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Status and phylogeny of Milyeringidae (Teleostei: Gobiiformes), with the description of a new blind cave-fish from Australia, *Milyeringa brooksi*, n. sp.

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Abstract

A phylogeny of Milyeringidae is reported and a new species, *Milyeringa brooksi*, is described from Cape Range National Park in the North West Cape (Cape Range Peninsula) of Australia. This species is distinguished on the basis of morphological and molecular characters from its only congener *Milyeringa veritas*. These diagnostic characters are related to a unique pattern of sensory papillae on the body and synapomorphies in three genes (cytochrome c oxidase I, cytochrome b, and NADH dehydrogenase 2). The new species is known only from the southern portion of the North West Cape spanning roughly 50 kilometers of subterranean habitat. This habitat is exceedingly rare and measures to preserve it and its fauna should be taken.

Key words: blind, cave, stygobites, taxonomy, troglodytic

Introduction

Whitley (1945) erected Milyeringidae, to contain the monotypic genus *Milyeringa*. He noted that the only morphological feature separating this family from the Eleotridae was the lack of eyes and this feature was the main diagnostic characteristic distinguishing it from other Gobioidei. He conjectured that *Milyeringa* had a close phylogenetic association with *Carassiops* (=*Hypseleotris*) and *Typhleotris* with the caveat that, "the interesting implications of this new form must await fuller elaboration later when I can refer to more literature than is available to me here in Western Australia." Unfortunately, the comparative data was lacking until recently.

The lone member of the monotypic family, *Milyeringa veritas* Whitley 1945, was described from Milyering Well on the North West Cape of Western Australia. *Milyeringa veritas* was described without many details about habitat and ecology; these details were produced much later by the meticulous studies of Humphreys and colleagues (2001, 2006; among others). At the time of its description, *M. veritas* was only known from the type locality. Humphreys and colleagues have subsequently observed this taxon from over two dozen sites throughout the North West Cape and adjacent Barrow Island (Humphreys, 2001). Adams and Humphreys (1993) conducted a population level study of *M. veritas* using allozymes and found significant population level structure among the fishes collected from these sites and hypothesized that some populations may represent undescribed species. Collections made by Darren Brooks and the author in May of 2009 confirm that members of the southern most range of *Milyeringa* constitute a new species that is described herein.

Materials and Methods

Specimens included in comparative analyses are listed in the species description and Materials Examined. Institutional abbreviations are as follows: LSUMZ (Louisiana State University Museum of Zoology, Baton Rouge), SAM (South Australia Museum, Adelaide); WAM (Western Australia Museum, Perth).

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Morphometric measurements, following Hubbs and Lagler (2004), were recorded to the nearest 0.1 mm using dial calipers and included: standard length (SL), head length (HL), head width, upper jaw length, body depth, pectoral-fin length, caudal-fin length, pelvic-fin length, first dorsal-fin base, second dorsal-fin base, anal-fin base, caudal peduncle length, caudal peduncle width, caudal peduncle depth, pre-1st dorsal length, pre-2nd dorsal length, prepelvic length, and preanal length. Other traditional measurements, such as, snout length, interorbital width and orbit diameter are excluded because of the lack of eyes in this taxon.

Specimens were collected using dipnets in caves and wells on the North West Cape of Australia. Tissues (fin clips, or flank muscle) were sampled and preserved in 95% ethanol. Tissue, GenBank, and voucher catalog numbers for all taxa examined are listed in Table 1.

Table 1: Genbank data and locality information for sequences .

