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Molecular phylogeny of *Thaia subrufa* (Hemiptera: Cicadellidae) based on the Mitochondrial Cytochrome Oxidase Subunit I (COI) gene

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Abstract
Leafhoppers of the family Cicadellidae are small wedge shaped insects widely distributed and many of its members are serious pests and vectors of diseases of many economic crops. These insects are distinguished morphologically by examining the colour, size, genital characters and also by examining the rows of small spines extending the length of hind tibia. *Thaia subrufa* (Motsch.) the orange headed leafhopper (OHLH) is one among them and is frequently found in the paddy field. The effective management of pest species cannot be undertaken without the accurate identification. Taxonomic literature dealing with the identification of these tiny insect is scattered in many journals and monographs published over for many years and found difficult to obtain. Molecular analyses using COI gene is evolving rapidly as part of taxonomy where accurate identification is possible examining the gene of the insect. Here in this study, partial segment of COI gene of sample was sequenced. The sequences were compared with those retrieved from NCBI system and the GenBank through BLASTn. The results showed that the COI sequence obtained in this study is a novel one and can facilitate the separation of the species. Sequence obtained in the study were submitted to the DNA Data Bank of Japan (DDBJ) under the accession number LC005702. Based on the relationships between the study insect and the closely related one were clearly distinguished.

Keywords: Molecular phylogeny, *Thaia subrufa*, DNA Data Bank of Japan (DDBJ), Accession number LC005702, Cytochrome Oxidase I (COI).

1. Introduction
The Hemipteran subfamily Typhlocybinae constitutes a vital group with regard to its morphology, biology and economic influence on the agricultural and forest wealth. The orange headed leafhopper *Thaia subrufa* (Motsch.) comes under this subfamily in the order Hemiptera, family Cicadellidae. Throughout Asia over 20 species of *Thaia* are identified so far and out of these species 5 are considered as pests. These five species are *Thaia subrufa*, *Thia ghaurii, Thia assamensis, Thia longipenia and Thia oryzivora*. *T. subrufa* are frequently found on paddy fields as a pests [1]. The adult and nymphs are found in clusters on leaf blades. They suck on leaf under surface, resulting white specks on the upper surface [1]. Sometimes the white specks turn brown and give the plant a burnt appearance [2]. Naik and Belavadi [3] recorded *T. subrufa* in the hill zone of Karnataka as an important pest and also reported on summer paddy [4]. These tiny insects not only cause physical damage but also act as transmitters of pathogens and virus from one plant to other during their feeding. The accurate taxonomy is the basis of retrieving biological information of species, without which there is a risk of ascribing wrong biological information for a species under study. It is the discipline of biology producing the classification of the diversity found in the biological domain [5]. Classification and phylogenetics mainly depends on observable morphology. It requires the subjective judgement of a specialist and also demands good taxonomic knowledge to recognise subtle differences between closely related species. Sometimes the morphological features may be variable with environmental factors which creates problems to the accurate analysis [6, 7]. It is estimated that for critical identification of 10-15 million species, based upon morphological judgements about 15,000 taxonomist is required with centuries for a preliminary level identification [8, 9]. As the existence of diversity of insect species and its complicated developmental stages, egg, larva, pupa and imago the identification using existing methods in certain species at the larva stage takes too much time and effort, as it has to be

raised into a full grown up. Molecular approach in insect taxonomy by using DNA sequence diversity to identify organisms is now recognized as essential to overcome taxonomic impediments [10]. With this DNA barcode method, a researcher can now identify crossbreeds and new types of insects that have not yet been discovered, as to the human eye they look quite similar to existing species.

A variety of molecular markers are available for characterization of genetic diversity of insects [11]. The usage of cytochrome oxidase subunit I (COI) as a universal identification marker utilising a single gene sequence is good enough to differentiate vast majority of animals species [9]. The cytochrome oxidase gene encoding the mitochondrial genome have conserved sequences whose evolutionary rate is appropriate to resolve phylogenetic relationship between small taxonomic units [12, 13].

Here we have partially sequenced the COI, the protein coding mitochondrial gene, for barcoding approach to assemble all available related species of *T. subrufa*, the leafhopper pest. Species clusters were identified using ClustalW tool with other Hemiptera species sequences in the NCBI nucleotide databank and a tree based approach was used here to study the phylogenetic relationship.

