論 文 の 内 容 の 要 旨

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Studies on genetic relationship between two closely related pufferfish, torafugu *Takifugu rubripes* and karasu *T. chinensis*
（近縁種トラフグおよびカラスの遺伝的関係に関する研究）

Among different pufferfish species, members of the genus *Takifugu* are most prominent and the species belonging to this genus are closely related. This is especially true for two pufferfish species such as torafugu *T. rubripes*, the most prominent member of this genus and also the most expensive among pufferfish, and karasu *T. chinensis*, both of which share their morphological characters and occupy the same habitation around the Yellow Sea and the East China Sea. However, the two species have different external color patterns. *T. rubripes* has some irregular black marks on a dorsal half of the posterior body part with white or pinkish anal fin. On the other hand, *T. chinensis* has no black marks on that body part with black anal fin. Despite such differences, the availability of hybrid-like individuals having intermediate color patterns between the two species leads to disputes arising in the Japanese fish markets during trade on species identification and classification. It is, therefore, necessary to provide definitive information about the two species by obtaining species-specific data and based on the analysis of genetic variation. Furthermore, the detection of genetic variation at the species level as well as genetic relationships among different *Takifugu* species is of great importance for sustainable aquaculture practices to reply on the expansion of aquaculture of *T. rubripes* in Japan, China and other countries. The present study was, therefore, undertaken to elucidate genetic relationship of *T. rubripes* and *T. chinensis* by sequence analyses of several mitochondrial (mt) and nuclear genes. Efforts were also made to identify the gene(s) responsible for the evident color-pattern differences between *T. rubripes* and *T. chinensis* by using two differential analysis methods, mRNA arbitrarily primed reverse transcription-polymerase chain reaction (RAP RT-PCR) and suppression subtractive hybridization (SSH). Finally genetic diversity was analyzed at the population level using microsatellite markers and mt control region (CR).

1. Studies on mitochondrial and nuclear genes of *Takifugu rubripes* and *T. chinensis*

Based on the mt genome data of *T. rubripes* reported by Elmerot et al. (2002), mt genes including those encoding 16S ribosomal RNA (rRNA), adenosine triphosphatase 6 (*ATPase 6*), nicotinamide adenine dinucleotide dehydrogenase subunit 4 (*ND4*), *ND5* and cytochrome *b* (cyt *b*) were compared between *T. rubripes* and *T. chinensis* and several hybrid-like individuals having skin and anal fin color patterns of both species. CR was selected because noncoding regions have generally evolved faster than coding regions. In addition, the nuclear genes such as internal transcribed spacer 1 (ITS1) and ITS2 were used to compare their genetic elements.

Primers were designed for mt genes based on the reported sequence of *T. rubripes* (DDBJ/EMBL/GenBank accession no. AJ421455) to amplify the segments of 640 bp in 16S rRNA, 308 bp in *ATPase 6*, 344 bp in *ND4*, 554 bp in *ND5*, 697 bp in cyt *b* and 1020 bp in CR. The sequences determined covered 20% in mtDNA. Informative sites were composed of one for 16S rRNA (position 1886), two each for *ATPase 6* (8427 and 8559), *ND4* (11 346 and 11 394) and *ND5* (12 219 and 12 298), and three for cyt *b* (14 762, 14 858 and 14 978) among 24 wild *T. rubripes*, 24 wild *T. chinensis* and six hybrid-like samples. Among 24 wild *T. rubripes* identified by external color patterns, 15% possessed nucleotide sequences consistent with *T. chinensis* registered in the DDBJ/EMBL/GenBank databases for *T. chinensis* (AP009534), whereas the sequences of 60% *T. chinensis* individuals were consistent with those registered for *T. rubripes* (AJ421455). As for the hybrid-like samples, two possessed *T. chinensis*-specific sequences in some base positions and *T. rubripes*-specific sequences in others. The remaining hybrid-like samples possessed *T. rubripes*-specific sequences. CR showed the intra-specific nucleotide sequence identity of 89 – 90% which was the lowest level among the mtDNA fragments analyzed for the three groups distinguished with their external color patterns. The rest of the mt genes showed 99 – 100% sequence identity with the respective gene fragments.