Scientific Name	Locality	Voucher	COI	ND1	CYTB
Milyeringa veritas 357	Woburi Rockhole	No voucher	HM590592	HM590604	HM590598
Milyeringa veritas 358	Kudumurra Well	No voucher	HM590593	HM590605	HM590599
Milyeringa veritas 383	Milyering Well	LSUMZ 13636	HM590594	HM590606	HM590600
Milyeringa veritas 385	New Moubowra Cave (C-495)	LSUMZ 13638	HM590596	HM590608	HM590602
Milyeringa veritas 386	Kuburu Well (C-27)	LSUMZ 13639	HM590597	HM590609	HM590603
Milyeringa brooksi 384	Pilgonoma Well (C-274)	LSUMZ 13637	HM590595	HM590607	HM590601
Milyeringa brooksi	Javis Well (C-263)	ABTC22891, SAM	AY722169	AY722306	AY722241
Milyeringa brooksi	Javis Well (C-263)	ABTC22891, SAM	AY722169	AY722305	AY722240
Outgroups					
Perccottus glennii			AY722171	AY722308	AY722244
Odontobutis potamophila			AY722174	AY722311	AY722247

A total of 2707 bp of DNA sequence was obtained from the following genes: cytochrome c oxidase I (COI; 1107 bp), cytochrome b (cytb; 626 bp), and NADH dehydrogenase 2 (ND2; 972 bp). Total DNA was extracted using a Qiagen DNEasy tissue extraction kit following the manufacturer's protocol. Gene fragments were amplified and sequenced using primers and protocols from Thacker and Hardman (2005): for COI the primers were GOBYL6468 and GOBYH7127; for ND2 the primers were GobyL4035, GobyL4041 and GobyH5258; and for cyt B the primers were Glu-2 and THr-R1. The annealing temperature used for all runs was 55° C and all sequence data were collected at Louisiana State University's Museum of Natural Science Molecular Lab.

Contigs were built in Sequencher version 4.8 (Gene Codes, Ann Arbor, MI) using DNA sequences from the complementary heavy and light strands. Sequences were edited in Sequencher. All novel sequences are deposited in GenBank under the accession numbers listed in Table 1. Outgroup taxa, *Perccottus glennii* and *Odontobutis potamophila* were selected because of their proximity to members of *Milyeringa* in the goby phylogeny of Thacker (2009). Anatomical terminology for the sensory papillae and other features are based on Wongrat and Miller (1991) and Miller (1973).

Maximum likelihood analyses and bootstrap calculations were conducted in RaxML 7.0.4 (Stamatakis et al., 2008) using the Cipres Portal V 1.15 implementing a GTR (General Time Reversible) + gamma (estimated in analysis) model for all molecular data. Individual gene partitions were analyzed, as was a concatenated data set. A total of 10,000 bootstrap replicates were run under a partition based on gene

fragments and coding regions (1st, 2nd, 3rd). An exhaustive parsimony search and a 10,000 replicate jackknife search was conducted in PAUP* 4.0 (Swofford, 2001).

Results

Two distinct clades of *Milyeringa* are recovered with high support values in phylogenetic analyses conducted under maximum likelihood and parsimony frameworks (trees were identical in topology, only the ML tree with parsimony jackknife values is shown; Fig.1). Sequence divergence between the two *Milyeringa* clades ranged from 2.7% to 3.2%. Sequence divergence within each of the two major clades (representing both species) was less than 1%.

