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Molecular Phylogeny of *Myllocerus viridanus* (Fabricius) by mitochondrial Cytochrome B gene Sequencing

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Abstract: **In order to create accurate control techniques against insect pests, correct taxonomic identification is critical. This paper describes the molecular phylogeny of Grey weevil, *Myllocerus viridanus* (Fabricius) by the sequencing of theirmitochondrial cytochrome B gene. The development of a simple and most elegant tools of DNA barcoding has made taxonomy and phylogeny studies a much facile process. *M.viridanus* is among the important polyphagous pests of plants of agricultural and horticultural importance. The sequencing of Cyt B gene yielded a 273 bp nucleotide product. Phylogentic analysis was carried out in Mega 11 software. GenBank deposition indicated the novel and first time records of cytochromeb gene barcode of *M.viridanus* and first time deposit of a species from the genus *Myllocerus*. The deposit was provided with an accession number OM223090. Further analysis showed that the partial sequence of mitochondrial cytochrome B of *M.viridanus* has 88.89% similarity with that of *Romualdius bifoveolatus* (KX087356) .The identity of *M.viridanus*(OM223090)ranged from 88.17 to 83.68% when nucleotide BLASTwas carried out with nearest 21 species of curculionids (NCBI). The result indicated that cytochrome b gene barcoding can be successfully used to resolve species level identification of the members of the family Curculionidae and the genus *Myllocerus* of the order Coleoptera. Thus mitochondrial Cytochrome b gene like mitochondrial COX 1 gene ,can be used as an efficient tool for the species level identification of the members of Coleopterans. Further interpretation on phylogeny was done by constructing neighbour joining tree using Kimura -2 Parameter. This approach was very informative and it helped in the better understanding of genetic variation among closely related species of Curculionids.**

***Index Terms*: Cytochrome B, DNA barcoding, *Myllocerus viridanus* (Fabricius), Phylogeny.**

I. I.INTRODUCTION

Weevil species of the genus *Myllocerus* (Curculionidae: Coleoptera) are often envisioned as plant pests of agricultural and horticultural significance which damage seeds, fruits, grains and leaves of crops (Khairmode & Sathe,2013). According to O'Brien *et al* (2006) the genus *Myllocerus* harbors a total of 336 species spread all over from Asia especially from south East Asia, the Indian subcontinent, Africa, Australia, the Palearctic and North America. From Indian subcontinent Ramamurthy and Ghai (1988) reported 73 species of *Myllocerus*.Most of them enjoy a broad spectrum of host range and hence they are all polyphagous. In India, several species of *Myllocerus* including cotton grey weevil (*Myllocerus undecimpustulatus* subsp. maculosusDebrochers des. Loges), apple weevil (*Myllocerus discolor* Boheman), almond weevil (*Myllocerus letivirens*Marshall), coconut ash weevil (*Myllocerus curvicornis* (F)) cocoa green leaf weevil (*Myllocerus viridanus* (Fabricius)) and mango leaf weevil (*Myllocerus sabulosus* Marshall) has established themselves as defoliators and very so often as significant pest (Joseph rajkumar et al., 2011).

Myllocerus viridanus (Fabricius) (fig.1 a-d) is a distinguished plant defoliator with broad host range comprising several plants of economic importance like cash crops, forest trees, agriculture crops and shrubby vegetation (Ahmad, 1989). *M.viridanus* was reported at first in many localities in Kerala and Tamil Nadu from teak plantations (Stebbing, 1914).Later defoliations by *M.viridanus* were reported on *Ipomea batatus* (L.) Lam.

(Rajamma, 1982), *Rosa odorata (*Andrews) Sweet (Tewari, 1983), *Citrus reticulata* Blanco and *Citrus aurantifolia* (Christm.) Swingle (Nagalingam, 1983). Report by Ahmed (1989) revealed the infestation of *M.viridanus* on *Acacia auriculiformis* Benth, *Anacardium occidentale* L., *Calliandra calothyrsus* Meisen, *Cassia auriculata* L., *Cassia fistula* L., *Cassia hirsuta* L., *Cassia tora* L., *Cassia. aurantifolia, Citrus reticulate* Blanco, *Eucalyptus robusta* Smith, *Eugea gambolana* Lam., *Eupatorium odoratum* L., *Helianthus annus* L., *Helicteres isora* L., *Ipomea batatus* (L.) Lam., *Ponagamia pinnata* L, *Populus deltoids* W. Bartram ex Marshall, *Sapindus tripliatus* L., *Solanum violacium* Ortega, *Tamarindus indica* L. and *Tectona grandis L.* A study by Hanumanthaswamy and Rajagopal (1995) reported *M.viridanus* as a pest of *Gloriosa superba* L. It was also reported on mulberry, *Morus alba* L. (Jayaswal et al, 1997).Based on field surveys conducted throughout in Padannakkad, Kerala, Rajan & Ghosh (2019) revealed 21 plant species from 13 families as new hosts of *M.viridanus*. Of these 13 plants were new report to India which included medicinal plants like Jackal jujube, Jelly leaf, Kamala tree, tick-trefoil, Schizomussaenda, common wire weed and curry leaves, ornamental plants like *Senna* sp and cup and saucer plant and trees like sandpaper tree, West Indian cherry, Macaranga and flame tree.

