
403 Bioproject PRJDB18283.

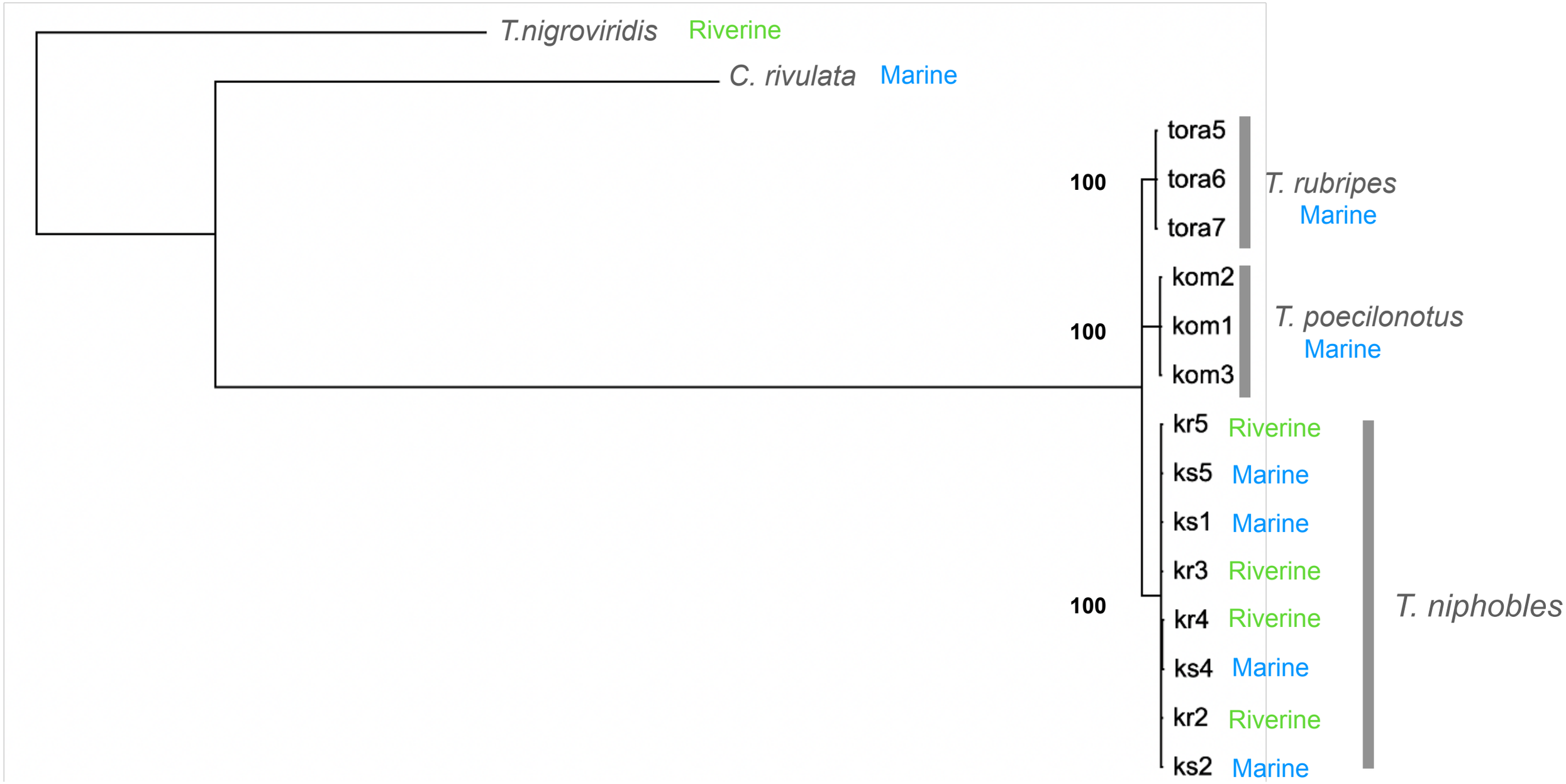

Fig. 1 Phylogenetic relationships between Takifugu species.
Maximum likelihood tree based on 116,941 SNPs. Node labels indicate bootstrap replicates.

