Characterization of the mitochondrial genomes of *Diuraphis noxia* biotypes

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Declaration

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Abstract

Diuraphis noxia (Kurdjumov, Hemiptera, Aphididae) commonly known as the Russian wheat aphid (RWA), is a small phloem-feeding pest of wheat (*Triticum aestivum* L). Virulent *D. noxia* biotypes that are able to feed on previously resistant wheat cultivars continue to develop and therefor the identification of factors contributing to virulence is vital. Since energy metabolism plays a key role in the survival of organisms, genes and processes involved in the production and regulation of energy may be key contributors to virulence: such as mitochondria and the NAD^+/NADH that reflects the health and metabolic activities of a cell. The involvement of carotenoids in the generation of energy through a photosynthesis-like process may be an important factor, as well as its contribution to aphid immunity through mediation of oxidative stress. The complete mitochondrial genome of global *Diuraphis noxia* populations was characterised using Next Generation sequencing, and was found to be 15 721bp in size and consisting of 38 genes typically found within most insects. Single nucleotide polymorphism (SNP) analyses of the genomes of nine populations revealed 125 SNPs in the protein coding genes with the majority of the SNPs occurring in the *ND* genes, and the least in the *ND4L* gene. Low SNP variant frequency was found for the *atp6* and *atp8* genes, which differed from other reports in the Hemiptera. Variable *ND5* expression levels were observed among the biotypes, although no correlation was apparent between *ND5* expression and the virulence associated with each biotype. Whereas *atp6* transcription was higher in the highly virulent biotype (SAM) under normal and stressful conditions in comparison to the least virulent biotype (SA1). A significantly higher NAD^+/NADH ratio was also observed for the SAM biotype under stressful conditions in comparison to the lesser virulent biotypes. UPLC-MS analysis did not reveal any lycopene or β-carotene due to low compound concentrations in the extracted samples but various hydrophobic compounds were present in different concentrations among the biotypes. The *carotene desaturase* expression profile revealed that SA1 had the lowest relative expression of the gene involved in carotenoid products, while SAM had the highest, under normal and stressful conditions. The results indicate that sequence conservation in mitochondrial genes are associated with key energy processes to maintain a state of homeostasis under variable conditions and that the generation of energy is a contributing factor to the virulence development of *D. noxia.* The results also show that carotenoids may possibly contribute to fitness of *D. noxia* through reactive oxygen species scavenging or the production of additional energy, but further investigation is needed for confirmation.

Uittreksel

Diuraphis noxia (Kurdjumov, Hemiptera, Aphididae) algemeen bekend as die Russiese koringluis (RWA), is ‘n klein floëem-voedende pes van koring (*Triticum aestivum* L). Virulente *D. noxia* biotipes wat instaat is om op voorheen bestande koring kultivars te voed gaan ontwikkel voortdurend, en daarom is die identifisering van faktore wat kan bydrae tot virulensie so belangrik. Omdat energie-metabolisme ‘n sleutelrol in die oorlewing van organismes speel, kan gene en prosesse wat by die produksie en regulering van energie betrokke is belangrike bydraers tot virulensie lewer: soos onder andere mitokondria en die NAD^+/NADH-verhouding wat die gesondheid en metaboliese aktiwiteit van ‘n sel reflekteer. Die betrokkenheid van karotenoïede in die produksie van energie deur 'n fotosintese-verwante proses kan 'n belangrike faktor bydraend tot luis fiksheid wees, asook die bydra daarvan tot plantluis-immuniteit deur bemiddeling van oksidatiewe stres. Die volledige mitochondriale genoom van globale *Diuraphis noxia* populasies is met behulp van volgende generasie DNA volgordebepaling gekarrakteriseer, en daar is bevind dat dit 15 721 bp in grootte is en uit 38 gene bestaan wat tipies binne insekte voorkom. Enkelnukleotied-polimorfisme (SNP) ontleding van die genome van nege populasies het onthul dat daar 125 SNPs in die proteïen-koderende gene voorkom, met die meerderheid van die SNPs in die *ND*-gene, en die minste in die *ND4L*-geen. Lae SNP-frekwensies is gevind vir die *atp6*- en *atp8*- gene, wat verskil van verslae oor ander Hemiptera. Veranderlike *ND5*-uitdrukkingsvlakke onder die biotipes is waargeneem, alhoewel geen korrelasie duidelik was tussen *ND5*-uitdrukking en die virulensie geassosieer met elke biotipe nie. Die transkripsie van *atp6* was hoër in die hoogs virulente biotipe (SAM) onder normale en stresvolle toestande in vergelyking met die minste virulente biotipe (SA1). ‘n Aansienlike hoër NAD^+/NADH-verhouding is ook waargeneem vir die SAM-biotipe onder spanningsvolle omstandighede in vergelyking met die minder virulente biotipes. UPLC-MS-analise het geen likopeen of β-karoteen geïdentifiseer nie as gevolg van lae verbinding konsentrasies in die onttrekte monsters, maar verskeie hidrofobiese verbindings was in verskillende konsentrasies tussen die biotipes teenwoordig. Die *karoteen desaturase*-uitdrukkingsprofiel het aangetoon dat SA1 die laagste relatiewe uitdrukking van gene betrokke by karotenoïed produksie het, terwyl SAM die hoogste relatiewe uitdrukking onder normale en spanningsvolle omstandighede het. Die resultate van die studie dui daarop dat die volgorde bewaring in mitochondriale gene verband hou met die sleutel energie prosesse om 'n toestand van homeostase onder wisselende omstandighede te handhaaf en dat die produksie van energie 'n bydraende faktor tot die ontwikkeling van virulensie in *D. noxia* is. Die resultate toon ook aan dat karotenoïede moontlik kan bydra tot fiksheid van *D. noxia*

deur reaktiewe suurstofspesies te aas of deur die produksie van addisionele energie, maar verdere ondersoeke word benodig ter bevestiging.

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List of Abbreviations

ºC – Degrees Celcius

ADP – adenosine diphosphate

ATP – adenosine triphosphate

atp6 – ATP subunit a

Bp – Base pair(s)

BLAST – Basic local alignment search tool

COI – Cytochrome oxidase 1

COII – Cytochrome oxidase 2

COIII – Cytochrome oxidase 3

Cytb – Cytochrome B

Dn – Diuraphis noxia

ET – ethylene

ETC – electron transport chain

$FADH_2$ – reduced flavin adenine dinucleotide

Gb – gigabase(s)

H – hours

HR – hypersensitive response

JA – jasmonic acid

kb – kilobases

L32 – ribosomal protein L32

Mb – megabase(s)

Min – minutes

mL – millilitre(s)

mm – millimetre

MS - mass spectrometry

NAD – nicotinamide adenine dinucleotide

NADH – reduced nicotinamide adenine dinucleotide

NCBI – National Centre for Biotechnology Information

ND1 – NADH dehydrogenase subunit 1

ND2 – NADH dehydrogenase subunit 2

ND3 - NADH dehydrogenase subunit 3

ND4 – NADH dehydrogenase subunit 4

ND4L - NADH dehydrogenase subunit 4L

ND5 – NADH dehydrogenase subunit 5

ND6 - NADH dehydrogenase subunit 6

NGS – next-generation sequencing

NILs – near isogenic lines

nm – nanometre

NRF – National Research Foundation

PCD – protein coding gene

PCR – polymerase chain reaction

qPCR –quantitative polymerase chain reaction

R – ressistance genes

ROS – reactive oxygen species

RWA – Russian wheat aphid

SA – salicyclic acid

SA – South Africa

Sec – seconds

SNP – Single Nucleotide Polymorphism

µl – microliter(s)

µm – micrometre(s)

UPLC – ultra high performance liquid chromatography

US – United States

UV – ultraviolet

v/v – volume/volume

Chapter 1

Introduction

1.1 Introduction

Diuraphis noxia (Kurdjumov, Hemiptera, Aphididae) commonly known as the Russian wheat aphid (RWA), is a serious agricultural pest of wheat (*Triticum aestivum* L) and cause significant economic damage on wheat in most wheat producing countries. *Diuraphis noxia* is characterized by the development of virulent biotypes that are able to feed on previously resistant wheat cultivars (Burd *et al.,* 2006) and therefor counteracts the host's defensive responses. Understanding the on-going evolutionary battle between *D. noxia* and their wheat hosts (Botha 2013) and contributing factors to virulence development in *D. noxia* are thus essential for scientists and plant breeders.

Since energy metabolism plays a key role in the survival of an organism, as well as in adaptation and tolerance to stress (Sokolova *et al.,* 2012), it's conceivable to propose that available energy levels may play a vital role in the virulence of *D. noxia* biotypes. Feeding stressors such as a low concentration of nutrients, chemical compounds present in the host phloem, resistance genes; and environmental stressors such as temperature and light, therefor likely affects the energy balance of an aphid and strains systems that are involved in energy acquisition, conversion and conservation, to maintain a state of homeostasis (Sokolova *et al.,* 2012).

Two main processes are responsible for the acquisition and conversion of energy from the environment: i.e., photosynthesis and the eukaryotic energy pathway. The presence of a photosynthesis-like process (phototrophy) in the pea aphid that results in an increase in available energy through the utilization of carotenoid molecules was reported in 2012 by Valmalette *et al*. Not only is this process involved in the acquisition and conversion of energy, but also in the scavenging of reactive oxygen species (ROS) produced by the host. A hypersensitive response is initiated by the plant in response to aphid feeding that produces toxic ROS to combat aphid infestation. To ensure survival during these stressful conditions, the aphid needs to produce ROS scavenging enzymes or antioxidants (carotenoids) responsible for the detoxification of ROS (Heidari *et al.,* 2012). These carotenoids can protect insects from ROS, and may thus contribute to the immune response in arthropods (Heath *et al.,* 2012) and subsequently to the virulence of *D. noxia*. The eukaryotic energy pathway on the other hand entails glycolysis, the citric acid cycle and oxidative phosphorylation, for which the mitochondrion is essential in the generation and conversion of energy molecules, and as such a comparison of these processes among the *D. noxia* biotypes may provide some clues relating to the virulence of *D. noxia*.

The energy costs associated with attack, defensive and elusive mechanisms of *D. noxia* are expensive, such as protein synthesis of elicitors against the resistance genes of the host plant. Thus, certain metabolic adaptations may be present in virulent biotypes that help them overcome stressful conditions such as flexible allocation of energy, metabolic control or the utilization of different metabolic processes responsible for energy acquisition and conversion (Sokolova *et al.*, 2012).

We therefor want to know if mitochondria contributes to the virulence associated with D. noxia biotypes through energy generation and if carotenoids are involved in enhancing aphid fitness in the scavenging of ROS as produced by the plants defences and by the production of energy through phototrophy. In order to answer these research questions, the aim of the present study was to determine if energy metabolism contributes to the virulence of *D. noxia* biotypes, and to what extent. Therefor the objectives were firstly, to observe mitochondrial sequence diversity using a bio-informatics approach utilizing Next-Gen sequencing, in order to derive a consensus mitochondrial genome for *D. noxia*. Secondly, to establish if mitochondrial sequence diversity influences mitochondrial gene regulation, as well as the production of energy and whether it has fitness consequences. Thirdly, to elucidate if virulent biotypes display a higher degree of metabolic regulation in comparison to lesser virulent biotypes. And lastly, whether variable carotenoid levels are found among *D. noxia* biotypes, which may indicate some level of involvement in virulence through increased immunity by ROS scavenging and through the production of energy by phototrophy.

Chapter 2 of this dissertation contains a brief background on *D. noxia,* its relationship to wheat, virulent biotypes, the mitochondrion, energy metabolism, and adaptations of aphids to deal with stress.

Chapter 3 focusses on mitochondrial sequence variation among the members of the Hemiptera, Sternorrhyncha (whiteflies, aphids and psyllids) and more importantly, amongst nine global *D. noxia* populations, in order to resolve some of the questions relating to the sequence diversity and difference in fitness of *D. noxia* biotypes.

Chapter 4 examines the energy metabolism of *D. noxia* biotypes by determining whether metabolic adaptations and certain trends in energy regulation are apparent amongst the biotypes and whether it plays a significant role in the virulence of *D. noxia* biotypes.

Chapter 5 consists of a summary of the main findings of this study and the significance thereof.

Appendix A contains tables of primer sequences, and of the sequences used in mitochondrial comparisons, and contains supplementary figures of results relevant to chapter 3.

Appendix B contains standard curves, melting curves, qPCR primer sequences and supplementary figures of results from the spectrometry analysis relevant to chapter 4.

1.2 Preface

The findings obtained and presented in this dissertation are the results of a study undertaken between January 2013 and October 2014 in the Department of Genetics, University of Stellenbosch, under the supervision of Prof AM Botha-Oberholster.

Research outputs:

De Jager, L., Burger, N.F.V. and Botha-Oberholster, A-M. 2014**.** Complete mitochondrial genome of *Diuraphis noxia* (Hemiptera: Aphididae) from nine populations, SNP variation between populations, and comparison with other Aphididae species. *African Entomology,* 22(4): 847-862.

De Jager, L. and Botha-Oberholster, A-M. 2014**.** Biotypic sequence diversity of the mitochondrial genome of *Diuraphis noxia*. Biennial International Plant Resstance to Insects (IPRI) Conference. Internationsal Oral Presentation, Marrakesh, Morocco.

De Jager, L. and Botha-Oberholster, A-M. 2014. Mitochondria of aphids – What can we learn? NRF/Kenyan bilateral bioinformatics workshop. Local Oral Presentation, Stellenbosch, South Africa**.**

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Chapter 2

Literature Review

2.1 Aphids

Approximately 5000 species of aphids (Remaudière and Remaudière 1997) are spread across the world with several pest aphid species colonizing major world crops such as wheat, resulting in considerable economic damage worldwide (Smith and Chuang 2013). Aphids cause severe plant damage either directly by their feeding activities by reducing plant growth or indirectly by transmitting devastating plant viruses to their host (Tagu *et al.,* 2008). This exceptional ability to transmit disease, coupled with a high reproductive rate make aphids some of the most devastating pests known in agriculture to date (Song and Liang 2009).

Aphids are small soft-bodied insects that belong to the order Hemiptera (Sternorrhyncha), which also includes whiteflies, mealybugs and psyllids. The members of this order are exclusively adapted to utilize phloem sap as a dominant or sole food source (Douglas 2006). Furthermore, aphids are grouped into the Aphididae family that consists of “true” aphids and the subfamily Aphidinae which comprises of many widespread aphid pests of food crops. Aphids are highly adapted to environmental conditions and are host specific, for example, the generalist feeders *Schizaphis graminum* (Greenbug) feeds on spring grain and *Acyrthosiphon pisum* (Pea aphid) feeds on legume crops, whilst *D. noxia* (Russian wheat aphid) is known as a specialist and prefers feeding on wheat and barley, all of which are considered as agricultural pests.

Aphids contain salivary glands and modified mouthparts called stylets that are fundamental in their complex relationship with host plants, through suppression or subvertion of host plant defences in order to establish phloem sap feeding (Will *et al.,* 2007). Whilst feeding, aphids penetrate the leaf tissue intracellularly with its stylet to reach the phloem, while saliva is continually excreted until feeding stops. Aphids use their saliva to modulate plant defense responses and thus two distinct types of saliva are secreted during the feeding process, namely a proteinaceous gelatinous saliva and a soluble watery saliva (Miles 1959). The gelling saliva consists of a mixture of proteins such as phenoloxidases and glucosidases and creates a semi rigid sheath around the stylet and facilitates leaf penetration into the phloem through this sheath path. This salivary sheath insulates the aphid’s stylet from making contact with the plant and thus avoids activation of plant defenses (Miles 1999). To successfully continue feeding, the aphid secretes watery saliva consisting of numerous amino acids and enzymes such as oxidases, hydrolases, pectinases and cellulases to modify phloem chemistry and to avoid further elicitation of plant defenses (Nicholson *et al.,* 2012; Goggin 2007; Will *et al.,* 2007). Aphids use their stylets to deliver salivary proteins or

ellictors into the phloem to deceive their hosts and disguise them by influencing their hosts' defense-signalling pathways, wound healing and volatile emissions (Walling 2008).

Phloem sap is known as an exclusive diet for animals and generally contains a high concentration of sugars, but low concentrations of nitrogenous compounds, particularly certain essential amino acids (Dadd 1985). Douglas (2006) found that the phloem sap's essential amino acid content is insufficient to support the exponential growth rate that has been observed in aphids. According to Goggin (2007) aphids are able to manipulate the nutritional quality of susceptible host plants by inducing foliar damage and thus mobilizing resources to their feeding sites. Protein breakdown occurs in the infected leaves and the essential amino acid concentration increases, improving the nutritional quality of the phloem sap (Sandström *et al*., 1999). Telang *et al.,* (1999) provided proof that Russian wheat aphid feeding results in an increased concentration of the ten essential amino acids along with prolonged infestation time and increased feeding damage, providing a nutritionally enhanced phloem diet. Although the aphid benefits from this manipulation process, it still does not fulfill their amino acid requirements.

Aphids have overcome this dietary deficit through the acquisition of bacterial endosymbionts that co-evolved with them to form a mutualistic symbiotic relationship over 150 million years ago (Botha 2013). This essential amino acid deficiency is compensated for by the biosynthetic contribution of their endosymbiotic bacteria. Endosymbionts are gamma-proteobacteria that are localized within specialized insect cells called bacteriocytes inside the abdomen of aphids. Few aphid species possess only a primary endosymbiont known as *Buchnera aphidicola,* while most aphids also contain a secondary endosymbiont such as the pea aphid that is able to host at least seven genera of facultative endosymbionts (Oliver *et al.,* 2010; Koga *et al*., 2003). *Buchnera* supplies its aphid host with essential amino acids and in return it receives a stable and nutrient-rich intracellular environment. The bacterial endosymbiont thus enables the aphid to maintain this limiting diet and still multiply generously (Tagu *et al*., 2008). *Buchnera* no longer possess the genes/metabolic pathways to synthesize non-essential amino acids and is thus in turn, dependent on its insect host for the constant supply of these amino acids and other essential metabolites (Itoh *et al*., 2002). These findings illustrate that this mutualistic relationship is based on a compulsory nutritional exchange between the aphid and *Buchnera* (Wernegreen and Moran 2000) and that symbiosis was a prerequisite for the colonization of specialized ecological niches and for the evolution of aphids (Moran and Degnan 2006).

This mutualistic symbiosis coupled to the aphids' ability to evade detection, suppress plant defense responses and alter plant metabolism has enabled aphids to become some of the worlds' most widespread devastating agricultural pests known today. Various economically important aphid pests of wheat exist today such as the English grain aphid, *Sitobion avenae* (*F.*), greenbug, *S. graminum* (Rondani), *Metopolophium dirhodum* (Walker), bird cherry-oat aphid, *Rhopalosiphum padi* (L.) and last but not least, the Russian wheat aphid, *D. noxia* (Schotzko and Bosque-Perez 2000).

2.2 *Diuraphis noxia* (*D. noxia*)

2.2.1 *D. noxia* host

Common bread wheat (*Triticum aestivum L*) is a highly versatile cereal grain that has been domesticated over ten thousand years and is now the second most cultivated crop worldwide (Gill *et al*., 2004). The hexaploid nature of wheat makes it extremely difficult to study and understand plant-pest interactions, since it is a combination of three genomes (A, B and D) with a total of 42 chromosomes and a haploid genome size of 16,000 Mb (Gill *et al*., 2004; Brenchley *et al.,* 2012). Wheat consists of winter and spring varieties that self-pollinate, simplifying selection of beneficial cultivars and so has made conventional breeding the main source for crop improvement.

Wheat is the second most important source of carbohydrates for human consumption and global yields declined by 1% from 1997 to 2007 (Ainsworth and Ort 2010) while the global population increased from 5.8 billion to 6.6 billion people. According to Edgerton (2009), the demand for wheat is expected to increase with 15%, or approximately 200 million tons per year, within the next decade. This also applies to South Africa where it forms the staple diet of many South Africans, and because of its high nutritional value it is classified as a vital field crop. Wheat grain is used in the manufacturing of flour, pasta, brewing, feeds and has also been used as a source for bio-fuel.

In South Africa hard winter wheat is produced in the central and eastern Free State, while spring wheat varieties are produced in the Western Cape. An ever increasing need for wheat coupled with a growing global population necessitates the development of wheat varieties with increased tolerance to stressors. Wheat production is influenced predominantly by abiotic stressors (drought and heat stress) and biotic stressors (i.e., pathogens and pests) of whom aphids feature prominently (Botha *et al.,* 2006). Aphid feeding results in large scale damage to wheat crops and subsequent yield losses (Marasas *et al.,* 2005). Understanding

the aphid-wheat interaction is thus of critical importance to develop resistant cultivars against this pest.

2.2.2 *D. noxia* overview

The Russian Wheat Aphid (RWA), *D. noxia* (Kurdjumov), is a small (<2.0 mm), spindle shaped gray-green phloem-feeding insect that is believed to have co-evolved with wheat since its original domestication 15 000 years ago (Botha 2013). *D. noxia* belongs to the family Aphididae which are known as serious agricultural pests since their rapid population growth allows for quick infestation of their host, *T. aestivum*, and causing significant damage. *D. noxia* feeding on susceptible plants results in longitudinal white and yellow chlorotic streaking (Fig. 2.1.A), leaf rolling (Fig. 2.1.B), leaf and head trapping (Fig. 2.1.C) and stunted growth which leads to the eventual death of the host under heavy infestation (Botha *et al*., 2006; Lapitan *et al.,* 2007). Ultrastructural damage is present in the wheat leaves and involves the disruption of chloroplasts, chlorophyll content loss and emptying of cells (Fouche *et al.,* 1984) which leads to reduced photosynthetic ability of the host plant. Individual plant losses can be as high as 90% due to *D. noxia* infestation (Du Toit and Walters 1984).

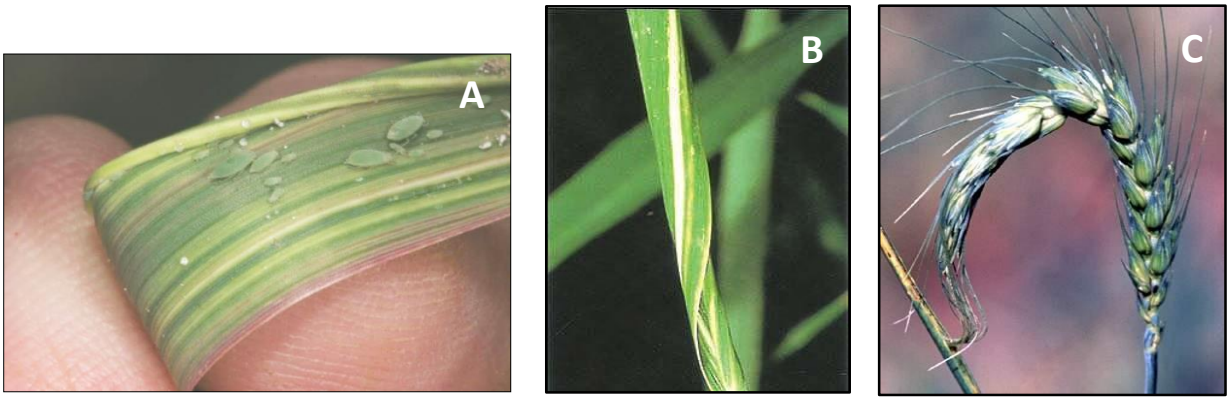

Fig. 2.1. *D. noxia* feeding symptoms in susceptible wheat plants. A.) Chlorotic streaking (http://entomology.k-state.edu/extension/current-topics/05.html). B.) Leaf rolling (http://ucanr.org). C.) Head trapping (http://platwise.org)

The earliest published reference to *D. noxia* as a pest was in 1901 (Kovalev *et al*., 1991) in Crimea and by 1990 it has spread to all cereal producing countries (Tolmay 2006), except Australia, with South Africa and the United States suffering the most severe losses to wheat production (Botha *et al.,* 2006). Economic losses in the United States alone have exceeded $650 million for the time period from 1986 to 1990, due to aphid infestations (Reed *et al*., 1992). Chemical control proved insufficient in defending the crops against this pest due to

leaf rolling that protects the aphid from contact pesticides. Thus, resistant wheat cultivars were developed to replace the chemical control of *D. noxia* infestations due to the low cost of host plant resistance and the ease of production. These resistant wheat cultivars containing *Dn* resistance genes proved an ideal method of managing *D. noxia* populations in wheat production areas prone to infestations (Burd *et al*., 2006).

2.2.3 *D. noxia* - Wheat Interaction

Durable resistant wheat cultivars are developed through the incorporation of a combination of *Dn* resistance genes (gene pyramiding) into *D. noxia*-resistant varieties to provide sufficient and improved protection against *D. noxia* feeding. As little as 14 *Dn* genes have been identified in wheat and its relatives, which confer resistance to various *D. noxia* biotypes although their exact function and mode of action is still largely unclear. These genes are designated as follows: *Dn1* and *Dn2* (Du Toit 1987, 1988, 1989), *Dn3* (Nkongolo *et al.,* 1991a), *Dn4* (Nkongolo *et al.,* 1991b), *Dn5* (Marais and Du Toit 1993), *Dn6* (Saidi and Quick 1996), *Dn7* (Marais and Du Toit 1993), *Dn8* and *Dn9* (Liu *et al.,* 2001), *Dnx* (Harvey and Martin 1990), *Dny* (Smith *et al.,* 2004), *Dn2414 (*Peng *et al.,* 2007), *Dn626580* (Valdez 2012), and *DnCI2401* (Fazel-Najafabadi 2014, (unpublished)).

Previous studies providefmuru evidence for the existence of elicitors, also known as effector proteins, from *D. noxia* that are recognized by the resistance (*R*) genes' products of the plant receptor, following the gene-for-gene model (Flor 1971; Keen 1990; Botha *et al.,* 2006; Lapitan *et al*., 2007). Defence signalling pathways of the plant are activated once the presence of the aphid is detected and trigger structural and biochemical defense mechanisms to deter aphid infestation (Botha *et al*., 2006, 2010; Boyko 2006; Murugan and Smith 2012; Smith *et al.,* 2005, 2010; Smith and Boyko 2007; Smith and Clement 2012; Van der Westhuizen *et al.,* 1998) either non-specifically through general effectors or specifically through protein effectors or elicitors (Johal *et al.,* 1995; Argrawal and Fishbein 2006). These elicitors may trigger the plants primary immune response, also known as effector triggered immunity (ETI) (Chisholm *et al*., 2006). A hypersensitive response is initiated that makes invaded cells or tissues commit suicide as part of a process known as programmed cell death (PCD) to stop the aphid infestation. Once PCD is activated, cells or tissues are flooded with toxic chemical compounds through elevated levels of reactive oxygen species (ROS) such as H_2O_2 that results in necrosis or cell death (Botha *et al*., 2005, 2006, 2010; Boyko 2006; Smith *et al.,* 2005; Smith and Boyko 2007; Van der Westhuizen *et al.,* 1998). Other biochemical pathways also employed as effective defense against the RWA include the jasmonic acid (JA)/ethylene (ET)-mediated pathway, salicyclic acid (SA)-mediated

pathway and even intermediate pathways of the aforementioned, based on the type of resistance gene present and the *D. noxia* biotype (Botha *et al.,* 2010; Smith *et al.,* 2010).

Wheat responds to *D. noxia* feeding in a gene-for-gene manner (Botha *et al.,* 2005; Boyko *et al.,* 2006) that results in either a compatible or incompatible interaction. A resistant cultivar recognizes an aphid elicitor by a R-gene protein product and this enables an array of defence responses such as the hypersensitive response in for example *Dn1*, *Dn5* and *Dn7* containing cultivars, which results in an incompatible interaction between the host and its pest and thus significantly decreases the RWA population and thus prevent infestation (Botha *et al.,* 2005; Liu *et al.,* 2011). Compatible interactions however results in the aphid overcoming the host defensive responses and leads to the successful feeding and reproduction of the aphid on its host (Botha *et al.,* 2005; Giordanengo *et al.,* 2010). It is speculated that these eliciting agents are injected into the feeding site of *D. noxia* to counteract plant defence responses and also results in the breakdown of chloroplast machinery (chlorophyll and carotenoid loss) and cellular membranes (Botha *et al.,* 2006; Liu *et al*., 2011). These elicitors thus possibly participate in the manipulation of plant metabolism to allow compatible interactions between *D. noxia* and its host. If these protein-containing eliciting agents can be identified, a novel integrated pest management strategy (i.e., that will enable control of the infestation of aphids or minimalize the aphids' negative effect on the wheat plants) might be developed. Currently, resistant wheat cultivars are the favoured control tactic for *D. noxia* infestations due to its sustainability and because it does not increase the cost of production (Schotzko and Bosque-Perez 2000).

Various sources of resistance have been identified and advanced resistant varieties have been developed that confers different categories of resistance to *D. noxia* feeding. Resistant host varieties supress the aphid abundance or elevate the damage tolerance level of the plants. The relationship between the plant and the aphid is thus affected by the kind of resistance, which is antibiosis, antixenosis or tolerance. These mechanisms can be used solely or in combination within resistant wheat cultivars to combat *D. noxia* infestation with the use of products of molecular pathways. Antibiosis resistance negatively affects the biology of the aphid and decreases pest abundance and subsequent damage (Painter 1958). It often results in increased mortality and reduced reproduction of the aphid. Antixenosis resistance affects the behaviour of the aphid and is expressed as a non-preference host plant in terms of food, shelter, or reproduction and the aphids prefer to avoid these plants (Painter 1951, 1958; Botha *et al.,* 2008). While tolerance is when the plant is able to withstand or recover from damage caused by the aphid that would normally severly injure or kill a susceptible plant (Painter 1958; Basky 2003). Unlike antibiotic plants, tolerant plants do not employ oxidative bursts associated with the hypersensitive response that

results in elevated levels of reactive oxygen species which results in cell death and necrosis. Instead, tolerant plants retain active photosynthesis and prevent chlorosis from occurring through up regulation of components of the photosystems. While antixenotic plants use volatile organic compounds to deter aphid feeding (Botha 2013).

2.2.4 *D. noxia* biotypes

An aphid biotype is defined as a new population that is able to overcome host-plant resistance (i.e., an aphid's virulence) of a previously resistant host. In other words, a *D. noxia* biotype can be classified as an infraspecific population that is able to injure a host plant that contains specific *Dn* resistance genes that are resistant to other infraspecific populations (Burd *et al.,* 2006).

D. noxia biotypes are classified exclusively on their ability to damage resistant and susceptible wheat plants (*T. aestivum*) while feeding. Two types of classification systems exist for the differentiation of biotypes. A two-category system classifies the aphid as virulent or avirulent. The three-category system classifies the aphid as virulent, intermediate or avirulent. The classification is based solely on the phenotypic response of the plant (i.e., foliar chlorosis damage) as a direct result of aphid feeding; susceptible, intermediate or resistant (Puterka *et al.*, 2012; Burd *et al.,* 2006). The measurement of the ability of a pest to infest a new host is termed virulence (Botha 2013). Different levels of virulence are thus attributed to different biotypes using these systems. This mode of classifying aphids according to their virulence and ability to feed on hosts containing different *Dn* genes have proved useful to a certain extent.

D. noxia was first reported in South Africa and the United states in 1978 (Walters 1984) and 1986 respectively (Morrison and Peairs 1998), and resistant wheat cultivars have been available since 1993 in South Africa (Marasas *et al.,* 2005) and since 1994 in the United States (Quick *et al.,* 1996) to control the infestation of this pest. However, in 2003 and 2005 new *D. noxia* biotypes emerged in the United States and South Africa respectively, which were able to feed on these previously resistant cultivars (Haley *et al*., 2004; Tolmay *et al*., 2006). The South African *D. noxia* biotype 1 (RWASA1) described by Du Toit (1989) was susceptible to the Tugela-*Dn1* cultivar containing the *Dn1* resistance gene, but the newly emerged biotype designated as RWASA2 overcame this resistance (Jankielsohn 2011). Likewise, the new US biotype overcame the *Dn4* resistance of the cultivars used in Colorado to successfully combat the first RWA US biotype. These new resistance-breaking biotypes raised immense concerns regarding the durability of future *D. noxia* resistant wheat cultivars.

At present, there exist three wild type South African *D. noxia* biotypes (SA1 SA2 and SA3) (Jankielsohn 2011) varying in virulence levels and a highly virulent laboratory-developed (mutant) RWA biotype (SAM) (Van Zyl *et al.,* 2005) that evolved under *Dn* resistant selective pressure from the SA1 biotype. Jankielsohn (2011) screened the *D. noxia* SA1, SA2 and SA3 biotypes on previously established resistant cultivars containing the designated resistance genes *Dn1, Dn2, Dn3, Dn4, Dn5, Dn7,* and *Dn9* to designate virulence to each biotype according to the three-category system. SA1 biotype caused susceptible symptoms to cultivars containing only the *Dn3* gene and is thus the least virulent South African biotype. Cultivars containing the *Dn1, Dn2, Dn3* and *Dn9* genes were shown to be susceptible to the SA2 biotype. While the SA3 biotype is shown to be the most virulent of the field populations due to resistance against the same *Dn* genes as the SA2 biotype, but with added resistance to the *Dn4* gene. Randolph *et al.,* (2009) screened seven of the RWA biotypes found in the United States for virulence and classification. The results demonstrated that the *D. noxia* US1-7 biotypes are indeed separate biotypes and differ in virulence from one another. US1 had low virulence, while US4, US5, US6 and US7 displayed intermediate virulence, and the US2 and US3 biotypes were shown to be highly virulent. Fortunately, the *Dn7* gene remains highly resistant to all the wild South African and American *D. noxia* biotypes, but problems with grain yield quality have been associated with the wheat-rye translocation that carries the *Dn7* gene. Cultivars that contain the *Dn7* gene such as Gamtoos R exhibits antixenotic properties (Marais *et al*., 1994; Anderson *et al*., 2003; Kogan and Ortman 1978), while cultivars carrying the *Dn5* gene display antixenotic and moderately antibiotic properties against South African biotypes (Wang *et al.,* 2004) and reduces RWA populations (Smith *et al.,* 1992).

By 2006, a total of 26 resistant wheat cultivars had been released that formed part of an integrated pest control strategy against *D. noxia* that also included cultural practices and natural predators (Tolmay *et al.,* 2006). To date, a total of 16 global *D. noxia* biotypes have been identified conferring different levels of virulence and thus resistance to various combinations of *Dn* genes within resistant wheat cultivars. The continuous development of virulent *D. noxia* biotypes requires the identification and introduction of resistance genes into susceptible wheat cultivars (Bouhsinni *et al.,* 2011; Jankielsohn 2011) in combination with biological, chemical, and cultural control strategies (Botha *et al.,* 2013) in order to protect crops against this pest and prevent large scale agricultural losses.

D. noxia biotypes present in South Africa, and in many other international locations, reproduce through facultative parthenogenesis, unlike in areas where *D. noxia* is considered endemic where they can also reproduce sexually. Parthenogenesis is thus a form of asexual reproduction which does not require any male aphids and results in the birth of live female

young/nymphs. This means of reproduction allows for non-recombinant vertical gene transfer from mother to offspring which allows for little genetic variation of the aphid, and the aphid's endosymbiont through successive generations (Tagu *et al*., 2008). Despite the lack of sexual recombination, biotypes still develop and the genetic basis for biotype development is still unclear. Possible explanations include random mutation, genetic drift and evolutionary adaptation to biotic stressors, such as the large scale cultivation of resistant wheat cultivars in different locations (Brinza *et al*., 2009). However, no correlation has been identified between Greenbug biotype development and deployment of resistant cultivars (Porter *et al*., 1997). Puterka *et al.,* (2007) suggests that various ecological and biological factors are associated with biotype development, not just resistant cultivars. Many aphid species use alternative hosts between harvest and planting and Weiland *et al.,* (2008) suggested that biotypes have emerged due to strong selection pressure of these non-cultivated grasses. Porter *et al.,* (1997) considered these non-cultivated hosts as an important factor in the maintenance of Greenbug genetic diversity and biotypification. *D. noxia* colonizes non-cultivated hosts such as volunteer wheat, rescue grass, wheat regrowth and common wild oat for at least a part of the year. Therefore, *D. noxia* sample collections should include populations feeding on cultivated and non-cultivated grass species in order to comprehensively monitor the diversity and distribution of *D. noxia* biotypes to manage this pest successfully (Jankielsohn 2011).

Prior to 2003, US *D. noxia* biotypes were believed to be biotypically and genetically uniform. After the detection of a new US biotype in 2003, the biotypes were investigated for genetic diversity and differences in the mitochondrial *cytochrome oxidase subunit 1* (*COI)* gene was found among biotypes. Biotypes grouped together (US3, US4 and US7) according to the two-category classification system was shown to differ genetically, regardless of their grouping based on virulence (Liu *et al.,* 2010). This demonstrates how a single biotype category can be represented by a number of genotypes, and that a new *D. noxia* introduction which differs in genotype can go undetected if it doesn't differ in biotype as classified by these two systems. Genetic differences among aphid biotypes are now recognized and it has been suggested that these differences may affect the feeding behaviour of the aphid and in return alter the phenotypic response of the host plant to feeding (Burd *et al.,* 2006). A study by Puterka *et al.,* (1992) found a high degree of biotypic diversity in a global collection of *D. noxia* samples. Reed and Frankham (2003) have associated biotypic genetic variation with increased species fitness, implying that increased biotypic variation of *D. noxia* may increase the aphids' ability to adapt to novel wheat cultivars. Therefor it is suggested that *D. noxia* will eventually overcome any resistant source

produced and this may be worrisome for plant breeders due to resistant wheat cultivars being the most economically and ecologically attractive control method.

Burd *et al.,* (2006) noted that it is pivotal to the future success of *D. noxia* resistance to assess the amount of *D. noxia* virulence and to understand the ecological and genetic basis of biotypic variation and its relationship to *D. noxia* fitness. *D. noxia* populations are able to rapidly diversify and it's important to monitor the biotypic and genetic structure of *D. noxia* populations (Puterka *et al.,* 2012). Genotype characterization and biotype identification of aphids is thus the first step in any cereal-breeding program to discover resistance genes and to develop resistant wheat cultivars (Kharrat *et al*., 2012).

2.3 Phylogeny

Selected gene sequences that reside in insect mitochondrial genomes have become the most commonly used molecular marker for population genetics, phylogeography, molecular diagnostics and phylogenetic studies, in the past two decades (Song and Liang 2009). Mitochondrial DNA gene sequences are particularly attractive for developing phylogenies of recently diverged taxa due to their rapid evolution and their higher likelihood of tracking a short internode. Construction of phylogenetic trees is also made easier due to the maternal inheritance of mitochondrial genes and the absence of intermolecular genetic recombination (Xu *et al.,* 2011; Anstead *et al*., 2002). Many mitochondrial genes, or parts of them, have been utilized to infer phylogenetic relationships among aphid species and to estimate genetic diversity among aphid biotypes.

