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Genetic characterization and DNA barcoding of the coffee white stem borer, *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae) infesting Robusta coffee (*Coffea canephora*)

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ABSTRACT: The coffee white stem borer (CWSB), *Xylotrechus quadripes* (Chevrolat) is one of the most destructive pests of Arabica coffee plants (*Coffea arabica*), while, Robusta coffee (*Coffea canefora*) is free from CWSB attack for long time. However in recent years, CWSB infestation is also noticed on Robusta coffee in Polibetta, Mekur, Siddapura and Sunitikoppa in Kodagu district of Karnataka. Hence, the present study was aimed to confirm the infestation of CWSB infesting Robusta and comparing its population infesting Arabica coffee by performing DNA barcoding. The grubs of CWSB were collected by split-opening the infested Arabica and Robusta stems and persevered in 70% ethanol for molecular identification. In addition, infested stems were covered with nylon mosquito net to collect emerging adults and were preserved. The CWSB genomic DNA was extracted and the DNA concentration varied from 615.9 to 1110.6 ng/µL. The CWSB genomic DNA was amplified and was sequenced the mtCOI region. CWSB mtCOI genes were cloned and sequenced and they matched with high similarity in nucleotide Basic Local Alignment Search Tool search and Barcode of life (BOLD) identification. Neighbour joining (NJ) and Maximum likelihood (ML) phylogenetic tree analysis showed that the individuals of the same species clustered together based on the mtCOI sequence similarity, regardless of their collection site and host.

Keywords: COI; DNA barcode, meta barding, maximum likelihood, Robusta Coffee, white stem borer, *Xylotrechus quadripes,*

INTRODUCTION

Coffee is one of the most important plantation crops in India. Arabica (*Coffea arabica*) and Robusta (*Coffea canefora*) are the two commercially grown coffee species in India. Cultivated area of Robusta and Arabica coffee was 226814 (49%) and 233081 hectares (51%), respectively in India (Anon., 2019). During 1951-61 the area under Arabica was 73 per cent as against only 23 per cent area under Robusta in India. The state of Karnataka is the largest Robusta coffee cultivator in India, with over 136 thousand hectares of cultivated area in 2019. Karnataka is the country's largest producer of coffee, accounting for more than 70 per cent (3 lakh tons) of India's production. Coffee production in India is hampered by various insect pests such as white stem borer, shot-hole borer, berry borer and mealy bugs.

The coffee white stem borer (CWSB), *Xylotrechus quadripes* (Chevrolat) is one of the most destructive pests of Arabica coffee plants. While, Robusta coffee is free from CWSB attack for a long time. The CWSB larvae bore into the stem of the plant which resulting in the death of young plants. The older plants though survive, but the yield is drastically reduced and secondarily prone to diseases. The adults are slender, elongate beetles (2-2.5cm in length) with a pair of long antennae. The forewings are black with characteristic white bands. Males are generally smaller than females (Anon., 2014). Stokes (1838) reported the first incidence of CWSB from Karnataka in India. The pest causes crop losses up to 93.6 percent in plantations with poor and untimely management (Basavaraj *et al.*, 2005).

Though the Arabica coffee is the most preferred and principal host, it also rarely attack and breed on other host plants like Robusta coffee, tree coffee, teak, *Olea* sp., etc. The incidence and emergence of adult of CWSB was noticed for the first time on the Robusta coffee

plants in three estates in Coorg region. Even though the infestation was noticed in Robusta, the life cycle was not completed as evidence of no adult emergence in most of the places except Polibetta area of Coorg. In this context, the presence of species complex was suspected in borer infested Robusta coffee in Coorg region. Hence, the present study was conducted to confirm the infestation of CWSB on Robusta and possible variations compared to CWSB infesting Arabica coffee through DNA barcoding as DNA barcoding is generally considered as a reliable, cost-effective and easy molecular identification tool with a wide applicability across metazoan taxa (Hebert *et al.*, 2003; Hajibabaei *et al.*, 2007).

