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Molecular ecology of the green mirid *Creontiades dilutus* Stål (Hemiptera: Miridae) - movement and host plant interactions across agricultural and arid environments.

James Peter Hereward

BSc. (Hons) University of Manchester

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Abstract

Creontiades dilutus, the green mirid, is endemic to Australia and widely distributed across the continent. These bugs have been recorded on a broad range of host-plants including native species, weeds and several crops, particularly cotton, lucerne, and soy. The economic relevance of green mirids to the Australian cotton industry increased in recent years in response to the uptake of transgenic cotton, which controls Lepidopteran pests but is ineffective against Hemiptera. In this thesis I combined several molecular and ecological approaches to develop a better understanding of the species status of this insect, its use of multiple hosts and its long distance movement.

Creontiades dilutus had reputedly been recorded in the USA during 2006. With collaborators at the USDA, I used sequence data (Cytochrome Oxidase I (COI) and 28S ribosomal gene) to establish that the insects concerned were highly unlikely to be *C. dilutus*. Subsequent taxonomic work confirmed that the USA species was indeed a separate species, *Creontiades signatus.* Using *C. signatus* as an out-group, further phylogenetic analyses showed that *C. dilutus* and *C. pacificus* are well differentiated according to the sequence of both genes. The COI sequences also indicated low levels of genetic diversity in *C. dilutus* (Pi = 0.0006), especially in comparison to *C. pacificus* (Pi = 0.0026). The low COI diversity indicated that more variable markers would be required for further analyses of gene flow in this species, and consequently 12 microsatellites were developed by enrichment.

To understand the use of multiple hosts by *C. dilutus*, all the available host plant data were analysed. Most of the putative host plants recorded prior to this thesis were crop species or introduced weeds. As *C. dilutus* has not been recorded outside of Australia it was evident that a more thorough investigation of potential native hosts was necessary. Over three seasons of field surveys in central and eastern Australia I added an additional 25 species to the list of potential hosts, 22 of which are native to Australia. The presence of nymphs indicates that *C. dilutus* is indeed able to feed and reproduce on 46 host plant species, most in the family Fabaceae. Quantitative sampling, however, revealed a strong association between *C. dilutus* and two plant species in the genus *Cullen.* These two species are thus likely the primary host plants for green mirids. To test whether green mirid individuals show a strong preference for *Cullen* under field conditions I amplified Chloroplast DNA from DNA extracted from whole insects. These diet analyses demonstrated that *C. dilutus* individuals do feed on alternative host plants to the one from which they were collected, even when that was a *Cullen* species, and multiple host use by individuals was not infrequent.

Green mirids are found, sometimes in large numbers, in arid parts of central Australia, and Miles (1995) suggested that this might be the main source of the mirids invading cotton and other crops in sub coastal eastern Australia. Green mirid abundance is seasonally inverse between the central arid regions and eastern cropping areas, and they likely experience different selective pressures in each region. The population genetic consequences of these dynamics were assessed by sequencing a mitochondrial COI fragment from individuals collected over 24 years, and screening microsatellite variation for 32 populations across two

seasons. A single COI haplotype predominated in samples from 2006/2007, but in the older collections (1983 and 1993) a different haplotype was most prevalent. This is consistent with successive population contractions and expansions, likely in response to alternate periods of drought and flood in the arid interior of Australia. The microsatellite data showed genetic differentiation between populations, evidence for movement between sites, and also genetic signatures of bottleneck events. The Simpson Desert, in central Australia was identified as a source of recent immigrants to populations in Biloela (m = 0.15, BAYESASS), eastern Australia, supporting the view that long distance migration is, indeed, a regular part of the ecology of this species. Together, these data highlight that since the advent of agriculture in Australia, green mirid dynamics are still shaped by its adaptations to arid, spatiotemporally variable environments.

Previous ecological studies presented evidence that *C. dilutus* may be a complex of cryptic species, and that two such cryptic species may be associated with cotton and lucerne crop hosts. Further, *C. dilutus* has a reported preference for lucerne over cotton, leading to the proposal of lucerne as a trap crop in cotton production systems. To test this proposition I sampled *C. dilutus* individuals from adjacent cotton and lucerne crops at three geographically separate sites within a single season. Individual-based clustering analyses using microsatellite data showed that gene flow was high across these crop hosts. Further, gut content analysis indicated that a relatively high proportion of individuals collected from one crop host had fed on the alternate host and several individuals had fed on both. These data support the presence of one species associated with cotton and lucerne, but also show that green mirids will readily move between these two hosts despite their relative preference for lucerne.

The findings outlined above are discussed in relation to ecological perceptions of generalist habits, the application of genetic techniques to the solution of ecological problems involving multiple host use, and the management and research implications arising from the data presented in this thesis.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer Reviewed Papers

Coleman RJ, Hereward JP, De Barro PJ, Frohlich DJ, Adamczyk JJ, Goolsby JA. (2008) Molecular comparison of *Creontiades* plant bugs from south Texas and Australia. *Southwestern Entomologist* **33**, 111-117.

Characterisation of 12 polymorphic microsatellites in the green mirid, *Creontiades dilutus* Stål (Hemiptera: Miridae) Authors: James P. Hereward, Michael G. Gardner, Cynthia Riginos, Paul J. DeBarro, Andrew J. Lowe. Published as on-line PDF http://tomato.biol.trinity.edu/manuscripts/10-6/mer-10-0251.pdf with summary published in:

Andris M, Aradottir GI, Arnau G, *et al.* (2010) Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2010-31 July 2010. *Molecular Ecology Resources* **10**, 1106-1108.

Hereward JP, Walter GH. (2012) Molecular interrogation of the feeding behaviour of field captured individual insects for interpretation of multiple host plant use. *PLoS One* **7**, e44435.

The following two papers were produced while I was under part time candidature and were the result of paid work, the subject of each was relevant to my research, and their production contributed to the methods used in this thesis.

Ridley AW, Hereward JP, Daglish GJ, Raghu S, Collins PJ, Walter GH. (2011) The spatiotemporal dynamics of *Tribolium castaneum* (Herbst): adult flight and gene flow. *Molecular Ecology* **20**, 1635-1646.

Carter KD, Seddon JM, Carter JK, Goldizen AW, Hereward JP (2012) Development of 11 microsatellite markers for *Giraffa camelopardalis* through 454 pyrosequencing, with primer options for an additional 458 microsatellites. *Conservation Genetics Resources* **4**, 943-945.

Publications included in this thesis

Chapter 2.1

Characterisation of 12 polymorphic microsatellites in the green mirid, *Creontiades dilutus* Stål (Hemiptera: Miridae) Authors: James P. Hereward, Michael G. Gardner, Cynthia Riginos, Paul J. DeBarro, Andrew J. Lowe. Published as on-line PDF http://tomato.biol.trinity.edu/manuscripts/10-6/mer-10-0251.pdf with summary published in:
Andris M, Aradottir GI, Arnau G, *et al.* (2010) Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2010-31 July 2010. *Molecular Ecology Resources* **10**, 1106-1108. – incorporated as appendix 2.

Contributor	Statement of contribution
Hereward J	Designed experiments (70%)
	Wrote the paper (90%)
Gardner M	Designed experiments (30%)
	Wrote the paper (10%)
Riginos C	Edited manuscript
De Barro P	
Lowe A	

Chapter 3.

Hereward JP, Walter GH. (2012) Molecular interrogation of the feeding behaviour of field captured individual insects for interpretation of multiple host plant use. *PLoS One* **7**, e44435.

Contributor	Statement of contribution
Hereward, JP	Designed experiments (90%)
	Wrote the paper (90%)
Walter, GH	Designed experiments (10%)
	Wrote the paper (10%)

Appendix 1.1

The following publication is reproduced in this thesis as an appendix, it was an early collaboration with colleagues in the USDA (United States Department of Agriculture). My role was to generate and edit the sequence data for the specimens. This is included as an appendix because it is relevant to Chapter two of the thesis, and some of the data arising from this paper is re-used in different analyses as part of chapter two.

Coleman RJ, Hereward JP, De Barro PJ, Frohlich DJ, Adamczyk JJ, Goolsby JA. (2008) Molecular comparison of Creontiades plant bugs from south Texas and Australia. *Southwestern Entomologist* **33**, 111-117. - Incorporated as Appendix 1.

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Coleman R	Designed experiments (40%)
	Wrote the paper (70%)
Hereward J	Designed experiments (40%)
	Wrote the paper (10%)
De Barro P	Designed experiments (10%)
	Wrote and edited paper (5%)
Frolich D	Conducted phylogenetic analyses
Additional Authors	Collected samples, edited drafts, designed experiments

Contributions by others to the thesis

Gimme Walter and Cynthia Riginos made significant contributions to the conception and design of the project, and provided advice and guidance regarding analyses throughout. Andrew Lowe and Paul DeBarro also contributed in this regard, particularly in the initial stages of the project. S. Raghu assisted with the statistical analyses reported in Chapter three.

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None

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"Good luck with your PhD, I imagine it will be quite different to when I did mine, in London during the Blitz".

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Mirid, microsatellite, CO1, generalist, polyphagy, secondary pest, *Creontiades dilutus*.

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ANZSRC code: 060808 Invertebrate Biology, 15%

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Please allocate the thesis a **maximum of 3** Fields of Research (FoR) Codes at the **4 digit level** and include the descriptor and a percent weighting for each code. Total percent must add to 100.
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FoR code: 0604, Genetics, 60%

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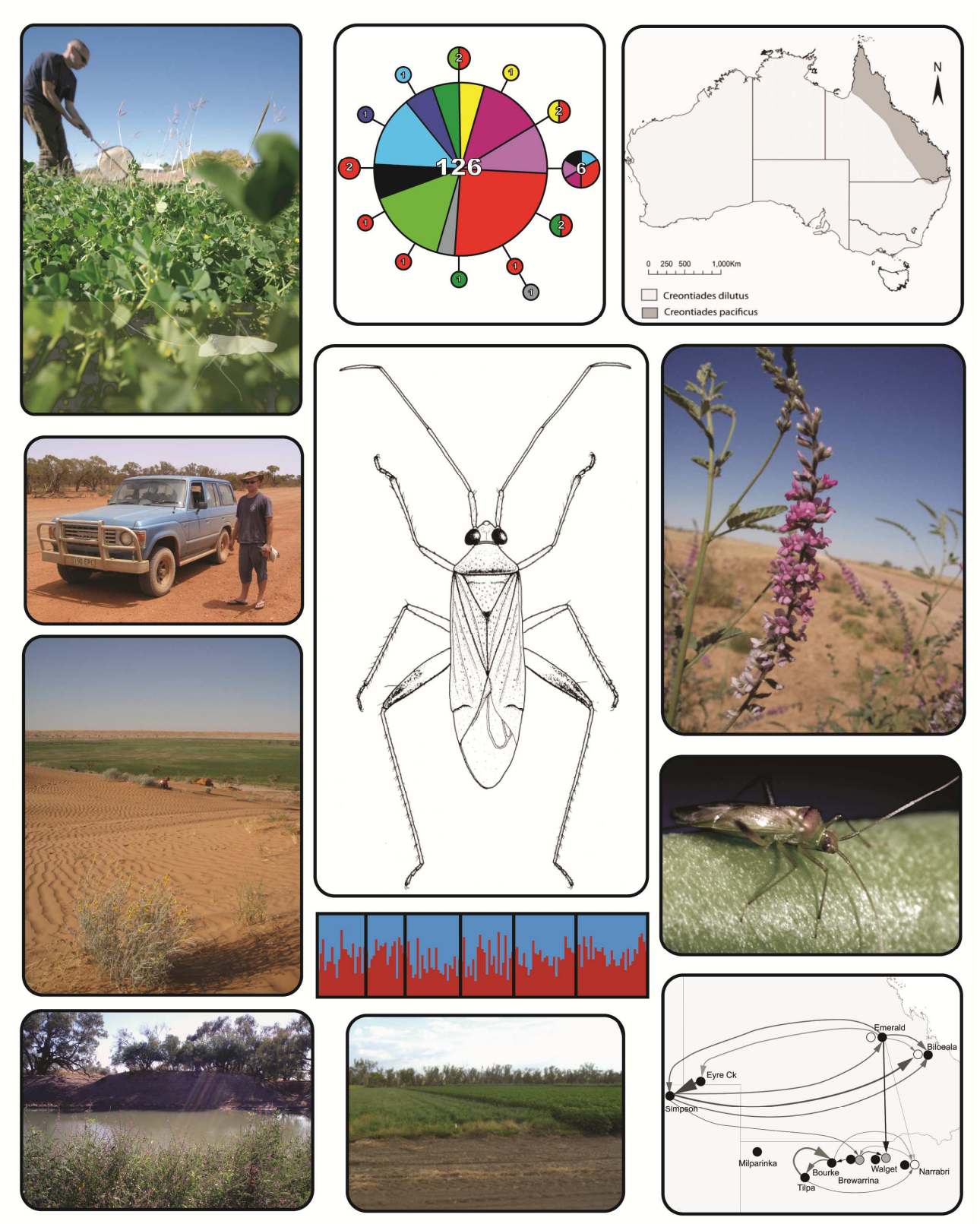

Fronticepiece. Centre: *Creontiades dilutus*. Clockwise from top left; Sampling mirids, *C. dilutus* COI haplotype network, Australian distribution of *C. dilutus* and *C. pacificus*, *Cullen australasicum*, *C. dilutus* on green bean, inference of recent migration using BAYESASS, adjacent cotton and lucerne (and lack of genetic differentiation (above)), *Cullen cinereum* on river bank, arid Australia, fieldwork.

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List of Tables

Tables are numbered according to their position within each chapter, for example Table 2.2.1 is the first table in the second section of chapter two.

List of Abbreviations used in the thesis

HWE	Hardy Weinberg Equilibrium
IBD	Isolation By Distance
IBDWS	Isolation By Distance Web Service
USA	United States of America
COI	Cytochrome Oxidase Subunit One
28S	28Sr Ribosomal RNA
DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
mtDNA	Mitochondrial DNA

Chapter One - Introduction

1.1 General background and pest status

The heteropteran family Miridae is the largest true bug family, with some 10,000 species (Schuh 1995). Although the bugs in this family are commonly referred to as plant bugs, the family spans unparalleled trophic diversity, with species that are even strictly predacious and some of the plant feeders being omnivorous. Further, the herbivorous habits range from monophagous to widely polyphagous (Wheeler 2001). The green mirid (*Creontiades dilutus*) is endemic to Australia (Malipatil & Cassis 1997) and was likely restricted to the arid interior (Fig. 1.1) prior to European settlement (before land was cleared to establish agriculture). This species is best known as a pest of cotton (McColl *et al.* 2011). Based on incidence records, however, the green mirid appears to be highly polyphagous, with 72 recorded host plants. Included in this host list are mainly introduced weeds, and several agricultural crops, such as cotton, lucerne, sorghum, soy, grapes, stone fruits, cucurbits, parsnips and potatoes (Foley & Pyke 1985; Hely *et al.* 1982; Hori & Miles 1993;

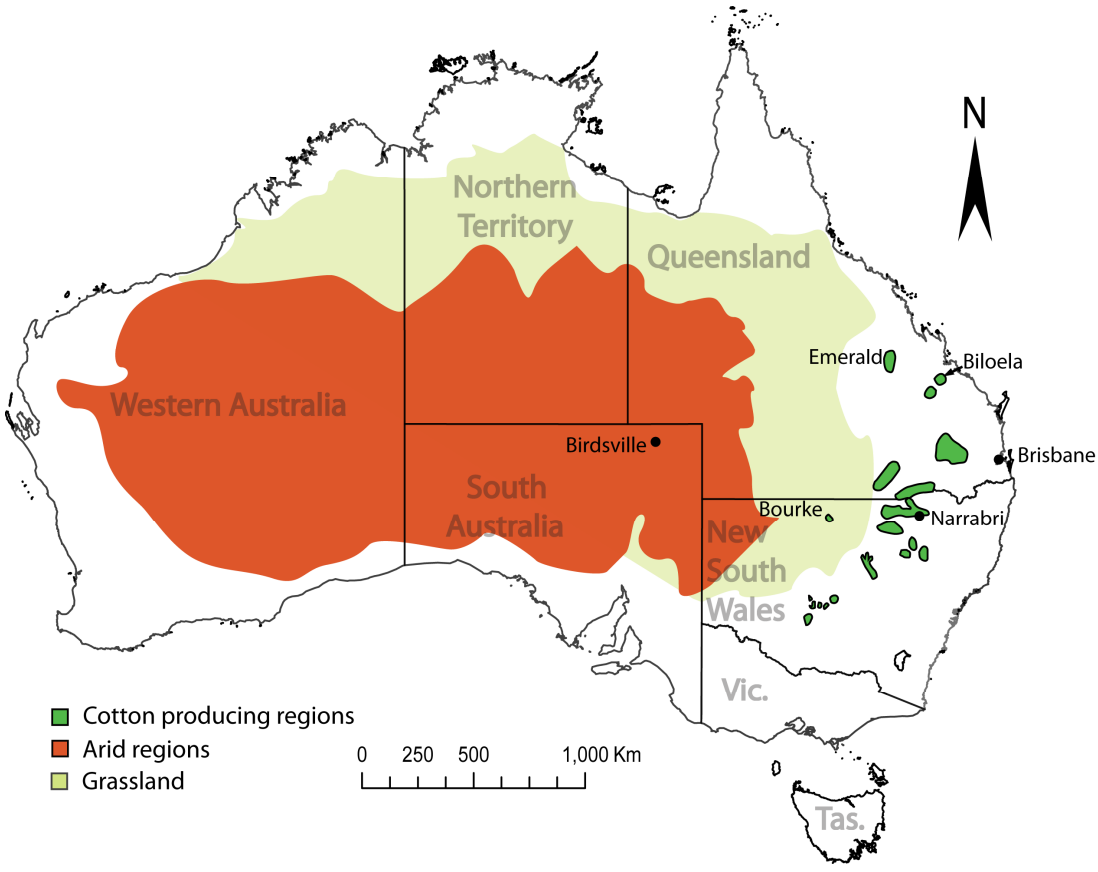


Figure 1.1 Schematic map of Australia showing the subcoastal cotton producing regions of eastern Australia, the arid zone of inland Australia and the grassland region in between (adapted from Bureau of Meteorology).

Until recently, *C. dilutus* was only considered a secondary pest of cotton, and the concern was not so much direct damage to the crop or yield reduction, but rather delays in crop maturity caused by compensatory cotton growth. The relative importance of the green mirid as a cotton pest in Australia has since increased as a direct consequence of the widespread uptake of transgenic cotton expressing Cry1Ac/Cry2Ab toxins from the soil bacterium *Bacillus thuringiensis* (Fitt *et al.* 1994). These toxins are lethal to bollworm caterpillars (*Helicoverpa armigera* and *H. puntigera*), the primary pests of Australian cotton (Fitt *et al.* 1994; Tabashnik 1997). Phytophagous mirids tend to feed using a lacerate and flush method, in which they target pockets of cells (Miles 1972). *Creontiades dilutus* feeds in this manner on the growing tips, squares and young bolls of cotton. Although these tissues express relatively high levels of Cry1Ac/Cry2Ab toxins (Sivasupramaniam *et al.* 2008). Like other heteropterans, *C. dilutus* is like other heteropterans in being unaffected by them (Torres & Ruberson 2006, 2008; Whitehouse *et al.* 2005).

Green mirids had previously been controlled incidentally by the broad-spectrum insecticides applied to control bollworm populations, but since the introduction of transgenic cotton the application of these insecticides has fallen by as much as 85% (Whitehouse 2011). Currently, almost all Australian cotton planted is transgenic and mirids have therefore become the main insect target of chemical control (Khan *et al.* 2004). Whereas progress towards the uptake of IPM approaches and techniques has been made in mirid management, “insurance sprays” are still applied when mirid densities are below threshold. Further, there is a strong reliance on fipronil (63% of managers consulted (n = 38)) raising concerns about the possibility of insecticide resistance developing in this pest (Whitehouse 2011).

The emergence of mirid pests following the uptake of transgenic cotton is not limited to Australia; this pattern has been repeated in China with *Apolygus lucorum* (Li *et al.* 2011; Lu *et al.* 2010), and in the USA with *Lygus hesperus* (Gross & Rosenheim 2011). Other species in the genus *Creontiades* have also been noted as emerging pests of transgenic cotton, for example *C. biseratense* in India (Rohini *et al.* 2009; Udikeri *et al.* 2010), *C. pallidus* in the Middle East (Hosseini *et al.* 2002; Stam 1987), and *C. signatus* in the USA (Armstrong *et al.* 2009). The latter was rumoured to be the Australian green mirid *C. dilutus* prior to the start of this thesis (a situation dealt with in Chapter two).

1.2 Previous research - morphological species identification and economic damage

Relatively little has been published on *C. dilutus*, although three PhD's have been produced on green mirid ecology and economic damage. The focus on integrated pest management grew in the late 1970s and 1980s following the development of resistance in *H. armigera*, initially to DDT and then to synthetic pyrethroids. This led to consideration of other pests as research priorities (including those controlled incidentally). At this time green mirids were simply included in the so-called "sap-sucking bug complex", which included five species of Heteroptera. They were dealt with as a single pest entity for which a single economic threshold was set (Chinajariyawong 1988). This broad classification not only hid the fact that little was known in general about the constituent species, but also that the exact mode of feeding and the diet of these species was not clear. Even the role of green mirids as cotton pests was obscure. Two of these bugs were subsequently demonstrated to be predatory (Chinajariyawong *et al.* 1989) and two could not reproduce on cotton and caused no damage to this crop (Chinajariyawong & Harris 1987; Chinajariyawong & Walter 1990). Further, the one species that did have an economic impact on cotton, green mirid, was clearly demonstrated to be two species, based on morphology alone (Chinajariyawong 1988). Miles (1995) further clarified the taxonomic distinction of these two mirids. Clear morphological differences were found in the juveniles, adults and eggs. The first species was recognised as *C. dilutus.* The second as *C. pallidifer*, later synonymised as *C. pacificus* (the brown mirid) by (Malipatil & Cassis 1997). Inspection of the eggs revealed that those of *C. dilutus* have a short respiratory horn on the operculum in contrast to the long respiratory horn of *C. pacificus*, perhaps because *C. dilutus* is adapted to a low rainfall environment.

Cage and field trials established that feeding damage from *C. dilutus* caused little yield loss, but that their feeding on the growing tips of seedling cotton and on young bols causing shedding and subsequent compensatory growth, this delayed crop maturity by about seven days (Chinajariyawong 1988). Khan (1999) calculated the economic injury level of *C. dilutus* based on the maturity delay observed following experimental manipulation of field populations, and proposed a threshold at a rate of 1 mirid per metre of row.

1.3 Host plant relationships

A host record list was initiated by (Chinajariyawong 1988) and comprised principally crop plants and their associated weeds, mostly introduced species. (Miles 1995) extended this substantially with an inland survey of green mirids. Nevertheless, relatively few native plant species feature on the host plant lists available, with only 15 out of 72 potential hosts regarded as native to Australia, and only seven of these having juvenile mirids recorded (Table 1.1). It is also evident from Table 1.1 that many of the host plants did not have juveniles present at the time of sampling. *Creontiades dilutus* is nevertheless regarded as a generalist in its use of resources. The majority of these records were generated in studies around the eastern cropping regions of Australia. Given that green mirid is an indigenous species, the host plant relationships outside of agriculture clearly require further investigation.

Table 1.1 Host records for green mirids available in the literature, Status: C = Crop, I = Introduced, N = Native, whether juveniles have been recorded, and Reference: 1 = Chinajariyawong 1987, 2 = Miles 1995, 3 = Malipatil and Cassis 1997, 4 = Khan 1999.

Family	Species	Common Name	Status	Juveniles	Reference
Molluginaceae	*Glinus lotoides*	Hairy carpet weed	I	Y	4
Aizoaceae	*Tetragonia tetragonoides*	New Zealand spinach	N	N	4
	Trianthema portulacastrum	Desert horse purslane	I	Y	1
Amaranthaceae	*Alternanthera nodiflora*	Common joyweed	I	Y	4
Apiaceae	*Trachymene glaucifolia*	Blue parsnip	N	Y	2
Asteraceae	*Calotis multicaulis*	Burr daisy	N	N	2
	Flaveria australasica	Speedy weed	N	N	2
	Helianthus annuus	Sunflower	C	Y	1, 3, 4
	Ixiolaena chloroleuca	Ixiolaena	N	N	2
	Rhodanthe floribunda	White pepper daisy	N	Y	2
	Senecio glossanthus	Slender groundsel	N	N	2
	Silybum marianum	Variegated thistle	I	Y	4
	Verbesina encelioides	Wild sunflower	I	Y	4, 2
	Xanthium occidentale	Noogoora burr	I	N	4
Boraginaceae	*Echium plantagineum*	Paterson's curse	I	NR	3
Brassicaceae	*Rapistrum rugosum*	Wild turnip	I	Y	4, 2
	Sisymbrium thellungii	African turnip weed	I	N	1
Cactaceae	*Aporocactus flagelliformis*	N/A	I	NR	3
Chenopodiaceae	*Salsola kali*	Salwort	I	N	1
Compositae	*Carthamus tinctorius*	Safflower	C	Y	4
	Gnaphalium luteo-album	Jersey cudweed	N	N	1
	Sonchus oleraceus	Common sow-thistle	I	N	1
Cucurbitaceae	*Citrullus vulgaris*	Melon	C	NR	3
	Cucumis sativus	Cucumber	C	NR	3
Fabaceae	*Cajanus cajan*	Pigeon pea	C	Y	1, 3
	Crotalaria sp.	Rattlepod	N	Y	2, 3
	Cullen cinereum	Annual verbine	N	Y	2
	Glycine max	Soy bean	C	Y	1
	Indigofera hirsute	Hairy indigo	N	Y	2
	Lupinus sp.	Lupine	I	Y	4, 3
	Macroptilium atropurpureum	Siratro	I	N	2
	Medicago polymorpha	Burr medic	I	Y	1
	Medicago sativa	Lucerne	C	Y	1, 2, 3, 4
	Melilotus indicus	Hexham scent	I	Y	1, 2
	Phaseolus vulgaris	Green bean	C	Y	4, 3
	Pisum sativum	Pea	C	NR	3

Continued overleaf

Table 1.1 (Continued)

Family	Species	Common Name	Status	Juveniles	Reference
	Rhynchosia minima	Rhyncosia	N	Y	2, 3
	Sesbania cannabina	Sesbania	N	Y	2, 4
	Vigna radiata	Mung bean	C	Y	1, 2, 3, 4
	Vigna unguiculata	Cowpea	C	NR	3
Goodeniaceae	*Goodenia heterophylla*	Variable-leaved Goodenia	N	N	2
Gramineae	*Avena sativa*	Oats	C	NR	3
	Dactylis glomerata	Cocksfoot	I	NR	3
	Echinochloa crus-galli	Barnyard grass	I	N	1
	Ehrharta erecta	Panic veldt grass	I	N	1
	Paspalum dialatum	Paspalum	I	N	1
	Sorghum bicolour	Sorghum	C	N	1
	Triticum aestivum	Wheat	C	NR	3
Haloragaceae	*Haloragis glauca*	Glauca		Y	4
Liliaceae	*Asparagus officinalis*	Asparagus	C	NR	3
Malvaceae	*Gossypium hirsutum*	Cotton	C	Y	1, 2, 3, 4
	Malva parviflora	Marshmallow	I	N	3, 4
	Melaleuca spp.	Teatree	N	NR	3
Polygonaceae	*Rheum rhabarbarum*	Rhubarb	C	NR	3
Rosaceae	*Malus pumila*	Apple	C	NR	3
	Prunus persica	Peach	C	NR	3
	Pyrus communis	Pear	C	NR	3
	Rosa sp.	Rose	C	NR	3
	Rubus idaeus	Raspberry	C	NR	3
Rutaceae	*Citrus limon*	Lemon	C	NR	3
	Citrus sinensis	Orange	C	NR	3
Solanaceae	*Datura inoxia*	Thornapple	I	N	4
	Lycopersicon esculenum	Tomato	C	NR	3
	Solanum nigrum	Black berry nightshade	I	Y	4
	Solanum tuberosum	Potato	C	NR	3
Umbellifereae	*Coriandrum sativum*	Coriander	C	N	4
	Umbellifereae			NR	3
Verbenaceae	*Verbena litoralis*		I	Y	1, 2
	Verbena supina	Trailing verbena	I	Y	4
	Verbena tenuisecta	Mayne's pest	I	Y	1, 2, 3
Vitaceae	*Vitis vinifera*	Grape	C	NR	3
Zygophyllaceae	*Tribulus terrestris*	Caltrop	I	Y	1, 2

1.4 Source of mirids invading cotton

Through field surveys and interviews with crop consultants (Miles 1995), it became evident that mirids tend to arrive in early season cotton in a sharp influx. Miles considered three aspects of green mirid ecology that may influence movement into cotton, namely diapause, host plant relationships and long distance migration. In lucerne there was evidence that pre-mated females enter reproductive diapause as an overwintering strategy, but lucerne did not appear to be the source of mirids invading cotton because densities in lucerne did not decrease when the influx to cotton occurred. Populations with high densities of individuals per plant sampled were found during spring in the inland arid zone of Australia (Fig. 1.1), when cotton is invaded. Potentially high densities were noted on *Cullen cinereum* (Fabaceae). Miles hypothesised that green mirids might invade cotton through long distance migration from these inland sources. The areas of grassland that separate the subcoastal cropping regions and the arid interior support few of the recorded hosts for *C. dilutus* (Fig. 1.1). Some suitable host plants can be found along road verges in these regions, but rarely enough to support high densities so it is likely that long distance dispersal would be the only mechanism for this source of *C. dilutus* to invade cotton. Miles also conducted an electrophoretic study of gene flow in green mirids using allozyme electrophoresis, and the results of this analysis indicated gene flow (low differentiation) between cotton and lucerne, and some degree of genetic differentiation ($F_{ST} > 0.1$) between western Queensland and more eastern sites, but these studies were not conclusive as not all allozyme loci could be successfully screened against all individuals.

Khan (1999) documented the life cycle of green mirids under laboratory conditions, showing that green mirids complete their life cycle from egg to egg laying in under 25 days and each female lays around 40 eggs, with fecundity and growth highest at 30^{o}C. Based on field surveys of overwintering hosts in Narrabri (northern New South Wales), Khan (1999) suggested that mirids invading cotton most likely originate from numbers building up on such overwintering hosts in early spring. This contrasted strongly with Miles' (1995) hypothesis that inland populations of green mirids are the likely source of mirids invading cotton in Biloela (central Queensland). If inland and coastal populations of mirids are indeed isolated from one another, with little movement between the two, the localised populations in cropping regions might be expected to be more likely to develop resistance due to the strong selective pressure from insecticides applied to control them.

Resistance concerns led Mensah & Khan (1997) to suggest that lucerne might be used as a trap crop for green mirids, when interplanted into cotton fields, or cultivated along the edges of fields. This development was based on their finding that green mirids show a preference for lucerne over cotton

in cage trials, this result was consistent with Miles' (1995) demonstration that lucerne was not the source of mirids invading cotton. Their field trials indicated, however, that these bugs do not appear to move into cotton when lucerne is mowed in the interplanted cotton. This pattern of host plant use in the field does not appear *prima facie* to be consistent with the presence of a single species' gene pool across both host plants. Despite the reported preference for lucerne, green mirids would be expected to move into cotton when lucerne was mowed in Mensah and Khan's (1997) trials if they were a single species that is able to use both hosts.

All researchers that have worked on green mirids report that they are not easy organisms to work with. Establishing the number of these bugs within a crop can be difficult, as they tend to aggregate in patches within a field (JPH *pers. obs.*). This is further complicated by the "flighty" behaviour of green mirids; they are easily disturbed, and move with considerable speed. Even the wingless nymphs move rapidly and evade capture easily. Attempts to maintain laboratory cultures for more than one generation have been relatively unsuccessful, and even keeping field collected bugs alive on the way back to the laboratory is problematic (M. Khan and A. Quade, Queensland Dept. of Agriculture Forestry and Fisheries, *pers. comm.*).

1.5 Research Questions

The material reviewed above suggests that specific information is required to answer the following questions relating to multiple host plant use by green mirids and the source(s) of mirids that invade cotton.

1. What are the host plant relationships of green mirids in central Australia?
2. Do these bugs undertake long distance dispersal between central Australia and coastal cropping regions?
3. Can the apparent difference in the use of cotton and lucerne by green mirids be explained by the presence of host associated cryptic species?

1.6 Approach

My approach to answering the questions above has been to use molecular ecology techniques within an autecological approach, keeping a strong focus on the interaction between the organism and its environment. The difficulty of rearing mirids in the laboratory for more than one generation, and the distances that they might cover in long distance migration mean that some of these problems

would have been intractable using standard ecological approaches. Further, the flighty nature of these bugs makes it difficult to determine whether they have indeed been feeding on a plant on which they are found.

Although the need for population genetics research was highlighted in a recent review of green mirids (McColl *et al.* 2011) it was only done so in the context of panmixia promoting the spread of resistance (Endersby *et al.* 2006). It is clear, however, that the interaction between gene flow and the spread of resistance is more complex than this (Caprio & Tabashnik 1992). Instead, in this thesis I use molecular techniques to answer a series of structured questions regarding the biology, ecology and genetic relationships of green mirids, as outlined below.

1.7 Thesis outline

This thesis is presented in the form of one preliminary genetic data chapter (Chapter two) and three inter-related papers intended for publication in peer-reviewed journals (one published and two submitted for review) as outlined below. These papers are presented largely unaltered from their manuscript format; as a consequence some degree of repetition exists among chapters, particularly within the introduction sections. References, however, have been combined into a single list at the end of the thesis.

Chapter two investigates the genetic relationships between green and brown mirids, and the *Creontiades* species that was found in the USA and reported to be *C. dilutus*, were investigated using mitochondrial and nuclear genes. Based on the low diversity observed in *C. dilutus* at the CO1 mitochondrial locus it was evident that a different molecular approach would be required to assess gene flow in this species. The rest of this chapter describes the work undertaken to construct a microsatellite library by enrichment (as this was conducted prior to the commercial availability of high throughput sequencing technology). This work was published as a primer note in Molecular Ecology Resources (Andris *et al* 2010).

The third chapter addresses question one (the host plant relationships of green mirids) by developing an approach that combines structured and quantified field sampling with an analysis of chloroplast intron markers. These plant markers were amplified from whole insect DNA extractions of green mirids to infer the recent feeding behaviour of this polyphagous insect under field conditions. This combination of ecological sampling and molecular diet analysis provided further insight to the use of multiple hosts by green mirids than either single approach would have.

I addressed question two (long distance dispersal) in chapter four, by screening green mirids from a wide geographic area (several thousand kilometres) and host plant range using the microsatellite markers that developed in chapter two to assess gene flow and genetic differentiation across green mirid populations sampled from the arid inland and the eastern cropping regions of Australia. I also investigated the temporal stability of haplotype frequencies through the analyses of CO1 haplotypes from samples spanning three decades.

The third question (whether there are cryptic species associated with two crop hosts) was investigated in chapter five. I tackled this question with a combined analysis of gene flow (using microsatellite genotypes) and recent feeding behaviour (and hence local movement among host species) with chloroplast intron markers. These analyses were conducted across adjacent cotton and lucerne plots at three geographically distant sites (up to 900km).

Taking the approach outlined above has extended insight into the biology and ecology of green mirids. Management options and future research can thus be set accordingly, and this is covered in chapter six, the general discussion. Current perceptions of generalist habits in the ecology of phytophagous insects are also discussed, and a conceptual and methodological framework for investigating multiple host plant use by herbivorous insects is presented and fully justified in this chapter.

Chapter 2: Preliminary genetic work – gene sequencing and microsatellite development.

2.1 Phylogenetic relationships between three species of *Creontiades*

2.1.1 Introduction

Green mirids (*Creontiades dilutus*) are endemic to Australia and have been recorded from across the continent, including the arid regions. In contrast, the brown mirid, *C. pacificus* has been recorded from China, several other countries in the Oriental region, and various islands in the south-west Pacific (Malipatil & Cassis 1997). Records of *C. pacificus* within Australia indicate that it is restricted to sub coastal areas of eastern Australia, where annual rainfall is much higher (Fig. 2.1). It should be noted that, despite the lack of formal museum records, *C. pacificus* is present in northern Australian cropping regions where rainfall is also high, for example Katherine (Northern Territory) (Ward 2005). It was not present, however, in any of the extensive surveys of the arid regions that I conducted during this thesis (Chapters 3 & 4). This distribution appears to support Miles' (1995) hypothesis that *C. dilutus* eggs appear to be adapted to dry conditions and *C. pacificus* to wetter environments.

In 2006 an emergent mirid pest was noticed in cotton in Texas, USA, it had been assigned to *C. dilutus* by a taxonomist and rumours were circulating that somehow *C. dilutus* had managed to invade American cotton from Australia. Through the sequencing and analysis of COI data I helped to establish that this was not the case, the COI fragments of the American mirid were 10% different to those from *C. dilutus*, indicating significant divergence between the two. The Texan samples were also represented by nine unique haplotypes (out of 13 individuals), a pattern not consistent with a recent introduction (Coleman *et al.* 2006, reproduced as Appendix 1). Further taxonomic investigation of the Texan samples confirmed that it was *Creontiades signatus*, a native of the Americas.

The two Australian *Creontiades* species are well separated morphologically, in particular by the egg opercula (Miles 1995), but within each there can be high variability in colour, especially in *C. pacificus* (Malipatil & Cassis 1997). In the first section of this chapter I reanalyse the data presented in Coleman *et al.* (2006) with additional CO1 and 28S sequences from *C. dilutus* and *C. pacificus* from Australia to further confirm the phylogenetic relationships between these species. In the second, I describe work undertaken to develop a set of microsatellite markers for *C. dilutus*.

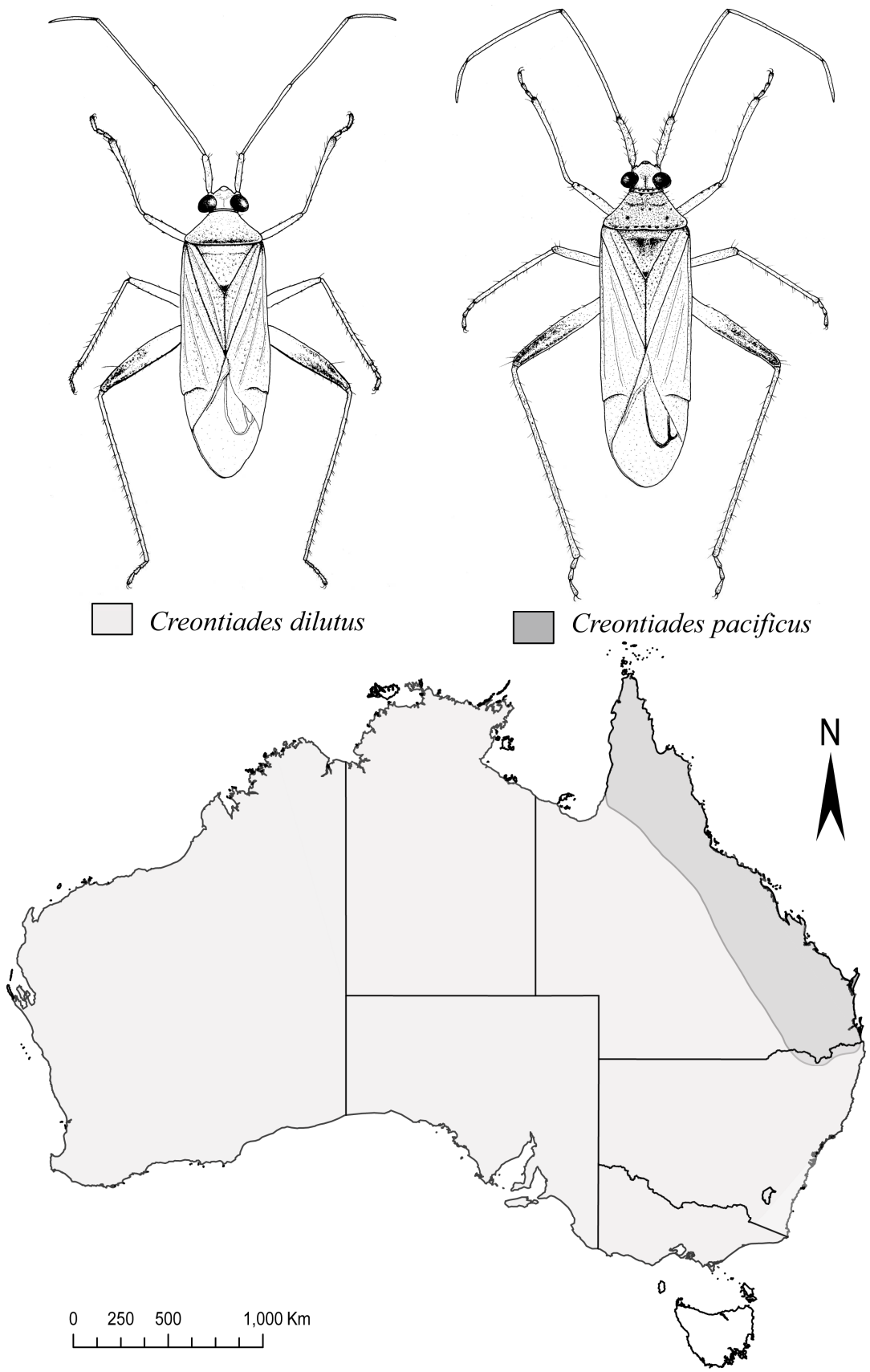


Figure 2.1 Above - Morphology of green mirid, *C. dilutus* (left) and brown mirid, *C. pacificus* (right) reproduced with permission from Malipatil and Cassis (1997). Below - Map showing the distribution of green and brown mirids (adapted from data in Malipatil and Cassis (1997) and online databases).

2.1.2 Materials and methods

The sequences for *C. dilutus* and *C. pacificus* used in the analyses presented in this chapter include the samples of Coleman *et al.* (2006) (Appendix A1), and additional samples of each species (Table 2.1.1). The Texan material (*C. signatus*) is reanalysed but no additional samples have been added (see Appendix A1 for collection data).

DNA was extracted using a modified salt precipitation protocol based on that of Miller *et al.* (1988). A fragment of the mitochondrial Cytochrome Oxidase 1 (COI) was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994). PCR was performed using Mango Taq (Bioline), 0.2 µM of each primer, and 2.5 mM of MgCl. PCR cycling conditions were: initial denaturation at 95ºC for 10min followed by 35 cycles of 95ºC denaturation (30s) 50ºC annealing (30s), and 72ºC elongation (45s). The D2–D3 region of the nuclear large-subunit ribosomal RNA gene (28S) was amplified using primers S3660 (28SF, Dowton & Austin, 1998) and A335 (28Sb, Whiting *et al.* 1997). PCR conditions were similar to those described for CO1, except that the annealing temperature was 52ºC. Amplicons for both CO1 and 28S samples were sequenced bidirectionally on an ABI 3730 (Macrogen). Sequences were edited using CodonCode Aligner.

Sequences were aligned using the program Geneious (Drummond et al 2010), and outgroups obtained from GenBank using the *blastn* algorithm to search the nucleotide (nr/nt) database. After trimming the alignements, the length of the CO1 fragment used for phylogenetic analysis was 565bp (Fig. 2.4). The 28S fragment used in the analysis that included a single outgroup was 706bp (Fig 2.2). This 28S phylogeny did not provide resolution of the *C. signatus* and *C. pacificus* clades (Fig. 2.2). The addition of more closely related outgroups resolved this relationship, but there was a relatively small region of overlap between the sequences generated in this study and those available on GenBank, so this analysis used 271bp of sequence (Fig. 2.3). Neighbour joining phylogenetic trees were constructed using the HKY genetic distance model in Geneious, with 1,000 bootstrap replicates. Haplotype networks were constructed for *C. dilutus* and *C. pacificus* using the R package TempNet (Prost & Anderson 2012), and nucleotide diversity (Pi) calculated in DNAsp v. 5 (Librado & Rozas 2009).

Table 2.1.1 Collection data for the *C. dilutus* and *C. pacificus* samples used in the construction of the haplotype network, and the phylogenetic trees presented in this chapter.

Location	Date	Lat. (S)	Long. (E)	Host plant	N.	Genbank Acessions
Creontiades dilutus						
Adelaide	2/12/2006	-34.82081	138.86996	*Polygonum convolvulus*	8	JX186015 to JX186022
Balingup	14/09/2007	-33.78890	115.97597	*Solanum nigrum*	8	JX186023 to JX186030
BarcLong	16/08/2006	-23.53322	145.07654	*Cullen cinereum*	8	JX186031 to JX186038
Biloela	10/01/2007	-24.37389	150.51298	*Gossypium hirsutum*	10	JX186039 to JX186048
Biloela	10/01/2007	-24.37389	150.51298	*Medicago sativa*	8	JX186049 to JX186056
Emerald	14/08/2006	-23.49576	148.18842	*Verbesina enceliodes*	8	JX186057 to JX186064
Emerald	15/08/2006	-23.57219	148.10006	*Verbesina enceliodes*	4	JX186065 to JX186068
Emerald	15/08/2006	-23.46627	148.09175	*Vicia sativa*	8	JX186069 to JX186076
Kununurra	28/08/2006	-15.64590	128.69688	*Gossypium hirsutum*	5	JX186077 to JX186081
Longreach	17/08/2006	-23.41773	144.22744	*Cullen cinereum*	8	JX186082 to JX186089
Longreach	17/08/2006	-23.40377	144.22121	*Cullen cinereum*	8	JX186090 to JX186097
Longreach	17/08/2006	-23.43817	144.24575	*Medicago polymorpha*	8	JX186098 to JX186105
Longreach	17/08/2006	-22.89413	143.78673	*Swainsona galegifolia*	8	JX186106 to JX186113
Narrabri	22/01/2007	-30.20075	149.57236	*Gossypium hirsutum*	6	JX186114 to JX186119
Narrabri	22/01/2007	-30.20075	149.57236	*Medicago sativa*	7	JX186120 to JX186126
Walget	31/08/2006	-29.91241	146.91791	*Rapistrum rugosum*	8	JX186127 to JX186134
WintJun	18/08/2006	-22.41200	143.05851	*Cullen cinereum*	7	JX186135 to JX186141
WintJun	19/08/2006	-23.78104	142.46578	*Cullen cinereum*	4	JX186142 to JX186145
WintJun	19/08/2006	-23.73375	142.42869	*Senna Artemisioides*	5	JX186146 to JX186150
Byee	14/03/2006	-26.25660	151.85388	*Cajanus cajan*	10	EF016724 to EF016733
Total number of samples					**146**	
Creontiades pacificus						
Balingup	14/09/2007	-33.78890	115.97597	*Solanum nigrum*	8	N/A
Byee	14/03/2006	-26.25660	151.85388	*Cajanus cajan*	18	N/A
Byee	14/03/2006	-26.32222	152.06833	*Medicago sativa*	7	N/A
Biloela	10/01/2007	-24.37389	150.51298	*Gossypium hirsutum*	3	N/A
Biloela	10/01/2007	-24.37389	150.51298	*Medicago sativa*	2	N/A
Emerald	14/08/2006	-23.49576	148.18842	*Medicago sativa*	14	N/A
Brookstead	28/02/2006	-27.73134	151.47476	*Sorghum bicolor*	21	N/A
Kingaroy	14/03/2006	-26.80250	151.97694	*Glycine max*	6	N/A
Total number of samples					**79**	

2.1.3 Results and Discussion

Coleman *et al.* (2006) (Appendix; A1) established that the three *Creontiades* taxa considered in this chapter were likely to be distinct species based on the degree of divergence observed at the CO1 locus. However, the phylogenetic relationship between the three was not clear due to the use of a single locus and the lack of suitable outgroups in the analysis. The preliminary genetic work described in this chapter resolves this issue through the analysis of a fragment of the 28S gene region, and the inclusion of suitable outgroups.

