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Molecular Phylogenetic Relationships of Puffer Fish Inferred from Partial Sequences of Cytochrome *b* Gene and Restriction Fragment Length Polymorphism Analysis

YU-WEN HSIEH AND DENG-FWU HWANG*

Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, Republic of China

Phylogenetic relationships among puffer fish were investigated by comparing cytochrome *b* gene sequences and restriction endonuclease assays of 16 species from Taiwan. DNA was prepared for sequencing by PCR. No variation in sequences was detected among individuals within each species. Direct estimates of mitochondrial cytochrome *b* gene sequence divergence among 16 puffer fish were from 3.41 to 31.78%. Different restriction patterns were found among 16 puffer fish with 10 restriction endonucleases, whereas no variation in patterns was detected among individuals within each species. The polymorphisms obtained by RFLP have provided a new set of genetic markers for the accurate identification of sibling puffer species. It is the first molecularly based study of puffer diversity and sheds light on the evolution and taxonomy of this major puffer fish family.

KEYWORDS: Phylogeny; puffer fish; cytochrome *b* gene; PCR-RFLP; species identification; mitochondrial DNA

INTRODUCTION

Identification of species of puffer fish is relevant for economical, religious, or public health concerns (*1*, *2*). Species detection is also relevant for wildlife management, which so far has not received the same level of attention as food inspection or human forensic science. Modern methods for species identification are based on DNA analysis, especially the PCR-RFLP analysis of mitochondrial DNA (*3*−*7*). Recently, it has been shown that analysis of mitochondrial DNA successfully differentiated species of fish (*8*−*12*). Polymerase Chain Reaction (PCR) amplification and restriction enzyme analysis of the cytochrome *b* gene have also been used for identification of fish species (*13*, *14*). PCR-RFLP allows the amplification of a conserved region of DNA sequence using PCR and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes. This technique has been used for speciation by exploiting DNA sequence variation within the mitochondrial D-loop region (*15*) and cytochrome *b* gene (*16*). In the latter study, DNA fingerprints were generated for 16 puffer fish species using 10 restriction enzymes. We evaluated the potential for the Cyt *b* PCR-RFLP method, as developed by Meyer et al. (*16*), to be used as a routine analytical tool for the identification of puffer fish species.

There are more than 30 kinds of puffer fish in Taiwanese seawaters. Over 10 kinds of puffer fish are easily confused. Therefore, puffer fish poisoning has sporadically been reported about two or three times per year (*17*, *18*). We recently reported that puffer fish meat and their products could be identified by species by using PCR amplification and restriction enzyme analysis of the cytochrome *b* gene (*19*−*23*). However, the data on the gene base in the cytochrome *b* gene of puffer fish are still very sparse (*24*). To compare most puffer fish species and establish the data bank, we collected 16 kinds of puffer fish that are commonly seen in Taiwanese seawaters. The specimens of the most common puffer fish, including *Lagocephalus gloveri*, *Lagocephalus lunaris*, *Lagocephalus wheeleri*, *Lagocephalus sceleratus*, *Takifugu niphobles*, *Takifugu oblongus*, *Takifugu pardalis*, *Takifugu poecilonotus*, *Takifugu stictonotus*, *Takifugu vermicularis*, *Takifugu xanthopterus*, *Arothron stellatus*, *Diodon holocanthus*, *Ostracion cubicus*, *Sphoeroides pachygaster*, and *Takifugu rubripes*, were collected and used for analysis of mitochondrial DNA. Then, PCR amplification of mitochondrial DNA sequence and restriction enzyme analysis were used to identify the species of these 16 puffer fish. Therefore, the present study was undertaken to differentiate the fish species among 16 Taiwanese puffer fish.

MATERIALS AND METHODS

Materials. The raw materials of 12 specimens for each puffer species including *L. gloveri*, *L. lunaris*, *L. wheeleri*, *L. sceleratus*, *T. niphobles*, *T. oblongus*, *T. pardalis*, *T. poecilonotus*, *T. stictonotus*, *T. vermicularis*, *T. xanthopterus*, *A. stellatus*, *D. holocanthus*, *O. cubicus*, and *S. pachygaster* were collected from fish piers in Keelung, Ilan, Tainan, and Kaohsiung County in Taiwan in the spring of 2002 and immediately transferred to the laboratory with ice. Twelve specimens of *T. rubripes* were collected from agricultural ponds in Taipei County and also immediately transferred to the laboratory with ice.

* Address correspondence to this author at the Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung, 202, Taiwan (telephone +886-2-2462-2192-5103; fax +886-2-2462-6602; e-mail dfhwang@mail.ntou.edu.tw).

