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Genomic identification of catfish species by polymerase chain reaction and restriction enzyme analysis of the gene encoding the immunoglobulin M heavy chain constant region

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Abstract

Nuclear DNA was isolated from the blood cells of catfish representing three families (clariidae, pangasiidae and ictaluridae) for analysis by polymerase chain reaction (PCR) and restriction enzymes. Primers specific for the CH_4 exon of the gene encoding the immunoglobulin M heavy chain of channel catfish (*Ictalurus punctatus*) were used. Nuclear DNA amplified with these primers yielded a single band of about 300 base pairs (bp) for *Clarias macrocephalus*, *Pangasius gigas*, *Pangasius hypophthalmus* and the hybrid of *P. gigas* × *P. hypophthalmus*. However, the same primers yielded two DNA bands of about 300 and 340 bp in *Clarias gariepinus* and in the hybrid of *C. macrocephalus* × *C. gariepinus*. Nucleotide sequences of the amplified DNA were determined for *I. punctatus*, *C. macrocephalus*, *P. gigas* and *P. hypophthalmus*. Based on the DNA sequence data, the restriction enzyme *Hpa*I was used to further characterize the PCR products of *P. gigas*, *P. hypophthalmus* and their hybrid. Digestion with this restriction enzyme yielded one DNA band (300 bp) for *P. gigas*, two bands (100 and 200 bp) for *P. hypophthalmus* and three bands (100, 200 and 300 bp) for the hybrid. These findings would aid in identifying

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genetic contributions in hybrid, androgenetic, gynogenetic and polyploid catfish. © 1997 Elsevier Science B.V.

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1. Introduction

The Mekong giant catfish (or 'Buk' in Thai) (*Pangasius gigas*, formerly *Pangasianodon gigas* Chevey) as well as other catfish of the family pangasiidae (e.g., *Pangasius bocourti* and *Pangasius krempfi*) of Mekong origin are important in capture fisheries in Laos, Cambodia and Thailand. The scarcity of *P. gigas* became apparent by a reduced number of fish captured in successive years. For example, only seven migrating spawners were caught in Chiengkong, Thailand in 1996, half of the number caught the previous year and only 10% of the largest catch in 1989 (Department of Fisheries, Thailand, unpublished data). Thus, several organizations have attempted to protect this species from extinction. Sperm cryopreservation was developed, which made sperm available for in vitro fertilization (Mongkonpunya et al., 1992, 1995). The fertilization ability of postthaw sperm was satisfactory for research purposes, but cryopreservation has not been scaled-up for use in hatchery production. Because *P. gigas* eggs are scarce, fertilizing capability of cryopreserved sperm was tested with the eggs of other catfish, e.g., *Pangasius hypophthalmus*, *Clarias gariepinus* and *Clarias macrocephalus*. It was found that fertilization rates of all crosses were comparable to those of control crosses (fresh eggs × fresh sperm of each species). The *P. gigas* × *P. hypophthalmus* cross resulted in normal embryo development and normal hybrid fry, and the growth rate of the hybrid (*P. gigas* × *P. hypophthalmus*) is better than that of *P. hypophthalmus* itself (Mongkonpunya et al., 1996; T. Jerdnapapun, Pataraphun Farm, personal communication 1993–1994). The hybrid is currently used for aquaculture in Nakorn Sawan, Thailand. However, the other crosses of *P. gigas* resulted in abnormal fry that died within 7 days after hatching (Mongkonpunya et al., 1995).