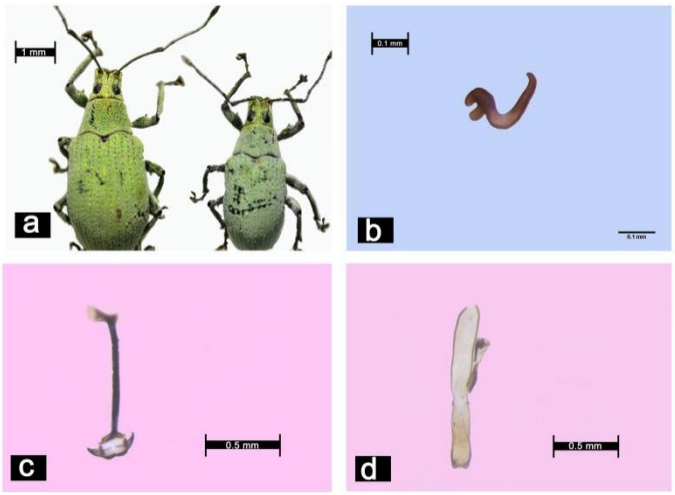

Figure 1 (a-d)

Figure 1.a:*Myllocerus viridanus adult*(female-left and male - right;1b:Spertheca(female);1c:Spiculam gastrae(male);1d: Aedeagus(male)

For years experts were in search of a silver bullet for accurate taxonomic identification that would be helpful even in the recognition of exotic pests as in biodiversity, community ecology, bio-monitoring, and systematic investigations. (Glover et al., 2010; Rivera & Currie, 2009).DNA barcoding is a multifunctional method that can improve taxonomic studies, and when combined with traditional taxonomy, it can become an indispensable and demanding tool for identifying species and assisting in their conservation (Hebert et al., 2003). Factors like phenotypic variation, developmental stages, and so on create hurdles for morphological feature-based identification systems (Murugan etal., 2016).The introduction of invasive species might have negative economic and environmental implications (Pimentel et al., 2000, 2005). The invasion of *Erionota toru*s Evans in various parts of Kerala has been studied using mitochondrial COX 1 gene barcode (Abdul and Ghosh, 2021).Mitochondrial Cytochrome Oxidase 1 gene barcode has been effictively used for species level identification of *Erionota* (Jaleeletal.,2019)and*Myllocerusviridanus* (Coleoptera :Curculionidae) (Ranjini et al., 2019, 2020 .The correct identification of the species is highly necessary for the successful management of the pest.

Struggling to overpower the insects that have undesired effects on crops and developing management strategies to control them depends entirely on the possibility of accurate and rapid identification. Factors such as the range and reliability of accessible barcode libraries often affect the accuracy of species identification. The lack of reference data is one of the main defect encountered in DNA barcode-based identification. The integration of different types of information of the species, including ecological, morphological, physiological, and molecular data, will improve species identification and accountability (Ekrem et al., 2007; Virgilio et al.,2010; Waugh,2007; Padial et al., 2010).

Even though phylogeny analysis studies of insects using COI gene were largely done, review of literature indicated that considerable progress has not been made in this field using the partial sequencing of mitochondrial Cytochrome b, since the literature available were scarce. Among the available literature, there are a few that are worth noting. Simmons & Weller, (2001) studied the degree of phylogenetic resolution portrayed by Cyt-b to analyse the relationships between genera within the tiger moth tribes Ctenuchini and Euchromiini and found that thesequence variation and A/T bias of Cytochrome b and COI are almost same. Ndong et al., (2015) compared the potential of the two genes Cytochrome b and COI in identifying and analysing the intra-specific variation of two species of *Sitophilu*s genus viz S*itophilus zeamais* Motschulsky and *Sitophilus oryzae* L.(Coleoptera: Curculionidae)and reported that the cyt-b has proven to be effective as a gene marker in studying the intra-specific variability, as it shows high haplotype and nucleotide diversity. Seddigh, & Darabi (2018) reported the structural, functional and phylogenetic analysis of cytochrome b (cytb) in selected insectsby interpreting the neighbor joining tree and concluded that several insects shared a common ancestor as they have high identity in their cytochrome b.