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                    1                                                         60
T. poecilonotus     TTACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTACTAAAAA
T. vermicularis     TCACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTGCTAAAAA
T. rubripes         TTACTCAACTACAAAAACATT-AATGGCCAGCTTACGCAAAACCCACCCCCTACTAAAAA
T. xanthopterus     TTACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTACTAAAAA
T. niphobles        TTACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTATTAAAAA
T. stictonotus      TTACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTACTAAAAA
T. pardalis         TTACTCAACTACAAAAACACT-AATGGCCAGCCTACGCAAAACCCACCCCCTACTAAAAA
T. oblongus         TCACTCAACTACAAAAACTCT-AATGGCCAGCCTACGCAAAACCCATCCCCTACTAAAAA
D. holocanthus      TTAATTAACTATAAGAACCCT-AATGGCAAGCCTTCGCAAAACCCACCCGCTACTAAAAA
A. stellatus        TTATTCAACTACAAGAAC-AT-AATGGCAAGCCTACGCAAAACCCACCCACTAATAAAAA
L. sceleratus       TACTTCTACTACAAGAAC-CT-AATGGCCAGCCTACGCAAAACGCACCCACTTATAAAAA
S. pachygaster      TTATTCAACTACAAGAAC-AT-AATGGCAAGCCTACGCAAAACTCATCCCCTAATAAAAA
L. gloveri          TGTCTCAACTACAAGAAC-CT-AATGGCCAGCCTACGCAAGTCCCATCCCCTTATGAAAA
L. wheeleri         TGCCTCAACTACAAGAAC-CT-AATGGCCAGCCTACGCAAATCGCATCCCCTCATGAAAA
L. lunaris          TGCTTCCACTACAAGAACTTT-AATGGCCAGCCTACGCAAGACCCATCCGCTCCTAAAAA
O. cubicus          TTATTCAACTATAAGAACCCTAAATGGCAAGCCTCCGTAAAACCCACCCCCTACTAAAAA
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                    61                                                       120
T. poecilonotus     TCGTAAACGACATAGTAATTGACCTCCCTACCCCCTCAAACATTTCCGCCTGATGAAACT
T. vermicularis     TCGTAAATGACATAGTAATTGACCTTCCTACCCCCTCAAACATTTCCGCCTGATGAAACT
T. rubripes         TCGTAAACGACATAGTAATTGACCTTCCTACCCCCTCAAACATTTCCGCCTGATGAAACT
T. xanthopterus     TCGTAAACGACATAGTAATTGACCTTCCCACCCCCTCAAATATTTCCGCCTGATGAAACT
T. niphobles        TCGTAAATGACATAGTAATTGACCTTCCTACCCCCTCAAACATCTCCGCCTGATGAAACT
T. stictonotus      TCGTAAATGACATAGTAATCGACCTTCCTACCCCCTCAAACATTTCCGCCTGATGAAACT
T. pardalis         TCGTAAACGACATAGTAATTGACCTTCCCACCCCCTCAAACATTTCCGCCTGATGAAACT
T. oblongus         TCGTAAACGACATAGTAATTGACCTCCCTACCCCCTCAAACATTTCCGCCTGATGAAACT
D. holocanthus      TCGCAAATGACATGGTTGTGGACCTTCCAACCCCGTCCAACATTTCAGCCTGATGAAACT
A. stellatus        TCGCAAACGACATAGTTATCGACCTCCCCACTCCATCAAACATCTCTGCCTGATGAAACT
L. sceleratus       TTGCCAACGACATGGTAATTGACCTACCAGCCCCCTCCAACATCTCTGCCTGATGAAACT
S. pachygaster      TCACTAACGACATAGTCGTCGACCTTCCAACCCCTGTAAACATTTCCGCCTGATGGAATT
L. gloveri          TTACTAATGACATAGTAATTGACCTCCCCACCCCTTCGAATATTTCCGCCTGGTGAAACT
L. wheeleri         TTGTAAACGACATAGTCATTGATTTACCAACCCCCTCAAACATCTCTGCATGGTGAAACT
L. lunaris          TCGTAAACGACATAGTTATTGACCTCCCAACCCCATCAAACATCTCAGCATGGTGAAATT
O. cubicus          TTGCTAATGACGCAGTAGTAGACCTCCCCACCCCCTCAAATATTTCTGTATGATGAAACT
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                    121                                                      180
T. poecilonotus     TCGGCTCCCTACTCGGATTATGCCTTATTGCACAAATCATCACAGGACTGTTCCTTGCAA
T. vermicularis     TTGGCTCCCTACTCGGATTATGCCTTATTGCACAAATCATCACAGGACTGTTCCTTGCAA
T. rubripes         TTGGCTCTCTACTCGGATTATGCCTTATTACACAAATCATCACAGGACTGTTCCTTGCAA
T. xanthopterus     TTGGCTCCCTGCTCGGACTATGCCTTATTACACAAATCATCACAGGACTATTCCTTGCAA
T. niphobles        TTGGCTCCCTACTCGGATTATGCCTTATTACACAAATCATCACAGGACTATTCCTTGCAA
T. stictonotus      TTGGCTCCCTACTCGGATTATGCCTTATTACACAAATCATCACAGGGCTGTTCCTTGCAA
