Strict conservation of the ITS regions of the ribosomal RNA genes in Northern snakehead (Channa argus)

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Abstract

The Northern Snakehead (Channa argus) is widely distributed and extremely resistant to hypoxia, it is now an aquaculture species in China and has heritable diversity of white body colour populations with two phenotypes of "grey fin rays" and "gold fin rays". In this study, the genetic diversity and molecular phylogenetics of C. argus with different body colours and geographical distribution are investigated based on their nuclear ITS1-5.8S-ITS2 sequences. The ITS1-5.8S-ITS2 sequences of the C. argus are conservative, with 1/1000 of mutation sites and 0.000-0.014 genetic distance indicates the C. argus that was studied has the same ITS1-5.8S-ITS2 sequences and genetic distance, all of which show that it is the same species. The intraspecific variation sites show that it was mainly transformation, white C. argus with "grey fin rays" individuals have more variation sites than "gold fin rays" individuals, and the variation associated with different geographical distributions is bigger than that associated with different body colors. The nuclear ITS1-5.8S-ITS2 sequences identified the differences between C. argus in different areas, which indicates that nuclear ITS1-5.8S-ITS2 sequences can be used to study molecular phytogeography.

Introduction

Channa argus of the Channidae family of Perciformes are freshwater air-breathing fish [1, 2] that are found in China, Russia, Korea, Japan, USA, Uzbekistan, Kyrgyzstan and Kazakhstan with black and white skin [3-8]. White C. argus are a naturally different phenotype population of *C. argus*, which have the gold fins and grey fins (most noticeable at the tail fin), and their distinct white body colour can be stably inherited, so white *C. argus* are thought to be unique in some studies and use *Ophicephalus argus* var. Kimnra as the scientific name [9-12]. White *C. argus* is distributed in Jialing River system of China and has some special traditional Chinese medicinal value because its meat can be used to promote lactation and is good for wound healing [9, 11, 13].

Lactate dehydrogenase (LDH) isozyme patterns of white *C. argus* (*O. argus* kimurai) and *C. argus* were studied and showed no difference [14]. The mitochondrial cytochrome oxidase subunit 1 (COI) of white *C. argus* was studied and showed that white *C. argus* should serve as an albino of *C. argus* [10]. Morphological characters and X-ray imaging studies also showed that white *C. argus* (*O. argus* kimurai) and *C. argus* have no significant morphological differences [12]. The DNA content of white *C. argus* and *C. argus* was also compared, without difference [15]. These results support the hypothesis that white *C. argus* and *C. argus* are the same species. White *C. argus* is now a farmed species and can be bred artificially. The displacement loop (D-loop) of *C. argus* and white *C. argus* was studied and showed genetic distance among white *C. argus* populations is very close and should avoiding inbreeding [11].

Ribosomes play a crucial role in gene expression and protein synthesis in eukaryotic cells, and are encoded by three genes of 18S, 5.8S and 28S, and two internal transcribed spacers of ITS1 and ITS2, of the order of “18S-ITS1-5.8S-ITS2-28S” [16]. 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, so ITS

is usually used for identification and phylo-genetic analysis of closely related species [17, 18]. but the genetic diversity of white *C. argus* and *C. argus* has not been studied by internal transcribed spacer (ITS).

There are two directions of ITS sequence diversity from the same species of fish. The first is conservative direction, the lake trout (*Salvelinus namaycush*) reported the minimal intraspecific variation of ITS with only one intra-individual polymorphism found [19]. The second is unconservative direction, the stone flounder *Kareius bicoloratus* revealed a high level of intra-individual of 18S rDNA, ITS1 and 5.8S rDNA with Type I, II and III [20]; ITS2 in *Cynoglossus zanzibarensis* was three types with high degree of polymorphism and non-concerted evolution [21]; the ITS1-5.8S-ITS2 of *Symphurus plagiusa* found to have two divergent types with marked intra-genomic variation [22]; the 18S-ITS1-5.8S of eleven species in Soleidae had little variation in six species but much variation in other five [23]; ITS of *Paraplagusia japonica* intra-individual showed high level of polymorphism [24]; ITS regions of *P. blochii* was also have intra-genomic variability with two types of highly divergent [25] and the *C. melampetalus* 18S-ITS1-5.8S was found two distinct types of 18S and 5.8S and five types of ITS1 sequence [26]; mostly the unconservative direction of reported ITS sequence diversity fishes was from Pleuronectiformes. The white *C. argus* and *C. argus* intra-genomic variability of ITS was not studied. In this study, the ITS1-5.8S-ITS2 of white C. *argus* (gold fins and grey fins types) and *C. argus* were sequenced and analyzed.

