Journal of Economic Entomology, XX(XX), 2018, 1–11
doi: 10.1093/jee/toy357
Research

Molecular Entomology

Infestation Levels and Molecular Identification Based on Mitochondrial COI Barcode Region of Five Invasive Gelechiidae Pest Species in Kenya

G. Kinyanjui,[1,2] F. M. Khamis,[1,3] F. L. O. Ombura,[1] E. U. Kenya,[2] S. Ekesi,[1] and S. A. Mohamed[1]

[1]Plant Health Theme, International Centre of Insect Physiology and Ecology (*icipe*), P.O. Box 30772-00100 GPO, Nairobi, Kenya, [2]Department of Biological Sciences, University of Embu, P.O. Box 6-60100, Embu, Kenya, and [3]Corresponding author, e-mail: fkhamis@icipe.org

Subject Editor: Raul Medina

Received 26 June 2018; Editorial decision 26 October 2018

Abstract

Invasive Gelechiidae pest species, namely *Tuta absoluta*, *Phthorimaea operculella*, *Aproaerema simplixella*, *Sitotroga cerealella*, and *Pectinophora gossypiella* are among the major constraints hampering agricultural economy in Kenya. Infestation levels were determined on respective host crops sampled from different localities and *P. operculella* recorded the highest infestation of 68.00 ± 4.92% on stored potato. *Aproaerema simplixella* and *T. absoluta* accounted for 61.33 ± 5.35% and 51.56 ± 5.22% maximal infestation on groundnuts and tomato leaves, respectively. Stored maize was significantly infested by *S. cerealella* (54.33 ± 5.31%) while no infestation was observed on the freshly harvested grains. Infestation on open bolls by *P. gossypiella* was relatively low (6.11 ± 3.46%) compared to *Anatrachyntis simplex* (45.67 ± 7.84%) that emerged as the key pest of cotton. The species were discriminated based on sequence similarities, evolutionary divergences, and phylogenetic analyses. A 658-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) gene was obtained from 302 specimens. Generally, genetic variations were low within and between Gelechiid populations, with an average of 0.02% and all intraspecific divergences were less than 2% except for *S. cerealella*. The Gelechiids data set generated eight Barcode Index Numbers (BINs), five of which were concordant and three belonging to *S. cerealella* were singleton. All species were separated into distinct clusters on a maximum likelihood tree. Data on infestation levels will be useful in defining the pest status of these Gelechiids in Kenya. DNA barcoding is also presented as a valuable tool to complement traditional taxonomy for rapid and accurate identification of these species of agronomic interest.

Key words: invasive Gelechiidae species, infestation levels, species identification, DNA barcoding

Increasing globalization of agricultural industry and trade has resulted in significantly increased accidental introductions of exotic pest species into Kenya. This has raised considerable concern in the agricultural sector because of severe crop damages, huge economic losses, and associated environmental damage attributable to these pests. Among the invasive species present in Kenya, there are several gelechiids. More than 250 species belonging to the family Gelechiidae have been reported as economically important pests that attack numerous agricultural and horticultural crops (Karsholt et al. 2013). Gelechiid larvae are oligophagous, often restricted to few host plant species. They exhibit diverse feeding techniques such as leaf mining, boring, and feeding internally on various plant parts including stems, seeds, grains, tubers, and fruits as well as inducing galls. This study laid emphasis on five invasive Gelechiidae species that are of great importance and have become a major impediment to agriculture in Kenya. These include *Phthorimaea operculella* (Zeller), *Aproaerema simplixella* (Walker), *Sitotroga cerealella* (Olivier), *Pectinophora gossypiella* (Saunders), and *Tuta absoluta* (Meyrick).

Phthorimaea operculella originates from South America and is among the most destructive pests of field and stored potato (*Solanum tuberosum* L.) (PSA 2015). This potato tuber moth is oligophagous and also attacks other solanaceous crops and weeds such as tomato (*Lycopersicon esculentum* (Mill.)), eggplant (*Solanum melongena* L.), sweet pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.), black nightshade (*Solanum nigrum* L.), and jimson weed (*Datura stramonium* L.) (Chandel et al. 2005). Tunnels made by the larvae on potato tubers reduce their market value and allow entry of secondary pathogens such as fungi and bacteria, making them unfit as seeds or for human consumption. The pest has the potential to cause up to 100% yield losses on untreated stored tubers and field infestation of about 62% has been observed in the neighboring Eastern Ethiopia (Sileshi and Teriessa 2001). However, despite its

reported presence in Kenya (CABI 2018), data on the levels of infestation and economic impact are scanty. *Aproaerema simplixella* is an invasive pest of Asian origin (Buthelezi et al. 2012). This groundnut leafminer attacks leguminous crops such as groundnuts (*Arachis hypogaea* L.), soyabeans (*Glycine max* L.), and lucerne (*Medicago sativa* L.) (Kenis and Cugala 2006). Tunneling and feeding of larvae inside leaves causes large scale defoliation and significant yield losses. In Africa, *Aproaerema modicella* is the widely reported groundnut leafminer but was later redefined as *A. simplixella* based on mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene analysis (Buthelezi et al. 2012). It was first reported in Uganda and believed to subsequently invade Malawi, Mozambique, Democratic Republic of Congo, and South Africa (Du Plessis 2003, Buthelezi et al. 2012). The pest is associated with over 50% yield losses of pods and its reported high abundance beyond the set economic threshold on South African groundnuts indicates its potential economic impact (Logiswaran and Mohanasundaram 1985, Shanower et al. 1993, van der Walt et al. 2009).

Sitotroga cerealella is a primary pest of stored grains such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), millet (*Pennisetum glaucum* L.), and common wheat (*Triticum aestivum* L.) (Trematerra 2015). This Angoumois grain moth is believed to have originated from the Angoumois province of France and has dispersed worldwide to subtropical and warm temperate regions (Akter 2013). The larvae bore and feed within kernels causing a significant reduction in nutritive value, weight, and quality of grains. In addition, heavily infested grains lose their market value and are unpalatable to humans. *Sitotroga cerealella* is found alongside other storage pests such as weevils, rodents, and moths, which collectively could cause up to 50% of grain losses under hot and humid weather conditions coupled with poor storage facilities. This pest has been reported in Kenya (CABI 2018), but little information is available with regards to its levels of infestation and damage. *Pectinophora gossypiella* is native to Asia and considered among the most destructive pests of cotton (*Gossypium* spp.) worldwide (Busck 1917). This pink bollworm has the potential to cause up to 61% yield losses when control measures are not implemented and economic losses of about US$1.14 billion have been realized on Indian cotton (Schwartz 1983, Singh 2016). The larvae tunnel into green bolls, feed on developing seeds, and cause improper boll opening. However, the most serious damage is linked to the presence of stained lint in open bolls that is of poor quality and unmarketable. The pest is oligophagous and attacks other plants such as lucerne (*M. sativa*), okra (*Abelmoschus esculentus* L.), and hibiscus (*Hibiscus* spp.) (Ballou 2014, CABI 2018). In addition, *P. gossypiella* has been previously reported in Kenya on *Gossypioides kirkii* Mast (Miller et al. 2014).

