Genetic factors associated with variation in abundance of the invasive yellow crazy ant (Anoplolepis gracilipes)

by

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Abstract

A key component of successful invasion is the ability of an introduced population to reach sufficient abundance to persist, spread, and alter or dominate the recipient biological community. Genetic diversity is one of many factors that may contribute to population dynamics, but has important ramifications for biological fitness, and thus invasion success in the long term. I explored genetic factors associated with variation in abundance (i.e., differential invasion success) of the yellow crazy ant *Anoplolepis gracilipes* in the Indo-Pacific region, primarily focussing on Arnhem Land in Australia's Northern Territory.

I explored five aspects that I hypothesised could contribute to variation in the abundance of this ant: 1) I investigated the unusual reproductive mode of A. gracilipes, and tested whether it involved dependent-lineage genetic caste determination (DL GCD) in Arnhem Land. In DL GCD systems populations require hybridisation between genetically distinct groups to produce both reproductive and worker castes. Asymmetry in the ratio of different lineages may result in low abundance and population collapse. I found no evidence for a DL GCD system in A. gracilipes, and thus its abundance in Arnhem Land does not appear to be constrained by any lineage ratio asymmetry. Worker reproduction (either of males or asexual production of other workers) also appeared unlikely. The reproductive mode of the species remains fascinating but enigmatic; 2) I explored whether multiple source populations were responsible for the observed variation in abundance in Arnhem Land (i.e., is abundance associated with propagule pressure, or populations from different sources), and if the population has diverged since introduction. The A. gracilipes population in Arnhem Land stemmed from a single source, and thus propagule pressure was apparently not responsible for variation in abundance. In contrast to many invasive ants, population divergence has occurred since introduction; 3) I tested the hypotheses that genetic variation was associated with variation in abundance in Arnhem Land, and that ecological success was density-dependent. While the population divergence found in Chapter 3 was not related to variation in abundance, genotypic diversity was higher in more abundant nest clusters. These more abundant nest clusters were in turn associated with lower native ant species diversity, and a difference in composition of the invaded ant community (i.e., greater ecological success); 4) I revisited the invasion of the yellow crazy ant in Tokelau to determine whether a haplotype that was linked to greater abundance and dominance of the ant community has increased in distribution. Although ants of the inferred dominant haplotype were implicated in most new invasions, their abundance was substantially lower than previously observed in Tokelau; 5) I conducted a preliminary analysis of the metagenomic diversity of *A. gracilipes* endogenous parasites and symbionts among populations from Christmas Island, Okinawa, Samoa and Arnhem Land. Bacterial community composition and diversity differed between the study populations. Variation in abundance among *A. gracilipes* populations in Arnhem Land was not due to parasite load on populations with low abundance. However, low abundance of *A. gracilipes* was correlated with lower microbial diversity overall, and higher prevalence of some groups, notably two that confer antibiotic properties.

Together, my findings suggest that propagule pressure, reproductive mode and haplotype-specific effects do not appear to be associated with variation in *A. gracilipes* abundance. Other genetic factors I investigated do appear to be associated with the variation in *A. gracilipes* abundance and effects on the invaded ant communities. Genotypic diversity was positively related to the abundance of *A. gracilipes* in Arnhem Land, and this relationship may be affected by population divergence through population bottlenecks. In addition, differences in bacterial diversity among populations highlighted several candidate bacteria that could be associated with variation in abundance, which would be a topic of future work. Although genetic factors are often implicated in the successful establishment of invasive species, my thesis demonstrates that genetic factors may also be associated with post-establishment population dynamics.

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Statement of authorship

Chapters 2 to 6 of this thesis were written as independent manuscripts intended for publication. As these chapters included contributions of collaborators, they were written in first person plural tense. In all cases I wrote the manuscripts. All three supervisors provided advice throughout and contributed to editing of the manuscripts, and are therefore all co-authors, with the exception of Chapter 5. The contributions of other co-authors to each chapter are outlined below, indicated by author initials³.

