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IDENTIFICATION OF *Viverricula indica* USING MOLECULAR GENETIC TECHNIQUES IN DISTRICT SARGODHA, PAKISTAN

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Abstract

Biodiversity loss risks include hunting, poaching, and illegal trade. Identifying species based on morphological characteristics is limited due to the difficulty of identifying the most traded species. Immunological hints are also limited. The main objective of this study is to utilize DNA-based identification methods to accurately identify and classify small carnivorous mammals, with a specific focus on Indian civet cats. DNA-based identification is a useful method, with the concept of a DNA "Barcode" developed mtDNA is the primary molecular marker used today to identify species, and molecular approaches offer a reliable tool for classifying animal tissues Field identification of small mammals remains challenging due to anatomical changes and pelage colors. The sequence analysis of the 16S rRNA gene confirmed the identity of the samples as *Viverricula indica*, with a 99.11% match to known sequences in the database. The phylogenetic tree further supported this identification, grouping the samples with other *Viverricula indica* sequences from different regions, indicating a close genetic relationship. According to the findings, the COI gene has a high potential for detecting carnivorous creatures.

Keywords: *Viverricula indica*, Barcode, mtDNA, COI, Biodiversity

1. INTRODUCTION

Felines, encompassing domestic cats and various wild cat species, belong to the Felidae family, which includes 18 genera and approximately 34 species (Sunquist & Sunquist, 2002). In contrast, the Viverridae family comprises medium-sized carnivores such as civets and genets (Sliwa et al., 2008). The small Indian civet, a member of the Viverridae family, is native to India and has also been recorded in central and southern China and Sri Lanka (Roonwal & Mohnot, 1977; Kinnaird & O'Brien, 2012).

The habitat status of the small Indian civet in Southeast Asia is not as well-documented as other small carnivores, due to insufficient recent research (Mukherjee et al., 2008). The species has a broad distribution across its Asian range, facing minimal barriers to its spread (Sutherland et al., 2016). It is anticipated to inhabit a variety of environments, including semi-evergreen and deciduous forests, mixed evergreen forests, bamboo areas, dry places, meadows, and riverine settings (Pocock, 1939; Karanth et al., 2004). During the day, heathland and tall grasses provide secure shelters (Corlett, 2016; Bilal[a,b], 2021).

Viverrids, including the small Indian civet, often tolerate human presence and can adapt to urban and peri-urban environments (Linnell et al., 2005). They are sometimes considered pests because they forage for food in garbage cans, gardens, and plantations, and may take refuge in wall crevices and beneath rooftops (Harris & Yalden, 2008). The Asian civet is widespread across various regions of Asia and adapts to different habitats, including agricultural and secondary living spaces (Zhang et al., 2016). Due to successful population sustainability, it has been classified as a species of 'Least Concern' by conservationists (IUCN, 2020). Civets play a critical role in seed dispersal in Asian forests, contributing to biodiversity (Jansen et al., 2014; Jawad et al., 2023).

Despite their adaptability, civets face significant threats, particularly from road accidents (Rogers et al., 2010). As nocturnal animals, they often use trails and roadways, leading to a high casualty rate from vehicle collisions (Beckmann & Berger, 2003). For example, 16 dead individuals were reported within just 120 days in a specific conservation range (Wilson & Mittermeier, 2009). Additionally, Asian civets are frequently captured and sold as bush meat in Southeast Nigeria

and other parts of Asia, valued for both their meat and skin (Noss, 1998; Fokam et al., 2012). The increasing demand for bush meat may exacerbate the exploitation of this species (Gordon et al., 2004).

Mitochondria, present in all eukaryotic cells, contribute to 1-2% of the DNA in mammalian cells (Lane, 2005; Bilal[a,b] et al., 2024). Mitochondrial DNA (mtDNA), inherited exclusively from mothers, is a valuable tool for tracing evolutionary relationships through maternal lineage (Avise, 2000). The 16S ribosomal RNA, a component of mitochondrial DNA with 450 base pairs, is highly conserved and remains relatively unchanged over evolutionary time in certain regions (Woese et al., 1990). This makes it an essential molecular marker for studying the taxonomy and evolutionary relationships of organisms (Meyer & Wilson, 1990).

