



TITLE:

Phylogenetic placement and diet of homalopsid snake *Miralia alternans* (Ruess, 1833)

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Letter to the editor

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Phylogenetic placement and diet of homalopsid snake *Miralia alternans* (Ruess, 1833)

DEAR EDITOR,

Miralia alternans (Ruess, 1833) is distributed in Borneo, Sumatra, and Java in Southeast Asia. The species is morphologically similar to *Raclinia indica*, a monotypic genus known from Peninsular Malaysia. Here, we collected a juvenile specimen of *M. alternans* from Borneo, and report on its coloration in life and first prey item recovered from the species. We also explored the phylogenetic position of the genus using molecular phylogenetic analysis of mitochondrial DNA (mtDNA) gene cytochrome *b* (*cyt b*) and nuclear gene prolactin receptor (*PRLR*), confirming *M. alternans* as sister to *R. indica*, with the two genera exhibiting relatively high genetic divergence in *cyt b* (13.0%–13.1%).

Mud snakes (Homalopsidae), which include 56 species in 29 genera, are primarily distributed in Southeast Asia (Uetz et al., 2022). *Enhydryis* Sonnini & Latreille, 1802, the formerly largest genus in the family, was divided into 15 genera by Murphy & Voris (2014). While several recent studies have inferred the phylogenetic relationships within the family, the positions of certain genera were not investigated, as half of the genera are only known from museum specimens (Bernstein et al., 2021). The monotypic genus *Miralia* Gray, 1842 was previously considered a synonym of *Enhydryis* (e.g., Gyi, 1970), but later resurrected by Murphy & Voris (2014). Several studies have identified morphological similarities between *Miralia alternans* and *Raclinia indica*, suggesting they may be closely related (Gyi, 1970). However, molecular analysis has not yet been conducted due to the lack of *M. alternans* tissues (Murphy, 2007; Quah et al., 2018). In 2010, we collected a juvenile *M. alternans* snake in Kuching, Sarawak, Malaysian Borneo, with tissue from this specimen used to clarify its phylogenetic position.

The *M. alternans* specimen was collected during a field

survey at the Matang Wildlife Centre, Kuching, Sarawak, Malaysia, in August 2010. Tissue samples were taken for genetic analyses and deposited with the voucher specimen in the Sarawak Research Collection, Sarawak Forest Department (SRC). In addition to the new specimen, we examined the morphological characters of previously deposited specimens in the Museum Zoologicum Bogoriense (MZB), Sarawak Museum (SM), and Zoological Reference Collection of the Lee Kong Chian Natural History Museum at the National University of Singapore (ZRC). Scale terminology and measurements followed Murphy & Voris (2014) and Quah et al. (2018).

For molecular analyses, DNA was extracted and fragments of the mitochondrial gene *cyt b* (1 053 bp) and nuclear gene *PRLR* (573 bp) were amplified by polymerase chain reaction (PCR). The PCR products were sequenced with PCR primers and BigDye v3.1 using Sanger sequencing methods, and the obtained sequences were deposited in GenBank under accession numbers LC667473 (for *cyt b*) and LC667474 (for *PRLR*). In addition to the newly sequenced data for *M. alternans*, we used Homalopsidae sequencing data from Bernstein et al. (2021) to identify the phylogenetic position of *M. alternans* among other homalopsid genera (Supplementary Table S1). Maximum-likelihood (ML) and Bayesian inference (BI) methods were used to conduct phylogenetic analyses. Uncorrected *P*-distances for the *cyt b* region among sequences were calculated. Details are provided in the Supplementary Materials and Methods.

The specimen (SRC 00064) was collected on 30 August 2010 in lowland primary forest at the Matang Wildlife Centre, Kuching, Sarawak, Malaysia. The specimen was found at 1930h under leaf litter, close to a small, shallow, sandy-

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bottomed stream (1–2 m wide). To date, only three specimens have been reported from Borneo (Gyi, 1970; Murphy, 2007; Murphy & Voris 2014). Thus, the new specimen represents the fourth record of the species from Borneo.

