



## Genetic and morphological evidence of a single species of bronze featherback (*Notopterus notopterus*) in Sundaland

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### ABSTRACT

The bronze featherback (*Notopterus notopterus*) is an iconic species of freshwater fish commonly found in South-East Asia and of great commercial and patrimonial importance. At present, the genus *Notopterus* comprises of two distinct species, *Notopterus notopterus* (Pallas, 1769) and *Notopterus synurus* (Bloch & Schneider 1801). On the Indonesian archipelago, genetic diversity and morphology of the bronze featherback were investigated for conservation purposes. The use of DNA-based species delimitation methods, applied to 165 Cytochrome oxidase I sequences of Notopteridae (121 belonging to *N. notopterus*), evidence a concordance between species and Molecular Operational Taxonomic Unit (MOTU) and the two species of *Notopterus* are recognized. In *N. notopterus*, 9 haplotypes are detected among the 121 sequences analyzed, and three are restricted to Sundaland. These three haplotypes had distinct geographic distribution with a haplotype observed in Java, Sumatra and Borneo, another haplotype restricted to South Sumatra and a third haplotype only found in Northern Sumatra. The analyze of 21 morphometric and 9 meristic variables revealed two groups within *N. notopterus*, which were only supported by subtle differences in measurements with overlapping distributions between groups. The present study

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supports the validity of *Notopterus notopterus* in Sundaland and the remarkable genetic continuity among populations across its range distribution.

## 1. Introduction

The Indonesian archipelago has among the most biodiverse freshwater fish fauna on the planet (Hoffman et al., 2010; Darwall and Freyhof, 2016). The high species diversity (>1218 species) and endemism (>630 species) are similar to those of the Mekong and Amazon systems in terms of species density (Hubert et al., 2015). The reasons behind such a rich ichthyofauna include: (i) the exceptional fragmentation and isolation of rivers across the world's largest archipelago (approx. 17,000 islands), and (ii) major biogeographical boundaries fragment the archipelago into several biodiversity hotspots with ancient links to either Eurasia or Australia (i.e., the Wallace Line; Lohman et al., 2011, Hutama et al., 2016). Understanding variations in fish species composition among different areas is an important knowledge to better manage and preserve biodiversity (Kong et al., 2017). Diversity of habitats and fragmentation may induce intraspecific variations in functional morphology and genetic diversity (Willis et al., 2005; Shuai et al., 2018). While new species are still frequently discovered, there is ongoing work to determine whether fish species, which display substantial morphological variations across catchments and islands represent a single species or whether there is a richer biodiversity waiting to be revealed by comprehensive taxonomic revisions (Kottelat, 2013; Wibowo et al., 2016).

The bronze featherback, *Notopterus notopterus* (Pallas, 1769), belongs to the family Notopteridae, an iconic family of tropical freshwater fishes inhabiting the low-lands of Africa and Asia. The family is comprised of four genera, *Chitala* and *Notopterus* occurring in Asia from India to Southeast Asia, and *Xenomystus* and *Papyrocranus* occurring in West and Central Africa (Roberts, 1992; Inoue et al., 2009; Froese and Pauly, 2023). The origin of the Notopteridae is tracing back to the Early Cretaceous when the African (*Papyrocranus* and *Xenomystus*) and Asian (*Notopterus*, *Chitala*) clades split (Inoue et al., 2009). *N. notopterus* is largely distributed in South-East Asia (Roberts, 1992). For long assumed to be distributed from Pakistan in the west to Vietnam in the east, it has been shown recently based on a comprehensive analysis of available mitochondrial cytochrome oxidase 1 sequences, that western populations correspond to a distinct species (Lavoué et al., 2020; Takagi et al., 2010), namely *Notopterus synurus* (Bloch & Schneider 1801). The broad geographic distribution of *N. notopterus* (Vietnam, Thailand, Malaysia and Indonesia) is not continuous and extends over the fragmented Indonesian archipelago as it occurs in the large islands of Sumatra and Java (Roberts, 1992; Lavoué et al., 2020; Nelson, 2006), and has been more recently reported from East Borneo (Wibowo et al., 2009). This part of the Indonesian archipelago corresponds to the large biodiversity hotspot of Sundaland, characterized by a high level of endemism and major anthropogenic threats (Myers et al., 2000, 2010).

The bronze featherback can grow up to 60 cm of Standard Length (SL) (Roberts, 1992) but individuals usually measure 25 cm (Froese and Pauly, 2023). The species can be identified by its tapered tail, a straight to slightly convex craniodorsal profile, and jaws not extending posteriorly beyond the eye unlike in the genus *Chitala* (Fig. 1). The bronze featherback feeds on insects, small fish, crustaceans and occasionally roots of aquatic plants (Gupta and Ray, 2022). From an ecological perspective, the bronze featherback appears to undertake local migrations with a preference for standing waters and wetlands for breeding (Rainboth, 1996). Adults breed during the monsoon season, moving into wetlands to lay their eggs in submerged grass and vegetation before returning to permanent riverine habitats (Talwar and Jhingran, 1991; Rainboth, 1996), a trend that makes this species particularly vulnerable to land conversion and forest fragmentation. Besides, the bronze featherback is widely distributed across Sundaland but he is rarely abundant and usually occurs in multiple small isolated patches (Dahrudin et al., 2017; Lavoué et al., 2020; Kottelat et al., 1993). As such, the bronze featherback is at high risk of population declines due its fragmented distribution, which amplifies the impact of overfishing, industry, pollution and monsoon delay (Darwall and Freyhof, 2016; Dahrudin et al., 2017; Ng, 2020; Gupta and Ray, 2022). The Food and Agricultural Organization (FAO) categorizes this commercially important species as a significant source of animal protein for daily human consumption and also for trade as an ornamental fish (Casavas et al., 1996; Kottelat and Widjanarti, 2005; Abbas et al., 2013). In Indonesia, all Notopteridae species are fully protected by the Ministry of Marine Affairs and Fisheries Regulation. As such, a careful examination of its taxonomic status and population genetic structure in Sundaland is urgently required.

