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Maternal identification of hybrid eggs in *Hexagrammos* spp. by means of multiplex amplified product length polymorphism of mitochondrial DNA

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ABSTRACT: Hybridization has been frequently observed among 3 species of greenlings (genus *Hexagrammos*) common in waters off Japan. In order to estimate the frequency of hybridization events from egg masses collected from male territories, efficient maternal identification of numerous egg masses is required. A novel streamlined approach for maternal identification of 3 *Hexagrammos* spp. was developed using multiplex amplified product length polymorphism (APLP) analysis of the mitochondrial cytochrome *b* (Cyt*b*) and the 12S and 16S ribosomal RNA (12-16S rRNA) regions. Concurrent use of species-specific primer sets permits the amplification of different-sized PCR products, diagnosing each species through one procedure of PCR in a single reaction tube. The APLP method produced more rapid, reliable, and cost-efficient species identifications compared to those from an established restriction fragment length polymorphism (RFLP) protocol.

KEY WORDS: APLP · Maternal identification · Hybridization · Mitochondrial DNA · Multiplex PCR · *Hexagrammos* spp. · Cytochrome *b* · 12S and 16S ribosomal RNA

INTRODUCTION

Hybrid zones have been recognized in various groups of fishes (Arnold 1997) and their study provides an opportunity to understand evolutionary processes of reproductive isolation and speciation (Jiggins & Mallet 2000). Most studies of hybrid zones are restricted to freshwater species (e.g. Scribner et al. 2000) and there is little current information regarding marine fishes (Nielsen et al. 2004). Generally, hybrid zones are relatively broader in the sea than on land, a result consistent with higher realized dispersal of marine populations (van Herwerden & Doherty 2006). Genetic screening will require substantially more specimens for analysis; thus low-cost and efficient methods of hybrid identification are necessary prerequisites for advancing the study of hybrid zones in marine fishes.

Species in the genus *Hexagrammos* are endemic to the North Pacific Ocean and widely distributed along coastal areas (Rutenberg 1970). *H. otakii*, *H. agrammus* and *H. octogrammus* were recently reported to hybridize with one another in the southern Hokkaido and Tohoku districts of Japan (Balanov & Antonenko 1999, Brykov & Podlesnykh 2001). Males of these congeneric species establish breeding territories and care for egg masses deposited by multiple females until hatching (Crow et al. 1997). Although such a breeding system readily permits paternal identification by collection of eggs from the male breeding territories, maternal identification by direct observation is difficult because females leave the territories soon after spawning.

Genetic variation of mitochondrial DNA (mtDNA) is a powerful tool for determining female parentage in teleost fishes, due to rapid nucleotide sequence evolu-

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tion and asexual maternal inheritance in most vertebrates (Avise & Lansman 1983, Nei 1987). The nonrecombining mtDNA genome experiences an effective rate of genetic drift 4-fold greater than that for nuclear DNA because of haploid maternal transmission, leading to faster accumulation of fixed differences among closely related species (Nei 1987). A polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method developed for the region spanning the mitochondrial 12S and 16S ribosomal RNA genes (Yanagimoto 2003) permits unequivocal maternal identification of these *Hexagrammos* species. Although the PCR-RFLP method is highly effective, it involves 2 procedures: amplification of a target region by PCR, and subsequent digestion by restriction enzymes. More efficient methods are necessary for the high sample throughput required to study hybrid zones in marine species.

In this study, we report the development of a multiplex amplified product length polymorphism (APLP) protocol for determining species maternity in 3 *Hexagrammos* species. This method is based upon PCR amplification of diagnostic fragments using species-specific primers. Concurrent use of these primer sets generates different-sized PCR amplicons, diagnosing each species simultaneously. Since this method requires only one PCR reaction within a single reaction tube per sample, it is likely to substantially reduce both cost and time for hybrid identification. This method has recently been applied in studies of humans (Umetsu et al. 2005), identification of fungal pathogens (Dendis et al. 2003) and ecological field studies supporting conservation management that required screening thousands of samples (Pank et al. 2001, Hosseini et al. 2007). Here we confirm the reliability and efficiency of the APLP method in direct comparison with an existing PCR-RFLP protocol.

