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DNA bar-coding confirmed the occurrence of *Stromatium barbatum* (Fabricius) on grape vines in Karnataka, India

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Abstract
Sromatium barbatum (Fabricius) has wide distribution from its native Asia. Being a dry wood infesting species, it is capable of developing in more than 350 host species including conifer and hardwood trees as well as bamboo and some woody vines. During 2019, *S. Barbatum* was observed for the first time infesting grape vine orchards of Northern Karnataka (India). Nevertheless, no further information is hitherto available from other parts of the world on its pest status on grapevines except from State Maharashtra (India).Morphological data are usually time consuming and need specialists, DNA bar-coding techniques are a uniform and practical method of species identification of insects. Therefore, we characterized *S. barbatum* at the molecular level and developed species specific DNA barcodes by using mitochondrial cytochrome oxidase gene. The sequences of *S. barbatum* have been deposited to the NCBI with accession numbers MT280795 and MT280796.Studies on other aspects of the pest are under progress.

Keywords: DNA bar coding, new pest, *Vitis vinifera, Stromatium barbatum*

1. Introduction
Grape (*Vitis sp.*) belonging to family Vitaceae is a commercially important fruit crop of India. It is a temperate crop which has got adapted to sub-tropical climate of peninsular India. In Karnataka, Vijayapura district ranked first with an area and production of 10,652 ha and 211.64 MT respectively (Anon, 2018) [1].

Many workers reported on the damage caused by Cerambycid beetles in vineyards from different parts of the world. [Galet, (1982) Azam (1979); Ashihara, (1982) Goodwin and Pettit (1994); Ocete and DelTio (1996) FAO(2001) Goodwin (2005)] [2, 3, 4, 5, 6, 7, 8].

In India, grapevine stem borer, *C. scabrator* was observed for the first time in Pune by Upasani and Phadnis (1968) [9] and subsequently in other parts of Maharashtra (Gandhale *et al.*, 1983) [10]; Mani *et al.*, 2008) [11] and 2012) [12], Andhra Pradesh (Azam, 1979) [3]; Rao *et al.*, 1979;) [13], Karnataka (Balikai *et al* 2003) [14]., Jagginavar *et al*., 2006) [15], Kariyanna *et al.* (2017) [16] and Sunitha *et al.* (2017) [17]] and Tamil Nadu (Chandrasekaran *et al.*, 1990) [18]].Till 2011 *C.scabrator* was the only Cerambycid stem borer reported on grapevines from India. Later Salini and Yadav (2011) [19] for the first time reported *S.barbatum* on grape vines in Maharastra state (19.75º N, 75.71º E.). The symptoms produced were different from another wood borer *C. scabrator*. The grubs of *S. barbatum* made winding tunnels by boring their way inside the wood. The tunnels were tightly packed with fine floury wood dust and excreta, which hampers the translocation of nutrients and in turn seriously reduces the bearing capacity or leading to complete drying of the affected cordons. The gnawing sound could be heard in the plantations where the infestation is severe. Pupation occurred inside the tunnel. The adults came out of the plants by making oval or near rectangular holes. The number of holes in one vine varied from 4-8 or even more than 8 occasionally. Presence of more than one larva was observed in all the examined cases. The pest was also found feeding on live green vines. Jadhav *et al*. (2017) [20] observed size polymorphism among the *S. barbatum* beetles and conducted morphometric analysis and Deoxyribonucleic Acid Bar-coding of *S.barbatum*. It was the first report on DNA bar coding of *S. barbatum* infesting grape vines. During 2019 grape orchards of Vijayapura district of Northern Karnataka State(13º 17' N and 77º 48'E) of variety Thompson Seedless on Dogridge rootstock were diagnosed with a cerambycid wood borer damage and live green grapevines were detected with large and numerous larval.

Galleries especially on branches and each branch of a vine harbored 4 - 12 grubs inside. (Figure 1). One could see the overlapping generations also. No external symptoms were observed immediately after the pest incidence, except for sound produced by the grub due to its feeding on wood. Other symptoms like cordons becoming weaker and nonbearing of fruit bunches were manifested only at the later stage.

Pest species must be correctly identified before adequate control measures are contemplated. Armed with identification, one can automatically know about the biology, distribution, bio ecology of the pest under study.

