Genetic variation in South African *Diuraphis noxia* biotypes

by

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Declaration

I, Evandré Annemari Minnaar, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification Magister Scientiae in Botany at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

I, furthermore, cede copyright of the dissertation in favour of the University of the Free State.

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ARC-SG	Agricultural Research Council-Small Grain
ATP	Adenosine triphosphate
Avr	Avirulent
bp	Base pairs
BSA	Bovine serum albumin
DMDC	Dimethyl dicarbonate
ΔK	Delta K
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
FS	Free State
K	“True” number of subpopulations
kb	Kilo base pairs
MCMC	Monte Carlo Markov Chain
MLG	Multilocus Genotypes
PIC	Polymorphic information content
RAPD	Randomly Amplified Polymorphic DNA
RNase A	Ribonuclease A
RWA	Russian wheat aphid
SA	South Africa
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA

Tris-HCl	Tris(hydroxymethyl)-aminomethane hydrochloride
USA	United States of America
USSR	Union of Soviet Socialist Republics
Vir	Virulent

List of SI Units

°C	Degrees Celsius
%	Percentage
g	Gravitational acceleration
cm	Centimetre(s)
h	Hour(s)
M	Molar(s)
min	Minute(s)
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar(s)
ng	Nanogram(s)
sec	Second(s)
U	Unit(s)
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar(s)
V	Volts
kV	Kilovolts
v/v	Volume per volume
W	Watt(s)
w/v	Weight per volume

Abstract

Wheat (*Triticum aestivum* L.) is an important winter and spring crop in South Africa with average dryland yields of 2.5 t/ha, while irrigated wheat yields 5 t/ha. *Diuraphis noxia* (Kurdjumov) commonly referred to as the Russian wheat aphid was recorded in 1978 to South Africa and has become a major pest in the wheat industry. It can cause over 80% yield loss in the summer rainfall region if not controlled. There are five described RWA biotypes in SA, namely RWASA1 to RWASA5. We investigated the genetic variation of 24 specimens which are representative of four South African RWA biotypes using three molecular techniques, AFLP, cDNA-AFLP and microsatellites.

With AFLP and cDNA-AFLP, we detected variation among specimens within a biotype. The cDNA-AFLP results increased the amount of the variation detected compared to the genomic AFLP results, but it was not sufficient to be linked to a specific biotype. With microsatellites, we were able to detect variation among biotypes and distinguish between them. We were unable to identify markers unique to a biotype. We found evidence that the genetic diversity may be linked to geographic location of the RWA, but further investigation is required to confirm this.

Keywords: *Diuraphis noxia*, wheat, AFLP, cDNA-AFLP, microsatellites, South Africa, genetic variation

Chapter 1

General Introduction

Wheat (*Triticum aestivum* L.) is one of the most cultivated crops in the world (Food and Agriculture Organization-FAO, 2020). It is grown on every continent apart from Antarctica, on an estimated 220.4 million hectares. According to the FAO, updated projections showed that global wheat production in 2020 will be 758.3 million tonnes (Food and Agriculture Organization-FAO, 2020). Wheat is considered a major source of plant protein for humans in the world, thus having a greater protein content than other important cereals (Community Research and Development Information Service, 2016).

All wheat types can be separated in two main groups, namely spring and winter wheat. Derived from the name, spring wheat is planted throughout spring and harvested during summer, whereas winter wheat is planted during autumn and harvested during spring. Winter wheat requires vernalisation to progress from vegetative state to a reproductive state. Wheat bears fruit in the form of kernels that consist of three parts: the outer layer called the bran, the nutritive called the endosperm and the germ, which is the embryo. Certain types of wheat can be as tall as 210 cm when fully grown, but cultivars used in industry are between 60 and 120 cm in length. Wheat is considered a healthy food source of numerous nutrients when consumed as whole grain and is included as fibre in the everyday diet of children and adults. For gluten sensitive people, wheat protein can trigger coeliac disease, a disease that impacts about 1% of the global population in developed countries.

Environmental factors such as unfavourable weather conditions, pests, and diseases negatively impact sustainable yields. Therefore, the continuation of wheat research contributes an important role to obtain new ways to improve wheat production and reduce the effects of pathogens and pests such as the Russian wheat aphid (RWA). RWA was first reported by Mordvilko in 1900 from barley fields in southern Russia (Jones *et al.,* 1989). This insect has affected cereal crops worldwide, including barley (*Hordeum vulgare* L.) and wheat (Miller *et al.,* 2001). Comparative studies across the native and introduced ranges of the RWA have been undertaken, but still have not clearly resolved the patterns or pathways of the worldwide distribution and spread of the RWA, primarily due to the limited polymorphic nature of the used markers (Starý, 1999; Liu *et al.,* 2010).

This is a common problem in distribution analysis: maternally inherited markers are ideal for tracking lineages, but there is often insufficient genetic variability in mitochondrial genes. Microsatellite marker similarities on the other hand have limited resolving power in species exhibiting obligate parthenogenesis, such as invasive populations of RWA.

To maintain the wheat population in South Africa (SA) is critical, both from an economic and food security viewpoint. Therefore, by limiting the production risk through better quality and more pest-resistant wheat cultivars will ensure that the local wheat producers will continue planting the crop in the future.

The first objective of this study was to obtain a clear understanding of the possible genetic variation within and between *Diuraphis noxia* biotypes in SA and how they are related to each other. The second objective was to identify a genetic marker to distinguish between the different biotypes. Through this project, it is believed that our research will give us insight on the development of the RWA population in SA that could aid with the effort to control RWA infestation.

Chapter 2

Literature Review

2.1. Wheat:

Wheat belongs to the genus *Triticum* (family Poaceae) and is one of the oldest and most important cereal crops worldwide. Wheat probably originated in the "cradle of civilization" in the Tigris and Euphrates river valley, near what is today known as Iraq. Its common name, cereal, was derived from the name of the Roman goddess, Ceres, who was believed to be the protector of the grain (National Association of Wheat Growers, 2019). The discovery of domesticated wheat grains in the archaeological remains of Ali Koshi in Iranian Khusistan (6 500 BC), as well as in Anatolia in Turkey (5 500 BC) (Morojele and Burger, 2009), confirmed its importance to early civilizations.

According to Morojele and Burger (2009), the most important members of the wheat genus are common wheat (*Triticum aestivum* L.) that is used for bread, durum wheat (*T. turgidum* L. ssp. *durum*) which is used in the production of pasta, and club wheat (*T. aestivum* ssp. *compactum*) which is a softer form used for cakes, crackers, cookies, pastries, and flours. Wheat is also used in the production of starch, glue, malt, dextrose, gluten and liquor, and for the production of biofuel.

Wheat cultivation initially spread from its origin to India, Pakistan and China in the east, to the Mediterranean region in the west and regions of the Union of Soviet Socialist Republics (USSR) in the north. As of 2020, the following are the top ten wheat producing regions/countries in the world: European Union, China, India, Russian Federation, United States of America (USA), Canada, Ukraine, Pakistan, Turkey and Argentina (Index Mundi, 2020). South Africa is the thirtieth largest producer of wheat.

The production of wheat is influenced by both abiotic (drought, heat etc.) and biotic (pathogens and pests) stress (Botha *et al.*, 2006). Abiotic stress extensively affects yield by decreasing seed size and number, which ultimately reduces seed quality (Sehgal *et al.*, 2018). It also causes a disturbance in germination and vegetative growth, reduces tiller production, and decreases grain filling (Britz *et al.*, 2007; Prasad *et al.*, 2011; Sehgal *et al.*, 2017).

Organisms such as viruses, bacteria, fungi, nematodes, and insects cause biotic stress. RWA infestation results in large-scale damage to wheat crops, followed by yield losses (Botha *et al.*, 2006). These losses could occur either directly (35-40%) when the aphid is feeding on the plants or indirectly (20-80%) when feeding aphids transmit viral and fungal diseases (Khan *et al.*, 2012).

2.2. Physical description of the Russian wheat aphid:

Diuraphis noxia (Mordvilko) (Hemiptera: Aphididae), also known as the RWA, is a relatively small light-green (except for the dark end of the antennae and legs) insect that ranges from 1.6 to 2.1 mm in length (Hodgson and Karren, 2008). Their bodies have an elongated cigar- or spindle-shaped form that appears wider in the middle while tapering off at each end (Stoetzel, 1987; Michaud and Sloderbeck, 2005). Most commonly distinguishable characteristics are shortened antennae (about one-quarter of the body length), reduced cornicles at the end of the abdomen and a piercing-sucking stylet on its head. When the RWA is observed from the side, it has a “double cauda” (Fig. 2.1), which looks like a double tail (Hodgson and Karren, 2008). Siphunculi, which are pores on the aphid’s stomach for extruding waxy defensive fluids, are absent in RWA but present on other cereal aphids (Aalbersberg *et al.*, 1987).

2.3. Global distribution:

The first published reference to *D. noxia* as a pest was in the Crimea (Mokrzhetsky, 1901 as quoted by Kovalev *et al.*, 1991). Random outbreaks of RWA occurred in the former USSR in 1901 (Halbert and Stoetzel, 1998). In 1962, the Konya Province in Turkey declared a RWA epidemic, where yield losses of 25-50% occurred (Elmali, 1998). From southern Russia, RWA spread to Spain in 1945 (Kazemi *et al.*, 2001). RWA was first found in central Mexico in 1980 (Puterka, 1992). In 1986, RWA was first reported in Texas and since then it spread westwards and northwards to the Pacific Coast and Canadian border, respectively (Merchant, 2014). In July 1988, RWA was first detected in the Canadian provinces of Alberta, British Columbia, and Saskatchewan (Morrison and Peairs, 1998). RWA was also reported in Chile in 1988 and Argentina in 1992 (Ortego and Delfino, 1994).

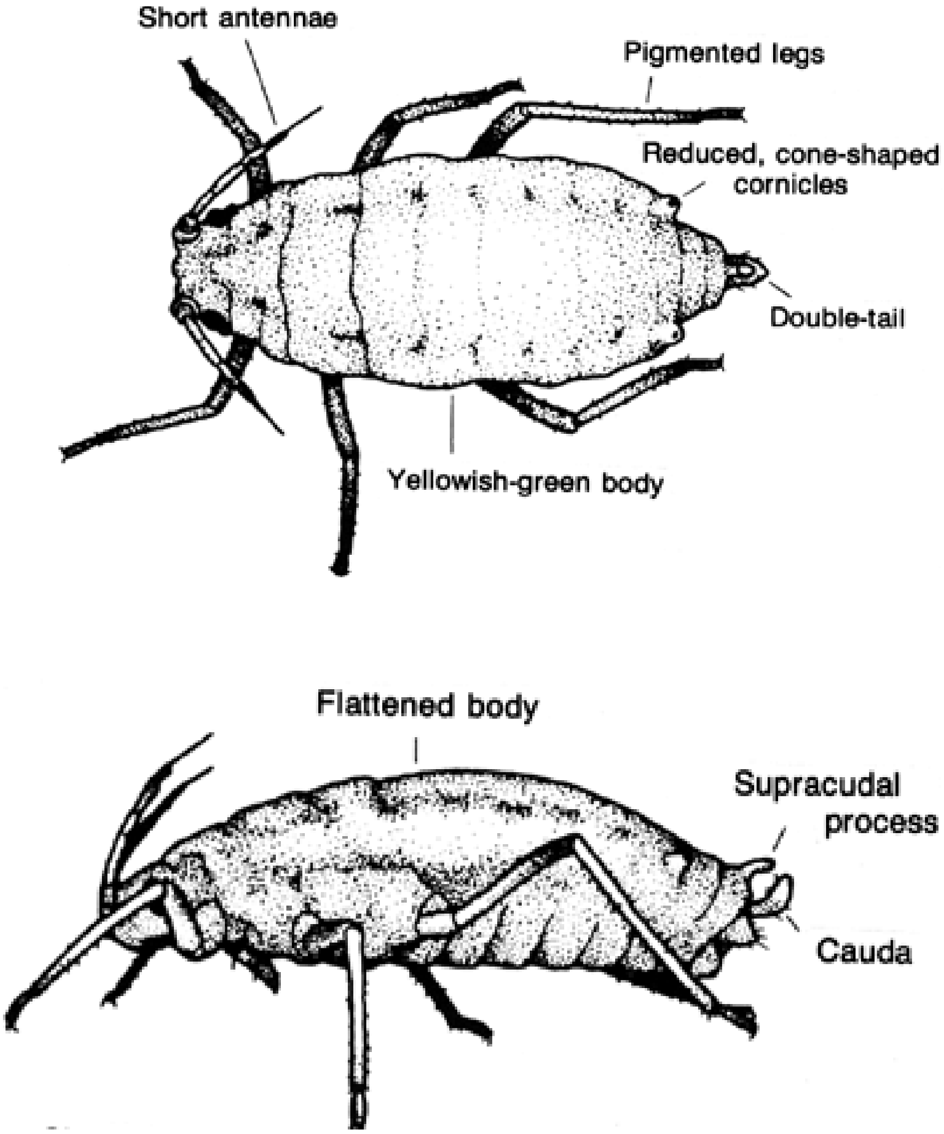

Figure 2.1. The top and side view of the Russian wheat aphid (Van Helden and Baker, 2017).

In Europe, the RWA has been recorded in Hungary (Basky, 1993), Serbia, Slovakia, Croatia, Romania, Austria, Portugal, Belgium, Bulgaria, Czech Republic, Poland, Turkey, Yugoslavia, and France (Zhang *et al.*, 2012).

The RWA was recently recorded in a wheat field near Tarlee, South Australian Mid North region (Grains Research and Development Corporation, 2017). It was also found in western Victoria at Kaniva, Nhill, Yaapeet and McKenzies Creek (Food and Agriculture Organization of the United States, 2016). The RWA was first reported in Africa in the Wukro and Adigrat regions of Ethiopia in 1972/73 and then in the western Wollo region in 1974 (Haile, 1981). RWA was first reported in South Africa in 1978 (Dürr, 1983) (Fig. 2.2).

2.4. Host plants:

The RWA is capable of feeding and surviving in a number of different habitats because they can tolerate a wide temperature range (Dolatti *et al.*, 2005). The host plants for RWA can be divided into either primary or secondary hosts (Armstrong *et al.*, 1991). The primary host supports the aphid's whole life cycle, including reproduction. The most common primary agricultural hosts are wheat and barley, but also include jointed goat grass (*Triticum cylindricum* Ces.) (Kindler and Springer, 1991). As for the secondary hosts, these are plants that allow the aphid to survive but not reproduce. These include oat, wild rye, triticale (*T. aestivum x Secale cereale* L.) and tall wheatgrass (Michaud and Sloderbeck, 2005). Complete lists of primary and secondary hosts are provided in Tables 2.1 and 2.2.

2.5. Natural enemies:

All insects have natural enemies, which limit their population growth. The RWA has a number of natural enemies and predators (Table 2.3) (Knutson *et al.*, 1997). These include the convergent lady beetle (*Hippodamia convergens* Guérin-Méneville), seven-spotted lady beetle (*Coccinella septempuctata* L.) and the Scymnus beetle (*Scymnus auritus* Thunberg) (Adisu *et al.,* 2003). The *Aphelinus hordei* female wasp lays her eggs inside the RWA. When the eggs hatch, the larvae feed on the aphid from the inside (Gonzaléz *et al.*, 1992). To escape their enemies, RWAs hide in the rolled leaves of host plants, making it difficult for larger predators to reach them. However, some of the natural predators are small enough to crawl into the rolled leaves to feed on the aphids.

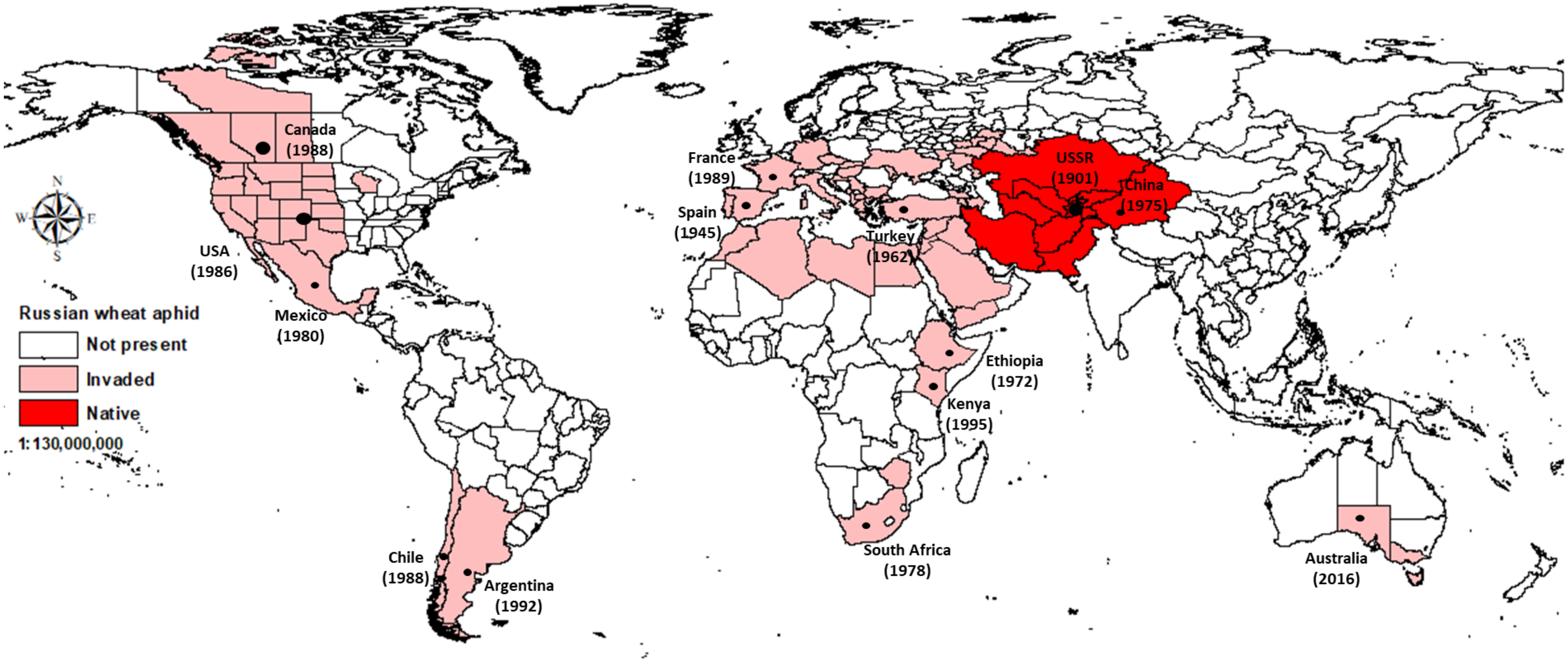

Figure 2.2. The global distribution of the Russian wheat aphid according to the year of first detection for selected countries. Russian wheat aphid was first found in the Ukraine and then spread to other parts of the world (Biosecurity New Zealand, 2020).

