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Abundance and identity of red spider mite species on Brachiaria grass in Kenya and its worldwide comparative phylogeny

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Abstract

Brachiaria grass in Kenya offers a vigorous and nutrient rich-forage option. Invasion of introduced Brachiaria grass in Kenya by red spider mite (RSM) indicates considerable damage during the dry season. A survey on the level of mite infestation and identification on various Brachiaria grass cultivars was carried out in August and October in 2016 in coastal lowlands, eastern, central, and western regions of Kenya. DNA-based identification was carried out using universal primers of internal transcribed spacer (ITS2) and cytochrome oxidase I (COI) gene regions. The abundance and damage by RSM were severe in eastern and western areas of the country with cvs. Marandu and Mulato II showing the highest level of infestation. BLAST results from the NCBI database revealed the red spider mite species damaging Brachiaria grass to be *Tetranychus urticae* (Koch.). The countrywide sequence samples did not show a much intraspecific genetic difference on COI region from the local populations but a marked wide phylogeny difference from the similar ITS2 region. The large divergence difference (> 0.10) calls for a repeat of the work in future to ascertain species delineation.

Keywords: Brachiaria grass, Red spider mite, Identity, Genetic diversity

Introduction

Agropastoral systems in Kenya have limited livestock feed sources of nutritional quality [1]. The newly introduced Brachiaria grass in Kenya is reported to provide high-quality forage for increased milk production [2]. During the last four years, Brachiaria grass varieties sourced from South America and introduced in Kenya were devastated by unknown red spider mite (RSM) species during the dry season lasting between two to four months. The RSM species could only be assumed to be any of the red species of the polyphagous species of genus *Oligonychus* or *Tetranychus*. In order to develop control measures or management options, it was important to identify the mite species.

Several species of RSM of Tetranychidae family are of major economic importance on a different field and greenhouse crops worldwide [3, 4]. These mites cause major damage in horticulture, industrial crops such as tea and coffee, pulses like beans, and vegetable crops of Solanaceae family [5, 6]. In horticultural production systems, use of bio-control agents has been reported but chemical control is also common [7, 8, 9]. Mite control on ornamental and flower industrial crops, both in open fields and greenhouses, take the direction of chemical control due to the market requirement of cosmetic appearance of the crop product. Conversely, classical biological control of cassava green mites was rated successful due to the presence of host-specific predatory mites and socio-economic factors of the growers in Africa [10, 11]. These control methods are readily considered in relation to the crop product and production systems.

Spider mite species can be misidentified when only morphological identity is considered [12, 13]. Nevertheless, new emerging molecular techniques have been tested and could be reliably used to identify the species where morphological expertise is not available. On morphology, an

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experienced taxonomy expert is required to correctly identify these pest mites after tedious slide mounts of the specimens [4, 13, 14].

Recently, various DNA markers have been developed for the diagnostic identification of the Tetranychidae species [12]. The two main genomic DNA regions recently targeted to identify *Tetranychus* species from other taxa of red spider mite species are the internal transcribed spacer (ITS2) rRNA gene region of the long fragments of 714-817 bp and cytochrome oxidase I (COI) of the mitochondria of 900bp [12]. Cryptic species among both *Tetranychus* and *Oligonychus* genera which bear the red appearance exist; thus the aim of the current work was to elucidate the true identity of the red mite species attacking Brachiaria grass in Kenya using molecular tools and explore infestation levels on cultivars and site regions.

Materials and Methods

Mite survey and sample collection

The survey on mite abundance on Brachiaria grass was carried out in seven sites located in different agroecological zones of Kenya (Table 1). These sites were Nairobi (Uthiru), Katumani, and Ithookwe in eastern; Eldoret, Kitale, and Alupe in western; Ol Joro Orok in central highlands; and Mtwapa in the coastal lowlands. The red spider mite species diversity was imposed on an agronomy experiment on the evaluation of the performance of Brachiaria grass cultivars in the different regions. The grass cultivars *B. brizantha* cv. MG4, *B. brizantha* cv Marandu, *B. brizantha* cv. Xaraes, *B. ducembens* cv. Basilisk, *B. hybrid* cv. Mulato II, *B. brizantha* cv. Piata, and *B. hybrid* cv. Cayman were established in plots of 5 x 4 m in 4 replications. Mite counts were taken from four inner rows of the eight rows per plot. Spider mite count samples were taken from 10 randomly selected leaves per plant of the net plot targeting a single spot population (Fig. 1). In each grass variety, a single colony was identified and with the aid of a pouter device individual mites were collected from four replicates and preserved in 100% alcohol vials for later molecular analysis. All plant species within the vicinity of the Brachiaria plots were also sampled for the presence of mites.

