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Research Article

COI-Based DNA Barcoding and Genetic Diversity Analysis of *Euconocephalus pallidus* (Tettigoniidae: Orthoptera) from Bahawalpur, Pakistan

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ABSTRACT

Euconocephalus pallidus (Redtenbacher, 1891), a katydid species belonging to the family Tettigoniidae, plays a significant ecological role across diverse habitats in Pakistan. Despite its ecological importance, molecular data on its genetic diversity and evolutionary relationships remain scarce. To address this knowledge gap, the present study investigates the genetic variation of *E. pallidus* using DNA barcoding of the mitochondrial Cytochrome c Oxidase I (COI) gene. PCR amplicons were purified and sequenced unidirectionally using the Sanger sequencing method. Raw sequences were processed, aligned using ClustalW, and compared with reference sequences from the NCBI database via BLAST analysis. Phylogenetic relationships were inferred using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software. BLAST results showed high sequence identity between the obtained COI sequences and those of closely related species, supporting the morphological identification of *E. pallidus*. The resulting phylogenetic tree placed *E. pallidus* in a clade with congeners such as *E. nasutus* and *E. varius*, supported by moderate bootstrap values. Furthermore, the observed intra-population genetic variation suggests potential phylogeographic structuring within the sampled population. This study provides novel insights into the genetic diversity and evolutionary positioning of *E. pallidus* in Pakistan. It represents the first molecular assessment of this species using DNA barcoding in the ecological context of the Bahawalpur division, addressing a critical gap in molecular data for Orthopteran insects in the region.

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Introduction

Biodiversity serves as a fundamental indicator of ecosystem health, and understanding species diversity is crucial for effective conservation and ecological management (Wang et al., 2024; Sajina et al., 2025). Among various insect groups, katydids (Family: Tettigoniidae) play an important ecological role, particularly in regulating plant populations and serving as a food source for numerous predators (Xu et al., 2022; Saupi et al., 2025). *Euconocephalus pallidus*, a species of katydid, is widely distributed across tropical and subtropical regions and is characterized by its distinctive acoustic signaling and herbivorous feeding behavior (Sri-In and Yang, 2019; Dong et al., 2022). Despite its

ecological importance, *E. pallidus* remains understudied in many regions, including Pakistan, where katydid diversity and distribution are poorly documented.

The Bahawalpur division in southern Pakistan encompasses diverse habitats ranging from arid desert zones to riverine and agricultural landscapes, providing suitable environments for a wide range of insect fauna. However, the lack of comprehensive studies has resulted in significant knowledge gaps regarding the genetic diversity, spatial distribution, and ecological adaptations of species such as *E. pallidus*. Although some studies have focused on the morphological traits of katydids in Pakistan (Cheng et al., 2025; Jakhrani et al., 2025), their genetic variation across ecological gradients remains largely unexplored. There is an increasing need for region-specific molecular studies to generate baseline data and enhance understanding of local biodiversity (Takeuchi et al., 2021; Gonon et al., 2024).

To address this gap, the present study investigates the genetic diversity of *E. pallidus* across various habitats in Bahawalpur division using DNA barcoding, a molecular technique that has transformed biodiversity assessment worldwide (Norros et al., 2022; Kimta et al., 2025). DNA barcoding provides a rapid and reliable means of species identification and genetic differentiation, especially for cryptic or morphologically similar taxa. Its successful application in numerous insect taxa has yielded critical insights into species richness, population structure, and evolutionary history (Altermatt et al., 2025). Globally, DNA barcoding has proven particularly effective in cases where morphological identification is hindered by intraspecific variability or the presence of cryptic species (Bartolini et al., 2020; Marullo et al., 2020). Studies from India, China, and the United States have employed this approach to investigate katydid diversity and revealed substantial genetic variation within Orthoptera (Smith et al., 2005; Symes et al., 2019; Shashank et al., 2022).

