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Research Article

Molecular Characterization of the Grasshopper Fauna in the Malakand Region, Pakistan

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ABSTRACT

The first systematic inventory of grasshoppers (Orthoptera: Acrididae) in the Malakand Division of Pakistan was conducted from April 2022 to April 2023. A total of 2,395 specimens were collected from 26 sites across eight districts using standardized sweep-netting and hand-collection methods, followed by morphometric identification based on total body length (TBL), pronotal length (PL), abdominal length (AL), tegmen length (TL), and hind femur length (HFL). Thirty-one species were recorded, representing 22 genera, 15 tribes, and nine subfamilies of Caelifera. Acridinae, Oedipodinae, Cyrtacanthacridinae, and Gomphocerinae were the most diverse subfamilies. Species richness and encounter frequency were positively correlated with warm, dry conditions, peaking in mixed-cropping zones of Swat, Buner, and Bajaur. Morphometric data revealed significant size variation, with *Choroedocus robustus* (TBL 60 mm, HFL 33 mm) and *C. illustris* (TBL 52 mm, HFL 40 mm) as the largest species, contrasting with smaller taxa such as *Phlaeoba panteli* and *Aulacobothrus socius* (TBL 13-14 mm), reflecting habitat adaptations and escape behavior. COXI DNA barcoding confirmed most morphological identifications. The nucleotide composition showed an AT bias (≈60-68%), R-values ranged from 1.02 to 3.87, and interspecific genetic distances (d) varied from 0.01 to 0.37. Pairwise divergence among *Acrida*, *Phlaeoba*, and *Truxalis* indicated genetic coherence, whereas higher distances in *Leva indica* and *Crucinotacris decisa* suggested broader geographic variation. Phylogenetic analysis using the Kimura 2-parameter model supported subfamily-level clades. Ecologically, assemblages included pest species such as *Cyrtacanthacris tatarica* and beneficial taxa vital for nutrient cycling, underscoring the need for context-specific conservation strategies.

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Introduction

Grasshoppers belong to the Kingdom Animalia, Phylum Arthropoda, Class Insecta, Order Orthoptera, and Family Acrididae. The order Orthoptera comprises grasshoppers, katydids, crickets, and locusts. The name "Orthoptera" is derived from the Greek words ortho (straight) and ptera (wings), referring to the characteristic straight wings of its members (Riyaz, 2021). Grasshoppers, members of the family Acrididae, are among the most economically important insects due to their ability to inflict severe

damage on crops and pastures.

Orthopterans are broadly classified into two suborders. The first group includes the long-horned grasshoppers (Tettigonioidea) and various crickets, while the second group comprises the short-horned grasshoppers, encompassing Caelifera, Grylloidea, and Gryllacridoidea. Within Caelifera, the superfamily Acridoidea contains the true short-horned grasshoppers, whereas Tetrigoidea and Tridactyloidea include pygmy grasshoppers and mole crickets, respectively. This taxonomic structure highlights the remarkable diversity within Orthoptera.

Pakistan, with its predominantly agrarian economy, where nearly 75% of the population depends directly or indirectly on agriculture, is particularly vulnerable to pest outbreaks. Despite cultivating a wide variety of cereal crops, vegetables, and fruits, farmers often experience declining profit margins due to pest infestations, especially those caused by grasshoppers and locusts. The family Acrididae, which includes both locusts and short-horned grasshoppers, is phytophagous and widely distributed, particularly thriving in arid and semi-arid regions (Jannat et al., 2018).

Globally, the family Acrididae comprises 31 subfamilies, 1,412 genera, and more than 6,832 described species. Of these, approximately 2,481 species occur in Asia, 890 restricted to tropical regions and 1,591 to temperate zones. Notably, 387 species are confined to the Indian subcontinent, underscoring its biodiversity significance. Among the Acrididae, five subfamilies, Acridinae, Gomphocerinae, Oedipodinae, Melanoplinae, and Cyrtacanthacridinae, have a cosmopolitan distribution, whereas the remaining subfamilies exhibit more localized geographical ranges (Guzmán et al., 2024).

Members of the family Acrididae are characterized by short antennae, typically less than three-quarters of the body length, and short ovipositors. This family also includes locusts, which are essentially grasshoppers capable of forming swarms under favorable environmental conditions (Ramakantha Rao, 1984). Sexual dimorphism is evident: males generally measure around 31 mm in length, whereas females are larger, ranging from 35 to 49 mm (Singh and Kumari, 2021). These morphological traits are important for taxonomic identification and for understanding their ecological roles and reproductive strategies.

Globally, grasshoppers represent one of the most ecologically significant insect groups, functioning as grazers, prey, and key participants in nutrient cycling. However, they are also among the most agriculturally damaging pests, causing substantial economic losses worldwide. The global and regional diversity of Acrididae, coupled with their dual ecological roles, both autecological and agroecological, underscores the need for further research into their biodiversity, systematics, and management within integrated pest management (IPM) frameworks.

The present study aimed to conduct the first comprehensive inventory and integrative taxonomic evaluation of grasshoppers (Orthoptera: Acrididae) in the Malakand Division of Pakistan, combining morphological and molecular approaches to enhance understanding of their diversity, distribution, and ecological significance.

Materials and Methods

Study area

The Malakand region, located in northern Pakistan along the Hindu Kush mountain range (35.303° N, 72.000° E), covers an area of approximately 952 km^2 and supports an estimated population of 720,296. The elevation varies from about 3,049 m in the west to 4,876 m in the northeast. The region is bordered by Afghanistan to the north, Hazara Division to the east, Mardan to the southeast, Charsadda to the southwest, and Mohmand Agency to the west (Ali et al., 2019).

The climate of Malakand is characterized by four distinct seasons: winter (November to mid-March), spring (March to April), summer (May to August), and autumn (September to October). Summer temperatures range between 16°C and 31°C (61°F to 88°F), with June being the hottest month (mean maximum 40°C or 104°F). August is relatively cooler due to higher humidity. Winters are cold, with minimum temperatures dropping to around -2°C (28°F). The mean annual rainfall is approximately 1,415 mm, increasing by about 100 mm from east to west under the influence of the monsoon (Ullah et al., 2016).

Agriculture is the predominant source of livelihood in the region, with sugarcane, wheat, and maize serving as the main crops (Ullah et al., 2016). Administratively, the Malakand Division consists of eight districts viz., Swat, Buner, Malakand, Shangla, Lower Dir, Upper Dir, Bajaur, and Chitral, each representing distinct ecological habitats.

Field surveys on grasshopper fauna were conducted across these districts, with representative sampling sites located at the following coordinates: Swat (35.2227° N,

72.4258° E), Buner (34.3943° N, 72.6151° E), Malakand (34.5030° N, 71.9046° E), Shangla (34.8872° N, 72.7570° E), Lower Dir (34.9161° N, 71.8097° E), Upper Dir (35.3356° N, 72.0468° E), Bajaur (34.7865° N, 71.5249° E), and Chitral (36.1113° N, 72.1416° E). These localities collectively provide comprehensive coverage of the region's topographical and ecological diversity (Ullah et al., 2016; Ali et al., 2019) (Figure 1).

