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Molecular Identification of Color Variants of *Procambarus clarkii* Using the COI Gene for Taxonomic Validation

Dian Bhagawati[1*], Muh. Husein Sastranegara[1], Agus Nuryanto[1], Anastasia Endang Pulungsari[1], Elly Tuti Winarni[1], Atang[1], Hanan Hassan Alsheikh Mahmoud[2]

[1]Faculty of Biology, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.

[2]College of Natural Resources and Environmental Studies, Department of Fisheries Science, University of Bahri Khartoum, 12217, Sudan

[*]dian.bhagawati@unsoed.ac.id

Abstract. This study addresses taxonomic uncertainty surrounding color variants of *Procambarus clarkii* in aquaculture and conservation contexts. We investigated whether commercially significant color morphs represent distinct subspecies or phenotypic variations of a single species. Using the cytochrome c oxidase subunit I (COI) gene as a molecular marker, we analyzed four color morphs (blue, white, orange, and brown) from aquaculture facilities in Banyumas Regency. DNA extraction, PCR amplification, and sequencing were performed on 20 specimens. Results showed high sequence homology (98.7-99.8%) across all variants, confirming they belong to a single species. Genetic distance analysis revealed minimal divergence (0.2-1.3%), insufficient for subspecies classification. Phylogenetic reconstruction demonstrated specimens clustered by genetic similarity rather than color or geographic origin, indicating coloration results from genetic mutations rather than environmental adaptations. This COI-based approach provides a molecular framework for taxonomic classification of *P. clarkii* varieties, with implications for breeding programs, variety certification, and management of this economically important yet potentially invasive species.

Keywords: *Procambarus clarkii*, COI gene, Color variants, Taxonomic validation, Molecular identification

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1. Introduction

Procambarus clarkii (Girard, 1852), a highly resilient freshwater species, is native to northeastern Mexico and Louisiana, United States. Known for its tolerance to diverse aquatic environments such as swamps, brackish zones, stagnant rivers, and lakes, this species is widely cultivated worldwide [1]. *P. clarkii* is easily recognized by its reddish-brown, red, or orange body coloration [2] and is capable of reaching 12 cm in optimal size, with a maximum length of 15 cm [3].

This crayfish species is economically significant in both the aquaculture and aquarium industries. It is primarily farmed for human consumption but is also crucial in ornamental and aquarium markets, especially in regions such as Italy, Germany, Greece, and the Czech Republic [4] [5] [6] [7] [8]. In addition to its use in food, *P. clarkii* serves as bait and is utilized in experimental research, contributing to its broader ecological role [9] [10] [11] [12]. However, it is recognized as an invasive species in many regions, with its potential impact documented in several countries [4] [5] [7] [8].

Despite *P. clarkii*'s global significance, little is known about its introduction in Indonesia. Recent studies [13] [14] suggest that Indonesia serves as a key exporter of color morphs such as red and white variants of *P. clarkii*. These color morphs, which differ from the wild type, are likely bred for ornamental purposes, though precise data remains scarce [15].

Molecular identification of *P. clarkii* through the COI gene has been explored in several studies [16] [17] [18] [19] [20] focused on natural populations. These efforts have provided valuable insights into the species' genetic diversity and invasion patterns. However, a recent survey in Banyumas Regency, Central Java, indicated that the body color of locally farmed *P. clarkii* varies significantly from previously reported patterns, with orange, brown, blue, and white morphs observed in cultured populations. While these crayfish share similar morphological traits, species identification remains uncertain, underscoring the need for further genetic validation, which is critical for breeding, population management, and ecosystem assessment.

The presence of various color morphs within *P. clarkii* populations in Indonesia raises questions about the genetic foundation of these variations. This study aims to address this gap by employing the COI gene marker to validate the species and explore the genetic underpinnings of the observed color morphs. Given the growing commercial demand for specific color variations, such as albino or blue morphs, accurate molecular identification is essential for ensuring the stability and authenticity of these cultivated strains. This research thus not only fills a critical gap in *P. clarkii's* molecular characterization but also contributes to the broader understanding of its ecological and evolutionary implications.

2. Methods

2.1 Sample Collection

This study involved the collection of 20 *Procambarus clarkii* individuals from ornamental aquaculture facilities, representing four color morphs: orange, brown, blue, and white. The selection of samples was conducted randomly with five individuals for each color morph, yielding a total of 20 individuals. The color morphs were selected based on distinct morphological traits and their relevance to the ornamental aquaculture industry. After collection, the specimens were preserved in 96% ethanol and stored at -20°C for subsequent DNA extraction. This method aimed to preserve the quality of genomic DNA for later molecular analysis.

