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**บทความวิจัย**

# ความแตกต่างทางพันธุกรรมของปลาโจกทรายในแม่น้ำโขง จังหวัดหนองคาย ประเทศไทย

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*ความแตกต่างทางพันธุกรรม*

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**บทคัดย่อ**

การศึกษานี้นำเสนอการประเมินความหลากหลายทางพันธุกรรมครั้งแรกของ Cyclocheilichthys enoplos ซึ่งเป็นปลาชนิดหนึ่งที่อยู่ในภูมิภาคเอเชียตะวันออกเฉียงใต้ และมีบทบาทสำคัญในเศรษฐกิจท้องถิ่นเป็นแหล่งอาหารสำหรับประชาชนในพื้นที่ ในการศึกษานี้ได้ใช้เครื่องหมายสก๊อต (Start Codon Targeted, SCoT, 9 primers) เพื่อประเมินความหลากหลายทางพันธุกรรมของปลาโจกทรายสามกลุ่มประชากรจากธรรมชาติในแม่น้ำโขง จังหวัดหนองคาย ประเทศไทย ตัวอย่างปลาที่เก็บมาทั้งหมด 28 ตัวอย่าง ผลการทดสอบพบว่าให้แถบดีเอ็นเอที่ให้โพลิมอร์ฟิซึมรวม 158 แถบ และมีค่า % of polymorphic loci (% P) อยู่ระหว่าง 48.80-85.54 % ประชากรจาก อำเภอรัตนวาปี (RP) มีความแตกต่างทางพันธุกรรมของประชากรสูงที่สุด ในขณะที่ประชากรจาก อำเภอสังคม (SK) มีระดับการแตกต่างทางพันธุกรรมของประชากรที่ต่ำที่สุด ค่าความหลากหลายทางพันธุกรรม (heterozygosity; He) และค่าดัชนีความหลากหลายทางพันธุกรรม (Shannon's Information index; I) ของทั้งสามประชากรอยู่ระหว่าง 0.195-0.296 และ 0.285-0.444 ตามลำดับ และแผนภาพจัดกลุ่มความสัมพันธ์จากค่า Nei's genetic distance และแผนภาพความสัมพันธ์เชิงวิวัฒนาการจากโปรแกรม NTSYS-PC จัดแบ่งประชากรออกเป็น 2 กลุ่มที่แตกต่างกันอย่างชัดเจน การวิเคราะห์ความแปรปรวนทางพันธุกรรมของประชากรระดับโมเลกุล (AMOVA) ได้แสดงให้เห็นว่าส่วนใหญ่ของความแตกต่างเกิดขึ้นภายในกลุ่มประชากร (87 %) ในขณะที่ความแตกต่างระหว่างกลุ่มประชากรมีค่าน้อย (13 %) โดยการวิจัยนี้จะเป็นข้อมูลเบื้องต้นสำหรับการจัดการประชากรปลาโจกทรายในภูมิภาคนี้

## Introduction

The Mekong River is the main river in Southeast Asia and one of the world's longest rivers, flowing approximately 4400 kilometers from the Tibetan plateau in China to Vietnam after crossing Thailand 1520 kilometers and through Nongkai Province 210.6 kilometers. The Mekong River possesses the world's second-highest inland fish variety with around 1100 species (Baran et al., 2005; Coates et al., 2006; Kang & Huang, 2021). It also provides habitat for one of the most productive inland fisheries (Hortle, 2009). *Cyclocheilichthys enoplos*, a freshwater fish in the Cyprinidae family, has a wide distribution range in Thailand, Vietnam, Laos, Cambodia, Malaysia, and Indonesia (Luo et al., 2018) with adult fish ranging in length from 35 to 80 centimeters and weight from 0.45 to 8 kilograms. This fish species is economically valued, fetching a high price of 150-200 baht per kilogram in the Northeast region of Thailand, making it a lucrative source of revenue for local fishermen earning 1000-2000 baht per day. *C. enoplos* is a renowned fish species found in the Nan River that serves as the provincial fish of Uttaradit Thailand (Seel-audom et al., 2021).

