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# Identification of Fruit Flies (Diptera: Tephritidae) at Species Level: The Molecular Technique is an Effective Tool

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## Abstract

Today's technology enables us to precisely identify any living thing using a single cell. This technology uses molecular probes that bind only to the DNA of targeted organisms, is based on gene amplification that multiplies a million-fold the DNA of interest, and involves comparing specific pieces of DNA between organisms. There are two important advantages of molecular identification over conventional techniques of microscopic examination that means morphological identification. Identification can be made using a very small amount of material and is much more accurate than with previous methods- a species, a population, or even an individual can be identified. So in accurate species identification, no doubt about the molecular method. For fruit fly identification, the molecular method based on PCR-RFLP, DNA barcoding of the *COI* gene and/or polymerase chain reaction (PCR) with species-specific primers is the commonly used and accurate method of identifying species level.

**Keywords:** Fruit fly, DNA barcoding, *COI* gene, economic important and species specific primers.

## Introduction

Fruit flies (Family Tephritidae) constitute a group of agricultural pests of worldwide importance that attack a wide range of fruits and vegetables (White *et al.*, 1992). Numerous fruit fly species cause enormous threats to fruit and vegetable production globally, exerting both quantitative and qualitative losses. However, few insect species have a more significant impact on world trade in agricultural produces than tephritids fruit flies (FAO/IAEA 2013). Direct damage of fruit flies are associated with dropping of fruits and rendering of inedible fruits. Besides, the direct damage to fruits and indirect losses are associated with quarantine restrictions imposed by importing countries to prevent the entry and establishment of exotic fruit fly species (Ekesi, 2012). There are nearly 5000 described species of tephritids fruit fly, of which approximately 350 are considered economically significant. These primarily belong to five genera: *Anastrepha* Schiner, *Bactrocera* Macquart, *Ceratitis* Macleay, *Dacus* Fabricius and *Rhagoletis* Loew (Van Houdt *et al.*, 2010). More than 70 fruit fly species are considered as important worldwide agricultural pests, and many others are minor or potential pests (White and Elson-Harris, 1992). The genus *Bactrocera* is the most economically significant fruit fly genus, with at least 50 species considered important pests, many of which are highly polyphagous (White *et al*.,1992).

A critical aspect of prediction and also monitoring is the ability to accurately identify any intercepted specimen to the species level (Armstrong and Ball, 2005). Therefore, accurate identification is essential in host fruit surveys and for species found in fruits destined for export, distinguishing exotic from native fauna (Armstrong and Ball, 2005). These limitations associated with morphological identification have been an impediment in decision-making regarding fruit fly infestations at

quarantine checkpoints and implementing appropriate management programs for tephritids fruit flies. In effect, researchers have developed other valuable alternative ways of fruit fly identification involving molecular markers. Currently, PCR-RFLP (Polymerase Chain Reaction Restriction Fragment Length Plymophism) and DNA barcoding of the *cytochrome oxidase c subunit I* (*COI)* gene are becoming the preferred molecular method to assist in precise and rapid identification of fruit fly species at any stage of development (PHA, 2011). Some recent studies have also used polymerase chain reaction (PCR) with species-specific primers to overcome the need for post-amplification digestion and DNA sequencing, thus allowing more rapid identification (Chua *et al.,* 2010; Zhang *et al.,* 2012). In this review, we discussed which method(s) is/are suitable for identifying fruit fly species.

**Limitations of Morphological identification method**

Many species of these tephritid fruit flies are morphologically similar. Still, they differ in their behavior, such as reproductive potentials, competitive abilities, and dispersive power (Duyck *et al.,* 2004). They thus have different potential impacts on food production and implications for biosecurity and market access. In addition, the immature stages of different genera are morphologically indistinguishable and are the most likely life stages to be intercepted in food produce (Blacket *et al.,* 2012). Moreover, in host fruit surveys, immature stages of different genera have been found to share the same fruit (Copeland *et al.,* 2006; Ekesi *et al.,* 2006) yet with no morphological diagnostic features. Uncertainty in species limits based on the traditionally used morphological features, together with overlapping host and geographic ranges, significantly impacts quarantine, pest management, and general biological studies (Clarke *et al.,* 2005).