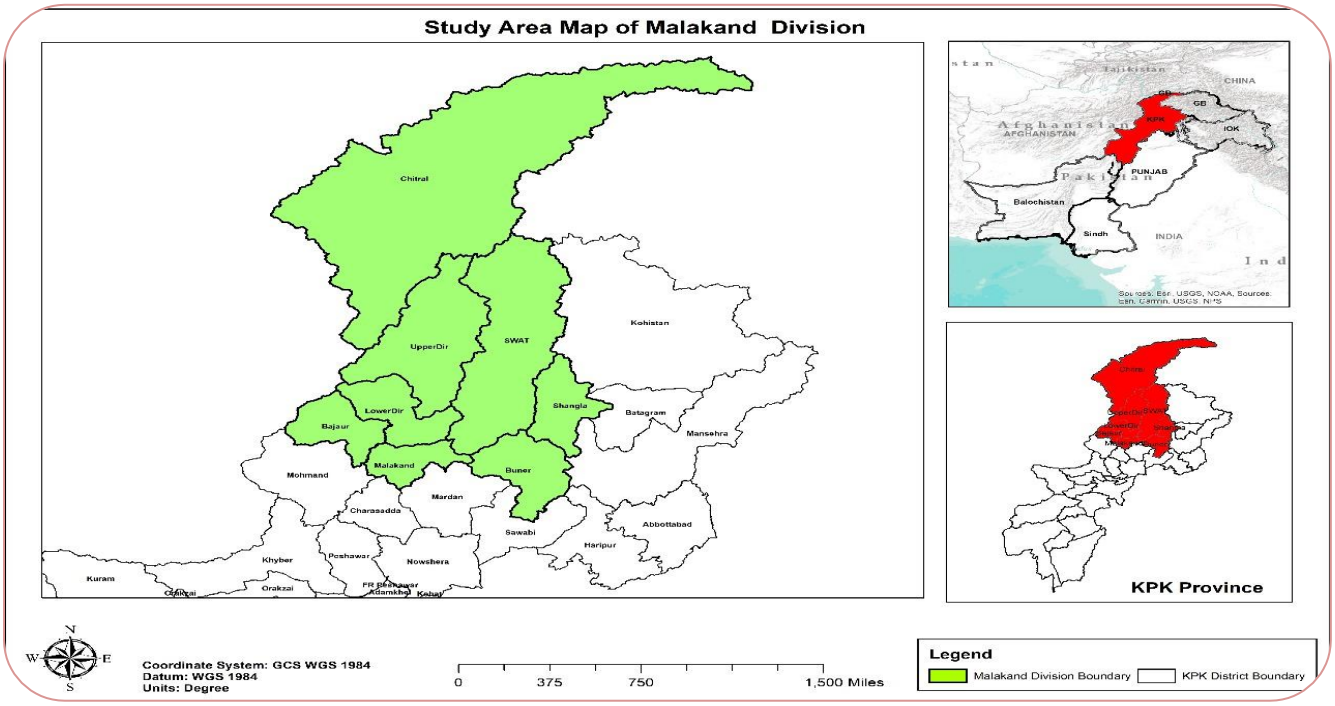


Figure 1. Map of Malakand division (prepared using QGIS software).

Sampling

Samples were collected monthly from eight different localities within the study area between 9:00 a.m. and 4:00 p.m. Grasshoppers were captured using circular aerial nets by sweeping through vegetation and by hand-picking, following the methods described by Akhtar et al. (2024). After capture, specimens were transferred into plastic bottles containing cotton soaked with ethyl acetate (serving as killing jars), each measuring approximately 4 cm in width and 6 cm in height. A cotton swab was placed inside to absorb excess fluid, and the specimens were later transferred to separate boxes to prevent discoloration.

Once immobilized, each specimen was pinned with stainless steel entomological pins (size 1, Bioequip, USA) inserted through the right side of the pronotum. The legs and antennae were carefully arranged to facilitate morphological identification, and the prepared specimens were preserved in wooden insect boxes. Field data, including collector's name, collection site, host plant, and geographical coordinates, were recorded at the time of sampling.

For morphological identification, specimens were compared with reference material available at several institutions, including the Department of Zoology, University of Swabi; Department of Zoology, University of Sindh, Jamshoro; Department of Zoology, University of Buner, Khyber Pakhtunkhwa; the National Agricultural Research Centre (NARC), Islamabad; and the National Insect Museum (NIM), Islamabad.

Morphological identification and preservation

Pinned specimens were examined for morphological identification using a stereomicroscope and a dissecting binocular microscope, with diagnostic keys developed by Riffat and Wagan (2015), supplemented by published taxonomic descriptions. Diagnostic characters studied included the morphology of the antennae, ovipositor, tympanum on the first abdominal tergum, and the three-segmented tarsi, all of which are critical for taxonomic differentiation within the family Acrididae.

To ensure identification accuracy, specimens were cross-verified with pre-identified reference material housed at

the Department of Zoology, University of Sindh, Jamshoro, and the National Insect Museum, NARC, Islamabad. For preservation, specimens intended for molecular analyses were stored in 70% ethanol, while voucher specimens for morphological study were mounted in insect boxes and tagged with complete collection details, including date, locality, and collector's name. To prevent fungal growth and ant infestation, naphthalene balls were placed inside the boxes, and anti-ant powder was applied as a precautionary measure.

Imaging and documentation

Specimen illustrations were produced using a camera lucida attachment on the stereomicroscope. When required, freehand drawings were prepared to supplement morphological observations. For photographic documentation, a digital camera coupled with a compound microscope was used to capture high-resolution images of adult specimens and diagnostic structures.

Morphometric measurements

Standardized morphometric analyses were performed using a millimeter-scale ruler to measure head length, antenna length, pronotum length, tegmen length, and total body length. These quantitative data were used for interspecific and intraspecific comparisons.

Field data and cataloguing

Comprehensive field data, including date, time, locality, host plant, and geographical coordinates, were systematically recorded in a field notebook. Each specimen was assigned a unique alphanumeric code to ensure traceability between the physical sample and corresponding field records. The same coding system was maintained for specimens designated for molecular analysis.

Specimen codes for molecular research

Specimens collected from various localities were assigned unique alphanumeric codes for molecular analyses. Specimens from Dargi were labeled MD 01 to MD 08, while those from Barikot were designated Brk 01, Brk 02, Brk 03, Brk 04, Brk 06, Brk 07, Brk 08, and Brk 10. Samples obtained from Mtta were coded Mta 01, Mta 02, Mta 04, Mta 06, Mta 09, and Mta 10. Finally, a single specimen from Malakand was labeled MK 01.

Molecular study

For DNA isolation and molecular analysis, the following reagents and equipment were used: Taq DNA polymerase, PCR buffer, $MgCl_2$, dNTPs, DNA template, distilled water, centrifuge, agarose gel, ethidium bromide, gel extraction kit (Bioer Technology Co., Ltd.), GeneBank database access, and ethanol (Chen et al., 2018).

Phylogenetic study based on DNA barcoding

DNA isolation

Leg tissue from an individual grasshopper was collected for genomic DNA extraction using the WizPrep™ gDNA Mini Kit (Cell/Tissue) from Wizbio Solutions, Korea. The leg samples were ground into a fine powder prior to extraction to enhance DNA yield and purity. High-quality genomic DNA was successfully isolated and assessed by electrophoresis on a 1.5% agarose gel. The mitochondrial cytochrome c oxidase subunit I (COX1) gene was selected as the target region for amplification via PCR.

Primer selection

Universal COX1 primers were utilized in the present study for DNA barcoding to facilitate molecular species identification and subsequent phylogenetic analysis. The primers used were as follows:

COX1-2: 5'-TAACTTTGGRTCCAAARAATC-3'

COX1-1: 5'-TTCAACAAAYCATAARGAATTGG-3' (Chen et al., 2018).

PCR Amplification

Genomic DNA was subjected to PCR amplification targeting the COX1 region. The 50 µL reaction mixture comprised:

22.5 µl PrimeSTAR Max DNA polymerase,

22.5 µl double-distilled water (ddH_2O),

2 µl DNA template,

1.5 µl forward primer and

1.5 µl reverse primer

The amplification protocol was as follows: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 95°C for 10 sec, annealing at 45-60°C for 15 sec, and extension at 65°C for 1 min/kb; followed by a final extension at 72°C for 10 min. PCR products were purified and sequenced using the primer walking method at Islamabad, Pakistan.

Polymerase chain reaction (PCR) conditions

The PCR mix was provided as a pre-formulated master mix with a standardized chemical composition optimized for high-fidelity amplification. The buffer contained 1% Triton X-100, 100 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 300 mM $(NH_4)_2SO_4$, 300 mM KCl, and 6% glycerol-providing optimal ionic strength and enzyme stability during DNA polymerization. The uncloned PCR amplicons were sequenced by Macrogen Inc. (Korea) or another internationally accredited sequencing facility to ensure high-quality nucleotide reads.

Molecular analysis

Molecular analyses were conducted using PhyloSuite

(Zhang et al., 2020) and MEGA 11 (Kumar et al., 2016) to construct phylogenetic trees employing both Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Reading frame conservation was maintained, and mitochondrial protein-coding genes were translated into amino acid sequences and aligned using the MUSCLE algorithm (Zahid et al., 2021). The aligned sequences were subsequently back-translated to nucleotides to preserve codon integrity for comparative analysis.

Further refinement included saturation analysis and codon position filtering to minimize substitutional biases, as described by Jayaprasad et al. (2024). This rigorous bioinformatic workflow ensured the robustness and reliability of the DNA barcode dataset and the resulting phylogenetic inferences. The complete methodological workflow is illustrated in Figure 2.