Preliminary analysis of the 701bp 28S gene fragment (Fig. 2.2) did not resolve the phylogenetic relationship between the *C. signatus* and *C. pacificus* clades, as suitable outgroups could not be obtained that covered the whole fragment. Restricting the analysis to a 271bp region well represented in the Miridae sequences available on GenBank resolved this relationship (Fig. 2.3). This analysis, together with the CO1 phylogeny (Fig. 2.4), provide support for the monophyly of each of the three species and resolve *C. signatus* and *C. pacificus* as sister clades in relation to *C. dilutus*. The genus is, however, globally widespread (McColl *et al.* 2011) and further phylogenetic analysis across the whole genus would be required to further clarify this relationship.

The phylogenetic analysis of the COI fragment indicated that the majority of *C. dilutus* individuals shared the same haplotype. This was investigated in relation to the other Australian species, *C. pacificus* through the construction of haplotype networks (Fig. 2.5). Nucleotide diversity in *C. dilutus* COI sequences was indeed low (Pi = 0.00058, n = 144), as 124 out of 144 *C. dilutus* individuals were represented by a single haplotype. Conversely, *C. pacificus* had higher nucleotide diversity (Pi = 0.00261, n = 79) and a more even distribution of haplotypes (Fig. 2.5).

The difference in genetic diversity between these two species at the COI locus might be explained by the different environments they inhabit. *Creontiades dilutus* is distributed across the continent (Fig. 2.1), but is strongly associated with arid environments, whereas *C. pacificus* is only found in coastal regions. The ephemeral resources that *C. dilutus* evidently relies upon for survival in these arid regions might result in an increased probability of population bottlenecks. Conversely, the relatively more stable resources in the coastal regions that *C. pacificus* inhabits might support higher numbers of individuals over longer periods of time.

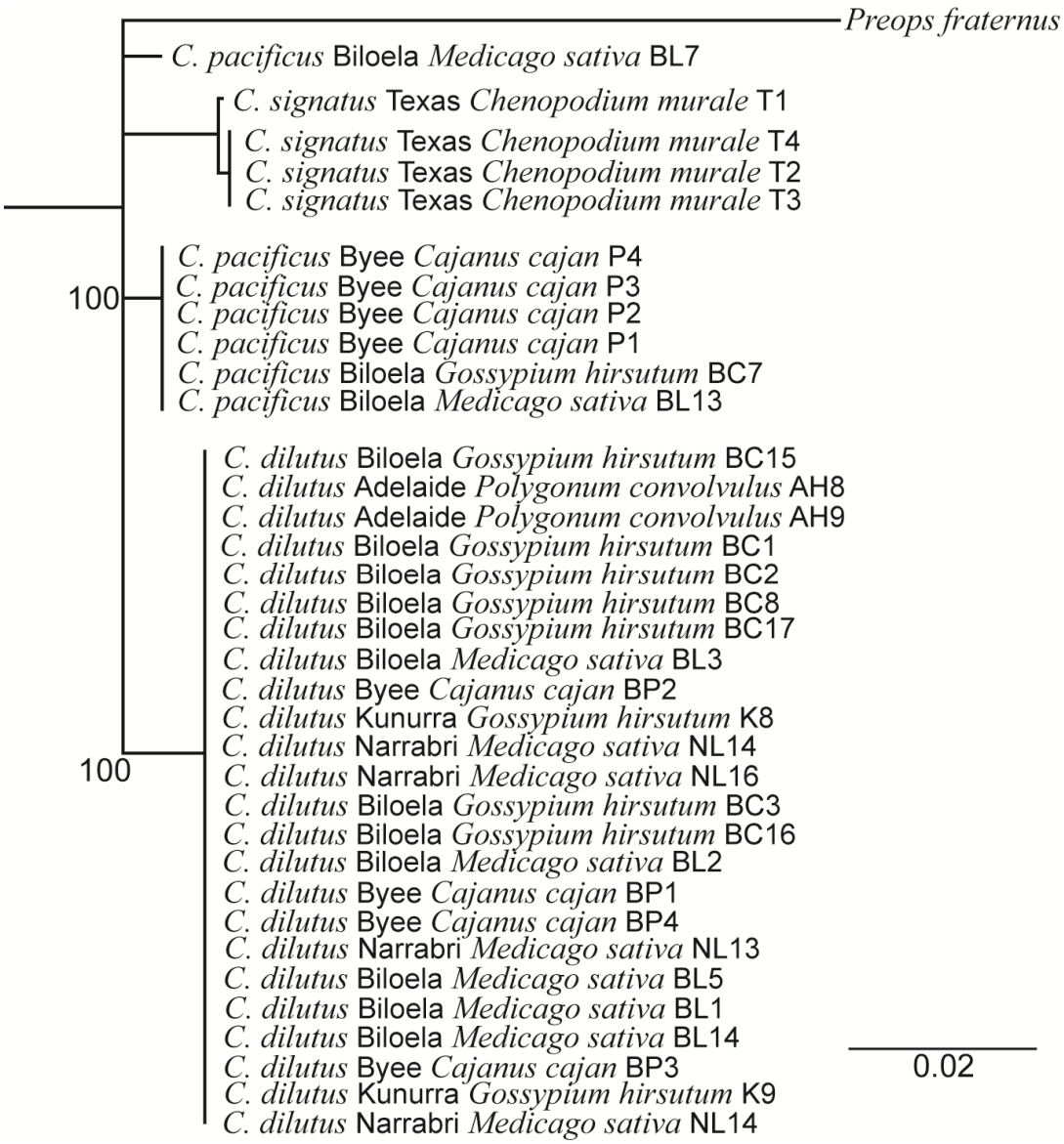


Figure 2.2 Phylogenetic neighbour joining tree showing the relationship between three *Creontiades* species as determined through the analysis of a 706bp fragment of the 28s gene region, using *Preops fraternus* (GenBank Accession: HQ676940) as an outgroup (Numbers represent the bootstap support for each clade based on 1000 replicates).

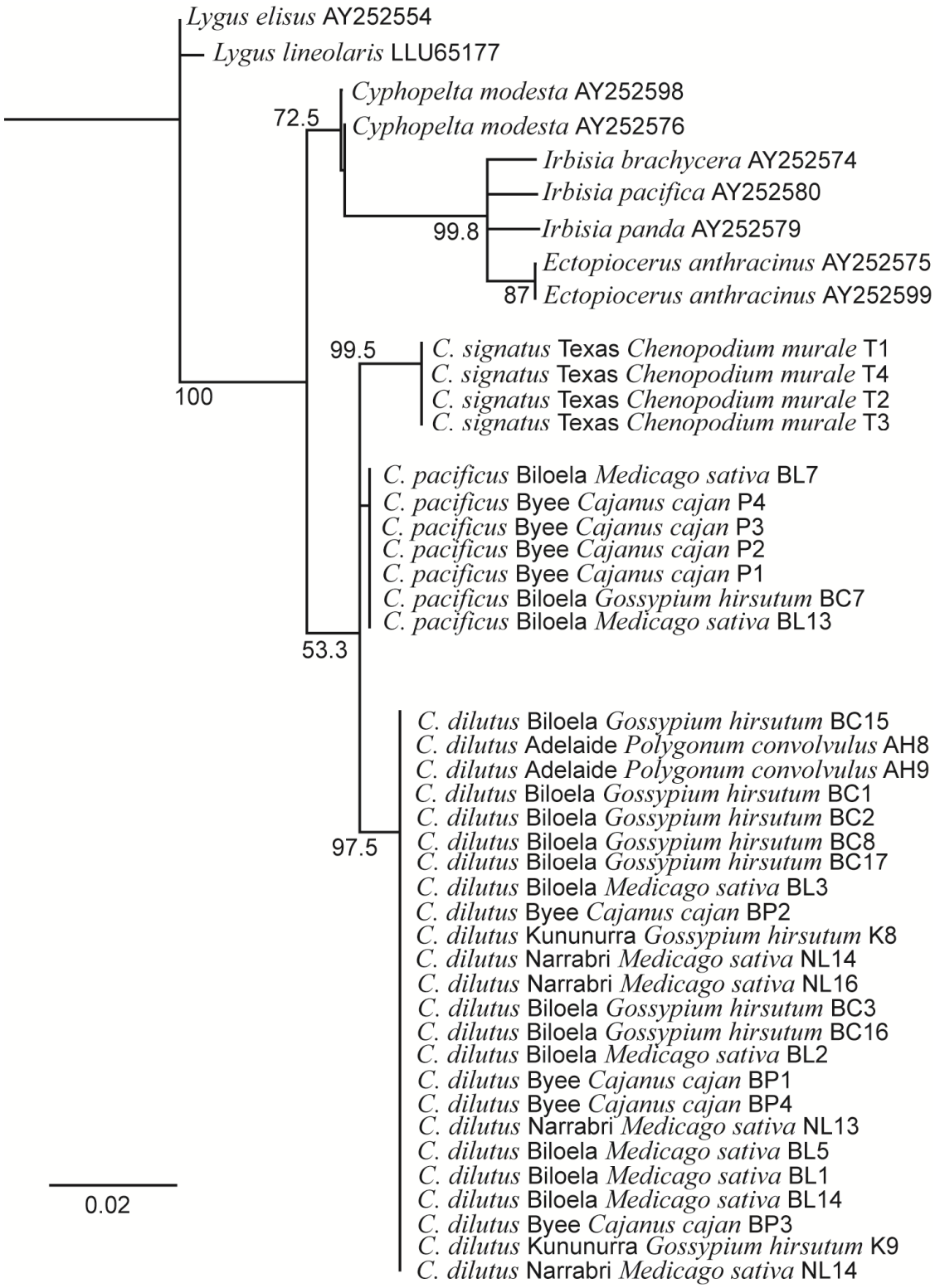


Figure 2.3 Phylogenetic neighbour joining tree showing the relationship between three *Creontiades* species as determined through the analysis of a 271bp fragment of the 28s gene region, using *Lygus elisus* as the rooted outgroup. Additional outgroup sequences were obtained from GenBank and the accession numbers are labelled. (Numbers represent the bootstap support for each clade based on 1000 replicates).

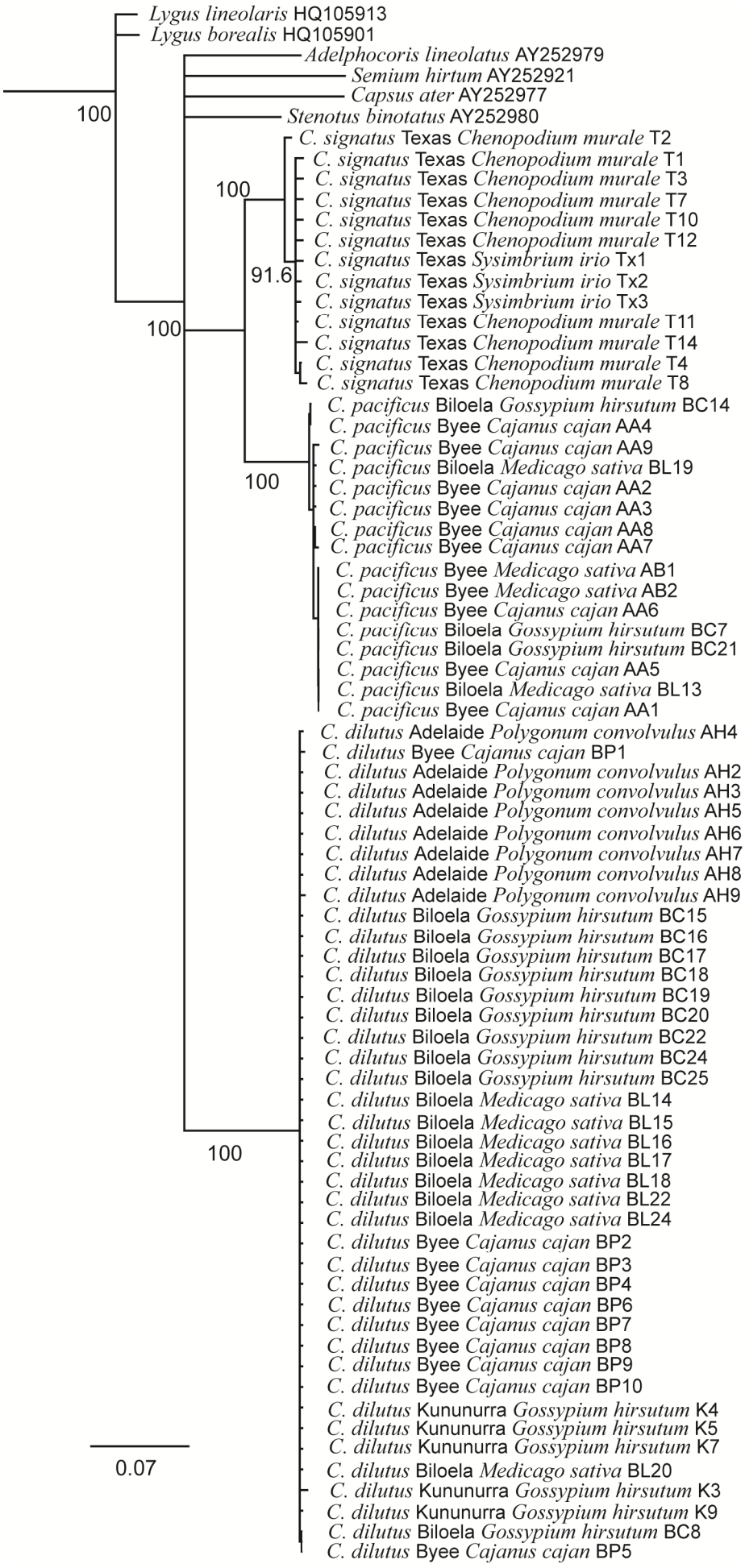


Figure 2.4 Phylogenetic neighbour joining tree showing the relationship between three *Creontiades* species as determined through the analysis of a 565bp fragment of the mitochondrial CO1 gene region, using *Lygus lineolaris* as the rooted outgroup. Additional outgroup sequences were obtained from GenBank and the accession numbers are labelled. (Numbers represent the bootstap support for each clade based on 1000 replicates).

The green mirid, *C. dilutus*, is considered to be the major pest of cotton in Australia, and is the main focus of this thesis. The questions highlighted in chapter one required that extensive sampling be conducted across the arid regions of Australia that this species inhabits. It was therefore beyond the scope of this thesis to conduct exhaustive sampling of brown mirid, *C. pacificus*. Detailed examination of genetic differentiation, gene flow and host plant relationships of this understudied species across the different islands and countries that it inhabits would undoubtedly further illuminate the mechanisms by which this genus has repeatedlty emerged as agricultural pests (Rohini *et al.* 2009; Udikeri *et al.* 2010; Hosseini *et al.* 2002; Stam 1987; Armstrong *et al.* 2009)

The possible reasons for the low mitochondrial diversity in *C. dilutus* are examined and interpreted in more detail in chapter four, with the aid of sequence data from samples collected in 1983 and 1993. It was clear, however, based on the low mitochondrial diversity observed in *C. dilutus* that mtDNA alone was not going to be sufficient to address issues of gene flow and dispersal in green mirid, and the next section of this chapter details the development of microsatellite markers by enrichment for this purpose.

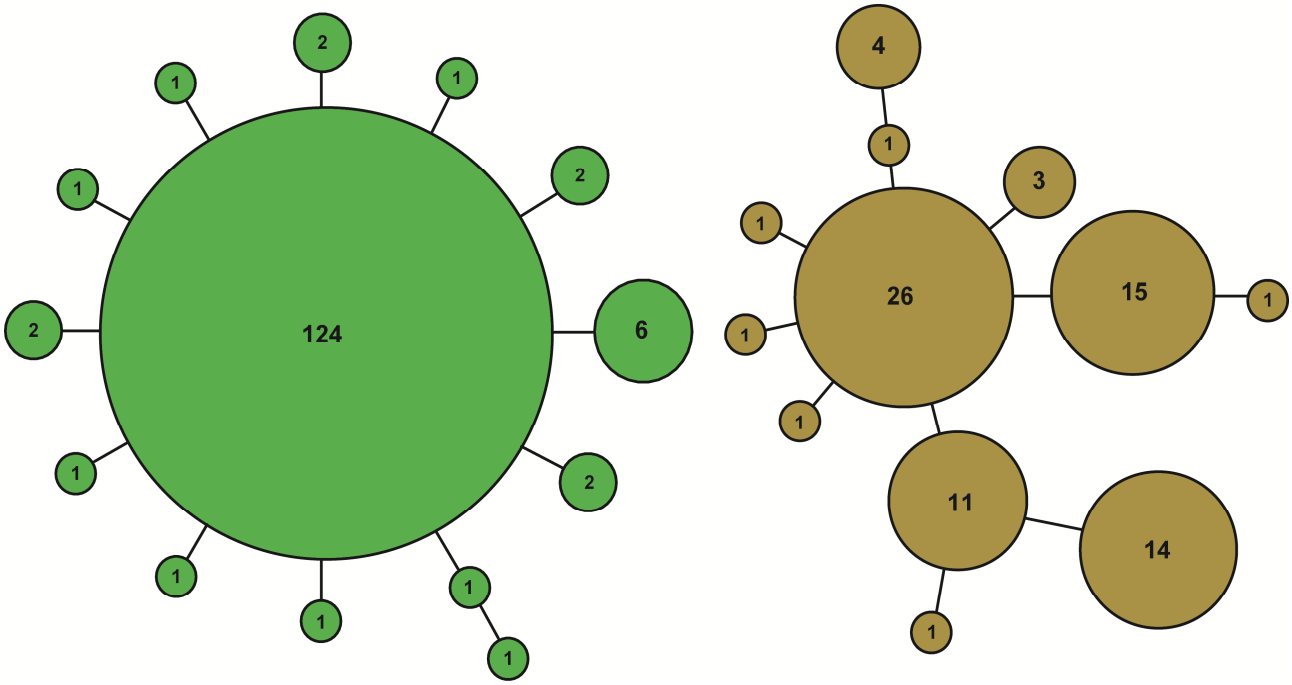


Figure 2.5 CO1 haplotype networks for *C. dilutus* (left) and *C. pacificus* (right) the size of each haplotype (circle) is proportional to the number of individuals (shown within) that were represented by that haplotype.

2.2 Characterisation of 12 polymorphic microsatellites in the green mirid, *Creontiades dilutus* Stål (Hemiptera: Miridae)

Creontiades dilutus is a widely distributed Australian mirid bug (Malipatil & Cassis 1997). It has been recorded on a broad range of host plants, including crops such as cotton, lucerne, sorghum, soy, grapes, stone fruits, cucurbits, parsnips, and potatoes (Malipatil & Cassis 1997; Miles 1996). *Creontiades dilutus* has also been recorded on a number of invasive and native plants, both in the eastern cropping regions and in arid central Australia (Chinajariyawong 1988; Khan 1999; Malipatil & Cassis 1997; Miles 1996). Here we report the development of 12 polymorphic microsatellite markers as a tool to investigate gene flow and species limits within this species.

Genomic DNA was extracted from one male and one female insect using QIAGEN blood and tissue kits. The DNA was pooled and a library was enriched for AC, AG, AAC and AAAG repeats following Gardner *et al.* (2008). Cleaned products (MoBio) were ligated into a pGEM-T Vector (Promega) and transformed into competent *Escherichia coli* JM109 cells (Promega). Insert positive colonies were PCR screened for the presence of enriched motifs using M13 vector primers and the appropriate repeat oligonucleotides (AC + AG or AAG + AAAC) in 10µl reactions as per Gardner *et al.* (1999). Eight hundred colonies were screened for repeats, with 95 positive colonies sequenced (by Macrogen, Korea); repeat motifs were present in 90% of the sequences. Primers were designed for 24 unique loci using PRIMER3 (Rozen & Skaletsky 2000) and M13 universal tails (5'GTAAAACGACGGCCAG) were added to the 5' end of forward primers for subsequent efficient fluorescent labelling (Schuelke 2000). Using the same pooled DNA, sequence tagged microsatellite (STM) libraries were prepared (Hayden *et al.* 2006) using compound probes $(AC)_5(AG)_6$, $(AC)_5(TC)_6$ and $(AC)_5(AT)_8$. A total of 96 insert positive colonies were sequenced, 76 contained the target microsatellite, and primers were designed for 32 loci.

Twenty one *C. dilutus* individuals were collected from a field of lucerne (*Medicago sativa* L. Fabaceae) at Brewarrina, New South Wales, Australia (Lat: -29.962E, Long: 146.850S) on 11/03/2008. Genomic DNA was extracted using high throughput salt precipitation based on the methods of Miller *et al.* (1988). All loci were amplified in 10µl reactions containing 1x MangoTaq buffer (Bioline), 2.5 mM MgCl2, 0.8 mM total dNTP's, 200 nM each primer, 0.25 U MangoTaq (Bioline), and 10–30 ng DNA. Amplification conditions were: initial denaturation of 94^{o}C for 10 min followed by 35 cycles of 94^{o}C for 30s, 50^{o}C for 45s, and 72^{o}C for 45s, with a final extension of 72^{o}C for 5 min. Products were visualised on a GelScan 2000 (Corbett Research) acrylamide gel

system with ethidium bromide staining. Primer pairs were further optimised by modification of annealing temperature and MgCl2 concentration (see Table 1 for details). Polymorphic loci were further screened by PCR as above but with the inclusion of a fluorescently labelled (HEX, FAM or NED) M13 primer (Schuelke 2000). Fragments were separated on a Megabace 1000 Fragment Analyzer (General Electric), and sizes determined using ET400R (ROX) size standard. Fragment Profiler (GE) was used to score alleles with manual confirmation of flagged peaks.

STM microsatellites have proved successful in several plant species to date, but most of the *C. dilutus* loci (78%, 25 loci) tested resulted in non specific amplification, perhaps because transposable elements have generated multiple copies of these loci (Zhang 2004). Only 4 out of the 32 loci tested (12.5%) amplified polymorphic loci specific enough to be useful in multiplex genotyping runs (mirsat-A1, mirsat-G8, mirsat-D4 and mirsat-G4), while the enriched loci resulted in 8 polymorphic loci. These STM loci amplify using one primer located in a compound join and therefore there is a potential for mis-priming. Furthermore, the screening results indicate that there is a possibility that these compound microsatellites may be associated with transposable elements in *C. dilutus.* For these two reasons, the four STM loci were further screened to ensure reliable genotyping results. Four individuals were selected from the screening population and separate PCR reactions were used to amplify and genotype each of the four individuals in 10 separate reactions (40 reactions per locus). Reproducibility was high for the four loci (mirsat-1A1 = 97.5%, mirsat-G8 = 97.5%, mirsat-D4 = 95% and mirsat-G4 = 95%), and a maximum of two alleles per locus was observed in all cases. This indicates that for these four loci, potential mis-priming / presence of multiple copies does not affect the accuracy of genotyping.

Frequency and Hardy-Weinberg equilibrium (HWE) calculations were computed using GenAlEx 6.1 (Peakall & Smouse 2006), the presence and frequency of null alleles estimated with MICROCHECKER (Van Oosterhout *et al.* 2004), and linkage disequilibria tested using GENEPOP (Rousset 2008). The number of alleles per locus ranged from 2 to 10 with a mean of 5.5 and expected heterozygosities from 0.09 to 0.84 with a mean of 0.49 (Table 1). There was no evidence of stutter errors or large allele dropout at any locus. Null alleles may occur at four loci as indicated by a general excess of homozygotes based on the 95% confidence intervals calculated in MICROCHECKER (mirsat-3E, mirsat-6H, mirsat-D4 and mirsat-G4). Null allele frequencies reported in Table 1 are estimated using the Brookfield 2 method implemented in MICROCHECKER under the assumption that PCR failure indicates a null homozygote. Five loci deviated significantly from HWE in the population screened (mirsat-3E, mirsat-G8, mirsat-6H, mirsat-D4 and mirsat-G4); p-values are reported in Table 2.2.1. It should be noted that the

individuals used to screen these loci were sampled from an agricultural crop and may have been subject to pesticide-mediated selection which may cause deviations from HWE at some loci. There is also the potential for overlapping generations in this species and migration between crops which would violate assumptions of the HWE model. No significant linkage disequilibria were detected among the 12 loci ($\alpha = 0.05$).

Table 2.2.1. Characteristics of 12 polymorphic microsatellites isolated from *Creontiades dilutus* when screened in 21 individuals from one site.

Locus[a]	Sequenced Motif	Primer Sequence (5'-3')[b]	Size Range	Ta	MgCl2 Mm	N	N_a	H_o	H_e	NF	Genbank Accession
mirsat-2F	$(TTA)_6$, $(TTG)_7$	F: *GTTCCGTGATGAAGTCTTGA	151-172	56	2.5	21	3	0.381	0.534	0.0998	GU937077
		R: CGTACAGAAGGTTCAACAAT									
mirsat-4B	$(AC)_7$	F: *CGGGTAGTTTCTCGGTTGAA	331-337	54	2.5	18	2	0.143	0.133	-0.0169	GU937080
		R: ATTGATGCAGCAGACATGGA									
mirsat-3H	$(TG)_{32}$	F: *GATTCGTGCCTAAGGTTCAA	146-178	50	3.5	20	10	0.750	0.640	-0.0671	GU937079
		R: GGACTAGGGCTAGAGGACGG									
mirsat-A1	$(CT)_6$	F: *TTCGTCAAAGCGGTCAC	155-167	50	3.5	19	4	0.158	0.150	-0.0072	GU937085
		R: ACACACACACAGAGAGAGAGAG									
mirsat-6B	$(AAC)_5$	F: *GAGAAGTGGAAGTCATCGCC	137-146	60	2.5	21	2	0.048	0.046	-0.0011	GU937082
		R: TGTTCTTCTGCTGAGTGGTATGA									
mirsat-5C	$(TG)_{20}$	F:*CCAAGTGTTTCCAATACGCC	260-288	56	2.5	21	10	0.857	0.785	-0.0407	GU937081
		R:GTTCGGAACCTCTTGTCAAA									
mirsat-G8*	$(TA)_5(GA)_7$	F: *ATTGGCCAAATAATCGAAG	111-129	49	3.5	18	3	0.210	0.345	0.1045	GU937088
		R: ACACACACACTCTCTCTCTCTC									
mirsat-3E**	$(TC)_8$	F: *ACAGTCGTGCCTTCCTCTTCTCT	201-213	51	3.5	17	2	0.000	0.420	0.2392	GU937078
		R: GTTCTCAGGTTTTGGGGAATGGATAG									
mirsat-7G	$(GA)_{15}$	F: *GGCACGTGGTCATAACACAA	131-159	50	3.5	19	7	0.800	0.838	0.0206	GU937084
		R: TCAGACGTGATTCCATTCCA									
mirsat-D4*	$(TC)_6$	F: *CGAATCTATCTATAGGCAGC	113-119	49	3.5	18	4	0.278	0.480	0.1163	GU937086
		R: ACACACACACAGAGAGAGAGAG									
mirsat-6H*	$(GA)_{14}$	F: *GCACGAAACGAAAGTTGTCA	371-409	52	3.5	20	8	0.400	0.651	0.1399	GU937083
		R: CGTTGCATAGCTCCTTGTGA									
mirsat-G4**	$(CT)_8$	F: *GGTCGGTATCAAATGACAG	190-204	49	3.5	20	10	0.350	0.785	0.1655	GU937087
		R: ACACACACACAGAGAGAGAGAG									

[a] Deviations from HWE * = P<0.05, ** = P<0.01, [b] asterisk indicates position of M13 tail, Ta = annealing temperature, N = individuals amplified, Na = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, NF = Null allele frequencies.

Cross species amplification was tested on the closely related species *Creontiades pacificus*, and also *Taylorilygus pallidulus* Blanchard (Hemiptera:Miridae). Reaction conditions for all amplifications were as reported in Table 1. A 700bp fragment of the mitochondrial CO1 gene exhibits 10% sequence divergence between *C. pacificus* and *C. dilutus* (Coleman *et al.* 2008). Forty three individuals were collected from soy (*Glycine max* L. Fabaceae) located in Kingaroy, Queensland, Australia (Lat: -26.803E, Long:151.977S) and screened for all loci. Five of the loci (mirsat-A1, mirsat-3H, mirsat-5C, mirsat-G8 and mirsat-7G) amplified and were polymorphic with numbers of alleles and *He* ranging from 4-11, and 0.216-0.747 respectively (for details see Table 2). Three of these loci show significant deviations from HWE and evidence of null alleles (mirsat-A1, mirsat-3H and mirsat-G8). Twenty four individuals of *Taylorilygus pallidulus* were collected from wild sunflower (*Verbesina encelioides* Cav. Asteraceae) in Emerald, Queensland, Australia (Lat: -23.495E, Long: 148.188S). *Taylorilygus pallidulus* is in the same tribe as *C. dilutus* and is found on many of the same host plants, but none of the described loci amplified in the population screened.

Table 2.2.2. Characteristics of 5 loci that amplify in 43 *Creontiades pacificus* individuals collected at one site.

Locus[a]	Size Range	N	Na	Ho	He	NF
1A1**	153-161	38	4.000	0.118	0.216	0.0808
3H***	151-175	42	9.000	0.190	0.320	0.0983
5C	264-272	42	4.000	0.703	0.482	-0.1488
2G8**	105-129	34	11.000	0.500	0.747	0.1414
7G	139-157	43	7.000	0.698	0.726	0.0161

[a] Deviations from HWE * = P<0.05, ** = P<0.01, *** = P<0.001, N = individuals amplified, Na = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, NF = Null allele frequencies.

Chapter 3: Molecular interrogation of the feeding behaviour of field captured individual insects for interpretation of multiple host plant use

3.1 Abstract

The way in which herbivorous insect individuals use multiple host species is difficult to quantify under field conditions, but critical to understanding the evolutionary processes underpinning insect - host plant relationships. In this study we developed a novel approach to understanding the host plant interactions of the green mirid, *Creontiades dilutus*, a highly motile heteropteran bug that has been associated with many plant species. We combine quantified sampling of the insect across its various host plant species within particular sites and a molecular comparison between the insects' gut contents and available host plants. This approach allows inferences to be made as to the plants fed upon by individual insects in the field. Quantified sampling shows that this "generalist" species is consistently more abundant on two species in the genus *Cullen* (Fabaceae), its primary host species, than on any other of its numerous listed hosts. The chloroplast intergenic sequences reveal that *C. dilutus* frequently feeds on plants additional to the one from which it was collected, even when individuals were sampled from the primary host species. These data may be reconciled by viewing multiple host use in this species as an adaptation to survive spatiotemporally ephemeral habitats. The methodological framework developed here provides a basis from which new insights into the feeding behaviour and host plant relationships of herbivorous insects can be derived, which will benefit not only ecological interpretation but also our understanding of the evolution of these relationships.

3.2 Introduction

A clear understanding of the behaviour of individual insects is crucial to interpreting many ecological and evolutionary phenomena, for it informs about the extent and limits of variation

within a population (or species) and about differences between populations or species. Ascertaining the feeding behaviour of herbivorous insect individuals under natural conditions is difficult, especially in those species that use multiple hosts, but it is crucial to defining host-plant interactions accurately. Although laboratory studies of host plant use do provide insight into how individuals use host plants of alternative species, they suffer several compounding limitations, including the difficulty of incorporating and testing long range host searching mechanisms, the exclusion of environmental influences, and the difficulty of reconciling behaviour observed in the laboratory with that observed in the field (Manners & Walter 2009). To determine what individuals feed on in the field requires not only observations of an insect on a host plant, but often a method of testing the feeding history of that individual relative to alternative host plants in the area. In this paper we elucidate the feeding behaviour of individual green mirids (*Creontiades dilutus*), a species of bug recorded from multiple host plants, under natural conditions. This required that a methodological approach be developed, based on a combination of structured sampling in the field and gut content analysis, as expanded below.

The use of multiple host plant species by an insect herbivore is usually determined through the scrutiny of host plant lists, but these comprise, at best, summary statements. Many such records are simply incidence records. The observed occurrence of an insect on a host plant does not necessarily confirm regular feeding or reproduction on that plant. This shortcoming can be overcome to some extent by using the presence of juveniles as an indication that a host is significant to the life cycle of that insect species. However, for species with highly motile juvenile stages (such as lepidopteran caterpillars and many orthopteran and hemipteran species) it can be difficult to be sure that juvenile presence on a plant truly represents feeding on that host. Furthermore, the relative importance of the host plant species to the ecology of the herbivore in question may be distorted by such incidence records, and their summary into host plant lists (Walter & Benfield 1994).

In this study we interrogate the feeding behaviour of green mirid individuals under field conditions. Not only is this species usually characterised as a “generalist” on the basis of both adults and

juveniles commonly being found on many host plant species (Chinajariyawong 1988; Khan 1999; Malipatil & Cassis 1997; Miles 1995), but it is also highly motile. Sampling of crops to establish patterns of invasion into cotton (Miles 1995), and microsatellite based analyses of migration (JPH Unpublished data) indicate that these insects move long distances (at a scale of at least 2000km) between the arid interior of Australia and eastern cropping regions. They are also highly motile within a locality (both adults and juveniles), fleeing in response to any disturbance (pers. obs. JPH). Although this particular mirid species is endemic to Australia (McColl *et al.* 2011), and was likely restricted to the arid interior prior to European settlement when land was cleared to establish broad scale agriculture, it has been recorded from only a few native Australian host plants (based on those incidence records that are available). An initial survey in this region did, however, implicate the native leguminous forb *Cullen cinereum* as a major host (Miles 1995), based on relatively high numbers on this plant. Thus, the use of multiple hosts by green mirid individuals in inland Australia, in particular, warranted further investigation.

Whereas mirids are often regarded as 'sap sucking bugs', they do not feed on phloem or xylem, as many hemipterans do. Instead, phytophagous mirids like *C. dilutus* use their stylets and watery saliva to lacerate and macerate a pocket of cells (Miles 1972). *Creontiades dilutus* saliva has a complex mix of proteases, and pre-oral digestion of plant tissue is evidently an important aspect of their feeding (Colebatch *et al.* 2001). The resultant mix of semi-digested plant cells and tissue is then consumed, which makes it probable that chloroplasts are consumed by *C. dilutus*.

A few studies have used chloroplast sequences to recover the gut contents of herbivorous insects, and thus determine directly which host plant species have been fed upon. To this end, chloroplast markers have been amplified from DNA obtained from dry coleopteran material in museum collections (Jurado-Rivera *et al.* 2009), and also from wild caught beetles (Navarro *et al.* 2010). These studies could not, however, relate dietary information directly to putative host plants because they relied on publically held database records of chloroplast sequences. The taxonomic resolution

of host plants has thus depended on the somewhat limited taxonomic coverage of records in these databases.

Through quantitative sampling of mirids on known host plants as well as potential host plants growing together across different localities, we were able to quantify the relative importance of each host species. We collected tissue samples from the range of plant species from which *C. dilutus* had been collected in each locality. These plants were identified and DNA extracted from both the plants and also the mirids collected from them. We then amplified chloroplast intergenic sequences from the plants and from individual insects to provide a direct link between insect individuals and the plants on which each had fed (within about 48hr prior to capture).

We show how this combination of ecological sampling data and molecular diet analysis provides new insights in understanding the ecology of insect feeding behaviour and for interpreting their host plant relationships in the field. Use of the proposed methodological and conceptual framework will therefore develop broader understanding of the ecological and evolutionary significance of the use of multiple plant species by herbivorous insects.

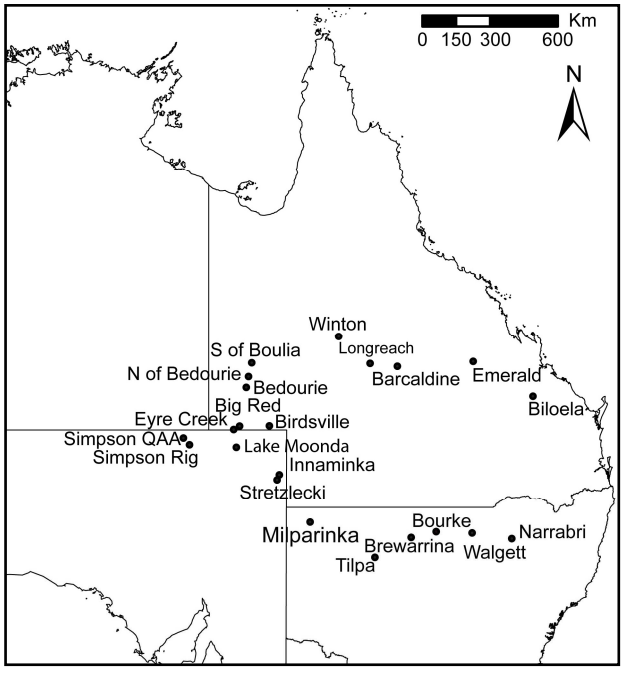


Figure 3.1. Map of northeastern Australia showing the sampling locations for the field survey.

3.3 Results

The extensive host plant survey in this study (Fig. 3.1, Appendix; A3.2) revealed 26 new putative host species, 22 of which are listed as Australian native species (Australian Virtual Herbarium http://www.ersa.edu.au/avh/, Appendix; A3.1). When combined with existing records, a total of 97 potential host plant species has now been recorded for *C. dilutus* (Appendix; A3.1). When hosts that have no record of *C. dilutus* nymphal presence are removed (54% of the total), this list is reduced to 45 host plant species across 15 families, but primarily Fabaceae (42% of those host species with nymphs recorded) and to some extent Asteraceae, with 16% (Fig. 3.2).

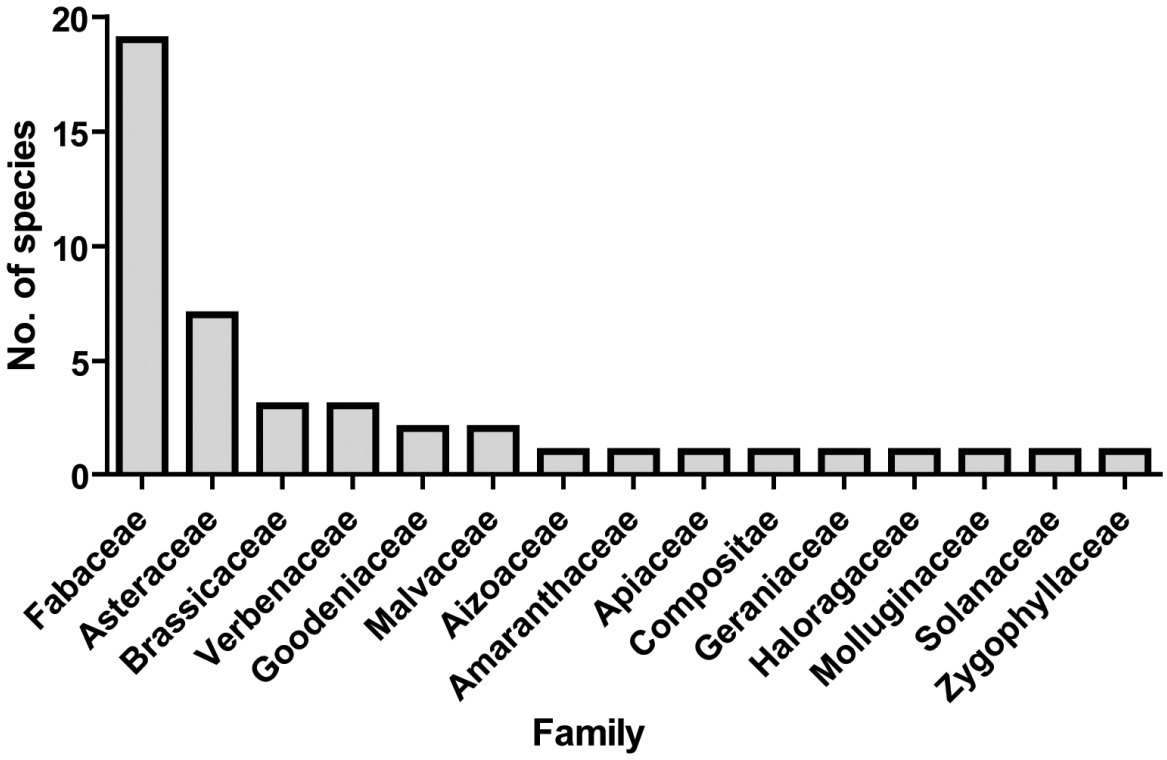


Figure 3.2. Number of host plant species per family for which records exist of *Creontiades dilutus* nymphal presence. Data from the survey reported in this paper and from records in the literature (see methods and Appendix; A S3.1 for details).

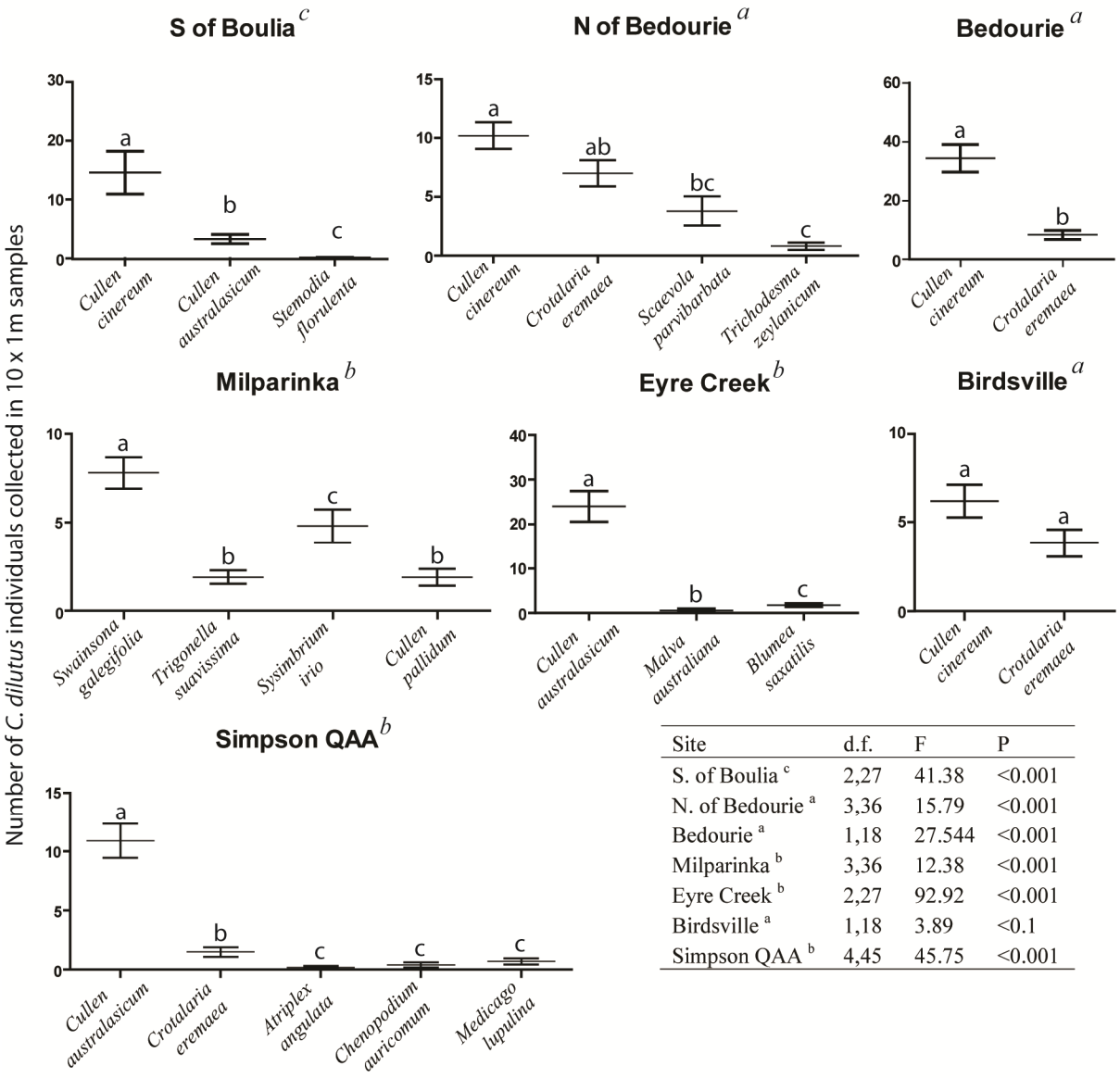
The field survey of *C. dilutus* host associations and abundance covered a circular transect of 6000km through central Queensland, the southeast corner of the Northern Territory, and northern

New South Wales. The area was selected because green mirids had been collected there before, it supports the ephemeral vegetation that typifies green mirid hosts, and other insects associated with such plants are known to invade sub coastal agriculture from there (Zalucki & Furlong 2005). As is typical of these arid regions (Morton *et al.* 2011) rainfall was temporally and spatially patchy during the season of this sampling. Suitable host plants (forbs and herbs) generally require more than one rainfall event (and this is usually highly localised) to flourish, adding to their patchy occurrence. Such localities are typically interspersed with large areas (often several hundred kilometers) of barren land.

Our quantitative sampling at the 22 sites where *C. dilutus* was present (of 82 likely sites investigated) revealed that the five plants on which green mirids were most numerous are all in the genus *Cullen* (Appendix; Table A3.2), and the highest number of mirids collected from a *Cullen* host (344 total, $5m^2$ sweep-net samples, n = 10) was over 4 times higher than the highest number retrieved from a non-*Cullen* host (*Crotalaria eremaea,* 80). However, not all *Cullen* species hosted large numbers of these bugs, as site-specific factors such as temperature extremes and time since colonisation also affect insect abundance. As with available potential hosts, *C. dilutus* was patchily distributed across the inland sites sampled, but most abundant where *Cullen* plants occurred (Appendix; A3.2). This could indicate that the presence of green mirids on adjacent plants may be spill-over from *Cullen* hosts. We therefore assessed the abundance of *C. dilutus* on a site by site basis, for those sites where *C. dilutus* had been sampled from *Cullen* host plants as well as other plant species.

Creontiades dilutus abundance was statistically different across potential host plant species at six of these seven sites, with only Birdsville returning no significant difference at $P < 0.05$ (Fig. 3.3). Abundance was consistently higher on *Cu. australasicum* and *Cu. cinereum* than on alternative hosts (Fig. 3.3). However, the third *Cullen* species sampled, *Cu. pallidum*, at Milparinka, had a significantly lower abundance of *C. dilutus* (mean 1.9 +/- 0.48) in comparison to the two host plants

with the highest abundance there (*Swainsona galegifolia* 7.8 +/- 0.89, *Sysimbrium irio* 4.8 +/- 0.92) at that site.



Site	d.f.	F	P
S. of Boulia [c]	2,27	41.38	<0.001
N. of Bedourie [a]	3,36	15.79	<0.001
Bedourie [a]	1,18	27.544	<0.001
Milparinka [b]	3,36	12.38	<0.001
Eyre Creek [b]	2,27	92.92	<0.001
Birdsville [a]	1,18	3.89	<0.1
Simpson QAA [b]	4,45	45.75	<0.001

Figure 3.3. Abundance of *C. dilutus* across host plants at seven sites in northeastern Australia where this bug was located and both *Cullen* and alternate host plants grew together (bars represent the mean and the error bars are +/- 1SE, n = 10). For a given site, bars with the same letter above them are not significantly different from one another, per ANOVA and Fisher's LSD test with a Benjamini-Hochberg correction for multiple pair wise comparisons. * no transformation required, **log transformation applied, *** log(log) transformation applied.