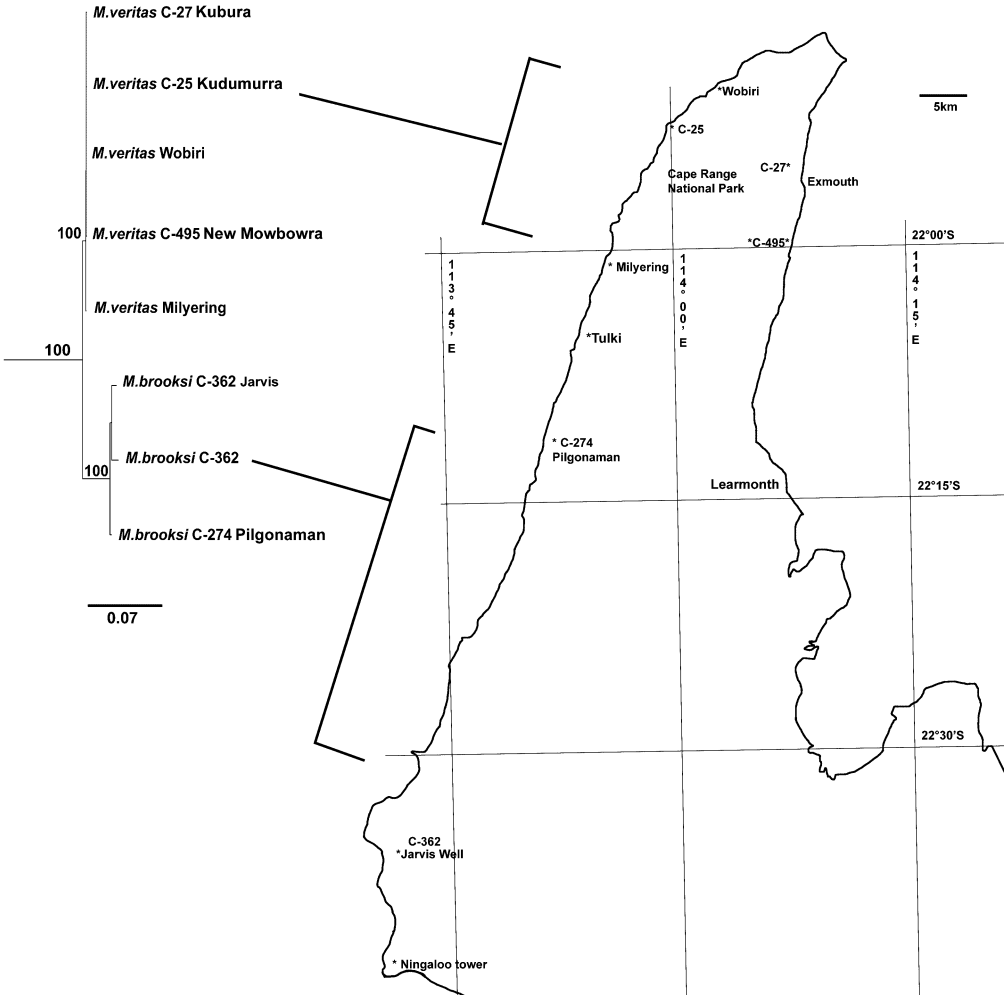

FIGURE 1. Phylogeny of Milyeringidae and map of the North West Cape of Australia. Phylogeny shown is a maximum likelihood analysis (parsimony analyses recover identical clades of *Milyeringa*), with parsimony jackknife values shown on ingroup clades (outgroups trimmed for illustration). Numbers or names not in italics next to scientific names represent localities.

GenBank sequences of *Milyeringa* created by Thacker and Hardman (2005) represent specimens from site C-362 or Javis Well (22° 36'S, 113° 41'E), in the North West Cape of Australia. This site has now dried out and has lacked fish for many years (Humphreys pers. comm.; tissue samples from SAM). These are the only GenBank sequences previously available for *Milyeringa.* New GenBank sequences from this study include data from Wobiri (21° 50'S, 114° 04'E), Kubura (21° 55' S, 114° 07'E), Milyering (22° 01'S; 113° 55'E),

New Mowbowra (21° 59’ S, 114° 07’E), Kudumurra (21° 52’ S, 114° 00’E) and Pilgonaman (22° 11’ S, 113° 52’E).

In this study no phylogenetic structure was recovered between the “Northeast” group of *Milyeringa* (sampled here from New Mowbowra C-27) and specimens from the “Northwest” which includes Milyering Well (the type locality of *M. veritas*) and Kudumurra C-25 (both sampled here); this contradicts the finding of Humphreys (2001; Adams and Humphreys 1993), who recognized these groups as distinct lineages based on allozyme data. Sequence divergence was less than 1% among samples from the northern range of *Milyeringa*. Members of the northern clade recovered in this analysis include specimens from the type locality of *M. veritas* (Milyering Well) and therefore represents *M. veritas* sensu strictu (Fig. 1). A “Southern clade” (Jarvis C-362 and Pilgonaman C-274) recovered in this study was also recovered by Humphreys (2001; Adams and Humphreys 1993) and corresponds to the new species described below (Fig.1).

***Milyeringa brooksi*, new sp.**

Holotype:— WAM P28330-001, Pilgonaman Well, 22°11’S, 113° 52’E, 8 July 1984 collected by M. Newton (Fig. 2, 3; Table 2).