*C. enoplos* commonly known as "Ta-Kok" is notable for its huge ovary and great fecundity, allowing it to produce a large

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number of eggs during its spawning season, which occurs between July-August (Ratanatrivong et al., 1993). Notability, a considerable decrease in fish quantity has been documented in the Nan River, dropping from 127 tons to 5 tons between 2007-2016 (Seel-audom et al., 2021). Analogous to the situation in the Mekong River, *C. enoplos* is particularly vulnerable to variations in water levels, especially during the start of the rainy season, when the Mekong water recedes swiftly, rendering reproduction more challenging. Additionally, the use of both organic and inorganic chemicals in fish cage farming has intensified in the Mekong River. The application of various fishing tools has enhanced fishing efficiency, affecting the Mekong River's ecosystem and contributing to a reduction in the number and diversity of Mekong fish species (Uawonggul et al., 2019).

The Start Codon Targeted Marker (SCoT) is a dominant marker that employs an ATG specific primer sequence (Collard & Mackill, 2009). SCoT has emerged as a preferred marker for low DNA concentrations since it does not require prior knowledge of DNA sequence genome. SCoT amplified fragment produced distinct and sharp bands that are convenient, rapid, and cost-effective for genetic studies. The genetic diversity of SCoT markers has been studied in a variety of fish species, including *Dicentrarchus punctus, Cheilinus trilobatus, Cheilinus quinquecinctus,* and *Chlorurus sordidus* (Hassan et al., 2020) *Dicentrarchus labrax* (Almaaty, 2020) *Mugil cephalus, Liza ramada, Liza grana,* and *Valamugil seheli* (Elian et al., 2021).

The purpose of the study was to evaluate genotypes using SCoT markers in order to investigate the genetic diversity and population structure of *C. enoplos*. The work is pioneering attempt to explore *C. enoplos* wild populations inside the Mekong River in Thailand's Nong Khai Province from a molecular perspective. Its findings are crucial for the region's conservation and governance of this species.

## Materials and methods

*Ethics approval and consent to participate*

The research procedures outlined in this study, including animal experimentation, were approved by the Ethics Committee for the Institutional Animal Care and Use Committee of Khon Kaen University (Record No. IACUC-KKU-24/66, Reference No. 660201.2.11/168 (28)), in accordance with the National Research Council of Thailand's Ethic of Animal Experimentation guidelines. To safeguard endangered species, only a little amount of fin tissue was obtained for each sample.

*Animal and sample collection*

The specimens of *C. enoplos* analyzed in this research were obtained from the collections of Mapanao et al. (2023), conducted from June 2021 to May 2022 and subsequently from February 2023 to July 2023. These specimens were sourced by local fishermen from the Mekong River in Nong Kai Province, Thailand, specifically from the locations of SK (Sangkhom, n = 4), M (Muang, n = 9), and RP (Rattanawapee, n = 15) (Figure 1). Additionally, *Cosmochilus harmandi* and *Bagarius bagarius* were included as outgroups for phylogenetic analysis. Rainboth's (1996) classification was used to identify the gathered samples as *C. enoplos* based on their morphological characteristics. Each deceased fish specimen, which was generously supplied by a fisherman, had a tissue segment taken that included the fin and/or muscle. The tissue was then preserved in 95 % ethanol to facilitate future DNA isolation.


**Figure 1** Location of *C. enoplos* populations sampled along the Mekong River in Nong Kai Province of Thailand

*Genomic DNA extraction*

Genomic DNA was extracted from fin clips or muscle tissues utilizing a modified salt extraction protocol (Lopera-Barrero et al., 2008). The quality of the DNA extracts was assessed by determining the ratio of wavelengths at 260 and 280 nm (Genova nano 737-501, Jenway, United Kingdom). The quality of the DNA samples was also evaluated through agarose gel electrophoresis, which was carried out in 1X TBE buffer at a voltage of 120 V for 30 minutes. The gel was stained with ethidium bromide for visualization.

