CHAPTER 3

LIMITED ENDOSYMBIONT VARIATION IN *DIURAPHIS NOXIA* (HEMIPTERA: APHIDIDAE) BIOTYPES FROM THE USA AND SOUTH AFRICA

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Introduction

Diuraphis noxia Kurdjumov (Russian wheat aphid, RWA), originally from Central Asia through to the Middle East (Stary 2000; Stary & Lukasova 2002), has increased its distribution to all cereal producing countries except for the region of Australasia (Blackman & Eastop 2000; Baker *et al.* 2003). Introduction to these new areas were shown to have low biotypic diversity (Puterka *et al.* 1993), probably due to a small founder effect. Recently new RWA biotypes appeared in the USA and South Africa (Haley *et al.* 2004; Burd *et al.* 2006; Tolmay *et al.* 2007; Weiland *et al.* 2008) implying either new introductions or adaptation and diversification of existing populations. Molecular analysis of the new biotypes supports a diversification theory (Lapitan *et al.* 2007). However, these new aphid biotypes still have little nuclear and mitochondrial sequence variation (Lapitan *et al.* 2007; Shufran *et al.* 2007). RWA biotypes are distinguished on their ability to overcome resistance, their fecundity and the damage that they cause to a plant differential, but not on aphid morphology (Puterka *et al.* 1992; Jyoti & Michaud 2005; Burd *et al.* 2006; Jyoti *et al.* 2006; Weiland *et al.* 2008). However, little information on these biotypes' bacterial endosymbionts exists.

Symbiosis enabled aphids to exploit a nutritionally imbalanced food source such as phloem (Srivastava 1987; Douglas 1998). The usual partner in this relationship, the bacterium *Buchnera aphidicola*, is maintained transovarially and maternally between generations inside aphid produced cells called mycetocytes (bacteriocytes) (Munson *et al.* 1991; Baumann *et al.* 1995; Dixon 1998; Douglas 1998). The *Buchnera*-host relationship is not only complex but also obligatory to both parties, with this gram-negative bacterium found in almost all aphid species. Removal of the endosymbiont from its host usually leads to sterile aphid offspring (Munson *et al.* 1991; Baumann *et al.* 1995; Douglas 1998). The dependency of the endosymbiont on its host was clearly illustrated by genome sequencing that showed many genes from key pathways were absent in the bacterium (Shigenobu *et al.* 2000; Tamas *et al.* 2002; Van Ham *et al.* 2003; Zientz *et al.* 2004).

Pathways retained by *B. aphidicola*, like the essential amino acid biosynthetic pathway, suggest that this endosymbiont still plays a crucial role in aphid nutrition (Shigenobu *et al.* 2000; Tamas *et al.* 2002; Van Ham *et al.* 2003; Nakabachi *et al.* 2005). The type of pathway genes retained appear to be diet dependent (Tamas *et al.* 2002; Zientz *et al.* 2004), *i.e. B. aphidicola* is responsible for the production and recycling of certain essential amino acids found in low quantities in the aphid's diet (Mittler 1971; Douglas & Prosser 1992; Douglas 1998). The production of these essential amino acids in sufficient quantities is accomplished by duplicating genes and by moving rate limiting enzymes (anthranilate synthase, *ptrpEG*), or even whole

pathways (leucine biosynthetic pathway, *pleuABCD*), to single or multiple copy plasmids (Lai *et al.* 1994; Van Ham *et al.* 1997; Baumann *et al.* 1999). These plasmid copy numbers are known to differ between biotypes of the same species (Moran *et al.* 2003).

However, lower plasmid copy numbers and the presence of pseudogenes have led to the belief that the symbiotic relationship between *D. noxia* and *B. aphidicola* is concurrently diverging from essential amino acid production (Lai *et al.* 1996; Thao *et al.* 1998). This hypothesis is further supported by observations that the RWA alters protein profiles and up-regulates leucine and tryptophan levels in the phloem of susceptible wheat cultivars, but not in resistant lines (Van der Westhuizen & Pretorius 1996; Telang *et al.* 1999; Porter & Webster 2000; Sandstrom *et al.* 2000; Ni *et al.* 2001).

In addition to the primary endosymbiont, *B. aphidicola*, aphids may contain an independently acquired secondary (facultative) symbiont(s) (Unterman *et al.* 1989; Chen *et al.* 1996; Darby *et al.* 2001; Fukatsu 2001). Evidence suggests that these can be horizontally transferred when aphids feed on contaminated host plants (Sandstrom *et al.* 2001; Russell *et al.* 2003; Russell & Moran 2005) and that even horizontal plasmid transfer to *B. aphidicola* could be possible (Van Ham *et al.* 2000). In *Bemisia tabaci* (sweet potato whitefly) certain secondary symbionts are biotype specific (Chiel *et al.* 2007). Facultative symbionts can contribute to the well being of their aphid hosts through inferring resistance against predators such as parasitic wasps (Oliver *et al.* 2003; Ferrari *et al.* 2004) or pathogenic fungi (Scarborough *et al.* 2005), an ability to withstand higher temperatures (Chen *et al.* 2000; Montllor *et al.* 2002), higher fecundity (Chen *et al.* 2000) and in some instances could even compensate for the loss of *B. aphidicola* (Koga *et al.* 2003). Similarly, some may have a negative impact on aphids, manifested as slower growth rate, a decrease in reproduction and a shorter life span (Chen *et al.* 2000; Leonardo 2004; Ferrari *et al.* 2007).