Materials And Methods

Ethics statement

All fish handling procedures were approved by the Animal Care and Use Committee of the Conservation and Utilization of Fishes Resources in the Upper Reaches of the Yangtze River, Key Laboratory of Sichuan Province (Neijiang, Sichuan, China) and approval on 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.

Sampling, DNA extraction, amplification, cloning and sequencing

White *C. argus* (three types of gold fins and three grey fins) and one *C. argus* individuals were collected from the breeding base of the Yin Jinyou Family Farm, Central District of Neijiag, Sichuan Province, China. The seven experimental fish are numbered as GOF1~3, GRF1~3 and BW1 (Figure 1). The samples were stored at -20°C 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, as they are farmed commercial fish.

We used a pair (FITS: 5' -CCGTAGGTGAACCTGCGG-3'/RITS: 5' -TTAAATTCAGCGGGTT GTCT-3') to amplify the 18S (partial)-ITS1-5.8S-ITS2-28S (partial) sequence in the *C. argus* genome [27]. PCR was carried out in 25 μL reaction volumes containing 2.0 mM MgCl2, 0.4 mM of each dNTP, 0.5 mM of each

primer, 1.0 U of Taq polymerase (Takara, Beijing, China), 2.5 μL of 10 × Taq buffer and approximately 50 ng of DNA template. The PCR cycle conditions included an initial denaturation at 95 ° C for 3min, followed by 35 cycles of denaturation at 95 ° C for 30 sec, an annealing temperature of 52 ° C for 50 s, and elongation at 72 ° C for 1.5 min. The PCR reaction was completed by a final extension at 72 ℃ for 10 min. The resulting PCR products were purified using a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech) and then inserted into a pMD 19-T vector (Sangon Biotech). The recombined plasmids were then transferred to DH5a E. coli and checked with the universal primers M13F (-47)/M13R (-48). Positive clones were sequenced in both directions using an ABI 3730XL DNA sequencer (Applied Biosystems, USA).

The 29 complete ITS1–5.8S–ITS2 sequences were obtained and 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 for identify 18S-ITS1-5.8S, and KJ451611 to identify 5.8S-ITS2-28S) as sequence boundary identification information. The relevant sequences of this species from other parts of the region (Jining City, Shandong Province, China) were downloaded from GenBank. In general, 40 nuclear ITS1 to 5.8S to ITS2 were analysed (Table 1). In this phylo-genetic study we used *C. asiatica* as the outgroup.

Sequence alignment, polymorphism, and secondary structure

The sequenced fragments were assembled using CodonCode Aligner (version 3, CodonCode Corporation, Dedham, MA, USA). The boundaries of the coding genes and the ITS1 region were determined using BLAST-based querying (http://blast.ncbi.nlm.nih.gov) of the sequences of closely related species [27, 28]. Sequence alignment was performed by ClustalX 2.0 [29] and manually checked manually using BioEdit version 7 [30]. The GC content of the sequences and the polymorphic sites were calculated using Bioedit [30]. Haplotype diversity (Hd) and nucleotide diversity (p) were calculated using the DnaSP software version 6.10 [31]. 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 [32].

Phylogenetic studies of *C. argus*

The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model [33]. The initial tree for the heuristic search was obtained automatically by applying the neighbour-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 is drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 40 nucleotide sequences. There were a total of 1182 positions in the final data set. Evolutionary analyses were conducted in MEGA 11[34]. Arlequin 3.5.2.2 was used for the Analysis of Molecular Variance (AMOVA) and fixation index (FST) [35]. PopART version

1.7 (https://popart.maths.otago.ac.nz/download/) was used to construct a haplotype [36] network based on the TCS method [37].