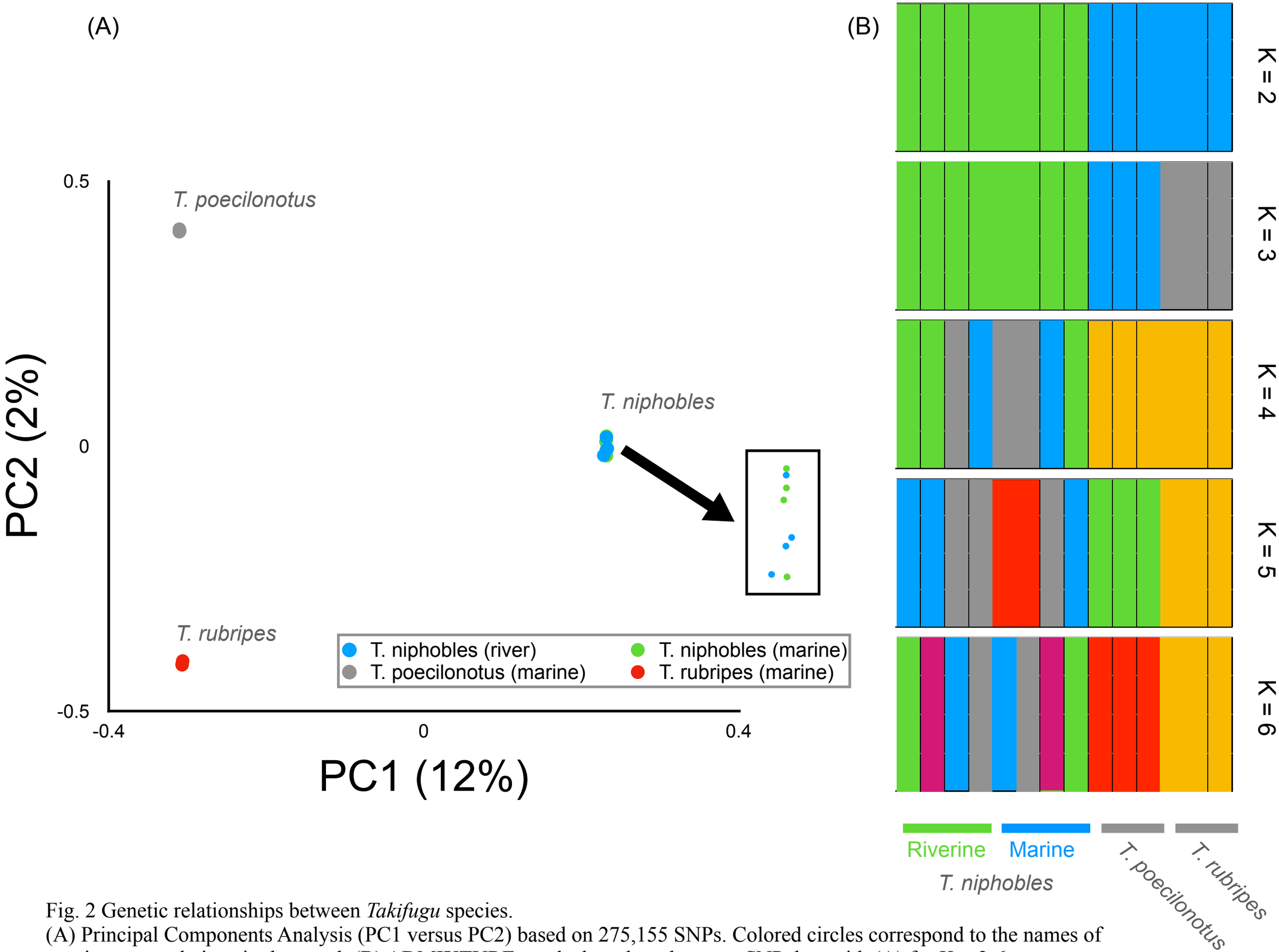

Fig. 2 Genetic relationships between *Takifugu* species.
(A) Principal Components Analysis (PC1 versus PC2) based on 275,155 SNPs. Colored circles correspond to the names of species or populations in the panel. (B) ADMIXTURE results based on the same SNP data with (A) for K = 2-6.

