Molecular Identification and Diversity of Helopeltis antonii and Helopeltis theivora (Hemiptera: Miridae) in India

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MOLECULAR IDENTIFICATION AND DIVERSITY OF *HELOPELTIS ANTONII* AND *HELOPELTIS THEIVORA* (HEMIPTERA: MIRIDAE) IN INDIA

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Abstract

The genus *Helopeltis* (Hemiptera: Miridae) includes some of the major pests of cashew (*Anacardium occidentale* L. [Anacardiaceae]). *Helopeltis* species severely damage tender shoots, inflorescences, immature cashew nuts and associated cashew apples (pseudocarps). In India cashew crop losses of more than 40% caused by *Helopeltis* species are often reported. One of the major limitations in meeting this problem has been the lack of taxonomic knowledge of the *Helopeltis* species complex, its distribution, host range, and the associated parasitoid complex. The two major pests of cashew, *Helopeltis antonii* and *Helopeltis theivora* closely resemble each other. Morphological identification of these species can be facilitated by the use of DNA barcoding of the mitochondrial cytochrome C oxidase subunit 1 (COX-1) and the development of species-specific molecular markers; and we accomplished both of these objectives in this study. These molecular markers also enable non-specialists to identify the above mentioned species in their various life stages, i.e., egg, nymph and adult. The molecular identification of *H. antonii* and *H. theivora* has been corroborated with morphological identification. We examined genetic differences in COX-1 sequences among 12 populations of *H. antonii* collected from different geographic locations in India for the first time. The phylogenetic analysis did not show any geographic or host-associated genetic differences in *H. antonii*, which were collected on different host plants. The present investigation resulted in the development of molecular technology to achieve quick, accurate and timely identification of *H. antonii* and *H. theivora*, a critical factor in understanding the epidemiology of the crop losses in cashew, resistance management and also in quarantine.

Key Words: *Helopeltis antonii, Helopeltis theivora,* COX1, species-specific marker

Resumen

El género *Helopeltis* (Hemiptera: Miridae) incluye algunas de las principales plagas del marañon (cajú) (*Anacardium occidentale* L. [Anacardiaceae]). Las especies de *Helopeltis* causan daño grave a los brotes tiernos, inflorescencias, las nueces inmaduras, y los pseudofrutos de marañon. En la India, a menudo se reportan pérdidas de los cultivos de marañón de más del 40% causado por las especies de *Helopeltis*. Una de las principales limitaciones en la resolución de este problema ha sido la falta de conocimiento taxonómico de las especies en el complejo de especies de *Helopeltis*, su distribución y rango de hospederos, asi como el complejo de parasitoides asociados. Las dos principales plagas de marañon, *Helopeltis antonii* y *Helopeltis theivora,* se parecen mucho. Se puede facilitar la identificación morfológica de estas especies por el uso del código de barras del ADN de la subunidad 1 mitocondrial del citocromo oxidasa C (COX-1) y el desarrollo de marcadores moleculares especificos al nivel de especies; en este estudio hemos logrado ambos objetivos. Estos marcadores moleculares también capacitan a los no especialistas para identificar las especies mencionadas en sus diversas etapas de la vida, es decir, el huevo, ninfa y adulto. La identificación molecular de *H. antonii* y *H. theivora* ha sido corroborada con la identificación morfológica. Se analizaron las diferencias genéticas en las secuencias de COX-1 en 12 poblaciones de *H. antonii* recolectadas en diferentes lugares geográficos en la India por primera vez. El análisis filogenético no mostró diferencias genéticas según el sitio geográfico o el hospedero asociado para *H. antonii*, que fue recolectado sobre diferentes plantas hospederas. La presente investigación resultó en el desarrollo de la tecnología molecular para lograr la identificación rápida, precisa y oportuna de la *H. antonii* y *H. theivora*, que es un factor crítico en la comprensión de

la epidemiología de las pérdidas de cosechas en el marañón, el manejo de la resistencia y también su cuarentena.

The tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) is one of the major pests of cashew, *Anacardium occidentale* L. (Sapindales: Anacardiaceae) in old world tropics, which include India, Brazil, Vietnam, Cote d'Ivoire, Tanzania, Guinea Bissau, Benin, Nigeria, Mozambique and Indonesia (Stonedahl, 1991). *Helopeltis antonii* causes an estimated yield loss of about 40% on cashew (Sundararaju & Sundarababu 1999). In addition to cashew, this pest also infests cocoa, *Theobroma cacao* L. (Malvales: Malvaceae); tea, *Camellia sinensis* L. (Ericales: Theacaeae) (Stonedahl, 1991), and neem, *Azadirachta indica* (Sapindales: Meliaceae) (Onkarappa & Kumar, 1997). Yet of late another mirid, *Helopeltis theivora* Waterhouse, which was a minor pest mainly attacking cocoa and tea, has become a serious pest of cashew in India. Nymphs and adults suck the sap from the young leaves and shoots of the host plants (Karmawati 2007) to cause discolored necrotic area or lesion around the point of entry of the labial stylets into the plant tissue (Stonedahl, 1991).

Stonedahl (1991) observed that many oriental species of *Helopeltis* are often misidentified due to the variations in size, coloration and the scutellar process. In this regard, identification based on morphology falls short, because it requires adult specimens, and the availability of advanced expertise in taxonomy. Identification is further complicated by the lack of morphological keys for immature stages, i.e., eggs, larvae and pupae. The problematic immature stages are most often encountered in import consignments. Thus, molecular species diagnostics based on COX-1 have become handy (von Dohlen et al. 2006), because they are not limited by developmental stage or gender (unpublished data). The mitochondrial cytochrome oxidase-I (COX-1) exhibits maternal inherited characteristics and reliable interspecific variation as compared to other markers (Savolainen et al. 2005), and therefore this same marker has been employed in molecular systematics of insects (Simon et al. 1994). The concept of DNA barcoding of species using mitochondrial cytochrome oxidase I gene was first reported by Hebert et al. (2003). Apart from this, various molecular markers have been employed for species identification and molecular phylogeny studies of insects, viz., Cytochrome b, 16S rRNA (von Dohlen & Moran 2000), 18S rRNA, 28S rRNA, 5.8S rRNA, ITS-Internal Transcribed Spacers, and EF1α (Elongation Factor) (Ji et al. 2003). Yet another method of resolving identification of closely related species is by developing species-specific primers producing specific amplicons.

Species-specific marker helps in discrimination of closely related species through the polymerase chain reaction, followed by agarose gel electrophoresis, and could be effectively used by a non-specialist in species diagnosis with a little training. Therefore in the present investigation, we sought to develop species-specific markers for *H. antonii* and *H. theivora* based on COX-1 partial sequences; and further carry out or the first time molecular diversity analyses employing COX-1 partial sequences for *H. antonii* to elucidate if biotypes or cryptic species exist.

Materials and Methods

Females of both *H. antonii* and *H. theivora* were collected with long glass tubes from cashew plantations at the Directorate of Cashew Research (DCR), Puttur, India (12°45'N/75°1'E). The females were allowed to lay eggs on potted cashew seedlings in the laboratory, and the seedlings with eggs were maintained in the laboratory. Seedlings were enclosed in perforated tubular transparent polyester film (175 μ thick; cage size: 30 × 7.5 cm). Caging was done from the fifth to the seventh d after oviposition to prevent migration of nymphs. Each seedling was labeled with the oviposition date and number of eggs deposited. Immediately after hatching the nymphs were transferred to aluminum nymphal rearing cages (Sundararaju & John 1992) as described below.

Four glass vials (5 mL capacity) were fixed on a small aluminum stand, which consisted of an aluminum plate (15 × 15 × 20 cm; 18 gauge) and a handle of 15 cm height fixed at the center with an adhesive (aluminum cage size: 15 × 15 × 20 cm;18 gauge). The rearing cage had 2 cloth sleeves to facilitate the removal of adults after final molting. Specimens at various stages, viz., eggs, nymphs and adults, were collected and preserved in 70% ethanol, and samples were preserved at -20 °C until needed. For molecular diversity studies, *H. antonii* and *H. theivora* were collected from different host plants and geographical locations in India (Table 1), and preserved in 70% ethanol at -20 °C until further use. Morphological identifications of *H. antonii* and *H. theivora* were carried out according to Stonedahl (1991) prior to molecular studies. In addition to these 2 species, closely related *Helopeltis* spp., viz. *H. bradyi* and *Pachypeltis maesarum* (Kirkaldy), were collected from DCR, Puttur and morphologically identified.

Table 1. Details of the specimens for which COX-1 markers were sequenced in the current study.

