Adaptive evolution of low salinity tolerance and hypoosmotic regulation in a euryhaline teleost, *Takifugu obscurus*

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Abstract

Background: The mechanism of osmoregulation is crucial for maintaining growth, development, and life activities in teleosts. *Takifugu obscurus*, the only euryhaline species in the genus *Takifugu*, is a proper model organism for studying the mechanism of low salt-tolerance and hypoosmotic regulation. **Results:** In this study, whole genome sequencing data were obtained from 90 pufferfish representing five species within this genus, *T. rubripes*, *T. obscurus*, *T. flavidus*, *T. niphobles*, and *T. bimaculatus*. Using a phylogeny, PCA, and population structure analyses, we observed similar amounts of population genetic differentiation among species. The five species are closely related to each other and have differentiated within a relatively short period, while *T. bimaculatus* and *T. flavidus* shared the most similar genetic backgrounds. We further identified hundreds of genes under selection related to hypoosmotic regulation between *T. obscurus* and other *Takifugu* species, including 16 representative genes involving ion transporters (*atp1a3*, *atp2a2*, *atp2a3*, *slc13a1*, *slc5a8*, *slc12a2*, *slc12a4*, *slc26a2*, *scn1b*, and *kcna2/3/10*), genes involved in hormone regulation (*fyn*, *prlr*, and *grb2*), and a gene associated with water absorption (*aqp3*). **Conclusions:** Our findings provide preliminary insight into the mechanism of osmoregulation and will facilitate follow-up validation of candidate genes related to osmoregulation in *T. obscurus*.

Background

Pufferfish are a group of migratory benthic teleost fishes belonging to the genus *Takifugu* (Tetraodontiformes, Tetraodontidae). They are naturally distributed in the Western Pacific Ocean, spanning the Bohai Sea, Yellow Sea, East China Sea, and the Sea of Japan. Some pufferfish, e.g., *Takifugu rubripes*, *Takifugu flavidus*, are commercially important fish in China, Japan, and Korea due to their high nutritional value and extraordinary flavor [1]. The genus *Takifugu* includes 25 species, most of which are closely related, that are able to produce fertile hybrids (e.g., *T. rubripes* × *T. niphobles*, *T. rubripes* × *T. flavidus*) [2]. Hybrids can occur in wild populations or in artificially-crossed breeding. The extensive existence of hybrids is mainly due to the relatively recent evolutionary time scale of *Takifugu* species diversification. *Takifugu* species underwent explosive speciation in the marine waters of East Asia within 1.8–5.3 Ma during the Pliocene [3]. This was consistent with the theoretical result [4] that more recently diverged species pairs are more likely to produce viable hybrids. It should not go unnoticed that *Takifugu* species display speciated-related phenotypic variations that could potentially improve our understanding of speciation mechanisms [5, 6].

Pufferfish have speciated and radiated under different salinity levels of the aquatic environment. What is noteworthy is that the habitats of most pufferfish are restricted to salt water, with only one exception that can survive and develop normally in freshwater. *T. obscurus*, as that exceptional case, is a euryhaline fish that can survive in both freshwater and saline water [7]. In its natural habitat, *T. obscurus* is an anadromous fish that migrates to freshwater to spawn, and newly hatched larvae live there for months [8]. Meanwhile, artificial culture of *T. obscurus* has developed rapidly in China, and the fish are able to grow and reproduce in freshwater over the entire life cycle [9]. Some pufferfish species have the potential

to adapt to low salinity to some extent; such species include *T. niphobles*, which is considered as a peripheral freshwater fish that can survive in freshwater for several days [10]. Fluctuating salinity, an important environmental factor, directly influences the fluid osmotic pressure of fish, in turn impacting the survival, growth, and reproduction of the fish [11, 12].

