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DNA sequence monomorphism of Indian spiny-tailed lizard *Saara hardwickii* suggests urgent conservation

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Abstract
The present study was carried out from January to June 2016 in Rajasthan that aimed to sequence the 16S rRNA gene of *S. hardwickii* to trace genetic variation. Non-invasive molecular techniques were used to isolate DNA from fecal matter. The sequences were edited and assembled using SeqMan computer program, aligned with six global sequences following Clustal W algorithm to ascertain single nucleotide polymorphisms (SNPs). Maximum-likelihood phylogenetic tree was constructed using MEGA v6.0 with 1000 bootstrap replicates. Multiple sequence alignment with global sequences showed single bp changes at four positions in Indian and two global samples. Phylogenetic analysis revealed shared ancestry between Indian and these two global samples with indels at seven bp positions 126-128 (insertion) and 249-252 (deletion), and largely due to the movement of specimens involved in illegal trading. The study though short term, quite promising and revealed monomorphic nature of Indian isolates that suggests urgent conservation of the species in India.

Keywords: *Saara hardwickii*, DNA, sequence, fecal pellet, non-invasive

1. Introduction
The recent accelerated population depletion of several species as a consequence of anthropogenic development has spawned a wide interest among conservationists to identify threats to endangered species. The knowledge of species occurrence and distribution patterns is crucial in this direction that helps to understand the underlying threats and prioritizing management decisions [1]. Species that exists in small populations and narrowly survive demographic contraction may undergo close inbreeding, genetic drift or loss of overall genomic variation due to allelic loss or reduction in homozygosis [2]. In recent times, the process of obtaining information on such animals which are either rare, endangered or threatened has become a tedious task and poses several logistical problems [3]. In the past two decades, the methods of molecular biology and DNA sequencing have been used to describe in precise detail the prospective status of several rare, endangered and threatened species [4]. Thus, genetic applications have transformed population genetics from an academic discipline to the forefront of global management decisions on wildlife conservation [2-4]. An array of molecular tools are available which includes recently developed invasive techniques for the analysis of hair, feathers, blood, saliva, skin and nails as a DNA source [5, 6]. However, their collection is quite difficult and provides less information [7]. In contrast, the use of fecal matter as a DNA source is a non-invasive approach and helps in surmounting many shortcomings of commonly used invasive techniques. It has also additional advantages such as ample availability of DNA source material and its easy detection based on certain morphological criterion [8]. The principle behind the utility of fecal matter is that it contains cells shed from the intestinal lining, thereby enabling the isolation of DNA. Thus, molecular approaches applied to feces, in collaboration with conventional analyses can be used for the identification of species, individuals, sex, life history, understanding their population dynamics and the assessment of genetic structure [9, 10]. Habitat destruction and illegal trading has devastated the survival and existence of many reptile species worldwide. One such reptile is the Indian spiny-tailed lizard *Saara hardwickii* (Gray 1827) (Figure 1 A). Being conservation dependent, this species comes under Schedule II of the Indian Wildlife (Protection Act), 1972 and Appendix II of CITES [11, 12]. It has a patchy distribution in India, Pakistan and Afghanistan [13, 14].

It is the only herbivorous lizard species of India and currently distributed mainly in small fragmented populations in the dry areas of northwestern India i.e. the Thar desert of Rajasthan and Gujarat [14-22]. Recently, one small population of this species is reported from the Sariska National Park [23], which at present is the only known population of this species in Rajasthan outside the Thar desert area. In India, there is a lack of intensive study on this species as all studies on it so far are either occasional observations or short term studies [14-23]. Due to lack of proper information on this species it has not been accessed by IUCN, though considered as Data Deficient Nationally [24, 25]. The main threat to this lizard is its commercial exploitation for meat, skin and oil, the later supposedly having great medicinal value. This species is also threatened due to illegal trading and habitat destruction [13, 18, 22]. The pocket population of *S. hardwickii* in India points towards an immediate genetic assessment of the species in order to understand the genetic variation among populations of this species so as to know the exact nature of evolutionary pressure on it. Herewith, sequence the 16S rRNA gene of *S. hardwickii* have attempted for the first time in Indian isolates, considering fecal pellets as the DNA source material to understand underlying genetic variation of this species.

