DNA based Identification of Food Pests: a Review

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Abstract: Food which is one of the basic requirements of humans have vital role in social health that should be authenticated for its quality, efficiency and efficacy to avoid spread of food born hazards caused by contaminating biological particles. Many insect species which are potent sources of food damage affecting consumer's health thrive in food materials originated from broad range of agricultural systems are not satisfactorily identified by existing morphological taxonomical keys causing impediments in their detection and eradication programs. Nonetheless, we have examined recently emerged DNA based identification systems such as species-specific PCR and real-time PCR using specific molecular marker, for instance, mitochondrial COI gene, which are powerful techniques for effective taxonomic investigations of food pest species that work with global comprehensive DNA sequence database. Moreover, we highlighted the molecular identification strategies for foodstuff infesting insect species in addition with bioinformatics tools that revolutionized the area of food entomology boosting up the effective implementation of global legislative laws making consumer's lives healthy and cheerful.

Index Terms **- Food, species-specific PCR, real-time PCR, mitochondrial COI gene, bioinformatics tools.**

INTRODUCTION:

The quality of stored food products determines human health which has gained considerable attention of food legislative agencies of many countries. During the period of last some years, the incidences of food infecting pests have been recorded with greater frequency (Kim 2011) may be due to increased food production to meet demands of increasing global population and insufficient storing and transportation facilities available for produced food materials. Since contamination of food materials by pests is result of infection by insect species, it becomes essential to take special care of food products while their harvesting, processing, packing, storing, transporting and distribution (Seo Young CHO et al., 2013). Entomology of stored products focuses on contamination of stored foods, for instance, invasion of cereals by insects, vegetables by caterpillars and maggots of flies in the sandwiches (Hall & Huntington, 2010). Insects belonging to order Arthropoda lead to damage the food materials and may be consumed coincidentally by humans giving rise to not only toxic but also various allergic effects along with health disorders and their defecate would be robust source of microbial load responsible for pathogenesis (Durden & Mullen, 2002).

In order to detect such pests in food materials, it is recommended to identify and remove them to avoid probable hazardous effects on human health. Nonetheless, in case of insect pest identification, one is required to depend on external characters related with their life stages and genders which are complex to identify, especially, in the case where specimens are immature, damaged or only tissue samples are available (Seo Young CHO et al., 2013) or immature members including eggs, pupae as well as larvae (Zhang, T. et al. 2016). Likewise, pests may be in the form of their body parts in the food material, which may be impractical to visualize, especially when the food materials such as stored grain, fruits to be inspected are in bulk amount. More to the point, the barriers in the identification process may be caused due to phenotypic variations among characters that are used for taxonomy (Ball & Armstrong 2006).

To solve confronted identification problem, concept of DNA barcoding that uses mitochondrial cytochrome c oxidase unit I (COI) gene as a primitive feature in many animal taxa (Hebert et al. 2003a, 2003b), has emerged, which can co-ordinate various stages of development and identify specimens that cannot be recognized by morphological examinations (Wheeler 2004; Vences et al., 2005; Will et al., 2005). The recently developed molecular identification system which is fast and perfect is gaining wide scale of appreciation for identification of insect pests (Huang, C. G., et al., 2009; Wang, Y. J. et al. 2014; Varadínová, Z., et al., 2015) and is authentic alternative technique for morphological taxonomy (Wang, Y. J. et al. 2014). Seo Young CHO et al. (2013) proposed that the insect pests associated with food have worldwide distribution; however, very fewer studies in relation with their DNA barcoding have been performed.

In this review, we assessed identification success of diverse food pests by mitochondrial COI gene using species-specific PCR and real time PCR and recorded the major gaps and their possible solutions for utility of molecular identification system for diagnosing food infested pests and their parts. Moreover, we highlighted DNA extraction strategies, PCR amplification conditions and various bioinformatics tools used for COI gene sequence analysis for food pest identification leading to qualitative and reliable examination of stored food products to ensure their durability, safety and efficiency.