The most widely used DNA sequence is that of the mitochondrial *COI* gene, containing both conserved sites and sequence differences among species, and can thus be used to study divergence among species within a genus (Anstead *et al.,* 2002; Footit *et al*., 2009; Ollivier *et al.*, 2011; Chen *et al*. 2012; Kharrat *et al*., 2012; Zhang *et al*., 2012; Lee *et al*., 2012). It may perhaps reveal differences among biotypes of the same aphid species. Studies using only single markers have limitations however, and the need to incorporate additional mitochondrial genes are important for the reliable identification of species or biotypes (Anstead *et al*., 2002; Kharrat *et al*., 2012).

The genetic variation of the grain aphid, *S. avenae* in China was determined by Xu *et al.,* (2011) using partial *COI* gene sequences due to a high degree of polymorphisms previously observed by Hu *et al.,* (2008). The study however showed that sequence divergence only varied from 0.2% (one nucleotide) to 1.7% (ten nucleotides) between the demographic populations of *S. avenae*. This finding of low sequence diversity for the *COI* gene is

congruent with that of Boulding (1998) that only observed 0.4% divergence of the pea aphid, *A. pisum*, suggesting that aphids generally have low mitochondrial DNA divergence.

Recent studies have integrated several mitochondrial genes to detect and resolve taxonomic diversity in aphids (Footit *et al*., 2009). The efficiency of three mitochondrial genes were examined as potential markers by Chen *et al* (2012) to distinguish between aphids species within the subfamily Lachninae. The sequence diversity between *COI*, *COII* and *cytochrome b* (*cytb)* genes was compared. It was found that *cytb* often led to ambiguous species identification, *COI* displayed the lowest divergence of all three markers and that *COII* yielded the most accurate identification of Lachninae species. A study by Kharrat *et al.,* (2012) showed that the mitochondrial genes, *cytb* and *NADH dehydrogenase* (*ND*), can successfully be used to detect DNA polymorphisms in *S. graminum*. This allowed for the examination of mitochondrial sequence divergence and the estimation of their relationship with the reported *S. graminum* biotypes.

Genetic methods are now employed to examine levels of migration, movement patterns and radiation of aphid biotypes. Possible dispersal methods and potential for future expansion of aphid populations into untarnished wheat-growing countries needs to be estimated using these mitochondrial genes. This is exactly what Zhang *et al.,* (2012) evaluated using the *COI*, *NADH dehydrogenase subunit 6* (*ND6*), partial *NADH dehydrogenase subunit 4L* (*ND4L*), 2 tRNA, total *ND6* and partial *cytb* genes. The study investigated the population genetics, demographic history and evolutionary adaptation of *D. noxia* in China, showing the promise of these mitochondrial genes as molecular markers for *D. noxia*.

It is essential to develop genetic markers to confidently differentiate between *D. noxia* biotypes without the use of phenotypic studies for rapid *D. noxia* biotype classification. Identification plays a vital role in the integrated management of pest aphids and early detection and risk analysis of newly introduced species or biotypes. Generating multiple complete mitochondrial genome sequences are thus necessary to gain a better understanding of species or biotype evolution/development and may provide insight into the biological functions of aphids.

2.4 The Mitochondrion

2.4.1 Endosymbiont Theory

The mitochondrion is known today as the energy-generating organelle present in eukaryotic cells, forming an essential part of life. These cytoplasmic organelles are responsible for the oxidative phases of cellular respiration and electron transport (Burger *et al.,* 2003) which provide each cell with the necessary energy to sustain life and perform routine processes.

Another cytoplasmic structure known as a chloroplast is a unique organelle associated with an energy-trapping process called photosynthesis. Chloroplasts are believed to have originated as endosymbiotic cyanobacteria, making them similar in this respect to mitochondria, although chloroplasts are found only in plants and protists.

Since the discovery of these extranuclear organellar genomes in the 1960's, the endosymbiont theory was raised. The hypothesis emphasized that eukaryotic cells started out as anaerobic organisms that lacked mitochondria and chloroplasts. Since establishing a symbiosis with a purple non-sulphur photosynthetic bacterium, the oxidative phosphorylation activities of the bacterium was beneficial to the eukaryotic cell and the photosynthetic process was no longer needed in the presence of atmospheric oxygen and was lost. The eukaryotic cell became dependent on the intracellular bacterium for survival and thus, the mitochondrion was formed. Despite the unorthodox nature of the endosymbiont theory, biologist accepted it as correct due to the substantial amount of supporting evidence. The genetic machinery of these organelles closely resembles that of prokaryotes, in that they possess the ability to duplicate themselves and transcribe and translate their genetic information. This gene expression processes that occurs in these organelles is similar to the equivalent processes found in bacteria. Furthermore, it has been found that organelle genes are more similar to the genes present in bacteria, than to eukaryotic nuclear genes (Klug *et al*., 2006). These observations thus led to the proposal that mitochondria and chloroplasts are the relics of once primitive free-living bacteria/prokaryotes that established a beneficial symbiotic association with primitive eukaryotic cells, billions of years ago.

2.4.2 Mitochondrial Structure

Mitochondria are double membrane cytoplasmic structures abundant in animal and plant cells (Burger *et al.,* 2003), especially in cells that are associated with active processes. Mitochondria consist of a porous outer membrane, selectively permeable inner membrane, intermembrane space, matrix space and cristae (Fig. 2.2.A). The outer membrane is a simple phospholipid bilayer that contains porins which makes it permeable to ions, nutrients, ATP, ADP and other small (<10 kilodaltons) molecules. These protein structures allow the passage of these important molecules with ease in and out of the mitochondria. Proteins that are involved in lipid synthesis and the conversion of lipid substrates are also present in this outer membrane. The inner membrane is a highly complex structure that is only permeable to oxygen, carbon dioxide and water. It is folded into numerous cristae which increase the total surface area of the inner membrane to hold vital protein complexes that are involved in the oxidation reactions of the electron-transport chain, ATP synthesis and the transportation of metabolites in and out of the matrix.

The region created by the outer and inner membranes is known as the intermembrane space and is important in oxidative phosphorylation, the primary function of mitochondria. It contains several enzymes that use ATP passing out from the matrix to phosphorylate other nucleotides. The matrix is a large internal space that contains the enzymes responsible for the citric acid cycle reactions and the oxidation of pyruvate and fatty acids. Dissolved water, oxygen, carbon dioxide and intermediate molecules that shuttle energy are also present inside the matrix. The matrix contains several identical copies of the mitochondrial genome, ribosomes, tRNAs and the enzymes responsible for expression of mitochondrial genes. The matrix is in close proximity to the inner membrane which allows for short diffusion time of these matrix components to inner membrane components. It is clear that these dynamic organelles are structurally and functionally engineered to produce energy as efficiently as possible, and are of great importance to scientists.

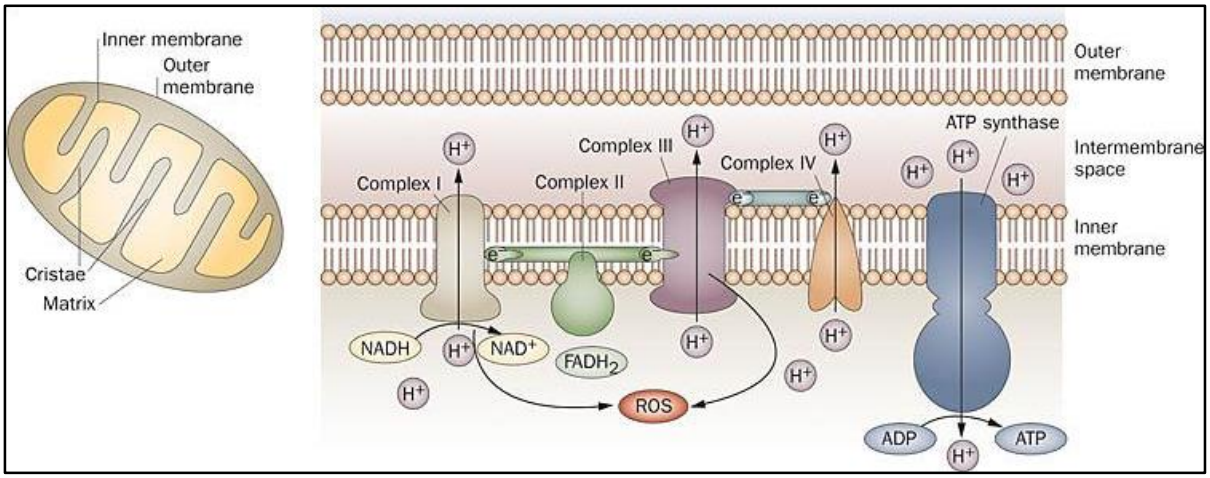

Fig.2.2. The internal structure of a mitochondrion (A) and the electron transport chain and ATP synthesis (B) (http://www.nature.com/nrrheum/journal/v7/n3/images/nrrheum.2010.213-f1.jpg)

2.4.3 Mitochondrial Genome Structure

The mitochondrial genome is characterized by its relatively small size, maternal inheritance, lack of recombination, stable and relatively short structure, and the frequent accumulation of polymorphisms. It is believed to consist of a single chromosome that is circular, although we now know that certain eukaryotes contain linear mitochondrial genomes, such as *Paramecium* and *Chlamydomonas*.

The mitochondrial genome is a double-stranded unit of genetic information with compact genomic organization and it retains limited gene contents. Mitochondrial genome sizes are variable however, and are unrelated to the complexity of the organism or the amount of

genes retained (Klug *et al*., 2006). Differences in size are mostly attributed to variations in length and organization of intergenic regions which consists of tandem repeats or stem-loop motifs. Mitochondrial genomes play an important role in metabolism, ion homeostasis, disease, apoptosis and aging, and have become a major data source for comparative genomics (Burger *et al*., 2003) due to its well conserved gene complement and rapid rate of substitutions. This also made the mitochondrial genome an informative tool for diverse evolutionary studies of animals (Chai *et al.,* 2012).

The mitochondrial genomes of insects are closed double stranded circular chromosomes generally ranging from 13-20kb in size, their gene content are well conserved and they have a low rearrangement rate. These characteristics make them a useful data set to study deep divergences of insects and molecular evolution (Hu *et al*., 2009). The mitochondrial genome typically encodes 37 genes that includes 13 protein-coding genes: Two subunits of ATPase (*atp6 and atp8*), three cytochrome c oxidase subunits (*COI, COII* and *COIII*), one cytochrome B (*cytB*), seven NADH dehydrogenase subunits (*ND1, ND2, ND3, ND4, ND5, ND6* and *ND4L*). The remaining genes encode 2 ribosomal RNA and 22 transfer RNA that are necessary to translate the protein-coding genes. Insects contain a large non-coding AT-rich region in their mitochondrial genomes that is also referred to as the control region, which is involved in the initiation and regulation of mitochondrial transcription and replication (Chai *et al.,* 2012). Despite the control region, the whole insect mitochondrial genome is compactly arranged with no introns, overlapping neighbouring genes and very limited intergenic nucleotides (Hu *et al*., 2009). The total AT content in insect mitochondrial genomes ranges from 63-88%, their start codons vary from ATA, ATG and ATAA (for the *COI* gene) and their stop codons are known as TAA and TAG. However, protein-coding genes that are followed by a downstream transfer RNA terminate with a T or TA. It is further known that the gene order of the mitochondria of aphids, psylids and many whiteflies resembles the proposed Insecta ancestral gene order and the amount of mitochondrial sequence divergence for aphids is only 13.1% which is considerable less than that of psylids and whiteflies (Baumann *et al*., 2004).

Nucleotide diversity in the mitochondrial genome of aphids has been widely studied, although only two complete mitochondrial genomes of aphids are available to date: that of *A. pisum* and *S. graminum*. Mitochondrial DNA gene sequences are particularly attractive for developing phylogenies of recently diverged taxa due to their rapid evolution and their higher likelihood of tracking a short internode, thus enabling the widespread use of mitochondrial genome sequences as molecular markers for aphid species and biotypes. Recent studies have integrated several mitochondrial genes to detect and resolve taxonomic diversity in aphids (Footit *et al*., 2009). Access to the information enclosed in the mitochondrial genome

of *D. noxia* could enhance several areas of aphid research that would include polymorphic markers to analyse population structure and migration patterns, phylogenetic studies, evolutionary biology, energy metabolism and possibly virulence associated studies.

2.4.4 Functionality of Mitochondria

The production of energy within cells is achieved through the process of cellular respiration. Cellular respiration consists of various metabolic reactions and processes and aims to convert biochemical energy from nutrients into an energy-rich molecule, adenosine triphosphate (ATP) through glycolysis and the aerobic or anaerobic pathway. Since aphids are eukaryotes that live in the presence of oxygen, the anaerobic pathway is not relevant and will thus not be discussed. Thus, energy is produced in aphids through the eukaryotic energy pathway that consists of glycolysis, the citric acid cycle and oxidative phosphorylation, with the latter two processes occurring within mitochondria in eukaryotic cells (Chai *et al*., 2012), thus making mitochondria the active sites of ATP synthesis.

The first process in the energy pathway consists of ten chemical reactions known together as glycolysis that occur in the cytoplasm of the cell, and results in the splitting and conversion of glucose into pyruvate through various phosphorylation and redox reactions. A net gain of two ATP molecules and two NADH molecules are produced per molecule of glucose converted to pyruvate (Nelson and Cox 2004). Pyruvate is then imported into the matrix of the mitochondrion where it is converted into acetyl-CoA in the presence of oxygen and generates one NADH. Acetyl-CoA enters the second energy process, the citric acid cycle, where acetyl-CoA gets oxidized to CO_2 through a process of eight steps involving different enzymes and co-enzymes. The citric acid cycle generates three more NADH molecules through the reduction of NAD^+ to NADH, and two other electron carrier molecules known as flavin adenine dinucleotide ($FADH_2$) and guanosine-5'-triphosphate (GTP). These reduced co-enzymes serve as electron carriers for biochemical reactions in the cell and carry-over hydrogen ions (protons) and high energy electrons into the respiratory electron transport chain (ETC) that is located in the inner mitochondrial membrane. The ETC and ATP synthesis are together known as oxidative phosphorylation, which is the third and last major energy process. The electrons are sequentially shuttled through a series of four protein complexes of the ETC that are imbedded in the inner mitochondrial membrane as can be seen from Fig 2.2.B, and ultimately to oxygen, where they combine to form water (Nelson and Cox 2004). Meanwhile the protons are exported from the matrix through the membrane to the intermembrane space to enhance the proton gradient. The enzyme ATP synthase utilizes the electrochemical proton gradient and undergoes a conformational

change to allow the protons to diffuse back down their gradient and leads to the production of ATP from ADP through the addition of a phosphate group (Nelson and Cox 2004).

This ATP molecule is used in cells as a coenzyme and transports chemical energy that exists within the high energy bonds between two phosphate groups (Nelson and Cox 2004). Through ATP hydrolysis, the cells are able to release this energy and other cells can then harness this energy through phosphorylation. Proteins are able to convert the chemical energy of ATP into osmotic, mechanical or synthetic cellular work. This process provides the cell with the necessary energy to fuel vital biological processes and thus to sustain life.

2.5 Adaptations of aphids

2.5.1 Carotenoids

Turning the sun's light into usable energy is a skill thought to be limited only to plants, algae, certain bacteria and solar panels. Researchers have recently discovered an aphid species, the pea aphid, which may possess this incredible ability. The process does not involve the transformation of carbon dioxide into organic compounds such as with photosynthesis, but suggests that the pea aphid holds the ability to harvest the sun's light energy and convert it to chemical energy the aphid can utilize. Valmalette *et al.,* (2012) have provided proof of an increased production of ATP molecules coupled with an increase in available sunlight and increased carotenoid levels in the pea aphid. Carotenoids are pigments that most animals obtain through their diet, however, like plants, fungi and microorganisms, aphids are also equipped to synthesize these pigments. This can be justified by the fact that an aphid's diet excludes the uptake of these hydrophobic carotenoid molecules from phloem sap (Valmalette *et al*., 2012; Novakova and Moran 2012).

Carotenoids can be divided into two groups: carotenes and xantophylls. While xantophylls are located primarily in plants, Valmalette *et al.,* (2012) observed that carotene molecules are situated in an optimal location to harvest light energy. They are found as a bilayer underneath the cuticle from 0 to 40µM in depth in the pea aphid. Carotenoids play a critical role in photosynthesis through harvesting light energy with their chromophore centres which generates free electrons. This results in the photo induced electron transfer from excited chromophores to acceptor molecules. These $NAD(P)^+$ molecules are then reduced to NAD(P)H, which drives the proton pump and sets mitochondrial ATP synthesis in motion. This energy generating process in the aphid can at this stage only be referred to as phototrophy (Lougheed 2012).

Despite the role of carotenoids in photosynthesis, carotenoids also play important parts in the mate choice, signalling, vision, diapause and photoperiodism of insects (Heath *et al*., 2012). The degradation of these carotenoid compounds leads to the formation of volatile apocarotenoids which can have hormonal properties and play an important role in ecological interactions in insects. Cleavage of carotenoid derivatives leads to retinal, which is part of important eye photoreceptors present in all taxa (Valmalette *et al*., 2012). The most important property of carotenoids however, would be its ability to protect chlorophyll from reactive oxygen species (ROS) during plant stress. ROS are toxic molecules that cause oxidative damage to DNA, proteins and lipids, and are produced at a low concentration during normal growth conditions (Heidari *et al*., 2012). However, during stressful conditions, the formation of ROS is dramatically increased, and the plant and pest suffers in the process. This antioxidizing property of carotenoids and their derivatives is responsible for the mediation of oxidative stress and are likely a key feature in modulating insect-plant interactions (Heath *et al.,* 2012). Heath *et al.,* (2012) reports that carotenoids may protect insects from both exogenous and endogenous sources of ROS generated from the plants' response to wounding, and may thus contribute to the immune response in arthropods. Carotenoids may be an adapted mechanism of protection for aphids and the reason why carotenoid biosynthetic genes have been retained in the aphid genome.

Research shows that an aphid ancestor acquired this pigment-producing power from a fungus following a single lateral gene transfer event, which was followed by duplication within the aphid genome (Moran and Jarvik 2010). Novakova and Moran (2012) investigated the diversification of genes involved in the biosynthesis of carotenoids in 34 aphid species and determined that aphid species differ in the amount of copies of *carotene desaturase* genes (responsible for inducing double bonds between neighbouring carbon residues; Novakova and Moran 2012) and it was suggested that multiple copies of these genes may be linked to the capacity for evolving novel carotenoid profiles to adapt to certain ecological circumstances. Since minor changes in the enzyme can lead to the production of novel carotenoids such as torulene (Schmidt-Dannert *et al*., 2000). It was also observed that the carotenoid composition of aphid species differ dramatically in the types and amounts of carotenoids present as can be seen from Fig. 2.3. Carotenoids are associated with the body colour of aphids that vary with environmental conditions. High levels of carotenoids are associated with green body colour in optimum conditions, very low levels are associated with white or pale colour when faced with limited resources, and those in-between carotenoid levels are associated with orange body colour (Lougheed 2012). *D. noxia's* carotenoid profile consists mainly of trans-alpha-carotene, trans-beta-carotene and epsilon carotene. Small amounts of trans-lycopene, 9-cis -beta-carotene, 13-cis-beta-carotene, gamma-carotene,

delta-carotene and alpha-cryptoxanthin were also identified in *D. noxia*. A small amount of xanthins that include lutein, cis-lutein, zeaxanthin and cis-zeaxanthin, were also found in *D. noxia*, and could be due to the induction of degradation of host plant cells that could possibly release carotenoids into the ingested ploem sap.

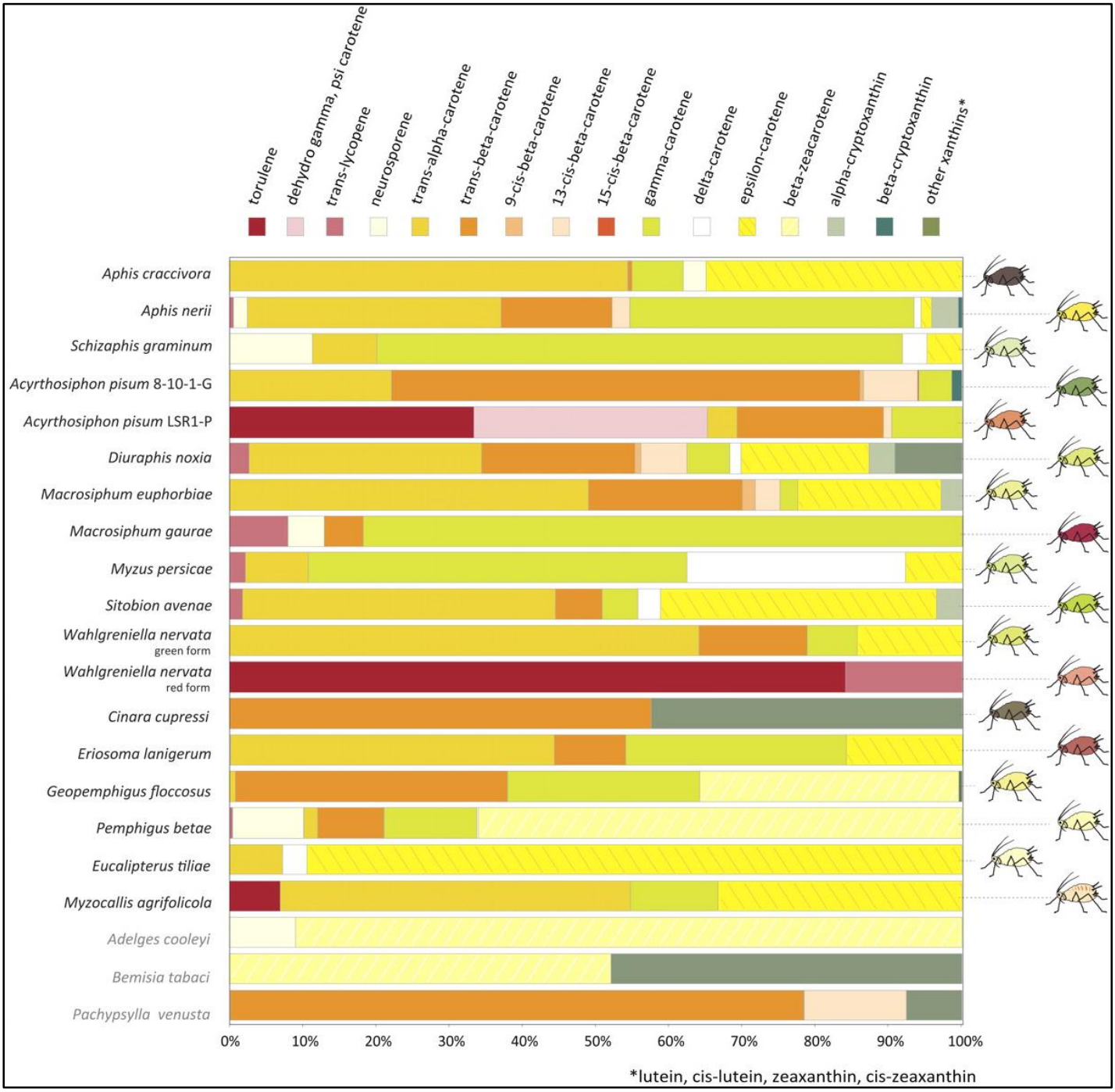

Fig. 2.3. Carotenoid profiles of different aphid species. The width of the bar indicates the proportional representation of carotenoids found within the aphid species (Novakova and Moran 2012).

Several other studies have documented lateral gene transfer between organisms, such as those discovered by Rumpho *et al.,* (2010) between two eukaryotes, the alga *Vaucheria litorea* and its predator, the sea slug *Elysia chlorotica*. The sea slug obtains the algal plastids through feeding and then the captured plastids are retained intracellularly where they are allowed to photosynthesize for months inside the sea slug. This phenomenon is referred to as kleptoplasty. Following predation, the algal nuclear genome is lost and the incorporation and utilization of algal genes to maintain functional chloroplasts inside the sea slug are highly motivated to provide increased amounts of energy. The lateral transfer of carotenoid biosynthetic genes to the aphid genome thus provide a selective advantage that may lead to increased fitness and is thus the reason they have been retained in the aphid genome for all these years.

2.5.2 Phenotypic polyphenism

Phenotypic polyphenism such as differences in colour and body size of individuals of the same species are widespread in animal taxa and also present in various aphid species (Smith 1998). A wide range of genetically identical individuals termed morphs that differ in colour and life cycles are present in aphids that may differ in biological properties such as host range, growth rate and susceptibility to natural enemies (Markkula 1963; Losey *et al*., 1997; Braendle and Weisser 2001; Blackman and Eastop 1984; Dixon 1998). The general understanding is that genetic and environmental factors, light intensity and host plant species are associated with the formation of colour morphs (Alkhedir *et al.,* 2010). Each colour morph provide a selective advantage and has specifically evolved to maximise their fitness in terms of dispersal, defence, reproduction and survival. The pea aphid displays an excellent example of phenotypic polymorphisms with its red, green and pale colour morphs as a result of environmental pressures. Red morphs are more susceptible to natural predators and has evolved a strategy to escape their predators by falling off their host plants and display greater dispersal abilities (Losey *et al.,* 1997; Farhoudie 2012). The higher lipid and carbohydrate content found in the red morph allows for abundant energy for dispersal through flying (Ahsaei *et al.,* 2013). Green morphs on the other hand are vulnerable to parasitic wasps (Losey *et al.,* 1997) but has adapted to live in cold conditions due to a lower abundance of parasitoids at low temperatures (Valmalette *et al*., 2012). Green morphs contains higher protein and glycogen content which is not seen as an energy source, but rather used for maintenance, development, morphogenesis and reproduction (Ahsaei *et al*., 2013) and thus display higher fecundity than red morphs (Markulla 1963). Caillaud and Losey (2010) have determined that the colour in *A. pisum* is determined by a single two-allelic locus, and that the red is dominant to the green. Some individuals of *A. pisum* lose

their colour shade and become pale yellowish under conditions such as crowding or poor nutrition due to low quality hosts. These pale aphids are expected to have a significantly lower reproductive fitness than red morphs due to their lower energy reserves (less carbohydrates and lipids than the red aphid) and body weight (Tabadkani *et al.,* 2013). This loss of colour is associated with a loss of carotenoids but is reversible if a high quality host is found and their nutrient rich diet is restored (Valmalette *et al.,* 2012). These pale aphids display significantly higher activity than red aphids by walking around to find high quality hosts (Tabadkani *et al.,* 2013). Tabadkani *et al.,* (2013) provided proof that starvation or deprivation of food sources most definitely induce dispersal in aphids as has been suggested for many insects (Perez-Mendoza *et al*., 1999). It is thus evident that polyphenism allows the aphid to respond appropriately to environmental changes so as to maximize their fitness and ensuring their survival.

2.5.3 Wing polyphenism

The mortality, wing development and behaviour and metabolism of insects have been widely studied under various environmental conditions. The capacity of insects to fly is perceived to be a key feature in their ecological and evolutionary success and has become an important field of study. Wing polyphenism is suggested to be an adaptation to fluctuating environmental conditions such as host quality, crowding and natural enemies, and allows the insect to escape its undesirable environment by increasing its dispersal capabilities. This beneficial process however is known as one of the most expensive activities performed by insects and potentially demand great metabolic, reproductive and survival costs.

A study by Castaneda *et al*., (2010) found that differential energy investments between the winged and wingless morphs were mainly reflected in growth or body mass rather than metabolic costs. Winged aphids are smaller and have a longer development time than wingless aphids, suggesting that an energy trade-off takes place between growth and wing development. In contrast, wingless aphids' show higher fecundity and a higher developmental rate that allows for an increase in colony size (Braendle *et al.,* 2006; Schwartzberg *et al.,* 2008). The results also showed that metabolic rate in the winged aphids varied more than that of the wingless aphids in response to different wheat hosts. Since considerable difference exist in the morphological, behavioural and physiological traits between winged and wingless aphid morphs, it is likely that these morphs also differ in their ability to cope with stressful environments (Braendle *et al.,* 2006). It is hypothesized that wingless aphids have fewer metabolic resources to deal with stress than winged aphids and are thus not adapted to varying environmental conditions.

A study suggested that winged aphids have an increase in energy production to fuel the costs of functional flight muscles and a complex sensory system for host selection. Using a genomic approach, Brisson *et al.,* (2007) has observed that winged pea aphids over-expressed genes involved in energy metabolism compared to wingless morphs. By summarizing these results, energy is either expended to flight apparatus and fuel instead of body growth and reproduction, or the amount of energy produced is increased to meet the energy demands of both.

2.5.4 Heat stress

Fluctuations in temperature are notorious for causing environmental stress to insects due to their high degree of sensitivity to air temperature (Neven 2000). Growth and fitness traits such as survival, development and fecundity, are affected by the insects altered behaviour, metabolic rate, cellular and biological processes, due to exposure to varying temperature (Bale *et al.,* 2002; Sorensen *et al.,* 2003). Environmental stress, especially heat stress is able to cause mitochondrial disturbances that affect respiration and oxidative phosphorylation (Neven 2000; Korsloot *et al.,* 2004; Kültz 2005). Various studies have shown a reduction in performance and reproduction of aphids that were exposed to high temperatures, such as the pea aphid *A. pisum* (Ontaka and Ishikawa 1991; Russell and Moran 2006), the rose grain aphid *Metopolophium dirhodum* (Ma *et al.,* 2004), the rumex aphid *Aphis rumicis* (Bayhan *et al.,* 2006), and the corn leaf aphid *Rhopalosiphum maidis* (Kuo *et al.,* 2006).

A recent study by Nguyen *et al.,* (2009) investigated the impact of heat and solar ultraviolet B (UV-B) radiation on the potato aphid *Macrosiphum euphorbiae* and found noticeable fitness and behavioural effects, but no significant effect on the survival of the aphids. Reduced performance, development and growth rate was evident as a reduction in aphid biomass was observed in both the winged and wingless aphids under heat stress, although wingless aphids were more dramatically affected. Various mitochondrial enzymes were altered under heat stress in the winged aphids, such as three enzymes that are part of the citric acid cycle, aconitase A, isocitrate dehydrogenase and malate dehydrogenase, were significantly reduced while, cytochrome oxidase one and NADH dehydrogenase subunit 5 which are involved in the electron transport chain were significantly increased under heat stress (Nguyen *et al.,* 2009). The winged aphids showed a reduction in glycolytic enzymes and down regulation of protein synthesis that may result in considerable bioenergetic savings as part of an energy conservation strategy to survive stressful conditions. These findings suggested that respiration and energy generation were disrupted in heat stressed aphids (Nguyen *et al.,* 2009).

2.6 DNA Sequencing Technologies

The age of DNA sequencing began when Frederick Sanger and his colleagues developed the first method of accurately determining the sequence of single stranded DNA molecules in 1977. This method termed Sanger sequencing, is based on the selective incorporation of chain terminating dideoxynucleotides by DNA polymerase during in vitro replication (Sanger and Coulson 1975; Sanger *et al.,* 1977). This method has been modified and optimized throughout the years and has evolved from using autoradiography and UV light to visualize the DNA bands to electronic DNA sequence peak chromatograms. The most recent method includes dye-terminator sequencing that utilizes the labelling of each of the four dideoxynucleotide chain terminators with different coloured fluorescence dyes that emit light at different wavelenghts. These dye-terminators are attached to all DNA fragments after replication and correspond to the dideoxy bases. The DNA fragments are then seperated by capillary electrophoresis and the flouresecnece is detected by laser excitation as the dye-labeled fragments migrate through the capillary array. The DNA sequence can then be read on a chromatogram containing peaks consisting of different colours, each of which corresponds to a specific nucleotide.

Sanger sequencing has been the most widely used method for approximately two decades and is still used today for small projects and when single sequence reads are required. More recently, a new age of Next-Generation Sequencing has begun that allows the user to produce enormous volumes of data cheaply. This has enabled large-scale automated genome analyses and variant detection that has facilitated comparative and evolutionary studies, and thus have widespread diagnostic applications among others. A wide variety of high throughput Next-Gen sequencing technologies are available today, but for the purpose of this study only the Roche GS-FLX Plus 454, ABI SOLiD, Illumina Hiseq 2000 and Ion torrent sequencing platforms will briefly be discussed.

Roche 454 was the first commercially available successfull NGS-system and has been utilized in a variety of whole genome projects such as the Neanderthal genome (Green *et al.,* 2006). 454 Sequencing is a large-scale parallel system that employs pyrosequencing technology which relies on the detection of pyrophosphate release during nucleotide incorporation, instead of chain termination with dideoxynucleotides. The GS-FLX Plus 454 system's advantages include long read lengths that can reach up to 700 bp with extreme accuracy (99.9%) and a fast runtime of 10 hours from sequencing start to finish. However, the system is low throughput with 0.7 Gb data output in 24 hours at a high cost (Liu *et al.,* 2012).

SOLiD (Sequencing by Oligo Ligation Detection) adopts the two-base sequencing based on ligation sequencing technology and allows the user to efficiently uncover single nucleotide polymorphisms and structural variations with modest coverage levels at low costs. The system's major shortcoming is that it generates short reads of up to 85 bp and thus only enables resequencing projects. The quality of reads however are excellent (99.99%) and the system's high throughput capabilities are better than the 454 system with 30 Gb data output per run within 7 days (Liu *et al.,* 2012).

The Illumina Hiseq 2000 platform relies on the sequencing by synthesis principle and generates short reads of up to 150 bp (Quail *et al.,* 2012) with an accuracy of 98%. It is a high throughput system that is able to generate 600 Gb per run in only eight days (Liu *et al.,* 2012), and at a low cost no less (cheaper than the 454 and SOLiD systems).

Ion Torrent's PGM DNA sequencer utilizes semiconductor sequencing technology which detects a change in pH (voltage) as a proton is released when a nucleotide is incorporated into the DNA molecule (Liu *et al.,* 2012). Because fluorescence chemistry and camera scanning equipment are unnecassary, a high speed of 20 Mb – 1 Gb (depending on chip size) data output in 2 hours are possible (Quail *et al.,* 2012) with 99% accuracy and approximately 200 bp per read. Another advantage is that the Ion Torrent technology costs less than the 454, SOLiD and Illumina platforms.

Although sequencing technology is evolving rapidly and improving constantly, all NGS systems still have their strenghts and limitations, and must be selected based on the project's needs. The 454 sequencing supplied large reads and enabled the assembly of overlapping fragments, while the Ion Torrent and Illumina sequencing supplied coverage deph, and the SOLiD sequencing was used to detect variants. Thus fullfilling in the current project's need.

2.6 Reference list

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Chapter 3

Complete mitochondrial genome of *Diuraphis noxia* (Hemiptera: Aphididae) from nine populations, SNP variation between populations, and comparison with other Aphididae species

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3.1 Introduction

Aphids cause severe plant damage either directly through their feeding activities or indirectly by transmitting plant viruses to their host (Tagu *et al*., 2008). The Russian Wheat Aphid (RWA), *Diuraphis noxia* (Kurdjumov), belongs to the family Aphididae which are known as serious agricultural pests, due to their rapid population growth which allows for the quick infestation of their host resulting in significant damage (Botha *et al.,* 2006; Lapitan *et al.,* 2007). *D. noxia* populations present in South Africa, and in many other international locations, reproduce through facultative parthenogenesis, unlike in areas where *D. noxia* is considered to be endemic where they can also reproduce sexually. This asexual means of reproduction allows for non-recombinant vertical gene transfer from mother to offspring affording little genetic variation to the aphid, and the aphid's endosymbiont, through successive generations (Tagu *et al*., 2008). Despite this lack of sexual recombination, new populations with varying levels of virulence still develop, enabling these populations to overcome host-plant resistance and feed on a previously resistant host. This continuous development of virulent *D. noxia* populations requires the identification and introduction of new resistance genes into susceptible wheat cultivars (Bouhssini *et al*., 2011) in order to protect crops against this pest and prevent large scale agricultural losses (Jankielsohn 2011). *D. noxia* populations are classified by the varying degree of damage that they cause through feeding on wheat plants (*Triticum aestivum* L.) containing different resistance (*Dn*) genes. This virulence classification is based on the plant's response to aphid feeding being either susceptible, intermediate or resistant (Puterka *et al.,* 2012). These designations are assigned to the plants based on foliar damage as a direct result of *D. noxia* feeding and thus group aphids as a certain biotype according to their virulence (Burd *et al*., 2006). Prior to 2003, US *D. noxia* populations were believed to be biotypically and genetically uniform. After the detection of a new biotype in 2003 (Haley *et al.* 2004), the populations were investigated for genetic diversity and differences in the mitochondrial *cytochrome oxidase subunit 1* (*COI)* gene were found among populations. Populations grouped together (US populations 3, 4 and 7) were shown to differ genetically, regardless of their grouping based on virulence (Lapitan *et al*., 2007; Liu *et al*., 2011). This demonstrates how a single biotype category can be represented by a number of genotypes, and that a new *D. noxia* introduction which differs in genotype can go undetected if it doesn't differ in biotype as classified by the phenotypic response of the host plant. Genotype characterization and biotype identification of aphids is thus the first step in any cereal-breeding program to discover resistance genes and to develop resistant wheat cultivars (Kharrat *et al.,* 2012). It is thus evident that genetic differences among the populations are of importance and is an area of study that needs more exploring (Shufran *et al.,* 2007, 2009).

The mitochondrial genomes of insects are circular chromosomes generally ranging from 13-20 kb in size with well conserved gene content and order. These characteristics make them useful to study deep divergences of insects and their molecular evolution (Hu *et al.,* 2009). The mitochondrial genome typically encodes 37 genes that include 13 protein-coding genes (PCGs): two subunits of ATPase (*atp6* and *atp8*), three cytochrome c oxidase subunits (*COI*, *COII* and *COIII*), one cytochrome B (*CytB*) and seven NADH dehydrogenase subunits (*ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *ND6* and *ND4L*). The remaining genes encode for 2 ribosomal RNAs and 22 transfer RNAs that are necessary to translate the protein-coding genes.

Insects contain a large non-coding AT-rich region in their mitochondrial genome that is also referred to as the control region, which is involved in the initiation and regulation of mitochondrial transcription and replication (Chai *et al.,* 2012). Despite the control region, the whole insect mitochondrial genome is compactly arranged with overlapping neighbouring genes, very limited intergenic nucleotides and no introns (Hu *et al*., 2009). The total AT content in insect mitochondrial genomes ranges from 63-88 %, their start codons vary between ATA, ATG and ATAA (for the *COI* gene) and their stop codons are known as TAA and TAG. However, PCGs that are followed by a downstream transfer RNA terminate with a T or TA. It is further known that the gene order of the mitochondria of aphids, psyllids and many whiteflies resembles the proposed Insecta ancestral gene order and the amount of mitochondrial sequence divergence for aphids is only 13.1 %, which is considerably less than that of psyllids and whiteflies (Baumann *et al*. 2004).

