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Short Communication

The Species Identity of the Two Color Morphs of Northern Snakehead (*Channa argus*) Based on mtDNA Control Region Sequences

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JZ, SX and AZ presented the methodology, analysed the data, wrote the manuscript and acquired funds. DS, SL and YF collected the samples. YZ reviewed and edited the manuscript. YC did data curation.

Key words
***Channa argus*, Haplotype, Genetic diversity, mtDNA control region**

ABSTRACT

The control region for mitochondrial DNA (mtDNA) has been considered as one of the most effective molecular markers in the study of identification for fish species, which has a fast evolutionary rate. And thus, in the present study, investigation of genetic comparison was performed based on the complete sequences of mtDNA control region for "Bicolor" and "White" types of northern snakehead (*Channa argus*) due to an uncertain classification of them. The results showed that the genetic distance for the inter-species ranged from 0.000 to 0.492 for the Channidae family. And the analysis of sequences showed that there were nine haplotypes in 60 individuals, which were eight unique and one shared haplotypes. In addition, the genetic distance for inter-species of all the haplotypes ranged from 0.000 to 0.004. And the mean pair-wise genetic distance between the two color morphs were estimated to be 0.001. This indicated that the "Bicolor" and "White" types of northern snakehead belong to the same species at the molecular level. Moreover, all the haplotypes were gathered together by the Neighbour-Joining (NJ) tree, further confirming that the two kinds of snakehead morphs belong to one species.

One characteristic of the great northern snakehead is gradient variation from north to south of China, which is usually caused by the difference of temperature, light density, and etc. In other words, some areas show a relatively steep performance, but some areas show gentle performance (Wang *et al*., 1992; Zhou *et al*., 2015). Accordingly, there are two distinct color morphs of northern snakehead *Channa argus*, the "bicolor" type that was widely distributed in China, and the monochromatic "white type" that was white without any blotches and only found in Jialing waters (Ding, 1994). Some researchers have classified them as two distinct species (Kimura, 1934), however, others have treated them as color varieties of one single species; these judgments were based solely on the morphological aspects (Wang *et al*., 1992).

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In order to elucidate the genetic relationship between the two color morphs and clarify genetic relatedness among the family Channidae, we herein determined the level of sequence divergence between the two colored morphs based on the complete sequences of mtDNA control region.

Materials and methods

Northern snakeheads were collected in 2014 and 2015 from three locations, in Jialing river systems in China. Basic characteristics of the sites for sampling were given in Table I. Fish were captured using lift and seine nets. All samples were identified according to morphological characters (Courtenay and Williams, 2004). Fin clips were taken as tissue samples and preserved in 95% ethanol.

Total genomic DNA was extracted from the caudal fin using a standard extraction kit (DNeasy tissue kit, Baitaike Biotech Co., Ltd, China). The genes for mitochondrial control region and partial adjacent regions were amplified using the following primers:

F: 5′-ATCGGACAAGTCGCTCTTTCCTCT-3′ and R: 5′-TGCGGATACTTGCATGTGTAAGT-3′ (Zhou *et al*., 2016). The PCR amplification was performed in a PE 9700 thermocycler (PerkinElmer Co. Ltd., USA). The amplification reaction was carried out in 50µl volumes consisting of 25µl of 2× PCR mix buffer, 0.5µl of 2.5 U/µl Taq DNA polymerase, 2µl of 100 ng/µl DNA template, 2µl of 10 mM of each primer, and 20.5µl of sterile ultrapure water (Dongsheng Biotech Co., Ltd, China). Thermal cycling condition were 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 60 sec for annealing, and 72°C for 90 sec for extension, and then followed by 72°C for 10 min for a final extension. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden). Sequencing was conducted on an ABI377 automatic sequencer with both forward and reverse primers.

The nucleotide sequences of mtDNA control region and adjacent regions were aligned using Clustal-X (Thompson *et al*., 1997) and then were edited and connected using BioEdit (Hall, 1999). The number of haplotypes and its frequencies, number of polymorphic sites, nucleotide composition, as well haplotype diversity and nucleotide diversity were estimated by Arlequin 3.0 (Excoffier *et al*., 2005). The dendrogram of nine populations was constructed using Kimura 2-parameter model in MEGA 6.0 based on the pairwise genetic distance (Tamura *et al*, 2013).

All of the procedures and animal handling were performed in accordance with the guide for the Chinese Association for Laboratory Animal Sciences. Approval of the study was obtained from the Animal Ethics Committee of South China Agricultural University.

Results and discussion

A total of 1060 bp was sequenced for 75 individuals of mtDNA control region. The complete sequences were aligned unambiguously with 907bp. The variation sites (510), parsimony informative polymorphic sites (282) and conversion/transversion ratio (0.90) were obtained.

