Molecular diagnostic markers of *Tachysurus fulvidraco* and *Leiocassis longirostris* and their hybrids

Hongwei Liang¹,², Shanshan Guo¹, Xiangzhong Luo¹, Zhong Li¹ and Guiwei Zou¹*

**Abstract**

**Background:** Bagridae is an important family of catfishes and has a high market demand. Recently, more cultivable Bagridae fishes are being exploited in China, and hybridization of some species has been carried out to achieve better growth performance, favorable sex ratios and better disease resistance. Yet, these hybrids have further increased the difficulties of taxonomy identification due to morphological indistinguishableness.

**Results:** In this study, the molecular identification technologies for *Tachysurus fulvidraco*, *Leiocassis longirostris* and their hybrids were successfully established by using mitochondrial COI and nuclear ITS sequences to identify the maternal and paternal lineage, respectively.

**Conclusion:** These molecular diagnostic methods could also be used to manage breeding plans of hybrids, monitor and minimize the negative impacts of hybridization programs in aquaculture. Furthermore, our study could also provide a reference for establishing detection technique for hybrids in other groups of fishes.

**Keywords:** Molecular diagnostic markers, COI, ITS, *Tachysurus fulvidraco*, *Leiocassis longirostris*

---

**Background**

Bagridae is an important family of catfishes which belongs to Order Siluriformes and consists of more than 220 species of 21 genera (Ferraris 2007). It is commonly found in Africa and Asia and has abundant species diversity. In China, the family includes approximately 30 species of 4 genera which inhabit in Yangtze River, Pearl River, Heilongjiang River and Yellow River (Qin et al. 2010). In the past decades, their populations have decreased rapidly and almost disappeared in many river systems due to over fishing, environmental pollution and other human disturbances (Luo et al. 2000; Wang et al. 2006; Xiao et al. 2014). In the meantime, an increasing number of Bagridae fishes have been cultured due to a high market demand. And to improve the growth performance and survival rate, some crossbreeding programs have been conducted, such as *Pseudobagrus ussurienensis* × *Pelteobagrus vachelli* (Cai et al. 2011; Qin et al. 2012; Wang et al. 2013), *Tachysurus fulvidraco* × *Pelteobagrus vachelli* (Wang et al. 2012; Zhang et al. 2016), *Tachysurus fulvidraco* × *Leiocassis crassilabrus*, and *Pelteobagrus vachelli* × *Leiocassis crassilabrus* (Wang 2013). The taxonomy of Bagridae is confusing and the validity of some catfishes should be further investigated and identified, what is more, taxonomic identification of hybrids is even more difficult. The main intention of crossbreeding is to obtain commercial advantages. Both yellow catfish (*Tachysurus fulvidraco*) and Chinese longsnout catfish (*Leiocassis longirostris*) in Bagridae have become important economic freshwater fishes with great market demand and high price in China due to their tender flesh, rich nutrition, few bones, convenient cooking and good taste (Liang et al. 2012; Xiao et al. 2014; Shen et al. 2014). To further improve the growth traits, they have also been used to produce hybrids.

Although crossbreeding could improve the genetic traits of animals, releases and escapes of hybrid...
individuals from fish farms have potential environment impacts. Hybrids can lead to the habitat changes for some fish species and change species composition in wild populations due to the change of diet or survival competition. Moreover, if the hybrids are fertile, the harmful effects of hybridization may cause pollution of gene pool and the extinction of some populations and species (Allendorf et al. 2001). Therefore, accurate identification of fish hybrids is critical to the sustainable aquaculture development. Reliable identification methods of hybrids can not only serve for the effective management of cross-breeding practices but also are used to monitor their negative impacts. Hybrid authentication is usually difficult and uncertain by morphological methods. However, molecular identification method is an ideal and alternative method to the traditional morphological discrimination in hybrid identification (Scribner et al. 2000). In the present study, we will try to establish an effective identification method of *T. fulvidraco*, *L. longirostris* and their hybrids using the polymerase chain reaction (PCR) based on mitochondrial and nuclear molecular marker.

**Methods**

**Experimental samples**

In this study, 30 *T. fulvidraco* individuals and 30 *L. longirostris* individuals were used to analyze parental lineages. For the interspecific hybrid, 30 specimens of *F_TL* (crosses using *T. fulvidraco* as female and *L. longirostris* as male) and *F_LT* (crosses using *L. longirostris* as female and *T. fulvidraco* as male) were genetically analyzed. All samples were obtained from the experimental farm of Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (YFI). The fish specimens were stored in the fish museum of YFI. All experimental procedures for the target fish were carried out in accordance to the standards of the Animal Care Policy of YFI.

