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Morphological and Molecular Classifications of Genus *Pholis*

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Abstract: Morphological and molecular classifications were attempted in an effort to establish species-specific classifications of three species of the genus *Pholis* in Korea; these species were subjected to morphological and molecular methodologies using body measurements, RFLP, RAPD, and phylogenetic trees using the nucleotide sequences of mitochondrial 16S and 12S ribosomal DNAs, cytochrome c oxidase I, and cytochrome b. The data demonstrated that the three species of genus *Pholis* are distinct from each other, both morphologically and genetically.

Key words: morphology, classification, mtDNA, gunnel, RFLP, RAPD, 16S and 12S ribosomal DNAs, cytochrome c oxidase I, cytochrome b, phylogenetic tree

INTRODUCTION

The tidepool gunnel (*Pholis nebulosa*), white gunnel (*Pholis fangi*), and mottled gunnel (*Pholis crassispina*) are members of the genus *Pholis*, family Pholidae, order Perciformes. The tidepool gunnel is distributed broadly along all Korean coasts, whereas the white and mottled gunnels are distributed throughout the western and southwestern seas, respectively (Kim et al., 2005). Juvenile white gunnels caught in the spring are considered to be an important food resource because of marked consumer preference for the flaked product, and the tidepool gunnel is also consumed raw in southeastern Korea (Hur et al., 1984).

Mitochondrial DNA (mtDNA), which was used to determine the genetic characteristics of the three species, codes for 13 polypeptides, 22 tRNAs, and 2 rRNAs. Based on the results of nucleotide substitution, the evolutionary speed of the mtDNA is 5 to 10 times as fast as that of nucleic DNA, and a series of intra-species variations were detected (Brown et al., 1979; Cann and Wilson, 1983). Moreover, gene recombination was not observed owing to maternal inheritance, such that a series of gradual changes can be observed on both inter- and intra-species bases (Zhu et al., 1994). It has been determined that obtaining data on inter- and intra-specific relationships is more efficient than obtaining protein data (Upholt and Dawid, 1977; Brown 1980).

Several studies have thus far been conducted regarding the family Pholidae; age, growth, and spawning behavior (Kang et al., 1996), feeding habits of tidepool and white gunnels (Hur and Kwak, 1997; Kim et al., 1985), morphological comparisons of tidepool and white gunnels (Hur et al., 1983), and the diet of the larval white gunnel (Kim et al., 1985) have been the subjects of these studies.

The three industrial species belonging to the genus *Pholis* in Korea can be morphologically distinguished in adult stage, but it is difficult to differentiate between them at the juvenile stage, owing to their similar external morphology. Accordingly, the principal objective of this study was to provide fundamental data for ecological research into the genus *Pholis* via analyses of morphological characteristics, body measurements, and genetic traits.

MATERIALS AND METHODS

Experimental fishes

20 tidepool gunnels (*Pholis nebulosa*) were purchased from the Seoho market, Tongyeong-si, Gyeongsangnamdo in Oct., 2007, 13 white gunnels (*Pholis fangi*) were purchased from Sinjindo Harbor, Taean-gun, Chungcheongnamdo in

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Jun., 2008, and 11 mottled gunnels (*Pholis crassispina*) were caught near Saryangdo, Gyeongsangnamdo in Mar., 2008. The sampled fishes were maintained at –20°C until use.

Morphological characteristics and body measurements

In order to determine the morphological characteristics, the tidepool, white, and mottled gunnels were applied to exterior body measurements, including total length (TL), body length (BL), body depth (BD), head length (HL), dorsal fin length (DL), anal fin length (AL), anal fin origin length (AOL), eye diameter (ED), and snout length (SnL), using a pair of digital calipers (Mitutoyo Co., Japan). The percentages of BL, BD, DL, AL, and AOL against TL and the percentages of ED and SnL against HL were calculated.

The number of spinous and soft rays in the ventral fin of the white gunnel were counted by staining the ventral fin with Alizarin red (SIGMA, USA), and destaining the stained parts, except for the spinous and soft rays, with mall solution (0.05% NaOH, 20% glycerine). Finally, the spinous and soft rays of the ventral fin were photographed with an OLYMPUS SI×9 camera (OLYMPUS, Japan).

Isolation of genomic DNA

Small pieces (0.5 cm×0.5 cm) of the tail fins from tidepool, white, and mottled gunnels were cut and solubilized with lysis buffer (10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 5 M urea, 0.1 mg/mL proteinase K), and were purified with an *Accuprep* Genomic DNA Extraction Kit (Bioneer, Korea). The concentrations of isolated DNA were calculated with a spectrophotometer (NanoDrop Technologies, USA), and were maintained at - 20°C until use.

Amplification and analysis of mitochondrial DNA

PCR amplification was conducted with a 10 pmol primer set; 5'-GCGATAGAAACGGGACACCG-3', 5'-TAGGTAA GGGGGAGGCGTGC-3' for 16S rDNA, 5'-AAAGGCTT GGTCCTGACTTTA-3', 5'-TTCCAAGTGCACCTTCCG GT-3' for 12S rDNA, 5'-GCATGAGCCGGAATAGTGGG-3', 5'-TATTCCAGCAAGCCCCAGGA-3' for cytochrome c oxidase subunit I (COI), 5'-CCTCCGATATCGCAACTG CC-3',5'-CCAAGGGCCTTATTTTCCG C-3' for cytochrome b (cytb) regions based on the complete genome of *Enedrias crassispina* (accession number, AP004449; Miya et al., 2003). PCR was conducted using an EX Taq DNA polymerase kit (Takara, Japan) and a PTC-20 apparatus (MJ Research, USA). The conditions utilized were as follows: initial denaturation for 1 cycle of 94°C for 5 min, 40 cycles (denaturation, 94°C, 30 s, annealing, 59°C, 30 s, extension, 72°C, 1 min). Amplified fragments of the expected size were confirmed by ethidium bromide-contained agarose gel (1.5%).

The single bands were purified using a PCR purification kit (DyneBionic, Korea), and the PCR fragments were subjected to direct sequencing in both directions using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). The nucleotide sequences of 16S and 12S rDNAs, COI, and cytb were analyzed using Chromas223, Bioedit, and BLAST software.

PCR-RFLP of mitochondrial DNA

The amplification products of 16S and 12S rDNAs, COI, and cytb on three species of genus *Pholis* were subjected to restriction fragment length polymorphism (RFLP). The products were then incubated for 3 hrs at 37°C with a variety of restriction enzymes (*Afa*I, *Dde*I, *Hae*III, *Hph*I, *Hsp*92II, *Mbo*I, *Msp*I, and *Xsp*I; Takara, Japan) and the recommended buffers. The digested fragments were visualized using ethidium bromide-contained agarose gel (2.5%).