2. Materials and Methods

2.1. Study area

The study area spotted along the paddy fields of northern Kerala extending from Thrissur to Kannur districts. An extensive variety of situations is spoken to, including lowland, highland and marshy areas where exhibited a greater diversity of organisms. The insects were collected during the rice cultivation season of Virippu and Puncha in agro climatic zones the year 2011- 2013.

2.2. Collection and identification of samples

By employing the sweep net technique and aspirator the specimens of *Thaia subrufa* were collected from the paddy fields of northern Kerala. Collected adult specimen were identified morphologically to species level by consulting published taxonomic keys and related literatures [14, 15]. To validate the identification the abdomen of each specimen was dissected to study the genitalia [16]. The collected specimens were stored at -20 °C until the DNA was extracted.

2.3. DNA extraction, amplification and sequencing

DNA was extracted from the tissue of the 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. The mitochondrial genomic DNA was amplified for COI gene using the specific forward and reverse primer pairs. 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 products were resolved on a 2% TAE- agarose gel, stained with Ethidium Bromide [17]. To remove unincorporated primers and dNTPs, the resultant PCR product was column purified using the nucleic acids purification kit of Gene JET of Fermantas Life Science. The purified PCR product was sequenced with forward as well as reverse primers using an ABI 3730XLgenetic analyser in Sanger's sequencing method [18].

2.4. Alignment and analyses

Chromatogram was analyzed and forward and reverse sequences were checked and annotated. Annotated sequences were imported and primer sequences were removed from the start and the end of the obtained sequence and sequence ambiguities were resolved. The COI sequences obtained were multiply aligned using ClustalW [19]. 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.

2.5. Phylogenetic analyses

Nucleotide sequences were analyzed using MEGA6 [20]. The matrix of corrected DNA distances was generated using Kimura two parameter model, and a phylogenetic tree was generated using the neighbor-joining algorithm [21]. Bipartitions in the neighbor-joining tree were examined by bootstrap analyses over 1,000 replicates [22]. Percentage nucleotide distances calculation were performed using MEGA6.

3. Results

The 466bp COI sequence obtained in the study were submitted to the DNA Data Bank of Japan (DDBJ) under the accession number LC005702. *T. subrufa* species from Cicadellidae family were used for partial sequence analysis of COI genes and further these sequences were used to elucidate the genetic relationship in *T. subrufa* species. Species identities were performed from the DNA sequence using the BLASTn of NCBI. The resulted sequences showed significant alignments of which the maximum identity ranged from 82% to 78%. The maximum score ranged from 431 to 274. The query coverage was found to be as 100-83% whereas the BLASTp result shows 90% identity to *Anzygina sp*.

Neighbourhood joining (NJ) method of Phylogenetic tree construction was preferred for accurate establishment of phylogenetic relationship and to trace out the presence of phylogenetic signals in the DNA sequences [23]. The distance was calculated between every pair of sequences and used to construct the phylogenetic tree which guided the final multiple alignment. The NJ tree comparison of the study organism, *T. subrufa* showed evolutionary similarity with *Anzygina sp*. (Fig.1).

