Review Article

COI-BASED DNA BARCODING OF PAKISTANI FAUNA: PROGRESS, CHALLENGES, AND FUTURE DIRECTIONS, A REVIEW

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ABSTRACT

DNA barcoding using the cytochrome oxidase I (COI) gene has revolutionized species identification and biodiversity research in Pakistan since 2006. This review assesses the progress, trends, and challenges of COI-based DNA barcoding in Pakistani fauna from 2006 to 2025. A total of 120 articles were downloaded, of which 91 were retained based on strict relevancy using PRISMA flow. Research efforts are unevenly distributed, with insects, particularly mosquitoes and fruit flies, receiving the most attention. However, mammals, amphibians, marine organisms, fungi, and microbes remain largely underrepresented. Geographic coverage and sample sizes vary widely, affecting statistical reliability and species representation. Methodological inconsistencies, such as unreported collection sites and varying trapping techniques, limit reproducibility and comparative analysis. Genetic divergence data reveal inconsistencies, with conspecific distances typically between 0-2% but sometimes reaching extreme values (e.g., 67.10%), suggesting sequencing errors, mislabeling, or reflecting data quality problems. Congeneric distances also vary significantly, emphasizing the need for taxon-specific barcoding thresholds. Phylogenetic analyses predominantly use MEGA software, with Neighbor-Joining and Maximum Likelihood methods being most common, while Bayesian inference remains underutilized, which provides statistical robustness and rate heterogeneity among models. The publication trend was slow from 2006 to 2012 but showed steady growth from 2013 to 2021 and a sharp rise from 2022 onwards, driven by increased funding and technological advancements. Research is mainly published in international journals, with some contributions to national journals such as the Pakistan Journal of Zoology. To enhance DNA barcoding in Pakistan, improvements such as expanded taxonomic and geographic coverage, standardized methodologies, increased data sharing, and integration with multigene approaches are necessary. Addressing these gaps will improve the accuracy and global relevance of COI-based DNA barcoding.

Keywords: DNA barcoding, COI gene, Biodiversity, Species identification, Pakistan fauna, Genetic divergence, Review

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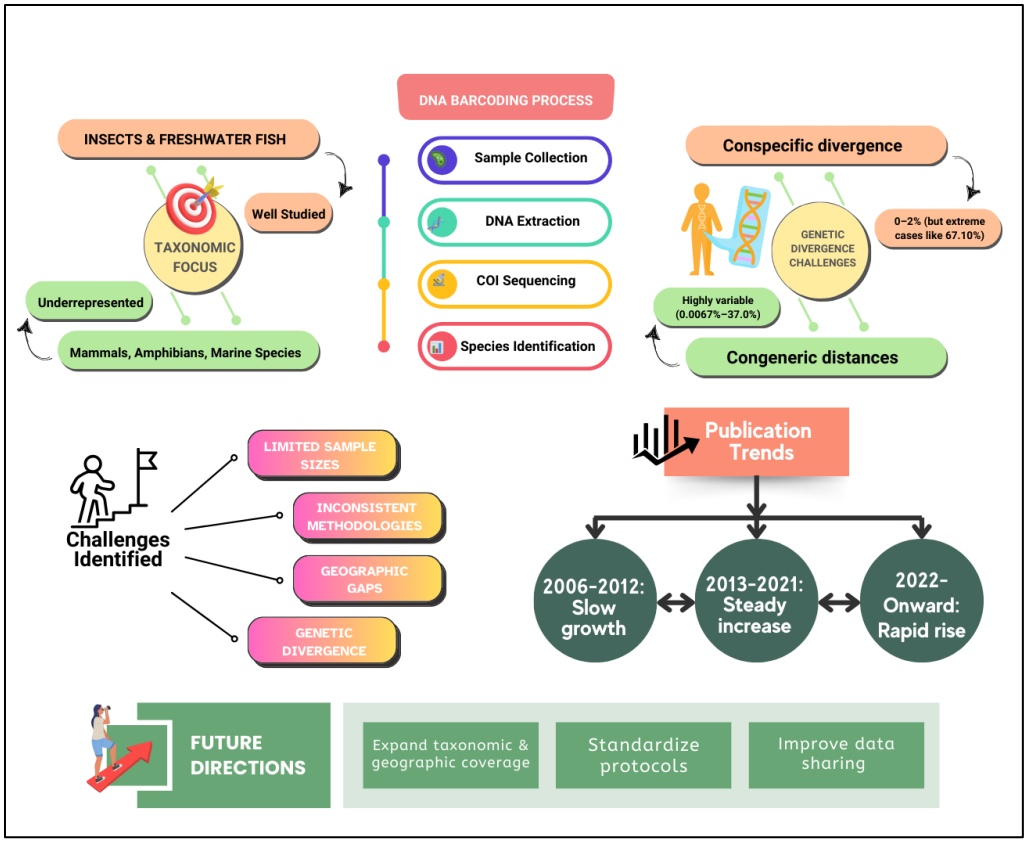

Graphical Abstract

INTRODUCTION

DNA barcoding has revolutionized taxonomy and systematics, enabling rapid and accurate identification of species. In the DNA barcoding approach, a short, standardized DNA fragment, such as the COI gene, is used for species identification. It has facilitated the discovery of new species, invasive species, and the monitoring of threatened populations (Antil *et al*., 2023). Pakistan harbors significant faunal diversity, which requires accelerated DNA barcoding efforts (Baig and Al-Subaiee, 2009). As Pakistan is located at the crossroads of the oriental and Palaearctic zoogeographic regions (Dikshit and Dikshit, 2025; Mirza, 1975) it boasts a unique diversity. Tropical and temperate forests, deserts and grasslands, mountains, and water bodies are rich in species both in diversity and richness.

Despite the rich diversity, Pakistan faces challenges that hinder the application of DNA Barcoding (Joly *et al*., 2014). The limited availability of taxonomic expertise, limited resources, and lack of infrastructure limit the use of DNA barcoding. Furthermore, this biodiversity is facing threats such as the destruction of habitat, climate change, overexploitation of resources, and pollution. These challenges can be covered with the valuable tool of DNA barcoding, lessening gaps and providing opportunities, such as cutting off laboratory facilities at the national level. International collaboration and knowledge sharing enable researchers to compare species from different regions.

Globally, researchers have made considerable progress in applying COI-based DNA barcoding for species identification (Emerson, 2025), however, research efforts in Pakistan remain limited and fragmented. Large portions of the country's rich fauna are still uncharacterized and limited to a few taxonomic groups. Moreover, there is a lack of a comprehensive assessment of the DNA barcoding status initiatives in Pakistan. Furthermore, little information is available on taxonomic coverage, institutional contributions, and publication trends. The absence of coordinated research networks and centralized databases has further widened the gap. Therefore, these gaps highlight the need for a systematic review that synthesizes current knowledge, identifies achievements and limitations, and outlines future directions for strengthening DNA barcoding research in Pakistan.

This study is needed because it brings together, for the first time, a systematic overview of COI-based DNA barcoding in Pakistan. Additionally, it documents history, limitations, achievements, and emerging trends in DNA barcoding. Earlier studies focused on isolated groups or were limited to restricted locations; this review provides a

broader national-level perspective. Moreover, no published review study is available on COI-based DNA barcoding from Pakistan. The current study is a review article that compiles, synthesizes, and critically analyzes published research on COI-based DNA barcoding in Pakistan from 2006 to 2025. This review aims to provide a comprehensive overview of the history, progress, challenges, and limitations of COI-based DNA barcoding in Pakistan, with the goal of guiding future research priorities and the acceleration of the use of DNA barcoding as a tool for conservation of biodiversity and management in Pakistan.

MATERIALS AND METHODS

The current review has focused on the COI-based DNA barcoding in Pakistan. The first published article was in 2006, and it continued through 2025. The data reveal that the COI-based DNA barcoding accelerated over the years. The study focused on articles on DNA barcoding, specifying COI as the gene of choice. The geographic focus is on Pakistan, including articles on barcoded animals from different regions, including the Provinces of Sindh, Balochistan, Punjab, Khyber Pakhtunkhwa, and Gilgit Baltistan, further explored in the following sections.

In the current review, a total of 120 articles were downloaded from research sites such as Google Scholar, PubMed, Web of Science, and Scopus using predefined Boolean search strings. The primary search string was: ("DNA barcoding" OR "DNA barcode" OR "COI-based DNA barcoding") AND ("COI gene" OR "cytochrome c oxidase I") AND "Pakistan" AND ("fauna" OR "animals" OR "insects"). Additional searches were performed using modified combinations of these keywords to ensure comprehensive coverage. Some of the articles (21 articles) were excluded from the study because.

- The current review article focuses on the COI gene only.
- 12S rRNA gene and Cyt-b genes are not the focus of the study, and
- Review articles on single species.

Finally, only 91 articles were retained for further study (**Supplementary Figure 1**). The PRISMA model was used for the screening process, which included steps like identification, screening, eligibility, and inclusion. The whole data was summarized in a Microsoft Excel Sheet (MS Office). Each article was studied for data extraction, with specialized columns for each. The columns for data extraction included Classification, Common Name, Congeneric Divergence, Conspecific Divergence, Most Abundant, Total Species Identified, No. of Samples, Total No of Collection Sites, Sites, Article Type, Trapping Method, Study Duration, Phylogenetic Analysis, Primer Pair, DNA Extraction Organ, Journal Name, Published Year, and Reference. Tables were also designed using the same Excel Sheet due to its ease of use. Figures were designed using R, Microsoft Excel, and GIS packages.

Research Progress and Publication Trends: DNA barcoding has advanced significantly in Pakistan over the past two decades. A closer examination reveals a shift from initial slow adoption to a recent surge in research activity (**Fig. 1**). It can be further observed through three main stages of development as follows.