Scale counts of *M. alternans* showed variation in supralabials 7–8, with 3rd–4th, 4th, or 4th–5th bordering orbit, supralabial formula 2-2-3, 3-1-3, 3-2-2, 3-1-4, or 3-2-3, 1st or 2nd supralabials contacting nasal cleft, infralabials 8–11, preoculars 0–1 (when no preocular prefrontals touching

anterior side of orbit), postoculars 1–2, mid dorsal scale rows 19–20, ventrals 127–164 in males and 120–152 in females, and subcaudals 28–39 in males and 23–36 in females (Supplementary Tables S2, S3). In life, dorsum glossy dark purple brown with narrow, transverse orange bands about one dorsal scale in length; first band on occiput lighter in color than other bands, about three dorsal scales in length; venter glossy dark purple brown, with yellow white bands about two ventrals in length, most interrupted by dark areas on midline

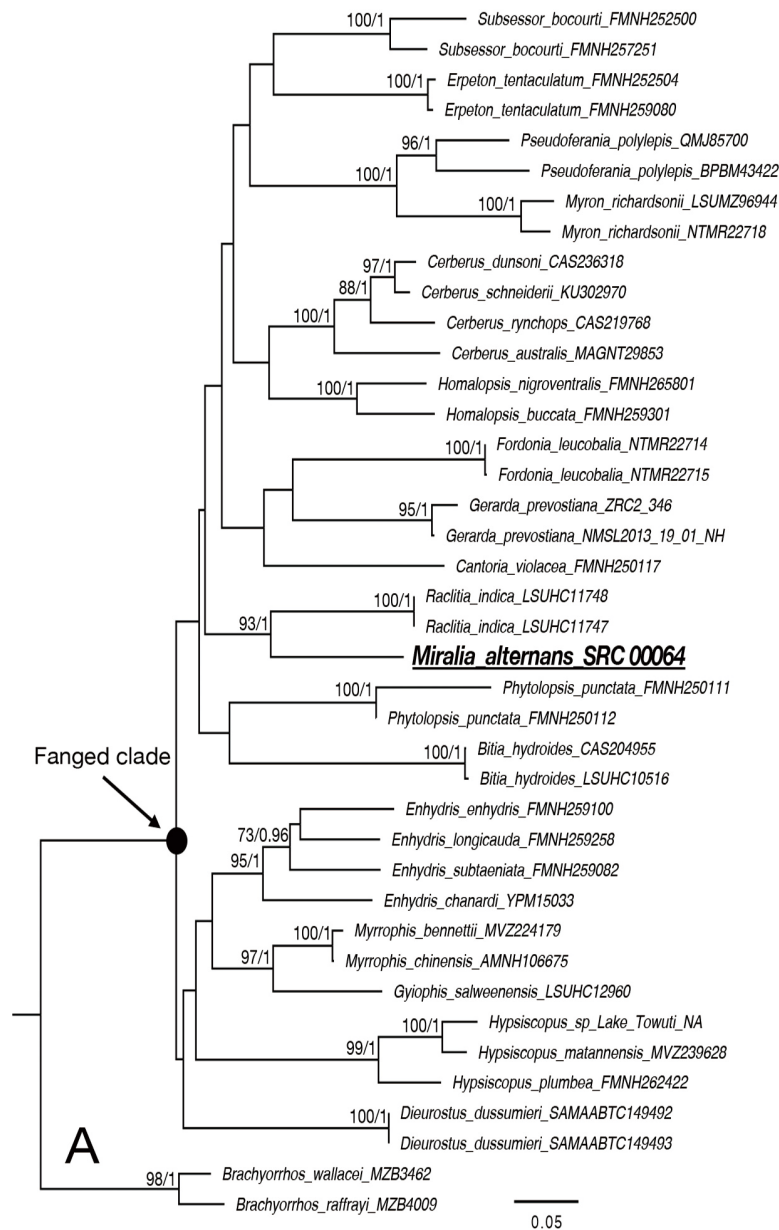


Figure 1 Phylogenetic relationships of Homalopsidae and *Miralia alternans* specimen

A: Maximum-likelihood tree based on mitochondrial gene *cyt b* and nuclear gene *PRLR* for samples of Homalopsidae. Numbers above branches represent bootstrap supports (not shown if below 70%) for ML (left) and posterior probabilities (not shown if below 0.95) for BI (right). B–D: Lateral profile of head (B), dorsal (C), and ventral (D) aspects of *Miralia alternans* specimen (SRC 00064). Scale bar: 10 mm (B) and 50 mm (C, D). Photos by K. Nishikawa.

