The Cat-eyed Snakes of Madagascar: Phylogeny and Description of a New Species of *Madagascarophis* (Serpentes: Lamprophiidae) from the Tsingy of Ankarana

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The cat-eyed snakes of the genus *Madagascarophis* are among the most commonly encountered snake species in Madagascar. Yet despite their broad distribution and frequent occurrence in human-disturbed habitat, *Madagascarophis* still contains unrecognized species diversity. Here, we describe a new species of Malagasy cat-eyed snake from a specimen found in the tsingy karst system of Ankarana in northern Madagascar. Using multiple loci from all currently described species, including the never-before-sequenced *M. ocellatus*, we delimit a new species and also determine its placement within the genus in a Bayesian coalescent framework, using BPP and *BEAST, respectively. Our results indicate that molecular data are sufficient to delimit this new taxon. These data also support its placement as the sister taxon to the recently described *M. fuchsi* which is endemic to the Montagne des Français karst massif also in northern Madagascar. We also provide a morphological description of this new snake species, which can be readily diagnosed based on external morphological characters, and include a species identification key for the entire genus based on external morphology.

WITHIN the tropical regions of the world, the discovery of squamate species new to science is still common (amphisbaenids, Teixeira et al., 2014; snakes, Ramadhan et al., 2015; and lizards, Colli et al., 2015). In particular, the isolated island of Madagascar, with 100% endemicity of colubroid snakes, regularly yields species new to science (Vieites et al., 2010; Glaw et al., 2013a, 2013b). Among Malagasy snakes, the cat-eyed snakes of the genus *Madagascarophis* are among the most commonly encountered species throughout many habitat types. All species of *Madagascarophis* are crepuscular/nocturnal, both terrestrial and semi-arboreal (Glaw and Vences, 2007), and spend their daylight hours concealed, such as in the axils of bromeliads (Lehtinen, 2002). These snakes' generalist diets include frogs, lizards, mammals, birds, and even other snakes (Bloxam et al., 1996; Andreone and Luiselli, 2000; Glaw and Vences, 2007).

Species of *Madagascarophis* are distributed across much of the island (except at elevations above 1700 m) and within a variety of habitats, including, for example, taxa such as *M. colubrinus* regularly found in anthropogenically disturbed areas (Kaloloha et al., 2011). The close association of *Madagascarophis* with human-inhabited areas may be significant from a medical standpoint; while not dangerously venomous, *M. colubrinus* is one of the few Malagasy species of opisthoglyphous snake known to cause envenomation and acute localized effects, which includes pain, swelling, blistering, and tissue necrosis (Domergue, 1989). Despite *Madagascarophis* being both common and of potential medical importance, new species of Malagasy cat-eyed snakes that are both genetically and morphologically distinct are still being discovered. Recently, Glaw et al. (2013a) described a possible microendemic restricted to Montagne des Français in northern Madagascar. This brings the number of species in the genus to a total of four: 1) the widespread *M. colubrinus*, 2) the southern/southwestern *M. meridionalis*, 3) the southwestern *M. ocellatus*, and 4) the northern microendemic from Montagne des Français, *M. fuchsi*.

Here we describe a new, possibly microendemic, species of *Madagascarophis* from the tsingy karst massif of Ankarana National Park in Antsiranana Province (Figs. 1–3). Ankarana is a limestone massif, with pinnacle-shaped karstic limestone formations (tsingy), narrow canyons, and massive underground cave networks. The natural vegetation is deciduous forest, which becomes more mesic in the bottom of canyons with water bodies. The surrounding habitat consists of relict deciduous forest and degraded grasslands with scrub. Many Malagasy reptiles are endemic to the northern region (Brown et al., 2016), with some known solely from Ankarana, including the rarely encountered snake *Alluaudina mocquardi*, the gecko *Lygodactylus expectatus*, and the chameleon *Brookesia confidens* (Glaw and Vences, 2007; Glaw et al., 2012).

During a 2014 expedition to northern Madagascar, we found a single specimen of a morphologically unusual *Madagascarophis* while surveying a tsingy rock plateau at night in Ankarana National Park. Using multiple loci, we examine species boundaries of this new Malagasy cat-eyed snake in a Bayesian coalescent framework and provide a morphological description that discriminates this new species from others in the genus. We also determine the placement of the new species within *Madagascarophis*, infer a species tree that includes all currently recognized species, and produce a taxonomic key for all species in the genus.

MATERIALS AND METHODS

Collection and morphological data.—Snakes included in this study from the 2014 expedition (Appendix 1) were euthanized using an anesthetic, fixed in 80% ethanol, and stored in 70% ethanol, generally following the protocols of Simmons (2002). Whenever possible, hemipenes were everted by injection and tied-off at their base. All vouchered specimens were assigned a RAX (C. J. Raxworthy field series)

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Fig. 1. Photos of *Madagascarophis lolo* in life; scale bar applies to lower panel of head only. Full body photo CJR, head SR.

field tag and number and deposited at the American Museum of Natural History or the University of Antananarivo. Tissue samples were taken from the liver and muscle tissue along the midbody wall from specimens immediately after death and stored in 95% ethanol, with long term-storage at –80°C. Several of the included tissue samples were from individuals collected on prior expeditions, collected under similar conditions except that the vouchers were preserved in 10%

Fig. 2. Tsingy habitat where *Madagascarophis lolo* was found in Ankarana National Park. Photos by SR.

formalin (Appendix 1). Collecting localities were recorded with GPS units, and photographs were taken of living specimens (to record natural coloration) and surveyed habitats.