Variation in salinity could lead to a series of physiological responses in osmoregulation-related organs and tissues, including kidney, gill, and intestine [7, 13, 14]. The mechanism of salinity adaptation in teleosts is a complex physiological procedure that cannot be examined using freshwater fish. As an ideal model, the mechanisms of low salinity tolerance differences between *T. obscurus* and other *Takifugu* species have been studied from different aspects. In general, *T. obscurus* has a solid osmotic adjustment capability and a superior osmoregulatory mechanism. In regard to physiological structure, *T. obscurus* has a freshwater fish-type nephron with a distal tubule in the kidney, while other *Takifugu* species have a seawater fish-type nephron without the distal tubule [7]. Two cotransporters, $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter 2 (NKCC2) and $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC), have been demonstrated to be differentially expressed in the distal nephrons of *T. obscurus* and other *Takifugu* fishes during the process of NaCl reabsorption [15]. The gill is another important osmoregulatory organ for maintaining ion gradients in teleosts [16]. $\text{Na}^+/\text{K}^+\text{-ATPase}$ (NKA), as a key enzyme of ion transport maintaining the Na^+ and K^+ gradient, is mainly distributed in the chloride cells of the gills and has a crucial function in the ion exchange and osmoregulation in *T. obscurus* [17]. The activity of NKA changes dramatically with the variation of environmental salinity [18]. In hypoosmotic environments, the intestine plays a pivotal osmoregulatory role in absorbing water and balancing ions through the intestinal epithelia [14]. Meanwhile, kidneys and gills respond to hypoosmotic conditions by participating in the excretion of hypotonic urine and transport of ions. *Slc26* has been demonstrated to be an important gene family for exchange of anions such as HCO_3^- and SO_2^- [19]. HCO_3^- and SO_2^- transporters, which are mainly located in the intestinal epithelial cells and the proximal tubules of the kidney, respectively, are two genes closely related to the adaptation to a seawater environment in *T. obscurus* [20, 21].

In brief, the mechanism of osmoregulation plays a vital role in maintaining life activities in teleosts and has been studied from various aspects at the organ and tissue levels as well as at the cellular and ion channel protein levels. However, the specific genes and co-regulators within their upstream and downstream pathways regulating body fluid osmotic pressure in teleosts have not been well characterized. Therefore, *T. obscurus* could serve as an ideal experimental model for investigating the molecular mechanisms of osmoregulation and adaptation to low-salinity environments in teleosts. Considering that the complete genome sequence of *T. obscurus* is not yet available, given the high genomic similarity among *Takifugu* species, the genome of its closely-related species *T. rubripes* could be considered as an alternative. *T. rubripes* was one of the earliest model species for vertebrates because of its compact genome size (~400 Mb) and genetic repertoire in comparison to other fishes [22]. A draft sequence of the pufferfish was generated using a whole-genome shotgun strategy for *T. rubripes* [23]. A comprehensive genetic map of *T. rubripes* [24] has been generated using 1220 microsatellites that facilitated anchoring the assembled scaffolds to the 22 chromosomes of *T. rubripes* on the basis of

preliminary research [25]. This consolidated genome map covered 86% of the genome assembly, and 72% of the scaffolds have been oriented by integrating with the genetic linkage map (<http://www.fugu-sg.org/>). This genome has been widely used as a valuable resource for comparative genomic and subsequent genetic analysis [26-28]. Furthermore, high-throughput sequencing has been developing rapidly during the last decade. Whole genome re-sequencing is already widely used as a routine tool for identifying genomic differences of samples and for detecting genetic variation.

In this study, the whole genomes of five closely-related pufferfish species were sequenced using Illumina HiSeq X Ten platform. Using the high-quality genomic sequencing data, we elaborated the population genetic differences among the five species. Then, a selective sweep analysis using salt water versus freshwater species was performed to explore the genetic architecture underlying the adaptive evolution of osmoregulation. This study may assist in delineating the genetic diversity of pufferfish and in identifying candidate genes and pathways involved in the regulation networks of low salinity tolerance.

