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Morphological and molecular identification of acridid grasshoppers (Acrididae: Orthoptera) from Poonch division, Azad Jammu Kashmir, Pakistan

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Abstract: The present study was conducted to resolve conflicts in the identification of grasshopper species of the family Acrididae (Orthoptera) on the basis of morphology and DNA barcoding. Grasshoppers representing 26 species of the family Acrididae were collected from different habitats and host plants from Poonch division of Azad Jammu Kashmir, Pakistan. Specimens were identified taxonomically and DNA sequenced for the cytochrome c oxidase (COI) barcode region. Barcodes of 19 morphological species were successfully obtained and the sequence data was used to separate species by Neighbor-Joining cluster analysis. Barcode data successfully discriminated 18 species, while two: *Patanga japonica* (Bolivar, 1898) and *P. succincta* (Johannson, 1763) could not be distinguished since they shared the barcode sequence and clustered together on the Neighbor-Joining (NJ) tree. Morphologically, specimens of *Shirakiacris shirakii* (Bolívar, 1914) were identified as one species, but barcode data revealed that in addition to *Shirakiacris shirakii* (Bolívar, 1914) two other species of the genus *Shirakiacris* are present in the region. Similarly, on the basis of morphological characters two species were indentified in subfamily Catantopinae, *Catantops erubescens* (Walker, 1870) and *Xenocatantops brachycerus* (Willemse, 1932), but barcode data suggest the presence of an additional *Catantops* species in the region. These findings show the usefulness of barcode data in discriminating grasshopper species and indicate that such data can be reliably used for developing reference libraries for species identification via sequence matches.

Keywords: Acrididae, COI, DNA barcoding, Kashmir, morphological identification.

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Author Contribution and Details: Naila Nazir - The principle author, it was her MSc (Hons.) research work. Now she is working as a lecturer. Khalid Mahmood - chairman and supervisor during the study. He is an orthopterist and currently working on some genera of Acrididae. Muhammad Ashfaq - co-supervisor, he was working as foreign professor in NIBGE Faislabad, Pakistan. His research interests are molecular biology and DNA barcoding of arthropods. He contributed in planning, carrying out the study and analyzing the sequence data. Junaid Rahim - assisted during the research in all aspects.

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INTRODUCTION

Grasshoppers are the most prevalent pests in all sorts of vegetation in pastures and grasslands. Family Acrididae encompasses the short-horned grasshoppers and locusts, phytophagous insects that are widely distributed throughout the world and considered ruinous in the arid zone (Watts et al. 1982). Taxonomists generally use morphological identification for studies used to plan control strategies, but this method of identification has several limitations (Scotland et al. 2003). Cryptic species (sibling species) may be incorrectly identified due to phenotypic malleability. Morphologically enigmatic taxa are common in many groups neglected by this approach (Jarman & Elliott 2000). Morphological keys are often limited to particular life stages, limiting the effectiveness of identification. Finally, a high level of proficiency is required to use the keys to avoid misdiagnoses. This has led to the use of molecular data to resolve cryptic species (Xiao et al. 2010). In micro genomic identification, system differences among DNA sequences are used to identify the different organisms (Wilson 1995). In fact these sequences are genetic barcodes enclosed in each cell. The barcode region, a 658-bp nucleotide fragment of mitochondrial COI has been accepted by scientists for identification of animal species (Hebert et al. 2003). The use of short standardized gene regions as internal species tag to recognize the species is an accurate, reliable, and rapid method. Due to copious benefits in identification, DNA barcoding is getting considerable concentration in the field of science (Hebert et al. 2004). The basic scientific advantage of DNA barcoding is fast and digital species identification at any life stage or piece of an organism, and the simplification of species explorations (Janzen et al. 2005). The selected DNA sequence precisely separates the species on the basis of interspecific and intraspecific variations (Matz & Nielsen 2005). Barcoding has helped in resolving cryptic species complexes (Burns et al. 2007; Deng et al. 2012) and performing ecological studies on various animal phyla (Valentini et al. 2009). The generated data is also being used to construct barcode reference libraries for identification of unknowns by matching sequences with the known species (Guralnick & Hill 2009; Janzen et al. 2009). A combination of molecular and morphological data can produce reliable data sets to be used in barcode libraries (Emery et al. 2009). Use of PCR as a tool to amplify and sequence genes and then exploit the nucleotide data for phylogenetic analysis and develop evolutionary relationships among grasshopper species has previously been practiced by a number of researchers (Colgan 1991; Chapco & Litzenberger 2003; Rowell & Flook 2004). Several researchers have used DNA data in phylogenetic analysis to identify grasshopper species (Chapco & Litzenberger 2002; Mukha et al. 2001; Song & Wenzel (2007) Ketmaier et al. 2010). Use of DNA data has also been used in combination with morphological data to establish species relationships (Brust 2008).

Keeping in view the economic importance of grasshoppers and their damage to crops in Azad Kashmir, a need for correct identification of this group of pests has emerged. Azad Jammu & Kashmir lies between 73–75^{0}N and of 33–36^{0}E and comprises an area of 5,134m^2 (13,297km^2) (Fig. 1). Poonch division of Azad Jammu Kashmir comprises an area of 2,792km^2. Its topography is mainly hilly, climatic conditions and floristic composition significantly varies from place to place. Administratively, this division consists of four districts, Bagh, Poonch, Sudhnoti, and Haveli. A survey was conducted to identify grasshopper species of family Acrididae from Poonch division. Major contributions to the Acrididae fauna of Kashmir have been provided by some entomologists like Kirby (1914), Fletcher (1919), Mahmood (1995), Mahmood & Yousaf (1999), Mahmood & Yousaf (2000); Mahmood et al. (2002);

