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Investigations into the perplexing interrelationship of the Genus *Takifugu* Abe, 1949 (Tetraodontiformes, Tetraodontidae)

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The phylogenetic relationships within the genus *Takifugu* Abe, 1949 (Tetraodontiformes, Tetraodontidae) remain unresolved. Because of the use of *Takifugu* as model organisms, the resolution of these relationships is crucial for the interpretation of evolutionary trends in biology. Pufferfishes of this genus are comprised of a comparatively small number of species and are mainly distributed along the coastal region of the western part of the Sea of Japan and the coastline of China. Mitochondrial gene sequences were employed to test the phylogenetic hypotheses within the genus. Seventeen species of the genus were examined. Molecular phylogenetic trees were constructed using the maximum parsimony, neighbor-joining, maximum likelihood and Bayesian methods. Our hypothesis of internal relationships within the genus differs from previous hypotheses. Our results indicate that (1) the genus *Takifugu* is a monophyletic assemblage; (2) the genus is divided into 6 subgroups based on the molecular data; and (3) there is low genetic diversity among the species within this genus. In addition, speciation within *Takifugu* appears to be driven by hybridization and isolation by distribution. Our results also suggested that the taxonomy in the genus should be clarified based on both molecular and morphological data.

phylogeny, cytochrome *b* gene, 12S rRNA gene, *Takifugu* Abe

Pufferfishes of the genus *Takifugu* Abe, 1949 (Tetraodontiformes, Tetraodontidae) occur in Southeast Asia and the genus is composed of comparatively few species. There are 22 recognized species, and all of which have been recorded in China (including two previously unknown forms) according to the description in Fauna Sinica[1] (cf. 23 Valid taxonomic names could be found in records in fishbase: http://www.fishbase.org). It consists of primarily brackish and freshwater species but also includes species that are reef-dwelling, a few that are entirely pelagic, and a small number of benthic slope-dwellers species. Members of this genus are mainly distributed along the coastal regions of the western part of the Sea of Japan and the East China Sea from the Yellow Sea northward to Muroran, Hokkaido, Japan. Additionally, a few species of the genus are also distributed in marine regions of the Indian Ocean[2−4] (Figure 1). Most of species can be found along coastal regions of the Yellow Sea, Bohai Sea, East China Sea and South China Sea, with some species entering the estuaries of rivers in China[1,4,5].

Due to patchy distribution, it is difficult to collect the specimens of *Takifugu.* Few molecular phylogenetic analyses have been conducted to date. The published phylogenetic studies were based on morphological[5], cytogenetic[6] and isozyme variations[7]. Additionally, some researchers have investigated the genus-level taxonomy for the purpose of species identification[4,8−14].

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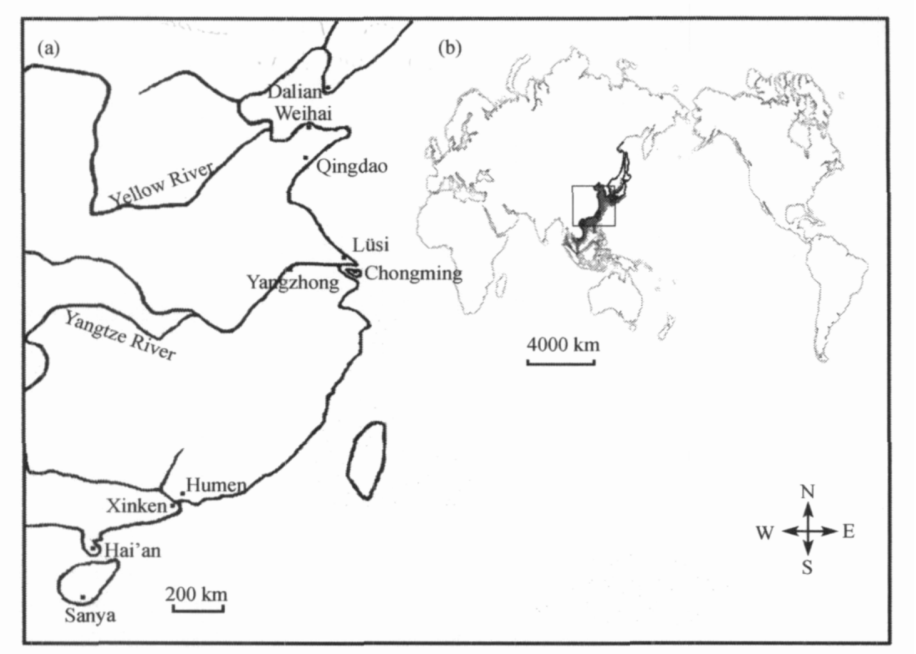

Figure 1 The sampling localities of the pufferfishes used in this study (a), and the distribution of the *Takifugu* Abe (shaded, (b)).

Before the 1950s, members of *Takifugu* Abe were ascribed to *Sphoeroides*. Based on their taxonomic work, Fraser-Brunner[8], Abe[9,10] and Whitley[11] showed the morphological diversity of these fishes was distinct and a new name *Takifugu* was given to discriminate them from *Sphoeroides*. Abe[9] also indicated that there were 6 subgenera comprised of 18 species, all of which occurred in marine habitats near China and Japan. Cheng et al.[5] examined the classification and interspecific relationships of 15 species (including 2 new species), found in China using morphological characters. Wang et al.[7] examined interspecific differences among 13 species within *Takifugu*, using allozyme variation of myogen. Miyaki et al.[6] performed karyological analyses for 6 species of *Takifugu* and revealed their remarkably similar chromosome complements. More recently, DNA-based analyses such as random amplified polymorphic DNA (RAPD) have been used to analyze the phylogenetic relationships among: (1) *T. pseudommus*, *T. obscurus*, and two forms of *T. rubripus* (from Japan and China)[12]; (2) *T. pseudommus*, *T. rubripus, T. xanthopterus*, *T. vermicularis* and *T. niphobles*; and (3) the population structure of two species, *T. pseudommus* and *T. rubripus*[4,13]. Song et al.[4] contributed topological phylogenetic trees for *T. pseudommus*, *T. rubripus*, *T. xanthopterus*, *T. vermicularis and T. niphobles*, which were generated from neighbor-joining analysis based on the data set of RAPD analysis and sequences of mitochondrial 16S rRNA (572 bp). Up to now, little systematic effort has been put forth to generate a DNA-based complete phylogeny and species identification of the genus *Takifugu*.

