# CHARACTERIZATION OF DARK AND PALE FORMS OF *FRANKLINIELLA SCHULTZEI* (TRYBOM) – AN ECOLOGICAL, BIOLOGICAL, MORPHOLOGICAL AND MOLECULAR APPROACH

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# Characterization of dark and pale forms of *frankliniella schultzei* (trybom) – an ecological, biological, morphological and molecular approach

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature..... Date.....

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This thesis has been submitted for examination with our approval as University supervisors.

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## DEDICATION

I would like to dedicate this thesis to God, my family and my supervisors for the intellectual, moral and emotional support they provided for me throughout my masters studies.

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| | |
|---|---|
| **RFLP** | Restriction fragment length polymorphism |
| **ITS** | Internal transcribed spacer |
| **PCR-RFLP** | PCR-restriction fragment length polymorphism |
| **TSWV** | *Tomato spotted wilt virus* |
| **TCSV** | Tomato chlorotic spot virus |
| **GRSV** | Groundnut ringspot virus |
| **CSNV** | Chrysanthemum stem necrosis virus |

## ABSTRACT

*Frankliniella schultzei* Trybom is an important pest of ornamental and vegetable crops worldwide and a vector of Tospoviruses, which are plant viruses belonging to the family Bunyaviridae. It occurs as two color forms (dark and pale) which are morphologically very similar hence has been considered as one species. However, differences in vector competency and geographic spread has led to debate on whether they are one or separate species. Moreover, evidence is lacking on whether these two forms can interbreed to qualify as one species. Hence to ascertain the taxonomic status of the dark and pale forms of *Frankliniella schultzei* laboratory reared dark and pale forms maintained at *icipe* were characterized using ecological, biological, morphological and molecular approaches. Differences in ecological preferences among the colour forms were determined by analyzing the host plants and ecological characteristics of the collection sites. Biological characterization was undertaken by assessing the parthenogenetic reproduction strategies of the two color forms and assess their potential to interbreed between colour forms. The two colour forms were also differentiated through morphometrics of character states. Molecular characterisation was undertaken through Restriction fragment length polymorphism (RFLP) analysis and further sequencing of ITS 2 sequences. The results indicated that ecologically, the dark forms were observed predominantly in the western regions of Kenya, while the pale forms predominated the coastal and eastern regions of Kenya. The two colour forms differed in their mode of parthenogenetic reproduction with the pale forms being theletokous while the dark forms being arrhenotokus. The dark male × pale female cross consistently resulted only in pale females indicating parthenogenesis and absence of interbreeding between the two colour forms. Pale males were absent in the colony and further field samples of *F. schultzei* males in different host plants indicated only presence of dark males in the environment. The two colour forms also differed in their morphological features such as length of third ocellar setae, setae on tergite 10 and distance of the third ocellar setae from tangent. The two colour forms exhibited significant molecular differences, by having consistent sequence differences resulting in their clading separately on a phylogenetic tree, with a sequence divergence of up to 23% between the two colour

forms. The RFLP banding profile of the two colour forms of *F.schultzei* showed the two forms having different banding patterns. Considering the distinct differences between the dark and pale colour forms of *Frankliniella schultzei* for ecological preference, parthenogenetic reproduction, in ability to interbreed, differences in morphological and molecular characteristics, the dark and pale colour forms of *F. schultzei* needs to be considered as two separate species.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

*Frankliniella schultzei* Trybom (Thysanoptera: Thripidae), known as the common blossom thrips (CBT) is one of major thrips pest affecting French beans production in Kenya (Nyasani *et al.*, 2009; Nyasani *et al.*, 2012). Thrips contribute to 63 – 68% yield loss in French beans due to direct feeding (Nyasani *et al.*, 2012) and a further 20% loss from rejection of produce damaged by thrips (Lohr, 1996). The common blossom thrips is widely distributed globally and cause economic losses to various crop pests (Kakkar *et al.*, 2014). They cause direct damage during feeding and oviposition and indirect damage as a vector of Tospoviruses (Sakimura, 1969). Thrips causes an annual loss of over 1 billion $ worldwide just from vectoring Tospoviruses (Prins & Goldbach, 1998).

*Frankliniella schultzei* occurs in two colour forms; dark and pale. The dark form is mainly found south of the Equator, while the pale form is mainly found north of the Equator (Sakimura, 1969). However,the two forms of CBT coexist in the same habitat with overlapping host ranges in India, Philippines, New Guinea, Northern Australia and East Africa (Mound, 1968; Sakimura, 1969). Overlapping habitats, host ranges and very similar morphological features apart from the differences in colour, suggests their grouping as one species. Hence, the pale form which was originally described as *Frankliniella sulphurea* (Schmutz, 1913) has been synonymised with *F. schultzei* (Mound, 1968).

Most thrips species reproduce both sexually (through mating) and asexually (through virgin females laying viable eggs without mating, a process called parthenogenesis) (Moritz, 1997). Although mixed colonies of both dark and pale colour forms have been reported on some host plants (Mound, 1968), no official reports on the possibility of interbreeding between the two forms are available and differences

between the two forms are not well established at morphological and molecular levels.

The colour forms are known to differ in their vector competence with the dark form considered to be a good vector of at least four Tospoviruses, whereas the pale form is a weak vector of only two (Sakimura, 1969; Cho *et al.*, 1988; Wijkamp *et al.*, 1995; Nagata & de Ávila, 2000). On the contrary, the pale forms are good vectors of *Maize chlorotic mottle virus* as compared to the dark forms (Nyasani *et al.*, 2015).

Therefore, this study aims to characterize the dark and pale colour forms of *F. schultzei* through combined assessment of morphological, molecular, biological and ecological differences This study emphasizes the importance of 'integrative taxonomy' in accurate species identification and nomenclature (Dayrat, 2005), which is very critical in quarantine pest diagnostic and management (Garza *et al.*, 2005).

### 1.2 Statement of the problem

Females of both dark and pale forms of *F. schultzei* are morphologically similar except for their body colour, and length of the III ocellar setae. Further they are observed in the similar crop ecologies and hence taxonomically classified as one species (Mound, 1968). Further, it has been postulated that in the crops where both the colour forms are often found, specimens with intermediary character states have been observed infrequently indicating the possibility of interbreeding (Subramanian *et al.*, 2012). Therefore, the question on whether they are the same species or different can only conclusively be answered through evaluation of their interbreeding potential, as well as their morphological and molecular differences which has not been done.

Hence this study aims to clearly distinguish and characterise the dark and pale forms of *Frankliniella schultzei* using morphological and molecular techniques and further using the same to reliably evaluate interbreeding potential and answer the question on their species status.

### 1.3 Justification of the study

Using previous literature, the two forms of *F. schultzei* are taxonomically considered as one species. Even though the pale type is often treated as the species *F. sulphurea*, this name is listed as a synonym, meaning they are still considered as one species (Wang *et al.*, 2010). However, using molecular identification methods, *F. schultzei* insects are seen to produce different banding patterns (Moritz *et al.*, 2004). This raises the need for accurate distinction and classification between the two forms. The occurrence of intermediary forms has been postulated (Subramanian *et al.*, 2012), but this has not been proven. Hence there is the need to observe and evaluate the occurrence of interbreeding between the two forms.

Eventually, accurate identification of *F. schultzei* is important in its management (Garza *et al.*, 2005) as reliable identification is crucial in applying early control measures (Toda & Komazaki, 2002).

### 1.4 Hypothesis

Dark and pale forms of *F. schultzei* are not morphotypes of the same species and cannot interbreed. 1.5 Objectives

#### 1.5.1 General objective

The general objective of this study is to characterize the dark and pale forms of *Frankliniella schultzei* Trybom using ecological, biological, morphological and molecular approaches

#### 1.5.2 Specific objectives:

The specific objectives of this study were to:

i. Determine the ecological distribution and host plant preferences of the dark and pale forms of *F. schultzei*

ii. Evaluate the possibility of interbreeding between the dark and pale forms of *F. schultzei*
iii. Analyze the morphological features of the dark and pale forms of *F. schultzei*
iv. Characterize the dark and pale forms of *F. schultzei* using ITS2-RFLP markers and sequence analysis.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction of Frankliniella schultzei

*Frankliniella schultzei* Trybom is a species of thrips (Thysanopterans) belonging to the family Thripidae . They are very small insects, with adult females being 1.1-1.5 mm long while adult males are 1.0-1.6 mm long (Kakkar *et al.*, 2014). These insects are highly polyphagous hence it feeds on a wide variety of vegetables and ornamental plants (Johansen, 2002). This species is considered of economic importance as it is a known vector of Tospoviruses (Wijkamp *et al.*, 1995) leading to losses of up to 80% on tomatoes in Kenya (Wangai *et al.*, 2001)

### 2.1.1 Life cycle of F. schultzei

Like many other thrips species, the life cycle of *F. schultzei* consists of an egg, two active feeding larval instars and two relatively inactive pre-pupa and pupal stages. Generally, *F. schultzei* females lay 30-300 smooth shelled eggs (fertilized or unfertilized) which are inserted into plant tissue with a saw like ovipositor. The complete life cycle usually lasts between 10-30 days depending on the temperature; warm temperatures favor regeneration while cooler temperatures favor dormancy (Lewis, 1997). The eggs are laid inside plant tissue and last for 2-4 days before they hatch into $1^{st}$ instar larvae, which develop to $2^{nd}$ instar larvae after 2 days. The $2^{nd}$ instar larvae then develops into a pre-pupa which lasts for 1-2 days and then the pupal stage which lasts for about 3 days before eventually emerging to an adult. The adult life span is about 30-45 days, under favorable conditions (Zitter *et al.*, 1989).

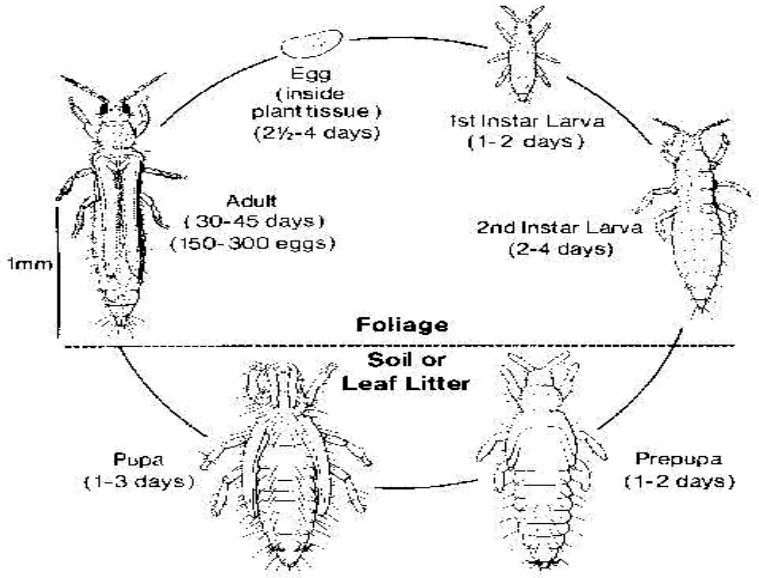



**Figure 1: The life cycle of F. schultzei illustrating the life stages and time of each stage (Source: http://vegetablemdonline.ppath.cornell.edu/factsheets/Virus_SpottedWilt.htm, 2013)**

### 2.1.2 Reproduction and mating behavior of *F. schultzei*

Thrips exhibit two types of reproductive behavior, where they can lay either fertilized eggs or unfertilized eggs (parthenogenesis). Fertilized eggs develop into females which have full number of diploid chromosomes while unfertilized eggs develop to males which are haploid. There are three types of parthenogenesis: arrhenotoky, where unfertilized eggs develop into males while fertilized eggs develop into females, thelytoky where unfertilized eggs develop into females and deuterotoky, where unfertilized eggs develop into both sexes (Moritz, 1997). *Frankliniella occidentalis* (Pergande), *Echinothrips americanus* Morgan and *Suocerathrip slinguis* (Mound and Marullo) exhibit arrhenotoky while *Parthenothrips dracaenae* (Heeger) and *Hercinothrips femoralis* (Reuter) are thelytokous parthenogenetic (Kumm & Moritz, 2008). Deuterotoky is known to occur in *Apterothrips apteris* (Daniel) population (Mound, 1992). Parthenogenetic reproduction in species belonging to Hymenoptera has been postulated to be caused

by the presence of a bacteria of endosymbionts from *Wolbachia* genus in the thrips (Stouthamer & Werren, 1993). *F. schultzei* is considered to be arrhenotokous-parthenogenetic (Sakimura, 1969).

### 2.1.3 Geographical distribution of *F. schultzei*

*Frankliniella schultzei* is mainly allopatric in distribution, with the dark form mainly found south of the Equator, while the pale form is mainly found north of the Equator and mixed colonies of both color forms are found in India, Philippines, New Guinea, and Northern Australia and in Sudan-Uganda-Kenya region (Mound, 1968; Sakimura, 1969). The distribution of *Frankliniella schultzei* is pan African and in east Africa it is widely distributed across Kenya and Uganda (Moritz et al., 2013)

### 2.1.4 Host Preferences of *F. schultzei*

*Frankliniella schultzei* is highly polyphagous, feeding on a wide variety of vegetables and ornamental plants (Moritz *et al.*, 2013). They are observed to aggregate and mate on corollas of rose of China (*Hibiscus rosasinensis* L.), cotton (*Gossypium hirsutum* L.), wax mallow (*Malvaviscus arboreus* Cav.), and morning glory *Ipomoea indica* (Burm.) Merril flowers (Milne *et al.*, 2002), and like other flower thrips, this has made them likely pollinators of a wide range of crops (Kirk, 1997). *Frankliniella schultzei* feeds on pollen and other plant parts such as petals and leaves (Milne *et al.*, 1996) as well as on *Tetranychus urticae* (C. L. Koch), commonly known as the two-spotted spider mites on cotton plants (Milne & Walter, 1997).

## 2.2 Economic importance of *F. schultzei* in Kenya

In Kenya, by the year 2007, French beans contributed 60% of the income from horticultural produce (HCDA, 2007) and thrips are the major pests affecting its production leading to 63-68% loss (Nyasani *et al.*, 2012). This is due to direct damage caused by feeding on plant leaves and flowers and indirect damage due to being vectors of Tospoviruses (Cho *et al.*, 1988; Lewis, 1997c). Therefore, thrips damage reduces household income, government and foreign exchange earnings

(Kasina *et al.*, 2009). In Kenya, crops that suffer great losses due to *Frankliniella schultzei* infestation are French beans, tomatoes, potatoes and tomatoes (Moritz et al., 2013; Nyasani *et al.*, 2009).