(Figure 1B, C, D). Color pattern was generally similar in all preserved specimens, but dorsal transverse bands were less clear and first band on occiput was shorter in adult specimens than in juvenile specimens.

The phylogenetic tree topologies using ML and BI were identical, except that the basal lineage relationships were not strongly supported in both analyses. Therefore, we only present the ML tree (Figure 1A). Analysis indicated that *M. alternans* was nested within the fanged clade of Homalopsidae, although clade monophyly was not well supported. The sister relationship between *M. alternans* and *R. indica* was strongly supported, with an uncorrected *P*-distance of 13.0%–13.1% in *cyt b* between the two species (Supplementary Table S4).

One examined specimen (MZB Ophi. 4877) was fixed as it was in the process of regurgitating an Asian swamp eel (*Monopterus cf. albus*) (Supplementary Figure S1).

The present study revealed huge morphological variation within *M. alternans* (Supplementary Tables S2, S3), indicating that the species may include multiple cryptic species. We only used samples from Sarawak and Java for morphological examination and from Sarawak for molecular analysis. Thus, taxonomic assessment of the species using wider morphological and phylogenetic sampling is needed.

Phylogenetic analysis indicated and confirmed a sister relationship between *M. alternans* and *R. indica*, as inferred in previous studies based on morphology (Gyi, 1970; Murphy et al., 2011). Although *M. alternans* and *R. indica* are morphologically similar, their genetic distance based on the mitochondrial *cyt b* gene was relatively high (13.0%–13.1%). Maximum interspecific genetic distances in *cyt b* within a genus are about 9%–12% and minimum intergeneric genetic distances within a family are about 10%–14% (Supplementary Table S4), comparable to the genetic divergence between *M. alternans* and *R. indica*.

Gray (Gray, 1849) suggested the possibility that *M. alternans* may be a variety of *R. indica*. Although the two species are morphologically similar, the number of ventrals generally differs (120–164 in *M. alternans* vs. 152–175 in *R. indica*), and the two species are deeply genetically divergent. Thus, we confirmed distinct species status for both species based on molecular and morphological differences.

However, the generic status of the two species may be reconsidered, as we could not find morphological differences between *Miralia* and *Raclitia* comparable to those of different genera. Both these genera were described in Gray (1842), and thus *Miralia* could be treated as a junior synonym of *Raclitia* or vice versa. To determine their generic treatment, additional morphological data, such as maxillary teeth and hemipenial morphology, as well as genetic material, especially topotypic *M. alternans* from Java, are crucial.

Murphy et al. (2011) suggested that *M. alternans* and *R. indica* may be fossorial and vermivorous. We found *Monopterus cf. albus* (Supplementary Figure S1) as a food item of *M. alternans* for the first time, and one specimen (SRC 00064) was collected very close to a stream. Therefore, we can infer that *M. alternans* is at least partly aquatic and feeds

on fish. However, more information on its natural history is required to reveal the dietary and behavioral habits of this rare species.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Field surveys and specimen collection were approved by the State Government of Sarawak (Research Permit No. NPW.907.4.2(III)-68).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

I.F., T.K., and K.E. conceived and designed the study. I.F. performed the experiments, measured the specimens, and wrote the manuscript. M.M., K.N., and K.E. revised the manuscript. M.Y.H. arranged the field survey. M.Y.H., M.M., K.N., and K.E. collected the specimens in the field. All authors read and approved the final version of the manuscript.

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Supplementary Materials

Materials and Methods

Morphology: All measurements were taken using a ruler to the closest 1 mm. Scale terminology and measurements were adopted from Murphy & Voris (2014) and Quah et al. (2018), and included snout-vent length (SVL), tail length, total length, number of supralabials, number of supralabials contacting eye, number of supralabials contacting nasal cleft, number of infralabials, number of preoculars, number of postoculars, number of supraoculars, dorsal scale rows (at neck/mid-body/before vent), number of ventrals, number of subcaudals, and cloacal plate single or divided. Values for paired head characters are given in left/right order. Sex was determined by dissecting or observing ventral tail base. Information on morphological characters of *M. alternans* and *R. indica* was obtained from Bergman (1960), Boulenger (1896), Gyi (1970), Iskandar (1987), Murphy (2007), and Quah et al. (2018) for comparisons.

