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Molecular identification of fruit flies, *Bactrocera* spp. (Diptera: Tephritidae) infesting guava fruits by using mitochondrial COI gene

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Abstract
Molecular identification of fruit flies, *Bactrocera* spp. (Diptera: Tephritidae) using mitochondrial COI gene using primer COI F, COI R, UEA7 and UEA10 and restriction enzymes (*Mse*l and *Alu*l) infesting guava fruits was carried out. The banding profiles in the electrophoresis gel were analysed. For the molecular studies, two set of *mt*DNA gene specific primer pairs i.e. COI F and COI R and UEA 7 F and UEA 10 R were used for polymerase chain reaction amplification of cytochrome oxidase I gene. In COI primer, samples yielded bands from all five *Bactrocera* spp. except *B. zonata* within the range of 1000-1500 bp, while UEA primer pair fragments were amplified PCR fragments from all five species within the range of 500-750 bp. The sequencing results showed total nucleotide length was 450-680 bases. Alignment of above sequences with NCBI database revealed that *B. dorsalis* and *B. cucurbitae* showed 97-99% similarity while, *B. zonata, B. verbascifoliae* and *B. correcta* showed 85-94% similarity. In *B. correcta* where there was deletion of 28 bp nucleotides as compared to sequence reported earlier. The restriction sites of DNA sequences were examined by Insilico approach in Bioedit program by selecting two restriction enzymes i.e. *Alu*l and *Mse*l which cut the sequence according to their sites and form a specific restriction pattern within five *Bactrocera* spp.

Keywords: *Bactrocera* spp., molecular identification, PCR, COI, restriction enzymes

1. Introduction
The Tephritid fruit flies are almost ubiquitous and found in all regions of the world. Some of these fruit flies possess a great potential to cause damage to agricultural and horticultural production [3, 11]. Among horticultural crops, fruits are of immense importance because of their economic value and return. Guava, *Psidium guajava* L. is one of the most important fruit crops known for its pleasant taste and nutritive value [7]. It is called as an apple of the tropics. As compared to other nations India has very low productivity, which is mainly due to pest problem [5]. It is host for many insects particularly during rainy and winter season. Amongst these, a fruit fly (*Bactrocera* spp.) is most important insect pests. The infestation of fruit fly is a major limiting factor in the production of guava. It is in the range of 20 - 46% with losses of 16 - 40% [5], 13.40 to 46.60 per cent and 12.50 to 42.86 per cent, respectively on weight basis and number basis [10]. There are about 325 species of fruit flies occurring in the Indian subcontinent, of which 205 are from India alone [8]. The major pest species belong to the genus *Bactrocera*: *B. cucurbitae*, *B. dorsalis* and *B. zonata*, while other species, such as *B. correcta, B. diversa* and *B. latifrons* are still localized in their distribution [8]. In quarantine work, it is quite common to encounter immature life stages or body parts of suspected pests that require species determination. The current method of identification, based on morphological characters of adult insects may encounter difficulty in separating sibling species of *Bactrocera* spp. or identifying immature stages [12]. In these cases, molecular determination of fruit flies would be an advantage. Polymerase Chain Reaction (PCR) amplification of DNA sequences, we can show how species identifications can be made from DNA sequences for both known and unknown material. We also show how molecular systematic relationships among *Bactrocera* spp. can be analyzed using DNA sequences [6]. In the present studies, Molecular identification of fruit flies, *Bactrocera* spp. (Diptera: Tephritidae) using mitochondrial COI gene using primer COI F, COI R, UEA7 and UEA10 and restriction enzymes (*Mse*l and *Alu*l) infesting guava fruits was carried out.

2. Materials and Methods

Fruit fly traps with cotton wicks treated with methyl eugenol and cue lure were installed in guava orchard of the Department of Horticulture, MPKV, Rahuri. The experimental study was conducted during 2010 to 2011.

2.1 Fruit fly collection and handling

Adult specimens of the five *Bactrocera* species were collected from infested fruits of guava in the experimental farm using chemical lures i.e. methyl eugenol and cue lure (Table 1). Morphological identification of the five species was carried out prior to molecular studies. The specimens were either stored at −20 °C in a freezer or preserved in 96% alcohol prior to analysis.