We took the following measurements: snout–vent length from the tip of the snout to the anterior of the cloaca; total length from snout to tail tip; tail length from the posterior of the cloaca to tail tip; head length from snout tip to end of jaw; head width at the widest point of the head; these measurements were taken to the closet mm. Scale measurements were taken using calipers and all measurements recorded in mm, to the closest 0.1 mm. We counted ventrals following Dowling (1951). We included circumoculars as all scales surrounding and in contact with the eye and included the supraocular in this count. Bilateral scale counts, when different between left and right sides, are given as left/right.

Molecular data and phylogenetic analyses.—We included the sample of the putative new species, all currently recognized species of *Madagascarophis* ($n = 14$ individuals), and *Phisalixella tulearensis* as an outgroup ($n = 2$ individuals). For all taxa except *M. fuchsi* and the putative new species, we had at least

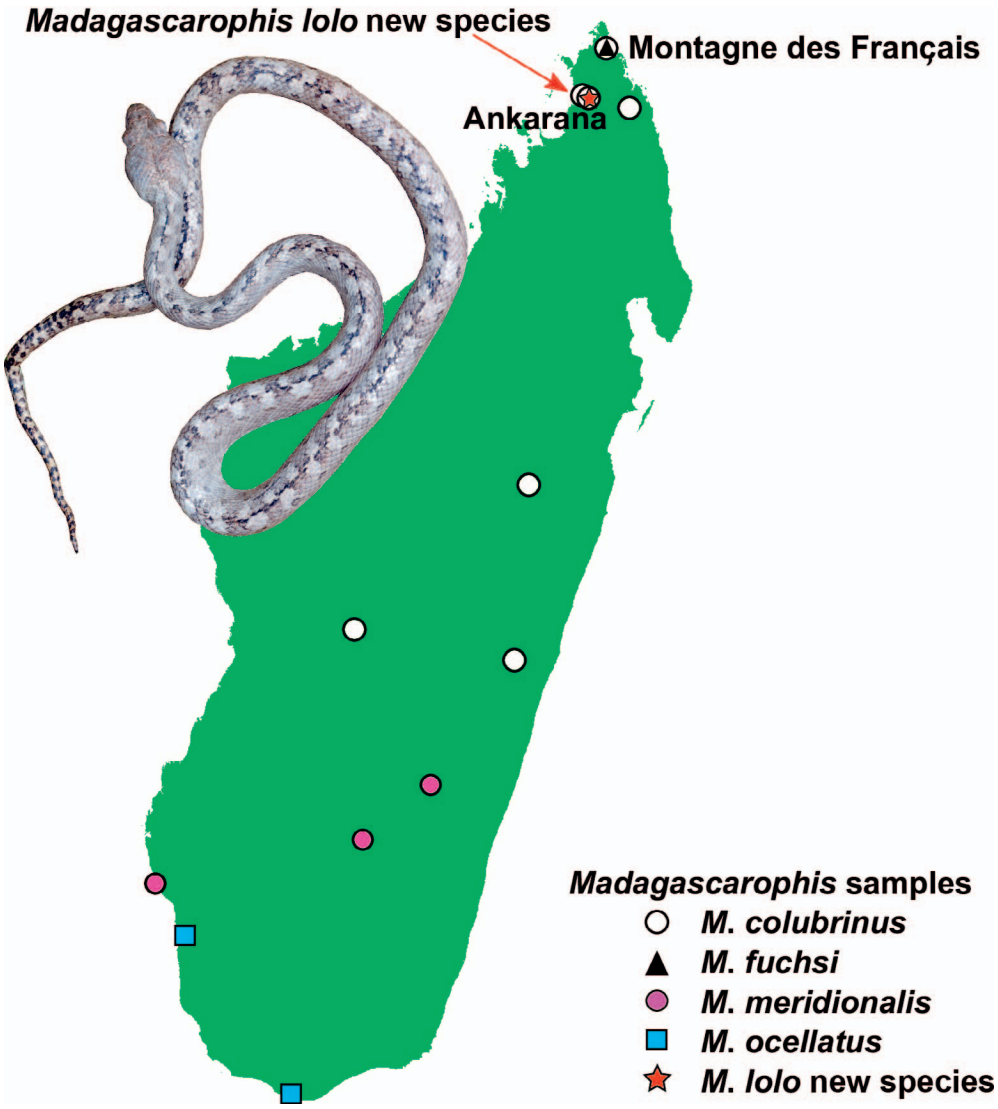

Fig. 3. Map showing localities for individuals of *Madagascarophis* included in this study. Photo of *Madagascarophis lolo* by SR.

Table 1. Genetic similarity of *Madagascarophis* by maximum percent uncorrected pairwise identity between/within species by gene; locus order is COI/Cmos/Rag2. Note that as *M. fuchsi* and *M. lolo* are represented by one individual, there is no maximum pairwise identity available for within species variation.

	M. colubrinus	*M. meridionalis*	*M. ocellatus*	*M. fuchsi*	*M. lolo*
M. colubrinus	94.1/99.8/99.5	—	—	—	—
M. meridionalis	88.0/99.6/99.7	98.9/100/100	—	—	—
M. ocellatus	87.5/98.5/99.4	86.4/98.9/99.7	99.7/99.8/99.7	—	—
M. fuchsi	87.3/99.3/99.2	85.2/99.4/99.5	87.6/99.1/99.2	N/A	—
M. lolo	86.1/99.3/99.4	85.2/99.6/99.7	87.0/99.4/99.4	90.4/99.8/99.5	N/A

two individuals per species (Fig. 3; Appendix 1). We included individuals of each species from multiple locations to capture genetic variation within species, particularly for the widely distributed *M. colubrinus* (Fig. 3), to ensure that our putatively new species is not simply an aberrant example of this widespread taxon. Additionally, we included *M. colubrinus* from the same locality as the new species, and from the same northern region of Madagascar (Fig. 3; Appendix 1). Previously named subspecies of *M. colubrinus* (see Domergue, 1987) have proven difficult to delimit using morphological data or even geographic range (Nagy et al., 2007); however, we review these subspecies with respect to locality, morphology, and genetic evidence to eliminate them as synonymous with the new taxon described in the discussion.