**Molecular identification**

Genetic markers and sequences from the mitochondrial genome, in particular, have proven to be very informative in the study of species diversity and evolutionary processes (Xie *et al.,* 2006). This is due to some of its peculiarities, such as strictly maternal inheritance, absence of recombination, a relatively high mutation rate, and last but not least, the availability of efficient PCR primers (Simon *et al.,* 1994) and a wealth of comparative data (Xie *et al.,* 2006). Mitochondrial *cytochrome oxidase subunit I (COI)* sequences were shown to be appropriate for intra-specific analysis because of the high degree of polymorphism observed.

Additionally, *COI* sequences are at the base of the barcoding identification system (Hajibabaei *et al.,* 2006). Besides being a valuable tool for species identification and discovery, it has been proposed as a powerful methodology in biosecurity and invasive species identification (Armstrong and Ball, 2005). Currently, this tool has been applied in pest monitoring and quarantine (Armstrong and Ball, 2005). Its usefulness has been confirmed in several hexapod orders: Coleoptera (Lobl and Leschen, 2005), Diptera (Scheffer *et al.,* 2006), Ephemeroptera (Ball *et al.,* 2005), Hemiptera (Lee *et al.,* 2011), Hymenoptera (Smith *et al.,* 2008) and Lepidoptera (Hajibabaei *et al.,* 2006). Species identification is achieved by comparing the sequence of an unknown sample to a reference database through similarity methods such as BLAST (Altschul *et al.,* 1990). The reliability of identification depends on the extent of taxonomical coverage of the group of interest and an understanding of the degree of variation within species (Lee *et al.,* 2011).

A case study on tephritid fruit flies (Armstrong and Ball, 2005) reported high rates of success, but also mentioned some difficulties with the identification of a few species (e.g. *B. dorsalis, B. cucurbitae, A. fraterculus*), where the occurrence of cryptic species, inadequate sampling of all genetic subgroups, and high levels of geographic differentiation might complicate identification. However, broader ad hoc surveys of the phylogeography and geographic variability in species might provide valuable additions to the barcoding dataset and its applicability in difficult groups. Modern control strategies, such as semiochemicals, sterile insect techniques, and foreseeable genetic tools, are strictly species/strain-specific and require a deep knowledge of taxonomy and population structure of the target. This necessity becomes even more sensible when dealing with insect groups characterized by sibling species, such as mosquitoes and tephritid fruit flies (Hu *et al.,* 2008).

Zhang *et al.* (2010) studied 689 bp nucleotide sequences of the mitochondrial *cytochrome oxidase I gene* of thirty-five individuals representing 7 *Bactrocera* species found in the Chongqing region in China and GenBank submitted sequences for another 20 *Bactrocera* species and 2 tephritid species, *Anastrepha ludens* and *Ceratitis capitata*, which were used as outgroups for the phylogenetic analysis. They reported *Bactrocera* (*Tetradacus*) *minax* and *Bactrocera* (*Zeugodacus*) *diaphora* sequences for the first time, and the subgenus *Bactrocera* (*Tetradacus*), represented by *B. (T.) minax* and *B. (T.) tsuneonis*, was included for the first time in an analysis of the genus *Bactrocera* phylogeny.