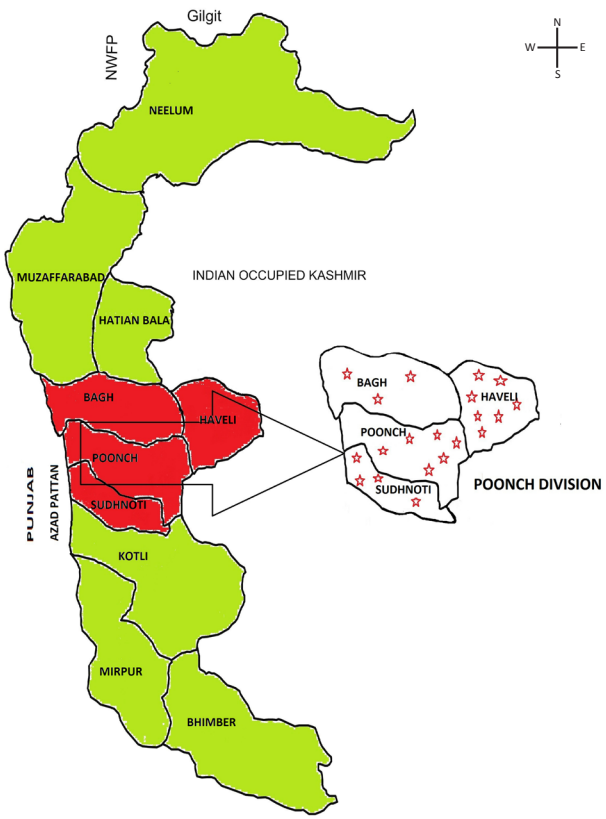


Figure 1. Map of Azad Kashmir illustrated Poonch division with highlighted collection localities

Table 1. Characteristics of collection localities of study area

District	Locality	Altitude(ft)	Latitude	Longitude
Poonch	Rawalakot	5393	33.8^{0}N	73.8^{0}E
	Hajeera	3167	33.6^{0}N	73.3^{0}E
	Jandali	6785	33.3^{0}N	73.3^{0}E
	Banjosa	6212	33.0^{0}N	73.9^{0}E
	Tolipeer	8800	33.0^{0}N	73.9^{0}E
	Abbaspur	4261	33.6^{0}N	73.0^{0}E
Sudhnoti	Mang	4842	33.2^{0}N	73.9^{0}E
	Plundari	4000	33.2^{0}N	73.9^{0}E
	Baloch	5304	33.8^{0}N	73.0^{0}E
	Trarkhel	5600	33.9^{0}N	73.7^{0}E
Haveli	Khautta	5189	33.6^{0}N	74.5^{0}E
	Degwar	5000	33.1^{0}N	74.4^{0}E
	Hajipeer	8221	33.5^{0}N	74.9^{0}E
	Kalamola	8207	33.3^{0}N	74.0^{0}E
	Aliabad	8606	33.8^{0}N	73.1^{0}E
	Bedori	12229	33.0^{0}N	74.9^{0}E
	Lasdana	8069	33.5^{0}N	73.4^{0}E
Bagh	Bagh City	6000	33.8^{0}N	73.5^{0}E
	Dherkot	5657	34.6^{0}N	73.3^{0}E
	Dhulli	6082	33.6^{0}N	73.3^{0}E

Mahmood & Rizwan (2002); Mahmood & Shah (2003) Mahmood et al. (2004); Reshi & Azim (2008); Azim & Reshi (2010) but nobody has made any effort to identify them on a molecular level either by DNA barcoding or by using any other marker. To remove identification conflicts among 26 morphological species of the family Acrididae from Poonch, and to add species sequences to the international barcode reference library, studies were performed to identify the grasshoppers morphologically and by DNA barcoding. Nevertheless, our knowledge of the grasshopper fauna of Azad Jammu Kashmir is still insufficient, particularly of species living in natural habitats and being commonly distributed over small areas.

MATERIAL AND METHODS

The collection of grasshoppers was carried out from the maximum floristic composition and cultivated crops like rice, maize, soybean, etc. A detailed survey of grasshoppers from the 19 localities of the study area (Table 1) from the year 2010–2011 and the collections were carried out with the help of a sweep net (24 inches diameter). The collected specimens were killed by cyanide and stretched out on the stretching board with the help of standard entomological pins (No. 16–40). The specimens were dried, examined with the use of a Leica MZ6 microscope and identified using keys (Bie-Bienko & Mischenko (1951), Drish (1961), Ritchie (1982), and Mason (1973), Suhail (1994), Mahmood (1995). The terminology of Kirby (1914) and Bie-Benko & Mischenko (1951) were used in this identification process. The specimens of each identified species were confirmed from (Eades et al. 2011).

Sequencing/ DNA barcoding

Morphologically identified grasshopper specimens were transferred to the Insect Molecular Biology Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad for DNA barcoding for their identification at the molecular level. The specimens were processed following standard DNA barcoding protocols as outlined previously (Hebert et al. 2003). In brief, labeled specimens were arrayed in a 96-well PCR plate fashion to correspond with the location of tissue samples in the plates. Specimen data on field identification, taxonomic identification, identifier, voucher type, collectors, collection date, province, region, locality, latitude, longitude and elevation was entered on a spreadsheet. Specimen data and images were uploaded to the Barcode of Life Data System (BOLD) (www.boldsystems.org) hosted by the Biodiversity Institute of Ontario, University of Guelph, Canada. Tissue sampling was performed by removing a small part of the insect's leg and transferring it into the labeled 96 well PCR plate in the corresponding well. Six copies of each species were used for molecular studies.

DNA isolation

A small part of the leg from individual grasshoppers was transferred to the PCR plate and genomic DNA was extracted following protocols described by Ivanova et al. (2006) at the Canadian Centre for DNA Barcoding within the Biodiversity Institute of Ontario.