DNA extraction

Four single female mites from the collected leaf sample were randomly used for DNA extraction according to the protocol by Matsuda et al. [12]. The DNA was extracted using Qiagen Kit (DNeasy Blood & Tissue Kit) where single mites were crushed in 1.5 ml centrifuge tubes and homogenized with Buffer ATL (100 µl). Then Proteinase K-enzyme (10 µl) was added and the homogenate was incubated for about one hour in 56.3°C hot-water bath. Addition of Buffer AL (100 µl) and alcohol (100 µl) with sample DNA cleaning was carried out as per Qiagen protocol. Final elution of 50 µl/g DNA was acquired for the PCR optimization process on the selected ITS2 (rDNA) and COI (mtDNA) gene regions.

PCR amplification

The primers used for the selected regions of rDNA and mtDNA are shown in Table 2. The optimum amplification of the ITS region was achieved at 94°C for 3 minutes (initial denaturation) followed by 35 cycles of further denaturation at 94°C (45 seconds), annealing at 52.5°C (1 minute), and extension at 72°C (1 minute). A further extension of 10 minutes at 72°C was allowed on the master cycler for final elongation. Similarly, the COI region's optimization was carried out at 94°C for 3 minutes (initial denaturation) followed by 35 cycles of further denaturation at 94°C (1 minute), annealing at 56.9°C for 1 minute, and extension at 72°C for 1 minute. A further extension of 10 minutes at 72°C was carried out for final elongation. The PCR products were separated in 1.5% agarose gel and purified with GeneJet PCR Purification kit (Thermo-Scientific) of Bioneer Co. Later, the elution was done with pure water to 40 µl and the products were stored at -20°C for the next step of sequencing.

DNA sequencing and nucleotide alignment

22 PCR products of both ITS2 and COI were successfully sequenced by the use of the Bioneer-3730xl sequencer. The forward and reverse sequences of both ITS and COI regions

Table 1: Brachiaria sample sites and their respective agroclimatic conditions.

Site	Altitude (m asl)	Mean temp range (◦C)	Annual rainfall (mm)	AEZ description
Nairobi (Uthiru)	1882	17-21	1200-1600	Cool-wet
Katumani	1600	16-20	600-800	Warm-dry
Ithookwe	1160	22-24	800-1200	Warm-wet
Eldoret	2153	16-18	1200-1600	Cool-wet
Kitale	1894	18-20	1200-1600	Cool-wet
Alupe	1189	22-24	1600-2000	Warm-wet
Ol Joro Orok	2393	14-16	1200-1600	Cold-wet

were trimmed and assembled by the use of the CLC Main Workbench (Version 7.8) software. BLASTs of nucleotide sequences were carried out on the NCBI database to determine the species identity by match percentage levels. Later, the MEGA6 software was used to align by ClustalW for pairwise and multiple alignments as well as to calculate diversity distances of sample nucleotide among close related accessions from NCBI databases.

Data analysis

Analysis of variance (ANOVA) was carried out to compare mite density per leaf from the different sites by using Statistical Analysis Software (SAS). Maximum Likelihood statistical method of model Tamura-Nei was selected for the analysis of trimmed and assembled nucleotides. Two outgroup taxa of *Oligonychus coffeae* from Japan were selected for nucleotide alignment and phylogeny tree construction on both COI (AB683671.1) and ITS2 (AB683734.1) gene regions alongside other samples. Bootstrap values based on 1,000 replications were calculated using the MEGA6 software to show specific nucleotide distances.