Despite these advancements, comparable molecular studies in Pakistan remain limited, particularly concerning *E. pallidus* in ecologically diverse regions such as Bahawalpur. Several recent works have significantly advanced Orthopteran research in the country. Jakhrani et al. (2025) reported the distribution of *Hieroglyphus oryzivorus* in upper Sindh, while Talpur and Sultana (2025) reviewed the life history and ecological potential of *Acheta domesticus* as an edible insect. A major taxonomic milestone was the annotated catalogue of Pakistani Acrididae published by Sultana and Song (2024). Behavioral diversity, including mating strategies, was explored by Shah and Sultana (2024). Bhanger et al. (2024) and Bozdar et al. (2024) contributed to the understanding of Gryllidae and Acridomorpha fauna, respectively, while Sultana et al. (2024) focused on the systematics of Oedipodinae in the Thar Desert. Molecular tools have gained traction, with Babar et al. (2023) utilizing COI gene barcoding for Acrididae identification, and Ashfaq et al. (2022) conducting a national-scale insect DNA barcoding survey. Foundational work by Sultana (2019), Sultana et al. (2015, 2021a, b), Kumar et al. (2022), and Irfan et al. (2025) laid the groundwork for these advancements, covering new species descriptions, mating behavior in Oxyinae, molecular differentiation of *Chrotogonus* spp., and the ecological/economic impacts of the 2019-2020 desert locust outbreak.

Collectively, these studies have significantly advanced our understanding of the biodiversity, taxonomy, behavior, and management of Orthoptera in the arid and agricultural landscapes of Pakistan.

The present study aims to bridge the existing knowledge gap by employing COI-based DNA barcoding to assess the genetic diversity and distribution of *E. pallidus* across different ecological zones of Bahawalpur division. By analyzing genetic patterns, we aim to uncover potential cryptic diversity, clarify phylogeographic relationships, and contribute to a more comprehensive understanding of local insect biodiversity. Moreover, this research highlights the utility of molecular tools for biodiversity monitoring in underexplored regions of Pakistan and may serve as a foundation for future studies on other insect taxa in the country.

Materials and Methods

Sampling

Specimen Collection and Preservation

Adult specimens of *E. pallidus* were collected from various ecological zones within the Bahawalpur division, Pakistan, between March and September 2024. Sampling sites were strategically selected to encompass a range of habitats, including agricultural fields, riverine environments, and arid landscapes, to capture the ecological and genetic variability of the species. A total of 117 adult individuals, comprising both males and females, were collected.

The collection sites spanned three districts, Bahawalpur, Bahawalnagar, and Rahimyar Khan, each representing

distinct ecological conditions (Figure 1). Geographic coordinates (latitude and longitude) for each sampling locality are provided in Table 1. Notable collection sites included Karaniwala, Jaunwala, Yazman, and Ahmedpur, among others.

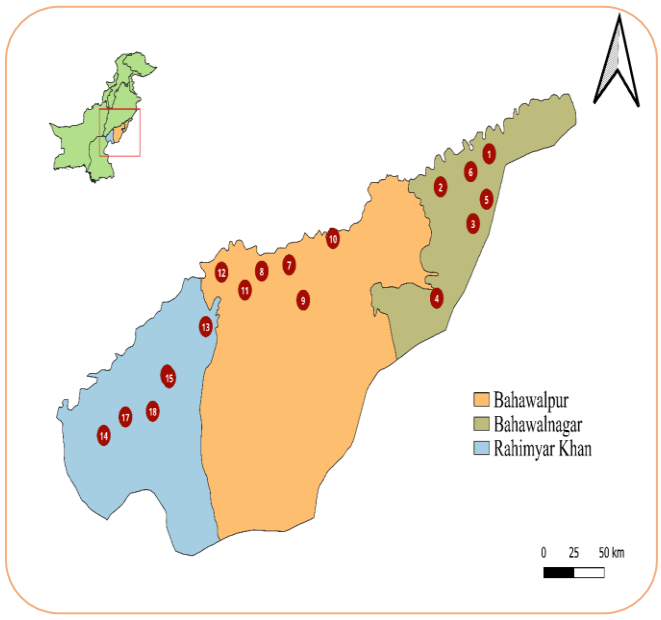

Figure 1: Sampling sites from Bahawalpur Division.