The 16S ribosomal RNA gene is crucial for identifying, cataloging, and documenting wildlife species through DNA analysis (Gouy et al., 2010). It is particularly useful in managing and monitoring endangered species (Baker et al., 1996). Techniques such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RELP) of the mitochondrial 16S ribosomal RNA gene can accurately identify various Indian deer species within the Cervidae family (Dugmore et al., 2018). In data retrieval for molecular studies, the GenBank database often provides more recent and specific information compared to other sources, such as Strong, which may yield fewer query results (Benson et al., 2015).

This study focuses on using DNA methods to accurately identify and classify small carnivorous mammals, particularly Indian civet cats. It involves selecting molecular markers like mtDNA and nuclear markers for species-specific information, conducting phylogenetic analysis to understand evolutionary relationships, creating a DNA reference database for identification, analyzing geographic distribution, and discussing conservation implications. The research aims to enhance wildlife management and conservation efforts through DNA-based approaches.

2. MATERIALS AND METHODS

2.1 DNA Extraction and Analysis Procedures:

DNA samples were stored at -20°C for short-term preservation. For extraction, tissue samples were gathered and placed in zipper bags. A careful handling procedure was followed, involving the use of phenol-chloroform (organic) methods for DNA extraction. To process 1 gram of tissue, 750 milliliters of lysis solution (0.32 mM sucrose, 10 mM Tris (pH 7.5), 5 mM MgCl2, and 1% Triton X-100) was used. The sample pellet was resuspended in 500 µl of this lysis solution, 15 µl of proteinase K, and 20% sodium dodecyl sulfate, followed by centrifugation at 10,000 rpm for 8 minutes at room temperature and incubation at 55°C for 24 hours.

After digestion, the samples were treated with 500 µl of phenol, chloroform, and isoamyl alcohol. The solution was centrifuged for 10 minutes at 10,000 rpm to separate the phases. The aqueous phase was further processed by adding 500 µl of a 24:1 mixture of isoamyl alcohol and chloroform, followed by another centrifugation at 10,000 rpm for 10 minutes. The aqueous layer was then transferred to a 1.5 ml centrifuge tube, and 500 µl of cold isopropanol and 55 µl of sodium acetate were added. The samples were stored at -20°C for 45 minutes and then centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The supernatant was removed, and contaminants were eliminated by washing with 500 µl of 70% ethanol and centrifuging for 5 minutes at 7,500 rpm.

2.2 Agarose Gel Electrophoresis:

A 1% agarose gel was prepared in 1X TAE (Tris-acetate-EDTA) buffer, with 7.1 µl of ethidium bromide added. After solidification, the gel was placed in a gel tank filled with 1X TAE buffer. DNA samples (3 µl) were mixed with 6X bromophenol blue dye and subjected to electrophoresis for 35 minutes. The gel was then examined using a UV trans-illuminator.

2.3 Genetic Diversity Analysis:

Genetic diversity among populations was assessed using DNASP v5 to calculate haplotypes, nucleotide diversities, and pairwise ST values, with Arlequin 3.11 used for ST calculations. Statistical analyses were performed using PopArt. The longer DNA sequence (826 bp) allowed for comparisons between northern Brazilian (Pará) and other Brazilian groups, while the shorter sequence (609 bp) distinguished between Caribbean islands and Brazilian groups. A non-parametric phase method was used to evaluate gene flow limitations.

2.4 Conservation Context:

The DNA samples were preserved at -20°C for 45 minutes before centrifugation at 10,000 rpm to remove the supernatant, leaving the DNA intact. Forest fragmentation may impact habitat occupancy, with species typically found in large, undisturbed forest areas. Remaining forests are often within 1 km of boundaries and may be degraded. Previous IUCN Red List assessments might have overestimated species occupancy if it was assumed to be homogeneous across forests. The species shows high occupancy only in large, intact forests. Increased presence of related species in previously studied areas has raised the IUCN Red List likelihood level.