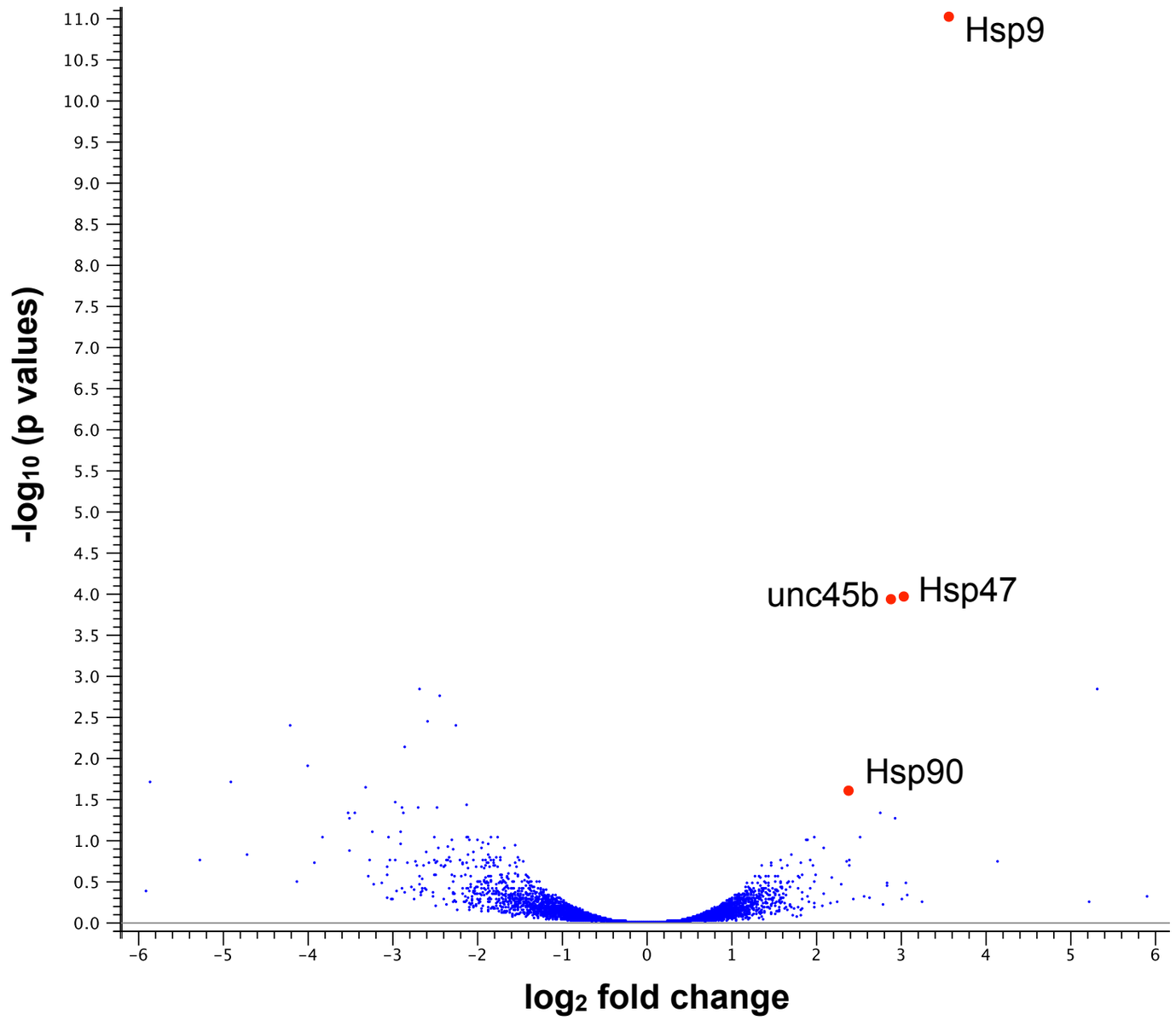

Fig. 3 A volcano plot for the gene expression differences between the riverine and marine grass puffer populations
Each blue dot represents the gene expressed in the eye. The log2 fold changes of average TPM values of the marine grass puffer population compared to those of the riverine grass puffer population 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.