Six haplotypes (WtcaHD1–D6) of mtDNA control region for "white" type and four haplotypes (BtcaHD1–D4) of "bicolor" type northern snakeheads that were defined from 60 nucleotide sequences were obtained (Table II). The nucleotide sequences of all the haplotypes were submitted in GenBank (KU852448-KU852457). From the haplotypes, the sequences of WtcaHD1 and BtcaHD2 were the common haplotypes, which have the largest number in terms of the distribution.

For genetic distance analysis *Lateolabrax maculatus* and *Epinephelus coioides* were used as outgroups. Based on DNA sequence of the mtDNA control region of *Channa* species in NCBI, sequences alignment via MEGA6.0 showed that all the haploids were gathered together as a branch. Genetic distance was calculated using Kimura 2-parameter model. The inter-specific genetic distances between nine kinds of Channidae were 0.000-0.496. And the maximum of genetic distance between the "white" and "bicolor" type *C. argus* was 0.004. The intra-species genetic distance based on haplotype sequences was 0.001. All the genetic distances and standard errors were shown in Table III.

The molecular phylogenetic trees were composed from the NJ tree. The phylogenetic tree showed that all haplotypes were clustered into a single group with a high confidence value between the "white" and "bicolor" type *C. argus*. And the fact that the progenies of *C. argus x C. maculate* was also clustered into one group provides a piece of evidence that the mtDNA belongs to maternal inheritance (Fig. 1).

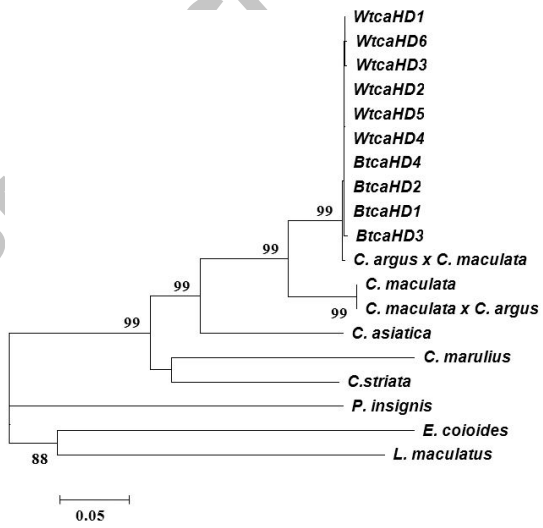

Fig. 1. Molecular phylogenetic tree constructed by NJ method based on mtDNA control region sequences using Kimura 2-parameter model. (High bootstrap values (>80%) in 1,000 resamplings are shown at the corresponding nodes).

The control region for mtDNA acts as a noncoding sequence between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes on the mitochondrion. The control region has the largest and fastest growing region of variations in the vertebrate mitochondrial sequences. And it generally used for phylogenetic analysis within and between populations (Nikolic *et al*., 2016). The analysis of sequences analysis showed that all the Channidaes family have rich mtDNA polymorphism. And the ratio of transition and transversion was 0.90, which means that the base variation in the Channidae family is high and the base substitution tends to saturation. This result was consistent with the characteristics how the sequence for the control region of mtDNA evolves.

Table I. Basic information of sampling sites and size (n) of *Channa* species.

Species	Location name	GPS coordinate	Altitude (m)	Date (Size)
Biocolor type *C. argus*	Neijiang city, Sichuan province, China	29°35'3.16"N 105° 2'49.95"E	332	2015(30)
White type *C. argus*	Neijiang city, Sichuan province, China	29°34'14.64"N 105° 4'1.95"E	332	2014(19)
	Ziyang city, Sichuan province, China	30° 6'28.77"N 104°38'39.46"E	357	2014(11)
C. maculata	San Jiaozhen, Zhongshan city, Guangdong province, China	22°39'14.31"N 113°25'43.95"E	1	2015(3)
C. maculata x *C. argus*	Guangzhou city, Guangdong province, China	23° 5'43.82"N 113°14'19.60"E	3	2015(3)
C. asiatica	Guangzhou city, Guangdong province, China	23° 5'48.06"N 113°14'5.13"E	3	2015(3)
C. micropeltes	Guangzhou city, Guangdong province, China	23° 5'28.31"N 113°14'1.34"E	3	2015(2)
C. striata	Vientiane city, Laos	17°57'52.27"N 102°35'17.97"E	167	2014(2)
C. lucius	Ha noi City, Vietnam	21° 1'41.45"N 105°51'48.94"E	12	2014(2)

Table II. Haplotype information of mtDNA control region among white and biocolor type *C. argus.*

Haplotype		Populations (number of the haplotypes and its frequencies)				Common haplotype
		white type	frequencies	biocolor type	frequencies	
mtDNA control region	WtcaHD1	23	0.77			WtcaHD1, BtcaHD2
	WtcaHD2	2	0.067			
	WtcaHD3	1	0.033			
	WtcaHD4	2	0.067			
	WtcaHD5	1	0.033			
	WtcaHD6	1	0.033			
	BtcaHD1			2	0.067	
	BtcaHD2			25	0.83	
	BtcaHD3			1	0.033	
	BtcaHD4			2	0.067	

WtcaH and BtcaH represent the Haplotype of white and biocolor type *C. argus.* D is mtDNA control region.

