Diversity of fall armyworm, *Spodoptera frugiperda* and their gut bacterial community in Kenya

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Abstract

The invasive fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) is a polyphagous pest that causes widespread damage particularly to maize and sorghum in Africa. The microbiome associated with *S. frugiperda* could play a role in their success and adaptability. However, these bacterial communities remain poorly studied, especially for *S. frugiperda* in Africa. We investigated the composition, abundance and diversity of microbiomes associated with larval and adult specimens of *S. frugiperda* collected from four maize growing regions in Kenya through high throughput sequencing of bacterial 16S rRNA gene. We identified Proteobacteria and Firmicutes as the most dominant phyla and lesser proportions of Bacteroidetes and Actinobacteria. We also observed differences in bacterial microbiome diversity between larvae and adults that are a likely indication that some prominent larval bacterial groups are lost during metamorphosis. Several bacterial groups were found in both adults and larvae suggesting that they are transmitted across developmental stages. Reads corresponding to several known entomopathogenic bacterial clades as well as the non-bacterial entomopathogen, *Metarhizium rileyi* (Farl.) Kepler, Rehner & Humber (2014), were observed. Mitochondrial DNA haplotyping of the *S. frugiperda* population in Kenya indicated the presence of both 'Rice' and 'Corn' strains, with a higher prevalence of the 'Rice' strain. Insights into the microbiota may ultimately provide alternative avenues for controlling of this pest.

Keywords: Fall armyworm, *Spodoptera frugiperda*, gut bacteria, 16S sequencing, corn strain, rice strain, mtDNA haplotype

Introduction

Invasions by exotic pests can have major detrimental effects on agricultural production and natural resources (Huber et al. 2002). The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is a polyphagous pest that is native to tropical regions of the western hemisphere, where it is known for its ability to cause economic damage to several crop species. In 2016, it was first detected in West Africa (Goergen et al. 2016), and since then this pest has rapidly spread across the continent (Goergen et al. 2016; Tindo et al. 2016; Day et al. 2017; Nagoshi et al. 2017, 2018; Cock et al. 2017; Rwomushana et al. 2018; Uzayisenga et al. 2018; Jacobs et al. 2018). By 2018, *S. frugiperda* was reported

present in all countries in Sub-Saharan Africa except Djibouti and Lesotho (Rwomushana et al. 2018). Furthermore, *S. frugiperda* has now also reached the Asian continent (Deole and Paul 2018; Sisodiya et al. 2018). Maize and other economically important food crops in these regions are extensively damaged by *S. frugiperda* larvae (Day et al. 2017) causing extensive economic losses threatening food security.

There is a lack of information about *S. frugiperda* - host plant interactions and other factors that may be leading to the rapid spread of *S. frugiperda* in the geographic regions that have recently been invaded. Many of the control measures used in the western hemisphere (e.g. transgenic maize, chemical insecticides) might not be readily available and economically viable for subsistence farmers in Africa. Further, the use of highly hazardous pesticides is not considered a sustainable long term control measure for any pest (FAO 2018). In addition, *S. frugiperda* is known to readily develop resistance to most chemical insecticides (e.g. pyrethroids, organophosphates and carbamates) and to transgenic maize that are used in its control (Yu 1991; Jakka et al. 2016; Banerjee et al. 2017; Flagel et al. 2018; Botha et al. 2019). In light of this, there is a great need for alternative, cost-effective control strategies for *S. frugiperda* (FAO 2018). A recent survey in Ethiopia, Kenya and Tanzania indicated that *S. frugiperda* has established interactions with indigenous parasitoid species (Sisay et al. 2018) that could be harnessed for biological control. A study on *S. frugiperda* host plant interactions in East Africa has also suggested a climate adapted push-pull system (Midega et al. 2018) and maize-legume intercropping (Hailu et al., 2018) for management of pests including fall armyworm on maize farms. However, many factors related to *S. frugiperda* rapid spread, host plant interactions, bio-ecology and insect-microbiome interactions in the African region remain poorly understood.

Insect microbiomes can have important consequences for the outcome of insect pest-natural enemies- host plant interactions (Ferrari et al. 2011). Strategies that involve modifying insect microbiomes are currently being evaluated for control and management of pests and vectors of plant diseases (Crotti et al. 2012; Perilla-henao and Casteel 2016; Arora and Douglas 2017; Beck and Vannette 2017). Insect microbiomes play a key role in the adaptation of insect to their environment and are therefore a major and often poorly understood determinant of the host plant and geographic range of insect pests (Su et al. 2013). In general, a greater diversity of microbial symbionts exist within the insect's gut lumen, while few others exist inside cells of the host, or on the cuticle (Douglas 2016). Gut microbial symbionts are known to influence their host's nutrition, usually by promoting digestion and availability of nutrients (Douglas 2009). These symbionts can also modulate the immune response and accessibility of the host to invading organisms, and therefore have direct or indirect effects on host susceptibility to parasites and pathogens (Dillon et al. 2005; Dong et al. 2009; Garcia et al. 2010; Vorburger et al. 2010; Narasimhan et al. 2014; Mclean and Godfray 2015; Vorburger and Rouchet 2016; Ubeda et al. 2017). Previous studies have also identified important roles of bacterial symbionts in the interactions between phytophagous insects and host plants (Frago et al. 2012; Biere and Bennett 2013; Brady and White 2013). In addition, microbial symbionts can break down complex molecules such as insecticides and promote insecticide resistance (Kikuchi et al.

2012; Xia et al. 2018). It is also notable that pathogenic bacteria can reside in host guts, only initiating or facilitating pathogenesis under certain conditions (Wei et al. 2017). Studying the gut microbiome is not only important from the standpoint of understanding mutualistic relationships but also for the potential development of microbial biocontrol agents.

There are an increasing number of studies examining the microbial diversity of lepidopterans. While in some of the assessed species, consistent bacterial communities have been observed in both field and laboratory collected populations as well as in insects reared on different diets (Broderick et al. 2004; Xiang et al. 2006; Pinto-Tomás et al. 2011), other studies reported no host specific resident communities that occurred, regardless of the insect diet (Hammer et al. 2017). It is possible that lepidopterans are less prone to forming robust 'core' microbiomes due to several factors: 1) very high pH in the midgut, 2) low retention time of food, 3) lack of microbe housing structures in the intestinal tract, and 4) continual replacement of the peritrophic matrix (Hammer et al. 2017). Nevertheless, bacterial communities do continually associate with lepidopterans and influence a variety of important host processes (Broderick et al. 2006; Anand et al. 2010; Wang et al. 2017).

Few studies have assessed the *Spodoptera*-associated gut microbiome. In a recent study, the microbial diversity of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) was examined by 16S rDNA sequence profiling (Gao et al. 2018). In *Spodoptera exigua,* the dominant bacterial clades are Proteobacteria and Firmicutes, with the predominant genus in larvae being *Enterococcus*. In *S. frugiperda*, previous studies have isolated several bacterial strains using culture-dependent methods (De Almeida et al. 2017; Acevedo et al. 2017). In this study, we used 16S rDNA sequence profiling to characterize the diversity of bacteria associated with populations of *S. frugiperda* in Kenya and assessed the prevalence of the corn and rice strains in these populations using Mitochondrial COI gene sequences.

