ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645
Indian Journal of Science and Technology, *Vol 8(1), 61–64, January 2015* DOI : 10.17485/ijst/2015/v8i1/52842

Molecular Phylogeny and Genetic Analysis of Green Leafhopper - *Nephotettix virescens* (Distant) using Mitochondrial COI Gene

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Abstract

Accurate and rapid diagnosis of taxa, is of crucial importance to quarantine, pest management and analysis. Advances in DNA technology has resulted in an exceedingly vary of molecular techniques which might be employed in phylogenetically focussed approaches. Use of DNA sequence data derived from region of mitochondrial cytochrome oxidase I gene (mtCOI) has been widely used to estimate phylogenetic relationships at different taxonomic levels across insects. The present study investigates the molecular evolution of the *Nephotettix virescens* using COI gene (DDBJ accession No. AB976528; ENA accession No. LM994675) and its usefulness for reconstructing phylogenetic relationships within and among the leafhopper species.

Keywords: DNA Sequencing, Leafhopper, Molecular Evolution, Mitochondrial COI, Nephotettix virescens, Phylogenetics

1. Introduction

The Order Hemiptera comprises over 20,000 species globally; most of them are economically important insects groups. They were widely distributed and many of its members are serious pests and vectors of diseases of many economic crops. The effective management of pest species damaging the crop cannot be undertaken without accurate identification[1]. The green leafhopper, *Nephotettix virescens* (Distant), coming under the subfamily Deltocephalinae of the family Cicadellidae, is considered as a serious pest of rice in many rice growing countries. Among the reported *Nephotettix* species, six are known to Asian regions and two in Africa. Species differentiation is comparatively tedious as the only reliable morphological character is the structure of male genitalia.

In *Nephotettix virescens* the vertex is unmarked with distinctive furrows which is longer in the middle than next to eyes and is pointed in most specimens (Figure 1). In some males usually head, pronotum and scutellum are green in colour but some have black marking nearer to ocelli. The colour of subgenital plate of the male genitalia is off-white and is partly black. Male pygofer is rounded with one long and four small spines. The middle of the shaft of the aedeagus bears 3–5 pairs of spines. The number of spines on the male aedeagus is a distinctive character of the *Nephotettix virescens* which separates it from other *Nephotettix* species[2].

The insect feeds mainly on the adaxial surface of the leaf blade and rarely on the leaf sheath[3]. The insect causes direct damage to the rice plant by sucking the sap from vascular tissues and reduces the vigour, number of tillers and yield of rice[4, 5]. Of the leafhopper vectors of the plant diseases 63% of the species comes under the subfamily Deltocephalinae[6]. These insects acts an efficient vector of rice tungro virus[7, 8]. Heavy infestations cause complete drying of rice plants a condition commonly known as "hopperburn".

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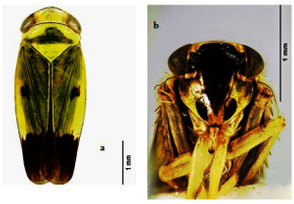

Figure 1. N. virescens. (a) Dorsal view. (b) Frontal view.

Despite of their economic importance, the phylogenetic relationships of Deltocephalinae has never been stable. The presence of vector species associated with the crop ecosystem must be monitored with care and if possible at the nymphal stage itself in order to avoid economic loss, for which all species must be identified unambiguously. Molecular identification method using mitochondrial DNA (mtDNA) has been widely utilized in phylogenetic studies of animals because it evolves rather more speedily than nuclear DNA, leading to the build-up of variations between closely connected species[9]. DNA barcoding has become a promising tool for the rapid and accurate identification of various taxa, and it has been used to reveal unrecognized species in several animal groups. DNA barcoding has the potential to improve the way researchers relate to wild biodiversity[10]. The introduction of DNA barcoding has highlighted the expanding use of COI as a genetic marker for species identification[11].

2. Materials and Methods

2.1 Collection and Identification of Samples

The area selected for the present study stretches along the paddy fields of northern Kerala extending from Thrissur to Kannur. A diverse geographical status is spoken to, including lowland, highland and marshy areas, and the area exhibits a high diversity of organisms. The specimens of *Nephotettix virescens* were collected from the paddy fields by employing the sweep net technique and aspirator. Collected adult specimens were identified morphologically by consulting published taxonomic keys and related literatures[12, 13]. To validate the identification, the abdomen of each specimen was dissected and the structure of the genitalia was assessed[14]. The collected specimens were stored at -20°C until the DNA was extracted.

