DOI : <http://doi.org/10.22438/jeb/42/2/MRN-1322>

Development of species-specific marker and molecular diversity of *Conogethes punctiferalis* and *Conogethes sahyadriensis* (Lepidoptera: Crambidae)

V. Kammar¹, D. Sagar^{2*}, R.K. Chandel², P.R. Shashank² and A.K. Chakravathy³¹Department of Entomology, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra, Bangalore-560 065, India²Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India³Division of Entomology and Nematology, ICAR-Indian Institute of Horticultural Research, Bangalore-560 089, India*Corresponding Author Email : garuda344@gmail.com

Received: 06.11.2019

Revised: 29.07.2020

Accepted: 24.10.2020

Abstract

Aim: The aim of this study was to develop species specific marker for quick identification of two *Conogethes* species and to study their molecular diversity.

Methodology: Species-specific markers were developed using mitochondrial cytochrome oxidase 1 (COI) partial gene sequences of *Conogethes punctiferalis* and *C. sahyadriensis* and they were validated. Phylogenetic tree was constructed using MEGA X program, tree robustness was evaluated by bootstrapping with 1000 replicates with all sequences of *C. punctiferalis* and *C. sahyadriensis*.

Results: Bioinformatic analysis of *C. punctiferalis* and *C. sahyadriensis* COI sequences revealed that they have sufficient genetic variability to develop species specific marker. The COI based species-specific markers amplified an expected fragment size of 333 bp for *C. punctiferalis* and 522 bp for *C. sahyadriensis*, which clearly differentiate these two species.

Interpretation: The species specific marker developed in this study will be useful in quick identification of *C. punctiferalis* and *C. sahyadriensis*. The use of COI gene as a marker can provide a better estimate of genetic diversity, as it is maternally inherited and can be helpful to understand evolutionary processes.

Key words: *Conogethes punctiferalis*, *C. sahyadriensis*, MtCOI, Molecular diversity, Species-specific marker

How to cite : Kammar, V., D. Sagar, R.K. Chandel, P.R. Shashank and A.K. Chakravathy: Development of species-specific marker and molecular diversity of *Conogethes punctiferalis* and *Conogethes sahyadriensis* (Lepidoptera: Crambidae). *J. Environ. Biol.*, **42**, 271-279 (2021).

Introduction

The shoot and fruit borers of genus *Conogethes* are lepidopteran pests infesting developing shoots and fruits. The genus *Conogethes* Meyrick, (1884) (Lepidoptera: Crambidae) is a large, taxonomically complex taxon of crambids distributed throughout the Oriental, Australian and tropical Asian regions (Shaffer *et al.*, 1996, Inoue and Yamanaka 2006). *Conogethes punctiferalis* (Guénee) commonly called as yellow peach moth is one such species. Larvae of this moth are considered polyphagous attacking more than 120 wild and cultivated plants (Sekiguchi 1974). Type locality of *C. punctiferalis* is India and the species complex of *Conogethes* is poorly understood. Hampson (1896) reported 20 species of *Dichocrocis* and for distinguishing the species of *Conogethes* he studied the characters of wing venation and arrangements of black spots on the wings which are now considered of little value.

In absence of precise identification of *C. punctiferalis*, Koizumi (1960) in Japan for the convenience identified two types of *C. punctiferalis* viz., the Fruit Tree Type and Conifer Type from angiosperms and Pinaceae gymnosperms, respectively. Most of the studies on the *Conogethes* moths are in progress in Japan, China and Australia, and the species have been barcoded (Armstrong, 2010). There is a dire need for similar work on the Indian species of *Conogethes* considering their economic importance. Similarly, *C. punctiferalis* larvae in India infesting castor (*Ricinus communis* L.) and cardamom (*Elettaria cardamomum* Maton) have been identified as two different lineages based on mass-rearing techniques (Chakravarthy *et al.*, 1991). Subsequently, Shashank *et al.* (2014a,b) accurately indicated differences in morphology, morphometry and molecular identification of male and female genitalia, larvae and pupae and in behaviour of above two species. Effect of interbreeding on offsprings between the two types of *C. punctiferalis* was also determined. Achille Guenee first described *C. punctiferalis* in 1854 and Sir George Hampson in 1896 included all species under the genus *Dichocrocis* based on morphological features. Since then *Conogethes* populations feeding on castor and cardamom have been included under the species *punctiferalis*. But in 2018, a new species *C. sahyadriensis* feeding on cardamom was delineated from closely related species of *Conogethes* (Shashank *et al.*, 2018).