PCR amplification and sequencing

Amplification of the COI-5' (barcode) was performed with primer pair LCO1490_t1/ HCO2198_t1 (TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG / CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA) following the PCR conditions; 94^{0}C (1 min), five cycles of 94^{0}C (40 s), 45^{0}C (40 s), 72°C (1 min); 35 cycles of 94^{0}C (40 s), 51^{0}C (40 s), 72^{0}C (1 min) and final extension of 72^{0}C (5 min). PCRs were carried out in 12.5µL reactions containing standard PCR ingredients and 2µL of DNA

template. PCR products were analyzed on 2% agarose E-gel® 96 system (Invitrogen Inc.). Amplicons were sequenced bidirectionally using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems 3730XL DNA Analyzer. Sequences were assembled, aligned and edited using CodonCode Aligner (CodonCode Corporation, USA). Obtained barcode sequences were edited and analyzed and uploaded to the BOLD for further analysis and storage. Specimens used for tissue sampling were saved as voucher specimens for future reference.

Sequence data analysis

Sequence similarity analysis to determine the matching species in the DNA/barcode databases were performed by using "Blast" and "Identification Request" tools of the NCBI and BOLD. Currently barcodes of 3008 specimens representing 421 Acridid species are readily available on BOLD for sequence comparisons. ClustalW nucleotide sequence alignments (Thompson et al. 1994) were performed using MEGA V5 (Tamura et al. 2011) under default parameters. Patterns of sequence divergence among taxa were visualized using the neighbor-joining method (Thompson et al. 1994). Evolutionary distances were computed using the maximum composite likelihood method based upon the units of the number of base substitutions per site after all positions containing gaps and missing data were eliminated from the dataset (Complete deletion model). To perform pairwise distance analysis and to generate distance histograms and distance ranks we used an online version of Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012).

RESULTS

Morphological identification and distribution of acridid species in Poonch

Details of the specimen collection habitats and their host plants are outlined in Table 2. The studies resulted in the morphological identification of 26 species under 15 genera of nine subfamilies of the family Acrididae (Table 2). Among subfamily Oedipodinae species of the genus *Gastrimargus* were found to be abundant at a higher altitude while *Sphingonotus longipennis* (Saussure, 1884), *Aiolopus thalassinus tumulus* (Fabricius, 1798), *Trilophidia japonica* (Sassure, 1888), *Trilophidia turpis* (Walker, 1870) were not abundant; only a few specimens of these species were collected during the survey. The species of genus *Acrida* of subfamily Acridinae were found to be abundant in areas of higher elevation while their population declined in lower elevations. *Spathosternum parsiniferum parsiniferum* (Walker, 1871) of subfamily Spathosterninae was found to be abundant at higher elevations while the species of genus *Hieroglyphus*, *Hieroglyphus nigroreplatus* (Bolivar, 1912), *Hieroglyphus banian* (Fabricius, 1798), *Hieroglyphus concolar* (Walker, 1870) and *Hieroglyphus oryzivorus* (Carl, 1916) were found on rice crops abundantly. Their population was restricted to lower elevations. While the species of subfamily Oxyinae particularly genus *Oxya* was recorded to be most abundant throughout the surveyed area, among them *Oxya fuscovittata* (Marschall, 1836) and *O. hyla hyla* (Serville, 1831) were most abundant over all sorts of vegetation. Subfamily Calliptaminae with the single species *Acorypha glucopsis* (Walker, 1870) was recorded as abundant at higher elevations. Eyprepocnemidinae also with the species *Shirakiacris shirakii* (Bolívar, 1914) and according to barcode results two more species (morphologically identified as *Shirakiacris shirakii* (Bolívar, 1914) but barcode results showed them to be different species under the same genus were found to be abundant at higher altitudes. The species of subfamily Catantopinae *Pachyacris vinosa* (Walker, 1870) was found to be very rich in higher altitudes and moderately in lower areas, while the population of *Paraconophyma kashmiricum* (Mischenko, 1950) was restricted only to the higher elevations of the surveyed area. The population of *Catantops erubescens* (Walker, 1870) and *Xenocatantops brachycerus* were not very plentiful but recorded from some higher areas from grasses, while *Catantops innatobalis* (Walker, 1871) was very rare with only a single specimen collected. Species of subfamily Cyrtacanthacridinae *Patanga succincta* (Johannson, 1763) and *Patanga japonica* (Bolivar, 1898) were most abundant in the surveyed area.

Barcode analysis

DNA barcodes of 85 specimens of 21 species were successfully sequenced and the size of the barcode was uniform among all the species producing successful barcodes. The sequences have either been allocated GenBank accession numbers or have been submitted to the European Molecular Biology Laboratory (EMBL)/ (DDBJ)/Gene Bank databases for assignment of accessions. We performed identity analysis of the species based on barcode sequence matches with those of other species already deposited in the Barcode of Life Data System (BOLD) and National Center for Biotechnology Information (NCBI) databases. From the database searches we found that only one species,

