DIFFERENTIATION OF TWO CLARIAS SPECIES (*Clarias macrocephalus* AND *C. gariepinus*) AND THEIR HYBRIDS BASED ON PCR-RFLP ANALYSIS

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Received date: 08.05.2015 Accepted date: 25.08.2015

ABSTRACT

Catfish hybrids (*Clarias macrocephalus x C. gariepinus*) have been popularly cultured in Viet Nam and have possibly escaped to the wild. Identification of hybrid individuals has become important in fishery resource management and aquaculture, but hybrid differentiation based on morphology is highly uncertain. This study employed PCR-RFLP method using a mitochondrial (cytochrome C oxidase subunit I, COI) marker and a nuclear (rhodopsin, *rho*) marker to differentiate hybrids from the parental species. Two genes were sequenced from 12 samples of two species (6 of each species) and 3 samples of the culutred hybrid. Sequences of the two species were aligned to find species-specific restriction enzymes. Restriction enzymes of SpeI and XcmI were selected to digest at species-specific sites of COI and *rho* genes, respectively. Results confirmed that C. macrocephalus is maternal lineage of the cultured hybrid. Sequence chromatogram and fragments after XcmI digestion of *rho* gene of the hybrid revealed intermediate patterns between two parental species. Therefore, PCR-RFLP analysis of COI and *rho* genes is an effective and accurate method for identification of catfish hybrid individuals.

Keywords: *Clarias*, hybrid, molecular marker, PCR-RFLP.

Phân biệt hai loài cá trê (*Clarias macrocephalus* và *C. gariepinus*) và con lai của chúng bằng phương pháp PCR-RFLP

TÓM TẮT

Cá trê lai (*Clarias macrocephalus* x *Clarias gariepinus*) được nuôi phổ biến ở Việt Nam và có thể thất thoát ra ngoài môi trường tự nhiên. Việc xác định đúng cá thể con lai trở nên quan trọng trong quản lý nguồn lợi cũng như trong nuôi trồng thủy sản. Phân biệt con lai dựa vào hình thái thường không chính xác. Nghiên cứu này sử dụng phương pháp PCR-RFLP đối với gien ti thể (Cytochrome C oxidase subunit I, COI) và gien trong nhân (Rhodopsin, *rho*) để phân biệt con lai với hai loài bố mẹ. Mười hai mẫu cá (6 mẫu cho mỗi loài) và 3 mẫu cá trê lai nuôi được giải trình tự 2 gien trên. Sau đó, trình tự gien của hai loài được sắp xếp thẳng hàng để tìm enzym cắt giới hạn đặc trưng cho loài. Hai enzym *Spe*I và *Xcm*I được chọn để cắt hai gien tương ứng, COI và *rho*. Kết quả khẳng định cá trê vàng *C. macrocephalus* là loài cá mẹ của con lai đang được nuôi hiện nay. Chromatogram và phân đoạn gien *rho* sau khi bị cắt bởi enzym *Xcm*I của con lai thể hiện đặc điểm trung gian của hai loài bố mẹ. Như vậy, phương pháp phân tích PCR-RFLP gien COI và *rho* là phương pháp hiệu quả và chính xác để xác định từng cá thể cá trê lai.

Từ khóa: *Clarias*, con lai, chỉ thị phân tử, PCR-RFLP.

1. INTRODUCTION

African catfish, *Clarias gariepinus* (*Cg*), was introduced to Viet Nam in the mid 1970s and also in some other Southeast Asia countries (FAO 1997). African catfish males have been hybridized with native walking catfish (*Clarias macrocephalus*, *Cm*) females to produce hybrids for aquaculture (Teugels et al., 1998). The *Cm* x *Cg* hybrids have been considered one of the

most successful inter-specific hybridization used in aquaculture. They have been cultured widely and yielded high production in Viet Nam and Thailand (Bartley et al., 2000). However, widespread farming of hybrids also raises concerns of genetic degradation of the native catfish gene pool if hybrids escape into the wild. In Thailand, Na-Nakorn et al. (2004) reported that typical alleles (based on allozyme) of the African catfish found in 12/25 wild populations and 1/1 hatchery population of walking catfish, indicating genetic introgression of African catfish into the native walking catfish. In efforts to conserve native catfish, it is important to identify hybrids at the individual level.

