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16S rRNA gene sequencing of the most regnant and pernicious pests *Brevundimonas diminuta, Craterium leucocephalum* and *Diachea leucopodia* associated with dried preserved forensically valuable Calliphorids

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Abstract
Calliphorids are forensically most valuable insects. These are collected from the crime site as circumstantial evidence and preserved for the purpose of identification. Though the prima facie of these preserved specimens seems to be in the perfect well maintained state but molecular analysis on these specimens reveal that these are harboured by various pests that are internally calamitous to these specimens and degrade the intact DNA of these specimens into small fragments. *Brevundimonas diminuta* (bacterium), slime molds *Diachea leucopodia, Craterium leucocephalum* have been identified as the most regnant and pernicious pests and approximately 500bp of *I6S rRNA* gene of these have been sequenced for the correct identification of these pests from preserved insect specimens. The author has also attempted to espy the most inimical traits that these pests are equipped with which would in turn help in calibrating effective measures to avoid their infestation and in safeguarding these subtle specimens.

Keywords: *16S rRNA*, blow fly, PCR

1. Introduction
Forensic Entomology holds a wide diapason. It is widely defined as the application and study of insects and other arthropods` biology to criminal matters. There are various approaches contributing to this field like morphologically and morph metrically identifying the type of insect species collected from the corpse. Molecular and genetical aspect dealing with the accurate identification of these specimens is much more informative and reliable than the morphological aspects because most of the time only fragmented remains or pupal cases are available for identification. But such samples are more prone to microbial attack. Thus to preserve such copacetic samples, it is important to identify the detrimental microbes colonising these preserved samples and to avoid their infestation. When isolation of DNA is tried on such samples, most of the times we fail to generate results as these microbes badly hamper the DNA extraction and amplification process. The insect tissue actually gets ruined by decomposing bacteria and only traces of fragmented insect DNA is left in the specimens available for DNA extraction. It becomes mandatory to tab the microbes that harbour and damage the insect collections stacked in various museums. And to fend off these microbes it becomes important to identify the group of microbes that are worst affecting these valuable specimens. *16S rRNA* gene is one of the key conserved genes among bacteria and has species specific signature sequences. *16S rRNA* gene has been used to study bacterial community structure in *Apis* [1]. It also accumulates mutations more rapidly than the nuclear *rDNA* genes and can decipher relationships even below family level [2]. It also shows valuable inter and intra specific divergences. All these traits make this gene an important molecular marker for identification. However for Calliphorids, mostly cytochrome oxidase subunit I gene has been used for identification [3, 4]. However no significant relevant citation is known that evinces symbiotic relationship among Calliphorids and *Brevundimonas diminuta*, *Craterium leucocephalum*, *Diachea leucopodia*. Also absence of probable symbiotic association of these pests with the Calliphorids was tested by amplifying fresh samples of Calliphorids under similar conditions, which yielded no positive result (Figure 3). The aim was to check for the microbes that actually are detrimental to these dry collections.

2. Materials and Methods

3-10 years old specimens were chosen randomly from the museum collection. These were then washed in distilled water to remove any superficial contaminants. Only legs were dissected out and used for DNA isolation, to avoid any chance of endosymbiotic microbe in insect gut or sequencing of any pathogen. Precautionary measures were strictly followed to avoid any further environmental contamination during DNA extraction. The tissue was crushed using liquid nitrogen. The DNA extraction was carried out using Qiagen Puregene Tissue extraction kit, following manufacture's protocol. This DNA was then dissolved in Hydration solution and stored at -20 °C. The same protocol was followed for extracting DNA from fresh counter specimens (choosing the same species).

DNA Amplification

The extracted DNA was amplified using microbe specific 16S rRNA primers, namely 16S SarF (5` CGCCTGTTTATCAAAAAAT3`) and 16S SarR (5`CCGGTCTGAACTCAGATCACGT3`) which is known as barcode for the microbes. All polymerase chain reactions were performed using Bio-rad T100TM thermal cycler. The thermal cycler conditions were the following: initial denaturation at 98 °C for 2 minutes followed by 40 cycles at 98 °C for 30 seconds, annealing at 55 °C for 40 seconds, elongation at 75 °C for 1 minute and final elongation at 75 °C for 7 minutes. 50 µl PCR cocktail constituted of Phusion DNA polymerase enzyme 1U/50 µl reaction, 5X Buffer 10 µl, 10 pm dNTP, 50 mM $MgCl_2$1 µl, 10 pm primers 1 ul each and MQ water (Thermo Fisher Scientific, India). 1-4 µl DNA was used for each PCR reaction, depending on the concentration of purified DNA.

