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MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF GRASSHOPPER PESTS OF RICE INFERRED FROM MITOCHONDRIAL COI GENE SEQUENCES

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Abstract: *Oryza sativa* (L.) is one of the world's most important crop providing staple food for nearly half of the global population. Rice is subjected to considerable damage by a number of grasshoppers. The accurate identification of pest species is a crucial step for the appropriate application of control measures. Molecular characterization and DNA barcoding of grasshopper pests of rice was performed in the present research work. Six species of grasshopper pests were identified from different paddy fields using COI gene sequences. Among the sequences the A+T content (64.2%) was higher than the G+C (35.8%) content. The interspecific genetic distance ranged between 13 and 34%. The haplotype analysis of *Conocephalus exemptus* showed highest (110) mutated sites among the analyzed species. Both the Maximum likelihood and Neighbor-joining phylogenetic tree construction showed two distinct clades of short-horned and long-horned grasshoppers. *C. exemptus* was found in another monophyletic group. Our results demonstrate that DNA barcoding is likely to be a powerful tool for identifying and understanding grasshopper evolution.

***Key words*:** Grasshopper, Rice pest, COI gene, DNA barcoding, molecular characterization.

INTRODUCTION

Grasshoppers of the order Orthoptera are considered as major injurious pest of rice fields, grasslands, rangelands, corns, small grains and vegetable crops all over the world (Branson and Sword 2009, Ciplak *et al.* 2008). The temperate and tropical species of short-horned grasshoppers can cause economical damage to postures and agriculture across the world. In the case of heavy infestations, they attack any plants like trees, shrubs and herbs (Arya 2013). In Bangladesh, the rice production is attacked by number of grasshopper pests. Plant physiology is disrupted by damage caused by such insects, resulting in reduced crop yields (Ane and Hussain 2016, Nasiruddin and Roy 2012). When

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they attack crops severely, they can eat entire leaves and reduce the midrib of plant stems (Riffat and Wagan 2009).

Accurate taxonomic identification is a key issue in attempting any integrated pest management program. Traditionally, identification of pests at the species level has been based on morphological analysis. The method of DNA barcoding has become the choice for fast, dependable and cost-effective identification of pest species. DNA barcoding not only correctly diagnoses adult specimens, but also identifies eggs and larvae at the species level. DNA barcodes are intended to identify species using a short, standardized gene region, the cytochrome c oxidase subunit I (COI), as a species label (Hebert *et al.* 2003, Hebert and Gregory 2005). A 658-bp fragment of COI gene is amplified using primers which are efficacious at amplifying this region from metazoan mitochondria (Chapco 1999, Mukha *et al.* 2002, Song and Wenzel 2008). The mitochondrial DNA which contained the structure and composition of genetic information can be used to characterize a population, phylogenetic and make it possible to reconstruct evolutionary relationships (Hebert *et al.* 2004, Lessinger *et al.* 2000).

DNA barcoding in grasshopper pests can be challenging, but well worthwhile, as it may pursue several evolutionary processes. This study is the first assiduity to construct a DNA reference dataset employing COI genes of grasshopper pests and their molecular characterization and other related bio-informatic analyses. Reference database that are stored in the GenBank can be used even by non-entomologists for easy identification and take appropriate decision for control measure.

MATERIALS AND METHODS

Sampling of grasshopper: The specimens (Fig. 1) were collected using insect net from different paddy fields of Savar area. Preliminary identification was carried out based on their morphology using identification keys (Srinivasan and Prabakar 2013, Sultana and Wagan 2008, Morris 2002, Thakkar *et al.* 2015). Somatic tissue rich in mitochondria (e.g. legs and wings) were separated carefully from the specimens, cleaned by ethanol and stored in a -20°C freezer for molecular analysis. The rest of the body of each grasshopper specimen was stored as a voucher specimen.