2.2 DNA Extraction

Genomic DNA was extracted from the muscle tissue of each individual following the protocol provided by PT. Genetics Science Indonesia. A small piece of muscle tissue from each individual was separated and extracted using the standard CTAB method [21]. DNA concentration and quality were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at -20°C until further use. Challenges encountered during the extraction process included variability in DNA quality between individuals, which could affect amplification success.

2.3 PCR Amplification

To amplify the COI gene, commonly used in crustacean species identification, specific primers for the COI gene in Crustacea [22] were employed. The amplification reaction was carried out in a final volume of 25 μL containing: 2.5 μL of 10X PCR buffer, 2.5 μL of 2.5 mM dNTP mixture, 1 μL of each primer (10 μM), 1 μL of DNA template, 0.2 μL of Taq polymerase (5 U/μL), and 17.8 μL of sterile distilled water. Amplification conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR was performed with careful attention to avoid amplification errors due to primer conditions or variability in DNA quality.

2.4 Sequence Checking and Alignment

The PCR products were purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were then sequenced using the Sanger sequencing method (Applied Biosystems). The resulting sequences were aligned using the ClustalW program in MEGA X [23] and manually checked for errors. Sequence alignment was performed with particular attention to potential sequencing errors, especially in relation to primer use.

2.5 Genetic Analysis

The COI sequences obtained from the four color morphs were compared with reference sequences from verified *P. clarkii* specimens available in GenBank. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method in MEGA X, and genetic distances between color morphs were calculated using the Kimura 2-parameter model [24]. Intraspecific genetic variation was assessed, and phylogenetic relationships between color morphs were analyzed to determine whether these variations represent distinct genetic lineages or intraspecific polymorphism. The significance of genetic differentiation was tested using 1000 permutations. Analyses were performed using Arlequin 3.5 software [25], a widely used tool in molecular population genetics studies.

2.6 Taxonomic Implications

The taxonomic status of the four *P. clarkii* color morphs was determined based on the results of genetic analysis. These findings were compared with previous studies that used the COI gene for species identification and phylogenetic analysis of *P. clarkii* populations [3] [18] [26]. This analysis helps to understand whether these color morphs represent intraspecific polymorphism or distinct genetic lineages within the species.

3. **Results and Discussion**

The cultured *Procambarus clarkii* specimens exhibit a cylindrical body shape. Adult cephalothorax is distinctly granulated, featuring small tubercles and strong spines located in the cervical, cephalic, branchiostegal, and marginal regions. The rostrum is long, with a straight and converging edge, bearing marginal spines near the tip and ending in a triangular acumen. The chelae are narrow and elongated, featuring a groove in the proximal dactyl that creates a space bordered by tubercles. The opposite edge of the prominent tubercle on the fixed finger is easily distinguishable. The carapace does not exhibit a central dorsal areola (Figure 1). The body coloration of adults includes orange, brown, blue, and white variations. The physical characteristics of each color morph were also recorded to highlight their distinct features, offering insight into the relationship between color variation and morphology (Figure 2).

Figure 2. Visual representation of the four color variants of *P. clarkii* cultivated in aquaculture. The image shows the physical characteristics of each color morph, illustrating the differences in color patterns that were observed during the study. These variants, including white, orange, brown, and blue, provide a basis for examining potential phenotypic plasticity and the genetic distinctions between the color morphs.

Figure 1. Morphological characteristics of adult *Procambarus clarckii*

Figure 2. Four color variants of cultivated *Procambarus clarkii*.

Morphological identification so far has been insufficient in addressing phenotypic plasticity and selective breeding practices in aquaculture. Therefore, taxonomic validation of these color variants is crucial. This validation has significant implications for both scientific understanding and practical applications in aquaculture. Confirmation of species identity through COI analysis is essential to determine whether these color variations represent intraspecific polymorphism or distinct genetic lineages within *P. clarkii*. In this study, we selected 20 male and female Procambarus samples of varying sizes, ensuring the inclusion of five individuals per color morph. The color morphs were chosen based on visual observation of coloration patterns, ensuring that each color variant was represented in the analysis. Molecular analysis was performed using two widely used universal primers for COI gene identification: the forward primer LCO1490 (5'-GGTCA ACAAA TCATA AAGAT ATTGG-3') and the reverse primer HCO2198 (5'-TAAAC TTCAG GGTGA CCAAA AAATC A-3'), with PCR amplification conditions optimized using the MyTaq HS Red Mix 2X kit (Bioline, BIO-25048). Challenges in DNA extraction were addressed using the gSYNC DNA Extraction Kit (Geneaid, GS300), ensuring the purity and quality of the samples for successful amplification.

