

Phylogenetic analysis and chemical composition of siniperoid fishes from the Northern mountainous area of Vietnam

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Abstract

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The siniperoids are freshwater fishes endemic to East Asia, mainly in China, bordering Vietnam. Phylogenetic studies of the siniperoid species have made great progress over the past decades in the world, but there is no published record of exactly how many species are present in Vietnam. This study aimed to identify species belonging to the siniperoids in the northern mountainous region of Vietnam by molecular biology method. In addition, we analyzed chemical composition of the species collected in Vietnam, which is the basic information for the development of this species' farming. The results of species identified by molecular markers of 45 samples of the siniperoids obtained showed that 25/45 samples (55.6%) were *Siniperca kneri* species, and 20/45 samples (44.4%) were *Siniperca chuatsi* species. The results of the chemical composition of these species showed that they contained all essential amino acids. Lipids and proteins were the main source of these species, in addition, they were rich in omega-3 and -6 fatty acids. This study confirmed that there are two species, *S. chuatsi* and *S. kneri*, belonging to the genus *Siniperca* in Vietnam. Preliminary investigations also provided useful information on the basic chemical composition of the siniperoid fishes for development and promotion of aquaculture.

Keywords: phylogenetic analysis; chemical composition; siniperoid fish; *Siniperca kneri*; *Siniperca chuatsi*

Introduction

The siniperoid fishes (perch fish) belong to a group of lower percoid fishes distributed only in East Asia. The siniperoid fishes, consisting of three genera and 12 species, are endemic freshwater fish in East Asia (Zhou et al., 1988). Interspecific and intergeneric morphological differences and phylogenetic relationships are important research subjects in studies about adaptation and evolutionary biology of freshwater fishes in East Asia (Zhao et al., 2006; Cao et al., 2021). It was known that geographical isolation and a high number of ecological niches are one of the factors leading to forma-

tion and divergence (Cao et al., 2021; Zhao et al., 2006). Zhou et al. (1988) proposed that these species have been divided into three genera, *Siniperca*, *Coreoperca*, and *Coreosiniperca*, based on some skeletal characteristics and LDH isozyme pattern. It was reported that *Siniperca chuatsi*, *S. kneri*, and *S. scherzeri* are three major species in Siniperoidinae (Chen et al., 2012). Increased demand for production of these species has accelerated extensive aquaculture of them. Using the morphological and skeletal comparison and ecological adaptability of siniperoid fishes, Cao et al. (2021) revealed that the morphological differences between *S. chuatsi* and *S. kneri* are closely related to the spatial position of the

skeleton. This indicated that phenotypic differences have reflected the ecological adaptation of these species to different natural environments. Other previous molecular studies greatly provided the interrelationships among major clades of the Siniperoidae based on mitochondrial DNA gene sequence, interspecific relationships among species produced controversial results (Chen et al., 2007; Chen et al., 2010; Li et al., 2010; Chen et al., 2012). Among those studies, it was confirmed there was a sister relationship between *S. chuatsi* and *S. kneri*, whereas the other relationships were either inconsistent or had low support (Chen et al., 2007; Chen et al., 2010; Li et al., 2010; Chen et al., 2012).

The siniperoids are a group of freshwater perciform fish endemic in East Asia, but widely distributed in southern China, next to the Northern mountainous area of Vietnam. Thus, this has stimulated an interesting group for biogeographic studies on freshwater fish fauna in Vietnam. However, more problem challenging the systematics of the siniperoids is that the species limits remain still unclear in Vietnam's northern mountains.

It was well known that fish has provided an important natural resource as a health food because several species have been identified as rich in easily digestible protein containing all the essential amino acids, therapeutically important polyunsaturated fatty acids, in addition to calcium, iodine, vitamins, and many other nutrients (González et al., 2006; Petricorena, 2015). It was considered that climate change might negatively affect marine fisheries and aquaculture in the coming years. This has led to changes in feeding behavior, food intake, and the chemical composition of fish (He et al., 2013). Although there are intensive research efforts on feeding behavior, food intake, some qualitative properties of common fish from the different aquatic environments, and chemical composition of fish and fishery products (González et al., 2006; Morton et al., 2006; Petricorena, 2015) with other nutritional components that are positively attributed to health. Some micronutrients are in general more abundant in aquatic animals and plants than mammalian meats or terrestrial vegetables. Since ancient times, fish and shellfish have been used as food, but only in the second period of the twentieth century has aquatic food gained enormous relevance. Chemical composition of fish varies greatly among species and from an individual fish to another, depending on age, sex, environment, and season. Fish is the only protein source that contains all the essential amino acids. Lipids and proteins are the major components of fish food; meanwhile carbohydrates are usually detected at very low levels (<0.5 %), there is a lack of information on chemical composition of these species. Taking these into account, in the current study, we investigated the number of the species and their relation-

