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*Full Length Research Paper*

# Identification of *Bactrocera invadens* (Diptera: Tephritidae) from Burundi, based on morphological characteristics and DNA barcode

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***Bactrocera (Bactrocera) invadens*** **Drew (Diptera: Tephritidae) is a new species of fruit fly in 2005. It belongs to the *Bactrocera dorsalis* complex, but is difficult to diagnose based on solely morphological identification. It occurs in India, Bhutan and some countries of Africa. In this study, 14 adult samples of fruit flies were collected from Bujumbura and Cibitoke in Burundi District. Microscopic observations showed morphological character states that were congruent with the diagnosis of *B. invadens*. The mitochondrial DNA (mtDNA) cytochrome c oxidase I (*COI*) gene sequence alignment demonstrated the similarity between specimens 1 and 2 and *B. invadens* is 99.47%, between specimen 3 and *B. invadens* 98.77%, between specimen 4 and *B. invadens* 99.82%, and between the other 10 specimens and *B. invadens* 100%. Therefore, all samples were identified as *B. invadens* based on morphological characteristics and DNA barcode of *COI* gene. This study represented the first report of *B. invadens* in the Burundi District.**

**Key words**: *Bactrocera (Bactrocera) invadens*, identification, morphological characteristics, mtDNA *COI* gene, DNA barcode.

## INTRODUCTION

*Bactrocera (Bactrocera) invadens* Drew is a new species recorded for the first time in Kenya in 2003, but it had always been recognized as a species belonging to Asian species complex (Lux et al., 2003). This species spreads quickly throughout the African continent, and has invaded many countries, including Kenya, Tanzania, Sudan, Cameroon, Uganda, Senegal, Togo, Comorin, Benin, Congo, Ghana, Nigeria, India and Bhutan (Mwatawala et al., 2004; Drew et al., 2005). The main host plant for this insect pest is mango, but it also invades loquat, guava, shaddock, orange, cucurbit, papaya, avocado, and many other fruits (Mwatawala et al., 2006). Due to a broad range of plant hosts and a high invasive ability, this species causes much damage throughout the world. It has displaced a native mango fruit fly *Ceratitis cosyra* through resource competition, larval competition and interference competition through aggressive behaviors by adults (Ekesi et al., 2009).

Traditionally, the identification method of Tephritidae species is based on the morphological characteristics of adult. *B. invadens* belongs to *Bactrocera dorsalis*-complex, which includes many cryptic species (Drew and Hancock, 1994) and regarded as the most difficult complex to identify with morphological data.

Recently, the molecular identification has become an effective complementarity of morphological identification (Ball et al., 2005; Timm et al., 2007, 2008), especially when DNA barcoding was proposed as a tool for identification of species (Hebert et al., 2003). DNA

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barcodes are short and standardized DNA sequences suitable to identify species. For this method, the mitochondrial gene and cytochrome c oxidase subunit I (*COI* gene) are often used for animal identification (Hebert et al., 2003). Because DNA barcoding meets or exceeds the minimum standards required for diagnostic protocols under ISPM No. 27 (FAO, 2006), it has been proposed to be developed into a standard governing diagnosis workflow for rapid identification of plant quarantine pests; the typical protocol of this method has been described in detail (Floyd et al., 2010). Barcode of Life Data (BOLD) system is an informatics workbench that helps researchers to acquire, store, and analyze DNA Barcode records, especially for *COI* gene. The molecular, morphological and distributional data from BOLD could be used to accomplish the identification of many biological individuals (Ratnasingham and Hebert, 2007).

The Republic of Burundi is a landlocked country, which lies south of the equator on the Nile-Congo watershed. It is bordered by Tanzania in the southeast, the Democratic Republic of the Congo (DRC) in the west, and Rwanda in the north. Lake Tanganyika forms part of its border with the DRC. Many of its neighbor countries (e.g., Tanzania, Congo, Kenya and Uganda), have reported the presence of *B. invadens*, but there is no record of this species in Burundi. Though *B. invadens* is distributed in many African countries, no data are available on its invasion pattern in Africa (Khamis et al., 2009). The distribution of *B. invadens* in Burundi is part of its colonization process in the Africa continent and would be very important for further study on the invasion pattern of this species.

