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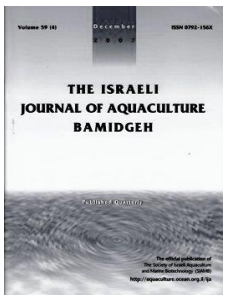

DNA Barcoding Analysis of Commercial Freshwater Fish Species Cultured in China

Qian Wang[1], Wei Dai[1], Xiaomei Wang[1], Jianxin Yang[2], Wenli Zhou[1]*

[1] *Tianjin Key Laboratory of Aqua-Ecology and Aquaculture, Department of In lineFisheries Science, Tianjin Agricultural University, Tianjin 300384, P.R. China*

[2] *Tianjin Huanxin Seed Multiplication Farm of freshfish, Tianjin 300384, P.R. China*

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Abstract

In this study, thirteen commercial freshwater fish species cultured in China, and one hybrid F1 (*Megalobrama amblycephala* ♀ × *Erythroculter ilishaeformis* ♂), were barcoded for a 860 bp region of the mitochondrial cytochrome oxidase subunit I (COI) gene. The results showed that a neighbor-joining (NJ) tree was constructed based on COI sequence clustered species in accordance with their taxonomic classification. Hybrid F1 could not be differentiated from *M.amblycephala*j in the neighbor-joining (NJ) tree. There was a significant negative correlation between interspecific hybrid compatibility and genetic distance (R^2 = 0.96). Hybrid compatibility can be predicted by the interspecific genetic distance.

Corresponding author. e-mail: wqgt1999@163.com

Introduction

Interspecific hybridization programs have been applied in fish farms with the purpose of producing animals that perform better than the parental species (hybrid vigor) (Bartley et al., 2001). However, the hybrid incompatibility caused by reproductive isolation always impedes interspecific hybridization. There is a correlation between parental divergence and reproductive isolation (Edmands, 2002). If interspecific hybrid compatibility could be predicted based on the genetic divergence of parents, this would be of great use to fish breeders. Genetic distance, representing interspecific genetic divergence, is often used and is a popular tool for species classification and evolutionary studies (Shirak et al., 2009; Wang et al., 2001) using molecular markers, including DNA barcoding.

DNA barcoding uses sequence diversity within a short and standardized gene region as a molecular diagnostic tool for species-level identification (Toffoli et al., 2008). DNA barcode sequences are very short relative to the entire genome and can be obtained reasonably quickly, and cheaply (Ardura et al., 2010). Hebert et al. (2003) proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of, animal species. The mitochondrial cytochrome oxidase subunit I (COI) gene has been employed as a global bioidentification sequence to catalogue the world's animal taxa, including fish (Ward et al., 2008). Combined with the potential for automated, rapid sample processing (Garland and Zimmer 2002), DNA barcoding could soon provide a powerful foundation for accurate and unambiguous fish species identification from eggs to adults, as well as analysis of phylogenetic relationship (Ward et al., 2009; Ardura et al., 2010; Zhang and Hanner, 2011). Moreover, it provides a tool for authentication of commercial fish products like fish slices and fillets that could not be morphologically identified, (Galal-Khallaf et al., 2014).

In this study, we examined sequence divergence at COI in 13 freshwater fish species and one hybrid. All of these are important commercial species cultured in China. The investigation may provide a further test of COI barcode for freshwater fish identification.

Materials and Methods

Fish specimens and DNA extraction. One hybrid F1 (*M.amblycephala* ♀ × *E. ilishaeformis* ♂) and 11 freshwater fish species belonging to 10 genera, 3 families, and 2 orders were provided by Tianjin Huanxin Seed Multiplication Farm of fresh fish, Tianjin, China(Table 1).