T. pardalis         TTGGTTCCCTACTGGGATTATGCCTTATCACACAAATCATCACAGGGCTCTTCCTTGCAA
T. oblongus         TTGGCTCCCTACTCGGATTATGTCTTATTACACAAATCGTCACAGGACTGTTCCTTGCAA
D. holocanthus      TCGGCTCCTTACTCGGTTTATGCCTAATTGCTCAAATCCTCACGGGCCTCTTCCTCGCCA
A. stellatus        TTGGCTCCCTGCTCGGACTTTGCCTAGTCGCCCAAATCCTAACAGGCCTATTTCTAGCAA
L. sceleratus       TTGGCTCCCTACTATCTGTCTGTCTAATCGCCCAGATCCTCACAGGCCTCTTTTTAGCGA
S. pachygaster      TTGGCTCCCTCCTAGGACTCTGCTTAATCGCCCAAATCTTAACAGGACTTTTCCTGGCAA
L. gloveri          TCGGCTCACTATTAGGACTATGTCTTATCGCACAAATCCTAACAGGATTATTCCTAGCCA
L. wheeleri         TTGGCTCACTACTAGGACTATGCCTTATCGCACAAATCCTAACAGGACTCTTCCTGGCAA
L. lunaris          TCGGCTCGCTGCTCGGACTATGTCTTATTGCCCAAATCGCAACAGGGCTCTTCCTAGCAA
O. cubicus          TTGGCTCCCTACTAGGTCTTTGCTTAGTAGCCCAAATTCTCACAGGCCTATTCTTAGCTA
                    * ** **  *  *     * **  *  *  * ** **    ** **  * **  * ** *
                    181                                                      240
T. poecilonotus     TACATTACACATCCGACATCTCTACTGCCTTTTCATCCGTAGCCCATATTTGCCGAGACG
T. vermicularis     TGCACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCATATTTGCCGAGACG
T. rubripes         TACACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCACATTTGCCGAGACG
T. xanthopterus     TACACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCATATTTGCCGAGACG
T. niphobles        TACACTACACATCCGACATTTCTACCGCCTTTTCATCCGTAGCCCATATTTGCCGAGACG
T. stictonotus      TACACTACACATCCGACATCTCTACCGCCTTTTCATCCGTGGCCCATATTTGCCGAGACG
T. pardalis         TACACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCATATTTGCCGAGACG
T. oblongus         TACACTACACATCCGATATCTCTACCGCCTTTTCATCCGTAGCACATATTTGCCGAGACG
D. holocanthus      TACATTACACATCTGACATTTCCACAGCCTTCTCGTCCGTTGCCCACATCTGCCGAGACG
A. stellatus        TACACTACACCTCTGACATCGCCACCGCCTTTTCATCCGTCGCCCACATCTGCCGCGACG
L. sceleratus       TACACTACACCTCCGACATCGCTACCGCCTTTTCATCCGTCGCCCACATCTGTCGGGATG
S. pachygaster      TACACTACACCTCTGATATCACTACAGCCTTCTCATCTGTCGCACACATCTGTCGAGACG
L. gloveri          TACACTACACCTCTGACATCGCTACAGCCTTTTCCTCAGTCACCCACATTTGCCGAGACG
L. wheeleri         TACACTACACCTCTGATATTGCCACGGCCTTCTCCTCAGTCGCCCACATCTGCCGAGATG
L. lunaris          TACACTACACCTCTGACGTTGCCACAGCTTTTTCATCAGTTGCCCACATCTGCCGAGATG
O. cubicus          TACATTATACACCAGATATCACCACAGCCTTCTCATCAGTCGCCCACATCTGCCGCGACG
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                    241                                                                  300
T. poecilonotus     TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCGTTCTTTTTTATCT
T. vermicularis     TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCGTTCTTTTTCATCT
T. rubripes         TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCATTCTTTTTTATTT
T. xanthopterus     TAAACTACGGCTGACTAATTCGTAATCTACACGCAAACGGTGCCTCATTCTTTTTTATCT
T. niphobles        TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCGTTCTTTTTTATCT
T. stictonotus      TAAACTACGGCTGACTAATTCGTAATCTACACGCAAATGGTGCCTCATTCTTTTTTATCT
T. pardalis         TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCGTTCTTTTTTATCT
T. oblongus         TAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGCCTCATTCTTTTTTATTT
D. holocanthus      TAAACTACGGATGACTGATCCGCAACCTTCACGCCAACGGCGCCTCCTTCTTCTTTATCT
A. stellatus        TTAACTATGGCTGACTAATTCGCAACCTCCACGCAAATGGGGCCTCCTTCTTCTTTATCT
L. sceleratus       TCAACTATGGCTGACTGATTCGGAACCTCCATGCAAACGGAGCCTCCTTCTTCTTTATCT
S. pachygaster      TTAACTATGGCTGACTTATCCGGAACCTCCATGCAAACGGAGCCTCCTTCTTCTTCATCT