In this study, we collected 14 adult samples of fruit flies in the Burundi District, and finished the identification of all the samples using morphological characteristics and BOLD. On the basis of the morphological and molecular diagnosis, we discussed the efficacy of mitochondrial DNA (mtDNA) *COI* gene-based identification and the significance of discovery of this pest insect for Burundi.

## MATERIALS AND METHODS

### Sample collection

Four adult fruit fly specimens (specimens 1 to 4) were collected in Mango trees from Bujumbura (29°21'E 3°22'S, the capital of the Republic of Burundi, located at the north bank of eastern Lake Tanganyika) during September, 2009, and the other 10 specimens (specimen 5 to 14) from Mango trees in Rugombo country (29°04'E 2°48'S) in Cibitoke (a province locating at the northwest of Burundi) (Table 1) during October, 2009. All samples were kept in 100% ethanol and stored at -80°C before DNA extraction.

### Morphological identification

The whole samples were observed using Stereo Optical Microscope (Olympus SZX7, Japan). The figures of samples, especially for the mesonotum were captured using Confocal Optical Imaging System (UV-CTS, China). Identification followed Drew et al. (2005). All specimen images which were captured before DNA extraction were kept in Plant Quarantine Laboratory in Department of Entomology of China Agriculture University.

### DNA extraction and *COI* gene amplification

DNA was extracted from the whole adult fruit fly using the commercial tissue/cell DNA mini kit (Tiangen, China). Each polymerase chain reaction (PCR) was completed in a final volume of 52 µl containing 32.6 µl sterilized distilled water, 6 µl 10 × reaction buffer, 3 µl $MgCl_2$ (25 mM), 2 µl nucleic acid DNA extraction (115.8 ng/µl) as a template and 0.4 µl *Taq* polymerase (2.5 U/µl, Tiangen, China); 2 µl dNTP mixture (2.5 mM), 3 µl forward and reverse primer (10 µM), respectively. Reaction condition can be described as follows: 94°C for 3 min, followed by 39 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min and then a final incubation at 72°C for 10 min. The reaction was performed on Veriti TM 96-well Thermal Cycler (ABI, USA). The universal *COI* gene primers were LCO-1490 (5'-GGTCAACAAATCATAAAGATATTG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer, 1994). The presence of bands of correct size was detected using gel electrophoresis. DNA samples of all specimens were also deposited at -80°C in Plant Quarantine Lab in Department of Entomology of China Agriculture University.

### DNA sequencing

The DNA was purified using a DNA purification kit (Tiangen, China) and then sequenced directly using a 3730XL DNA genetic analyser (ABI, USA). To get correct sequences, every sample was sequenced in two directions, using the same primers that were used for PCR.

### Sequence analysis

Sequence assembly was performed with DNAMAN 5.2.2.0. After multiple alignments of sequences using Clustal X (Version 1.83), the forward and reversed nucleic acid sequence with low quality was deleted. The obtained sequences of *COI* gene were submitted to GenBank for the accession numbers and to BOLD for making alignment with the standard sequences online and for species identification.

### Phylogenetic analysis

In order to verify the above identification results, neighbor joining (NJ) tree was used for phylogenetic analysis among the sequences of samples and related species from the GenBank database (Table 2). It was constructed using MEGA Version 4.0.2 (Tamura, 2007) with NJ method (Saitou and Nei, 1987) and Kimura 2-Parameter (Nei and Kumar, 2000; Hebert et al., 2003). The pair wise genetic distances based on Kimura 2-Parameter were also computed using MEGA Version 4.0.2. The relationships were inferred based on genetic distances.