Results

Results of PCR amplification of nuclear ITS1-5.8S-ITS2 in *C. argus*

In this study we amplified the ITS1-5.8S-ITS2-28S fragment from seven *C. argus* individuals. A total of 29 isolated clones were randomly selected and determined (GenBank accession numbers ON667943 ~ ON667971). The complete ITS1-5.8S-ITS2 sequences of this study and five sequences of C. argus (WL1 ~ 5) from other study were extremely conservative in length and nucleotide composition (Table 2). The sequence polymorphism indicated that full length ITS1-5.8S-ITS2 exhibited 22 haplotypes and 32 variable sites, The most polymorphism is from ITS2 with 17 haplotypes and 18 variable sites, The least polymorphism is from 5.8S with 4 haplotypes and 3 variable sites. However, the full length GC content, ITS1, 5.8S and ITS2 shows inconsistency, with GC content of 68%, 72%, 56-57% and 70-71%, manifested in the higher GC content of ITS. Polymorphism of the sequences is 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.

Detailed variant sites of the the alignment of ITS1-5.8S-ITS2 sequences in C. argus

The twenty-four ITS1-5.8S-ITS2 sequences in the six white *C. argus* (GOF1.1~ 3.4 and GRF1.1~ 3.4) have 23 variable sites. Those sequences have two identical variation sites (T→C, C→G), and other twenty one variation sites were randomly distributed in GOF1 .2 (C→T, T→C), GOF1 .3 (C→T), GOF2 .1 (G→A, C→T), GOF2 .2 (C→T, T→C,), GOF2 .4 (T→C), GOF3 .1 (C→T), GOF3 .2 (T→C, T→C), GOF3 .4 (G→A), GRF1 .1 (C→T), GRF2 .1 (T→C), GRF2 .3 (A→G, G→C), GRF2 .4 (T→C, T→C), GRF2 .5 (G→T, G→T), and GRF3 .2 (T→C). The GRF2 individual has the most variation sites (7 sites), but GOF group has more variation sites than GRF group. The transitions and transversions sites are 18 and 5 (Supplement Figure. 1). Comparing the variation sites of ten ITS1-5.8S-ITS2 sequences in the *C. argus* of Neijiang (BW1.1~1.5) and Jining (WL1~WL5) can be seen, except two same variation sites (T→C, C→G) from BW1.1 to others C. argus, other seven variation sites randomly distributed in BW1.4 (C→T), WL2 (T→C), WL3 (A→G, A→G), WL4 (A→G), WL5 (T→C, G→A). The transitions and transversions sites are 8 and 1 (Supplement Figure 1).

The prediction of the secondary structure of 159 bp nuclear 5.8S sequences in *C. argus* showed the corresponding minimum free energy of -59.8 kcal/mol. The structures formed include six perfect loops and six perfect stems (Figure. 2).

Phylogenetic analysis of *C. argus*

The clustering of *C. argus* from different geographical distributions and in different body colors was studied in the phylogenetic analysis by using the ML method. Based on the tree topology, the *C. argus* from the same geographical distributions clustered into one clade, *C. argus* of the Neijiang group and *C.*

argus of the Jining group were sister-group with high support values of 95% and 97% respectively (Figure. 3), 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 color in *C. argus* of BW, GOF or GRF from Neijiang does not form a monophyletic cluster (Figure. 3), indicating that these sequences cannot separate fish with different body color traits from the same region.

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

Discussion

The nuclear ITS1-5.8S-ITS2 is commonly used in parasites, fungus, plants, and other organism phylogenetic relationship research, such as in *Clonorchis sinensis* [38], *Atriplex halimus*, *Momordica* [39, 40], *Lentinus edodes* and *Isaria spp*. [41, 42]. In this study, the white *C. argus* and the *C. argus* phylogenetic relationship were analyzed. The sequence polymorphism shows 32 variant sites from 38012 sites (1/1000) but no regular variant type. This result differs from that of Pleuronectiformes fish [21-26]. However, since intraspecies ITS sequences in some species appear in several types, detection of polymorphisms of the intraspecies ITS sequences should be considered first when used for species identification, and unlike the COI gene [43, 44], conclusions should be drawn based on several cloned sequences. The genetic distance of *C. argus* based on nuclear ITS1-5.8S-ITS2 is 0.000-0.014 indicating that the white *C. argus* and *C. argus* are the same species. This is consistent with other previous research findings [10, 12, 14, 15]. Combining the results of the mutation sites with the genetic distance of *C. argus*, the ITS sequence polymorphism of *C. argus* is in a conserved direction.