DNA extraction strategies:

Diverse methods are used for DNA extraction of insect pest originated from food products as shown in table no.1. Although DNeasy Blood and Tissue Kit, TIANamp Genomic DNA kit, QIAgen's DNEasy extraction kit were used by various researchers (table 1), Wizard® Promega Kit method (Promega Corporation, Maidison, USA) may also work effectively for DNA extraction of insect species.

Sr.no	Method	Manufacturer	Target pest	Origin	Reference
1	DNeasy Blood and Tissue Kit	Qiagen Genomics Inc., Dusseldolf, Germany.	Insect pests	Food products	Seo Young CHO et al., 2013
2	TIANamp Genomic DNA kit	(TIANGEN, China)	*Tribolium spp.*	Flour	Zhang, T. et al. 2016
3	QIAgen's DNEasy extraction kit	QIAGEN,Valencia,Califor-nia,USA	Oryzaephilus surinamensis (sawtoothed grain beetle)	Stored grains	Jouni Sorvari et al., 2012

Table 1: Genomic DNA extraction kits used for food pests DNA extraction.

PCR amplification:

Primer designing:

The extracted genomic DNA is passed for PCR amplification targeting to specific molecular marker (658 bp mitochondrial COI gene sequence for animals) using specifically designed primer pairs. For example, the primers for *T. castaneum* and *T. confusum* are developed using ITS (internal transcribed spacer) region coded by rDNA and mitochondrial gene cytochrome C oxidase subunit I (COI) (Nowaczyk, K. et al. 2009). Designing such primers is very crucial step that determines success rate of DNA barcoding for species identification of food contaminating insects. However, the development of primers depends on certain factors (table 2a, table 2b and table 2c) that potentially affect their efficacy and efficiency. Zang T. et al., (2016) successfully designed and tested species-specific, TaqMan probe and real time PCR primers for six species (*T.destructor*, *T. brevicornis*, *T. madens*, *T. freeman*, *T. castaneum*, *T. confusum*) of genus *Tribolium* belonging to order Coleoptera and family Tenebrionidae which are flour habituating beetles. Some important characteristics that affect during PCR amplification are GC composition of primers that control their melting temperature. Therefore, their frequency in the primer is needed to be monitored. Moreover, the presence of secondary structures in primer sequences hinders the performance of Taq DNA polymerase enzyme leading to insufficient amplification of molecular marker under study. The length of primer should be less enough to bind its complementary sequence on target DNA and this primer sequences are trimmed at the time of sequence alignments.

Majority of researchers preferred Folmer primer pair (LCO1490, forward; HCO2198, reverse) for amplification of mitochondrial COI gene as these primers are used as universal invertebrate primers (O. Folmer et al., 1994) (table 3). Although Zang T et al. (2016) developed species-specific and real time PCR primers for molecular identification of 6 species of genus *Tribolium*, these are not tested on global scale including vertebrate and invertebrate animals covering wide range of taxa that would advocate authenticity of their universal applicability. If these primers are approved as universal ones, it would be great landmark in molecular taxonomy as these would be used to amplify COI sequences in the case where Folmer primer pair fails making the molecular identification system easier due to availability of optional primer assembly.

Sr.no	Character	Quantity
1	Length	18bp-30bp
2	Absolute value of Delta G (ΔG)	9
3	3′-end composition	1 or more specific bases
4	distinct hairpin structure	Absent
5	GC%	30% -70%
6	Ability to differentiate various species	Present
7	False priming	<100%
8	Annealing temperature	Optimum

Table 2a: Species-specific primer designing parameters for amplification COI gene belonging to Tribolium species (Zhang T. et al. 2016).

Sr.no	Character	Quantity
1	length	18bp-30bp
2	GC%	C%>G%; otherwise complementary use, GC content 30%-80%
3	5′-end composition	No G bases
4	Repeats of same types of bases, particularly when series of 4 G bases arrive	This condition was avoided
5	Probes	Close to the primers
6	Complementary secondary structures	Absent

Table 2b: Parameters for TaqMan probe designing used in real time PCR for molecular identification of Tribolium species (Zhang T. et al. 2016).