## RESULTS

### Morphological characteristics of *B. invadens*

The stereo optical microscope observation showed the

**Table 1.** Collection information on specimens identified in this study.

| Specimen | Collection locality | Date | Longitude, latitude | Host plant | GenBank accession number | DNA vouchers number |
|---|---|---|---|---|---|---|
| Specimen 1-4 | Bujumbura, Burundi | September 2009 | 29°21'E 3°22'S | Mango | HQ689655-HQ689658 | CAU00347-CAU00350 |
| Specimen 5-14 | Rugombo, Cibitoke, Burundi | October 2009 | 29°04'E 2°48'S | Mango | HQ689659-HQ689668 | CAU00351-CAU00360 |

**Table 2.** Species information from GenBank for phylogenetic tree construction.

| Specie | Collection locality | Submission time | Accession number | Reference |
|---|---|---|---|---|
| *Bactrocera invadens* | Azaguié, Ivory Coast | 11-August-2008 | FJ009202 | Virgilio et al., 2009 |
| *Bactrocera papayae* | Khorat, Thailand | 04-July-2005 | DQ116326 | |
| *Bactrocera zonata* | Hyderabad, India | 04-July-2005 | DQ116362 | |
| *Bactrocera philippinensis* | Nga, Palau | 04-July-2005 | DQ116319 | |
| *Bactrocera correcta* | Khon Kaen University, Thailand | 04-July-2005 | DQ116265 | Armstrong and Ball, 2005 |
| *Bactrocera dorsalis* | Tanzania | 04-July-2005 | DQ116281 | |
| *Bactrocera dorsalis-2* | Tanzania | 04-July-2005 | DQ116279 | |
| *Bactrocera carambolae* | Suriname | 04-July-2005 | DQ116253 | |
| *Ceratitis capitata* | Sao Paulo, Brazil | 04-July-2005 | DQ116368 | |

main morphological features of the whole adult (Figure 1). For the Thorax, the base color of the scutum mostly was dark orange-brown and occasionally black. There were two narrow parallel-sided lateral post-sutural vittas, ending at or just behind intra-alar seta. The scutellum was yellow, and had a narrow black basal band. For the abdomen, there was a "T" pattern across tergite III to V, and with pecten present on tergum III. The visible features of the thorax were the different markings on the scutum, most of which were a lanceolate dark pattern. There were also variations (Figure 2). Based on the morphological characteristics, all the samples were identified as *B. invadens*, in accordance with the description by Drew et al. (2005). The identifications were confirmed by the fruit fly taxonomic expert, Fan Liang (Inspection and Quarantine Technology Center of Guangdong Entry and Exit Inspection and Quarantine Bureau) in China.

## DNA sequence alignments

PCR amplification was successfully achieved, and 14 *COI* gene sequences of 570 bp in length were obtained. The sequences were deposited in GenBank under the accession numbers from HQ689655 to HQ689668 (Table 1). All the sequences were submitted to BOLD for species identification. By comparison with all the *COI* sequences in BOLD, the highest sequence similarity occurred between the 14 fruit fly samples and *B. invadens*. The *COI* sequence similarity between specimen 1, 2, 3, 4 and *B. invadens* was 99.47, 99.47, 99.82 and 98.77%, respectively. The sequence similarity between the last 10 specimens and *B. invadens* was 100%. Besides, the taxonID trees based on BOLD showed that all 14 specimens were within the same cluster with *B. invadens*. Therefore, all the unknown fruit fly samples were determined as *B. invadens*.

## Phylogenetic analysis

Because the sequences of specimen 7, 13 and 14 were the same, and so were that of specimen 5, 6, 8, 9, 10, 11 and 12, we chose 2 (HQ689661 of specimen 7 and HQ689659 of specimen 5) of the 10 *COI* sequences from Rugombo as representations for NJ tree construction. The corresponding parameters for NJ analysis were listed in Figure 3.

The NJ tree based on *COI* gene sequences showed that all 14 samples clustered in the genus *Bactrocera* with a high bootstrap value (Figure 3). They were within the same cluster with *B. invadens*, and formed sister clusters to *B. dorsalis*, *B. papayae*, and *B. carambolae*. Sequences from Bujumbura formed a sub-cluster, while samples from Rugombo kept two individual branches in the cluster, which meant that samples from Rugombo showed the closest relationship with known *B. invadens.*

The *COI* gene sequence Kimura 2-parameter distance between 14 samples and *B. invadens* ranged from 0 to 0.015. There was a high genetic distance value between the 14 samples and *B. zonata* (0.101 to 0.116) and *B.*


**Figure 1**. The morphological features of *Bactrocera invadens*, A: Male adult; B: Lanceolate black markings on the scutum; C: Female adult; D: Marking on the scutum.

