The occurrence of the strongyloid nematodes, Kalicephalus viparae viparae (Nematoda: Diaphanocephalidae), in viper Snake, Macrovipera lebetina (Reptilia: Viperidae), Southwestern Iran

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The occurrence of the strongylid nematodes, *Kalicephalus viparae viparae* (Nematoda: Diaphanocephalidae), in viper Snake, *Macrovipera lebetina* (Reptilia: Viperidae), Southwestern Iran

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Author Contributions

All authors contributed to the study conception and design. Methodology (Material preparation, data collection and analysis) were performed by Sara Larki, Alireza Alborzi, Kia Bahramnejad, Zeinab Asadi; Molecular study were performed by Sara Larki and Zeinab Asadi; The first draft of the manuscript was written by Sara Larki and Kia Bahramnejad; All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript including Sara Larki, Alireza Alborzi, Kia Bahramnejad, Zeinab Asadi.
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Abstract

An ancylostomatid *Kalicephalus* spp. is the common parasitic intestinal nematode of reptiles. West-Asian blunt-nosed viper, a venomous snake found in extensive areas of Iran. Between June - September 2017, two dead viper snakes were referred to the parasitology laboratory and examined for intestinal parasites. Several white elongated roundworms were collected and fixed to identify under light and scanning electron microscopes (SEM) based on morphological and molecular characteristics. For the molecular survey, some parts of identified worms were extracted and the ITS of nuclear ribosomal DNA (rDNA) amplified by PCR reaction. All collected hookworms were taxonomically identified as *Kalicephalus viperae viperae* and exposited in an individual branch far from of Ancylostomatidae species and close to *A. braziliense* with 88% discrepancies in the phylogenetic tree. The morphological characteristics and a large part of *K. viperea viperea* rDNA nucleotide sequence was reported in viper snakes for the first time in the world and Iran.

Keywords: *Kalicephalus viparae*; viper snake; *Macrovipera lebetina*; Iran

Introduction

*Kalicephalus*, the common hookworm-like nematode parasite usually seen in the small intestine and sometimes esophagus snake and rarely lizard species (Anderson 2000; Goldberg et al. 2002). Although this strongylid nematode belongs to the superfamily Diaphanocephaioidea, some morphological characteristics, development, and life cycle is quite similar to that of the hookworms of Ancylostomatidea and is located in a separate branch of hookworms in the phylogenetic tree (Anderson 2000). More than 50 species of *Kalicephalus* have been identified in a wide range of different species of snake as well as humans throughout the world which do not have any host specificity (Chai et al. 2003; Santoro et al. 2013; Matt et al. 2020). Although this reptile intestinal nematode has been reported from various Asian countries including India (Kavitha et al. 2014), the Philippines (Kuzmin et al. 2013), Nepal (Pun and Maharjan 2016), China (Liu et al. 2017), Republic of Korea (Choe et al. 2016), Indonesia (Purwaningsih and Mumpuni 2011), Iraq (Anah et al. 2019) and Turkey (Düşen et al. 2010), there were no reports of *Kalicephalus* spp. from Iran. The thin-shelled eggs containing morula are excreted with reptile feces and developed quickly. After hatching these eggs, the third-stage larvae developed under suitable environmental conditions (Anderson 2000). It appears that snakes are infected mostly orally by this hookworm species (Schad 1956), then the infective larvae encyst within the gastrointestinal track's wall and develop into adults (Anderson 2000). In most reptiles, gastrointestinal nematodes infect the digestive system at a high rate, particularly in captive snakes (Pasmans et al. 2008; Rataj et al. 2011; Hallinger et al. 2018). This kalicephalid nematode causes various gastrointestinal symptoms, ranging from mild enteritis to anorexia, dyspnoea, dysentery, and even death when intensely involved with secondary bacterial infections in hosts (Junker et al. 2009). Therefore, the infections caused by *Kalicephalus* spp. can affect breeding snakes and cause economic losses (Lie et al. 2019).
In the past decade, Khuzestan Province in Iran has undergone major urban development and destruction of wildlife due to the growth of suburbanization in cities. Meanwhile, the effects of global warming and water tensions have led to a migration of terrestrial reptiles into urban areas to survive. Consequently, more dead snakes are being raised intensively in these areas. *Macrovipera lebetina* (Linnaeus, 1758) (Ophidia: Viperidae) has been reported in wide geographical distribution throughout central Asia and the Middle East (Moradi et al. 2014). *M. lebetina* is one of the most poisonous snakes on the Iranian plateau. *Vipera lebetina obtusa* is commonly known as a West-Asian blunt-nosed viper found in extensive areas of Iran (Nasiri et al. 2014). According to recent parasitic studies, intestinal helminths, Acanthocephala, intestinal protozoa, and haemoparasites are common endoparasites that infected *Vipera lebetina* (Nasiri et al. 2014). Although nearly 50 species of *Kalicephalus* have been identified throughout the world, there is no morphological and molecular information of this gastrointestinal hookworm parasite from Iran.