African catfish and walking catfish are morphologically different, especially in occipital process shape, color, body size, etc. External morphology of hybrids show intermediate characteristics between the two parental species (Teugels et al., 1998). In many cases, such as early life stages or post-F_1 hybridization, hybrids cannot be distinguished from their parents based on morphology. Another method of hybrid identification is karyological analysis. Karyotype of *Cg* is 2n = 56 (Teugels et al., 1992), and that of *Cm* is 2n = 52 (Sittikraiwong, 1987). *Cm* x *Cg* hybrid has an intermediate karyotype (2n = 54) from their two parent species (Visoottiviseth et al., 1997). Similarly, hybrid between *Clarias gariepinus* and *Heterobranchus longifilis* (2n = 52) also has karyotype of 2n = 54 (Teugels et al., 1992). Nowadays, karyological method has been used less often due to time and expertise requirements (Garte, 1993).

PCR-RFLP (Polymerase chain reaction-Restriction fragement length polymorphism) is one of the effective and simple DNA-based methods that are commonly used in inter-specific hybrid identification (do Prado et al., 2012; Hashimoto et al., 2010; Vaini et al., 2014). The principle of this method is based on single nucleotide polymorphims of mitochondrial and/or nuclear genes and the use of restriction enzymes that cut at species-specific sites. When mitochondrial DNA (mtDNA) is digested by restriction enzymes, a RFLP pattern of a hybrid is similar to that of maternal species due to maternal inheritance. On the other hand, RFLP of nuclear genes (nDNA) produce intermediate patterns of two parental species (Hashimoto et al., 2010). This method has been used successfully in identifiying hybrids, for example, of (female) *Leporinus macrocephalus* x (male) *Leporinus elongatus* (Hashimoto et al., 2010) or *Pseudoplatystoma corruscans* x *P. reticulatum* (Vaini et al., 2014).

The objectives of this study were to develop PCR-RFLP of mitochondrial and nuclear genes to distinguish two *Clarias* species and identify their hybrids, making effective contribution to fisheries management and culture of *Clarias* species.

2. MATERIALS AND METHODS

2.1. Fish sampling locations

Walking catfish of 58.4 - 103.5 g in weight were collected in conservation areas of Long An (Lang Sen Wetland Reserve), Dong Thap (Tam Nong), Ca Mau (U-Minh) and Kien Giang (U-Minh Thuong) provinces. Different populations of walking catifsh were sampled to ensure the coverage intra-species genetic variation. African catfish of 02 - 1,680 g and hybrids of 168 - 340 g were sampled in a hatchery located in Chau Thanh District, Hau Giang province. Hybrids were indentified based on the shape of the occipital process (Teugels et al., 1998) and on the hatchery manager's information.

2.2. DNA extraction, PCR and sequencing

DNA was extracted from fish fin clips using QIAGEN kit. Concentrations and quality of DNA were measured by Nanodrop (2000), ranging from 31 to 498 ng/µL with A260/A280 from 1.82 to 2.03 across samples. DNA extracts were then diluted into 20 ng/µL for PCR.

One mitochondrial gene (Cytochrome C Oxidase Subunit I, COI) and one nuclear gene (Rhodopsin, rho) were amplified using universal primers (Table 1). Final concentrations of 25 µL

Table 1. Primer sequences for PCR and sequencing of COI and *rho* genes

Gene	Primer	Sequence 5' - 3'	References
COI	Fish F2-t1	TGTAAAACGACGGCCAGTCGACTAATCATAAA GATATCGGCAC	(Ivanova et al., 2007; Ward et al., 2005)
	Fish R2-t1	CAGGAAACAGCTATGACACTTCAGGGTGACC GAAGAATCAGAA	
Sequencing	M13-F	TGTAAAACGACGGCCAGT	Ivanova et al., 2007
	M13-R	CAGGAAACAGCTATGAC	
Rho	RH193-F	CNTATGAATAYCCTCAGTACTACC	Chen et al., 2003
	RH1039-R	TGCTTGTTCATGCAGATGTAGA	
Sequencing	RH193-F and RH1039-R for 2 sequence directions		