L. gloveri          TAAGCCACGGATGACTAATTCGAAACCTCCACGCAAACGGAGCCTCTTTCTTCTTCATCT
L. wheeleri         TCAACTACGGCTGACTAATTCGAAACCTGCATGCAAATGGAGCCTCCTTCTTCTTCATTT
L. lunaris          TTAATTACGGCTGACTCCTCCGCAACCTCCACGCAAACGGGGCCTCTTTCTTCTTCATTT
O. cubicus          TTAACTACGGCTGACTAATCCGAAACCTTCACGCAAACGGTGCATCATTCTTCTTCATCT
                    * *   * ** *****  * ** ** ** ** ** ** ** ** ** ***** ** ** *
                    301                                                                  360
T. poecilonotus     GCTTATACTCCCACATCGGCCGAGGCCTCTACTATGGCTCTTACCTAAGTAAAGAAACCT
T. vermicularis     GCTTATACTCCCACATCGGCCGAGGACTCTACTATGGCTCTTACCTGAGTAAAGAAACCT
T. rubripes         GCTTATACTCCCACATCGGCCGAGGTCTTTACTATGGCTCTTACCTAAGTAAAGAAACCT
T. xanthopterus     GCTTATACTCCCACATCGGCCGAGGTCTCTACTATGGCTCTTACCTAAGTAAAGAAACCT
T. niphobles        GCTTATACTCCCATATCGGCCGAGGTCTCTACTATGGCTCTTACCTAAGTAAAGAAACCT
T. stictonotus      GCTTATACTCCCACATCGGCCGAGGCCTCTACTATGGCTCCTACCTGAGCAAAGAAACCT
T. pardalis         GCTTATACTCCCATATCGGCCGAGGTCTCTACTACGGCTCTTACCTAAGTAAAGAGACTT
T. oblongus         GCCTATATTCCCATATCGGTCGAGGCCTCTACTATGGCTCTTACCTAAGTAAAGAAACCT
D. holocanthus      GCATTTACCTGCATATCGGACGGGGTCTATACTACGGCTCATACCTGAATAAAGAAACTT
A. stellatus        GCATCTACTTCCACATCGGACGAGGACTCTACTACGGCTCCTACCTCAACAAAGAAACCT
L. sceleratus       GCATCTACTTTCACATTGGACGAGGACTATACTACGGCTCTTACCTTAACAAAGAGACCT
S. pachygaster      GCATCTATCTCCATATCGGACGAGGCCTCTACTACGGCTCCTATCTCAATAAAGAAACCT
L. gloveri          GTATCTACCTCCACATTGGGCGCGGCCTGTACTACGGCTCCTTTCTCAACAAGGAAACCT
L. wheeleri         GTATTTACCTTCATATTGGACGTGGCCTATACTATGGCTCATTCCTTAACAAAGAAACAT
L. lunaris          GCATCTACTTCCACATCGGACGTGGCCTCTACTACGGTTCCTACTTAAACAAAGAAACCT
O. cubicus          GCCTTTACCTCCATATTGGACGAGGGCTTTATTATGGCTCCCATCTCTCAAAAGAAACTT
                    *  * **    ** ** ** ** ** ** ** ** ** **     *    ** ** ** *
                    361                                                                  425
T. poecilonotus     GAAACGTAGGTGTAGTCCTCTTACTTTTAGTAATGGCTACCGCTTTCGTAGGATACGTCCTTCCA
T. vermicularis     GAAACGTAGGTGTAGTCCTCTTACTTTTAGTAATGGCCACCGCTTTCGTAGGGTACGTCCTCCCA
T. rubripes         GAAACGTAGGGGTAGTCCTCTTACTTTTAGTAATGGCCACCGCTTTCGTAGGCTACGTTCTCCCA
T. xanthopterus     GAAACGTAGGTGTAGTCCTCTTACTTTTAGTAATGGCTACCGCTTTCGTAGGATACGTTCTTCCA
T. niphobles        GAAACGTGGGCGTAGTCCTCTTACTTCTAGTAATGGCCACCGCTTTTGTAGGATACGTCCTTCCA
T. stictonotus      GAAACGTAGGTGTAGTCCTCTTACTTTTAGTAATGGCCACCGCTTTTGTAGGATACGTTCTTCCA
T. pardalis         GAAACGTAGGTGTAGTCCTCTTACTTTTAGTGATAGCCACCGCTTTCGTAGGGTACGTTCTTCCA
T. oblongus         GAAACGTAGGAGTAGTCCTCTTACTCCTAGTAATGGCCACCGCTTTCGTAGGGTACGTCCTTCCA
D. holocanthus      GAAACGTTGGGGTCGTCCTACTACTTCTAGTAATAGCAACAGCCTTCGTAGGTTACGTCCTCCCC
A. stellatus        GAAATATTGGAGTGGTCCTGCTACTACTCGTCATAGCCACCGCCTTCGTGGGCTACGTTCTCCCC
L. sceleratus       GAAACATCGGAGTAGTCCTTCTTCTCCTCGTAATGGCAACCGCCTTTGTGGGCTACGTCCTCCCC
S. pachygaster      GAAACATTGGAGTCATCCTCCTACTTCTTGTAATAGCCACTGCCTTTGTAGGATACGTACTACCT
L. gloveri          GAAACGTCGGAGTAGTCCTACTGCTTTTAGTTATAGCCACAGCCTTCGTGGGCTACGTCCTTCCC
L. wheeleri         GAAACGTAGGAGTAATCCTCCTGCTCTTAGTAATAGCCACAGCCTTCGTGGGCTATGTCCTTCCC
L. lunaris          GAAACGTCGGGGTAGTCCTTCTGCTTCTAGTCATAGCCACTGCCTTCGTCGGCTACGTCCTCCCC
O. cubicus          GAAATATTGGGGTCGTACTTTTTTTACTAGTTATAATTACTGCCTTTGTTGGTTACGTCCTACCC
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Figure 1. Sequence comparison of a partial cytochrome *b* gene of 16 puffer fish species.

