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Research Article

DNA Barcode Reference Library for the African Citrus Triozid, *Trioza erytreae* (Hemiptera: Triozidae): Vector of African Citrus Greening

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Abstract

Citrus (*Citrus* spp.) production continues to decline in East Africa, particularly in Kenya and Tanzania, the two major producers in the region. This decline is attributed to pests and diseases including infestation by the African citrus triozid, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae). Besides direct feeding damage by adults and immature stages, *T. erytreae* is the main vector of '*Candidatus* Liberibacter africanus', the causative agent of Greening disease in Africa, closely related to Huanglongbing. This study aimed to generate a novel barcode reference library for *T. erytreae* in order to use DNA barcoding as a rapid tool for accurate identification of the pest to aid phytosanitary measures. Triozid samples were collected from citrus orchards in Kenya, Tanzania, and South Africa and from alternative host plants. Sequences generated from populations in the study showed very low variability within acceptable ranges of species. All samples analyzed were linked to *T. erytreae* of GenBank accession number KU517195. Phylogeny of samples in this study and other *Trioza* reference species was inferred using the Maximum Likelihood method. The phylogenetic tree was paraphyletic with two distinct branches. The first branch had two clusters: 1) cluster of all populations analyzed with GenBank accession of *T. erytreae* and 2) cluster of all the other GenBank accession of *Trioza* species analyzed except *T. incrustata* Percy, 2016 (KT588307.1), *T. eugeniae* Froggatt (KY294637.1), and *T. grallata* Percy, 2016 (KT588308.1) that occupied the second branch as outgroups forming sister clade relationships. These results were further substantiated with genetic distance values and principal component analyses.

Key words: Citrus greening, DNA barcoding, reference library, rapid species identification

Globally, citrus (*Citrus* spp.) is one of the most important fruit crops, with an average annual production of over 128 million metric tons (MT) (FAOSTAT 2012). Species of commercial importance include sweet orange (*Citrus sinensis* Osbeck), lemon (*Citrus limon* [L.] Burm. f.), lime (*Citrus aurantifolia* [Cristm.] Swingle), grapefruit (*Citrus paradisi* Macfad), and mandarin (*Citrus reticulata* Blanco). Among the various species, C. *sinensis* is the most popular and accounts for more than half of the world's total production (FAOSTAT 2012). In Africa, the highest citrus-producing countries are South Africa and Egypt (FAOSTAT 2016) with production destined for local, regional, and export markets. In East Africa, Kenya and Tanzania produce 251,459 and 415,203 MT respectively, from an estimated 13,000 ha (FAOSTAT 2013). However, this level of production is far below the domestic demand in both countries, resulting in bulk importation of fruits and related processed products particularly from South Africa, Egypt, and the Middle East. Paradoxically, citrus production is on a gradual decline in Kenya and Tanzania (Kilalo et al. 2009, Nyambo 2009) despite its huge production potential and the high demand for fruits.

Worldwide, citrus production is constrained by various economic, biological, and environmental factors. Ranking high among these are pests and diseases, of which, the African citrus triozid (ACT), *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), is considered as one of the most damaging pests (Kilalo et al. 2009, Ekesi 2012). It has a wide geographical distribution in Africa with reports from Angola, Kenya, Ethiopia, Eritrea, Madagascar, Malawi, Mauritius, La Réunion, South Africa, Sudan, Swaziland, St. Helen, Tanzania, Uganda, Zambia, DR Congo, Rwanda, Comoros, and Cameroon (Aubert 1987, EPPO/CABI 2006, EPPO 2014). ACT normally prefers cool

areas and higher altitudes where young flushes thrive longer (Green and Catling 1971) as its reproduction and development mainly occur on young expanding leaves. Direct feeding by *T. erytreae* causes leaf curling and notching and deposition of honeydew on infested plants favor the growth of sooty mould, which reduce plant vigor and productivity and affect the aesthetic value of leaves. Similarly, the feeding activity of its related counterpart Asian citrus psyllid *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae) may kill the developing plant terminals or cause leaf abscission (Michaud 2004). *D. citri* pest has recently been reported in East Africa (Shimwela et al. 2016, Rwomushana et al. 2017). ACT infestations of leaf clusters in the highlands of Kenya can be as high as 65%, and these distorted leaves provide refuge for other pests (Ekesi 2012).

Although direct damage to the plant can be significant, ACT is most known for the transmission of the phloem-limited bacterium, '*Candidatus* Liberibacter africanus' (CLaf), the causative agent for African Citrus Greening disease (ACG) (Bové 2006). This bacterium is a close relative of '*Candidatus* Liberibacter asiaticus' (CLas) and '*Candidatus* Liberibacter americanus' (CLam) that are causal agents of Huanglongbing(HLB) disease; CLas is transmitted by *D. citri* in Asia and North America and CLam in Brazil (Halbert and Manjunath 2004, Hall et al. 2012, Grafton-Cardwell et al. 2013). HLB occurs worldwide and is the most devastating disease of citrus for which there is still no known cure (McClean and Schwarz 1970, Halbert and Manjunath 2004, Bové 2006, Saponari et al. 2010). The yield of affected trees is not only considerably reduced by continuous fruit drop, dieback, and tree stunting but also by the poor quality of fruits that remain on the trees and which are inedible. In Kenya and Tanzania, ACG is reported to have had the greatest impact on citrus production especially in the highlands, causing yield losses of 25–100% (Swai et al. 1992, Pole et al. 2010). However, HLB has not yet been reported in both countries but only found in northern Ethiopia (Saponari et al. 2010).