While morphological data are usually time consuming and need specialists, DNA bar-coding techniques are a uniform and practical method of species identification of insects and can be used for the identification of all developmental stages of insects, their food webs and biotypes and this may not be possible with morphology-based taxonomy. DNA bar-coding has the potential of being a valuable tool to biologists. (Jalali *et al*., 2015) [21]. DNA Bar-coding as a reliable tool for identification of Coleoptera was reported by Greenstone (2005) [22]. DNA bar- coding utilizes a short genetic sequence of about 650 base pairs from the cytochrome coxidase subunit1(COI) of the mitochondrial gene (Folmer *et al*., 1994) [23] and Jinbo *et al*., 2011) [24]. The high specificity of the COI region makes it an ideal tool for diagnostics (Herbert *et al*., (2004) [25] and Jalali *et al*., (2015) [21]. Owing to the seriousness of this new pest which resulted in 20.00 - 100.00% grape vine damage, the present investigation was undertaken to provide DNA barcode for the correct and quick identification of the new pest of grape vines in Karnataka state.

Fig 1AB: Destructive sampling to collect grubs of *S.barbatum*

Fig 1C: Grubs of *S. barbatum* collected from grapevines

2. Materials and Methods

The grubs of different sizes were collected from two orchards of Devarahipparagi village of Vijayapura district, Karnataka, India (16° 91' N, 76°23' E) during first week February 2020 randomly from infested grapevine branches of variety Thompson Seedless on Dogridge rootstock aged 8 years and spaced at 11 × 6 feet. Two hundred grubs were collected from each orchard and collected grubs were put separately into individual vials. From two hundred grubs of each orchard ten grubs were picked and put into perforated zip lock polythene bags of size 5 × 10 cm individually and live grubs were shifted to Barcode Biosciences an ISO9001: 2015 Certified Company, Bengaluru, Karnataka, India within 24 hours of collection for DNA bar-coding after labeling as sample one and sample two. Single grub from each sample was selected for extraction of DNA.

2.1 Experimental Method

DNA was isolated using MN (Macherey-Nagel) kit and its quality was evaluated on1.0% agarose gel. Fragment of COI gene was amplified by LCO (LCO-1490: GGT CAA CAAATC ATA AAG ATA TTG G and HCO (HCO-2198:TAA ACT TCA GGG TGA CCAAAA AAT CA) primers. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with LCO and HCO primers using BDT v3.1 Cycle sequencing kit on ABI 3730×l Genetic Analyzer. Consensus sequence of COI gene was generated from forward and reverse sequence data using aligner software. The C O I gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. (Figure 2 and Figure 3). Based on maximum identity score (Table 1 and Table 2) first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP data base and the phylogenetic tree was constructed using MEGA 6. (Figure 4 and Figure 5). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model). The tree with the highest log likelihood (- 2333.23) is shown. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor- Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11nucleotide sequences. Codon positions included were1st+2nd+3rd+Noncoding. There were a total of 819 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2]. [Tamura *et al*. (2013) Tamura and Nei (1993)] [26, 27].

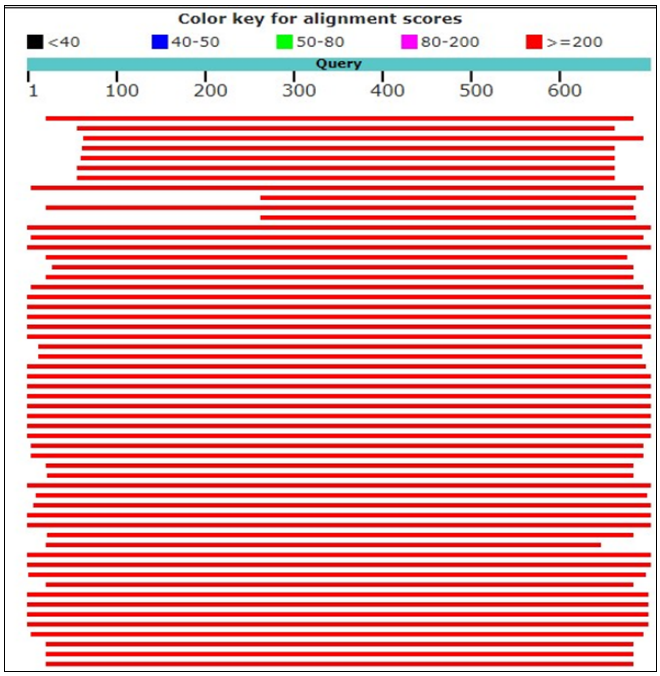

Fig 2: Distribution of 100 Blast Hits on the Query Sequence (sample 1)