MATERIALS AND METHODS

Specimen collection. Adult specimens of 3 *Hexagrammos* spp. were captured by gill nets and traps at a coastal reef off Usujiri, Japan, where interspecific *Hexagrammos* hybrids have been reported, and at Mie and Akkeshi, Japan, where hybrids have not been reported (Fig. 1; Yabe et al. 1991). Fishes were identified by external morphology based upon the number of lateral lines, frap pairs, and caudal fin shape, following Shinohara (1994) and Nakabo (2000). Individuals possessing intermediate characters were excluded from DNA analysis as putative hybrids. Egg masses were collected from the male territories of 3 *Hexagrammos* spp. at the coast of Usujiri using SCUBA (Fig. 1). Each egg mass was kept separate until hatching, and one larva was randomly

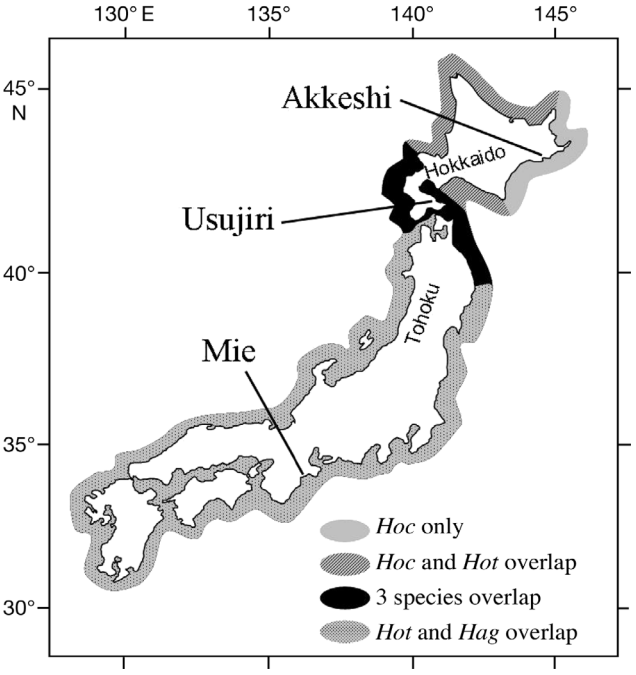

Fig. 1. *Hexagrammos* spp. Geographic distributions of 3 *Hexagrammos* species (Hot: *H. otakii*, Hag: *H. agrammus*, Hoc: *H. octogrammus*; overlap of 2 or more species also indicated) and locations where specimens were sampled. Adult specimens were sampled from Usujiri, Mie and Akkeshi. All egg masses were sampled from Usujiri

selected from each for genetic analysis. All tissues from adults and larvae were preserved in 99% ethanol in a freezer at –30°C. The number of specimens of adults and larvae are shown in Table 1.

DNA extraction, PCR reaction and sequencing. Total genomic DNA was extracted using the Puregene DNA purification kit (Gentra Systems) according to manufacturer's instructions and stored in a refrigerator at 4°C until use. The primer sets, Cyt*b*-F, 5′-ATGGC-

Table 1. *Hexagrammos* spp. Numbers of specimens of adults and larvae used for analysis

	Site	N
Adults		
H. otakii	Usujiri	6
	Mie	6
H. agrammus	Usujiri	6
	Mie	6
H. octogrammus	Usujiri	6
	Akkeshi	6
Total		36
Larvae of territorial male		
H. otakii		11
H. agrammus		11
H. octogrammus		10
Total		32

AAGCCTACGAAAAA-3′ (forward primer); Cyt*b*-R, 5′-TCCTAAGGCCTTGTTTTCTA-3′ (reverse primer); 12S-F, 5′-CGGGAACTACGAGCAAAAG-3′ (forward primer); and 16S-R, 5′-TCTTTTAGTCTTTCCCTGGGG-3′ (reverse primer), were designed from sequences of the cytochrome *b* region (Cyt*b*, AF087409, AF087410 and AF087412) and the 12S and 16S rRNA regions (12-16S rRNA, AB084628, AB084629 and AB084631) reported by Yanagimoto (2003) and Yanagimoto & Kobayashi (2004). PCR amplification was carried out with an ABI 9700 thermal cycler (Applied Biosystems) using the following thermal cycle profile: denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 2 min, followed by extension at 72°C for 7 min. PCR reactions were conducted with 1 µl of template DNA (20 to 40 ng µl^{-1}), 24 µl of PCR buffer (pH 8.0) containing of 0.5 µl of each primer (50 µM), 2.5 µl of dNTP mix (2.5 mM each), 2.5 µl of 10× Ex *Taq* buffer (TaKaRa Bio), 0.13 µl of TaKaRa Ex *Taq* HS DNA polymerase (5 U µl^{-1}; TaKaRa Bio) and 17.87 µl of sterile distilled water.

After purification with a GFX filter (Amersham Biosciences), PCR products were used as templates for direct cycle sequencing reactions using the same primers as described above and the ABI Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems) following manufacturer's instructions. Automated DNA sequencing of both strands was conducted with an ABI PRISM 3130 XL DNA sequencer (Applied Biosystems).