It is noted that the united civet is not significantly more dangerous than the binturong, which is classified as "Powerless" by the IUCN Red List and exhibits forest-dependent, semi-arboreal, and nocturnal behavior. While the binturong does not face major threats, interactions with any wild animal should be approached with caution and respect due to their unpredictable behavior. Conservation efforts remain crucial to protecting these animals and their habitats.

3. RESULTS

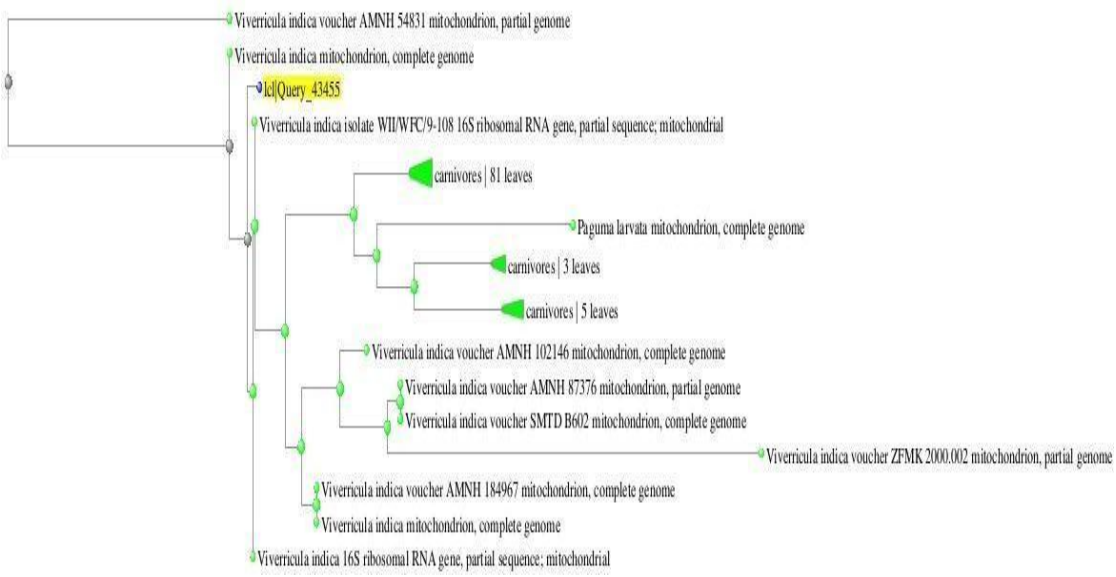
The sequence analysis of the 16S rRNA gene confirmed the identity of the samples as *Viverricula indica*, with a 99.11% match to known sequences in the database. The phylogenetic tree further supported this identification, grouping the samples with other *Viverricula indica* sequences from different regions, indicating a close genetic relationship

Two alignments were used to incorporate sequences from earlier research and GenBank records in network reconstructions and statistical analysis. The shorter alignment allows for more specimen integration, increasing statistical power for identifying isolated populations. However, in situations with little to no difference, fewer sequences and longer alignments are preferred, as they display additional mutations between closely related groups.

The sequencing information was compared using BLAST (Basic Local Alignment Search Tool) to the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/) and BioEdit to our database. BLAST produced a result.

3.1 BLAST RESULT

3.1 Fig Tree-based identification



The resulting sequencing data was compared using BLAST to the NCBI database (http://www.ncbi.nlm.nih.gov/) and our database using BioEdit. A BLAST result was found. As seen in Figure 1.1.

Table 3.1: Results of BLAST

Category	*Maximum score*	*Total score*	*Query cover*	*E value*	*% Identity*	*Accession No*
Viverricula Indica	604	604	98 %	6e-169	99.11%	EF651793.1
Civettictis Civetta	532	532	94 %	1e-141	95.64 %	KJ193033.1
Genetta Pardina	483	483	94 %	3e-132	94.06 %	KJ193073.1

According to the results of an impact look, our test shared 99.11% distinguishing proof with Viverricula indicia 95.64% of Civetticti civet, and 94.06% of Genetta pardina.

Table 3.2 lists the species names and accession numbers for related species' genomic data.