Table 2. PCR cycles of COI and *rho* genes

PCR steps	COI		Rho		Number of cycles
	Temperature	Time	Temperature	Time	
1. Initial denaturation	95°C	2 min.	95°C	4 min.	1
2. Denaturation	94°C	30 sec.	94°C	40 sec.	
3. Annealing	52°C	40 sec.	55°C	40 sec.	35
4. Extension	72°C	1 min.	72°C	1 sec.	
5. Final extension	72°C	10 min.	72°C	7 min.	1

PCR for both genes include 1 X buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 5 pmoles of each forward and reverse primer (Table 1), 1.25 U Taq (Choice-Taq™ DNA Polymerase, Denville Scientific Inc.), and 100 ng DNA template. PCR cycles were based on previous studies (Chen et al., 2003; Ward et al., 2005) with the increase of annealing time (Table 2).

PCR products were purified using QIAGEN kit and then sent to the Genomic Center (Michigan State University, USA) for sequencing. Primers for 2-direction sequencing of COI were M13F and M13R (Ivanova et al., 2007), and those of *rho* were the forward and reverse primers of amplified reactions (RH193F and RH1039-R) (Chen et al., 2003). Twelve samples of *Cm* and *Cg* (6 samples for each species) and 3 samples of the cultured hybrid were sequenced for 2 genes.

2.3. Sequence alignment and selection of restriction enzymes

We aligned 12 sequences (6 sequences for each species) of COI gene from our study and 99 sequences (66 of *Cg* and 23 of *Cm*) from Genbank to find conserved sequences of each species. For rhodopsin gene, 12 sequences of *Cm* and *Cg* from this study were aligned. The program MEGA 6.0 (Tamura et al., 2013) was used for sequence alignment and estimation of nucleotide composition and Kimura 2- parameter genetic distance between two catfish species.

After alignment, 650 bp of COI gene and 795 bp of *rho* gene were used to search for restriction sites at conserved sequences by using Restriction mapper available at http://www.restrictionmapper.org/. Enzymes were selected based on following criteria: (i) species-specific cutting sites (work in only one species), (ii) cutting sites at positions not less than 100 bp from the two ends so that fragments can be visualized easily by agarose gel electrophoresis, and (iii) and only a single cutting site.

2.4. Digestion PCR products and checking RFLP

PCR products were digested by restriction enzymes, *Spe*I for cutting COI sequence and

*Xcm*I for *rho* (New England Biolabs, 10 U/µL) in 12 µL total volume containing 1X enzyme buffer, 5 U enzyme, and 4 µL PCR products. Enzyme reactions were incubated at 37°C for 150 minutes. Restriction fragments were checked by agarose gel 1.4%. Fragment sizes were estimated based on 100 bp DNA ladder (Invitrogen™). Fifteen COI and 30 *rho* products (5 COI and 10 *rho* for each fish group) were used for enzyme digestion tests.

3. RESULTS

3.1. Comparison of COI and *rho* sequences between *Cm* and *Cg*

COI sequences of *Cm* and *Cg* samples in this study were aligned 99% with sequences of the same species reported in Genbank. Nucleotide compositions and GC% (42.5%, 32.2% and 55.5% of 1st, 2nd and 3rd codon bases, respectively) were similar between two species (Table 3). There were 83 single nucleotide polymorphisms of COI sequences, resulting in Kimura 2-parameter genetic distance between the two species of 0.16 ± 0.019. High variation in COI sequences between two species helps easily identify species-specific restriction sites for restriction enzymes.