Androgenesis is a technique of producing offspring with all-paternal inheritance of nuclear DNA (Bongers et al., 1994). Androgenetic offspring of common carp (*Cyprinus carpio*) yielded 7% to 18% hatching and 78% to 89% survival for at least 24 days. The method involved fertilization of ultraviolet (UV)-irradiated eggs (150–300 mJ cm^{-2}) with fresh sperm, followed by a 40°C shock for 2 min at 26–30 min after fertilization (Bongers et al., 1994). In order to obtain homozygous offspring of *P. gigas*, androgenesis has been developed in our laboratory by using eggs from other commonly cultured species (e.g., *C. gariepinus* and *P. hypophthalmus*). However, individuals produced by interspecific androgenesis may differ from *P. gigas* due to mitochondrial inheritance from the eggs, if mitochondrial DNA was not inactivated. Despite this, interspecific androgenesis is a more direct way to revive extremely endangered or extinct species than by upgrading through hybridization. Subsequently, the fry resulting from this technique have to be characterized to assure their breeding identity is truly androgenetic before further use. One of the most reliable methods for genetic identification is the use

of specific DNA markers. The polymerase chain reaction (PCR) is a rapid and sensitive procedure for in vitro amplification of specific DNA sequences using appropriate primers.

In this study, DNA was isolated from catfish blood and analyzed by PCR using primers designed to target the CH_4 exon of the channel catfish (*Ictalurus punctatus*) gene encoding the constant region of the immunoglobulin M heavy chain (Zhang et al., 1994). The potential for observable differences in catfish of the families pangasiidae, clariidae and ictaluridae in terms of polymorphic DNA patterns related to the immunoglobulin heavy chain were evaluated. Further analysis of the amplified genomic DNA involved direct DNA sequencing, which led us to the use of restriction enzyme analysis for further characterization of these catfish.

2. Materials and methods

2.1. Blood collection

Parental species and hybrids of the following fish were raised at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand: *P. gigas, P. hypophthalmus*, *P. gigas* × *P. hypophthalmus*, *C. macrocephalus*, *C. gariepinus*, *C. macrochepalus* × *C. gariepinus* and *I. punctatus*. *I. punctatus* from a research population at Louisiana State University also provided samples for the DNA sequence analysis.

Blood samples (0.2 ml) were collected with sterile syringes from the caudal vessels, immediately plunged into liquid nitrogen (to prevent clotting and to aid in cell lysis) and stored for further analysis. All glassware, pipette tips, centrifuge tubes, glass pipettes and solutions were autoclaved to avoid DNA recontamination. Molecular biology-grade reagents were used.

2.2. DNA isolation

Thawed samples (100 μl) of whole blood were placed into 2.5-ml microcentrifuge tubes, to which 200 μl of DNAZOL reagent (Gibco-BRL, Gaithersburg, MD) was added, and the mixture was vortexed for 1–2 min to lyse cells. 500 μl of 95% ethanol was added to the samples, gently mixed for 5 min and centrifuged at 7000 g for 10 min to precipitate the genomic DNA. This step was repeated two times to remove debris. DNA was transferred to 1.5-ml microcentrifuge tubes and air-dried for 3–5 min. 200 μl of sterilized water was added, mixed well and heated at 45°C in a water bath for 10 h to solubilize the DNA. Samples were centrifuged at 7000 g for 10 min and the supernatant was collected. Purity and concentration of DNA was measured with a spectrophotometer (Beckman DU series 60). Purity was estimated by calculating the ratio of the absorbance measured at 260 nm (A260; maximum absorbance of DNA) and the absorbance at 280 nm (A280; maximum absorbance of protein).