Phylogenetic analysis: For molecular analyses, DNA was extracted from tissues preserved in 99% ethanol using a Qiagen DNeasy Blood and Tissue Kit (Valencia, CA, USA), following the manufacturer's protocols. Sequences of the mtDNA fragment of *cyt b* and nuclear DNA (nuDNA) fragment of PRLR were amplified by polymerase chain reaction (PCR) using primers Glu-5' eeg (5'-TGATATGAAAACCACCGTTG-3') (Suzuki & Hikida, 2011) and H16064 (5'-CTTTGGTTTACAAGAACAATGCTTTA-3') (Burbrink et al., 2000) for *cyt b*, and PRLR_f1 (5'-GACARYGARGACCAGCAACTRATGCC-3') and PRLR_r3 (5'-GACYTTGTGRACCTTCYACRTAATCCAT-3') (Townsend et al., 2008) for PRLR. The PCR products were sequenced with the PCR primers and BigDye v3.1 by Sanger sequencing methods, and the obtained sequences were deposited in GenBank under accession numbers LC667473 (for *cyt b*) and LC667474 (for PRLR). In addition to the newly sequenced data for *M. alternans*, we used the Homalopsidae sequencing data in Bernstein et al. (2021) to identify the phylogenetic position of *M. alternans* among other homalopsid genera (Supplementary Table S1). Sequences were aligned using the MAFFT program v7.490 (Katoh et al., 2005) with default settings. We used maximum-likelihood (ML) and Bayesian inference (BI) methods to conduct phylogenetic analyses. The most suitable substitution model was selected using the ModelTest-NG program v0.1.7 (Darriba et al., 2020) based on Bayesian information criteria (BIC). ML analysis was conducted using IQ-TREE v1.6.12 (Nguyen et al., 2015). Support values of internal branches for the ML tree were evaluated based on 1 000 bootstrap replicates. The BI tree was generated based on 10 million generations of Markov chain Monte Carlo (MCMC) iterations using MrBayes v3.2.6 (Ronquist et al., 2012). The first 25% of generations were discarded as burn-in, and we sampled one per 100 remaining generations. Convergence of the MCMC runs was verified using TRACER v1.6 (Rambaut et al., 2014). Robustness of the BI tree topology was assessed using Bayesian posterior probabilities. Uncorrected pairwise distances (*p*-distances) for *cyt b* among sequences were calculated using MEGA X (Kumar et al., 2018).

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Supplementary Figure S1 Specimen of *Miralia alternans* (MZB Ophi. 4877) regurgitating an Asian swamp eel (*Monopterus cf. albus*).

Supplementary Table S1. Samples used for molecular analysis in this study together with the information on voucher specimens, collection locality, and GenBank accession numbers.

