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IMMUNOGENETIC INSIGHTS AND CONSERVATION OF THE ENDEMIC LEMPUK FISH (GOBIIFORMES: *Gobiopterus*)

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Abstract
The health and conservation of the endemic lempuk fish (*Gobiopterus* sp.) are closely linked to their genetic diversity and immunological function. Assessing immune gene expression offers a robust indicator of fish health and environmental stress. This study examines the morphological characteristics, immune gene activity, and genetic variation of lempuk fish from Ranu Grati, East Java, Indonesia. Males had larger bodies and longer dorsal fins than females, which were shorter and stockier. Expression of immune genes (TNF-α, IL-6, IFN-γ) was quantified using RT-qPCR, while mitochondrial COI sequences were analyzed to determine genetic relationships. TNF-α was markedly upregulated, suggesting a strong pro-inflammatory response to environmental or pathogenic stimuli. COI analyses revealed moderate genetic divergence between East Javan and adjacent populations, with a close affinity to Malaysian *Gobiopterus* and clear separation from Australian species. Preserving genetic variation is essential for resilience against environmental stressors, and is necessary for the conservation of this endemic species.

Keywords: Gene expression, genetic diversity, immunological response, *Gobiopterus*, Ranu Grati

Introduction

Fish population health reflects both genetic diversity and immune competence, two pillars of resilience against environmental stress and disease. Immune gene profiling provides a direct assessment of physiological condition and pathogen resistance (Ali *et al.* 2014, Szitenberg *et al.* 2012). Genes such as TNF-α, IL-6, and IFN-γ mediate inflammation, immunoregulation, and antiviral defence, making their expression patterns useful biomarkers of stress and disease exposure (Morrissey 2010).

Genetic variation supports species survival under changing conditions (Kovach *et al.* 2013, Wu *et al.* 2021, Chen *et al.* 2019). The lempuk fish (*Gobiopterus* sp.), endemic to Ranu Grati Lake in East Java, represents a keystone in the lake's trophic web and local biodiversity (Anitasari *et al.* 2021, Hertika 2024). Its restricted range and ecological role highlight its conservation significance (Faqih *et al.* 2021, Anitasari 2024). Beyond its value as an endemic, the lempuk fish contributes to local fisheries and nutrient cycling (Prihanto *et al.* 2022).

Understanding the genetic and immunological dynamics of this population is crucial for long-term conservation. Genetic monitoring—combining DNA sequencing and immune gene expression—provides essential data on adaptability, reproductive viability, and environmental stress tolerance (Osborne *et al.* 2012, Mastrochirico-Filho *et al.* 2019). Such approaches strengthen management of hatchery-wild interactions and guide biodiversity protection (Ukenye *et al.* 2019, Harris *et al.* 2014).

This study integrates morphological, immunological, and genetic analyses to evaluate the health and diversity of *Gobiopterus* sp. in Ranu Grati. It aims to (1) describe morphological dimorphism, (2) quantify immune gene expression (TNF-α, IL-6, IFN-γ), and (3) determine genetic divergence through COI sequencing to inform conservation strategies and population management.

Material and Methods

Study area. Sampling took place from April to August 2024 in Ranu Grati Lake, Ranuklindungan Village, Grati District, Pasuruan Regency, East Java. The lake was divided into five stations—four peripheral and one central—to capture spatial heterogeneity in limnological and biological conditions. This lacustrine system hosts diverse endemic fauna, including *Gobiopterus* sp., making it ideal for assessing genetic and immunological variation (Fig. 1).

Fish sampling. Between 50 and 100 lempuk fish (1–2.5 cm total length) were collected across the five stations (Sup. Table 1). Specimens were immobilized on ice immediately after capture and frozen until molecular analysis. This standardized collection ensured representative coverage of the population and minimized degradation of RNA and DNA.

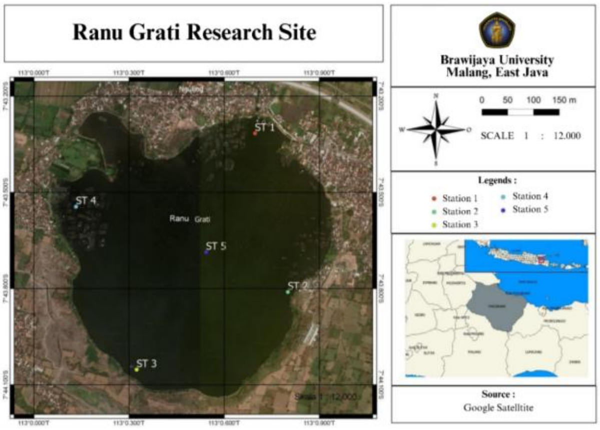

Figure 1. Sampling location stations in Ranu Grati

RNA extraction and reverse transcription. Total RNA was isolated from whole-body tissue using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocols. Purity and concentration were verified with an Implen NanoPhotometer. Complementary DNA (cDNA) was synthesized from 1 µg total RNA using the RevertAid cDNA Synthesis Kit (Thermo Scientific, Germany) to obtain 20 µL of cDNA per sample.