The importance of this symbiont-host relationship was recently illustrated in the *B. aphidicola* found in *Acyrthosiphon pisum*, where a single point mutation proved to be crucial for the survival of the host at different temperatures (Dunbar *et al.* 2007). Changes in symbiont diversity or small changes within the symbiont(s) can therefore have major effects on the aphid's viability and adaptation potential. The possible role of symbiont(s) on RWA biotype development and the reverse, *i.e.* the effect of biotype formation on the symbiont(s), have to date not been investigated. Here these interactions are investigated in ten *D. noxia* biotypes by determining the complete endosymbiont assemblages, as well as sequence variation and copy numbers of the leucine and tryptophan plasmids from their primary endosymbiont, *B. aphidicola*.

Materials and Methods

Diuraphis species and biotypes

The originally introduced South African biotype (SA) (Du Toit 1989) was obtained from a colony established on field collected parthenogenetic females at the ARC-Small Grains Institute, Bethlehem, South Africa and sustained on a RWA susceptible wheat cultivar, Scheepers. A mutated form of the South African biotype (SAM), laboratory induced by *Dn* resistant selective pressure, was maintained on the resistant cultivar, TugelaDN (Van Zyl & Botha 2008). Females of both biotypes were kept in insect cages at 20 ± 2 ºC with continuous artificial fluorescent lighting. Samples of the eight USA biotypes were obtained from Prof. N.L.V. Lapitan (Colorado State University, Fort Collins, USA). Biotype USA1 is the original USA 1986 RWA introduction and USA2 is characterized by its virulence to *Dn4* resistant winter wheat cultivars (Haley *et al.* 2004). The first five USA biotypes were from cultivated wheat; USA6 from both volunteer wheat and downy brome; USA7 collected from volunteer wheat, crested wheatgrass, Canada wildrye, green foxtail and intermediate wheatgrass, while USA8 was collected from crested wheatgrass and smooth brome (Lapitan *et al.* 2007; Weiland *et al.* 2008). USA biotypic identifiers are described in detail by Weiland *et al*. (2008). All USA biotypes were isofemale lines maintained on a mixed diet of susceptible wheat and barley cultivars under greenhouse conditions (Lapitan *et al.* 2007). Individuals of *D. mexicana* and *D. tritici* were collected on mountain brome in Colorado, USA, and kindly provided by Dr. G.J. Puterka (USDA-ARS, Stillwater, Oklahoma, USA). Apterae (wingless) morphs were collected with a brush, washed with 70 % (v/v) ethanol and rinsed twice with sterilized distilled water before DNA extraction. Total DNA was extracted using the DNAzol extraction protocol (Molecular Research Centre). DNA of the USA biotypes was extracted as described in Lapitan *et al*. (2007) and shipped at -20 °C to South Africa. All DNA samples were treated with RNase, cleaned (DNeasy cleanup kit, Qiagen, USA) and quantified (Nanodrop ND-1000 Spectrophotometer, Thermo Scientific, RSA).

Biotypic endosymbiont investigation

A portion of the *16S rDNA* gene was amplified for use in denaturing gradient gel electrophoresis (DGGE) analysis using two universal primers, one containing a GC-clamp (*Table Appx 3.1*, Appendix Chapter 3). All PCR reagents and conditions used in this research are described in *Table Appx 3.1*. The GC-clamp increases fragment separation, enabling the detection of up to single point mutation (Myers *et al.* 1985; Sheffield *et al.* 1989; Muyzer & Smalla 1998). Amplicons, after verification on agarose gels, were run in duplicate on 8 %

polyacrylamide gels with a denaturing gradient of 25-55 % urea-formamide. DGGE was performed as described in Surridge (2007) and stained with SYBR Gold (Molecular Probes, USA). Bands were viewed under a blue light trans-illuminator (model CCR DR-88M DR, Inqaba-Biotec), excised, re-amplified and sequenced.

Buchnera aphidicola sequence variation amongst biotypes

The leucine plasmid, *pleuABCD* and parts of the *ptrpEG* plasmid, *16S rDNA* and *trpB*, were amplified and sequenced (ABI BigDye v3.1. System, Applied Biosystems, USA) (*Table Appx 3.1*). Fragments were aligned using ContigExpress (Vector NTI Advance 9, Invitrogen, USA) (Lu & Moriyama 2004), with cloned fragments first vector clipped using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/). Inconsistencies in assemblies were manually investigated on the corresponding chromatograms and resolved with further sequencing. All BLAST analyses (Altschul *et al.* 1990; Altschul *et al.* 1997) were against the non-redundant Genbank database (NCBI, http://www.ncbi.nlm.nih.gov/).

Structural analysis

Bacterial promoters on the leucine plasmid were predicted with BPROM (http://softberry.com). Quickfold (Markham & Zuker 2005) and the Kinefold server (Xayaphoummine *et al.* 2005), using simulations of stochastic folding pathways from different random seed events, were used to predict the free energy values of the inverted repeat region upstream of *leuA*. The plasmids were screened for *Rho*-independent terminators using FindTerm (http://softberry.com).

Plasmid copy numbers

Plasmid copy numbers were determined according to Plague *et al*. (2003). External standards were amplified (*Table Appx 3.1*), cleaned (QIAquick PCR purification kit, Qiagen) and cloned into pGEM-T Easy vector (Promega, USA). Plasmid isolations were done with the Concert Rapid Plasmid Miniprep System (GibcoBRL, Life Technologies, USA) and single copy amplicon inserts confirmed with sequencing. Plasmid copy number for cloned external standards were determined spectrophotometrically in triplicate and converted to copy number per µg DNA (Plague *et al.* 2003). Real-time qPCRs were performed using forward and nested reverse primers of the different target genes (*Table Appx 3.1*). Primer optimization and qPCR reactions were done in accordance with the manufacturer's protocols (Bio-Rad, USA). The iQ SYBR Green Supermix (Bio-Rad) was used for quantitative PCRs and reactions performed on an iCycler with

an iQ Real-Time PCR Detection System (Bio-Rad). Single amplicons were confirmed for each qPCR with melting curve analysis and on 2 % TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.3)) agarose gels. The iCycler Optical System Interface v2.3 was used to calculate the copy number for each sample using calibrated external standard curves of each gene. Plasmid number per chromosome gene was calculated for the leucine plasmid using *leuB*:*trpB* ratio (Plague *et al.* 2003).

