University of Reading

Morphological and Molecular Characterization of the House Dust Fauna and its Forensic Importance for the Kingdom of Saudi Arabia

A thesis submitted by

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Dedication

*To my parents**, Salha** and **Hussain** who gave me great support and encouragement throughout my life, thank you for always being there for me during the good and bad. Thank you for always believing in me, even when I didn't believe in myself. I could have never accomplished this without you.*

*To my brilliant wife, **Alaa**, and my children **Rital**, **Ramiz** and **Rifal**, for their sacrifice and motivation to continue on this path. You have been a constant source of support and encouragement throughout these years. You have pushed me in ways I could not have imagined and made me stronger. Without you, none of this would have been possible.*

*To all my friends, specially **Dr Hani**, **Dr Othman**, **Dr Faris, Dr Ali, Dr Abdurahman** and **Dr Mohammed**. Whose friendship, motivation and positivity kept me going.*

*To my country **Saudi Arabia**, that gave me the chance and financial support to follow my dream.*

Declaration

I, Riyadh Aeban, confirm that this is my own work and that the use of all material from other sources has been properly and fully acknowledged.

Riyadh Aeban

Reading, 2020

Abstract

Over 200 mite species, known collectively as House Dust Mites (HDM), are highly synanthropic, living specifically in human dwellings, in mattresses and other upholstered furniture. Their forensic value is enhanced by their microhabitat specificity. Therefore, mites are omnipresent, yet very environmental specific. This applies also but not exclusively to rape or sexual assault cases, since they have a preference for human secretions, including semen and saliva.

The correct and accurate identification of mites is key in the analysis of trace evidence. House dust mite species can be linked to indoor crimes and may provide clues on the crime itself. The acaro-fauna of dwellings in the Kingdom of Saudi Arabia (KSA), its biodiversity in general and for Pyroglyphydae (Astigmata) mites in particular have been studied in this research in three climatic regions. The highest abundance of a diverse fauna of Pyroglyphidae mites was found in the Coastal Area and the Highlands. One particular morpho-species was identified as new and it is described in chapter 2. *Dermatophagoides saudi* sp. nov, was specific to one geographical region in KSA (chapter 3). When associated with a crime, this mite can act as a forensic marker of location, provenance.

Moreover, in the investigation of crime scenes and during the forensic analysis of traces the use genetics tools, and specifically microsatellites to identify populations that can link to suspects, victims or locations use more often non-human biological trace evidence; and mites (Acari) are the most ubiquitous traces in crime scenes. Microsatellites are genetic markers characterised by multiple repeats of short nucleotide strings. Perhaps the most challenging step into utilising the house dust fauna as trace evidence is the collection of reasonable number of mites as well as the amounts of mite DNA, that will allow down-stream molecular reactions -especially considering the small size of the mites and their samples. This study addressed this challenge and three preservation methods of HDM, for their further DNA extraction and microsatellite studies were considered (Chapter 4). Clearly the method that considers the direct homogenisation of mites from dust, without fixatives was the most promising. DNA of mite specimens was extracted successfully from one and multiple mites (pooled) (Chapter 5). However, the quality and quantity of DNA varied and is positively correlated to the number of mites pooled.

Microsatellite markers previously described for the most ubiquitous domestic mite, *Dermatophagoides farinae* (Acari, Pyroglyphidae) have been therefore tested in laboratory reared *D. farinae* (*Df*), and in *D. farinae*, *D. pteronyssinus* and *D. saudi* sp. nov. from wild colonies, from UK and KSA (Chapter 5). Therefore, cross species specificity was also tested. Primers and their microsatellite regions (as identified previously on the genome) were tested using 125 previously defined primer pairs for *D. farinae*. Varying PCR conditions were tried. Sixty three percent (63%) of the primers correctly amplified, for the first time microsatellites for *D. farinae*. A lower number of primers were also successful for the other two species of *Dermatophagoides*, including the new species from KSA. The idea is to enable future research to determine if mite population genotypes can be linked to specific houses, localities and to hosts from different cultural and geographical backgrounds.

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Foremost, all praises to **Allah** for his merciful guidance throughout my life and during this PhD.

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Finally, I wish to express my genuine appreciation to the **general department of Medical services – Ministry of interior, Royal Embassy of Saudi Arabia and Saudi Cultural bureau in London** for funding and supporting my PhD studies at the University of Reading.

Table of Contents

List of Figures

List of Tables

List of abbreviations

HDM	**House Dust Mites**
D. farinae / Df	*Dermatophagoides farinae*

D. pteronyssinus / Dp	*Dermatophagoides pteronyssinus*
T. longior / Tl	*Tyrophagus longior*
T. putrescentiae / Tp	*Tyrophagus putrescentiae*
Dermatophagoides sp. nov.; Dermatophagoides saudi sp. nov.; D. saudi sp. nov.	Putative new species of mite, from KSA- with similar characteristics to *D. pteronyssinus and D. evansi (Dermatophagoides saudi sp. nov.)*
Lab-*Df*	Colony (laboratory) reared *Dermatophagoides farinae*
UK-5 ….	Mite sample 5 from the UK
C1, C2 ….	The colonies code
MS	Microsatellite / Microsatellites
MS #	Microsatellite number
N-ALL	Null Allele
E.EXT	Ethanol extraction
F.EXT	Freezing extraction
L.EXT	Living ATL extraction
KSA	Kingdom of Saudi Arabia
UK	United Kingdom of Great Britain and Northern Ireland
Conventions used when referring to MS primers	
1F1, 1F2 …	*Df* microsatellite no. 1, Forward primer 1, 2, etc
1R1, 1R2 …	*Df* microsatellite no. 1, Reverse primer 1, 2, etc
5F1R2 ….	*Df* microsatellite no. 5, Forward primer 1, Reverse primer 2, etc
45bFR ….	*Df* microsatellite no. 45b, Forward primer, Reverse primer , etc
CA	Correspondence Analysis

Work related to this research

- Oral presentation at the University of Reading for Saudi society on 23th February 2018, titled "Characterization of Microsatellite loci developed for *Dermatophagoides farinae*".

- Oral presentation at University of Reading for SBS and PhD students on 13th December 2018, titled "Forensic Medicine" and "Characterization of Microsatellite loci developed for *Dermatophagoides farinae* and novel identification of Saudi House Dust Mites of forensic importance".

- A number of medical and biological activities at the University of Reading organized for PhD students during while I was a volunteering member of the Saudi society, such as poster competition (18/03/2019).

- Genetic, Biological, Forensic, Medical and Molecular courses from WHO, Harvard University, open university and virtual medical academy.

- I received the Red award prize 2018/19 at University of Reading for these activities.

List of presentations

Research Poster competition 20 June 2017, Doctoral Research Conference 2017, University of Reading, titled "Microsatellites and population genetics of house dust mites and their application un forensic analyses".

Three-Minute Thesis (3MT) Competition 20 June 2017, Doctoral Research Conference 2017, University of Reading, titled "Microsatellites and population genetics of house dust mites and their application un forensic analyses".

SBS Symposium 13th June 2017 (Poster session), University of Reading, titled "Microsatellites and population genetics of house dust mites and their application un forensic analyses".

Oral presentation at the SBS Symposium, School of Biology, University of Reading on 26th June 2018, titled "Characterization of Microsatellite loci developed for *Dermatophagoides farinae* and novel identification of Saudi House Dust Mites".

Oral presentation at the SBS Symposium, School of Biology, University of Reading on 25th June 2019, titled "Characterization of microsatellite loci developed for *Dermatophagoides farinae* and identification of Saudi House-Dust-Mites (HDM) of potential forensic importance".

Poster presentation at the doctoral Research Conference 2019, University of Reading on 19th June 2019, titled "Characterization of microsatellite loci developed for *Dermatophagoides farinae* and novel identification of Saudi House-Dust-Mites (HDM) of forensic importance". I was awarded the prize for best poster presentation.

Aeban, R., & Perotti, A. (2019). Characterization of microsatellite loci developed for *Dermatophagoides farinae* and novel identification of Saudi House-Dust-Mites (HDM) of forensic importance. A poster presentation at the Genetics society, A century of Genetics conference, 2019 Genetics Society Meeting, Royal College of Physicians, Edinburgh.

Chapter 1 : Morphological and Molecular studies of House Dust Mites

1.1 House-dust fauna

With the improvement of domestic living conditions and the presence of domestic pets, the incidence of micro-arthropods in homes is rising worldwide, and as a consequence, they are causing harm to humans, being the causative agents of allergies (Colloff, 2010). Arthropods are invertebrates from the phylum Arthropoda, they have segmented bodies, exoskeleton, and paired jointed appendages that include mouthparts, legs and antennae. Arthropoda is the largest animal phylum representing a percentage of 82% (Zhang, 2013). They include insects, millipedes, arachnids, crustaceans, and centipedes. It has been reported that insects are the first most abundant class in dwellings, representing a high percentage of 80% of house dust fauna, followed by arachnids with a percentage of 8.77% (Zhang, 2013, Madden et al., 2016). Although arthropods coexist and evolve with humans in the houses, we know little about them historically (Bertone et al., 2016). Only a few studies have been conducted on arthropod associated with houses in order to explore their diversity (using morphological differences and biodiversity) (Bertone et al., 2016, Madden et al., 2016, Mullen and OConnor, 2019).

1.1.1 Acari

Acarology is the study of Acari, which includes ticks and mites. They are Arachnids and they belong to the phylum Arthropoda (Colloff, 2010, Krantz and Walter, 2009). Mites are generally less than a millimetre in size, whereas ticks are slightly larger (up to 3 cm). Due to their minute size, mites are often overlooked and not obvious to the naked eye without the use of a microscope (Navajas et al., 2010, Mullen and OConnor, 2019, Nicholson et al., 2019).

Mites inhabit dust, water and soil, and are sometimes present as parasites on humans, other animals and plants (Frost et al., 2009, OConnor, 1982, OConnor, 2009, Perotti et al., 2009b). Mites are found throughout our environment and are mostly impossible to

eliminate. The 200+ species of House Dust Mites (HDM) known are widespread globally and inhabit domestic textiles, dust and upholstered furnishings such as beds, clothes, carpets and sofas (Colloff, 2010). Two of the most common species of HDM present in European houses, particularly the United Kingdom, are *Dermatophagoides farinae* and *D. pteronyssinus*. Colloff (2009) reported that HDM are present in all UK houses when suitable environmental conditions for their growth are present. HDM are present in all human dwellings, however, their abundance depends on the ratio of humidity to temperature (Arlian and Platts-Mills, 2001, Solarz, 2001). Robert Hooke first noticed and discovered microscopic living organisms, such as his own house dust mites on a sliver of cork, in 1665, through a microscope lens (Schneider, 2018).

Potential predators of HDMs include other mite species, particularly cheyletid mites (*Cheyletus* spp., Cheyletidae). These predatory mites are not found in all houses and exist in smaller numbers, therefore, are likely a minor constraint on HDM populations (Bochkov et al., 2002, Colloff, 2010).

1.2 House-dust fauna from the KSA

The Kingdom of Saudi Arabia (KSA) is geographically the largest state in the Middle East, with a land area of about 2,160,500km2. It encompasses the Rub'al Khali desert and spans from the Persian Gulf to the Red Sea. Most regions of Saudi Arabia are characterised by persistently hot temperatures with some presenting higher records of humidity than expected, and so have an abundance of mites, particularly HDM. Few studies of mites in KSA have been conducted (Indraganti and Boussaa, 2017); hence many aspects of the results of is research are likely to be novel, describing new mite populations and species.

Because Saudi Arabia is a vast land, there are large variations in climate and geographic factors from coastal, urban, forest, mountain and desert climates (Indraganti and Boussaa, 2017).

Predominant domestic arthropods (domestic insects and HDM species) may be distinct in different Saudi areas. Previous studies on HDM distribution in the kingdom of Saudi

Arabia were limited in most cities (Alotaibi et al., 2018, Negm et al., 2012, Alatawi and Kamran, 2018). The data are old and limited, without systemic studies of the local HDM. Ibid

Al-Khalifa and Bayoumi (1983) studied Saudi soil in the Riyadh regions and identified Mesostigmatic mites, Prostigmatic mites, Astigmatic mites and Cryptostigmatic mites (Al-Khalifa and Bayoumi, 1983). Al-Frayh et al. (1997) demonstrated the first detailed information on Saudi house dust analysis and detection. According to Al-Frayh et al. Ibid, the most common arthropods known up to now in Saudi house dust (mainly in humid cities) are cockroach and arachnids, such as dust mites. The other widespread contents and arthropods of Saudi house dust are fungal spores, beetles (*Coleoptera*), pollens, fungal spores, and body particles of human and pets, such as dander (Al-Frayh et al., 1997, Abdel-Dayem et al., 2017, Aldhafer et al., 2016). In terms of families of house dust mites, Pyroglyphidae (such as *D. farinae* and *D. pteronyssinus*) and Acaridae (stored food mites, like *Acarus*) are known to be prevalent in Saudi houses (Al-Frayh et al., 1997). Al-Atawi (2011) investigated the predatory and phytophagous mites associated with crop plants in Saudi Arabia, especially in Riyadh. The investigated mites were Prostigmatic mites (Family: Eriophyidae, Tetranychidae, Stigmaeidae, Eupalopsellidae) and Mesostigmatic mites (Family: Ascidae) (Al-Atawi, 2011). Negm et al. (2013) conducted a study which showed that new species of Astigmatic mites (family: Avenzoariidae) associated with birds (feather mites) were identified in Saudi regions (in Umm Al-Malik island, Farasan island and Arabian Gulf), (Negm et al., 2013). Asiry and Fetoh (2014) stated that HDMs are a diverse element of the Saudi Arabia fauna and Laelapidae species are ectoparasitic mites and one of the most common abundant Mesostigmatic mites in north western Saudi Arabia (Asiry and Fetoh, 2014).

Until today, little is known on mites from KSA. There is a large gap in the field of acarology in general and HDM in particular.

1.3 Mites and their forensic implications

Mites have a record of over 140 years in forensic investigations and estimations of time of death (Perotti, 2009), they were one of the earliest forensic tools providing important

evidence about post mortem intervals in human cadavers. In 1878, Jean Pierre Mégnin studied and reported the presence of 2.4 million mites in the skull of a cadaver in Paris; he suggested that their time of arrival to the carcass by insect carriers was around eight months, which was later reanalysed by Perotti (2009), who estimated the same time length than the original pathologist, 5 months (Perotti et al., 2009b, Perotti, 2009). Mites colonize corpses and participate in all decomposition stages up to the skeletal stage (Braig and Perotti, 2009). Mites can also provide relevant evidence for postmortem relocation, from the crime site, because they are abundant, widespread and diverse (Perotti et al., 2009b). In a variety cases, such as lower temperature conditions, mummification, and in concealed cadavers, mites may represent the only helpful, forensic evidence available (Braig and Perotti, 2009, Perotti and Braig, 2009).

HDM can be particularly important in forensic analysis, for example, to detect the offender within a household (Solarz, 2009); mite species vary from one host to an other, and sometimes between body parts within the same host. Saloña-Bordas and Perotti (2014) highlight their characteristics and adaptations to the particular environment of hung bodies, including their micro-locality and habitat specificity (Perotti et al., 2009b).

1.4 Molecular characterization of populations

1.4.1 About microsatellites

The initial work on genetic markers was in the fields of haematology and serology when Karl Landsteiner discovered the ABO blood system and determined that People can be placed into various groups depending on their blood type. In 1978, May, Weber and Tautz identified DNA polymorphisms through the Southern blot technique. A highly polymorphic locus was identified in 1980 (Abdul-Muneer, 2014). It was in 1984 when Alec Jeffreys studied, described, and created most of the applications of genetic sciences (De la Fuente et al., 2000). With this progress, the variable number tandem repeats technique was the first powerful tool developed for human identification. Genetics studies developed, thanks to the work of the chemist Kary Mullis, in 1983, when he determined and developed the PCR technique (Carbonnelle et al., 2007). In 1990, short tandem repeats were analyzed by Alec Jeffreys, who was the first to utilize them as the ‘working’

tool, short tandem repeats are the marker used in forensic genetics analysis such as determining kinship (Sun et al., 2012).

Molecular genetic procedures are becoming increasingly significant in acarology. They have proven their usefulness in evolutionary and systematic acarology, and they have been utilized with success to study the phylogeny, taxonomy and population genetics of mites. These significant developments were carried out over 13 years (from 1993 to 2006). In 1993, 49 DNA sequences, originating from mites and reported in the GenBank nucleotide sequence database, were implemented, and in 2006 up to 90,000 DNA sequences were executed (Dabert, 2006). Myriads of applications have gone into conservation biology. Living species affected by geographic barriers, selection, genetic drift or migration leading to loss of genetic diversity, inbreeding and survival rate of the population. Mainly, microsatellite analyses generate significant information on the population genetic structure, genetic variation and genetic diversity. This information is helpful in the species management by identifying the intraspecific genetic structure and/or controlling the inbreeding, reproductive fitness and high genetic diversity (Wright and Bentzen, 1995)**.** They comprise short, tandemly repeated segments of repetitive DNA. Motifs may be repeated 2-50 times and range from 2-3 (4-10) bp in length, which are found at hundreds to thousands of loci in the genome (Goldstein, 1999, Richard *et al.*, 2008). MS have many features which make them useful in genetic studies, including their occurrence in all eukaryotic genomes, potentially high levels of allelic variation, co-dominant inheritance so that hetero- and homo-zygotic alleles can be differentiated, suitability to automated analysis, small locus size hence readiness of amplification, often high levels of polymorphism, meaning they can often be diagnostic characteristics. These features have led to their use in diverse and widespread applications in the last few decades, including cloning; genotyping and mapping, genetic evolution and ecology, and population and forensic studies (Learmount et al., 2003, Nishimura et al., 2003). They are very well understood, and a wide range of literature is available to guide in their use.

The benefits of MS continue to result in the new development of scientific applications for researchers, such as automated revealing systems. However, microsatellites also have some drawbacks. Their *de novo* isolation for any given taxon requires relatively high effort with commensurate financial costs to identify loci and sequence flanking regions for primer design (Liu and Cordes, 2004). Often the identification of numerous loci is necessary, and they are generally applicable to only a single or small number of closely related species. In 2011, Lepais and Bacles supported that and stated that microsatellites

have some problems, including that the requirement for large numbers of markers and individuals from large population genotyping, time, cost, and microsatellites and species isolation (particularly that are difficult to transfer) limit the uses of microsatellites in some organisms. To face these problems at the present time, the technique needs to decrease the cost and time of microsatellites development and genotyping to adjust the data generation to the requirements for population genetics (Lepais and Bacles, 2011). In 2014, Abdul-Muneer also proved that MS have disadvantages. Generally, however, the advantages overcome the disadvantages (Queller et al., 1993, Rakoczy-Trojanowska and Bolibok, 2004).

Regarding Microsatellites composition, they have different types of repeated motifs, based on a specific sort of formulae (Table 1.1).

1. Microsatellites can be comprised as perfect, imperfect, and complex repeated pattern according to their repeated pattern.
2. They could be comprised repeates of mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, hexanucleotide, heptanucleotide, octanucleotide, nanonucleotide, and decanucleotide based on the number of their base-pair repeats (Singh et al., 2011, Singh et al., 2012, Singh, 2016, Bhukya, 2016).

Table 1.1: Compositions of Microsatellites.

DNA satellites		**Sort formula**	**Examples**
Microsatellites	***A- Based on repeat pattern***	Perfect	- CACACACACACACACA-
		Imperfect	CACACAACACACATCACACA-
		Complex	-CACACACA AATAATAAT-
	B-Based on number of base pairs	Mono-nucleotide	TTTTTTT or CCCCCCCC
		Di-nucleotide	TATATATATATATATA
		Tri-nucleotide	CCA CCA CCA CCA CCA
		Tetra-nucleotide	GATA GATA GATA
		Penta-nucleotide	TCTCATCTCATCTCA

The formulas and examples of microsatellites (Singh et al., 2011, Singh et al., 2012, Singh, 2016, Bhukya, 2016).- A brief description of each type is given below.

1.4.1.1 The perfectness condition of microsatellites

The perfectness status of MS was first characterised by Oliveira et al. (2006). There are two ways to classify microsatellites. The first method, through computing the degree of

the perfectness of the microsatellites. Weber (1990) recognises three classes of perfectness. First, perfect repeats class, which consists of a single, uninterrupted array of a particular motif (an accurate and consistent alignment of primers to flanking regions); to clarify, in a given sequence, a tandem repeat of a size n, a perfect repeat of a size n is a subsequence which repeats (continuously) twice or more in the sequence, which is specified by the user. Also, the DNA molecules of this class are subject to a variety of mutational events. One of the less well understood is perfect repeats duplication in a stretch of DNA, which is called the pattern, converted into two or more copies, each following the preceding one in a contiguous fashion. For example, there could be … TCGGA … → … TCGGCGGCGGA … in which a single occurrence of triplet CGG has been transformed into three identical, adjacent copies. So, CGG is a perfect repeat.

The second class is known as imperfect repeats, which has an array that interrupted by one or several out-of-frame bases. This means, imperfect repeat is the extension of the nucleation sites of the motif as long as some termination criteria are satisfied. The number of imperfections between the individual repeats the copy, and the perfect repeats the motif the more than its limit (denoted by k parameter, which is set by the user) and the percentage of imperfection is more than the limit set by the user and denoted by p parameter). The third class is known as compound repeats, which intermingles between perfect or imperfect arrays of several motifs.

1.4.1.2 Repeat and number of nucleotides

Another way to classify microsatellite is the number of base pairs. Microsatellite markers, or short tandem repeats (polymorphic DNA loci) are comprised of a short segment and domain piece of repetitive DNA in which a particular DNA motif is repeated 5-50 times, and are found at hundreds to thousands of various loci in the genome (Goldstein, 1999, Richard et al., 2008). The repeated DNA motifs range from 1 to 6. In order to clarify, the Number per each genome ranges from 1000 to ≥100000, the motif size ranges from 1 to 6, the total length ranges from ≥1 to 100. The most common kind of microsatellites present are dinucleotides. In the vertebrate genome, (-AC-) is the most common class of dinucleotides, while (-AT-) is the second most common class of dinucleotides repeats. Poly (-A/T-) in mononucleotides motifs are the most common microsatellites, while

trinucleotides, tetranucleotides, pentanucleotides, and hexanucleotides are the least common kinds of microsatellites in the genome (Chistiakov et al., 2006). In trinucleotide repeats, (-CGG-) and (-CCG-) are comparatively abundant in most exons of a vertebrate; this is the opposite of the intron, which has very low numbers of these repeats (Tóth et al., 2000). Toth et al. (2000) added that the most frequent kinds are trinucleotides and the second are hexanucleotides, whereas the tetranucleotides are rare in the exon and common in the intron (Tóth et al., 2000).

A number of characteristics of DNA repeats are known to influence their degree of instability. These include the length of the repeat unit, the base composition, the number of DNA repeats, the sequence context and the degree of 'perfection' of the repeat tract (Campregher et al., 2010). Such studies were based on the use of selective media. When quantitating mutation rates, the presence of pre-existing mutations within such assays cannot be precluded. In 1990, Weber showed that the level of polymorphism exhibited by PCR-amplified (CA)n microsatellites is positively correlated with the number of uninterrupted, perfect repeats at a given locus. The following figure shows some examples of the short tandem repeats (trinucleotides) (Figure 1.1).

a

Participant 1GTACAAGACTACTACTACTACTACTACTACATCTGGTG (Trinuclueotide–8 repeats)

Participant 2 GTACAAGACTACTACTACTACTACTACATCTGGTG (Trinuclueotide–7 repeats)

Participant 3 GTACAAGACTACTACTACTACTACATCTGGTG (Trinuclueotide–6 repeats)

Participant 4 GTACAAGACTACTACTACTACATCTGGTG (Trinuclueotide–5 repeats)

b

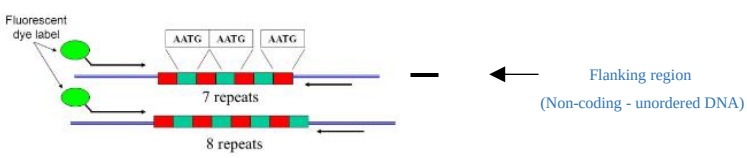


Figure 1.1: Short tandem repeats or STRs. a) example, list of microsatellite alleles; b) example, location of repeats within the genome.

In order to be more specific about the terminology described above, we provide a short definition of each term as follows:

- **Repeat region** is variable between samples while the flanking regions where PCR primers bind are constant.
- **Homozygote** is equal to both alleles, which are the same length.
- **Heterozygote** is equal to alleles that differ and can be resolved from one another.
- **Primer positions** define PCR product size.

1.4.2 Molecular acarology and microsatellites

Genetic markers in mites offer valuable tools for the analysis of forensic evidence, and if genetic profiles, particularly MS, can be shown to differ within and among populations of mites and according to the environment and their geographical origins, then their significance may potentially be greatly enhanced. The gene flow, the time periods and new alleles may gain entry into the populations. These are considered major factors in the development of genetic differences and allele's losses and all these diagnoses are carried out by microsatellite markers (Slatkin, 1985, Delaney et al., 2009). Commonly, genetic variability is utilized to help investigators to identify the species genetically; it deals with the variability of the environment in the current period. Ge et al. (2014) reported that genetic differentiation of Acari species is high, indicating high biodiversity and that there is low gene flow. Acari is prevalent around its hosts, due to its parasitic relationship, such relationships have been reported to lead to genetically differentiated host races in mite species (Magalhães et al., 2007).

The development of a genetic marker has provided an opportunity to assess individual host species based on their DNA genotype. The Acari and their host genomics, such as the profiles of gene expression, are likely to facilitate studies addressing the functional investigations and sequencing data of their entire genomes.

Population variation of the Acari has also been studied for describing the differences in the molecular genetic markers among individuals (genetic differences in the same species) and that may due to the genetic recombination or mutation. The procedures used to study these species have much in common, such as microsatellites studies. These studies permit the flexibility in the genetic management by assessing and maintaining the genetic variability and to understand the Acari population survival on the host (Chistiakov et al., 2006, Li et al., 2017, Litt and Luty, 1989, Lepais and Bacles, 2011, Liu and Cordes, 2004).

The molecular genetic markers are frequently used to assess genetic relationships between individuals and populations through the genetic distance's estimation. Mites are widespread in different geographical locations and in some habitats; they have become a major threat to their hosts (worldwide pest). Population genetic studies utilizing genetic molecular markers have proven to be tremendously informative to find out host-specific pests, to address questions about genetic variations, genetic diversity at both intra-specific and inter-specific, genetic differences, host preferences, phylogeography and population genetic structure (Navajas and Fenton, 2000). Genetic molecular markers in Acari have been used in forensic genetics, genetic diversity analyses and phylogenetic analysis such as mitochondrial and nuclear genetic markers. However, microsatellites in Acari have been extensively used in a few species of economic importance and described due to their features such as their existence in all eukaryotic genomes, high allelic variation level, high capability for automated analysis, high mutation rate, highly polymorphic characteristics, and their codominant nature permit the estimation of within and among breed genetic diversity even if they are closely related (Chistiakov et al., 2006, Li et al., 2017, Litt and Luty, 1989, Lepais and Bacles, 2011, Liu and Cordes, 2004). In this review, there are some examples of published molecular genetic markers of Acari reviewed in Table 1.2. Currently, microsatellites discussed below are the most popular markers in host genetic characterization studies (Sunnucks, 2000).

MS were reported to be under-represented in two mite species, *Tetranychus urticae* and *Amblyseius fallacis* (Navajas et al., 1998). However, more recently, genetic structure in populations of *T. urticae* has been successfully studied using MS, (Aguilar-Fenollosa et al., 2015, Hada et al., 2016), and in another pest-mite, *Varroa destructor* (Kelomey et al., 2017), while in *Halotydeus destructor*, microsatellite analysis has revealed climate combined with host plant associations in cryptic species that may also be regionally

segregated (Hill et al., 2016). Genetic similarity was found between a lineage, which originated in Cape Town, South Africa, and invasive populations found in Australia.

Mites have been studied at the population level, individual level and species level. They are the most diverse group of Acari. Ticks have been studied to the same degree as the mites, but they have a less diverse group than mites. Most of the Acari studied in mites are Phytoseiidae (predatory mites), Pyroglyphidae, Eriophyidae and Tetranychidae (spider mites), while most of the Acari studied in ticks are Ixodidae.

Several experts have researched the microsatellites of different mite species that are utilised as controls of pests, such as the spider mite that damage agricultural plants as well as of mite species that cause a variety of damage to their host organisms for example; destruction of agricultural crops, allergenic diseases in humans and parasitic diseases in animals (Table 1.2).

Table 1.2: Overview of published studies on microsatellite of mites. List arranged alphabetically by Family name. 1st column: Acari species (by family); 2nd column: Microsatellite information; 3rd column: host/habitat; 4th column: disease produced if any, and references.

Acari Species	Microsatellites (MC) related studies	Host origin	Issues-related mites Reference
Acaridae Flour mite *Acarus siro*	13 Micrs-Developed Polymorphism level detection Genetic divergence (Genetic changes, mutation) identified 12 Micrs-Developed	Grain and flour	Pest of stored grain (Learmount et al., 2003)

Tyrophagus putrescentiae Stored food *Aleuroglyphus ovatus*	Detect Genetic Diversity Investigate the genotypic variability Micrs analysis (ISSR) Detection of genetic diversity Polymorphism level detection The microsatellite (AT) sequence in the largest non-coding region was observed Polymorphism level detection Studies of MICA polymorphism with asthma and rhinitis	High fat and protein content Stored food In nest & grasslands Storage food	Stored products pest& Mycology pest Copra itch, dermatitis, respiratory allergies (Zou et al., 2011) Allergenic diseases (Sun et al., 2014)
Cheyletidae Predatory mite *Cheyletus malaccensis*	Mitochondrial COI and 12S rRNA genes Population genetic structure Analysis of genetic differentiation	Grain storages	Important biological control agent for pest mites (Yang et al., 2016)
Demodecidae Folicular mite *Demodex canis* *Demodex folliculorum*	3-Micrs-Developed Investigation of Micrs linked to DLA class II DLA: Dog Leukocyte Antigen To prevent JGD JGD: Juvenile generalized demodicosis Identification of specific chromosomal regions before to genotype candidate genes (Genetic diversity of the functional immune response) 1st column: Acari species (by family); 2nd column: Microsatellite information; 3rd column: host/habitat; 4th column: disease produced if any, and references.	Dog Human	Parasitic skin disease Demodicosis (It et al., 2010) Demodicosis (Daneshparvar et al., 2017)
Eriophyidae Coconut mite *Aceria guerreronis*	32 Micrs used - 9 Micrs-Identified Breeding program-Mite resistance Genetic pest management 90 expressed polymorphism Analysing the genetic diversity	Coconut	Coconut palm Scarring & distortion of coconut pests Premature fall

	Used for the genetic characterization		Reduction in fruit size/weight/water volume Aesthetic damages (necroses) (Barreto, 2017) (Shalini et al., 2007)
Pigeon pea mite *Aceria cajani*	5 Micrs-Developed Identifying the genomic regions associated with resistance Resistance breeding of SMD	Pigeon pea	Vector of Pathogen Sterility mosaic disease by PPSMV (SMD) Fail to produce flowers
Wheat curl mite *Aceria tosichella*	9-Micrs-Developed & evaluated population genetic analysis and provide information on the genetic structure Assessed genetic differentiation	Wheat	Cereal pest[j] and a vector for spreading and transmission of vir uses (Miller et al., 2012)
Rust mite *Calepitrimerus vitis* Blister mite, Bud mite *Colomerus vitis*	10 Micrs-Isolated (polymorphic 4 blister+3 bud mite) Breeding biology, Genetic differentiation Population genetic structure	Grapevine	Grapevine pests (Carew et al., 2004)
Glycyphagidae Stored food mite *Lepidoglyphus destructor*	47 Micrs-identified Using ISSR fingerprinting Assessing genetic diversity & differentiation	Stored grain	Stored grain pest Allergy (Ge et al., 2014)
Hydrachnidia Water mite *Hygrobates fluviatilis*	13 Micrs-Developed Studying intra-specific variation Testing the possible correlation between DNA polymorphism & environmental heterogeneity	Water	Water pollution (Asadi et al., 2009)
Laelapidae Rodent mites *Laelaps muricola* *Laelaps giganteus*	mtDNA COI & nuclear ITS1 phylogeography approach Evolutionary history, Taxonomic status, Intraspecific diversity, Genetic and morphological diversity (assessments)	Rodents	Biting and blood sucking Skin rash (Engelbrecht, 2016)

Phytoseiidae Predators of spider mites *Amblyseius swirskii* *Amblyseius cucumeris* *Amblyseius andersoni* *Amblyseius stipulatus* *Neoseiulus barkeri* *Phytoseiulus persimilis* *Typhlodromus phialatu*	5 Micrs-Developed Cloning, Genetic management Population genetic studies Genetic structure studies Testing cross amplifications of Micrs	Plant	Treat *Tetranychus* species (Biological control) (Sabater-Muñoz et al., 2012)
Neoseiulus californicus	5 Micrs-Developed Screening genetic polymorphism Screening allelic variations	Plant Temperate & subtropical regions	Control the spider mite on various crops (Perrot-Minnot et al., 2000)
Neoseiulus womersleyi	3 Micrs-Developed Lack of genetic markers Examining genetic diversity Studying intra-specific variation	Plant In temperate & subtropical regions Leaves of plants	Control Crop pests The important natural enemy of the spider mite (Hinomoto and Taro, 2005)
Podapolipidae Ectoparasitic mite of insects *Coccipolipus hippodamia*	7 dinucleotide &1 trinucleotide-Isolated Genetic diversity and variability	European two-spot ladybird *Adalia bipunctata*	Ectoparasites (Gordon et al., 2009)
Psoroptidae Mange mite *Psoroptes* spp.	9 Micrs-Isolated Polymorphism	*Oryctolagus cuniculus* (Rabbit) *Ovis aries* (Sheep)	Sheep scab, Rabbit scab (Evans et al., 2003)
Pyroglyphidae House dust mite *Dermatophagoides farinae* *D. pteronyssinus*	The microsatellite (AT) sequence in the largest non-coding region was observed Polymorphism level detection Studies of MICA polymorphism with asthma and rhinitis	House Dust	Asthma Rhinitis (Balog et al., 2005)
Sarcoptidae Itch mite *Sarcoptes scabiei*	12+22+13 Micrs identified (3 populations) hypervariable microsatellite loci (Comparison + Genotyped) Population genetic studies	Human	Crusted scabies (Walton et al., 1999)