Nucleotide diversity in the mitochondrial genome of aphids has been widely studied, particularly those of *Acyrthosiphon pisum* and *Schizaphis graminum*. Mitochondrial DNA gene sequences are particularly attractive for developing phylogenies of recently diverged taxa due to their rapid evolution and their higher likelihood of tracking a short internode, thus enabling the widespread use of mitochondrial genome sequences as molecular markers for aphid species and populations. Recent studies have integrated several mitochondrial genes to detect and resolve taxonomic diversity in aphids (Foottit *et al*., 2009).

Despite the extensive use of the mitochondrial *cytochrome oxidase subunit 1* (*COI*) gene, studies using only single markers have limitations and the need to incorporate additional mitochondrial markers is important for the reliable identification of aphid species or populations (Anstead *et al*., 2002; Kharrat *et al.,* 2012). Recent studies have examined several mitochondrial genes, or parts of them, as potential markers. A study by Kharrat *et al.,* (2012) demonstrated that the mitochondrial genes, *cytochrome B* (*CytB*) and *NADH-dehydrogenase* (*ND*), have successfully been utilized to detect DNA polymorphisms and estimate relationships among *S. graminum* populations. Zhang *et al.,* (2012) has also

revealed the potential of the mitochondrial genes *COI*, *NADH dehydrogenase subunit 6* (*ND6*), partial *NADH subunit 4L* (*ND4L)*, 2 tRNAs, total *ND6* and partial *CytB* genes as molecular markers for *D. noxia*. These mitochondrial sequences were employed to investigate population genetics, demographic history and evolutionary adaptation of *D. noxia* in China. Generating multiple complete mitochondrial genome sequences is thus necessary to gain a better understanding of species or biotype evolution and/or development and may provide insight into certain biological functions of aphids. Access to the information enclosed in the mitochondrial genome of *D. noxia* could enhance several areas of aphid research that would include polymorphic markers to analyse population structure and biotypic diversification, phylogenetic studies, evolutionary biology, energy metabolism and possibly virulence associated studies.

In the present study we sequenced the mitochondrial genomes from nine global *D. noxia* populations to derive a consensus mitochondrial genome for *D. noxia*. This consensus was then used to compare sequence variation amongst these populations in an effort to resolve some of the questions relating to their sequence diversity and difference in fitness. We conducted phylogenetic analyses to confirm the organisation of the *D. noxia* mitochondrial genome to that of other members of the Hemiptera, Sternorrhyncha (whiteflies, aphids and psyllids). And lastly, we compared the complete consensus genome of *D. noxia* to that of its closest phylogenetic taxa, also economical pests, namely *A. pisum* and *S. graminum.*

3.2 Materials and Methods

3.2.1 Aphid samples and DNA Extraction

Genomic DNA from adult aphids of the South African *Diuraphis noxia* biotypes SA1 and SAM (laboratory biotype mutated from an SA1 biotype (Van Zyl 2007; Van Zyl and Botha 2008)) and a pooled sample of nine *D. noxia* biotypes, collected from Argentina, the Czech Republic, South Africa, Syria and the USA (Botha *et al*., 2012) were used for next generation sequencing during the study. The genomic DNA extraction of aphid DNA was conducted as follows; aphids were flash frozen in liquid nitrogen, ground and DNA was extracted using the Qiagen DNeasy Blood and Tissue kit® according to the manufacturer's protocol (Botha *et al*., 2012).

3.2.2 Library construction and sequencing

Whole genomic DNA from the SAM biotype was utilized in a variety of next generation sequencing technologies to provide sequence reads for the assembly of the mitochondrial

genome of *D. noxia*. Genomic DNA was used to construct a 365 bp long library using the Ion Xpress Fragment Kit for sequencing on the Ion Torrent platform to perform paired-end sequencing. A shotgun library consisting of 1 642 bp long fragments was also created and sequenced using the GS-FLX Plus 454 sequencing platform. Lastly, the Illumina Hiseq 2000 platform was used to sequence a library consisting of 500 bp long fragments to supply paired-end reads for the assembly.

Whole genomic DNA obtained from the pooled sample was used to construct a 50 bp long, mate-paired library and was sequenced using the SOLiD platform to utilize in the discovery of single nucleotide polymorphisms (SNPs) among the *D. noxia* biotypes. In all instances, the SNPs occurring in the PCGs that resulted in either truncations or frame shift mutations were verified by Sanger sequencing.

3.2.3 Sequence assembly, annotation and analysis

Raw sequences from the 454, Illumina and Ion Torrent platforms were trimmed and filtered for quality prior to their alignment to the reference. The complete mitochondrial genome of *Acyrthosiphon pisum* (NC_011594) was used as reference for mapping due to close phylogenetic positioning and high homology of its protein coding genes to *D. noxia* (Table 3.1, Fig. 3.1, Figs. A1-A6). A total of 20 116 reads were mapped to the reference sequence to create a contiguous sequence with an overall Q40 (*Chance of an erroneous base call is 1 in 10 000*) Phred score of 98.8%. The genome mapping and nucleotide composition calculations were performed with the Geneious software v6.1.6 (Kearse *et al.,* 2012). The constructed contig was confirmed through the use of Sanger based capillary sequencing using multiple PCR primers designed with the NCBI's primerBLAST (Table S1) (Ye *et al*. 2012; http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) that utilizes the primer3 (Untergrasser *et al*., 2012; Koressaar and Remm 2007) platform, so as to cover stretches of the newly assembled genome. These amplified regions were cloned using the Thermo Scientific InsTAclone® cloning kit and were subjected to capillary based Sanger sequencing for assembly confirmation and circularization of the mitochondrial genome. The final mitochondrial genome of *D. noxia* had an overall Q40 Phred score of 99.1 % and a mean coverage of approximately x348.8 when the 454, Illumina and Ion Torrent sequences were re-mapped back to the assembled mitochondrial genome. MAFFT v7.0.17 (Katoh *et al*., 2013) was used for whole genome alignments and comparisons. The FFS-NS-2 algorithm was used with a gap open penalty of 1.53 and an offset value of 0.123.

The locations and orientation of protein-coding genes, tRNA genes and rRNA genes were identified by determining sequence similarities to available sequence data from *A. pisum* (NC_011594) and *S. graminum* (NC_006158) through multiple alignments using the NCBI's

BLAST program v2.2.28 (Altschul *et al.,* 1990). The transfer RNA genes were also identified using the tRNAscan-SE software (Lowe and Eddy 1997; available online at http://lowelab.ucsc.edu/tRNAscan-SE), and only tRNA genes with a cove score above 20 were recognized as genuine tRNA genes. The protein-coding gene nucleotides were manually curated and were translated on the basis of the Invertebrate Mitochondrial Genetic Code and were compared to the protein-coding genes of other aphid species. Consequently, the data obtained from the mitochondrial genome of *D. noxia* showed no ambiguous nucleotide bases within protein coding genes. The A+T rich region was subjected to a search for tandem repeats using the Phobos (Mayer 2006-2010) software.

SOLiD sequences obtained from sequencing a pooled sample of nine different biotypes, were reference mapped to the assembled mitochondrial genome of the *D. noxia* SAM biotype using Geneious v6.1.6. The assembled reads were then assessed for SNPs when compared to the reference sequence using Geneious's built-in software. SNPs were classified if at least 20% of the sequences held the alternate base at that position with a minimum coverage of 50x and a minimum *P*-value (probability of substitution due to sequencing error) of 10^{-6} when comparing the sequence quality of the mapped reads in the SNP region. At least 10 of the predicted SNPs were confirmed through the use of Sanger based capillary sequencing using multiple PCR primers.

Table 3.1. Number of sequenced mitochondrial genomes for the Hymenoptera and Hemiptera, Sternorrhyncha. In this study, the complete (consensus) mitochondrial genome of *D. noxia* (Kurdjumov) was added to the available data set.

Genera	Accession numbers	Platform	Reference
Acyrthosiphon pisum	NC_011594	PCR, Cloning Sanger Sequencing	Moran *et al*. 2008 (Unpublished)
Apis mellifera ligustica	NC_001566	Restriction digests, PCR/Sanger sequencing	Crozier and Crozier 1993
Aleurochiton aceris	NC_006160	PCR, Cloning, Sanger Sequencing	Thao *et a*l. 2004
Aleurodicus dugesii	NC_005939	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Bemisia tabaci	NC_006279	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Cavariella salicicola	KC332935	PCR, Sanger sequencing	Wang *et al.* 2013
Diuraphis noxia		PCR/Sanger sequencing	Zhang *et al*. 2014
		NextGen sequencing, PCR, Sanger sequencing	This study
Neomaskellia andropogonis	NC_006159	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Pachypsylla venusta	NC_006157	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Schizaphis graminum	NC_006158	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Tetraleurodes acaciae	NC_006292	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004
Trialeurodes vaporariorum	NC_006280	PCR, Cloning, Sanger Sequencing	Thao *et al*. 2004

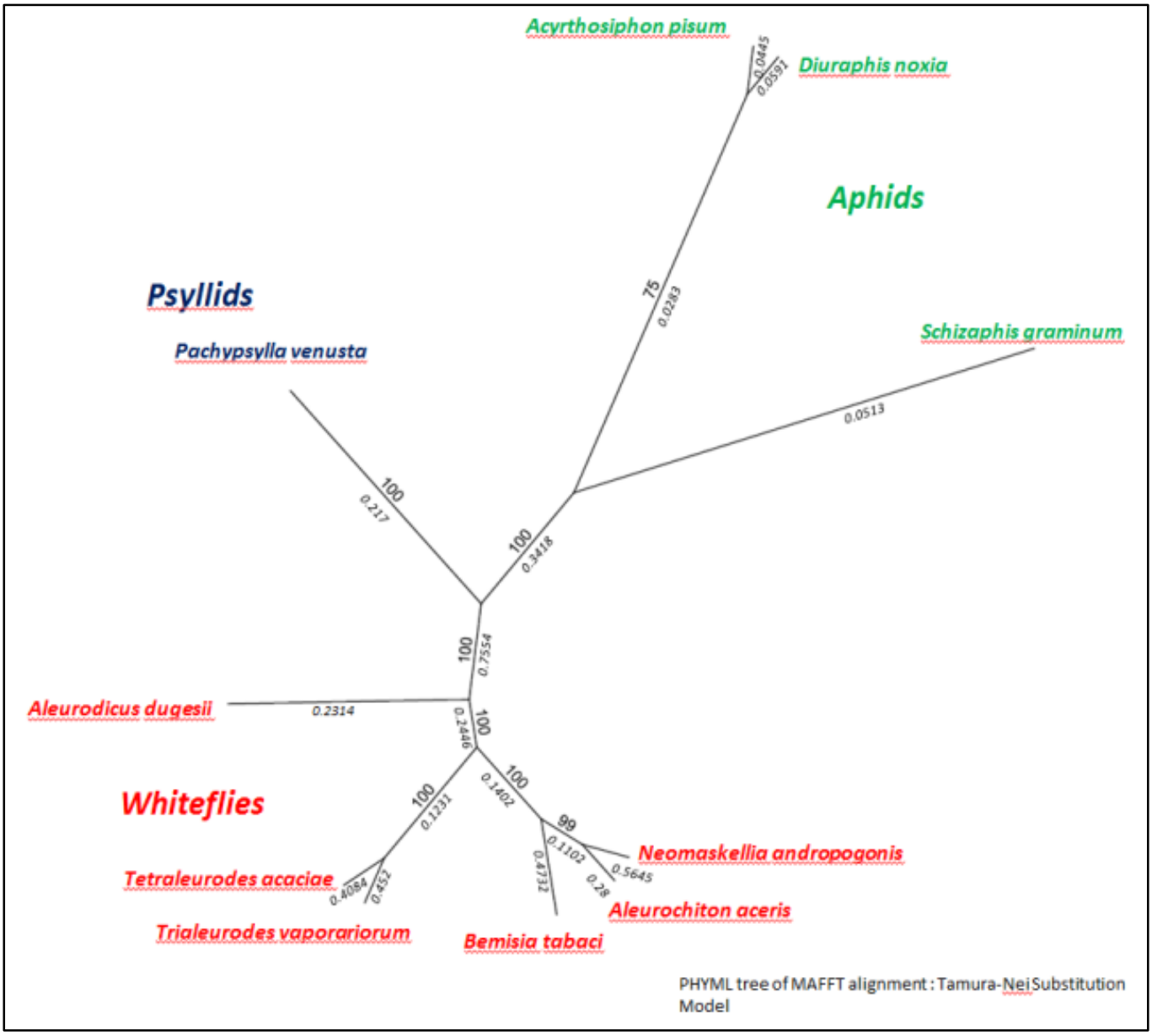


Fig. 3.1. Unrooted phylogenetic tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) after analysis using the Tamura-Nei Substitution Model (MAFFT, Geneious v6.1.6). The tree is based on alignment of the complete mitochondrial genomes of the whiteflies, *Aleurochiton aceris*, *Aleurodicus dugesii, Bermisia tabaci, Neomaskellia andropogonis, Tetraleurodes acaciae* and *Trialeurodes vaporariorum*, *Pachypsylla venusta* (psyllid), and the aphids, *A. pisum*, *S. graminum* and *D. noxia.*

3.2.4 Phylogenetic Analyses

The protein-coding and rDNA genes were identified by BLAST searches (Altshul *et al.,* 1990) of Genbank. The methods used for the phylogenetic analyses were conducted as previously described using parsimony (bootstrapping values were calculated based on a 1 000

replicates, Swofford 1998) (Moran *et al*., 1999; Thao *et al*., 2004) based on monophyly (Simon and Hadrys 2013) and used *Apis mellifera ligustica* (Hymenoptera) (NC_001566) as an outgroup. The genera included in the analysis are presented in Table A2. Whole genome analysis was conducted using PHYML (Guindon *et al*., 2010) (Available at http://www.atgc-montpellier.fr/phyml/) with both the Tamura-Nei and Jukes-Cantor substitution models performed with 100 bootstraps. The gamma distribution parameter and the Transition/Transversion ratio were used as estimated values. The number of substitution rate categories was left at the default of 4 and the proportion of invariable sites was set to 0. The trees were optimized for branch length and topology with the type of improvement set to NNI. Analyses using whole genomes were also performed using MrBayes v3.2.1 (Huelsenbeck and Ronquist 2001) making use of the Jukes-Cantor substitution model with a gamma rate variation set at four categories and with *D. noxia* set as outgroup. The rest of the parameters were left at default values.

3.3 Results

3.3.1 Phylogenetic analysis of the Hemiptera, Sternorrhyncha

Phylogenetic analyses that included a selection of Hemiptera taxa, including the ancestral aphid species (*Daktulosphaira vitifoliae*) to confirm the evolutionary association of *D. noxia* relative to other taxa in the Aphididae, were performed for direct comparison of genomic composition and gene order (Figs. A2-A8). Table 3.1 lists all Hemiptera, Sternorrhyncha mitochondrial DNA genomes sequenced to date, while Table A2 provides the accession numbers of mitochondrial DNA genome sequences used in this study for the phylogenetic analyses. An unrooted phylogenetic tree provides the relationships of whiteflies, psyllids and aphids based on the whole mitochondrial genomes of 10 Sternorrhyncha (Fig. 3.1, Appendix Fig. A1). Since the complete genome of *Apis mellifera ligustica*, Aphidae, Hymenoptera is available in GenBank, it was used as outgroup during the phylogenetic analyses of the rooted trees. The rooted phylogenetic trees based on the mitochondrial *COI*, *CytB*, *ND1*, *ND2*, *12S rDNA* and *16S rDNA* are presented in the supplementary Figs. A2-A7. In all the trees, three taxonomic groupings were evident, i.e., psyllids, whiteflies and aphids with the Phylloxera that group distant to the aphids and *A. pisum* and *S. graminum* that are closest to *D. noxia*.

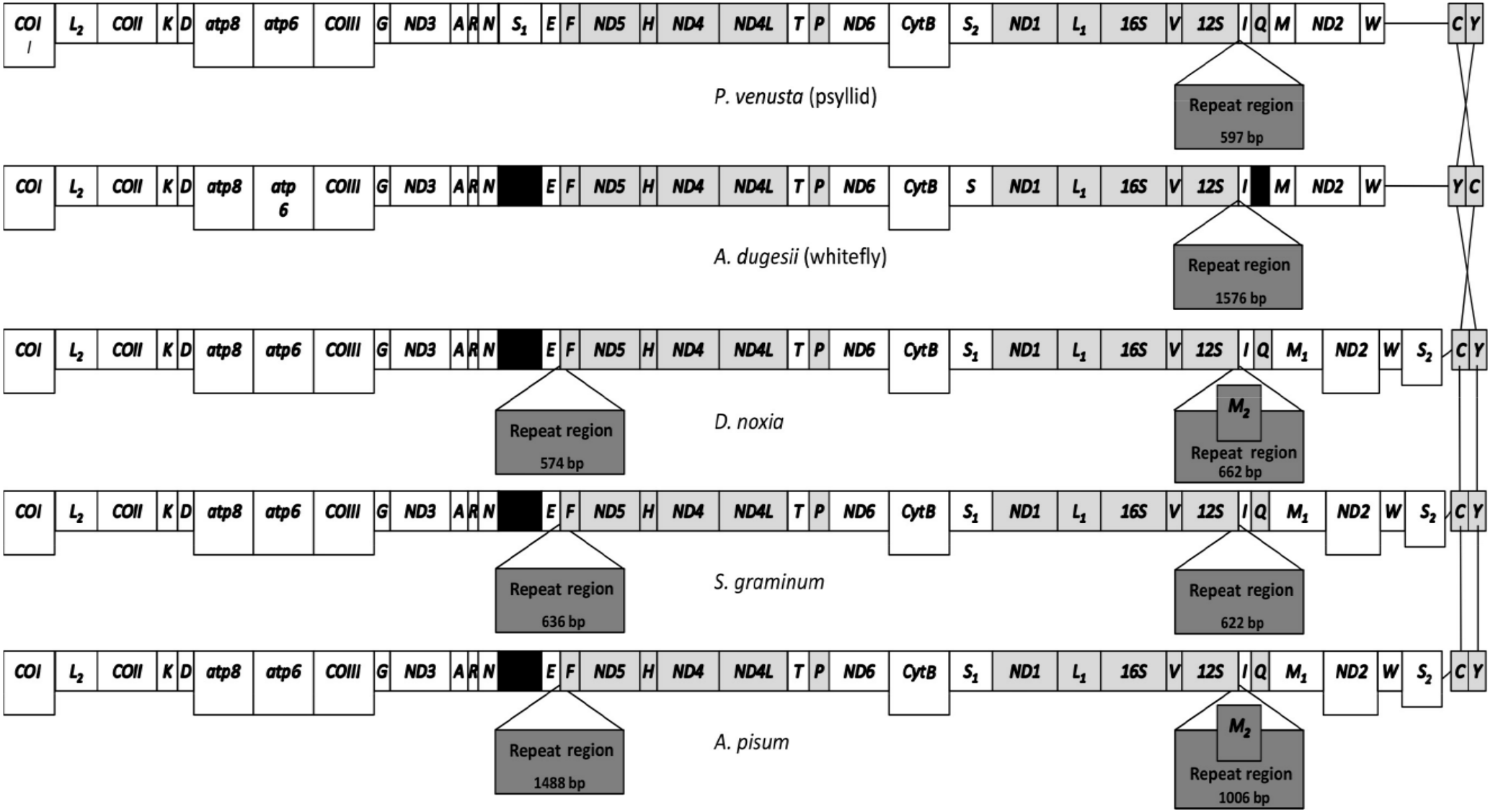


Fig. 3.2. A comparison in mitochondrial gene arrangement between *Pachypsylla venusta* (psyllid), *Aleurodicus dugesii* (whitefly) and the aphids, *A. pisum*, *S. graminum* and *D. noxia.*

3.3.2 Genome organization, and composition

The mitochondrial genome of *D. noxia* is a circular molecule (15 721 bp in length) and consists of 38 genes typically found within most insects: 20 different transfer RNA genes (with duplicates of $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Met}$) (Fig. 3.3), 13 protein-coding genes, and 2 ribosomal genes (Fig. 3.4, Table 3.2). Of these genes, 24 are transcribed on the majority-coding strand (H-strand) and the remaining 14 genes are transcribed on the minority-coding strand.

The nucleotide composition of the mitochondrial genome of *D. noxia* is AT-rich (with A = 45.8% and T = 38.8%) with an accumulated AT content of ~84.7% across the entire genome. The remaining ~15.3% genomic content is represented by 9.5% C and 5.8% G (Table 3.3).

A total of 3 631 codons, excluding termination codons are present in *D. noxia* (Tables A3 and A4). All PCGs in *D. noxia* are initiated with the ATN start codon (with ATG used for *COIII*, *CytB*, *ND4;* with ATA used for *Atp6*, *COI*, *COII*, *ND4L*; and the rest used ATT) while TAA, a single T (for *ND2*, *COI* and *ND4*) and TA (for *atp6* and *COIII*) are used as termination codons (Table 3.2). The amino acid usages for PCGs revealed that Leu (UUR; 15.1%) was the most frequently used amino acid, followed by Phe (13.5%) and Ile (13.5%) (Tables A3 and A4). The AT-bias in the *D. noxia* mitochondrion genome is also reflected by the absence of 10 GC-rich codons (GCG, GGC, CCG, CGG, AGG, TCG, ACG, CTC, CTG, TAG), and low usage of TGC, CGC, TCC, ACC, GTC, TGG (occurring only once) and CCC, CAG, AGC, GTG (occurring only twice). Whilst codons TTT (464), ATT (472), TTA (494), and ATA (302) are abundantly in use.

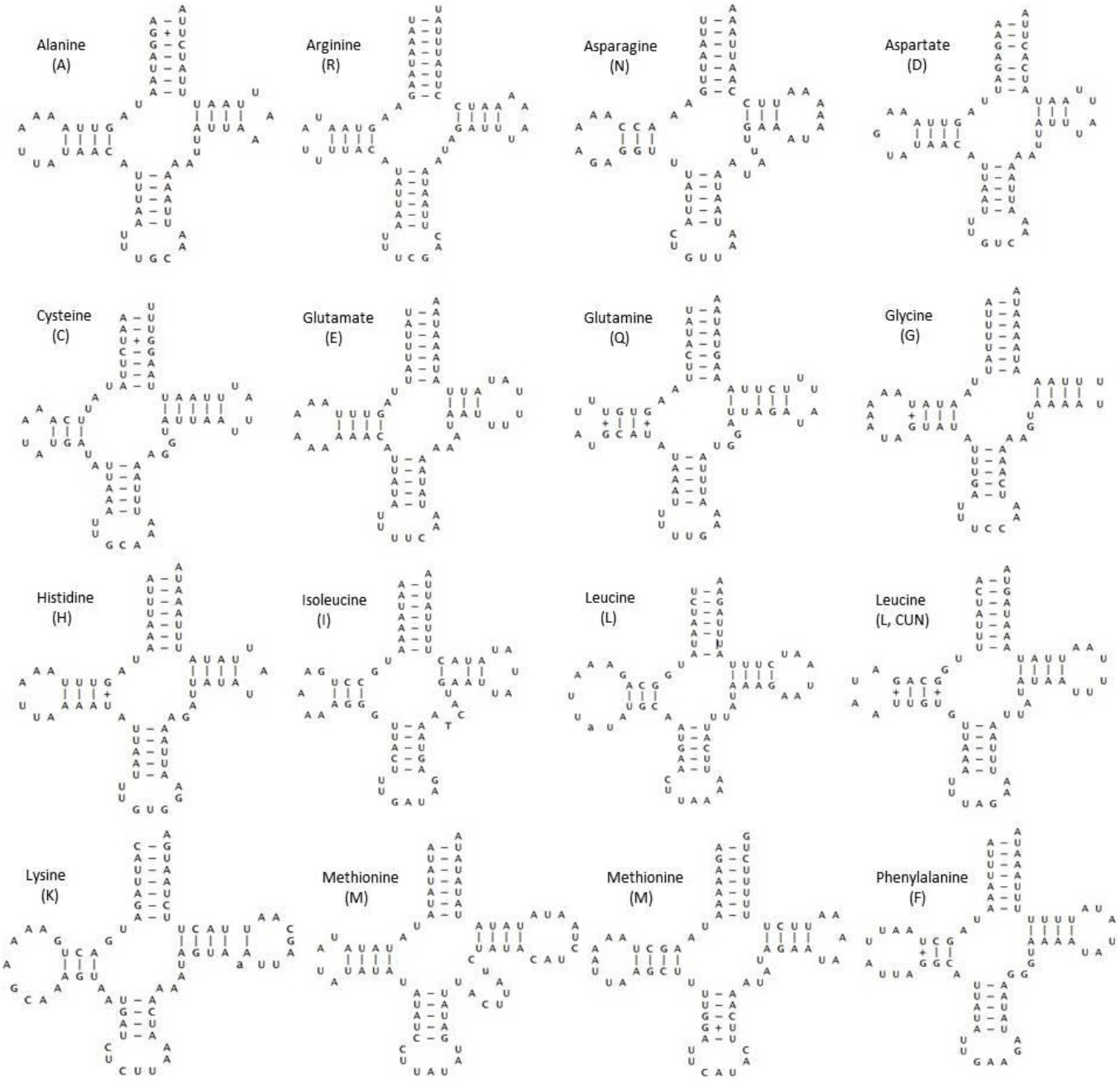
There are two major non-coding regions of 574 bp and 662 bp located at the gene junctions $tRNA^{Glu}$/$tRNA^{Phe}$ and *s-rRNA*/$tRNA^{Ile}$ respectively, the latter being the AT-rich region or putative control region that is responsible for the initiation and regulation of mitochondrial transcription and replication. Except for these non-coding regions, the entire genome comprises of compactly arranged coding sequences with no introns and with very few intergenic nucleotides. *D. noxia's* mitochondrial genes overlap at 11 locations, varying from 1 - 67 bp with the largest protein-coding gene overlap located between *atp8* and *atp6* (14 bp) and the largest tRNA-gene overlap located between *$tRNA^{Ser}$* and *$tRNA^{Trp}$* (67 bp).

Table 3.2. List of mitochondrial genes of *D. noxia* indicating orientation, size and codons.

Gene	Direction	Region (bp)	Size (bp)	Anticodon	Start codon	Stop codon
COI	forward	1-1,531	1,531		ATA	T-tRNA
tRNA^{Leu}	forward	1,532-1,599	68	TAA		
COII	forward	1,603-2,274	672		ATA	TAA
tRNA^{Lys}	forward	2,277-2,349	73	CTT		
tRNA^{Asp}	forward	2,350-2,411	62	GTC		
atp8	forward	2,421-2,570	150		ATA	TAA
atp6	forward	2,557-3,204	648		ATA	TAA
COIII	forward	3,204-3,989	786		ATG	TAA
tRNA^{Gly}	forward	3,989-4,052	64	TCC		
ND3	forward	4,050-4,406	357		ATA	TAA
tRNA^{Ala}	forward	4,407-4,470	64	TGC		
tRNA^{Arg}	forward	4,469-4,534	66	TCG		
tRNA^{Asn}	forward	4,534-4,599	66	GTT		
tRNA^{Glu}	forward	4,664-4,728	65	TTC		
tRNA^{Phe}	reverse	5,303-5,371	69	GAA		
ND5	reverse	5,372-7,042	1,671		TAA	ATT
tRNA^{His}	reverse	7,094-7,156	63	GTG		
ND4	reverse	7,157-8,465	1,309		TAT	A-tRNA
ND4l	reverse	8,474-8,764	291		TAT	ATT
tRNA^{Thr}	forward	8,766-8,827	62	TGT		
tRNA^{Pro}	reverse	8,829-8,895	67	TGG		
ND6	forward	8,897-9,388	492		ATT	TAA
cytb	forward	9,392-10,510	1,119		ATG	TAA
tRNA^{Ser}	forward	10,515-10,579	65	TGA		
ND1	reverse	10,589-11,524	936		TAA	ATT
tRNA^{Leu}	reverse	11,525-11,589	65	TAG		
lrRNA	reverse	11,591-12,849	1,259			
tRNA^{Val}	reverse	12,848-12,908	61	TAC		
srRNA	reverse	12,924-13,691	768			
tRNA^{Met}	forward	14,011-14,082	72	TAT		
tRNA^{Ile}	forward	14,354-14,417	64	GAT		
tRNA^{Gln}	reverse	14,415-14,480	66	TTG		
tRNA^{Met}	forward	14,486-14,552	67	CAT		
ND2	forward	14,553-15,530	978		ATT	TAA
tRNA^{Trp}	forward	15,529-15,594	66	TCA		
tRNA^{Ser}	forward	15,530-15,596	67	TGA		
tRNA^{Cys}	reverse	15,588-15,652	65	GCA		
tRNA^{Tyr}	reverse	15,655-15,720	66	GTA		

3.3.3 Transfer RNA and ribosomal RNA genes

The mitochondrial genome of *D. noxia* contains 23 *tRNA* genes consisting of 20 different transfer RNA genes, of which *tRNA*^{Leu}, *tRNA*^{Ser} and *tRNA*^{Met} appears in duplicate (Fig. 3.4, Table 3.2). The sizes of the *tRNA* genes in *D. noxia* ranges from 61 (*tRNA*^{Val}) to 73 bp (*tRNA*^{Lys}). All these tRNAs displayed the typical clover-leaf structure synonymous to arthropod mitochondrial genomes. Differences observed between the folded secondary structures were mostly located in the dihydrouridine (DHU) and TψC loops (Fig. 3.3). The obtained DHU arm structures were also reported for *A. pisum* and *S. graminum*, and other insect genomes (Cameron and Whiting 2008; Wan *et al*., 2012).



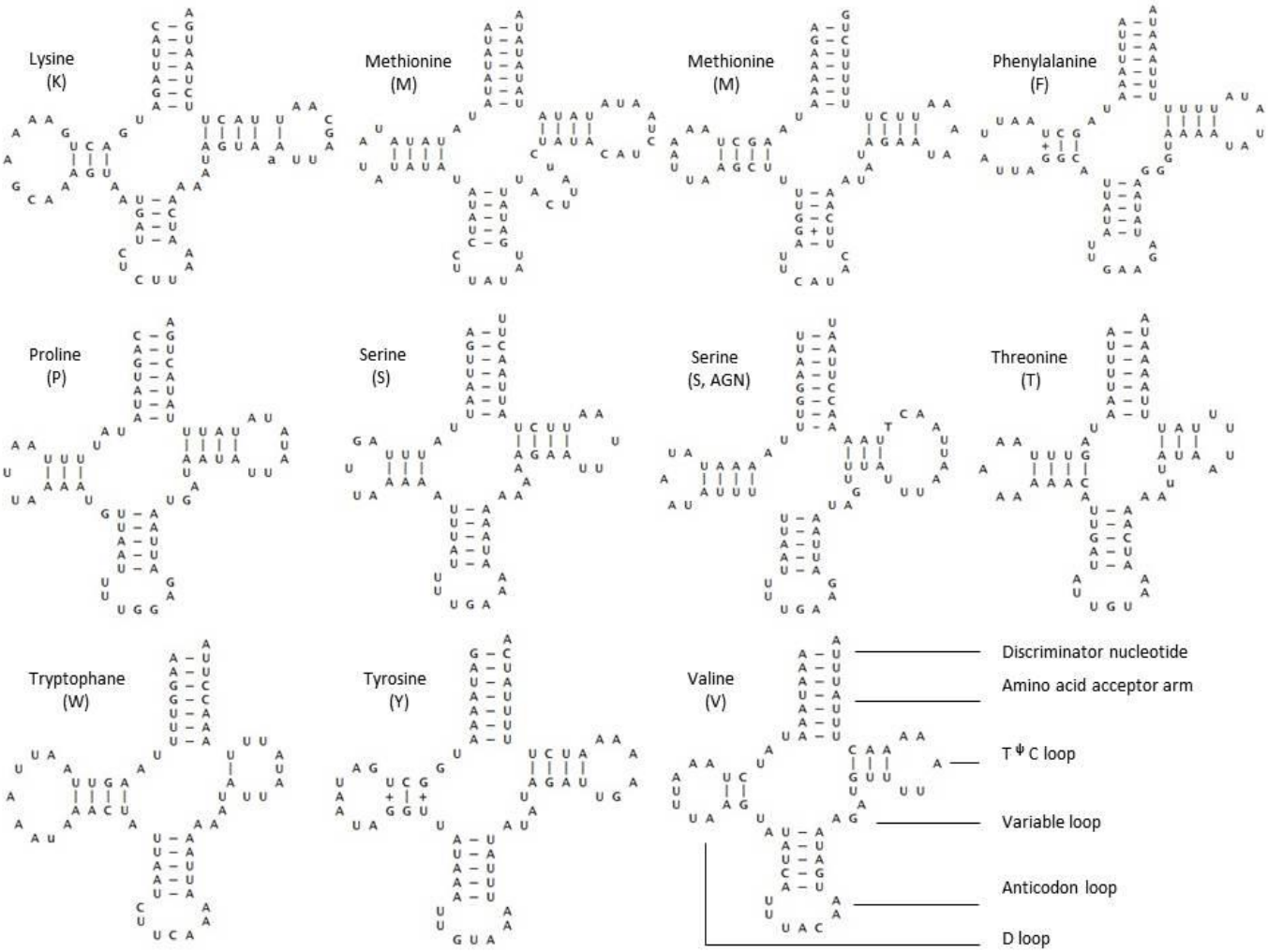


Fig. 3.3. Inferred secondary structure of the tRNAs present in the *D. noxia* mitochondrial genome. The tRNAs are labelled with the abbreviations of the corresponding amino acids. The tRNAs for Leu and Ser are identified in the figure by the codons recognized, rather than by the anticodon present in the tRNA itself. Dashes (-) indicate Watson-Crick base pairing and (+) indicate G-U base pairing.

Table 3.3. Comparison between the mitochondrial genomes of *D. noxia* to the two closest related Aphididae genera (*A. pisum* and *S. graminum*). Termination codons were excluded in total number of codons.

Aphid species	Whole genome		PCGs*		L-rRNA		S-rRNA		AT-rich region	
	Size (bp)	AT%	No. of codons	AT%	Size (bp)	AT%	Size (bp)	AT%	Size (bp)	AT%
Diuraphis noxia	15,721	84.7	3,631	84.2	1,259	85.1	768	84.6	662	86.4
Schizaphis graminum	15,721	83.9	3,655	83.3	1,259	85.4	766	84.1	622	86.8
Acyrthosiphon pisum	16,971	84.7	3,631	83.7	1,259	85	767	84	1,006	89.2

* PCGs: Protein-coding genes

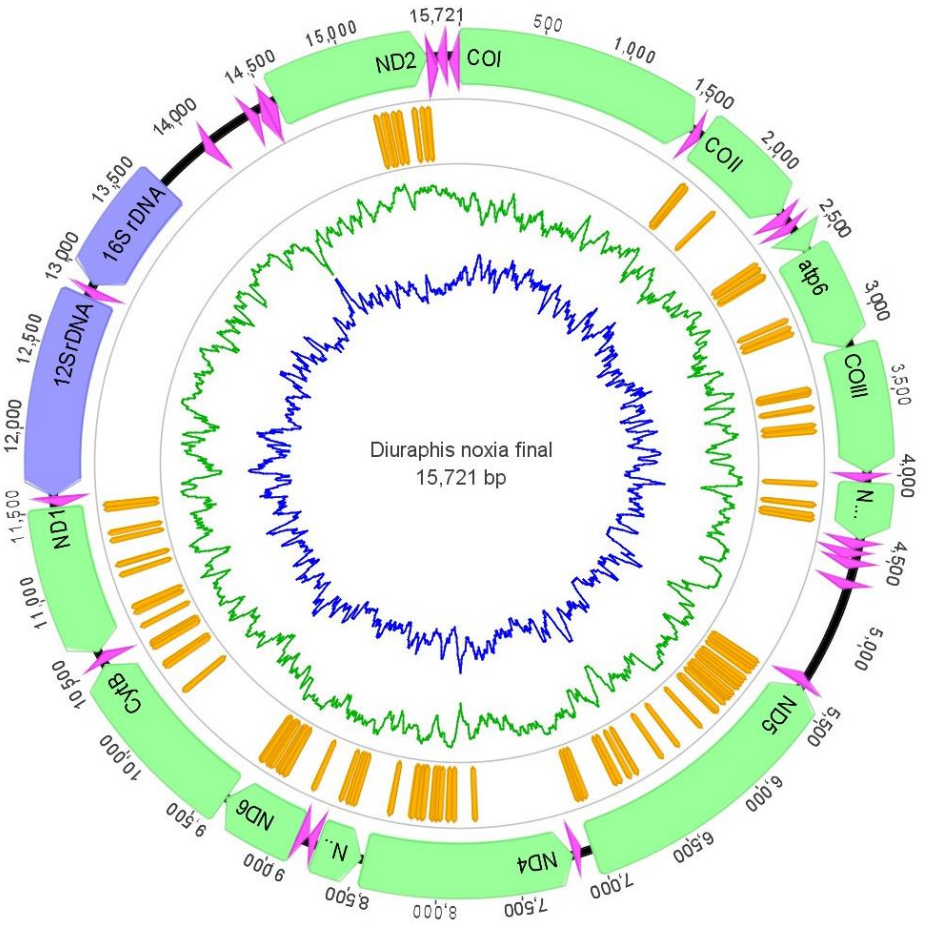


Fig. 3.4. Organization of the mitochondrial genome of *D. noxia.* Genes are transcribed in a clockwise direction, except where otherwise indicated. tRNA genes are indicated in pink and single nucleotide polymorphisms among the populations are indicated in orange. The percentage of AT content across the whole mitochondrial genome is indicated in green while GC content is indicated in blue.

Table 3.4. Comparison between the percentage identities obtained from whole genome alignments of *A. pisum*, *S. graminum* and *D. noxia* using MAFFT.

	Diuraphis noxia	*Acyrthosiphon pisum*	*Schizaphis graminum*
Diuraphis noxia		85.30%	88.20%
Acyrthosiphon pisum	85.30%		84.10%
Schizaphis graminum	88.20%	84.10%	

3.3.4 Comparison between *D. noxia* and its closest relatives *S. graminum* and *A. pisum*

When comparing the mitochondrial genomes of *D. noxia* to that of *S. graminum* and *A. pisum* (Table 3.3), it was found that the *D. noxia* mitochondrial genome is similar in size to that of *S. graminum,* but smaller than that of *A. pisum* (16 971 bp *vs* 15 721 bp). When comparing the mitochondrial genomic composition of *D. noxia* to that of *S. graminum* and *A. pisum* (Fig. 3.5, Tables 3.4 and A4) expressed in the percentage identities, it was found that the *D. noxia* mitochondrial genome was more similar to that of *A. pisum* than to *S. graminum* when defined by similarity on PCGs. Other distinct differences between the genomes of *D. noxia*, *A*. *pisum* and *S. graminum* include the number of PCGs, length and AT-richness of the *12S rDNA* and repeat region. Direct comparison in the percentage AT-richness revealed that both *D. noxia* and *A. pisum* had an AT-richness of 84.7%, while the genome of *S. graminum* is 83.70% (Table 3.3).

