Indian J. Fish., 70 (4): 82-88, 2023 doi:10.21077/ijf.2023.70.4.132316-08

Available online at: epubs.icar.org.in/index.php/IJF

Molecular phylogenetics of albino phenotypes of northern snakehead (*Channa argus*) from Neijiang City, Sichuan Province, China

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Abstract

The northern snakehead *Channa argus* is widely distributed and extremely resistant to hypoxia; which is cultivated in China as an aquaculture species and has heritable albino body colour populations. Here, two phenotypes of albino *C. argus* of "gray fin rays" and "gold fin rays." were studied. The genetic diversity and molecular phylogenetics of *C. argus* with different body colours and geographical distributions were investigated based on their nuclear ITS1-5.8S-ITS2 sequences. The ITS1-5.8S-ITS2 sequences of the *C. argus* were conservative, with 1/1000 of mutation sites and 0.000-0.014 genetic distance, which showed that the studied phenotypes of *C. argus* belong to the same species. The intraspecific variation sites of the studied sequences showed that it was mainly nuclear transformation; with the sequences from albino *C. argus* with "gold fin rays" individuals having more variation sites than "gray fin rays" individuals. The difference in the geographical distributions of *C. argus* were distinguished in phylogenetic relationship studies, which supported that nuclear ITS1-5.8S-ITS2 sequences can be used to study molecular phylogeography.

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Keywords:
Albino *Channa argus*, ITS, Mutation sites, Molecular phylogeography

Received : 16.01.2023
Accepted : 03.12.2023

Introduction

Normal *Channa argus* of the Channidae family of Perciformes is freshwater air-breathing fish (Ishimatsu and Itazawa, 1981; Nelson *et al*., 2016) with black and white skin that is found in China, Russia, Korea, Japan, the USA, Uzbekistan, Kyrgyzstan and Kazakhstan (Lee, 1988; Ivantsoff, 2006; Wegleitner *et al*., 2016). Albino *C. argus* populations have distinct albino body colour and can be inherited and therefore, these were thought to be unique by some authors and mentioned the scientific name as *Ophicephalus argus* var *kimurai* (Zhou *et al*., 2016; Zhou *et al*., 2019; Fan *et al*., 2022; Zhou *et al*., 2022). Albino phenotypes *C. argus* are distributed in the Jialing River system of China and has some special traditional Chinese medicinal value and used to promote lactation and wound healing (Zhou *et al*., 2016; Jiang *et al*., 2018; Fan *et al*., 2022).

The lactate dehydrogenase (LDH) isozyme patterns of albino *C. argus* and normal *C. argus* were studied which showed no difference (Wang, 1992). The mitochondrial cytochrome oxidase subunit 1 (COI) of albino *C. argus* was also studied, which concluded that these are albino forms of *C. argus* (Zhou *et al*., 2019). The morphological characters and X-ray imaging studies also showed albino *C. argus* (*O. argus kimurai*) and *C. argus* have no significant morphological differences (Zhou *et al*., 2022). The DNA content of albino *C. argus* and *C. argus* was also compared, with no difference (Li *et al*., 2016). These results suggest that albino *C. argus* and *C. argus* are the same species. Albino *C. argus* is now a farmed species and can be artificially bred. The displacement loop (D-loop) of *C. argus* and albino *C. argus* was studied and showed that the genetic distance among albino *C. argus* populations is very close and should avoid inbreeding (Fan *et al*., 2022). However, the different phenotypes of albino *C. argus* have not been reported in previous studies and the genetic diversity of albino *C. argus* and normal ones has not been verified using internal transcribed spacer (ITS) sequence analysis.

Ribosomes play a crucial role in gene expression and protein synthesis in eukaryotic cells and coded by three genes of 18S, 5.8S and 28S and two internal transcribed spacers of ITS1 and ITS2, in the order of "18S-ITS1-5.8S-ITS2-28S" (Nazar, 2004). Compared with coding genes (18S, 5.8S and 28S), ITS1 and ITS2 face less selective pressure, have close to neutral evolution and have a faster evolution rate; thus, ITS is usually used for identification and phylogenetic analysis of closely related species (Sun *et al*., 2007; Kehie *et al*., 2016).