### 2.2.1 Direct damage caused by thrips

Thrips pierce plant cells with their mouthparts and feed on the plant sap resulting in the formation of deformed flowers, necrotic leaves, stems, shoots, abscissions and distorted fruit (Cloyd, 2009; Kirk, 1997; Lewis, 1997c; Nderitu *et al.*, 2008). The females also cause damage, using the ovipositor while laying the eggs (Moritz, 1997). Thrips damage also often results in qualitative losses and reduces the marketability of fruits and vegetables (Funderburk, 2009). *Frankliniella schultzei* can cause direct damages to crops by feeding on pollen and floral tissue, leading to flower abortion (Kakkar *et al.*, 2014).

### 2.2.2 Indirect damage caused by *F. schultzei*

So far, 20 Tospovirus species have been identified globally along with 14 thrips species in the family *Thripidae* that can serve as vectors (Riley *et al.*, 2011). The dark form of *F. schultzei* is considered to be a vector of at least four tospoviruses: *Tomato spotted wilt virus* (TSWV), Tomato chlorotic spot virus (TCSV) Groundnut ringspot virus (GRSV) and Chrysanthemum stem necrosis virus (CSNV), while the pale form is a weak vector of TSWV and TCSV (Kakkar *et al.*, 2014; Cho *et al.*, 1988; Wijkamp *et al.*, 1995; Sakimura, 1969). Plants infected by TSWV display chlorosis, necrosis, rings-potting and other symptoms. Fruits of infected plants are not marketable (Funderburk, 2009).

## 2.3 Management of thrips

Thrips are difficult to control because of the secluded habitat, wide host range, short lifecycle and ability to resist insecticides. Therefore, growers often resort to repeated and indiscriminate use of several groups of insecticides to control thrips (McDonald *et al.*, 1998; Nyasani *et al.*, 2012). Migration of thrips has also made management difficult. Within a local area thrips migration is facilitated by wind; their fringed

wings enable them to fly for a longer distance (Lewis, 1997b). International trade in vegetables and flowers has also led to migration of thrips hence becoming invasive alien (Reitz, 2009). Control measures for thrips infestation include, chemical, cultural resistance and biological strategies (Ravi *et al.*, 2012).

### 2.3.1 Chemical management of thrips

Thrips management through chemicals (insecticides and pesticides) has been extensively used through time and the chemicals used range from thiocyanates, nicotine sulphates, chlorinated hydrocarbons, carbamates, cholinesterase inhibitors (Lewis, 1997a) to carbofurans, endosulfans (Rao & Swami, 1986) and imidacloprid (Morse & Hoddle, 2006). Chemical insecticides offer the most widely adopted control strategy of thrips in Kenya and farmers depend on them heavily (Nderitu *et al.*, 2008). However foliar sprays of chemical insecticides often fail to manage thrips because of their cryptic feeding behaviour, ability to pupate in the soil and resistance to pesticides (Berndt *et al.*, 2004). Recently, Pinheiro *et al.*(2013) studied the use of botanical pesticides such as essential oils of Citronella grass and its main components (citronellal, citronellol and geraniol) were insecticidal to *F. schultzei*

### 2.3.2 Cultural control of thrips

Intercropping with non-host crops such as white clover, sunflower and baby corn could substantially reduce thrips infestation on the host plant by either attracting thrips predators or reducing the intensity of light getting to the plant, hence reduce visibility of the plant to the insect and enhance the market yields (Parrella & Lewis, 1997; Trdan *et al.*, 2006; Foote *et al.*, 2010; Nyasani *et al.*, 2012). Furthermore, intercropping encourages the presence of natural enemies such as *Orius tristocolor* (white) and *Ceranisus spp*. which are major thrips predators (Parrella & Lewis, 1997; Nyasani *et al.*, 2012). *F. schultzei* have a preference for Irish potatoes and Sunflowers compared to baby corn and French beans in the field. Therefore, intercropping sunflowers and irish potatoes with French beans would assist in reducing the infestation of French beans, as the insects would be first attracted to the

Irish potatoes and sunflower, providing an early opportunity for spraying them, before the French beans blossom (Nyasani *et al.*, 2012).

### 2.3.3 Biological control of thrips

Thrips can be controlled through the application of entomopathogenic fungi. For instance *Metarhizium anisopliae* (Metsch) Sorok and *Beauveria bassiana* L. have been reported to be effective on *Megalurothrips sjostedti* (Trybom) infesting cowpea, *Thrips tabaci* Lindeman infesting onions and *F. occidentalis* Pergande infesting French beans (Ekesi *et al.*, 1998; Maniania *et al.*, 1998).

Natural enemies such as predatory bugs *Orius laevigatus* and the predatory mites *Stratio laelaps* (Hypoaspis) *Miles* and *Gaeo laelaps* (Hypoaspis) *aculeifer*are have also been reported to be effective against thrips (Bennison *et al.*, 2001). Parasitoids such as *Ceranisus menes* (Hymenoptera: Eulophidae) parasitize larval thrips however; they have not been proven to be very effective for thrips control (Murai *et al.*, 2000; Loomans, 2003).

### 2.3.4 Behavioural control of thrips

Thrips are known to be attracted to each other and their host plants through a combination of colour and pheromone cues (Muvea, 2011; Hamilton, 2005; Terry, 1997). Response to such cues varies with thrips species for instance, during open field experiments *F. occidentalis* and *F. schultzei* are attracted to yellow sticky traps, *F. intonsa, F. schultzei* and *Thrips tabaci* are attracted to blue traps while *Thrips palmi* and *Thrips imaginis* are attracted to white traps (Lewis, 1997c; Muvea, 2011). Similar to colour, there are several semiochemicals which are thrips attractants such as ethyl nicotinate, anisaldehyde, euginol, geraniol, myrcene, benzaldehyde (Terry, 1997) and methyl anthranilate (Murai *et al.*, 2000). Pheromones such as (R)-lavandulyl acetate and neryl (S)-2-methylbutanoate, specific to thrips *F. occidentalis* males, enhance their attraction (Hamilton, 2005). Methyl isonicotinate branded as LUREM-TR in combination with a coloured sticky trap are known to enhance thrips captures from 2 – 20 folds (Davidson *et al.*, 2007; Teulon *et al.*, 2007; van Tol *et al.*,

2012; Muvea et al., 2014). A combination of sticky traps with pheromones has been shown to greatly control thrips, as demonstrated by Sampson & Kirk (2013) where the use of blue sticky traps reduced the number of adult *F. occidentalis* thrips by 61% and addition of the pheromone neryl (S)-2-methylbutanoate reduced the adult thrips number per flower by 73%, resulting in increased returns by £2200 per hectare. Another example is the control of *F. occidentalis* on pepper flowers using sticky traps with pheromones, where 10 traps were able to effectively control the insects in one week, and the pheromone had a double-attraction effect of increasing aggregation as well as attracting males to the traps (Elimem *et al.*, 2014)

### 2.4 Morphological identification of thrips

Traditionally, the identification of thrips has been based on external morphological features, such as body shape, length and colour (Moritz, 1994; Mound & Kibby, 1998) utilizing diagnostic aids such as dichotomous keys (Moritz *et al.*, 2005). Over time, more matrix based keys which are user-friendly, pictorial and versatile in information provision have been adopted. Several modern and computerized keys are developed for thrips identification. Pest thrips of the world (Moritz et al., 2004); Thrips of California (Hoddle *et al.* 2008); Pest thrips of North America (Moritz et al., 2008) are keys that combine an interactive computer based system with current taxonomic methods and images that enable rapid identification. A more recent example is the Lucid Key developed for Pest thrips of East Africa (Moritz *et al.*, 2013) which is useful in identifying over 100 pest thrips in east Africa and is user-friendly with provisions for expert route for non-thrips taxonomists.

The morphological features that set apart *F. schultzei* from the other species within its genus are; its 3$^{rd}$ocellar setae are very close together between the anterior margins of hind ocelli, its metanotal campaniform sensilla are absent and its 8$^{th}$tergite has the postero-marginal comb of microtrichia weakly developed or completely absent (Mound, 1968; Moritz *et al.*, 2013; Sartiami & Mound, 2013).

## 2.5 Molecular Identification

Recently, molecular procedures have been used for the identification of species at both adult and immature stages (Farris *et al.*, 2010). Most of these molecular procedures are PCR based (Toda & Komazaki, 2002; Moritz et al., 2004) and used for thrips species diagnostics as well as for population studies (Farris *et al.*, 2010). These have included DNA sequencing (Brunner *et al.*, 2002) and PCR-based methods such as real-time PCR (Walsh *et al.*, 2005), PCR-restriction fragment length polymorphism (PCR-RFLP) (Toda & Komazaki, 2002; Rugman-Jones *et al.*, 2006; Moritz *et al.*, 2004), random amplified polymorphic DNA (RAPD) (Fang *et al.*, 2005), and simple sequence repeat (SSR-PCR) (Brunner & Frey, 2004).

Molecular approaches have been utilized in thrips identification (Inoue & Sakurai, 2007; Brunner *et al.*, 2002) and DNA barcoding is one of the molecular techniques used in identifying insect species using short genetic markers (Hebert *et al.*, 2003; Waugh, 2007). The most widely used barcode loci in insect identification are the mitochondrial cytochrome oxidase 1 (COI) (Hebert *et al.*, 2003; Brunner *et al.*, 2002; Macharia *et al.*, 2015; Timm *et al.*, 2008) gene and the internal transcribed spacer (ITS) ribosomal DNA region (Moritz *et al.*, 2001; Campbell *et al.*, 1993; Toda & Komazaki, 2002; Moritz *et al.*, 2004; Rugman-Jones *et al.*, 2006; Farris *et al.*, 2010).

### 2.5.1 DNA barcoding

DNA barcoding is a molecular biological identification technique which utilizes short mitochondrial DNA sequences (usually the Cytochrome C oxidase I gene) or the highly conserved nuclear DNA sequences (especially the Internal Transcribed Spacer (ITS) regions of ribosomal genes or elongation factor-1α genes) to group unknown individuals. It relies on sequence similarity, where an individual is identified based on previously defined taxonomic entities. In using DNA barcoding, first the organism has to be classified taxonomically using its morphological features then its DNA sequence is aligned with its taxonomic relatives. Inexact matches are either grouped with taxa present in the database or identified as new to the database based on whether they fall within a threshold of sequence similarity. This is justified

by the observation that variation among species is normally lower than interspecies variations (Simon, 1991; Schlötterer *et al.*, 1994; Hebert *et al.*, 2003; Hajibabaei *et al.*, 2006). The sequences of interest (COI, ITS or EF-1α) are analysed in combination with various molecular biological methods and markers, such as RFLP, SSR, AFLP and RAPD (Brunner *et al.*, 2002; Mehle & Trdan, 2012; Inoue & Sakurai, 2007). Therefore, DNA barcoding has been selected as the standardized tool for molecular taxonomy and identification (Galtier *et al.*, 2009; Armstrong *et al.*, 1997; Armstrong & Ball, 2005; Hebert *et al.*, 2003).

#### 2.5.1.1 Cytochrome C Oxidase I gene in DNA barcoding

Cytochrome C oxidase I (COI) is a subunit of a large trans-membrane protein found in the mitochondrion, which is highly conserved across species that employ oxidative phosphorylation in metabolism (Waugh, 2007). The presence of COI region in the mitochondrial DNA, maternal inheritance makes it an appropriate marker of molecular biodiversity. Maternal inheritance makes COI region to behave as a single, non-recombining locus and share a common genealogy. This reasonably simplifies the representation and analysis of within-species variations data. Secondly, mitochondrial DNA is supposed to be evolved in a nearly neutral fashion, involved in basic metabolic functions (respiration) and is less likely than other genes to be involved in adaptive processes. Finally, the evolutionary rate of mitochondrial DNA has been frequently assumed to be clock-like in the absence of any mutations spreading through positive selection, only neutral (and slightly deleterious) mutations accumulate in time, so that mitochondrial DNA divergence levels should roughly reflect divergence times (Galtier *et al.*, 2009).

*Frankliniella schultzei* was among species that were used in studies that utilized COI gene markers (Timm *et al.*, 2008; Kadirvel *et al.*, 2013; Macharia *et al.*, 2015) and the findings suggested possible genetic differences in the population of *F. schultzei*.

### 2.5.1.2 Internal Transcribed Spacer Region of the ribosomal DNA

The ITS region includes the ITS1 and ITS2 regions, separated by the 5.8S gene, and is situated between the18S (Small Subunit) and 28S (Large Subunit) genes in the nuclear DNA repeat unit. The entire ITS region typically ranges between 450 and 700 base pairs. In most studies, either the ITS1 or the ITS2 region have been targeted in high-throughput sequencing, because the entire ITS region is considered too long for sequencing (Bellemain *et al.*, 2010). It is easily amplified by PCR from even miniscule amount of DNA and is easy to sequence (Young & Coleman, 2004; Ashfaq *et al.*, 2010). The ITS2 gene is a suitable choice for the identification of closely related species or sympatric populations as the ITS region has a high variability between different species and extremely low variability between individuals of the same species (Moritz *et al.*, 2001).

The ITS2 primer pairs 28Z (5´AGACTCCTTGGTCCGTGTTTC 3´) and P1 (5´ATCACTCGGCTCGTGGATCG 3') have been used in thrips identification (Moritz *et al.*, 2004). The ITS1 primers CS249 (5'-TCGTAACAAGGTTTCCG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') together with ITS2 primers ITS2-F (5'-TGTGAACTGCAGGACACATG-3') and CS250 (5'-GTTRGTTTCTTTTCCTC-3') have been used for characterising *Scirtothrips spp.* populations (Rugman-Jones *et al.*, 2006). Toda & Komazaki (2002) utilised the ITS2 region of ribosomal DNA to conduct a study on identification of nine thrips species; *F. occidentalis* Pergande, *F.intonsa* Trybom, *Thrips hawaiiensis* Morgan, *Thrips coloratus* Schmutz, *Thrips flavus* Schrank, *Thrips palmi* Karny, *Thrips tabaci* Lindeman, *Thrips setotus* Moulton and *Scirtothrips dorsalis* Hood in Japan and they used the primer pair 5' CGAGTATCGATGAAGAACGCAGC 3' and 5' AATGCTTAAATTTAGGGGGTA- 3' for evaluating intra and inter-species divergence.