Spinturnicidae Bat wing mites *Spinturnix myoti*	9+5 Micrs linkage disequilibrium studies (coadaptation and coevolution) Population genetic & Genetic association studies	Bat wings	The strong interaction between bat wings and their parasites (Van Schaik et al., 2011)
Tenuipalpidae False spider mite *Brevipalpus phoenicis*	Genotyping by Micrs Lack recombination Population genetic studies (Distinct)	Plant Crop	Crop pest (Groot et al., 2005)
Tetranychidae Spider mites *Tetranychus cinnabaribus*	31 Micrs- identified Polymorphism level detection By MISA software	Plant Crop Tomatoes, peppers, cucumbers, strawberries, maize, soy, apples, grapes, and citrus Plant	Damage fruit trees, vegetables, ornamentals, agricultural crops, and weeds (Li et al., 2009) (Bu et al., 2015)
Tetranychus evansi	16 micrs-developed Population genetic diversity & structure Genetic clustering	Plant Under the surface of leaves Plant	Crop pest (Boubou et al., 2012)
Tetranychus kanzawai	7 Micrs-Isolated Behavioral ecology, population genetics, and genome mapping	Plant	Damaged leaves (yellow and dry) (Nishimura et al., 2003)
Tetranychus ludeni, Tetranychus piercei, Tetranychus phaselus and Tetranychus puearicola	101 Micrs-identified Population genetics studies Genetic variation in the numbers of repeats	Plant	Agricultural pests (Zhang et al., 2016)
Tetranychus truncatus	4 Micrs-identified Genetic diversity Studying intra-specific variation	Plant	Pests of agricultural crops (Hinomoto et al., 2009)
Tetranychus turkestani	5 Micrs-used (genetic diversity) Population genetic structure	Citrus orchards	Plant damage and pests (Bailly et al., 2004)

Tetranychus urticae	42 Micrs identified Polymorphism level detection		Crop pests (Sauné et al., 2015)
Eutetranychus banksi *Eutetranychus orientalis* *Tetranychus okinawans* *Oligonychus perseae*	5 Micrs-Developed Cloning, Genetic management Population genetic studies Genetic structure studies Testing cross amplifications of Micrs		Citrus orchards pest (Sabater-Muñoz et al., 2012)
Mononychellus tanajoa *Tetranychus tanajoa*	500 Micrs analysed-5 expressed polymorphism Identification of some chromosomal regions that confer resistance to the mites	Plant Leaves Cassava	Pest of cassava (Macea Choperena et al., 2012)
Panonychus citri	15 Micrs-Isolated 12 of 15 loci deviated from *HWE*. Presence of inbreeding, Wahlund effect and null alleles: potential sources of HWE *deviations*. Linkage disequilibrium studies	Citrus orchards	Citrus pests (Sun et al., 2014)
Trombiculidae Blood sucking mites *Leptotrombidium* sp.	Microsatellite analysis Polymorphism level detection	Human In Damp areas & Grasslands	Irritation Severe itching & dermatitis (Zhang et al., 2008)
Varroidae Honeybee mite *Varroa destructor*	19 Micrs loci used Amount of gene flow provides robust estimates of mutation parameters for each locus 16 Micrs-Developed Polymorphism level detection	Western honeybees *Apis mellifera*	Attack the Western honeybee, *Apis mellifera* *Apis carena* (Cornuet et al., 2006) Apicultural pest Beekeeping pest (Solignac et al., 2003)
Varroa jacobsoni	15-Micrs-Developed Polymorphism level detection Genotypic variation Estimating genetic structure Helpful for population-genetic studies Gene flow estimation of mite	*Apis cerana* (Honeybee)	Bee pest (Evans, 2000)

Oribatida Ptyctimous mites	Determining the genetic variability and patterns of this variation	Soil and litter Litter-topsoil Tapeworm host	Bio indication of pollution (Niedbała and Dabert, 2013)

Mites are organisms that can provide useful and strong evidence in forensic studies for legal investigations. This is as a result of many of their features and adaptations, including their micro-locality/habitat specificity (Perotti et al., 2009b). Mites can be important tools in criminal analysis, for example, to detect the offender (Solarz, 2009). To clarify, mite species are different from one host body part and from one host body to another**.** According to their features, their micro-habitat specificity refers to their choosing a particular individual that hosts them, while their micro-locality refers to their choosing a particular place in the body(Perotti, 2009). Guzinski et al. (2008) and other authors supported that mites contribute to the diagnosis of forensic crimes (Medeiros-Silva et al., 2015, Van Houtte et al., 2013, Estrada-Peña et al., 2012, Guzinski et al., 2008, Ludwig, 2015). The history of forensic acarology is about 140 years old. Forensic acarology started developing by the first case that had been analyzed by helping mites to give important evidence about the case to assess the post mortem interval (Perotti, 2009). This is defined as the 'time since death' estimation. In 1878, Jean Pierre Megnin studied and identified the presence of 2.4 million mites in the skull of a newborn body in Paris, and he suggested that the mites' time of arrival to the carcass by the carrier (insects) was around 8 months (Perotti, 2009, Perotti et al., 2009b). Therefore, forensic diagnosis uses these micro-arthropods as indicators of time of death (Perotti et al., 2009b). Mites can also provide relevant evidence and proof information for body relocation, movement, or the suspect location from the offense site, as a result of the mites' high abundance, widespread status, diversity and high levels of interaction with criminal events (Perotti et al., 2009b). Mites colonize host corpses and participate in all stages of their decomposition, i.e., from the initial fresh stage to the skeletal stage in the decomposition step (Braig and Perotti, 2009). Therefore, mite studies are imperative to forensic diagnosis. Mites are one of the most dominant arthropod groups that coexist on host carcasses. Under some specific situations, such as lower temperature conditions, mummification conditions, and concealment cases, mites may be the only markers or evidence available, and these conditions, in turn, are helpful for forensic diagnosis (Braig and Perotti, 2009, Perotti and Braig, 2009). It has

been clearly demonstrated that most of the forensic implications can be detected by the mites analysis, because of their features (Frost et al., 2009).

1.4.3 Microsatellites in forensic studies using non-human DNA

Microsatellites repeat sequences can vary from one person to another, and this genetic polymorphism can be used in DNA fingerprinting and other forensic applications (Mahtani and Willard, 1990, Goldstein, 1999). For this reason, MS are considered to hold one of the essential roles in forensic diagnosis, which in turn is focused on the study of DNA in a specific place for object identification (Campobasso et al., 2005, Jones et al., 2002, Urquhart et al., 1994). Genetic variability is routinely utilized to help investigators to identify species, populations and individuals.

The word forensic derives from the Latin: *forum* + *ensis* = public debate, in open court (Lynch, 1993). It signifies practical knowledge which may be used in the investigation of crime obtained from the analysis of particular bodies, items or matters of trace evidence (Inman and Rudin, 2000). It may identify the offender, victim or other significant factors at the crime scene (Christian et al., 2000, Saferstein, 2002). A major field of forensic science is forensic genetics (Campobasso et al., 2005, Jellreys, 1985, Gill et al., 1985, Jeffreys et al., 1985a, Jeffreys et al., 1985b).

For further details, microsatellites are considered to hold one of the essential roles in forensic diagnosis, which in turn is focused on the study of DNA in a specific place for object identification (Campobasso et al., 2005). Microsatellites have many advantages, such as a higher degree of polymorphism that can be utilized in forensic determination and diagnosis (Veselinović, 2005).

1.4.4 *Dermatophagoides farinae* microsatellites

Microsatellites markers have been developed for mites (Alasaad et al., 2012). The testing of microsatellites, especially primer specificity in this present work, was based upon previously identified but unpublished microsatellite loci for *Dermatophagoides farinae* (*Df*) (Pyroglyphidae) and primers developed to amplify them (Al-Khalify, 2018), which

have only recently been made available. Genome sequences in which the loci were identified, were assembled from 11,600 overlapping, consensual DNA sequences (contigs) assembled in 495 scaffolds (Al-Khalify, 2018). This atypical and rarely evaluated approach to microsatellite characterisation is reported to avoid genomic regions of low complexity, where primers are less likely to be locus-specific. Furthermore, it identifies regions with greater overall length and repeats than those traditionally isolated, and is likely, therefore, to identify, since polymorphism is dependent on length and number of repeats in microsatellite loci, so more genotypes. Repeats in these MS vary in length from 2 to 10 base pairs, so are in some cases outside the typical MS range. The microsatellite markers and putative primer sequences were supplied by Bangor University (Al-Khalify, 2018), and are highly suited to the purposes of this study.

The available documentation shows they exhibit a lot of variation and that a large number of potential primers have been identified and sequenced. These MS represent an opportunity to study the distribution and relative abundance of distinct genotypes within and between populations of *D. farinae*. Several benefits may also accrue to future research, if, in this investigation, systematic analyses illuminate and quantify: MS characteristics, primer alignment and amplification success distinct in the target species; allelic polymorphism; genotypic discriminatory power; and transferability to, and associated amplification of MS in phylogenetically related taxa.

The correct design of primer sets is fundamental for successful DNA amplification. The supplied primers are suitable because of their length; they are, relative to many other MS, long (16-24 bases) which can affect binding at the selected annealing temperature, but significantly contributes to binding specificity (Abd-Elsalam, 2003). Their melting temperature, 55°C-60°C, is appropriate for the PCR reactions and equipment available.

A universal tag was added to the 5'-end of the forward primer, which bears no sequence homology to the mite genome (Nicot et al., 2004), this facilitates detection of the amplicons by the sequencer or microsatellite analyser (Neilan et al., 1997, Nicot et al., 2004, Al-Khalify, 2018).

Chapter 2 : Biodiversity of the house-dust fauna and its forensic implications in Saudi Arabia

Abstract

This study is a first examining the diversity and abundance of the house dust fauna identified by classic taxonomic methods, as previous research in the Kingdom of Saudi Arabia has only gathered information on mite species by studying the presence of allergens of 2 to 3 Astigmata species.

The composition of the fauna of arthropods inhabiting houses, with emphasis in house dust mites, within three different climatic regions of the Kingdom of Saudi Arabia were studied. The three areas were established as: 'Desert Cities' for Al-Kharj and Riyadh; the 'Coastal Area', Jeddah city and surroundings; and the 'Highlands, considering the cities of Shafa and Taif.

The most numerous mite species were those from the family Pyroglyphidae, followed by mite species from the Acaridae and Cheyletidae, and this was clear for Coastal Area and Highlands; while the Desert Cities were characterised by the dominance of Cheyletidae mites.

The abundance of a diverse fauna of Pyroglyphidae mites in Coastal Area and Highlands is supported by higher humidities when compared to the desert, where Cheyletidae species seem to dominate. A variety of cheyletids, from predators to skin parasites of domestic and wild birds and mammals were identified. Wild fauna is more common in the country side, in the desert, justifying the occurrence of these mite species.

Association of mites from a variety of other families is congruent with the different habitats, for example, there was a positive correlation between Astigmata families in general (Glycyphagidae, Pyroglyphidae and Acaridae) with the Coastal Area.

2.1 Introduction

Mites and ticks (Acari: Ixodida) associated with house dust and humans have been extensively studied (Colloff, 2010, Solarz, 2009, Krantz and Walter, 2009, Hughes, 1976). Over 150 species of mites have been identified and associated with human dwellings; including botanical, zoological, and predatory mites as well as oribatid (soil), and storage mites. However, the frequently encountered mites that are directly associated with the presence of humans and are found in large numbers in the house dust, are members of the family Pyroglyphidae (Astigmatina) (Colloff, 2010). The main three species of this mite family are the cosmopolitan *Dermatophagoides pteronyssinus, D. farinae* and *Euroglyphus maynei* (Arlian and Morgan, 2003, Wharton, 1976, Neal et al., 2002, Solarz, 2009, Colloff, 2010).

Mites are omnipresent, however sensible to the surrounding ecological changes, therefore, habitat specific (Colloff, 2010, Krantz, 1978). House dust mites colonise and thrive in new houses via humans habitation (Warner et al., 1999), and different species inhabit different rooms of the house (Frost et al., 2009). These properties make mites of high importance to forensic investigations of indoor crime scenes.

Very few indoor scenes of crimes have reported mites as evidence (Russell et al., 2004). Following Locards' principle, every contact leaves a trace (Locard, 1931). The contact may occur between two people, between a person and an object or between two objects (Taupin and Cwiklik, 2010). Mites as trace evidence may link objects or people to a precise habitat at a specific point in time. Hani et al. (2018) have highlighted the importance of mites as trace evidence and markers of location. In a case involving illegal money, mites being the only trace evidence found, they acted as markers of location and linked the bags of banknotes to a precise location in the world, being Australasia (Hani et al., 2018).

Mite evidence can be easily transferred between individuals, or objects coming in contact during a crime. Mites offer a significant amount of evidence on location, relocation and the time elapsed since a crime has happened as well as DNA or pathological traces on the victims such as skin lesions or allergic reactions (Perotti et al., 2009b, Frost et al., 2009, Turner, 2009, Saloña-Bordas and Perotti, 2014, Hani et al., 2018, Kamaruzaman et al.,

2018, Solarz, 2009, Szelecz et al., 2017, Perotti and Braig, 2009). The minute size of mites makes them easily overlooked to the untrained eye during the collection of forensic evidence.

The indoor acaro-fauna occurrence and diversity depends primarily on the permanent presence of individuals in homes or working areas (Warner et al., 1999). Therefore, indoor environments may have an important impact on the diversity and abundance of these mites. Habits and lifestyle of individuals such as; their pets, their indoor plants, their activities or the food stored in their kitchens may directly affect the mite-faunal composition inside their homes (Frost et al., 2009). Because the surrounding conditions in houses have an influence on the faunal variety, each location may have its own biodiversity, which makes mites a useful forensic tool and an important marker of location during crimes.

Forensic acarology can make a good tool in crimes scene investigations and develop into a valuable alternate input into forensic analysis (Perotti et al., 2009b, Frost et al., 2009, Turner, 2009, Perotti and Braig, 2009).

This research project aims to i) investigate the biodiversity of house-dust fauna in the Kingdome of Saudi Arabia (KSA), from which five main representative regions were selected; ii) evaluate the impact of regional climate on the composition of the house-dust fauna, particularly mites; and to iii) preliminary assess its potential use as forensic marker/s of location.

2.2 Materials and methods

2.2.1 General information

House dust samples studied in this research were collected over the period of two years, starting in December 2016 and finishing in January 2018. The samples, in this study, originated from the total of 37 houses, located in five different cities, which are Al-Kharj, Jeddah, Riyadh, Shafa, and Taif (Figure 2.1).

The five different cities were selected based on geographical and environmental characteristics. In KSA, Al-Kharj and Riyadh being urbanised areas and located in the

middle of the Saudi Arabian desert, they were categorised as desert cities; Jeddah was categorised as a coastal area; and Shafa and Taif are both located in the highlands of Saudi Arabia, thus, these two were grouped under highlands. Therefore:

Desert Cities: Al-Kharj and Riyadh

Coastal Area: Jeddah

Highlands: Shafa and Taif

Figure 2.1: The five sampling areas displayed on the map of the Kingdom of Saudi Arabia (KSA) with red dots representing; Desert Cities: Al-Kharj and Riyadh; Coastal Area: Jeddah; Highlands: Shafa and Taif.

2.2.2 Sampling and processing regimes

The dust samples in this research were collected from human dwellings by their owners or inhabitants with the aid of their own vacuum cleaner devises. The house dust was extracted from floors, carpets and rugs at a rate of $1m^2$ per minute for 5min. The owners transferred the samples into 96% Ethanol, dried them afterwards, and the collected preserved/fixed dust was put into carefully labelled plastic bags for their transport or mail to the laboratory for further analysis.

Once in the laboratory, at the University of Reading, UK, the samples were processed by rehydrated at 70% alcohol (v/v) and subsamples were examined under the stereomicroscope for the extraction of any arthropods present.

The extracted specimens were mounted permanently on glass slides in Hoyer's medium for further microscopic examination following Hani et al. (2018), Krantz (1978) and Anderson (1954).

A Nikon Optiphot phase contrast microscope at up to 1000X was utilised for identification. The morphological observations and measurements were made in micrometres (µm) at suitable magnifications. Images were captured using Motic Moticam 3 Plus (microscope digital camera).

Keys and descriptions were used for mite identifications (Michaud et al., 2012, Krantz and Walter, 2009, Wharton, 1976, Hughes, 1976, Colloff, 1998, Fain, 1967).
The taxonomic classification of the house dust mite species was done following description and keys provided by Colloff (2010) and Hughes (1976) (Hughes, 1976, Colloff, 2010).

2.2.3 Data examination

In order to check for dependency between the mite species and their locations, the Kendall rank correlation coefficient was utilised. It is a non-parametric test and do not depend on assumptions founded on the type of distribution. The test was conducted using Stata13 (StataCorp, 2014).

2.2.3.1 Exploratory data analysis

In order to picture the data of micro-arthropods' species and geographical locations freely, multivariate analyses, such as Correspondence Analysis (CA) and cluster analysis were used. The CA was performed to graphically present the connection between species and areas; and to permit the visual inspection of data. The test was performed using Stata13 software package (StataCorp, 2014).

Cluster analysis utilizing correlation similarity index were used as an investigative technique. This method is useful to group invertebrates based on the similarities of their abundance in each particular location. This was performed using the software package PAST3.

Pie charts were generated as basic EDA (exploratory data analysis) to visually explore the biodiversity of houses in the different cities. Also, to observe the differences between regions based on dust fauna composition. This was performed using Stata13 (StataCorp, 2014).

2.3 Results

2.3.1 Biodiversity of the house-dust fauna

Samples of house-dust collected from households in KSA appear to vary between regions. They appear to have a fairly diverse arthropod composition (Figure 2.2). A high proportion of the fauna retrieved from dust appears to be mite species from the family Pyroglyphidae, followed in number by mite species from the families Acaridae and Cheyletidae. Oribatid mites were surprisingly present in the house-dust from KSA in a relatively significant proportion. Data of mite numbers per location is provided in Table A.1 (Appendix).

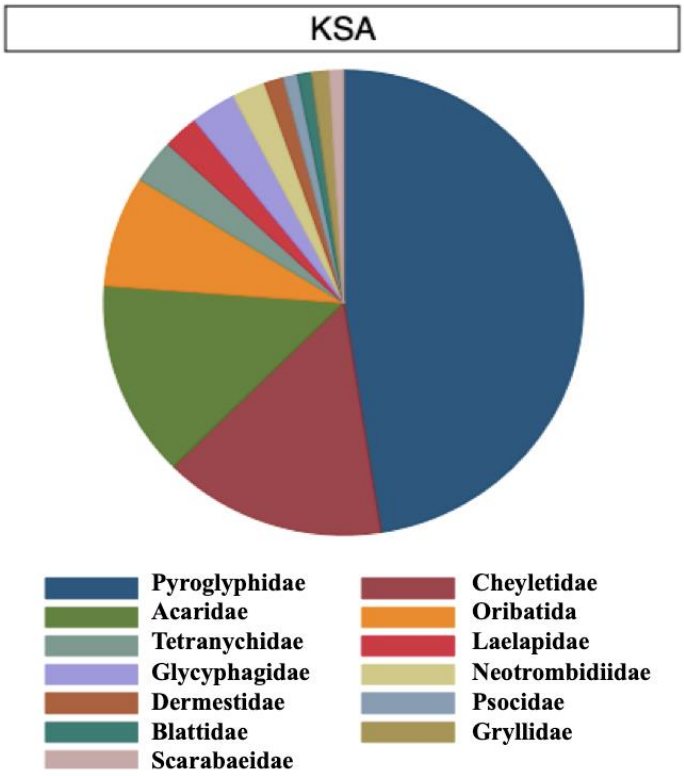


Figure 2.2: The arthropods' composition of house dust.

The fauna found in house dust seems to vary notably between the five different cities. Al-Kharj and Riyadh are both located in the desert and their house-dust fauna is largely composed of mites from the Cheyletidae family. Samples from Jeddah, Shafa and Taif appear to contain a large number of mites from the family Pyroglyphidae, followed by Acaridae and Cheyletidae (Figure 2.3).

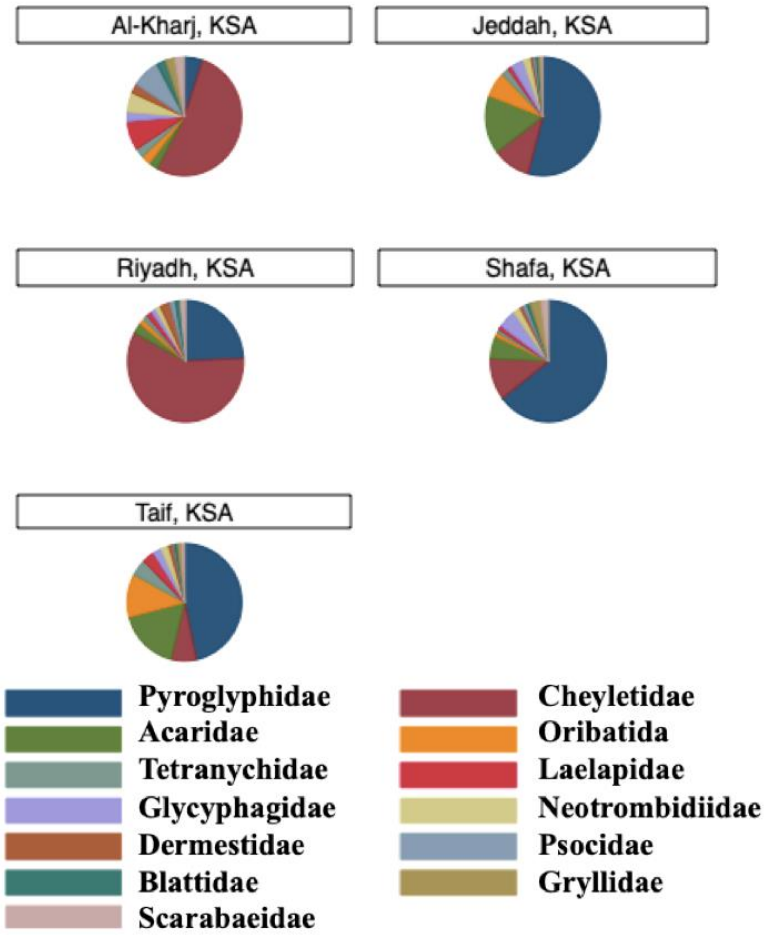


Figure 2.3: The Arthropods' composition of house dust in five major cities in KSA. Desert Cities: Al-Kharj and Riyadh; Coastal Area: Jeddah; Highlands: Shafa and Taif.

Visual assessment of the dendrogram (Figure 2.4) demonstrates a good support for the association between mite families based on correlation with a similarity index. This test was performed to group invertebrates according to similarities of abundance by location. All the mite families were grouped together at a level of similarities greater than 75% with a bootstrap of 68% and 3 major groups are supported. One cluster is formed by mites from the families Acaridae, Neotrombidiidae, Glycyphagidae and Pyroglyphidae and seemed to appear at similar sampling locations with low support (29%). Oribatid mites alongside the spider mites, Tetranychidae, were grouped with the predatory Laelapidae in 45% of the cases. Cheyletidae mites showed no similarities with mites from the other families, in terms of sampling location.

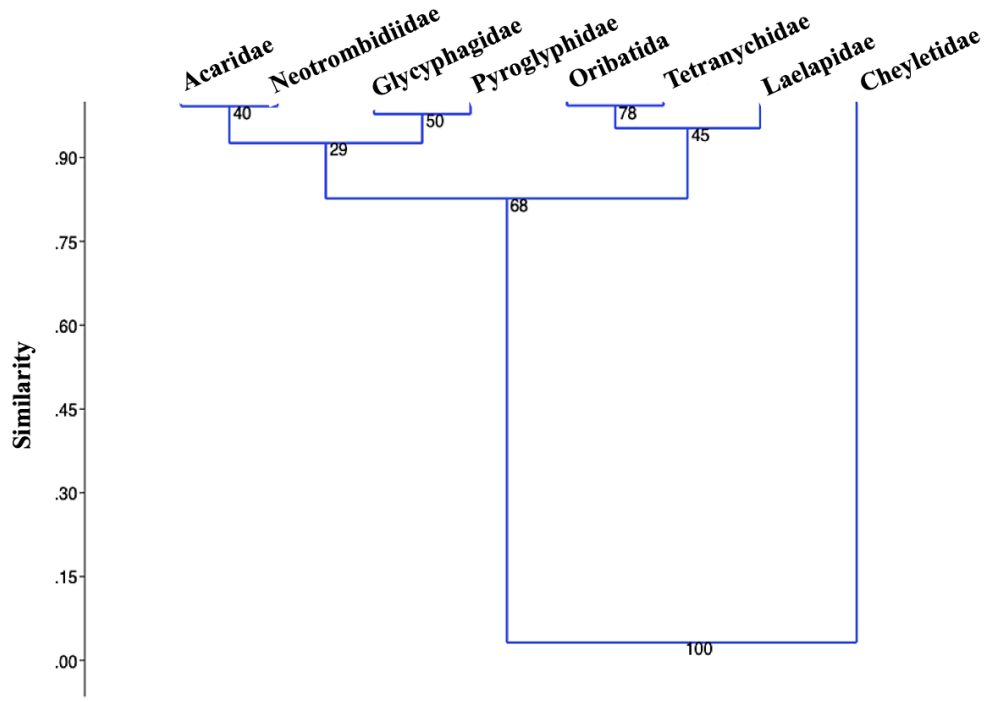


Figure 2.4: Dendrogram including eight mite families: Bootstrapped cluster analysis (UPGMA), using correlation similarity coefficient matrix. Numbers at the nodes indicate the probability of the branching (1000 iterations).

2.3.2 Association between mite families and geographical regions in KSA

Particular mite species are specific to different environments. In this case, the different geographical areas, coast area, desert cities and the highlands of Saudi Arabia seem to be associated with certain species of mites (Figure 2.5).

There is a high positive correlation between dust samples from houses located in the coastal area of Saudi Arabia the mite families Glycyphagidae, Acaridae and Pyroglyphidae (Figure 2.5). Oribatid and Tetranychidae mites are associated to the highlands on the CA graph. And the desert cities are grouped with individuals of the Cheyletidae family. Mites from the Neotrombidiidae are located in the center of the graph and close to the origin, showing no variation. Whereas, species from the family Laelapidae did not associate in particular with any of the locations.

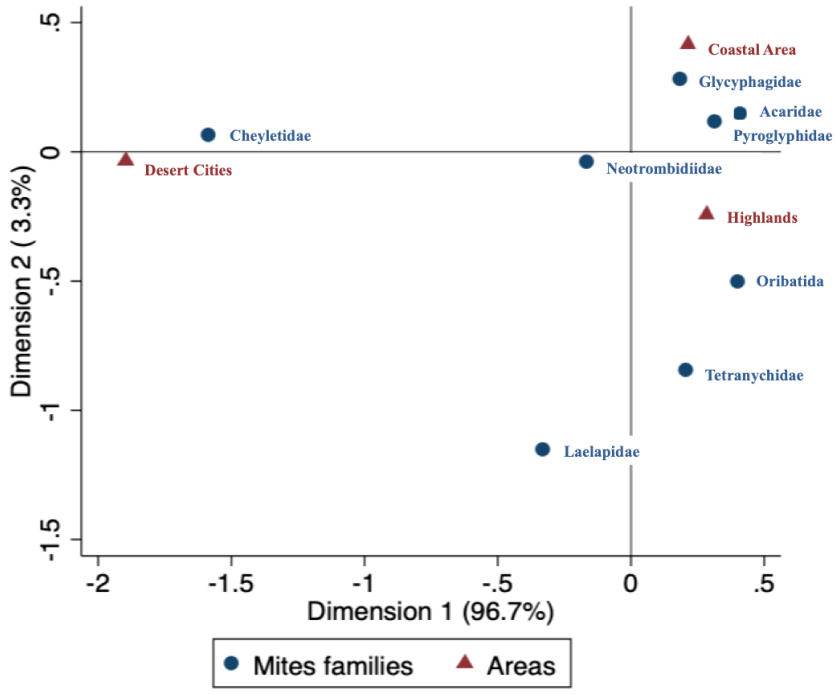


Figure 2.5: Correspondence analysis biplot for three sampling regions and mite families. Desert Cities: Al-Kharj and Riyadh; Coastal Area: Jeddah; Highlands: Shafa and Taif.

2.3.3 Diversity of mites species from the genus *Dermatophagoides*

The yet unknown *Dermatophagoides* mite species (described in Chapter 3) is only found in house-dust samples collected from the coastal area of Saudi Arabia (Appendix data Table A-2). *Dermatophagoides saudi* sp. nov. seems to be directly associated with the coastal households (Jeddah) and it represents around the third of dust mites, members of the Pyroglyphidae family in this region.

In the desert cities of Saudi Arabia (Al-Kharj and Riyadh), the Pyroglyphidae family in house-dust is represented by *Dermatophagoides pteronyssinus* and *D. farinae* equally (Figure 2.6). While in the cities of Shafa and Taif, in the highlands of Saudi Arabia, the species *D. pteronyssinus* represents the majority, with two thirds of all pyroglyphid mites retrieved from households in this region. The other third corresponding to mites from *D. farinae* (Figure 2.6).

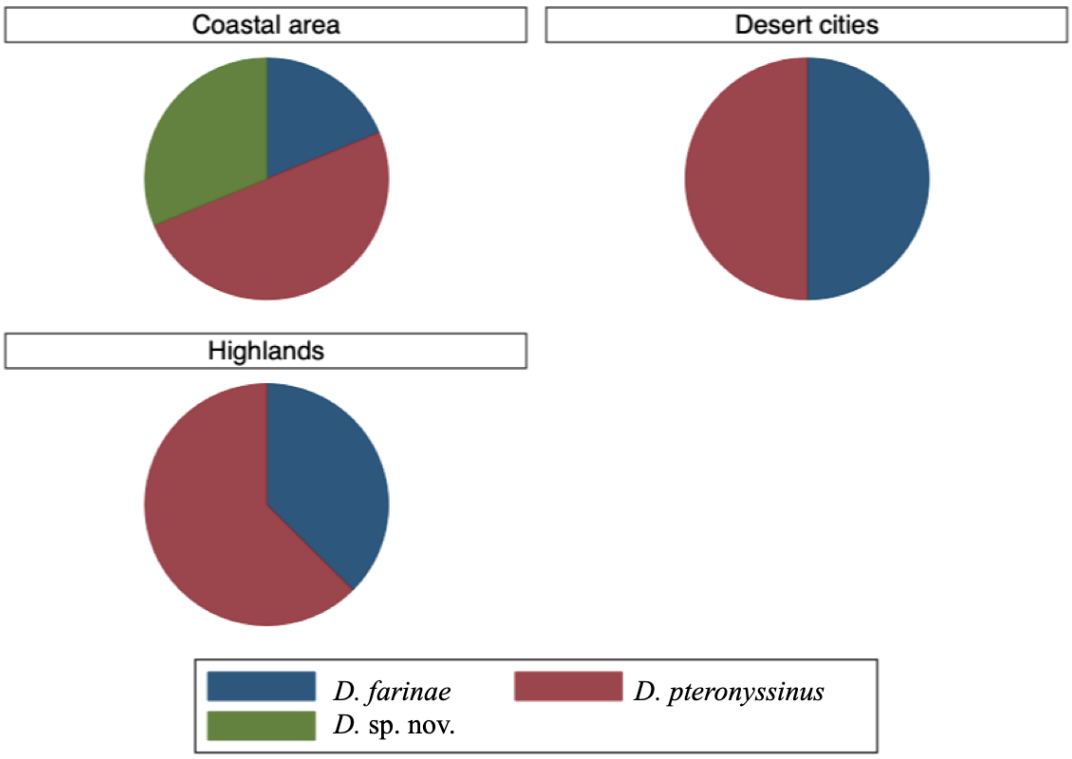


Figure 2.6:The diversity of mites' species from the genus *Dermatophagoides* found in house-dust sampled the three main regions of KSA. *D.*sp.nov.: *Dermatophagoides* sp.nov.

Based on the Kendall correlation coefficient, in KSA the mite species *Dermatophagoides pteronyssinus* and *D. farinae* have a positive correlation. They appear together in dust samples collected from Saudi Arabian households. The new species *Dermatophagoides* sp. nov. does not show correlation with the other two species from the family Pyroglyphidae (r = 0.2) (Table 2.1).

Table 2.1:Kendall correlation coefficient to test for independency between three mites species from the genus *Dermatophagoides* in KSA. (obs=5). *D.*sp. nov.: *Dermatophagoides* sp.nov.

	D. farinae	*D. pteronyssinus*	*D.* sp. nov.
D. farinae	1.0000		
D. pteronyssinus	0.8000	1.0000	
D. sp. nov.	0.2000	0.2000	1.0000

2.4 Discussion

House-dust may contain a variety of micro-arthropods surviving in a particular environment and building a relationship with humans (Colloff, 2010). Dwellings in the Kingdom of Saudi Arabia appear to comprise a wide range of arthropods. This can be due to the environmental conditions, or because of the habits, lifestyle and culture of the individuals. Mites' life cycle and colony dynamic, may be influenced by a range of elements, including abrupt environmental changes like temperatures, humidity, pH and food (Krantz, 1978, Krantz and Walter, 2009, Colloff, 2010).

The geographical locations studied in the KSA have two climatic variations with a difference in land elevations (Indraganti and Boussaa, 2017). The region of Riyadh and Al-Kharj both categorized as desert cities, have a hot-dry climate with a desert subzone. The region of Jeddah, Taif and Shafa is covered by a subtropical climate with a Mediterranean subzone (Indraganti and Boussaa, 2017); with the difference of Jeddah, being a coastal city laying directly on the coast; and both of Taif and Shafa located on the mountains.

Variations in temperatures and humidity affect the growth and development of many mite species, like members of the family Acaridae (Klien and Walzl, 2010).
The most encountered mites species extracted from vacuum cleaners contents are from the family Pyroglyphidae (Colloff, 2010, Wharton, 1976). Other mites are also associated with humans or their stored foods in houses, such as mites from the families; Acaridae, Neotrombidiidae and Glycyphagidae (Zhang, 1998, Hughes, 1976, Colloff, 2010). Mites from the family Acaridae are associated with stored food; they favor indoor environments with high levels of humidity (Klien and Walzl, 2010). Neotrombidiidae, or velvet mites are associated with domestic plants, thus they have developed a close relationship with humans and their dwellings (Hughes, 1959). Members of the family Glycyphagidae were reported to be associated with rodents but they also have been extracted from house dust and display a close relationship with humans (Van Bronswijk et al., 1973).
In this study, these four families of mites showed a close relationship in terms of locations, where they have been sampled and were clustered together (Figure 2.4 and 2.5).

