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Description of a new species of *Phenacostethus* (Atheriniformes: Phallostethidae) endemic to Kalimantan Selatan, Indonesian Borneo, reveals deep mtCOI divergence among miniature species

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Abstract. *Phenacostethus sikat,* new species, is described from coastal drainages of Kalimantan Selatan, the southeastern province of Indonesian Borneo, that flow into the Java Sea and the Straits of Makassar. This discovery and description brings the number of valid species of priapiumfishes, the atheriniform subfamily Phallostethinae, to 24 and extends the known distribution of the phallostethines to drainages on the eastern margin of the modern Sunda Shelf. *Phenacostethus sikat* differs from congeners in features of the priapium, the complex bilaterally asymmetric, subcephalic copulatory organ of males, and is readily distinguished by a unique, brush-shaped seminal papilla in males, as opposed to a simpler, ruffled seminal papilla in *Phe. smithi,* its inferred sister species based on morphology, and a smooth seminal papilla in males of *Phe. posthon* and *Phe. trewavasae*. Males of *Phe. sikat* and *Phe. smithi* are either sinistral or dextral, the hooked toxactinium arises on the right or left side of the head and curves strongly under the head towards the aproctal side of the body. Males of *Phe. posthon* and *Phe. trewavasae* are exclusively dextral or sinistral, respectively. Males of *Phe. sikat* have an ossified inner pulvinular bone which is cartilaginous or absent in *Phe. smithi*. Males of *Phe. sikat* and *Phe. smithi* have a curved and pointed or blunt second ctenactinium as opposed to a highly reduced second ctenactinium in males of *Phe. posthon* and *Phe. trewavasae*. Females of the new species lack rudimentary pelvic fins which are present in adult females of *Phe. smithi*, *Phe. posthon,* and *Phallostethus*. The new species is also diagnosed by a colour pattern with few or no melanophores along the horizontal septum anterior to the anal-fin origin. The mtCOI sequences of *Phe. sikat* differ from those of congeners by 13.85–24.23%, with an average of 21.54%, an extraordinarily high level of divergence comparable to that of other diminutive fishes. Like congeners, *Phe. sikat* is a miniature species. The largest specimen known is an adult female just 15.3 mm SL.

Key words. allopatry, DNA barcode, endemism, *Phenacostethus sikat*, *Phenacostethus smithi,* Sunda Shelf

INTRODUCTION

Priapiumfishes are a group of small to minute fishes that live in coastal, brackish, and freshwater habitats throughout Southeast Asia. The largest species reaches up to 40 mm SL and many mature at less than half that size (Parenti, 1989, 2014). Priapiumfishes comprise the subfamily Phallostethinae of the atheriniform family Phallostethidae (Table 1). Phallostethine monophyly and morphological homology of the bilaterally asymmetric, subcephalic copulatory organ—the priapium—were reviewed by Parenti (1989). Although the elaborate modifications of the priapium, unique to and diagnostic of the Phallostethinae, have been addressed in detail, questions remain about mode of reproduction and other aspects of life history in this subfamily (Roberts, 1971a; Munro & Mok, 1990; Grier & Parenti, 1994; Mok & Munro, 1997). These tiny fishes are translucent in life, and most go unrecorded in the field. Comparative morphology of females has received little attention but may inform questions of priapial homology (Parenti, 1986a). Study of phallostethines has the potential to elucidate the developmental mechanisms that lead to asymmetrical body plans (e.g., Palmer, 2004).

This description of *Phenacostethus sikat* brings the number of valid species of priapiumfishes, the subfamily Phallostethinae, to 24 (Table 1). The tribe Phallostethini comprises two genera, *Phallostethus,* with three species, and *Phenacostethus*, with four species including the new taxon, recorded from localities around the South China Sea, plus the Indian Ocean coast of the Malay Peninsula, and eastern Borneo that drain into the Java Sea and the Straits of Makassar (Fig. 1). A second tribe, Neostethini, with one genus, *Neostethus,* and 12 valid species, is known from southwestern Sulawesi, eastern Borneo, and the southern Philippines, as well as localities around the South China Sea. A third tribe, Gulaphaliini, with one genus, *Gulaphallus*, and five valid species, is endemic to the Philippines (Table 1).

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Table 1. Classification of the family Phallostethidae (Atheriniformes), following Parenti (2014).

Family Phallostethidae
Subfamily Denatherininae
Genus *Dentatherina* Patten & Ivantsoff, 1983
Dentatherina merceri Patten & Ivantsoff, 1983
Subfamily Phallostethinae
Tribe Phallostethini
Genus *Phallostethus* Regan, 1913 (3 valid species)
Phallostethus dunckeri Regan, 1913
Phallostethus lehi Parenti, 1996
Phallostethus cuulong Shibukawa et al., 2012
Genus *Phenacostethus* Myers, 1928 (4 valid species)
Phenacostethus smithi Myers, 1928
Phenacostethus posthon Roberts, 1971a
Phenacostethus trewavasae Parenti, 1986a
Phenacostethus sikat Parenti, Lumbantobing & Haryono, new species
Tribe Neostethini
Genus *Neostethus* Regan, 1916 (12 valid species)
Neostethus lankesteri Regan, 1916
Neostethus bicornis Regan, 1916
Neostethus amaricola (Villadolid & Manacop, 1935)
Neostethus palawanensis (Myers, 1935)
Neostethus thessa (Aurich, 1937)
Neostethus ctenophorus (Aurich, 1937)
Neostethus borneensis Herre, 1939
Neostethus villadolidi Herre, 1942
Neostethus zamboangae Herre, 1942
Neostethus robertsi Parenti, 1989
Neostethus djajaorum Parenti & Louie, 1998
Neostethus geminus Parenti, 2014
Tribe Gulaphallini
Genus *Gulaphallus* Herre, 1925 (5 valid species)
Gulaphallus eximius Herre, 1925
Gulaphallus mirabilis Herre, 1925
Gulaphallus falcifer Manacop, 1936
Gulaphallus bikolanus (Herre, 1926)
Gulaphallus panayensis (Herre, 1942)

Species of the genera *Phallostethu*s and *Phenacostethus* are well documented from localities around the South China Sea including northwestern Borneo (Fig. 1; Parenti, 2014: fig. 1). A coherent phallostethine biota of the South China Sea was noted by Parenti (2014) who anticipated the discovery of new phallostethine taxa distributed along margins of the Sea (Parenti, 1996). The recent descriptions of *Phallostethus cuulong* from the Vietnamese Mekong (Shibukawa et al., 2012) and of *Neostethus geminus* from coastal Brunei in northwestern Borneo (Parenti, 2014) corroborate that prediction. The current description of a new species extends the known distributional limits of the phallostethines beyond areas surrounding the South China Sea to include those bordering the Java Sea and the Straits of Makassar at the eastern margin of the modern Sunda Shelf. Thus, as well as to describe a new species of *Phenacostethus* based on comparative morphology of males and females and comparison of a DNA locus, an equally important purpose of this report is to spotlight the understudied, endemic coastal fish fauna of the eastern margin of the Sunda Shelf.

MATERIAL AND METHODS

Specimen collection. Specimens were collected in Kalimantan Selatan (Borneo) and Riau (Sumatra) between 2007 and 2009 with the cooperation of the Indonesian Institute of Sciences (LIPI), currently Badan Riset dan Inovasi Nasional (BRIN), the National Research and Innovation Agency. Specimens were collected in accordance with the Guidelines for the Use of Fishes in Research (UFR Committee, 2014) using standard field techniques including seining and dipnetting. Specimens were fixed in 10% formalin and then transferred through graded series of alcohol to 75% ethanol for long-term storage or fixed in 95% ethanol. Specimens reported on herein are housed in the Museum Zoologicum Bogoriense, Cibinong, Indonesia (MZB); National Museum of Natural History, Washington, D.C. (USNM); Zoological Reference Collection, Lee Kong Chian Natural History Museum, National University of Singapore (ZRC); and the Australian Museum, Sydney (AMS).

Specimen preparation and character homology. Counts were made from cleared-and-counterstained preparations, radiographs and microCT scans; the value for the holotype is in brackets. Alcian blue and alizarin red counterstained