DNA extraction: For genomic DNA extraction, the leg or wing tissue samples were taken and used Wizard® Genomic DNA Purification Kit, USA, following the manufacturer's protocol after little modification as narrated in Aslam *et al.* (2019a, 2019b) and Rain *et al.* (2019). The yield and integrity of DNA was measured using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) which was extracted from the samples.

Gene Amplification and PCR products visualization: Extracts were amplified by polymerase chain reaction (PCR) following standard protocols. Primer set used in this study was LCO 1490 (5′- GGTCAACAAATCATAAAGATATTGG- 3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.* 1994). PCR was performed using a Veriti® Thermal Cycler from Thermo Fisher Scientific, USA. Amplified PCR products were analyzed using 1% agarose gel electrophoresis and the DNA was exposed to a gel recording system (BioDoc Analyzer, Biometra, Germany).

Gene sequencing: After purification (by PCR clean up system of Promega Corporation- USA) and quality checkup (by NanoDrop Spectrophotometers of Thermo Scientific spectrophotometer, USA) of PCR products sequencing was performed to determine the nucleotide sequence of COI region. The sequencing was carried out using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA).

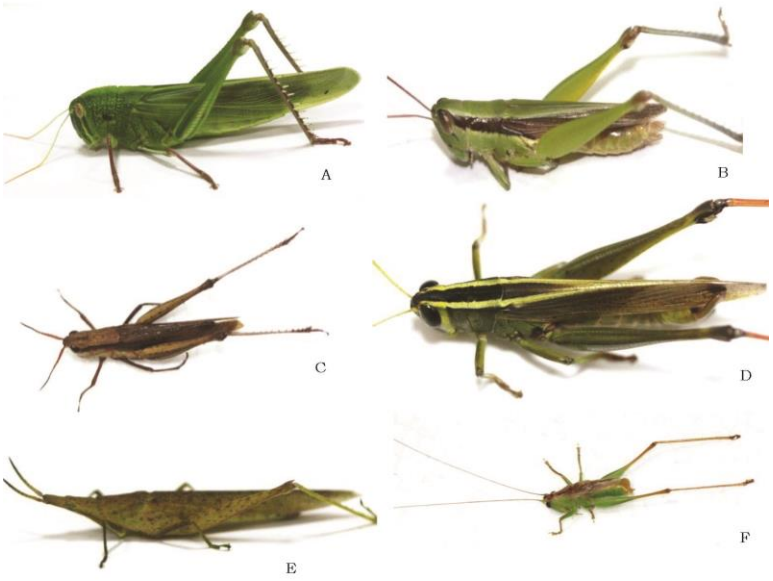

Fig. 1. The morphology of grasshopper species (A. *Chondracris rosea,* B. *Oxya fuscovittata,* C. *Phlaeoba tenebrosa*, D. *Choroedocus violaceipes*, E. *Atractomorpha lata*, F. *Conocephalus exemptus*).

Sequence analysis: Bio Edit v.7.0.5 software was used to check quality of the chromatogram. Sequences were aligned by ClustalW with the help of MEGA (Molecular Evolutionary Genetic Analysis) tools (version 10) (Kimura 1980, Kumar *et al.* 2018). Nucleotide compositions were estimated and summarized, and pairwise distances were assessed using Kimura 2 Parameter (K2P) model with the MEGA10 program. Jalview, version 2.9 was used for preparing multiple

sequence alignment (Von der Schulenburg *et al*. 2001). Popart1.7 software was used to perform Haplotype networking. Maximum likelihood (ML) and neighbor-joining (NJ) methods were used for reconstruction of phylogeny using bootstrap with 1000 times of repetition in MEGA software program v.10.0. Some relevant sequences were downloaded from NCBI GenBank for phylogenetic tree analysis.