In *C. dilutus*, the chloroplast *trnL* intron used by (Jurado-Rivera *et al.* 2009) did not amplify with a high success rate, probably as a result of degradation by extra-oral digestion in these bugs, which is likely to reduce the number of larger DNA fragments remaining in the insects gut. We therefore selected the *trnL-trnF* intergenic spacer which is generally a smaller region (158-438bp as opposed to 389-614bp) (Taberlet *et al.* 1991) and therefore more amenable to PCR amplification from degraded DNA. The *trnL-trnF* intergenic spacer amplified in 100% of our host plant DNA samples (21 species in 8 families) amplifying fragments from 161 to 567bp. These sequences were highly variable across families with many insertions and deletions and a single alignment could not be produced to assess sequence divergence. Five separate alignments were produced that correspond to the 5 families for which we had sequenced more than one species, leaving three sequences un-aligned. With the exception of the three species of *Cullen* (which only differed from one another by one bp substitution) the closest sequences in our data were those of *Senecio gregorii* and *S. depressicola* which had 2.1% base difference, for all other species it was considerably higher. We therefore set a threshold of 2% difference to define a *trnL-trnF* match for the insect derived fragments, but this value is arbitrary and a match should not be considered a robust plant species identification.

The amplification success rate of the chloroplast marker in insect-derived DNA was relatively low (28.5%, 288 insect samples), yielding 82 good sequences (length = 80-398bp after poor quality sequence was removed). This likely represents a limitation of gut content analysis in mirids by means of PCR, because their extra-oral digestion probably degrades DNA. The size variation in this fragment was such that it allowed more than one sequence to be recovered from each of 5 insect samples by agarose gel recovery representing feeding on more than one host plant. These five included two of the individuals from Eyre Creek (Table 3.1) one returned both *Cullen* and *Sysimbrium irio* fragments and one returned both *Cullen* and *Chenopodium auricomum* fragments. The other 3 samples for which multiple feeding was detected were from sites that had low numbers of mirids sequenced, and were not included in the analysis presented here. Specifically; one

individual from Simpson desert that had both *Crotalaria eremea* and *Cullen australasicum* fragments, one individual from Simpson that had *Senecio gregorii* and *Blennodia pterosperma* fragments, and one from Milparinka that had both *Cullen* and *Phlegmatospermum cochlearinum*. In addition, 4 of the sequences were of poor quality and probably also represented feeding on multiple hosts. These four sequences were not recovered by cloning, and instead were discarded from the analysis. It is also possible that closely related plants that were not sampled in our plant dataset may not have been diagnosed with the *trnL-trnF* fragment used (as for the *Cullen* sequences, see below). Our results are therefore conservative in underestimating the use of multiple hosts by individuals of this species.

The fragment amplified from both the plants and the mirids collected from these plants was diagnostic for all plant species that we had sequenced using a threshold of 2% difference, with the exception of the three species of *Cullen*. The sequences from these three host species differed by only one site toward the *trnF* end of the plant sequences and this site was absent from many of the *Cullen* sequences obtained from insect DNA. More than one species of *Cullen* was, however, never present at the same site, so the gut derived sequences could be assigned to host species based on the availability of that host at any given site. There were only two instances where the host plant detected in the mirid was not in our set of plant-amplified chloroplast sequences, in the first instance (C2721, Genbank accession JX134164) a Genbank search indicated that this might be *Sysimbrium irio*, pair wise alignment with this sequence (Genbank accession DQ180275.1) gave 1.4% difference and we defined this as a match although the 2% threshold used is arbitrary and this identification should be considered provisional. In the second (C1501, JX134132) *Panicum virgatum* was the closest sequence available on Genbank (e-value = 2E-116). When our sequence was pair wise aligned to the *P. virgatum* complete chloroplast genome (Genbank accession HQ731441.1) there was 4.2% difference, which is outside of our 2% threshold, and we assigned this sequence to the genus *Panicum*.

Host plant collections for which less than 5 sequences had amplified successfully were excluded from this analysis leaving 66 insect-derived chloroplast sequences (Genbank accessions JX134132–JX134197). Of these 66 sequences, 10% showed that the green mirid individuals had fed on a plant other than the one that they had been collected from. Even when collected from *Cullen* hosts a high proportion of individuals had fed on a different plant species (Table 3.1).

Table 3.1 Gut-derived chloroplast sequences from the green mirid *Creontiades dilutus* showing the number of sequences that match the host (N host) from which the insects were sampled, and the number that match a plant other than the one from which the insects were sampled (N different). Plant species on which *C. dilutus* had fed but was not collected from are listed (Species recovered).

Host plant sampled	Site	N host	N different	Species recovered
Blumea saxatilis	Birdsville	9	1	*Panicum*
Cullen australasicum	Simpson QAA	8	1	*Chenopodium auricomum*
Calotis plumulifera	Simpson QAA	9	1	*Cullen australasicum*
Cullen australasicum	Eyre Creek	6	3	*Chenopodium auricomum*
				Calotis plumulifera
				Sisymbrium irio
Brachysome campylocarpa	Lake Moonda	11		
Senecio gregorii	Stretzlecki	17	0	

3.4 Discussion

To explore the host plant relationships of this highly motile insect, with a broad reported host range, we developed a framework that integrates quantified spatial host plant sampling with molecular analyses of recent plant food intake. This framework goes beyond incidence records, allowing inference into the rates of host plant species use and recent feeding behaviour of individual insects. The ability to make this inference for field collected insects means that a critical assessment of the relationship between an insect and multiple hosts can be made without the limitations of laboratory studies. We discuss the findings of this approach specifically in relation to *C. dilutus*, then consider the implications of our results and approach more broadly.

3.4.1 Host plant relationships of C. dilutus

Creontiades dilutus is highly motile and is endemic to Australia (McColl *et al.* 2011). A large number of incidence records demonstrate that these bugs feed on multiple hosts. Green mirids were likely restricted to the arid interior of Australia prior to European settlement and the spread of agriculture. This implies, in turn, that the species has close evolutionary relationships to plants in this area (see introduction). Before this study the host plant relationships of *C. dilutus*, in particular outside of agricultural areas, was not fully resolved. Our aim, therefore, was to investigate the use of multiple hosts by this species, particularly in central Australia.

Our data do confirm that *C. dilutus* uses many host plant species, most of which are in the family Fabaceae (Fig. 3.2). However, the abundance of *C. dilutus* is consistently higher on plants in the genus *Cullen* than on other host plant species surveyed. Specifically, the Australian native species *Cu. cinereum* and *Cu. australasicum* are identified as primary hosts for green mirids by the quantitative host plant sampling presented here. Not only is the highest mirid abundance recorded on these species, but on a site by site basis these two *Cullen* host plants have significantly higher abundance of *C. dilutus*, across six sites, compared to other plant species sampled locally (Fig. 3.3). *Cullen australasicum* and *Cu. cinereum* are morphologically similar to one another, but *Cu. pallidum* is densely covered in hairs, which may explain why this latter species seems to be a relatively poor host for green mirids (Fig. 3.3). Alternatively, the chemical cues used by *C. dilutus* for host location and feeding initiation may well differ across these *Cullen* species, but this requires further investigation.

Simultaneous sampling of the insect and the host plants available locally allowed a molecular comparison of the insect gut contents (at the time of sampling) with the host plant from which it was collected. This underpins an inference of feeding behaviour beyond just incidence of the insect on a plant. Our molecular analysis of host plant feeding in *C. dilutus* shows that this species often feeds on host plant species other than the one from which it had been collected, even when they

were collected from their primary host (Table 3.1). The behaviour that this represents is particularly striking considering that fragments of the length that we amplify here can evidently be detected for only as long as 12 to 48 hrs post ingestion (Fournier *et al.* 2008; Gariepy *et al.* 2007; Hoogendoorn & Heimpel 2001; Muilenburg *et al.* 2008).

The behavioural implications for the mirids appear somewhat contradictory, however. Whereas green mirid abundance is much higher on *Cu. australasicum* and *Cu. cinereum* than on other host plants nearby, individuals collected from these primary hosts evidently do move between different plant species locally and feed on these other hosts, even species that are relatively insignificant in terms of mirid abundance. The host use of generalist species is often viewed in the context of optimisation strategies (Scheirs *et al.* 2000) and enemy free space (Mulatu *et al.* 2004). Optimal diet mixing, for example, has been suggested to favour resource generalisation through individual fitness gains. However, feeding trials on *Nezara viridula*, a heteropteran that uses multiple host species in a similar way to *C. dilutus*, show that diet mixing does not provide direct fitness benefits. The use of multiple hosts does, however, allow this species to persist on sub-optimal plant species when their primary host species are not available (Velasco & Walter 1993). In the arid interior of Australia, *C. dilutus* is associated with spatially and temporally patchy resources that are highly dependent on recent rainfall. Except in years of unusual rainfall, precipitation events and plant growth tend to be localised. We suggest that the use of multiple hosts represents a similar behavioural adaptation to that of *N. viridula*, and this allows these bugs to survive and reproduce within a patchy and ephemeral environment.

This study has focussed on the relationships between *C. dilutus* and native host plants in the arid interior of Australia. At the time of sampling (winter) green mirids were present only in very low numbers on agricultural crops sampled; effectively zero in our standardised sampling (Appendix; A3.2). In the summer, by contrast, green mirids are very difficult to locate in the arid interior, as it is far too hot and dry to support plant growth, but in agricultural regions they reach much higher densities on lucerne (*Medicago sativa*, Fabaceae) than on any other crops (Miles 1995), densities on

lucerne reach almost as high as on the *Cullen* primary hosts (JPH unpublished data 2007-2008), with irrigation in agricultural areas being significant in this respect.

Our confirmation that *Cullen* species are primary hosts, and the revelation of multiple-host feeding over a short time, highlights several questions regarding the higher abundance of *C. dilutus* on these two species relative to other host plants in arid Australia, and its relationship to lucerne where that is cultivated. The specific cues (olfactory or visual) that *C. dilutus* uses to locate hosts and initiate feeding may be shared across *Cu. cinereum*, *Cu. australasicum* and lucerne. Alternatively, green mirids may perform better on these hosts in comparison to other plant species. Targeted research into the host searching behaviour of green mirids and the specific cues to which they respond would begin to answer these questions. Host performance testing is difficult in this species, as it has proved impossible to maintain a laboratory culture for more than three generations; the research presented here indicates, however, that using *Cullen* hosts in the laboratory may be a possible solution to this problem.

3.4.2 Future use of this framework

Molecular techniques are increasingly being employed to analyse the diet of wild organisms (Pompanon *et al.* 2012; Valentini *et al.* 2009; Yoccoz 2012). In insects such studies have tended to focus on predation, requiring that specific assays are developed (Fournier *et al.* 2008; Hoogendoorn & Heimpel 2001; Northam *et al.* 2012; Traugott *et al.* 2012). The use of chloroplast sequences provides a general approach to assessing herbivorous insect diets (Jurado-Rivera *et al.* 2009), although it has not been applied to answer specific questions about polyphagous species until now. Some of the most significant agricultural pests are polyphagous insects, and polyphagous habits are difficult to explain in evolutionary terms (Jaenike 1990; Jermy 1984). The conceptual and methodological framework we propose here provides a targeted approach to interrogating the recent feeding history of individuals under field conditions. It does so by combining the quantified spatial sampling of insect abundance across multiple hosts in the field with a molecular comparison

between the gut contents of these samples and the locally available host plants. By contrast, a bar-coding only approach to diet analysis could not have highlighted the contrast between insect abundance across different host plant species and individual behaviour in the same way. The work presented here is a "proof of concept" evaluation of the combined approach. Through it we illustrate how this combination of techniques can illuminate host use in a way that incidence records cannot, for it reveals where insects have actually been feeding in the field. Getting such information in any other way would be intractable without molecular techniques, principally because these insects cannot be reliably followed in the field for observation purposes.

The amplification success of plant DNA from mirids was low (28%), probably because of DNA degradation through extra-oral digestion. Nevertheless, valuable insights into the feeding behaviour of individual bugs could still be made. When using chloroplast sequences for diet analyses a trade-off between amplification success and host plant resolution is evident. Indeed, consensus has not been reached on the best regions to use as a plant DNA barcode, and no single region fits all the requirements (Hollingsworth *et al.* 2011; Pettengill & Neel 2010). Shorter regions such as the P6 loop of the *trnL* intron provide better amplification success from degraded DNA but lower resolution of host species (Valentini *et al.* 2009). As recommended for the broader plant bar-coding effort, diet analyses would most likely benefit from the use of more than one region to balance this trade-off.

Future studies of insects recorded from multiple plant species should evaluate their feeding on 'incidental hosts', ones that have no records of juveniles, or from which few insects have been collected. If no evidence of feeding is found then a scientific basis for the removal of such species from host plant lists can be made. Not only can incidence records be refined in this way to represent the ecology of the herbivore more realistically but, conversely, insect feeding on hosts where no observations of insect presence have been made can be detected when an insect collected on a specific plant has indeed fed on another one recently. This is important for applied entomological research, not only in cases such as biocontrol, where the accurate establishment of host plant

relationships in the field is critical (e.g. Manners *et al.* 2011), but also in the study of agricultural pests. An insect that is sampled from a particular crop may have fed on another crop or non crop host plant prior to moving onto the crop in question, and this approach provides a means to recognise this aspect of individual insect behaviour.

Evolutionary studies have increasingly used insect herbivores as systems to investigate speciation (Borer *et al.* 2011; Feder *et al.* 2003; Matsubayashi *et al.* 2010; Nosil *et al.* 2009), and cases are often portrayed as incipient or ongoing speciation events driven by ongoing selection across two alternative host plant species. There is, however, an alternative explanation for many of these patterns. Speciation may well have occurred in geographically separate populations and, under natural conditions now, host use is differentiated across the two species and gene flow is effectively zero. Evaluating such examples requires, first, that variation in host plant use can be attributed to the individual, the population or the species, and, second, where differences in host use are observed between populations, that contemporary levels of gene flow between these populations is quantified accurately. Both aspects must be evaluated under field conditions because both feeding and mating trials in the laboratory often give equivocal results, probably because of the unintended removal of long range aspects of host and mate searching mechanisms (Walter 2003). Our approach provides a way to evaluate the first of these two factors through the analysis of feeding by individuals in the field and their relative abundance on each host. The second can only be accomplished through the sampling of multiple insects from different hosts in the field, and the quantification of contemporary gene flow using multiple loci (Malausa *et al.* 2007).

We hope that the methodological approach developed here will enable not only a more thorough testing of host plant interactions under field conditions, but also a deeper understanding of the evolutionary processes pertaining to insect – host plant relationships.

3.5 Materials and Methods

Host records were collated from the available literature on *C. dilutus* (Chinajariyawong 1988; Khan 1999; Malipatil & Cassis 1997; Miles 1995). Field surveys of host plants and *C. dilutus* abundance were conducted during July and August 2007 in the eastern cropping regions of Australia and the arid interior (Fig. 3.1). Permits were not required for the collection of this species as it is an economically significant pest, and collections were made at road verges. Sites were dictated by the availability of plants suitable for sampling, which was patchy at best. At each site stands of possible host plants were located for sampling, with each having to consist mostly of one species (>95%), and cover at least 10m by 10m. In 6000km travelled only 22 such sites were located; the remaining terrain was too dry.

Creontiades dilutus abundance was quantified using a standardised sweep net sample with an area of $5m^2$,ten replicates. The adults and juveniles of *C. dilutus* are highly motile, and sweep net sampling has been shown to be a reliable and repeatable method to sample this species (Threlfall *et al.* 2005). Abundance was recorded, and *C. dilutus* individuals were collected and stored in 96% ethanol for subsequent DNA analysis (up to a maximum of 50). Herbarium specimens of each putative host were collected for identification, and leaf tissue was collected and stored in silica gel for DNA analysis. Herbarium samples were identified using the public reference centre of the Queensland Herbarium (Department of Environment and Resource Management, Brisbane). Putative host plants recorded from the survey in this study were integrated into the list of host plants so far reported in the literature (Appendix; A3.1). This list was then reduced to those records that specified nymphal bugs had been recorded on the plant in question, and the number of host plant species in each family was plotted (Fig. 3.2).

Plants in the genus *Cullen* had the highest relative abundance of green mirids (Appendix; A3.2), but the abundance of an insect on a host plant is also affected by site-specific factors. We therefore analysed *C. dilutus* abundance on a site by site basis, considering only sites where *Cullen* hosts

were sampled and more than two *C. dilutus* individuals had been sampled on another host using the standardised sampling outlined above. The abundance of green mirids across different host plants at each of these seven sites (of 22 sites in total) was compared using a one-way ANOVA. Appropriate transformations were applied to the data to conform to ANOVA assumptions (Fig. 3.3). *Post hoc* pair wise comparisons of means were made using Fisher's LSD test, with the experiment-wise alpha-level (0.05) maintained using a Benjamini-Hochberg correction (Benjamini & Hochberg 1995).

To investigate the immediate feeding history of bugs relative to the plant species from which they had been sampled, we amplified chloroplast intergenic spacers from both the insects and plants sampled. We selected sites where sufficient *C. dilutus* had been collected from several hosts including *Cullen*, and we extracted DNA from all plants that had returned at least one mirid in the quantified sampling. DNA was extracted from these putative host plants using a CTAB protocol (Doyle & Dickson 1987), and from *C. dilutus* thorax and abdomens using QIAGEN DNeasy tissue kits (Qiagen). The *trnL-trnF* intergenic spacer was amplified for both putative hosts, and insect gut contents, using the *trnL* e (B49873: GGTTCAAGTCCCTCTATCCC) and *trnF* f (A50272: ATTTGAACTGGTGACACGAG) primers (Taberlet *et al.* 1991). PCR was performed using Platinum Taq (Invitrogen), 0.2-0.4 µM of each primer, and 1.5-3µM of MgCl. PCR cycling conditions were similar to those detailed by (Jurado-Rivera *et al.* 2009), with a touchdown of one degree per cycle (18 cycles) from 60^{o}C to 43^{o}C annealing temperature (60s), and 27 additional cycles at 42^{o}C. Denaturation was 94^{o}C for 30s, and elongation was 72^{o}C for 45s. Amplicons were sequenced bi-directionally on an ABI 3730 (Macrogen). Sequences were edited using CodonCode Aligner. Plant derived sequences were used to construct a local BLAST database in Geneious (Drummond AJ 2010), and insect-gut derived sequences were batch blasted (*blastn*) against this database, and against the nr/nt database (NCBI, Genbank). When the BLAST search indicated a hit the insect-derived sequence was pair wise aligned with the plant-derived sequence using ClustalW [40], and a hit was defined using a 2% base difference threshold (Table 3.1). Host plant sequences

(JX134198 – JX134221), and gut content sequences (JX134132 – JX134197), were deposited in Genbank.

Chapter 4. Gene flow in the green mirid, Creontiades dilutus (Hemiptera: Miridae), across arid and agricultural environments with different host plant species.

4.1 Abstract

Creontiades dilutus (Stål), the green mirid, is a polyphagous herbivorous insect endemic to Australia. Although common in the arid interior of Australia and found on several native host plants that are spatially and temporally ephemeral, green mirids also reach pest levels on several crops in eastern Australia. These host associated dynamics, distributed across a large geographic area, raise questions as to whether (i) seasonal fluctuations in population size result in bottlenecks and drift, (ii) arid and agricultural populations are genetically isolated, and (iii) the use of different host plants results in genetic differentiation. We sequenced a mitochondrial COI fragment from individuals collected over 24 years and screened microsatellite variation from 32 populations across two seasons. The predominance of a single COI haplotype and negative Tajima D in samples from 2006/2007 fit with a population expansion model. In the older collections (1983 and 1993) a different haplotype is most prevalent, consistent with successive population contractions and expansions. Microsatellite data indicates recent migration between inland sites and coastal crops and admixture in several populations. Altogether, the data suggest that long distance dispersal occurs between arid and agricultural regions, and this, together with fluctuations in population size, lead to temporally dynamic patterns of genetic differentiation. Host associated differentiation is also evident between mirids sampled from plants in the genus *Cullen* (Fabaceae), the primary host, and alternative host plant species growing nearby in arid regions. Our results highlight the importance of jointly assessing natural and agricultural environments in understanding the ecology of pest insects.

4.2 Introduction

Many insects that damage agricultural crops have invaded the resources provided by agriculture across wide areas and this has generated alternative predictions as to their evolutionary trajectories. Although the provision of novel resources by agriculture might promote host-adaptation (Via 1990), it has also been argued that gene flow will increase among populations of native insects when their range is expanded through the anthropogenic spread of potential hosts, making local adaptation less

likely (Oliver 2006). Insects that use both native and introduced hosts thus provide a "natural experiment" to explore the likely consequences of ongoing anthropogenic change in plant distribution and abundance.

Few genetic studies have examined the interactions of insects between both native host plants and agricultural resources simultaneously, but the available evidence indicates that several outcomes are possible, including geographic differentiation, host-associated differentiation and widespread gene flow. Both the rice mirid *Stenotus rubrovittatus* (Hemiptera: Miridae), native to Japan, and Queensland fruit fly *Bactrocera tryoni* (Diptera: Tephritidae) show strong geographic differentiation (Kobayashi *et al.* 2011; Yu *et al.* 2001). In the former it indicates divergence across Pleistocene refuges and the latter divergence since invading crop and fruit hosts outside its original Queensland distribution. Furthermore, an isolated population in inland Australia (Alice Springs) showed strong genetic evidence of a population bottleneck. Host-associated differentiation has also been recorded, in the corn leafhopper *Dalbulus maidis* (Hemiptera: Cicadellidae), and this has been associated with a shift from wild hosts to maize (*Zea mays*) within the last 9000 years since domestication (Medina *et al.* 2011). In contrast to the above examples, a lack of isolation by distance was found across 1700km in the migratory moth *Trichoplusia ni* (Lepidoptera: Noctuidae), between its native range in California, and crops that it seasonally invades in Canada (Franklin *et al.* 2010). Clearly the patterns found to date are strongly influenced by the biology and life history of the organism in question, as well as the environment it inhabits.

Strong regional differentiation, as found in *Stenotus rubrovittatus* (Kobayashi *et al.* 2011), and Queensland fruit fly (Yu *et al.* 2001), might be expected in species that do not regularly disperse long distances (Bohonak 1999). Conversely, high gene flow, as documented for the migratory moth *Trichoplusia ni* (Franklin *et al.* 2010), has also been reported in many widespread agricultural pests (Endersby *et al.* 2007; Endersby *et al.* 2006; Margaritopoulos *et al.* 2009), and even in pest species thought to be relatively sedentary (Voudouris *et al.* 2012). Anthropogenic and unassisted dispersal can both allow the invasion of novel resources by insect populations (Stone *et al.* 2007; Stone & Sunnucks 1993). Such anthropogenic dispersal was thought to be the primary mechanism allowing colonisation of grain storages by *Tribolium castaneum*, as this species was considered relatively sedentary (Drury *et al.* 2009). Active dispersal by flight has subsequently been shown to better explain patterns of regional genetic differentiation (Ridley *et al.* 2011; Semeao *et al.* 2012), highlighting that the capacity of organisms to disperse can be underestimated.

Another important aspect of pest insect dynamics is fluctuations in population size, which are expected based on the seasonal availability of most agricultural crops and the occurrence of pest outbreaks. Temporal fluctuations in gene frequencies are tied to the number of effective breeders (Waples & Teel 1990), and the temporal stability of regional genetic structure recorded across 5 years of sampling in the Queensland fruit fly implies that populations of sufficient size persist across seasons, despite the occurrence of regional outbreaks of this species (Yu *et al.* 2001). Regional differences in outbreak propensity in the migratory locust (*Locusta migratoria*) have allowed an empirical evaluation of its effects (Chapuis *et al.* 2009; Chapuis *et al.* 2008). No difference was found in genetic diversity between outbreak and non-outbreak populations (indicating that non outbreak populations persist in sufficient size) but regional differentiation was much higher for non-outbreak populations (Chapuis *et al.* 2009; Chapuis *et al.* 2008). Spatial and temporal variance in population size, migration rates, and extinction rates are predicted to not only affect mean $F_{ST,}$ but also result in large fluctuations in the genetic differentiation between populations over time (Whitlock 1992).

Patterns of host associated differentiation might be obscured by migration, bottlenecks and population expansion, and interpreting the relative effects of demographic processes remains a challenge for empirical population genetics (Li *et al.* 2012; Pavlidis *et al.* 2008). Furthermore, host plant associated differentiation following host shifts is considered more likely in host specialists (Funk *et al.* 2002). Few studies report host associated differentiation in insects that use multiple host plants (but see (Sword *et al.* 2005)). Geographic differentiation generally appears to be higher in host plant specialists than insects that use multiple hosts (Gaete-Eastman *et al.* 2007; Groot *et al.* 2011; Habel & Meyer 2009; Kelley *et al.* 2000; Zayed *et al.* 2005). This correlation is usually interpreted as a consequence of the spatial patchiness of a single resource in comparison to the more widespread availability of multiple resources. Resources can, however, be temporally patchy and it is not clear whether this correlation would hold under these circumstances. In this study we examine dispersal, fluctuations in population size, and the use of multiple host plants in an insect herbivore across both its native range (and host plants) in arid regions of Australia, and novel hosts (agricultural crops) that it has invaded within the last 200 years.

Creontiades dilutus is a mirid bug that is endemic to Australia and is a major pest of cotton (Malipatil & Cassis 1997; McColl *et al.* 2011). This species has been associated with numerous host plant species and, prior to the advent of agriculture in Australia, was probably restricted to the relatively open interior. Here, the temporal variability of rainfall events is higher than in most other globally comparable desert systems with similar mean annual rainfall (Morton *et al.* 2011).

Variability is compounded by years of drought (Letnic & Dickman 2006; Nicholls 1991). The availability of herbaceous plants is consequently ephemeral and often spatially patchy. During the summer months of November to February *C. dilutus* persists in this region in low numbers despite temperatures in excess of 45^{o}C and the availability of few host plants (JPH *pers. obs.*, Jan. 2007). Host plants persist longer in winter (June-August), but only if sufficient rain falls. With the exception of "flood years", when host plants may be unusually widespread and persistent, suitable hosts generally require two or more successive rain events to thrive. These rain events usually occur only locally and typically the hosts are spatially patchy, with large areas of barren land between. Inland temperatures are close to optimal for mirid development during winter, allowing a generation time of around 25 days (Khan *et al.* 2009). Rapid population expansion is thus possible and large numbers of *C. dilutus* can be found where conditions are suitable.

Despite being associated with 37 native herbaceous plant species in arid Australia, *C. dilutus* is consistently more abundant on two species in the genus *Cullen* (Fabaceae), *Cu. australasicum* and *Cu. cinereum* (Hereward & Walter 2012). These two plant species are thus the likely primary hosts of these bugs. However, molecular analysis of chloroplast sequences from the gut contents of individual green mirids showed that even when collected from these primary hosts a significant proportion had fed from other plant species (Hereward & Walter 2012). This suggests that the capacity to feed across multiple host plant species might be a behavioural adaptation that enables survival when the primary hosts are not available.

Since European settlement of Australia, the introduction of widespread agriculture and associated land-clearing in sub-coastal regions has enabled herbaceous plants to grow more widely and abundantly. Green mirids are supported in agricultural systems throughout the year but mostly in association with crops and introduced plant species, especially lucerne (*Medicago sativa*, Fabaceae) (Miles 1995). Summer temperatures in sub-coastal Australia are close to optimal for mirid development, but low winter temperatures support only slow rates of development and mirid abundance is low; this is the reverse of what happens in arid regions where they are most abundant during winter months. The movement of *C. dilutus* onto cotton crops in late spring has been characterised by a sudden synchronous increase in numbers early in the growing season, and the source of these insects remains unknown, but does not appear to be lucerne or other local hosts (Miles 1995).

Creontiades dilutus presumably relies primarily on dispersal to cope with adverse conditions by locating suitable patches of host plants when local conditions become unfavourable, because

diapause (based on current evidence) is a facultative winter reproductive phenomenon (Miles 1995). Whereas *C. dilutus* persists throughout the year in the arid interior and sub-coastal agricultural regions of Australia, its abundance is seasonally inverse between the two. It also uses different host plant resources, and is likely to experience different selection pressures, across these two regions. The potential for *C. dilutus* to move between central Australia and the eastern cropping regions, perhaps on prevailing winds or storm fronts, has been suggested (Miles 1995). An alternative model is that discrete populations occur in the inland and coastal regions, with little gene flow between them. The extent of gene flow between these two regions is likely to affect any response of this species to selection, and thus the extent to which adaptation to novel hosts is likely, and this has yet to be determined.

In inland Australia *C. dilutus* relies on host plants that are both spatially and temporally highly variable, even within a single season. Local population extinctions and founder effects might be expected, especially during dry years, as local resources die off and new patches are located. Conversely, when inland Australia experiences floods the increased host abundance together with the short generation time of this multivoltine insect are likely to allow massive increases in population size across large areas. Pesticides applied in agricultural regions also have the potential to cause localised population contractions, and although green mirids are present on lucerne throughout the year in agricultural regions their abundance during winter months is low (Miles 1995).

We sampled mirids across both arid and agricultural regions in Australia, covering most of the geographic distribution of this endemic species and including the major host plants. We genotyped microsatellites from samples spanning two seasons and sequenced a mitochondrial COI fragment from green mirids collected over 24 years. Given the ecology of green mirids outlined above, and the challenges posed by both agricultural and arid environments we structured our analyses according to the following three questions: (i) Do seasonal fluctuations in population size in both arid and agricultural regions result in genetic signatures of bottlenecks and drift? (ii) Does long distance dispersal occur between arid and agricultural populations? (iii) Is genetic differentiation associated with the use of multiple host plants in the (arid) native range? We found that genetic patterns in *C. dilutus* are temporally dynamic, consistent with spatial and temporal heterogeneity in its arid range. Long distance dispersal between arid and agricultural populations is evident from the data, and host associated differentiation was found between the primary host plants and alternative hosts in arid regions. Together, these results highlight the importance of considering ecological and evolutionary processes across the distribution of an organism.

4.3 Materials and Methods

4.3.1 Mitochondrial DNA sequencing and analysis

Ten population samples were taken during 2006 and 2007 from across Australia, and 146 of these individuals were sequenced for the COI fragment using the Folmer primers LCOI490 and HC02198 (Folmer *et al.* 1994) and a standard PCR protocol with an annealing temperature between 47^{o}C and 50^{o}C. We also obtained pinned specimens retained at The University of Queensland from previous research on this species. We were able to amplify the same fragment from 16 individuals collected in Gatton (Queensland) from lucerne in 1983 and 25 individuals collected in Biloela (Queensland) from lucerne in 1993. DNA was extracted from the pinned specimens using Qiagen DNeasy columns after being soaked in TE buffer overnight. The PCR protocol was the same as for the ethanol preserved specimens. The COI fragments were sequenced bi-directionally at Macrogen (Korea) on an ABI3730, and then aligned, edited and trimmed using Codon Code Aligner v4.0. Details of the sample locations and Genbank accession numbers are given in Table 4.1.

Haplotype networks were constructed using the R package TempNet (Prost & Anderson 2012). The temporal haplotype network was restricted to sites in the eastern Queensland cropping region (48 samples from 2006/2007) where the samples from 1983 and 1993 had been collected. Nucleotide and haplotype diversity were calculated in DnaSP v. 5 (Librado & Rozas 2009).

The utility of mitochondrial markers for phylogeographic studies in insects could be compromised by fixation induced by endosymbionts that generate cytoplasmic incompatibility (Ballard & Whitlock 2004; Hurst & Jiggins 2005). We therefore screened 24 individuals from 2006/2007 that returned the most prevalent COI haplotype (see results) for the presence of *Wolbachia* using the wsp81 and wsp691 primers (Zhou *et al.* 1998), in case this common symbiont was present in our samples.

Table 4.1 – Sampling locations, host plants and genbank accession numbers for the COI sequences used in the analyses presented in this chapter.

Location	Date	Latitude (S)	Longitude (E)	Host plant	N	Genbank Acessions
Adelaide	2/12/2006	-34.82081	138.86996	Polygonum convolvulus	8	JX186015 to JX186022
Balingup	14/09/2007	-33.78890	115.97597	Solanum nigrum	8	JX186023 to JX186030
BarcLong	16/08/2006	-23.53322	145.07654	Cullen cinereum	8	JX186031 to JX186038
Biloela	10/01/2007	-24.37389	150.51298	Gossypium hirsutum	10	JX186039 to JX186048
Biloela	10/01/2007	-24.37389	150.51298	Medicago sativa	8	JX186049 to JX186056
Emerald	14/08/2006	-23.49576	148.18842	Verbesina enceliodes	8	JX186057 to JX186064
Emerald	15/08/2006	-23.57219	148.10006	Verbesina enceliodes	4	JX186065 to JX186068
Emerald	15/08/2006	-23.46627	148.09175	Vicia sativa	8	JX186069 to JX186076
Kununurra	28/08/2006	-15.64590	128.69688	Gossypium hirsutum	5	JX186077 to JX186081
Longreach	17/08/2006	-23.41773	144.22744	Cullen cinereum	8	JX186082 to JX186089
Longreach	17/08/2006	-23.40377	144.22121	Cullen cinereum	8	JX186090 to JX186097
Longreach	17/08/2006	-23.43817	144.24575	Medicago polymorpha	8	JX186098 to JX186105
Longreach	17/08/2006	-22.89413	143.78673	Swainsona galegifolia	8	JX186106 to JX186113
Narrabri	22/01/2007	-30.20075	149.57236	Gossypium hirsutum	6	JX186114 to JX186119
Narrabri	22/01/2007	-30.20075	149.57236	Medicago sativa	7	JX186120 to JX186126
Walget	31/08/2006	-29.91241	146.91791	Rapistrum rugosum	8	JX186127 to JX186134
WintJun	18/08/2006	-22.41200	143.05851	Cullen cinereum	7	JX186135 to JX186141
WintJun	19/08/2006	-23.78104	142.46578	Cullen cinereum	4	JX186142 to JX186145
WintJun	19/08/2006	-23.73375	142.42869	Senna Artemisioides	5	JX186146 to JX186150
Biloela	20/09/1993	-24.37389	150.51298	Medicago sativa	12	JX186151 to JX186162
Biloela	5/05/1993	-24.37389	150.51298	Medicago sativa	13	JX186163 to JX186175
Gatton	16/01/1983	-27.58760	152.36181	Medicago sativa	16	JX186176 to JX186191
Byee	14/03/2006	-26.25660	151.85388	Cajanus cajan	10	EF016724 to EF016733

4.3.2 Microsatellites - sample collection and genotyping

A total of 32 population samples was collected from 17 different host plant species in inland Australia and sub-coastal eastern Australia between January 2007 and March 2008 (Table 4.2). Individual insects were preserved in 96% ethanol. DNA was extracted using a modified salt precipitation protocol based on that of Miller *et al.* (1988). Nine microsatellites (mirsat-2F, mirsat-4B, mirsat-3E, mirsat-A1, mirsat-3H, mirsat-6B, mirsat-5C, mirsat-G8, and mirsat-7G) were PCR amplified and genotyped on a Megabace capillary electrophoresis system (Amersham Biosciences) as per (Andris *et al.* 2010). Microsatellite peaks were confirmed and binned manually. In total, 768 specimens were genotyped; the DNA extractions of individuals that failed to amplify at more than six loci were assumed to be low quality and were discarded, leaving 665 genotyped individuals (Table 4.2).

Table 4.2 Population codes, number of individuals genotyped, collection details and host plant species for population samples genotyped with microsatellites in this chapter.

Code	N	Location	Date	Host Plant	Family
BIL-GH	19	Biloeala	9/01/2007	*Gossypium hirsutum*	Malvaceae
BIL-MS1	16	Biloeala	9/01/2007	*Medicago sativa*	Fabaceae
BIL-MS2	15	Biloeala	28/07/2007	*Medicago sativa*	Fabaceae
EMR-VE1	24	Emerald	10/01/2007	*Verbesina encelioides*	Asteraceae
EMR-MS	29	Emerald	10/01/2007	*Medicago sativa*	Fabaceae
EMR-GH	26	Emerald	10/01/2007	*Gossypium hirsutum*	Malvaceae
EMR-CA	21	Emerald	29/07/2007	*Cicer arietinum*	Fabaceae
EMR-VE2	26	Emerald	29/07/2007	*Verbesina encelioides*	Asteraceae
BIR-BS	12	Birdsville	3/08/2007	*Epaltes cunninghamii*	Asteraceae
SIM-BP1	19	Simpson Desert	4/08/2007	*Blennodia pterosperma*	Brassicaceae
SIM-GC	13	Simpson Desert	4/08/2007	*Goodenia cycloptera*	Goodeniaceae
SIM-SG1	13	Simpson Desert	4/08/2007	*Senecio gregorii*	Asteraceae
SIM-BP2	9	Simpson Desert	5/08/2007	*Blennodia pterosperma*	Brassicaceae
SIM-SG2	11	Simpson Desert	5/08/2007	*Senecio gregorii*	Asteraceae
SIM-CE	23	Simpson Desert	6/08/2007	*Crotalaria eremaea*	Fabaceae
SIM-CA	29	Simpson Desert	7/08/2007	*Cullen australasicum*	Fabaceae
EYR-CA	28	Eyre Creek	7/08/2007	*Cullen australasicum*	Fabaceae
MIL-TS	29	Milparinka	10/08/2007	*Trigonella suavissima*	Fabaceae
MIL-SG	26	Milparinka	10/08/2007	*Swainsona galegifolia*	Fabaceae
MIL-SI	29	Milparinka	10/08/2007	*Sisymbrium irio*	Brassicaceae
MIL-CP	17	Milparinka	10/08/2007	*Cullen pallidum*	Fabaceae
TIL-EC	26	Tilpa	11/08/2007	*Erodium cygnorum*	Geraniaceae
BOU-EC	31	Bourke	11/08/2007	*Erodium cygnorum*	Geraniaceae
WAL-MP	26	Walget	11/03/2008	*Malva parviflora*	Malvaceae
BRE-MP	19	Brewarrina	11/03/2008	*Malva parviflora*	Malvaceae
BRE-MS1	25	Brewarrina	11/03/2008	*Medicago sativa*	Fabaceae
BRE-EC	26	Brewarrina	12/08/2007	*Erodium cygnorum*	Geraniaceae
BRE-PC	10	Brewarrina	12/08/2007	*Phlegmatospermum cochlearinum*	Brassicaceae
BRE-MS2	12	Brewarrina	12/08/2007	*Medicago sativa*	Fabaceae
NAR-MP	9	Narrabri	13/08/2007	*Malva parviflora*	Malvaceae
NAR-GH	24	Narrabri	20/01/2007	*Gossypium hirsutum*	Malvaceae
NAR-MS	23	Narrabri	20/01/2007	*Medicago sativa*	Fabaceae

4.3.3 HWE, genetic diversity, genetic differentiation, and tests for recent bottlenecks

We estimated null allele frequency using the expectation maximisation algorithm of Dempster *et al.* (1977) implemented in FreeNA (Chapuis & Estoup 2007) with 10,000 bootstrap resamplings. Deviations from Hardy Weinberg Equilibrium (HWE) were calculated using the exact probability test (Guo & Thompson 1992) implemented in Genepop (Rousset 2008) and a sequential Bonferroni correction was applied per locus to account for multiple tests. Locus mirsat-3E showed deviations

from HWE in many samples and was consequently shown to have relatively high frequencies of null alleles (Table 4.3) and was discarded. The total number of alleles per locus, average number of alleles per locus, and (Nei 1987) unbiased gene diversity (per locus and sample) were calculated using FSTAT (Goudet 2001). Expected (He), observed (Ho) and unbiased expected (UHe) heterozygosities were computed using Genalex (Peakall & Smouse 2006). Exact tests for linkage disequilibria were carried out in Genepop (Rousset 2008).

The proportion of genetic variance that can be attributed to within population comparisons and between population comparisons was estimated using an analysis of molecular variance (AMOVA) in Genalex (Peakall & Smouse 2006). Unbiased pairwise and locus specific F_{ST}'s (Weir 1996) were computed with and without the algorithm for the exclusion of null alleles (ENA) implemented in FreeNA (Chapuis & Estoup 2007). Pairwise exact tests of genotypic differentiation were computed using Genepop (Rousset 2008), as this estimator is more appropriate in situations where gene frequencies may deviate from HWE expectations, and a sequential Bonferroni adjustment was performed to account for multiple population comparisons.

To test for signatures of recent demographic bottlenecks in the microsatellite data, the Wilcoxon test for heterozygote excess (under the two-phase mutation model) and the allele frequency mode shift analysis were performed using the program BOTTLENECK (Piry *et al.* 1999) for all 32 populations, and a sequential Bonferroni adjustment applied.

4.3.4 Spatiotemporal patterns of genetic differentiation and tests for migration

We tested for the presence of isolation by distance (IBD) to explore gene flow in relation to the temporal and geographic aspects of the sampling strategy. Initially this analysis was restricted to the sampling period of July to August 2007 (when samples were widespread geographically and collected over a short period from both agricultural hosts and native inland hosts). Subsequently, all samples were analysed to assess the temporal stability of the August 2007 pattern, these additional samples represented the same agricultural crops sampled in 2007. The presence of an IBD effect was investigated by regressing ENA corrected genetic distance (F_{ST} / (1-F_{ST})) against geographic distance (Rousset 1997). A Mantel test of matrix correspondence was used to test for significance using the Isolation By Distance Web Service (IBDWS) 3.15 (Jensen *et al.* 2005).

Patterns of genetic differentiation and admixture, which may be obscured by statistics that assume the correct *a priori* identification of populations, were clarified with the individual-based Bayesian

clustering algorithm implemented in the program STRUCTURE (Pritchard *et al.* 2000). Low levels of null alleles are unlikely to affect the overall outcome of assignment testing such as the one implemented in the STRUCTURE algorithm (Carlsson 2008). The 'admixture' model was used as the most appropriate for a species in which dispersal is likely. Initially, values of K from 1 to 14 were used, with a burn-in of 50000 and a run length of 500000, and each K value was replicated 3 times. These results were exported to the program STRUCTURE HARVESTER (Earl & VonHoldt 2011) and the most likely value of K for the data set was inferred using the ΔK method of Evanno *et al.* (2005). The data were then analysed using this value for K with a burn-in of 100,000 and 1,000,000 subsequent iterations; this was replicated 10 times. The results were permuted with CLUMPP (Jakobsson & Rosenberg 2007) and the mean of the permuted results plotted using DISTRUCT (Rosenberg 2004).

Recent migration between arid inland sites and the eastern cropping regions was tested with the Bayesian-assignment based algorithm implemented in BAYESASS, which estimates rates of recent migration (m) (Wilson & Rannala 2003). This algorithm represents a major advance in the analysis of recent migration events as it does not assume that each designated population is in Hardy-Weinberg equilibrium, and produces reasonable estimates of actual migration in an experimental setting (Mardulyn *et al.* 2008). The results of the IBD test indicated that genetic differentiation is unstable across seasons, so we restricted our analysis of gene flow to estimators of recent migration rather than coalescent approaches to estimating long term averages of migration such as MIGRATE (Beerli & Felsenstein 2001). Population samples were grouped by location and then split by the time of collection, because the results of the STRUCTURE analysis indicated significant temporal shifts in cluster assignment within sites. The BAYESASS computation was performed 10 times with different starting seeds to assess convergence across runs. The results of the 10 runs were converted to tabular format using a custom Perl script (Appendix; A4.2) for comparison. The number of times each outcome was achieved over the 10 runs was recorded, and the mean migration rates were calculated for each of these outcomes. Migration rates with lower 95% confidence intervals below m = 0.02 were not considered significant and were also omitted. We used the lower 95% CI to assess the significance of migration rates because experimental tests in *Caenorhabditis remanei* indicate that actual migration rates tend to be lower than the inferred rates but within the 95% CI (Mardulyn *et al.* 2008).

4.3.5 Host plant associated differentiation

Hierarchical AMOVA was performed across all 32 populations, with the higher order defined as host-plant in Genalex (Peakall & Smouse 2006). The host plants in central Australia, however, are completely different species to those used by green mirids in eastern cropping regions. To test the hypothesis that differentiation might be associated with host plant usage we tested for genetic differentiation across host plants with respect to two inland localities within which multiple host plants had been sampled (namely Simpson Desert and Milparinka). The STRUCTURE algorithm was run using the admixture model with a burn-in of 100,000 and 500,000 subsequent iterations, with K = 2 "population" clusters.

4.4 Results

4.4.1 Mitochondrial

Genetic diversity was low for the mitochondrial COI sequences, with one haplotype dominating each of the three temporal samples. The 2006/2007 samples (n = 146) had a nucleotide diversity (Pi) of 0.00055, and a haplotype diversity of 0.278. Tajima's D was -2.26 ($P < 0.01$) indicating an excess of low frequency polymorphisms likely due to population expansion. This pattern was similar in 1983 (n = 16), where nucleotide diversity was 0.00021, haplotype diversity 0.125, and Tajima's D -1.16, but not significant ($P>0.10$), and in 1993 (n = 25), Pi = 0.00026, haplotype diversity 0.153, and Tajima's D -0.69, also not significant ($P>0.10$).

In the eastern cropping regions of Queensland, where a comparison of haplotype frequencies could be made across three temporal samples spanning 24 years, there has been a shift in the dominant haplotype between 1993 and the more recent samples (Fig. 4.1, bottom). The haplotype that was prevalent across the whole of Australia in 2006/2007 (Fig. 4.1 middle) was present at much lower frequency in the earlier samples (1983 and 1993). Our screen for *Wolbachia* was negative in all cases (data not shown).