For these samples we extracted DNA using a Quiagen DNEasy kit, following the tissue protocol. We sequenced three loci: one mitochondrial gene (*COI*, 667 bp) and two nuclear loci (*Cmos*, 539 bp; *Rag2*, 663 bp) performed at the American Museum of Natural History on an ABI 3730 (PCR and sequencing details Appendix 2). Sequences were edited and aligned using the Geneious alignment algorithm in Geneious v.6.1.4 and checked by eye to ensure that these three protein coding loci did not contain stop codons. We confirmed the identity of all samples of the species of *Madagascarophis* (excepting *M. ocellatus*, which has never been sequenced, but which is the most morphologically distinct and easily identified) using the BLAST function from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the barcoding gene *COI*. This gene is considered the barcode gene for species, and a match for each individual with its labeled name ensured that all field identifications (especially for any *M. colubrinus* and *M. meridionalis* where the voucher was unavailable) were correct. All resulting sequences are deposited on GenBank (KU925295–KU925345) with alignments available in the Dryad Digital Repository (Ruane et al., 2016).

To delimit species of *Madagascarophis,* we used BPP v.3.1 (Yang and Rannala, 2010). BPP is a multi-species coalescent-based method that takes multiple independent loci and uses a reversible-jump Markov chain Monte Carlo (rjMCMC) to examine probability of species delimitation while using estimates of Θ_a (effective population N_e*mutation rate μ for each species), τ_a (the time of origin for each species), and τ_d (the timing of diversification into two descendent species). Support for species hypotheses were generated using posterior probability distributions (Pp), where values >95% of the distribution indicate strong support for delimiting taxa. We ran BPP using the non-fixed guide tree option (requires no guide tree). This option (joint species delimitation and species tree inference of unguided species delimitation; Yang and Rannala, 2014) attempts to collapse all combinations of the pre-assigned species into single species, but does not split any of the pre-fixed species into multiple species. We used the rjMCMC algorithm 0 and adjusted the fine-tuning parameters between 0.30 and 0.70 for each parameter, which best allows mixing of the rjMCMC among the species-delimitation models. Zhang et al. (2011) demonstrated BPP may be sensitive to the prior distributions of ancestral population size (Θ) and root age (τ_o), and so similar to previous studies (e.g., Ruane et al., 2014) we parameterized Θ and τ_o using a gamma (Γ) distribution (α, β) for a variety of parameter combinations, including large ancestral population sizes and deep divergences, Γ (1, 10); small ancestral population sizes and shallow divergences, Γ (2, 2000); large ancestral population sizes, Γ (1, 10) and shallow divergences, Γ (2, 2000); and intermediate population sizes, Γ (2, 100) with deep divergences, Γ (1, 10). For each of these population size/divergence time scenarios, we ran three separate analyses using different starting seeds, each run for 1 million generations with a burnin of 100,000 generations, and a sample frequency of every five generations.

To estimate the placement of the new species of *Madagascarophis* among congenerics, we ran a *BEAST analysis (Drummond and Rambaut, 2007; Heled and Drummond, 2010) with the species of *Madagascarophis* supported by the BPP analyses as the terminal taxa and *Phisalixella tulearensis* as the outgroup, resulting in a phylogeny for the genus. For the *BEAST analysis, we used a birth-death speciation prior, and model of sequence evolution for each locus was determined in the program JmodelTest2 (Darriba et al., 2012) by first calculating the likelihood scores among 88 different models and then using the Bayesian information criterion to determine the best-supported model of sequence evolution (*Rag2* = HKY+I, *Cmos* = HKY, *COI* = HKY+G). Each *BEAST analysis was run for 50 million generations, sampled every 1000 generations, and we assessed stationarity of the runs using Tracer v.1.5 (Rambaut and Drummond, 2009). Analyses were repeated three times to assess consistency among results.

RESULTS

Molecular data, phylogeny, and delimitation.—The three target loci amplified and sequenced successfully for all individuals (Appendix 1), and the uncorrected pairwise divergences between species of *Madagascarophis* showed substantial genetic differences between species (Table 1). One *M. colubrinus* (RAN42379) was found to have a single heterozygous site for *RAG2* and both alleles for this individual were included in the BPP and *BEAST analyses. The BLAST search verified that all individuals most closely matched previous identifications 95–100% for COI, which is within the range of variation known for mtDNA in these taxa (Nagy et al., 2007). BLAST showed a 90% match between *M. fuchsi* and the potentially new taxon.