Zhang *et al.,* (2010) observed that nucleotide diversity within subgenus ranged from 9.1 to 19.0% among the subgenera, and the net divergence among subgenera ranged from 4.6 to 12.7%. Phylogenetic analysis based on maximum parsimony method supported that subgenus

*Bactrocera* (*Bactrocera*) and *Bactrocera (Zeugodacus)* are paraphyletic. The subgenus *Zeugodacus*, *Bactrocera (Zeugodacus) caudate, Bactrocera (Zeugodacus) diaphora, and Bactrocera (Zeugodacus)* scutellata are closely related to *Bactrocera (Zeugodacus) tau* and *Bactrocera (Zeugodacus) cucurbitae*. These results indicated that subgenus Austrodacus and *Zeugodacus*, which attack cucurbit plants, are closely related to the subgenus Afrodacus, *Bactrocera*, and *Gymnodacus*, which attack plants of numerous families.

Earlier phylogenetic relationships among 24 *Bactrocera* species belonging to 9 subgenera were studied by Smith *et al.* (2003) with DNA sequence of portions of the mitochondrial 16S rRNA, *cytochrome oxidase II*, *tRNALys*, and *tRNAAsp* genes suggested (1) the genus *Bactrocera* is monophyletic, (2) the subgenus *Zeugodacus* is paraphyletic, (3) the subgenus *Daculus* is a sister group to subgenus Bactrocera and (4) the subgenus *Bactrocera* is monophyletic. Asokan et al. (2011) reported the mt*COI* based identification of three fruit flies, *B. dorsalis*, *B. correcta* and *B. zonata* where molecular identification has corroborated the morphological identification. A single fragement of approximately 500 bp was amplified for *B. dorsalis*, *B. correcta* and *B. zonata*. Sequencing results showed that the total nucleotide length obtained was 440 bases for all three species of fruit flies. The alignment of the above sequences in Bioedit revealed 92% similarity between *B. dorsalis* and B. correcta and between *B. correcta* and *B. zonata.* The number of nucleotides that were different between *B. dorsalis* and *B. correcta* and between *B. correcta* and *B. zonata* was 32 and 28, respectively. The highest variation (11%) was observed between *B. dorsalis* and *B. zonata*, where there was difference in 45 nucleotides. *Bactrocera cucurbitae* populations sampled throughout Southern China, Thailand, and the Philippines by Hu *et al.* (2008). They observed that these populations were genetically very similar and most likely constitute a single phyletic unit with no sign of cryptic species or historical separation based on the mitochondrial *cytochrome oxidase I* gene analysis. They also observed that a single haplotype predominates throughout this region. However, interspecific distances with outgroups ranged from 0.051 between *B. cucurbitae* and *B. tau* to 0.167 between *B. cucurbitae* and *B. dorsalis.*

Shi *et al.* (2005) conducted an analysis of population genetic structure of *B. dorsalis* from China using mitochondrial *cytochrome oxidase* (*COI*) gene sequences. They observed twenty-eight haplotypes among 37 individuals with up to 13 mutations, and genetic distances reached 2.2% between haplotypes. They also observed many haplotypes were missing in the sampled populations in the haplotype network. However, 43 haplotypes were observed in the six *Bactrocera dorsalis* populations (71 individuals) with up to 12 mutations from China using *COI* gene sequences by Liu *et al.* (2009). *B. tau* is a major cucurbit pest. Morphologically, members of the *B. tau* complex show differences in the three yellow stripes on the thorax, along with the size and shape of dark bands on the dorsal abdomen. However, some species of the *B. tau* complex could not be easily distinguished morphologically. Mitotic karyotype and electrophoresis analyses of the *B. tau* complex have been useful tools for separating these closely related species. However, the methods are somewhat tedious and time-consuming (Baimai *et al.*, 2000). Analysis of mitotic karyotypes of the larvae belonging to the same species of adult fruit flies morphologically identified as *B. tau* s.s. and *B. tau*-like species has revealed seven distinct chromosomal forms which are most likely to represent seven closely related species within the *B. tau* complex. All members of the *B. tau* complex in this study exhibited mitotic karyotype 2n=12, conforming to the other species groups of the genus Bactrocera as previously described (Baimai *et al.*, 2000). Jamnongluk *et al.* (2003) compared sequences of the mitochondrial *cytochrome oxidase I* gene of eight species of the *Bactrocera tau* complex from Thailand using *Bactrocera dorsalis, Bactrocera pyrifoliae, Ceratitis capitata, Anopheles gambiae,* and *Locusta migratoria* as outgroups. The sequence divergence between species in the *B. tau* complex ranged from 0.06 to 28%, and up to 29% between the complex and its tephritid outgroups, *B. dorsalis* and *C. capitata*.