2. Materials and methods

2.1 Genomic DNA isolation, PCR amplification and Automated DNA sequencing

The present study was carried out from January to June 2016. In field, fresh fecal pellets of *S. hardwickii* (Figure 1 A, B) were collected from three locations in Rajasthan namely, Jorbeer, Bikaner, Tal Chhapar, Churu and Desert National Park (DNP), Jaisalmer (Figure 1 C). Ten to fifteen pellets of each individual of *S. hardwickii* was collected near its burrow in zipper bags and kept at room temperature for four to five days at field station. Genomic DNA was isolated from these pellets using MolBio HIMEDIA MB544 HiPurA stool DNA purification kit with certain modifications to the standard protocol. Four to five pellets of each individual were taken and a cotton applicator was used to remove the outer layer of the pellet. The handle of the applicator was removed and the head was preserved in 1.5 ml vials containing PBS. Sterile gloves and forceps were used to avoid contamination. To each vial containing the applicator, 300 µl alkaline lysis buffer and 30 µl proteinase kinase were added. Vials were incubated overnight at room temperature. Next day, they were vortexed for 30 seconds. Cotton applicators were removed from each vial and 300 µl stool lysis buffer 1 was added. Following this 330 µl 100% ethanol was added. Then, vortexed and allowed to spin for 30 seconds at 12000 rpm. Lysate was loaded onto the spin column and allowed to spin for 30 seconds at 12000 rpm. Supernatant present in the collection tube was discarded. About 500 µl of wash solution was added to the spin column followed by centrifugation at 12000 rpm for 30 seconds. Flow through was discarded and the wash step was repeated once more. Centrifugation was done for 3 minutes at 12000 rpm. Spin columns were transferred to the labeled eppendorfs, 100 µl of TE buffer was added and the samples were incubated for 10 minutes. This was followed by final centrifugation at 13000 rpm for 2 minutes. The step involving the addition of TE buffer was repeated again. The elute was transferred to a fresh capped 2ml collection tube for longer DNA storage. The integrity of the isolated DNA was checked by running the samples on 2 % agarose gel and visualizing it under UV trans-illuminator. PCR was conducted using the designed primers for 16S rRNA gene (Figure 2, Table 1). Each 25 µl PCR reaction mixture contained 0.2 mM concentration of each deoxyribonucleotide triphosphate (dNTP), 10 pmol of each primer (forward and reverse), 1 U of Taq DNA polymerase (Bangalore Genei, India) with 1X polymerase buffer and 1 µl template DNA. Annealing temperature for each DNA fragment was standardized using the temperature-gradient PCR. PCR gradient thermal cycling included an initial denaturing step of 5 min at 95 ºC, followed by 35 cycles of 1 min at 95 ºC, 1 min at 45 ºC–65 ºC for annealing (gradient) and 1 min at 72 ºC for extension, and a final extension cycle of 10 min at 72 ºC. Five µl of PCR amplified products were mixed with two µl of bromophenol blue and were allowed to run on a 2 % agarose gel in TBE buffer, stained with ethidium bromide (EtBr), and visualized under UV illumination.

The amplified PCR products were subjected to PCR purification using exo-SAP (Fermentas, Life Sciences). One unit each of exonuclease (0.025 µl) and shrimp alkaline phosphatase (0.5 µl) were mixed with 10 µl of PCR products and was allowed to run in a thermal cycler at 37 ºC for 60 min and 85 ºC for 15 min. Further, 1-2 µl exo-SAP purified products were utilized for preparing sequencing reaction with 6 µl of big dye terminator (BDT) ready reaction mix and 0.8 pmol of each primer (both forward and reverse). Cycle sequencing was performed in a thermal cycler as follows; initial denaturation at 95ºC for 5 min, followed by 25 cycles of final denaturation at 95 ºC for 10s, annealing at 50 ºC for 5s and extension at 60 ºC for 4 min. The probes were subjected to automated DNA sequencing from both the directions, i.e. forward and reverse (2X coverage) on an automated DNA analyzer, ABI 3730XL (Applied Biosystems), under the facility of the National Institute of Malaria Research (NIMR), New Delhi, India.

2.2 DNA sequence editing, alignment and SNP identification

DNA sequencing of 16S rRNA gene was performed for six samples (two samples each from three locations) (Table 1). The obtained sequences were edited and assembled using the SeqMan computer program incorporated in the Lasergene software (DNASTAR, Madison, WI, USA). Homologous DNA fragments of six individual sequences (Accession Number KY393426 to KY393431) were then aligned along with six global sequences (Accession Number FJ639587.1 to FJ639591.1 and AB474756.1) (Table 2) following the Clustal W algorithm incorporated in the MegAlign computer program (a part of the Lasergene v.7 computer program) to ascertain single nucleotide polymorphisms (SNPs) and to carry out phylogenetic analysis. Furthermore, MEGA v6.0 was used to reconstruct maximum-likelihood (ML) tree [26] with 1000 bootstrap replicates taking *Leiolepis reevesii* (Accession Number AF215262.1) as an out group.