MATERIALS AND METHODS

Collection of CWSB beetles infesting Robusta coffee

During 2019, three adults of *X. quadripes* infesting robusta coffee from Coorg and 15 adults infesting arabica coffee from different locations (eight from Chikkamaglur, three each from Coorg, and Hassan districts of Karnataka, two from Tamilnadu and one from Andrapradesh) were used for DNA analysis. The CWSB infested robusta and arabica stems were collected from field and brought to the laboratory caged under net for emergence of adults (Fig.1-6). The emerged adults were preserved in 70 percent ethyl alcohol until they were used for molecular studies

CWSB genomic DNA isolation

Total genomic DNA was extracted by using the Cetyl Trimethyl Ammonium Bromide (CTAB) method with some modification. The *X. quadripes* specimens were washed with sterile distilled water to remove alcohol prior to homogenization. Rest of the collected specimens were used as voucher specimen and deposited in the Department of Entomology, Regional Coffee Research Station, Balehonnur, Karnataka. The adult beetles

Table 1: Per cent pair wise nucleotide identity of MCoI gene sequences of *X quadripes* infecting coffee with other MCoI gene sequences of stem borers available in the NCBI database

Insect species	Accession numbers	MH758752	MH758751	MH758750	MH758749	MH758748	MH75874	MH758746	MH758745	MH758744	MH758743	MH758742	MH758741	MH758740	MH758739	MH758738
X. quadripes-	KY379322	99.6	996	99.6	99.6	996	996	100	99.6	996	100	90.5	99.6	99.6	99.6	99.6
X. quadripes	KY379321	99.3	99.3	99.3	99.3	99.3	99.3	99.6	99.3	99.3	99.6	90.2	99.3	99.3	99.3	99.3
X. quadripes	KY379320	99.6	99.6	99.6	99.6	99.6	99.6	100	99.6	99.6	100	90.5	99.6	99.6	99.6	99.6
X. quadripes	KU321048	91.0	91.0	91.0	91.0	91.0	91.0	91.3	91.0	91.0	91.3	82.7	91.0	91.0	91.0	91.0
X. quadripes	KU321045	91.0	91.0	91.0	91.0	91.0	91.0	91.3	91.0	91.0	91.3	82.7	91.0	91.0	91.0	91.0
X. quadripe-	MZ379256	95.4	95.4	95.4	95.4	95.4	94.8	95.1	94.8	94.8	95.1	86.7	94.8	94.8	94.8	94.8
X. quadripe	MW008763	99.5	99.5	99.5	99.5	99.5	99.5	99.8	99.5	99.5	99.8	90.4	99.5	99.5	99.5	99.5
X. quadripes	MW008762	99.3	99.3	99.3	99.3	99.3	99.3	99.6	99.3	99.3	99.6	90.2	99.3	99.3	99.3	99.3
X. chinensis	MK098127	87.0	87.0	87.0	87.0	87.0	87.0	87.3	87.0	87.0	87.3	80.3	87.0	87.0	87.0	87.0
X. yanoi	MN905265	79.7	79.7	79.7	79.7	79.7	79.7	80.0	79.7	79.7	80.0	75.1	79.7	79.7	79.7	79.7
X. antilope	KM446368	85.7	85.7	85.7	85.7	85.7	85.7	86.0	85.7	85.7	86.0	78.1	85.7	85.7	85.7	85.7
X. colonus-	MN315194	85.8	85.8	85.8	85.8	85.8	85.8	86.1	85.8	85.8	86.1	79.7	85.8	85.8	85.8	85.8
X. antilope	KM446967	77.9	77.9	77.9	77.9	77.9	77.9	78.2	77.9	77.9	78.2	70.3	77.9	77.9	77.9	77.9
X. grayii	MN905261	85.9	85.9	85.9	85.9	85.9	85.9	86.2	85.9	85.9	86.2	79.7	85.9	85.9	85.9	85.9
X. pantherinu	KJ966163	85.5	85.5	85.5	85.5	85.5	85.5	85.8	85.5	85.5	85.8	80.2	85.5	85.5	85.5	85.5
X. buqueti	KY357555	85.4	85.4	85.4	85.4	85.4	85.4	85.7	85.4	85.4	85.7	79.7	85.4	85.4	85.4	85.4
X. undulatus-	KM845651	84.0	84.0	84.0	84.0	84.0	84.0	84.3	84.0	84.0	84.3	78.2	84.0	84.0	84.0	84.0
X. buqueti-	KY357555	85.4	85.4	85.4	85.4	85.4	85.4	85.7	85.4	85.4	85.7	79.7	85.4	85.4	85.4	85.4
X. arvicola-	KU915557	84.9	84.9	84.9	84.9	84.9	84.9	85.2	84.9	84.9	85.2	78.8	84.9	84.9	84.9	84.9
X. yanoi	MN905265	79.7	79.7	79.7	79.7	79.7	79.7	80.0	79.7	79.7	80.0	75.1	79.7	79.7	79.7	79.7
X. undulatus	KM846304	84.1	84.1	84.1	84.1	84.1	84.1	84.4	84.1	84.1	84.4	78.4	84.1	84.1	84.1	84.1
Rusticoclytus rusticus	MN315200	83.7	83.7	83.7	83.7	83.7	83.7	84.0	83.7	83.7	84.0	78.4	83.7	83.7	83.7	83.7

