Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i2.4263

MOLECULAR CHARACTERIZATION OF GRASSHOPPER SPECIES FROM TEHSIL OGHI

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Abstract:
Grasshopper belongs to the order Orthoptera, under the class Insecta. Grasshoppers are pests of vegetation and crops. The research was conducted on the molecular study and phylogenetic relationship of collected species of Grasshoppers, within families Tettigoniidae, Acrididae, and Dericorythidae, collected from tehsil, oghi. A total of 70 samples was collected and morphological identification was done using an Orthopteran Taxonomic key. The taxonomic analysis revealed 9 species, under 8 genera, and spreading into 3 families. The 9 identified species revealed i-e *Melanoplus bivittatus*, *Anacridium aegyptium, hieroglyphs Banian*, *Kosciuscola tristis tristis*, *stenobothrus stigmatics*, under the family Acrididae. Two species i-e *Omocestus viridulus* and *Conocephalus fuscus* belong to the family Tettigoniidae. While the two species i-e *Dericorys albidula*, *Dericorys tibialis* resulted under the family *Dericorythidae*. DNA was isolated from Grasshoppers' legs using the Gervay protocol. The amplification of 16 Sr RNA was performed through polymerase chain reaction (PCR). Amplified 16 Sr RNA gene was set for sequencing. After successful sequencing, samples were further identified by BLAST in the Gene Bank, NCBI. The molecular study was performed with various parameters i-e Genetic distances, Molecular Clock, and Phylogenetic relationship. The genetic distance was estimated to be 0.168-0.187. The value of the molecular clock revealed 0.168. The phylogenetic tree was based on the Neighbor-Joining tree, the tree length was revealed 0.00 to 0.021. The evolutionary relationship among collected species and these species retrieved from GeneBank were clustered together and their similarities were recorded. The estimated phylogenetic tree of the species was also clustered based on the Neighbor-Joining tree. The samples collected and processed contain no novel species; all of the species are pre identified but first time recorded from tehsil oghi.

Introduction

Grasshopper belongs to the order Orthoptera, under the class Incesta, A total of 20,000 species has been recorded throughout the world (Jana *et al*., 2015). There are 1.4 million species of arthropods on earth, over 53% belong to the class Insecta (Hassan *et al*., 1994). Grasshoppers belong to Order Orthoptera, of class Insecta, which is the 2nd largest group of familiar organisms worldwide (Sonnenfeld *et al*., 2009). Orthoptera is the oldest lineage of living insects, with 28,418 species, and consist half of the arthropods are orthoptera (Pocco and Cigliano, 2020). The family Acrididae is a diverse lineage of orthoptera with 6,700 recognized species (Song *et al*., 2018). The locusts appear at night and lay eggs on crops while the grasshoppers attack crops. Most species that do not damage crops are bio indicators (Hochkirch, 2001). Grasshoppers live in terrestrial habitats (Cigliano *et al*., 2000). They have significant impacts on ecology, mostly in the recycling of nutrients in grasslands and invertebrates are a great source of nutrition (Joern, 1979) and vertebrates (Gandar, 1982). They are sensitive to the environment because found everywhere in the world, which is why they act as bio-indicator (Bazelet and Samways, 2014). Grasshoppers are excellent monitors of landscape because they are sensitive to the environment (Bazelet and Samways, 2014).

The specie identification is done by DNA barcoding, for specimens, they use standardized short regions in genes (McClenaghan *et al*., 2015). The subunit I of cytochrome oxidase (COI) is used in a system of identification, in the birds (Hebert *et al*., 2003), in the pieces (Zemlak *et al*., 2009), in the Lepidoptera (Hebert *et al*., 2003); in the fruit flies (Hajibabaei *et al*., 2007), and in the arachnids (Barrett and Hebert, 2005). Recently DNA barcoding system is also used in plants (Kress *et al*., 2005), macro algae (Saunders, 2005), in fungi (Summerbell *et al*., 2005), in sponges (Scicluna *et al*., 2006). The variation among species is due to a gap that has a 95 to 98% success rate in species identification (Kandul *et al*., 2004).

Taxonomists use morphological identification for the identification of species (Scotland *et al*., 2003). Because of phenotypic similarity, the sibling is misperceived (Jarman and Elliott, 2000). The important method of identification is to study the stages of metamorphosis. The molecular identification resolve issue of kinfolk species (Xiao *et al*., 2010).