The families Pyroglyphidae, Acaridae and Glycyphagidae seemed to show an association with the coastal area, suggesting that house dust samples collected from homes in Jeddah, KSA, contained a larger number of mites from these three families (Figure 2.5); this is perhaps due to the frequent humidity bursts in the climatic regions, due to the proximity to the sea.

Mites from the family Pyroglyphidae, found in the house dust in this study, were all from the genus *Dermatophagoides*. Three main species were recovered from samples collect from Saudi Arabian house dust, they are *D. pteronyssinus* and *D. farinae* in addition to a new species, *Dermatophagoides* sp. nov which description forms Chapter 3 (Figure 2.6).

The new mite's species *Dermatophagoides* sp. nov. was found only in samples collected from houses of the coastal area with subtropical climate and Mediterranean subzones. The unique environmental factors of this geographical region seem to provide the required conditions for these mites to survive. In this case, with adequate knowledge, the new species of *Dermatophagoides* may be utilized as a forensic marker of location and can possibly link a crime to a particular area in the world, similarly to the case described by Hani et al. (2018).

Other opportunistic mites like those from the families; Laelapidae, Cheyletidae, Tetranychidae and the oribatid mites have been repeatedly found in human houses preying on other micro-arthropods, moving indoor via phoresy and pets, and on domestic plants or on the humans themselves. Although, species of the Cheyletidae were abundantly found in the house-dust collected from desert cities covered by a dry hot climate (Figure 2.3 and 2.5). Preliminary identification of the species in this family showed that two types of mites are found, predatory and bird/mammal skin, cheyletids. Their habit, especially those from birds, justifies their presence in the desert, as these houses are more prompted to the visit of wild birds and mammals.

Because the majority of crimes happen in a closed environment, indoor crime scenes are important scenarios to simulate and study (Taupin and Cwiklik, 2010, Coyle, 2016). Phoretic mesostigmatid mites have been linked to decomposition of corpses, carcasses and their associated arthropods outdoors and indoors. They have been successfully used to estimate time elapsed since death or to prove the presence of their associated arthropods

in a number of crime cases and forensic studies (Hanifah et al., 2015, Hartini et al., 2003, Saloña-Bordas and Perotti, 2014, Perotti et al., 2009b, Perotti, 2001, Perotti et al., 2009a, Wade and Rodriguez, 1961, Leclercq, 1978, Kamaruzaman et al., 2018).

Also mites from the family Acaridae, like the ones found in this study, have been reported as forensically important. The species *Tyroglyphus longior* has been found in large numbers associated with indoor decomposition of a newborn corpes and they have been used to estimate the time elapsed since death. Mégnin (1894) mistakenly thought that these mites were phoretic and might have reached the decomposing cadaver 5 months before the body was discovered and an autopsy was conducted. Hundred and thirty years later, Perotti (2009) reanalyzed the case and based on new information the time of death was estimated to 8 months before it was discovered (Perotti, 2009).

Mites are able to give clues on locations of origin; they can be linked to specific environments, specific plants, soils, and regions in the world. By using their life cycle, they can also help estimating the time elapsed since they came in contact with decomposing cadavers. There is a lack of adequate knowledge with regards to the indoor environments. Studies like this one may facilitate the understanding of their association with human dwelling and make a first stepping-stone for future studies of decomposition and crime scenes in indoor environments.

Chapter 3 : A new species of house dust mite, *Dermatophagoides saudi* sp. nov. (Acari: Pyroglyphidae) from Saudi Arabia.

Abstract

Dermatophagoides saudi sp. nov. is a new domestic mite species from the family Pyroglyphidae. Its description is based on females and males found in house dust collected from human dwellings in the city of Jeddah and its outskirts, Saudi Arabia. The new species can be easily distinguished from other *Dermatophagoides* mites by unique diagnostic characters in both sexes, such as the dorsal idiosoma striation, prodorsal shield, apodemes, and chaetotaxy in general; additional sclerotized plates on both lateral sides of the hysteronotal shield, and variations of the epimera in males. Similarities in morphologies, like in the receptaculum semini of females, locate this mite within the *pteronyssinus* species group.

Dermatophagoides saudi sp. nov. becomes highly relevant in health as a newly described allergen causative, prompting the development of regional allergy treatments. New species of mites from the indoor environment are important markers in forensic analysis, especially for analyses of provenance.

Keywords

Acari, Pyroglyphidae, *pteronyssinus* species group, house dust, allergens, traces, forensics.

This paper is under review for the Saudi Journal of Biological Sciences with the submission number: SJBS-S-21-00061

3.1 Introduction

House dust mites are micro-arthropods that are associated with human dwellings and are worldwide the focus of allergy studies as major producers of indoor allergens (Hughes, 1976, Colloff, 2010, Fain, 1967, Van Bronswijk and Sinha, 1971, Mumcuoğlu and Özkan, 2020). They can be found in every conceivable human-inhabited environment, in carpets, beds, curtains, furniture, wardrobes as well as kitchen cupboards. Their primary source of food is human skin dander and shedding, which later become contaminated by bacteria and mould (Hughes, 1976, Colloff, 2010, Hubert et al., 2020, Arlian, 1992, Arlian and Morgan, 2003). The most common mites found in houses belong to the family Pyroglyphidae (Astigmata); in the wild are found in nests of Icteridae (*Quiscalus quiscula*), Tyrannidae (*Sayornis*), Hirundinidae (*Petrochelidon fulva*) and other birds; in nests of *Peromyscus* and on Hominidae (Colloff, 2010, Van Der Hoven et al., 1992, Van Bronswijk and Sinha, 1971, Racewicz, 2000, Fain, 1967).

The genus *Dermatophagoides* was first described by Bogdanove in 1864 (Fain, 1967). In 1966, Fain redescribed *D. pteronyssinus.* He suggested that there were no apparent differences between the species *D. pteronyssinus* (Trouessart), *Visceroptes saitoi* Sasa and *Mealia toxopei* Oudemans, and consequently described them as being the exact same species. Specimens of the species *D. scheremetewskyi* were lost and the drawings were not complete for the diagnosis, therefore, Fain (1966) could not completely exclude this species from being an additional synonym of *D. pteronyssinus.* He presented the cosmopolitan characteristics of this species and its occurrence in various habitats and environments. Fain (1966) was the first to report that *D. pteronyssinus* is mainly found in house dust and its presence is strongly associated with humans, feeding on skin shedding found in the house dust. Despite their high occurrence in homes and direct association with humans, mites from this family have rarely been described morphologically; and up-to-date no new species has been reported from the Arabian peninsula. Only a study in 1997 by Al-Frayh, et al. (1997) indirectly reported the presence of house dust mites in varied climatic regions in Saudi Arabia (by studying allergens), emphasising the prevalence of *Dermatophagoides* species, especially in coastal areas like the city of Jedda. Due to their suspected causative role of asthma, some skin and some respiratory allergies (Ade et al., 2020, Warner et al., 1999, De Souza et al., 2020), intensive research on these mites and their allergens has been carried out mainly in Europe and America, collecting, over the course of more than four decades a considerable amount of data. Up to 2% of the

world population develop allergic reaction to these ubiquitous mites, which leads to an interest in understanding their biology, physiology, ecology and specifically their allergens (Ade et al., 2020, Warner et al., 1999, De Souza et al., 2020, Erban et al., 2020, Hubert et al., 2020).

In general, mites (the Acari) are omnipresent and can be found everywhere, however, they are subtle to the surrounding ecological changes, which can make many species environmentally quite specific (Colloff, 2010, Arlian and Morgan, 2003, Neal et al., 2002). Mites colonise new houses via humans, pets, plants, arthropods or other micro-arthropods through phoresy (Perotti and Braig, 2009); but interestingly, house-dust mites do not thrive into large colonies without the permanent presence of humans (Warner et al., 1999). It has been found that mite species on bed linen are different from those found underneath the beds. Also, mites colonising wardrobes are different from those found on curtains, rugs and other fabrics inside houses (Frost et al., 2009). Mite species vary significantly between one household to another, or even within the same household, different microhabitats (Colloff, 2010, Warner et al., 1999, Van Bronswijk and Sinha, 1971, Soltani et al., 2011, Wharton, 1976, Neal et al., 2002). These properties make mites of high importance in forensic investigations of indoor crime scenes, as indoor markers (Perotti et al., 2009a, Frost et al., 2009, Solarz, 2009). So far, mites encountered in indoor crime scenes, have been directly linked to decomposing human corpses. Leclercq and Verstraeten (1988), Russell et al. (2004) and Pimsler et al. (2016) have reported the abundance of astigmatid mites on indoor decomposing human bodies, which were solely colonised by mites. The absence of any other arthropods raise the importance of some, unique indoor mite species in forensic settings, becoming suitable markers for origin/location of a crime, illegal trade of goods, and even of victims or perpetrators (Prichard et al., 1986, Hani et al., 2018, Szelecz et al., 2017).

Identifying and describing new species is essential for the understanding of allergens' chemistry and their associated allergies. In terms of knowledge of the diversity of the dust fauna from different parts of the world, a new species of arthropod from inside houses, endemic or specific to a particular biogeographical region becomes a new biological marker impacting the way new treatments for allergies should be conducted, as well as adding to the scarce list of regional, indoors forensic markers. Here we describe a new species of the genus *Dermatophagoides* located within the cosmopolitan *pteronyssinus* species group. All descriptions were carried out from adult specimens collected in house dust from the city of Jeddah, Saudi Arabia.

3.2 Materials and Methods

The specimens examined and described in this paper were extracted from house dust collected from 15 households located in the urban area of Jeddah (the city and its surroundings), Saudi Arabia. The dust was vacuumed from carpets, rags and floors, by the household owners, who immediately preserved approx. 150g in tubes containing 750 ml of 96% ethanol. The samples were dried for transportation and later shipped to the University of Reading, United Kingdom, where their contents were further processed and examined. Once in the forensic acarology lab, the samples were re-hydrated in 75% (v/v) ethanol (Ethanol Vol/Water Vol).

Mite specimens were identified from the dust and manually extracted using a fine paintbrush (5/0) and a micro-dissecting needle (0.125 mm, ultra fine, 1 Micron tip) under a stereo-microscope (LEICA M125).

The selected specimens were permanently mounted in Hoyer's medium for further microscopic examination following Hani et al. (2018), Henderson (2001), Amrine and Manson (1996), Krantz (1978), Anderson (1954).

The mounted specimens were examined with a Nikon Optiphot phase contrast microscope at up to 1000X (10X ocular used with a 100X objective). The morphological observations and measurements were made in micrometres (µm) and at appropriate magnifications. The digital imagery was captured using Motic Moticam 3 Plus (microscope digital camera).

The setal nomenclature system (chaetotaxy) used in this study follows that of Griffiths et al. (1990).

The comparison of main morphological characters was done using main identification keys from Fain (1966) and Fain (1967), keys to the genera published by Hughes (1976), and keys to the species groups and species that offer more detail, and follow a comprehensive system, by Colloff (2010).

3.3 Results

3.3.1 Genus: *Dermatophagoides* Bogdanov, 1864

3.3.1.1 Species group: *pteronyssinus*

Until present, the *pteronyssinus* species group was composed of two mite species; *Dermatophagoides evansi* Fain, Hughes & Johnston, 1967 and *Dermatophagoides pteronyssinus* Trouessart, 1897 (Colloff, 2010, Hughes, 1976). This study describes a third novel species of mites from the genus *Dermatophagoides* (this group) that has significant morphological similarities as well as differences with both mite species from the group. The substantial differences of these mites from the previously described species is sufficient to define them as a novel species, yet their resemblance to mites from the *pteronyssinus* species group merits their inclusion in this group.

3.3.2 *Dermatophagoides saudi* sp. nov. (Figures 3.1- 3.16)

Dermatophagoides saudi sp. nov. in this study, is represented by 2 holotypes (female and male) and 43 paratypes (24 females and 19 males), deposited in the University of Reading. Descriptions are based on the holotypes plus a handful of paratypes. *Dermatophagoides saudi* sp. nov. diagnosis is summarised in Tables 1 and 2, which aim to aid with the future identification of the three species within the *pteronyssinus* group.

3.3.2.1 Diagnosis of adult female (Table 3.1)

3.3.2.1.1 Dorsal side of the idiosoma

The idiosoma (Figure 3.1, ventral view) is 350-380 µm in length and 220-260 µm in width (n=4).

Dorsally, striations of the idiosoma are similar in shape, size and density to those of *Dermatophagoides evansi* and *D. pteronyssinus* (Colloff, 2010, Hughes, 1976, Fain, 1967). The medial part of the dorsal idiosoma is longitudinally striated, starting anteriorly to seta d_1 and ending posteriorly to seta e_1, presenting longer striations than in *D. evansi* and *D. pteronyssinus* where the longitudinal striations are generally limited between d_1 and e_1 (Colloff, 2010, Fain, 1967).

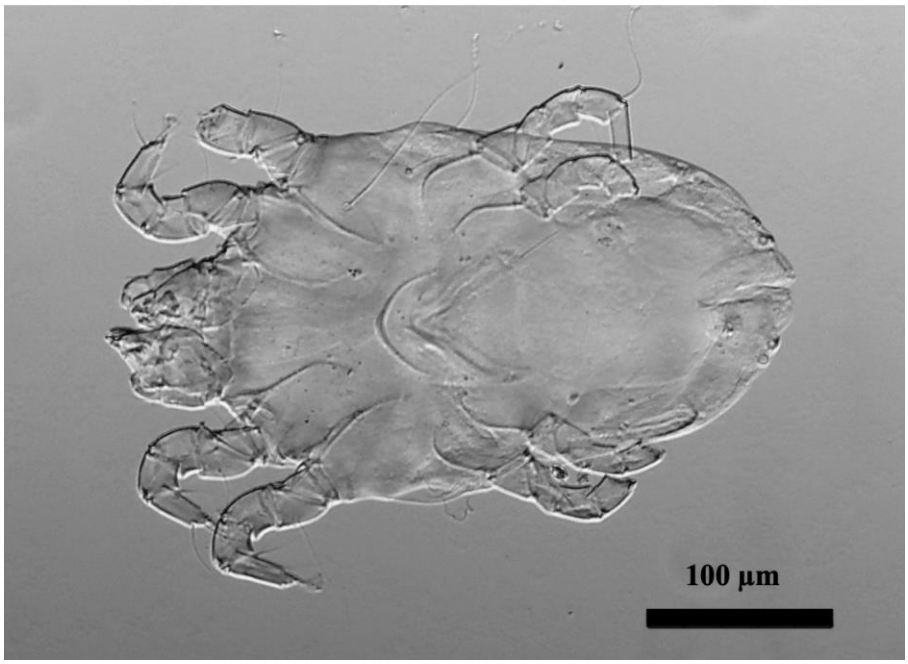


Figure 3.1: *Dermatophagoides saudi* sp. nov. Female.

The prodorsal shield is longer than wider, it has two variations in terms of shapes in the examined specimens (Figures 3.2 and 3.3) and is slightly different in shape from those of the species *D. evansi* and *D. pteronyssinus.* The base of seta *sce* is surrounded by a small, oval shaped, sclerotized and punctuated plate (Figure 3.4).

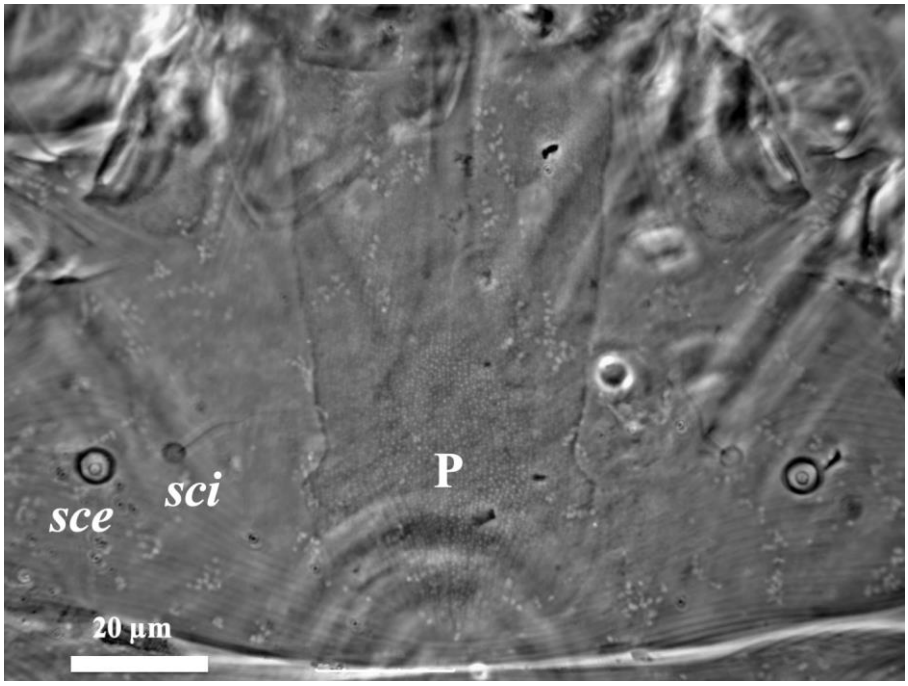


Figure 3.2: *Dermatophagoides saudi* sp. nov. Female. Prodorsal shield (p), variation A).

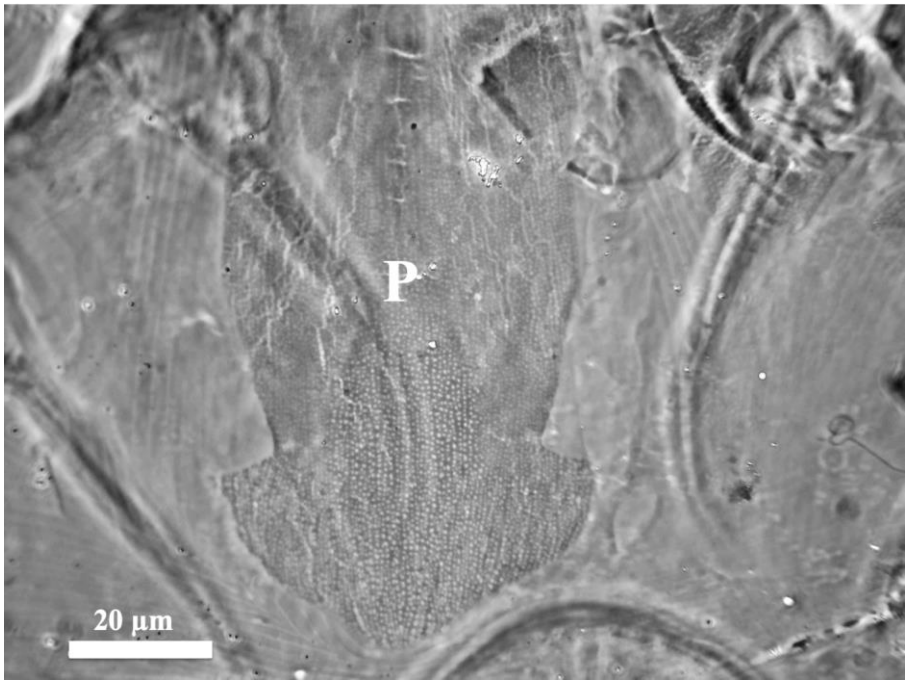


Figure 3.3: *Dermatophagoides saudi* sp. nov. Female. Prodorsal shield (p), variation B).

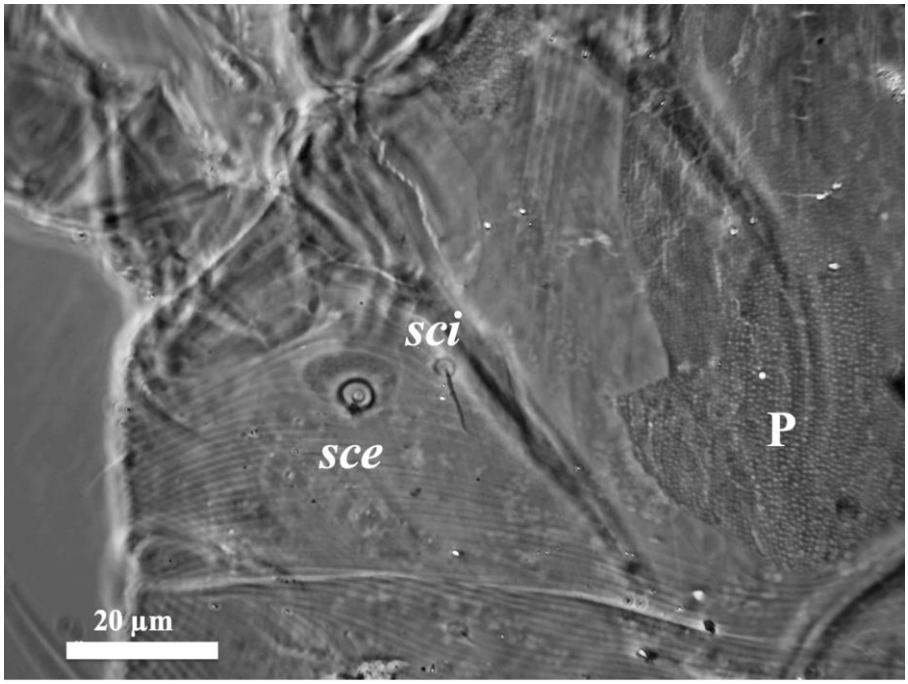


Figure 3.4: *Dermatophagoides saudi* sp. nov. Female. Details of alveoli of setae *sce* and *sci* and the oval punctuated plates.

3.3.2.1.2 Ventral side of the idiosoma

The ventral region of the idiosoma in the female *Dermatophagoides saudi* sp. nov. is less sclerotized than in *D. evansi* and *D. pteronyssinus.*

Epimera are well defined (Figure 3.5, showing epimera I and II) and the apodemes are well sclerotized reaching the dorsal side of the idiosoma around leg I. Receptaculum seminis is circular with 10 to 12 lobes; it is U–shaped in the cross section and much broader at the apex than the base, in comparison to that of the species *D. pteronyssinus.*

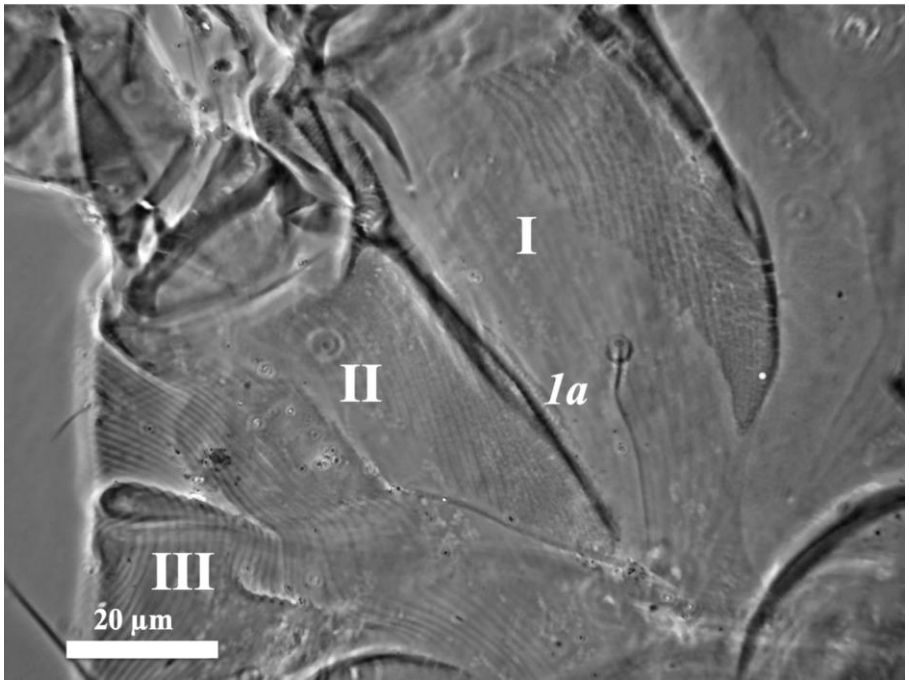


Figure 3.5: *Dermatophagoides saudi* sp. nov. Female. Details of epimera I, II and their sclerotized borders (apodemes). Seta *1a* laying outside of the sclerotized region of epimera I.

The ductus bursae is uniform in thickness and the bursa copulatrix is cone-shaped, enclosed by a sclerotized plate on the posterior region of the opisthosoma (Figure 3.6).

Epigynium is located between legs I and IV, it has an inverted, bigger U-shape in comparison to both other species, it is strongly sclerotized and anteriorly to it, the cuticles are longitudinally striated (Figure 3.7).

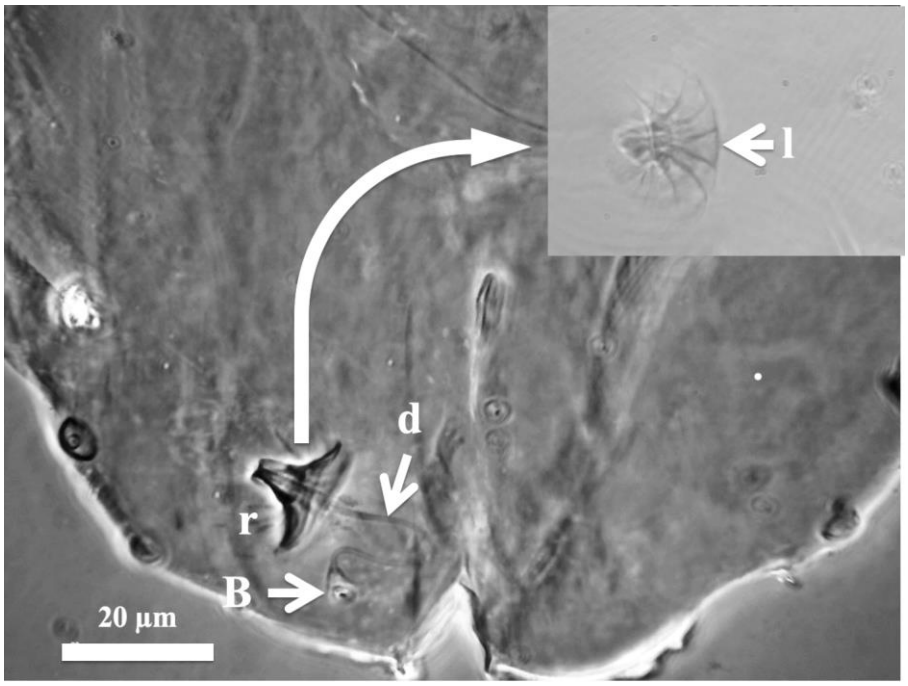


Figure 3.6: ***Dermatophagoides saudi*** **sp. nov. Female. Details to the U-shaped sclerotized receptaculum seminis (r) in the cross section, composed of 10-12 lobes (l) when viewed from the top (thick arrow), ductus bursae (d) and bursa copulatrix (B).**

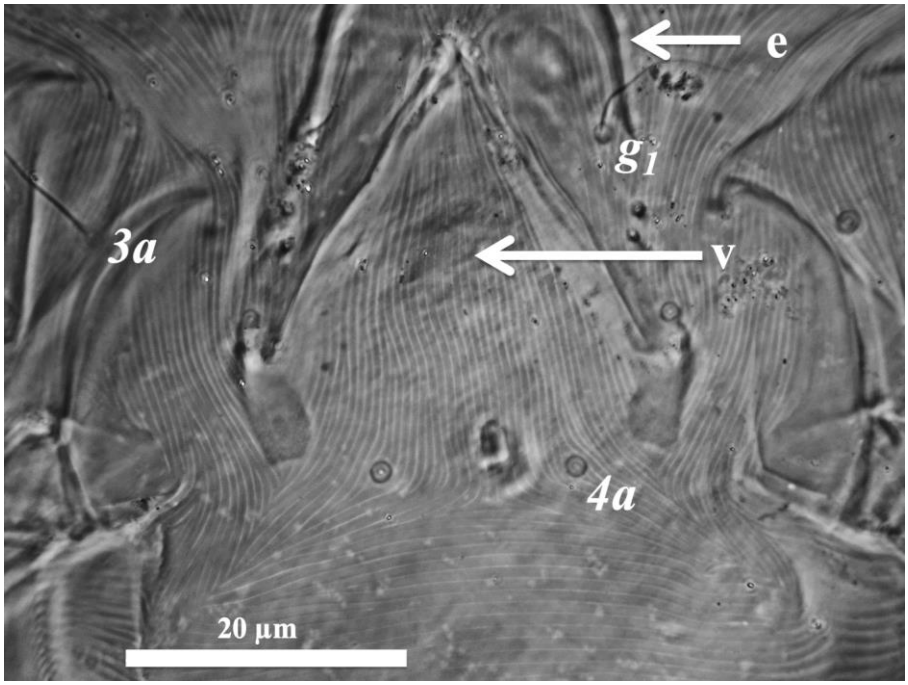


Figure 3.7: ***Dermatophagoides saudi*** **sp. nov. Female. Sclerotized epigynium (e), details of vulva (v) and setae** ***3a, g1*** **and the vestigial setae** ***4a*** **(only the alveoli are present).**

3.3.2.1.3 Legs

Legs I, II, III and IV are relatively equal in width and length, with leg III being slightly longer than leg IV.

3.3.2.1.4 Chaetotaxy

Generally, the chaetotaxy of the idiosoma of *Dermatophagoides saudi* sp. nov., presents some similarities to that of *D. evansi*. The main differences are observed dorsally in *sce* being shorter; and ventrally in *1a* not being located in the sclerotized region of epimera I (the apodemes) nor in the edge of it (its position is rather similar to that of the species *D. pteronyssinus*), *1a* is 27-28 µm (n=3) (Figure 3.5). Seta g_1 is longer, it is 17-18 µm in length (n=3); g_2 is also longer and exceeds the alveoli of seta *4a*, it is 26-28 µm long (n=3). At the base of the vulva, setae *4a* is vestigial (only alveoli are present) (Figure 3.7).

Setae h_1 and h_2 in the novel species (not shown) are similar to those of *D. pteronyssinus* and different from *D. evansi* in that they are not found closer to each other and are not located in the same sclerotized perianal region.

Chaelotaxy of the legs is similar to the two species, *D. evansi* and *D. pteronyssinus*, with the exception of a few dissimilarities:

Leg I: σ_1 absent, σ_2 longer measure of ~9µm, seta of genus I is absent in the specimens studied (Figure 3.8)

Leg II: σ is similar to that of *D. pteronyssinus* and much longer than that of *D. evansi*

Leg III: tibia is thicker and the tarsus is thinner than those of the two species from the *pteronyssius* group. ϕ is shorter reaching less than two thirds of tarsus III (~45µm) (Figure 3.9).

Leg IV: similar to both other species.

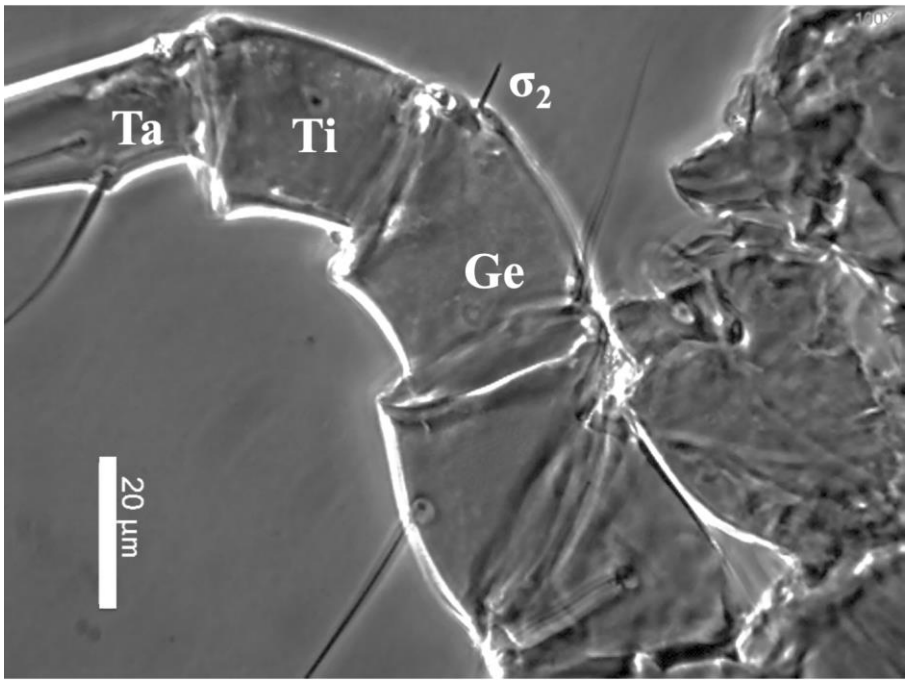


Figure 3.8: ***Dermatophagoides saudi*** **sp. nov. Female. Details of leg I; setae σ_1 absent and σ_2 longer than the other species.**

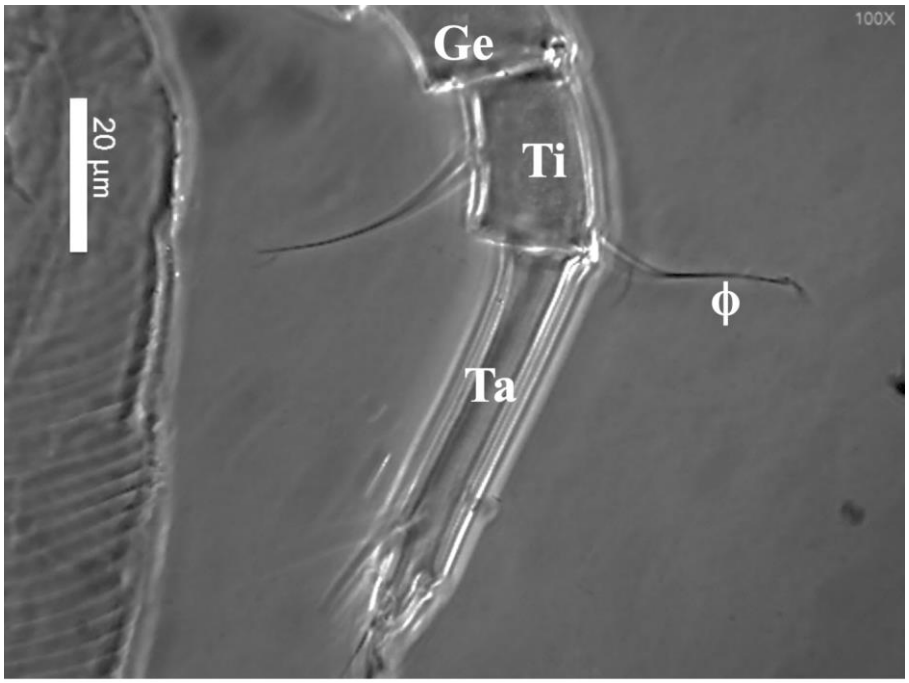


Figure 3.9: ***Dermatophagoides saudi*** **sp. nov. Female. Leg III with details of tarsus (Ta) and tibia (Ti). Setae ϕ shorter, reaching less than two thirds of tarsus III.**

Table 3.1: A summary table of the main morphological differences of females from the three species, members of the *pteronyssinus* apecies group.