### 2.5.2 Recent advances in molecular markers used for thrips identification

Advances into DNA barcoding has developed universal primers for amplification of the COI and ITS regions. The most common ones are the COI primers designed by

Folmer *et al.*(1994); LCO1490: 5' GGTCAACAAATCATAAAGATATTGG 3' and HC02198: 5' TAAACTTCAGGGTGACCAAAAAATCA-3' which consistently amplified a 710-bp fragment of COI across the broadest array of invertebrates (80 invertebrate species from 11 phyla). Another set of universal primers for insects are the LepF1: 5'-
ATTCAACCAATCATAAAGATATTGG 3' and LepR1: 5' TAAACTTCTGGATG TCCAAAAAATCA-3' used by Hebert *et al.*, (2004). In a later study, Hajibabaei *et al.*(2006) modified the LepR/LepF insect primers by adding MLepF1 (5' GCTTTCCCACGAATAAATAATA 3') LepR1 and MLepR1 (5' CCT GTTCCAGCTCCATTTTC-3')-LepF1. This modification enabled amplification of Lepidopteran samples that were older than ten years.

### 2.5.3 Advantages and constrains of morphological and molecular identification techniques

Species identification using morphological features has some significant limitations as the species might have minimal or no phenotypic changes but have high genetic variability. Morphological identification overlooks cryptic features, which are common in many groups and the use of keys requires a high level of expertise as misdiagnosis is common (Hebert *et al.*, 2003; Armstrong & Ball, 2005). A constraint in thrips morphological identification is that larval stages cannot be identified with most available keys and they exhibit fewer characters of diagnostic value than the adults (Glover *et al.*, 2010).

DNA barcoding enables the identification of closely related species using genetic distances computation (Sbordoni, 2010). It also provides a high degree of taxonomic resolution (>95%) for most species. Moreover, it enhances the robustness of any key and assignment of species identification by incorporating the range of intra-specific polymorphism and adding as many reference sequences as possible (Armstrong & Ball, 2005; Hajibabaei *et al.*, 2006; Waugh, 2007)

The dependence of DNA barcoding on taxonomic databases introduces a limitation because if a particular species is not represented in the reference database, it could be wrongly identified as another species (Virgilio *et al.*, 2010). Therefore, it is not predictive, and is limited to a comprehensive, reliable and near complete reference database. Thus, this approach can function only as an identification tool, complementing morphological classification but not as a standalone taxonomic system (Elias *et al.*, 2007; Vogler & Monaghan, 2007)

In using DNA barcoding, a major contentious issue has been designating a mean of 2-3% divergence to infer intra- and inter-specific genetic differences between two sequences as indicative of different insect species (Hebert *et al.*, 2003). This threshold level assumes a constant mutation rate across different taxa. However, the mitochondrial genome of Thysanoptera are subject to particularly high levels of rearrangements (Shao & Barker, 2003), therefore 2% divergence between species might be appropriate for one group of taxa but 6% is more accurate for another; so, drawing species boundaries based solely on COI becomes ambiguous (Rubinoff *et al.*, 2006). It is also not advisable to infer general patterns of an organism's genetic identity only from COI as it is a very small fraction of the genome. Therefore this should be backed up withother data such as morphology, biology, ecology, or nuclear DNA sequences (e.g. EF1-α or ITS) so as to avoid phylogenetic inferences that are not broadly supported (Rubinoff & Holland, 2005; Elias *et al.*, 2007).

On the other hand, ITS is also a challenging marker technically as it is present in multiple distinct copies, and has a high likelihood of containing high intra and inter-genomic variation. Moreover, ITS may only be useful for species identification in taxon-specific studies as it produces alignment overlaps in the genus-specific range (Dentinger *et al.*, 2011; Stern *et al.*, 2012). However, the fact that ITS2 sequences are potential markers for general phylogenetic studies and that they have been widely used for phylogenetic reconstructions at both the genus and the species levels makes them ideal for species differentiation (Miao *et al.*, 2008; Schultz & Wolf, 2009).

Economically, morphological identification is much cheaper than molecular identification as the materials and equipment used in morphological identification

require less expenditure (Hillis, 1987; Wiens, 2004). However both molecular and morphological identification techniques need to be used in a complementary manner to clearly identify specimens. Therefore, a more general, simple, accurate, and large-scale identification method would be helpful to facilitate identification of thrips species occurring in a cropping system where multiple species co-exist, and the population dynamics are influenced by numerous factors (Kadirvel *et al.*, 2013)

In conclusion, putting into consideration all the previous literature, in this study, *Frankliniella schultzei* was first characterised on the basis of their ecological distribution. This was so as to give a preview of the current distribution in Kenya and more so in regards to the two colour forms of *F. schultzei*, as the distribution of the two colour forms in Kenya had not been well documented. Interbreeding experiments were then conducted so as to determine whether the two colour forms of *F. schultzei* interbreed and can produce intermediary forms as postulated by Sakimura (1969) and to also determine each colour form's parthenogenetic reproduction as this had not been previously documented. The morphological differences between the two forms were determined, so as to ascertain their identity and add on to the previously documented differences between the two colour forms. Finally, molecular techniques were employed in differentiating the two colour forms so as to determine their genetic divergence. ITS2 was utilized in this study because it had been previously used effectively to differentiate other thrips species (Moritz et al., 2004).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study site

This study was conducted at the International Centre of Insect Physiology and Ecology *icipe*, Duduville, Nairobi, Kenya, in the Animal Rearing and Quarantine Unit. The dark forms of *F. schultzei* originated from a population on *Malvaviscus grandiflorus* Kunth from Kasarani, Nairobi County (GPS coordinates: -1.221, 36.897) and has been in culture for approximately 100 generations. The pale form of *F. schultzei* originated from a population on *Phaseolus vulgaris* (Linnaeus) in Kitui county (GPS coordinates: -1.298, 37.944) and has also been in culture for approximately 100 generations. The breeding experiments were conducted in the Thrips Laboratory while the molecular experiments were conducted in the Arthropod Pathology Unit, both of which are in *icipe*, Duduville, Nairobi, Kasarani.

## 3.2 Research Design

To test the hypothesis, the experiments conducted in this study were categorised into four groups: ecological, biological (breeding), morphological and molecular.

First, the ecological analysis was conducted as a preliminary study to evaluate the difference in distribution of the two colour forms in different host plants as well as in different altitudes. This analysis was conducted on data based on thrips that had been previously collected from various locations within Kenya and was analysed using quantitative analysis.

Then the biological experiments were conducted to determine whether the dark and pale forms of *F. schultzei* interbreed. Insects used in these experiments were selected using simple random sampling method. This study was conducted in four treatments (Table 1) with insects used being selected from the egg stage (20 female insects per treatment) and each treatment was repeated in triplicate. Data was analysed based on morphology and molecular differences.

On the morphological aspect, selected morphological features were measured and were analysed quantitatively using differences in mean. In the molecular aspect, only the parents were analysed through DNA amplification, Restriction Fragment Length Polymorphism (RFLP) and sequence analysis (Jeffreys *et al.*, 1985).

### 3.3 Ecological characterisation of the two forms of *F. schultzei*

A preliminary study was conducted to determine the host plant and ecological preferences of the two colour forms of *F. schultzei*. This was done by analysing the geo-referenced database maintained in the Biosystematics unit, *icipe*. The data used was from a collection of thrips samples undertaken between August 2008 and July 2009 from over 88 locations in Kenya. This collection was made by Dr. Subramanian Sevgan and Mr. Benard Muia of *icipe* (Kasarani, Nairobi). A total of 1383 *F. schultzei* specimens were assessed in this study from this collection. The samples were collected from 21 counties; Bungoma, Busia, Kakamega, Homabay, Tharaka-Nithi, Trans-Nzoia, Nairobi, Kilifi, Lamu, Baringo, Kirinyaga, Nakuru, Meru, Kiambu, Muranga, Mombasa, Embu, Makueni, Machakos and Kitui county. They were collected from a variety of host plants. At each location, sampling was undertaken at random from 10 - 20 plants/location using standard "Plant tapping method" (Pearsall & Myers, 2000; Mfuti *et al.*, 2015). The identity of the *F. schultzei* insects collected was confirmed using the Lucid key (Moritz *et al.*, 2013) prior to archiving them by slide mounting. The geo-referenced database included information on the Global Positioning System (GPS) co-ordinates of each sampling location, host plant records, dates of collection and thrips species information.

### 3.4 Rearing of *F. schultzei* thrips

Both colour forms of *F. schultzei* were reared in ventilated jars (16 × 16 × 6cm) with thrips-proof nets and filter paper in cover and fed on French bean pods *Phaseolus vulgaris* (L.) var. *Samantha* at 28 ± 2°C, 60–80% relative humidity with a 12 h darkness and 12 h light photoperiod (12 L: 12 D). Each jar contained 7-8 pods. The pods were sterilised before use by soaking them in a solution of iodized salt, 10% sodium hypochlorite and water then rinsed in distilled water. Adult thrips were

allowed to lay eggs on the pods; three days later, the pods containing eggs were removed and placed in another clean container. Filter paper and paper towel were placed below the pods in the container to facilitate pupation (Loomans and Murai, 1997). Filter paper and paper towel with pupae were maintained for adult emergence and these adults were subsequently used for furthering the generations. The dark and pale forms of *F .schultzei* were reared in separate rooms. Their morphological identity was assessed using the Lucid key (Moritz *et al*., 2013). Their larval instars were identified based on the number of days after oviposition and the size of the individuals. These instars were then used in the biological characterization.

### 3.5 Biological characterisation - interbreeding analysis of *F. schultzei*

The sample size was determined using Mead's Resource equation (Mead, 1988); E=N-B-T (N = the total number of individuals in the study minus 1, B = the environmental factors allowed in the design minus 1, T = the number of treatment groups including the controls in the study minus 1 and E = the degrees of freedom of the error component and should be between 10 and 20). Using this formula, considering that there were 4 treatments, T = 3, B = 0 and E = 20, resulting in N = 24 which when divided by the number of treatments (4) would provide the number of insects to be used per treatment to be 6. However, due to the minute size of the insect and so as to increase precision, 20 insects were used per treatment.

In order to generate virgin parents, larvae of the different colour forms were transferred individually into 90 mm Petri dishes lined with paper towel and fed with a piece of French bean pod (about 7cm long) until adult emergence. The sex of emergent adults was determined as described by Moritz *et al.* (2013) under a dissecting microscope (Leica Microsystems®) at X40 magnification and utilized in interbreeding experiments. 20 dark and 20 pale *F. schultzei* females were placed individually in 10ml glass vials for mating with dark males (80 males for each colour form treatment) in a female to male ratio of 1:4 for 3 days. After mating, all females were placed in separate Petri dishes containing sterile French bean pod for oviposition. Recently emerged larvae (F1) were transferred to separate Petri dishes and fed as described above until adult emergence. Concomitantly, treatments meant

to test the parthenogenesis of virgin dark and virgin pale females were conducted with twenty females of each colour form held in the absence of males (Table 1). Each experiment was replicated three times. There was an absence of pale males in the colony; therefore, the pale male × dark female cross was not conducted.

Twenty (20) parental specimens of both forms (dark and pale) and their respective F1 offspring (from virgin dark female, virgin pale female, dark male x dark female and dark male x pale female) were prepared for mounting on slides by placing them in 1.5ml Eppendorf tubes containing 5% Sodium hydroxide (NaOH) for 12 hours. They were then placed in distilled water 3 times for 5 minutes each and then taken through an alcohol dehydration series. The cleared specimens were stored in clove oil for at least 30 minutes before mounting on slides using Canada balsam method as outlined by Moritz *et al.* (2013) for further morphological characterization.

**Table 1. Interbreeding treatments used for *F. schultzei* and their respective objectives**

| **Treatments** | **Female** | **Male** | **Purpose** |
|---|---|---|---|
| Dark Virgin | 20 | - | Determine the type of parthenogenesis |
| Pale Virgin | 20 | - | Determine the type of parthenogenesis |
| Cross 1 (dark female × dark male) | 20 | 80 | Assess the breeding potential of dark forms |
| Cross 2 (pale female × dark male) | 20 | 80 | Assess the interbreeding potential |

### 3.5.1 Confirmation of Pale *F. schultzei* males in the wild

Due to the absence of pale *F. schultzei* males in the colony, it was essential to confirm their presence in the wild. Thirteen (13) male *F. schultzei* were collected from *Ipomoea setosa* Ker-Gawl flowers within *icipe* (Duduvillei, Nairobi) which hosted both pale and dark *F. schultzei*. All males were treated as tentative candidates as it has been postulated that males from both colour forms are similar in colour (Sakimura, 1969). They were subjected to morphological and molecular characterization as earlier described.

### 3.6 Morphological characterisation of dark and pale *F. schultzei*

The identity of the specimens mounted on the slides were determined using morphological features outlined in the Lucid key on "Pest thrips of East Africa" (Moritz *et al.*, 2013). Eight morphological features were selected to further characterize 20 of each the colour forms parents and 20 of their F1 offspring based on previous literatures (Johansen, 2002; Hamodi & Abdul-Rassoul, 2004; Retana-salazar *et al.*, 2010; Wang *et al.*, 2010; Subramanian *et al.*, 2012; Moritz *et al.*, 2013). The features selected included the length of third ocellar setae (*Sl*), distance of the third ocellar setae from the tangent of the anterior margin of the hind ocellar (*DfT*), the lengths of 1$^{st}$ocellar setae (*Set1*), 2$^{nd}$ocellar setae (*Set2*), metanotal lateral setae (*Mls*), major setae on tergite X (*Terg10*), width of the forewings (*Fw*) and the appearance of posteromarginal comb of microtrichia on tergite VIII (*Comb*) (Fig. 2). Morphometric measurements were made on the specimens using a Leica DM LB® microscope fitted with Leica EC3® camera and Leica Application Suite Version 3.0.0 software (Leica Microsystems, Switzerland). The length of third ocellar setae (*Sl*) and distance of the third ocellar setae from the tangent of the anterior margin of the hind ocellar (*DfT*), had been previously described by Subramanian *et al.* (2012) as differentiating morphological features between the two colour forms, hence were further used to characterise 20 of the F1 offspring from the crosses, (dark female × dark male and pale female × dark male crosses) in comparison to their respective parents.

