Delineation of Undescribed, Morphologically Cryptic Cave Beetles of the Pseudanophthalmus pubescens Species-Group (Coleoptera: Carabidae: Trechinae)

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DELINEATION OF UNDESCRIBED, MORPHOLOGICALLY CRYPTIC CAVE BEETLES OF THE *PSEUDANOPHTHALMUS PUBESCENS* SPECIES-GROUP (COLEOPTERA: CARABIDAE: TRECHINAE)

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Master of Science

By
Jedidiah John Nixon
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DELINEATION OF UNDESCRIBED, MORPHOLOGICALLY CRYPTIC CAVE BEETLES OF THE PSEUDANOPHTHALMUS PUBESCENS SPECIES-GROUP (COLEOPTERA: CARABIDAE: TRENCHINAE)

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Associate Provost for Research and Graduate Education
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The genus *Pseudanophthalmus* Jeannel is a widespread and extremely diverse taxon of troglobiont beetle endemic to the karst of eastern North America, with its distribution centered in Tennessee and Kentucky. Despite lying near the heart of this region, the *pubescens* species-group was thought to contain many undescribed species. In this study, the validities of several of the late Dr. Thomas Barr’s unpublished putative new species in the *pubescens*-group were tested both morphologically and molecularly. Body measurements (length and width for head, thorax, and abdomen), as well as male genital morphology were compared to see if they reflected theorized species limits.

Genes (COI and 28S) were used to infer parsimony and Bayesian phylogenies to see if clades supported species hypotheses. Seven taxa, found valid, are formally described as full species for the first time, and are presented with COI barcodes. This more than doubles the *pubescens*-group, bringing it from six full species to 13. Of the described taxa, at least five are suspected to require revision. Additionally, four new species were discovered molecularly, but will require further collecting, morphological examination, and characterization before description. It is now apparent that morphology in general should not be relied upon solely when delineating *Pseudanophthalmus* species.
INTRODUCTION

Background

Lineage, Life History, and Diversity

Carabidae (ground beetles) along with Staphylinidae (rove beetles) and Leiodidae (round fungus beetles), are the three beetle families most commonly encountered in caves (Peck and Thayer 2003). Carabidae comprise 23% of all described troglobitic arthropods in eastern North America (Culver et al. 2000). Of the Carabidae, the globally distributed subfamily Trechinae is one of the lineages most prone to adoption of cavernicolous. Trechines’ typical habitat of wet gravel banks along streams may have predisposed them to the colonization of karst caves throughout their global range. Indeed, they have had many independent evolutions of cavernicolous in karst areas across the globe (Faille et al. 2011; Maddison et al. 2019).

The Trechinae are among the most speciose terrestrial troglophiles. These cave organisms, along with many others, are drastically undersurveyed, and likely contain much hidden biodiversity. New genera and species of cave trechine are described every year or so from all across the globe, (e.g., Lohaj and Lakota 2010; Casale et al. 2012; Sun and Tian 2015; Zhao and Tian 2016; Niemiller et al. 2017). This eastern North American clade in particular has much to reveal.

The *Pseudanophthalmus* Jeannel trechine carabids endemic to eastern North America are true troglobionts: obligate cave denizens that carry out their entire life cycle in caves. They are morphologically convergent in classic troglomorphic fashion –they lack eyes and flight wings, and have reduced pigmentation. Additionally, they may be less sclerotized than their epigean brethren and have lengthy specialized sensory discal
elytral setae. This genus is particularly likely to hold undescribed biodiversity, from lack of both sampling and prior molecular phylogenetic analyses (Fišer et al. 2018).

Undescribed terrestrial troglophile biodiversity is strongly correlated with sampling effort, and the rate of new species discovery in eastern North America has not yet plateaued (Niemiller and Zigler 2013). More than half of described *Pseudanophthalmus* species are only known to occur in single caves (Barr 1960b), and many caves have yet to be sampled. Similarly, in a broad Tennessee troglobiont survey, over half of all single-site endemic troglobionts were beetles (largely *Pseudanophthalmus*; Niemiller and Zigler 2013).

**Distribution and the Effects of Geologic Isolation**

The limestone karst of eastern North America is a hotspot of cave carabid diversity, with 145 described, and up to an estimated 250 species of *Pseudanophthalmus* alone (Peck 1998; Barr 2004). Centered in distribution on the karst of the Interior Low Plateaus, they predominantly occupy the karst of Mississippian Plateau I & II (MP I & II) in Kentucky and Tennessee. However, *Pseudanophthalmus* are also found on the periphery of MP I as it extends to touch the southeastern corner of Illinois and as it stretches northward into the Mitchell Plain of southcentral Indiana (Barr 1960a) and northward into southcentral Ohio with the tip of the MP II karst (Krekeler 1958). The *Pseudanophthalmus* range also extends to the east, following the Virginia/West Virginia border in the karst of the Appalachian Valley (Barr 1981). The farthest west *Pseudanophthalmus* have been documented is the southeastern corner of Missouri (R. H. Neidlinger unpublished).

The late Dr. Thomas Barr suspected that the western portion of Kentucky,
particularly the karst bordering the southern edge of the western Kentucky coal fields, held many undescribed species with limited distributions. His reasoning was that this area, the western edge of the MP I karst plateau, has many isolated karst knobs, surrounded by areas devoid of karst, that perhaps can best be described as “karst islands,” (Culver 1970). This contrasts with the central and eastern parts of Kentucky, which contain a more complete sandstone cap with underlying broadly interconnected MP I and MP II karst (Simpson and Florea 2009); thus this region is home to cave trechines with much larger ranges. For example, the monotypic genera *Neaphaenops* Jeannel and *Darlingtonea* Valentine have populations separated by as much as ca. 80 miles (but see (Boyd et al. 2020) with regard to the latter genus and isolated genetic clusters).

In the *Ptomaphagus* Hellwig cave leiodid beetle of Tennessee and Alabama, geographically varied rates of erosion have controlled degree of isolation and thus vicariance (Leray et al. 2019), much like what I suspect is the case in the *pubescens-* group *Pseudanophthalmus* in the fragmented western reaches of MP I karst. A Climatic Relict Model with multiple cave colonization events and surface travel between caves during interglacial periods has traditionally been used to explain distributions of both *Pseudanophthalmus* and *Ptomaphagus* (Culver and Pipan 2009). However, this fails to correspond well to the time of *Ptomaphagus’* speciation, which appears to be much more ancient. Comparing cave systems produces a better temporal match – the oldest lineages of *Ptomaphagus* hail from the fragmented western periphery of the southern Cumberland Plateau, while more recent speciation has been observed in the more intact and widely connected centra region of the southern Cumberland Plateau (Leray et al. 2019). Barr and Holsinger (1985) observed similarly higher abundance of short range endemics in the
discontinuous karst of the Appalachian Valley than in the broadly connected eastern portions of MP I and MP II karst, but didn’t come to the same conclusion, that speciation had been primarily driven by karst fragmentation.

Possible Effects of Glaciation

The cool, moist climate along the borders of glacial ice sheets is hypothesized to have favored the geographic expansion of the surface-dwelling ancestor of these cave trechines (Jeannel 1949a). Barr (1965) thought that these ancestors either retreated up into the soils of mountains and adopted edaphic lifestyles or down into cool, moist caves along the periphery of the receding ice sheets during the warmer, drier interglacial periods (Barr 1960a). Alternatively, one can easily envision predominantly riparian trechine beetles following surface water flow into karst and becoming isolated in these below surface ecosystems, and continuing to occupy much the same niche (Barr 1967).

In the years since the glaciers receded, erosion has worn away at the sandstone capstone, more so in the western reaches of Kentucky. When a sandstone cap is present, water flows through the fractures and pores in the sandstone, until it reaches the limestone, where it dissolves the rock, forming conduits and, eventually, interconnected cave systems (Palmer 1991; White et al. 1995). When the sandstone cap is lost, the underlying limestone is then subjected to more complete dissolution, fragmenting the contiguous karst layer, which in turn disconnects cave passages, eventually exposing underlying non-karst bedrock (Deike 1989). As a result, caves located in western Kentucky tend to be far less interconnected than those in the central and eastern parts of
the state where such famously long systems as Mammoth Cave and the Flint Ridge cave system are found (Haney et al. 1985).

**Possible Effects of River drainages**

Since the early days of cave biology, it has been thought that some troglobites might disperse via solutional channels. “Inasmuch as genital type can be correlated with river drainage systems; it is reasonable to suppose that anophthalmid distribution is conducted along river valleys perhaps in part by subterranean streams.” (Valentine 1932). It has been proposed that cave trechines may be capable of using underground rivers to disperse (Barr 1959; Barr 1960b), as they can survive several hours immersed (Barr and Peck 1965). However, as observable *Pseudanophthalmus* numbers increase following flooding events (presumably due to beetle movement into larger passages in an attempt to avoid high water), transportation downstream may be a rare occurrence (McKinney 1975). Even if an uncommon phenomenon, and historically suspected to be mostly if not entirely unidirectional (Krekeler 1959), transportation by streams may set up long-term population connectivity dynamics, and may make the task of finding related populations and differentiating species somewhat less arduous. Small surface streams generally will not serve as barriers to cave beetle dispersal if there are abundant caves on either side of the stream (Barr 1959; Barr and Peck 1965). However as meander frequency decreases, and size increases (Krekeler 1959; Barr 1959), rivers become capable of serving as a barrier to gene flow and producing reproductive isolation (Boyd et al. 2020).
Speciation

Isolation due to cavernicoly combined with an already reduced capacity for surface dispersal due to troglomorphy tends to lead to dramatically high endemicity and low gene flow among troglobitic arthropods (Kane et al. 1992a). Even if some troglobitic taxa are able to survive on the surface, they will likely be at a competitive disadvantage to more generalist surface taxa. (Howarth 1983).

Troglotic members of *Pseudanophthalmus* are likely unable to survive outside of their cool, moist cave systems. Currently, much of the eastern United States may be too warm for the continued survival of even their few edaphic congeners (Barr 1967). These restrictions to dispersal and isolation within relatively small cave systems will decrease gene flow and increase genetic drift, pushing *Pseudanophthalmus* to diversify in fragmented landscapes (Krekeler 1959). This will lead to decreases in within-site diversity and increases in between-site diversity (Kane et al. 1992a). Even amphipods, which may be found both in cave and surface streams and are capable of traveling between the two (Kane et al. 1992b), have markedly lower genetic variation in their cave populations than in their surface populations (Carlini et al. 2009). Therefore, cave denizens, especially troglobites, may be predisposed to developing genetically distinct sibling species by virtue of their relatively small population size and isolation (Laing et al. 1976).

Externally morphologically indistinguishable, internally morphologically different

Despite their close external morphological similarity, *Pseudanophthalmus* species have previously been delineated into 26 species groups based on morphology, especially
genitalic characteristics (Barr 2004). These genitalic morphospecies reflect hypothesized barriers to gene flow, some of which may prove to be true, as multiple species that coexist in single caves may exhibit niche separation (McKinney 1975). Furthermore, when distributions overlap, at least some *Pseudanophthalmus* also display morphological character displacement in mean body length (Van Zant *et al.* 1978), as well as differences in behavior (in sympatry *menetriesi* preferentially forages in organic material, *pubescens* preferentially forages on riparian silt banks; in allopatry, both occupy a wider riparian foraging niche (Barr and Crowley 1981)) and phenology (McKinney 1975). Studies of this nature have only examined two species in detail, *Pseudanophthalmus pubescens* and *P. menetriesi* (McKinney 1975; Barr and Crowley 1981) which have long been classified in different species groups (Jeannel 1949b), though those species groups are thought to be closely related sister clades (Barr 2004). Examination of less closely related *Pseudanophthalmus* taxa may reveal more pronounced differences.