Takifugu rubripes's role has become more important not only in commerce, but also in functional genome studies as the model organism since the 1990s[15]. A draft sequence covering about 95% of the genome, using the "whole-genome shotgun" sequencing strategy, results of the assembly annotation, and a preliminary analysis of the genome was reported in *Science*[15], and later amended in July 2005 (http://www.fugu-sg.org/index.html). It has a uniquely compact genome with unusually small introns and lacks extensive repetitive sequences and pseudo-genes, making it a useful model for gene discovery, annotating the human and other vertebrate genomes[4,16,17]. A reliable phylogeny of the group, which is crucial for a better interpretation of the evolutionary trends for speciation of the group, is still absent[18]. Although the phylogenetic relationships among the genera of the family have been recently discussed by Holcroft[19,20], in which the relationships of the order Tetraodontiformes and the interrelationships of tetraodontiform fishes were laid out, it is essential to determine the phylogenetic position of *T. rubripes* by identifying its sister species and to place *Takifugu* Abe

into a comparative context.

The purpose of this study is to infer the phylogenetic relationships of the species within the genus *Takifugu*, to evaluate the monophyly of the group, and to estimate the validity of some of the currently recognized species within this genus. We used molecular data to infer the phylogeny of *Takifugu* from mitochondrial cytochrome *b* (Cyt *b*) and 12S rRNA genes. Cyt *b* gene has been considered one of the most useful genes for phylogenetic reconstruction, and is probably the best-known mitochondrial gene with respect to structure and function of its protein product[21]. Cyt *b* gene contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains overall[22−24]. Moreover, in order to add more informative sites, complete sequences of the 12S rRNA gene from 16 species were added to the Cyt *b* gene sequences to generate a robust phylogeny of the genus *Takifugu*. Two species, *Tetraodon nigroviridis* and *Lagocephalus gloveri*, were included as outgroups. They are the putative closest relatives to *Takifugu* according to recent studies[19,20].

1 Materials and methods

1.1 Species examined and sample collection

Due to recent over-fishing, wild stocks of *Takifugu* have been decreasing rapidly[25], which increased the difficulties of sampling; therefore, *T. porphyreus*, *T. pardalis*, *T. guttulatus*, *T. plagiocellatus* and *T. reticularis* were not included in this study. 17 spececies of *Takifugu* Abe, about three fourths of all described species (according to Su and Li[1]; Figure 1), were collected, including the Chinese coastal samples, in an effort to represent the diversity of this genus as much as possible (Table 1). All samples were preserved in 95% ethanol, and deposited in the Freshwater Fish Museum of the Institute of Hydrobiology, the Chinese Academy of Sciences.

1.2 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from fins following phenol/chloroform extraction procedure[26]. Target regions of the mitochondrial DNA were amplified from the total DNA extracts using the polymerase chain reaction (PCR). The primers for both Cyt *b* and 12S rRNA, Cyt *b* F (5′-GGCGTGAAAAACCATCGTTG-3′) and Cyt *b* R (5′-CCCCGACATTCGGTTTACAAGAC-3′), and 12sF (5′-GCAGAGTACTGAAGATGCTAAG-3′) and 12sR (5′-CGTCAACTCGGTGTAAGG-3′) were adapted from Xiao et al.[27] and Liu[28], modified with the mitochondrial sequence of *T. rubripes*[16] (GenBank Accession No. AJ421455). The PCR contained approximately 100 ng of template DNA, 1.5 μL of each primer, 6 μL of 10× reaction buffer, 1.5 μL dNTPs (each 2.5 mmol/L), and 1.5 U *Taq* DNA polymerase in total 60 μL in a total volume. The reaction conditions were conducted as the following: an initial 94℃ denaturation for 4 min; fol-

Table 1 Pairwise p-distance of the complete of Cyt *b* gene data matrix of 19 taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
T. rubripes																		
T. pseudommus	0.003																	
T. stictonotus	0.005	0.003																
T. flavidus	0.034	0.037	0.04															
T. bimaculatus	0.032	0.034	0.037	0.018														
T. ocellatus	0.042	0.045	0.042	0.034	0.042													
T. variomaculatus	0.042	0.045	0.042	0.034	0.042	0												
T. alboplumbeus	0.045	0.048	0.051	0.048	0.051	0.042	0.042											
T. stictonotus	0.056	0.059	0.062	0.042	0.051	0.051	0.051	0.059										
T. xanthopterus	0.031	0.034	0.037	0.029	0.032	0.037	0.037	0.043	0.051									
T. orbimaculatus	0.026	0.029	0.032	0.035	0.032	0.032	0.032	0.034	0.051	0.026								
T. fasciatus	0.026	0.029	0.032	0.037	0.034	0.031	0.031	0.034	0.056	0.026	0							
T. coronoidus	0.026	0.029	0.032	0.037	0.034	0.031	0.031	0.034	0.056	0.026	0	0						
T. vermicularis	0.032	0.034	0.037	0.037	0.034	0.034	0.034	0.034	0.056	0.037	0.016	0.016	0.016					
T. niphobles	0.051	0.054	0.056	0.054	0.051	0.056	0.056	0.059	0.076	0.062	0.037	0.043	0.042	0.048				
T. oblongus	0.054	0.056	0.059	0.057	0.054	0.059	0.059	0.062	0.079	0.065	0.04	0.045	0.045	0.051	0.003			
T. poecilonotus	0.04	0.042	0.045	0.037	0.034	0.04	0.04	0.045	0.054	0.034	0.029	0.034	0.034	0.034	0.054	0.056		
T. nigroviridis	0.199	0.196	0.199	0.186	0.189	0.216	0.216	0.195	0.203	0.195	0.191	0.196	0.196	0.206	0.209	0.206	0.192	
L. gloveri	0.234	0.237	0.241	0.23	0.233	0.233	0.233	0.244	0.259	0.256	0.24	0.245	0.245	0.245	0.23	0.226	0.244	0.216

lowed by 35 cycles of 94℃ denaturation for 45 s, 52℃ annealing for 1 min 45 s, 72℃ extension for 45 s; and a final 72℃ extension for 8 min. Amplified DNA was fractionated by electrophoresis on a 0.8% low-melting agarose gels, recovered from the gels and purified using BioStar Glassmilk DNA purification Kit according to manufacturer's instructions. Fragments of Cyt *b* and 12S rRNA genes were sequenced by using gel-purified PCR products. The same primers were used for sequencing. The sequences have been deposited in GenBank (Accession numbers were listed in Table 2). Cyt *b* and 12S rRNA sequences for *T. nigroviridis* were downloaded from GenBank (GenBank Accession No. DQ019313) for inclusion in the analysis.