Name of the insect	Voucher number	Host plant	Site of collection	GenBank accessions
H. antonii	DCR-01	Cashew	Puttur, Karnataka	HM 142602
H. antonii	DCR-02	Cashew	Pelicode, Kerala	HM 142603
H. antonii	DCR-03	Cashew	Madakkathara, Kerala	HM 142604
H. antonii	DCR-04	Cashew	Chintamani, Karnataka	HM 142605
H. antonii	DCR-05	Cashew	Paria, Gujarat	HM 142606
H. antonii	DCR-06	Cashew	Vengurle (Inorg. farm), Maharashtra	HM 142607
H. antonii	DCR-07	Cashew	Vengurle (Org. farm), Maharashtra	HM 142608
H. antonii	DCR-08	Cocoa	Vittal, Karnataka	HM 142609
H. antonii	DCR-09	Guava	IIHR, Bangalore	HM 142610
H. antonii	DCR-10	Neem	Chintamani, Karnataka	HM 142611
H. antonii	DCR-11	Cashew	Kasaragod, Kerala	HM 990966
H. antonii	DCR-12	Cocoa	Puttur, Karnataka	HM 990967
H. theivora	DCR-20	Acalypha	Payyannur, Kerala	HM 142613
P. maesarum	DCR-32	Cashew	Puttur, Karnataka	HM142612

DNA Isolation and Polymerase Chain Reaction

To isolate the genomic DNA using the modified CTAB method, an individual mirid was taken and a small part its abdomen was used (Saghai Maroof et al. 1984). The rest of the insect body was used as the specimen voucher in the Division of Biotechnology, Indian Institute of Horticultural Research, Bangalore. Briefly, a part of the abdomen was ground with 1.0 ml of cetyl trimethyl ammonium bromide buffer (CTAB) 2%, 100 mM Tris-HCl (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol (added just prior to use) and suspended in the same buffer. The suspension was incubated at 65 °C for 2 h and then an equal volume of chloroform: isoamylalcohol (24:1) was added. The suspension was centrifuged at 10,000 rpm for 10 min at 8 °C. The upper aqueous layer was transferred to a fresh micro centrifuge tube taking care not to disturb the middle protein interface. DNA was precipitated by adding an equal volume of ice-cold 95% ethyl alcohol. The precipitated DNA was spun at 10,000 rpm and the resultant DNA pellet was washed with 70% ethanol and dissolved in 50 µL DNase-, RNase- and Protease- free molecular biology water (5 PRIME). Extracted DNA was further purified for free of RNA contaminants by addition of 10 µL/100 µL of RNase. The intact genomic DNA was visualized using a 0.8% agarose gel and quantified using a fluorometer (DyNa quant 200, Hoefer, www.hoeferinc.com/) following standard procedures. Depending upon the concentration, the DNA samples were diluted with sterile water to get a working solution of 20-25ng/µL. A portion of the total DNA was preserved in glycerol (10%) in -80 °C for future reference purpose.

Polymerase Chain Reaction was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells) with the following cycles; 94 °C for 4 min as initial denaturation followed by 35 cycles of 94 °C 30 s, 48 °C for 45 s, 72 °C for 45 s and 72 °C for 20 min as final extension. The universal barcode primer by Hebert *et al*., (2003) (LCO - 5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3'; HCO- 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3') specific to mitochondrial cytochrome oxidase I (COX-1), which results in the amplification of approximately 700 bp fragment were used in the present study. PCR was performed in 25-µL total reaction volume containing 20 picomoles of each primer, 10 mM Tris HCl (pH-8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5U of Taq DNA polymerase (Fermentas Life Sciences). The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10µg/mL) and visualized in a gel documentation system (UVP). For the species-specific primers mentioned in Table 1, PCR mix and PCR cycle parameters were the same except for annealing temperature, viz., 63 °C for 45 s for both *H. antonii* and *H. theivora.*