Materials and methods

Insect collection

Spodoptera frugiperda larvae were collected from infested maize fields in Kenya between June and December 2017 at the following locations: Ngeria (N00.37024 E035.9862) and Burnt Forest (N00.22505 E035.42479) in Uasin Gishu County; Msamia, Kitale (N00.98009 E034.97170) in Trans Nzoia County; Shimba Hills (S04.33228 E039.34361) in Kwale County and Chala Irrigation Scheme (S03.27338 E037.13816) and Wundanyi (S03.337538 E038.33612) in Taita Taveta County. Part of the field collected insects from each sampled region in Kenya were reared on fresh maize leaves in ventilated cages to pupation and eclosion at 27 °C and 60% humidity, while the rest were stored in absolute ethanol at -20°C.

DNA extraction and 16S rDNA sequencing

Guts from 9 live stage 5-6 larvae and 9 one-day old emerging adults from the Kenya collected samples were dissected separately in phosphate buffered saline (PBS) following surface sterilization and used for DNA extraction. Insects were surface sterilized in 70% ethanol, in 5% v/v sodium hypochlorite solution followed by 3 washes in PBS for 3 minutes in each solution. DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's instructions. DNA extracted from gut samples was submitted for high throughput sequencing targeting the v4 region of the bacterial 16s rRNA gene using the Illumina Miseq platform (Center for Integrated Genomics, University of Lausanne, Switzerland). Sequence reads were checked for quality using FastQC v 0.11.28 (Andrews 2010) and pre-processed to remove adapters and sequencing primers using Cutadapt v1.18 (Martin 2011). Forward and reverse reads were imported into the QIIME2-2018.11 (Boylen et al. 2018). The deblur plugin (Amir et al. 2017) was used to further filter the reads based on per base quality scores, merge the paired-end-reads and cluster reads into operational taxonomic units (OTUs). A total of 457501 sequence reads were retained after removal of spurious reads and all reads shorter than 220 nucleotides in length for further analysis. These sequences clustered into 1796 OTUs. Of these, 197 OTUs survived low count and interquartile range-based variance filtering to eliminate OTUs that could arise from sequencing errors and contamination. Taxonomic assignment was done using the blast classifier against the Silva132 reference database (Quast et al. 2013) at a 99% identity cut-off. OTUs initially characterized as "*Candidatus Hamiltonella*" by comparison to the Silva132 reference database were re-analyzed by homology searches against the NCBI nr nucleotide database through blast (Altschul et al. 1990) and found to be *Pseudomonas*, highlighting a potential incorrect assignment in the reference database. OTU prevalence and variance based filtering as well as alpha and beta diversity measures were applied to the data in the Microbial Analyst Marker Data Profiling (Dhariwal et al. 2017). Shannon diversity indices were applied along with Mann-Whitney and analysis of variance statistics in profiling alpha diversity between sets of samples. Beta diversity was evaluated using Bray-Curtis and unweighted Unifrac distances. Significance testing was done using analysis of group similarities (ANOSIM) and non-metric multidimensional scaling (NMDS) used for ordination. The empirical analysis of digital gene expression data in R (edgeR) algorithm (Robinson et al. 2009) was used to evaluate differential abundance of bacterial genera reads between sample groups. All sequence reads were archived in the Sequence Read Archive (SRA) under the BioProject: PRJNA521837.

mtDNA haplotyping

DNA was extracted from surface-sterilized whole insects using the ISOLATE II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's instructions. Mitochondrial COI gene sequences were amplified from insect DNA by PCR using the primer LCO1490 and HCO2198 (Folmer et al. 1994). Reactions were set up in total volumes of 10 µl each, containing 5× MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline, London, UK), 2 µM of each primer, 0.25 mM MgCl2 (Thermo Fischer Scientific, Massachusetts, USA), 0.125 µl MyTaq DNA polymerase (Bioline, London, UK), and 7.5 ng/µl of DNA template. These reactions were set up in a Master cycler Nexus gradient thermo-

cycler (Thermo Fischer Scientific, Massachusetts, USA) 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 50.6 °C and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. PCR products were run through 1% agarose gel electrophoresis and visualized by ethidium bromide staining and UV trans-illumination. Direct sequencing was done for all host mtCOI gene and the sequences deposited in the GenBank.

Results

We profiled the bacterial microbiome for 18 samples from 4 different locations in Kenya. In addition, samples were collected from these 4 sites plus two additional sites for mtDNA haplotyping (Fig. 1).

