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Identification and phylogenetic analysis of fruit borer species of litchi using DNA barcode sequences

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ABSTRACT

Fruit borers are the major insect pests of litchi in humid orchard conditions at the time of fruit ripening in Indian subcontinent. The major constraint faced in formulating any management strategies against the borer is the difficulty in identification of the correct species. The litchi fruit borer has low intraspecific variation among the existing borer populations. Molecular approaches have been used to identify various species. In the present study, partial cytochrome oxidase I (COI) sequences were used to understand the phylogenetic relationship among borer complex, and assess their usefulness to identify and classify unknown borer species collected from litchi orchards. All together, 150 specimens of litchi fruit borer from Bihar and Jharkhand were examined and 2 morphologically similar moths of each genus were used for further analysis. Sequence analysis revealed that the intraspecific and interspecific variations ranged from zero to 10.0% and 4.4 to 20.3%, respectively among fruit borer complex. The phylogenetic analysis showed that the borers specimen used in this study clustered in to distinct species-groups designated as *Conopomorpha sinensis* Bradley, *C. litchiella* Bradley, *Cryptophlebia ombrodelta* Lower and *Gatesclarkeana* spp. Higher intraspecific genetic variation was observed in *Conopomorpha* species complex as compared to *Cryptophlebia* species complex. Present study has clearly demonstrated that DNA barcoding is an efficient and accurate method for identification of coexists of borer complex infestation in litchi fruits. Hence, this approach can play important role in formulating viable pest management strategies.

Key words: *Litchi chinensis*, borer species, intraspecific & interspecific variation, phylogenetic analysis, genetic variability.

INTRODUCTION

Litchi is a subtropical fruit tree native to Southern China, one of the highly-prized fruits in South-East Asia because of its excellent flavour and refreshing taste. Being, environmentally sensitive fruit tree, litchi requires dry and cool but frost-free winters with daily maximum temperature below 20-22°C, long and hot summers with daily maximum temperature above 35°C. The areas receiving high rainfall (1200 mm) resulting into high humidity has been considered ideal for litchi cultivation (Srivastava *et al.*, 18). Litchi crop is affected by number of biotic and abiotic factors but pests' incidence and physiological disorders are the major obstacles in achieving constantly high-quality fruit yields (Srivastava *et al*., 19). The damage caused by insect pests involves multiple species acting at the same time and their high putting the litchi orchards under lot of threat. In India, there are nearly 42 insect and mite pest species which attack litchi in different stages of growth. The majority of them belong to Lepidoptera and Coleoptera, followed by the Homoptera and Hemiptera. Among all, fruit borers are major and regular pests of litchi and longan (*Dimocarpus longan* Lour.: Sapindaceae) that causes significant economic losses. Infestation to seed and fruits of litchi is reported by number of lepidopteran species which includes *Conopomorpha sinensis* Bradley, *Conopomorpha litchiella* Bradley, *Conopomorpha cramerella* Snellen (Lepidoptera: Gracillariidae), *Cryptophlebia ombrodelta* Lower, *Cryptophlebia illepida* Buttler, *Gatesclarkeana erotias* Meyrick (Lepidoptera: Tortricidae) and *Blastobasis* sp. (Li *et al*., 7; Bhatia *et al*., 1; Kumar *et al*., 6). Species composition of fruit borer population in litchi ecosystems has been found to affect the level of damage and timing of incidence in litchi fruits. Information on species complex, distribution, bio-ecology and natural enemy complex of litchi fruit borers is key to formulate management practices. One of the major constraints in formulating the management practices for litchi fruit borer is the difficulty to identify the borer species. The poor collection of moths in pheromone traps used in India could also be attributed to wrong identification of the species, as they are primarily meant for cocoa borer, *C. cramerella* Snellen (Reddy *et al*., 14). Various morphological keys have been established to identify fruit borer insect species of litchi at adult stages (Bradley, 2). However, morphological identification of borer species is difficult because of morphological examination of moths up to species

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level is restricted to certain life stages. No reliable keys for the identification of the immature stages, cryptic speciation and scarcity of solid morphological characters need development of taxonomic expertise. In addition, identification becomes more difficult because of high intraspecific variation found in borer moth populations.