ship belonging to the siniperoid fishes presented in Vietnam using genetic sequence analysis. In addition, an important issue of this study is to determine the nutritional composition of the species investigated in the Northern mountainous area of Vietnam. The current study may provide useful information on the species and their relationship presented in Vietnam and their chemical composition values, stimulating the aquaculture industry to increase perch production to satisfy regional demand.

Materials and Methods

Fish samples were collected in Lang Son, Ha Giang, Bac Kan and Cao Bang province in the northern mountainous region of Vietnam, bordering China, and stored in 96% ethanol (EtOH) before experimentation. Forty-five fish samples were coded from RM1 to RM45.

Chemicals and reagents

Sodium hydroxide (NaOH), sulphuric acid (H₂SO₄), hydrochloric acid 6N (HCl), ethanol (EtOH), and methanol (MeOH) were obtained from Merck. All other reagents were of analytical reagent grade and were used without further purification.

Total DNA extraction

Total genomic DNA is extracted from the fish fins using a modified salt precipitation method (Sambrook & Russell, 2001). The extracted DNA product was tested on agarose gel at a concentration of 0.8 - 1%. The purity of DNA was determined by NanoDrop. The principle of the method is based on the absorption of light at the wavelength of 260 nm and 280 nm purine and pyrimidine bases. One unit of OD (Optical Density) at 260 nm is equal to a concentration of 50 µg/ml for the double-stranded DNA solution calculated by the formula: Concentration of DNA (µg/mL) = OD_{260nm} × 50 × dilution coefficient. The DNA solution was considered to be clean (without protein) when the ratio OD_{260nm}/OD_{280nm} is between 1.8–2.0.

Gene amplification PCR reaction (PCR1)

Gene amplification PCR reaction (PCR1) is based on the PCR technique, which allows rapid cloning of a DNA sequence many times in a few hours. Primers were used to amplify COI gene fragments of mtDNA of this species according to Ward et al. (2005), including FishF1 (F: 5'TCAACCAACCACAAAGACATTGGCAC3') and FishR1 (R: 5'TAGACTTCTGGGTGGCCAAAGAATCA3'). The PCR1 reactions were carried out in a total volume of µL containing 25 µL of MyTaq™ Mix 2× (Bioline),

2 μL of forward primer at 10 μM , 2 μL of reverse primer at 10 μM , 2 μL of ADN structure (200 ng/mL), and 19 μL of deionized water. The amplification program included denaturation for 2 min at 94°C and 35 cycles for 30 s at 95°C, 15 s annealing at 58°C, and 30 s extension at 72°C. The final extension was 4 min at 72°C and stored at 4°C.

The amplified products were evaluated by agarose gel electrophoresis at a concentration of 1.5%.

Purification of the amplified product (PCR1)

The amplified products (PCR1) were purified using QI-Aquick PCR Purification Kit from Qiagen, following the protocol described by the supplier.

PCR sequencing reaction (PCR2)

The components including 8 μL of DTCS Quick Start Kit, 0.5-10 μL of the purified PCR1 product at 50 ng/100 fmol, and 2 μL of Primer at 1.6 μM were mixed and then distilled water was added to obtain 20 μL of the mixture. The heat cycle for the PCR1 sequencing reaction (repeating 30 cycles) was as follows: 96°C for 20 s, 50°C for 20 s, 60°C for 4 min, and storage of product at 4°C.

