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COI based molecular identification of mango leaf hoppers (Hemiptera: Cicadellidae) in India

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Rapid, accurate and timely identification of invasive pest insects, such as, mango leafhoppers, is important and a challenge worldwide. In this regard, DNA barcoding employing a 658 bp fragment of 5□□' region of the mitochondrial cytochrome oxidase I (COI) gene is an effective tool in addressing the above. In the present study, we developed DNA barcodes for five species of mango leaf hoppers, *viz.*, *Amritodus atkinsoni*, *A. brevistylus*, *Idioscopus clypealis*, *I. nagpurensis* and *I. niveosparsus*, which were collected from Karnataka, India. Our study will help in providing a rapid, convenient and precise tool for species discrimination in mango leaf hoppers, regardless of their life stages and polymorphism.

Keywords: DNA barcoding, mango leafhoppers, mitochondrial cytochrome oxidase I (CO-I)

Introduction

Mango leaf hoppers are notorious sap suckers and are pan tropical in nature, which belong to Cicadellidae. Cicadellideans are ranked as one of the largest families that are ascribed till date[1]. However in India, the most common species of mango leaf hoppers are *Amritodus atkinsoni* Leth., *Idioscopus niveosparsus* Leth. (*I. nitidulus* Walker), *I. clypealis* Leth. and *Amrasca splendens* Ghauri from Kerala; *Busoniominus manjunathi* Viraktamath, *I. anasnyal* Viraktamath and *I. jayshriae* Viraktamath from Karnataka; *A. brevistylus* Viraktamath from South India; and *I. nagpurensis* Pruthi from plain regions of India[2-8]. They cause the most severe and devastating effects since they are monophagous to mango[5,9]. Apart from Indian subcontinent, they attribute their incidence across South East Asia and Papua New Guinea, where major cultivation of mango is carried out. Symptomatically, hoppers infestation on mango results in direct crop loss depending on the population size by premature fruit drop and withering and shedding of flowers and flower buds, thereby reducing the overall yield as well as its marketability. At peak infestation, leaf hoppers excrete copious amount of honey dew, which forms a thin shiny layer on the surface of leaves and in turn facilitates the fungal growth causing sooty leaf and improper photosynthesis. Considering the pest potential and invasiveness, it is necessary to identify them rapidly and accurately at the port of entry in a given time. Though classical taxonomy proves its reliability but has limitations, such as, requirement of adult specimens for morphological analysis, inadequate skilled personals etc. At this juncture, molecular species identification based on mitochondrial cytochrome oxidase I (COI) becomes handy, since the technique is neither dependent on developmental stage nor on gender with high degree of accuracy[10,11].

Molecular markers have paved the way for vital data in species identification and phylogenetic studies. Adjunct with classical taxonomy, molecular markers assist in various aspects like rapid and accurate identification of species at a given time with ease. In the recent past, mitochondrial gene is a choice of use in most of the molecular systematic studies because of its maternal inheritance and reliable interspecific variation at par with other markers[1]. The concept of DNA barcoding is proposed by Hebert *et al*[12]. This method employs a short

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standardized region (658 bp) of mitochondrial cytochrome oxidase I (mtCOI) providing highly effective method of species identification across animal kingdom. Currently, international consortium for barcode of life (iBOL) advocates the use of COI for species identification, as it exhibits reliable inter-specific variation[1]. In the present study, we employed CO-I specific primers for molecular species identification of five mango leaf hoppers species, *viz.*, *A. atkinsoni*, *A. brevistylus*, *I. clypealis*, *I. nagpurensis* and *I. niveosparsus*, which were collected from Karnataka, India.

Materials and Methods

Mango leaf hoppers were collected from various regions in Karnataka, preserved in 70% ethyl alcohol and stored at –20°C until further work.