RESULTS AND DISCUSSION

Grasshopper identification: A total of 754 grasshopper samples were collected from various rice fields of Bangladesh and morphologically classified (Fig. 1). The COI genes of these species were sequenced and the sequences consisted of >600bp to <700bp. BLAST analysis (Basic Local Alignment Search Tool) of retrieved sequences showed almost 100% similarity with the previously deposited sequences of the NCBI GenBank. It confirms the accuracy of identification of these grasshopper pests. They were found belong to 6 species namely *Atractomorpha lata, Phlaeoba tenebrosa, Choroedocus violaceipes, Chondracris rosea, Conocephalus exemptus, Oxya fuscovittata*. Among them, *P. tenebrosa, C. violaceipes, C. rosea, C. exemptus, O. fuscovittata* were reported for the first time from Bangladesh.

The successful sequences were submitted to GenBank database and gained accession numbers (Table 1).

Table 1. Voucher ID, GenBank accession number and the GPS location of the sequenced grasshopper species

Voucher ID	Species name	GPS Position	GenBank Acc. no.
GH0E2	*Chondracris rosea*	23.954910N,90.279522E	MK007252.1
GH0K	*Oxya fuscovittata*	23.199978N,90.726010E	MH999493.1
GH0Q	*Phlaeoba tenebrosa*	23.873019N,90.267504E	MG587919.1
GH0L3	*Choroedocus violaceipes*	23.887267N,90.271425E	MG587923.1
GH0R	*Atractomorpha lata*	23.771946N,90.375230E	MH898871.1
GH0W2	*Conocephalus exemptus*	23.878203N,90.265187E	MK026737.1

Nucleotide composition of COI sequence: The COI sequences were analyzed for nucleotide composition (Table 2). Total nucleotide composition of the COI gene varied among the specimens but all the sequences showed expected maximum AT biasness. As expected, average AT content (64.20%) was found significantly higher than the GC content (35.8%) (Table 2). The highest percentage of thiamine (T= 34.4%), guanine (G= 19.3%) and cytosine (C= 21.2%) found in *C. exemptus,* adenine (A= 35.8%) in *A. lata*. On the contrary, the lowest percentage of thiamine (T= 31.5%) and guanine (G= 14.4%) found in *A. lata,* cytosine (C=17.8%) in *O. fuscovittata* and adenine (A= 25.1%) in *C. exemptus*.

Table 2. Nucleotide composition of COI sequence of grasshopper pests

Species	Position	T (%)	C (%)	A (%)	G (%)	(A+T)%	(G+C)%
A. lata	1st	42.1	26.9	15.2	15.7	67.3	32.7
	2nd	28.6	10.7	58.7	2.0		
	3rd	23.9	17.3	33.5	25.4		
C. violaceipes	1st	41.0	28.8	14.2	16.0	62.3	37.7
	2nd	33.3	12.7	49.8	4.2		
	3rd	20.8	19.8	27.8	31.6		
C. rosea	1st	41.2	28.4	14.7	s15.6	64.4	35.6
	2nd	38.1	9.0	49.5	3.3		
	3rd	21.8	19.4	28.0	30.8		
C. exemptus	1st	40.3	28.4	14.7	16.6	59.5	40.5
	2nd	40.8	15.2	35.5	8.5		
	3rd	22.3	19.9	25.1	32.7		
O. fuscovittata	1st	41.6	28.0	14.5	15.9	66.2	33.8
	2nd	35.5	7.9	55.1	1.4		
	3rd	23.8	17.3	28.0	30.8		
P. tenebrosa	1st	42.5	27.4	14.2	16.0	65.6	34.4
	2nd	35.2	8.7	53.4	2.7		
	3rd	24.3	18.3	27.1	30.3		

Sequence alignment: Alignment was performed among the 6 grasshopper pest species. The alignment result (Fig. 2) showed that the species had high conserved region and low non- conserved region among them. So, all of the grasshoppers species were closely related. The *C. exemptus* had highest degree of non- conserved region and they were mostly diverse among them.