1.3 Sequence variation and phylogenetic analysis

Because Cyt *b* is a protein-coding gene, and a previously obtained 12S rRNA sequence template was used, alignment of the sequences was straightforward and unambiguous. The Cyt *b* and 12S rRNA nucleotide sequences of *T. rubripes*[16] (GenBank Accession No. AJ421455) were downloaded for alignment, and also included in the subsequent analyses. Sequence editing and multiple alignments were performed using different alignment editors, such as Editseq in DNAstar (http://www.dnastar.com/web/index.php), CLUSTAL X[29], and SEAVIEW[30], and verified by eye. Alignment was performed in an NEXUS file by organizing the *T. rubripes* nucleotide sequence into codons according to the published amino acid translation. The consensus light strand sequences generated for this study were then aligned by eye to the *T. rubripes* template sequence.

Wiens[31] suggested that unless the proportion of missing data was large, addition of incomplete data sets was more likely to improve phylogenetic accuracy than if they were discarded. A Cyt *b* sequence for *T. poecilonotus* (424 bp) was included although there was no corresponding 12S rRNA sequences for this species. Alignment gaps in 12S rRNA sequences were treated as missing characters. The analyses were performed using the combined data set of Cyt b and 12S rRNA genes, which included seventeen species of *Takifugu* and two outgroups (Table 2).

Phylogenetic congruence of Cyt *b* and 12S rRNA data sets were performed by the partition homogeneity test of Farris et al.[32] with PAUP* 4.0b10[33]. The partition homogeneity test supported the combination of the Cyt *b* and 12S rRNA data sets ($P = 0.962$). The TN93[34] distance was used to analyze and examine potential mutational site saturation across the sequenced dataset as a

Table 2 Listing of species examined in this study, following classification of Su and Li[1] with GenBank Accession numbers

Taxon	Voucher No.	Collection locality	GenBank Accession No.	
			Cyt *b*	12s rRNA
T. nigroviridis			DQ019313	DQ019313
L. gloveri	IHB0411227	Qingdao, ShanEast	EF126108	EF126128
T. poecilonotus			AY267360	
T. oblongus	IHB0411173	Sanya, Hainan	EF126107	EF126110
T. niphobles	IHB0411175	Sanya, Hainan	EF126106	EF126109
T. alboplumbeus	IHB0411177	Sanya, Hainan	EF126097	EF126111
T. ocellatus	IHB0411191	Humen, GuangEast	EF126095	EF126113
T. variomaculatus	IHB0411195	Humen, GuangEast	EF126096	EF126114
T. orbimaculatus	IHB0411198	Humen, GuangEast	EF126100	EF126122
T. orbimaculatus	IHB0411207	Xinken, GuangEast	EF126104	EF126126
T. fasciatus	IHB0411203	Yangzhong, Jiangshu	EF126102	EF126123
T. fasciatus	IHB0411203	Xinken, GuangEast	EF126101	EF126124
T. flavidus	IHB0411217	Yangzhong, Jiangshu	EF126093	EF126117
T. flavidus		Lvsi, Jiangshu	EF126092	EF126116
T. coronoidus	IHB0411223	Lvsi, Jiangshu	EF126103	EF126125
T. xanthopterus	IHB0411224	Qingdao, ShanEast	EF126099	EF126115
T. rubripes	IHB0411226	Lvsi, Jiangshu	EF126089	EF126119
T. rubripes			AJ421455	AJ421455
T. basilevskianus	IHB0507170	Weihai, ShanEast	EF126091	EF126121
T. stictonotus	IHB0507172	Weihai, ShanEast	EF126098	EF126112
T. vermicularis		Dalian, Liaoning	EF126105	EF126127
T. pseudommus	IHB0507101	Dalian, Liaoning	EF126090	EF126120
T. bimaculatus	IHB0411214	Chongming, Shanghai	EF126094	EF126118

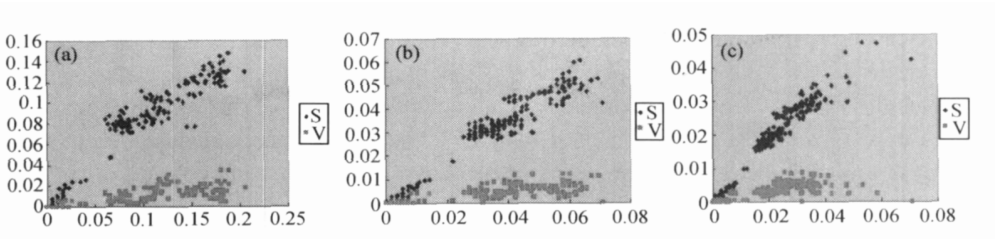

Figure 2 Saturation tests. Numbers of transition and transversion substitutions vs. the TN93 distances in pairwise comparisons of 21 sequences (excluded outgroups) for the 3rd position of Cyt *b* gene (a), Cyt *b* gene (b) and combined dataset (c). s, Transition; v, transversion.

whole and in the third position of Cyt *b* gene (outgroups excluded), as implemented in DAMBE[35]. The amount of sequence saturation was inferred from the shape of the trend line, with a linear relationship indicating that the sequence was unsaturated and an asymptotic relationship indicating the presence of saturation[36]. All plotting of the analysis indicated relatively linear relationships between substitution rate and genetic distance (Figure 2) demonstrating that transition and transversion were not saturated.

Measures of nucleotide composition were obtained using PAUP* 4.0b10[33]. Following Irwin et al.[37], base compositional bias was calculated and a chi-square (χ^2) test was also calculated for different genes and for each codon position and for all codon positions of Cyt *b* gene, as implemented in PAUP*.

Different phylogenetic analyses were performed using different methods to gauge the robustness of our resulting hypotheses. Neighbor-joining distance (NJ), maximum parsimony (MP)[38], maximum likelihood (ML)[39] were used to analyzed the aligned data matrix as implemented in PAUP*, and a Bayesian approach[40] as implemented in MrBayes 3.04b[41]. Node supports were assessed using ML, MP, and NJ bootstraps (BS)[42] with 250, 1000, and 1000 replicates, and Bayesian posterior probabilities (PP) in Bayesian analysis.

The best-fit model was determined as implemented in MODELTEST 3.06[43], employed to calculate the likelihood ratio test (LRT), to determine the fit nucleotide substitution as implemented in PAUP*. Based on the LRT, the ML was performed with the GTR + G + I evolution model for the alignment (GTR = General Time Reversible model; G = gamma distributed site-to-site variation; and I = proportion of invariable sites)[44]. In the ML analysis, we used heuristic searches with TBR (tree-bisection-reconnection) branch swapping with 10 times of the addition sequence (addseq = asis). In NJ analysis, the distance method was executed using TBR heuristic search with 10 random-addition-sequences. In MP analysis, The TI bias was estimated with ML and an associated transversion (TV)/transition (TI) ratio was calculated in Mega 3.0[45]. Weighting scheme (TV∶TI = 8∶1) was implemented to the third position of Cyt *b* gene, and equal weights corresponding to others position for Cyt *b* gene and 12S rRNA gene in MP analysis. The MP method was performed using heuristic searches with 10 random-addition-sequence replicates and TBR branch swapping.

