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Molecular Evidence for Divergence in the Shoot and Fruit borer, *Conogethes spp.* (Lepidoptera: Crambidae) Infesting Select Host-Plants in India

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ABSTRACT

The Shoot and fruit borer, *Conogethes* moths on many host-plants appear to be similar in wing pattern and color. However, behavioral studies elicited a doubt that there may be differences in host specialization. Documenting and recognizing the differences are crucially important for the management of this polyphagous pest. A study was conducted on genetic diversity of *Conogethes* species infesting select host plants based on COI genes. The pairwise genetic distance analysis between the individuals varied from 0.000 to 0.076, indicating high genetic divergence. The nearest neighbour distance between *Conogethes* bred within the 15 populations was 5.32 per cent, indicating wide genetic variability between two *Conogethes* populations. Sequence length showed significant variation from 477 bp (castor) to 726 bp (gingiberaceae) and per cent G+C content for COI showed low variations (0.17%) than cardamom of *Conogethes* species. In addition, topologies of neighbor-joining tree indicated that *Conogethes* sp. breeding on castor, mango, pear, peach, plum, guava, sapota, belong to *C. punctiferalis* while those on cardamom, turmeric and ginger are of a separate clade. Further genetic analysis revealed significant genetic differentiations among the two sampled populations, reflecting limited gene flow. The results of an analysis of molecular variance (AMOVA) indicated the existence of significant genetic variation among the examined host races. Suggesting that the variations in *Conogethes* populations are genetically heterogeneous.

YELLOW peach moth, *Conogethes punctiferalis* (Guenée) (Lepidoptera: Crambidae), is widely distributed in South and East Asia, Australia and Papua New Guinea (CAB International, 2011). The larva of *C. punctiferalis* is a typical generalist and feeds on a broad range of hosts, including peach, chestnut, apple, pear, plum, apricot, durian, citrus, papaya, cardamom, ginger, eggplant, and maize (Sekiguchi, 1974; Waterhouse, 1993).

The accurate identification of insect species is one of the important aspects in entomological science. In most groups, traditional taxonomic research is based on morphological characters. It is difficult to identify cryptic and polymorphic species through conventional taxonomy. However, there are difficulties for want of experts. For this purpose, molecular methods have been found valuable in discriminating cryptic species of insects (Jackson and Resh, 1998; Pilgrim *et al.,* 2002). Cryptic species are defined as two or more distinct species classified as a single nominal species because they are morphologically indistinguishable (Bickford *et al.,* 2007). One of the mechanisms thought to promote speciation in phytophagous insects is shifts to new hosts that lead to the establishment of new species via an intermediate step of host-race formation (Bush, 1969; Dres and Mallet, 2002). The occurrence of insect host races exhibit recently evolved genetic differentiation with respect to host-plant use (Dres and Mallet, 2002). The *Conogethes* larvae reared on castor bean and cardamom required two different mass-rearing techniques (Chakravarthy *et al.,* 1991). Visual observations showed distinct differences in the size of an adult and in the feeding behavior of larvae infesting two hosts. *Conogethes punctiferalis* is considered as a major pest on the two crops that causes up to 63 per cent yield loss in castor and more than 20 per cent yield loss in cardamom (Kapadia, 1996). The differences in habitat of castor and cardamom and in the morphological characteristics of *C. punctiferalis* moths infesting both crops elicit a doubt that the *C. punctiferalis* bred on castor (CBR) and on cardamom (CBE) may be different host races or cryptic species (Shashank *et al.,* 2014).

The cytochrome oxidase subunit I (COI) region of *mt*DNA is useful for determining intra- and interspecific phylogenetic relationships at the genus and

species level (Caterino and Sperling, 1999; Trewick, 2000) and within families (Logan, 1999), studies on differences between host of *C. punctiferalis* reared on castor, mango, pear, peach, plum, guava, sapota, and the COI region of *C. punctiferalis* reared on cardamom in India, along with demographic analysis have been conducted.

Fully matured *Conogethes* larvae were collected from diverse regions of India. The Cetyl trimethyl ammonium bromide (CTAB) procedure for isolating DNA was simplified from one proposed by Doyle and Doyle (1990). The extracted DNA was quantified using both Nanodrop DNA quantifier and also by electrophoresis on 0.8 per cent agarose gel with diluted uncut k DNA as standard. The universal barcode primer described by Folmer *et al.* (1994) (LCO-50-GGT CAA CAA ATC ATA AAG ATA TTG G-30; HCO-50-TAA ACT TCA GGG TGA CCA AAA AAT CA-30) specific to mitochondrial cytochrome oxidase I (COI) was used in the present study. Isolated sample solution was preserved at -20º C for subsequent DNA isolation and PCR reaction. Total DNA fraction from the sample was obtained using a standard CTAB kit protocol. All samples were screened for variation at each of the COI primers. PCR reaction was performed under fixed condition. The reaction mixture was set up in sterile 0.2 ml microfuge tubes. The PCR reaction process was as follows: 94 ºC pre-denaturation, 94 ºC denaturation for 1 min., annealing at 52 ºC for 30 s, and primer extension at 72 ºC for 1 min for 35 cycles of polymerization and a final primer extension at 72 ºC for 5 min. The amplification product was preserved in the refrigerator at 4 ºC. A 5µl aliquot of PCR product were resolved on a 1 per cent agarose gel that contained 0.5 lg/ml ethidium bromide at 100 V for 30 min, and data was acquired under a UV Tran-illuminator and photographed immediately for further interpretation using the Gel-Doc system (BioRad, Hercules, CA). The remaining 45 µl of PCR product was purified using Cetyl trimethyl ammonium bromide kits (CTAB) following the manufacturer's protocols. Purified PCR products are sequenced in an automated sequencer (ABI Prism 3730; Applied Biosystems, USA) at the specific commercial facilities (SciGenome, India).The PCR products were resolved by electrophoresis using 3 percent agarose gel in1X Tris borate EDTA buffer for about 2 h at 110V with 100 bp ladders. The gel was stained with ethidium bromide (0.5 ìg / ml), viewed under UV Tran-illuminator and photographed for further interpretation using Gel-Doc system (Bio Rad).