The nuclear genes consisting of linearized partial 18S rRNA, complete internal transcribed spacer 1 (ITS1), 5.8S rRNA and partial ITS2 showed the same levels of sequence identity (99 – 100%) between *T. rubripes* and *T. chinensis*. These results indicate a very low level of variation between the two *Takifugu* species.

2. Studies on the genes responsible for color differentiation between *Takifugu rubripes* and *T. chinensis*

To shed light on the events on how these two pufferfish species show differentiation in their skin and anal fin colors, the nuclear genes encoding melanocortin receptor (*MC1R* and *MC4R*) and pro-opiomelanocortin (*POMC*) were analyzed for their sequences, since these genes have been reported to be very closely associated with pigmentation and related functions in human and other vertebrates. The determined complete nucleotide sequences of 903 bp and 969 bp for *MC1R* and *MC4R*, respectively, and a partial sequence of 374 bp for *POMC* showed 99 – 100% inter- and intraspecific sequence identity, declining their roles in the observed color pattern differences.

Then two differential analysis techniques, RAP RT-PCR and SSH, were employed by using skin and anal fin tissues which show different color patterns between *T. rubripes* and *T. chinensis*. RAP RT-PCR was performed with cDNAs from anal fin tissues of the two

target species using an arbitrarily chosen primer A-5 (5′-AATCTAGAGCTCCCTCCA-3′) from the RAP-PCR kit (Stratagene, La Jolla, CA, USA). SSH, on the other hand, was performed for skin tissues from two different locations and anal fin tissues, and three SSH libraries enriched in differentially expressed transcripts were constructed using PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA, USA). Reverse transcription-PCR confirmed species-specific expression for serine pamitoyl transferase subunit 2 gene (*sptlc2*) obtained by RAP RT-PCR and 17 different genes obtained by SSH which include those encoding secreted frizzled-related protein 4 (*sfrp4*), vomeronasal 1 receptor F4 (*v1rf4*), DEAH (Asp-Glu-Ala-His) box peptide 16 (*dhx16*), E74-like factor 2 (*elf2*), protocadherin2 (*pcdh2*), LRRGT00033 protein, immunoglobin super family 21 (*ifsf21*), kelch repeat and BTB (POZ) domain containing 3 (*btb3*), c15orf24 protein, h2A histone family member Y (*h2afy*), s100 calcium binding protein a11 (*s100a11*), mKIAA1506 protein (*mll3*), annexin 2a (*anxa2a*), c1q-like adipose specific protein, IgM heavy chain constant region (*ighm*), MGC108117 protein and 40S ribosomal protein S17 (*rps17*).

Sptlc2 notified by RAP RT-PCR in the present study has been reported to act as an epidermal barrier in skin and up-regulated by UVB (λ, 290 – 320 nm) irradiation in human keratinocytes. The significantly higher levels of *sptlc2* expression in *T. chinensis* anal fin tissues compared to that of *T. rubripes* indicates its possible role in controlling the movement of melanosomes to maintain melanocytes confined in the anal fin region in *T. chinensis*. Among the differentially expressed SSH transcripts, calcium ion binding proteins including s100a11 and anx2a, cell adhesion molecules including pcdh2, proteolytic proteins including c15orf24 and LRRGT00033, and Wnt signaling pathway related proteins including sfrp4 and v1fr4 could play vital roles for creating the observed color pattern differences between *T. rubripes* and *T. chinensis*.