Molecular cloning and Sequencing

The PCR amplified fragments were eluted using Nucleospin® *Extract II according to the manufacturer's protocol (MN, Germany) and ligated into the general purpose-cloning vector,* InsT/Aclone (Fermentas GmBH, Germany) according to the manufacturer's protocol. Five micro liters of the ligated vector was cloned into 200 µL of competent Escherichia coli (DH5α) cells by heat treatment at 42 °C for 45 s and the whole content was transferred into a tube containing 800 µL of SOC (tryptone-2% w/v, yeast extract-0.5% w/v, NaCl-8.6mM, KCl-2.5mM, MgSO4-2.0mM, Glucose-20mM in 1000 mL water, pH7.0) and rotated at 150 rpm, 37 °C for 1 h; 200 µL of the above culture was spread on Luria-Bertani agar (LBA) (tryptone-10 g, yeast extract-5 g, NaCl-5 g, agar-15 g in 1000 mL of water, pH 7.0) containing ampicillin (100 g/mL), IPTG (4 mg/mL) and X-gal (40 mg/mL) and were incubated at 37 °C for 16 h. Blue/white selection was carried out and all the white colonies (colonies harboring

the insert) were maintained on LBA containing ampicillin (100 mg/mL), incubated at 37 °C overnight and stored at 4 °C until further use. Plasmids were prepared from the overnight culture of the positive colonies cultured in LB broth (enzymatic casein- 10 g, yeast extract-5 g, NaCl-5 g in 1000 water, pH 7.0) using modified alkali lysis method (Birnboim & Dolly, 1979). Plasmids were resolved in 1.0% agarose gel, stained with ethidium bromide (10 mg/mL) and visualized in a gel documentation system. Clones that had 2.3 kb as compared to control plasmid (1.8 kb) were selected for sequencing. For the purpose of sequencing, plasmids were isolated using GeneJET™ Plasmid Miniprep Kit (Fermentas, Germany) according to manufacturer's protocol, from overnight cultures of the 5 randomly selected clones multiplied in LB broth. Sequencing was carried out in an automated sequencer (ABI Prism 310; Applied Biosystems, USA) using M13 universal primers both in forward and reverse directions. Homology search was carried out using BLAST (http://www.ncbi.nlm.nih.gov), and the differences in COX-1 sequences of *H. antonii* and *H. theivora* were determined using the sequence alignment editor 'BioEdit'. Sequences for *H. antonii* and *H. theivora* were deposited with the NCBI database. For the development of species-specific markers for *H. antonii* and *H. theivora*, 8 sets of forward and reverse primers were synthesized based on the variable regions in the aligned sequences of both these species. The primers thus designed were tested both on identified *H. antonii* and *H. theivora* (2 specimens each of *H. antonii* and *H. theivora* in various developmental stages viz. eggs, nymphs and adults) and 3 each of unidentified adult specimens (later specimen vouchers were identified and results were corroborated) collected from cashew at DCR, Puttur. The PCR amplified fragments resulting from species-specific markers for *H. antonii* and *H. theivora* were further cloned, sequenced and analyzed as above. In order to validate the species-specific marker specificity, we carried out the cross amplification through PCR for both the developed primers with different species of mirids, viz., *H. bradyi* and P. *maesarum*, which were morphologically identified and collected from DCR, Puttur, Karnataka.

Results

Mitochondrial Cytochrome Oxidase I (COX-1) sequencing yielded a 709 bp fragment for *H. antonii* and *H. theivora*. A comparison of the replicate sequences for all mirids sequences showed no mismatch, indicating there were no sequencing errors. Pair wise alignment of HM142602 and HM142613 showed that there were variations in 72 nucleotides out of 658 bp, amounting to a 10.94% difference between *H. antonii* and *H. theivora* (Fig. 1).

A total of 10 primer sets were identified each for *H. antonii* and *H. theivora* based on the variation in the sequences. Out of 10, one primer set viz. Rebi AF & Rebi AR and Rebi-TF & Rebi-TR, could successfully identify *H. antonii* and *H. theivora,* respectively (Table 2 and Fig. 2). These species-specific markers were verified by sequenc-