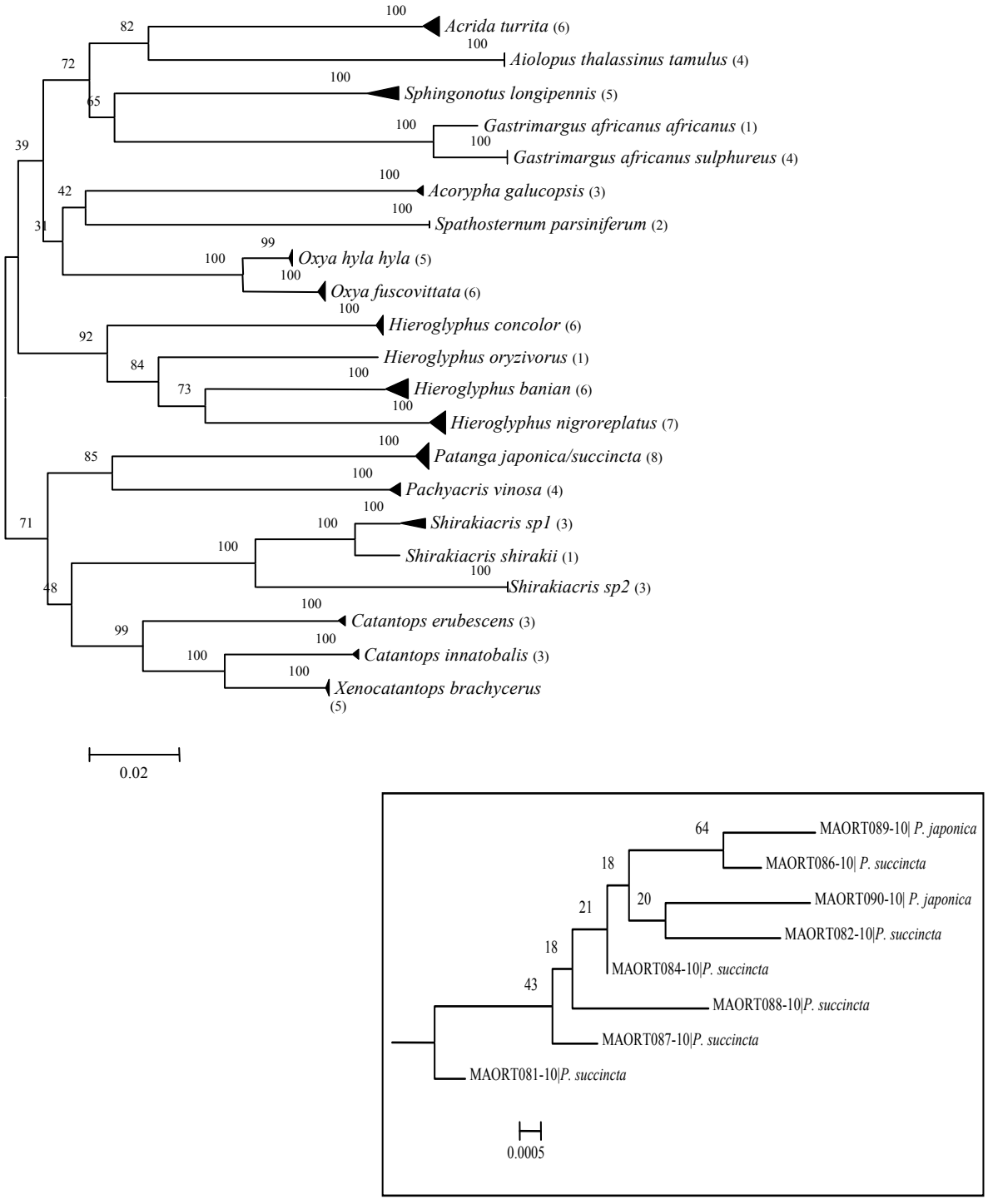
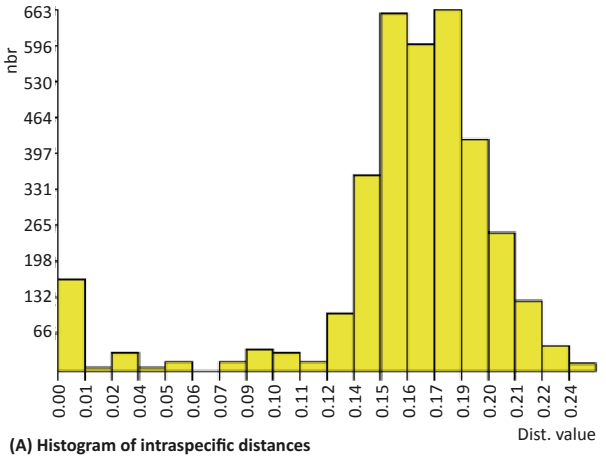


Figure 2. DNA barcode-based neighbor joining cluster analysis of species belonging to family Acrididae collected from Poonch. The tree is based on 85 sequences derived from 22 species. Bootstrap values (500 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those used to infer the phylogenetic tree. Tree nodes are collapsed for each species. Numbers in brackets next to each species names represent the number of individuals analyzed. Sequence distances were computed using the K2P method and are shown as in base substitutions per site. All positions containing gaps and missing data were eliminated using pairwise deletion option. Analyses were conducted in MEGA5. Sub-tree in a box indicates branching pattern of two species, *Patanga Japonica* and *P. succincta* which show no pattern of genetic difference in the COI barcode region.

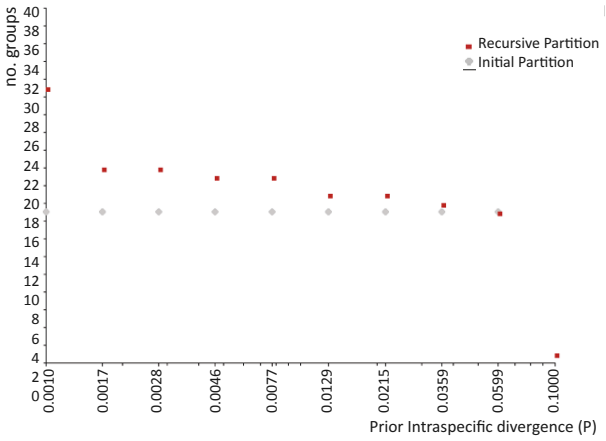
Aiolopus thalassinus tumulus (Fabricius, 1798) shared the barcode with conspecifics from Kenya and South Africa. Barcodes of none of the other species from our studies matched with those from any other country in