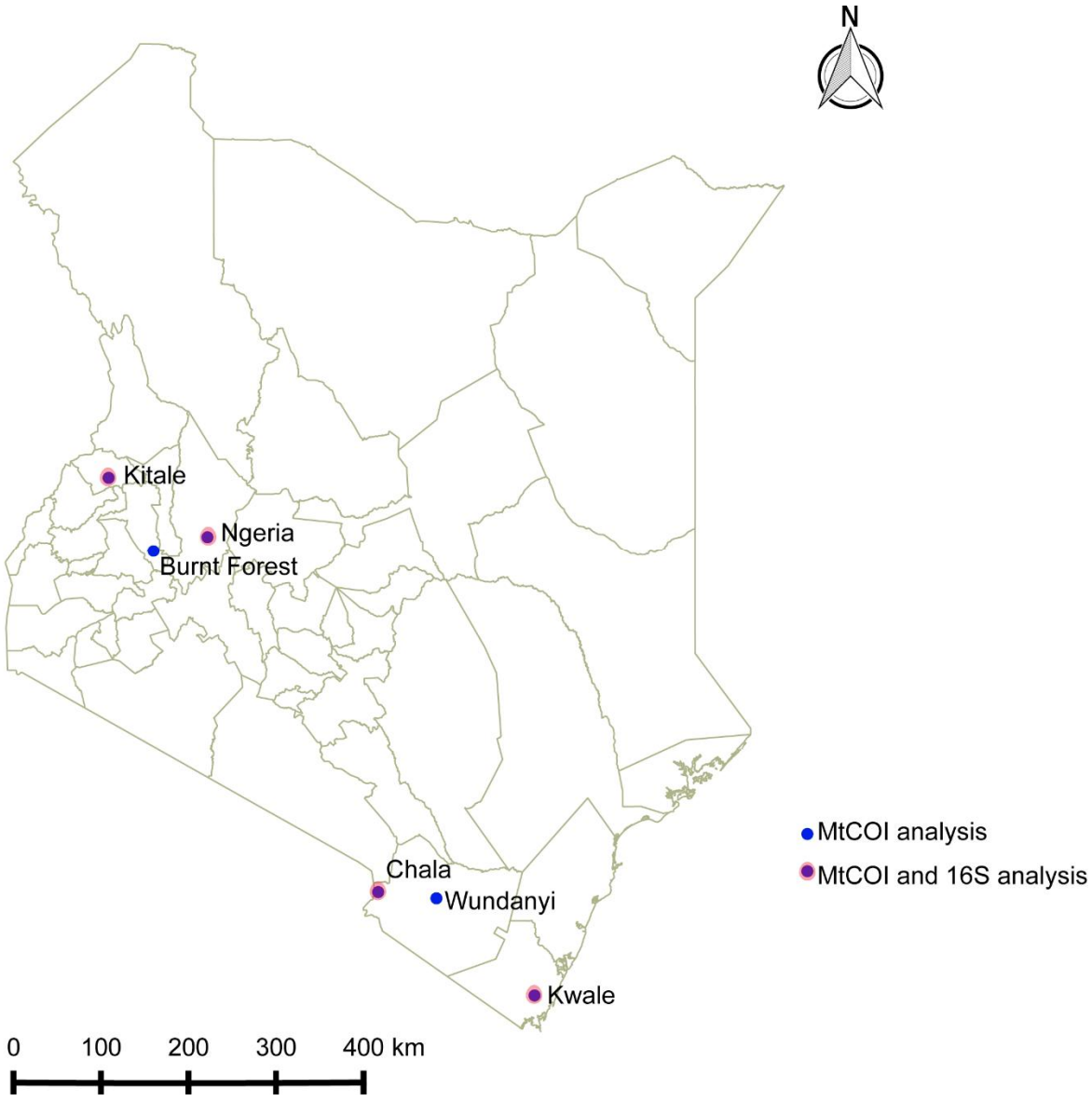
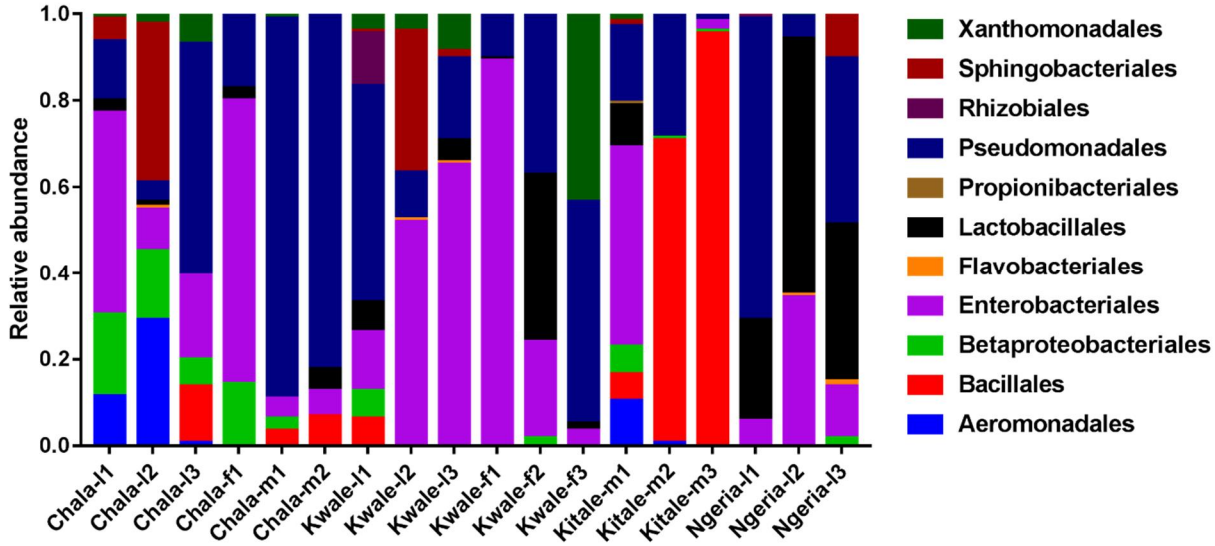


Fig. 1 Sites from which *Spodoptera frugiperda* larvae were collected in Kenya

152 The most abundant bacterial Phyla observed across the fall armyworm gut samples were Proteobacteria,
153 Firmicutes, Bacteroidetes and a small proportion of Actinobacteria (Fig.10, Supplementary material). OTUs
154 clustering in the orders Enterobacteriales and Pseudomonadales were predominant in the majority of the
155 samples (Fig. 2).



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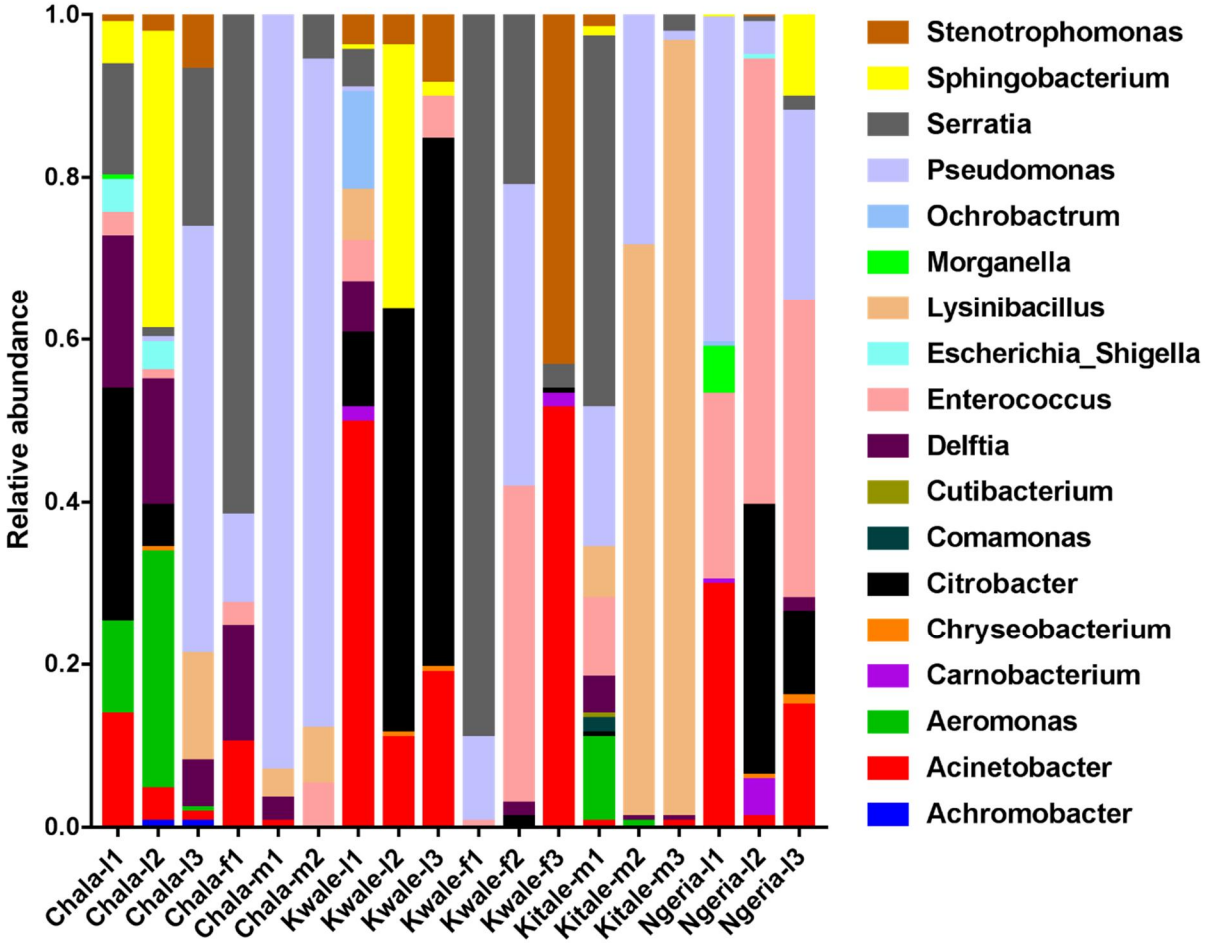
157 **Fig. 2** Composition of bacterial OTUs at Order level in screened *Spodoptera frugiperda* samples