Electrophoresis

PCR products were detected by gel electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Figure 1 shows the amplified DNA bands for *Diachea leucopodia, Craterium leucocephalum* while figure 2 depicts the amplicons for *Brevundimonas diminuta* on dry preserved samples. Figure 3 shows no amplification, thus confirming the absence of these pests on the fresh samples.

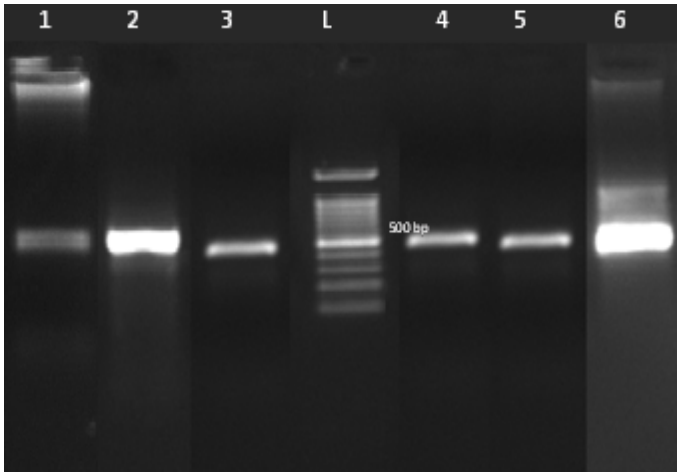

Fig 1: Gel image showing the DNA amplification bands for the *Diachea leucopodia, Craterium leucocephalum* and *Brevundimonas diminuta* on dry preserved samples (Lane 1-6)

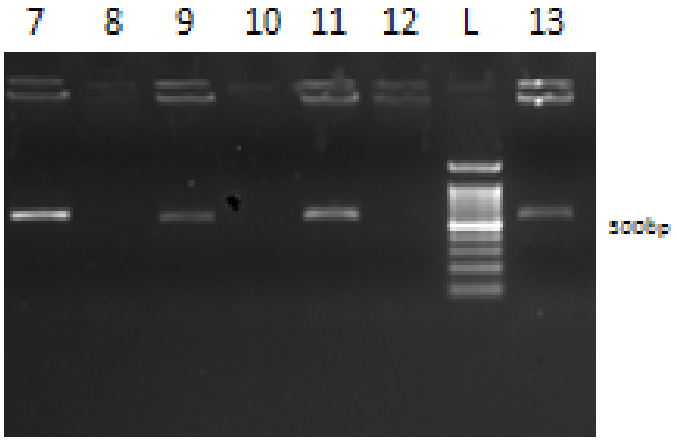

Fig 2: Gel image showing the DNA amplification bands for the *Diachea leucopodia, Craterium leucocephalum* and *Brevundimonas diminuta* on dry preserved samples (Lane 7-13)

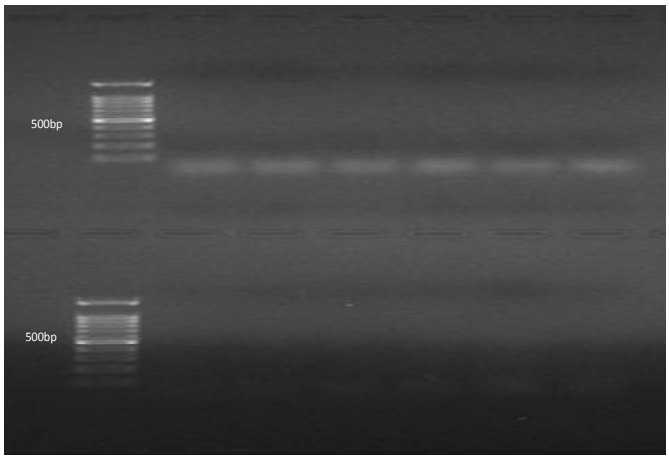

Fig 3: Gel image showing no relevant DNA amplification bands in case of fresh samples.

Sequencing

Using ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the purified PCR amplicons were sequenced. Sequencing was performed on 3130 Genetic analyser Automated DNA sequencing machine. The softwares used for sequence analysis were Sequencing Analysis 5.1; Chromas Pro v3.1. Table 2 enlists the DNA sequences so obtained.

BLAST

BLAST search was carried out to compare these obtained sequences with the database of sequences in the NCBI (National Centre for Biotechnology Information). These organisms were then correctly identified as *Brevundimonas diminuta, Craterium leucocephalum* and *Diachea leucopodia.* These were then successfully submitted in the Genbank. The accession numbers so obtained for them are mentioned in Table 1.