Individual sequences were aligned with DNA Space v3.5 (Hitachi Software Engineering), and Arlequin v3.1 (Excoffier et al. 2005) was used for detection and enumeration of haplotypes. Haplotype sequences have been deposited in DDBJ GeneBank with the following accession numbers (Cyt*b*, AB290730-AB2907969; 12-16S rRNA, AB290770-AB290802). Phylogenetic analysis among haplotypes was carried out using MEGA 3.1 software (Kumar et al. 2004). Nucleotide divergences were computed using the Kimura 2-parameter model (Kimura 1980) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei 1987). Robustness of topology nodes was tested using the bootstrap method with 1000 replications (Felsenstein 1985).

Design of species-specific primers and multiplex APLP analysis. Three species-specific forward primers were designed from the haplotype sequences of the Cyt*b* and 12-16S regions such that at least 1 nucleotide at the 3′-end of the primer was unique to 1 target species in the alignment (Fig 2). Primers designed for both the Cyt*b* and 12-16S rRNA regions (Table 2) were positioned internal to the forward and reverse primer used for sequencing so as to generate unambiguously diagnostic-sized fragments for each

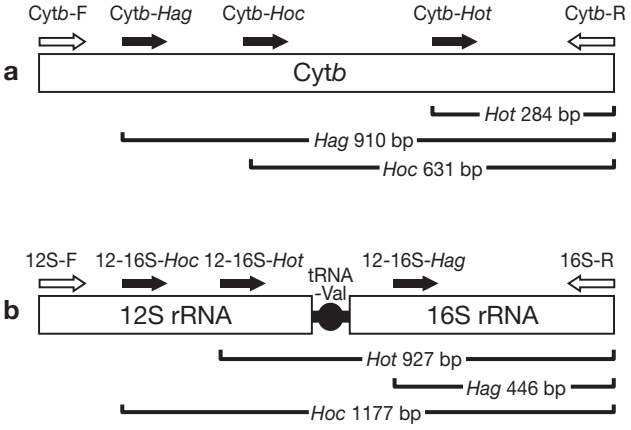

Fig. 2. *Hexagrammos* spp. Schematic representation of (a) Cyt*b* and (b) 12-16S rRNA regions, indicating relative annealing sites and orientation of the primers used for DNA sequencing (open arrows) and species specific primers (solid arrows). Right- and left-pointing arrows: forward and reverse primers, respectively. The expected fragment sizes for each species are indicated below. Species abbreviations are given in Table 2

Table 2. *Hexagrammos* spp. Multiplex primer sets, melting temperatures (T_m) and product size for cytochrome *b* (Cyt*b*) and the 12-16S rRNA amplified product length polymorphism. Sequences of 3 species-specific primers (forward primers) and the single reverse primer are shown. *Hot*, *Hag* and *Hoc* refer to *H. otakii, H. agrammus,* and *H. octogrammus*, respectively. Sequences of the non-target species are shown below the sequence of each species-specific primer (orthologue). Nucleotide differences between each species-specific primer and its ortholog are indicated in boldface type

Locus	Primer	Sequence (5′-3′)	T_m (°C)	Product size
Cyt*b*	Cyt*b*-*Hot* (ortholog)	TCCC**T**AACAA**G**CTAGG**G**GG**T**	63.5	284 bp
		TCCC**C**AACAA**A**CTAGG**A**GG**C**		
	Cyt*b*-*Hag* (ortholog)	CGGCTGACTTATCCGTAAT**C**	61.0	910 bp
		CGGCTGACTTATCCGTAAT**T**		
	Cyt*b*-*Hoc* (ortholog)	CTCAGTTGACAA**C**GCCACA**T**	63.8	631 bp
		CTCAGTTGACAA**T**GCCACA**C**		
	Cyt*b*-R (reverse)	TCCTAAGGCCTTGTTTTCTA	58.0	
12-16S rRNA	12-16S-*Hot* (ortholog)	ACACTGAGAAGTCACCCGT**T**	61.2	927 bp
		ACACTGAGAAGTCACCCGT**G**		
	12-16S-*Hag* (ortholog)	AG**C**CTGACCCCTATTGATG**T**	62.3	446 bp
		AG**T**CTGACCCCTATTGATG**C**		
	12-16S-*Hoc* (ortholog)	ACAGCGAA**T**ACGGA**G**GGTGT**T**	65.6	1177 bp
		ACAGCGAA**C**ACGGA**A**GGTG**C**		
	16S-R (reverse)	TCTTTTAGTCTTTCCCTGGGG	63.1	

species. For each amplicon, a single reverse primer (described in the previous section) was used concurrently with the 3 species-specific forward primers in a single tube for PCR amplification.