Results

Biotypic endosymbiont investigation

Most aphids contain one or more endosymbionts. In order to establish the relationship between the aphid *D. noxia* and its particular endosymbiont, we have investigated the occurrence of mono-or-multisymbiosis in RWA biotypes. DGGE of the bacterial-*16S rDNA* (*Table 3.1*) amplicons showed that the ten *D. noxia* biotypes, *D. tritici* and *D. mexicana*, were all monosymbiotic.

The obtained *16S rDNA* DGGE gene fragments, from both the biotypes and other *Diuraphis* species, were sequenced to confirm the identity of the endosymbiont. Genbank database searches (Altschul *et al.* 1990; Altschul *et al.* 1997) had highly significant homologies with *B. aphidicola* (E-values: 0 to $7e^{-140}$), *Sodalis glossinidius* ($1e^{-67}$), *Erwinia chrysanthemi* ($2e^{-66}$) and an *Arsenophonus* endosymbiont ($3e^{-66}$). A Ribosomal Database (release 9.51) (Cole *et al.* 2006) search and phylogenetic analysis (*Figure Appx 3.1*) confirmed the endosymbiont as *B. aphidicola*, although the biotypes had homologous sequences that differed by four indels from the Genbank accession for *B. aphidicola* of *D. noxia* (*Table 3.1*).

Buchnera aphidicola sequence variation amongst biotypes

The importance of *Buchnera* in aphid nutrition is well known. However, the current hypothesis states that the relationship between the RWA and its endosymbiont is degrading. We have therefore targeted genes or plasmids involved in nutrition to investigate the aphid-endosymbiont relationship. To this end more than 10 kb was sequenced for *B. aphidicola* from each *D. noxia* biotype (*Table 3.1*). Although numerous differences to sequences on Genebank were found (*Table 3.1*), only a single CCC insertion on the leucine plasmid differs between the *Buchnera* sequences of RWA biotypes.

This CCC-insert was upstream of the *leuA* gene (*pleuABCD*) in an inverted repeat region of biotypes SA, SAM, USA3 and USA7. The biotypes' *pleuABCD* sequences also increased in

length, from 7768 (Genbank) to 7771 (without insert) and 7774 bp (with insert). Other differences were also observed between the leucine plasmid sequences of the biotypes and previously submitted Genbank accessions (AF041837, NC001911 and *Figure Appx 3.2*). These include various insertions, deletions or point mutations, for example a single nucleotide insertion in a non-coding region between the *leuA* and *leuB* genes transposed them into the same open reading frame (*Figure Appx 3.2*). The differences in the sequenced leucine plasmids also altered the AT-contents causing an increase from 74.11 % to 74.39 % and 74.36 %, when compared with the previously deposited Genbank accessions (AF041837, NC001911).

Table 3.1 Sequences analyzed and the differences observed against homologues on Genbank and between the different biotypes.

Fragment	***Size in base pairs***	***Genbank differences***	***Biotype differences***
Bacterial chromosome			
16S rDNA[#]	460	4	0
trpB[#]	342	0	0
Bacterial plasmids			
pleuABCD[#]	7768-7774	60	1 (CCC-insert)
ptrpEG[#]	~2100	3	0
Aphid mitochondrion sequences			
COI across the distribution range	Variable	1	-

[#]Fragments sequenced in this project and used in the comparisons (Genbank accession numbers FJ705277-FJ705296; FJ705299-FJ705318). Genebank accessed November 2008.

Primary and secondary structural analysis

The primary and secondary structures of DNA are known to play a crucial role in gene expression. Thus, changes in it may alter secondary structures and therefore functionality of the gene region. To date, no differences within a species were shown for this region (*Figure 3.1*). We therefore simulated the secondary folding patterns to predict free energy values for the inverted repeat region upstream of *leuA*.

Analyses revealed that the most stable stemloop structure produced by this inverted repeat region is formed in biotypes without the insert, with a predicted free energy of -63.4 kcal mol^{-1} (Kinefold server, Xayaphoummine *et al.* 2005), while biotypes containing the CCC-insert gave a free energy value of -57.9 kcal mol^{-1}. Similarly, Quickfold (Markham & Zuker 2005) also predicted lower free energy values for DNA from the biotypes without the CCC-insert (*Figure 3.2*), though both values were lower than those predicted by Kinefold server.