Table 1: The table enlists the pernicious microbes and their Genbank accession numbers.

Species	Type	Genbank Accession number
Brevundimonas diminuta	Bacterium	KP720659
Diachea leucopodia	Myxomycetes	KR013194
Craterium leucocephalum	Myxomycetes	KR013153

Table 2: The table enlists the nucleotide sequence so obtained by sequencing.

Species	Sequence
Brevundimonas diminuta	GAATTCCCCGTGAAGATGCGGGGTTCCCGCGGTCAGACGGAAAGACCCTATGAACCTTTACTAT AGCTTCGCCTTGGCGTTAGCGACCGTATGTGTAGGATAGGTGGGAGGCTATGAAGCCGGGGCGC CAGCTCTGGTGGAGCCATCCTTGAAATACCACCCTTACTGTCGTTGACGTCTAACCGAGGGCCGT TATCCGGTCCCGGGACATGGCGTGGTGGGTAGTTTGACTGGGGCGGTCGCCTCCCAAAGTGTAA CGGAGGCGCGCGATGGTTAGCTCAGACCGGTCGGAAATCGGTCGTCGAGTGCAATGGCATAAGC TAGCCTGACTGCGAGACTGACAAGTCGAGCAGAGACGAAAGTCGGCCATAGTGATCCGGTGGTC CCGCGTGGAAGGGCCATCGCTCAACGGATAAAAGGTACTCTAGGGATAACAGGCTGATTTTGCC CAAGAGTCCATATCGACGGCAAAGTTTGGCACCTC
Diachea leucopodia	GGTGTTTATTAGAGGCACCGCCTGCCCAGTGACACATGTTTAAACGGCCGCGGTACCCTAACCGT GCAAAGGTAGACATAATCACTTGTTCCTTAAATAGGGACCTGTATGAATGGCTCCACGAGGGTT CAGCTGTCTCTTACTTTTAACCAGTGAAATTGACCTGCCCGTGAAGAGGCGGGCATGACACAGC AAGACGAGAAGACCCTATGGAGCTTTAATTTATTAATGCAAACAGTACCTAACAAACCCACAGG TCCTAAACTACCAAACCTGCATTAAAAATTTCGGTTGGGGCGACCTCGGAGCAGAACCCAACCT CCGAGCAGTACATGCTAAGACTTCACCAGTCAAAGCGAACTACTATACTCAATTGATCCAATAA CTTGACCAACGGAACAAGTTACCCTAGGGATAACAGCGCAATCCTATTCTAGAGTCCATATCAA CAATAGGGTTTACGACCTCGATGTTGGATCAGGACATCCCGATGGTGCAGCCGCTATTAAAGGTT CGTTTGTTCAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGAGGC
Craterium leucocephalum	CCTGGTTTGCCACCGAAACTTTAATAGCGGCTGCACCATCGGGATGTCCTGATCCAACATCGAGG TCGCTAAACCCTATTGTTGATATGGACTCTAGAATAGGATTGCGCTGTTATCCCTAGGGTAACTT GTTCCGTTGGTCAAGTTATTGGATCAATTGAGTATAGTAGTTCGCTTTGACTGGTGAAGTCTTAG CATGTACTGCTCGGAGGTTGGGTTCTGCTCCGAGGTCGCCCCAACCGAAATTTTTAATGCAGGTT TGGTAGTTTAGGACCTGTGGGTTTGTTAGGTACTGTTTGCATTAATAAATTAAAGCTCCATAGGG TCTTCTCGTCTTGCTGTGTCATGCCCGCCTCTTCACGGGCAGGTCAATTTCACTGGTTAAAAGTAA GAGACAGCTGAACCCTCGTGGAGCCATTCATACAGGTCCCTATTTAAGGAACAAGTGATTATGC TACCTTTGCACGGTTAGGGTACCGCGGCCGTTAAACATGTGTCACTGGGCAGGCGGTGCCTCTAA TACTGGTGATGCTAGAGGTGATGTTTTTGATAAAACAGGCGAGCGA