Each multiplex PCR was carried out with an ABI 9700 thermal cycler (Applied Biosystems) following the thermal cycle profile: denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min, followed by extension at 72°C for 7 min. Reactions were conducted by adding 1 µl of template DNA (20 to 40 ng μl^{-1}) to 24 µl of PCR mix buffer (pH 8.0) containing of 0.5 µl of each primer (50 µM), 2.5 µl of dNTP mix (2.5 mM each), 2.5 µl of PCR buffer, 0.13 µl of *Taq* polymerase (5 U μl^{-1}) and 16.87 µl of sterile distilled water. Ampli *Taq* Gold (Applied Biosystems) DNA polymerase was used to assure high annealing stringency of species-specific primers to complementary diagnostic sites. When PCR amplification was inconsistent, possibly because of poor storage conditions and potentially degraded DNA, TaKaRa Ex *Taq* HS (TaKaRa Bio) DNA polymerase was used instead of Ampli *Taq* Gold. PCR products were separated by electrophoresis on 1.5% agarose gels for 20 min at 100 V, then visualized under UV light following staining with ethidium bromide.

PCR-RFLP analysis. To compare reliability and efficiency of the APLP method with an existing PCR-RFLP method, the same individuals were analyzed again using the latter method. The enzymes *Dde* I and *Msp* I were used to restrict the 12-16S rRNA gene fragment following Yanagimoto (2003). DNA Space v3.5 (Hitachi Software Engineering) was used to select the restriction enzymes *Hin*f I and *Rsa* I to screen variation in the Cyt*b* gene fragment. Digestions were performed in 10 µl volumes containing 8 µl of PCR product, 4 U of restriction enzyme, and 1 µl of 10× buffer supplied by the manufacturer at 37°C for 5 h or longer. The reaction was stopped by heat inactivation by 15 min incubation at 65°C. Restriction fragments were separated by electrophoresis on 2.0% agarose gels for 40 min at 100 V and visualized following ethidium bromide staining.