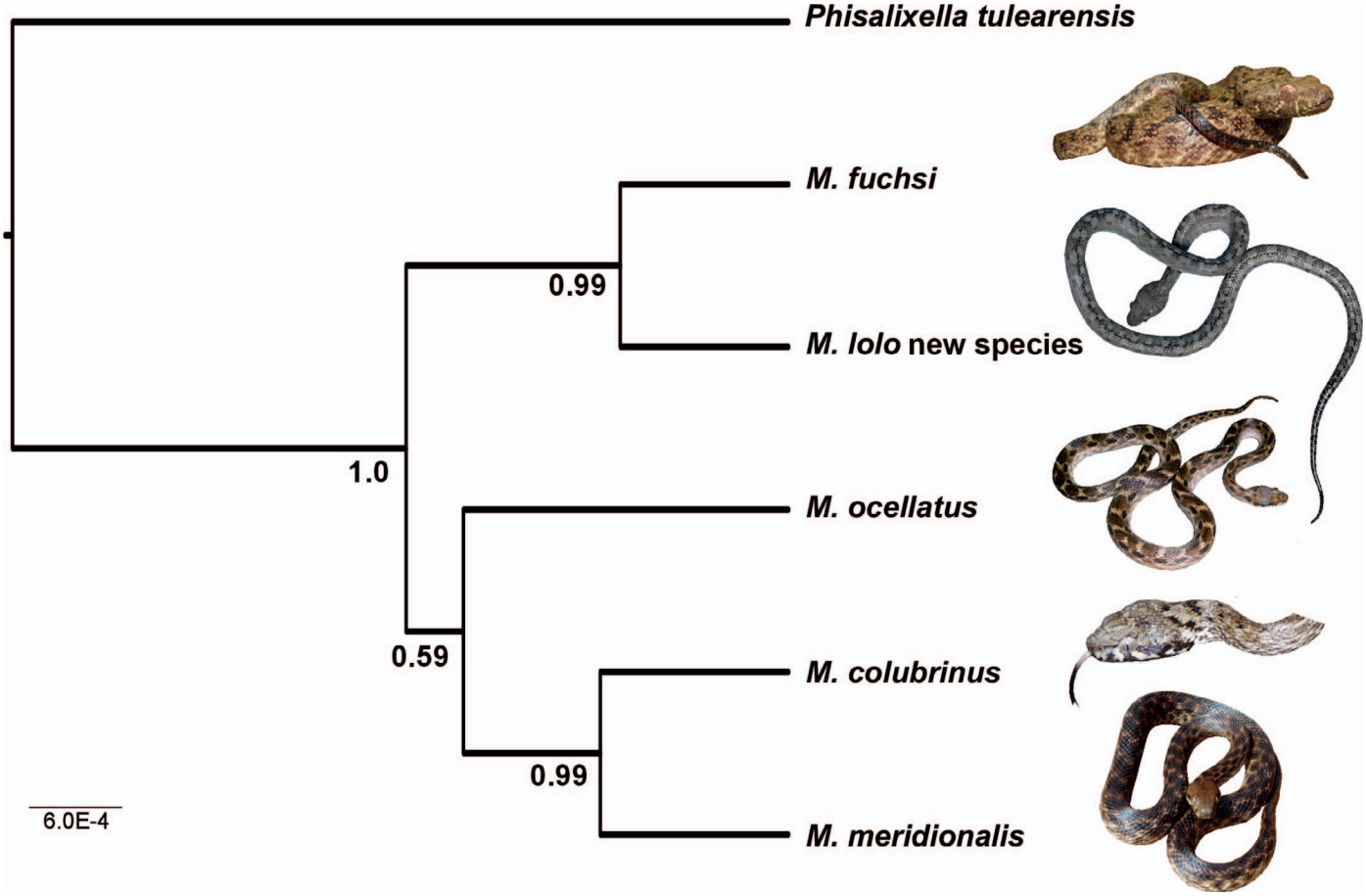

Fig. 4. Species tree from *BEAST showing the relationships of species of *Madagascarophis* with posterior probability support for each node indicated; scale bar indicates substitutions per site. Photos from top to bottom from FTB, SR, CJR, SR, SR.

Our BPP analyses delimited the putatively new *Madagascarophis* from its sister taxon, *M. fuchsi* (Fig. 4), under all ancestral population sizes and divergence times. We found that the new species is best supported as distinct from *M. fuchsi* when parameterized for small ancestral population sizes/shallow divergences (Pp = 1.0) and intermediate ancestral populations sizes/deep divergences (Pp = 0.99); it is least supported for large ancestral populations/deep divergences (Pp = 0.87) and large ancestral population sizes/shallow divergences (Pp = 0.82). For the two latter parameterizations with lower support values, the next best-supported delimitations collapsed the new taxon and *M. fuchsi* together as a single species distinct from the remaining species of *Madagascarophis* (Pp = 0.12 and 0.10, respectively). The BPP results also confirmed the validity of the other species (*M. ocellatus*, *M. meridionalis*, and *M. colubrinus*) in the genus, with high support (Pp > 0.95) for divergences for these taxa and all parameterization combinations. For each of the BPP parameterizations, all three independent runs of each analysis gave the same delimitation results. The BPP analyses also produced a species tree that even when using different parameterizations of population size and divergence time typically resulted in a sister taxon relationship between the new species and *M. fuchsi* and between *M. colubrinus* and *M. meridionalis*, which was similar to the *BEAST analysis (described below). The placement of *M. ocellatus* was not well supported for any given analysis (Pp < 0.70).

For the *BEAST analyses, the first 25% of samples were discarded as burnin after assessment in Tracer, and the effective samples sizes were >200 for all parameters. The *BEAST species tree had high support for the placement of taxa with the exception of *M. ocellatus* (Pp = 0.59) and, like the species tree generated during the species delimitation in BPP analyses, placed the new taxon as the sister species to *M. fuchsi* (Fig. 4). All runs resulted in the same tree, which we consider the species tree for the genus (Fig. 4). Examination of the individual gene trees from the *BEAST shows that that for any given locus, the new species and *M. fuchsi* have longer branches connecting them when compared to the intraspecific lengths within any of the other species of *Madagascarophis*, which might also indicate that the new taxon and *M. fuchsi* are distinct species (trees available in the Dryad Digital Repository; Ruane et al., 2016).

Madagascarophis lolo, new species

urn:lsid:zoobank.org:act:E94496B3-AC04-4FE2-AFA6-8CC7DBE3A63A

Figures 1, 2, 3, 4, 5; Table 1

Holotype.—AMNH 176422 (RAX 12626), adult male (Fig. 1), Madagascar, Antsiranana Province, Diana Region, Ankarana National Park, ~4 km northwest of the village of Mahamasina, tsingy karst trail, 102 m elevation, 49.11507°E, 12.94210°S, 9 February 2014, 1930 hours, B. Randriamahatantsoa, C. Raxworthy, S. Ruane.