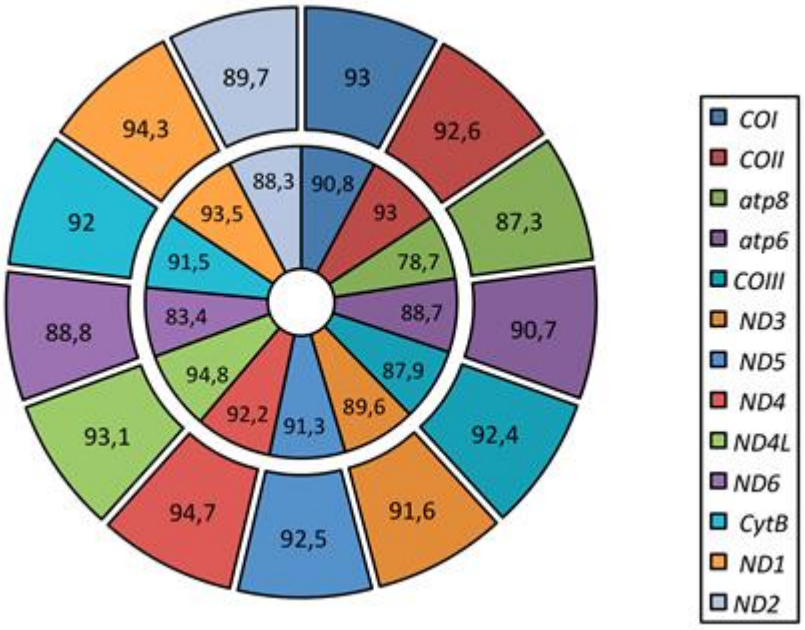


Fig. 3.5. A comparison of protein coding genes between *A. pisum*, *S. graminum* and *D. noxia.* The percentage identity between the protein coding genes of *D. noxia* and *A. pisum* (outer circle) and *S. graminum* (Inner circle).

The *D. noxia* genes/regions are arranged in the same order as the ancestral insect genome, which differ to the arrangement in other groups within the Sternorrhyncha (i.e., psyllids and whiteflies) (Boore 1999) (Figs. 2, S8). A unique feature to the mitochondrial genome of *D.*

noxia and its close aphid relatives, *A. pisum* and *S. graminum*, is the presence of an additional non-coding repeat region when compared to other groups within the Sternorrhyncha (i.e., psyllids and whiteflies). This region occurs consistently between the $tRNA^{Glu}$ and $tRNA^{Phe}$. In the genomes of *D. noxia* (this study) and *A. pisum* (Wang *et al.* 2013) an additional $tRNA^{Met}$ is located in the large repeat region. The *D. noxia* mitochondrial genome, like with *A. pisum* and *S. graminum*, contains an additional $tRNA^{Ser}$ located after $tRNA^{Trp}$. Direct comparisons of gene/region order with *D. noxia*'s closest relatives, *A. pisum* and *S. graminum*, revealed a similar gene/region order with the additional repeat region (Figs. 3.2 and A8).

When comparing the PCGs between these taxa, it found that *D. noxia* and *A. pisum* were more similar, than *D. noxia* and *S. graminum* (Fig. 3.5) despite their size difference and regards to their AT-richness. Direct comparisons between the PCGs revealed, with the exception of the *COI* and *ND4L* genes (where these gene sequences were more similar between *D. noxia* and *S. graminum)*, that all other genes had higher similarities between *D. noxia* and *A. pisum*. Interestingly, the 12S rDNA gene of *D. noxia* contains the highest AT-richness, while *A. pisum* has the longest AT-rich repeat region. When comparing codon numbers and codon usage, comparable codon numbers were found in *D. noxia* (3 631), *A. pisum* (3 631) and *S. graminum* (3 655) (Table 3.3). Also, in all these taxa ATN served as initiation codon, while TAA, a single T, and TA were used as termination codons.

3.3.5 Genetic variants amongst *D. noxia* populations

SOLiD sequences obtained from sequencing a pooled sample of nine populations, were reference mapped to the assembled mitochondrial genome of the *D. noxia* SAM biotype using Geneious v6.1.6. After mapping, a total of 125 SNPs were called using the above qualifiers and these occurred within the PCGs (*Atp8*, *Atp6*, *COII*, *COIII*, *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *DN6, CytB*). The majority of these SNPs resulted in amino acid changes via substitutions, but five resulted in truncations and two deletions induced frame shift mutations (Tables 3.5 and A5). The SNP variant frequency varies between 20 and 63.8 %, with the most variant SNP being 63.8 % for a deletion (-C) that results in a frame shift at position 20 in *ND5*. This PCG also displayed the highest variability with the highest number of SNPs (30). Eight of these SNPs however had no effect on the protein and are predicted to be silent. The observed nucleotide changes further supported the AT-bias in the *D. noxia* genome, since with a few exceptions (22) the nucleotide changes either resulted in a C (10.4 %) or G (7.2 %), while in all other instances the change resulted in an A or T, with most changing into adenine (50.4 %). The PCG with the least amount of SNPs was *ND4L* (4). A

subset of the SNPs obtained through SNP calling were confirmed using Sanger sequencing (Figs. A9 to A11).

Table 3.5. Single nucleotide polymorphisms (SNPs) obtained amongst nine *D. noxia* populations that resulted in truncation and frame shift type mutations with a minimum variant frequency of 20% using the Geneious software v6.1.6. Indicated are the gene, SNP position in the CDS, nucleotide change, protein effect, coverage and variant frequency.

CDS Position (Bp)	Polymorphism Type	Nucleotide Change	Gene	Protein Effect	Coverage	Variant Frequency
			COII			
382	Transversion	G -> T		Truncation	92	21.70%
			ND3			
177	Substitution	CTTA -> AGAG		Truncation	56	29.20%
			ND5			
20	Deletion	- C		Frame Shift	376	63.80%
			ND4			
331	Transversion	C -> A		Truncation	165	25.50%
			ND6			
254	Transversion	T -> A		Truncation	52	21.20%
261	Transversion	T -> A		Truncation	50	22.00%
			ND2			
854	Deletion	-AT		Frame Shift	91	36.30%

3.4 Discussion

The mitochondrial genome of the class Insecta showed considerable variation in gene order in the Hemipteroid assemblage (i.e., orders Phthiraptera, Psocoptera, and Thysanoptera), but a high level of conservancy in gene order in the Hemiptera (including the Sternorrhyncha) indicating an ancestral gene order for this group (Boore 1999; Shao and Baker 2003; Thao *et al.,* 2004). From the phylogenetic analyses using all the coding genes (Figs. 3.1, A1-A7), it can be inferred that *A. pisum* and *S. graminum* are more closely related to *D. noxia* in the Hemiptera (Sternorrhyncha), than any other species included in the study. Based on the conserved gene order in this group, these members can therefore be used for mapping and genome comparison as was demonstrated in the present study.

The complete mitochondrial genome of *Diuraphis noxia* is 15 721 bp in length and consists of 38 genes typically found within most insects: 20 different transfer RNA genes (including a duplicate $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Met}$), 13 PCGs, and 2 ribosomal genes (Fig. 3.4, Table 3.2). The obtained genome size of *D. noxia* falls in the range of animal mitochondrial genomes (~16 000 bp long) (Crozier and Crozier 1993). As previously shown, with some exceptions, the mitochondrial gene order in *D. noxia* is similar to that previously reported for aphids and psyllids (Figs. 3.2, A8), and corresponds to that of the ancestral aphid species (*Daktulosphaira vitifoliae*) supporting previous reports on high conservancy within this order (Wolstenholme 1992; Crozier and Crozier 1992, 1993; Boore 1999; Thao *et al.,* 2004).

The mitochondrial genomes of insects are very compact with a general consensus that the principal non-coding segments of the genome are low in G+C content usually following *12S rDNA* (Boore 1999; Scheffer 1999; Wolstenholme 1992; Thao *et al*., 2004). The consensus mitochondrial genome obtained in this study is slightly smaller in size (64 bp) than that previously reported by Zhang *et al.,* (2014, 15 784 bp) for *D. noxia*. However, this size difference may be ascribed to the different strategies followed. In the case of the Zhang *et al*., (2014) study, PCR amplification of genomic regions using 7 primers designed from *A. pisum* (GenBank accession no.'s NC_011594, Moran *et al.,* 2008 unpublished) and *S. graminum* (GenBank accession no.'s NC_006158, Thao *et al*., 2004) was used as template for primer design to obtain the mitochondrial genomic regions, and thus represented low sequence depth. The data in the paper is also based on a single aphid population from China. In the present study, sequence data was obtained from nine global *D. noxia* populations after using several Next Generation sequencing platforms and Sanger sequencing providing more than 348x coverage of the mitochondrial genome of *D. noxia* (Botha *et al.,* 2012; Botha 2013).

Variation in mitochondrial genome size is mostly the consequence of the length of the non-coding repeat regions as observed in the present study (e.g., when comparing the genomes of *A. pisum* and *S. graminum*, with that of *D. noxia*), and to a lesser extent due to the increase/decrease in number or length of structural genes (Table 3.3). Even though the genomes of *D. noxia* and *S. graminum* were found to be similar in size, distinct differences were observed in the PCGs. Also, the *12S rDNA* genes of *D. noxia* are more AT rich, but with a lower AT-richness in the *16S rDNA*. The genome of *A. mellifera* were also found to have an AT-bias, with 84.9% of the nucleotides being either A or T, having guanine as the rarest nucleotide (Crozier and Crozier 1993). Direct comparison in the percentage identities obtained from whole genome alignments between *D. noxia, A. pisum* and *S. graminum* using MAFFT, revealed the highest similarity in the mitochondrial genomes of *D. noxia* and *S.*

graminum (88.20%, Table 3.4)., which is likely the result of the size of the AT-rich region (662 bp *vs* 622 bp, respectively, Table 3.3).

In the present study, it was found that 24 of the genes are transcribed on the majority-coding strand (H-strand) and the remaining 14 genes are transcribed on the minority-coding strand. Zhang *et al.,* (2014) found 22 tRNA genes in the mitochondrial genome of *D. noxia* as opposed to the 23 tRNA genes found in the present study. The *D. noxia* mitochondrial genome contains 20 different transfer RNA genes, with $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Met}$ in duplicate, and all of these displaying the typical clover-leaf structure synonymous to arthropod mitochondrial genomes (Crozier and Crozier 1993). These findings are congruent with the tRNAs present in the mitochondrial genome of *A. pisum*. While the location and length of tRNA genes are similar in *D. noxia, A. pisum* and *S. graminum,* the latter contains one less $tRNA^{Met}$ positioned after the $tRNA^{Val}$. When comparing *D. noxia* to that of Zhang *et al.,* (2014), their *D. noxia* had a translocated $tRNA^{Ser}$. Thus, showing that findings in the present study are more similar to that of *A. pisum* and *S. graminum* than that reported by Zhang *et al*., (2014). Since the conclusions drawn in the present study is based on analyses of a much larger sequence data set, the findings are likely to be more conclusive.

Generally aphids have low mitochondrial DNA divergence with divergence in the order of 0.4% for the mitochondrial *COI* gene (Boulding 1998; Sunnucks and Hales 1996), but can be as high as 1.7% (i.e., *Sitobion avaenae,* Xu *et al*., 2011) for migratory pest insects that cover wide ecological regions like *D. noxia.* These findings suggest that gene flow among aphid populations may prevent natural populations from diverging by genetic drift. To understand the diversity of *D. noxia* populations so as to infer observed fitness, we sequenced the genomes of nine global *D. noxia* populations expressing different levels of virulence and compared the variant SNP frequency amongst them. Using SOLiD sequences obtained from sequencing a pooled sample of nine distinct populations, a total of 125 SNPs were called within the PCGs. Of these, 15.2% (19) were silent mutations resulting in no amino acid change, while the majority (80%) produced amino acid changes via substitutions, but with 3.2% (5) resulting in truncations and 1.6% (2) in deletions inducing frame shift mutations (Table 3.5). Much higher levels of substitutions than any other form of mutation were also reported for *A. mellifera* (honeybee) PCGs (Crozier and Crozier 1992, 1993). The SNP variant frequency varies between 20% and 63.8%, with the average frequency of most of the SNPs being 24.8 $\pm$ 5.3%. The observed high SNP variant frequency was expected, as previous studies based on mitochondrial DNA sequence analysis of the *COI* gene from *D. noxia* from several US and South African populations suggested limited variation (Shufran *et al.,* 2007, 2009; Lapitan *et al.,* 2007). In the present study, using stringent parameters, we

didn't find SNPs in the COI gene of our samples either, and therefore the current data is supportive of their findings.

We observed SNP variant frequencies in the PCGs in the following order *ND5* > *ND6* > *ND4* > *ND1* > *ND2* > *CytB* > *ND3*, *COIII*, *COII, atp6* > *atp8* > *ND4L* with the majority of the SNPs (75.2%) occurring in the *ND* genes. In a Metazoan mitochondrial context, the *ND* genes are the protein backbone of the respiratory chain Complex I (CI, NADH: ubiquinone oxidoreductase) - a gaint multiheteromeric enzyme located in the inner membrane of the mitochondrion, consisting of a peripheral arm protruding into the mitochondrial matrix, where electron transport takes place, and a membrane-embedded arm, where proton translocation takes place. Complex I contains seven of the 13 mitochondrial encoded proteins (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), with ND2, ND4 and ND5 that seem to function as electron transporters, while ND1 and ND2 play an important structural role between the membrane-embedded and peripheral arms of the Complex (Da Fonseca *et al.,* 2008). Complex II consists entirely of nuclear encoded genes and Complex III of CytB (containing the essential catalytic active site). In the CO Complex (Complex IV), COI catalyzes electron transfer to the ultimate acceptor, molecular oxygen. COII and COIII also belong to the catalytic core of this complex. While Complex V (ATP synthase) harbours atp6 and atp8, where atp6 is a key component of the proton channel and atp8 the apparent regulator of the complex assembly (Da Fonseca *et al*., 2008; Castellana *et al.,* 2011). In *D. noxia* the majority (24%) of SNP variants occurring in the PCGs were observed in the *ND5* gene. Unfortunately, little is known about the specific causal effects of mutations occurring in the *ND5* gene in the mitochondrial Complex I of Insecta. In mammalian mitochondrial genomes, however mutations in these genes are known to have dire consequences, such as early age onset in young and encephalopathy (Janssen *et al.,* 2006; Carroll *et al.,* 2006; Malfatti *et al.,* 2007) clearly reducing the fitness of the affected individuals. The high SNPs variant frequency in the *ND5* gene may also have similar fitness consequences for a migratory pest like *D. noxia* living under varying ecological regions.

Purifying selection plays a major role on all mitochondrion genes, with *CO* and *CytB* genes revealing the most stringent selection, while selection is more relaxed for ATP synthase and some NADH dehydrogenase complex genes. It is suggested that mutation and genetic drift is governed by synonymous changes in mitochondrial PCGs, where gene function is strongly preserved through purifying selection. Especially removal of mutations which cause amino acid changes in subunits possessing crucial functions, such as *COI, COII, COIII*, and *CytB* (Castellana *et al.,* 2011). Our findings concurred with previous observations about the *CO* and *ND* genes (Wang *et al*., 2013), but differed with regards to the *CytB* and ATP synthase genes, with the latter showing limited variation. This is contradictory of recent findings that

suggest the highest evolutionary rates in *atp8* for the Hemipterans (Aphididae) in general (Wang *et al*., 2013). The conservancy in the ATP synthases may point towards increased importance of the *atp6* and *atp8* genes in aphid fitness.

Mitochondrial genes are also known to be under natural selection pressures, suggesting the minimising of non-functional gene length, elimination of redundant regions and shrinkage or reduction in genome sizes (Schneider and Ebert 2004; McKnight and Shaffer 1997; Rand 1993). In the present study, findings on the mitochondrial genome of *D. noxia* suggest otherwise with increased repeat regions and additional tRNA genes. These findings also concurred with previous findings on *A. pisum* and *S. graminum* (Thoa *et al.,* 2004), which suggests that the mitochondrial genome of *D. noxia* continues to increase over time.

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3.6 Appendix A

Table A1. Primer sequences used for confirmation of contig assembly in the repeat regions, and SNPs obtained between selected *D. noxia* biotypes.

Target region	Primer ID	Sequence
Repeat region 1	RR1_F	TATGTTTAACCGCAACTGCTG
	RR1_R	CATGATTTACTCTATCAAAGTAACCCT
Overlapping - Start and end regions	BE_F	CCTTAATTAAATAATTAATACCTTTAAATTTGC
	BE_R	GATCCAATTATACCTGATCAAATTCC
Repeat region 2	RR2_F	AGAAGCTGCTAACTATCTTTTAAAGC
	RR2_R	GAGCATTATATTGAAGATATAAGGGTAA
COI	COI_F	ACCACGACGATATACAGATT
	COI_R	TGAATGTTCTAATGGTGGTAA
COII	COII_F	ACTAAGATTTCAAAATAGAAATTCTC
	COII_R	GTGTAAAGATGGTATAGCAATA
atp8	atp8_F	ACTTAATTCCTCAAATAGC
	atp8_R	GTTGATGGATCAAAAATATTA
atp6	atp6_F	TTCAATAACTTTAGCTTTACCATTTTG
	atp6_R	GCAGTAAGACGAATTGATAAAGATA
COIII	COIII_F	TAAACTTATTCATAATTATTAGTATTTC
	COIII_R	GATGGTGCTAATGAGTTAT
NR3	ND3_F	ATCATAATAAATCAGCACCATTCG
	ND3_R	CAATTTAAAGATCCAAATTTTCATTCA
ND4	ND4_F	TGAACAACAGAAGAATAAGCA
	ND4_R	AGTGGTTCAATAATTTTAGCTGG
ND6	ND6_F	ATTATGAAAATAAATTTATATTTGAAG
	ND6_R	CGAATAGGCCCCTTATTA
CytB	CytB_F	TCATTCCATGCTAATGGAGC
	CytB_R	CTGTGGCTCCTCAGAATGATA
ND1	ND1_F	AGGAGAACGATTTATTTCAGC
	ND1_R	CTAATTCAAATTATTCAGTTTTAGGA
ND2	ND2_F	CTATATCATCAATTAATAATACTACATG
	ND2_R	TAATATAGGAGGTAAACCTATAATTG

Table A2. Number of mitochondrial genome sequences for the Hymenoptera and Hemiptera, Sternorrhyncha. In this study, the complete (consensus) mitochondrial genome of *D. noxia* (Kurdjumov) was added to the available data set. Asterisk indicates available complete mitochondrial genomes.

Genera	Genbank entries	Accession numbers
Acyrthosiphon pisum *	23 OCT 2008	NC_011594
Adelges laricis	21 OCT 2005	DQ256161
Adelges laricis	05 JUN2000	AF275215
Adelges laricis	04 DEC 2008	FJ502429

Aphis gossypii	19 DEC 2007	EU358872
Aphis gossypii	16 NOV 2009	GU205351
Aphis gossypii	05 JUN 2009	AB506731
Apis mellifera ligustica *	08 SEP 1999	NC_001566
Aleurochiton aceris *	27 DEC 2003	NC_006160
Aleurodicus dugesii *	27 DEC 2003	NC_005939
Bactericera cockerelli	16 APR 2004	AY601890
Baizongia pistaciae	06 MAY 2008	EU701529
Bemisia tabaci *	29 DEC 2003	NC_006279
Calophya schini	16 APR 2004	AY601891
Cinara longipennis	08 JAN 2010	GU457829
Cinara longipennis	08 JAN 2010	GU457835
Cinara terminalis	15 MAY 2003	AY300193
Cinara villosa	15 MAY 2003	AY300207
Cinara wahtolca	15 MAY 2003	AY300204
Daktulosphaira vitifoliae	28 APR 2005	DQ021446
Diuraphis noxia	This study	This study
Diuraphis tritici	08 OCT 2010	HQ392589
Glycaspis brimblecombei	16 APR 2004	AY601889
Hyalopterus pruni	19 DEC 2007	EU358890
Hyalopterus pruni	08 JAN 2010	GU457815
Hyalopterus pruni	17 SEP 2012	JX844371
Melaphis rhois	05 JUN 2000	AF275223
Melaphis rhois	20 SEP 1995	MRU36747
Melaphis rhois	31 AUG 2013	KF601668
Myzus persicae	19 DEC 2007	EU358893
Myzus persicae	01 NOV 2010	HQ528298
Myzus persicae	05 JUN 2009	AB506740
Neomaskellia andropogonis *	12 MAR 2004	NC_006159
Pachypsylla venusta *	16 APR 2003	NC_006157
Pemphigus betae	07 APR 2010	HM064549
Pineus pini	26 JAN 2010	GU571089
Pineus pini	20 OCT 2006	EF073216
Rhopalosiphum maidis	19 DEC 2007	EU358894
Rhopalosiphum maidis	01 NOV 2010	HQ528300
Rhopalosiphum maidis	10 MAY 2013	KF022221
Rhopalosiphum padi	19 DEC 2007	EU358896
Rhopalosiphum padi	08 APR 2004	AY594671
Rhopalosiphum padi	07 AUG 2001	AJ315884
Schlechtendalia chinensis	20 SEP 1995	SCU36737
Schlechtendalia chinensis	01 DEC 2001	AF454628
Schlechtendalia chinensis	31 AUG 2013	KF601669
Schizaphis graminum *	21 JAN 2004	NC_006158
Tetraleurodes acaciae *	29 DEC 2003	NC_006292
Tetraneura chinensis	02 APR 2007	EF534368
Trialeurodes vaporariorum *	29 DEC 2003	NC_006280
Uroleucon ambrosiae	16 APR 1998	AF059686
Uroleucon ambrosiae	03 APR 1998	AF057047
Uroleucon leonardi	01 NOV 2010	HQ528302
Uroleucon rurale	16 APR 1998	AF059685
Uroleucon rurale	03 APR 1998	AF057046

Table A3. Synonymous codon usage in the *D. noxia* mitochondrial genome.

Amino Acid / Percentage (%)*	Codon	Number	Amino Acid / Percentage (%)	Codon	Number
Ala/2.0	GCA	28	Pro/3.0	CCA	51
	GCC	6		CCC	2
	GCG	0		CCG	0
	GCT	39		CCT	55
Cys/0.9	TGC	1	Gln/1.3	CAA	47
	TGT	32		CAG	2
Asp/1.4	GAC	4	Arg/1.1	CGA	24
	GAT	46		CGC	1
Glu/1.9	GAA	61		CGG	0
	GAG	7		CGT	14
Phe/13.5	TTC	29	Ser/7.7	AGA	59
	TTT	464		AGC	2
Gly/3.7	GGA	69		AGG	0
	GGC	0		AGT	33
	GGG	6		TCA	107
	GGT	61		TCC	1
His/1.5	CAC	8		TCG	0
	CAT	47		TCT	78
Ile/13.5	ATC	20	Thr/3.3	ACA	70
	ATT	472		ACC	1
Lys/3.9	AAA	132		ACG	0
	AAG	11		ACT	49
Leu/15.1	CTA	17	Val/2.0	GTA	24
	CTC	0		GTC	1

	CTG	0		GTG	2
	CTT	23		GTT	44
	TTA	494	Trp/2.3	TGA	84
	TTG	16		TGG	1
Met/8.8	ATA	302	Tyr/4.5	TAC	11
	ATG	15		TAT	154
	ATT	4	Stop/0.3	TAA	10
Asn/8.2	AAC	21		TAG	0
	AAT	279			

*The percentage next to each amino acid three letter codes indicate the percentages of the amino acids found among all the protein coding genes.

Table A4. Codon usage in the *D. noxia* mitochondrial genome.

Amino Acid	Frequency	Percentage
A	73	2.00%
C	33	0.90%
D	50	1.40%
E	68	1.90%
F	493	13.50%
G	136	3.70%
H	55	1.50%
I	492	13.50%
K	143	3.90%
L	550	15.10%
M	321	8.80%
N	300	8.20%
P	108	3.00%
Q	49	1.30%
R	39	1.10%
S	280	7.70%
T	120	3.30%
V	71	2.00%
W	85	2.30%
Y	165	4.50%
*	10	0.30%

Table A5. Single nucleotide polymorphisms (SNPs) (excluding SNPs resulting in truncations and frame shifts) obtained amongst nine *D. noxia* populations with a minimum variant frequency of 20% using the Geneious software v6.1.6. Indicated are the gene, SNP position in the CDS, nucleotide and amino acid change, type of change, coverage and variant frequency. SNPs indicated in grey represent positional overlapping PCGs.

CDS Position (Bp)	Polymorphism Type	Nucleotide Change	Amino Acid Change	Codon Change	Protein Effect	Coverage	Variant Frequency
			COII				
102	Transversion	A -> T	M -> I	ATA -> ATT	Substitution	161	26.70%
104	Transversion	C -> A	S -> Y	TCT -> TAT	Substitution	119	21.80%
104	Transition	C -> T	S -> F	TCT -> TTT	Substitution	119	23.50%
107	Transition	C -> T	T -> M	ACA -> ATA	Substitution	80	30.00%
111	Transition	C -> T		ATC -> ATT	None	57	29.80%
132	Transversion	T -> G	I -> M	ATT -> ATG	Substitution	74	20.30%
			atp8				
42	Transition	C -> T		TTC -> TTT	None	113	22.10%
133	Transversion	T -> A	Y -> N	TAT -> AAT	Substitution	259	24.70%
140	*Transversion*	*A -> T*	*K -> M*	*AAA -> ATA*	*Substitution*	*93*	*21.50%*
140	*Transversion*	*A -> T*	*N -> Y*	*AAT -> TAT*	*Substitution*	*93*	*21.50%*
146	*Transversion*	*T -> A*	*I -> N*	*ATT -> AAT*	*Substitution*	*51*	*21.60%*
146	*Transversion*	*T -> A*	*F -> I*	*TTT -> ATT*	*Substitution*	*51*	*21.60%*
			atp6				
4	*Transversion*	*A -> T*	*K -> M*	*AAA -> ATA*	*Substitution*	*93*	*21.50%*
4	*Transversion*	*A -> T*	*N -> Y*	*AAT -> TAT*	*Substitution*	*93*	*21.50%*
10	*Transversion*	*T -> A*	*I -> N*	*ATT -> AAT*	*Substitution*	*51*	*21.60%*
10	*Transversion*	*T -> A*	*F -> I*	*TTT -> ATT*	*Substitution*	*51*	*21.60%*
353	Transition	G -> A	C -> Y	TGT -> TAT	Substitution	96	21.90%
405	Transition	T -> C		ATT -> ATC	None	246	28.90%
447	Transition	T -> C		ATT -> ATC	None	230	27.80%
			COIII				
206	Transversion	G -> C	G -> A	GGT -> GCT	Substitution	213	26.30%
226	Substitution	ATA -> TTT	M -> F	ATA -> TTT	Substitution	124	29.00%
245	Transversion	G -> T	S -> M	AGA -> ATA	Substitution	60	20.00%
342	Transition	A -> G		TTA -> TTG	None	143	23.10%
345	Transition	T -> C		AAT -> AAC	None	131	22.90%
462	Transversion	T -> A	N -> K	AAT -> AAA	Substitution	101	25.70%
509	Transversion	G -> T	S -> I	AGT -> ATT	Substitution	90	22.20%
			ND3				
30	Transversion	A -> T	L -> F	TTA -> TTT	Substitution	69	20.30%
185	Transversion	C -> A	A -> E	GCA -> GAA	Substitution	64	21.90%
254	Transition	C -> T	S -> F	TCT -> TTT	Substitution	153	24.20%
257	Transversion	A -> T	N -> I	AAT -> ATT	Substitution	182	21.40%
279	Transversion	A -> T	L -> F	TTA -> TTT	Substitution	192	29.70%
281	Transversion	T -> A	F -> Y	TTT -> TAT	Substitution	134	23.90%

				ND5			
44	Transversion	T -> A	K -> M	AAA -> ATA	Substitution	260	20.80%
56	Transversion	A -> T	I -> N	ATT -> AAT	Substitution	373	21.70%
83	Transition	G -> A	S -> F	TCT -> TTT	Substitution	142	26.80%
309	Transition	C -> T		GGG -> GGA	None	227	28.20%
348	Transition	T -> C		AAA -> AAG	None	121	29.80%
417	Transversion	T -> A		TCA -> TCT	None	151	20.50%
473	Transversion	C -> A	S -> M	AGA -> ATA	Substitution	88	28.40%
475	Transversion	T -> A	N -> Y	AAT -> TAT	Substitution	110	20.90%
681	Transition	C -> T		TTG -> TTA	None	75	20.00%
822	Transition	T -> C		AAA -> AAG	None	118	22.90%
1,035	Transition	T -> C		AAA -> AAG	None	90	20.00%
1,182	Transversion	T -> G	L -> F	TTA -> TTC	Substitution	425	26.10%
1,184	Transversion	A -> T	M -> K	ATA -> AAA	Substitution	294	20.10%
1,200	Substitution	TC -> AA	ES -> DW	GAG, AGA -> GAT,TGA	Substitution	77	30.50%
1,266	Transition	G -> A		TTC -> TTT	None	105	24.80%
1,351	Transition	T -> C	I -> V	ATT -> GTT	Substitution	106	26.40%
1,406	Transition	G -> A	S -> F	TCT -> TTT	Substitution	99	21.20%
1,408	Transversion	A -> T	L -> M	TTA -> ATA	Substitution	132	22.70%
1,432	Transition	T -> C	M -> V	ATA -> GTA	Substitution	111	24.30%
1,439	Transversion	C -> A	W -> L	TGA -> TTA	Substitution	68	20.60%
1,461	Transition	C -> T		GTG -> GTA	None	129	26.40%
1,483	Transition	G -> A	L -> F	CTT -> TTT	Substitution	171	24.60%
1,547	Transversion	G -> T	T -> N	ACT -> AAT	Substitution	86	20.90%
1,582	Transition	A -> G	F -> L	TTT -> CTT	Substitution	87	26.40%
1,585	Substitution	CT -> AA	S -> F	AGT -> TTT	Substitution	119	27.70%
1,606	Transversion	T -> A	S -> C	AGT -> TGT	Substitution	175	21.10%
1,614	Transversion	C -> A	K -> N	AAG -> AAT	Substitution	205	27.30%
1,638	Transversion	T -> A	L -> F	TTA -> TTT	Substitution	304	33.60%
1,640	Transversion	C -> A	W -> L	TGA -> TTA	Substitution	218	20.20%
				ND4			
101	Transversion	T -> A	K -> M	AAG -> ATG	Substitution	177	22.00%
103	Transversion	T -> A	I -> F	ATT -> TTT	Substitution	127	20.50%
255	Transversion	C -> A	M -> I	ATG -> ATT	Substitution	221	29.00%
261	Transversion	T -> A	M -> I	ATA -> ATT	Substitution	416	27.90%
300	Transversion	A -> T		AGT -> AGA	None	176	28.40%
305	Transversion	C -> A	S -> M	AGA -> ATA	Substitution	176	23.30%
329	Transversion	T -> A	Y -> F	TAT -> TTT	Substitution	185	22.70%
401	Transversion	C -> A	G -> V	GGA -> GTA	Substitution	246	21.10%
424	Transversion	T -> A	I -> F	ATT -> TTT	Substitution	296	27.70%
434	Transition	G -> A	S -> F	TCT -> TTT	Substitution	122	26.20%
461	Transversion	C -> A	S -> I	AGT -> ATT	Substitution	69	21.70%
517	Transversion	T -> A	N -> Y	AAT -> TAT	Substitution	218	22.50%
557	Transversion	A -> T	V -> E	GTA -> GAA	Substitution	123	21.10%
704	Transversion	T -> A	N -> I	AAT -> ATT	Substitution	91	23.10%

ND4L							
44	Transition	C -> T	G -> E	GGA -> GAA	Substitution	131	21.40%
94	Transversion	C -> A	G -> C	GGT -> TGT	Substitution	64	20.30%
122	Transversion	G -> T	S -> Y	TCT -> TAT	Substitution	120	23.30%
147	Transversion	T -> A	M -> I	ATA -> ATT	Substitution	84	21.40%
ND6							
33	Transition	T -> C		ATT -> ATC	None	68	27.90%
253	Transversion	T -> A	L -> M	TTA -> ATA	Substitution	60	23.30%
283	Transversion	G -> T	V -> L	GTA -> TTA	Substitution	86	24.40%
289	Substitution	ATA -> TTC	M -> F	ATA -> TTC	Substitution	70	20.00%
294	Transition	T -> C		TAT -> TAC	None	74	23.00%
313	Transversion	T -> A	L -> M	TTA -> ATA	Substitution	79	30.40%
358	Substitution	TT -> GG	F -> G	TTT -> GGT	Substitution	54	34.90%
380	Transversion	T -> G	I -> S	ATT -> AGT	Substitution	77	26.00%
385	Substitution	CCT -> TCA	P -> S	CCT -> TCA	Substitution	53	20.80%
387	Transversion	T -> A		CCT -> CCA	None	56	32.10%
389	Transversion	A -> T	N -> I	AAT -> ATT	Substitution	53	26.40%
406	Transversion	A -> T	M -> L	ATA -> TTA	Substitution	174	20.10%
435	Transversion	A -> T	L -> F	TTA -> TTT	Substitution	238	21.00%
449	Transversion	G -> T	W -> L	TGA -> TTA	Substitution	77	20.80%
451	Transversion	A -> T	M -> L	ATA -> TTA	Substitution	74	20.30%
CytB							
687	Transition	C -> T		TTC -> TTT	None	54	44.40%
688	Transition	A -> G	M -> V	ATA -> GTA	Substitution	57	26.30%
895	Transversion	C -> A	P -> T	CCT -> ACT	Substitution	79	20.30%
915	Transversion	T -> A	N -> K	AAT -> AAA	Substitution	55	23.60%
916	Transversion	T -> A	F -> I	TTT -> ATT	Substitution	53	20.80%
1,053	Transversion	T -> A	I -> M	ATT -> ATA	Substitution	233	29.20%
1,072	Transversion	A -> C	I -> L	ATT -> CTT	Substitution	252	25.00%
1,091	Transversion	A -> T	K -> M	AAA -> ATA	Substitution	64	21.90%
ND1							
13	Transition	C -> T	V -> I	GTT -> ATT	Substitution	74	23.00%
19	Transversion	C -> A	V -> F	GTT -> TTT	Substitution	162	21.60%
48	Transversion	T -> A	M -> I	ATA -> ATT	Substitution	427	22.00%
211	Transversion	T -> A	N -> Y	AAT -> TAT	Substitution	111	23.40%
276	Transversion	A -> T	I -> M	ATT -> ATA	Substitution	124	20.20%
446	Transversion	C -> A	S -> M	AGA -> ATA	Substitution	61	23.00%
448	Transversion	T -> A	M -> L	ATA -> TTA	Substitution	76	23.70%
524	Transversion	T -> A	N -> I	AAT -> ATT	Substitution	94	20.20%
759	Transversion	C -> A	K -> N	AAG -> AAT	Substitution	89	34.80%
799	Transversion	C -> A	V -> F	GTT -> TTT	Substitution	166	25.90%
908	Transversion	C -> A	S -> I	AGT -> ATT	Substitution	78	24.40%
ND2							
580	Transversion	T -> A	L -> M	TTA -> ATA	Substitution	111	27.90%
583	Transversion	A -> T	I -> F	ATT -> TTT	Substitution	133	29.30%
634	Transversion	A -> T	I -> F	ATT -> TTT	Substitution	110	20.90%

658	Transversion	T -> A	F -> I	TTT -> ATT	Substitution	153	31.40%
705	Transversion	T -> A	I -> M	ATT -> ATA	Substitution	59	20.30%
750	Transversion	A -> T	K -> N	AAA -> AAT	Substitution	57	22.80%
857	Transversion	T -> G	F -> C	TTT -> TGT	Substitution	89	38.20%
919	Transversion	A -> T	N -> Y	AAT -> TAT	Substitution	125	20.80%
959	Transition	G -> A	S -> N	AGT -> AAT	Substitution	51	23.50%

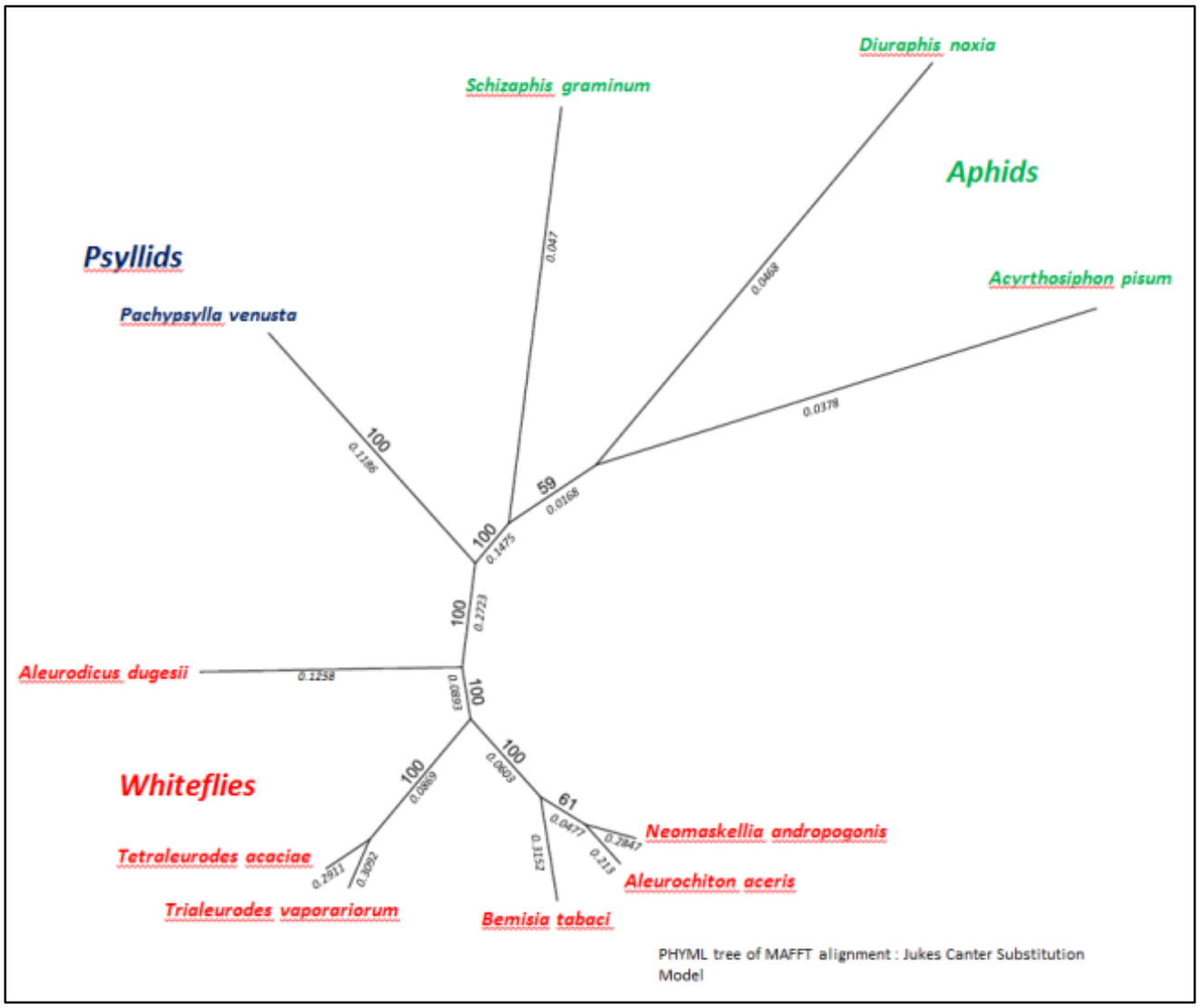

Fig. A1. Unrooted phylogenetic tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) after analysis using the Jukes-Cantor Substitution Model (MAFFT, Geneiousv6.16). The tree is based on alignment of the complete mitochondrial genomes of the whiteflies, *A. aceris*, *A. dugesii, Bermisia tabaci, N. andropogonis, T. acaciae* and *T. vaporariorum*, *P. venusta* (psyllid), and the aphids, *A. pisum*, *S. graminum* and *D. noxia.*