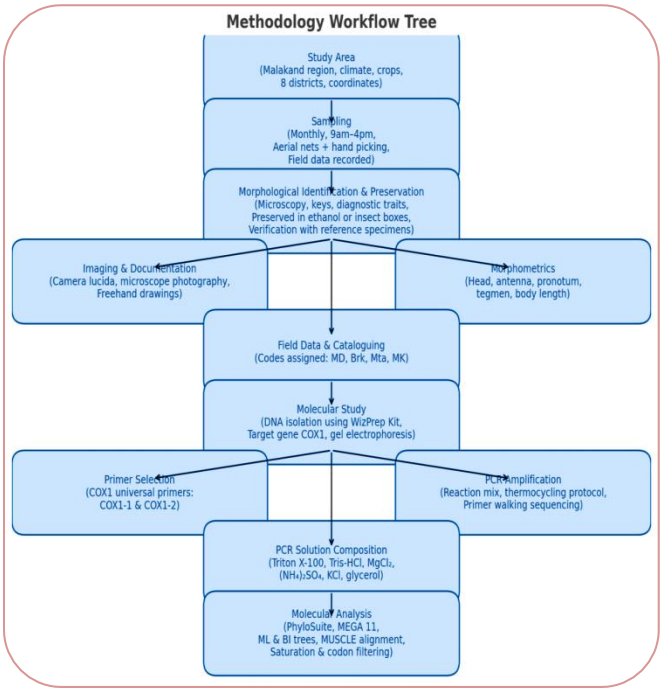


Figure 2. Research design map showing the study area, sampling, morphological identification, molecular study, and molecular analysis, with branches for imaging, morphometrics, primer selection, PCR amplification, and PCR solution composition.

Results

A total of 31 grasshopper species were recorded from the Malakand region during 2022-2023, all belonging to the suborder Caelifera within the family Acrididae (Table 1). These species were distributed across nine subfamilies and fifteen tribes, with Acridinae, Oedipodinae, and Cyrtacanthacridinae emerging as the most dominant groups (Table 2; Figure 3). Characteristic species such as *Acrida exaltata*, *Truxalis nasuta*, *Hieroglyphus banian*, and *Oxya hyla hyla* reflected the ecological adaptability of Acrididae to the diverse agro-ecological zones of Malakand. The co-occurrence of pest-associated species like *Cyrtacanthacris tatarica tatarica* alongside grassland-specialized taxa such as *Phlaeoba* spp. underscores both the agricultural and ecological importance of the regional grasshopper fauna.

Species-level identification was validated through molecular barcoding of collected specimens using the mitochondrial cytochrome oxidase subunit I (*COXI*) gene. The corresponding accession numbers and GenBank codes are provided in Table 3. Several taxa viz., *Aulacobothrus socius*, *Leva indica*, and *Cyrtacanthacris tatarica tatarica*, were represented by multiple accessions, thereby improving molecular coverage. In contrast, a few species (*Coryphistes ruricola* and *Phlaeoba infumata*) lacked accession numbers or specimen codes, highlighting partial gaps in molecular confirmation. The integration of morphological taxonomy with DNA barcoding strengthens the phylogenetic framework of Acrididae and provides a basis for future comparative analyses (Figure 4).

Morphometric analysis (Table 4) revealed significant variation in body size among the recorded taxa. The largest species were *Choroedocus robustus* (TBL 60 mm, HFL 33 mm) and *C. illustris* (TBL 52 mm, HFL 40 mm), whereas the smallest were *Phlaeoba panteli* and *Aulacobothrus socius* (TBL 13–14 mm). Hind femur length (HFL), a key parameter influencing jumping ability, ranged from 8 mm in *P. panteli* to 40 mm in *Choroedocus illustris*, suggesting distinct ecological specializations between smaller grasshoppers and larger locusts.

The molecular characterization of the *COXI* gene revealed a conserved nucleotide composition bias, with A+T content ranging from 60-68% and C+G content from 32-40%. R-values varied between 1.02 (*Chrotogonus homalodemus*) and 3.87 (*Aiolopus thalassinus thalassinus*), while net average genetic distances (d) ranged from 0.01 (*Sphingonotus rubescens rubescens*, *Trilophidia annulata*) to 0.37 (*Leva indica*) (Table 5). High sequence similarity in taxa with the lowest mean dissimilarities contrasted with the greater divergence observed in *Leva indica* and *Crucinotacris decisa*.

The Maximum Likelihood phylogenetic tree (Figure 5), constructed under the Kimura 2-parameter model (Kimura, 1980), grouped species into distinct clades

corresponding to their respective subfamilies, corroborating both morphological and molecular systematics. Overall, this integrated approach combining morphometrics, classical taxonomy, and molecular barcoding proved effective in elucidating the diversity and evolutionary relationships of Acrididae species in the Malakand region.

A total of 31 grasshopper species, all belonging to the suborder Caelifera within the family Acrididae, were recorded from the Malakand region during 2022-2023 (Table 6). These species were distributed across nine subfamilies and fifteen tribes, with Acridinae, Cyrtacanthacridinae, Oedipodinae, and Gomphocerinae exhibiting the greatest diversity. The findings identify Malakand as a rich hotspot for acridid fauna, comprising species that include both important agricultural pests and ecologically significant grassland inhabitants.

Table 7 presents the morphometric analysis of grasshopper species from the Malakand region, revealing considerable size variation among species. *C. robustus* (TBL: 60 mm; HFL: 33 mm) and *C. illustris* (TBL: 52 mm; HFL: 40 mm) were the largest species, whereas *P. panteli* and *A. socius* were among the smallest (TBL: 13-14 mm). The hind femur length, an important trait associated with jumping ability, was highest in *C. illustris* (40 mm) and lowest in *P. panteli* (8 mm). Overall, the data indicate marked interspecific variation in body proportions, reflecting adaptations to diverse ecological habitats.

Table 8 and Figure 7 present the nucleotide composition and mean genetic distance (d) among grasshopper species identified from the Malakand region during 2022-2023. The data include the R-value, representing the ratio of transition to transversion mutations, along with the percentages of adenine (A), thymine/uracil (T/U), cytosine (C), and guanine (G) bases in the mitochondrial cytochrome c oxidase subunit I (COXI) gene. These results provide insights into the genetic diversity among the species and highlight interspecific variations within the region's grasshopper populations. The observed genetic distances reflect evolutionary relationships among species, contributing to a clearer understanding of phylogenetic patterns and evolutionary divergence within the family Acrididae.

Molecular characterization of grasshoppers via the COXI gene

The *Cytochrome Oxidase I* (COXI) gene, located in the mitochondrial genome, plays a crucial role in oxidative phosphorylation, where electrons from cytochrome *c* are transferred to molecular oxygen (O_2) to drive ATP synthesis (Povea-Cabello et al., 2024). The COXI gene is highly conserved among eukaryotic organisms, exhibiting only minor interspecific sequence variations.

Grasshopper samples were validated through the presence of distinct DNA bands observed on agarose gel electrophoresis (Figure 4). The PCR-amplified mitochondrial DNA products were sequenced through a commercial molecular biology service. Post-sequencing, the obtained reads were aligned and edited using BioEdit software, and phylogenetic dendrograms were constructed to visualize genetic clustering.

The finalized sequences were subsequently submitted to the GenBank database, where accession numbers were assigned to each species. The reconstructed phylogeny (Figure 6) represents the evolutionary relationships among the grasshopper species identified from the Malakand region.

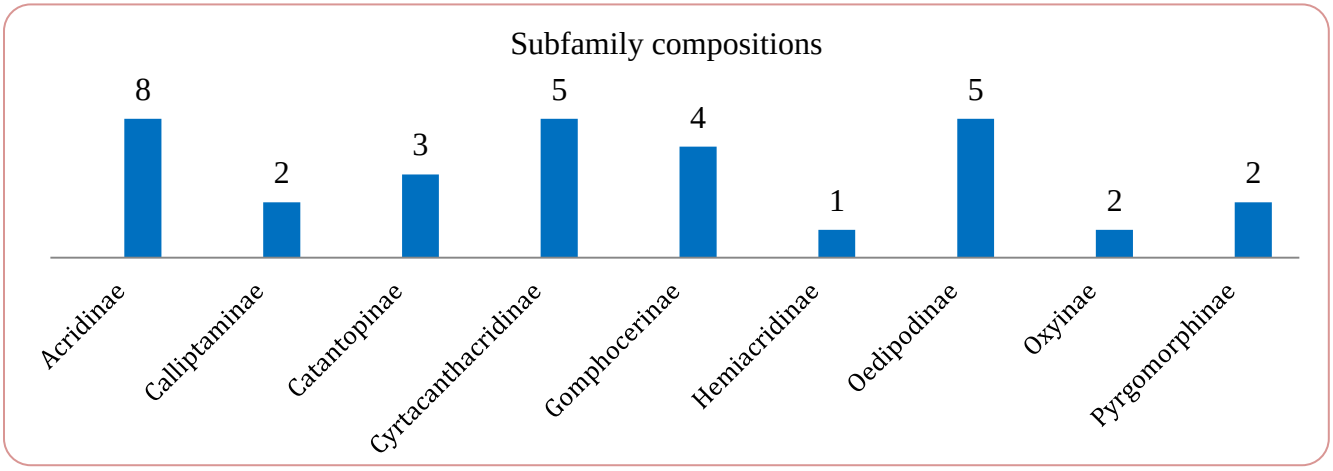


Figure 3. Subfamily composition of grasshopper fauna in the Malakand region, Pakistan.