All New World cave trechines (with the exception of the distantly related, exclusively North American *Xenotrechus* Barr and Krekeler) form a single, molecularly-supported clade falling within Jeannel’s “*Trechoblemus series*” (Jeannel 1928; Maddison *et al.* 2019). This clade is morphologically supported as well: all its members are “anisotopic,” (i.e., males have their genital apparatus oriented sideways; Barr 2004). Male genital morphology has successfully been used to characterize trechines to the species-group level, but finer-scale relationships remain unclear (Barr 2004). Prior work with other arthropod groups has found that sufficiently detailed phylogenies based on male genital morphology may produce similar results to molecular phylogenies in some cases (e.g., Hedin 1997; Slaney and Blair 2000), though this is far from the only outcome,
and convergence may readily lead to morphological homoplasy and misleading associations (e.g., Bennik et al. 2016; Bocek and Bocak 2017; Pardo-Diaz et al. 2019).

**Purpose & Hypotheses**

The North American cave trechines present a fascinating system for phylogenetic examination by virtue of their vast diversity, restricted distribution, varying levels of isolation, and uncertain evolutionary relationships. Barr (1969) thought that “there are no immediate, epigean ancestral genera now present in North America” (for *Pseudanophthalmus*) and blamed this, along with their extreme within-genus morphological similarity, for stymying attempts at phylogenetic classification (Barr 2004). The increased accessibility of molecular phylogenetics has provided excellent phylogenetic resolution on European cave trechines (Faille et al. 2010; Ribera et al. 2010; Faille et al. 2011; Faille et al. 2013) and one lineage of the North American cave trechine (Boyd 2015; Boyd et al. 2020), but much is yet unknown about the diversity, distribution, and speciation of most genera of North American and Asian cave trechines.

Using both morphological and molecular evidence, this study tests the validity of and hypothesizes species limits for Barr’s nine putative new taxa in the *pubescens* species-group. The *pubescens* species-group is one of the larger constituent lineages of the *Pseudanophthalmus* as defined by Barr (2004), and may become much larger. In particular members of other clades, such as the *menetriesi* species-group, may be supported as being part of the *pubescens* species-group. This study also elucidates the relationships between Barr’s nine putative new *pubescens*-group taxa to the six previously described *pubescens*-group species (one with two subspecies). I consider
species, as defined in this study, as independent evolutionary units (i.e., monophyletic groups) that are reproductively isolated as determined/supported by phylogenetic evidence, and ideally exhibiting unique aedeagal (male genitalic) morphologies. The pubescens-group’s validity (i.e., monophyly) is also tested phylogenetically by the inclusion of members of other Pseudanophthalmus species groups: the geographically close tenuis (P. barberi) and menetriesi-groups (undetermined putative new P. sp. collected from Sharon Grove Cave, Kentucky), and the more geographically distant engelhardtii (P. wallacei) and intermedius- or robustus- groups (undetermined P. sp. collected from Nunley Mountain Cave, Tennessee). This study also assesses whether male genital morphology is a reliable factor for species delimitation among the Pseudanophthalmus cave trechines. Lastly, this work provides a framework for further Pseudanophthalmus research, by testing different primers and optimizing PCR protocols. Notably, all hypothesized new species were described not only morphologically but also molecularly via COI barcodes.

Specific questions

1. I divided the pubescens group species into three morphological groupings based on aedeagal morphology. The ciliaris-like grouping (ciliaris, colemanensis, caledonicus n. sp., crassus n. sp., riparius n. sp. and tentatively longi n. sp.); the loganensis-like grouping (loganensis, ciliaris orlindae, lacustris n. sp., lancasteri n. sp., loganensis thomasi n. ssp., and tentatively “amicus n. sp.” and longi n. sp.); as well as the pubescens intrepidus-like grouping (pubescens intrepidus, pubescens pubescens, crinitus n. sp.,
tentatively “amicus n. sp.” and tentatively princeps). Do these aedeagal morphological groupings correspond to phylogenetic predictions?

2. Is the pubescens-group monophyletic, or are some of its presumed members more closely related to other species groups?

3. Is the widely distributed Pseudanophthalmus pubescens a single lineage (i.e., are the subspecies pubescens intrepidus and pubescens pubescens worthy of elevation to full species status)?

4. Pseudanophthalmus ciliaris occupies a vast range, and, in addition to P. ciliaris ciliaris and P. ciliaris orlindae, it has two described subspecies that have since been elevated to full species status (P. loganensis, P. colemanensis). Are these species monophyletic? Is there a need for further partitioning of Pseudanophthalmus ciliaris?

5. Is Barr’s putative unpublished subspecies Pseudanophthalmus loganensis thomasi truly a subspecies? Many of Barr’s previous pubescens-group subspecies were subsequently elevated to full species status (e.g., Pseudanophthalmus (ciliaris) loganensis, P. (ciliaris) colemanensis, P. ciliaris orlindae). Morphologically, I remain unconvinced that the putative P. loganensis thomasi is valid; the aedeagal morphology in this taxon does not appear like either P. loganensis or P. ciliaris but is more similar to that seen in P. longi n. sp. and P. lacustris n. sp.

6. Do Kinnin Cave (Type locality for Pseudanophthalmus crassus n. sp.) and Yates Cave (separated by 5.3 linear miles) share the same species as Barr hypothesized? While Kinnin Cave does have northward water flow, is it truly hydrologically connected to Yates Cave, which has a surface drainage as its primary inflow?
7. Carpenter Cave (Type and only locality for *Pseudanopthalmus crinitus* n. *sp.*) is at the physiographic level of the Central Basin of Tennessee, and is thought to be stratigraphically isolated from the Kentucky Pennyroyal Plateau, i.e., connected to no other caves in the region (Harker and Barr 1980). Does this result in *Pseudanopthalmus crinitus* n. *sp.* to appear basal in the topology of *pubescens*-group species, or more closely related to a central Tennessee lineage of *Pseudanopthalmus* such as the *intermedius* or *robustus* species groups?

8. Do Potter (*née* Haddon) Cave (Type locality for *Pseudanopthalmus longi* n. *sp.*) and Buck Fork Creek Cave (both outflow caves, Buck Fork Cave 9.6 linear miles WNW of Potter) share the same species, or does the undescribed *P. menetriesi*-group beetle from Sharon Grove Cave, a mere 4.7 miles N, be more closely related to the Potter Cave species? *Pseudanopthalmus pubescens*, though later given its own species-group (Jeannel 1949b; Barr 1980), was included in the *menetriesi*-group of Valentine (1932), and the *menetriesi* and *pubescens* groups are still considered to be closely related.
MATERIALS AND METHODS

Cave Beetle Sampling

I sampled across western and central KY, collecting *Pseudanophthalmus* from caves indicated by the late Dr. Thomas Barr (Harker and Barr 1980) to potentially contain undescribed species of the *pubescens*-group (Figure 2), and from additional promising caves in the vicinity that might be new localities for the same or additional undescribed *Pseudanophthalmus* species (Table 1).

All individuals involved in sampling followed precautions to avoid spread of *Pseudogymnoascus destructans*, the introduced European fungus responsible for White Nose Syndrome (Gargas *et al.* 2009; Lorch *et al.* 2011) and the associated widespread North American bat die-offs (Leopardi *et al.* 2015). These efforts were taken as a precaution, despite evidence that bats may be far better vectors of White Nose Syndrome than are cavers (Frick *et al.* 2010).

Caving protocol was as follows: Cave gear and clothing were washed between caves, disposable cotton caving suits were worn over clothing to prevent possible fungal spore transfer, shoes were spritzed with Lysol IC before and after caving. Lysol IC has been found to be more effective at killing *Pseudogymnoascus destructans* spores than ammonia or alcohol (Shelley *et al.* 2013). Notably, the National Park System initially used this fluid, but employees are now forbidden from non-label uses and use ammonia and/or ethanol instead (United States Fish & Wildlife Service 2018).

In the field, beetle specimens were collected by hand into scintillation vials containing 95%-100% EtOH. Trapping was attempted via the aboveground pitfall trap designed by Chris DeWildt (see DeWildt 2007). Unfortunately, the traps proved
ineffective due to a more biodiverse system than Mammoth Cave, featuring raccoons, packrats, and Hadenoecus “cave crickets”, all of which disturbed our traps. Prior attempts at using pitfall traps to collect Pseudanophthalmus have also found manual collection to be simpler (Krekeler 1958; Keith 1975). Upon return to the lab, samples were transferred into clean 100% EtOH, and stored at -80°C for long-term DNA preservation.

**DNA extraction and amplification**

DNA was extracted at the WKU Biotechnology Center, following the protocol developed by Valkanas and Philips in 2012 (unpublished), and subsequently modified or refined in some cases by Boyd (2015).

Samples were prepared from individual beetles by extraction of total DNA. For described cave beetle species (and some females of abundant putative new species), bodies were destructively ground to increase amount of DNA extracted. For males of suspected new species, the head, prothorax, and abdomen were disarticulated from the body and the abdomen opened further via piercing with clean insect pins. Body parts were kept as voucher specimens. If the digestive tract was found to be full, it was excluded to the extent possible, to prevent amplification of foreign DNA from prey items, as in Maddison *et al.* (1999).

Samples were either ground in 1.5 mL tubes with sterile plastic pestles or were placed directly in 1.5 mL tubes. CTL Buffer (350 μL) and of Proteinase K (25 μL) were added, the tubes vortexed, and then incubated at 60° C for 1-4 hours, with periodic agitation.

DNA was extracted using an E.Z.N.A. Insect DNA kit from Omega Bio-Tek.
Nucleic acid concentration and purity was quantified using a NanoDrop 2000 spectrophotometer. DNA extractions were stored at -80°C for long-term preservation.

Initial attempts at COI amplification using the reverse primer “Pat” and the forward primer “Jerry” (Simon et al. 1994) were almost entirely unsuccessful, presenting few and, at best, weak bands. Upon consultation with Dr. Karen Ober, we used the standard primer “Pat” along with her modification (for use with *Pseudanophthalmus*) of the forward primer “Jerry” (the modification here called “COIF Ober”). Amplification of 28S used Ober’s primer codes “D1” and “D33’Alt.” Primer sequences may be viewed in (Table 2).

PCR was conducted using an Eppendorf™ Mastercycler™ Pro machine using TaKaRa PreMix Ex Taq Version 2.0. After running a temperature gradient for each gene, we decided upon the PCR temperature protocols listed in (Table 3). By gel ladder estimation, our COI PCR resulted in a ~1100 bp target region, and our 28S PCR resulted in a ~1000 bp target region.