Slow Progress (2006–2012): The first publication in the current dataset goes back to 2006 (Memon *et al*., 2006), marking the introduction of DNA barcoding in Pakistan. After that, no study appeared until 2010 (Ashfaq *et al*., 2010), suggesting isolated early efforts and a large gap (**Fig. 1**). A lack of widespread consistency and very limited institutional support during this era can be observed, for example, as evidenced by a study (Iftikhar*et al*., 2016).

Gradual Growth (2013–2021): The frequency of publications increased soon after 2013, with intermittent research activity through 2016, with a modest rise in output, with five publications. The period shows an increase in interest in DNA barcoding, likely driven by global recognition of its role in biodiversity conservation and species delimitation. During the years between 2017 and 2021, the output remained steady with multiple publications for each year. This duration shows the prominent research focus on DNA barcoding in Pakistan. Institutions and researchers recognized the importance of DNA barcoding during this phase.

Rising Research Trends (2022-2025): The dataset shows a sharp increase in publications starting in 2022. Peak publication occurred during 2023 and 2024 (**Fig. 1**), marking the most productive years. This is possibly fueled by increased funding, greater understanding of taxonomic and ecological applications, and an advancement in sequencing technologies. The dataset marks 2024 as an all-time high in research output with 10+ publications, while the momentum is sustained for 2025 (Anjum *et al*., 2025), indicating a robust and expanding research community in the field.

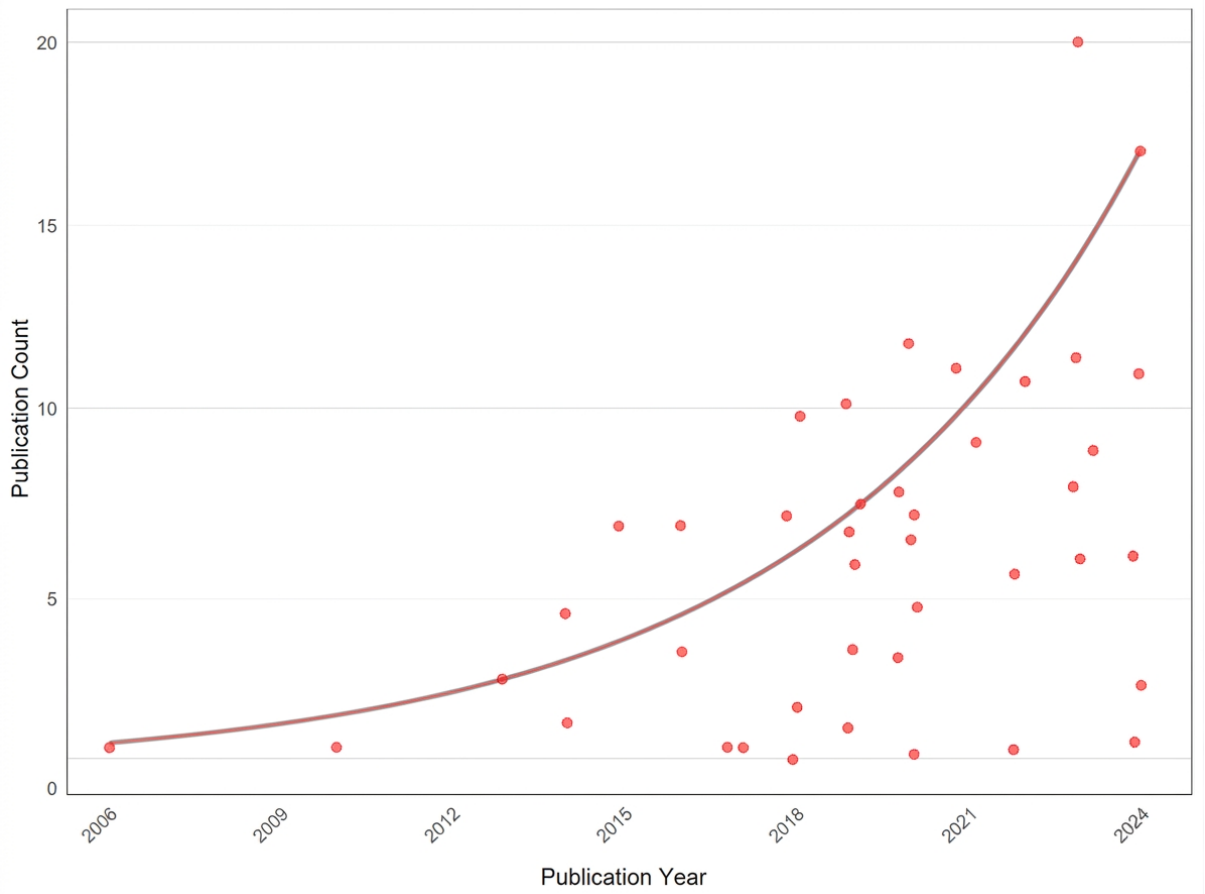

Fig. 1. Temporal Distribution of DNA Barcoding Publications: A scatter plot illustrating individual publications across the years, highlighting trends in research activity over time.

Factors Affecting Growth: Several factors have contributed to the recent acceleration in DNA barcoding in Pakistan. Bioinformatics tools and sequencing platforms have likely facilitated this domain. It further shows the increase in government and private funding for this sector, as noted in the funding section of many articles. The Canadian Centre for DNA barcoding is credited with global influence and collaboration in Pakistan. Finally, as Pakistan is blessed with rich biodiversity (Baig and Al-Subaiee, 2009), therefore, conservation efforts need rapid identification, which is a growing factor in accelerating studies. This shift from foundational studies to more applied research indicates a promising future for molecular taxonomy and biodiversity assessment in the country (Akhtar and Ali, 2016). Further investigation into specific research themes, institutional contributions, and funding trends would provide deeper insights into the progress and future trajectory of DNA barcoding research in Pakistan (Ruedi *et al*., 2023). The publication trend, as is evident from the dataset, highlights the diverse range of journals, and a closer look reveals key insights into the scope, impact, and reach of DNA barcoding research in the country (**Table 1**).

***Table 1*. Methodology, study, and publication details (Number of Samples, Number of Collection Sites, Article Type, Study Duration, DNA Extraction Source, Journal, Reference). (See the complete Table in Supplementary Table 1).**

No. of Samples	Total No of Collection Sites	Article Type	Study Duration	DNA Extraction Source	*Journal Name*	Reference
1684	491	Research Article	2010-2013	Single Leg	*PLOS One*	(Ashfaq *et al*., 2014a)
4503	329	Research Article	2010-2013	Single Leg	*PLOS One*	(Ashfaq *et al*., 2017)
589	255	Research	2010-2013	Single Leg	*PLOS One*	(Ashfaq *et al*.,

		Article				2014b)
60273	1858	Research Article	2010-2019	Single Leg	*PeerJ*	(Ashfaq *et al*., 2022)
495	158	Research Article	2009-2012	Not Provided	*PLOS One*	(Iftikhar *et al*., 2016)

International Dominance: Internationally recognized high-impact journals have published a significant portion of articles on DNA barcoding. A well-regarded open-access platform, PLOS One, appears frequently, indicating it has accepted well-reputed research studies. The overview shows that PLOS One accepts articles with a greater sample size (Ashfaq *et al*., 2014b; Iftikhar *et al*., 2016). *PeerJ, Scientific Reports, Frontiers in Physiology,* and *Molecular Ecology Resources* are the most frequently appearing journals with a high number of publications in DNA barcoding from Pakistan, helping in increasing visibility (Ashfaq *et al*., 2022). Other journals, such as *Mitochondrial DNA Part A and B,* and *Molecular Biology Reports,* reflect a strong focus on these studies. It is also evident that Mitochondrial DNA-like journals should focus on the acceptance of DNA barcoding, a central theme of the mitochondrial genome. National and local journals have contributed significantly to the research on DNA barcoding, as evidenced by the inclusion of articles in the *Pakistan Journal of Zoology, Punjab University Journal of Zoology, Journal of Bioresource Management,* and others (**Table 1**).

Multidisciplinary and Applied Research Trends: Some journals, such as *Applied Ecology and Environmental Research, Journal of Freshwater Ecology,* and *Journal of Materials and Environmental Science,* highlight the importance of DNA barcoding beyond pure taxonomy (Anjum *et al*., 2025; Khan *et al*., 2023). These journals have addressed ecological and environmental concerns, including species conservation, ecosystem health assessment, and biodiversity monitoring. More broad fields, such as veterinary sciences, livestock, and agriculture, are also in focus as barcoding is used in these fields, as evidenced by publications in the *Journal of Applied Research in Plant Sciences* and *Frontiers in Veterinary Sciences* (Ali *et al*., 2024a). The presence of greater data accessibility on open-access platforms like *Research Square* suggests that data presentation is also necessary for broader studies within the scientific community (Asif *et al*., 2023)

Publication trends indicate that DNA barcoding should be a focus of interdisciplinary journals to be fruitful for the wider scientific community. Both national and international journals are placing greater focus on DNA barcoding research in Pakistan (**Table 1**). A shift toward biodiversity conservation, ecology, and agriculture is an indicator of practical applications in the fields. Notably, the assessment of journal metrics, such as impact factor and citation metrics, should be carried out by institutions to understand how Pakistan's research is influencing global barcoding studies. This rapid growth should also be assessed to determine the funding sources and affiliations of publications.

Sources of DNA Extraction: Two important aspects of DNA barcoding are the dependence of success on the quality and reliability of DNA extracted from biological samples. In the current review, **Table 1** summarizes a diverse range of organs and tissues used to extract DNA. Variation among samples is a factor in species type, research objectives, and ethical considerations.

Arthropod Studies: Dominance of Leg Samples: The dataset shows single or multiple legs of arthropods as the primary source of DNA (**Table 1**). This organ yields sufficient genetic material for analysis, while remaining nonlethal for insects. Studies show that only one or two legs of adults were used, indicating minimal species damage and preservation for morphological assessment, thereby integrating genetic and morphological methods for identification.