Molecular techniques integrated with morphological observations can provide valuable insights into the taxonomy of freshwater fish species (see Pilipenko et al., 2012; Liu et al., 2021). DNA barcoding, the use of a standardized gene fragment for species delimitation and specimen identification, is increasingly used in taxonomic studies (Schlick-Steiner et al., 2010; Hausmann et al., 2011;



Fig. 1. Photograph of a specimen of *Notopterus notopterus* collected in Sumatra (BIF3689, SL=238 mm).

Hubert and Hanner, 2015; Abdulmalik-Labe et al., 2022). It proved to be particularly effective in Sundaland to clarify species identity in multiple fish groups (Beck et al., 2017; Conte-Grand et al., 2017; Hubert et al., 2019; Sholihah et al., 2020; Dahruddin et al., 2021), as well as help discover new species (Keith et al., 2017; Mennesson et al., 2021). In this context, our study presents the results of a genetic characterization of the bronze featherback in Sundaland using DNA barcodes together with a morphometric assessment of the bronze featherback morphological variability. Our goal is to provide insights into the taxonomic status of *N. notopterus*, its genetic diversity and phylogeographic pattern to provide recommendations for its sustainable management and conservation. In particular, sea levels have fluctuated widely during the Pleistocene, causing islands of the Sunda Shelf to repeatedly separate and merge (Lohman et al., 2011; de Bruyn et al., 2013; Dixon, 2015; Hutama et al., 2016; Sholihah et al., 2021a, 2021b). Ancient river systems have been documented in Sundaland during Pleistocene low sea levels, which impact on species dispersal, hence population genetic structure, is still largely debated (Woodruff, 2010; de Bruyn et al., 2013; Sholihah et al., 2021a,b; Woodruff, 2010). The impact of past river system geography on phylogeographic structure is examined here for *N. notopterus*, and implications for conservation are discussed.

## 2. Materials and methods

### 2.1. Ethics statement

All fish capturing and handling procedures complied with the laws and guidelines of the Ministry of Marine Affairs and Fisheries, Republic of Indonesia (PER.10/MEN/2010 and Kepmen KP No. 1/2021) and the study was approved by the Agency for Marine and Fisheries Research and Human Resources, Ministry of Marine Affairs and Fisheries, Republic of Indonesia (2023/BRSDM/XII/2021).

### 2.2. Sampling locations and sample preparation

Sampling was carried out at 28 locations across Sumatra, Java and Borneo (Fig. 2; Table S1). Fish were collected using fish traps, fishing rods, nets and traditional fishing gear or purchased from fishermen on-location. Tissue biopsies (approximately 1 cm<sup>3</sup> from each specimen) were performed with a sterile scalpel, placed in separate 1.5 mL vials and preserved in an absolute ethanol solution for



**Fig. 2.** Collecting sites in Sundaland for the 121 DNA barcode records of *Notopterus notopterus* analyzed in this study. The color gradient refers to depth.

later genetic analysis. Specimens were stored in a 5% formalin solution, and were marked with a label containing a unique number and sample location.

### 2.3. Taxonomy of *N. notopterus*

Until recently, the genus *Notopterus* was believed to be monotypic. However, [Lavoué et al. \(2020\)](#) recognized two distinct lineages based on mitochondrial sequences between eastern Asian and western Asian populations, and resurrected *Notopterus synurus* for the western Asian *Notopterus*. Currently, the most extensive morphological description of *N. notopterus* available is a compilation of characters from both species, *N. notopterus* and *N. synurus*, by [Roberts \(1992\)](#). This publication is still the main reference for the morphology of *Notopterus* species used e.g., [Ng \(2020\)](#) or [Froese and Pauly \(2023\)](#). [Roberts \(1992\)](#) description suggests a low morphological diversity within *Notopterus*, already mentioned by [Lavoué et al. \(2020\)](#). These authors highlighted the need of a detailed morphological comparison of *N. notopterus* and *N. synurus*. In subsection 3.2. we give a compilation of diagnostic characters of *N. notopterus* from Sundaland.

### 2.4. Morphometric and meristic analysis

A total of 21 measurements were recorded using a dial caliper, including: standard length (SL), from the tip of snout to the caudal fin's central base; head length (HL), from the tip of the snout to the occiput's posterior border; snout length (SNL), from the tip of the snout to the anterior front edge of eye; head width (HW), from left to right of the jaw; interorbital width (IW), distance between the left and right eyes; head depth (HD), measured along a line traversing perpendicularly the top of the head just above the eye and passing through the middle of the eye, to the bottom of the lower jaw; upper jaw length (UJL), from the tip of the snout to the posterior edge of the pre-maxilla; lower jaw length (LJL), from the chin tip to the posterior border of the mandible; eye diameter (ED), from the upper to lower border of the orbital cavity; body depth (BD), highest distance between dorsal and ventral; body width (BW), from left to right of the origin of the pectoral fins; pre-dorsal length (PDL), from the tip of the snout to the bottom of first dorsal fin; pre-pelvic length (PPL), from the tip of the snout to the bottom of anterior pelvic fin; pre-ventral length (PVL), from the tip of the snout to the bottom of anterior ventral fin; pre-anal length (PAL), from the tip of the snout to the base of anterior anal fin; dorsal base length (DFBL), from first to last dorsal fin ray; dorsal fin length (DFL), from anterior to posterior of dorsal fin; ventral fin length (VFL), from anterior to posterior of ventral fin; anal fin base length (ABL), from first to last anal fin ray; anal fin length (AFL), highest distance of anal fin; and pelvic fin length (PFL), from base to the tip of first pelvic fin.

All measurements were transformed as percentage of SL, and all measurements of the head are presented as a percentage of HL. All morphometric data were analyzed by Principal Component Analysis (PCA) using SPSS version 23. Prior to analysis, all measurements were transformed using a covariance matrix (within-groups) and stepwise analysis before PCA was run to check for the homogeneity of variance ([Turan et al., 2006](#); [Stella, 2019](#);). The meristic count includes the number of dorsal fin rays (D), pectoral fin rays (P), anal fin rays (A), the number of scale rows on the preoperculum (SPO), the number of perforated scales along the lateral line (LL), the number of scales between LL and the dorsal fin (SLD), and the number of abdominal scutes (SCT). We also examined the presence of a dark spot at the base of the pectoral fin, as it has been previously used to distinguish *Notopterus* species in Southeast Asia.