Table 1. Overall abundance of grasshopper species of the family Acrididae in the Malakand region, Pakistan, during 2022-2023.

Subfamily	Tribe	Species
Acridinae	Acridini	*Acrida exaltata* (Walker, 1859)
		A. gigantea (Herbst, 1786)
		A. indica (Dirsh, 1954)
		A. turrita (Linnaeus,1758)
	Phlaeobini	*Phlaeoba infumata* (Brunner von Wattenwyl, 1893)
		P. panteli (Bolivar, 1902)
		Ph. tenebrosa (Walker, 1871)
		P. brachyptera (Caudell,1921)
Calliptaminae	Truxalini	*Truxalis eximia eximia* (Eichwald, 1830)
		T. nasuta (Linnaeus, 1758)
Catantopinae	Catantopini	*Diabolocatantops innotabilis* (Walker, 1870)
		Xenocatantops humilis (Serville, 1838)
Cyrtacanthacridinae	Cyrtacanthacridini	*Anacridium aegyptium* (Linnaeus, 1764)
		Cyrtacanthacris tatarica tatarica (Linnaeus, 1758)
		Choroedocus illustris (Walker, 1870)
		C. robustus (Serville, 1838)
	Eyprepocnemidini	*Heteracris littoralis* (Rambur, 1838)
Gomphocerinae	Arcypterini	*Aulacobothrus luteipes luteipes* (Walker, 1871)
		Aulacobothrus socius (Bolivar, 1902)
		Crucinotacris decisa (Walker, 1871)
	Dociostaurini	*Leva indica* (Bolivar, 1902)
Hemiacridinae	Hieroglyphini	*Hieroglyphus banian* (Fabricius, 1798)
Oedipodinae	Acrotylini	*Acrotylus insubricus insubricus* (Scopoli, 1786)
	Epacromiini	*Aiolopus thalassinus thalassinus* (Fabricius, 1781)
	Locustini	*Oedaleus abruptus* (Thunberg, 1815)
	Phlaeobini	*Sphingonotus rubescens rubescens* (Walker, 1870)
	Trilophidiini	*Trilophidia annulata* (Thunberg, 1815)
Oxyinae	Oxyini	*Oxya hyla hyla* (Serville, 1831)
		O. velox (Fabricius, 1787)
Pyrgomorphinae	Pyrgomorphini	*Atractomorpha crenulata* (Fabricius, 1793)
	Chrotogonini	*Chrotogonus homalodemus* (Blanchard, 1836)

Table 2. Subfamilies, number of genera, and number of species of grasshopper fauna from the Malakand region, Pakistan.

Sr. No.	Subfamily	No. of genera	No. of species
01	Acridinae	2	8
02	Calliptaminae	1	2
03	Catantopinae	2	2
04	Cyrtacanthacridinae	4	5
05	Gomphocerinae	3	4
06	Hemiacridinae	1	1
07	Oedipodinae	5	5
08	Oxyinae	1	2
09	Pyrgomorphinae	2	2
Total	09	21	31

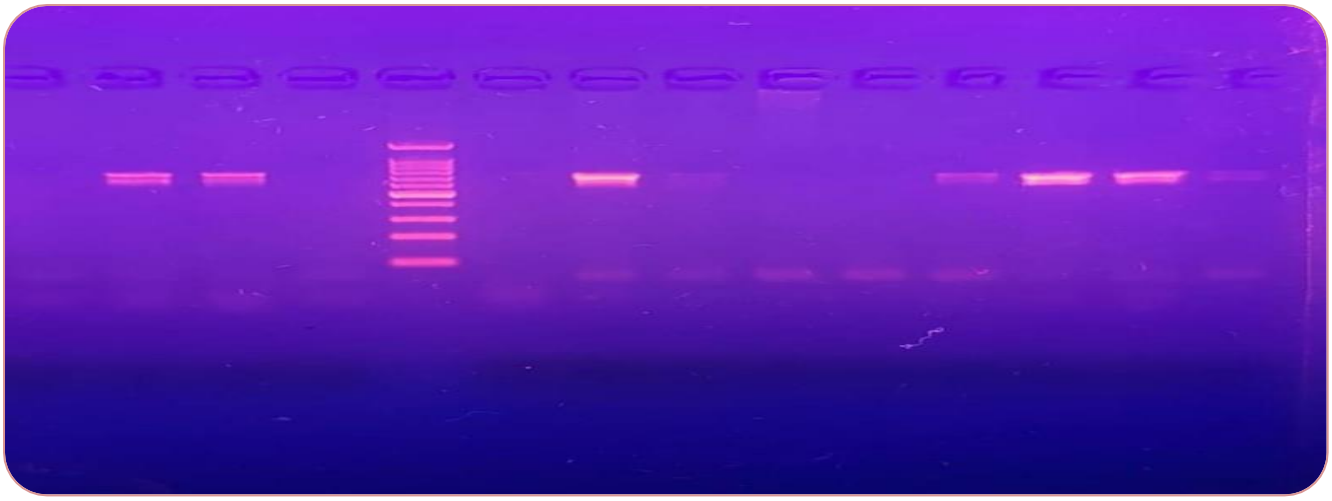


Figure 4. Confirmation of DNA by gel electrophoresis.

Table 3. Name of species with assigned code and accession number.

Sr. No.	Species Name	Code Assign	Accession No.
01	*A. exaltata*	Brk08	PV802563
02	*A. gigantea*	MD06	PX061801
03	*A. indica*	MD05	PX067301
04	*A. turrita*	MD08	PV731347
05	*P. infumata*	MD 06	PV802652
06	*P. panteli*	Brk03	PV740199
07	*P. tenebrosa*	MD06	PV695205
08	*P. brachyptera*	MD02	PX067742
09	*T. eximia eximia*	MD 07	PV802649
10	*T. nasuta*	MK07	PV687496
11	*D. innotabilis*	Mta06	PV731405
12	*X. humilis*	Brk06	PV802651
13	*A. aegyptium*	Brk07	PV802579
14	*C. tatarica tatarica*	Brk10/MD01	PV687494/PV694996
15	*C. illustris*	Mta01	PV802682
16	*C. robustus*	Brk09	PV802623
17	*H. littoralis*	Mta08	PV802625
18	*A. luteipes luteipes*	Mta02	PV802593
19	*A. socius*	Mta02/Brk04/Brk11	PV687460/PV687456/PV695570
20	*C. decisa*	Brk02	PV687458
21	*L. indica*	Mta01/Brk05/Mta07	PV695550/PV687457/PV731352
22	*H. banian*	Mk03	PV802624
23	*A. insubricus insubricus*	Mta09	PV802577
24	*A. thalassinus thalassinus*	MK01	PV695489
25	*O. abruptus*	Mta04	PV731350
26	*S. rubescens rubescens*	Mk02	PV802635
27	*T. annulata*	Mta10	PV731346
28	*O. hyla hyla*	MD07	PV731349
29	*O. velox*	MD03	PX061776
30	*A. crenulata*	MD04	PV695471
31	*C. hemolatus*	Brk 01	PV698157

Table 4. Morphological characteristics of grasshopper species collected from the Malakand region, Pakistan, during 2022-2023.

Sr. No.	Species	TBL	PL	AL	TL	HFL
01	*A. exaltata*	33	04	11	26	19
02	*A. gigantea*	29	04	08	24	16
03	*A. indica*	33	04	11	26	19
04	*A. turrita*	30	04	10	24	18
05	*P. infumata*	15	04	06	17	09
06	*P. panteli*	13	03	05	15	08
07	*P. tenebrosa*	14	04	06	17	10
08	*P. brachyptera*	13	04	06	17	08
09	*T. eximia eximia*	37	05	12	31	23
10	*T. nasuta*	34	05	11	28	20
11	*D. innotabilis*	25	04	07	23	11
12	*X. humile*	22	04	06	16	11
13	*A. aegyptium*	30	5	07	18	23
14	*C. tatarica tatarica*	38	05	07	17	20
15	*C. illustris*	52	10	11	51	40
16	*C. robustus*	60	09	15	43	33
17	*H. littoralis*	25	03	09	20	14
18	*A. luteipes luteipes*	15	02	06	10	11
19	*A. socius*	14	03	06	09	10
20	*C. decisa*	21	03	06	17	10
21	*L. indica*	15	3	5	8	9
22	*H. banian*	15	03	06	10	11
23	*A. insubricus insubricus*	15	04	06	17	09
24	*A. thalassinus thalassinus*	14	03	05	17	09
25	*O. abruptus*	14	04	06	14	17
26	*S. rubescens rubescens*	22	03	07	23	15
27	*T. annulata*	14	03	05	17	09
28	*O. hyla hyla*	22	04	08	17	12
29	*O. velox*	21	03	06	17	10
30	*A. crenulata*	21	04	07	07	14
31	*C. homalodemus*	25	06	07	07	15

TBL (Total Body Length); PL (Pronotum Length); AL (Antenna Length); TL (Tegmina Length); HFL (Hind Femur Lenght).