### Material and Methods

#### Snakes collection and study area

Iran, due to its climatological diversity, is considered a country rich in a wide range of species biodiversity. The northern and western regions of Iran are the most abundant centers of local endemism (Gholamifard 2011). The Khuzestan plain, southwest of Iran, is the richest hotspot of endemic species with an average of 56 different species of reptiles throughout the country (Hosseinzadeh et al. 2014). Ahvaz the center of Khuzestan province is located between 48.67 °E longitudes and 31.33 °N latitudes. The annual mean of maximum summer temperatures is 48°C (in July) and the minimum winter temperature is 19.3°C (in January) with semi-arid to the arid climate. The annual amounts of rainfall are 0–50 mm (13.66 mm) and humidity are 9-43% (23.83%) in several months.

In the present study, two dead viper snakes due to various incidents were referred to the parasitology laboratory of shahid Chamran University of Ahvaz between June to September 2017. All snakes originated from farms and residential yards of the suburban areas of Ahvaz. The snake’s body wall was dissected by a longitudinal incision and the visceral organs and lumen of the digestive tract were examined visually for the presence of helminths (Fig 1).

#### Collection and morphological analysis of parasites

Flowing snake gastric and intestinal autopsy, some elongated cylindrical roundworms with a white head (>1.5 cm long) were found embedded in the mucosal gastrointestinal tract of the snakes and collected. The isolated worms were cleared carefully, fixed in 70% ethanol, and mounted in lactophenol for light microscopy examination. Based on morphological characteristics, the recovered helminths were identified to species (Schad 1962; Santoro et al. 2013).

**Fig 1** Dissection of the alimentary canal of *Macrovipera lebetina* snake from Iran

The middle part of identified specimens was stored at -20°C for molecular investigations and one specimen was prepared and referred to Scanning Electronic Microscope (SEM). For the ultrastructural study, the Ronghang and Roy (2015) and Kumar and Kumar (2016) methods were used with some modifications (Ronghang and Roy 2015;
Kumar and Kumar 2016). Briefly, after washing the specimens in PBS, the anterior and posterior ends of worms were cut and fixed in 5% glutaraldehyde (Sigma, USA) for 24h at 4 °C. The dehydration process was performed with a gradient series of ethanol, then the samples were dried using Tetramethylsilane (Sigma, USA). Finally, specimens were mounted on aluminum stubs, coated with a thin layer of gold, and examined with Leo 1455 VP SEM (Carl-Zeiss, Germany) at 18-23 KV.