### 2.5. DNA extraction and sequencing

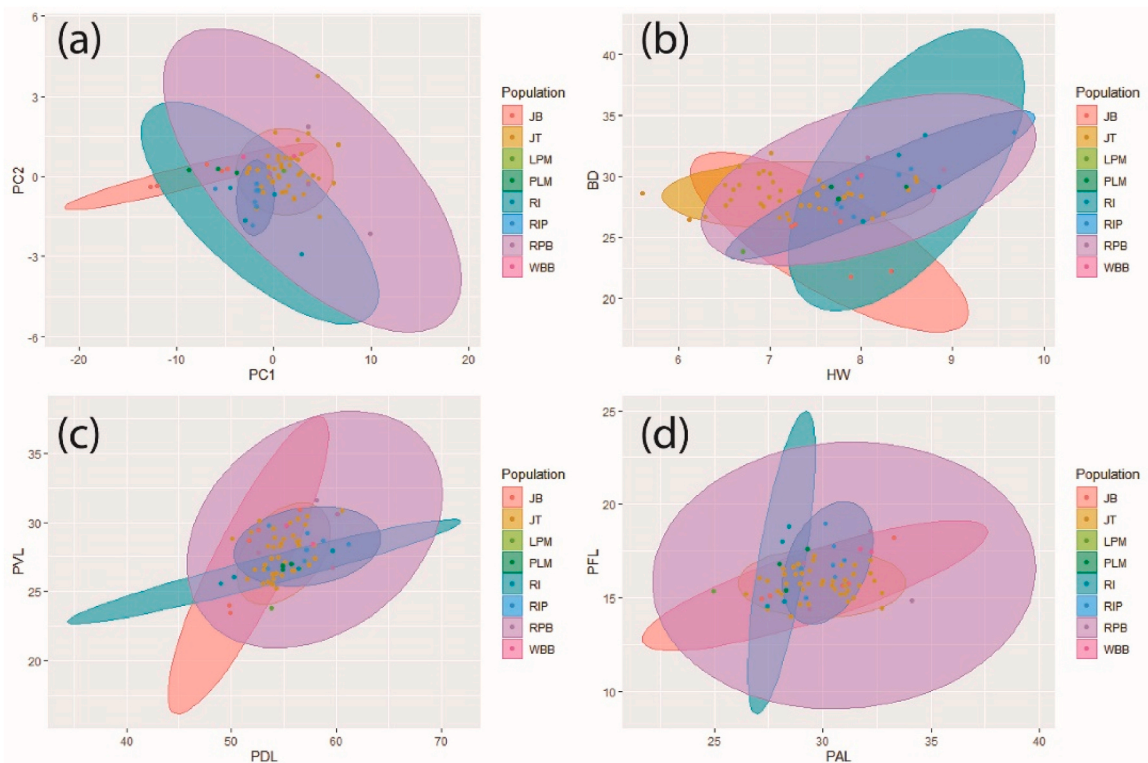
DNA extraction were performed on 87 specimen tissues using the DNA Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions. Extracted A 652-bp segment from the 5' region of the cytochrome oxidase I gene (COI) was amplified using the primer pair FishF1 (5' TCAACCAACCACAAAGACATTGGCAC-3'), and FishR1 (5' TAGACTTCTGGGTGGCCAAAGAATCA-3') ([Ward et al., 2005](#)) or the primer cocktail C.FishF1t1-C.FishR1t1 ([Ivanova et al., 2007](#)). The PCR reactions for the primer cocktail had a final volume of 10.0 µl containing 5.0 µl Buffer 2X, 3.3 µl ultrapure water, 1.0 µl each primer (10 µM), 0.2 µl enzyme Phire Hot Start II DNA polymerase (5 U) and 0.5 µl of DNA template (~50 ng). PCR amplifications with the primer pair FishF1/FishR1 had a final volume of 25.0 µl containing 12.5 µl of Taq ready mix, 9.5 µl ultrapure water, 1.0 µl each primer (10 µM) and 1 µl of DNA template. The thermal cycling conditions for the primer cocktail consisted of an initial denaturation at 98 °C for 5 min followed by 30 cycles denaturation at 98 °C for 5 s, annealing at 56 °C for 20 s and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. The thermal cycling conditions for the primer pair FishF1/FishR1 consisted of an initial denaturation at 95 °C for 10 min followed by 35 cycles denaturation at 94 °C for 60 s, annealing at 48 °C for 60 s and extension at 72 °C for 20 s, followed by a final extension step at 72 °C for 7 min. The PCR products were visualized using 1% agarose gel and further purified with ExoSap-IT® (USB Corporation, Cleveland, OH, USA) and sequenced in both directions. Sequencing reactions were performed using the "BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction" and sequencing was performed on an automatic sequencer ABI 3130 DNA Analyzer (Applied Biosystems). Sequences were deposited in GenBank and BOLD ([Ratnasingham and Hebert, 2007](#)).

### 2.6. Genetic species delimitation and phylogenetic inferences

Several methods for species delineation based on DNA sequences have been proposed ([Pons et al., 2006](#); [Ratnasingham and Hebert, 2013](#); [Kapli et al., 2017](#); [Puillandre et al., 2021](#)). Each of these have different properties, particularly when dealing with singletons (i.e. lineages represented by a single sequence) or heterogeneous speciation rates among lineages ([Luo et al., 2018](#)). A combination of different approaches is increasingly used to overcome potential pitfalls arising from uneven sampling ([Kekkonen and Hebert, 2014](#);