Diagnostic morphologies	*D. saudi* sp. nov.	*D. pteronyssinus*	*D. evansi*
Idiosoma			
-Length	350-380 µm	305-400 µm	360-440 µm
-Width	220-260 µm	201-240 µm	240-295 µm
Dorsal striations	Longitudinal, starting anteriorly to setae d_1 and ending posteriorly to setae e_1.	Longitudinal, starting between setae d_1 and e_1 but slightly different in patterns than the two other species.	Longitudinal, starting between setae d_1 and e_1.
Prodorsal shield	Two variations observed	Nine variations documented	One variation documented
Alveoli of setae *sce*	Surrounded by a slightly larger oval sclerotized and punctuated plate.	Surrounded by a small round sclerotized region/ ring.	Surrounded by a small round sclerotized plate.
Receptaculum seminis	Sclerotized, circular with 10-12 lobes. U–shaped in the cross section and much broader at the apex than the base.	Sclerotized, circular with 10-13 lobes. U–shaped in the cross section and slightly broader at the apex than the base.	Sclerotized base with no lobes. U–shaped in the cross section and broader at the base than the apex.
Ductus bursae	Uniform in thickness.	Uniform in thickness.	Twice as thick in the posterior part when compared to the anterior part.
Bursa copulatrix	Cone-shaped, enclosed by a sclerotized plate on the posterior region of the opisthosoma.	Cone-shaped, but it has not been documented weather it is enclosed by a sclerotized plate or not.	Indistinct from the ductus bursae.
Epigynium	U–shaped with a much bigger curve than both other species.	U–shaped.	U–shaped.
Chaetotaxy			
- Setae *1a*	-Not located in the sclerotized region of epimera I (apodemes).	-Not located in the sclerotized region of epimera I.	-Its alveoli are located in the margin of the sclerotized region of epimera I.
- Setae g_1			

- Setae g_2	-longer than in both other species, it measure 17-18 µm. - longer than both other species and exceeds the alveoli of seta *4a*, it is 26-28 µm long.	- Does not exceed the alveoli of seta *4a*.	-Does not exceed the alveoli of seta 4a.
- Setae *4a*	- Vestigial (only alveoli are present).	- Setae present	- Setae present
- Setae *h1* and *h2*	-Not found closer to each other and are not located in the same sclerotized perianal region.	-Not found closer to each other and are not located in the same sclerotized perianal region.	-They are close to each other and are both found in the same sclerotized perianal region.
- Setae of leg I	- σ_1 absent, σ_2 longer measuring ~9µm.	- σ_1 present.	- σ_1 present.
- Setae of leg II	- σ is long.	- σ is long.	- σ is shorter than both other species.
- Setae of leg III	- ϕ is shorter reaching less than two thirds of tarsus III	- ϕ is longer exceeding the tip of tarsus III.	- ϕ is longer exceeding the tip of tarsus III.

3.3.2.2 Diagnosis of the adult male (Table 3.2)

3.3.2.2.1 Dorsal side of the idiosoma

The idiosoma (Figure 3.10) measures 265-290 µm in length and 168-180 µm in width (n=4).

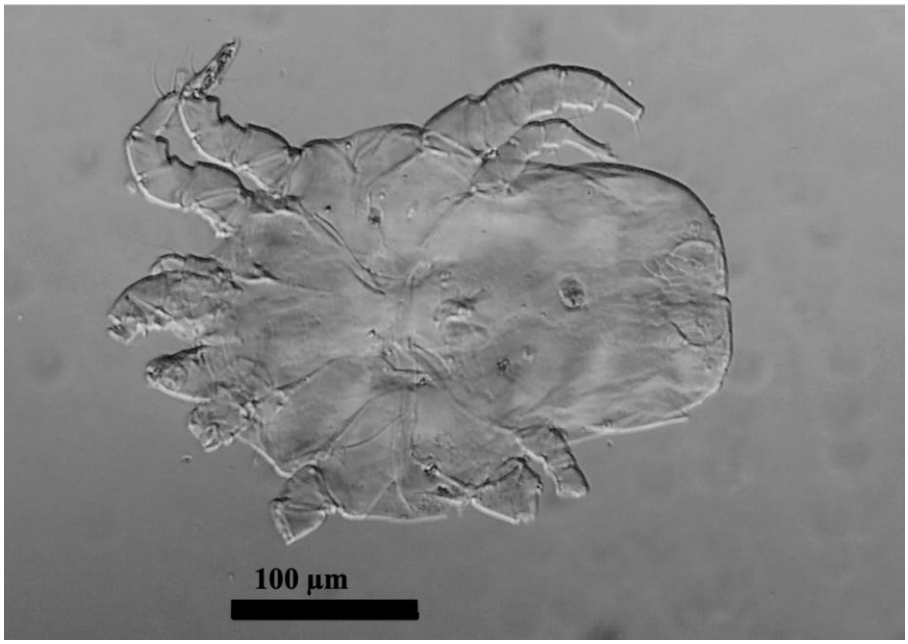


Figure 3.10: *Dermatophagoides saudi* sp. nov. Male.

In *Dermatophagoides saudi* sp. nov. the dorsal striations and the form of the idiosoma are similar to those of *D. evansi*, with minor differences to *D. pteronyssinus*. The prodorsal shield is slightly different in shape from both species (Figure 3.11) and the sclerotized regions of the epimera (apodemes) reach the dorsum and are slightly bigger than those of *D. evansi*. Alveoli of setae *sce* are located in an oval shaped plate as in females, alveoli of setae *sce* and *sci* are surrounded by a thin sclerotized ring (Figure 3.12).

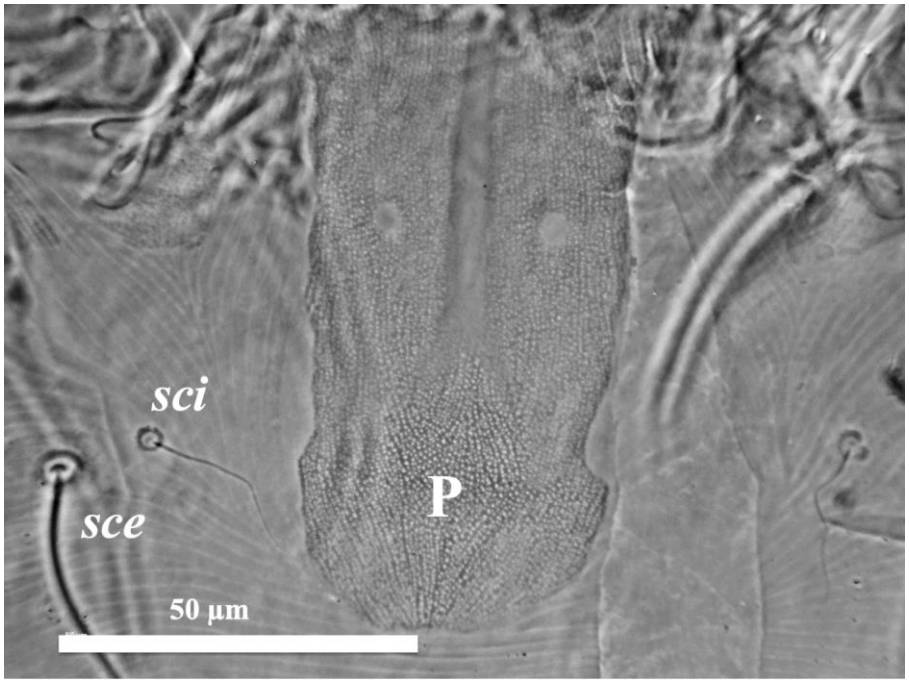


Figure 3.11: ***Dermatophagoides saudi*** **sp. nov. Male. Prodorsal shield (p). Details of setae** ***sce*** **and** ***sci.***

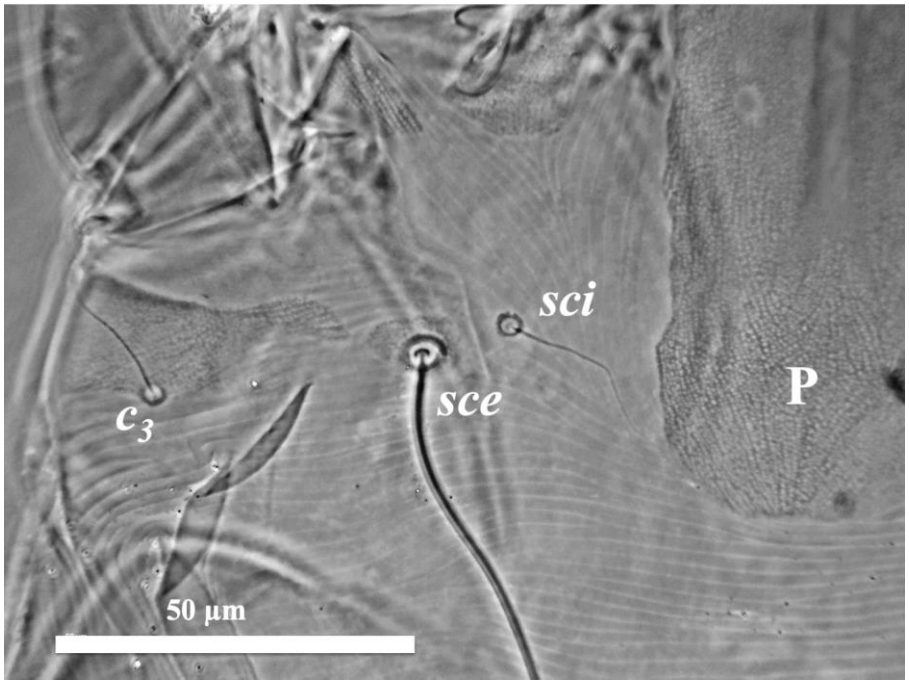


Figure 3.12: ***Dermatophagoides saudi*** **sp. nov. Male. Details of setae** ***sce, sci*** **and** c_3 **with the punctuated plates.**

Setae d_1 (not shown) are shorter than both of the other two mite species. The Hysteronotal shield is similar in shape to those of *D. evansi* and *D. pteronyssinus* but it is much smaller in size, measuring on average 90 µm in length and 45 µm in width at its largest part, which is located in the posterior portion (n=4). Posteriorly, on each of the lateral sides of the Hysteronotal shield, there is an additional lateral sclerotized and punctuated shield, named Para-shield (PS), which is separated from it by only a few striations (Figure 3.13), never observed in the other two species.

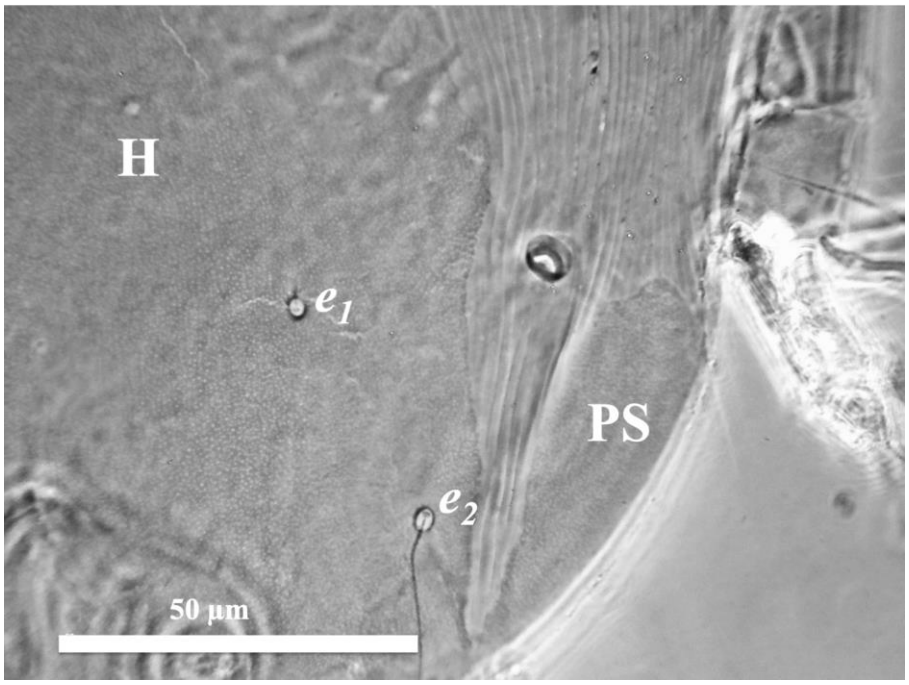


Figure 3.13: *Dermatophagoides saudi* sp. nov. Male. Hysteronotal shield (H) with details of setae e_1 and e_2. The sclerotized region is visible on one side of the Hysteronotal shield, named here Para-shield (PS).

3.3.2.2.2 Ventral side of the idiosoma

Ventrally, the male of *Dermatophagoides saudi* sp. nov. combines a mixture of characteristics of both *D. evansi* and *D. pteronyssinus.*

Epimera I, II, III and IV are well defined and not fused at all (Figure 3.14), unlike the other two species where the epimerae II and III are fused together. Apodemes are largely sclerotized, reaching the dorsal side of the idiosoma around legs I, II and III but not as well developed ventrally as the species *D. evansi*.

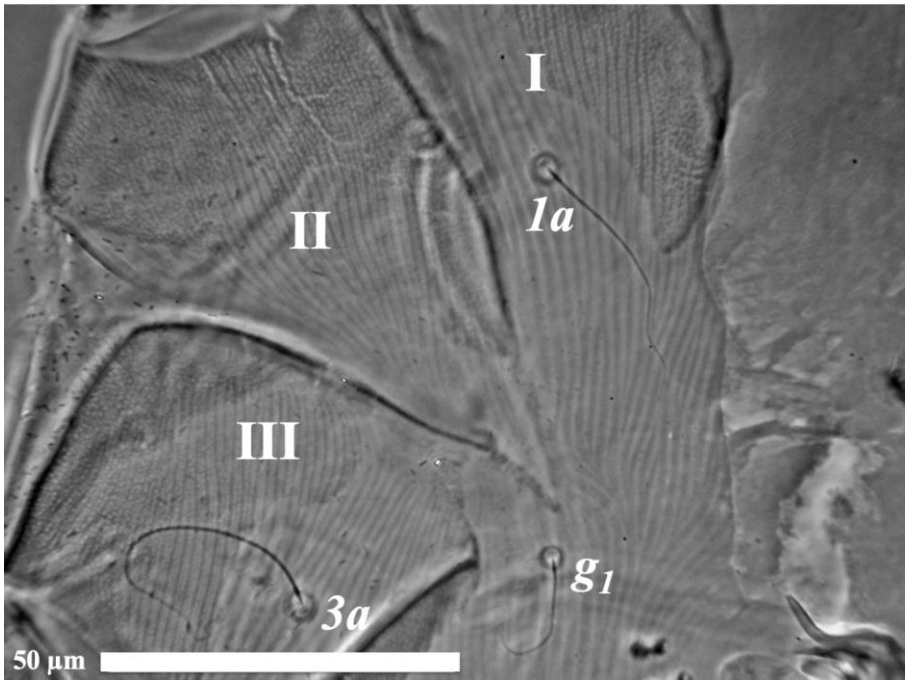


Figure 3.14: ***Dermatophagoides saudi*** **sp. nov. Male. Details of epimera I, II and III not fused and their sclerotized borders (apodemes). Setae** ***1a*** **and** ***3a*** **located outside of the sclerotized regions of epimera I and III respectively.**

The sexual organ is located between two sclerotized shields that in contrast with the other two species are further out (separated) anteriorly than posteriorly (Figure 3.15).

The perianal region is narrow and surrounded by striations, which extend posteriorly reaching and merging with the dorsal striations (Figure 3.16), unlike both species *D. evansi* and *D. pteronyssinus*, where the posterior ventral striations do not exceed the alveoli of setae ps_2. Another difference with these two species is found in the anal plate, that in the new species is broader than long, measuring on average 40 µm in length and 61 µm in width (n=4), and it is located in the middle of the perianal region (Figure 3.16).

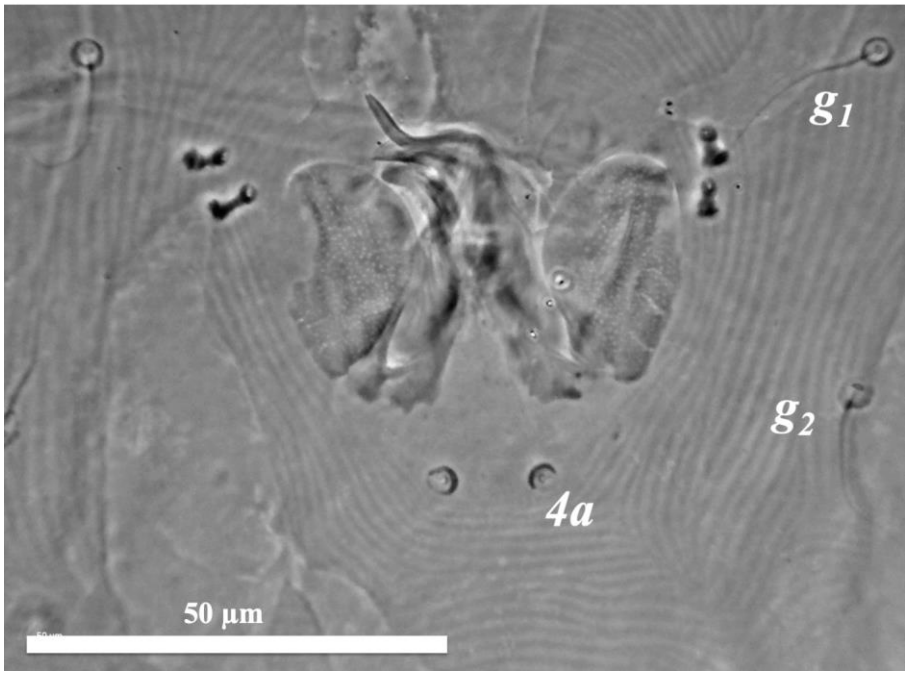


Figure 3.15: ***Dermatophagoides saudi*** **sp. nov. Male. Genitalia with details of setae** g_1 **,** g_2 **and** ***4a.***

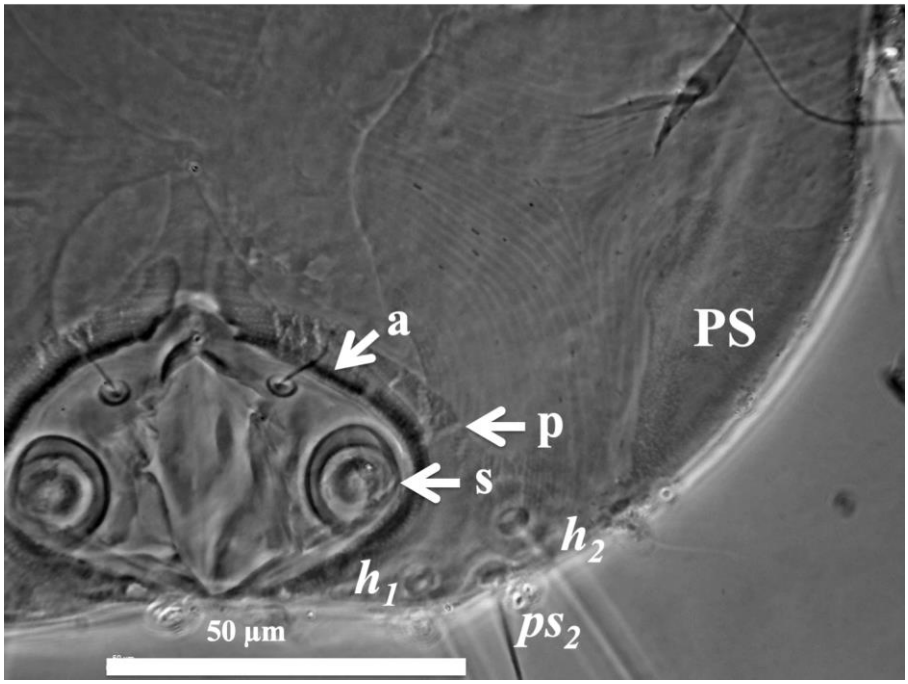


Figure 3.16: ***Dermatophagoides saudi*** **sp. nov. Male. Details of anal plate (a), anal suckers (s), the perianal region (p), para-shield (PS) and setae** h_1**,**h_2 **and** ps_2 **.**

3.3.2.2.3 Legs

Legs I, II, III and IV are rather similar to the legs of the species *Dermatophagoides evansi*. Leg III is almost 1.8 broader and 1.7 longer than leg IV (Figure 3.10).

3.3.2.2.4 Chaetotaxy

In the new species, the main differences in chaelotaxy are observed ventrally in setae *1a* and *3a*, their alveoli are located outside of the sclerotized regions of the epimera (apodemes) I and III respectively (Figure 3.14). This is different from *D. evansi* where the sclerotized apodemes cover most of coxa I and coxa III entierly, but it is quite similar to that of *D. pteronyssinus* .

Dorsally, setae *f2* (not shown) in the new species are longer by a third in comparison with the other species.

Chaetotaxy of the legs is identical to that of *D. evansi.*

Table 3.2: A summary table of the main morphological differences of males from the three species, members of the *pteronyssinus* apecies group.

Diagnostic morphologies	***D. saudi* sp. nov.**	***D. pteronyssinus***	***D. evansi***
Idiosoma			
-Length	265-290 µm	240-309 µm	285-315 µm
-Width	168-180 µm	168-212 µm	189-204 µm
Alveoli of setae *sce*	Surrounded by a slightly larger oval sclerotized and punctuated plate	Surrounded by a small round sclerotized region, which blends with the apodemes II that reach the dorsal side laterally	Surrounded by a small round sclerotized plate
Hysteronotal shield	Much smaller in size, measuring on average 90 µm in length and 45 µm in width at its largest part, which is located in the posterior portion	It measures 150 µm in length and 106 µm in width at its largest part	It measures 180 µm in length and 120 µm in width at its largest part
Sclerotized, punctuated region named para-shield	Present and separated from the hysteronotal shield by only a few striations	Absent	Absent

Epimera I, II, III and IV	Not fused at any level and apodemes less scletorised than other species	Epimera II and III fused together forming a Y-shaped oblique structure	Epimera II and III fused together Y-shaped oblique structure
Perianal region	Sclerotized, narrow and surrounded by striations, which extend posteriorly reaching and merging with the dorsal striations	posterior ventral striations do not exceed the alveoli of setae ps_2	posterior ventral striations do not exceed the alveoli of setae ps_2
Anal plate	Broader than long	Longer than broad	Longer than broad
Chaetotaxy			
- Setae *1a* and *3a*	-Not located in the sclerotized region of epimera I and epimera III	- Not located in the sclerotized region of epimera I and epimera III	- Alveoli of *1a* are located in the margin of the sclerotized region of epimera I and *3a* are in apodemes III
- Setae f_2	-Longer by a third than in both other species		

3.4 Discussion

The arid climate of the Arabian peninsula is not the preferred habitat of pyroglyphid house dust mites (HDM), which require a minimum humidity of about 65% RH for thriving (Brandt and Arlian, 1976, Colloff, 2010). In a study of allergens in houses, to detect the presence of *D. farinae* and *D. pteronyssinus* in four different climatic regions of Saudi Arabia, it was found that the house dust from the city of Jeddah, carry significant higher numbers of the species *D. farinae* (Al-Frayh et al., 1997). The lower yield of *D. pteronyssinus* allergens was perhaps the result of the presence of *Dermatophagoides saudi* sp. nov., which excretes different allergens, not described at the time of the experiment and, therefore, not detected. Up to now, due to the poor occurrence of HDM in the Arabian peninsula in general, no major studies of the diversity of indoor acari species have been conducted. This work brings novel knowledge adding a new species of major HDM unique to Saudi Arabia. *Dermatophagoides saudi* sp. nov. main features fit well with mites from the cosmopolitan species group *pteronyssinus*, except for a few characteristics reported in this study (Colloff, 2010, Hughes, 1976). One of the main

features is the extended or extra sclerotization of tegumentary areas, having extra sclerotised shields like sce and para-shield plates, perhaps an adaptation to the arid climate of the Arabian peninsula, helping preventing water loss.

The presence and abundance of this newly described mite species in house dust collected from dwellings in Jedda, might also provide an answer to allergies that are unique to this geographical area. A more thorough study on the biology, ecology and specific allergens (of this new species) should be considered by practitioners addressing allergies, for a better understanding and future effective treatment for atopic people in the region.

Aside from the subject of allergies, new mite species, like *Dermatophagoides saudi* sp. nov. is a key link to a location/origin. Because HDM are so synchronized with the life of their 'hosts', human beings, they can connect people or goods to other people, to specific habitats, environments, plants, soils, or regions in the world. Because they are living organisms, and not just a piece of an abiotic element or a dust particle, valuable information can be extracted from their complex natural history, offering themselves as basic informative tools in forensic investigations (Braig and Perotti, 2009, Pimsler et al., 2016, Prichard et al., 1986). For example, in a crime scene happening indoors, they can be the only evidence providing time estimations, since they can come in contact with human cadavers from before death.

Studies like this one help increase our understanding of the micro-arthropod fauna living in human dwellings dust in disparate biogeographical regions, generating novel knowledge of the unique and long term association between mites and humans.

3.5 Conclusions

A new species of a major house dust mite, unique to Saudi Arabia is described here. *Dermatophagoides saudi* sp. nov. has unique characteristics becoming a new biological marker of the domestic micro-arthropod fauna of the Arabian peninsula. Further work on allergy should consider the presence of *Dermatophagoides saudi* sp. nov. especially in the coastal areas, where the mite seems to abound.

Morphological diagnostic features like idiosomal striation, shape of prodorsal shield and apodemes, and chaetotaxy in general; as well as unique additional lateral shields in males additional, plus parallelisms with either *D. pteronyssinus* and/or *D. evansi* add this new species to the *pteronyssinus* species group.

3.6 Acknowledgements

The authors thank the house owners in Saudi Arabia for donating the samples of mites. Riyadh Aeban was funded by the Saudi government.

3.7 Conflicts of interest

The authors declare no conflicts of interests

Chapter 4 : Testing preservation methods of mites for the best DNA extraction in quality and quantity.

Abstract

Very few species of house dust mites (HDM) are known to have been molecular characterised. This is due to two main factors. One, the lack of taxonomists able to sort out species from dust; two, due to the lack of standardised protocols for the conservation of specimens, in a way that small amounts of DNA can be properly retrieved for any further molecular analysis.

In this work, different methods of preservation of specimens from two different sources: Laboratory maintained *Dermatophagoides farinae* and field collected *Dermatophagoides* species (from UK and KSA house dust) were explored. This was followed by DNA extractions carried out of the varied-preserved specimens. Three preservation methods were tested: 1. ethanol immersion: E.EXT, 2. freezing to ≈-20 ˚: F. EXT, 3. not preserved, instead directly disrupted by Qiagen ATL (lysis buffer): L.EXT

The most reliable preservation method for DNA extraction was L.EXT (mainly based on results of tests with the laboratory populations), which is the lising in buffer of specimens directly from their medium, with no fixation nor freezing. It was observed a positive correlation between the number of mites used and the DNA yield obtained.

4.1 Background and objectives

Preservation of specimens for optimal extraction of DNA is a crucial step for downstream molecular applications, as many factors can affect the purity, quality and concentration of extracted DNA (Demeke and Jenkins, 2010, Jakubowska et al., 2012, Desloire et al., 2006). Considering that mites are particularly microscopic, most standard DNA extraction protocols designed for invertebrates fail to acquire a reasonably yield for further molecular characterisations, for example, simple SANGER sequencing (Alasaad et al., 2009). Therefore, for this study on HDM it was necessary to investigate, 1st, preservation methods, that were followed by alternative DNA extraction techniques in order to secure enough yield of DNA from a single *Dermatophagoides* specimen, of

suitable concentration of DNA of known purity and quality. The objective of this chapter is crucial to conduct the microsatellite testing, presented in the following Chapter 5.
Will differences in mite preservation methods effect on the results of DNA extraction, its quality and quantity?

4.2 Materials and methods

4.2.1 Sample preservation

Mites originated either from laboratory reared *Dermatophagoides farinae* colonies (Lab-*Df)*, and from the 'wild', provided by house owners, from the dust of their houses in the Kingdom of Saudi Arabia (KSA) and in the United Kingdom (UK).
To test mite preservation methods, 15 batches, all of 4 mites each were isolated from Lab-*Df* into sterilised Petri-dishes. Five mite batches were preserved in the each of following three 'treatments':
1. ethanol immersion: E.EXT
2. freezing to ≈-20 ˚: F. EXT
3. not preserved, instead directly disrupted by Qiagen ATL (lysis buffer): L.EXT

For the specimens from KSA, all dust samples and therefore, the mites extracted from them were preserved in ethanol immersion (E.EXT). The dust was preserved in ethanol in the KSA, then dried and sent to UK where it was rehydrated in ethanol again.

For the specimens from UK, the majority of the dust samples were preserved in ethanol (E.EXT), while a few were preservedby freezing (F.EXT). These two methods of preservation were used arbitrarily depending on the number of samples provided opportunistically. Because the majority of samples were provided by the house owners, they were preserved already by the owners in ethanol. Only a few samples were frozen by the house owners.

4.2.2 DNA extraction

The following whole genomic DNA extraction method was applied to all samples.
Lab-*Df* colony: 15 samples
UK: 25 samples
KSA: 25 samples

Mites were extracted from the preserved tubes and washed thoroughly in distilled H2O. In the case of samples carrying ethanol, E.EXT, these were first dried overnight to remove ethanol. Samples free of ethanol, from the 3 preservation methods were then transferred into 20µL of ATL buffer in a 1.5 ml Eppendorf tube and crushed, the small ATL volume aided homogenisation. When thoroughly crushed, 160µL of ATL buffer was added followed by 20µL of proteinase K. The solution was pipette-mixed prior to 10-second vortexing then incubated overnight at 56 °C. If a gelatinous layer was visible after incubation an extra 50µL of ATL and 10µL of proteinase K was added followed by further incubation at 56°C for one hour, to ensure full lysis and prevent clogging of the spin column during subsequent procedures. Samples were re-mixed and re-vortexed and incubation continued until the gelatinous layer was dispersed. The manufacturer's recommended protocols were then followed (Qiagen DNA extraction kit protocol). After elution, DNA was stored at -20°C until needed.

4.2.3 DNA quantification

To accurately quantify extracted DNA (for further comparisons between preservation methods), a DeNovix DS-11 FX Spectrophotometer / Fluorometer was used, applying the high sensitivity spectrophotometric assay. Prior to this assay, a working solution was prepared (avoiding any prolonged exposure to light) comprising 100 µL dye (DeNovix dsDNA High Sensitivity Dye 100x), 100 µL enhancer (DeNovix dsDNA High Sensitivity Enhancer 100x), and 10 mL buffer (DeNovix dsDNA broad range buffer). Hundred and ninety (190) µL of working solution and a 10 µL DNA were added to two labelled sets of thin-walled 0.5 mL PCR tubes, which were incubated for five minutes. After incubation, measurements were taken with the spectrophotometer.

4.2.4 PCR methods

All Polymerase Chain Reaction methods will be described in the following chapter (5). PCR were performed, to confirm that the quality of DNA, despite the low yield was good enough to carry on with amplifications and sequencing. For this, primer set 90FR2 (described in Chapter 5) was used in PCRs with one sample of each of the three preservation methods, all tests using lab-*Df* (L.EXT, F.EXT and E.EXT). The expected fragment length of the amplified DNA is 183 bp (base pairs).

4.2.5 Statistical Analysis.

Data were reported as the sample mean for measurements from DENOVIX (Figure 5.2). Comparisons between L.EXT, F.EXT and E.EXT were performed using ANOVA, Dunn's posthoc, and for comparisons between yield of DNA obtained and nr of mites used per DNA extraction, Pearsons r, linear correlation. This was performed using the software package PAST3. Microsoft Excel was also used for graphs.

4.3 Results

4.3.1 Lab-*Df* samples: Comparison of preservation methods and DNA quality

Genomic DNA quantification and qualification data from the different preservation methods are presented in Table 4-1. Quantities of DNA are homogeneous within the preservation methods as groups (normally distributed), while quality varies, and A260/280 ratios (cleanliness of DNA) are not normally distributed for E. EXT (ethanol) and F. EXT (freezer). Mean concentrations per extraction method, E.EXT, L.EXT and F.EXT (means: 5.21, 10.26, 8.03 ng/μL respectively) showed significant difference ($F=8.178$, $df=2$, $P<0.005$), particularly between ethanol and directly used (E.EXT, L.EXT, $P>0.01$, Dunn's post hoc), suggesting the preservation method is influential.

In the following Figure 4-1, results of the electrophoresis (gel) run are the confirmation, after amplification (PCR using primer 90FR2), of the quality of the DNA. The highest concentrations of DNA were those extracted from L.EXT, Table 4.1, which coincide with the results of the gel electrophoresis, lane n4. Following these results, L.EXT was

consistently used for lab-*Df* whereas, for wild collected mite samples (that were immediately preserved into ethanol) followed a different extraction method (following section). Samples preserved as E.EXT produced the weakest band.

Table 4.1: lab-*Df*: Whole genomic DNA spectrophotometry results of genomic DNA comparing the three preservation methods.

Sample number	ng/uL	A260/280 Ratio	Preservation Method
1	5.93	2.54	E.EXT
2	4.92	1.72	E.EXT
3	5.08	0.77	E.EXT
4	1.27	1.28	E.EXT
5	8.89	0.97	E.EXT
6	9.97	1.98	L.EXT
7	9.34	1.79	L.EXT
8	8.60	2.01	L.EXT
9	10.24	1.81	L.EXT
10	13.15	2.79	L.EXT
11	7.14	1.60	F.EXT
12	8.33	1.52	F.EXT
13	6.90	2.81	F.EXT
14	8.05	1.70	F.EXT
15	9.75	1.82	F.EXT
Preservation methods	**E.EXT**	**L.EXT**	**F.EXT**
Concentration of DNA: ng/µL; A260/280 ratios.			