Molecular analysis of parasites

For the molecular survey, the Genomic DNA of collected samples was extracted using a DNA extraction Kit (SinaClon Bioscience, Iran) according to the manufacturer’s instruction. The PCR amplification was performed on tandemly repeated DNA using NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') primers (Zhu et al. 1999). The internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) of Kalicephalus spp. were amplified to the molecular and phylogenetic analysis. PCR reactions were executed in a 25-µL system composed of 12.5 µl of Taq DNA polymerase master mix Red (Amplicon, Denmark, MgCl2: 1.5 mM), 1 µl of each primer (10 µM) (Bioneer, South Korea), 7 µl of DNA template (99.4 ng), and 3.5 µl of DNase free water. PCR cycling included an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for the 30s, specific annealing at 55 °C for 30s, and extension at 72 ° C for 1 min. Lastly, a final extension at 72°C for 5 min was also performed. The PCR reaction for negative control included all components of the system except DNase-free water instead of template DNA. PCR products were electrophoresed on 1.5% agarose (SinaClon Bioscience, Iran) in Tris-acetate-EDTA (TAE) buffer, stained with SafeStain (SinaClon Bioscience, Iran), and then visualized under ultraviolet light. Positive PCR products of Kalicephalus Sp. were sequenced by Bioneer Lab (South Korea) in both directions for morphologic confirmation. Then the data were analyzed using BioEdit V. 7.0.5.3 software, the basic local alignment search tool (BLAST) program, and NCBI databases (National Center for Biotechnology Information, Bethesda, MD, USA). The alignment sequences were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 10, using the default parameters for the molecular phylogenetic study of the species.

Phylogenetic relationships were inferred based on analysis using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-1209.56) was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 315 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The topological stability of the tree was evaluated by 1000 bootstrap replications.

Results
The species of studied snakes belonged to *Macrovipera lebetina* in Viperidae Family with 115 and 100 cm in length. The identification of snake species was performed by Iranian Plateau Herpetology Research Group (IPHRG), from Razi University of Kermanshah, Iran.

The morphological and morphometric characteristics under a light microscope showed that all collected worms from the stomach and intestine of snakes belonged to *Kalicephalus viperae viperine*, Rudolph 1819, Lichtenfels 1980 species. This identified parasitic species was confirmed by Stephen Goldberg, from the Department of Biology, Whittier College, Whittier, California 90608, USA. Five roundworms were found in one snake and three worms with similar morphological characteristics in another one. The average length of female worms was 1.1 cm. The morphometric data are provided in Table 1. Unfortunately, probably due to some tissue necropsy associated with studied snake death, all the collected worms were female and we couldn’t isolate and describe the male worm’s features.

**Table 1** Measurements of *Kalicephalus viperae* were collected from the Iranian *Macrovipera lebetina* host (μm)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Description</th>
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<tbody>
<tr>
<td>Body long, cylindrical, filiform; anterior end obliquely truncated; buccal capsule was bivalvular, compressed laterally, and supported by thick cuticular bases. Head small and narrow with three anterior chitinoid ridges narrow, usually with elongated narrow-necked esophagus ending in a rounded muscular bulb (club-shape), and their dorsal gutters well developed. The posterior chitinoid pieces of <em>K. viperae chitinous</em> were connected weakly by superficial sclerotization. The esophagus was short, stout, and strongly bulbed (Fig 2). The vulval pore opened in the ventral mid-body into simple divergent uterine branches (Amphidelphic uterus) (Fig 3).</td>
<td></td>
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<td>Fig 2 <em>Kalicephalus viperae</em> recovered from Turan blunt-nosed viper snake (<em>Macrovipera lebetina</em>) in Iran. Anterior part with the esophageal region. The buccal capsule is symmetrical and consists of 4 anterior plates (stars), 4 anterior chitinoid ridges (arrows), and 3 papillae (arrowheads), and Dorsal gutter (DG), and the buccal cavity opens directly anterior (Face)</td>
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<td>Fig 3 The vulval pore (arrowhead) opened in the ventral mid-body, Uterus with embryonated eggs (Amphidelphic uterus in left picture). The tail of the female with a blunt end and a terminal spike protruding from the end of the tail (right)</td>
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| The ultrastructural findings (SEM) showed that the worm’s body was elongated and its anterior end was slightly wider than the width at mid-length and slowly slims down to the tail. The body cuticle was finely longitudinally striated. As seen in only the perfect lateral view, the characteristics of the hookworm’s head were considered. The head was small, almost rounded, and had the three dorsal, ventral, and middle circumoral papillae. The spike-like process found on the median papilla of *K. viperae Amphidiploid* distinguishes this parasite from other species in this family. The buccal capsule was bivalvular and included two lateral valves consisting of several chitinoid pieces. Externally, three longitudinal parenchymatous bands (ridges) on each lateral wall of valves protrude from the basal collar and terminate in the papillae around the mouth. The entire structures of the ridge formed arches as seen in lateral view (Fig 4). The anterior chitinoid ridge was curved and narrow. The posterior chitinoid pieces of the buccal capsule and the transverse connection between them were important taxonomically. The posterior chitinoid pieces of *K.*
were seen triangular with the rounded external posterior corner. The tail of the female worm was slim, long with a blunt end and a terminal spike at the end (Fig 5).