Selected molecular markers viz., Cytochrome b, 16S rRNA, 18S rRNA, 28S rRNA, 5.8S rRNA, internal transcribed spacers, elongation factor-1 α , mitochondrial cytochrome oxidase-I (mtCOI) (Von Dohlen and Moran, 2000, Ji *et al.*, 2003, Raboudi *et al.*, 2005) have been employed by researchers for species identification and molecular phylogeny studies in *Conogethes* spp. Mitochondrial genes, because of maternal inheritance and reliable inter-specific variation (Savolainen *et al.*, 2005), have been widely employed in studying molecular insect systematics (Simon *et al.*, 1994). This is a first attempt to develop species-specific markers of *Conogethes* spp. in India. In the current study, molecular diversity analyses were carried out deploying mitochondrial (mitochondrial cytochrome oxidase-1) gene. The objective of the present study was to develop species-specific markers for lepidopteran pest, *C. punctiferalis* and *C. sahyadriensis* based on mtCOI for life-stage independent identification and determination of phylogenetic relationship.

Materials and Methods

Stock cultures of two species of *Conogethes* populations were maintained in the laboratory at 23 $\pm$ 2°C, 70-85% relative humidity and 16 h light and 8 hrs dark period in Department of Entomology, UAS, GKVK, Benagaluru. Morphological identification of *C. punctiferalis* and *C. sahyadriensis* were carried out according to Shashank *et al.* (2018). For developing species-specific markers, the *C. punctiferalis* and *C. sahyadriensis* larvae were collected from castor (*R. communis*) and cardamom (*E. cardamomum*), in Karnataka, South India. For molecular diversity studies, *C. punctiferalis* and *C. sahyadriensis* larvae were collected on select host plants from different locations in India (Table 1, 2) and samples were preserved in ethyl alcohol and stored at -20°C.

Total DNA was isolated by DNeasy blood and tissue kit (QIAGEN GmbH, Germany) from individuals of *Conogethes* collected from diversified locations in India. The original specimens were preserved as voucher specimens in the Entomology Division, ICAR-Indian Agricultural Research Institute, Pusa campus, New Delhi. For development of species-specific marker and molecular diversity analyses, mitochondrial cytochrome oxidase-I (COI) gene was used. The genomic DNA was used as template in polymerase chain reaction (PCR) to amplify COI gene region with primers LCO1490 (F) 5' GGT CAA CAAATC ATAAAG ATA TTG G-3' and HCO 2198 (R) 5' TAAACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer *et al.*, 1994). Polymerase chain reaction was carried out in a thermal cycler (C1000, Bio-Rad, USA) reaction with the following cycling parameters; initial denaturation at 94 °C for 4 min, 35 amplification cycles [94°C for 1 min, 48°C or optimal annealing temperature (Tm °C) for 1 min, 72 °C for 1 min], followed by a final extension step at 72 °C for 5 min. PCR was performed in 25 μ l total reaction volume containing 12.5 μ l of Thermo Scientific Dream Taq PCR Master Mix (2X), 10 μ M of primer (1 μ l each), nuclease free water (8.5 μ l) and DNA template (2 μ l).

The amplified products were electrophoresed on 1.0% agarose gel, stained with ethidium bromide with 100 bp DNA ladder (Invitrogen, Thermo Fisher Scientific, USA) and visualized in a UV gel documentation unit (Alpha Imager HP, USA). For the species-specific primers, as mentioned in Table 3, PCR mix and PCR cycling parameters were the same, except for the annealing temperature viz., 40 °C for 60 sec. The PCR amplified single band was purified using a QIAquick PCR purification kit (QIAGEN GmbH, Germany). Purified PCR products were sequenced in both forward and reverse directions with an automated sequencer (ABI Prism® 3730 XL DNA Analyzer; Applied Biosystems, Maryland, USA). After obtaining the sequences, they were aligned using sequence alignment editor BioEdit version 7.0.5.3 (Hall 1999) and the homology search was carried out using BLAST (<http://www.ncbi.nlm.nih.gov>) to confirm the species identity. Sequences of *Conogethes* populations were deposited with the National Center for Biotechnology Information (NCBI) database and the accessions numbers were obtained (Table 1, 2). In order to develop the species-specific primer for *C. punctiferalis* and *C. sahyadriensis*, the differences in COI sequences of both the species were determined using the sequence alignment editor BioEdit version 7.0.5.3 (Hall, 1999). Five sets of forward and reverse