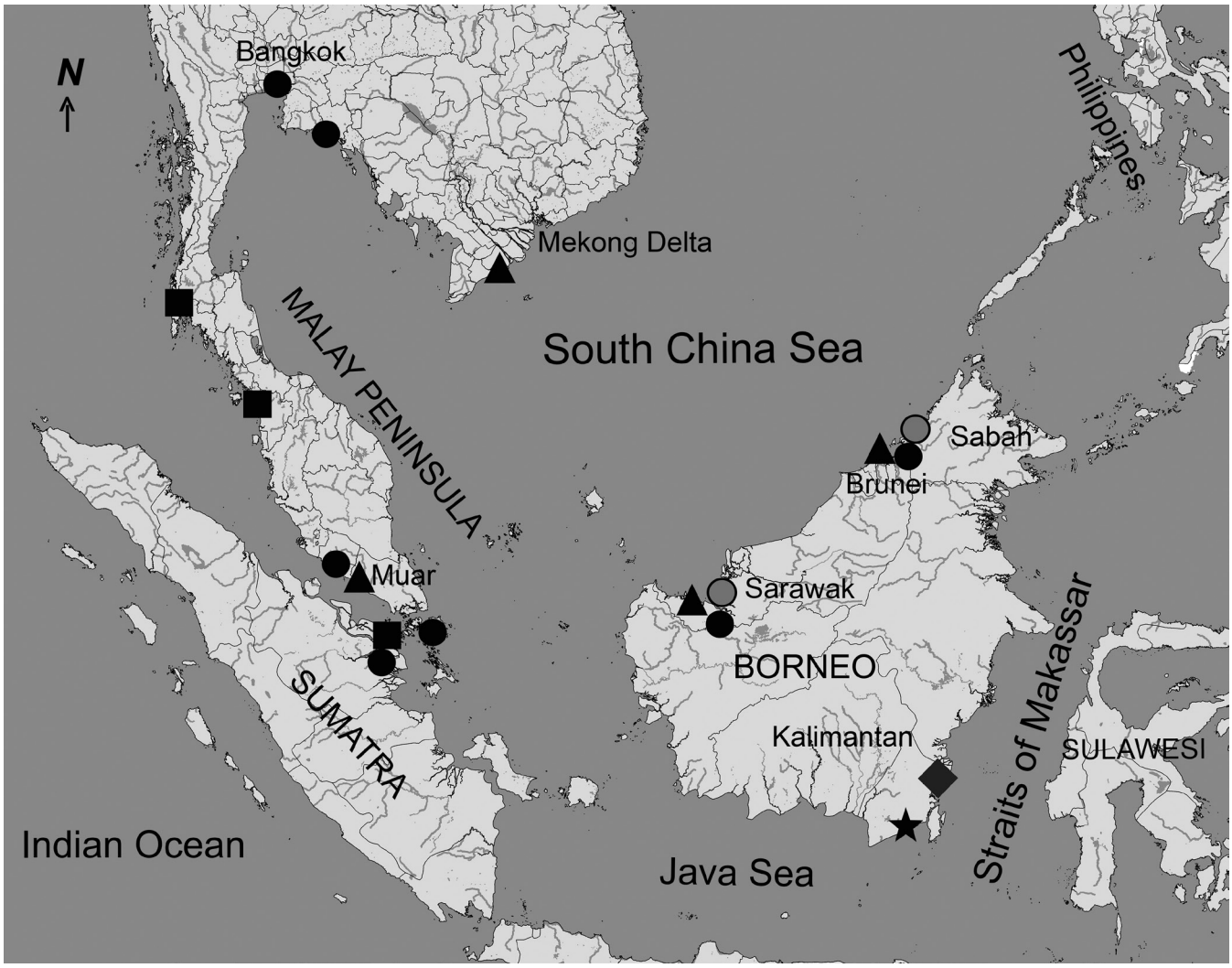

Fig. 1. Distribution of the phallostethin fishes discussed herein. The genus *Phenacostethus*: *Phe. sikat* (black star, southeastern Borneo), *Phe.* sp. 1 (black diamond, southeastern Borneo), *Phe. trewavasae* (gray circles, Sarawak and Brunei, northwestern Borneo), *Phe. smithi* (black circles, Thailand, Cambodia, Sumatra, Peninsular Malaysia, Sarawak, and Brunei), and *Phe. posthon* (black squares, Thailand, Peninsular Malaysia, and Sumatra). Details of the collection localities of *Phe. sikat* are given in the text. The genus *Phallostethus* (black triangles): *Pha. dunckeri* (Muar, Peninsular Malaysia), *Pha. cuulong* (Vietnamese Mekong), and *Pha. lehi* (Sarawak and Brunei, northwestern Borneo). Map includes information from Roberts (1971a: fig. 1). Each symbol may represent more than one collection.

specimens were prepared according to the protocol of Dingerkus & Uhler (1977). A synonymy and homology proposal of the priapial bones and muscles by Parenti (1989) is followed here with qualifications as noted. Regan (1916: 23) first used the terms sinistral and dextral to describe the orientation of the bilaterally asymmetric priapium: in a sinistral male, the anal opening is on the right, the proctal side. The left side of the body of a sinistral male is the aproctal side. The reverse is true for a dextral male. Abbreviations: SL, standard length; asl, above sea level; imm., immature. Other abbreviations are clear in context.

Scanning, reconstruction, and segmentation. The mature male holotype of *Phe. sikat* (MZB 25501) and a mature male *Phe. smithi* (USNM 329581) were scanned on a GE Phoenix v|tome|x m 240/180kV Dual Tube micro-computed tomography (μCT) scanner at the National Museum of Natural History, Smithsonian Institution. Scans were performed using a molybdenum target with the following "Fast Scan" settings: 80 kV, 125 μA, 1000 ms exposure time, and 3.5–3.8 μm voxel size. The resulting x-ray projections were reconstructed into three-dimensional image stacks using the software package datos|x reconstruction v2.4.0. Resulting three-dimensional image stacks were uploaded to MorphoSource (www.morphosource.org). Media identification numbers are listed in the Comparative material section. Image stacks were visualised and segmented using the SlicerMorph module (Rolfe et al., 2021) within 3D Slicer v5.2.2 (Fedorov et al., 2012) and the protocol described in Girard et al. (2022a).

DNA barcoding. To test our morphological species hypothesis, we targeted a 654-bp fragment of the 5′-end of the mitochondrial cytochrome oxidase-c subunit I (mtCOI) locus using Sanger-sequencing. DNA extraction, amplification, and sequencing were all carried out in the Laboratory of Analytical Biology (LAB), National Museum of Natural History, Smithsonian Institution. We extracted the whole genomic DNA from tissue samples (i.e., the caudal portion of the trunk posterior to the first dorsal fin or a muscle biopsy from the right side of the body dissected from ethanol-fixed

voucher specimens) using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We amplified the fragment using the Fish-BCL (TCAACYAATCAYAAAGATATYGGCAC) and Fish-HCL (TAAACTTCAGGGTGACCAAAAAATCA) primer pair (Baldwin et al., 2009) via the polymerase chain reaction (PCR) in a total 25 μL solution containing: 4 μL of the template genomic DNA, 0.5 μL of each primer (10μM), 12.5 μL GoTaq® Hot Start Polymerase (Promega Corporation, Madison, Wisconsin, USA), 0.4 μL $MgCl_2$ (50 mM), 0.16 mg/mL BSA, and 6.7 μL sterile water. For difficult reactions, we added up to 1.2 μL $MgCl_2$ (50 mM) and up to 0.8 mg/mL BSA, while the sterile water was proportionally adjusted to reach the total 25 μL. PCR amplification was performed using a thermal cycler with the following steps: 1 cycle of initial denaturation at 95°C for 6 min; 38 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 45 s; 1 cycle of final extension at 72°C for 7 min. PCR products were visualised on a 1.5% agarose gel stained with GelRed™ (Biotium Inc., Fremont, California, USA) to verify whether the targeted fragment successfully amplified (amplicon) and also to confirm the negative controls. To remove excess primers and nucleotides, the amplicon was cleaned by adding 3 μL of the 9:1-diluted ExoSAP-IT® (Affymetrix Inc., Santa Clara, California, USA) exonuclease enzyme into the 15–20 μL sample, then incubated at 37°C for 30 min using a thermal cycler, followed by heating the sample at 80°C for 15 min to deactivate the enzyme. To sequence the amplicon, we performed a cycle sequencing reaction separately for the forward and reverse directions (bidirectional sequencing)—each in a total 10 μL solution containing: 1 μL of the clean amplicon, 0.5 μL of the pertinent primer (10 μM), 1.75 μL of the sequencing buffer, and 0.5 μL of the fluorescent dye terminators (BigDye™ Terminator Kit v3.1, Applied Biosystem Inc. Foster City, California, USA)—using the thermocyler with the following profile: 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension 60°C for 4 min, and then held at 10°C. Cycle sequencing products were purified through a gel filtration with Sephadex® G-50 (MilliporeSigma, Burlington, Massachusetts, USA) following the manufacturer's protocol. Purified cycle sequencing products were analyzed by automated fluorescence detection on a 3730*xl* DNA Analyzer (Applied Biosystems Inc., Waltham, Massachusetts, USA) sequencer.

Sequence assembly and quality control. To analyse individual sequences and control quality, the resulting trace files from the sequencer were exported into and managed with Geneious Prime v.2019.1.1 (Kearse et al., 2012). Using Geneious, we trimmed both ends of each chromatogram, followed by assembling the forward and reverse strands of each sample to create a contig sequence, which then was examined for stop codons and edited as necessary to be finally generated as a consensus sequence. By BLASTing each of the consensus sequences against the NCBI database (Altschul et al., 1990), we verified whether the sample identity matches any previously generated sequences of phallostethids as listed in the resulting multiple BLAST hits. We downloaded additional mtCOI sequences of potential phallostethid fishes from the BLAST results as well as by querying pertinent taxonomic names using the NCBI Taxonomy Browser (Sayers et al., 2021). We also queried the consensus sequences against the Barcode of Life Database (BOLD; Ratnasingham & Hebert, 2007), from which potential phallostethid mtCOI sequences were also downloaded. To confirm the identity of the downloaded mtCOI sequences from both GenBank and BOLD, we cross-checked them against the GenBank database using the BLASTn search. We aligned the available mtCOI sequences of the verified phallostethids using MUSCLE v.3.8.425 (Edgar, 2004) implemented in Geneious. We inspected the aligned sequences for their reading frames using AliView v.1.26 (Larsson, 2014) and adjusted them manually based on the inferred amino acid translation. The alignment dataset containing only phallostethid taxa was trimmed to yield a 654 bp consensus length. We determined the genetic divergence among the aligned mtCOI sequences by generating a pairwise nucleotide distance matrix based on Kimura 2-parameter distances (Kimura, 1980) using MEGA X v.10.2.6 (Kumar et al., 2018) with missing data treated under the pairwise deletion option.

Molecular Phylogenetic Analysis. The purpose of our molecular phylogenetic analysis was to test our morphological hypothesis that *Phe. sikat* is a valid species. We evaluated the genetic divergence of *Phe. sikat* relative to other phallostethid taxa (i.e., using the pairwise nucleotide distances of mtCOI sequences). We had low taxon sampling for molecules at both intraspecific and interspecific levels. Therefore, we were limited to procedures that do not allow reconstruction of intraspecific clusters and generation of molecular profiles for the resulting assemblages, routine steps when assessing species status via the DNA barcode (Hebert et al., 2003), or similarly via other methods of DNA-based species delimitation, such as GMYC (Pons et al., 2006). Despite this, we performed an analysis using the resulting branch lengths as proxies to: (1) evaluate species validity based on genetic divergence as shown by the depth of the split between species (external branch lengths); and (2) investigate the approximate molecular rates of phallostethid taxa relative to other atheriniform fishes. For the molecular phylogenetic analysis, a new dataset containing representatives of atheriniform lineages (hereafter 'the atheriniform dataset') was assembled by aligning all the available phallostethid mtCOI sequences with those of several additional atheriniform taxa and of two non-atheriniform atherinomorph outgroups (the cyprinodontiform *Aplocheilus lineatus* and the beloniform *Strongylura* sp.) retrieved from GenBank and BOLD (Supplementary Table 1). For the additional atheriniform sequences, representative species with complete or almost-complete length coverage (approximately 654 bp) were chosen from all available atheriniform families to maximise taxonomic coverage. Following assembly of the atheriniform dataset, we conducted model testing and maximum likelihood (ML) phylogenetic inference for the dataset in IQ-Tree version 2.1.2 (Minh et al., 2020). For model testing, we partitioned the dataset based on codon position (i.e., three partitions) and the best-fit model for each partition was estimated using ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in IQ-Tree.