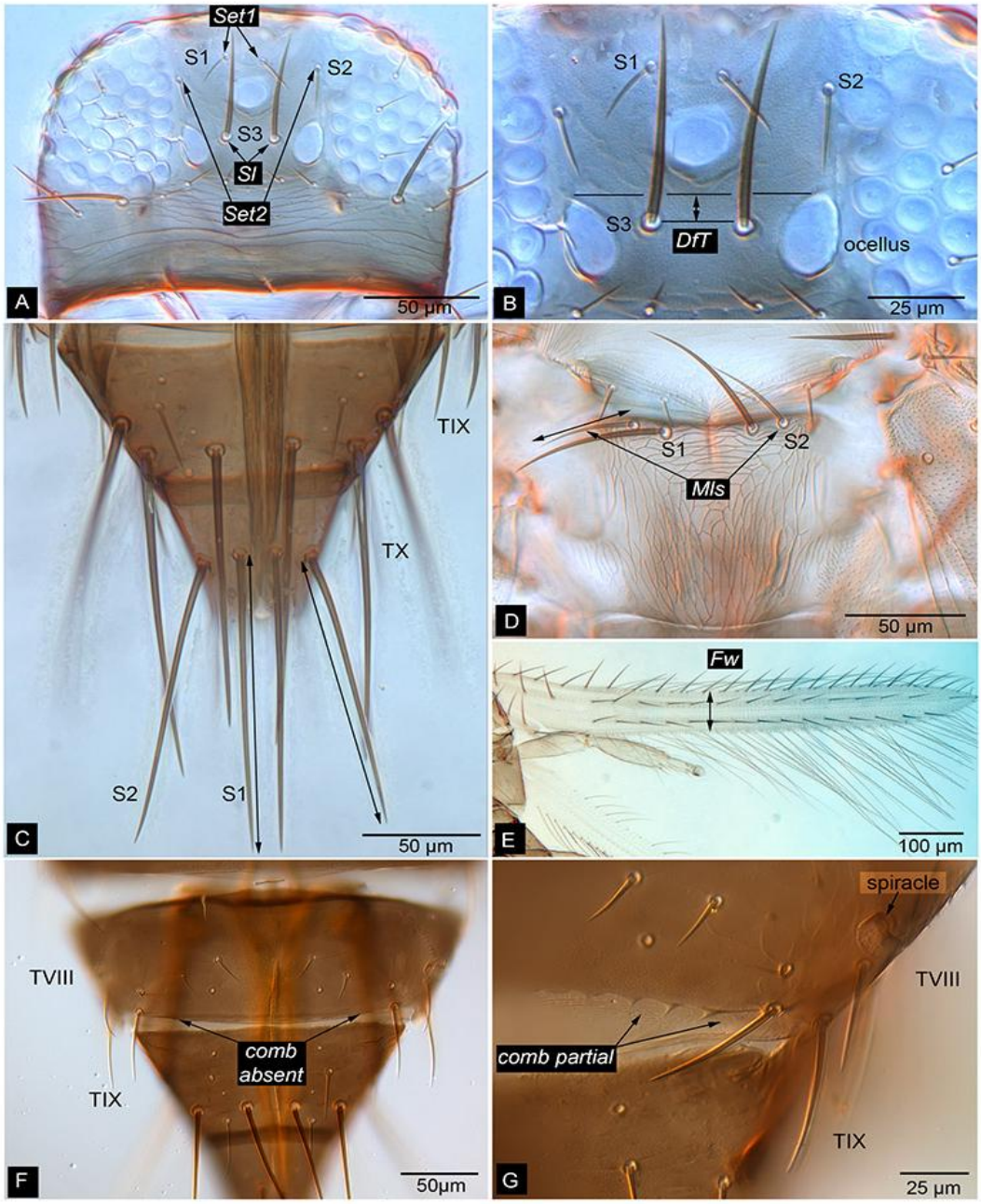



**Figure 2. Morphological features used in morphometric study. (A) *Set1*, *Set2* and *Sl*, 1st, 2nd and 3rd ocellar setae length respectively; (B) *DfT*, Distance of 3rd ocellar setae from tangent of anterior margin of hind ocellar; (C) *Terg10*,Tergite 10 major setae length; (D) *Mls*, Metanotal lateral setae Length; (E) *Fw*, Forewing width; (F-G) *Comb*, absence of comb and presence of partial comb respectively. (Photograph courtesy of Prof. Gerald Moritz, 2015)**

### 3.7 Molecular Characterization of *F. schultzei*

#### 3.7.1 DNA Extraction of thrips samples

Individual parental specimens of *F. schultzei* stored in 95% ethanol were removed from 1ml Eppendorf tubes and allowed to air dry for 2 min. Each specimen was then placed on a sterile microscope slide and their abdomen dissected using a sterile surgical blade. For ease of dissection, female insects were selected as candidate specimen as they are larger in size compared to the males. Total DNA was extracted from the dissected abdomen using ISOLATE II Genomic DNA Bioline Kit (Bioline Inc©, MA, USA). Following the manufacturer's instructions, the abdomen of the individual insects was placed in a 1.5ml microfuge tube and a pre-lysis was done by adding 180µl of the pre-lysis buffer together with 25 µl of 0.02mg/µl proteinase K. The solution was then vortexed and incubated in a hot water bath at 56°C overnight. The lysed sample was vortexed again briefly and 200µl of the lysis buffer added. The solution was then vortexed vigorously and incubated at 70°C for 10 minutes. After incubation, 210µl of 100% ethanol was added into the solution and vortexed vigorously again. The entire solution was then loaded into a spin column with a 2ml collection tube and centrifuged for 1 minute at 11,000 x g. The flow through was discarded and the DNA in the column membrane was washed by adding 500µl of the wash buffer and centrifuging for 1 minute at 11,000 x g then adding 600µl of the wash buffer into the column for the second wash and centrifuging for 1 minute at 11,000 x g. The membrane was then dried by centrifuging the empty column at 11,000 x g for 1 minute, so as to remove any residual ethanol. The column was then taken out of the collection tube and placed in a 1.5ml microfuge tube. There was a slight modification in that the DNA was then eluted by adding 30µl of the elution buffer preheated at 70°C onto the centre of the membrane. The membrane was then incubated at room temperature for 5 minutes before centrifuging the eluted DNA into the microfuge tube at 11,000 x g for 2 minutes.

The concentration and quality of the DNA was measured using a nanodrop spectrophotometer (Thermo Scientific© Nanodrop 2000™) based on the absorption of light at 260:280 wavelengths ratio. This was done by first thoroughly sterilising

the upper and lower pedestals of the nanodrop, with emphasis on the sample placement point. Then the elution buffer was used as the blank to standardize the readings by placing 2µl of the buffer on the lower measurement pedestal point then the upper sampling arm of the nanodrop machine was lowered over the 2µl drop, ensuring the upper and lower pedestal points are in contact with the drop. The machine was then run and the readings were generated on the computer screen, in the preinstalled Nanodrop 2000 program. This procedure was then repeated using 2µl of the extracted DNA samples and sterilizing the arms after every sample.

The remaining body parts of the *F. schultzei* insects, that largely consisted of head, thorax and a section of the abdomen were cleared and mounted on slides for morphological identification as earlier described (Moritz et al., 2013).

### 3.7. 2 PCR Amplification of the Internal Transcribed Spacer 2 region

The Internal Transcribed Spacer region (ITS2) was amplified from the DNA obtained using the primer pair ITS2 F 5'-CGAGTATCGATGAAGAACGCAGC- 3' (Despres *et al.,* 1993) and ITS2 R5'-AATGCTTAAATTTAGGGGGTA- 3' (Campbell *et al.,* 1994). Amplification was performed in 30µl total reaction volumes, comprising 3µl of genomic DNA solution, 10 pmol of each primer, 10mM dNTPs, 1.25mM $MgCl_2$, 5X Phusion HF buffer, 100% DMSOand 5 units of Phusion DNA Polymerase. The amplification was carried out in an Arktik Thermo Scientific® Thermocycler. The DNA was initially denatured at 98˚C for 1 minute 10 seconds followed by 35 cycles of denaturation for 20 seconds at 98˚C, annealing for 40 seconds at 57.2˚ and elongation for 45 seconds at 72˚C. The last cycle was followed by a 10 minutes incubation period at 72˚C to complete any partially synthesized strands. The amplification products were separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized under UV imaging system.

Amplified DNA products were purified using ISOLATE II PCR and Gel Bioline Kit (Bioline Inc©, MA, USA) following the manufacture's instructions. This was done by first excising individual bands out of the agarose gel, under UV light. The pieces of gel containing the bands were individually transferred into 1.5ml microfuge tubes.

The weight of each gel piece was determined used a weighing scale, and using an empty microfuge tube to tare the scale. The binding buffer was then added into each tube, depending on the weight of the gel; 200µl of buffer per 100mg of agarose gel. The tubes were then incubated at 50°C for 10 minutes, with regual gentle swirling to ensure the gel was completely dissolved. The solution was then transferred to the gel column placed in a 2ml collection tube and centrifuged for 30s at 11,000 x g. The bound PCR product in the membrane was then washed twice using 700µl of the wash buffer per wash and centrifuged at 11,000 x g for 1 min after each wash. The membrane was then dried by centrifuging the column while empty for 1 minute at 11,000 x g. The column was then placed in a 1.5ml microfuge tube and the PCR product was eluted out by placing 30µl of the elution buffer onto the membrane, incubated at room temperature for 5 minutes and centrifuged for 2 minutes at 11,000 x g. The concentration and quality of the purified PCR product was measured using a nanodrop (Thermo Scientific© Nanodrop 2000™) as earlier described.

The purified amplicons were then analysed using two techniques; sequence analysis and Restriction fragment length polymorphism analysis (RFLP) (Jeffreys *et al.*, 1985).

### 3.7.2.1 ITS2 Sequence Analysis

Five (5) Purified DNA samples from the two colour forms of *F. schultzei* were selected for sequencing based on their concentration. They were then sent to Macrogen Inc© (Netherlands) for sequencing.

The sequences were then edited and deposited in GenBank and accession numbers were obtained. Homology searches of the ITS2 sequences against DNA databases were performed using nBLAST. Multiple sequence analysis of both dark and pale *F. schultzei* parental ITS2 sequences were conducted using Clustal W2 (Larkin *et al.*, 2007) and Pairwise sequence divergence was calculated using Kimura two parameter model (Kimura, 1980) in MEGA 6 (Tamura *et al.*, 2013).

### 3.7.2.2 Restriction fragment length polymorphism (RFLP) analysis of ITS2 amplicons

ITS2 fragments obtained from amplification using ITS2F-ITS2R primer pair were also subjected to Restriction Fragment Length Polymorphism (RFLP) analysis (Jeffreys *et al.*, 1985) using four restriction enzymes; *Alu*1, *Hinf*1, *Rsa*1 and *Hae*III (Thermo Scientific, Inc).These four enzymes were selected as they had been used previously to distinguish other thrips species (Moritz *et al.*, 2001; 2004). Each enzyme reaction was conducted separately. The reactions were carried out in 18µl reaction volumes, comprising of 10 X Fast Digest green buffer, 2 units of restriction enzyme and 5µl purified PCR products. The DNA was first digested at 37˚C for 14 hours and then the reaction was stopped by denaturing the enzyme at 65˚C for 5 minutes. The restricted fragments were separated by electrophoresis in 2% agarose gels containing ethidium bromide at 70V for 1.5 hours and visualized under UV imaging system. Analysis of the fragments was then conducted by observing the differences in banding patterns and noting any unique marker fragments found in each colour form.

## 3.8 Data analysis

Analysis of data obtained from the morphometrics study was performed using R version 3.1.3. Normality of data was checked using the Shapiro-Wilk test. Multiple comparisons of mean of the morphometric features between parents (dark and pale) and respective F1 offspring (dark male × dark female and dark male × pale female) were made using ANOVA in Microsoft® Excel 2007/XLSTAT©-Pro Version 6.1.9,2003 (Addinsoft, Inc., Brooklyn, NY, USA). The level of significance was fixed at 95%. Principal component analysis (PCA) was then conducted using R 3.1.3 (R Core Team, 2015) to determine the relationship between dark and pale *F. schultzei* parental populations, as well as between dark *F. schultzei* males from the colony and *F. schultzei* males from *I. setosa*. This was determined, based on the clustering of the eight morphometric variables measured. ITS2 DNA sequences were analysed by first editing and aligning them using Geneious® version 8.0.2 (Kearse *et al.*, 2012). Consensus sequences were deposited in GenBank and accession numbers

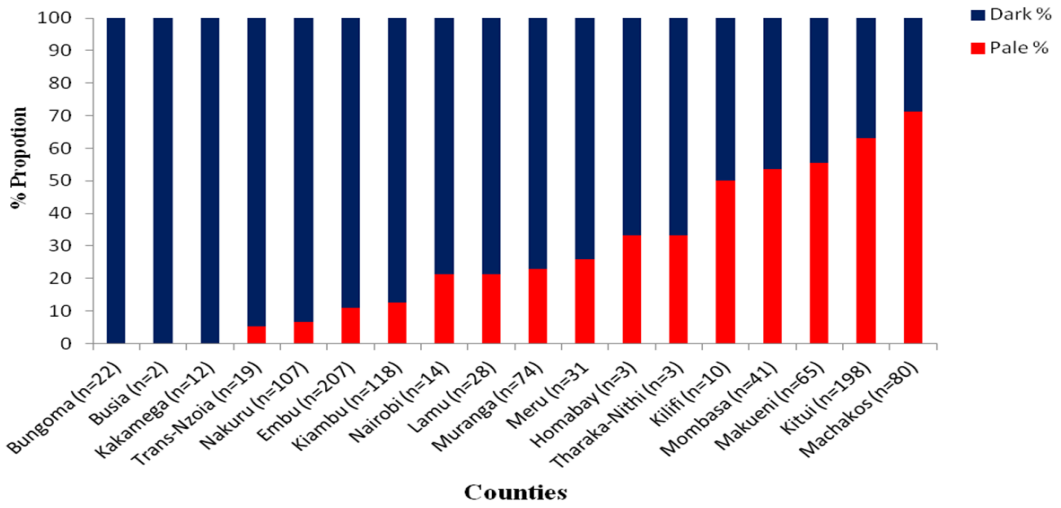
were obtained. Homology searches of the ITS2 sequences against DNA databases were performed using nBLAST (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence analysis of both dark and pale *F. schultzei* parental ITS2 sequences were conducted using Clustal W2 (Larkin *et al.*, 2007). Pairwise sequence divergence was calculated using Kimura two parameter model (Kimura, 1980) in MEGA 6 (Tamura *et al.*, 2013). To visualize the patterns of divergence, a neighbour-joining tree (Saitou & Nei, 1987) was constructed from the evolutionary distances using a bootstrap of 1000 replicates and complete deletion option. The genetic distances were also used to generate a principal component plot using GenAlEx 6.41 (Peakall & Smouse, 2006). The descriptive statistical analysis on host plant and geographic preference were performed using Microsoft® Excel 2007/XLSTAT©-Pro (Version 6.1.9,2003, Addinsoft, Inc., Brooklyn, NY, USA).