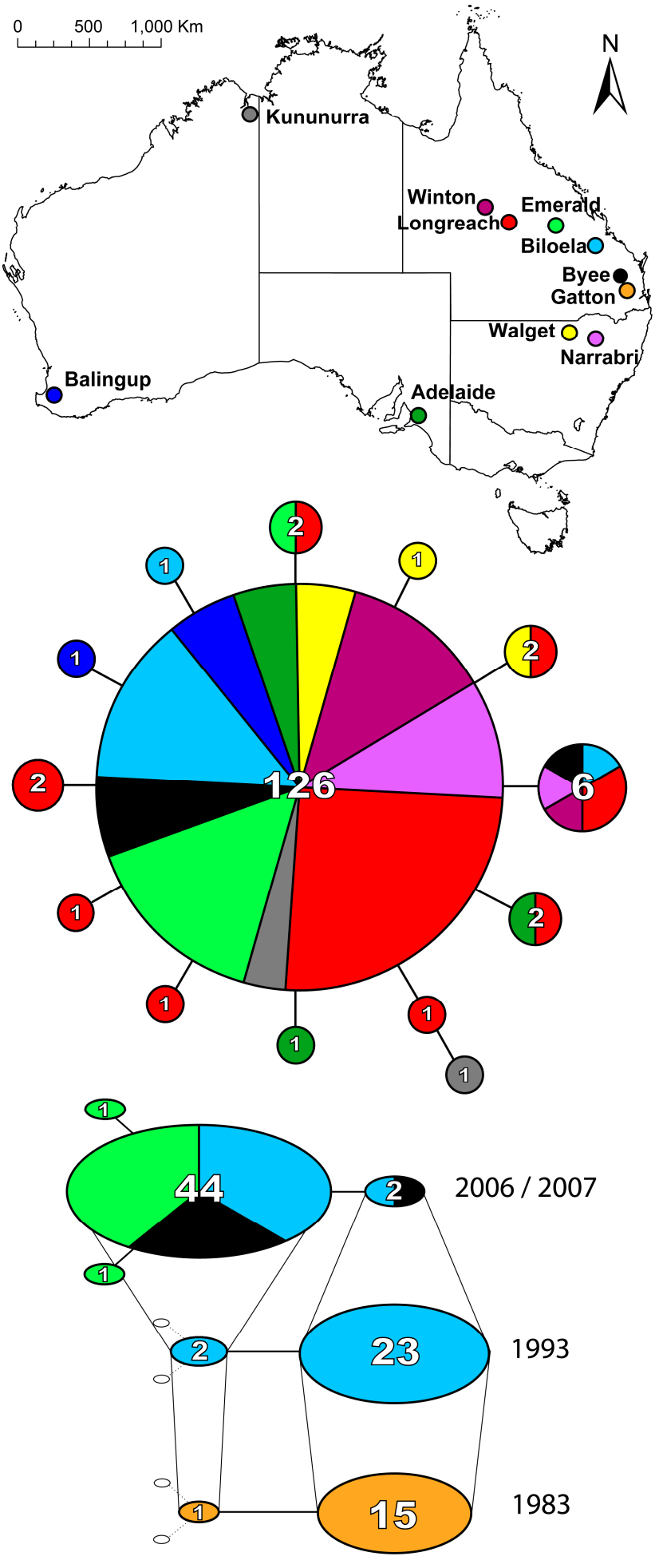


Figure 4.1 Map (top) showing sampling locations for the COI sequences obtained for this study (location colours maintained throughout the figure). Haplotype network (middle) showing all sequences from 2006/2007, and temporal haplotype network (bottom) showing the change in haplotype frequencies in Eastern Queensland between 1983 and 2006/2007. The area of each haplotype (circles and ellipses) represents the number of individuals having that haplotype (numbers inside haplotypes), empty ellipses (bottom figure) show haplotypes present in the 2006/2007 samples but not in 1993 or 1983.

4.4.2 HWE, genetic diversity, genetic differentiation, and tests for recent bottlenecks

A total of 105 alleles was scored across all loci and all populations, once the null-allele prone locus mirsat-3E had been removed (Table 4.3). Unbiased gene diversity for each population (Nei 1987), when averaged across loci, ranged between 0.32 and 0.79 (mean 0.51) and was not significantly different between samples from agriculture and those taken inland (Fig. 4.2, two tailed permutation test, P = 0.275). Four of 32 population samples, three from inland and one from agriculture, showed genetic signatures indicating a recent bottleneck in the allele mode shift analysis (BIR-BS, SIM-SG1, SIM-SG2, BIL-GH, Fig. 4.2), although only two of these showed a significant heterozygote excess in the Wilcoxon test (BIR-BS, P = 0.0117 and BIL-GH, P = 0.0078). In addition, three of the four populations showed indications of admixture, and neither of the Wilcoxon tests were significant (with an alpha probability of P > 0.05 after Bonferroni correction).

Table 4.3 Locus specific details for microsatellites used in this study; ∑Na, total number of alleles, $\hat{N}a$, average number of alleles per population sampled, Ho, observed and He, expected heterozygosities, HWD (number of population samples deviating from Hardy Weinberg Equilibrium), null allele frequencies, and locus specific global F_{ST} without and with the exclusion of null alleles.

Locus	∑Na	$\hat{N}a$	Ho	He	HWD	Null alleles	gF_{ST} Null	gF_{ST} No Null
mirsat-2F	11	4.88	0.52	0.56	2	0.051	0.17	0.17
mirsat-4B	10	3.61	0.31	0.46	2	0.080	0.27	0.25
mirsat-3E	16	5.97	0.25	0.56	13	0.199	0.21	0.18
mirsat-A1	9	2.52	0.19	0.26	5	0.074	0.19	0.21
mirsat-3H	21	5.48	0.41	0.42	1	0.048	0.09	0.08
mirsat-6B	6	1.76	0.03	0.09	0	0.067	0.03	0.09
mirsat-5C	20	7.94	0.82	0.77	0	0.034	0.04	0.04
mirsat-G8	13	4.67	0.30	0.54	6	0.174	0.03	0.03
mirsat-7G	15	5.91	0.69	0.68	2	0.038	0.06	0.06

Deviations from HWE were inferred in all loci for some populations, and the presence of null alleles was also inferred (Table 4.3). HWE deviations might, however, be expected in recently admixed populations due to the Wahlund effect. We took three approaches to assess and minimise the effects of null alleles: 1) estimation of F_{ST} values using null allele corrected and non corrected data, 2) removal of the two loci that had the greatest effect on HWE (mirsat1A1 and mirsat2G8), and then comparing results across the 6 locus and 8 locus data sets, and 3) selection of analyses that are more robust to low frequencies of null alleles and small deviations from HWE (see methods for details). Evaluating the effects of null allele/HWE deviations using these three methods revealed that the low frequencies of null alleles inferred in some population samples for some loci did not dramatically affect the overall signal in the data, and all results shown are for the 8-locus dataset. Tests for genotypic linkage disequilibria returned no significant associations between pairs of loci for any of the 32 population samples after sequential Bonferroni correction for multiple comparisons.

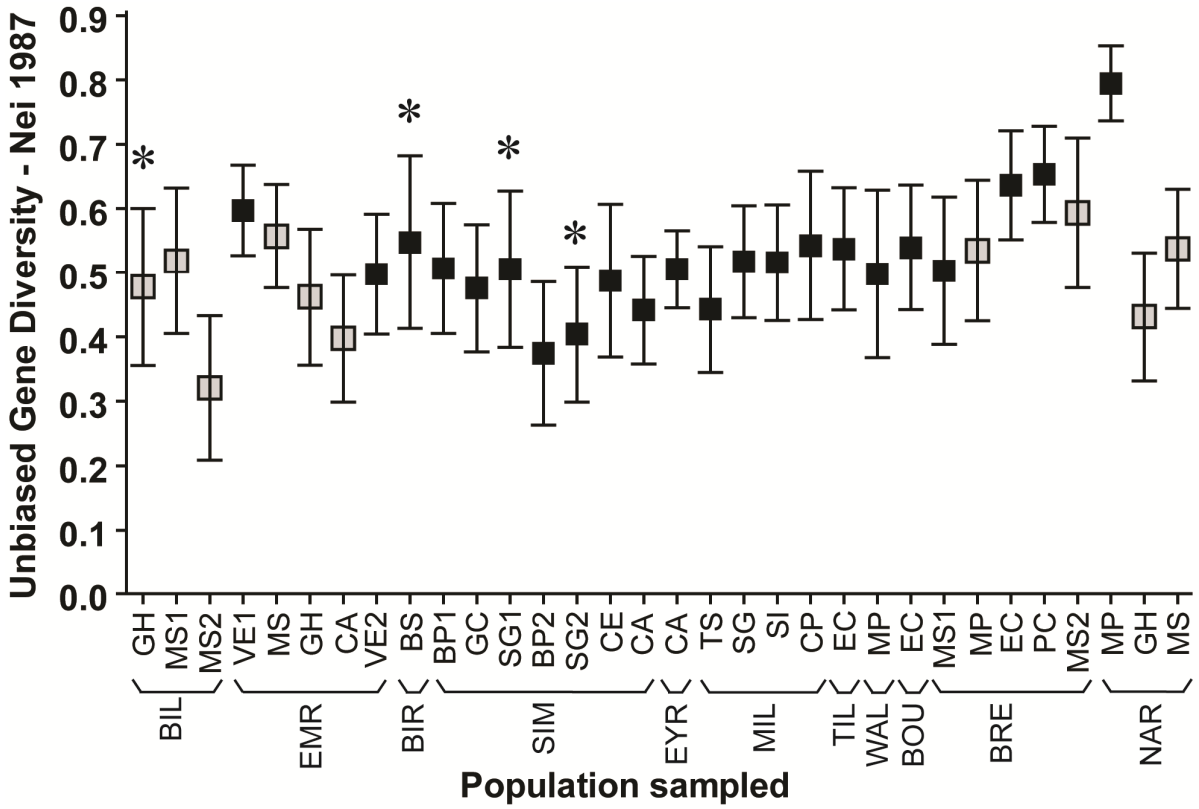


Figure 4.2 Nei's unbiased gene diversity averaged across loci for all populations. Grey boxes represent samples collected from agricultural crops; black boxes represent samples collected from non-crop hosts. Asterisks indicate population samples for which there is some evidence of a recent bottleneck (see results for details).

The AMOVA apportioned 19% of all molecular variance to among-population comparisons (Φ_{PT} = 0.188, P = 0.001). The global F_{ST} estimates were similar with or without the elimination of null alleles, with the uncorrected data returning only a slightly higher estimate (F_{ST} = 0.122 using the ENA algorithm and 0.128 without ENA correction). Pairwise F_{ST}'s ranged from 0.0019 to 0.329 (mean = 0.112), with 374 of 528 comparisons of genotypic differentiation being significant after sequential Bonferroni correction (Appendix; A4.3).

4.4.3 Spatiotemporal patterns of genetic differentiation and tests for migration

The Mantel test of correspondence between geographic distance and genetic differentiation revealed a significant isolation by distance pattern when the analysis was restricted to the broad-scale geographic sampling of July to August 2007 (Fig. 4.3, r = 0.2897, P = 0.0099). In contrast, when all sampling events were included in the analysis (January 2007 – March 2008) no isolation by distance effect was evident (r = 0.0076, P = 0.4465). The inclusion of these additional samples represented the same host plants that were sampled in agricultural regions during the July - August 2007 collections.

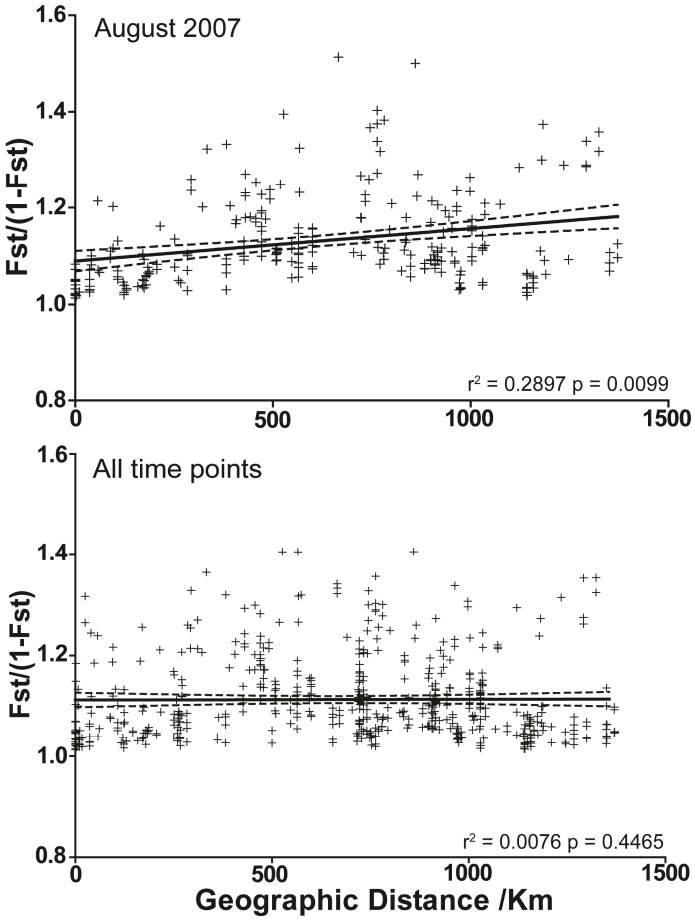


Figure 4.3 Results of Mantel test for isolation by distance. Above: Samples collected during August 2007 (r = 0.2897, p = 0.0099). Below: All samples collected (January 2007 – March 2008) (r = 0.0076, p = 0.4465). Solid line shows the regression, and dashed lines show 95% confidence intervals.

The ΔK method (Evanno *et al.* 2005) indicated that K = 3 was the most likely number of genetic "clusters" for this data set. The combined and permuted results of the subsequent 10 runs of the STRUCTURE algorithm with K set at 3 are shown in relation to the geographic origin of the population samples (Fig. 4.4). A broad geographic pattern is discernible in the assignment of individuals to clusters; individuals from Milparinka, Tilpa and Bourke are mostly assigned to one cluster with high posterior probabilities, and these populations yielded the highest pair-wise F_{ST} values when compared to the other sites (F_{ST} = 0.047-0.307; mean = 0.132; 148 of 156 tests of genotypic differentiation significant). Admixture was evident in several populations, and the proportion of admixed individuals and their cluster assignment shifted between January 2007 and July 2007 in both Biloela and Emerald, and between January 2007 and August 2007 in Narrabri (Fig. 4.4.)

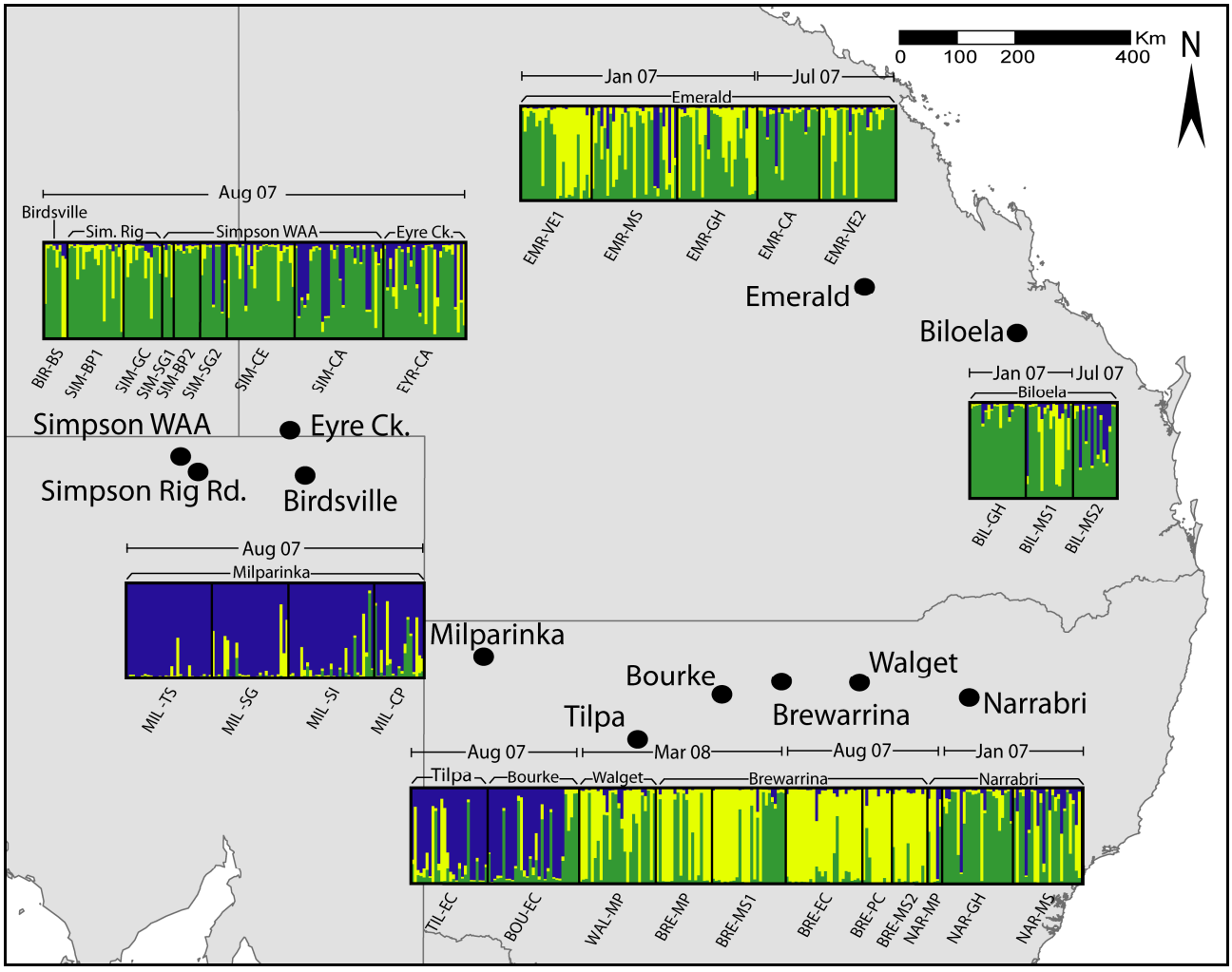


Figure 4.4 Results of STRUCTURE clustering analysis, separated into blocks showing the geographic origin and date of sampling. Each bar represents one individual; the proportion of each colour represents the posterior probability of assignment to one of three clusters.

We detected significant levels of recent migration, at the full geographic extent of sampling, using the BAYESASS algorithm. Variability was detected across runs of the algorithm, but this was characterised as a reversal of the inferred direction of migration between sites rather than changes in the sites between which dispersal was inferred. Figure 4.5 is a graphical representation of the migration rates and the frequency that each migration outcome was reached over ten runs of the algorithm using different starting seed (See Appendix; A4.3 for the full results, including 95% CI's). Significant migration was inferred between the Simpson Desert sites, in the arid interior, and the sub-coastal agricultural areas in Queensland (Biloela and Emerald) e.g. Simpson (August 2007) to Biloela (January 2007), m = 0.14, lower 95% CI = 0.08, upper 95% CI = 0.21, 6/10 runs. Although the direction of migration was most often towards agricultural regions, the direction was not always consistent across runs and strong inference cannot be made as to the direction of dispersal from this result. Migration between Milparinka and other sites was not inferred from the genetic data, which is consistent with the outcomes of the STRUCTURE analysis and pairwise F_{ST}'s.

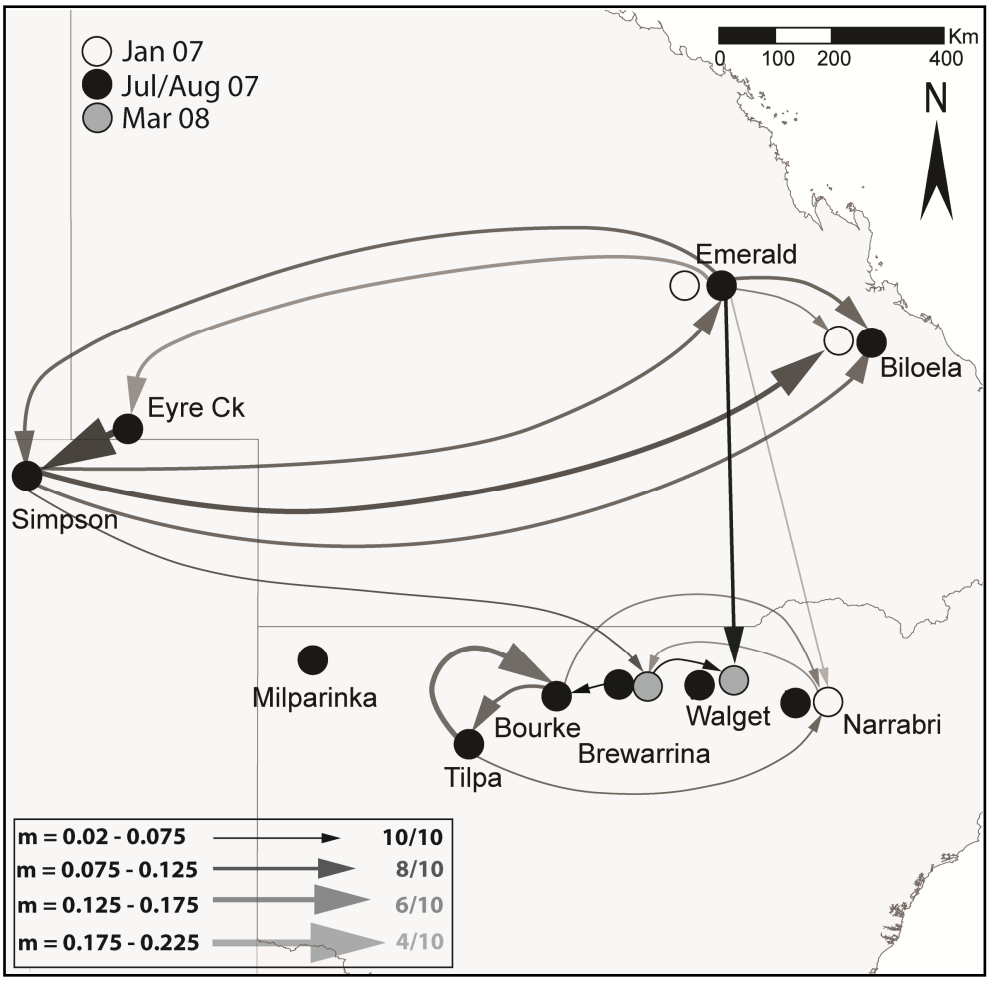


Figure 4.5 Graphical representation of migration rates inferred using the Bayesian assignment algorithm in BayesAss. The size of the arrows indicates the migration rate (m) whereas the shade of the arrows indicates the number of times this outcome was reached over 10 runs with varying starting seeds. Outcomes that were reached less than 4 times, and migration rates lower than 0.02 are not shown. (See text and Appendix; A4.3 for 95% CI's).

4.4.4 Host plant associated differentiation

The Hierarchical AMOVA indicated an effect of host plant on molecular variance (Φ_{RT} = 0.078, P = 0.001), however, host plant species were not sampled consistently across the whole of the sampling area (because each has a restricted distribution relative to the scale of the study). Genetic differentiation in relation to host plant species was therefore evaluated across two sites where several species could be sampled at each. At both of these, some degree of genetic differentiation was associated with plants in the genus *Cullen* relative to all the other host plants sampled, although this was more pronounced at the Simpson Desert sites than at Milparinka in western New South Wales (Fig. 4.6) (but note that at Milparinka *C. dilutus* was sampled from *Cu. pallidum* and at the Simpson Desert/Eyre creek sites from *Cu. australasicum*). Further examination of the genotyping data at these two sites revealed that the genetic differentiation indicated by the STRUCTURE analysis appears to stem from the higher occurrence of rare alleles on *Cullen* host plants than on alternative hosts.

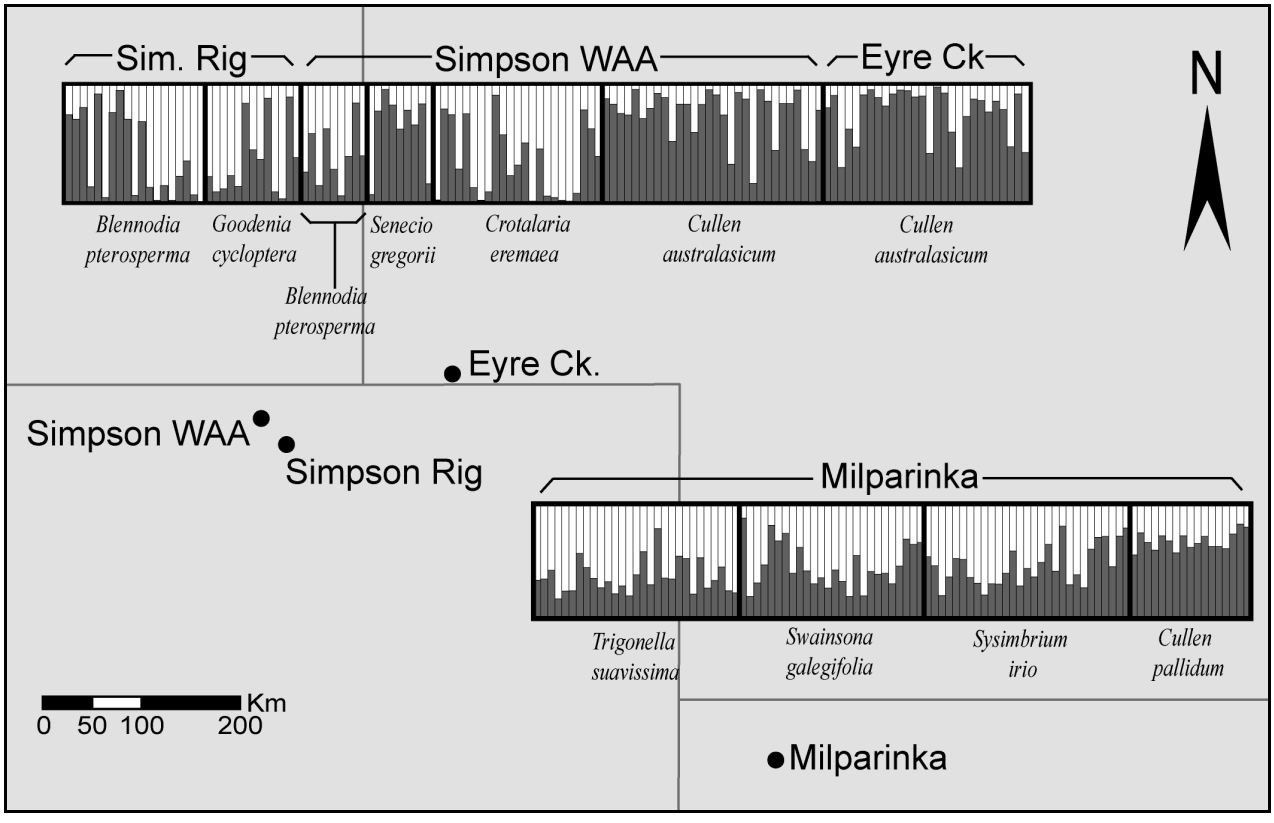


Figure 4.6 STRUCTURE analysis restricted to sites where multiple host plants were present and sampled. Each bar represents the posterior probability that the individual belongs to one of two clusters. The host plant species from which the particular samples were collected is listed below each population.

4.5 Discussion

Genetic patterns in green mirids reveal strong departures from equilibrium expectations, including changes in population size, admixture, and recent migration. Rather than fitting an equilibrium island model (Latter 1973; Wright 1931) our results are more consistent with a model of complex spatiotemporal dynamics (Wegmann *et al.* 2006; Whitlock 1992). These dynamics are likely to arise from the extreme spatial and temporal environmental heterogeneity that typify dry years in this bug's native range in arid Australia. Heterozygote excesses and allele frequency mode shifts indicate that several populations may have passed through recent localised bottlenecks, despite the short generation time and high reproductive rate of this species. The prevalence of a single COI haplotype across the whole of the continent (from 2006/2007 samples), negative Tajima D values, and the shift in the most prevalent haplotype between 1993 and 2006/2007 (Fig. 4.1) are consistent with recurrent reductions in population size over a longer period. These past reductions in population size may reflect alternate periods of drought (when resources are ephemeral and spatially patchy) and flood, which cause widespread environmental homogeneity in terms of host plant availability. The present broad geographic prevalence of the one COI haplotype, inference of recent migration from the microsatellite data (Fig. 4.5), and admixture in agricultural populations (Fig. 4.4) demonstrate that populations in arid and agricultural regions are connected by gene flow. Whereas weak genetic differentiation was detected locally (within arid regions) across their primary host plants (in the genus *Cullen*) and alternative plant species, it was mostly the presence of rare alleles that was responsible for this pattern. We suggest that rare alleles on the primary hosts (*Cu. cinereum* and *Cu. australasicum*) could be a consequence of higher abundance of *C. dilutus* relative to their abundance on alternative host species. These points are expanded and justified below.

4.5.1 Genetic diversity and founder effects

We assessed genetic diversity and possible founder effects across inland and agricultural sites to determine whether ephemeral host availability (inland) or pesticide use (in agriculture) affected the temporal stability of patterns of genetic differentiation between mirid populations. We found no significant difference in microsatellite diversity between *C. dilutus* collected from crop hosts and those collected from non-crop hosts (Fig. 4.2). Genetic signals of recent bottleneck events (heterozygote excess) were present in the microsatellite data in three inland and one agricultural population of *C. dilutus* (Fig. 4.2). None of the tests, however, were significant after Bonferroni correction, so we can only tentatively infer localised contractions. Lucerne, the primary crop host of

C. dilutus, is often grown without pesticides in Australia, and insecticide-induced bottlenecks are less likely to be driving patterns of genetic differentiation than the arid dynamics of this mirid species.

Genetic differentiation among *C. dilutus* populations was higher than generally reported for pest insect species surveyed within an agricultural context (Endersby *et al.* 2007; Endersby *et al.* 2006; Kim *et al.* 2009; Torres & Azeredo-Espin 2009). The highest F_{ST} values were attributed to pair-wise comparisons between three arid inland sites in New South Wales (Milparinka, Tilpa, and Bourke), and other populations. The Structure analysis also clearly differentiated populations at these three sites from others. Although no heterozygote excess was detected at these sites (indicative of a recent bottleneck) we suspect that a combination of spatial heterogeneity and founder effects could contribute to strong genetic drift (and therefore high genetic differentiation) given that our study was conducted during a dry period when patches of host plants were separated by large areas of barren land. Elevated F_{ST}'s are predicted (even when migration rates are high) under spatiotemporally dynamic population models if environmental heterogeneity contributes to a large variance in local population size (Wegmann *et al.* 2006), a scenario consistent with the ecology of *C. dilutus*.

The low nucleotide diversity (pi = 0.00055) and change in predominant mitochondrial haplotype over the last 13 years was striking. Mitochondrial DNA is expected to suffer a more extreme loss of alleles than nuclear markers during demographic bottlenecks due to the uniparental inheritance of the plastid and the reduced effective population size of its genome (Simon *et al.* 1994; Wilson *et al.* 1985). For example, a local population founded by a single gravid female would have one mitochondrial haplotype, but potentially 4 microsatellite alleles. Similar shallow "star shaped" genealogies and negative values of Tajima's D have been reported in agriculturally damaging insects with documented dispersal capacity (Albernaz *et al.* 2012), and in the case of the widespread noctuid pest *Helicoverpa armigera* this pattern even spans continents (Behere *et al.* 2007). Human assisted range expansion of pest insects through the provision of agricultural resources is the scenario that typically explains widespread haplotypes (Grapputo *et al.* 2005). In green mirids, however, the dominant haplotype not only occurs across both agricultural and native arid regions, but has changed within the last 24 years, indicating that the alternation between dry and wet years in arid regions could be responsible, rather than the introduction of agricultural resources over the last 200 years.

An alternative explanation for low mitochondrial diversity would be selection against certain mitochondrial haplotypes due to cytoplasmic incompatibility caused by endosymbionts such as *Wolbachia* (Ballard & Whitlock 2004; Hurst & Jiggins 2005). Our screen for *Wolbachia* was negative in all 24 samples. There are, however, many other known symbionts of arthropods and likely yet more to be discovered, so it is not possible to rule out their presence with simple PCR tests (Hurst & Jiggins 2005). If endosymbionts were affecting mtDNA haplotype frequencies for *C. dilutus*, the pattern of temporal shift recorded in the most dominant haplotype must have been caused by more than one such event. These patterns are therefore most parsimoniously explained by successive bottlenecks caused by drought periods, followed by range expansion during wetter seasons.

4.5.2 Long distance dispersal between arid and agricultural populations

That one haplotype is now dominant across the 5000 km width of Australia indicates that dispersal in *C. dilutus* has been widespread, whether the loss of other haplotypes was drought or symbiont induced. Geographic differentiation was higher in the microsatellite dataset, but the geographic distribution was not stable over time. This is evident from the temporary nature of the isolation by distance effect (Fig. 4.3), the temporal shifts in cluster assignment in the STRUCTURE analysis at Biloela, Emerald, and Narrabri (Fig. 4.4), and by the change in most prevalent COI haplotype between 1993 and 2006. Admixture across large geographic distances most likely results from dispersal, and this is evidenced by the inference (BAYESASS; Fig. 4.5) of significant migration rates across distances over 1500 km. The direction of inferred migration was not consistent across multiple runs of the algorithm, so conclusions regarding the directionality of dispersal remain tentative. Return migration from agricultural regions back to Central Australia by pest populations that derived originally from central desert areas (as postulated for *Helicoverpa punctigera*, which is also an Australian arid adapted species) is thought to be unlikely based on prevailing wind directions and because positive evidence of its existence has never been found (Downes *et al.* 2010).

The sites between which migration was inferred were consistent across runs of the BayesAss algorithm, and are likely to represent regular movement paths. *Creontiades dilutus* populations can expand rapidly, and abundance is seasonally inverse between inland and eastern regions. A migration event in late spring/early summer, when numbers are high in inland areas and low in cropping regions, might therefore result in a much higher inferred migration rate than the actual number of individuals migrating and establishing successfully. The regular seasonal influx of *C.*

dilutus to cotton crops, which does not appear to be derived from local lucerne populations (Miles 1995), indicates that dispersal from inland populations may be a regular occurrence, the microsatellite data support this hypothesis, but it does require further direct testing.

4.5.3 Host plant associated genetic differentiation in arid regions

We found weak genetic differentiation between *C. dilutus* from *Cu. australasicum* and alternative hosts in the same geographic area in the structure analysis (Fig. 4.6), which may partly account for the significant role of host plants implicated by the hierarchical AMOVA. Plants in the genus *Cullen* maintain a significantly higher density of *C. dilutus* than other available hosts, indicating that plants in this genus are primary hosts for green mirids (Hereward and Walter 2012). However, analyses of gut contents using chloroplast intron markers revealed that a substantial proportion of *C. dilutus* individuals collected from the *Cullen* primary host plants had recently fed on other host plants (Hereward and Walter 2012). The use of multiple plant species by *C. dilutus* is perhaps best understood as a behavioural adaptation to survive in an arid environment where host plants are ephemeral and the primary host species not always available (Velasco & Walter 1993). The physiological and behavioural processes that underpin their multiple host use warrants investigation in association with the movement of individuals.

Previous quantified sampling showed that *C. dilutus* abundance was significantly higher on the *Cullen* hosts, *Cu. cinereum* and *Cu. australasicum*, than alternative hosts locally, but not *Cu. pallidum* (Hereward & Walter 2012). We detect genetic differentiation in green mirids between *Cu. australasicum* and other hosts locally, but not for *Cu. pallidum* (Fig. 4.6). More rare microsatellite alleles were present in green mirid populations from *Cu. australasicum* than from alternative hosts. This may be a consequence of a much greater proportion of green mirids being attracted to these plants (perhaps from refuges provided by alternate hosts) and surviving. The relative absence (and perhaps even loss) of rare alleles on alternative hosts needs to be investigated directly if these patterns are to be understood mechanistically.

4.5.4 Conclusions and implications

Creontiades dilutus shows evidence of widespread dispersal in both the mitochondrial and microsatellite datasets examined here, despite this species using different plant resources (both locally and regionally), having seasonally inverse abundance between inland and agricultural regions, and presumably experiencing different selective pressures in these regions of Australia.

The change in the most prevalent mitochondrial haplotype over 24 years is consistent with successive population contractions and expansions, likely in relation to fluctuations between dry periods and wet periods in the arid regions of Australia. Dispersal appears to be the major mechanism by which *C. dilutus* is able to survive on the ephemeral resources in this region, and the data provide no indication that large numbers of these bugs persist through dry periods by diapause. The spatiotemporal dynamics and changing gene frequencies outlined above contrast with the lack of differentiation found in the same agricultural regions over several years for the highly dispersing *H. armigera* (Endersby *et al.* 2007) and the temporal stability recoded for Queensland fruit fly (Yu *et al.* 2001). These dynamics also differ from the stepwise founder effects associated with insects that colonise new temporally stable habitat "islands" through human movement (Stone & Sunnucks 1993). Patterns of genetic differentiation and gene flow in green mirids seem to be driven instead by the spatial and temporal heterogeneity of their native hosts, but these same effects have spread to agricultural regions. This fits with Oliver (Oliver 2006)'s hypothesis that the expansion of host resources is likely to increase gene flow in native insects.

With such spatiotemporal dynamics, adaptation to novel host plants is unlikely. We nevertheless found weak host associated differentiation between green mirids on their primary host plants and those on alternative hosts growing locally, despite establishing previously that these individuals will feed on alternative host species even when in the nearby vicinity of the primary host (Hereward & Walter 2012). Many herbivorous insects that use multiple hosts have been shown, by thorough quantitative sampling, to have a similar closer affinity to one host species than others that it may use (Manners & Walter 2009; Milne & Walter 2000; Rajapakse *et al.* 2006). Assessing gene flow and genetic diversity in more of these instances might further our understanding of multiple host use by herbivorous insects.

Our results highlight the importance of assessing evolutionary and ecological processes across the distribution of an organism that uses both native and human altered habitats simultaneously. If our analyses had been restricted to either agricultural areas or localised parts of the arid range of this species, our interpretations might be quite different. For example, broader geographic analyses of *Rhagoletis pomonella*, perhaps the most famous example of host associated differentiation following the human introduction of novel hosts (cultivated apple) (Bush 1993), to include native hosts in Mexico, indicates that the differences in host plant use had an allopatric rather than sympatric origin (Feder *et al.* 2003; Michel *et al.* 2007). In *C. dilutus* we find that the spatiotemporal dynamics in its arid native range continue to drive genetic patterns across both arid and agricultural environments. The adaptations that allow it to persist despite the spatio-temporal

heterogeneity of host resources in arid regions (migration and the use of alternative hosts) appear to have not only facilitated the colonisation of new agricultural habitats but also maintain gene flow across large distances.

Chapter 5: Resolving multiple host use of an emergent pest of cotton with microsatellite data and chloroplast markers (Creontiades dilutus Stål; Hemiptera, Miridae).

5.1 Abstract

Following the global uptake of transgenic cotton several Hemipteran pests have emerged as primary targets for pesticide control. Previous research on one such emergent pest; *Creontiades dilutus*, indicated differential use of two crop hosts, cotton (*Gossypium hirsutum*, Malvaceae) and lucerne (alfalfa) (*Medicago sativa*, Fabaceae). Green mirids invading cotton in Biloeala (Queensland, Australia) did not appear to have come from adjacent lucerne fields. Further, when lucerne strips interplanted to cotton in New South Wales were mown, numbers of green mirids in cotton did not increase. One explanation for this apparent demographic independence of lucerne and cotton inhabiting mirids would be the presence of cryptic species within the taxonomic species *C. dilutus* associated with these two crops. To test this hypothesis we assessed gene flow using microsatellite markers across adjacent cotton and lucerne crops at three geographically separated sites (up to 900km apart). We also analysed the recent feeding behaviour of these insects by amplifying chloroplast markers from their gut contents. We find high gene flow between these two crops, and no evidence of cryptic species. Further, the gut analyses revealed evidence of substantial recent movement between these two crops. We discuss the implications of these results for interpreting multiple host use in this species and the management of this economically important pest.

5.2 Introduction

Creontiades dilutus (Hemiptera: Miridae), the green mirid, is an endemic Australian bug, recorded from a broad range of host plants including many crops (Foley & Pyke 1985; Miles 1995; Malipatil & Cassis 1997). Prior to the adoption of transgenic cotton that expresses *Bt* toxins *C. dilutus* was controlled incidentally by the application of pesticides targeted at the noctuid moths *Helicoverpa armigera* and *H. punctigera* (Khan *et al.* 2004; Whitehouse 2011). In Australia, the application of broad spectrum pesticides has fallen by as much as 85% in transgenic cotton (Whitehouse 2011), and *C. dilutus* has consequently emerged as the main insect target of chemical control (Khan *et al.* 2004) because heteropterans are unaffected by the Cry1Ac and Cry2Ab toxins expressed by transgenic cotton (Whitehouse *et al.* 2005; Torres & Ruberson 2006, 2008).

The widespread adoption of *Bt* cotton globally has resulted in a similar shift in primary pests towards a number of mirid species, including *Apolygus lucorum* in China (Lu *et al.* 2010; Li *et al.* 2011) and *Lygus hesperus* in the USA (Gross & Rosenheim 2011). Other *Creontiades* species are now emerging pests of transgenic cotton, for example *C. biseratense* in India (Rohini *et al.* 2009; Patil *et al.* 2010), *C. pallidus* in the Middle East (Stam 1987; Hosseini *et al.* 2002) and *C. signatus* in the USA (Coleman *et al*. 2008; Armstrong *et al.* 2010; 2011). All of these mirids use multiple host plants. Such "generalists" are frequently found to comprise suites of cryptic species (Hebert *et al.* 2004; Burns *et al.* 2008; Bonebrake *et al.* 2011). Interpreting the consequences of multiple host use thus requires that the species status across hosts is determined accurately (Paterson 1991; Walter 2003). Failure to recognise cryptic species in economically important insects can result in catastrophic errors and wasted resources in pest management or biological control, (Clarke & Walter 1995; Paterson 1991; Walter 2003; Bickford *et al.* 2007). The development of integrated pest management programs aimed at controlling polyphagous mirid pests needs to include the correct resolution of species status across host plants as a research priority.

Molecular techniques feature prominently in the investigation of species boundaries, but careful application of these techniques is required to establish the limits of mating and thus gene flow between the populations concerned (Paterson 1991; Bickford *et al.* 2007). Increasingly, single locus makers are recognised as poor choices for delimiting closely related species, especially those from uniparentally inherited genomes (such as mitochondria and chloroplasts) (Petit & Excoffier 2009). Such markers are liable to over-represent low frequency hybridisation and suffer from incomplete lineage sorting, both of which undermine the accurate assessment of contemporary levels of gene flow in the system of interest (Powell 1983; Berthier *et al.* 2006; Nevado *et al.* 2009). The results of such studies may be unequivocal when reciprocal monophyly is found between populations from different resources in sympatry, but they still suffer from the problem that "absence of evidence is not evidence of absence". Thus, when reciprocal monophyly is not detected the claim cannot be made that there is only one species.

A multi-locus multi-allelic approach to species delineation is therefore required, but even with multiple markers the specific approach and study design are critical, as classic measures of differentiation such as F_{ST} rely on the correct *a priori* designation of "populations". Discrepancies in the biology of the organism must therefore drive the study design (Walter 2003); such discrepancies would generally signal that the organisms would be doing something unexpected if only one species were involved. In herbivorous insects, for example, an unexpected difference in the use of host plants or resources might provide the basis for structuring such studies. The insects must be

sampled in sympatry across the respective host species as close to within a generation as possible. Multi-locus assignment methods such as STRUCTURE (Pritchard *et al.* 2000), NEWHYBRIDS (Anderson & Thompson 2002), or parentage analyses can then be used to infer contemporary rates of gene exchange across the different populations to be tested. The strength of this approach is revealed by the resolution of the presence of cryptic species in *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae) from maize and, on the other hand mugwort and hops (Malausa *et al.* 2007), where mitochondrial analyses had found no such differentiation across hosts (Martel *et al.* 2003).

Previous research on green mirids in Australia indicates that there are discrepancies in the use of two crop species, cotton (*Gossypium hirsutum*, Malvaceae) and lucerne (alfalfa) (*Medicago sativa*, Fabaceae). Field surveys of *C. dilutus* in central Queensland indicated that the influx to cotton is characterised by wide expanses of cotton (across 10s to100s of km) being colonised within a short time by relatively uniform low densities of these insects (Chinajariyawong 1988; Miles 1995). Numbers in adjacent lucerne fields, which cover very much smaller areas than cotton, did not appear to decrease dramatically at the same time, so lucerne was apparently not the source of mirids that had moved into cotton (Miles 1995). If lucerne was not the source of mirids that invaded cotton crops, then what was? And what was the underlying cause of the apparent difference in use of the two crop hosts by this species?

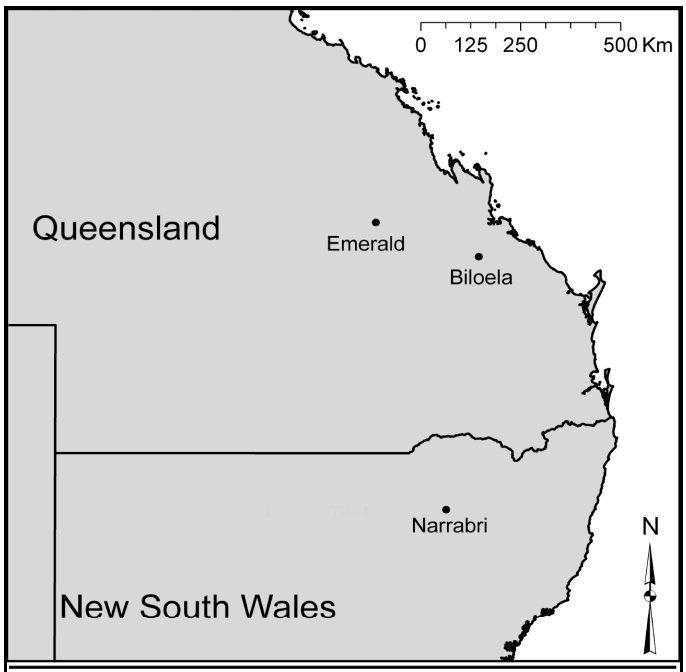
Lucerne has been proposed as a trap crop for *C. dilutus* when interplanted to cotton (Mensah & Khan 1997). A greater attraction to lucerne was indicated in mesh-cage tests but, in no-choice tests, oviposition and survival were similar across cotton and lucerne. Under field conditions, however, *C. dilutus* was much more abundant in lucerne strips than in the similar sized inter-planted cotton strips. Following mowing of lucerne, however, *C. dilutus* numbers did not increase in the inter-planted cotton. *Creontiades dilutus* may have moved onto weeds adjacent to the field site, as increased numbers were sampled from there (Mensah & Khan 1997). Another explanation is that the mirid population on lucerne is a different (cryptic) species to that which occurs on cotton. To interpret resource use by the green mirid, which is renowned for its use of multiple hosts, thus requires that this apparent difference in host plant use be explained. Can it be explained by the presence of two species using different host plants? If not, then how can the differential use of these two host species be explained? Resolving these questions is critical to the effective management of these pests. For example, lucerne may not prove a successful trap crop for green mirids if host associated cryptic species are present across these two crop hosts.

The evidence for cryptic species within *C. dilutus* was explored by sampling mirids from adjacent patches of cotton and lucerne at three geographically separated sites. Microsatellites were used to assess gene flow between the insects in adjacent crop hosts. In addition, host feeding was determined by amplifying chloroplast intron markers from the gut of a subsample of individual mirids (Hereward & Walter 2012) to determine whether bugs from each of the crops had fed upon the alternative crop. This method previously revealed that whereas two particular plant species in the genus *Cullen* are likely the primary hosts for green mirids in the arid interior of Australia, individuals collected from these hosts had often fed on other plant species as well (Hereward & Walter 2012). With this approach, we could thus test whether green mirid individuals move between cotton and lucerne. A lack of movement combined with a lack of gene flow (mating) between hosts would provide strong evidence for the presence of cryptic species. Alternatively, movement across the two hosts by members of a single species would require a better understanding of the functional relationship of green mirids with each of these hosts. In either case, the resolution provided by such an approach provides a sound basis from which further ecological research can be developed to underpin pest management options for this pest, should be applicable to many other insect pests, and is expanded in the discussion.

5.3 Materials and Methods

5.3.1 Sampling

Mirids were collected at three sites separated by 100's of km. At two of them cotton and lucerne grow within 50m of each other – Biloela (24.38^{o}S, 150.52^{o}E) in central Queensland, and Narrabri (30.20^{o}S, 149.57^{o}E) in central New South Wales (Fig. 5.1). At these two sites, about 750km apart, 30 individuals were collected from each host plant. Juveniles and adults of both sexes were collected. The third sample was collected at Emerald (23.57^{o}S, 148.21^{o}E), which is about 250km from Biloela, and about 900km from Narrabri, where cotton was separated from lucerne by about 5km. These samples were all collected in January 2007 (Fig. 5.1), and stored in ethanol.