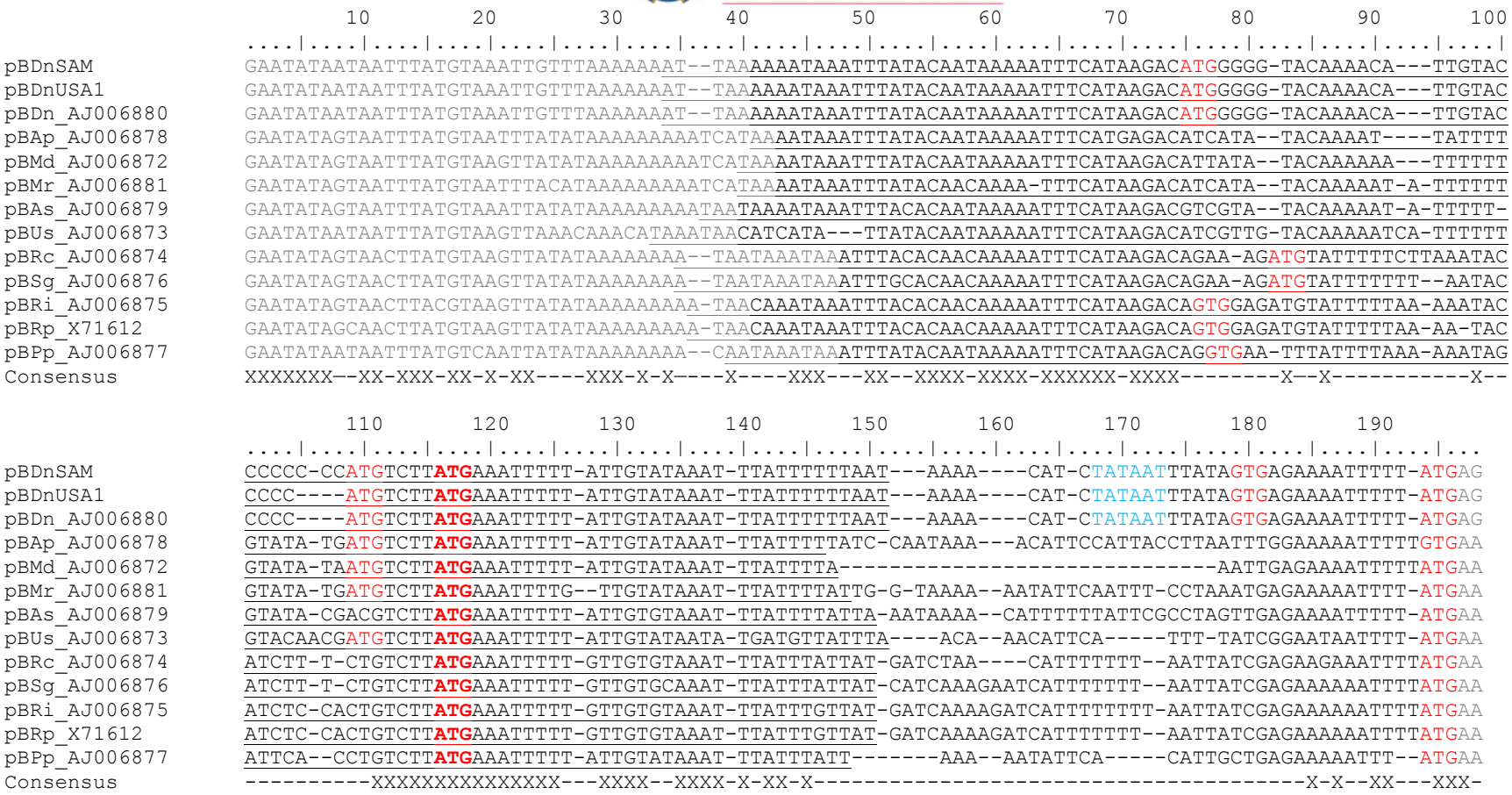

Figure 3.1 The inverted repeat region between *repA2* and *leuA* on leucine plasmids of the Aphididae. The end of *repA2* and the start of *leuA* are indicated in grey, with possible start codons indicated in red. Abbreviations: 'pB' indicates the leucine plasmid of *B. aphidicola* which is then followed by the aphid species, 'Dn' and biotype 'SAM': pBDnSAM, *D. noxia* biotype SAM; pBDnUSA1, *D. noxia* biotype USA1; pBDn, *D. noxia* (Genbank); pBAp, *Acyrthosiphon pisum*; pBMd, *Metapolophium dirhodum*; pBMr, *Macrosiphum rosae*; pBAs, *Aulacorthum solani*; pBUs, *Uroleucon sonchi*; pBRc, *Rhopalosiphum cerasifoliae*; pBSg, *Schizaphis graminum*; pBRi, *R. insertum*; pBRp, *R. padi*; pBPp, *Ptercomma populeum*.