2.3. Polymerase chain reaction

PCR primers were developed (Zhang et al., 1994) to target the CH_4 exon of the gene encoding the constant region of the *I. punctatus* immunoglobulin M heavy chain, based on the published sequence of the gene (Wilson et al., 1990). These primers were synthesized by the LSU gene probes and Expression Systems Laboratory at Baton Rouge, LA. The primer sequences were (5′ to 3′): TCCCCAAGGTT-TACTTGCTCGCTCC and CGATGGATCTGGATATGTGGCGCAC. From these primers, a DNA fragment of 303 base pairs (bp) was expected to be amplified from channel catfish DNA. PCR was performed in reaction mixtures containing 1 μg (2–3 μl, depending on the concentration) of target DNA, 0.5 μl (15–19μM) of each primer, 5 μl of 10 × PCR buffer, 1 μl of deoxyribonucleotide triphosphates mixture (10.0mM each of dATP, dTTP, dCTP and dGTP) and sufficient sterilized water to make a final reaction volume of 50 μl. Each reaction mixture was covered with 50 μl mineral oil. The PCR tubes were placed into the thermal cycler and denatured at 95°C for 2 min. Then 1 unit of Taq DNA polymerase (Gibco) was added under the layer of mineral oil and 30 cycles of PCR were performed using the conditions of: denaturation at 95°C for 30 s; annealing at 59°C for 30 s; elongation at 72°C for 30 s. The PCR products were stored at 4°C until analysis by electrophoresis. Each sample (12 μl) was loaded in a 2% agarose gel (Amresco, Solon, OH) in TBE buffer (0.089M tris, 0.089M borate and 0.002M EDTA) and electrophoresed at 5 V cm^{-1} for 1.5 h. DNA was stained with 0.5 μg ml^{-1} ethidium bromide, and the banding of DNA fragments was visualized and photographed on an ultraviolet light transilluminator (Photodyne UV 26, Hartland, WI). The fragment sizes (bp) were estimated graphically by the use of a standard curve derived from data on the mobility of the DNA markers (123-bp DNA ladder) (Gibco).

2.4. DNA sequencing

All samples were sequenced using a Perkin-Elmer Applied Biosystems 310 genetic analyzer and the protocol of the ABITM dye terminator cycle-sequencing ready-reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer, Foster City, CA). The templates used for sequencing reactions were the PCR products obtained from amplification with the CH_4 primers as detailed in Section 2.3. Template DNA was purified using the Qiagen PCR Purification Kit (Qiagen, Chatsworth, CA) and was resuspended in 50 μl of 10 mM tris HCl. For sequencing, 0.5 μg of template DNA was amplified under the following conditions: 96°C for 1 min (initial denaturation); followed by 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Samples were maintained at 4°C until sequencing. Each sample was assayed three times to yield a consensus sequence for computer analysis. DNA sequence data were aligned with the published channel catfish sequence for the CH_4 exon (Wilson et al., 1990) and with sequences obtained from channel catfish maintained at Kasetsart University and Louisiana State University using the Sequence Navigator software (Perkin-Elmer).

2.5. Restriction enzyme analysis

The DNA used for restriction enzyme analysis was the PCR products amplified as described in Section 2.3. Each reaction contained 1 μl (5 units) of *Hpa*I (New England

Biolabs, Beverly, MA), 0.5 μg DNA, 5 μl buffer and sufficient distilled water for a final volume of 50 μl. Digests were performed at 37°C for 90 min and samples were stored at 4°C until electrophoresis. Digested DNA was electrophoresed for 2.5 h through 4% agarose gels in TBE buffer at 6 V cm^{-1} as described in Section 2.3. The gels were stained with ethidium bromide, illuminated with UV light and photographed. Fragment sizes were estimated in relation to 100-bp ladder DNA marker (New England Biolabs).

3. Results and discussion

3.1. Phenotype of parental species and the hybrids

The mature parental species used in this experiment are easier to identify morphologically than are their fry which are almost identical. The *P. gigas* and the hybrid of *P. gigas* × *P. hypophthalmus* have truncated snouts, lunated caudal fins and their eye positions are below the jaw level. *P. hypophthalmus*, however, has rounded snout and forked caudal fin and the eye position is above the jaw level (Mongkonpunya et al., 1996; Roberts and Vidthayano, 1991). As for clariids, the shape of the occipital condyle is distinct in *C. gariepinus* and the hybrid of *C. gariepinus* × *C. macrocephalus*. The occipital bone of *C. macrocephalus* is round or without prominent condyle.