Location	Host Plant	Date	n
Biloela	cotton	Jan-07	19
Biloela	lucerne	Jan-07	16
Narrabri	cotton	Jan-07	24
Narrabri	lucerne	Jan-07	23
Emerald	cotton	Jan-07	26
Emerald	lucerne	Jan-07	29

Figure 5.1. Sample locations and number of individuals (n) from each sampling event that were used in the microsatellite analyses.

5.3.2 Microsatellite genotyping

DNA was extracted using a modified salt precipitation protocol based on that of Miller *et al.* (1988). Nine microsatellites (mirsat-2F, mirsat-4B, mirsat-3E, mirsat-A1, mirsat-3H, mirsat-6B, mirsat-5C, mirsat-G8, and mirsat-7G) were amplified and separated on a MegaBACE 4000 capilliary electrophoresis system (Amersham Biosciences), as per Andris *et al.* (2010). Microsatellite peaks were confirmed and binned manually.

5.3.3 Gut content analysis

Fifty individuals were selected from each host plant at Biloela and Narrabri. Chloroplast *trnL* intron markers were PCR-amplified from whole insect derived DNA (as above) using the primers: c A49325 (5'CGAAATCGGTAGACGCTACG) and d B49863 (5'GGGGATAGAGGGACTTGAAC) (Taberlet *et al.* 1991). PCR conditions comprised: 25µl reactions using Platinum Taq (Invitrogen), 0.2 µM each primer and 1.5 mM $MgCl^2$ amplified with the touchdown cycling conditions described by (Jurado-Rivera *et al.* 2009). These primers yield different sized PCR products for cotton (600bp) and lucerne (400bp). Selected products were

sequenced on the ABI 3730xl platform (Macrogen) to ensure that each fragment was from the correct plant. Subsequently, these fragments were separated by agarose gel electrophoresis and scored for each individual bug.

5.3.4 Statistical analyses of microsatellite data

The presence of null alleles was inferred from our data using the expectation maximization algorithm of Dempster *et al.* (1977), and global F_{ST} (Weir 1996) computed with and without the ENA correction in FreeNA (Chapuis & Estoup 2007). The number of alleles and heterozygosity (observed and expected) were computed in GenAlEx6.

We used the clustering algorithm as implemented in STRUCTURE (Pritchard *et al.* 2000), which uses gene frequencies to assign individuals to any specified number of clusters (K) within a Markov Chain Monte Carlo framework. We used both the "admixture" and "no-admixture" models. In the former, individuals are allowed shared ancestry between populations. This model deals better with the complexity of many biological systems, and deals with hybrids in a more natural way. The "no-admixture" model assumes that populations are discrete, and is less appropriate for mirids in the cotton/lucerne context, but is better able to detect subtle structure. We ran these models with all nine loci "with nulls", and for the seven loci that showed little evidence of null alleles ("no nulls") to test for the effect of null alleles on the inference of this algorithm. We used a burn-in of 50,000 iterations and a further 500,000 iterations and did not allow the use of population designations for the inference of cluster membership. Under each scenario the algorithm was run 5 times for each value of K (K = 2 to K = 5). The results were permuted and averaged using CLUMPP (Jakobsson & Rosenberg 2007) and plotted using "distruct" (Rosenberg 2004).

We used NewHybrids to infer whether the genetic data indicate the presence of separate gene pools (i.e. species) and, if so, whether F1 or F2 hybrids could be detected. This algorithm also uses an MCMC approach to determine the posterior probability of individuals belonging to five classes, but uses an explicit genetic model for hybridisation. The approach does not require that parental gene frequencies are known, or that separate pure parental species have been genotyped (Anderson & Thompson 2002). We ran this algorithm on both the "nulls" and "no nulls" datasets. Several runs were initiated for each dataset to ensure that the same results were converged on each time, then used a burn-in of 500,000 iterations, followed by 250,000 iterations. The results were plotted using "distruct" (Rosenberg 2004).

5.4 Results

Two loci (mirsat-3E and mirsat-1A1) showed evidence of null alleles (Table 4.1) and our analyses were run with and without these loci (see below). (Weir 1996) Global F_{ST} was 0.063 with all 9 loci and without ENA correction (Chapuis & Estoup 2007), but 0.058 with ENA correction. With just the seven loci with low null allele frequencies, global F_{ST} was 0.041 without correction, and 0.41 with correction. The loci were variable across the populations sampled, with a total of 104 alleles when the null-prone loci were included and 83 when not included (Table 5.1).

Table 5.1. The specific microsatellite loci (left hand column) amplified across all samples. Given for each of these is the number of alleles (NA), mean frequency of null alleles (Null), global F_{ST} without ENA correction, global F_{ST} with ENA correction, observed (H_o) and expected heterozygosity (H_e).

	NA	Null	GF_{ST} w/o ENA	GF_{ST} w ENA	Ho	He
Mirsat-2F	11	0.058	0.02	0.03	0.55	0.61
Mirsat-46B	8	0.108	0.02	0.02	0.25	0.54
Mirsat-3E	16	0.228	0.23	0.21	0.27	0.65
Mirsat-1A1	5	0.142	0.03	0.02	0.07	0.20
Mirsat-3H	19	0.103	0.04	0.03	0.38	0.48
Mirsat-66B	3	0.032	0.09	0.19	0.02	0.05
Mirsat-5C	17	0.049	0.01	0.01	0.70	0.80
Mirsat-2G8	13	0.061	0.02	0.02	0.28	0.51
Mirsat-7G	12	0.078	0.11	0.10	0.61	0.69

When the two loci with higher null allele frequencies were excluded and the admixture model was used, no difference was detected in cluster assignment using the structure algorithm (Fig. 5.2A). When all 9 loci were included and the no admixture model used, some weak structure seems present, with Emerald samples having greater assignment to one cluster than the other samples (Fig. 5.2B). This pattern is not evident, however, under the same model with the “no nulls” dataset (Fig. 5.2C). With this same dataset and K increased to three, the admixture results show some individuals have a higher posterior probability of belonging to a third cluster, with the rest having an even assignment to all three (Fig. 5.2D). When the NEWHYBRIDS algorithm was used with the “no null” dataset these same individuals were assigned with some ambiguity to either the second parental species category or the pure F2 category (Fig. 5.2E, see individuals marked with an asterisk). In analyses with all nine loci, these individuals were assigned with higher posterior probability to the second parental category (data not shown).

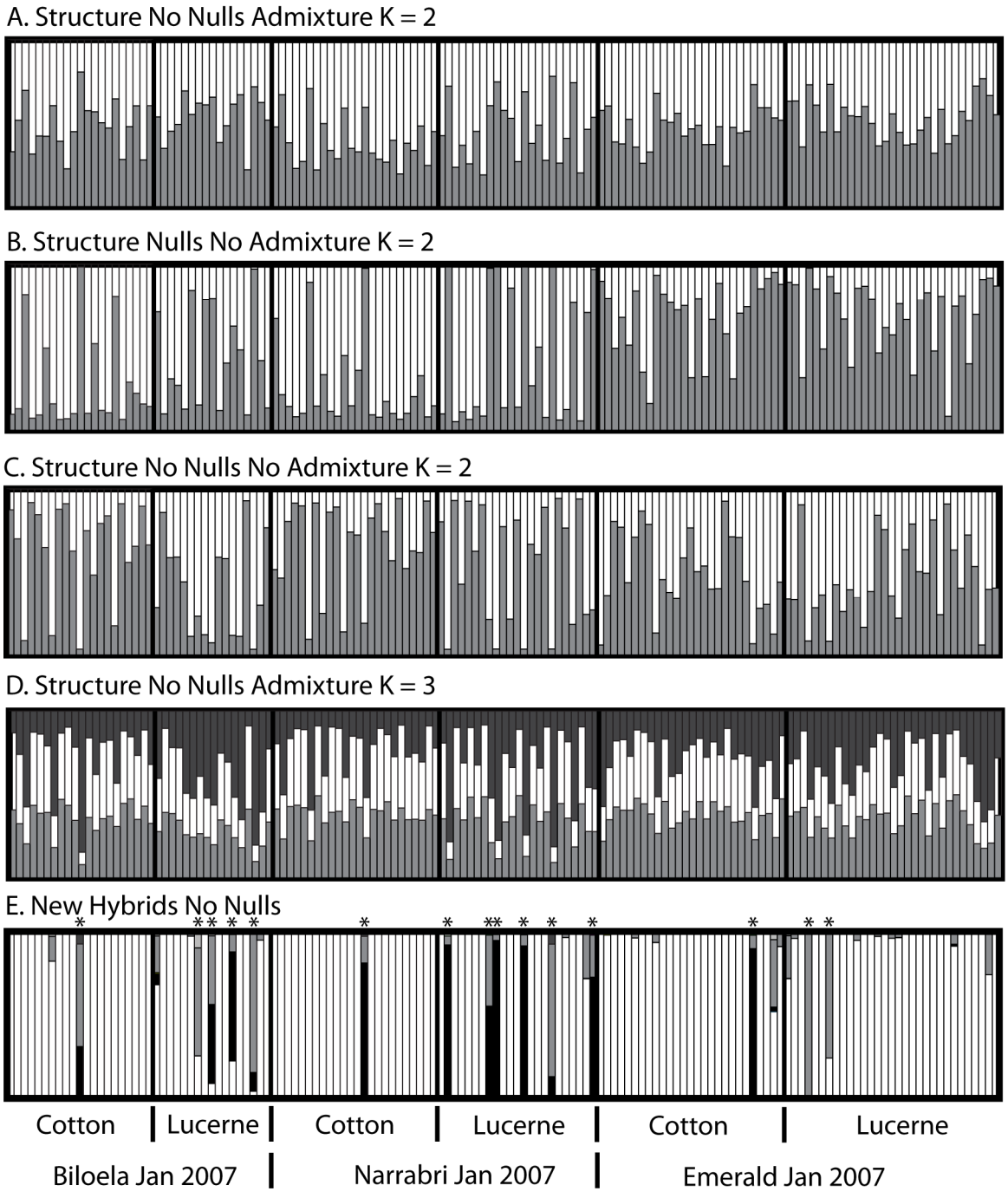
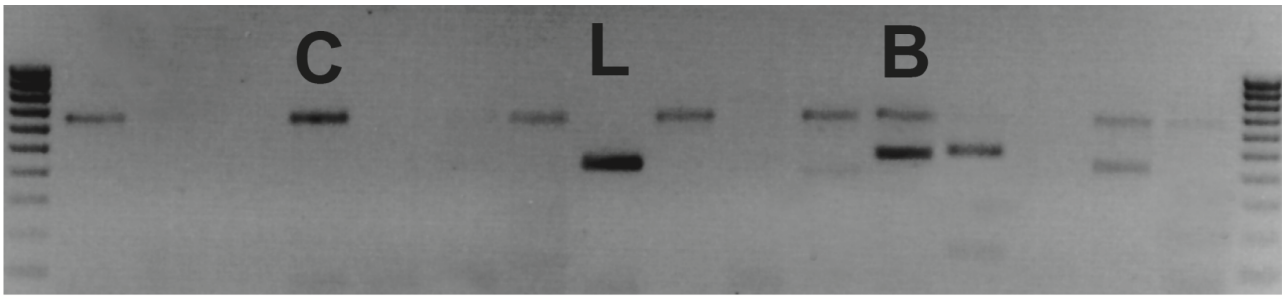


Figure 5.2. Results of the clustering analyses performed with Structure and NEWHYBRIDS. Each bar represents one individual and the shading represents the posterior probability that the individual concerned belongs to each of K clusters (which is given above the bar diagrams). Asterisks above the bottom diagram (E) indicate the 15 individuals that have a greater than 50% posterior probability of assignment to a separate parental species (black) or an F2 hybrid (grey) in the NEWHYBRIDS analysis.

The chloroplast intron markers amplified from mirid gut contents resulted in two different sized products, 600bp (cotton) and 400bp (lucerne), 5 random cotton-sized products and 5 lucerne-sized products were sequenced to confirm their identity. The cotton-sized sequences were all identical and are an exact match for *Gossypium hirsutum* (GenBank, *blastn* search). The 5 lucerne-sized sequences were also identical, and matched *Medicago sativa* with a single base pair difference. One representative sequence of each crop species was deposited in GenBank (accession numbers: cotton = submitted, awaiting accession lucerne = submitted, awaiting accession). From our analyses of gut contents of individuals from adjacent cotton and lucerne fields, 35% of mirids sampled in cotton had also fed on lucerne (n = 98), and 18% of those mirids collected from lucerne had also fed on cotton (n = 99) (Fig. 5.3). Of those collected in cotton, 24% returned evidence of having fed only on lucerne (n = 98). Those from lucerne indicated 12% had fed only on cotton (n = 99) (Fig. 5.3).



	Narrabri - Cotton	Narrabri - Lucerne	Biloela - Cotton	Biloela - Lucerne
N Individuals	50	49	48	50
C - Fed Cotton	68.0 %	10.2 %	62.5 %	14.0 %
L - Fed Lucerne	24.0 %	85.7 %	22.9 %	78.0 %
B - Fed Both	8.0 %	4.1 %	14.6 %	8.0 %

Figure 5.3. Gel (top) shows typical results of the gut content analysis of 14 bugs (numbers below). Clearly evident is the separation of cotton-amplified PCR products (C), lucerne-amplified products (L), and individuals that had both cotton and lucerne DNA in their guts (B). The table (below) shows the number of individuals (%) from each collection from which these products were amplified.

5.5 Discussion

Before this study, green mirids had shown every indication of comprising cryptic species across cotton and lucerne. Interpreting the host use of these insects in terms of them belonging to a single species was not straightforward. The novel application of these two different molecular approaches within a sampling strategy based on prior observations of the ecology of these bugs has resolved the problem. We detected high gene flow between cotton and lucerne, and even evidence, based on their feeding, of frequent movement between these two crops. Our results therefore indicate that the

unusual patterns of host plant use recorded for *C. dilutus* are not a consequence of hidden host-specific cryptic species. Furthermore, fragments of the length that we amplified from the mirid gut contents can evidently be only detected within 12 to 48 hrs post ingestion (Hoogendoorn & Heimpel 2001; Gariepy *et al.* 2007; Fournier *et al.* 2008; Muilenburg *et al.* 2008). Out gut content analysis therefore shows that individual mirids will move between these hosts frequently when they are planted nearby, to the extent that a quarter of all bugs investigated from cotton had evidence only of lucerne in their guts, and with 12% of those from lucerne having fed only on cotton at the time of capture (Fig. 5.3). Below, we interpret our findings in relation to previous studies, and then outline the implications for further research on green mirids and the consequences for their management.

5.5.1 Gene flow across host plants

Genetic differentiation was low across all our samples (global F_{ST} = 0.041 in the "no nulls" dataset), consistent with high gene flow. Under the most conservative parameters (the admixture model and the "no nulls" dataset), the STRUCTURE algorithm indicates a lack of genetic structure across host plants and across geography from our microsatellite data (Fig. 5.2A). This finding is broadly in line with preliminary allozyme work undertaken by (Miles 1995), where no differentiation was found between cotton and lucerne. Conversely, the NewHybrids analysis implicated 15 individuals as being either a second species or pure F2 crosses (Fig. 5.2E), but these individuals are not associated predominantly with cotton or lucerne.

In situations with low differentiation the model based methods that we used in this study can sometimes infer structure that might not be biologically relevant. NewHybrids specifically relies on the presence of gene frequency differences between species (Anderson & Thompson 2002). We used the Structure algorithm with and without the null-prone loci under different model conditions to determine whether this same pattern would be inferred. Although it has been shown that clustering analyses, such as STRUCTURE, are insensitive to low frequencies of null alleles (Carlsson 2008), when we included loci with relatively high frequencies of null alleles (0.14 and 0.22, Table 2) quite different outcomes that were biologically reasonable were returned (Fig. 5.2B). When the number of clusters, K, was set to 3 these same individuals were assigned to the third cluster. On closer inspection of the genotypes, the 15 individuals that clustered differently to the other samples mostly had a higher number of loci that are homozygous for a single allele, and this may have led to the clustering algorithms separating them. Possible causes of this pattern of homozygosity are discussed in the following subsection.

5.5.2 Multiple host use and management of green mirids

Green mirids are endemic to Australia and found in high numbers in the arid interior in association with two native central Australian legumes *Cu. cinereum* and *Cu. australasicum* (Fabaceae) (Hereward & Walter 2012). Using the same microsatellite loci as used here (Table 1) we found genetic evidence of bottlenecks and long distance migration between the arid interior and more eastern sub-coastal cropping regions (JPH, unpublished data). The individuals with increased homozygosity in Fig. 5.2E may be the result of immigrants from inland populations, or their offspring. The gut contents analysis showed that green mirid individuals will move frequently between cotton and lucerne and a significant proportion of individuals will feed on both when they are planted close to each other (Fig. 5.3). Together, these data indicate that green mirid individuals form a single gene pool (or species), with individuals that associate with both cotton and lucerne.

The influx of mirids to cotton has been characterised as sudden and widespread, with these bugs appearing across most cotton crops in the relatively extensive cotton growing region Biloela (for example) within a 24 hr period, as demonstrated by crop consultant surveys and field sampling (Miles 1995). Green mirids are present in lucerne in high numbers at this time, but sticky trap data showed that numbers of mirids in lucerne did not drop appreciably at the time of mirid influx to cotton (Miles 1995).

Lucerne supports consistently higher numbers of mirids than cotton, which is a relatively poor host for them (even though low densities are enough to cause damage (Chinajariyawong 1988; Khan *et al.* 2004)). This difference appears to have a sensory basis, because more mirids colonise lucerne in choice tests, but the survival of adults and nymphs in no choice tests did not differ between the two hosts (Mensah & Khan 1997). Further, lucerne is more closely related to the primary hosts of green mirids, namely *Cu. cinereum* and *Cu. australasicum* which are also Fabaceae, than is the malvaceous cotton (Hereward & Walter 2012). However, performance testing has been largely limited to a single generation of these bugs, because rearing them under laboratory conditions for much longer than that is difficult. The use of *Cullen* to rear mirids may provide a solution to this problem. Green mirids are highly mobile and we have no notion at what distance the flying bugs detect potential host plants and respond to them, or even the cues to which they respond. Most likely, the mirids invade both crops from a distant source at the same time, but the high numbers already in lucerne mask their arrival. The host searching mechanism in green mirids, particularly long range cues, clearly require directed research.

The field trials of (Mensah & Khan 1997) indicated that when lucerne is mowed, mirid numbers do not increase appreciably in adjacent cotton, but our data show that when these two crops are planted next to each other, a relatively high proportion of mirid individuals do move between the two crops and a substantial proportion feed on both. Mowing lucerne is likely to be enough of a disturbance to cause these insects to move further than adjacent cotton strips, and possibly a considerable distance given their high propensity for "startle" flight and their obvious flight capacity (JPH, unpublished data). Our collective results indicate that green mirids are likely to move a lot, both within sites (even when the primary host is present (Hereward & Walter 2012)) and across distances up to 2000 km (JPH, unpublished data). The former may be a consequence of disturbance, the latter related to prevailing weather, but these aspects require further investigation if the invasion of cotton by these insects is to be understood.

Our results indicate that lucerne may not be an ideal trap crop for green mirids on the basis of movement and preference alone. The results of Mensah and Khan (1997) may instead be a consequence of higher predator abundance in the unsprayed lucerne strips. *Cullen* species may prove to be a better option and it is likely that the opportunity to test this proposition will arise as both *Cu. cinereum* and *Cu. australasicum* are under investigation as drought tolerant pasture crops (Lori *et al.* 2009; Bennett *et al.* 2010; Suriyagoda *et al.* 2010; Bell *et al.* 2012). Should these species be domesticated and planted as crops it is also likely that they will support large numbers of green mirids that are likely to move into cotton, given that they support very high numbers of *C. dilutus* (Hereward & Walter 2012), and that gene flow between *Cullen* in the arid interior and cotton in eastern regions has been documented (JPH, unpublished data). Future attempts to rear green mirids under laboratory conditions should, however, investigate the use of *Cullen* as a host, as a reliable method of maintaining more than a couple of generations would enable experimental approaches to understanding host detection and localisation.

5.5.3 Conclusions

Although previous ecological data raised the possibility of cryptic species under the single taxon *C. dilutus* differing mainly in their use of cotton and lucerne hosts, we find no evidence to support this suggestion. Their unusual pattern of host use relative to these crops thus needs an ecological explanation, the beginnings of which are offered here. Our approach to testing for the presence of cryptic species in green mirids, a combination of molecular analysis of gene flow and gut contents analysis using chloroplast markers, has allowed this issue to be clarified. Previous data can now be interpreted in a new light, and future research directions set accordingly. However, our results also provide a methodological lesson; care must be taken when analysing microsatellite data that null

alleles are dealt with adequately and that appropriate analytical models are chosen. The approach outlined in this study should be widely applicable to herbivorous insect pests, and understanding the species status of economic pests is critical to interpreting their ecology and thus setting accurate management guidelines.

Chapter 6: General Discussion

Creontiades dilutus, the green mirid, is considered a major pest of cotton although it is only ever found in relatively low numbers on this crop. Cotton may be a relatively poor host for this species or it may simply not be attractive to them. Nevertheless green mirids are targeted for pesticide control because low numbers are sufficient to cause economic damage to cotton. The current economic threshold of one mirid per metre of row (Khan 1999; Whitehouse 2011) is an order of magnitude lower than the abundance reached on host plants that attract the highest numbers of these bugs, lucerne (up to 30/m (Miles 1995)) and *Cullen* (about 34/m, Appendix A3.1) (both Fabaceae). Understanding the ecology of this bug, and the basis of its use of cotton, therefore required that the association of green mirids with multiple host plants be assessed outside of the cotton agricultural system as well as within, and that methodologies be developed to assess host plant use under field conditions.

In this discussion I outline current perceptions of generalist habits in herbivorous insects, and the theoretical and methodological problems that purported generalists pose. I then propose a conceptual and methodological framework for investigating multiple host plant use, with particular reference to the results presented here on green mirids. Finally I summarise the implications for the management of green mirids in cotton that arise from these results, and highlight aspects of green mirid ecology that warrant further investigation.

6.1 Perceptions of ecological generalists

In the 420 million years or so since the emergence of the first insects (Engel & Grimaldi 2004), they have become the most diverse class of organisms in the history of life. This diversity has long intrigued biologists. Most insects are herbivorous and are host specialists (Bernays 1998), and there is evidence that phytophagy has favoured diversification (Mayhew 2007; Mitter *et al.* 1988). To some extent the diversity of insects might therefore be explained by adaptation to specific host plant defences, or to specific host plant cues that would maximise the chances of localising an appropriate food source, oviposition site or mating partner.

Host plant generalists, by contrast, are difficult to understand in terms of specific adaptations. Such generalist species and their host use are often viewed in the context of optimisation strategies (Scheirs *et al.* 2000) or enemy free space (Jeffries & Lawton 1984; Mulatu *et al.* 2004). Optimal diet mixing has also been proposed to favour generalisation, but there is no evidence that mixed

diets provide direct nutritional benefits in the polyphagous heteropterans investigated thus far (Bernays & Minkenberg 1997; Velasco & Walter 1993).

An ability to use multiple resources appears to be an obvious advantage in terms of resource availability (Futuyma & Moreno 1988). If that assumption is correct, why do so many insects have a narrow diet? Many explanations rely on the concept of "trade-offs" in performance on different hosts. The basic principle behind the trade-off paradigm is that there must be a fitness cost to maintaining a generalist strategy, and therefore a fitness benefit to developing a specialist strategy. The fitness benefit of specialist feeding habits was central to Ehrlich and Raven's (1964) co-evolutionary theory of insect-host plant interactions, with the evolution of adaptations (like detoxification mechanisms) to deal with plant secondary metabolites representing the fitness cost. Subsequent phylogenetic analyses have mostly not, however, supported the generality of co-evolutionary theory with respect to insect-plant interactions (Lopez-Vaamonde *et al.* 2003; Mitter *et al.* 1991; Percy *et al.* 2004; Weintraub *et al.* 1995). The lack of phylogenetic support for strict co-evolution even extends to the close relationship between internal parasites and their host organisms (Garcia *et al.* 2011; Jan *et al.* 2012) and even some obligate pollination mutualisms (Machado *et al.* 2005).

At the core of these explanations for generalist habits is an assumption that intraspecific competition (optimisation strategies) or interspecific competition (enemy free space) is the predominant determinant of the behaviour and thus abundance of insect herbivores. This perception prevails despite the early recognition that plant resources are rarely fully exploited by herbivorous insects and that scant evidence exists for direct competition between them (Jermy 1984), or for such competition having influenced their evolution (Lawton & Strong 1981). This perception of generalist species might, to some extent, be attributed to anthropomorphism and the unrivalled ingenuity of humans in exploiting resources well beyond those necessary for our survival. Whatever the underlying philosophical basis, interpretations that relate to optimisation strategies, the acquisition of "enemy free space", and performance trade offs all implicitly assume that fitness is increased by efficiency gains though the use of multiple hosts. Under this scenario, herbivorous insects that use multiple host plants are expected to show flexibility in host plant preference and use across their distribution as they switch to alternative host species and adapt to local conditions as they maximise fitness within the range of resources available (Fox & Morrow 1981).

A general interpretation of insect-host plant relationships requires that multiple systems be assessed because a pattern observed in one system may not hold when extended to others. This is highlighted by the subsequent phylogenetic re-assessments of what is now referred to as Ehrlich and Raven's

(1964) classic co-evolutionary theory (Thompson 2005), described above. For example, a "double dated" phylogeny of monophagous psyllids and their legume host plants indicates a host plant radiation around 5 million years prior to the psyllid radiation, with over 60% of the psyllid radiation being attributed to subsequent host switching rather than to co-speciation (Percy *et al.* 2004). But what should focus such studies of insects that use multiple host plants?

Statistical meta-analyses of published datasets have become a popular tool for biologists to tease out patterns across multiple studies. However, if the primary data in the studies that are eventually incorporated into such an analysis were not collected in a manner that renders them comparable methodologically, a confident interpretation cannot be made. A further stricture is that "the study of patterns must be free from any assumptions about processes, if they are to be used to test hypotheses dealing with the mechanisms of evolution" (Chapleau *et al.* 1988). In the case of adaptation and speciation, however, process has been implicitly elevated above the primary pattern observed in nature (Walter 2013). Interpretation of multiple host use by herbivorous insects has tended to rely on the practice of generating lists of host plants with little justification for the inclusion of each. Such lists generally include a wide range of plants, many of which are incidental to the ecology of the herbivore in question. The plants involved are thus treated typologically rather than quantitatively (Walter & Benfield 1994), obscuring the primary pattern of multiple host use. Although some attempts have been made to categorise host lists into 'reproductive hosts' or 'incidental hosts', the typological approach continues to hinder our ability to understand *how* an insect uses different host plants let alone *why*.

The elevation of process over pattern (which is often considered merely descriptive) has also filtered through to the study of multiple host use by herbivorous insects. For example, Fox & Morrow (1981) interpreted some herbivore species as being generalists through the evolution of specialist host relationships locally. Data were presented to support this hypothesis, but the species status of the insects concerned was determined taxonomically (rather than in population genetics terms (Chapter five)), and the geographic distribution and host species range of the insect herbivores were not documented quantitatively. Their review therefore failed to provide a method to assess the overall pattern of multiple host use accurately. The spatial variability of host use was, instead, assumed, and multiple explanations provided. These include availability of suitable hosts (both spatially and temporally), community characteristics such as the relative abundance of hosts vs non hosts, rapid shifts in the genetic basis of preference, direct or indirect competitive interactions with other herbivorous insects, differences in host physiology, presence of predators, and the predictability of host abundance. To develop a general understanding of multiple host use in

insects thus requires a method that is capable of determining the primary pattern of host use. Only then can the underlying functional processes and mechanisms be investigated and interpreted.

To date, a consistent methodology has not been presented to assess these patterns in nature. More accurate general interpretations of multiple host use clearly require that individual herbivorous species must be assessed quantitatively in terms of their interactions with their environment, and this must be conducted across their geographic distribution and with respect to all host plants with which it associates (so that the different host species can be assessed relative to one another). This point forms the basis of the approach recommended in this chapter. In brief, the species limits of the herbivore must be defined accurately and the association of the insects with each host must be ascertained quantitatively across the geographic range of the species so that the stability of these patterns can be assessed across space and through time. These aspects are outlined more completely and justified further below.

6.2 Conceptual and methodological framework

6.2.1 Species limits

The evolutionary significance of host plant use can be determined only if the variation in host use can be ascribed to the level of the individual, the population, or the species. The misinterpretation of multiple resource use as a consequence of the presence of undetected cryptic species in the taxon under investigation has received more attention recently (Jenkins *et al.* 2009; Bonebrake *et al.* 2011; Burns *et al.* 2008; Hebert *et al.* 2004; Loxdale *et al.* 2011), and was a possibilty not acknowledged by Fox and Morrow (1981). The contribution of molecular approaches to understanding ecological problems has undoubtedly played a role in the prominence given to cryptic species recently (Bickford *et al.* 2007), although the appropriate theory and other techniques have long been available (Paterson 1981; 1991; Walter 2003).

To deal most realistically with the adaptations and ecology of organisms, species must be defined in population genetics terms, simply because sexual reproduction and gene flow play dominant roles in the distribution of genes and thus adaptations. Therefore, an understanding of diet breadth and variation in host use is reliant on the prior establishment of the limits of the species in question. Here a species is defined as a species gene pool, within which individuals have the potential to recognise and localise appropriate mating partners and thus complete fertilisation under natural conditions (Paterson 1981). Species limits must be assessed within the context of the natural

environment because the mechanism by which individuals detect, localise and interact with appropriate mating partners (the specific mate recognition system of Paterson) can be heavily dependent on environmental context, and results from cage trials can be ambiguous (Fernando & Walter 1997). Many insects have such an intimate relationship with a specific host plant, or a subset of specific host plants, they may never encounter members of closely related species under field conditions despite mating and producing viable offspring with them under laboratory conditions (Claridge *et al.* 1985, 1988; Drosopoulos 1985).

The importance of environment in defining the limits of a species gene pool is central to the recognition concept of species (Paterson 1986). Explicit tests of rates of gene flow across the host species used by insect herbivores under natural conditions have, however, remained relatively rare. In one notable exception, presumed 'host races' of *Ostrinia nubilalis*, which are able to hybridise and produce viable offspring under laboratory conditions (Liebherr & Roelofs 1975), were shown to cross-mate at frequencies below 1% under natural conditions using a model-based analysis of microsatellite markers (Malausa *et al.* 2007). Surprisingly, such explicit tests of gene flow rates have not been conducted on host associated populations of the apple maggot (*Rhagoletis pomonella*), the textbook example of sympatric speciation. Current rates of gene flow across apple and hawthorn populations of *R. pomonella* remain untested with a model that infers the rate of F1 and F2 hybrid production, despite the recent recognition that the origins of the major differences between these putative races were allopatric. Each of these races has been associated with different mountain ranges in Mexico (Feder *et al.* 2003; Feder *et al.* 2005; Michel *et al.* 2007). Further, fixed differences in plant volatile recognition are evident between genetically differentiated populations of *R. pomonella* that attack different hawthorn species in the USA (Cha *et al.* 2012). That these flies mate only on or near their natal host fruit under natural conditions is widely recognised, but the status of host associated populations continues to be addressed through reproductive isolation criteria as determined through laboratory crosses (Rull *et al.* 2010).

Recent research on the *Rhagoletis pomonella* complex has also clearly demonstrated the critical importance of geography in understanding species limits. The geographic context of hybridisation, when it does occur, is critical to assessing the likely origins of differences in host plant relationships, and associated genetic differentiation. The *Pauropsalta annulata* species complex of cicadas in eastern Australia illustrates this point; each of the three component species in this complex is strongly associated with a small number of eucalypt tree species. A spatially explicit analysis of the calling songs of the males, a critical component of the specific mate recognition system of these insects, revealed that these adaptations were stable across the entire distribution of each species, but that one of these species is represented by two sub-species that hybridise where

their ranges overlap. Each subspecies maintains consistency in their calling song structure, host plant associations and ovipositor length across the rest of their respective distributions, despite this localised hybridisation. This pattern is more parsimonious with allopatric divergence and subsequent range expansion than with sympatric speciation, as sympatric divergence would fail to explain the presence and stability of these behavioural features over such vast areas of allopatry. If only the zone of overlap between these two subspecies had been investigated the pattern would fit that of sympatric ecological speciation (Rundle & Nosil 2005). This example not only highlights the importance of geographical context in determining species limits, but also in assessing possible modes of speciation.

The careful application of theory and technique, and the interplay between them, is therefore crucial to defining species limits accurately, and it must be done in this way before diet breadth can be determined. In the case of the green mirid, geographically replicated tests of gene flow between cotton and lucerne (Chapter five, Fig. 5.2) did not support the hypothesis that cryptic species explain the reported differential use of these two host plants by this bug (Mensah & Khan 1997; Miles 1995). Analyses of gene flow across most of the geographic distribution of *C. dilutus* (Chapter four) also indicate that it is most likely a single species across all host plants tested. The low diversity (high similarity) of CO1 sequences (Fig. 4.1), admixture and gene flow evident from the microsatellite data (Figs. 4.4 & 4.5) and the weak differentiation between populations on *Cullen* and alternative hosts (Fig. 4.6) all support this proposition. Without further exhaustive sampling it is difficult to rule out the possibility that cryptic species do exist under the name *C. dilutus*, but were not encountered during this thesis. It is clear, however, that a single species, *C. dilutus*, does use multiple host plants. The following section outlines an approach to testing interpretations of multiple host use once the species status of the organism in question has been clarified, and it is clear that individuals from a single species are using multiple host plants.

6.2.2 Testing and interpreting multiple host use across the distribution of species

Optimisation-based interpretations of multiple host use tend to assume that each of the potential hosts within a given list can and will be readily used by individuals of a herbivorous insect as they maximise their fitness relative to one another and in relation to environmental change (and even as species optimise in relation to one another). The logical prediction that follows from this line of reasoning is that the rates at which a generalist species uses a given host plant species should vary across space and time in response to competition and other selective pressures envisaged to influence host relationships of these insects.

The prediction outlined above can be tested only if host plant use by a particular species is quantified systematically across its geographic range, and can be falsified if host plant relationships are spatially and temporally stable. Despite a general paucity of systematic quantitative studies of this nature in the literature, a striking trend is evident in those that do exist. Although broadly regarded as generalists, the herbivorous insects with multiple hosts that have been quantitatively assessed tend to have a particularly close association with only a small subset of those hosts (Clarke *et al.* 2001; Manners *et al.* 2011; Milne & Walter 2000; Popple & Walter 2010; Velasco *et al.* 1995; Walter & Benfield 1994; Zalucki *et al.* 1994). This observation has lead to the designation of primary hosts. These are the plants with which these insects are most regularly associated, and on which they are found at highest abundance, or, in evolutionary terms, the hosts to which the species is primarily adapted. Extensive research into the spatio-temporal stability of these patterns would provide key insights into the general applicability of interpretations based on optimisation.

Establishing the pattern of host use in terms of species limits of the herbivore concerned and its primary hosts, as well as the geographic and temporal stability of these patterns, is therefore critical to scientific hypothesis testing among alternative processes. Defining the primary host of an insect herbivore (that uses multiple hosts) is also critical to structuring subsequent research into host use. Tests of the variability of host plant relationships, the relevance of "enemy free space", or the physiological responses of the insects to alternative host plants might have only limited value if they are conducted solely on secondary or incidental hosts, which is not an uncommon practice.

Helicoverpa armigera, a widespread species that is considered a generalist, has a strong attraction to pigeon pea (*Cajanus cajan*) but also accepts other plant species for oviposition (Rajapakse & Walter 2007). This strong attraction to the primary host has lead to the adoption of pigeon pea as a trap crop for *H. armigera* in transgenic cotton systems, with the aim of slowing the development of resistance (Sequeira & Playford 2001). Analyses of plant volatiles and sensory responses to those volatiles show that each of these alternative hosts emits only a subset of the volatiles emitted by pigeon pea, indicating that the mechanism of multiple host use in this species is likely due to a reduced threshold of host acceptance (Rajapakse *et al.* 2006). Researchers continue to ignore this result when designing tests of oviposition behaviour (Zalucki *et al.* 2012), gene expression responses to different host plants (Kotkar *et al.* 2012; Maria de la Paz *et al.* 2012), nutritional value of hosts (Hemati *et al.* 2012) and trade-offs between adult oviposition and larval performance (Liu *et al.* 2012). Despite the strong negative selection assumed to be provided by transgenic cotton, *H. armigera* oviposition preference in relation to tobacco, cotton and cabbage has, however, remained consistent across three decades (Zalucki *et al.* 2012).

Although laboratory based behavioural tests can provide valuable insights into the host location mechanism of herbivorous insects, they almost inevitably exclude environmental context and long-distance host-location cues. This can lead to a discrepancy between the observed host range under laboratory conditions, and the realised host range under field conditions, a situation that is commonly reported (e.g. Manners *et al.* 2011; Rafter *et al.* 2008). It is therefore desirable to assess host-use in relation to the natural environment of the organism in question (Manners *et al.* 2011).

Chloroplast markers extend ability in this respect as they provide a method of detecting past feeding behaviour in field collected insects. This approach has been applied to various organisms, including herbivorous insects (Handeler *et al.* 2010; Jurado-Rivera *et al.* 2009; Navarro *et al.* 2010; Valentini *et al.* 2009). This approach has, to date, been predominantly applied typologically to refine host lists generated previously. In this thesis I developed an approach to understanding multiple host use by insects by building on these technical advances. Specifically, I combined them with a quantified field survey of the relative insect abundance across plant species available locally and the relative regularity with which the different host plant species were used. This combined approach has already yielded new insights into green mirid host plant relationships.

The consistently higher abundance of green mirids on two species in the genus *Cullen* highlighted that these Australian native legumes are the primary hosts of *C. dilutus* (Chapter three, Fig. 3.3). Further comprehensive testing of the temporal stability of this pattern should be a research priority as discussed above. Miles (1995) did note, however, that large numbers of green mirids were found on *Cullen* during her surveys some 15 years prior to this thesis, indicating that green mirid host-use has remained stable over this period. The gut content analysis showed, however, that *C. dilutus* will feed on other plant species, and that a proportion of those collected from these primary hosts had done so (Table 3.1).

Quantitative analyses of the stability of insect host plant relationships must be conducted systematically before predictions following from optimisation based interpretations of multiple host use can be falsified. It is clear, however, that such explanations fail to account for the proximate mechanisms that might lead to multiple host use by the introduction of the unnecessary intermediate of “efficiency” (Finlay-Doney & Walter 2012). In the case of green mirids, it seems most plausible, based on the evidence presented in this thesis and the arid environment to which this species is primarily adapted, that multiple host plant use is a fixed adaptation to survive ephemeral conditions. This has been demonstrated experimentally in *Nezara viridula*, a heteropteran that also uses multiple hosts (Velasco & Walter 1993). If *C. dilutus* individuals were using different host plants to maximise their fitness relative to each other, or to other species, then the pattern of host use would

be expected to vary across space and time. Instead, *Cullen* has likely been the primary host of green mirids for some time, and in agricultural areas lucerne hosts the highest abundance of green mirids. This crop is visually similar, and phlyogentically closer to *Cullen* than other crop species that *C. dilutus* uses, suggesting that the observed pattern of host use is driven by the primary adaptations of this bug rather than inter or intra-specific competition.

6.3 Implications for management of green mirids and future research priorities arising from this thesis

The sequence data and phylogenetic trees presented in chapter two (Figs. 2.2, 2.3, 2.4) confirmed that green and brown mirids (*C. dilutus* and *C. pacificus*) are well separated species, as realised by earlier morphological studies on green mirid (Chinajariyawong 1988; Malipatil & Cassis 1997; Miles 1995). *Creontiades dilutus* and *C. pacificus* are closely related (Chapter 2.1), but the genus is globally widespread and has not received much phylogenetic attention. Sequence data also helped determine that the emergent mirid pest in the USA, originally believed to be the Australian *C. dilutus*, was actually a different species (Coleman *et al.* 2008). Subsequent taxonomic examination of the USA mirid revealed it was in fact *C. signatus*, indigenous to the Americas, and this species has since received considerable research interest (Armstrong 2010; 2010a Armstrong *et al.* 2009a; Armstrong *et al.* 2010; Armstrong *et al.* 2009b; Armstrong *et al.* 2009c; Brewer *et al.* 2012). Given that a number of *Creontiades* species are agricultural pests and use multiple hosts, a thorough phylogenetic analysis in combination with detailed analyses of host use across this ecologically poorly understood genus may provide an evolutionary perspective to host use within this group and thus contribute to interpreting the functional aspects of multiple host use.

Creontiades dilutus had significantly lower genetic diversity in the mitochondrial CO1 gene sequences than *C. pacificus* (Fig. 2.5). Miles (1995) noted differences in the morphology of the egg opercula of these two species that possibly indicated adaptations to relatively dry (*C. dilutus*) or wet (*C. pacificus*) conditions. This hypothesis is apparently borne out by the distribution of these two bugs, with brown mirids restricted to the relatively wet coastal regions of Australia, and green mirids present throughout the arid regions (Fig 2.1). The difference in genetic diversity between these species likely correlates to the stability of populations in these two habitats, a point that was explored more fully in Chapter four (also see below).

Given that *C. dilutus* is endemic to Australia, and in particular has adaptations and a distribution that indicate a close association with the arid interior of Australia, it seemed strange that so few

native host plants had been recorded. During two years of field surveys I added 22 native host plants to this already extensive list (currently 97 plant species). As outlined in the previous section, however, our understanding of multiple host use has been hindered, rather than aided by host plant lists. *Creontiades dilutus* is found more regularly and at consistently higher densities on two species in the genus *Cullen*, namely *Cu. australasicum* and *Cu. cinereum* (Chapter three, Fig. 3.3). Gut content analyses demonstrate, however, that green mirid individuals will move between other plant species and feed on these different species, even when collected from their primary hosts (Chapter three, Fig 3.4).

The green mirid has been notoriously difficult to maintain, under laboratory conditions, for more than two generations (M. Khan, Queensland Department of Agriculture Forestry and Fisheries, *pers. comm.* 2009). By establishing that these two species are primary hosts for *C. dilutus* I have provided a new option for laboratory rearing that may enable future studies to explore, more fully, the cues used for host location and acceptance. Furthermore, no effective biological control agents have been found for green mirids to date. Some individuals have been observed carrying mites but these cause little mortality (M. M. Miles, Queensland Department of Agriculture Forestry and Fisheries, *pers. comm.* 2010). Future attempts to locate biological control agents for use in the integrated pest management of this bug (for example egg parasitoids) would be best directed towards the original geographic distribution and primary host plant species, namely *Cullen* species growing in inland areas of Australia, rather than the expanded range of this pest in eastern agricultural cropping regions. Perhaps more worrying in terms of mirid abundance in agricultural regions is the proposal to use *Cullen* species as pasture crops in Australia (Bell *et al.* 2012; Bennett *et al.* 2010; Lori *et al.* 2009; Suriyagoda *et al.* 2010).

I assessed gene flow between *C. dilutus'* native arid range and host plants and the eastern cropping regions (where it causes economic damage) using the microsatellites that I developed (Chapter two), and CO1 sequences of recent material and samples collected much earlier, in 1983/1993. The analysis of these data revealed a spatiotemporally dynamic pattern of genetic differentiation and gene flow. The low diversity of CO1 haplotypes and the shift in predominant haplotype over time (Chapter four, Fig. 4.1) indicate that population bottlenecks (perhaps severe) followed by expansion may not be uncommon in this species, likely the result of fluctuations between dry and wet periods in the arid interior. The microsatellite data showed that although some differentiation is detectable between regions, strong evidence exists for admixture and migration (across vast distances) between the arid interior and subcoastal cropping regions (Chapter four, Figs. 4.3 & 4.4).

Genetic differentiation across host plants was not strong, but there were more rare alleles present in samples collected from the primary host. This caused a pattern of weak differentiation as revealed by the STRUCTURE analysis (Fig. 4.5). If this pattern is observed in other species that use multiple hosts, it highlights the importance of quantifying host use in studies that assess host associated genetic differentiation in herbivorous insects. In green mirids, the adaptations to the arid environment (multiple host use, and dispersal) have likely not only allowed the colonisation of new environments, but continue to be the predominant determinant of current patterns of genetic differentiation. Currently a single pesticide (Fipronil) is used heavily to control green mirids in cotton (Whitehouse 2011). Although no resistance to Fipronil or other pesticides has been reported to date in *C. dilutus*, the American mirid pest *Lygus lineolaris* has developed increasing levels of resistance to organophosphates (Zhu *et al.* 2012). Continuing gene flow from arid inland populations into the eastern cropping regions would be expected to dilute resistance genes, and therefore reduce the ability of *C. dilutus* to develop fixed resistance to pesticides. It was, however, expected that *Helicoverpa punctigera* (which also disperses into cotton from inland sites) would be less likely than *H. armigera* (which is mostly restricted to cropping regions) to develop resistance to *Bt* toxins (Gunning & Easton 1994).

Previous research highlighted discrepancies in the use of cotton and lucerne by green mirids. The tests of gene flow presented in this thesis show that these discrepancies are not caused by the presence of cryptic species on these two crop hosts (Chapter five, Fig. 5.2). Further, the analyses of chloroplast sequences showed that when cotton and lucerne are adjacent, a proportion of green mirid individuals will move between these crops and feed on both (Fig. 5.3). Miles (1995) surveyed green mirid abundance in lucerne plots prior to, and following the influx of green mirids to cotton, and concluded that lucerne was not the source of mirids that invade cotton crops. These results can be explained, however, by green mirid dispersal from inland sites (Fig. 4.5), if they settle at the same frequency on cotton and lucerne. Anticipating the number of mirids likely to turn up in cotton in any given year is difficult. We first need to understand what causes them to take long distance flight, and whether it is a controlled behaviour, at least on leaving the plant initially. Although the isolation by distance analyses (Chapter four, Fig. 4.3) indicates recent dispersal, the frequency with which such events might take place is still an open question.

The chloroplast analysis (Chapter five, Fig. 5.3) indicates that attractiveness may not be the reason that lucerne trap crops appeared to be effective in Mensah & Khan's (1997) study, because of the considerable movement between these two crops by *C. dilutus* demonstrated in this thesis. The presence of unsprayed lucerne strips possibly boosts the numbers of predators of green mirids, such

as spiders (*Oxyopes molarius*) and damsel bugs, (*Nabis kinbergii*) (Whitehouse *et al.* 2011a), which might explain the observed efficacy of these strips in Mensah & Khan's (1997) work. Species of *Cullen*, specifically *Cu. cinereum* and *Cu. australasicum*, may prove to be better trap crops than lucerne, but green mirids do move between these primary hosts and other plant species (Chapter three). Without structured research into the host searching and acceptance mechanisms in green mirids it is therefore difficult to predict how effective this approach would be. Both *Cu. cinereum* and *Cu. australasicum* are currently under investigation, in Australia, as drought tolerant pasture crops (Lori *et al.* 2009; Bennett *et al.* 2010; Suriyagoda *et al.* 2010; Bell *et al.* 2012). Together, the results presented in this thesis indicate that green mirids disperse readily, even over long distances, and will accept hosts other than their primary host plant species as they terminate their dispersal flight and even as part of their local movement. It is therefore possible that the introduction of these species as widespread pasture crops would instead produce large numbers of green mirids that may subsequently move into the cotton system. Clearly, the reason for their movement and acceptance of alternative hosts needs to be addressed.