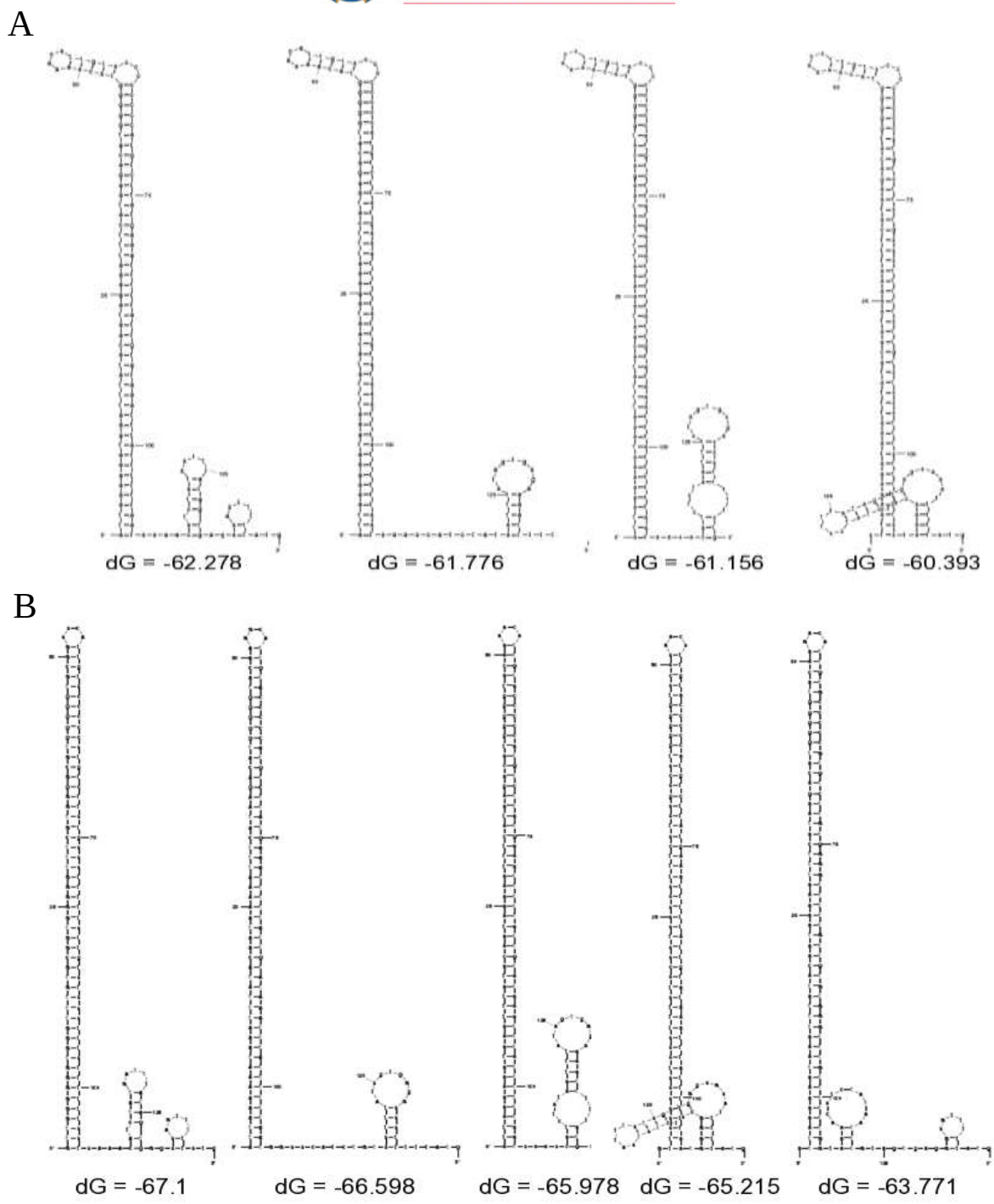

Figure 3.2 Minimum free energy diagrams for the inverted repeat region up to the start codon of *leuA* of *D. noxia* biotypes as predicted with Quickfold (DNA and temperature 25 °C, DINAMelt Server) (Markham & Zuker 2005), with (*Figure 3.2A*) and without (*Figure 3.2B*) CCC-insert. Similar structures and energy values were also obtained for predicted RNA structures of the same region. In both cases the CCC-insert resulted in an increase of ~5 kcal mol^{-1} free energy between the predicted structures. All free energy values (dG) are given as kcal mol^{-1}.

Interestingly, *B. aphidicola* has only two predicted sigma factors, σ^{32} (*rpoH*) and σ^{70} (*rpoD*) (Shigenobu *et al.* 2000). A BPROM promoter search of the regions adjacent to the CCC-insert predicted that it could form a new transcription factor (TF) binding site with homology to *rpoH3* TF binding site. The first predicted BPROM promoter has homology with two σ^{70} TF binding sites and a better linear discriminant function (LDF) score than the new σ^{32} binding site that is formed by the CCC-insert.

The leucine plasmid has two predicted *Rho*-independent terminators, one following *leuD* and the other, following the *repA2* gene. FindTerm (http://softberry.com) predictions high-lighted the second inverted repeat region, the one in which the CCC-insert is located, as the best *Rho*-independent terminator on the plasmid. The CCC-insert is located upstream of the *leuA* gene (*Figure 3.3*) and downstream of a predicted Sigma70 promoter (BPROM, http://softberry.com), TATAAT box and start codon, and could therefore be transcribed.