Molecular identification is the sequence of DNA used to differentiate species of grasshopper, by identification of micro genes (Wilson, 1995). The sequence used for identification is a sequence of genetic barcodes, which are conserved in each cell of the body. The DNA barcode region is a COI mitochondrial fragment of 658 base pairs that are used for molecular identification of all organisms (Boyan *et al*., 2008). DNA barcoding, is the quick or accurate identification of species, at any stage of metamorphosis and DNA barcoding is also used to differentiate, intraspecific species and interspecific species (Janzen *et al*., 2005).

The Survey from the literature shows grasshoppers, in different regions of the world, however, the biodiversity and pest behaviour of grasshopper's reported in many regions of the World. While Phylogenetic history and molecular study of grasshopper is not done, yet in different regions of Pakistan. They are herbivores and bio indicators of crops (Lanes et al., 2020). Only one family lives in forests, which are Ommatolampidinae (Sergeev, 2021). On the other hand, the family Melanoplinae is alpine and has a rich diversity, in mountainous regions (Sundararaj and Selvi, 2021). The shape and size of the body and feeding behavior, ecology, and history of life are much diverse (Sergeev, 2021). The identification by the molecular study is more accurate than the morphology, embryology, ecology, and identification by anatomy (Popova *et a*l., 2021). The literature review show that There have only been a few historical attempts in Pakistan to use molecular techniques like ITS and rDNA to determine the type of grasshopper. The DNA analysis of grasshoppers and their variety from the Tehsil Oghi KPK, Pakistan, has not, however, been sufficiently explored. DNA barcoding techniques are essential for an organism's identification and taxonomic placement. The current study looks into the diversity of grasshoppers in Tehsil Oghi, KP, Pakistan, utilising both morphological and molecular characterization methods. This endeavour will help identify grasshoppers, which will ultimately result in the compilation of Pakistan's grasshopper inventory.

Materials and methods

Study area

The current research study area, was Tehsil Oghi, District Mansehra Pakistan. Oghi is one of the tehsils of District Mansehra, in the KP province of Pakistan. Its territory consisted of Agror Valley, and some region's headquarters is Oghi. The other tehsils are formed of the different union councils (Ahmed et al., 2019). In August 2017, the three councils of Shanaya Darband, Nika, and Pani were separated into tehsil of Darband (Ahmed et al., 2019). In 2020-2021 material was collected from mountainous, crops, and deserted, areas covered with grasses, herbs, and shrubs. The collection of grasshoppers from Tehsil Oghi, District Mansehra (Figure.1)

Morphological Identification and preservation

Morphological identification was done by Orthopeterian key (Name of key and author with the year). For identification, other available data was used. The Storing and killing methodology was used. For a collection of the insects, the insect net is used, grasshoppers were killed by the insecticide spray. After 2 hours of insecticide spray, the sample has to be dry. Then the sample was labelled and preserved. The common Pins were placed in the thorax region and other regions like antennae, legs, wings, for taxonomic study. The Naphthalene balls were used for preservation, for the protection of the sample from microbes and insects (Vickery and Kevan, 1983).

Molecular characterization

DNA extraction

DNA was extracted by protocol given by Guryev (Van Heesch *et al.*, 2013). DNA is extracted from the leg of a grasshopper. The Legs were ground in powder and stored in 1.5ml Eppendorf tubes. 500ul of lysis buffer was added. Then add proteinase, K of 4.5 ul was added, and then add Beta Mercaptoethanol (BME) of 3ul were added. Then the samples were placed in an incubator for 2 hours, the temperature was set at 60˚C, samples were vortex after 20 minutes. The phenol-chloroform isoamylase of 400ul was added. After that centrifuge for 15 min at 12000 rpm. Two layers were formed, in a sample, the upper layer is supernatant while the lower layer is the palate 500ul of the upper layer is transferred into another Eppendorf tube while the lower layer was discarded with the tube. Then the cooled chilled Isopropanol of 4500ul was added. The tubes were preserved in the refrigerator for 1 night. The next day sample was centrifuged for 15 min at 12000 revolutions per minute. The liquid was discarded from Eppendorf tubes. 70% ethanol of 400ul was added. The samples were centrifuged for 5 min at 8000 rpm. Then ethanol was wasted. Let the tubes be left to dry for 1 night. When the tubes are fully dry add, 30ul of double distilled water was added. In molecular biology, gel electrophoresis is used for the extraction of DNA.