RAPD analysis

The DNAs were then subjected to random amplified

Table 1. Comparison of the morphological characteristics in Genus *Pholis*

Analysis	*Pholis nebulosa*		*Pholis fangi*		*Pholis crassispina*	
	Range	Mean±S.D	Range	Mean±S.D	Range	Mean±S.D
Number of samples	20		13		11	
In hundredths of Total length (%)						
BL	92.3~95.5	93.5±0.7	90.7~92.3	92.0±0.6	93.8~96.8	95.2±1.2
BD	9.8~12.9	11.5±0.8	11.4~13.1	12.6±1.3	11.5~14.0	12.7±0.8
DL	78.1~88.5	82.5±2.2	72.6±78.9	76.8±2.2	76.4~83.7	81.4±2.7
AL	37.3~42.3	40.6±1.5	39.9~43.0	42.3±1.4	35.7~41.5	39.5±1.6
AOL	46.6~54.2	51.8±2.1	47.1~52.2	49.3±1.6	48.9~56.5	52.8±2.5
In hundredths of Head length (%)						
ED	15.4~20.0	17.6±1.3	18.5~22.7	19.9±1.5	14.1~22.9	17.6±5.8
SnL	15.9~22.2	19.0±1.7	18.8~28.9	23.6±3.0	15.0~21.1	17.2±5.4

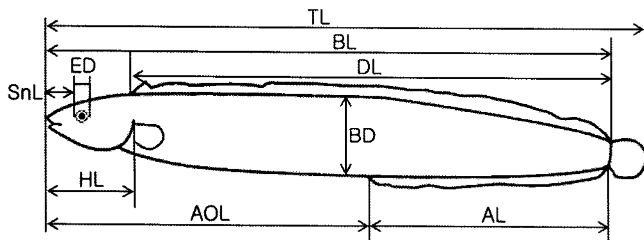

Fig. 1. The illustration shows the genus *Pholis* including body measurements, TL: total length, BL: body length, BD: body depth, HL: head length, DL: dosal fin length, AL: anal fin length, AOL: anal fin origin length, ED: eye diameter, and SnL: snout length

polymorphic DNA (RAPD). PCR amplification was conducted with 1 μL of 0.5 mM random primers; OPA-5, 5'-AGGGGTCTTG-3', OPA-10, 5'-GTGATCGCAG-3', and OPA-19, 5'-CAAACGTCGG-3' (Operon Technologies, USA). PCR was conducted with EX Taq DNA polymerase kit (Takara, Japan). The conditions utilized were as follows: initial denaturation for 1 cycle of 94°C for 5 min, 40 cycles (denaturation, 94°C, 1 min, annealing, 40°C, 1 min, extension, 72°C, 2 min). The amplified fragments were verified with ethidium bromide-stained agarose gel (1.5%).

RESULTS

Morphological characteristics and body measurements

The genus *Pholis* is typically characterized by a compressed form, with a high top and bottom and narrow left and right. The tidepool gunnel is long and ribbon-shaped, with a scattered black and dark-brown splotchy pattern on a light brown background. The dorsal fin contains short spines in a long, triangle pattern that lines up regularly with the origin, with a white outline at the end of the round shaped-tail fin. The white gunnel is similar to the tidepool gunnel, but can be distinguished from other species by the white H-type pattern lined up on the origin of the dorsal fin. The end of the tail fin was vertically straight, without any discernible pattern. The mottled gunnel also resembles the tidepool gunnel, but with a stick pattern containing white sections and I-type stripes lined up on the dorsal fin origin, and a yellow, round-shaped tail fin.

The average total lengths and body weights of the tidepool, white, and mottled gunnels were 22.0±1.7 cm, 43.1±11.8 g (n=20); 15.2±1.1 cm, 14.6±1.1 g (n=13); 12.7±1.5 cm, 7.8±2.5 g (n=11), respectively. In the tidepool, white, and mottled gunnels, the spinous rays of the dorsal fin were numbered LXXVI~LXXXIII, LXXVIII~LXXXI, and LXXIII~LXXXI, the spinous and soft rays of the anal fin were numbered II, 35~42; II, 42~45; II, 34~41, respectively. The spinous and soft rays of the ventral fin were I, 1 for the tidepool and mottled gunnels, and were I, 0~1 for white gunnel (Fig. 2).

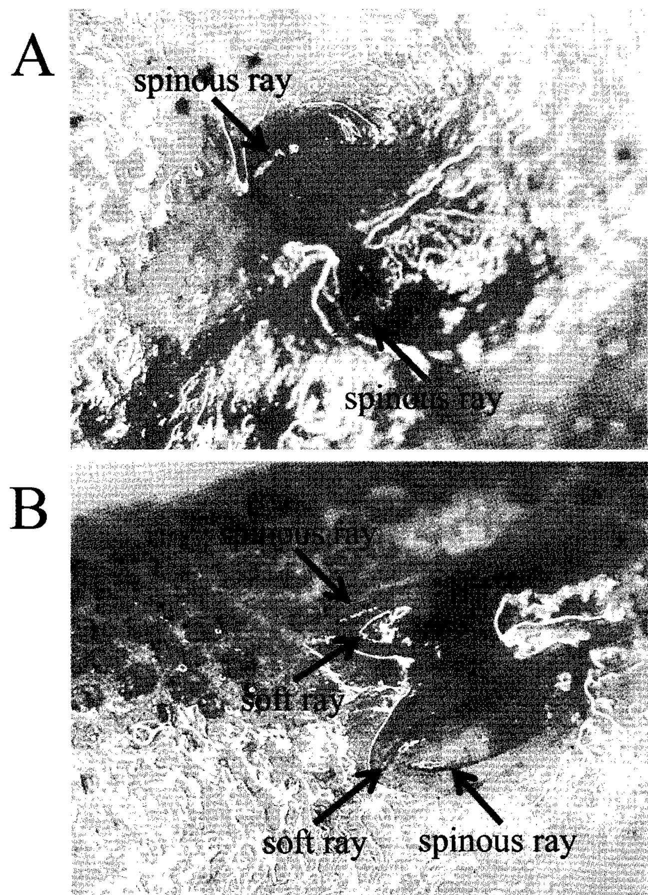

Fig. 2. Pictures show Alizarin red staining on the spinous and soft rays of white gunnel (*Pholis fangi*). A and B mean I, 0 and I, 1, respectively.

Table 2. Similarity percentages of 12S and 16S rRNAs, COI, and cytb in Genus *Pholis*

Species gene	*P. nebulosa*: *P. fangi*	*P. nebulosa*: *P. crassipina*	*P. fangi*: *P. crassispina*
12S rRNA	99.6%	99.0%	99.1%
16S rRNA	99.3%	99.2%	99.1%
COI	98.1%	96.5%	96.1%
cytb	97.0%	93.1%	91.8%

As for the percentages of BL, BD, DL, AL, and AOL against TL and the percentages of ED and SnL against HL (Fig. 1, Table 1), the white gunnel had the smallest percentage of BL against TL (92.0%), and the percentages of BD against TL were similar among the three, with the tidepool, white, and mottled gunnels at 11.5, 12.6, and 12.7%, respectively. The percentage of DL against TL was 82.5, 81.4, and 76.8%, in order, for the tidepool, mottled, and white gunnels. The mottled gunnel had the smallest percentage of AL against TL (39.5%). The percentages of AOL against TL in the mottled, tidepool, and white gunnels were 52.8, 51.8, and 49.3%, in order. With regard to the percentage of ED against HL, the white gunnel (19.9%)