Vertebrate Studies: Muscle and Whole-body samples: Muscles, dorsal muscle tissue, muscle pieces, and pectoral fins were terms used by different articles for DNA barcoding. Fish, amphibians, and mammals are shown to be barcoded using sources from muscle tissue. Insects, larvae, and certain aquatic species have been barcoded using whole-body tissue. Some studies have used the whole body except for the removal of 1cm of the anal region, a possible focus to ensure uncontaminated DNA samples.

Mammalian and Avian Barcoding: Blood and Internal Organs: Several studies have used blood samples for DNA barcoding in birds and mammals (Asif *et al*., 2023; Rehman *et al*., 2015). It is a non-invasive or minimally invasive sampling method. Many studies have used internal organs such as the spleen, liver, and tail tip, which may involve integrating disease studies, population genetics, and molecular diagnosis. Chiropteran biodiversity has been extensively considered, as evidenced by DNA extraction from patagium and hind leg tissue samples specific to this group. Keel tissues and feather follicles are extensively reported from birds; the latter are used non-invasively for barcoding studies.

Aquatic and Reptilian Species: Fins, Scales, and Skin Samples: Studies involving fish and reptiles have been reported with DNA extraction from fins, scales, and skin. DNA barcode studies have extensively used caudal and pectoral fins

because it provides high quality DNA. Skin and scale samples are good sources of DNA when collected fresh with little or no harm to the species.

Primer Selection: The effectiveness of the COI gene as a standard for DNA barcoding largely depends on the selection of universal primers. Several primers utilized in Pakistan are being discussed in the text that follows.

Universal Primers are designed to facilitate DNA barcoding across a broad range of taxa (Ali *et al*., 2024b; Ullah *et al*., 2018). These primers (**Table 2**) were originally developed for arthropods' barcoding, but are now applied extensively to fish, reptiles, insects, and mammals because of high success. Some primers are used for samples that have degraded DNA, such as museum specimens or forensic samples (Hussain et al., 2025).

Table 2. Details of Primers used in different studies from Pakistan.

Primer Name	Primer Length	Reference
LCO1490 and HCO2198	Forward primer: 5'-GGTCAACAAATCATAAAGATATTGG-3' Reverse primer: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	(Ahmad *et al*., 2019a; Khan *et al*., 2020)
RepCOI-F and RepCOI-R	Forward primer): 5'-TNTTMTCAACNAACCACAAAGA-3' Reverse primer: 5'-ACTTCTGGRTGKCCAAARAATCA-3'	(Hussain *et al*., 2025)
C_LepFolF and C_LepFolR LepF1 and LepR1	Forward primer: ATTCAACCAATCATAAAGATATTGG Reverse primer: TAAACTTCTGGATGTCCAAAAAATCA	(Naz *et al*., 2023a; Sajid *et al*., 2021)
LepF2_t1 and LepR1	Forward primer: TGTAAAACGACGGCCAGTAATCATAARGATATYGG Reverse primer: TAAACTTCTGGATGTCCAAAAAATCA	(Naz *et al*., 2023a)
FishF1 and FishR1	Forward primer: 5'-TCAACCAACCACAAAGACATTGGCAC-3' Reverse primer: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	(Karim *et al*., 2024; Sajjad *et al*., 2023)
FishF2 and FishR2	Forward primer: 5'-TCGACTAATCATAAAGATATCGGCAC-3' Reverse primer: 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'	
BirdF1 and BirdR1	Forward primer:TTCTCCAACCACAAAGACATTGGCAC Reverse primer: ACGTGGGAGATAATTCCAAATCCTG	(Awan *et al*., 2013)
UniMinibarF1 and UniMinibarR1	Forward primer:TCCACTAATCACAARGATATTGGTAC Reverse primer: GAAAATCATAATGAAGGCATGAGC	(Khan *et al*., 2018)
L-turtCOIF and H-turtR	Forward primer: TACCTGTGATTTTAACCCGTTGAT Reverse primer: TGGTGGGCTCATACAATAAAGC	(Ali *et al*., 2024b)

Lepidoptera is one of the most diverse orders of the class Insecta. Lepidopteran primers have yielded good results in moths and butterflies, ensuring reliability in amplification and sequencing (Naz *et al*., 2023b; Sajid *et al*., 2021) (**Table 2**). In Pakistan, two sets of primers have been used successfully for DNA barcoding of fish (Karim *et al*., 2024; Sajjad *et al*., 2023). Birds, the drivers of the ecosystem, have been a focus of DNA barcoding in Pakistan. Several species have been identified and analyzed using the bird primers given in **Table 2** (Awan *et al*., 2013).

Mini barcodes are special primers that are the focus of museum specimens, environmental DNA (eDNA), and forensic studies (Khan *et al*., 2018). A known successful primer for degraded DNA barcoding is presented below. Notably, this primer is ideal for shorter DNA sequences. Specific primers designed for the identification and characterization of sea turtles (Ali *et al*., 2024b) are also the key focus of turtle-based studies. Some of the primer sets are specialized for only a known species, and no universal study has declared them to be universal or applicable to other groups. Moreover, many of the studies have designed primer sets for their own. These were designed with the Primer3 application (Akhtar and Ali, 2016).

The selection of primers is crucial to the success of DNA barcoding. Universal primers like LCO1490/HCO2198 remain widely used across taxa, while group-specific primers (e.g., FishF1/FishR1 for fish, LepF1/LepR1 for Lepidoptera) offer higher amplification success for organisms. Mini-barcoding primers such as UniMinibarF1/UniMinibarR1 have expanded the application of DNA barcoding to degraded samples (Meusnier *et al*., 2008). The continuous development and optimization of primers will further enhance the effectiveness of DNA barcoding in taxonomy, biodiversity monitoring, and conservation efforts.

Phylogenetic Models and Softwares: One of the purposes of DNA barcoding is to find evolutionary linkages between species and to study their phylogenetic relationships. Several studies have used software packages and models to asses evolutionary relationships and phylogenetic tree construction, summarized in **Fig. 2**. Each of these has its own strengths and weaknesses. In the current review, the used packages include MEGA (various versions), Bayesian Inference, DNAStar, DnaSP, DNAMAN, and Fast Minimum Evolution Algorithm, along with phylogenetic models such as Jukes-Cantor and Neighbor-Joining (NJ) methods illustrated in **Fig. 2**.

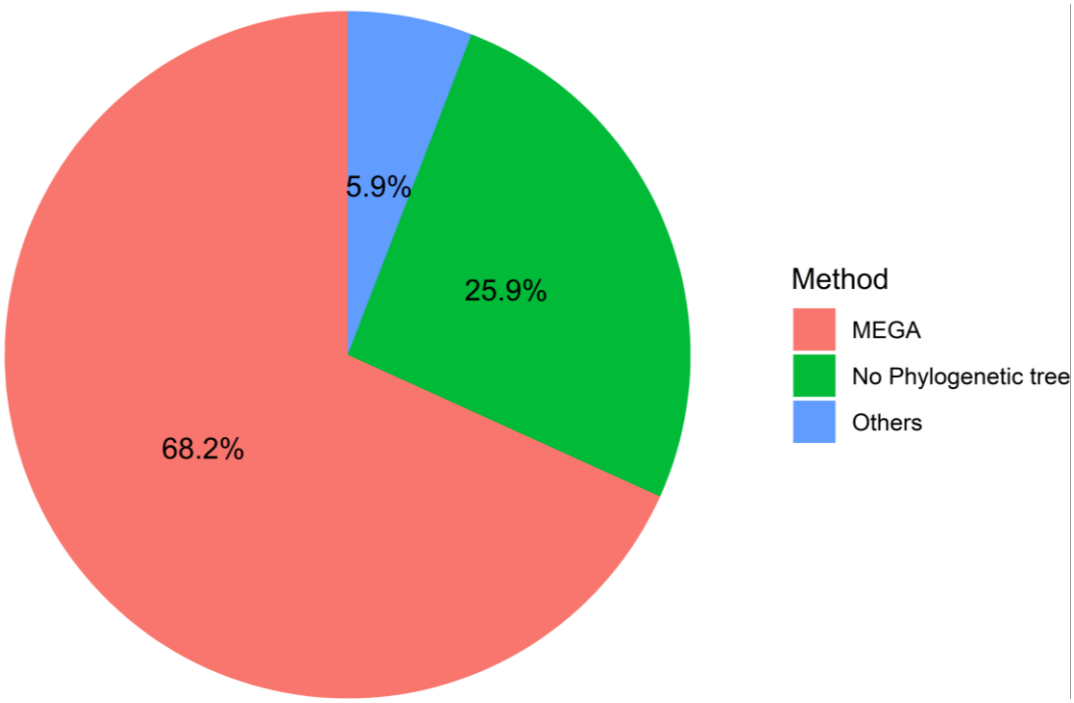

Fig. 2. Distribution of phylogenetic analysis methods used in the study. The majority of analyses were conducted using MEGA (58%) and 'No Phylogenetic Tree' (22%).

MEGA Software: In the current dataset, MEGA is one of the most used software with multiple versions, including MEGA 5, MEGA 6, MEGA 7, MEGA X, and MEGA 11 (Kumar *et al*., 1994). This software package includes several user-friendly options. Firstly, it is user-friendly, with a graphical user interface that is easy to navigate, making it easy for beginners. Secondly, it supports comprehensive features like multiple sequence alignment, construction of phylogenetic trees, evolutionary model selection, and provides ways for statistical validation. Thirdly, MEGA supports multiple algorithms, including Maximum Likelihood (ML), Minimum Evolution (ME), Neighbor-Joining (NJ), and Maximum Parsimony (MP). Fourthly, MUSCLE and ClustalW, both integrated alignment tools, are supported by MEGA. Finally, it runs on Windows, Mac, and Linux systems, works offline, and is updated regularly with new features and bug fixes (Keklik, 2023). However, MEGA is not optimized for high-performance computing, lacks flexibility, consumes significant RAM with large sequences, lacks Bayesian inference, and is less efficient for intensive analyses (Hall, 2013).