Results

Mite infestation levels

The level of infestation of Brachiaria by red spider mite infestations was significantly ($P < 0.05$) different among sites on specific cultivars (Table 3). There were no mite infestations at Ol Joro Orok and Mtwapa sites. However, some red spider mites were observed on cassava plants bordering the Brachiaria grass plots at Mtwapa. The seven Brachiaria cultivars - MG4, Marandu, Xaraes, Basilisk, Mulato II, Piata, and Cayman showed varied infestation levels of RSM at the different sites. Katumani, Ithookwe, and Kitale had the highest mite infestation levels. Mite grass

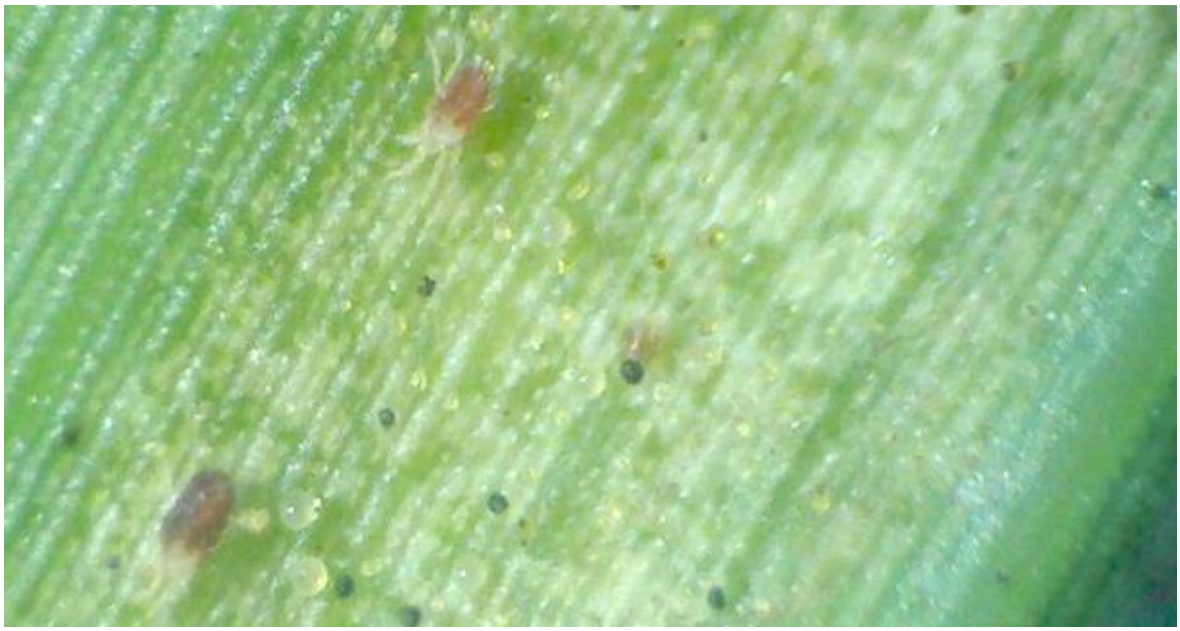

Fig. 1: Red spider mite colony (Mag.x40) on Brachiaria grass.

Table 2: Primers used for the polymerase chain reaction (PCR) amplification of internal transcribed spacer 2 (ITS2) and the cytochrome c oxidase subunit I (COI) regions.

Primer name		Sequence (5' to 3')	Reference
ITS2 region			
rDO2	Forward primer	GTCGTAACAAGGTTTCCGTA	[15]
HC2	Reverse primer	ATATGCTTAAGTTCAGCGGG	[16]
COI region			
CI-J-1718	Forward primer	GGAGGATTTGGAAATTGATT	[17]
COI REVA	Reverse primer	GATAAAACGTAATGAAAATG	[18]

cultivar preference was significantly ($P < 0.0001$) highest on Marandu and Mulato II among the sites. Sites of Alupe, Kitale, and Eldoret had lowest mite densities on the cultivars of Marandu, Mulato II, and Basilisk at < 13 mites per leaf. The cv. Piata incidentally had lowest infestation levels across all the sites.

