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Review

Mitochondrial Cytochrome c Oxidase Subunit I (COI) Gene for Identification of Stink Bugs: Review

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Abstract

Crop losses by plant pests have been a major global concern and hence various techniques have been introduced the control such pests. Pests like stink bugs have known to cause considerable damage to crops. As a result, understanding their taxonomy is crucial, hence DNA based techniques like DNA barcoding have been introduced to augment the identification of stink bugs. A short fragment of mitochondrial Cytochrome c Oxidase subunit I (COI) gene can be used for host-specific identification of the stink bugs. Though this methodology is not fully reliable, the arena is open for research with appropriate changes in current data and hence can help to identify these bugs and probably other similar arthropods. In this review, species identification of stink bugs by assessing COI gene has been described briefly along with statistical methods like neighbor-joining for biomonitoring the pests.

1. Introduction:

About 20-40% of crops are damaged due to pests or plant diseases [1]. Many natural calamities are held accountable for huge agro-ecological losses within the world ensuing in undersupply of food for the growing population. Some of the natural causes of crop loss are heat waves, droughts, flooding, and water-logging [2,3,4] along with the wild animals [5,6] These drawbacks are more noticeable and severe in developing countries where agriculture drives the economy. The common influential pests destroying agriculture include plant bugs like true bugs, aphids, grasshoppers, and beetles [7].

Insects are major threats to crops due to their large diversities. Almost two-thirds of insects feed on plants [1]. The fifth-largest insect order is represented by sting

bugs belonging to the Order Hemiptera. Sting bugs themselves include about 1,00,000 species, 5800 genera, and 140 families and are representative of the largest group of hemimetabolous insects [7,9]. They have a diverse metabolic pool and can utilize varied food resources. Sting bugs can survive in disparate environments including water and land. With agricultural plants being an amicable source of food for sting bugs and economic losses to farmers, losses expected are exorbitant [8]. They are generally classified into many groups such as water striders, leafhoppers, planthoppers, and aphids depending on their host, physical appearance, and habitat. Several losses to agricultural fruit and crop have been associated with them. For example, these species led to the loss of around $37 million to apple farmers in the Mid-Atlantic States [10]. Mustard bug, a type of sting bug, has caused vegetable and economically important crops in parts of India, the Middle East, and Africa. North America also suffered extensive losses in the Brassica crop [17,18,19].

In India, *TessaratomaJavanica,* a species of stink bug significantly affected the lichi produce. The bug was found to feed on sap from fruits and flowers affecting its concerned economy [11]. *Halyomorphahalys,* also known as a brown marmorated stink bug, is an invasive insect species. It has invaded regions of the United States and Europe with a target on fruits row crops and vegetables [20]. Thus, stings bugs are found to affect more than 300 host species in major parts of the Northern hemisphere, Europe, and Southern hemisphere [21,22]. Thus, as described, sting bugs damage the agro-economic system severely affecting the average economic growth of cultivators and food supply. In this review, we examined the status of the mitochondrial cytochrome c oxidase subunit I (COI) gene for the identification of stink bugs and reported that although COI gene-based stink bug species identification has attracted potential researchers, it has not shown a 100% effective method for the species identification.

2. Research Gaps:

Previous studies were performed to identify valid bug species and hence determined their phylogenetic relationship using mitochondrial Cytochrome c oxidase I (COI) gene fragment [12,13,14,15]. However, no study has been undertaken to validate heteropteran groups using DNA-based analysis generating probabilities of uncertainties in the authentication of their grouping pattern. Moreover, the linked platforms of the evolution of these species groups using molecular markers were not highlighted. These areas are open for further research.

3. Research opportunities:

The control of sting bugs is the need of the hour which is possible only when their taxonomy is known. Unfortunately, these are poorly understood due to impediments in morphological taxonomic identifications. In many cases, the availability of external properties of damaged or immature specimens is hard for taxonomic identification. These species are challenging to identify owing to polymorphism in colors [23, 24]. However, DNA-based techniques such as DNA barcoding have fastened the arena of biological diversity studies by meeting taxonomic standards.

We can analyze species of stink bugs belonging to various genera and families along with study their host specificity using a short fragment of mitochondrial Cytochrome c oxidase subunit I (COI) gene. There are several advantages of using this method. Firstly, mitochondrial DNA is small in size and thus a favored genetic marker for species identification. It is present in cells in a large amounts making amplification easier. This DNA is conserved across various animals, with no introns [25,26,27].COI gene has been used to identify several worms, insects, and animal species [16,25,26]. A study based on the difference in mitochondrial cytochrome oxidase sequence was conducted to identify different species of *A. aegypti* [25]. It was also successfully used for the identification of species of teat fish [27]. *Bemisiata baci,* which is a major pest of commercial vegetables and plants, has many distinguishable biotypes. This makes its morphological identification impossible. Molecular methods like COI are now considered for their identification [28]. Thus, we can analyze species of stink bugs belonging to various genera and families along with study their host specificity using a short fragment of mitochondrial Cytochrome c oxidase subunit I (COI) gene. There is an urgent need to explore the genetic diversity of stink bugs on a broad scale globally to generate sufficient molecular marker-based libraries that can be used for further taxonomic identifications of various species. Moreover, we can find maximum intraspecific genetic distance among studied stink bug species with standard error with a distinct barcode gap. Mitochondrial COI