**Figure 2.** Different markings on the scutum of *B. invadens.*

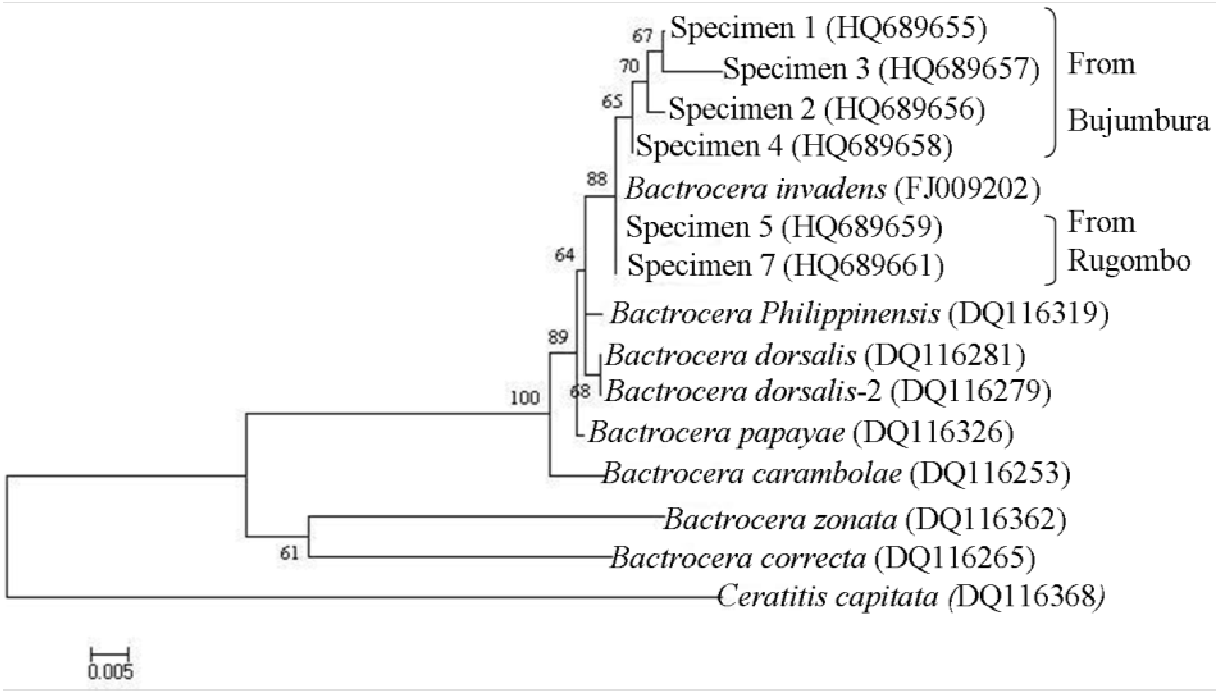


**Figure 3.** Neighbour-joining phylogenetic tree based on *COI* gene sequences, showing the phylogenetic positions of 14 fruit fly specimens, other *Bactrocera* species and represents genus *Ceratitis*. *Ceratitis capitata* was used as outgroup. The number of sites are 487, and scale bar = 0.005 substitutions per nucleotide position. Percent bootstrap values above 50 (1000 replicates) are indicated at notes.

*correcta* (0.099 to 0.111). However, 14 samples showed a relatively lower distance value from *B. carambolae* (0.017 to 0.029), *B. Papayae* (0.006 to 0.019), *B. dorsalis* (0.006 to 0.021), and *B. philippinensis* (0.006 to 0.021) (Table 3).