Quantitative real-time PCR (RT-qPCR) and data analysis. Expression of TNF-α, IL-6, and IFN-γ was quantified with the Maxima SYBR Green qPCR Kit (Thermo, USA) on a QuantStudio™ Real-Time System (Applied Biosystems). Cycling conditions comprised an initial denaturation at 94 °C (5 min), followed by 40 cycles of 94 °C (10 s), 55 °C (30 s), and 72 °C (40 s). Melting-curve analysis confirmed product specificity. β-actin served as the housekeeping gene for normalization, and relative expression was computed using the $2^{-}(\Delta\Delta Ct)$ method (Livak & Schmittgen, 2001). Statistical significance was set at $p < 0.05$.

DNA sequencing. Genomic DNA was extracted with the PureLink™ Genomic DNA Mini Kit (Invitrogen). The mitochondrial cytochrome oxidase I (COI) gene was amplified using primers (Sup. Table 2) of Knüpfer *et al.* (2020). PCR reactions (25 µL) contained 3 µL DNA template, 11 µL PCR Supermix, 0.5 µL of each primer (0.01 mM), and 10 µL ddH$_2$O. Thermal cycling: 95 °C (2 min) → 35 cycles of 94 °C (30 s), 54 °C (30 s), 72 °C (1 min) → final extension 72 °C (10 min). Products were run on 2 % agarose gel and visualized with GelDoc. Purified amplicons (~723 bp) were sequenced at 1st Base Pte Ltd, Malaysia.

Data analysis. All data are expressed as mean ± SD. One-way ANOVA followed by LSD posthoc tests evaluated differences among groups (SPSS v 20.0, Chicago, USA). Graphs were produced with GraphPad Prism 7.

Results

Morphology of Lempuk Fish. Both sexes of *Gobiopterus* sp. are minute (2–3 cm), transparent, and laterally compressed. The short dorsal fin lies close to the rounded caudal fin, which allows agile movement in shallow waters. The head is proportionally large with dorsally placed eyes. Sexual dimorphism is evident: males have longer bodies, extended dorsal fins, and more protruding snouts, whereas females are smaller and stockier (Fig. 2). These distinctions correspond with patterns described for other *Gobiopterus* species inhabiting lentic systems.

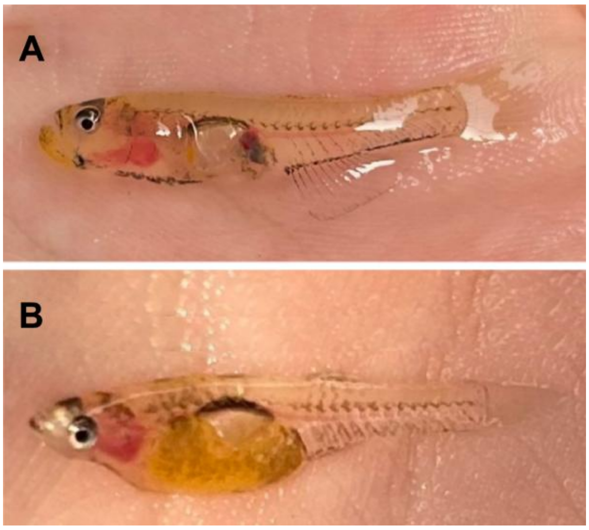

Figure 2. (A) Male and **(B)** female lempuk fish (*Gobiopterus* sp.) in Ranu Grati, East Java

Immune Gene Expression (TNF-alfa, IL-6, IFN-Gamma) Analysis. Immune profiling revealed distinctive cytokine responses. TNF-α (Fig. 3) expression was markedly elevated (60–80-fold) across all groups, indicating a pronounced pro-inflammatory reaction, probably triggered by environmental stressors or microbial challenge. IL-6 (Fig. 4) levels remained moderate (20–25-fold) and uniform, implying a regulated inflammatory process that limits tissue damage. IFN-γ expression (25–35-fold) was consistent among groups, signifying sustained antiviral readiness. Melting-curve analyses displayed single peaks, confirming primer specificity. Collectively, these data suggest an active yet balanced immune system, reflecting adaptive immune vigilance under fluctuating lake conditions.