The recent invasion of *T. absoluta* builds up the list of invasive Gelechiidae pest species and further compounds their negative economic impact in Kenya (Tonnang et al. 2015). This tomato leafminer is native to South America and causes significant damage to tomato by inducing rotting in fruits, rendering them inedible and reducing their marketability. It is oligophagous and has a great potential to cause damage to other crops in Solanaceae, Fabaceae, and Cucurbitaceae families (Desneux et al. 2010, Mohamed et al. 2015). A recent study on Kenyan tomato observed a serious leaf infestation of about 92% and fruit damage of 60% (G. Kinyanjui et al., unpublished data). Accurate knowledge of the actual levels of infestation by these invasive taxa on their respective primary host crops will provide insight into their pest status, necessity of control, and potential economic risks to agriculture in Kenya. In addition, a rapid and accurate identification tool for these invasive pests will be useful for effective and sustainable integrated pest management (IPM) initiatives and could facilitate prompt implementation of phytosanitary measures in the country.

Traditionally, identification of Gelechiidae species is solely based on examination of external morphological features of adults such as segmentation of antennae, structure of labial palpi, and wing venation as well as musculature morphology of male genitalia (Brambila et al. 2010, Karsholt et al. 2013). While genital features are extremely important in identification of these species, dissection of genital structures is not commonly exercised due to limited taxonomic expertise. Besides, adults of species such as *T. absoluta*, *P. operculella*, *A. simplixella*, and *Keiferia lycopersicella* Walsingham (tomato pinworm) are difficult to distinguish using naked eyes while on the fields and especially when key diagnostic characters such as antennae and wing scales are lost or damaged (Brambila et al. 2010, Roditakis et al. 2010). Trapping systems based on synthetic sex pheromones could also attract more than one species and especially in mixed species cropping thus rendering accurate identification of closely related species problematic (Roditakis et al. 2010). In addition, immature life stages and mutilated specimens encountered at quarantine checkpoints are unsuitable for morphological identification due to insufficient taxonomic keys. For accurate diagnosis therefore, intercepted larvae have to be reared to adults, which is time-consuming and sometimes unreliable when they die. These limitations hinder the implementation of prompt and appropriate pest control measures and also delay quarantine decisions with consequent serious losses in the national economy. In such cases, molecular diagnostic tools not limited by developmental stages, sex, and type of specimens offer an added advantage.

DNA barcoding has become instrumental in species identification of several insect pests (Hajibabaei et al. 2006; Barr et al. 2012; Khamis et al. 2012, 2017; Karthika et al. 2016; Kinyanjui et al. 2016). It presents a valuable approach for rapid and accurate identification of species based on the principle that a short standardized sequence of mtCOI gene could be used as an internal molecular identification tag to identify and delineate species (Hebert et al. 2003a, Sperling and Roe 2009). DNA barcoding assigns unknown specimens by comparing target to reference sequences of known species and thus, its success depends on the availability of a comprehensive database of DNA sequences for comparison of species and analysis (Hebert et al. 2003a). This molecular tool could be applied as a complementary alternative for morphological taxonomy and help overcome its inherent limitations. The objectives of the study were to 1) determine the levels of infestation of five invasive Gelechiidae pest species on their respective host crops in Kenya, and 2) identify these Gelechiidae pests using DNA barcoding.

Materials and Methods

Field Surveys, Sampling Sites, and Methods

Field surveys for the five Gelechiids were conducted in 12 localities across Kenya, between April 2015 and October 2016 (Fig. 1). These localities were selected based on host plants' availability and each species was sampled in five localities. In each locality, three sampling sites for each species were selected for the study. Generally, agricultural crops such as maize, potato, and tomato are widely cultivated across Kenya (MoALF 2015). However, crops such as groundnuts and cotton are grown in few selected localities in the eastern (Embu, Mwea, and Meru) and western (Homa Bay, Siaya, and Migori) regions of Kenya. The sample units were considered as individual leaves, fruits, tubers, bolls, or grains based on the particular