Table 1: Species of fruit fly recorded from guava orchards in the traps

Sr. No.	Traps	Fruit fly species collected	Family /subfamily
1.	Methyl eugenol	*Bactrocera zonata* (Saunders)	Tephritidae/Dacinae
2.		*Bactrocera dorsalis* (Hendel)	Tephritidae/Dacinae
3.		*Bactrocera correcta* (Bezzi)	Tephritidae/Dacinae
4.		*Bactrocera verbascifoliae* (Bezzi)	Tephritidae/Dacinae
5.	Cue lure	*Bactrocera cucurbitae* (Coquillett)	Tephritidae

2.2 DNA extraction, amplification and sequencing

Total DNA was extracted using HiPurA^{TM} insect DNA purification spin kit (HiMedia) following the manufacturer's protocol for insect tissue with slight modifications to increase DNA yield [2]. Either a whole body or one leg was used for each extraction. Two sets of primers were used for polymerase chain reaction (PCR) amplification of cytochrome oxidase I (COI) markers (Table 2). PCR amplifications for the first set of primers (COI F and COI R) were performed following the conditions as described by [2], an initial denaturation at 95 °C for 3 minutes, 40 cycles at 93 °C for 1 minute, 55 °C for 1 minute, extension at 72 °C for 2 minutes and final extension at 72 °C for 15 minutes. The PCR conditions for the second set of primers (UEA7 and UEA10) were as follows: an initial denaturation at 94 °C for 3 minutes, 35 cycles at 94 °C for 1 minute, 50 °C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 30 minutes.

Table 2: Oligonucleotides used for DNA amplification and sequencing of the *Bactrocera* spp.

Set	Primer	Type	Sequence	Reference
1	COI	Forward	5'-TACAATTTATCGCCTAAACTTCAGCC-3'	Chua *et al*. 2009
	COI	Reverse	5'-CATTTCAAGTTGTGTAAGCATC-3'	
2	UEA 7	Forward	5'-TACAGTTGGAATAGACGTTGATAC-3'	
	UEA 10	Reverse	5'-TCCAATGCACTAATCTGCCATATTA-3'	

The presence of genomic DNA was confirmed when bands were successfully visualized as high molecular weight bands by resolved by 2.0% agarose gel electrophoresis. The DNA fragment sizes were estimated by comparing with 1 kb markers. The band corresponding to the target PCR product was excised and purified using HiPer^{TM} gel elution spin kit (HiMedia). All samples were then sent to M/s. Bangalore-Genei Company for sequencing. The sequence of five *Bactrocera* spp. amplified using both primer sets i.e. COI (F/R) and UEA (F/R) were aligned using Chromas lite software and examined for their recognition sites by two restriction enzymes using the Bioedit program. Two restriction enzymes i.e. *Alu*I and *Mse*1 (New England, Biolabs) were then chosen for further work.

3. Results and Discussion

3.1 Confirmation of genomic DNA from fruit flies

The genomic DNA of identified fruit flies species were successfully extracted by following the manufacturer protocol given in the HiPurA^{TM} insect DNA purification spin kit (HiMedia). The degree of DNA purity and yield was varied accordingly to species to species. The quality of DNA was much improved by using HiPurA^{TM} insect DNA purification spin kit (HiMedia). No smearing was observed in the bands. The DNA purity was within the range of 1.6-2.0 and DNA yield was 20-145 ng. The presence of genomic DNA was confirmed when bands were successfully visualized as high molecular weight bands by resolved by 0.8% agarose gel electrophoresis.

3.2 Polymerase Chain Reaction Analysis (PCR) of fruit flies DNA

After confirmation of the genomic DNA from fruit fly species, two primers sets COI (F/R) and UEA (F/R) was used for the PCR amplification of cytochrome oxidase I (COI) gene. The expected 1.3 kb DNA fragment was successfully visualized in 2% agarose gel by PCR.