Results

Sample collection and genotyping

A total of five *Takifugu* populations comprising 90 individuals were collected from three sites in China (**Figure 1, Supplementary Table 1**). After quality control of extracted DNA, all of the individuals were re-sequenced to explore the population structure and the selective signals under adaptive evolution. In total, 438.67 Gb of sequencing data were generated, while for each individual, 4.87 Gb sequences with an average of ~12.43-fold depth per sample were obtained. From 2.92 G raw reads, 2.57 G reads were mapped to the reference genome for SNP calling with an average mapping ratio of 87.87%. After the sequence alignment and filtering, a total of 11,344,115 candidate SNPs were identified for further analysis (**Supplementary Table 2**).

Population structure

To investigate the genetic relationships of different *Takifugu* species, a phylogenetic tree was constructed using all of the informative SNPs (**Figure 2A**). Each population formed a distinct clade that reflected the evolutionary differences among species as expected. Similar results were observed in the plot of the PCA (principle components analysis) analysis. Overall, the populations of *T. rubripes*, *T. niphobles*, and *T. obscurus* clustered separately and tightly, while the individuals of *T. flavidus* and *T. bimaculatus* were closely clustered together and overlapped. This was consistent with the results of phylogenetic analysis in that the clades of *T. flavidus* and *T. bimaculatus* shared one main branch point and were adjacent to each other. Four clusters from the PCA were far apart from each other with no confusing admixture. Population structure of the five species was evaluated using the Bayesian clustering program STRUCTURE. As the model K=4 obtained the highest log likelihood value, the population structure for K=4 is shown in **Figure 2C**. Unsurprisingly, similar results were observed; the five populations were separated into three subgroups. Outside the *T. rubripes* and *T. obscurus* subgroups, *T. flavidus*, *T. niphobles*, and *T. bimaculatus* clustered and shared a common ancestry. *T. rubripes*, *T. niphobles*, and *T. obscurus* showed

a simplex inheritance background, while *T. flavidus* and *T. bimaculatus* reflected a more admixed genetic structure. *T. flavidus* and *T. bimaculatus* have a very similar genetic structure and shared some ancestral sequences with *T. obscurus*. When K=2, 3, or 5, only two subgroups existed, *T. rubripes* and the others (Figure S1). This information, which was consistent with the phylogenetic analysis, also reflected the relatively earlier differentiation time of *T. rubripes* and the close genetic relationships and relatively recent species differentiation of the other four species.

LD decay and pattern of haplotype blocks

LD (Linkage Disequilibrium) decay was investigated for five populations, and R^2 values were calculated by PLINK and sorted by distance ranges. *T. flavidus* showed the highest R^2 value, while the other four populations obtained significantly lower R^2 values (**Figure 3A**). When the distance was greater than 1 kb, the mean R^2 values were 0.55 in *T. flavidus*, 0.40–0.45 in *T. rubripes*, *T. niphobles*, and *T. obscurus*, and 0.38 in *T. bimaculatus* (**Supplementary Table 3**). LD decay of whole samples and saline samples showed higher R^2 values than most single populations except for *T. flavidus*. Because of the relatively small sample sizes for *T. flavidus* (N=8) and *T. bimaculatus* (N=8), haplotypes were constructed only for the populations of *T. rubripes*, *T. niphobles*, and *T. obscurus* using the PLINK “-block” parameter. The distribution of haplotype block lengths was calculated using the R software ggplot2 package (**Figure 3B**). The block patterns were similar in three populations, while all samples and saline samples showed higher densities in smaller blocks. The maximum block lengths were 146.67 kb, 36.51 kb, and 34.22 kb in *T. rubripes*, *T. niphobles*, and *T. obscurus*, respectively, while the corresponding mean block sizes were 3.81 kb, 2.17 kb, and 2.42 kb (**Supplementary Table 4**).