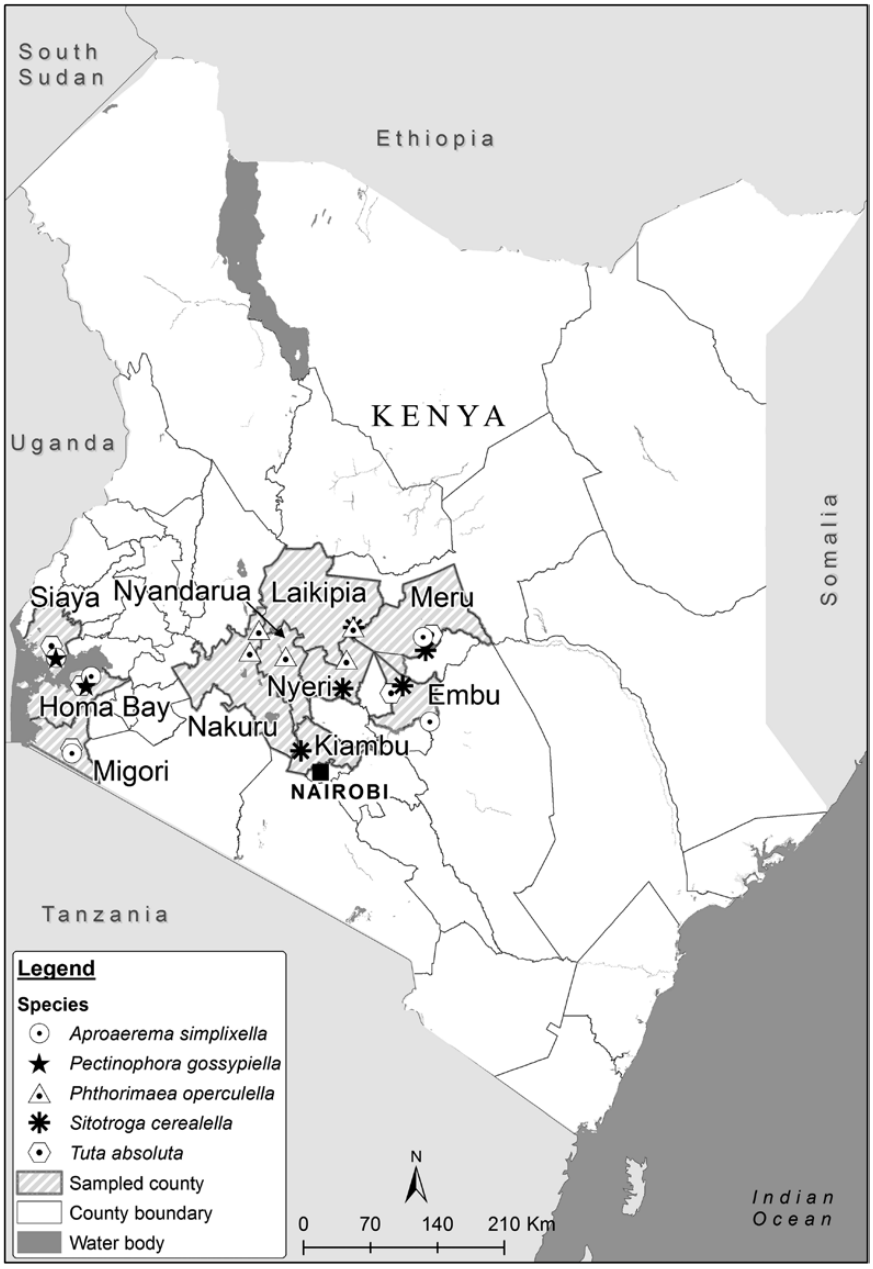

Fig. 1. Map of Kenya showing the sampling localities of five invasive Gelechiidae pest species.

preference for each species. Factors such as crop variety planted and pest management practices used by farmers in the studied sites were not considered. The global positioning system (GPS) coordinates were recorded for all the surveyed sites. *Phthorimaea operculella* was sampled from stored and freshly harvested potatoes (*S. tuberosum*). From each site, 60 tubers were selected at random from a 20 liter full container (debe), commonly used by traders in the local market. They were placed in plastic containers (20 × 13 × 15 cm) with lids containing fine muslin cloth (16 × 9 cm) and transported to the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, where the number of infested tubers as exhibited by the presence of tunnels was recorded. The species was identified based on external morphological characters and damage symptoms described in literature (Chandel et al. 2005, Brambila et al. 2010, PSA 2015). *Aproaerema simplixella* was sampled from groundnuts (*A. hypogaea*) whereby from each site, two leaves were picked at random from 30 plants in a transversal zigzag sampling pattern. They were placed in plastic containers (20 × 13 × 8 cm) lined with moist paper towels to prevent drying of leaves, covered with lids containing fine muslin cloth (16 × 9 cm) and transported to the laboratory. The number of infested leaves was recorded. The species was identified based on descriptions and damage symptoms as reported by Buthelezi et al. (2012).

Sitotroga cerealella was sampled from freshly harvested and stored maize (*Z. mays*). Sixty grains were randomly selected from 1 kg of maize, placed in plastic paper bags, and taken to the laboratory for observation. They were checked visually using a hand lens and all grains with mines were recorded as infested (Saikia et al. 2014). Care was taken on the stored maize to differentiate grains that were damaged by weevils. However, it was not possible to differentiate the damage symptoms of *S. cerealella* from other moths and to confirm the species; therefore, infested grains were

individually kept in closed Petri dishes (9.2 cm diameter × 1.7 cm height). Emerging adults of the target species were identified based on morphological characteristics described by Shamsudeen (2013). *Pectinophora gossypiella* was sampled from cotton (*Gossypium hirsutum*). From each site, two open cotton bolls were randomly picked from 30 plants in a transversal zigzag sampling pattern. They were kept in plastic containers (20 × 13 × 15 cm) with lids containing fine muslin cloth (16 × 9 cm) and transported to the laboratory. The number of infested bolls was recorded and the species was identified based on descriptions and damage symptoms reported in literature (Vennila et al. 2007, Singh 2015). *Tuta absoluta* was sampled from tomato (*L. esculentum*) plant at flowering/fruiting stage. Two leaves from the middle stratum and two fruits were randomly picked from 30 plants in a transversal zigzag sampling pattern. The leaves were kept in plastic containers (20 × 13 × 8 cm) containing damp paper towels and were covered with lids containing a fine muslin cloth (16 × 9 cm). They were transported to the laboratory, checked under a stereomicroscope (Leica Microsystems Ltd, Milton Keynes, UK), and the total number infested was recorded. Fruits were kept in plastic containers (20 × 13 × 15 cm) with lids containing fine muslin windows (16 × 9 cm), transported to the laboratory and the number of infested recorded.

Sample Collection and Processing

Some larvae present on the infested plant materials (tubers, leaves, fruits, bolls, and grains) were collected per sampling site using a soft camel hair brush and preserved in 95% ethanol. These infested plant materials were then maintained in the laboratory at ambient temperatures of 25–28°C and 60 ± 10% RH until emergence of adults. They were stored in plastic rearing containers (20 × 13 × 8 cm) with lids containing muslin windows (16 × 9 cm) and the larvae were provided with fresh plant materials as required. The containers were checked daily and emerging adults were aspirated and preserved in 95% ethanol. The molecular analysis was done at the *icipe*, Nairobi, Kenya, and included both adults and larvae specimens. Five samples were selected randomly from each site. Dorsal, ventral, and lateral images were taken at 10× magnification using LAS EZ4D stereomicroscope (Leica Microsystems Limited, Germany). However, samples of *P. gossypiella* and *P. operculella* were relatively bigger and thus the images were taken at 8× magnification. All samples were surface sterilized in 3% bleach, rinsed thrice with distilled water, and put individually into sterile 1.5 ml Eppendorf tubes. Four samples of *Anatrachyntis simplex* (Walsingham, 1891) and additional populations of *T. absoluta* that had been previously collected from Kenya, Tanzania, Uganda, and Peru were also included in the molecular analysis (Table 2). Voucher specimens for each collection site have been deposited at the Molecular Pathology Laboratory, Arthropod Pathology Unit in *icipe*.

DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from individual insect using Isolate II Genomic DNA Kit (Bioline, London, UK) as per the manufacturer's instructions. Extracted DNA was quantified using Nanodrop 2000/2000c spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and stored at −20°C. An approximately 700-bp fragment of mitochondrial COI gene was amplified using LepF1 (5′-ATT CAA CCA ATC ATA AAG ATA TTG G-3′) and LepR1 (5′-TAA ACT TCT GGA TGT CCA AAA AAT CA-3′) (Hajibabaei et al. 2006). All PCR reactions were carried out in a final 20 µl volume containing 5× MyTaq reaction buffer (Bioline) (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers, and enhancers), 0.5 pmol µl^{-1} of each primer, 0.25 mM $MgCl_2$, 0.0625 U µl^{-1} MyTaq DNA polymerase (Bioline), and 15 ng µl^{-1} of DNA template. PCR reactions were set up in a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) and the cycling conditions were 2 min at 95°C, then 35 cycles of 30 s at 95°C, 40 s at 53°C and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. Successfully amplified COI regions were purified using Isolate II PCR and Gel Kit (Bioline) as per the manufacturer's instructions and sent to a commercial sequencing facility (Macrogen Inc Europe Laboratory, Amsterdam, the Netherlands) for bidirectional sequencing using ABI 3700 sequencer.