2.2 DNA Extraction, Amplification and Sequencing

DNA was extracted from the tissue of thoracic leg of the specimen, using NucleoSpin® tissue Kit of Macherey-Nagel as per the manufactures guidelines. The DNA isolated was confirmed using 1% agarose gel and 2ng was amplified for COI gene using the appropriate forward (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse (5'-TAAACTTCAGGGTGACCAAAAAAT-CA-3')primers. The PCR reaction mixture comprised of 2ng of genomic DNA (1µ1), 1µ1 each of forward and reverse primer (Table. 01) at a concentration of 5µM, 1µ1 of dNTPs (2.5mM), 2µ1 10X reaction buffer, 0.20µ1 *Taq* polymerase (5U/µ1) and 13.8µ1 H_2O. The PCR profile consisted of initial denaturation step of 5min at 95°C followed by 30 cycles of 10 sec at 95°C, 1 min at 55°C and 45 sec at 72°C and ending with a final phase of 72°C for 3 min. The PCR product was resolved on a 2% TAE-agarose gel, stained with ethidium bromide[15]. To remove unincorporated primers and dNTPs the resultant PCR product was column purified using the nucleic acids purification kit of Gene JET™ of Fermentas Life Science. The purified PCR product was sequenced by Sanger's method using an ABI 3730XL genetic analyser. The sequences were submitted to DNA Bank of Japan (DDBJ) and European Nucleotide Archive (ENA) are accessible under accession numbers AB976528 and LM994675 respectively.

2.3 Alignment and Analyses

Chromatogram was analyzed and forward and reverse sequences were checked and annotated. Annotated sequences were trimmed off primer sequences and sequence ambiguities were resolved. The COI sequence obtained was aligned using ClustralW[16]. The aligned COI sequences were translated to amino acids to check for the presence of premature stop codons that indicate the presence of nuclear pseudogenes or sequencing errors.

2.4 Phylogenetic Analyses

Nucleotide sequences were analyzed using MEGA6 software[17]. The matrix of corrected DNA distances was generated using Kimura Two parameter model, and a phylogenetic tree was generated using the neighbor-joining algorithm[18]. Bipartitions in the neighbor-joining tree were examined by bootstrap analyses over 1,000

replicates[19]. Percentage nucleotide distances calculation were performed using MEGA6.

3. Results and Discussion

The mitochondrial Cytochrome Oxidase I region of the sample was successfully amplified using PCR. The NCBI blast database result revealed that partial COI gene sequence of *N. virescens* isolated from Kerala showed 96% similarity with *N. virescens* isolated from Orissa. The composition of nucleotides of both species of *Nephotettix* showed clear bias to nucleotide 'AT'. The nucleotide composition analysis revealed the high AT content in the COI gene of *N. virescens* (73.0%). The nucleotides T, C, A and G present in the COI sequence in the following concentrations 34.10%, 12.0%, 39.0% and 15.30% respectively. But in the case of *N. virescens* (BOLD ID-BIPR006-13) isolated from Orissa, the nucleotide composition is 33.30%, 13.70%, 37.80% and 14.90% respectively *i.e.* 1.2% difference in the concentration of nucleotide 'A' between these two species. There is 4.0% decrease in the concentration of nucleotide 'A' in the first position of the codon of *N. virescens* (Orissa) compared to *N. virescens* which is 39.80% and 43.40% respectively (Table 1).

The phylogeny analysis using NJ tree revealed the sharing of common ancestor of these two species (Figure 2). The *Macrosteles sp*. is found to be an outgroup of *N. virescens* isolated from Kerala. The BLAST result obtained in this study revealed that the sequence product obtained for *N. virescens* Kerala is novel. The branch length of *N. virescens* isolated from Kerala is 0.032 and that isolated from Orissa is 0.010, which was less compared to the *N. virescens* isolated from Kerala indicating less divergence time taken by Orissa species from their ancestor.