The lengths of ITS1 and ITS2 are 608 bp, and 293 bp respectively, the lengths of which are distributed in teleost ITS1 and ITS2 of the teleost [45]. There is a positive correlation between the GC content and DNA molecular structure [46], and whether the GC content of coding genes 18S, 5.8S, 28S or spacers ITS1, ITS2 of fishes is greater than 50% [45], the GC content of *C. argus* nuclear ITS1-5.8S-ITS2 is 68%, which is the same as with other fishes [47]. The GC content of *C. argus* nuclear ITS1and ITS2 is 72% and 70-71%, which is the same as ITS coevolution and GC balance [48]. The length, minimum free energy is of *C. argus* 5.8S is almost the same with *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 of secondary structure, but not same with *P. pavoninus*, *S. plagiusa* (Type B), *P. japonica* (Type B) [21-25]. The type of base variation sites of alignment of ITS1-5.8S-ITS2 in *C. argus* is a lot of transitions and few transversions, which is the same as in *Tridacna crocea*, *Kareius bicoloratus*, genus *Nymphaea*, *Aphanomyces astaci* and *S. plagiusa* [20, 22, 49-51]. And the variation sites in ITS1 are more than ITS2, the variation sites of 5.8S are the least.

Compared with the polymorphisms of different individuals of *C. argus,* white *C. argus* with gold fin (GOF) individuals have more variation sites than white *C. argus* with grey fin (GRF) individuals, indicating that GRF is more mutated than GOF compared to *C. argus*; *C. argus* (Neijiang) and *C. argus* (Jining) individuals have fewer variation sites, indicating that the nuclear variation ITS1-5.8S-ITS2 variation accompanied by body colour variation exceeds the variation accompanied by geographical difference.

The present study on molecular phylogeography used some gene to conduct analysis. Those gene include mtDNA, ITS, and SSR, multiple genes [52-55]. Based on nuclear ITS1-5.8S-ITS2, the different geographical distributions of C. argus are also successfully distinguished.

Conclusion

We report for the first time the different heritable body colors of white northern snakehead (*Channaargus*). The diversity of studied is conservative ITS, and the results support that white northern snakehead and northern snakehead are the same species and that ITS can be used in molecular phylogeography.

Declarations

Author Contributions

Li Ao, Tingjuan Zhao, Sha Li and Jie Li carried out experiments and get the data for this article, Shixi Chen analyzed the data, prepared figures, then with Zakaria Hossain Prodhan write and revised this article, Yuanchao Zou gave some suggestions for this article. All authors reviewed the manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability Statement

we confirm that the datasets generated or analyzed during the current study are available in the NCBI repository (https://www.ncbi.nlm.nih.gov/), GenBank accession number: ON667943 ~ ON667971, KJ451597 ~ KJ451601, and KJ451602 ~ KJ451609, the ON667943 ~ ON667971.

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Tables

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

species name	Phenotype	Serial number	Geographic origin	Aligned length	n	GenBank accession number
Channa argus	White-gold fins	GOF1.1~3.5	Neijiag City, Sichuan Province, China.	1118 bp	10	ON667948~~ON667961
	White-grey fins	GRF1.1~3.4	Neijiag City, Sichuan Province, China.	1118 bp	14	ON667962~~ON667971
	Black and white	BW1.1~1.5	Neijiag City, Sichuan Province, China.	1118 bp	5	ON667943~~ON667947
	Black and white	WL1~WL5	Jining City, Shandong Province, China	1118 bp	5	KJ451597~~KJ451601
Channa asiatica		Outgroup	Guangdong Province, China	1118 bp	6	KJ451602~KJ451609

n, number of sequences.