Data Analysis

The percentage of infestation was calculated as the number of infested plant materials (leaves, fruits, tubers, bolls, and grains) divided by the total number of sampled plant materials sampled per site and multiplied by 100. Data were subjected to a generalized linear model assuming a quasi-binomial distribution error and logit link. Infestation levels were compared between species, host crops, and between localities. For *T. absoluta*, *P. operculella*, and *S. cerealella*, data on individual species were subjected to a one-way ANOVA in order to compare percentage infestation between different sampling units (i.e., stored and freshly harvested tubers and grains as well as tomato leaves and fruits). The data were first arcsine-transformed to comply with normality assumptions and homogeneity of variances before analyses. All statistical analyses were carried out in R v3.2.3 software (R Development Core Team 2017). DNA sequences were edited using Chromas v2.1.1 (Technelysium Pty Ltd, South Brisbane, Australia). They were trimmed to a final length of 658 bp and all gaps were removed using Jalview v2.8.2 (Waterhouse et al. 2009). The sequences were compared to reference sequences on GenBank database using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990, Clark et al. 2016) and the Barcode of Life Data systems database (BOLD) using the species identification engine tool http://www.boldsystems.org/index.php/IDS_OpenIdEngine (Ratnasingham and Hebert 2007). The Barcode Index Number (BIN) system in BOLD was used to confirm concordance between submitted barcode sequences and species designation. Sequence divergences and nucleotide composition were analyzed using the 'distance summary' and 'sequence composition' tools in BOLD, respectively. Evolutionary genetic divergences over sequence pairs between species were calculated using the p-distance model in MEGA 6.0 (Tamura et al. 2013). Intra- and interspecific sequence divergences were analyzed using the barcode gap analysis tool in BOLD. Multiple sequence alignments were generated using MUSCLE v3.8.31 (Edgar 2004). A maximum likelihood (ML) analysis was performed based on GTR+G model in MEGA 6.0. Selection of the best-fit model for our data set was based on the lowest Bayesian Information Criterion (BIC) value using jModeltest v2.1.7 (Darriba et al. 2012). Phylogenetic clustering was assessed by bootstrapping with 1,000 replicates.

Results

Infestation Levels by Gelechiids

Damage symptoms were confirmed by examining the presence of larvae, mines, or frass indicative of infestation on respective crops. The studied Gelechiid pests except *P. gossypiella* were present in all the sampled localities. However, infestation levels varied significantly between species ($F = 7.72$, df = 4, $P < 0.0001$) and between host crops ($F = 42.72$, df = 7, $P < 0.0001$). *Phthorimaea operculella* recorded the highest infestation on stored potato tubers (68.00 ± 4.92) followed by *A. simplixella* on groundnuts

(61.33 ± 5.35), *S. cerealella* (54.33 ± 5.31) on stored maize, and *T. absoluta* on tomato leaves (50.56 ± 5.22) (Table 1). The lowest levels of infestation were recorded on freshly harvested potatoes by *P. operculella* (3.89 ± 1.70), whereas no infestations by *S. cerealella* were observed on freshly harvested maize grains (Table 1). The percentage infestation by *S. cerealella* on stored maize (54.33 ± 5.31) was significantly higher when compared with freshly harvested maize (0.00 ± 0.00) ($F = 188.80$, df = 1, $P < 0.0001$). Similarly, infestation levels recorded on stored potato (68.00 ± 4.92) by *P. operculella* were found to be significantly higher compared to freshly harvested tubers (3.89 ± 1.70) ($F = 153.30$, df = 1, $P < 0.0001$). On tomato, significantly higher levels of infestation were recorded on leaves (50.56 ± 5.22) than fruits (15.56 ± 3.32) ($F = 32.22$, df = 1, $P < 0.0001$). There were no significant differences in the infestation levels by the five Gelechiids when compared across different localities ($F = 1.19$, df = 11, $P = 0.31$). *Pectinophora gossypiella* recorded an average infestation of 6.11% and was only present in Homa Bay and Siaya. However, an additional species *A. simplex* became a pest of interest in our study because it was present in all the sampled localities and recorded relatively high levels of infestation on cotton (45.67 ± 7.84).

Molecular Identification of Gelechiids

A partial sequence of 658 bp of mitochondrial COI gene was obtained from 298 specimens representing five species. The percentage identities obtained from GenBank and BOLD were high for all the species ranging between 98 and 100% with an exception of *S. cerealella*. Most sequences of *S. cerealella* displayed a percentage sequence similarity ranging from 89 to 93% and 98 to 100% with GenBank and BOLD sequences, respectively. DNA sequences displaying BLAST hits between 89 and 91% with GenBank sequences were not matched in the BOLD database. All sequences were uploaded to the BOLD with associated trace files and images and submitted to NCBI GenBank. Accession numbers for *T. absoluta* (KU565496–KU565720) and other Gelechiidae pest species (MF121833–MF121905) are provided in Table 2. The five Gelechiid species were assigned to eight BINs, five of which were taxonomically concordant. All individuals within a species were assigned to one BIN. However, out of the 23 samples of *S. cerealella*, 20 were assigned to one concordant BIN and three were assigned to three singleton BINs. The sequences displayed low or no intra- and interpopulation divergences and in most cases, sequences within a species were or nearly identical except *S. cerealella*. For instance, 225 specimens belonging to 45 populations of *T. absoluta* from Kenya, Uganda, Tanzania, and Peru displayed a genetic divergence of 0.0%. A total of 18 specimens representing five Kenyan populations of *P. operculella* also showed a genetic variation of 0.0%. *Sitotroga cerealella* was represented by 23 specimens belonging to five Kenyan populations and displayed genetic divergence of 0.016%. The four specimens of *P. gossypiella* recorded an intrapopulation variation of 0.0%, while 28 specimens representing six Kenyan populations of *A. simplixella* showed an intra- and interpopulation divergence of 0.0%.