Table 2.1. Primary host plants of Russian wheat aphid.

Scientific name	**Common name**	**Reference**
Hordeum vulgare L.	Barley	Stoetzel (1987)
Triticum aestivum L.	Wheat	Stoetzel (1987)
Triticum turgidum L. ssp. durum	Durum wheat	Hughes (1988)
Triticum cylindricum Ces.	Jointed goatgrass	Kindler and Springer (1989)
Bromus hordeaceus L.	Blando bromegrass	Kindler and Springer (1989)
Vulpia myuros (L.) C.C.Gmel.	Rattail fescue	Kindler and Springer (1989)
Leymus arenarius (L.) Hochst.	European dunegrass	Kindler and Springer (1989)
Elymus canadensis L.	Canada wildrye	Armstrong *et al.* (1991)
Hordeum pusillum Nutt.	Little barley	Kindler and Springer (1989)
Bromus arvensis L.	Field bromegrass	Kindler and Springer (1989)
Agropyron intermedium (Host) P.Beauv.	Intermediate wheatgrass	Kindler and Springer (1989)
Agropyron cristatum (L.) Gaertn.	Creased wheatgrass	Armstrong *et al.* (1991)

Table 2.2. Secondary host plants of Russian wheat aphid.

Scientific name	**Common name**	**Reference**
Avena sativa L.	Oat	Kindler and Springer (1989)
Secale cereale L.	Rye	Kindler and Springer (1989)
Agropyron elongatum (Host) P.Beauv.	Tall wheatgrass	Kindler and Springer (1989)
Elymus triticoides Buckley	Beardless wildrye	Kindler and Springer (1989)
Oryzopsis hymenoides (Roem. & Shultz) Ricker	Indian ricegrass	Kindler and Springer (1989)
Cynodon dactylon (L.) Pers.	Bermuda grass	Kindler and Springer (1989)
Phalaris canariensis L.	Canary grass	Stoetzel (1987)
Phleum pratense L.	Timothy grass	Stoetzel (1987)
Hordeum murinum L. ssp. *murinum*	Wall barley	Stoetzel (1987)
Bromus madritensis L.	Spanish brome	Stoetzel (1987)
Oryza sativa L.	Rice	Stoetzel (1987)
Avena fatua L	Wild oats	Jankielsohn (2013)
Bromus catharticus L	Rescue grass	Jankielsohn (2013)

Table 2.3. Natural enemies of *Diuraphis noxia* in South Africa.

Scientific name	**Type**	**Distribution**	**Reference**
Adalia bipunctata	Predator	South Africa	Aalbersberg *et al.* (1988)
Aphelinus asychis	Parasite	South Africa	Aalbersberg *et al.* (1988)
Cheilomenes lunata	Predator	South Africa	Carver (1989)
Cheilomenes propinqua	Predator	South Africa	Aalbersberg *et al.* (1988)
Diaeretiella rapae	Parasite	South Africa	Aalbersberg *et al.* (1988)
Eupeodes corrolae	Predator	South Africa	Carl (1989)
Exochomus concavus	Predator	South Africa	Aalbersberg *et al.* (1988)
Hippodamia convergens	Predator	South Africa	Carl (1989)
Lioadalia flavomaculata	Predator	South Africa	Aalbersberg *et al.* (1988)
Scymnus morelleti	Predator	South Africa	Aalbersberg *et al.* (1988)

2.6. Communication:

The RWA communicates by producing an alarm pheromone called (*E*)-β-farnesene (Hardie *et al.*, 1999). They release droplets of the pheromone from the cornicles that are located at the end of their abdomen, which produce an odour repellent to other aphids (Fig. 2.3). This is a protection mechanism to warn the colony against predators. When the pheromone is released, the aphids respond by removing their stylets from the leaf and falling off the host plant to escape (Becker, 2000).

2.7. Lifespan of the Russian wheat aphid and optimal temperature for surviving:

Temperature appears to be a dominant factor influencing reproductive and developmental rates of RWA (Aalbersberg *et al.*, 1987). The optimal temperature range for reproduction is between 5 and 30°C. If the temperature falls below 25°C, the aphids mature at a slower rate but above 25°C, they mature at a much faster rate (Merchant, 2014). Many other variables such as the host plant's growth stage (Girma *et al.*, 1990), host quality (Merrill *et al.*, 2008), aphid biotype (Randolph *et al.*, 2008), population density (Michaud *et al.*, 2006), and feeding site on the plant (Starý, 1999), play important roles in the reproduction of the RWA.

2.8. Life cycle, reproduction, and development of the Russian wheat aphid:

The RWA has a complicated life cycle. With environmental changes in temperature, day-length or the quality of available nutrients, the life cycle of the RWA adapts to become more complex (Dixon, 1998). The RWA has the capability to reproduce both sexually and asexually (holocyclic) or just asexually (anholocyclic) (Tolmay, 2006).

In most holocyclic species, there are anholocyclic clones that are parthenogenic, which means that the female gamete develops into a new individual without fertilization by a male gamete. The RWA reproduces holocyclic in countries such as Hungary, Spain, and Russia (Basky and Jordaan, 1997). There are also designated intermediate clones that produce sexual individuals that over-winter parthenogenically (Llewellyn *et al.*, 2003). The asexually reproducing RWA populations consist of only females, where the adults give birth to live identical nymphs (Gustafson *et al.*, 2009).

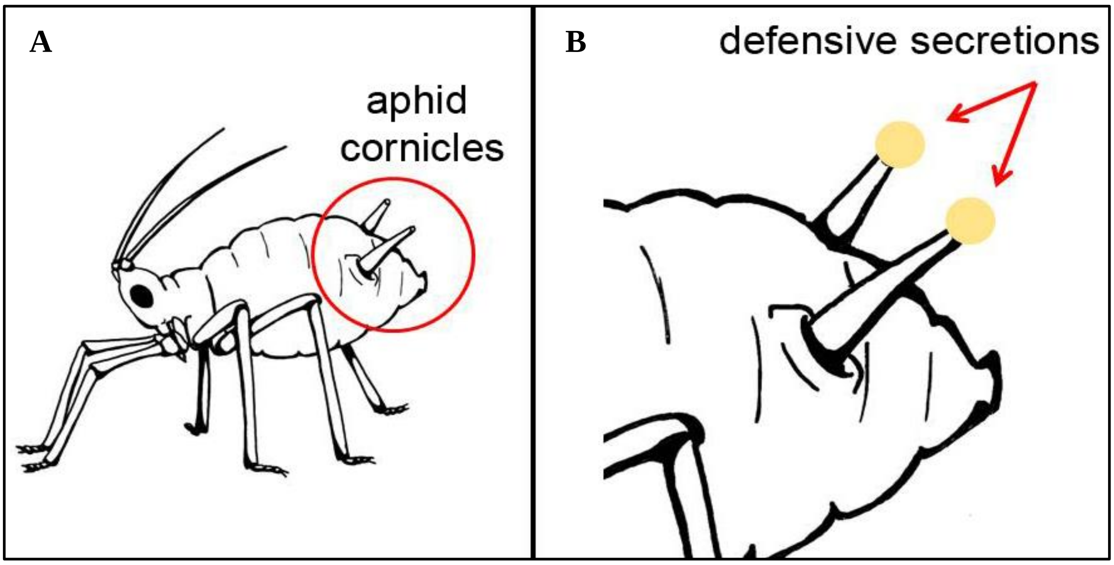

Figure 2.3. Illustrated in A) is the placement of the Russian wheat aphid cornicles, while B) shows the release of pheromone droplets from the two cornicles (The Bug Chicks, 2020).

These young nymphs are similar to the adults, except that they are covered with a white waxy exudate.

After multiple moultings, the nymphs develop into either wingless (apterous) or winged (alate) adults (Harvey and Kofoid, 1993) (Fig. 2.4). Wingless aphids are more abundant because they have a higher reproductive capability and can produce 4-5 nymphs per day for a 3-4-week period. The overall maturation time is between seven to ten days. During winter, reproduction continues, but at a reduced rate where the aphids remain active and continue feeding (Michaud, 2010).

In the case of sexual reproduction, the RWA is holocyclic and thus reproduce both sexually (eggs that overwinter) and asexually (generally in the warmer months). After fertilization, females lay 8-10 eggs and die after a few days. In early spring, the eggs hatch and the population increase through parthenogenesis (Puterka *et al.*, 1993). As a result of genetic recombination during sexual reproduction, new biotypes can be produced that are well adapted for survival on previously resistant crops. Sexual reproduction allows the aphids to overwinter in the egg stage, whereas in the asexual reproductive stage, the aphids need host plants to continue feeding during winter (Tolmay, 2006).

2.9. Alate Russian wheat aphids (flight behaviour):

The main role for winged aphids is to fly to plants in new areas where they can establish new colonies. They feed for numerous days on the plants, before reproduction starts (Michaud and Sloderbeck, 2005). Winged aphids produce very few daughters, because they are not known to procreate and therefore have small reproductive organs. Because they are poor fliers, they most likely rely on wind currents to travel to new habitats (Hodgson and Karren, 2008; Merchant, 2014). When flying, they only respond to the green or yellow-green colour of young plants, since they choose only young and healthy plants to start a new colony upon (Michaud and Sloderbeck, 2005). They have significant wing muscles and fat bodies to store more energy for flights.

Figure 2.4. Winged Russian wheat aphid (Source image: Edgar Schliephake) (Schliephake, 2013).

2.10. Feeding method:

Phloem-feeding insects provide extra challenges to plants as they reduce photosynthates, introduce viruses and chemical and/or protein effectors that alter the plant defence signalling, induce infestation symptoms, and reduce plant growth and development (Kaloshian and Walling, 2005). Knowledge of the salivary gland and its secreted substances is critical to understand aphid-host plant interactions (Madhusudhan and Miles, 1998; Burd, 2002).

The RWA uses a piercing-sucking stylet, which is inserted between the mesophyll cells, to absorb phloem sap from the plant (Burd, 2002) (Fig. 2.5).

As RWA establishes feeding sites, it discharges two types of saliva: the salivary sheath (gel) and soluble (watery) saliva (Cooper *et al.*, 2010). The roles of both the stylet sheath and watery saliva is to suppress the plant defence responses (Miles, 1999).

The salivary sheath contains 1,4-glucosidases that hydrolyse phenolic glycosides (Cooper *et al.*, 2010). The watery saliva induces changes in the physiology of the plant that benefit aphid feeding and nutrition, while also affecting the plant epidermal cells, causing white and yellow streaks on leaves, as well as leaf rolling (Will *et al.*, 2007). The watery saliva contains several enzymes, which establish and maintain the feeding sites. Included are pectinases, cellulases, amylases, oxidases, phenolic glycosides, glucose dehydrogenase, and enzymes that hydrolyse sucrose (Miles, 1999; Harmel *et al.*, 2008). Proteins have been discovered in the body and saliva of aphids that may act as effectors, which affect the plant's metabolism (Harmel *et al.*, 2008).

Effectors in the saliva of the green peach aphid *Myzus persicae*, suppress defence responses in *Arabidopsis thaliana* (Pitino and Hogenhout, 2013). The Mp10 effector from *Myzus persicae* suppresses basal immunity pathways in *Nicotiana benthamiana* and triggers a suppressor of the G-2 allele of SKP1-CUL1-F-box protein-dependent chlorosis response. According to a study by Elzinga *et al.* (2014), specific *Myzus persicae* (Sulzer) effector proteins stimulate aphid fertility. Salivary proteins Mp56, Mp57 and Mp58 decrease aphid reproduction, by activating the plant defence responses, while MpC002 and Mp55 increase aphid fertility.

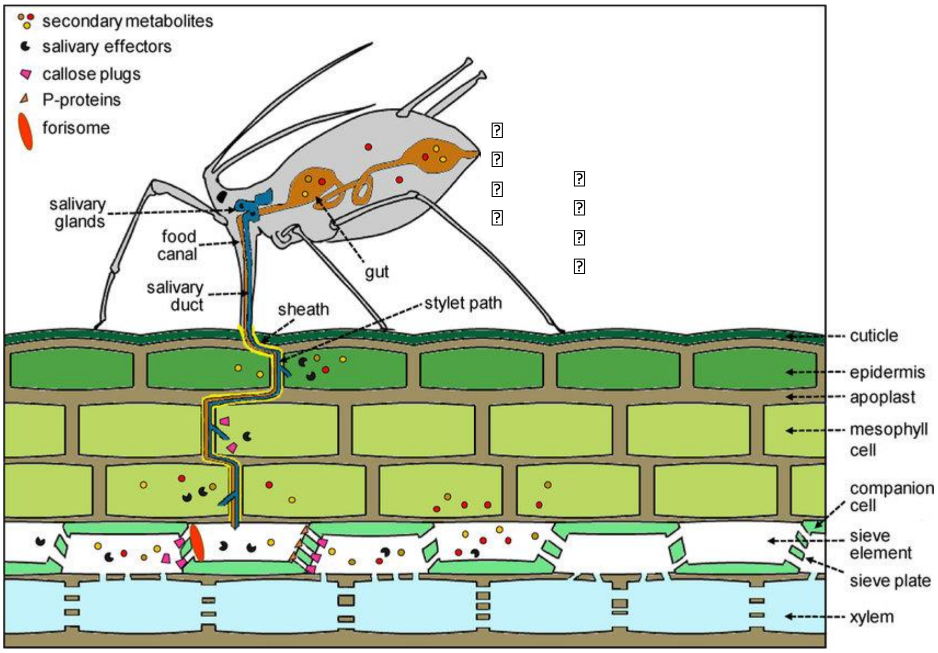

Figure 2.5. A diagram indicating how aphids feeds on plants (Nalam *et al.*, 2019).

Nicholson *et al.* (2012) did a study on the proteomic analysis of secreted salivary proteins of RWA biotypes in the USA. Proteins that were distinctive to the RWA salivary proteome varied among biotypes and deviated from the well-known salivary proteomes of non-phytotoxic aphids. RWA2USA saliva contained protein effectors or phytotoxins that were not present in the saliva of other biotypes, while important masking proteins were not present in RWA5USA. This could play a major role in the gene-for-gene resistance in aphid-plant interactions.

According to Mohase and van der Westhuizen (2002), glycoprotein elicitors occurred in the intercellular wash fluids of RWASA1-infested resistant wheat. These glycoproteins induced different defence responses in resistant and susceptible plants. Mohase and Taiwe (2015) showed that elicitors from RWASA1, but not RWASA2 saliva, induced high levels of defence responses in the *Dn1*-containing wheat cultivar. The reason for this could be that RWASA2 saliva contained effectors that suppress plant defences within the Tugela *Dn1* wheat cultivar.

2.11. Plant damage and symptoms:

The symptoms of RWA infestation are very unique. The RWAs feed on the upper surface of young leaves (Walters *et al.*, 1980). Discoloration of the leaves is caused by a drastic reduction in chlorophyll content when the chloroplast membranes are destroyed (Fouché *et al.*, 1984). Once the plant loses nutrients, leaf rolling occurs that protects the aphid colony from danger (Hein *et al.*, 1989) (Fig. 2.6). Leaf rolling traps the flag leaf and prevents the head from developing properly. Undeveloped heads have a “fish-hook” appearance, which implies that 30-50% of the head may not fill properly because of poor pollination (Burd *et al.*, 1998) (Fig. 2.7).

Other characteristics of RWA infestation are typical white, yellow, and purple to reddish-purple longitudinal streaks on the leaves (Pike, 1991) (Fig. 2.8). Eventually, RWA infestation can cause plant death or yield reduction (Hein, 1992).

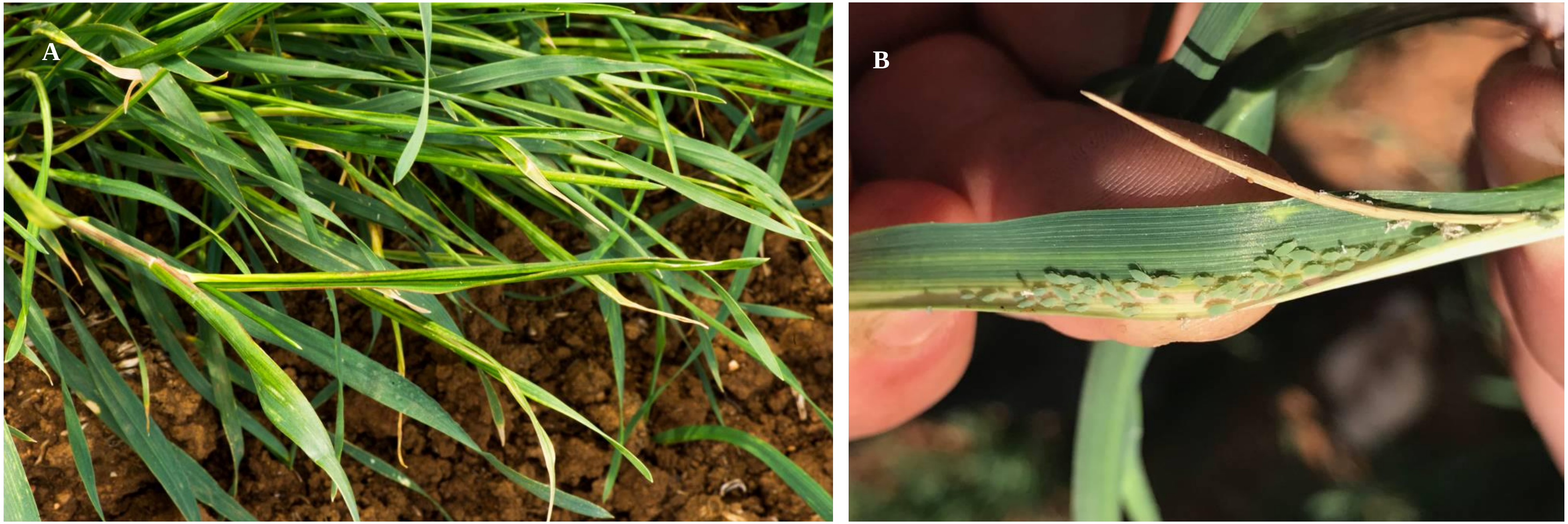

Figure 2.6. A) illustrates rolled leaves that are caused by Russian wheat aphid infestation (Source image: MA Nash) (Perry and Kimber, 2016), while B) is where Russian wheat aphid colonies can be found inside the rolled leaf (Source image: Ali Kuchel) (Kuchel, 2017).

Figure 2.7. A) Normal wheat heads compared to B) “fish hook” deformation caused by Russian wheat aphid feeding (Njuguna *et al.*, 2016).

Figure 2.8. Typical white and purple to reddish-purple longitudinal streaks of Russian wheat aphid infested wheat. The streaks on the leaf are a result of heavy infestation of Russian wheat aphid (Photo by E. Bynum) (Bynum, 2015).