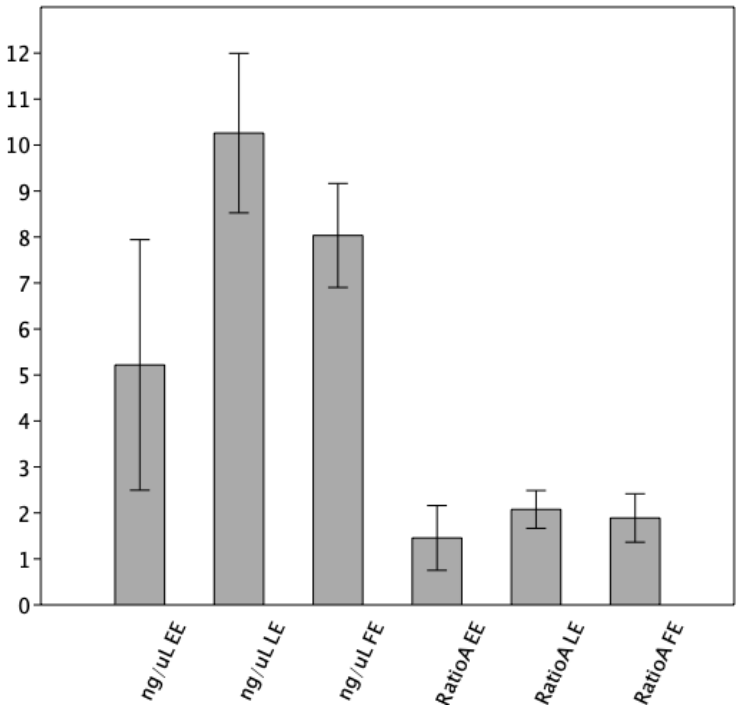


Figure 4.1: Visual comparison between L.EXT, F.EXT, F.EXT extraction methods of Lab-D*f*, showing mean DNA concentrations (ng/uL EE, LF and FE) and Ratio of absorbance values (RatioA- etc.) (data from table 4-1). [x axis are numerical values; lines showing Standard Deviation]

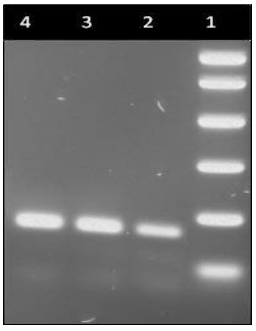


Figure 4.2: Separation of DNA bands with Primer set 90FR2, for 3 Preservation Method representative samples. Lane 1: Ladder. Lane 2: ethanol preservation-E.EXT. Lane 3: freezing preservation-F.EXT. Lane 4: ATL disruption (Lysis), no fixation: L.EXT). [A 2% [agarose gel]]

4.3.2 UK and KSA samples: Comparison of preservation methods and DNA quality

Results of the genomic DNA quality analysis using a DeNovix dsDNA high sensitivity spectrophotometric assay for the 50 collected samples of *Dermatophagoides*, 25 from the

UK and 25 from KSA, indicate that whole genome DNA was successfully extracted from all 50 samples, regardless of the preservation method. However, the quantity and quality was highly variable, as shown by the concentrations of DNA in the results and the variable ranges of the A260/280 ratios. The A260/280 ratios for KSA are higher than the threshold of 1.8 (N=19/24), while for UK lower (N=8/24), Table 5.2.

For the UK samples (Fig. 4.3), a high linear correlation was found between DNA concentration and number of mites/extraction sample (r = 0.8944, Linear-Pearson). Almost no correlation is observed between either absorbance ratio and nr of mites, or concentration (r > 0.39, Linear-Pearson).

For the KSA samples (Fig. 4.4.), a linear correlation was found between DNA concentration and number of mites/extraction sample (r = 0.656, Linear-Pearson). A negative low correlation is observed between absorbance ratio and nr of mites (r = -0.32, Linear-Pearson), and no correlation between absorbance ratio values and concentration (r = -0.07, Linear-Pearson).

Table 4.2: Collected Samples -DNA Quantification Results

UK sample

No.	ng/ul	260/280	Mite no.
1	1.32	6.4	3
2	3.64	2.18	7
3	4.98	3.84	8
4	1.7	-3.2*	5
5	7.91	1.99	8
6	4.47	1.73	5
7	1.59	2.16	4
8	1.65	4.22	4
9	1.11	0.23	4
10	0.72	0.99	2
11	0.52	-1.16*	2
12	0.93	0.59	2
13	0.87	0.37	2
14	2.71	0.95	4
15	0.09	1.21	1
16	0.59	-0.32*	1
17	1.09	0.77	2
18	0.47	0.24	1
19	0.12	0.02	1
20	0.68	0.39	2
21	0.74	1.07	2
22	1.51	1.78	4
23	2.58	2.72	4
24	1.33	0.16	2
25	0.26	-1.8*	1

KSA samples

No.	ng/ul	260/280	Mite no.
1	5.85	1.86	6
2	3.602	3.13	4
3	1.733	1.36	3
4	2.078	3.03	4
5	1.975	1.37	4
6	3.604	2.8	6
7	4.18	1.15	4
8	4.198	5.25	6
9	2.206	2.07	5
10	1.401	2.54	4
11	2.113	4.34	5
12	2.487	2.73	5
13	3.025	6.11	4
14	2.049	5.33	2
15	6.063	2.19	7
16	1.704	-2.6*	5
17	4.76	2.42	6
18	2.837	3.84	4
19	5.471	1.79	5
20	2.118	9.2	3
21	2.56	6.22	4
22	1.81	4.21	4
23	3.17	2.1	4
24	3.91	4.79	4
25	2.07	1.43	4

(*) DENOVIX reading error (negative readings)

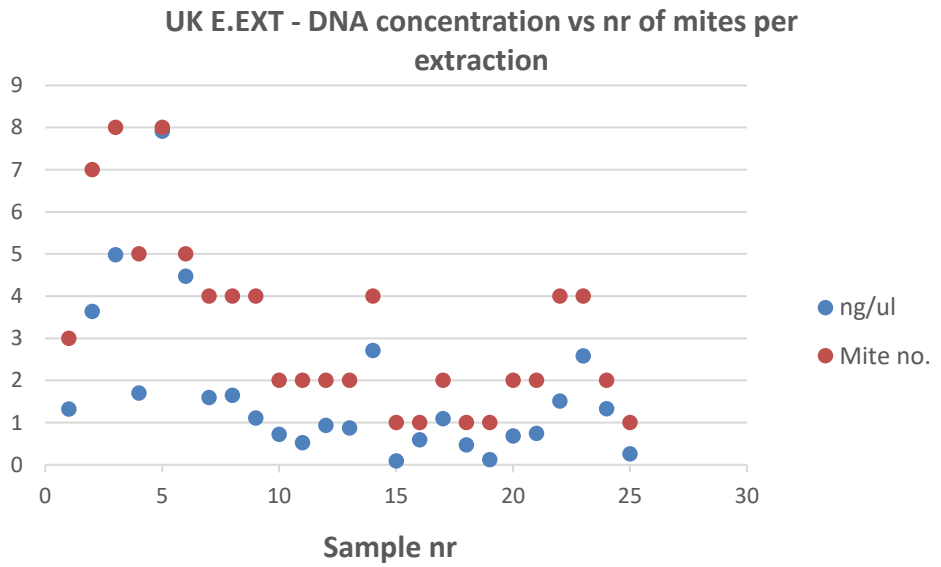


Figure 4.3: Samples from the UK. Scatterplot of concentration of DNA vs mite number/extraction sample

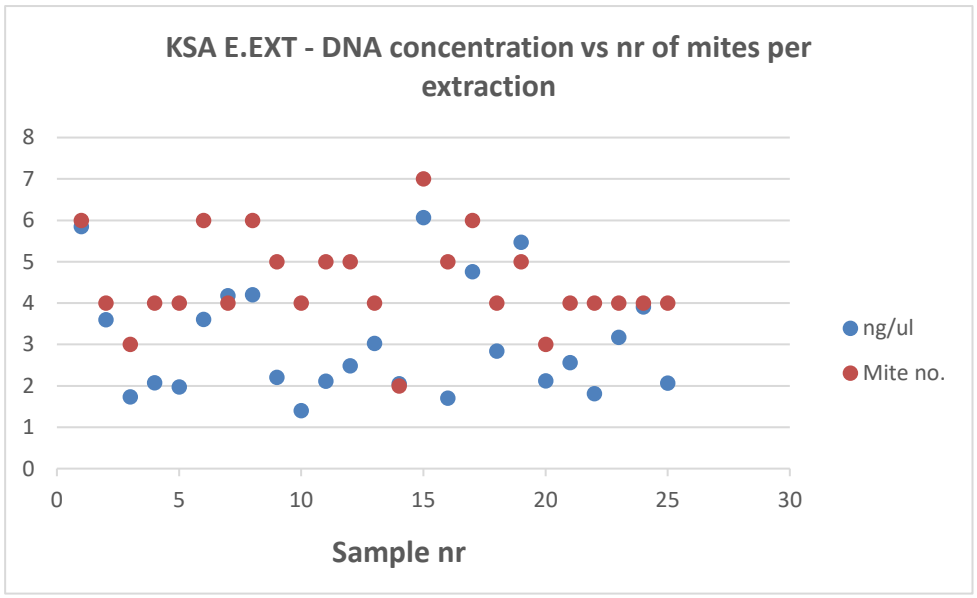


Figure 4.4: Samples from the KSA. Scatterplot of concentration of DNA vs mite number/extraction sample

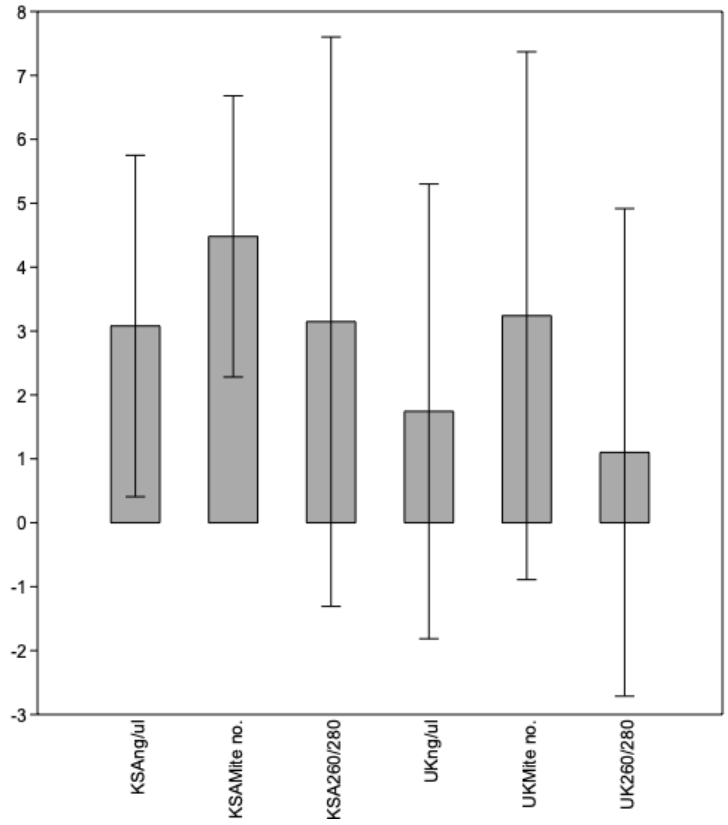


Figure 4.5: Bar chart of all data from KSA and UK (data detailed in Table 4.2), showing the great dispersion of values. [x axis are numerical values; lines showing Standard Deviation].

No comparisons were abled between the two wild sources as the number of mites used in each extraction greatly differed and the data for each measurement varied considerably, being not normally distributed (Fig. 4.5). This was due to the unpredictable availability of specimens.

4.4 Discussion

4.4.1 DNA extractions

Genomic DNA was successfully extracted from all samples of lab-*Df*, not fixed and from all UK and KSA samples (mainly Ethanol and Frozen) tested using pooled mites, as evidenced by spectrophotometric DNA quantification. However, results were very variable in respect of DNA quantities and quality, A260/A280 ratios differed in some cases from the published parameter value/threshold of ~1.8 (1.8-2.0) for the spectrophotometer used. When this value is lower, this suggests the presence of notable

quantities of proteins, salts, carbohydrates or solvents in the extractions, and this might be the case for samples collected in UK, and might relate to humidity levels of households, with much more dump within houses when compared to KSA. The KSA is settled in a desert. If the absorbance ratio value is much higher, it might also suggest issues relating to calibration of the blank. The samples from KSA showed the highest values. However, the manufacturers recommendation, is that, despite such anomalies, samples are best evaluated by downstream applications, in this case, PCRs.

Mite preservation methods had an impact in the amount of DNA extracted, and this was observed in the controlled experiments with the lab colonies, lab-*Df* mites. A significant difference in yields is clear between ethanol preserved and directly used (E.EXT and L.EXT). The mites fixed into ethanol yielded less DNA (lower concentrations) that when went directly into a lysis buffer, however, this does not appear to have affected the performance of subsequent PCRs, since visualisations of Lab-*Df* amplifications were generally similar in band strength and fragment length. Based on the visualisation in Figure 4.1 there is a clear difference between the groups, E.EXT, L.EXT and F.EXT.

Of more significant finding, and requiring further methodological investigation, extraction of sufficient DNA quantity from single mite extractions was not achieved, and, therefore, variably pooled samples of 2-8 mites were used per DNA extraction. Other researchers have achieved extractions from a single mite (Colloff, 1987, Gomi et al., 1997, Al-Khalify, 2018) which outcome clearly preserves the individuality of subsequent genotypifications, very valuable for further MS analyses. However, following this point on number of specimens required, there was a clear positive linear correlation between the nr of mites used and the DNA yielded, the more mites included in the DNA extraction the higher the concentration. UK yielded higher concentrations than KSA and this difference in correlation might be due to the extra step of the preserved mites, having to be dried during transport from KSA to UK. In the following Chapter (5), the value of the pooled DNA extractions proved valuable for testing the 100s of primer sets with their corresponding tags. The availability of high quality DNA must be considered when designing microsatellites (Abd-Elsalam, 2003, Neilan et al., 1997).

Chapter 5 : Testing newly developed microsatellites in a reared colony of *Dermatophagoides farinae* and field *Dermatophagoides* species (UK and KSA houses)

Abstract

New proposed microsatellites (MS) for *Dermatophagoides farinae* (Acari: Pyroglyphidae), together with a number of variable primer sets, new to science, were studied. Hundred and twenty five microsatellite markers were designed (elsewhere) for the mite species *Dermatophagoides farinae.* To check these microsatellites a large number of potential primers, over 200, has been designed and both DNA of lab colonies of this species as well as wild, house dust mites were used. Tests were carried out under different polymerase chain reaction (PCR) cycling conditions to gather the best conditions for their amplification.

One hundred and 58 primer sets were tested, sixty three percent (63%) of them amplified MS successfully for *D. farinae*, a few only were successful with other *Dermatophagoides* species checked, for which the concentration of DNA used were increased, compared to *D. farinae*.

5.1 Introduction

The present study is based upon previously identified (Al-Khalify, 2018) microsatellite loci for *Dermatophagoides farinae* (*Df*) (Acari, Pyroglyphidae) and their designed primers. Al-Khalify (2018) work followed the gathering of the repeats from the whole genome sequence of the species, made available only recently.

Testing the accuracy of the primer sets and studying and testing the microsatellites (MS) will allow future work to genetically differentiate populations, and, for example, to estimate the distribution and relative abundance of distinct genotypes within and between populations, which can have implications in forensic analyses, as these mites are major House Dust Mites (HDM). Several benefits may also accrue to future research: analyses on mite MS characteristics, primer specificity and amplification success in the target species and closely related. As well as giving new insights into

allelic polymorphisms of MS and transferability to, of MS, phylogenetically related taxa.

The genome sequences of *Df* from which the loci were identified were assembled had 11,600 overlapping, consensual DNA sequences (contigs) into 495 scaffolds (Al-Khalify, 2018). This modern atypical approach to microsatellite characterisation, was considered here instead of classic PCR fishing, to avoid isolating genomic regions of low complexity where primers are less likely to be locus-specific. Furthermore, the genomic approach identifies regions of tandem repeats as well as number of repeats in each, with greater overall length than those isolated using traditional methods (Abdul-Muneer, 2014, Zane et al., 2002, Tanizawa et al., 1992). The microsatellite markers and putative primer sequences were obtained from the genome sequence of *Df* by Bangor University (Al-Khalify, 2018), and resulted, after proceeding with the different tests that form this project, highly suited to the purposes of this study.

The correct design of primer sets is fundamental for successful DNA amplification. The length, roughly of 16-24 bases, especially when working with the fluorescent tags to amplify MS, can affect binding at the selected annealing temperature, but significantly contributes to binding specificity (Abd-Elsalam, 2003). Perhaps defining the correct melting temperature, is the most challenging step to amplify the correct genome fragments, the correct MSs especially when multiplexing (Altshuler, 2006, Abd-Elsalam, 2003, Rahman et al., 2000).

For the primers to amplify properly and the MS to be correct and able to be read, a universal tag is added to the 5'-end of the forward primer, which bears no sequence homology to the mite genome (Nicot *et al.*, 2004), this facilitates detection of the amplicons by the sequencer or microsatellite analyser (Neilan *et al.*, 1997, Nicot *et al.*, 2004).

5.2 Objectives

This work looked at testing 101 primer pairs or sets, designed to amplify 125 microsatellites of the house dust mite, *Dermatophagoides farinae*. The primer sets were gathered from the sequences of the characterised microsatellites together with

their flanking sequences. The idea was to determine amplification success of targeted microsatellite loci in our laboratory cultured *D. farinae.* All the experiments required testing the conditions for PCR amplification, such as PCR cycling temperatures, and efficacy of a variety of tags and tails. All with the aim to obtain the most successful primers than can then be utilised in multiplex reactions for microsatellite characterization. A second objective was to test amplification success on DNA extracted from wild *D. farinae* and *D. pteronyssinus,* from samples collected from a variety of dwellings in disparate geographic locations, in the United Kingdom and in the Kingdom of Saudi Arabia. Thus, to ascertain if the microsatellites and primers are transferable between populations and species, if the amplification is repeatable, and to assess the quality of amplification.

5.3 Materials and methods

5.3.1 Subject organisms

All PCRs were undertaken with mites sampled from the reared lab colony of *D. farinae* Df, plus UK samples and KSA samples of house dust mites, specifically *D. farinae* and *D. pteronyssinus*. Details of the DNA extractions are provided in Chapter 4.

5.3.2 Primers

Microsatellite detection is based on PCRs using specific microsatellite primer sequences tagged with dyes, that the sequencer or microsatellite analyser reads the tags and identifies the dye (Neilan et al., 1997, Nicot et al., 2004). The primers were tagged with a universal tag at the **5'-end** of the forward primer (Nicot et al., 2004).
Prior to carry on the testing over 200 primers, a total of ten tags were studied, tried or analysed for a sampled set of primers and the two with the least correspondence to the target genome were found to be M13 mod B, 5'- CAC TGC TTA GAG CGA TGC -3'. Plus the additional tag, pig-Tail 8 to allow good annealing (Botta et al., 2004, Neilan et al., 1997, Rampling et al., 2001, Ballard et al., 2002). The tags range in length between 15 and 18 nucleotides (Al-Khalify, 2018). The reverse primers were amended at the 5'-end with a partial of half PIG tail (to add adenosine for accuracy of annealing temperature during the second part of the PCR reaction) of 5'- GTT T -3'

(Altshuler, 2006, Rahman et al., 2000, Abd-Elsalam, 2003). An example of tagged forward primer is: cactgcttagagcgatgcACAACCCAATTCAAATGGATCCA (in capital letters is the Primer 1F). Primers were supplied by Al-Khalify (2018) here (see Table 5.1). For correct design of primer sets, there were many significant parameters to consider during their design:

- Length

An appropriate length, particularly a short length (around 16-24 bases), was enough in length and specificity, to bind easily to the template at the annealing temperature.

- Melting Temperature

The preferred melting temperature for primer was around 55°C-60°C. In the case of high GC content, however, a primer requires a high melting temperature (around 75°C-80°C).

- Specificity

The specificity of designed primers to the target sequences during the preparation was substantial.

Table 5.1: Microsatellite primers (as in (Al-Khalify, 2018)): List of 251 primers, Forward: F, and Reverse: R. Oligosequence names are listed along with the reference numbers applied in the text. Base pair sequences for each primer are listed as supplied.

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-1-M13B_F	1F	cactgcttagagcgatgcACAACCCAATTCAAATGGATCCA
Df-MS-1_R	1R	gttTGTGGTTCGAGAGATGGAAA
Df-MS-2-M13B_F	2F	cactgcttagagcgatgcTCCGCCTCATCATCATCAACA
Df-MS-2_R	2R	gttTCCCTGTTGTACCGTCAAGT
Df-MS-3-M13B_F1	3F1	cactgcttagagcgatgcACCGTTCCATAGTCCTCCCA
Df-MS-3-M13B_F2	3F2	cactgcttagagcgatgcTTCAAGCCCGGTACACACAA
Df-MS-3_R	3R	gtTTGCGCACGATTCTTTCTGC
Df-MS-4-M13B_F	4F	cactgcttagagcgatgcTTCAGGCAGTCAACAAGTGA
Df-MS-4_R	4R	gtttAGAGAGAAATGCATTTGACTTGA
Df-MS-5-M13B_F1	5F1	cactgcttagagcgatgcTGTCTTCAAGTTTCATAGATTTCCA
Df-MS-5-M13B_F2	5F2	cactgcttagagcgatgcTCATAGATTTCCAATGAATCACC
Df-MS-5_R1	5R1	gttTCCACTTGCTTTGGGCTTCT
Df-MS-5_R2	5R2	gtTTGGGGGCAATATGAAGGGG

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-6-M13B_F	6F	cactgcttagagcgatgcTGGCCAAGCTAAACAACACA
Df-MS-6_R1	6R1	gttTCACGACAACAGTATGATCGA
Df-MS-6_R2	6R2	gttTGCTATTGAATCACGACAACAGT
Df-MS-7-M13B_F	7F	cactgcttagagcgatgCGGATTCTGCGTCCTGTGTA
Df-MS-7_R	7R	gtTTGGTGGCAGCAACAACAAC
Df-MS-8-M13B_F	8F	cactgcttagagcgatgcTCATCCGACTCGTGCAACTC
Df-MS-8_R	8R	gtttAGTTGGTAGTGGTGGCCAAA
Df-MS-9-M13B_F	9F	cactgcttagagcgatgcAAAAACAACAGCCACCGTGG
Df-MS-9_R1	9R1	gtttGTTGACGATCCAGAACGGGA
Df-MS-9_R2	9R2	gtttGCGAATGCGTAAATCGTCGT
Df-MS-10-M13B_F1	10F1	cactgcttagagcgatgcAGGTTGCCGACATCTGTTCA
Df-MS-10-M13B_F2	10F2	cactgcttagagcgatgcGTGTGTGCCGCGGAAAATAT
Df-MS-10-M13B_F3	10F3	cactgcttagagcgatgcTAGTATCACGTGTGTGCCGC
Df-MS-10_R	10R	gtttAAAATCCTAACCGCACGCAC
Df-MS-11-M13B_F	11F	cactgcttagagcgatgcACACGCAGCATCTCAAATGAC
Df-MS-11_R	11R	gttTGACATTCATACACACGCACA
Df-MS-12-M13B_F	12F	cactgcttagagcgatgcTCGTCAATACACCGACTCGA
Df-MS-12_R	12R	gtttAGAGAGTGGGCGCAAAAGAA
Df-MS-13-M13B_F	13F	cactgcttagagcgatgCCGGTTGTTTGGCATCATCG
Df-MS-13_R	13R	gtttAGAGAGTGGGCGCAAAAGAA
Df-MS-14-M13B_F	14F	cactgcttagagcgatgcTACCGTAATCATCATCATGA
Df-MS-14_R	14R	gttTGATGATCATGGTTTAGAATGACAGA
Df-MS-15-M13B_F1	15F1	cactgcttagagcgatgcTCGGATGATGGCGATGATGG
Df-MS-15-M13B_F2	15F2	cactgcttagagcgatgcGGCGATGATGGGGAATGGAA
Df-MS-15_R	15R	gttTCTTCGGAGCTATTCTGCAACA
Df-MS-16-M13B_F	16F	cactgcttagagcgatgcTGTTGGAGTTTGATTCATCACA
Df-MS-16_R	16R	gtttCGAACAACATCATCGCCAACA
Df-MS-17-M13B_F	17F	cactgcttagagcgatgcTCATTTTTAGATTTTGTCGATGA
Df-MS-17_R	17R	gtttTCGTTTTGATTATGCAGCACA
Df-MS-18-M13B_F	18F	cactgcttagagcgatgcGTGTGCGCGCGATTATTTGA
Df-MS-18_R	18R	gtttACGTAAATCAAGCTCCGCCT
Df-MS-19-M13B_F	19F	cactgcttagagcgatgcTCAGTGGTGGTCCATTTTGGA
Df-MS-19_R1	19R1	gtttACCTATCTGCACTGTCGTAA
Df-MS-19_R2	19R2	gttTCTTGGTGGTCAAATCAATCGA
Df-MS-20-M13B_F1	20F1	cactgcttagagcgatgcAACTCAATGTCCAGCAGCGA
Df-MS-20-M13B_F2	20F2	cactgcttagagcgatgcTGTCCAGCAGCGATGACATT
Df-MS-20_R	20R	gttTGCTGATGCTGATGCTGCTA

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-21-M13B_F	21F	cactgcttagagcgatgcTCTCCTATTCTCACCATACTCACA
Df-MS-21_R	21R	gtttCCGTTTTTCATCACTCCAGACC
Df-MS-22-M13B_F	22F	cactgcttagagcgatgCCACCATCAACATCACCATCG
Df-MS-22_R1	22R1	gttTGATGATGATTGCGTTTGTTTCA
Df-MS-22_R2	22R2	gttTGAATGATGATGATTGCGTTTGT
Df-MS-23-M13B_F	23F	cactgcttagagcgatgcTCATTGTTGTCGTTGTTTGTTCA
Df-MS-23_R	23R	gttTCGTGGAAACAAATATCGCAACC
Df-MS-24-M13B_F	24F	cactgcttagagcgatgcGGTGCTCGTCGATGGACAAA
Df-MS-24_R1	24R1	gttTCGTCACTGCTTTGATCAACA
Df-MS-24_R2	24R2	gttTCAACATCTTCATCGTCACTGC
Df-MS-25-M13B_F	25F	cactgcttagagcgatgcTCATTCAATGTTTTTATCGTTGA
Df-MS-25_R1	25R1	gtttAGTGATAATATTGAAAGTCGA
Df-MS-25_R2	25R2	gtttACGAATACAACAAATTTAACAA
Df-MS-26-M13B_F	26F	cactgcttagagcgatgcTCCGTGATGATATGATGTTCCA
Df-MS-26_R	26R	gttTGAAAAGAATTTCGTCAACAACA
Df-MS-27-M13B_F1	27F1	cactgcttagagcgaTGCATTCACATTCAGCAGCG
Df-MS-27-M13B_F2	27F2	cactgcttagagcgatgcGGCTACTGCACCACCAGTTA
Df-MS-27_R	27R	gtttTCTGTTGTTGTTGGGCGACT
Df-MS-28-M13B_F	28F	cactgcttagagcgatgCATCATCAACATCGGCTGCG
Df-MS-28_R1	28R1	gtttGAGCAAATCTTGATCAATTAA
Df-MS-28_R2	28R2	gtttATTGAGCAAATCTTGATCAATTAA
Df-MS-29-M13B_F	29F	cactgcttagagcgatgCCACCACAGTTCTTGTTCC
Df-MS-29_R	29R	gtttCAGCATTGGCAGCGTTAGTG
Df-MS-30-M13B_F1	30F1	cactgcttagagcgatgcACATGAACGAAGACGACGAGA
Df-MS-30-M13B_F2	30F2	cactgcttagagcgatgcACAACATGAACGAAGACGACG
Df-MS-30_R	30R	gtttACCCGTTGTCCATTAACTTTCA
Df-MS-31-M13B_F	31F	cactgcttagagcgATGCGGGCGATCAATATGGT
Df-MS-31_R1	31R1	gttTCTTGTGAATCCGATTGTGCT
Df-MS-31_R2	31R2	gttTCCGATTGTGCTAATTGTTGCT
Df-MS-31-M13B_F	31F	cactgcttagagcgATGCGGGCGATCAATATGGT
Df-MS-31_Rb	31Rb	gtttAGGCAGGTTTATGATGAGCA
Df-MS-32-M13B_F	32F	cactgcttagagcgatgcAGAAAACATTGACCTTATCATGCA
Df-MS-32_R	32R	gtttCGTGAACGTGCGTAGGTAAA
Df-MS-33-M13B_F	33F	cactgcttagagcgatgcTGCATTGAGGGAAAGCAAACA
Df-MS-33_R	33R	gttTGACAAACAACCAGCTGCAT
Df-MS-34-M13B_F	34F	cactgcttagagcgatgcGATGTTGTTTCCACACCGCC
Df-MS-34_R1	34R1	gtttAAAAACGACAATGTCCGGCC

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-34_R2	34R2	gttTCCGGCCGTTAGTGGAAAAT
Df-MS-34_R3	34R3	gtttGACAATGTCCGGCCGTTAGT
Df-MS-35-M13B_F	35F	cactgcttagagcgatgcTCCCTTTCGTTACTGATTTGTTTGT
Df-MS-35_R	35R	gtttCGGTACTCCAAACACCTGGA
Df-MS-36-M13B_F	36F	cactgcttagagcgatgcACAAGAATCGAATCTTCTCCA
Df-MS-36_R1	36R1	gttTCATTGATTTCACGATCGATTTGA
Df-MS-36_R2	36R2	gttTCATTTCATTGATTTCACGATCGA
Df-MS-37-M13B_F	37F	cactgcttagagcgatgcAGGTCTTTTGTTCCGTCATGT
Df-MS-37_R	37R	gtttACAAGGATTTCATGTATTAGGGCC
Df-MS-38-M13B_F	38F	cactgcttagagcgatgCGGATGCTGGCCGTTATTTG
Df-MS-38_R	38R	gttTCAACATCACCATCATCAATCGA
Df-MS-39-M13B_F1	39F1	cactgcttagagcgaTGCACGTGCTAATAGCCTTGA
Df-MS-39-M13B_F2	39F2	cactgcttagagcgatgcTGTTGCACGTGCTAATAGCC
Df-MS-39_R	39R	gtttGGTCAAATGCACACAGGTAGC
Df-MS-40-M13B_F	40F	cactgcttagagcgatGCAATCACAGCTTCCTGATCC
Df-MS-40_R1	40R1	gttTGCCTTTGTATCTGATGCAGA
Df-MS-40_R2	40R2	gtttGAGCCATCCGAATTTTTGATCCT
Df-MS-41-M13B_F1	41F1	cactgcttagagcgatGCCATGTATAATGATAATTCGTTTGC
Df-MS-41-M13B_F2	41F2	cactgcttagagcgatgcTTCAATTCGATTCGTTGAAA
Df-MS-41_R	41R	gtttCCATCATCGTCATCAACAAGTGG
Df-MS-42-M13B_F	42F	cactgcttagagcgaTGCATAGACCGTGACGACAA
Df-MS-42_R	42R	gttTCGGAACAGGCTTGACAGAC
Df-MS-43-M13B_F	43F	cactgcttagagcgatgCAGAAAAGAAGTGTGATTCCT
Df-MS-43_R1	43R1	gttTGGATAATTCAACATCAGATGGA
Df-MS-43_R2	43R2	gtttAGATGGATAATTCAACATCAGATGGA
Df-MS-44-M13B_F1	44F1	cactgcttagagcgatgcTGACCGACATTGACTTGTTTGAC
Df-MS-44-M13B_F2	44F2	cactgcttagagcgatgcTGATGACCGACATTGACTTGT
Df-MS-44_R	44R	gtttACAAGATTTTGGATCAATCGCCA
Df-MS-45-M13B_F	45F	cactgcttagagcgaGCCATTCGCAAATTTTCGTGAC
Df-MS-45_Ra	45Ra	gtttGCTCTTGTTGCTTTTGTGGCT
Df-MS-45-M13B_F	45F	cactgcttagagcgaGCCATTCGCAAATTTTCGTGAC
Df-MS-45_Rb	45Rb	gtttGCCAACCAGTTTGCTGTTGT
Df-MS-46-M13B_F	46F	cactgcttagagcgatgcTCCAACCTAACACGACCAGT
Df-MS-46_R1	46R1	gtttGAGTCTAGTTCGGCATGCGT
Df-MS-46_R2	46R2	gttTCAATGTAATCTTTTCCGTTT
Df-MS-47-M13B_F	47F	cactgcttagagcgatgcTGTGGTTCAATCCAACATTCTGT
Df-MS-47_R	47R	gttTGGGTGATCATCAACATCGTCA