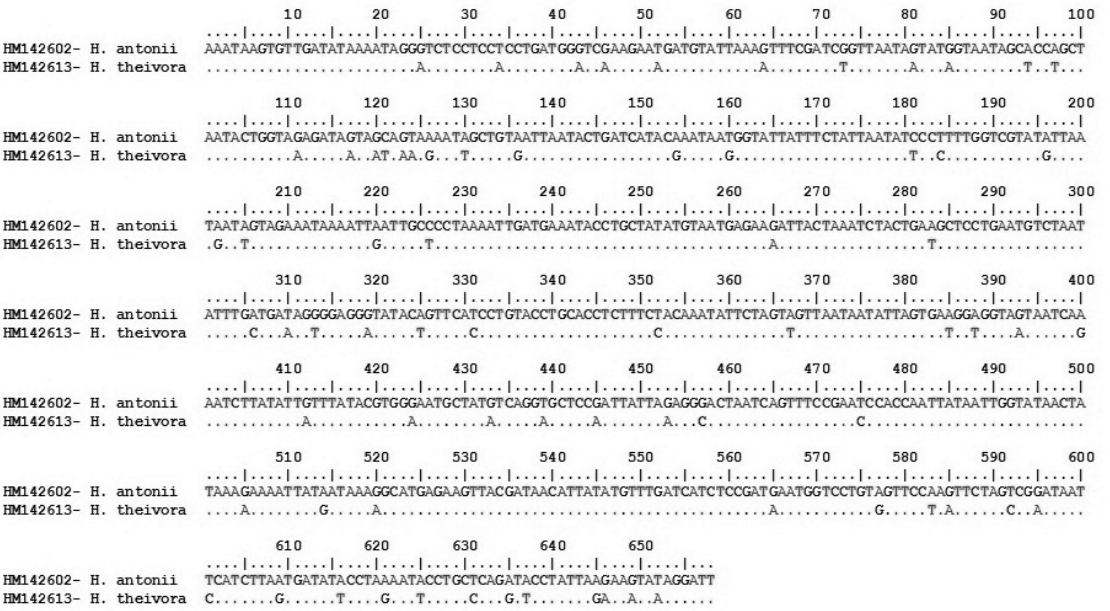

Fig. 1. COX-1 sequence comparison for *Helopeltis antonii* and *H. theivora* from the cytochrome oxidase I (COX-1) gene showing difference. Analyses performed using Bioedit v.7.0.

TABLE 2. SPECIES-SPECIFIC MARKERS OF *HELOPELTIS ANTONII* AND *H. THEIVORA*.

Species	Primer	Binding Region (bp)	Product size (bp)
H. antonii	Rebi-AF- 5'- GATGATAGGGGAGGGTATACAGTTCAT -3'	305-331	347
	Rebi-AR- 5'- ATACTTCTTAATAGGTATCTGAGCAGGT -3'	625-652	
H. theivora	Rebi-TF- 5'- AAGATAATAATAAAAGAATTGCTGTG -3'	111-136	541
	Rebi-TR- 5'- TTATTTTCTAATAGGAACCTGGGCAG -3'	627-652	

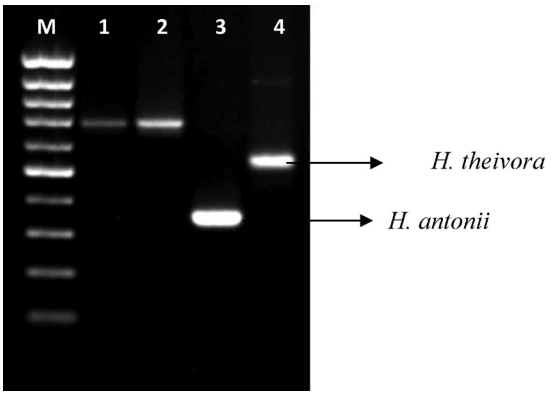

Fig. 2. Validation of species-specific markers for *Helopeltis antonii* and *H. theivora*. Legend: M - 100 bp DNA ladder (Thermo Scientific, Fermentas), 1 & 2 - PCR Amplified product (COX-1) *H. antonii* and *H. theivora* respectively, 3 - *H. antonii*- specific marker, 4 - *H. theivora*- specific marker.

ing and had product sizes of 347bp and 541bp for *H. antonii* and *H. theivora*, respectively, for both identified and test specimens.

BLAST search for the sequences obtained using species-specific marker for both *H. antonii* and *H. theivora* showed the highest hits for the respective species. Hence these species-specific markers can be useful to identify *H. antonii* and *H. theivora* in various life stages, viz., egg, nymph or adults, without the aid of sequencing. However the applicability of these primers to other populations of *H. antonii* and *H. theivora* within and outside India depends on the variation in the nucleotide sequences both in forward (305-331 and 111-136 for *H. antonii* and *H. theivora,* respectively) and reverse primer binding regions (625-652 and 627-652 for *H. antonii* and *H. theivora,* respectively).

Comparisons of forward and reverse primer binding regions for *H. antonii* (HM142602) with other 11 existing COX-1 (658bp) deposits showed that there were no variations in any of the GenBank accessions. In case of *H. theivora,* only one GenBank accession was available (present study), from which the primers developed.