**DNA extraction, PCR amplification and sequencing**

Total genomic DNA was extracted from muscle tissue using the traditional phenol–chloroform extraction method (Taggart et al. 1992). DNA quality was determined by electrophoresis in a 1% agarose gel. Cytochrome C Oxidase subunit I (COI) sequences were obtained by the described primer pairs in Table 1 which were designed from the COI sequences of *T. fulvidraco* (GenBank accession no. HM641815) and *L. longirostris* (GenBank accession no. NC014586). For identifying the hybrid, the nuclear ITS sequences of *T. fulvidraco* (10 individuals) and *L. longirostris* (10 individuals) were firstly obtained by the published primer pairs ITSF/ITSR in Table 1 (Yang et al. 2010). Then the differentiation of two catfish species was analyzed and the primer pairs ITSPF/ITSPR were designed to amplify the specific regions (ITSP) of different sequences length based on the obtained ITS sequences.

DNA amplifications were carried out by the polymerase chain reaction (PCR) in a total reaction volume of 25 μL. Each 25 μL PCR reaction system for COI and ITS contained 1 μL of 10 mM each primer, 1 U *Taq* DNA polymerase (TaKaRa, Japan), 2.5 μL 10 × PCR buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM MgCl₂; TaKaRa), 2 μL 10 mM dNTP (TaKaRa, Japan) and about 50 ng genomic DNA template. The PCR reaction for ITSP contained 1 μL of 10 mM each primer, 1.25 U *LA Taq* (TaKaRa, Japan), 12.5 μL 2 × GC bufferI (5 mM MgCl₂, TaKaRa), 4 μL 10 mM dNTP (TaKaRa, Japan) and about 50 ng genomic DNA template. All PCR reactions were performed on S1000™ Thermal Cycler (BIO-RAD, USA) based on the different conditions. The procedures for COI: pre-denaturing at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, and extending at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The procedures for ITS: pre-denaturing at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s, and extending at 72 °C for 45 s; and a final extension at 72 °C for 10 min. The procedures for ITSP: pre-denaturing at 94 °C for 5 min; 30 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s, and extending at 72 °C for 5 min. The PCR products (COI and ITS) were run on 2.0% agarose gels for 1 h at 80 V and then purified by a DNA

| Locus | Primer sequences (5′–3′) | PCR products sizes (bp) |
|-------|-------------------------|------------------------|
|       |                         | *T. fulvidraco* | *F_TL* | *L. Longirostris* | *F_LT* |
| COI   | F-CTACAATCCACCGCTTAA    | 1515                 | 1515   | 1515              | 1515   |
|       | R-TAGAAGAAAGTGACAGAGGG  |                        |        |                    |        |
| ITS   | F-GTATGGTGAACCTGGGAAGGATCA | 1106              | –      | 1018              | –      |
|       | R-GAGTTAACACCGGTGTGGGCTGCATT |                    |        |                    |        |
| ITSP  | F-CGTAACAGGTTCGTCGATGTTG | 678                  | 602 and 678 | 602              | 602 and 678 |
|       | R-ATCCACCGCTAAGAGTTGTCAG |                        |        |                    |        |
Agarose Gel Extraction Kit (Axygen, USA). The purified PCR products were cloned into pMD 18-T vector and sequenced by ABI 3730 automated sequencer (Applied Biosystems, USA).

Data analysis
The obtained COI and ITS sequences were edited and aligned using the Clustal W (Hall 1999). A neighbor-joining (NJ) tree was constructed based on the COI gene sequences of all individuals from each group using MEGA Version 6 (Tamura et al. 2013). Silurus meridionalis was used as outgroup. The uncorrected p-distances model was carried out and node support was assessed based on 1000 bootstrap replicate. The different regions of ITS sequences in length were identified using MEGA Version 6 (Tamura et al. 2013). The primer pairs ITSPF/ITSPR (Table 1) were designed to amplify the specific ITS sites of T. fulvidraco and L. longirostris.

DNA fragment size analysis
DNA fragment sizes of ITSP were determined by electrophoresis on 2% agarose gels with ethidium bromide (1 ng/mL) for 1 h at 80 V. The agarose gels were observed and captured by Gel document system (Clinx, China).