3.2. DNA purity and concentration

DNA from blood samples of different catfish species isolated by this one-step method were found to be acceptable for PCR analysis. The ratios of the absorbance measured at 260 nm and 280 nm were in the range of 1.8 to 2.1 which indicated relative purity and which were comparable to those typically isolated by using phenol–chloroform extraction (Sambrook et al., 1989; Ausubel et al., 1995). Concentrations were found to be in a useful range for PCR (~ 0.5 μg μl^{-1}).

3.3. PCR and genome identification

Amplification of specific DNA sequences by the use of CH_4 primers allowed us to distinguish between the two species within the family clariidae but not the two species within pangasiidae. A single DNA fragment of about 300 bp (Fig. 1) was found in *C. macrocephalus*, *P. gigas*, *P. hypophthalmus* and the hybrid of *P. gigas* × *P. hypophthalmus*, similar in size to the fragment amplified from DNA of channel catfish (Zhang et al., 1994). However, two fragments of DNA ($\sim$ 300 and 340 bp) were detected in *C. gariepinus* and the hybrid of *C. macrocephalus* × *C. gariepinus*. Because DNA of *C. macrocephalus* yielded only a single band while the hybrid with *C. gariepinus* yielded a doublet, this enabled us to identify the androgenetic offspring produced from *C. macrocephalus* or *P. gigas* sperm with *C. gariepinus* eggs (the putative androgenetic offspring would yield only the paternal single band pattern of genomic DNA). The presence of double bands would indicate contribution of maternal genetic materials from the eggs, and hence, failure of the induced androgenesis process (data not shown).

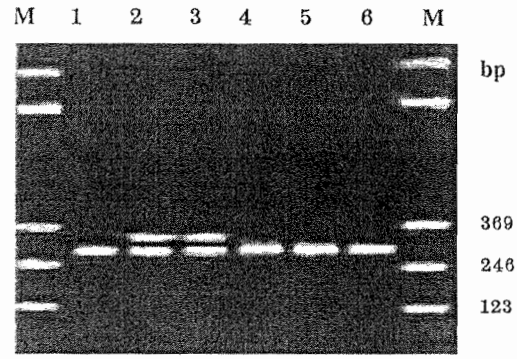

Fig. 1. Amplification of genomic DNA from catfish blood by PCR. Primer sequences targeted a 300-bp fragment of the CH_4 exon of the gene encoding the immunoglobulin M heavy chain of *I. punctatus*. Lane 1, *C. macrocephalus*; lane 2, *C. gariepinus*; lane 3, hybrid (*C. marcrocephalus* × *C. gariepinus*); lane 4, *P. gigas*; lane 5, *P. hypophthalmus* and lane 6, hybrid (*P. gigas* × *P. hypophthalmus*). Marker (M) was 123-bp DNA ladder.

However, identification of putative androgenetic offspring from crosses among pangasiid species could not be performed by using CH_4 primers alone, because the PCR products of paternal, maternal and hybrid DNA showed the same single band. Therefore, in the absence of restriction enzyme analysis (described in Section 3.5), the eggs of *C. gariepinus* should be used in attempting to produce androgenetic *P. gigas* because, if successful, the offspring would only show the paternal meristic characters, for examples, colorless, truncated snout and lunated caudal fin which are easy to identify and could be confirmed by the presence of the single paternal band pattern of genomic DNA.

3.4. DNA sequencing

Observation of DNA fragments amplified by PCR (Fig. 1) did not allow us to distinguish species within the family pangasiidae. It was necessary, therefore, to determine the nucleotide sequence of the amplified DNA products to aid in selecting an appropriate restriction enzyme for the identification of catfish. The complete sequence of the immunoglobulin M heavy chain constant region gene of the channel catfish has been reported (Wilson et al., 1990). The gene contains four constant regions, domain-encoding exons (CH_1 to CH_4) and two transmembrane exons (TM1 and TM2). We found complete agreement among the published DNA sequence for CH_4 and the DNA amplified from channel catfish maintained in Thailand and the United States. The DNA sequence of the CH_4 region of channel catfish and the three other catfish species were compared (exclusive of primer sequences), and found to possess an overall agreement of 78.2% (Fig. 2). Moreover, within the two species of pangasiidae, 96.4% of the nucleotide sequences were identical, confirming their closely related genomic identities.