Individual mango leaf hopper was taken and a small portion of the abdomen was used for DNA extraction. Rest of the specimen was used as voucher specimen and deposited in the Division of Entomology, University of Agricultural Sciences, GKVK, Bangalore. Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method[13], which follows tissue homogenization using liquid nitrogen with subsequent addition of STE buffer [100 mM NaCl, 10 mM Tris HCl (pH 8.0), and 1.0 mM EDTA (pH 8.0)]. The homogenate was incubated at 65°C for 1 h, followed by centrifugation at 8000 rpm for 15 min at room temperature. Ethanol precipitated DNA was dissolved in 20 µL of nuclease free water (Eppendorf, Germany). Quantification was carried out on a fluorometer (DyNa quant 200, Hoefer, San Franciso, CA) according to the standard protocol. Depending on the initial concentration, the DNA samples were further diluted using nuclease free water (Eppendorf, Germany) such that the working solution reaches 20-25 ng/µL concentration. PCR was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells, USA) with the parameters: 94°C for 4 min as initial denaturation, followed by 35 cycles of 94°C for 40 sec, annealing 47°C for 40 sec, extension at 72°C for 45 sec, and 72°C for 20 min as final extension employing the universal CO-I primers: LCO-1490; 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO-2198; 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA-3'[12,14]. The PCR was performed in 25 µL of total reaction volume containing 2.0 µL of DNA template, 10 pico mole of both the primers, 0.25 mM of dNTP mix, 1.5 mM of $MgCl_2$, 1.0 U of Taq DNA polymerase, 2.5 mM of Taq buffer. The amplicons were resolved in 1.0% agarose gel with [10 µg/mL] ethidium bromide and visualized under gel documentation system [UVP]. The PCR products were eluted with Nucleospin extract II kit [MN, Germany] and sequencing was performed using M13 universal primer in both forward and reverse directions in an automated sequencer (ABI Prism 3730 XL DNA Analyzer-Applied Biosystems, USA). Homology search was performed by BLASTX (http://www.ncbi.nlm.nih.gov) and the nucleotide variation in the sequence is determined by sequence alignment editor BioEdit version 7.0.5.3[15]. Neighbor-joining trees were constructed using Kimura-2-parameter (K2P) distance model[16,17] employing MEGA.5.0[18].

Results and Discussion

The COI from all five species (Fig. 1) were successfully sequenced and the comparison of the triplicate sequences for respective mango leaf hopper species showed no mismatches, thus no sequencing errors. Sequence analysis revealed that 69 characters were variable and 74 characters were parsimony informative. No nuclear copies were amplified as indicated by the absence of stop codons within the sequences and the base composition was almost similar with no indels. All sequences generated in this study were deposited to NCBI-GenBank with acc. no.: *I. clypealis* (HQ268815), *I. niveosparsus* (HQ268816), *A. brevistylus* (HQ268817), *I. nagpurensis* (HQ268818) and *A. atkinsoni* (HQ268819). The reliability of the clustering pattern in the trees was determined using the bootstrap test with 1,000 replications employing MEGA 5.0[18] (Table 1). The nucleotide frequencies of these five mango leaf hoppers were 28.30 (A), 40.85 (T), 15.02 (C), 15.84 (G) (cumulative). The base composition of the COI gene fragment was biased toward adenine (A) and thymine (T), which constituted 69.1% of the total. The overall transition (ti)/transversion (tv) bias was found to be (R=) 1.086.

Rapid, accurate and timely identification of invasive insects, such as, mango leaf hopper, is important and challenging, as these particular pests outnumber all other insects in terms of both number and diversity[19]. In this connection, molecular identification employing COI barcoding has an advantage of not being limited by polymorphism,