Table 2. Nucleotide diversities of 34 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

Figures

Figure 1

C. argus used in this study, GOF1 ~ 3: white gold fins *C. argus*, GRF1 ~ 3: grey fins white *C. argus*, BW1: black and white *C. argus*.

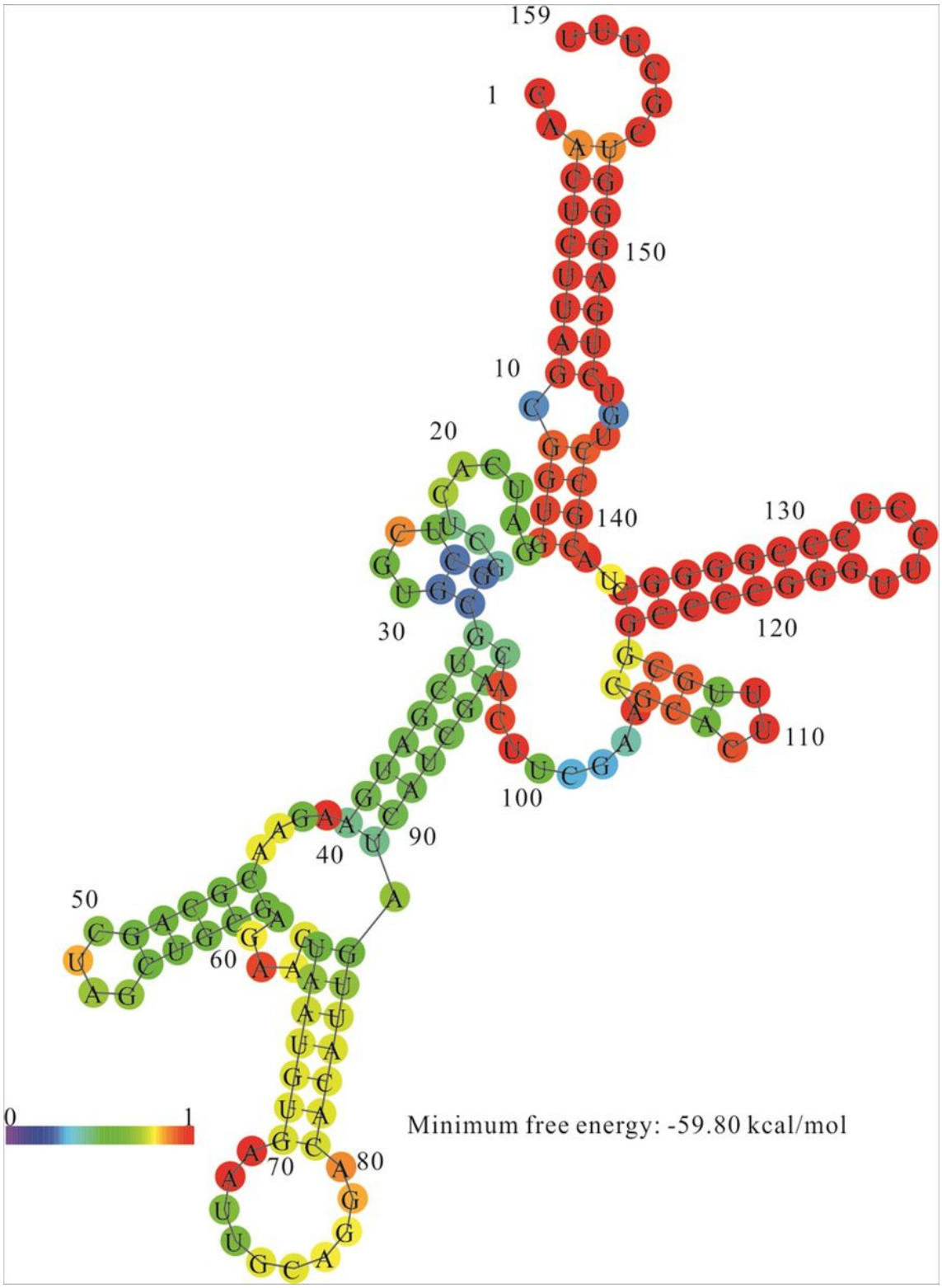

Figure 2