3.5. Restriction enzyme analysis

An attempt to differentiate the genomic DNA of the two pangasiid species was made through the use of restriction enzyme analysis. The complete sequences of CH_4 for *P.*

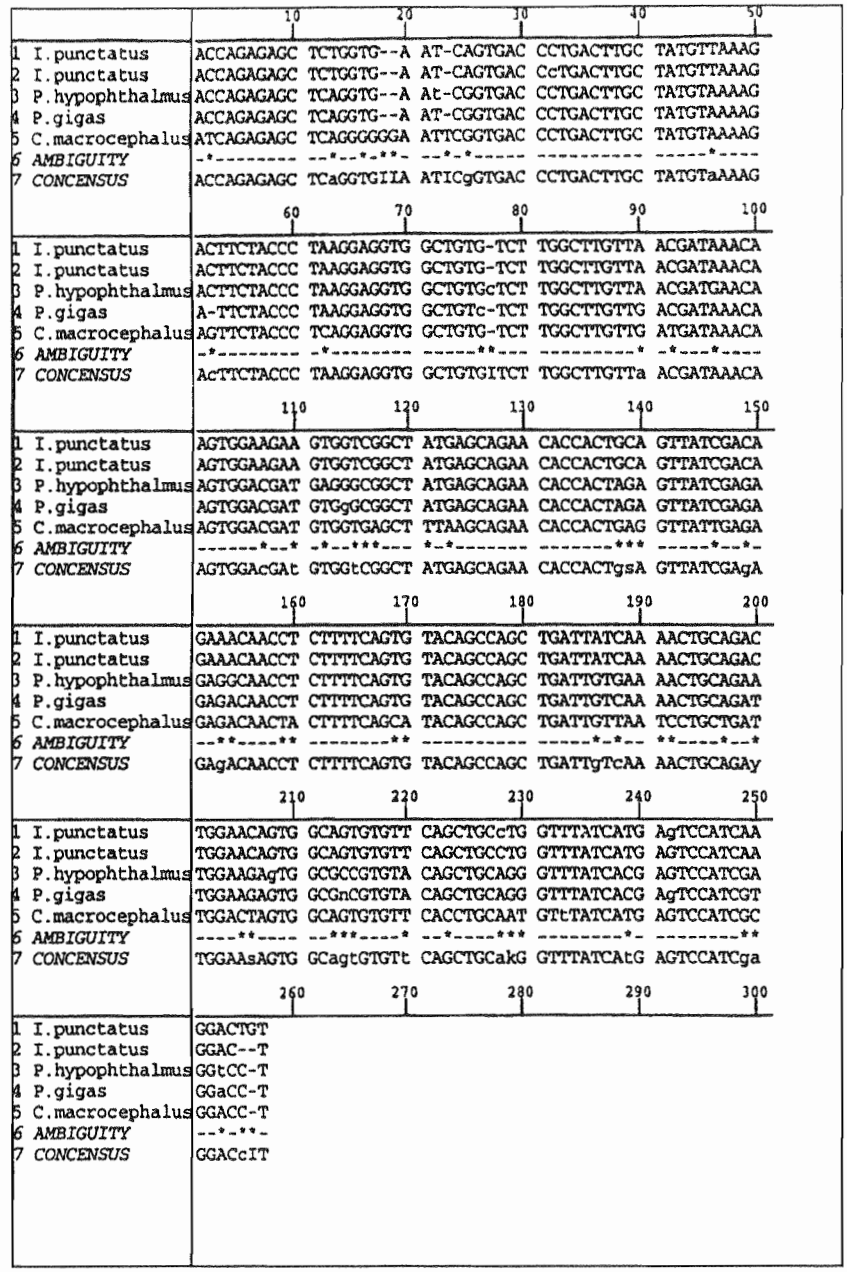

Fig. 2. Nucleotide sequences of catfish DNA fragments corresponding to the CH_4 exon of the immunoglobulin gene of *I. punctatus*. Sequences were aligned by computer to yield the greatest agreement among DNA samples from the following catfish species: *I. punctatus*, *P. hypophthalmus*, *P. gigas* and *C. macrocephalus*. Lower case letters indicate a concensus of two out of three analyses of a particular sample. Letter codes (in addition to the standard A, G, C and T abbreviations for nucleotides) were: K = G or T; S = C or G; Y = C or T; and I = inserted space. Asterisks indicate variability in the base sequence among species. Inclusion of primer sequences at each end (a total of 50 bp) would yield the expected nucleotide sequence of 303 bp for *I. punctatus*.

gigas and *P. hypophthalmus* were computer-analyzed for use of various restriction enzymes. It was hypothesized that *Hpa*I would be an appropriate enzyme for this purpose because it could cut specifically at one site in *P. hypophthalmus* DNA but at

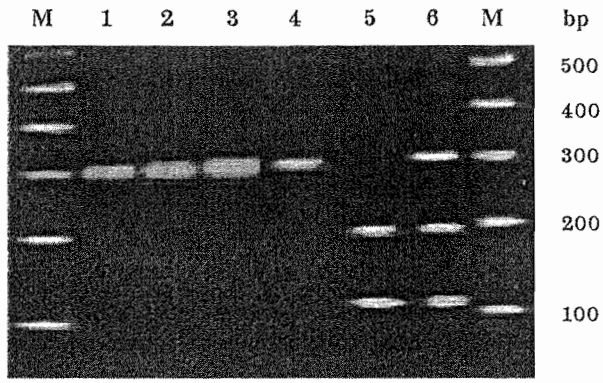

Fig. 3. Restriction enzyme analysis with *Hpa*I of catfish DNA amplified by PCR using primers targeted to the CH_4 exon of the immunoglobulin gene of *I. punctatus*. Digestion with the restriction enzyme cuts DNA only at a specific recognition sequence. Lane 1, *P. gigas* × *P. hypophthalmus*, nondigested; lane 2, *P. hypophthalmus*, nondigested; lane 3, hybrid (*P. gigas* × *P. hypophthalmus*), nondigested; lane 4, *P. gigas*, cut; lane 5, *P. hypophthalmus*, cut and lane 6, hybrid (*P. gigas* × *P. hypophthalmus*), cut. Marker (M) was 100-bp ladder.

none in *P. gigas* DNA. DNA restriction fragments resulting from digestion with *Hpa*I for these catfish and their hybrid were compared to those of nondigested DNA. All uncut samples yielded the expected single-band pattern (Fig. 3). Digested samples revealed a single band of 300 bp for *P. gigas*, two bands of 100 and 200 bp for *P. hypophthalmus* and three bands of 100, 200 and 300 bp for the hybrid. Hence, species identities within the family were clarified, and the hybrid revealed a combination of the parental genotypes.

Considering the computer analysis of these various restriction enzymes, *Hpa*I is not the only enzyme capable of serving this specific purpose. For example, *Bsm*AI would be predicted to cut at two sites (at nucleotide positions 75 and 140 of the amplified fragment) for *P. gigas* DNA, but at none for *P. hypophthalmus* DNA. The electrophoresis pattern resulting from *Bsm*AI digestion would be three bands for *P. gigas*, a single band for *P. hypophthalmus* and four bands for the hybrid.

4. Conclusion

The findings of this study are not only useful for the genetic identification of hybrid, androgenetic, gynogenetic or polyploid catfish, but also provide more information on genetic diversity in the form of gene sequences, taxonomy, and preservation of fish species through androgenetic breeding.

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