2.12. Control strategies for the Russian wheat aphid:

2.12.1. Cultural control:

Cultural control is a technique to modify the growing environment to reduce the occurrence of unwanted pests. This could include changes in planting date, furrow orientation, irrigation, fertilization, and manipulation of crop residue (Walker and Peairs, 1994). To reduce late RWA infestation, the planting date of the crop should be earlier with 1-2 weeks (Halbert *et al.*, 1990). Running irrigation furrows, in the northern hemisphere, from north to south decreases RWA infestation in the winter compared to east to west oriented furrows. This is due of the reduced solar insolation (Hammon and Peairs, 1992).

Another control method is crop rotation where wheat alternates with a summer crop such as cotton (*Gossypium hirsutum* L.). This method can prevent RWA populations from surviving on wheat fields. Since RWA must survive the period between harvest of the current season and planting of the next, management of volunteer plants in and around the fields such as a rescue grass, false barley and wild oats can help to control infestation. This requires the ploughing under of all crop residues and control of non-crop plants with herbicides (Kindler *et al.*, 1991).

Venter *et al.* (2014) showed that plants treated with potassium phosphate delayed RWA development and decreased numbers of aphids. This study indicated that potassium phosphate has a negative effect on the aphid population, which decreased the effect of aphid feeding on wheat. Potassium phosphate induced higher levels of phenylalanine ammonia lyase and lipoxygenase enzyme activities in the plants. In another study by Mitchell and Walters (2004), potassium phosphate treatment on barley increased activities of peroxidase, lipoxygenase, and phenylalanine ammonia lyase.

2.12.2. Chemical control:

Chemical insecticides can control the RWA, but this method is problematic since it provides insufficient penetration of pesticides to aphids living in rolled up leaves (Hayes, 1998). Therefore, systemic insecticides are required, with the active ingredients, disulfuton, dimethoate or demeton-S-methyl. Vapour-action insecticides with active ingredients chlorpyriphos or parathion, are also effective against RWA (Nel *et al.*, 2002).

Variation in RWA susceptibility to insecticides containing chlorpyrifos implies that there is a greater possibility that RWA may develop resistance against them (Brewer and Kaltenbach, 1995).

2.12.3. Biological control:

This control strategy uses natural enemies of RWA, such as hoverflies, parasitoids, lacewings, and ladybirds to manage infestation (Carver, 1989; Bernal *et al.*, 1993; Hughes *et al.*, 1994; Lee *et al.*, 2005). One advantage of this approach is that when the predators are present on the plants, they will attack the RWA as soon as they appear, without any further input required from the farmer.

2.13. Glasshouse screening process of the phenotypic response of Russian wheat aphid against wheat resistance genes:

A general screening process to identify virulence of RWA against deployed resistance genes includes the following steps (Lindeque, 2008):

Step 1: Precondition aphids before infestation of test plants. Preconditioning refers to the similar rearing of all aphid colonies on susceptible host plants (Schotzko and Smith, 1991) (Fig. 2.9).

Step 2: Infestation and evaluation of wheat seedlings. Seedling evaluation produces a consistent response and is the preferred growth stage for mass evaluation (Robinson, 1992; Porter *et al.*, 1993). The evaluation of resistance levels is done 2-4 weeks after infestation.

Step 3: Score seedlings for resistance. Simple scoring systems are used to determine resistance. In some instances, researchers have applied independent scoring to separate the symptoms of infestation based on leaf rolling, chlorosis (Formusoh *et al*., 1992) (Fig. 2.10). A score of 1-9 or 1-6 would be given based on the observed plant symptoms. Scale of screening = 1-3: Resistant (R); 4-6.5: Medium Resistant (MR); 6.5-10: Susceptible (S) (Tolmay et al., 1999). According to Miller *et al.* (1994) chlorosis was the visual symptom of RWA damage, which had the best correspondence to physiological measures of susceptibility (Lindeque, 2008).

Figure 2.9. Screening method of the phenotypic response of Russian wheat aphid against wheat resistance genes. A) Clonal colonies of Russian wheat aphid biotypes and B) rearing and seedling evaluation for identification of Russian wheat aphid resistance (Richter, 2011).

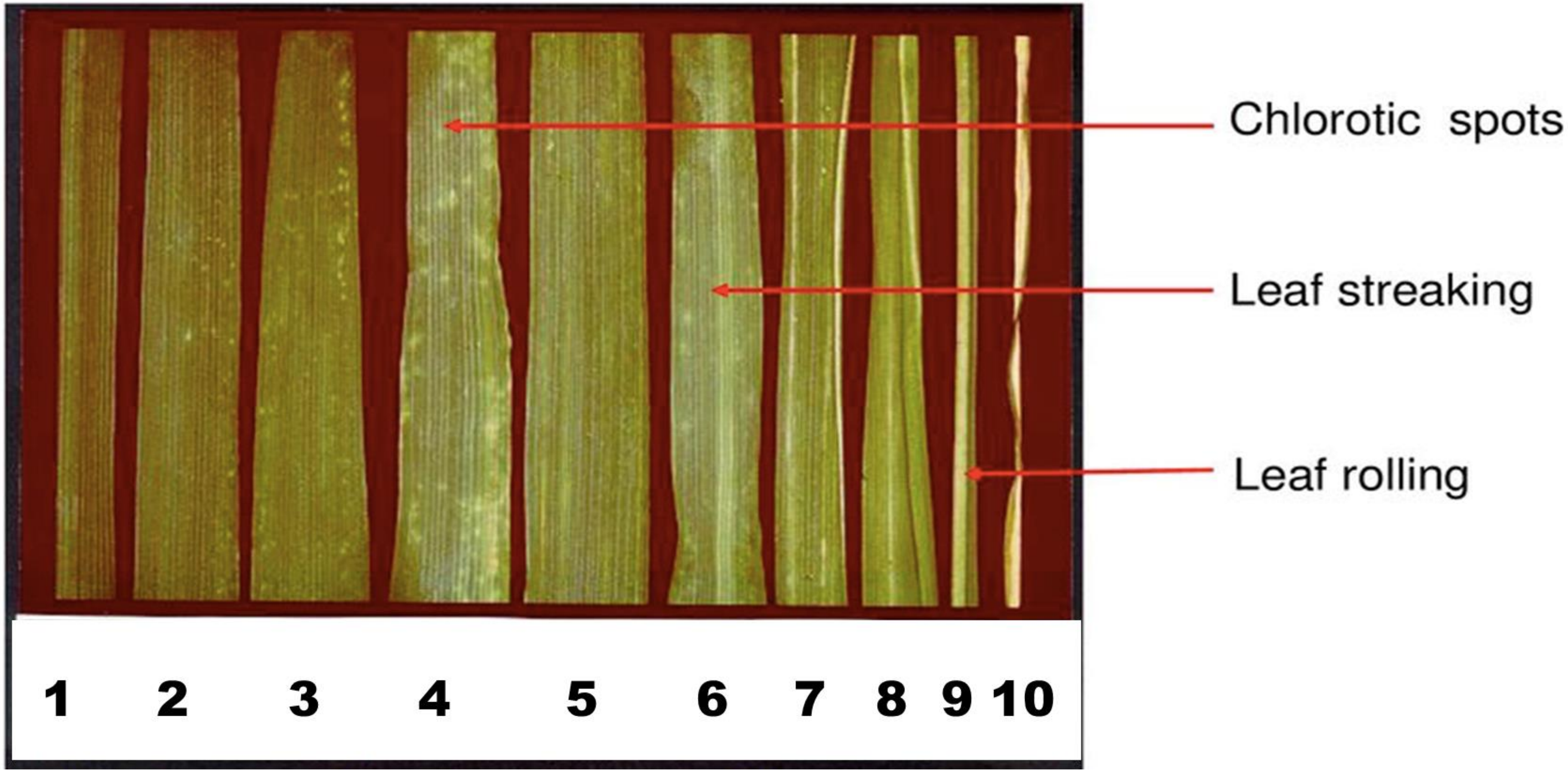

Figure 2.10. Russian wheat aphid symptoms on wheat leaves used for screening. 1-4: leaf chlorosis, 5-6: striping on leaves and 7-10: leaf rolling.

Scale of screening = 1-3: Resistant (R); 4-6.5: Medium Resistant (MR); 6.5-10: Susceptible (S) (Tolmay *et al.*, 1999).

2.14. Global genetic studies done on Russian wheat aphid:

Modern genetic markers such as enzyme variants (allozymes), microsatellites, Amplified Fragment Length Polymorphisms (AFLP) and Randomly Amplified Polymorphic DNA (RAPD) have been used in genetic studies of invasive species (Zhang, 2012).

Most RWA research in previous years have concentrated on discovering resistance genes in wheat and barley cultivars rather than documenting the biology and population genetics of the RWA itself (Zhang, 2012). There has been a limited number of studies done on the population genetics of RWA and very little is known about RWA in its native range (Shufran *et al.* 2007; Shufran and Payton 2009).

Puterka *et al.* (1992) stated that the absence of regional genetic variation in *Myzus persicae* and *Acyrithosiphon pisum* aphid populations was due to directional selection acting on parthenogenetic clones (Wool *et al.*, 1978; Simon *et al.*, 1982; Blackman, 1985). According to Puterka *et al.* (1993), diversity in allozyme and RAPD-PCR profiles found in RWA populations between the eastern and north-eastern Mediterranean regions was due to the holocyclic life cycle of RWA in these regions.

The RWA populations in the Ukraine are also holocyclic (Kiriac *et al.*, 1990) and because of this, they have two distinct allelomorphs. Puterka *et al.* (1993) however had no explanation for the lack of genetic diversity found within these RWA populations.

They stated that the presence of distinct biotypes within the same allelomorphs indicated that biotypes are new developments in the evolutionary history of RWA.

The reason for the absence of similarity between geographical and genetic distances in USA biotypes is due to random formation of clones through commerce rather than through migration (Puterka *et al.*, 1992). In a study conducted in the USA, a similar pattern was seen in the mosquito *Aedes albopictus* (Black *et al.*, 1988). It was found that the variation in local populations of *Aedes albopictus* in the USA was not related to recent colonization but rather due to the natural breeding structure of the species.

Gong *et al.* (2012) identified variation in methylation levels of four putative salivary-gland effector genes transcribed within two USA RWA biotypes. This was possible evidence that methylation could be a contributing factor to increased RWA virulence between the biotypes.

2.15. The Russian wheat aphid in South Africa:

As one of the most invasive agricultural species in the world, RWA has a widespread impact on the ecosystems they invade. Since its introduction in 1978, the RWA has become the major pest of wheat in SA, where it can cause over 80% yield loss in the summer rainfall region if not controlled (Du Toit and Walters, 1984; Jankielsohn, 2014). It was reported that the RWA in SA is anholocyclic (Tolmay, 2006). Because of the mild climate in SA, the RWA multiples asexually, with the sexual cycle being absent. This means that the populations are essentially clonal with very little genetic differentiation. Therefore, the different biotypes should have very little genetic differences between them.

2.15.1. Wheat production in South Africa:

Upon his arrival in the Western Cape in 1652, Jan van Riebeeck introduced wheat to SA (Morojele and Burger, 2009). Other regions in the Western Cape and Free State (FS) provinces adopted this crop. In SA, winter wheat is currently produced in the central and eastern FS and Western Cape, whereas spring wheat is produced in the Northern Cape. About 75% of wheat in SA is produced under dryland conditions and 25% under irrigation. The planting dates for wheat vary depending on the cultivar and area. In the western FS, winter wheat is normally planted during April/May, in the eastern FS during June/July and in the Western Cape during March/April. This all depends on weather conditions during the specific season. Spring and facultative wheat is mostly planted during September/October. Winter irrigation wheat is planted in the western FS and some areas in the eastern FS. Dry land wheat generally yields on average 2.5 t/ha in SA, while irrigation wheat yields 5 t/ha.

2.15.2. Russian wheat aphid distribution in South Africa:

It was thought that RWA was distributed by wind and humans from the Mediterranean region across mid-Africa to SA (Walters, 1984). In 1978, the RWA was first reported in the Orange Free State in SA (Dürr, 1983). The distribution was limited to the Bethlehem area during 1978, but in 1979 spread to other wheat producing areas in the country (Walters *et al.*, 1980). The current distribution of RWA in SA is in the Western Cape, Northern Cape, eastern FS (most problematic), western FS, and central FS (Fig. 2.11).

Figure 2.11. The current distribution of Russian wheat aphid biotypes in South Africa (A. Jankielsohn, *personal communication*).

2.15.3. Host-plant resistance against Russian wheat aphid:

Host-plant resistance is a useful control strategy against RWA infestation in SA (Van Niekerk, 2001; Tolmay and Van Deventer, 2005). This method is cost effective and environmentally sustainable (Marasas, 1999).

One of the most important advantages of host-plant resistance is the fact that it occurs independently of the management ability and resource level of the producer (Tolmay, 2001). The RWA resistant cultivars released for commercial use, had a yield advantage above susceptible cultivars in farmers' fields (Smit *et al.*, 2010).

Up to 2006, 27 RWA resistant wheat cultivars were released in SA. These cultivars were, Caledon, Elands, Gariep, Komati, Limpopo, Matlabas, Nossob, PAN 3235, PAN 3364, SST 124, SST 322, SST 333, SST 334, SST 347, SST 363, SST 367, SST 399, SST 935, SST 936, SST 946, SST 966, SST 972, SST 983, Tarka and Tugela-*Dn* (Tolmay, 2006).

The following wheat lines CO 03797, CO 03804, CO 03811, Yumar, CO 9500043, CO 960223, 94M370, Karee-*Dn*8, Betta-*Dn*9, PI586955 and Stanton can be used by breeders as new sources of resistance to RWA (Jankielsohn, 2017). According to the "Production of small grains in winter rainfall region 2020", the following is the newest resistant wheat cultivars on the market, namely Snel, US2014, AgBeacon, US2007 and Ibis (Nienaber and Killian, 2020).

Crop resistance against RWA biotypes raises a big concern because the virulence of RWA appears to differ within and between geographical regions (Bush *et al.*, 1989; Puterka *et al.*, 1992; 1993). Therefore, new biotypes may appear that can overcome the defences of resistant cultivars that carry the *Dn*-resistance genes used to control outbreaks (Botha and Venter, 2000).

2.15.4. Different Russian wheat aphid biotypes in South Africa:

An aphid biotype is described as a population of aphids that can damage wheat by overcoming the resistance gene carried within a previously resistant cultivar (Burd and Porter, 2006). Since the RWA develops new biotypes to overcome deployed resistance genes, it threatens the durability of plant resistance (Burd and Porter, 2006).

At present, there are five described RWA biotypes in SA (Table 2.4), namely RWASA1 to RWASA5 (Jankielsohn, 2019). RWASA1 described by Du Toit (1989) was the first biotype that was established in 1978. It was virulent against the *Dn*3 resistance gene. The RWASA2 was first noted in 2005 in the wheat producing areas of the eastern FS and was virulent against the *Dn*1, *Dn*2, *Dn*3, *Dn*8, *Dn*9, and *Dny*2006 resistance genes (Jankielsohn, 2014). The third biotype RWASA3 found in 2009, was virulent against the *Dn*1, *Dn*2, *Dn*3, *Dn*4, *Dn*8, *Dn*9, and *Dny*2006 resistance genes. In 2011, RWASA4 was discovered in Bethlehem in the eastern FS. This biotype was virulent against the *Dn*1, *Dn*2, *Dn*3, *Dn*4, *Dn*5, *Dn*8, *Dn*9, and *Dny*2006 resistance genes (Jankielsohn, 2014).

According to Jankielsohn (2019), RWASA5 was discovered in SA in 2019 and is currently the most virulent recorded biotype with virulence against the *Dn*1, *Dn*2, *Dn*3, *Dn*4, *Dn*5, *Dn*8, *Dn*9, *Dnx*2006, and *Dny*2006 resistance genes. It has only been noted in the eastern FS.

2.15.5. Genetic studies done on Russian wheat aphid in South Africa:

Genome sequencing of RWASA1 and the South African RWA biotype SAM, a highly virulent mutant biotype, which is strictly quarantined in the laboratory, was published in 2017 (Burger and Botha, 2017). Both genomes were sequenced to determine the genomic factors liable for biotypification. In addition, a pooled sample containing geographically separated RWA populations (MixIX) was included to determine the possibility of heterogeneity of the whole species. A comparison of the genic complements between RWA biotypes SAM and RWASA1 illustrated no differences. Both biotypes had similar genome sizes, utilizing genome characteristics estimation and *k*-mer counting.

The low variation across the genomes of the RWASA1 and SAM biotypes was similar to previous studies (Puterka *et al.*, 1993; Swanevelder *et al.*, 2010), which explained the limited genetic variation between the different biotypes (Burger and Botha, 2017).

Buchnera aphidicola Munson et al. 1991, sp. *nov* is gammaproteobacterium that has a symbiotic association with aphids. They play essential roles in the aphid’s metabolism and development. A study done by Swanevelder *et al.* (2010) suggested that a specific CCC trinucleotide insertion on the leucine plasmid of *B. aphidicola* varied between the different RWASA biotypes. This trinucleotide is located upstream of the *leuA* gene in a reversed repeat region of the SA biotypes.

Table 2.4. The five different Russian wheat aphid biotypes' phenotypic response against wheat resistance genes (Jankielsohn, 2019).

Resistance gene	**RWA Biotype**				
	RWASA1	RWASA2	RWASA3	RWASA4	RWASA5
*Dn*1	Avr	Vir	Vir	Vir	Vir
*Dn*2	Avr	Vir	Vir	Vir	Vir
*Dn*3	Vir	Vir	Vir	Vir	Vir
*Dn*4	Avr	Avr	Vir	Vir	Vir
*Dn*5	Avr	Avr	Avr	Vir	Vir
*Dn*7	Avr	Avr	Avr	Avr	Avr
*Dn*8	Avr	Vir	Vir	Vir	Vir
*Dn*9	Avr	Vir	Vir	Vir	Vir
*Dnx*2006	Avr	Avr	Avr	Avr	Vir
*Dny*2006	Avr	Vir	Vir	Vir	Vir

Avirulent (Avr) and Virulent (Vir)

According to Sydenham and Tolmay (2017), methylation sensitive molecular techniques can be used to identify different methylation patterns in the DNA of RWA. This method could play an important role to identify biomarkers for RWA biotypes around the world, which could ultimately lead to a potential identification tool to track RWA biotype introductions. Presently the identification of different biotypes depends phenotypic screening and so far, no notable molecular results were obtained to distinguish between South African RWA biotypes.

Chapter 3

Materials and Methods

3.1 Materials

Dr. A. Jankielsohn of the Agricultural Research Council-Small Grain (ARC-SG), Bethlehem, provided twenty-four Russian wheat aphid clone specimens representative of four South African RWA biotypes (Table 3.1). Four specimens from Lesotho was included as they occur close to a major wheat production area and they may influence the South African population. Three control aphid specimens (*Sitobion avenae* Fabr., *Rhopalosiphum padi* L. and *Metopolophium dirhodum* Walk.) were provided by Dr. G. Prinsloo of the ARC-SG.