Table. 2 Morphologically identified species and their hosts

Subfamily	Species	Habitat description and host plants
Oedipodinae	*Gastrimargus africanus africanus* (Saussure, 1888)	Kalamola in grassy meadows, 8 males, 1 female, 13.ix.2010; 3 females, 04.ix.2010; Khautta on grasses 1 male, 03.x.2010; Trarkhel on maize 3 males, 24.vii.2010; Jandali on maize, 1 female, 08.x.2010.
	Gastrimargus africanus sulphureus (Bie- Bienko, 1951)	Kalamola on grasses 3 males, 3 females, 13.x.2010; 1 female, 18.ix.2010; Banjosa on grasses 2 males, 4 females, 06.viii.2010; Khautta on grasses 1 male, 1 female, 23.ix.2010; Trarkhel on shrubs 3 males, 1 female, 12.xi.2010.
	Aiolopus thalassinus tamulus (Fabricius, 1798)	Hajipeer, 5 males, 4 females, 23.ix.2011
	Trilophidia japonica (Saussure, 1888)	Hajipeer on wild herbs, 6 males, 23.ix.2011; Hajeera on cultivated plant nursery, 3 females, 4 males, 02.ix.2010
	Trilophidia turpis (Walker, 1870)	Hajipeer on wild herbs, 5 males, 4 females, 23.ix.2011
	Sphingonotus longipennis (Saussure, 1884)	Hajipeer on grassy meadows, 6 females, 5 males, 23.ix.2011
Acridinae	*Acrida turrita* (Linne, 1758)	Rawalakot on grasses, soybean and maize fields, 3 females, 20.x.2010; 1 male, 24.x.2010; Hajeera on rice field and grasses, 4 females, 1 male, 24.x.2010; 2 females, 1 male, 20.x.2010.
	Acrida gigantea (Herbst, 1786)	Mang on grasses, 4 females, 24.x.2010; Plundari on maize fields and grasses, 3 females, 4 males, 24.x.2010; Rawalakot on soybean field 1 female, 6 males, 25.x.2010.
Spathosterninae	*Spathosternum parsiniferum parsiniferum* (Walker, 1871)	Degwar on grasses, 1 female, 06.vii.2010; 1 female, 3 males, 20.ix.2010; 2 females, 20.ix.2011; 1 male, 04.ix.2011; 1 male,, 19.ix.2011; 1 male, 20.ix.2011; 1 male, 3 females, 23.ix.2011. Khautta on maize fields and grasses, 1 female, 05.viii.2011; 1 female, 04.viii.2011
Hemiacridinae	*Hieroglyphus nigrorepletus* (Bolivar, 1912)	Hajeera on rice fields, 4 females, 6 males, 30.ix.2010
	Hieroglyphus banian (Fabricius, 1798)	Hajeera on rice fields, 5 males, 7 females, 20.x.2010
	Hieroglyphus concolor (Walker, 1870)	Hajeera on rice fields, 5 females, 7 males, 09.ix.2010
	Hieroglyphus oryzivorus (Carl, 1916)	Hajeera on rice fields, 5 males, 6 females, 20.x.2010
Oxyinae	*Oxya fuscovittata* (Marschall, 1836)	Rawalakot on grasses and soybean fields, 20 females, 5 males, 19.viii.2010; Banjosa on wild shrubs, 9 females, 2 males, 14.viii.2010; Tolipeer on wild grasses, 6 females, 04.ix.2010; Jandali on maize field and grasses, 17 females, 14 males, 16.viii.2010
	Oxya hyla hyla (Serville, 1831)	Hajeera on grasses and rice fields, 32 females, 2 males, 20.x.2010; Baloch on grasses 12 females, 07.viii.2011.
	Oxya hyla intricata (Stål, 1861)	Hajeera on rice fields and grasses, 3 females, 7 males, 20.x.2010.
	Oxyina bidentata (Willemse, 1925)	Rawalakot on grasses and maize field, 3 females, 4 males, 19.viii.2010.
Calliptaminae	*Acorypha glaucopsis* (Walker, 1870)	Degwar on wild grasses and herbs, 4 females, 20.ix.2009; 1 female, 04.ix.2010; 2 females, 06.ix.2010; 1 female, 19.ix.2010; 3 males, 04.viii.2010 (N. Nazir)
Eyprepocnemidinae	*Shirakiacris shirakii* (Bolívar, 1914)	Rawalakot on grasses and maize fields, 2 females, 3 males, 19.viii.2010; Khautta on grasses and plant nursery, 2 females, 23.viii.2010; 3 males, 03.iii.2010; Degwar on wild plants and grasses, 3 females, 22.viii.2010; 2 males, 03.viii.2010 (N. Nazir)
Catantopinae	*Pachyacris vinosa* (Walker, 1870)	Degwar on wild grasses and herbs, 2 females, 01.x.2010; 1 female,, 06.vii.2010; 1 female, 09.x.2010; 2 males, 10.x.2010; Khautta on maize fields and grasses, 3 females, 10.x.2010; Abbaspur on maize fields, plants nursery and grasses, 2 females, 08.ix.2010 (N. Nazir)
	Paraconophyma kashmirica (Mishchenko, 1950)	Degwar on grasses and wild herbs, 3 females, 19.vii.2010; 2 males, 21.vii.2011; Kalamola on wild grasses and herbs, 3 males, 15.ix.2011; 4 females, 13.ix.2010; Bedori on grazing meadows, wild grasses, 2 females, 19.vii.2010; Aliabad on grasses, 2 females, 12.viii.2010 (N. Nazir)
	Catantops erubescens (Walker, 1870)	Khautta on grasses and maize fields, 2 females, 4 males, 07.vii.2010; 4 females, 20.vii.2010 (N. Nazir)
	Xenocatantops brachycerus (Willemse, 1932)	Hajipeer on wild flower plants, 3 females, 2 males, 23.ix.2011; Halan Shumali on wild plants, 1 female, 5 males, 23.ix.2011; Bagh on grasses, 2 females, 1 male, 23.ix.2011 (N. Nazir)
	Catantops innatobalis (Walker, 1871)	Khautta on wild flowers and grasses, 4 females, 6 males, 07.vii.2010 (N. Nazir)
Cyrtacanthacridinae	*Patanga succincta* (Johannson, 1763)	Rawalakot on grasses and soybean field, 3 females, 2 males, 03.ix.2010; Bagh on grasses 4 females, 23.ix.2011; Trarkhel on maize fields, 2 females, 1 male, 16.x.2010; Jandali on dry residues of maize plants, 6 females, 4 males, 18.x.2010 (N. Nazir)
	Patanga japonica (Bolivar, 1898)	Rawalakot 4 females, 1 male, 03.ix.2010; Trarkhel 5 females, 3 males, 16.x.2010. (N. Nazir).