**DNA sequencing**

The PCR products were mailed to GENEWIZ for sequencing. The COI gene due to its relatively long length was sequenced with both the forward and reverse primers while 28S were sequenced only in the forward direction. Sequences were edited, aligned, and concatenated in Geneious 7.1.9 (Kearse et al. 2012; http://www.geneious.com n.d.). The DNA sequences introduced in this paper will be submitted to GenBank, and accession numbers are pending (Table 6).
Morphology

Morphological characters were recorded for each each of the seven described *pubescens*-group taxa, as well as Barr’s nine putative *pubescens*-group taxa, based on measurements of his preserved specimens on loan from the Carnegie Museum of Natural History. All characters recorded were based on those found to be diagnostic by Barr in previous *Psuedanophthalmus* descriptions.

All body length measurements were taken from the midline, all body width measurements were taken from the widest points (head, pronotum, elytra). Antennal lengths were not included, as few specimens were pinned with straightened antennae, and I wished to avoid risking damage to Barr’s historical specimens. Body length/width measurement ratios were used to generate PCAs in R (Figures 3-5).

Some aedeagal characters were excluded (e.g. paramere setal count) due to damage in Barr’s preserved material, and the potential to record incorrect information. While all of Barr’s *pubescens*-group aedeagi were imaged, only those deemed best and most representative (Table 4) were imaged (Figures 6-7). In the A simple, unweighted character-state matrix was generated from the aedeagi (Table 5). Unfortunately, the parsimony tree this character state matrix yielded was entirely pectinate and uninformative. Despite this unresolved tree, it is possible to see similarity in male genitalia between certain described and putative new species, and so the *pubescens*-group was divided into four subgroups based on unweighted aedeagal morphology, three of which contain putative new species (Table 6).
Treebuilding:

I used *Lionepha erasa* (COI & 28S), a distantly related trechine, as an outgroup for molecular phylogenetic analyses. Four other *Pseudanophthalmus* species groups were included to test the monophyly of the *pubescens*-group. This included two taxa from central Tennessee (the *engelhardtii*-group (represented by *Pseudanophthalmus wallacei* of Weaver Cave) and the *intermedius*- or *robustus*-group (represented by *P.* undetermined of Nunley Mountain Cave)) as well as two species groups from Kentucky (the *tenuis*-group (represented by *P.* *barberi* of Penitentiary and Bandy Caves) and the *menetriesi*-group (represented by *P.* *menetriesi* of Crumps Cave, and *P.* *n.* *sp.* of Sharon Grove Cave)).

After manual editing in Geneious 7.19 (Kearse et al. 2012), sequences of both genes were aligned according to the Geneious 7.19 defaults for the embedded ClustalW software (Larkin et al. 2007) with a gap open cost of 15 and a gap extend cost of 6.66). Geneious was also used for concatenating sequences.

A preliminary neighbor-joining tree examining only untrimmed (i.e., sequences of varied length) COI from the putative new species (and some non-*pubescens* *Pseudanophthalmus* to serve as a root) was built (Figure 8). A subsequent neighbor-joining trees were constructed in Geneious for COI, 28S, and the concatenated trimmed (i.e., cut to equal length) sequences, but of these only the COI was deemed to be especially informative (Figure 9).

Parsimony analyses were performed for COI, 28S, and the concatenated sequences using the NONA program in WinClada 1.00.08 (Nixon 1999). Final parsimony trees were imaged in FigTree 1.4.4 (Rambaut 2007) (Figures 10-14).
Bayesian analyses were performed for single gene and concatenated COI and 28S sequences using MRBAYES 3.2 (Ronquist et al. 2012). Optimal models for sequence evolution were first determined using JModelTest 2.1 (Darriba et al. 2012), based on Bayesian information criterion (BIC). For COI, general time reversal (GTR) with an inverse gamma distribution. For 28S, the GTR model was used with a gamma distribution. Two simultaneous searches were run, each with four Markov chain Monte Carlo (MCMC) chains (three hot and one cold), with a sample frequency of 1000. These were run for as many generations as were needed to reduce the standard deviation of split frequencies below 0.01.

**Descriptions**

Species descriptions were written for the nine putative new *Pseudanophthalmus* species in the *pubescens*-group, drawing on previous descriptions of other eastern North American cave trechines as a descriptive template (Horn 1868; Valentine 1937; Barr 1959; Barr 1979; Barr and Crowley 1981). The Barr material on loan from the Carnegie Museum of Natural History was used for morphological examination and description. Newly collected specimens were used for acquisition of DNA barcodes. All body length measurements were made along the midline of the body from the anterior edge of the clypeus to the elytral apex, and all body width measurements were made at the widest points of each examined body region (head, pronotum, elytra). All body measurements were made by ocular micrometer.

I measured all of Barr’s mounted *pubescens*-group specimens on loan from the CMNH had length and width measured for heads, pronota, and elytra. Length/width
ratios from these three body regions were used to build a Principal Component Analysis in RStudio (Figures 3-5). The first graph of this PCA grouped individuals by species (both putative new and described), and exhibited severe overlap – clear distinctions were not apparent (Figure 3). A second graph of this same PCA grouped individuals based on the four aedeagal subgroups (ciliaris-like, loganensis-like, pubescens-like, and princeps-like) instead of species, and this seemed to better delineate groups (Figure 4). In the above PCA, Principal Component 1 was responsible for the vast majority of the variance, followed by Principal Component 2 (Figure 5), therefore examination of the remaining principal components was unnecessary and presumed to be of little utility.

While body measurements were taken, the species descriptions are primarily based on male genitalia due to their (often greater) diagnostic utility for the differentiation of species and/or species clusters, as has been found in other beetle taxa (Sasakawa and Kubota 2007; Medina et al. 2013). The undescribed species were sorted into three groups – those species most morphologically similar to Pseudanophthalmus pubescens male genitalia (pubescens-like), those species most similar to P. ciliaris male genitalia (ciliaris-like), and those most similar to P. loganensis male genitalia (loganensis-like) (Table 5).

COI DNA barcodes are included for each of these species, as this seems likely to be, at least in the many cases of cryptic speciation, the future of taxonomy (Hebert et al. 2003a; Waugh 2007; Kress et al. 2015). However, this gene fragment may be of less utility in the differentiation of certain taxa (Hebert et al. 2003b; Yusseff-Vanegas and Agnarsson 2017).
RESULTS AND DISCUSSION OF PHYLOGENETIC ANALYSES

COIF Preliminary Neighbor-Joining Tree

Our preliminary UPGMA neighbor-joining tree based on 46 COIF (CO1 forward) sequences constructed in Geneious (Figure 8) included eight of the nine putative new *pubescens*-group species and was rooted on two taxa of non-*pubescens*-group *Pseudanophthalmus*. Putative new species are indicated in the same colors as they were in Figure 2 as follows: *P. crassus* in red, *P. caledonicus* in orange, *P. riparius* in yellow, *P. longi* in green, *P. lancasteri* in navy, *P. lacustris* in sky blue, *P. amicus* in pink, and *P. crinitus* in dark purple. *Pseudanophthalmus loganensis thomasi* was not included, as none of its COI sequences amplified successfully. This preliminary tree supported the monophyly of the *pubescens*-group, and the integrity of almost all of Barr’s putative new species.

*Pseudanophthalmus longi* n. sp. was an exception to this, with the Potter/Hadden Cave *P. longi* n. sp. individuals coming out basal to both the Buck Fork Creek Cave *P. longi* n. sp. individuals and the clade of *P. riparius* n. sp.. Five of the putative new species supported in this tree were found to have range extensions from what Barr had recorded (*Pseudanophthalmus amicus*, *P. crinitus*, *P. lacustris*, *P. lancasteri*, *P. riparius*).

*Pseudanophthalmus riparius* n. sp. looked as if its ranges extensions might turnout to be a sister species, as node support joining the two was only 58%. *Pseudanophthalmus caledonicus* n. sp. and *P. crassus* n. sp. both had 100% node support for their clades and no discovered range extensions. There were no results for *Pseudanophthalmus loganensis thomasi* n. ssp., as its CO1 sequence did not amplify.
COI Neighbor-Joining Tree, all taxa

A subsequent UPGMA neighbor-joining tree of 91 COI sequences was next constructed in Geneious (Figure 9). This and all subsequent trees use *Lionepha erasa* as their rooted outgroup and include additional described species of the *pubescens*-group previously sequenced by Dr. Karen Ober.

Notably, the presence of two ostensible sequences of *Pseudanophthalmus princeps* and *P. pubescens intrepidus* from Jack Johnson Cave detrimentally impacted the tree. Those two individuals came out basal to all *Pseudanophthalmus* examined, distant from any *pubescens*-group members, and are even basal to included *tenuis*-, *menetriesi*-, *engelhardtii*-, and *robustus*-*intermedius* species groups. Sequence difference from all other *Pseudanophthalmus* is sufficiently extreme that I suspect that those two sequences may not even represent trechine DNA or alternatively they are nuclear copies of COI (K. Ober personal communication). Both Jack Johnson cave individuals were excluded from further analyses as their presence increased the non-resolution of the topologies discovered.

Inclusion of new sequences revealed that the described *Pseudanophthalmus ciliaris ciliaris* came out in two clades. From here on, they will be referred to as *Pseudanophthalmus ciliaris* Clade A: (*P. ciliaris* from Silvey Cave, with new locality Elk Fork Creek Cave) and *P. ciliaris* Clade B: (*P. ciliaris* from Durham Cave, Jeter River Cave, Bloody Murder Cave (Montgomery County, Tennessee), and *P. “ciliaris” colemanensis* from Clarksville Lake Cave). However, the type locality (Coleman Cave, TN) of *Pseudanophthalmus colemanensis* still presents an apparently separate lineage and valid species, if with a basal connection to the *engelhardtii*-group instead of the
Pseudanophthalmus loganensis also contains an ostensible *P. "ciliaris orlindae"*, which may either indicate that *P. ciliaris orlindae* is merely a morphologically variant *P. loganensis*, or that our examined specimen of *P. ciliaris orlindae* had been misidentified and its species may represent a distinct morphological form rather than a single lineage.

*Pseudanophthalmus "amicus n. sp"*. splits into two clades, although this may be due to the influence of the long branches of the Jack Johnson Cave sequences. Interestingly, the smaller “amicus” clade based on one specimen from Bear Cave (hereafter “*P. amicus*” clade A) comes out as sister to an ostensible *Pseudanophthalmus “ciliaris”* from Bloody Murder Cave and basal to the *pubescens*-group node, potentially indicating misidentifications or the possibility that Bear Cave is shared by two *Pseudanophthalmus* species. The larger “*P. amicus n. sp.*” clade contains L&N Railroad Cave, a site known only to contain one species of *P*, the type locality of *P. pubescens*. Therefore, this clade is hereafter labeled as *P. pubescens* (“*P. amicus*” clade B).