Seed of RWA susceptible wheat cultivar SST387 used to multiply the RWA specimens, was provided by Dr. L. Mohase of the University of the Free State.

3.2 Methods

3.2.1. Propagation of Russian wheat aphid (*Diuraphis noxia*):

SST387 seed was germinated for 24 h at 18°C in the dark on Advantec Grade No. 5A filter paper soaked with distilled water in 9 cm petri dishes. Twenty Germinated seeds were planted in 18 cm pots containing soil (red soil mixed with peat in a 1:1 ratio) and grown under greenhouse conditions maintained at 24°C day and 19°C night temperatures using natural light. The seedlings were watered daily. From Zadoks 12 (two-leaf stage) (Zadoks *et al*., 1974), the plants were fertilized with Efekto Multifeed® Classic 19:18:16 (43) fertilizer, was applied by weekly as per manufacturer's instruction. After 10 days of planting (Zadoks 21), the plants were infested with approximately ten RWA that were placed on each plant with a soft paintbrush. A population of each colony was kept in separate isolation net cages to avoid cross-contamination.

Table 3.1. Aphid specimens used in the current study.

Biotype	**Colony codes**	**ARC-SG clone colony code**	**Date sampled**	**Locality**	**Coordinates**	**Host plant**
1	RWA1.1	3	30/10/2012	Florisbad	S28° 2' 48.066" E26° 2' 5.792"	Irrigation wheat
1	RWA1.2	13	16/09/2015	Vaalharts	S27° 51.325' E24° 48.888'	Irrigation barley
1	RWA1.3	24	28/06/2016	Clanwilliam	S31° 59' 2.89" E19° 14' 58.37"	Dryland wheat
1	RWA1.4	26	9/06/2016	Prieska	S29° 29' 46.752" E23° 14' 59.027"	Irrigation wheat
1	RWA1.5	6	30/08/2017	Heidelberg	S34° 14' 59.028" E20° 42' 24.228"	Dryland wheat
1	RWA1.6	7	Unknown	Lesotho	S29.54977°E28.16147°	Dryland Barley
2	RWA2.1	1	10/09/2012	Fouriesburg	S28° 39' 53.8" E28° 09' 29.8"	Volunteer barley
2	RWA2.2	11	06/11/2014	Bethlehem	S28° 9' 1.184" E28° 18' 18.359"	RAS nursery
2	RWA2.3	14	19/10/2015	Leribe, Lesotho	S29° 1' 52.14" E27° 44' 40.596"	Dryland wheat
2	RWA2.4	15	22/10/2015	Reitz	S28° 2' 42.864" E28° 22' 3.107"	Dryland wheat
2	RWA2.5	18	15/09/2016	Kirklington	S28° 50' 13.092" E27° 43' 20.027"	Nursery
2	RWA2.6	62	11/10/2017	Clarens	S28° 33' 51.048" E28° 23' 59.35"	Volunteer wheat
3	RWA3.1	12	16/10/2015	Fouriesburg	S28° 29' 59.064" E28° 11' 3.768"	Volunteer wheat
3	RWA3.2	14	19/10/2015	Roma, Lesotho	S29° 26' 40.092" E27° 43' 9.983"	Dryland wheat
3	RWA3.3	19	25/10/2016	Bethlehem	S28° 20' 4.56" E28° 16' 16.463"	Unknown
3	RWA3.4	70	30/10/2017	Clocolan	S29° 0' 03.98" E27° 38' 12.91"	Cultivar evaluation
3	RWA3.5	68	19/10/2017	Arlington	S28° 1' 34,23" E27° 47' 33,79"	Cultivar evaluation
3	RWA3.6	51	10/10/2017	Ficksburg	S28° 49' 2.892" E28° 0' 24.984"	Dryland wheat
4	RWA4.1	6	18/10/2011	Aberfeldy	S28° 10' 48.072" E28° 52' 22.476"	Irrigation wheat
4	RWA4.2	7	06/11/2012	Pannar	Unknown	Dryland wheat
4	RWA4.3	16	22/10/2013	SGI (Dankbaar)	S28° 9' 23.868" E28° 17' 28.176"	Dryland wheat
4	RWA4.4	20	20/10/2015	Ficksburg	S28° 48' 49.068" E27° 56' 22.775"	Dryland wheat
4	RWA4.5	45	9/10/2017	Petrus Steyn	S27°42'27.108"E28°6'34.019"	Volunteer wheat
4	RWA4.6	4	19/10/2015	Unknown, Lesotho	S29.03115°E27.74461°	Dryland wheat
Control	*Sitobion avenae*	-	Unknown	Bethlehem SGI	Unknown	Unknown
Control	*Rhopalosiphum padi*	-	Unknown	Bethlehem SGI	Unknown	Unknown
Control	*Metopolophium dirhodum*	-	Unknown	Bethlehem SGI	Unknown	Unknown

3.2.2. Genomic DNA extraction from dried aphids:

Aphid specimens (25-50 mg) were sampled in 50 ml centrifuge tubes and dried for 24 h using silica gel. The dried aphids were ground to a fine powder for 60 sec using the TissueLyser II (Qiagen, Hilden, Germany, https://www.qiagen.com/).

Total genomic DNA was extracted from the twenty-four RWA and three control aphid specimens using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA).

A volume of 900 µl cell lysis solution was added to the finely ground tissue and incubated for 10 min at room temperature. The samples were centrifuged at 16 000 *g* for 1 min and the supernatant discarded. A volume of 300 µl nuclei lysis solution [75 mM NaCl, 24 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0] and 3 µl Ribonuclease A (RNase A; 4 mg/ml) were added to the pellet. After re-suspending the pellet, the tubes were incubated for 15 min at 37°C. After adding 100 µl protein precipitation solution, each tube was vigorously vortexed and centrifuged for 5 min at 16 000 *g*. The supernatant was transferred to a new 1.5 ml Eppendorf tube containing 300 µl isopropanol and the tube centrifuged for 3 min at 16 000 *g*. The DNA pellet was washed once with 300 µl 70% (v/v) ethanol and air-dried. The DNA pellet was dissolved overnight in 50 µl TE (10 mM tris(hydroxymethyl)-aminomethane hydrochloride [Tris-HCl], 1 mM EDTA pH 8.0) at 4°C.

DNA concentration and purity were determined using the NanoDrop™ spectrophotometer (ThermoFisher Scientific, Massachusetts, USA). The DNA was diluted to 10 ng/µl with TE and 100 ng DNA of each sample separated on an agarose gel.

3.2.3. Agarose gel electrophoresis:

A 1.2% (w/v) agarose gel was prepared in 0.5x Tris-acetic acid-EDTA (TAE), a twenty-fold dilution from 10x TAE buffer (0.4 M Tris-HCl pH 8.0, 5.71 ml glacial acetic acid, 10 mM EDTA) (Sambrook *et al.*, 1989). A loading buffer containing 0.04% (w/v) bromo-phenol blue and 2.5% (w/v) ficoll was added to 100 ng DNA of each sample before loading on the gel. Electrophoresis was performed at 100 V for 25 min in 0.5x TAE running buffer. A GelDoc RX$^+$ system (Bio-Rad Laboratories, Inc., Hercules, California, United States) was used to visualize the bands on the gel.

3.2.4. Amplified Fragment Length Polymorphism analysis of the Russian wheat aphid population

3.2.4.1. DNA digestion, ligation, and PCR amplification:

Two microgram DNA of each aphid colony was digested for 5 h with 4 U *MseI* (Promega Corporation, Madison, WI, USA) in 1x *Mse*-buffer at 37°C. The DNA was subsequently digested overnight with 5 U *EcoRI* at 37°C after the addition of NaCl to a final concentration of 50 mM. A 10 µl ligation mix containing specific *MseI*- and *EcoRI*-adaptors (Table 3.2) was added to the 50 µl digestion reaction mix and incubated at 16°C overnight. The ligation reaction consisted of 1x ligase buffer, 0.4 mM adenosine triphosphate (ATP), 50 pmol *MseI*-adaptor, 5 pmol *EcoRI*-adaptor and 1 U T4 DNA ligase.

The pre-selective PCR amplification of all samples was done using 5 µl of the undiluted adaptor-ligated DNA along with 1x GoTaq® MDx Hot Start buffer (Promega Corporation, Madison, WI, USA), 2 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphates (dNTPs), 30 ng *MseI*-primer + A, 30 ng *EcoRI*-primer + C (Table 3.2) and 1 U GoTaq® DNA polymerase. The PCR conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec, with a final elongation of 10 min at 72°C.

Amplified fragments were separated on a 1.5% (w/v) agarose gel (3.2.3) to confirm successful amplification, after which 1:50 dilutions of all DNA samples were made using ddH_2O.

The selective PCR amplification reaction was done using 5 µl of the diluted pre-selective PCR products with 1x GoTaq® DNA polymerase buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 100 µg/ml bovine serum albumin (BSA), 30 ng *MseI*-primer, 30 ng *EcoRI*-primer (Table 3.3) and 0.75 U GoTaq® DNA polymerase. The following program was used: 94°C for 5 min followed by 94°C for 30 sec, 65°C for 30 sec, decreasing by 1°C every cycle and 72°C for 60 sec for 9 cycles followed by 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec for 25 cycles with a final 2 min elongation at 72°C. A total of eight different *MseI*- and *EcoRI*-primer combinations were used (Table 3.3).

Table 3.2. *MseI*- and *EcoRI*-adaptor and primer sequences used for AFLP analysis in the current study. The selective nucleotides in each primer are indicated in bold (Herselman, 2003).

Type	**Process involved**	**Sequence (5'-3')**
MseI-adaptor forward	Ligation	GACGATGAGTCCTGAG
MseI-adaptor reverse	Ligation	TACTCAGGACTCAT
EcoRI-adaptor forward	Ligation	CTCGTAGACTGCGTACC
EcoRI-adaptor reverse	Ligation	AATTGGTACGCAGTCTAC
MseI-primer + A	Pre-amplification	GACGATGAGTCCTGAGTA**A**
EcoRI-primer + C	Pre-amplification	CTCGTAGACTGCGTACCAATT**C**
MseI-primer + 2 / 3	Amplification	GACGATGAGTCCTGAGTAA**NN / NNN**
EcoRI-primer + 2 / 3	Amplification	CTCGTAGACTGCGTACCAATTC**NN / NNN**

Table 3.3. Different *MseI*- and *EcoRI*-primer combinations used for AFLP selective PCR amplification in the current study. The selective nucleotides in each primer are indicated in bold (Herselman, 2003).

Combination	***MseI*-primer sequences (5'-3')**	***EcoRI*-primer sequences (5'-3')**
1	GACGATGAGTCCTGAGTAA**CAA**	CTCGTAGACTGCGTACCAATTC**ACT**
2	GACGATGAGTCCTGAGTAA**CGC**	CTCGTAGACTGCGTACCAATTC**ACC**
3	GACGATGAGTCCTGAGTAA**CG**	CTCGTAGACTGCGTACCAATTC**AC**
4	GACGATGAGTCCTGAGTAA**TT**	CTCGTAGACTGCGTACCAATTC**TG**
5	GACGATGAGTCCTGAGTAA**AT**	CTCGTAGACTGCGTACCAATTC**TG**
6	GACGATGAGTCCTGAGTAA**CG**	CTCGTAGACTGCGTACCAATTC**CC**
7	GACGATGAGTCCTGAGTAA**CAC**	CTCGTAGACTGCGTACCAATTC**AG**
8	GACGATGAGTCCTGAGTAA**CTA**	CTCGTAGACTGCGTACCAATTC**ACA**

3.2.4.2. Separation and scoring of amplified AFLP alleles on poly-acrylamide gels:

To visualize the amplified AFLP fragments, the PCR products were separated on 5% (w/v) denaturing polyacrylamide gels [19:1 acrylamide:bis-acrylamide, 7 M urea, 1x Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA)]. The 20 µl PCR samples were mixed with 10 µl formamide loading buffer [98% (v/v) formamide, 10 mM EDTA pH 8.0, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol] and denatured for 5 min at 94°C. Electrophoresis was performed at a constant power of 80 W for 2 h using 1x TBE as running buffer.

AFLP alleles were visualized in the acrylamide gel with silver staining as described by the Silver Sequence^{TM} DNA Sequencing System (Promega Corporation, Madison, WI, USA). After electrophoresis, the gel was first soaked in 10% (v/v) glacial acetic acid for 30 min, followed by three wash steps with deionized water for 10 min each. The gel was then soaked in a 0.01 M $AgNO_3$ solution for 30 min followed by a quick rinse in deionized water to remove excess $AgNO_3$. The gel was then developed in 0.36 M sodium carbonate (Na_2CO_3) until the bands on the gel were clearly visible. The reaction was terminated with 10% (v/v) glacial acetic acid.

Stained gels were left upright overnight to air dry and photographed by exposing photographic paper (Kodak Polymax II RC) directly under the gel to dim light for approximately 20 sec. The photograph was a negative copy of the stained gel.

3.2.4.3. Genetic analysis of the South African Russian wheat aphid population using AFLP data:

A binary data matrix, that was scored manually, for the amplified AFLP alleles was prepared in Excel where each row represented the scored amplicons per AFLP marker and each column the different biotypes. The binary data matrix was prepared by scoring each amplified fragment as either present (1) or absent (0), while missing data was scored as “9”. The scored data was used to prepare a phylogram with DARwin 5.0.158 software (Perrier *et al.*, 2003). The percentage dissimilarity was first calculated from the data set with the minimum proportion of data set at 50%. The software then constructed an unrooted phylogram with the unweighted neighbor-joining cluster method using a total of 30 000 bootstraps.

The polymorphic information content (PIC) value is used to measure the polymorphism of a marker locus used in linkage analysis. In this study, the PIC was calculated from the binary data using iMEC: Online Marker Efficiency Calculator developed by Amiryousefi *et al.* (2018). A PIC >0.5 suggested high diversity, a PIC <0.25 suggested low diversity and a PIC between 0.25 and 0.5 suggested intermediate diversity as described in Botstein *et al.* (1980).

3.2.5. RNA extraction using TRIzol®:

Twenty RWA and three control aphid specimens were ground to a fine powder in liquid nitrogen. About 100 µg frozen tissue was transferred to a 1.5 ml Eppendorf and total RNA extracted by adding 500 µl TRIzol® Reagent (ThermoFisher Scientific, Massachusetts, USA) to the frozen tissue. After vortexing each sample for 10 sec, the samples were incubated for 10 min at room temperature. To this was added 100 µl chloroform, where after the tubes were inverted 15 times before incubating them at room temperature for 5 min. The samples were centrifuged at 12 000 *g* for 15 min at 4°C. The total RNA was precipitated from 250 µl of the cleared supernatant using 250 µl isopropanol. The samples were mixed by inversion and left for 10 min at room temperature. After centrifugation at 12 000 *g* for 10 min at 4°C, the supernatant was removed, and the pellet washed with 500 µl 70% (v/v) ethanol. The samples were again centrifuged at 7 500 *g* for 10 min at 4°C, the supernatant then removed, and the pellet air-dried. After 5 min, 200 µl dimethyl dicarbonate (DMDC)-treated water (Sambrook *et al.*, 1989) was added to each pellet and incubated for 1 h on ice to dissolve the RNA. The samples were now centrifuged at 12 000 *g* for 5 min at 4°C and the concentration and purity of the RNA determined using the NanoDrop™ spectrophotometer. The quality and quantity of the RNA was confirmed by separating 300 ng RNA on a 1.2% (w/v) agarose gel (3.2.3).

3.2.6. Complimentary DNA-Amplified Fragment Length Polymorphism analysis of the Russian wheat aphid population:

3.2.6.1. cDNA synthesis:

One microgram total RNA and 500 ng random primers (Promega Corporation, Madison, WI, USA) were in combined in a total volume of 5 µl, denatured for 5 min at 70°C and immediately placed in ice-water for at least 5 min. cDNA was synthesized by adding 4 µl ImProm-II™ (Promega Corporation, Madison, WI, USA) reaction buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs and 100 U ImProm-II™ reverse transcriptase in a 20 µl reaction. The reaction was incubated at 25°C for 5 min followed by 42°C for 60 min and 70°C for 15 min.

To confirm that the success of cDNA synthesis, two RWA reference genes (Table 3.4) were PCR amplified from the synthesized cDNA. The composition of the PCR reaction was 5 µl diluted cDNA (1:2.5 dilution in ddH_2O), 1x KAPATaq ReadyMix (Kapa Biosystems, Massachusetts, USA) and 1 µM of the specific primer pair. The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec, with a final elongation step of 10 min at 72°C. Amplified fragments were separated on a 1.5% (w/v) agarose gel (3.2.3) to confirm amplification.

3.2.6.2. cDNA-AFLP analysis of the Russian wheat aphid population

Two microgram cDNA of each aphid colony was digested for 5 h with 4 U *MseI* in 1x *Mse*-buffer at 37°C. The DNA was subsequently digested overnight with 1 U *TaqI* enzyme at 37°C in the same buffer solution. A ligation mix containing specific *MseI*- and *TaqI*-adaptors (Table 3.5) was added to the 50 µl digestion reaction mix and incubated at 16°C overnight. The ligation mix consisted of 1x ligase buffer, 0.4 mM ATP, 50 pmol *MseI*-adaptor, 50 pmol *TaqI*-adaptor and 1 U T4 DNA ligase.

The pre-selective PCR amplification of all samples was done using 5 µl of the undiluted adaptor-ligated cDNA along with 1x Promega GoTaq® MDx Hot Start buffer (Promega Corporation, Madison, WI, USA), 2 mM $MgCl_2$, 200 µM dNTPs, 30 ng *MseI*-primer + A, 30 ng *TaqI*-primer + A (Table 3.5) and 1 U GoTaq® DNA polymerase. The PCR conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec, with a final elongation of 10 min at 72°C. Amplified fragments were separated on a 1.5% (w/v) agarose gel (3.2.3) to confirm successful amplification, after which DNA of all samples was diluted (1:50) using ddH_2O. The selective PCR amplification reactions were done using 5 µl of the diluted pre-selective PCR products with 1x GoTaq® DNA polymerase buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 100 µg/ml BSA, 30 ng *MseI*-primer, 30 ng *TaqI*-primer (Table 3.6) and 0.75 U GoTaq® DNA polymerase.

Table 3.4. The two control reference genes used to test for successful *Diuraphis noxia* cDNA synthesis (Sinha and Smith, 2014).

Primer	**Forward primer sequence (5'-3')**	**Reverse primer sequence (5'-3')**	**T_m (°C)**	**Amplicon size (bp)**
RTActin	TGCCTGATGGTCAAGTCATC	TCAAGGAATGCTTTGAGCTG	55	553
RTTEF2	CACATGGGTCACCTGGGAATA	TCGGATTCACTGCTGATTGC	55	220

Table 3.5. *MseI*- and *TaqI*-adaptor and pre-amplification primer sequences used for cDNA-AFLP analysis. The selective nucleotide in each primer is indicated in bold.