(A) Histogram of intraspecific distances



(B) Partition analysis.

Figure 3. Pairwise distance divergence analysis of Acridid grasshopper species as performed by ABGD.

BOLD or NCBI databases.

Cluster analysis of the barcode data showed that 18 of the 21 species included in the dataset formed distinct and non-overlapping monophyletic clusters (Fig. 2). Tree nodes for each morphological species with multiple specimens were collapsed which appear as vertical lines or triangles in the tree indicating the level of intraspecific divergence. Two species, *Patanga japonica* (Bolivar, 1898) and *Patanga succincta* (Johannson, 1763) shared the same cluster on the dendrogram. The subtree (Fig. 2A) indicates the minor genetic distances among the specimens of these two species but with no clear pattern of species grouping. Specimens of the species, *Eyprepocnemis shiriaki* produced three separate clusters with significant bootstrap support (100%) indicating that the species is a complex of at least three species (Fig. 2). The species *Gastrimargus africanus* is represented by two subspecies, *G. africanus africanus* (Saussure, 1888) and *Gastrimargus africanus sulphureus* (Bie- Bienko 1951). Both the subspecies made monophyletic clusters with strong bootstrap support (Fig. 2). *Pachyacris vinosa* lies on the same branch as on the *Patanga succincta* (Johannson, 1763) and *Patanga japonica* (Bolivar, 1898) while according to Orthoptera Specie File (OTS) *Patanga succincta* (Johannson, 1763) and *Patanga japonica* (Bolivar, 1898) are under the subfamily Cyrtacanthacridinae (Kirby, 1902) and *Pachyacris vinosa* (Walker, 1870) under the subfamily Catantopinae Bie-Bienko & Mischenko (1951). According to barcoding results both of them share the same genus and subfamily which supports Bie-Bienko & Mischenko (1951) who kept *Pachyacris vinosa* (Walker, 1870), *Patanga succincta* (Johannson, 1763) and *Patanga japonica* (Bolivar, 1898) under the same subfamily Catantopinae.

The distance data and the groups produced by recursive and initial partitions generated by ABGD are presented in Fig. 3A and 3B. In the dataset 18 species are represented by two or more than two specimens. The distributions of distances show a gap between the intraspecific and the interspecific distances (Fig. 3A). The partitions analysis shows the presence of 19 groups by recursive partition at a divergence level of 2.15% in the dataset (Fig. 3B).

DISCUSSIONS

The variability in the genus *Gastrimargus* was found in two subspecies and when they were barcoded their sequence data show a considerable variation among the two morpho subspecies. Some of the species were collected from a very low altitude to very higher altitudes showing a wide range of distribution. In the present study 26 species of family Acrididae were identified and subjected to DNA barcoding made comparisons with the nucleotide data among species and phylogenetic analysis performed. Out of 26 species, barcoding results of 21 species were obtained. The remaining five species either did not yield amplification or the sequences were not of good quality/were contaminated. Among these sequenced species morphologically identified two same subspecies of genus *Gastrimargus* shown in the phylogenetic tree represents a lot of variation which requires further taxonomic expertise to resolve this confusion. Similarly, two species of genus *Patanga* also require taxonomic expertise and it is in the process of removal by the taxonomist first author and co-authors. Nucleotide data of the gene sequenced in these studies did not match perfectly with any of the other grasshopper species in the Gene Bank. Similarly, there were significant nucleotide variations among all the

sequenced genes of the 18 species. The DNA barcode region of COI (COI-5') showed significant nucleotide differences among grasshopper species and came out as a promising region to be used for grasshopper species identification. The phylogenetic analysis based on the barcode region of COI also provided better relationships among various grasshopper species. DNA barcoding is a new phenomenon and is not only being used to identify species but is also being used to study species relationships and to investigate genetic diversities among insect populations (Mondal et al. 1999; Hajibabaei et al. 2006; Emery et al. 2009; Ashfaq et al. 2011). In conclusion, the use of nucleotide data from the barcode region of COI supported the grasshoppers, identifications and phylogenetic relationships performed on the basis of morphological characters. The nucleotide data, however, could not be used to make comparisons with other such sequences in the gene bank databases as sequences from the same region of COI were not available in the gene bank. This shows the limitation of the use of DNA data for species identification. Sequences produced from the grasshopper species in the current studies and their submission in the gene bank database will be a good addition to the sequence database as well as to the barcode reference library.

REFERENCES

Ashfaq, M., J. Ara, A.R. Noor, P.D.N. Hebert & S. Mansoor (2011). Molecular phylogenetic analysis of a scale insect (*Drosicha mangiferae*; Hemiptera: Monophlebidae) infesting mango orchards in Pakistan. *European Journal of Entomology* 108(4): 553–559.

Azim, M.N. & S.A. Reshi (2010). Taxonomic notes on the tribe Acridini Latreille (Acridinae: Acrididae: Orthoptera) of Kashmir, India. *Acta Zoológica Mexicana (nueva serie)* 26(1): 219–222.

Bie-Bienko, G.Y. & L.L. Mischenko (1951). *Locust and Grasshoppers of USSR and Adjacent Countries*. Pt I & II, Monson, Jerusalem, 691pp.

Brust, L.M. (2008). Taxonomy and distribution of Acridid grasshoppers in Nebraska and effect of temperature and immersion on grassland pests. PhD Thesis, University of Nebraska.