Recently, molecular approaches have provided potent tools for correct identification of species and investigate phylogenetic relationships among insects. DNA polymorphisms at mitochondrial and nuclear genes level have been used for insect molecular systematic and diagnostics (Gariepy *et al*., 3). DNA based methods for species identification has advantages as they are technically more precise, non recombinant, high copy numbers, accelerated rate of evolution, simple maternal inheritance, applicable to large-scale screening, and requires no taxonomic expertise. Sequences of the mitochondrial cytochrome oxidase I (mtCOI) coding gene from different lepidopteran species were used to detect several haplotypes (Shapiro *et al*., 15). Hebert *et al*. (4) proposed that partial COI sequences could be a universal barcode for the identification of insects at the species level rapidly and in precise way. DNA barcoding can be employed as a useful approach for molecular identification of species in their various life stages and forms, host associated genetic differences, discrimination of cryptic species, as well as biotypes. Using DNA barcoding even a non-taxonomist can easily identify and discriminate a matrix containing a mixture of biological species. To better implement an Integrated Pest Management (IPM) program, a more general, simple, accurate, and large scale method would be helpful to facilitate identification of borer species occurring in litchi ecosystem where multiple species co-exist, and the population dynamics are influenced by numerous factors.

Hence, our aim in this study was to use partial COI sequences to understand the phylogenetic relationship among fruit borer species, and assess their usefulness to identify the correct species infesting the litchi fruits which could be useful in formulating effective management practices against borer complex in litchi.

MATERIALS AND METHODS

For this study, borer moths were collected from infested fruits of litchi (Table 1). Freshly borer infested litchi fruits under field conditions were kept individually in insect rearing cages (10×20 cm) at 25±1°C, RH=60±5%, and 16:8 hr (light: dark phase) at the insectary of the ICAR Research Complex for Eastern Region, Research Centre, Ranchi and ICAR-NRCL, Muzaffarpur (India) for adult moth emergence. Adult individuals were preliminary identified based on the morphological characters described by Bradley (2) and Hu *et al*. (5). Among 150 specimens, 2 similar moths from each sample were used for further analysis. In order to understand and document inter and intraspecific variations in the barcoding region of each species (Meyer and Paulay, 10), we analysed all the sequences of fruit borers of litchi to other closely related available sequences from NCBI GenBank and a total of 58 sequences set were used for final analysis. Selection of close related sequences were made based on high similarity score and E-values equal to zero, and only non-redundant species sequences were retained for further analysis.

Total DNA was extracted from individuals of litchi fruit borer moth following the protocol described by Prabhakar *et al*. (12) with minor modifications. Fragment of the mitochondrial genome was amplified from each individual using primer pairs, 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′ (Hebert *et al*., 4). PCR amplifications were performed by using the standard cycling parameters which includes; an initial denaturation step at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, an annealing step at 47°C for 45s, an extension step at 72°C for 45s and a final extension step at 72°C for 20 min. The PCR products were separated in 2% (w/v) agarose gel using TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Amplified PCR products were freeze dried and sent for custom sequencing using *CO1* primers (Xcelris Labs Limited, India). Forward and reverse sequences were obtained using corresponding PCR primers on both the DNA strands.

DNA sequences were aligned using ClustalW programme implemented in MEGA ver. 6.0. Software (Tamura *et al*., 20). A homology search was done using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) and all the sequences generated were deposited in NCBI-GenBank (Supplementary material 1). Fourteen fruit borer species collected from different locations and fruits *viz*., *C. sinensis*, *C. cramerella*, *C. litchiella*, *C. ombrodelta*, *C. illepida*, *C. wraggae*, *Cryptophlebia* sp *C. peltastica*, *C. semilunana*, *C. stigmata*, *C. pallifimbria*, *C. iridosoma*, *Gatesclarkeana* sp. and *G. tenebrosa* were used as reference species based homology search and their most authenticated COI sequences were obtained from GenBank (Table 2). The aligned sequences were used for phylogenetic analysis using MEGA version 6.0. Pairwise genetic

Table 1. Details of COI sequences of fruit borer species of litchi and their related species.