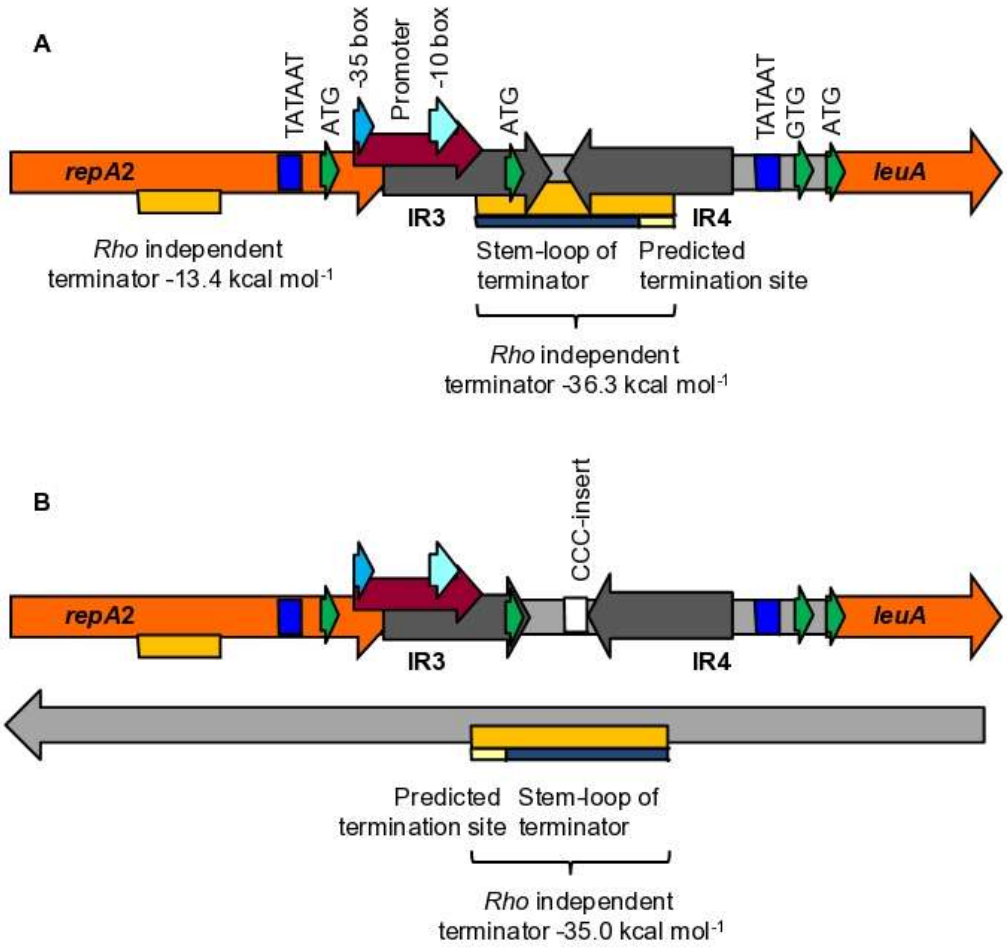

Figure 3.3 The region between *repA2* and *leuA* indicating predicted promoter and *Rho* independent terminator regions of RWA biotypes without (*Figure 3.3A*) and with (*Figure 3.3B*) the CCC-insert. The CCC-insert shifts the predicted *Rho* independent terminator from the coding to the non-coding strand (*B*). This increases the predicted free energy value of the terminator structure, suggesting a less stable terminator.

Plasmid copy numbers

In order to investigate the possible role of the endosymbiont in the aphid-endosymbiont relationship, we have studied the plasmid copy numbers in *Buchnera*. It is suggested that individuals with higher copy numbers may have advantages in terms of fitness, as higher copy numbers supply the aphid with more essential amino acids, while lower copy numbers and pseudogenes indicate a degenerating relationship with reduced access to essential amino acid availability (Lai *et al.* 1996; Thao *et al.* 1998).

We have found that an average of 0.9 copies/bacterial chromosome for *pleuABCD*. The South African RWA biotypes had lower *pleuABCD* copies than the USA biotypes (*Figure 3.4*), while USA8, had more than double the previously published number of copies (2 copies per bacterial genome, *Figure 3.4*).

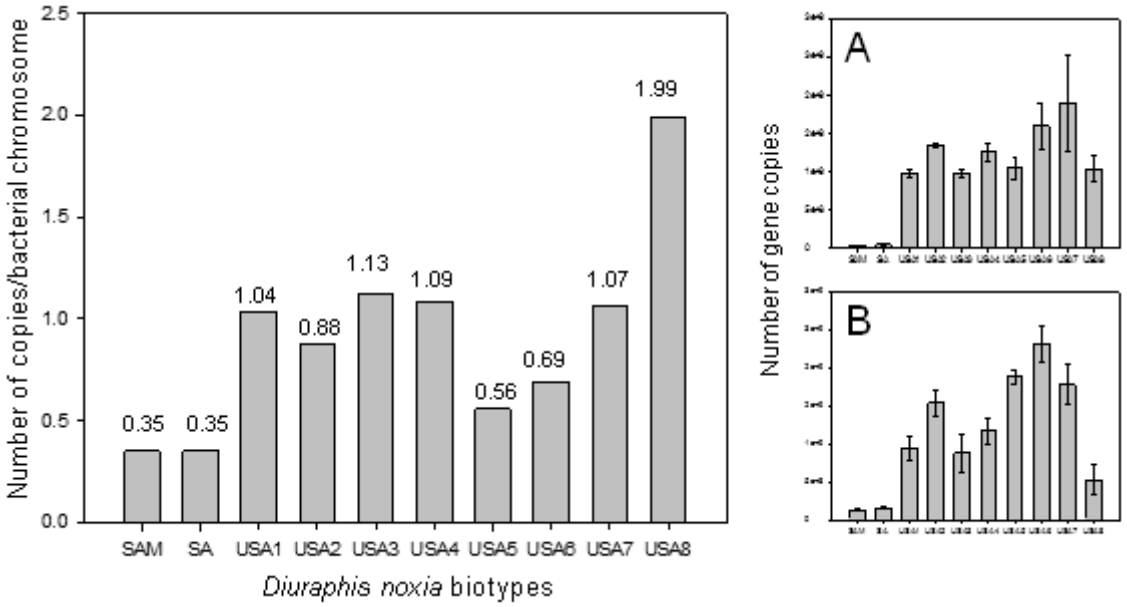

Figure 3.4 The ratio *leuB:trpB* was used to calculate the copy number of the different biotypes. The mean copy numbers for *leuB* (insert A, n=4) and of *trpB* (insert B, n=4) were determined for the different biotypes and then used to calculate the *leuB:trpB* ratio. South African biotypes had lower copy numbers than their USA counterparts which is supported by literature. The average across all the biotypes was 0.91 copies per chromosomal gene.

Discussion

Biotypic endosymbiont investigation

DGGE has been used to investigate associated microbial endosymbionts in a variety of insects, including wasps (Reeson *et al.* 2003), whiteflies (Gottlieb *et al.* 2006), ants (Stoll *et al.* 2007), ticks (Moreno *et al.* 2006) and aphids (Haynes *et al.* 2003). In the present study the DGGE fragments supplied sufficient phylogenetic resolution to determine *B. aphidicola* accession up to the host level (*Figure Appx 3.1*). Although aphids have been shown to contain secondary endosymbionts in addition to *B. aphidicola*, the contribution of a secondary symbiont in RWA biotype development was not supported by DGGE-based data since the *Diuraphis* species and biotypes analyzed were all monosymbiotic.