3. Results

Genomic DNA isolation was performed for each of the ten different samples from three locations in Rajasthan. Successful PCR amplification was observed in five samples from Churu and seven samples each from Bikaner and Jaisalmer. Following this, two PCR amplified products were taken from each population and the sequencing protocol was performed. Sequencing could not be performed for all the PCR amplified products due to time and resource constraints. DNA sequencing of all the six samples (two samples from each of the three locations) produced a 364 bp of 527 bp region of 16S rRNA gene of *S. hardwickii* (Table 1). Edited sequences were compared with each other as well as with the

reference sequence (Accession Number FJ639591.1) to obtain multiple sequence alignment (MSA) using MegAlign software. MSA represented all the sequences generated from our study to be monomorphic (Figure 3 A). Thus, no statistical test could be applied to study the genetic variation or the nature of evolutionary forces.

In order to carry out the phylogenetic analysis and to infer the position of Indian isolates among those from across the world, six global sequences (Accession number FJ639587.1 to FJ639591.1 and AB474756.1) (Table 2) were downloaded from NCBI (www.ncbi.nlm.nih.gov) and aligned with those from our study (Accession Number KY393426 to KY393431) to obtain a global alignment (Figure 3A). On comparison with global samples, single base pair changes at four positions 132 (C>T), 229 (T>A), 233 (A>C) and 293 (C> T) were observed in Indian as well as two global samples (Accession Number FJ639590.1 and FJ639591.1) (Figure 3 A). The alignment further showed indels at seven bp positions, 126-128 (insertion) and 249-252 (deletion) in sequences from this study and same two global sequences. The ML phylogenetic tree showed the clustering of all the global isolates into a single clade except the two global sequences that found to be closer to the Indian samples and formed a separate clade along with the Indian isolates (Figure 3 B).

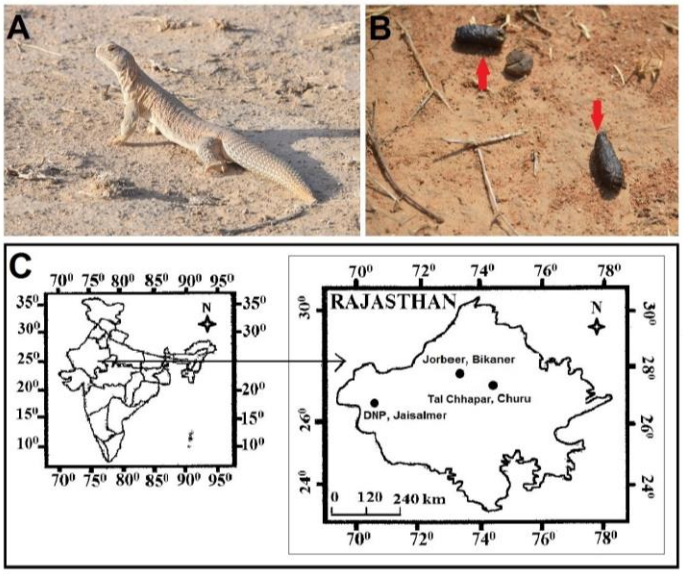

Fig 1: A. Adult spiny-tailed lizard (female), B. Pellets (marked in red arrow), C. Study area showing pellet collection sites.