T. erytreae is the principal vector for CLaf (McClean and Oberholzer 1965), though Massonie et al. (1976) demonstrated (albeit experimentally) that it also has the ability to transmit the CLas. Furthermore, it is hypothesized that in Mauritius and La Réunion, where both CLaf and CLas are present, *T. erytreae* could be transmitting both bacteria (EPPO/CABI 2006). Therefore, ACT represents a great threat to citrus production in Africa, so significant that it is classified as an A1 quarantine pest by the European and Mediterranean Plant Protection Organisation (OEPP/EPPO 1988).

T. erytreae has a wide host range within the Rutaceae family, including *Zanthoxylum capensis* Thunb. Harv., *Clausena anisata* (Willd) Hook, and *Vepris lanceolata* (Lam.) G. Don (van der Merwe 1923, Catling and Annecke 1968, Moran 1968, van Bruggen and Yilma 1985, Cocuzza et al. 2016). Although *T. erytreae* is a pest of commercial citrus in Africa and on the Indian Ocean islands of Madagascar and Mauritius, it is also reported to successfully complete its development on other indigenous host plants, a fact that was known long before citrus was introduced into the region (Anon 1967). Therefore, managing the indigenous reservoirs that act as alternative hosts also has direct implications in planning and executing effective control measures against the pest in citrus orchards.

Even though proper identification and understanding of ACT's bio-ecology is crucial for its proper management (Moran 1968, Samways and Manicom 1983, van den Berg et al. 1991), there is a significant gap with regard to its molecular identification and barcoding—information that are important to boost bio-ecological and morphological data of *T. erytreae* and enhance its management. A GenBank search (National Center of Biotechnology Information, NCBI) showed that there is limited sequence and genome data for *T. erytreae*. Given the quarantine status and expanding range of the pest in recent years, there is a challenge in proper identification, especially in newly invaded areas, and obstacles in first time detection could hinder or delay deployment of adequate management measures and containment of the pest species to curb its spread. Morphological identification may be fraught with inaccuracy as Hollis (1984) mentioned 49 Afrotropical species within the genus *Trioza*, often occupying the same niche. Moreover, African *Trioza* spp. are notoriously difficult to separate taxonomically using morphological keys only (Aubert 1987). Therefore, the development of molecular techniques (e.g., using barcoding) to distinguish *T. erytreae* from closely related species could rapidly facilitate its identification and management.

ACT control has a direct link to the management and containment of citrus Greening. van Vuuren and da Graça (1978) indicated that *T. erytreae* needs only 1 h to feed on a suitable host plant to transmit the bacterium. This suggests that time is critical in *T. erytreae* identification, underlining the need for efficient and accurate analytical tools for fast pest identification. In this study, we focused on the use of mitochondrial DNA (*mt*DNA) and in particular, the cytochrome *c* oxidase subunit I (COI), for identifying *T. erytreae* (adults and nymphs) collected from citrus and other alternative host plants in Kenya, Tanzania, and South Africa. This technique was preferred since the *mt*DNA contains a high proportion of nucleotide substitutions that have evolutionary significance, lack introns, have limited exposure to recombination, and exhibit haploid mode of inheritance (Avise et al. 1987, Saccone et al. 1999). Use of robust universal primer sets that work for many genera has enabled the routine recovery of specific segments of the mitochondrial genome (Folmer et al. 1994; Simmons and Weller 2001; Hebert et al. 2003a,b). The COI region has been proposed as the barcode region of choice (Hajibabaei et al. 2007), and this has brought new valuable insights into species identification. The COI region can be used not only for species identification, delineation, and discrimination of closely related species but also for construction of phylogenetic distribution of groups within a species (Cox and Hebert 2001, Wares and Cunningham 2001). Furthermore, a growing database of validated sequences is publicly accessible for reliable identification of species and storage of data produced globally (Hebert et al. 2003a,b; Ratnasingham and Hebert 2007). Though DNA barcoding is a rapid and reliable tool for identification and species delineation, some studies have questioned the reliability of this tool as it relies on the use of the COI gene. This is because of the presence of nonfunctional copies of this gene called nuclear mitochondrial pseudogenes (Numts) that are present in several groups of organisms (Hazkani-Covo et al. 2010). Furthermore, DNA barcoding study hypotheses, purpose of study, and barcode data analyses methods should be keenly deliberated for successful use of this tool (Collins and Cruickshank 2012). Hence, in this study primer, optimization and robustness were critically taken into consideration.

Several studies have reported the utility of using DNA barcoding in different insect groups (Hebert et al. 2003a,b ; Hebert et al. 2004; Ball and Armstrong 2006; de León et al. 2011; Khamis et al. 2012; Kinyanjui et al. 2016) and demonstrated that more than 95% of species possess unique COI barcode sequences; thus, species-level identifications are often attained (Hajibabaei et al. 2007). Our study was consequently undertaken to generate a barcode reference library for *T. erytreae* and propose DNA barcoding as a rapid tool to be used for identification of the pest, as well as strengthened the phytosanitary management and guide the implementation of control measures. We present the preferred primers and methodological approach for fast identification of ACT and show sequence divergence within the targeted region.