Red spider mite identity

Nucleotide lengths of ITS2 and COI were between 850 and 1060 bp which were subjected to NCBI BLASTs. Nucleotide similarity matches of 88-90% were scored on COI region indicating the mite species to be *Tetranychus urticae* (Koch.) with divergence distance levels ranging from 0.105 to 0.136 from the other world site populations (Table 4). Using the NCBI-worldwide database, the genetic population based on the nearest COI region was found to be in Tunisia with 0.105 divergence distance. On the other hand, ITS2 region showed higher intra-country population nucleotide divergence unlike the highly conserved COI gene region. ITS2 region divergence ranged from 0 to 0.046 among the sites of Kitui, Uthiru (Nairobi), Alupe, and Mtwapa. The red spider mite sample on cassava at Mtwapa in coastal Kenya was also identified as *T. urticae* and displayed closely related genetic relationship to another in-country site of Kitui. The closest nucleotide genetic similarity of Kenya's *T. urticae* was with that of China, Spain, and France with the divergence levels of 0.111, 0.182, and 0.184, respectively.

Table 3: Mite density on Brachiaria grass cultivars in Kenya site regions in 2016.

No. mites per grass leaf at the sites								
Site	MG4	Marandu	Xaraes	Basilisk	Mulato II	Piata	Cayman	*P-value*
Katumani	-	24.0a^{A}	11.0a^{C}	15.0a^{B}	21.0a^{A}	4.0b^{D}	11.0a^{C}	< 0.0001
Ithookwe	8.0a^{E}	18.0b^{B}	9.0b^{D}	16.3a^{C}	19.5a^{A}	7.0a^{F}	0b^{G}	< 0.0001
Eldoret	3.0bc^{D}	5.0d^{C}	2.0d^{E}	8.8b^{A}	7.0c^{B}	2.0cd^{E}	-	< 0.0001
Kitale	2.0cd^{C}	12.0c^{A}	7.0c^{B}	7.5b^{B}	11.0b^{A}	3.0bc^{C}	-	< 0.0001
Alupe	3.8b^{BC}	6.0d^{AB}	8.0bc^{A}	5.3b^{ABC}	8.0b^{A}	3.0bc^{C}	-	0.0046
Ol Joro Orok	0d	0e	0d	0c	0d	0d	0b	-
Mtwapa	0d	0e	0d	0c	0d	0d	0b	-
df	6, 26	6, 27	6, 27	6, 27	6, 27	6, 27	3, 15	
F-value	19.8	58.6	64.1	23.3	35.5	35.5	66.7	
P-Value	< 0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Table 4: Pairwise distances of mitochondrial cytochrome oxidase subunit I (COI) and internal transcribed spacer 2 among other nearest nucleotide NCBI databases of *Tetranychus urticae* (Koch) of worldwide scope.

Region COI	**Sequence code**	MG4-Ke	Basil-Ke	Mara-Ke	Xara-Ke	Bz	Tun	Spn	Jpn	Mxc	Iran	.Frc
	MG4-Ke	-	0	0	0	0.133	0.105	0.136	0.125	0.125	0.122	0.129
	Basil-Ke	0	-	0	0	0.133	0.105	0.136	0.125	0.125	0.122	0.129
	Mara-Ke	0	0	-	0	0.133	0.105	0.136	0.125	0.125	0.122	0.129
	Zara-Ke	0	0	0	-	0.133	0.105	0.136	0.125	0.125	0.122	0.129
	Bz	0.133	0.133	0.133	0.133	-	0.047	0.008	0.056	0.029	0.014	0.008
	Tun	0.105	0.105	0.105	0.105	0.046	-	0.049	0.029	0.029	0.023	0.047
	Spn	0.136	0.136	0.136	0.136	0.003	0.049	-	0.059	0.059	0.059	0.008
	Jpn	0.125	0.125	0.125	0.125	0.056	0.029	0.059	-	0.029	0.029	0.056
	Mxc	0.125	0.125	0.125	0.125	0.029	0.009	0.013	0.056	-	0.009	0.013
	Iran	0.122	0.122	0.122	0.122	0.013	0.023	0.049	0.029	0.029	-	0.013
	Frc	0.129	0.129	0.129	0.129	0.008	0.049	0.056	0.056	0.056	0.056	-
Region ITS	**Sequence code**	Xara-KT-Ke	Mul II-KM-Ke	MG4-UT-Ke	Mara-KT-Ke	Cobra-KT-Ke	Cass-MP-Ke	Piat-Al-Ke	Bz	Spn	Jpn	Frc
	Xara-KT	-	0.005	0.000	0.046	0.000	0.005	0.005	0.189	0.189	0.189	0.189
	Mul II-KM	0.003	-	0.003	0.046	0.000	0.000	0.008	0.185	0.182	0.185	0.184
	MG4-UT	0.000	0.003	-	0.046	0.000	0.005	0.005	0.189	0.187	0.189	0.189
	Mara-KT	0.046	0.043	0.046	-	0.046	0.044	0.048	0.232	0.232	0.232	0.232
	Cobra-KT	0.000	0.003	0.000	0.000	-	0.005	0.005	0.189	0.189	0.189	0.189
	Cass-MP	0.005	0.002	0.005	0.044	0.005	-	0.008	0.187	0.187	0.187	0.186
	Piat-Al	0.005	0.008	0.005	0.005	0.005	0.009	-	0.191	0.189	0.191	0.187
	Bz	0.189	0.185	0.189	0.232	0.189	0.187	0.191	-	0.002	0.011	0.002
	Spn	0.189	0.182	0.187	0.232	0.189	0.187	0.189	0.003	-	0.014	0.005
	Jpn	0.189	0.185	0.189	0.232	0.189	0.187	0.191	0.003	0.014	-	0.009
	Frc	0.189	0.184	0.189	0.232	0.189	0.186	0.187	0.002	0.005	0.003	-