MrBayes calculates Bayesian posterior probabilities by using a Metropolis-coupled, Markov chain Monte Carlo (MCMCMC) sampling approach. Intermixed model of Bayesian analyses was used, with model determination implemented in MODELTEST 3.06[43]. The data matrix was partitioned by gene (Cyt *b* and 12S rRNA) and by codon position. Starting trees were random, phylogenetic constraints were not used, and four simultaneous Markov chains were run for 2000000 generations, with trees sampled every 200 generations for a total of 10000 trees in the initial sample. The first 1000 trees were discarded as burnin and the posterior probabilities of the nodes were determined from the remaining 9000 trees. A 50% majority rule consensus tree of these combined samples was constructed.

In order to determine the best topology recovered under the different methods (NJ, MP, ML and Bayesian analysis) of analysis, two-tailed Wilcoxon signed-ranks test[46] and Shimodaira-Hasegawa test[47] were conducted, using 1000 bootstrap replicates with RELL (resampling estimated log-likelihood) optimization as implemented in PAUP*.

Test for substitution rate constancy (molecular clock test) of the combined data (outgroups excluded), was also performed under likelihood criteria, by constraining the mean rate of substitution to be constant among lineage and comparing this tree to the ML estimate obtained when this constraint was relaxed. The test compared the

log-likelihood of the most likely tree with and without a molecular clock enforced. If the topologies were equivalent and the likelihoods are not significantly different (likelihood-ratio test, $\alpha = 0.05$, d$f = S–2$, where S = number of taxa), the molecular clock hypothesis cannot be rejected[41].

2 Results

2.1 Sequence analysis

The final combined data matrix consists of 2095 aligned base positions (i.e. characters) which consisted of 1143 characters of the complete Cyt *b* gene and 952 characters from the 12S rRNA gene. All sequences were deposited in GenBank (Accession numbers listed in Table 2).

Within the ingroup, Cyt *b* sequences of 1137 nucleotides in length (coding for 378 aa) with nucleotide base composition showed low level of G (14.4% across all sites all taxa and only 6.8% at third position), which is characteristic for the mitochondrial genome and similar to those previously reported for Actinopterygii fish[48,49] (Table 1). There is little variation in nucleotides within the ingroup especially in 12S rRNA gene. In comparison to the outgroup species *T. nigroviridis*, two deletion base-sites (6 bp coding for 2 aa) were found within the Cyt *b* gene. Significant compositional biases existed at the 2nd and 3rd codon position, where there was a marked under representation of guanine (12.6% and 6.8%, respectively), and the overall base compositional bias was 0.14 for Cyt *b* gene. There was significant difference in base compositional bias between the ingroup and the outgroup species. The highest bias was found in the 3rd codon position (0.33) followed by the first codon position (0.20) and the smallest observed bias was in the 2nd codon position (0.02), similar to those previously reported for sisorid catfishes[24]. Nucleotide composition among taxa was homogenous at all three codon positions (1st codon position, $\chi^2 = 8.56$, df=69, $P = 1.00$; 2nd codon position, $\chi^2 = 8.73$, df=69, $P = 1.00$; and 3rd codon position, $\chi^2 = 51.04$, d$f = 69$, $P = 0.95$), indicating that base composition across taxa was stationary in the DNA data.

The average base composition of 12S rRNA gene was similar to those previously reported for cypriniform fishes[28]. Nucleotide composition of the gene among taxa was calculated as a whole: $\chi^2 = 7.46$, d$f = 66$, $P =$ 1.00, further indicates that the data are conserved, suggesting that 12S rRNA gene perform poorly at this level of analysis relative to Cyt *b* gene and morphological characters.

The uncorrected *p*-distance matrix obtained from the analysis of the alignment of all Cyt *b* sequences is shown in Table 1. The value of pairwise distance among members of the ingroup ranged from 0 (between *T. orbimaculatus* and *T. fasciatus*) to 0.079 (between *T. oblongus* and *T. stictonotus*), and distances between ingroup and outgroup species ranged from 0.189 (between *T. nigroviridis* and *T. bimaculatus*) to 0.259 (between *L. gloveri* and *T. stictonotus*).

2.2 Phylogenetic relationships

The monophyly of *Takifugu* is recovered with strong support in all analyses. The ML tree (Figure 3) constructed from a set of 23 combined sequences, confirmed the monophyly of the genus *Takifugu* (BS = 99). Maximum likelihood analysis yielded one tree with likelihood $-\ln L = 6627.22$. The other analysis yielded similar topologies (not shown). Bayesian analysis resulted in a consensus tree (50% majority rule) with great posterior probabilities at a majority of nodes. Monophyly of *Takifugu* was also supported by the Bayesian, MP, and NJ topology (PP=100, BS=100, and BS=100, respectively). Six major clades within the group are identified (Figure 3). In clade A, *T. ocellatus* is sister to *T. variomaculatus* with strong support (BS=100). Clade B contains 4 species: *T. poecilonotus*, *T. xanthopterus*, *T. alboplumbeus* and *T. stictonotus* with moderate support (BS>50). Within clade B, *T. alboplumbeus* is sister to *T. stictonotus* (BS=65). In clade C, *T. flavidus* is sister to *T. bimaculatus* with strong support (BS=94). Clade D, *T. basilevskianus* and *T. pseudommus* is composed of sister taxa plus *T. rubripes* with moderate support value (BS=78). *Takaifugu niphobles* is sister to *T. oblongus* with strong support (BS=100), forming clade E. Clade F contains 4 species: *T. orbimaculatus*, *T. coronoidus*, *T. fasciatus*, and *T. vermicularis* with robust support (BS=92). Clades A—F were also supported in MP, NJ and Baysian analyses. In contrast to the topologies, we found that clades D, E and F had a closer relationship than other clades in all analysis (Table 3). These three clades assembled in different analysis, clade F formed a sister-group relationship with clade E, and then together plus clade D formed a monophyly.