FIGURE 2. Holotype of *Milyeringa brooksi*, WAM P28330.

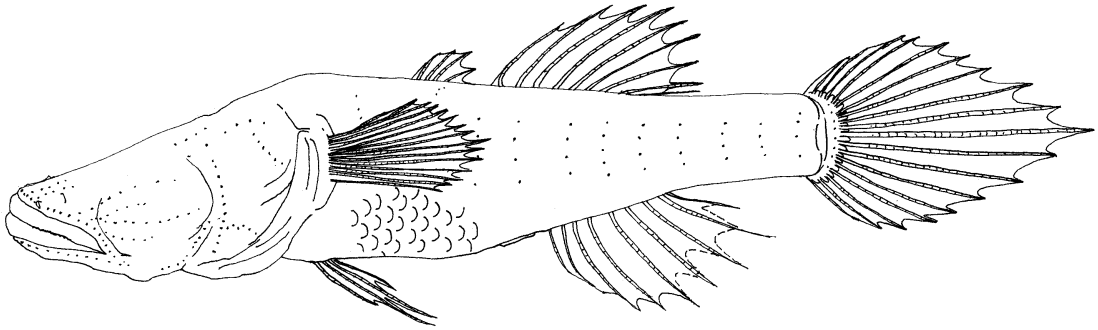

FIGURE 3. Illustration of the holotype of *Milyeringa brooksi*, WAM P28330 showing expanded fins, selected scales and sensory papillae. Illustration by Jill Meredith Dowling.

Paratypes:— LSUMZ 13637, Pilgonaman Well, 22° 11’ 30.5"S, 113° 52’ 00.1"E, 21 May 2009, AUS-6-2009, 1:15-1:40pm, collected by P. Chakrabarty, D. Brooks, and Annemarie Noël; WAM P29242 (n=2), 22° 12’S, 113° 51’E, 19 May 1983 collected by B. Vine et al.

TABLE 2. Morphometric comparison of *Milyeringa brooksi* and *M. veritas* (including holotype).

	M. brooksi **holotype**	**M. *brooksi*** **N=7**	***M. veritas*** **N=16**
Standard length (mm)	36.38	21.1–38.33	30.7–52.6
Head length % SL	38.8	37.2–39.5	29.9–45.4
Body depth % SL	20.6	15.3–20.6	15.8–25.4
Pectoral-fin length % SL	27.1	16.7–27.7	15.3–27.2
Pelvic-fin length % SL	18.6	11.9–20.9	10.5–26.1
Caudal-fin length % SL	13.6	13.6–23.3	21.5–39.3
1st Dorsal-fin base % SL	5.7	5.7–10.2	5.0–9.3
2nd Dorsal-fin base % SL	16.2	15.6–22.1	12.8–23.5
Anal-fin base % SL	17.0	12.5–17.0	12.1–18.6
Caudal-peduncle length % SL	17.8	15.7–23.6	17.3–25.1
Caudal-peduncle depth % SL	10.5	9.8–12.6	8.8–13.2
Caudal-peduncle width % SL	8.7	5.0–9.1	5.1–10.0
Pre-1st dorsal length % SL	51.7	47.7–53.2	50.1–57.7
Pre-2nd dorsal length % SL	61.8	61.1–66.4	59.8–65.1
Preanal length % SL	65.1	65.1–68.9	62.7–71.3
Prepelvic length % SL	38.2	35.7–39.0	21.5–43.9
Head width % HL	70.8	54.2–70.8	48.2–72.8
Upper jaw length % HL	48.9	41.4–48.9	36.1–46.9

