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Molecular investigation of Cavernicoles from Kotumsar Cave in Northern Eastern Ghats, India

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Abstract

The Kotumsar cave is situated in the Eastern Ghats and has been reported by the existence of 14 different organisms morphologically. To reassess the living taxa and hitherto unreported organisms, intervention of molecular tool is required to corroborate the exact faunal diversity. In the present study, we dealt with the environmental samples and opportunistically encountered living specimens from both deep and transition zones of the Kotumsar cave. The morphological and integrated approach confirmed the existence of *Rhinolophus rouxii* (Medellin *et al*., 2017) (bat), *Kempiola shankari* Sinha and Agarwal, 1977 (cricket), *Heteropoda leprosa* Simon, 1884 (spider). Further, the collected environmental DNA (eDNA) samples were successfully identified as *Fejervarya pierrei* (Dubois, 1975) (frog), *Indoreonectes evezardi* (Day, 1872) (fish), *Metrocoris* sp. (true bug), *Barytelphusa cunicularis* (Westwood, 1836) (crab), *Trigoniulidae* sp. (millipede), and *Megascolecidae* sp. (worm). Hence, the present investigation through combined approaches by both morphological and molecular data helps to add six more organisms to the faunal checklist of Kotumsar cave. The study also contributed the genetic information of cavernicoles in the global database from India. This genetic information would further help to pursuing other biological studies and adopt better conservation strategies of cave-dwelling organisms and restoration of the colligated ecosystem.

Keywords: Cave Fauna, Conservation, DNA Barcoding, Environmental DNA (eDNA), New Record

Introduction

Caves are the unique ecosystem on the earth holding several hidden biological components that are yet to be described (Barr, 1968). Besides an innumerable biological importance, the caves often allow for cultural, architectural, and geological aspects as well as ecotourism (Sponsel, 2015). These subterranean ecosystems have receiving low inputs of energy and light but high humid conditions, offering habitats for the specified organisms (Pricop & Negrea, 2009; Biswas, 2010). Caves also provide unique natural confined condition for studying biological and geological processes (Buhlmann, 2001). Due to the endemism, extraordinary adaptation, and dire risk of extinction, the cave-dwelling organisms (cavernicoles) are now a focus of intense research throughout the world (Culver & Pipan, 2009; Beron, 2015). Many new species, new genera and even new families have been described from different caves in India (Prasad, 1996; Kottelat *et al*., 2007; Harries *et al*., 2008; Disney, 2009; Biswas & Shrotriya, 2011; Banafar & Biswas, 2016).

The Eastern Ghats region in India is bestowed with a large number of historical caves (Biswas, 1992b). However, the exploration of cavernicoles in India has been mostly limited to morphological descriptions. The Kotumsar cave is regarded as one of the safest homes of several endemic faunal components and has gained attention by the researchers from all over the world (Skalski, 1990). A recent review has listed 14 different organisms in this cave, including three unidentified species with incomplete taxonomic information (Biswas, 2010). Nevertheless, these cavernicoles are surviving with

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limited habitat resources, and are facing anthropogenic disturbances that lead to decline the extant diversity (Biswas, 1992a; Biswas, 2009). Hence, to review the living taxa and hitherto unreported organisms in this ecosystem warrants employing of molecular tool to substantiate the exact faunal diversity.

In recent past, the metabarcoding has gained success towards inventorying and monitoring the biotic components from environmental DNA (eDNA) (Sato *et al*., 2017; Kundu *et al*., 2018). The eDNA is a genetic material coalesce in the environment, derived from an living organism as different forms like shed of epidermal cells, and other body secretions like feces, urine, gametes (Valentini *et al*., 2016; Deiner *et al*., 2016). These physiological behavior open opportunities to this approach to reveal the occurrence of organisms that cannot be effortlessly sampled due to the legitimate constraint in wildlife accessibility (Sutherland *et al*., 2013). However, the assessment of eDNA is meagerly attempted to monitor the diversity in cave ecosystem. Hence, a pilot survey was conducted in Kotumsar cave in northern Eastern Ghats which led to collect the environmental samples (water, moist gravels, and detritus) and investigate through partial mitochondrial Cytb and COI gene fragments to identify the extant faunal diversity. The aimed study would be beneficial for systematics research and speleology as well as helpful to formulate the policies for sustainable management and conservation for the cave fauna and colligated ecosystem.