gene will be extensively used to detect Indian stink bug species with required reliability only when a sufficient DNA sequence database would be available. Recently, a combined COI-COII haplotype sequence was successfully used to identify 59 haplotypes of brown marmorated stink bugs out of which 54 were novel [29]. Another study conducted in Thailand used Multiplex PCR based on COI sequence for differentiating different species of *Anopheles barbirostris* [26,30].

4. Automated Barcode Gap Discovery (ABGD) analysis:

DNA barcoding is a widely used tool in ecology and taxonomy studies. It is used in areas such as biodiversity conservation and the identification of invasive species. Due to advancements in sequencing technology in combination with new bioinformatics databases, for example, NCBI, and BOLD Systems, biodiversity studies have advanced from barcoding at the individual level to metabarcoding. However, metabarcoding depends on large quantities of reference libraries. There are various algorithms, for example, Automated Barcode Gap Discovery (ABGD), that may generate different operational taxonomic units that define species based on different boundaries. The analytical method used for the analysis of DNA barcodes can greatly affect the outcomes of biodiversity studies. However, ABGD can recognize the distance between the distribution of divergence that relates to variance between Interspecific distances and intraspecific distances [32].
ABGD is an automatic procedure that can sort short DNA sequences into hypothetical species depending upon the barcode gap. It is observed when the divergence among organisms associated with different species is larger than the divergence among organisms of the same species. For this, a range of precedent intraspecific divergence is used to deduce a model-based one-sided confidence limit from the data for intraspecific divergence [33].

ABGD then identifies the first significant gap beyond the aforesaid limit by detecting the barcode gap and then uses it for the data partition. To obtain finer partitions, deductions of the gap and limit detections are applied recursively to prior obtained groups up to no further partitioning [33]. The stink bug specimens can be studied by Automated Barcode Gap Discovery (ABGD) [33] which is a fast, simple tool and is freely available online for understanding histogram distances with initial as well as recursive partitions. However, it is found that the presence of subspecies owing to mutations of the COI gene makes it difficult for ABGD analysis with the NJ method to give clear results. This can be solved by using a more compressive method that uses a multilocal strategy to eliminate taxonomic uncertainty [31].

5. Species identification:

Traditionally, species identification was done by comparing various morphological/phenotypic characters but these methods could not classify organisms when the specimens were incomplete, and many times led to inaccurate classifications. Currently, molecular techniques are most widely used for identification. They are fast, reliable and don't suffer from drawbacks like traditional methods. Internal Transcribed Spacer ITS, COI I and COI II have been successfully used for taxonomic classification. DNA barcoding makes use of COI fragments to identify organisms at the species level. By this method, unknown sequences can be compared with the database to conclude the closest match [24]. Barcode of Life Data systems BOLD and GenBank are two of the most important databanks for DNA barcodes. Many criteria must be satisfied before a sequence is accepted as a barcode in BOLD [24]. Barcode Index Number is commonly used in the BOLD system. It generates a Neighbor-joining (NJ) tree based upon varied Distance metrics [32]. In a study for insect diversity from the Sahara-Arabian region, DNA barcoding in conjunction with Barcode Intex Number (BIN) system was used to differentiate each specimen into species. The study showed that the BIN system can be used to sidestep the limitations that occur due to the low availability of a taxonomic specialist. This system can also be used to circumvent the errors that arise due to non-described insect species. Once the DNA databases are properly expanded, it will also be possible to undertake metabarcoding studies [35]. In another study, insect pests were identified by DNA barcoding. The results were then compared with morphological identification. It was observed that morphological methods could not accurately differentiate between species if specimens were immature. Their result showed that the nearest neighbor distance was greater than the largest value of intra-sequence divergence for all species. Identical results were obtained for Hemiptera and Tussock moth species [36]. Thus, the BOLD system has immense scope for species identification. Currently, species of stink bugs are identified using NCBI and BOLD DNA sequence

databases, and their images are uploaded on BOLD systems. The species identification success varies and is based on the availability of sufficient DNA sequences in DNA databases and accuracy in species identification. This approach is supported by Shen et al. 2013 [37] who state that the DNA barcode database of GenBank has inaccurate taxonomic identifications of which assessment is not performed [38]. Additionally, difficulties in morphological identifications of some voucher specimens due to damage or lack of identification expertise using their images led to less species identification success. Nevertheless, Coeur d'acier et al (2014) [38] have argued that such errors can be dissolved by analyzing voucher specimens. In contrast, 91.5% identification success for 418 species of true bugs belonging to Central Europe was reported by Raupach et al (2014) [12].