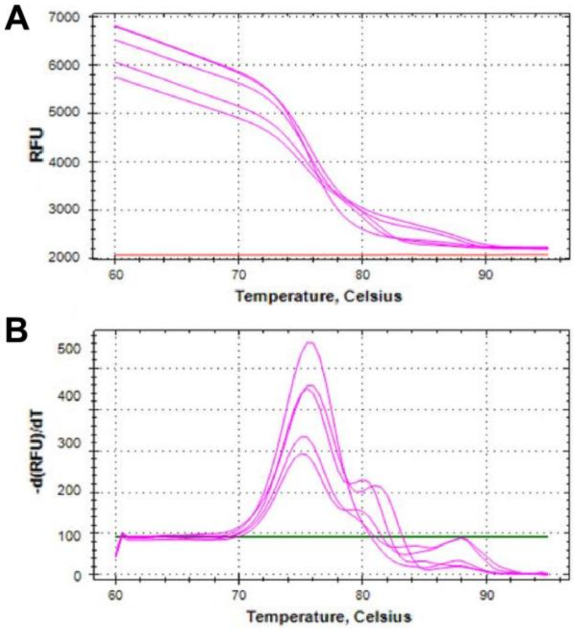

Figure 3. (A) Melting curve and **(B)** melt peak of the Lempuk fish (*Gobiopterus* sp.) IFN-Gamma gene; the generated PCR product showed a melting curve with a single peak

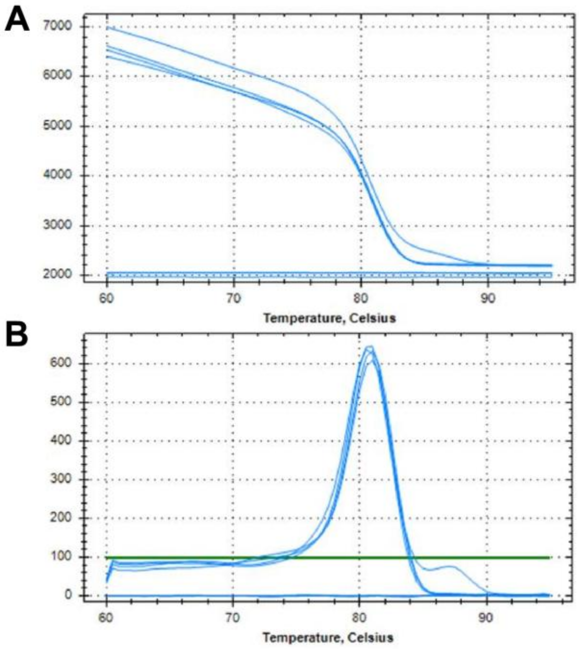

Figure 4. (A) Melting curve and **(B)** melt peak of Lempuk fish (*Gobiopterus* sp.) IL-6 gene; the generated PCR product showed a melting curve with a single peak

DNA Sequencing Result. Amplification of the mitochondrial COI gene yielded a clear 723 bp product. Absence of bands in the no-template control confirmed reaction purity. Successful amplification enabled downstream phylogenetic and genetic-distance analyses.

Phylogenetic tree. COI-based phylogenetic reconstruction demonstrates clustered relationships among *Gobiopterus* populations (Fig. 5). Specimens from Central Java (*G. brachypterus*) formed a tight clade with near-zero bootstrap divergence, reflecting minimal intraspecific variation. *G. lacustris* from China and the Philippines were grouped closely (bootstrap ≤0.006), revealing cross-regional connectivity. Indian and Malaysian *G. brachypterus* populations also exhibited close affinity (bootstrap ≈ 0.005–0.006). In contrast, the East Java lempuk population formed a distinct branch (bootstrap 0.123), suggesting geographic isolation. The most distant taxon, *G. semivestitus* from Australia (bootstrap 0.139), underscores deep lineage divergence. *Danio rerio* was used as the outgroup, validating tree topology. Overall, the East Javan population's separation supports its recognition as a genetically unique lineage requiring targeted conservation.

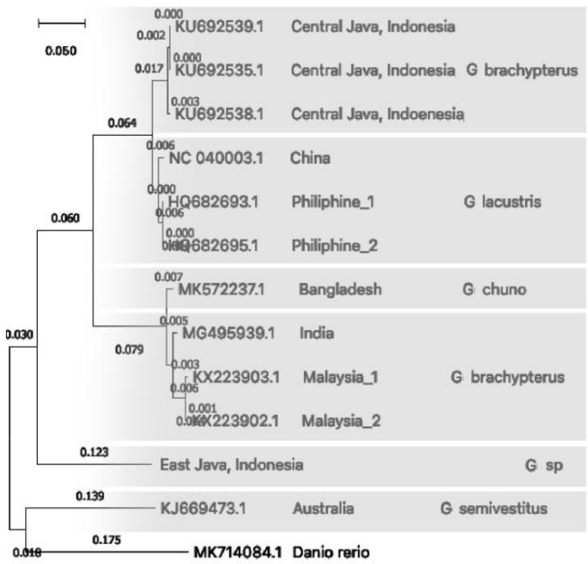

Figure 5. The phylogenetic tree of lempuk fish at Ranu Grati, East Java, Indonesia