Estimates of evolutionary divergence over sequence pairs between species are as shown in Table 3. The lowest divergence was observed between *T. absoluta* and *P. operculella* (0.110) while *P. gossypiella* and *P. operculella* were the most genetically divergent (0.161). The BOLD results showed *T. absoluta* as the nearest neighbor of *P. operculella* and vice versa, whereas *A. simplixella* and *P. gossypiella* were the distantly related (Table 3). Sequence divergences were <2% except *S. cerealella* and all species displayed values of >2% to the nearest neighbor. The intraspecific sequence divergences ranged from 0.0 to 6.67%, with an average of 0.02%, while the interspecific divergences ranged from 10.83 to 20.5%, with an average of 14.38% (Table 4). ML analysis separated 302 COI sequences of Gelechiids and *A. simplex* into distinctive clusters (Fig. 2). High genetic homogeneity was observed between populations of *T. absoluta*, *P. operculella*, and *A. simplixella*. Individuals within *S. cerealella* displayed intraspecific divergences but the cluster was strongly supported.

Discussion

Data on infestation levels of four Gelechiids, namely *S. cerealella*, *T. absoluta*, *P. operculella*, and *A. simplixella* indicate that they cause significant damage to key agricultural crops including maize, potato, tomato, and groundnuts, thereby constituting the greatest threat to agriculture and food security in Kenya. These pests were recorded in all the sampled localities indicating their widespread distribution across the country. Infestations of 68% on stored potato tubers by *P. operculella* are particularly distressful considering that potato is the most important vegetable for many livelihoods in Kenya (AFA 2014). Based on our findings therefore, we can conclude that the pest highly contributes to the huge postharvest losses experienced by potato farmers and hence a major constraint to successful potato production in Kenya. Our results also showed very low or no infestation of freshly harvested tubers when compared with the stored produce. The differential levels of infestation could be attributed to lack of effective postharvest pest control measures in storage sheds and farm stores in contrast to frequent application of pesticides on field potato. However, Hanafi (1999) observed that although freshly harvested tubers may lack the damage symptoms, they could have

Table 1. The percentage infestation (mean ± SE) on different host crops sampled across Kenya by five invasive Gelechiidae pest species and *Anatrachyntis simplex*

Species name	Host crop	Percentage infestation (mean ± SE)
Phthorimaea operculella	Stored potato tubers	68.00 ± 4.92
	Freshly harvested tubers	3.89 ± 1.70
Sitotroga cerealella	Stored maize grains	54.33 ± 5.31
	Freshly harvested grains	0.00 ± 0.00
Tuta absoluta	Tomato leaves	50.56 ± 5.22
	Tomato fruits	15.56 ± 3.32
Aproaerema simplixella	Groundnut leaves	61.33 ± 5.35
Pectinophora gossypiella	Open cotton bolls	6.11 ± 3.46
Anatrachyntis simplex	Open cotton bolls	45.67 ± 7.84

The number of sampling units (n) was 60 for all host crops.

Table 2. Collection data with species, sample codes, locality, latitude/longitude, and GenBank accession numbers of five Gelechiidae pest species sampled from different localities in Kenya between April 2015 and October 2016.

Species name	Sample codes	Locality	Latitude/Longitude	GenBank accession numbers
Phthorimaea operculella	PC1-PC5	Subukia	00°03′02.9″N/036°13′42.6″E	MF121867, MF121866, MF121865, MF121881, MF121880
Phthorimaea operculella	PB1	Bahati	00°09′05.8″S/036°08′41.6″E	MF121868
Phthorimaea operculella	PD1-PD5	OlKalou	00°11′47.9″S/036°28′48.5″E	MF121879, MF121878, MF121877, MF121876, MF121875
Phthorimaea operculella	PE1, PE2	Nanyuki	00°04′39.9″N/037°07′00.0″E	MF121874, MF121882
Phthorimaea operculella	PA1-PA5	Nyeri	00°13′26.6″S/037°03′08.9″E	MF121873, MF121872, MF121871, MF121870, MF121869
Sitotroga cerealella	AR1-AR5	Nyeri	00°29′55.0″S/037°01′08.3″E	MF121892, MF121893, MF121894, MF121895, MF121896
Sitotroga cerealella	AE1-AE5	Embu	00°28′16.0″S/037°34′53.0″E	MF121897, MF121898, MF121899, MF121900, MF121901
Sitotroga cerealella	AM1-AM5	Meru	00°08′23.0″S/037°47′50.8″E	MF121883, MF121884, MF121885, MF121886, MF121887
Sitotroga cerealella	AN1-AN4	Nanyuki	00°04′33.6″N/037°07′17.4″E	MF121888, MF121889, MF121890, MF121891
Sitotroga cerealella	AL1-AL4	Limuru	01°05′01.3″S/036°37′29.1″E	MF121902, MF121903, MF121904, MF121905
Pectinophora gossypiella	CD1, CE1, CE2	Siaya	00°12′36.3″S/034°19′23.4″E	MF121864, MF121863, MF121862
Pectinophora gossypiella	CF2	Homa Bay	00°27′59.5″S/034°36′32.6″E	MF121861
Aproaerema simplixella	E1-E3	Embu	00°48′37.6″S/037°50′10.8″E	MF121847, MF121848, MF121849
Aproaerema simplixella	M1-M5	Meru	00°00′57.2″S/037°46′24.1″E	MF121860, MF121838, MF121839, MF121840, MF121833
Aproaerema simplixella	T1-T5	Tharaka Nithi	00°10′31.9″S/37°49′58.5″E	MF121846, MF121845, MF121844, MF121843, MF121842
Aproaerema simplixella	G1-G5	Migori	01°06′21.2″S/034°28′18.2″E	MF121850, MF121851, MF121852, MF121853, MF121854
Aproaerema simplixella	H1-H5	Homa Bay	00°23′00.3″S/034°39′08.5″E	MF121855, MF121856, MF121857, MF121858, MF121859
Aproaerema simplixella	S1-S5	Siaya	00°12′36.3″S/034°19′23.4″E	MF121841, MF121834, MF121835, MF121836, MF121837
Tuta absoluta	Me1-5	Meru	00°00′21.6″N/037°51′03.9″E	KU565710, KU565709, KU565708, KU565707, KU565706
Tuta absoluta	Eb1-5	Embu	00°32′33.9″S/037°28′20.8″E	KU565640, KU565639, KU565638, KU565637, KU565636
Tuta absoluta	Mg1-5	Migori	01°03′47.0″S/034°28′09.3″E	KU565570, KU565569, KU565568, KU565567, KU565566
Tuta absoluta	Hb1-5	Homa Bay	00°28′23.1″S/034°33′45.5″E	KU565625, KU565624, KU565623, KU565622, KU565621
Tuta absoluta	Mw1-5	Mwea	00°36′24.8″S/037°22′30.0″E	KU565675, KU565674, KU565673, KU565672, KU565671
Tuta absoluta	Gi1-5	Gichugu	00°27′55.1″S/037°18′36.8″E	KU565685, KU565684, KU565683, KU565682, KU565681
Tuta absoluta	Ki1-5	Kisii	00°40′41.8″S/035°03′14.4″E	KU565720, KU565719, KU565718, KU565717, KU565716
Tuta absoluta	Tav1-5	Taveta	03°26′55.4″S/037°39′22.9″E	KU565665, KU565664, KU565663, KU565662, KU565661
Tuta absoluta	Ltk1-5	Loitoktok	02°50′57.9″S/037°32′15.9″E	KU565680, KU565679, KU565678, KU565677, KU565676
Tuta absoluta	Is1-5	Isiolo	00°19′33.0″N/037°33′14.1″E	KU565715, KU565714, KU565713, KU565712, KU565711
Tuta absoluta	Na1-5	Nakuru	00°03′02.9″N/036°13′42.6″E	KU565705, KU565704, KU565703, KU565702, KU565701
Tuta absoluta	Nya1-5	Nyahururu	00°03′32.6″N/036°21′41.4″E	KU565700, KU565699, KU565698, KU565697, KU565696
Tuta absoluta	Nye1-5	Nyeri	00°27′44.0″S/036°55′48.5″E	KU565695, KU565694, KU565693, KU565692, KU565691
Tuta absoluta	Kw1-5	Kwale	04°23′26.0″S/039°29′43.6″E	KU565590, KU565589, KU565588, KU565587, KU565586
Tuta absoluta	Mld1-5	Malindi	03°05′04.8″S/040°06′14.5″E	KU565565, KU565564, KU565563, KU565562, KU565561
Tuta absoluta	Kf1-5	Kilifi	03°36′35.2″S/039°49′57.2″E	KU565610, KU565609, KU565608, KU565607, KU565606
Tuta absoluta	Mrg1-5	Murang'a	00°45′20.9″S/037°08′45.4″E	KU565560, KU565559, KU565558, KU565557, KU565556
Tuta absoluta	Ka1-5	Kabete	01°14′24.5″S/036°43′53.6″E	KU565620, KU565619, KU565618, KU565617, KU565616
Tuta absoluta	Lim1-5	Limuru	01°09′33.1″S/036°38′35.2″E	KU565585, KU565584, KU565583, KU565582, KU565581
Tuta absoluta	Kmb1-5	Kiambu	01°05′18.0″S/036°49′09.2″E	KU565600, KU565599, KU565598, KU565597, KU565596
Tuta absoluta	Mch1-5	Machakos	01°09′47.4″S/037°31′52.6″E	KU565575, KU565574, KU565573, KU565572, KU565571
Tuta absoluta	Kti1-5	Kitui	00°50′56.5″S/038°00′21.4″E	KU565595, KU565594, KU565593, KU565592, KU565591
Tuta absoluta	Th1-5	Thika	01°03′31.0″S/037°07′27.0″E	KU565525, KU565524, KU565523, KU565522, KU565521
Tuta absoluta	Ser1-5	Sergoit	00°41′18.2″N/035°25′49.7″E	KU565530, KU565529, KU565528, KU565527, KU565526
Tuta absoluta	Eld1-5	Uasin Gishu	00°52′21.6″N/035°21′53.1″E	KU565635, KU565634, KU565633, KU565632, KU565631
Tuta absoluta	Kkg1-5	Kakamega	00°46′29.1″N/035°04′37.5″E	KU565605, KU565604, KU565603, KU565602, KU565601
Tuta absoluta	Bug1-5	Bungoma	00°48′47.1″N/034°29′15.3″E	KU565645, KU565644, KU565643, KU565642, KU565641