Identification of specimens

Specimen identification was performed using a stereoscopic binocular microscope (Model Z730, China) equipped with magnification capabilities of 100X × 10X. This setup allowed for detailed examination of morphological characteristics critical for accurate species determination. The identification process was guided by the comprehensive taxonomic keys provided by Cigliano et al. (2025), accessible through the Orthoptera Species File database (http://www.orthoptera.org), which offers an up-to-date and globally recognized reference for Orthoptera taxonomy.

In addition to the digital keys, standard morphological identification protocols described by Uvarov (1977) and Sultana and Wagan (2015) were employed. These references provided essential diagnostic features for distinguishing among various families, genera, and species of Orthoptera. Key identification characteristics included body coloration, size, structure and venation of the wings, shape and segmentation of antennae, and morphology of the hind legs and genitalia. The integration of classical taxonomic literature with modern digital resources ensured a thorough and reliable identification process.

The broad geographic distribution of these sampling locations enhanced the representativeness of the dataset and was essential for assessing the genetic diversity and phylogenetic relationships of *E. pallidus*. All specimens were preserved in 70% ethanol and stored at -20 °C until further molecular analysis.

Table 1: All localities and coordinates of Bahawalpur division.

Sr. No.	Localities	Coordinates Latitude	Longitude
1	Karaniwala	28.30866	70.12830
2	Jaunwala	29.47168	70.52837
3	Yazman	29.12422	71.74831
4	Lalsohanra	29.46919	71.97828
5	Ahmedpur	29.15324	71.26113
6	Uch	29.23895	71.06148
7	Bahawalnagar	29.99359	73.25276
8	Chishtian	29.79456	72.85777
9	Haroonabad	29.61049	73.13888
10	Fortabas	29.19305	72.85745
11	Donga Bonga	29.74729	73.24409
12	Islampur	29.88926	73.10421
13	Liaquatpur	28.93930	70.94566
14	Sadiqabad	28.30866	70.12830
15	Khanpur	28.64774	70.65600
16	Zahirpir	28.66138	70.63813
17	Alanpur	28.41760	70.30365
18	Chandpur	28.46020	70.52837

DNA isolation and purification

Muscle tissues from *E. pallidus* specimens were used for genomic DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's protocol. Tissue homogenization was carried out using lysis buffer, and proteinase K was added to digest the tissues. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), while DNA integrity was verified via electrophoresis on a 1% agarose gel. Only samples with an A260/A280 ratio between 1.8 and 2.0 were used for downstream analyses.

For PCR amplification of a 652-bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, the following primers were used:

Forward: TTTATACTTCATCTTTGGAG

Reverse: TAAATGTTGATATAAAATTG

PCR reactions (25 µl total volume) consisted of 2 µl genomic DNA (25 ng/µl), 2.5 µl of 10× PCR buffer, 3 µl of

$MgCl_2$ (50 mM), 2.5 µl of dNTPs (2 mM each), 1 µl of each primer (10 pmol), 0.8 µl of Taq polymerase (5 U/µl), and 13.2 µl of PCR-grade water. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 sec, annealing at 47 °C for 30 sec, and extension at 72 °C for 30 sec; followed by a final extension at 72 °C for 5 min.

PCR products were visualized on a 1% agarose gel to confirm amplification of the target 652-bp fragment. Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced unidirectionally. Chromatograms were edited and cleaned using Chromas software (Technelysium Pty Ltd, Australia) to ensure sequence quality. Sequence alignment was performed using ClustalW, and consensus sequences were compared with the NCBI nucleotide database using the BLAST tool to confirm species identity and identify closely related taxa.

Phylogenetic analysis

To assess the phylogenetic position of *E. pallidus*, 11 reference COI sequences, including outgroup taxa, were retrieved from the NCBI database. Multiple sequence alignment was performed using ClustalW implemented in MEGA version 11 to identify conserved and variable regions within the COI gene. Phylogenetic relationships were inferred using the Maximum Likelihood (ML) method based on the Tamura and Nei (1993) model, which was identified as the best-fitting substitution model via model selection in MEGA11.