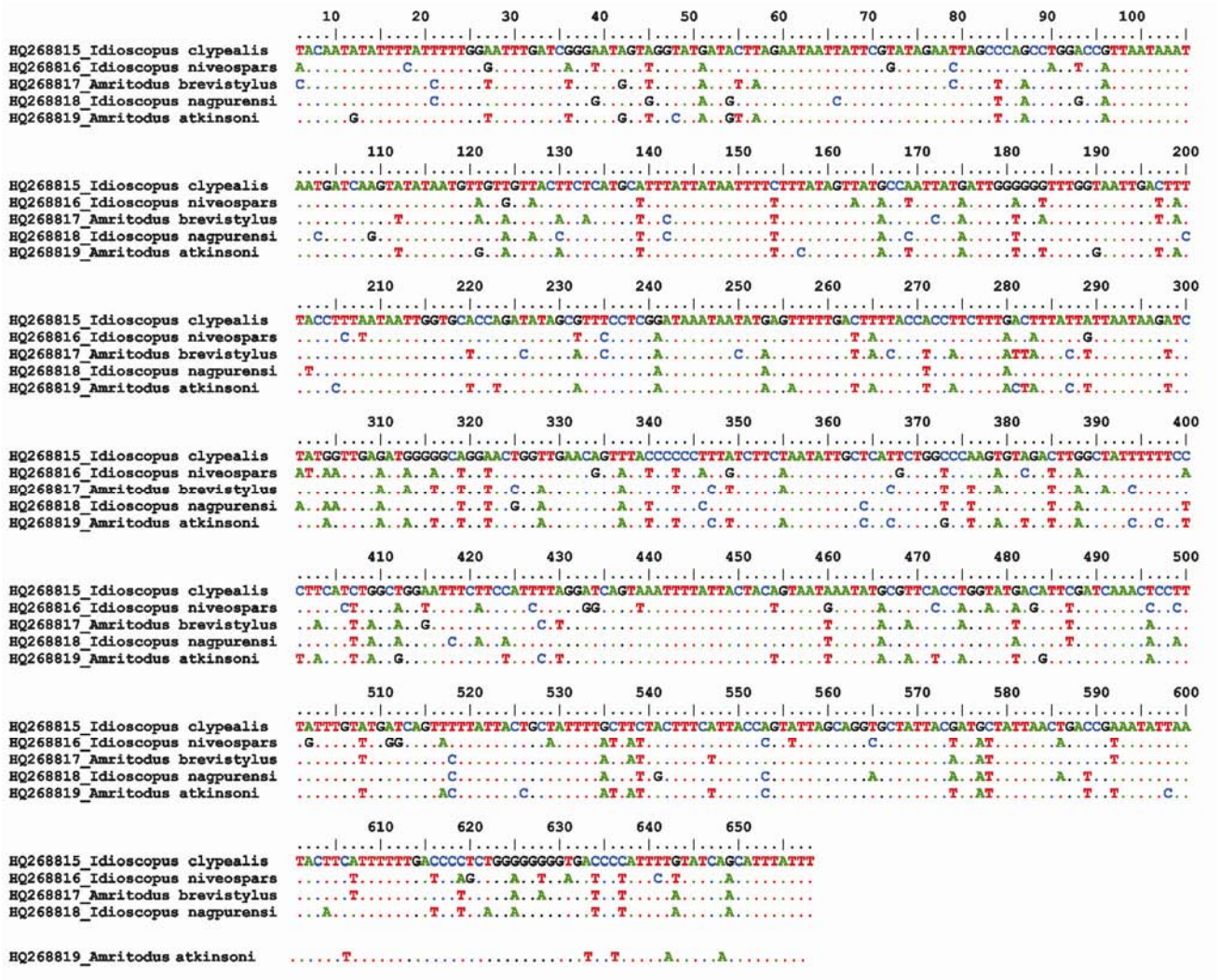

Fig. 1—Consensus sequence of 658 bp from the mitochondrial cytochrome oxidase I (COI) gene for mango leaf hopper species, *viz.*, *I. clypealis*, *I. niveosparsus*, *A. brevistylus*, *I. nagpurensis* and *A. atkinsoni*.

sexual form (asexual/sexual) and life stages of the target species[10]. Apart from these, its versatility is because of the high copy number as compared with nuclear genes in the cell, highly conserved with short intergenic region, maternally inherited and absence of intron[20]. In addition to species identification, COI may be suitably employed to elucidate the prevalence of biotypes[21] and for the discovery of new species[22]. Barcoding is also an invaluable tool when polymorphism is shown by insects, such as *Ceratitis capitata* Wiedemann and *Anastrepha fraterculus* Wiedemann[23].

All the species of mango leaf hoppers employed in the present study were differentiated clearly on the basis of DNA barcodes generated, which proved to be a valuable tool for the identification of these serious insect pests. The whole process complements the classical taxonomy. The Neighbor-Joining (NJ) tree revealed two clades, in which the first clade corresponds to the genus *Idioscopus* and the second clade represents the genus *Amritodus* (Fig. 2). Insect pest management programmes require a clear

Table 1—Maximum composite likelihood estimate of the pattern of nucleotide substitution from five mango leaf hopper species, *viz.*, *I. clypealis*, *I. niveosparsus*, *A. brevistylus*, *I. nagpurensis* and *A. atkinsoni*.

	A	T	C	G
A	-	*9.11*	*3.53*	**8.2**
T	*6.31*	-	**8.87**	*3.35*
C	*6.31*	**22.89**	-	*3.35*
G	**15.46**	*9.11*	*3.53*	-

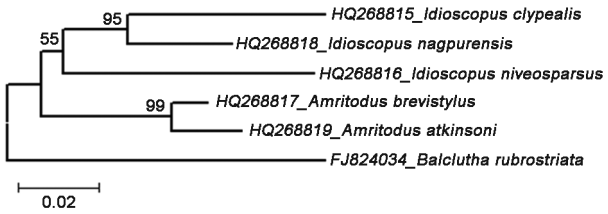

Fig. 2—Neighbor-Joining (NJ) tree with bootstrap support (1000 replicates) showing clusters of five different leaf hopper species, *viz.*, *I. clypealis*, *I. niveosparsus*, *A. brevistylus*, *I. nagpurensis* and *A. atkinsoni*, which were collected from Bengaluru, Karnataka. *Balclutha rubrostriata*, an invasive red-streaked leaf hopper (FJ824034) was used as an out group.

understanding on the pest species in question. In a nutshell, present work will help in providing a rapid, convenient and precise tool for species discrimination in mango leaf hoppers, regardless of their life stages and polymorphism.

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