6.4 Concluding remarks

The debate about the evolutionary significance of ecological specialisation vs generalisation in terms of resource use has intensified recently, with special reference to insect host-plant interactions. Loxdale *et al.* (2011) considered the improbability of generalist habits evolving given the reduction in competition that should follow when specialist habits evolve. Conversely, Dennis *et al.* (2011) argued that true generalists not only exist, but are an essential precursor to the evolution of species with specialist habits, and that turnover between the two states (especially given the increased likelihood of extinction in specialist species) can explain a balance between the two habits. In contrast to these two perspectives, an alternative argument has been developed to indicate that our perception of "generalists" might not have been accurately developed from the outset, mainly because the functional relationship of the insects with their hosts (or prey) has been ignored to a large extent, and that a more mechanistic interpretation is needed as a basis for developing robust generalisations (Finlay-Doney & Walter 2012).

The research presented here on *C. dilutus* extends this latter perspective, for it indicates that we do need to know much more about individual species that use multiple hosts, how they do so and why. Understanding the primary patterns of multiple host use under natural conditions (and the stability or variability of these patterns) is critical to the evaluation of alternative processes, and appropriate testing of alternative hypotheses. To this end, the approach outlined and justified in this chapter will

contribute to the development of more robust generalisations about the ecological and evolutionary significance of the “generalist” way of life in herbivorous (and other) insects.

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Appendices

Appendix 1.1:

Molecular Comparison of *Creontiades* Plant Bugs from South Texas and Australia

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Authors: R. J. Coleman1, J. P. Hereward[2], P. J. De Barro[2], D. R. Frohlich[3], J. J. Adamczyk, Jr.[1], and J. A. Goolsby[1]

[1]USDA-ARS, Beneficial Insects Research Unit, 2413 E. Hwy 83, Weslaco, TX 78596

[2]CSIRO Entomology and CRC for Cotton Catchment Communities, 120 Meiers Road, Indooroopilly, Queensland 4068, Australia

[3]University of St. Thomas, Department of Biology, 3800 Montrose Blvd., Houston, TX 77006

Abstract.

Research was conducted to evaluate the possibility that a plant bug damaging cotton, *Gossypium hirsutum* L., in south Texas is actually green mirid, *Creontiades dilutus* Stål, which is the primary plant bug pest of cotton in Australia. Molecular comparisons targeting a fragment of the CO1 region of mitochondrial DNA were made on *Creontiades* specimens collected from the Lower Rio Grande Valley of Texas and specimens of green mirid and brown mirid, *C. pacificus* Stål, collected from Queensland, Australia. The emerging south Texas cotton pest is neither of the species tested from Australia; rather it is a closely related, possibly indigenous species. Further morphological systematics work is needed to identify the *Creontiades* species from Texas, and collection of additional specimens from several locations where it is known to occur is ongoing.

Introduction

The mirine genus *Creontiades* Distant is widely distributed in temperate, subtropical and tropical regions of the world (Yasunaga 1997). Several species are reported to damage a number of vegetable and field crops, particularly legumes and cotton, *Gossypium hirsutum* L. A mirid in the genus *Creontiades* that feeds on cotton terminals, squares and small bolls is considered an annual pest in the Lower Rio Grande Valley and Coastal Bend regions of Texas (Norman and Sparks 2002, Fromme 2006, Parker 2006). Feeding damage can result in abscission of squares and small bolls. Treatment thresholds have yet to be experimentally determined, however, recommendations concerning action levels and insecticide choices generally follow guidelines developed for *Lygus* spp. (Norman and Sparks 2002). Specimens the senior author collected from the Lower Rio Grande

Valley and provided to taxonomists were identified as *C. debilis* Van Duzee, (J.C. Schaffner, Department of Entomology, Texas A&M University) or *C. dilutus* Stål (M.D. Schwartz, Agriculture and Agrifood Canada, Ottawa, Ontario, Canada). Green mirid, *C. dilutus*, is the primary plant bug pest of cotton in Australia (Khan et al. 2004). The D2 gene has proven to be useful in a molecular analytical approach for characterization at the species level in arthropods (De Barro et al. 2000, Goolsby et al. 2006). De Barro and Goolsby (unpublished data) used the D2 expansion domain sequence of the 28S rRNA to compare specimens collected from the Lower Rio Grande Valley with specimens of *C. dilutus* from Australia. Preliminary evidence indicated that the Texas mirid could be *C. dilutus*.

With a divergence of opinions as to the true taxonomic identity of the mirid from south Texas, our objective was to use a molecular analytical approach targeting a fragment of the COI region of mitochondrial DNA to determine the degree of nucleotide sequence divergence between *Creontiades* sp. from the Lower Rio Grande Valley and *C. dilutus* from Queensland, Australia and infer phylogenetic relationship between the two populations. Such knowledge can provide insight into the potential need for search of natural enemies and/or biological control options for this pest.

Materials and Methods

Collections of *Creontiades* sp. adult specimens were obtained with a sweep net from nettleleaf goosefoot, *Chenopodium murale* L., and London rocket, *Sisymbrium irio* L., at two locations in the Lower Rio Grande Valley, Texas, U.S.A. Collections of green mirid, *C. dilutus* StÂl, and brown mirid, *C. pacificus* StÂl, were collected with a sweep net from a commercial planting of pigeonpea, *Cajanus cajan* (L.) Millsp., near Byee, Queensland, Australia. For the molecular comparison, 10 specimens each of *Creontiades* sp. and *C. dilutus* and five specimens of *C. pacificus* were used in this study. Adult insects were preserved in 95% ethanol at - 20o C prior to extraction. Collection and specimen data (including GenBank accession numbers) for the individuals used in the molecular comparison are shown in Table 1. The head, abdomen and legs of each specimen was removed and only the thorax used for DNA extraction to avoid inclusion of inhibitors from the head and possible protein contamination from the gut. The remaining body parts were stored at the University of Queensland for future analyses. Genomic DNA was extracted with the DNEasy extraction kit from Qiagen, with an elongated lysis of 12 hours. PCR was performed on the genomic DNA using the Folmer primer set: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3', HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al 1994). These primers amplify a 658 b.p. fragment of the COI region of mitochondrial DNA. PCR 25µl reactions were set up using Qiagen *Taq* polymerase according to the manufacturerís instructions in reactions with a 1.5mM Mg2+ concentration. Cycling conditions were as follows: 3 minutes of initial denaturation

at 95^{O} C followed by 30 cycles of denaturation for 1 minute at 95^{O} C, 1 minute of annealing at 50^{O} C and 1-minute extension at 72^{O} C. Product checking was carried out on a 1% agarose gel, visualized by ethidium bromide staining and ultra-violet illumination. PCR products of the expected size were recovered from all samples and sequenced bidirectionally by MACROGENTM on a 3730xl DNA analyser using the above primers. Sequences were edited using Chromas Pro (Technelysium Pty. Ltd.) and BioEdit (Hall 1999), and aligned using ClustalW (Thompson et al 1994); this produced a 567-bp-processed fragment. PAUP (Phylogenetic Analysis Using Parsimony [*and Other Methods]) (Swofford 2002) was used for neighbor-joining, parsimony, and maximum likelihood estimation. For parsimony, 101 nucleotide positions were informative (of 567). Bootstrapping was performed for 1,000 replications (10 random addition sequence replicates, tree-bisection-reconnection (TBR) and MulTrees in effect). For maximum likelihood, MODELTEST (Posada and Crandall 1998) indicated that the most appropriate model of nucleotide substitution was GTR+G where the proportion of invariable sites (I) equaled zero and the gamma shape parameter (G) equalled 0.2252. Nucleotide frequencies were A = 0.3406, C = 0.1613, G = 0.1715, T = 0.3266. Bootstrapping was performed for 100 replications (10 random addition sequence replicates, tree-bisection-reconnection (TBR) and MulTrees in effect). Three unique *C. pacificus* haplotypes were used as the outgroups in all analyses.

Table A1.1.1. Collection and Specimen Data for the *Creontiades* spp. Individuals Used in Molecular Comparison.

Location	Date	Host plant	Species	Specimen code	GenBank accession number	Sex
Byee Queensland Australia	14 Mar. 2006	Pigeonpea	C. dilutus	QLDbyeeFWD1	EF016724	M
				QLDbyeeFWD2	EF016725	M
				QLDbyeeFWD3	EF016726	F
				QLDbyeeFWD4	EF016727	F
				QLDbyeeFWD5	EF016728	F
				QLDbyeeFWD6	EF016729	F
				QLDbyeeFWD7	EF016730	M
				QLDbyeeFWD8	EF016731	M
				QLDbyeeFWD9	EF016732	M
				QLDbyeeFWD10	EF016733	F
Byee Queensland Australia	14 Mar. 2006	Pigeonpea	C. pacificus	QLDbyeePACFWD1	EF016734	M
				QLDbyeePACFWD2	EF016735	F
				QLDbyeePACFWD3	EF016736	M
				QLDbyeePACFWD4	EF016737	M
				QLDbyeePACFWD5	EF016738	F
Hidalgo Co. Texas	15 Feb. 2005	Nettleleaf goosefoot	C. sp	TXFWD1	EF016739	F
				TXFWD2	EF016740	M
				TXFWD3	EF016741	M
				TXFWD4	EF016742	M
				TXFWD7	EF016743	F
				TXFWD8	EF016744	F
				TXFWD10	EF016745	M
				TXFWD11	EF016746	F
				TXFWD12	EF016747	F
				TXFWD14	EF016748	F
Cameron Co. Texas	9 Mar. 2005	London rocket	C. sp	TX1FWD1	EF016749	F
				TX1FWD2	EF016750	M
				TX1FWD3	EF016751	F

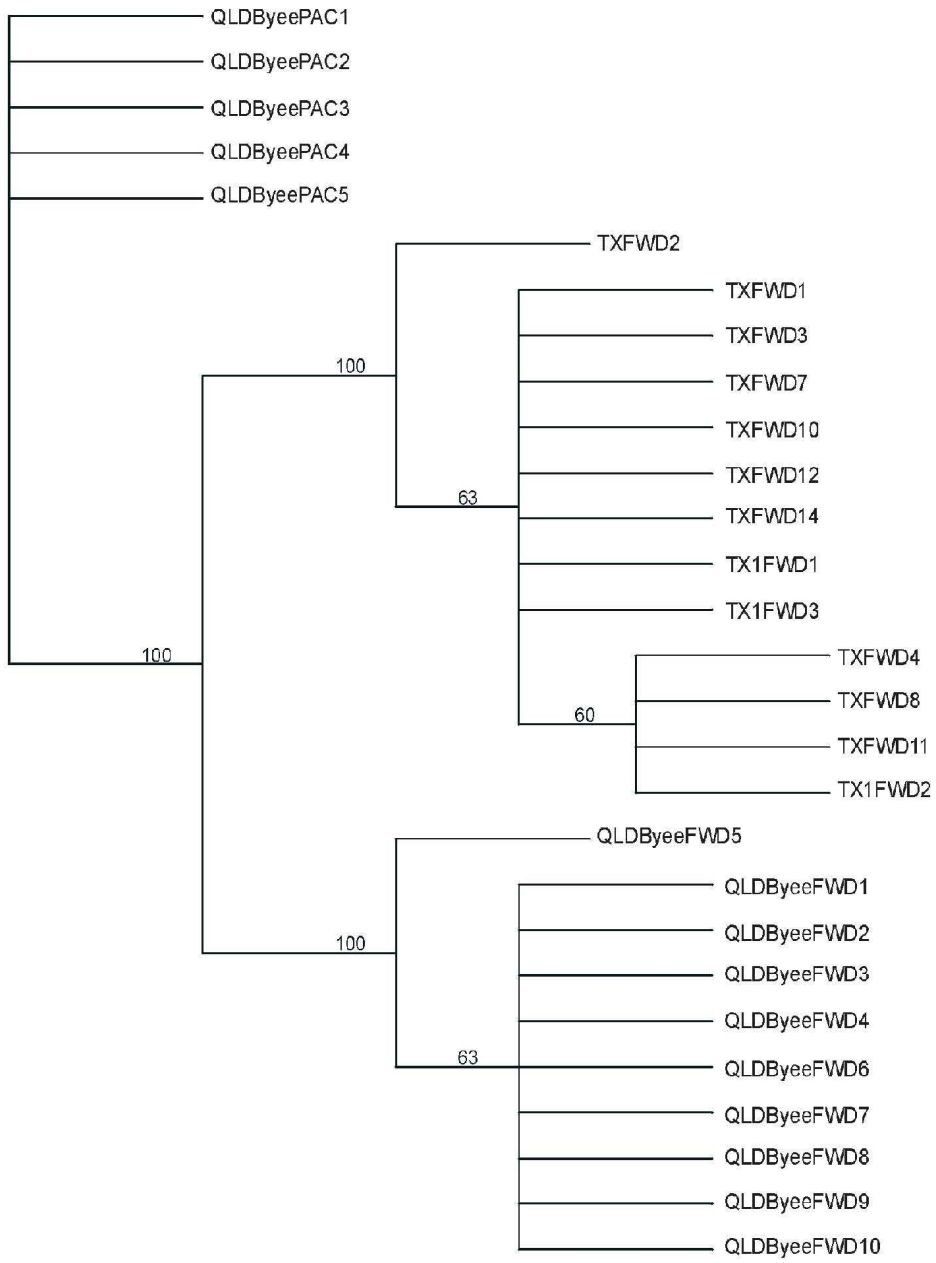

Fig. 1. COI maximum likelihood tree with bootstrap values for *Creontiades* spp. QLDByeePAC = *C. pacificus* from Byee Queensland, Australia (outgroup); QLDByeeFWD = *C. dilutus* from Byee Queensland, Australia; TXFWD + TX1FWD = *Creontiades* sp. from South Texas, USA.

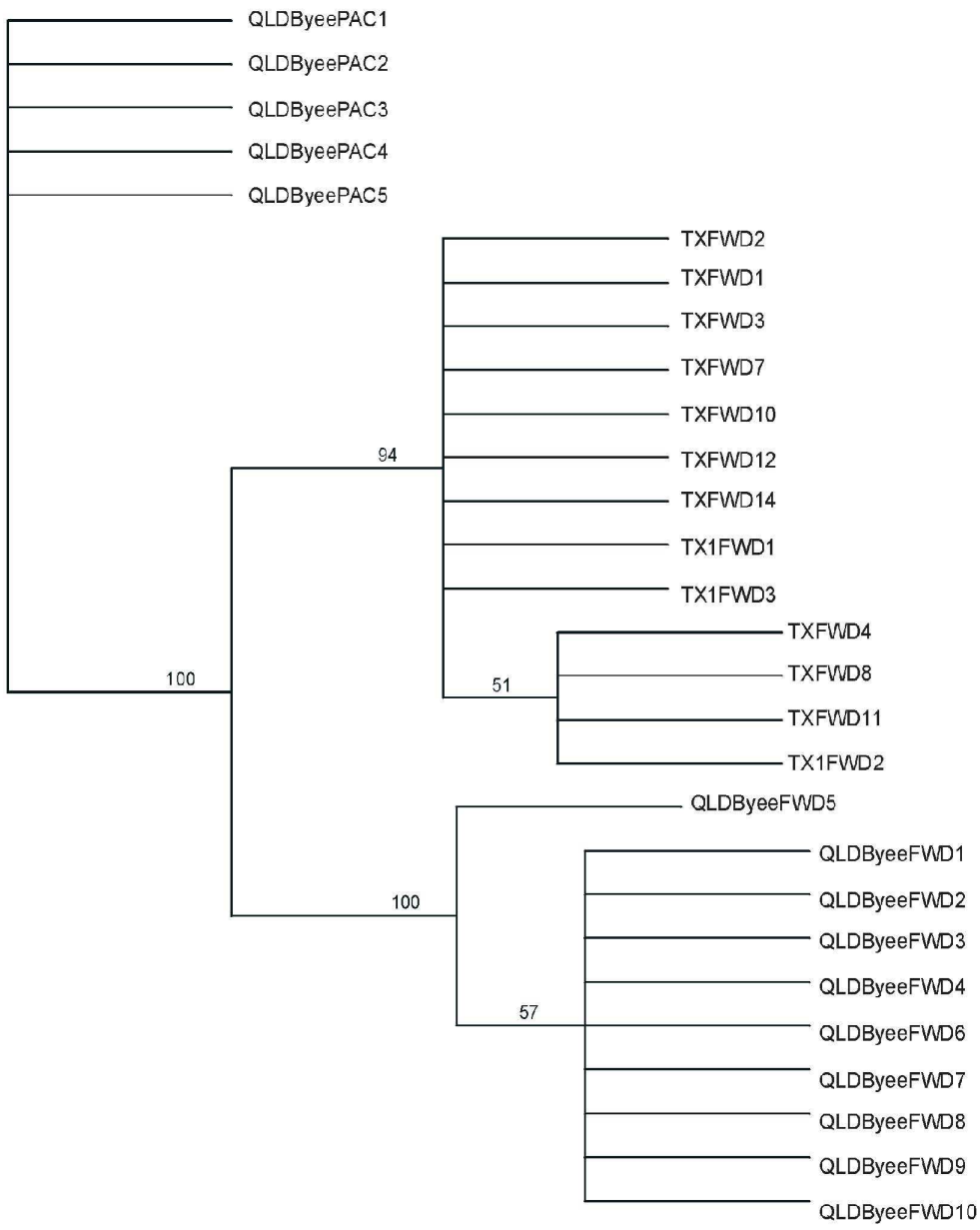

Fig. 2. COI parsimony tree with bootstrap values for *Creontiades* spp. QLDByeePAC = *C. pacificus* from Byee Queensland, Australia (outgroup); QLDByeeFWD = *C. dilutus* from Byee Queensland, Australia; TXFWD + TX1FWD = *Creontiades* sp. from South Texas, USA.

Results

From the total of 28 individuals examined, the 13 specimens from Texas contained nine unique haplotypes, the 10 *C. dilutus* specimens only three and the five *C. pacificus* also three unique haplotypes. All three analytical methods resulted in identical reconstructions. Fig. 1 shows the relationships resulting from maximum likelihood analysis. The Texas and Australia groups were separated by bootstrap values of 100 on each branch. For parsimony, an identical tree was produced with only small differences in bootstrap values (Fig. 2). The branch leading to all of the Australia sequences was supported by a bootstrap value of 100 and the Texas branch was supported by a value of 94. Neighbor-joining provided the same relationship. The Texas mirids all had a within group similarity of 99%. *Creontiades dilutus* from Australia had a within group similarity of 99%. The between group similarity was 89-90%. In CO1, this suggests they might be different species because a 2-5% difference equates to approximately 1 million years (Brower 1994).

Discussion

The fact that there are nine unique haplotypes in the 13 Texas specimens sequenced suggests that a recent invasion of Texas by *C. dilutus* is unlikely because invasions generally involve a small number of founders with low genetic variability. The CO1 is a relatively conservative gene and the great diversity observed in this study suggests this *Creontiades* species has been in Texas for a long time.

Other evidence supports the molecular analyses that the mirid from Texas is not *C. dilutus*. In the Texas mirid, the egg operculum turns a jet black color after incubating for approximately 48 hours at 250 C, whereas the operculum of green mirid eggs from Australia remain an opaque color until eclosion regardless of incubation temperature (M. Khan, personal communication). *Creontiades dilutus* eggs hatch in 4 to 5 days at 30-350 C (Khan et al. 2004), whereas the least time recorded from oviposition to egg hatch for the Texas mirid is 8 days. Also, in the Texas mirid, 1st-3rd instars have multiple red bands on the antennae rather than the single band on the distal segment as in *C. dilutus*. Generalist polyphagous species are prime candidates for harboring specialist cryptic species that cannot be distinguished by morphology alone. It is possible that this is the case with *C. dilutus* in Australia. Further genetic analysis of specimens from a geographic spread and range of host species is ongoing. This will reveal the presence and extent of any cryptic divergence, which can be examined in relation to the Texan *Creontiades*.

Differences in species characterization at the molecular level found in this study may indicate that a revision is warranted to define a new morphological character set to separate species in this taxon. We are collecting additional material from Texas locations to support this taxonomic need.

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Appendix 3.1 Incidence records of the green mirid *Creontiades dilutus* obtained from a survey of the literature and field survey results presented in Chapter 3. Refernces: A = Chinajariyaong 1987, B = Miles 1996, C = Malipatil and Cassis 1997, D = Khan 1999, E = This thesis.

Family	Species	Common Name	Juveniles Present	Reference
Aizoaceae	*Tetragonia tetragonoides*	New Zealand spinach	N	D
Aizoaceae	*Trianthema portulacastrum*		Y	A
Amaranthaceae	*Alternanthera nodiflora*	Common joyweed	Y	D
Apiaceae	*Trachymene glaucifolia*	Blue parsnip	Y	B
Asteraceae	*Calotis multicaulis*	Burr daisy	N	B
Asteraceae	*Flaveria australasica*	Speedy weed	N	B
Asteraceae	*Helianthus annuus*	Sunflower	Y	A, C, D, E
Asteraceae	*Ixiolaena chloroleuca*		N	B
Asteraceae	*Rhodanthe floribunda*		Y	B
Asteraceae	*Senecio glossanthus*	Slender groundsel	N	B
Asteraceae	*Silybum marianum*	Variegated thistle	Y	D
Asteraceae	*Verbesina encelioides*	Wild sunflower	Y	D, B, E
Asteraceae	*Xanthium occidentale*	Noogoora burr	N	D
Asteraceae	*Blumea saxatilis*		Y	E
Asteraceae	*Brachyscome campylocarpa*		Y	E
Asteraceae	*Epaltes cunninghamii*		Y	E
Asteraceae	*Polycalymma stuartii*	Poached-egg daisy	N	E
Asteraceae	*Senecio depressicola*		N	E
Asteraceae	*Senecio gregorii*	Annual yellowtop, fleshy groundsel	Y	E
Boraginaceae	*Echium plantagineum*	Paterson's curse	NR	C
Boraginaceae	*Trichodesma zeylanicum var. zeylanicum*	Camel bush, cattle bush	N	E
Brassicaceae	*Rapistrum rugosum*	Wild turnip	Y	D, B, E
Brassicaceae	*Sisymbrium thellungii*	African turnip weed	N	A
Brassicaceae	*Blennodia pterosperma*	Wild stock, native stock	Y	E
Brassicaceae	*Phlegmatospermum cochlearinum*		Y	E
Brassicaceae	*Sysimbrium irio*	London rocket	N	E
Cactaceae	*Aporocactus flagelliformis*		NR	C
Chenopodiaceae	*Salsola kali*	Salwort	N	A
Chenopodiaceae	*Atriplex angulata*	Angular saltbush	N	E
Chenopodiaceae	*Chenopodium auricomum*	Queensland bluebush	N	E
Compositae	*Carthamus tinctorius*	Safflower	Y	D
Compositae	*Gnaphalium luteo-album*	Jersey cudweed	N	A
Compositae	*Sonchus oleraceus*	Common sow-thistle	N	A, E
Cucurbitaceae	*Citrullus vulgaris*	Melon	NR	C
Cucurbitaceae	*Cucumis sativus*	Cucumber	NR	C
Fabaceae	*Cajanus cajan*	Pigeon pea	Y	A, C, E
Fabaceae	*Crotalaria sp.*	Rattlepod	Y	B, C
Fabaceae	*Cullen cinereum*	Annual verbine	Y	B, E
Fabaceae	*Glycine max*	Soy bean	Y	A
Fabaceae	*Indigofera hirsuta*	Hairy indigo	Y	B
Fabaceae	*Lupinus sp.*	Lupine	Y	D, C

Continued overleaf

Appendix 3.1 Continued

Family	Species	Common Name	Juveniles Present	Reference
Fabaceae	*Macroptilium atropurpureum*	Siratro	N	B
Fabaceae	*Medicago polymorpha*	Burr medic	Y	A, E
Fabaceae	*Medicago sativa*	Lucerne	Y	A, B, D, C, E
Fabaceae	*Melilotus indicus*	Hexham scent	Y	A, B
Fabaceae	*Phaseolus vulgaris*	Green bean	Y	D, C
Fabaceae	*Pisum sativum*	Pea	NR	C
Fabaceae	*Rhynchosia minima*	Rhyncosia	Y	B, C
Fabaceae	*Sesbania cannabina*	Sesbania	Y	B, D
Fabaceae	*Vigna radiata*	Mung bean	Y	A, B, D, C
Fabaceae	*Vigna unguiculata*	Cowpea	NR	C
Fabaceae	*Cicer arietinum*	Chick pea	N	E
Fabaceae	*Crotalaria eremaea*	Loose flowered rattlepod, bluebush pea	Y	E
Fabaceae	*Crotalaria dissitiflora*	Grey rattlepod, plains rattlepod	Y	E
Fabaceae	*Cullen australasicum*	Cullen	Y	E
Fabaceae	*Swainsona galegifolia*	Gilgai darling pea, down's pea bush	Y	E
Fabaceae	*Trigonella suavissima*	Channel clover	Y	E
Fabaceae	*Vicia sativa subsp nigra*	Narrow leaf vetch	Y	E
Geraniaceae	*Erodium cygnorum*	Blue storksbill, wild geranium	Y	E
Goodeniacea	*Goodeniacea heterochila*		N	B
Goodeniaceae	*Goodenia cycloptera*		Y	E
Goodeniaceae	*Scaevola parvibarbata*		Y	E
Gramineae	*Avena sativa*	Oat	NR	C
Gramineae	*Dactylis glomerata*	Cocksfoot	NR	C
Gramineae	*Echinochloa crus-galli*	Barnyard grass	N	A
Gramineae	*Ehrharta erecta*	Panic veldt grass	N	A
Gramineae	*Paspalum dialatum*	Paspalum	N	A
Gramineae	*Sorghum bicolor*	Sorghum	N	A
Gramineae	*Triticum aestivum*	Wheat	NR	C
Haloragaceae	*Haloragis glauca*	Glauca	Y	D
Liliaceae	*Asparagus officinalis*	Asparagus	NR	C
Malvaceae	*Gossypium hirsutum*	Cotton	Y	A, B, D, C, E
Malvaceae	*Malva parviflora*	Marshmallow	N	C, D, E
Malvaceae	*Malva australiana*	Australian hollyhock, flood mallow	Y	E
Molluginaceae	*Glinus lotoides*	Hairy carpet weed	Y	D
Myrtaceae	*Leptospermum/ Melaleuca spp.*	Teatree	NR	C
Polygonaceae	*Rheum rhabarbarum*	Rhubarb	NR	C
Rosaceae	*Malus pumila*	Apple	NR	C
Rosaceae	*Prunus persica*	Peach	NR	C
Rosaceae	*Pyrus communis*	Pear	NR	C
Rosaceae	*Rosa sp*	Rose	NR	C
Rosaceae	*Rubus idaeus*	Raspberry	NR	C
Rutaceae	*Citrus limon*	Lemon	NR	C

Continued overleaf

Appendix 3.1 Continued

Family	Species	Common Name	Juveniles Present	Reference
Rutaceae	*Citrus sinensis*	Orange	NR	C
Scrophulariaceae	*Stemodia florulenta*		N	E
Solanaceae	*Datura inoxia*	Thornapple	N	D
Solanaceae	*Lycopersicon esculenum*	Tomato	NR	C
Solanaceae	*Solanum nigrum*	Black berry nightshade	Y	D
Solanaceae	*Solanum tuberosum*	Potato	NR	C
Umbelliferae	*Coriandrum sativum*	Coriander	N	D
Umbelliferae	*Umbelliferae*		NR	C
Verbenaceae	*Verbena litoralis*		Y	A, B
Verbenaceae	*Verbena supina*	Trailing verbena	Y	D
Verbenaceae	*Verbena tenuisecta*	Mayne's pest	Y	A, B, C
Vitaceae	*Vitis vinifera*	Grape	NR	C
Zygophyllaceae	*Tribulus terrestris*	Caltrop	Y	A, B
Zygophyllaceae	*Zygophyllum lodocarpum*		N	E

Appendix 3.2. *Creontiades dilutus* collection data showing all sites sampled during the field survey reported in Chapter 3. Juv = Juveniles present?

Common Name	Family	Date	Lat	Long	Elev	Total	Mean	Juv
Burr medic	Fabaceae	28/07/2007	150.60706	24.5043	161	0	0	NA
Lucerne	Fabaceae	28/07/2007	150.51276	24.37395	169	0	0	NA
Lucerne	Fabaceae	28/07/2007	150.52171	24.37525	176	0	0	NA
Lucerne	Fabaceae	28/07/2007	150.52314	24.37698	171	0	0	NA
Blue storksbill, wild geranium	Geraniaceae	28/07/2007	150.51912	24.37488	172	0	0	NA
Chick Pea	Fabaceae	29/07/2007	148.0923	23.46574	190	0	0	NA
Wild Turnip	Malvaceae	29/07/2007	148.0923	23.46574	190	0	0	NA
Vetch	Fabaceae	29/07/2007	148.0923	23.46574	190	0	0	NA
Spiked malvastrum	Malvaceae	29/07/2007	148.0923	23.46574	190	1	0.1	N
Chick Pea	Fabaceae	29/07/2007	148.0923	23.46574	190	0	0	NA
Burr medic	Fabaceae	29/07/2007	148.16142	23.5013	183	0	0	NA
Asteraceae	Malvaceae	29/07/2007	148.19818	23.49852	170	0	0	NA
Blue storksbill, wild geranium	Malvaceae	29/07/2007	148.19818	23.49852	170	0	0	NA
Lucerne	Fabaceae	29/07/2007	148.20941	23.57894	191	0	0	NA
Wild Sunflower	Asteraceae	29/07/2007	148.11391	23.55583	163	2	0.2	N
Blackberry nightshade	Solanacae	30/07/2007	145.28114	23.55155	256	0	0	NA
Burr medic	Fabaceae	30/07/2007	145.28114	23.55155	256	0	0	NA
Muellers salltbush	Chenopodiaceae	30/07/2007	145.13105	23.54188	270	0	0	NA
Burr medic	Fabaceae	30/07/2007	144.22381	23.43804	188	0	0	NA
Muellers salltbush	Chenopodiaceae	30/07/2007	144.22381	23.43804	188	0	0	NA
Bullamon lucerne, white scurfpea	Fabaceae	31/07/2007	142.45028	22.23838	169	0	0	NA
Grey rattlepod, plains rattlepod	Fabaceae	31/07/2007	142.45028	22.23838	169	2	0.2	N
Coffee senna, arsenic bush	Caesalpiniaceae	31/07/2007	142.11104	22.21488	226	0	0	NA
Annual Verbine, native lucerne	Fabaceae	1/08/2007	139.67076	23.41438	192	146	14.6	Y
Blue-rod	Plantaginaceae	1/08/2007	139.6707	23.41618	115	2	0.2	N
Galvanised burr	Chenopodiaceae	1/08/2007	139.66935	23.41635	114	0	0	NA
Native verbine	Fabaceae	31/07/2007	139.65477	23.41563	119	33	3.3	Y
Galvanised burr	Chenopodiaceae	31/07/2007	139.65477	23.41563	119	0	0	NA
Annual Verbine, native lucerne	Fabaceae	1/08/2007	139.54743	23.94401	105	102	10.2	Y
Galvanised burr	Chenopodiaceae	1/08/2007	139.54743	23.94401	105	0	0	NA
Loose flowered rattlepod, bluebush pea	Fabaceae	1/08/2007	139.54537	24.12115	99	70	7	Y
Small-beard Fanflower	Goodeniaceae	1/08/2007	139.54537	24.12115	99	38	3.8	Y
Camel bush, cattle bush	Boraginaceae	1/08/2007	139.54537	24.12115	99	8	0.8	Y
Annual Verbine, native lucerne	Fabaceae	1/08/2007	139.46623	24.37087	79	344	34.4	Y
Green Pussytail	Amaranthaceae	1/08/2007	139.46506	24.37405	86	0	0	NA
Loose flowered rattlepod, bluebush pea	Fabaceae	1/08/2007	139.46506	24.37405	86	84	8.4	Y
Annual Verbine, native lucerne	Fabaceae	2/08/2007	139.05527	25.87944	37	62	6.2	Y
Loose flowered rattlepod, bluebush pea	Fabaceae	2/08/2007	139.0538	25.87966	56	38	3.8	N
N/A	Asteraceae	3/08/2007	139.07843	26.67069	21	5	0.5	Y
N/A	Asteraceae	3/08/2007	139.07843	26.67069	21	3	0.3	Y
Wild stock, native stock	Brassicaceae	4/08/2007	137.27917	26.57427	18	31	3.1	Y
N/A	Goodeniaceae	4/08/2007	137.27917	26.57427	18	4	0.4	Y
Annual yellowtop, fleshy groundsel	Asteraceae	4/08/2007	137.2745	26.57424	17	3	0.3	N

Continued overleaf

Appendix 3.2 continued

Common Name	Family	Date	Lat	Long	Elev	Total	Mean	Juv
Wild stock, native stock	Brassicaceae	5/08/2007	137.04221	26.32323	67	12	1.2	N
Green mulla mulla	Amaranthaceae	5/08/2007	137.04221	26.32323	67	0	0	NA
Tangled mulla-mulla	Amaranthaceae	5/08/2007	137.04221	26.32323	67	0	0	NA
Poached-egg daisy	Asteraceae	5/08/2007	137.04221	26.32323	67	1	0.1	Y
Annual yellowtop, fleshy groundsel	Asteraceae	5/08/2007	137.04221	26.32323	67	14	1.4	Y
Loose flowered rattlepod, bluebush pea	Fabaceae	6/08/2007	138.81915	25.9011	29	15	1.5	Y
Tangled mulla-mulla	Amaranthaceae	6/08/2007	138.81915	25.9011	29	0	0	NA
Camel bush, cattle bush	Boraginaceae	6/08/2007	138.81915	25.9011	29	0	0	NA
Tall Verbine, Georgina lucerne	Fabaceae	7/08/2007	138.81915	25.9011	29	109	10.9	Y
Fan saltbush, angular saltbush	Chenopodiaceae	7/08/2007	138.81915	25.9011	29	2	0.2	Y
Queensland bluebush	Chenopodiaceae	7/08/2007	138.81915	25.9011	29	4	0.4	N
Wooly headed Burr daisy	Asteraceae	7/08/2007	138.81915	25.9011	29	7	0.7	Y
Tall Verbine, Georgina lucerne	Fabaceae	7/08/2007	138.85397	25.9002	36	240	24	Y
Australian Hollyhock, flood mallow	Malvaceae	7/08/2007	138.85397	25.9002	36	6	0.6	N
N/A	Asteraceae	7/08/2007	138.85397	25.9002	36	18	1.8	Y
N/A	Asteraceae	8/08/2007	140.34479	25.84281	51	8	0.8	Y
Tall Verbine, Georgina lucerne	Fabaceae	9/08/2007	140.73364	27.74113	30	30	3	Y
Annual yellowtop, fleshy groundsel	Asteraceae	9/08/2007	140.65095	27.93677	41	7	0.7	N
Wild stock, native stock	Brassicaceae	9/08/2007	140.18757	28.49232	21	48	4.8	Y
Annual yellowtop, fleshy groundsel	Asteraceae	9/08/2007	140.18757	28.49232	21	8	0.8	N
Gilgai Darling pea, Down's pea bush	Fabaceae	10/08/2007	141.91533	29.5757	160	78	7.8	Y
Coopers clover	Fabaceae	10/08/2007	141.91533	29.5757	160	19	1.9	Y
Pop saltbush	Chenopodiaceae	10/08/2007	141.91533	29.5757	160	0	0	NA
London rocket	Brassicaceae	10/08/2007	141.91533	29.5757	160	48	4.8	N
Bullamon lucerne, white scurfpea	Fabaceae	10/08/2007	141.91533	29.5757	160	19	1.9	Y
Blue storksbill, wild geranium	Geraniaceae	11/08/2007	144.41601	30.93653	83	21	2.1	Y
Burr medic	Fabaceae	11/08/2007	144.41601	30.93653	83	0	0	NA
London rocket	Brassicaceae	11/08/2007	144.41601	30.93653	83	0	0	NA
Blue storksbill, wild geranium	Geraniaceae	11/08/2007	145.81435	30.1743	100	7	0.7	NA
Blue storksbill, wild geranium	Geraniaceae	12/08/2007	146.7766	29.95203	77	42	4.2	Y
Common sowthistle	Asteraceae	12/08/2007	146.7766	29.95203	77	0	0	NA
Lucerne	Fabaceae	12/08/2007	146.35038	29.9615	115	2	0.2	N
Wild Turnip	Malvaceae	12/08/2007	148.15509	29.99946	132	5	0.5	N
Common sowthistle	Asteraceae	12/08/2007	148.15509	29.99946	132	32	3.2	Y
Vetch	Fabaceae	13/08/2007	149.78924	30.32028	201	0	0	NA
Common sowthistle	Asteraceae	13/08/2007	149.78924	30.32028	201	0	0	NA
Burr medic	Fabaceae	13/08/2007	149.78924	30.32028	201	0	0	NA
Wild Turnip	Malvaceae	13/08/2007	149.79217	30.32563	207	2	0.2	Y
Vetch	Fabaceae	13/08/2007	149.77808	30.31509	207	2	0.2	Y

Appendix 4.1. Pairwise Fst's for all sites at which microsatellites were genotyped in Chapter 4. NS = Not significant, * P = 0.00001-0.000095, ** P = 0.000001-0.00001, *** P < 0.000001.

	BIL-GH	BIL-MS1	BIL-MS2	EMR-VE1	EMR-MS	EMR-GH	EMR-CA	EMR-VE2	BIR-BS	SIM-BP1	SIM-GC	SIM-SG1	SIM-BP2	SIM-SG2	SIM-CE	SIM-CA	EYR-CA	MIL-TS	MIL-SG	MIL-SI	MIL-CP	TIL-EC	BOU-EC	WAL-MP	BRE-MP	BRE-MS1	BRE-EC	BRE-PC	BRE-MS2	NAR-MP	NAR-GH	NAR-MS
BIL-GH		0.074	**0.120**	**0.098**	**0.085**	**0.057**	0.061	0.070	**0.077**	0.050	**0.030**	0.046	0.046	0.065	**0.081**	**0.104**	**0.093**	0.193	**0.188**	**0.137**	**0.096**	**0.111**	**0.121**	0.056	**0.173**	**0.119**	**0.133**	**0.223**	0.192	**0.169**	0.030	**0.071**
BIL-MS1	NS		**0.182**	**0.061**	**0.077**	**0.111**	0.162	0.063	0.064	0.097	0.091	0.069	0.128	0.129	0.137	**0.154**	**0.131**	0.194	**0.151**	**0.111**	**0.075**	**0.105**	**0.122**	0.055	**0.137**	**0.083**	**0.106**	**0.138**	0.134	0.148	**0.073**	**0.061**
BIL-MS2	***	**		**0.181**	**0.168**	**0.130**	0.046	0.098	**0.119**	0.099	0.089	0.079	**0.124**	**0.167**	**0.145**	0.062	**0.102**	**0.193**	**0.188**	**0.174**	**0.193**	**0.192**	**0.214**	0.157	**0.226**	**0.190**	**0.203**	**0.278**	0.329	**0.298**	**0.114**	**0.146**
EMR-VE1	***	*	***		0.012	0.048	**0.117**	**0.039**	0.034	**0.044**	0.060	0.047	0.076	0.076	**0.077**	**0.125**	**0.084**	**0.185**	**0.142**	**0.117**	**0.072**	**0.062**	**0.081**	0.028	**0.086**	**0.053**	**0.064**	**0.109**	0.084	0.073	**0.074**	**0.067**
EMR-MS	***	**	***	NS		0.021	**0.108**	**0.047**	0.032	**0.053**	0.056	0.064	0.065	0.048	0.051	**0.115**	**0.081**	**0.162**	**0.148**	**0.100**	**0.053**	**0.050**	**0.051**	0.045	**0.141**	**0.068**	**0.070**	**0.114**	0.114	0.070	**0.072**	**0.066**
EMR-GH	***	***	***	NS	NS		**0.079**	**0.055**	0.031	**0.039**	0.042	0.048	0.042	0.050	**0.026**	**0.102**	**0.077**	**0.186**	**0.185**	**0.132**	**0.086**	**0.076**	**0.085**	0.048	**0.143**	**0.089**	**0.091**	**0.158**	0.150	0.081	**0.067**	**0.085**
EMR-CA	NS	NS	NS	**	**	***		0.044	0.073	0.029	**0.025**	0.039	0.006	0.068	0.067	0.053	0.062	**0.173**	**0.168**	**0.147**	**0.149**	**0.143**	**0.124**	0.109	**0.214**	**0.154**	**0.173**	**0.269**	0.277	**0.234**	0.067	**0.114**
EMR-VE2	NS	NS	NS	**	***	**	NS		0.014	0.013	0.051	0.021	0.025	0.083	0.050	**0.052**	**0.041**	**0.153**	**0.117**	**0.099**	**0.098**	**0.103**	**0.080**	0.059	**0.131**	**0.073**	**0.103**	**0.162**	0.165	0.156	0.054	**0.061**
BIR-BS	*	NS	*	NS	NS	NS	NS	NS		0.012	0.048	0.014	0.049	0.065	0.024	**0.088**	**0.070**	**0.132**	**0.107**	**0.073**	**0.057**	**0.080**	**0.082**	0.042	**0.114**	**0.051**	**0.087**	**0.106**	0.139	0.077	**0.075**	**0.068**
SIM-BP1	NS	NS	NS	**	**	***	NS	NS	NS		0.027	0.004	0.010	0.061	0.036	**0.050**	**0.038**	**0.170**	**0.148**	**0.117**	**0.103**	**0.095**	**0.097**	0.044	**0.125**	**0.072**	**0.090**	**0.149**	**0.155**	0.126	0.054	**0.079**
SIM-GC	***	NS	NS	NS	NS	*	NS	NS	NS	NS		0.008	0.007	-0.009	0.043	**0.073**	**0.067**	**0.169**	**0.151**	**0.113**	**0.065**	**0.071**	**0.082**	0.039	**0.163**	**0.112**	**0.107**	**0.184**	0.156	0.106	0.046	**0.072**
SIM-SG1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.020	0.069	0.053	0.074	0.058	**0.204**	**0.181**	0.157	0.115	**0.114**	0.128	0.069	0.074	0.094	0.122	0.184	0.213	0.169	0.035	0.062
SIM-BP2	NS	NS	***	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.034	0.025	**0.077**	0.058	**0.164**	**0.157**	**0.125**	**0.103**	**0.104**	**0.072**	0.064	**0.195**	**0.122**	**0.130**	**0.230**	**0.218**	0.181	0.063	0.094
SIM-SG2	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.031	**0.126**	**0.096**	**0.177**	**0.170**	**0.119**	0.047	**0.061**	**0.062**	0.069	**0.212**	**0.130**	**0.117**	**0.199**	**0.179**	0.096	0.090	0.096
SIM-CE	***	NS	***	*	NS	*	NS	NS	NS	NS	NS	NS	NS	NS		**0.122**	**0.100**	**0.178**	**0.178**	**0.132**	**0.100**	**0.113**	**0.092**	0.078	**0.201**	**0.124**	**0.132**	**0.202**	**0.194**	0.105	**0.112**	**0.129**
SIM-CA	***	***	NS	***	***	***	NS	*	***	***	**	NS	**	***	***		0.002	**0.147**	**0.144**	**0.118**	**0.150**	**0.132**	**0.140**	**0.132**	**0.205**	**0.135**	**0.120**	**0.192**	**0.232**	**0.217**	**0.081**	**0.100**
EYR-CA	***	***	***	***	***	***	NS	***	***	***	**	NS	NS	*	***	NS		**0.153**	**0.142**	**0.114**	**0.120**	**0.099**	**0.110**	**0.106**	**0.174**	**0.102**	**0.097**	**0.166**	**0.187**	**0.168**	**0.069**	**0.078**
MIL-TS	***	***	***	***	***	***	***	***	***	***	***	*	***	***	***	***	***		0.013	0.015	**0.126**	**0.144**	**0.128**	**0.169**	**0.307**	**0.197**	**0.188**	**0.223**	**0.247**	**0.245**	**0.178**	**0.149**
MIL-SG	***	***	***	***	***	***	***	***	***	***	***	*	***	***	***	***	***	NS		0.012	**0.114**	**0.132**	**0.129**	**0.138**	**0.248**	**0.158**	**0.166**	**0.187**	**0.198**	**0.220**	**0.168**	**0.129**
MIL-SI	***	***	***	***	***	***	***	***	***	***	***	NS	***	***	***	***	***	NS	NS		**0.064**	**0.087**	**0.084**	**0.100**	**0.239**	**0.130**	**0.125**	**0.154**	**0.168**	**0.175**	**0.121**	**0.088**
MIL-CP	***	***	***	***	***	***	***	***	***	***	***	NS	***	NS	***	***	***	***	***	**		0.009	0.038	**0.056**	**0.203**	**0.096**	**0.084**	**0.123**	**0.116**	0.073	**0.082**	**0.049**
TIL-EC	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	NS		0.027	**0.068**	**0.179**	**0.091**	**0.070**	**0.125**	**0.110**	0.070	**0.078**	**0.052**
BOU-EC	***	***	***	***	***	***	***	***	***	***	***	NS	***	*	***	***	***	***	***	***	NS	NS		**0.090**	**0.242**	**0.113**	**0.102**	**0.184**	**0.172**	**0.131**	**0.087**	**0.065**
WAL-MP	***	**	***	NS	NS	*	*	***	NS	NS	NS	NS	NS	NS	NS	***	***	***	***	***	***	***	***		0.090	0.038	**0.075**	**0.136**	**0.082**	0.081	0.050	**0.053**
BRE-MP	***	***	***	***	***	***	***	***	**	***	***	NS	***	***	***	***	***	***	***	***	***	***	***	NS		0.066	**0.174**	**0.213**	**0.191**	**0.210**	**0.132**	**0.125**
BRE-MS1	***	***	***	***	***	***	***	***	**	***	***	NS	*	***	***	***	***	***	***	***	***	***	***	NS	NS		**0.066**	**0.091**	**0.105**	0.102	**0.083**	**0.064**
BRE-EC	***	***	***	***	***	***	***	***	***	***	***	NS	**	***	***	***	***	***	***	***	***	***	***	***	***	***		**0.024**	**0.045**	0.048	**0.104**	**0.090**
BRE-PC	***	***	***	***	***	***	***	***	**	***	***	NS	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***		0.091	0.096	**0.185**	**0.149**
BRE-MS2	***	**	***	**	***	***	***	***	NS	***	**	NS	*	*	***	***	***	***	***	***	***	***	***	***	***	***	***	NS		0.066	**0.173**	**0.148**
NAR-MP	***	NS	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	**	**	***	***	*	NS	NS	**	NS	*	NS	NS	NS	NS		**0.180**	**0.156**
NAR-GH	NS	*	*	***	***	***	NS	NS	**	NS	NS	NS	NS	NS	***	***	***	***	***	***	***	***	***	NS	***	***	***	***	***	**		0.010
NAR-MS	***	***	***	***	***	***	**	*	**	***	**	NS	NS	NS	***	***	***	***	***	***	**	***	*	***	**	***	***	***	***	*	NS	

Appendix 4.2. Custom Perl script for converting the output of BayesAss into a tabular format (tab delimited text) that can be read into excel.

```
#!/usr/bin/perl

use warnings;
use strict;

#ask the user for the name of the intput and output files
print "Please enter the name of the file you would like to sort:";
my $inputfile =<>;
chomp $inputfile;
my $fileextension = "_table.txt";
my $outputfile =$inputfile.$fileextension;

#open file handle to GMALL2output, and output file

open (IN, "$inputfile") or die ("could not open file \n");
open (OUT, ">$outputfile") or die ("could not open output file \n");

#define results,INTO,FROM and confidence arrays

my @POPS;
my %FROM;
my $pop;

#set population counter to zero
my $countINTO = 0;
#read in file line by line,chomp, and split on spaces

while (my $line = <IN>){
	chomp $line;
	my @words = split (/\s/, $line);
	#use if else loop to retrieve the data and push into arrays
		if ($line =~ /migration rates into/){
			push (@POPS, "$words[5]");
			#count the number of populations using the INTO Hash
			$countINTO++;
		}
		#take the from population and assign to the pop variable to use as a key for the hash
```

```
		elsif ($line =~ /From\s+population/){
			$pop = $words[5];
		}
		#take the mean and push into the hash using the key defined in $pop above
		elsif ($line =~ /mean for this distribution/){
			#substitute the comma at in the mean value for nothing
			$words[5] =~ s/,//;

			push (@{$FROM{$pop}}, "$words[5]");
		}
		#take the 2 confidence intervals and push into the same hash-key as above
		elsif ($line =~/Confidence interval: \(([\d\.\-e]+), ([\d\.\-e]+)/) {
			push (@{$FROM{$pop}}, $1);
			push (@{$FROM{$pop}}, $2);
		}

		else{
		}
}
# print to screen the number of populations using the counter and list them from the INTO array
print "The number of populations read in from this file is $countINTO: \n";
print join ("\n", @POPS),"\n";
#print to screen the data array for the first key in the hash to make sure the data has been read in correctly
print "The data for the first population are:\n ";
print $POPS[0], "\t";
print join ("\t", @{$FROM{$POPS[0]}}),"\n";

#print the title lines to the output file
print OUT "\tINTO\nFROM\t";
print OUT join ("\tlower 95% CI\tupper 95% CI\t", @POPS),"\tlower 95% CI\tupper 95% CI","\n";
#print the relevant data to the output file using foreach loop on the keys stored in the @POPS array
foreach my$element(@POPS){
	print OUT $element, "\t";
	print OUT join ("\t", @{$FROM{$element}}), "\n";
}

close IN;
close OUT;

exit;
```

Appendix 4.3. Results of 10 runs of the BayesAss Algorithm using the microsatellite data generated in Chapter 4, with averages and total number of times the result was achieved.