***Table1**.List of analyzed specimens with associated sample.*

Species	*Genera*	*Families*	*Orders*
Aristichthys nobilis	*Aristichthys*	Cyprinidae	Cypriniformes
Carassius auratue pengze （PZ）	*Carassius*	Cyprinidae	Cypriniformes
Carassius auratus red var (R)			
Carassius curatus cuvieri (W)	*Carassius*	Cyprinidae	Cypriniformes
Ctenopharyngodon idellus	*Ctenopharyngodon*	Cyprinidae	Cypriniformes
Cyprinus carpio (G)	*Cyprinus*	Cyprinidae	Cypriniformes
Cyprinus carpio (BL)			
Cyprinus carpio haematopterus (WK)			
Cyprinus carpio specularis (KL)			
Erythroculter ilishaeformis	*Erythroculter*	Cyprinidae	Cypriniformes
Hypophthalmichthys molitrix	*Hypophthalmichthys*	Cyprinidae	Cypriniformes
Ictiobus cyprinellus	*Ictiobus*	Catostomidae	Cypriniformes
Megalobrama amblycephala	*Megalobrama*	Cyprinidae	Cypriniformes
Myxocyprinus asiaticus	*Myxocyprinus*	Catostomidae	Cypriniformes
Oreochromis niloticus	*Oreochromis*	Cichlaidae	Perciformes

Abbreviations of subspecies name used in China are in parentheses.
WK:Russian carp; G: Germany mirror carp; BE: Birnur carp; KL: mirror carp; W: Japanese crucian carp; R: red crucian carp; PZ: Pengze carp.

Total sample size was 88 specimens. Tissue samples from each of the specimens were collected aseptically and preserved in 95% ethanol. A small piece of alcohol-preserved tissue was dissolved in 200 μL of STE buffer (1mM Tris HCl, 1mM EDTA and 10 mM NaCl) in a microcentrifuge tube. Total DNA was extracted using Phenol-Chloroform-

Isoamylalcohol method. The DNA samples were then dissolved in nuclease-free water and stored at -20℃ until further use.

PCR amplification and sequencing. The COI gene (860 bp) was amplified using the set of published primers (Peng et al., 2009) as follows: L5956-COI (5'-CACAAAGACATTGGCACCCT-3') and H6855-COI (5'-AGTCAGCTGAAKACTTTTAC-3'). The amplification reactions were performed in a total volume of 50 μl comprising 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5μM of each primer, 0.25 mM of each dNTPs(TAKARA ™), 1-2 U of Taq polymerase (TAKARA ™) and 100 ng of DNA template. The thermocycler conditions for the amplification were: an initial denaturation at 94℃ for 4 min, then 35 cycles of denaturation at 94℃ for 45 s, annealing at 48℃ for 30 s and extension at 72℃ for 1 min 30 s, followed by a final extension at 72℃ for 5 min. The PCR-amplified products were analyzed in 1.8% agarose gels. Those products with a single uniform band were purified, and then sequenced in an automated DNA sequencer (ABI 3730, Applied Biosystems Inc., CA, USA) at Sangon Biotech(Shanghai) Co., Ltd.

Analyzing correlation between hybrid compatibility and genetic divergence. According to the definition of Xu et al. (2010), hybrid compatibility was scored as follows: 1, both negative and positive cross F1 viable; 0.5, either negative or positive cross F1 viable; 0, both negative and positive cross F1 inviable. Interspecific hybridizations among some freshwater fish species investigated in this study have been performed by Tianjin Huanxin Seed Multiplication Farm of freshfish (Tianjin, China) (Jin, 2009). Interspecific hybrid compatibility among 8 freshwater fish species reported by Jin (2009) and genetic distance estimated using COI barcodes are summarized in Table 2. The linear correlation between hybrid compatibility and genetic divergence (genetic distance) was analyzed using Excel 2007.