Results
To identify the maternal parentage, all individuals were amplified using the primers COIF/COIR for gene COI. For the COI sequences, the fragment sizes were approximately 1500 bp and there was no obvious difference in length among T. fulvidraco, L. longirostris, FTL and FLT (Table 1). Sequence analysis indicated that all individuals were divided into two groups, with one group including all the individuals of T. fulvidraco and hybrid FTL, and the other containing the rest L. longirostris and hybrid FLT. Similarly, the results showed that all individuals were divided into two clades as the former sequences analysis from phylogeny tree analyses (Fig. 1). The hybrid FTL and T. fulvidraco were grouped into one clade, and the hybrid FLT and L. longirostris into the other clade. These results suggested that all individuals came from two different maternal parentages, T. fulvidraco and L. longirostris, due to the maternal inheritance characteristics of mitochondrial. However, paternal line could not be identified from the sequences of COI gene.

To detect the paternal line, ITSF/ITSR was firstly used to amplify the ITS sequences and to differentiate between T. fulvidraco and L. longirostris. By comparing the specific sites, some valuable regions were found. Then the primer pairs ITSPF/ITSPR were designed to obtain the amplification of the specific region for ITS (ITSP) based on their different sequences. Furthermore, all individuals were amplified and then detected by electrophoresis in 2% agarose gel. The results showed that there was only one band for T. fulvidraco individuals (about 670 bp) and L. longirostris individuals (about 600 bp) (Fig. 2), while a heterozygous pattern with two bands (600 and 670 bp) was observed for hybrids FTL and hybrids FLT, respectively. Together with the result of maternal line, the paternal line could thus be inferred (Fig. 2).

In conclusion, a method based on sequence variation was established for taxonomic identification of hybrids. Firstly, we used the mitochondrial COI primers to obtain the sequences of the individuals and construct phylogeny tree to determine their maternal parentage by analyzing their homology and phylogenetic relation. Then we used the nuclear marker ITSPF/ITSPR to detect the hybrids and their paternal line. The application of this mitochondrial COI and internal transcribed spacer ITS marker has been proved to be feasible to identify the hybrids.

Discussion
Hebert et al. (2003) proposed a molecular technique named DNA barcoding to identify species and COI gene has then been commonly used as a standardized marker. Though COI gene is widely used as a genetic marker for fish species authentication, it is not an appropriate marker for all aspects of species identification (Clark 2015). The COI gene sequences only could not determine a hybrid because mtDNA exhibits maternal inheritance. The COI gene of hybrid F1 showed identical characteristic with its maternal parentage and can not represent different alleles (Rubinoff et al. 2006). Thus, hybrid individuals could be misidentified as their maternal parent species based solely on COI gene. Other genetic markers must be further considered due to the potential problem for accurate identification based on single COI gene (Rubinoff et al. 2006).

Recently, some researches have been carried out to identify hybrids and detect the hybridization events based on the mitochondrial and nuclear molecular markers. Hashimoto et al. (2011) established the molecular appraised technology for Serrasalmid fish and their hybrids based on mitochondrial genes (COI and Cytb) and nuclear genes (RAG2). The molecular diagnostic method was also established for hybrids between Netroplus catfish species Pseudoplatystoma corruscans and Pseudoplatystoma reticulatum by mitochondrial 16S and nuclear genes RAG2 (Prado et al. 2011). Dio et al. (2015) identified the hybridization of burrfish between Chilomycterus antillarum and Chilomycterus schoepfii using COI gene sequences and AFLP technology. The interspecies hybridization in the freshwater stingrays Potamotrygon motoro and P. falkneri was revealed by mitochondrial gene (COI and Cytb) and nuclear microsatellite markers (Cruz et al. 2015). The results show that the established
identification methods can be rapidly implemented and effectively determine the hybrid individuals. The ITS region exhibited low variations within species and high variations between species (Yu et al. 2006). What is more, it has several advantages due to its rapid evolution, easy isolation and non-coding structure (Chow et al. 2009). It has been widely used in the molecular identification and systematics studies for species discrimination (Chow et al. 2009; Yang et al. 2010; Zhu et al. 2011). As a result of hybridization, the nuclear ITS locus is a heterozygote consisted of heterologous alleles, one of which comes from maternal line and the other from paternal line for diploid individuals. Hence, it could be used to successfully identify hybrids by the heterozygous pattern in this study.

Primer design must be considered as a robust diagnostic technology. First, the conservative and consistent sites were used to design the primer pairs to simultaneously achieve the specific ITSP region sequences of *T. fulvidraco* individuals, *L. longirostris* and their hybrids. Second, there were different length PCR products by
amplification of designed species-specific primers, and the species-diagnostic bands could be easily distinguished (Pank et al. 2001). In this study, the amplification sequences gained for *T. fulvidraco* individuals and *L. longirostris* individuals by primers ITSPF/ITSPR were approximately 670 and 600 bp, respectively, which could be easily and directly observed from the agarose gel. Another consideration factor was to ensure the high efficiency of the amplification reaction (Pank et al. 2001). In our study, we increased the amplification success rates and simultaneously obtained two different-sized products by utilization of *LA Tag* and GC buffer.