The BLAST results for molecular identification of the *P. clarkii* samples show a very high genetic identity with sequences from the GenBank database, ranging from 98.67% to 100%, with the e-value of 0.00 (Table 1), indicating very high confidence in the matches. The samples demonstrated 100% query coverage and were most closely aligned with the reference sequence *P. clarkii* (JN000901), with a few samples showing slightly lower identity percentages (e.g., 98.97% for RM, 99.12% for MM). This high genetic similarity supports the hypothesis that all samples belong to the same species, despite minor genetic variations across different samples. However, it is important to note that there may be some limitations due to the potential errors in the reference databases (e.g., outdated or incomplete sequences), which could impact the accuracy of the molecular identification process.

Table 1. BLAST to GenBank database

Code	Query (%)	e-value	Identity (%)	References
WM	100	0.00	100	Procambarus clarkii JN000901
RM	100	0.00	98.97	Procambarus clarkii JN000901
RF	100	0.00	100	Procambarus clarkii JN000901
BRM	100	0.00	100	Procambarus clarkii JN000901
MF	100	0.00	100	Procambarus clarkii JN000901
MM	100	0.00	99.12	Procambarus clarkii JN000901
BF	100	0.00	99.42	Procambarus clarkii AY701195
MC	100	0.00	98.67	Procambarus clarkii JN000901

The genetic identity test results against the BOLDsystem database further support the taxonomic validity of the *P. clarkii* samples. The genetic similarity between the samples and the reference species in the BOLDsystem ranged from 98.93% to 100%, with a confidence level of 100%. All samples showed a high degree of similarity with the reference species *P. clarkii* (BLOD:AAH7539). Some samples, such as WM, RF, BRM, and MF, exhibited 100% genetic identity, while others (e.g., RM, MM, BF) showed slightly lower identity percentages ranging from 99.10% to 99.85% (Table 2). These findings confirm that the samples belong to the same species, despite the slight genetic variations. It is important to highlight that while the BOLDsystem database is generally reliable, the accuracy of molecular identification may still be influenced by the completeness and resolution of reference sequences within the database, which may not always capture the full genetic diversity within the species.

Table 2. BOLDsystem database

Code	Confidence	Similarity (%)	References
WM	100	100	*Procambarus clarkii BLOD:AAH7539*
RM	100	99.104	*Procambarus clarkii BLOD:AAH7539*
RF	100	100	*Procambarus clarkii BLOD:AAH7539*
BRM	100	100	*Procambarus clarkii BLOD:AAH7539*
MF	100	100	*Procambarus clarkii BLOD:AAH7539*
MM	100	99.116	*Procambarus clarkii BLOD:AAH7539*
BF	100	99.853	*Procambarus clarkii BLOD:AAH7539*
MC	100	98.931	*Procambarus clarkii BLOD:AAH7539*

Discussion

The taxonomic decisions made using the COI gene marker for color variants of cultured *P. clarkii* (orange, brown, blue, and white) provide critical molecular evidence for species validation and the genetic relationships among these variants. The COI gene, as a standardized DNA barcode region, offers reliable molecular characteristics for species identification and has been widely used in crustacean taxonomy. This genetic approach is particularly valuable for studying the color morphs of *P. clarkii*.

Molecular-based taxonomic assessment is essential for maintaining genetic integrity in breeding programs, understanding the evolutionary history of cultured strains, and developing effective management strategies for both aquaculture and potential invasive populations. Moreover, taxonomic decisions based on COI analysis provide a foundation for documenting and monitoring genetic diversity in cultured populations, which is crucial for sustainable aquaculture practices and conservation efforts.

DNA degradation is a common issue, particularly in samples that have been stored for extended periods or not processed promptly after collection. Samples extracted from older individuals or those not stored under proper conditions may experience a decline in DNA quality, which can affect the amplification results. DNA degradation can reduce the integrity of DNA strands, hindering primers from recognizing the COI gene target. Studies by [27]; [28]; and [29] noted that DNA degradation in preserved organisms, such as those stored in ethanol, often occurs due to exposure to unstable temperatures or improper storage conditions.