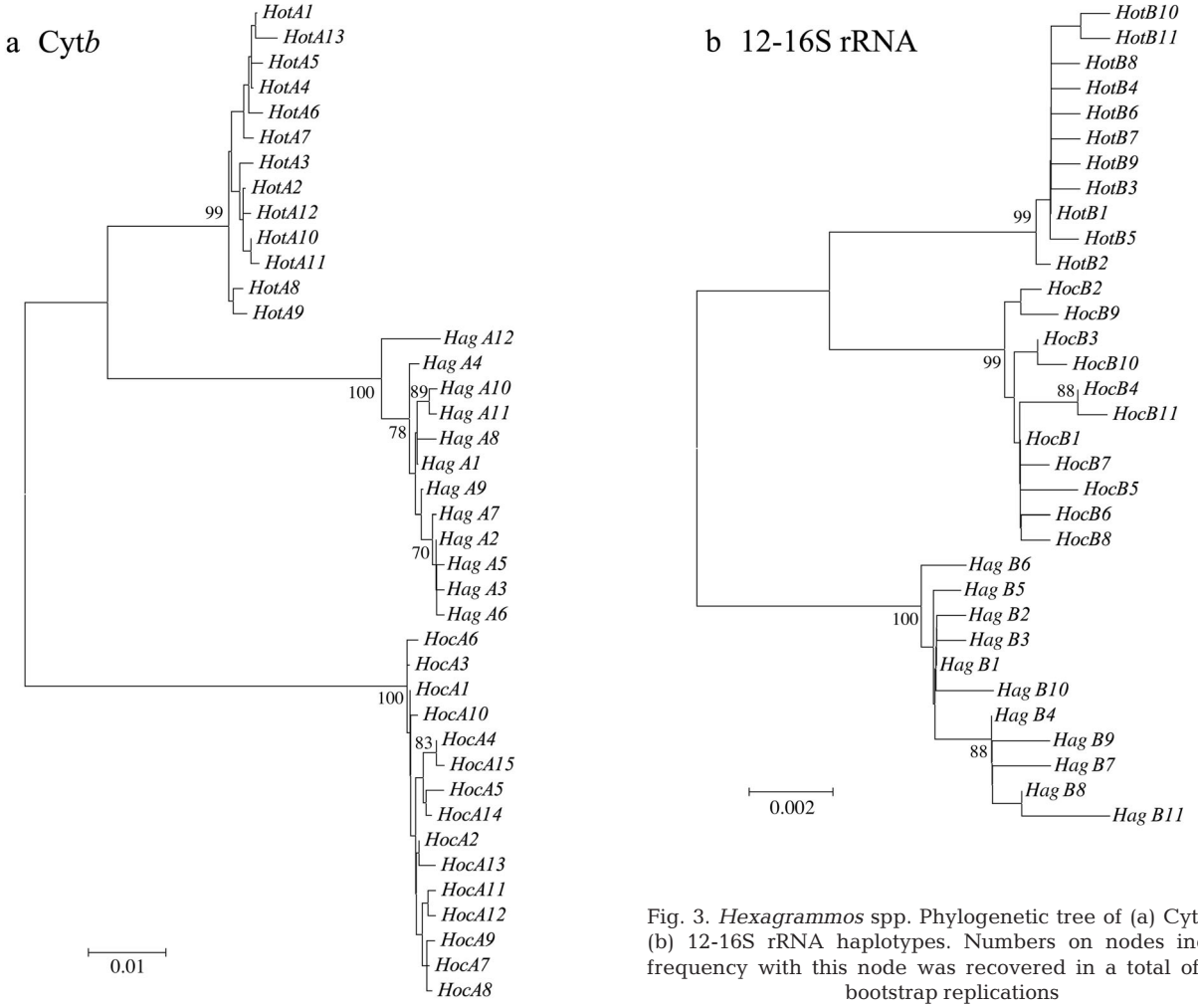

Fig. 3. *Hexagrammos* spp. Phylogenetic tree of (a) Cyt*b* and (b) 12-16S rRNA haplotypes. Numbers on nodes indicate frequency with this node was recovered in a total of 1000 bootstrap replications

RESULTS

Nucleotide sequences of 3 species

Nucleotide sequences were obtained for 986 base pairs (bp) of the Cyt*b* region and 1421 bp of the 12-16S rRNA region. A total of 40 and 33 haplotypes were detected in the Cyt*b* and 12-16S regions, respectively, and sequences of variable nucleotide sites of these haplotypes are shown in Appendix 1 (Cyt*b*) and Appendix 2 (12-16S rRNA), both available as Supplementary Material at www.int-res.com/articles/suppl/b001p187_app.pdf. Haplotypes from both regions fell into 3 distinct clusters in neighbor-joined phylogenetic tree (Fig. 3), corresponding to morphological identifications (Appendix 3, available as Supplementary Material at www.int-res.com/articles/suppl/b001p187_app.pdf). Cyt*b* haplotypes *Hot* A1-13, *Hag* A1-12 and *Hoc* A1-15 were considered to be species-specific for *Hexagrammos otakii*, *H. agrammus* and *H. octogrammus*, respectively. Haplotypes for the 12-16S rRNA region, *Hot* B1-11, *Hag* B1-11 and *Hoc* B1-11, were specific for *H. otakii*, *H. agrammus* and *H. octogrammus*, respectively.

APLP band patterns of the Cyt*b* and the 12-16S rRNA regions

PCR amplicons of the Cyt*b* fragment in the multiplex APLP assay were expected to be 284 bp (*Hexagrammos otakii*), 910 bp (*H. agrammus*) and 631 bp (*H. octogrammus*) in length and electrophoresis of these products confirmed diagnostic fragment patterns for the 3 species (Fig. 4a,b). In the 12-16S rRNA region, fragments of 927 bp (*H. otakii*), 446 bp (*H. agrammus*) and 1177 bp (*H. octogrammus*) in length (Fig. 4c,d) were produced by APLP. All the results from the APLP analysis corresponded with haplotype identifications and unexpected banding patterns were not observed in either the Cyt*b* or the 12-16S fragments. APLP analysis for 32 larval specimens produced a band pattern specific to each species (Fig. 4b,d). In several cases, band patterns from larvae differed from the observed species of territorial male guarding the egg mass (Fig. 4b,d): 2 and 5 larvae from egg masses guarded by *H. otakii* and *H. agrammus* males were identified as having *H. octogrammus* mothers, respectively; and one larva from an egg mass guarded by a *H. octogrammus* male was identifies as having a *H. agrammus* mother (Appendix 3).