Run 1	INTO													
FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6784	(0.66683-0.707641)	0.0070	(7.39965e-08-0.0452114)	0.0004	(5.02067e-19-0.003253)	0.0042	(3.32989e-07-0.024694)	0.0018	(1.53536e-10-0.00997136)	0.0033	(7.11051e-09-0.0228437)	0.0010	(9.83695e-10-0.00673681)
Biloeala Jul07	0.0037	(1.97263e-07-0.0218262)	0.6860	(0.667002-0.73877)	0.0004	(1.19479e-19-0.00417796)	0.0020	(1.82551e-09-0.0124083)	0.0007	(7.23795e-12-0.00459683)	0.0031	(1.21336e-08-0.0197063)	0.0010	(2.16905e-10-0.006328)
Emerald Jan07	0.0719	(4.89443e-06-0.202911)	0.0099	(5.81098e-08-0.0625765)	0.9951	(0.979833-0.999842)	0.0220	(0.000111429-0.0678772)	0.0008	(3.73778e-12-0.00565865)	0.0167	(3.19796e-08-0.0734202)	0.0012	(4.08876e-10-0.00736538)
Emerald Jul07	0.0039	(5.71019e-07-0.0208117)	0.0067	(1.05101e-08-0.0412441)	0.0003	(5.11782e-20-0.00376354)	0.6756	(0.666905-0.69682)	0.0010	(1.85787e-11-0.00677542)	0.0030	(3.44023e-09-0.0206527)	0.0011	(4.33423e-10-0.0070801)
Simpson Aug07	0.2042	(0.0777035-0.313396)	0.2363	(0.118365-0.314413)	0.0005	(3.86987e-19-0.00438976)	0.2705	(0.214859-0.313468)	0.9884	(0.972231-0.99779)	0.2628	(0.181072-0.319667)	0.0010	(1.53769e-10-0.00663297)
Eyre Aug07	0.0043	(3.11746e-07-0.0261688)	0.0060	(4.3607e-08-0.0355629)	0.0004	(5.69822e-18-0.00396491)	0.0033	(8.96927e-08-0.0189611)	0.0013	(3.79801e-11-0.00854976)	0.6785	(0.666881-0.709454)	0.0010	(9.75528e-09-0.00662263)
Milparinka Aug07	0.0067	(1.53908e-07-0.0338581)	0.0077	(3.45161e-08-0.0525173)	0.0004	(1.75299e-18-0.00358529)	0.0020	(1.27571e-08-0.0138799)	0.0006	(2.10314e-12-0.00430358)	0.0082	(8.82275e-09-0.0519965)	0.9499	(0.908962-0.983226)
Tilpa Aug07	0.0037	(7.83322e-07-0.0237166)	0.0059	(1.56725e-07-0.0379281)	0.0004	(2.18338e-20-0.00376541)	0.0024	(4.76872e-08-0.0146492)	0.0006	(1.0503e-11-0.00519674)	0.0034	(1.48855e-08-0.0224988)	0.0009	(8.65633e-10-0.00589077)
Bourke Aug07	0.0043	(4.63701e-07-0.0244251)	0.0079	(4.24141e-08-0.0534275)	0.0004	(1.00669e-19-0.00452112)	0.0025	(1.74425e-08-0.0162082)	0.0006	(1.76089e-12-0.00447045)	0.0059	(3.57543e-09-0.0323383)	0.0388	(0.00753945-0.0776278)
Walget Mar08	0.0038	(3.24112e-07-0.0210224)	0.0065	(1.35444e-08-0.0425569)	0.0004	(1.90086e-18-0.00316062)	0.0022	(8.7395e-09-0.0130997)	0.0006	(4.78229e-12-0.00411709)	0.0030	(3.83433e-09-0.0194245)	0.0011	(2.48078e-09-0.00782098)
Brewarrina Mar08	0.0036	(3.98688e-07-0.0185661)	0.0071	(2.3886e-08-0.0483844)	0.0004	(1.30596e-18-0.00364622)	0.0032	(2.34841e-08-0.0222034)	0.0016	(9.15475e-12-0.0107518)	0.0036	(3.72919e-09-0.0275986)	0.0011	(3.68338e-09-0.00751781)
Brewarrina Aug07	0.0063	(9.20529e-07-0.0341372)	0.0066	(8.01388e-08-0.045363)	0.0005	(3.44704e-18-0.00456329)	0.0073	(2.24617e-06-0.0316891)	0.0009	(2.64693e-11-0.00633113)	0.0052	(2.63357e-09-0.0290449)	0.0008	(8.11857e-10-0.00557596)
Narrabri Jan07	0.0052	(2.05186e-06-0.0300278)	0.0064	(2.29592e-08-0.04102)	0.0004	(7.11133e-21-0.00343414)	0.0027	(1.42722e-07-0.0171501)	0.0013	(2.93519e-12-0.00916102)	0.0033	(4.7969e-09-0.0225865)	0.0010	(6.83574e-11-0.00733938)

Run 1 Continued	INTO											
FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0037	(4.432e-09-0.0258471)	0.0012	(4.91797e-18-0.0108073)	0.0047	(3.33056e-06-0.0278879)	0.0021	(2.27126e-08-0.0135338)	0.0008	(7.45878e-19-0.00694764)	0.0029	(2.2976e-05-0.0157413)
Biloeala Jul07	0.0034	(5.8575e-09-0.0229855)	0.0012	(3.22533e-20-0.011788)	0.0038	(2.63161e-06-0.02177)	0.0019	(1.32199e-08-0.0126379)	0.0007	(2.0626e-18-0.00686865)	0.0032	(2.08972e-05-0.0179983)
Emerald Jan07	0.0067	(9.43397e-09-0.0453171)	0.0012	(5.34879e-19-0.011044)	0.1384	(0.0630721-0.221493)	0.0604	(1.29902e-05-0.142269)	0.0009	(4.46801e-20-0.00864056)	0.0844	(0.0219378-0.159718)
Emerald Jul07	0.0036	(2.5182e-08-0.0273294)	0.0012	(4.99194e-21-0.0114505)	0.0036	(1.01852e-05-0.018035)	0.0020	(2.01118e-09-0.013271)	0.0007	(7.31168e-19-0.00737683)	0.0029	(5.17117e-06-0.0145041)
Simpson Aug07	0.0072	(1.69573e-08-0.0471737)	0.0013	(5.50922e-18-0.0132781)	0.0705	(0.0164464-0.154175)	0.0573	(0.00808063-0.126239)	0.0009	(7.4827e-19-0.00805367)	0.1306	(0.0529714-0.211686)

Eyre Aug07	0.0033	(3.87404e-09-0.0230904)	0.0010	(3.61669e-20-0.00947349)	0.0041	(1.09456e-05-0.0200717)	0.0021	(5.15959e-09-0.01388)	0.0007	(9.79551e-18-0.00665668)	0.0031	(1.11705e-05-0.015068)
Milparinka Aug07	0.0169	(3.44355e-07-0.0761808)	0.0010	(3.39566e-19-0.00992565)	0.0048	(4.19911e-06-0.0282772)	0.0022	(5.95276e-08-0.0144668)	0.0007	(6.24208e-19-0.00592589)	0.0035	(5.14545e-06-0.0163889)
Tilpa Aug07	0.6793	(0.667085-0.713382)	0.0011	(2.88559e-18-0.00936205)	0.0042	(6.33709e-06-0.0227416)	0.0021	(7.28799e-09-0.0135378)	0.0007	(1.92337e-18-0.00696427)	0.0029	(8.84213e-06-0.0156315)
Bourke Aug07	0.2602	(0.165902-0.320459)	0.9867	(0.951875-0.999637)	0.0062	(6.61631e-06-0.0313009)	0.0029	(2.22932e-08-0.0172517)	0.0008	(1.79062e-18-0.00965827)	0.0737	(0.0340398-0.125298)
Walget Mar08	0.0036	(1.41282e-08-0.0241292)	0.0008	(2.85425e-18-0.00794715)	0.6762	(0.666885-0.70235)	0.0021	(2.7291e-08-0.0150908)	0.0008	(2.04544e-19-0.00652754)	0.0030	(8.07701e-07-0.0145779)
Brewarrina Mar08	0.0045	(5.07069e-08-0.0305915)	0.0010	(5.16844e-19-0.0100938)	0.0732	(0.0274727-0.133212)	0.8600	(0.793613-0.942955)	0.0008	(1.42597e-19-0.00788308)	0.0046	(6.48631e-06-0.0213492)
Brewarrina Aug07	0.0040	(3.01588e-09-0.0232465)	0.0013	(3.86581e-20-0.013302)	0.0058	(6.88528e-06-0.0301522)	0.0027	(9.10149e-09-0.0166124)	0.9907	(0.965468-0.999684)	0.0118	(0.00104914-0.0342328)
Narrabri Jan07	0.0035	(7.03016e-09-0.0234638)	0.0010	(6.34511e-20-0.0104022)	0.0045	(6.71325e-06-0.0226859)	0.0022	(5.82435e-08-0.0146461)	0.0007	(1.55851e-19-0.00637227)	0.6736	(0.66689-0.690011)

Run 2 INTO

FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.9865	(0.954951-0.9997)	0.0120	(5.90459e-08-0.0653415)	0.0601	(0.00417827-0.132157)	0.0477	(0.00022126-0.1237)	0.0018	(2.76346e-11-0.0130852)	0.0051	(2.70211e-09-0.0348814)	0.0015	(5.34219e-09-0.00984498)
Biloeala Jul07	0.0009	(1.11362e-18-0.00890129)	0.6856	(0.66708-0.730908)	0.0010	(8.70454e-10-0.00699945)	0.0031	(3.28449e-07-0.0172556)	0.0007	(3.16723e-12-0.00447911)	0.0033	(6.28802e-09-0.0218479)	0.0012	(1.64709e-09-0.00773857)
Emerald Jan07	0.0013	(7.72332e-18-0.0124838)	0.0086	(3.84104e-08-0.0621388)	0.9190	(0.850401-0.976448)	0.0208	(0.00174517-0.0589488)	0.0010	(1.77687e-11-0.00677185)	0.0117	(4.3254e-08-0.0699237)	0.0011	(1.34476e-09-0.00802118)
Emerald Jul07	0.0010	(5.02287e-20-0.0096683)	0.0068	(3.00629e-08-0.0393727)	0.0011	(2.11757e-09-0.00717368)	0.6761	(0.666987-0.697091)	0.0010	(6.18685e-11-0.00744035)	0.0031	(7.99794e-09-0.023465)	0.0010	(2.8009e-10-0.00649983)
Simpson Aug07	0.0012	(1.69832e-18-0.0117442)	0.2220	(0.109963-0.31357)	0.0071	(1.7107e-07-0.0312564)	0.2231	(0.138912-0.295368)	0.9865	(0.967738-0.997156)	0.2658	(0.183584-0.319393)	0.0012	(3.87504e-09-0.00881507)
Eyre Aug07	0.0012	(7.98796e-19-0.0100057)	0.0064	(4.07603e-08-0.0412507)	0.0011	(9.12125e-09-0.0072763)	0.0035	(4.34825e-07-0.0186059)	0.0017	(3.51331e-11-0.00997775)	0.6778	(0.666926-0.706475)	0.0011	(3.26422e-09-0.00696023)
Milparinka Aug07	0.0013	(3.51499e-18-0.0112523)	0.0182	(2.6266e-07-0.0857426)	0.0013	(9.76777e-10-0.00884785)	0.0034	(1.72323e-07-0.01846)	0.0007	(5.19443e-12-0.00562813)	0.0109	(4.38797e-08-0.0551714)	0.9442	(0.896961-0.985922)
Tilpa Aug07	0.0014	(1.87635e-20-0.0121909)	0.0069	(2.16713e-08-0.0434949)	0.0021	(4.31412e-09-0.012885)	0.0032	(3.52202e-07-0.0171371)	0.0007	(3.49596e-11-0.00489462)	0.0042	(8.92449e-09-0.029606)	0.0433	(0.00395543-0.090001)
Bourke Aug07	0.0010	(2.74341e-18-0.00973747)	0.0070	(2.10052e-08-0.0477471)	0.0010	(2.90762e-10-0.00710459)	0.0035	(3.12142e-07-0.0207034)	0.0012	(1.0838e-11-0.00917725)	0.0034	(9.643e-09-0.0230656)	0.0012	(2.29047e-09-0.00809027)
Walget Mar08	0.0010	(4.45446e-17-0.010039)	0.0060	(1.97104e-08-0.0405809)	0.0012	(1.3322e-08-0.00843872)	0.0029	(5.29329e-08-0.0178794)	0.0009	(1.02925e-11-0.00667198)	0.0034	(1.67497e-08-0.0208099)	0.0012	(1.99511e-09-0.00827524)
Brewarrina Mar08	0.0012	(4.74948e-18-0.0107358)	0.0063	(8.51671e-08-0.0390482)	0.0016	(3.78594e-09-0.0107513)	0.0032	(1.01676e-06-0.0184683)	0.0008	(8.32887e-12-0.00648072)	0.0032	(2.8004e-08-0.0210253)	0.0010	(4.3838e-09-0.00659539)
Brewarrina Aug07	0.0012	(4.17225e-18-0.0109634)	0.0071	(1.68727e-08-0.0482734)	0.0020	(3.20617e-08-0.0125959)	0.0063	(2.36909e-06-0.0273453)	0.0011	(4.70869e-11-0.00834013)	0.0050	(2.73705e-08-0.0315993)	0.0010	(4.205e-11-0.00698376)
Narrabri Jan07	0.0009	(2.14552e-18-0.00909438)	0.0070	(8.98612e-08-0.0436237)	0.0013	(3.32343e-09-0.00820967)	0.0031	(2.05574e-07-0.016452)	0.0018	(1.12961e-10-0.0102919)	0.0032	(2.37607e-09-0.0223057)	0.0010	(3.19328e-10-0.00644287)

Run 2 Continued INTO

FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0040	(2.38381e-13-0.0276249)	0.0045	(8.40484e-09-0.0293299)	0.1055	(0.0300598-0.19982)	0.0977	(0.0314034-0.161095)	0.0008	(2.31467e-19-0.00647386)	0.1910	(0.0991774-0.255107)
Biloeala Jul07	0.0016	(5.22242e-18-0.0124843)	0.0034	(8.34836e-09-0.0236336)	0.0045	(4.29755e-06-0.0218253)	0.0020	(1.2822e-08-0.0130774)	0.0007	(4.87205e-17-0.00597417)	0.0030	(3.2081e-06-0.0165191)
Emerald Jan07	0.0025	(2.51821e-14-0.0221432)	0.0043	(6.72289e-09-0.028804)	0.0834	(0.0265199-0.163774)	0.0094	(6.70137e-08-0.0508573)	0.0016	(3.99811e-16-0.0154494)	0.0149	(8.4376e-06-0.0549971)
Emerald Jul07	0.0019	(8.80926e-15-0.0159398)	0.0033	(3.71928e-09-0.0208111)	0.0042	(5.0172e-06-0.0212336)	0.0020	(3.98446e-09-0.0146249)	0.0008	(3.72119e-18-0.00717352)	0.0027	(1.64007e-06-0.0146112)
Simpson Aug07	0.0032	(4.49144e-15-0.0261432)	0.0040	(3.38467e-09-0.0264104)	0.0254	(0.000175141-0.087457)	0.0214	(3.88434e-07-0.0782688)	0.0008	(6.54583e-18-0.00838049)	0.0232	(1.12486e-05-0.112894)
Eyre Aug07	0.0017	(9.43531e-15-0.0152225)	0.0035	(3.20993e-08-0.0220505)	0.0044	(6.94118e-06-0.023226)	0.0021	(4.73013e-09-0.0135494)	0.0007	(6.54489e-18-0.00607682)	0.0025	(7.36414e-08-0.0133866)
Milparinka Aug07	0.0048	(1.3735e-14-0.0346777)	0.0058	(1.19241e-08-0.035729)	0.0041	(3.65116e-06-0.0206226)	0.0020	(1.17813e-09-0.0127389)	0.0008	(2.08744e-18-0.00754672)	0.0038	(9.51159e-06-0.0201285)
Tilpa Aug07	0.9706	(0.913669-0.99831)	0.2777	(0.213738-0.323458)	0.0051	(5.41368e-06-0.0274136)	0.0035	(2.02422e-08-0.0203142)	0.0008	(9.79347e-19-0.0073249)	0.0559	(0.0168423-0.099413)
Bourke Aug07	0.0016	(2.57571e-15-0.0129408)	0.6786	(0.667019-0.709265)	0.0048	(7.54108e-06-0.0269005)	0.0019	(1.24647e-08-0.012874)	0.0008	(4.84461e-19-0.00763267)	0.0028	(2.1531e-06-0.0150628)
Walget Mar08	0.0017	(1.04252e-14-0.0143552)	0.0037	(9.06935e-09-0.0240351)	0.6768	(0.666925-0.704447)	0.0021	(5.11691e-09-0.0128952)	0.0007	(4.70753e-18-0.0066355)	0.0027	(7.13965e-07-0.014192)
Brewarrina Mar08	0.0022	(3.67018e-14-0.0198974)	0.0040	(2.95066e-09-0.026408)	0.0715	(0.028629-0.12658)	0.8507	(0.797521-0.903651)	0.0012	(9.89643e-19-0.0106339)	0.0127	(2.79514e-06-0.057358)
Brewarrina Aug07	0.0020	(5.75483e-15-0.0167414)	0.0037	(3.01315e-09-0.024314)	0.0058	(1.00617e-05-0.0286027)	0.0030	(4.91413e-09-0.0196337)	0.9896	(0.95546-0.999673)	0.0115	(0.00103022-0.0357363)
Narrabri Jan07	0.0022	(1.00477e-16-0.0198347)	0.0034	(4.38736e-09-0.0218781)	0.0043	(1.02957e-05-0.0210079)	0.0022	(5.45458e-09-0.0171179)	0.0007	(1.87154e-18-0.00550571)	0.6734	(0.666919-0.691638)

Run 3 INTO

FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6776	(0.666935-0.706581)	0.0067	(2.48922e-08-0.0432526)	0.0004	(1.58731e-19-0.00374523)	0.0035	(2.20498e-07-0.0194674)	0.0018	(2.07471e-09-0.0115501)	0.0038	(7.72975e-09-0.0250801)	0.0011	(1.26202e-09-0.00766756)
Biloeala Jul07	0.0026	(1.14399e-08-0.0161266)	0.6871	(0.667123-0.734561)	0.0004	(4.02294e-19-0.0035515)	0.0030	(3.4961e-09-0.0170887)	0.0011	(3.65975e-09-0.0070737)	0.0033	(5.34208e-09-0.0208433)	0.0010	(1.37684e-09-0.00726986)
Emerald Jan07	0.0180	(5.06629e-07-0.0745712)	0.0096	(6.12676e-08-0.0558869)	0.9938	(0.975847-0.999777)	0.0202	(3.65095e-06-0.0759413)	0.0023	(4.1475e-10-0.0154752)	0.0247	(2.87235e-07-0.100332)	0.0012	(2.64774e-09-0.00859573)
Emerald Jul07	0.2718	(0.20192-0.320958)	0.0106	(5.55966e-08-0.0621027)	0.0007	(2.27191e-18-0.00633218)	0.7898	(0.715497-0.86852)	0.1424	(0.0773207-0.210695)	0.0049	(4.68431e-08-0.0315693)	0.0016	(5.24295e-09-0.0101117)
Simpson Aug07	0.0048	(3.2594e-08-0.0307719)	0.2297	(0.120479-0.310017)	0.0009	(2.54434e-18-0.00850962)	0.1568	(0.0831345-0.230774)	0.8426	(0.779404-0.902871)	0.2545	(0.167544-0.317731)	0.0014	(2.11168e-08-0.00863958)
Eyre Aug07	0.0029	(9.41118e-09-0.0178093)	0.0068	(2.17369e-08-0.0441288)	0.0004	(2.63006e-18-0.00438113)	0.0033	(1.53331e-08-0.0203782)	0.0017	(2.46875e-08-0.01128)	0.6778	(0.667021-0.706077)	0.0011	(2.44259e-09-0.00768856)
Milparinka Aug07	0.0045	(1.17787e-08-0.0270276)	0.0072	(4.14387e-08-0.0450145)	0.0005	(4.42603e-20-0.00429708)	0.0026	(6.33374e-08-0.0168456)	0.0011	(6.55783e-10-0.00777743)	0.0035	(2.67862e-08-0.0228838)	0.9367	(0.899661-0.968666)

Tilpa Aug07	0.0030	(3.44708e-08-0.020523)	0.0065	(7.81155e-09-0.0415572)	0.0005	(2.18184e-19-0.0043442)	0.0031	(2.31295e-08-0.0186648)	0.0009	(1.08253e-10-0.00619956)	0.0031	(1.19008e-08-0.0224313)	0.0009	(8.50291e-10-0.00568245)
Bourke Aug07	0.0032	(2.19717e-08-0.0217835)	0.0091	(1.4599e-08-0.0513049)	0.0004	(1.5294e-18-0.00317575)	0.0024	(2.79358e-08-0.0157512)	0.0008	(2.76613e-09-0.00588644)	0.0039	(2.26305e-08-0.0249892)	0.0505	(0.0204204-0.0881708)
Walget Mar08	0.0028	(1.61701e-08-0.0184114)	0.0064	(2.88407e-08-0.0397932)	0.0004	(2.1898e-18-0.00412222)	0.0029	(6.99581e-09-0.0200819)	0.0011	(4.68536e-10-0.00693086)	0.0034	(9.8697e-09-0.0241535)	0.0011	(8.58732e-10-0.00669061)
Brewarrina Mar08	0.0027	(2.22126e-08-0.0195502)	0.0072	(2.40132e-08-0.0466579)	0.0005	(7.08945e-19-0.00464387)	0.0020	(1.08174e-08-0.0133311)	0.0011	(1.49649e-09-0.007684)	0.0079	(4.61398e-08-0.0467712)	0.0013	(1.02283e-09-0.00800539)
Brewarrina Aug07	0.0035	(1.03523e-08-0.023118)	0.0062	(4.76778e-08-0.0406736)	0.0006	(2.43113e-18-0.00616473)	0.0070	(1.51103e-07-0.0294442)	0.0011	(1.44994e-08-0.00672133)	0.0055	(4.50746e-08-0.0329697)	0.0011	(9.84312e-11-0.00749659)
Narrabri Jan07	0.0025	(1.22766e-08-0.0163957)	0.0070	(1.70983e-08-0.0435271)	0.0004	(4.18374e-18-0.00409658)	0.0034	(4.5068e-08-0.0209452)	0.0019	(7.27412e-11-0.0106821)	0.0036	(3.13131e-08-0.0218526)	0.0010	(7.58956e-10-0.00693942)

Run 3 Continued INTO

FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0035	(3.29157e-09-0.0222645)	0.0012	(9.88382e-19-0.0109641)	0.0042	(4.95965e-06-0.022542)	0.0022	(4.00028e-09-0.0137382)	0.0008	(2.62601e-20-0.00786542)	0.0023	(4.88165e-07-0.0119745)
Biloeala Jul07	0.0033	(3.99296e-09-0.0208357)	0.0010	(2.76369e-19-0.00972194)	0.0045	(3.65723e-06-0.023624)	0.0020	(7.61379e-09-0.0131952)	0.0007	(3.31704e-18-0.0080098)	0.0026	(1.03744e-05-0.0135637)
Emerald Jan07	0.0077	(9.11648e-09-0.0450204)	0.0013	(1.66486e-18-0.0127155)	0.1193	(0.0428196-0.207208)	0.0196	(4.02323e-07-0.0827867)	0.0008	(2.59836e-17-0.00648281)	0.1565	(0.0534298-0.244883)
Emerald Jul07	0.0036	(6.56429e-09-0.026541)	0.0015	(3.3944e-18-0.0139871)	0.0863	(0.0212659-0.182084)	0.1095	(0.0490378-0.16976)	0.0007	(1.75224e-19-0.00674684)	0.0182	(7.87321e-05-0.0642509)
Simpson Aug07	0.0061	(1.36277e-08-0.042974)	0.0014	(3.80359e-18-0.0125528)	0.0121	(4.8185e-06-0.0577301)	0.0066	(2.73069e-08-0.0419629)	0.0009	(4.04625e-18-0.00757031)	0.0569	(0.00609551-0.137783)
Eyre Aug07	0.0038	(4.66093e-09-0.0237834)	0.0011	(9.55227e-19-0.011059)	0.0040	(1.14926e-05-0.0216457)	0.0020	(7.1513e-09-0.0128084)	0.0007	(3.80762e-18-0.00718857)	0.0025	(4.22208e-06-0.0144815)
Milparinka Aug07	0.0056	(1.59578e-08-0.0358339)	0.0011	(1.09284e-17-0.0124173)	0.0040	(3.07948e-06-0.0224434)	0.0021	(2.69451e-08-0.0146072)	0.0008	(8.22775e-18-0.00794572)	0.0033	(5.38252e-06-0.0168202)
Tilpa Aug07	0.6779	(0.666911-0.707086)	0.0013	(1.89484e-19-0.013312)	0.0037	(3.52942e-06-0.0197355)	0.0022	(1.47397e-08-0.0135226)	0.0008	(1.13124e-18-0.00796319)	0.0025	(8.885e-06-0.012486)
Bourke Aug07	0.2699	(0.196051-0.321202)	0.9852	(0.943898-0.999621)	0.0042	(2.23659e-06-0.0223648)	0.0022	(4.67138e-08-0.0151171)	0.0007	(2.03556e-18-0.00754128)	0.0531	(0.0144588-0.0981385)
Walget Mar08	0.0039	(4.38178e-09-0.0297438)	0.0010	(3.31706e-18-0.0097565)	0.6766	(0.667052-0.704876)	0.0020	(1.63046e-08-0.0146923)	0.0006	(1.45536e-18-0.00583872)	0.0027	(1.85892e-06-0.0146777)
Brewarrina Mar08	0.0070	(1.38754e-07-0.0429632)	0.0013	(9.67378e-21-0.0133183)	0.0715	(0.0260042-0.131082)	0.8441	(0.7914-0.896564)	0.0008	(3.65837e-19-0.00796899)	0.0150	(4.13321e-05-0.0509539)
Brewarrina Aug07	0.0047	(5.9285e-08-0.0308338)	0.0013	(4.76681e-21-0.0126806)	0.0051	(6.97529e-06-0.0254944)	0.0034	(3.83624e-09-0.0220463)	0.9909	(0.96713-0.999673)	0.0108	(0.00103245-0.0328752)
Narrabri Jan07	0.0030	(2.34029e-09-0.0205431)	0.0013	(4.32216e-18-0.0115642)	0.0045	(3.53845e-06-0.0258018)	0.0021	(1.12342e-08-0.0153025)	0.0007	(2.9322e-18-0.00699518)	0.6734	(0.666905-0.690607)

Run 4	INTO															
FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07			
Biloeala Jan07	0.6757	(0.66691-0.697521)	0.0058	(5.57301e-08-0.0345112)	0.0005	(2.16579e-17-0.00387732)	0.0030	(2.96628e-08-0.0185481)	0.0006	(5.07571e-12-0.00486177)	0.0034	(4.70958e-09-0.022532)	0.0012	(1.58645e-10-0.00733286)		
Biloeala Jul07	0.0035	(1.16144e-07-0.0203149)	0.6863	(0.667157-0.739502)	0.0005	(2.50837e-17-0.00486531)	0.0026	(1.13559e-07-0.015076)	0.0009	(7.07755e-11-0.00634611)	0.0038	(1.60468e-09-0.0258752)	0.0013	(1.33847e-10-0.0086867)		
Emerald Jan07	0.0654	(2.44569e-05-0.170931)	0.0083	(3.10668e-08-0.0512769)	0.9941	(0.978387-0.999847)	0.0234	(0.000471537-0.0717736)	0.0010	(1.76863e-11-0.00714236)	0.0109	(1.51081e-08-0.0603181)	0.0006	(7.72363e-11-0.00411975)		
Emerald Jul07	0.0029	(2.06594e-07-0.015522)	0.0063	(3.08684e-08-0.0392054)	0.0004	(1.09365e-17-0.00370773)	0.6777	(0.667335-0.700728)	0.0031	(5.79907e-10-0.0140277)	0.0034	(8.50647e-09-0.0229445)	0.0011	(8.20774e-12-0.00727245)		
Simpson Aug07	0.2198	(0.113043-0.31455)	0.2381	(0.134611-0.317023)	0.0008	(4.39061e-17-0.00710488)	0.2675	(0.207258-0.310195)	0.9867	(0.971371-0.996664)	0.2736	(0.2007-0.32264)	0.0009	(1.56996e-11-0.00650295)		
Eyre Aug07	0.0033	(6.11893e-08-0.0193541)	0.0055	(6.74309e-09-0.0362836)	0.0005	(3.5474e-19-0.00439302)	0.0024	(4.13333e-08-0.0156648)	0.0009	(1.77716e-11-0.00614931)	0.6779	(0.666936-0.709025)	0.0010	(2.69522e-13-0.00749486)		
Milparinka Aug07	0.0076	(3.19537e-07-0.0375459)	0.0073	(7.89403e-08-0.0441968)	0.0005	(7.49563e-19-0.00424549)	0.0025	(4.10111e-08-0.0165171)	0.0006	(3.27498e-11-0.00456622)	0.0032	(5.3999e-09-0.021221)	0.9734	(0.920224-0.998259)		
Tilpa Aug07	0.0033	(7.80407e-08-0.0191204)	0.0064	(3.19672e-08-0.0450379)	0.0004	(1.72357e-19-0.00323328)	0.0024	(2.08655e-08-0.0144822)	0.0012	(4.38213e-11-0.00891984)	0.0030	(3.86776e-09-0.0191896)	0.0011	(5.46316e-11-0.00828139)		
Bourke Aug07	0.0032	(3.23205e-07-0.0170881)	0.0063	(9.6655e-09-0.0399627)	0.0004	(1.0016e-20-0.0048428)	0.0023	(1.17668e-08-0.0161365)	0.0013	(1.28048e-10-0.00806898)	0.0037	(6.43794e-09-0.0232732)	0.0011	(3.94087e-12-0.00711179)		
Walget Mar08	0.0033	(6.65802e-08-0.0183704)	0.0059	(1.55411e-08-0.0374841)	0.0004	(3.69334e-18-0.00411436)	0.0027	(2.4513e-08-0.016455)	0.0010	(1.39825e-10-0.00734378)	0.0031	(1.01827e-08-0.0191778)	0.0008	(1.99082e-12-0.00587612)		
Brewarrina Mar08	0.0033	(3.19286e-07-0.0196149)	0.0063	(8.9183e-09-0.0435783)	0.0004	(7.10363e-18-0.0040032)	0.0024	(1.50901e-07-0.0148986)	0.0009	(1.92652e-11-0.00662368)	0.0037	(9.60447e-09-0.0249067)	0.0007	(1.57839e-11-0.0052044)		
Brewarrina Aug07	0.0040	(4.89217e-08-0.0217073)	0.0070	(1.09442e-08-0.0457426)	0.0006	(5.98628e-18-0.00521129)	0.0073	(1.11692e-05-0.0279648)	0.0010	(4.62219e-11-0.00772024)	0.0057	(2.33602e-08-0.0359302)	0.0007	(1.15554e-11-0.00547741)		
Narrabri Jan07	0.0047	(1.21593e-07-0.0249114)	0.0106	(6.59263e-08-0.0609149)	0.0005	(1.18175e-18-0.00479808)	0.0039	(3.55785e-08-0.0224816)	0.0007	(1.36469e-11-0.00584383)	0.0045	(1.21142e-08-0.0290372)	0.0161	(2.7043e-10-0.0666864)		

Run 4 Continued	INTO											
FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0040	(6.75428e-08-0.0246372)	0.0037	(8.47695e-09-0.0269133)	0.0039	(4.70748e-06-0.019397)	0.0019	(2.57691e-08-0.014067)	0.0008	(1.835e-18-0.00631325)	0.0017	(2.96143e-08-0.010583)
Biloeala Jul07	0.0034	(2.32002e-09-0.0237507)	0.0037	(6.06413e-08-0.0256108)	0.0044	(1.04114e-05-0.0226455)	0.0022	(5.3699e-08-0.0136573)	0.0007	(1.20288e-17-0.00760656)	0.0021	(1.26961e-07-0.0123854)
Emerald Jan07	0.0037	(4.32765e-09-0.0257449)	0.0048	(1.25745e-09-0.031848)	0.1415	(0.0631889-0.230191)	0.0380	(2.62733e-06-0.101627)	0.0016	(3.83759e-18-0.0132786)	0.0364	(0.000163013-0.108569)
Emerald Jul07	0.0037	(3.10535e-08-0.0250733)	0.0034	(1.85344e-08-0.0240836)	0.0041	(2.11409e-06-0.0213464)	0.0021	(7.66318e-08-0.0135137)	0.0008	(1.80431e-18-0.00807041)	0.0019	(7.10381e-08-0.0127622)
Simpson Aug07	0.0054	(9.40672e-09-0.0331588)	0.0046	(1.4813e-09-0.0314559)	0.0700	(0.0183718-0.142344)	0.0803	(0.0238666-0.146857)	0.0012	(2.30888e-17-0.0114102)	0.1169	(0.0195283-0.195573)
Eyre Aug07	0.0038	(6.64408e-09-0.026303)	0.0037	(8.56613e-09-0.0248771)	0.0041	(3.10716e-06-0.0222038)	0.0020	(7.26917e-09-0.0126514)	0.0009	(1.21854e-16-0.00831602)	0.0020	(4.3952e-08-0.0118627)
Milparinka Aug07	0.0157	(3.27315e-07-0.0786484)	0.0050	(2.13262e-08-0.0309656)	0.0043	(1.43635e-05-0.0231864)	0.0022	(4.4147e-08-0.0160398)	0.0007	(5.72404e-19-0.00668882)	0.0021	(5.65289e-08-0.0125694)

Tilpa Aug07	0.6787	(0.667105-0.707753)	0.0037	(3.6628e-09-0.0260303)	0.0042	(1.91229e-06-0.0217243)	0.0023	(1.86249e-08-0.0146696)	0.0008	(7.7436e-19-0.0063767)	0.0022	(8.6992e-08-0.013968)
Bourke Aug07	0.0035	(1.06393e-08-0.0236285)	0.6782	(0.666933-0.707095)	0.0041	(9.91644e-07-0.0225918)	0.0020	(6.47567e-09-0.0127302)	0.0007	(9.02311e-19-0.00689742)	0.0018	(2.73816e-07-0.0114333)
Walget Mar08	0.0035	(3.48184e-08-0.0224624)	0.0034	(6.17515e-09-0.0250354)	0.6767	(0.666957-0.702575)	0.0020	(1.16203e-08-0.0131452)	0.0009	(1.92964e-18-0.00799671)	0.0021	(4.01195e-07-0.011674)
Brewarrina Mar08	0.0043	(1.19183e-08-0.0268295)	0.0033	(3.15127e-09-0.0219513)	0.0704	(0.0279858-0.127418)	0.8598	(0.798493-0.930592)	0.0010	(1.63098e-17-0.00786025)	0.0030	(7.9804e-08-0.0197099)
Brewarrina Aug07	0.0039	(1.09223e-08-0.0284158)	0.0032	(1.13133e-08-0.0217919)	0.0050	(6.29585e-06-0.0260856)	0.0024	(1.48757e-08-0.0166367)	0.9889	(0.962568-0.999727)	0.0148	(0.0014256-0.0428689)
Narrabri Jan07	0.2665	(0.183436-0.322196)	0.2793	(0.212482-0.324261)	0.0071	(6.58473e-06-0.038141)	0.0028	(3.60706e-08-0.017437)	0.0010	(9.91048e-17-0.00910549)	0.8130	(0.746147-0.906572)

Run 5 INTO

FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6764	(0.666905-0.698717)	0.0025	(2.90563e-17-0.0258233)	0.0008	(1.02042e-16-0.00725718)	0.0039	(9.2965e-07-0.023644)	0.0023	(6.28329e-08-0.0152858)	0.0031	(4.18648e-10-0.0216447)	0.0010	(1.99772e-11-0.00783408)
Biloeala Jul07	0.2627	(0.187928-0.312641)	0.9695	(0.886576-0.99939)	0.0046	(5.20699e-17-0.0354621)	0.2404	(0.161203-0.30017)	0.2629	(0.197728-0.302943)	0.0047	(2.54301e-08-0.0304373)	0.0013	(1.85142e-10-0.00875736)
Emerald Jan07	0.0165	(1.30421e-06-0.0725366)	0.0025	(4.25196e-18-0.0210169)	0.9870	(0.945006-0.999867)	0.0128	(2.00726e-05-0.0481261)	0.0013	(2.56578e-09-0.00789725)	0.0083	(9.42007e-09-0.0480697)	0.0008	(1.68983e-11-0.00625521)
Emerald Jul07	0.0026	(3.36247e-08-0.0165585)	0.0022	(2.22627e-18-0.0210664)	0.0006	(1.80046e-16-0.00575463)	0.6743	(0.66687-0.695677)	0.0019	(2.99724e-08-0.0110414)	0.0033	(1.4475e-08-0.0226154)	0.0013	(2.08401e-10-0.00933527)
Simpson Aug07	0.0051	(3.9938e-09-0.0324971)	0.0055	(3.52187e-17-0.0501935)	0.0009	(1.47937e-16-0.00706763)	0.0361	(0.000421076-0.100851)	0.7184	(0.683715-0.784436)	0.2756	(0.209395-0.319898)	0.0010	(2.92316e-10-0.00706574)
Eyre Aug07	0.0028	(3.6926e-08-0.0177012)	0.0019	(3.13084e-18-0.0202902)	0.0006	(2.27219e-16-0.00564269)	0.0031	(5.62476e-08-0.0176047)	0.0018	(1.59483e-09-0.0113322)	0.6785	(0.66705-0.709771)	0.0015	(4.0763e-10-0.0101682)
Milparinka Aug07	0.0141	(3.17839e-05-0.0518967)	0.0023	(5.08153e-18-0.0246805)	0.0007	(1.14781e-15-0.00615767)	0.0027	(1.44072e-06-0.014671)	0.0011	(4.03472e-09-0.00751598)	0.0062	(3.50726e-08-0.0328331)	0.9762	(0.944397-0.997151)
Tilpa Aug07	0.0027	(3.53043e-09-0.0173711)	0.0022	(6.64004e-19-0.0208477)	0.0006	(1.01915e-15-0.00572243)	0.0034	(9.47208e-07-0.0189951)	0.0019	(2.74677e-10-0.0123972)	0.0030	(4.82369e-09-0.0202977)	0.0009	(1.62824e-10-0.00711407)
Bourke Aug07	0.0027	(2.60914e-09-0.0176218)	0.0027	(4.0528e-18-0.0268002)	0.0008	(4.83875e-17-0.00676219)	0.0028	(4.48921e-07-0.0168495)	0.0009	(1.59419e-08-0.00590808)	0.0033	(1.84299e-08-0.0216574)	0.0120	(9.80941e-08-0.0426702)
Walget Mar08	0.0028	(4.34026e-08-0.0185858)	0.0019	(6.96031e-19-0.0174648)	0.0006	(8.84991e-17-0.00492116)	0.0028	(2.32401e-07-0.0164295)	0.0015	(6.81725e-08-0.00924866)	0.0032	(1.2104e-08-0.0221852)	0.0013	(1.01279e-10-0.00941984)
Brewarrina Mar08	0.0030	(1.52782e-08-0.0190516)	0.0022	(8.86332e-18-0.0201912)	0.0011	(5.47473e-17-0.00999651)	0.0062	(7.89308e-08-0.0306434)	0.0012	(1.07575e-08-0.00780079)	0.0033	(1.01084e-08-0.0207933)	0.0006	(1.5969e-11-0.00462373)
Brewarrina Aug07	0.0058	(3.7598e-08-0.0301853)	0.0021	(4.11381e-17-0.0210353)	0.0009	(4.08595e-17-0.00715051)	0.0075	(6.67658e-06-0.0288731)	0.0025	(1.12898e-08-0.0147088)	0.0042	(1.24617e-08-0.0280267)	0.0008	(4.37105e-11-0.00519624)
Narrabri Jan07	0.0029	(3.89695e-08-0.0182107)	0.0024	(8.8119e-17-0.0215604)	0.0007	(2.53627e-16-0.0060846)	0.0039	(1.07287e-06-0.0208037)	0.0023	(1.56378e-08-0.0133291)	0.0033	(2.5963e-09-0.0219791)	0.0013	(1.59002e-10-0.00920547)

Run 5 Continued	INTO											
FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0041	(5.42049e-09-0.0279136)	0.0010	(8.51159e-19-0.00949119)	0.0044	(1.06284e-05-0.0249448)	0.0017	(4.02549e-09-0.0126523)	0.0008	(6.70853e-17-0.00686752)	0.0026	(3.5461e-06-0.0129222)
Biloeala Jul07	0.0083	(4.43242e-08-0.0446696)	0.0012	(4.49253e-19-0.0126766)	0.1006	(0.0366398-0.176834)	0.1094	(0.0504364-0.163602)	0.0015	(1.47724e-18-0.0109274)	0.1682	(0.092873-0.230979)
Emerald Jan07	0.0061	(4.84399e-08-0.0358003)	0.0011	(4.45036e-19-0.0106137)	0.0931	(0.0220171-0.177597)	0.0150	(1.97888e-07-0.0727327)	0.0012	(1.92799e-17-0.0126405)	0.0541	(0.0127569-0.116371)
Emerald Jul07	0.0038	(2.42209e-08-0.0224873)	0.0010	(4.25979e-19-0.00945908)	0.0044	(2.86222e-06-0.0252974)	0.0023	(6.65311e-09-0.0160113)	0.0007	(1.09834e-16-0.0062879)	0.0026	(2.28267e-06-0.0141561)
Simpson Aug07	0.0042	(4.67955e-08-0.0277973)	0.0011	(2.09813e-19-0.0104933)	0.0242	(1.48374e-05-0.0921064)	0.0027	(7.67201e-09-0.0167738)	0.0014	(1.1958e-18-0.0125394)	0.0065	(6.47372e-06-0.0325098)
Eyre Aug07	0.0038	(4.36983e-08-0.0234447)	0.0009	(2.44369e-19-0.00907717)	0.0045	(1.11717e-05-0.0231552)	0.0023	(8.60942e-09-0.0144023)	0.0008	(2.44312e-17-0.00773644)	0.0025	(2.68655e-06-0.0130749)
Milparinka Aug07	0.0239	(3.74941e-07-0.0940226)	0.0010	(2.51181e-20-0.00908064)	0.0047	(3.48465e-06-0.0237182)	0.0022	(9.73483e-09-0.0155385)	0.0010	(7.07331e-18-0.0079354)	0.0028	(4.3512e-06-0.0162835)
Tilpa Aug07	0.6790	(0.667034-0.709113)	0.0009	(5.04562e-19-0.0100621)	0.0043	(3.31314e-06-0.0215213)	0.0020	(3.00697e-09-0.015348)	0.0008	(1.53098e-17-0.00731657)	0.0026	(1.7207e-06-0.0137842)
Bourke Aug07	0.2498	(0.155057-0.316868)	0.9877	(0.955531-0.99943)	0.0044	(1.08107e-05-0.0229985)	0.0026	(3.02691e-09-0.0169832)	0.0008	(5.64281e-17-0.00759304)	0.0551	(0.0202937-0.102757)
Walget Mar08	0.0037	(3.92645e-08-0.0230338)	0.0009	(1.02988e-19-0.0090525)	0.6765	(0.666915-0.701091)	0.0018	(3.44225e-09-0.0133322)	0.0009	(6.09026e-18-0.0094092)	0.0027	(5.47656e-06-0.0149305)
Brewarrina Mar08	0.0056	(8.51052e-08-0.0372869)	0.0011	(7.01071e-19-0.0092886)	0.0696	(0.0273157-0.122399)	0.8525	(0.799141-0.903492)	0.0015	(2.95376e-16-0.0128966)	0.0143	(2.55348e-05-0.0497664)
Brewarrina Aug07	0.0043	(2.49163e-08-0.0263031)	0.0010	(8.71749e-19-0.010516)	0.0052	(7.95131e-06-0.028161)	0.0034	(2.62481e-08-0.0216043)	0.9879	(0.95572-0.999683)	0.0117	(0.00145107-0.0342077)
Narrabri Jan07	0.0035	(8.56803e-09-0.0251549)	0.0011	(6.72822e-19-0.0102561)	0.0042	(1.0918e-05-0.0246953)	0.0021	(3.00859e-08-0.0140529)	0.0007	(1.89725e-17-0.00567167)	0.6744	(0.666812-0.695431)