*The Start Codon Targeted Marker (SCoT) analysis*

Nine of the thirty-six *SCoT* markers described in Table 1 were selected for the genotyping of specimens. The PCR reaction mixture (10 µl) contains 10 ng of DNA template, 2 µl of 5X HOT FIREPol® Blend Master Mix (Solis Biodtne, Estonia), 1 µl of 10 pM of each primer, and distilled water added to reach a final volume of 10 µl. The PCR cycling conditions were as follows: a 12-minute initial denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 50-55°C for 30 seconds, and extension at 72°C for 30 seconds, with a 5-minute final extension step at 72°C. The samples were examined on a 2 % agarose gel and stained with ethidium bromide to visualize the PCR amplification results. A 100-base pair (bp) molecular size ladder (Vivantis, Malaysia) was used to estimate fragment sizes.

**Table 1** The SCoT primers used for amplification, as well as the best conditions for each primer and the polymorphic DNA band (T = temperature, Pb = Polymorphic bands, PIC, polymorphism information content)

| NO. | Primer Name | Primers Sequence | T (°C) | PB | PIC |
|---|---|---|---|---|---|
| 1. | SCoT-2 | CAACAATGGCTACCACCC | 55 | 15 | 0.38 |
| 2. | SCoT -4 | CAACAATGGCTACCACCT | 50 | 23 | 0.38 |
| 3. | SCoT -6 | CAACAATGGCTACCACCT | 55 | 18 | 0.37 |
| 4. | SCoT -7 | CAACAATGGCTACCACGA | 55 | 18 | 0.40 |
| 5. | SCoT -11 | AAGCAATGGCTACCACCA | 50 | 16 | 0.37 |
| 6. | SCoT -12 | ACGACATGGCGACCAACG | 55 | 19 | 0.34 |
| 7. | SCoT -17 | ACCATGGCTACCACCGAG | 55 | 19 | 0.36 |
| 8. | SCoT -31 | CCATGGCTACCACCGCCT | 55 | 17 | 0.27 |
| 9. | SCoT -34 | ACCATGGCTACCACCGCA | 55 | 13 | 0.32 |
| Total | | | | 158 | |
| | | **Average** | | **17.50** | **0.35** |

*Data analysis*

The SCoT bands were encoded using binary characters, with 1 representing presence and 0 representing absence. These binary characters were utilized subsequent investigation. A UPGMA dendrogram was

calculated and established using the NTSYSpc 2.1 software (Rohlf, 2000) to assess the genetic link between the samples tested. The genetic diversity of both intra-population and inter-cultivar was evaluated by computing several genetic diversity characteristics such as the number of monomorphic bands, polymorphic bands, and % of polymorphism (%). Additionally, expected heterozygosity (*He*) and Shannon's information index (*I*) were calculated by GenAlEx 6.5 program (Peakall & Smouse, 2012). Moreover, the diversity within the population (Hs), total species diversity (Ht), and gene flow (Nm) were also computed using the POPGENE software (Version 1.31) (Yeh et al, 1999). To describe the distribution of genetic variability among and within populations, the Analysis of Molecular Variance (AMOVA), Principal Coordinate Analysis (PCoA) and Mantel test were performed using the GenAlEx 6.5 program.

## Results and Discussion

*SCoT primer evaluation*

After the preliminary screening of 36 SCoT markers, nine SCoT markers were selected. This selection was based on the demonstration of distinct polymorphic band patterns and the consistency in reproducing results across various assessments. A total of nine markers generated 158 fragments with an average of 17.5 fragments per primer. SCoT-4 produced the most bands, with a maximum of 23, while SCoT-34 produced the fewest bands, with a minimum of 13. The mean Polymorphism Information Content (PIC) value was 0.35, ranging from 0.27 for SCoT-31 to 0.40 for SCoT-7 (Table 1). According to the current investigation, the selected markers have moderate polymorphisms (PIC = 0.356), where 0.25 < PIC < 0.5 reflect moderate polymorphisms and PIC values < 0.25 indicate a low rate of polymorphism (Zhang et al., 2020).