Purification of PCR2 product

The above mixture of purified PCR2 product (20 μL) was transferred to new eppendorf containing 2 μL of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2$) at 3M, pH 5.2; 2 μL of EDTA at 100 mM, pH 8.0; and 1 μL of glycogen at 20 mg/mL. Then, 75 μL of 95% ethanol was added, and the tubes were centrifuged at 13000 g for 20 min at 4°C. The supernatants were removed to obtain the precipitated DNA in the bottom. The precipitates were washed with 200 μL of 70% EtOH, vortexed, and centrifuged at 13000 g for 3.5 min at 4°C. After centrifugation, the EtOH was removed to obtain the DNA pellets. The DNA pellets were air-dried and then diluted with 40 μL of sodium lauryl sulfate (SLS).

Gene sequencing analysis

Gene sequencing analysis was performed on Genome-Lab GeXP. The DNA solutions were transferred to the well on the running plate. Each well was poured with one drop of mineral oil, which was provided together with the DTCS Quick Start Kit, on top to prevent sample evaporation. For data analysis, the gene sequences were evaluated for quality using Finch TV 1.4.0 software (<http://www.geospiza.com>). The program ClustalW Multiple Alignment in BioEdit 7.1.9 was used to compare and align the sequences, then evaluated and determined the similarity. The COI gene sequences of the fish samples were compared with the DNA sequence of this fish published on the International Gene Bank using the

BLAST tool on NCBI (National Center for Biotechnology Information) or BOLD (National Center for Biotechnology Information) Barcode of Life Data System) to identify similar species.

Chemical composition analysis

For analysis, individual samples were prepared from fish musculature (the lateral muscle) taken from the same position after removal of skin and then homogenized in ice-cold.

Fish fillets ($n = 5$ per treatment) were triplicate for lipid (Soxhlet method; AOAC, 2000), moisture (AOAC, 2000) and Kjeldahl protein, which was calculated using the Kjeldahl nitrogen and conversion factor 6.25 ($N \times 6.25$) (AOAC, 2000).

The amino acid composition was determined using AccQ-Fluor reagent for derivatization as previously described (Marten & Naguschewski, 2011). This method used a free amino acid column (symmetry C_{18} (5 μm , 4.6 x 250 mm), Water, USA). Namely, amino acids were subjected to precolumn derivatization using AccQ-Fluor reagent. Each sample was homogenized, weighed (1 g) in a test tube with a screw cap, and 9 mL of HCl 6N was added. Test tubes were sealed and autoclaved for 16 h at 125°C. Samples were then introduced into 100 mL volumetric flasks and brought to volume with purified water. A 10 μL aliquot of the sample was added with 70 μL AccQ-Fluor Borate buffer and 20 μL AccQ-Fluor Reagent for derivatization. Derivatives were then injected and analyzed using high-performance liquid chromatography (HPLC).

The fatty acid composition of lipids is determined by applying gas chromatography (GC) after transmethylation of the respective sample with 2% H_2SO_4 in absolute methanol (MeOH) at 50°C (Ivanova et al., 2018; ISO 5509: 2000). Identification of fatty acids (ω -3 and ω -6 fatty acids) was performed by comparison of retention times with those of a standard mixture of fatty acids subjected to GC under identical experimental conditions (ISO 5508: 2004). All these data are presented as arithmetic means \pm standard deviation (\pm SD).

Results and Discussion

COI gene sequencing

After optimizing the conditions for PCR to amplify the COI gene fragment in the fish samples, we performed PCR to amplify this gene fragment in all the obtained samples and conducted electrophoresis to evaluate the product by 1.5% gel agarose. The results showed that the PCR product band was clear and no by-products appear, and the amplified gene fragment size was in the range of 700 bp (Figure 1). This