Run 6	INTO											
FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6760	(0.66703-0.700239)	0.0067	(1.30005e-08-0.0403811)	0.0006	(3.18374e-16-0.00495337)	0.0009	(9.78274e-19-0.00849844)	0.0016	(5.34927e-09-0.0110317)	0.0036	(1.21069e-08-0.0237899)	0.0010	(2.13226e-10-0.00676863)
Biloeala Jul07	0.0027	(3.29867e-08-0.0191)	0.6856	(0.667128-0.735848)	0.0005	(6.22365e-17-0.00384864)	0.0007	(3.63018e-19-0.00668691)	0.0016	(8.96435e-09-0.0096589)	0.0032	(1.35532e-08-0.0206083)	0.0009	(1.33134e-09-0.00598321)
Emerald Jan07	0.0171	(1.09283e-07-0.0789792)	0.0081	(4.20218e-08-0.0540048)	0.9914	(0.966305-0.999811)	0.0016	(6.59782e-16-0.0140419)	0.0012	(3.26916e-10-0.00876739)	0.0150	(4.6864e-09-0.0754654)	0.0011	(1.06839e-10-0.00718275)
Emerald Jul07	0.0063	(1.27184e-07-0.0376837)	0.2271	(0.109272-0.311928)	0.0014	(2.33889e-16-0.0127076)	0.9882	(0.959286-0.999817)	0.2559	(0.202686-0.298859)	0.2678	(0.183621-0.320535)	0.0012	(3.36713e-09-0.00755101)
Simpson Aug07	0.2698	(0.194455-0.317538)	0.0199	(1.74082e-08-0.111699)	0.0016	(2.23996e-17-0.0130723)	0.0013	(3.7217e-17-0.0131295)	0.7262	(0.684463-0.777204)	0.0061	(1.31329e-08-0.0366052)	0.0020	(1.13426e-09-0.0123666)
Eyre Aug07	0.0028	(1.33654e-08-0.017794)	0.0068	(3.82957e-08-0.0419391)	0.0005	(3.64424e-18-0.00444894)	0.0008	(7.61063e-16-0.00761328)	0.0015	(1.46849e-08-0.00974833)	0.6779	(0.667013-0.709346)	0.0009	(9.08295e-10-0.00662)
Milparinka Aug07	0.0081	(2.35201e-07-0.0376775)	0.0067	(3.73214e-08-0.0420011)	0.0007	(6.81308e-18-0.00585345)	0.0011	(4.89511e-17-0.00974463)	0.0010	(1.03195e-09-0.00651755)	0.0046	(1.32597e-08-0.0308575)	0.9441	(0.903664-0.980653)

Tilpa Aug07	0.0030	(4.57315e-08-0.0202685)	0.0065	(1.94786e-08-0.0413772)	0.0005	(2.94983e-17-0.00465551)	0.0008	(2.14459e-17-0.0071951)	0.0036	(8.31022e-08-0.0161878)	0.0033	(3.92193e-09-0.0220524)	0.0009	(5.44401e-09-0.00617428)
Bourke Aug07	0.0031	(1.96261e-09-0.0190056)	0.0076	(5.86363e-08-0.0444885)	0.0006	(2.48413e-16-0.00419755)	0.0008	(4.41669e-17-0.00767175)	0.0012	(4.52541e-09-0.00811989)	0.0040	(5.32896e-08-0.025543)	0.0443	(0.0102875-0.0830111)
Walget Mar08	0.0029	(2.17465e-08-0.0172398)	0.0062	(4.03088e-08-0.0407172)	0.0005	(2.2821e-16-0.0044897)	0.0008	(2.14173e-17-0.00755747)	0.0015	(4.21423e-08-0.00930209)	0.0034	(8.75438e-09-0.0215088)	0.0010	(4.61589e-10-0.00611738)
Brewarrina Mar08	0.0027	(1.747e-08-0.0170407)	0.0063	(2.04321e-08-0.0392476)	0.0006	(4.45507e-16-0.00514386)	0.0010	(6.37457e-17-0.00930613)	0.0015	(2.741e-08-0.00907062)	0.0037	(2.20178e-08-0.0211873)	0.0009	(2.06272e-09-0.00622584)
Brewarrina Aug07	0.0029	(1.04097e-08-0.0186534)	0.0063	(3.65732e-08-0.0411432)	0.0007	(3.45953e-16-0.00583819)	0.0012	(3.32196e-16-0.011291)	0.0019	(2.54756e-08-0.0109495)	0.0042	(3.77231e-08-0.0287965)	0.0009	(1.14431e-09-0.00655309)
Narrabri Jan07	0.0025	(1.86056e-08-0.0149095)	0.0063	(6.61509e-09-0.0417635)	0.0005	(3.99519e-19-0.00505148)	0.0008	(5.29169e-17-0.0072949)	0.0013	(6.49043e-09-0.00816574)	0.0033	(7.441e-09-0.0219462)	0.0009	(1.51256e-09-0.00647065)

Run 6 Continued INTO

FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0039	(7.82492e-09-0.0275591)	0.0011	(1.7741e-19-0.012815)	0.0043	(7.19432e-06-0.0219191)	0.0021	(2.0846e-08-0.0147351)	0.0008	(2.73637e-16-0.00734488)	0.0029	(1.14022e-05-0.0138358)
Biloeala Jul07	0.0038	(5.94392e-09-0.0281701)	0.0013	(8.1254e-21-0.0123478)	0.0040	(6.83808e-06-0.021692)	0.0021	(6.98492e-09-0.0152025)	0.0007	(1.49893e-17-0.00700385)	0.0030	(3.37429e-06-0.0149813)
Emerald Jan07	0.0052	(6.97359e-09-0.0337473)	0.0012	(2.29987e-20-0.0108861)	0.1043	(0.0325658-0.199657)	0.0101	(2.71056e-08-0.054833)	0.0010	(1.21385e-17-0.00990038)	0.0763	(0.0085731-0.162542)
Emerald Jul07	0.0048	(3.6928e-09-0.0313842)	0.0011	(1.54892e-18-0.0111211)	0.0248	(4.28793e-05-0.0897573)	0.0079	(4.57706e-08-0.0433932)	0.0012	(4.13115e-19-0.0113748)	0.0648	(0.000518781-0.170425)
Simpson Aug07	0.0057	(4.54241e-08-0.0346368)	0.0012	(1.32833e-19-0.011466)	0.0861	(0.00941528-0.187575)	0.1082	(0.0482984-0.169799)	0.0009	(1.86021e-18-0.00703396)	0.0754	(5.91531e-05-0.211209)
Eyre Aug07	0.0035	(1.06517e-08-0.0241413)	0.0011	(9.76675e-21-0.0105999)	0.0043	(5.05814e-06-0.0211913)	0.0021	(8.55746e-10-0.0131835)	0.0007	(5.65142e-18-0.00699956)	0.0028	(6.13096e-06-0.0128813)
Milparinka Aug07	0.0077	(5.97856e-08-0.0436596)	0.0012	(7.38463e-20-0.0122557)	0.0041	(1.75217e-06-0.0208407)	0.0019	(1.77211e-08-0.0126891)	0.0008	(3.62502e-19-0.00670929)	0.0032	(6.70222e-06-0.0152744)
Tilpa Aug07	0.6782	(0.667066-0.706217)	0.0010	(4.42347e-21-0.00964697)	0.0041	(2.46106e-06-0.0238906)	0.0023	(6.0728e-09-0.0156211)	0.0007	(7.38132e-19-0.00747824)	0.0029	(7.87476e-06-0.014386)
Bourke Aug07	0.2721	(0.193676-0.322281)	0.9865	(0.954714-0.999449)	0.0061	(6.69603e-06-0.0289999)	0.0024	(8.11571e-09-0.0161364)	0.0009	(2.28609e-19-0.00786552)	0.0752	(0.0365695-0.122757)
Walget Mar08	0.0034	(1.03288e-08-0.0215646)	0.0010	(4.95781e-19-0.00990706)	0.6767	(0.667055-0.700872)	0.0018	(8.71358e-09-0.0122026)	0.0008	(5.609e-21-0.00802264)	0.0031	(6.03119e-06-0.0151715)
Brewarrina Mar08	0.0041	(8.83867e-09-0.027406)	0.0010	(3.65309e-19-0.0114169)	0.0701	(0.0290896-0.123698)	0.8542	(0.796367-0.9094)	0.0010	(9.0333e-19-0.00987081)	0.0042	(1.77578e-06-0.0206683)
Brewarrina Aug07	0.0040	(5.03365e-09-0.0251826)	0.0010	(1.48037e-18-0.00927669)	0.0064	(8.91787e-06-0.0319318)	0.0029	(7.85726e-09-0.01785)	0.9898	(0.964467-0.999586)	0.0131	(0.00185135-0.0348381)
Narrabri Jan07	0.0035	(2.27058e-09-0.0249804)	0.0012	(1.00606e-17-0.0116549)	0.0046	(3.98079e-06-0.0237729)	0.0021	(2.30901e-09-0.0146391)	0.0008	(2.95754e-19-0.00816686)	0.6732	(0.666813-0.690527)

Run 7	INTO															
FROM		Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6774	(0.667006-0.703924)	0.0068	(4.55734e-08-0.0392622)	0.0008	(2.36393e-15-0.00564456)	0.0034	(2.08785e-06-0.0196435)	0.0016	(9.95934e-11-0.0112402)	0.0034	(4.05264e-09-0.021957)	0.0006	(3.9149e-16-0.00498159)
Biloeala Jul07	0.0041	(5.38628e-07-0.0219406)	0.6861	(0.667119-0.733527)	0.0009	(2.0211e-13-0.00686356)	0.0037	(1.67521e-07-0.020592)	0.0008	(9.49345e-11-0.00570174)	0.0033	(1.66616e-08-0.0238624)	0.0006	(1.71789e-15-0.00477097)
Emerald Jan07	0.0219	(0.00014555-0.0777118)	0.0074	(2.66575e-08-0.0440968)	0.9412	(0.801706-0.99918)	0.0248	(0.00177931-0.0672501)	0.0010	(3.66445e-11-0.00661539)	0.0112	(6.99197e-08-0.058273)	0.0008	(4.45161e-18-0.00699364)
Emerald Jul07	0.0042	(4.39297e-07-0.0272528)	0.0070	(2.5799e-08-0.0477385)	0.0009	(1.29952e-15-0.00682916)	0.6752	(0.666815-0.696975)	0.0015	(2.30625e-10-0.0100123)	0.0032	(5.47853e-10-0.0240278)	0.0005	(4.26457e-16-0.00451673)
Simpson Aug07	0.0645	(0.00190563-0.148277)	0.2238	(0.109497-0.314799)	0.0042	(4.76342e-12-0.0231465)	0.2111	(0.124615-0.292756)	0.9872	(0.970663-0.997025)	0.2721	(0.201853-0.321724)	0.0011	(6.67374e-17-0.0100368)
Eyre Aug07	0.0043	(1.19192e-06-0.0211698)	0.0065	(7.02303e-08-0.037535)	0.0010	(5.60917e-14-0.0065931)	0.0031	(1.27013e-06-0.0184587)	0.0009	(5.09438e-11-0.00644901)	0.6779	(0.666925-0.704362)	0.0005	(1.05073e-15-0.00486974)
Milparinka Aug07	0.0124	(0.000185188-0.0399971)	0.0076	(3.88271e-08-0.0506189)	0.0012	(1.98321e-13-0.00908353)	0.0030	(8.82857e-07-0.0164252)	0.0005	(1.34827e-11-0.00393443)	0.0035	(2.8982e-09-0.0255359)	0.9848	(0.940351-0.999816)
Tilpa Aug07	0.0041	(1.44387e-06-0.0232285)	0.0067	(2.13418e-08-0.0391033)	0.0014	(6.82261e-13-0.0128372)	0.0032	(9.71631e-07-0.0193973)	0.0006	(2.19475e-11-0.00441047)	0.0035	(9.16206e-09-0.0242529)	0.0077	(7.57591e-17-0.0425673)
Bourke Aug07	0.0037	(2.25379e-06-0.0214586)	0.0065	(4.21435e-08-0.0398117)	0.0009	(1.1005e-13-0.00659129)	0.0035	(5.78505e-07-0.0178499)	0.0009	(1.91444e-11-0.00616564)	0.0039	(1.78779e-08-0.0281995)	0.0005	(3.26586e-16-0.00469714)
Walget Mar08	0.0037	(9.37256e-07-0.0217426)	0.0067	(1.66745e-08-0.0420751)	0.0008	(2.6637e-13-0.005632)	0.0034	(4.15215e-07-0.0197082)	0.0013	(1.5235e-10-0.00849561)	0.0034	(1.56456e-08-0.0233989)	0.0005	(3.37996e-15-0.00402841)
Brewarrina Mar08	0.0037	(2.11565e-06-0.0213899)	0.0068	(4.28461e-08-0.0403791)	0.0013	(6.1199e-13-0.00913394)	0.0030	(7.63208e-07-0.0157043)	0.0011	(5.72718e-12-0.0077674)	0.0041	(3.10842e-09-0.0276045)	0.0006	(4.11836e-17-0.00641986)
Brewarrina Aug07	0.0056	(1.85776e-06-0.0291481)	0.0062	(1.16214e-08-0.0405243)	0.0020	(1.57378e-13-0.0135856)	0.0066	(7.97198e-07-0.0282014)	0.0016	(1.24802e-09-0.0102913)	0.0049	(4.84736e-09-0.0323107)	0.0006	(4.67027e-17-0.00498408)
Narrabri Jan07	0.1905	(0.104353-0.280247)	0.0221	(1.02732e-07-0.114828)	0.0436	(9.20874e-13-0.173904)	0.0560	(0.00504818-0.12642)	0.0009	(3.29197e-11-0.00672738)	0.0056	(2.21892e-08-0.0369035)	0.0011	(6.3245e-14-0.00946904)

Run 7	INTO											
FROM		Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07
Biloeala Jan07	0.0016	(2.87805e-15-0.0124651)	0.0039	(6.6302e-10-0.0252873)	0.0043	(6.40317e-06-0.0200636)	0.0020	(9.26322e-10-0.0141895)	0.0008	(1.68945e-19-0.00755709)	0.0017	(9.10325e-09-0.0109264)
Biloeala Jul07	0.0020	(1.01717e-13-0.0153169)	0.0036	(1.22029e-08-0.0232089)	0.0041	(3.60414e-06-0.0222111)	0.0021	(5.94442e-09-0.0128666)	0.0007	(9.53031e-19-0.00667842)	0.0016	(4.06326e-09-0.0116397)
Emerald Jan07	0.0033	(8.53394e-15-0.0276086)	0.0038	(9.27123e-09-0.0264175)	0.0784	(0.0265076-0.15561)	0.0115	(3.01746e-08-0.0630259)	0.0016	(1.22721e-18-0.0190034)	0.0121	(2.9503e-08-0.05605)
Emerald Jul07	0.0018	(1.54066e-14-0.013423)	0.0037	(4.70314e-09-0.0241349)	0.0039	(2.5248e-06-0.0191069)	0.0021	(7.75915e-09-0.0125117)	0.0008	(6.4509e-19-0.00755803)	0.0017	(2.21595e-08-0.0122617)
Simpson Aug07	0.0032	(1.47658e-14-0.0230451)	0.0047	(4.46658e-08-0.0315346)	0.0457	(0.00272095-0.120314)	0.0075	(4.58032e-08-0.0410133)	0.0014	(4.66193e-19-0.0144489)	0.0076	(1.37618e-09-0.0423647)
Eyre Aug07	0.0019	(9.54798e-16-0.0156341)	0.0040	(9.64204e-09-0.0249401)	0.0041	(6.60669e-06-0.020109)	0.0021	(2.88952e-08-0.01378)	0.0008	(9.36626e-20-0.00702097)	0.0018	(5.9072e-08-0.0116317)
Milparinka Aug07	0.0091	(3.932e-13-0.0544858)	0.0049	(1.44009e-08-0.0312933)	0.0046	(1.19779e-05-0.0233218)	0.0027	(6.15169e-09-0.0180108)	0.0006	(4.83373e-19-0.00618395)	0.0020	(5.24934e-09-0.0114086)

Tilpa Aug07	0.9630	(0.893732-0.998771)	0.2763	(0.207336-0.322722)	0.0042	(3.65819e-06-0.0228204)	0.0023	(2.50898e-08-0.0157759)	0.0009	(5.85616e-20-0.00844325)	0.0551	(0.0177225-0.103581)
Bourke Aug07	0.0021	(1.65971e-13-0.0169757)	0.6784	(0.666853-0.709385)	0.0042	(3.90416e-06-0.0219181)	0.0020	(4.86603e-09-0.0151384)	0.0007	(1.94575e-18-0.00741336)	0.0018	(5.36225e-09-0.0125094)
Walget Mar08	0.0018	(4.23582e-14-0.015709)	0.0036	(3.96913e-09-0.0241279)	0.6768	(0.666985-0.700796)	0.0021	(6.07574e-09-0.0127604)	0.0006	(2.97796e-19-0.00665412)	0.0022	(1.49181e-08-0.0144993)
Brewarrina Mar08	0.0026	(9.19241e-15-0.0210684)	0.0037	(3.05829e-09-0.0266693)	0.0719	(0.0313682-0.129079)	0.8510	(0.800269-0.901729)	0.0009	(3.04794e-18-0.00882642)	0.0027	(3.14374e-09-0.0180918)
Brewarrina Aug07	0.0027	(1.57756e-14-0.0217892)	0.0038	(3.89703e-09-0.0233741)	0.0050	(4.67968e-06-0.0266319)	0.0035	(9.23413e-08-0.0199239)	0.9893	(0.948865-0.999625)	0.0132	(0.000993287-0.0393007)
Narrabri Jan07	0.0050	(4.53928e-14-0.0363974)	0.0056	(2.45539e-09-0.0344135)	0.0929	(0.0337313-0.173563)	0.1091	(0.054482-0.16274)	0.0008	(9.80979e-19-0.00850577)	0.8963	(0.832836-0.946208)

Run 8 INTO

FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6760	(0.66703-0.700239)	0.0067	(1.30005e-08-0.0403811)	0.0006	(3.18374e-16-0.00495337)	0.0009	(9.78274e-19-0.00849844)	0.0016	(5.34927e-09-0.0110317)	0.0036	(1.21069e-08-0.0237899)	0.0010	(2.13226e-10-0.00676863)
Biloeala Jul07	0.0027	(3.29867e-08-0.0191)	0.6856	(0.667128-0.735848)	0.0005	(6.22365e-17-0.00384864)	0.0007	(3.63018e-19-0.00668691)	0.0016	(8.96435e-09-0.0096589)	0.0032	(1.35532e-08-0.0206083)	0.0009	(1.33134e-09-0.00598321)
Emerald Jan07	0.0171	(1.09283e-07-0.0789792)	0.0081	(4.20218e-08-0.0540048)	0.9914	(0.966305-0.999811)	0.0016	(6.59782e-16-0.0140419)	0.0012	(3.26916e-10-0.00876739)	0.0150	(4.6864e-09-0.0754654)	0.0011	(1.06839e-10-0.00718275)
Emerald Jul07	0.0063	(1.27184e-07-0.0376837)	0.2271	(0.109272-0.311928)	0.0014	(2.33889e-16-0.0127076)	0.9882	(0.959286-0.999817)	0.2559	(0.202686-0.298859)	0.2678	(0.183621-0.320535)	0.0012	(3.36713e-09-0.00755101)
Simpson Aug07	0.2698	(0.194455-0.317538)	0.0199	(1.74082e-08-0.111699)	0.0016	(2.23996e-17-0.0130723)	0.0013	(3.7217e-17-0.0131295)	0.7262	(0.684463-0.777204)	0.0061	(1.31329e-08-0.0366052)	0.0020	(1.13426e-09-0.0123666)
Eyre Aug07	0.0028	(1.33654e-08-0.017794)	0.0068	(3.82957e-08-0.0419391)	0.0005	(3.64424e-18-0.00444894)	0.0008	(7.61063e-16-0.00761328)	0.0015	(1.46849e-08-0.00974833)	0.6779	(0.667013-0.709346)	0.0009	(9.08295e-10-0.00662)
Milparinka Aug07	0.0081	(2.35201e-07-0.0376775)	0.0067	(3.73214e-08-0.0420011)	0.0007	(6.81308e-18-0.00585345)	0.0011	(4.89511e-17-0.00974463)	0.0010	(1.03195e-09-0.00651755)	0.0046	(1.32597e-08-0.0308575)	0.9441	(0.903664-0.980653)
Tilpa Aug07	0.0030	(4.57315e-08-0.0202685)	0.0065	(1.94786e-08-0.0413772)	0.0005	(2.94983e-17-0.00465551)	0.0008	(2.14459e-17-0.0071951)	0.0036	(8.31022e-08-0.0161878)	0.0033	(3.92193e-09-0.0220524)	0.0009	(5.44401e-09-0.00617428)
Bourke Aug07	0.0031	(1.96261e-09-0.0190056)	0.0076	(5.86363e-08-0.0444885)	0.0006	(2.48413e-16-0.00419755)	0.0008	(4.41669e-17-0.00767175)	0.0012	(4.52541e-09-0.00811989)	0.0040	(5.32896e-08-0.025543)	0.0443	(0.0102875-0.0830111)
Walget Mar08	0.0029	(2.17465e-08-0.0172398)	0.0062	(4.03088e-08-0.0407172)	0.0005	(2.2821e-16-0.0044897)	0.0008	(2.14173e-17-0.00755747)	0.0015	(4.21423e-08-0.00930209)	0.0034	(8.75438e-09-0.0215088)	0.0010	(4.61589e-10-0.00611738)
Brewarrina Mar08	0.0027	(1.747e-08-0.0170407)	0.0063	(2.04321e-08-0.0392476)	0.0006	(4.45507e-16-0.00514386)	0.0010	(6.37457e-17-0.00930613)	0.0015	(2.741e-08-0.00907062)	0.0037	(2.20178e-08-0.0211873)	0.0009	(2.06272e-09-0.00622584)
Brewarrina Aug07	0.0029	(1.04097e-08-0.0186534)	0.0063	(3.65732e-08-0.0411432)	0.0007	(3.45953e-16-0.00583819)	0.0012	(3.32196e-16-0.011291)	0.0019	(2.54756e-08-0.0109495)	0.0042	(3.77231e-08-0.0287965)	0.0009	(1.14431e-09-0.00655309)
Narrabri Jan07	0.0025	(1.86056e-08-0.0149095)	0.0063	(6.61509e-09-0.0417635)	0.0005	(3.99519e-19-0.00505148)	0.0008	(5.29169e-17-0.0072949)	0.0013	(6.49043e-09-0.00816574)	0.0033	(7.441e-09-0.0219462)	0.0009	(1.51256e-09-0.00647065)

Run 8	INTO											
FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0039	(7.82492e-09-0.0275591)	0.0011	(1.7741e-19-0.012815)	0.0043	(7.19432e-06-0.0219191)	0.0021	(2.0846e-08-0.0147351)	0.0008	(2.73637e-16-0.00734488)	0.0029	(1.14022e-05-0.0138358)
Biloeala Jul07	0.0038	(5.94392e-09-0.0281701)	0.0013	(8.1254e-21-0.0123478)	0.0040	(6.83808e-06-0.021692)	0.0021	(6.98492e-09-0.0152025)	0.0007	(1.49893e-17-0.00700385)	0.0030	(3.37429e-06-0.0149813)
Emerald Jan07	0.0052	(6.97359e-09-0.0337473)	0.0012	(2.29987e-20-0.0108861)	0.1043	(0.0325658-0.199657)	0.0101	(2.71056e-08-0.054833)	0.0010	(1.21385e-17-0.00990038)	0.0763	(0.0085731-0.162542)
Emerald Jul07	0.0048	(3.6928e-09-0.0313842)	0.0011	(1.54892e-18-0.0111211)	0.0248	(4.28793e-05-0.0897573)	0.0079	(4.57706e-08-0.0433932)	0.0012	(4.13115e-19-0.0113748)	0.0648	(0.000518781-0.170425)
Simpson Aug07	0.0057	(4.54241e-08-0.0346368)	0.0012	(1.32833e-19-0.011466)	0.0861	(0.00941528-0.187575)	0.1082	(0.0482984-0.169799)	0.0009	(1.86021e-18-0.00703396)	0.0754	(5.91531e-05-0.211209)
Eyre Aug07	0.0035	(1.06517e-08-0.0241413)	0.0011	(9.76675e-21-0.0105999)	0.0043	(5.05814e-06-0.0211913)	0.0021	(8.55746e-10-0.0131835)	0.0007	(5.65142e-18-0.00699956)	0.0028	(6.13096e-06-0.0128813)
Milparinka Aug07	0.0077	(5.97856e-08-0.0436596)	0.0012	(7.38463e-20-0.0122557)	0.0041	(1.75217e-06-0.0208407)	0.0019	(1.77211e-08-0.0126891)	0.0008	(3.62502e-19-0.00670929)	0.0032	(6.70222e-06-0.0152744)
Tilpa Aug07	0.6782	(0.667066-0.706217)	0.0010	(4.42347e-21-0.00964697)	0.0041	(2.46106e-06-0.0238906)	0.0023	(6.0728e-09-0.0156211)	0.0007	(7.38132e-19-0.00747824)	0.0029	(7.87476e-06-0.014386)
Bourke Aug07	0.2721	(0.193676-0.322281)	0.9865	(0.954714-0.999449)	0.0061	(6.69603e-06-0.0289999)	0.0024	(8.11571e-09-0.0161364)	0.0009	(2.28609e-19-0.00786552)	0.0752	(0.0365695-0.122757)
Walget Mar08	0.0034	(1.03288e-08-0.0215646)	0.0010	(4.95781e-19-0.00990706)	0.6767	(0.667055-0.700872)	0.0018	(8.71358e-09-0.0122026)	0.0008	(5.609e-21-0.00802264)	0.0031	(6.03119e-06-0.0151715)
Brewarrina Mar08	0.0041	(8.83867e-09-0.027406)	0.0010	(3.65309e-19-0.0114169)	0.0701	(0.0290896-0.123698)	0.8542	(0.796367-0.9094)	0.0010	(9.0333e-19-0.00987081)	0.0042	(1.77578e-06-0.0206683)
Brewarrina Aug07	0.0040	(5.03365e-09-0.0251826)	0.0010	(1.48037e-18-0.00927669)	0.0064	(8.91787e-06-0.0319318)	0.0029	(7.85726e-09-0.01785)	0.9898	(0.964467-0.999586)	0.0131	(0.00185135-0.0348381)
Narrabri Jan07	0.0035	(2.27058e-09-0.0249804)	0.0012	(1.00606e-17-0.0116549)	0.0046	(3.98079e-06-0.0237729)	0.0021	(2.30901e-09-0.0146391)	0.0008	(2.95754e-19-0.00816686)	0.6732	(0.666813-0.690527)

Run 9	INTO											
FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6799	(0.666952-0.710616)	0.0067	(1.89176e-08-0.0459342)	0.0004	(1.85022e-20-0.00346316)	0.0026	(1.41336e-11-0.0188414)	0.0023	(5.62743e-09-0.0131269)	0.0036	(7.25359e-09-0.0252859)	0.0006	(7.22374e-16-0.00448712)
Biloeala Jul07	0.0029	(8.50543e-09-0.0179512)	0.6862	(0.66709-0.736257)	0.0004	(1.43946e-18-0.00381314)	0.0024	(1.12705e-10-0.0179755)	0.0010	(1.33401e-09-0.00783382)	0.0035	(1.50264e-09-0.0254407)	0.0010	(8.59598e-17-0.00776907)
Emerald Jan07	0.0310	(1.40485e-06-0.101248)	0.0074	(3.06568e-08-0.0501905)	0.9942	(0.976007-0.999923)	0.0099	(3.21779e-09-0.0459188)	0.0017	(6.27379e-09-0.0104006)	0.0076	(4.93599e-09-0.0425737)	0.0006	(3.66068e-15-0.00555756)
Emerald Jul07	0.2528	(0.169418-0.315714)	0.2008	(0.0279181-0.310448)	0.0009	(5.4098e-19-0.00785901)	0.9497	(0.870558-0.993691)	0.2456	(0.0741082-0.307091)	0.0051	(1.53081e-08-0.0330662)	0.0006	(4.68331e-16-0.00518353)
Simpson Aug07	0.0035	(3.54679e-08-0.021963)	0.0319	(2.792e-07-0.188979)	0.0006	(7.28558e-18-0.00441412)	0.0167	(2.90717e-09-0.0728093)	0.7375	(0.677714-0.911556)	0.2755	(0.205011-0.323432)	0.0006	(4.79767e-16-0.00471635)
Eyre Aug07	0.0032	(3.71214e-08-0.0196447)	0.0068	(1.40047e-07-0.0375229)	0.0004	(7.2644e-19-0.0040535)	0.0018	(2.14688e-10-0.0136078)	0.0015	(2.41062e-08-0.00944787)	0.6781	(0.666841-0.706828)	0.0006	(5.77428e-16-0.0053943)
Milparinka Aug07	0.0058	(1.62227e-07-0.0310253)	0.0142	(5.92919e-08-0.0754497)	0.0004	(1.06178e-19-0.00370478)	0.0018	(7.26245e-11-0.0137644)	0.0014	(6.13581e-10-0.00881104)	0.0044	(4.67616e-09-0.0310586)	0.9904	(0.967608-0.999658)

Tilpa Aug07	0.0040	(6.34573e-08-0.0261132)	0.0068	(2.65944e-08-0.0384774)	0.0004	(8.3932e-19-0.00332265)	0.0019	(1.10205e-11-0.0119965)	0.0011	(1.83729e-09-0.00667136)	0.0033	(8.31445e-09-0.0235478)	0.0005	(5.56069e-17-0.00443966)
Bourke Aug07	0.0041	(1.08693e-08-0.0213334)	0.0067	(7.84442e-08-0.047061)	0.0004	(3.37556e-19-0.00377089)	0.0016	(1.50598e-10-0.012564)	0.0015	(3.28674e-08-0.00889934)	0.0032	(9.67599e-09-0.0210851)	0.0008	(9.2423e-16-0.00717666)
Walget Mar08	0.0034	(4.01181e-08-0.0214151)	0.0066	(2.33356e-08-0.0414257)	0.0004	(2.19912e-18-0.00366605)	0.0015	(1.84239e-11-0.0106586)	0.0011	(2.44758e-09-0.0065518)	0.0030	(7.09918e-09-0.0216946)	0.0005	(3.44212e-16-0.00446466)
Brewarrina Mar08	0.0030	(1.07318e-08-0.0202593)	0.0057	(6.7197e-08-0.037389)	0.0004	(1.17706e-18-0.00408564)	0.0026	(9.00957e-12-0.0185534)	0.0009	(3.8955e-10-0.0063699)	0.0028	(8.76625e-10-0.018864)	0.0005	(3.84083e-15-0.00433213)
Brewarrina Aug07	0.0032	(7.7841e-08-0.0210247)	0.0065	(6.6239e-08-0.0433736)	0.0006	(4.32176e-19-0.00427766)	0.0056	(1.20445e-09-0.0283118)	0.0034	(1.66174e-08-0.0150252)	0.0041	(6.99425e-09-0.0272572)	0.0005	(4.59188e-17-0.00363526)
Narrabri Jan07	0.0032	(3.49217e-08-0.0201787)	0.0137	(2.50858e-08-0.0809826)	0.0006	(2.30931e-18-0.00537811)	0.0019	(3.65503e-11-0.0149032)	0.0012	(1.41865e-08-0.00787019)	0.0058	(1.02566e-08-0.0343329)	0.0028	(3.88387e-15-0.0188761)

Run 9 Continued INTO

FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0035	(1.0837e-07-0.0244625)	0.0034	(8.25947e-09-0.0236313)	0.0044	(3.52331e-06-0.0238328)	0.0020	(5.33837e-09-0.013249)	0.0007	(4.91316e-18-0.0061687)	0.0025	(2.02882e-07-0.0152945)
Biloeala Jul07	0.0036	(1.14879e-08-0.0238233)	0.0036	(2.62476e-09-0.0245277)	0.0047	(4.30054e-06-0.0267685)	0.0019	(6.17427e-09-0.0115059)	0.0008	(2.22669e-17-0.00663577)	0.0021	(1.08655e-07-0.0129281)
Emerald Jan07	0.0063	(1.12993e-07-0.0412388)	0.0039	(7.17661e-09-0.0256121)	0.0875	(0.0285451-0.171266)	0.0177	(1.11689e-07-0.0852856)	0.0012	(1.92928e-18-0.00991923)	0.0276	(0.000299932-0.0909662)
Emerald Jul07	0.0043	(2.07475e-07-0.0305911)	0.0055	(1.72267e-09-0.0353728)	0.0878	(0.0342033-0.16263)	0.1069	(0.0501875-0.167002)	0.0010	(5.52079e-19-0.00839797)	0.1329	(0.0583277-0.200723)
Simpson Aug07	0.0046	(2.8849e-08-0.0323108)	0.0038	(1.27818e-09-0.025814)	0.0427	(0.000583793-0.115242)	0.0036	(4.92204e-08-0.0213158)	0.0009	(6.29003e-20-0.0085151)	0.0086	(2.71861e-07-0.0429138)
Eyre Aug07	0.0038	(1.80493e-08-0.0232177)	0.0032	(1.56638e-09-0.0187872)	0.0039	(2.01313e-06-0.0200747)	0.0022	(1.50645e-08-0.0160413)	0.0008	(1.95981e-18-0.00664273)	0.0019	(3.03953e-08-0.0123082)
Milparinka Aug07	0.0276	(5.90149e-06-0.089455)	0.0063	(1.14764e-08-0.0394561)	0.0044	(7.34224e-06-0.0227076)	0.0023	(7.4965e-09-0.0146927)	0.0008	(1.22315e-16-0.00749146)	0.0022	(7.71432e-08-0.0133982)
Tilpa Aug07	0.6789	(0.666983-0.708299)	0.0038	(1.50656e-09-0.0255124)	0.0047	(1.07123e-05-0.0234516)	0.0023	(1.25181e-08-0.0148101)	0.0008	(9.6957e-19-0.00838551)	0.0025	(2.58348e-07-0.0140615)
Bourke Aug07	0.0033	(1.47006e-08-0.0208028)	0.6777	(0.666901-0.706744)	0.0042	(4.91548e-06-0.020336)	0.0021	(1.18036e-08-0.0149773)	0.0008	(2.36109e-19-0.0077044)	0.0023	(3.26613e-08-0.0148723)
Walget Mar08	0.0035	(1.8303e-08-0.0215778)	0.0038	(2.02049e-08-0.0252442)	0.6765	(0.666875-0.701595)	0.0019	(4.63611e-09-0.0136559)	0.0007	(9.86723e-18-0.00616168)	0.0021	(1.39584e-07-0.0118574)
Brewarrina Mar08	0.0076	(1.88582e-07-0.0399318)	0.0038	(5.85587e-10-0.0261171)	0.0676	(0.0267119-0.122428)	0.8495	(0.796088-0.897664)	0.0010	(8.46819e-18-0.0084289)	0.0027	(5.29987e-07-0.017618)
Brewarrina Aug07	0.0039	(4.7216e-08-0.0239582)	0.0054	(1.88242e-08-0.0336261)	0.0055	(9.89769e-06-0.0253594)	0.0048	(5.81959e-09-0.0286208)	0.9896	(0.963653-0.999737)	0.0135	(0.00161339-0.0394967)
Narrabri Jan07	0.2490	(0.156585-0.31185)	0.2759	(0.208881-0.322649)	0.0061	(9.74449e-06-0.0320197)	0.0028	(8.28724e-08-0.019166)	0.0009	(1.93481e-18-0.00781884)	0.7991	(0.744236-0.858755)

Run 10	INTO															
FROM	Biloeala_Jan07		Biloeala_Jul07		Emerald_Jan07		Emerald_Jul07		Simpson_Aug07		Eyre_Aug07		Milparinka_Aug07	
Biloeala Jan07	0.6784	(0.66683-0.707641)	0.0070	(7.39965e-08-0.0452114)	0.0004	(5.02067e-19-0.003253)	0.0042	(3.32989e-07-0.024694)	0.0018	(1.53536e-10-0.00997136)	0.0033	(7.11051e-09-0.0228437)	0.0010	(9.83695e-10-0.00673681)
Biloeala Jul07	0.0037	(1.97263e-07-0.0218262)	0.6860	(0.667002-0.73877)	0.0004	(1.19479e-19-0.00417796)	0.0020	(1.82551e-09-0.0124083)	0.0007	(7.23795e-12-0.00459683)	0.0031	(1.21336e-08-0.0197063)	0.0010	(2.16905e-10-0.006328)
Emerald Jan07	0.0719	(4.89443e-06-0.202911)	0.0099	(5.81098e-08-0.0625765)	0.9951	(0.979833-0.999842)	0.0220	(0.000111429-0.0678772)	0.0008	(3.73778e-12-0.00565865)	0.0167	(3.19796e-08-0.0734202)	0.0012	(4.08876e-10-0.00736538)
Emerald Jul07	0.0039	(5.71019e-07-0.0208117)	0.0067	(1.05101e-08-0.0412441)	0.0003	(5.11782e-20-0.00376354)	0.6756	(0.666905-0.69682)	0.0010	(1.85787e-11-0.00677542)	0.0030	(3.44023e-09-0.0206527)	0.0011	(4.33423e-10-0.0070801)
Simpson Aug07	0.2042	(0.0777035-0.313396)	0.2363	(0.118365-0.314413)	0.0005	(3.86987e-19-0.00438976)	0.2705	(0.214859-0.313468)	0.9884	(0.972231-0.99779)	0.2628	(0.181072-0.319667)	0.0010	(1.53769e-10-0.00663297)
Eyre Aug07	0.0043	(3.11746e-07-0.0261688)	0.0060	(4.3607e-08-0.0355629)	0.0004	(5.69822e-18-0.00396491)	0.0033	(8.96927e-08-0.0189611)	0.0013	(3.79801e-11-0.00854976)	0.6785	(0.666881-0.709454)	0.0010	(9.75528e-09-0.00662263)
Milparinka Aug07	0.0067	(1.53908e-07-0.0338581)	0.0077	(3.45161e-08-0.0525173)	0.0004	(1.75299e-18-0.00358529)	0.0020	(1.27571e-08-0.0138799)	0.0006	(2.10314e-12-0.00430358)	0.0082	(8.82275e-09-0.0519965)	0.9499	(0.908962-0.983226)
Tilpa Aug07	0.0037	(7.83322e-07-0.0237166)	0.0059	(1.56725e-07-0.0379281)	0.0004	(2.18338e-20-0.00376541)	0.0024	(4.76872e-08-0.0146492)	0.0006	(1.0503e-11-0.00519674)	0.0034	(1.48855e-08-0.0224988)	0.0009	(8.65633e-10-0.00589077)
Bourke Aug07	0.0043	(4.63701e-07-0.0244251)	0.0079	(4.24141e-08-0.0534275)	0.0004	(1.00669e-19-0.00452112)	0.0025	(1.74425e-08-0.0162082)	0.0006	(1.76089e-12-0.00447045)	0.0059	(3.57543e-09-0.0323383)	0.0388	(0.00753945-0.0776278)
Walget Mar08	0.0038	(3.24112e-07-0.0210224)	0.0065	(1.35444e-08-0.0425569)	0.0004	(1.90086e-18-0.00316062)	0.0022	(8.7395e-09-0.0130997)	0.0006	(4.78229e-12-0.00411709)	0.0030	(3.83433e-09-0.0194245)	0.0011	(2.48078e-09-0.00782098)
Brewarrina Mar08	0.0036	(3.98688e-07-0.0185661)	0.0071	(2.3886e-08-0.0483844)	0.0004	(1.30596e-18-0.00364622)	0.0032	(2.34841e-08-0.0222034)	0.0016	(9.15475e-12-0.0107518)	0.0036	(3.72919e-09-0.0275986)	0.0011	(3.68338e-09-0.00751781)
Brewarrina Aug07	0.0063	(9.20529e-07-0.0341372)	0.0066	(8.01388e-08-0.045363)	0.0005	(3.44704e-18-0.00456329)	0.0073	(2.24617e-06-0.0316891)	0.0009	(2.64693e-11-0.00633113)	0.0052	(2.63357e-09-0.0290449)	0.0008	(8.11857e-10-0.00557596)
Narrabri Jan07	0.0052	(2.05186e-06-0.0300278)	0.0064	(2.29592e-08-0.04102)	0.0004	(7.11133e-21-0.00343414)	0.0027	(1.42722e-07-0.0171501)	0.0013	(2.93519e-12-0.00916102)	0.0033	(4.7969e-09-0.0225865)	0.0010	(6.83574e-11-0.00733938)

Run 10	INTO											
FROM	Tilpa_Aug07		Bourke_Aug07		Walget_Mar08		Brewarrina_Mar08		Brewarrina_Aug07		Narrabri_Jan07	
Biloeala Jan07	0.0037	(4.432e-09-0.0258471)	0.0012	(4.91797e-18-0.0108073)	0.0047	(3.33056e-06-0.0278879)	0.0021	(2.27126e-08-0.0135338)	0.0008	(7.45878e-19-0.00694764)	0.0029	(2.2976e-05-0.0157413)
Biloeala Jul07	0.0034	(5.8575e-09-0.0229855)	0.0012	(3.22533e-20-0.011788)	0.0038	(2.63161e-06-0.02177)	0.0019	(1.32199e-08-0.0126379)	0.0007	(2.0626e-18-0.00686865)	0.0032	(2.08972e-05-0.0179983)
Emerald Jan07	0.0067	(9.43397e-09-0.0453171)	0.0012	(5.34879e-19-0.011044)	0.1384	(0.0630721-0.221493)	0.0604	(1.29902e-05-0.142269)	0.0009	(4.46801e-20-0.00864056)	0.0844	(0.0219378-0.159718)
Emerald Jul07	0.0036	(2.5182e-08-0.0273294)	0.0012	(4.99194e-21-0.0114505)	0.0036	(1.01852e-05-0.018035)	0.0020	(2.01118e-09-0.013271)	0.0007	(7.31168e-19-0.00737683)	0.0029	(5.17117e-06-0.0145041)
Simpson Aug07	0.0072	(1.69573e-08-0.0471737)	0.0013	(5.50922e-18-0.0132781)	0.0705	(0.0164464-0.154175)	0.0573	(0.00808063-0.126239)	0.0009	(7.4827e-19-0.00805367)	0.1306	(0.0529714-0.211686)
Eyre Aug07	0.0033	(3.87404e-09-0.0230904)	0.0010	(3.61669e-20-0.00947349)	0.0041	(1.09456e-05-0.0200717)	0.0021	(5.15959e-09-0.01388)	0.0007	(9.79551e-18-0.00665668)	0.0031	(1.11705e-05-0.015068)
Milparinka Aug07	0.0169	(3.44355e-07-0.0761808)	0.0010	(3.39566e-19-0.00992565)	0.0048	(4.19911e-06-0.0282772)	0.0022	(5.95276e-08-0.0144668)	0.0007	(6.24208e-19-0.00592589)	0.0035	(5.14545e-06-0.0163889)

Tilpa Aug07	0.6793	(0.667085-0.713382)	0.0011	(2.88559e-18-0.00936205)	0.0042	(6.33709e-06-0.0227416)	0.0021	(7.28799e-09-0.0135378)	0.0007	(1.92337e-18-0.00696427)	0.0029	(8.84213e-06-0.0156315)
Bourke Aug07	0.2602	(0.165902-0.320459)	0.9867	(0.951875-0.999637)	0.0062	(6.61631e-06-0.0313009)	0.0029	(2.22932e-08-0.0172517)	0.0008	(1.79062e-18-0.00965827)	0.0737	(0.0340398-0.125298)
Walget Mar08	0.0036	(1.41282e-08-0.0241292)	0.0008	(2.85425e-18-0.00794715)	0.6762	(0.666885-0.70235)	0.0021	(2.7291e-08-0.0150908)	0.0008	(2.04544e-19-0.00652754)	0.0030	(8.07701e-07-0.0145779)
Brewarrina Mar08	0.0045	(5.07069e-08-0.0305915)	0.0010	(5.16844e-19-0.0100938)	0.0732	(0.0274727-0.133212)	0.8600	(0.793613-0.942955)	0.0008	(1.42597e-19-0.00788308)	0.0046	(6.48631e-06-0.0213492)
Brewarrina Aug07	0.0040	(3.01588e-09-0.0232465)	0.0013	(3.86581e-20-0.013302)	0.0058	(6.88528e-06-0.0301522)	0.0027	(9.10149e-09-0.0166124)	0.9907	(0.965468-0.999684)	0.0118	(0.00104914-0.0342328)
Narrabri Jan07	0.0035	(7.03016e-09-0.0234638)	0.0010	(6.34511e-20-0.0104022)	0.0045	(6.71325e-06-0.0226859)	0.0022	(5.82435e-08-0.0146461)	0.0007	(1.55851e-19-0.00637227)	0.6736	(0.66689-0.690011)

Appendix 3.3.a Summary table of mean migration rate and N (Numer of runs out of 10 (with different starting seeds) that the migration rate was significant (lower 95% CI above m=0.02))

	Biloeala Jan07		Biloeala Jul07		Emerald Jan07		Emerald Jul07		Simpson Aug07		Eyre Aug07		Milparinka Aug07		Tilpa Aug07		Bourke Aug07		Walget Mar08		Brewarrina Mar08		Brewarrina Aug07		Narrabri Jan07	
	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N	Mean m	N
Biloeala Jan07		0	0.006786	0	0.006485	0	0.007424	0	0.001723	0	0.003629	0	0.000989	0	0.003588	0	0.002215	0	0.0144809	1	0.0115805	1	0.000783	0	0.0213416	1
Biloeala Jul07	0.028941	2		0	0.000957	0	0.026068	1	0.027178	1	0.003442	0	0.001015	0	0.003647	0	0.002159	0	0.0138438	1	0.0127783	1	0.0007984	0	0.0192098	1
Emerald Jan07	0.033185	9	0.007962	0		0	0.01593	6	0.001232	0	0.01379	8	0.000968	0	0.005371	0	0.002398	0	0.1088559	9	0.0252101	8	0.0011852	0	0.0623106	10
Emerald Jul07	0.055572	3	0.070120	5	0.000801	0		0	0.090922	4	0.056453	3	0.001072	0	0.003594	0	0.002302	0	0.0247706	4	0.0244745	2	0.000863	0	0.0295291	3
Simpson Aug07	0.124693	6	0.146345	9	0.001864	0	0.145489	8		0	0.215485	8	0.001205	0	0.005257	0	0.002472	0	0.0533265	10	0.0453133	6	0.0010023	0	0.0631603	6
Eyre Aug07	0.003187	0	0.005940	0	0.000584	0	0.002546	0	0.001414	0		0	0.000971	0	0.003243	0	0.002067	0	0.0041842	0	0.0020952	0	0.0007332	0	0.0024851	0
Milparinka Aug07	0.007549	0	0.008548	0	0.000669	0	0.002214	0	0.000868	0	0.005729	0		0	0.013591	6	0.002864	0	0.0043839	0	0.0021688	0	0.0007601	0	0.0029653	0
Tilpa Aug07	0.003197	0	0.006038	0	0.000717	0	0.002365	0	0.001482	0	0.003361	0	0.005817	0		0	0.056801	2	0.0042793	0	0.0023157	0	0.0007878	0	0.0132387	3
Bourke Aug07	0.003271	0	0.006931	0	0.000603	0	0.002273	0	0.001023	0	0.004107	0	0.023237	8	0.159479	7		0	0.0050576	0	0.002342	0	0.0007946	0	0.0414505	6
Walget Mar08	0.003045	0	0.005886	0	0.000562	0	0.002217	0	0.001104	0	0.003219	0	0.000966	0	0.003205	0	0.002005	0		0	0.0019757	0	0.0007659	0	0.002654	0
Brewarrina Mar08	0.002960	0	0.006117	0	0.000735	0	0.00278	0	0.001206	0	0.003972	0	0.000868	0	0.004675	0	0.002128	0	0.0709096	9		0	0.001021	0	0.0067927	0
Brewarrina Aug07	0.004181	0	0.006101	0	0.000902	0	0.005736	0	0.001617	0	0.00482	0	0.000814	0	0.003741	0	0.002311	0	0.0056218	0	0.0031758	0		0	0.0125443	9
Narrabri Jan07	0.022002	0	0.008825	0	0.004884	0	0.007927	0	0.001405	0	0.0039	0	0.00271	0	0.054309	2	0.057085	2	0.0137231	0	0.012976	1	0.0007773	0		0