Diagnosis.—A new species of *Madagascarophis* than can be diagnosed from its congeners by the following combination

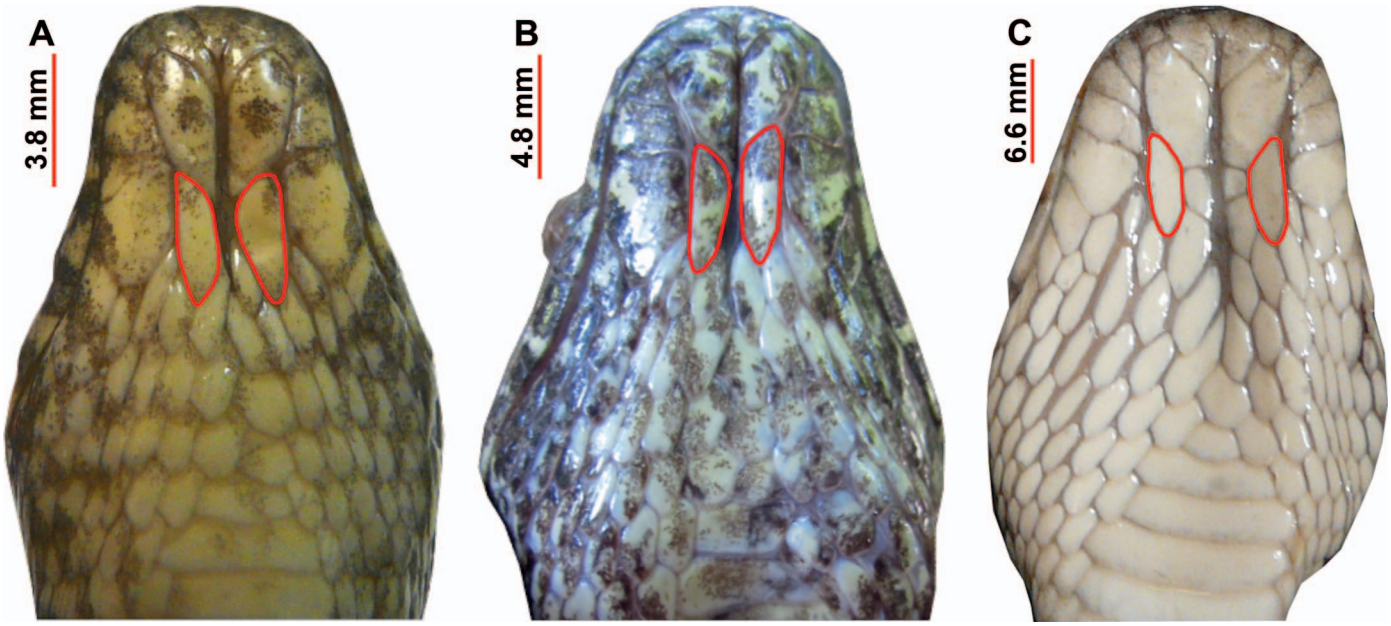

Fig. 5. Ventral head view, showing the posterior infralabial scale contact of *Madagascarophis lolo* (A; RAX12626), *M. fuchsi* (B; RAX12424), *M. colubrinus* (C; RAX10540); a scale bar is provided for each species. Photos by SR (A, C) and FTB (B).

of characters: an overall gray body color with a black vertebral stripe and alternating light gray blotches down the dorsum, 25 scale rows at midbody, 189 ventral scales and 56 divided subcaudal scales, with extended contact of the posterior inframaxillary scales. *Madagascarophis lolo,* new species, differs from all other species of *Madagascarophis* having a gray body color combined with an alternating pattern of pale gray blotches along the vertebral column and the presence of black scales on the vertebral row scales, giving the appearance of a thin black dorsal stripe (Figs. 1, 3, 4). This overall combination of color and pattern is unique among species of *Madagascarophis* (see Domergue, 1987:fig. 4 for comparison). *Madagascarophis lolo,* new species, differs from all other species with the exception of *M. fuchsi* by having extended contact of the posterior inframaxillary scales (Fig. 5). We note the specimen of *M. lolo,* new species, has a single gular scale that infringes on the posterior end of the posterior inframaxillaries. However, with the exception of *M. fuchsi*, the posterior inframaxillary contact of *M. lolo,* new species, is still much greater than for the other species of *Madagascarophis* (Fig. 5; see Glaw et al., 2013a for additional examples).

Madagascarophis lolo, new species, differs from *M. fuchsi* by having a lower number of infralabial scales (10 *M. lolo* vs. 12–13 *M. fuchsi*) and a higher ventral scale count (171–174 *M. fuchsi* vs. 189 *M. lolo*). However, this 15 ventral scale difference falls within the intraspecific range of other species (e.g., 35 ventral scales in *M. meridionalis*). It differs from the other species of *Madagascarophis* except *M. colubrinus* by having a lower ventral scale count (189 *M. lolo*): 183–209 in *M. colubrinus*, 205–224 in *M. ocellatus*, and 197–232 in *M. meridionalis*. A general difference between *M. lolo,* new species, and most other *Madagascarophis* is the dorsal scale count at midbody. *Madagascarophis lolo,* new species, has 25 dorsal scale rows as does *M. fuchsi*, in contrast to the 27–29 typically found in *M. colubrinus* (rarely 25, and not syntopically), 29–33 in *M. meridionalis*, and 29–31 in *M. ocellatus* (Glaw and Vences, 2007; Glaw et al., 2013a). It also differs genetically from all other species in the genus, e.g., *M. lolo* vs. *M. fuchsi*, *COI* uncorrected pairwise distance = 9.6% (Table 1). For specimens not examined here (Appendix 1), additional data were used from Domergue (1987) and Glaw et al. (2013a) for the diagnosis.