Sr.no	Character	Quantity
1	length	<4 bases for primer pair; 18bp-30bp for single base primers
2	GC%	40%-60%
3	3′-end composition	No A bases as the first base, no use of 3 G or C bases in a row
4	Repeats of same types of bases, particularly when series of 4 G bases appears	This condition was avoided
5	Complementary primers	Absent

Table 2c: Factors considered for Real-time PCR primer designing identification of Tribolium species (Zhang T. et al. 2016).

Sr. no	Primer	Sequence	Target gene and organism	Primer size (bp)	Reference
1	LCO1490 HCO2198	5′-GGTCAACAAATCATAAAGATATTGG-3′ 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Food Insects; COI	25 26	Seo Young CHO et al., 2013
2	LCO1490 HCO2198	5′-GGTCAACAAA TCATAAAGATATTGG-3′ 5′ -TAAACTTCAGGGTGACCA AAAAATCA-3′	Flour beetles; COI	25 26	Zhang, T. et al. 2016
3	LCO1490 HCO2198	5’-GGG TCA ACA AAT CAT AAA GAT ATT GG-3’ 5’-TAA ACT TCAGGG TGACCAAAAAAT CA-3’	*Oryzaephilus surinamensis* (sawtoothed grain beetle)	26 26	Jouni Sorvari et al., 2012

Table 3: The primers used for PCR amplification of various food pests.

Sr. no	Species	Primer	Sequence(5′-3′)	Size (bp)	Tm (°C)	Product length (bp)
1	*T.destructor*	Tde25F20 Tde451R24	5′-ATGAGCAGGAATAGTTGGTA-3′ 5′-ATAGGTCGTATATTGATTACTGTG-3′	20 24	51.6 51.7	450
2	*T. brevicornis*	Tbr63F23 Tbr394R23	5′-TTCGAGCAGAATTAGGTAATCCC-3′ 5′-TTCCTGCTAAATGTAATCTAAAG-3′	23 23	55.8 50.7	354
3	*T. madens*	Tma41F22 Tma508R23	5′-GGAACCTCTTTAAGATTATTAG-3′ 5′-TTGCTGTAATTACCACAGCTCAG-3′	22 23	49.7 57.2	490
4	*T. freemani*	Tfr379F22 Tfr526R23	5′-CGTAGATTTAGCAATTTTCAGG-3′ 5′-GTGAAAGAAGTAGAAGAATAGCG-3′	22 23	53.3 52.2	170
5	*T. castaneum*	Tca33F26 Tca346R24	5′-GAATAGTAGGCACTTCATTAAGACTC-3′ 5′-CCATGTGCAATGTTTGATGAGAGG-3′	26 24	56.3 57.9	337
6	*T. confusum*	Tco261F23 Tco474R25	GGCTCCTGCCACCCTCATTAAGA GGTATTCGTTCAAATGATATTCCTG	23 25	61.7 55.7	238

Table 4: Species-specific primers designed for amplification of COI gene sequences of *Tribolium* spp. (Zhang, T. et al. 2016)

Moreover, PCR amplification conditions and cycles are also crucial for amplification of targeted molecular marker and various researchers used different parameters to standardize PCR protocol for COI gene amplification of various food pests (table 6). The range of annealing temperature varied between 45°C -52°C. However, data provided by Zang et al. (2016) is lacking in certain areas as authors have not reported either annealing temperature or extension temperature (table 6). The comprehensive PCR amplifying condition database would assists in molecular taxonomic identifications of broad range taxa of food pests in variety of agricultural food materials.

Data analysis:

Using COI gene sequences of 6 *Tribolium* species, proper regions for specific primers were identified using Bioedit v7.2.0 and Beacon Designer 8.12 was employed for designing of TaqMan probes and real-time PCR primers which were developed for molecular identification of species under study as shown in table 4 and table 5, respectively. This study has elaborated the regime of DNA based identification systems by analyzing DNA of food pests with real time PCR connecting DNA barcoding approach with other supportive assembly. Furthermore, in case of ambiguous species identification by DNA barcode approach due to availability of insufficient reference library (Nakakita, H. 1983; Virgilio et al. 2012), real time PCR analysis can be proved as additional tool for species identification. In contrast, real time PCR has certain limitations in case of accuracy and reliability due to absence of provision for verification of obtained results in contrast to DNA barcoding technology.