The phylogenetic relationships among the 28 genotypes were analyzed using the UPGMA method in the NTSYSpc program. The genotypes were found to be separated into two distinct clusters with a similarity range of 0.50-0.80 (Figure 2). The dendrogram clearly distinguished between the two groups. The first group included RP1, RP2, RP4, RP3, RP8, RP12, RP13, RP10, RP11, RP14, RP15, M2, M4, M6, RP6, M8, RP16, M3, M5, RP7, and RP9, while the second group included M7, M9, M10, SK1, SK2, SK3, and SK4. *Cosmochilus harmandi* and *Bagarius bagarius* made comprised the outgroup.

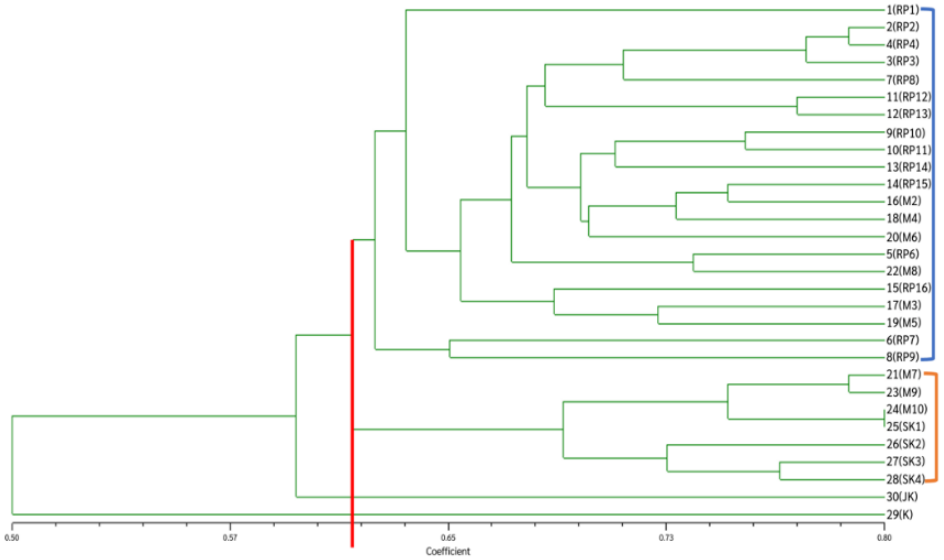


**Figure 2** Dendrogram shows the diversity relationship between the study specimens analyzed with NTSYS 2.1 *C. enoplos*; SK=Sangkhom, M=Muang, RP=Rattanawapee and outgroup; JK= *Cosmochilus harmandi*, K= *Bagarius bagarius*

*Genetic diversity*

The genetic diversity of the population was analyzed using the GenAlEx 6.5 program, which revealed a % of polymorphic loci (% P) range of 48.80-85.54 %, expected heterozygosity (He) range of 0.195-0.296, and Shannon's Information index *(I)* range of 0.285-0.444. The RP site had the highest diversity P = 85.54 %, He = 0.296, I = 0.444 followed by the M site P = 74.10 %, He = 0.276, I = 0.408 and the SK site population had the lowest diversity P = 48.80 %, He = 0.195, I = 0.285. The overall genetic diversity of the *C. enoplos* population was P = 69.48 %, He = 0.256 and I = 0.379 (Table 2).

**Table 2** Genetic diversity of *C. enoplos* (N = numbers of samples, P (%) = % of polymorphic loci, He = expected heterozygosity and I = Shannon's information index)

| Population | N | P (%) | He | I |
|---|---|---|---|---|
| RP (Rattanawapee) | 15 | 85.54 % | 0.296±0.014 | 0.444±0.018 |
| M (Muang) | 9 | 74.10 % | 0.276±0.015 | 0.408±0.021 |
| SK (Sangkhom) | 4 | 48.80 % | 0.195±0.017 | 0.285±0.024 |
| Total Population | 28 | 69.48 % | 0.256±0.009 | 0.379±0.013 |