*Pseudanophthalmus crassus n. sp.*, *P. crinitus n. sp.*, and *P. lancasteri n. sp.* were still entirely supported. *Pseudanophthalmus riparius n. sp.* continues to have either two new localities or a sister taxon. *Pseudanophthalmus longi n. sp.* comes out in a basally unresolved trichotomy, and with *P. ciliaris* Clade B appearing as an unresolved comb. Similarly, *Pseudanophthalmus lacustris n. sp.* comes out in a basally unresolved comb with *P. ciliaris* Clade A.
COI Parsimony Trees, all taxa sans Jack Johnson Cave

Parsimony analyses (Figures 10 & 11) discovered 1880 equally parsimonious trees in NONA before tree-swapping was indicated as unnecessary (89 taxa, 721 characters, 581 uninformative, 140 informative, length = 710, CI = 72, RI = 83). The two Jack Johnson Cave individuals which came out basally to all other *Pseudanophthalmus* in Figure 9 were excluded, on the premise that they are unreliable sequences and may be affected by long branch attraction. In the strict consensus tree (Figure 10), *Pseudanophthalmus ciliaris* Clade A once again contains the Elk Fork Creek Cave individual, *P. ciliaris* Clade B contains “*P. colemanensis*” Clarksville Lake Cave and comes out as sister to *P. riparius n. sp.* clade, *P. loganensis* continues to subsume “*P. ciliaris orlindae*,” and *P. colemanensis* falls outside of the *pubescens*-group as sister to the *engelhardti*-group.

Interestingly, *Pseudanophthalmus lacustris n. sp.* (here paraphyletic because of the inclusion of Gorham Cave) is either sister to *P. ciliaris* Clade A, or may merit subsuming it.

*Pseudanophthalmus “amicus n. sp.”* continues to be split between two clades—one outside of the *pubescens*-group composed of the single 2015 *P. “amicus n. sp.”* Bear Cave individual and an individual from Bloody Murder Cave (from now on referred to as the *P. “amicus n. sp.”* Clade A) and the other inside the *pubescens*-group consisting of a partially resolved polytomy of seven individuals that includes L&N Railroad Cave and Wright Cave as new localities. The strict consensus tree shows no separation between *P. pubescens* L&N Railroad Cave and the *Pseudanophthalmus “amicus n. sp.”* collected from both Bear Cave and Friendship Cave and, which occur together in a polytomy. As L&N Railroad Cave is the type locality for *Pseudanophthalmus pubescens pubescens*,

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this supports *P. “amicus n. sp.”* Clade B being subsumed into that subspecies (hereafter *P. pubescens* [*“P. amicus” Clade B*]). Based on the increased resolution that NONA’s phylogenetic inference model lends us, *Pseudanophthalmus riparius* n. sp. continues to either have two new localities (Chandler and Flemonds Caves) or is sister to a new species of *Pseudanophthalmus* from those localities. All *P. longi n. sp.* come out in an unresolved polytomy basal to *P. loganensis*. *Pseudanophthalmus crassus* n. sp., *P. crinitus* n. sp. and *P. lancasteri* n. sp. continue to be monophyletic. Also of interest, *P. menetriesi* Crumps Cave and the undescribed *menetriesi*-group species from Sharon Grove Cave come out within the *pubescens*-group instead of basally, though this may be a consequence of our inclusion of insufficient *menetriesi*-group taxa. In addition, both Mutters Cave and Devils Den Cave (both Kentucky localities) appear to have new undescribed species.

In the majority rules topology (*Figure 11*), *Pseudanophthalmus crassus* appears as sister to the two included *menetriesi*-group taxa and the two new *Pseudanophthalmus* species, as opposed to their unresolved polytomy in *Figure 10*.

### 28S Parsimony Tree, all taxa

This analysis (*Figure 12*) yielded a single tree (84 taxa, 929 characters, 890 uninformative, 39 informative, Length = 203, CI = 93, RI = 91) after 1000 reps in NONA, with no further swapping of subtrees required. As only a single shortest tree resulted, this is a strict consensus tree by default.

After the rooted *Lionepha* outgroup, the *tenuis*-group *Pseudanophthalmus* come out in a basal clade, followed by another clade consisting of the undescribed
Pseudanophthalmus from Mutters Cave and Devils Den Cave. This is followed by a clade containing a basal \( P. \ crinitus \ n. \ sp. \) to a group of \( P. \ pubescens \) ("\( P. \ amicus \)" of both COI clade A and B) which contains "\( P. \ princeps \)" COI B). This is followed by two entirely resolved clades, one of \( P. \ crassus \ n. \ sp. \) and one of \( P. \ lacustris \ n. \ sp. \) (no longer paraphyletic, as the Gorham Cave locality is being considered as an entirely new species).

Beyond this point, the tree is rather unresolved. \( P. \ loganensis \) appears as a resolved clade within it, as do two \( P. \ longi, \ n. \ sp. \) Besides these two exceptions, all taxa present (\( P. \ ciliaris \) of both COI clades A & B, \( P. \ colemanensis \), \( P. \ caledonicus \ n. \ sp. \), \( P. \ lancasteri \ n. \ sp. \), and \( P. \ riparius \ n. \ sp. \)) are interspersed with little resolution.

The association of \( Pseudanophthalmus \ crinitus \ n. \ sp. \), \( P. \ pubescens \ pubescens \), and "\( P. \ princeps \)" COI B is in agreement with my predictions based on genitalic morphology (Table 5).

**COI & 28S Parsimony Tree, only taxa with both genes**

After 100,000 reps in NONA, seven equally parsimonious trees were found and further swapping of subtrees was indicated as unnecessary (76 taxa, 1650 characters, 1482 uninformative, 168 informative, Length = 849, CI = 78, RI = 83) (Figure 13). All 76 taxa present in this strict consensus tree were represented with both 28S and COI sequences.

As per previous trees, the tenuis-group, "\( Pseudanophthalmus \ princeps \)" A, "\( P. \ amicus \)" A, and \( P. \ colemanensis \) all fall outside of the pubescens-group. Within the pubescens-group, \( P. \ crassus \ n. \ sp. \) is entirely resolved and basal, followed by \( P. \ caledonicus \ n. \ sp. \) which again comes out by itself. Two of the new undescribed
*Pseudanophthalmus* taxa (Mutters Cave and Devils Den Cave) come out as sister to the clade containing resolved *P. crinitus n. sp.*, resolved *P. pubescens* (“*P. amicus*” clade B), and “*P. princeps*” B. This differs from Figure 10 in which those two new taxa came out with the *menetriesi*-group as sister to *P. crassus*. Beyond this there is a trichotomy, with the first branch being *P. lancasteri* entirely resolved. The second branch of this trichotomy is the clade consisting of the new Gorham Cave species, resolved *P. lacustris n. sp.*, and resolved *P. ciliaris* clade A. The final branch of the above trichotomy breaks into a tetrachotomy consisting of entirely resolved *P. longi n. sp.*, entirely resolved *P. riparius n. sp.*, entirely resolved *P. loganensis*, and entirely resolve *P. ciliaris* clade B.

**COI & 28S Parsimony Tree, taxa with either or both genes**

3060 trees were found before NONA deemed further subtree swapping unnecessary (89 taxa, 1650 characters, 1477 uninformative, Length = 875, CI = 77, RI = 84). Due to the vast number of topologies found, majority rules (Figure 14) was more informative than strict consensus. 85 of the taxa included in this majority rules tree were represented by COI sequences, 80 by 28S sequences.

Outside of the *pubescens*-group, the *tenuis*-group is basal to the *Pseudanophthalmus*. It is followed by the clade containing “*P. princeps*” A and “*P. amicus*” Clade A, then the clade containing *P. colemanensis*. Inside of the *pubescens*-group, most basal is the clade formed of *menetriesi*-group *P. n. sp.*, sister to the entirely supported *P. crassus n. sp.*. This is followed by the entirely supported clade of *P. caledonicus n. sp.*. This is followed by a dichotomy with a clade comprised of the two new undescribed *Pseudanophthalmus* species and *menetriesi*-group *P. menetriesi* Crumps
Cave, sister to a clade containing resolved *P. crinitus* n. sp., resolved *P. pubescens* (“*P. amicus*” Clade B), and “*P. princeps*” B. Sister to the above dichotomy is a tetrachotomy: One, the undescribed *P. n. sp.* Gorham Cave. Two, the resolved *P. lancasteri* n. sp. clade. Three, *P. n. sp.* Gorham Cave basal to the clade containing resolved *P. lacustris* n. sp. and *P. ciliaris* Clade A. Four, a pentachotomy containing *P. longi* n. sp. Haddon/Potter Cave, *P. longi* n. sp. Buck Fork Creek Cave, resolved *P. riparius* n. sp. resolved *P. loganensis*, and resolved *P. ciliaris* Clade B.

With regard to the described *pubescens*-group species, *Pseudanophthalmus ciliaris* Clade A, *P. ciliaris* Clade B, and *P. loganensis* (including Cross Plains Cave) are behaving as in previous analyses. *Pseudanophthalmus pubescens pubescens* (containing what was formerly labeled as *P. “amicus n. sp.”* Clade B) comes out as a sister clade to *P. crinitus* n. sp. again. *Pseudanophthalmus princeps* comes out in two separate places again.

*Pseudanophthalmus caledonicus* n. sp., *P. crassus* n. sp., *P. crinitus* n. sp., *P. lancasteri* n. sp., *P. longi* n. sp. (both previously known localities), and *P. riparius* n. sp. (including Chandler and Flemonds Cave as new locality records) are all distinct clades. *Pseudanophthalmus lacustris* n. sp. (subsuming *P. l. thomasi*) is supported but not as thoroughly, but this is likely due to two orphan sequences (a COI without a 28S and vice versa), which can decrease resolution. In light of this, the node support of 86% in this majority rules topology (Figure 14) is likely sufficient evidence to split it from its sister group of *Pseudanophthalmus ciliaris* Clade A.

In addition to the putative new species described herein, there also appear to be several additional undescribed species, with several occurring within the pubescens-
group, collected from Mutters Cave, Devil’s Den Cave, and Gorham Cave. Based on the geographical distribution of these caves and the phylogeny, I would predict that these three localities represent three to four new species.

**COI & 28S Bayesian tree, excluding Jack Johnson Cave’s *P. princeps* and *P. pubescens intrepidus***.

After 5,000,000 generations MrBayes arrived at a nearly identical topology (Figure 15, 16) to that seen in the parsimony trees. The virtue of Bayesian methods is that they utilize Maximum Parsimony systems and use a model of molecular evolution that take the current best prediction into account, and so may arrive at better hypotheses of evolution with less time than a completely random search method.

The only differences in phylogenetic relationships between the Bayesian (Figure 15, 16) and maximum parsimony majority rules trees (Figure 14) were the placement of three singleton new species (*P. n. sp. Mutters Cave, P. diabolus n. sp. Devils Den Cave, P. n. sp. Crumps Cave*) in a clade sister to the *crinitus n. sp. + pubescens pubescens* clade in the Bayesian analysis, and in a clade sister to the *crassus n. sp. + P. n. sp. Sharon Grove Cave* clade in the maximum parsimony analysis. The Bayesian analysis results more closely match the geographic distribution of *Pseudanophthalmus* populations. Additionally, the Bayesian analysis found *P. longi n. sp.* to be sister to *P. loganensis* and the *P. ciliaris B + P. riparius n. sp.* clade, whereas the maximum parsimony analysis only found *P. longi n. sp.* to be sister to *P. loganensis*.