Table 1. Collection data and references for *T. erytreae* samples

Code name	Host plants	Country	GPS co-ordinates	Elevation (m)	GenBank accessions
TeKe1-A, B, C, D	Citrus	Kenya	S00°10′30.0″ E035°05′15.3″	1297	KY754656, KY754655, KY754654, KY754653
TeKe2-A, B, D, E	Citrus	Kenya	S00°14′04.9″ E035°08′26.7″	1405	KY754652, KY754651, KY754650, KY754649
TeKe3-B, C, D	Citrus	Kenya	S00°11′43.3″ E035°34′47.7″	2303	KY754648, KY754647, KY754646
TeKe4-A, B, C, D, E	Citrus	Kenya	S00°13′24.1″ E035°08′05.0″	1390	KY754645, KY754644, KY754643, KY754642, KY754641
TeKe5-A, B, C, D, E	*Stephania abyssinica*	Kenya	S00°19′22.5″ E035°13′38.7″	1997	KY754640, KY754639, KY754638, KY754637, KY754636
TeKe7-A, B, C, D, E	Citrus	Kenya	S01°19′04.3″ E037°24′40.7.3″	1710	KY754635, KY754634, KY754633, KY754632, KY754631
TeKe8-A, B, C, D, E	Citrus	Kenya	S00°32′47.9″ E037°35′08.6″	1223	KY754630, KY754629, KY754628, KY754627, KY754626
TeKe9-A, B, C	Citrus	Kenya	N00°06′27.0″ E037°46′01.7″	1275	KY754625, KY754624, KY754623
TeKe10-B	Citrus	Kenya	N00°08′24.2″ E037°50′23.7″	1438	KY754622
TeKe11-A, B, C	Citrus	Kenya	S00°33′47.4″ E037°23′03.2″	1333	KY754621, KY754620, KY754619
TeKe12-CA-1, 2, 3	*Clausena anisata*	Kenya	S00°27′45.8″ E037°05′27.3″	1778	KY754618, KY754617, KY754616
TeKe12-1, 2, 3	Citrus	Kenya	S00°27′45.8″ E037°05′27.3″	1778	KY754615, KY754614, KY754613
TeKe13-A, B, C	Citrus	Kenya	S00°51′38.8″ E037°07′49.5″	1395	KY754612, KY754611, KY754610
TeTz2	Citrus	Tanzania	S06°51′17.9″ E037°40′36.8″		KY754588
TeSA 1, 3, 5, 6, 7, 10	Citrus	South Africa			KY754594, KY754593, KY754592, KY754591, KY754590, KY754589
TeKe14-A, B, C, D, E, F, G, H, I	*Murraya koenigii*	Kenya	S01°16′09.3″ E036°49′26.2″	1683	KY754609, KY754608, KY754607, KY754606, KY754605, KY754604, KY754603, KY754602, KY754601
TeKe15-A, B, C, D, E, F	Citrus	Kenya	N00°05′00.8″ E037°50′20.3″	1147	KY754600, KY754599, KY754598, KY754597, KY754596, KY754595
TeCA 2, 5, 6, 9	*Clausena anisata*	Kenya	S00°27′45.5″ E037°05′27.5″	1777	KY754587, KY754586, KY754585, KY754584

Methodology

Sample Collection

T. erytreae samples were collected through a survey from citrus plants at various locations in Kenya, Tanzania (one specimen), and South Africa (Table 1). Since ACT is known to complete its development also on other hosts of the Rutaceae family (Aubert 1987), additional samples (mainly immature stages) were also collected from *C. anisata* (Willd.) Hook.f., and curry tree *Murraya koenigii* (L.) Spreng., and included in our analysis. During the survey, we encountered several unidentified *Trioza* sp. feeding on *Stephania abyssinica* Walp. (Menispermaceae) in fields adjacent to a citrus orchard and the nymphs were sampled and also included in the analysis. For *C. anisata*, samples consisted of 1) insects collected from a single plant shading a section of a citrus orchard, and 2) insects collected from trees in indigenous vegetation and not within the vicinity of any citrus orchard. Georeferenced data (GPS coordinates and elevations) of the sampling points were taken and a map generated (Fig. 1).

Insect sampling involved collection of plant parts with nymphal stages, which were held in an aerated lunch box with moist tissue paper to prevent desiccation. *T. erytreae* can easily be detected on citrus and other Rutaceae due to the clustering of nymphs in pit-like galls, where the developing stages nest. The nymphs were taken to the laboratories of the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus in Nairobi, Kenya, where they were maintained on seedlings of their respective host plants in cages until adult emergence. A minimum of five *T. erytreae* adults were randomly selected from each collection point, photographed dorsally, ventrally, and laterally at 25× using a Leica LAS EZ4D stereomicroscope (Leica Ltd., Switzerland) prior to DNA extraction. Voucher specimens are deposited at *icipe*'s Molecular Pathology Laboratory in the Arthropod Pathology Unit.