## DISCUSSION

### Morphological features

For morphological identification, one can usually distinguish between *B. invadens* and *B. dorsalis*. It has been reported that there are three obvious differences between the two species (Drew et al., 2005). Firstly, the base color of the scutum of *B. invadens* is dark orange-brown, and mostly with lanceolate dark brown or black markings. Secondly, the post-sutural vitas of this invasive fruit fly are narrower than that of *B. dorsalis*. Finally, the aedeagus of *B. invadens* is longer than that of *B. dorsalis*. Our observations were consistent with these reported morphological features of *B. invadens*, which are important for rapid identification of this new invasive fruit fly species. Moreover, as shown in Figure 2, we also found additional marking variations.

### DNA alignments using BOLD

Although molecular methods such as restriction fragment length polymorphisms (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), species-specific primers, and inter simple sequence repeat (ISSR), etc. (Bardakci and Skibinski, 1994; Muraji and Nakahara, 2002; Nakahara et al., 2002; Diaz et al., 2003; Martins-Lopes et al., 2007) have been used for species identification for a long time, the operations of DNA techniques are difficult and the identification standard is not uniform. Recently, DNA barcodes approach has become a useful method for diagnosing different species of Hymenoptera, Lepidoptera, Diptera (Tephritidae and Culicidae), and Trichoptera, etc. (Janzen et al., 2005; Hajibabaei et al., 2006; Cywinska et al., 2006; Smith et al., 2005; Crywinska et al., 2010; Foottit et al., 2008, 2009; Armstrong and Ball, 2005). As an informatics workbench of DNA barcodes, BOLD is a species identification tool that accepts DNA sequences from the barcode region, returns a taxonomic assignment to the species level when possible. We could accomplish the fast identification of species based on BOLD.

In this work, all 14 fruit fly samples were identified as *B. invadens* based on the DNA alignments results using BOLD. The 14 fruit flies belonged to 2 different geographical populations, Bujumbura population (specimens 1 to 4) and Rugombo population (specimens 5 to 14). As mitochondrion gene, *COI* gene has variations between different individuals of one species. For the 14 *COI* gene

**Table 3.** Genetic distance based on Kimura 2-parameter.

| Specie | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. capitata* | 0.000 | | | | | | | | | | | | | | |
| *B. zonata* | 0.192 | 0.000 | | | | | | | | | | | | | |
| *B. correcta* | 0.165 | 0.089 | 0.000 | | | | | | | | | | | | |
| *B. carambolae* | 0.167 | 0.106 | 0.099 | 0.000 | | | | | | | | | | | |
| *B. papayae* | 0.170 | 0.101 | 0.094 | 0.010 | 0.000 | | | | | | | | | | |
| *B. dorsalis* | 0.173 | 0.104 | 0.092 | 0.015 | 0.004 | 0.000 | | | | | | | | | |
| *B. dorsalis*-2 | 0.173 | 0.104 | 0.092 | 0.015 | 0.004 | 0.004 | 0.000 | | | | | | | | |
| *B. philippinensis* | 0.173 | 0.104 | 0.097 | 0.015 | 0.004 | 0.004 | 0.004 | 0.000 | | | | | | | |
| *B. invadens* | 0.175 | 0.101 | 0.099 | 0.017 | 0.006 | 0.006 | 0.006 | 0.006 | 0.000 | | | | | | |
| HQ689655 | 0.183 | 0.108 | 0.106 | 0.023 | 0.012 | 0.012 | 0.012 | 0.012 | 0.006 | 0.000 | | | | | |
| HQ689656 | 0.183 | 0.108 | 0.106 | 0.023 | 0.012 | 0.012 | 0.012 | 0.012 | 0.006 | 0.004 | 0.000 | | | | |
| HQ689657 | 0.191 | 0.116 | 0.111 | 0.029 | 0.019 | 0.021 | 0.021 | 0.021 | 0.015 | 0.008 | 0.012 | 0.000 | | | |
| HQ689658 | 0.178 | 0.104 | 0.102 | 0.019 | 0.008 | 0.008 | 0.008 | 0.008 | 0.002 | 0.004 | 0.004 | 0.012 | 0.000 | | |
| HQ689659 | 0.175 | 0.101 | 0.099 | 0.017 | 0.006 | 0.006 | 0.006 | 0.006 | 0.000 | 0.006 | 0.006 | 0.015 | 0.002 | 0.000 | |
| HQ689661 | 0.175 | 0.101 | 0.099 | 0.017 | 0.006 | 0.006 | 0.006 | 0.006 | 0.000 | 0.006 | 0.006 | 0.015 | 0.002 | 0.000 | 0.000 |