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P. fangi        ACTCCTGTGAGAATGCCCTAATAGTTCCCCGCCCGGGAACAAGGAGCTGGTATCAGGCAC
P. nebulosa     ACTCCTGTGAGAATGCCCTAATAGTTCCCCGCCCGGGAACAAGGAGCTGGTATCAGGCAC
P. crassispina  ACTCCTGTGAGAATGCCCTAATAGTTCCCCGTCCGGGAACAAGGAGCTGGTATCAGGCAC
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P. fangi        AACCCTAGTTAAGCCCACGACGCCTTGCTTAGCCACACCCTCAAGGGAACTCAGCAGTGA
P. nebulosa     AACCCTAGTTAAGCCCACGACGCCTTGCTTAGCCACACCCTCAAGGGAACTCAGCAGTGA
P. crassispina  AACCCTAGT-GAGCCCACGACGCCTTGCTTAGCCACACCCTCAAGGGAACTCAGCAGTGA
                *********  *************************************************

P. fangi        TAAACGTTAAGCCCTAAGTGAAAACTTGACTTAGTCAAAGCTAAGAGGGCCGGTAAAACT
P. nebulosa     TAAACGTTAAGCCCTAAGTGAAAACTTGACTTAGTCAAAGCTAAGAGGGCCGGTAAAACT
P. crassispina  TAAACATTAAGCCCTAAGTGAAAACTTGACTTAGTCAAAGCTAAGAGGGCCGGTAAAACT
                ***** ******************************************************

P. fangi        CGTGCCAGCCACCGCGGTTATACGAGAGGCCCAAGTTGACAGACAGCGGCGTAAAGAGTG
P. nebulosa     CGTGCCAGCCACCGCGGTTATACGAGAGGCCCAAGTTGACAGACAGCGGCGTAAAGAGTG
P. crassispina  CGTGCCAGCCACCGCGGTTATACGAGAGGCCCAAGTTGACAGACATCGGCGTAAAGAGTG
                ********************************************* **************

P. fangi        GTTAAGTTAAAATTGTACTAAAGCCGAACACCCTCCAGGCTGTTATACGCATCCGAAGGT
P. nebulosa     GTTAAGTTAAAATTGTACTAAAGCCGAACACCCTCCAGGCTGTTATACGCATCCGAAGGT
P. crassispina  GTTAAGTTAAAATTGTACTAAAGCCGAACATCCTCCAGGCTGTTATACGCATCCGAAGAT
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P. fangi        AAGAAGTTCAACCACGAAGGTGGCTTTATTTAATCTGAACCCACGAAAGCTACGACACAA
P. nebulosa     AAGAAGTTCAACCACGAAGGTGGCTTTATTTAGTCTGAACCCACGAAAGCTACGACACAA
P. crassispina  AAGAAGTTCAACCACGAAGGTGGCTTTATTTAGTCTGAACCCACGAAAGCTACGGCACAA
                ******************************** ********************* *****

P. fangi        ACTGGGATTAGATACCCCACTATGCCTAGCCCTAAACATTGATAGTATTATACACCCACT
P. nebulosa     ACTGGGATTAGATACCCCACTATGCCTAGCCCTAAACATTGATAGTATTATACACCCACT
P. crassispina  ACTGGGATTAGATACCCCACTATGCCTAGCCCTAAACATTGATAGTATTATACACCCACT
                ************************************************************

P. fangi        ATCCGCCTGGGAACTACGAGCATTAGCTTAAAACCCAAAGGACTTGGCGGTGCTTTAGAT
P. nebulosa     ATCCGCCTGGGAACTACGAGCATTAGCTTAAAACCCAAAGGACTTGGCGGTGCTTTAGAT
P. crassispina  ATCCGCCTGGGAACTACCAGCATTAGCTTAAAACCCAAAGGACTTGGCGGTGCTTTAGAT
                ***************** ******************************************

P. fangi        CCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTTTCCTTGTT
P. nebulosa     CCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTTTCCTTGTT
P. crassispina  CCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTTTCCTTGTT
                ************************************************************

P. fangi        TTTCCCGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCTAAATAGTAAGCAAAA
P. nebulosa     TTTCCCGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGCTAAATAGTAAGCAAAA
P. crassispina  TTTCCCGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGTCAAATAGTAAGCAAAA
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P. fangi        CTGGTAGAACCCAAAACGTCAGGTCGAGGTGTAGCGTATGGGAAGGGAAGAAATGGGCTA
P. nebulosa     CTGGTAGAACCCAAAACGTCAGGTCGAGGTGTAGCGTATGGGAAGGGAAGAAATGGGCTA
P. crassispina  CTGGTAGAACCCAAAACGTCAGGTCGAGGTGTAGCGTATGGGAAGGGAAGAAATGGGCTA
                ************************************************************

P. fangi        CATTCGCTAGCATAGCGAATACGGACGATGCACTGAAACGTTCATCTGAAGGAGGATTTA
P. nebulosa     CATTCGCTAGCATAGCGAACACGGACGATGCGCTGAAACGTTCATCTGAAGGAGGATTTA
P. crassispina  CATTCGCTAGCATAGCGAATACGGACGATGCACTGAAACGTTCATCTGAAGGAGGATTTA
                ******************* *********** ****************************

P. fangi        GCAGTAAGCAGGAAATAGAGTGTTCCGCTGAAATTGGCCCTGAAGCGCGCACACACCGCC
P. nebulosa     GCAGTAAGCAGGAAATAGAGTGTTCCGCTGAAATCGGCCCTGAAGCGCGCACACACCGCC
P. crassispina  GCAGTAAGCAGGAAATAGAGTGTTCCGCTGAAATTGGCCCTGAAGCGCGCACACACCGCC
                ********************************** *************************

P. fangi        CGTCACTCTCCCCAAGCCTACCAACCTAATTAATTAAACCACAATAATCGCAAAGGGGAG
P. nebulosa     CGTCACTCTCCCCAAGCCTACCAACTTAATTAATTAAACCACAATAATCGCAAAGGGGAG
P. crassispina  CGTCACTCTCCCCAAGCCTACCAACCTAATTAATTAAACCATAATAATCGCAAAGGGGAG
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P. fangi        GCAAGTCGTAACATGGTAAGTGTACCGG
P. nebulosa     GCAAGTCGTAACATGGTAAGTGTACCGG
P. crassispina  GCAAGTCGTAACATGGTAAGTGTACCGG
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Fig. 3. Nucleotide sequence alignment of mitochondrial 12S ribosomal DNA from three species of the genus *Pholis*. The dashed lines indicate gaps inserted to provide the best alignment. Asterisks symbolize the fully conserved nucleotide sequences. The NCBI accession numbers of the nucleotide sequence are FJ687230 for *Pholis nebulosa*, FJ687231 for *Pholis fangi*, and FJ687232 for *Pholis crassispina*.