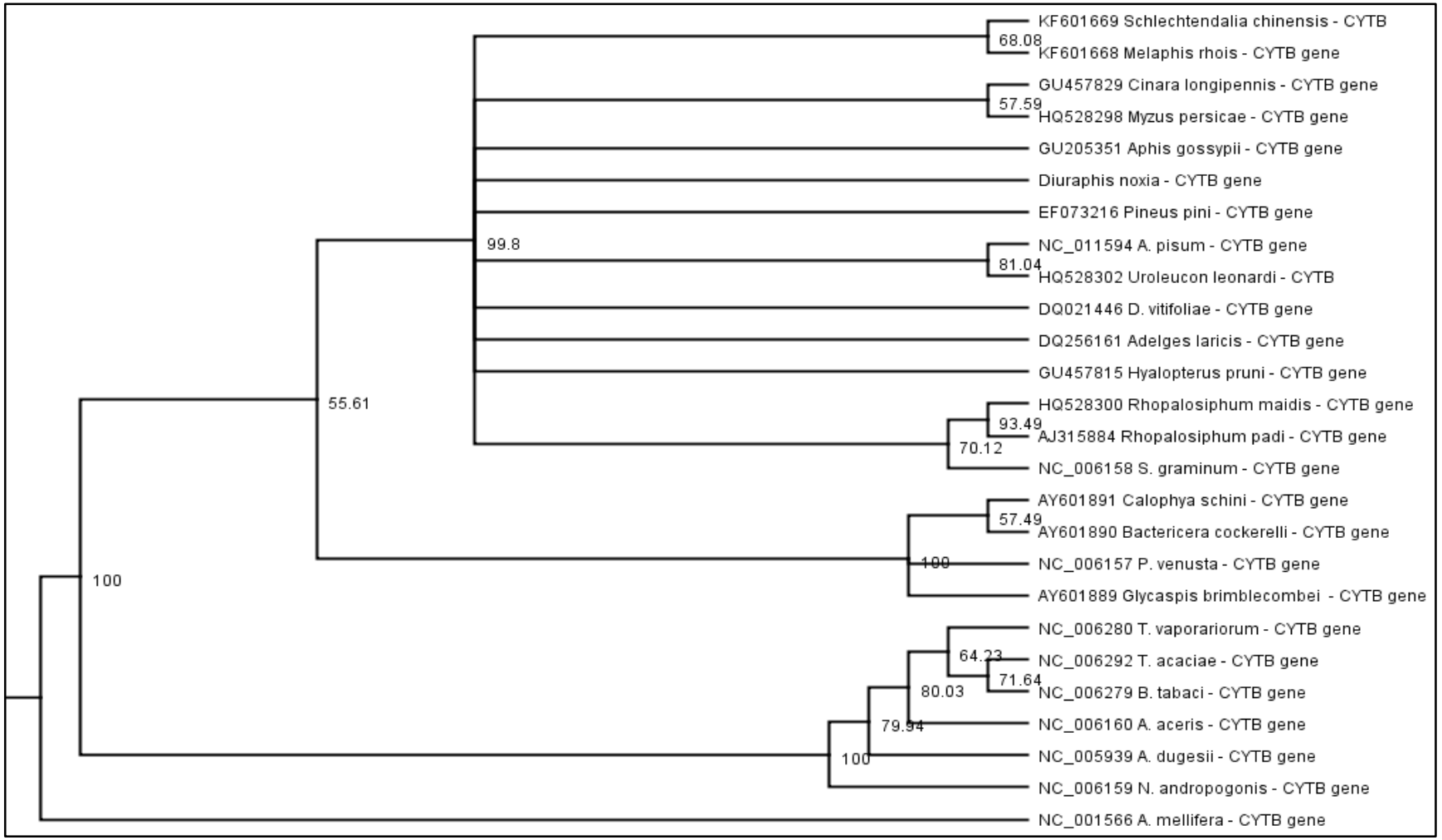

Fig. A2. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the mitochondrial *CytB* sequences. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

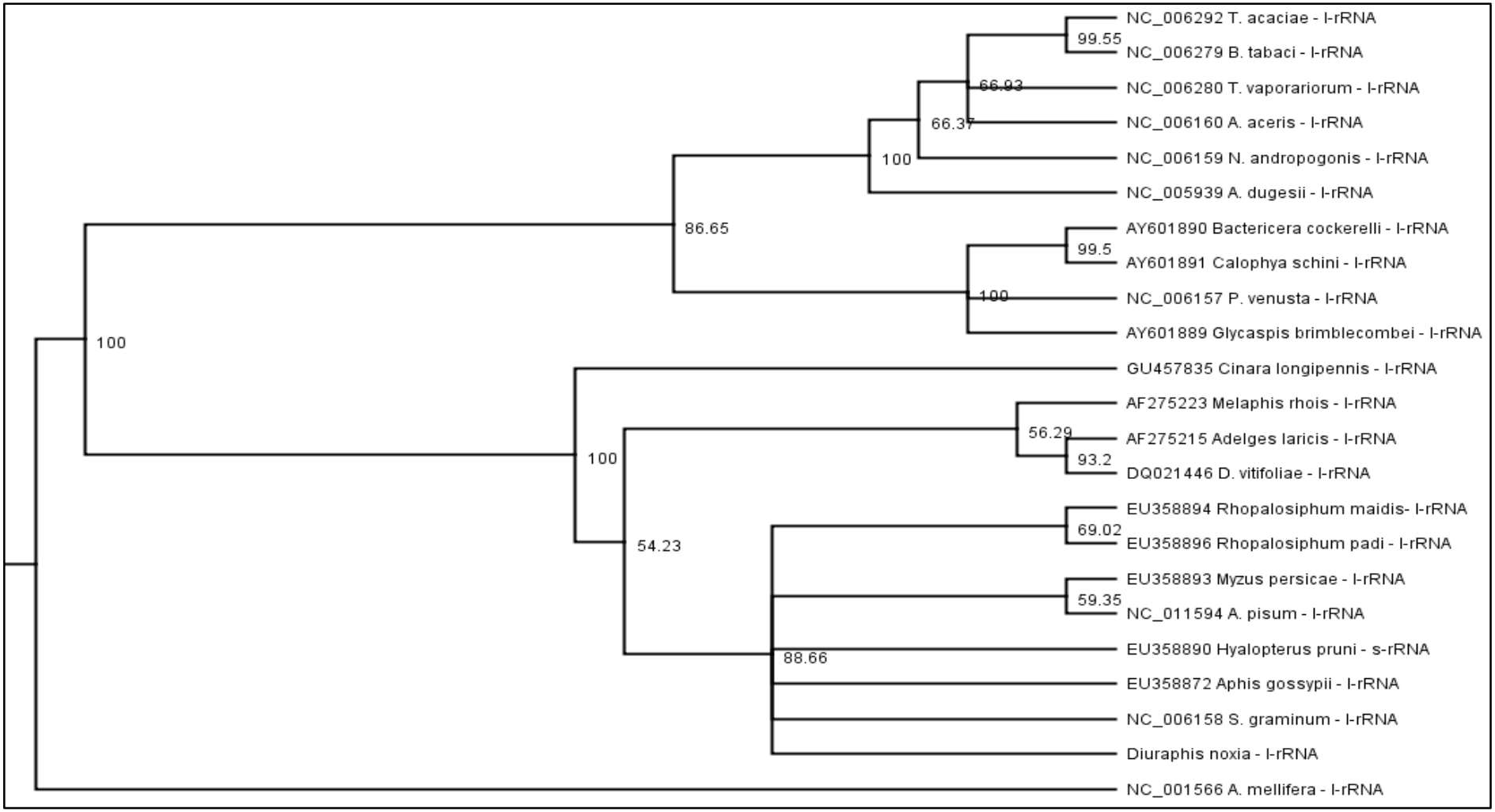

Fig. A3. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the mitochondrial *16S rDNA* sequences. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

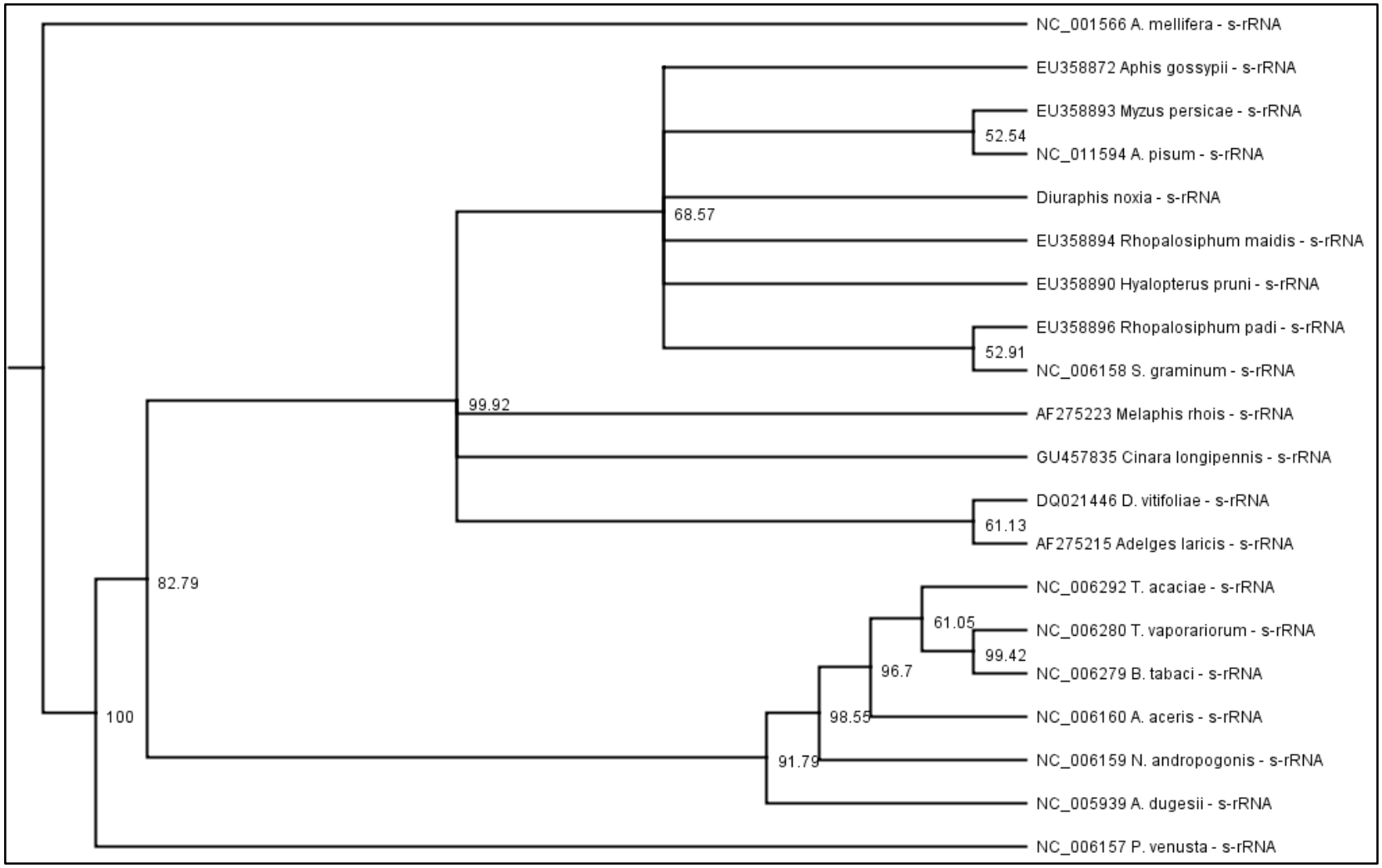

Fig. A4. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the *12S rDNA* mitochondrial sequences. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed.. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

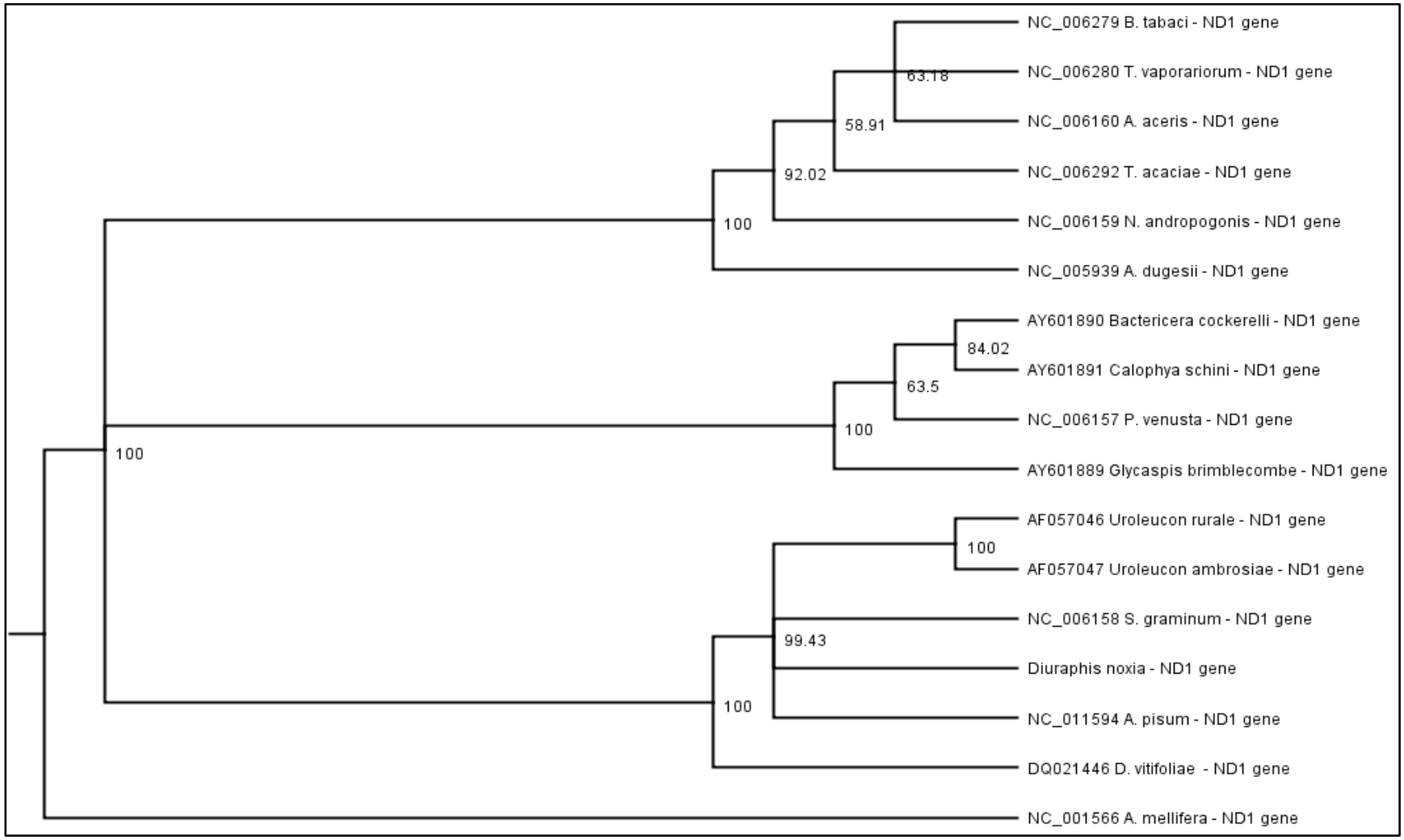

Fig. A5. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the *ND1* mitochondrial sequences. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

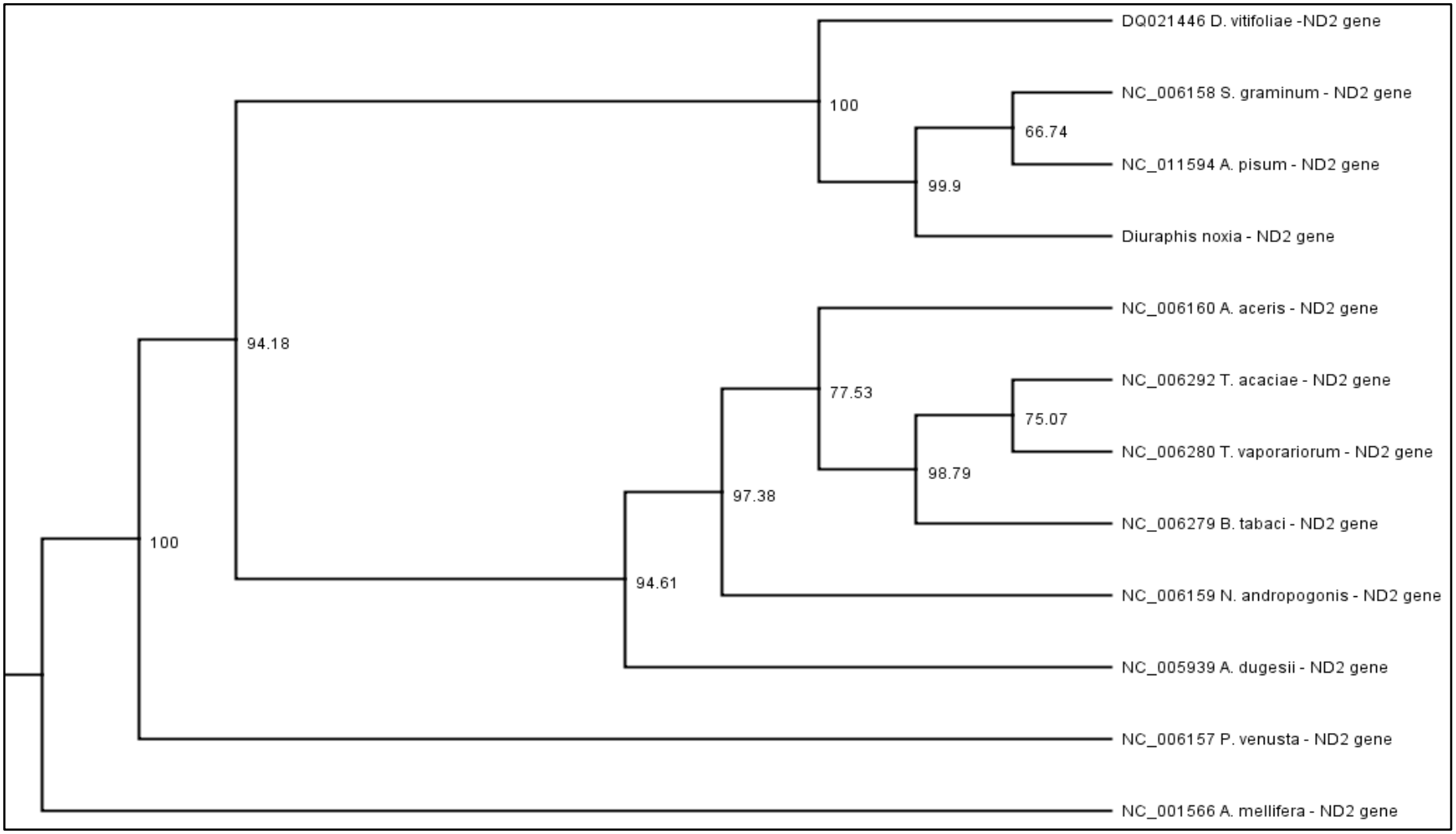

Fig. A6. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the *ND2* mitochondrial sequences. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed.. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

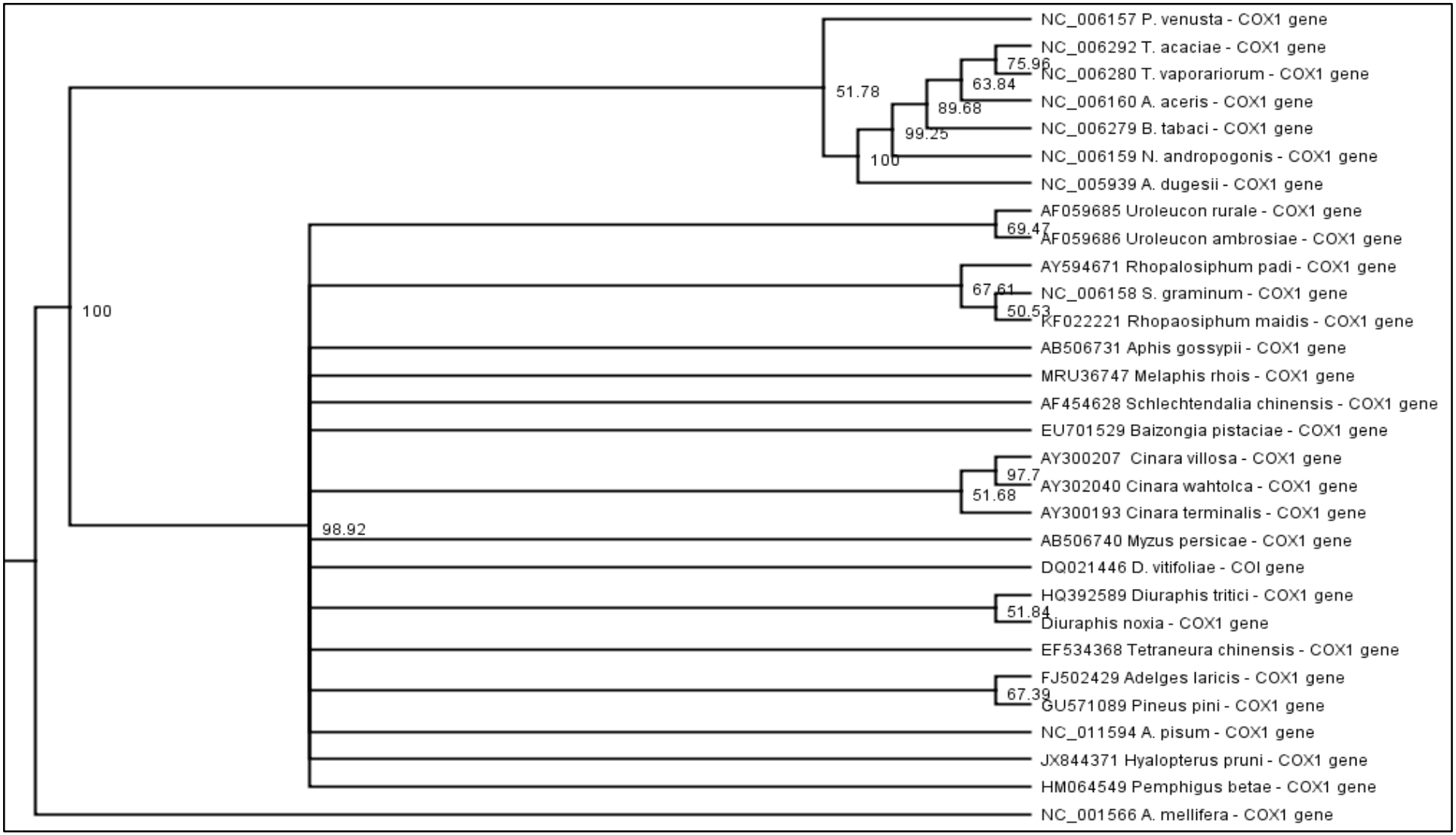

Fig. A7. Maximum Parsimony analysis tree showing the relationships of members of the Sternorrhyncha (whiteflies, aphids and psyllids) with *Apis mellifera* as out group. The tree is based on the *COI* mitochondrial sequences. The bootstrap consensus tree inferred from 1 000 replicates is taken to represent the evolutionary history of the taxa analysed. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.

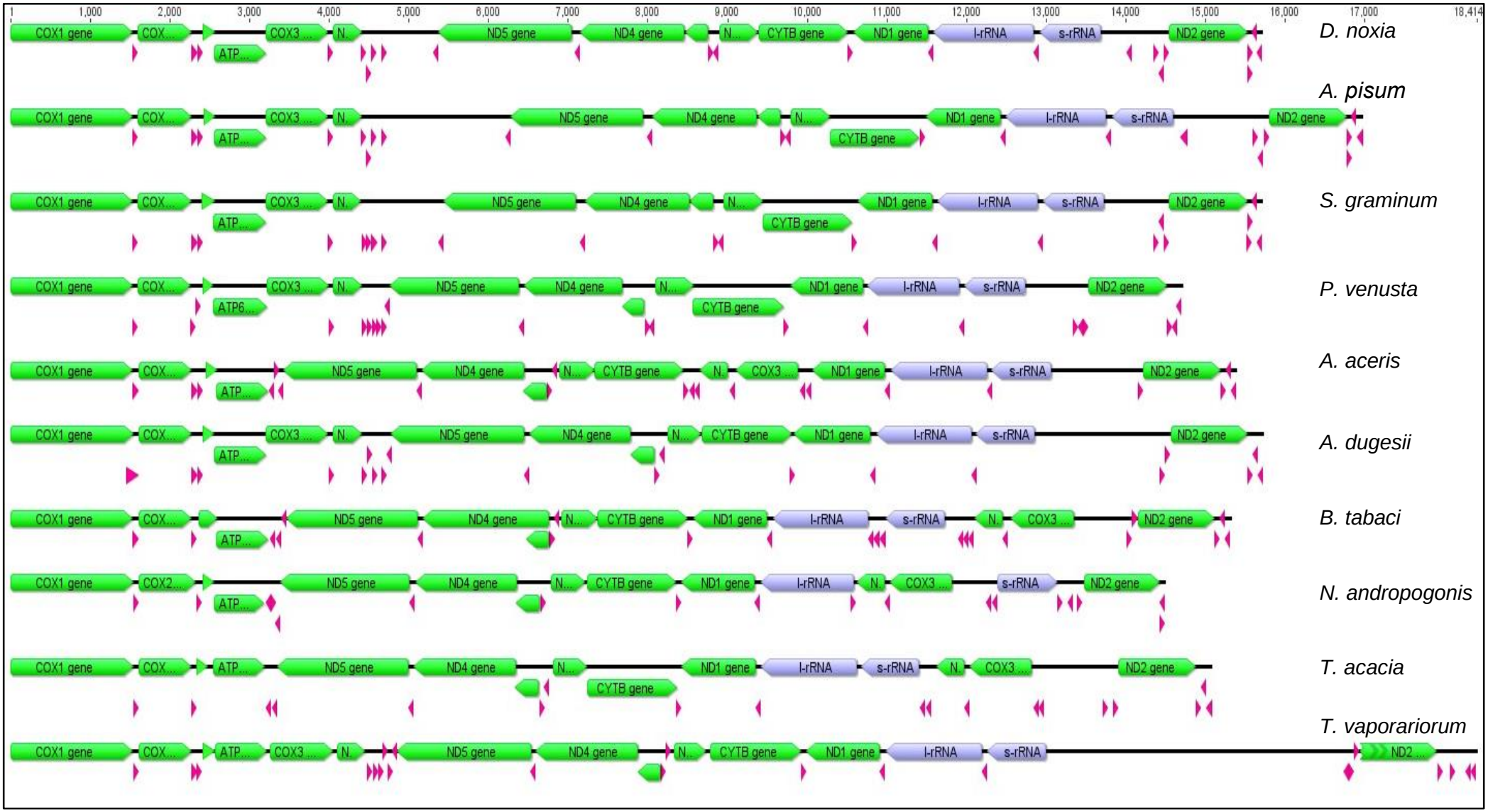

Fig. A8. A comparison in mitochondrial genomic composition and gene order between the whiteflies*, A.aceris*, *A. dugesii, B. tabaci, N. andropogonis, T.acaciae* and *T. vaporariorum*, *P. venusta* (psyllid), and the aphids, *A. pisum*, *S. graminum* and *D. noxia.*

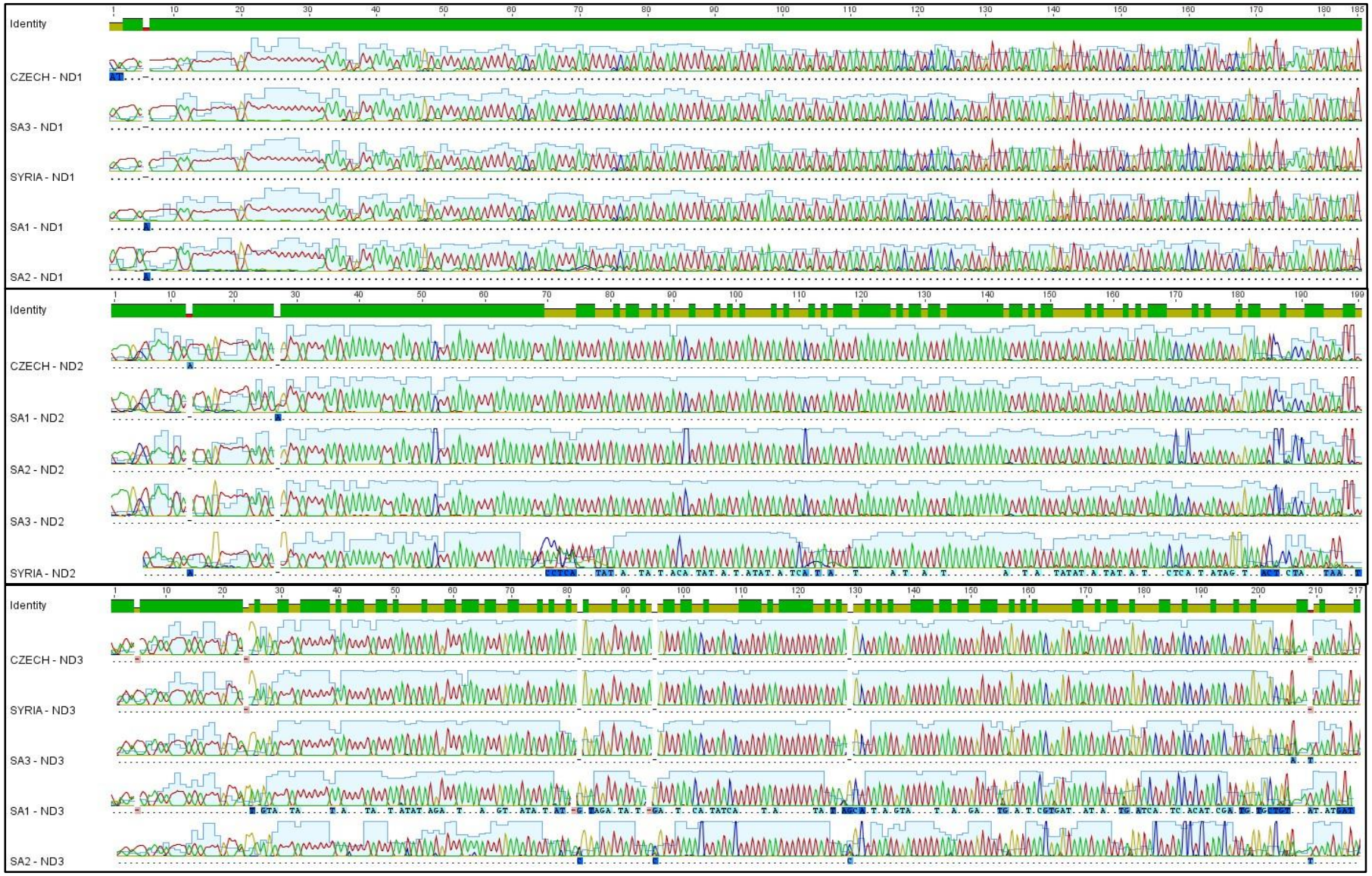

Fig. A9. Alignment of the mitochondrial genes *ND1*, *ND2* and *ND3* illustrating the single nucleotide polymorphisms (SNPs) between five *D. noxia* populations originating from Syria, Czech Republic and South Africa. Indicated are disagreements from the consensus sequence.

Fig. A10. Alignment of the mitochondrial genes *COI*, *COII* and *COIII* illustrating the single nucleotide polymorphisms (SNPs) between five *D. noxia* populations originating from Syria, Czech Republic and South Africa. Indicated are disagreements from the consensus sequence.

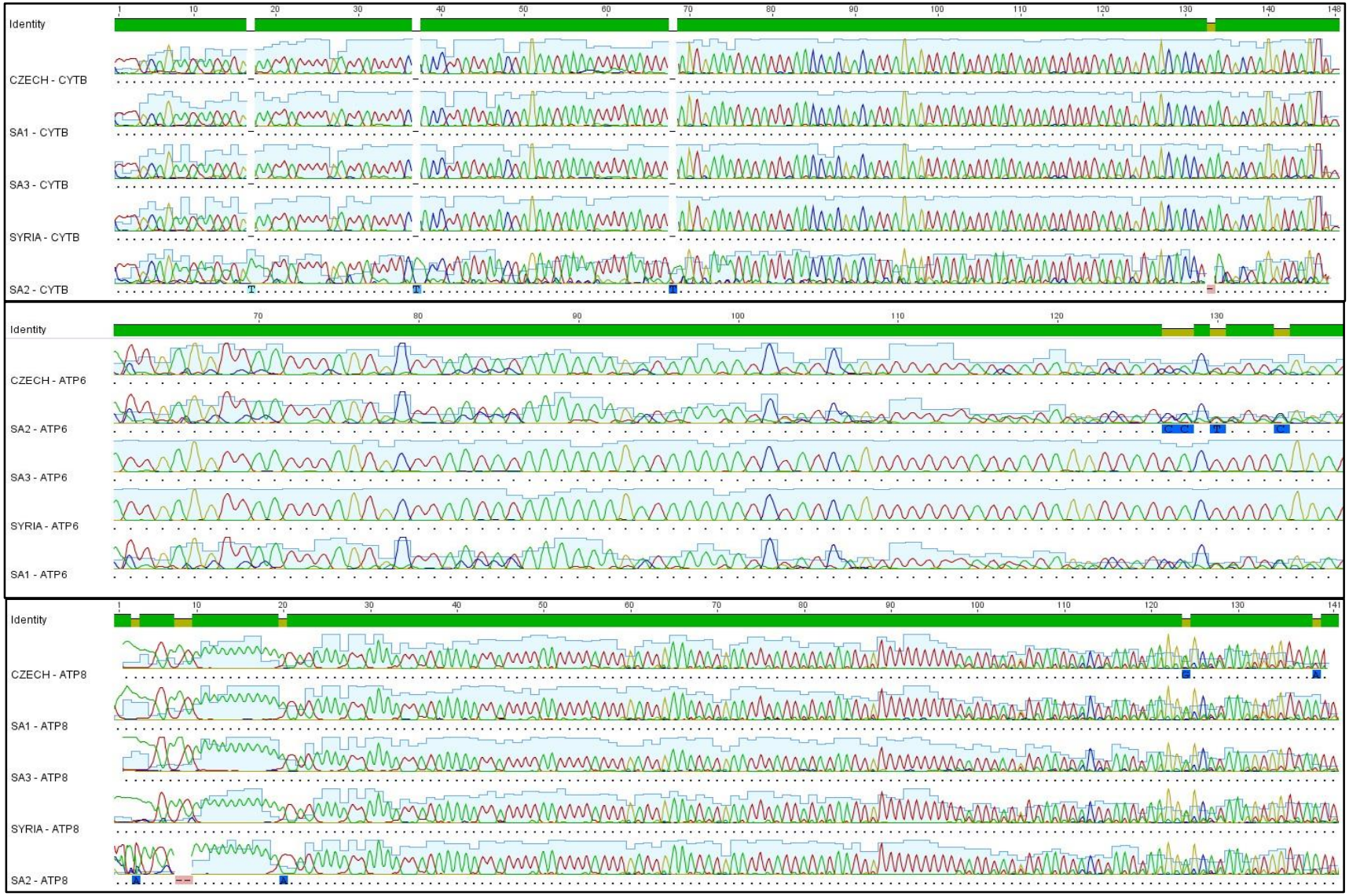

Fig. A11. Alignment of the mitochondrial genes *CytB, atp6* and *atp8* and illustrating the single nucleotide polymorphisms (SNPs) between five *D. noxia* populations originating from Syria, Czech Republic and South Africa. Indicated are disagreements from the consensus sequence.

Chapter 4

Association of mitochondrial and carotenoid genes to the energy metabolism of *D. noxia* biotypes and their relation to virulence

4.1 Introduction

All living organisms need energy to fulfil vital biological processes such as growth, metabolism and reproduction. Thus, energy sources must be obtained from the environment in the form of organic food molecules and sunlight. Eukaryotic cells transform the biochemical energy held in the chemical bonds of food molecules into a more readily usable form such as the energy-rich carrier molecule adenosine triphosphate (ATP) that stores energy within a high-energy bond between its second and third phosphate (Nelson and Cox 2004). Significant quantities of ATP are generated through the eukaryotic energy pathway that consists of glycolysis, the citric acid cycle and oxidative phosphorylation.

Glycolysis and the citric acid cycle entails the breakdown of carbon sources to release and convert energy into small energy-rich molecules such as ATP, flavin adenine dinucleotide ($FADH_2$), guanosine-5'-triphosphate (GTP) and nicotinamide adenine dinucleotide hydride (NADH), that serve as co-factors or electron carriers for biochemical reactions in the cell (Nelson and Cox 2004). The electron transport chain (ETC) and ATP synthesis are together known as oxidative phosphorylation. This process involves carrier molecules that transport reducing equivalents (electrons and hydrogen ions) to the ETC to regenerate NAD^+ for its use in glycolysis and induces the formation of an electrochemical proton gradient across the membrane that drives the synthesis of ATP through phosphorylation, and is catalysed by ATP synthase (Nelson and Cox 2004). Cells are able to harness and convert the chemical energy of ATP into osmotic, mechanical or synthetic cellular processes. This energy pathway thus provides the cell with the necessary energy to fuel biological processes and thus to sustain life.