Chapter 2 author contributions: MG designed the research and formulated the research questions. MG and BH collected the samples, and BH conducted initial dissections of workers. MG conducted the genetic analysis, remainder of dissections, analysed the data, and interpreted the results.

Chapter 3 was accepted for publication on June 2nd 2011 and is published in *Diversity and Distributions* **18**:323-333. Author contributions: MG, PL and BH designed the research and formulated the research questions. MG and BH collected the samples. MG conducted the behavioural assays, genetic analysis and data analyses, and interpreted the results.

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Chapter 6 author contributions: MG designed the research and formulated the research questions. AF wrote Perl scripts as specified by MG. CJ wrote the function SolexaStats as specified by MG. MG, PL and BH collected samples. MG conducted lab work and analysis, and interpreted the results.

Chapter 1. General introduction

Populations colonising a new area, whether they are invasive species, natural arrivals, or populations translocated for conservation, face similar challenges. Founding population sizes are often small, and the new environment is likely to differ in some way from the environment in which the organism evolved, which together may present major obstacles. Extrinsic factors such as climate, habitat and ecological interactions, and intrinsic factors such as genetic variation and life history traits, may influence not only the successful establishment and persistence of the population, but also on-going population dynamics. It has long been speculated that genetic variation and evolutionary changes may contribute significantly to the success of invasive species (Baker 1965; Lewontin 1965; Mayr 1965). My thesis focuses on how genetic factors may affect the variation in abundance (i.e., differential invasion success) of the yellow crazy ant, *Anoplolepis gracilipes* (Smith 1857).

1.1 Abundance and invasion success

Ideally, invasions are best studied in the context of how populations in the introduced range differ from those in the native range (Elton 1958; Hierro *et al.* 2005; Suarez and Tsutsui 2008; Sanders and Suarez 2011). This enables inferences to be made regarding how ecological differences and evolutionary changes between the ranges may have contributed to invasion success. Invasive ants exhibit many differences in ecology, population structure and behaviour between their native and introduced ranges, which have made it possible to identify factors that have contributed to their success in novel environments (Ross *et al.* 1996; Porter *et al.* 1997; Suarez *et al.* 1999; Tsutsui and Case 2001; Tsutsui *et al.* 2003a; Foucaud *et al.* 2006; LeBrun *et al.* 2007). However, comparisons between the ranges are impossible when the native range is not known. This is unfortunately the case for *A. gracilipes*. Although its congeners are African, the ant may have Asian origins (Wetterer 2005), which is supported by some evidence (Drescher 2011; Sebastien *et al.* 2012). For example, the closest relatives of some endosymbionts of *A. gracilipes* or the hosts of those endosymbionts appear to be from Asia (Sebastien *et al.* 2012), and lower diversity at mitochondrial markers in Indo-Pacific populations suggests

that these may have arisen from populations in Asia (Drescher 2011). However, the entire range is not known and has not been sampled. In particular, no information exists regarding the population genetic structure or behaviour of the ant in India (Drescher 2011; H. Feldhaar, personal communication).

So without reference to the native range how can we define and measure invasion success? Without the ability to compare with the native range, it is not possible to determine changes that may have facilitated invasion. In addition, merely the fact that a species is apparently successful in the introduced range is not, on its own, a sufficient criterion to judge any specific characteristics as factors contributing to invasion success. Although invasion success is difficult to definitively quantify, and impossible to predict over the long term, the ability to reach sufficient abundance to persist, spread, and alter or dominate the recipient biological community is a key component to the success of many invasive species (Ehrlich 1986; Crawley 1987; Mack et al. 2000). This is clearly true of invasive ants. Overwhelming evidence suggests that the ecological success of invasive ants is owing to their ability to attain significantly high numbers, and thus dominate the novel ecological community (Macom and Porter 1996; Ross et al. 1996; Holway 1999; Human and Gordon 1999; Morrison 2000; Hill et al. 2003; O'Dowd et al. 2003; Abbott 2005; Le Breton et al. 2005; Rowles and O'Dowd 2007; Bos et al. 2008; Krushelnycky and Gillespie 2008; Lester et al. 2009; Sagata and Lester 2009; Krushelnycky et al. 2010). Mechanistically, higher abundance of invasive ants confers stronger direct competitive ability relative to native ants (e.g., Human and Gordon 1999; Morrison 2000), typically through reduced intra-specific aggression (e.g., Holway et al. 1998; Suarez et al. 1999; Steiner et al. 2007). Thus, for the purpose of this thesis I define invasion success at a given point in time as the ability of the invading population to reach sufficient abundance to cause negative ecological effects in the community to which it is introduced.