3.3 PCR amplification of cytochrome oxidase I gene of 5 identified fruit fly DNA samples with COI primer and UEA primer

Total five DNA samples of fruit flies *viz., B. zonata, B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae* were used during PCR amplification. The PCR reaction mixture was prepared. Total 20 µl PCR product quantity of each DNA sample was used for PCR amplification. In COI primer, out of 5 DNA samples of fruit flies, 4 DNA fragments were amplified successfully in PCR but except *B. zonata* all remaining flies i.e., *B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae* was amplified. The DNA fragments measured with 1 kb ladder (GeneRuler^{TM}, Fermentas Company). All amplified DNA fragments was seen on 2% agarose gel and depicted in Fig. 1. The DNA fragment of fruit flies in COI primer was amplified in the range of 1000-1500 bp and molecular weight of the amplified DNA fragment are, *B. dorsalis* (1398 bp), *B. correcta* (1320 bp), *B. verbascifoliae* (1320 bp) and *B. cucurbitae* (1335 bp). While in, UEA primer, total five DNA fragments *viz., B. zonata, B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae* were successfully amplified by combination of reaction mixture. In UEA primer, the entire DNA was successfully amplified and

seen on 2% agarose gel. Amplified DNA fragments were measured with 1 kb ladder (GeneRuler™, Fermentas Company) and are depicted Fig. 2. Five DNA bands with UEA primer were amplified in the range of 500 bp - 750 bp and molecular weight of each amplified fragment are *B. zonata* (596 bp), *B. dorsalis* (570 bp), *B. correcta* (576 bp), *B. verbascifoliae* (583 bp) and *B. cucurbitae* (576 bp).

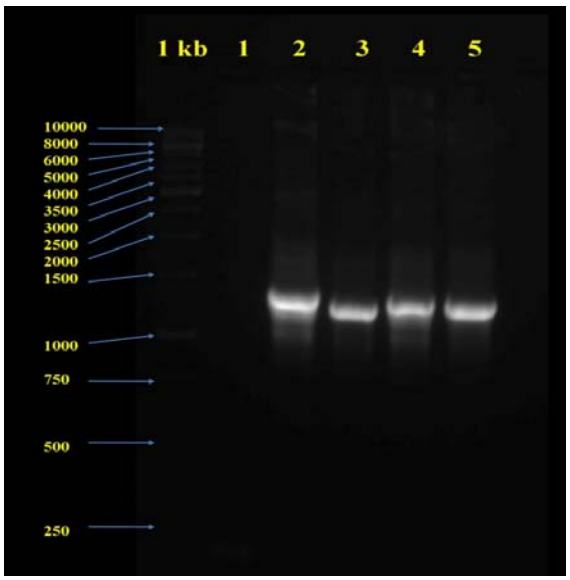

Fig 1: DNA amplification of 5 *Bactrocera* species in primer COI (F/R) using PCR (1. *B. zonata* (not amplified) 2. *B. dorsalis*, 3. *B. correcta*, 4. *B. verbascifoliae*, 5. *B. cucurbitae*).

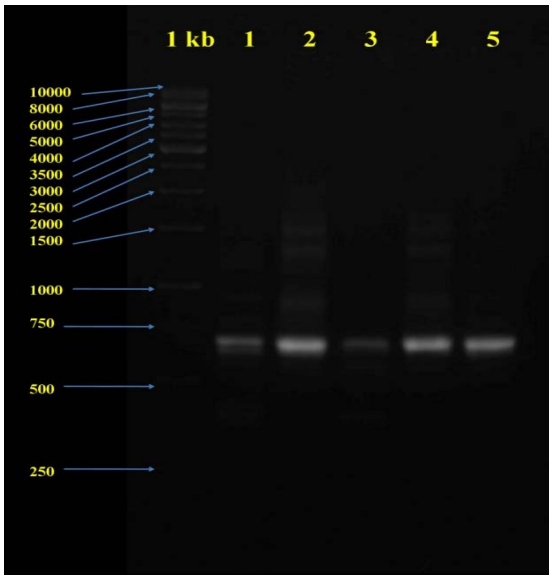

Fig 2: DNA amplification of 5 *Bactrocera* species in primer UEA (F/R) using PCR (1. *B. zonata*, 2. *B. dorsalis*, 3. *B. correcta*, 4. *B. verbascifoliae*, 5. *B. cucurbitae*).