Genetic distance. The COI-based distance matrix (Sup. Table 3) indicates moderate differentiation between the East Java lempuk and Central Java *G. brachypterus* (0.29) but near identity with Malaysian populations (0.00–0.29). Distances from the Philippine *G. lacustris* averaged 0.24 and from *G. semivestitus* (Australia) 0.36, confirming greatest divergence from the latter. Populations within Central Java, Malaysia, and the Philippines exhibited minimal internal variation (0.00–0.03), consistent with restricted dispersal or recent divergence. These values highlight moderate overall heterogeneity within *Gobiopterus*, with the East Javan lempuk maintaining sufficient genetic distinctness to merit regional management attention.

Discussion

Immune response gene expression. The expression profiles of TNF-α, IL-6, and IFN-γ in *Gobiopterus* sp. reveal a coordinated and adaptive immune response to environmental pressures. The markedly elevated TNF-α (60–80-fold) indicates strong pro-inflammatory activation, consistent with earlier studies linking this cytokine to mucosal defence and pathogen-induced stress in fish (Ma *et al.* 2014, Rojas *et al.* 2012). Such activation likely reflects exposure to fluctuating limnological conditions or microbial agents within Ranu Grati. Stable IL-6 expression (20–25-fold) across groups suggests controlled inflammation aimed at maintaining tissue integrity while preventing chronic immune activation. IL-6 functions as both a pro- and anti-inflammatory mediator, balancing cytokine cascades to preserve homeostasis (Jiang *et al.* 2015, Zhang *et al.* 2013). This moderation is essential for long-term survival in dynamic habitats, as sustained inflammation can lead to tissue damage and energy depletion. Consistent IFN-γ levels (25–35-fold) confirm active antiviral readiness and heightened immune preparedness. IFN-γ enhances macrophage activation and cytokine signaling, providing a critical line of defense against viral pathogens (Sheng *et al.* 2010, Zhang *et al.* 2023). Collectively, these gene-expression patterns indicate that the lempuk fish maintain immune equilibrium—a state of readiness without overreaction—vital for species thriving in variable freshwater environments.

Phylogenetic and Genetic Insights. The COI-based phylogeny delineates both close and divergent relationships within *Gobiopterus* lineages. Tight clustering of *G. brachypterus* populations from Central Java with minimal genetic distance (0.00) implies recent divergence or continuous gene flow. Similarly, *G. lacustris* populations from China and the Philippines group together, reflecting conserved lineages possibly shaped by shared ecological pressures (Faqih *et al.* 2021, Roesma *et al.* 2019). Conversely, the East Javan *Gobiopterus* sp. forms a distinct branch, separated by a bootstrap value of 0.123 and a genetic distance of 0.29 from Central Java, signifying geographic isolation and restricted dispersal. The strong affinity (0.00–0.29) with Malaysian populations suggests historical connectivity via regional hydrological systems. The greatest divergence occurs with *G. semivestitus* from Australia (0.36), emphasizing long-term evolutionary separation driven by continental barriers. These phylogenetic relationships highlight how spatial and ecological isolation in Ranu Grati has produced a genetically unique population—a pattern consistent with endemic species that evolve under closed basin conditions. Such distinctiveness warrants local conservation measures focusing on genetic integrity and habitat stability.

Conservation and Genetic Diversity. Genetic diversity underpins resilience to disease, climatic fluctuations, and anthropogenic disturbances (Khal 2023, Castro *et al.* 2023). Populations with low variability are more vulnerable to pathogens and environmental shifts (Vandegrift *et al.* 2011). The moderate divergence of the East Javan lempuk suggests an adaptive equilibrium—enough differentiation to preserve uniqueness without severe genetic bottlenecking. Preserving this diversity is crucial for ecological stability, as it strengthens ecosystem resilience and enhances adaptive potential under climate change (Thilakarathna *et al.* 2022, Godley *et al.* 2010). Integrating molecular monitoring into conservation frameworks enables the detection of declining genetic variation and informs restocking or translocation programs that avoid genetic homogenization.

Ecological Implications. The immune activation observed in this study may reflect environmental stressors—temperature variability, eutrophication, or pathogen prevalence—within Ranu Grati. Monitoring cytokine expression thus provides a sensitive biomarker for assessing population health and habitat quality. Coupled with genetic data, it offers a holistic diagnostic system for managing endemic freshwater fish. As Ranu Grati faces increasing anthropogenic pressure, conservation strategies must balance community livelihood with biodiversity protection. Maintaining habitat quality, regulating nutrient input, and preventing overfishing will complement molecular-based conservation measures to secure the future of *Gobiopterus* sp. populations.

Author contributions

All the authors contributed equally.

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Research permits

No permit required

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Supplemental data

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