Comparative nucleotide phylogeny

The COI region showed two clear clades of varied *T. urticae* phylogenetic relationships (Fig. 2). The first clade had sub-clades 1a, 1b, and 1c of the samples from Japan-Mexico-Iran, China, and Kenya, respectively. Another Japan nucleotide appeared to be grouped in the second clade (Clade 2) together with populations from the other countries like Brazil, Spain, France, and the Canary Islands. It was noted that the Kenyan sample nucleotide had no intra-population divergence on the COI nucleotide. The comparative ITS2 region of Kenya and

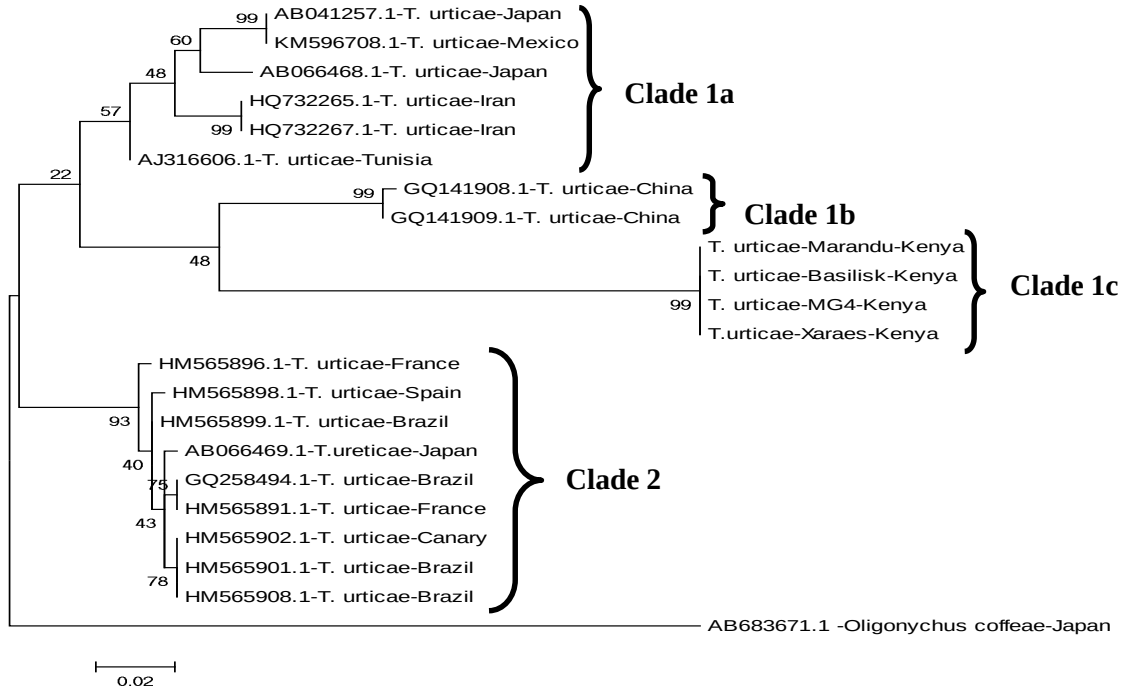

Fig. 2: Maximum Likelihood (ML) tree based on the cytochrome c oxidase subunit I (COI) nucleotide divergences of *Tetranychus urticae* (Koch) on varied Brachiaria grass cultivars in Kenya and genetic differences among related databases from NCBI. Bootstrap values based on 1000 replications are shown at branch nodes.