DNA Extraction and PCR Amplification of a Fragment of the Cytochrome *b* Gene. Total cellular DNA was extracted from the muscle of each puffer fish species essentially according to the method of DeSalle and Birstein (*11*). The PCR amplification reactions were performed in a total volume of 100 μL. Each reaction mixture contained 1000 ng of extracted template DNA, 0.4 μM of each primer, 200 μM of each dNTP, and 2.5 units of Pro Taq DNA polymerase (Amresco, Solon, OH) in a buffer containing 20 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 1% Triton X-100, 500 mM KCl, and 0.1% (w/v) gelatin.

PCR was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA) with a denaturation step of 95 °C for 10 min, followed by 30 cycles consisting of 1 min at 95 °C, 1 min at 50 °C, and 2 min at 72 °C. The last extension step was 10 min longer. The set of primers used for PCR amplification was designated as follows:

L14724

5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′

and

H15149

5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′

The pair of primers corresponded with those described by Kocher et al. (*25*). The products of PCR amplification were analyzed by agarose gel electrophoresis.

Table 1. Sequence Divergence of a Part of the Cytochrome *b* Gene of 16 Puffer Species (1, *T. poecilonotus*; 2, *T. vermicularis*; 3, *T. rubripes*; 4, *T. xanthopterus*; 5, *T. niphobles*; 6, *T. stictonotus*; 7, *T. pardalis*; 8, *T. oblongus*; 9, *D. holocanthus*; 10, *A. stellatus*; 11, *L. sceleratus*; 12, *S. pachygaster*; 13, *L. gloveri*; 14, *L. wheeleri*; 15, *L. lunaris*; 16, *O. cubicus*)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0.00	3.41	4.42	3.42	4.18	4.44	5.19	5.44	20.27	20.41	24.19	24.00	22.32	23.29	22.60	28.38
2		0.00	4.41	4.68	4.43	4.43	5.44	5.95	20.90	20.43	23.53	24.32	22.63	23.26	23.25	29.55
3			0.00	3.91	4.93	4.93	5.43	5.97	21.29	19.80	24.24	24.70	24.09	23.34	22.31	29.97
4				0.00	4.18	3.92	4.42	6.50	22.24	19.12	24.53	23.65	22.66	23.97	23.96	28.01
5					0.00	4.69	5.19	5.97	20.27	20.08	23.51	23.31	24.03	23.29	23.62	28.75
6						0.00	5.72	7.03	21.90	19.12	24.53	23.31	23.34	23.63	24.31	28.75
7							0.00	7.27	20.92	19.72	24.24	24.06	23.68	23.68	24.31	29.50
8								0.00	23.27	21.39	24.19	22.68	23.68	21.94	22.28	29.55
9									0.00	20.43	23.97	22.61	25.69	24.53	22.60	23.49
10										0.00	15.81	16.85	21.48	21.65	17.04	22.77
11											0.00	22.87	23.81	20.48	23.43	28.96
12												0.00	23.12	22.43	23.50	24.38
13													0.00	16.39	20.26	28.92
14														0.00	19.33	29.75
15															0.00	31.78
16																0.00

Table 2. Expected Lengths of Restriction Fragments (BasePair, bp) Generated by Digestion of Mitochondrial Cytochrome *b* Gene PCR Products with 10 Restriction Enzymes (PCR-RFLP)

	*Bsa*JI	*Hin*fI	*Bsa*I	*Sap*I	*Taq*I	*Eco*RI	*Ssp*I	*Ear*I	*Stu*I	*Bsr*DI
L. gloveri	214 + 271									
L. wheeleri	228 + 257									
S. pachygaster	157 + 328									
T. stictonotus	103 + 139 + 244									
L. lunaris	×	206 + 280		201 + 285						
T. oblongus	×	×			139 + 347					
L. sceleratus	×	203 + 283		×						
O. cubicus	×	×			×	225 + 261				
D. holocanthus	×	×			×	×				
A. stellatus	×	135 + 351								
T. xanthopterus	139 + 347		129 + 357				128 + 358			
T. rubripes	139 + 347		×						×	×
T. niphobles	139 + 347		129 + 357				×	×		
T. pardalis	139 + 347		129 + 357				×	201 + 285		
T. poecilonotus	139 + 347		×						134 + 352	×
T. vermicularis	139 + 347		×						×	211 + 275

Cleanup and Sequencing of the PCR Products. PCR product was purified by using the method of DeSalle and Birstein (*11*). Purified PCR products from each puffer fish species were sequenced at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) in an ABI PRISM 377-96 DNA Sequencer (Perkin Elemer/Applied Biosystems Division). Two replicate sequences were obtained from each sample. Sequence analysis was performed using the Genetics Computer Group Wisconsin Package, version 10.3 (*26*).