Bayesian Inference: It is a more sophisticated and alternative method for phylogenetic analysis. It is often implemented in MrBayes, BEAST, and PhyloBayes (Huelsenbeck and Ronquist, 2001). It has several advantages, such as robust statistics, being suitable for handling evolutionary rate heterogeneity, and being able to be utilized with complex evolutionary models. It is limited too due to its intensive computational requirements, and requires expertise in Markov Chain Monte Carlo (MCMC) methods.

Fast Minimum Evolution Algorithm: A rapid phylogenetic tree can be constructed with this package. It is faster than ML and NJ methods and is sufficient for the assessment of biodiversity on a large scale (Desper and Gascuel, 2002). Its use is limited due to underestimating evolutionary distances and its potential for less accuracy than Bayesian inference and ML.

Jukes-Cantor Model: It is a simple substitution model that is fast and easy, but it is only useful for basic phylogenetics. Its results are often unrealistic due to the assumption of equal base frequencies and are less accurate than General Time Reversible models (Erickson, 2010).

DNAMAN and DNA Star: These are commercial tools used for DNA sequence analysis. These are user-friendly and support multiple alignments; however, they are not publicly accessible and are not as feature-rich as MEGA or others.

Analysis of Study Duration: The aim of DNA barcoding is to analyze species richness and diversity, genetic variability, and patterns of evolution over time. Therefore, the duration of study or period of sampling is a crucial factor in the reliability and robustness of DNA barcoding (Skalak *et al*., 2012). In the present dataset, a significant inconsistency has been observed, such as the unviability or unevenness of duration (**Table 1**).

Importance of Study Duration DNA Studies: Several aspects of the study on DNA barcoding are affected by the duration. Many species are available only at a specific time or season of the year. Species vary in behavior, population structure, and genetic diversity. For example, insects and birds often have specific breeding seasons. Short-term research studies in DNA barcoding may lead to failure in capturing fluctuations and are biased. Moreover, long-term studies may help in detecting genetic changes over the years, adaptation, and genetic drift. In the current review, studies that spanned between 2010 and 2019 (Ashfaq *et al*., 2022) and 2017-19 (**Table 1**) have provided more insights than those that were limited to a season or so. Climate change and habitat alteration are also factors of influence. They affect genetic diversity and species distribution. Studies that span from June 2014 to October 2017 or from August 2018 to July 2022 allow researchers to actively study climate impacts and habitat factors, whereas short-term studies fail to do so. Short-term studies provide a limited snapshot of genetic diversity. While studies that sample for years provide a robust, reliable view of diversity.

Patterns Observed: Based on the study duration in the current dataset, four major trends can be observed. Firstly, short-term studies lasted only a few months (March-August 2023, October-February, May, and October 2020) (**Table 1**). These studies contribute valuable data but lack seasonal representation. Secondly, medium-term studies lasting one to three years offer more insights. Thirdly, long-term studies that spanned from 2010 to 2019 and 2018 to 2022 provide stronger and more reliable datasets that capture multi-year genetic variations and environmental influences. Finally, some studies collected samples at irregular intervals, such as selected months from 2009, 2012, 2013, 2014, and 2015 (Liu *et al*., 2017) which may affect comparability and consistency in DNA barcoding.

Future studies may incorporate mandatory reporting of study durations, long-term monitoring, seasonal considerations, and standardized methodologies.

Analysis of Trapping Methods: The choice of trapping method is always associated with sample diversity, data reliability, and accuracy of species delimitation (Massey *et al*., 2022). A wide range of trapping methods has been actively used by COI-based DNA barcoding studies in Pakistan (**Table 3**) (Hussain *et al*., 2024). Despite a wide range of trapping methods, the data reveal inconsistencies and gaps in research.

Table 3. **Observed Trapping Methods with their Advantages and Limitations**

Trapping Method	Advantages	Limitations	Examples in Data
Nets (Sweep Nets, Hand Nets, Fish Nets, Cast Nets, Drag Nets, Cannon Nets)	Mobile species can be captured easily, like fish and insects.	The specimen may be damaged	Casting, sweep, and fish nets
Light Traps (with CO_2, UV Illuminated Sheets, Methyl Eugenol Lures)	Beneficial for nocturnal and flying species	Biased toward light-sensitive species	CO_2, coupled with UV sheet or use of methyl eugenol traps
Aspirators	helps to capture beetles and mosquitoes	Larger and evasive species may be missed	Portable and battery-operated suction devices
Pitfall Traps	Useful for ground-thriving arthropods.	weather-dependent effectiveness	Pitfall traps
Malaise Traps	Useful for flying insects like flies and wasps	It is expensive and requires monitoring	Malaise traps
Beating Vegetation Over White Paper	Helps to capture arboreal and hidden insects	Difficult for ground-living insects	Beating vegetation over white paper
Hand Collection	Effective for visible species.	It is laborious work	Snake sticks etc.
Blood Sampling (Jugular Vein Collection)	Blood collection from vertebrates	Requires an expert.	Use of syringes
Already Preserved Specimens	Historical studies	DNA quality may be low	Preserved specimen

Spray Procedures and Suction Devices	Valuable for cryptic species	May affect DNA quality	Spray procedures, mosquito nets
Transect-Based Sampling	Helpful in systematic data collection	Takes a long time	Line transect method

Why are Trapping Methods Important? For the collection of specimens, trapping methods are necessary to minimize bias and ensure a representative sample. To what extent is a method effective? This largely depends on the preservation of genetic material, as some trapping methods may damage DNA; specialized techniques are required for habitat types like terrestrial, freshwater, etc., summarized in **Table 3** with advantages and limitations. Moreover, some behaviours are specific to a species, like insects which are attracted to light traps, while fish require nets. The present dataset reveals a combination of passive (trap-based) and active methods (manual collection), each with advantages and limitations summarized and presented in **Table 3**.

Bias in Trapping Methods: Light traps, malaise traps, and hand collection are favorite methods for insect capturing (**Table 3**), which possibly lead to underrepresentation of non-flying or nocturnal insects. Cast nets, gill nets, and drag nets have been used for aquatic species; however, mesh size and sampling depth have not been mentioned, which may influence species selectivity. In the present data set, researchers have relied greatly on hand collection and jugular vein blood sampling from mammals, but there is a lack of details on mammalian, amphibian, and reptilian-specific traps like Sherman traps for rodents (Anthony *et al*., 2005) and drift fences for amphibians. Moreover, there is a lack of standardization in reporting, as some studies use vague descriptions like nets and hand collection, which don't reflect standardization in trapping mechanisms.

Future Recommendations: Future research should focus on standardizing trapping protocols, ensuring species-specific sampling strategies, and incorporating non-invasive genetic sampling methods to improve biodiversity assessments in Pakistan. Each publishing journal should set an exact trapping technique as a requirement for publication, along with mesh size, lure types, and collection duration. Additionally, a single method may misrepresent or underrepresent species; therefore, incorporating techniques should be used. Ethical considerations should be a priority in studies where animals are involved; besides, eDNA should be a focus for researchers on DNA barcoding.

Analysis of Types of Articles: The present review reveals that the majority of the articles utilized are research articles, while some as Rapid Communications, Short Communications, MITO Communications, and Review Articles.

Dominance of Research Articles: The dataset is heavily skewed towards Research Articles, which are detailed studies presenting original findings based on field or laboratory investigations. These articles present data that lead to reproducibility, have undergone peer review, and are more reliable and accurate. However, these articles have focused on specific case studies, are limited to a few species in many cases, and are limited to a certain geographic area.

Short and Rapid Communications: These are types of articles that briefly discuss new discoveries, methodological improvements, or preliminary results. The data set shows that these articles have provided timely insights, are focused, and concise. However, these articles are limited to data and lack statistical analysis. Moreover, these are not extensively peer-reviewed, which may compromise quality control.

MITO Communications: In mitochondrial genome sequencing and its applications in phylogenetics, these two article types play a key role. These are highly relevant to DNA barcoding and provide genome-level insights. However, these have a narrow focus as ecological and taxonomic discussions are missing and in the format is short, lacking extensive methodological comparisons.

Review Articles: Critical analysis has been provided by review articles that take a specific perspective or section of DNA barcoding. These types of studies are limited in the present dataset and discuss only specific parts or aspects of DNA barcoding (Hanif *et al*., 2023; Tahir and Akhtar, 2016a). A lack of multiple review articles indicates a lack of multiple and broader meta-analyses.

DNA Barcoding Sampling in Pakistan: The currently available data on COI-based DNA barcoding reveal that extensive geographic coverage has been established across Pakistan. However, a critical analysis reveals notable gaps, biases, and underrepresentation of specific taxa and ecosystems.

Geographic Bias: In DNA barcoding studies, unequal geographic distribution is observed in sampling (**Table 4**). The dataset shows that Punjab dominated sampling efforts with significant locations in Lahore, Faisalabad, Sargodha, and Multan (**Fig. 3**). However, several locations like Baluchistan, Gilgit-Baltistan, Khyber Pakhtunkhwa, and most of Sindh have gained less attention despite their ecological importance. Multiple cities and river systems like the Indus, Ravi, and

Chenab have provided more data, which could be due to accessibility in Punjab, and it can be due to a large number of samples from Punjab, mainly due to easy accessibility. So, this gap can be addressed through collaborative approaches in less-explored areas such as Baluchistan and Gilgit-Baltistan. Baluchistan, despite being the largest province with unique ecosystems covering mountains, deserts, and marine, has only a few locations in Quetta, Ziarat, and Hazarganji-Chiltan National Park that have been covered in DNA barcoding. There seems to be a lack of surveys due to logistical challenges, political instability, and limited research funding. The Broader Karakoram, Himalayas, and Hindu Kush regions have been underreported, despite being biodiversity hotspots (**Table 4**). Only Skardu has received a small approach from Gilgit Baltistan. The omission of biodiversity hotspots such as distinct alpine and glacial regions constitutes a significant research gap. Pakistan has relatively fewer publications compared to neighboring countries like India, Iran, China, and Bangladesh (**Fig. 4**). China is leading in this scenario with more than a thousand publications.