# CHAPTER FOUR

# RESULTS

## 4.1 Ecological characterisation - Host plant and geographical preference

Among the 1383 *F. schultzei* specimens observed from the thrips collections at *icipe*, the proportion of dark *F. schultzei* (67.46%) exceeded that of the pale *F. schultzei* form (32.53%). The dark colour form was predominant in Bungoma, Busia and Kakamega Counties, which are dry to sub-humid mid altitude regions. The proportion of pale *F. schultzei* population increased gradually towards the coast with high proportions in the low altitude hot and humid areas such as Mombasa and Kilifi. Counties with the highest propotions of pale *F. schultzei* were in the warm and dry medium altitude region such as in the Kitui, Makueni, Machakos Counties (Fig. 3).




n = Total number of insects per county

The host plants harbouring the highest proportion of dark *F. schultzei* were

**Figure 3.Proportion of dark and pale *Frankliniella schultzei* in different Counties of Kenya**

*Phaseolus* sp., *Cucurbita sp*., and *Ipomoea sp*. while those with the highest populations of pale forms were *Capsicum sp.*, *Senna sp.* and *Ajuga remota* Benth (Labiatae). Host plants that had nearly equal proportions of both colour forms of *F. schultzei* were *Bidens pilosa* L. 1753, *Brassica oleracea* L., *Citrulluslanatus* (Thunb.) Matsum & Nakai and *Solanum lycopersicum* L. (Fig. 4).

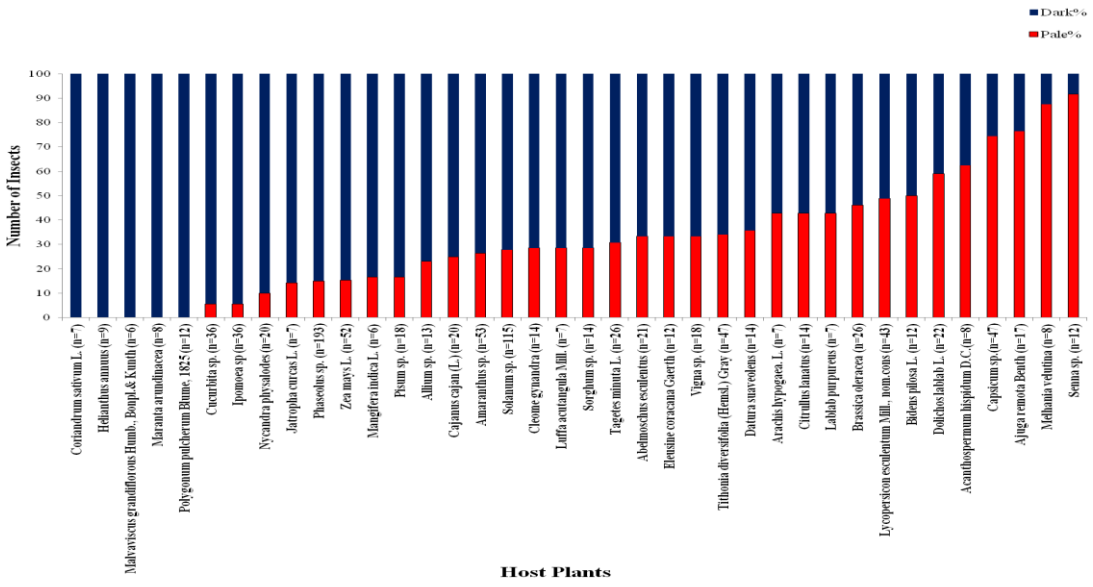



**Figure 4.Proportion of dark and pale forms of *Frankliniella schultzei* collected from different host plants in Kenya.**

The population of the dark colour form increased with altitude and was most prominent at altitudes of between 700-1900m. Above 1300m, the population of the pale colour form decreased as altitude increased (Fig. 5).

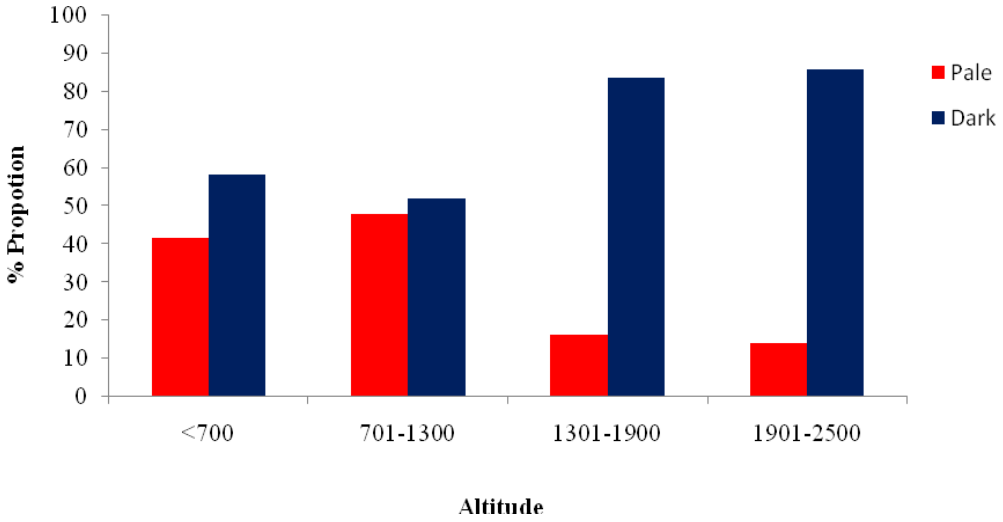



**Figure 5. Propotions of dark and pale forms of *Frankliniella schultzei* collected from plants at different altitudes in Kenya**

### 4.2 Biological characterisation - interbreeding

In asexual reproduction, virgin dark *F. schultzei* females produced only male F1 offspring while virgin pale *F. schultzei* produced only female F1 offspring, indicating a difference in parthenogenesis between the two forms. The control dark male × dark female cross produced both male and female dark F1 offspring at a ratio of 1:1 showing interbreeding, while pale female × dark male cross produced only female F1 offspring which resembled their pale mother; suggesting a lack of interbreeding (**Table** 2).

**Table 2. Interbreeding of *Frankliniella schultzei* colour forms**

| Parents | Sex of F1 offspring | Sex Ratio (Female: male) of F1 offspring | Colour of F1 offspring | *Sl*and *DfT*of F1 offspring |
|---|---|---|---|---|
| **Virgin dark female** | Male | 0:1 | All dark | Identical to parents |
| **Virgin pale female** | Female | 1: 0 | All pale | Identical to parents |
| **Dark male × dark female** | Female and male | 1:1 | All dark | Identical to parents |
| **Dark male × pale female** | Female | 1:0 | All pale | Identical to pale mother |

### 4.3 Morphological characterisation of *F. schultzei*

#### 4.3.1 Comparison of morphological features means

Analysis of the female parents revealed that there was significant difference between the mean of the two colour forms. Comparative analysis using Tukey HSD test indicated that the dark *F. schultzei* parents had significantly longer *Set1* (0.108 µm ± 0.004), *Set2* (0.123 µm ± 0.003), *Sl* (0.337 µm ± 0.005), *Terg10* (1.019 µm ± 0.012) as compared to the pale parents *Set1* (0.078 µm ± 0.005), *Set2* (0.086 µm ± 0.004), *Sl* (0.240 µm ± 0.007) and *Terg10* (0.900 µm ± 0.017). It was further observed that the dark parents had significantly shorter *Mls* (0.130 µm ± 0.002), *Fw* (0.117 µm ± 0.002) and *DfT* (0.059 µm ± 0.003) as compared to the pale parents *Mls* (0.145 µm ± 0.003), *Fw* (0.130 µm ± 0.003) and *DfT* (0.103 µm ± 0.004) (Table 3 & Fig. 6).

**Table 3. Mean length (± Standard Error) of seven quantitative morphological features of parental populations of the two colour forms of *Frankliniella schultzei***

| **Morphological features** | Mean length (µm) ± Standard Error* | |
|---|---|---|
| | **Dark parents** | **Pale parents** |
| 1st ocellar setae (*Set1)* | **0.108** ± 0.003 | **0.078±** 0.005 |
| 2nd ocellar setae *(Set2)* | **0.123** ± 0.003 | **0.086±** 0.004 |
| 3rd ocellar setae *(Sl)* | **0.337±** 0.005 | **0.240±** 0.007 |
| Major setae on tergite 10 *(Tegr 10)* | **1.019±** 0.012 | **0.900±** 0.017 |
| Metanotal lateral setae *(Mls)* | 0.130± 0.002 | 0.145± 0.003 |
| Width of the forewings *(Fw)* | 0.117± 0.002 | 0.130± 0.003 |
| Distance of the third ocellar setae from the tangent of anterior margin of the hind ocellar *(DfT)* | **0.059±** 0.003 | **0.103±** 0.004 |

*All the seven features were significantly different between the two forms of *F.schultzei* (P<0.0001); the most significantly different features are in bold

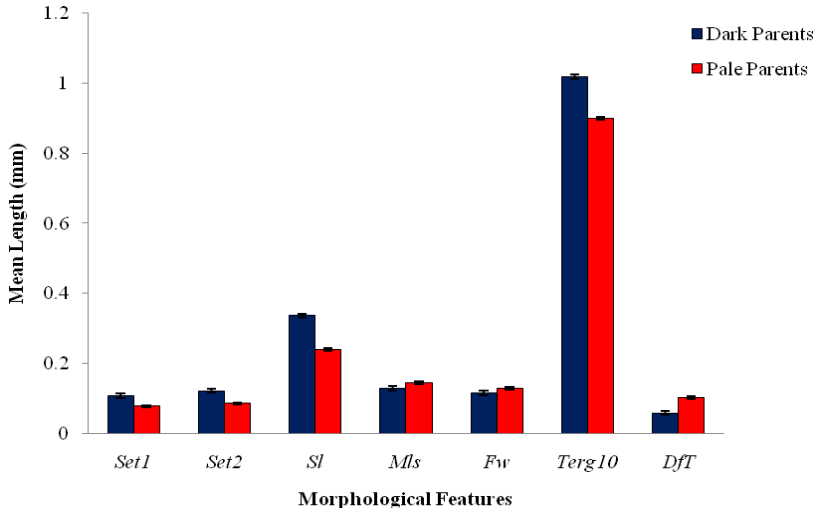



**Figure 6.Mean length (± Standard Error) of seven quantitative morphological features of parental populations of the two colour forms of *Frankliniella schultzei.***

Further analysis of parents compared to their F1 offspring revealed significant differences among the parents and F1 in *Sl* and *DfT*. Comparative analysis using the Tukey HSD test indicated that *Sl* of dark parents (0.337µm ± 0.005) was significantly longer than that of the F1 offspring of dark male × pale female cross **(**0.249 µm ± 0.012). Pale parents (0.240 µm ± 0.007) had significantly shorter *Sl* compared to F1 offspring of the dark male × dark female cross (0.353 µm ± 0.009). *Sl* was also significantly longer in the F1 offspring of the dark male × dark female cross (0.353 µm ± 0.009) than those from the dark male × pale female cross **(**0.249 µm ± 0.012). No significant differences were found in *Sl* between dark parents (0.337 µm ± 0.005) and dark male × dark female F1 offspring (0.353 µm ± 0.009) as well as between pale parents (0.240 µm ± 0.007) and dark male× pale female F1 offspring (0.249 µm ± 0.012) (Fig. 3).

The *DfT* was significantly shorter in dark *F. schultzei* parents (0.059 µm ± 0.003) as compared to the F1 offspring from the dark male × pale female cross (0.106 µm ± 0.009). Pale parents (0.103 µm ± 0.004) had significantly longer *DfT* than the F1 offspring of the dark male × dark female cross (0.062 µm ± 0.007). Dark male × dark female offspring (0.062 µm ± 0.007) had significantly shorter *DfT* than dark male × pale female F1 offspring (0.106 µm ± 0.009). No significant difference in *DfT* was found between dark parents (0.059 µm ± 0.003) and F1 offspring of the dark male × dark female cross (0.062 µm ± 0.007) or between pale parents (0.103 µm ± 0.004) and F1 offspring of the dark male × pale female cross (0.106 µm ± 0.009) (Table 4 & Fig. 7).

**Table 4. Mean length (± Standard error) of contrasting morphological features of dark and pale** *F. schultzei* females compared to their respective F1 offspring

| **Morphological features** | **Dark parents (µm)** | **Dark male × dark female F1** | **Pale parents (µm)** | **Dark female × pale female F1** |
|---|---|---|---|---|
| 3rd ocellar setae *(Sl)* | 0.337 ± 0.005 | 0.353 ± 0.009 | 0.240 ± 0.007 | 0.249 ± 0.012 |
| Distance of the third ocellar setae from the tangent of anterior margin of the hind ocellar *(DfT)* | 0.059 ± 0.003 | 0.062 ± 0.007 | 0.103 ± 0.004 | 0.106 ± 0.009 |

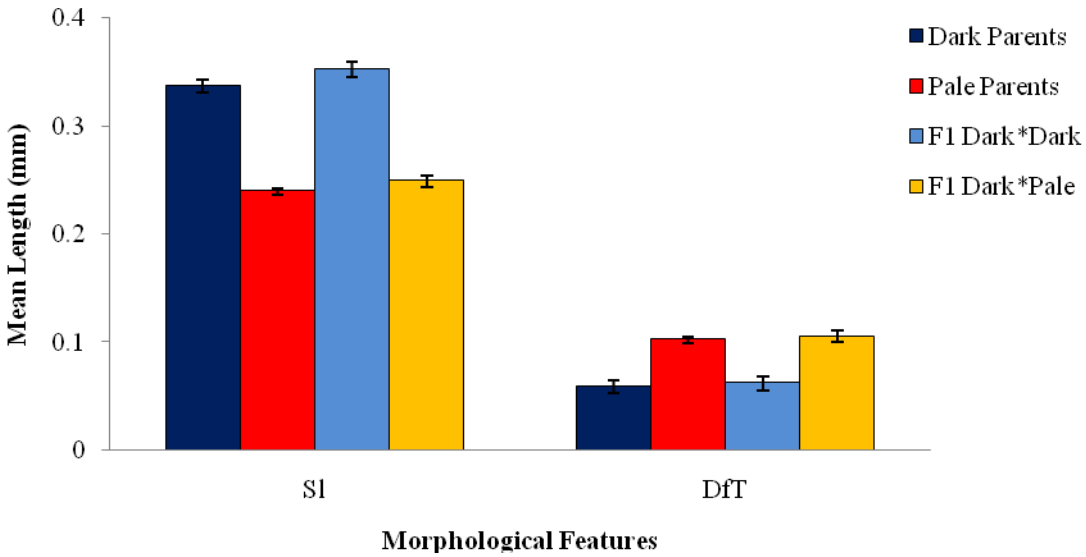



**Figure 7. Mean length (± Standard error) of contrasting morphological features of dark and pale *F. schultzei* females compared to their respective F1 offspring.**

Appearance of *Comb* in the dark form was either absent or incomplete with one or two pairs of marginal microtrichia while the pale form had an incomplete marginal *Comb* with three pairs of microtrichia (Fig. 2).