Restriction Site Analysis of PCR Products. For the restriction site analysis of the cytochrome *b* region, the PCR products were extracted and purified the same way as in DNA extraction. Satisfactory results were also obtained without previous purification of the amplified DNA fragments. The endonucleases *Bsa*JI, *Hin*fI, *Bsa*I, *Sap*I, *Taq*I, *Eco*RV, *Ssp*I, *Ear*I, *Stu*I, and *Bsr*DI (Promega, Madison, WI) were searched from the GCG system by inputting our sequences and tested for restriction analysis of the amplified PCR products. Digests were performed in 10 μL volumes with 100−200 ng of amplified DNA, 5 units of enzyme, and 1:10 dilution of the manufacturer's recommended 10× digestion buffer and bovine serum albumin (BSA). Digestions were incubated for 2 h at 65 °C (*Taq*I and *Bsr*DI), 60 °C (*Bsa*JI), 50 °C (*Bsa*I), 37 °C (*Hin*fI, *Sap*I, *Eco*RV, *Ssp*I, *Ear*I, and *Stu*I). The resulting fragments were separated by electrophoresis in a 1.5% agarose gel containing 1 μg/mL ethidium bromide for 1 h at 100 V. The sizes of the resulting DNA fragments were estimated by comparison with a commercial 100-bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS

DNA extracts from the muscle of *L. gloveri*, *L. lunaris*, *L. wheeleri*, *L. sceleratus*, *T. niphobles*, *T. oblongus*, *T. pardalis*, *T. poecilonotus*, *T. stictonotus*, *T. vermicularis*, *T. xanthopterus*, *A. stellatus*, *D. holocanthus*, *O. cubicus*, and *S. pachygaster* were tested for amplification using the L14724/H15149 primers, which should produce a 486-bp fragment. These sequences were submitted to GenBank for accession numbers AY128527 (*L. lunaris*), AY128528 (*T. niphobles*), AY128529 (*T. oblongus*), AY128530 (*L. gloveri*), AY128531 (*L. wheeleri*), AY267356 (*L. sceleratus*), AY267357 (*O. cubicus*), AY267358 (*S. pachygaster*), AY267359 (*T. pardalis*), AY267360 (*T. poecilonotus*), AY267361 (*T. rubripes*), AY267362 (*T. stictonotus*), AY267363 (*T. vermicularis*), AY267364 (*T. xanthopterus*), AY267365 (*A. stellatus*), and AY267366 (*D. holocanthus*) (http://www.ncbi.nlm.nih.gov). Electrophoretic analysis of the PCR products from these samples exhibited the same 486-bp fragment. **Figure 1** shows the comparison of DNA sequences in 16 puffer fish species (accession number: ALIGN_000549, http://www.ebi.ac.uk/embl/Submission/align_top.html). The sequence divergence of a part of the cytochrome *b* gene of 16 puffer species is shown in **Table 1**. Percent divergences of genus *Takifugu* were <8% and of genus *Lagocephalus* were <24%, but percent divergences of different genera are >15%. The genetic distances between the different puffer fish species ranged from 3.41 to

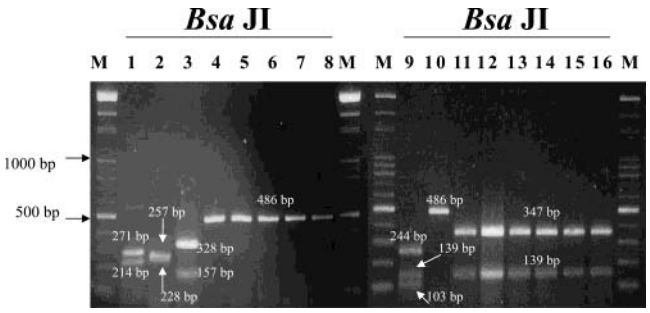

Figure 2. Electrophoretic analysis of PCR products of the 486 bp cytochrome *b* gene digested with *Bsa*JI on 2.0% agarose gel. M, molecular weight marker, Bio 100-bp DNA ladder. Lanes: 1, *L. gloveri*; 2, *L. wheeleri*; 3, *S. pachygaster*; 4, *L. lunaris*; 5, *L. sceleratus*; 6, *A. stellatus*; 7, *T. oblongus*; 8, *D. holocanthus*; 9, *T. stictonotus*; 10, *O. cubicus*; 11, *T. xanthopterus*; 12, *T. niphobles*; 13, *T. pardalis*; 14, *T. rubripes*; 15, *T. poecilonotus*; 16, *T. vermicularis*.