There are two directions of ITS sequence diversity in the same fish species. The first is the conservative direction; as reported in lake trout (*Salvelinus namaycush*) with minimal intraspecific variation of ITS with only one intra-individual polymorphism found (Zhuo *et al*., 1994). The second is non-conservative direction, as observed in the stone flounder *Kareius bicoloratus* with a high level of intra-individual variation of 18S rDNA, ITS1 and 5.8S rDNA with Type I, II and III (Xu *et al*., 2009); ITS2 in *Cynoglossus zanzibarensis* having three types with high degree of polymorphism and non-concerted evolution (Gong *et al*., 2016); the ITS1-5.8S-ITS2 of *Symphurus plagiusa* having two divergent types with marked intra-genomic variation (Gong *et al*., 2018a); the 18S-ITS1-5.8S of eleven species in Soleidae with little variation in six species but much variation in the other five species (Gong *et al*., 2018b); ITS of *Paraplagusia japonica* intra-individual showing high level of polymorphism (Gong *et al*., 2019); ITS regions of *Paraplagusia blochii* having intra-genomic variability with two highly divergent types (Gong *et al*., 2020) and *C. melampetalus* 18S-ITS1-5.8S with two distinct types of 18S and 5.8S and five types of ITS1 sequence (Gong *et al*., 2021). Mostly the unconservative direction of ITS sequence was reported from fishes belonging to Pleuronectiformes. Intra-genomic variability of ITS between albino *C. argus* and normal *C. argus* has not been studied so far. In this study, the ITS1-5.8S-ITS2 of albino *C. argus* belonging to to two colour morphs *i. e.,* gold fin and grey fin types and normal *C. argus* were sequenced and analysed for the first time.

Materials and methods

Six albino *C. argus* (three gold fin and three grey fin types) and one normal *C. argus* individuals were collected from the Yin Jinyou Family Farm Breeding Base, Central District of Neijiag, Sichuan Province, China. The experimental fish in the three different categories were numbered as GOF1~3, GRF1~3 and BW1 (Fig. 1). The specimens were transported to the laboratory in oxygen filled bags immediately after collection. A portion of the musculature from the base of the dorsal fin was excised and total genomic DNA was extracted using a Fine Pure Universal Genomic DNA Kit (GENFINE BIOTECH, Beijing, China) following the manufacturer's protocol. No specific permits were required for the specimens used in the present study. All fish handling procedures were approved by the Animal Care and Use Committee of the Conservation and Utilisation of Fisheries Resources in the Upper Reaches of the Yangtze River, Key Laboratory of Sichuan Province (Neijiang, Sichuan, China) and the approval No. is UFR010018. The experiments were also approved by the Academic Committee of the College of Life Sciences, Neijiang Normal University. At the same time, all methods were carried out in accordance with the recommendations in the Global Code of Conduct for Research in Resource-Poor Settings guidelines and ARRIVE guidelines.

PCR was performed in 25 µl reaction volumes containing 2.0 mM $MgCl_2$, 0.4 mM each of dNTPs, 0.5 mM of each primer, 1.0 U of Taq polymerase (Takara, Beijing, China), 2.5 µl 10 × Taq buffer and approximately 50 ng of DNA template. PCR cycling conditions included an initial denaturation at 95^{0}C for 3 min, followed by 35 cycles of denaturation at 95^{0}C for 30 s, annealing at 52^{0}C for 50 s, and elongation at 72^{0}C for 1.5 min. The PCR reaction was completed with a final extension at 72^{0}C for 10 min. The primer pair, FITS: 5' -CCGTAGGTGAACCTGCGG-3'/RITS: 5' -TTAAATTCAGCGGGTT GTCT-3' was used to amplify the 18S (partial)-ITS1-5.8S-ITS2-28S (partial) sequence in the *C. argus* genome (Zhu *et al.,* 2015).

Fig. 1. Phenotypes of *C. argus* used in the study. GOF1~3: Gold fin albino *C. argus;* GRF1~3: Grey fin albino *C. argus;* BW1: Normal *C. argus*

PCR products were purified using a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech) and inserted into a pMD 19-T vector (Sangon Biotech). Recombined plasmids were transferred into DH5a *E. coli* and checked using the universal primers M13F (-47)/ M13R (-48). Positive clones were sequenced in both directions using an ABI 3730XL DNA sequencer (Applied Biosystems, USA).

The ITS1–5.8S–ITS2 sequences were deposited in the GenBank database under accession numbers ON667943~~ON667971. To identify the sequence of the various regions, we searched the NCBI database with keywords "Channidae 18s" and "Channidae 28s" and found two gene annotations (EU216546 to identifying 18S-ITS1-5.8S, and KJ451611 for limiting 5.8S-ITS2-28S) as sequence boundary identification information. The sequences of normal *C. argus* from other region (Jining City, Shandong Province, China) were sourced from GenBank (Table 1). In this phylogenetic study, *Channa asiatica* was used as outgroup.