Heuristic search strategies such as Neighbor-Joining (NJ) and BioNJ were applied for tree construction. The robustness of the tree topology was evaluated using 1000 bootstrap replicates. The final ML tree with the highest log-likelihood value (-1892.25) was selected and visualized using MEGA11. Bootstrap values were displayed at the corresponding nodes to indicate statistical support for each clade.

The phylogenetic analysis successfully placed *E. pallidus* within its respective clade, along with other closely related katydid species in the family Tettigoniidae, thereby providing insight into the genetic diversity and evolutionary relationships of the species.

Results

All adult specimens of *E. pallidus*, including both males and females, were identified based on their external morphological characteristics using standard taxonomic keys. Diagnostic features such as body coloration, size, and distinctive structures were carefully examined to confirm species identity. A representative image of *E. pallidus* is provided in Figure 2.

Figure 2: A representative image of the captured *E. pallidus*.

Phylogenetic analysis

The phylogenetic analysis of *E. pallidus* (accession no. PQ740473.1) was conducted using reference sequences retrieved via NCBI BLAST to elucidate its evolutionary relationships within the Tettigoniidae family. The resulting phylogenetic tree revealed a well-supported clustering pattern, highlighting both genetic similarities and divergences among the analyzed taxa.

The query sequence (*E. pallidus*, PQ740473.1) clustered closely with two other *E. pallidus* sequences (OQ283712.1 and MW009066.1), forming a distinct clade with a bootstrap value of 66, indicating moderate support for their shared ancestry (Figure 5). This clade was nested within the broader *Euconocephalus* lineage, reflecting species-level genetic variation.

Adjacent to the *E. pallidus* clade, sequences of *E. nasutus* (OQ283711.1 and PQ675603.1) and *E. varius* (OQ283713.1 and OL663230.1) formed separate, yet closely related clades, supporting the phylogenetic coherence of the genus (Figures 3 and 4). Bootstrap values within these clades ranged from 38 to 100, indicating variable levels of support. Notably, the branching point separating *E. varius* from *E. pallidus* exhibited a high bootstrap value of 100, suggesting a well-resolved divergence between the two species.

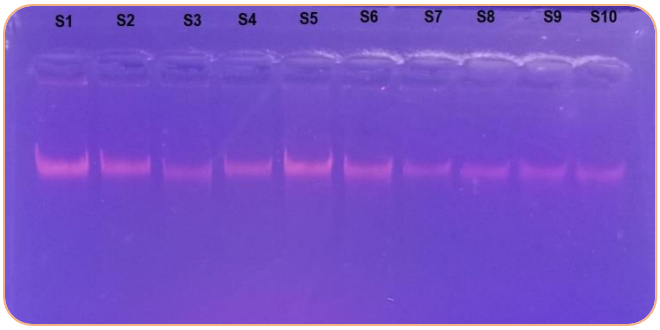

Figure 3: Representative Agarose gel electrophoresis image of some DNA samples extracted from *E. pallidus.*

Outside the *Euconocephalus* cluster, more distantly related taxa such as *Banza nihoa* (DQ649491.1 and DQ649492.1) and *Ruspoliia dubia* (JQ793668.1) formed a distinct and well-supported group (bootstrap = 100), reinforcing their monophyletic relationship and taxonomic separation from *Euconocephalus* species. The outgroup, comprising *Neoconocephalus robustus* (KJ208581.1) and *Gampsocleis buergeri* (LC619087.1), was positioned separately from the ingroup taxa, confirming its evolutionary divergence (Figure 5).

Figure 4. Representative agarose gel electrophoresis depicting PCR results of product size of 652 bp with a 200 bp DNA ladder used as size marker.