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P. Crassispina  GCATCATGATTTAGCAAGTGTAACCCAAGCGAAGCGTGCTTTAGTTTGGTATCCCGAAAC
P. nebulosa     GCATCATGATTTAGCAAGTGTAACCCAAGCGAACCGTGCTTTAGTTTGGTGTCCCGAAAC
P. fangi        GCATCATGATTTAGCAAGTGTAACCCAAGCGAAGCGTGCTTTAGTTTGGTATCCCGAAAC
                ********************************* **************** *********

P. crassispina  TGGGTGAGCTACTCCAAGACAGCCTATTAATAGGGCACACCCGTCTCTGTGGCAAAAGAG
P. nebulosa     TGGGTGAGCTACTCCAAGACAGCCTATTAATAGGGCACACCCGTCTCTGTGGCAAAAGAG
P. fangi        TGGGTGAGCTACTCCAAGACAGCCTATTAATAGGGCACACCCGTCTCTGTGGCAAAAGAG
                ************************************************************

P. crassispina  TGGGAGGAGCTTTGAGTAGAGGTGACAGACCTACCGAACCTAGTTATAGCTGGTTGTTCA
P. nebulosa     TGGGGGGAGCTTTGAGTAGAGGTGACAGACCTACCGAACCTAGTTATAGCTGGTTGTTCA
P. fangi        TGGGGGGAGCTTTGAGTAGAGGTGACAGACCTACCGAACCTAGTTATAGCTGGTTGTTCA
                **** *******************************************************

P. crassispina  AGAAATGAATAGAAGTTCAGCCTCCTGGCTTCTCTTTTCACTTTAGTTTAACCCCTATTG
P. nebulosa     AGAAATGAATAGTAGTTCAGCCTCCTGGCTTCTCTTTTCACTTTAGTTTAACCCCTATTG
P. fangi        AGAAATGAATAGTAGTTCAGCCTCCTGGCTTCTCTTTTCACTTTAGTTTAACCCCTATTG
                ************ ***********************************************

P. crassispina  ATGTGCTTAAGAAACCGAGAGAGTTAGTCAAAGGGGGTACAGCCCCTTTGAACCAAGACA
P. nebulosa     ATGTGCTTAAGAAACCGAGAGAGTTAATCAAAGGGGGTACAGCCCCTTTGAACCAAGACA
P. fangi        ATGTGCTTAAGAAACCGAGAGAGTTAATCAAAGGGGGTACAGCCCCTTTGAACCAAGACA
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P. crassispina  CAACTTTATCAGGAGGGTAAAGATCATAATAAACCAAAGGTAAATATTTGGGTGGGCCTA
P. nebulosa     CAACTTTATCAGGAGGGTAAAGATCATAATAAACCAAAGGTAAATATTTGGGTGGGCCTA
P. fangi        CAACTTTATCAGGAGGGTAAAGATCATAATAAACCAAAGGTAAATATTTGGGTGGGCCTA
                ************************************************************

P. crassispina  AAAGCAGCCATCCCCTTAGAAAGCGTTAAAGCTCAGATATACCGTCAACCCTTCTTATTC
P. nebulosa     AAAGCAGCCATCCCCTTAGAAAGCGTTAAAGCTCAGATATACCGTCAACCCTTCTTATTC
P. fangi        AAAGCAGCCATCCCCTTAGAAAGCGTTAAAGCTCGAATATACCGTCAACCCTTCTTATTC
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P. crassispina  TGATCACATAATCTTATCCCCCTAAACATACTGAACCATCCCATGCCTGCATGGGAGTGA
P. nebulosa     TGATCACACAATCTTATCCCCCTAAACATACTGAACCATCCCATGCCCACATGGGAGTGA
P. fangi        TGATCACACAATCTTATCCCCCTAAACATACTGAACCATCCCATACCTATATGGGAGTGA
                ******** *********************************** **   **********

P. crassispina  TTATGCTAATATGAGTAATAAGAGAGCCTTTGGCTCTCTCCCTGCACACGTGTACGTCGG
P. nebulosa     TTATGCTAATATGAGTAATAAGAGAGCCTTCGGCTCTCTCCCTGCACACGTGTACGTCGG
P. fangi        TTATGCTAATATGAGTAATAAGAGAGCCTTCGGCTCTCTCCCTGCACACGTGTACGTCGG
                ****************************** *****************************

P. crassispina  AACGGACACCCCGCCGACCATTAACGGCCCCAATCAAAGAGGGCACTGAATAATAGATTA
P. nebulosa     AACGGACACCCCGCCGACCATTAACGCCCCCAATCAAAGAGGGTACTGAATAATAGATTA
P. fangi        AACGGACACCCCGCCGACCATTAACGGCCCCAATCAAAGAGGGCACTGAATAACAGATTA
                ************************** **************** ********* ******

P. crassispina  AACAACAAGAAAAGCGTTCAGGAATTAACCGTTAACCCCACACAGGTGTGCCCACAGGGA
P. nebulosa     AACAACAAGAAAAGCATTCAGGAATTAACCGTTAACCCCACACAGGTGTGCCCACAGGGA
P. fangi        AACAACAAGAAAAGCATTCAGGAATTAACCGTTAACCCCACACAGGTGTGCCCGTAGGGA
                *************** *************************************  *****

P. crassispina  AAGGCTAAAAGAGAGAGAAGGAACTCGGCAAACACATCAAAGCCTCGCCTGTTTACCAAA
P. nebulosa     AAGGCTAAAAGAGAGAGAAGGAACTCGGCAAACACATCAAAGCCTCGCCTGTTTACCAAA
P. fangi        AAGACTAAAAGAGAGAGAAGGAACTCGGCAAACACATCAAAGCCTCGCCTGTTTACCAAA
                *** ********************************************************

P. crassispina  AACATCGCCTCTTGCAAACTTAATAAATAAGAGGTCCCGCCTGCCCTGTGACTATTAGTT
P. nebulosa     AACATCGCCTCTTGCAAACTTAATAAATAAGAGGTCCCGCCTGCCCTGTGACTATTAGTT
P. fangi        AACATCGCCTCTTGCAAACTTAATAAATAAGAGGTCCCGCCTGCCCTGTGACTATTAGTT
                ************************************************************

P. crassispina  TAACGGCCGCGGTATTTTGACCGTGCGAAGGTAGCGCAATCACTTGTCTTTTAAATGAAG
P. nebulosa     TAACGGCCGCGGTATTTTGACCGTGCGAAGGTAGCGCAATCACTTGTCTTTTAAATGAAG
P. fangi        TAACGGCCGCGGTATTTTGACCGTGCGAAGGTAGCGCAATCACTTGTCTTTTAAATGAAG
                ************************************************************

P. crassispina  ACCTGTATGAATGGCATAACGAGGGCTTAACTG
P. nebulosa     ACCTGTATGAATGGCATAACGAGGGCTTAACTG
P. fangi        ACCTGTATGA--TGCATAACGAGGGCTTAACTG
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Fig. 4. Nucleotide sequence alignment of mitochondrial 16S ribosomal DNA from three species of the genus *Pholis*. The dashed lines and asterisks are the same as in Fig. 3. The NCBI accession numbers of the nucleotide sequence are FJ687233 for *Pholis nebulosa*, FJ687234 for *Pholis fangi*, and FJ687235 for *Pholis crassispina*.

had a higher percentage than other two species (tidepool and mottled gunnels, 17.6%), and the percentage of SnL against HL in the white gunnel (23.6%) was higher than that measured in the other two species (tidepool gunnel,