At very high abundances the effects of invasive ants can be severe. Disassembly of resident ant communities may occur (Porter and Savignano 1990; Sanders *et al.* 2003; Lester *et al.* 2009), and in extreme cases invasions can negatively affect ecosystem functioning (O'Dowd *et al.* 2003; Davis *et al.* 2008). However, when in low abundance, invasive ants may not be able to compete successfully with native ants or dominate

resources (Oliver *et al.* 2008; Sagata and Lester 2009). Hence, any factors that positively or negatively affect the abundance of the invading ant population, and thus its competitiveness, will affect invasion success. The comparison of more and less abundant populations in the invaded range may provide insight into why some populations persist while others fail (or decline).

A number of factors contribute to the ability of invasive ants to reach high abundance (reviewed by Krushelnycky *et al.* 2010). One of the key drivers of the ecological success of ants is their advanced social organization (Wilson 1987). This social organisation can be viewed as a continuum from multicoloniality, where colonies are made up of a single nest, whose members behave aggressively to all outsiders, to unicoloniality, where ants display little or no aggression among many nests over the entire population, over a wide geographical area (Bourke and Franks 1995; Pedersen *et al.* 2006; Suarez *et al.* 2008). Although unicoloniality is found among ants that are not invasive, many invasive ants are unicolonial. Indeed, unicoloniality is considered to be one of the most important traits promoting the abundance and ecological success of many invasive ants (Helanterä *et al.* 2009; Krushelnycky *et al.* 2010).

1.2 Genetic factors and the success of invasive ants

Introduced populations face significant genetic challenges, and it has long been speculated that genetic variation and evolutionary changes may contribute to the success of invasive species (Baker 1965; Lewontin 1965; Mayr 1965). The success of invasive species has been called a genetic paradox (Allendorf and Lundquist 2003). This is because initial founding populations may experience extreme genetic bottlenecks, or founder effects, owing to a limited number of colonisers carrying only a small proportion of the genetic variation of the source population (Mayr 1942). Small founding populations are susceptible to the effects of a random change in allele frequencies over several generations (i.e., genetic drift), which most often leads to the rapid loss of rare, but potentially important genetic variation (Fisher 1930; Wright 1931; Nei *et al.* 1975). Founder effects and strong or repeated bottlenecks have a number of ecological and evolutionary consequences (Frankel and Soulé 1981; Sakai *et al.* 2001). In the short term, the probability of inbreeding, which potentially leads to reduced fitness owing to

inbreeding depression (Keller and Waller 2002), increases with a loss of allelic diversity as individuals become more closely related (Lande 1988; Frankham 2005). Susceptibility to disease may also increase through a loss of resistance conferred by variation at immune-related loci (Piertney and Oliver 2005; Luquet *et al.* 2012), and genetic diversity is known to enhance disease resistance among invertebrates (O'Brien and Evermann 1988; Altermatt and Ebert 2008; Lefèvre *et al.* 2011). The rate of evolutionary change in response to natural selection is proportional to the amount of additive genetic variance in the population (Fisher 1930). As bottlenecks are expected to decrease additive genetic variance (Chakraborty and Nei 1982; Lynch and Hill 1986), the population's adaptive potential is hindered (Willi *et al.* 2006), and extinction risk is increased (Frankham 2005). Despite these apparent disadvantages, invasive populations sometimes even undergo adaptive evolution (reviewed by Lee 2002; Dlugosch and Parker 2008), or potentially experience greater success owing directly to genetic bottlenecks (Suarez *et al.* 1999; Tsutsui *et al.* 2000).