The Popgen 32 program was used for Nei's gene diversity analysis, which revealed that the Nei's gene diversity among populations (Ht) was 0.3315±0.0251, the Nei's gene diversity within subpopulations (Hs) was 0.2558±0.0192, and the coefficient of differentiation (Gst) was 0.2283. The variance between populations was 13 %, while the intra-population variance was 87 %. Furthermore, the PhiPT value was 0.129 ($p>0.001$) (Table 3), indicating that the *C. enoplos* population had moderate genetic diversity (Mir et al., 2021). The genetic linkages between and within populations were also evaluated using principal coordinates analysis (PCoA), which indicated that the two fish groups were clustered (Figure 3). The first and second coordinates explained 12.67 % and 8.16 % of the total molecular variance, respectively (Figure 4). The Nm value of 1.690 indicated a population gene flow rate.

**Table 3** Analysis of molecular variance (AMOVA) in *C. enoplos* (Df = degrees of freedom; SS = sum of squared observations; MS = mean of squared observations; Est. Var. = estimated variance; PhiPT = proportion of total genetic variance between individuals within populations)

| Source of variation | Df | SS | MS | Est. Var. | Value % |
|---|---|---|---|---|---|
| Among pops | 2 | 118.902 | 59.451 | 3.969 | 13 % |
| Within Pops | 25 | 667.633 | 26.705 | 26.705 | 87 % |
| Total | 27 | 786.536 | | 30.675 | 100 % |
| PhiPT = 0.129, $p < 0.001$ | | | | | |

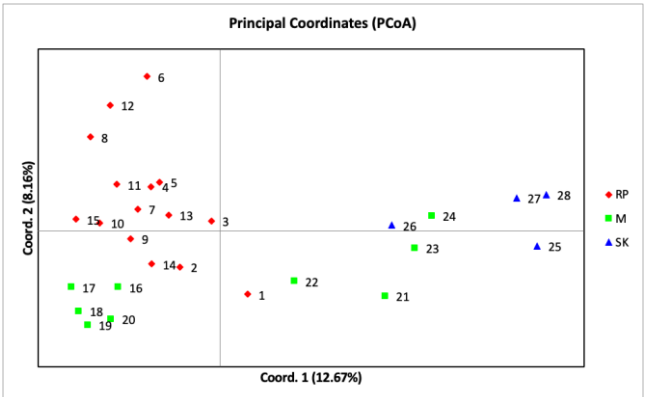


**Figure 3** Principal coordinate analysis and structural analyses of three populations of *C. enoplos* RP = Rattanawapee, M = Muang, SK = Sangkhom

The Nei Genetic Distance analysis produced a pairwise population matrix that revealed the genetic distance across populations ranged from 0.067-0.229. The highest genetic distance of 0.229 was observed between the RP and SK populations, while the smallest distance of 0.067 was between the RP and M populations (Table 4). A dendrogram was constructed using the UPGMA relationship approach, which showed that the RP and M populations were the most closely related. The greatest genetic divergence was discovered between RP and SK (Figure 4). In addition, Mantel tests demonstrated a positive correlation between geographic and genetic distance among populations ($R^2 = 0.6044$, p-value = 0.0048) (Figure 5).

**Table 4** Nei's unbiased genetic distance (below diagonal) and identity (above diagonal) among three populations of *C. enoplos* from RP (Rattanawapee), M (Muang) and SK (Sangkhom) estimated from SCoT marker

| | Rattanawapee | Muang | Sangkhom |
|---|---|---|---|
| RP (Rattanawapee) | 0.000 | 0.935 | 0.795 |
| M (Muang) | 0.067 | 0.000 | 0.820 |
| SK (Sangkhom) | 0.229 | 0.198 | 0.000 |

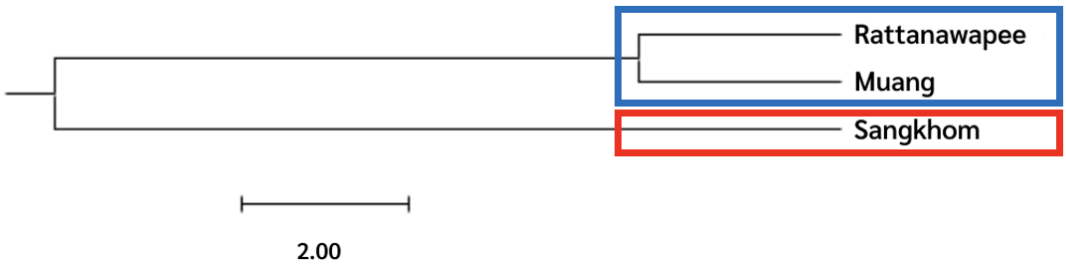


**Figure 4** Dendrogram produced following the unweighted pair group method with an arithmetic mean of *C. enoplos* populations based on the genetic distance of Nei (1972)