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P. Fangi        CCAGCCCGGCGCCCTACTAGGCGACGACCAAATTTATAATGTAATTGTTACAGCACATGC
P. nebulosa     CCAGCCCGGCGCCCTACTAGGCGACGACCAAATTTATAATGGAATTGGTACAGCACATGC
P. crassispina  CCAGCCCGGCGCCCTACTAGGCGACGACCAAATTTATAATGTAATTGTTACAGCACATGC
                ***************************************** ***** ************

P. fangi        ATTCGTAATAATTTTCTTTATAGTAATGCCAATCATGATCGGGGGTTTCGGAAACTGGCT
P. nebulosa     ATTCGTAATAATTTTCTTTATAGTAATACCAATCATGATCGGGGGCTTCGGAAACTGGCT
P. crassispina  ATTCGTAATAATTTTCTTTATAGTAATACCAATCATGATCGGGGGCTTCGGAAACTGACT
                *************************** ***************** *********** **

P. fangi        TATTCCCCTAATGATCGGGGCCCCAGACATGGCATTTCCCCGTATAAATAACATGAGTTT
P. nebulosa     TATCCCCCTAATGATCGGAGCCCCAGACATGGCATTCCCCCGTATAAATAATATGAGTTT
P. crassispina  TATTCCCCTAATGATCGGGGCCCCAGACATGGCGTTTCCCCGTATAAATAACATGAGTTT
                *** ************** ************** ** ************** ********

P. fangi        TTGGCTTCTTCCTCCTTCTTTCCTTCTTCTCCTTGCCTCTTCTGGGGTTGAGGCAGGAGC
P. nebulosa     TTGGCTTCTTCCCCCTTCTTTCCTTCTTCTCCTTGCCTCTTCTGGGGTTGAGGCGGGAGC
P. crassispina  TTGGCTTCTCCCCCCTTCTTTCCTCCTCCTCCTTGCCTCTTCTGGGGTTGAGGCGGGAGC
                ********* ** *********** ** ************************** *****

P. fangi        TGGTACAGGGTGAACGGTGTACCCGCCCCTTTCTGGTAATTTAGCGCACGCCGGGGCCTC
P. nebulosa     TGGCACAGGGTGAACGGTATACCCGCCCCTTTCTGGTAATTTAGCGCACGCCGGGGCCTC
P. crassispina  TGGTACAGGGTGGACGGTATACCCGCCCCTTTCTGGTAATTTAGCGCACGCCGGGGCCTC
                *** ******** ***** *****************************************

P. fangi        TGTTGACTTAACAATCTTTTCTCTTCACCTGGCGGGGATTTCTTCAATTCTTGGGGCAAT
P. nebulosa     TGTTGACTTAACAATCTTTTCTCTTCACCTGGCGGGGATTTCTTCAATTCTTGGGGCAAT
P. crassispina  TGTTGATTTAACAATCTTTTCTCTTCACCTGGCGGGGATTTCTTCAATTCTTGGAGCAAT
                ****** *********************************************** *****

P. fangi        TAATTTTATCACAACCATTATTAACATGAAACCCCCTGCCATTTCTCAGTATCAAACACC
P. nebulosa     TAATTTTATCACAACCATTATTAACATAAAACCCCCTGCCATTTCTCAGTATCAAACACC
P. crassispina  TAATTTTATCACAACGATTATTAACATAAAACCTCCTGCCATTTCTCAATACCAGACACC
                *************** *********** ***** ************** ** ** *****

P. fangi        GCTCTTCGTTTGATCCGTACTCATTACCGCCGTTCTTCTGCTCCTCTCACTCCCCGTGCT
P. nebulosa     GCTCTTCGTTTGATCCGTGCTCATTACCGCCGTTCTTCTGCTCCTCTCACTCCCCGTGCT
P. crassispina  GCTCTTCGTTTGATCCGTACTCATTACCGCCGTTCTTCTGCTCCTTTCACTTCCCGTGCT
                ****************** ************************** ***** ********

P. fangi        TGCAGCCGGAATCACCATACTCCTAACAGACCGTAATCTGAACACCACCTTTTTCGACCC
P. nebulosa     TGCAGCCGGAATCACCATACTCCTAACAGATCGTAATCTAAACACCACCTTTTTTGACCC
P. crassispina  TGCAGCCGGGATTACTATGCTCCTAACAGATCGTAATCTAAACACTACCTTTTTTGACCC
                ********* ** ** ** *********** ******** ***** ******** *****

P. fangi        CGCAGGAGGGGGCGACCCGATTCTTTACCAACACTTGTTCTGATTTTTTGGCCACCCTGA
P. nebulosa     CGCAGGAGGGGGCGACCCAATTCTTTACCAACACTTGTTCTGATTTTTTGGCCACCCTGA
P. crassispina  GGCAGGAGGGGGTGACCCAATTCTTTACCAACACTTGTTCTGATTTTTCGGCCATCCTGA
                 *********** ***** ***************************** ***** *****

P. fangi        GGTATATATTCTTATTCTTCCCGGCTTCGGGATAATTTCACATATTGTTGCCTACTATTC
P. nebulosa     GGTATATATTCTTATTCTTCCCGGCTTCGGGATAATTTCACATATTGTTGCCTACTATTC
P. crassispina  AGTGTACATTCTTATTCTTCCTGGCTTCGGAATAATTTCACACATTGTTGCCTACTATTC
                 ** ** ************** ******** *********** *****************

P. fangi        GGGCAAAAAAGAGCCTTTTGGCTATATAGGAATGGTATGGGCAATGATGGCCATCGGTCT
P. nebulosa     TGGCAAAAAAGAGCCTTTTGGTTATATAGGAATGGTGTGGGCAATGATGGCCATCGGTCT
P. crassispina  TGGTAAAAAGGAGCCTTTTGGCTATATAGGAATGGTGTGGGCAATGATGGCCATCGGTCT
                 ** ***** *********** ************** ***********************

P. fangi        TCTCGGGTTTATCGTATGGGCCCACCACATGTTTACAGTCGGGATGGACGTAGATACACG
P. nebulosa     TCTCGGGTTTATCGTATGGGCCCACCACATATTTACAGTCGGGATAGACGTAGACACACG
P. crassispina  TCTAGGATTTATCGTATGGGCCCACCACATGTTTACAGTCGGAATAGACGTAGACACACG
                *** ** *********************** *********** ** ******** *****

P. fangi        TGCATATTTCACCTCCGCCACCATGATCATCGCAATTCCTACGGGGGTGAAGGTCTTTAG
P. nebulosa     TGCATATTTTACCTCCGCCACCATGATCATTGCAATTCCTACGGGG-TGAAGGTCTTTAG
P. crassispina  GGCATATTTTACCTCCGCCACCATGATCATCGCAATTCCTACGGGGGTGAAGGTCTTTAG
                 ******** ******************** *************** *************

P. fangi        CTGACTTGCAACCCTTCACGGAGGCTCTATTAAGTGG-AAACCCCCCTTCTCTGAGCCCT
P. nebulosa     CTGGCTTGCAACCCTTCACGGAGGCTCTATTAAGTGGGAAACCCCCCTTCTCTGAGCCCT
P. crassispina  CTGACTTGCAACCCTTCACGGAGGCTCTATTAAGTGGGAGACCCCGCTTCTTTGAGCACT
                *** ********************************* * ***** ***** ***** **