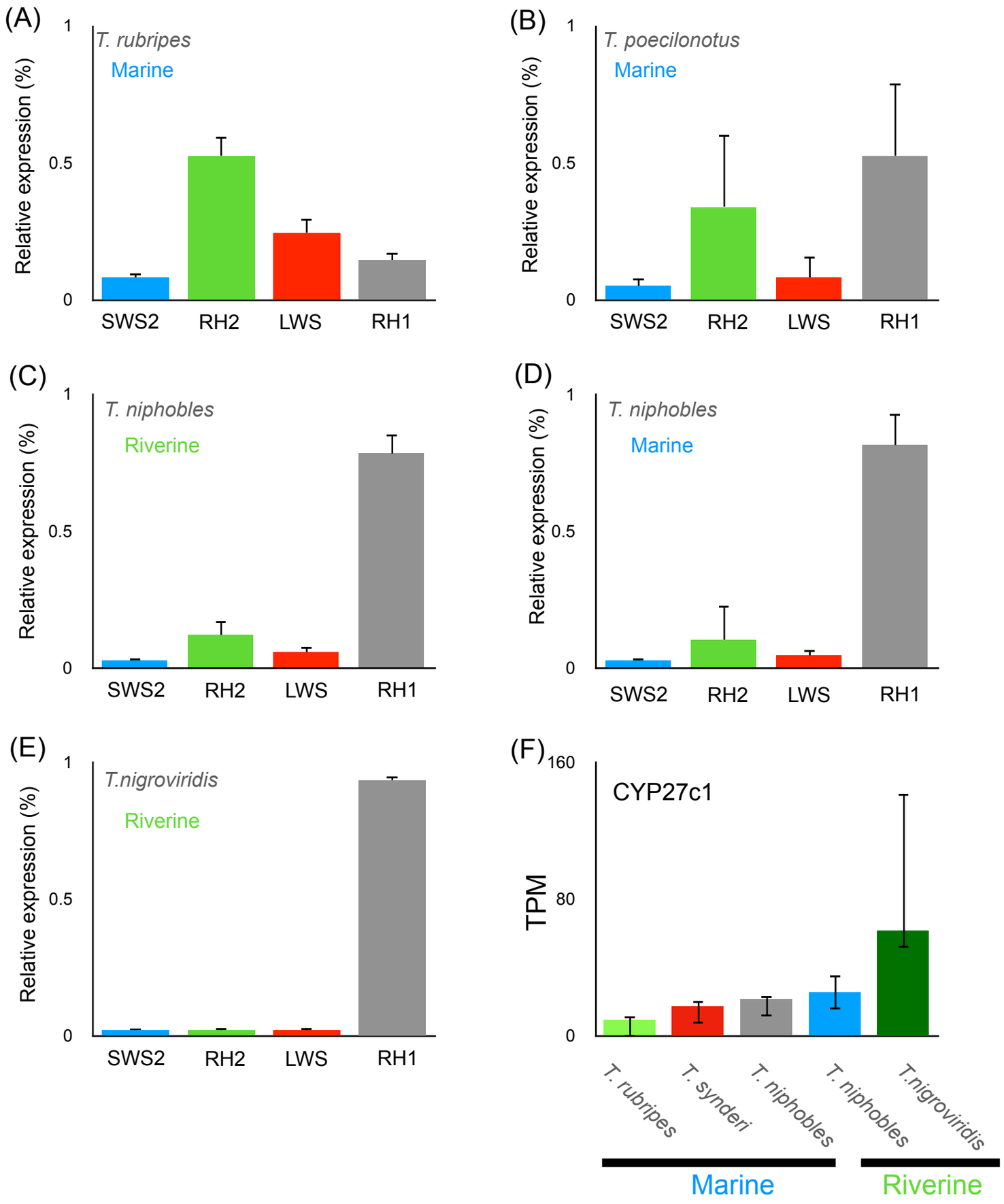

Fig. 4 Expression of opsin and CYP27c1 genes
The relative expression of opsin genes in (A) *T. rubripes*, (B) *T. poecilonotus*, (C) a riverine population of grass puffer, (D) a marine population of grass puffer, and (E) *D. nigroviridis*. (F) the expression of CYP27c1 gene.