The sequenced fragments were assembled using the CodonCode Aligner (version 3, CodonCode Corporation, Dedham, MA, USA). The boundaries of the coding genes and ITS1 region were determined using BLAST-based (http://blast.ncbi.nlm.nih.gov) querying of sequences of closely related species (Kumar *et al.,* 2013; Zhu *et al.,* 2015). Sequence alignment was performed using ClustalX 2.0 (Larkin *et al.,* 2007) and checked manually using BioEdit version 7 (Hall, 1999). The GC content of the sequences and polymorphic sites were calculated using Bioedit. Haplotype diversity (Hd) and nucleotide diversity (p) were calculated using DnaSP software version 6.10 (Rozas *et al.,* 2017). Then secondary structure was predicted using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The minimum free energy (G at 37) was estimated for stability comparison (Michael and Patrick, 1981).

The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with the superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendent clade is shown next to each internal node in the tree. Evolutionary analyses were conducted in MEGA 11 (Tamura *et al*., **2021)**. Arlequin 3.5.2.2 was used for the Analysis of Molecular Variance (AMOVA) and fixation index (FST) (Excoffier and Lischer, 2010). PopART version 1.7 (https://popart.maths.otago.ac.nz/download/) was used to construct a haplotype (Leigh *et al*., 2015) network based on the TCS method (Clement *et al*., 2002).

Results

In this study, the three types of *C. argus* were sourced from the same location of Yin Jinyou Family Farm Breeding Base. The albino golden fin type fishes have albino skin with golden fins, while albino grey fin types also have albino skin but with grey fins and the different colour of fins are apparently located in caudal fin, however, the normal *C. argus* do not have albino skin but with white and black skin (Fig. 1). The albino gold fins, albino grey fins and normal *C. argus* appears distinctly different form each other. The ITS1-5.8S-ITS2-28S fragment from seven *C. argus* individuals were amplified by PCR. Isolated clones were randomly selected and sequenced (GenBank accession numbers ON667943~~ON667971). The *C. argus* ITS1-5.8S-ITS2 sequences from this study (GOF1.1~ 3.5, GRF1.1~ 3.4, BW1.1~1.5) and other studies (WL1~5) were conservative in length and nucleotide composition (Table 2). Sequence polymorphism indicated that the full-length ITS1-5.8S-ITS2 of *C. argus* exhibited 22 haplotypes and 32 variable sites; the most polymorphism was obtained for ITS1 with 17 haplotypes and 18 variable sites; while the least polymorphism was observed for 5.8S with four haplotypes and three variable sites. The GC contents of full-length, ITS1, 5.8S and ITS2 showed inconsistency, with GC content of 68, 72, 56-57 and 70-71, manifested in the higher GC content of ITS. Polymorphism of the sequences was also indicated by pair distances with full length, ITS1, 5.8S and ITS2 of 0.000-0.004, 0.000-0.007, 0.000-0.013 and 0.000-0.014, respectively.

The ITS1-5.8S-ITS2 sequences in the six albino *C. argus* (GOF1.1~ 3.4 and GRF1.1~ 3.4) had 23 variable sites. These sequences had two identical variation sites (T→C, C→G) and the other twenty-one variation sites were randomly distributed. The GRF2 individual had the most variable sites (7 sites), but GOF individuals had more variable sites than GRF individuals. The number of transition and transversion sites was 18 and 5, respectively. Comparing the variation sites of ten ITS1-5.8S-ITS2 sequences in the *C. argus* of

Table 1. Nuclear ITS1–5.8S–ITS2 sequences investigated in this study

Species name	Phenotype	Serial number	Geographic origin	Aligned length	GenBank accession No.
Channa argus	Albino-gold fins	GOF1.1~ 3.5	Neijiag City, Sichuan Province, China.	1118 bp	ON667948~~ ON667961
	Albino-grey fins	GRF1.1~ 3.4	Neijiag City, Sichuan Province, China.	1118 bp	ON667962~~ ON667971
	Normal	BW1.1~1.5	Neijiag City, Sichuan Province, China.	1118 bp	ON667943~~ ON667947
	Normal	WL1~WL5	Jining City, Shandong Province, China	1118 bp	KJ451597~~ KJ451601
Channa asiatica		Outgroup	Guangdong Province, China	1118 bp	KJ451602~KJ451609