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-48-M13B_F1	48F1	cactgcttagagcgatgcTGACTGGGGAGGTGGAAAGA
Df-MS-48-M13B_F2	48F2	cactgcttagagcgatgcTTTGGCCAATTGACTGGGGA
Df-MS-48_R	48R	gtttAGATAGAATCCGGACCAGAA
Df-MS-49-M13B_F	49F	cactgcttagagcgatgCGTACACGTTTTGTTGGCGA
Df-MS-49_R1	49R1	gtttGCAGCATCGGCATCATCATC
Df-MS-49_R2	49R2	gtttATCAGCAGCATCGGCATCAT
Df-MS-50-M13B_F	50F	cactgcttagagcgatgcTGTGGTCGTTGTAGTTGGTGT
Df-MS-50_R	50R	gtttCGTTCAATTCGGGCCGTTTT
Df-MS-51-M13B_F	51F	cactgcttagagcgatgcACGCATCTAAAAATGTGAAGGGT
Df-MS-51_R	51R	gtttAGTGATGATAATTAGTGGCA
Df-MS-52-M13B_F	52F	cactgcttagagcgatgCACACAAGTGGGGAAAGGAGA
Df-MS-52_R	52R	gtttAGAGAGCAAAAGTGAGAGGACA
Df-MS-53-M13B_F	53F	cactgcttagagcgatgcTCAAAATCAGCATATCATGATGGT
Df-MS-53_R1	53R1	gtttAGATGTGCGAACATTTTAACA
Df-MS-53_R2	53R2	gtTTCATAAAGATGTGCGAACA
Df-MS-54-M13B_F	54F	cactgcttagagcgatgcTTGTTGTCTACTTTTCTCACT
Df-MS-54_R	54R	gttTGATGGCCATCGATTAACATGT
Df-MS-55-M13B_F	55F	cactgcttagagcgatgCGCACACACCCGAAACATTC
Df-MS-55_R	55R	gtttACAAAATCAAGTTCGACATCG
Df-MS-56-M13B_F	56F	cactgcttagagcgatgCCGGATCTTCAGCTTATCCGA
Df-MS-56_R	56R	gttTGCCAATTCACCGATTCATCA
Df-MS-57-M13B_F	57F	cactgcttagagcgatgcACCTTCGATGTAACTGTTTCATCG
Df-MS-57_R	57R	gttTCAACACCAAGTTCAATCTCCA
Df-MS-58-M13B_F	58F	cactgcttagagcgatgcTCGTTACAGTCGTTGTTCAACT
Df-MS-58_R	58R	gtttACGGATGAAAAATCTGCCTGA
Df-MS-59-M13B_F1	59F1	cactgcttagagcgatgcTGGATGGGTGGAAATGGTGG
Df-MS-59-M13B_F2	59F2	cactgcttagagcgatgcATTGGGGAATGGATGGGTGG
Df-MS-59_R	59R	gtttGGGGGTGTCTATTTCTCTCT
Df-MS-60-M13B_F	60F	cactgcttagagcgatgcAACCGACTAACCAGCCAACC
Df-MS-60_R	60R	gtttAGTAGCGGCAAAACTCCACA
Df-MS-61-M13B_F	61F	cactgcttagagcgatgcACCCTCTAGCTATATGGCGA
Df-MS-61_R1	61R1	gtttGCTGCAAATTGTTTGGTTTTGGT
Df-MS-61_R2	61R2	gttTGAGCTGCAAATTGTTTGGT
Df-MS-62-M13B_F	62F	cactgcttagagcgatgcAAAGACTTGCAGCCGGATGA
Df-MS-62_R1	62R1	gtttGCGGCAACAACACCATCATC
Df-MS-62_R2	62R2	gttTCAATGTCAGCAATGTCGGC
Df-MS-63-M13B_F1	63F1	cactgcttagagcgatgcAAGCCGAAAGTTCACACGGT

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-63-M13B_F2	63F2	cactgcttagagcgatgcCGAAAGTTCACACGGTCGAC
Df-MS-63_R	63R	gttTGGACGATGAAAAGAATGACAAACA
Df-MS-64-M13B_F	64F	cactgcttagagcgatgcAGCTTAGAAATCAACATTTGCCA
Df-MS-64_R	64R	gttTGCAACAGAATTGAAAGTGGGA
Df-MS-65-M13B_F	65F	cactgcttagagcgatgcACCCTCTAGCTATATGGCGA
Df-MS-65_R1	65R1	gtttGCTGCAAATTGTTTGGTTTTGGT
Df-MS-65_R2	65R2	gttTGAGCTGCAAATTGTTTGGT
Df-MS-66-M13B_F	66F	cactgcttagagcgatgcAGAATGTGCGAGATTCTGCA
Df-MS-66_R	66R	gtttGAGAACCCACCACTCTCTTCA
Df-MS-67-M13B_F	67F	cactgcttagagcgatgcAGCTACAACAACAGTGAAACACA
Df-MS-67_R	67R	gttTATTGATGGCCTGGCGGTTT
Df-MS-68-M13B_F	68F	cactgcttagagcgatgcCGCCTTGAACACCATGATGC
Df-MS-68_R	68R	gttTCCAAAACATTAACTTTCCAATGTGT
Df-MS-69-M13B_F1	69F1	cactgcttagagcgatgCTGTATTGTGCACGTGCTAA
Df-MS-69-M13B_F2	69F2	cactgcttagagcgatgcTGTGCACGTGCTAAATTTCT
Df-MS-69_R	69R	gttTGTTCAAACCTAGACTATTCTGT
Df-MS-70-M13B_F1	70F1	cactgcttagagcgatgcTGAGCCCAACACAACATTTT
Df-MS-70-M13B_F2	70F2	cactgcttagagcgatgcAAAATGAGCCCAACACAACA
Df-MS-70_R	70R	gttTGTTTTGGCTTGAAATGGGA
Df-MS-71-M13B_F	71F	cactgcttagagcgatgCGTGTCACATGTAAATTCCGGT
Df-MS-71_R	71R	gtttAGCCTGACAATTTGAAAATGGT
Df-MS-72-M13B_F	72F	cactgcttagagcgatgcACACTGCACTTTGAAATTGAACA
Df-MS-72_R	72R	gtTTCATTCCCCATACGTTACA
Df-MS-73-M13B_F	73F	cactgcttagagcgatgcACAGTGAGATAAAGAATCCAAGA
Df-MS-73_R1	73R1	gttTCTTGTCTAGCTTCATCATCTTCA
Df-MS-73_R2	73R2	gttTGTCTAGCTTCATCATCTTCATTCA
Df-MS-74-M13B_F	74F	cactgcttagagcgatgcTGATTTTCTTCACCACCACCA
Df-MS-74_R1	74R1	gtttAGGATAAATCATCGTGAACAAGCT
Df-MS-74_R2	74R2	gttTCATCGTGAACAAGCTAAAAGAGA
Df-MS-75-M13B_F	75F	cactgcttagagcgatgcAGTAAGTTTTCAATCTTGGAA
Df-MS-75_R	75R	gttTGCGACAACGATGACGATGA
Df-MS-76-M13B_F	76F	cactgcttagagcgatGCCATGTCCGGCGATTAAAT
Df-MS-76_R	76R	gttTCGTCATCTTCATTCTGGTGACA
Df-MS-77-M13B_F	77F	cactgcttagagcgatgcACAAGTTGATGAGACATTACATCGT
Df-MS-77_R1	77R1	gtttCCTTGATGGCCGGAAAAGTG
Df-MS-77_R2	77R2	gttTGATGGCCGGAAAAGTGAGA
Df-MS-78-M13B_F	78F	cactgcttagagcgatgcTGAGAGTCAATCATCATTAAGTTGA

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-78_R	78R	gtttCGTTTTCATTTTTATCGTCCAACCA
Df-MS-79-M13B_F1	79F1	cactgcttagagcgatgcTCATCATAATCAATCCGTTCAGCT
Df-MS-79-M13B_F2	79F2	cactgcttagagcgatgcACCCGAATCCATCTAAAGAATGA
Df-MS-79_R	79R	gtttAATCACATGTGTGTCTGCGC
Df-MS-80-M13B_F1	80F1	cactgcttagagcgatgcAACATCTGTTTTGCTTGACAAGC
Df-MS-80-M13B_F2	80F2	cactgcttagagcgatgcAGCAGCAACAATTTTTCAGTGT
Df-MS-80_R	80R	gtttACTCACTGCCATAGTCGACG
Df-MS-81-M13B_F	81F	cactgcttagagcgatgcTCGCATGACAAGATCAAAA
Df-MS-81_R	81R	gTTTTTCTTCGTTGTTTGTT
Df-MS-82-M13B_F	82F	cactgcttagagcgatgcACCAAATGATCAATGTATCGGACT
Df-MS-82_R	82R	gttTCGTTGATTATTAGGCCATGGT
Df-MS-83-M13B_F	83F	cactgcttagagcgatgcTGTGCAATTTAGGTGTATCCCGA
Df-MS-83_R	83R	gttTGTTGTCAGAATTAAACGACGACA
Df-MS-84-M13B_F	84F	cactgcttagagcgatgcTTGAGTGTCGCTAAATGCCA
Df-MS-84_R	84R	gtttATACTAGGTCGCGTGCTGTG
Df-MS-85-M13B_F	85F	cactgcttagagcgatgcAGGCATGGCTTAGAAATGTCCT
Df-MS-85_R	85R	gtttAGGTGACCAAATGCCATTCA
Df-MS-86-M13B_F	86F	cactgcttagagcgatgcGGCACCACAAGAAGAAATGATGA
Df-MS-86_R	86R	gtttAACTCGAGCCTACAACCTACA
Df-MS-87-M13B_F1	87F1	cactgcttagagcgatgcTGACTCAATGAAAGACTAACCT
Df-MS-87-M13B_F2	87F2	cactgcttagagcgatgcTCCAGATATGACTCAATGAAAGACT
Df-MS-87_R1	87R1	gttTCAAATTTCGTGCATCAAGAGT
Df-MS-87_R2	87R2	gttTGTCGATATCAAATTTCGTGCA
Df-MS-88-M13B_F	88F	cactgcttagagcgatgcTGGACCACCAGAATGAAATGGA
Df-MS-88_R	88R	gttTCATCAAACGTTTGTGATTGGCA
Df-MS-89-M13B_F	89F	cactgcttagagcgatgcTGTCAGTTAAAATGGCAGGCAA
Df-MS-89_R	89R	gttTGCCAAAGGGGTAATGTCAGT
Df-MS-90-M13B_F	90F	cactgcttagagcgatgcTCCATGGACGATTGATTTGTTCA
Df-MS-90_R1	90R1	gttTCGGGCGTTTCAAGTGGATT
Df-MS-90_R2	90R2	gtttCCAAAATGTCGGGCGTTTCA
Df-MS-91-M13B_F1	91F1	cactgcttagagcgatgcTGGAGATATGAACACATAGCAACT
Df-MS-91-M13B_F2	91F2	cactgcttagagcgatgcACCACTCTATTGGAGATATGAACACA
Df-MS-91_R	91R	gtttCCAAAATGTCGGGCGTTTCA
Df-MS-92-M13B_F	92F	cactgcttagagcgatgCATGTCTGGCACTCGGAAGT
Df-MS-92_R	92R	gtttACCCTTGGTCTGATGACTGC
Df-MS-93-M13B_F	93F	cactgcttagagcgatgcTGGATCGATCAATTTGGATGGA
Df-MS-93_R	93R	gtttCCAACGATTCACATTTCGGCA

OLIGONAME	DfMS Reference	SEQUENCE (including tag and tail sequences)
Df-MS-94-M13B_F	94F	cactgcttagagcgatgcTGATGAATTCCAGAGTTTGTGGT
Df-MS-94_R	94R	gtttAGTGAATTGATTTTACAACAGGA
Df-MS-95-M13B_F	95F	cactgcttagagcgatgcAAGCCGAAAGTTCACACGGT
Df-MS-95_R	95R	gttTGGACGATGAAAAGAATGACAAACA
Df-MS-96-M13B_F	96F	cactgcttagagcgatgcTTCTCCTTCCATATTCAATGA
Df-MS-96_R	96R	gttTCGGGTTAGAATTGCAGACA
Df-MS-97-M13B_F	97F	cactgcttagagcgatgcTCACTAGACATGATCTGTAAAA
Df-MS-97_R	97R	gttTCGGGTTAGAATTGCAGACA
Df-MS-98-M13B_F	98F	cactgcttagagcgatgcTTGCACACAGGGCAATTTGC
Df-MS-98_R1	98R1	gttTCATCAACACAATTGGAAATGTTGA
Df-MS-98_R2	98R2	gttTGGAAATGTTGAATTGAAACGA
Df-MS-99-M13B_F	99F	cactgcttagagcgatgcACCATTTTGGATCCTAGAACATCCT
Df-MS-99_R	99R	gtttGCACAAAGAGGCAAGGCATT
Df-MS-100-M13B_F	100F	cactgcttagagcgatgcAGCCATCCATTCATCTCATCCA
Df-MS-100_R	100R	gtttAGTTTCGAATCAAATACTGTATCCGT
Df-MS-101-M13B_F	101F	cactgcttagagcgatgcGGTCGGTTGCTAGCGTGTAA
Df-MS-101_R	101R	gTTTCATTCTTCGGCGCTTCA

5.3.2.1 PCR Amplifications

Initial PCRs were carried out using 5 µl of Q5 High Fidelity 2x Master Mix (New England Biolabs #M0492S), 1.25 µl of each primer (forward and reverse from 20 µM of 100 micromolar stock solution with 80 µl H2O.), 0.65 µl of Df genomic DNA and 16.85 µl dH2O in standard PCR tubes. A peQSTAR 96 Universal Gradient (Isogen Life sciences, The Netherlands) thermocycler was used for PCRs and tubes were subjected to the following cycles. Denaturation at 95°C (2 minutes), 30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), extension at 72°C (1.5 min), final extension at 72°C (5 minutes).

PCR products were visually inspected by gel electrophoresis.

PCR conditions were varied in subsequent assays, to comply with the primer supplier's instructions in respect of annealing temperature. Annealing temperatures and DNA templates were investigated to identify optimal conditions, as described below (5.3.5).

Table 5.2:Optimised volumes used for PCR

Total Volume	**~25 µl**
PCR Master Mix	5 µl
Primer F	1.25 µl
Primer R	1.25 µl
d-H2O	16.85 µl
Template	0.65µl

5.3.3 Gel electrophoresis

5.3.3.1 Gel preparation

To visualise the amplifications a 1.5-2% agarose gel was prepared (1.5-2 g of agarose) (Fisher BioReagents, PB1356-500) in 100 mL of TAE (Tris-acetate EDTA) buffer. After measuring and combining components in an Erlenmeyer, the solution was microwaved for about 85 seconds. Next, the solution was allowed to cool by pouring cold water on it for about 30-35 seconds, and 2 µL of Ethidium bromide solution was finally added (stain to visualise DNA, E1510-10ML SIGMA life science). The subsequent step was to pour the gel mixture on the tray, flaked on two sides by tapes and the insertion comb. The gel was left to set for one-third of an hour before filling the tray with the regulator buffer solution and removing the combs. The samples and ladder were then pipetted into the gel wells (created by the comb) and ran for 55 min at 105 volts.

5.3.3.2 Gel electrophoresis procedure:

When the gel was set, the plate was filled with a regulator buffer solution and the combs removed. A standard 100bp DNA ladder (Promega G210A) was pipetted into one well left by the comb and 7 µL of PCR product individually into the remaining wells (PCR products and ladder were prepared from 6 µL of PCR product/ladder and 1 µl of loading dye (6x loading dye Promega biocompare G190A BLUE/ORANGE)). The power to run the gel was at 105 volts for ≈55 minutes to facilitate electrophoretic separation of the DNA fragments (i.e., electric current was applied to pull them

forward across the gel from the negative electrode (cathode) to the positive ones (anode), because PCR products (DNA molecule) have negative charge). When a gel is stained with Ethidium Bromide (DNA binding dye), the DNA molecules can be seen as bands. Each band representing a set of DNA molecules. After gel electrophoresis, UV light (UVP BIODOC-IT IMAGING SYSTEM BENCHTOP UV TRANSILLUMINATOR) was utilized to visualize the fragments of DNA.
On completion of primer testing and amplification, a second agarose gel was produced for just the successful primers, as a reference for subsequent analyses.

5.3.4 Laboratory Df primer testing

5.3.4.1 PCR optimization – testing a selected number of primers

All PCR optimisation assays used the lab-*Df* genomic DNA, extracted as previously described (Chapter 4). Optimal PCR conditions were assayed with a number of trials.

Trial 1. DNA templates were varied, by increasing the number of mites used in the extraction. Mite numbers and resultant DNA concentrations are shown in Table 5.3. Annealing temperature of 53 °C. All 4 combinations of primer 5, forward and reverse variants, 5F1R1, 5F2R1, 5F1R2 and 5F2R2, were used with each DNA template.

Table 5.3:Trial 1: Mite numbers and DNA concentrations.

Mite number	DNA concentration (ng/ul)
1	0.18
2	2.68
3	5.93

Trial 2. Trial 1 was repeated, but using annealing temperature of 57.7 °C and using only DNA concentrations of 2.68 and 5.93, and the same 4 combinations of primer 5.

Trial 3. Tested 5 annealing temperatures, 53.6°C, 54.5°C, 55.6°C, 56.9°C and 57.8°C. All 4 variants of primer 5 were used at each temperature, and the DNA concentration was only at 0.18 ng/µl.

5.3.4.2 Testing all primers

PCR based MS amplifications using all 101 primers, in 252 combinations of forward (123) and reverse (129) variants, as supplied, were undertaken to measure their individual amplification and alignment success, at various annealing temperatures. Where multiple forward or reverse primers for a given MS locus were supplied, all combinations were amplified.

First, all primers were tested against DNA extractions using L.EXT preservation (Chapter 4, just lysing and no ethanol or freezing) of Lab-*Df*.

5.3.5 Collected UK and KSA mites and Primer testing

To test primer amplification for the target MS on a broad range of HDM collections of wild specimens from geographically separate populations, PCRs were undertaken with genomic DNA extracted from the UK and KSA collected mite samples, only *D. farinae Df, D. pteronyssinus Dp* and *D. saudi* sp. nov. *Ds* were included in this assay. Genomic DNA was extracted from a total of 68 mite samples as shown in Table 6-4, and a number of mites extracted each containing 1-8 mites of either *Df*, *Dp* or *Ds*, using the described method in Chapter 4 (Wild Mite samples were fixed in ethanol, E.EXT).

Table 5.4:Summary of HDM collection for PCR analysis, by species. *Df*: *Dermatophagoides farinae*; Dp: D. pteronyssinus; Dsp: *Dermatophagoides* sp. nov.

Country	***Df***	***Dp***	***Dsp***
UK	21	10	0
KSA	14	19	4
Totals	35	29	4

Overall Total	68		

5.3.5.1 Testing primers on UK mites

The following PCR Trials were conducted (table 5.5)

1. Using genomic DNA from 9 UK collected samples of *Df*, 18 PCRs were undertaken, one for each of 18 primers already identified to amplify successfully and further selected to have produced strong signal and estimated correct lengths in gels (See Table 6-5). PCR conditions matched optimal settings already determined for lab-*Df* mites. This trial included new PCRs of lab-*Df* for the same primers, for comparison.

2. MS # 27 was amplified using Primer set 27F2R for 9 UK samples of *Df* (one duplicated), and 2 of *Dp*, to compare results by using for this primer designed for *Df*.

3. Using genomic DNA from 1 UK collected sample of *Dp*, 25 PCRs were undertaken, one for each of 25 primers already identified to amplify successfully and further selected to have produced strong signal and estimated correct lengths in gels. The results were promising the application of the MS and their primers in other species of *Dermatophagoides*. PCR conditions matched optimal settings already determined for the lab-*Df* mites.

5.3.5.2 Testing primers on KSA mites

The following PCR Trial was conducted (Table 5.5.)

MS # 45, Primer 45bFbR was used to amplify for 9 KSA samples *Df*, to compare results for this primer, and 4 samples *Ds*, also for comparison. Following an absence of bands in gels for all *Ds*. PCRs, the assay was repeated with 11 PCRs for 6 samples of *Ds* using various genomic DNA concentrations (1.85, 2.4, 3.0 µl) in the template, not all samples amplified for all concentrations.

Table 5.5: UK and KSA Mites tested - PCR trials.

UK Mites			
Primer No.	Sample No.	Species	Trial No.
30F1R 33FR 95FR 24FR2 36F1R1 36F1R2 47FR 32FR 80F2R 93FR 31bFbR 31aFR1 45aFaR 45bFbR 92FR 49FR2 84FR	UK-1	*Df*	1
27F2R	UK-1-2 UK6 (x2) UK-7 UK16 UK21-22 UK-25	*Df*	2
27F2R	UK-3-4	*Dp*	
47FR 30F1R 93F1R 30F2R 36FARA 80FR 31FAR1 31FBRB 36bfr1B 36Bfr2 49FR1 24FR2 92FR 84FR 45FARA 45FBRB 49FR2 32FR 33FR	UK-5	*Dp*	3

KSA Mites			
Primer No.	Sample No.	Species	Trial No.
45bFbR	KSA-1 KSA-2 KSA-3 KSA-4 KSA-5 KSA-16 KSA-17 KSA-18 KSA-19	*Df*	4
45bFbR	KSA-1 KSA-2 KSA-3 KSA-4	*Ds*	
45bFbR	KSA-1 KSA-2 KSA-3 KSA-4 KSA-19 KSA-20	*Ds*	

List of primers, samples and associated trial number.

Trials 1-3 UK Mite Collections

Trial 4 KSA Mite Collections

5.3.6 DNA purification of PCR products

To purify PCR products, the GeneJET PCR purification kit (#k0702 Fermentas LIFE SCIENCES) was used. The protocol was carried as by manufacturers recommendations: 50 µL of binding buffer was added to the PCR product (50 µl) and mixed. After that, the mixture was checked for the correct colour, meaning that the mixture must be yellow. If instead, the solution presents a violet or orange colour, 10µL of 3M sodium acetate must be added so that the colour becomes yellow (pH adjustment). Next, 50 µL of isopropanol was added to the mixture (with a ratio of 1:2 binding buffer/PCR product as the DNA fragments less than 500 bp). The subsequent step was to transfer all mixture into a GeneJET column (purification column membrane) followed by centrifugation at 12500 x g for 1 minute. After that, the supernatant was discarded and the column was transferred to a new 1.5 collection tube. Next, 700 µL of wash buffer was added followed by centrifugation at 12500 x g for 1 minute and discarding the supernatant. Again, the column was transferred to a new 1.5 mL collection tube and centrifuged directly without adding any solutions to eliminate any residual of wash buffer. Next, the column was transferred to a 1.5 mL Eppendorf tube, 50 µL of elution buffer was carefully added to the centre of column, followed by centrifugation, and discarding the column; finally, the purified PCR product was stored at -20 ˚C.

5.3.7 Sequencing of PCR products

PCR products of Lab-*Df*, *Df* of UK, *Dp* of UK, *Df* of KSA and *Ds*, were sent to Eurofins Genomics Sequencing (Germany). Samples were first purified as described above. Purified products were quantified by Denovix dsDNA spectrophotometry as described above.

Prior to sequencing, PCR products were prepared by placing PCR product samples were placed in Mix2Seq barcoded tubes containing ddH2O and 2 µL primer (Forward/Reverse). Volume PCR product was calculated from the concentration and the overall volume was made up to 22 µL with and dH_2O.

5.4 Results

The successful samples comprised combinations of all successfully amplified primers for lab-*Df*: 18 primers for nine collected UK *Df* samples; one primer for one collected *Dp* sample; three primers for six collected KSA *Ds*.

Of the tested primers with tags/tails, primer combination 5F1R1 produced successful amplifications. Correspondence with the microsatellite loci was confirmed by amplification using lab-*Df*. Microsatellite Loci were amplified successfully with 100 (63%) primer pairs, while 58 (37%) failed to amplify.

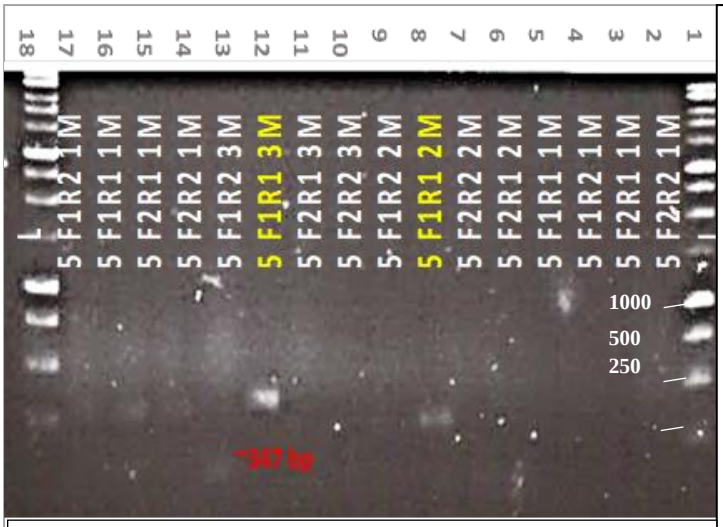
UK Df sample populations were successfully amplified at 35 loci, using optimal annealing temperatures (results not shown), providing scope to identify informative loci in due course. Eighteen primers with genomic DNA of collected sample UK-1 and lab-Df (Trial 1) resulted in similar amplifications, indicating that these primers are functional beyond the gene pool for which they were designed.

This was also confirmed in Trial 2 and with the KSA samples. By increasing the template concentration, interspecific transferability was observed by Microsatellite primer testing on DNA extracted from Dermatophagoides species: D. pteronyssinus (27 F2R-Trial 3) and D. saudi sp. nov. (45FbRb, 32FR and 36FbR2-Trial 4)).

5.4.1 PCR Optimisation

5.4.1.1 Results of the PCR trials

Trial 1. According to Figure 5.1, using different concentrations of DNA in the template, primer 5 in all its combinations failed to amplify to any clear extent, except, 5F1R1, which showed bands at higher DNA concentrations of 2.68 and 5.93 at ≈347bp (yellow in Figure 5.1), as expected.

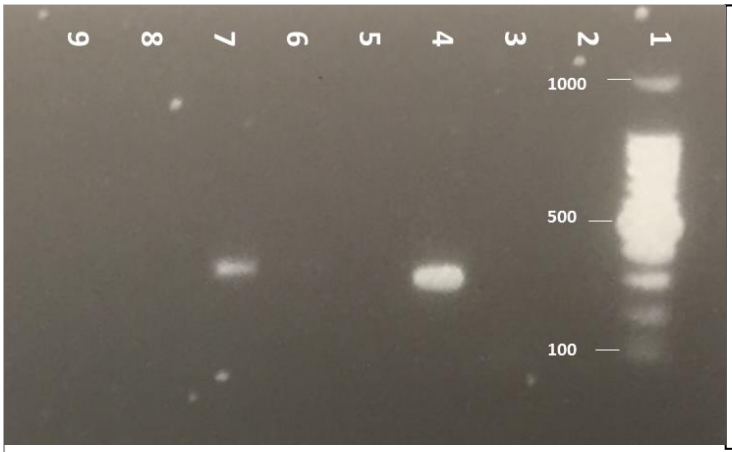


Lanes 8 and 12 only show bands indicating DNA amplification. Fragment length ≈347bp. Template concentrations: 1M = 0.18, 2M = 2.68, 3M = 5.93 *ng*/*L*.

Agarose gel 2%.

Lane No.	Primer No. / FR Combination	Template DNA Concentration
1	Ladder	-
2	5F2R2	0.18
3	5F2R1	0.18
4	5F1R2	0.18
5	5F1R1	0.18
6	5F2R1	2.68
7	5F2R2	2.68
8	5F1R1	2.68
9	5F1R2	2.68
10	5F2R2	5.93
11	5F2R1	5.93
12	5F1R1	5.93
13	5F1R2	5.93
14	5F2R2	0.18
15	5F2R1	0.18
16	5F1R1	0.18
17	5F1R2	0.18
18	Ladder	

Figure 5.1: Comparison of PCRs for lab-*Df*, various DNA template concentrations, and 4 primer 5 combinations (F and R), annealing temperature 53.0 ˚C. M: mite numbers used; L Ladder.



Lane No.	Primer No. / FR Combination	Template DNA Concentration
1	Ladder	-
2	5 F2R1	5.93
3	5 F1R2	5.93
4	5 F1R1	5.93
5	5 F2R1	2.68
6	5 F1R2	2.68
7	5 F1R1	2.68
8	5F2R2	5.93
9	5F2R2	2.68

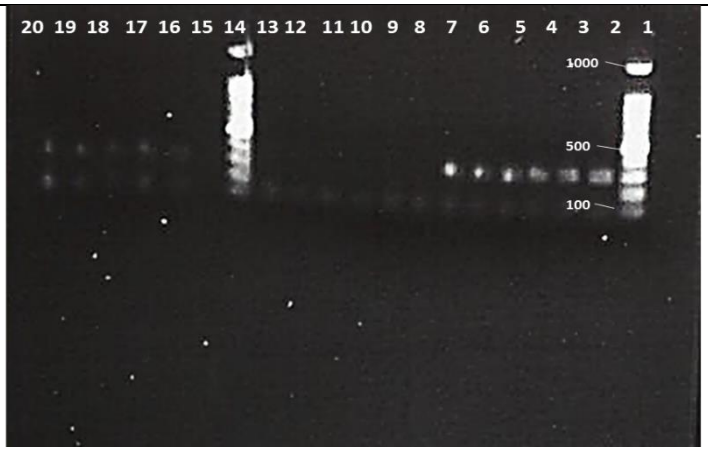
Only lanes 4 and 7 only show bands indicating DNA amplification. Fragment length ≈347bp. Template concentrations *ng*/µ. Lanes 8 and 9 (5F2R2) not shown.

Agarose gel 2%.

Figure 5.2: Comparison of PCRs for lab-*Df* with 2 DNA template concentrations, and 3 variants of primer 5, annealing temperature 57.7 ˚C.

Trial 2,Figure 5.2, a higher annealing temperature and different concentrations of DNA in the template demonstrated that primer 5 continued to fail to amplify except combination 5F1R1, which amplified with DNA concentrations of 2.68 and 5.93 producing amplicon lengths of ≈347bp, as expected. Combination 5F2R2, not shown, entirely failed to amplify.

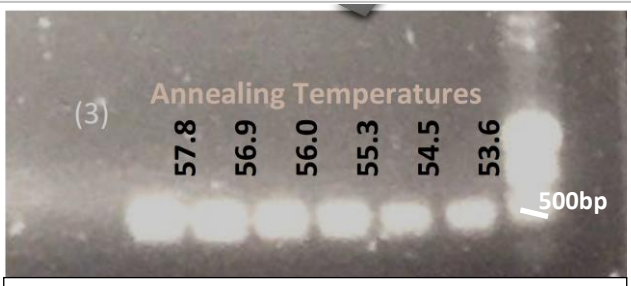
Trial 3 showed, Figure 5.3, that the primer combination 5F1R1 in fact produced successful amplifications at all tested annealing temperatures. Primer 5F1R1, as the most successful variant, was re-analysed at the same temperatures and the resultant products compared by gel electrophoresis with an agarose solution of 2% (Figure 5.4). A clear gradient emerged, with stronger, but less condensed banding as temperature increased.



Lane No.	Primer No. / FR Combination	Annealing Temperatures °C
1	Ladder	-
2	5F1R1	53.6
3	5F1R1	54.5
4	5F1R1	55.3
5	5F1R1	56.0
6	5F1R1	56.9
7	5F1R1	57.8
8	5F1R2	53.6
9	5F1R2	54.5
10	5F1R2	55.3
11	5F1R2	56.0
12	5F1R2	56.9
13	5F1R2	57.8
14	Ladder	-
15	5F2R1	53.6
16	5F2R1	54.5
17	5F2R1	55.3
18	5F2R1	56.0
19	5F2R1	56.9
20	5F2R1	57.8

Primer 5F1R1 (Lanes 2-7), 5F1R2 (Lanes 8-13), 5F2R1 (Lanes 15-20)

Figure 5.3: Comparison of PCRs with 3 primer variants, and 6 varying annealing temperatures as shown. 3 primer variants 5F1R1 (347bp).



Primer 5F1R1 (347bp), annealing temperatures are shown above lanes.

Agarose gel 2%.

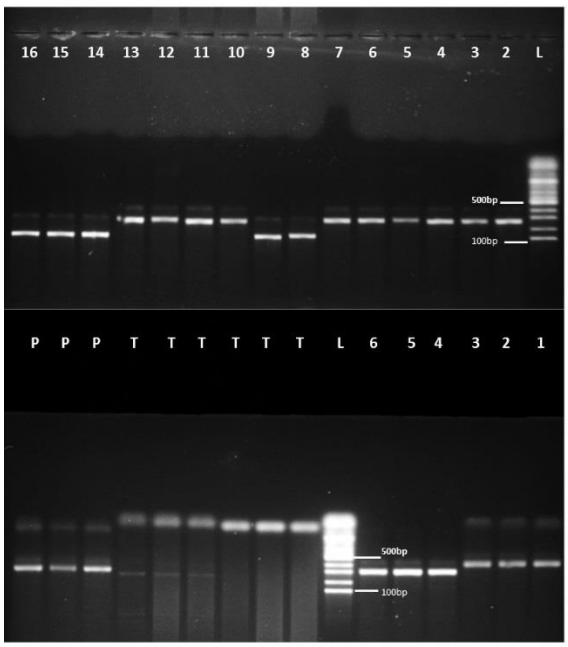
Figure 5.4:Results for Primer 5F1R1 at 6 annealing temperatures.

5.4.2 Results from testing all primers

5.4.2.1 Lab-*Df* Primer Tests

Electrophoresis visualisations of the results of PCRs of all the primers with lab-*Df*, at manufacturer's recommended annealing temperatures, in some cases optimised as shown in Figure 5.5, for primers 31 to 33. Chapter 5, in Appendix presents the gel runs with the bands (App. Figs 3.1-3.16), results of all amplifications, using the remaining primer sets.

Table 5.6 summarises the results, both positive and failed (negative) amplifications are shown, some primers produced strong bands which appeared to be the wrong length, the actual fragment lengths as estimated are highlighted red, the expected lengths given alongside. Success was adjudged from strong bands developed in the gel tests. Many loci produced bands of multiple or ambiguously multiple fragment lengths.



Locus analysis for Primers 31 to 33, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

Upper Gel

Lanes 2 – 4		**31FaR1**	
2	53.4	✓	341bp
3	56.5	✓	341bp
4	58.7	✓	341bp
Lanes 5 – 7		**31FaR2**	
5	53.4	✓	332bp
6	56.5	✓	332bp
7	58.7	✓	332bp
Lanes 8– 9		**31FaRb**	
8	53.4	✓	130bp
9	58.7	✓	130bp
Lanes 10-11		**31FbR1**	
10	53.4	✓	341bp
11	57.5	✓	341bp
Lanes 12 - 13		**31FbR2**	
12	53.4	✓	332bp
13	57.5	✓	332bp
Lanes 14 – 16		**31FbRb**	
14	53.4	✓	132bp
15	56.5	✓	132bp
16	57.5	✓	132bp

Lower Gel

Lanes 1 – 3		**32FR1**	
1	53.4	✓	405bp
2	56.5	✓	405bp
3	58.7	✓	405bp
Lanes 4-6		**33FR**	
4	53.4	✓	284bp
5	56.5	✓	284bp
6	58.7	✓	284bp

T: Testing some primers

Figure 5.5:Lab-*Df*, example of PCR results for primers 31 to 33.