3. Results and Discussion

The aim of this study was to identify the most detrimental pests that invade these preserved specimens in insect boxes (insect boxes were fumigated with naphthalene powder). Analysing the preserved dry Calliphoridae samples on a random basis, we could conclude that majority of the worst affected specimens were found to be invaded by these three most pernicious pests and likewise suggests towards stringent methods to eliminate and avoid these specific pests that harbour the valuable insect collection. *Brevundimonas diminuta* is considered one of the major contenders in the process of tissue decomposition as are most common organism in the soil and other moist environments [5]. Also *Brevundimonas diminuta* shows resistance to fluoroquinolones [6]. This bacterium has an *opd* gene which can synthesise an organophosphate hydrolase, OPH, EC3.1.8.1 [7, 8]. This enzyme is also capable of hydrolysing a wide variety of organophosphorus insecticides like paraoxon, ethyl parathion, methyl parathion etc. [9]. Thus, this bacterium is found to be highly inimical. While the plasmodial slime molds *Diachea leucopodia* and *Craterium leucocephalum* secrete hydrolytic enzymes like proteinase and peptidase, thus destroying the insect tissue and exposing the DNA for environmental degradation. Thus these further add to the process of DNA fragmentation.

Thus there is a sincere need to switch over to better and more suitable methods for insect preservation, to keep the collection undamaged for long time. We suggest dipping or spraying Ticarcillin Sodium/ Clavulanate Potassium Powder on such specimens along with naphthalene powder, as this would prevent most of the microbial invasions and would preserve the specimens for long. Also failed amplification results in case of fresh blow fly samples validate, no relevant symbiotic association of this bacterium thus highlighting its major role as detrimental bacterium. We could also explore the feasibility of using *16S rRNA* gene sequences as the standard for classification and identification of microbes; because it is present in most microbes and shows proper changes [10]. Our results indicate that identification of these microbes based on *16S rRNA* is mostly in good agreement with the identification based on traditional biochemical approaches. Also presence of this bacterial DNA in the isolated insect DNA hampers the correct molecular typing of the subject organism. Particularly it proves to be a big drawback in the case of DNA barcoding, where universal primers are applied, thus mistakenly amplifying the wrong organism. This is one of the biggest reasons researchers avoid working on old preserved dry insect samples that lie piled up in museums since years and instead prefer to go for fresh collection.

4. Acknowledgements

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5. Disclosure

The authors are not conversant of any memberships, financial holdings, affiliations that could raise a conflict of interest.

6. References

1. Saraithong P, Li Y, Saenphet K, Chen Z, Chantawannakul P. Bacterial community structure in *Apis florea* larvae analyzed by denaturing gradient gel electrophoresis and *16S rRNA* gene sequencing. Insect Science, 2014; doi: 10.1111/1744-7917.12155.
2. Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Entomological Society of America 1994; 87: 651-701.
3. Liu QL, Cai JF, Chan YF, Gu Y, Guo YD, Wang XH *et al.* Identification of forensically important blow fly species (Diptera: Calliphoridae) in China by mitochondrial cytochrome oxidase I gene differentiation.

Insect science 2011; 18(5):554-564.
4. Priya Bhaskaran KP, Sebastian CD. Molecular barcoding of green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) using COI gene sequences. Journal of Entomology and Zoology Studies 2015; 3(1):10-12.
5. Kang SJ, Choi NS, Choi JH, Lee JS, Yoon JH, Song JJ. *Brevundimonas naejangsanensis* sp. nov., a proteolytic bacterium isolated from soil, and reclassification of *Mycoplana bullata* into the genus *Brevundimonas* as *Brevundimonas bullata* comb. nov. International Journal of Systematic and Evolutionary 2009; 59:3155-3160.
6. Xiang YH, Roberto AA. *Brevundimonas diminuta* infections and its resistance to fluoroquinolones. Journal of Antimicrobial Chemotherapy 2005; 55:6.
7. Mulbry WW, Kearny PC, Nelson JO, Karns JS. Physical comparison of parathion hydrolase plasmids from *Pseudomonas diminuta* and *Flavobacterium* sp. Plasmid 1987; 18:173-177.
8. Serdar CD, Murdock DC, Rohde MF. Parathion hydrolase gene from *Pseudomonas diminuta* MG: subcloning, complete nucleotide sequence and expression of the mature portion of the enzyme in *Escherichia coli.* Biotechnology 1989; 7:1151-1155.
9. Dumas DP, Cladwell SR, Wild JR, Raushel FM. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. Journal of Biological Chemistry 1989; 264:19659-19665.
10. Ting L, Peter GS, Daniel BO. Reverse Transcription of *16S rRNA* to Monitor Ribosome-Synthesizing Bacterial Populations in the Environment. Applied and Environmental Microbiology 2009; 75 (13):4589-4598. doi:10.1128/AEM.02970-08. PMC 2704851.