Description.—Adult male in excellent state of preservation, tail complete, short ventral slit midbody for DNA tissue sample, lower body slit for assessing gonad development (fully formed mature testes; 10 mm length, 2 mm width). Snout–vent length 426 mm, tail length 65 mm, tail short (13% of total body length). Head length 20 mm, width 12 mm. Head distinct from neck. Eyes large, 3 mm horizontal diameter, pupil vertically elliptical. Supralabials 8, not in contact with the eye. Infralabials 10, first pair in contact behind mental, infralabials 1–5 in contact with inframaxillaries. Rostral broader than high, 3 mm wide/1.5 mm high, visible from above. Nasal divided below nostril, in contact with 1st and 2nd supralabials. Single loreal, in contact with nasal, preoculars, prefrontal, and supralabials 2 and 3. Circumoculars 9, 1 supraocular, 2 preoculars, 3 suboculars, and 3 postoculars. Temporals 4 + 4/4 + 5. Dorsal surface of head includes pair of internasals (width 1.7 mm/length of suture 2 mm), pair of prefrontals (width 2 mm/length of suture 2.1 mm), pair of supraoculars (width 2.8 mm/length 4.8 mm), frontal longer than wide (length 5.7 mm/anterior width 2.9 mm), pair of parietals (length of suture 4.6 mm). Two pairs of inframaxillaries (anterior inframaxillary length 4.9 mm, posterior inframaxillary length 3.1 mm), posterior inframaxillaries substantially in contact with each other excepting small gular scale at posterior end (Fig. 5). Dorsal scale rows 23-25-19 at 10th ventral from anterior, midbody, and 10th ventral anterior to cloaca.

Coloration and pattern.—Dorsal ground color gray in life, alternating lighter gray blotches/squares alongside vertebral column, many vertebral row scales black in coloration, giving general appearance of black dorsal line interrupted occasionally by gray scales (Fig. 1). At roughly the posterior 1/3 of the body, dorsal scale rows 7 and 8 occasionally black, giving spotted appearance in lateral view. Overall coloration pale

gray in preservation. Color of iris in life gray/silver with gold flecking (Fig. 1), opaque gray in preservation. Dorsum of head, including rostral, internasals, prefrontals, frontal, supraoculars, and parietals gray. Supralabials 4–8 with gray and white mottling, infralabials with gray and white mottling. Slightly darker diffuse brown line runs from the posterior of eye to posterior margin of mouth. Tail gray with black mottling, darker and more contrasting compared to body, with slight flush of pale orange towards tail tip. Ventral scales cream with no pattern anteriorly, small amounts of gray flecking on ventral scales beginning at the posterior 1/3 of the body, continuing and increasing in intensity onto the subcaudal scales.

Natural history.—Similar to other species of *Madagascarophis*, *M. lolo* appears to be crepuscular/nocturnal; the specimen was found active on the ground at 1930 hours on tsingy karst rocks, in an exposed area with low scrub habitat. This is very similar to what has been described for *M. fuchsi* (Glaw et al., 2013a), and our own observation of the *M. fuchsi* sample included here, which we found outside a small cave in the karst system of the Montagne des Français massif, approximately 70 km away. By contrast, the other species of *Madagascarophis* found at Ankarana, *M. colubrinus*, was common in canyon forests and surrounding relict forests, as well as in anthropogenically disturbed habitat. We suspect the reason that *M. lolo* has gone undetected for so long at Ankarana is that the exposed tsingy plateau has been poorly surveyed at night in previous expeditions due to problems of gaining safe access to these areas. *Madagascarophis lolo* may be endemic to the karst habitats of Ankarana, and possibly Analamerana, which is the closest karst system to the east.

Etymology.—The species name, *lolo*, is taken from the Malagasy word for ghost; it is a noun in apposition to the genus name. This name refers to 1) the ghostly pale gray color of the holotype, and 2) that *M. lolo* has eluded discovery for so long at Ankarana, arguably one of the better surveyed sites in Madagascar.

DISCUSSION

High levels of microendemicity may be common for certain regions in Madagascar (Brown et al., 2016), such as Ankarana, where several reptile genera have endemic representatives (e.g., *Alluaudina*, *Brookesia*, *Lygodactylus*; Glaw and Vences, 2007). The new species of *Madagascarophis* described here is, as far as we currently know, restricted to the tsingy habitat of Ankarana. It seems unlikely that this snake has never been recorded previously, given that this particular area of Ankarana is one of the most accessible areas in the national park and a popular ecotourist destination in Madagascar. However, the trail where we collected this snake was only created in the last ten years and traverses a previously inaccessible area of exposed tsingy plateau which is otherwise difficult to access, especially at night. Because this new snake is found in a national park, and its habitat is naturally well-protected from anthropogenic degradation, we do not consider this species to be vulnerable to extinction. However more survey work is needed to establish the population size and distribution limits of this snake.

Both the morphological and genetic results indicate a close/sister taxa relationship of *M. lolo* with *M. fuchsi* (Fig. 4). With just a single specimen, more individuals are needed to describe the intraspecific variation of *M. lolo*, which is also true for the recently described *M. fuchsi*, known from only four specimens. There is the possibility that these two sister taxa represent populations of the same species; however, the genetic differentiation between *M. lolo* and *M. fuchsi* (e.g., *COI* = 9.6%; Table 1) is higher than or similar to the mtDNA divergence found among many species of snakes, e.g., *Lachesis* (Zamudio and Greene, 1997), *Naja* (Slowinski and Wüster, 2000), and *Pantherophis* (Burbrink et al., 2000), and is beyond what is found within other species of *Madagascarophis*, including the highly variable *M. colubrinus* (Table 1; Nagy et al., 2007). *Madagascarophis lolo* can also be readily identified from all other species in the genus, including *M. fuchsi*, based on morphology alone (see Key to the species of *Madagascarophis*). Our coalescent species delimitation analyses also indicate *M. lolo* is a distinct species, although this is best supported under small ancestral population sizes and shallow divergences (Pp = 100%). This scenario may be the most realistic for the *Madagascarophis*, where populations of the range-restricted *M. lolo* as well as *M. fuchsi* are likely small.