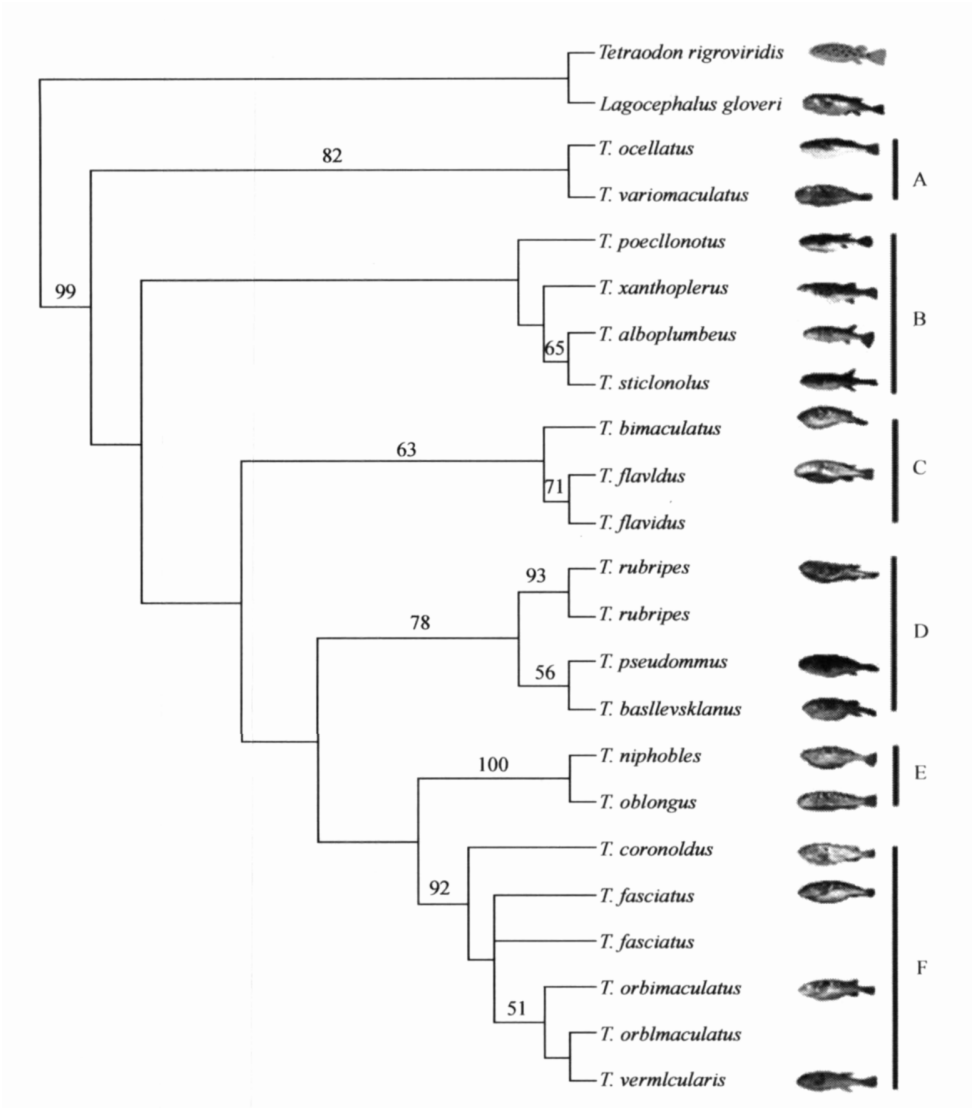

Figure 3 Reconstructed phylogeny of the *Takifugu* using Maximum Likelihood phylogenetic approaches based on combined Cyt *b* and 12S rRNA gene sequences. ML tree using GTR + G + I model; $\alpha = 0.5976$, $I = 0.5458$; $-\ln L = 6627.22335$. Bootstrap estimates are derived from 250 replicates. Numbers represent node supports inferred from bootstrap (only value above 50 are shown).

Table 3 Support values for MP, ME, and Bayesian nodes of clades shared by ML phylogeny[a)]

Clade	ML	MP	NJ	Bayesian
A	82	100	73	100
B				65
C	63	97		94
D	78	80	57	
E	100	100	94	100
F	92	98		99

a) The blank means BS or PP value lower than 50.

From our different analyses, we also could infer that the basal status among the clades is not yet resolved. Additionally, the details of the topological structure were not consistent among different analyses. Two-tailed Wilcoxon signed-ranks tests and Kishino-Hasegawa test were carried out to determine whether the topologies recovered under the different methods of analyses were significantly different from one another. The two tests did not reject the monophyly of *Takifugu* (Table 4), and the ML, MP, NJ, and Bayesian hypothesis were not significantly different, which covered similar log-likelihood score. The ML tree was recovered as the best tree based on Shimodaira-Hasegawa test. Buckley et al.[50] indicated that the SH test had two advantages over the more commonly used Kishino-Hasegawa (KH)[46] and Templeton[45] tests; therefore we considered the ML tree as the best topology in this study.

Table 4 Statistical comparison of alternative topologies including MP, NJ, ML, Bayesian trees, and various evolutionary hypotheses using Templeton[46] and Shimodaira-Hasegawa[47] tests[a)]

Tree	Templeton test				Shimodaira-Hasegawa test		
	length	*N*	*Z*	*P***	–ln*L*	Diff –ln*L*	*P*
MPc tree	801	1	1	–1	6658.0538	10.02293	0.263
ML tree	801	24	24	–0.193	6648.0308	best	
NJ tree	805	25	25	–1	6664.0564	16.02554	0.135
MP tree	800	Best			6654.9235	6.89267	0.397
Bayesian tree	803	5	–1.342	0.1797	6655.0058	6.975	0.443

a) Associated probabilities are given and significantly ($P < 0.05$) worse topologies are indicated by asterisks.

Due to among-lineage rate heterogeneity in the combined dataset, the molecular clock hypothesis was not supported. Specifically, the likelihood of the clock-like Cyt b tree with *L. gloveri* and *T. nigroviridis* as outgroups (–ln*L* = 4384.71189) and that of the unconstrained tree (–ln*L* = 4363.55237) were significantly different from each other ($\chi^2 = 42.32$, d*f* =22, $P = 0.006 < 0.05$). The result indicated that various lineages evolved at different rates and the use of molecular clock calibrations to estimate species divergence time appears statistically invalid for the data set.

3 Discussion

3.1 Monophyly of the genus *Takifugu* and relationships within it

The pufferfishes of the genus *Takifugu* are East Asian fishes, mainly distributed along the coastal region in western part of the Sea of Japan and the East China. The interrelationships of this genus had sparked controversy for some time[5,7,10,51]. Abe[9] first defined the genus *Takifugu* as a natural group. Most of the previous studies of this group were based on the descriptions of the adaptive characters, and no systematic work based on molecular data had been done. Therefore, we focused on comparing the cladogram from our molecular data with results of morphological[5] and myogen analyses[7]. Cheng et al.[5] and Wang et al.[7] suggested that *Takifugu* formed a monophyletic group (Figure 4). In their morphological analysis, Cheng et al.[5] focused on anatomical characters such as the skull, the dermal spines and the disposition of the spinose areas. They constructed their phylogenetic tree mainly based on the geographical distribution and the characters mentioned above. No other analysis method was adopted in their analysis. Wang et al.[7], investigated patterns of speciation by examining the electrophoretic patterns of the myogen via cluster analysis method. The methods and characters used in those studies were not comprehensive. Results of the phylogenetic analyses of our data indicate explicitly that *Takifugu* is a monophyletic group with robust support of various analyses.