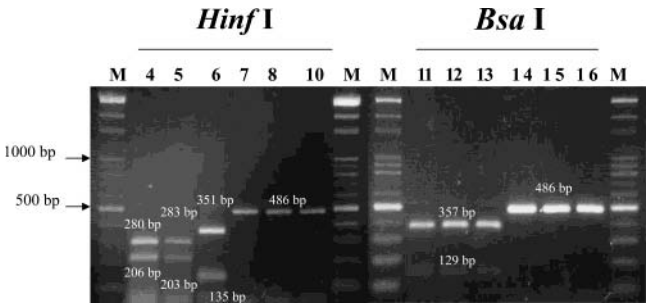

Figure 3. Electrophoretic analysis of PCR products of the cytochrome *b* gene digested with *Hin*fI and *Bsa*I on 2.0% agarose gel. Lane legends are as in **Figure 2**.

31.78%. These numbers were generated by the Kimura two-parameter distance program of the GCG computer package.

The restriction enzymes *Bsa*JI, *Hin*fI, *Bsa*I, *Sap*I, *Taq*I, *Eco*RV, *Ssp*I, *Ear*I, *Stu*I, and *Bsr*DI were found to be potentially useful because of their convenience and speed in identifying the 16 puffer fish species, and different restriction patterns were obtained from the 16 puffer fish species with these 10 restriction endonucleases. The expected lengths of restriction fragments generated by digestion of mitochondrial cytochrome *b* gene PCR products with the 10 restriction enzymes are shown in **Table 2**. The restriction enzyme *Bsa*JI could first differentiate the species of *L. gloveri* (271 bp + 214 bp), *L. wheeleri* (257 bp + 228 bp), *S. pachygaster* (328 bp + 157 bp), and *T. stictonotus* (244 bp + 139 bp + 103 bp) from the other 12 puffer species and could distinguish the others into two groups (486 bp and 347 bp + 139 bp). Next, using *Hin*fI could differentiate the species of *A. stellatus* (351 bp + 135 bp), *L. lunaris* (206 bp + 280 bp), and *L. sceleratus* (203 bp + 283 bp) from the other three puffer species (486 bp), but the restriction bands of *L. lunaris* and *L. sceleratus* are too similar, and the other three puffer fish could not be distinguished, so we used *Sap*I to differentiate the previous two kinds of puffer fish and used both *Taq*I and *Eco*RV for the differentiation of *T. oblongus* (139 bp + 347 bp) and *O. cubicus* (225 bp + 261 bp). Two kinds of restriction fragments (486 bp and 129 bp + 357 bp) could be differentiated by *Bsa*I among the leftover six kinds of puffer fish. Then, the differentiation of *T. xanthopterus* (128 bp + 358 bp) and *T. pardalis* (201 bp + 285 bp) could be finished by *Ssp*I and *Ear*I. The last, using *Stu*I and *Bsr*DI, could differentiate the species of *T. poecilonotus* (134 bp + 352 bp) and *T. vermicularis* (211 bp + 275 bp). All cleavage patterns predicted from the sequence were checked experimentally. Results following digestion of the puffer fish products showed that band sizes obtained by electrophoresis in 1.5% agarose gel were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis (**Figures 2**−**5**). The rapid analysis of the partial sequence of the cytochrome *b* gene and PCR-RFLP could be applied to identify unknown puffer fish species during 24 h. A phylogenetic tree of the 16 puffer fish species is shown in **Figure 6**. It was constructed by the neighbor-joining method and based on the substitutions per 100 bases. Therefore, the polymorphisms obtained by restriction fragment length polymorphism and sequence analysis have provided a new set of genetic markers for accurate identification the sibling species and morphospecies of 16 puffer fish.

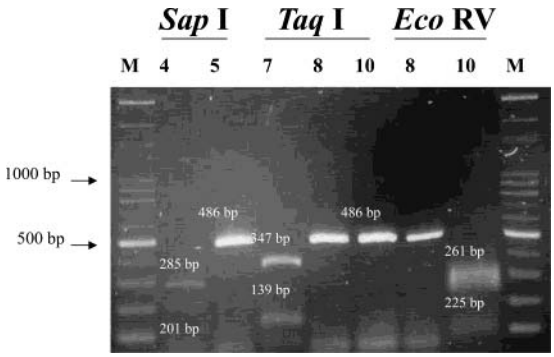

Figure 4. Electrophoretic analysis of PCR products of the cytochrome *b* gene digested with *Sap*I, *Taq*I, and *Eco*RI on 2.0% agarose gel. Lane legends are as in **Figure 2**.

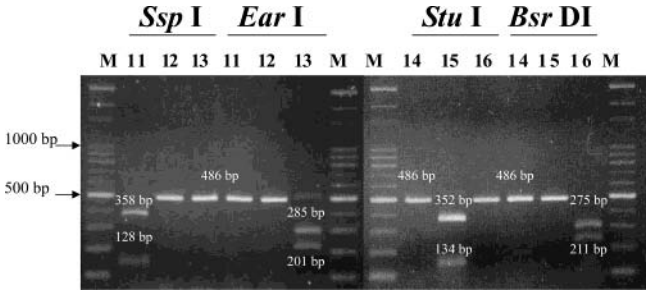

Figure 5. Electrophoretic analysis of PCR products of the cytochrome *b* gene digested with *Ssp*I, *Ear*I, *Stu*I, and *Bsr*DI on 2.0% agarose gel. Lane legends are as in **Figure 2**.