Interspecific genetic distance: The analysis was done using 6 nucleotide sequences. Codon positions comprised were 1st+2nd+3rd+Noncoding. The number of base substitutions in each site among the sequences is shown in Table 3. The percentage of interspecific genetic divergence of grasshopper was ranged from 13 to 34%. The lowest percentage (13%) of pairwise distance was observed between *O. fuscovittata* and *P. tenebrosa* and highest distance (34%) was found between *A. lata* and *C. exemptus*.

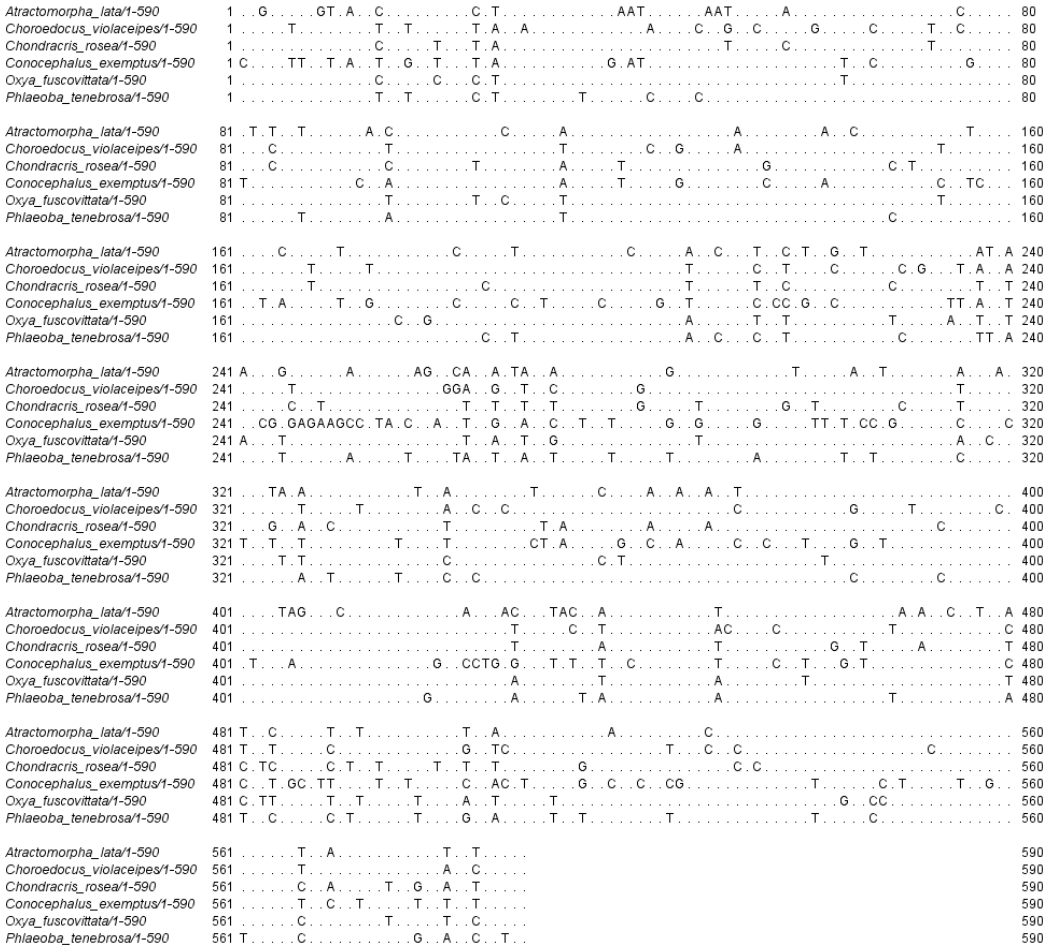

Fig. 2. Multiple sequence alignment of COI genes of grasshoppers. Dots indicate the conserved and the base pairs denote the non- conserved region.