Species	Host plant	Number of samples	Origin	Year of collection	GenBank accession name
Conopomorpha sinensis	Litchi fruits	2	Bihar: India	2015	
	Litchi fruits	2	Jharkhand: India	2015	
	Rambutan and Cocoa	2	Australia	-	HQ824810*; HQ824811*
Conopomorpha cramerella	Rambutan and Cocoa	3	Australia	-	HQ824809*; HQ824807*; HQ824805*
	Unknown	1	USA	-	KJ657669*
	Unknown	1	Malaysia	-	EU644524*
	Unknown	1	Indonesia	-	EU644599*
Conopomorpha litchiella	Litchi fruits	2	Jharkhand: India	2015	
	Litchi fruits	2	Bihar: India	2015	
Cryptophlebia ombrodelta	Litchi fruits	2	Jharkhand: India	2015	
	Litchi fruits	2	Bihar: India	2015	
	Unknown	1	Australia	2004	KF401637*
	Unknown	2	Australia	1995	KF400426*; KF402024*
	Unknown	1	Australia	2003	KF403816*
	Unknown	1	Australia	1999	KF397678*
Cryptophlebia illepida	Unknown	1	Hawaii: USA	-	KF491660*
Cryptophlebia wraggae	Unknown	1	Australia	2003	KF395486*
	Unknown	1	Australia	2004	KF401333*
	Unknown	1	Australia	2007	KF400014*
	Unknown	1	Australia	1972	KF399046*
Cryptophlebia sp.	Unknown	1	Papua New Guinea	1905	HM422451*
	Unknown	4	Papua New Guinea	2010	KP850758*; KP850176*; KP850127*; KP850108*
Cryptophlebia peltastica	Unknown	2	Kenya	2000	KJ592352*; KJ592123*
Cryptophlebia semilunana	Unknown	1	Kenya	2003	KJ592119*
Cryptophlebia stigmata	Unknown	2	Australia	1990	KF397258*; KF398556*
	Unknown	1	Australia	1988	KF401264*
Cryptophlebia pallifimbria	Unknown	1	Australia	1998	KF402831*
	Unknown	1	Australia	1964	KF402818*
Cryptophlebia iridosoma	Unknown	1	Australia	1993	KF399232*
	Unknown	1	Australia	2000	KF395448*
Gatesclarkeana sp.	Litchi fruits	2	Bihar: India	2015	
	Unknown	1	Indonesia	1985	KF399587*
	Unknown	3	Australia	2007; 2008; 1981	HM372975*; KF397980*; KF399431*
	Unknown	1	Papua New Guinea	2010	KP850226*
	Unknown	1	Australia	1988	KF395803*
	Unknown	1	Australia	1992	KF405615*
Gatesclarkeana tenebrosa	Unknown	3	Australia	1981; 1988	KF396067*; KF401335*; KF397166*

*Represent the data is previously published sequence obtained from GenBank

distance between fruit borers of litchi and their related species were obtained based on Kimura's two parameter model and used for estimating intraspecific or interspecific genetic distances (K2P) across species. A neighbor-joining (NJ) tree of fruit borers of litchi and their related species was constructed using the K2P distances (Saitou and Nei, 29) with bootstrapping of 1,000 times as implemented in MEGA version 6.0.