Type	**Process involved**	**Sequence (5’-3’)**
MseI-adaptor forward	Ligation	GACGATGAGTCCTGAG
MseI-adaptor reverse	Ligation	TACTCAGGACTCAT
TaqI-adaptor forward	Ligation	CTCGTAGACTGCGTAC
TaqI-adaptor reverse	Ligation	CGGTACGCAGTCT
MseI-primer + A	Pre-amplification	GACGATGAGTCCTGAGTA**A**
TaqI-primer + A	Pre-amplification	GTAGACTGCGTACCG**A**

Table 3.6. The ten different *MseI*- and *TaqI*-primer combinations used for selective PCR amplification of cDNA-AFLP fragments in the current study. The selective nucleotides in each primer are indicated in bold.

Combination	***MseI*-primer sequence (5'-3')**	***TaqI*-primer sequence (5'-3')**
1	GACGATGAGTCCTGAGTAA**AC**	GTAGACTGCGTACCGA**GA**
2	GACGATGAGTCCTGAGTAA**AC**	GTAGACTGCGTACCGA**TC**
3	GACGATGAGTCCTGAGTAA**AC**	GTAGACTGCGTACCGA**CT**
4	GACGATGAGTCCTGAGTAA**AC**	GTAGACTGCGTACCGA**AG**
5	GACGATGAGTCCTGAGTAA**AC**	GTAGACTGCGTACCGA**GT**
6	GACGATGAGTCCTGAGTAA**TC**	GTAGACTGCGTACCGA**GA**
7	GACGATGAGTCCTGAGTAA**TC**	GTAGACTGCGTACCGA**TC**
8	GACGATGAGTCCTGAGTAA**TC**	GTAGACTGCGTACCGA**CT**
9	GACGATGAGTCCTGAGTAA**TC**	GTAGACTGCGTACCGA**AG**
10	GACGATGAGTCCTGAGTAA**TC**	GTAGACTGCGTACCGA**GT**

The following program was used: 94°C for 5 min followed by 94°C for 30 sec, 65°C for 30 sec, decreasing by 1°C every cycle and 72°C for 60 sec for 9 cycles followed by 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec for 25 cycles with a final 2 min elongation at 72°C. A total of ten different *MseI* and *TaqI*-primer combinations were used (Table 3.6).

3.2.6.3. Separation and scoring of amplified cDNA-AFLP alleles on the QIAxcel ScreenGel® system:

The amplified cDNA-AFLP alleles were separated on the QIAxcel ScreenGel® system (Qiagen Hilden, Germany) using a DNA High Resolution Gel cartridge. The OM800 method was used for separation, which consisted of a sample injection voltage of 5 kV for 10 sec, and 6 kV separation voltage with a separation time of 920 sec. Both the QX 15 bp / 1 kb alignment marker and QX 50-800 bp v2.0 DNA size marker were used to calculate the sizes of amplified alleles. Both a binary and allelic data matrix was prepared in Excel for the ten cDNA-AFLP primer pairs as previously described (3.2.4.3). The allelic data matrix was based on allele sizes and machine assisted scoring was done.

The cDNA-AFLP data matrices were used to prepare phylograms within DARwin 5.0.158 as previously described (3.2.4.3).

3.2.7. Microsatellite marker analysis of the Russian wheat aphid population

3.2.7.1. PCR amplification:

Of the seventeen microsatellite markers initially tested (Table 3.7), only fifteen were used to genotype the twenty-four RWA and three control aphid specimens: *Sm11, S17b, Sa4.Σ* (Simon *et al*., 1999); *S16b, S23, S49* (Wilson *et al*., 2004); *Dn1, DnE1, Dn5, Dn6, Dn13, Dn16, Dn22, Dn25* and *Dn27* (Zhang *et al*., 2012). The first six markers (*Sm11, S17b, Sa4.Σ, S16b, S23, S49*) were developed for aphid species other than the RWA, including *Sitobion avenae* Fabr. and *Sitobion miscanthi* Takahashi. These primers successfully cross-amplified loci in other aphids, including the RWA. The remaining nine markers were developed for RWA.

Table 3.7. Microsatellite markers used for Russian wheat aphid phylogenetic study.

Locus	Primer code	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	T_m (°C)	Amplicon Size (bp)	Reference
Sm10	RWA_SSR_1	TCTTCTCTATACACCTATAAAC	TTATGCTAATCTCACAATAC	40	250	Wilson *et al*., 2004
Sm11	RWA_SSR_2	GGTACCCCTATGTTATTACGCG	AACCCTACGGGTAACGCC	39	250	Simon *et al*., 1999
Sm12	RWA_SSR_3	TTCGGTATAATAGTGCGTG	GGCGATGCGACTAAAC	44	270	Wilson *et al*., 2004
S16b	RWA_SSR_4	ATAAAACAAAGAGCAATTCC	GTAAAAGTAAAGGTTCCACG	54.6	175	Wilson *et al*., 2004
S17b	RWA_SSR_5	TTCTGGCTTCATTCCCGGTCG	CGTCGCGTTAGTGAACCGTG	62	250	Simon *et al*., 1999
S23	RWA_SSR_6	GGTCCGAGAGCATTCATTAGG	CGTCGTTGTCATTGTCGTCG	54.6	275	Wilson *et al*., 2004
S49	RWA_SSR_7	CGCATTTAGGAGGTTTCGAC	CATGTGCAGTGGACGAGGAA	39	180	Wilson *et al*., 2004
Sa4.Σ	RWA_SSR_8	GTGACGTATACGCGATGCG	GACGTCGATATTAGCCTAGCC	62	175	Simon *et al*., 1999
Dn1	RWA_SSR_9	GCAGGGTTACCAATGTTTC	TGAGTAGCAGGTAATTCAGGAG	61.1	218-242	Zhang *et al*., 2012
DnE1	RWA_SSR_10	ATACTATGCGTCCGTCGTCC	GCTGGACTTGTTGATGGTGA	61.1	130-154	Zhang *et al*., 2012
Dn5	RWA_SSR_11	AATCGCACCCTGGGCAAC	GTGGGATTCTAAACTGAGGGC	62	205-277	Zhang *et al*., 2012
Dn6	RWA_SSR_12	TGATCGGCTCCATAAAAAC	GTAGCAAGTTTGACCCTAAA	59.6	331-451	Zhang *et al*., 2012
Dn13	RWA_SSR_13	AGATTCTGCCGTATGTGATTC	CGCAGCCAACAAGCTATTA	59.6	158-258	Zhang *et al*., 2012
Dn16	RWA_SSR_14	GTCCTCGTGGATACTCATCAT	AATCGGTGTCAGGTTTCG	59.6	116-178	Zhang *et al*., 2012
Dn22	RWA_SSR_15	ACGGATTTAACGCAAATTTTA	CGAATGTAATGCGATGTTGC	57.3	176-242	Zhang *et al*., 2012
Dn25	RWA_SSR_16	GCGTGATCCGAGGTCTTT	GACGATTAGGGAGAAGTGAA	57.3	102-124	Zhang *et al*., 2012
Dn27	RWA_SSR_17	TTCTGTGGTAGTGGTCCCG	GACCACTCACCCTATCTCAC	61.1	180-206	Zhang *et al*., 2012

PCR reactions were carried out in 10 μl volumes. Each reaction contained 1x KAPATaq ReadyMix, 5 ng aphid DNA and 1 μM of the specific primer pair.

PCR reactions were run using the following program: one cycle of 95°C for 3 min followed by 40 cycles of 94°C for 30 sec, optimal annealing temperature of each primer pair (Table 3.7) for 30 sec and 72°C for 30 sec. A final cycle of 72°C for 5 min was included. The PCR products were separated on an agarose gel (3.2.3) to verify successful amplification.

3.2.7.2. Separation and scoring of amplified microsatellite alleles on poly-acrylamide gels:

To visualize the fifteen amplified microsatellite markers, PCR products were separated on both 5% (w/v) denaturing polyacrylamide gels (3.2.4.2) and the QIAxcel ScreenGel® system (3.2.6.3).

A binary data matrix was prepared for the silver stained gels, and an allelic data matrix based on allele sizes for the QIAxcel ScreenGel® system where each individual was scored as a diploid containing two alleles.

3.2.7.3. Genetic analysis of the South African Russian wheat aphid population using microsatellite data:

Both binary and allelic microsatellite data matrices were used to prepare phylograms within DARwin 5.0.158 software as previously described (3.2.4.3).

To determine the genetic structure of the RWA population based on the microsatellite data, a Bayesian model-based cluster analysis was done within STRUCTURE 2.2 (Jombart *et al.*, 2010; Pritchard *et al.*, 2000) using the allelic data matrix. A total of ten *K*-value runs (*K* = 1 to 10) were performed with a burn-in period of 10 000, 10 000 Monte Carlo Markov Chain (MCMC) repetitions after burn-in with 10 iterations per "True" number of subpopulations (*K)* value.

The analysed data from STRUCTURE was then used within CLUMPAK (http://clumpak.tau.ac.il; Jakobsson and Rosenberg, 2007) to determine the optimal number of predicted sub-populations using the ad-hoc *ΔK* statistic (Evanno *et al.*, 2005). Once the optimal number of sub-populations were confirmed, STRUCTURE analysis was repeated using the optimal number of sub-populations, and 100 000 for both burn-in and MCMC repetitions. To conclude, a bar plot was drawn which illustrated the placement of the individual RWA and control aphid specimens within the predicted sub-populations.

The genetic diversity between sub-populations was determined using Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 2005) within ARLEQUIN 3.5.12 software. A total of 16 000 permutations were used to test the significance level of both the AMOVA components and the F-statistics parameter F_{ST}. The F_{ST} value was used to evaluate the validity of the population structure where an F_{ST} value of 0 implied no genetic variation between the sub-populations and a value of 1 suggested 100% genetic variation. F_{ST} values between 0 and 0.05 suggested low significance, between 0.05 and 0.15 moderate significance, between 0.15 and 0.25 high significance and above 0.25 a very high significance (Wright, 1951).

Chapter 4

Results

4.1. Genomic DNA extraction with Wizard® Genomic DNA Purification Kit:

Russian wheat aphids were collected in 2 ml Eppendorf tubes and dried overnight with silica gel. DNA was extracted from the dried tissue with the Wizard® Genomic DNA Purification Kit. DNA extraction was repeated for samples with degraded DNA as indicated by a smear on an agarose gel. For RNA contamination that was present as a low molecular weight smear, an RNase treatment was included to eliminate all RNA. Acceptable DNA samples with a band of genomic DNA in excess of 10 000 bp without any smear (Fig. 4.1), were used for subsequent AFLP and microsatellite analyses.

4.2. AFLP analysis of the South African Russian wheat aphid population:

The AFLP method is a genetic fingerprinting technique, which targets the whole genome based on selective PCR amplification of modified restriction fragments. Fragments between 100 and 1 000 bp in size, were scored. Eight primer combinations with an average of eleven AFLP loci per primer pair yielded a total of 93 scored AFLP loci. These eight primer combinations were chosen because of their polymorphic nature. The primer combinations had an average PIC of 0.32, which suggested that the used primer combinations had an intermediate level of diversity. The primer combinations with the highest PIC values were primer combinations 2 and 3 with a PIC value of 0.36 each.

Based on the generated phylogram (Fig. 4.2), it was clear that some polymorphisms were found between the 24 RWA specimens.

Four clades were formed. The control aphid specimens were divided into two clades where clade C contained *Rhopalosiphum padi* and *Sitobion avenae* and clade D contained *Metopolophium dirhodum*, which had the closest genetic similarity value of 39.11% to RWA biotype 1, 38.39% to RWA biotype 2, 38.52% to RWA biotype 3 and 39.46% to RWA biotype 4. All the RWA specimens formed clade A except for RWA4.3 and RWA4.4 which separated from the rest to form clade B. RWA1.6, RWA2.1, RWA2.6 and RWA3.1 formed a smaller sub-clade within clade A.

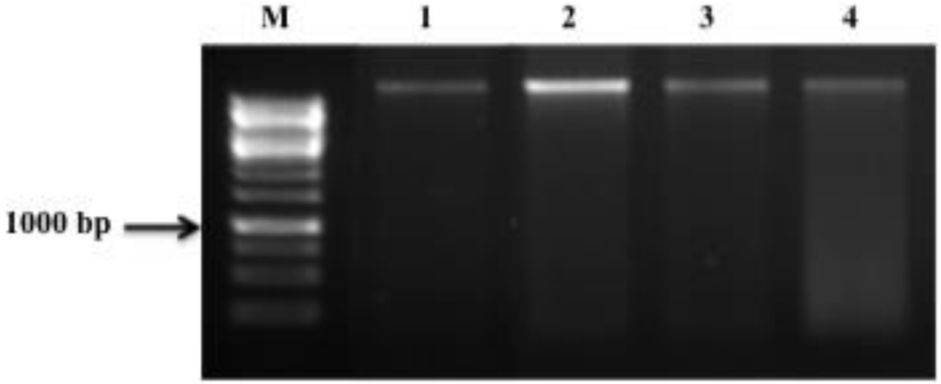

Figure 4.1. A 1.2% (w/v) agarose gel illustrating genomic DNA extracted with the Wizard® Genomic DNA Purification Kit from Russian wheat aphid samples. Lane M represents the molecular ladder (1 kb Gene Ruler, Thermo Fisher Scientific), while lanes 1 to 4 represent genomic DNA extracted from RWA1.2, RWA2.1, RWA3.2 and RWA4.3, respectively.

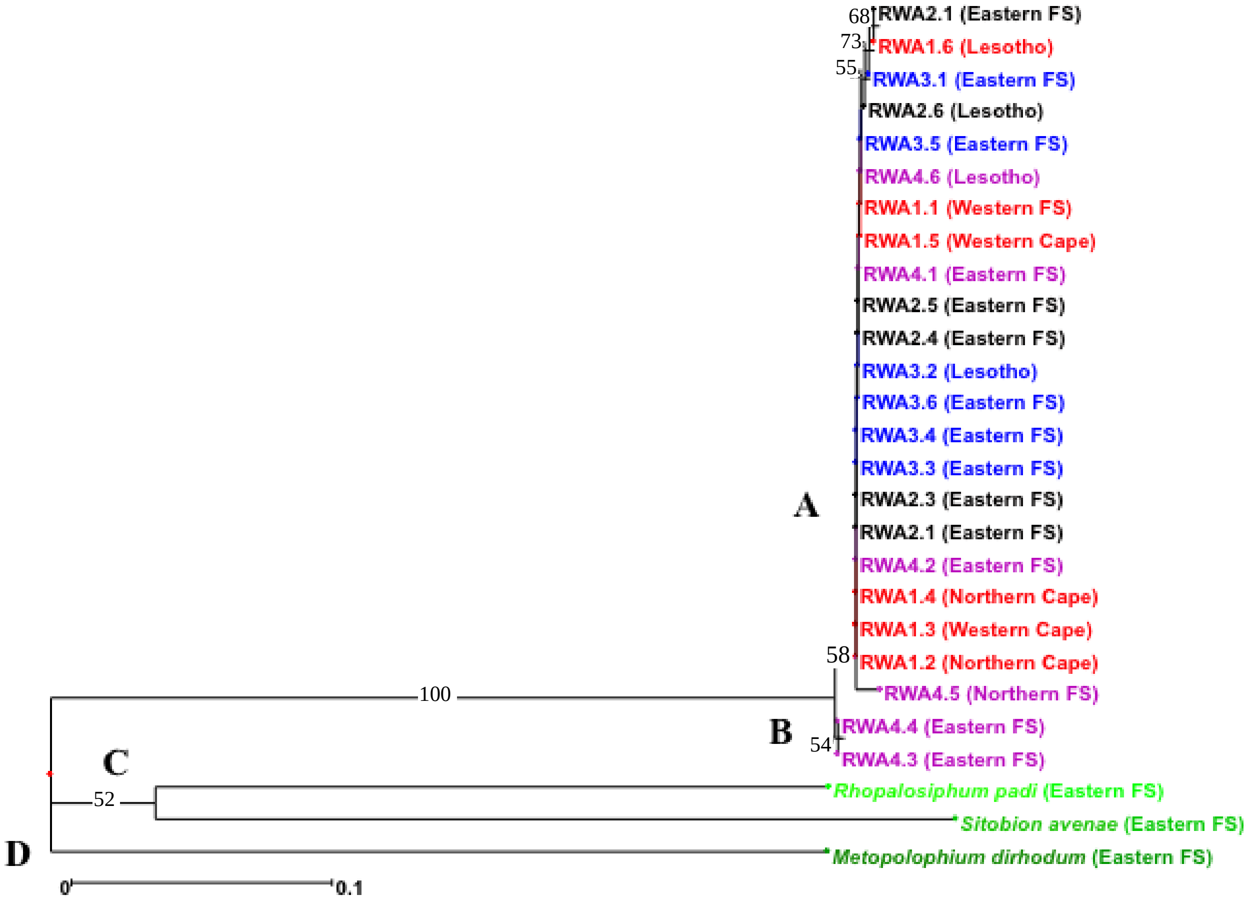

Figure 4.2. A phylogenetic analysis of twenty-four Russian wheat aphid and three control specimens using AFLPs. The red font represents biotype RWASA1 (RWA1.1-RWA1.6), black biotype RWASA2 (RWA2.1-RWA2.6), blue biotype RWASA3 (RWA3.1-RWA3.6) and purple biotype RWASA4 (RWA4.1-RWA4.6). The three control aphids *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum* were indicated in green. Bootstrap values are indicated on branches.

Since the main aim of the study was to genetically differentiate between the four RWASA biotypes, it was clear that AFLPs would not provide sufficient polymorphic data to separate the four biotypes from one another or to identify a specific marker linked to a specific biotype.

4.3. cDNA-AFLP analysis of the South African Russian wheat aphid population:

cDNA-AFLP uses cDNA that was synthesized from RNA for analysis, rather than total genomic DNA. The motivation for using cDNA is that it targets all expressed genes, including those that could be responsible for virulence on susceptible wheat cultivars. This ultimately results in less generated information compared to genomic DNA. It was thus decided to use cDNA-AFLP analysis to try to distinguish between the four RWASA biotypes.