On the basis of the eight morphological features measured, no intermediary measurements were observed between dark and pale parents as well as between F1 offspring of both crosses.

### 4.3.2 Principal Component Analysis of Parents Morphometrics data

The morphological features measured were then subjected to Principle Component Analysis, where the first two principal components contributed to 94.1% of the total variance (PC1=86.9 and PC2=7.2). The scatter plot developed using the loadings generated by PC1 and PC2 showed significant difference between dark and pale *F. schultzei* parents as the two forms separated into two distinct clusters (Fig. 8). The four most different contributing features were the length of the major setae at tergite 10, length of the 3$^{rd}$ocellar setae, 2$^{nd}$ocellar setae length and distance of 3$^{rd}$ocellar

setae from tangent of anterior margin of hind ocellar, each contributing 79%, 52.5%, 19.3% and 18% towards the distinct clustering of the colour forms, respectively.

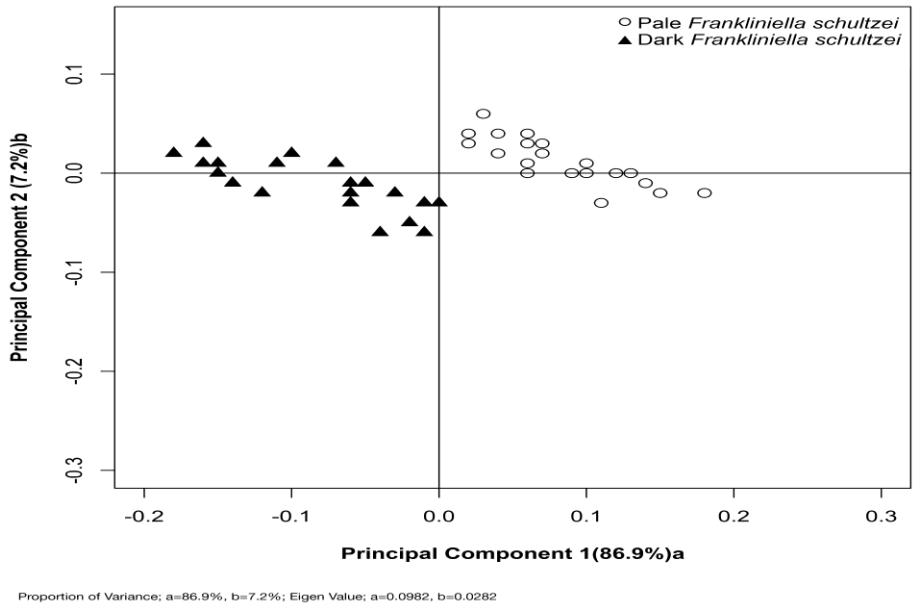



**Figure 8. Principal component analysis of morphometric characters states of parental dark and pale forms of *Frankliniella schultzei***

### 4.4 Morphological confirmation of Pale *F. schultzei* males in the wild

The morphology of the 13 *F. schultzei* males which were collected in the wild from *Ipomoea setosa* flowers was analyzed using Principal Component Analysis. From the graph that was generated, there was no significant difference between dark *F. schultzei* males from the colony of and those found in *I. setosa* flowers, as the two populations clustered together (Fig. 9).

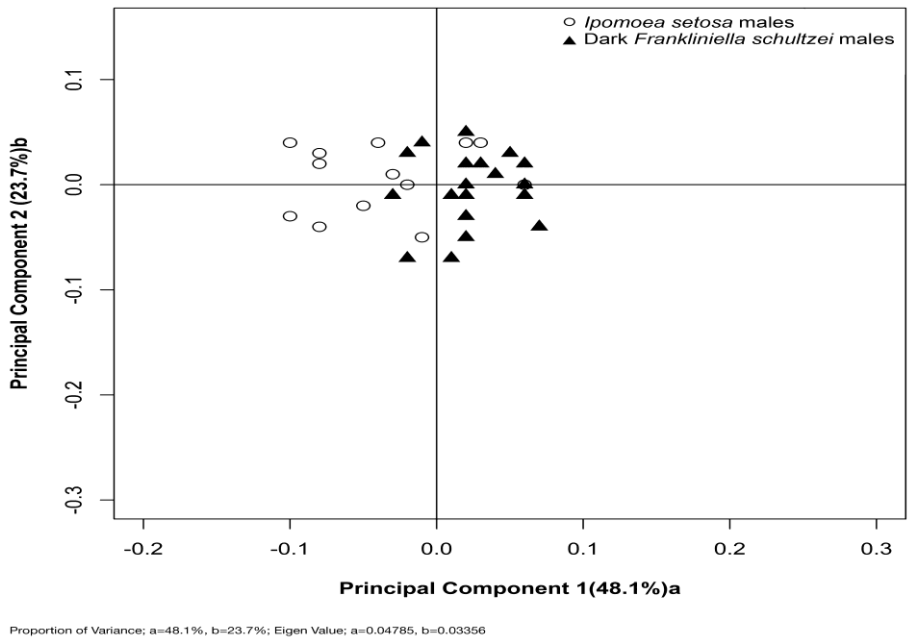



**Figure 9. Principal component analysis of eight morphometric characters of males from *Ipomoea Setosa* (ipom) and dark *Frankliniella schultzei* males from the laboratory colony (dark)**

### 4.5 Molecular characterisation

#### 4.5.1 DNA Extraction

DNA was successfully extracted from insects of both colour forms. The purity and concentration at 260:280 wavelengths was on average 1.85 and 10ng/µl respectively. However, a DNA pellet was not visible at the bottom of the microfuge tubes, possibly due to each sample being extracted from one insect, which is minute in size.

### 4.5.2 PCR analysis

The ITS2 region of both dark and pale *F. schultzei* parents produced fragments of ~700bp (Fig. 10). The concentration of the amplified product at 260:280 wavelengths was on average 38.34ng/µl and the purity of the product was 1.93.

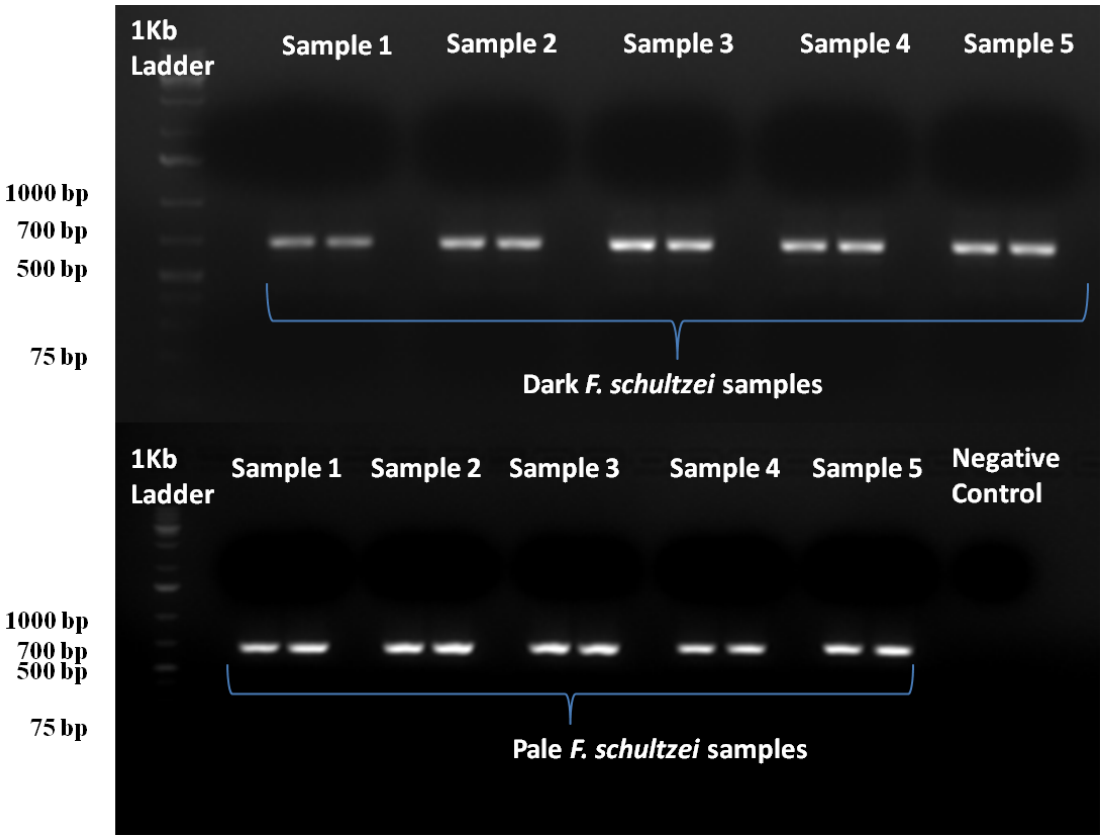



**Figure 10. Gel electrophoresis image of Dark F. schultzei and pale F. schultzei samples amplified using ITS2 primer pair ITS2F/ITS2R producing a 700 bp band. Each sample was loaded in the gel in duplicates.**

#### 4.5.2.1 ITS2-RFLP analysis

Digestion of amplicons both colour forms using *Alu*1, *Hinf*1, *Rsa1* and *Hae*III restriction enzymes yielded differences in banding patterns and fragment sizes between the two colour forms (Fig. 11).

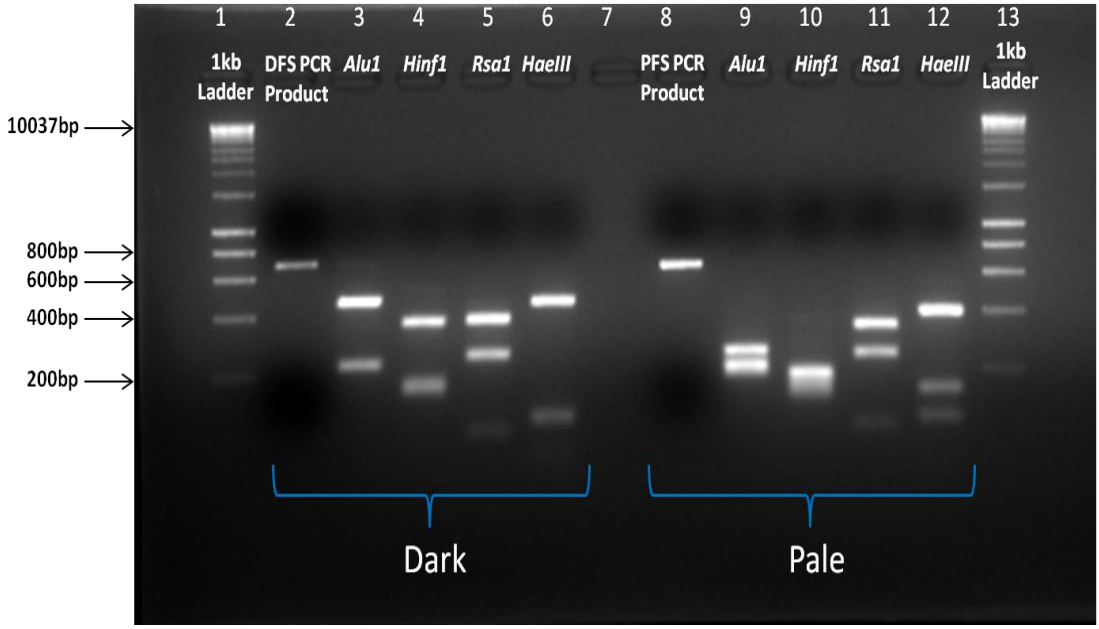



**Figure 11.ITS2-RFLP banding profile of dark and pale forms of Frankliniella schultzei.**

Where; Lanes 1 & 13 – 1Kb ladder; lanes 2 & 8 – ITS2 PCR Product of dark and pale forms respectively; lanes 3-6 & 9-12 – restriction of ITS2 region of dark and pale forms respectively using restriction enzymes Alu1, Hinf1, Rsa1 and HaeIII.