Genomic DNA Extraction, Polymerase Chain Reaction, and Sequencing

Adult insects were surface-sterilized using 3% sodium hypochlorite and rinsed with distilled water. Genomic DNA was extracted from individual insects using the Isolate II genomic DNA Kit (Bioline, United Kingdom), following the manufacturer's instructions. The resultant DNA was eluted with deionized water to a final volume of 50 µl with quality and quantity checked using Nanodrop 2000/2000c Spectrophotometer (Thermo Scientific, USA) by measuring optical density at $A_{260\ nm}$ and $A_{280nm,}$ respectively. Samples that had a ratio range of $A_{260\ nm}/A_{280nm}$ below 1.7 were discarded. Polymerase chain reaction (PCR) was done to amplify the barcode region using the

Fig. 1. Map of Kenya and Tanzania showing the sampling sites/regions for *T. erytreae* samples.

primer pairs LCO1490 and HCO2198 (Folmer et al. 1994) and LepF1 and LepR1 (Hebert et al. 2004), in separate reactions. PCR was carried out in a total reaction volume of 20 µl containing 5× MyTaq reaction buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers, and enhancers) (Bioline), 10 µmole of each primer, 0.5 mM $MgCl_2$ (Thermo Scientific), 0.25 µl MyTaq DNA polymerase (Bioline), and 15 ng/µl of DNA template. These reactions were set up in the Master cycler Nexus gradient (Thermo Scientific) using the following cycling conditions: initial denaturation for 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at annealing temperature of 50.6°C (for LCO1490 and HCO2198 primer set) and 52°C (for LepF1 and LepR1), and 1 min at 72°C, then a final elongation step of 10 min at 72°C. The target gene region for both primer sets was between 650 and 700 bp.

The amplified PCR products were resolved through a 1.2% agarose gel. DNA bands on the gel were analyzed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp, USA). Successfully amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline) following manufacturer's instructions. The purified samples were bidirectional sequenced by Macrogen Inc Europe Laboratory, the Netherlands.

Sequence Data Analysis

Sequences were assembled and trimmed to remove primer sequences using Geneious v8.1.5 (http://www.geneious.com, Kearse et al. 2012) and Chromas Lite v2.1.1 (Techelysium Pty Ltd, Queensland, Australia). Closest sequence identities were determined using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and multiple alignments of the assembled, trimmed sequences were done using Clustal X software (version 2.1) (Thompson et al. 1997). The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al. 2016). Maximum Likelihood method was applied as the tree-building algorithm to visualize the patterns of divergence among the triozid samples. The reliability of the clustering pattern in the tree was evaluated using a bootstrap analysis with 1,000 replicates. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (Saitou and Nei 1987, Gascuel 1997) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with the superior log likelihood value (Tamura et al. 2013). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site involving 73 nucleotide sequences that included sequences generated from this study and 21 available GenBank accessions of *Trioza* sp. [*T. erytreae* (KU517195), *T. urticae* (Linnaeus 1758) (JX987965.1), *T. aylmeriae* Patch, 1912 (KR042632.1), *T. eugeniae* Froggatt (KY294637.1), *T. zimmermani* Tuthill, 1942 (KY294169.1), *T. vitiensis* Kirkaldy, 1907 (KY294168.1), *T. tricornuta* Taylor, Jennings, Purcell, and Austin, 2013 (KY294165.1), *T. remota* Foerster, 1848 (KY294163.1), *T. percyae* Taylor, 2013 (KY294161.1), *T. pallida* (Uichanco, 1919) (KY294160.1), *T. outeiensis* Yang, 1984 (KY294159.1), *T. obunca* Fang and Yang, 1986 (KY294158.1), *T. malloticola* (Crawford, 1928) (KY294156.1), *T. magnoliae* (Ashmead) (KY294155.1), *T. kuwayamai* Enderlein, 1914 (KY294154.1), *T. anceps* Tuthill, 1944 (KY294149.1), *T. alipellucida* Klyver, 1932 (KY294147.1), *T. adventicia* Tuthill, 1952 (KY294146.1), *T. barrettae* Taylor and Moir, 2014 (KP709055.1), *T. grallata* Percy, 2016 (KT588308.1), and *T. incrustata* Percy, 2016 (KT588307.1)].

Pairwise nucleotide sequence divergences and overall transition/transversion ratio were calculated using Kimura 2-parameter (K2P) distance model (Kimura 1980) in MEGA 7 (Kumar et al. 2016), and principal component plot was then developed from the genetic distance matrix by GenAlEx 6.5 (Peakall and Smouse 2006, 2012). For the genetic distance matrix, a single representative sequence for each homologous group was used. Therefore, the following groups were considered: all samples from the study (*T. erytreae*), GenBank reference sequences of *T. erytreae* (TeKU517195), *T. urticae* (Turticae), *T. aylmeriae* (Talymeriae), *T. eugeniae* (Teugeniae), *T. zimmermani* (Tzimmermani), *T. vitiensis* (Tvitiensis), *T. tricornuta* (Ttricornuta), *T. remota* (Tremota), *T. percyae* (Tpercyae), *T. pallida*

(Tpallida), *T. outeiensis* (Touteiensis), *T. obunca* (Tobunca), *T. malloticola* (Tmalloticola), *T. magnoliae* (Tmagnoliae), *T. kuwayamai* (Tkuwayamai), *T. anceps* (Tanceps), *T. alipellucida* (Talipellucida), *T. adventicia* (Tadventicia), *T. barrettae* (Tbarrettae), *T. grallata* (Tgrallata), and *T. incrustata* (Tincrustata). The COI sequences were submitted to the Barcode of Life Database (BOLD) (http://www.boldsystems.org/) and subsequently deposited in GenBank. The project was uploaded to BOLD with the project code TEBP, assigned BOLD accessions TEBP001-16.COI-5P to TEBP073-16.COI-5P and in GenBank, accession numbers KY754584 to KY754656.