RNA was extracted from dried and ground tissue of twenty RWA and three control specimens with the TRIzol® extraction method. From Fig. 4.3, it was clear that good quality RNA was extracted with the two visible rRNA bands at 1000 bp and 1250 bp, being intact. The DNase treatment eventually eliminated any DNA contamination, such as that which was visible in the *Rhopalosiphum padi* sample.

cDNA synthesis was performed on the purified RNA from twenty RWA and three control aphid specimens using the ImProm-II™ Reverse Transcription System. Control PCR reactions were done for two RWA reference genes (*RTAct* and *RTTEF2*) to confirm that cDNA synthesis was successful, and that the cDNA could be used for AFLP analysis. As seen in Fig. 4.4, both reference genes (*RTAct* – 553 bp; *RTTEF2* – 220 bp) were successfully amplified from both RWA samples. This confirmed that the cDNA synthesis from total RNA was successful. The successful cDNA synthesis from all RWA and control specimens were then confirmed using the *RTAct* reference gene (Fig. 4.5). cDNA-AFLP analysis was performed for the twenty RWA and three control aphid specimens using ten AFLP primer pairs. The PCR products were separated with the QIAxcel ScreenGel® system and the data scored as either a binary or an allelic data matrix. A binary data matrix was based on the presence or absence of an amplified allele, whereas the allelic data matrix was based on size differences between the amplified loci. The reason for using both matrices was to determine which scoring method gave the more accurate phylogram that correlated closest to the previous AFLP results.

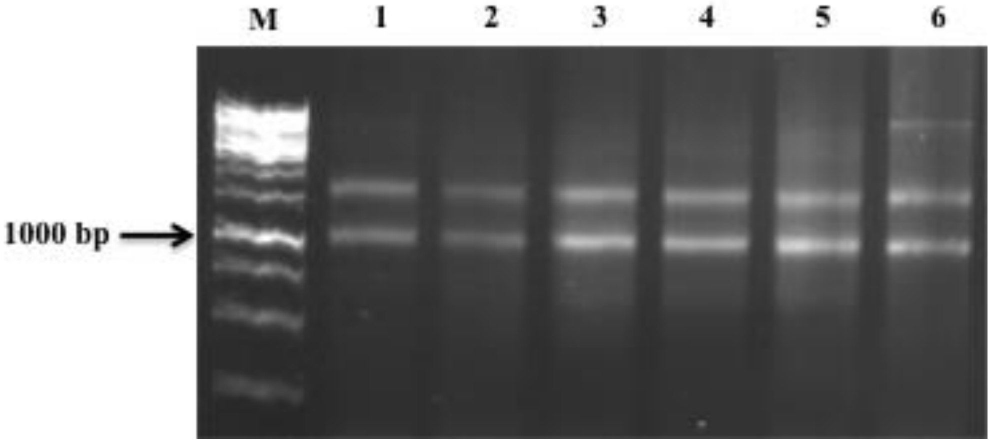

Figure 4.3. A 1.2% (w/v) agarose gel illustrating total RNA extraction using the TRIzol® extraction method from twenty Russian wheat aphid and three control specimens. Lane M represents the molecular ladder (1 kb Gene Ruler, Thermo Fisher Scientific), while lanes 1 to 6 represent RNA extracted from RWA2.4, RWA2.5, RWA3.4, RWA3.5, RWA4.1 and *Rhopalosiphum padi*, respectively.

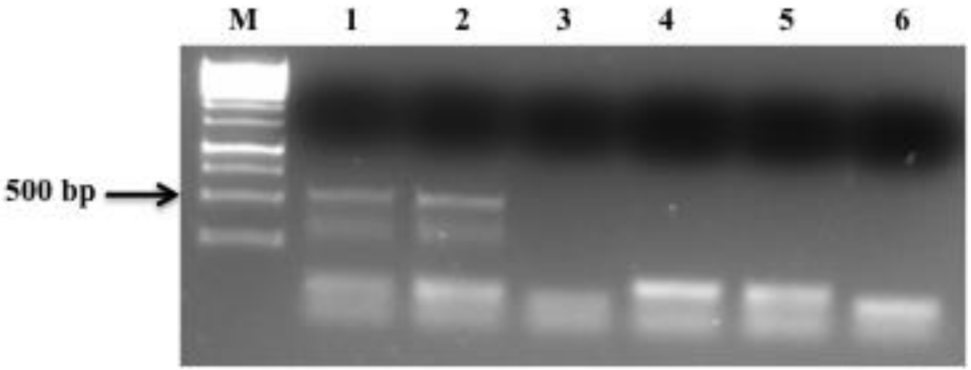

Figure 4.4. A 1.2% (w/v) agarose gel illustrating the PCR amplification of two Russian wheat aphid reference genes (*RTAct* - 553 bp, *RTTEF2* - 220 bp) from synthesized cDNA of RWA2.4 (lanes 1 and 4) and RWA2.5 (lanes 2 and 5), respectively. Lane M represents the molecular ladder (1 kb Gene Ruler, Thermo Fisher Scientific) and lanes 1 to 6 the PCR amplification products of *RTAct* (lanes 1-3) and *RTTEF2* (lanes 4-6), respectively. Lanes 3 and 6 were negative control reactions.

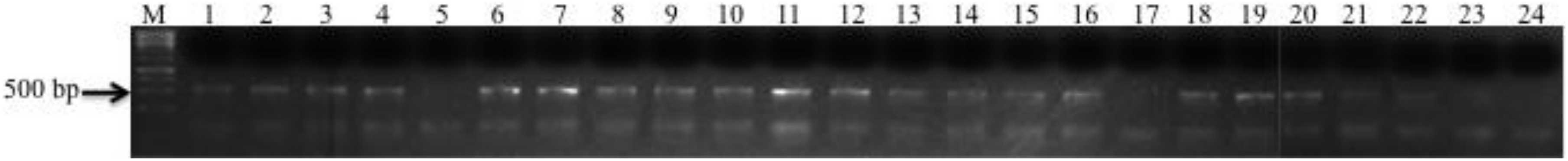

Figure 4.5. A 1.2% (w/v) agarose gel illustrating the successful amplification of the Russian wheat aphid *RTAct* (553 bp) reference gene from synthesized cDNA for all tested aphid samples. Lane M represents the molecular ladder (1 kb Gene Ruler, Thermo Fisher Scientific), while lanes 1 to 23 represent cDNA synthesis from twenty Russian wheat aphid and three control aphid specimens. Lane 24 represents the negative control.

An average of seven AFLP loci per primer pair (78 cDNA-AFLP loci in total) were scored for the ten primer combinations within the binary data matrix. The cDNA-AFLP primer combinations had an average PIC of 0.36, which suggested that the used primer combinations had an intermediate level of diversity. The primer combination with the highest PIC value was primer combination 2 with 0.39.

Based on the phylogram (Fig 4.6), it was clear that there were polymorphic differences between and within the four RWA biotypes. The reason for the excessive variation between specimens was that bands were scored as either present or absent and since there were a number of bands that appeared only in one or two specimens and not in the others, this created the diversity.

Five clades were formed with four RWA specimens (RWA3.3, RWA3.2, RWA3.1 and RWA1.5) in clade A, fifteen mixed RWA specimens in clade B, RWA4.2 in clade C, two control specimens (*Metopolophium dirhodum* and *Sitobion avenae*) in clade D and *Rhospalosiphum padi* in clade E (Fig. 4.6).

The main reason why the binary data matrix did not result in an accurate phylogram, was because there were amplified loci present in only one of the specimens, which is referred to as an outlier. These outliers comprised most of the data, because the phylogram scored these outliers as variation between and within biotypes, but it did not follow the pattern from the AFLP analysis.

It was consequently decided to test the allelic data matrix based on the same data set. Loci were now scored as fragment sizes and not as either present or absent. All the bands that were present in most of the samples were scored. Bands that differed slightly in fragment size were scored as the same. For instance, alleles in RWA1.1 (181 bp), RWA1.2 (182 bp) and RWA1.3 (179 bp) were all scored as 180 bp.

With the allelic data matrix, an average of four AFLP loci per primer pair (48 AFLP loci in total) was scored for the ten primer pairs. Seven clades were formed with fourteen RWA specimens in clade A, RWA3.2 in clade B, RWA3.1, RWA3.3 and RWA4.6 in clade C, RWA2.1 in clade D, RWA4.2 in clade E, *Metopolophium dirhodum* and *Sitobion avenae* in clade F and *Rhospalosiphum padi* in clade G (Fig. 4.7). The grouping of the control specimens differed from the AFLP phylogram (Fig. 4.2). RWA1.5, RWA2.4, RWA2.6 and RWA4.5 formed a sub-clade within clade A and was not linked to a specific biotype.

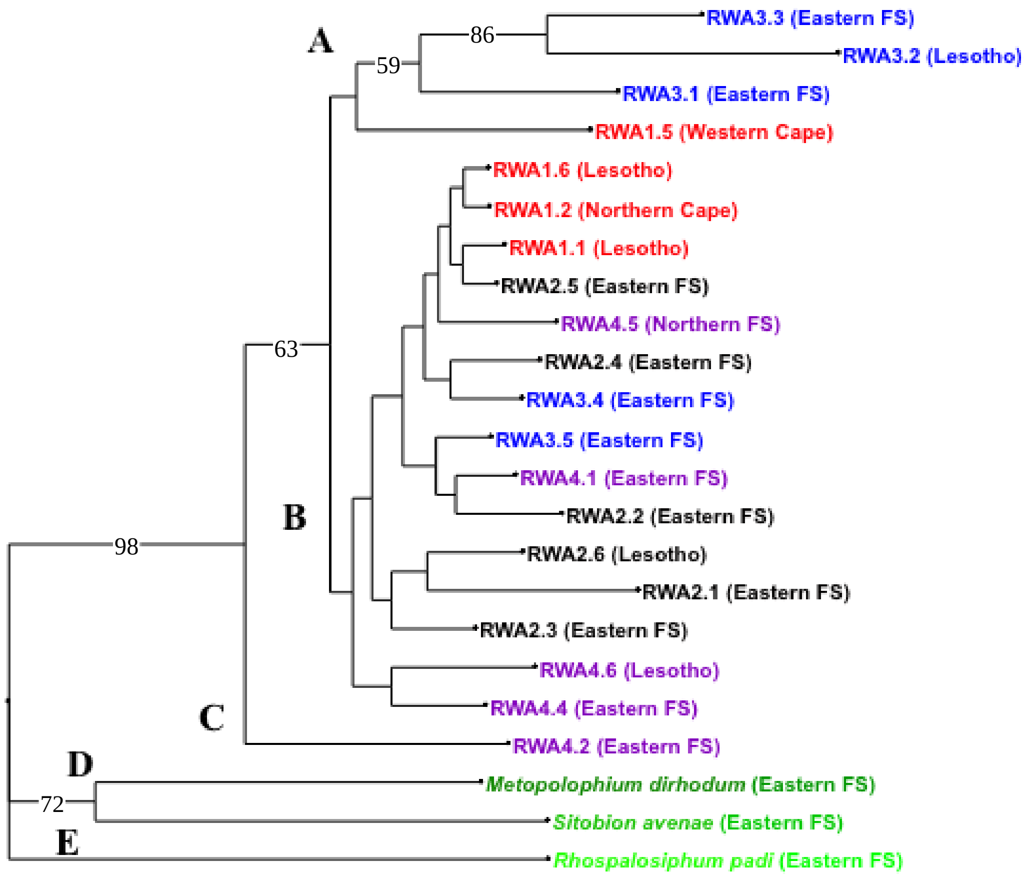

Figure 4.6. Phylogenetic analysis of twenty Russian wheat aphid and three control specimens using cDNA-AFLP data scored as a binary data matrix. Red font represents biotype RWASA1 (RWA1.1, RWA1.2, RWA1.5 and RWA1.6), black biotype RWASA2 (RWA2.1-RWA2.6), blue biotype RWASA3 (RWA3.1-RWA3.5) and purple biotype RWASA4 (RWA4.1, RWA4.2, RWA4.4-RWA4.6). The three control aphids *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum* were indicated in green. Bootstrap values are indicated on branches.

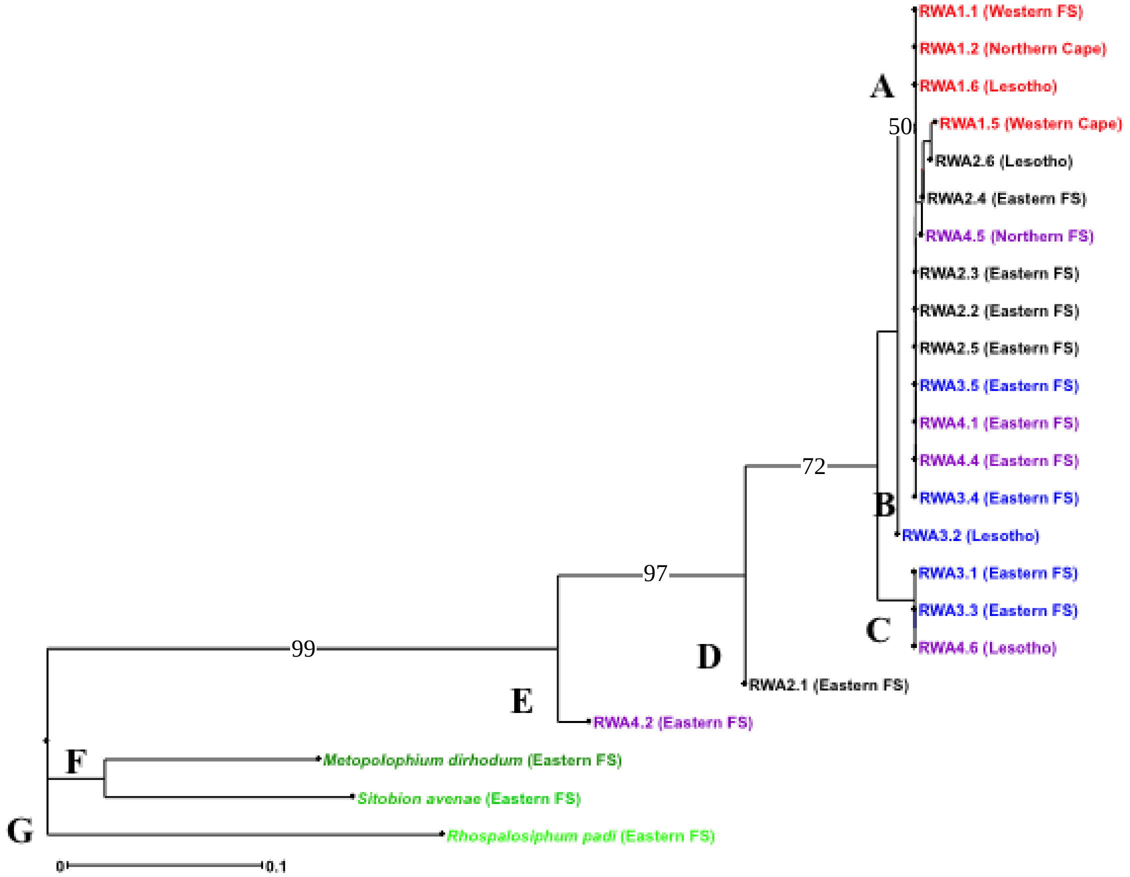

Figure 4.7. Phylogenetic analysis of twenty Russian wheat aphid and three control specimens using the allelic data matrix of the cDNA-AFLP analysis. Red font represents biotype RWASA1 (RWA1.1, RWA1.2, RWA1.5 and RWA1.6), black biotype RWASA2 (RWA2.1-RWA2.6), blue biotype RWASA3 (RWA3.1-RWA3.5) and purple biotype RWASA4 (RWA4.1, RWA4.2, RWA4.4-RWA4.6). The three control aphids *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum* were indicated in green. Bootstrap values are indicated on branches.

It was concluded that, although cDNA-AFLP analysis based on allele sizes gave a similar phylogram to that of the original AFLP analysis, there were still not enough supportive data to distinguish between the four RWASA biotypes, or any allelic bands that were linked to a specific biotype.

4.4. Microsatellite analysis of the South African Russian wheat aphid population:

In order to improve the genetic separation of the four RWA biotypes into well-defined sub-populations, microsatellite analysis was investigated as a suitable alternative. Fifteen microsatellite markers were initially chosen to fingerprint the four RWA biotypes and three control aphid species. The optimal annealing temperatures for all primer pairs were verified with a gradient PCR reaction. The optimal annealing temperatures for the different primer pairs were 39°C (*Sm11*), 54.6°C (*S16b*, *S17b*, *S23*), 57.3°C (*S49*, *Dn22*, *Dn25*), 59.6°C (*Dn6, Dn13, Dn16*), 61.1°C (*Dn1*, *DnE1*, *Dn27*) and 62°C (*Sa4.Σ*, *Dn5*) (Fig. 4.8).

Three of the cross-species microsatellite markers (*Sm11, S23* and *S49*) were eventually excluded from the analysis, since no reliable and repeatable PCR amplification was obtained using these primer pairs.

Two different methods were used to separate and score the amplified microsatellite markers to illustrate the genetic relationships amongst the four RWA biotypes and three aphid controls.

For the first method, the amplified DNA was separated on polyacrylamide gels and visualized with silver staining. These amplified fragments were visually scored as a binary data matrix that was used to construct a phylogram with the unweighted neighbor-joining method. A total of 44 amplified fragments were scored from the 12 primer pairs that were used.

Eleven of the twelve markers produced monomorphic fragments, with only primer pair *S17b* producing polymorphic fragments.

The RWA and three control aphid specimens were split into three different clades containing RWA1.1 – RWA2.4, RWA2.5 – RWA4.6, and the three control specimens respectively (Fig. 4.9).

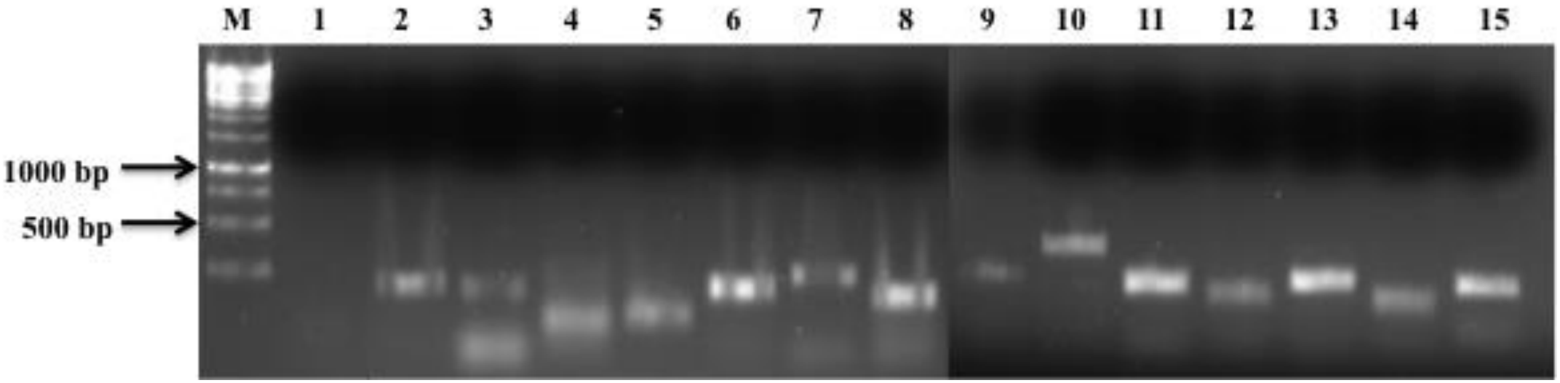

Figure 4.8. A 1.2% (w/v) agarose gel illustrating the different PCR amplification products of the fifteen microsatellite primer sets at their respective optimal annealing temperatures. Lane M represents the molecular ladder (1 kb Gene Ruler, Thermo Fisher Scientific), while Lanes 1 to 15 represent primer sets *Sm11*, *S16b*, *S17b*, *S23*, *S49*, *Sa4.Σ*, *Dn1*, *DnE1*, *Dn5*, *Dn6*, *Dn13*, *Dn16*, *Dn22*, *Dn25* and *Dn27*.