Table 1. Comparison BLAST on the BOLD database

Sample code	Similar species	Code on BOLD	Degree of similarity, %	Sample code	Similar species	Code on BOLD	Degree of similarity, %
RM1	<i>Siniperca knerii</i>	KU049656	99.85	RM24	<i>Siniperca knerii</i>	KU049658	100
RM2	<i>Siniperca knerii</i>	KU049657	99.85	RM25	<i>Siniperca chuatsi</i>	MW402975	100
RM3	<i>Siniperca knerii</i>	KU049658	99.85	RM26	<i>Siniperca chuatsi</i>	MW402975	100
RM4	<i>Siniperca chuatsi</i>	MW402975	100	RM27	<i>Siniperca chuatsi</i>	MW402975	100
RM5	<i>Siniperca knerii</i>	KU049658	100	RM28	<i>Siniperca knerii</i>	KU049658	100
RM6	<i>Siniperca chuatsi</i>	MW402975	100	RM29	<i>Siniperca chuatsi</i>	MW402975	100
RM7	<i>Siniperca chuatsi</i>	MW402975	100	RM30	<i>Siniperca knerii</i>	KU049656	99.85
RM8	<i>Siniperca chuatsi</i>	MW402975	100	RM31	<i>Siniperca knerii</i>	KU049656	99.85
RM9	<i>Siniperca knerii</i>	KU049658	100	RM32	<i>Siniperca chuatsi</i>	MW402975	100
RM10	<i>Siniperca chuatsi</i>	MW402975	100	RM33	<i>Siniperca knerii</i>	KU049658	100
RM11	<i>Siniperca knerii</i>	KU049656	99.85	RM34	<i>Siniperca knerii</i>	KU049658	100
RM12	<i>Siniperca knerii</i>	KU049656	99.85	RM35	<i>Siniperca knerii</i>	KU049658	100
RM13	<i>Siniperca chuatsi</i>	MW402975	100	RM36	<i>Siniperca chuatsi</i>	MW402975	100
RM14	<i>Siniperca knerii</i>	KU049658	100	RM37	<i>Siniperca chuatsi</i>	MW402975	100
RM15	<i>Siniperca knerii</i>	KU049658	100	RM38	<i>Siniperca knerii</i>	KU049658	100
RM16	<i>Siniperca knerii</i>	KU049658	100	RM39	<i>Siniperca chuatsi</i>	MW402975	100
RM17	<i>Siniperca chuatsi</i>	MW402975	100	RM40	<i>Siniperca knerii</i>	KU049656	99.85
RM18	<i>Siniperca chuatsi</i>	MW402975	100	RM41	<i>Siniperca knerii</i>	KU049656	99.85
RM19	<i>Siniperca knerii</i>	KU049658	100	RM42	<i>Siniperca knerii</i>	KU049658	100
RM20	<i>Siniperca chuatsi</i>	MW402975	100	RM43	<i>Siniperca knerii</i>	KU049658	100
RM21	<i>Siniperca chuatsi</i>	MW402975	100	RM44	<i>Siniperca knerii</i>	KU049658	100
RM22	<i>Siniperca chuatsi</i>	MW402975	100	RM45	<i>Siniperca chuatsi</i>	MW402975	100
RM23	<i>Siniperca knerii</i>	KU049658	100				

when comparing the nucleotide sequences of 2 haplotypes of the *S. knerii* species. The results also showed that *S. knerii* and *S. chuatsi* were replaced at Cytosine (C) and Thymine (T) at position 466 belonging to red circled area when comparing sequences of three haplotypes. In there, two haplotypes were represented for *S. knerii* species and the other for *S. chuatsi* species. It has been presumed that *S. chuatsi* and *S. knerii* have evolved from a common ancestor 4.3 million years ago (Chen et al., 2010; Song et al., 2017), and evolved certain adaptive characters to different environments.

In general, fish species exhibit greater variation in morphological features both within and between species and between populations than any other vertebrate (Jawad et al., 2022). This may be due to changes in feeding environment, prey type, food availability and other abiotic aspects (Cao et al., 2021; Jawad et al., 2022). The natural distribution of *S. chuatsi*, and *S. knerii* is different in geographical location and water system, but there are overlaps and cross-distributions. It was found that *S. chuatsi* is distributed in the northern areas, *S. knerii* in the southern areas of China, bordering

Fig. 3. Nucleotide differences in the genus *Siniperca*

Vietnam. In overlapping distribution areas, *S. knerii* can be present in the upper, middle, and lower reaches and tributaries, while *S. chuatsi* lives in the middle and lower reaches or lakes (Zhao et al., 2006; Chen et al., 2010; Cao et al., 2021). It is presumed that *S. chuatsi* and *S. knerii* differentiated as a result of adaptation to different freshwater environments.