primers were synthesized based on the variable regions in the aligned sequences of the same. The primers thus designed were validated both on identified *Conogethes* populations collected on the select host plants from field. PCR amplified fragments resulting from species-specific markers for *Conogethes* populations were further sequenced and analyzed as above. To validate the species-specific primer specificity, cross amplification was tried through PCR for both the primer sets developed in the current study. All the sequences established were analysed corresponding to COI, using BioEdit. 7.0 program using Clustal W. The aligned sequences were further analysed using MEGA version X (Kumar et al., 2018). Neighbour-Joining (NJ) trees were constructed with Kimura-2-parameter (K2P) distance model (Kimura 1980). All the corresponding sequences of mitochondrial markers for *Conogethes* were deposited in the NCBI-GenBank.

Results and Discussion

The PCR amplicon of the same size (Approx. 700 bp) was amplified for *C. punctiferalis* and *C. sahyadriensis* and the sequences showed that the total nucleotide length was 587 bp for the two candidate species. The BLAST search showed that the sequences had the similarity for the respective species. Alignment of the COI sequences for *C. punctiferalis* (JX064420) and *C. sahyadriensis* (JX064423) in BioEdit v.7.0 revealed that among 587 in 31 nucleotide positions showed variations amounting to approximately 5% difference between *C. punctiferalis* and *C. sahyadriensis* (Fig. 1). Species-specific markers require only PCR that is rapid, inexpensive and accessible. Of the 5 primer sets identified each for *C. punctiferalis* and *C. sahyadriensis*, 1 primer set, viz., SD (CP) -F and SD (CP) -

R and SD (CS) - F and SD (CS) -R, successfully identified *C. punctiferalis* and *C. sahyadriensis*, respectively (Table 3, Fig. 2). These species-specific markers amplified an expected fragment size of 333 bp for *C. punctiferalis* and 522 bp for *C. sahyadriensis*. These same fragments were sequenced and results showed that species-specific markers exhibited the similarity for respective species. Similar amplicon sizes were obtained from test specimens of *Conogethes* populations derived from select host plants and geographical position in India.

Validation of the species-specific primers so developed were made deploying PCR using SD (CP) -F and SD (CP) -R and SD (CS) - F and SD (CS) -R primers and genomic DNA isolated from two test species of *Conogethes*, morphologically identified and deposited at the Division of Entomology, Indian Agricultural Research Institute, Pusa, New Delhi. No PCR reaction produced amplification, including non-specific amplicons, where the PCR mix and PCR cycling conditions were same, including annealing temperature, viz., 40°C for 60 sec for all the reactions undertaken. However, the applicability of these primers on other populations of *C. punctiferalis* and *C. sahyadriensis* within and outside India is based on the variations in the nucleotide sequences in forward (6-23 for both *C. punctiferalis* and *C. sahyadriensis*) and reverse primer binding regions 321-339 for *C. punctiferalis* and 508-528 for *C. sahyadriensis*. A comparison of forward primer binding regions for *C. punctiferalis* (JX064420.1) with other existing COI sequences (sequences generated in the current study) showed that there were variations at 5th position. Similarly, in the reverse primer binding region, there were variations at 285th position. One accession had T instead of C in another accession. A comparison of forward primer binding region for *C. sahyadriensis* with existing COI sequences showed that there were no variations in any of the nucleotide positions in either regions.

Table 1: Analyzed samples of *C. punctiferalis* with description of host plant, site of collection and NCBI-Genbank accession numbers

Species	Host plant	Site of collection	NCBI-GenBank accessions
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Junagadh, Gujarat, India	Jx064411
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Junagadh, Gujarat, India	Jx064412
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Ludhiana, Punjab, India	Jx064413
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Ludhiana, Punjab, India	Jx064414
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Chhindwara, Madhya Pradesh, India	Jx064415
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Chhindwara, Madhya Pradesh, India	Jx064416
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Hyderabad, Andhra Pradesh, India	Jx064418
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Hassan, Karnataka, India	Jx064419
<i>C. punctiferalis</i>	<i>Ricinus communis</i> L.	Hassan, Karnataka, India	Jx064420