5.4.2.2 Summary of the results

Table 5.6:Summary Results of PCR amplifications of lab-*Df*, using all primer pairs and variants, negative and positive bands with the expected, unexpected sizes and optimal annealing temperatures

Successful Amplifications				Failed Amplifications
Primer No.	**Opt. Anneal. Temperat.**	**Sizes (bp)**	**Expected sizes**	**Primer No.**
5F1R1	57.8	347		**1FR**
8FR	54.6	259		**2FR**
9FR2	58.2	320		**3F1R**
9FR1	58.2	386		**3F2R**
10F1R	58.2	498	249	**4FR**
10F2R	58.2	334		**5F1R2**
11FR	53.9	230	389	**5F2R1**
12FR	54.6	254		**5F2R2**
13FR	56.9	361		**7FR**
14FR	58.2	475	291	**10F3R**
15F1R	58.2	263		**15F2R**
16FR	54.6	291		**18FR**
17FR	58.2	224		**20F1R**
18FR	58.2	193		**24FR1**
19FR1	55.3	322		**34FR2**
19FR2	53.9	251		**36F1R1**
20F2R	59.6	351		**36F1R2**
21FR	58.5	295		**38FR**
22FR1	56.1 & 57.5	432		**40FR1**
22FR2	56.1	436		**40FR2**
23FR	53.9	271		**41F1R**
24FR2	58.2	242		**41F2R**
25FR1	49.8	155		**43FR1**
25FR2	48.5	212		**43FR2**
26FR	53.1	490	273	**44F1R**
27F1R	58.2	363		**44F2R**
27F2R	58.2	329		**46FR1**
28FR1	51.4	300	251	**46FR2**
28FR2	51.4	282	254	**48F1R**
29FR	54.0	325		**48F2R**
30F1R	55.3	417		**53FR1**
30F2R	55.3	420		**53FR2**
31FAR1	58.7	341		**55FR**
31FARB	58.7	130	473	**57FR**
31FAR2	58.7	332		**62FR**
31FBR1	57.5	341		**66FR**
31FBRB	57.5	132	473	**67FR**
31FBR2	57.5	332		**68FR**
32FR	56.5	405	273	**69F1R**
33FR	56.5	284		**69F2R**
34FR1	57.7	279		**70F1R**
34FR3	57.7	273		**70F2R**
35FR	58.7	290		**73FR2**
36FAR1A	56.1	202		**74FR1**
36FAR1B	56.1	204		**74FR2**
36FAR2	54.8	207		**75FR**
36FBR1A	54.8	341		**76FR**
36FBR1B	56.1	135	473	**77FR**
36FBR2	54.8	332		**78FR**
37FR	56.5	144		**79F2R**
39F1R	59.8	270		**79F1R**
39F2R	59.8	273		**81FR**
45FARA	59.8	302		**85FR**

Successful Amplifications				Failed Amplifications
Primer No.	Opt. Anneal. Temperat.	Sizes (bp)	Expected sizes	Primer No.
45FBRB	59.8	434		86FR
45FARB	59.8	434		91F2R1
45FBRA	59.8	302		91F2R
47FR	56.5	220		94FR
49FR1	59.8	293		97FR
49FR2	56.5	297		
50FR	56.5	401		
51FR	52.3	183		
52FR	52.3	184		
54FR	50.5	215		
55FR	57.7	173		
56FR	50.5	214		
58FR	58.7	341		
59F1R	56.5	155		
60FR	52.3	171		
61FR1	56.1	177		
61FR2	52.4	180		
63F2R	56.5	277		
63F1R	52.4	281		
64FR	55.4	204		
65FR2	56.0	180		
65FR1	59.8	177		
71FR	57.7	147		
72FR	50.1	279	247	
80F1R	56.5	221		
80F2R	56.5	201		
82FR	50.0	191		
83FR	59.6	184		
84FR	61.2	177		
87F1R1	58.0	133	133	
87F1R2	58.0	195		
87F2R2	58.0	195	141	
87F2R1	54.6	195	133	
88FR	50.0	275	130	
89FR	58.7	150		
90FR1	58.7	175		
90FR2	58.7	183		
92FR	59.7	310		
93FR	58.7	158		
95FR	55.5	281		
96FR	51.1	211		
98FR2	55.5	147	153	
98FR1	58.7	160		
99FR	60.4	133	161	
100FR	57.5	150		

Successful (left) and failed (negative, right) amplifications as determined by results of electrophoretic gels.
Actual annealing temperatures are shown, which were optimised.
All amplicon lengths matched the expected lengths, except those in red (expected lengths shown alongside).

5.4.3 Collected Mite UK and KSA Amplification of MS Loci

As previously stated, a number of samples were collected from different buildings in the UK and KSA. The aim was to use successful primers on field *Dermatophagoides* species from both the UK and KSA, especifically on *D. farinae* and *D. pteronyssinus* DNA collected from various dwellings in widely distributed geographic locations in the United Kingdom and Saudi Arabia.

After optimising the PCR conditions for all primers (above), the primers that successfully amplified microsatellites from the lab-*Df* colony were used on mite samples collected from UK and KSA. The same methods were used as those described above for PCR and gel electrophoresis, except for the DNA template used for *D. saudi* sp. nov., which was increased from 0.65 µL to 1.85 µL, 2.4 µl and 3 µl to ensure that the primers could amplify from the template DNA. The PCR annealing temperature was 59.8˚C and the primer for DfMS 48 was the best performing.

5.4.3.1 UK sample Amplification

Trial 1. Results of PCRs for 18 primers in Figure 5.6 show highly comparable, positive amplifications in all cases of sample UK-1 in comparison to Lab-*Df*, both in respect of MS signal strength and estimated fragment length, which is also, consistent with the expected lengths. All primers amplified, only primer 24FR2 produced a weak signal for UK-1 only.

Trial 2. Results of evaluation of primer 27F2R with selected UK samples of *Df* and *Dp.* Strong DNA bands are visible in the electrophoresis at ≈329bp for all *Df* samples. Bands varied in intensity, both samples of *Dp* failed to amplify. (Figure 5.7).

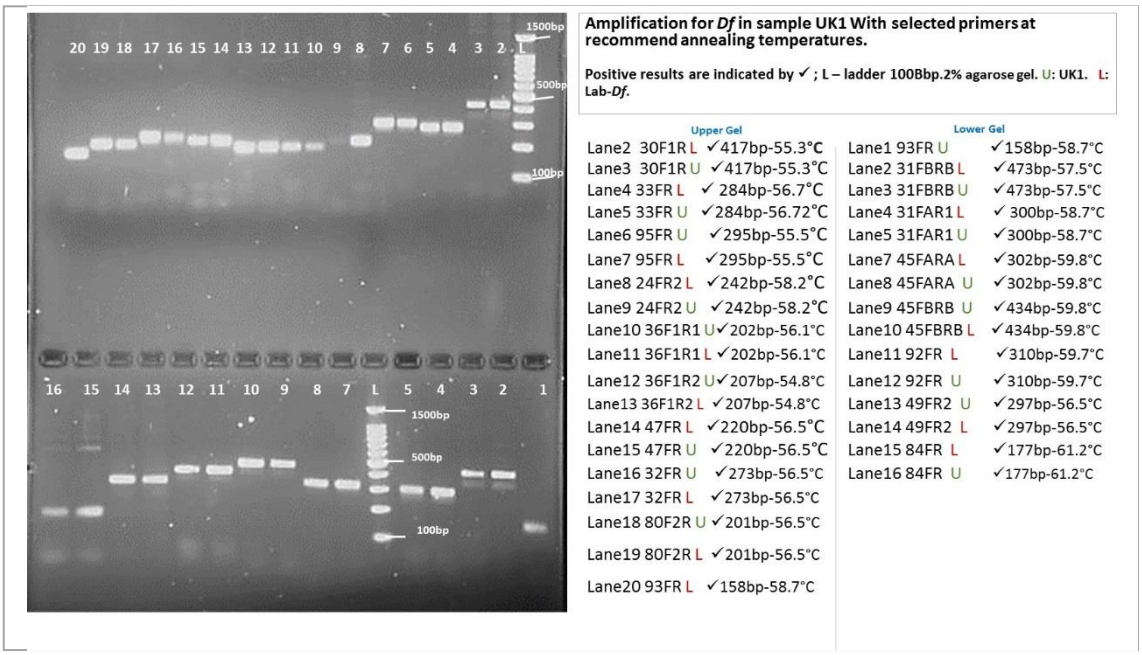


Figure 5.6: 18 Primer amplifications of reared *Df* compared to a single UK collected sample. PCR results of lab-*Df* (L) and UK-2 collected sample (S). Primers show highly comparable amplification. Agarose gel 2%.

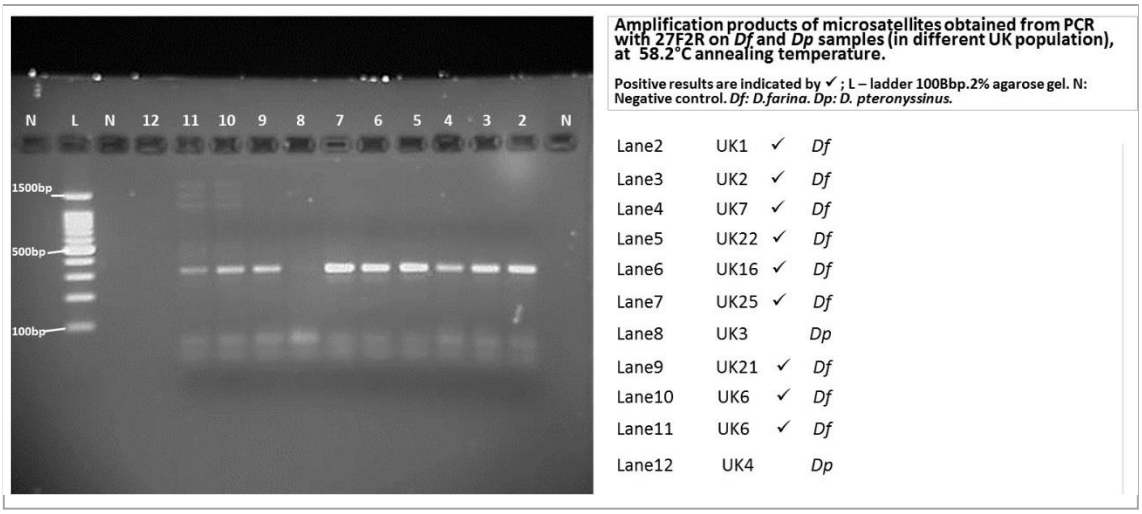


Figure 5.7: Primer 27F2R amplification of selected UK *Df* samples. This figure illustrates various population samples of *D. farinae* with primer 27F2R. Most bands appear to accumulate around 329 bp. Agarose gel 2%.

Trial 3. Results of evaluation of 25 primers with sample UK-5 of *Dp.* (Figure 5.8) shows a failure to amplify for all primers, except 95FR, which produced a strong signal, and 47FR, 93FR, 36aFR1, 31aFR1, 49FR1, 92FR, 45aFaR and 33FR which produced weak and ambiguous signals.

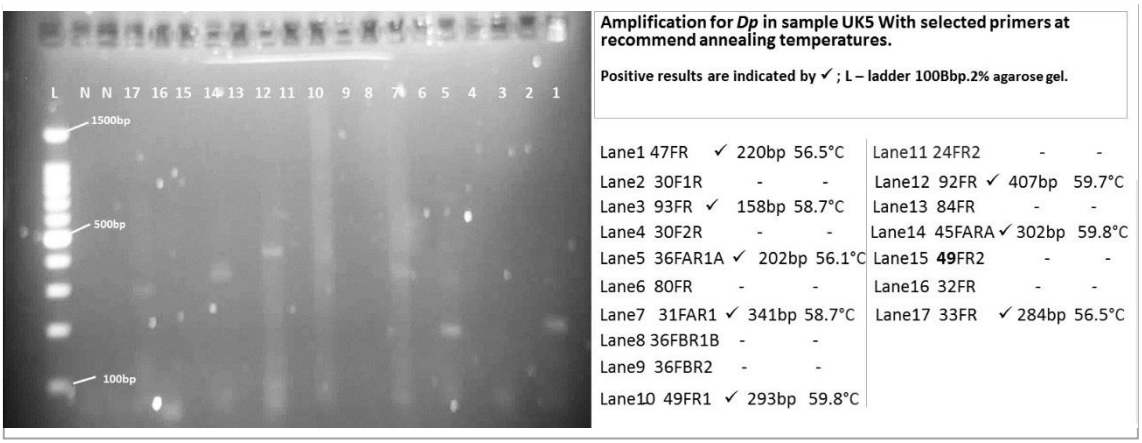
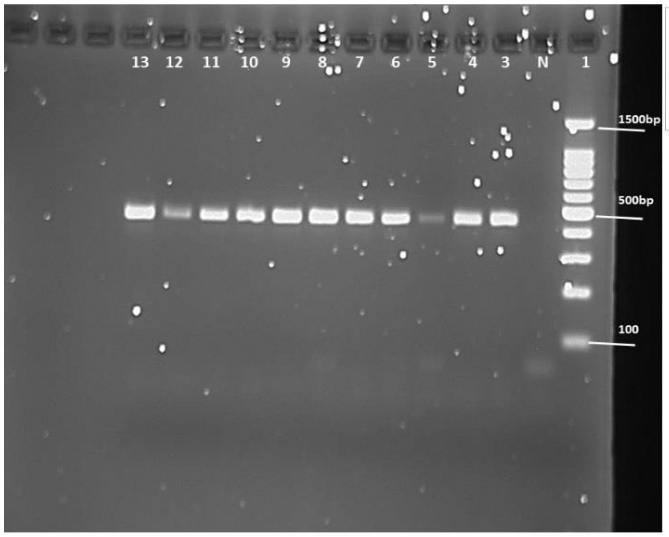


Figure 5.8: Various primer amplifications on selected UK *D. pteronyssinus* samples. UK collected samples of *Dp* with various primers. A total of 9 bands were produced indicating successful amplification. The table shows expected fragment sizes for the MS. Agarose gel 2%.

5.4.3.2 KSA HDM Sample Amplification

Results for KSA correspond to Trial 4, evaluation of primer 45bFbR with 9 KSA samples of *Df* and 4 of *Ds* (Figure 5.9). Using equivalent amounts of DNA in the template, all *Df* samples amplified strongly with bands of the expected length (≈434 bp), whereas the 4 *Ds* samples all failed.

Re-amplified *Ds* samples with higher concentrations of genomic DNA, resulted in amplifications which appeared to strengthen in line with increasing DNA concentration and appear to be the expected length (434 bp).

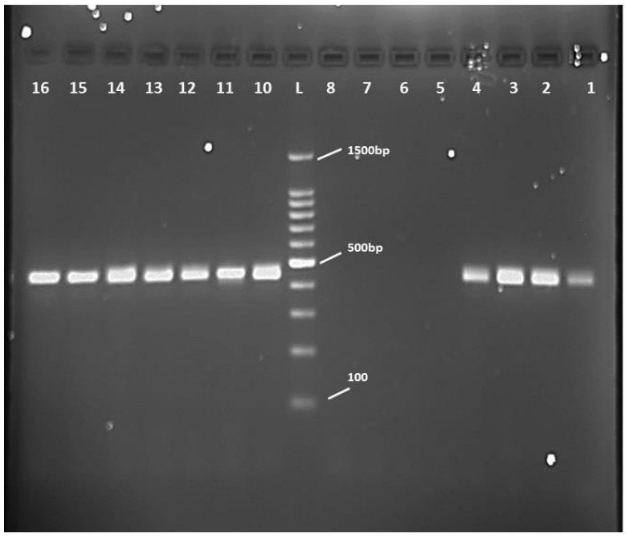


PCR of Primer 45FBRB, showing amplification with varying DNA concentrations

Annealing temperature 59.8°C. Estimated fragment size~434 bp.

Only positive results are indicated by ✓ ; Amount of DNA template in PCR reaction is highlighted green; L – ladder 100bp, N – negative control. 2% agarose gel.

Lane 3	KSA19	✓ 3µl
Lane 4	KSA20	✓ 2.4µl
Lane 5	KSA1	✓ 1.85µl
Lane 6	KSA1	✓ 2.4µl
Lane7	KSA2	✓ 2.4µl
Lane8	KSA2	✓3µl
Lane9	KSA3	✓ 3µl
Lane10	KSA3	✓2.4µl
Lane11	KSA4	✓ 2.4µl
Lane12	KSA4	✓ 1.85µl
Lane13	KSA1	✓ 3µl



PCR of Primer 45FBRB, showing amplification with varying DNA concentrations for I_{Ds} and *D.farinae* species.

Annealing temperature 59.8 °C. Estimated fragment size ~434 bp.

Only positive results are indicated by ✓ ; Amount of DNA template in PCR reaction is highlighted green; L – ladder 100bp, P – positive control, N – negative control. 2% agarose gel.

Lane 1	KSA16 (***D.farinae***)		✓ 0.65µl
Lane 2	KSA17 (***D.farinae***)		✓ 0.65µl
Lane 3	KSA18 (***D.farinae***)		✓ 0.65µl
Lane 4	KSA19 (***D.farinae***)		✓ 0.65µl
Lane 5	KSA1		0.65µl
Lane 6	KSA2	*Ds*	0.65µl
Lane7	KSA3		0.65µl
Lane8	KSA4		0.65µl
Lane10	KSA1 (***D.farinae***)		✓ 0.65µl
Lane11	KSA2 (***D.farinae***)		✓ 0.65µl
Lane12	KSA3 (***D.farinae***)		✓ 0.65µl
Lane13	KSA4 (***D.farinae***)		✓ 0.65µl
Lane14	KSA5 (***D.farinae***)		✓ 0.65µl
Lane15	KSA4 (***D.farinae***)		✓ 0.65µl
Lane16	KSA5 (***D.farinae***)		✓ 0.65µl

Figure 5.9: PCR amplifications of KSA *Df* and *Ds* (Df like/sp. nov) using primer 45bFR. Variation of the band intensities reflects different DNA concentrations. 45bFR estimated size 434 bp. Upper Figure: lanes 5,6,7,8 very low concentration genomic DNA (0.65 µl). Lower Figure: lanes 5 & 12 low concentration genomic DNA (1.85 µl); lanes 6,7,10,11 medium concentration DNA (2.4 µl); lanes 8,9 high concentration (3 µl). Agarose gel 2%. Annealing temperature for all: 59.8 ˚C.

5.5 Discussion

In this study we aimed to test newly developed microsatellite markers for *D. farinae*, to determine which of the validated primer pairs (158, (Al-Khalify, 2018)) could successfully amplify, and the appropriate PCR conditions for amplification. Results were as expected, as the optimal conditions for PCR might have been still affected, and this was obvious with some primers specificity, for example, at the selected annealing temperature (Abd-Elsalam, 2003).

5.5.1 Lab-*Df* Amplifications

Correspondence to the microsatellite loci was tested by amplification using lab-*Df* (Figure 5-5 and App. Figs 3.1-3.16). MS Loci were amplified successfully with 63% of the primers utilised, summarised in Table 5.3. The figures show that, in addition, several primers, such as 6FR1 and 6FR2, amplified their loci weakly. Many loci produced bands of different amplicon fragment lengths, and some gel visualisations are ambiguous, however, this may be initial evidence of allelic polymorphism. Failures to amplify may arise from differences in the sequences of flanking regions or variations in the repeated sequence at the locus.

Amplicon fragments which do not conform to the expected lengths may be an indication of primers binding to sites which are not at the target locus. Several causes of non-specific primer binding are widely reported, and include low annealing temperature, high primer concentration, too low template concentration or, improper primer design and/or manufacturing. Figures 5.5 and App. Figs 3.1-3.16, highlight primers which produced unexpected amplicon lengths or dual bands. In general, it is accepted that longer primers result in higher alignment specificity (Abd-Elsalam, 2003), and by comparison, those used here are long (19-26 bp) (Al-Khalify, 2018). Hence, they are likely to be specific, nevertheless, given their novelty, samples of all primers will be sequenced to ensure alignment to the expected locus.

These results, conducted at a range of annealing temperatures, produced clearly differing strengths of product, and the optimal temperature for future PCRs is highlighted. Further optimisations of PCR conditions were trialled. Primer 5, with a dinucleotide repeat, was investigated thoroughly in 3 trials: Trial 1 - recommended annealing temperature, 53.0 °C, varying DNA template concentrations, Trial 2 - similar, with a higher annealing temperature, 57.7 °C, Trial 3 – with varying annealing temperatures. This demonstrated that only primer combination 5F1R1 amplified successfully, at any temperature, and with any DNA concentration; none of the variants was successful. The trials also showed the benefits of higher DNA concentrations, and, at least for this primer, sensitivity to annealing temperatures, since stronger amplifications were achieved as temperature increased.

In regards to the amplification and visualization of microsatellite -MS products, a universal tag was added to the 5'-end of the forward primer, as described and supported by Nicot et al., 2004. The two tags with the least correspondence in the target genome are M13 mod B, 5' and Tail A were used with more optimisation processes, to avoid any non-specific amplification (stutter bands), and more MS troubleshooting that mentioned above. In addition, the reverse primer was amended at the 5'-end with a partial of half PIG tail to achieve optimal annealing conditions. Overall, these changes and optimisation practices led to very significant and favourable precise amplifications. These actions were supported and reported in Al-Khalify (2018), in which she reported mostly the same changes and amendments to the primers from the genome sequence as a suggestion to obtain better PCR reaction amplifications.

5.5.2 Amplifications from different mite populations

The comparison of 18 primers with genomic DNA of collected sample UK-1 and lab-*Df* (Trial 1) resulted in similar amplifications, indicating that these primers are functional beyond the gene pool for which they were designed. This was confirmed in Trial 2, since primer 27F2R successfully amplified for 9 collected UK samples (1 duplicated) of *Df*, suggesting functionality across a potentially wide genotypic range in this species, which it was essential to establish as it facilitates evaluation of inter-population genetic divergence, a key objective of this study (Funk et al., 2009, Ciofi et al., 2002).

Trials with *Dp*, (Trials 2 and 3) showed limited success, Primer 27F2R failed to amplify for *Dp* from 2 UK collected samples (Figure 6-23), under similar conditions to those applied to 8 different collected *Df* samples. However, when 26 primers were tried (Figure 6-24) weak amplifications were achieved in 9 of them, suggesting that with higher DNA concentrations, and adjustments to annealing temperatures, some or all of these primers may be transferable to *Dp*, an important finding for this study (Ollitrault et al., 2010).

All UK *Df* sample populations were successfully amplified at 35 loci, using optimal annealing temperatures (results not shown), providing scope to identify informative loci in due course. To obtain these informative loci, the universal tag tail was included in order to amplify amplicons created by microsatellite adaptation (Xu et al., 1999). Whereas, for those unproperly amplified loci some corrective actions were conducted, such as set up for an optimal temperature with certain PCR reaction modifications were adapted. Therefore, successful MS loci products were gained. These technical alterations were adapted from previous studies (Payne, 1997, Roux, 2009).

Primer 45bFbR was shown to successfully amplify 9 KSA genomic samples; however, under the PCR same conditions *D. saudi* sp. nov. failed to amplify, so this primer may not readily transfer to this new species. On the other hand, when the template DNA was increased for *D. saudi* sp. nov., amplifications were successful. Therefore, it is necessary for both this species and *Dp*, to use higher DNA concentrations in future PCRs. There are few studies in KSA on HDM supporting this hypothesis (Alotaibi et al., 2018, Negm et al., 2012, Alatawi and Kamran, 2018), the number of mites for molecular studies was small, following the sample size that was very small (Mirza et al., 2020, Fallatah et al., 2019).

Chapter 6 : Conclusions

This research focuses on studying the most abundant species of HDM in the Kingdom of Saudi Arabia, KSA, followed by the microsatellite primer testing of the DNA extracted from the *Dermatophagoides* species found in different UK and KSA geographical areas, and from laboratory colonies. The implications of this study are helpful for their future application in forensics as they facilitate the identification of the most prevalent and unique marker mite species around humans in the KSA.

Molecular studies using microsatellite markers can reveal the genetic structure of a population, elucidating genetic differences or characterizing different populations. Environmental modification, geographical location, their impact to other small microorganisms (such as fungi), and their interaction with various hosts, all influence the genetic variation and differentiation of the most common HDM, *Dermatophagoides* species.

Pyroglyphidae mites are known to be the two of the most widespread HDMs in the UK and the KSA, their distribution and abundance are affected by environmental factors, such as temperature and humidity (Nordenfors et al., 1999), but also by ecology and domestic conditions. The low numbers of mites in collected dust samples might also result from methodological limitations, including unsystematically selected and inadequate dust collections, delays in transfer from dust to culture conditions, or failure to extract all mites from the dust. Colloff (1987), achieved much higher volumes, 97 / 100 mg mites averaged across 124 collections from 23 dwellings in Glasgow, using mattresses and carpets as source, and from just carpets found 37.2 / 100mg, with *D. pteronyssinus* overwhelmingly the most common species; this despite the Glasgow climate being more northerly than any of the other locations, so likely to have a cooler climate.

With very little published information about the mite fauna of KSA, the species list from KSA dust samples presented here is comprehensive enough, representing a significant contribution to our knowledge of this fauna. Several allergenic studies (KSA) have confirmed the presence and some diversity amongst common pathogenic species, principally *D. farinae* and *D. pteronyssinus*. The higher diversity found in KSA compared to UK samples, and the reversed proportions of *D. farinae* to *D.*

pteronyssinus may reflect climatic, environmental or local domestic differences, but the same methodological limitations may also apply. In some cases, additional species found, may be parasitic on pet animals such as parrots or cats, which were present in the dwelling from which the sample was taken, notably, two putative species of *Cheyletus* (Cheyletidae) a family which is known to parasitise birds and animals. The explored taxa are summarized in Chapter 2. This work encountered the possibility of one or more novel species of HDM, which constitute valuable additions to the KSA house fauna known. They might also, following further investigation, be found to be more widespread in the middle East than just the KSA. These HDMs were detected in different houses and in diverse environmental conditions, and there seems to be a relationship between some species and their locations which will be investigated systematically later as a follow up of this study. In particular, *Dermatophagoides* sp. nov, a mite with affinity to *D. pteronyssinus* but with distinct morphology, was only found from Jeddah, a coastal city. Species with narrow geographic ranges have potential in forensic analysis, being able to localise the origins of evidential samples (Perotti et al., 2009b).

Chapter 2 described the collection and diversity of HDM in the KSA. Regarding the identification of Saudi HDM, novel species were discovered *(Dermatophagoides saudi* sp. nov.) in the KSA samples from Jeddah city (coastal area). *Dermatophagoides saudi* sp. nov. and another unusual *Cheletopsis*-like (still under identification, data not shown) are similar to known taxa, *Dermatophagoides evansi* and *Cheletopsis* respectively, but have distinctive morphological characteristics. *Cheletopsis*-like are known to parasitise birds (Sinai rosefinches (*Carpodacus synoicus*, Fringillidae)). This is helpful for both forensic diagnoses and forensic genetic diagnoses.

In the conclusion of Chapter 5, the PCR assays show that a high proportion of the MS loci can be amplified using the defined primers. However, care needs to be taken with the selection of the forward and backward variants, annealing temperatures, and concentrations of DNA in the template. This indicates, in so far as they have been tested to date, that effective methodologies have now been established that can be applied, with modifications as discussed, to the assays required for the second part of

this research. As mentioned, the DNA extraction of *D. farinae* was optimized successfully using QIAGEN ATL lysis (Chapter 4).

The functionality of these useful primers extends across the range of both the UK and the KSA *D. farinae* populations sampled for this study. This renders the loci highly suited to the analysis of population genetic divergence, providing that I am able to establish a sufficient degree of allelic polymorphism. Furthermore, there is inter-specific transferability of the loci to *D. pteronyssinus* and *D. saudi* sp. nov., though, as might be expected, with seemingly lower quantities of PCR product and not consistently. The establishment of inter-population and -species transfer is a very important milestone in the development of the MS loci and this will be explored at length in the second part of this study.

6.1 Limitations:

A number of limitations faced in this research include: 1. Time. 2. issues with Cost. 3. DNA extraction from the mites. 4. Sample collection from Saudi Arabia (different geographical location). 5. Mite identification that required difficult to obtain literature to revise and much time dedication from my supervisor and Dr. M. Hani to train me in taxonomy. 6. PCR optimization, which involved several variants (time consuming). 7. Mite rearing, that was a challenge due to fungi growth.

6.2 Future research

6.2.1 Future Multiplexing

Microsatelites can be multiplexed (multiple primers combined with a DNA sample in a single PCR). To be successful, multiple primers must anneal at the same temperature and they must previously have been labeled with appropriate non-ambiguous dyes. Issues involved in the interpretation of the resultant chromatograms must also be considered to ensure that the allelotypes can be readily and unambiguously distinguished. This is often achieved by including non-overlapping fragment lengths in the multiplex configuration. Suitable multiplex configurations will be considered and used if practical.

6.2.2 Forensic analysis

HDM may be a valuable source of information as trace evidence, particularly in indoor crime scenes; therefore microsatellite markers represent a potentially powerful new tool in forensic analysis. By genetically characterizing the mites, it may be possible to find the differences in the populations and to pinpoint the environmental and geographical origins of the samples.

The results obtained so far show that it is possible to amplify the new MS markers for *D. farinae* and to a lesser extent, *D. pteronyssinus*. Genetic analyses are possible, given the sample collections, at the national, regional and even individual dwelling levels. If differences can be detected, it may become possible to link the house of a suspect with that of a victim. I will assess and discuss, using the outcomes of the population analysis, the implications of the genotypic characteristics of these MS loci for future forensic analysis.

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List of appendices

Appendix 1: Biodiversity of the house-dust fauna and its forensic implications

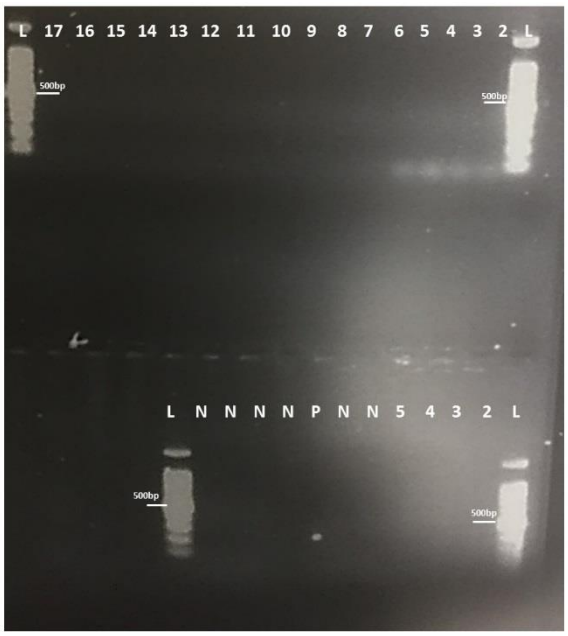
Table A-1: Total number of mites from each family collected in the five different cities in KSA

Cities	Pyroglyphidae	Cheyletidae	Acaridae	Oribatida	Tetranychidae	Glycyphagida	Laelapidae	Neotrombidiid	Dermestidae	Psocidae	Blattidae	Gryllidae	Scarabaeidae
Jeddah	149	29	44	19	6	10	3	6	2	2	2	2	1
Al-Kharj	2	20	1	1	1	1	3	2	1	3	1	1	1
Riyadh	17	41	2	1	1	1	1	1	2	1	1	0	1
Taif	196	26	62	43	17	9	12	8	5	1	3	4	3
Shafa	64	11	6	1	1	5	1	2	1	1	1	3	2

Table A-2: Total number of mites of each species from the genus Dermatophagoides collected in the five different cities.

Cities	*D. farinae*	*D. pteronyssinus*	*D. saudi* sp. nov.
Jeddah	9	24	15
Al-Kharj	1	2	0
Riyadh	4	3	0
Taif	30	45	0
Shafa	3	10	0

Appendix 2: Figures of agarose gel results of all the tested primer sets with lab-Df DNA.



Locus analysis for Primers 1 to 4, showing amplification at different annealing temperatures.

L – ladder 1kb, P – positive control, N – negative control.2% Agarose gel.

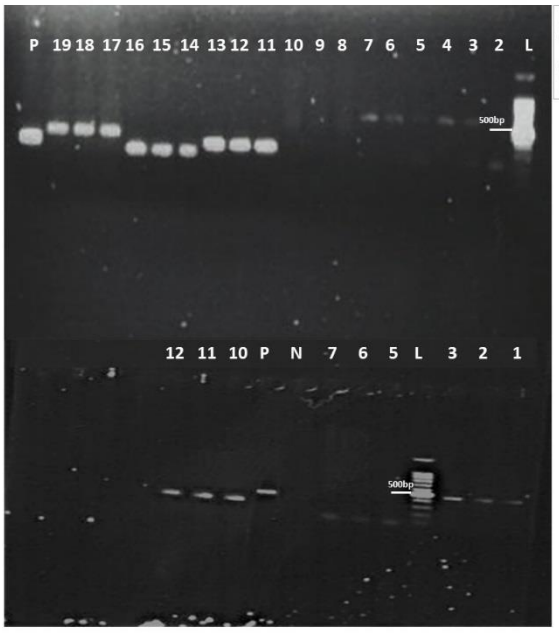
Upper Gel

Lanes 2 – 5		**1FR**
2	53.1	
3	54.6	
4	56.1	
5	57.5	
Lanes 6 - 9		**2FR**
6	53.1	
7	54.6	
8	56.1	
9	**57.5**	
Lanes 10 - 13		**3F1R**
10	53.1	
11	54.6	
12	56.1	
13	57.5	
Lanes 14 - 17		**3F2R**
14	53.1	
15	54.6	
16	56.1	
17	57.5	

Lower Gel

Lanes 2 – 5		**4FR**
2	53.1	
3	54.6	
4	56.1	
5	57.5	

Figure 2.1: Lab-*Df*, PCR results for primers 1 to 4.