Genome-wide selective sweep analysis

The genetic diversity in certain regions of the genome might be dramatically decreased as a result of natural selection or domestication. Among the five species in this study, the *T. obscurus* population (Ob group) is more strongly euryhaline compared with the other four species (the Saline group), which is probably due to its unique genetic background. To identify genome regions under selection in the *T. obscurus* genome, we scanned the genome-wide variation and allele frequency spectra based on the approximately 13.69 million SNPs. The π ratios ($\pi_{\text{Saline/Obcurus}}$) were calculated using a 100 kb sliding-window approach with 10 kb steps. In comparison to the Saline group, the Ob group had a significantly lower level of diversity (median $\pi_{\text{Saline/Obcurus}} = 2.860$), reflecting the fewer recombination events in the Ob population. We identified a total of 1,548 significant windows (top 5%, empirical π ratios ≥ 5.856) with median $\pi_{\text{Saline/Obcurus}} = 8.230$ that included 554 candidate genes (**Supplementary Table 5**) based on the π ratio analysis. To further validate the genome regions under strong selective pressure in the Ob population, the genome regions with F_{st} greater than 0.232 (top 5%) were also identified, corresponding to 497 candidate genes (**Supplementary Table 5**). A total of 379 candidate genes shared by both the π ratio and F_{st} analyses were recognized as potential genes under selection (**Figure 4A**). Among these selected genes, many are involved in ion transportation and ATPase related pathways; those that have been reported in previous studies include *atp1a3* (sodium/potassium-transporting ATPase), *slc13a1*

(sodium/sulfate symporter), *kcna2/3/10* (potassium voltage-gated channel subfamily A member 2/3/10), *slc5a8* (sodium-coupled monocarboxylate transporter), and *scnb1* (sodium channel subunit beta-1) (**Figure 4B**). The diversity in the neighboring regions of these selected genes showed significant decrease in the Ob population compared with the Saline group, indicating possible selective sweeps adapting to the long-term changing osmotic environment. For example, in the *kcna* gene family, *kcna2*, *kcna3*, and *kcna10* in the same sweep region (>500 kb) were identified, while another gene, *slc5a8*, was also found in this region. To better understand these candidate genes and their potential functions, further GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses also identified several key genes related to osmotic regulation. Two genes, *fyn* and *atp2a3*, were enriched in the GO term “ATP binding,” and *grb2* was enriched in the “Gap junction” pathway (**Figure 4B**, **Figure S2**, and **Supplementary Table 6**). The results based on Tajima’s D analysis also provided supporting evidence identifying strong selective sweep signals (**Figure 5**). The results suggested that the genomes of the Ob population have significantly evolved adaptations to the euryhaline environment.

As the Saline group contains four species, the genetic divergence among species could well reduce the common background noise when used as a whole. The comparison would then be focused on the species’ shared lower osmoregulation ability in contrast to the Ob population. Still, it might be useful to identify more potential genes under selection by one-to-one comparisons between the Ob population and the other four populations (shown as Bi, Fl, Ni, Ru). The π ratios and Fst values for the four comparisons are shown in **Figure 6A**, and genes within selected regions are summarized in **Supplementary Tables 7-10**. The most abundant selected genes (211 genes) were found in the comparison Fl vs Ob, while 41 genes, 132 genes, and 92 genes were identified in Bi vs Ob, Ni vs Ob, and Ru vs Ob, respectively. Despite the result that similar genes were found in all four comparisons of Saline vs Ob (e.g., *atp1a3*, *atp2a3*, *fyn*, and *scnb1*), other important ion transporter genes were also identified (**Figure 6B**), including *slc12a4* (potassium/chloride transporter) from the Bi vs Ob group and *slc12a2* (sodium/potassium/chloride transporter) and *slc26a2* (anion exchanger) from the Ru vs Ob group, indicating their vital roles in regulating osmotic balance. Additionally, *atp2b2* (calcium-transporting ATPase 2) and *aqp3* (aquaporin 3) were also discovered in selective sweep regions from three comparisons (Bi vs Ob, Fl vs Ob, and Ru vs Ob). The *prlr* (prolactin receptor), which was reported to be involved in salinity tolerance of Nile tilapia [29], was also identified in Bi vs Ob and Fl vs Ob comparisons. GO and KEGG enrichment analyses identified crucial pathways including “neuromast development” (contains *slc26a2*), “integral component of plasma membrane” (contains *aqp3*), and “neuroactive ligand-receptor interaction” (contains *prlr*), suggesting involvement of core functions of the neural system in the regulation of salinity (**Supplementary Table 11**). Further analyses based on Tajima’s D differences also provided evidence for previous selective sweep signals (**Figure S3**). These results suggested that in pairwise comparisons between the Ob population and the other four populations, the genome of the Ob population displays significant selection signatures indicating enhancement of the species’ euryhaline ability.