3.4 Elution of amplified DNA fragment of COI and UEA primer

Total nine DNA fragments i.e. four 1000-1500 bp DNA fragments from COI primer and five 500-750 bp DNA fragments from UEA primer were eluted successfully by following the manufacturer protocol given in HiPer™ gel elution spin kit (HiMedia). All amplified bands have been separated and excised the DNA fragment carefully using a UV light box. All eluted DNA fragments samples were sent to M/s. Bangalore-Genei Company for the sequencing of each amplified fragments.

3.5 Sequence alignment of COI and UEA primer

The first primer set (COI F and COI R) had successfully amplified a 1.3 kb long sequence of the COI gene only from *B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae* except *B. zonata*. However, using second primer set (UEA 7 F and UEA 10 R), a shorter sequence of COI gene was amplified successfully for all five fruit fly species i.e. *B. zonata, B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae*. An approximately 700 bp long nucleotide sequence was obtained using this primer sets. This primer was used successfully to amplify DNA fragments from dried specimens of fruit flies.

Raw nucleotide data of DNA fragments obtained from M/s Bangalore Genei Pvt. Ltd, Bangalore were analysed using Chromas lite program. The sequences obtained from both the primers were aligned through BLAST (Basic Local Alignment Search Tool) with sequence data of *Bactrocera* previously reported in NCBI (National Centre of Biotechnology Information) site. The reference accession numbers of sequences used for alignment were *B. dorsalis* (DQ845759.1, DQ917577), *B. correcta* (DQ845759.1, AY530905.1 and GU323781.1), *B. verbascifoliae* (DQ845759), *B. zonata* (DQ845759.1 HQ446513.1) and *B. cucurbitae* (AY945051.1, JN635562.1).

In multiple sequence alignment, homology of five *Bactrocera* spp. was compared with previously reported NCBI database through BLAST option. In homology study, *B. dorsalis* and *B. cucurbitae* showed 98-99% similarity with NCBI GenBank database while *B. correcta, B. zonata* and *B. verbascifoliae* showed 85-94% homology. In *B. correcta*, deletion of 28 nucleotides was observed with homology search. This result showed that there was a mutation occurred in *B. correcta*.

3.6 Insilico restriction digestion analysis of COI gene (*Alu*I and *Mse*I) using by Bioedit program for *Bactrocera* spp.

By using Bioedit program, restriction enzymes cut the sequence of fruit flies in to fragments and showing the different banding patterns. The species wise cut fragments with restriction positions in COI and UEA fragments is shown in Fig. 3, 4, 5 and 6.