Table 3. Interspecific genetic distances among 6 species of grasshopper pest

Species name	1	2	3	4	5	6
Atractomorpha lata						
Choroedocus violaceipes	24					
Chondracris rosea	22	17				
Conocephalus exemptus	**34**	30	30			
Oxya fuscovittata	20	16	14	30		
Phlaeoba tenebrosa	22	15	16	32	**13**	

Haplotype: A broadly used approach for measuring levels of genetic variation within and between species is through the construction of haplotype. TCS can also be used to visualize and estimate relationships among organisms

using diagrams. Here, the haplotype analysis was performed among mitochondrial gene of 6 grasshopper species (Fig. 3). The haplotype result of mitochondrial COI gene of grasshopper indicated that all the species were highly genetically diverse. *O. fuscovittata* and C*. rosea* separated from its immediate common ancestors by 12 and 45 mutational steps. The *P. tenebrosa* showed 39 mutated sites from its immediate ancestor and the immediate ancestor among them showed 8 mutational steps. Also, the immediate ancestor of *P. tenebrosa* and *C. exemptus* and *C. violaceipes* separated from 13 mutational sites. *C. violaceipes* genetically remote from its common ancestor by 38 mutational numbers and *C. exemptus* showed highest (110) amount of mutated sites among the grasshopper species.

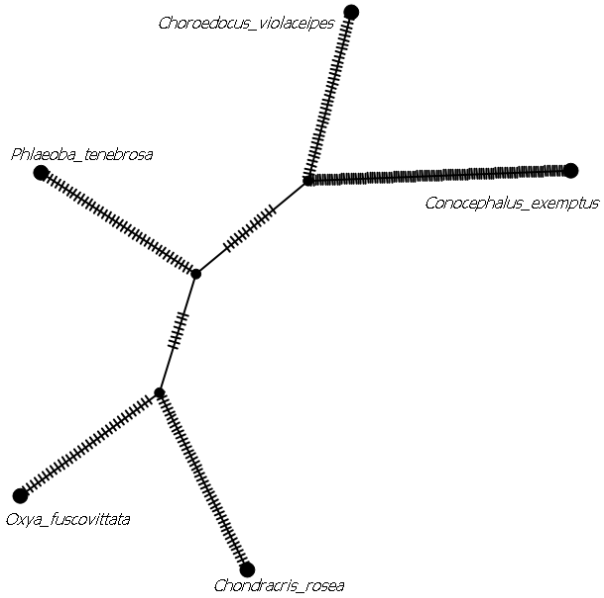

Fig. 3. Haplotype analysis. Large circles denote the haplotype and small circles the immediate common ancestors. Mutational steps are marked hatch marks.

Phylogenetic analysis: Phylogenetic relationships were constructed using the COI sequences of this study to examine whether the evolution of all grasshopper species occurred in a common ancestor and the species were closely related. The same species from GenBank was taken along with to confirm the relationship. The hymenopteran species *Apis mellifera* (Acc. no. AF250946.1) used as outgroup (Fig. 4 and 5).

The maximum likelihood tree (Fig. 4), indicates that the short-horned species of Acrididae family namely *C. violaceipes, P. tenebrosa*, *O. fuscovittata,* C.

rosea and *A. lata* were originated from one monophyletic group and the long-horn species *C. exemptus* under Tettigoniidae family from another monophyletic group. The species *A. lata* showed 64% identity with *C. violaceipes* forming a major subclade. All other species formed many sub-branched among them with different bootstrap value. *C. violaceipes* and *P. tenebrosa* diverge from a common ancestor with 79% similarity, the C. *rosea* and *O. fuscovittata* also originated from an immediate ancestor of *P. tenebrosa* with 26% similarity and showed 41% homology between them. All the individual species group showed 100% similarity among them. The neighbor- joining tree (Fig. 5) also showed the same result.