158 We note that despite the high genus-level diversity between samples (Fig. 3), there were some similarities
159 based on developmental stage and location. For example, there was a very high proportion of: 1)
160 *Pseudomonas* in the two adult male samples from Chala, 2) *Citrobacter* in two larval samples from Kwale,
161 3) *Lysinibacillus* in two male samples from Kitale and 4) *Enterococcus* in two larval samples from Ngeria.
162 It was noted that *Stenotrophomonas*, *Sphingobacterium*, *Serratia*, *Pseudomonas*, *Morganella*,
163 *Enterococcus* and *Delftia* were observed in both larvae and adult samples.



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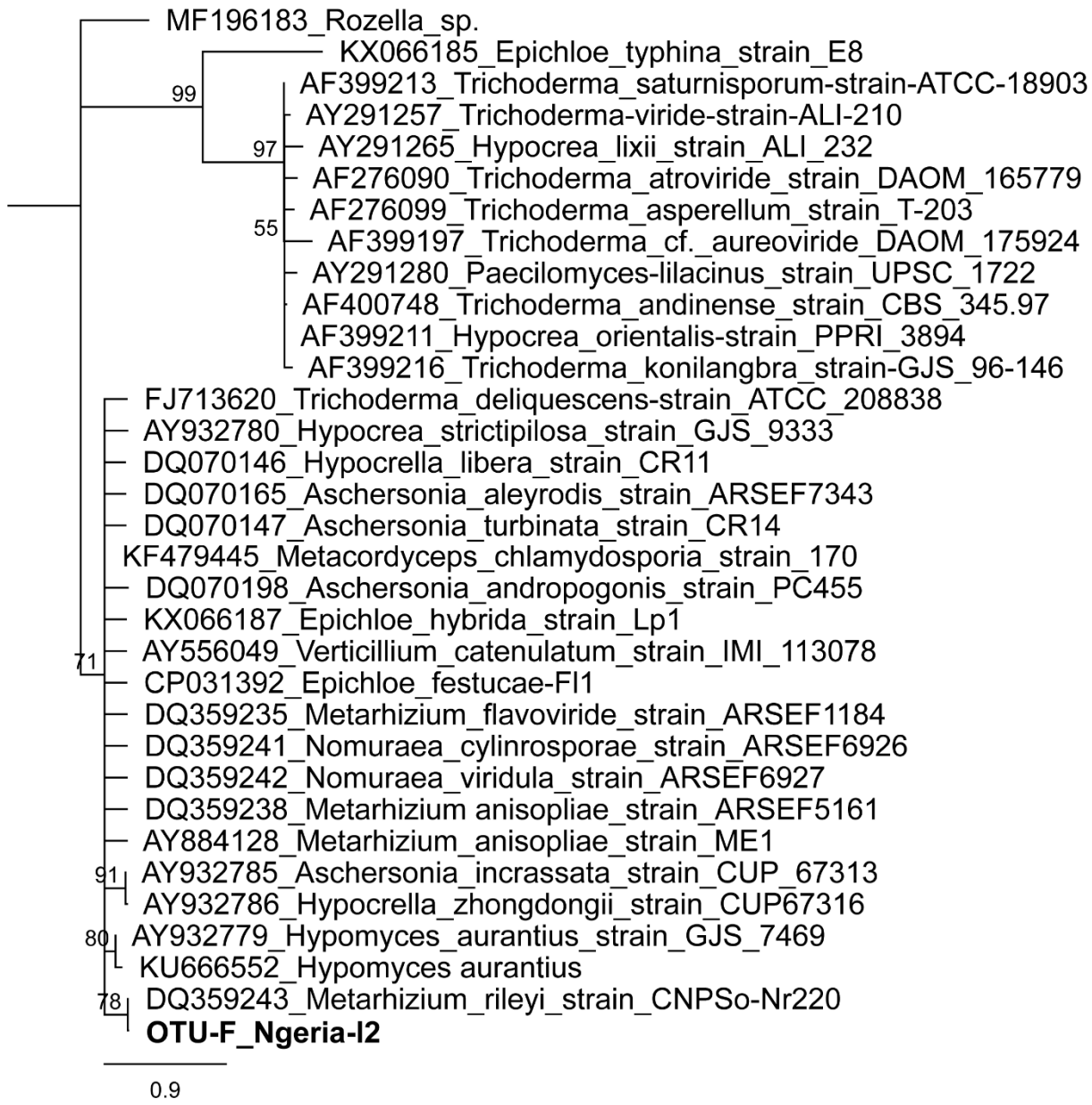
165 **Fig. 3** Genus level composition of (% of OTUs) in the different samples of *S. frugiperda*. The relative
166 abundances of the 19 most abundant genera are represented here

167 In one of the larval samples from the Ngeria site (Ngeria-l2), we observed an excessive number of non-
168 bacterial reads. Upon closer inspection, these were found to be closely related to *Metarhizium rileyi* (Farl.)
169 Kepler, Rehner & Humber (2014) (formerly *Nomuraea rileyi*), an entomopathogenic fungi that is known to
170 infect *S. frugiperda* (Fig. 4).



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172 **Fig. 4** Neighbor-joining tree of fungus OTU detected in *S. frugiperda* sample (Ngeria-l2; in bold) and
173 GenBank accessions of small subunit ribosomal RNA gene sequences from related fungi. Sequences are
174 labelled by their GeneBank Accessions followed by genus, species and strain where available. Bootstrap
175 values are indicated above the branches. Branches with a bootstrap value less than 50 are collapsed. A
176 sequence from a species in the genus *Rozella* is included as an out-group

177 The bacterial OTU richness appeared to be higher in *S. frugiperda* larvae than adults, however this
178 difference was not statistically significant (p-value: 0.062526; [Mann-Whitney] statistic: 19) using Shannon
179 diversity metrics (Fig. 5a). In addition, no significant variation in OTU richness and abundance was
180 observed between larvae from different sampling sites (p-value: 0.32834; [ANOVA] F-value: 1.3486) (Fig.
181 5b).