ML tree reconstruction was performed in IQ-Tree with the best-fit models edge-linked (Chernomor et al., 2016), the perturbation strength (-pers) set to 0.1, the number of unsuccessful iterations to stop (-nstop) set to 5000, and the nodal support measured by 1000 pseudo-replicates of ultrafast bootstrap approximation (UFBoot2; Hoang et al., 2018). The resulting tree was visualised with FigTree version 1.4.4 (Rambaut, 2018).

All supplementary material is provided as an attachment within this PDF document. They can be accessed via the Attachment panel (the 'paperclip' icon; accessed by View>Show/Hide>Navigation Pane>Attachments). For optimal compatibility, please use the Adobe Acrobat Reader (free-to-use; download here).

TAXONOMY & SYSTEMATICS

Genus *Phenacostethus* Myers, 1928

Type species. *Phenacostethus smithi* Myers, 1928, by original designation and monotypy.

Differential Diagnosis. *Phenacostethus* differs from the genus *Phallostethus* in having a lower oral jaw that protrudes beyond the upper jaw and a reduced second ctenactinium (priapial bone) which is prominent and serrated in *Phallostethus*. *Phenacostethus* has fewer anal-fin rays (14–15 as opposed to 26–28), second dorsal-fin rays (5–7 as opposed to 8–10) and vertebrae (33–35 as opposed to 40) than in *Phallostethus* (Parenti, 1986: table 1). *Phenacostethus* has a first dorsal fin with one unsegmented ray versus first dorsal fin absent in *Phallostethus*. *Phenacostethus* and *Phallostethus* differ from all other phallostethines (*Neostethus* and *Gulaphallus*) in having a priapium with a prominent, hooked toxactinium and a shield-like pulvinular pad, structures present in other priapiumfishes but not as well-developed.

Composition and distribution. Four species, including the new species described herein (Table 1): *Phenacostethus smithi* Myers, 1928, fresh and brackish waters, Thailand, Cambodia, Sumatra, Peninsular Malaysia, Sarawak, and Brunei; *Phe. posthon* Roberts, 1971a, fresh and brackish waters, Thailand and Peninsular Malaysia, and Sumatra; *Phe. trewavasae* Parenti, 1986b, freshwater, Sarawak, Malaysian Borneo, and Brunei; *Phe. sikat*, new species, freshwater, Kalimantan Selatan, Indonesian Borneo.

Phenacostethus sikat, new species
(Figs. 2–5, Tables 1–5)

Holotype. MZB 25501, sinistral male, 13.4 mm SL, Indonesia, Provinsi Kalimantan Selatan, Kabupaten Tanah Laut, Kecamatan Jorong, Jorong River, along Jalan Ahmad Yani, road from Pelaihari to Simpangempat, (Field no. TGK 01), 3°58.794 S, 114°56.375 E, elevation 20.7 m asl, collected by D. Lumbantobing, D. Rudaya, and A. Daely, 7 August 2007.

Paratypes. MZB 25502, 10: 4 dextral males, 12.5–15.2 mm SL; 2 sinistral males, 11.8–12 mm SL; 4 females, 11.5–13.6 mm SL. ZRC 64468, 3: dextral male 13.5 mm SL; 2 females, 11–11.4 mm SL; USNM 443824, 12: 3 dextral males, 12.2–14.5 mm SL; 2 sinistral males, 11.2–12 mm SL; 4 females, 14.3–15.3 mm SL, from which 4: 1 sinistral male, 12 mm SL, 1 dextral male, 14.5 mm SL, and 2 females, 14.7–14.8 mm SL, have been cleared-and-counterstained for bone and cartilage, collected with the holotype. USNM 393660, 7: 3 dextral males, 11.5–12.6 mm SL, 4 females, 12–15.1 mm SL, Jorong River, along Jalan Ahmad Yani, the road from Pelaihari to Simpangempat, (Field no. TGK 32), 3°58.48 S, 114°56.23 E, elevation 20.7 m asl, collected by D. Lumbantobing, D. Rudaya, and A. Daely, 25 August 2007; USNM 443826, 1 sinistral male fixed in 95% ethanol, of which the posterior portion of the body was used for DNA extraction. USNM 393627, 1 possibly dextral, immature male, 11 mm SL, Asem-asem River, along Jalan Ahmad Yani, the road from Pelaihari to Simpangempat, (Field no. TGK 02), 3°54.419 S, 115°04.828 E, elevation 18.1 m asl, collected by D. Lumbantobing, D. Rudaya, and A. Daely, 8 August 2007.

Differential diagnosis. *Phenacostethus sikat* is distinguished from all other species of phallostethids by a unique, brush-shaped seminal papilla in males with some 15 folds of tissue in the holotype (Figs. 2, 3, 5). This differs from the skin at the distal end of the genital or seminal papilla of male *Phe. smithi*, its inferred sister species based on morphology, which forms prominent ruffles or crenulations, but these are fewer in number—6 to 9 and more distantly spaced than the folds of *Phe. sikat.* Males of *Phe. sikat* and *Phe. smithi* are either sinistral or dextral: the toxactinium, the prominent, curved holding or clasping component of the priapium (Bailey, 1936), arises on the right or left side of the head and curves strongly under head towards the aproctal side of the body. The seminal papilla is on the aproctal side opposite the articulation point of the toxactinium. In congeners *Phe. posthon* and *Phe. trewavasae,* the distal portion of the seminal papilla is smooth, not ruffled, and males are exclusively dextral (*Phe. posthon*) or sinistral (*Phe. trewavasae*). Females of *Phe. sikat* lack rudimentary pelvic fins, as does *Phe. trewavasae*; these are present in female *Phe. posthon* and *Phe. smithi* (see below) as well as *Phallostethus* (Shibukawa et al., 2012: fig. 3). *Phenacostethus sikat* has a second ctenactinium (one of the elongate priapial bones) that is curved and pointed and extends beyond the ventral body profile (Figs. 2, 3, 5). Males of *Phe. smithi* may have either a pointed or blunt second ctenactinium (Figs. 3, 5). Males of *Phe. sikat* differ further from those of *Phe. smithi* in having an ossified, as opposed to cartilaginous or absent, inner pulvinular bone (Parenti, 1989: fig. 2). The new species has a unique pigmentation pattern with few or no melanophores along the horizontal septum anterior to the anal-fin origin (Figs. 2, 4) as opposed to a more complete line of melanophores in congeners, including a putative undescribed species.

Fig. 2. *Phenacostethus sikat*. A, Holotype, MZB 25501, male, 13.4 mm SL. The caudal fin is damaged and incomplete. B, Paratype, USNM 443824, female, 13.8 mm SL. Bars = 1 mm.

Description. Meristic data for the new species are summarised in Table 2. A small, slender laterally compressed species, maximum size recorded 15.3 mm SL. No vestigial pelvic-fin rays or bones in adult females; males with parts of pelvic and pectoral fins modified into a bilaterally asymmetric priapium that is either sinistral (4 male specimens, including holotype) or dextral (11 male specimens): prominent externalised subcephalic bone a toxactinium arising on right or left side of body, articulates with proctal axial bone via a broad base, and curves under the head towards left or right side of body. Priapium fully developed only in largest males. The seminal papilla large and prominent with 15 folds of tissue in the holotype. Cartilaginous pulvinular pad lateral to and covering articulation point of toxactinium and proctal axial bone; small inner pulvinular bone just anterior to point of articulation (Fig. 5). Cartilaginous pulvinular pad suboval. Curved and pointed second ctenactinium articulates with posterior base of aproctal axial and posterior infrasulcar bones (Fig. 5). Papillary bone large with a broad base that articulates with basipenial and prepapillary bones. Papillary and slender penial bone support seminal papilla. Paired priapial ribs (ribs that do not articulate with a vertebra in adults) each with a posterior flange, elongate dorsoventrally, meet just dorsal to the proctal or aproctal axial bone and adjacent to the antepleural cartilage. First articulated rib on fourth vertebra. Ventral dermal keel extends from base of priapium in males or urinary opening in females, to just before anal-fin origin (Fig. 2). Posttemporal bone simple, no ventral limb. Preamaxillary ascending process thin and elongate; rostral cartilage rectangular, longer in anterior-posterior axis. Jaw teeth unicuspid, pointed, in a single uneven row: small medial, teeth separated by a gap from larger, distal teeth. Paradentary bone edentulous, largely cartilaginous. Ventral gill arches with three ossified basibranchials. Hyoid bar with four to five [four] branchiostegal rays. Caudal fin forked, dorsal and ventral rays form incipient lobes. Caudal skeleton with two thin epurals, autogenous parhypural, and a dorsal and a ventral hypural plate.

Principal caudal fin rays i,6–7/7,i [i,7/7,i]. Pectoral fin narrow and elongate, with 9 rays. Two dorsal fins, the first with one short, thickened unsegmented ray supported by a single, elongate pterygiophore; the second dorsal fin with 6 rays. Anal-fin rays 14–15 [14], the first ray short and unsegmented. Vertebrae 32–34 [34] (precaudal 13–15 + caudal 19–21, including ural centrum). Scales on body small, deciduous, not counted as many are lost.

Table 2. Select meristic data for holotype (MZB 25501) and cleared-and-counterstained paratypes (USNM 443824) of *Phenacostethus sikat*, new species. Mean includes holotype.

Character	Holotype	Paratypes	Mean
1st dorsal-fin rays	1	1 (n=4)	1
2nd dorsal-fin rays	6	6 (n=4)	6
Anal-fin rays	14	14–15 (n=4)	14.6
Pectoral-fin rays	9	9 (n=4)	9
Principal caudal-fin rays	i,7/7,i	i,6–7/7,i (n=4)	i,6.4/7,i
Vertebrae	34(15+19)	32–34(13–14+19–21) (n=4)	33.4(13.6+19.8)
Branchiostegal rays	4	5 (n=4)	4.8
Standard length (mm)	13.4	12–14.8 (n=4)	13.9

Colour in life. *Phenacostethus sikat* is translucent in life with an orange spot at base of caudal fin. Melanophore pigmentation pattern as in preservative (below).