P. fangi        TGG
P. nebulosa     TGG
P. crassispina  CGG
                 **
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Fig. 5. Nucleotide sequence alignment of cytochrome c oxidase I from three species of the genus *Pholis*. The dashed lines and asterisks are identical to those shown in Fig. 3. The NCBI accession numbers of the nucleotide sequence are FJ687236 for *Pholis nebulosa*, FJ687237 for *Pholis fangi*, and FJ687238 for *Pholis crassispina*.

```
P. Fangi        GCCGAGACGTGAACTACGGGTGGCTTATCCGTAATCTGCATGCTAACGGAGCTTCCTTCT
P. nebulosa     GCCGGGACGT-AACTACGGGTGGCTTATCCGCAATCTCCATGCTAACGGAGCTTCCTTCT
P. crassispina  GCCGGGACGT-CACTACGGATGACTCATCCGCAACCTACATGCTAACGGAGCCTCCTTTT
                **** *****  ******* ** ** ***** ** ** ************** ***** *

P. fangi        TCTTTATCTGCCTTTACATGCACGTCGGTCGTGGACTATACTATGGCTCTTACCTTTATA
P. nebulosa     TCTTTATCTGCCTTTACATGCACGTCGGTCGTGGACTATACTATGGCTCTTACCTTTATA
P. crassispina  TCTTTATCTGCCTTTATATACACATCGGCCGTGGGCTATACTACGGCTCTTACCTTTATA
                **************** ** *** **** ***** ******** ****************

P. fangi        AGGAAACGTGAAACGTTGGGGTCATCCTCCTCCTCCTTGTAATAATGACTGCTTTCGTTG
P. nebulosa     AGGAAACGTGAAACGTTGGGGTCATCCTCCTCCTTCTTGTGATAATAACTGCTTTCGTTG
P. crassispina  AGGAGACATGGAACGTGGGGGTTATCCTATTCCTTCTTGTGATGATAACTGCTTTCGTTG
                **** ** ** ***** ***** *****  **** ***** ** ** *************

P. fangi        GTTATGTCTTGCCCTGAGGACAAATGTCGTTTTGAGGGGCAACAGTCATTACCAACCTCC
P. nebulosa     GTTATGTCTTGCCCTGAGGACAAATATCGTTTTGGGGAGCAACGGTCATTACCAACCTCC
P. crassispina  GCTATGTTTTGCCTTGAGGACAAATATCGTTCTGAGGAGCAACAGTCATTACTAACCTTC
                * ***** ***** *********** ***** ** ** ***** ******** ***** *

P. fangi        TCTCCGCGGTACCCTATATCGGCAGTTCTCTCGTTCAGTGAATTTGAGGGGGCTTCTCAG
P. nebulosa     TCTCCGCGGTGCCCTATATCGGCAGTTCTCTCGTTCAGTGAATTTGAGGGGGCTTCTCAG
P. crassispina  TCTCCGCAGTTCCCTATATCGGTAGTTCTCTCGTTCAATGGATTTGAGGGGGCTTCTCAG
                ******* ** *********** ************** ** *******************

P. fangi        TAGACAACGCCACCCTGACCCGCTTTTTCGCATTCCACTTCCTATTTCCATTCGTTGTTG
P. nebulosa     TCGACAACGCTACCCTGACCCGCTTTTTCGCCTTCCACTTCCTGTTCCCATTCGTTGTTG
P. crassispina  TCGATAACGCTACTCTAACCCGCTTTTTTGCCTTCCACTTCCTATTCCCGTTCGTTGTTG
                * ** ***** ** ** *********** ** *********** ** ** **********

P. fangi        CGGGGGCCACTTTCGTCCACCTCATCTTCCTACACCAGACAGGCTCGAACAACCCCCTAG
P. nebulosa     CGGGAGCCACTTTCGTCCACCTCATCTTCCTGCACCAGACAGGCTCGAACAACCCCCTAG
P. crassispina  CAGGGGCCACTTTCGTCCACCTTATCTTCCTACACCAGACGGGCTCGAATAACCCTCTGG
                * ** ***************** ******** ******** ******** ***** ** *

P. fangi        GCCTGAACTCAGATGCTGACAAAATCTCGTTTCACCCCTACTTTTCATATAAAGACCTTC
P. nebulosa     GCCTGAACTCGGATGCTGACAAAATCTCGTTTCACCCCTACTTTTCATATAAAGACCTTC
P. crassispina  GCCTGAACTCAGATGCTGACAAAATCTCATTTCACCCCTACTTTTCATATAAAGACCTTC
                ********** ***************** *******************************

P. fangi        TGGGCTTTGCAGCCCTACTCACCGGCCTCACAGCCCTCGCCCTGTTCTCACCAAATCTCT
P. nebulosa     TGGGCTTTGCAGCCCTACTCACTGGCCTCACAGCCCTCGCCCTGTTCTCACCAAATCTCT
P. crassispina  TGGGCTTTGCAGCTCTACTTATTGGCCTCACAGCCCTCGCTCTATTTTCACCAAATCTCT
                ************* ***** *  ***************** ** ** *************

P. fangi        TGGGAGACCCTGACAACTTTACCCCAGCAAACCCCTTAGTCACTCCGCCCCACATCAAGC
P. nebulosa     TGGGAGACCCTGACAACTTTACCCCGGCGAACCCCCTAGTTACTCCGCCCCACATCAAGC
P. crassispina  TGGGAGACCCTGACAACTTTACCCCGGCAAACCCCTTGGTTACTCCGCCCCACATCAAGC
                ************************* ** ****** * ** *******************

P. fangi        CCGAGTGATACTTCCTGTTTGCCTATGCAATCCTCCGCTCAATCCCCAACAAGCTAGGCG
P. nebulosa     CCGAGTGATACTTCCTGTTTGCCTATGCAATCCTCCGCTCGATCCCCAACAAACTAGGCG
P. crassispina  CCGAATGGTACTTTCTGTTTGCCTATGCTATCCTACGCTCCATCCCCAACAAACTAGGCG
                **** ** ***** ************** ***** ***** *********** *******

P. fangi        GAGTTTTGGCCCTCCTTGCTTCTATCCTTGTGCTCATGGTTGTCCCCATTCTCCACACCT
P. nebulosa     GAGTTTTAGCCCTCCTTGCTTCTATCCTTGTGCTTATGGTTGTCCCCATTCTCCACACCT
P. crassispina  GAGTTTTAGCCCTCCTTGCCTCCATCCTTGTACTTATAGTTGTCCCCATTCTCCACACCT
                ******* *********** ** ******** ** ** **********************

P. fangi        CTAAGCAACGGGGCCTGACCTTTCGCCCGGTCACACAATTCCTATTTTGGACCCTTATCG
P. nebulosa     CTAAGCAGCGGGGCCTAACCTTTCGCCCCGTCACACAATTCCTATTTTGGACCCTTATCG
P. crassispina  CTAAGCAGCGGGGCCTAACCTTCCGCCCGGTCACACAATTCCTATTTTGAACTCTTATCG
                ******* ******** ***** ***** ******************** ** *******

P. fangi        CGGATCTCGCCATCTTAACGTGAATCGGAGGCATGCCCGTAGAACACCCGTACATTATTA
P. nebulosa     CAGATCTCGCCATCTTGACATGAATCGGAGGCATGCCTGTAGAACACCCCTACATTATTA
P. crassispina  CAGACGTCGCCATCTTGACATGAATCGGGGGCATGCCTGTAGAACACCCGTACATTATTA
                * **  ********** ** ******** ******** *********** **********

P. fangi        TTGGCCAAGTCGCATCCGTCTTATATTTTTCCCTCTTTCTCGTCCTCTCTCCACTAGCGG
P. nebulosa     TTGGCCAAGTCGCATCCGTCTTGTATTTTTCCCTCTTTCTCGTCCTCTCTCCGCTAGCGG
P. crassispina  TTGGCCAAGTCGCATCCGTCTTATATTTTTCCCTCTTTCTCATCCTCTCTCCGCTGGCGG
                ********************** ****************** ********** ** ****
```