The validation of the species-specific markers which were developed in the present study were carried out employing PCR using both these primers and the genomic DNA isolated from the closely related mirid spp. viz., *H. bradyi* and *P. maesarum*, which were morphologically identified and collected from the Directorate of Cashew Research, Puttur. None of the PCR reactions produced any amplification, including a non-specific amplicon, where the PCR mix and PCR cycling conditions were kept same, including the annealing temperature viz. 63 °C for 45 s for all reactions.

Considering the pest potential, it is necessary to analyze the molecular diversity in *H. antonii* and *H. theivora* in order to elucidate the presence of biotypes/cryptic species, if any. In this regard, *H. antonii* samples were collected from 12 different geographical locations in India and corresponding COX-1 sequences were deposited in NCBI-GenBank (Table. 1). The BLAST search of the above COX-1 sequences showed that all of them had maximum similarity only for the genus, *Helopeltis*. Multiple sequences of COX-1 were aligned using Clustal-X (Version 1.83, Thompson et al 1997) with default settings. The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for *H. antonii* sequences was performed using MEGA 4.0 (Tamura et al. 2007). The reliability of the clustering pattern in the trees was determined by the bootstrap test, with 1000 replications. Each entry showed the probability of substitution from one base (row) to another base (column) instantaneously (Table 3). The rates of different transitional substitutions are indicated in bold, while transversional substitutions are shown in italics. The *H. antonii* nucleotide frequencies were 0.331 (A), 0.349 (T), 0.18 (C) and 0.14 (G). The base composition of the COX-

TABLE 3. MAXIMUM COMPOSITE LIKELIHOOD ESTIMATE OF THE PATTERN OF NUCLEOTIDE SUBSTITUTION FROM 12 POPULATIONS OF *HELOPELTIS ANTONII*.

	A	**T**	**C**	**G**
A	—	*5.08*	*2.62*	**5.6**
T	*4.82*	—	**17.69**	*2.03*
C	*4.82*	**34.33**	—	*2.03*
G	**13.3**	*5.08*	*2.62*	—

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

1 gene fragment was biased toward adenine (A) and thymine (T), which together constituted 68.0% of the total. The overall transition (ti)/ transversion (tv) bias of *H. antonii* nucleotide sequence was R= 1.632, where $R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]$. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the datasets.

Comparison of the COX-1 sequences of 12 populations of *H. antonii* showed 0-2% variation among them (Fig. 3). Maximum Parsimony (MP) and Maximum Likelihood (ML) analysis were performed with PAUP 4.0b10 (Swofford 1998), using the heuristic search procedure with 1000 random additions of sequences and 10 trees held at each pseudo-replicate and the tree bisection reconnection (TBR) branch swapping method, all characters were treated as unordered and equally weighted for the MP analysis. For Maximum Likelihood (ML) analysis, the best fit model of nucleotide evolution was evaluated using Modeltest v.3.7 (Posada & Crandall, 1998) based on the Akaike Information Criterion (AIC). The HKY model of nucleotide substitution was selected for the ML analysis. Bootstrapping of the MP analysis (1000 replications) and ML analysis (100 replications) was also implemented in the heuristic search procedure with a maxtree setting of 100 trees.

The phylogram for the listed 12 NCBI accessions (Table 1 and Fig. 4) of *H. antonii* revealed that there were 2 clades: Clade I, associated with the populations of *H. antonii* collected from Madakkathara (cashew) and Puttur (cocoa) and the Clade II, which is associated with rest of the populations of *H. antonii* collected from different geographical locations. On the other hand the analysis did not reveal any host- associated genetic differentiation in *H. antonii* as observed in *Thrips tabaci* Lindeman (Brunner et al. 2004) and *Pentalonia* spp. aphids (Foottit et al. 2010).

Discussion

In case of Miridae, little is known about the genetic background (Latip et al. 2010). Of the 41 species of *Helopeltis* recorded in the old world tropics, only 3 species are confined to India viz. *H. antonii*, *H. theivora* and *H. bradyi* (Stonedahl 1991). Stonedahl (1991) reported that many species of Miridae are similar in coloration and general morphological structures and accurate identification is possible only by examining the male and female genital structures. Hence molecular identification using species-specific markers is an advantage where there is morphological polymorphism is absent in the target species. Development of species-specific markers for *H. antonii* and *H. theivora* is of immense value to identify these pests in various life stages, viz., egg, nymph or adult. Even after 4-5 decades of research no molecular identification or diversity analysis has been reported on mirids.