Table 2. *Continued*

Species name	Sample codes	Locality	Latitude/Longitude	GenBank accession numbers
Tuta absoluta	Bs1-5	Busia	00°26′53.2″N/034°06′38.5″E	KU565650, KU565649, KU565648, KU565647, KU565646
Tuta absoluta	Nrb1-5	Nairobi	01°13′26.6″S/036°53′49.1″E	KU565555, KU565554, KU565553, KU565552, KU565551
Tuta absoluta	Nyk1-5	Nanyuki	00°04′39.9″N/037°07′00.0″E	KU565670, KU565669, KU565668, KU565667, KU565666
Tuta absoluta	Kbz1-5	Makueni	02°23′18.1″S/038°00′03.0″E	KU565615, KU565614, KU565613, KU565612, KU565611
Tuta absoluta	Nrk1-5	Narok	00°54′57.0″S/034°56′55.6″E	KU565550, KU565549, KU565548, KU565547, KU565546
Tuta absoluta	Nv1-5	Naivasha	00°37′35.7″S/036°22′49.5″E	KU565545, KU565544, KU565543, KU565542, KU565541
Tuta absoluta	Ok1-5	Nyandarua	00°11′38.7″S/036°28′29.5″E	KU565540, KU565539, KU565538, KU565537, KU565536
Tuta absoluta	Lmu1-5	Lamu	02°23′09.8″S/040°26′58.2″E	KU565580, KU565579, KU565578, KU565577, KU565576
Tuta absoluta	Nz1-5	Kaplamai	00°58′47.9″N/035°07′14.6″E	KU565630, KU565629, KU565628, KU565627, KU565626
Tuta absoluta	TzMo1-5	Moshi (Tz)	03°23′17.3″S/037°31′58.9″E	KU565690, KU565689, KU565688, KU565687, KU565686
Tuta absoluta	TzLu1-5	Lushoto (Tz)	04°50′52.6″S/038°20′07.9″E	KU565520, KU565519, KU565518, KU565517, KU565516
Tuta absoluta	TzAr1-5	Arusha (Tz)	03°18′20.0″S/036°41′40.6″E	KU565660, KU565659, KU565658, KU565657, KU565656
Tuta absoluta	TzMw31-35	Nyaholongo (Tz)	02°43′54.4″S/033°1′21.0″E	KU565505, KU565504, KU565503, KU565502, KU565501
Tuta absoluta	TzMw21-25	Mwagala (Tz)	02°41′59.6″S/033°4′28.4″E	KU565510, KU565509, KU565508, KU565507, KU565506
Tuta absoluta	TzMw41-45	Nyamalolo (Tz)	02°41′14.0″S/033°3′28.3″E	KU565500, KU565499, KU565498, KU565497, KU565496
Tuta absoluta	TzMw11-15	Nyamle (Tz)	02°42′52.3″S/033°2′57.2″E	KU565515, KU565514, KU565513, KU565512, KU565511
Tuta absoluta	Ug1-5	Mukono (Ug)	00°28′34.4″N/032°48′19.2″E	KU565655, KU565654, KU565653, KU565652, KU565651
Tuta absoluta	Sa1-5	Lima (Peru)	12°4′39.0″S/076°56′53.0″W	KU565535, KU565534, KU565533, KU565532, KU565531