Colour in preservative. Ground colouration pale yellow. Colour pattern in alcohol like that of congeners (as in Roberts, 1971a): discrete melanophores scattered on dorsal surface of head and anterior portion of body, along horizontal septum, around orbit, on operculum and priapium, and along basal portion of anal fin, dorsal midline, and ventral midline. Orange spot at base of caudal fin not preserved. Few to no melanophores along the horizontal septum just anterior to a point above the anal-fin origin; from that point, a discrete row of dash-shaped, evenly spaced melanophores continues posteriorly to the caudal-fin base. A translucent, membranous dome on top of the head, flattened in most alcohol specimens.

Distribution and habitat. Known from three collections in southeastern coastal drainages of Kalimantan Selatan, as detailed above, with field numbers TGK 01, TGK 02, and TGK 32 (Figs. 6, 7a). These three coastal stream habitats were at altitudes of 18.1 to 20.7 m asl. Other species collected at these sites and catalogued in the USNM include members of other exclusively freshwater or estuarine taxa in the families Anabantidae, Aplocheilidae, Bagridae, Balitoridae, Osphronemidae, Channidae, Cyprinidae, and Tetraodontidae. Another specimen, *Phe.* sp. 1 (USNM 443825; Fig. 4), that was highly divergent from the new species in mtCOI sequence data (see below), was collected from a coastal stream habitat at 7 m asl (field no. TGK 18; Fig. 7b). Other species collected with it and catalogued in the USNM included those in the family Cyprinidae but are otherwise largely estuarine or coastal taxa in the families Clupeidae, Eleotridae, Gobiidae, and Zenarchopteridae. See discussion under Biogeography, below, for more details on habitat.

Etymology. The specific epithet *sikat*, Bahasa Indonesia for brush, to refer to the distinct, brush-shaped external morphology of the seminal papilla of adult males of the new species.

Remarks. Specimens of the new species are in fair to good condition. The alizarin red bone stain in the four cleared-and-counterstained specimens faded soon after preparation and the skeleton could not be restained. We suspect that the specimens are partially decalcified. Likewise, radiographs of the specimens did not clearly reveal characters of the skeleton. MicroCT scans are used to illustrate the priapial skeleton and some details of external anatomy. Meristic data are recorded for the holotype and cleared-and-counterstained paratypes.

RESULTS

Comparative morphology

Size and Colour Pattern. Species of *Phenacostethus* are the smallest of the priapiumfishes. The new species, *Phe. sikat*, is minute: the largest specimen recorded is an adult female 15.3 mm SL. *Phenacostethus posthon*, at a maximum recorded size of 18 mm SL, is near the maximum size for the genus (Roberts, 1971a, 1971b). The largest specimens of *Phe. smithi* reported by Mok & Munro (1997) are 17 mm SL. The holotype of *Phe. trewavasae*, a mature male, is 14.1 mm SL (Parenti, 1986b), although specimens in USNM 329580, a subsequent collection from Brunei identified as this species, reach 19.0 mm SL. These are delicate fishes with small, deciduous scales in contrast to specimens of *Phallostethus*, *Neostethus,* and *Gulaphallus* which are larger, more robust, and fully scaled.

Specimens of *Phe. sikat* are also distinguished by a unique colour pattern: there are few to no melanophores along the horizontal septum anterior to a point above the anal-fin origin. Posterior to this point, there is a discrete row of evenly spaced, dash-shaped melanophores, that ends at the caudal-fin base.

Males. Male phallostethids in the tribe Phallostethini and the gulaphallin *Gulaphallus bikolanus* have a relatively large, conspicuous seminal papilla. Male *Phe. sikat* and *Phe. smithi* are unique in having a fleshy seminal papilla that has prominent folds at its distal end as opposed to being smooth

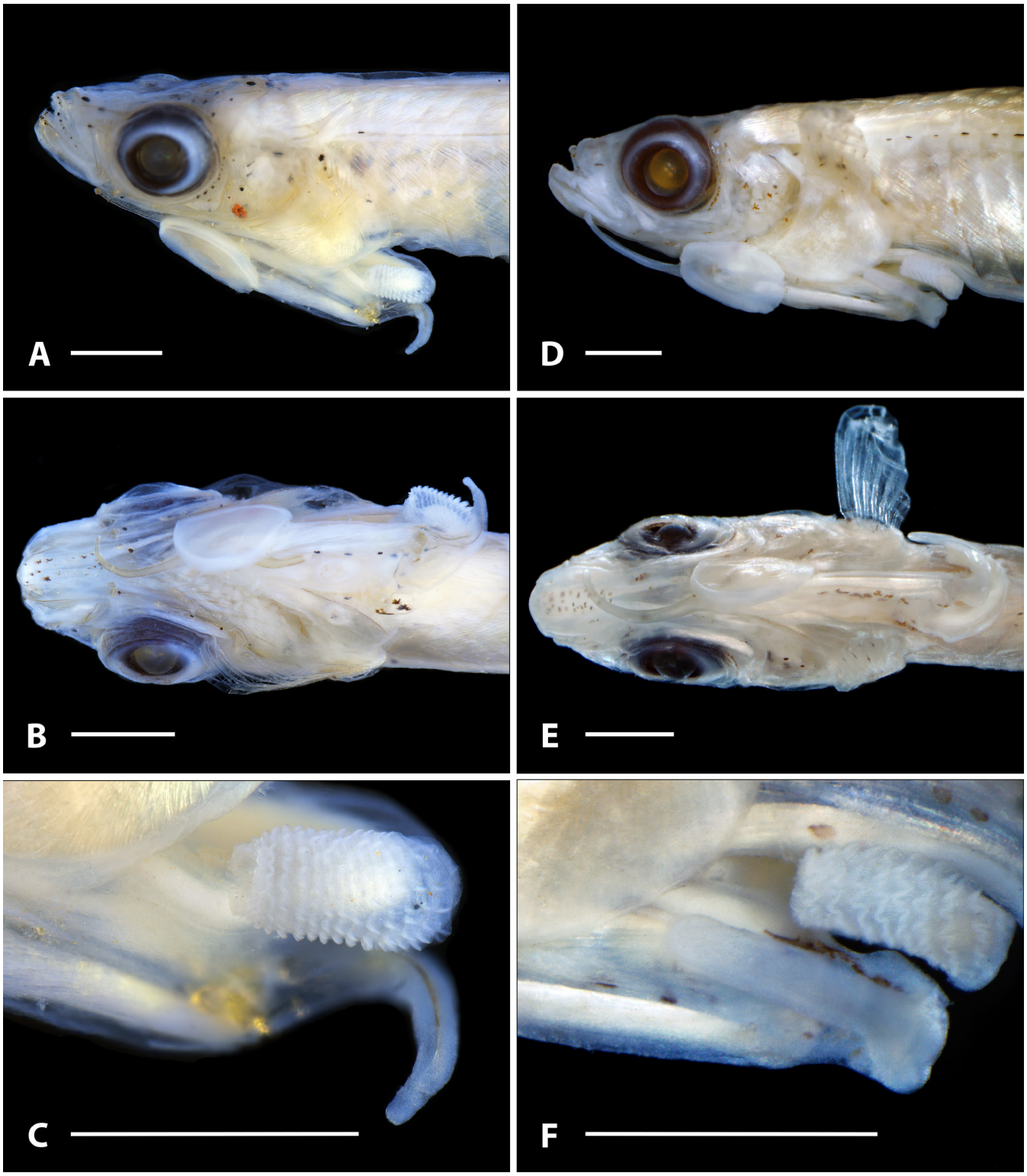

Fig. 3. *Phenacostethus sikat*, holotype, MZB 25501, male, 13.4 mm SL. A, lateral view of head and priapium; B, ventral view of head and priapium; C, lateral view of priapium focused on brush—like seminal papilla. *Phenacostethus smithi*, USNM 329582, male, 16.1 mm SL. D, lateral view of head and priapium; E, ventral view of head and priapium; F, lateral view of priapium focused on ruffled seminal papilla. Bars = 1 mm.

and without ornamentation. We consider this character a synapomorphy and the basis for the hypothesised sister group relationship of the two species. The seminal papilla of *Phe. smithi* is reported to have "…a series of a half-dozen or more crenulated radial folds or extensions resembling a set of ruffled lace cuffs…" (Roberts, 1971a: 10, 12) or "…eight folds of tissue…" (Munro & Mok, 1990: 225). We count 9 in the male specimen that we examined using microCT (Fig. 5). The folds are thicker, more numerous, and more closely placed in *Phe. sikat*—we count 15 in the holotype—and give the distal tip of the seminal papilla species a distinct round brush-like appearance (Figs. 2, 3, 5). The 15 male specimens of *Phe. sikat* ranged in size from 11.5–15.2 mm SL. Of these, only the holotype—which is not the largest

Table 3. Specimens of phallostethids used in the mtCOI analysis with their GenBank accession and museum catalog numbers. See Supplementary Table 1 for a complete list of non-phallostethid atheriniform mtCOI sequences along with the corresponding GenBank/BOLD accession numbers used in this study.