***Table 2**. K2P genetic distance (%) within various taxonomic levels.*

Comparisons within	*Numbers of comparisons*	*Genetic distance (%)*				
		Taxa	*Minimum*	*mean*	*maximum*	*SE*
Species	84	13	0	0.34	5.5	0.009
Genera	84	10	0.1	5.00	12.6	0.018
Families	84	3	0.5	14.5	19.9	0.015
Orders	84	2	17.1	32.2	63.6	0.028

Data analyses. Two COI sequences of *Cyprinus pellegrini* and *Cyprinus multitaeniata* were referenced from the Genebank (accession numbers: JX042166 and HM536896). All sequences we sequenced and referenced were aligned using Clustal X 1.8 software. Sequence divergences were calculated using the Kimura two parameter (K2P) genetic distance model (Kimura 1980). Neighbour-joining (NJ) tree of K2P genetic distance was created to provide a graphic representation of the patterning of divergence between species (Saitou and Nei 1987). Bootstrapping was performed in MEGA 4.0 (Tamura et al. 2007) with 1000 replications.

Results

The average K2P genetic distance of individuals within species was 0.34% compared with 5.00 % for species within genera (table 2). There was 15-fold more variation among congeneric species than among conspecific individuals. Mean divergence among species within families and classes increased to 14.5% and 32.2%, respectively (table 1). Overall nucleotide frequencies of COI sequence were C (26.4%), T (29.1%), A (26.2%), G (18.4%), respectively.

As showed in the NJ tree (Fig. 1), *O. niloticus* which showed the greatest genetic divergence was placed at the base of the tree. The different genera were derived and formed branches respectively. All COI sequences formed species units were clustered in monophyletic groups at the genus level. Hybrid F1 (*M.amblycephala* ♀ × *E.ilishaeformis* ♂) could not be differentiated from *M.amblycephala*j in the NJ tree. Genetic distance between hybrid F1 and *M.amblycephala* ♀ (0.2%) was much closer than that between hybrid F1 and *E.ilishaeformis* ♂ (6.1%). This indicated that hybrid F1 had no hereditary similarity.

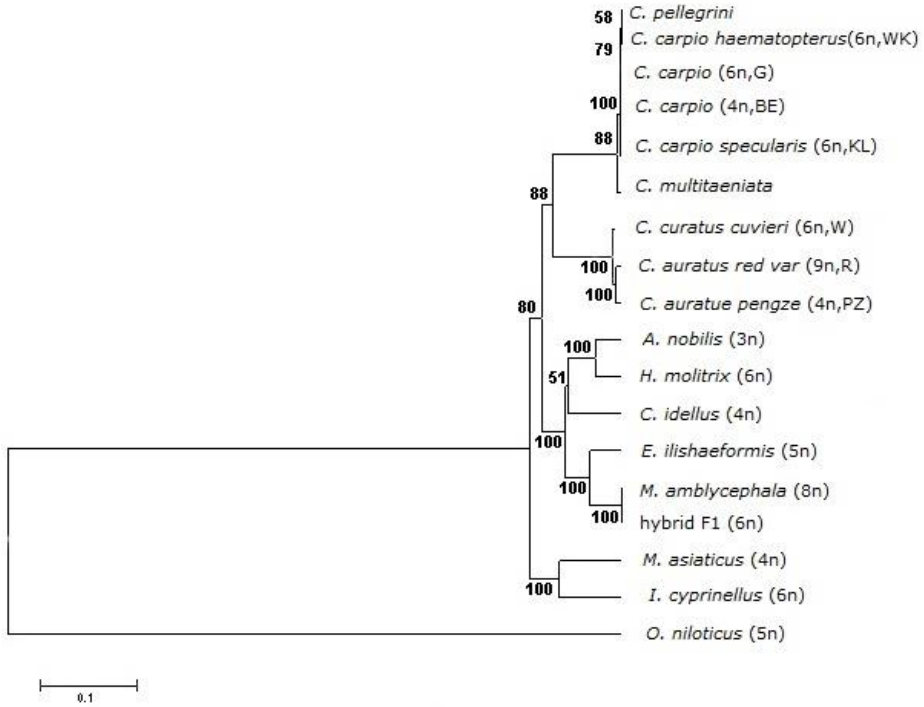

Fig. 1. Neighbor-joining tree of 90 COI sequences from 13 freshwater fish species and one hybrid F1 (*M.amblycephala* ♀ × *E. ilishaeformis* ♂), using K2P distances. In parentheses are the numbers of specimens sequenced.