Ingroup	Voucher Number	Locality	Source	cyt b	PRLR
<i>Miralia alternans</i>	SRC: 00064	Malaysia, Sarawak, Kuching	This Study	LC667473	LC667474
<i>Bitia hydroides</i>	CAS: 204955	Myanmar, Ayeyarwady Division, vic Mwe Hauk Village	Alfaro et al., 2008	EF395896	NA
<i>Bitia hydroides</i>	LSUHC: 10516	Malaysia, Penang, Pulau Penang, Monkey Beach	Bernstein et al., 2021	MT802645	MT807938
<i>Brachyorrhos raffrayi</i>	MZB: 4009	Indonesia, Ternate, Desa Maliaro	Murphy et al., 2012a	JX139713	NA
<i>Brachyorrhos wallacei</i>	MZB: 3462	Indonesia, Halmahera, Jailolo	Murphy et al., 2012a	JX139715	NA
<i>Cantoria violacea</i>	FMNH: 250117	Thailand, Phuket Prov	Alfaro et al., 2008	EF395897	NA
<i>Cerberus australis</i>	MAGNT: 29853	Australia, Darwin, Buffalo Creek	Bernstein et al., 2021	MT802647	MT807940
<i>Cerberus dunsoni</i>	CAS: 236318	Palau Islands, Babeldaob Island	Bernstein et al., 2021	MT802648	MT807941
<i>Cerberus rynchops</i>	CAS: 219768	Myanmar, Ayeyarwady Division	Bernstein et al., 2021	MT802657	MT807949
<i>Cerberus schneiderii</i>	KU: 302970	Philippines, Cebu Province	Bernstein et al., 2021	MT802660	MT807952
<i>Dieurostus dussumieri</i>	SAMA: ABTC 149492	India, Kerala	Kumar et al., 2012	JX463014	NA
<i>Dieurostus dussumieri</i>	SAMA: ABTC 149493	India, Kerala	Kumar et al., 2012	JX463015	NA
<i>Enhydris chanardi</i>	YPM15033	No Locality, Pet Trade	Bernstein et al., 2021	MT802679	MT807968
<i>Enhydris enhydris</i>	FMNH: 259100	Vietnam, Kien Giang Province	Bernstein et al., 2021	MT802681	MT807970
<i>Enhydris longicauda</i>	FMNH: 259258	Cambodia, Kampong Chhnang Province	Bernstein et al., 2021	MT802689	MT807975
<i>Enhydris subtaeniata</i>	FMNH: 259082	Vietnam, Kien Giang Province	Bernstein et al., 2021	MT802692	MT807977
<i>Eripton tentaculatum</i>	FMNH: 252504	Thailand, Phattalung Province	Bernstein et al., 2021	MT802699	MT807984
<i>Eripton tentaculatum</i>	FMNH: 259080	Vietnam, Kien Giang	Bernstein et al., 2021	MT802698	MT807983
<i>Fordonia leucobalia</i>	NTM R: 22714	Australia, Northern Territory, Darwin	Alfaro et al., 2008	EF395914	NA
<i>Fordonia leucobalia</i>	NTM R: 22715	Australia, Northern Territory, Darwin	Alfaro et al., 2008	EF395915	NA
<i>Gerarda prevostiana</i>	NMSL: 2013.19.01.NH	Sri Lanka, Mannar	Ukuwela et al., 2017	KY206891	NA
<i>Gerarda prevostiana</i>	ZRC2.346	Singapore, Lim Chu Kang mangroves	Alfaro et al., 2008	EF395916	NA
<i>Gyioptis salweenensis</i>	LSUHC: 12960	Myanmar, Kayin State	Bernstein et al., 2021	MT802702	MT807985
<i>Homalopsis buccata</i>	FMNH: 259301	Cambodia, Koh Kong Province	Bernstein et al., 2021	MT802703	MT807986
<i>Homalopsis nigroventralis</i>	FMNH: 265801	Thailand, Nong Khai	Bernstein et al., 2021	MT802709	MT807992
<i>Hypsiscopus matanensis</i>	MVZ: 239628	Indonesia, Sulawesi, South Sulawesi	Bernstein et al., 2021	MT802713	MT807996
<i>Hypsiscopus plumbea</i>	FMNH: 262422	Thailand, Phu Province	Karns et al., 2010	GU997208	GU997224
<i>Hypsiscopus</i> sp.-Lake-Towuti	NA	Indonesia, Sulawesi, South Sulawesi, Lake Towuti	Alfaro et al., 2008	EF395919	NA
<i>Myron richardsonii</i>	LSUMZ: 96944	Papua New Guinea, Daru Island	Bernstein et al., 2021	MT802721	MT808001
<i>Myron richardsonii</i>	NTM R: 22718	Australia, Northern Territory, Darwin	Alfaro et al., 2008	EF395918	NA
<i>Myriophis bennetti</i>	MVZ: 224179	Vietnam, Vinh Phuc Province	Bernstein et al., 2021	MT802722	MT808003
<i>Myriophis chinensis</i>	AMNH: 106675	Vietnam	Bernstein et al., 2021	MT802726	MT808005
<i>Phytolopsis punctata</i>	FMNH: 250111	Malaysia, Selangor	Bernstein et al., 2021	MT802728	NA
<i>Phytolopsis punctata</i>	FMNH: 250112	Malaysia, Selangor	Bernstein et al., 2021	MT802729	MT808008
<i>Pseudoberania polylepis</i>	BPBM: 43422	Indonesia, Papua Province, Fakfak Division	Bernstein et al., 2021	MT802730	MT808009
<i>Pseudoberania polylepis</i>	QM: J85700	Australia, Queensland, Mt. Isa	Bernstein et al., 2021	MT802732	MT808011
<i>Radlitia indca</i>	LSUHC: 11747	Malaysia, Pahang	Bernstein et al., 2021	MH981308	NA
<i>Radlitia indca</i>	LSUHC: 11748	Malaysia, Pahang	Bernstein et al., 2021	MH981307	MT808012
<i>Subsessor bocourti</i>	FMNH: 252500	Thailand	Alfaro et al., 2008	EF395902	NA
<i>Subsessor bocourti</i>	FMNH: 257251	Cambodia, Siem Reap Province	Bernstein et al., 2021	MT802733	MT808013
Outgroup					
<i>Laticauda laticaudata</i>	AM: EBU13932	unknown	Lukoschek and Avise (Unpublished)	FJ587153	FJ587126