Species	Tissue sample code/ abbreviation	Catalog number	Locality	GenBank	BOLD	References
Phenacostethus sikat	PHES2-I62	USNM 443826	Jorong River, Southeastern Borneo, Indonesia	OP379711	n/a	This study
Phenacostethus sp. 1	PHES1-G77	USNM 443825	Sampanahan River, Southeastern Borneo, Indonesia	OP379712	n/a	This study
Phenacostethus sp. 2	PHE1-ES36	MZB 26075	Reteh River, Riau, Sumatra, Indonesia	OP379713	n/a	This study
Phenacostethus sp. 2	PHE2-ES38	MZB 26075	Reteh River, Riau, Sumatra, Indonesia	OP379714	n/a	This study
Phenacostethus smithi	PHSMI	n/a	n/a	AP006773	n/a	Miya, M. (unpublished)
Neostethus geminus	NGEMI	USNM 389674	Belait, Brunei Darussalam	KR059866	BMIN119100-17	Hagedorn et al. (2018)
Neostethus lankesteri	NLAN1	USMFC (108)0001	Kedah, Peninsular Malaysia	MW498698	DBMR282-19	Abidin et al. (2021)
Neostethus lankesteri	NLAN2	USMFC (108)0001	Kedah, Peninsular Malaysia	MW498699	DBMR283-19	Abidin et al. (2021)
Neostethus lankesteri	NLAN3	USMFC (108)0001	Kedah, Peninsular Malaysia	MW498697	DBMR284-19	Abidin et al. (2021)

Fig. 4. Anterior portion of the body of specimens from which the caudal portion was removed in the field. DNA was extracted from the caudal portion for mtCOI (barcode) analysis. Above, *Phenacostethus sikat*, USNM 443826; below, *Phenacostethus* sp. 1, USNM 443825. Bars = 1 mm.

male—has a fully developed seminal papilla. Seminal papillae of the other males are less elaborate. This is seen in other phallostethin populations (e.g., Roberts, 1971a).

The pointed second ctenactinium of *Phe. sikat* extends beyond the body profile in our mature male specimens (Figs. 3, 5). In contrast, our mature male *Phe. smithi* (Fig. 3, 5) has a straight, blunt second ctenactinium. Males of *Phe. sikat* differ further from those of *Phe. smithi* in having an ossified rather than cartilaginous or no inner pulvinular bone.

Females. The anus and urogenital pores of female phallostethines lie ventral to the pectoral-fin base and are in line from anterior to posterior: anus, oviduct aperture or genital pore, and urinary pore or aperture (Regan, 1913: fig. 2; TeWinkel, 1939: fig. 3; Mok & Munro, 1997). In both *Phe. smithi* and *Phe. posthon*, females have a rudimentary pelvic-fin girdle represented by small, paired chondral ossifications covered by skin (Parenti, 1986a: figs. 4, 5) which Myers (1928: 8) called "post-anal papillae." The pelvic girdle, including fin-rays, is absent in females of *Phe. sikat* and *Phe. trewavasae*.

DNA Barcoding

Database searches and verification. New sequence data of the targeted 654-bp mtCOI were successfully generated for four individuals belonging to three morphologically distinct species of *Phenacostethus* (Table 3; GenBank accession numbers OP379711–OP379714): one individual of *Phe. sikat,* new species (PHES2-I62; OP379711); one individual

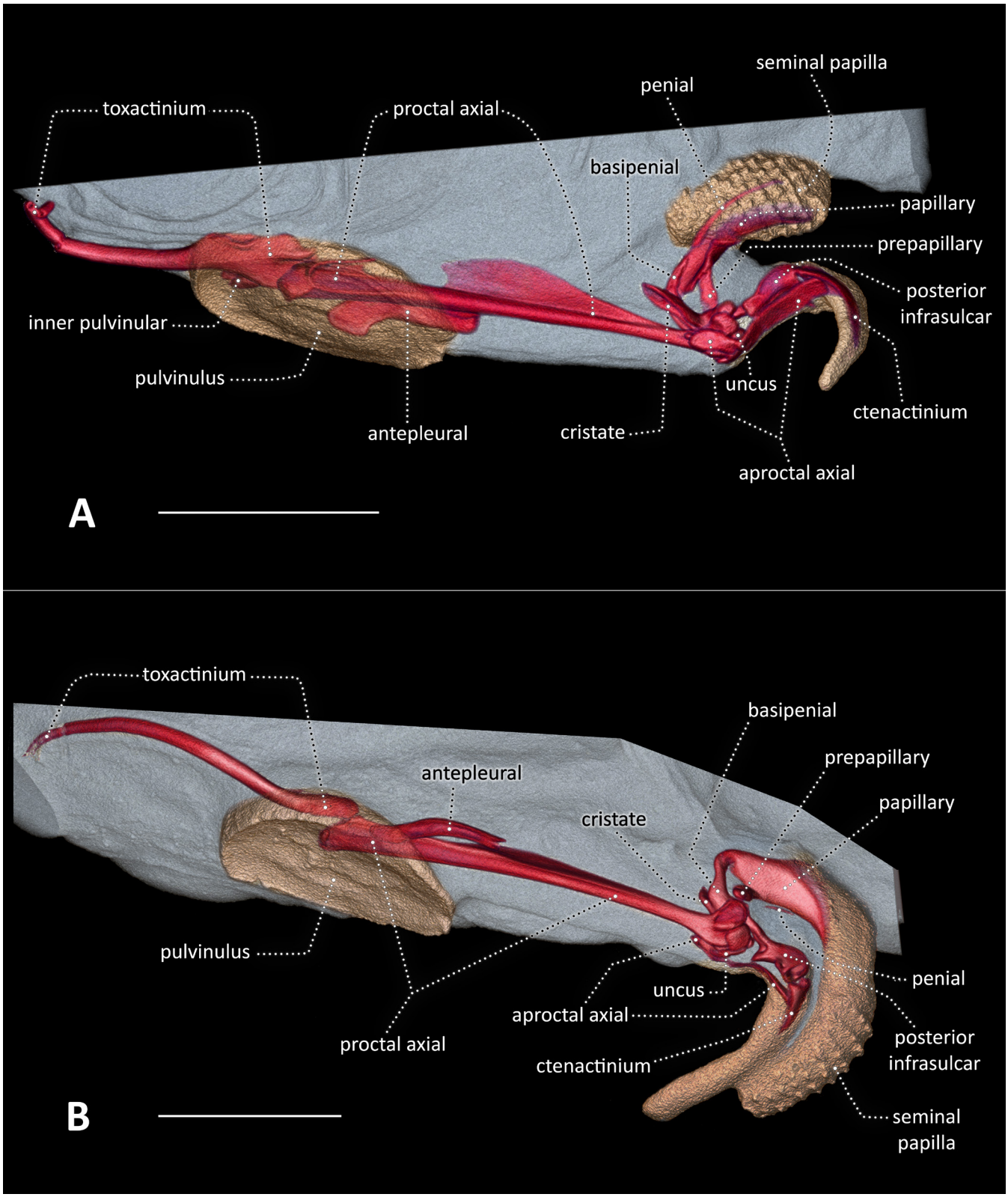

Fig. 5. MicroCT scans to illustrate skeletal and some soft tissue anatomy of the priapium in A, *Phenacostethus sikat*, MZB 25501, holotype, left lateral view of a sinistral male; and B, *Phenacostethus smithi*, USNM 329581, left lateral view of a dextral male. Names of priapial components follow Parenti (1989). The distal tip of the toxactinium, the prominent, anterior, hooked bone, is damaged in both specimens. Bars = 1 mm.

of *Phenacostethus* sp. 1 (PHES1-G77; OP379712); and two individuals of *Phenacostethus* sp. 2 sharing the same haplotype (PHE1-ES36 [OP379713]; and PHE2-ES38 [OP379713]). As we queried the newly generated mtCOI sequences of *Phenacostethus* against the GenBank and BOLD databases, we noted an exceptionally low availability of molecular data for phallostethid taxa. Only two additional sequences from other phallostethid individuals were obtained from GenBank via searches using BLASTn alone. Among the four newly generated mtCOI sequences, only those

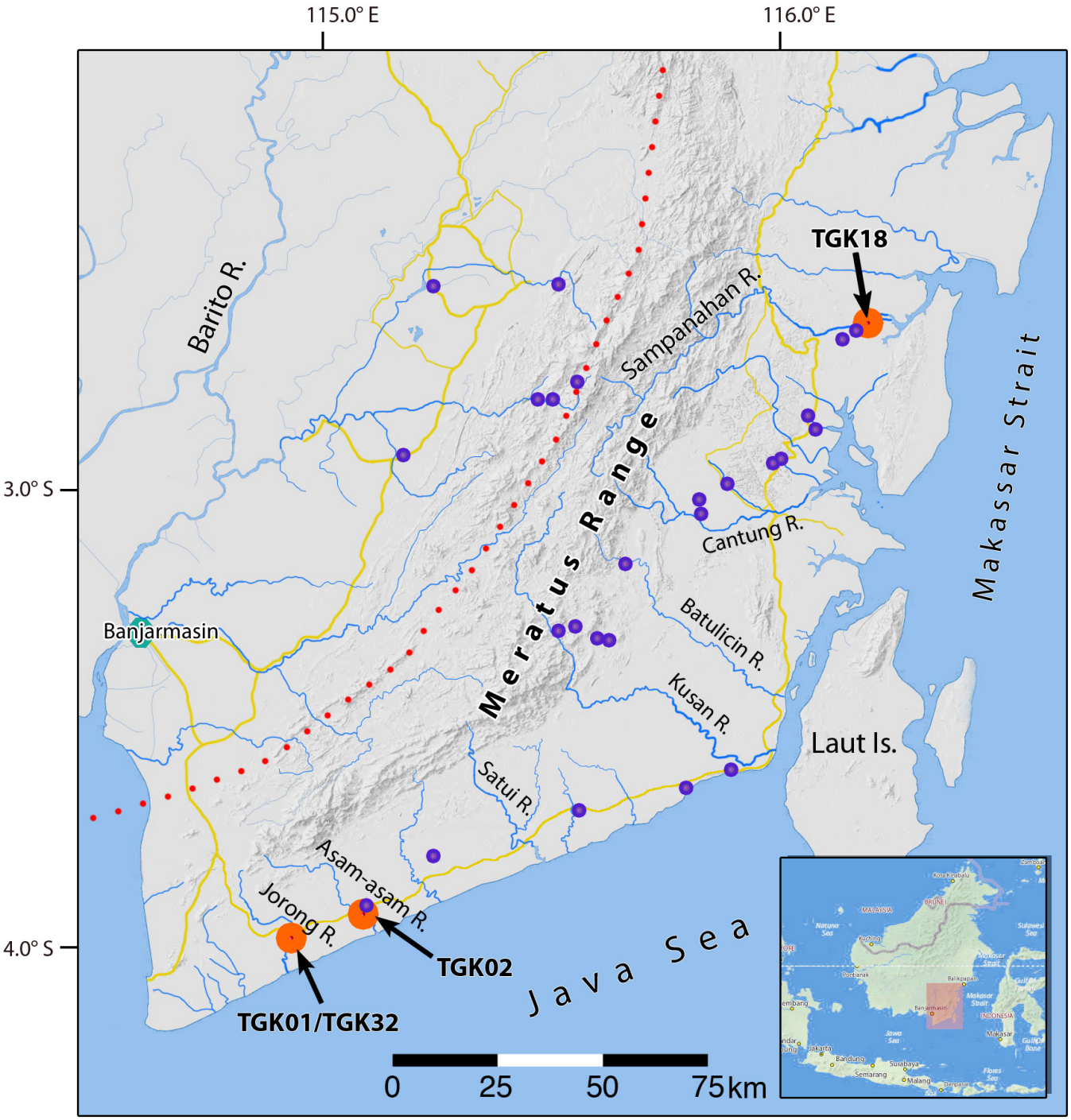

Fig. 6. Collection localities of freshwater and coastal fishes from Kalimantan that included specimens of the genus *Phenacostethus*. TGK01/TGK32 and TGK02, localities of *Phenacostethus sikat*. The holotype was collected at TGK01. TGK18 is the locality of *Phenacostethus* sp. 1, represented by one specimen (USNM 443825) distantly separated from *Phe. sikat* in mt COI data (see Table 4 and Discussion). Blue dots represent other localities sampled in August 2007 which included no phallostethid specimens. Yellow lines are roads. Base map from Microsoft Encarta Interactive World Atlas 2001.