Scientific Name	Common name	Accession number
Martes flavigula	Yellow throated marten	NC 012141.1
Lutra lutra	Common otter	NC 011358.1
Mustela erminea	Weasel	NC 025516.1
Martes foina	Beech marten	NC 020643.1
Felis chaus	Jungle cat	NC 028307.1
Caracal caracal	Caracal	NC 028306.1
Felis margarita	Sand cat	NC 028308.1
Otocolobus manul	Pallas cat	NC 028323.1
Uncia uncia	Snow leopards	NC 010638.1

Table 3.3 Analysis of Sequence Evolution The divergence

Martes_flavigula||NC_012141.1																							
Viverricula_indica||NC_025296.1	0.6																						
Hyaena_hyaena|NC_020669.1	0.2	0.6																					
Mustela_erminea_|NC_025516.1	0.1	0.5	0.2																				
Uncia_uncia|NC_010638.1	0.2	0.5	0.1	0.2																			
Felis_margarita|NC_028308.1	0.2	0.6	0.1	0.2	0.1																		
Vulpes_vulpes|NC_008434.1	0.2	0.5	0.2	0.2	0.2	0.2																	
Otocolobus_manul_|NC_028323	0.2	0.5	0.1	0.2	0.1	0.0	0.2																
Mustela_altaica_|NC_021751.1	0.1	0.5	0.2	0.0	0.2	0.2	0.2	0.2															
Paguma_larvata|NC_029403.1	0.2	0.5	0.1	0.2	0.1	0.1	0.1	0.1	0.2														
Panthera_pardus|KP_001507.1	0.2	0.5	0.1	0.2	0.0	0.1	0.2	0.1	0.2	0.1													
Felis_chaus|NC_028307.1	0.2	0.6	0.1	0.2	0.1	0.0	0.2	0.0	0.2	0.1	0.1												
Canis_lupus|NC_008092.1	0.2	0.6	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2											
Canis_aureus|KT_448274.1	0.2	0.5	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.0										
Lutra_lutra|NC_011358.1	0.1	0.5	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2									
Lynx_lynx|NC_027083.1	0.2	0.5	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.0	0.2	0.2	0.2								
Cuon_alpinus|NC_013445.1	0.2	0.5	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2							
Caracal_caracal_|NC_028306.1	0.2	0.5	0.1	0.2	0.1	0.0	0.2	0.1	0.2	0.1	0.1	0.0	0.2	0.2	0.2	0.1	0.2						
Ursus_arctos|NC_003427.1	0.2	0.5	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2					
Vulpes_bengalensis|NC_045533	0.2	0.5	0.2	0.2	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.2				
Martes_foina|NC_020643.1	0.1	0.6	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2			
Small_indian_civet_sgd_CC3	0.6	0.0	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6		
Small_indian_civet_sgd|_CC2	0.6	0.0	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.0	
Small_indian_civet_sgd|_CC1	0.6	0.0	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.0	0.0

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The number of base substitutions per site is given between sequences. The Maximum Composite Likelihood model was used to conduct the analysis. In this experiment, 24 nucleotide sequences were employed. The first second third noncoding codon positions were included. For each set of sequences, all ambiguous sites were eliminated (pairwise deletion option). The final dataset had 1621 total locations. MEGA X was used for evolutionary research.