RESULTS AND DISCUSSION

Rapid and timely identification of insects such as fruit borers is important and challenging worldwide particularly at immature stages (Sperling and Hickey, 17). The classification of closely related lepidopteran species based on morphological features alone presents several difficulties and the risk of inaccuracy because the function of certain attributes differs in different environments, leading to the prevalence of several biotypes (Linares *et al*., 8). Because of this, DNA barcoding employing the CO-I gene sequence has become an alternative and effective tool for species identification (Silva *et al*., 16). The partial COI region of moths emerged from litchi fruit were successfully sequenced. Further analysis revealed that 118 characters were variable out of which, 4 singlton and 114 characters were parsimony informative from the 635bp regions investigated in present study. The nucleotide sequences have been deposited in the National Centre for Biotechnology Information (NCBI) database under the accession numbers KP979792, KP997222, KP997223, KP997224, KP997225, and KP997226. The sequences were compared against species those previously deposited in the NCBI database, and showed 99-100% similarity, as sequences available in database. No pseudogenes were amplified as indicated by the absence of stop codons within the sequences and the base composition was similar with no indels. Reliability of the clustering pattern in the tree was determined using the bootstrap test with 1000 replications employing MEGA 6.0 (Tamura *et al*., 20) (Table 2). Nucleotide frequencies were 32.0% (A), 39.1% (T), 14.1% (C) and 14.8% (G). There was a strong AT bias (71.1%), which is in accordance with previous work carried out on insect mitochondrial genomes (Win *et al*., 21). The overall transition (ti)/ transversion (tv) bias of nucleotide sequence was R= 2.69.

The range and mean of Intra- and interspecific genetic distances across all 13 species groups were computed by averaging the K2P distances of all possible combinations of COI sequence variation in a pair-wise manner (Table 3 and Table 4). The mean intra- and interspecific genetic distances of *C. sinensis* were 0.1% (range, 0.0-0.3%) and 13.4% (range, 04.4-18.1%), respectively. As these ranges indicate, there is no overlap between intra- and inter-specific genetic distances in *C. sinensis.* In the case of *C. litchiella*, there was also no overlap between mean intra- and inter-specific genetic distances with values 0.1% (range, 0.0-0.3%) and 13.9% (04.4-18.0%), respectively. The mean intra- and inter-specific genetic distances of *C. ombrodelta* are 0.8% (range, 0.0-2.3%) and 10.0% (range, 02.10-18.6%). The mean intra- and inter-specific genetic distances of *Gatesclarkeana* spp. are 6.5% (range, 0.0-10.0%) and 14% (range, 07.4-20.3%), respectively. These values of intra- and interspecific genetic distances of *C. ombrodelta* and *Gatesclarkeana* spp. indicated slight overlap between them. Widely distributed species exhibited high intraspecific divergence. The 0.00% sequence divergence showed that no DNA sequences overlapped, possibly due to low variation among individuals of the same species from different geographical locations. In contrast, the maximum intraspecific nucleotide divergence of 2.3% indicated that there is a higher degree of variation among individuals of the same species from different locations. Similarly, Lukhtanov *et al.* (9) concluded that geographical distance is often associated with

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution from 56 individuals of 13 species of fruit borers of litchi and their related species.

	A	T	C	G
A	-	4.57	1.65	17.83
T	3.73	-	5.36	1.72
C	3.73	14.88	-	1.72
G	38.6	4.57	1.65	-

Table 3. Intraspecific genetic distance (K2P) of fruit borers of litchi based on partial COI sequences that have two or more sequences of fruit borers with minimum, maximum and average values.

Species groups	No. of individual	Intraspecific genetic distance (%)		
		Min.	Max.	Average
C. sinensis	6	0.00	0.30	0.10
C. cramerella	6	0.00	0.30	0.10
C. litchiella	4	0.00	0.30	0.10
C. ombrodelta	9	0.00	2.3	0.80
Gatesclarkeana spp.	9	0.00	10.00	6.50

Table 4. Inter-specific genetic distance (K2P) of fruit borers of litchi and their related species based on partial COI sequences that have two or more sequences of fruit borers with minimum, maximum and average values.