The concatenated MrBayes tree found support for *P. longi* (63%) and *P. lacustris* (93%) comparable to the results of the parsimony tree. Branch length revealed that while
*P. crassus* and the new species from Sharon Grove are nearest neighbors, they are far from the same species. The same can be said of all other undescribed new species detected (one new species for each of Mutters Cave, Devils Den Cave, Crumps Cave, one to two new spp. for Hoy Cave, one tentative new sp. for Gorham Cave).
DESCRIPTIONS

Bearing in mind the “seven deadly sins of DNA barcoding” (Collins and Cruickshank 2012), molecular delimitation of species limits is still of clear value in this system. Morphology has proven deceptive in this and other lineages of cryptic cave trechine species, (a la early lumping of New World trechines into Old World Aphaenops, Barr’s faulty classification of Darlingtonea and Ameroduvalius (Maddison et al. 2019)) and other little brown beetles have been similarly plagued (Blair et al. 2005; Hulcr et al. 2015; Paterson et al. 2016).

Notably, the pubescens-group contained a mere seven described taxa (including two subspecies) prior to this study, so this work fully doubles the number of known pubescens-group Pseudanophthalmus. I anticipate that further new pubescens-group species will be discovered as more cave beetle records are acquired for the poorly-known caves on the periphery of the western Kentucky coal fields, and as the two most widely distributed species, P. pubescens and P. ciliaris ciliaris undergo genetic examination. Indeed, reaching identifications via a COI barcode library will likely be easier and more accurate than reliance upon morphological characteristics in some groups of species, especially when non-specialists are involved (Schindel and Miller 2005). That said, the only way for taxonomy to make true progress is an integrative approach, as neglecting either approach will be to the detriment of the other (Will et al. 2005). And so, without further ado, here are morphological species descriptions for the seven putative Barr taxa that I found supported.
Brief generic summary:


*Diagnosis* – *Pseudanophthalmus sensu stricto* (excluding the other genera of *Trechoblemus* series North American cave trechines – *Ameroduvalius, Darlingtonena, Neaphaenops*, and *Nelsonites*) range in length from ≈3.5 mm – 7.5 mm. Color ranges from castaneous-testaceous (chestnut-brick) to rufotestaceous (red-brick), with teneral individuals exhibiting paler hues and some level of transparency.

The mandible in *Pseudanophthalmus* is 2-4 tuberculate (in the *pubescens*-group), not 5- as in *Ameroduvalius*, and lacks a deep gap separating anterior and posterior teeth. *Pseudanophthalmus* elytra are generally less convex than those of *Darlingtonena*, and the two can often be separated by size as well – *Darlingtonena* is 6-8 mm long, at the upper limits of most *Pseudanophthalmus* species groups. The first two segments of the maxillary palps are subequal, not as in the closely related *Neaphaenops*, in which the terminal segment of the maxillary palp is markedly shorter than the penultimate. The head is routinely ¾ the width of the pronotum, unlike in *Nelsonites*, where the head is very large, nearly subequal to the pronotum. For more characteristics to differentiate these genera, see (Barr and Krekel 1967). Troglobitic *Pseudanophthalmus*, such as those in Kentucky and Tennessee, will be truly aphaenopsian (with elongated heads and abbreviated frontal grooves that end blindly near the occiput, not continued around behind the genae). Accordingly, *Pseudanophthalmus* lack eyes, aside from the single known edaphic *Pseudanophthalmus* species of West Virginia which has eye rudiments (Barr 1967).
The first step to within-genus identification is to consider collection locality – *Pseudanophthalmus* is a broadly distributed genus, and distribution may be used to filter some morphologically similar species that belong to different species groups. In western Kentucky, there are three main species groups: the *pubescens*-group, the *menetriesi*-group, and the *tenuis*-group, but even these can be separated by distribution to some extent (Figure 1). The *tenuis*-group is situated along the eastern edge of the western Kentucky coal fields, and follows its border south, its distribution sometimes overlapping with those of *menetriesi*- and *pubescens*-group species. The *menetriesi*-group is primarily located in central Kentucky, extending east to Mammoth Cave National Park, and extending west along the southern border of the western Kentucky Coal fields, and its distribution often overlaps with both the *pubescens*- and *barberi*- species groups. The *pubescens*-group was first described from along the Kentucky-Tennessee border, but extends eastward to Mammoth Cave, and the putative new species (Figure 2) extend its distribution north to the border of the western Kentucky Coal fields and west to Trigg County, nearly to the Cumberland River.

I divided the seven new *pubescens*-group species into three morphological groupings – those most morphologically similar in their aedeagi – male copulatory organs (Figure 5) – to *Pseudanophthalmaus ciliaris*, *P. loganensis*, and *P. pubescens*. 
The *ciliaris*-like morphological subgroup of the *pubescens*-group.

Beetles of this morphological subgroup are moderate in size for the *pubescens*-group, ranging from 4-6 mm in length. They are distributed all across northwestern and north-central Tennessee (Stewart, Montgomery, Cheatham, Montgomery, Robertson, and Sumner counties) and the adjacent portions of western Kentucky (Crittenden, Caldwell, Trigg, Christian, Todd, Logan, and Simpson counties). Described species are *Pseudanophthalmus ciliaris* (Valentine 1937; Barr 1959), *P. colemanensis* (Barr 1959), and possibly *P. ciliaris orlindae* (Barr 1959; Barr 1979), though each of these species may be in need of revision and/or reclassification (see Discussion). The new species with similar male genitalia are *Pseudanophthalmus riparius*, *P. caledonicus*, *P. crassus*, and *P. longi*. 
**Pseudanophthalmus riparius**, new species

*Etymology.* – Latin *riparius*, “of the riverbank.”

*Diagnosis.* – Best identified as a toptype, secondarily by the lunate median lobe of its aedeagus. Its median lobe is similar to that of *Pseudanophthalmus caledonicus n. sp.*, but subapically expanded. Internal sac spinulose.

*Description.* – Aedeagus with a strong basal keel, as in *Pseudanophthalmus ciliaris*. Basal bulb of median lobe sharply, strongly deflexed. Median lobe expanded subapically, apex sharp and sinuate in left lateral view compared to *P. ciliaris*. Left copulatory piece straight, slightly slenderer than in *Pseudanophthalmus ciliaris ciliaris*, right copulatory piece with folded knobbed tip. Internal sac markedly spinulose. Elytra are widest at midpoint, and have less pronounced pubescence.

*Measurements (mm).* – Total length 4.70-5.35, mean 5.00±0.24 (N = 6); head 1.05 long X 0.86 wide; pronotum 0.86 long X 0.95 wide; elytra 3.00 long X 1.84 wide. Aedeagal length 0.97 mm, mean 0.97 mm (N=2).

*Distribution* – Ranges from central to eastern Christian County, Kentucky. Known from Glovers Cave (six individuals examined), Carter Cave, Chandler Cave, and Flemonds Cave of Christian County, Kentucky.
**Pseudanophthalmus. caledonicus, new species**

*Etymology.* – Latin *caledonicus*, “of Caledonia,” after a town near the proposed type locality.

*Diagnosis.* – Best identified as a topotype, secondarily by the lunate median lobe of its aedeagus, similar to *Pseudanophthalmus riparius n. sp.*, but apically tapered.

*Description.* – Aedeagus with a variable basal keel, basal bulb generally large, median lobe thick and sharply deflexed above paramere attachment point, but overall lunate. Median lobe comes to a sharp point apically. Left copulatory piece thin and rodlike, slightly curved toward parameres. Right copulatory piece very broad, without clear clublike or spatulate structure. Parameres with 4-5 setae. Elytra widest at midpoint, pronounced elytral pubescence.

*Measurements (mm).* – Total length 4.81-5.35, mean 5.16±0.18 (N = 8); head 1.11 long X 0.89 wide; pronotum 0.98 long X 1.18 wide; elytra 3.07 long X 1.86 wide. Aedeagal length 1.00-1.03 mm, mean 1.02 (N=2).

*Distribution* – Known from Lawrence (*aka* Husk) Cave (8 examined) of northeast Trigg County, Kentucky. Purported to be found in several other hydrologically connected caves in the area.
**Pseudanophthalmus crassus, new species**

*Etymology.* – Latin *crassus*, “stout.” Descriptive, after its proportionally broader pronotum than other *pubescens*-group species.

*Diagnosis.* – Readily identified as a topotype, secondarily by its proportionally broader pronotum and elytra than other members of the *pubescens*-group.

*Description.* – Aedeagus with a strong basal keel, as in *Pseudanophthalmus ciliaris*, basal bulb of median lobe is deflexed, while median lobe itself is slightly constricted on both sides. Left copulatory piece very straight and thin, expanded basally, not clearly spinulose, nor curved towards right copulatory piece. Right copulatory piece broad, folded, forming a spatulate tip. Parameres with 4 setae. Pronotum exceptionally broad (mean PW/PL = 1.26, as opposed to a more typical 1.0-1.2 in other *pubescens*-group species Elytra are widest at posterior 1/3, and are covered with pronounced pubescense.

*Measurements* (mm). – Total length 4.54-4.92, mean 4.68±0.17 (N = 4); head 0.99 long X 0.82 wide; pronotum 0.88 long X 1.11 wide; elytra 2.81 long X 1.82 wide. Aedeagal length 1.00 mm (N=1).

*Distribution* – Known from Yates Cave (three examined) and Kinnin Cave (one examined) of northeast Crittenden County, Kentucky.
Pseudanophthalmus longi, new species

Etymology. – Latin longi “lengthy.” Descriptive, after its proportionally lengthier pronotum than other pubescens-group species.

Diagnosis. – Readily identified as a topotype. Lengthy pronotum. The small and slender aedeagus appears similar to those of the loganensis subgroup, with the exception of its strong basal keel.

Description. – Aedeagus with a strong basal keel, as in Pseudanophthalmus ciliaris, basal bulb of median lobe sharply deflexed. Left copulatory piece short, weakly curved towards right copulatory piece, as in the loganensis subgroup, though more clearly spinulose. The right copulatory piece is large, rounded with an apical knob, and broader than in Pseudanophthalmus ciliaris ciliaris. Parameres with 6 setae. Pronotum tends to be proportionally longer than other pubescens-group (mean PL/PW = 0.97, as opposed to a more typical PL/PW of 0.82). Elytra are widest at their midpoint, and have less pronounced pubescense.

Measurements (mm). – Total length 4.05-5.03 mm, mean 4.64±0.29 (N = 14); head 1.02 long X 0.83 wide; pronotum 0.83 long X 0.86 wide; elytra 2.76 long X 1.73 wide. Aedeagal length 0.86-0.94 mm, mean 0.90 mm (N=2).

Distribution – Ranges from northwest to central Todd County, Kentucky. Known from Buck Fork Creek Cave (8 examined) and Potter (aka Haddon) Cave (6 examined) of Todd County, Kentucky.
The *loganensis*-like morphological subgroup of the *pubescens*-group.

Most beetles of this lineage are small in size, ranging from 4-5 mm in length. Their distribution is centered in south-central Kentucky (Logan, Butler, Simpson, Warren, and Allen County) and extends downward to touch north-central Tennessee (Robertson and Sumner counties). There is only one currently described species, *Pseudanophthalmus loganensis* (Barr 1959; Barr 1979), though the vastly larger *P. ciliaris orlindae* (Barr 1959; Barr 1979) may merit inclusion (see Discussion). New species are *Pseudanophthalmus lacustris* (which has subsumed the putative *P. loganensis thomasi*) and *P. lancasteri*. 
Pseudanophthalmus lacustris, new species

Etymology. – Latin, lacustris, “of the lake”. Likely after Escue Cave’s location along Sumner Sportsman Club Lake, Portland, Tennessee.