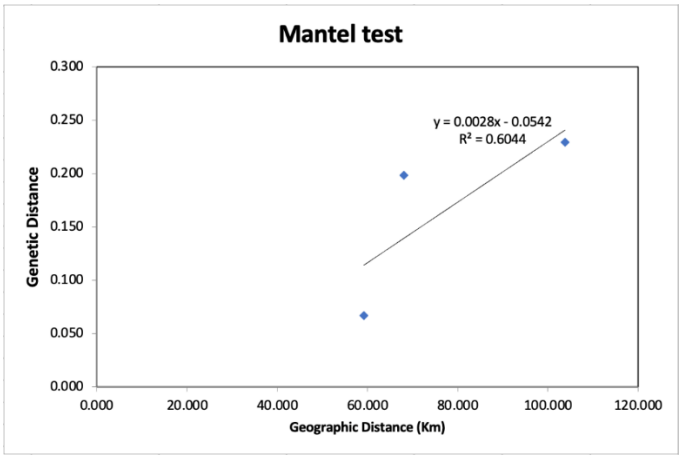


**Figure 5** Correlation test of genetic distance (GD) and geographic distance (Km)

SCoT markers are a set of reproducible markers that were developed based on short and conserved regions in plant genomes positioned near translation initiation codons (Collard & Mackill, 2009). These markers have been utilized to assess genetic diversity, population structure, and to distinguish cultivars. However, their application in animal studies remains limited (Hassan et al., 2020). Nevertheless, recent studies have demonstrated that SCoT markers can be used successfully in DNA fingerprinting, identification of cultivars, and estimation of genetic variation and structure in aquatic animals such as *Neverita josephinia*, *Hexaplex trunculus*, and *Murex altispira*. Ten SCoT primers yielded 115 amplicons spanning from 120 to 1500 bp in one study,

with a polymorphism % of 20-78 % (Almaaty, 2020). Furthermore, the results of utilizing SCoT and ISSR markers did not differ considerably in terms of genetic diversity. For ISSR and SCoT markers, *Cheilinus trilobatus*, *Cheilinus quinquecinctus*, and *Chlorurus sordidus* specimens from Saudi Arabia's Farasan had expected heterozygosity (Hexp) of 0.470 and 0.435, respectively, and an average polymorphism information content (PIC) of 0.359 and 0.339, respectively (Hassan *et al.*, 2020). In addition, studies have shown that the use of ISSR and SCoT markers in assessing genetic variation and structure in *Mugil cephalus*, *Liza ramada*, *Liza grana*, and *Valamugil seheli* samples collected from four different locations in Egypt produced amplified amplicons, with 176 and 132 for SCoT and ISSR markers, respectively. 153 and 111 of these amplicons were polymorphic, resulting in polymorphism % of 86.90 % and 84.10 % polymorphic amplicons/primer, respectively. The similarity indices with SCoT and ISSR markers ranged from 0.47 to 0.84 and 0.54 to 0.92, respectively (Elian et al., 2021). Both markers produced comparable findings.