Fig 4 Ultrastructural characteristics of the anterior end of *K. viperae viperae* (SEM) in lateral view. The spike-like processes were observed on the median papilla (arrowhead in right picture).

Fig 5 The posterior end of the female *K. viperae viperae* with a blunt tail and a terminal spike protruding from the end of it.

The internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) falls in between 18S, 5.8S, and 28S rDNA sequences, including ITS-1 and ITS-2 sequences, were amplified in PCR reaction. ITS genes amplification corresponding to a band size of about 850 bp was identified as *K. viperae Amphidiploid*. The amplicons of samples were subjected to 1% agarose gel electrophoresis. The PCR product was deposited in the GenBank (Accession no. MT465450). The explanation of the sequencing results showed that the considered sequence had more than 80% similarity to *Ancylostoma* and *Uncinaria* spp. sequences of hookworms in the Ancylostomatidae family. ITS gene rDNA phylogeny analysis of *K. viperae* sequence showed the isolated species has high similarity to *Ancylostoma* species deposited in GenBank from around the world. The phylogenetic tree consisted of a large clade of Ancylostoma species. Dendrograms based on ITS gene sequences representing different isolates aligned on an accordant length of 314 bp and constructed using NJ with building strategies and/or distance models were similar and in the sister relationship of *A. braziliense* with 88% discrepancies in bootstrap values (Fig 6).

Fig 6 Neighbor-joining tree (1000 bootstrap replications) showing the phylogenetic relationship of *K. viperae viperae* from Iranian viper snake and ITS sequences deposited in Genbank of authentic strains of *Ancylostoma* species. The tree scale (0.2) represents evolutionary distances in units of base substitutions per site as computed by the Kimura-2 parameter method.

Discussion

The species of *Kalicephalus* was described from the stomach and intestine of a venomous snake, *Macrovipera lebetina*, for the first time in Iran. In the past, the gastrointestinal hookworm, *Kalicephalus* spp., had been primarily found in Colubrid snakes and was mainly found in the small intestine. However, recent studies have shown that this ancylostomatid worm may also be found in other snake species across the world (Schad 1962; Frank 1985). Strongylida Hookworms *Kalicephalus* spp. can infect the esophagus, stomach, and small intestinal lumen of American corn snakes (*Pantherophis guttatus*) (Matt et al. 2020). According to a recent study in Iraq, the *Kalicephalus* species infected 75% of the Iraqi *Eryx jaculus* snakes (Anaha and Al-Mayali 2019). They were also found primarily in the small intestine of Arabian sand spotted snakes while in northwest Turkey the predilection site of *Kalicephalus* species was the stomach, small and large intestine, and even the rectum of 18% of *Coronella austriaca* snakes (Dusen et al. 2010). Also, *K. brachycephalus* and *K. sinensis* were detected from the rectum and stomach of Korean terrestrial snakes, respectively (Choe et al. 2016). Most of the *Kalicephalus* spp. were found to be attached to the mucosa of the stomach and intestines and rarely seen in the esophagus (Junker et al. 2009) or rectum (Choe et al. 2016), whereas
some species were observed to be free inside the alimentary canal lumen. Therefore, _Kalicephalus_ were able to adapt to the physiological conditions of their hosts for a long time and can inhabit snakes throughout the gut.