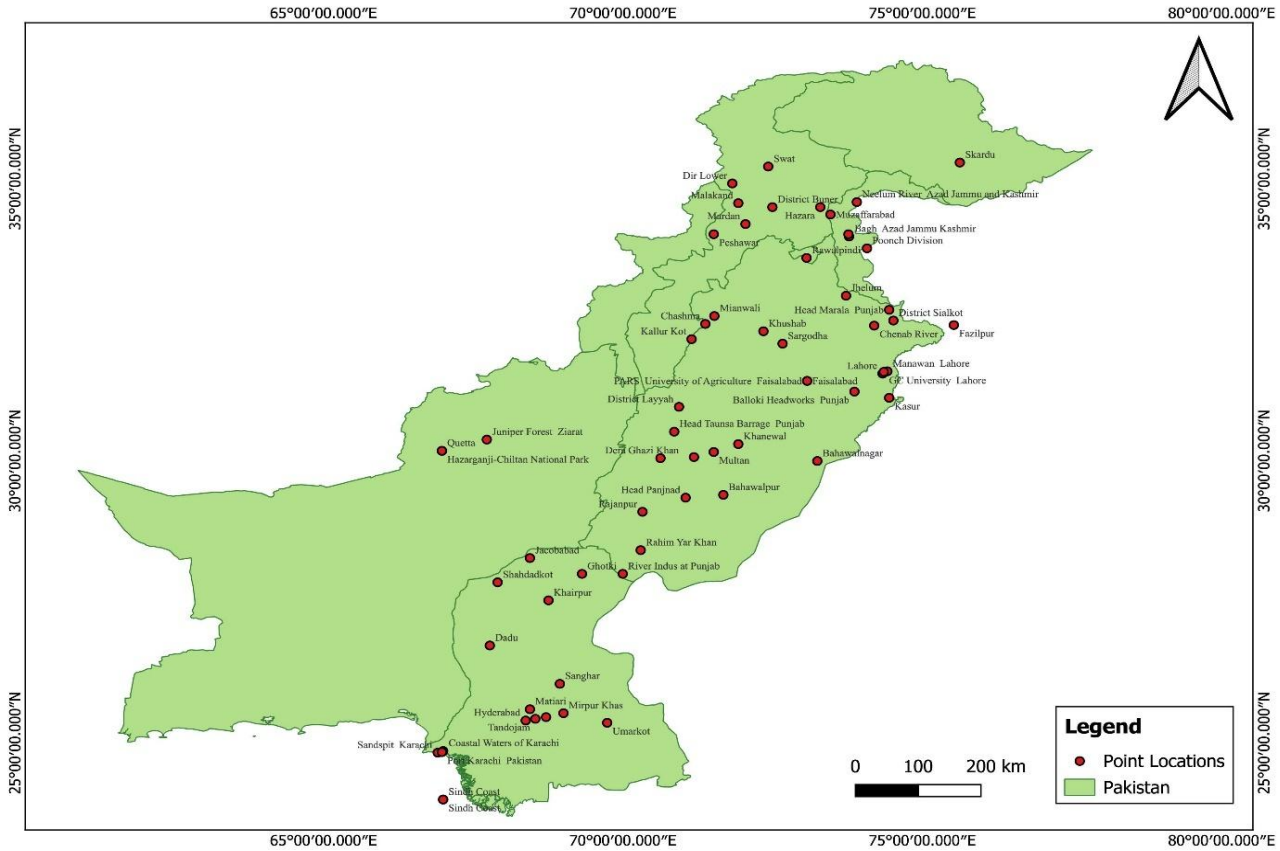

Fig. 3. Map of Pakistan showing sampling areas. A map generated using R (Leaflet package), displaying key locations in Pakistan with small circles for easy identification.

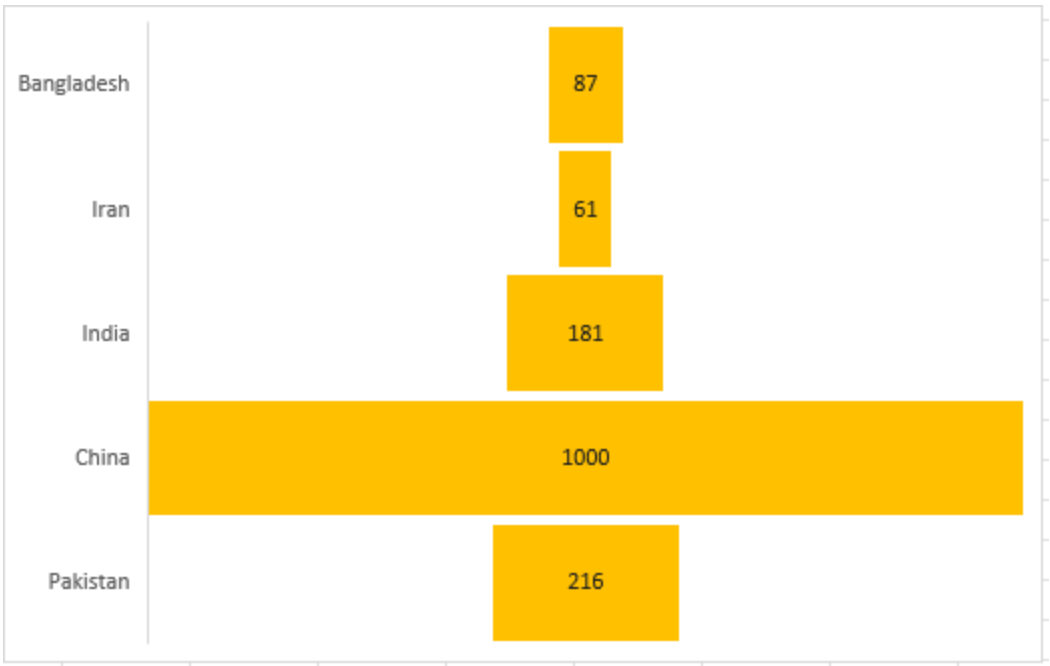

Fig. 4. A comparison of the number of DNA barcoding studies on COI genes among Neighboring countries accessed through Google Scholar, Scopus, Web of Science, and PubMed.

Table 4. Geographic and Taxonomic Biases in COI-Based DNA Barcoding in Pakistan

Category	Observations	Implications	Recommendations
Geographic Bias	More research output in Punjab, while less focus on Balochistan, Gilgit-Baltistan, especially	Accessible areas were represented more, while unique regions were ignored.	Work should be expanded to remote and unexplored areas.
Ecosystem Coverage	Marine, alpine, and deserts have been neglected, while rivers and agricultural areas have been explored potentially	Less data about Marine and high-altitude species.	Protecting areas, mountains, and unique ecosystems should be focused on.
Taxonomic Bias	More focus has been given to fish and insects while less to fungi, mammals, and microbes.	Key species remain understudied. Limited data about conservation strategies.	Neglected groups like mammals and fungi should be focused.
Institutional Constraints	Most research from Punjab-based institutes (UAF, GCU, AARI Faisalabad). Very few studies from other regions or remote regions.	Institutional bias skews results and questions funding.	Collaboration should be made in the universities of Balochistan and Khyber Pakhtunkhwa.
Freshwater vs. Marine Sampling	Focal point has been established in rivers like the Indus and the Chenab, while no focus on mangroves and corals	Marine species are underrepresented despite a long coastline.	Marine biodiversity should be focused.
Protected Areas	Limited barcoding from national parks (except Hazarganji-Chiltan, Juniper forests, Dodhial Pheasant Center, and zoos).	Conservation areas have been ignored.	National parks, Ramsar sites, and wildlife reserves should be sorted out.
Cross-Border Research	International collaboration with China, Saudi Arabia, and Egypt	Shared areas not accessed, including borders and the Indus Delta.	Cross-border diversity research should be encouraged with Iran, India, Afghanistan, and China.

Policy and Conservation Impact	Endemic and endangered species remain unstudied. Conservation efforts are comparatively less.	Data on many species is missing; therefore, inadequate policies are in place.	Threatened, endemic, ecologically important species should be focused.

Underrepresentation of Certain Ecosystems: Some of the critical ecological zones have been underrepresented in DNA barcoding studies. In Punjab, rivers like the Indus, Chenab, Ravi, Jhelum, and Satluj have been sampled widely, and coastal and marine biodiversity is underrepresented despite their importance with respect to mangroves, coral reefs, estuaries, and deep-sea ecosystems. In Sindh, few sites like Sandspit (Karachi), Fish Harbor Road (Karachi), and Sindh Coast have been sampled, while the nearby marine species are still underreported, showing a bias towards certain ecosystems due to the unavailability of facilities and funds. Some of the most important spots, such as the Cholistan desert, are sampled; however, the Thar desert is still missing, despite its unique xeric biodiversity. Moreover, the Kharan and Makran deserts are also missing from the dataset. When protected areas are compared to open areas, Hazarganji-Chiltan National Park, Juniper forests in Ziarat have been discussed and sampled, while northern forests, alpine meadows, and temperate ecosystems are overlooked. The Margalla Hills, Deosai National Park, Fairy Meadows, and other ecologically rich areas of Gilgit-Baltistan and Khyber Pakhtunkhwa remain absent from barcoding studies (**Fig. 3, Table 4**).

Overrepresentation of some areas: As compared to other ecosystems, agricultural and urban ecosystems have been studied and explored. Pests, pollinators, and invasive species have been barcoded, with the Ayyub Agricultural Research Institute and PARS (UAF Faisalabad) as the dominant. An overrepresentation has been shown for Lahore, Karachi, and Faisalabad, which thrive in human-modified habitats.