The most important genes under selection are summarized in **Supplementary Table 12**. Ion transporters comprise a large proportion of the candidate genes, while growth and neural development-related genes might play upstream roles in the regulation networks.

Discussion

Previous phylogenetic analysis of 15 *Takifugu* species [3] indicated that the main lineage has undergone explosive speciation within 2.4–4.7 Ma, meaning that all species are closely related. From the combined results of the phylogeny, PCA and population structure analysis in our study, four main clades were identified: *T. rubripes*, *T. obscurus*, *T. niphobles* and the group of *T. bimaculatus* and *T. flavidus*. This result is consistent with the ML analysis of 24 *Takifugu* species using *cyt b*, 12s rRNA gene sequences and mitogenomic data [3, 30] showing that *T. bimaculatus* and *T. flavidus* were clustered together in one branch, while *T. rubripes*, *T. obscurus*, and *T. niphobles* were assigned to different branches.

The genes discovered in selective sweep regions included ion transporters, neural regulators, and growth-related genes, most of which have been previously reported. The *T. obscurus* population possesses stronger low-salt tolerance than the other four species, and thus the comparisons were made by grouping the four salt-water species together and searching for potential genes under selection in *T. obscurus*. As various characteristic differences may be revealed by selection analysis, including growth, body shape, body color, and low-salinity tolerance, the genetic divergence within the saline populations could well attenuate the genetic background noise of other traits, allowing us to focus on their common features such as weakened low-salinity tolerance. Similar strategies have been reported in a previous study by Feng Cheng, who identified selective sweeps for leaf-heading trait in the two species *Brassica rapa* and *Brassica oleracea* [31]. Chinese cabbage has a unique heading-trait accession in *B. rapa*, while cabbage is the only accession with the heading trait in *B. oleracea*. The non-heading accessions in each species were grouped together to compare with the Chinese cabbage and cabbage. Thus, genetic divergence of other traits among the non-heading accessions would be balanced and would probably not appear in selective sweep analyses. In our study, pairwise comparisons between *T. obscurus* and the other four species were made as a supplementary analysis to the group comparisons in order to identify additional candidate genes. We identified a number of *slc* genes in the Ob population that were embedded in selected regions. These *slc* genes encode various transmembrane transporters of anions and cations (e.g., *slc13a1*, *slc5a8*, *slc12a2*, *slc12a4*, *slc26a2*). The apical membrane Na⁺-sulfate cotransporter encoded by *slc13a1* mediates sulfate reabsorption across the renal proximal tubule and intestinal epithelia, promoting osmoregulation in low-salinity waters [32, 33]. Similarly, a considerable number of genes were identified encoding energy-dependent ATP-binding transporters (*atp1a3*, *atp2a2*, *atp2a3*) and voltage-gated ion channels (*scn1b*, *kcna2/3/10*). Among these genes, *atp1a3* that codes Na⁺/K⁺-ATPase plays a key role in the Na⁺ and K⁺ gradient balance, and this gene has been reported as being differentially expressed in salinity-varying environments in *T. obscurus* [18]. The voltage-gated ion channel genes (*scn1b* and *kcna* family) have been discovered by differential gene expression during spawning [34] and selective sweeps adapting freshwater and alkaline waters in Amur Ide [35, 36]. Therefore, the genes encoding transcellular ion transporters and channel proteins are reshaped by natural selection from the variable euryhaline environment. Adapting to lower-salinity environments can lead to varied secretion of hormones (e.g., *prrl*, *ghr*, *igf*) that modulate permeability of cells and prevent ion loss and water absorption [37, 38]. The genes *fyn* and *grb2* were embedded in our enriched pathways of