Diagnosis. – May be identified as a topotype in its part of Sumner County, Tennessee, though in parts of Allen County, Kentucky, this may not be possible due to an abundance of other Pseudanophthalmus species. Similar to other loganensis subgroup members, the left copulatory piece gently curves towards the right copulatory piece. Can be separated from Pseudanophthalmus lancasteri n. sp. by lack of a basal keel. May be distinguished from Pseudanophthalmus loganensis by a slight apical broadening of the shorter median lobe.

Description. – Median lobe slender, broadest subapically, coming to a gentle point. Basal bulb only gently deflexed, unlike Pseudanophthalmus lancasteri n. sp. Left copulatory piece small with no spinules observed, tapering apically, curved towards right copulatory piece. Right copulatory piece comparatively wide, folded without any sort of knob at its tip – negligibly spatulate. Elytra are widest at their posterior 1/3, and have dense pubescence and punctate elyral striae.

Measurements (mm). – Total length 4.03-4.95 mm, mean 4.46±0.27 (N = 10); head 0.98 long X 0.77 wide; pronotum 0.83 long X 1.00 wide; elytra 2.65 wide X 1.66 long. Aedeagal length 0.79-0.84 mm, mean 0.82 mm (N=3).

Distribution – Ranges from north central Sumner County, Tennessee, to central Allen County, Kentucky. Known from Escue Cave (6 examined), Sumner County, Tennessee,
to Lynn Cave (new record, 4 examined) and Sommer Cave (new locality), Allen County, Kentucky.

*Remarks* – Subsumes Barr’s putative unpublished subspecies of *Pseudanophthalmus loganensis thomasi.*
*Pseudanophthalmus lancasteri*, new species


*Diagnosis.* – Similar to other *loganensis* subgroup members, the left copulatory piece gently curves towards the right copulatory piece. Unlike others *loganensis* subgroup members, its median lobe is straight and strongly deflexed at the basal bulb and bears a keel reminiscent of the *ciliaris* subgroup but it has a longer and straighter aedagus. Also of note, its elytra tend to not meet at their apex.

*Description.* – Median lobe slender, expanded slightly subapically. The left copulatory piece gently curves toward the right copulatory piece. The left copulatory piece is small, slender, shorter than the right piece and nondescript, with no spinules clearly visible, though it appears rough in texture. The right copulatory piece is slender, with a small spatulate tip, though far less developed than spatulate tips of the *pubescens* subgroup. Basal bulb strongly deflexed, almost forming a right angle, and a basal keel is present. The widest point of the elytra is at the midpoint, and elytral pubescense is pronounced.

*Measurements* (mm). – Total length 4.32-5.03 mm, mean 4.70±0.28 (N = 17); head 1.02 long X 0.82 wide; pronotum 0.85 long X 1.04 wide; elytra 2.82 long X 1.74 wide. Aedeagal length 0.87-0.98 mm, mean 0.93 mm (N=4).

*Distribution* – Ranges from northwest Warren County to southeast Butler County, Kentucky. Known from Thomas Cave (9 examined), Jacks Cave (2 examined), and Helen Morgan Cave (new locality) of Warren County, as well as Jesse Stewart Cave (6 examined) of Butler County, Kentucky.
The *pubescens*-like morphological subgroup of the *pubescens*-group.

Most beetles of this subgroup are large in size, ranging from 5-7 mm in length, and their distribution is localized to central and south-central Kentucky (Simpson, Warren, Edmonson, Hart, Allen, Barren, and Metcalfe counties). Current species are

*Pseudanophthalmus pubescens pubescens* (Horn 1868; Barr and Crowley 1981), *P. pubescens intrepidus* (Barr 1985), and possibly *P. princeps* (Barr 1979). The new species is *Pseudanophthalmus crinitus*. Notably, this study finds that *P. p. pubescens* subsumes the *P. “amicus n. sp.”* Clade B).
*Pseudanophthalmus crinitus*, new species

*Etymology.* – Latin *crinitus*, “wooly.” Descriptive, after its dense pubescense.

*Diagnosis.* – Head exceptionally broad and rounded in comparison to other *pubescens*-group species, dense elytral pubescense. Body rufous, darker than other members of the *pubescens* morphological subgroup.

*Description.* – Aedeagus with strongly sculptured copulatory pieces. Left copulatory piece apically attenuate in lateral view and spinulose, if less pronounced than in *Pseudanophthalmus princeps*. Right copulatory piece strongly spatulate with broad apical knob and spinulose internal sac. Medial lobe thick and arcuate, basal bulb with a level of deflexion intermediate to *Pseudanophthalmus pubescens pubescens* and *P. princeps*. Elytra are widest at midpoint and are covered with a dense pubescense.

*Measurements* (mm). – Total length 5.41-5.46 mm, mean 5.42±0.03 (N = 3); head 1.21 long X 0.95 wide; pronotum 1.03 long X 1.26 wide; elytra 3.19 long X 1.96 wide. Aedeagal length 1.06 mm (N=1).

*Distribution* – Known from Carpenter Cave (3 examined) and Goodrum Cave (new locality) of central Allen County, Kentucky

*Remarks* – Based on aedeagal morphology, *Pseudanophthalmus crinitus* n. sp. is most similar to *P. pubescens pubescens* and *P. p. intrepidus*. 
ADDITIONAL NEW PUTATIVE SPECIES

Evidence for four or perhaps even five new undescribed species was discovered from molecular data. However, morphological species descriptions are not possible at this time – further collections must be made to ensure that these genetically distinct populations (see below for discussions on four of these) are indeed well-supported genetic clusters, as opposed to single aberrant, individuals, yielding contaminated sequences.

Putative new non-pubescens-group species, *P. “amicus”*, known from Bear Cave, Allen County, Kentucky and Bloody Murder Cave, Montgomery County, Tennessee. It was herein referred to as n. sp. *Pseudanophthalmus “amicus”* Clade A and appears to fall outside of the pubescens-group, though in the distribution of Barr’s defunct *P. amicus*. (While the name “*P. amicus*” was proposed by Barr, his *amicus* was of a member of the pubescens-group, here suspected to be what was referred to as “*P. amicus* Clade B”, a synonym of *P. p. pubescens*). To reduce confusion associated with this past, it is possible that another open species name should replace “*amicus*” when this group is formally described.

Putative new pubescens-group species, *P. diabolus*, from Devils Den Cave, Metcalf County, Kentucky; Crumps Cave, Hart County, Kentucky; Mutters Cave, Barren County, Kentucky. A basal member of the *Pseudanophthalmus pubescens*-group clade, sister to *P. crinitus n. sp.* and *P. pubescens pubescens*, yet sufficiently distinct from both that it may deserve elevation to full species status, if morphology proves distinctive.

Based on unequal branch length (Figure 16), I suspect that further molecular examination may reveal that Mutters Cave houses a distinct species from Devils Den and Crumps Cave. Examination of the aedeagal morphology of this clade promises to be very
interesting, based on the marked morphological distinction between *Pseudanophthlamus pubescens pubescens* and *P. crinitus* n. sp. (Figure 7) and the broad east-west distribution of these three caves.

**Putative new pubescens-group species, undescribed**, Gorham Cave, Logan County, Kentucky. It appears to be basal to *P. ciliaris* Clade A (Silvey Cave, Tennessee) and *P. lacustris n. sp*. From the single specimen, it seems sufficiently molecularly distant to justify elevation to full species status, but it lacks a representative sampling to understand morphological variation.

**Putative new menetriesi-group species, undescribed**, known from Sharon Grove Cave, Todd County, Kentucky. The final undescribed putative species that is strongly supported by molecular data, it was first mentioned in Harker and Barr (1980). Barr thought it to be a member of the *menetriesi*-group, a clade he deemed sister to the *pubescens*-species group. As this Sharon Grove Cave beetle comes out basally to *P. crassus* n. sp. in our phylogenies, we must ask ourselves which species group these two species belong in, a question that will require further collection and examination of *menetriesi*-group specimens.
DISCUSSION

NEED FOR REVISION

This research highlighted the currently rampant nature of non-monophyly in the delimited *pubescens*-group *Pseudanophthalmus*. Of seven currently described taxa, five were found to be non-monophyletic (*P. ciliaris ciliaris*, *P. ciliaris orlindae*, *P. colemanensis*, *P. loganensis*, *P. princeps*), and the remaining two (*P. pubescens pubescens* and *P. pubescens intrepidus*) were underrepresented in our study. Of the five previously described *pubescens*-group species (*P. pubescens* and *P. ciliaris* both have two subspecies), each is thought to have a distribution spanning several counties, despite most falling to the west of the more widely connected eastern portion of the MP I karst and within more disjunct formations. All described taxa examined with sufficient representation were found to be more representative of morphotypes than of reproductive isolation as reflected by genetic divergence.

We are left with the sense that morphology is deceptive, and not necessarily reflective of reproductive boundaries. This study has exposed paraphyly via *P. ciliaris orlindae* coming out within *P. loganensis* and *P. colemanensis* coming out inside *P. ciliaris ciliaris*. Likewise, polyphyly has been discovered in *P. ciliaris ciliaris*, *P. colemanensis*, and *P. princeps*, which all exhibit multiple genetically distinct and genetically distant clades. It is my suspicion that further research will find that other *Pseudanophthalmus*, especially those described species occupying more fragmented habitats, such as the western peripheral portions of MP I karst, are much nearer to topotypes than their kin in more connected karst regions such as MP II karst and the eastern portions of MP I.
RESEARCH QUESTIONS AND CONCLUSIONS

1. The aedeagal morphological groupings did not correspond to phylogenetic relationships, for the most part. The exception to the rule was my grouping of *P. pubescens* together with *P. “amicus n. sp.”* and *P. crinitus n. sp.* For all others, aedeagal morphological similarity appears to not have been synapomorphic. Hence examination of internal male genitalic morphology did not result in accurate predictions of relatedness.

2. The *pubescens*-group as it stands is not strictly monophyletic. *P. colemanensis* appears to be a member of the *engelhardti* species-group of central Tennessee, and the one new species we molecularly determined was present in Bear Cave, the type locality of Barr’s putative *P. amicus*, was also determined to fall outside of the *pubescens* species-group. Tissue samples should be extracted from Barr’s own *P. amicus* and sequenced to see if they are of this separate species-group (here *P. amicus* Clade A) or were merely a morphologically variant *P. pubescens*.

3. In regards to the hypothesis that the widely distributed *Pseudanophthalmus pubescens* constitutes a single lineage, I will disregard the presumably incorrect *P. pubescens intrepidus* sequences in answering this question. The included samples from Crumps Cave (a known locality for both *P. menetriesi* and *P. pubescens*) fell outside of the *P. pubescens pubescens* clade centered on L&N Railroad Cave, and I suspect it is worthy of elevation to species status. I also suspect that the widely distributed “*P. pubescens*” does indeed contain some cryptic diversity, however, the eastern reaches of *P. pubescens*’ range were not included in my study, and so I am as yet unable to provide any well supported examples.
4. There appear to be two lineages of *P. ciliaris* within the *pubescens* species-group (here discussed as *P. ciliaris* Clade A and *P. ciliaris* Clade B) and I suspect that there are others. The trend towards geographical topotypes will likely become more pronounced in *P. ciliaris*’ heartland, along the western reaches of Kentucky and Tennessee’s shared border (Figure 1). This is hypothesized due to less consistently present sandstone cap, which has led to the development of more fragmented karst islands and more isolated populations that are thus more prone to diverge into what can be considered separate species.