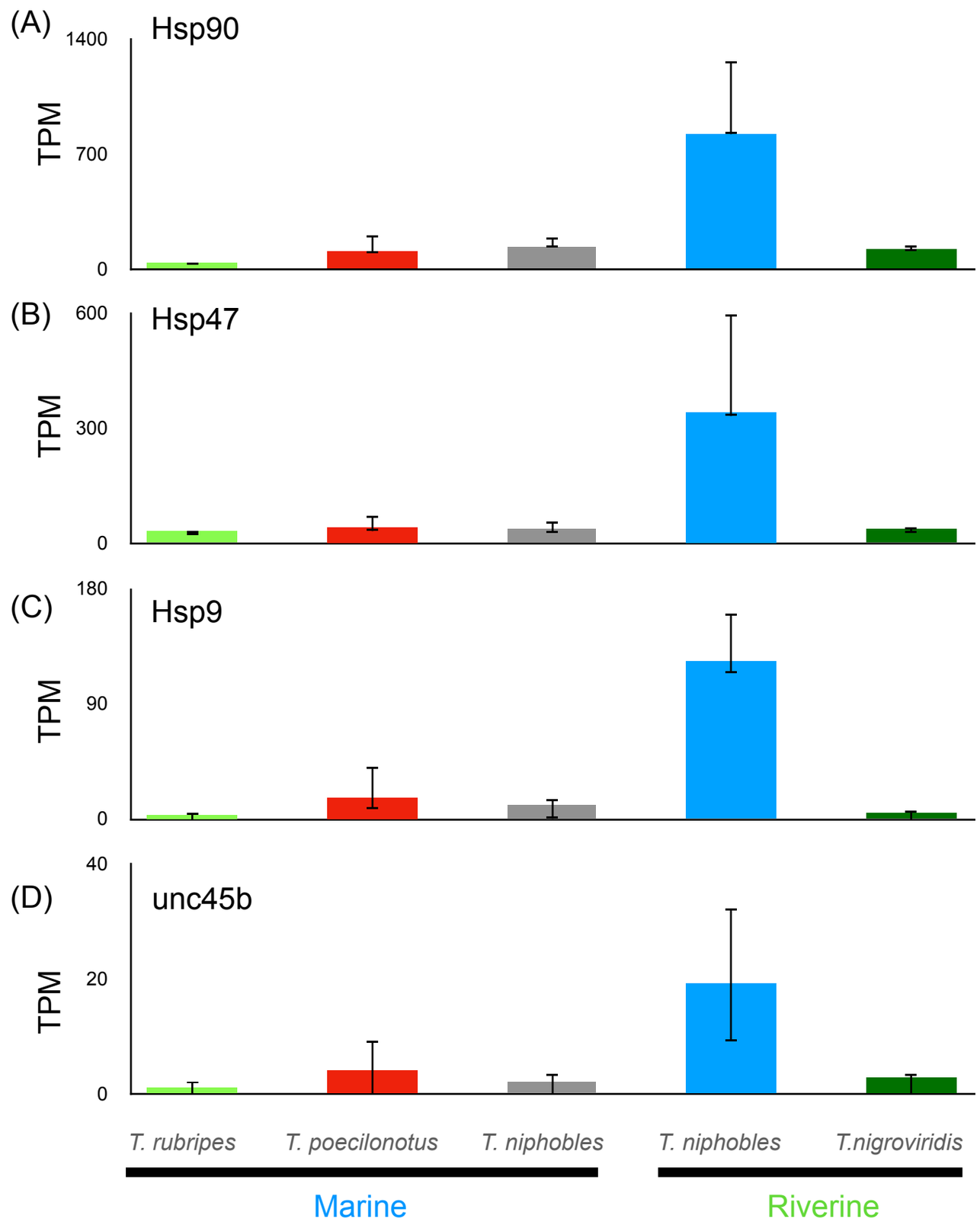

Fig. 5 Expression of heat shock protein and related genes
The expression of (A) Hsp90, (B) Hsp47, (C) Hsp9, and (D) unc45b in *T. rubripes*, *T. synderi*, riverine and marine populations of grass puffer, and *T.nigroviridis*