Material and Methods

Kotumsar cave is located near Jagdalpur in the Indian state of Chhattisgarh in northern Eastern Ghats. It is situated near the bank of the River Kanger in Kanger Valley National Park. The entrance coordinates are 18.86 N 81.93 E, and it lies at an altitude of 560 meter above the sea level (Figure 1A). The field survey was conducted by the Freshwater Biology Regional Centre (FBRC), Zoological Survey of India (ZSI), Hyderabad, to collect the environmental samples from the cave. The cave is divided into three distinct zones, the twilight zone, intermediate zone and deep zone. Environmental samples were collected from the intermediate zone and deep zone in the present study with precise manner without much disturbance to its natural condition. During the environmental sampling, we opportunistically encountered some organisms (Crickets and Spider). The environmental samples were stored in a sterile container and live specimens were preserved in 70% ethanol. The moist gravels and detritus samples were stirring with distilled water for the downstream process. The environmental samples were screened through different pore sieves and segregate the possible body parts (skin shade, leg parts etc.) through microscopic screening. The genetic materials were preserved in 70% alcohol for DNA based investigation. Morphological identifications of the live organisms were confirmed by practising taxonomists of the respective group and co-authors.

The genomic DNA was extracted through QIAamp DNA Investigator Kit (QIAGEN Inc., Germantown, MD) as per the standard protocol and stored at FBRC. Based on naked-eye observation of the extant fauna inside the cave and previous reports, both vertebrates and invertebrate specific three sets of primer pairs were used to amplify the desired mitochondrial gene fragments (Cytb and COI) (Folmer *et al*., 1994; Verma & Singh, 2003; Barrett & Hebert, 2005). The PCR amplification was performed as per previously cited protocols (Kumar *et al*., 2019; Kundu *et al*., 2018; Chatterjee *et al*., 2017). The amplicons were checked in 1% agarose gels containing ethidium bromide (10 mg/ml) and purified using QIAquickR Gel extraction kit (QIAGEN Inc., Germantown, MD). Each purified products were bi-directionally sequenced in 48 capillary arrays 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) following Sanger sequencing methods at the in-house sequencing facilities at Centre for DNA Taxonomy laboratory, ZSI, Kolkata.

The forward and reverse chromatograms of each generated COI and Cytb genes were checked thoroughly in Sequence Scanner software (Applied Biosystems, Foster City, CA). To check the quality of the generated sequences, mismatches, and gaps; the online tools, Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) were used with appropriate genetic code for amino acids. The consensus sequence of each biological sample was built after the alignment of 'forward sequence' and reverse complementary of the 'reverse sequence' through ClustalX software (Thompson *et al*., 1997). The generated sequences were submitted in GenBank for acquiring the accession numbers. To identify the environmental samples and cavernicole organisms, the global BLAST was performed to obtain the similarity search results in