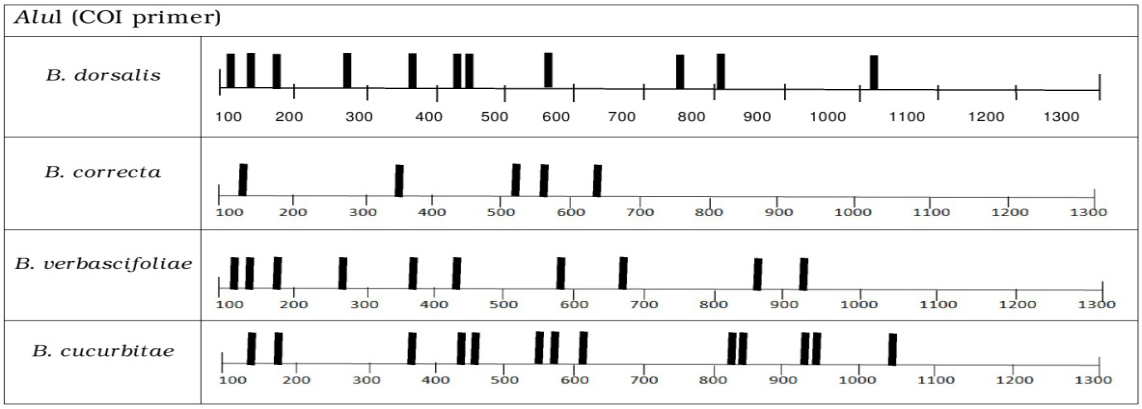

Fig 3: Restriction sites by *Alu*1 enzyme in COI PCR product

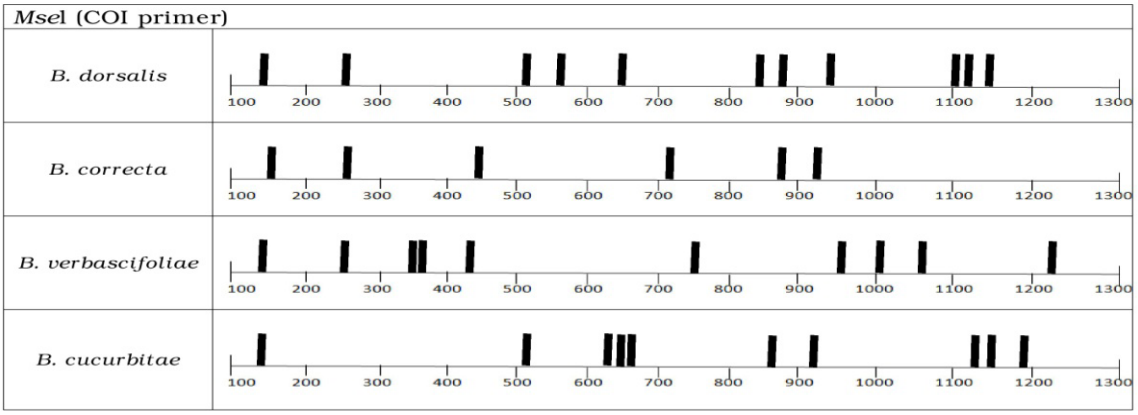

Fig 4: Restriction sites by *Mse*1 enzyme in COI PCR product

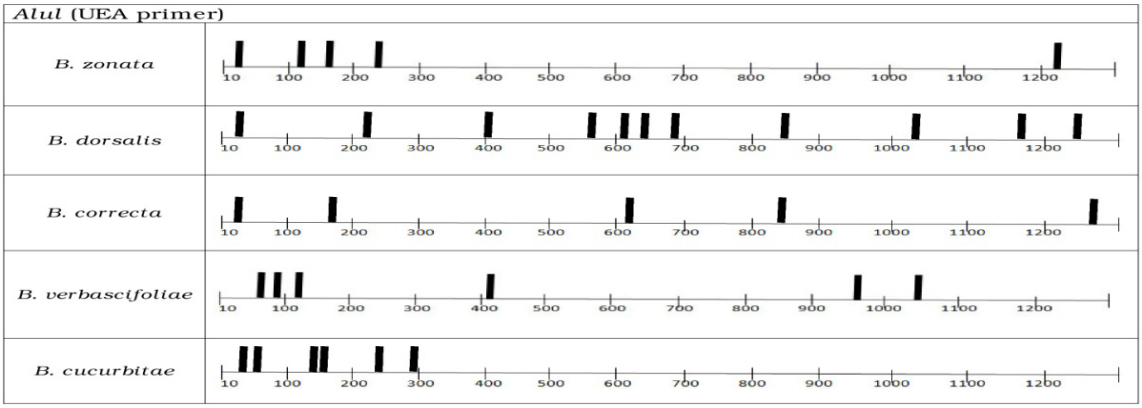

Fig 5: Restriction sites by *Alu*1 enzyme in UEA PCR product

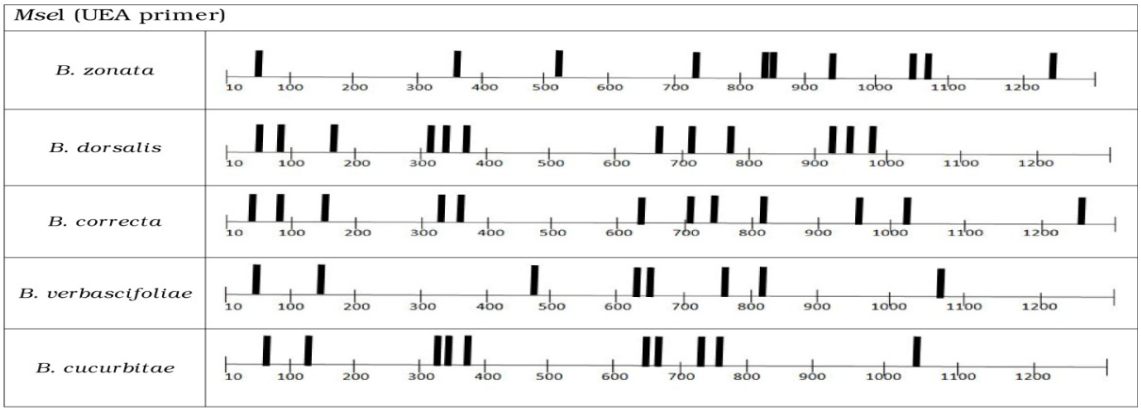

Fig 6: Restriction sites by *Mse*1 enzyme in UEA PCR product

The present study demonstrated, the use of cleaved amplified polymorphic sequence (CAPS) markers for the differentiating the five *Bactrocera* species i.e. *B. zonata, B. dorsalis, B. correcta, B. verbascifoliae* and *B. cucurbitae* on the basis of COI sequences. The molecular technique described here is useful for identifying *Bactrocera* accurately, especially in quarantine work situated at national borders or airports. Currently identification of *Bactrocera* species complex using morphological characters are time consuming and technically difficult for closely related species. Diagnostic morphological characters for adult and immature stages are also lacking for most tephritids [11].

[9] also employed PCR methods for identification of *Bactrocera* spp. using mitochondrial DNA. They analysed 18 *Bactrocera* spp. and successfully indentified most of the *Bactrocera* spp. except for two closely related species, *B. carambolae* and *B. papayae* while, [1] studied the molecular identification of three *Bactrocera* species using mitochondrial cytochrome oxidase I and found sequencing result of three species of fruit flies i.e. *B. dorsalis, B. correcta* and *B. zonata*, total nucleotide length obtained was 440 bases. Alignment of sequences of fruit fly species showed 92% similarity between *B. dorsalis, B. correcta* and *B. zonata*. Similarly, [2] studied species identification of *Bactrocera* spp. and showed COI gene in *Bactrocera* spp. was successfully amplified by COI (F/R) and UEA (F/R) and a distinct banding profile for *B. tau, B. latifrons, B. cucurbitae, B. umbrosa, B. carambolae* and *B. papayae* were observed.