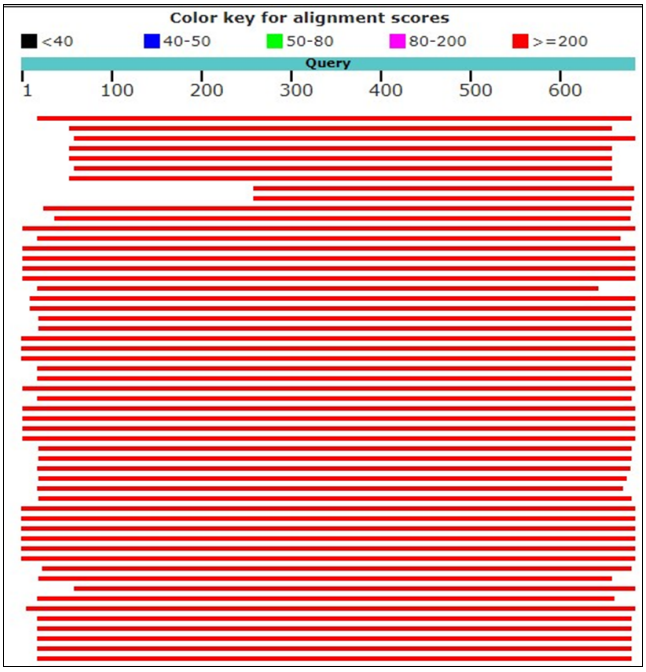

Fig 3: Distribution of 100 Blast Hits on the Query Sequence (Sample 2)

3. Results and Discussion

High molecular weight single band DNA was observed when evaluated on 1.0% agarose gel after isolating using MN kit. Fragment of COI gene, on amplification by LCO and HCO primers resulted in a single discrete PCR amplicon band of 700 bp when resolved on agarose gel. A BLASTN search of 700bp sequence as a query in NCBI for sample I showed 96.51-99.00% sequence match(Percent Identity) with *S. barbatum* sequences deposited at NCBI gene Bank [M921773.1(96.51%),KM921771.1(96.68%),MF939090.1(96.91%).MK689217.1(96.97%),KM921774.1(96.99%),KM921770.1(97.32%),MF939098.1(97.96%) and KM921772.1(99.00%)] Query sequence match was limited to 86.17% with *S. longicorne* (FJ558998.1) and 86.34% with *Stromatium* sp(KY357579.1) (Table 1) which indicated different species. Similarly BLASTN search for sample 2 showed 96.67-99.17% homology with *S. barbatum* sequences of NCBI gene bank Accession numbers KM921773.1(96.67%), KM921774.1(96.84%),MK689217.1(97.10%),KM921771.1(97.14%),KM921770.1(97.17%),MF939090.1(97.39%), MF939089.1(97.62%) and KM921772.1(99.17%).The query match of 84.98% (Accession number HM433527.1) and 85.25% (Accession number HQ559066.1) suggested the difference from *Anelaphus parallelus* and *Cerambycinae* sp respectivel (Table 2). Phylogenetic analysis (Fig 4 and Fig 5) confirmed the BLASTN search results and occurrence of *S. barbatum* on grape vines in Karnataka state (India). The sequences of *S. barbatum* have been deposited to the NCBI with accession numbers MT280795 and MT280796 and voucher specimen were deposited at Dept of Agricultural Entomology, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India

Table 1: Sequences producing significant alignments (Sample 1)