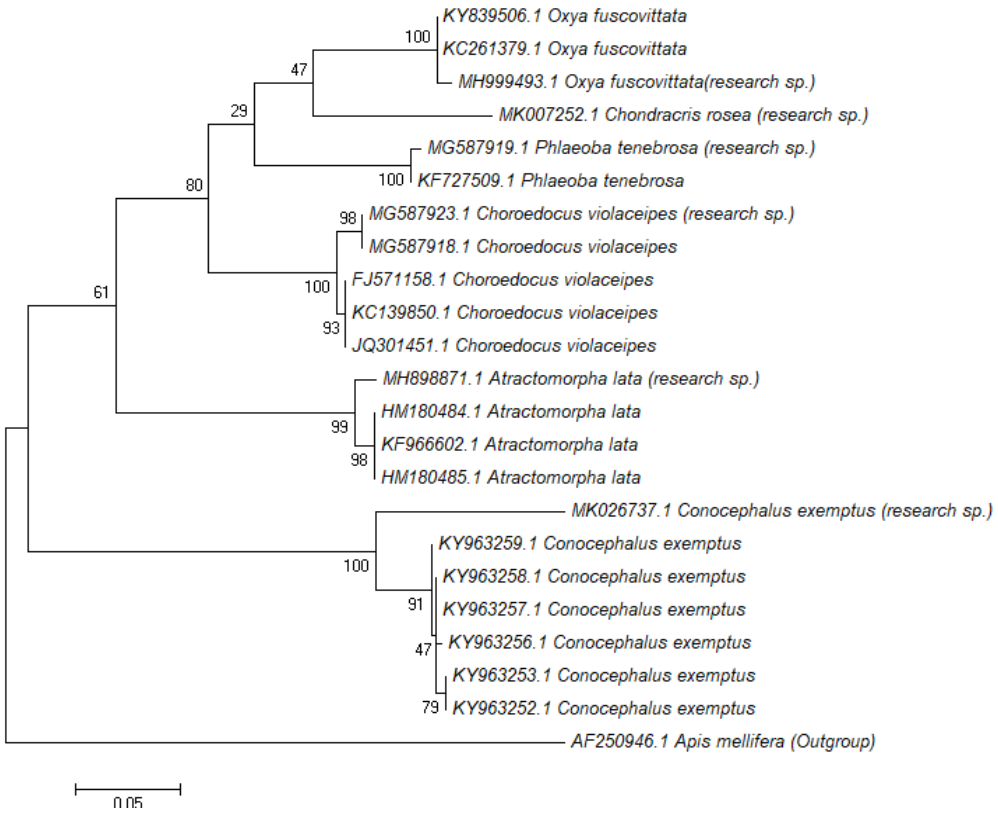

Fig. 4. The evolutionary chronicle of grasshopper species - deduced by Maximum Likelihood (ML) method.

DNA barcoding is an effective tool for molecular identification of insects, especially grasshoppers (Cywinska *et al.* 2006). The fundamental purpose of

DNA barcoding is to standardize molecular methods for complementary morphological species identification (Azpurua *et al.* 2010, Gutierrez *et al.* 2014, Adeniran *et al.* 2019). Six grasshopper species were identified that were collected from different paddy fields. Nasiruddin and Roy (2012) reported nine species of grasshopper pests from paddy fields from two areas of Hathazari, Chittagong, but they have no molecular evidence.

Various parameters are used to characterize DNA sequences (Dwivedi and Gadagkar 2009). Among the sequence the average AT (64.2%) content is higher than the GC (35.8%) content. The GC content of grasshopper is analogous to the typical values of 33–53% in other animal lineages (Min and Hickey 2007). This result shows identical pattern as reported by Zhang *et al.* (2010), Muraji and Nakahara (2001), Muraji and Nakahara (2002). Previously different COI sequence analysis from Bangladesh showed similar maximum AT content (Das *et al.* 2020, Aslam *et al.* 2019a and 2019b, Sarker *et al.* 2019a and 2019b).