Sr .no	Species	Primer	Sequence(5′-3′)	Size (bp)	Tm(°C)	Product length (bp)
1	*T.destructor*	TdeF TdeR TdeP	CGTACAGAACTAGGAAAC CCGATTATTATAGGTATTACTATG FAM-TCCTTAATCGGGAATGACCAAAT-BHQ	18 24 23	58.1 57.8 65	116
2	*T. brevicornis*	TbrF TbrR TbrP	GAGCAGTAGCAATTACAG TTCGGTCGGTTAATAATATAG FAM-TCACTTCCAGTGTTAGCCGGTG-BHQ	18 21 22	58.9 58.7 69.6	84
3	*T. madens*	TmaF TmaR TmaP	TCCTGGTTCTCTAATTGG GCTCCTAGTATAAGTGGAA FAM-AATGTAATTGTCACAGCCCATGC-BHQ	18 19 23	59.3 59.1 67.1	138
4	*T. freemani*	TfrF TfrR TfrP	CGTAGATTTAGCAATTTTCAGG TGAAAGAAGTAGAAGAATAGCG FAMAGCTGGTATCTCATCAATTCTTGGAGC-BHQ	22 22 27	61.7 61.9 69.8	169
5	*T. castaneum*	TcaF TcaR TcaP	GATCCTCTGTTGATCTTG CAGGAAGAGATAAGAGAAG FAMTCTGGGAGCAGTTAATTTCATTACAAC-BHQ	18 19 27	58.1 57.5 66.8	183
6	*T. confusum*	TcoF TcoR ToP	CAGGATGAACTGTTTACC GTAGGTCGTATATTAATTACTG FAM-ATCATCTAATATCGCTCACGGAGGAG-BHQ	18 22 26	58.7 57.3 68.6	151

Table 5: TaqMan probes and primers designed for six *Tribolium* species. (Zhang, T. et al. 2016)

We evaluated efficacy of various tools used for data analysis of COI sequences belonging to insects found in food materials (table 7). Species-specific primers eligible for *Tribolium* spp. COI gene amplification, were designed using Primer Premier 5.0 (Zang et al. (2016). In addition to various tools used by scientists for COI gene sequence editing using chromatogram file such as Contig Express program, Geneious Pro 5.3.6 (table 7), Codon Code Aligner (Codon Code Corporation, USA) may be used as effective software. Further, Muscle (Robert C. Edgar, 2004) and Clustal W (J D Thompson et al., 1994) algorithms may be used for wide range of DNA sequence alignments in addition with Clustal X (Larkin et al. 2007) and DNAMAN 7.0.2 used for sequence alignment of food product pests and flour beetles, respectively (table 7).

Sr. no	Initial Denaturation	Denaturaion with cycles	Annealing	Extension	Final extensio n	Target animal and gene	Reference
	Species-specific PCR						
1	94°C for 3 min	40 cycles at 94°C (30 sec)	45–48°C (1 min)	72°C (30 sec)	72°C for 10 min	Food Insects; COI	Seo Young CHO et al., 2013
2	94 °C for 3 min	35 cycles of 94 °C for 1 min	52 °C for 1 min	72 °C for 1 min	72 °C for 10 min.	Flour beetles; COI	Zhang, T. et al. 2016
	Real time PCR						
1	95 °C for 30 s	35 cycles of 95 °C for 5 s	60 °C for 34 S	Data not availbale	72 °C for 10 min	Flour beetles; COI	Zhang, T. et al. 2016
2	95°C for 5min	then 40 cycles of 94°C for 30 sec	50°C for 30 sec	72°C for 1 min 30 sec	10 min at 72°C	*Oryzaephilus surinamensis* (sawtoothed grain beetle)	Jouni Sorvari et al., 2012

Table 6: PCR amplification conditions used by various researchers for PCR amplification for their molecular identification

Genetic variation pattern:

Seo Young CHO et al. (2013) compared COI sequences of insects found in food products of Korea and stated that mean intra specific and interspecific divergences were 0.84%, 20.10% respectively for order Coleoptera and 0.20%/15.90%, respectively for order Diptera. This result is congruent with the minimum criteria for maximum intraspecific distance (3%) proposed by Paul D. N. Hebert et al., (2003a) for individuals of both orders. Furthermore, interspecific distances reported by authors in both cases matched with the barcode gap rule (Hebert PDN et al., 2004) which suggest that for species identification with standard barcode gap, mean interspecific distance must be 10 times more than mean intraspecific distance. Thus, it is clear that authors explicitly succeeded to identify the food pests found in Korea reliably meeting criteria of existing scientific standards.