Kekkonen et al., 2015; Shen et al., 2019; Sholihah et al., 2020; Arida et al., 2021;). We used four different algorithms of sequence-based species delimitation to delimitate Molecular Operational Taxonomic Unit (MOTU): (1) Refined Single Linkage (RESL) as implemented in BOLD and used to generate Barcode Index Numbers (BIN) (Ratnasingham and Hebert, 2013), (2) Assemble Species by Automated Partitioning (ASAP) (Puillandre et al., 2021), (3) Poisson Tree Process (PTP) in its single (sPTP) and multiple rates version (mPTP) as implemented in the stand-alone software mptp\_0.2.3 (Zhang et al., 2013; Kapli et al., 2017), (4) General Mixed Yule-Coalescent (GMYC) in its single (sGMYC) and multiple threshold version (mGMYC) as implemented in the R package Splits 1.0–19 (Fujisawa and Barraclough, 2013). Both mPTP and GMYC use phylogenetic trees as input file. We reconstructed a maximum likelihood (ML) tree for the former using IQ-TREE (Nguyen et al., 2015) with the most-likely substitution model according to ModelFinder following the Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al., 2017) available at <http://iqtree.cibiv.univie.ac.at> (Trifinopoulos et al., 2016). For the GMYC algorithm we calculated an ultrametric, fully resolved tree using the Bayesian approach implemented in BEAST 2.6.2 (Bouckaert et al., 2014). Sequences were collapsed into haplotypes prior to reconstructing the ultrametric tree using the ALTER online portal (<http://www.sing-group.org/ALTER/>), and Bayesian reconstructions were based on a strict-clock prior of 1.2% per million year (Bermingham et al., 1997). Two Markov chains of 20 million each were ran independently using Yule pure birth and GTR+I+ $\Gamma$  substitution models. Trees were sampled every 5000 states, after an initial burnin period of 5 million. Both runs were examined using Tracer 1.7.1 (Rambaut et al., 2018) (ESS>200), and combined using LogCombiner 2.6.2, and the maximum credibility tree was constructed using TreeAnnotator 2.6.2 (Bouckaert et al., 2014).

A final COI gene tree was reconstructed using the SpeciesTreeUCLN algorithm of the StarBEAST2 package (Ogilvie et al., 2017). This approach implements a mixed-model including a coalescent component within species and a diversification component between species that allows accounting for variations of substitution rates within and between species (Ho and Larson, 2006). SpeciesTreeUCLN jointly reconstructs gene trees and species trees, and as such requires the designation of species, which were determined using the consensus of our species delimitation analyses. The SpeciesTreeUCLN analysis was performed with the same parameters as mentioned above.



**Fig. 3.** Principal component analysis based on 21 morphometric variables collected on the 75 individuals of *Notopterus notopterus* sampled in Sumatra and Java. (a), Plot and centroid of PCA between *N. notopterus* from West Java (JB), East Java (JT), Lampung (LPM), Palembang (PLM), Riau (RI and RIP), Rawa Pening Lake (RPB), and Bening Widias Reservoir (WBB). (b), Relationship between head width (in % head length) and body depth (in % head length) of *N. notopterus* from West Java (JB), East Java (JT), Lampung (LPM), Palembang (PLM), Riau (RI and RIP), Rawa Pening Lake (RPB), and Bening Widias Reservoir (WBB). (c), Relationship between pre-dorsal length (in % standard length) and pre-ventral length (in % standard length) of *Notopterus* species from West Java (JB), East Java (JT), Lampung (LPM), Palembang (PLM), Riau (RI and RIP), Rawa Pening Lake (RPB), and Bening Widias Reservoir (WBB). (d), Relationship between pre-anal length (in % standard length) and pelvic fin length (in % standard length) of *Notopterus* species from West Java (JB), East Java (JT), Lampung (LPM), Palembang (PLM), Riau (RI and RIP), Rawa Pening Lake (RPB), and Bening Widias Reservoir (WBB).

## 2.7. Genetic diversity and phylogeographic inferences

We reconstructed a maximum likelihood (ML) tree of *N. notopterus* sequences using IQ-TREE (Nguyen et al., 2015) with the most-likely substitution model selected by ModelFinder following the Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al., 2017) available at <http://iqtree.cibiv.univie.ac.at> (Trifinopoulos et al., 2016). Bootstrap proportions were estimated with 1000 replicates.

A Bayesian tree of *N. notopterus* haplotype was reconstructed using the Bayesian approach implemented in BEAST 2.6.2 (Bouckaert et al., 2014). Sequences were collapsed into haplotypes using the ALTER online portal (<http://www.sing-group.org/ALTER/>), and Bayesian reconstructions were based on a lognormal clock prior of 1.2% per million year (Bermingham et al., 1997). Two Markov chains of 20 million each were ran independently using a Yule pure birth model and the best substitution model as identified by ModelFinder in IQtree. Trees were sampled every 5000 states, after an initial burnin period of 5 million. Both runs were examined using Tracer 1.7.1 (Rambaut et al., 2018) (ESS>200), and combined using LogCombiner 2.6.2, and the maximum credibility tree was constructed using TreeAnnotator 2.6.2 (Bouckaert et al., 2014).

Several parameters of genetic diversity were estimated using the R package pegas 1.0 (Paradis, 2010) including the number of haplotypes (h), haplotype diversity (Hd) (Nei and Tajima, 1981), nucleotide diversity ( $\pi$ ) (Nei, 1987), genetic diversity based on the number of segregating sites ( $\theta$ ) (Watterson, 1975), and Tajima's D test of neutrality (Tajima, 1989). The spatial distribution of genetic diversity was characterized using the Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) as implemented in pegas with two levels of spatial structure including groups of populations and populations. Two alternative schemes of spatial partitioning were considered: (i) populations within islands, (ii) populations within paleorivers (de Bruyn et al., 2013; Sholihah et al., 2021a, 2021b). Average genetic distances among islands or among paleorivers were calculated based on the distance matrix generated by the AMOVA in pegas using the R package *usedist* (Bittinger and Bittinger, 2022) with the function `dist_multi_centroids`.