Table 2: Analyzed samples of *C. sahyadriensis* with description of host plant, site of collection and NCBI-Genbank accession numbers

Species	Host plant	Site of collection	NCBI-GenBank accessions
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Mudigere, Karnataka, India	Jx064422
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Mudigere, Karnataka, India	Jx064423
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Mudigere, Karnataka, India	Jx064424
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Bengaluru, Karnataka, India	Jx064425
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Bengaluru, Karnataka, India	Jx064426
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Idduki, Kerala, India	Jx064427
<i>C. sahyadriensis</i> sp. nov.	<i>Elettaria cardamomum</i> Maton	Idduki, Kerala, India	Jx064428

Table 3: Species specific molecular markers developed for *C. punctiferalis* and *C. sahyadriensis*

Species	Primer	Binding region (bp)	Product size (bp)
<i>C. punctiferalis</i>	SD (CP) -F - 5'-AGGAACTTCCTTAAGTTT-3'	6-23	333
	SD (CP) -R - 5'TCCACCATGTGCAATATTA'3	321-339	
<i>C. sahyadriensis</i>	SD (CS) -F - 5'-GGGAACTTCCTTAAGTTT-3'	6-23	522
	SD (CS) -R - 5'TGCTAATACTGGTAAAGATAA'3	508-528	

Table 4: Maximum Composite Likelihood Estimate of the pattern of nucleotide substitution from 11 populations of *C. punctiferalis* and 8 populations of *C. sahyadriensis*

	A		T		C		G	
	<i>C. punctiferalis</i>	<i>C. sahyadriensis</i>	<i>C. punctiferalis</i>	<i>C. sahyadriensis</i>	<i>C. punctiferalis</i>	<i>C. sahyadriensis</i>	<i>C. punctiferalis</i>	<i>C. sahyadriensis</i>
A	-	-	1.05	0.09	0.45	0.04	4.31	32.29
T	0.8	0.07	-	-	24.5	0.04	0.4	0.03
C	0.8	0.07	57.28	0.09	-	-	0.4	0.03
G	8.57	67.13	1.05	0.09	0.45	0.04	-	-

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

Molecular identification using a species-specific marker is an advantage where there is cryptic species complex in the target species population. In *Conogethes*, there is a suspicion that within *C. punctiferalis* complex, the population feed on a wide range of host plants leading to host-mediated speciation across diversified habitats. In such species complex (there may be more than one species) in lepidoptera, the development of species-specific markers for *C. punctiferalis* and *C. sahyadriensis* are of immense significance. The species-specific markers enable even a non-specialist or non-taxonomist to identify the target species, i.e., *C. punctiferalis* and *C. sahyadriensis* at any developmental stage without sequencing and elaborate examination and time.

The maximum composite likelihood defines the sum of related log-likelihoods among the species components (Tamura et al., 2004). The biological data sets for COI were utilised for maximum composite likelihood estimate pattern of nucleotide substitution for *C. punctiferalis* sequences using MEGA.X (Kumar et al., 2018). The reliability of clustering pattern in the phylogenetic trees was determined by bootstrap test, with 1000 replications. Rates of different transition substitutions are shown in bold and those of transversion substitutions are shown in italics. The *C. punctiferalis* nucleotide frequencies were 0.343 (A), 0.359 (T), 0.164 (C) and 0.133 (G). The base composition of these gene fragments was biased toward Adenine (A) and Thymine (T), which together constituted 70.2% of the total nucleotides. The overall transition (ti)/ transversion (tv) bias of *C. punctiferalis* nucleotide sequence was $R = 1.007$, where $R = [A^*G^*k1 + T^*C^*k2] / [(A+G)^*(T+C)]$. Codon positions included were 1st + 2nd + 3rd + Noncoding. For *C. sahyadriensis*, the nucleotide frequencies were 0.344 (A), 0.361 (T), 0.155 (C) and 0.14 (G) (Table 4).

The base composition of COI gene sequences was biased towards Adenine. Current analysis conducted using the sequences generated for both *C. punctiferalis* and *C.*

sahyadriensis, revealed sequence variations. This suggests that genetic structuring in terms of species, host-associated or habitat-related genetic differences are associated with both *C. punctiferalis* and *C. sahyadriensis*. This was evidenced using COI sequences of other *Conogethes* populations. As discovered, genital variations of both sexes between *C. punctiferalis* and *C. sahyadriensis* are reflected in their genetic structural differences and can be the result of host-mediated speciation with associated environmental factors affecting the growth and development of *Conogethes* population.