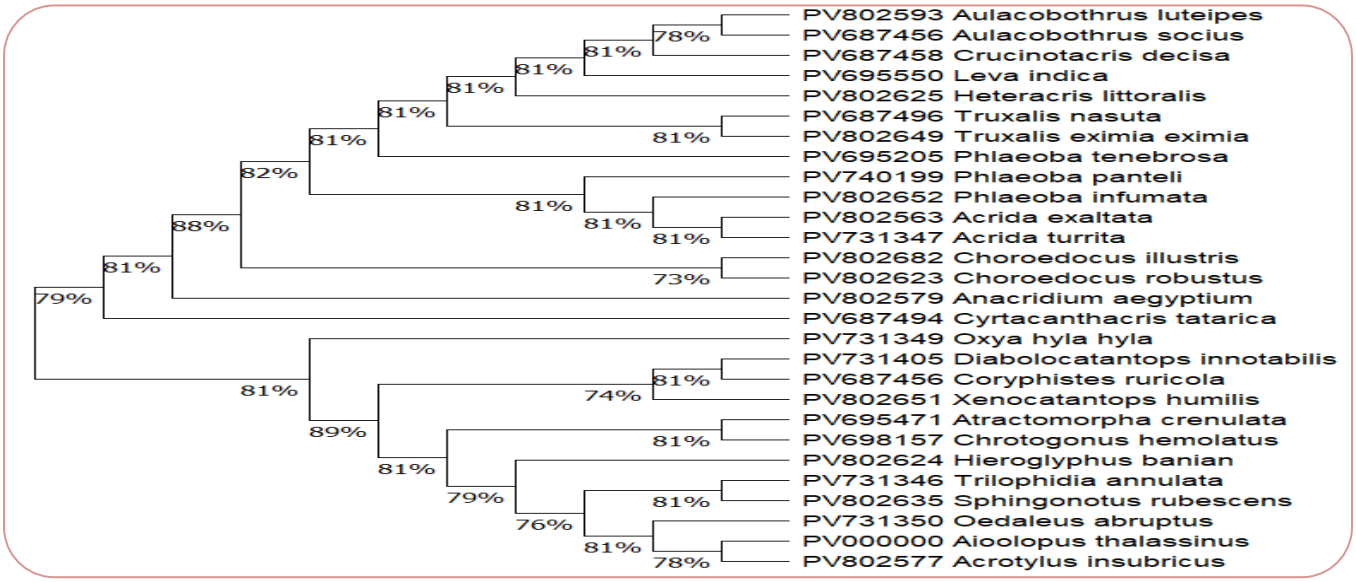


Figure 5. Phylogenetic tree of species reported from the study area.

Table 5. Overall mean genetic distance and R-values of the reported species.

Sr. No.	Species	Access No.	R value	A%	T/U%	C%	G%	Over all mean distance (d)
01	*A. exaltata*	PV802563	1.67	32.28	32.29	19.01	16.33	0.06
02	*A. gigantea*	PX061801	1.13	38.03	32.52	16.24	13.21	0.23
03	*A. indica*	PX067301	1.38	32.33	33.83	18.29	15.54	0.48
04	*A. turrita*	PV731347	1.31	33.28	32.59	19.12	16.23	0.13
05	*P. infumata*	PV802652	1.54	32.33	33.83	18.29	15.54	0.05
06	*P. panteli*	PV740199	1.36	31.60	33.57	18.24	16.69	0.09
07	*P. tenebrosa*	PV695205	1.23	31.13	34.40	18.91	15.56	0.07
08	*P. brachyptera*	PX067742	1.27	32.23	33.90	17.76	16.11	0.15
09	*T. eximia eximia*	PV802649	1.77	32.34	34.43	17.38	15.85	0.10
10	*T. nasuta*	PV687496	1.97	34.30	32.59	17.37	15.74	0.04
11	*D. innotabilis*	PV731405	1.77	31.85	33.08	19.76	15.31	0.15
12	*X. humile*	PV802651	1.96	30.01	34.04	19.78	16.17	0.06
13	*A. aegyptium*	PV802579	2.67	30.01	33.30	19.67	17.04	0.07
14	*C. tatarica tatarica*	PV687494 PV694996	1.17	32.28	34.11	17.85	15.75	0.06
15	*C. illustris*	PV802682	2.37	29.86	32.20	20.10	17.84	0.05
16	*C. robustus*	PV802623	1.44	29.24	33.24	19.28	18.04	0.07
17	*H. littoralis*	PV802625	3.06	28.29	34.82	19.54	17.35	0.03
18	*A. luteipes luteipes*	PV802593	2.77	31.54	33.92	17.70	16.84	0.02
19	*A. socius*	PV687460 PV687456 PV695570	1.37	32.03	32.78	18.68	16.52	0.06
20	*C.decisa*	PV687458	1.20	32.12	32.87	18.60	16.41	0.34
21	*L. indica*	PV69550 PV687457 Pv731352	1.33	31.59	33.46	18.07	16.88	0.37
22	*H. banian*	PV802624	1.25	30.96	33.98	18.35	16.71	0.12
23	*A. insubricus insubricus*	PV802577	1.34	31.65	33.43	19.62	15.30	0.15
24	*A. thalassinus thalassinus*	PV695489	3.87	34.05	32.03	18.40	15.52	0.02
25	*O. abruptus*	PV731350	1.85	30.38	33.25	19.13	16.78	0.07
26	*S. rubescens rubescens*	PV802635	2.93	32.83	30.70	19.58	16.89	0.01
27	*Trilophidia annulata*	PV731346	2.12	32.57	33.40	17.67	16.36	0.01
28	*O. hyla hyla*	PV731349	1.23	31.92	33.59	18.05	16.43	0.13
29	*O. velox*	PX061776	1.44	31.17	34.36	18.30	16.17	0.30
30	*A. crenulata*	PV695471	1.92	35.06	31.72	1793	15.28	0.08
31	*C.homalodemus*	PV698157	1.02	32.57	33.40	17.67	16.36	0.12

Table 6. Grasshopper species of the suborder Caelifera (family Acrididae) documented from the Malakand region, Pakistan (2022-2023).

Subfamily	Tribe	Species
Acridinae	Acridini Phlaeobini	*Acrida exaltata* (Walker, 1859); *Acrida turrita* (Linnaeus, 1758) *Phlaeoba infumata* (Brunner von Wattenwyl, 1893); *Phlaeoba panteli* (Bolívar, 1902); *Phlaeoba tenebrosa* (Walker, 1871)
Calliptaminae	Truxalini	*Truxalis eximia eximia* (Eichwald, 1830); *Truxalis nasuta* (Linnaeus, 1758)
Catantopinae	Catantopini	*Diabolocatantops innotabilis* (Walker, 1870); *Coryphistes ruricola* (Burmeister, 1838); *Xenocatantops humilis* (Serville, 1838)

Cyrtacanthacridinae	Cyrtacanthacridini	*Anacridium aegyptium* (Linnaeus, 1764); *Cyrtacanthacris tatarica tatarica* (Linnaeus, 1758); *Choroedocus illustris* (Walker, 1870); *Choroedocus robustus* (Serville, 1838)
	Eyprepocnemidini	*Heteracris littoralis* (Rambur, 1838)
Gomphocerinae	Arcypterini	*Aulacobothrus luteipes luteipes* (Walker, 1871); *Aulacobothrus socius* (Bolívar, 1902); *Crucinotacris decisa* (Walker, 1871)
	Dociostaurini	*Leva indica* (Bolívar, 1902)
Hemiacridinae	Hieroglyphini	*Hieroglyphus banian* (Fabricius, 1798)
Oedipodinae	Acrotylini	*Acrotylus insubricus insubricus* (Scopoli, 1786)
	Epacromiini	*Aiolopus thalassinus thalassinus* (Fabricius, 1781)
	Locustini	*Oedaleus abruptus* (Thunberg, 1815)
	Phlaeobini	*Sphingonotus rubescens rubescens* (Walker, 1870)
	Trilophidiini	*Trilophidia annulata* (Thunberg, 1815)
Oxyinae	Oxyini	*Oxya hyla hyla* (Serville, 1831)
Pyrgomorphinae	Pyrgomorphini	*Atractomorpha crenulata* (Fabricius, 1793)
	Chrotogonini	*Chrotogonus homalodemus* (Blanchard, 1836)

Table 7. Morphometric measurements (mm) of grasshopper species collected from the Malakand region, Pakistan (2022-2023).