All of *P. ciliaris*’ current and former subspecies (with the potential exception of *P. ciliaris orlindae*) are valid species, though not all are of the *pubescens* species-group. The case of mistaken *P. ciliaris* vs. *P. colemanensis* identification is interesting, as it not only highlights that cryptic diversity exists in a widespread *Pseudanophthalmus* species (*P. ciliaris*), but also shows that morphological variation (the basis for the identification of *P. colemanensis* Clarksville Lake Cave) does not necessarily correspond to genetic variation. As the presence of *P. ciliaris ciliaris* clades A and B in this study attests, morphology should not be trusted when delineating *Pseudanophthalmus* species, and many extant taxa likely require revision.

5. The putative new species *P. loganensis thomasi* was not a valid subspecies, nor, indeed, was it a near relative of *P. loganensis*. Instead, it came out in the putative *P. lacustris* clade, sister to *P. ciliaris* Clade A.

6. Kinnin Cave and Yates Cave, despite their apparent geographical separation, do share one species. They have formed a strongly supported clade in every molecular phylogenetic tree discovered.
7. Carpenter Cave is not stratigraphically isolated from the Kentucky Pennyroyal Plateau as thought by Barr (1980), or, if so, it has not been isolated for long enough for its cave beetles to diverge from their kin in nearby Goodrum Cave. Despite ostensibly being on the physiographic level of the Central Basin of Tennessee, Carpenter Cave’s putative new species of beetle is not located nearer the Tennessee taxa included in my trees. Instead, *P. crinitus* appears to be a strongly supported sister species to *P. pubescens pubescens*, a Kentucky-centered species whose range does not extend very far into Tennessee.

8. Potter (*aka* Haddon) Cave and Buck Fork Creek Cave, both sites for *P. longi* *n. sp.*, do appear to share the same species, though with comparatively low support. I suspect that the main driver behind poor support of this clade is presence of a few comparatively low quality DNA sequences. I would like to remedy this with further collection from caves in the region – perhaps an intermediate cave would result in a bridge between the two and stronger support, as was the case when I added Helen Morgan Cave to my *P. lancasteri* *n. sp.* analyses. Regardless, neither of these caves has the same species as nearby Sharon Grove Cave.
SUMMARY

Barr’s putative new species are all somewhat distinct when one examines aedeagal morphology. Of his nine putative *pubescens*-group *Pseudanophthalmus*, seven were molecularly supported and herein described as new species. Five of these (*Pseudanophthalmus caledonicus* n. sp., *P. crassus* n. sp., *P. crinitus* n. sp., *P. lancasteri* n. sp., *P. riparius* n. sp.) were very strongly supported, with 100% clade support in both concatenated COI & 28S Bayesian and parsimony phylogenies. The remaining two taxa elevated to full species status were *P. lacustris* n. sp. (93% clade support in concatenated COI & 28S Bayesian and parsimony phylogenies) and *P. longi* n. sp. (63% clade support in Bayesian, and 64% clade support in parsimony concatenated COI & 28S phylogenies). These latter two new species likely had reduced clade support due to the absence of COI or 28S sequences for several individuals.

In addition to these formal descriptions, this research found the need for further examination of *Pseudanophthalmus* populations in general and the the *pubescens*-group in particular. Tantalizing connections between the *menetriesi* and *pubescens* group were observed that require further sampling. There is a minimum of four undescribed species revealed by this research, and more shall doubtless be found by additional sampling and further molecular investigation. While morphology should be recorded in future species descriptions, it is not useful when delineating *Pseudanophthalmus* species and inferringing their evolutionary relationships.
FIGURES

Figure 1. Distribution of all known taxa for the four *Pseudanophthalmus* species groups known from western Kentucky. The *tenuis*-(blue diamonds), the *cumberlandus*-(green squares), the *menetiesi*-(red downwardpointing triangles), and the *pubescens*-(purple upwardpointing triangles). Black points indicate the localities sampled for this study.
Figure 2. (A) Distribution of Barr’s nine putative new species in the *pubescens* species-group. (red *P. crassus*, orange *P. caledonicus*, yellow *P. riparius*, green *P. longi*, navy *P. lancasteri*, cyan *P. lacustris*, pink *P. amicus*, violet *P. crinitus*, black *P. loganensis thomasi*). (B) Distribution of the seven supported new species in the *pubescens* species-group. Note that *P. amicus* and *P. l. thomasi* were not supported, but *P. crinitus, P. lacustris*, and *P. riparius* had impressive range extensions.
Figure 3. Morphological PCA of head, thorax, and elytral length and width measurements for the *pubescens*-group, sorted by species (putative new and described). Ellipses indicate normal distributions in which 68% of the members of each species would be expected to fall, based on prior data points. Ellipses and datapoint numbers correspond in color to the species they represent (see key at right).
Figure 4. Morphological PCA of head, thorax, and elytral length and width measurements for the *pubescens*-group, sorted by described species’ presumed relatedness (dark green includes *P. c. ciliaris*, *P. c. orlindae*, and *P. (c.) colemanensis*; gray includes *P. loganensis* and the putative n. ssp. *P. l. thomasi*; pink contains *P. p. pubescens*, *P. p. intrepidus*, and their presumed hybrid; and in purple *P. princeps* stands alone). No assumptions are made about the remaining eight putative new species’ relatedness, and they are plotted together in blue. Ellipses indicate normal distributions in which 68% of the members of each cluster of species might be expected to fall, based on prior data points, and correspond in color to the points they encircle (see key below). Species are indicated by the same numbers as they were in the prior PCA’s key (see Figure 3).
Figure 5. Explanatory power of each Principal Component for Figure 3 & Figure 4.
Figure 6. Labeled aedeagi from *Pseudanophthalmus ciliaris*, both uneverted (A) and everted (B).
Figure 7. Aedeagi for all currently known *pubescens*-group *Pseudanophthalmus*, imaged from aedeagal slides in Barr’s collection using standardized magnification (relative sizes are accurate, scale bars = 200 μm). Species names given are those recorded by Barr on his slide labels. Notes about species’ support in this study has been included below – n. sp. indicates Barr’s new species, syn. indicates a junior synonym. Aedeagi have been roughly sorted based on geographical location, with those species found in the northeast at the upper right of the page, those found in the southwest of the *pubescens*-group’s range at the lower left, etc. Barr’s specimen numbers for these taxa and the caves they were collected from may be viewed in Table 4.
Figure 8. Neighbor-joining tree of COIF sequences, rooted on non-\textit{pubescens}-group \textit{Pseudanophthalmus} (\textit{robustus}-/\textit{intermedius}-group and \textit{tenuis}-group). Colors indicate Barr’s putative new species, and correspond to those in (Figure 2, 16).
Figure 9. Neighbor-joining consensus tree of COI sequences.

Rooted outgroup: Linephea erasa

"P. amicus" clade A (falls outside of the pubescens group)
Outgroup: intermedius or robustus group P. sp.
& "P. princeps" A (falls outside of the pubescens group)
Outgroup: tenuis group P. barbieri
Outgroup: engelhardtii group P. wallacei
P. colemanensis (falls outside of the pubescens group)
Undescribed: P. n. sp. #2 (Devils Den)
Outgroup: menetriesi group P. menetriesi
Outgroup: menetriesi group P. n. sp. (Sharon Grove)

P. crassus, n. sp.

Undescribed: P. n. sp. #1 (Mutters Cave)
F. caledonicus, n. sp.
P. crinitus, n. sp.
P. pubescens ("P. amicus" clade B)

"P. princeps" B

F. lancasteri, n. sp.
P. lacustris, n. sp.
P. ciliaris clade A
P. riparius, n. sp.
P. longi, n. sp.
P. loganensis
(includes P. orlindae Cross Plains Cave)
P. ciliaris clade B
(includes P. colemanensis Clarksville Lake Cave)

Ingroup: pubescens group
Figure 10. Parsimony COI strict consensus tree.

Rooted outgroup: *Lionepha erasea*

*P. amicus* clade A (falls outside of the *pubescens* group)

Outgroup: *engelhardtii* group *P. wallacei*

*P. colemanensis* (falls outside of the *pubescens* group)

Outgroup: *intermedius* or *robustus* group *P. sp.

&P. princeps* A (falls outside of the *pubescens* group)

Outgroup: *tenensis* group *P. barbieri*

*P. caledonicus*, n. sp.

Outgroup: *menetriesi* group *P. n. sp. (Sharon Grove)*

Undescribed: *P. n. sp. #1 (Mutters Cave)*

Outgroup: *menetriesi* group *P. menetriesi*

Undescribed: *P. n. sp. #2 (Devils Den)*

*P. crassus*, n. sp.

*P. crinitus*, n. sp.

*P. pubescens* (*P. amicus* clade B)

*P. lancasteri*, n. sp. (paraphyletic)

*P. lacustris*, n. sp.

*P. ciliaris* clade A

*P. princeps* B

*P. longi*, n. sp.

*P. loganensis* (includes *P. orlindae* Cross Plains Cave)

*P. riparius*, n. sp.

*P. ciliaris* clade B (includes *P. colemanensis* Clarksville Lake Cave)
Figure 11. Parsimony COI majority rules tree.
Figure 12. Parsimony 28S strict consensus tree.
Figure 13. Parsimony concatenated COI and 28S strict consensus tree.
Figure 14. Parsimony concatenated COI and 28S majority rules consensus tree. Node support values indicate the percentage of trees which included that topology.
Figure 15. Bayesian tree of concatenated COI and 28S sequences. Proportional-transformed image with node support labels produced in FigTree v1.4.4. Colors correspond to those in (Figure 4, 10).
**Figure 16.** Bayesian tree of concatenated COI and 28S sequences. Untransformed image produced in FigTree v1.4.4. Colors correspond to those in (Figure 4, 10). Rooted outgroup (*Lionepha*) not shown. Scalebar = 0.01.
Table 1: Collection results for 10 of Barr’s putative new *Pseudanophthalmus* species, 9 of the *pubescens* -group, from at 14 historical caves and two caves with newly discovered populations in KY and TN, USA. To protect sensitive cave habitats and species, precise cave locations are not included. Please contact the authors or the KSS for data requests.