Genetic changes in populations in the introduced range of invasive ants have been hypothesised to promote or magnify unicoloniality, whereby ants that are genetically similar perceive each other as related, although they are not. This may occur through a general loss of genetic diversity (Tsutsui et al. 2000), a specific loss of variation at recognition loci (Giraud et al. 2002; Ross et al. 2003), or the action of green beard (Hamilton 1964; Dawkins 1976) alleles (Keller and Ross 1998). For example, it has been suggested that in the invasive Argentine ants (Linepithema humile), a general loss of genetic diversity owing to population bottlenecks has promoted large-scale unicoloniality, and thus the ecological success of these ants (Suarez et al. 1999; Tsutsui et al. 2000; Tsutsui and Case 2001; Tsutsui et al. 2003b; Brandt et al. 2009). Alternatively it has been suggested that bottlenecks have resulted in genetic cleansing at specific recognition alleles, which may have led to unicoloniality (Giraud et al. 2002). Others disagree that a loss of genetic diversity has stimulated unicoloniality in this species (Vogel et al. 2010), based on the fact that unicoloniality also occurs in the native range (Pedersen et al. 2006). However, there are clear differences in the scale of colony size, genetic diversity, behaviour and ecology of this species throughout the introduced range (Suarez et al. 1999; Giraud et al. 2002; Corin et al. 2007; Brandt et al. 2009; Suhr et al. 2009; van Wilgenburg et al. 2010). Such changes or plasticity in social structure are not limited to

Argentine ants (Ross *et al.* 1996; Cremer *et al.* 2008; Suarez and Tsutsui 2008). In a group such as ants, where genetic factors may govern sociality and aggression (Hamilton 1964), it is intuitive that genetic and evolutionary factors should play an important role in the invasion success of these species (Ross *et al.* 1996; Cremer *et al.* 2008; Suarez and Tsutsui 2008).

A loss of genetic diversity may provide a number of other positive benefits in the invaded range. Typically, rare alleles are most likely to be lost through genetic drift following the founder event (Nei *et al.* 1975), which may be of benefit to the population if deleterious alleles are purged (Schmid-Hempel *et al.* 2007). Bottlenecks may also result in an increase in adaptive potential by disrupting the evolutionary inertia of co-adapted gene networks, which has been suggested as a mechanism for a number of models of speciation and adaptation, including Wright's shifting balance theory (Wright 1931), and founder flush speciation models (Mayr 1963; Carson 1968; Templeton 1980). These models predict that bottlenecks and genetic drift enable populations to reach new adaptive peaks, but they are controversial (Cheverud and Routman 1996; van Heerwaarden *et al.* 2008). In addition, depending on whether serial bottlenecks occur, the effects of additive variance and epistatic variance may differ (Goodnight 1987), as may the conversion from non-additive to additive variance (Goodnight 1988).

Genetic diversity may also be unimportant if asexual reproduction enhances invasion success. In asexually reproducing species, highly adapted clonal lineages can be shielded from potentially harmful sexual recombination events by population bottlenecks (Liu *et al.* 2006; Mergeay *et al.* 2006). Clonal reproduction is found in some populations of the invasive little fire ant (*Wasmannia auropunctata*), while other populations reproduce sexually (Fournier *et al.* 2005; Foucaud *et al.* 2006; Foucaud *et al.* 2007). As clonal populations of this ant are more prevalent in the introduced range, this reproductive mode may contribute to invasion success (Foucaud *et al.* 2010). It has been suggested that the ecological success of other invasive ants may also be facilitated by clonal reproduction (Kellner and Heinze 2010; Pearcy *et al.* 2011), although these studies have not investigated the native and introduced ranges, often an important comparison to make when assessing invasion success (Elton 1958; Hierro *et al.* 2005; Sanders and Suarez 2011).