Description	Max Score	Total Score	Query Cover	E value	Percent Ident	Accession
Stromatium barbatum voucher NRCG-SB-3	1077	1077	86%	0	99.00%	KM921772.1
Stromatium barbatum voucher GB369_CERDBT13	1053	1053	89%	0	96.97%	MK689217.1
Stromatium barbatum voucher NRCG-SB-1	1013	1013	85%	0	97.32%	KM921770.1
Stromatium barbatum voucher NRCG-SB-5	1005	1005	85%	0	96.99%	KM921774.1
Stromatium barbatum voucher NRCG-SB-2	1000	1000	86%	0	96.68%	KM921771.1
Stromatium barbatum voucher NRCG-SB-4	994	994	86%	0	96.51%	KM921773.1
Stromatium longicorne voucher Cer0016	741	741	98%	0	86.17%	FJ558998.1
Stromatium barbatum isolate COL005	728	728	60%	0	97.86%	MF939089.1
Stromatium sp. CA12_3.02r	717	717	94%	0	86.34%	KY357579.1
Stromatium barbatum isolate COL006	706	706	60%	0	96.91%	MF939090.1

Table 2: Sequences producing significant alignments (Sample 2**)**

Description	Max Score	Total Score	Query Cover	E value	Percent Identity	Accession
Stromatium barbatum voucher NRCG-SB-3	1083	1083	88%	0	99.17%	KM921772.1
Stromatium barbatum voucher GB369_CERDBT13	1048	1048	91%	0	97.10%	MK689217.1
Stromatium barbatum voucher NRCG-SB-1	1016	1016	88%	0	97.17%	KM921770.1
Stromatium barbatum voucher NRCG-SB-5	1005	1005	88%	0	96.84%	KM921774.1
Stromatium barbatum voucher NRCG-SB-2	1005	1005	87%	0	97.14%	KM921771.1
Stromatium barbatum voucher NRCG-SB-4	1000	1000	88%	0	96.67%	KM921773.1
Stromatium barbatum isolate COL005	723	723	61%	0	97.62%	MF939089.1
Stromatium barbatum isolate COL006	717	717	61%	0	97.39%	MF939090.1
Cerambycinae sp. BOLD:AAM7695	671	671	95%	0	85.25%	HQ559066.1
Anelaphus parallelus voucher BIOUG	647	647	93%	0	84.98%	HM433527.1

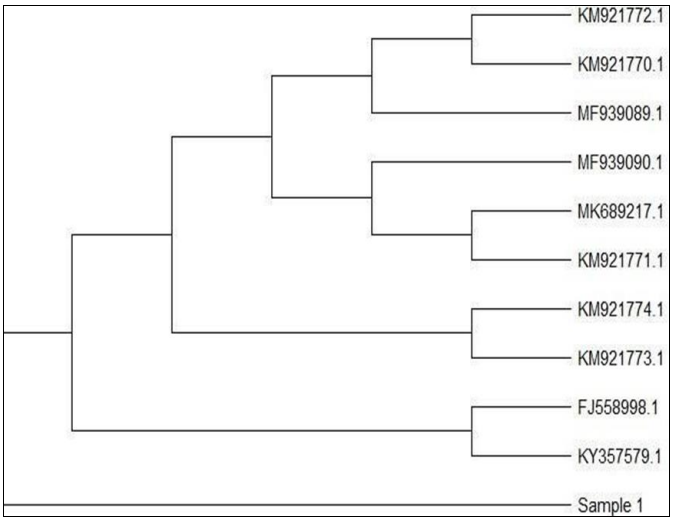

Fig 4: Molecular Phylogenetic analysis by Maximum Likelihood method (Sample 1)

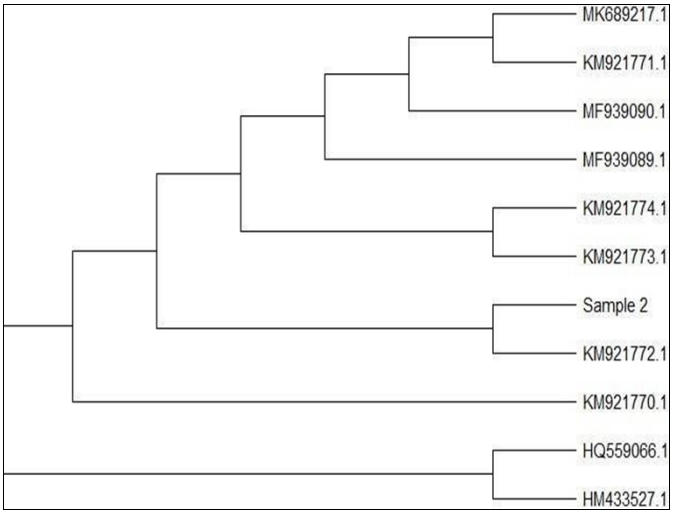

Fig 5: Molecular Phylogenetic analysis by Maximum Likelihood method (Sample 2)