Taxonomic Bias and Underreporting: In the current dataset, some species or ecosystems have been focused on more than others. For instance, there has been a great focus on economically significant species, while a holistic approach to biodiversity is missing. When discussing taxonomically, some groups like insects, freshwater fish, and agricultural pests have been widely reported, while amphibians, reptiles, fungi, and invertebrates have been underreported. Species of terrestrial vertebrates like birds, reptiles, and mammals have been focused less on than occupied and protected places like national parks and zoos. Microbial DNA barcoding is nearly missing despite its importance and omnipresence in soil, water, and the gut, which are considered crucial for ecological and health studies.

Institutional and Logistical Constraints: Punjab and Sindh have gained greater focus, which suggests the research facilities, funding, and role of academic institutions. Security concerns, political instability, and limited infrastructure may explain less exploration of Balochistan, Gilgit-Baltistan, and interior Sindh. Moreover, cross-border and multi-geographic sampling is rare, which may limit regional comparative studies on biodiversity.

Conservation and Policy Implications: As DNA barcoding is successful when two major biases are removed, the geographic and taxonomic constraints limit its applications in conservation biology. The dataset reveals that many endemic and threatened species remain unstudied in marine, desert, and high-altitude biodiversity, affecting conservation policies. Biodiversity monitoring programs are negatively affected when fewer species are barcoded from national parks and protected areas. More balanced DNA barcoding efforts are needed in Pakistan, a richly cultural and ecologically diverse country.

Future Recommendations: To achieve a balanced focus on rich biodiversity barcoding of Pakistan, major gaps and biases should be addressed. Sampling should be expanded to underrepresented locations of Baluchistan, Gilgit-Baltistan, Khyber Pakhtunkhwa, and parts of Sindh. Marine and high-altitude ecosystems should be focused on species that focus on neglected groups like reptiles and mammals. Multinational collaborative research should be the primary focus of future research, and international partnerships should be integral to study designs for cross-border studies.

Sampling Effort and Species Identified

Analysis of the Number of Sampling Sites: Substantial variation has been observed in the number of sampling sites across studies included in this review, ranging from multi-site investigations to studies with extremely limited geographical coverage (**Table 1**). The inconsistency highlights several key points regarding the comprehensiveness, representativeness, and potential limitations of DNA barcoding efforts in the country. Some studies have demonstrated extensive sampling efforts with 1858, 491, and 329 sites (**Table 1**). These studies and others like them provide robust datasets that help in species identification and genetic diversity assessments. These types of studies are rare across 80-plus articles of the current review. In contrast to this, a high number of studies have reported few sampling sites, such as

ten or fewer, reaching only one site in a few studies. This can lead to the underrepresentation of species and highlight biases in barcoding, thereby reducing the effectiveness of the studies.

A large number of studies do not mention the number of sites and are labeled here as "Not Mentioned" (**Table 1**). It raises concerns about scientific rigor and data reproducibility. Therefore, results can be questioned, and it is difficult to draw conclusions. Future research may be constrained by data limitations, and it will be difficult to compare, reproduce, or validate the data. Besides the above, several studies have mentioned multiple sites that fall into a similar category of ambiguity. The lack of exact figures limits meaningful comparison and meta-analyses.

Analysis of Number of Samples: The number of samples was analysed across different studies on DNA barcoding in Pakistan. These numbers ranged from single-digit figures to over 60000 samples (Ashfaq *et al*., 2022). Some of the studies reported high numbers of samples, such as 60,273, 53,092, 10,792, 10,653, and 8,641 (**Table 1**), which provide a robust dataset that enhances species identification and reporting, biodiversity assessments, and increases the reliability and validity of DNA barcoding databases. These studies have been supported by external funding, which is why they supported a high number of samplings. Besides these, many studies reported small sample sizes such as 1, 3, 4, or 5. These studies contribute well to DNA barcoding studies; however, they are often inadequate for biodiversity assessments and conclusions. They are often coupled with poor statistical analysis, potential misidentification, and incomplete representation when at species-level biodiversity is considered.

Analysis of the Number of Species Identified: The country's DNA barcoding efforts are supported by studies that extended from one species to thousands of species across different articles. These variations are associated with several factors like comprehensiveness, scope, and methodological approaches.

Large-Scale Studies: Comprehensive Biodiversity Assessment: Extensive taxonomic coverages have been demonstrated in some of the studies, such as 1,364 species across 1,375 (Ashfaq *et al*., 2022) genera in a study, while another with 379 (Ashfaq *et al*., 2017) species spanning 52 families. These studies contribute significantly to the importance of DNA barcoding for species delimitation, evolutionary studies, and conservation strategies. Another study with 254 families, which fits in 17 orders, also adds to the broad taxonomic range. These studies demonstrate well-designed sampling strategies, which are crucial for DNA barcoding. These studies have improved large-scale efforts, encouraged others to follow suit in the same range, and improved Pakistan's representation at the BOLD and GenBank levels.

Moderate Species Coverage studies: Limited Impact: Many of the studies have reported a handful of species, genera, and families. Some of these have 1 to 5 species, with certain studies even focusing on a single species or genus (**Table 1**). These are valuable for specific taxonomic groups; however, they provide only a limited contribution to overall efforts. Small sample sizes reduce the ability to assess intraspecific variation, which is crucial for differentiating cryptic species and understanding population dynamics. Moreover, some of the studies have focused on specific taxonomic groups, such as one limited to 86 pheretimoid species complex (Hussain *et al*., 2022) and the great gap between morphologically and molecularly identified species, as 50 species were identified morphologically, but only 8 species were identified molecularly (Khan *et al*., 2024a). Some studies do not specify the exact number of species, which creates ambiguity for taxonomic researchers. This lack of clarity reduces the usefulness of such studies for comparative analyses and biodiversity assessments. Additionally, the use of BINs (Barcode Index Numbers) instead of species names, as seen in the study reporting 15 BINs (Ashfaq *et al*., 2014b). may indicate that taxonomic validation was incomplete, limiting the practical application of the data.

New Species: Promising but Underutilized: Out of the studied articles, a few studies report the discovery of new species, such as one that reported 11 species, including 6 new ones, and another study with 2 species, with 1 new species (**Table 5**) (Memon *et al*., 2006; Sajid *et al*., 2021). This highlights the potential of DNA barcoding in uncovering previously unrecognized biodiversity in Pakistan. However, the low number of articles reporting new species can be seen as underexplained or underexplored. There also seems to be a lack of expertise in species validation through DNA barcoding and molecular phylogenetics. In conclusion, there is a need for both expansion and standardization of sampling protocols across the country. Smaller studies should focus on poorly studied taxa to fill the gap, while extensive studies should be encouraged for biodiversity assessment. Efforts should be made to expand taxonomic coverage and species validation.

Table 5. Taxonomic and genetic data (Classification, Congeneric and Conspecific Divergence, Most Abundant and Total Species Identified) of the articles studied (See the complete Table in Supplementary Table 2)

Classification	Congeneric Divergence	Conspecific Divergence	Most Abundant Species	Total Species Identified
Mosquitoes	2.3-17.8%	0-2.4%	Culex quinquefasciatus (61%)	32
Lepidoptera, moths	6.4% (2.7-16.0%)	0.0-3.3 (<2%)	N/A	379 species, 52 families
Hemiptera: Whitefly *Bemisia tabaci* Complex	N/A	0.0-19.9% (mean 4%)	N/A	15 BINs
Insects	N/A	N/A	N/A	1364 species, 1375 genera
Thrips (Thysanoptera)	5.6-27% (19% mean)	0.00-7.6% (mean 7.6%)	N/A	43

Analysis of Most Abundant Species: Although the main purpose of DNA barcoding isn't to assess species abundance, the inclusion of abundance patterns can still provide meaningful insights into the ecological context, aiding in highlighting sampling biases, research priorities, and the dominant taxa studied within Pakistan. This analysis also supports future studies by indicating which groups are well-documented and which remain underrepresented, guiding more balanced biodiversity assessments. These studies also provide insights into gaps where ecologically important or understudied groups remain poorly represented. Many diversity indices, such as the Shannon Diversity Index and Simpson's Diversity Index, are extensively used to estimate species diversity and richness (**Table 5**). However, in barcoding studies, it is still very scarce, limited to a few studies (Anjum *et al*., 2025). Therefore, it is necessary to carry out biodiversity estimation in parallel to identification. In the current scenario, reporting of the most abundant species is inconsistent, with many studies fail to provide such information. Some studies have mentioned the dominant species, such as *Culex quinquefasciatus (61%)*, *Bactrocera zonata*, *Hypophthalmichthys molitrix*, and *Anopheles stephensi.* The Majority of the list is labeled as Not Available (**Table 5**). Species that are abundant in any ecosystem play a crucial role in ecosystem stability or functioning. For example, *Culex quinquefasciatus* and *Aedes aegypti* are not just dominant mosquito species but also significant vectors for diseases, such as dengue and malaria (Rodriguez, 2005). Understanding their abundance and distribution can help in vector control and public health strategies. Similarly, dominant agricultural pests such as *Bactrocera zonata* can have serious economic implications.

At the other end of the spectrum, less abundant species may indicate rare or threatened taxa requiring conservation attention. Species such as *Gazella bennettii* and *Vulpes vulpes* are notable mentions, as their population trends could indicate habitat disturbances or conservation success. If studies do not mention the most or least abundant species, critical information about species decline or dominance is lost. When dominant species are reported, this provides insights into potential biotic homogenization, where a few species outcompete and replace native biodiversity. The presence of invasive species such as *Hypophthalmichthys molitrix* (silver carp) and *Oxya hyla hyla* (a grasshopper species) suggests ecological shifts that could affect native species and ecosystems. Without data on species abundance, it is difficult to assess the impact of invasive species or take appropriate management actions. Accurate reporting improves DNA barcoding and its utility in DNA databases. Without explicitly stating the true status, the databases may not accurately reflect the true ecological importance of each species.