were grounded in liquid nitrogen using a mortar and pestle. The sample material was collected in 1.5 mL microcentrifuge tube and re-suspended in 300 µl lysis buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 0.02M ethylene di amine tetra acetic acid, 2X CTAB, 2X PVP and 2 µl β-mercaptoethanol was added to the extraction buffer just prior to use). The homogenised mixture was incubated at 65°C for 15 min. Occasional mixing was done during incubation by inverting the tube. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added. Kept the homogenised mixture for 5-10 min at room temperature. The homogenate was centrifuged at 12000 rpm for 15 min. The supernatant was transferred into a new 1.5 mL centrifuge tube, followed by addition of equivalent volume of ice cold isopropanol. The tubes were centrifuged at 13000 rpm for 15 min. The pellet was dried and dissolved in 30µl of TE buffer (1M Tris, 0.5M EDTA pH 8). The quality and quantity of sample genomic DNA was determined by using NanoDrop spectrophotometer and agarose gel electrophoresis.

The DNA obtained from *X. quadripes* beetle samples was used for amplifying a portion of mitochondrial *mtCOI* gene fragment, using forward primer (**F:** 5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer (**CO1 R:**5'-TAAACTTCAGGGTGACCAAAAAATCA-3')(Folmer *et al.,* 1994) .Each reaction mixture of 25 µl consisted of 2.5 µl of 10X PCR buffer, 2.0 µl $MgCl_2$ (2.5 mM), 0.2 µl dNTPs (200 µM), 0.5 µl of *Taq* Polymerase (3U/ µl), 1 µl of each of forward and reverse primer sequences, 1 µl of DNA, and 16.3 µl of distilled water. The amplification was carried out in thermal-cycler (Peqlab, Germany), following PCR conditions of denaturation at 94°C for 60 sec; annealing at 47°C for 45 sec and extension at 72°C for 50 sec (30 cycles, plus an initial denaturation at 94°C for 3 min and a final extension at 72°C for 10 min). The PCR negative control contained the identical amount of PCR mixture with 3 µl distilled water instead of DNA template. A PCR positive control was also included, containing the PCR mixture plus DNA that had been successfully put through the PCR reaction on previous studies in the laboratory. To ensure repeatability, three replications for each of the reactions were conducted. The *X. quadripes* amplified mtCOI products were resolved on 1.5% agarose gel, stained with ethidium bromide (10 µg/ml) to check for successful amplification and visualized in a gel documentation system.

Cloning of CWSB mtCOI PCR fragments and sequencing

The PCR-generated Robusta *X. quadripes* mtCOI amplicons were eluted using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Germany), ligated to pTZ57R vector with 3'-ddT overhangs for TA cloning with blue/white screening according to manufacturer's instructions and cloned in *Escherichia coli* competent cells, strain DH5α (Sambrook and Russell, 2001). Cloning was confirmed by colony PCR, plasmid mobility check and restriction analysis of recombinant plasmid DNA containing *X. quadripes* infesting Robusta coffee mtCOI. Plasmid DNA was isolated from an overnight bacterial culture, using a reliable plasmid miniprep method (Gene JET Plasmid Miniprep Kit, #K0502 - Thermo Fisher Scientific, USA) according to kit guidelines. Selected recombinant plasmids were sequenced using M13F/R primers. At least three independent clones per sample were sequenced.