## 3. Results

### 3.1. Morphometric and meristic analysis

A total of 75 specimens of *N. notopterus* (range of size in total length: 16.7 – 32.7 cm) from Sumatra and Java (15 specimens from Sumatra, and 60 specimens from Java) were analyzed and compared to assess possible morphometric and meristic differences. The PCA was performed on 21 morphological characters and no particular grouping was observed among individuals and/or populations (Fig. 3a). All individuals belong to a single group. The fishes from Sumatra (RI, RIP, PLM, LPM) and from Java (JB, JT, RPB, WBB) differ in some morphological characters like head width and body depth (Fig. 3b), pre-dorsal and pre-ventral length (Fig. 3c), and pre-anal in pelvic fin length (Fig. 3d). In terms of meristic characters (Table 1), the mean number of dorsal fin rays was 8.9 in Java and 6.7 in Sumatra, while the mean number of pectoral fin rays was 14.2 in Java and 12.1 in Sumatra. In Java, the mean number of anal fin rays was 110 and 103.3 in Sumatra. In terms of number of scale rows on the preoperculum, individuals from Java had a mean number of 7.8, while individuals from Sumatra had a mean number of 5.9. The number of lateral line scales in longitudinal series was in the mean value 161 in Java and 173.5 in Sumatra. The mean number for the transverse scales rows was 22.3 in Java and 23 in Sumatra. The mean number of abdominal scutes was 31.71 in Java and 28.1 in Sumatra. All specimens had no dark spot at the base of the pectoral fin.

### 3.2. Diagnosis of *Notopterus notopterus* (Pallas 1769) (Fig. 1, Table 1)

Numbers are mean. Adults generally uniformly plain colored, no dark spot at base of pectoral fin; cranio-dorsal profile convex; jaws extending posteriorly to a vertical through the posterior edge of the eye; dorsal fin rays 7.8; anal fin rays 106.7; pectoral fin rays 13.0; scale rows on preoperculum 6.8; scales along lateral line 167.3; scales between lateral line and dorsal fin 22.7; number of abdominal scutes 29.9; head length and head depth 20.7% and 15.2% of SL, respectively; predorsal length and preanal length 53.5% and 28.6% of

**Table 1**

Meristic counts for the two morphometric groups detected and collected from the 75 individuals of *N. notopterus* analyzed in this study. Values correspond to the minimum and maximum and means are given in parenthesis.

Group	Java	Sumatra
Number	n = 60	n = 15
Characters:		
Size (in mm) (TL)	167.44-327 (132.40)	159.76-264.24 (212.07)
Dorsal-fin rays	8-9 (8.93)	6-8 (6.73)
Anal-fin rays	98-111 (110.01)	89-111 (103.33)
Pectoral-fin rays	11-14 (13.78)	10-14 (12.13)
Scales rows on preoperculum	7-8 (7.75)	7-13 (7.86)
Scales along the lateral line	160-165 (161)	130-229 (173.46)
Scales in dorsal transverse series / between Lateral Line and Dorsal Fin	22-24 (22.33)	21-26 (23)
Blackspot on pectoral fin base	No	No
Abdominal scutes	28-34 (31.71)	21-34 (28.13)

SL, respectively; dorsal fin base length and anal fin base length 3.1% and 72.1% of SL, respectively; snout length 22.2% of head length; eye diameter 21% of head length; upper jaw length and lower jaw length 42.5% and 36,1% of head length, respectively.

### 3.3. Species delimitation and phylogenetic analysis

A total of 87 COI sequences were generated from the 28 locations visited in Sumatra, Java and Borneo (Fig. 2; Table S1). All the newly produced sequences were 652 bp in length and no stop codons were detected, suggesting that the sequences collected represent functional coding regions. In addition, 34 sequences of *N. notoapterus* were mined from GenBank as well as 39 sequences of *N. synurus* and 5 sequences of *Chitala blanci*, *C. lopis*, *C. chitala* and *C. ornata* (Table S1). Several COI sequences of *N. notoapterus* mined from Genbank were 500 bp long and the final alignment was trimmed to 500 bp to match the length of the shortest sequences. In total, 165 COI sequences were analyzed, with 121 sequences belonging to *N. notoapterus*. DNA-based species delimitation methods resulted in congruent delimitation schemes with six MOTUs for ASAP, sPTP and mGMYC, three MOTUs delimited by mPTP and five MOTUs delimited by sGMYC (Fig. 4; Table S1). The final consensus scheme consisted of seven MOTUs, including two MOTUs in the genus *Notopterus* for *N. notoapterus* and *N. synurus*. The divergence of the mitochondrial lineages of *N. notoapterus* and *N. synurus* is dated around 4 Million years ago (Ma), and the MRCA (Most Recent Common Ancestor) of *N. notoapterus* haplotypes is dated around 0.8 Ma (Fig. 4).

### 3.4. Genetic diversity and phylogeography of *N. notoapterus*

The ML tree of the 121 sequences of *N. notoapterus* was reconstructed using the HKY+F+ $\Gamma$  substitution model as indicated by the BIC in ModelFinder (Fig. 5, Fig. S1), and rooted with two sequences of *N. synurus*. The maximum and minimum HKY+F+ $\Gamma$  genetic distances were 0,11671 and 0,0974 between the two species, respectively. The ML tree recognizes nine haplotypes, with varying spatial distribution. The most basal haplotypes are located in Asia Mainland and three haplotypes are observed in Sundaland, including two haplotypes restricted to Sumatra and a third haplotype widely distributed in Sumatra, Java and Borneo.

Both haplotype (Hd) and nucleic ( $\pi$ ) diversity are higher among samples from Continental Asia (Thailand, Peninsular Malaysia,