In the present study, PCR analysis with two primers i.e. COI and UEA have been performed on *Bactrocera* spp. DNA sequences. The present results showed that five species of *Bactrocera* spp. can be separated from one another by combination of COI (F/R) and UEA (F/R) primers and by using two restriction enzymes *Alu*I and *Mse*I in Bioedit program. Bioedit program cut the DNA sequence of fruit flies according to the restriction sites of *Alu*I and *Mse*I enzymes which give the specific banding patterns according to *Bactrocera* spp. sequences.

The restriction profile of *Bactrocera* spp. using DNA sequence obtained from whole adult insect body in Bioedit program showed the robustness of using molecular techniques for species identification even only when broken pieces or parts of insect are available [4]. have also shown that parts of an insect could produce sufficient PCR product for species identification. This would be useful tool in quarantine work.

As the identification by molecular techniques presented here was conducted on samples from experimental farm of MPKV, Rahuri, the possibility exists that they might not be applicable to individuals from outside this geographical area. Nevertheless, as great similarity between the present alignment exercise of COI sequences from Ahmednagar district and those from other countries has been found, it is likely that this method might also be useful for individuals of the same species from other countries. Whether this method is applicable to other *Bactrocera* species not tested here remains to be seen, but the authors believe that the banding profiles produced from combination of the primer sets and restriction enzymes could help in identifying at least five species of fruit fly.

4. Conclusion

In molecular identification, COI gene was successfully amplified in five *Bactrocera* spp. by using COI (F/R) but except *B. zonata* in COI (F/R) and by UEA primers. The DNA sequence of *B. correcta* shows 28 bp deletion of nucleotide, probably suggesting occurrence of mutation in *B. correcta*. *B. verbascifoliae* showed the divergence from various fruit flies reported in GenBank accession with highest homology with *B. dorsalis, B. papayae* and *B. cucurbitae*. By using restriction enzyme *Alu*I and *Mse*I, five *Bactrocera* species showed different restriction patterns in their DNA sequences. Using PCR amplification of DNA sequences, we can be able to show species identification from DNA sequences which will be helpful in quarantine work irrespective of their growth stage.

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