“Focal adhesion,” while *prlr* was enriched in “Neuroactive ligand-receptor interaction.” The *aqp3* gene was included in the “integral component of plasma membrane” in our selective sweep analysis; this gene has been reported to be involved in regulating water absorption in renal tubules of Atlantic salmon [39]. The main categories of osmoregulation genes have been covered in our study; however, the detailed mechanisms of gene interaction and regulation of gene expression are not yet fully understood. Further experimental evidence in view of expression or methylation would also be necessary in the future to support our potential candidate genes related to osmoregulation in *T. obscurus*.

Conclusions

Using whole genome sequencing data, we analyzed the population relationships of five *Takifugu* species. The selective signatures of the *T. obscurus* population were screened for effects on low salt-tolerance and hypoosmotic pressure adjustment capacity. Phylogenetic analysis, PCA, and population structure analysis were conducted, and interspecific genetic relationships among five species within the genus *Takifugu* were established. In addition, a number of genes within selected regions have indicated potential function in osmotic regulation. Among the results, 16 genes were identified as potential candidate genes that could possibly be associated with hypoosmotic pressure regulation in *T. obscurus*. These results provide an empirical foundation for further insights into the genetic mechanism of osmoregulation in *T. obscurus*.

Methods

Sample collection

A total of 90 samples from five populations were collected, with 29 *T. rubripes*, 30 *T. obscurus*, 8 *T. flavidus*, 8 *T. bimaculatus*, and 15 *T. niphobles*. The samples of *T. rubripes*, *T. flavidus*, and *T. bimaculatus* were from the Tanghai breeding base, Beidaihe Central Experiment Station, Chinese Academy of Fishery Sciences (119°31'32"E, 39°48'58"N). A natural population of *T. niphobles* was obtained from the open seas of Bohai Bay located near Beidaihe, Qinhuangdao, Hebei in China (119°32'55"E, 39°48'56"N). *T. obscurus* were sampled from the Zhongyang pufferfish manor in Hai'an, Jiangsu, China (120°53'58"E, 32°36'44"N). The salinity of the corresponding water environment was measured using a salinometer. The water salinity for *T. obscurus* was 6 ppt, while the water salinity for the other *Takifugu* species was equivalent to the natural salinity of seawater, 30 ppt.

Whole genome sequencing and SNP discovery

The collected fish were euthanized in MS222 solution (30mg/L) for three minutes before tissue collection. Genomic DNA of 90 fish was extracted from the caudal fin using DNeasy 96 Blood & Tissue Kits (Qiagen, Shanghai, China) in accordance with the manufacturer's instructions. The integrity of DNA was checked via electrophoresis on 1.0% agarose gels. Genomic DNA was quantified by Nanodrop-1000 (Thermo Scientific, Wilmington, DE, USA), qualified by the fluorometer Qubit 4.0 (Thermo Scientific) and

diluted to 50 ng/μL for sequencing. The total amount of DNA for whole genome sequencing was no less than 2 μg per sample. After DNA quality verification, all individuals belonging to *T. rubripes* (N=29), *T. obscurus* (N=30), *T. flavidus* (N=8), *T. bimaculatus* (N=8), and *T. niphobles* (N=15) were used for genome re-sequencing. DNA libraries were constructed and barcoded using the DNA Library Prep Reference Guide (Illumina). Sequencing was performed on the Illumina HiSeq X Ten platform with 150 bp paired-end reads. The average sequencing depth was 10× coverage per sample. The quality of raw reads was assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The sequences were filtered by the following steps: (1) Potential remnants of adapter sequences were removed; (2) low-quality paired reads with higher than 10% unrecognized bases in a single-end read were eliminated; and (3) low-quality bases with a Phred quality score lower than 20 were trimmed. After filtering, clean sequencing data were used for SNP calling. The sequencing data from each individual were aligned to the genome assembly of *T. rubripes* FUGU5 [24] using the Burrows-Wheeler Aligner (BWA) [40]. SNP calling was conducted using SAMTOOLS and BCFTOOLS with default parameters [41].