Gel electrophoresis

The 1% gel is prepared by 450 ul of TAE (50X) buffer, with 0.1g powder of agarose were mixed with 45ml of dd H2O and then heat for 2 minutes. The Ethidium bromide of 3ul was added to the gel after cooling. In the gel were tray combs were adjusted, for the creation of wells for loading of the sample. The dd water of 300ml and TAE buffer solution of 5ml were added inside the tank of gel, gel tray was put in the gel tank. 2 ul of sample and dye bromophenol blue of 2ul were mixed, and then loaded in wells of gel tray. The accurate movement of DNA, the voltage, set at 70 to 75 volts, for 35 min. Then the gel tray was removed from the gel tank and observed beneath the UltraViolet Trans illuminator. The results are stored in form of a photograph.

PCR (DNA amplification)

For the amplification of mit16sr RNA, PCR is done. Polymerase chain reaction reagents were Taq 10X buffer 2.5ul, Taq DNA polymerase 0.4ul, template DNA 2ul, Taq DNA polymerase 0.4ul, primers 1ul forward and 1ul reverse, dNTPs 2ul, MgCl2 2ul, ddH2O 10ul, the final volume was 21µl

Table 1: Conditions of PCR for 16sr RNA

Sr.no	Temperature	Time	Cycles
	Pre PCR-Denaturation (94°C)	5 minutes	1
	Denaturation (94°C)	45 sec	
	Annealing (57°C)	45 sec	
	Elongation (72°C)	45 sec	35
	Post Elongation (72°C)	5 minutes	1
	Holding (4°C)	∞	4

Primers

The Primer used is 16Sar. The two primers are used are forward and Backward (Table 3.3).

Table 2: Primer sequence forward and Reverse

16Sar	**Forward**	**CGCCTGTTTAACAAAAACAT**
16Sbr	**Backward**	**CCGGTCTGAACTCAGATCACGT**

Phylogenetic analysis

A total of ten samples, of PCR product, was sent to the Korea, for sequencing, to purify and sequence. A total of 9 sequences from which nine samples were sequenced successfully, except G3. The 16sr RNA one of the genes of mitochondria (Coghlan *et al*., 2012).The different software of bioinformatics software is used to evaluate, the sequenced data, and to a comparison of sequences with published data. The NCBI BLAST (Basic local alignment search tool) was used for searching similar sequences, which is submitted to NCBI by a different researcher. The analysis of the phylogeny of the datasheet was evaluated in MEGA7, MEGA7 open file and then upload into Fasta file, then the file was interpreted by bootstrap 1000, that shows the branch of phylogeny, by their percentages (Tamura *et al*., 2013). The phylogenetic tree formation and evolutionary relationships, of the datasheet, were evaluated by two methods: Maximum Likelihood and NJ Tree (Saitou and Imanishi, 1989).

Results

A total of 70 samples of grasshopper were collected from District Mansehra, tehsil Oghi. Identification of specimens resulted in 9 species *Melanoplus bivittatus*, *Schistocerca piceifrons piceifrons, Hieroglyphus banian*, *Kosciuscola tristis tristis*, *Dericorys albidula*, *Dericorys tibialis*, *Conocephalus fuscus, Omocestus viridulus, stenobothrus stigmaticus.* Under 8 genera and 3 families. The molecular study and Phylogenetic study of explored species were done by 16sr RNA and analysis of phylogeny. Photograph of the identified species provided in figure 4

Combine Phylogenetic Relationship

The rate of evolution selected was done by the NJ method. The distances between them are determined by the Maximum Composite Likelihood and are measured based on substitutions per site. A total of 500 sequences of nucleotide is used.

Tree Cluster

There are five clusters in the combined phylogenetic tree, 7 species in cluster one i-e Melanoplus bivittatus, Kosciuscola tristis tristis, Anacridium egyptium, Hieroglyphus banian, Dericorys albidula, Conocephalus fuscus, Dericorys tibialis. One specie is in Cluster two Omocestus viridulus. One species in Cluster three is stenobothrus stigmaticus.