Additional samples of *Tuta absoluta* were collected from Tanzania (Tz), Uganda (Ug), and Peru.

a considerable quantity of *P. operculella* eggs and first instar larvae carried along to the storage facilities. Therefore, we cannot conclude fully that freshly harvested tubers are less targeted by the pest and it is highly likely that damage symptoms would show several weeks after storage. Furthermore, our findings suggest that these storage facilities act as a harborage of *P. operculella* and special attention with regard to pest control is required since most growers store a high percentage of harvested potatoes for future food use, seed, or market sale. Despite its recent invasion, observed infestation by *T. absoluta* indicates that it is becoming a pest of great concern to tomato production in Kenya. However, our data estimates are low when compared to recent reports of 100% maximal damage (Mohamed et al. 2012, Chidege et al. 2016). Nevertheless, Abbes and Chermiti (2011) observed that the levels of *T. absoluta* infestation on Tunisian greenhouse tomato depended on plant phenology often increasing in subsequent growth stages. Thus, given that our sampling was conducted on the flowering/fruiting stage, it is highly probable that the pest could achieve even higher infestations in the late growth stages. The differences observed between infested leaves and fruits could also be attributed to the particular growth stage at which sampling was done, which is usually dominated by eggs and first instar larvae of *T. absoluta*, with accompanying high infestation levels on leaves and low or no infestation on fruits (Chermiti et al. 2009).

To our knowledge, this is the first report of *A. simplixella* in Kenya. The species identity and damage symptoms correspond to descriptions reported for *A. simplixella* populations in South Africa and the rest of Africa, although previously reported as *A. modicella* (Buthelezi et al. 2012). Given that groundnut leafminer was first detected in Uganda in 1998 and later spread to other East and South African countries (Du Plessis 2003), our data could be an indicator that the species also invaded Kenya at the time but was surprisingly unnoticed or not reported. Despite the fact that the economic impact of its invasion has not been studied, observed infestation rates reaching up to 61% on groundnut leaves are likely to cause significant yield and financial losses to growers. Heavy infestation of stored maize by *S. cerealella* highlights among the most severe postharvest losses encountered by maize farmers. Our results therefore demand urgent management strategies of the pest given the importance of maize as a staple food crop as well as manifold contribution to the Kenyan economy. *Sitotroga cerealella* is also known to attack field grains during harvest but the damage symptoms are usually not visible (Akter 2013). The percentage infestation of the pest reported herein may therefore represent the minimum estimates because they are only based on the visible damage symptoms at advanced stages. These include fully developed mines inside grains, frass, and open holes that symbolized outlets of emerged adults. At early stages of infestation, damage symptoms are usually not detectable even by the use of hand lens and this could probably explain the absence of infestation on freshly harvested maize grains. However, it is expected that signs of infestation could be observed after few weeks of storage and these data therefore are important to farmers and stakeholders to implement and maintain appropriate pest control practices for maize and other target cereals even after storage. Most farmers usually store their grains inside sacks that are not hermetically closed and thus act as breeding sites for most pests. There is continued females oviposition and reproduction inside these sacks that could lead to up to 100% infestation depending on the duration of grains storage. With regard to *P. gossypiella*, our results suggest that it is probably not a key pest of cotton in Kenya when compared with India (Singh 2015), because it was only recorded in Siaya and Homa Bay with low infestation levels of 6.11%. However, *A. simplex* was present

Table 3. Estimates of evolutionary divergence (K2P) over sequence pairs between five Gelechiidae species as inferred using the p-distance model in MEGA 6.0 (Tamura et al. 2013)

	Tuta absoluta	*Phthorimaea operculella*	*Pectinophora gossypiella*	*Sitotroga cerealella*	*Aproaerema simplixella*	NN	Distance to NN
Tuta absoluta	0					*Phthorimaea operculella*	10.83
Phthorimaea operculella	**0.110**	0				*Tuta absoluta*	10.83
Pectinophora gossypiella	0.139	**0.161**	0			*Sitotroga cerealella*	11.69
Sitotroga cerealella	0.150	0.150	0.126	0		*Pectinophora gossypiella*	11.69
Aproaerema simplixella	0.159	0.157	0.135	0.151	0	*Pectinophora gossypiella*	13.52

NN = nearest neighbor. In bold are the lowest and highest evolutionary distances values between the species.

Table 4. Sequence divergences of five Gelechiidae species at the taxonomic level

	Min distance (%)	Mean distance (%)	Max distance (%)	SE distance (%)
Within species	0	0.02	6.67	0
Within family	10.83	14.38	20.5	0

There were a total of 298 sequences in the analysis that represented five taxa.

in all the sampling sites and recorded significantly higher infestation levels on cotton. To date, this is also the first report of *A. simplex* and adds to the list of devastating invasive species in Kenya. This pest is commonly regarded to as the false pink bollworm and has previously been observed feeding on cotton bolls in Florida (Halbert 2015). It is still unclear why *P. gossypiella* was not present in Embu, Mwea, and Meru when compared to *A. simplex*, but it is possible that availability of alternative host crops could be a key contributing factor. Indeed, *Pectinophora gossypiella* had previously been reported in Kenya on a native fruit, the *G. kirkii* Mast (Miller et al. 2014) and this is probably its preferred host. Lack of significant differences on the levels of infestations of all the studied pests when compared across localities could indicate that their distribution was not limited by geographical parameters. It is also possible that factors such as crop variety and pest management practices used in the studied sites did not have an influence on the observed levels of infestations.

Reliable diagnostic tools are a prerequisite for effective IPM initiatives and plant quarantine systems. COI-based barcoding was able to distinguish the five pest species based on sequence similarity, evolutionary divergences, and phylogenetic analyses. These results contribute to literature documented on DNA barcodes of notorious agricultural pests present in Kenya (Khamis et al. 2012, 2017; Kinyanjui et al. 2016). High percentages of sequence similarity obtained from BOLD and GenBank databases enabled identification of individual specimen to the species level. In addition, intraspecific divergences were lower than interspecific divergences, enabling effective discrimination of the species (Hebert et al. 2003b). Based on sequence similarity, the groundnut leafminer was identified as *A. simplixella* with 100% match and nucleotide search of both databases revealed no COI sequences of *A. modicella*. This finding may suggest further studies on molecular identification of *A. modicella* in order to determine the genetic relationship between the two species. Populations of *T. absoluta* clustered together irrespective of sampling sites and geographical location, indicating low genetic divergences which were also observed in *A. simplixella*. Our findings are also consistent with results observed on populations of *T. absoluta* from South American and Mediterranean Basin countries using COI and internal transcribed spacers (ITS) 1 and 2 markers (Cifuentes et al. 2011). Indeed, Buthelezi et al. (2012) also observed low intra- and interpopulation genetic variations in *A. simplixella* sampled from South Africa. This phenomenon is commonly associated with introduced species as a consequence of founder events and could possibly be a contributing factor toward the invasion success of these pests (Tsutsui et al. 2000, Cifuentes et al. 2011).