CWSB mtCOI Sequence analysis

Using DNASTAR software (DNASTAR, Madison, Wisconsin, USA), *X. quadripes* mtCOI, gene chromatograms were tested, assembled and edited. With BIOEDIT version 7.0.9.0, the primer sequences were removed manually. Multiple sequence alignments were performed by CLUSTAL W. In order to ensure the correct target gene fragment, the Basic Local Alignment Search Tool (BLAST) was checked for all sequences in the NCBI database.Using MEGAX and DNA to protein translation (http://insilico.ehu.es/translate/) nucleotide sequences were converted into amino acids. This was done to ensure that stop codons and pseudo genes did not exist (Kumar *et al.*, 2016). *X. quadripes* mtCOI sequences having reference sequences in the NCBI-GenBank alone were used in the homology analysis. The *X. quadripes* mtCOI sequences deposited in the NCBI-GenBank and BOLD were included in the homology analysis.

Homology search

NCBI-Basic Local Alignment Search Tool (BLAST 2.2.20) was performed with mega blast as the default settings against the non-redundant nucleotide collections of NCBI database. All the study sequences were used as queries and the homologous sequences were identified. BLAST was run to determine the identity of the query sequences by comparing with the known DNA sequences in the GenBank records.

Genetic distance and Phylogenetic analysis

Genetic distances of each Robusta and Arabica *X. quadripes* were conducted in MEGAX (Kumar *et al.*, 2016) using Kimura 2 Parameter and *p*- distance model. All the ambiguous positions were removed for each sequence pair. Codon positions, first, second, third and non-coding sequences were included. Phylogenetic analysis was conducted in MEGAX (Kumar *et al.*, 2016) using Neighbour-Joining method (NJ) and Maximum likelihood method (ML) (Saitou and Nei, 1987). Bootstrap replicates were set to 1000 replicates. Codon

Fig. 1-6. Coffee white stem borer *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae) infested Robusta coffee (*Coffea canephora*) garden Polibetta, Coorg. 1-Collection of adult beetles; 2 & 3- Tunneling behavior of *X. quadripes* infestation in Robusta coffee plant; 4- Adult of *X. quadripes* ready for the emergence. 5 & 6- Adult of *X. quadripes* emerged from Robusta coffee stems characterized by a hole. The collected beetles were used for laboratory studies.

position first, second, third and non-coding sequences were included in the analysis. Gaps were treated through partial deletion. All the *X. quadripes* mtCOI sequences from GenBank, NCBI were extracted in FASTA format. MUSCLE tool was used to align these nucleotide sequences.

RESULTS

Morphological variations in coffee stem borer

The morphological variation was observed in adult CWSB beetles infesting robusta coffee. Coffee stem borer was identified as *X. quadripes* based on the taxonomic keys published by Hiremath (2015). By studying adult beetle morphology, the pest was recognized as *X. quadripes*. The morphology of *X. quadripes* beetles was identical to that of Arabica. The beetles also exhibited characteristically long antennae of the family Cerambycidae. Both male and female beetles were identified. The presence of the ovipositor identified the females (Seetharama *et al.*, 2005). There were no morphological differences in coffee stem borer adults collected from Arabica and Robusta.

CWSB molecular identification and mtCOI sequence analysis

The genomic DNA from the individual specimen of *X. quadripes* was successfully isolated. The genomic DNA concentration of *X. quadripes* ranged from 615.9 to 1110.6 ng/µL. The absorption ratios of 260:280 met pure DNA criteria and ranged from 1.79 to 1.82.