Diagnosis:— *Milyeringa brooksi* is distinguished from its only congener (*M. veritas*) by the following features: (1) 10–12 vertical lines of sensory papillae from pectoral-fin base to caudal fin base (Fig. 3, 4) versus inconspicuous and/or dispersed sensory papillae on body) and (2) by molecular synapomorphies listed in Table 3. In addition, *M. brooksi* adults are typically shorter than *M. veritas* individuals (max length recorded < 40 mm SL vs. up to 53 mm SL), posterior nostril tubular in *M. brooksi* with a skin flap (versus posterior nostril typically simple and round in *M. veritas*) and by typically having conspicuous papillae on the dorsal aspect of head. Pattern of sensory papillae in *M. brooksi* present in four lines on lateral and medial edge of frontal bones near posterior edge of head and by four rows of sensory papillae between left and right nostril (two rows on each side of midline) running in parallel, these anterior papillae extend only slightly past posterior nostril on each side of head (versus variable condition found in *M. veritas* individuals which have either inconspicuous pore like papillae on head or conspicuous papillae where lateral most papillae near nostrils form a continuous line to papillae on lateral edge of frontal bones).

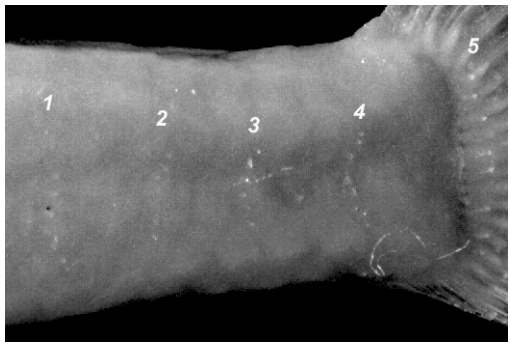

FIGURE 4. Close-up view of vertical lines of sensory papillae on the caudal peduncle in *M. brooksi*. Image shows the last five rows of papillae on the body of the holotype.

TABLE 3: Synapomorphic changes of *Milyeringa brooksi*. Number in column refers to nucleotide position followed by the character state in *M. veritas* and an arrow showing the transition in *M. brooksi*.

	ND2 (1–972)	CYTB (973–2080)	COI (2081–2707)
Milyeringa brooksi molecular synapomorphies	20 (T→C); 73 (A→G); 74 (A→G); 84 (A→G); 146 (A→G); 151 (C→T); 195(G→A); 223 (A→C); 283(G→A); 286(G→A); 340(G→A); 355 (A→G); 424 (G→A); 430 (A→G); 469(G→A); 508(G→A); 518(A→G); 532(G→T); 533(G→T); 646(A→G); 652(A→G); 665(A→G); 817(T→C); 826(G→A); 838(G→A); 883(G→A); 896(A→G); 938(A→G); 940(T→C)	1048(C→T); 1092 (G→A); 1155 (T→C); 1164 (T→C); 1191 (T→C); 1260 (T→C); 1282 (G→A); 1293 (C→A); 1302 (A→T); 1374 (A→G); 1437 (A→G); 1454 (A→G); 1503 (C→A); 1539 (T→C); 1590 (A→G); 1626 (C→T); 1782(C→T); 1851 (C→T); 1918 (G→A); 1984 (G→A); 2016 (T→C); 2019 (C→T); 2025 (C→T); 2029 (G→A); 2052 (G→C)	2221 (C→T); 2227 (C→T); 2236 (G→A); 2287 (G→A); 2299 (G→A); 2419 (A→G); 2425 (A→G); 2455 (C→T); 2467 (A→C); 2522 (T→C); 2560 (T→C); 2566 (T→C); 2590 (A→C); 2620 (T→C)