The Neighbor-Joining tree was constructed using MEGA X (Kumar et al., 2018) with bootstrap support (1000), and *Marwitzia dichocrocis* (Hampson) was used as an out-group in the species analysis of *Conogethes* sp. *M. dichocrocis* is a closely related genus of *Conogethes* from Africa. In *C. punctiferalis*, NJ tree was constructed from nine generated sequences of COI gene across the host plants with one out-group. Phylogeny results showed that *C. punctiferalis* sequences formed a separate clade showing high diversity within the species (Fig. 3A). It clearly indicated that *C. punctiferalis* populations are undergoing genetic pressure in response to its wide host range, may lead to host-mediated speciation within the *C. punctiferalis* complex in India and other country as well. In *C. sahyadriensis*, eight COI based sequences were generated from a group of select host plants and utilised for construction of Neighbor-Joining tree with one out-group. Phylogeny tree clearly revealed no genetic diversity within *C. sahyadriensis* and formed a single clade in the phylogeny (Fig. 3B). This clearly indicated that *C. sahyadriensis* is an oligophagous pest restricted to few zingiberaceous host plants without exhibiting any selection pressure due to host plants.

Rapid, accurate and timely identification of insects is important and challenging. Developing species-specific marker is

	10	20	30	
<i>C. punctiferalis</i> (JX064420.1)	A T A G T A G G A A C T T C C T T A A G T T T A T T A A T T	30		
<i>C. sahyadriensis</i> (JX064423.1)	A T A G T G G G A A C T T C C T T A A G T T T A T T A A T T	30		
Clustal Consensus	* * * * *	29		
	40	50	60	
<i>C. punctiferalis</i> (JX064420.1)	C G A G C T G A A T T A G G T A A T C C A G G A T C A T T A	60		
<i>C. sahyadriensis</i> (JX064423.1)	C G A G C T G A A T T A G G T A A T C C A G G A T C A T T A	60		
Clustal Consensus	* * * * *	59		
	70	80	90	
<i>C. punctiferalis</i> (JX064420.1)	A T T G G A G A T G A T C A A A T T T A T A A T A C A A T T	90		
<i>C. sahyadriensis</i> (JX064423.1)	A T T G G A G A T G A T C A A A T T T A T A A T A C A A T T	90		
Clustal Consensus	* * * * *	89		
	100	110	120	
<i>C. punctiferalis</i> (JX064420.1)	G T A A C A G C T C A T G C T T T T A T T A T A A T T T T T	120		
<i>C. sahyadriensis</i> (JX064423.1)	G T A A C A G C T C A T G C T T T T A T T A T A A T T T T C	120		
Clustal Consensus	* * * * *	118		
	130	140	150	
<i>C. punctiferalis</i> (JX064420.1)	T T T A T A G T A A T A C C T A T T A T A A T T G G A G G T	150		
<i>C. sahyadriensis</i> (JX064423.1)	T T T A T A G T A A T A C C T A T T A T A A T T G G A G G T	150		
Clustal Consensus	* * * * *	148		
	160	170	180	
<i>C. punctiferalis</i> (JX064420.1)	T T T G G A A A T T G A T T A G T T C C T C T A A T A T T A	180		
<i>C. sahyadriensis</i> (JX064423.1)	T T T G G A A A T T G A T T A G T T C C T T T A A T G T T A	180		
Clustal Consensus	* * * * *	176		
	190	200	210	
<i>C. punctiferalis</i> (JX064420.1)	G G G G C C C C A G A T A T A G C T T T C C C T C G A A T A	210		
<i>C. sahyadriensis</i> (JX064423.1)	G G A G C T C C A G A T A T A G C C T T T C C C C G A A T A	210		
Clustal Consensus	* * * * *	201		
	220	230	240	
<i>C. punctiferalis</i> (JX064420.1)	A A T A A T A T A A G A T T T T G A T T A C T T C C C C C T	240		
<i>C. sahyadriensis</i> (JX064423.1)	A A T A A T A T A A G A T T T T G A C T A C T T C C C C C T	240		
Clustal Consensus	* * * * *	230		
	250	260	270	
<i>C. punctiferalis</i> (JX064420.1)	T C A C T A A C T C T T T T A A T T T C C A G A A G A A T T	270		
<i>C. sahyadriensis</i> (JX064423.1)	T C A T T A A C T C T T T T A A T T T C T A G A A G A A T T	270		
Clustal Consensus	* * * * *	258		