Buchnera aphidicola sequence variation amongst biotypes

A single CCC insertion on the leucine plasmid differs between the sequences of the RWA biotypes after more than 10 kb was sequenced for *B. aphidicola* from each *D. noxia* biotype. This insert is located upstream of the *leuA* gene (*pleuABCD*) in an inverted repeat region in the South African biotypes and two US biotypes, namely USA3 and USA7. Interestingly, the CCC-insert is located within the variable region of the inverted repeat stem and is consistent with sequence variation found within the family Aphididae (Silva *et al.* 1998). The insert also results in an increase in sequence length, this increase in size is in sharp contrast with other publications where it was shown that plasmids were shrinking in accordance with the genome reduction usually observed in *B. aphidicola* (Gil *et al.* 2006). Mutations, *e.g.* single nucleotide insertion in non-coding region between the *leuA* and *leuB* genes that transposed them into the same open reading frame, were also observed. The CCC-insertion could change the expression levels of *leuA*, and thus would also change the expression levels of the other portions of the operon, *i.e. leuB*.

The rate of sequence change in endosymbiotic bacteria is higher than in free living bacteria (Baumann 2005), therefore more sequence divergence is expected. This is supported by the genome comparison of *B. aphidicola* from different aphid species that showed extremely stable genomes with no re-arrangements or gene acquisitions, but with substantial sequence evolution and few gene losses (Tamas *et al.* 2002; Van Ham *et al.* 2003). In contrast, very little sequence variation is usually found within *B. aphidicola* from the same population (Funk *et al.* 2001; Abbot & Moran 2002; Van Ham *et al.* 2003). These observations are supported by data in the present study where only a single insert has been found to vary among the different biotypes.

Similarly, limited sequence variation was previously found for these biotypes' mitochondrial cytochrome oxidase subunit I (*COI*) gene (*Table 3.1*) (Lapitan *et al.* 2007; Shufran *et al.* 2007). Phylogenetic analysis of these and sequences in *Table 3.1* support the same relationships as determined by aphid and mitochondrial genes (*Figure 3.5*).

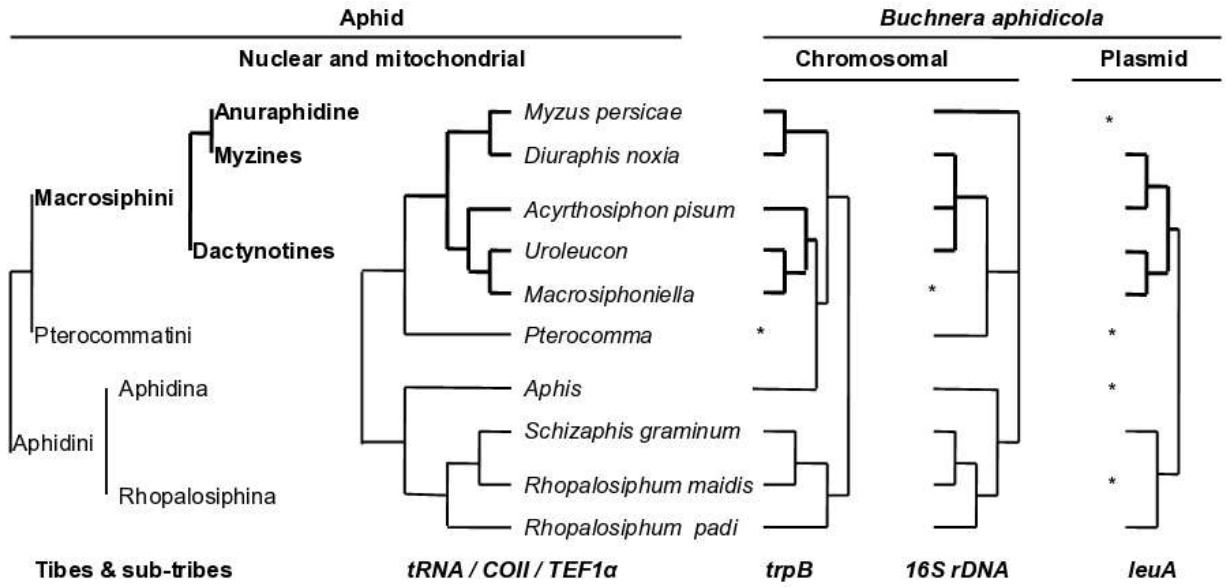

Figure 3.5 Phylogenetic relationships between tribal representatives of the subfamily Aphidinae. The first two phylogenies are an abbreviated representation of a combined nuclear and mitochondrial dataset of *tRNA*, *COII* and *TEF1α*, from von Dohlen *et al.* (2006). Bold lines indicate members of the tribe Macrosiphini. Species names are given where data are available for the phylogenies and genus names when different species of the same genus were used. An asterisk indicates absent genera.

Primary and secondary structural analysis

The inverted repeat region, where the insert is located, has known secondary structural differences and is conserved within the family Aphididae (Silva *et al.* 1998). The most stable secondary structure is the one with the lowest free energy, which is usually the structure with the most bases paired. To this end, most secondary structure prediction algorithms identify the best secondary structure by trying to minimize free energy (Wuchty *et al.* 1999). Since both these prediction algorithms (Quickfold and Kinefold server) awarded higher free energy values for the CCC-insert containing regions, it is highly likely that this region forms a less stable structure.