Supplementary Table S2. Morphology of *Miralia alternans* and *Raclinia indica*. *:Data from this study and previous studies (See Supplementary Materials and Methods.). +: Data from Quah et al. (2018).

Characters	<i>Miralia alternans</i> *	<i>Raclinia indica</i> +
Tail length/SVL	Male: 0.14–0.17, Female: 0.09–0.11	Male: 0.14, Female: -
Supralabials	7 or 8	7 or 8
Supralabials contacting the eye	4th or 4–5th	4th
Supralabials contacting the nasal cleft	1st or 2nd	1st
Infralabials	8–11	8 or 9
Preoculars	0 or 1	0 or 1
Postoculars	1 or 2	1
Supraoculars	1	1
Dorsal scales rows	19/19–20/17–19	19/19–20/17–19
Ventrals (Male)	127–164	152–173
Ventrals (Female)	120–152	162–175
Subcaudals (Male)	28–39	34–36
Subcaudals (Female)	23–36	28–34
Cloacal plate	Divided	Divided

Supplementary Table S3. Morphological information for the specimens of *Miralia alternans* examined. Voucher abbreviations: MZB = Museum Zoologicum Bogoriense; SM = Sarawak Museum; SRC = Sarawak Research Collection, Sarawak Forest Department; ZRC = Zoological Reference Collection of the Lee Kong Chian Natural History Museum at The National University of Singapore.

Characters	SRC 00064	ZRC 2.3523	SM no number	MZB Oph. 583	MZB Oph. 1744	MZB Oph. 1746	MZB Oph. 4877	MZB Oph. 1805-1	MZB Oph. 1805-2
locality	Kuching, Sarawak	Lundu, Sarawak	Kuching, Sarawak	Sukabumi, West Java	Java	Java	Java	Java	Java
Sex	Male	Male	Female	Female	Male	Male	Female	Female	Male
	154	139	549	404	402	306	407	393	512
Tail length	22.5	19.9	51	41	68	45	40	39	76
Tail length/Total length	0.13	0.13	0.09	0.09	0.14	0.13	0.09	0.09	0.13
Supralabials	8/8	7/7	7/7	8/8	8/8	8/8	7/7	8/8	7/8
Supralabials contacting the eye	4–5th/4–5th	4–5th/4–5th	4th/3–4th	4th/4th	4th/4th	4th/4th	4th/4th	4th/4th	4th/4th
Supralabials contacting the nasal cleft	2nd/1st	2nd/1st	1st/1st	1st/1st	1st/1st	-	1st/1st	1st/1st	1st/1st
Infralabials	10/11	10/10	9/9	9/9	9/9	9/9	8/8	9/9	9/9
Preoculars	1/1	1/1	no/no	1/1	1/1	1/1	1/1	1/1	1/1
Postoculars	2/2	2/2	2/2	1/1	2/2	2/2	2/2	1/2	2/2
Supraoculars	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Dorsal scales rows	19/19/17	19/19/17	19/19/18	19/19/18	19/19/17	19/19/19	19/19/17	19/19/17	-/19/17
Ventrals	137	140	-	137	134	136	133	132	132
Subcaudals	35	37	29	26	36	32	25	24	34
Cloacal plate	Divided	Divided	Divided	Divided	Divided	Divided	Divided	Divided	Divided