Table 2. Nucleotide diversities of the nuclear ITS1-5.8S-ITS2 sequences in *C. argus*

Compare items	Full length	ITS1	5.8S	ITS2
Aligned length (bp)	1118 bp	608	159	293
Variable site	32	18	3	8
Number of Haplotypes	22	17	4	7
GC content (%)	68	72	56-57	70-71
Pair distances	0.000-0.004	0.000-0.007	0.000-0.013	0.000-0.014

Neijiang (BW1.1~1.5) and Jining (WL1~WL5), except two same variation sites (T→C, C→G) from BW1.1 to the other *C. argus*, seven variation sites were randomly distributed. The number of transition and transversion sites was 8 and 1, respectively. The predicted 159 bp 5.8S secondary structure in *C. argus* showed a minimum free energy of -59.8 kcal mol^{-1} and formed six loops with six stems (Fig. 2).

The clustering of *C. argus* from different geographical distributions and body colours was studied by the phylogenetic analysis using the ML method. Based on the tree topology, *C. argus* from the same geographical distributions clustered into one clade and the *C. argus* of the Neijiang group and the *C. argus* of the Jining group were found to be sister-groups with high support values of 95 and 97%, respectively, suggesting that the phylogenetic relationships based on these nuclear ITS1-5.8S-ITS2 sequences can separate *C. argus* that differ in geographic distribution. Consistent body colour in *C. argus* of BW, GOF, or GRF from Neijiang did not form a monophyletic cluster, indicating that these sequences cannot separate fish with different body colour traits from the same region (Fig. 3).

The haplotype network of the 28 haplotypes (labelled H1 to H28) was examined for the presence of population structure (Fig. 4). The constructed haplotype network showed that the outgroup and *C. argus* formed two groups and the 22 haplotype types of *C. argus* were in the same clade. This further supports the fact that the *C. argus* samples were from a genetically homogenous population.

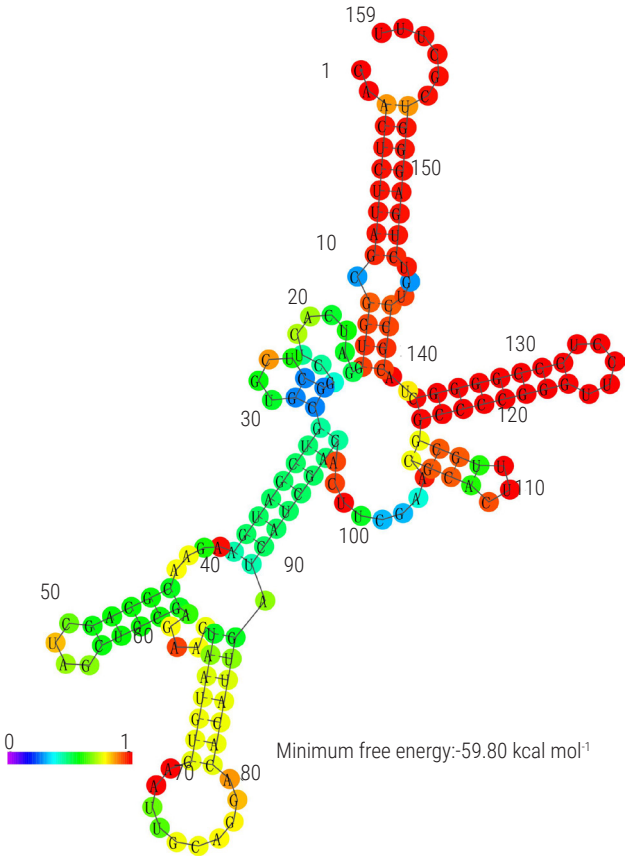

Fig. 2. Inferred secondary structures of nuclear 5.8S in *C. argus* from results for minimum free energy (MFE) prediction

Discussion

Nuclear ITS1-5.8S-ITS2 is commonly used to study phylogenetic relationship of parasites, fungi, plants and other organisms, such as *Clonorchis sinensis* (Tatonova *et al*., 2012), *Atriplex halimus*, *Momordica* (Elframawy *et al*., 2016; Ghosh *et al*., 2021), *Lentinus edodes* and *Isaria* spp. (D'Alessandro *et al*., 2014; Song *et al*., 2018). The phylogenetic relationships of albino *C. argus* and normal