Burns, J.M., D.H. Janzen, M. Hajibabaei, W. Hallwachs, P.D.N. Hebert (2007). DNA barcodes of closely related (but morphologically and ecologically distinct) species of skipper butterflies (Hesperiidae) can differ by only one to three nucleotides. *Journal of the Lepidopterists Society* 61: 138–153.

Chapco, W. & G. Litzenberger (2002). A molecular phylogenetic study of two relict species of melanopline grasshoppers. *Genome* 45(2): 313–318; http://dx.doi.org/10.1139/g01-156

Chapco, W. & G. Litzenberger (2003). A molecular phylogenetic analysis of the grasshopper genus *Melanoplus* Stål (Orthoptera: Acrididae) - an update. *Journal of Orthoptera Research* 11(1): 1–9; http://dx.doi.org/10.1665/1082-6467(2002)011[0001:AMPAOT]2.0.CO;2

Colgan, D.J. (1991). Phylogenetic studies of acridoid grasshoppers comparing 2n2- and 4-base recognizing endonucleases. *Journal of Evolutionary Biology* 4(4): 575–591; http://dx.doi.org/10.1046/j.1420-9101.1991.4040575.x

Deng, J., F. Yu, T.X. Zhang, H.Y. Hu, C.D. Zhu, S.A. Wu & Y.Z. Zhang (2012). DNA barcoding of six *Ceroplastes* species (Hemiptera: Coccoidea: Coccidae) from China. *Molecular Ecology Resources* 12(5): 791–796; http://dx.doi.org/10.1111/j.1755-0998.2012.03152.x

Drish, V.M. (1961). A preliminary revision of the families and subfamilies of Acridoidae (Orthoptera: Insecta). *Bulletin of British Museum (Natural History) Entomology* 10: 351–419.

Eades, D.C., D. Otte, M.M. Cigliano & H. Braun (2011). *Orthoptera Species File*. Version 5.0/5.0. [may 2013]. <http://Orthoptera.SpeciesFile.org>.

Emery, V.J., L.J. Eckert & G. Christopher (2009). Combining DNA barcoding and morphological analysis to identify specialist floral parasites (Lepidoptera: Coleophoridae: Momphinae: *Mompha*). *Molecular ecology Resource* 9(s1): 217–223; http://dx.doi.org/10.1111/j.1755-0998.2009.02647.x

Fletcher, J.B. (1919). Annotated list of Indian crop pest. Report of Proceedings of 3rd Entomological Meeting, Pusa, 1: 306pp.

Guralnick, R. & A. Hill (2009). Biodiversity informatics: automated approaches for documenting global biodiversity patterns and processes. *Bioinformatics* 25(4): 421–428; http://dx.doi.org/10.1093/bioinformatics/btn659

Hajibabaei, M., D.H. Janzen, J.M. Burns, W. Hallwachs & P.D.N. Hebert (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of National Academy of Science* 103(4): 968–971; http://dx.doi.org/10.1073/pnas.0510466103

Hebert, P.D.N., A. Cywinska, S.L. Ball & J.R. deWaard (2003). Biological identifications through DNA barcodes. *Proceedings of Royal Society, London, B.* 270(1512): 313–321; http://dx.doi.org/10.1098/rspb.2002.2218

Hebert, P.D.N., M.Y. Stoeckle, T.S. Zemlak & C.N. Francis (2004). Identification of birds through DNA barcodes. *PLoS Biology* 2: 1657–1663.

Ivanova, N.V., J.R. deWaard, P.D.N. Hebert (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes* 6(4): 998–1002; http://dx.doi.org/10.1111/j.1471-8286.2006.01428.x

Janzen, D.H., M. Hajibabaei, J.M. Burns, W. Hallwachs, E. Remigio & P.D.N. Hebert (2005). Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical Transactions of Royal Society B: Biological Sciences* 360(1462): 1835–1845; http://dx.doi.org/10.1098/rstb.2005.1715

Janzen, D.H., W. Hallwachs, P. Blandin, J.M. Burns, J.M. Cadiou, I. Chacon, T. Dapkey, A.R. Deans, M.E. Epstein, B. Espinoza, J.G. Franclemont, W.A. Haber, M. Hajibabaei, J.P.W. Hall, P.D.N. Hebert, I.D. Gauld, D.J. Harvey, A. Hausmann, I.J. Kitching, D. Lafontaine, J.F. Landry, C. Lemaire, J.Y. Miller, J.S. Miller, L. Miller, S.E. Miller, J. Montero, E. Munroe, S.R. Green, S. Ratnasingham, J.E. Rawlins, R.K. Robbins, J.J. Rodriguez, R. Rougerie, M.J. Sharkey, M.A. Smith, M.A. Solis, J.B. Sullivan, P. Thiaucourt, D.B. Wahl, S.J. Weller, J.B. Whitfield, K.R. Willmott, D.M. Wood, N.E. Woodley & A.J. Wilson (2009). Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. *Molecular Ecology Resources* 9: 1–26; http://dx.doi.org/10.1111/j.1755-0998.2009.02628.x

Jarman, S.N. & N.G. Elliott (2000). DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae, 'living fossils' from the Triassic. *Journal of Evolution Biology* 13(4): 624–633; http://dx.doi.org/10.1046/j.1420-9101.2000.00207.x

Ketmaier, V., H. Stuckas, J. Hempel, I. Landeck, M. Tobler, M. Plath & R. Tiedemann (2010). Genetic and morphological divergence among Gravel Bank Grasshoppers, *Chorthippus pullus* (Acrididae), from contrasting environments. *Organism Diversity and Evolution* 10(5): 381–395; http://dx.doi.org/10.1007/s13127-010-0031-1

Kirby, W.F. (1914). *Orthoptera (Acrididae). Fauna of British India including Ceylon and Burma.* Taylor and Francis, London, 276pp.