Sr. no	Software used for sequence editing	Software used for sequence alignment	User	Organism
1	BioEdit Sequence Alignment Editor ver. 7.1.3,Geneious ver.5.5.7(Hall 1999)	Clustal X (Larkin et al. 2007)	Seo Young CHO et al., 2013	Insects of food products
2	Contig Express program, DnaSP v.5.1 (for haplotype identification)	DNAMAN 7.0.2	Zhang, T. et al. 2016	Flour beetles
3	Geneious Pro 5.3.6 (Drummond et al. 2011)	-	-	-

Table 7: Bioinformatics tools used for data analysis of COI sequences of food pests

Furthermore, authors stated that intra-specific and inter-specific distances of individuals belonging to family Muscidae which are foremost food invasive pests (Highland 1984, 1991; Campbell et al. 2004), were recorded as 0.1% and 9.9%, correspondingly. Similar observations were recorded by Yu et al. (2007) who stated that intra-specific distance of Muscidae family was less than 0.6% while inter-specific variation was found to be in the range of 9.7–14.4%. In contrast, Seo Young CHO et al. (2013), reported less than 70% success rate in Dipteran identification based on phylogenetic analysis of DNA barcodes and stated that incorrect identifications were due to overlapping between intra and inter specific variations. It is because, distinct barcode gap is required between intra and inter-specific distances for reliable species identification using DNA barcoding technology and it will accelerate the effectiveness of taxonomic differentiation. This issue was further elaborated by Meier et al. (2006, 2008) who stated that even if the two DNA barcodes are identical, these may belong to various species of Diptera. However, development of complete DNA barcode sequence database would assist in truthful identification of species belonging to Diptera (Seo Young CHO et al. 2013).

Seo Young CHO et al. (2013) reported low success in the species identification of genus *Lucilia* belonging to family Calliphoridae in Diptera with maximum intra-specific distance as 0.4% and minimum inter-specific variation as 0.5%. Reason of this aspect was provided by Whitworth et al. (2007) who reported that this genus from order Diptera has non-monophyly in its taxa giving low identification success. Similarly, Funk and Omland (2003) reported low intraspecific and interspecific variations as <0.2% and in the range of 0.7% –2.4%, respectively suggesting common existence of not only paraphly but also polyphly in the species that are close to each other.

Seo Young CHO et al. (2013) suggested that re-evaluation of external characters and further investigations related with taxonomy may be used in cases of less genetic variations found in Diptera with particular emphasis to *Lucilia caesar* as well as

Lucilia sericata along with subspecies of genus *Culex* like *Culex pipiens*. In addition, Kimura 2-parameter method (K2P) (Kimura 1980) as a distance model has been used by many researchers for analyzing pairwise distances among COI sequences of food pests (table 8) whereas, NJ tree was preferred for construction of phylogenetic tree with MEGA (Tamura et al. 2011) as a platform for phylogeny analysis. Finally, Automated Barcode Gap Discovery (ABGD) (Puillandre, N., et al., 2012) which is freely available web based tool for analyzing distance histograms and species clusters with distinct gap was used by Zhang, T. et al. (2016) for analyzing species distinctness using COI sequences of flour beetles.