Table III. Pairwise distances calculated using Kimura 2-parameter model for mtDNA control region.

	Wtca HD1	**Wtca HD2**	**Wtca HD5**	**Wtca HD6**	**Wtca HD4**	**Wtca HD3**	**Btca HD4**	**Btca HD2**	**Btca HD1**	**Btca HD3**	***C. argus* x *C. maculata***	***C. maculata* x *C. argus***	***C. asiatica***	***C. marulius***	***C. striata***	***P. insignis***	***E. coioides***	***L. maculatus***
WtcaHD1		0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
WtcaHD2	0.000		0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
WtcaHD5	0.000	0.000		0.001	0.000	0.001	0.000	0.000	0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
WtcaHD6	0.001	0.001	0.001		0.001	0.002	0.001	0.001	0.001	0.003	0.002	0.012	0.012	0.019	0.025	0.022	0.035	0.036	0.035
WtcaHD4	0.000	0.000	0.000	0.001		0.001	0.000	0.000	0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
WtcaHD3	0.001	0.001	0.001	0.003	0.001		0.001	0.001	0.001	0.003	0.002	0.012	0.012	0.019	0.025	0.022	0.035	0.036	0.035
BtcaHD4	0.000	0.000	0.000	0.001	0.000	0.001		0.000	0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
BtcaHD2	0.000	0.000	0.000	0.001	0.000	0.001	0.000		0.000	0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
BtcaHD1	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000		0.002	0.002	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
BtcaHD3	0.003	0.003	0.003	0.004	0.003	0.004	0.003	0.003	0.003		0.003	0.012	0.012	0.018	0.025	0.022	0.034	0.036	0.035
C. argus x *C. maculata*	0.003	0.003	0.003	0.004	0.003	0.004	0.003	0.003	0.003	0.006		0.011	0.011	0.018	0.025	0.022	0.035	0.036	0.035
C. maculata	0.090	0.090	0.090	0.092	0.090	0.092	0.090	0.090	0.090	0.094	0.089		0.000	0.019	0.026	0.023	0.034	0.035	0.037
C. maculata x *C. argus*	0.090	0.090	0.090	0.092	0.090	0.092	0.090	0.090	0.090	0.094	0.089	0.000		0.019	0.026	0.023	0.034	0.035	0.037
C. asiatica	0.207	0.207	0.207	0.209	0.207	0.209	0.207	0.207	0.207	0.209	0.209	0.217	0.217		0.024	0.022	0.032	0.037	0.036
C. marulius	0.335	0.335	0.335	0.337	0.335	0.337	0.335	0.335	0.335	0.335	0.337	0.347	0.347	0.309		0.023	0.035	0.044	0.037
C.striata	0.272	0.272	0.272	0.274	0.272	0.274	0.272	0.272	0.272	0.272	0.272	0.289	0.289	0.273	0.296		0.036	0.043	0.032
P. insignis	0.487	0.487	0.487	0.490	0.487	0.490	0.487	0.487	0.487	0.487	0.490	0.486	0.486	0.473	0.484	0.496		0.037	0.039
E. coioides	0.528	0.528	0.528	0.532	0.528	0.532	0.528	0.528	0.528	0.532	0.525	0.527	0.527	0.551	0.616	0.597	0.509		0.038
L. maculatus	0.513	0.513	0.513	0.516	0.513	0.516	0.513	0.513	0.513	0.516	0.516	0.531	0.531	0.526	0.535	0.468	0.536	0.493	

For different species in family or genus category, a larger inter-specific distance is a prerequisite for accurate identification of species (Peng *et al*., 2009). Hebert *et al*. (2003) analyzed 13320 species of 11 phylums in the animal kingdom, indicating that the genetic distances within the species were generally less than 0.020 and that most of the genetic distances for intra-specific were less than 0.010. The results of this study showed that the average genetic distance within two color morphs of northern snakeheads was 0.001. The maximum genetic distance between different haplotypes is 0.004, which was less than 0.010, these results indicated that they belonged to the same species and they are not subspecies at the molecular level. And compared with other Channidae species, the genetic distances among them ranged from 0.092 to 0.483. Previous studies showed that many subspecies were at intermediate values as expected, but several had levels of divergence equivalent to populations, resulting in classifying errors (Rosel *et al*., 2017). The control region of mtDNA has been used successfully to determine the phylogeography and structure of populations within species (Craig *et al*., 2016). From NJ tree, all the Channidae species clustered together and all the haplotypes clustered in a clade solely with *C. argus x C. maculate*, which is consistent with maternal inheritance. These results indicated that the "white" type is probably an albino of "bicolor" type *C. argus*. This conclusion is consistent with that of our previous studies (Zhou *et al*., 2016, 2017, 2018). In summary, these results have certain application value in phylogenetic and germplasm analysis.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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