Species[a]	*C. c*	*C. l*	*C. o*	*C. w*	*C. sp*	*C. p*	*C. s*	*C. st*	*C. pl*	*C. i*	*G. sp*	*G. t*
C. s[b]	5.80-6.50 (6.30)	4.20-4.80 (4.40)	16.50-17.40 (16.30)	16.90-17.40 (17.00)	13.30-14.80 (14.30)	14.10-14.40 (14.10)	14.10	12.50-13.20 (12.80)	12.50-12.50 (12.50)	15.30-16.20 (15.70)	14.40-18.20 (15.30)	17.80-18.30 (18.10)
C. c		4.50-6.40 (6.20)	15.70-19.60 (18.50)	17.40-18.70 (18.60)	13.70-15.30 (15.20)	16.10-16.90 (16.70)	15.70-16.10 (16.00)	15.30-16.50 (16.20)	14.10-15.30 (14.80)	16.10-17.40 (16.90)	13.70-18.70 (16.70)	19.70-20.60 (20.30)
C. l			16.50-17.80 (17.00)	1.40-2.70 (2.10)	14.50-16.50 (16.00)	16.10-17.00 (16.60)	16.10-16.50 (16.30)	16.10-16.50 (16.30)	14.10-14.40 (14.30)	16.50-17.80 (16.90)	14.00-19.50 (15.90)	19.10-20.00 (19.50)
C. o				1.40-2.70 (2.10)	6.10-7.80 (06.50)	6.80-7.20 (7.00)	5.50-6.20 (5.90)	5.10-6.80 (6.40)	6.10-7.50 (6.90)	7.20-7.80 (7.70)	13.70-18.70 (16.70)	9.90-14.60 (11.50)
C. w					6.40-6.80 (6.50)	7.90-8.30 (7.70)	6.90	6.90-7.90 (7.30)	6.50-7.50 (6.70)	8.20-8.60 (8.30)	11.40-14.60 (12.30)	13.80-14.20 (13.90)
C. sp						5.10-5.50 (5.10)	6.10	6.10-7.50 (6.50)	5.10-5.10 (5.10)	6.80-7.50 (7.10)	8.80-12.50 (10.30)	11.80-12.20 (11.90)
C. p							5.50-6.10 (5.30)	6.40-7.50 (7.40)	4.50-5.10 (5.00)	7.50-7.90 (7.60)	8.80-12.50 (10.90)	11.80-12.50 (12.10)
C. s								7.10-7.50 (7.20)	4.80-5.40 (5.10)	7.20-7.90 (7.60)	8.50-12.50 (11.90)	11.80-12.20 (12.00)
C. st									6.80-7.80 (7.00)	7.90-8.60 (8.30)	9.90-13.40 (11.20)	12.20-12.60 (12.10)
C. pl										6.80-6.80 (6.80)	8.10-13.30 (11.40)	13.00-13.40 (13.20)
C. i											10.60-14.20 (11.40)	12.20-12.60 (12.40)
G. sp												1.40-8.60 (7.40)

[a]*C. s, C. sinensis; C. c, C. cramerella; C. l, C. litchiella; C. o, C. ombrodelta; C. w, C. wraggae; C. sp, Cryptophlebia* sp.; *C. p, C. peltastica; C.s, C. semilunana; C.st, C. stigmata; C. pl, C. pallifimbria; C. i, C. iridosoma; G. sp, Gatesclarkeana* sp.; *G. t, G. tenebrosa*

[b]Range and mean of pair-wise genetic distances of fruit borers of litchi and their related species. The mean values are indicated in parenthesis. The actual value is provided wherever only single pair is involved.

an increased genetic divergence, but the increase is too small to impede the identification of species. The overall range and mean of interspecific genetic distance of fruit borer species presented in Table 4 reveal the values for different species as *C. sinensis* (04.4-18.1, 13.4), *C. cramerella* (06.2-20.3, 16.0), *C. litchiella* (04.4-18.0, 13.9), *C. ombrodelta* (02.10-18.6, 10.0), *C. wraggae* (06.5-18.0, 10.3), *Cryptophlebia* spp. (05.1-16.7, 09.4), *C. peltastica* (05.3-16.6, 09.4), *C. semilunana* (05.1-16.3, 09.8), *C. stigmata* (05.1-16.3, 09.2), *C. pallifimbria* (07.0-16.9, 10.3), *C. iridosoma* (04.4-18.0, 13.9), *Gatesclarkeana* spp.