Restriction by *Alu1* produced two bands in both colour forms, with a common ~200bp band but the dark form had a second ~500bp band, while the second band for the pale form was ~250bp band. There were also differences in the bands produced by *Hinf1* and *Rsa1* in both colour forms, with both colour forms having similar first bands of ~150bp and ~250bp in these two enzymes, respectively. Restriction with *Hinf1* resulted in a second band of ~350bp for the dark form, while the pale form had a second band of ~200bp. Similarly, in

*Rsa1* the dark form had a second band of ~400bp while the pale form had a second band of ~350bp. Restriction with *HaeIII* resulted in a distinct band of ~150bp for the pale form which was absent in the dark form (Table 5)

#### 4.5.2.2 Nucleotide sequence analysis of ITS2 from dark and pale *F. schultzei*

**Table 5: Restriction enzymes and size of fragments produced by Dark and Pale**

| RESTRICTION ENZYME | COLOUR FORM | FRAGMENT SIZE (BASE PAIRS) | COMMENTS |
|---|---|---|---|
| *ALU1* | Dark *Frankliniella Schultzei* | ~200, 500 | Restricted |
| | Pale *Frankliniella Schultzei* | 200, ~250 | Restricted |
| *HINF1* | Dark *Frankliniella Schultzei* | ~150, 400 | Restricted |
| | Pale *Frankliniella Schultzei* | ~180, ~200 | Restricted |
| *RSA1* | Dark *Frankliniella Schultzei* | ~100, ~300, 400 | Restricted |
| | Pale *Frankliniella Schultzei* | ~100, ~300, ~400 | Restricted |
| *HAEIII* | Dark *Frankliniella Schultzei* | 100, 500 | Restricted |
| | Pale *Frankliniella Schultzei* | 100, ~150, 400 | Restricted |

**F.** *schultzei*

The 700bp amplicons of the 5 dark and 5 pale *F. schultzei* females were edited using Geneious version® 8.0.2. Upon querying their sequence similarity to previously known sequences in the NCBI nucleotide database using nBLAST (http://www.ncbi.nlm.nih.gov/blast), 4 samples from each colour form were found to have an identity of greater than 90% as well as an E-value of less than 0.00, hence were selected for further analysis. These 4 samples were then deposited in GenBank and were given the accession numbers KR105630 - KR105633 for dark forms and