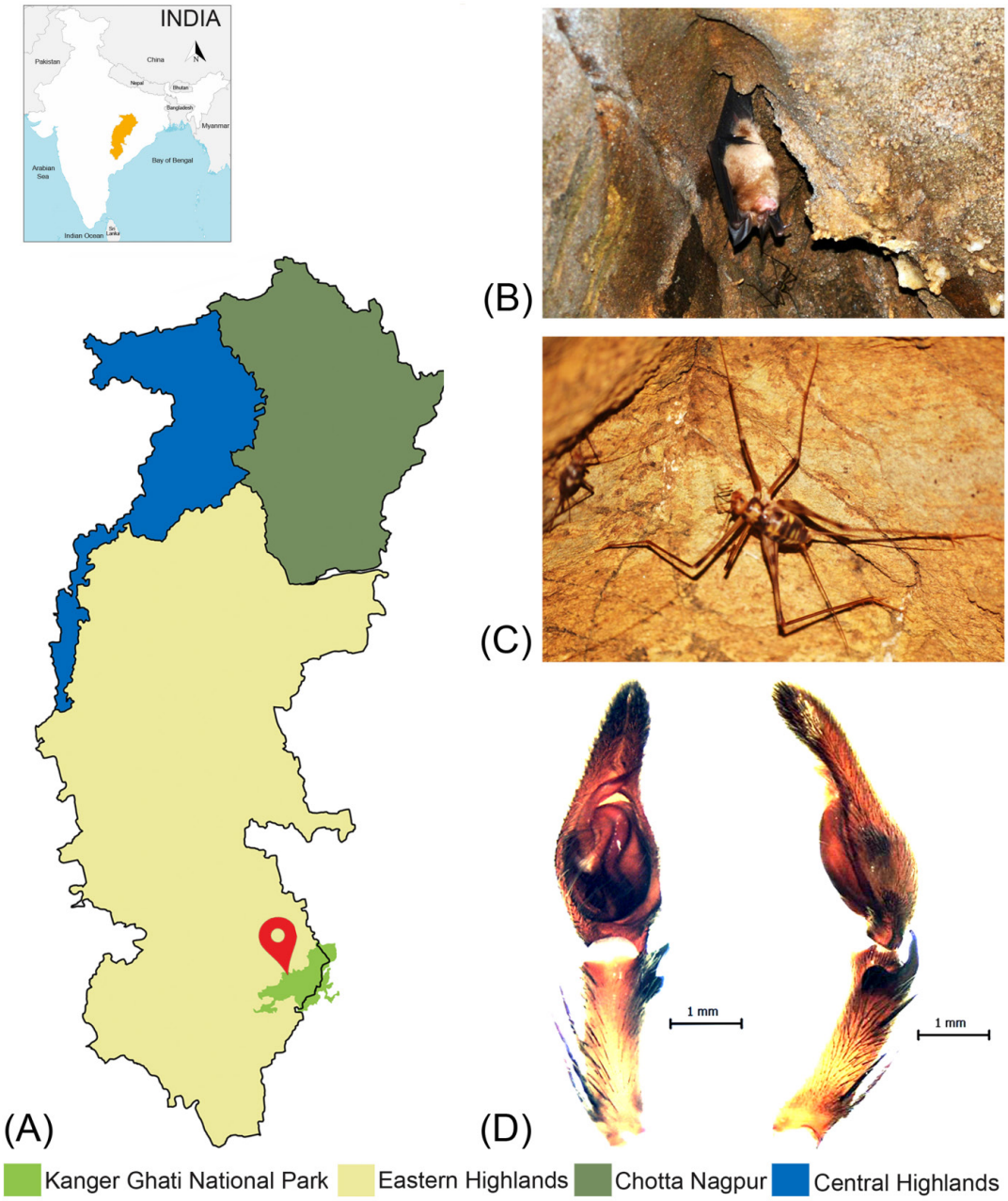

Figure 1. **(A)** Map showing the different geographical regions of Deccan Peninsula in Chhattisgarh state of India. Red pin showing the collection localities of eDNA and cavernicoles in Kotumsar cave. **(B-C)** Morphologically encountered organisms, **(D)** Ventral and lateral view of *H. leprosa* genitalia.

GenBank database. Further, to check the monophyletic criteria in cluster-based phylogenetic analysis, 'Blast Tree View' was performed in NCBI webserver (https://www.ncbi.nlm.nih.gov/blast/treeview) with Neighbour-Joining (NJ) tree method, maximum sequence difference value=0.75, and maximum target sequences-10 algorithm parameter. To validate the phylogenetic outcomes, the same data were further acquired from GenBank and analyzed through MEGA6 (Tamura *et al.*, 2013) with Kimura 2 Parameter (K2P).

Results and Discussion

A total of three different groups of organisms (bats, spiders, and crickets) were encountered morphologically during the survey (Table 1). The present survey was unable to spot 11 previously reported taxa from the surveyed ecosystem. Based on the visual identification and photographic record, we confirmed the bat species as the previously reported Rufous horseshoe bat, *Rhinolophus rouxii* (Medellin *et al*., 2017) (Figure 1B). Two opportunistically encountered invertebrate species were identified as crickets, *Kempiola shankari* (Figure 1C), and spider, *Heteropoda leprosa* based on taxonomic characters (Gravely, 1931; Sinha & Agarwal, 1977). Further, the genital characters (male pedipalps) were dissected from the studied sample (ZSI_FBRC_DNA142) with the help of sterile surgical scalpel blades and acquired the photographs by using Leica M205A for taxonomic confirmation. The huntsman spiders, *H. venatoria* was previously reported from this cave, however the studied sample of *H. leprosa* can be easily differentiated by the below-mentioned characters: In *H. venatoria*, tibial apophysis short and broad with two teeth with semi-circular notch, lower side strongly convex, anterior median eyes not much smaller than posterior medians, ocular quadrangle not very narrow in front. However, in the studied specimen, anterior median eyes much smaller than posterior, ocular quadrangle extremely narrow in front, tibial apophysis stouter, variable, sometimes truncate, often with a more or less strong process below (Figure 1D). Both the morphologically identified invertebrates were further investigated with molecular data. The generated COI sequence (605bp) of morphologically identified *H. leprosa* showed 91.33% similarity with *H. venatoria* in GenBank. The NJ phylogeny showed *H. leprosa* is the sister taxa of *H. venatoria* with 9% to 9.4% genetic distance. Further, *H. leprosa* also showed 10.4% to 10.6% genetic distance with *H. maxima* in the studied dataset (Figure 2A). Further, due to the lack of reference library of crickets, the generated COI sequence (677bp) of *K. shankari* showed 84.68% similarity with *Pimelia scabrosa* (Coleoptera). The NJ phylogeny showed the distinct clade of *K. shankari* with 14.1% to 18.6% genetic distance with other species of Crickets, and Beetles (Figure 2B).