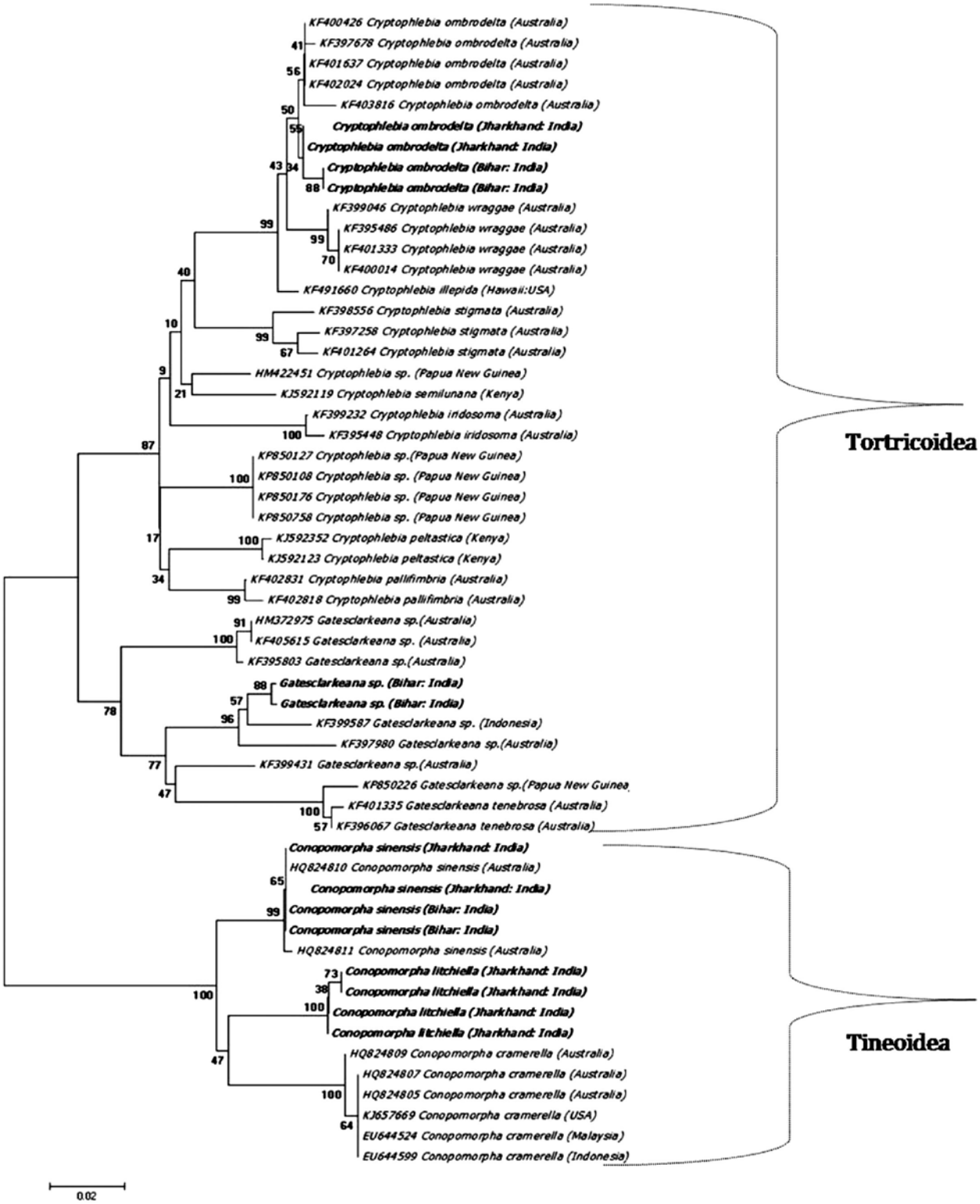

Fig. 1. Neighbour joining tree showing genetic relationships of fruit borers of litchi and their related species collected from litchi and different crops based on partial COI sequences. Numbers shown next to the branches are bootstrap values (1,000 replicates) obtained with Kimura 2-paramter (K2P) distance.

(07.4-20.3, 14.0) and *G. tenebrosa* (07.1-20.3, 15.9). In most cases, the mean genetic distance of a particular species with the rest of the species was >10.0%. The lowest mean genetic distance between species was 9.20% (*C. stigmata*) and the highest was 16.0% (*C. cramerella*). Sequence divergence at COI of >2% is used for species discrimination in lepidopterans (Hebert *et al.,* 4). Low sequence divergences (not in present study), ranging from 0% to 1.2% have been found within many species of lepidopteran species (Zakharov *et al.,* 22; Win *et al*., 21) which may be due to presence of intraspecific hybridization (Hebert *et al*., 4). Moreover, the gap between maximum intraspecific and minimum interspecific distances has been used for species delinitation in various animal groups (Meyer and Paulay, 10; Puillandre *et al*., 13). Variation in the nucleotide sequence is a fundamental property of all living organisms, and may be used for their identification and phylogenetic status.

Molecular phylogenetic tree was constructed for the COI gene using the NJ method (Fig. 1). The NJ tree showed that all sequences from 13 fruit borer species unambiguously clustered into four separate groups with already published sequences in Genbank and identified as *C. ombrodelta, Gatesclarkeana* spp., *C. sinensis* and *C. litchiella* (Fig. 1). Clear groups of the species belonging to *Conopomorpha*, *Cryptophlebia* and *Gatesclarkeana* genera were observed. Genera *Cryptophlebia* and *Gatesclarkeana* are closely related from superfamily Tortricoidea distinguished it to superfamily Tineoidea which includes genera *Conopomorpha.*