DISCUSSION

The cytochrome *b* locus has been well characterized among different vertebrate groups (*27*, *28*). Unseld et al. (*29*) described the cytochrome *b* gene as a useful molecular marker for investigating phylogenetic relationships within vertebrates. First, the cytochrome *b* gene is the gene that is perhaps most extensively sequenced to date for the vertebrates. Second, the evolutionary dynamics of the cytochrome *b* gene and the biochemistry of the protein product are better characterized than most other molecular systems. Finally, levels of genetic divergence typically associated with sister species and congeners usually are in a range in which the cytochrome *b* gene is phylogenetically informative and unlikely to be severely compromised by saturation effects involving superimposed nucleotide substitutions. These studies have revealed that the level of cytochrome *b* gene sequence variation is suitable for addressing general questions on species-specific diversity. However, because DNA sequence analysis is costly, the PCR-RFLP approach as applied by Meyer et al. (*16*) provides a more practical approach for detecting genetic variation between

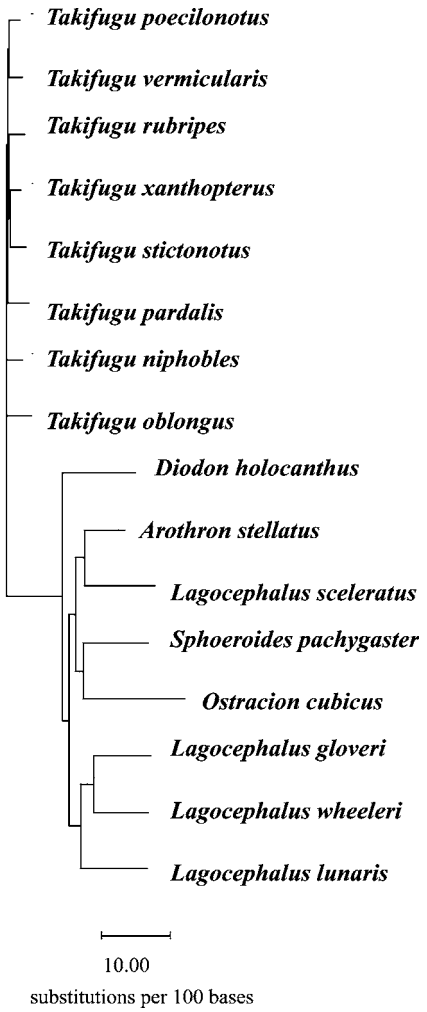

Figure 6. Phylogeny tree of 16 puffer fish species based on the partial sequence of cytochrome *b* gene was constructed by the neighbor-joining method.

species. This method was therefore evaluated in the current study to assess its suitability as a routine analytical method for determining the species of puffer fish.

In this study, we found that application of direct sequence analysis and restriction enzyme could be available for identifying 16 puffer fish from Taiwanese seawaters. A 486 bp region within the cytochrome *b* gene was successfully amplified from different species of 16 puffer fish and could be available for differentiating each specific species. According to our data, the sequence divergences of 16 puffer fish were from 3.41 to 31.78%. Assuming the rate of 2% substitution per base pair per million years of differentiation time (*30*), these substitutions correspond to 1.71−15.89 million years. The exact estimation of differentiation time should be difficult, because several factors including the substitute position at the codon, transition or transversion type change, and kinds of animals, etc., are known to change the speed of the molecular clock. Although intraspecific genetic variability is present in wild populations (*31*), individual variation of the 486-bp gene from each puffer fish species collected from Taiwan was not found in this study. Thus, analysis of the partial sequence of cytochrome *b* gene adopted in this study was more efficient and less laborious than complete sequencing of gene, with little loss of information. The cytochrome *b* PCR-RFLP species identification assay was determined to be a suitable method for the identification of 16 puffer fish collected from Taiwanese seawaters. The identification of species following restriction enzyme digestion was shown to be simple and straightforward by judicious choice of 10 restriction enzymes. Furthermore, the detected time could be limited within the limits of 24 h and four steps. Therefore, the use of the resulting DNA sequence and PCR-RFLP analyses could be valuable to wildlife officers requiring identification of the animal samples, particularly when trade in endangered unknown puffer fish uses slice samples of meat. This result was similar to those reported by D'Amelio et al. (*32*) and Partis et al. (*6*). Hence, the utilization of restriction endonuclease in the PCR product of cytochrome *b* gene in the puffer fish is useful for differentiating the 16 Taiwanese puffer fish. Furthermore, to improve the confidence in gene and restriction site analyses, further studies of more puffer fish specimens collected from different waters are needed.

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