Different species of hookworms are the most prevalent gastrointestinal parasites in reptiles. In this study, only _Kalicephalus viperea_ nematodes were found in the digestive tract of studied snakes. In captive snakes taken from German households and zoological gardens, ancylostomatid _Kalicephalus_ spp. were the most prevalent alimentary nematode species (3.3%) (Hallinger et al. 2020). Among the gastrointestinal helminths of Indian pit viper snakes ( _Bothrops jararaca_), _K. inermis_ was the most commonly identified kalicephalid species (Grego et al. 2004). Also, three species of _Kalicephalus_, namely _K. indicus_, _K. bungari_, and _K. brachycephalus_, were reported in six species of snakes ( _Elaphe carinata_, _Zaocys dhumnade_, _Naja najaatra_, _Elaphe taeniura_, _Bungarus multicinctus_, and _Dinodon rufozonatum_) from China, which _K. indicus_ was evaluated as the most common nematode species with the highest prevalence of 72.8% (Liu et al. 2017).

Identification of many isolated _Kalicephalus_ species from different regions throughout the world snakes has mainly been based on morphological features and taxonomically. In Nepal, the nematode _Kalicephalus_ sp. was isolated from the intestinal tract of _Amphiesma stolatum_, a non-venomous snake population (Shyam and Mahendra 2016). Also, in India, one species of _Kalicephalus_ was found in the stomach and intestine of a maintained Russell’s viper snake (Kavitha et al. 2014), with no parasitic species detection. Whereas, _K. colubri colubrid_ was reported in a captive mole snake ( _Pseudadaspis cana_) in South Africa, and described its morphological features in detail (Junker et al. 2009). Also, _K. indicus_, _K. bungari_, and _K. assimilis_ were found in the intestine of three species of Indonesian snakes, _Ophiophagus hannah_, _Ptyas mucosus_, and _Naja Sputatrix_, and their morphological findings were recorded (Purwaningsih and Mumpuni 2011). The findings of these studies were described using light and stereo microscope, while in previous years, a scanning electron microscope (SEM) was also applied to better describe the ultrastructural characteristics of _Kalicephalus_ species.

The species of _K. subulatus_ from Wagler’s snake, _Xenodon merremi_ in Argentina (Gonzalez et al. 2018), _K. indicus_, _K. bungari_, and _K. brachycephalus_ from six snake species (Liu et al. 2017) and _K. brachycephalus_ and _K. sinensis_ were isolated from the rectum and stomach of Korean terrestrial snakes (Cho et al. 2016) and identified by light and scanning electron microscope (SEM) in detail, which well-defined some structural features specific to the species.

According to Nasiri et al.’s (2014) survey on parasitic infections of Iranian snakes, 50% of _Vipera lebetina obtuse_ collected from various provinces (during 2012) were positive for intestinal parasites (Nasiri et al. 2014). In the present study, we investigated the case of _K. viperae marram_ in the intestine and stomach of two studied West-Asian blunt-nosed viper snakes and additionally manifested part of the genomic information for the first time in the world by molecular assay. _K. viperae_ is commonly found in various old-world snakes, especially vipers and _Elaphe_ spp. (Schad 1962). In European vipers, the _K. viperae_ were reported from the intestine of Western whip snake _Hierophis viridiflavus carbonarius_ (Colubridae) from Southern Italy (Santoro et al. 2013) and the esophagus of _Vipera aspis_ and _V. latastei_ snakes from several areas of the Iberian Peninsula, Spain (Santos et al. 2006). Recently, Liu et al., (2019) presented the rDNA ITS of _Kalicephalus_ spp. as a suitable genetic marker for interspecies variation in the taxa of the
phylogeny. They obtained ITS rDNA sequences of *K. indicus*, *K. bungari*, and *K. brachycephalus* with 98.4% intra-
and 80%–89%, interspecific identities (Liu et al. 2019).