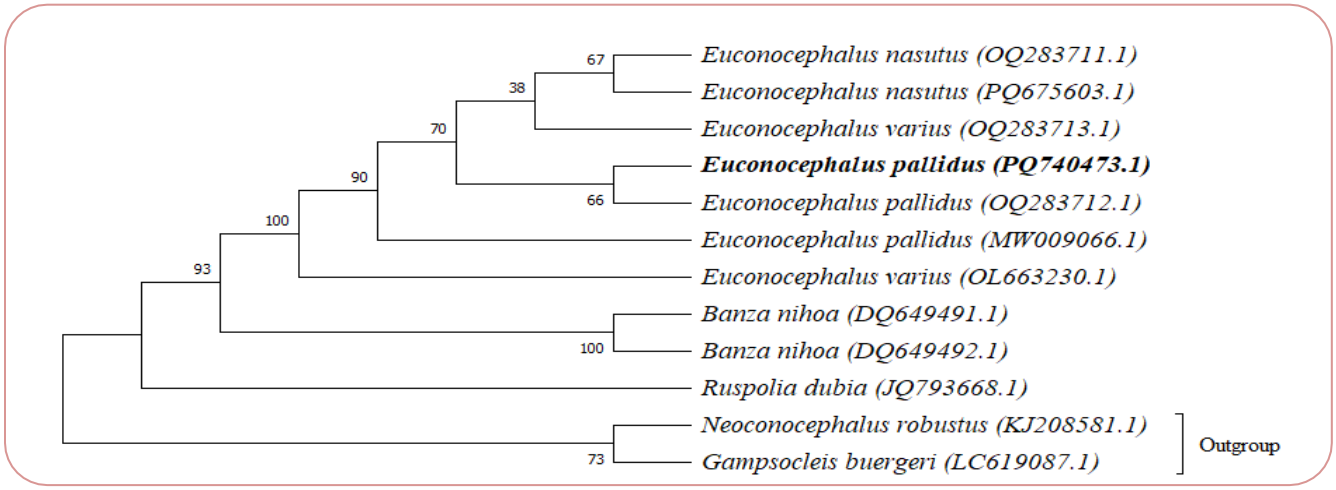

Figure 5. Maximum Likelihood phylogenetic tree based on COI sequences of *E. pallidus*, constructed with 1000 bootstrap replicates.

The sequence labeled in bold, *E. pallidus* (PQ740473.1), represents the specimen analyzed in this study. Its close clustering with two other *E. pallidus* sequences (OQ283712.1 and MW009066.1), supported by a bootstrap value of 66%, confirms its molecular identity and phylogenetic placement. The genus *Euconocephalus* formed a distinct monophyletic clade comprising *E. pallidus*, *E. nasutus*, and *E. varius*, reflecting close genetic relationships. Within this clade, *E. nasutus* sequences clustered with relatively low support values (67% and 38%), suggesting either recent divergence or intraspecific variation. The *E. varius* clade clustered near *E. pallidus* with a bootstrap value of 70%, indicating a close but distinct genetic separation.

Bootstrap values across the tree indicated varying levels of phylogenetic confidence: high values (e.g., 100, 93, and

90) reflected strong support; moderate values (e.g., 70, 66) indicated acceptable support, while lower values (e.g., 38) suggested weak resolution at certain nodes. *Gampsocleis buergeri* and *Neoconocephalus robustus* served as appropriate outgroup taxa (bootstrap = 73%), anchoring the tree for evolutionary interpretation. Meanwhile, *B. nihoa* formed a separate, well-supported lineage (bootstrap = 100), and *Ruspoliia dubia* emerged as an independent branch, highlighting distinct evolutionary trajectories within Tettigoniidae.

Overall, the phylogenetic analysis corroborates the molecular identification of *E. pallidus*, supports its taxonomic placement within *Euconocephalus*, and provides insights into the evolutionary relationships and genetic diversity among closely related species within the genus.

Discussion

E. pallidus, a member of the family Tettigoniidae, plays a significant ecological role and serves as a key representative for katydid diversity studies in Pakistan. Recent advances in molecular biology have enabled the exploration of genetic diversity and evolutionary relationships using mitochondrial markers, particularly the Cytochrome c Oxidase I (COI) gene. In the present study, COI-based molecular analyses, including Sanger sequencing, BLAST comparisons, and phylogenetic reconstruction, were employed to investigate the genetic variation of *E. pallidus* across various habitats in the Bahawalpur District.