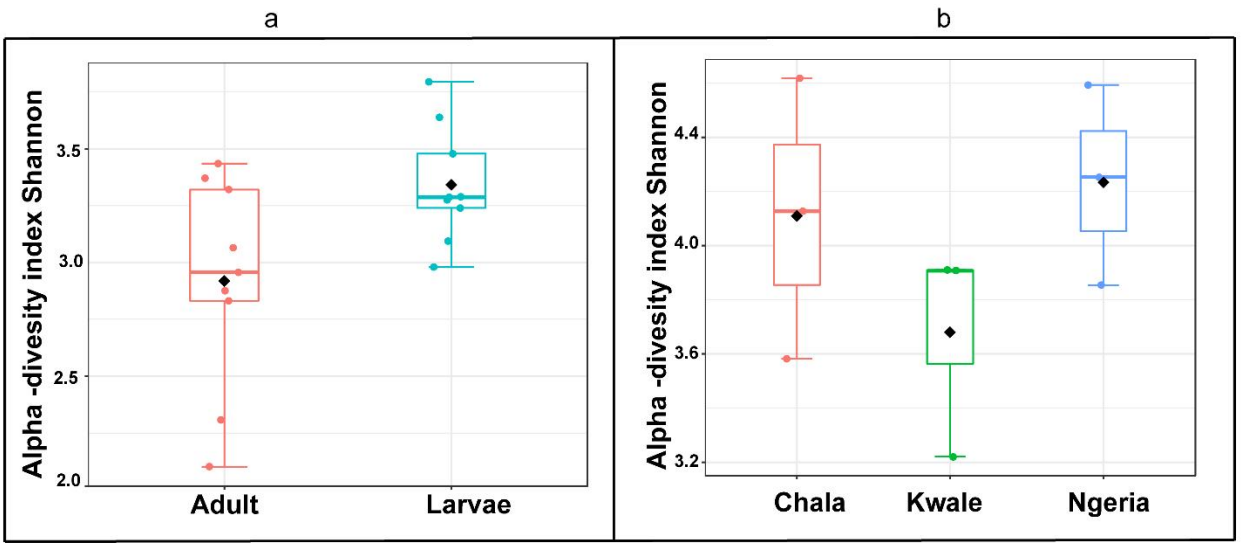
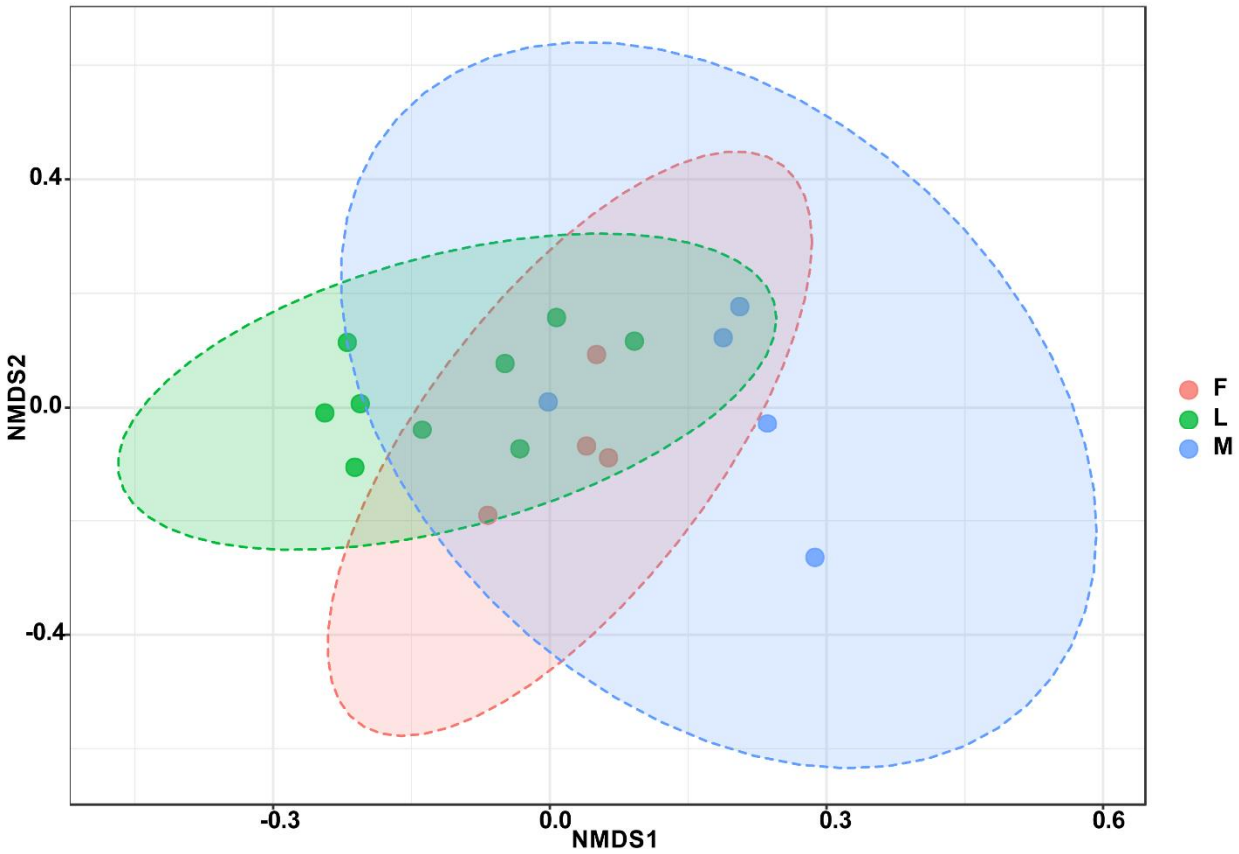


Fig. 5 A comparison of the Shannon diversity indices for: **(a)** adult and larval samples from all sites and **(b)** larvae collected from different sites. The Shannon diversity index (H') was calculated based on the OTU-level of classification. The boxplots show the distribution of H' values across all samples

The composition of bacterial OTUs between larvae and adult *S. frugiperda* was observed to overlap, with no significant dissimilarity ([ANOSIM] R=0.17365; p-value < 0.081 stress=0.14876) (Fig. 6a). However, OTU composition was observed to vary significantly among larval samples from different sites ([ANOSIM] R: 0.45679; p-value < 0.017 stress=0.05711) (Fig. 6b).