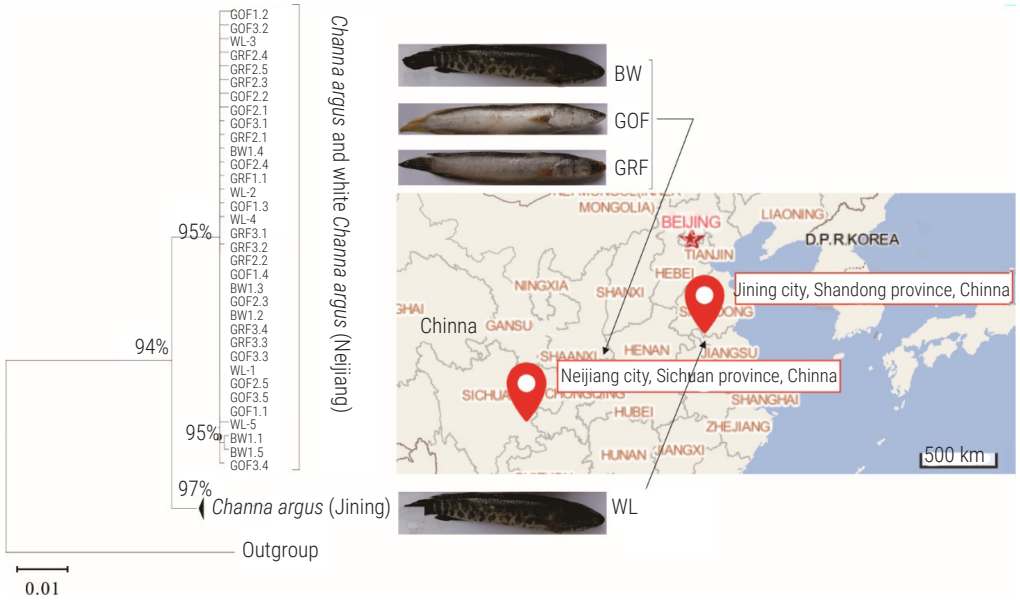

Fig. 3. The ML phylogenetic tree of *C. argus*. Phylogenetic tree was reconstructed based on ITS1-5.8S-ITS2 sequences of *C. argus* with gold fins albino *C. argus* (GOF1~3), grey fins albino *C. argus* (GRF1~3) and normal *C. argus* (BW1) from Neijiang City or Jining City (WL1~WL5), China. The map is used with permission of MAPWORLD (https://map.tianditu.gov.cn/)

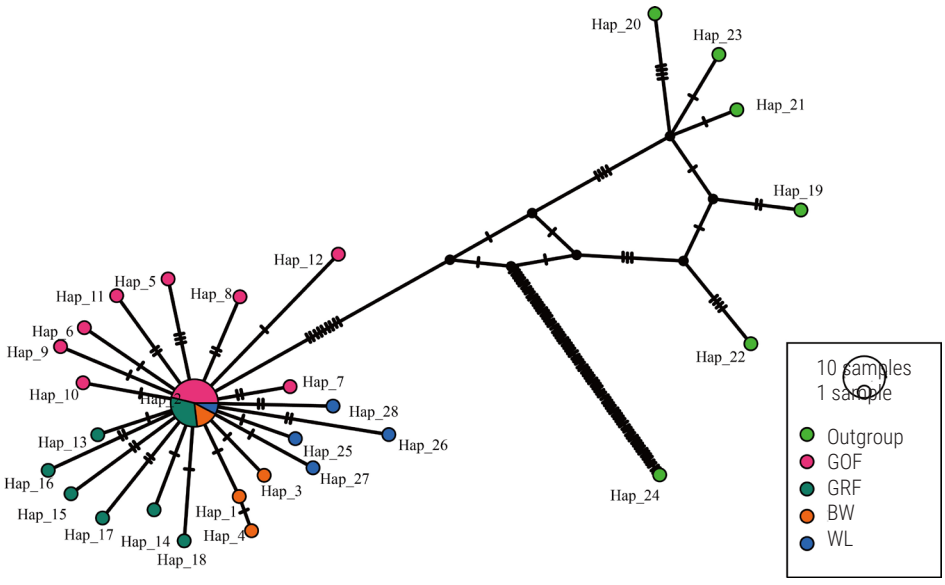

Fig. 4. Haplotype network of *C. argus* ITS1-5.8S-ITS2 sequences. Different colours represent different locations. The circle size is proportional to the sample number. Each dash on the line symbolises one mutational event