Species relationships with each other

The evolutionary relationship between the species Melanoplus bivittatus, Kosciuscola tristis tristis, Anacridium egyptium, Hieroglyphus banian, Dericorys albidula, Conocephalus fuscus, Dericorys tibialis, Omocestus viridulus, stenobothrus stigmaticus cluster together. The bootstrap value of Melanoplus bivittatus is 0.012 with Locusta migratoria, while, Kosciuscola tristis tristis, Anacridium

egyptium, Hieroglyphus banian, Dericorys albidula, Conocephalus fuscus, Dericorys tibialis bootstrap value is 0.00 with Locusta migratoria. The Conocephalus fuscus has the bootstrap value of 0.002 with Dericorys tibialis. The stenobothrus stigmatics have a bootstrap value of 0.008 with Choroedocus robustus. The Omocestus viridulus has a bootstrap value of 0.005 with Acrida Ungarica.

Discussion

The molecular characterization of grasshopper's species into class Insecta, from District Mansehra, Tehsil Oghi. A total of 9 species i.e. *Melanoplus bivittatus, Anacridium aegyptium, Hieroglyphus banian, Kosciuscola tristis tristis, Dericorys albidula, Dericorys tibialis, Conocephalus fuscus, Omocestus viridulus, stenobothrus stigmaticus* were documented. Molecular markers are proven as a reliable method for taxonomic studies (Patwardhan *et al*., 2014). The molecular features are helpful for Thesil oghi, for the fauna of Orthoptera. The Molecular analyses are based on the 16Sr RNA. (ML), (NJ) and (MP), are used to determine the credibility of each method during this phylogenetic study. A total of 9 Species under the family *Melanoplus bivittatus, Anacridium aegyptium, Hieroglyphus banian, Kosciuscola tristis tristis, stenobothrus stigmaticus,* from the family *Acrididae, Omocestus viridulus,* and *Conocephalus fuscus* from family *Tettigoniidae,* while *Dericorys albidula, Dericorys tibialis* from family *Dericorythidae.* The outcome the supported by previous research (Bughio *et al*., 2013). The morphological studies of 25 species of grasshoppers from Sindh Pakistan (Sultana *et al*., 2013). Total 30 from the Punjab-based agroecological and 35 species for the morphological features (Hebert *et al*., 2003).

The analysis of the genotype of grasshopper is done by, 658-bp nucleotide of 16Sr RNA and mitochondrial Cytochrome Oxidase I (Wilson *et al*., 1995). The related work was done on the DNA barcodings for the genotypes of grasshopper identification (Dowle *et al.*, 2014). The related work on DNA is information in the use, in phylogenetic studies to differentiate species of the grasshopper species (Ketmaier *et al*., 2010). The information in DNA is combined by the morphological characterization, for building species relations between genotypes of grasshopper (Dowle *et al.*, 2014). The alignment of Cytochrome Oxidase I fragment (660 bp) has 30% variation in sites and 21% parsimony informative sites. The study on *Tettigonidae* is done in East African Meconematinae, shows an alignment of *16S* rRNA (499 bp) has 19% variation in sites and 18% parsimony informative sites, and the *H3* alignment (335 bp) has 10% variation in sites and 7% parsimony informative sites (Hemp et al., 2020). The study of Acrididae is done in Eurasian podismines. The factors that vary like the presence of autapomorphies, of invariant sites (Chintauan-Marquier *et al*., 2014). The interspecific relationships, *Locusta migratoria* resemble *Melanoplus bivittatus* and *Conocephalus fuscus* were clustered together in the molecular tree with the close genetic distances is (0.003). The *Locusta migratoria* resemble *Dericorys tibialis* were clustered together in the molecular tree, which are at close genetic distances that is (0.000). The *Locusta migratoria* resemble *Hieroglyphus banian Dericorys albidula* clustered in the molecular trees shows close genetic distances of (0.005). The *Locusta migratoria* resemble *Anacridium aegyptium,* clustered on the molecular tree, which is at close genetic distances of (0.015). The *Locusta migratoria* resemble *Kosciuscola tristis tristis* clustered in the molecular tree by the close genetic distances of (0.006). The *Acrida Ungarica* resemble *Omocestus viridulus* clustered on the molecular tree by the close genetic distances of (0.003). The *Choroedocus robustus* resemble *stenobothrus stigmaticus* clustered on the molecular tree by the close genetic distances of (0.006). In conclusion, that study investigate the relationships on the bases of phylogeny of grasshopper species, that is the sequence the mtDNA 16Sr RNA, a gene from 9 species in 8 genera and 3 families.(Chen et al., 2018). In the same way, the findings on the molecular levels in many groups of the grasshoppers are concluded (Song et al., 2018).