190

191 **Fig. 6a** Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities in
192 bacterial communities detected in the *S. frugiperda* samples. Samples are colored according to their
193 developmental stage and sex as indicated on the legend where F=female, L=larvae and M=male

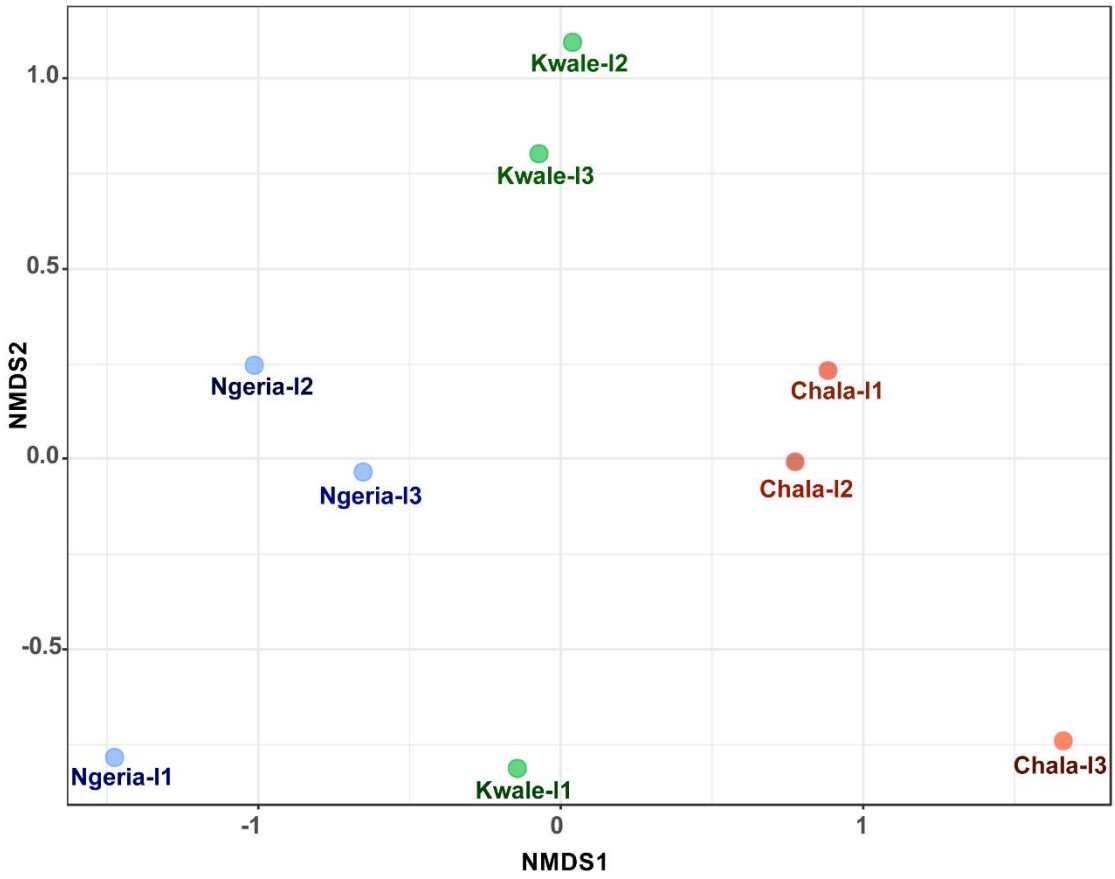


Fig. 6b Non-metric multidimensional scaling (NMDS) ordination based on Weighted Unifrac distance dissimilarities in bacterial OTU composition between larval sample pairs from different sites

A significant differential abundance was observed for 3 bacterial genera between larvae and adult *S. frugiperda* samples using the EdgeR algorithm at an adjusted p-value of 0.05. Two of these: *Citrobacter* (log2FC=4.4178, p value=3.6E-6, FDR=7.218E-5) and *Sphingobacterium* (log2FC=3.625, p value=1.01E-4, FDR=0.0010118) were more abundant in larvae whereas the third: *Lysinibacillus* (log2FC=-3.2247, p value= 4.4E-3, FDR=0.029375) was more abundant in adults (Fig. 7).

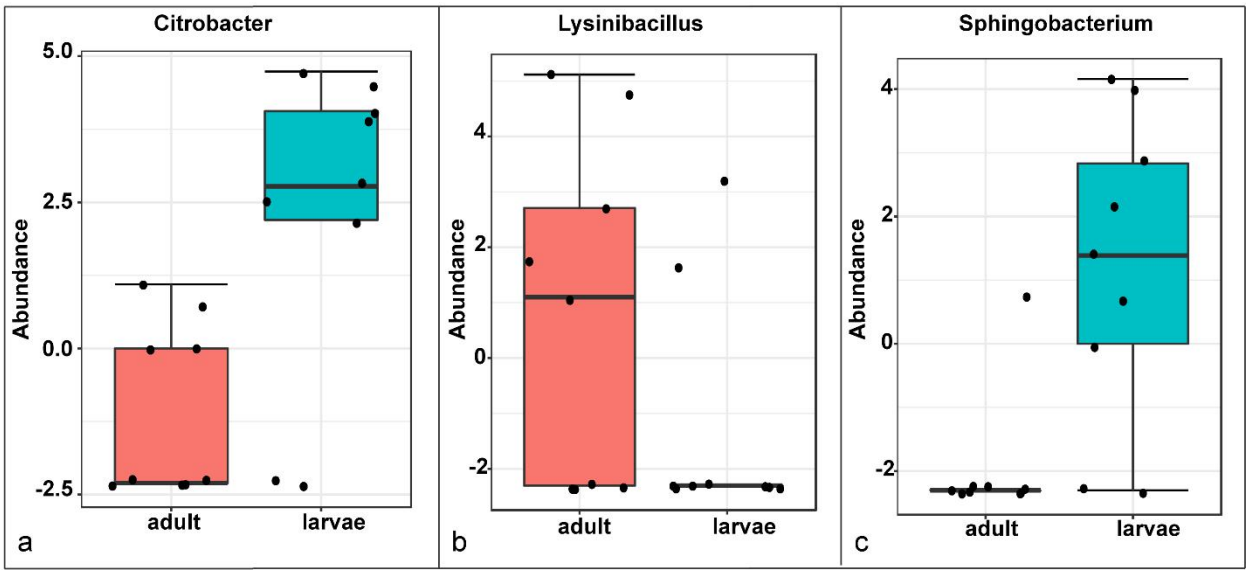


Fig. 7 Comparative abundance of A) *Citrobacter,* B) *Lysinibacillus* and C) *Sphingobacterium* between adults and larvae of *S. frugiperda.* Abundance is shown on a log transformed scale of original counts

Based on mtDNA sequences, the *S. frugiperda* strains detected in this study were identical to strains from Canada, USA and Brazil, as well as strains that were recently reported in Kenya and other parts of Africa and India (Fig. 8).