	280	290	300	
<i>C. punctiferalis</i> (JX064420.1)	G T T G A A A A T G G A G C T G G A A C A G G A T G A A C A	300		
<i>C. sahyadriensis</i> (JX064423.1)	G T T G A A A A T G G A G C A G G A A C A G G A T G A A C A	300		
Clustal Consensus	* *	287		
	310	320	330	
<i>C. punctiferalis</i> (JX064420.1)	G T A T A C C C C C C T C T T T C A T C T A A T A T T G C A	330		
<i>C. sahyadriensis</i> (JX064423.1)	G T A T A C C C C C C C C T T T C A T C C A A T A T T G C T	330		
Clustal Consensus	* *	314		
	340	350	360	
<i>C. punctiferalis</i> (JX064420.1)	C A T G G T G G A A G A T C T G T T G A T C T T G C T A T T	360		
<i>C. sahyadriensis</i> (JX064423.1)	C A T A G T G G T A G A T C T G T T G A T C T C G C T A T C	360		
Clustal Consensus	* *	340		
	370	380	390	
<i>C. punctiferalis</i> (JX064420.1)	T T T T C C C T T C A T T T A G C G G G A A T T T C T T C T	390		
<i>C. sahyadriensis</i> (JX064423.1)	T T T T C C C T T C A T C T A G C G G G A A T T T C T C T	390		
Clustal Consensus	* *	368		
	400	410	420	
<i>C. punctiferalis</i> (JX064420.1)	A T T T T A G G A G C G A T T A A T T T C A T T A C A A C A	420		
<i>C. sahyadriensis</i> (JX064423.1)	A T T T T A G G A G C A A T T A A T T T C A T C A C A A C A	420		
Clustal Consensus	* *	396		
	430	440	450	
<i>C. punctiferalis</i> (JX064420.1)	A T T A T C A A T A T A C G A A T T A A T G G A T T A T C A	450		
<i>C. sahyadriensis</i> (JX064423.1)	A T T A T T A A T A T A C G A A T T A A T G G A C T A T C A	450		
Clustal Consensus	* *	424		
	460	470	480	
<i>C. punctiferalis</i> (JX064420.1)	T T T G A T C A A A T A C C T C T T T T A T T T G A G C T	480		
<i>C. sahyadriensis</i> (JX064423.1)	T T T G A T C A A A T A C C T C T T T T A T T T G A G C T	480		
Clustal Consensus	* *	454		
	490	500	510	
<i>C. punctiferalis</i> (JX064420.1)	G T A G G A A T T A C A G C T T T A T T A C T T C T T C T A	510		
<i>C. sahyadriensis</i> (JX064423.1)	G T A G G A A T T A C A G C T T T A T T A C T T C T T T T A	510		
Clustal Consensus	* *	483		
	520	530	540	
<i>C. punctiferalis</i> (JX064420.1)	T C T C T G C C A G T A T T A G C G G G T G C T A T T A C T	540		
<i>C. sahyadriensis</i> (JX064423.1)	T C T T T A C C A G T A T T A G C A G G T G C T A T T A C T	540		
Clustal Consensus	* *	510

Fig. 1: Consensus sequence of 587 bp from the mitochondrial cytochrome oxidase I (COI) gene for *C. punctiferalis* and *C. sahyadriensis*.

Fig. 2: Validation of species-specific markers for *C. punctiferalis* and *C. sahyadriensis*. M - 100bp DNA ladder (Invitrogen, Thermo Fisher Scientific) 1 and 2 - PCR amplified product (COI gene) of *C. punctiferalis* and *C. sahyadriensis*, respectively; 3: *C. punctiferalis* - specific molecular marker; 4: *C. sahyadriensis* - specific marker.

an easy, economical way of species discrimination in closely related species. This method is handy and independent of life stage, sex, polymorphism and other related factors. Rebijith *et al.* (2012) developed species-specific marker for identification of tea mosquito bug, *Helopeltis* species in India. The results showed that using species-specific marker *Helopeltis antonii* Signoret and *Helopeltis thievora* Waterhouse populations were differentiated. DNA barcoding and species-specific marker can also be used for identification of different species of cryptic mired bugs in India.