Conclusion

About 70 samples belongs to *3* families and the 8 genera. The orthopteran fauna of Tehsil oghi, District Mansehra, molecular characteristics give us useful documentation. The molecular analysis of the 16Sr RNA, give us the proficient results. By (ML), (MP), and (NJ) we reach the reliability of each

approach in the research of Phylogenetic analysis. The difference among these approaches are few nodes, by the minor differences by their bootstrap values, they get familiar phylogenetic trees. The species of Orthopteran discovery, show significantly potential.

References

1. Bazelet, C. S., & Samways, M. J. (2014). Habitat quality of grassland fragments affects dispersal ability of a mobile grasshopper, Ornithacris cyanea (Orthoptera: Acrididae). *African Entomology*, *22*(4), 714-725. https://hdl.handle.net/10520/EJC163604
2. Barrett, R. D., & Hebert, P. D. (2005). Identifying spiders through DNA barcodes. *Canadian Journal of Zoology*, *83*(3), 481-491. https://doi.org/10.1139/z05-024
3. Boyan, G. S., Williams, J. L. D., & Herbert, Z. (2008). Fascicle switching generates a chiasmal neuroarchitecture in the embryonic central body of the grasshopper Schistocerca gregaria. *Arthropod structure & development*, *37*(6), 539-544. https://doi.org/10.1016/j.asd. 2008.07.005
4. Bughio, B. A., Sultana, R., Wagan, M. S., Ullah, F., & Rafi, M. A. (2013). Studies on the tribe Acrotylini (Acrididae: Orthoptera) from Pakistan. *Int. J. Biol*, *3*, 180-191. http://dx.doi.org/10.12692/ijb/3.4.180-191
5. Chen, Y. Z., Deng, W. A., Wang, J. M., Lin, L. L., & Zhou, S. Y. (2018). Phylogenetic relationships of Scelimeninae genera (Orthoptera: Tetrigoidea) based on COI, 16S rRNA and 18S rRNA gene sequences. *Zootaxa*, *4482*(2), 392-400. http://zoobank.org/urn:lsid:zoobank. org:pub:799E8FBF-3EDD-4070-B78C-C9E8CAEB2E38
6. Chintauan-Marquier, I. C., Amédégnato, C., Nichols, R. A., Pompanon, F., Grandcolas, P., & Desutter-Grandcolas, L. (2014). Inside the Melanoplinae: New molecular evidence for the evolutionary history of the Eurasian Podismini (Orthoptera: Acrididae). *Molecular phylogenetics and evolution*, *71*, 224-233.https://doi.org/10.1016/j.ympev.2013.09.009.
7. Cigliano, M. M., De Wysiecki, M. L., & Lange, C. E. (2000). Grasshopper (Orthoptera: Acridoidea) species diversity in the Pampas, Argentina. *Diversity and distributions*, *6*(2), 81-91. https://doi.org/10.1046/j.1472-4642.2000.00077.x
8. Coghlan, M. L., Haile, J., Houston, J., Murray, D. C., White, N. E., Moolhuijzen, P., ... & Bunce, M. (2012). Deep sequencing of plant and animal DNA contained within traditional Chinese medicines reveals legality issues and health safety concerns. *PLoS genetics*, *8*(4), e1002657. https://doi.org/10.1371/journal.pgen.1002657
9. Dowle, E. J., Morgan-Richards, M., & Trewick, S. A. (2014). Morphological differentiation despite gene flow in an endangered grasshopper. *BMC Evolutionary Biology*, *14*(1), 1-15. http://www.biomedcentral.com/1471-2148/14/216
10. Gandar, M. V. (1982). The dynamics and trophic ecology of grasshoppers (Acridoidea) in a South African savanna. Trophic ecology of grasshoppers in South African savanna. *Oecologia*, 370-378. https://www.jstor.org/stable/4216777
11. Hajibabaei, M., Singer, G. A., Hebert, P. D., & Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *TRENDS in Genetics*, *23*(4), 167-172. https://doi.org/10.1016/j.tig.2007.02.001
12. Hassan, S. A., Bigler, F., Bogenschütz, H., Boller, E., Brun, J., Calis, J. N. M., ... & Vogt, H. (1994). Results of the sixth joint pesticide testing programme of the IOBC/WPRS-working group «pesticides and beneficial organisms». *Entomophaga*, *39*, 107-119.
13. Hebert, P. D., Ratnasingham, S., & De Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(suppl_1), S96-S99. https://doi.org/10.1098/rsbl.2003.0025
14. Hemp, C., Scherer, C., Brandl, R., & Pinkert, S. (2020). The origin of the endemic African grasshopper family Lentulidae (Orthoptera: Acridoidea) and its climate-induced diversifica tion. *Journal of Biogeography*, *47*(8), 1805-1815. https://doi.org/10.1111/jbi.13880