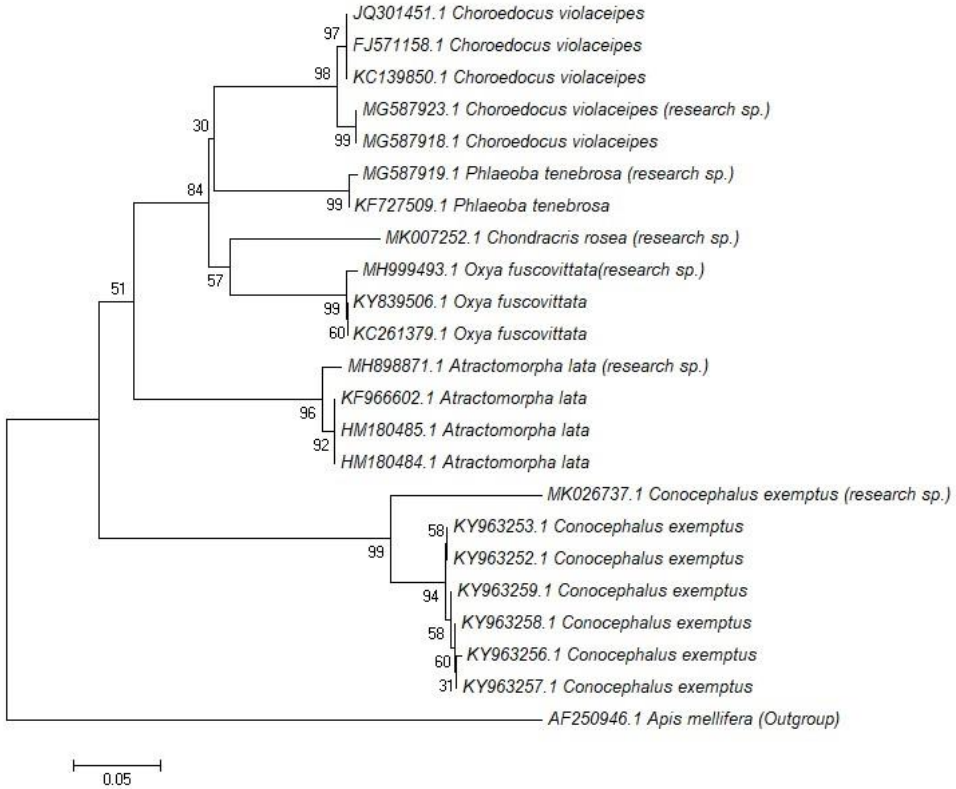

Fig. 5. The evolutionary relationship among grasshopper species was reckoned using the Neighbor-Joining (NJ) method.

In percentage terms, the range of intraspecific and interspecific COI variation is found in other life groups, such as moths, beetles, birds which typically exhibit <3% intraspecific variation and >2% interspecific variation (Rivera and Currie 2009, Pentinsaari *et al.* 2014, Schmidt *et al.* 2015, Huemer *et al.* 2018). The interspecific genetic distance of grasshopper ranges between 13.8 and 34.5% which supports the above range. Guo *et al.* (2011) reported interspecific variation in sarcophagus species ranging from 7 to 10%.

The phylogenetic analysis provided better relationships among various grasshopper species. The short-horned species and long horned- species were grouped in two different clades. Our findings support the results of Hawlitschek *et al.* (2017) and Nazir *et al.* (2014).

Moreover, all the grasshopper species were correctly identified with highest identity values. The properties of the DNA sequences were precisely studied and subsequently utilized for inferring the evolutionary data and the genetic divergence was appropriately congruent. Therefore, this study may provide deeper insights into the knowledge of grasshopper pests in rice and other economically valuable crops based on their nucleotide sequences and evolutionary history. In conclusion, the proper identification of these grasshopper will help in control them from the injury of crops.

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