Rhodopsin gene between species had similar nucleotide compositions (Table 3). GC content in *rho* gene was higher in 2nd (64.6 ± 0.20%) compared to those of the 1st and 3rd codon bases (40.7 ± 0.24% and 46.9 ± 0.44%, respectively), which is different from that of COI gene. No within genetic distance among samples of each species was found, indicating that COI sequence is highly conserved within each species. Compared to COI gene, *rho* gene was more similar between 2 species. They differed in 22 variable sites with genetic distance (Kimura 2-parameter) of 0.027 ± 0.0006. Based on variable sequences, restriction enzymes specific for each species were found.

COI and *rho* sequences of hybrid were similar to *Cm*. However, chromatograms of *rho* sequences of hybrid showed two peaks at every nucleotide that is different between *Cg* and *Cm*. In Fig. 1, for example, *Cg* and *Cm* differ in 2 nucleotides at sites 393 and 399 bp (G and C in *Cg*, A and A in *Cm*). Hybrid samples had two peaks of A and G at site 393, and A and C at site 399. At these sites, maternal peaks were higher than paternal peaks, resulting in sequences reading as the same as *Cm*. Restriction enzymes cutting at these sites were predicted to produce fragment patterns that are intermediate between 2 parental species.

3.2. RFLP of two genes of *Clarias* species

Based on criteria for choosing restriction enzymes, three enzymes were found to cut COI conserved sequences of *Cm* and two enzymes worked only on *Cg*. Enzyme *Spe*I was selected for cutting COI sequence of *Cg* at the recognition size 5'-A CTAGT-3', resulting in two fragments of approximately 250 and 550 bp (sizes of PCR products approx. 800 bp). Digestion with *Spe*I enzyme showed that *Cm*

Table 3. Nucleotide compositions and within-group genetic distances based on COI and *rho* genes of two *Clarias* species

Species	T	C	A	G	Within group difference*
COI					
C. gariepinus	29.0 ± 0.17	25.9 ± 0.11	27.7 ± 0.15	17.4 ± 0.12	0.010 ± 0.002
C. macrocephalus	29.2 ± 0.20	26.1 ± 0.17	27.4 ± 0.09	17.3 ± 0.15	0.007 ± 0.002
Rho					
C. gariepinus	29.0 ± 0.0	27.3 ± 0.0	20.2 ± 0.0	23.5 ± 0.0	0
C. macrocephalus	28.7 ± 0.0	27.5 ± 0.0	20.6 ± 0.0	23.2 ± 0.0	0

*Note: * Within-group difference based on Kimura 2-parameter method*

and the hybrid had only one band of approx. 800 bp (the same size of undigested COI), meanwhile *Cg* had 2 bands as predicted sizes of 250 and 550 bp (Fig. 2). The result confirmed that the hybrids inherited maternally from *Cm*.

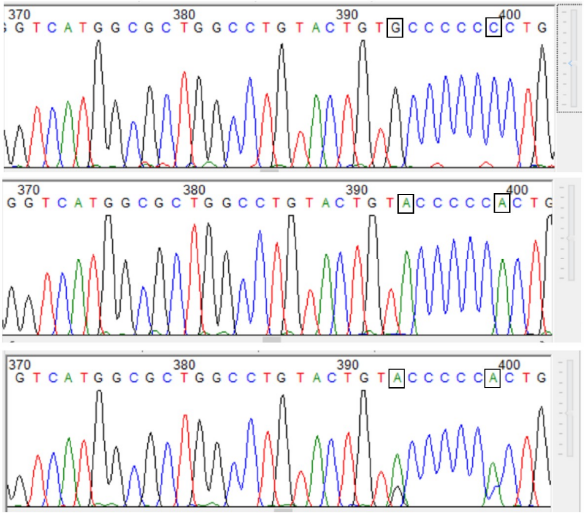

Fig. 1. Chromatograms of a short rhodopsin segment that is polymorphic (at positions 393 and 399 as labeled) between two *Clarias* species

Note: top: C. gariepinus; middle: C. macrocephalus; bottom: Cm x Cg hybrid.