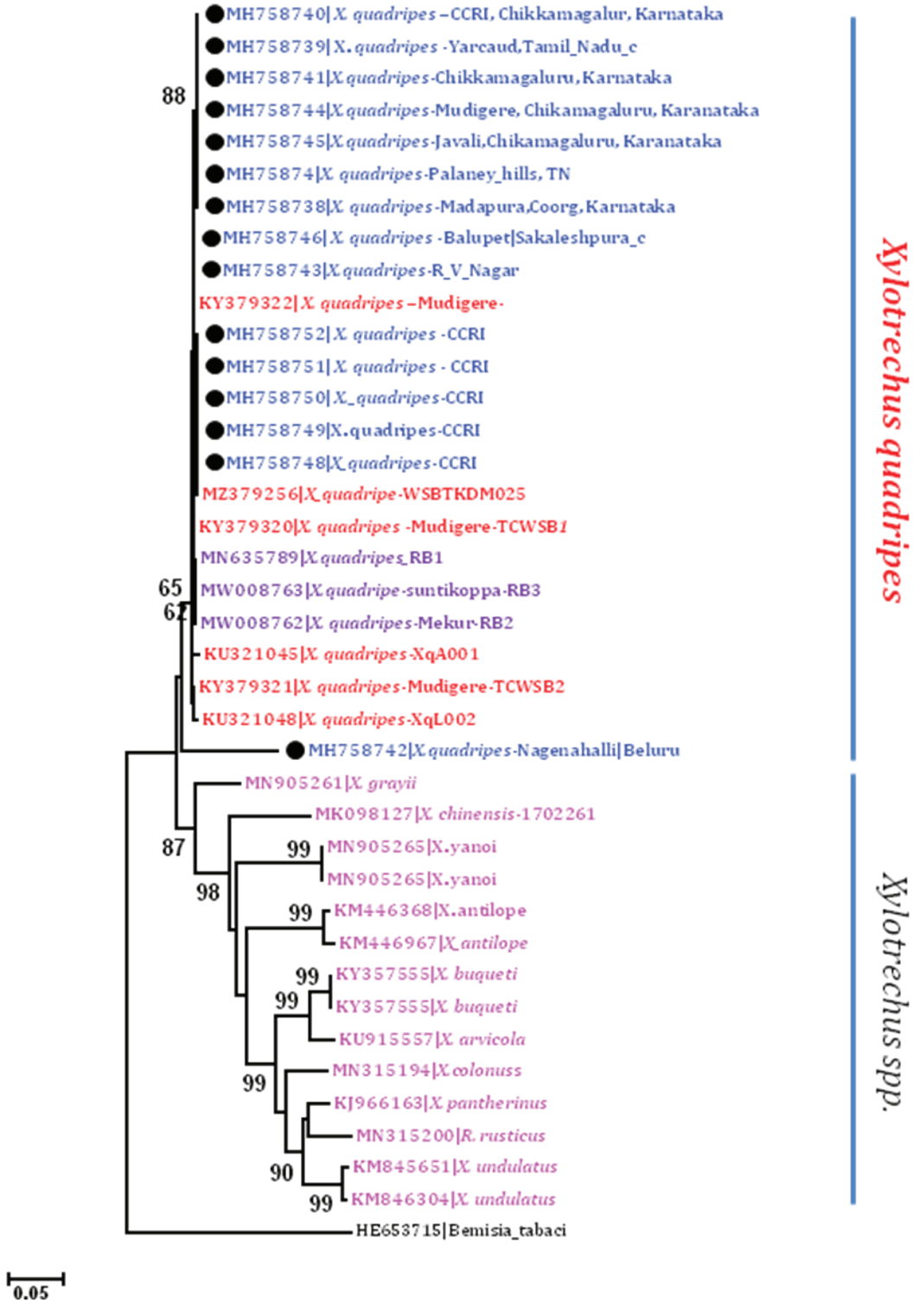

Fig. 7. Cladogram derived from analyses of the mitochondrial cytochrome oxidase subunit I (mtCOI) marker demonstrating the phylogenetic relationships based on neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML), of the combined dataset (658bp) under partition strategies. Evolutionary analyses were conducted in phylogeny.fr. NCBI HE653715 *Bemisia tabaci* served as an out group.