Functional analysis was performed with zebrafish using morpholino oligonucleotide (MO) for two genes among the differentially expressed ones obtained by SSH including *dhx16* and *s100a11* since these two genes are thought to be closely related to coloration events in fish. Zebrafish embryos injected with *dhx16* MO showed little difference in the coloration until 48 hours post fertilization (hpf) in comparison with the control batch. Several larvae injected with MO showed chromatophore diffusion on the lateral side of the body at 72 hpf. Furthermore, there were some deformities in the body shape with hatched larvae at 48 hpf. *Dhx16* is involved in pre-mRNA splicing and thought to possess a complex role in various physiological pathways. As for zebrafish embryos injected with *s100a11* MO, no evident differences in the color pattern were observed when compared with those in the control batch up to 8 days post fertilization, indicating the formation of pigment granules in zebrafish to be independent of *s100a11*. A comprehensive study of all differentially expressed genes is required to elucidate the event of color differentiation between *T. rubripes* and *T. chinensis*.

3. Genetic diversity analysis between *Takifugu rubripes* and *T. chinensis*

The discovery of overlapping mt gene sequences in partial 16S rRNA, *ATPase 6*, *ND4*, *ND5* and cyt *b* between *T. rubripes* and *T. chinensis* rendered it essential to estimate the degree of relatedness of the two species at the population level. Genetic variation was, therefore, surveyed with four microsatellite loci at the nuclear gene level and with CR

(561 bp) at the mt gene level among two wild *T. rubripes* populations [collected at Tsushima Island in 2003 (TT) and Soneshinden, Kitakyushu in 2008 (ST)] and one wild *T. chinensis* population [collected on the east coast of Korea in 2004 (KK)].

Microsatellite genotyping, among which three were reported by Furukawa et al. (2004) and one unpublished, was carried out by PCR for the total genomic DNA from 150 pufferfish individuals, and the amplified PCR products were analyzed using QIAxcel system (QIAGEN, Irvine, CA, USA). The sample size was 50 each for the three populations. Data analyses were performed using Genepop v3.4 (Raymond and Rousset, 1995), TFPGA (Miller, 1997) and Fstat softwares (Goudet, 1995). All four microsatellite loci were polymorphic and yielded a total of 138 different alleles. Total number of alleles per locus ranged from 25 to 47. The genetic diversity index values of TT, ST and KK populations were 0.9505, 0.9350 and 0.9335, respectively. The values of genetic distance and genetic differentiation (G_{ST}) between TT and KK (0.0543 and 0.0189, respectively) were smaller than those between TT and ST (0.0857 and 0.0194, respectively). UPGMA dendrogram using microsatellite data also showed that TT formed one clade with KK, whereas ST was separated from this clade.

Population dynamics study using CR for the same 150 pufferfish specimens was performed by direct sequencing of this gene fragment which yielded 161 variable sites (28.87%) and resulted in 106 haplotypes. Haplotype diversity (h) was highest in ST population (0.99837 ± 0.004) and lowest in KK (0.91184 ± 0.026). Sequence diversity (π) also showed a similar trend among the three *Takifugu* populations (0.02914 ± 0.019175 for ST and 0.00546 ± 0.004993 for KK). Overall h and π values were 0.98192 ± 0.018 and 0.01756 ± 0.00130, respectively. The genetic distance using CR among the three *Takifugu* populations was consistent with those obtained using microsatellite loci where the distance between TT and KK populations was smaller (0.0821) than those between TT and ST populations (0.1473). Average pairwise difference also showed a similar trend of genetic relationship where the value was lowest between TT and KK (7.49040) and largest between TT and ST populations (17.76480). These results suggest that *T. rubripes* and *T. chinensis* are indeed very closely related and probably regarded as the same species.

Conclusion

A very close genetic relationship was found between *T. rubripes* and *T. chinensis* in the present study, showing the possibility that these two species are the same species. Their evident difference in color may be due to regional or site variations where samples were collected, although it is uncertain whether or not the flesh texture and taste would be different between the two species. In addition, the differentially expressed genes obtained in the present study are also important as they are potential candidates for color pattern variation between the two species. From the data obtained so far, a scheme on the phylogeography as well as admixture of these two *Takifugu* species around the Sea of Japan can be concluded which may contribute to the studies on the mechanisms involved in speciation of the members of *Takifugu* genus.