The efficiency of DNA barcodes for food pest identification:

Seo Young CHO et al. (2013) assessed efficacy of DNA barcoding technology for identification of 17 species of insect pests belonging to 10 genera found in food products of Korea and stated that barcode gaps could reliably identify taxa above species level in majority of investigated species. Moreover, authors stated that this technique fails to work when reference sequence data would not be available in the sequence database and in case of identification of heterogeneous organisms, building of their reference database would be more difficult. Unfortunately, reference sequence data of near about 90% of known insect species is not available (Sujeevan Ratnasingham and Paul D. N. Hebert, 2007). Additionally, the organism whose reference sequence is not available in the sequence database will be inaccurately assigned to heterospecific sequence of DNA barcode showing maximum similarity (Virgilio et al. 2012). In addition, 658bp COI genes were used Y.J. Wang et al., (2014) to overcome the errors of *Cryptolestes* species identification by morphological analysis and these species are pests of stored food products that were found in China, Czech Republic and the USA. Moreover, Varadínová, Z., et al., (2015) established COI based identification systems for five species (*Cryptolestes capensis*, *Cryptolestes ferrugineus*, *Cryptolestes pusilloides*, *Cryptolestes pusillus* and *Cryptolestes turcicus*) of *Cryptolestes* stored food pests.

Sr. no	Distance model used for pairwise sequence variation analysis	Statistical method used for phylogeny construction	Distance histograms	Target organisms	References
1	Kimura two parameter (K2P) model (Kimura 1980).	Neighbor-joining (NJ) using MEGA 5.05 (Tamura et al. 2011)	NA	Insects of food products	Seo Young CHO et al., 2013
2	Kimura 2-parameter method (K2P) (Kimura 1980)	Neighbour-joining (NJ) phylogenetic Trees were constructed in MEGA 6.017 (Tamura et al. 2011)	Automatic Barcode Gap Discovery (ABGD) (Puillandre, N., et al., 2012).	Flour beetles	Zhang, T. et al. 2016
3	Kimura 2-parameter method (K2P) (Kimura 1980)	-	-	*Cryptolestes* species	Y.J. Wang et al., 2014.

Table 8: Genetic distance model, statistical method for phylogeny construction and online available bioinformatics tool used for molecular identification of food pests.

Species identification has been performed using molecular biology technology with increased rate (Zhang T. et al. 2016). In addition, Zhang T. et al. (2016) proposed that 6 species of genus *Tribolium* can be identified by DNA barcodes, species-specific Polymerase Chain Reaction (PCR) in addition to real time PCR. Furthermore, authors tested the DNA extraction efficacy of *Tribolium* spp. and concluded that enough DNA can be extracted from the insects in question that can be used for generation of DNA barcodes, species -specific PCR and real-time PCR systems for their identification. As well, authors observed that the universal primers designed for DNA barcoding using mitochondrial COI gene with assistance of species specific primers along with TaqMan probe as well as primers sets used for *Tribolium* species identification can be used to identify ontogenetic stages of the beetle species under study. Besides, they suggested that COI sequences provided strong infrastructure for 6 species identification of *Tribolium* beetles found in stored food product with distinct barcode gap. Additionally, authors proposed that the clades of 6 *Tribolium* species formed by NJ tree were congruent with their morphological identifications. Further, authors argued that species-specific PCR is superior technique for species identification over DNA barcoding approach as it does not require DNA sequencing and merely extraction of genomic DNA followed by PCR and electrophoresis are enough to identify species. Furthermore, the PCR assay can be completed within 3 hours utilizing DNA of unidentified *Tribolium* species.

Although Zhang T. et al. (2016) claimed that real-time PCR is beneficial over traditional PCR due to feasibility of its use for without melting curve study, it has certain demerits such as high cost and requirements of reaction kits in addition to TaqMan probes and suggested for wide scale collection of specimens, testing of molecular markers that are used commonly along with development of multiplex PCR system. In addition, authors claimed that internally transcribed spacer (ITS) region can be used for identification of 6 species *Tribolium* beetles from stored food products more effectively than COI gene. Finally, authors argued that species-specifc primers as well as real-time PCR may be substantially used in multiplex PCR system.

CONCLUSION:

All these investigations clearly suggest that the food associated pests causing harms to customers can be successful identified by partial mitochondrial COI gene with required efficiency and future studies relating with its use in identification of wide range of raw as well as cooked agricultural food materials including fruits, vegetable, cereals, pulses etc. would aid for effective eradication of pests from diet of consumers making their lives happier and healthy.

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