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

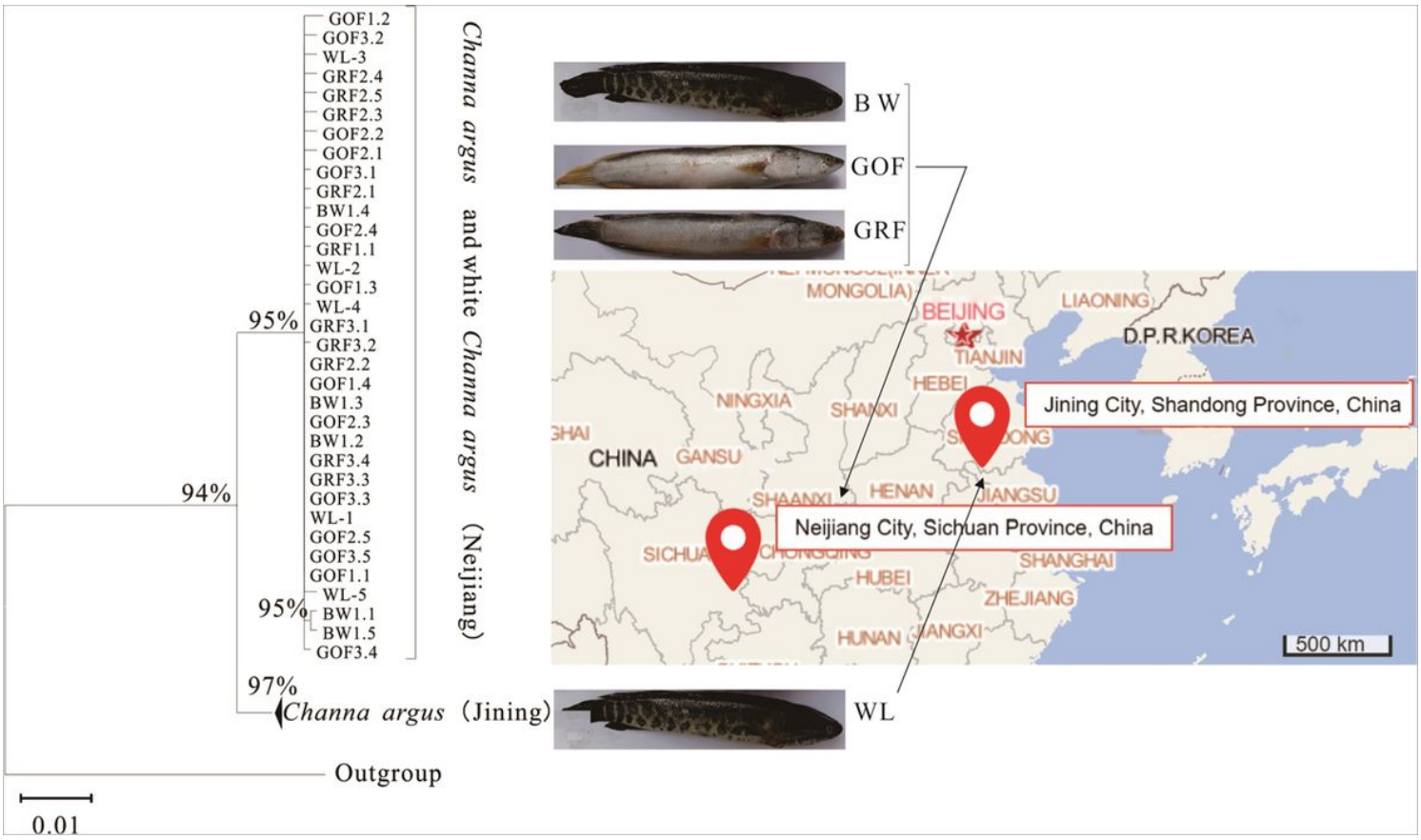

Figure 3

The ML phylogenetic tree reconstructed based on ITS1-5.8S-ITS2 sequences of eight *C. argus* with three phenotypes from two places The map is used with permission of MAPWORLD (https://map.tianditu.gov.cn/)