It is therefore clear that ATP synthase plays a critical role in oxidative phosphorylation and thus the generation of ATP. This membrane-bound complex is also known as complex V and is composed of two main structural domains, F_0 and F_1. The F_0 domain is embedded in the innermembrane of the mitochondrion and allows the flow of protons across the membrane, while the F_1 domain protrudes into the matrix and contains the catalytic parts necessary to convert ADP into ATP (Nelson and Cox 2004). The mitochondrial *atp6* gene encodes ATP6 (subunit a) of the F_0 domain of ATP synthase and plays a vital role in the efficiency of ATP synthesis (Lenaz *et al.*, 2004). It was concluded from chapter 3 that the mitochondrial ATP synthase genes (*atp6* and *atp8*) may point towards increased importance in aphid fitness due to the low frequency of SNPs detected and will be investigated further in this chapter.

In contrast to the low SNP frequency observed for the ATP synthase genes, the occurrence of SNPs was abundant in the mitochondrial *ND* genes, with the highest SNP frequency observed for the *ND5* gene which will also be further investigated in this chapter. The *ND5*

gene encodes for the NADH dehydrogenase 5 and is a core subunit of a large enzyme known as complex I (NADH-ubiquinone oxidoreductase) that is localized in the innermembrane of the mitochondrion. Complex I is responsible for the first step in the ETC, i.e., electron transfer from NADH to ubiquinone, and is thus essential in the ETC to provide energy for the generation of ATP. Mutations occurring in the human orthologs of mitochondrial genes such as *atp6* and *ND5* can cause cell death due to insufficient energy and a variety of neurological, endocrinological, and muscular diseases (DiMauro 2004), thus decreasing the fitness and longevity of an individual.

Though the structural components of the ETC are important, one can thus recognize that electron carriers or co-enzymes such as nicotinamide adenine dinucleotide (reduced, NADH; oxidized, NAD^+) plays a crucial role in shuttling electrons and thus energy from one molecule to another (Nelson and Cox, 2004). Co-enzymes can be used throughout the cell to power metabolism and construct new cellular components through redox reactions such as the reduction of NAD^+ to NADH through catabolic reactions during glycolysis and the citric acid cycle (Matthew *et al.,* 2000) or the oxidation of NADH to NAD^+ during oxidative phosphorylation where NADH serves as a substrate for the NADH dehydrogenase complex of the mitochondrial ETC, which transfers electrons to coenzyme Q, generating NAD^+ in the process (Matthew *et al*., 2000).

The balance between the reduced and oxidized forms of NAD is known as the NAD^+/NADH ratio (Nelson and Cox 2004).This ratio plays an important role in the regulation of the intracellular redox state, and reflects the metabolic state of a cell. NADH needs to be re-oxidised constantly to maintain a proper redox state, which is achieved primarily by the ETC during respiratory growth (Bakker *et al.,* 2001; Cruz *et al*., 2001). The NAD^+/NADH ratio is an important measurement that reflects both the health and metabolic activities of a cell (Schafer and Buettner 2001). The effects of the NAD^+/NADH ratio are rather complex and controls the activity of several key enzymes vital in energy metabolism such as the glycolytic enzyme glyceraldehyde 3-phosphate and the pyruvate dehydrogenase complex that is responsible for the production of pyruvate and its conversion to acetyl-CoA, a substrate for the TCA cycle (Matthew *et al.,* 2000). Despite NAD's essential role as a coenzyme in the regulation of energy metabolism (Matthew *et al*., 2000), NAD also participates in various biological processes that include transcription (Zhang *et al.,* 2002; Smith and Boeke 1997; Anderson *et al.*, 2002; Rutter *et al*., 2001), telomere maintenance and DNA repair (Wilkinson *et al.,* 2001; Burkle 2001). In addition to serving as a coenzyme, NAD can also be utilised by enzymes as a substrate. These enzymes include NAD-dependent oxidoreductases (Matthew *et al.,* 2000), NAD-dependent DNA ligases (Wilkinson *et al*., 2001), poly(ADP-ribose) polymerase (Burkle 2001) that are involved in post-translation modification and the Sir2p

family, which are NAD-dependent deacetylases that affect age regulation, longevity and transcriptional silencing by using NAD^+ to remove acetyl groups from proteins (Imai *et al.,* 2000; Landry *et al.,* 2000; Smith *et al.,* 2000; Vaziri *et al.,* 2001; Luo *et al.,* 2001). Any variations in the energy status of a cell are associated with changes in the ratio of free NAD^+ to NADH and this ratio can thus be used to indicate which individual manages stressful environments more successfully regarding energy generation.

An alternative way for eukaryotes to generate energy is through the process of photosynthesis, however insects are unable to do so due to the lack of chloroplasts. Nevertheless, a study has recently proposed that carotenoids are involved in an archaic photosynthetic mechanism in insects and found that levels of carotenoids in the pea aphid correlated with the levels of ATP present in the different colour morphs. ATP levels rose and sunk as the aphids' exposure to sunlight was increased and decreased, suggesting that the aphid utilizes sunlight to produce ATP through a photosynthesis-like process (Valmalette *et al*., 2012). The presence of this phototrophic event may be a support mechanism the aphid can utilize when exposed to stressful environments such as inhabiting a new niche to evade predation (Lougheed 2012). The production of additional energy may not be the only advantage of carotenoid biosynthetic genes in the aphid genome. Carotenoids and their derivatives are known for their antioxidizing ability that is responsible for the mediation of oxidative stress and is likely a key feature in modulating insect-plant interactions. Heath *et al.,* (2012) reports that carotenoids may protect insects from both exogenous and endogenous sources of reactive oxygen species generated from the plant's response to wounding, and this may contribute to the immune response in arthropods. It can thus be theorized that some aphids or biotypes can utilize carotenoid genes to acquire an advantage above other aphids or biotypes. Whatever the advantage of these carotenoid biosynthetic genes may be, aphids have integrated and maintained these genes in their genome for a reason, making their contribution worth exploring.

The objectives of this study were firstly to determine whether differences in energy homeostasis (as expressed in the NAD^+/NADH ratio and expression of the *atp6* and *ND5* genes) correlate to the observed virulence of *D. noxia* biotypes. Secondly, to determine whether differences in immune regulation (deducted from the expression and quantification of carotenoids) are associated with the virulence attributed to different *D. noxia* biotypes.

4.2 Materials and Methods

4.2.1 Aphid rearing

The aphids included in this part of the study were the South African *D. noxia* biotypes: SA1, SA2, SA3 and SAM. The SA1, SA2 and SA3 biotypes were obtained from separate colonies established from field-collected parthenogenetic females at the ARC-Small Grains Institute, Bethlehem, South Africa. The SA1 biotype was maintained on a *D. noxia* susceptible wheat cultivar, Tugela. The SA2 and SA3 biotypes were maintained on the resistant cultivar, Tugela-*Dn1*. A hypervirulent mutant form of the South African biotype (SAM), was obtained by selective pressure via long-term force-feeding on resistant germplasm in the laboratory, and maintained on the resistant cultivar Tugela-*Dn1* (Van Zyl and Botha 2008). Females of all four biotypes were kept in insect BugDorm Insect cages (MegaView Science Co., Ltd) at 20 ± 2 °C with continuous artificial fluorescent lighting to prevent cross-contamination. Only adult aphids were used in the experiments conducted.

4.2.2 Plant material

Hexaploid wheat (*Triticum aestivum* L.) germplasm of the near-isogenic lines (NILs) Tugela, Tugela-*Dn1* (Tugela*4/SA1684), Tugela-*Dn5* (Tugela*4/SA463) and Gamoos-*Dn7* (94M370) was obtained from the Small Grain Institute, Bethlehem, South Africa (Liu *et al.,* 2001; Tolmay *et al.,* 2006). The NILs were selected based on their reported resistance phenotypes: Tugela (*D. noxia* susceptible); Tugela-*Dn1* (antibiotic; Du Toit 1989a); Tugela-*Dn5* (antixenotic and moderately antibiotic; Wang *et al.,* 2004); and Gamtoos-*Dn7* (antixenotic; Marais *et al.,* 1994). Seeds were sown into pots and thinned to three seedlings per pot after 5 days. Plants were grown for 14 days (2-3 leaf stage) under greenhouse conditions in a 1:2:2:1 mixture of perlite (Chemserve, Olifantsfontein, South Africa), sifted bark compost, loam and sand at 25°C ± 2°C.

4.2.3 Evaluation of energy homeostasis

4.2.3.1 Aphid stress

In order to observe and quantify energy differences (i.e., NAD^+/NADH) among the biotypes, aphid feeding cages were manually constructed to provide aphids with an artificial feeding media to ensure constant and identical parameters across the experiment for each biotype. Aphid feeding cages and feeding media were prepared according to Cooper *et al.,* (2010) with small modifications. The cages were constructed using a 65 mm diameter sterile disposable petri dish, parafilm, a plastic O-ring and feeding media (Fig. 4.1.A). The bottom side of the petri dish contains a bevelled stacking ridge which was covered with thinly stretched parafilm to create a shallow space to pipette 2-3 ml of feeding media into. The plastic rings were placed on top of the parafilm layer to provide a feeding surface for the aphids. The petri dish lid is placed on top of the plastic ring to prevent the aphids from

leaving the feeding surface (Fig.4.1.C). To increase aphid feeding, the aphids were kept at 4ºC for 5 hours prior to the experiment to increase hunger and subsequent feeding. The aphid cages were also placed on sheets of yellow paper to stimulate aphid feeding (Cooper *et al.,* 2010) (Fig. 4.1.B).

The *D. noxia* aphids fed on an artficial diet containing a 15% sucrose solution as evaluated by Cooper *et al*., (2010) as an optimal feeding media for *D. noxia*. The amount of stress the aphids could tolerate was evaluated by exposure to growth lights for a prolonged period of time as follows: 18H light/ 6H dark, 24H light. High light intensity, heat from the growth lights and a deficiency in nutrients can result in oxidative stress. The light measurement was approximately 2000 lux. Despite high mortality rates, three biological samples of 0.01 g per sample were collected for each biotype for the NADH assay.

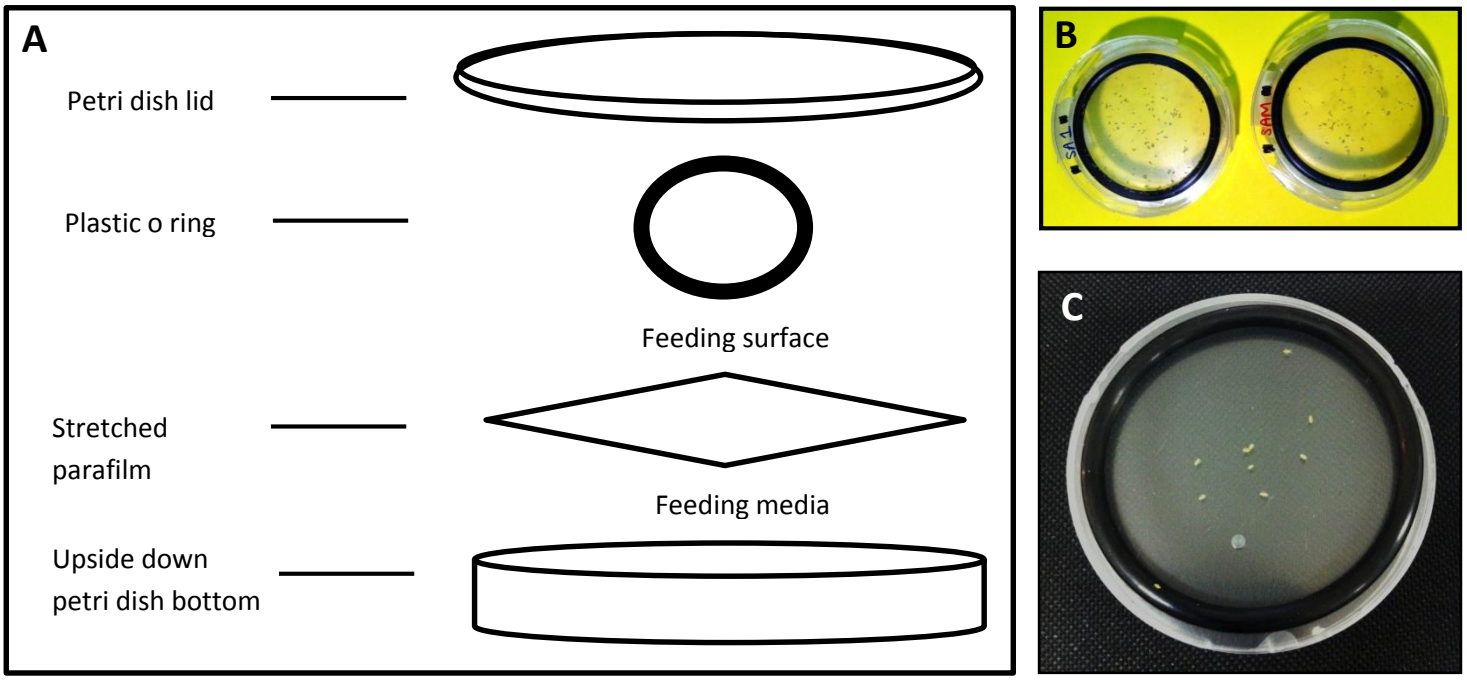

Fig. 4.1. Construction of an aphid feeding cage (A) and the finished product containing aphids (B and C).

4.2.3.2 NAD$^+$/NADH Colorimetric Assay

The measurement of the intracellular nicotinamide nucleotides were achieved through the use of the BioVision NAD+/NADH Quantification Colorimetric Kit (Bioassay Systems, Hayward, CA, USA) and the Promega Glomax Multi Detection System. The assay was performed to quantify the NAD$^+$/NADH balance in the cytoplasm among the South African *D. noxia* biotypes (SA1, SA2, SA3 and SAM) to provide insight into the energy transformation

and redox state of the different biotypes. The assay was performed in triplicate and according to the manufacturer's protocol, and determinations were normalized with total protein content.

Protein concentration was determined according to the method of Bradford (1976) using the Bio-Rad protein assay reagent with bovine albumin (Bio-Rad) as a standard. The Glomax Spectrophotometer, following the method described by Rybutt and Parish (1982) was used for this purpose.

4.2.4 Contribution of carotenoids to energy production and immunity.

4.2.4.1 Carotenoid extraction for spectrometry analysis

The carotenoid extraction protocol was adapted from Mertz *et al.,* (2010), Taungbodhitham *et al.,* (1998) and Dhuique-Mayer *et al.,* (2005), and was conducted as previously described for the pea aphid (Valmalette *et al.,* 2012). Aphids were collected from their preference hosts under laboratory conditions from the SA1, SA2, SA3 and SAM biotypes. These aphids were flash frozen in liquid nitrogen and kept at -80ºC until the carotenoid extraction was performed. The aphids were ground using a mortar and pestle with liquid nitrogen. 15 ml of extraction solvent A (ethanol/hexane, 4:3 v/v, containing 0.1% of butylated hydroxytoluene as antioxidant) and 80 mg of $MgCO_3$ was added to neutralize the acidity of the mixture and the mixture was stirred for 5 min. The residue was separated from the liquid phase by filtration with a filter funnel n°2 (volume 50 ml) and the precipation was washed twice with 15 ml of solvent A and 15 ml of ethanol. Thereafter 15 ml of hexane was added to recover most of the lipophilic compounds. The organic phases were then transferred to a separating funnel (volume 100 ml) and were washed once with 40 ml of 10% sodium chloride and twice with 40 ml of distilled water. The aqueous layer was then removed. The hexanic phase was recovered in a beaker and dried with 1 g of anhydrous sodium sulphate and filtered with cotton glass in a 100 ml conical ball. The dried organic phase was evaporated using a rotavapor and the residue was recovered with 250 µl of dichloromethane and 250 µl of tert-Butyl methyl ether/methanol (80:20, v/v). The extract was transferred to amber vials before chromatopgraphic analysis. The input concentration for spectrometry analysis of the SA1, SA2 and SA3 biotypes were equal, whereas the input concentration of the SAM biotype was half of that of the other biotypes.

4.2.4.2 Ultra high performance liquid chromatography coupled to mass spectrometry (UPLC-MS) analysis of carotenoids

The instrumentation used for analysis was a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-orthogonal acceleration-time of flight (TOF) mass spectrometer (SYNAPT G2; Waters, Milford, MA, USA) using an APCI interface. The chromatographic separation was carried out on the Waters UPLC BEH C18 column (2.1 mm × 100 mm × 1.71 µm) with a column temperature set at 32ºC. The mobile phases were water/1% formic acid as eluent A, and acetonitrile/methanol (7:3, v/v) as eluent B. The gradient was performed according to table 4.1 and the flow rate was fixed at 0.5 ml/min. The injection volume was 3 µl and the detection was monitored from 230-700 nm. A standard dilution series was prepared from β-carotene and lycopene for comparison.

Table 4.1. Concentration of solvents in the eluting gradient

Time (min)	Flow (ml/min)	% A	% B
0	0.5	25	75
0.6	0.5	25	75
6.5	0.5	5	95
7.5	0.5	0	100
13.6	0.5	0	100
14.1	0.5	25	75

Note: A, 1% formic acid in water. B, acetonitrile/methanol (7:3, v/v).

4.2.5 Quantitative real-time PCR analysis

The expression of *atp6, ND5* and *carotenoid desaturase,* was measured at two time points; 0 hours on their respective preference hosts which are susceptible to feeding, Tugela for SA1 and Tugela-*Dn1* for SA2, SA3 and SAM; 72 hours on resistant cultivars, Gamtoos–*Dn7* and Tugela-*Dn5.* Gamtoos-*Dn7* and Tugela-*Dn5* were used as non-preference hosts for all four South African *D. noxia* biotypes to induce stress due to the different *Dn* genes of these two cultivars exhibiting different modes of resistance that provide a stressful environment for the aphids. Adult *D. noxia* aphids were placed inside a 15 ml falcon tube along with one leaf from the non-preference cultivar at the 3-4 leaf stage and were left to feed and collected after 72 hours.

Total RNA was extracted from these aphids using the Zymospin Direct Zol RNA Miniprep and a *DNase* treatment was performed to remove any DNA contamination. First strand

cDNA synthesis was conducted using the Bio-Rad iScript Select cDNA synthesis kit and primed with random hexamers according to the manufacturer's protocol.

Each real-time quantitative PCR (qPCR) amplification reaction contained 2 µl of cDNA template (2 ng), 0.5 µl of each specific primer (10 pmol/µl) (Table S1), and 5 ul of 2x SYBR and Fluoroscein qPCR mix reagent (Bioline) in a final volume of 10 µl. The amplification profile for all reactions were as follows: Initial denaturation (50.0°C) for 2 minutes and 95 ºC for 10 min, followed by 40 cycles of denaturation (95.0°C) for 15 seconds, annealing (primer Tm) for 30 seconds and extension (72.0°C) for 30 seconds. All qPCR reactions were performed on the Bio Rad CFX96 Real-Time System (Bio-Rad, USA) and the threshold cycle (CT) values were calculated using the Bio-Rad CFX ManagerTM software (Bio-Rad). Single-fragment amplification was verified by melt curve analysis (Appendix B, figure B3-B7). Gene expression values were standardized across three independent biological replicates, with each sample amplified in triplicate with a PCR efficiency of at least within the 95th percentile assuring almost perfect doubling per amplification cycle (Appendix B, figure B8-B12). Relative transcript abundance was calibrated to the mean expression of the SA1 biotype that fed on Tugela, and normalized against the reported expression of *L32* and *B-Tubulin* in each sample (Pfaffl 2001; Vandesompele *et al.,* 2002) due to constitutive expression in aphid species such as *A. pisum* (Shakesby *et al.*, 2009; Whyard *et al.*, 2009) and *M. persicae* (Pitino *et al.*, 2011; Pitino and Hogenhout 2012). T-tests were used to determine the significance of the observed differences in expression among samples using a *P*-value of 0.05.

NCBI's Primer Blast with the primer3 (Untegrasser *et al*., 2012; Ye *et al.*, 2012) platform was utilized in the design of the qPCR primers. The atp6 and ND5 primers (table B1) were designed from the consensus mitochondrial genome sequence obtained in chapter three. The *carotene desaturase* primers (table B1) were designed from conserved sequence regions obtained from multiple alignments of carotenoid desaturase genes of *D. noxia* (HM235780 and HM235781) and *D. noxia* genome contigs using the Geneious software v6.1.6 (Kearse *et al*., 2012). Primers for *L32* and *B-Tubulin* (table S1) were obtained from Shakesby *et al.*, (2009).

4.3 Results

4.3.1 Evaluation of energy homeostasis

To quantify the available free energy levels between the South African *D. noxia* biotypes, the concentration of NAD^+ and NADH were measured. The concentration of NAD^+ ranged from

approximately 1.49 nmol/g protein for the SA2 biotype to 2.23 nmol/g protein for the SAM biotype as can be seen in figure 4.2 and table 4.2. The t-tests indicated significant differences in total NAD^+ in the SA1 and SA2 biotypes and also between the SA2 biotype and the SA3 and SAM biotypes. No significant difference was observed between the SA1 and SA3 and SAM biotypes. The concentration of NADH ranged from roughly 0.275 nmol/g protein for the SAM biotype to 0.363 nmol/g for the SA1 biotype. No significant differences were observed in the concentration of NADH per g protein among the biotypes.

Table 4.2. The concentration of NAD^+ and NADH among the South African *D. noxia* biotypes normalized with protein content. Values are expressed in nmol per gram protein.

Biotypes	SA1	SA2	SA3	SAM
Total NAD+ and NADH	2.157361881	1.797129584	2.361954585	2.502631148
NADH	0.362600363	0.307323574	0.304385014	0.275880599
NAD+	1.794761519	1.48980601	2.057569571	2.226750549
Ratio	4.949695874	4.847678917	6.75975976	8.071428571

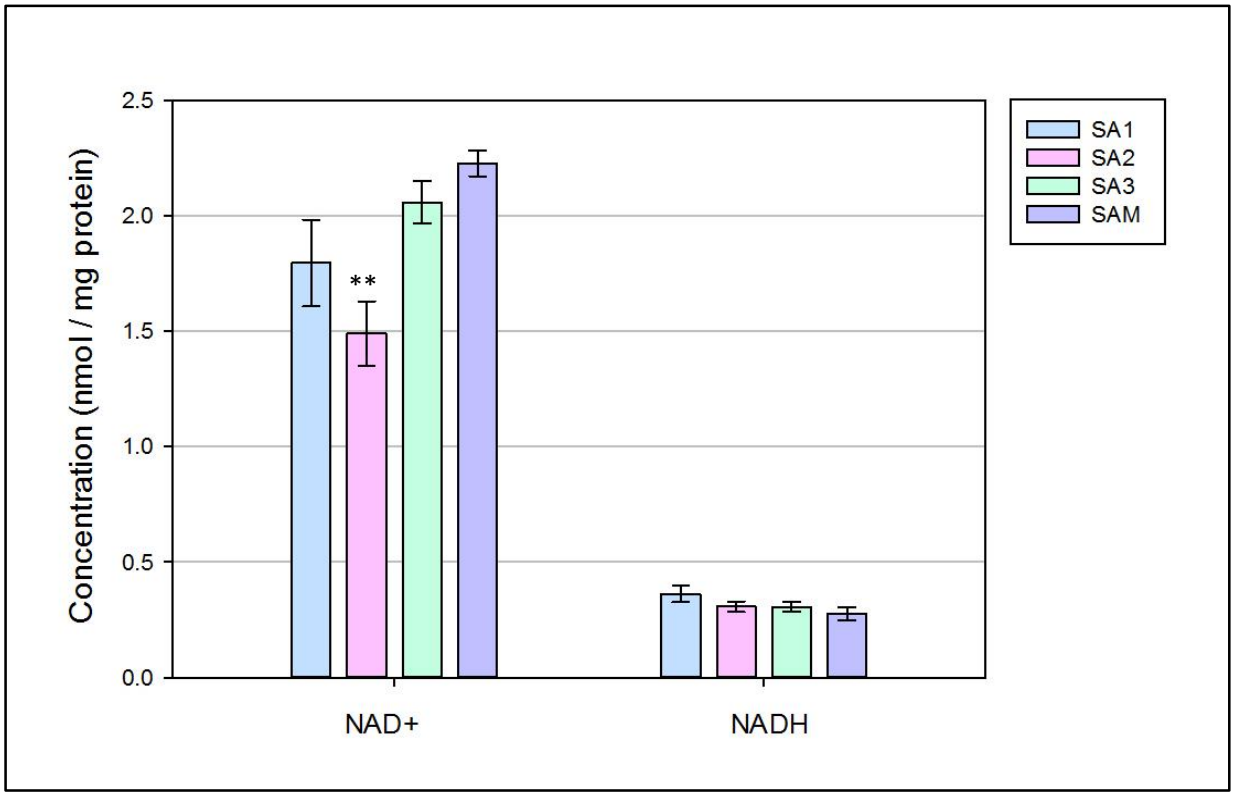

Fig. 4.2. The concentration of cytosolic NAD^+ and NADH expressed as nmol per mg protein in the South African *D. noxia* biotypes.

* * Indicates significant differences among the biotypes.

Since the cytosolic free NAD^+/NADH ratio is important for a wide variety of cellular processes, this was calculated for the four South African *D. noxia* biotypes. The results showed that the SAM biotype displayed the highest NAD^+/NADH ratio of 8.07, and the SA3 biotype the second highest ratio of 6.76 (Fig. 4.3)(Table 4.2). The SA2 biotype contained the lowest NAD^+/NADH ratio of approximately 4.85, followed closely by the SA1 biotype with a ratio of 4.95. The t-tests indicated no significant difference in the NAD^+/NADH ratio between the SA1 and SA2 biotypes. However, these biotypes differed significantly from the SA3 and SAM biotype. A significant difference was also observed between the SA3 and SAM biotype.

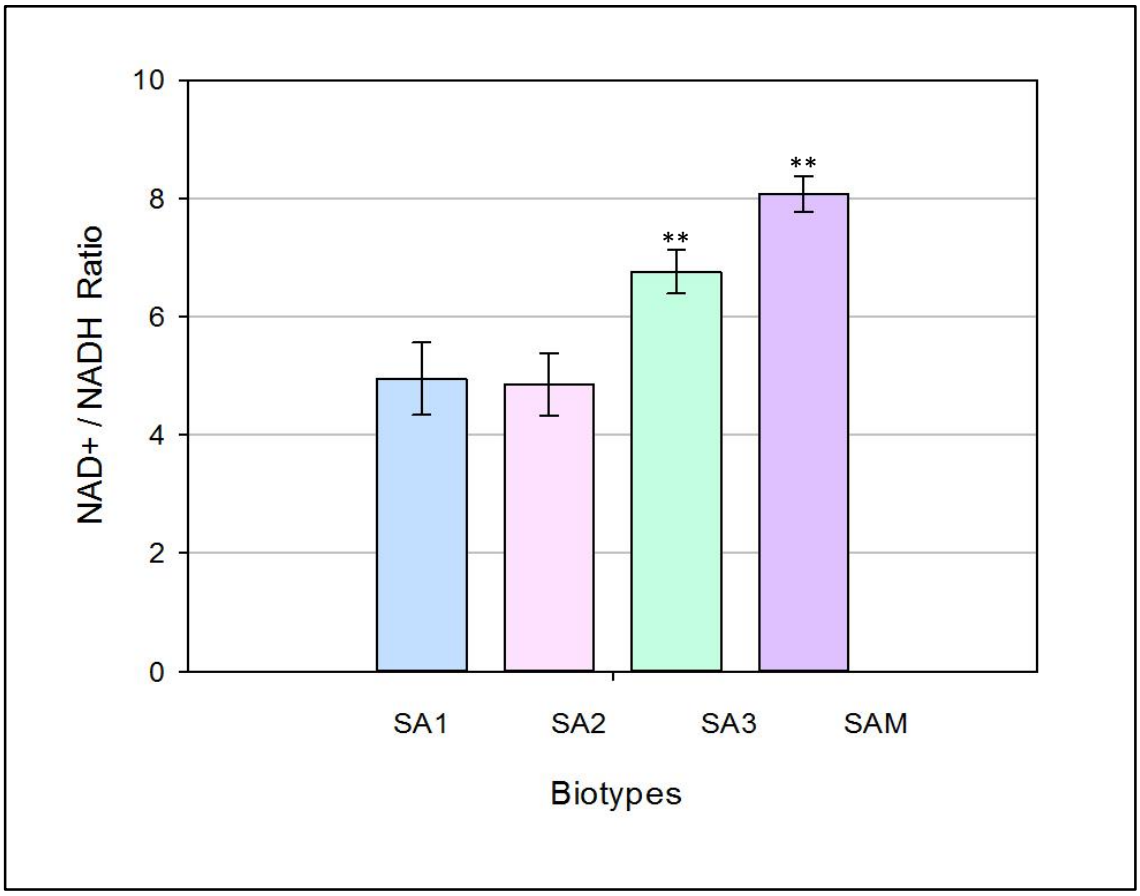

Fig. 4.3. The cytosolic free NAD^+/NADH ratio of the South African *D. noxia* biotypes.

* * Indicates significant differences among the biotypes.

A qPCR analysis was conducted on the South African *D. noxia* biotypes after exposure to stress on the resistant cultivars, Gamtoos-*Dn7* and Tugela-*Dn5*, in order to measure differential gene regulation of the *atp6* and *ND5* to establish whether these genes contribute significantly to the regulation and production of energy in *D. noxia.*

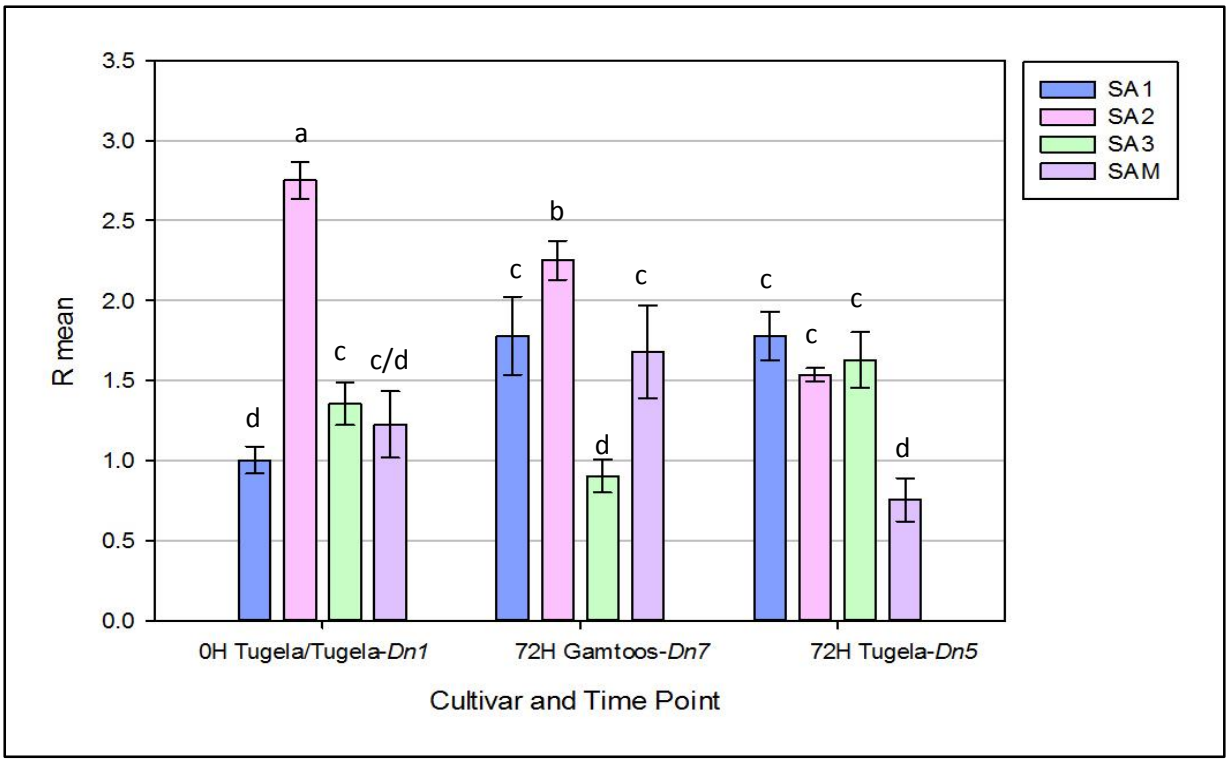

Fig. 4.4. A comparison of the relative expression (Rm) of *ND5* after *D. noxia* feeding on preference and non-preference wheat hosts at 0 hours and 72 hours. The expression measured was normalized with *L32 and β-tubulin* and was calibrated with SA1 0 hours.

Letters a-d indicate that peaks containing different letters differ significantly from each other according to t-tests.

No obvious trend in *ND5* expression was observed among the biotypes (figure 4.4), i.e., the expression profiles differed among all four biotypes with the highest expression measured in the SA2 biotype. The SA1 biotype displayed an increase in *ND5* expression when transferred to both non-preference cultivars, whereas the SA2 biotype's *ND5* expression decreased when transferred to both non-preference cultivars. *ND5* expression in the more virulent biotypes (SA3 and SAM) did not change significantly irrespective of their preference host and Tugela-*Dn5*.

The relative expression results of the *atp6* gene (figure 4.5) showed that the least virulent SA1 biotype had the lowest level of *atp6* expression of all the biotypes while feeding on both the preference and non-preference hosts, while SA2 and the most virulent SAM biotype displayed the highest *atp6* expression levels on their preference hosts. The SA2 and SAM biotypes' expression is approximately 2 fold that of the SA1 biotype on their preference hosts, and the SA3 biotype is 1.5 fold that of the SA1 biotype. When comparing the

expression of the biotypes on their non-preference hosts, no significant differences were observed between the biotypes except that SA1 showed lower expression levels than SA2, SA3 and SAM in all instances. Interestingly, the expression of *atp6* was slightly lower in the SA2 and SAM biotypes when fed on their non-preference hosts.

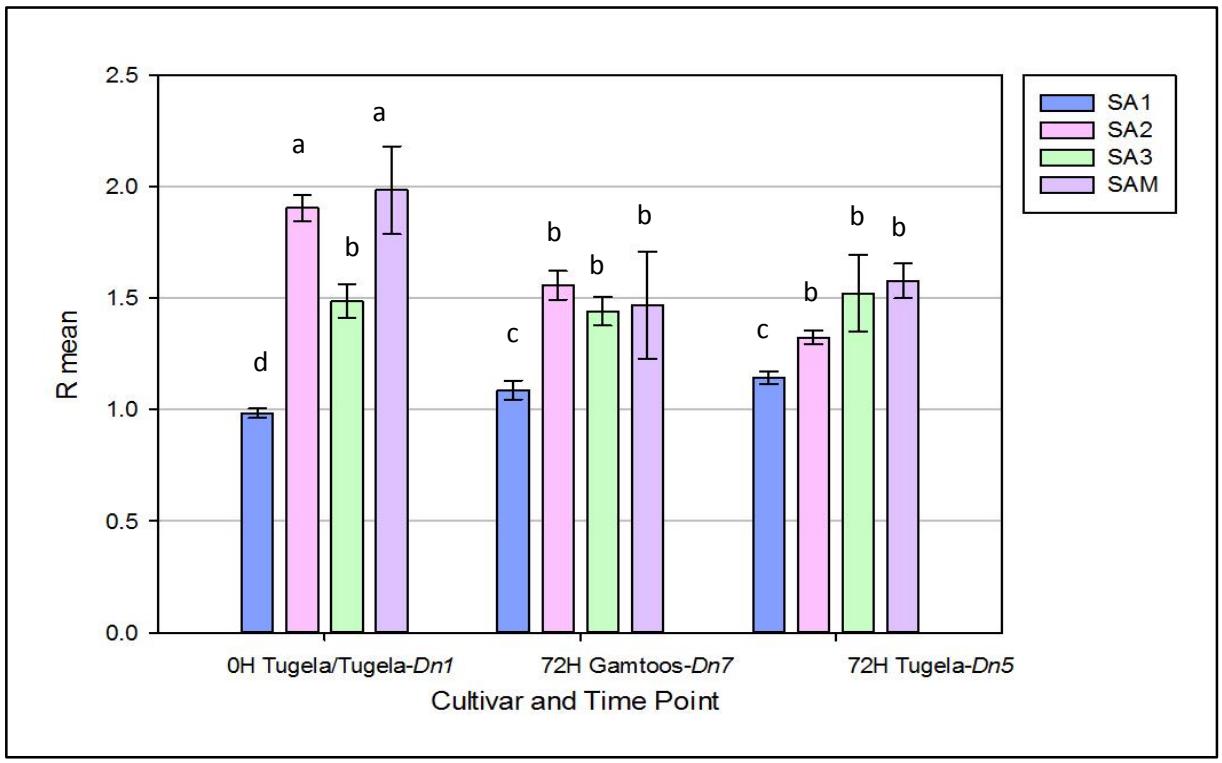

Fig 4.5. A comparison of the relative expression (Rm) of *atp6* after *D. noxia* feeding on preference and non-preference wheat hosts at 0 hours and 72 hours. The expression measured was normalized with *L32 and β-tubulin* and was calibrated with SA1 0 hours.

Letters a-d indicate that peaks containing different letters differ significantly from each other according to t-tests.

4.3.2 Contribution of carotenoids to energy production and immunity

It is suggested that aphids use carotenoids as non-enzymatic ROS scavenging antioxidants. Thus to assess whether carotenoids and the expression of *carotene desaturase* differ between the four South African *D. noxia* biotypes, the composition of carotenoids were assessed (Figs. 4.7-4.9 and B13-B16) and the expression of carotene desaturase were measured (Fig. 4.6). When comparing the carotene desaturase expression between the *D. noxia* biotypes, the least virulent SA1 biotype had the lowest relative expression of carotene

desaturase, while the most virulent SAM biotype had the highest (figure 4.6). Both these biotypes displayed a relatively constant level of *carotene desaturase* expression while feeding on their preference and non-preference cultivars. When the aphids were transferred from their preference hosts to the non-preference host, Gamtoos-*Dn7*, no change in *carotene desaturase* expression was observed. However, when feeding on the antibiotic Tugela-*Dn5*, a significant decrease in carotene desaturase expression was measured in the SA2 and SA3 biotypes.