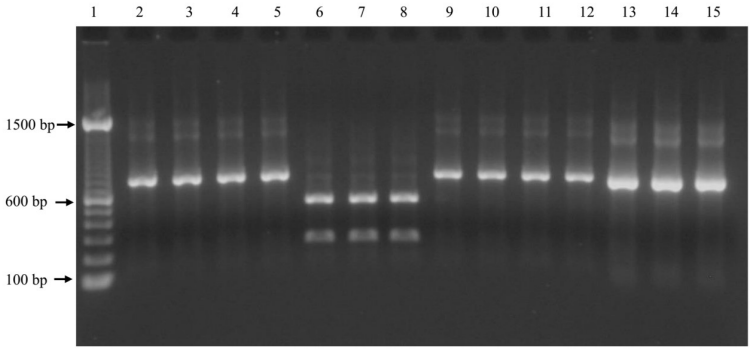

Fig. 2. PCR - RFLP patterns of COI gene digested with *Spe*I.

Note: Lanes 1: 100 bp ladder; 2 - 5: C. microcephalus (Cm); 6 - 8: C. gariepinus (Cg); 9 - 12: hybrids; 13 - 15 undigested COI of Cm, Cg, and the hybrid, respectively.

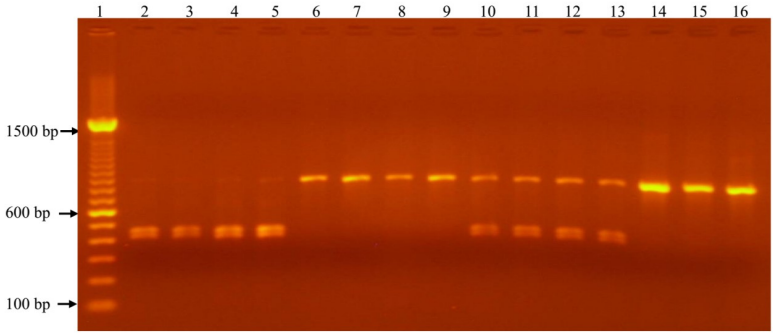

Fig. 3. PCR - RFLP patterns of rhodopsin gene digested with *Xcm*I.

Note: Lanes 1: 100 bp ladder; 2 - 5: C. microcephalus (Cm); 6 - 9: C. gariepinus (Cg); 10 - 13: hybrids; 14 - 16 undigested rhodopsin of Cm, Cg, and the hybrid, respectively.

Similar procedure was applied on nuclear *rho* gene. Among four restriction enzymes found (three for *Cg* and one for *Cm*), *Xcm*I was used. *Xcm*I cut the *Cm rho* at the recognition sequence 5'-CCACTGG T CGGCTGG-3', producing 2 fragments with similar sizes, 420 and 430 - 450 bp. Variation in sizes of these two bands depended on the effectiveness of amplification at the two ends of the *rho* gene. Rhodopsin products digested by *Xcm*I showed different bands between *Cm* and *Cg*, in which one band was of approx. 850 bp in *Cg*, 2 bands with similar size of 420 - 450 bp in *Cm*. The hybrids had 3 bands, one of 850 bp and two bands of 420 - 450 bp, representing for both parental species (Fig. 3).

4. DISCUSSION

The important contribution from this study is the finding of PCR - RFLP markers to distinguish the *Clarias* catfish hybrid from their two parent species, which solves uncertainty in morphological classification. Restriction enzymes selected based on sequence alignment between two parental species digested genes at species - specific sites and produced predictable DNA fragments. In addition, the use of both mitochondrial and nuclear genes could set two reciprocal hybrids apart and differentiate them from their parental species (Hashimoto et al., 2010). The same fragments of COI gene (mtDNA) digested by *Spe*I restriction enzyme between *Cm* and the hybrid confirm that *Cm* is of maternal lineage of the cultured hybrid. If *Cg* x *Cm* hybrids were tested, their COI - RFLP patterns would be the same as *Cg*. However, COI - RFLP cannot differentiate a hybrid from its maternal species due to maternal inheritance of mtDNA. On the other hand, RFLP of nuclear genes (such as rhodopsin in this study) of both reciprocal hybrids was intermediate between two parental species. Therefore, using nuclear markers can distinguish hybrids from their parental lineages. The intermediate pattern of rhodopsin - RFLP of the hybrid was confirmed by polymorphisms at segregating sites in hybrid sequences (Fig. 1).