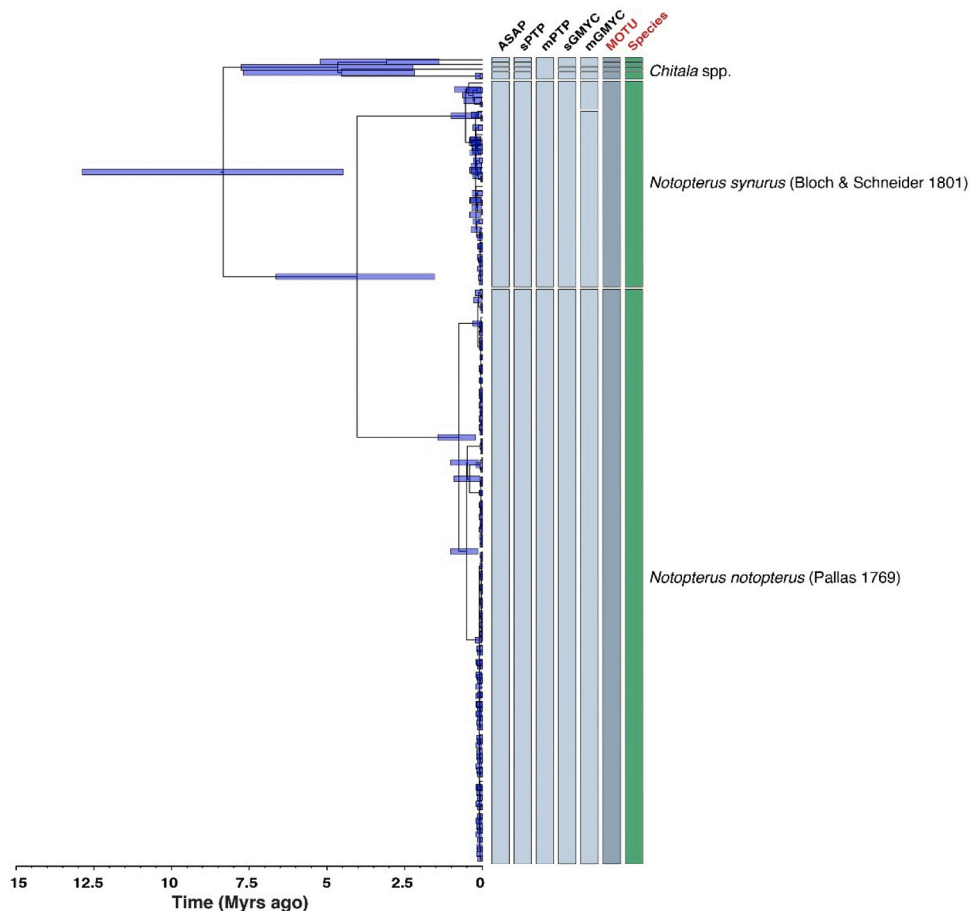


Fig. 4. Mitochondrial gene tree for the 165 DNA barcodes inferred with SpeciesTreeUCLN, including 95% Highest Posterior Density (HPD) interval for node age estimates, genetic species delimitation results for the five methods and their 50% consensus, and species names.

Myanmar) and Sumatra with  $\pi$  and  $\theta_w$  above 0.008 and 2, respectively (Table 2). Genetic diversity is particularly low among Java samples as a single haplotype was detected. The Tajima's D tests was significant only for Sumatra with a positive, significant D value (Table 2), indicative of an excess of rare haplotype.

The Bayesian tree of *N. notopterus* haplotypes, reconstructed with the HKY+F+  $\Gamma$  substitution model, is consistent with a derived position of Sundaland haplotypes in the tree (Fig. 6). Two well supported mitochondrial lineages are identified, with high posterior probabilities and a divergence estimated to happen around 1 Ma. The first mitochondrial lineage (lineage I) is distributed in Asia mainland and Sumatra, and the second mitochondrial lineage (lineage II) is distributed in Asia mainland, Sumatra, Java and Borneo. Lineage I is distributed in two paleorivers, namely Siam and Malacca strait, and lineage II is widely distributed in Siam, North Sunda and East Sunda, but is absent from Malacca strait (Fig. 6).

The AMOVA performed among islands and populations indicates that island partitioning poorly account for the molecular variance as the Sum of Square Deviation (SSD) is  $4.37 \times 10^{-3}$  and fixation index ( $\Phi_{CT}$ ) is 0.062 among islands, and SSD is  $8.79 \times 10^{-3}$  and  $\Phi_{SC}$  is 0.994 among populations within islands, and only the partitioning level among populations within island is significant (Table 3). The result of the AMOVA performed among paleorivers and populations is markedly different. Paleoriver partitioning better account for the molecular variance than islands. The SSD is  $1.14 \times 10^{-2}$  and  $\Phi_{CT}$  is 0.729 among paleorivers, and SSD is  $1.181 \times 10^{-3}$  and  $\Phi_{SC}$  is