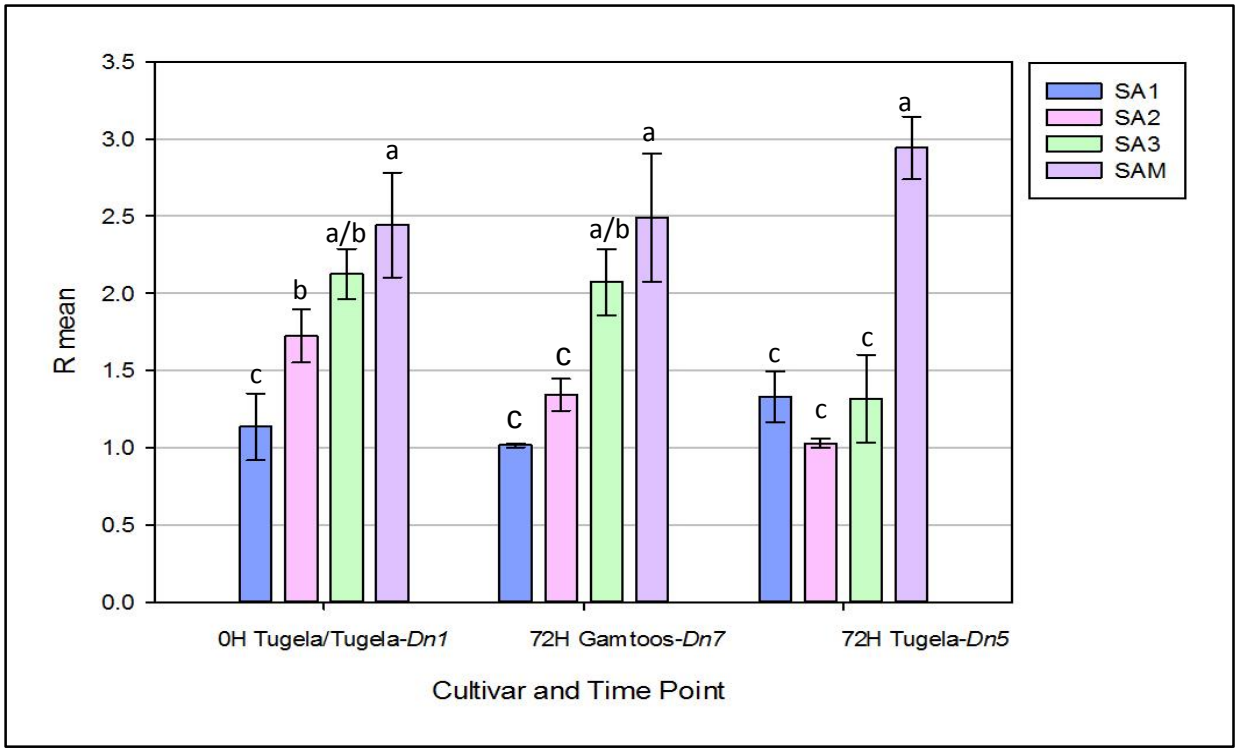

Fig. 4.6. A comparison of the relative expression (Rm) of *carotene desaturase* after *D. noxia* feeding on preference and non-preference wheat hosts at 0 hours and 72 hours. The expression measured was normalized with *L32 and β-tubulin* and was calibrated with SA1 0 hours.

Letters a-c indicate that peaks containing different letters differ significantly from each other according to t-tests.

The composition of the extracted carotenoids from the South African *D. noxia* biotypes are represented as ion chromatograms and UV chromatograms. Fig. B13 contains the 8 ppm lycopene and β-carotene standards and displayed peaks at specific retention times with specific molecular weights for comparison with extracted samples. The UV chromatogram (Fig. 4.9) revealed a peak at retention time (RT) 10.94 for the lycopene standard and a peak

at RT 14.47 for the β-carotene standard. The ion chromatogram (Fig.4.9) revealed peaks at retention time (RT) 10.96 and 11.00 for the lycopene standard and peaks at RT 14.46, 14.49 and 14.56 for the β-carotene standard.

The ion chromatogram (Fig. 4.7) of the extracted samples from the different *D. noxia* biotypes showed various peaks at different concentrations and might have possible indicated the presence of lycopene and β-carotene among other extracted carotenoids. The UV chromatogram (Fig. 4.9) however revealed no peaks at RT10.94 for lycopene, but displayed peaks around RT 14.5 for the SA1, SA2 and SAM biotypes.

Closer inspection revealed that no lycopene or β-carotene was present in any of the extracted samples due to either no peaks present or RT times and molecular weights did not match up with those of the standards (Fig. 4.8 and Figs. B13 -16). It can thus be concluded that the *D. noxia* biotypes contains various highly hydrophobic compounds that are thus coloured and can be classified as carotenoids. These unidentified carotenoids differed in concentration among the different *D. noxia* biotypes as can be seen from the difference in height of the peaks in Fig. 4.7. This may indicate that carotenoid profiles differ slightly between *D. noxia* biotypes and may possibly play a role in aphid fitness. But unfortunately lycopene and β-carotene could not be identified in the *D. noxia* biotypes because of low concentrations of the samples. Due to time constraints and large sample sizes required for analyses, the process could not be repeated.

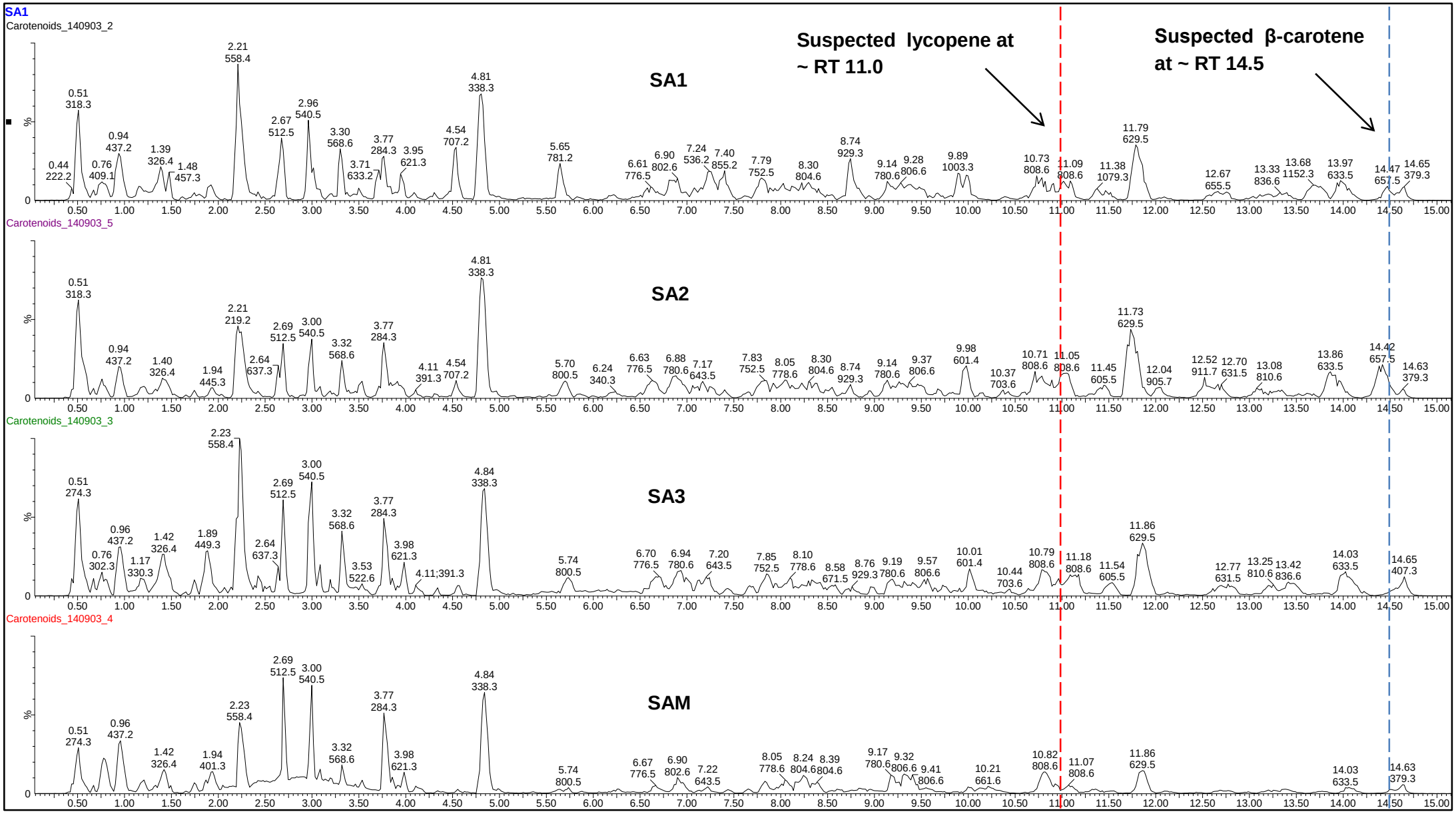

Fig. 4.7. Ion chromatograms of the extracted samples from the South African *D. noxia* biotypes. Red dashed line indicate suspected lycopene at RT 11.0. Blue dashed line indicate suspected β-carotene at RT 14.5.

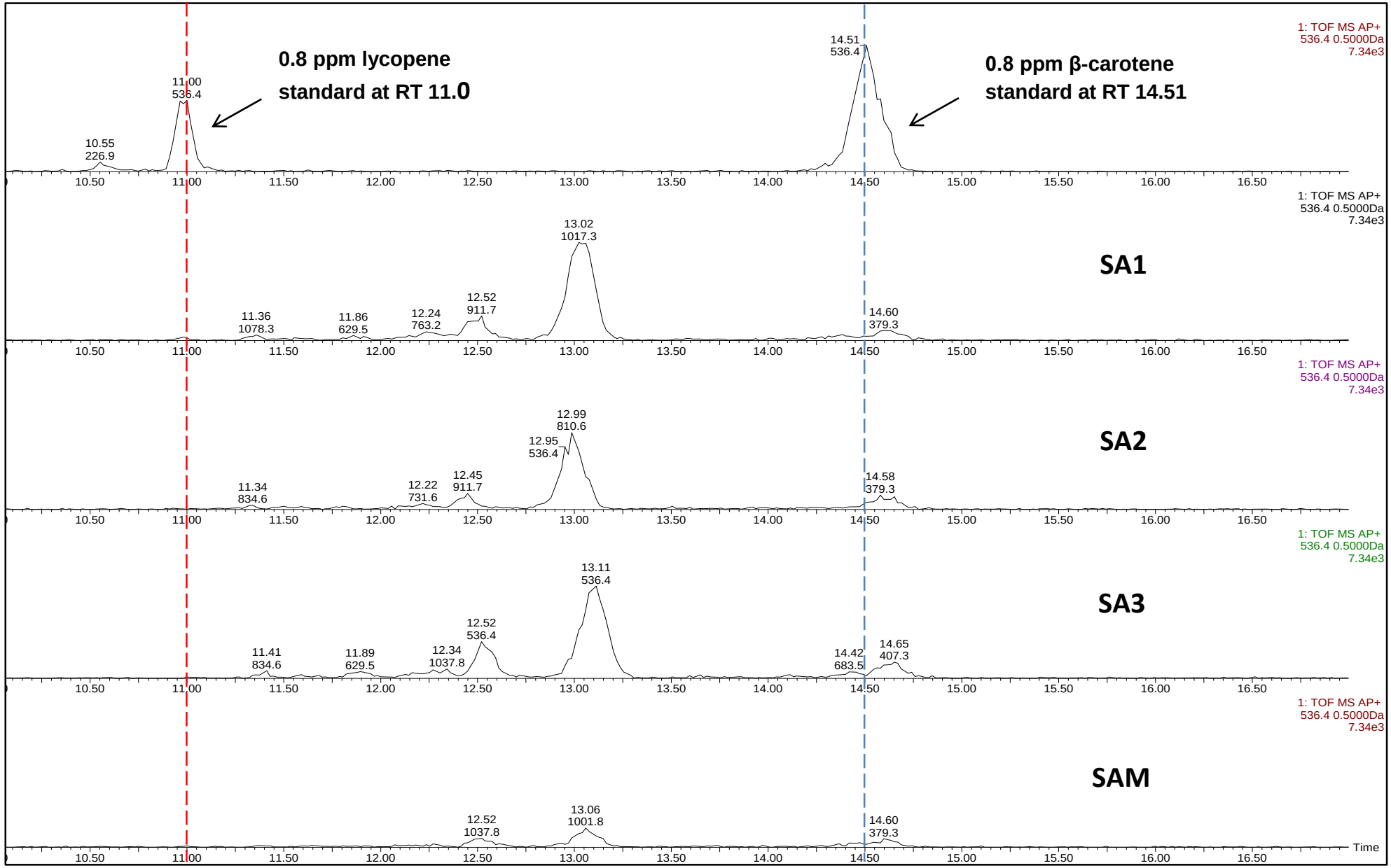

Fig. 4.8. Extracted ion chromatogram of the standards and the extracted samples from the South African *D. noxia* biotypes. Red dashed line indicate suspected lycopene at RT 11.0. Blue dashed line indicate suspected β-carotene at RT 14.5.

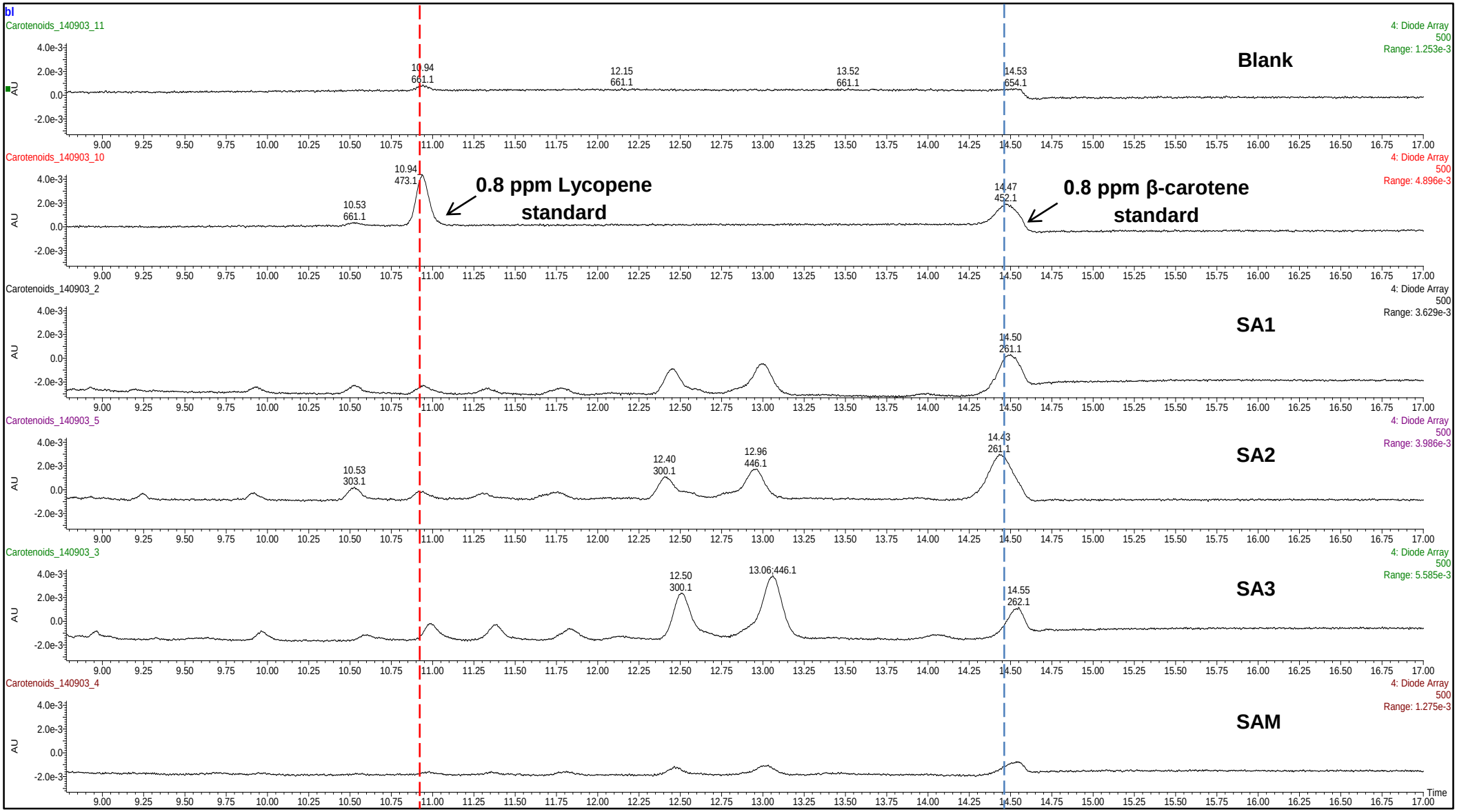

Fig. 4.9. UV chromatograms at 500 nm of the blank, standards and the extracted samples from the South African *D. noxia b*iotypes. Red dashed line indicate suspected lycopene at RT 10.94. Blue dashed line indicate suspected β-carotene at RT 14.47.

4.4 Discussion

Selye (1976) defined stress as a state of threatened homeostasis and anything that is apparent to be a threat of this homeostasis can be considered as a stressor. It is found that organisms that are stressed undergo various behavioural and physiological changes such as increased respiration, feeding suppression, reduced reproductive activities, avoidance reactions and endocrine secretions that increase the available energy of the organism (Charmandari *et al.,* 2005; Schneiderman *et al.,* 2005).

Resistant wheat varieties are known as a stressor of *D. noxia* and have been developed to contain different *Dn* genes and exhibit different modes of resistance to *D. noxia* feeding. The relationship between the plant and the aphid is thus affected by the kind of resistance and may alter the metabolism of *D. noxia* in various ways. A study by Liu *et al.,* (2011) showed that the metabolism of wheat slows down for several hours when under attack from *D. noxia,* to expend the energy needed to produce chemical and physical defences to feeding. This strategy can be adapted to *D. noxia* and one can speculate that virulent *D. noxia* biotypes may have the capacity to control their energy utilization like the plant did, by constraining energy from non-essential processes such as growth and reproduction, and relocating the energy to defensive, elusive or attack mechanisms. These virulent biotypes may also have the capability to utilize their energy pathways (or alternative energy pathways) more efficiently and increase the production of available energy, when compared to lesser virulent biotypes. The production of proteins (elicitors) associated with the virulence of *D. noxia* biotypes requires copious amounts of energy as protein synthesis are considered one of the most energy-consuming functions of a cell (Sokolova *et al.,* 2012) and would suggest that the latter is more suitable than an energy trade-off.

4.4.1 Evaluation of energy homeostasis

The expression of the selected target genes, *atp6* and *ND5*, involved in energy metabolism was measured while the aphids fed on their preference hosts displaying a basal level of metabolic regulation, and after feeding on the non-preference hosts, Gamtoos-*Dn7* and Tugela-*Dn5*, which subjected the aphids to a stressfull environment. Fig. 4.4 and 4.7 demonstrates that while feeding on their preference hosts under a state of homeostasis, the SAM biotype displayd a significantly higher level of expression than the SA1 biotype for the *atp6* gene, i.e., 2 fold that of SA1. Even under stressfull conditions while feedig on non-preference hosts, the SAM biotype still displayed a higher level of *atp6* transcription than the SA1 biotype (Figs. 4.4, 4.8-4.9). These observations are significant because the

hypervirulent SAM biotype was obtained by selective pressure from the least virulent *D. noxia* biotype, SA1. This data could indicate that virulent biotypes are able to produce more energy than lesser virulent biotypes under identical conditions through differential gene regulation. The data can also indicate that *atp6* is essential in aphid fitness and may be the reason why the sequence is conserved and SNP occurrence is minimal (de Jager *et al.,* 2014, Chapter 3: Fig. 3.4).

A slight decrease in *atp6* expression was observed when the SAM aphids fed on its non-preferance hosts in comparison to feeding on its preference host. This decrease in expression may be explained by the resistance genes present in Gamtoos-*Dn7* and Tugela-*Dn5* which employs different mechanisms of resistance to *D. noxia* feeding. The Tugela-*Dn5* cultivar contains the *Dn5* resistance gene and exerts antixenotic and moderately antibiotic effects (Wang *et al*., 2004), while Gamtoos-*Dn7* confers resistance through antixenosis (i.e., non-preference feeding) due to the *Dn7* resistance gene (Marais *et al*., 1994). Thus, due to the resistance genes present in both cultivars, the feeding behaviour of the SA2 and SAM biotype may have been affected by the antixenotic resistance effects. Which may have resulted in a decreased uptake of phloem sap, resulting in a decrease in available sugar for the production of ATP, and thus a decline in *atp6* expression as observed (Figs. 4.4, 4.7-4.9).

The *ND5* expression levels in all the biotypes differed mainly from that observed for the *atp6* gene, except for the SA2 biotype that displayed the same pattern (Fig. 4.4) with high expression levels at 0 hours that decrease as resistance is introduced by the non-preference cultivars (Fig. 4.5). The variable *ND5* expression levels among the biotypes could be attributed to the high SNP frequency observed (de Jager *et al.,* 2014, Chapter 3: Fig. 3.4) that results in variances in gene regulation of the *ND5* gene. Equal levels of *ND5* expression was observed in the SA1 and SAM biotypes while feeding on their preference hosts (Fig. 4.7) and after feeding on Gamtoos-*Dn7* (figure 4.8). While a lower *ND5* expression level was observed in the SAM biotype while feeding on Tugela-*Dn5* in comparison to the SA1 biotype (Fig. 4.9). The results indicate that *ND5* may not be essential in *D. noxia* fitness, as there was no correlation observed between *ND5* expression and the virulence associated with each biotype. The findings suggest that the mitochondrial complex I functions even under low levels of *ND5* expression and thus low levels of NADH dehydrogenase 5 subunit transcription in *D. noxia*.

The coenzyme nicotinamide adenine dinucleotide plays an important role as a redox carrier and substrate for a wide variety of processes, and has emerged as a key regulator of metabolism, stress resistance and longevity (Braidy *et al*., 2011). This coenzyme forms part

of the NAD^+/NADH ratio. A ratio that fluctuates in response to a change in metabolism (Sanni *et al.,* 2001; Gaikwad *et al.,* 2001; Ramasamy *et al.,* 1998; Mongan *et al.,* 2002; MacDonald and Marshall 2000) and reflects both the health and metabolic activities of a cell (Schafer and Buettner 2001). Many human diseases are associated with changes in the level of NAD and/or the NAD^+/NADH ratio. Thus, making NAD a putative metabolic regulator of transcription, longevity and several age-associated diseases that include diabetes, cancer and neurodegenerative diseases in humans (Dumollard *et al.,* 2007; Eto *et al.,* 1999; Anastasiou and Krek 2006; Garriga-Canut *et al.,* 2006; Lin and Guarente 2003). It can thus be inferred that the NAD^+/NADH ratio can reflect the fitness of an individual or *D. noxia* biotype.

The obtained cytosolic NAD^+/NADH ratio among the *D. noxia* biotypes is displayed in Fig. 4.2 and table 4.1 after exposure to multiple stressors such as limiting feeding media, captivity and prolonged exposure to high light intensity that increased the temperature significantly. The aphids fed on artificial feeding media inside aphid cages under growth lights to maintain a controlled environment to provide each *D. noxia* biotype with the same amount of stress: i.e., low concentration of sucrose, high light intensity and duration, increase in temperature and the inability to cope with these stressors by dispersal due to confinement. A controlled environment was needed due to differences observed in phloem content of different host plants (Winter *et al.,* 1992; Geiger and Servaites, 1997) and higher phloem sugar levels found during the day than at night, which illustrates that phloem sugar levels are influenced by light, temperature, plant species and developmental age (Winter *et al.,* 1992; Geiger and Servaites 1997).

A NAD^+/NADH ratio of approximately 4.9 was detected for the lesser virulent SA1 and SA2 biotypes. A higher NAD^+/NADH ratio of 6.8 was observed for the more virulent SA3 biotype, while an even higher NAD^+/NADH ratio of 8.1 was observed for the highly virulent SAM biotype. Interestingly, the SAM biotype has a NAD^+/NADH ratio of almost double that of the least virulent SA1 biotype, and demonstrates that the SAM biotype has double the redox potential of the SA1 biotype which enables it to produce or convert energy more efficiently than a lesser virulent biotypes.Thus, showing that the NAD^+/NADH ratio increases proportionally as the virulence of the *D. noxia* biotypes increases and must in essence be a relevant factor in the virulence associated with *D. noxia* biotypes. Environmental stress such as heat stress induce elevated ROS generation (Abele *et al*., 2007; Kakkar *et al.,* 2007) which requires energy-costly up-regulation of antioxidant defences to scavenge, transform and detoxify ROS (Halliwell and Gutteridge, 1999). A study by Braidy *et al.,* (2011) showed that oxidative stress in vertebrates induced NAD^+ depletion and plays a significant role in the aging process by compromising energy production, DNA repair and genomic surveillance. It

can thus be concluded that lowered intracellular NAD^+ levels and subsequently a lowered NAD^+/ NADH ratio in a *D. noxia* biotype may decrease longevity and the lifespan of the specific biotype, such as the lesser virulent biotypes SA1 and SA2.

Reduced mitochondrial activity of complex I to IV was also observed with aging individuals and impacts both redox status and ATP production (Braidy *et al.,* 2011). The low levels of *atp6* expression in the least virulent SA1 biotype can thus be associated with the low NAD^+/NADH ratio of 4.9 (Fig. 4.2 and table 4.1), in comparison to the higher *atp* expression levels observed in the SAM biotype that can be correlated with the highest NAD^+/NADH ratio observed (8.1). A correlation between redox potential and energy production is thus present in *D. noxia* and attributes to virulence of biotypes. An indirect connection between NAD^+, energy homeostasis and gene regulation is thus evident.

Unfortunately, confinement to the low sugar diet restricts the possible acquisition of resources by the *D. noxia* aphids and thus limits the uptake of available energy and the rate of its' metabolic conversions by the aphids, thereby making the mitigation of oxidative stress difficult. Trade-offs between basal maintenance and energy costs of fitness related functions such as growth, reproduction and development, are thus necessary to ensure survival under such stressful conditions (Sokolova *et al.,* 2012). Nguyen *et al.,* (2009) suggested that respiration and energy generation are disrupted in heat stressed potato aphids based on a reduction in glycolytic enzymes and down regulation of protein synthesis that may result in considerable bioenergetic savings as part of an energy conservation strategy.

4.4.2 Contribution of carotenoids to energy production and immunity

In contrast to this method of energy conservation and differential resource allocation, aphids may adapt a compensation stategy by which phototrophy can be utilized to enhance energy production as hypothesized by Valmalette *et al.,* (2012) or metabolic control can be executed by the aphids, allowing them to change the output of a metabolic pathway over time in response to a change in circumstance (Nelson and Cox 2004). A higher energy output would eliminate energy trade-offs and allow transcription of antioxidative compounds to deal with ROS due to heat stress. Because all the biotypes ingested the same concentration of sucrose solution, all the biotypes should in theory display the same NAD^+/NADH ratio. Though the virulent SAM biotype displayed a NAD^+/NADH ratio almost double that of the least virulent biotype, SA1, thereby suggesting that virulent biotypes' metabolic responses to stress are superior to that of lesser virulent biotypes: i.e., phototrophy and metabolic control can be utilized more efficiently and a higher tolerance to heat stress is present. Jyoti *et al.,* (2006) provided proof that increased virulence of *D. noxia* aphids can be associated with the enhanced ability to tolerate heat stress, as suggested by the current results. These results

show that the NAD^+/NADH ratio may be an indicator of the virulence of a *D. noxia* biotype and increased fitness in terms of metabolic adaptations can be attributed to increased NAD^+/NADH ratios in *D. noxia* biotypes.

Plants deal with a wide variety of abiotic (temperature, flooding, drought, salinity etc.) and biotic stresses (pathogens and pests), all of which directly or indirectly results in oxidative stress. Oxidative stress damage the plant cells through the formation of ROS which are produced by the excitation of oxygen or the transfer of electrons to oxygen (Contreras-Porcia *et al.,* 2011). A rapid increase in ROS concentration (oxidative burst) (Apostol *et al.,* 1989) is experienced by the plant during incompatible plant-pest interactions such as an aphid infestation. ROS is rapidly accumulated at the attack site in an attempt to prevent further colonisation or the spread of the aphid population either directly by the toxic ROS or indirectly by the hypersensitive response which results in host cell death (Lamb and Dixon 1997; Gechev *et al.,* 2006). ROS scavenging enzymes or antioxidants are the key essential molecules responsible for the detoxification of ROS to ensure survival during stressful conditions (Heidari *et al.,* 2012).

Carotene desaturase is involved in the biosynthesis of carotenoids, which act as non-enzymatic ROS scavenging antioxidants (Apel and Hirt 2004; Cheesman 2007; Gill *et al.,* 2010). The *carotene desaturase* expression (Fig. 4.6) observed at 0 hours on the preference hosts showed higher levels correlating to higher virulence levels of each biotype (SA1< SA2< SA3< SAM): i.e., the least virulent biotype, SA1, showed 1 fold relative expression levels compared to the most virulent biotype, SAM's 2.5 fold relative expression levels. The SA1 and SAM biotype both displayed constant carotene desaturase expression levels while feeding on their preference hosts (Fig. 4.7) and after feeding on their non-preference hosts, Gamtoos-*Dn7* and Tugela-*Dn5*, in a stressfull environment (Figs. 4.8-4.9). While Gamtoos-*Dn7* and Tugela-*Dn5* both employ antixenotic mechanisms of resistance against aphid feeding (Marais *et al.,* 1994; Wang *et al.,* 2004), Tugela-*Dn5* also employs antibiosis (Wang *et al.,* 2004) and thus it was expected that a difference in *carotene desaturase* expression should have been observed in virulent biotypes. Antibiotic plants employ oxidative bursts that are associated with the hypersensitive response and results in elevated levels of ROS which results in cell death and necrosis (Botha 2013) and are toxic to the aphids. Elevated H_2O_2 concentrations have been reported in wheat plants that were attacked by *D. noxia*, showing that ROS production is a response to insect attack (Moloi and van der Westhuizen 2006). Therefor the production of enzymes responsible for the biosynthesis of carotenoids and thus antioxidants must be essential in the survival of *D. noxia* biotypes under stressful conditions. A higher level of *carotene desaturase* expression was suggested to be present

due to an increased need for antioxidative compounds to quench the reactive oxygen species produced by the plant as a result of aphid feeding. High levels of antioxidants in plants under stressful conditions is a response to increased levels of ROS to alleviate deleterious effects (Heidari *et al.,* 2012). Although, this was not the case with *D. noxia.*

The results indicated that a higher level of *carotene desaturase* expression was not present as a result of stress from the non-preference cultivars or the production of ROS, but as a result of a higher level of virulence attributed to a certain biotype, such as with the SAM biotype. A possible explanation is that the least virulent SA1 biotype does not have the capacity to produce large quantities of energy under any circumstance and thus does not have the capability of transcribing higher volumes of carotenoids to defend the aphid against toxic ROS and subsequently results in lower fitness. Whereas the virulent SAM biotype is capable of producing or regulating energy more efficiently as seen from the high NAD^+/ NADH ratio (Fig. 4.2 and table 4.1) and higher levels of atp6 trancription (Figs. 4.4, 4.7 – 4.9), and thus already produces a high enough concentration of carotenoids to defend itself under oxidative stress conditions. Thus eliminating the need for increased expression of the *carotene desaturase* gene in the SAM biotype. This ability to transcribe high levels of carotenoids may contribute to the virulence of *D. noxia* biotypes by allowing longevity surpassing lesser virulent biotypes. The results undeniably showed that high *carotene desaturase* levels may attribute to increased fitness of an aphid biotype.

Despite the positive antioxidative effects of carotenoids, the high rate of transcription of *carotene desaturase* in the SAM biotype results in a high concentration of carotenoid molecules in comparison to the lesser virulent biotypes, and these carotenoids could also contribute to the production of additional energy as hypothesized by Valmalette *et al*., (2012). A higher potential for generating energy was observed in the SAM biotype when compared to the lesser virulent biotypes, as deducted by SAM's high NAD^+/NADH ratio (Fig. 4.3) This could demonstrate that exposure to high light intensity for prolonged periods of time could result in the increased production of carotenoids and subsequent additional energy in virulent *D. noxia* biotypes. A study conducted by Alkhedir *et al.,* (2010) found that the amount of carotenoids in the green morphs of the grain aphid, *Sitobion avenae F,* were higher when reared at high light intensity, and thus supports a portion of this finding. Unfortunately, the spectrometry analysis did not supply sufficient data to support this finding due to low carotenoid concentrations in the *D. noxia* samples. However, differences in concentrations of different unidentified carotenoids (Figs. 4.7 – 4.10) was observed among the biotypes, and can support this assumption.

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4.6 Appendix B

Table B1. Primer sequences for Real-Time quantitative PCR

Target region	Primer ID	Sequence
atp6	atp6_F	ACACCAATTTATTTAGCCCCTTT
	atp6_R	TGCAGTAAGACGAATTGATAAAGAT
carotene desaturase	CD_F	CATACATTGCCCGACGAACC
	CD_R	TCCTTCTGGTGCTGCTGTTG
ND5	ND5_F	GTAACTATACCTGAACTATAAGATT
	ND5_R	GGTTGAGATGGTTTAGGTAT
L32	L32_F	CGTCTTCGGACTCTGTTGTCAA
	L32_R	CAAAGTGATCGTTATGACAAACTCAA
B-tubulin	Bt_F	GGCCAAGGGTCATTACACTGA
	Bt_R	TGCGAACCACGTCCAACA

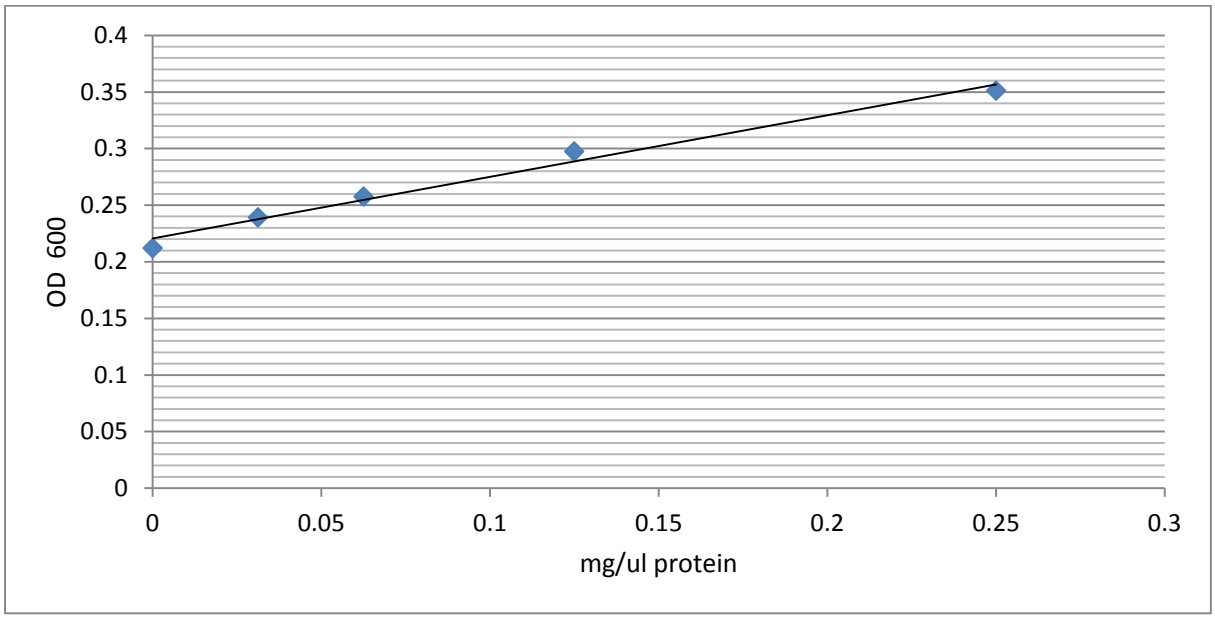


Fig. B1. The standard curve for protein determination utilized in the NAD+/NADH colorimetric assay. Protein content was measured at 600 nm and is expressed in mg/µl protein. The R^2 value is 0.9838

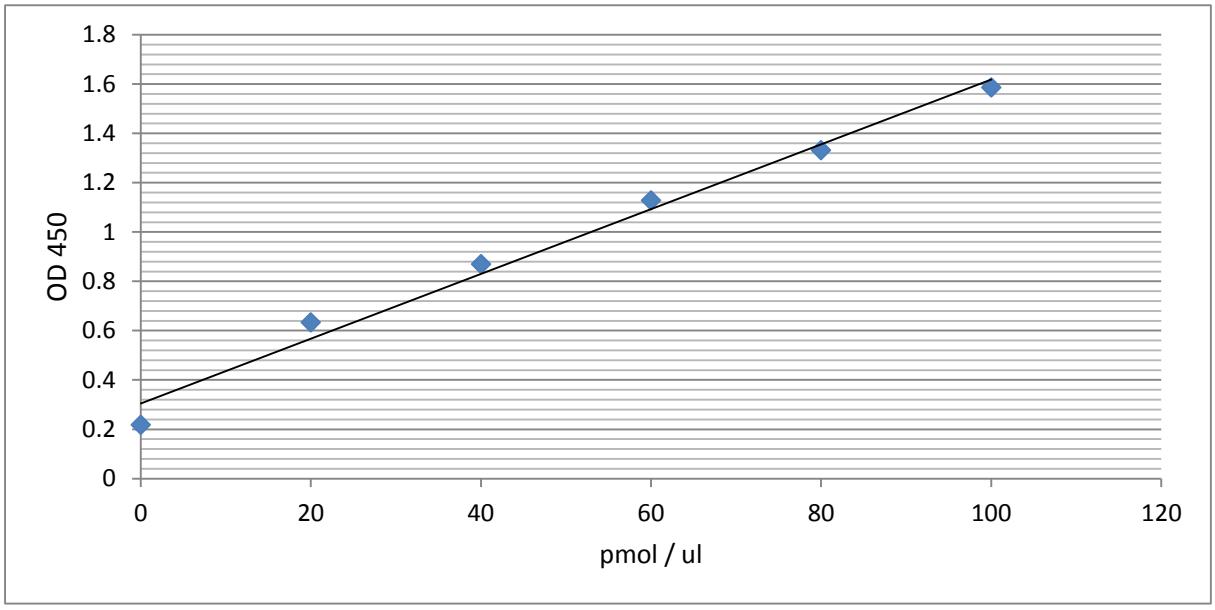
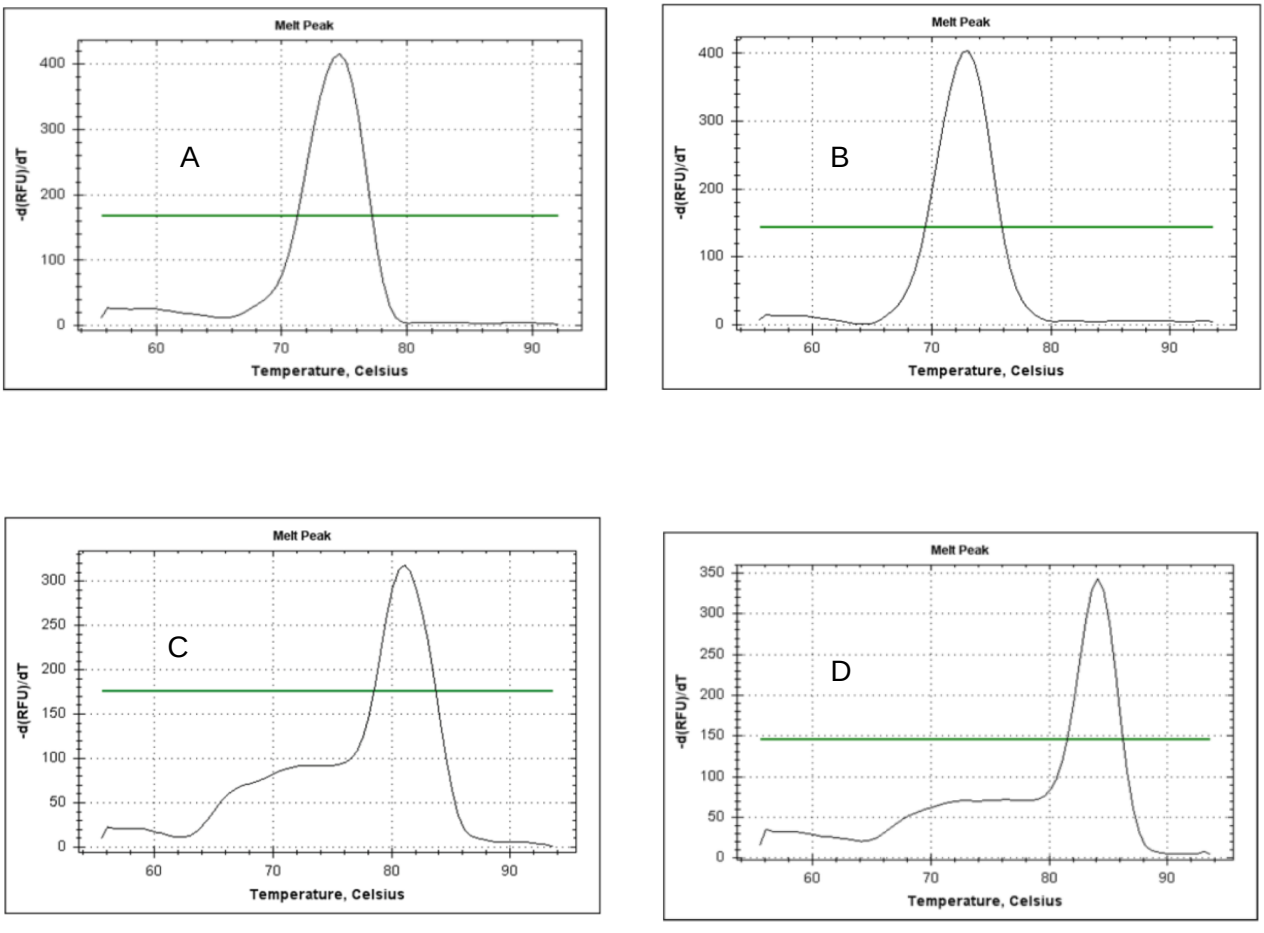


Fig. B2. The standard curve for NAD^+/NADH determination in the NAD^+/NADH colorimetric assay. The NAD^+/NADH content was measured at 450 nm and is expressed in pmol/ µl. The R^2 value is 0.9867.