For putting the adequate management strategies into practice, an identification system which is rapid and accessible at a global level is vital. In the current study we studied the phylogeny of *M.viridanus* (Coleoptera:Curculionidae) based on DNA sequencing of cytochrome b and further analysed its importance in curculionid taxonomy.

II. II MATERIALS AND METHODS

A. I)SPECIMEN COLLECTION

Adult weevils were collected from Acacia trees available in in Edat (12.1032° N, 75.2337° E), Payyanur of Kannur district. Specimens were collected by handpicking method and the collected insects were kept in clean and neat bottles. In the lab they were stored at -20^0 C for DNA extraction and as voucher specimen for future reference.

B. II)GENOMIC DNA ISOLATION

The genomic DNA of the weevil was isolated from the whole body of the weevil as the insect was small. Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. The quality of the DNA thus isolated was checked using agarose gel electrophoresis.1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose inTE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarosegel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidiumbromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel.Gels were visualised in UV trans illuminator

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR profile of temperature comprised of an initial denaturation of 95 ^{o}C for 5 minutes followed by 35 cycles at 95^o C /30 sec for denaturation, an annealing temperature of 48^o C for 40 seconds and elongation step at 72^0 C for 60 seconds. The final extension step was at 72^{o}C for 7 minutes.Universal primers of cytochrome B were used which include forward Mcb 398 5^{1}TACCATGAGGACAAATATCATTCTG3^1 and reverse Mcb869 5^{1}CCTCCT-AGTTTGTTAGGGATTGATCG 3^1 (Verma and Singh2002). The reaction mixture of PCR consisted of 0.25µL each of forward and reverse primers , genomic DNA in 1 µl, 2.5 µl reaction buffer 2.5 µl of dNTPs, 0.20 µl Taq polymerase and sterile water in a volume of 16.8 µl. Using Mo Bio Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) the PCR amplified product was column purified following the Manufacturer's instructions.

The amplified and purified PCR product was then resolved in 1.2% TAE Agarose Gel stained with Ethidium bromide and visualized on gel documentation system with trans illuminator.

Exo SAP -IT treatment

Unwanted primers and dNTPS from PCR product were removed by ExoSAP-IT (GE Health care) treatment.It consists of two hydrolytic enzymes ,Exonuclease I and shrimp alkaline Phosphatase(SAP) in a specially formulated buffer .

5 microlitres of PCR product is mixed with 0.5 microlitres of ExoSAP-IT and incubated at 37^{0}Cfor 15 minutes followed by enzyme inactivation at 85^0 C for 5 minutes .

III) SEQUENCING AND EVOLUTIONARY ANALYSIS

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. After sequencing both sequences were trimmed and assembled in Sequencher (Gene Codes Corporation, Arbor, MI, USA) to get a consensus sequences.

The quality of the sequence was checked using Sequence Scanner Software v1(Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010).

These sequences were then deposited in NCBI GenBank (KU871376&MG021103) aligned and the consensus sequence was used for analysis. The evolutionary analysis was done using theNCBInucleotide BLAST(http://www.ncbi.nlm.nih.gov/nucleotide/).Intra and interspecific distances were measured in MEGA XI (Tamura *et al.*,2021).

III. III RESULT AND DISCUSSION

The sequencing of mitochondrial cytochrome B with the universal primer yielded a 273 bp consensus sequence of DNA. The GenBank deposition of the sequence in NCBI indicated the first ever and new report of *M.viridanus* and was provided with accession number OM223090. Percentage of identity of the nucleotide sequences were interpreted with the help of BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi.). Genbank analysis revealed that the sequence *M.viridanus*has 88.89% identity with the cytochrome b sequence of *Romualdius bifoveolatus* (KX087356, Hunter,et al . 2017a) reported from London, United Kingdom . The nucleotide BLAST alignment of the Cytochrome b coding sequence of *Myllocerus viridanus* (OM223090) showed that 262 nucleotides of *M.viridanus* aligned from