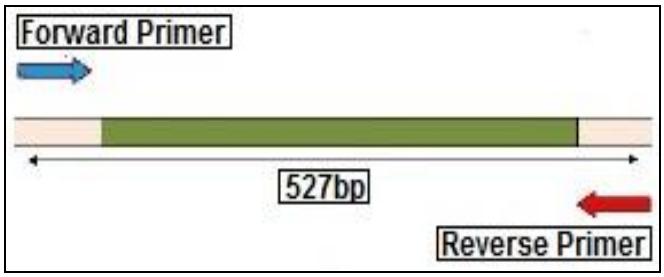

Fig 2: Structure of 16S RNA gene

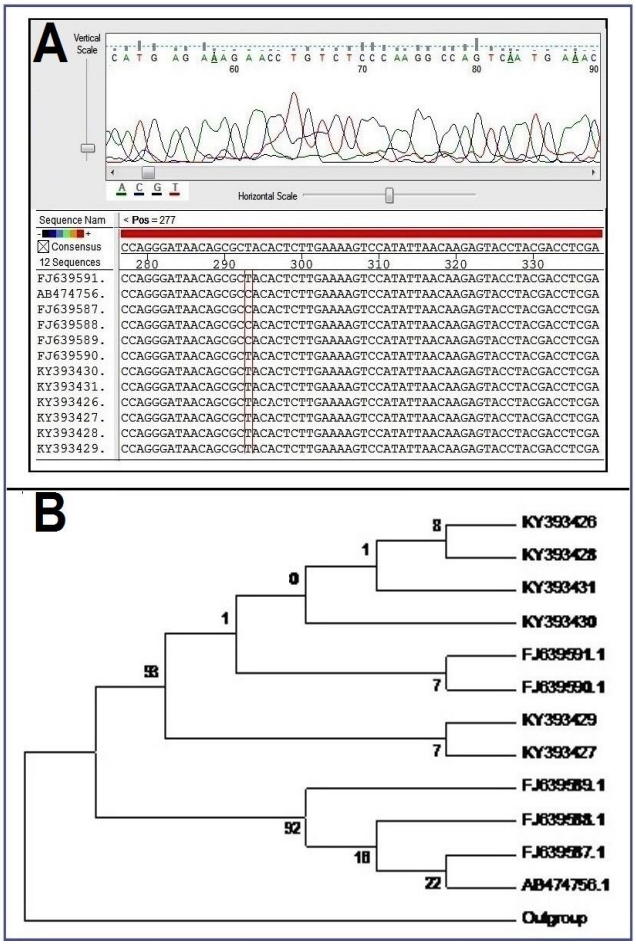

Fig 3: A. DNA sequence chromatogram in Seq Man and sequence alignment in Meg Align; **B.** Phylogenetic tree (numbers on the branches indicate percentage bootstrap replicates).

Table 1: Details of the primers used in amplifying 16S rRNA gene and sequences generated during this study

Primer	Primer sequence (5'-3')	Length (BP)	Annealing temp (°c)
Forward	5' TTAACGGCCGCAGAAATAAG 3'	20	63.7
Reverse	5' AGATCACGTAGGGCTTTAATCG 3'	22	63.1
Sample	Fragment size (BP)	Locality	GPS coordinate
KY393426; KY393427.	364	Tal Chhapar, Churu	27°48'36.6"N; 74°27'13.8"E.
KY393430; KY393431.	364	Jorbeer, Bikaner	27°59'04.3"N; 73°22'37.6"E.
KY393428; KY393429.	364	DNP, Jaisalmer	26°43'07.2"N; 70°35'45.0"E.

Table 2: NCBI retrieved 16Sr RNA sequences of *S. hardwickii* worldwide

Gene bank accession number	Geographic origin and locality	Reference
FJ639591.1	Unknown	Wilms *et al.* [14]
FJ639590.1		
FJ639589.1		
FJ639588.1		
FJ639587.1		
AB474756.1	Unknown	Amer and Kumazawa [27,28]

4. Discussion

Several studies in the past have been involved invasive techniques using the muscle tissue of *S. hardwickii* for DNA isolation and subsequent sequencing procedures [14, 27, 28]. However, acquiring genetic information through invasive techniques is challenging as they induce an unacceptable amount of stress to sensitive animals [29]. This study suggested the use of fecal matter more promising in comparison to the traditional DNA sources whose collection and use is quite difficult. While there is no tissue that can universally be applied to all research projects, fecal matter is a much more promising alternative to traditional invasive procedures which can be applied to study any species under threat [30]. In accordance to this, this study is a pioneer attempt on reptiles to use fecal pellets as source of DNA. The DNA from fecal samples yielded a good quality of amplifiable DNA and ultimately used for DNA sequencing and phylogenetic study.

Hitherto used all global isolates to trace phylogeney of *S. hardwickii* have unknown geographic origin and locality (Table 2). Five of these isolates (FJ639587.1 to FJ639591.1) have been used by Wilms *et al.* [14] to trace phylogeny of spiny-tailed lizards globally under genus *Uromastyx* in which *S. hardwickii*, *S. asmussi* and *S. loricata* were removed from the genus *Uromastyx* and resurrected under a new genus *Saara* based on both morphology and phylogeny. However, Wilms *et al.* [14] have also cleared that global distribution of *S. hardwickii* is restricted to India, Pakistan and Afghanistan. In this study, shared ancestry of two global isolates (Accession Number FJ639591.1 and FJ639590.1) used by Wilms *et al.* [14] was observed with Indian isolates which assures their Indian origin (Fig. 3 B). One of the reasons for this could be the high prevalence of *S. hardwickii* being involved in the pet trade and the movement of specimens worldwide for large scale poaching and commercial exploitation [13, 28]. Most importantly from MSA, all the sequences generated in this study found to be monomorphic. Since this species is conservation dependent and in India currently it exists in small pocket populations in northwestern part [14-23], DNA sequence monomorphism suggests that there is an urgent need of conservation of this species in its present distribution in northwestern India. However, this cannot be completely ascertained from this study because of the small sample size and thus study with large sample size might be useful for more accurate population genetic analysis.

5. Conclusion

The present study is one of the primary conservation attempts for Indian spiny-tailed lizard that shows non-invasive *techniques* can be used successfully for genetic study of any reptile in future. The study though short term, quite promising and revealed monomorphic nature of Indian isolates suggesting urgent conservation of the species in India. Results of this study further demand much more intensive studies taking into account a large sample size that might prove useful in giving a better picture on genetic variation and ancestral information of this species.

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