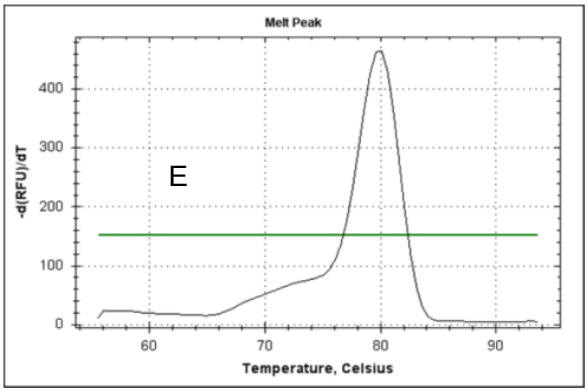


Fig. B3. The melt curve analysis of the *atp6* gene (A), *ND5* gene (B), *carotene desaturase* gene (C), *L32* gene (D) and the *β-tubulin* gene (E).

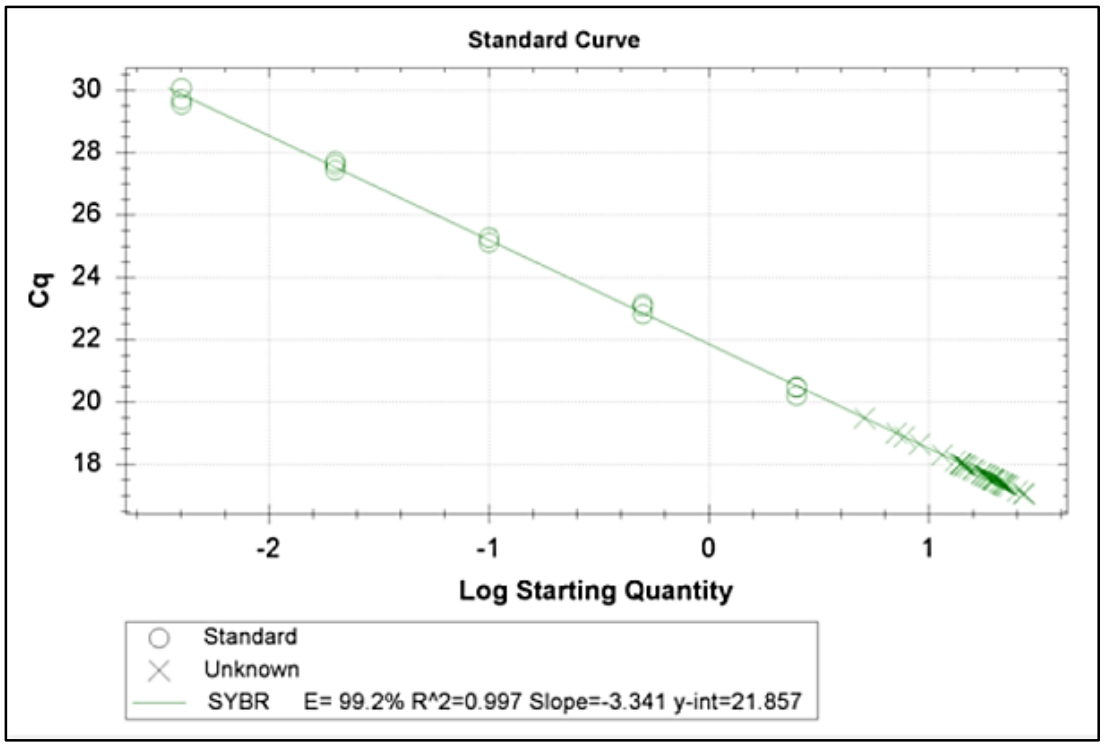


Fig. B4. The standard curve for the *atp6* gene.

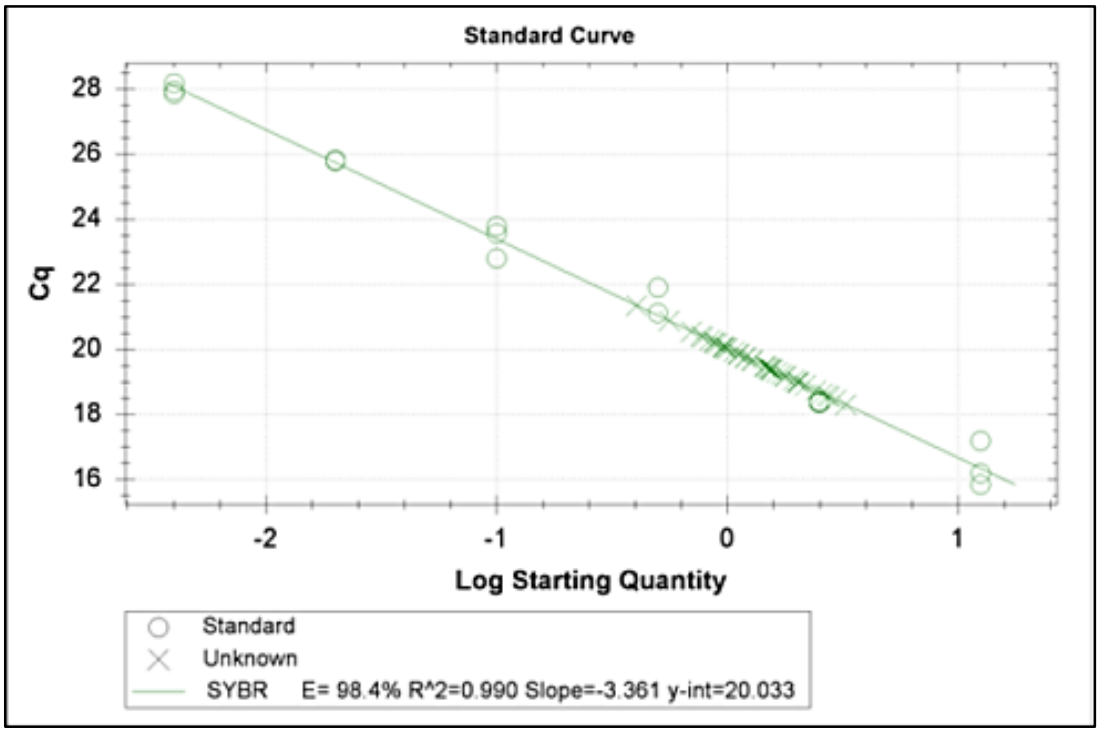


Fig. B5. The standard curve for the *ND5* gene

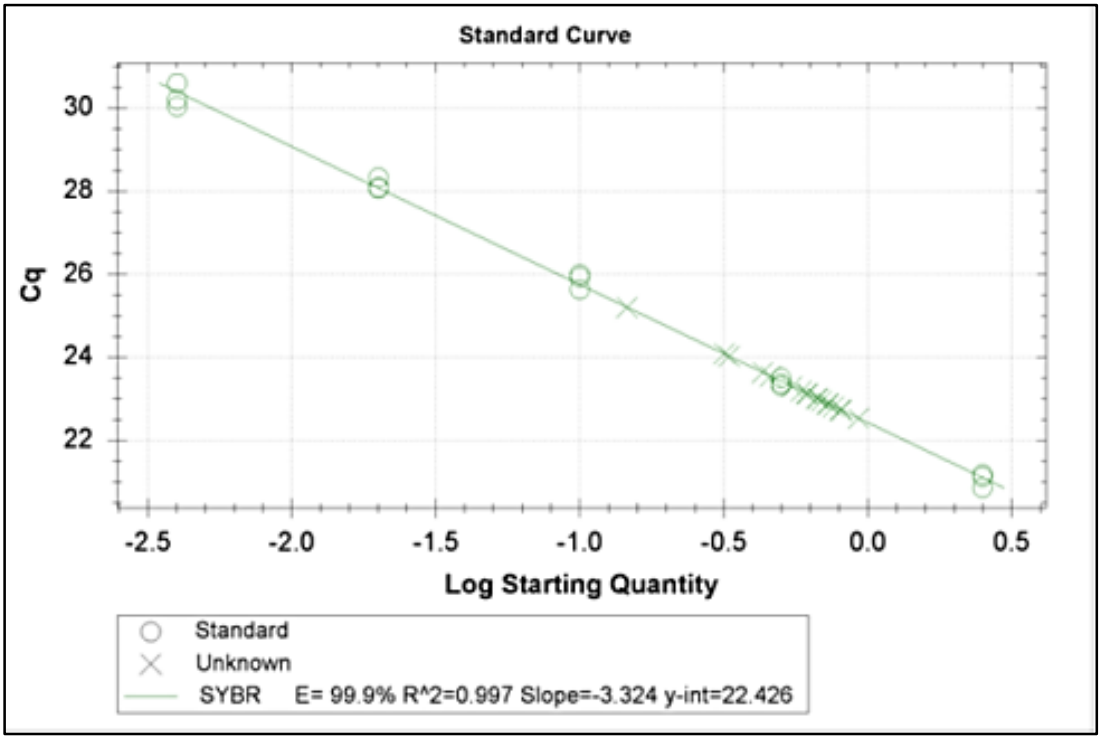


Fig. B6. The standard curve for the *carotene desaturase* gene

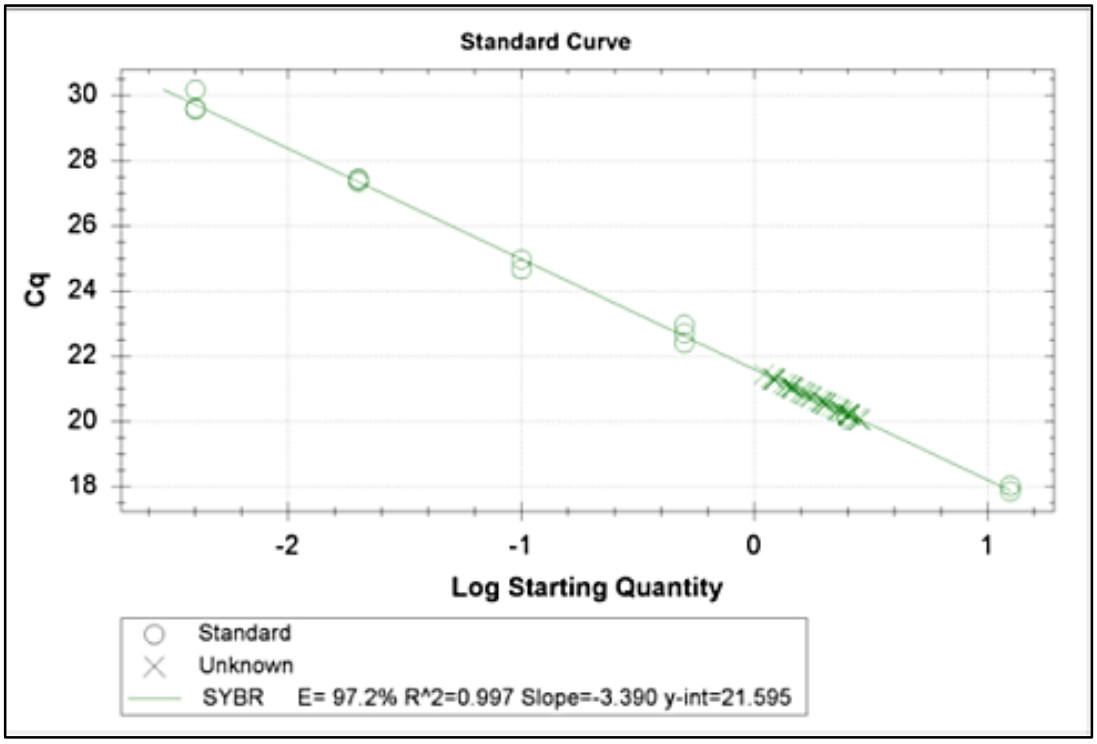


Fig. B7. The standard curve for the *L32* gene

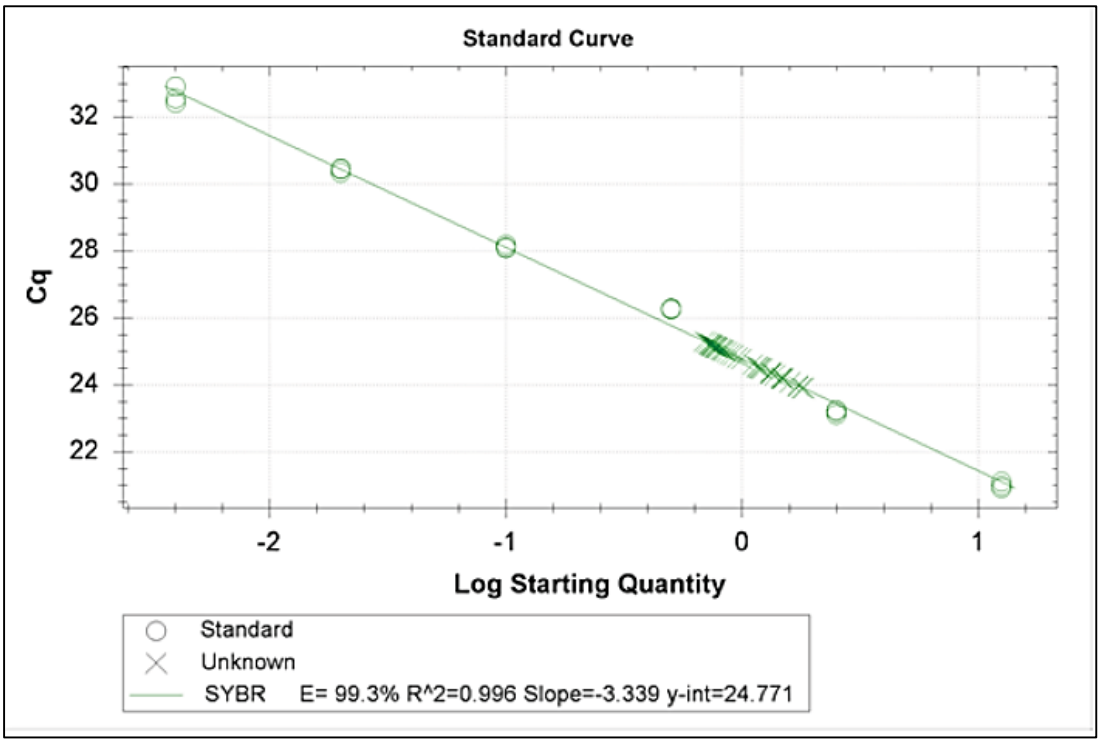


Fig. B8. The standard curve for the *β-Tubulin* gen

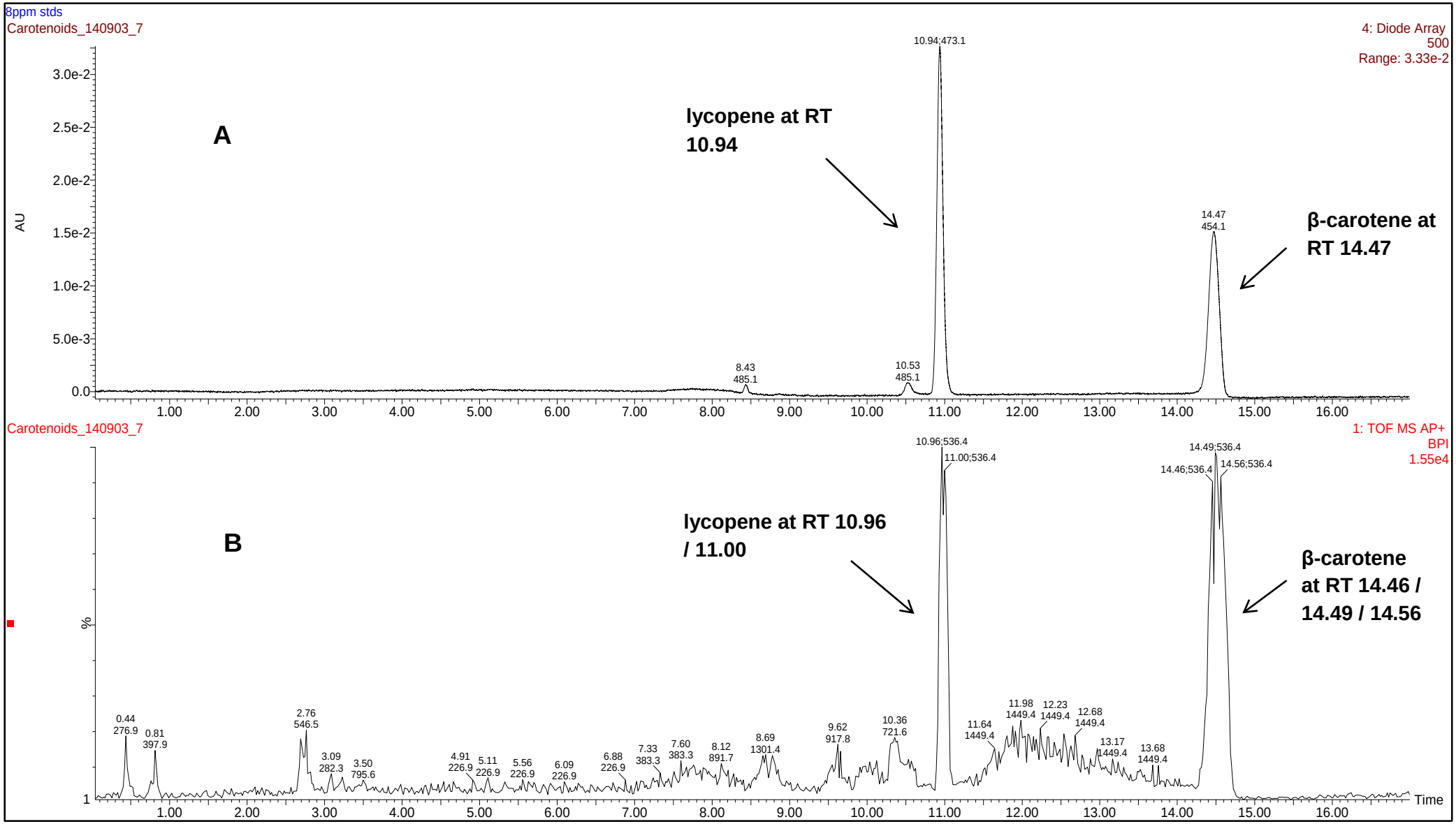


Fig. B9. UV chromatogram (A) and Ion chromatogram (B) of the 8 ppm β-carotene and 8 ppm lycopene standards. (AU: Absorbance units)

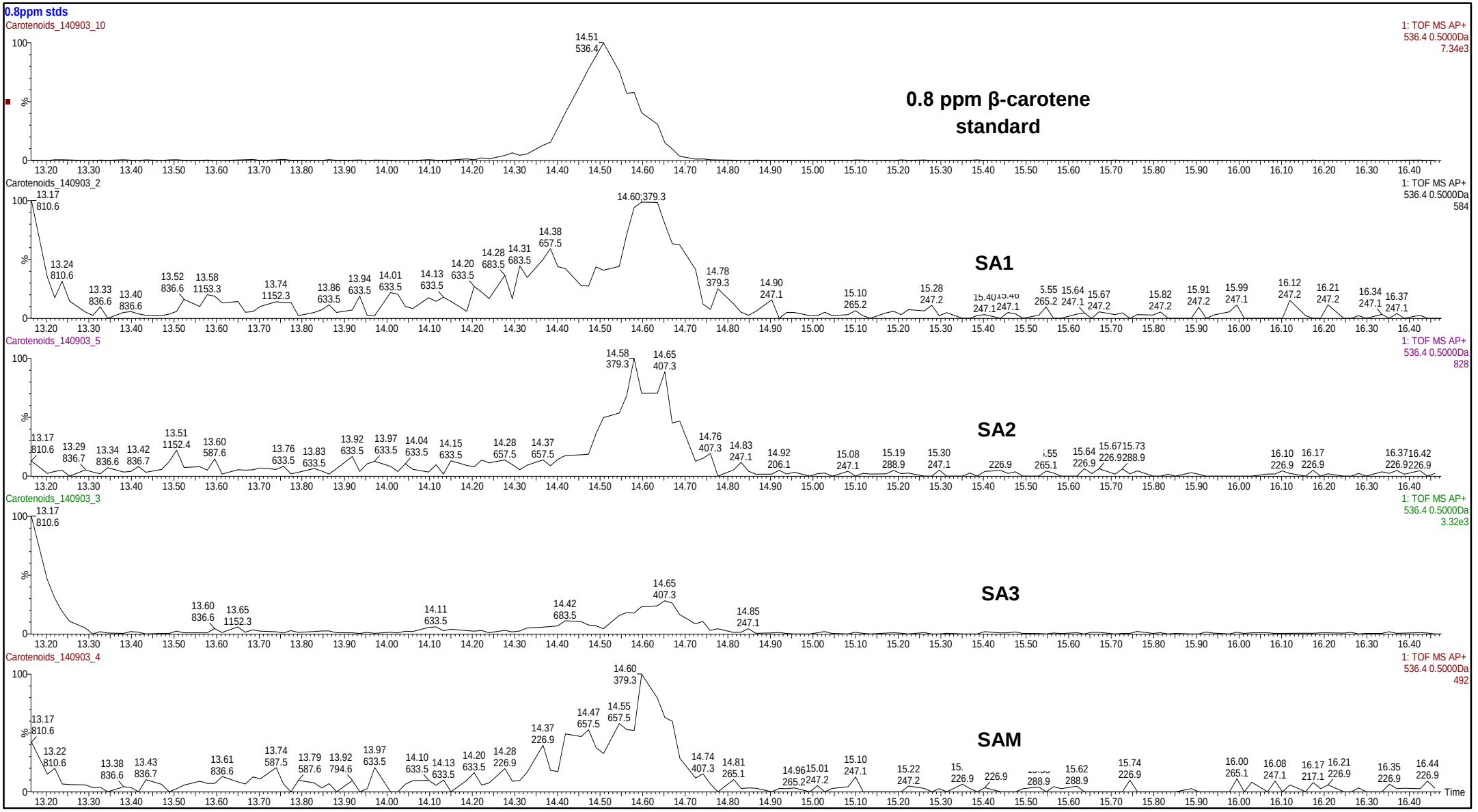


Fig. B10. Extracted ion chromatogram of β-carotene (m/z 536.4) of the standard and the extracted samples from the South African *D. noxia* biotypes.

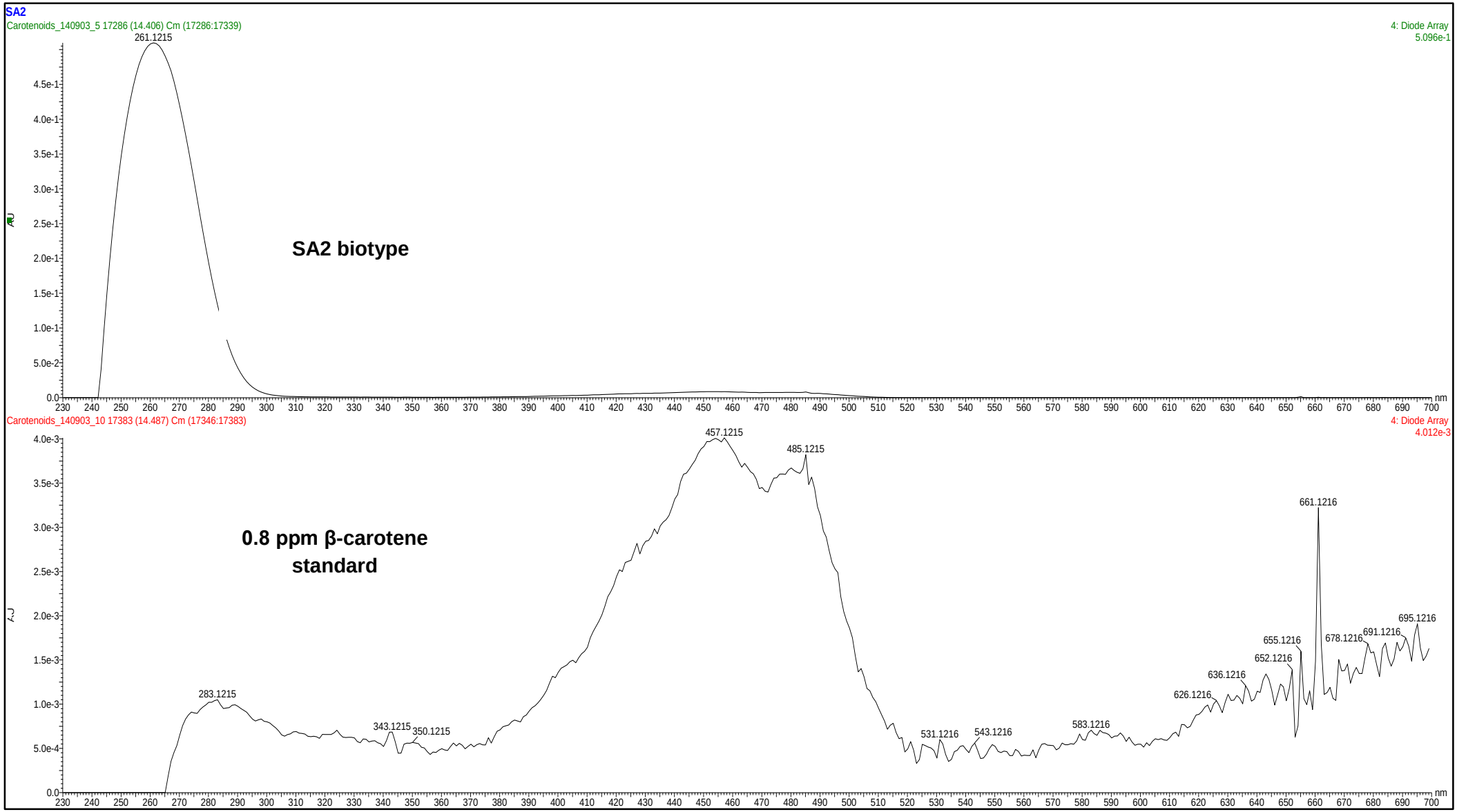


Fig. B11. UV chromatogram of the suspected β-carotene peak from the SA2 biotype and the β-carotene peak from the standard . (AU: Absorbance units)

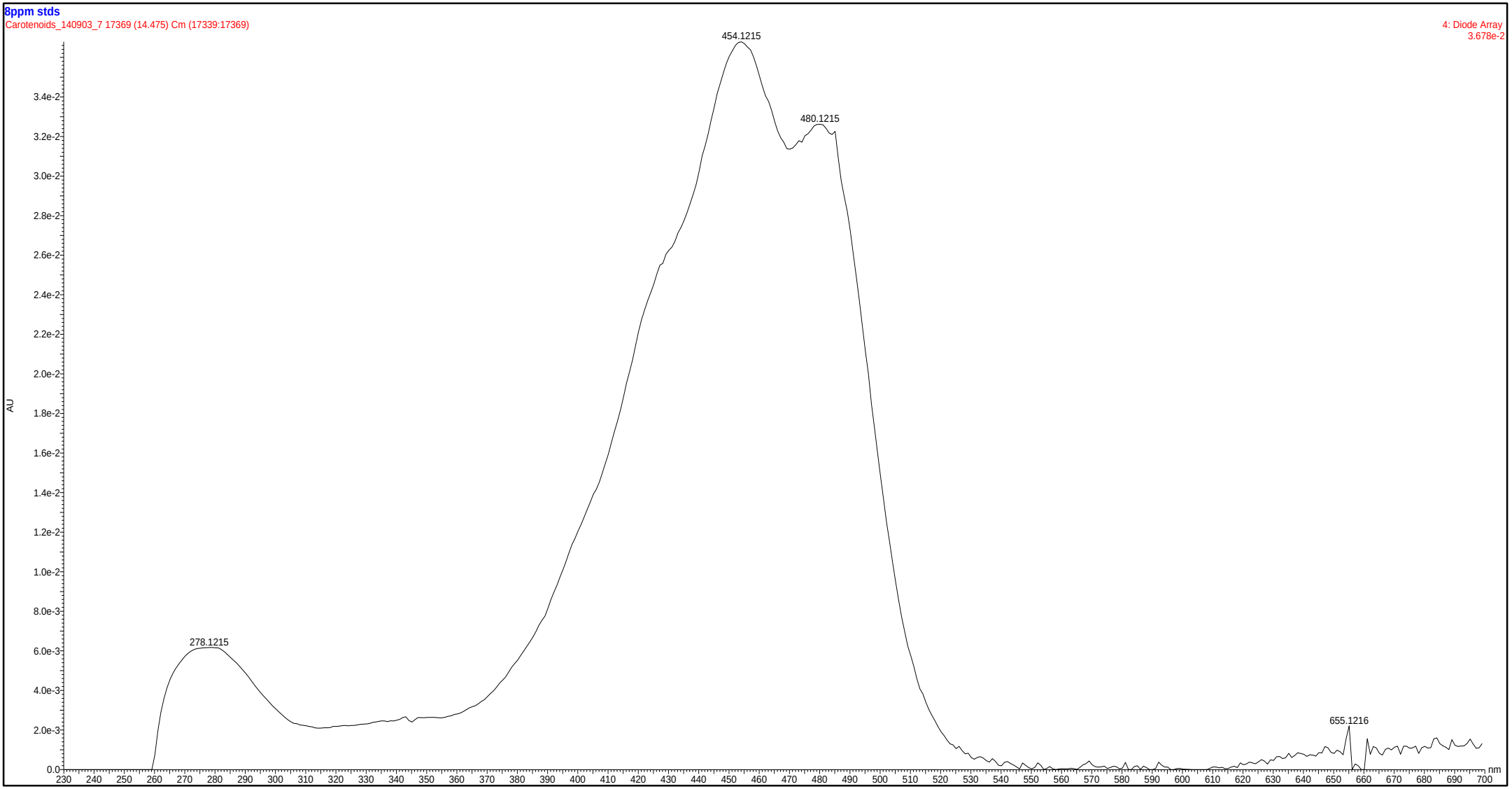


Fig. B12. UV chromatogram of the 8 ppm β-carotene standard. (AU: Absorbance units)

Chapter 5

Summary

5.1 Summary

Aphids are some of the worlds' most widespread devastating agricultural pests known today and cause significant economic losses worldwide. *Diuraphis noxia* (Kurdjumov, Hemiptera, Aphididae) commonly known as the Russian wheat aphid (RWA), is a small highly adaptable phloem-feeding insect and a pest of wheat (*Triticum aestivum* L). Since its classificatioon as a pest in 1901 (Kovalev *et al*., 1991), many reported virulent *D. noxia* biotypes have been described that are able to "break down" the resistance of *Dn* genes in previously resistant wheat cultivars. The continuous development of virulent biotypes necessitates the need to understand the molecular and genetic basis of biotypic variation and its relationship to *D. noxia* fitness.

The survival of an aphid is largely dependent upon the maintenance of homeostasis, which in the context of energy metabolism involves energy acquisition, conversion and conservation. Adaptation and tolerance to stressors affects the energy balance of an aphid and often has fitness consequences. Organisms that are stressed undergo various behavioural and physiological changes such as increased respiration that increases the available energy of the organism (Charmandari *et al.,* 2005; Schneiderman *et al.,* 2005) in order to allocate energy to adaptive or defensive mechanisms in response to stress (Sokolova *et al.,* 2012). The mitochondrion is most often responsible for the production of energy through the process of respiration, and must inevitably play a vital role in the survival and thus fitness of organisms. Aphids experience feeding stress from the defence response of resistant hosts, which includes the production of reactive oxygen species (ROS) as part of the hypersensitive response and is toxic to aphids (Lamb and Dixon 1997; Gechev *et al.,* 2006). Detoxification of these toxic molecules by ROS scavenging enzymes or antioxidants are thus needed to ensure the survival of the aphid (Heidari *et al.,* 2012). Carotenoids are non-enzymatic ROS scavengers and are believed to play a role in the immunity of insects by mediating oxidative stress (Heath *et al.,* 2012). Carotenoids have also been reported to play a role in a photosynthesis-like process (phototrophy) to help aphids produce energy (Valmalette *et al*., 2012).

In order to answer our first research question relating to mitochondria and its contribution to energy generation and the consequential virulence of *D. noxia*, a consensus mitochondrial genome of global *D. noxia* populations was characterised using NextGen sequencing, and was found to be 15 721bp in size and consisting of 38 genes typically found within most insects (De Jager *et al.,* 2014). A single nucleotide polymorphism (SNP) analysis of the

genomes of nine populations was conducted in order to observe sequence diversity among the mitochondrial genomes of the *D. noxia* biotypes. The analysis revealed 125 SNPs in the protein coding genes with the majority of the SNPs occurring in the *ND* genes, and the least in the *ND4L* gene. Low SNP variant frequency was also found for the *atp6* and *atp8* genes, which differed from other reports in the Hemiptera (De Jager *et al.,* 2014).

A targeted examination of the energy metabolism of *D. noxia* included the *atp6* gene (low SNP frequency) and *ND5* gene (high SNP frequency) to establish if sequence diversity influences transcription and subsequent energy production. The qPCR analysis revealed a higher level of transcription of the *atp6* gene in the highly virulent biotype (SAM) under normal and stressful conditions in comparison to the least virulent biotype (SA1). Variable *ND5* expression levels were observed, although no correlation was apparent between *ND5* expression and the virulence associated with each biotype. The results suggest that genes containing low SNP frequencies such as the *atp* gene could be vital in energy generation and aphid fitness. Whereas, a high SNP frequency within PCG's could indicate that these genes does not play a key role in the fitness of aphids, as observed in the *ND* genes. Hence, showing that sequence conservation is essential in genes associated with key energy processes to maintain a state of homeostasis under variable conditions.

The concentration of cytosolic free NAD^+ and NADH were measured among the biotypes to quantify their available free energy levels in order to compare metabolic regulation among the biotypes. The NAD^+/NADH ratio is used for comparison due to its importance as a regulator of metabolism, stress resistance and longevity, and thus reflects the health and metabolic activities of a cell. A significantly higher NAD^+/NADH ratio was observed for the virulent biotype (SAM) in comparison to the lesser virulent biotypes (SA1 and SA2). The NAD^+/NADH ratio can be correlated to the observed *atp6* expression and thus indicates that virulent biotypes may have an increased capacity for the generation and regulation of energy resources. A correlation between redox potential and energy production is thus present in *D. noxia* and contributes to virulence of *D. noxia.* An indirect connection between NAD^+, energy homeostasis and gene regulation is thus evident.

In order to answer our second research question concerning carotenoids and their involvement in *D. noxia* immunity and phototrophy, the composition of carotenoids were assessed and the expression of *carotene desaturase* was measured among the biotypes. The UPLC-MS analysis unfortunately did not reveal any lycopene or β-carotene due to low concentrations in the extracted samples. Various hydrophobic compounds were present in different concentrations in the extracted samples from the different biotypes that were coloured, and can be classified as carotenoids (Valmalette *et al.,* 2012). The *carotene*

desaturase expression profile revealed that the least virulent SA1 biotype had the lowest relative expression of *carotene desaturase*, while the most virulent SAM biotype had the highest. This ability of the virulent biotype to transcribe high levels of carotenoids may contribute to the aphids immune response by mediation of oxidative stress (ROS scavenging) or may provide the aphid with additional energy through a photosynthesis-like process (supported by SAM's high NAD^+/NADH ratio and highest *atp6* expression levels).

In conclusion; it is clear from the results obtained in this study that the generation of energy is a contributing factor to the virulence of *D. noxia*. The results also show that the frequency of SNPs within PCGs in the mitochondrial genome may indicate the relevance of a specific gene to aphid fitness. Mutations resulting from SNPs in the *ND5* gene of humans have dire fitness consequences (Janssen *et al.,* 2006; Carroll *et al.,* 2006; Malfatti *et al.,* 2007), whereas a high SNP frequency was observed in the *ND5* gene of *D. noxia* with limited negative functional effects. Genes that contain a low frequency of SNPs such as the *atp6* gene in *D. noxia* seems to be more important in aphid fitness. The results also indicated different levels of carotene desaturase transcriptional regulation between the highly virulent and less virulent biotypes, but unfortunately the composition and the quantity of carotenoids could not be determined. Therefor the contribution of carotenoids to the immune response and indirectly to the fitness of aphids is a field of study that needs further exploration.

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