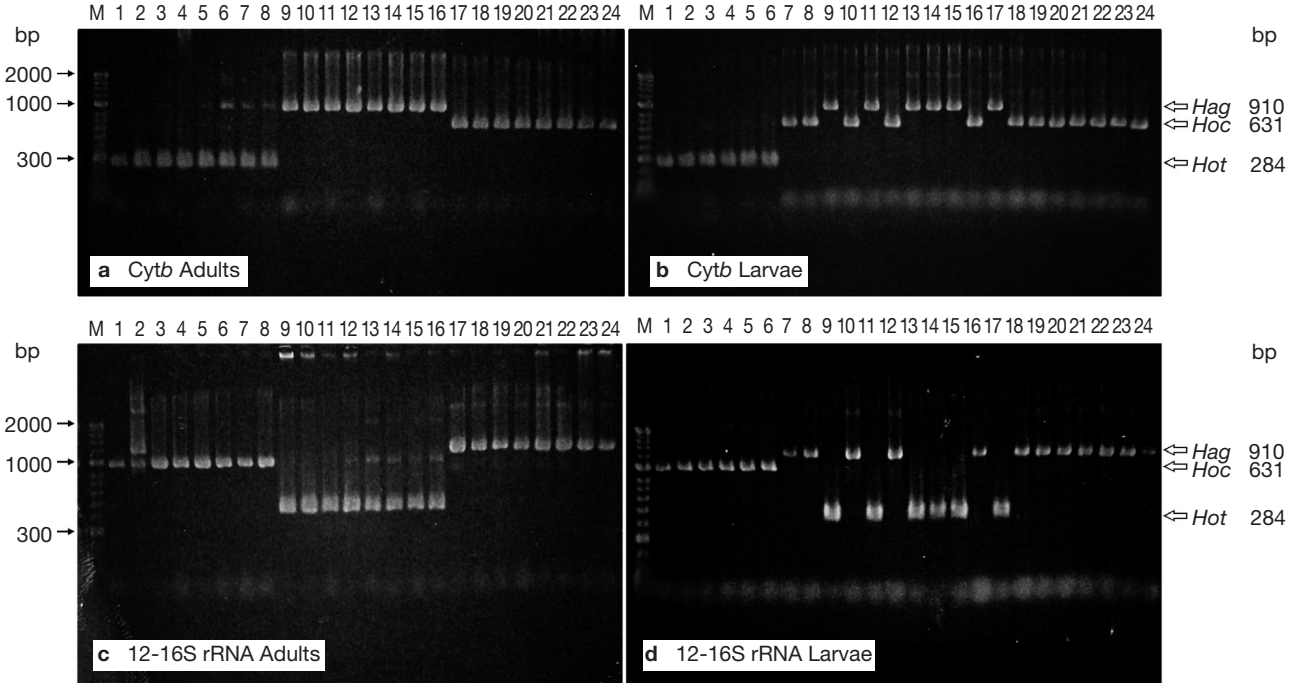

Fig. 4. *Hexagrammos* spp. Electrophoretic patterns of amplified product length polymorphism (APLP) from the (a,b) mitochondrial Cyt*b* and (c,d) 12-16S rRNA regions of 3 *Hexagrammos* species. Adult specimens: Lanes 1 to 4, *H. otakii* from Usujiri; Lanes 5 to 8, *H. otakii* from Mie; Lanes 9 to 12, *H. agrammus* from Usujiri; Lanes 13 to 16, *H. agrammus* from Mie; Lanes 17 to 20, *H. octogrammus* from Usujiri; and Lanes 21 to 24, *H. octogrammus* from Akkeshi. Larval specimens hatched from egg masses collected in the male territories of 3 *Hexagrammos* species: Lanes 1 to 8, egg masses in the territories of *H. otakii*; Lanes 9 to 16, those in *H. agrammus* territories; Lanes 17 to 24, those in *H. octogrammus* territories. Lane M contains molecular size standards. Open arrows labeled *Hot*, *Hag* and *Hoc* show the species-specific fragments of *H. otakii*, *H. agrammus* and *H. octogrammus*, respectively

Table 3. *Hexagrammos* spp. Size (bp) of restriction enzyme digestion fragments from the Cyt*b* and 12-16S rRNA regions for PCR-RFLP identification of 3 *Hexagrammos* spp. Species abbreviations are given in Table 2

Locus:	Cyt*b*						12-16S rRNA					
Enzyme:	*Hin*f I			*Rsa* I			*Dde* I			*Msp* I		
Species:	*Hot*	*Hag*	*Hoc*	*Hot*	*Hag*	*Hoc*	*Hot*	*Hag*	*Hoc*	*Hot*	*Hag*	*Hoc*
	480	489	1134	900	592	508	379	387	387	821	982	821
	303	480		234	308	392	345	379	379	439	439	439
	228	123			234	234	278	278	278	161		161
	123	30					152	152	152			
		12					93	93	93			
							88	88	88			
							42	27	27			
							27	17	17			
							17					

RFLP restriction profiles

Electrophoresis of PCR Cyt*b* products digested by *Hin*f I and *Rsa* I were expected to show diagnostic fragment patterns for the 3 species (Table 3). When the PCR Cyt*b* products were digested by *Hin*f I, 6 RFLP patterns were observed; Patterns A, D and F were the common patterns for *Hexagrammos otakii*, *H. agrammus* and *H. octogrammus*, respectively (Fig. 5a). Most RFLP results correctly reflected identification by phylogenetic analysis of haplotypes. However, several specimens produced exceptional band patterns; haplotypes of *Hot* A1, *Hot* A4, *Hot* A5, and *Hot* A13 (Appendix 1) gained a restriction site and exhibited Pattern B (Fig. 5a). Two haplotypes (*Hot* A10 and *Hot* A11, Appendix 1) lost a restriction site, exhibiting Pattern C, and haplotype *Hag* A7 gained a restriction site, resulting in Pattern E (Fig. 5a). PCR Cyt*b* products digested by *Rsa* I displayed 3 RFLP patterns; A, B and C were the common patterns to *H. otakii*, *H. agrammus* and *H. octogrammus*, respectively (Fig. 5b). Two haplotypes in *H. octogrammus* (*Hoc* A3 and *Hoc* A14, Appendix 1)