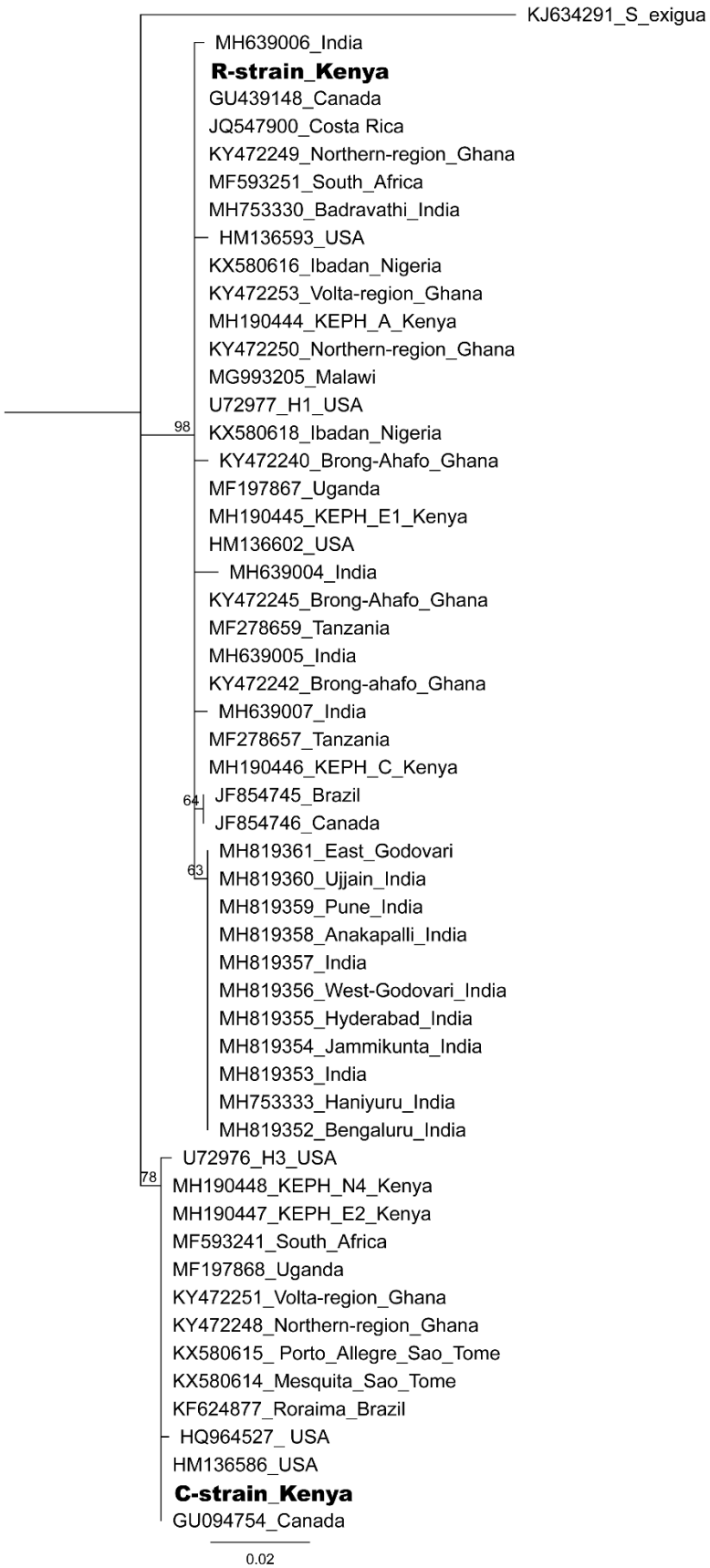


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Fig 8 Neighbor-joining tree based on mtCOI sequences of *S. frugiperda* from the GenBank and representative haplotypes from this study (in bold). Bootstrap values are indicated above branches. Branches with bootstrap values less than 50 are collapsed. A sequence from *Spodoptera exigua is* included as an out-group. Sequences are labelled with their GenBank accession numbers, collection site where available and country of collection

All the samples clustered in two major clades widely referred to as either the 'Rice' or the 'Corn' strain (hereafter referred to as R- strain and C- strain). We investigated the frequency of mtDNA haplotypes of *S. frugiperda* samples collected at several sites in Kenya. Overall, 90% of the samples (n=85) clustered as R-strain, whereas 10% (n=9) clustered as C-strain. Proportions of the R-strain in populations at the different sites were 100% (n=6) for Burnt Forest, 83% (n=6) for Chala, 86% (n=7) for Wundanyi, 82% (n=11) for Kitale, 91% (n=35) for Kwale and 82% (n=17) for Ngeria (Fig. 9).

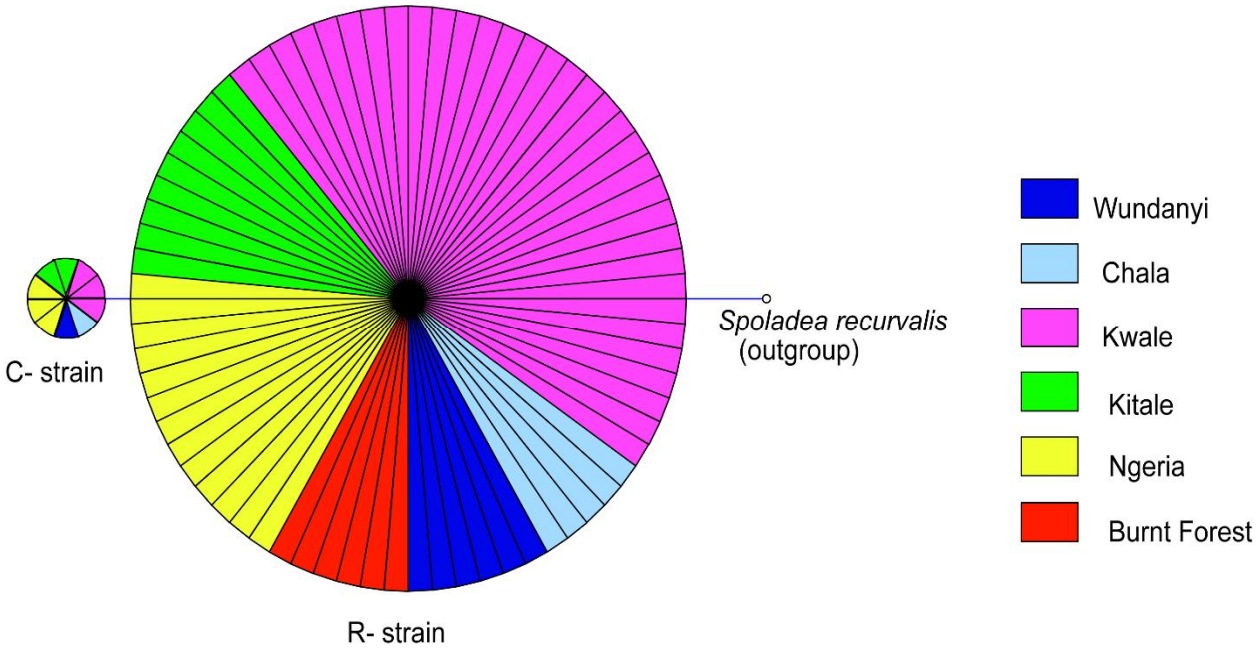

Fig. 9 Mitochondrial COI haplotype map of the *S. frugiperda* samples collected at various sites in Kenya. Node size is proportional to number of samples and individual samples are represented as fractions of the nodes. A sequence of *Spoladea recurvalis* is included as an out-group. Sequences for all samples are accessible from GenBank using the accessions: MK492929-MK493010