Results

DNA was extracted from the samples collected from the surveys in citrus orchards and other host plants in Kenya and from citrus orchards in Tanzania and South Africa. The DNA yields for the samples ranged between 10.6 and 56.7 ng/µl with the ratios of $A_{260\ nm}/A_{280nm}$ between 1.79 and 2.27; therefore, samples were of sufficient quantity and quality for routine PCR. In this study, we employed the primers based on Folmer et al. (1994) and the Lep F1 and Lep R1 primers (Hajibabaei et al. 2006) for amplification of the COI barcode region for *T. erytreae*. Despite being the recommended primers for universal barcoding for invertebrates, the Folmer et al. (1994) primers yielded short sequences of about 50–120 bp with numerous incongruences, while the Lep F1 and Lep R1 primer pairs consistently gave excellent amplifications with strong amplicons of approximately 650 to 700 bp. When the PCR products were excised and purified, the recovered pure amplicons were of high yield, mostly ranging between 23 and 58 ng/µl. On sequencing, almost all the reads were of high quality (≥98%), sufficient length, and almost no miss amplifications. Hence, the Lep F1 and R1 primers were used for DNA barcoding of this insect. Seventy-three COI barcode region sequences obtained through amplification with Lep primers were analyzed. The mean nucleotide frequencies were G=13.91%, C=13.68%, A=31.81%, and T=40.60%, with a clear AT-bias. The overall estimated transition/transversion bias (R) was R=0.90, and the summary of nucleotide substitution matrix is shown in Table 2. The maximum Log likelihood was –1,394.136, and the nucleotide frequencies were A=25%, T/U=25%, C=25%, and G=25%. The within-species mean pairwise sequence divergence was 1.14%, with a minimum of 0% and a maximum distance of 11.2%.

The COI barcode sequences obtained using the Lep primers for the samples from Kenya and Tanzania showed absolute homology (100%) with *T. erytreae* (KU517195) in a BLAST search. Sequences for *T. erytreae* samples from South Africa also showed absolute homology (100%) to *T. erytreae* (KU517195). These South African samples were identified taxonomically/morphologically and were used as references for comparison with the East African barcodes. All amplified PCR products had single bands. In addition, the DNA sequence data were of good quality and analyzed using the maximum likelihood approaches.

Table 2. Maximum likelihood estimate of substitution matrix from 73 *T. erytreae* samples

	A	T/U	C	G
A	–	*6.56*	*6.56*	**11.88**
T/U	*6.56*	–	**11.88**	*6.56*
C	*6.56*	**11.88**	–	*6.56*
G	**11.88**	*6.56*	*6.56*	–

Each entry is the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

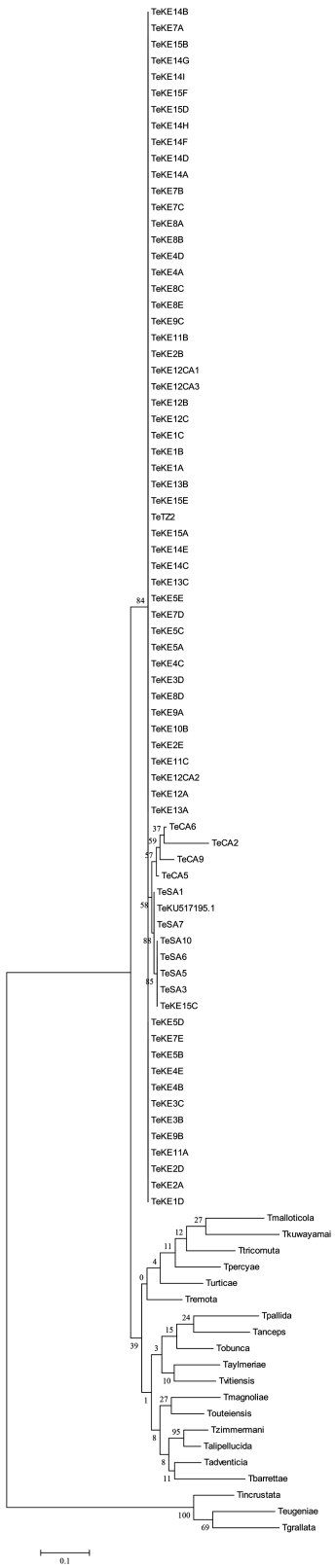

Fig. 2. Maximum Likelihood tree showing evolutionary relationships between *T. erytreae* samples from the study inferred by MEGA 7 (Kumar et al. 2016).