Species	TBL (Total	PL (Pronotum)	AL (Antenna)	TL (Tegmina)	HFL Femur)
A. exaltata	33	04	11	26	19
A. turrita	30	04	10	24	18
P. infumata	15	04	06	17	09
P. panteli	13	03	05	15	08
P. tenebrosa	14	04	06	17	10
T. eximia eximia	37	05	12	31	23
T. nasuta	34	05	11	31	20
D. innotabilis	25	04	07	23	11
C. ruricola	21	03	06	17	10
X. humilis	22	04	06	16	11
A. aegyptium	30	05	07	18	23
C. tatarica	38	05	07	17	20
C. illustris	52	10	11	51	40
C. robustus	60	09	15	43	33
H. littoralis	25	03	09	20	14
A. luteipes	15	02	06	10	11
A. socius	14	03	06	09	10
C. decisa	21	03	06	17	10
L. indica	15	03	05	08	09
H. banian	15	03	06	10	11
A. insubricus	15	04	06	17	09
A.thalassinus	14	03	05	17	09
O. abruptus	14	04	06	14	17
S. rubescens	22	03	07	23	15
T. annulata	14	03	05	17	09
O. hyla hyla	22	04	08	17	12
A. crenulata	21	04	07	07	14
C. homalodemus	25	06	07	07	15

Table 8. Nucleotide composition and overall mean genetic distance (d) among grasshopper species from the Malakand region, Pakistan (2022-2023).

Species	Accession Number	R value	A%	T/U%	C%	G%	Mean Distance (d)
A. exaltata	PV802563	1.67	32.28	32.29	19.01	16.33	0.06
A. turrita	PV731347	1.31	33.28	32.59	19.12	16.23	0.13
P. infumata	PV802652	1.54	32.33	33.83	18.29	15.54	0.05
P. panteli	PV740199	1.36	31.60	33.57	18.24	16.69	0.09
P. tenebrosa	PV695205	1.23	31.13	34.40	18.91	15.56	0.07
T. eximia eximia	PV802649	1.77	32.34	34.43	17.38	15.85	0.10
T. nasuta	PV687496	1.97	34.30	32.59	17.37	15.74	0.04
D. innotabilis	PV731405	1.77	31.85	33.08	19.76	15.31	0.15
C. ruricola	PV687456	1.29	31.52	33.70	18.12	16.67	0.11
X. humilis	PV802651	1.96	30.01	34.04	19.78	16.17	0.06
A. aegyptium	PV802579	2.67	30.01	33.30	19.67	17.04	0.07
C. tatarica tatarica	PV687494, PV694996	1.17	32.28	34.11	17.85	15.75	0.06
C. illustris	PV802682	2.37	29.86	32.20	20.10	17.84	0.05
C. robustus	PV802623	1.44	29.24	33.24	19.28	18.04	0.07
H. littoralis	PV802625	3.06	28.29	34.82	19.54	17.35	0.03
A. luteipes luteipes	PV802593	2.77	31.54	33.92	17.70	16.84	0.02
A.s socius	PV687460, PV687456, PV695570	1.37	32.03	32.78	18.68	16.52	0.06
C. decisa	PV687458	1.20	32.12	32.87	18.60	16.41	0.34
L. indica	PV69550, PV687457, PV731352	1.33	31.59	33.46	18.07	16.88	0.37
H. banian	PV802624	1.25	30.96	33.98	18.35	16.71	0.12
A. insubricus insubricus	PV802577	1.34	31.65	33.43	19.62	15.30	0.15
A. thalassinus thalassinus	PV695489	3.87	34.05	32.03	18.40	15.52	0.02
O. abruptus	PV731350	1.85	30.38	33.25	19.13	16.78	0.07
S. rubescens rubescens	PV802635	2.93	32.83	30.70	19.58	16.89	0.01
T. annulata	PV731346	2.12	32.57	33.40	17.67	16.36	0.01
O. hyla hyla	PV731349	1.23	31.92	33.59	18.05	16.43	0.13
A. crenulata	PV695471	1.92	35.06	31.72	17.93	15.28	0.08
C. homalodemus	PV698157	1.02	32.57	33.40	17.67	16.36	0.12

The phylogenetic relationships among the grasshopper species were further clarified through molecular analysis of the COXI gene. The phylogenetic trees (Figures 8 and 9) illustrate the clustering of species according to their genetic distances.

Discussion

The current year-long survey (April 2022-April 2023) documented the grasshopper fauna of the Malakand region across its eight districts viz., Buner, Malakand, Bajaur, Swat, Shangla, Lower Dir, Upper Dir, and Chitral, yielding 31 species belonging to 22 genera, 17 tribes, 9 subfamilies, 1 family, and 1 order. The assemblage was taxonomically dominated by the family Acrididae, with subfamilies Acridinae**,** Calliptaminae**,** Catantopinae**,** Cyrtacanthacridinae**,** Gomphocerinae**,** Hemiacridinae**,** Oedipodinae**,** Oxyinae, and Pyrgomorphinae represented. The recorded species included *A. exaltata* (Walker, 1859), *A. turrita* (Linnaeus, 1758), *P. infumata* (Brunner von Wattenwyl, 1893), *P. panteli* (Bolivar, 1902), *P. tenebrosa* (Walker, 1871), *T. eximia eximia* (Eichwald, 1830), *T. nasuta* (Linnaeus, 1758), *D. innotabilis* (Walker, 1870), *C. ruricola* (Burmeister, 1838), *X. humilis* (Serville, 1838), *A. aegyptium* (Linnaeus, 1764), *C. tatarica tatarica* (Linnaeus, 1758), *C. illustris* (Walker, 1870), *C. robustus* (Serville, 1838), *H. littoralis* (Rambur, 1838), *A. luteipes luteipes* (Walker, 1871), *A. socius* (Bolivar, 1902), *C. decisa* (Walker, 1871), *L. indica* (Bolivar, 1902), *H. banian* (Fabricius, 1798), *A. insubricus insubricus* (Scopoli, 1786), *A. thalassinus thalassinus* (Fabricius,

1781), *O. abruptus* (Thunberg, 1815), *S. rubescens rubescens* (Walker, 1870), *T. annulata* (Thunberg, 1815), *O. hyla hyla* (Serville, 1831), *A. crenulata* (Fabricius, 1793), and *C. homalodemus* (Blanchard, 1836).

Parallel to taxonomic documentation, this study assessed distribution, diversity, morphology, seasonal occurrence, and host specificity across the representative agroecological zones of Malakand. Consistent with subtropical, montane mosaics, taxonomic richness was concentrated within the Acrididae, reflecting their broad ecological amplitude and habitat generalism.

The observed seasonal patterns align with previous findings that interannual variation in grasshopper assemblages is influenced by atmospheric variability, moisture regimes, vegetation phenology, and microhabitat structure (Du et al., 2022). In habitats with diverse vegetation, grasshopper richness typically peaks during warmer months when soft plant tissue and biomass are abundant (Panhwar et al., 2024). Our findings corroborate this trend: species richness and encounter rates were highest under relatively warm and dry conditions, indicating that temperature and rainfall are the principal abiotic factors influencing emergence, survival, and voltinism (Blanco-Sierra et al., 2024). Field observations further suggest that elevated temperatures accelerate hatching and enhance early survivorship, especially in arid microhabitats (Chaudhary et al., 2025). Such interactions, combined with crop cycles and irrigation, likely explain the site-specific variations observed in Swat, Buner, and Bajaur.