Conservation of this entire region between *repA2* and *leuA*, including the inverted repeats, within *B. aphidicola* found associated with the family Aphididae (*Figure 3.1*) suggests a functional constraint (Silva *et al.* 1998). A start codon (methionine), preserved in all the

members of the family, is located in the conserved region of the stemloop just following the CCC-insert (*Figure 3.1*). Destablization of this region by the CCC-insert, as predicted by free energy values (*Figure 3.2*) could thus allow easier access to this start codon.

BPROM predictions suggest that the first promoter has homology to two σ^{70} TF binding sites and a better linear discriminant function (LDF) score than the new σ^{32} binding site that is formed by the CCC-insert. However, predicting promoters accurately in AT-rich genomes, such as those of *Buchnera*, is extremely difficult (Baumann *et al.* 1995). If the new CCC-insert-induced σ^{32} binding site is functional, then biotypes with this insert could produce more leucine. Since the CCC-insert also forms an imperfect stemloop (*Figure 3.2*) which destabilizes the secondary DNA structure, the TF binding site would be easier accessed during transcription.

Interestingly, the FindTerm program predicted that the terminating site of this *Rho*-independent terminator following the *repA2* gene would change from the coding strand, in the plasmids without the CCC-insert, to the non-coding strand in the presence of the CCC-insert (*Figure 3.3*). If the CCC-insert moves the termination site to the non-coding strand, transcription termination could be deactivated and upstream promoters could come into play in regulating *leuA*. Because of the location of the CCC-insert, and the possibility that this region may be transcribed, the stemloop/hairpin structure formed by the inverted repeats could act in stabilising the mRNA of either *repA2* or *leuA*. Both 5'- and 3'-terminal hairpin structures are known to act as mRNA stabilizers in bacteria (Wong & Chang 1986; Emroy *et al.* 1992). In all cases this region could play a role in gene expression, mRNA stabilization or translational regulation.

Plasmid copy numbers

The observed average of 0.9 plasmid copies/bacterial chromosome for *pleuABCD* was higher than the previously published value of 0.8 (Thao *et al.* 1998). Interestingly, the South African biotypes had lower *pleuABCD* copies than the US biotypes. This, together with the CCC-insert that is absent in the original US biotype, suggests that the US RWA introduction did not originate from South Africa, thus supporting previous findings of multiple introductions (Lapitan *et al.* 2007; Smith 2009). In contrast, USA8 a RWA biotype only collected from species other than wheat, *i.e. Agrophyron cristatum*, had more than double the previously published number of copies (2 copies per bacterial genome, *Figure 3.4*). The data on the copy numbers of the leucine plasmid supports the hypothesis that the CCC-insert is a functional mutation. The South African biotypes, with the CCC-insert, have lower leucine copy numbers than their US counterparts. One may argue that the US biotypes with the inserts (USA3 and USA7) have only recently developed, and as such have not yet had sufficient time to lower their copy numbers.

Gene amplification allows for adaptation by enabling the over expression of specific gene products necessary for survival in a changing environment or biological interaction (Romero & Palacios 1997). This state would only remain while selective conditions were in effect (Lai *et al.* 1994; Romero & Palacios 1997). Furthermore, having only a single transcriptional regulator for essential amino acid biosynthesis known to exist in a single *B. aphidicola* accession, plasmid copy number is the most likely regulatory mechanism (Moran *et al.* 2005). Plasmid copy numbers are highly variable between and within aphid species (Plague *et al.* 2003). The same tendency was observed for the *pleuABCD* plasmids in *D. noxia*.

Wide-host range selective pressure may explain the high *pleuABCD* copy number of the USA8 biotype. SAM, though kept under selective pressure by feeding aphids resistant wheat cultivars, developed out of the original SA biotype. The low copy number observed here shows the close relationship between SA and SAM. The observed *pleuABCD* copy numbers for the South African biotypes are similar to the 0.3 copies per bacterial genome obtained by Moran *et al*. (2003) for the wild type SA biotype. Copy number suggests that the symbiosis could be involved when the RWA feeds on hosts other than wheat, but no differences in copy number or sequence structure exist between biotypes feeding on resistant and susceptible wheat cultivars.

The observation that genetic diversity of *B. aphidicola* cannot explain the ecological diversity observed between aphids holds here for the different biotypes, and *B. aphidicola*'s initial contribution in *Diuraphis* adaptation is still supported (Tamas *et al.* 2002; Van Ham *et al.* 2003). The CCC-insert and lower copy numbers may support the idea of symbiotic degradation between *D. noxia* and *B. aphidicola* for the South African biotypes, as suggested by Wernegreen and Moran (2000), but the opposite can be argued for one of the US biotypes (USA8) where higher copy numbers for the leucine plasmid have been observed, and for those biotypes where the CCC-insert occurs.

Conclusions

Aphids feeding on different plant species or cultivars have different requirements of their endosymbionts since the essential amino acid content of plants varies (Sandstrom & Moran 1999). This research shows that *B. aphidicola* of different RWA biotypes showed little variation in sequence, but differed in plasmid copy numbers. However, small variations in *B. aphidicola* have major implications for host viability (Dunbar *et al.* 2007). Varying *B. aphidicola* plasmid copy numbers may allow some measure of adaptation to the host where essential amino acids cannot be altered in the plant. Therefore, the role of the CCC-insert is unclear: it may confirm the suggested degeneration of the symbiotic relationship between the RWA and *B. aphidicola* if it

causes down-regulation of subsequent genes; or the opposite could occur and the genes may be up-regulated. It could also be non-functional indicating only normal variation for the region as observed within the family. Keeping symbiosis in mind may still prove to be the key in understanding biotype development within aphids.

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