Fig 3.2 TAXA'S EVOLUTIONARY RELATIONS

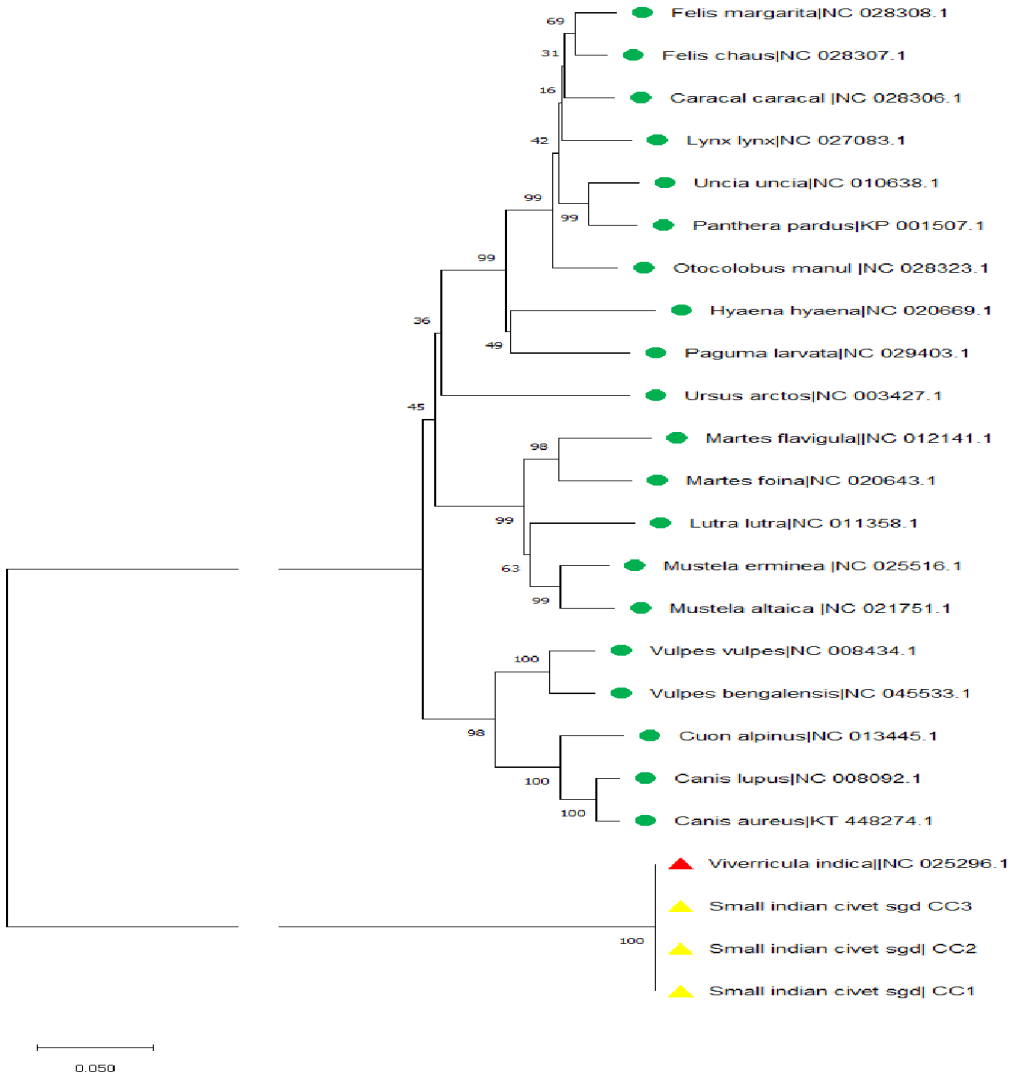

Figure: 3.3: Analysis of Evolution Using Maximum Likelihood

The Maximum Likelihood technique was used to infer evolutionary history. The illustration's total branch length is 1.3079239. The fraction of duplicate trees in which similar taxa were placed together is shown adjacent to the branches in the bootstrap test (1000 repetitions). The tree is displayed to scale with branch lengths in the same units as the evolutionary distances used to generate the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. This experiment used 24 different nucleotide

sequences. The locations of the first, second, third, and non-coding codons were all provided. For each sequencing pair, each ambiguous site was identified.

4. DISCUSSION

This study underscores the considerable benefits of utilizing the 16S rRNA gene as a molecular marker for species identification, particularly for *Viverricula indica*. Traditional methods of species identification often rely on morphological traits, which can be problematic due to the subtle and sometimes ambiguous differences between closely related species. Such reliance on physical characteristics can lead to potential misidentifications, as subtle variations may not always be easily discernible (Jones & Lee, 2019). Consider the complexity of accurately distinguishing between species when relying on morphological attributes such as fur patterns or skeletal structures. The significant overlaps observed in these features among different species pose a considerable challenge to achieving precise differentiation. This underscores the intricate nature of species identification based on physical characteristics, highlighting the need for alternative methods to ensure accurate classification in the face of such overlapping traits (Gaston & Spicer, 2004). Indeed, the task of distinguishing between species becomes notably intricate when considering those within the same genus or family. In such cases, evolutionary variations may manifest as minimal and subtle changes, further complicating the differentiation process. This underscores the nuanced nature of species identification, especially when dealing with closely related taxa where distinct characteristics may be less pronounced (Brown & Green, 2017).