from *Phenacostethus* sp. 1 (PHES1-G77) revealed two additional sequences of phallostethids in the top 100 hits of the search. It matched its congener *Phe. smithi* (AP006773) with 82.14% identity and the other phallostethid *Neostethus geminus* (KR059866) with 84.35% identity (but with a shorter fragment of 607 bp, thus a lower, 92%, query cover), while recovering the gobioid *Awaous grammepomus* as the best hit with up to 83.26% identity (99% query cover; KU692316). In contrast, particularly for the sequences of *Phe. sikat* (PHES2-I62) and *Phenacostethus* sp. 2 (PHE1-ES36 and PHE2-ES38), no phallostethid taxa were among the top 100 matching records of the BLASTn searches. Vouchers identified as the sparid *Dentex* sp. were the best matching records with 85.74% (MK628423) and 91.19% (MK628424) similarities. Identification of these two larval voucher specimens from an upper Mekong tributary as *Dentex* by Panprommin et al. (2020) was based solely on overall sequence similarity and is doubtful.

Table 4. Genetic distances among species of phallostethids based on the mitochondrial COI gene.

	PHES-I62	PHES-G77	PHE1-ES36	PHE2-ES38	PHSMI	NGEMI	NLAN1	NLAN2	NLAN3
PHES2-I62 (*Phenacostethus sikat*)	–								
PHES1-G77 (*Phenacostethus* sp. 1)	22.04	–							
PHE1-ES36 (*Phenacostethus* sp. 2)	13.85	23.57	–						
PHE2-ES38 (*Phenacostethus* sp. 2)	13.85	23.57	0	–					
PHSMI (*Phenacostethus smithi*)	24.23	21.01	25.47	25.47	–				
NGEMI (*Neostethus geminus*)	24.26	18.46	24.98	24.98	12.68	–			
NLAN1 (*Neostethus lankesteri*)	23.15	19.95	22.45	22.45	19.51	21.14	–		
NLAN2 (*Neostethus lankesteri*)	23.38	19.73	22.22	22.22	19.29	20.91	0.16	–	
NLAN3 (*Neostethus lankesteri*)	23.38	19.73	22.22	22.22	19.29	20.91	0.31	0.16	–

By querying Phallostethidae on the NCBI Taxonomy Browser, we retrieved one additional mtCOI sequence of a potential phallostethid species identified as *Neostethus bicornis* (AY655527). While cross-checking this sequence against the GenBank database, we found that it instead matched the gobioid *Gobiopterus lacustris* with 100% similarity; therefore, we excluded it from our subsequent analysis (see below). From the BOLD database, three additional sequences of phallostethid individuals identified as *Neostethus lankesteri* (MW498697–MW498699) were obtained. We incorporated six additional mtCOI sequences that represent three phallostethid taxa (i.e., *Neostethus geminus*, *N. lankesteri*, and *Phe. smithi*)—with initial lengths ranging from 607 to 655 bp—from the GenBank and BOLD records in our multiple alignment dataset containing only phallostethid taxa. Thus, we analysed a total of nine mtCOI sequences representing six phallostethid taxa (Table 4).

Sequence characterisation. In our final 654-bp mtCOI dataset of the phallostethid taxa, no frameshifts, insertions, deletions, or premature stop-codons were detected in any of the sequences, suggesting that they represent functional gene fragments, while also ruling out the possibility of our amplifying nuclear mitochondrial pseudogenes or NUMTs. The alignment of our mtCOI dataset revealed that 208 (31.8%) were variable and 169 (25.8%) of those were parsimony-informative, with 39 singleton sites. These variable sites represent substitutions mostly at the third codon position in up to 195 sites (29.8%), while overall resulting in only four non-synonymous mutations. Based on the pairwise statistical analyses, these substitutions consist of 61 transitional (si) and 42 transversional (sv) pairs, all of which are at the third codon position except for 10 transitions at the first one.

Genetic divergence. We detected remarkably high genetic divergence among the phallostethid taxa—the genera *Phenacostethus* (five individuals in four species) and *Neostethus* (four individuals in two species) (Table 4). The congeneric-interspecific pairwise K2P genetic distance in the genus *Phenacostethus* was 21.46% on average (range 13.85–25.47%) and that of *Neostethus* was 20.99% (range 20.91–21.14%). In particular, the new species *Phe. sikat* exhibited a relatively deep divergence as compared to the other six phallostethid species with an average K2P distance of 21.54% (range 13.85–24.26%). The congeneric-interspecific distance among *Phenacostethus* was 20.04% (range 13.85–24.23%). These values are exceptionally high compared to other fishes of the Indo-West Pacific region assessed with mtCOI given that the average species–within–genus (congeneric interspecific) distances are, for example: 9.93% (range 0–20.63%) among 207 marine fishes in Australia (Ward et al., 2005), 6.5% (range 0–21.7%) among 85 fish genera in the Taiwan Strait (Bingpeng et al., 2018), and 16.7% (range 1.11–23.59%) among 134 mangrove-associated fish species (94 genera) in Peninsular Malaysia (Abidin et al., 2021). Whereas within atherinomorphs, the average K2P distance of *Phenacostethus* for mtCOI sequences (20.04%) is nearly equal to those values at the interfamilial level among three other atheriniform families (i.e., Atherinidae, Atherinopsidae, and Melanotaeniidae; range 20.85–22.37%)

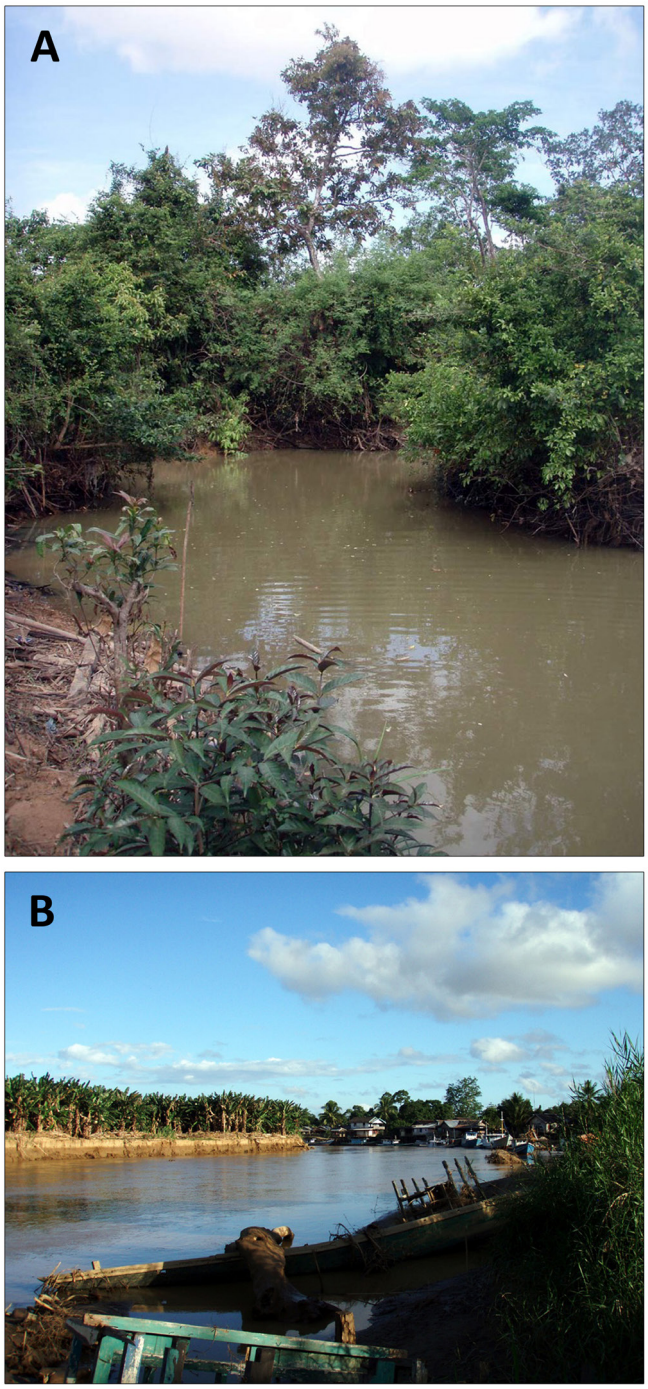

Fig. 7. Two stream habitats in Kalimantan Selatan sampled in 2007: A, Type locality of *Phenacostethus sikat,* the Jorong River at 20.7 m above sea level (3°58.794 S, 114°56.375 E). B, Sampanahan River at Sampanahan Hulu at 7 m above sea level (2°37.745 S, 116°11.170 E).

as reported by Heras & Roldán (2011). Of note, such a high genetic divergence in *Phenacostethus* overlaps the overall range shared by some fishes also characterised by their diminutive body size, such as the danionid minnow *Danionella* (range 10.25–25.50%; Britz et al., 2021) and the characid tetra *Priocharax* (range 8.80–31.8%; Mattox et al., 2020; 2021; 2023).