12085th nucleotide to 12346th nucleotide of *Romualdius bifoveolatus*(accession no. KX087356) from GenBank nucleotide database, a complete mitochondrial genome sequence having16327 nucleotides.Thus the alignment showed 88.17%identity with the cytochrome b part of the genome of *Romualdius bifoveolatus.* (fig.2)

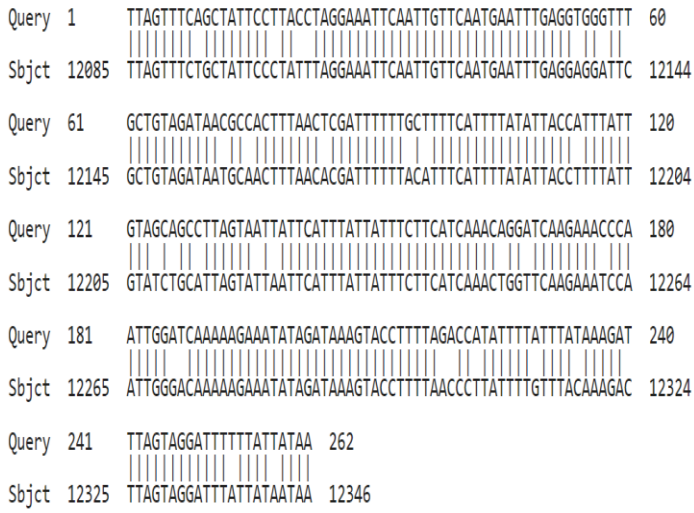

Fig.2 The nucleotide BLAST alignment the Cytochrome b partial coding sequence of *M.viridanus* (262 nucleotides) with the complete mitochondrial genome sequence (16327 nucleotides) of *Romualdius bifoveolatus*

For further analysis of phylogeny, sequences of *M. viridanus* and 25 other sequences that showed 83% to 88.89% identity in BLAST analysis were retrieved from NCBI .88.89 % was the highest %identity available for cytochrome b gene in Genbank NCBI blast for Curculionid species . The sequences are enlisted in table 1.

SI No	Insect name (family)	NCBI Accession number,(Authors)
1	*Romualdius bifoveolatus* (Curculionidae)	KX087356 (Hunter,et al ., 2017a)
2	*Bothrometopus daviesi* (Curculionidae)	MT701332 (Baird etal.,2021)
3	*Bothrometopus elongatus* (Curculionidae)	MT701341 (Baird et al.,2021)
4	*Bothrometopus randi* (Curculionidae)	MT701364(Baird et al.,2021)
5	*Ectemnorhinus viridis* (Curculionidae)	MT701400 (Baird et al.,2021)
6	*Bothrometopus gravis* (Curculionidae)	MT701350 (Baird et al.,2021)
7	*Bothrometopus crozetensis* (Curculionidae)	MT701331(Baird et al.,2021)
8	*Bothrometopus parvulus* (Curculionidae)	MT701358 (Baird et al.,2021)
9	*Bothrometopus gracilipes* (Curculionidae)	MT701349 (Baird et al.,2021)
10	*Palirhoeus eatoni* (Curculionidae)	MT701410(Baird et al.,2021)
11	*Pantomorus viridicans*(Curculionidae)	GU565287 (Rosas et al., 2011)
12	*Pantomorus stupidus*(Curculionidae)	GU565283 (Rosas et al., 2011)
13	*Hypera meles (Curculionidae)*	KY796721 (Sanaei and Kim 2017).
14	*Listronotus bonariensis* (Curculionidae)	KF905632 (Winder 2013)
15	*Bothrometopus angusticollis*(Curculionidae)	MT701322 (Baird et al.,2021)
16	*Bothrometopus fasciatus* (Curculionidae)	MT701343 (Baird et al.,2021)
17	*Myllocerinus aurolineatus* (Curculionidae)	NC040931 (Chen et al.,2019)
18	*Polydrusus inustus* (Curculionidae)	FJ442927 (Kajtoch et al., 2008)
19	*Brachypera zoilus* (Curculionidae)	KY796938 (Sanaei and Kim 2017 b)
20	*Conicobruchus flabellicornis* (Chrysomelidae)	KM378120 (Le Ru et al.,2014)
21	*Melanterius interstitialis* (Curculionidae)	KP774188 Pinzon Navarro (2015)
22	*Hypera viciae* (Curculionidae)	KY796942 (Sanaei, E. and Kim,I.2017a)
23	*Micrambe villosus* (Cryptophagidae)	KX087317 (Hunter et al.,2017b)
24	*Staphylinidae sp* (Staphylinidae)	KP774187 (Pinzon Navarro 2016)
25	*Bruchidius cinerascens* (Chrysomelidae)	KT696199 (Andujar et al., 2016)