6. Demerits:

The errors in identifications using DNA barcoding are due to misleading taxonomic identifications in GenBank databases including NCBI and BOLD systems and singleton barcodes shown to be belonging to many species [40]. Similarly, specimens can be identified up to only family level (Pentatomidae) which has shown the highest matching with multiple species of either the same genus or different genera. A study to classify 17 insect species using DNA barcoding and BOLD led to the misidentification of 4 species. The sequence showed almost 95 to 100 % match to samples belonging to taxa of a different order. This could have occurred due to errors in the reference library or cross-contamination of a sample. We know that some of the records present in Gene Bank and the BOLD database are indeed sourced from misidentified species, but this error cannot be solved easily. In yet another study, 60% of 4977 species of European Lepidoptera were misidentified due to errors in the database and improper taxonomy. Thus, there is a need for methods that can avoid such errors. The existence of mitochondrial pseudogenes or the presence of infection due to *Wolbachia* can affect the species identification by DNA barcoding [17]. Collins and Cruickshank, (2013) [41] stated that NJ trees can be ambiguous, particularly when used with an incomplete reference library and problems related to NJ trees could not be solved by leftover tree inference methods, for instance, maximum likelihood or parsimony.

This may be due to insufficient generation of DNA barcode library because of inadequate work performed by researchers on a global scale and is recommended to widen the zone of biodiversity genomics to fasten the species identification of stink bugs using DNA-based identification systems. Tembe (2014) [14] generated new records of 111 COI sequences of 73 species of Pentatomorpha bugs in the barcode database. The above demerits can be resolved by using the BIN system as it objectively registers lineages that are genetically diverse [39].

7.Genetic divergence:

More than 5% divergence in DNA barcode is indicative of a strong separation between two hypothetical species. However not all congeneric invertebrate species show similar divergence. This is mainly true in the case of insects as they are known to have lower interspecies divergence. Non-winged arthropods have higher interspecific divergence [42]. In a study, DNA barcoding was unable to identify specimens of *L. dialects* because of its maximum intraspecific value (1.69%), which was higher than the mean intraspecific value. Further, its mean intra-specific value does not correspond with insect taxa like mayflies and black flies. Amongst species that have recently diverged from each other using simple criteria to delimit species may give satisfactory results [42].

An efficient way for analysis involves specimens studied at different ranks of taxonomy using COI sequences (Table 1). We can compare the sequences that belong within the species, genera, and families and obtain minimum, mean and maximum distances with standard errors in percentages. For example, 3-5% intraspecific divergence was reported in Hemiptera, suggesting the presence of cryptic species in the population [43,13,44]. This helps to investigate the genetic variation pattern among species under study.

8. Phylogenetic analysis:

The neighbor-joining method is used as a statistical method to construct a phylogenetic tree for stink bug species since it shows a clear barcode gap which may be used for identifying true bugs at the species level [14]. The reason for unexpected genetic variations, if any, may be evolutionary events that occurred in the genus in question. It may also be because of geographical barriers or speciation variance as a result of natural catastrophes as these regions are completely distinct and placed at longer distances in the state.

In a study of COI barcoding to classify samples from the family Miridae (true bugs), the neighborhood-joining

method was used to generate a phylogenetic tree. The authors found mean and average maximum interspecific genetic distance similar to previous studies. Thus, it can be concluded that DNA barcoding was useful for investigating true bug species [45]. This study is limited to related species because of nucleotide substitution [46]. Mitochondrial DNA was also useful in the identification of true bugs like Indian Pentatomid. This mitochondrial DNA acts as an excellent marker for phylogenetic study, description of geographical distribution, genetic variation, and phylogeny of unknown species [26].

Table 1: Genetic divergence at species, genus, and family levels.

	n	Taxa	Compa-risons	Min dist (%)	Mean dist (%)	Max. dis (%)	S.E (%)
Within species	30	10	77	0.01	0.33	1.88	0.02
Within genus	1	2	1	Data Unavai-lable	Data Unavai-lable	Data Unavai-lable	0.01
Within family	15	2	332	12.91	18.26	23.44	0.01

9.Conclusion:

To control and manage pests, it is essential to accurately identify causative species. Sampling in sufficiently large numbers and barcoding of stinkbugs of each species of various habitats will effectively uncover molecular diversity between populations, which is helpful for the identification of stink bugs [17]. The COI-based identification of stink bug species has received the potential attention of researchers. However, it is not a 100% effective method for species identification. Future research may be focused on increasing the genetic data in DNA libraries for precise identifications of unknown taxa of stink bug genetic variants.

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