Reza and Khadijeh (2018) developed species-specific primer and single nucleotide polymorphism (SNP) and found a

multiplex polymerase chain reaction as a molecular diagnostic tool to distinguish immature stages of most common and abundant species of cutworms viz., *Agrotis ipsilon* (Hufnagel), *Agrotis exclamationis* (Linnaeus) and *Peridroma saucia* (Hubner). There has been suspicion that at least two types of *Adoxophyes honmai* (Yasuda) exist in Korea. This moth attacks apple, peach, pear and tea. Moths have extreme morphological similarity and can be distinguished by developing new PCR primer sets.

Similarly species specific primers have also been developed and validated for different insect pests viz., two species of genus *Scolytus* (Amini and Hosseini, 2016), five stored

Fig. 3: Neighbour-joining phylogenetic tree of *C. punctiferalis* (A) and *C. sahyadriensis* (B) species based on the COI gene sequences. *Marwitzia dichocrocis* was used as an out group in the analysis.

product pests viz., *Rhyzopertha dominica*, *Sitophilus granarius*, *S. oryzae*, *S. zeamais* and *Sitotroga cerealella* (Sola et al., 2018) and four major thrips vectors viz., *Thrips palmi*, *T. tabaci*, *Scirtothrips dorsalis*, and *Frankliniella schultzei* (Jangra et al., 2020). These species specific primers are handy and quick tool in screening large number of samples and assessing the distribution profile of cryptic species.

This molecular identification technique with no enzyme digestion and sequencing step is convenient and rapid for identification of different species that are difficult to distinguish morphologically. The analysis of present study clearly showed that two species of *Conogethes* spp. remained skewed for a long time in India and also in neighbouring countries despite several studies on different aspects on two *Conogethes* populations. The importance of species-specific markers for species identification of *C. punctiferalis* and *C. sahyadriensis* amongst many other *Conogethes* species has been documented. From the present

study, it can be concluded that developed markers helps in rapid, accurate, morphological and life-stage independent identification of *C. punctiferalis* and *C. sahyadriensis* which are morphologically indistinguishable. Undoubtedly, it helps in precise identification and in formulating effective management practices and also aids in developing appropriate quarantine protocols.

Acknowledgments

The senior author whole heartedly acknowledge the help received from Dr. S. Subramanian, Principal Scientist, Division of Entomology, ICAR-Indian Agricultural Research Institute, Pusa Campus, New Delhi, for providing laboratory facilities to conduct this work and I profoundly thank Mr. Prabhulinga T. Scientist, ICAR-Central Cotton Research Institute, Nagpur and Dr. Rani A.T., Scientist, ICAR-Indian Institute of Vegetable Research, Varanasi for their valuable guidance for preparing of manuscript. We are also thankful to the Department of Entomology, UAS,

GKVK, Bengaluru as this work is a part of Ph. D thesis of first author.

Add-on Information

Authors' contribution: V. Kammar: Planning, execution of experiments; D. Sagar, P.R. Shashank: Planning, manuscript preparation; R.K. Chandel: Execution of experiments; A.K. Chakravarthy: Planning, final editing of manuscript.

Research content: The research content is original and has not been published elsewhere