The overall mean nucleotide base composition for the five mtCOI sequences in the genus *Phenacostethus* was: A (23.43%), C (29.45%), G (18.69%), and T (28.44%), with a slightly biased A+T content (51.87%) higher than the G+C (48.13%). These values show an obvious anti-G bias especially strong in the third codon position (10.83% in average), which is typical for other fishes and vertebrates (Table 5; Perna & Kocher, 1995; Satoh et al., 2016). Despite the overall slightly biased A+T content, *Phenacostethus* sp. 2 shows a slightly bias G+C content of 50.92% in stark contrast to the 44.19% observed in *Phenacostethus* sp. 1. This discrepancy in the G+C content—a 6.73% difference—at the intrageneric level is unusually high among fishes, even vertebrates. As a comparison, the maximum pairwise difference for the mitogenomic G+C content among four congeneric killifish species of *Fundulus* is 3.3% with the G+C content ranges from 38.9–42.2% (Whitehead, 2009; Wagner et al., 2016), whereas among 10 congeneric grouper species of *Epinephelus* the difference is 1.2%, with a range of G+C content from 43.8 to 45% (Zhuang et al., 2013). The average mitogenomic difference of G+C content among a total of 39 species in four genera of birds, for example, is 2.25% within a range of 2–3% (Clare et al., 2008).

For our atheriniform dataset, we obtained from GenBank and BOLD (Supplementary Table 1) 89 additional mtCOI sequences: 87 represent seven non-phallostethid atheriniform fishes, and two represent outgroups (i.e., the cyprinodontiform *Aplocheilus lineatus* and the beloniform *Strongylura* sp.). Given the length-coverage criterion, the shortest initial length for these additional sequences is 652 bp, except for some atheriniform bedotiid species with just 645 bp. After the final alignment that included the nine phallostethid mtCOI sequences, the atheriniform dataset contained a total of 98 terminal taxa. Phylogenetic inference with the atheriniform dataset resulted in a maximum likelihood tree that did not resolve the monophyly of the subfamily Phallostethinae (Supplementary Fig. 1). All the phallostethid taxa examined in our molecular analysis were recovered into two major clades, both resolved as polyphyletic and each embedded in the tree distantly with high nodal support (100% bootstrap value). The first phallostethid clade (hereafter 'phallostethid clade I') is composed of all the *Neostethus* taxa and two species of *Phenacostethus* (*Phenacostethus* sp. 1 and *Phe. smithi*); it is embedded in a larger clade also containing most of the Australian atherinid taxa that is not well supported (65% bootstrap value). The other phallostethid clade (hereafter 'phallostethid clade II') is composed of the two other species of *Phenacostethus* (*Phenacostethus* sp. 2 and *Phe. sikat*) and recovered as sister to all the atherinopsid taxa under analysis; phallostethid clade II and the atherinopsid clade together form a larger clade with moderate support (78% bootstrap value). Apart from being polyphyletic, phallostethid clades I and II are both marked by distinctly long internal and external branches. Particularly for phallostethid clade II, the long branches of the topology, which recovered *Phe. sikat* as sister to *Phenacostethus* sp. 2, demonstrate a deep divergence between the two species.

Mislabeled or misidentified sequences. We cross-checked all the downloaded mtCOI sequences that had potential taxonomic inaccuracies against the GenBank database using

Table 5. Comparison of nucleotide composition and external branch length among the species of phallostethids.

Taxa	Code/Codon	Total length (bp)	Base composition (%)				A+T (%)	G+C (%)	Branch length
			A	C	G	T			
Individual sample:									
Phenacostethus sikat	PHES2-I62	654	22.17	29.51	19.72	28.59	50.76	49.24	0.05
Phenacostethus sp. 1	PHES1-G77	654	24.16	26.15	18.04	31.65	55.81	44.19	0.25
Phenacostethus sp. 2	PHE1-ES36	654	23.24	31.96	18.96	25.84	49.08	50.92	0.15
	PHE2-ES38	654	23.24	31.96	18.96	25.84	49.08	50.92	
Phenacostethus smithi	PHSMI	654	24.31	27.68	17.74	30.28	54.59	45.41	0.12
Neostethus geminus	NGEMI	607	24.55	27.35	17.63	30.48	55.02	44.98	0.1
Neostethus lankesteri	NLAN1	639	23.16	28.48	18.47	29.89	53.05	46.95	
	NLAN2	639	23.16	28.64	18.47	29.73	52.9	47.1	0.2
	NLAN3	639	23.16	28.48	18.47	29.89	53.05	46.95	
Intrageneric mean:									
Phenacostethus spp.	1st position	218	25.23	25.96	30.55	18.26	43.49	56.51	–
	2nd position	218	15.14	27.98	14.68	42.20	57.34	42.66	–
	3rd position	218	29.91	34.40	10.83	24.86	54.77	45.23	–
	All positions	654	23.43	29.45	18.69	28.44	51.87	48.13	–

BLASTn as well as verified them with the pertinent references (Sparks & Smith, 2004; Kadarusman et al., 2012; Sasaki & Kimura, 2014, 2020; Jayadi et al., 2019). We found that a total of 29 previously generated sequences deposited in GenBank and BOLD were likely mislabeled or misidentified based on the most recent taxonomic revision (e.g., Sasaki & Kimura, 2020), comparisons of sequence identity (e.g., being identical vs. >6% different) or discrepancies between what was recorded in the GenBank database and that in the associated publication (e.g., Jayadi et al., 2019) as listed in Supplementary Table 2. These sequences were generated from published (Sparks & Smith, 2004; Aquilino et al., 2011; Zhao et al., 2016a, 2016b; Goodbody-Gringley et al., 2019; Jayadi et al., 2019; Panprommin et al., 2020; Sasaki & Kimura, 2020; Wang et al., 2020) and unpublished studies. To minimise the accumulation of multiple iterations of these potential taxonomic inaccuracies, we follow Girard et al. (2022b) and identify and provide updated identifications for these sequences. We did not examine the voucher specimens for these sequences; therefore, our revised identifications are tentative. Some of these revised identifications—22 sequences representing 14 species—were used for the terminal labels of the corresponding sequences on the resulting phylogenetic hypothesis and highlighted in red-coloured font (Supplementary Fig. 1). Although the identifications of their constituent sequences have been verified herein as possibly accurate, several non-phallostethid atheriniform genera are paraphyletic or polyphyletic in the tree, such as *Pseudomugil*, *Telmatherina*, *Paratherina*, *Rheocles*, *Melanotaenia*, *Atherinella*, *Menidia*, and *Atherina*. This may be due to insufficient data sampling.

DISCUSSION

Reproductive biology and morphology. Male phallostethins are fully mature when the toxactinium, the prominent curved priapial bone, is fully-grown and the sperm ducts that lead to the seminal papilla are full of sperm (Munro & Mok, 1990: 225). Fewer than one-third of the males from collections of *Phe. smithi* examined by Munro & Mok (1990) were fully mature. Further, they reported just a single male with a well-developed seminal papilla. Roberts (1971a) also reported that only the largest of the males he examined had a fully developed seminal papilla. Among our 15 male specimens of *Phe. sikat*, only the holotype has a fully developed, highly crenulated seminal papilla. Reasons for such a small proportion of mature males in samples of both species—and solely a single male with a well-developed papilla—are speculative and include increased mortality or a shift in habitat with maturity or seasonality (Munro & Mok, 1990) or a failure to collect mature males.

Fertilisation is internal in all phallostethine fishes, as far as known (Roberts, 1971a; Munro & Mok, 1990; Grier & Parenti, 1994; Mok & Munro, 1997). Males in the genera *Neostethus* and *Gulaphallus* form sperm bundles or spermatozuegmata that are transferred to females who

subsequently lay fertilised eggs. Sperm are abundant in the ovarian cavities of phallostethines from which we infer that nearly all viable oocytes are fertilised (Grier & Parenti, 1994). The seminal papilla of male *Neostethus* and *Gulaphallus* is smaller and simpler than that of male *Phenacostethus* which form transitory "sperm balls" that disperse into free sperm (Mok & Munro, 1997). The large and fleshy seminal papilla of males in the genus *Phenacostethus* is correlated with the efficient transfer of free sperm. This mirrors the reproduction among internally fertilising cyprinodontiform fishes: sperm bundles are well-developed in males of species that transfer the bundles to females through a groove in an elongate gonopodium, as in most viviparous poeciliids, for example. In contrast, male anablepids do not form sperm bundles, or form incomplete bundles, and transfer free sperm to females through a fleshy, tubular gonopodium that is continuous with the sperm duct (Grier & Parenti, 1994; Mok & Munro, 1997).

Deep molecular divergence in phallostethids. The deep molecular divergence of *Phe. sikat*—as reflected by pairwise nucleotide distances relative to the other phallostethid species (Table 4) and all the relatively long branches in phallostethid clade II (Supplementary Fig. 1)—supports our morphological hypothesis that *Phe. sikat* is a valid species. We had only one individual to assess species limits via DNA-based approaches. We cannot calculate an intraspecific distance necessary to estimate a cutoff value between intra- and interspecific variabilities (e.g., the barcoding gap; Meyer & Paulay, 2005) to compare with pertinent threshold values in various methods of DNA-based species delimitation (Lim et al., 2012; Ahrens et al., 2016). Despite this, a single specimen may be used to test validity of a species because of its highly distinct genetic makeup, in conjunction with ad hoc criteria that include complementary data concordant to this genetic differentiation (Lim et al., 2012). The genetic distances of *Phe. sikat* to its congeners (≥13.85%) are excessively high relative to most standard threshold distances common in animal barcode analyses for initial species diagnosis. The values for *Phe. sikat* are at least fourfold greater than the molecular threshold (3.5%) proposed by Ward et al. (2009) to provisionally recognise a new species of fish. With the average congeneric mtCOI distance of 20.04% (range 13.85–24.23%), *Phe. sikat* is roughly two times more divergent than congeners as compared to Australasian fishes (103 genera) with the average congeneric distance based on singletons being 9.64% (Ward et al., 2009). Further, the genetic divergence is over two times higher in *Phe. sikat* than in the atheriniform rainbowfish genus *Melanotaenia* (mean congeneric distance 9.20%; range 0–17%; Kadarusman et al. (2012).