The new species described here is unique with respect to coloration. Although species of *Madagascarophis* (with the exception of *M. ocellatus*) have extremely variable intraspecific color patterns, we have not observed any species or individuals with the same coloration seen in the specimen of *M. lolo*; it possesses a pattern that appears to be well matched to the tsingy rock habitat with varying and alternating shades of gray (Figs. 1, 2). By contrast, no other species of *Madagascarophis* has a predominantly gray dorsal ground coloration—they are either brown, blackish brown, orange, or yellowish brown.

Although it is beyond the scope of this study to validate the status of previously described subspecies of *Madagascarophis colubrinus*, we discuss them here to avoid problems with synonymy with respect to *M. lolo*. In the most complete examination of the genus, Domergue (1987) recognized five subspecies of *M. colubrinus* and an additional full species, *M. citrinus* (as well as *M. meridionalis* and *M. ocellatus*). More recent work on these snakes (Nagy et al., 2007; Glaw et al., 2013a) proposed: 1) *M. meridionalis* and *M. ocellatus* remain distinct species; 2) *M. c. occidentalis* is a junior synonym of *M. c. colubrinus*; 3) *M. c. insularis* is a junior synonym of *M. citrinus*, but *M. citrinus* is a subspecies of *M. colubrinus*; 4) There is some genetic structure and corresponding morphological variation indicating that *M. c. septentrionalis* and *M. c. citrinus* are distinct from the nominant *M. c. colubrinus;* and 5) *M. fuchsi* is a distinct species that occurs sympatrically with *M. colubrinus* at Montagne des Français.

Importantly, in our genetic analyses and in the aforementioned studies, all of the subspecies of *M. colubrinus* form a separate and distinct *M. colubrinus* clade, which is the sister taxon to *M. meridionalis*. Our study, like Glaw et al. (2013a), finds *M. fuchsi* falling outside the *M. colubrinus* + *M. meridionalis* clade, with the addition that *M. lolo* is the sister taxon to *M. fuchsi*. Therefore, all taxa within *M. colubrinus* are distinct from *M. lolo* and *M. fuchsi*. One subspecies, *M. c. pastoriensis,* has not been included in any molecular phylogenetic studies (due to a lack of genetic material); however, this taxon is restricted to the Antananarivo region in central Madagascar and is characterized by having a nearly black body and yellow eyes (Domergue, 1987), which does not correspond to *M. lolo*.

The other *Madagascarophis* taxon also found at Ankarana, *M. c. septentrionalis,* is distinct from *M. lolo* in that it does not typically have 25 dorsal scale rows (rather 27, or 29 from sites further north) or contact of the posterior inframaxillaries (see

Glaw et al., 2013a). We included *M. colubrinus* (which would correspond to *M. c. septentrionalis*) from the collecting locality of *M. lolo* in our genetic analyses and demonstrate that these are not sister taxa.

This study is the first to include genetic data for *M. ocellatus* and all other described species of *Madagascarophis* in a coalescent-based species tree (Fig. 4). This tree was well supported at almost all nodes (Pp $\geq$ 0.99), and importantly, it provides information on the sister taxa relationship of the two most recently described species, *M. lolo* and *M. fuchsi*. Each of these species occurs sympatrically with the widespread *M. colubrinus* (which is the sister taxon to the more southerly distributed *M. meridionalis*), but our tree indicates that no sister species of *Madagascarophis* occur sympatrically. These findings are similar to other studies that suggest that recently diverged sister taxa are typically allopatric and that similarity in niche may limit sympatry (e.g., Peterson, 1999; McCormack et al., 2010; Pigot and Tobias, 2013). The possible karst specialization of *M. lolo* and *M. fuchsi* may allow it to occur in the same area with the more distantly related generalist *M. colubrinus*.

The exception to the generally high support values across the species tree is the placement of *M. ocellatus* as the sister taxon to *M. colubrinus* + *M. meridionalis* (Pp = 0.62). Unlike the more widespread *M. colubrinus* and *M. meridionalis*, *M. ocellatus* is poorly known and is found only in the dry regions of southwestern Madagascar. Our results suggest *M. ocellatus* is the sister taxon to *M. colubrinus* + *M. meridionalis* (Fig. 4), though we expect future studies using larger genetic datasets may be able to provide more robust support for the phylogenetic placement of *M. ocellatus*.

Finally, this study demonstrates that snake species new to science are likely waiting to be discovered in Madagascar, even among commonly encountered taxa, and we expect there are still high numbers of endemic, undescribed squamates in Madagascar. As many of the currently recognized squamate species in Madagascar have very large ranges (e.g., *Geckolepis maculata*, *Zonosaurus madagascariensis*, *Phelsuma lineata*; Glaw and Vences, 2007), phylogeographic studies using modern genomic techniques in an integrative context with morphology and ecology will likely discover additional taxa. Future work in Madagascar and other tropical regions worldwide should focus not only on the discovery of obvious, morphologically differentiated species, but also consider widespread taxa with potential cryptic diversity as well.

KEY TO THE SPECIES OF *MADAGASCAROPHIS*

Information is based on samples in Appendix 1 and Glaw et al. (2013).