Locus analysis for Primers 6 to 10, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓ ; optimal temperature is highlighted red;
L – ladder 1kb, P – +ve control, N – -ve control. US: Un-Specific band.2% agarose gel

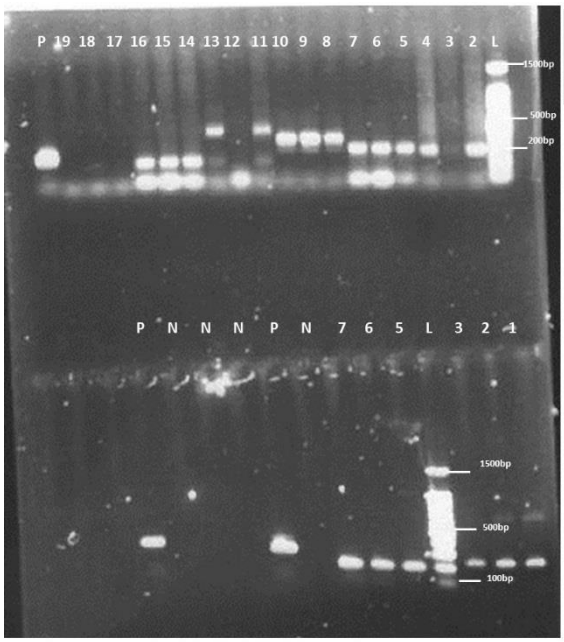
Upper Gel

Lanes 2 – 4			**6FR1**
2	53.9	US	
3	56.1	US	
4	57.7	US	
Lanes 5 – 7			**6FR2**
5	54.6	US	
6	56.9	US	
7	58.2	US	
Lanes 8– 10			**7FR**
8	54.6		
9	56.9		
10	58.2		
Lanes 11-13			**9FR1**
11	54.6	✓	386bp
12	56.9	✓	386bp
13	58.2	✓	386bp
Lanes 14 – 16			**9FR2**
14	54.6	✓	320bp
15	56.9	✓	320bp
16	58.2	✓	320bp
Lanes 17 – 19			**10F1R**
17	54.6	✓	498bp
18	56.9	✓	498bp
19	58.2	✓	498bp

Lower Gel

Lanes 1 – 3			**10F2R**
1	54.6	✓	465bp
2	56.9	✓	465bp
3	58.2	✓	465bp
Lanes 5 – 7			**10F3R**
5	54.6		
6	56.9		
7	58.2		
Lanes 10 – 12			**8FR**
10	54.6	✓	259bp
11	56.9	✓	259bp
12	58.2	✓	259bp

Figure 2.2: Lab-*Df*, PCR results for primers 6 to 10.



Locus analysis for Primers 11 to 17, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control.2%agarose gel

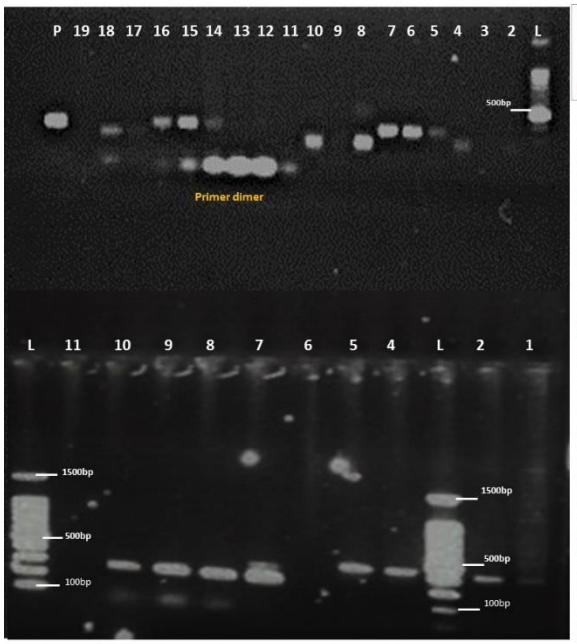
Upper Gel

Lanes 2 - 4			**11FR**
2	53.9	✓	230bp
3	56.1	✓	230bp
4	57.7	✓	230bp
Lanes 5 – 7			**12FR**
5	54.6	✓	254bp
6	56.9	✓	254bp
7	58.2	✓	254bp
Lanes 8- 10			**13FR**
8	54.6	✓	361bp
9	56.9	✓	361bp
10	58.2	✓	361bp
Lanes 11 -13			**14FR**
11	54.6	✓	475bp
12	56.9	✓	475bp
13	58.2	✓	475bp
Lanes 14 – 16			**15F1R**
14	54.6	✓	263bp
15	56.9	✓	263bp
16	58.2	✓	263bp
Lanes 17 – 19			**15F2R**
17	54.6		
18	56.9		
19	58.2		

Lower Gel

Lanes 1 - 3			**16FR**
1	54.6	✓	291bp
2	56.9	✓	291bp
3	58.2	✓	291bp
Lanes 5 - 7			**17FR**
5	54.6	✓	224bp
6	56.9	✓	224bp
7	58.2	✓	224bp

Figure 2.3: Lab-*Df*, PCR results for primers 11 to 17.



Locus analysis for Primers 18 to 23, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control.2%agarose gel.

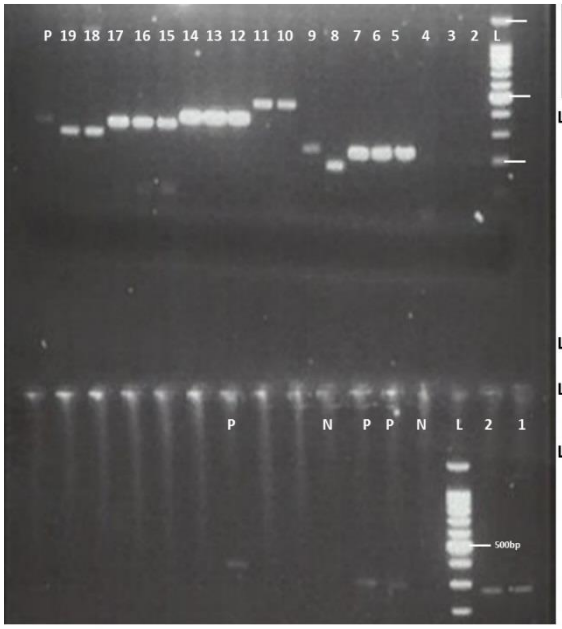
Upper Gel

Lanes 2 – 4			**18FR**
2	54.6		
3	56.1		
4	58.2	✓	193bp
Lanes 5 – 7			**19FR1**
5	53.9	✓	322bp
6	55.3	✓	322bp
7	56.9	✓	322bp
Lanes 8–10			**19FR2**
8	53.9	✓	251bp
9	54.6	✓	251bp
10	56.9	✓	251bp
Lanes 11-13			**20F1R**
11	53.9		
12	56.1		
13	57.7		
Lanes 14 – 16			**20F2R**
14	53.9	✓	351bp
15	56.1	✓	351bp
16	57.7	✓	351bp
Lanes 17 – 18			**21FR**
17	56.9	✓	295bp
18	58.5	✓	295bp

Lower Gel

Lanes 1 – 2			**22FR1**
1	53.9	✓	432bp
2	56.1	✓	432bp
Lanes 4 – 5			**22FR2**
4	53.9	✓	436bp
5	56.1	✓	436bp
Lanes 8-10			**23FR**
8	53.9	✓	271bp
9	56.1	✓	271bp
10	58.2	✓	271bp

Figure 2.4: Lab-*Df*, PCR results for primers 18 to 23.



Locus analysis for Primers 24 to 28, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓ ; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control.2%agarose gel.

Upper Gel

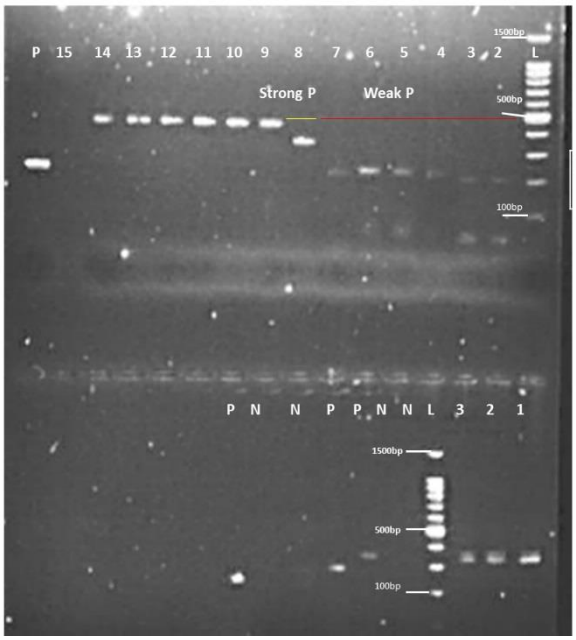
Lanes 2 – 4			**24FR1**
2	53.1		
3	55.3		
4	57.7		
Lanes 5 - 7			**24FR2**
5	53.9	✓	242bp
6	56.1	✓	242bp
7	58.2	✓	242bp
Lane 8			**25FR1**
8	49.8	✓	155bp
Lane 9			**25FR2**
9	48.4	✓	212bp
Lanes 10 -11			**26FR**
10	51.4	✓	490bp
11	53.1	✓	490bp
Lanes 12-14			**27F1R**
12	53.9	✓	363bp
13	56.1	✓	363bp
14	58.2	✓	363bp
Lanes 15-17			**27F2R**
15	53.9	✓	329bp
16	56.1	✓	329bp
17	58.2	✓	329bp

Lower Gel

Lanes 1 – 2			**28FR2**
1	51.4	✓	254bp
2	53.1	✓	254bp

Lanes 18 – 19			**28FR1**
18	51.4	✓	251bp
19	53.1	✓	251bp

Figure 2.5: Lab-*Df*, PCR results for primers 24 to 28.



Locus analysis for Primers 29 to 30, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓ ; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

Upper Gel

Lanes 9-11			**30F1R**
9	53.1	✓	417bp
10	55.3	✓	417bp
11	57.7	✓	417bp
Lanes 12 – 14			**30F2R**
5	53.1	✓	420bp
6	55.3	✓	420bp
7	57.7	✓	420bp

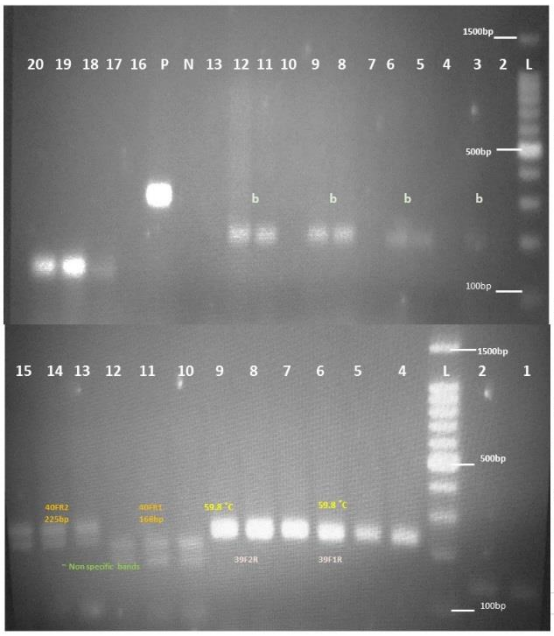
Lower Gel

Lanes 1 - 3			**29FR**
1	54.6	✓	325bp
2	56.9	✓	325bp
3	58.5	✓	325bp

Weak Positive -Lanes 2 – 7(Low conc of DNA)

Strong Positive – Lane 8 (High conc. of DNA)

Figure 2.6: Lab-*Df*, PCR results for primers 29 to 30.



Locus analysis for primers 36 to 40, showing amplification at different annealing temperatures.

Primer 36 – primer volume in PCR mixed increased to 30µL with 70µL dH_2O
Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2% agarose gel.

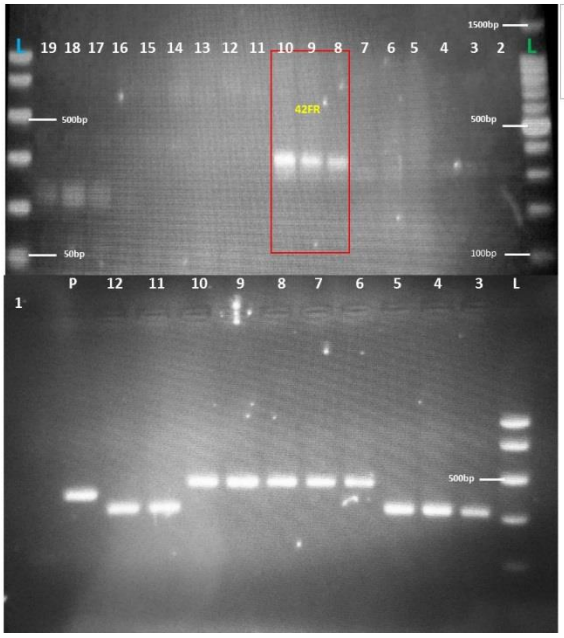
Upper Gel

Lane 2	**54.5**		**36FR1**
Lane 3			**36FR1B**
3	56.1	✓	202bp
Lane 4	**54.5**		**36FR2**
Lanes 5-7	**54.5**		**36FbR1**
5	50.5	✓	202bp
6	56.1	✓	202bp
7	58.7		
Lanes 8 - 10			**36FbR2**
8	50.5	✓	207bp
9	54.5	✓	207bp
10	58.7		
Lanes 11-13			**36FbR1b**
11	50.5	✓	202bp
12	56.1	✓	202bp
13	58.7		
Lanes 17 – 19			**37FR**
14	52.3	✓	144bp
15	56.5	✓	144bp
16	59.8	✓	144bp
Lane 20	**52.3**		**38FR**

Lower Gel

Lanes 1 – 2			**38FR**
1	56.5		
2	59.8		
Lanes 4 – 6			**39F1R**
4	52.3	✓	270bp
5	56.5	✓	270bp
6	59.8	✓	270bp
Lanes 7 - 9			**39F2R**
7	52.3	✓	273bp
8	56.5	✓	273bp
9	59.8	✓	273bp
Lanes 10 – 12			**40FR1**
10	52.3		
11	56.5		
12	59.8		
Lanes 13 – 15			**40FR2**
13	52.3		
14	56.5		
15	59.8		

Figure 2.7: Lab-*Df*, PCR results for primers 36 to 40.



Locus analysis for Primers 41 to 45, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

Upper Gel

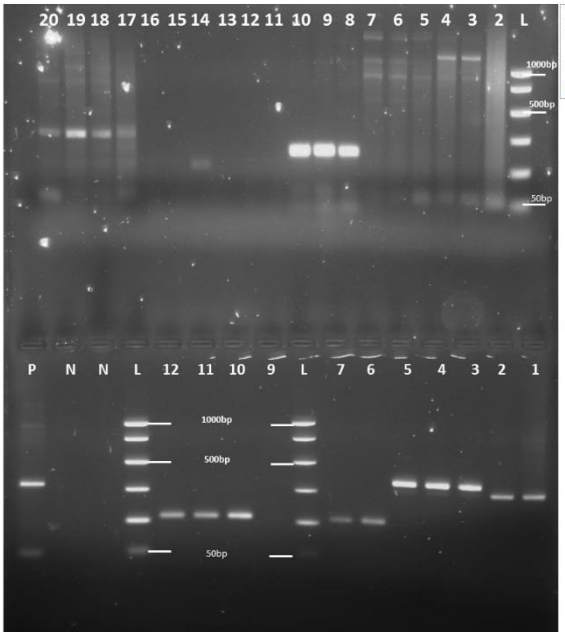
Lanes 2 – 4			**41F1R**
2	52.3		
3	56.5		
4	59.8		
Lanes 5 – 7			**41F2R**
5	52.3		
6	56.5		
7	59.8		
Lanes 8– 10			**42FR**
8	52.3	✓	276bp
9	56.5	✓	276bp
10	59.8	✓	276bp
Lanes 11-13			**43FR1**
11	51.2		
12	55.4		
13	59.5		
Lanes 14 – 16			**43FR2**
14	51.2		
15	55.4		
16	59.5		
Lanes 17 – 19			**44F1R**
17	52.3		
18	56.5		
19	59.8		

Lower Gel

Lanes 1			**44F2R**
1	59.8		
Lanes 3 – 5			**45FaRa**
3	52.3	✓	302bp
4	56.5	✓	302bp
5	59.8	✓	302bp
Lanes 6 – 7			**45FaRb**
6	55.4	✓	434bp
7	59.8	✓	434bp
Lanes 8 – 10			**45FbRb**
8	52.3	✓	434bp
9	56.5	✓	434bp
10	59.8	✓	434bp
Lanes 11 – 12			**45FbRa**
11	55.4	✓	302bp
12	59.8	✓	302bp

L 100pb marker
L PCR marker (bp)

Figure 2.8: Lab-*Df*, PCR results for primers 41 to 45.



Locus analysis for Primers 46 to 52, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓ ; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel%.

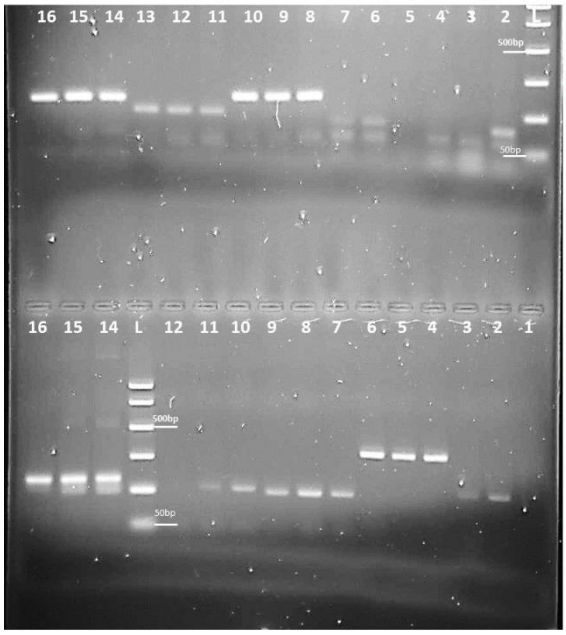
Upper Gel

Lanes 2 – 4			**46FR1**
2	53.4		
3	56.5		
4	59.8		
Lanes 5 – 7			**46FR2**
5	50.1		
6	51.2		
7	52.3		
Lanes 8– 10			**47FR**
8	52.3	✓	220bp
9	56.5	✓	220bp
10	59.8	✓	220bp
Lanes 11-13			**48F1R**
11	52.3		
12	56.5		
13	59.8		
Lanes 14 – 16			**48F2R**
14	52.3		
15	56.5		
16	59.8		
Lanes 17 – 18			**49FR1**
17	52.3	✓	293bp
18	56.5	✓	293bp
19	59.8	✓	293bp
Lane 20			**49FR2**
20	52.3	✓	297bp

Lower Gel

Lanes 1 – 2			**49FR2**
1	56.5	✓	432bp
2	59.8	✓	432bp
Lanes 3 – 5			**50FR**
3	52.3	✓	401bp
4	56.5	✓	401bp
5	59.8	✓	401bp
Lanes 6 - 9			**51FR**
6	52.3	✓	183bp
7	56.5	✓	183bp
9	59.8		
Lanes 10 - 12			**52FR**
10	52.3	✓	184bp
11	56.5	✓	184bp
12	59.8	✓	184bp

Figure 2.9: Lab-*Df*, PCR results for primers 46 to 52.



Locus analysis for Primers 53 to 60, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓ ; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarosege.

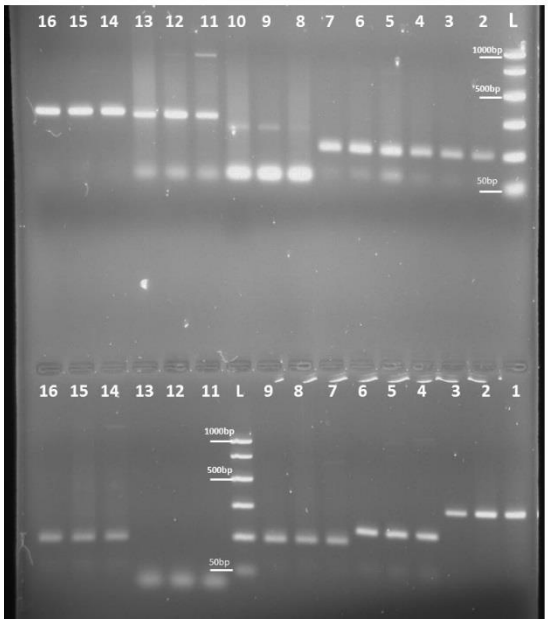
Upper Gel

Lanes 2 – 4			**53FR1**
2	50.1		
3	52.3		
4	55.4		
Lanes 5 – 7			**53FR2**
5	50.1		
6	51.2		
7	53.4		
Lanes 8– 10			**54FR**
8	50.5	✓	215bp
9	54.5	✓	215bp
10	58.7	✓	215bp
Lanes 11-13			**55FR**
11	50.1		173bp
12	53.4		173bp
13	57.7		173bp
Lanes 14 – 16			**56FR**
14	50.5		214bp
15	54.5		214bp
16	58.7		214bp

Lower Gel

Lanes 1 – 3			**57FR**
1	50.5		
2	54.5		
3	58.7		
Lanes 4 – 6			**58FR**
4	50.5	✓	341bp
5	54.7	✓	341bp
6	58.7	✓	341bp
Lanes 7 - 9			**59F1R**
7	52.3	✓	155bp
8	56.5	✓	155bp
9	59.8	✓	155bp
Lanes 10 - 12			**59F2R**
10	52.3	✓	168bp
11	56.5	✓	168bp
12	59.8	✓	168bp
Lanes 14- 16			**60FR**
14	52.3	✓	171bp
15	56.5	✓	171bp
16	59.8	✓	171bp

Figure 2.10: Lab-*Df*, PCR results for primers 53 to 60.



Locus analysis for Primers 61 to 66, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

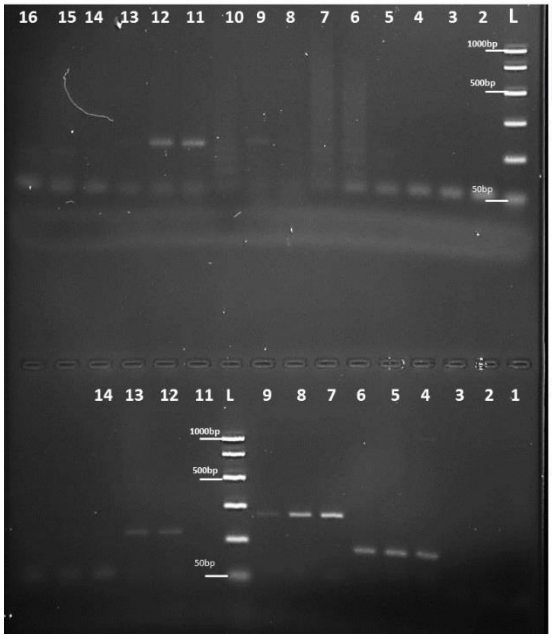
Upper Gel

Lanes 2 – 4			**61 FR2**
2	50.1	✓	180bp
3	52.4	✓	180bp
4	56.5	✓	180bp
Lanes 5 - 7			**61FR1**
5	50.1	✓	177bp
6	53.4	✓	177bp
7	56.5	✓	177bp
Lane 8 - 10			**62FR1**
8	52.3		
9	56.5		
9	59.8		
Lanes 11 -13			**62FR2**
11	52.3	✓	265bp
12	56.5	✓	265bp
13	59.8	✓	265bp
Lanes 14 - 16			**63F1R**
14	52.3	✓	281bp
15	56.5	✓	281bp
16	59.8	✓	281bp

Lower Gel

Lanes 1 – 3			**63F2R**
1	52.3	✓	277bp
2	56.5	✓	277bp
3	59.8	✓	277bp
Lanes 4 - 6			**64FR**
4	52.3	✓	204bp
5	55.4	✓	204bp
6	59.5	✓	204bp
Lanes 7 – 9			**65FR1**
7	53.4	✓	177bp
8	56.5	✓	177bp
9	59.8	✓	177bp
Lanes 11 – 13			**66FR**
11	52.3		
12	57.7		
13	59.8		
Lanes 14 – 16			**65FR2**
14	50.1	✓	180bp
15	53.4	✓	180bp
16	56.0	✓	180bp

Figure 2.11: Lab-*Df*, PCR results for primers 61 to 66.



Locus analysis for Primers 67 to 73, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarosege.

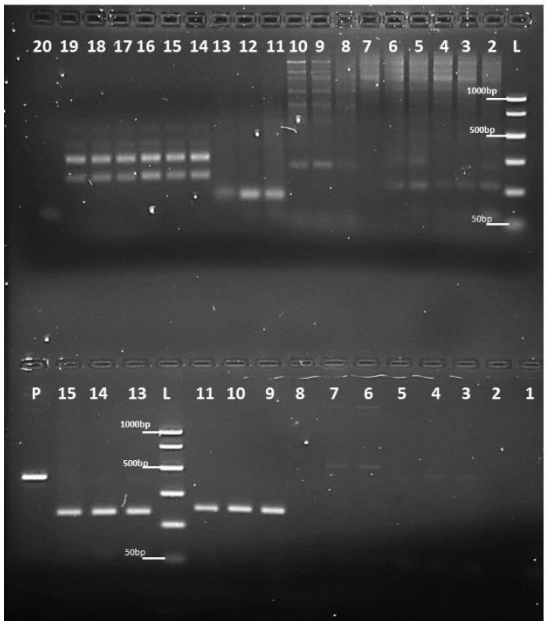
Upper Gel

Lanes 2 - 4			**67FR**
2	52.3		
3	56.5		
4	59.8		
Lanes 5 - 7			**68FR**
5	50.5		
6	54.5		
7	58.7		
Lanes 8 - 10			**69F1R**
8	50.1		
9	53.4		
10	57.7		
Lanes 11 -13			**69F2R**
11	50.1	Weak	204bp
12	53.4	Weak	204bp
13	57.7		
Lanes 14 – 16			**70F1R**
14	50.1		
15	53.4		
16	57.7		

Lower Gel

Lanes 1 – 3			**70F2R**
1	50.1		
2	53.4		
3	57.7		
Lanes 4 - 6			**71FR**
5	50.1	✓	147bp
6	53.4	✓	147bp
7	57.7	✓	147bp
Lanes 7 – 9			**72FR**
7	50.1	✓	279bp
8	53.4	✓	279bp
9	57.7	✓	279bp
Lanes 11 – 13			**73FR1**
11	51.2		
12	55.4		
13	59.8		
Lanes 14 – 16			**73FR2**
14	51.2		
15	55.4		
16	59.8		

Figure 2.12: Lab-*Df*, PCR results for primers 67 to 73.



Locus analysis for Primers 74 to 80, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2% agarose gel.

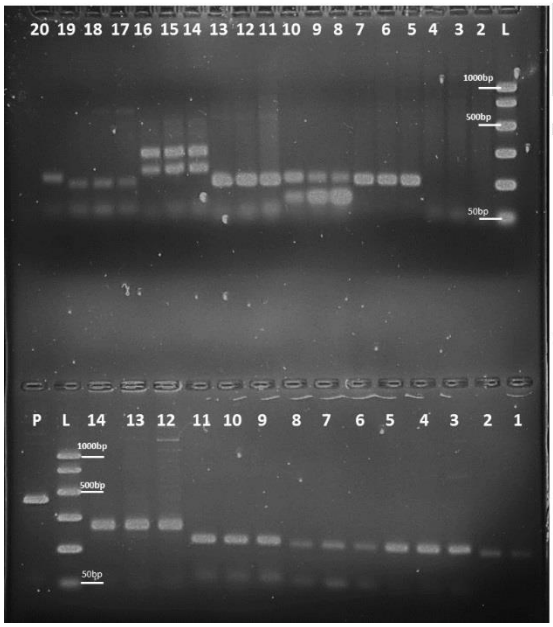
Upper Gel

Lanes 2 – 4			**74FR1**
2	52.3		
3	56.5		
4	59.8		
Lanes 5 - 7			**74FR2**
5	51.2		
6	55.4		
7	59.5		
Lane 8 - 10			**75FR**
8	52.3		
9	56.5		
10	59.8		
Lanes 11 -13			**76FR**
11	52.3		
12	56.5		
13	59.8		
Lanes 14 – 16			**77FR1**
14	52.3		
15	56.5		
Lanes 17-19			**77FR2**
17	51.2		
18	55.4		
19	59.5		
Lane 20			**78FR**
20	51.2		

Lower Gel

Lanes 1 – 2			**78FR**
1	55.4		
2	59.5		
Lanes 3 - 5			**79F1R**
3	52.3		
4	56.5		
5	59.8		
Lanes 6 – 8			**79F2R**
6	52.3		
7	56.5		
8	59.8		
Lanes 9 – 11			**80F1R**
9	52.3	✓	221bp
10	56.5	✓	221bp
11	59.8	✓	221bp
Lanes 13 – 15			**80F2R**
13	52.3	✓	201bp
14	56.5	✓	201bp
15	59.8	✓	201bp

Figure 2.13: Lab-*Df*, PCR results for primers 74 to 80.



Locus analysis for Primers 81 to 88, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

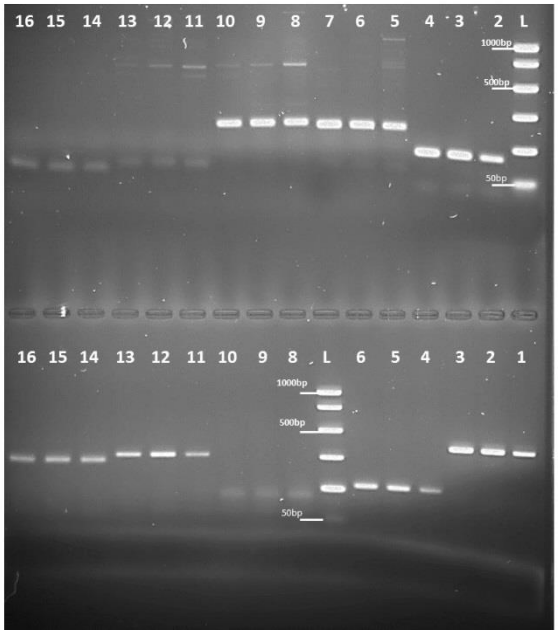
Upper Gel

Lanes 2 – 4				**81FR**
2	46.7			
3	47.4			
4	48.4			
Lanes 5 - 7				**82FR**
5	50.0	✓	191bp	
6	53.2	✓	191bp	
7	58.0	✓	191bp	
Lane 8 - 10				**83FR**
8	50.0		184bp	
9	53.2		184bp	
10	59.6	Double Bands	184bp	
Lanes 11 -13				**84FR**
11	51.6	✓	177bp	
12	56.3	✓	177bp	
13	61.2	✓	177bp	
Lanes 14 – 16				**85FR**
14	51.6			
15	54.6			
16	58.0			
Lanes 17 – 19				**86FR**
17	50.0	Double Bands 189bp-287bp		
18	54.6			
19	60.6			
Lane 20				**87F1R1**
20	51.6	weak	133bp	

Lower Gel

Lanes 1 – 2			**87F1R1**
1	54.6	weak	133bp
2	58.0	weak	133bp
Lanes 3 - 5			**87F1R2**
3	51.6	✓	195bp
4	54.6	✓	195bp
5	58.0	✓	195bp
Lanes 6 – 8			**87F2R1**
6	51.6	weak	133bp
7	54.6	weak	133bp
8	58.0	weak	133bp
Lanes 9 – 11			**87F2R2**
9	51.6	✓	195bp
10	54.6	✓	195bp
11	58.0	✓	195bp
Lanes 12 – 14			**80F2R**
13	50.0	✓	275bp
14	53.2	✓	275bp
15	59.5	✓	275bp

Figure 2.14: Lab-*Df*, PCR results for primers 81 to 88.

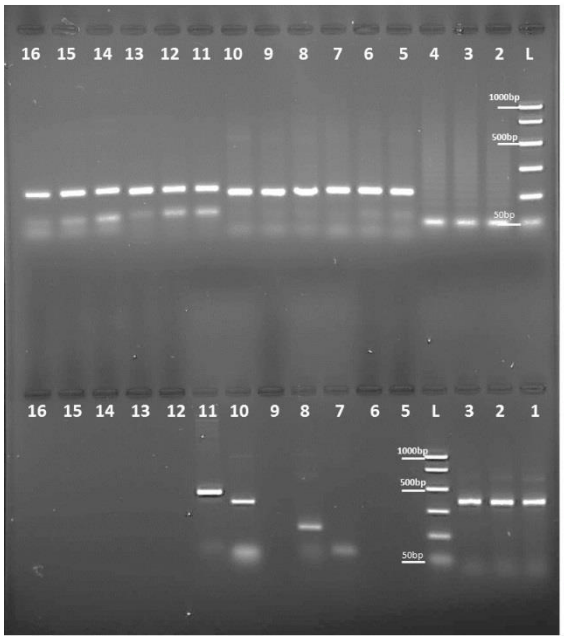


Locus analysis for Primers 89 to 96, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2%agarose gel.

Upper Gel				Lower Gel			
Lanes 2 – 4		**89FR**		**Lanes 1 – 3**		**92FR**	
2	51.6	✓	150bp	1	52.3	✓	385bp
3	55.5	✓	150bp	2	56.4	✓	385bp
4	58.7	✓	150bp	3	59.7	✓	385bp
Lanes 5 – 7		**90FR1**		**Lanes 4 - 6**		**93FR**	
5	51.6	✓	175bp	4	51.6	✓	158bp
6	55.5	✓	175bp	5	55.5	✓	158bp
7	58.7	✓	175bp	6	58.7	✓	158bp
Lanes 8– 10		**90FR2**		**Lanes 8 - 10**		**94FR**	
8	51.6	✓	183bp	8	51.1		
9	55.5	✓	183bp	9	53.3		
10	58.7	✓	183bp	10	56.4		
Lanes 11-13		**91F1R**		**Lanes 11 - 13**		**95FR**	
11	51.6			11	51.6	✓	281bp
12	55.5			12	55.5	✓	281bp
13	58.7			13	58.7	✓	281bp
Lanes 14 – 16		**91F2R**		**Lanes 14 - 16**		**96FR**	
14	51.6			14	51.1	✓	211bp
15	55.5			15	54.4	✓	211bp
16	58.7			16	57.5	✓	211bp

Figure 2.15: Lab-*Df*, PCR results for primers 89 to 96.



Locus analysis for Primers 97 to 101, showing amplification at different annealing temperatures.

Only positive results are indicated by ✓; optimal temperature is highlighted red; L – ladder 100bp, P – positive control, N – negative control. 2% agarose gel.

Upper Gel				Lower Gel			
Lanes 2 – 4		**97FR**		**Lanes 1 – 3**		**101FR**	
2	51.6			1	52.3	✓	319bp
3	55.5			2	55.5	✓	319bp
4	57.7			3	57.5	✓	319bp
Lanes 5 – 7		**98FR1**					
5	52.3	✓	160bp				
6	56.4	✓	160bp				
7	58.7	✓	160bp				
Lanes 8– 10		**98FR2**					
8	51.1	✓	153bp				
9	53.3	✓	153bp				
10	55.5	✓	153bp				
Lanes 11-13		**99FR**					
11	53.3	✓	161bp				
12	57.5	✓	161bp				
13	60.4	✓	161bp				
Lanes 14 – 16		**100FR**					
14	53.3	✓	150bp				
15	57.5	✓	150bp				
16	60.4	✓	150bp				

Figure 2.16: Lab-*Df*, PCR results for primers 97 to 101.