15. Hochkirch, A. (2001). *A Phylogenetic Analysis of the East African Grasshopper Genus Afrophlaeoba Jago, 1983:(Orthoptera: Acridoidea: Acridinae)*. Cuvillier.
16. Hotz, G., & Reuschl, H. (1967). Damage to deoxyribose molecules and to U-gene reactivation in UV-irradiated 5-bromouracil-DNA of phage T4 Bo ras influenced by cysteamine. *Molecular and General Genetics MGG*, *99*(1), 5-11.
17. Hotz, G., & Reuschl, H. (1967). Damage to deoxyribose molecules and to U-gene reactivation in UV-irradiated 5-bromouracil-DNA of phage T4 Bo ras influenced by cysteamine. *Molecular and General Genetics MGG*, *99*(1), 5-11.
18. Janzen, D. H., Hajibabaei, M., Burns, J. M., Hallwachs, W., Remigio, E., & Hebert, P. D. (2005). Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1462), 1835-1845. https://doi.org/10.1098/rstb.2005.1715
19. Jarman, & Elliott. (2000). DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae,'living fossils' from the Triassic. *Journal of Evolutionary Biology*, *13*(4), 624-633. https://doi.org/10.1046/j.1420-9101.2000.00207.x
20. Joern, A. (1979). Resource utilization and community structure in assemblages of arid grassland grasshoppers (Orthoptera: Acrididae). *Transactions of the American Entomological Society*, 253-300. https://www.jstor.org/stable/25078241
21. Kandul, N. P., Lukhtanov, V. A., Dantchenko, A. V., Coleman, J. W., Sekercioglu, C. H., Haig, D., & Pierce, N. E. (2004). Phylogeny of Agrodiaetus Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of COI and COII and nuclear sequences of EF1-α: karyotype diversification and species radiation. *Systematic Biology*, 278-298. https://www.jstor.org/stable/4135412
22. Ketmaier, V., Stuckas, H., Hempel, J., Landeck, I., Tobler, M., Plath, M., & Tiedemann, R. (2010). Genetic and morphological divergence among Gravel Bank Grasshoppers, Chorthippus pullus (Acrididae), from contrasting environments. *Organisms diversity & evolution*, *10*, 381-395. 10.1007/s13127-010-0031-1
23. Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences*, *102*(23), 8369-8374. https://doi.org/10.1073/pnas.0503123102
24. Lanes, G. O., Kawada, R., Azevedo, C. O., & Brothers, D. J. (2020). Revisited morphology applied for systematics of flat wasps (Hymenoptera, Bethylidae). *Zootaxa*, *4752*(1), 1-127.
25. P McClenaghan, B., Gibson, J. F., Shokralla, S., & Hajibabaei, M. (2015). Discrimination of grasshopper (O rthoptera: A crididae) diet and niche overlap using next-generation sequencing of gut contents. *Ecology and evolution*, *5*(15), 3046-3055.atwardhan, A., Ray, S. and Roy, A. 2014. Molecular markers in phylogenetic studies-a review. *Journal of Phylogenetics & Evolutionary Biology*, 2014. https://doi.org/10.1002/ece3.1585
26. Pocco, M. E., & Cigliano, M. M. (2020). The grasshoppers (Orthoptera, Acridomorpha) from the Mitaraka Mountain Range, French Guiana. *Zoosystema*, *42*(7), 105-114. https://doi.org/10.5252/zoosystema2020v42a7
27. Popova, K. V., Baturina, N. S., Molodtsov, V. V., Yefremova, O. V., Zharkov, V. D., & Sergeev, M. G. (2021, July). The handsome cross grasshopper Oedaleus decorus (Germ.)(Orthoptera: Acrididae) as a newly emerging pest in the south-eastern part of West Siberian Plain. In *Proceedings of the 1st International Electronic Conference on Entomology, online* (pp. 1-15). https://iece.sciforum.net/
28. Saitou, N., & Imanishi, T. (1989). Relative efficiencies of the Fitch-Margoliash, maximum-parsimony, maximum-likelihood, minimum-evolution, and neighbor-joining methods of phylogenetic tree construction in obtaining the correct tree.
29. Saunders, G. W. (2005). Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical transactions of the Royal Society B: Biological sciences*, *360*(1462), 1879-1888. https://doi.org/10.1098/rstb.2005.1719