Description:— *Milyeringa brooksi* are small (<40 mm SL), unpigmented, eyeless stygobitic fish (Fig. 2, 3). Comparative morphometric features are listed in Table 2. ***Head*:** Head large (about 40% of SL), broad (area posterior to cheeks widest part of animal) and nearly flat. Eyes absent, with no remnants of a lens (as determined by x-rays) or other features related to vision. Mouth large (about 45% of HL) curved ventrally and posteriorly at 45° from dorsal aspect of head. Lower jaw protrudes slightly anterior to upper jaw. Anterior nostril tubular immediately dorsal to upper lip. Posterior nostril oval, somewhat tube shaped, larger than anterior nostril with excess skin forming flap. Toungue flat and blunt anteriorly. Teeth unicuspid and strongly recurved. Teeth set in three to five irregular rows from most anterior edge to angle of mouth. Teeth on inner (medial) side of jaws larger than those on anterior edge with largest teeth near angle of mouth. ***Gills*:** Gill cover large (about 40% of HL) branchiostegal membranes almost completely exposed. Lower arm of gill arch very elongate (making counts difficult without dissection). Ten to 12 gill rakers present on ceratobranchial of first gill arch. Gill rakers long and thin, small denticles present on medial side of rakers. Rakers on medial edge of gill arch short, covered dorsally with denticles. Gill filaments thick and short (about same length as longest rakers). ***Sensory papillae:*** One line of papillae loosely follows shape of anterior edge of opercle and preopercle, opercular row of papillae continues on ventral side of head around gular region. Preopercular row of papillae are tightly bound to edge of preopercule in comparison to those on opercule. [These opercular rows are similar in location to nueromast rows *ot, os, oi* of Wongrat and Miller's (1991) study of *Perccottus glenii* and *Bostrychus urophthalmus*.] Four parallel rows of sensory papillae present on cheek, second row from top is longest extending to papillae on preoperculum [this row is similar to Wongrat and Miller's (1991)

nueromast row *b* with innervation from the ramus buccalis in *Perccottus glenii*], other rows less dense and do not reach preopercular row. Largest papillae on head present around lips; these sensory papillae follow closely the outline of lips on all sides of mouth including ventrally. Few papillae on dorsal aspect of head, those present are concentrated posteriorly and anteriorly. Two rows of papillae present on each side of posterior edge of head. Rows consist of about five papillae near lateral and medial edge of frontal bones. Four rows of papillae between left and right nostril (two rows on each side of midline) running in parallel [similar in their location to the rows in *Perccottus glenii* that are innervated by the truncus supraorbitalis in that species Wongrat and Miller (1991)]. These rows of papillae extend only slightly past posterior nostril on each side of head. Papillae on body less conspicuous than those on head. No lateral line. Ten to 12 vertical lines of papillae present on body. First vertical line of sensory papillae on body (most anterior column) found dorsal to pectoral-fin base. Most posterior line is on caudal-fin base just posterior to caudal flexure (with about eight large papillae). Posterior-most line has largest sensory papillae on body. Largest papillae along body concentrated near midline of each vertical line and become smaller dorsally and ventrally. All lines of papillae on body and head are composed of single well-defined papillae in a row. In some individuals papillae are more conspicuous on one side of body than the other. ***Body*****:** Body is slim relative to head becoming thinner and shorter posteriorly. A sulcus is present on dorsal surface of body between posterior end of head and first dorsal fin-base. There are less pronounced grooves between pelvic and anal fins, and along the midline of caudal peduncle. Twenty-four total vertebra (10 precaudal + 14 caudal, including last ½ centrum). Paired intermuscular elements are present suspended above precaudal centra 3 to 6. ***Scales:*** Body covered in thin, deeply embedded, transparent cycloid scales. Head naked and chest (region anterior to pelvic fins) generally naked (one paratype had chest scales). ***Fins:*** Two separate dorsal fins, first is short (less than 1/3 of height of second) with four thin and weak spines, second with nine unbranched but segmented rays. There are four rays in the pelvic fin, nine in the anal fin, 18 segmented unbranched principal caudal-fin rays and 14 pectoral-fin rays. Second dorsal, pectoral, pelvic, anal, and caudal fins all have elongated trailing rays to varying degrees. Vertical from anterior portion of anal-fin base reaches between third and forth soft ray of second dorsal fin. Posterior edge of anal-fin base is slightly posterior to vertical from termination of second dorsal-fin base. ***Color:*** In life, *M. brooksi* is depigmented, appearing almost uniformly pinkish white. Pink color is most conspicuous where blood is concentrated, such as over gills as seen through gill cover. Coloration uniform off-white cream in preservative. Area above brain transparent and brain case appears purplish in life and dark grey in preservative. Fins and gill covers are hyaline.

Etymology:— Named for Darren Brooks whose knowledge of these fishes and the caves that house them is unsurpassed, and whose efforts to help conserve these unique ecosystems have been invaluable.