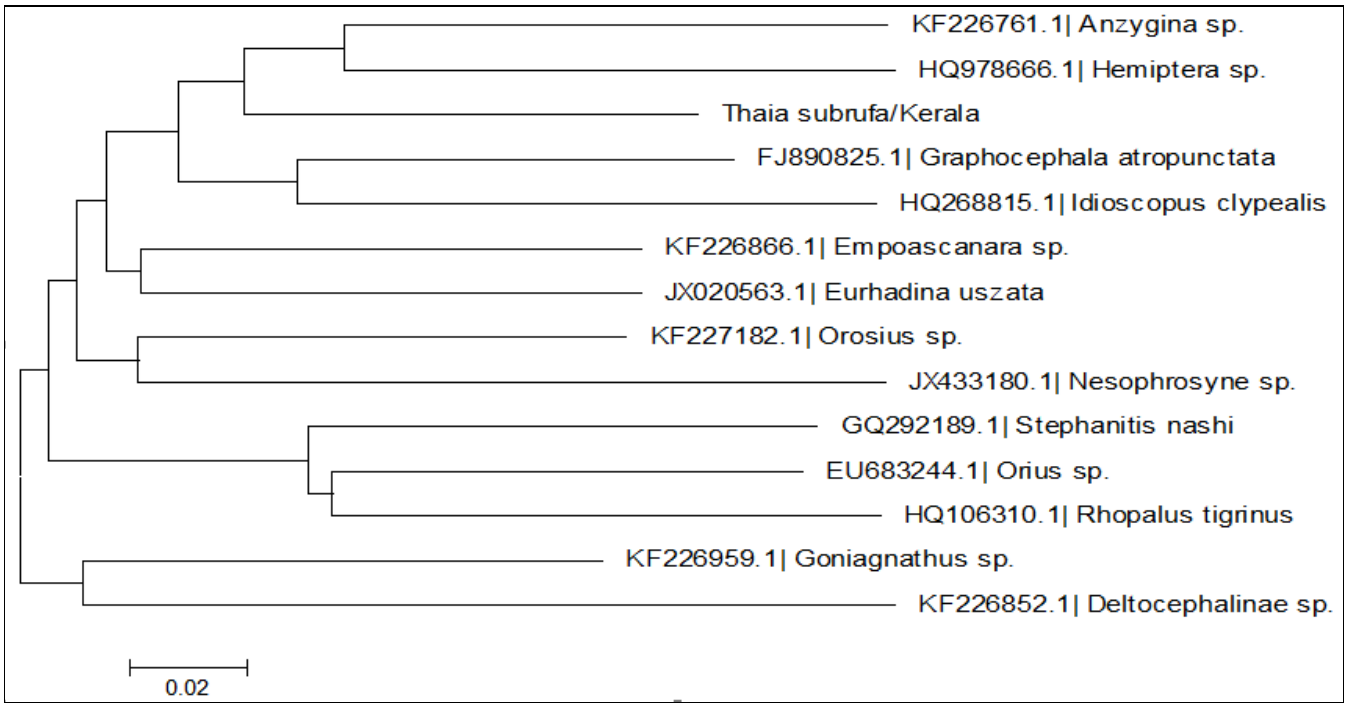

Fig 1: NJ tree constructed using COI gene sequence of *T. subrufa* from Kerala, with related species

T. subrufa displays a branch length of 0.08 when compared to the nearest relative the *Anzygina* species, which have a branch length 0.09. This indicates that the latter evolved earlier from the ancestor. Nucleotide composition summaries are shown in Table 1. The table represents the Molar concentration of DNA nucleotides in the COI region of *T. subrufa* sample from Kerala against closely related organisms of Order Hemiptera. Results showed that the Thymine content was high in all the samples. Adenine was the second predominant in molar concentration next to thymine followed by Cytosine. The average nucleotide frequencies of T, C, A, and G are 36.5, 17.1, 30.8 and 15.6 % respectively.

Table 1: The percentage of nucleotide frequencies in each position of codon of the COI sequence of *T. subrufa* and related species

Species name	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**
LC005702 *Thaia subrufa*	37.2	17.8	29.1	16.0	43	28.1	14.1	14.8	45	9.4	38.6	7.1	24	15.7	34.6	26
FJ890825 *Graphocephala sp.*	40.3	16.5	27.7	15.4	45	25.8	14.8	14.1	49	8.7	36.2	6.3	27	15.0	32.3	26
KF226761 *Anzygina* sp.	38.5	16.2	28.0	17.3	45	27.3	14.1	14.1	43	7.9	37.8	11	28	13.4	32.3	26
HQ978666 Hemiptera sp.	38.5	17.5	27.7	16.2	44	27.3	14.8	14.1	46	10	34.6	9.4	26	15.0	33.9	25
KF226866 *Empoascanara*	38.5	15.6	30.9	15.0	44	26.6	14.1	15.6	46	5.6	45.6	2.4	25	14.3	33.3	27
JX020563 *Eurhadina uszata*	34.6	16.8	34.0	14.7	44	25.8	14.8	15.6	39	7.9	51.2	2.4	21	16.5	36.2	26
KF227182 *Orosius* sp.	35.3	16.8	33.0	14.9	45	24.2	15.6	14.8	38	9.4	48.8	3.9	23	16.5	34.6	26
JX433180 *Nesophrosyne* sp.	35.9	20.4	28.0	15.7	44	26.6	14.8	14.8	42	16	34.6	7.1	22	18.1	34.6	25
GQ292189 *Stephanitis nashi*	37.4	15.2	33.0	14.4	45	25.0	14.1	15.6	44	6.3	48.0	1.6	23	14.2	37.0	26
HQ268815 *Idioscopus*sp.	40.8	15.7	26.4	17.0	45	26.6	14.8	14.1	51	5.5	31.5	11	27	15.0	33.1	25
KF226959 *Goniagnathus* sp.	35.6	16.5	34.8	13.1	45	25.8	14.8	14.1	36	8.7	51.2	3.9	25	15.0	38.6	21
EU683244 *Orius* sp.	36.9	14.4	33.0	15.7	44	26.6	14.1	15.6	43	2.4	51.2	3.1	24	14.2	33.9	28
HQ106310 *Rhopalustigrinus*	32.5	18.1	33.8	15.7	44	25.8	14.1	16.4	29	13	54.3	3.1	24	15.0	33.1	27
KF226852 *Deltocephalinae sp.*	29.6	22.3	31.2	17.0	43	27.3	14.1	15.6	22	22	45.7	10	24	17.3	33.9	25
Average	**36.5**	**17.1**	**30.8**	**15.6**	**44**	**26.3**	**14.5**	**15.0**	**41**	**9.6**	**43.5**	**6.0**	**24**	**15.4**	**34.4**	**25**