Table 1. A list of Cavernicoles in Kotumsar Cave is combined from the present and previous studies. VI= visual identification, MI= molecular identification, IA= integrated approaches by morphological and molecular data, NO= not observed

Sl. No.	Common name	Identified organism	Identified by	Voucher IDs (Accession No.)	Reference
(a) Vertebrates:					
1.	Frog	*Fejervarya pierrei*	MI	ZSI_FBRC_DNA136 (MK404058)	This Study, First Report
2.		*Hydrophylax malabaricus*	NO	-	Biswas, 2010
3.	Fish	*Indoreonectes evezardi*	MI	ZSI_FBRC_DNA138 (MK404059)	Bhargava *et al*., 1984; Biswas, 2010; This Study
4.	Bat	*Rhinolophus rouxii*	VI	-	Chakravorty, 2008; Biswas, 2010; This Study
5.		*Hipposideros cineraceus*	NO	-	Chakravorty, 2008; Biswas, 2010
(b) Invertebrates:					
6.	Spider	*Heteropoda leprosa*	IA	ZSI_FBRC_DNA142 (MK391565)	This Study, First Report
7.		*Heteropoda venatoria*	NO	-	Biswas, 2010
8.	Cricket	*Kempiola shankari*	IA	ZSI_FBRC_DNA139 (MK391564)	Sinha & Agarwal, 1977; Biswas, 2010; This Study

Sl. No.	Common name	Identified organism	Identified by	Voucher IDs (Accession No.)	Reference
9.	Guano-moth	*Kangerosithyris kotomsarensis*	NO	-	Skalski, 1992; Biswas, 2010
10.	Bugs	*Metrocoris* sp.	MI	ZSI_FBRC_DNA150 (MK391567)	This Study, First Report
11.	Copepod	*Parastenocaris kotumsarensis*	NO	-	Reddy & Defaye, 2009; Biswas, 2010
12.	Isopod/ Pillbugs	*Armadillidium* sp.	NO	-	Biswas, 2010
13.	Amphipod	*Kotumsaria bastarensis*	NO	-	Messouli *et al.*, 2007; Biswas, 2010
14.	Snail	*Opeas* sp.	NO	-	Biswas, 2010
15.	Crab	*Barytelphusa cunicularis*	MI	ZSI_FBRC_DNA132-34 (MK391560-62)	This Study, First Report
16.	Syncarida	*Chilibathynella kotumsarensis*	NO	-	Reddy, 2006; Biswas, 2010
17.	Centipede	*Thereuopoda longicornis*	NO	-	Biswas, 2010
18.	Millipede	Polydesmid	NO	-	Biswas, 2010
19.	Millipede	Trigoniulidae sp.	MI	ZSI_FBRC_DNA137 (MK391563)	This Study, First Report
20.	Worm	Megascolecidae sp.	MI	ZSI_FBRC_DNA158 (MK391566)	This Study, First Report