1a. Dorsal pattern consists of distinct brown/black ocellae, eye color red ..... ***Madagascarophis ocellatus***
1b. Dorsal pattern consists of anything other than brown/black ocellae, eye color not red 2
2a. Extended contact of the posterior inframaxillary (genial) scales, 25 dorsal scale rows 3
2b. Posterior inframaxillary scales not in contact, 25 or more dorsal scale rows 4
3a. Ventral scales ~171–174, infralabials 12 or 13, body color variable ..... ***Madagascarophis fuchsi***
3b. Ventral scales ~189, infralabials 10, gray body color with a thin black dorsal stripe ..... ***Madagascarophis lolo*, new species**
4a. Dorsal scale rows usually $\geq$ 29, ventral scale count 187–232 ..... ***Madagascarophis meridionalis***
4b. Dorsal scale rows $\leq$ 29, ventral scale count 180–209 ..... ***Madagascarophis colubrinus***

DATA ACCESSIBILITY

Data associated with this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.n10d7.

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Appendix 1. Information for genetic samples used in this study; both field numbers and associated museum numbers are included when available (tissue sample only/not yet vouchered individuals have only field numbers). Institutional abbreviations follow Sabaj Pérez (2014), with the addition of ELO (collection of E. Courtois), RAN (collection of R. A. Nussbaum, University of Michigan), RAX (collection of C. J. Raxworthy, American Museum of Natural History), and TJP (collection of T. Papenfuss). * Collected on the 2014 Madagascar Expedition.

Field number	Museum catalog number	Genus	Species	Latitude	Longitude
RAX10540	AMNH162786	*Madagascarophis*	*colubrinus*	−20.0667	48.2500
RAX9507	AMNH160089	*Madagascarophis*	*colubrinus*	−19.6898	46.1159
RAX10941	UADBA pending	*Madagascarophis*	*colubrinus*	−17.8539	48.4171
RAN42379	UMMZ209609	*Madagascarophis*	*colubrinus*	−13.0833	49.6667
RAX12636*	Tissue only	*Madagascarophis*	*colubrinus*	−12.9656	49.1386
RAX12613*	Tissue only	*Madagascarophis*	*colubrinus*	−12.9311	49.0559
RAX12427*	Tissue only	*Madagascarophis*	*colubrinus*	−12.3343	49.3582
RAX12424*	AMNH176425	*Madagascarophis*	*fuchsi*	−12.3343	49.3582
RAN65023	UADBA pending	*Madagascarophis*	*meridionalis*	−22.8671	43.3955
RAN65046	UADBA pending	*Madagascarophis*	*meridionalis*	−22.8671	43.3955
TJP28179	MVZ238848	*Madagascarophis*	*meridionalis*	−22.3402	46.2222
ELO011	Tissue only	*Madagascarophis*	*meridionalis*	−21.6466	47.1415
RAX8426	AMNH160077	*Madagascarophis*	*ocellatus*	−25.5275	45.1206
RAN48383	UMMZ218364	*Madagascarophis*	*ocellatus*	−23.4916	43.7623
RAX12626*	AMNH176422	*Madagascarophis*	*lolo,* new species	−12.9421	49.1150
RAX9843	AMNH162836	*Phisalixella*	*tulearensis*	−16.3741	47.0522
RAX9884	AMNH162837	*Phisalixella*	*tulearensis*	−16.1202	46.9521

Appendix 2. PCR and sequencing protocols. All PCR reactions were 10 μL reactions consisting of 5 μL of GoTaq® Green Master Mix, 3 μL of H_2O, 0.5 μL each of forward and reverse primers at a 10 μM concentration, and 1 μL of DNA extract. Samples were incubated at 96°C for 15 min initially, 96°C for 45 s, followed by 45 s at the appropriate temperature for the primer pair (see below), with a 72°C extension period for 1 min. This procedure, minus the initial 15 min incubation period, was repeated for 35 cycles. Reactions were cleaned with 2 μL of ExoSap-IT® following the Exosap-IT® protocol. Sequencing reactions used the same primers as for the PCR reactions. We used the BigDye® Terminator v3.1 Cycle Sequencing Kit; each sequencing reaction consisted of 0.2 μL of ABI BigDye® Terminator Ready Reaction Mix, 1.5 μL of ABI BigDye® 5X Sequencing Buffer, 4.3 μL of H_2O, 1 μL of the cleaned PCR reaction template, and 0.5 μL of the 10 μM primer for each direction. Sequencing reactions were incubated initially at 96°C for 1 minute, followed by 96°C for 10 s, 50°C for 5 s, and 60°C for 1 min 15 s and repeated for 15 cycles, minus the initial 1 min incubation period; the reaction was then incubated at 96°C for 10 s, 50°C for 5 s, and 60°C for 1 min 30 s and repeated for 6 cycles; the reaction was then incubated at 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min and repeated for 5 cycles. Sequencing reactions were cleaned prior to sequencing using ethanol precipitation.

Locus	PCR temp.	Primer		Reference
Cmos	48.0°C	S77	5′–CAT GGA CTG GGA TCA GTT ATG–3′	Lawson et al., 2005
		S78	5′–CCT TGG GTG TGA TTT TCT CAC CT–3′	Lawson et al., 2005
COI	48.0°C	RepCOI-F	5′–TNT TMT CAA CNA ACC ACA AAG A–3′	Nagy et al., 2012
		RepCOI-R	5′–ACT TCT GGR TGK CCA AAR AAT CA–3′	Nagy et al., 2012
Rag2	48.0°C	L562	5′–CCT RAD GCC AGA TAT GGY CAT AC–3′	Vidal and Hedges, 2005
		H1306	5′–GHG AAY TCC TCT GAR TCT TC–3′	Vidal and Hedges, 2005