sequences of 570 bp, there were 9 base substitution sites, including 7 transversion sites and 2 transition sites. Eight substitution sites existed in Bujumbura population. Four sequences from Bujumbura population yielded 4 haplotypes, while 10 sequences from Rugombo population yielded 2 haplotypes. We speculated that Bujumbura population has a higher nucleotide diversity and genetic variation than Rugombo population. Thus, *COI* gene sequences from Bujumbura population did not match the *B. invadens* sequence from BOLD and the other sequences from Rugombo population were perfectly matched.

## Efficacy of *COI*-based identification

In traditional classification, *B. invadens* has been distinguished from *B. dorsalis* by the scutum patterns variation; however, its morphological characteristics are quite similar to other species of *B. dorsalis*-complex (Drew and Hancock, 1994).

We demonstrated that mitochondrial *COI* gene-based identification method was effective for *B. invadens*, as many related familiar species were successfully clustered into different clades through the *COI* profile. Though genetic distances among all the 14 samples and *B. dorsalis*, *B. papayae* and *B. philippinensis* were very small (0.006 to 0.021), the NJ tree can distinguish all the specimens from them clearly. All the 14 fruit flies were in the same cluster within *B. invadens*, which revealed that they showed the closest relationship with *B. invadens*. *B. dorsalis* and *B. philippinensis* formed sister clusters to them and showed a relative distant phylogenetic relationship.

Through simple DNA extraction, *COI* gene amplification and sequencing process, the *COI* based identification was easier and quicker than that of morphological based identification. This is true for not well-studied species, very small organisms, immature stages, and other species without distinguished morphological characteristics (Armstrong and Ball, 2005; Ball et al., 2005; Greenstone et al., 2005; Barber and Boyce, 2006; Rowley et al., 2007). This method had been used for cryptic species complexes identification, such as

budworm species complex, *Bactrocera tau* complex and a species complex in the genus *Aphelinus* (Hymenoptera: Aphelinidae), before it was named DNA barcodes (Sperling and Hickey, 1994; Chen et al., 2002; Jamnongluk et al., 2003). *B. dorsalis*-complex includes more than 75 species, which have exiguous morphological and molecular differences (Drew and Hancock, 1994). It is unclear whether this method is effective for all the species in the complex. The small genetic distance between some species in the complex makes it difficult to determine an interspecific genetic distance threshold value for species identification within the *B. dorsalis*-complex. Further studies with more specimens are needed for the systemic analysis of species correlation and for the confirmation of the molecular identification methods.

## Significance for Burundi

The Republic of Burundi is a traditional agricultural country. This was the first report of *B. invadens* in Burundi. It is significant for plant protection practice in this country, such as eradication and integrated pest management (IPM). Male annihilation technique (MAT), trapping male adults with methyl eugenol, will be a useful method to heavily diminish their population. Killing pupa with environmental friendly insecticide would also be helpful when the MAT method is non-effective. Besides, biological control method using pupal parasitoid should also be considered. Potential geographical distribution of *B. invadens* in Africa has been predicted using two models (genetic algorithm for rule-set prediction (GARP) and maximum entropy method (Maxent)) (De Meyer et al., 2010). More attention should also be paid to the suitable distribution area and suitable outbreak season, especially in the mango orchard.

According to African invasion registration, there were at least two uncorrelated outbreaks of *B. invadens*, both starting from East Africa (Khamis et al., 2009). Further population genetics studies of *B. invadens* are necessary for Burundi, using more samples from more localities. Such studies would clarify the origin and pathway of this pest (Barr, 2009). Fruits from suitable distribution areas and where there are records of *B. invadens* should be quarantined and controlled strictly, to stop this destructive fruit fly's further invasion in the African continent.

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