Nuclear genes are powerful markers for hybrid identification. Nuclear genes such as rhodopsin, recombination activating genes RAG1 and RAG2, tropomyosin, etc., have been commonly used in previous studies (Chen et al., 2008; Kochzius et al., 2010; Larmuseau et al., 2010; López et al., 2004). Different genes have different substitution rates (Zhang et al., 2002) which also vary among taxa (Shen et al., 2013).

Among nuclear genes, rhodopsin is one of the most diversified markers. Chen et al (2008) found that rhodopsin in 2 species representive for 2 genera (*Leuciscinae* and *Rasborinae*) of the family Cyprinidae showed the highest substitution rates (resulting in genetic distance between two species of 0.160) compared to other nuclear genes (0.081 - 0.139). In the sand goby family Gobiidae, rhodopsin was also found of high variation (genetic distance 0.11) among 4 genera (Larmuseau et al., 2010). In our study, genetic distance of rhodopsin between two species within the genus *Clarias* is 0.027, comparable to other within - genera such as *Danio*, *Puntius*, and *Devario* belonging to the family Cyprinidae, ranging from 0.002 to 0.041 (Collins et al., 2012). Variation in rhodopsin sequences between *Cm* and *Cg* and highly conserved segregating sites within species indicated that this gene is reliable for catfish hybrid identification.

COI sequence has higher variation between two investigated species compared to the *rho* marker. Although it is not used to distinguish maternal *Cm* and the hybrid, it can be an important marker to identify *Cg* x *Cm* hybrid. In practice, *Cg* x *Cm* hybrid has not been used for aquaculture in Viet Nam or Thailand (Bartley et al., 2000) but India (Bernardo, 1996). The *Cm* x *Cg* hybrids are more preferred because of their fast growth, high survival rates, body color favored by consumers, etc. Similar to *Clarias* hybrids commercially farmed in Viet Nam, *Leporinus macrocephalus* x *Leporinus elongatus* hybrids are commonly cultured in Brazil (Hashimoto et al., 2014, 2010; Porto - Foresti et al., 2013). Hashimoto et al. (2010) used PCR - RLFP of cytochrome b (mtDNA) and tropomyosin (nDNA) to identify hybrids.

Accurate identification of catfish hybrid individuals is important in both aquaculture and fisheries management. In aquaculture, misuse of impure walking catfish can result in slow growth and reduced disease resistance of hybrids (Senanan et al., 2004). In fisheries, management of aquatic genetic recourses has been challenging. Nowadays, aquatic species have been introduced intentionally or unintentionally around the world (FAO, 1997; Gozlan et al., 2010), which causes both positive and negative effects. On one side, introduced species can increase aquaculture production for human consumption. On the other side, they can hybridize with native species and produce fertile hybrids, resulting in degradation of native species' gene pools (Cucherousset and Olden, 2011; Leprieur et al., 2009; Na-Nakorn et al., 2004). Escapes of catfish hybrids into the wild can be obvious in Viet Nam, especially in the Mekong Delta with the fact given that hybrid farming has expanded and flooding occurs annually in the rainy season. Therefore, applications of DNA markers such as PCR - RFLP of mitochondrial COI (maternal lineage) and nuclear rhodopsin genes (parental lineages) to identify hybrids are valuable tools in both aquaculture and fishery resource management of *Clarias* and also for other species.

5. ACKNOWLEDGEMENT

The author thanks Prof. Uthairat Na - Nakorn (Kasetsart University, Thailand), Prof. Kim Scribner and Jeannette Kanefsky (Michigan State University, USA) for valuable advice. This research was funded by the Viet Nam National Foundation for Science and Technology Development (NAFOSTED) under grant number: 106-NN.05-2014.86.

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