Phylogeny, principle component analysis, and genetic structure

To understand the evolutionary relationships of pufferfish species, maximum likelihood (ML) analysis was performed to construct a phylogenetic tree using RAxML version 8 [42]. The bootstrap analysis was conducted by the best-scoring topology with 1000 replicates. The iTOL software (<http://itol.embl.de/upload.cgi>) was used to display the phylogenetic tree. PCA was conducted using PLINK 1.9 [43] and Genome-wide Complex Trait Analysis (GCTA) software [44]. The population structure was investigated by the Bayesian clustering program STRUCTURE 2.3.1 [45] using all of the SNPs, with 2000 iterations. The number of genetic clusters K was predefined from two to five. The exported structure matrix was visualized by the StructurePlot 2.0 software [46].

LD decay and haplotype construction

LD decay for five populations, saline populations, and all samples was estimated within a range of 5 kb using PLINK 1.9 [43]. The average R^2 value per kb region was calculated and plotted against the physical distances of SNPs in units of kb. Haplotype blocks that showed strong linkages were constructed using the PLINK “-blocks” parameter for each population.

Identification of selective signatures between populations

Whole genome scanning between freshwater species (Ob: *T. obscurus*, n=30) and saline water species (Saline: *T. rubripes*, *T. flavidus*, *T. niphobles* and *T. bimaculatus*, n=60), as well as between *T. obscurus* and the other four species separately, was conducted using three methods: π , Fst, and Tajima's D tests. The π distribution was calculated using a sliding window method in VCFTOOLS [47]. The genome was divided into 100 kb sliding windows, and the stepwise distance was 10 kb. The π ratios of Saline/Ob as well as from *T. obscurus* and the other four species were calculated, and the ratios were sorted. Fst and Tajima's D test were conducted using VCFTOOLS “-weir-fst-pop” and “-TajimaD” parameters, respectively. The regions with the highest 5% π ratio and Fst values were identified and considered as candidate

regions under selective sweeps. The scatter plots of π ratios and F_{st} values were visualized using the R ggplot2 package (<http://cran.r-project.org/package=ggplot2>).

Enrichment analysis

Genes involved in the regions of selective sweeps were chosen for functional annotation. GO and KEGG pathway analyses were performed using DAVID 6.8 [48, 49] between *T. obscurus* and four saline water species, as well as between Saline and Ob.

List Of Abbreviations

SNP: single nucleotide polymorphism; PCA: principal component analysis; NKCC2: Na⁺-K⁺-Cl⁻ cotransporter 2; NCC: Na⁺-Cl⁻ cotransporter; NKA: Na⁺/K⁺-ATPase; LD: linkage disequilibrium; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the care and use of animals for scientific purposes set up by the Animal Care and Use Committee of the Chinese Academy of Fishery Sciences (ACUC-CAFS). The protocol was approved by the ACUC-CAFS.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during the current study are available in the NCBI SRA repository (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA522329/>).

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

JX initiated and coordinated the research project. HZ and JX conducted the analysis, and drafted the manuscript. JH, HL, HZ and GX engaged in sample collection and DNA extraction. All authors read and approved the final manuscript.

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Supplementary Material

Supplementary Table 1-12. Summary of sample information and re-sequencing data, LD decay and block size, gene lists under selective sweeps, GO and KEGG enrichment, and potential candidate genes related to osmoregulation.