**DNA barcodes and barcoding**

Hebert *et al.* (2003a, b) proposed a technique for amplifying a 648 bp region of the mitochondrial *cytochrome-c oxidase subunit 1* (*COI*) gene to ensure rapid and accurate identification of a wide range of biological specimens. Then, the Life Barcode project was introduced to encourage DNA barcoding as a global standard for sequence-based eukaryote identification. This project was formally launched in 2004 by the establishment of the Consortium for the Barcode of Life (CBOL), which aims to create a standard DNA barcode protocol and build a comprehensive DNA barcode library (Jinbo *et al.*, 2011).

Nowadays, DNA barcoding has become a powerful tool for successful identification of fruit flies. On the basis of the COI gene, several rapid diagnostic approaches have been established, such as real-time PCR, PCR-RFLP (restriction fragment length polymorphisms), microfluid dynamic array techniques and loop-mediated isothermal amplification (LAMP). Such methods were successfully implemented in identifying several economically important fruit fly species. *Armstrong et al. (2005)*

studied the COI sequences of fruit fly samples collected from New Zealand ports, and the findings were compatible with the previous results of the constraint fragment duration polymorphisms (RFLPs). However, identification using DNA barcodes detected species which could not be recognized by RFLP analysis (Armstrong *et al.,* 2005). The DNA barcoding (amplification of the sequence by COI gene) method was applied to identify the larvae which were collected from guava fruit of Thailand, resulting in the identification of *B. correcta* (Buahom *et al.,* 2011). *Barr et al., (2017)* only differentiated four species of *Anastrepha* (*Anastrepha grandis*, *A. serpentine*, *A. ludens*, and *A. striata*) based on barcode data, though they used 539 DNA sequence from 74 species. Out of 10 species of *Bactrocera*, eight species were easily distinguished by Manger *et al.* (2018) based on the standard DNA barcoding region of the *COI* gene. Moreover, the drawbacks of this approach are also noticeable, as it is costly and time-consuming.

**Species specific primers**

In some studies, polymerase chain reaction (PCR) with species-specific primers have also been used to resolve the need for post-amplification digestion and DNA sequencing that enables quicker identification (Chua *et al.*, 2010). The two pairs of species-specific primers were successfully developed by Chua *et al.* (2010) on the basis of 1517 bp of the mtDNA *COI* gene that could distinguish *B. carambolae* and *B. papayae* under normal PCR testing conditions. Asokan *et al.* (2011) developed species primers based on DNA barcode sequences for the identification of *B. dorsalis* and *B. zonata*, and this approach could accurately classify all life stages of the target species. Zheng *et al.* (2019) developed two pairs of species-specific primers that can easily identify all developmental stages of *B. minax* and *B. tsuneonis*, which is very cost-effective molecular method of identification.

**Conclusion**

Fruit fly species have been a serious threat to agriculture in worldwide. Morphological identification sometimes creates misidentification in adult stages, while in egg, larvae and pupal stages are totally difficult. Molecular techniques also offer a considerable advantage over traditional morphological forms of fruit fly discrimination as well as within host-parasitoid identification, which currently relies on dissection of immature parasitoids from the host, or lengthy and labor-intensive rearing methods. For rapid identification, polymerase chain reaction (PCR) with species-specific primers method is very effective, which can be used in quarantine stations. However, DNA barcoding proves an effective tool that can be employed for species identification, elucidation of cryptic species, biotypes, and the discovery of new species.

**Conflict of interest**

Authors have no conflict of interest.

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