Fig. 6. Nucleotide sequence alignment of cytochrome b from three species of the genus *Pholis*. The dashed lines and asterisks are the same as in Fig. 3. The NCBI accession numbers of the nucleotide sequence are FJ687239 for *Pholis nebulosa*, FJ687240 for *Pholis fangi*, and FJ687241 for *Pholis crassispina*.

19.0%; mottled gunnel, 17.2%). As a result of morphological observation, the white gunnel differed morphologically somewhat among the three species.

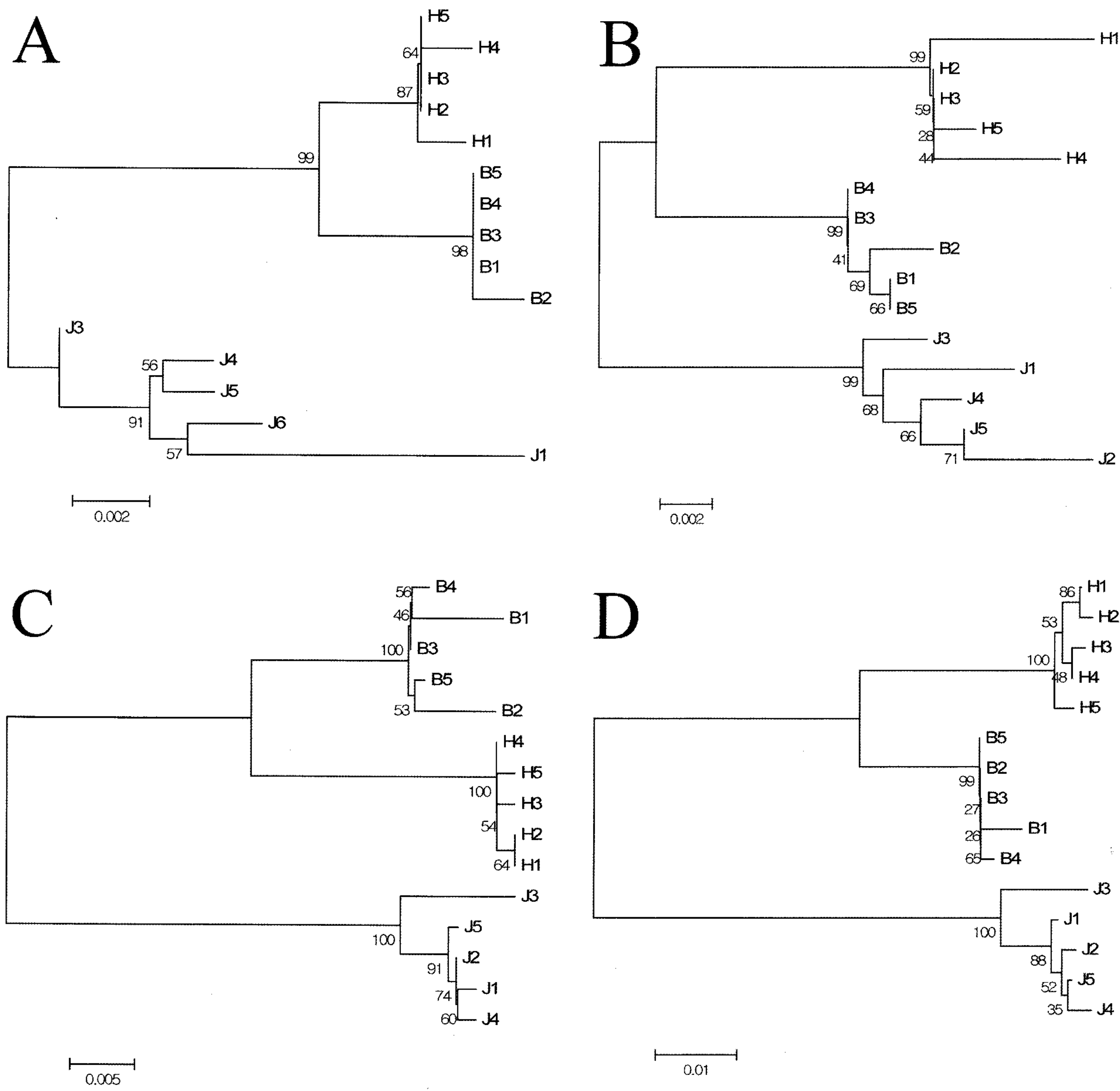

Fig. 7. Based on the nucleotide sequences of mitochondrial 12S (A) and 16S (B) ribosomal DNAs, cytochrome c oxidase I (C), and cytochrome b (D) DNAs from five individuals of three species in the genus *Pholis*, phylogenetic trees were conducted in MEGA4 (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Letters B, H, and J indicate *Pholis nebulosa*, *Pholis fangi*, and *Pholis crassispina*, respectively.

Genetic characteristics

The alignment of the nucleotide sequences (868 bp) of 12S rDNA in three species of genus *Pholis* is shown in Fig. 3. 17 genetic variations were confirmed among the species. The greatest similarity was noted between the tidepool and white gunnels, with a similarity of 99.6% (Table 2). With regard to the nucleotide sequence composition, all three species evidenced a slightly higher ratio of the A+T pair (tidepool, 50.8%; white, 51.2%; mottled, 51.6%) relative to the G+C pair (tidepool, 49.2%; white, 48.8%; mottled, 48.4%).

The alignment of 16S rDNA nucleotide sequences (873 bp) on three species of genus *Pholis* are provided in Fig. 4. 23 genetic variations were confirmed among the species. The greatest level of similarity was noted between the tidepool and white gunnels, evidencing a similarity of 99.3% (Table 2). With regard to the nucleotide sequence composition, all three species evidenced slightly higher ratios of the A+T pair (tidepool, 54.5%; white, 54.9%; mottled, 54.6%) to the G+C pair (tidepool, 45.5%; white, 45.1%; mottled, 45.4%).