Species of present study of genus *Gatesclarkeana* was not able to determine from morphological as well as Genbank query blast. Specimens of genus *Gatesclarkeana* were much closer to specimens collected from Indonesia (KF399587) and Australia (KF397980) than others. Phylogenetic tree analysis showed that species *C. litchiella* much closer to *C. cramerella* under family Gracillariidae. The *C. sinensis* is a sister group to the clade of *C. cramerella* + *C. litchiella* in tree. Thus, different borer species that we used in this study constitute a single lineage of closely related species. In present study, we have not observed adult moths of *C. cramerella* from infested fruits of litchi which is considered as one of the major fruit borer species of litchi (Bhatia *et al.*, 1; Nair and Sahoo, 11). This was confirmed through genetic variations and phylogenetic analysis among present moths emerged from infested fruit of litchi and NCBI deposited sequences. Therefore, it is concluded that *C. sinensis* is the major species causing infestation in litchi however; other borer species needs to be confirmed through large sampling from litchi infested fruits.

In conclusion, the main objective of this study was to validate COI sequence based method to identify borer complex that coexist on mature fruits of litchi. Insect pest management approaches require a clear understanding of the pest species in terms of their particular biology, ecology and population structure/genetics. The study demonstrated that partial COI sequences provide a simple and accurate means to identify major fruit borer species of litchi (*C. sinensis*, *C. litchiella*, *C. ombrodelta* and *Gatesclarkeana* spp.) out of which, *C. sinensis* was most potent species causing major infestation. However, future studies should be undertaken to confirm whether these populations having higher genetic distances within a species constitute a cryptic species complex to develop management schedule against them.

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