Conspecific Genetic Distance: Pakistan's Perspective: In the current review, the intraspecific divergence varies significantly across articles (**Table 5**). The standard threshold is 2%; however, an inconsistent gap has been shown by Pakistani studies. Some studies have provided clear ranges or their mean values, while others do not report them and are labeled as "Not Available". A broad range of genetic distances is available as low as 0.0% and as high as 67.10% (**Table 5**). This genetic distance is a crucial metric in DNA barcoding and helps establish a threshold. Generally, this value remains below 2%, but depending on evolutionary history, geographic separation, and species, it can vary. Critically, the large distance (i.e., 67.10%) shows an error of calculations, a mislabeling, or a data quality red flag, therefore, it should be re-examined or flagged in databases.

Wide Variability in Reported Values: Here, we report high inconsistency in intraspecific values from Pakistan based on the COI gene. Many of the studies have reported values between 0 2.4%, which aligns with standard barcoding

expectations (Dinh *et al*., 2019). For instance, the studies reporting 0-1.6%, 0.0-2.26%, and mean values around 0.2%-0.7% suggest expected intraspecific variation (**Table 5**). Some of the studies from the current scenario report high values as high as 32.18%, which exceed normal values, which may indicate the presence of cryptic species (Dinh *et al*., 2019), taxonomic errors, variable genetic markers, and geographic structuring. Some studies have reported no variation, reflecting low genetic diversity and limited sampling. Several studies have not reported conspecific genetic distances. This lack of data reduces comparability and creates a gap in establishing a threshold for Pakistan's biodiversity.

Implications for Species Delimitation and Accuracy: Absence of clear genetic distance may lead to misidentification and indicate overlooked taxonomic diversity. For species delimitation, the threshold is set to 2%; however, the variability in the dataset suggests that a single universal threshold may not be sufficient. Therefore, work on taxon-specific thresholds is necessary. Future studies should adhere to standardized methods to improve the consistency and reliability of reported values.

Congeneric Genetic Distance in DNA Barcoding: In the whole dataset, interspecific genetic divergence is inconsistent (**Table 5**). Few studies have provided well-defined ranges or their mean values from very low values of (0.0000–0.0067%) to exceptionally high distances (37.02%) (Islam *et al*., 2018). Congeneric distances help to define the boundary between species.

Wide Variation in Reported Values: Many studies report expected values such as 2.3–17.8%, 2.8–23.2% (mean 8.8%), and 7.93%, summarized and outlined in **Table 5**. These values are in line with global thresholds and successfully defined species boundaries.

Some studies have reported extreme values such as 37.02%, which may be attributed to deep genetic divergence, misidentification, and the presence of cryptic species. Some of the values are less than 1%, which may indicate recent speciation, hybridization, or taxonomic issues. Many of the studies have not presented values for congeneric distances, which limits the comparison. In the current review, this gap may be due to a focus on identification rather than evolutionary relationships, incomplete sampling, and the use of various markers rather than a single universal primer.

The Barcode Gap Challenge: The barcode gap, the distance that separates species, is crucial for distinguishing species. Congeneric distance should be more than conspecific distance. Some studies have reported overlapping values, which results in blurring identification. Therefore, there is a need for taxon-specific threshold adjustment, co-validation of morphological and molecular identification, and the integration of mitochondrial markers alongside nuclear markers. An international standard is inadequate; taxon-specific validation and multi-gene approaches are essential.

Focused vs. Ignored Taxa, Limitations, and Recommendations: In Pakistan, a wide range of taxa, including insects, fish, birds, mammals, arachnids, reptiles, and amphibians, have been covered through DNA barcoding. However, this tremendous focus over the past two decades is uneven across different groups and geographies (**Fig 5**).

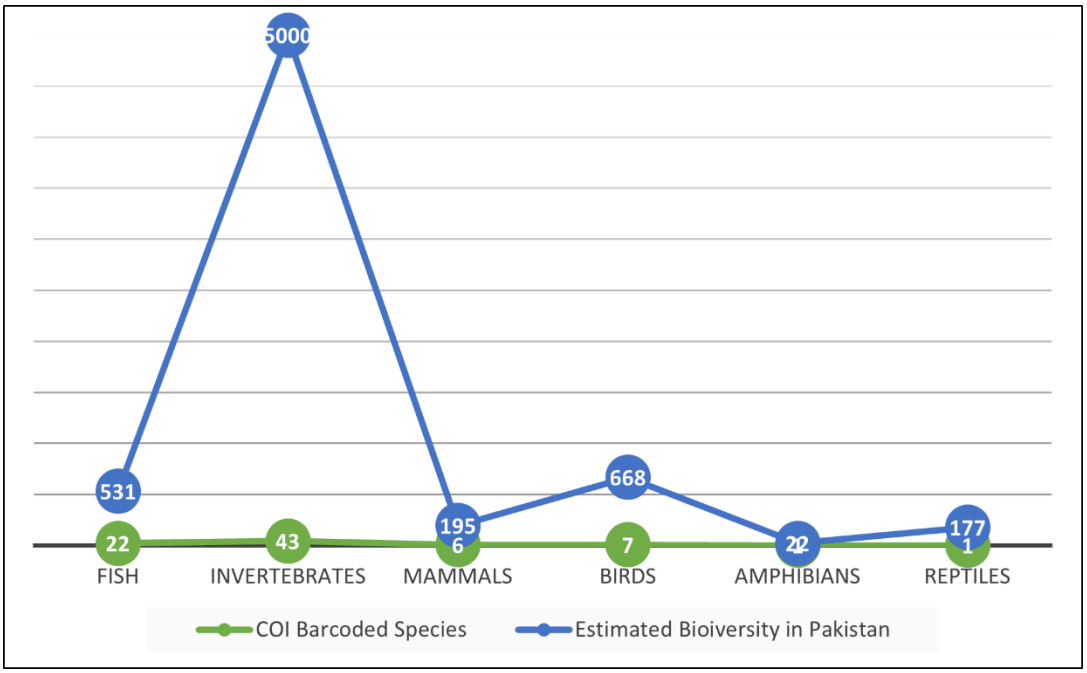

Fig. 5. A line chart comparing the number of barcoding studies per major taxonomic group vs. their estimated species richness in Pakistan

Groups Received More Attention: A significant portion of studies have focused on insect species, including species from Diptera, Hemiptera, Lepidoptera, Hymenoptera, and Orthoptera, among which pests and disease vectors are dominant. Species of mosquitoes like *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles* mosquitoes, pests like Thrips (*Thysanoptera*), whiteflies (*Hemiptera: Aleyrodidae*), stink bugs (*Hemiptera: Pentatomidae*), fruit flies (*Bactrocera spp.*), cotton bugs (*Dysdercus spp.*, *Oxycarenus hyalinipennis*), and mealybugs (*Phenacoccus solenopsis*), and beneficial insects like *Apis mellifera* (honeybee) and *Coccinellidae* (ladybird beetles, important for biological control) have gained special attention. Spiders and other arachnids belonging to the order Araneae have been well-documented for DNA barcoding. While talking about fishes, more focus has been given to economically important species like members of the Cyprinidae family, marine fishes (*Sillago indica*), and endangered species like *Tor putitora*. Few studies on Doves, pigeons, pheasants, and starlings, few mammals like ungulates including *Gazella bennettii*, *Moschus cupreus*, *Muntiacus muntjac*, *Capra hircus*, *Bos taurus*, and bats like Vespertilionidae, Emballonuridae, *Pipistrellus coromandra* have been barcoded. Some studies have examined turtles (*Lissemys punctata*) and toads (*Duttaphrynus spp.*), however, herpetofauna remain understudied.

Ignored or Understudied Taxa: When studied and understudied groups are compared, Pakistan's coastal and deep-sea biodiversity remains largely unexplored. Corals and molluscs and crustaceans have been ignored. Studies on carnivores such as foxes, hyenas, jackals, and leopards are missing. Rodents, shrews, and hedgehogs have not yet been studied. Pakistan is blessed with snake and lizard diversity (Khalid *et al*., 2019); however, only a few studies have focused on them. Molecular work on fungi and microorganisms is scarce across Pakistan.

Comparison of Pakistan's Biodiversity and Molecular Work: Currently, over 198 fish species, 198 mammal species, 666 bird species, 177 reptile species, and 22 amphibian species have been identified in Pakistan (**Fig. 6**), far more than the barcoded species, and there remains a large gap between the two. Moreover, approximately 6,000 plant species exist, but very few DNA barcoding studies have been published. While microorganisms are extremely underexplored at the molecular level.