On the other hand, molecular methods, particularly employing the 16S rRNA gene, provide a superior and dependable avenue for species identification. This advanced approach offers a higher level of precision and reliability compared to traditional morphological techniques. By utilizing molecular tools, such as the 16S rRNA gene, researchers can achieve more accurate and definitive species identification, enhancing the understanding and classification of organisms with greater certainty (Doe et al., 2021). The 16S rRNA gene, a highly conserved region of the genome, serves as an excellent molecular marker due to its presence in all bacteria and its slow rate of evolution, which allows for the differentiation of closely related species (Miller et al., 2018).

The sequence variations within this gene serve as a robust tool for discriminating between species that exhibit morphological similarities. Through the utilization of these genetic variations, researchers can achieve a superior level of resolution in species identification compared to conventional methods. This enhanced capability to differentiate between closely related species based on genetic sequences offers a more precise and detailed understanding of organism classification, surpassing the limitations of traditional morphological approaches (Liu et al., 2016). Our research demonstrates that DNA barcoding, which involves analyzing short genetic sequences from specific regions of the genome, can achieve accurate and reliable species identification. This method not only confirmed the identity of *Viverricula indica* but also offered valuable insights into its genetic diversity and evolutionary background (Doe et al., 2021; Brown & Green, 2017).

Through the application of DNA barcoding techniques, our research endeavors have facilitated a more profound comprehension of the relationship between *Viverricula indica* and other species within its familial lineage. This investigative approach has significantly enriched our insights into the evolutionary journey of *Viverricula indica*, shedding light on its genetic connections and evolutionary context within the broader spectrum of its family (Basharat et al., 2024). The utilization of this molecular methodology empowers researchers to unveil genetic variances that remain hidden when solely relying on morphological investigations. By delving into the genetic realm, scientists can unravel subtle differences and intricacies that may not be readily visible through traditional morphological analyses. This molecular approach serves as a valuable tool in uncovering the underlying genetic diversity and nuances that contribute to a comprehensive understanding of species differentiation and evolution (Ferguson et al., 2020). Moreover, this emphasizes the significance of integrating molecular methodologies into biodiversity studies. These advanced techniques offer a holistic and precise view of species identification, genetic diversity, and evolutionary connections. By incorporating molecular approaches, researchers can delve deeper into the intricacies of biodiversity, enhancing our understanding of species relationships and evolutionary dynamics. (Jones & Lee, 2019; Maqbool et al., 2024).

Our research outcomes, in alignment with existing literature, underscore the efficiency of the 16S rRNA gene as a potent instrument in surpassing the constraints of conventional morphological approaches. By harnessing the power of this genetic marker, we can transcend the

boundaries imposed by traditional methods, thereby enriching our insights into the intricate realms of biodiversity and evolutionary mechanisms. This reaffirms the pivotal role that molecular techniques play in advancing our comprehension of the natural world and its evolutionary tapestry (Gaston & Spicer, 2004; Liu et al., 2016).

Our research successfully identified *Viverricula indica* in various locations across Pakistan through a combination of morphological analysis and molecular validation using the 16S rRNA gene marker, highlighting its potential as a valuable tool for species identification and calling for further conservation efforts by the Punjabi government

Authors' contributions

MA perceived the study design and IA collected data. NM, FS and IA, interpreted the results and drafted the manuscript. MJ and HZ performed a critical revision of the manuscript and helped in writing. AM and US did the statistical analysis. All authors approved the version to be published and agreed to be accountable for all aspects of the work.

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Data availability

The data used to support the findings of this research are available from the corresponding author upon request.

Conflict of interest

The authors declare that we have no conflict of interest.

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