The generated DNA sequences of eight eDNA were distinctly identified up to species level for five samples, genus level for one sample, and family level for two samples. The generated Cytb sequence (447bp) of the studied sample (ZSI_FBRC_DNA136) showed >99% similarity with the *Fejervarya* species. Further, the NJ tree resulted close relationship of the generated DNA data with four species (*F. granosa*, *F. syhadrensis*, *F. caperata*, and *F. pierrei*) with 0.3% to 3.8% genetic distances (Figure 2C). Among two close database sequences, *F. caperata* sequence (MH423757) is unpublished, thus assumed as confusing for species discrimination. The previous studies have reported the occurrence and distribution of cave-dwelling frogs from different Indian caves (Suwannapoom *et al.*, 2018). Based on the close genetic distance (0.3%) with the published data of *F. pierrei* (AB488834) (Kotaki *et al.*, 2010), we confirmed the identity of eDNA and occurrence of *F. pierrei* in the Kotumsar cave. In addition, there was a report of *Hydrophylax malabaricus* from Kotumsar cave (Biswas, 2010). However, according to Padhye *et al.*, 2015, this species is restricted to the southern Western Ghats, and its congener *Hydrophylax bahuvistara* is distributed in the Eastern Ghats. Therefore, based on the geographical location of Kotumsar cave; we assumed the previously reported species may be *H. bahuvistara*. The generated Cytb sequence (426bp) of the studied sample (ZSI_FBRC_DNA138) showed 96.71% similarity with the different color forms of *Indoreonectes evezardi* in GenBank database. The NJ tree also showed close clustering of the studied and database sequences with 3.4% to 7.5% genetic distances (Figure 2D). Hence, we confirmed the identity of eDNA as *I. evezardi*. Although the previous studies assessed the physiological and behavioral changes of fish fauna in Kotumsar and other caves, the genetic investigation is anonymous till date (Bhargava *et al.*, 1984; Biswas, 1993). In the present study, the resulted in high intra-species genetic distances revealed possible cryptic diversity or distinct gene pool of *I. evezardi* in Kotumsar cave, which need further investigation with more molecular markers and sampling from this ecosystem and other known distribution localities.

The eDNA sample (ZSI_FBRC_DNA150) was identified up to the genus level, *Metrocoris* sp. due to lacking of sufficient molecular data in the global database.