Ethical approval: Not Applicable

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

References

- Amini, S. and R. Hosseini: A multiplex polymerase chain reaction based method for rapid identification of two species of the genus *Scolytus* Geoffroy (Col: Curculionidae: Scolytinae) in Iran. *J. Entomol. Acarol. Res.*, **48**, 11-15 (2016).
- Armstrong, K. F.: DNA barcoding: A new module in New Zealand's plant biosecurity diagnostic toolbox. *Bull. OEPP*, **40**, 91 – 100 (2010).
- Chakravarthy, A.K., H. Honda and N.E. Thyagaraj: Comparison of containers for larval rearing in stalk and fruit feeding type of *Conogethes punctiferalis* (Guen.) (Lepidoptera: Pyralidae). *Int. Sym. Plant. Crops*, **9**, 127– 131 (1991).
- Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek: DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Marine Biol. Biotechnol.*, **3**, 294-299 (1994).
- Hall, T.A.: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.*, **41**, 95-98 (1999).
- Hampson, G.F.: The Fauna of British India including Ceylon and Burma (Moths). Taylor and Francis, London, pp. 594 (1896).
- Inoue, H. and H. Yamanaka: Redescription of *Conogethes punctiferalis* (Guenée) and descriptions of two new closely allied species from Eastern Palaearctic and Oriental Regions (Pyralidae: Pyraustinae). *Tinea*, **19**, 80-91 (2006).
- Jangra, S., A. Mittal, H. Dhali, R. K. Jain and A. Ghosh: A multiplex PCR assay for rapid identification of major tospovirus vectors reported in India. *BMC Genom.*, **21**, 170 (2020).
- Ji, Y.J., D.X. Zhang and L.J. He: Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Mol. Ecol. Notes*, **3**, 581-585 (2003).
- Kimura, M.: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**, 111-120 (1980).
- Koizumi, K.: Two forms of *Dichocrocis punctiferalis* (Guenée) presumably representing separate species. The main purport of a lecture the 20th annual meeting of the Entomological Society of Japan., pp. 8–9 (1960).
- Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura: MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mole. Biol. Evol.*, **35**, 1547-1549 (2018).
- Meyrick, E.: On the classification of the Australian Pyralidina. *Trans. Ento. Soc. Lond.*, **1884**, 277-350 (1884).
- Raboudi, F., P. Chavigny, M. Marrakchi, H. Makni, M. Makni and M.F. Vanlerberghe: Characterization of polymorphic microsatellite loci in the aphid species *Macrosiphum euphorbiae* (Hemiptera: Aphididae). *Molec. Ecol. Notes*, **5**, 490-492 (2005).
- Rebijith, K.B., R. Asokan, N.K. Krishna Kumar, K.K. Srikumar, V.V. Ramamurthy and P. Shivarama Bhat: DNA barcoding and development of species-specific markers for the identification of tea mosquito bugs (Miridae: Heteroptera) in India. *Environ. Entomol.*, **41**, 1239-1245 (2012).
- Reza, H. and M. Khadijeh: A multiplex PCR method for identification of two common true cutworm species (Lepidoptera: Noctuidae) tested in the central plain of Guilan province, Iran. *Zootaxa*, **4420**, 243–250 (2018).
- Savolainen, V., R.S. Cowan, A. P. Vogler, G.K. Roderick and R. Lane: Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Phil. Trans. Roy. Soc. London, B, Biol. Sci.*, **360**, 1805-1811 (2005).
- Sekiguchi, K.: Morphology, biology and control of the yellow peach moth, *Dichocrocis punctiferalis* Guenée (Lepidoptera: Pyraustidae). *Bull. Ibaraki Hortic. Expe. Station, Special Issue.*, **89**, 1-90 (1974).
- Shaffer, M., E.S. Nielsen and M. Horak: Pyraloidea. In: Checklist of the Lepidoptera of Australia. *Monograph of Australia Lepidoptera*, **4**, 164–199 (1996).
- Shashank, P.R., A.K. Chakravarthy, R. Chandrashekharaiah and K.R.M. Bhanu: Behavioural studies on shoot and fruit borer, *Conogethes punctiferalis* Guenée, (Crambidae: Lepidoptera) host-associated populations reveal occurrence of cryptic species. *Entomol. Gener.*, **35**, 103–115 (2014a).
- Shashank, P.R., A.K. Chakravarthy, B.R. Raju and K.R.M. Bhanu: DNA barcoding reveals the occurrence of cryptic species in host-associated population of *Conogethes punctiferalis* (Lepidoptera: Crambidae). *Appli. Entomol. Zool.*, **49**, 283-295 (2014b).
- Shashank, P. R., V. Kammar, R. Mally and A.K. Chakravarthy: A new Indian species of shoot and capsule borer of the genus *Conogethes* (Lepidoptera: Crambidae), feeding on cardamom. *Zootaxa*, **4374**, 215–234 (2018).
- Simon, C., F. Frati, A. Bechenbach and B. Crespi: Evolution, weighting, and phylogenetic utility of mitochondrial gene sequence and compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.*, **87**, 651-701 (1994).
- Sola, M., J. Riudavets and N. Agusti: Detection and identification of five common internal grain insect pests by multiplex PCR. *Food Control*, **84**, 246-54 (2018).
- Von Dohlen, C.D. and N.A. Moran: Molecular data support a rapid radiation of aphids in the Cretaceous and multiple origins of host alternation. *Biol. J. Linn. Soc.*, **71**, 689-717 (2000).