Phylogenetic analysis

There are two propositions on the classification of the siniperoid fishes (Peng et al., 2020; Cao et al., 2021). In the *Siniperca* group, *S. chuatsi* was the sister species of *S. knerii*, both of them were then grouped with the other species in *Siniperca* along with *Coreosiniperca roulei* renamed as *Siniperca roulei*. In this study, we determined the genetic relationships among some species of the genus *Siniperca* based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA) consisting of 7 sequences, in which two sequences represented *S. knerii* and one sequence represented *S. chuatsi* species. In addition, the sequence of the species including *Siniperca scherzeri*, *Siniperca roulei*, *Siniperca fortis*, and *Siniperca obscura* was analyzed, and the sequence of these species was obtained from NCBI with corresponding GenBank numbers.

Association taxa grouped in the bootstrap test with 1000 replicates were shown next to the branches. The tree was plotted to scale, with branch lengths in units of evolutionary distance used to infer the phylogenetic tree. The evolutionary distance was determined using the Tajima-Nei method (Tajima & Nei, 1984) and units of the number of base substitutions per site. Evolutionary analyses were performed on MEGA X (Kumar et al., 2018). Kumar et al. (2018) reported a transformation of MEGA to enable cross-platform use on Microsoft Windows and Linux operating systems. MEGA X does not require virtualization or emulation software and provides a uniform user experience across platforms. MEGA X has additionally been upgraded to use multiple computing cores for many molecular evolutionary analyses. MEGA X is available in two interfaces (graphical and command line). As shown in Figure 4, the phylogenetic tree showed the genetic relationships of several species of the genus *Siniperca*. Namely, *S. knerii* and *S. chuatsi* identified in this study were closely related to each other, and they were distantly related to the other four species including *S. scherzeri*, *S. roulei*, *S. fortis*, and *S. obscura*. This was shown by the division into two large branches of the phylogenetic tree, in which the two species *S. knerii* and *S. chuatsi* were grouped into branch 1, and the other four species were grouped into branch 2 with the absolute high bootstrap value (100%). Our study confirmed that *S. chuatsi* and *S. knerii* are sister species, next joined by *S. obscura*, *S. roulei*, *S. scherzeri* and finally by

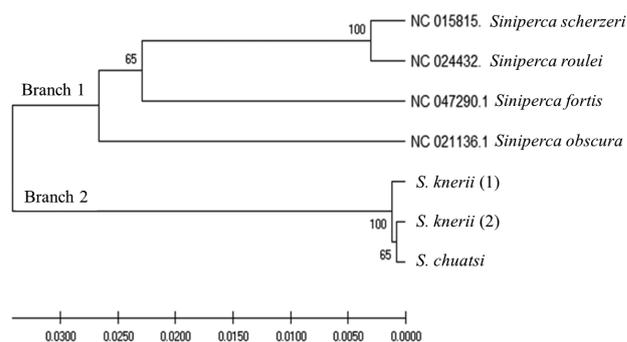


Fig. 4. Diagram of the genetic relationships between two species, *S. chuatsi* and *S. knerii*, and the others of the genus *Siniperca*

S. fortis. The phylogenetic study on the siniperoids resolved interspecific relationships and the evolutionary history of the siniperoids.

It was shown that *Siniperca chuatsi*, *S. knerii* and *S. scherzeri* inhabit large rivers and lakes, whereas *S. obscura*, *S. loonana*, *S. undulata*, *S. roulei* and *Coreoperca* were often found in tributaries or in relatively small drainage (Chen et al., 2010; Chen et al., 2012). *Siniperca scherzeri* is widespread in south China but occurs in scattered localities in Korea and Liaoning province (Song et al., 2017). The siniperoids are endemic to East Asia, and widely distributed in most Chinese river drainages, but the phylogenetic analysis is the first step to understanding the origin of the siniperoids present in Vietnam, the impact of past geological events on their evolutionary patterns, and the evolution of the freshwater fish fauna.