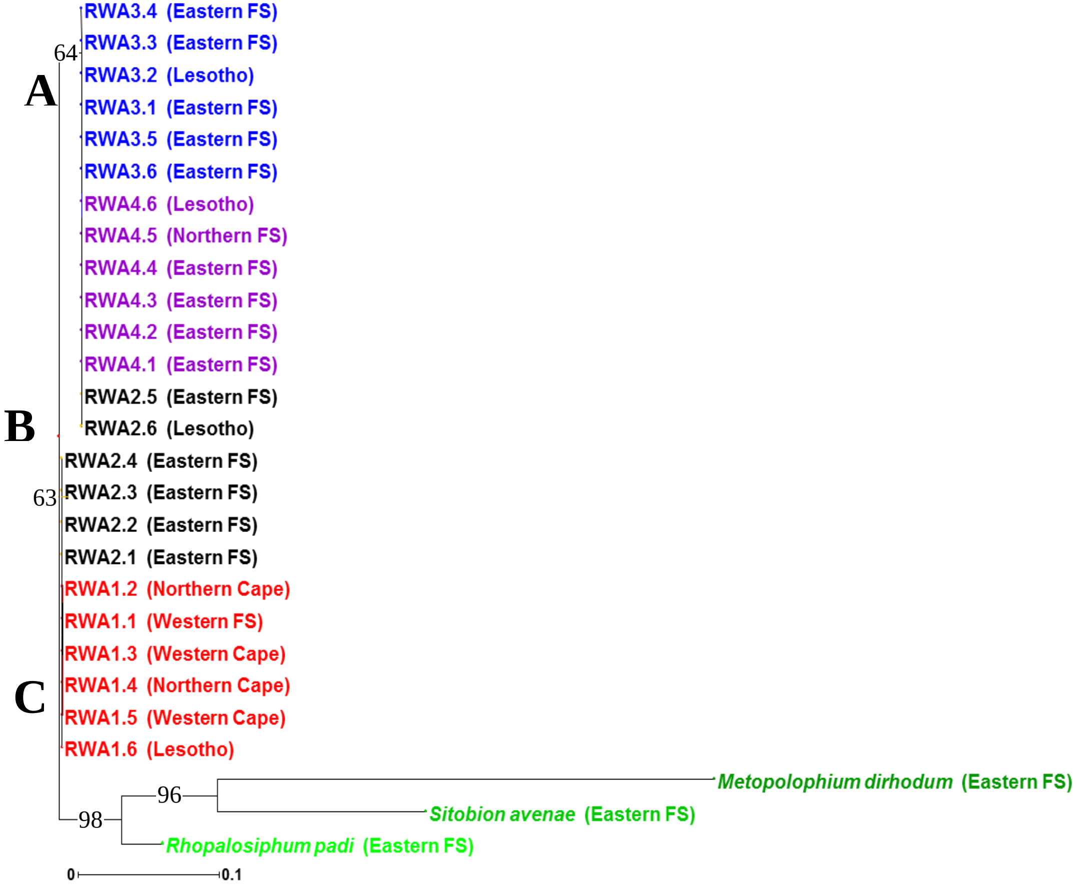

Figure 4.9. Phylogenetic analysis of twenty-four Russian wheat aphid and three control specimens. The phylogram was generated with microsatellite data using the unweighted neighbor-joining method from DARwin5.0 (Perrier *et al.*, 2003). Red font represents biotype RWASA1 (RWA1.1-RWA1.6), black biotype RWASA2 (RWA2.1-RWA2.6), blue biotype RWASA3 (RWA3.1-RWA3.6) and purple biotype RWASA4 (RWA4.1-RWA4.6). The three control aphids *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum* were indicated in green. Bootstrap values are indicated on branches.

The three controls were further separated into two sub-clades with *Sitobion avenae* and *Metopolophium dirhodum* grouping together. This division was supported by high bootstrap values. *Rhopalosiphum padi* was 93% similar to the four RWA biotypes, while *Sitobion avenae* had a similarity value of 74% and *Metopolophium dirhodum* 53% in relation to the RWA biotypes. The specimens of clade A were 99% similar to those of clade B.

To improve the resolution of the amplified microsatellite marker separation, a second separation method was tested when the PCR products were separated with the QIAxcel ScreenGel® system. Separation on the QIAxcel ScreenGel® system improved the resolution of the amplified markers significantly by displaying more polymorphisms compared to the polyacrylamide gels.

The twelve primer sets used generated 24 alleles that were scored using the allelic data matrix and were used to construct the phylogram. Primer sets *S16b*, *Dn6* and *Dn13* all produced polymorphic alleles that allowed the discrimination between the four RWA biotypes and three control specimens (Fig. 4.10).

Four clades were found with the three control specimens grouped into one clade (98% bootstrap support) containing two sub-clades, in agreement with previous results (Fig. 4.9). For the four RWA biotypes, clade A contained all six RWA biotype 1 specimens that formed two sub-clades. Clade B contained all biotype 2 and 3 specimens that were sub-divided into two and three sub-clades, respectively. The two biotypes were clearly distinguishable from each other. Biotype 4 that formed clade C was similarly divided into two sub-clades. Bootstrap support for the major branches was between 50 and 70%.

The following approximate percentage similarity values were found between the four biotypes: biotype 1 was 80% similar to biotype 2, 90% similar to biotype 3 and 85% to biotype 4, biotype 2 was 90% similar to biotype 3 and 80% similar to biotype 4 and biotype 3 was 90% similar to biotype 4. In general, based on locality (Table 3.1) specimens collected from the eastern FS and Lesotho (biotypes 2, 3 and 4) were more closely related to each other than to the biotype 1 specimens that were collected in the western parts of the wheat producing areas.

The model-based Bayesian clustering method STRUCTURE was used to determine the structure of the South African RWA population in relation to the three control aphid population representatives.

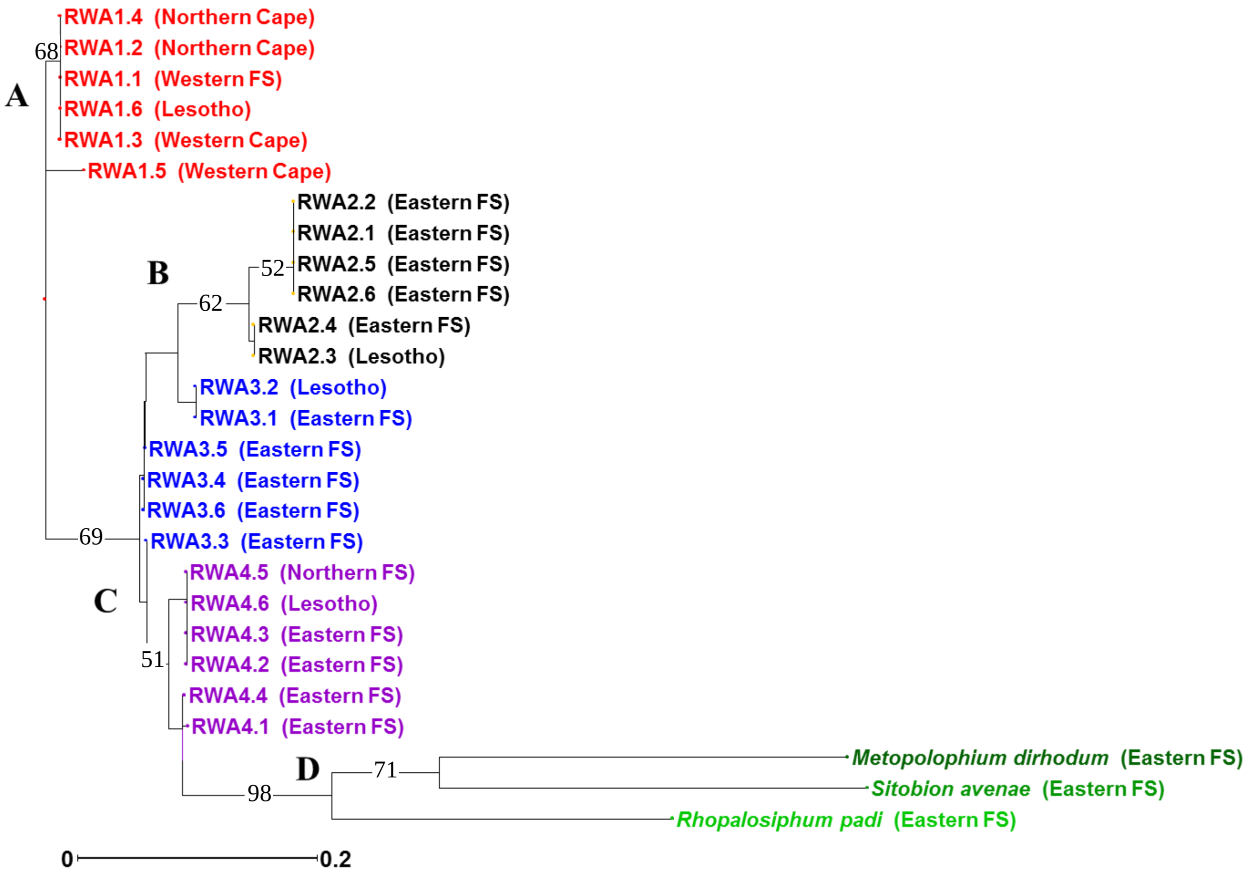

Figure 4.10. Phylogenetic analysis of twenty-four Russian wheat aphid and three control specimens using an allelic data matrix. The DARwin 5.0 software (Perrier *et al.*, 2003) was used to construct a phylogram with the allele size data matrix based on twelve microsatellite primer sets. Red font represents biotype RWASA1 (RWA1.1-RWA1.6), black biotype RWASA2 (RWA2.1-RWA2.6), blue biotype RWASA3 (RWA3.1-RWA3.6) and purple biotype RWASA4 (RWA4.1-RWA4.6). The three control aphids *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum* were indicated in green. Bootstrap values are indicated on branches.

The optimal number of sub-populations was calculated as $K = 2$ with some support at $K = 6$ (Fig. 4.11). When the K value was subsequently increased to 3, three well defined sub-populations with little admixture were found (Fig. 4.12). However, at $K = 4$ and $K = 5$, severe admixture within one sub-population was found without further division into more sub-populations. It was only at $K = 6$ that further subdivision into a fourth sub-population was found.

Based on these results, it was decided to re-analyse the data at $K = 3$ and $K = 6$ (Fig. 4.12).

At $K = 3$, RWA biotype 2 formed sub-population A, RWA biotype 1, 3 and 4 formed sub-population B and the control aphid specimens formed sub-population C (Fig. 4.13A). Within sub-population B, RWA biotype 4 shared slight admixture with sub-population A. The division of specimens into the three sub-populations was highly significant as indicated by $F_{ST} = 0.76882$. According to the AMOVA analysis in Table 4.1, the percentage variation amongst groups were 42.14%, among populations within groups 34.74% and within populations 23.11%.

When $K = 6$, RWA biotype 1 formed sub-population C separately from RWA biotype 3 and 4 (sub-population B), RWA biotype 2 (sub-population A) and the control specimens (sub-population D) (Fig. 4.13B). Limited admixture with sub-populations A and C was evident within sub-population B. This sub-division was again significant with an $F_{ST} = 0.76882$ where most of the variation was attributed to variation among groups (47.67%) and less among populations within groups (27.58%) and within populations (25.47%) (Table 4.2).

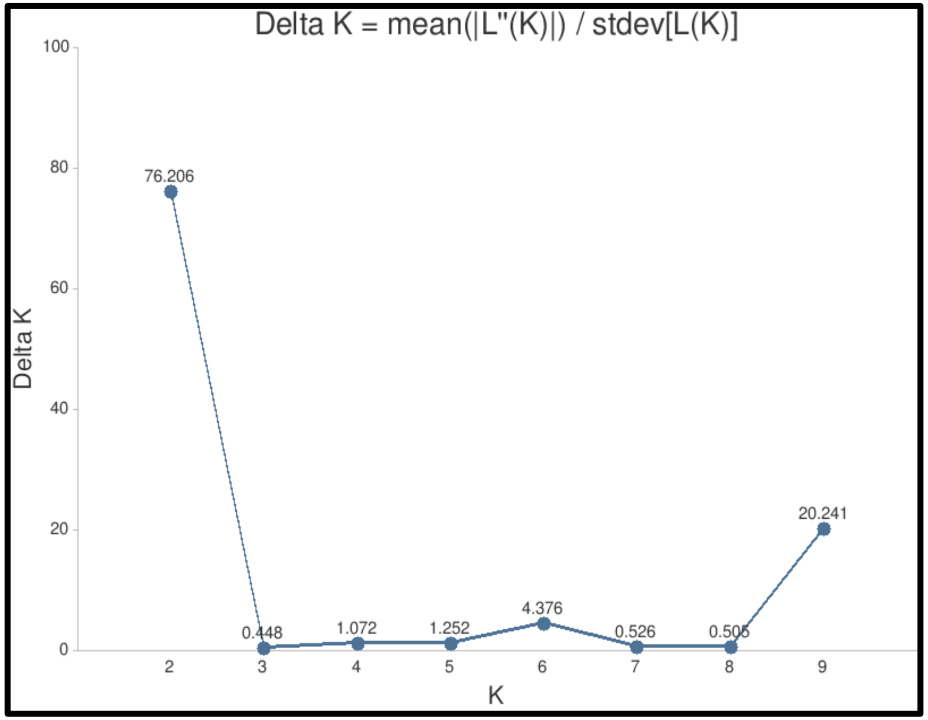

Figure 4.11. The Δ*K* graph generated in CLUMPAK indicating the optimal number of sub-populations for twenty-four Russian wheat aphid and three control specimens (Evanno *et al*., 2005).

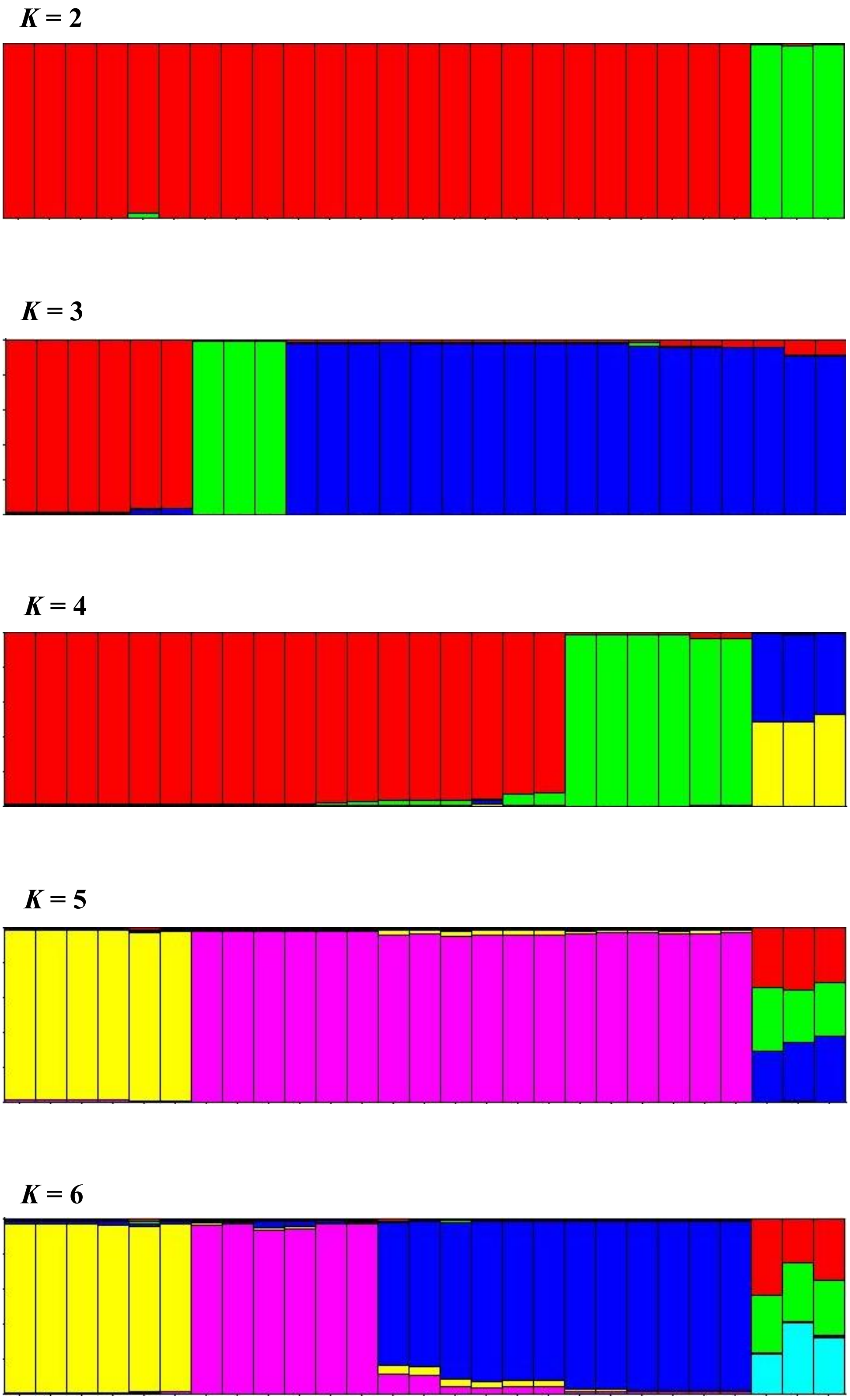

Figure 4.12. Division of twenty-four Russian wheat aphid and three control aphid specimens into sub-populations using STRUCTURE with *K* values ranging from 2 to 6.