Species names	Nucleotide Frequencies in percentage														
	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
AB976528| LM994675|Nephotettix virescens|Kerala	34.1	12.0	39.0	14.9	47	3.6	43.4	6.0	16	14.5	50.6	19.3	40	18.1	22.9	19.3
BIPR006-13|Nephotettix virescens|orissa	33.3	13.7	37.8	15.3	46	7.2	39.8	7.2	16	14.5	50.6	19.3	39	19.3	22.9	19.3
EU981862.1| Deltocephalinae sp.	35.7	18.5	32.5	13.3	40	8.4	49.4	2.4	23	15.7	36.1	25.3	45	31.3	12.0	12.0
EU981888.1| Macrosteles sp.	37.8	18.1	28.5	15.7	43	8.4	39.8	8.4	25	14.5	33.7	26.5	45	31.3	12.0	12.0
EU981894.1| Deltocephalinae sp.	37.8	16.5	32.9	12.9	42	3.6	54.2	.0	27	14.5	32.5	26.5	45	31.3	12.0	12.0
FJ824094.1| Baloluths rubrovittata	35.7	17.7	32.1	14.5	43	7.2	44.6	4.8	19	15.7	37.3	27.7	45	30.1	14.5	10.8
FR779766.1| Conosanus obsoletus	31.7	22.5	32.5	13.3	30	18.1	49.4	2.4	20	19.3	34.9	25.3	45	30.1	13.3	12.0
JX020552.1| Typhlocyba serrata	35.7	20.1	31.3	12.9	37	10.8	47.0	4.8	23	20.5	33.7	22.9	47	28.9	13.3	10.8
JX433170.1| Nesophrosyne sp.	35.7	18.9	31.3	14.1	42	7.2	45.8	4.8	20	19.3	34.9	25.3	45	30.1	13.3	12.0
JX433209.1| Scaphoideus sp.	36.1	19.7	29.3	14.9	41	10.8	41.0	7.2	23	16.9	34.9	25.3	45	31.3	12.0	12.0
JX433212.1| Mimotettix sp.	34.5	19.7	32.5	13.3	37	9.6	50.6	2.4	22	18.1	34.9	25.3	45	31.3	12.0	12.0
JX433213.1| Orosius sp.	32.1	20.1	31.7	16.1	33	10.8	48.2	8.4	19	20.5	33.7	26.5	45	28.9	13.3	13.3
KF226916.1| Exitianus nanus	34.5	20.5	30.9	14.1	36	12.0	48.2	3.6	23	18.1	32.5	26.5	45	31.3	12.0	12.0
KF227059.1| Ipeides haskeri	36.5	19.7	29.3	14.5	43	10.8	39.8	6.0	20	19.3	33.7	26.5	46	28.9	14.5	10.8
KF227152.1| Mayawa sp. I	35.3	16.1	34.9	13.7	34	7.2	56.6	2.4	25	13.3	34.9	26.5	47	27.7	13.3	12.0
KF227164.1| Orosius argentatus	32.1	20.1	31.7	16.1	33	10.8	48.2	8.4	19	20.5	33.7	26.5	45	28.9	13.3	13.3
KF227165.1| Orosius argentatus	32.1	20.1	31.3	16.5	33	10.8	47.0	9.6	19	20.5	33.7	26.5	45	28.9	13.3	13.3
Avg.	**34.8**	**18.5**	**32.3**	**14.4**	**39**	**9.3**	**46.6**	**5.2**	**21**	**17.4**	**36.3**	**25.2**	**44**	**28.7**	**14.1**	**12.9**

Table 1. Nucleotides frequencies in each position of codon of the COI sequence of *N.virescens* isolated from Kerala and in the related species

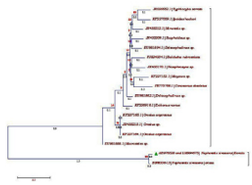

Figure 2. Phylogenic relationship of N. virescens isolated from Kerala, inferred by NJ tree method.

The partial coding sequence of COI was proved as a powerful tool for the accurate identification of organisms[20]. The partial COI sequence generated in this study showed considerable variation with other species. The variation in the codons 'A' nucleotide composition in second position of COI sequence of *N. virescens* and *N. virescens* (Orissa) when compared to the first position indicated that it has highest mutation rates. The first and the second position the nucleotide 'T' composition has high variation in the COI sequence of *N. virescens* isolated from Kerala and from that of Orissa. In both the species the COI sequence is highly biased to AT. The shift to AT compositional bias with low 'G' and the 'C' in the sense and template strand may have arisen through directed mutational pressure[21].

N. virescens isolated from India showed 0–0.04% intraspecific divergence with that of geographically isolated population. The geographical isolation may be the reason for this high divergence of the *N. virescens* between the populations. The high interspecific divergence observed in *N. virescens* may be due to their adaptation to Polyphagy. The intraspecific divergence observed here between the species of *N. virescens* of Kerala and Orissa revealed the occurrence of the *N. virescens* to this area have a common ancestor.

4. Conclusion

The COI sequence is an effective tool to identify the *N. virescens* in any stage of its life cycle. The collected population of *N. virescens* showed that the COI (DDBJ accession No. AB976528; ENA accession No. LM994675) sequence is highly biased towards the nucleotides A and T. But in the codon usage the concentration of each nucleotide in the each position of codon varied depending upon the population. Phylogenetically *N. virescens* (BIPR006-13) is the nearest relative of *N. virescens* analysed in this study. This study also revealed that the *N. virescens* of Kerala and Orissa were originated from a single ancestor.

5. Acknowledgement

The authors are thankful to Dr. C. A. Viraktamath for specimen identification and literature provision.

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