Chemical composition of fish filets

Generally, fish is a nutrient-rich food and possesses many components that have a positive impact on human health since it is a complete protein source that contains all essential amino acids. It was well known that the chemical composition of fish meat to a considerable extent depends on the species, type of food, age, and fish body size. This study gave an overview of the basic chemical composition of wild fish (*S. knerii* and *S. chuatsi*) with body weight of 500 ± 20 g (Table 2). The results showed that the wild fish contained $71.43 \pm 0.73\%$ moisture, $17.83 \pm 2.29\%$ protein, $1.17 \pm 0.76\%$ fat and $4.10 \pm 0.43\%$ ash. It was also found that the wild fish contained 18 amino acids including all essential amino acids. Amino acids can be produced by hydrolyzing proteins. The eight amino acids generally regarded as essential for humans are lysine (0.57 ± 0.04 g/100 g), methionine (0.33 ± 0.02 g/100 g), threonine (0.51 ± 0.03 g/100 g), tryptophan (0.62 ± 0.03 g/100 g), isoleucine (0.61 ± 0.04 g/100 g), leucine (0.52

Table 2. Chemical composition of the perch fish fillets

Moisture, fat, ash, protein and fatty acids			
Moisture (fresh sample, %)	71.43 ± 0.73	Ash, %	4.10 ± 0.43
Lipid (freeze-dried, %)	1.17 ± 0.76	Omega 3, mg/g	78.70 ± 5.10
Protein (freeze-dried, %)	17.83 ± 2.29	Omega 6, mg/g	63.91 ± 5.53
Amino acids, g/100 g			
Histidin	0.51 ± 0.03	Glutamic	1.60 ± 0.09
Threonine	0.51 ± 0.03	Serine	0.35 ± 0.03
Valine	0.30 ± 0.02	Glycine	0.39 ± 0.02
Methionine	0.33 ± 0.02	Alanine	0.29 ± 0.02
Lysine	0.57 ± 0.04	Arginine	0.72 ± 0.07
Leucine	0.52 ± 0.03	Proline	0.28 ± 0.02
Isoleucine	0.61 ± 0.04	Cystein	0.05 ± 0.01
Phenylalanine	0.44 ± 0.03	Tyrosine	0.36 ± 0.02
Aspartic	1.00 ± 0.06	Tryptophan	0.62 ± 0.03

± 0.03 g/100 g), phenylalanine (0.44 ± 0.03 g/100 g), and valine (0.30 ± 0.02 g/100 g).

An important parameter that has attracted the attention of consumers and researchers is the content of n-3 (ω-3) and n-6 (ω-6) fatty acids in these species (Ackman et al., 2002; González et al., 2006; Petricorena, 2015). In this study, high concentrations observed in n-3 and n-6 fatty acids were 78.70 and 63.91 (mg/g), respectively. The fatty acid composition of fish usually differs depending on various factors including species, age, diet, and whether they are farmed or wild (González et al., 2006; Petricorena, 2015; Bochert et al., 2023). Normally, diet may affect the fatty acid composition of fish, and with this, the aquaculture industry possesses an excellent tool for beneficially altering the fatty acid profile of fish (González et al., 2006; Petricorena, 2015) or eggs of fish (Bochert et al., 2023). González et al. (2006) showed that farmed yellow perch contained a significantly higher n-2:n-6 ratio, indicating that with appropriate dietary ingredients fatty acid profiles could be beneficially altered in farmed yellow perch. This important issue can stimulate the aquaculture industry to increase the production of perch fish in the near future because the increased amount of fatty acids, especially n-3 (ω-3) and n-6 (ω-6) fatty acids, must always be attractive to consumers.

Preliminary investigations provided useful information on the basic chemical composition of perch fish for the development and promotion of aquaculture of this species. In further study, it is necessary to evaluate the nutritional composition at different stages of development as well as identify the factors affecting the nutritional composition including feed, water source, environmental conditions, and so on to harvest the best source of nutrients.

Conclusions

We reported here for the first time the complete genetic sequence of the siniperca fishes in Vietnam's northern mountains. In this research, it was confirmed that there are two species, *S. chuatsi* and *S. knerii*, belonging to the siniperca fishes in the Northern mountainous area of Vietnam. These data provided useful information for further understanding of the phylogenetic classification, evolution and genetic sequence of the siniperca fishes. Preliminary investigations also provided useful information on the basic chemical composition of these species. This could be used as a basis for genetic studies and nutritional values of the species presented in Vietnam, stimulating the aquaculture industry to increase fish production to satisfy regional demand.

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