By comparing our results to those of previous studies, some conclusions can be drawn. In clade D, monophyly of *T. basilevskianus* and *T. pseudommus* plus *T. rubripes* is also supported by Cheng et al.[5], and Song et al.[4]. Additionally, the sister relationship between *T. flavidus* and *T. bimaculatus* was supported by Cheng et al.[5], however not by Wang et al.[7]. Based on Wang et al.[7], *T. flavidus* forms a sister-relationship with *T. oblongus,* while *T. bimaculatus* was more closely allied with *T. fasciatus,* while our data suggest that *T. bimaculatus* and *T. fasciatus* are distantly related. Additionally, our analyses support the conclusion that *T. stictonotus* is sister to *T. alboplumbeus*, forming a monophyletic clade with *T. xanthopterus* and *T. poecilonotus* and the monophyly of clade F (*T. fasciatus*, *T. coronoidus*, *T. orbimaculatus* and *T. vermicularis*), both of which do not agree with the results in Cheng et al.[5] and Wang et al.[7] However, the division into different skull types based on morphology is supported by our data.

The phylogenetic tree constructed from electrophoretic patterns of myogen[7] (Figure 4(b)) is only partially congruent with the topology acquired by Cheng et al.[5] (Figure 4(a)). The interrelationships inferred by our analysis are neither completely congruent with the previous morphological hypotheses nor conclusions drawn from allozyme analysis of myogen. However, from our molecular data analysis, phylogenetic resolution among the clades was generally limited to recent relationships, with deeper nodes receiving poor support values (Figure 3); thus we are unable to explicitly resolve relationships among the 6 subgroups. However, we are able to show support for several previously hypothesized subgroups.

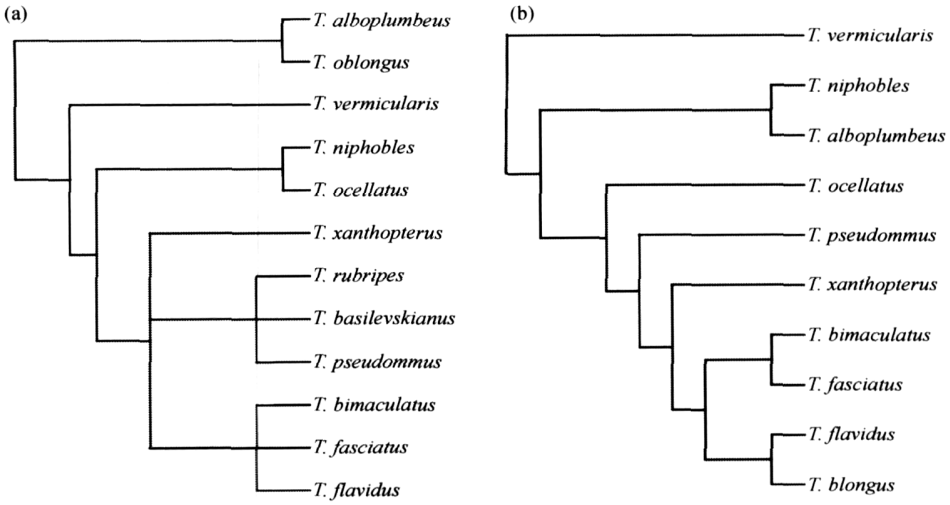

Figure 4 Cheng et al.[5] (a) and Wang et al.[7] (b) acquired hypothetical cladogram of the *Takifugu* fishes based on morphological characters and electrophoretic patterns of the myogen.

The mitochondrial Cyt *b* gene sequences generated in this study did not perform very well at this level phylogenetic analysis, although it has been widely used in systematic studies to resolve divergences at many taxonomic levels, particularly inter- or intra-specific relationships[52,53]. We are convinced that members of the genus are very closely related. The interrelationships among different subgroups were not resolved, suggesting that: (1) these subgroups might be engaged in parallel speciation; and (2) the data or the technique we used in this study do not provide the adequate information needed for this level of analysis.

However, our results conflict with many relationships suggested by the morphological analyses of Cheng et al.[5], who included some specialized characters directly derived from the primitive state such as geobiology, the skull, the dermal spines and the disposition of the spinose areas. All these characters might be advanced characters instead of a reliable phylogenetic character. In contrast to the morphological analysis, we consider that molecular data should be more phylogenetically informative.

3.2 Speciation and taxonomic implications of the *Takifugu* genus

Tetraodontoidea fishes might have originated in a warm tropical marine zone, and with differentiation and evolution transferred to lukewarm marine, brackish estuarine, and inland freshwater habitats[1]. The earliest emergence of Tetraodontidae fishes in the fossil record is in the Eocene[1]. They are considered to be the most differentiated group of fishes, and have a geobiological distribution following the path of ocean currents[1]. Tetraodontidae occur in tropical zone and temperate environments as well as inshore or estuarine habitats, suggesting the possibility that isolation may have been a driving pressure in their evolution. *Takifugu* fishes have small ranges, which is probably due to their poor swimming ability. *Takifugu* are distinguished from other genera by epidermal appearance and shape, swimming bladder and abdominal or dorsal spines.

Most of the species are found only near inshore areas. According to our molecular data, the mode of divergence for the group may be different from that of most members of Tetraodontidae with the divergence time of these species occurring over a short time frame. The fossil record of this group is scare and more details should be confirmed with more fossil evidence.

Our molecular data indicate that species within *Takifugu* are very closely related. Comparing morphological data with our molecular data, we found that morphological characters based on the comparative morphology of the skull, dermal spines, and the dispositions of the spinous areas are adaptive characters. The proportion elements of all these distinctive characters used for the reconstruction of the phylogenetic relationship may be subjective in morphological analysis and are not consistent with the molecular level evidence: e. g. *T. alboplumbeus* and *T. oblongus* forms a sister group in the

morphological analysis, united primarily by geographical distribution and strong spinous areas[5]; however, the Cyt *b* genetic uncorrected-*p* distance was about 0.01, which is moderately diverse for members of this genus.

We noted that some species were very similar morphologically and not easily distinguished from each other[1], such as *T. niphobles* and *T. flavidus*. The genetic distance among them was large (about 0.054) for members of the genus. Kadereit[54] suggested adaptive characters might have arisen by macro-mutation of the structural genes. As a result of coincidental selective advantages or occurrences in small isolated populations, these mutations were brought to fixation, and they most likely did not represent characters due to speciation. It may be inferred that some specialized species (e.g. *T. flavidus*) obtained some adaptive features due to macro-mutation, resulting in a rapid diversification within the genus. The route of differentiation and evolution is not the same among members of *Takifugu* as with the majority of species of Tetraodontidae fishes.