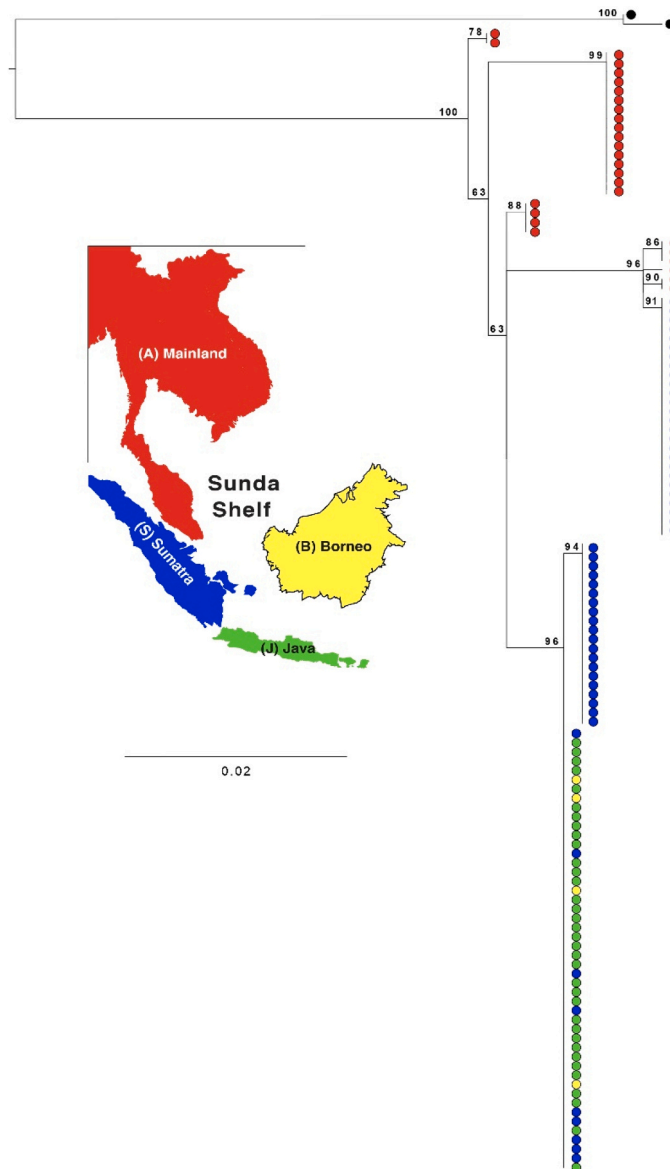


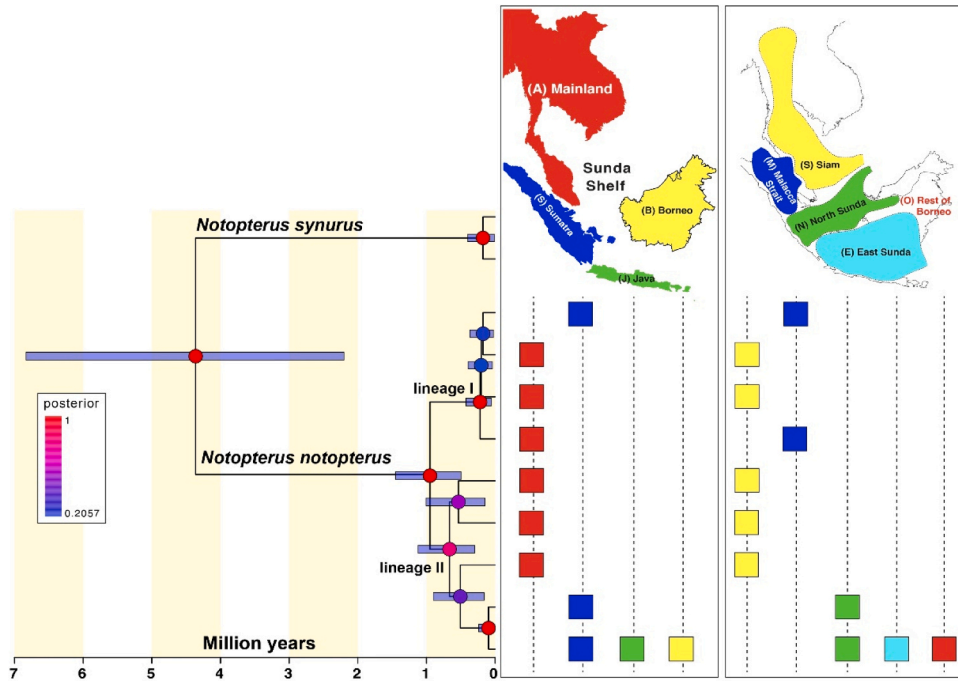
Fig. 5. Maximum Likelihood tree reconstructed with IQ-TREE based on the 121 individual sequences of *Notopterus notopterus*, rooted with two sequences of *Notopterus synurus* with a HKY+F+  $\Gamma$  substitution model and bootstrap proportion (numbers above branches) based on 1000 replicates. Circles represent individual sequences and the color chart illustrates individual sequences distribution across islands.



**Table 2**

Summary statistics of the genetic diversity among populations of *N. notopterus*. *N*, number of individuals; *h*, number of haplotypes; *Hd*, haplotypic diversity;  $\pi$ , nucleotide diversity;  $\theta_w$ , theta; Tajima's D test including D value and significance of the test (\*significant at 0.025 threshold).

	N	h	Hd	$\Phi$	$\theta_w$	D	p-value
Continental Asia	24	5	0.5398	0.0086	4.5524	0.2755	0.7830
Sumatra	55	3	0.6290	0.0103	2.6227	3.8098	0.0001
Java	34	1	0	0	0	-	-
Borneo	4	1	0	0	0	-	-



**Fig. 6.** Bayesian tree inferred with BEAST 2.6.2 based on the nine haplotypes of *Notopterus notopterus*, rooted with two individual sequences of *N. synurus* with a HKY+F+ $\Gamma$  substitution model, a lognormal clock prior of 1.2% per million year and a Yule pure birth model. Node circles illustrate posterior probabilities and node bars represent 95% highest posterior density. Squares illustrate the distribution of haplotypes across islands and paleorivers.

0.979 among populations within paleorivers (Table 3). Both partitioning levels are significant. This result is confirmed by the genetic distances, which are generally smaller between islands, ranging between 0 and 0.012 (Table 4), than between paleorivers, ranging between 0 and 0.02 (Table 5).

**Table 3**

Summary statistics of the Analysis of Molecular Variance (AMOVA) as implemented in pegas for two schemes of spatial partitioning including sites within islands and sites within paleorivers. SSD, Sum of Square Deviation; MSD, Mean Sum of Square; df, degree of freedom;  $\sigma^2$ , standard deviation; p-value obtained after 1000 random permutations;  $\Phi$ , fixation index measuring the correlation of haplotypes at each of the hierarchical levels; and variance coefficient.

	Islands			Paleorivers		
	Between groups (CT)	Within groups, between sites (SC)	Within sites (ST)	Between groups (CT)	Within groups, between sites (SC)	Within sites (ST)
SSD	4.369618e-03	8.798849e-03	-	1.135706e-02	1.811406e-03	-
MSD	1.456539e-03	8.798849e-04	-	2.839265e-03	2.012673e-04	-
df	3	10	-	4	9	-
s2	7.9538e-06	1.1949e-04	-	1.0127e-04	3.6880e-05	-
p-value	0.1683	0	-	0	0	-
F	0.06203826	0.99362414	0.99401969	0.7289951	0.9796334	0.9944806
Variance coefficient	7.357387	10.412015	26.611570	5.436637	13.970047	22.942149