30. Scicluna, S. M., Tawari, B., & Clark, C. G. (2006). DNA barcoding of Blastocystis. *Protist*, *157*(1), 77-85. https://doi.org/10.1016/j.protis.2005.12.001
31. Scotland, R. W., Olmstead, R. G., & Bennett, J. R. (2003). Phylogeny reconstruction: the role of morphology. *Systematic biology*, *52*(4), 539-548. https://www.jstor.org/stable/3651141.
32. Sergeev, M. G. (2021). Distribution patterns of grasshoppers and their kin over the Eurasian Steppes. *Insects*, *12*(1), 77. https://doi.org/10.3390/insects12010077
33. Song, H., Mariño-Pérez, R., Woller, D. A., & Cigliano, M. M. (2018). Evolution, diversification, and biogeography of grasshoppers (Orthoptera: Acrididae). *Insect Systematics and Diversity*, *2*(4), 3.https://doi.org/10.1093/isd/ixy008
34. Sonnenfeld, N., Schappert, S. M., & Lin, S. X. (2009). Racial and ethnic differences in delivery of tobacco-cessation services. *American journal of preventive medicine*, *36*(1), 21-28. https://doi.org/10.1016/j.amepre.2008.09.028
35. Sultana, R., Wagan, Y. S., & Wagan, M. S. (2013). Orthopteran biodiversity of Thar desert, sindh, Pakistan. *Pakistan Journal of Zoology*, *45*(2). http://www.zsp.com.pk/pdf45/299-304%2..
36. Summerbell, R. C., Lévesque, C. A., Seifert, K. A., Bovers, M., Fell, J. W., Diaz, M. R., ... & Crous, P. W. (2005). Microcoding: the second step in DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1462), 1897-1903. https://doi.org/10.1098/rstb.2005.1721
37. Sundararaj, V., & Selvi, M. (2021). Opposition grasshopper optimizer based multimedia data distribution using user evaluation strategy. *Multimedia Tools and Applications*, *80*, 29875-29891. https://doi.org/10.1007/s11042-021-11123-4
38. Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729. https://doi.org/10.1093/molbev/mst197
39. Van Heesch, S., Mokry, M., Boskova, V., Junker, W., Mehon, R., Toonen, P., ... & Guryev, V. (2013). Systematic biases in DNA copy number originate from isolation procedures. *Genome biology*, *14*(4), 1-9 http://genomebiology.com/2013/14/4/R33.
40. Vickery, V. R., & Kevan, D. K. M. (1983). A monograph of the orthopteroid insects of Canada and adjacent regions. Vols. I and II. *A monograph of the orthopteroid insects of Canada and adjacent regions. Vols. I and II.*, (13).
41. Wilson, K. H. (1995). Molecular biology as a tool for taxonomy. *Clinical infectious diseases*, *20*(Supplement_2),S117-S121. https://doi.org/10.1093/clinids/20.Supplement_2.S117
42. Wilson, M. R., DiZinno, J. A., Polanskey, D., Replogle, J., & Budowle, B. (1995). Validation of mitochondrial DNA sequencing for forensic casework analysis. *International journal of legal medicine*, *108*, 68-74.
43. Xiao, J.-H., Wang, N.-X., LI, Y.-W., Murphy, R. W., Wan, D.-G., Niu, L.-M., Hu, H.-Y., Fu, Y.-G., and Huang, D.-W. 2010. Molecular approaches to identify cryptic species and polymorphic species within a complex community of fig wasps. *PLoS One,* 5**,** e15067.
44. Zemlak, T. S., Ward, R. D., Connell, A. D., Holmes, B. H., & Hebert, P. D. (2009). DNA barcoding reveals overlooked marine fishes. *Molecular Ecology Resources*, *9*, 237-242. https://doi.org/10.1111/j.1755-0998.2009.02649.x