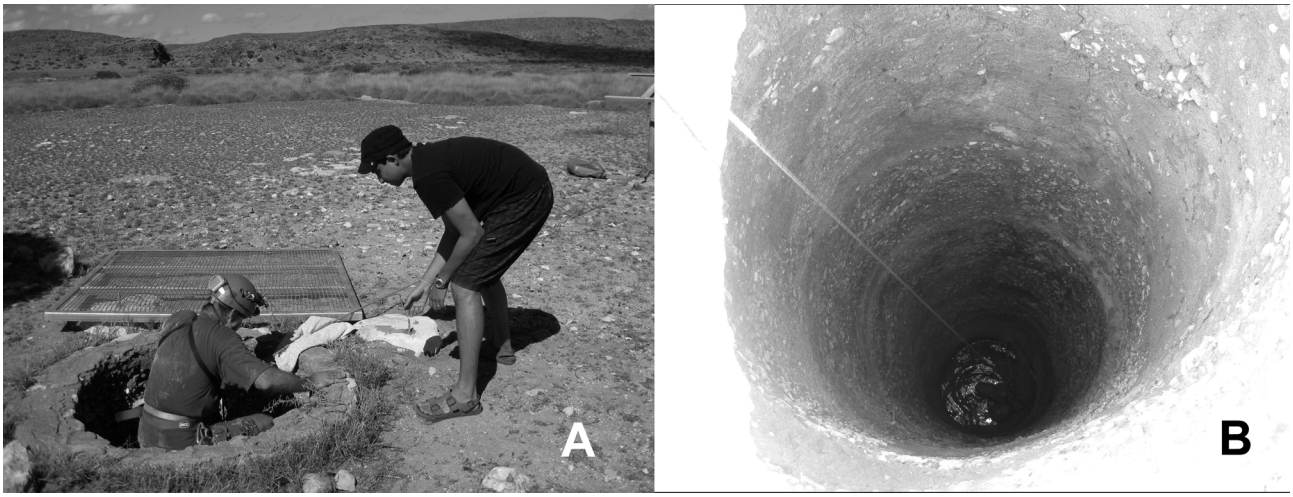

FIGURE 5. (A) The author and Darren Brooks at the type locality of *Milyeringa brooksi*, Pilgonaman Well, (B) Mr. Brooks at the bottom of the well approximately 10 meters below the surface.

Distribution:—This species is found in the karst systems and underground water channels from Tulki Well (22° 06'S. 113° 54'E) in the north, to an area near the abandoned Ningaloo tower (22° 42'S 113° 40'E; also called Point Cloates Lighthouse on local maps) in the southwestern portion of the Cape Range of Australia. The type locality is Pilgonaman Well (Fig. 5), which along with Tulki Well is located within Cape Range National Park. Jarvis Well, which is now filled in, and the Ningaloo Tower location are found outside of the Cape Range National Park. The distribution of these fishes spans approximately 75km along Yardie Creek road on the southeastern side of the North West Cape. The range likely spans underground interconnected waterways that incorporate these surface openings (wells and caves) separated from those belonging to *M. veritas* in the northern portion of the North West Cape. The specimens from Tulki Well and the Ningaloo tower (see Materials Examined) are not part of the type series and were not sampled in the molecular study; however, these formalin-fixed specimens exhibit the diagnostic morphological features of *M. brooksi*.

Discussion

Milyeringa was initially placed in its own family, Milyeringidae, by Whitley (1945). Members of *Milyeringa* have subsequently been considered as members of Eleotridae (Miller 1973; Humphreys 2001), and Odontobutidae (Thacker and Hardman 2005). Thacker's (2009) comprehensive study of Gobiiformes recovered *Milyeringa* as part of a trichotomy with Rhyacicthyidae and Odontobutidae, sister to all remaining Gobioidei. Thacker (2009) considered *Milyeringa* an odontobutid based on results from earlier work (Thacker and Hardman 2005); however, pending more detailed analysis, I recommend retaining Milyeringidae as a valid family to highlight its distinct position within the Gobiiformes and for its extreme ecological specializations.