accession numbers KR105634 - KR105637 for pale forms. Multiple alignments of sequences from the dark and pale forms of *F. schultzei* in Clustal W2 showed consistent nucleotide differences between the two forms (Fig. 12).

```
KR105632
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105633
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105631
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105630
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105634
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105635
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105636
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60

KR105637
TTTCGAACGCACATTGCGGCCCTGGGACTTGTTCCCGGGCCACACCTGACTGAGGGTCGG 60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KR105632
TAGACCATATCTCACGATACGACCAGACTGTCCTCTCTTCGGAGAGGCGAGAATGGAGTT 120

KR105633
TAGACCATATCTCACGATACGACCAGACTGTCCTCTCTTCGGAGAGGCGAGAATGGAGTT 120

KR105631
TAGACCATATCTCACGATACGACCAGACTGTCCTCTCTTCGGAGAGGCGAGAATGGAGTT 120
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KR105630
TAGACCATATCTCACGATACGACCAGACTGTCCTCTCTTCGGAGAGGCGAGAATGGAGTT 120

KR105634
TAGACTATATCTCACGATACGACCAGACTGTCCTCTCCTCGGAGAGGCGAGAATGGAGTC 120

KR105635
TAGACTATATCTCACGATACGACCAGACTGTCCTCTCCTCGGAGAGGCGAGAATGGAGTC 120

KR105636
TAGACTATATCTCACGATACGACCAGACTGTCCTCTCCTCGGAGAGGCGAGAATGGAGTC 120

KR105637
TAGACTATATCTCACGATACGACCAGACTGTCCTCTCCTCGGAGAGGCGAGAATGGAGTC 120

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KR105632                              TTCCGAGGCAAGATGGGCCCTCCCCGATTAGTTTCG-
GCGTGTGGGGCCCCTTGCGC-GG 178

KR105633                              TTCCGAGGCAAGATGGGCCCTCCCCGATTAGTTTCG-
GCGTGTGGGGCCCCTTGCGC-GG 178

KR105631                              TTCCGAGGCAAGATGGGCCCTCCCCGATTAGTTTCG-
GCGTGTGGGGCCCCTTGCGC-GG 178

KR105630                              TTCCGAGGCAAGATGGGCCCTCCCCGATTAGTTTCG-
GCGTGTGGGGCCCCTTGCGC-GG 178

KR105634                                        TTCCGAGGCGGGGAGAGG-------
GAGAGTTTTCATGCTCTCTCTCTCACTCGCCCTGG 173

KR105635                                        TTCCGAGGCGGGGAGAGG-------
GAGAGTTTTCATGCTCTCTCTCTCACTCGCCCTGG 173

KR105636                                        TTCCGAGGCGGGGAGAGG-------
GAGAGTTTTCATGCTCTCTCTCTCACTCGCCCTGG 173

KR105637                                        TTCCGAGGCGGGGAGAGG-------
GAGAGTTTTCATGCTCTCTCTCTCACTCGCCCTGG 173
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KR105632                                 TACTCTTAAAGCACGAGGCGTTGGCGCGGCGGAG--
GAGAGAGAAATTGATTTTTCTCCG 236

KR105633                                 TACTCTTAAAGCACGAGGCGTTGGCGCGGCGGAG--
GAGAGAGAAATTGATTTTTCTCCG 236

KR105631                                 TACTCTTAAAGCACGAGGCGTTGGCGCGGCGGAG--
GAGAGAGAAATTGATTTTTCTCCG 236

KR105630                                 TACTCTTAAAGCACGAGGCGTTGGCGCGGCGGAG--
GAGAGAGAAATTGATTTTTCTCCG 236

KR105634
TACTCTTAAAGCTCGAAGCGCTGCCGCGGCGGAGTTGAGAGAAAAAATTTTTTTTTTTTCT 233

KR105635
TACTCTTAAAGCTCGAAGCGCTGCCGCGGCGGAGTTGAGAGAAAAAATTTTTTTTTTTTCT 233

KR105636
TACTCTTAAAGCTCGAAGCGCTGCCGCGGCGGAGTTGAGAGAAAAAATTTTTTTTTTTTCT 233

KR105637
TACTCTTAAAGCTCGAAGCGCTGCCGCGGCGGAGTTGAGAGAAAAAATTTTTTTTTTTTCT 233

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KR105632                                                                 ----
TCTTCTCCGTACCGCGTCGGCGTCGCCGTTTGATCAGCCGTCTCTGCATTCCGGAG 292

KR105633                                                                 ----
TCTTCTCCGTACCGCGTCGGCGTCGCCGTTTGATCAGCCGTCTCTGCATTCCGGAG 292

KR105631                                                                 ----
TCTTCTCCGTACCGCGTCGGCGTCGCCGTTTGATCAGCCGTCTCTGCATTCCGGAG 292
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KR105630                                                              ----
TCTTCTCCGTACCGCGTCGGCGTCGCCGTTTGATCAGCCGTCTCTGCATTCCGGAG 292

KR105634              TTGCTCTTCTCCGTACCGCGGGGGTGTCCCCAATTGATCAGTCGTCTCT--
TGACCGGAG 291

KR105635              TTGCTCTTCTCCGTACCGCGGGGGTGTCCCCAATTGATCAGTCGTCTCT--
TGACCGGAG 291

KR105636              TTGCTCTTCTCCGTACCGCGGGGGTGTCCCCGATTGATCAGTCGTCTCT--
TGACCGGAG 291

KR105637              TTGCTCTTCTCCGTACCGCGGTGGTGTCGCCGATTGATCAGTCGTCTCT--
TGACCGGAG 291

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:******

KR105632
CGACTGAGTCCTGTGGCTTGTGAAACGAGCAGTGCGCGACGCAGTCACCTTCTGCGCCGC 352

KR105633
CGACTGAGTCCTGTGGCTTGTGAAACGAGCAGTGCGCGACGCAGTCACCTTCTGCGCCGC 352

KR105631
CGACTGAGTCCTGTGGCTTGTGAAACGAGCAGTGCGCGACGCAGTCACCTTCTGCGCCGC 352

KR105630
CGACTGAGTCCTGTGGCTTGTGAAACGAGCAGTGCGCGACGCAGTCACCTTCTGCGCCGC 352

KR105634                                                  CGACCGAGTCC-
GTGGCTTGTAAAATGGGCAGGGGGCAACGCAGTCCCCTTCTGCGTTGC 350

KR105635                                                  CGACCGAGTCC-
GTGGCTTGTAAAATGGGCAGGGGGCAACGCATTCCCCTTCTGCGTTGC 350

KR105636                                                  CGACCGAGTCC-
GTGGCTTGTAAAATGGGCAGTGGGCAACGCAGTCCCCTTCTGCGTTGC 350

KR105637                                            CGACCGAGTCC-GTGGCTTG-
TAAATGGGCAGTGCGCAACGCAGTCACCTTCTGCGTTGC 349

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```
KR105632                                                              GCCGCATCAG-
AAACACCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTTCCAGGACT 411

KR105633                                                              GCCGCATCAG-
AAACACCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTTCCAGGACT 411

KR105631                                                              GCCGCATCAG-
AAACACCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTTCCAGGACT 411

KR105630                                                              GCCGCATCAG-
AAACACCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTTCCAGGACT 411

KR105634
CCCCCATCAAAACCCCCCCCCACCGGTGTCCTGGCCCATCCTATCACCTTGGGTCGGGACT 410

KR105635
CCCCCATCAAAACCCCCCCCTACCGGTGTCCTGGCACATCCTATCAGCTTGGGTCGGGACT 410

KR105636
GCCGCATCAAAACCCCCCCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTGTCGGGACT 410

KR105637
GCCGCATCAGAACACCCCCTACCGGTGTCCTGGCACATCCTATCAGCTTGTGTCGGGACT 409

                  **   *****.   *..*.***   **************.**********   ****
*.*****



KR105632                                                                T---
GGGATATTCGTATTGATGCGTCGAGCCACACTCAAAAGGACTCTTTCCGTCTCTCG 468

KR105633                                                                T---
GGGATATTCGTATTGATGCGTCGAGCCACACTCAAAAGGACTCTTTCCGTCTCTCG 468

KR105631                                                                T---
GGGATATTCGTATTGATGCGTCGAGCCACACTCAAAAGGACTCTTTCCGTCTCTCG 468

KR105630                                                                T---
GGGATATTCGTATTGATGCGTCGAGCCACACTCAAAAGGACTCTTTCCGTCTCTCG 468
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KR105634
TTGGGGGATATTCGTATTGATGCTTCAGGCCACAAAAGGAAGGACTCGGTTGTTTTTTTG 470

KR105635
TTGGGGGATATTCGTATTGATGCTTCAGGCCACAAAAGGAAGGACTCGGTTGTTTTTTTG 470

KR105636
TTGGGGGATATTCGTATTGATGCTTCAGGCCACAAAAGGAAGGACTCGGTTGTTTTTTTG 470

KR105637
TTGGGGGATATTCGTATTGATGCTTCAGGCCACAAAAGGAAGGACTCGGTTGTTTTTTTG 469

                *   ****************** **..******.:...********  *   * *
* *



KR105632        CG---ATGC 474

KR105633        CG---ATGC 474

KR105631        CG---ATGC 474

KR105630        CG---ATGC 474

KR105634        CGGGCATGC 479

KR105635        CGGGCTTGC 479

KR105636        CGGGCATGC 479

KR105637        CGGGCATGC 478

                **   :***
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**Figure 12. Multiple sequence alignment of a 498bp section from the ITS2 region. Dark (KR105630 – KR105633) and pale(KR105634 – KR105637) *Frankliniella schultzei* sequences**

**Distance estimates from the sequences were then used to generate a Neighbour-joining tree which separated the dark and pale *F. schultzei* into two clades (Fig. 13).**

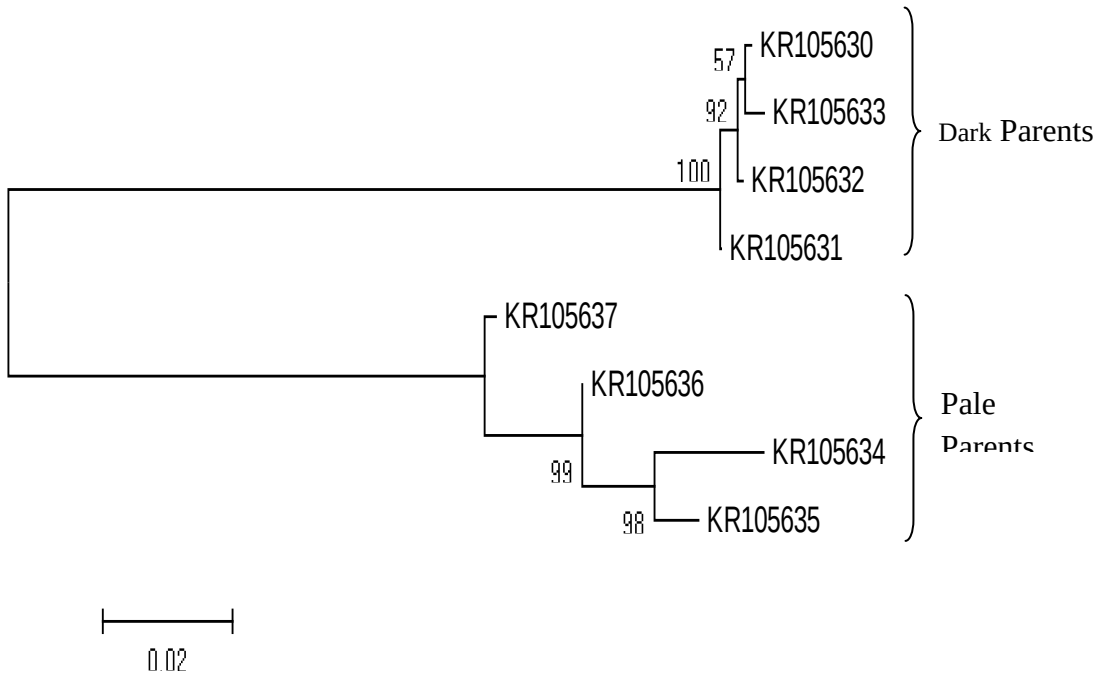


**Figure 13. Unrooted neighbour-joining tree of Frankliniella schultzei constructed from Kimura two-parameter distance model.** Dark (KR105630 – KR105633) and pale (KR105634 – KR105637).

The genetic distance between the two colour forms was also calculated and it ranged from 18% to 23%, which is a significant distance, indicating that they may be separate species. The average distance between the individuals within the same colour form was estimated between 0% and 1% among the dark forms and 0% and 4% among the pale forms, respectively (Table 6).

**Table 6. Genetic distances of *Frankliniella schultzei* ITS2 sequences. Dark (KR105630 – KR105633) and pale(KR105634 – KR105637).**

| | **KR105630** | **KR105632** | **KR105633** | **KR105631** | **KR105634** | **KR105635** | **KR105636** | **KR105637** |
|---|---|---|---|---|---|---|---|---|
| **KR105630** | 0.00 | | | | | | | |
| **KR105632** | 0.00 | 0.00 | | | | | | |
| **KR105633** | 0.00 | 0.01 | 0.00 | | | | | |
| **KR105631** | 0.01 | 0.00 | 0.01 | 0.00 | | | | |
| **KR105634** | 0.23 | 0.23 | 0.23 | 0.23 | 0.00 | | | |
| **KR105635** | 0.22 | 0.22 | 0.22 | 0.22 | 0.02 | 0.00 | | |
| **KR105636** | 0.20 | 0.20 | 0.20 | 0.20 | 0.03 | 0.02 | 0.00 | |
| **KR105637** | 0.19 | 0.19 | 0.19 | 0.18 | 0.04 | 0.04 | 0.02 | 0.00 |

For a better graphical view of the differences in genetic distance, the principal component analysis graph of the genetic distances (Fig. 14) shows the two colour forms separating into two distinct clusters.

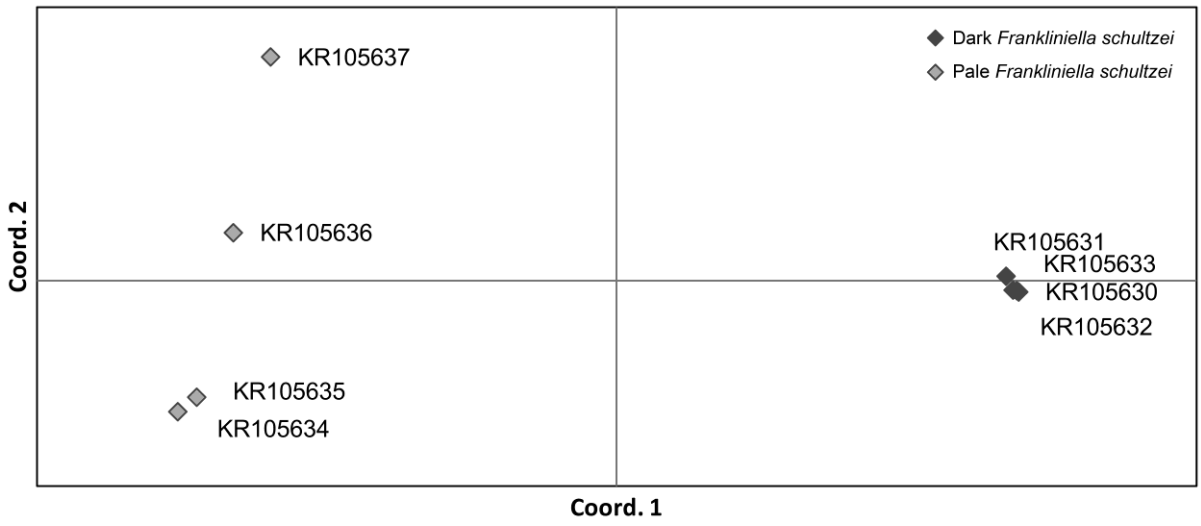


**Figure 14. Principal component analysis of genetic distances between dark (KR105630 – KR105633) and pale (KR105634 – KR105637) forms of *F. schultzei.***

### 4.6 Molecular confirmation of Pale *F. schultzei* males in the wild

The results of the morphological comparison of dark *F. schultzei* males in the colony to those in the wild described in section 4.4 were also supported using molecular techniques. This was by comparison of the ITS2-RFLP banding pattern produced by restriction of the ITS2 region of males collected from *I. setosa* flowers to those of males from the dark *F. schultzei* colony. This was done using restriction enzyme *Alu*1 (Thermo Scientific, Inc). All wild males had banding patterns identical to those of dark males in the laboratory colony (Fig. 15) indicating a possible absence of pale males in the wild.

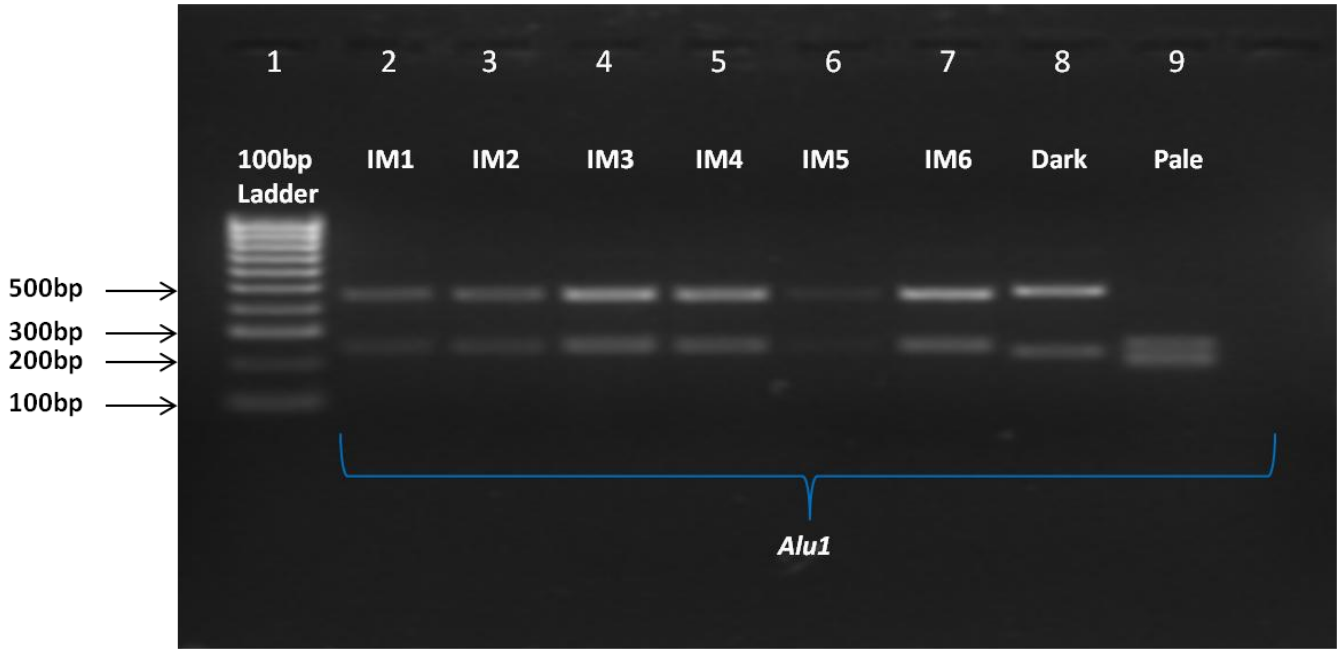


**Figure 15. ITS2-RFLP banding profile of males from Ipomoea setosa. Where; Lane 1 – 100bp ladder; lane 2-7 – Restricted ITS2 region of males from *Ipomoea setosa* digested using restriction enzyme *Alu1*; lane 8 – dark *F. schultzei* male from colony; lane 9 – pale *F. schultzei* female from colony.**

# CHAPTER FIVE

# DISCUSSION

This is the first study to characterise the dark and pale forms of *Frankliniella schultzei* based on ecological, biological, morphological and molecular methods. The ecological characterisation provided an initial understanding on the distribution of two colour forms as it appears to be influenced by the climate and host plant. The differences in agro-ecological preferences of the two colour forms could be related to their host plant preference. Host plants such as beans (*Phaseolus* spp) and Cucurbits (*Cucurbita* spp.) that are widely grown in high altitude regions with cool temperatures were predominantly infested by the dark forms of *F. schultzei*. However, plants such as *Capsicum* sp., *Senna* sp. and *Ajuga remota* Benth which thrive well in the low altitudes with warm and humid conditions were predominantly infested by the pale forms. Differences in prevalence of pale and dark *F. schultzei* could have been also influenced by variations in temperature in the different agro ecologies. For instance, Murai & Toda (2001) reported that at lower rearing temperatures, *Thrips tabaci* adults were darker as compared to paler thrips when reared at higher temperatures. Similarly in montane areas often characterized by cool temperatures, the dark forms of *F. occidentalis* were frequently observed (Hoddle *et al.*, 2008). Dark forms of *F. occidentalis* were more abundant during the cooler months, while pale forms were abundant during the warmer months (Elimem *et al.*, 2011).

Following the differences in geographical distribution, the breeding experiments revealed that the two colour forms of *F. schultzei* exhibit different parthenogenetic reproduction, as virgin dark females produced male offspring while virgin pale females produced only female offspring. The emergence of male progenies from virgin dark females indicates an arrhenotokous form of parthenogenetic reproduction in dark *F. schultzei* which could be due to endosymbionts such as *Wolbachia spp* (Kumm & Moritz, 2008) which are known to influence parthenogenetic reproduction. This needs to be further investigated with the two colour forms of *F. schultzei.* On the other hand, emergence of only female progenies similar to the

female parent in dark male × pale female crosses indicates the absence interbreeding between the colour forms and female progenies only similar to the mother indicates theletokous parthenogenetic reproduction. In this study, evidence of pale *F. schultzei* males was lacking both in the colony and in the wild. The absence of males needs to be further confirmed on other host plants and regions. Circumstantial evidence for the absence of males in *F. schultzei* is given by Wijkamp *et al.*, (1995) where the population of pale *F. schultzei* used for transmission studies had only females, while the population of dark *F. schultzei* had both males and females. The absence of pale males and emergence of female progenies from virgin pale female indicates a thelytokous form of parthenogenetic reproduction in pale *F. schultzei*. The allopatric distribution between the dark and pale forms of *F. schultzei* (Sakimura, 1969) make Kenya, Uganda, Sudan, India, New Guinea and Puerto Rico the regions where both colour forms can be found (Mound, 1968; Kakkar *et al.*, 2014) and this could be the reason behind the lack of potential to interbreed between the two colour forms. In allopatric populations, genetic divergence usually leads to evolution of barriers (Gavrilets, 2004) which in turn could become a hindrance to interbreeding and reproductive isolation, therefore contributing to speciation (Bush, 1975). The allopatric distribution of the two colour forms might have created differences in their mate-choosing response systems, creating a pre-mating barrier and eventually preventing interbreeding within the same ecosystem (Templeton, 1989; Grant & Grant, 2009) as observed in this study. In recent studies with *F. occidentalis*, Rugman-Jones *et al.*, (2010) found evidence of reproductive isolation in biologically and morphologically diverse sympatric populations of *F. occidentalis*. This was based on differences in nuclear as well as mitochondrial gene sequences, inferring that the diverse populations could be two cryptic species.

Differences in interbreeding between the two colour forms of *F. schultzei* were further supported by the analysis of eight morphometric character states undertaken in this study which revealed significant differences between the two colour forms. The length of the major setae at tergite 10, length of the 3$^{rd}$ and 2$^{nd}$ocellar setae and distance of 3rd ocellar setae from the tangent to the anterior margin of hind ocellar were key character states that differentiated the two colour forms. Subtle variations

in the morphological character states of thrips has been used to differenciate species (Mound, 2002). Within thrips species, a high degree of variability in morphological character states have been reported in *Frankliniella occidentalis* (Pergande) (Chumak, 2014; Cluever *et al.*, 2015) and *Scirtothrips dorsalis* Hood (Rugman-Jones *et al.*, 2006; Hoddle *et al.*, 2008; Dickey *et al.*, 2015). Similarly populations of some thrips species have been shown to differ in their modes of reproduction and host associations, as has been shown for *Thrips tabaci* (Jenser & Szénási, 2004; Jenser *et al.*, 2006; Westmore *et al.*, 2013; Nault *et al.*, 2014).

The common blossom thrips, *F. schultzei* also exhibits high degree of variability in terms of morphological characters (Johansen, 2002; Cavalleri & Mound, 2012) and populations have been grouped into pale and dark colour forms (Mound, 1968). However the taxonomic status of the two colour forms of *F. schultzei* has been a subject of debate among thrips biologists. Some biologist, such as Bhatti *et al.* (2009), considered the pale form of *F. schultzei* as a separate species, *Frankliniella sulphurea* Schmutz, 1913. Others consider the two colour forms as one species since they occur sympatrically with overlapping host plants (Hoddle *et al.*, 2008; Mound, 1968). From this study, clear morphological differences could be demonstrated between the pale and dark forms of *F. schultzei*.

v. In addition to differences for morphological character states, the two colour forms of *F. schultzei* exhibited significant variations at molecular level with genetic distances of up to 23% between them, grouping them into two distinct clades. This genetic distance is also higher than the 3% and 6% distances that have been previously documented by Hebert *et al.* (2003) and Rubinoff *et al.* (2006) to infer species differentiation, In addition, differences in genetic distance has been shown to clearly distinguish between thelytokous (parthenogenetically reproducing) populations of *Thrips tabaci* from the arrhenotokous (sexually reproducing) populations, irrespective of geographical distribution (Jacobson *et al.*, 2013). Sequence analysis has also been able to clearly distinguish 10 thrips species, noting that the genetic divergence between insects within the same species was

very low but genetic divergence between different species was high at 16-27.5% (Brunner *et al.*, 2002). In addition to the sequence divergence, the high level of molecular differences between the two colour forms of *F. schultzei* in the ITS regions strongly indicates that they are two different species. Considering the fact that the ITS region has a high inter-specific variability and low intra-specific variability (Moritz *et al.*, 2001), a change within this region is considered significant in differentiating the two colour forms. Moreover, the non-coding and highly evolving ITS2 region has been previously used for the identification of closely related thrips species such as *Frankliniella occidentalis*, *Frankliniella intonsa* (Trybom), *Thrips hawaiiensis* Morgan, *Thrips tabaci* Lindeman, and *Scirtothrips dorsalis* Hood (Toda & Komazaki, 2002; Rugman-Jones *et al.*, 2006; Farris *et al.*, 2010).

The morphometric, molecular, ecological and host plant differences between the dark and pale forms of *F. schultzei* together with their inability to interbreed lead us to conclude that the colour forms represent distinct species. Further investigation on the presence/absence of pale *F. schultzei* males in different regions and host plants, factors influencing the modes of parthenogenetic reproduction, differential response to climatic variables and vector competence can further provide valuable insights into the divergence of the two species.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATION

## 6.1 Conclusion

In conclusion, the results of this study found that:

1. The two colour forms of *F. schultzei* differ in their geographical distribution as well as in their host plant preferences, with the pale form being prevalent in the hot and humid lowlands, while the dark form is more prevalent in the cool, humid and wet regions.
2. The dark and pale forms also differ in their morphology as this study highlighted four key morphological features that are significantly different between the two forms; the length of the major setae at tergite 10, length of the $3^{rd}$ and $2^{nd}$ocellar setae and distance of 3rd ocellar setae from the tangent to the anterior margin of hind ocellar
3. The two forms differ in their parthenogenetic modes as dark virgins get only male offspring (arrhenotoky) while pale virgins have only female offspring (thelytoky). This is considered a significant difference in breeding patterns between the two forms. The lack of any males in the pale colony as well as the environment and the lack of interbreeding between the two forms is also a significant difference indicating that they are two separate species.
4. The molecular differences; the fact that the two colour forms of *F. schultzei* clade separately, have a genetic distance divergence of upto 23%, and the difference in RFLP banding pattern between the parents of the two forms is an indication that the two colour forms of *F. schultzei* are two different species.

## 6.2 Recommendations

This study therefore recommends that:

1. A more intense study on the distribution of *F. schultzei* and ecological variation between the two colour forms should be conducted, including other countries in the East African region.
2. Further investigation should be conducted on the presence of pale males in the wild; if it is a local or worldwide occurrence
3. Further morphological and molecular analysis needs to be conducted on the offspring generated from crossings of the two colour forms to establish whether there is any genetic transfer between the two forms as well as intermediate morphological features in the offspring.

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