Table 1 Curculionid Species showinghighest % identity with *M.viridanus*(in the descending order) retrieved from NCBI with Genbank accession numbers and name of the authors

Twenty five Species retrieved fron NCBI based on the nucleotide blast of *Myllocerus viridanus* (Fabricius)showing highest identity of 88.17 to lowest identity of 83.68 % in their descending order are as follows. *Romualdius bifoveolatus*(Beck), *Bothrometopusm daviesi* Chown&Kuschel) ,*Bothrometopus elongatus*(Jeannel),*Bothrometopus randi* (Jeannel), *Ectemnorhinus viridis*(Waterhouse) ,*Bothrometopus gravis* (Chown&Kuschel),*Bothrometopus crozetensis*(Enderlein), *Bothrometopus parvulus* (Waterhouse),*Bothrometopus gracilipes*(Waterhouse),*Palirhoeuseatoni* (Waterhouse), *Pantomorus viridicans* Sharp ,*Pantomorus stupidusn*Boheman, *Hypera meles* (Fabricius),*Listronotus bonariensis*(Kuschd),

Bothrometopus angusticollis (Waterhouse),*Bothrometopus fasciatus* Jeannel,*Myllocerinus aurolineatus* (Voss) *Polydrusus inustus* Germar,,*Brachypera zoilus* (Scopoli),*Conicobruchus flabellicornis* (Bohemann) *Melanterius interstitialis* Lea,*Hypera viciae* (Gallenhall),*Micrambe villosus*(Heer),*Staphylinidae sp,Bruchidius cinerascens*(Gyllenhal).All the twenty five species belong to the order Coleoptera .Of these first 19 species followed by 21st ,and 22nd species belong to the family Curculionidae, 20th and 25th species (*Conicobruchus flabellicornis* (Bohemann),*Bruchidius cinerascens*(Gyllenhal)) belong to Chrysomelidae and 23rd species(*Micrambe villosus*(Heer))belongs to Cryptophagidae ,24th species (*Staphylinidae sp)*belongs to Staphylinidae.Last three species showed lowest percent identity in nucleotide blast of *M.viridanus.*However Cytochrome b barcode of the species of the genus *Myllocerus* (Coleoptera:Curculionidae) except that of *M.viridanus* has not been deposited so far and the barcode with the NCBI accession number OM223090 is the new and first time deposit of the Genus *Myllocerus* .The NCBI nucleotide blast result showed that the percentage identity of *M.viridanus* (Genbank accession No. OM223090) with 25 species retrieved from NCBI ranged from 88.17 to 83.68 .Out of the 25 species 21 belong to the family Curculionidae and 2 species belong to the family Chrysomelidae and 1 species each belong to Cryptophagidae and Staphylinidae .

The phylogenetic tree was constructed using the Neighbor-Joining method (Kimura,1980,Saitou & Nei, 1987). The optimal tree is shown (Fig.3). The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) and are shown next to the branches (Felsenstein,1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling ,1965) and are in the units of the number of amino acid substitutions per site. This analysis involved 26 amino acid sequences. The coding data was translated assuming a Invertebrate Mitochondrial genetic code table. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 75 positions in the final data set. Evolutionary analyses were conducted in MEGA11(Tamura et al. ,2021).

The evolutionary history was inferred (fig.3) using the Neighbor-Joining method (Saitou and Nei ,1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed .(Felsenstein ,1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. This is due to the absence of related data sequences in the NCBI deposition .Therefore we recommend more deposition of sequence through further research in the order Coleoptera and the family curculionidae in particular .The result of the phylogenetic analysis (fig.4) indicate that *Myllocerus viridanus* forms a distinct clad in the phylogenetic tree .It can also for the species level identification ofthe genus *Myllocerus* .Thus Cytochrome b similar to mitochondrial