Based on high levels of variance in body shape and skin color and texture, classification of the group has been difficult[5]. Su and Li[1] described two species *Takifugu coronoidus* and *T. fasciatus* in their morphological description; however, the two species cannot be distinguished from each other based on principal component analysis using the method of morphometrics (unpublished data). In our study, *T. coronoidus* and *T. fasciatus* had two identical Cyt *b* and 12S rRNA sequences. Combined with the morphological evidence mentioned above, we suppose that these two species are in fact synonymous. However, more population level investigations should been performed to confirm this view.

The most interesting observation during the course of this study involved *Takifugu ocellatus* and *T. variomaculatus* in the Pearl River. Throughout our sampling, *T. variomaculatus* was only found in the estuary area of the Pearl River, and *T. ocellatus* was found along the South China Sea to the East China Sea. This distribution was also recorded by Su and Li[1]. The two species are morphologically similar, with the only difference between them being dermal texture on their back. They formed a sister-group relationship in our analysis. During our sampling course, morphological intermediates were found in estuary area of the Pearl River (unpublished data). A similar observation was made for species found in the estuary area of the Yangtze River and near shore area of the Bohai Sea. Widespread species and endemic species exited in these areas: *T. fasciatus* and *T. coronoidus* in the estuary area of the Yangtze River and *T. basilevskianus, T. rubripes* in the Bohai Sea[1] respectively. These three areas have the following features in common: (1) low genetic differentiation among resident species; (2) rich species diversity; and (3) intermediate specimens. Song et al.[4] indicated that intermediate characters could be produced by hybridization in *T. rubripes* and *T. pseudommus*. The distinguishing characters between these two species are the coloration of the body and the anal fin. Song et al.[4] found many intermediates that possessed intermediate features such as white anal fins with black fringe[4]. The occurrence of natural hybridization between *T. xanthopterus* and *T. vermicularis* was also described by Masuda et al.[51].

Isolated distributions, different salinity of marine habitats and poor swimming ability of *Takifugu* made isolation of species possible, creating conditions for speciation. Multiple species exist in the specific locality, suggesting that hybridization may have played a very important role in the speciation within this genus. Estuarine area of large rivers might have played a central role in *Takifugu* speciation over time, particularly in creating hybrid zones. Therefore, the speciation of *Takifugu* might be different from that of other Tetraodontoidea fishes, making it an interesting topic for further research to elucidate the interrelationship and differentiation within the group. In addition to the population-level investigations, a more comprehensive study of the interrelationship within the genus is warranted, especially considering that *Takifugu* is playing an increasingly important role in comparative genomics.

In conclusion, phylogenetic analyses suggest that *Takifugu* is a monophyletic clade divided into 6 subgroups. Phylogenetic resolution within the genus was generally limited to recent relationships and deeper nodes received poor support values (Figure 3). We can infer that species in the genus *Takifugu* are not highly divergent at the molecular level. The close relationships within the genus provide more experimental options for future investigations. Our results suggest that diversification in the group occurred rapidly, and speciation of the genus may still be in process corresponding to modern-day isolated inshore areas, where intermediate specimens were discovered during our sampling course. Due to the economic genome of *Takifugu* fishes, more

investigations of species evolution from the perspective of the genomic level would contribute to understanding genome evolution and species differentiation in vertebrates. Our data resolved some aspects of *Takifugu* phylogeny, but questions regarding subgroup interrelationships remain unresolved. The positions of the subgroups and their intermediates in different trees did not receive robust support values and different analyses were not convergent, suggesting the need for further investigations at subgroup level. Increased taxon sampling plus more sensitive methods, such as microsatellite markers, would certainly improve resolution and support. Future studies should also target other mitochondrial and nuclear genes such as D-loop and RAG1 gene in an effort to provide additional independent molecular data to determine the patterns and processes of speciation in *Takifugu* at population level.

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1 Su J, Li C. Fauna Sinica, Class Teleostei, Tetraodontiformes. Beijing: Science Press, 2002
2 Santini F, Tyler J C. A phylogeny of the families of fossil and extant tetraodontiform fishes (Acanthomorpha, Tetraodontiformes), upper cretaceous to recent. Zool J Linnean Soc, 2003, 139(4): 565－617
3 Masuda H, Amaoka K, Araga C, et al. The Fishes of the Japanese Archipelago, vol. Text and Plates. Tokyo: Tokai Univ Press, 1984
4 Song L, Liu B, Xiang J, et al. Molecular phylogeny and species identification of pufferfish of the genus *Takifugu* (Tetraodontiformes, Tetraodontidae). Mar Biotechnol (NY), 2001, 3(4): 398－406
5 Cheng Q, Wang C, Tian M, et al. Studies on the Chinese Tetraodonoid fishes of the genus *Fugu*. Acta Zool Sin (in Chinese), 1975, 21(4): 359－378
6 Miyaki K, Tebeta O, Kayano H. Karyotypes in six species of pufferfishes genus Takifugu (Tetraodontidae, Tetraodontiformes). Fish Sci, 1995, 61: 594－598
7 Wang K, Zhang P, Yin Q. Studies on the interspecific differences of myogen and cluster of the genus Fugu. Ocean Limnol Sin (in Chinese), 1984, 15(5): 493－500
8 Fraser-Brunner A. Notes on the plectognath fishes. VIII. The classification of the suborder Tetraodontoidea, with a synopsis of the genera. Ann Mag Nat Hist, 1943, 10(61): 1－18
9 Abe T. Taxonomic studies on the puffers (Tetraodontidae, Teleostei) from Japan and adjacent regions, V: Synopsis of the puffers from and adjacent regions. Bull Biogeogr Soc Japan, 1949, 14(1, 13): 1－15, 89－140, 141－142
10 Abe T. Taxonomic studies on the puffers (Tetraodontidae, Teleostei) from Japan andadjacent regions, VII: Concluding remarks, with the introduction of two new genera, *Fugu* and Boesemanichthys. Jpn J Ichthyol, 1952, 2(1, 2, 3): 35—44; figs. 31—33, 93—97, 117—127
11 Whitley G P. Studies in ichthyology, vol 16. Records of the Australian Museum, 1953
12 Chen C, Shi T, Sun S G, et al. Identification a phylogenetic relationships among four species of puffer fish in *Fugu* as determined by RAPD markers. Marine Fish Res (in Chinese), 2001, 22(3): 32－36
13 Song L, Li H, Cui Z, et al. Population genetic structure and genetic differentiation of the pufferfish *Takifugu rubripes* and *Takifugu pseudommus* revealed by RAPD Analysis. High Technol Lett, 2003, 9(1): 22－25
14 Song L S, Liu B, Wang Z, et al. Phylogenetic relationships among pufferfish of genus *Takifugu* by RAPD analysis. Chin J Oceanol Limnol, 2001, 19(2): 128－134
15 Aparicio S, Chapman J, Stupka E, et al. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science, 2002, 297(5585): 1301－1310
16 Elmerot C, Arnason U, Gojobori T, et al. The mitochondrial genome of the pufferfish, *Fugu rubripes*, and ordinal teleostean relationships. Gene, 2002, 295(2): 163－172
17 Brenner S, Elgar G, Sandford R, et al. Characterization of the pufferfish (*Fugu*) genome as a compact model vertebrate genome. Nature, 1993, 366(6452): 265－268
18 Chen W J, Orti G, Meyer A. Novel evolutionary relationship among four fish model systems. Trends Genet, 2004, 20(9): 424－431
19 Holcroft N I. A molecular test of alternative hypotheses of tetraodontiform (Acanthomorpha: Tetraodontiformes) sister group relationships using data from the RAG1 gene. Mol Phylogenet Evol, 2004, 32(3): 749－760
20 Holcroft N I. A molecular analysis of the interrelationships of tetraodontiform fishes (Acanthomorpha: Tetraodontiformes). Mol Phylogenet Evol, 2005, 34(3): 525－544
21 Esposti M D, De Vries S, Crimi M, et al. Mitochondrial cytochrome *b*: Evolution and structure of the protein. Biochim Biophys Acta, 1993, 1143(3): 243－271
22 Miya M, Nishida M. Use of mitogenomic information in teleostean molecular phylogenetics: A tree-based exploration under the maximum-parsimony optimality criterion. Mol Phylogenet Evol, 2000, 17(3): 437－455
23 Zardoya R, Meyer A. Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. Mol Biol Evol, 1996, 13(7): 933－942
24 Peng Z, He S, Zhang Y. Phylogenetic relationships of glyptosternoid fishes (Siluriformes: Sisoridae) inferred from mitochondrial cytochrome *b* gene sequences. Mol Phylogenet Evol, 2004, 31(3): 979－987