As shown in Fig. 2, there was a significant negative correlation between interspecific hybrid compatibility and genetic distance (R^2 = 0.96). Both negative and positive cross F1 were viable when genetic distance was equal to 6.1 (table 3).

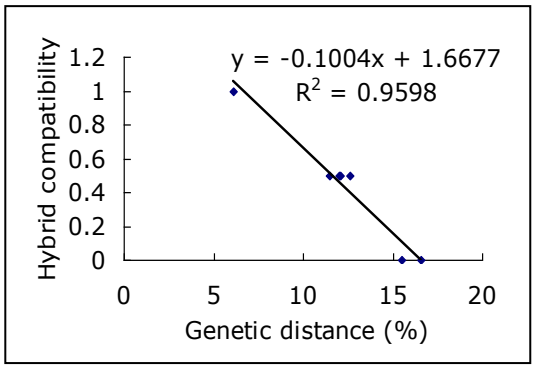

Fig. 2. The correlation between hybrid compatibility and genetic distance of freshwater fish species.

Table 3. Hybrid compatibility and K2P genetic distance among 8 freshwater fish species.

A	*B*	*A×B*	*B×A*	*Hybrid compatibility*	*Genetic distance (%)*
M. amblyceph	*E.ilishaeformis*	1	1	1	6.1
A. nobilis	*H. molitrix*	1	0	0.5	11.5
C. carpio haematopterus	*C. auratus red var*	1	0	0.5	12.0
C.carpio specularis	*C. auratus red var*	1	0	0.5	12.1
C. curatus cuvieri	*C. carpio*	1	0	0.5	12.6
C. auratus red var	*M.amblyceph*	0	0	0	15.5
I. cyprinellus	*C. auratus red var*	0	0	0	16.6

Discussion

We found the guanine-cytosine (GC) content of the COI region in 13 freshwater fish species of Osteichthyes was 44.8 %. The GC contents in 143 and 9 Osteichthyes species were 47.1% and 43.2%, respectively (Ward et al. 2005; Saccone et al. 1999). Higher GC content (above 40%) in mitochondrial genome might be a genetic feature of teleost.

A threshold to delimit species with DNA barcode data has been proposed (Hebert et al 2004a,b). At least 10 times the average intraspecies distance should be flagged as provisional new species. We found the average genetic distance within conspecific specimens (0.34 %) was 15 times lower than that found among congeneric species (5.00 %). These values were consistent with those found in freshwater fish from Canada (0.27% (conspecific) and 8.37% (congeneric)) (Hubert et al., 2008), Mexico and Guatemala (0.45% (conspecific) and 5.1% (congeneric)) (Valdez-Moreno et al., 2009),

Cuba (0.6% (conspecific) and 9.1% (congeneric)) (Lara et al., 2010). All these data suggest that DNA barcoding can be used as a useful tool for the identification of freshwater fish species.

Hybrid F1 (*M.amblycephala* ♀ × *E. ilishaeformis* ♂) could not be differentiated from *M.amblycephala* in NJ tree constructed with the COI barcode. Hybridization among species would create taxonomic uncertainty when mitochondrial DNA is maternally inherited because any hybrid or subsequent generation would have the maternal DNA only (Ward et al., 2005). Where species boundaries were blurred by hybridization, supplemental analyses of one or more nuclear genes will be required.

The positive relationship between reproductive isolation and genetic distance has been confirmed in angiosperm (Moyle et al., 2004), butterflies (Presgraves, 2002), birds (Lijtmaer et al. 2003), and fish (Russell 2003; Bolnick and Near, 2005). Consistent with above investigation results, hybrid compatibility among freshwater fishes decreased with the increasing genetic distances. To improve the success rate of interspecific hybridization, hybrid compatibility should be predicted first by assessing the genetic distances of the parents.

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