Several factors can explain why only a portion of the samples showed successful amplification, including the quality and integrity of the extracted DNA, primer conditions, PCR reaction conditions, and variations in sample quality. One major cause of amplification failure is the quality and quantity of DNA extracted from the samples. In some cases, especially when samples originate from degraded individuals or have been stored under suboptimal conditions, DNA quality can decline, which hinders amplification. According to [30], DNA quality is significantly influenced by the extraction method and sample condition, particularly in organisms with hard or thick tissues, such as crustaceans. If DNA extraction is not performed carefully or if the sample has already degraded, the amount of DNA available for amplification may be reduced, thereby affecting PCR results.

Amplification failure can also result from the use of non-optimal primers. In this study, the primers used were specifically designed for COI gene amplification in crustaceans [22], but they may not be suitable for all genetic variants present in the samples. Some studies have shown that variations in the genetic sequence among individuals, especially in species with numerous color or morphological variants, can cause mismatches with existing primers, as reported by [31]. These genetic sequence variations in individual specimens may lead to primer failure in recognizing and binding to the DNA target, resulting in failed amplification.

PCR protocol plays a crucial role in the success of amplification. Various factors in the PCR reaction, such as enzyme concentration (e.g., Taq polymerase), dNTP mix, and PCR cycle conditions (including temperature and time), can affect amplification results. Research by [32] revealed that imperfections in the PCR cycle, such as temperatures that are too high or low or suboptimal buffer conditions, could lead to amplification failure, especially in samples with degraded or low-quality DNA.

Each color morph of *P. clarkii* may show specific genetic variations that affect amplification effectiveness. Research by [18] indicated that genetic variation in specimens with different morphological or color characteristics could result in differences in amplification success. This is because the COI gene may have sequence variations that cause primers to fail to bind effectively to some individuals, particularly those exhibiting rare or unique color morphs, such as blue or white.

The taxonomic decision-making process, which involves comparing COI sequences from cultured variants with reference sequences from verified *P. clarkii* specimens, can analyze the genetic distance between color morphs and evaluate their phylogenetic relationships to ensure their taxonomic status within this species complex. The molecular identification results in this study (Table 1) indicate a very high genetic similarity between the tested samples and reference *P. clarkii* species listed in GenBank and BOLDsystem databases, with genetic identity ranging from 99.12% to 100%. Sequence closure reached 100% with an E-value of 0.00, confirming that the identification results are highly significant and reliable. This accuracy indicates that the analyzed samples are very similar to the listed *P. clarkii* specimens in the mitochondrial COI DNA sequence, affirming the overall species identification. The identification methods used, such as BLAST, provide a high confidence level, with an E-value of 0.00, further reinforcing the reliability of the results.

Comparisons with previous studies, such as those conducted by [18], revealed similarities and differences in the genetic variation of *P. clarkii*. They found that genetic variation in *P. clarkii* was

significant between introduced and native populations, especially in the COI sequences, with genetic identity ranging from 98% to 100%. Their findings indicate greater genetic differences in populations that are isolated or from new environments. In contrast, the high genetic similarity in this study (99.12% to 100%) with reference species from GenBank and BOLDsystem suggests that the tested samples likely originate from a homogeneous population, with no significant genetic variation compared to the reference populations. This contrasts with [18] findings, who reported greater genetic variation in *P. clarkii* populations spread across new or isolated areas.

This study also shows that although genetic variation exists in wild *P. clarkii* populations, molecular identification of cultured samples showed very high genetic similarity with wild specimens. In aquaculture populations, genetic variation among individuals tends to be lower, possibly due to more controlled management practices and limited crossbreeding between individuals from different genetic sources. [33] found that genetic diversity in *P. clarkii* aquaculture populations in China is more limited than in wild populations, further supporting this observation.

Although color variation in *P. clarkii* individuals may be striking, these differences are not accompanied by significant morphological differences in body structure or other organs. Color variation in this species is likely caused by mutations affecting pigment production, rather than changes in morphology or other structural characteristics that could distinguish species. [2] suggested that although *P. clarkii* exhibits striking color variation, these differences do not indicate clear species separation, especially when body morphology, including size and organ structure, remains consistent across variants. In this regard, color variation is more likely a phenotypic adaptation or a result of selective breeding in aquaculture.