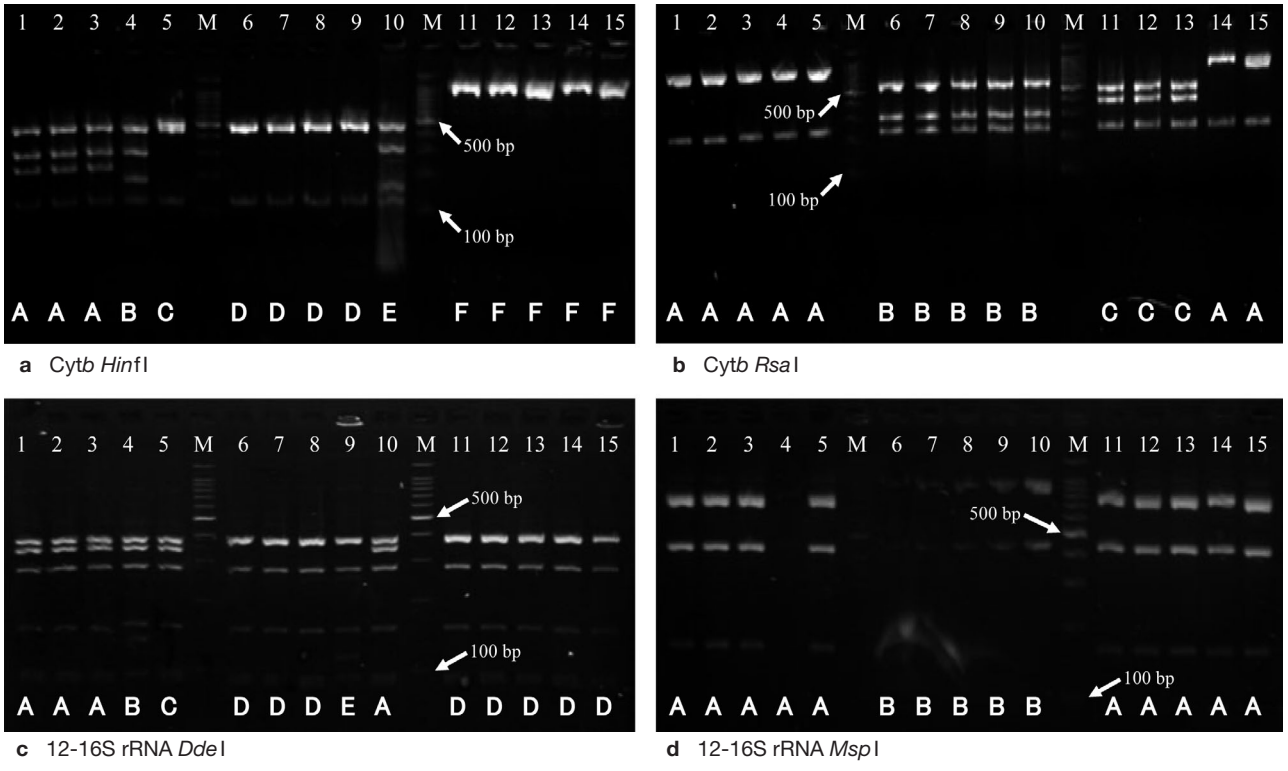

Fig. 5. *Hexagrammos* spp. Electrophoretic patterns of restriction enzyme fragment products in adult specimens from (a,b) Cyt*b* and (c,d) 12-16S rRNA regions digested with *Hin*f I (a), *Rsa* I (b), *Dde* I (c) and *Msp* I (d). Lanes 1 to 5, *H. otakii*; Lanes 6 to 10, *H. agrammus*; Lanes 11 to 15, *H. octogrammus*. Lane M contains molecular size standards. Alphabetic nomenclature at the bottom of each lane indicates each restriction pattern

lost a restriction site, resulting in Pattern A (Fig. 5b) and leading to misidentification of *H. octogrammus* as *H. otakii*.

RFLP profiles of the 12-16S rRNA PCR products restricted by *Dde* I and *Msp* I did not discriminate among the 3 species by use of either enzyme alone. Fragment patterns produced by *Dde* I digestion were identical for only *Hexagrammos otakii* but did not distinguish between *H. agrammus* and *H. octogrammus*; and those produced by *Msp* I digestion were the same for *H. otakii* and *H. octogrammus* (Table 3). Again, most RFLP results correctly reflected the phylogenetic placement of haplotypes, with several specimens producing atypical band patterns. Five RFLP patterns were observed when the 12-16S rRNA PCR products were digested by *Dde* I; Pattern A was the pattern common to *H. otakii* and Pattern D was shared by both *H. agrammus* and *H. octogrammus* (Fig. 5c). However, 2 haplotypes in *H. otakii* (*Hot* B10 and *Hot* B11, Appendix 1) lost 2 and 1 restriction sites, respectively, producing Patterns B and C (Fig. 5c). One *H. agrammus* haplotype (*Hag* B9) lost a restriction site, resulting in the unique Pattern E; and another (*Hag* B6) gained a restriction site resulting in the Pattern A common to *H. otakii*. When 12-16S PCR products were restricted by *Msp* I, 2 RFLP patterns were observed; Pattern A was shared by *H. otakii* and *H. octogrammus* and Pattern B was diagnostic for *H. agrammus* (Fig. 5d). All RFLP profiles from *Msp* I agreed with identifications based upon haplotype sequences.