Our studies demonstrate the development of species-specific markers, a significant step in identification of *H. antonii* and *H. theivora* at various life stages that occur on different crops in India. The major criteria taken into consideration while developing species-specific markers for *Helopeltis* species are intra and inter-specific variations (Bayar et al. 2002; Brunner et al. 2004).

Helopeltis is typically a low density pest with high damage. Plants are strongly affected by the toxin injected during feeding by nymphs and adults. However the chemical nature, potency of the toxin and the extent of damage by different species of *Helopeltis* vary depending on the species. Similarly there is a need to monitor natural enemies of *Helopeltis*, which requires their proper identification to the species level. Perusal of literature on chemical control of mirids reveals that endosulfan was the most effective. In the recent past, however the allegedly ecocidal episode involving endosulfan use in cashew production in the Kasargod and Kannur Districts of Kerala, has initiated an unabated debate on these issues, the related environmental-impact assessment, the 3 R's (Resistance, Residues, and Resurgence), and especially the human health hazards. In the quest to replace heavy reliance on endosulfan with an ecologically-based pest management strategy, it is essential to begin with the identification of the pest and beneficial insects especially in the cashew and cocoa ecosystems, where endosulfan has been used heavily. This study stands as a connecting link in the further development of such a identification tool. Indeed it is imperative that the tool can help identify *H. antonii* and *H. theivora* in all of their life stages; eggs, nymphs and adults.

Conclusion

This study represents a first attempt at molecular identification and diversity characterization of *Helopeltis* mirid bugs using the mitochondrial COX-1 DNA sequence. For the first time barcoding of both *H. antonii* and *H. theivora* has been successfully carried out by employing COX-1 primers; and from the same sequences, species-specific markers have been developed for the identification of the above 2 species independent of sexual gender or life stage. Thus this study represents a starting point for the molecular identification of *Helopeltis* mirid bugs. Future management of these pests can now be more accurately implemented with the accurate identification these and other mirid species.

Fig. 3. Consensus sequence of 658 bp from the mitochondrial cytochrome oxidase I (COX-1) gene for *Helopeltis antonii* populations collected from different geographical locations in India. Dots indicate nucleotides identical throughout the species compared.

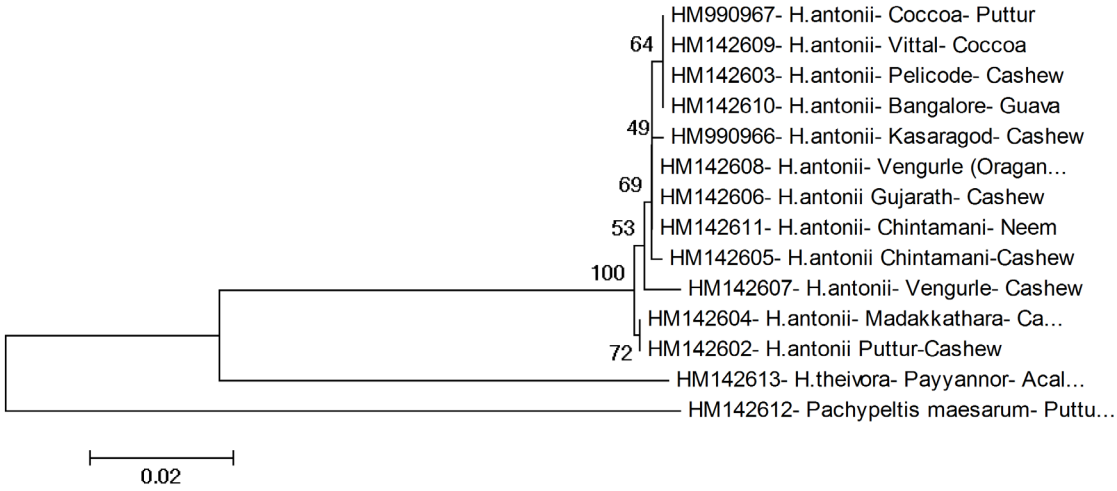

Fig. 4. Maximum Parsimony (MP) tree (MEGA. 4.0) with bootstrap support (1000 replicates) showing clustering of *Helopeltis antonii* for COX-I sequences. *Helopeltis theivora* (HM142613) and *Pachypeltis maesarum* (HM142612) were used as out groups.

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