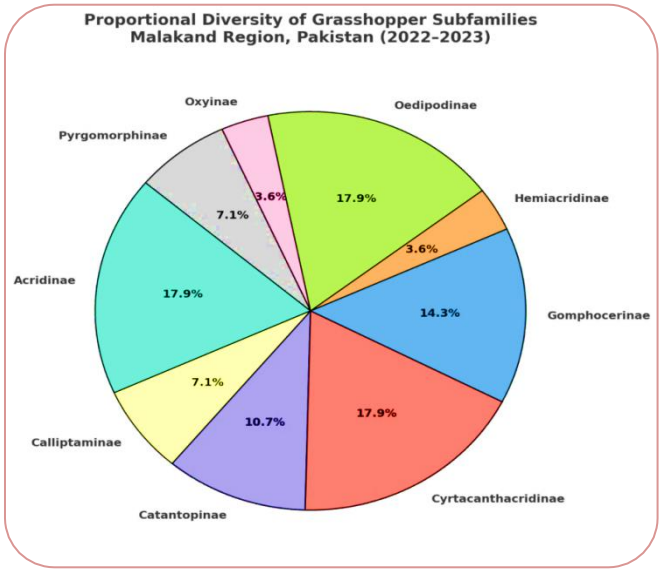


Figure 6. Proportional diversity of grasshopper subfamilies in the Malakand region (2022-2023).

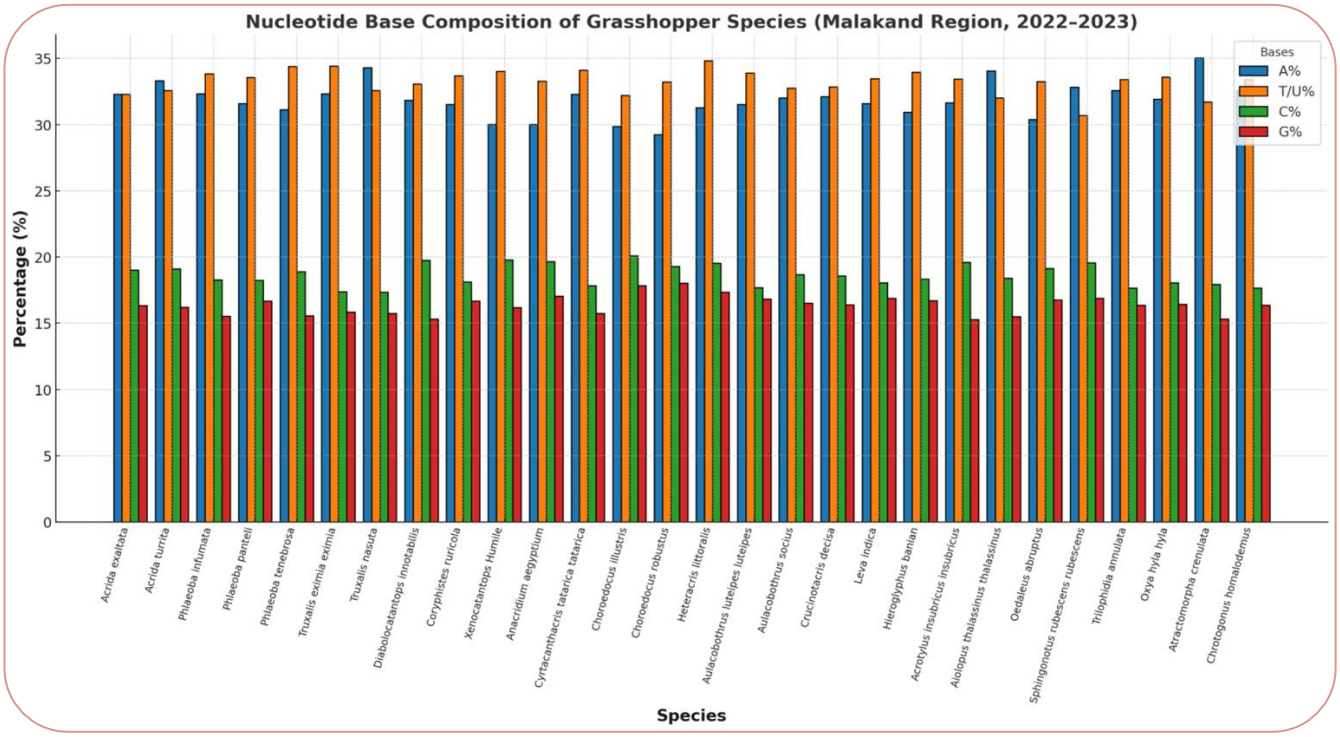


Figure 7. Nucleotide base composition (A, T/U, C, and G percentages) of grasshopper species from the Malakand region (2022-2023).

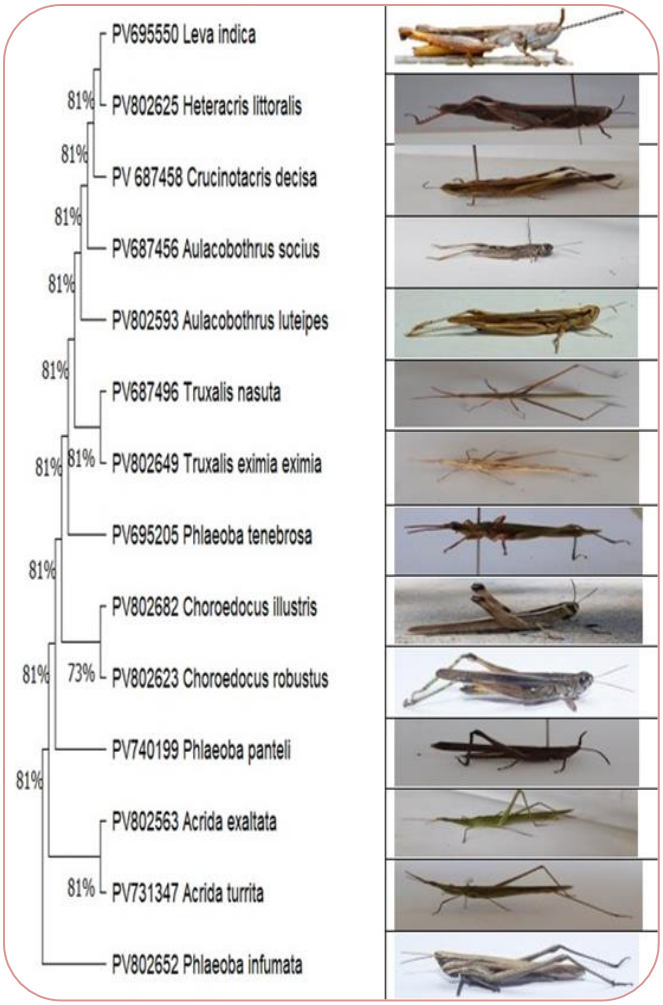


Figure 8. Phylogenetic tree illustrating the clustering of species based on their genetic distances.

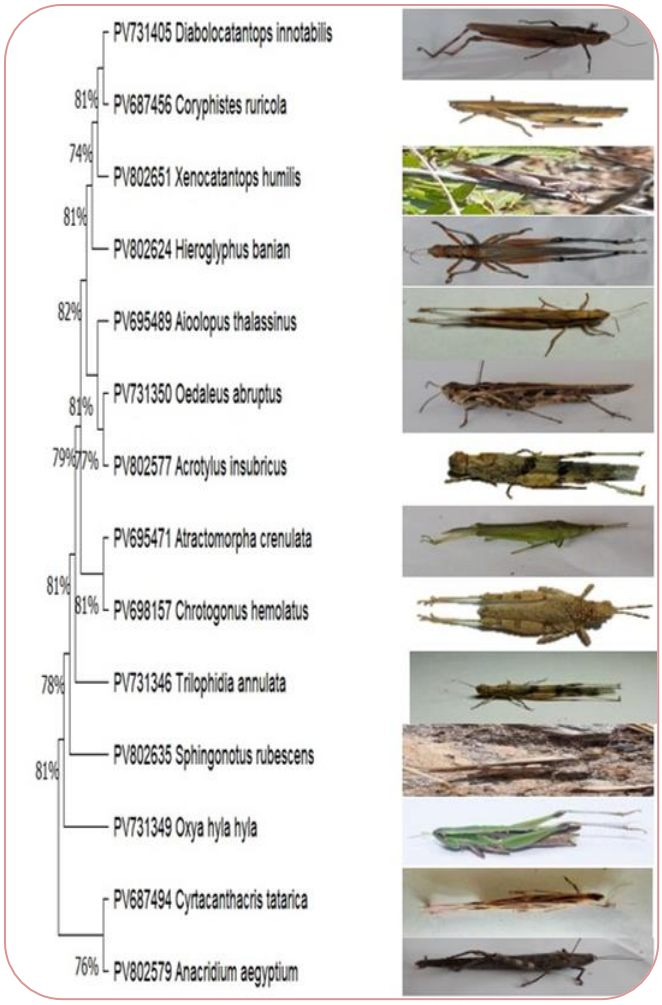


Figure 9. Phylogenetic tree illustrating the clustering of species based on their genetic distances.