Discussion

We found that the gut bacterial communities of most *S. frugiperda* samples were dominated by Proteobacteria. This observation is similar to proportions reported in other phytophagous insects, in particular lepidopterans (Belda et al. 2011; Xia et al. 2013, 2017; Landry et al. 2015; Ramya et al. 2016; Snyman et al. 2016; Strano et al. 2017; Chen et al. 2018). Only three samples, two adult males from Kitale (Kitale-m2 and Kitale-m3) and one larvae from Ngeria (Ngeria-l2) were dominated by Firmicutes. Four genera of bacteria, *Pseudomonas*, *Delftia*, *Enterococcus* and *Serratia* that were recorded in this study have

previously been isolated from *S. frugiperda* (De Almeida et al. 2017; Acevedo et al. 2017). Surprisingly, *Staphylococcus*, *Microbacterium*, *Arthrobacter* and *Leclercia* that were previously isolated from *S. frugiperda* in Brazil (De Almeida et al. 2017) were not found in any of the samples we profiled in Kenya. Similarly, *Pantoea*, *Enterobacter*, *Raoultella* and *Klebsiella* previously identified in oral secretions of *S. frugiperda* in Pennsylvania, USA (Acevedo et al. 2017) were not found in the profiled Kenyan samples.

We observed significant differences in OTU composition between larvae from different sites. This was most likely caused by complex biological and environmental factors in the diverse agro-ecological zones that were sampled. Diet is known to strongly influence the microbiome of lepidopterans (Strano et al. 2017; Sittenfeld et al. 2002; Priya et al. 2012; Montagna et al. 2016), however in this study all samples were collected from maize plants. Hence, the observed compositional differences are not likely to be caused solely by diet. We observed differences in bacterial OTU composition between larvae and adults, however with a relatively low number of samples these differences were not statistically significant. It is interesting that many of the detected bacterial genera such as *Stenotrophomonas*, *Sphingobacterium*, *Serratia*, *Pseudomonas*, *Morganella*, *Enterococcus* and *Delftia* are found in both life stages, which suggests that gut bacterial community members are transmitted across developmental stages. Bacteria that are continually transmitted across developmental stages (and across generations) may evolve a closer, mutualistic relationship with their hosts (Moran 2006). Future studies should investigate the effects of these microbes on host fitness and investigate the extent to which they are vertically transmitted from parents to offspring. In contrast, *Citrobacter* and *Sphingobacterium* were observed to be differentially abundant in larvae than in adults, a likely indicator that these two genera may be part of the fraction of bacterial communities that are lost during transition of *S. frugiperda* into the adult stage. *Lysinibacillus*, on the other hand, was more abundant in adults than in larvae and therefore could have an adult-specific function.

Notably, we identified *Serratia*, *Lysinibacillus* (formerly *Bacillus*) and *Pseudomonas*, species of which have been reported to have entomopathogenic properties (Castagnola and Stock 2014). In addition, one sample had a high number of reads attributed to a relative of a non-bacterial entomopathogen, *Metarhizium rileyi,* which has been previously isolated and tested for efficiency against *S. frugiperda* (Maniania and Fargues 1985; Mallapur et al. 2018). It will be worthwhile to explore the pathogenicity of these microbes for *S. frugiperda* and to determine if they could be incorporated into biological pest management strategies (Ruiu et al. 2015).

Based on the mtCOI gene sequence, we observed two mtDNA haplotypes in Kenya (C- and R- strains). These findings confirm that both haplotypes are present in Kenya, as has been demonstrated for other countries in Africa (Rwomushana et al. 2018). The majority of the *S. frugiperda* samples collected were characterized as R-strain suggesting that this strain is dominant in *S. frugiperda* populations in Kenya. These observations are in agreement with a previous study (Goergen et al. 2016) that observed C- and R-strains appear to have an East-West axis alignment in the African region with the Eastern Africa having

progressively lower frequencies of the mtCOI C-strain (Goergen et al. 2016). We observed that some variants of the rice strain have been reported in other places such as Ghana and India but those were not detected in this study. It is interesting to note that in addition to a similar rice strain as the one detected in Kenya, a variant differing by a single nucleotide polymorphism has been recorded from various locations in India (Fig. 8). This variant has however not been reported in Africa. It is therefore possible that the invasion into India may not have come directly from the African continent, or invasion could have included strains from Africa and elsewhere.

Symbiotic bacteria play a key role in the biology of insects. We characterized the gut bacterial communities in *S. frugiperda* larvae and adult samples collected from several locations in Kenya, finding some important differences and similarities across samples and in relation to other studies on this species (Acevedo et al. 2017; De Almeida et al. 2017). Understanding the gut microbial symbionts of this pest species may facilitate the development of novel, cost-effective control strategies.

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Author contributions

SE, SS, JH and FMK conceived and designed the research, JG conducted experiments and analyzed data, JG and JH wrote the manuscript, JVB and HP contributed materials. All authors read and approved the manuscript

Conflict of Interest

The authors declare that they have no conflict of interest.

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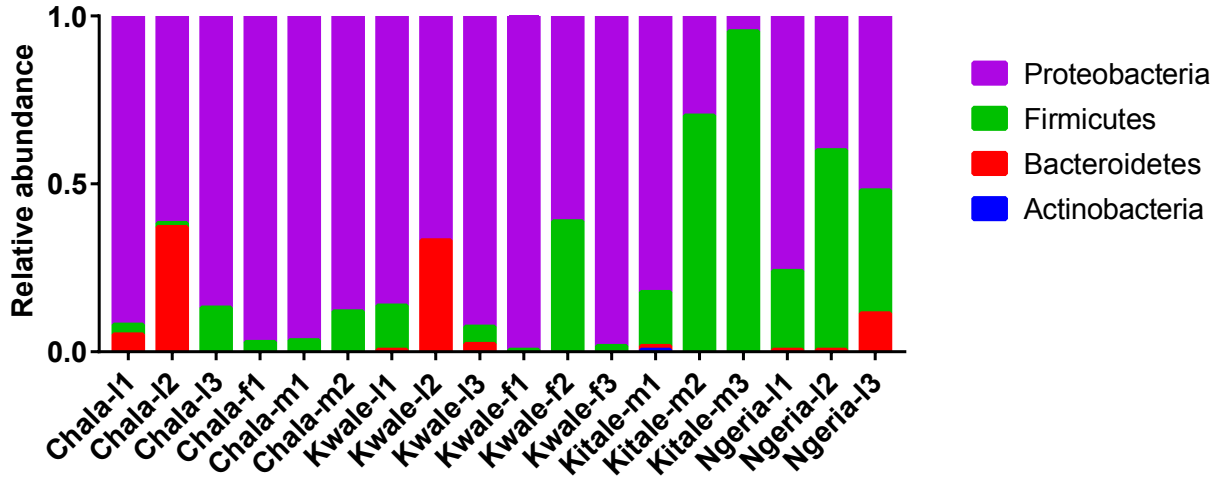
521 **Supplementary material**



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523 Fig. 10 Relative abundance of bacterial phyla detected in *S. frugiperda* samples