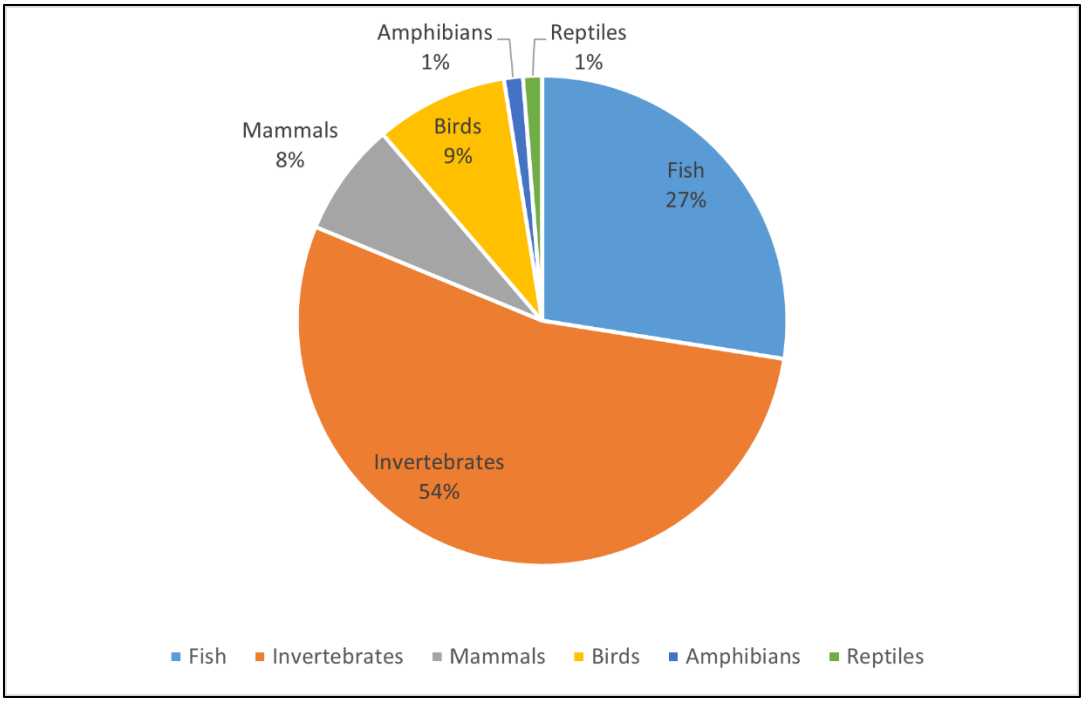

Fig. 6. Proportional representation of major species groups barcoded in Pakistan through DNA Barcoding with the COI gene.

Reporting Deficiencies and Standardization Gaps

Unspecified and Non-Traditional DNA Sources: In the current review, a notable portion of studies have not provided a DNA source, highlighting a potential gap in reporting and standardization of DNA barcoding (**Table 1**) (Awan *et al*., 2013; Sajid *et al*., 2021). Moreover, DNA extraction was carried out from animal-derived products like tanned skins, coats, and fur samples, ensuring a focus on illegal wildlife trade (Janjua *et al*., 2017). Some studies have utilized fecal samples, hair, and skin for DNA, which is a good application in non-invasive genetic monitoring of mammals (Naseem *et al*., 2020).

A diverse range of sources has been used for DNA barcoding in Pakistan, including fins and scales from fish, muscle tissues from vertebrates, legs from arthropods, coats and tanned skins from wildlife species (**Table 1**). A more robust focus on DNA extraction should be on using fecal matter, hair, blood drops, etc., for non-invasive sampling techniques.

Lack of Study Duration: A significant number of research studies in the current review did not report their study duration, indicated as (Not Provided) in **Table 1**. It makes it difficult for future researchers to replicate or validate current findings, and temporal trends cannot be compared to data from other times. Moreover, standardized guidelines for DNA barcoding studies are necessary to ensure methodological transparency. Given the lack of duration, it becomes challenging to assess whether a study sufficiently covered seasons or years to avoid sampling bias, such as the summer of 2022 (Gul *et al*., 2024) or selected months of 2020.

Lack of Trapping Method and Number of Samples: A notable number of studies did not report trapping methods, which affects data comparability and reproducibility, and raises questions about reliability. It makes data difficult for future use by researchers to assess the effectiveness of different trapping methods.

In several studies from Pakistan, the number of samples is missing (Not mentioned), which limits the impact of DNA barcoding. These studies conclude that there is a lack of transparency, data validation, and reproducibility. Future studies should focus on a standard number of samplings in DNA barcoding, while placing more emphasis on large-scale sampling.

Lack of Species Abundance: The unavailability of data on species abundance may be due to several issues, such as the focus of the study on species identification rather than ecological significance. It may also be associated with incomplete sampling or bias in specimen collection.

Limitations in DNA Barcoding Studies: One of the major limitations of DNA barcoding in Pakistan is the focus on economically important species while ignoring the ecologically significant species. Many studies are restricted to specific regions such as Punjab. However, diverse regions such as Balochistan and Khyber Pakhtunkhwa have been ignored. Moreover, many of the studies have focused on a few species, while other taxa are largely ignored. Finally, the availability of the universal or species-specific markers is also a significant problem.

Conclusion: The current study concludes that COI-based DNA barcoding in Pakistan has faced multiple challenges. These challenges include biased geographic studies, limited resources in terms of financial and laboratory resources, fragmented studies, ignored taxa (e.g., fungi, microbes, reptiles, and marine organisms), a missing barcode library, and limited availability of biodiversity data; therefore, these should be the focus of the next studies. Multinational collaboration should be strengthened to advance progress in DNA barcoding. Barcoding outcomes should be linked to policy and conservation strategies. Furthermore, ignored taxa can be studied if global and local collaboration between institutions and governments is established. The study further concludes the unavailability of expertise in COI-based DNA barcoding at the institutional level.

Recommendations: To address these taxonomic gaps, taxonomic coverage should be expanded to neglected taxa, including carnivores, rodents, small mammals, snakes, geckos, skinks, and frogs, and to aquatic life across Pakistan's water. Geographic sampling should be prioritized in the underrepresented regions, specifically Balochistan, Khyber Pakhtunkhwa, and Gilgit-Baltistan. The COI gene should be coupled with other genes, and molecular markers should be assessed alongside morphological characters. Moreover, barcoding outcomes should be linked to policy management and conservation strategies. Conservation strategies should be focused, and eDNA should be the top priority of DNA barcoding researchers with the use of Artificial intelligence for species delimitation and sequencing as well. We develop and propose a standardized checklist (**Table 6**) to enhance reproducibility and comparability of future DNA barcoding studies in Pakistan. Additionally, a three-tier proposal has been introduced to address the gaps in future studies (**Fig. 7**). Finally, combining MEGA with Bayesian inference will strengthen such studies and help maintain potential reproducibility.

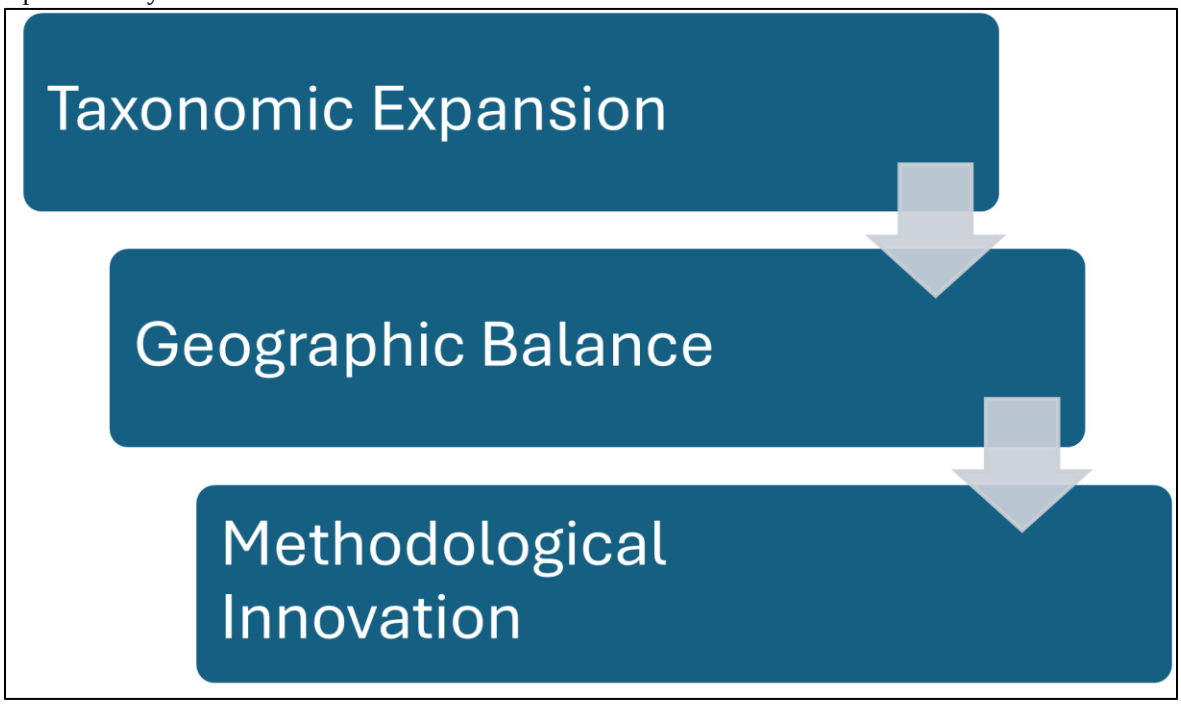

Fig. 7. Three-tier proposal for addressing the gaps in DNA Barcoding studies in the future.

Table 6. Proposed Checklist for COI-Based DNA Barcoding Studies in Pakistan

Category	Essential elements
Context and Design of Study	✓ Inclusion of Geographic locations ✓ Presence of coordinates of sampling sites ✓ Duration and seasons of study

	✓	Presence of clear objectives
Details of Sampling	✓	Number of samples per species
	✓	Site selection rationale and number of sites
	✓	Methods of trapping, such as Sherman and Longworth traps
	✓	Statements of ethical approval
Handling of Sampling	✓	Source of tissue for DNA extraction (muscle, fin, leg)
	✓	Method of preservation (ethanol, etc.)
	✓	Voucher specimen in a recognized database
Molecular Methods	✓	Extraction Protocol
	✓	Primer details
	✓	PCR conditions
	✓	Sequencing platform
Data management	✓	Explanation of sequence quality control
	✓	Submission of sequence/s to BOLD/GenBank
	✓	Provision of Accession Numbers
Analysis and interpretation	✓	Models/Software packages used
	✓	Genetic distance methods specified
	✓	Criteria for species delimitation
	✓	Phylogenetic/clustering method used
Reporting and transparency	✓	Limitations of the study clearly acknowledged
	✓	Methodological details for reproducibility and reliability
	✓	Supplementary data including metadata, alignments, and trees

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