Fourteen individuals of *Conogethes* were subjected to the DNA barcoding based CO I gene, infesting castor, cardamom, mango, pear, peach, plum, guava, sapota, ginger and turmeric supported with morphological studies indicated genetic differences in *Conogethes* populations. The pairwise genetic distance analysis between the individuals varied from Nucleotide variation within Fourteen samples is 0.00 – 5.32 per cent indicating high genetic divergence (Tables I, II and 3III. The maximum intraspecific pair-wise distance in *Conogethes* bred on castor was 0.010 than on cardamom 0.072. The nearest neighbour distance between *Conogethes* bred on castor, mango, pear, peach, plum, guava, sapota and cardamom was 5.32 per cent, indicating wide genetic variability between two *Conogethes* populations. Results of this studies demonstrated a clear differentiation of a cryptic lineage (cardamom), which can be viewed as a host-plant-based cryptic species on the basis of high (5.32 %) sequence divergence because genetic divergence of more than 5 per cent suggests the likely occurrence of a new species (Hebert *et al.,* 2004). The *Ostrinia* species complex is a model for speciation of Lepidoptera and provides a basis for comparison with observations from this studies on *C. punctiferalis* (Coates *et al.,* 2005; Kim *et al.,* 1999; Ohno *et al.,* 2006). Similar results were observed in the *Maruca vitrata* (Fabricius, 1787) complex (Margam *et al.,* 2011). Armstrong (2010) compared DNA barcoding of different populations of *Conogethes* and revealed that Australian and Asian specimens form separate clades that are divergent by *6 per cent, and their data successfully distinguished *C. punctiferalis* and *C. pluto*. The phylogenetic tree constructed using BOLD tool indicated two separate clades for *Conogethes* within 15 samples. When the sequences were compared across world *Conogethes* infesting castor was homologous to *C. punctiferalis*. However, the sequences from *Conogethes* infesting cardamom form a separate clade (Figure 1). This indicated that no sequences were present in database and it is genetically different from existing species in Bold.

TABLE I

Genetic distance within species and within genus using BOLD management and analysis system (K2P distances)

	n	Taxa	Min Distance (%)	Mean Distance (%)	Max Distance (%)	SE Distance (%)
Within Species	15	2	0.009	0.325	2.002	0.015
Within Genus	15	1	5.181	6.081	8.13	0.053
Within Species(Normalized)	15	2	0.295	0.412	0.879	0.411

TABLE II

Genetic distances (%) to the nearest–neighbours and mean intra-specific distance using BOLD system (K2P distance)

Spices	Mean Intra-Sp	Max Intra-Sp	Nearest Species	Nearest Neighbor	Distance to NN
Conogethes punctiferalis	0.592	2.57	*Conogethes* spp.	CONOG1.13	5.32
Conogethes spp.	0.173	1.07	*Conogethes punctiferalis*	CONOG2.13	5.32

TABLE III

Pairwise genetic distance among the 14 Conogethes population

1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.00													
5.02	0.00												
5.02	0.00	0.00											
0.61	4.86	4.86	0.00										
0.30	5.32	5.32	0.91	0.00									
0.76	5.02	5.02	0.15	1.06	0.00								
0.15	5.17	5.17	0.76	0.46	0.91	0.00							
0.15	5.02	5.02	0.46	0.46	0.61	0.30	0.00						
0.00	5.02	5.02	0.61	0.30	0.76	0.15	0.15	0.00					
0.00	5.02	5.02	0.61	0.30	0.76	0.15	0.15	0.00	0.00				
0.00	5.02	5.02	0.61	0.30	0.76	0.15	0.15	0.00	0.00	0.00			
0.76	5.02	5.02	0.15	1.06	0.00	0.91	0.61	0.76	0.76	0.76	0.00		
0.76	5.02	5.02	0.15	1.06	0.00	0.91	0.61	0.76	0.76	0.76	0.00	0.00	
0.46	4.86	4.86	1.06	0.76	1.22	0.61	0.61	0.46	0.46	0.46	1.22	1.22	0.00

Fig. 1. Neighbour-Joining of COI for *Conogethes* spp. breeding on castor, cardamom, mango, pear, peach, plum, cocoa, guava and sapota

In conclusion, the molecular evidence reveals to confirm the presence of cryptic species within *C. punctiferalis* breeding on select host-plants. Though further works are required to form a clear understanding in this regard, outcome results suggest the apparent existence of two species pairs, evidence of genetic divergence by >5 per cent. This is an interesting case and need further studies. Results from the current study strengthen information about the *Conogethes* species complex and help in developing appropriate integrated pest management strategies for these insect pests.

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