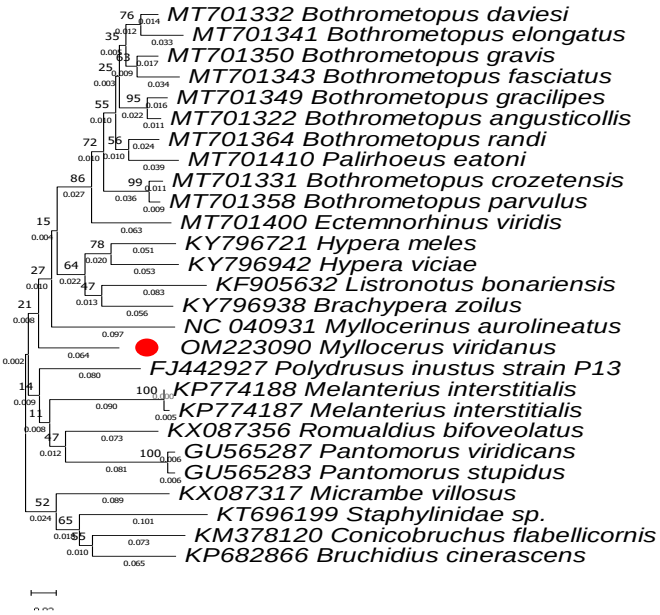

Fig.3 Neighbour joining tree for *M.viridanus* constructed using Kimura 2-parameter

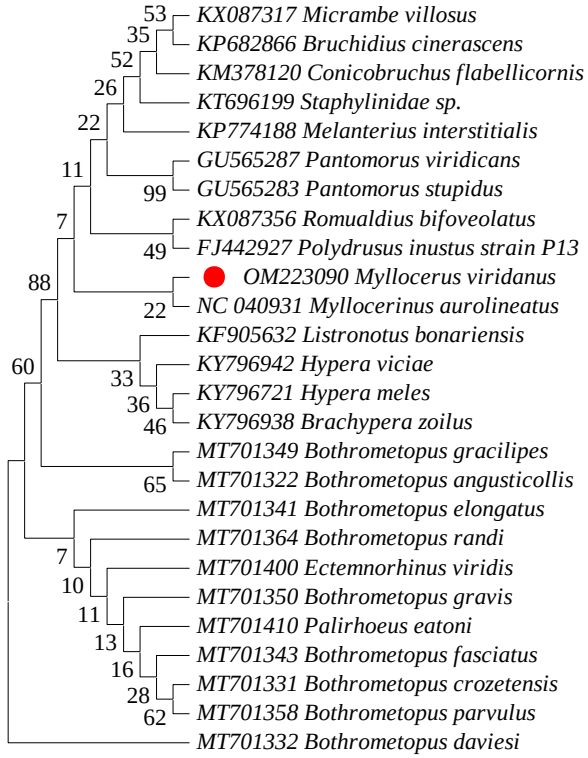

Fig.4.Evolutionary relationships of taxa

COX 1 gene ,can be used as an efficient tool for the species level identification of curculionids belonging to the Order Coleoptera.

De Pancorbo *et al.* (2004) studied the molecular identification of *Dermestes frischii* , *Lucilia sericata*, and *Piophila casei* by cytochrome b analysis . The result of the analysis included a DNA sequence of 358 bp of cyt b ,however they could not further interpret the inter specific diversity as no other cytb sequences of species were available in the database of GenBank. They further concluded that sequence analysis of cytb is also among the genes that could be explored competently for the refinement of molecular identification of insects.

Thus lack of a reference data set, is one of the biggest obstacles to DNA barcode identification (Virgilio et al., 2010). Generally a perspicuous and precise taxonomic identification may have to face the challenges of cryptic species, inaccurately categorization of taxa etc and all of which can be easily managed with a proper reference data set of barcodes (Pogue & Simmons 2008;Engstrand et al.,2010)

IV CONCLUSION

The present study indicated the first-time deposition of mitochondrial cyt b sequence of *Myllocerus viridanus* in the Genbank . Mitochondrial Cytochrome b barcode of *M.viridanus* using the universal primers showed distinct variationsand base substituitions in the nucleotide sequencesof closely related groups of curculionids (Coleoptera :Curculionidae) and therefore can be successfully used along with mitochondrial Cytochrome Oxidase 1 gene barcode, as an efficient tool for the analysis of the genera and species of the family curculionidae (Coleoptera :curculionidae) . The cyt b sequences ofno other species of the genus *Myllocerus* and even the other closely related genus in the order coleoptera are available in the database of GenBank. Thus when the tree is interpreted ,the species of *M.viridanus* do not have any other species to share its clad within the genus. The study could become highly significant and more coincidences could be observed if further mitochondrial cytochrome b sequence of related species were carried out and deposited in the Genbank through further research .

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