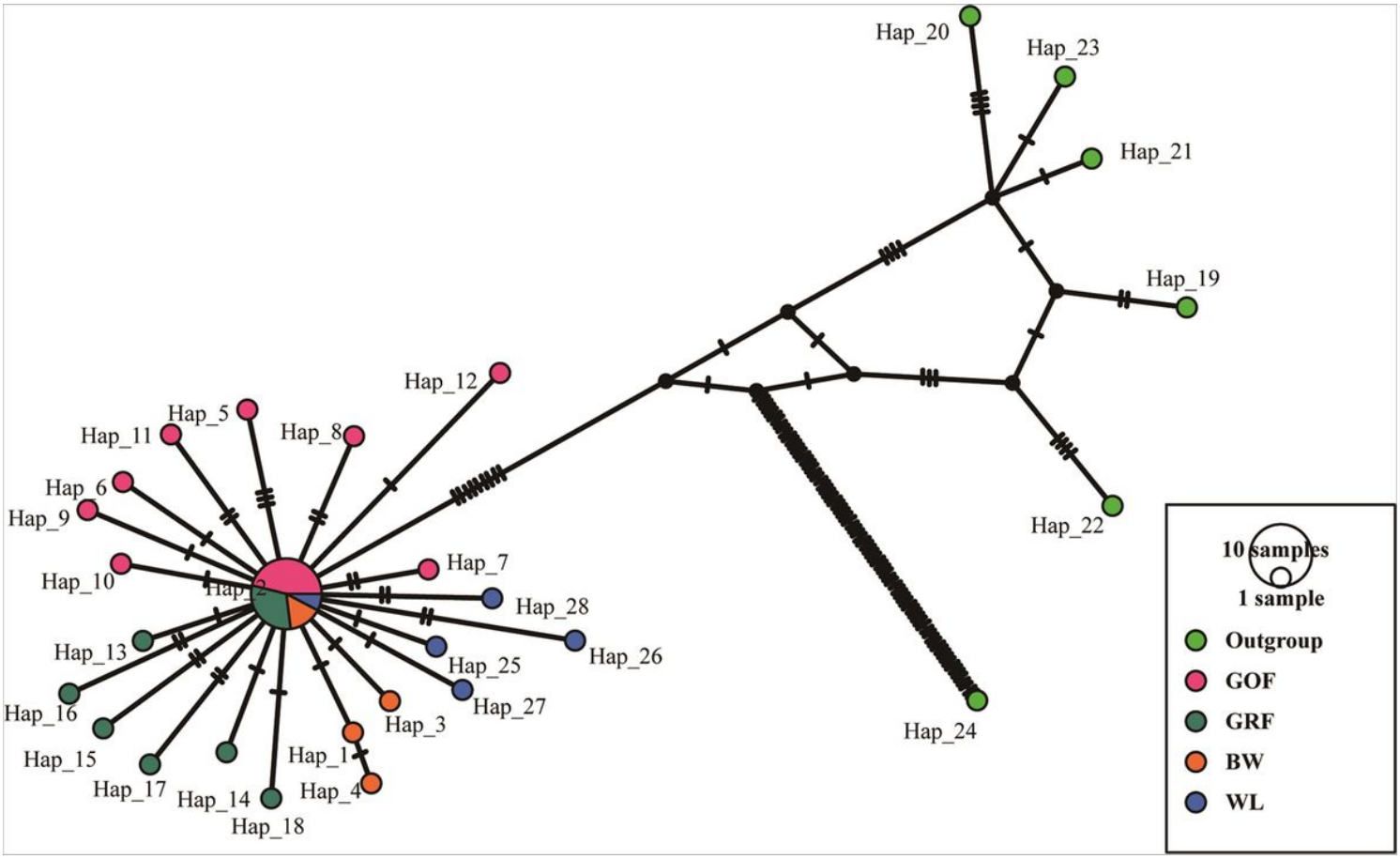

Figure 4

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

Supplementary Files

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- SupplementaryMaterial.docx