DISCUSSION

The novel multiplex APLP method presented in this study achieved accurate identification of 3 *Hexagrammos* spp. using either the Cyt*b* or 12-16S rRNA regions. APLP analysis provided unequivocal maternal identification of larvae hatched from egg masses guarded by territorial males (the presumed sire) and, in some cases, strongly inferred interspecific hybridization within this genus.

Important considerations for developing genetic assays applicable to studying hybrid zones of marine species are accuracy, speed and cost. Based upon these criteria, APLP appears to have substantial advantages over more conventional approaches, such as DNA sequencing and PCR-RFLP methods. Direct sequencing and phylogenetic identification should be the most reliable and informative method but requires more time (2 rounds of PCR) and expense than methods employing only a single PCR reaction. Although PCR-RFLP analysis is faster and less expensive, it requires the cost of enzymes plus the time for restriction digests. In addition, some identification errors due to mutations at restriction sites were evident in this study based upon RFLP patterns for both the Cyt*b* and 12-16S rRNA regions. In particular, the Cyt*b* region contained 9 haplotypes for the 3 species which showed variant RFLP patterns. This region is moderately variable in these *Hexagrammos* spp. (151 substitutions in 145 polymorphic sites within 986 bp, Appendix 1), resulting in the loss or gain of restriction sites in some haplotypes and potential misidentification due to intraspecific variation. On the other hand, the 12-16S region evolves most slowly in mtDNA (Mindel & Honeycutt 1990) and the numbers of diagnostic sites in this region are limited (25 sites within 1421 bp, Appendix 2), exhibit less variation (62 substitutions in 60 polymorphic sites within 1421 bp, Appendix 2), and do not provide unequivocal diagnostic patterns for 3 *Hexagrammos* spp. based upon a single restriction enzyme.

The multiplex APLP analysis thus represents improvements upon accuracy and efficiency compared to PCR-RFLP. Properly designed species-specific primers used in the APLP method correctly assigned all specimens into the 3 *Hexagrammos* species inferring that APLP is robust to the effects of intraspecific variation. Moreover, the concurrent use of 4 primers (3 species-specific forward primers and a single reverse primer) in single reaction tube simplifies the procedure. Indeed, only one process of PCR was required to identify 3 *Hexagrammos* species using either the Cyt*b* or the 12-16S rRNA regions.

To achieve accurate and rapid identification with APLP analysis, the following 5 points about designing species-specific primers and PCR conditions for the multiplex assay should be considered. (1) Each species-specific primer should be designed at sites that are fixed in one species and that differ from the other species, reducing the potential for misidentification caused by intraspecific variation. (2) All primers should have similar melting temperatures to amplify each PCR product consistently. When the melting temperatures of primers differ substantially, primers which have low melting temperatures fail to anneal at higher annealing temperatures, and primers with higher melting temperatures lose primer specificity at low annealing temperature. Therefore primer length and GC content should be adjusted to make them comparable in melting temperature. (3) Melting temperatures of all primers and the annealing temperature for PCR should be set comparatively high to achieve optimal primer specificity to the target sequences and to avoid primer-to-primer annealing (Pank et al. 2001). In this study, the sequences of 2 primers, Cyt*b*-*Hag* and 12-16S-*Hot*, differed from each other by only one base over a 20 bp annealing site (Table 2). Despite such small differences in specificity, those primers strictly amplified each species-specific amplicon under high-stringency PCR

conditions (annealing at 60°C). (4) The 3′-end of each primer should not have complementary sequence with other primers in order to avoid primer-to-primer annealing, a condition of primer design used in this study (Table 2). (5) The annealing sites of species-specific primers should be located sufficiently far apart to amplify unambiguously diagnostic fragments between species in order to increase the speed of gel interpretation (Pank et al. 2001). In this study, diagnostic APLP fragments for the 3 *Hexagrammos* spp. were clearly discriminated by visual observation of the gel after 20 min of electrophoresis.

Overall, the multiplex APLP protocol represents an efficient, reliable, easy, rapid, and economical method for maternal identification of 3 *Hexagrammos* spp. and should accelerate studies of hybridization in other marine species.

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