Establishing identification technology for hybrids is vital to monitor hybridization programs and manage crossbreeding progress (Alllendorf et al. 2001). Meanwhile, hybridization programs should be continuously monitored to assess their impacts and safeguard wild populations since it is almost impossible to recover the population characteristics if the wild populations were suffered from genetic contamination. In this study, molecular diagnostic method for the target fish species was successfully established, and it could be applied for identifying not only eggs, larvae and young individuals during the breeding procedure but also fish meat and fish products. It could also become a reference for establishing detection technique for hybrids in other groups of fishes.

**Conclusions**

The molecular identification methods for *T. fulvidraco*, *L. Longirostris* and their hybrids were successfully established. Meanwhile, these molecular diagnostic tools could also be used routinely to assess breeding plans of fish farms, better manage fish hybrids, as well as to monitor and minimize the negative impacts resulting from the implementation of hybridization projects in aquaculture industry.

**Authors' contributions**

HWL designed the study, carried out the genetic studies, interpreted the data and drafted the manuscript. SSG and XZL carried out the sample collection and participated in the sequences analysis. ZL and GWZ proposed suggestions and participated in revising of the manuscript. All authors read and approved the final manuscript.

**Author details**

1 Yangtze River Fisheries Research Institute, The Chinese Academy of Fisheries Sciences, No. 8, 1st Wudayan Road, Wuhan East-lake Hi-tech Development Zone, Wuhan 430223, China. 2 Freshwater Aquaculture Collaborative Innovation Center of Hubei Province, Wuhan 430070, China.

**Acknowledgements**

We thank James, Lin Zhang and Wei Ye for reviewing this manuscript. This study was supported by Special Scientific Research Funds for Central Non-profit Institutes, Chinese Academy of Fishery Sciences (2014A11JC05), Special Funds for National Science and technology Basic Work (2013FY110700) and National Infrastructure of Fishery Germplasm Resources of China (2015DKA30470).

**Competing interests**

The authors declare that they have no competing interests.

**References**

Alllendorf FW, Leary RF, Spruell P, Wenenburg JK (2001) The problem with hybrids: setting conservation guidelines. Trends Ecol Evol 16(1):613–622

Cai YX, Chen YM, Chen XH, Wang MH, Pan Y, Xia AJ (2011) Morphometric differences of the hybrid F1 of *Pelteobagrus vachelli* × *Pseudobagrus ussuriensis*. J Lake Sci 23(2):264–270

Chow S, Ueno Y, Toyokawa M, Oshara I, Takeyama H (2009) Preliminary analysis of length and GC content variation in the ribosomal first internal transcribed spacer (ITS1) of marine animals. Mar Biotechnol 11:301–306

Clark LF (2015) The current status of DNA barcoding technology for species identification in fish value chains. Food Policy 54:85–94

Cruz VP, VERA M, Mendonca FF, Parde BG, Martinez Oliviera AC, Foresti F (2015) First identification of interspecies hybridization in the freshwater stingrays *Potamotrygon motoro* and *P. tayleri* (*Myllobatiformes, Potamotrygonidae*). Conserv Genet 16:241–245

Dio H, Zengke Y, Takahashi H, Sakai H, Ishibashi T (2015) Hybridization of burrfish between *Chilomycterus antillarum* and *Chilomycterus schoepfi* in captivity revealed by AFLP and mtDNA sequence analyses. Ichthyol Res 62:516–518

Ferraris CJ (2007) Zooteixa: checklist of catfishes, recent and fossil (Osteichthyes: Siluriformes), and catalogue of Siluriform primary types. Magnolia NZ 14:1–628

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp 41:95–98

Hashimoto DT, Mendonca FF, Senhorini JA, Oliveira C, Foresti F, Porto-Foresti F (2011) Molecular diagnostic methods for identifying Serrasalmid fish (*Pacu, Pirapitinga, and Tambaqui*) and their hybrids in the Brazilian aquaculture industry. Aquaculture 321:49–53

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc B Biol Sci 270:313–321

Liang HW, Hu GF, Li Z, Zou GW, Liu XL (2012) Mitochondrial DNA sequence of yellow catfish (*Peleobagrus fulvidraco*). Mitochondrial DNA 23(3):170–172