Our preliminary molecular phylogenetic analysis based on a single mitochondrial locus recovered phallostethids as polyphyletic (Supplementary Fig. 1). Although we realise the limit of using only mtCOI sequences to resolve deep phylogenetic nodes (such as the interfamilial level) in fish systematics, there is an exceptionally broad range of overall G+C content within *Phenacostethus* (Table 5) worth highlighting here, considering the congeneric level still being at a relatively shallow standpoint, yet resulting in such a distantly polyphyletic topology. A high level of base composition heterogeneity—as reflected by large discrepancies in G+C content—is likely to represent a significant deviation from stationarity that can mislead phylogenetic analyses (Mooers & Holmes, 2000; Romiguier & Roux, 2017). The deviation in G+C content has been identified as responsible for topological incongruences recovered with low support (e.g., Betancur-R et al., 2013; Dornburg et al., 2017; Hirt et al., 2017; Kuang et al., 2018). We suspect that the polyphyly of phallostethid taxa (Supplementary Fig. 1) may largely be due to the highly biased G+C content among species of *Phenacostethus*. Phylogenetic studies on atherinomorph fishes using more genetic markers and more samples of phallostethids are needed not only to test this hypothesis about the biased G+C content (see Clare et al., 2008 on the sentinel approach based on mtCOI sequences), but also to reconstruct more rigorously the phylogeny of family Phallostethidae and its atherinomorph relatives.

Correlated with such a bias in G+C content, the evolutionary rate among phallostethid taxa may also be exceptionally rapid compared to other atheriniform fishes because of their extremely small size. In vertebrates, including ray-finned fishes, rates of molecular evolution strongly correlate with body size; rates tend to be faster in taxa with smaller bodies because body size correlates with several physiological and life history traits affecting molecular evolution, such as generation time, life span, population size, and metabolic rate (Martin & Palumbi, 1993; May et al., 2020; Tan et al., 2021). Hirt et al. (2017) identified a significant correlation between an increased rate of molecular evolution and small body size in actinopterygian fishes, especially in Cypriniformes as reflected by the long branch leading to the miniature *Paedocypris*, pointed out earlier by Britz et al. (2014). A similar pattern is observed in the diminutive ricefish *Oryzias setnai* (see Yamahira et al., 2021; Britz et al., 2022) and perhaps the miniaturised tetra *Priocharax* (see Souza et al., 2022). This rapid rate of molecular evolution may lead to long-branch attraction, further confounding our ability to reconstruct phylogeny.

Biogeography. The distribution of the seven species in the genera *Phallostethus* and *Phenacostethus* is marked by allopatry and endemism (Fig. 1; Parenti, 2014: fig. 1). The three species in the genus *Phallostethus* are distributed around the South China Sea in Muar, Peninsular Malaysia (*Pha. dunckeri*), the Vietnamese Mekong (*Pha. cuulong*), and northwestern Borneo (*Pha. lehi*). *Phenacostethus trewavasae* is a northwestern Borneo endemic. Its inferred sister species, *Phe. posthon,* is endemic to localities on the Indian Ocean coast of the Malay Peninsula and Sumatra (Parenti, 1989: fig. 1). *Phenacostethus smithi* is the most widely distributed species of phallostethin. It was described from Bangkok, Thailand, and has been collected from the Cambodian Mekong, Sumatra (Riau), Peninsular Malaysia, and northwestern Borneo (Sarawak and Brunei). We hypothesise, based on variation in morphology of the priapial elements and mtCOI data reported here, that it represents

more than one species and should be revised. The seventh species, *Phe. sikat*, was collected in coastal localities along the southeastern margin of Kalimantan (Figs. 1, 6). Its discovery extends the known distributional limits of the phallostethins beyond areas surrounding the South China Sea to include those bordering the Java Sea and the Straits of Makassar between eastern Borneo and western Sulawesi.

The eastern margin of Sundaland was extended by a mid-to Late Cretaceous accretion of Gondwanan terranes that is marked in eastern Kalimantan by the Meratus suture (Fig. 6; Metcalfe, 2011; Zahirovic et al., 2014). The biogeographic separation of modern Borneo and Sulawesi are recognised as Wallace's Line; the genera *Phallostethus* and *Phenacostethus* live west of that boundary, as far as known. *Phenacostethus sikat* and the potentially undescribed species that we call *Phenacostethus* sp. 1 (USNM 443825) live east of the Meratus Mountains, isolated from congeners. *Phenacostethus sikat* is genetically most similar to *Phenacostethus* sp. 2 (PHE1-ES36 and PHE2-ES38) from Sumatra, Indonesia, with an interspecific distance of 13.8% for mtCOI. In comparison, *Phe. sikat is* relatively more divergent from *Phenacostethus* sp. 1 (PHES1-G77), with an interspecific distance of 22.7%. *Phenacostethus sikat* and *Phenacostethus* sp. 1 were collected in different localities on the eastern versant of the Meratus Range, each in a separate drainage and habitat. The type locality of *Phe. sikat* is in the middle mainstream of the Jorong River—a small river basin draining the southernmost slope of the Meratus Range—characterised by a relatively narrow width (5–10 m), water with low turbidity and moderate current, a muddy substrate with organic materials, banks lined with tree vegetation, and an assemblage of stenohaline fishes, such as ostariophysans (e.g., *Nemacheilus* sp., *Rasbora* sp., and *Mystus* cf. *castaneus*) and anabantiforms (e.g., *Anabas testudineus*, *Channa striata*, and *Trichopodus trichopterus*). In contrast, *Phenacostethus* sp. 1 was collected from the lower mainstream of the Sampanahan River, a much larger river basin draining the eastern slope of the Meratus Range, marked by a broader width (30–50 m) with highly turbid water swiftly flowing over a sandy bottom mixed with thick, muddy sediment, with its banks lined by a more open, disturbed vegetation. This locality appears to be along a fluvio-tidal transition zone given that its species assemblage included not only fewer stenohaline cyprinoid species, but was also marked by euryhaline fishes, such as gobies (e.g., *Gobiopterus* sp., *Redigobius* sp., and *Stenogobius* sp.) and a sleeper (*Butis* sp.). These differences in habitat characteristics of the two species—*Phe. sikat* and *Phenacostethus* sp. 1— may explain their deep genetic divergence. Given that *Phenacostethus* sp. 1 is known only from one specimen, we cannot test this hypothesis.

The phallostethid fauna around the Java Sea and Straits of Makassar is poorly known. Species in the genus *Neostethus* are part of a biota that spans the Straits of Makassar, hence spanning Wallace's Line. *Neostethus lankesteri* (AMS I.19355-041) is known from Balikpapan Bay near the mouth of the Mahakam. *Neostethus borneensis* has been reported from eastern Sabah, Malaysian Borneo (Parenti, 1989: fig. 1). Sulawesi harbours just a single species of phallostethine, *Neostethus djajaorum*, described from a coastal drainage in the southwestern arm (Parenti & Louie, 1998).

A survey of the Mahakam River, eastern Kalimantan, by Kottelat (1994) reported no specimens of phallostethids, although that study focused on riverine, not coastal, habitats. More recent surveys in Indonesian Borneo have focused on peat swamps in Central Kalimantan, west of the Meratus range (Haryono & Wahyudewantoro, 2020) and include no phallostethids. Our study is the first to integrate molecular approaches to assess the taxonomic status and the diversity of phallostethid species. The results of our mtCOI DNA barcode analysis not only reflect the scarcity of genetic information available for phallostethid taxa in public databases such as GenBank and BOLD, but also the striking rarity of vouchered genetic samples available for the group. This is unfortunate given the unparalleled morphological evolution of priapiumfishes, much still unknown to science, and the deep molecular divergence and highly disparate nucleotide composition among species of phallostethid species that we report here. Continued surveys to better understand the levels of endemicity of the native fish fauna and phylogenetic analyses to understand relationships among the species are warranted.

CONCLUSIONS

The morphologically distinct *Phenacostethus sikat* is described from coastal localities in Kalimantan Selatan, the southeastern portion of Indonesian Borneo. Its discovery expands the known range limits of the phallostethin fishes. The divergence in mtCOI sequences, the DNA barcode, between the new species and its congeners is remarkably deep, ranging from 13.8–24.23%. Deep molecular divergences mark the evolution of phallostethins throughout their broad range. These deep divergences, coupled with a high degree of endemism, reflect a prolonged period of isolation among taxa, rapid rates of differentiation, or a combination of both. Additional collecting and study of phallostethins throughout Southeast Asia is needed to better understand the limits of their distribution, the extent of their endemism, and their phylogenetic relationships.

Comparative material. *Neostethus lankesteri*, AMS I.19355-041 (26), Indonesian Borneo: Kalimantan Timur: Balikpapan Bay. *Phallostethus cuulong*, Paratypes: USNM 404477 (1), USNM 404478 (1), USNM 404479 (1, cleared and stained), Vietnamese Mekong. *Phallostethus lehi,* USNM 365043 (4), Sarawak, Malaysia. *Phenacostethus* sp. 1, USNM 443825, 1 female fixed in 95% ethanol, posterior part of body used for extraction of DNA. Kalimantan Selatan, Kabupaten Kotabaru, Kecamatan Sampanahan, Sampanahan River at Sampanahan Hulu, (Field no. TGK 18), 2°37.745 S, 116°11.170 E, elevation 7 m asl, D. Lumbantobing, D. Rudaya, and A. Daely, 19 August 2007. *Phenacostethus* sp. 2, MZB 26075 (2), Riau, Sumatra, Indonesia. *Phenacostethus posthon,* USNM 229302 (150), Muar, Malaysia. *Phenacostethus smithi*, USNM 88667 (100), Thailand; USNM 326054 (10), Bintan Island, Riau Archipelago, Indonesia; USNM

329581 (73), USNM 329582 (16), Daerah Belait, Brunei. *Phenacostethus trewavasae*, USNM 329580 (15), Daerah Belait, Brunei. Image stacks of the two μCT-scanned specimens have been uploaded to MorphoSource as follows: *Phenacostethus sikat*, MZB 25501, holotype, media identifier 000529527; *Phenacostethus smithi*, USNM 329581, media identifier 000529510.

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