4. Discussion

The genetic and phylogenetic relationships among the *T. subrufa* specimen isolated from Kerala were also explored. The sequence identification with NCBI database found that no sequences for COI gene are available for the species *T. subrufa*. This result reveals that sequence generated is novel.

The COI sequence of *T. subrufa* isolated from Kerala showed 19.4% similarity with that of *Anzygina* sp. (Gen Bank Acession No. KF226761) isolated from Barrow Island, Australia. But it is observed a variation in genetic similarity of 18.6% to Hemiptera sp. (Acession No. HQ978666) isolated from Canada. A 0.2% difference is observed between the species when compared to *T. subrufa*. Among the different species of Hemipteran order, Deltocephalinae sp. (Acession No. KF226852) isolated from Australia showed highest variation (27.2%) with *T. subrufa* (Table 2).

Table 2: Percentage of evolutionary divergence of *T. subrufa* from Kerala with related species

Species name	% of divergence
FJ890825 *Graphocephala atropunctata*	18.4%
KF226761 *Anzygina* sp.	19.4%
HQ978666 *Hemiptera* sp.	18.6%
KF226866 Empoas canara	19.7%
JX020563 Eurhadina uszata	19.0%
KF227182 *Orosius* sp.	21.3%
JX433180 *Nesophrosyne* sp.	21.4%
GQ292189 *Stephanitisnashi*	23.7%
HQ268815 *Idioscopusclypealis*	20.4%
KF226959 *Goniagnathus* sp.	21.9%
EU683244 *Orius* sp.	25.3%
HQ106310 Rhopalu stigrinus	26.3%
KF226852 Deltocephalinae sp.	27.2%

Nucleotide composition summaries were obtained and shown in (Table 1). The table represents the Molar concentration of DNA nucleotides in the COI region of *T. subrufa* sample from Kerala against closely related organisms of Hemiptera. Results showed that the Thymine content was high in all the samples followed by adenine and cytosine. The average nucleotide frequencies of T, C A, and G are in the proportion 36.5%, 17.1%, 30.8% and 15.6% respectively. In the nucleotide triplet code, there is strong compulsion in the nucleotide changes in second position of all codons and first position of many codons. Due to the third base degeneracy character of the genetic code third position of many codons and first position of some codons is less constraint. The variations in the strong constraint positions lead to the variations in the amino acid sequence. But the variations in the less constraint position will not affect (silent) the phenotype and these less constrained codon positions evolved at high rate [24]. Sequence based identification has been used in many studies to identify the insect species, the closely related species showed 90% similarity and the distantly related species showed less than 90% similarity in the same gene sequence [25, 26].

5. Conclusion

The present study clearly shows that availability of DNA tools for diversity assessment which will greatly facilitate and complement taxonomic studies. The combination of DNA sequencing data with traditional taxonomy will serve as a model that can be applied across disciplines. It will increase the rate of species identification which will help to deal with rapid identification of pest species where species identification is difficult at the earlier life stage of the pest species. Our result reveal that COI barcoding permit the unambiguous identification of pest species *T. subrufa* the pest of paddy, thus there is need to look for integrated approach in taxonomy for quick identification of this insect biodiversity along the paddy fields.

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