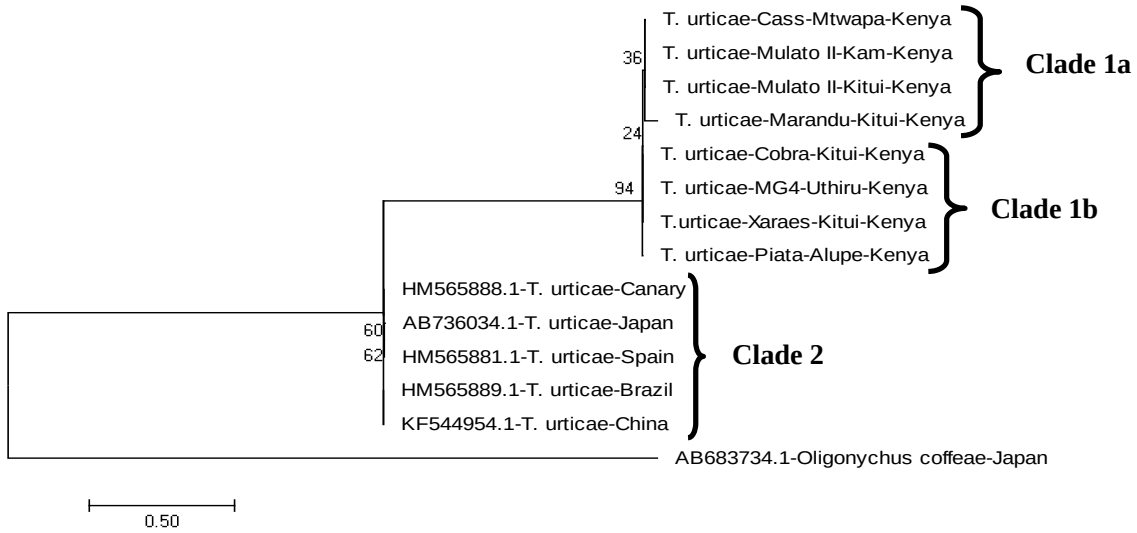

Fig. 3: Maximum Likelihood (ML) tree based on the internal transcribed spacer (ITS2) nucleotide divergences of *Tetranychus urticae* (Koch) on varied Brachiaria grass cultivars in Kenya and genetic distance differences among related databases from NCBI. Bootstrap values based on 1000 replications are shown at branch nodes.

the other world database indicated two distinct phylogenetic clades (Fig. 3). The Kenya population grouping appeared to be a mixture of two genetic materials, though of low genetic divergence of sub-clades 1a and 1b. Mtwapa, Katumani (Kam), and some Kitui populations were clustered together in sub-clade 1a while Kitui, Alupe, and Uthiru (ILRI-BecA, Nairobi Campus field) nucleotides were grouped in sub-clade 1b. The nucleotides from Japan, Spain, China, Canary Islands, and Brazil showed the distinct genetic difference from Kenya populations.