The genetic diversity of *C. enoplos* populations, using 28 samples from the Nong Khai Province along the Mekong River in Thailand. The previous studies within the Cypriniformes order haplotypic diversity in *Cobitis dalmatina* was analyzed with 20 samples (Buj et al., 2015), and *Squalius illyricus* with ten samples, identifying four haplotypes from the Cetina River (Buj et al., 2020). For *C. enoplos* showed high levels of genetic diversity, with an expected heterozygosity (*He*) of 0.256±0.009 and the Shannon index (*I*) of 0.379±0.013 (Table 2). In comparison, a prior study on *Labeo chrysophekadion* in the Mekong Delta of Laos using similar dominant markers, notably ISSR found mean estimates of *He* and *I* to be 0.300 and 0.436, respectively (Mashyaka & Duong, 2021). Another study using ISSR markers discovered that wild populations of bighead catfish (*Clarias macrocephalus*) in Vietnam's the Mekong Delta had He of 0.298±0.023 and I of 0.440±0.032 (Nguyen & Duong, 2022), which were relatively higher than those identified in *C. enoplos.*

In addition, the Mantel test revealed that the genetic connections among diverse groups were consistent with their respective geographic proximity. Geographic distance and genetic distance were shown to have a substantial positive correlation ($R^2 = 0.6044$, p-value = 0.0048) among these populations. The presence of geographic barriers has resulted in the emergence of two distinct gene groups within the *C. enoplos* species, which can be linked to genetic differentiation. The present study provides preliminary results indicating that the examined species have a moderate level of genetic diversity. Future research with greater sample numbers and more sampling sites, however, are required to gain thorough insights into the genetic structure of these species. Notably, the findings indicate that *C. enoplos* exhibits moderate genetic diversity. To preserve the current genetic diversity, it is necessary to effectively manage fishing activities and restrict the impact of human construction barriers along the Mekong River. Overfishing is a well-known phenomenon that affects population size and leads to the genetic diversity loss in a variety of fish species, as previously documented (Mashyaka & Duong, 2021).

## Conclusions

SCoT markers have been demonstrated to be useful in evaluating genetic diversity in wild populations as well as in examining population structure and genetic differentiation. The genetic diversity of *C. enoplos* population in Nong Khai Province was determined to be moderate. Both

phylogenetic trees and principal coordinate analysis (PCoA) were employed to investigate the relationships between individuals in the three populations, and both proved to be effective techniques. The dendrogram results indicated two comparable grouping patterns. The largest genetic diversity was found in the RP population whereas the lowest was found in the the M and SK populations. These findings can be employed in breeding programs to enhance fish culture and serve as crucial knowledge for the conservation of *C. enoplos*. The population in the RP area, which exhibits high genetic diversity, could be utilized as a hatchery area to encourage genetic variation in the population. Furthermore, it has the potential to serve as a rich genetic resource and a baseline stock for successful selective breeding systems.

## Acknowledgments

This project is partially supported by Research and Technology Transfer Affairs of Khon Kaen University. We extend our heartfelt dedication of this research to the local fishing communities within the research location, whose invaluable assistance greatly contributed to the successful completion of this study. Throughout the study period, the fishermen, fishmongers, and almost everyone in the area provided us with valuable support and cooperation.

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**Research article**

# Genetic variation of Cyclocheilichthys enoplos Mekong River, Nong Khai Province, Thailand

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**ABSTRACT**

This study presents the initial genetic diversity evolution of *Cyclocheilichthys enoplo*s, a fish species native to the Southeast Asia region that plays a vital role in the local economy as a food source. Start Codon Targeted (SCoT) markers (9 primers) were used to assess the genetic diversity of three wild populations located on the Mekong River in Thailand's Nong Khai province. A total of 28 specimens were analyzed, utilizing nine markers to examine genetic structure and diversity. The findings revealed 158 amplified and polymorphic loci, with polymorphic loci ratios ranging from 48.80 % to 85.54 % across the three groups. The Rattanawapee (RP) population had the highest amount of polymorphism, whereas the Sangkhom (SK) population had the lowest level. The values for Nei's gene diversity (He) and Shannon's Information index (I) were 0.195 to 0.296 and 0.285 to 0.444, respectively. The phylogenetic tree constructed using NTSYS-PC software and the dendrogram based on Nei's (1978) genetic distance indicated two different groups. The analysis of molecular variance (AMOVA) indicated that the majority of the variation occurred within populations (87 %), with relatively few differences between groups (13 %). This study provides preliminary information for the management of *Cyclocheilichthys enoplos* in this location.

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