Grape cultivation is one of the most profitable farming, not only in India but also at global level. Biotic and abiotic stresses play vital role on successful cultivation. Insect pests in general and cerambycid stem borers in particular pose a major threat to viticulture all over the world as reported by various workers. In most areas where grapes are grown, various endemic cerambycids will attack the vines (Linsley 1959) [28]. In the Lower Hunter Valley, New South Wales (NSW), an outbreak of a lamiine cerambycid, fig longicorn (Acalolepta vastator (Newman)), is causing serious damage to vineyards. (Goodwin and Pettit 1994) [5]. Some high-value vineyards in Spain are increasingly threatened by a xylophagous insect, Xylotrechus arvicola (Olivier), a polyphagous cerambycid that has become a grapevine pest during the last 10 years and damage caused by X. arvicola is severe both in terms of yield and wine composition. (Ocete *et al*.,2008) [29]. Mani *et al*. (2014) [30] created a worldwide list of pests of grape vine which indicated the occurrence of S.barbatum only in India. No reports are available on the same from other parts of the world.

The results of the investigation on occurrence of S.barbatum on grapevines are in line with the findings of Salini and Yadav (2011)) [19] and Jadhav *et al*. (2017) [20]. But no reports available on the exact quantitative and qualitative losses caused by this pest.

The present investigations on occurrence of this new pest and DNA bar coding of the pest resulted not only in quick and accurate identification of this new pest S. barbatum on grapevines in Karnataka State, one of the leading states in grape production but also revealed that the pest can cause 100.00 percent vine damage and significant yield loss in the absence of suitable prophylactic and control measures.

S. barbatum has wide distribution from its native Asia including Bangladesh, India, Myanmar, Nepal, Pakistan, Sri Lanka and Thailand as well as the Andaman and Nicobar Islands and the African countries of Somalia and Tanzania as well as various nearby islands in the Indian Ocean, including Madagascar, Mauritius, Reunion, Rodrigues, and the Seychelles (Vitali, 2015) [31]. Being a dry wood infesting

species, it is also capable of developing in a wide array of hosts, with more than 350 plant species, including both conifer and hardwood trees as well as bamboo and some woody vines such as grape(Duffy, 1968) [32] It is frequently transported in international trade (Cocquempot *et al*., 2014) [33]. According to Beeson and Bhatia (1939) [34] and Duffy (1953) [35], larvae of S. barbatum create powdery frass that is packed tightly in their galleries, which occur in both sapwood and heartwood. At times, the galleries are so numerous in individual pieces of wood that, only the exterior wood surfaces are left intact. S. barbatum is extremely polyphagous, with over 300 host plants recorded. It is of considerable economic importance because of its preference for seasoned timbers, being known to readily attack furniture, wooden structures, rafters, door and window frames, shelves and panels, etc. S.barbatum (Fabricius 1775), is able to develop to maturity in seasoned timber and is distributed worldwide due to human commerce. (M Jin *et al*., 2018) [36].

Added to this, this pest is having a very long life cycle from one several years on different hosts. For example, in India, Beeson and Bhatia (1939) [34] reported that adults of the same cohort emerged in one to seven years from the same host material, Albizia stipulata (DC.) Boivin, with most (93%) requiring two to four years. The longest development period reported was 10 years (Duffy, 1968) [32].

4. Conclusion

At the global level few species of cerambycid stem borers are increasingly threatening high value grape orchards. At present the *S. barbatum* is emerging as a major pest of grapevines in two leading grape producing states of India on Thompson seedless variety. Since it is able to develop to maturity in seasoned timber and is distributed worldwide due to human commerce, with nearly 350 alternate host plants and very long life cycle, it may turn out to be a pest on grapevines in other parts of the world also. Hence there is an urgent need to develop suitable management strategies before the epidemics of this pest occur in other parts of the country, through studies on possible routes of entry of this pest, bio ecology, alternate host plants and life tables. The present investigations also emphasize the need of strict quarantine measures both at regional and global level.

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