Phylogeny of all the *Trioza* samples was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the best log likelihood (−4,329.6280) is shown in Figs. 2 and 3. The phylogenetic tree was paraphyletic with two distinct branches. The first branch separated into two clusters, where the first cluster hosted all populations analyzed in the current study regardless of host-plant (*C. sinensis*, *M. koenigii*, *S. abyssinica*, or *C. anisata*) and sampling region (Kenya, Tanzania, or South Africa), and the GenBank accession of *T. erytreae* (Figs. 2 and 3). The other cluster was occupied by all the other GenBank accessions of *Trioza* species included in the analyses except for *T. incrustata*, *T. eugeniae*, and *T. grallata*. These three species branched separately as outgroups forming sister clade relationships (Figs. 2 and 3).

Estimates of evolutionary divergence over sequence pairs between groups were successfully generated from all sequenced samples. Numbers of base substitutions (obtained per site from averaging over all sequence pairs between groups) are presented as a genetic distance matrix (Table 3). Overall mean genetic distance of 0.011, which was within the acceptable ranges of species variability (Hebert et al. 2003a,b; Virgilio et al. 2010), was initially inferred for the 73 nucleotide sequences generated from the various populations in this study. The genetic distance between ACT samples from the study and the GenBank accessions of *T. erytreae* was 0.014, while all other species clearly separated from these two groups with more than 0.15 genetic distances, confirming the phylogenetic analyses. *T. grallata* exhibited a very distant relationship with the two *T. erytreae* groups with a genetic distance of 0.8. Other *Trioza* species closely related included *T. zimmermani* and *T. alipellucida* with a genetic distance of 0.085. Greatest genetic distance of 0.995 was observed between *T. kuwayamai* and *T. grallata*. Additionally, *T. eugeniae* seems to exhibit the most distant relationship with all the other *Trioza* species. The distance matrix was used to generate the principal component plot (Fig. 4) where the first two axes in the principal component analysis (PCA) explained 51.81% of the variation (the first axis 45.8%, and the second axis 6.01%) between all the *Trioza* samples analyzed from the study and GenBank accessions. The PCA clustered *T. erytreae* samples in the study with the GenBank accession of *T. erytreae*. *T. eugeniae*, in concurrence with genetic distance matrix, clustered on its own. Furthermore, *T. grallata* and *T. incrustata* were closely associated and occupied an axis separated from other *Trioza* species (Fig. 4).

Discussion

Rapid and accurate identification of pest species is a fundamental requirement for effective pest management and phytosanitary procedures. The main objective of this study was to establish and validate a rapid identification tool for *T. erytreae*, infer variability among populations, and generate an ACT reference barcode library to fast track identification for management of the pest. With the advances in molecular biology, most species identification levels are based on the use of both morphological and molecular methodologies to facilitate characterization and species delineation. Currently, the most widely used molecular tool is DNA barcoding (Hebert et al. 2003a,b; Ratnasingham and Hebert 2007). DNA barcoding offers a tool that can expedite species identification in the absence of taxonomic expertise, interception of immature stages or damaged specimen with reduced morphological features (Armstrong and Ball 2005).

We initially tested the DNA barcoding primers developed by Folmer et al. (1994) to recover the barcode segment of the mitochondrion genome of *T. erytreae* since we previously used this primer pair for similar studies (Khamis et al. 2012, Kinyanjui et al. 2016). However, these primers did not reliably amplify the gene region of interest. Hence, we resorted to the use of alternative primers, Lep primers, which provided satisfactory amplification of the psyllid mitochondrion genome. Therefore, for *T. erytreae*, we recommend Lep primers for reliable amplification of the COI barcode region. All the sequences generated in the study linked to a barcode of *T. erytreae* (KU517195.1) that had been recently uploaded (Hodgetts et al. 2016). This sample was collected from Gran Canaria, Spain, from *C. sinensis* whereas our study had a more comprehensive sampling course from East and South Africa, and from different host plants. Various studies have demonstrated the effectiveness of the DNA barcoding platform in identification of pest species of economic importance (Armstrong and Ball 2005, Ball and Armstrong 2006, Khamis et al. 2012, Kinyanjui et al. 2016), and our results concur with the findings of Hodgetts et al. (2016) on its use for *T. erytreae* identification.

Although *T. erytreae* is known to develop exclusively on host plants of Rutaceae family (Aubert 1987), the Maximum Likelihood model-based phylogenetic analysis results showed that the triozid found on *S. abyssinica*, Menispermaceae is also identified as *T. erytreae*. These results are conformed with a previous study by Kalyebi et al. (2015) which stipulated that *Ficus* spp. (Moraceae), *Diospyros mespiliformis* Hochst. ex A. DC (Ebenaceae) and *S. abyssinica* were host plants for *T. erytreae* though based on only pit gall formation. Our results clearly call for a more comprehensive documentation of *T. erytreae* host plants range, since this has potential implications for managing this important vector pest and associating plants outside of the family Rutaceae that could have serious consequences for its control and/ or containment. Therefore, this study has not only successfully identified *T. erytreae* attacking citrus but has also delineated the potential alternative host plants of the pest in Kenya. Closely related species exhibiting similar morphological traits have previously been identified based on their host plants association (Coeur d'acier et al. 2014), which is yet again complicated by the possible host range expansion by *T. erytreae*. However, this is an exploratory study and we are currently undertaking more surveys to include more localities, host plants, and multigene analyses of this pest to reliably give substantial insights into host specificity and host range of ACT in Africa.