Basically, intraspecific divergences are less than 2% (Avise 2000) and the exceptional case of *S. cerealella* is unclear. This species could also be associated with the reported maximum intraspecific divergences of 6.67%, which contributed to its splitting into one concordant and three singleton BINs. This genetic variation was observed in intra- and interpopulations thus ruling out the element of geographical isolation. Our results could possibly indicate differential response of individuals to insecticides or natural enemies. These evolutionary divergences were also displayed between study sequences and reference sequences in GenBank, where BLAST hits ranged from 89 to 93%. However, these high divergences did not compromise the use of COI-based barcoding in discriminating the species because all specimens of *S. cerealella* were matched to the species. In addition, phylogenetic analyses ruled out the possible existence of cryptic species because all the samples clustered strongly with bootstrap values of 99%. The species identity was further confirmed by BOLD results, where percentage identities ranged from 98 to 100%. Results on low evolutionary divergences between *T. absoluta* and *P. operculella* and being the nearest neighbors could be useful in the search for effective natural enemies given that populations of closely related species could be suppressed by a common biological control agent.

In conclusion, our data presented new information regarding the actual levels of infestation and identification of five invasive Gelechiidae pest species in Kenya. These are the first critical steps toward establishing their potential risks and defining effective IPM techniques. However, our study only provides baseline results and thus additional studies are required to assess the flight and infestation dynamics of these pests on their respective crops under different

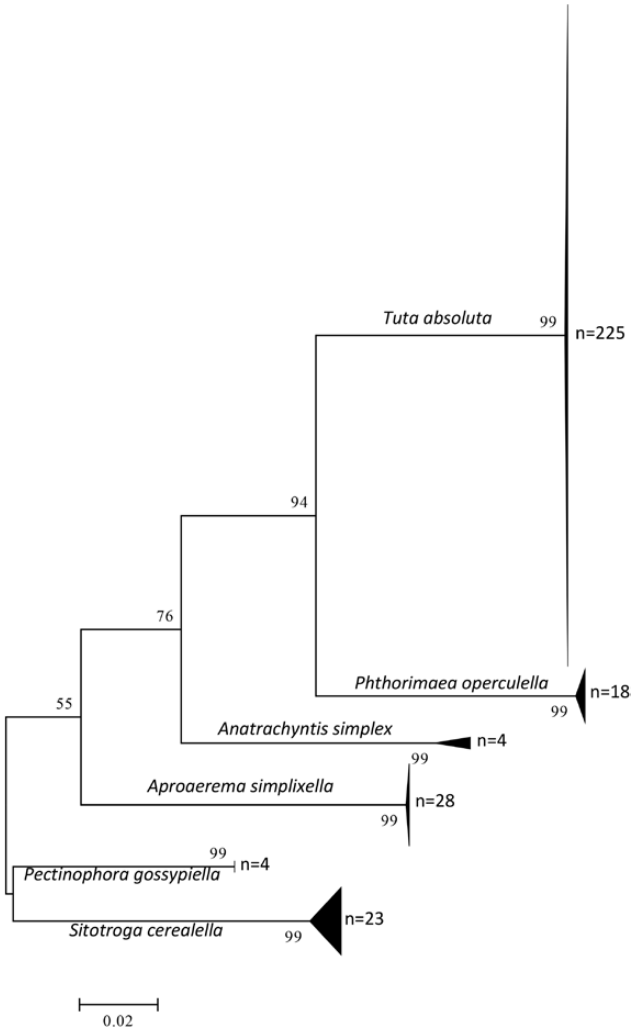

Fig. 2. Maximum likelihood tree depicting 658-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) of 302 specimens representing five Gelechiidae species and *Anatrachyntis simplex*. The clades have been collapsed and the number of sequences is provided. The tree was drawn to scale, and the branch lengths are in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Values at the nodes are bootstrap values for 1,000 replications.

cropping seasons. Notably, our data on the levels of infestation could have been influenced by the rates of parasitism and predation and thus the need to research on the coevolved natural enemies and evaluate their potential to suppress the populations of these invasive species. The presence of *A. simplex* also calls for more comprehensive studies on cotton to assess its economic impact.

COI-based barcoding has provided an efficient and accurate tool for identification of these pests and has an added advantage of not being limited by life stages of the target species. Specifically, the larval stages of *P. gossypiella* and *A. simplex*, both feeding on cotton bolls, are pink in color. Although *A. simplex* larvae are smaller in size, it is quite a challenge differentiating them from the early instars of *P. gossypiella* based on morphological identification. DNA barcoding could therefore play an important role in accelerating pest diagnostics in respect to easy monitoring as well as early detection of target and newly introduced species. It could also assist in formulating appropriate species-specific pest management programs that will effectively reduce agricultural losses. Generated sequences will contribute to the expansion of reference databases for future species-level identifications within the family Gelechiidae. Whereas mitochondrial COI displayed low genetic variation within species, more research on population genetic structure of these species and particularly, *S. cerealella*, using more robust molecular markers such as microsatellites is warranted.

Data Accessibility

COI sequence data used for this study were submitted to the Barcode of Life Database (BOLD) (TAEA001-16-TAEA225-16 and TAEA226-17-TAEA298-17) and deposited to GenBank (accession numbers KU565496–KU565720 and MF121833–MF121905).

Acknowledgments

We acknowledge the numerous farmers who allowed us to carry out sampling in their farms. The research was conducted with financial support by the following organization: Federal Ministry for Economic Cooperation and Development (BMZ), Germany, icipe-Tuta IPM project (Project No.: 12.1433.7-001.00). We also acknowledge *icipe* core funding provided by United Kingdom's Department for International Development (DFID); Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Ministry for Economic Cooperation and Development (BMZ), Germany; and the Kenyan

Government. Finally, we thank three anonymous reviewers for providing useful comments and discussions that improved the earlier version of the manuscript. F.M.K., S.E., and S.A.M conceived and designed the experiments. G.K. collected samples. G.K. and F.L.O.O. performed the experiments. G.K., F.L.O.O., and F.M.K. analyzed the data and drafted the manuscript. Supervision: F.M.K., S.A.M., S.E., and E.U.K. All authors read and approved the final draft of the manuscript.

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