Luo M, Jiang LK, Liu Y, Zhan QG, Xia ZS (2000) Comparative study on isoenzymes in *Leiocassis longirostris*. Chin J Appl Environ Biol 6(5):447–451

Pank M, Stanhope M, Natansom L, Kohler N, Shivji M (2001) Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (*Carcharhinidae*) using multiplex PCR. Mar Biotechnol 3:231–240

Prado FD, Hashimoto DT, Mendonca FF, Senhorini JA, Foresti F, Porto-Foresti F (2011) Molecular identification of hybrids between Neotropical catfish species *Pseudoplatystoma coruscans* and *Pseudoplatystoma reticulatum*. Aquac Res 42:1890–1894

Qin Q, Cai YX, Xu ZQ, Ge JC, Chen XH, Huo GM, Bian WJ (2010) Comparison of six species of four genera of *Bgrideae* in the lower Yangtze River based on 165 rDNA gene sequences. Jiangsu J Agri Sci 2:373–376

Qin Q, Liang DW, Wang MH, Chen XH, Liu WB, Cai YX (2012) Studies on embryonic development of crossbreed F1 of *Pseudobagrus ussuriensis* (♀) and *Pelteobagrus vachelli* (♂). J Nanjing Norm Univ 35(2):81–86

Rubinoff D, Cameron S, Will K (2006) A genomic perspective on the shortcomings of mitochondrial DNA for “barcoding” identification. J Hered 97:581–594

Scribner KT, Page KS, Barton ML (2000) Hybridization in freshwater fishes: a review of case studies and cytonuclear methods of biological inference. Rev Fish Biol Fish 10:299–323

Shen T, He XS, Lei ML, Wang JR, Li XM, Li JM (2014) Cloning and structure of a histocompatibility class IIA gene (*Lelo-DAA*) in Chinese longsnout catfish (*Leiocassis longirostris*). J Biomed Sci 21:13

Taggart JB, Hynes RA, Prodoh PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. J Fish Biol 40:963–965

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:7725–7729

**Funding**

Funds for National Science and technology Basic Work (2013FY110700).
Wang F (2013) Morphometric differences analysis of Peltobagrus vachelli, Pelteobagrus fulvidraco, Leiocassis crassilabrus and their hybrid F1, Chin Agric Sci Bull 29(2):36–43
Wang ZW, Zhou JF, Ye YZ, Wei QW, Wu QJ (2006) Genetic structure and low-genetic diversity suggesting the necessity for conservation of the Chinese longsnout catfish, Leiocassis longirostris (Pisces: Bagridae). Environ Biol Fish 75:455–463
Wang MB, Chen Q, Chen YB, Guo C (2012) Hybridization study of yellow catfish Pelteobagrus fulvidraco and Peltobagrus vachelli. Mod Agric Sci Technol 24:273–274
Wang MH, Cai YX, Chen JH, Qin Q, Chen YM, Zhong LQ, Bian WJ, Zhang MS (2013) Growth comparison of offsprings in Ussuri Bullhead Pseudobagrus ussuriensis and Darkbarble catfish Peltobagrus vachelli and their reciprocal Hybrids. Fish Sci 1:50–54
Xiao MS, Bao FY, Cui F (2014) Pattern of genetic variation of yellow catfish Pelteobagrus fulvidraco Richardson in Huaihe river and the Yangtze river revealed using mitochondrial DNA control region sequences. Mol Biol Rep 41:5593–5606
Yang G, Yu JH, Xu P, Tang YK, Li JL (2010) Phylogenetic relationship among 8 common species of catfish based on Ribosome 18S and ITS sequences. Chin J Zool 45(4):110–117
Yu DH, Jia XP, Chu KH (2006) Common pearl oysters in China, Japan, and Australia are conspecific: evidence from ITS sequences and AFLP Fish Sci 72:1183–1190
Zhang GS, Yin SW, Wang YY, Li L, Wang XL, Ding YD, Zang X, Zhang HW, Jia YH, Hu YL (2016) The effects of water temperature and stocking density on survival, feeding and growth of the juveniles of the hybrid yellow catfish from Pelteobagrus fulvidraco × Peltobagrus vachelli. Aquac Res 134(9):402–413
Zhu J, Zhong LQ, Zhang CF, Liu H, Li B (2011) Sequence variation and secondary structure analysis of the first ribosomal internal transcribed spacer (ITS-1) between Cyprinus carpio carpio and C. Carpio haematopterus. Biochem Genet 49:20–24

Submit your manuscript to a SpringerOpen journal and benefit from:
► Convenient online submission
► Rigorous peer review
► Immediate publication on acceptance
► Open access: articles freely available online
► High visibility within the field
► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com