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<th>Date visited</th>
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<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td>Calvert Spring</td>
<td></td>
<td>2019.07.12</td>
<td>X</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td><strong>P. lancasteri</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Esuce Cave (private sink entrance)</td>
<td>Hist.</td>
<td>2019.03.24</td>
<td>1</td>
<td>(B.Collins, J.J.Nixon, &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td>Cheek’s Stand Cave</td>
<td></td>
<td>2019.03.24</td>
<td>0</td>
<td>(B.Collins, J.J.Nixon, &amp; T.K.Philips)</td>
</tr>
<tr>
<td><strong>P. loganensis thomasi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY: Allen Co</td>
<td>Lynn Cave</td>
<td></td>
<td>2013.09.21</td>
<td>1</td>
<td>(O.F.Boyd &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>2019.02.23</td>
<td>0</td>
<td>(J.J.Nixon &amp; W.I.Payette)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2020.02.08</td>
<td>2</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2020.02.11</td>
<td>0</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2019.02.23</td>
<td>0</td>
<td>(J.J.Nixon &amp; W.I.Payette)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2020.02.08</td>
<td>1 obs</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2020.02.11</td>
<td>1 obs</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td>KY: Warren Co</td>
<td>Helen Morgan Cave</td>
<td>NEW</td>
<td>2019.02.13</td>
<td>0</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2019.02.16</td>
<td>4</td>
<td>(J.J.Nixon &amp; W.I.Payette)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2020.02.08</td>
<td>3</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td><strong>P. loganensis thomasi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. longi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY: Todd Co</td>
<td>Buck Fork Cave</td>
<td>Hist.</td>
<td>2018.05.24</td>
<td>4</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td><strong>P. riparius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY: Christian Co</td>
<td>Golvers Cave (burial site entrance) (TYPE)</td>
<td>Hist.</td>
<td>2018.05.22</td>
<td>0</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td>KY: Christian Co</td>
<td>Golvers Cave (river entrance)</td>
<td>Hist.</td>
<td>2018.05.22</td>
<td>0</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td>KY: Christian Co</td>
<td>Carter Cave (Golvers Cave System)</td>
<td>NEW</td>
<td>2018.05.22</td>
<td>4</td>
<td>(J.J.Nixon &amp; T.K.Philips)</td>
</tr>
<tr>
<td>n. sp. P. menetriesi group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY: Todd Co</td>
<td>Sharon Grove Cave</td>
<td>Hist.</td>
<td>2013</td>
<td>4</td>
<td>(T.K.Philips &amp; M.Mahan)</td>
</tr>
</tbody>
</table>
**Table 2:** Primers used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Primer</th>
<th>Binding region</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1</td>
<td>R</td>
<td>Pat</td>
<td>5'-TCCAATGCACTAATCTGCCATATTA-3'</td>
</tr>
<tr>
<td>CO1</td>
<td>F</td>
<td>CO1F Ober</td>
<td>5'-CGAATAAAATAATAAGATTTTGA-3'</td>
</tr>
<tr>
<td>28S</td>
<td>F</td>
<td>D1</td>
<td>5'-AAAGAAACTAACWAGGTT-3'</td>
</tr>
<tr>
<td>28S</td>
<td>R</td>
<td>D33'alt</td>
<td>5'-TTCACCATCTTTCGGTCC-3'</td>
</tr>
</tbody>
</table>

**Table 3:** PCR temperature protocol used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protocol</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1</td>
<td>98°C - 2 min initial denature</td>
<td>95°C - 5 min initial denature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>98°C - 10 sec</td>
<td>95°C - 30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51°C - 30 sec</td>
<td>49°C - 30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C - 1 min</td>
<td>72°C - 1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C - 2 min final extension</td>
<td>72°C - 3 min final extension</td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td>4°C - hold</td>
<td>4°C - hold</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Specimen information for the most representative *pubescens*-group aedeagi in the Carnegie Museum of Natural History’s collection.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Current Status</th>
<th>Barr's Specimen ID code</th>
<th>Collection Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. amicus</em></td>
<td>putative, unsupported</td>
<td>B1186F</td>
<td>Sulphur/Friendship Cave, Allen Co, KY</td>
</tr>
<tr>
<td><em>P. loganensis thomasi</em></td>
<td>putative, unsupported</td>
<td>B1562B</td>
<td>Piano Salt peter Cave, Warren Co, KY</td>
</tr>
<tr>
<td><em>P. caledonicus</em></td>
<td>new species</td>
<td>B1045C</td>
<td>Husk Cave, Trigg Co, KY</td>
</tr>
<tr>
<td><em>P. crassus</em></td>
<td>new species</td>
<td>B1542B</td>
<td>Yates Cave, Crittenden Co, KY</td>
</tr>
<tr>
<td><em>P. crinitus</em></td>
<td>new species</td>
<td>B1537B</td>
<td>Carpenter Cave, Allen Co, KY</td>
</tr>
<tr>
<td><em>P. lacustris</em></td>
<td>new species</td>
<td>B800C</td>
<td>Escape Cave, Sumner Co, TN</td>
</tr>
<tr>
<td><em>P. lancasteri</em></td>
<td>new species</td>
<td>B1560A</td>
<td>Thomas Cave, Warren Co, KY</td>
</tr>
<tr>
<td><em>P. longi</em></td>
<td>new species</td>
<td>B1044B</td>
<td>Buck Fork Creek Cave, Todd Co, KY</td>
</tr>
<tr>
<td><em>P. ciliaris ciliaris</em></td>
<td>previously described</td>
<td>B1179C</td>
<td>Keysburg Buzzard Cave, Logan Co, KY</td>
</tr>
<tr>
<td><em>P. ciliaris orlindae</em></td>
<td>previously described</td>
<td>B174D</td>
<td>Cook Cave, Logan Co, KY</td>
</tr>
<tr>
<td><em>P. colemanensis</em></td>
<td>previously described</td>
<td>BA170A</td>
<td>Coleman Cave, Robertson Co, TN</td>
</tr>
<tr>
<td><em>P. loganensis</em></td>
<td>previously described</td>
<td>B840E</td>
<td>Hoy Cave, Simpson Co, KY</td>
</tr>
<tr>
<td><em>P. princeps</em></td>
<td>previously described</td>
<td>B1562E</td>
<td>Salt peter Cave, Warren Co, KY</td>
</tr>
<tr>
<td><em>P. pubescens pubescens</em></td>
<td>previously described</td>
<td>B1543A</td>
<td>Pruitt Salt peter Cave, Warren Co, KY</td>
</tr>
<tr>
<td><em>P. pubescens intrepidus</em></td>
<td>previously described</td>
<td>B1249A</td>
<td>Edmonds Cave, Barren Co, KY</td>
</tr>
</tbody>
</table>

Table 5: Aedeagal character state matrix for the *P. pubescens* species-group.

| Character (p1,a0) | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganensis)? | Parameres excavate (a la loganansen)
Table 6: Aedeagal morphological groupings for the known *pubescens*-group species

<table>
<thead>
<tr>
<th>Species</th>
<th>Aedeagal morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. ciliaris ciliaris</em></td>
<td><em>ciliaris</em>-like</td>
</tr>
<tr>
<td><em>P. colemanensis</em></td>
<td><em>ciliaris</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. caledonicus</em></td>
<td><em>ciliaris</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. crassus</em></td>
<td><em>ciliaris</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. riparius</em></td>
<td><em>ciliaris</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. longi</em></td>
<td><em>ciliaris</em>-like or <em>loganensis</em>-like</td>
</tr>
<tr>
<td><em>P. ciliaris orlindae</em></td>
<td><em>ciliaris</em>-like or <em>loganensis</em>-like</td>
</tr>
<tr>
<td><em>P. loganensis</em></td>
<td><em>loganensis</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. lacustris</em></td>
<td><em>loganensis</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. lancasteri</em></td>
<td><em>loganensis</em>-like</td>
</tr>
<tr>
<td>n. ssp. <em>P. loganensis thomasi</em></td>
<td><em>loganensis</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. amicus</em></td>
<td><em>loganensis</em>-like or <em>pubescens</em>-like</td>
</tr>
<tr>
<td><em>P. pubescens pubescens</em></td>
<td><em>pubescens</em>-like</td>
</tr>
<tr>
<td><em>P. pubescens intrepidus</em></td>
<td><em>pubescens</em>-like</td>
</tr>
<tr>
<td>n. sp. <em>P. crinitus</em></td>
<td><em>pubescens</em>-like</td>
</tr>
<tr>
<td><em>P. princeps</em></td>
<td><em>pubescens</em>-like or <em>princeps</em>-like</td>
</tr>
</tbody>
</table>

Table 7: Genbank accession codes for this study’s new species and putative new species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cave</th>
<th>GenBank Accession Code(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n. sp. <em>P. caledonicus</em></td>
<td>Lawrence (nee Husk) Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. crassus</em></td>
<td>Yates Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. crassus</em></td>
<td>Kinnin Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. crinitus</em></td>
<td>Carpenter Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. crinitus</em></td>
<td>Goodrum Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lacustris</em></td>
<td>Escue Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lacustris</em></td>
<td>Lynn Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lacustris</em></td>
<td>Sommer Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lancasteri</em></td>
<td>Thomas Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lancasteri</em></td>
<td>Jacks Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lancasteri</em></td>
<td>Helen Morgan Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. lancasteri</em></td>
<td>Jesse Stewart Caves</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. longi</em></td>
<td>Buck Fork Creek Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. longi</em></td>
<td>Potter (nee Haddon) Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. riparius</em></td>
<td>Carter Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. riparius</em></td>
<td>Chandler Cave</td>
<td></td>
</tr>
<tr>
<td>n. sp. <em>P. riparius</em></td>
<td>Flemonds Cave</td>
<td></td>
</tr>
<tr>
<td>Putative n. sp. <em>P. amicus</em></td>
<td>Bear Cave (2015)</td>
<td></td>
</tr>
<tr>
<td>Putative n. sp. <em>P. amicus</em></td>
<td>Bloody Murder Cave (2015)</td>
<td></td>
</tr>
</tbody>
</table>
APPENDICES

Appendix I: Command Blocks

i. NONA commands (all analyses):

rs 0
hold 100000
mult*1000

ii. MrBayes commands (concatenated COI & 28S):

log start filename = nixonoberconcx.txt
execute nixonoberconcx.nex
charset COI = 1-1081;
charset 28S = 1082-2009;
partition favored = 2: COI, 28S;
set partition = favored;
lset applyto = (1) nst=6 rates=invgamma;
lset applyto = (2) nst=6 rates=gamma;
prset applyto=(all) ratepr=variable;
showmodel
mcmcp ngen=5000000 printfreq=1000 samplefreq=1000 nchains=4;
Appendix II: Final Trimmed Sequences

i. 81 Final 28S sequences:

**Lionepha_erasa**

GCACTGAGCCTGGCTGCTGAAATGGCCAATGGGCAATGTAGTGTTTAGGAGTGCCCGTTTAACCCGGATATTGTTAGCGTGTCCAAGTCTCTTCTTGAAGGGGGCCACTCGCCCATAGAGGGTGCCAGGCCCGTTGTTGACCGTTAATATGTTCGGGTGGGTTTTCTCCTAGAGTCGGTTGCTTGAGAGTGCAGCTCTAAGTGGGTGGTAAACTCCATCTAAGGCTAAATATAACCACGAGACCGATAGCGAACAAGTACCGTGA

**P_amicus_BearCaveFemale1**

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**P_amicus_BearCaveFemale2**

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**P_amicus_FriendshipCaveMale1**

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**P_amicus_FriendshipCaveMale2**

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**P_amicus_FriendshipCaveFemale1**

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**P_amicus_FriendshipCaveFemale2**

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**P_amicus_FriendshipCaveMale2**

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72
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P_lacustris_EscueCaveFemale1
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ii. Final COI sequences:

P_amicus_BearCaveFemale1
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P_ciliaris_JeterRiverCave3

P_ciliaris_JeterRiverCave4

P_ciliaris_JeterRiverCave5

P_ciliaris_JeterRiverCave6

P_ciliaris_SilveyCave1
P_ciliaris_SilveyCave6

P_colemanensis_ClarksvilleLakeCave1

P_colemanensis_ClarksvilleLakeCave2

P_colemanensis_ColemanCave1

P_colemanensis_ColemanCave2
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