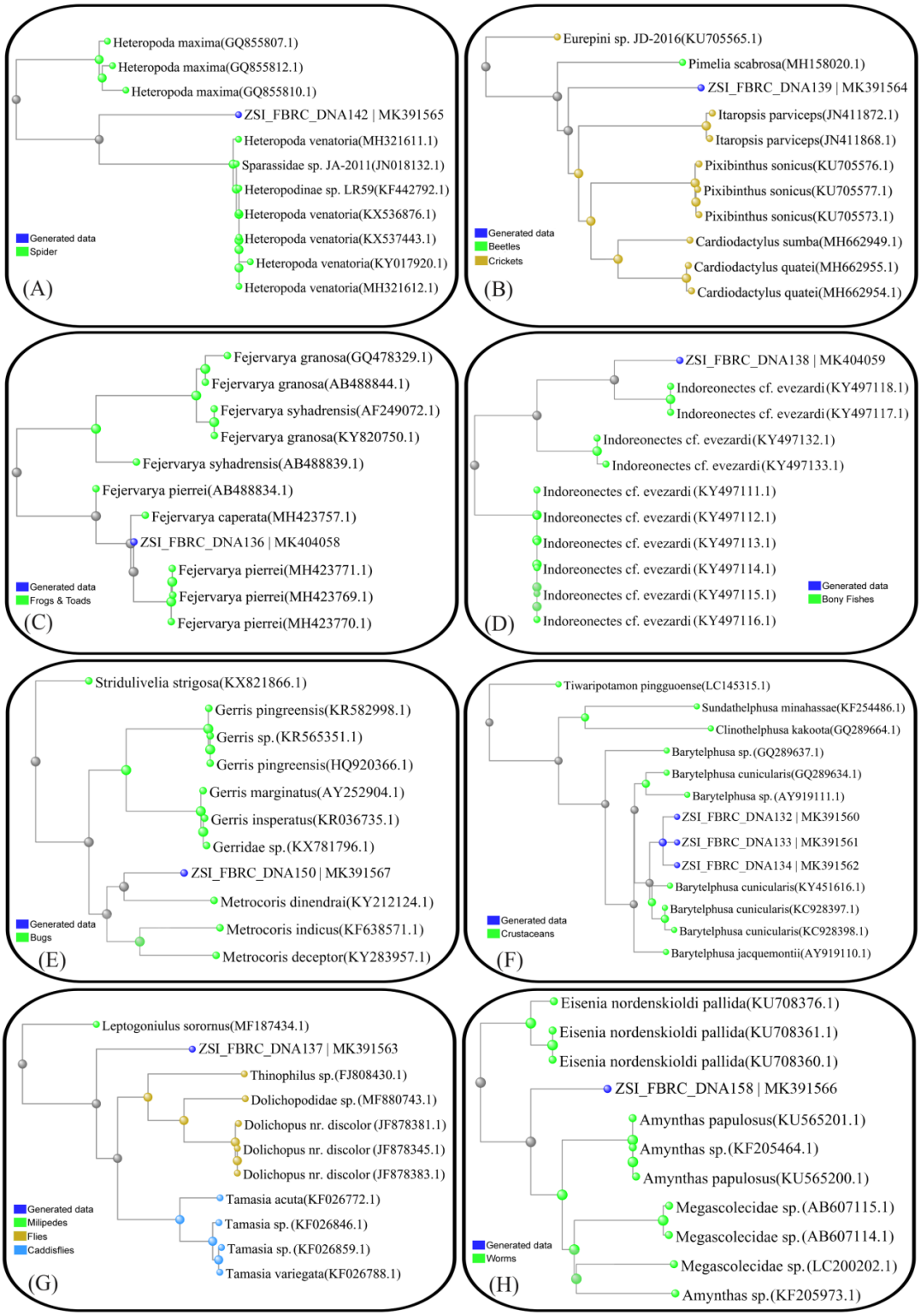

Figure 2. **(A-H)** Neighbour-joining trees inferred cluster-based identification of different eDNA and cavernicoles encountered in the present study based on both mitochondrial COI and Cytb gene.

The generated 681bp COI sequence showed 90.91% similarity with *Metrocoris dinendrai* (true bugs) in GenBank. Further, the NJ tree also showed close cladding of the resulted *Metrocoris* sp. with *M. dinendrai* with 10% genetic distance (Figure 2E). Three eDNA samples (ZSI_FBRC_DNA132-134) were identified as *Barytelphusa cunicularis*. The generated COI sequences (612bp) showed 97.39% similarity with *B. cunicularis* in GenBank database. Both the resulted and database sequences of *B. cunicularis* showed cohesive clustering in the NJ phylogeny with 2.5% to 6.2% genetic distance (Figure 2F). The database sequences of the previously collected specimens were from Amba River in Maharashtra and Musi River in state Telangana. However, the resulted high genetic variation of *B. cunicularis* within India provides further scope for molecular investigation to perceive the actual diversity. The generated 630 bp COI sequence of the eDNA sample (ZSI_FBRC_DNA137) showed 80.38% similarity with *Leptogoniulus sorornus* (millipede) species within the family Trigoniulidae. The NJ tree also showed a distinct clade of the investigate eDNA sample with other closely related species (millipedes, flies, and caddisflies) with 21.8% to 24.1% genetic distance (Figure 2G). Hence, we confirmed the identity up to the family level as Trigoniulidae sp. The generated 425 bp COI sequence of the eDNA sample (ZSI_FBRC_DNA158) showed 85.55% similarity with Megascolecidae sp. (worm) in GenBank. The NJ tree also showed a distinct clade of the investigate eDNA sample with other closely related warms with 16.5% to 18.7% genetic distance (Figure 2H). Hence, we confirmed the identity up to the family level as Megascolecidae sp.

Altogether, the present study added one vertebrate (*Fejervarya pierrei*) and five invertebrate species (*Heteropoda leprosa*, *Barytelphusa cunicularis*, *Metrocoris* sp., Trigoniulidae sp., and Megascolecidae sp.) in the faunal checklist of Kotumsar cave from both deep and transition zones (Table 1). In a cave, there may be three categories of cave-dwelling organisms occupying different habitat zones (Chirstman & Culver, 2001; Sket, 2008). However, the twilight zone was not encountered in the present effort, which may have influence from external conditions, and organisms may frequently or unexpectedly enter in the ecosystem (Biswas, 2010). Nevertheless, the conservation and restoration of these subterranean biospheres and associated living organisms is desperately necessitated through multiple biological and ecological approaches (Hildreth-Werker & Werker, 2006). Our primary objective was to generate DNA sequence data of eDNA from the cave ecosystem for accurate species identification. Thus, the generated sequence information not only helps to investigate the faunal diversity beyond previous reports but also helps to enrich the global database in terms of cavernicoles. The present effort with molecular information further allow studying of several other aspects of biology, behaviour, etc., of cave-dwelling organisms in different caves in India, and encourage acquiring effective management and conservation strategies.

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