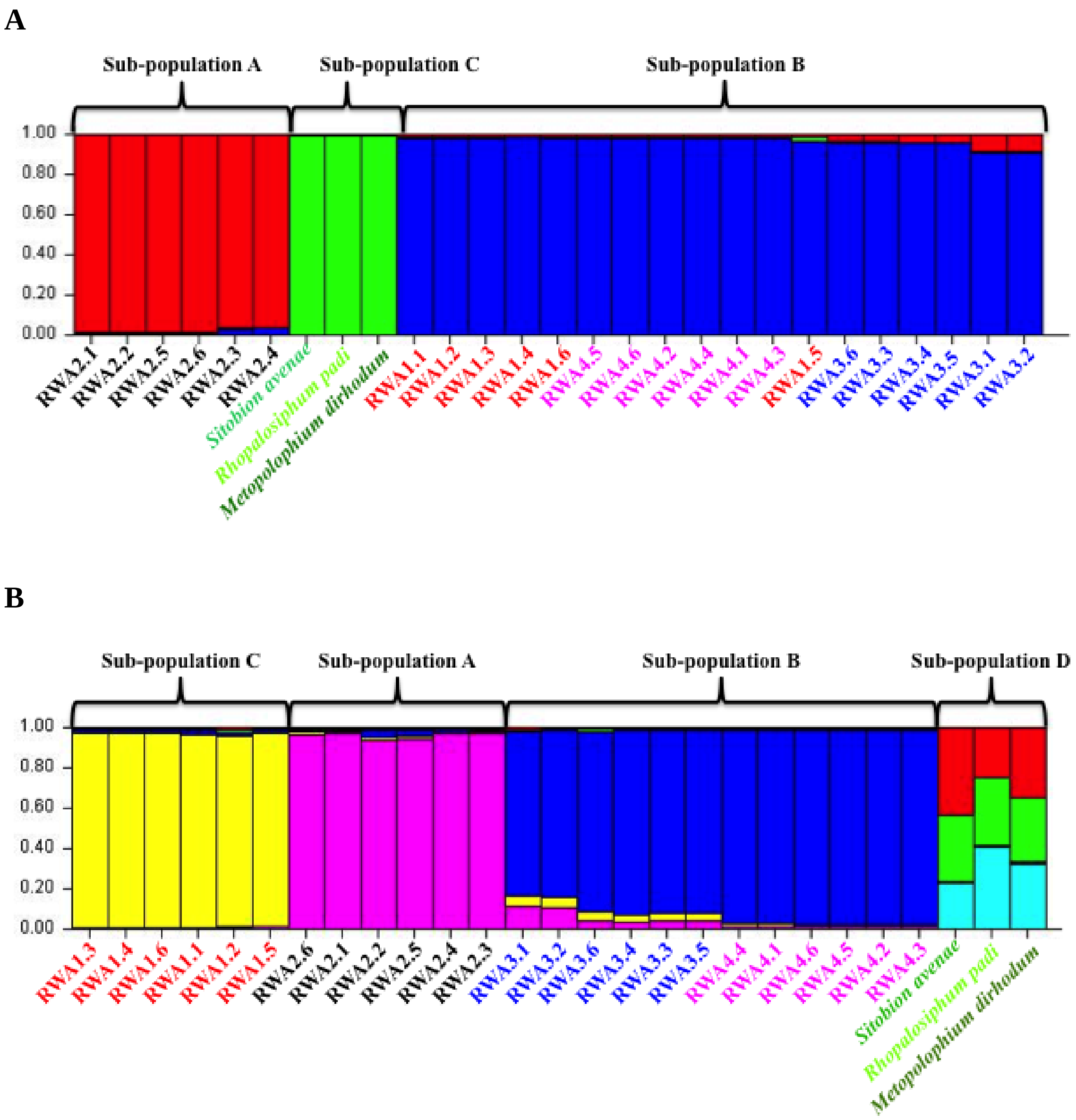

Figure 4.13. Division of twenty-four Russian wheat aphid and three control specimens into three (A) and six (B) sub-populations using STRUCTURE.

Table 4.1. Analysis of Molecular Variance results using Arlequin version 3.5.1.2 for *K* = 3.

***K* = 3**			
AMOVA design and results (average over 24 loci):			
Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	31.686	1.45816	42.14303
Among populations within groups	16.100	1.20199	34.73933
Within populations	16.833	0.79988	23.11764
Total	64.619	3.46004	

Table 4.2. Analysis of Molecular Variance results using Arlequin version 3.5.1.2 for *K* = 6.

***K* = 6**			
AMOVA design and results (average over 24 loci):			
Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	41.786	1.45034	46.67484
Among populations within groups	6.000	6.000	27.58351
Within populations	16.833	0.79988	25.74166
Total	64.619	3.10733	

Chapter 5

Discussion

The overall aim of this study was to develop a genetic marker that could rapidly distinguish between the four RWA biotype populations in SA. To achieve this, three different molecular marker techniques were investigated to determine their ability to distinguish between the biotypes. These marker techniques were AFLP, cDNA-AFLP and microsatellites. The successful completion of the project would allow us to increase the efficiency of identifying different biotypes. DNA fingerprinting is much faster than phenotyping, where a set of wheat differentials is used to identify the biotypes. In addition, since few aphids are required for fingerprinting, it is not necessary to increase the aphid colonies, thus minimizing the risk of contamination.

Vos *et al.* (1995) first described the AFLP technique, which differed from RAPD fingerprinting, since it is a more robust and repeatable technique that targets the whole genome of the organism. Restriction enzymes are used to recognize and digest DNA at short loci called palindromes to generate unique fingerprint patterns. Single nucleotide mutations can either create new palindromes or alter existing ones, thereby leading to changes in the number and/or location of these palindromes. The mutation rate per locus for AFLP was estimated to be between 10^{-6} and 10^{-4} per generation (Bonin *et al.* 2007). Fingerprint patterns unique to different individuals are thus created in this way.

Knight *et al.* (2006) were able to differentiate between drought and non-drought tolerant individuals of *Boechera holboellii* with cDNA-AFLP. They demonstrated that it was possible to link a phenotypic trait to a molecular marker. While in principle identical to AFLP, cDNA-AFLP is a refinement of AFLP, which targets the transcriptome of individuals that represents only the expressed genes. Isolated mRNA, which is used as a starting material, is first converted to cDNA before restriction enzyme digestion. Since the RWA biotypes displayed phenotypic differences in their responses on different differential wheat lines, it was expected that these differences could be detected in the expressed genes. Changes in cDNA-AFLP fingerprinting patterns occur in the same manner as for AFLP.

Microsatellite markers are much more repeatable than RAPD markers (Botha *et al.*, 2004). They are easy to visualize and less complex than AFLP while having the ability to detect codominant inheritance of alleles. Microsatellites are distributed across the whole genome with high levels of polymorphism (Guo *et al.*, 2017). Microsatellite markers are regions of tandem repeats of basic DNA motifs that are less than six base pairs in length. They have a mutation rate between 10^{-6} and 10^{-2} mutations per locus per generation, which is higher than the standard point mutation markers (Anmarkrud *et al.*, 2008). Changes in the number of tandem repeats of a microsatellite resulting in different lengths are due to DNA polymerase errors during DNA replication. This leads to the insertion or deletion of single or multiple repeat motifs that ultimately results in changes of microsatellite lengths, thus forming different alleles (Kruglyak *et al.*, 1998). Other sources of microsatellite length variation are when DNA strands separate and re-associate incorrectly, or nucleotide duplication or substitutions events (Anmarkrud *et al.*, 2008). The main advantage of microsatellites is that they can be highly diverse, even among closely related individuals.

Puterka *et al.* (1993) conducted a genetic study on RWA sampled from various parts of the world. They used seventeen enzymes, which represented twenty isozyme loci, to investigate 36 RWA populations from eleven different countries. Three of the seventeen enzymes (β-esterase; phosphoglucose isomerase; 6-phosphogluconate dehydrogenase produced polymorphic allozyme loci. These three loci grouped the populations from different countries into five distinct clusters. Cluster 1 consisted of RWA populations from Ukraine (Crimean Peninsula), Jordan and Syria. The Ukrainian (Kerson), Kirghizian, and Moldavian populations each formed their unique cluster, while populations from Turkey, France, Canada, Mexico, USA, and SA grouped in the last cluster. These clusters correlated well with their geographic region of origin, except for the populations from Ukraine, which had populations in two different clusters. The authors noted that the populations from countries (Ukraine and Moldava) in which the holocyclic life cycle was known to occur (Kiriac *et al.*, 1990) contributed the most to the RWA diversity. The presence of the holocyclic life cycle in Ukraine was also most probably responsible for the formation of its two distinct allelomorphs.

In addition to the allozyme markers, Puterka *et al.* (1993) also used RAPD fingerprinting to describe the genetic variation of eighteen of the RWA populations, which they previously used for the allozyme studies. No Ukrainian population were included. The RAPD analysis provided more polymorphisms than the allozyme markers but supported the allozyme marker data with the same derived phylogeny. The only difference was the placement of the Kirghizian and Moldavian populations with respect to those from Syria and Jordan. The probable reason for the French, South African, Mexican, and American populations grouping in both analyses, is that they most likely shared a common ancestor. Using either the RAPD or allozyme markers, minimal genetic variation was observed within each of the populations (Puterka *et al.*, 1993).

Black *et al*. (1992) used RAPD to detect polymorphisms between RWA samples from SA and Syria. The Syrian samples included ten specimens originating from a single clone and seven South African specimens. They found polymorphisms between the populations of both countries based on four RAPD primers that were used, while some amplified bands were shared between both populations. The South African population had a set of unique amplified bands, and it was noted there were two genotypes present within the South African samples with the genotypes differing only with the presence of one band and absence of another.

Shufran and Payton (2009) investigated the variation between the five RWA biotypes (RWA1 to RWA5) found in the USA using mitochondrial DNA sequencing and RAPD and microsatellite markers. Biotypes RWA3, RWA4, RWA5 and RWA7 (which was later described) grouped into one biotype namely RWA3/7 (Puterka *et al.*, 2014) as they shared the same virulence pattern on 16 cereal genotypes. When compared to the South African RWA biotypes, RWASA4 had a similar phenotypic profile to RWA2 and RWA3/7 (*Dn*6 was not used to characterize the South African biotypes). From a total of 58 RAPD primer sets, only two showed variation between the USA biotypes. The variation was the lack of a band for RWA2 and another missing band for RWA5. The mitochondrial DNA analysis showed no variation between the biotypes for the 525 bp sequenced cytochrome oxidase subunit I (*COI*) gene fragment. The microsatellite analysis tested eight loci, of which seven showed no allelic differences. The lack of variation observed by Shufran and Payton (2009) was also observed by Puterka *et al.* (1993) 16 years earlier.

A study by Malinga *et al.* (2007) revealed polymorphisms between tropical RWA populations in Kenya using three AFLP primers with the pair E-AAC/M-CAC showing the most variation. Their results showed that the variation within the Njoro-region populations was higher than the variation between clones of the Timau-region populations. They found two distinct genotypes, and the first consisted of two Njoro-region populations that displayed a high percentage of polymorphism and the remaining populations of both the Njoro and Timau-regions. They noted that genetic variation within RWA populations in tropical or mild climate environments is thought to be uncommon because these populations undergo the anholocyclic life cycle in these environments, where RWA reproduces asexually through parthenogenesis.

Zhang *et al.* (2012) combined microsatellite marker analysis with mitochondrial DNA sequencing to investigate the Chinese RWA population. Ten microsatellites were used on 1040 RWA populations, which generated 928 multilocus genotypes (MLG). They found different levels of genetic diversity between the northern and southern sites, with the former having significantly higher levels of diversity. They also noted that there was limited gene flow between the northern and southern populations along with traces of possible founder events that occurred in the southern population. Also, in this study, sequence data from 27 populations across eighteen different locations in China was generated when two mitochondrial DNA gene regions (partial cytochrome oxidase I and the nicotinamide adenine dinucleotide dehydrogenase subunit six) were sequenced. Eighteen different haplotypes were identified, of which one was shared across all regions, while one region had fourteen unique haplotypes. This provided evidence for the long-term existence in and expansion of RWA in western China. The mitochondrial genome of RWA has a rapid evolution rate, making it a prime candidate for investigating taxonomic diversity (Foottit *et al.*, 2008) and tracking RWA migration and gene flow between populations (de Jager, 2014). Mitochondrial DNA markers have been identified to evaluate the fitness of RWA, but it has not yet been able to distinguish between different biotypes.

Zhang *et al.* (2014) suggested that their microsatellite data illustrated that the same RWA population, which originated from the Middle East, colonized SA and USA. The data were generated from 504 single apterous adults, which were collected from native populations (China, Tajikistan, Turkey, Iran and Syria), North African populations (Ethiopia and Kenya) and suspected introduced populations (SA, Mexico, USA, Chile and Argentina). Ten microsatellite loci were used for this study. A total of 370 different MLG were generated, which split into two groups, with the native and North African populations forming one group and the suspected introduce populations the other group based on genetic distance. STRUCTURE analysis also indicated that numerous RWA individuals from Turkey were identical to the South African and USA RWA specimens, similar to what Puterka *et al.* (1993) found. They concluded that a single invasion from SA led to the establishment of RWA on the North American continent.

Using cDNA sequences of seventeen putative salivary gland transcripts from pea aphid, Cui *et al.* (2012) were able to distinguish between the North American RWA1 and RWA2 biotypes. The 17 transcripts were selected since they probably coded for secreted proteins due to the presence of hydrophobic regions at the N-termini of the polypeptides. The regions amplified by cDNA from the RWA were orthologs of the pea aphid transcripts. High levels of polymorphisms were found within thirteen transcripts, while there was no variation between the other four. Although the salivary gland transcripts exhibited high levels of variation, the authors noted that the transcripts, which are unique to a biotype, are less abundant than the transcripts shared among biotypes. These unique transcripts would only be effective if they were preserved across several generations. The problem with less abundant alleles is that they could arise and disappear relatively fast, making them less effective markers to distinguish biotypes.

RWA individuals within a population are generally distinct clones as invasive populations mostly reproduce asexually (Zhang *et al*., 2014). With the lack of sexual reproduction in the South African population, it was expected that the biotypes would share an extensive genetic similarity, similar to previous studies. In the current study, the AFLP analysis of the whole genome showed little variation between the South African specimens, similar to what Puterka *et al.* (1993) described for the USA and Mexican populations. The observed variation was present between individuals within a biotype, but not in all the individuals.

Two data matrices were used to score the cDNA-AFLP data: a binary data matrix and the allelic data matrix. The reason for using both matrices was to determine which scoring method gave the more accurate phylogram that correlated closest to the previous AFLP results. The use of cDNA-AFLP results increased the degree of variation detected compared to the AFLP results, but it was still not sufficient to be linked to a specific biotype.

It was only possible using microsatellite markers to detect variation between the RWA biotypes in SA. Microsatellite primers *S16b* and *S17b* developed for *Sitobion miscanthi,* and *Dn6* and *Dn13* developed for the RWA, were the only primers to generate polymorphic alleles. Cross-species amplification with primers is not uncommon with RWA, as Cui *et al.* (2012) demonstrated when using pea aphid transcripts to investigate the RWA cDNA.

When the binary microsatellite data set was used, DARwin and STRUCTURE divided the South African RWA population into only two major sub-populations, making it impossible to distinguish the biotypes from one another. This was based on the polymorphic nature of a single primer set, *Sb17b*. The variation was again between individuals from different specimens, with no variation unique to a specific biotype.

With the allelic data set, genetic variation was detected for primers *S16b*, *Dn6* and *Dn13* that allowed us to distinguish the four biotypes from each other. This increased observed variation was due to the higher resolution power provided by the QIAxcel fragment analyser to detect different allele sizes more efficiently. STRUCTURE (K=6) divided the RWA specimens into three sub-populations, namely RWA1, RWA2 and RWA3/RWA4. This data was supported by the allelic DARwin phylogram, where RWA1 and RWA2 were separated from RWA3/RWA4. This separation was supported by the AMOVA results that showed that the most variation was between the groups.

In SA, the four RWASA biotypes are found in the main wheat production areas of the Western Cape, Northern Cape and Free State provinces (A. Jankielsohn, *personal communication*). The original RWASA1 biotype occurs mostly in the Western Cape, Northern Cape, and western FS regions, whereas RWASA2 to RWASA4 occurs in the eastern FS (Jankielsohn, 2016). This geographical split correlated well with the observed grouping of the four biotypes in the DARwin tree. The split also aligns well with the main wheat production areas, with the Western Cape/Northern Cape/western FS sub-population showing less genetic diversity than the eastern FS group. While the first only had one putative sub-population, the eastern FS group had two. The genetic variation in the RWA sub-populations within the different geographical regions is similar to what Malinga *et al.* (2007) and Zhang *et al.* (2012) observed in the Kenyan and Chinese RWA populations, respectively. Malinga *et al.* (2007) attributed variation to the micro-regions in which RWA adapted to. Zhang *et al.* (2012) also noted that their more diverse population occurred in a mountainous region. This may also be true for SA, since while the eastern FS is not a mountainous region, it borders one, namely Lesotho. Jankielsohn (2011) stated that Lesotho could serve as a green bridge for RWA, which could have added to the diversity of the eastern FS sub-populations. Another reason for the difference of variation between the two regions may be due the methods used to control RWA. In the Western Cape province, chemical control is predominantly used, whereas in the eastern FS, chemical control is used alongside resistant cultivars (Dean, 2020).

RWA also possesses an endosymbiont called *Buchnera aphidicola*, which plays a role in essential amino acid production for the aphid. The endosymbiont is closely linked to the development of the biotypes as it plays a role in the survival of the RWA on hosts with resistance genes (Swanevelder *et al.*, 2010). Small regions of the endosymbiont genome could be studied in future to investigate the genetic relationships amongst RWA populations, as it was noted that endosymbionts of RWA biotypes showed sequence variation (Shufran *et al*., 2007).

In conclusion, we were able to detect varying levels of genetic variation within the South African RWA population based on the type of technique used. The AFLP analysis showed the least variation followed by cDNA-AFLP, with microsatellites showing the most genetic diversity between the four biotypes. While we were able to detect variation, we were unable to identify markers unique to each of the biotypes. We found that genetic diversity could be linked to the geographic location of the RWA, but further studies are required to confirm this. Future studies could include sequence analysis of loci specific to the endosymbionts, mitochondrial DNA, and transcriptomes to further investigate the genetic diversity within the four biotypes that form the South African RWA population.

Chapter 6

Conclusion and Recommendations

6.1 Conclusion:

This study gave us a better understanding of the structure of the RWA population is South Africa on a genetic level. We showed that different molecular techniques provide different levels of variation using the same set of specimens. While we did not identify markers unique to a biotype, we found three microsatellite primers that were polymorphic in nature. These revealed that there were two main genetic groups consisting of four sub-populations representing the four biotypes. The four sub-populations were unevenly divided between the two main groups. We noted that the two groups corresponded with the main wheat cultivation areas, namely the Western Cape and eastern FS provinces. Further studies are required to distinguish between RWASA3 and RWASA4.

6.2 Recommendations:

Future studies on the genetic diversity between the different biotypes, should include:

- the analysis of variation within salivary proteins between the four RWA biotypes, since it is proposed that differences in virulence reside in the saliva;
- genetic analysis of mitochondrial loci of the four RWA biotypes since this was shown to be effective to track migration of RWA in other countries. It will allow the possible detection of gene flow between the different wheat production areas in SA;
- genetic analysis of the RWA endosymbiont since it can help the RWA overcome host resistance and contributes to biotype development.

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