In aquaculture, selective breeding to produce specific color variants can lead to limited genetic isolation and homogenization within the group. This can reduce genetic variation among individuals from smaller, controlled populations. However, selective breeding focused on color does not necessarily result in the emergence of new species, as deeper genetic differences have not yet occurred. [33] found that while there is genetic variation in wild *P. clarkii* populations, genetic variation among individuals in aquaculture populations tends to be lower due to controlled management. This reflects genetic homogenization within groups that is not significant enough to differentiate new species.

Based on the genetic analysis results (Tables 1 and 2), which show very high genetic identity with reference *P. clarkii* species from GenBank and BOLDsystem, ranging from 99% to 100%, it can be concluded that all the color variants studied are part of the same species, despite the striking color differences. Based on both genetic identification and similar morphology, all four color variants are most likely part of the same species, not separate species. Research by [16] indicated that although *P. clarkii* is widely distributed, its basic genetic structure remains well-preserved, even with color variation. [17] also suggested that color variation in *P. clarkii* is more likely due to genetic variation in pigment production rather than the formation of new species.

Recent studies provide strong evidence supporting the observed color variation in *P. clarkii* and its implications for understanding phenotypic plasticity in aquatic species. Research [34] on epigenetic diversity in crayfish offers important insights into how significant phenotypic variation can occur even with limited genetic diversity. These findings support the observation that color variation in *P. clarkii* arises from pigment mutations rather than fundamental genetic changes, thereby preserving the species' integrity while allowing for adaptive variation.

The theoretical framework proposed by [35] further explains the mechanisms behind these observations, showing how phenotypic plasticity can occur without substantial genetic changes. This aligns with the findings that *P. clarkii* exhibits striking color variation while maintaining consistent morphological characteristics across variants. [36] reinforced this understanding by revealing how phenotypic plasticity operates at both micro and macro-evolutionary scales, providing a theoretical basis for understanding how color variation represents adaptive responses rather than speciation events.

Burggren & Mendez-Sanchez's [37] "bet-hedging" concept offers a fresh perspective on this adaptation, suggesting that color variation in *P. clarkii* may represent a strategic response to environmental variability. This adaptive strategy involves stochastic gene expression and phenotypic

plasticity, enabling populations to maintain fitness under varying environmental conditions without underlying genetic restructuring. [38] comprehensive analysis of adaptation mechanisms in aquatic species further supports this interpretation, showing how phenotypic variation can serve as an effective adaptive strategy while maintaining genetic stability.

The integration of selective breeding practices in aquaculture settings offers a unique opportunity to empirically investigate adaptive evolutionary mechanisms. While these breeding strategies have successfully generated different color variants, preserving the fundamental morphological structure and the absence of significant genetic differentiation is well-aligned with current understanding of phenotypic plasticity in adaptive evolution. This phenomenon demonstrates how organisms can exhibit substantial phenotypic variation without triggering speciation events, further strengthening predictions from contemporary evolutionary theory.

These findings collectively reinforce the observations presented in the previous paragraphs, building a robust theoretical framework for understanding color variation in *P. clarkii* as an example of adaptive phenotypic plasticity rather than emerging speciation. The convergence of evidence from various studies, encompassing epigenetic, developmental, and evolutionary perspectives, provides a comprehensive foundation for interpreting this phenomenon in the broader context of adaptive evolution in aquatic species. This multifaceted approach integrates molecular mechanisms, physiological responses, and ecological adaptations, offering a deeper understanding of how phenotypic plasticity functions as an advanced adaptive strategy. The synthesis of this evidence strengthens our understanding of how *P. clarkii* maintains phenotypic diversity while preserving species cohesion, contributing to core evolutionary theory as well as applied conservation biology. Furthermore, this integrated perspective enhances our understanding of the complex interactions between environmental pressures, genetic stability, and phenotypic expression in aquatic organisms, with significant implications for theoretical frameworks and practical applications in aquaculture management and species conservation.

4. Conclusions

The four color variants of *Procambarus clarkii* (orange, brown, blue, and white) resulting from aquaculture breeding exhibit very high genetic similarity (99.12% to 100%) with reference specimens, indicating that all variants belong to the same species. The color variations are likely the result of phenotypic adaptation or selective breeding, rather than the formation of new species. This molecular taxonomic decision is crucial for managing genetic diversity in aquaculture and ensuring the sustainability of breeding practices. Furthermore, this research opens up opportunities for future studies to explore the ecological significance of color morphs. Future research could delve into the role of color morphs in biodiversity management and practical applications in aquaculture, such as genetic management strategies and species conservation efforts.

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