Supplementary Figure 1. Genetic structure of pufferfishes for K=2, 3, and 5. Each individual has been indicated as a colored segment representing the proportion contributed by its ancestral population.

Supplementary Figure 2. Pie plot of the enriched pathways of osmotic regulation related genes between the Saline and *T. obscurus* populations.

Supplementary Figure 3. Box plot of the Tajima's D differences between selective sweep regions and the whole genome. Boxes denote the values between the 25th and 75th percentiles and the black transverse line inside the box denotes the median. Black vertical lines denote the values within 1.5 times of quartile values. Outliers are shown as black dots. (A) *T. rubripes* vs *T. obscurus*. (B) *T. flavidus* vs *T. obscurus*. (C) *T. bimaculatus* vs *T. obscurus*. (D) *T. niphobles* vs *T. obscurus*.

Figures

Figure 1
Geographic distribution of five Takifugu populations.

Figure 2

Phylogeny, PCA, and population structure of five Takifugu populations. (A) The maximum-likelihood phylogenetic tree. The tree was constructed from 11,344,115 SNPs of 90 pufferfish from five populations (red: *T. rubripes*, orange: *T. flavidus*, yellow: *T. bimaculatus*, green: *T. niphobles*, and purple: *T. obscurus*). (B) The principal components analysis (PCA) of five Takifugu populations. (C) Genetic structure of the pufferfish inferred by STRUCTURE analysis. The number of ancestral populations $K=4$ is shown. Each individual has been indicated as a colored segment representing the proportion of its ancestral population.

Figure 3

LD decay and haplo-block size distributions. (A) LD patterns of five Takifugu populations, saline populations, and all individuals. (B) Distribution of haplotype block length of *T. niphobles*, *T. obscurus*, *T. rubripes* populations, saline populations, and all individuals.

Figure 4

Pi, Fst, and selected genes from selective sweep analysis between the saline populations and T. obscurus. (A) Distribution of π ratios and Fst values. Red data points were identified as selective sweeps that passed the thresholds of π ratio (the 5% right tail of the empirical π ratio distribution, π ratio $\geq$ 5.856) and Fst (the 5% right tail of the empirical Fst distribution, Fst $\geq$ 0.232). (B) Representative genes with strong selective sweep signals in the T. obscurus population. Genome annotations are shown at the bottom, and red bars represent coding sequences.

Figure 5

Distribution of π ratios, F_{st} , and Tajima's D values for selective sweep regions between the saline populations and *T. obscurus*. (A) Distribution of π ratios and the absolute differences of Tajima's D values for genomic regions under selective sweeps in comparison with the whole genome. Blue dots denote the selected regions, while black dots denote the unselected regions. (B) Distribution of F_{st} and the absolute differences of Tajima's D values for genomic regions under selective sweeps in comparison with the whole genome. Blue dots denote the selected regions, while black dots denote the unselected

regions. (C) Box plot of the absolute differences of Tajima's D values for genomic regions under selective sweeps in comparison with the whole genome. Boxes denote the values between the 25th and 75th percentiles, and the black transverse line inside the box denotes the median. Black vertical lines denote the values within 1.5 times the quartile values. Outliers are shown as black dots.

Figure 6

Pi, Fst, and candidate genes identified under selective sweep analysis between *T. obscurus* and four other Takifugu populations. (A) Distribution of π ratios and Fst values. Red data points that passed the thresholds of π ratio and Fst (the 5% right tail of the empirical π ratio and Fst distributions) located to the upper right were identified as selected regions for the *T. rubripes*/*T. obscurus*, *T. flavidus*/*T. obscurus*, *T. bimaculatus*/*T. obscurus*, and *T. niphobles*/*T. obscurus* comparisons. (B) Candidate genes with strong selective sweep signals in the *T. obscurus* population. The π ratios were plotted using 10 kb sliding windows. Genome annotations are shown at the bottom, and red bars represent coding sequences.

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