The genetic distances observed between the populations studied were low, with an overall mean of 0.011, typical for variability within a species and in congruence with other studies that used DNA barcoding for species identification and delineation (Khamis et al. 2012, Kinyanjui et al. 2016). This further confirms the identity of the *T. erytreae* samples in the study and the fact that the pest can be found not only on Rutaceae but also on non-Rutaceae host plants. Moreover, pairwise intraspecific mean sequence divergence inferred for all the sequences generated in this study was 1.1% which is within adequate ranges reported previously (Kinyanjui et al. 2016). In a study on utility of DNA barcoding across insect orders, Virgilio et al. (2010) reported intraspecific sequence divergences of DNA barcodes belonging to 1,995 insect species ranging between 0.0 and 7.64%. Intraspecific sequence divergence should be lower than interspecific divergence to create a barcoding gap that enables assignment and delimitation of undescribed species (Hebert et al. 2003a, Meyer and Paulay 2005, Meier et al. 2008). Interspecific COI divergences among species pairs can range between 0.0 and 53.7% (Hebert et al. 2003b); and for a successful species identification based on genetic distances, there should be no overlap between intraspecific and interspecific sequence divergences.

Table 3. Estimates of evolutionary divergence of mitochondrial COI gene region over sequence pairs between groups as determined using p-distance model in MEGA 7 (Kumar et al. 2016)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Terytreae	0.000																					
2. TeKU517195.1	0.014	0.000																				
3. Turticae	0.167	0.179	0.000																			
4. Taylmeriae	0.198	0.203	0.191	0.000																		
5. Tzimmermani	0.167	0.171	0.207	0.203	0.000																	
6. Talipellucida	0.155	0.160	0.179	0.199	0.085	0.000																
7. Tmagnoliae	0.174	0.179	0.223	0.199	0.179	0.179	0.000															
8. Tadventicia	0.155	0.152	0.191	0.179	0.145	0.127	0.164	0.000														
9. Touteiensis	0.179	0.191	0.188	0.187	0.164	0.165	0.168	0.172	0.000													
10. Tpallida	0.262	0.268	0.242	0.271	0.242	0.247	0.271	0.260	0.234	0.000												
11. Tobunca	0.191	0.203	0.200	0.191	0.176	0.172	0.212	0.197	0.182	0.220	0.000											
12. Tvitiensis	0.166	0.171	0.175	0.179	0.203	0.164	0.223	0.164	0.224	0.259	0.152	0.000										
13. Ttricornuta	0.201	0.199	0.207	0.236	0.247	0.233	0.242	0.237	0.245	0.292	0.259	0.200	0.000									
14. Tanceps	0.232	0.238	0.221	0.200	0.234	0.221	0.243	0.238	0.241	0.246	0.203	0.211	0.224	0.000								
15. Tmalloticola	0.225	0.231	0.231	0.231	0.240	0.227	0.252	0.265	0.235	0.294	0.240	0.256	0.219	0.240	0.000							
16. Tpercyae	0.196	0.195	0.183	0.207	0.207	0.207	0.215	0.187	0.212	0.252	0.227	0.199	0.205	0.279	0.248	0.000						
17. Tkuwayamai	0.280	0.278	0.252	0.265	0.271	0.257	0.266	0.270	0.253	0.295	0.311	0.261	0.262	0.274	0.256	0.236	0.000					
18. Tbarrettae	0.226	0.232	0.231	0.257	0.233	0.199	0.245	0.204	0.207	0.275	0.259	0.258	0.249	0.277	0.270	0.227	0.292	0.000				
19. Tremota	0.137	0.141	0.156	0.203	0.179	0.164	0.171	0.167	0.164	0.223	0.199	0.219	0.207	0.207	0.223	0.175	0.297	0.211	0.000			
20. Teugeniae	0.767	0.751	0.909	0.858	0.847	0.889	0.857	0.942	0.933	0.961	0.887	0.909	0.925	0.922	0.910	0.949	1.050	1.037	0.848	0.000		
21. Tincrustata	0.752	0.751	0.795	0.839	0.839	0.842	0.829	0.892	0.834	0.979	0.871	0.821	0.862	0.873	0.823	0.899	0.944	0.923	0.865	0.245	0.000	
22. Tgrallata	0.802	0.793	0.847	0.897	0.887	0.866	0.877	0.951	0.917	0.982	0.838	0.857	0.944	0.877	0.897	0.982	0.995	0.960	0.917	0.257	0.248	0.000