Discussion

Brachiaria grass mite infestation levels at the different sites appeared to be highest where dry spells persist for long similar to the findings on cassava green mite [19]. Highest infestations were observed on grass cultivars Marandu, Mulato II, and Basilisk. Cultivars showing least damage were Piata and MG4. Sites noted for highest damage of brown-broached leaves were Katumani, Kitui, and Alupe corresponding to the high temperature regimes at the sites. The cool wet site of Ol Joro Orok was found to have no mite infestations probably due to low temperature and high precipitation. Likewise, the warm-humid Mtwapa site was completely free of the mite infestation. However, the cassava plants found next to the Brachiaria plots were infested with red spider mites which after analysis was found to be the similar species of *T. urticae* from Kitui and Uthiru (Nairobi). No clear explanation of *T. urticae* preference of cassava leaf tissue in Mtwapa to Brachiaria grass was deducible from the literature in that warm-humid environment.

The BLAST protocol was most reliable for quick results on species identity [20]. The results of the nucleotide molecule analysis showed that the polyphagous *T. urticae* was the mite pest damaging Brachiaria grass in Kenya. The species has diverse crop host and was also identified as the red spider mite on cassava at Mtwapa in the coastal lowlands of Kenya.

Limited inference could be made on the origin of *T. urticae* in Kenya as an invasive species and its relation to other African sequences from the phylogeny tree. The nearest genetic closeness of Kenya's *T. urticae* on ITS2 region from the phylogeny tree was observed to be that of Tunisia, and correctly so being in the same continent. Still the large divergence difference of 0.105 of Kenya sequences to the Tunisia one is not explainable as to being due to biogeographic genetic occurrence since NCBI database has sequences from far distances apart but comparably of low divergence [21, 22]. Similar problems of large divergence were observed where *T. urticae* and *T. turkestani* formed different clades on an ITS tree while the two species were not distinguishable in COI tree [15].

Whether the *T. urticae* species in Kenya could be a different species from earlier analyzed sequences from NCBI is subject to a repeat of similar work in future. Otherwise, other workers have reported fairy huge nucleotide variation of Tetranychid species from other analyzed sequences; partly due to the presence of reproductive parasites among other factors [23]. To prevent the presence of such contaminants, some treatments have been used to remove such effect which leads to likely misleading divergence [23]. Since we did not use the similar treatment, it is advisable in future for the work to be repeated for comparative study and see if the huge variation from other worldwide sequences is reduced.

Another viewpoint is that the phylogeny analysis results showed that the far distant Brazil nucleotide could be treated as a proof for the difference in the population source in Kenya and South America. Hence, the *T. urticae* species ought to be viewed as being in the country before the introduction of the Brachiaria grass.

The population overlap of *T. urticae* among the world sites, before country quarantine facilities were strengthened, was possible during the product trade of various fresh and green crops. However, there is no indication of this from the constructed phylogeny trees. The countrywide distribution of *T. urticae* populations in different localities of Kitui, Katumani, Alupe, Uthiru (Nairobi), and Mtwapa did not reflect much nucleotide genetic difference even on the more robust ITS region. This could be explained by the fact that the pest mite is polyphagous on both wild and cultivated crops [23, 24]. It could also be hypothesized that dispersal of the mite by various activities from one area to another is highly variable as people move across with green plant materials for consumption or planting. This could not have been accounted for by the quarantine regulations at national and international level due to the conspicuous way of farmers to exchange planting materials from one region of the country to another. Considering *T. urticae* biological potential for fast colonization of plant species and the optimum conditions in existence in places where Brachiaria grass is grown, high damage levels will continue to be experienced in specific warm localities in Kenya. An approach of suppressing mite damage would encompass the manipulation of pasture production field conditions and an integration of the biological and cultural techniques as applied in cassava [19, 25].

Conclusion

The present work of sample sequencing, conducting nucleotide BLASTs on NCBI database, and identifying the mite species was carried out in less than a week time. This presented an ideal faster approach to identify the pest species with little difficulties to the untrained morphological taxonomist of spider mite species. In the near future, we plan to repeat the present work using the red spider mites sourced from Brachiaria grass in similar sites and free of infection of

intracellular bacteria of the genera *Wolbachia* (alpha Proteobacteria) and *Cardinium* (Sphingobacteria), which can cause reproductive abnormalities or DNA contamination during extraction [18]. Probably inclusion of more diverse plant species infested with red spider mites will be a plus to unravel further mite diversity in Kenya.

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Conflict of Interest

None of the authors has any conflict of interest to declare.

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