C. argus were analysed in this study. The sequence polymorphism showed 32 variant sites from 38012 sites (1/1000), but no regular variant type. This result differs from that of Pleuronectiformes fish (Gong *et al*., 2016; Gong *et al*., 2018a; Gong *et al*., 2018b; Gong *et al*., 2019; Gong *et al*., 2020; Gong *et al*., 2021). However, since intraspecies ITS sequences in some species appear in several types, the detection of polymorphisms of the intraspecies ITS sequences should be considered first when used for species identification, unlike the conservative COI gene (Sultana *et al*., 2018; Hossain *et al*., 2019) and conclusions should be drawn based on several cloned sequences. The genetic distance of *C. argus* based on nuclear ITS1-5.8S-ITS2 was 0.000-0.014, indicating that the albino *C. argus* and normal *C. argus* are the same species. This is consistent with previous research findings (Wang, 1992; Li *et al.,* 2016; Zhou *et al.,* 2019; Zhou *et al.,* 2022) and suggests that the scientific name of albino northern snakehead and normal snakehead is *C. argus*. Combining the results of the mutation sites with the genetic distance of *C. argus,* the ITS sequence polymorphism of *C. argus* was conserved.

The lengths of studied ITS1 and ITS2 are 608 bp and 293 bp, respectively and their lengths are distributed in teleost ITS1 and ITS2 length ranges (Si *et al*., 2016). There is a positive correlation between GC content and DNA molecular structure (Yakovchuk *et al*., 2006). The GC content of the coding genes 18S, 5.8S, 28S, or spacers ITS1 and ITS2 of fishes is greater than 50% (Si *et al*., 2016) and the GC content of *C. argus* nuclear ITS1-5.8S-ITS2 is 68%, which is same as that of other fishes (Si *et al*., 2017). The GC content of *C. argus* nuclear ITS1 and ITS2 was 72 and 70-71%, which is the same as the ITS coevolution and GC balance (Torres *et al.,* 1990). The length and minimum free energy of *C. argus* 5.8S were almost the same as those of Zebrias crossolepis, *S. plagiusa* (Type A), *P. japonica* (Type A), *Pardachirus pavoninus*, *Solea ovata, Kareius bicoloratus, P. blochii* and some Cynoglossidae species with little difference in secondary structure, but differed from those of *P. pavoninus, S. plagiusa* (Type B) and *P. japonica* (Type B) (Gong *et al*., 2016; 2018a; 2019; 2020). The type of base variation sites of alignment of ITS1-5.8S-ITS2 in *C. argus* were a number of transitions and few transversions, which is the same as in *Tridacna crocea*, *Kareius bicoloratus,* genus *Nymphaea*, *Aphanomyces astaci* and *S. plagiusa* (Yu *et al.,* 2000; Xu *et al.,* 2009; Dkhar *et al.,* 2011; Makkonen *et al.,* 2011; Gong *et al.,* 2018a). The variation sites in ITS1 were more than ITS2 and the variation sites of 5.8S were the least.

Compared with the polymorphisms of *C. argus,* albino *C. argus* with gold fin (GOF) individuals have more variation sites than albino *C. argus* with grey fin (GRF) individuals. This indicates that GOF is more mutated than GRF compared with normal *C. argus.* In contrast, normal *C. argus* (Neijiang) and *C. argus* (Jining) individuals have fewer variation sites, indicating that the nuclear ITS1-5.8S-ITS2 variation accompanied by body colour variation exceeds the variation accompanied by geographical differences. Molecular phylogeography studies are based on genes, include mtDNA, ITS, SSR and multiple genes (Gilmore *et al.,* 2010; Kavalco *et al.,* 2011; Unmack *et al.,* 2012; Hassanien and Al-Rashada, 2021). Based on the nuclear ITS1-5.8S-ITS2 in the present study, the different geographical distributions of *C. argus* were also successfully distinguished.

This study report for the first time the two kinds of heritable body colours of albino northern snakehead (*C. argus*). The diversity of conservative ITS studied and the results support that the albino northern snakehead and normal northern snakehead are the same species and that ITS can be used in molecular phylogeography studies.

Acknowledgements

This study was supported by the Science and Technology Department of Sichuan Province Foundation (2022NSFSC1721 and 2021YFN0033); Science and Technology Program Projects of Sichuan Provincial Science and Technology Department

(2021YFN0028); Neijiang Normal University School-level Research project (2023YB12) and the Neijiang Normal University Science and Technology Foundation (2019FM05).

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