Additional species of *Milyeringa* are likely to be discovered. For example, the Barrow Island population of *Milyeringa* is notable for being (presumably) disjunct from mainland populations. This island is difficult to access and populations from Barrow Island have not been included in any molecular or morphological study. There is a possibility that this island population may represent a novel taxon.

It is somewhat surprising that no additional phylogenetic structure was detected among the *M. veritas* populations sampled here (Fig. 1). Among *M. veritas* (sensu strictu) that were sampled in previous studies (Adams and Humphreys 1993; Humphreys 2001) there was some population level structure detected from allozyme frequencies between specimens from the type locality (Milyering Well) and specimens from more northeastern localities of the North West Cape. The discrepancy between these analyses most likely reflects different data sources and levels of analysis. However, both analyses did recover a southwestern clade that corresponds to the new species described here. Notably, separate northern and southern populations roughly corresponding to the split between *M. veritas* and *M. brooksi* populations were also recovered for a subterranean crustacean (*Stygiocaris lancifera*) that is also endemic to the North West Cape (Page et al. 2008). This separation provides additional inferential evidence of a subterranean barrier that keeps both fishes and invertebrates isolated.

A blind synbranchid eel, *Ophisternon candidum*, lives in sympatry with populations of both species of *Milyeringa* throughout much of their range (Romero and Vanselow 2000; Humphreys 2001). A single specimen of the eel was observed at Kudumurra well with *M. veritas* specimens by the author. Specimens of *O. candidum* have been observed in the range of *M. brooksi* (Humphreys 2001). No phylogenetic data for *Ophisternon* is available and it would be very interesting to know if genetic or morphological differences exist among populations of the eel in the North West Cape.

The type localities of both species of *Milyeringa* are within Cape Range National Park (*M. veritas* – Milyering Well; *M. brooksi*—Pilgonaman Well). Nonetheless, the survival of both species remains tenuous. Introduced species (guppies, *Poecilia reticulata*, were observed in Dozer Cave; 21° 58'S, 114° 07'E), pollution, and other habitat degradation could rapidly cause extirpations and potentially extinction of these taxa. Little is known about the population numbers of these species and presumably most individuals live in

underground waterways where they cannot be observed. It is likely that the towns of Exmouth and Learmonth, which are growing rapidly in the arid landscape in the vicinity of these rare underground habitats, will one day encroach upon these systems. It is also likely that additional cave and underground systems remain undiscovered in this area. It will be important to maintain the isolated populations of *M. veritas* and *M. brooksi*. Currently these populations are separated by less than 10km within the Cape Range National Park (the distance between Milyering and Tulki Well). An area directly south of the Cape Range National Park is a prohibited Defense Reserve Area that may also restrict the habitat of *M. brooksi*. The endangered status of *M. veritas* under the Western Australia Conservation Act should be expanded to include the new species. It is vital that these rare and poorly-studied species be managed and protected.

Acknowledgments

Darren Brooks and his wife Jackie were wonderful hosts and guides and this work would not have been possible without their help. Sue Morrison, curator of fishes at the Western Australia Museum, helped a great deal arranging collections and making export possible. Bill Humphreys put me in contact with people in Western Australia and his work on the caves of this region was extremely valuable. The Department of the Environment and Conservation of Western Australia, particularly Brooke Halkyard, was also very accommodating with permits and permissions. Haw Chuan Lim (Graduate Research Assistant, LSU) was very helpful in carrying out aspects of the molecular analysis. Christine Thacker and Mike Hardman were gracious in providing primer sequences and answering queries. The illustration of the holotype was done by an undergraduate student at LSU, Jill Meredith Dowling. This work was supported by startup funds from Louisiana State University and the Museum of Natural Science as well as the National Science Foundation DEB 0916695.

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Materials Examined

Non-type material
Milyeringa brooksi (Non-type material) WAM 28329; WAM 29241; WAM 29243
Milyeringa veritas – LSUMZ 13636, LSUMZ 13638, LSUMZ 13639; WAM 2913 (holotype); WAM 4058/63, WAM 5635; WAM 5695; WAM 5729; WAM 5740; WAM 7158; WAM 28328; WAM 28331; WAM 29113; WAM 29239; WAM 29247.