Spatial variation was most pronounced in Swat, Buner, and Bajaur, where differences in crop diversity, irrigation intensity, and human activity were evident. The richness and relative abundance patterns observed here are consistent with regional studies across Pakistan, including reports from Sindh (Mustafa et al., 2024), Gujrat, Punjab (Hussain et al., 2024), the Mount Elum Piedmont in Buner (Khan et al., 2021), Tehsil Havelian (Waqar et al., 2024), and Kuchlak, Quetta (Khalil et al., 2025). In our study, higher species counts in mixed cropping systems and disturbed margins indicate that vegetation composition and anthropogenic activities such as mowing, grazing, fallow rotation, and pesticide use strongly influence grasshopper distributions (Ali et al., 2024). The co-occurrence of *A. thalassinus* and *Acrotylus* spp., as also reported from Sindh (Pitafi, 2024), reinforces the association of xeric-tolerant oedipodines and acridinines with open, sun-exposed habitats having sparse canopy cover.

Although regional diversity was moderate, the subfamily and tribal composition suggested distinct community assembly patterns. Acridinae, Oedipodinae, and Cyrtacanthacridinae were most prevalent, while Gomphocerinae dominated grass-rich habitats. The comparatively lower representation of Hemiacridinae and Oxyinae likely reflects either restricted local niches or under-sampling of preferred microhabitats. Seasonal turnover was marked by a high relative abundance of *O. hyla hyla* and *Acrida* spp. in lowlands, tracking resource availability (lush monocotyledons in irrigated plots) and temperature-dependent activity (Hussain et al., 2024). *A. turrita* was ubiquitous and stable, while *O. hyla hyla* and *P. panteli* exhibited broad ecological tolerance. Conversely, the absence of *H. banian* and *A. ungarica* in certain sites

may indicate localized habitat restrictions and stringent microclimatic requirements (Sarwar et al., 2023).

Our findings support an ecological filtering framework: (i) regional climate and elevation determine the potential species pool; (ii) land use and vegetation structure filter species based on dispersal ability, oviposition microhabitats, and dietary breadth; and (iii) seasonal weather influences phenology and cohort strength. Comparable conclusions were drawn by Kalhoro et al. (2019), who emphasized that plantation type and local environmental factors shape community composition, a result consistent with our cross-site comparisons.

Functionally, the Malakand grasshopper assemblage comprises species with diverse feeding habits, mobility, and life cycles, playing key roles in seed dispersal, nutrient cycling, and serving as prey for reptiles, spiders, and birds. Although certain species (e.g., *C. tatarica tatarica*) pose agronomic threats through defoliation and tiller damage, others contribute to weed suppression. Thus, the ecological impact of grasshoppers is context-dependent, traits conferring pest potential in monocultures may enhance ecosystem stability in heterogeneous landscapes.

Morphometric analysis revealed substantial interspecific variation in total body length, pronotal length, antennal length, tegminal length, and hind femur length. The largest species, *C. robustus* (TBL 60 mm, HFL 33 mm) and *C. illustris* (TBL 52 mm, HFL 40 mm), exhibited robust hind femora, indicative of strong jumping ability and adaptation to open habitats. In contrast, smaller taxa such as *P. panteli* and *A. socius* (TBL 13-14 mm) likely depend more on crypsis and microhabitat fidelity. These allometric trends align with habitat structure and predation pressures and are consistent with patterns documented in semi-arid Asian acridid communities (Hussain et al., 2024). Such morphological traits, combined with diagnostic features (antennae type, tympanum position, tarsal segmentation), remain critical for accurate species identification.

Mitochondrial COXI barcoding largely supported morphological species delimitations. The nucleotide composition exhibited the characteristic AT-rich bias (60-68% A+T) typical of insect mitochondrial genomes. R-values ranged from 1.02 (*C. homalodemus*) to 3.87 (*A. thalassinus thalassinus*), reflecting compositional variation among lineages. Mean genetic distances (d = 0.01-0.37) indicated high sequence conservation in *S. rubescens rubescens* and *T. annulata* (d = 0.01), but significant divergence in *L. indica* (d = 0.37) and *C. decisa* (d = 0.34). These results agree with established DNA barcoding evidence showing that COXI reliably distinguishes most grasshopper species, with low intraspecific distances among geographically connected populations (Barandica et al., 2024; Garimella et al., 2024; Letsiou et al., 2024; Rachakonda and Karanth, 2024).

Comparative analyses further confirmed lineage coherence across geographic regions. For instance, Pakistani *A. exaltata* (A+T 69.2%) clustered closely with Indian accessions (MW147492, MW392902, MW504301; A+T 64.6–68.8%), supporting a shared lineage (Sayers, al., 2024). Similarly, *A. turrita* from Pakistan and Canada (JN167791) showed 20.2% genetic distance but still formed a single clade, consistent with historical dispersal (Chapco and Contreras, 2011). Comparable cross-border coherence was observed for *P. infumata*, *P. panteli*, *P. tenebrosa*, and *T. eximia eximia*, confirming phylogenetic consistency and barcoding reliability (Wang et al., 2021; Das et al., 2022; Karso, 2022; Sukhikh et al., 2025).

Maximum likelihood analyses under the Kimura 2-parameter model supported subfamily-level clades congruent with traditional morphological taxonomy, validating the diagnostic value of features such as tympanal placement, pronotal configuration, and tarsal segmentation. The strong concordance between morphological and molecular datasets, despite localized intraspecific divergence (e.g., *L. indica*), supports the use of integrative taxonomy, where morphology provides hypotheses tested by DNA barcoding. In orographic ecotones like Malakand, combining both approaches is essential to differentiate recent radiations and cryptic species complexes.

Two limitations warrant mention. First, although sampling spanned eight districts, finer temporal replication and microhabitat-level sampling (e.g., wet margins, fallows, forest edges) would enhance understanding of species phenology and niche preferences. Second, molecular coverage, though extensive, was not exhaustive, some taxa lacked barcodes or had incomplete accessions. High intraspecific distances in a few species may result from divergent sequence lengths, nuclear mitochondrial pseudogenes (NUMTs), alignment errors, or population structure. These limitations can be addressed by sequencing longer COXI fragments, adding nuclear loci (e.g., 16S, 28S, ITS), and increasing geographic sampling density.

Overall, the convergence of (i) species richness and occurrence patterns linked to climate and land use, (ii) morphometric variation, and (iii) COXI-based clustering, provides a coherent picture of how grasshopper communities assemble and persist within the agricultural-montane mosaic of Malakand (Kalhoro et al., 2019; Du et al., 2022; Sarwar et al., 2023; Blanco-Sierra et al., 2024; Panhwar et al., 2024; Chaudhary et al., 2025).

Conclusion

This study presents the first integrated taxonomic and molecular assessment of grasshopper fauna (Family: Acrididae) in the Malakand region of Pakistan. The documentation of 31 species across nine subfamilies and fifteen tribes highlights the region's remarkable acridid diversity and establishes it as a hotspot for orthopteran fauna. The predominance of Acridinae, Oedipodinae, and Cyrtacanthacridinae indicates assemblages adapted to Malakand's diverse agro-ecological zones, comprising both grassland specialists and species with pest potential.

The research effectively demonstrates the strength of a combined morphological and molecular approach. Morphological identifications were well supported by DNA barcoding of the COI gene, which resolved most species with high confidence and produced a valuable genetic reference dataset for future studies. Morphometric analyses revealed considerable interspecific variation, particularly in hind femur length, suggesting diverse ecological adaptations and locomotor strategies ranging from small, cryptic forms to large, migratory locusts.

Although molecular data largely corroborated conventional morphology-based systematics, phylogenetic analyses revealed notable genetic divergence in species such as *L. indica* and *C. decisa*, suggesting potential cryptic species complexes. However, the absence of molecular data for a few taxa underscores persistent challenges in achieving complete molecular characterization. This study substantially advances the understanding of grasshopper biodiversity in northwestern Pakistan and provides a vital baseline for future monitoring in the face of climate change and land-use alteration. The findings have direct relevance to agricultural pest management and conservation biology by cataloguing key orthopteran components of the regional ecosystem. Future work should include broader spatial and temporal sampling, ecological niche modelling, and comprehensive genomic analyses of the divergent lineages identified here.

Authors' Contributions

AAZ conceptualized and designed the study. MSA prepared the experimental materials, collected the data, and performed the analyses. KS, IA, and SH provided technical guidance and supervised the overall research work. FK drafted and refined the manuscript. All authors critically reviewed, edited, and approved the final version of the manuscript for publication.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 13: Climate Action
SDG 15: Life on Land

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