The identified Iranian *K. viperae* latest was constructed in an individual branch, far from of *Ancylostomatidae*
species in the phylogenetic tree. The results of the phylogenetic analysis of amplified sequences in this study were
similar to the results of other identified *Kalicephalus* species from China The phylogenetic tree revealed that congener
*Kalicephalus* is located in the same branch, which is far apart from other branches of nematodes (Liu et al. 2019).
Unfortunately, there were no published data in NCBI to align the *K. viperae* sequence results to the chine's molecular
information of Liu et al., (2019) investigation. The present study was the first report of *Kalicephalus* sp. in viper
snakes from Iran using morphological and molecular assays. More comprehensive studies on the identification of
other common parasitic species in Iranian reptiles seem necessary.

Conclusion

In conclusion, the morphological characteristics, and nucleotide sequence including a large part of the nuclear
ribosomal DNA (rDNA) of *Kalicephalus viperae* latest was identified for the first time in the world and Iran. Also,
the results of the phylogenetic analysis showed that this species is taxonomically close to other carnivores’
hookworms, especially *A. braziliense*.

Conflict of interest statements

All authors declare that they have no conflict of interest.

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References


**Statements & Declarations**

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**Competing Interests**

The authors disclose all financial or non-financial benefits associated with the manuscript.

**Author Contributions**
All authors contributed to the study conception and design. Methodology (Material preparation, data collection and analysis) were performed by Sara Larki, Alireza Alborzi, Kia Bahramnejad, Zeinab Asadi; Molecular study were performed by Sara Larki and Zeinab Asadi; The first draft of the manuscript was written by Sara Larki and Kia Bahramnejad; All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript including Sara Larki, Alireza Alborzi, Kia Bahramnejad, Zeinab Asadi.

Data Availability

All data generated or analysed during this study are included in this published article. The molecular datasets found during the current study are available in the National Center for Biotechnology Information repository, [https://www.ncbi.nlm.nih.gov/nuccore/MT465450.1](https://www.ncbi.nlm.nih.gov/nuccore/MT465450.1).

Ethics approval

The ethical guidelines have been consistence during the designing the project, date collection, and preparation of results. All of authors gave their informed consent prior to their inclusion in the research. We also hereby declare all ethical standards in line with the principles of the Declaration of Helsinki respected in the submitted article.
Figures

Figure 1

Dissection of the alimentary canal of *Macrovipera lebetina* snake from Iran
Kalicephalus viperae recovered from Turan blunt-nosed viper snake (Macrovipera lebetina) in Iran. Anterior part with the esophageal region. The buccal capsule is symmetrical and consists of 4 anterior plates (stars), 4 anterior chitinoid ridges (arrows), and 3 papillae (arrowheads), and Dorsal gutter (DG), and the buccal cavity opens directly anterior (Face)
Figure 3

The vulval pore (arrowhead) opened in the ventral mid-body, Uterus with embryonated eggs (Amphidelphic uterus in left picture). The tail of the female with a blunt end and a terminal spike protruding from the end of the tail (right)

Figure 4

Ultrastructural characteristics of the anterior end of *K. viperae viperae* (SEM) in lateral view. The spike-like processes were observed on the median papilla (arrowhead in right picture)
Figure 5

The posterior end of the female *K. viperae viperae* with a blunt tail and a terminal spike protruding from the end of it.
Figure 6

Neighbor-joining tree (1000 bootstrap replications) showing the phylogenetic relationship of *K. viperae* from Iranian viper snake and ITS sequences deposited in Genbank of authentic strains of *Ancylostoma* species. The tree scale (0.2) represents evolutionary distances in units of base substitutions per site as computed by the Kimura-2 parameter method.

**Supplementary Files**

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- Table3.pdf