25 Cui J, Shen X, Yang G, et al. Genetic diversities of *T. rubripes* and *T. pseudommus* determined by microsatellites DNA variations. High Technol Commun, 2005, 15(12): 90－96
26 Sambrook J, Fritsch E F, Maniatis T. Molecular Cloning: A Laboratory Manual-2nd. New York: Cold Spring Harbor Laboratory Press, 1989
27 Xiao W, Zhang Y, Liu H. Molecular systematics of Xenocyprinae (teleostei: cyprinidae): Taxonomy, biogeography, and coevolution of a special group restricted in East Asia. Mol Phylogenet Evol, 2001, 18(2): 163－173
28 Liu H Z. Phylogenetic relationships of the cypriniformes tested by mtDNA 12S rRNA sequence variations. Acta Genet Sin, 2004, 31(2): 137－142
29 Thompson J D, Gibson T J, Plewniak F, et al. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res, 1997, 25(24): 4876－4882
30 Galtier N, Gouy M, Gautier C. SEAVIEW and PHYLO_WIN: Two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci, 1996, 12(6): 543－548
31 Wiens J J. Combining data sets with different phylogenetic histories. Syst Biol, 1998, 47(4): 568－581
32 Farris J S, Kallersjo M, Kluge A G, et al. Testing significance of incongruence. Cladistics, 1995, 10(3): 315－319
33 Swofford D L. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), 4th ed. Sunderland, Massachusetts: Sinauer Associates, 2002
34 Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol, 1993, 10(3): 512－526
35 Xia X, Xie Z. DAMBE. Software package for data analysis in molecular biology and evolution. J Hered, 2001, 92(4): 371－373
36 Guo X, He S, Zhang Y. Phylogeny and biogeography of Chinese sisorid catfishes re-examined using mitochondrial cytochrome *b* and 16S rRNA gene sequences. Mol Phylogenet Evol, 2005, 35(2): 344－362
37 Irwin D M, Kocher T D, Wilson A C. Evolution of the cytochrome *b* gene of mammals. J Mol Evol, 1991, 32(2): 128－144
38 Farris J S. Methods for computing Wagner trees. Syst Zool, 1970, 19(1): 83－92
39 Felsenstein J. Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol, 1981, 17(6): 368－376
40 Mau B. Bayesian phylogenetic inference via Markov chain Monte Carlo methods. Madison: University of Wisconsin-Madison, 1996
41 Huelsenbeck J P, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 2001, 17(8): 754－755
42 Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 1985, 39(4): 783－791
43 Posada D, Crandall K A. MODELTEST: Testing the model of DNA substitution. Bioinformatics, 1998, 14(9): 817－818
44 Lanave C, Preparata G, Saccone C, et al. A new method for calculating evolutionary substitution rates. J Mol Evol, 1984, 20(1): 86－93
45 Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform, 2004, 5(2): 150－163
46 Templeton A R. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution, 1983, 37(2): 221－244
47 Shimodaira H, Hasegawa M. Multiple comparisons of loglikelihoods with applications to phylogenetic inference. Mol Biol Evol, 1999, 16(8): 1114－1116
48 Cantatore P, Roberti M, Pesole G, et al. Evolutionary analysis of cytochrome *b* sequences in some Perciformes: Evidence for a slower rate of evolution than in mammals. J Mol Evol, 1994, 39(6): 589－597
49 Lydeard C, Roe K J. The Phylogenetic Utility of the Mitochondrial Cytochrome *b* Gene for Inferring Relationships Among Actinopterygian Fishes. San Diego: Academic Press, 1997
50 Buckley T R, Simon C, Shimodaira H, et al. Evaluating hypotheses on the origin and evolution of the New Zealand alpine cicadas (Maoricicada) using multiple-comparison tests of tree topology. Mol Biol Evol, 2001, 18(2): 223－234
51 Masuda Y, Shinohara N, Takahashi Y, et al. Occurrence of natural hybrid between pufferfishes, *Takifugu xanthopterus* and *T. vermicularis*, in Ariake Bay, Kyushu, Japan. Nippon Suisan Gakkaishi, 1991, 57(7): 1247－1255
52 Johnson J B, Jordan S. Phylogenetic divergence in leatherside chub (*Gila copei*) inferred from mitochondrial cytochrome *b* sequences. Mol Ecol, 2000, 9(8): 1029－1035
53 Near T J, Porterfield J C, Page L M. Evolution of Cyt *b* and the molecular systematics of Ammocrypta (Percidae: Etheostomatinae). Copeia, 2000, 3: 701－711
54 Kadereit J W. Molecules and morphology, phylogenetics and genetics. Bot Acta, 1994, 107: 369－373