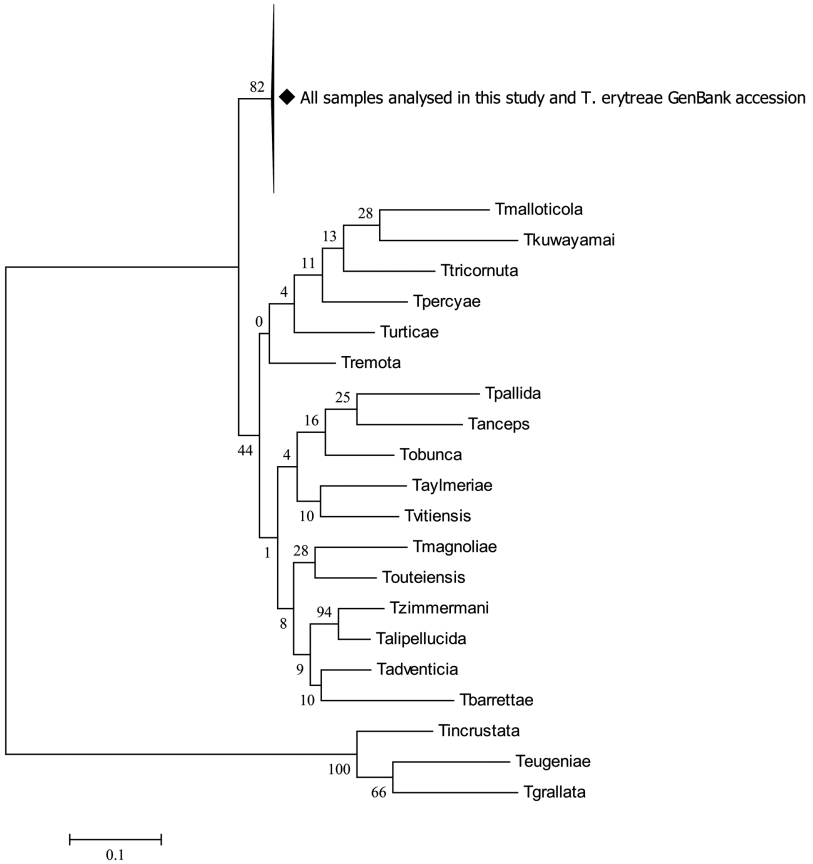

Fig. 3. Condensed maximum likelihood tree showing evolutionary relationships between *T. erytreae* samples from the study inferred by MEGA 7 (Kumar et al. 2016).

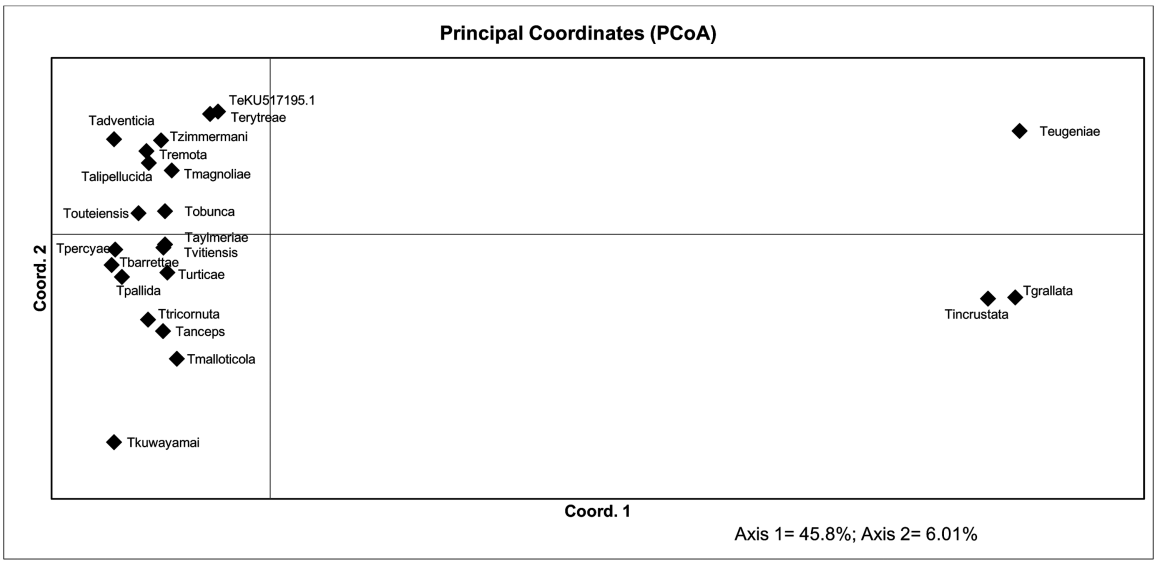

Fig. 4. Plots of the principal component analysis (PCA) for the *T. erytreae* samples and other *Trioza* species calculated using GenAlEx.

Interestingly, only one barcode accession reference could be found for *T. erytreae* in the GenBank which was deposited recently (28 October 2016) for which our samples were successfully linked to. Our study further adds the first reference barcode library of 73 *T. erytreae* sequences from Kenya, Tanzania, and South Africa that have been uploaded to BOLD and GenBank to bridge the knowledge gap in identification and management of this important citrus pest.

In conclusion, this study has shown that DNA barcoding based on the 5′ end of the mitochondrial COI gene is a suitable tool for

identification of *T. erytreae* attacking citrus and other alternative host plants. Moreover, the study provides the first comprehensive reference barcode library for this pest in Africa. With the availability of molecular tools like DNA barcodes, it is anticipated that rapid and accurate identification of ACT could be facilitated, consequently aiding its monitoring, detection, and successful management. Indeed, effective control of *T. erytreae* should in turn contribute to improved management of ACG in citrus, for which the pest is the primary vector. Hence our findings are not only useful for countries in which the pest has already established but also would enable countries that are at risk of invasion by ACT to strengthen their phytosanitary and quarantine measures. With the availability of the barcode reference library, throughput characterization of the pest should be enhanced.

Data Accessibility

DNA sequences were submitted to the Barcode of Life Database (BOLD) (TEBP001-16 – TEBP073-16) and deposited to GenBank (Accession numbers KY754584 to KY754656).

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