According to the manufacturer's protocol, the PCR products resulting from the amplification of *X. quadripes* mtCOI genes were eluted and cloned to InsTA cloning vector pTZ57R/T (Thermo Scientific, USA). The *X. quadripes* mtCOI, genes were successfully sequenced. The *X. quadripes* mtCOI plasmid comparison of the triplicate sequences for respective *X. quadripes* showed no mismatches, thus no sequencing errors. After alignment and trim, *X. quadripes* mtCOI sequences was 658 in length. Evidence of nuclear copies was not found in any of the sequences subjected to analyses, which was supported by the absence of stop codons and the base composition was similar with no indels. *X. quadripes* nucleotide sequences was deposited in GenBank for the first time. Using MEGAX (Molecular Evolutionary Genetics Analysis), phylogenetic analysis was carried out. The aligned sequences, accessible *X. quadripes* sequences in NCBI GenBank and Barcode of life (BOLD) have been used to build phylogenetic tree. The mtCoI gene sequences of 20 stem borer (*X. quadripes*) samples were characterized in the present study was compared with 19 mtCoI gene sequences of different *Xylotrechus* species retrieved from the NCBI database. The analysis showed that, 20 mtCoI gene sequences of stem borer (*X. quadripes*) collected in different location on coffee showed highest nucleotide identity of 90.1 to 100 % with stem borers infecting robusta and Arabica coffee and other crops reported from different places of south India (Table 2).Whereas different species of stem borer infecting diverse crops are showed very low sequence similarities with coffee infecting stem borers.

The evolutionary relationship of the mtCoI gene sequences of stem borer (*X. quadripes*) characterized in the present study together with 19 mtCoI gene sequences of different *Xylotrechus* species retrieved from the NCBI database were analysis using the neighbor joining method by MEGA X software (Kumar et al., 2016) with 1000 boot strapped replications using *Bemisia tabaci* (HE653715) as out group. The analysis showed that the mtCoI gene sequences of different stem borer samples are formed two major groups with supporting high bootstrap values.

The first group contains only coffee stem borers of Arabica and Robusta and all mtCoI gene sequences characterized in the present study are closed cluster with several stem borers (*X. quadripes*) infecting coffee reported so far in different places of Karnataka. The second group contains stem borers infecting of different crops are formed in to separate group. The analysis also showed that the stem borers (*X. quadripes*) infecting coffee are formed into monophyletic groups. Based on nucleotide identity and phylogentic analysis showed that the stem borers (*X. quadripes*) infecting coffee characterized in the present study have not much variations geographically and different host with respect mtCoI gene sequences. Further the phylogenetic tree showed that Robusta and Arabica CWSB populations are similar in gene sequence, being placed in one branch separated from the out-group (Fig. 7). The phylogenetic tree also emphasizes a very close genetic similarity among populations Robusta and Arabica CWSB.

DISCUSSION

In the present study, based on the fulfillment of the criteria, COI sequences of the study species were assigned to their taxonomic groups. This study contributes to a growing body of work that demonstrates the effectiveness of DNA barcodes in species identifications for members of the Class Robusta CWSB. The approach has now gained preliminary validation in all major lineages of ascidians. The current results extend prior barcoding investigations on ascidians by establishing its effectiveness in a group with an unusually high rate of mitochondrial evolution. Moreover, because of the limited intraspecific variation, an effective identification system can be created by analyzing just a few specimens of each species. However, Robusta coffee plant is resistant for CWSB, the beetles even feed initially but grubs won't develop in to adults but only in cut and dead stems of Robusta the CWSB completes its life cycle (Venkatesha *et al.* 1995). However, Kurian (2000) reported the emergence of adult beetles from Robusta plants from only Coorg. So from the study it is clear that the beetles found in Robusta is also *X. qudripes*. Further the factors responsible for infestation and completion of life cycle in robusta, the host plant intraction may a future line of work can be carried out.

There is no literature on molecular studies of *X. qudripes*, this is the first attempt to study the molecular aspects on this pest. Even there is no sequences available in NCBI data base expect very few from India only by Sridevi *et al.* (2016), and Gowda (2017).

CONCLUSION

According to our findings we can hypothesize and suggest that the phylogenetic tree analysis showed that the individuals of the same species clustered together based on the mtCOI sequence similarity, regardless of their collection host and site. The DNA barcoding for the CWSB identification revealed that identified species remains same for both Robusta and Arabica coffee. This rare incidence on Robusta coffee may be attributed to interplanting of Robusta with Arabica in those estates and also lower shade in Robusta coffee plantations. Further, decrease in shade probably could contribute for the rise in temperatures and ultimately favourable microclimate for the development of CWSB. Hence, in the interplanted Arabica and Robusta estates the more number of adult beetles emerged from Arabica plants and

may be responsible for increased incidence on Robusta plants.

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