The alignment of COI nucleotide sequences (902 bp) on

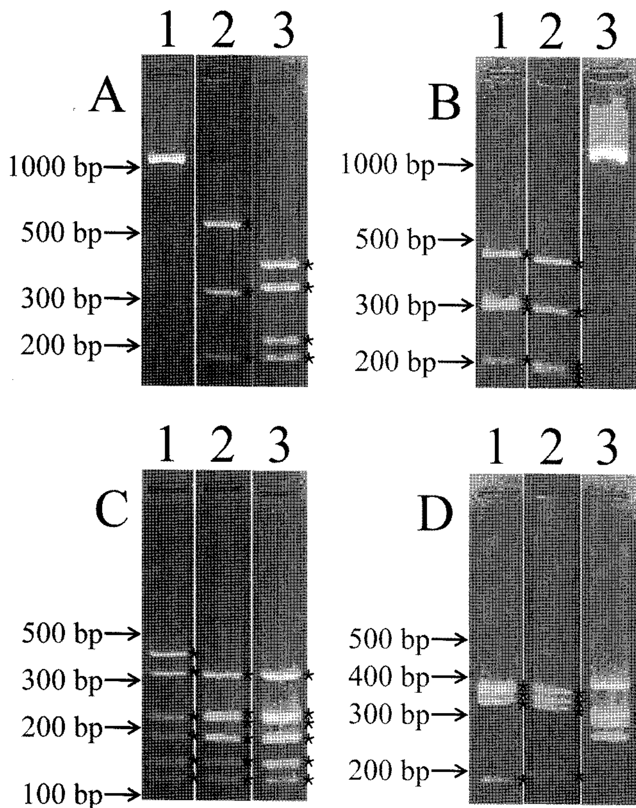

Fig. 8. Pictures show the results of RFLP in COI regions. COI-amplified fragments on three species of the genus *Pholis* were digested using different restriction enzymes (A: *AfaI*, B: *DdeI*, C: *HaeIII*, and D: *HphI*), along with the molecular markers (arrows). Lanes 1, 2, and 3 indicate *Pholis nebulosa*, *Pholis fangi*, and *Pholis crassispina*, respectively. The asterisks mean restriction enzyme digested fragments.

three species of genus *Pholis* are provided in Fig. 5. 70 genetic variations were verified among the species. The highest level of similarity was noted between the tidepool and white gunnels, with a similarity of 98.1% (Table 2). With regard to the nucleotide sequence composition, all three species evidenced slightly higher ratios of the A+T pair (tidepool, 52.2%; white, 51.9%; mottled, 52.8%) relative to the G+C pair (tidepool, 47.8%; white, 48.1%; mottled, 47.2%).

Nucleotide sequence alignments (899 bp) of cytb on three species of genus *Pholis* are provided in Fig. 6. 106 genetic variations were verified among the species. The greatest similarity was noted between the tidepool and white gunnels, with a similarity of 97.0% (Table 2). With regard to the nucleotide sequence compositions, the tidepool and white gunnels evidenced a slightly higher ratio of the G+C pair (tidepool, 51.7%; white, 51.2%) relative to the A+T pair (tidepool, 48.3%; white, 48.8%). However, the mottled gunnel evidenced a slightly higher ratio of A+T pair (50.9%) relative to the G+C pair (49.1%).

Based on the nucleotide sequences of 12s rDNA (Fig. 3), 16s rDNA (Fig. 4), COI (Fig. 5), and cytb (Fig. 6), phylogenetic analysis is performed to clarify the relationship among inter- and intra-species of genus *Pholis* as shown in Fig. 7. The phylogenetic tree revealed a close relationship between the tidepool and white gunnels with a slight genetic distance.

The PCR products of the COI regions of the tidepool, white, and mottled gunnels were subjected to RFLP for effective species classification. As a result of treatment with several restriction enzymes (*Afa*I, *Dde*I, *Hae*III, *Hph*I, *Hsp*92II, *Mbo*I, *Msp*I, and *Xsp*I), several restriction sites were verified as the result of treatment with *Afa*I, *Dde*I, *Hae*III, and *Hph*I (Fig. 8). In the treatments with *Afa*I, 3 and 4 restriction sites were verified in the white and mottled gunnels, respectively, but no restriction sites were detected in the tidepool gunnel (Fig. 8-1). 4 and 5 *Dde*I restriction sites were confirmed in the tidepool and white gunnels, respectively, but no restriction sites were detected in the mottled gunnel (Fig. 8-2). In the treatments with *Hae*III and *Hph*I, 6 and 4 restriction sites, respectively, were confirmed in all gunnels (Fig. 8-3 and 8-4).

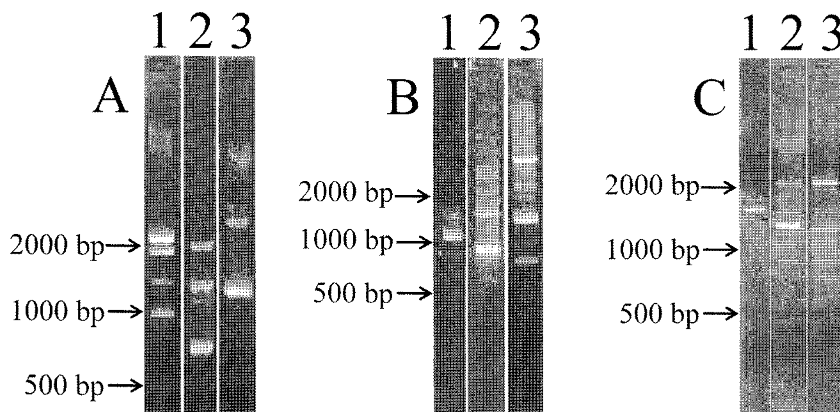

Fig. 9. Pictures show the results of RAPD in the genus *Pholis* using different primers (A: OPA-5, B: OPA-10, and C: OPA-19). Lanes 1, 2, 3, and arrows are the same as shown in Fig. 8.

RAPD amplified by 10-mer random primers (OPA-5, OPA-10, and OPA-19) was shown in Fig. 9. Each of the RAPD results revealed distinct species-specific patterns from the tidepool, white, and mottled gunnels.

DISCUSSION

With regard to morphological characteristics, the triangle, H-type, and I-type patterns on the dorsal fin origins of the tidepool, white, and mottled gunnels were distinct from each other, but all commonly exhibited a compressed body form. The spinous and soft rays of the dorsal, anal, and ventral fins evidenced differences indicating different levels of evolution and devolution in the genus *Pholis*. No clear differences were observed among the three species in terms of the percentages of BL, BD, DL, AL, and AOL against TL, but the percentages of white gunnel on ED and SnL against HL were higher than those of the other two tested species.

As a result of similarity analysis conducted on the three species, the cytb region evidenced the lowest level of similarity, indicating that the cytb region varies substantially inter- and intra-species (Brown et al., 1982; Palma and Spotorno, 1999). With regard to the RFLP method used to identify the three species, it is believed that *Afa*I and *Dde*I digestions on the COI region of genus *Pholis* proved efficient, since different sizes and numbers of fractions were noted after restriction enzyme digestion. Moreover, when classifications were conducted on the basis of RAPD assays, the OPA-19 primer was considered to be the most efficient, owing to its clear amplification pattern. RAPD is a much easier classification scheme than RFLP, which requires one additional step for restriction enzyme treatment.

The establishment of concise classifications among the genus *Pholis* is critically important to the Korean gunnel market, effective juvenile resource management, and fundamental ecological data accumulation. Moreover, the present study will save a great deal of effort and time for the classification of the genus *Pholis* with a high degree of accuracy.

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