#### 4. Discussion

By aggregating 165 mitochondrial sequences, originating from specimens largely distributed in Southeast Asia, the validity of the two species of *Notopterus* is corroborated as DNA-based species delimitation methods agreed on the recognition of two species within *Notopterus* (Froese and Pauly, 2023; Fricke et al., 2023). Within *N. notopterus*, three unique haplotypes were detected in Sundaland with a first haplotype observed in Java, South Sumatra and South Borneo and corresponding to the paleorivers of North and East Sunda, a second haplotype restricted to South Sumatra, and corresponding to the paleoriver North Sunda, and a third haplotype observed only in the Northern Sumatra and corresponding to the Malacca strait paleoriver. The genetic distances between these haplotypes are low and the six haplotypes observed in Asia mainland are placed in a basal position, suggesting a recent colonization of Sundaland from Asia mainland. This scenario of recent expansion in Sundaland from the continent is further corroborated by the highest genetic and morphologic variability in northern than southern populations. Although, *Notopterus notopterus* is economically exploited in Sundaland and fishing pressure might be responsible for this pattern of genetic diversity among *N. notopterus* populations (Smith et al., 1991; Thorpe, 1993; Pinsky and Palumbi, 2014), it is unlikely to account per se for this North-South gradient of genetic diversity as the fishing pressure is not equally distributed in Sundaland. An historical origin of this gradient is more likely, as ancient paleoenvironmental processes connecting and disconnecting river systems are well known in Sundaland, particularly during the Pleistocene (Lohman et al., 2011; Dixon, 2015; Hutama et al., 2016; de Bruyn et al., 2013; Sholihah et al., 2021a, 2021b). As such, the ancient connection of rivers in Southern Sumatra with rivers on Java Island through the North Sunda paleoriver (Yap, 2002; Hutama et al., 2016; Sholihah et al., 2021a, 2021b) likely explains the occurrence of the same haplotype at these locations. On the other hand, the haplotype exclusive to northern Sumatra, and corresponding to Malacca strait, had limited connectivity to Java Island in the past, and as such is limited to Northern Sumatra. A similar situation was previously depicted for several fish lineages with various distribution ranges in Sundaland (Dahruddin et al., 2021; Kottelat, 1989; Pouyaud and Paradis, 2009; Sholihah et al., 2021a, 2021b). Similar biogeographic patterns have also been described for invertebrates, where phylogenetic studies on freshwater shrimp (*Macrobrachium* spp.) also demonstrated the existence of genetic exchange or accumulation in Sundaland (de Bruyn et al., 2004).

During the late Pleistocene, the subsidence of Sundaland (Sarr et al., 2019; Husson et al., 2020) and volcanic activity partially explains the evolution of the rich Indonesian freshwater fish fauna (Olden et al., 2010). Nevertheless, we cannot exclude alternative and contemporary mechanisms including hydrodynamic effects shaping morphology as has been recently shown for another notopterid fish, the clown knifefish *Chitala chitala* (Chandran et al., 2022). These authors documented distinct intra-specific phenotypic variations among populations from several rivers of the Ganga catchment (India) with different hydrological flows which they linked to separate adaptation to specific environments. Here, the morphological variability observed was moderately structured spatially as some subtle differences were observed between the specimens collected in Java and Sumatra but morphological variability within islands was generally higher than between islands, a trend calling for a broader assessment of morphological variability across a diversity of habitat.

Intraspecific variation of genetic diversity is strongly influenced by biogeographic and environmental conditions, rapid changes during the geological history of a region may foster the development of dispersal routes, hence facilitating gene flow (Lohman et al., 2011; de Bruyn et al., 2012, 2013; Sholihah et al., 2021a, 2021b; Wen et al., 2022). Sundaland is well-known for its complex geological and paleoenvironmental history with intricate connections occurring between islands during the Pleistocene. The bronze featherback illustrates this complexity as islands poorly account for its genetic variability while ancient river systems significantly explain it (Gupta et al., 2013). This pattern has major implications for the conservation of *N. notopterus* and fisheries management for this particular species. Regarding mitochondrial genetic diversity, units of managements should not be defined based on current geography of Sundaland but following the contour of Paleorivers. However, a substantial portion of the genetic variability is observed between sites within paleorivers, suggesting that management units should account for both ancient river contours and sites, for instance by considering portions of paleorivers on different islands as distinct management units. This is best exemplified in Sumatra where the three haplotypes of Sundaland are observed and their distribution match the contours of paleorivers. This partitioning is also consistent with patterns previously described in other fish groups (Sholihah et al., 2020a, 2020b), where islands and paleorivers fragmentation interacted during the Pleistocene. The present study also evidences a higher genetic diversity in Sumatra than in Java and Borneo, suggesting that populations in Sumatra would be a better source for restocking and genetic rescue in Java and Borneo if required. However, defining such managements plans at the regional scale requires a broader assessment of the genetic variability of the bronze featherback at nuclear markers in order to distinguish between past and contemporary gene flow and further confirm the North-South gradient of genetic diversity.

#### 5. Conclusion

The present study reveals a low genetic differentiation among populations across the range distribution of *N. notopterus* in Sundaland, since only a single MOTU was delimited. The genetic and morphological evidence presented here support the recognition of a single valid species of *Notopterus* in Indonesia. However, the high morphological variability and complex partitioning of its mitochondrial genetic variability questions its origin and implication for management. The present study further confirm that *N. notopterus* has a wide distribution in Sundaland, which extend until Borneo to the East and requires a more detailed study of its genetic structure using nuclear markers.

**Table 4**  
Average pairwise uncorrected genetic distances between islands.

	Borneo	Java	Mainland
Java	0.000		
Mainland	0.012	0.012	
Sumatra	0.008	0.008	0.008

**Table 5**  
Average pairwise uncorrected genetic distances between paleorivers.

	East Sunda	Malacca	North Sunda	Borneo
Malacca	0.019			
North Sunda	0.001	0.020		
Borneo	0.000	0.019	0.001	
Sumatra	0.013	0.021	0.015	0.013

### CRedit authorship contribution statement

**Surbani Indah Lestari:** Formal analysis, Data curation, Project administration, Resources, Validation, Writing – review & editing. **Muslimin Boby:** Formal analysis, Validation, Visualization. **Prakoso Vitas Atmadi:** Conceptualization, Supervision, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Dahrudin Hadi:** Supervision, Data curation, Investigation, Methodology, Resources, Visualization, Writing – review & editing. **Vasemägi Anti:** Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Haryono Haryono:** Conceptualization, Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Hubert Nicolas:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **Kurniawan Kurniawan:** Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing. **Ahnelt Harald:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Writing – review & editing. **Wibowo Arif:** Supervision, Validation, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Funge- Smith Simon:** Conceptualization, Funding acquisition, Investigation, Supervision. **Stuart Ivor G.:** Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Jaya Yohanes Yudha P.:** Funding acquisition, Investigation, Project administration, Supervision. **Sudarsono Sudarsono:** Funding acquisition, Project administration, Supervision, Validation, Visualization.

### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

### Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2023.e02786](https://doi.org/10.1016/j.gecco.2023.e02786).

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