These findings contribute substantially to understanding the species' evolutionary history and genetic structure, laying the groundwork for future taxonomic and conservation efforts. Despite global progress in insect phylogenetics, molecular data on katydids, particularly *E. pallidus*, remain limited in South Asia. Traditional taxonomy has largely relied on morphological features, which, though informative, often lack the resolution needed to delineate closely related or cryptic species. Molecular approaches provide more robust tools for species identification and phylogenetic clarification.

Previous studies on Tettigoniidae have generally been restricted in either taxonomic breadth or geographic scope, limiting their broader applicability. By analyzing 117 specimens collected from multiple ecotypes across Bahawalpur, this study offers a more comprehensive and regionally representative genetic overview of *E. pallidus* in Pakistan. Historically, katydid research in the country has focused primarily on morphological taxonomy, with minimal integration of molecular data. Although earlier studies using mitochondrial markers like COI have offered preliminary phylogenetic insights into *Euconocephalus* species, these were often based on limited sample sizes and narrowly focused sampling areas.

Comparative studies by Hawlitschek et al. (2017) and De Jesús-Bonilla et al. (2020) demonstrated the utility of COI barcoding in delimiting orthopteran species; however, these did not include *E. pallidus* from South Asia. The current study expands on their findings by incorporating a broader geographic sampling from Pakistan and specifically focusing on the molecular delineation of *E. pallidus*. The COI sequences proved effective for species-level identification. BLAST results showed high sequence identity between the sampled *E. pallidus* individuals and reference sequences, supporting morphological identification. Phylogenetic analyses clustered *E. pallidus* with *E. nasutus* and *E. varius*, consistent with previous studies.

Notably, the moderate bootstrap support (66%) for the *E. pallidus* clade suggests potential intraspecific variation, possibly influenced by geographical or ecological factors. These observations align with findings by Tan et al. (2021) and Tiwari et al. (2022), though the present study contributes novel insights through a larger and ecologically diverse dataset from Pakistan. Importantly, this work represents the most extensive molecular dataset of *E. pallidus* from Pakistan to date. The diversity of sampling sites within Bahawalpur enhances the robustness of the dataset and provides a valuable opportunity to investigate the impact of environmental heterogeneity on genetic variation.

The phylogenetic placement of *E. pallidus* within the *Euconocephalus* clade, alongside closely related taxa, supports its current taxonomic classification and suggests possible phylogeographic structuring within the species. These findings highlight the need for further research into regional population dynamics, which could reveal cryptic lineages or localized genetic adaptations.

Although the study makes significant contributions, several limitations should be noted. Although 117 specimens were analyzed, this sample may not fully represent the genetic diversity of *E. pallidus* across its entire distribution in Pakistan. Furthermore, the exclusive use of mitochondrial COI sequences, though effective for species identification, may not capture more subtle genetic variations detectable through

nuclear DNA. Future studies incorporating nuclear markers or whole-genome sequencing would provide higher-resolution insights. Moreover, environmental variables such as habitat type, land use, and climate data were not integrated into this study but could enhance the ecological interpretation of the genetic patterns observed.

Conclusion

This study enhances the molecular understanding of *E. pallidus* in Pakistan and demonstrates the utility of the COI barcode for species identification and phylogenetic analysis of katydids. The evaluation of specimens from diverse habitats within the Bahawalpur division provides valuable insights into the species' genetic diversity and evolutionary relationships. These findings establish a foundational dataset for future research in taxonomy, ecology, and conservation of Tettigoniidae in South Asia.

The study also emphasizes the need for broader genomic investigations using nuclear markers and integrative approaches that combine genetic, morphological, and ecological data. Such initiatives are essential for the discovery of cryptic diversity, accurate species delimitation, and effective conservation planning for katydids and other orthopterans in underrepresented regions like Pakistan.

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Author's Contributions

MI collected the specimens, SK provided guidance, and analyzed the data, RS conceptualized the study and proofread the manuscript MTM & KN compiled the results.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 9: Industry, Innovation and Infrastructure
SDG 13: Climate Action
SDG 15: Life on Land

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