Mahmood, K. (1995). Taxonomic studies of Acridoidea (Orthoptera) of Azad Jammu and Kashmir. PhD Thesis. Department of Entomology, University of Agriculture Faisalabad, Pakistan.

Mahmood, K. & M. Yousaf (1999). New records of Oedipodinae (Acrididae: Orthoptera) from Azad Jammu and Kashmir with the description of a new species. *Journal of Orthoptera Research* 3:

271–275.

Mahmood, K. & M. Yousaf (2000). Taxonomic studies on Pyrgomorphidae and Catantopinae (Acridiodea; Orthoptera) from Azad Jammu and Kashmir. *Pakistan Journal of Biological Sciences* 3(11): 1914–1916.

Mahmood, K. & U. Rizwan (2002). Grasshoppers species composition and distribution pattern in District Poonch, Azad Jammu and Kashmir, Pakistan (conference paper).

Mahmood, K., Y. Mohammad & K. Abdul (2002). Taxonomic study of some Catantopinae (Acrididae: Orthoptera) from Azad Jammu and Kashmir. *Pakistan Journal of Zoology* 34(3): 233–237.

Mahmood, K. & W. Shah (2003). New records of Eyprepocnemidinae (Acrididae: Orthoptera) from Azad Jammu and Kashmir. *Pakistan Journal of Arid Agriculture* 6(1): 25–27.

Mahmood, K., K. Abbas & W.H. Shah (2004). A Preliminary Study of grasshoppers (Acrididae: Orthoptera) of Baltistan, Azad Jammu & Kashmir, Pakistan. *Pakistan Journal of Zoology* 36(1): 21–25.

Mason, J.B. (1973). A revision of the genera *Hieroglyphus* Krauss, *Parahieroglyphus* Carl and *Hieroglyphodes* Uvarov (Orthoptera: Acridiodea). *Bulletin of British Museum (Natural History) Entomology* 28(7): 509–560.

Matz, M.V. & R. Nielsen (2005). A likelihood ratio test for species membership based on DNA sequence data. *Philosophical Transactions of Royal Society B: Biological Sciences* 360(1462): 1969–1974; http://dx.doi.org/10.1098/rstb.2005.1728

Mondal, S.K., A.K. Hazra & S.K. Tandon (1999). Studies on taxonomy, biology and ecology of grasshoppers infesting field crops and vegetables with notes on nymphal taxonomy of some species in West Bengal. *Records of the Zoological Survey of India. Occasional Paper* 173: 1–178.

Mukha, D., W.M. Brian & C. Schal (2001). Evolution and phylogenetic information content of the ribosomal DNA repeat unit in the Blattodea (Insecta). *Journal of Insect Biochemistry and Molecular Biology* 32(9): 951–960; http://dx.doi.org/10.1016/S0965-1748(01)00164-3

Puillandre, N., A. Lambert, S. Brouillet & G. Achaz (2012). ABGD, automated barcode gap discovery for primary species delimitation. *Molecular Ecology* 21(8): 1864–1877; http://dx.doi.org/10.1111/j.1365-294X.2011.05239.x

Reshi, S.A. & N. Azim (2008). Studies on some aspects of biodiversity of Cyrtacanthacridini (Orthoptera: Acrididae) of Kashmir, Himalayas. *Annals of Plant Protection Sciences* 16(2): 393-395

Ritchie, J. (1982). A taxonomic Revision of the genus *Gastrimargus* Saussure (Orthoptera: Acrididae). *Bulletin of British Museum (Natural History) Entomology* 44: 293–329.

Rowell, C.H.F. & P.K. Flook (2004). A dated molecular phylogeny of the Proctolabinae (Orthoptera, Acrididae), especially the *Lithoscirtae*, and the evolution of their adaptive traits and present biogeography. *Journal of Orthoptera Research* 13(1): 35–56; http://dx.doi.org/10.1665/1082-6467(2004)013[0035:ADMPOT]2.0.CO;2

Scotland, R.W., R.G. Olmstead & J.R. Bennett (2003). Phylogeny reconstruction: The role of morphology. *Systematic Biology* 52(4): 539–548.

Song, H. & J.W. Wenzel (2007). Phylogeny of bird-grasshopper subfamily Cyrtacanthacridinae (Orthoptera: Acrididae) and the evolution of locust phase polyphenism. *Cladistics* 24(4): 515–542; http://dx.doi.org/10.1111/j.1096-0031.2007.00190.x

Suhail, A. (1994). Taxonomic studies on Acridoidea (Orthoptera) of Pakistan. PhD Thesis. Department of Agriculture Entomology, University of Agriculture Faisalabad, Pakistan, 343pp.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei & S. Kumar (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology Evolution* 28(10): 2731–2739; http://dx.doi.org/10.1093/molbev/msr121

Thompson, J.D., D.G. Higgins & T.J. Gibson (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22(22): 4673–4680; http://dx.doi.org/10.1093/nar/22.22.4673

Valentini, A., F. Pompanon & P. Taberlet (2009). DNA barcoding for ecologists. *Trends in Ecology and Evolution* 24(2): 110–117; http://dx.doi.org/10.1016/j.tree.2008.09.011

Watts, J.G., E.W. Huddleston & J.C. Owens (1982). Rangeland entomology. *Annual Review of Entomology* 27: 283–311; http://dx.doi.org/10.1146/annurev.en.27.010182.001435

Wilson, K.H. (1995). Molecular biology as a tool for taxonomy. *Clinical Infectious Diseases 20: 192–208.*

Xiao, J.H., N.H. Wang, Y.W. Li, R.W. Murphy, D.G. Wan, L.M. Niu, H.Y. Hu, Y.G. Fu & D.W. Huang (2010). Molecular approaches to identify cryptic species and polymorphic species within a complex of community of Fig Wasps. *PLoS ONE* 5: e15067; http://dx.doi.org/10.1371/journal.pone.0015067