Among the mechanisms invoked to explain why there is frequently no genetic paradox is propagule pressure, which is one of the most commonly observed factors associated with invasion success generally (Von Holle and Simberloff 2005; Colautti et al. 2006; Dlugosch and Parker 2008; Simberloff 2009; Wilson et al. 2009b). Propagule pressure owing to a large initial founding population size or multiple introduction events (Simberloff 2009; Wilson et al. 2009b) may result in higher genetic diversity in the introduced range than the native range, and thus promote invasion success (Dlugosch and Parker 2008). Multiple introduction events can increase the overall population size, thereby reducing the potential negative effects of genetic drift (Allendorf and Lundquist 2003; Roman and Darling 2007; Facon et al. 2008). Moreover, in some cases, multiple introductions can increase the genetic diversity of the introduced population relative to native populations through inter- or intra-specific hybridisation (Ellstrand and Schierenbeck 2000; Kolbe et al. 2004; Petit 2004; Facon et al. 2008). Although propagule size may affect the establishment of invasive ants (Holway et al. 2002; Sagata and Lester 2009; Yang et al. 2012), populations may also require only a few founders (Hee et al. 2000; Corin et al. 2007; Ross and Shoemaker 2008; Foucaud et al. 2010). To date, no studies have suggested that inter- or intra-specific hybridisation contributes to the success of invasive ants. By contrast, hybridisation may negatively affect invasion success. A number of ant species are known to hybridise, often with negative fitness consequences for the hybrid population (Feldhaar et al. 2008). Some ant species appear to avoid these fitness costs via dependent lineage genetic caste determination (DL GCD) (Feldhaar et al. 2008). The DL GCD mode of reproduction (Anderson et al. 2006) requires obligate hybridisation of two lineages (typically species) to produce workers, while pure lineages become queens (Helms Cahan et al. 2002; Volny and Gordon 2002; Helms Cahan and Vinson 2003). Queens must therefore mate with males from each lineage to produce both workers and reproductives. If the ratio of the two lineages present in the population is skewed, colony failure rates increase (Anderson et al. 2006; Schwander et al. 2006). Ants with DL GCD may thus have lower chances of invasion success if one lineage is underrepresented.

In some cases, specific biotypes or genotypes may be more invasive than others, while genetic diversity per se does not influence invasion success (Perring 2001; Saltonstall 2002; Petit 2004; Abbott et al. 2007). Non-native, genotypically distinct populations of the reed grass *Phragmites australis* in North America have faster rates of spread, and are able to outcompete native populations (Saltonstall 2002). Similarly, the whitefly (Bemisia tabaci species complex) has a number of genetic biotypes, of which only two have attained pest status (Perring 2001). Although invasive and non-invasive *Bemisia* biotypes carry different communities of endosymbionts, including the sex-manipulating Wolbachia, Rickettsia and Arsenophonus (Gueguen et al. 2010; Thierry et al. 2011), it is not known how these might affect fitness in these Bemisia study populations (Gueguen et al. 2010; Thierry et al. 2011). In other invasive whitefly populations Rickettsia has positive effects on both its own and its host fitness through host sex-ratio manipulation towards females, which increases the rate of host reproduction and spread (Himler et al. 2011). Genotype-specific invasion success may also be experienced by the invasive yellow crazy ant A. gracilipes, in which one distinct haplotype has been implicated in the high abundance of specific populations of the ant in Tokelau (Abbott et al. 2007). In addition, A. gracilipes harbours a number of endosymbionts, including the sex-ratio manipulators Arsenophonus and Wolbachia (Sebastien et al. 2012), although their function in ants is unknown.

Finally, the molecular markers typically used to estimate genetic diversity might not always directly relate to establishment success and evolutionary potential, as they might not accurately represent the total diversity in the genome, particularly regarding quantitative traits (Väli *et al.* 2008). The variation in quantitative traits may not be affected by bottlenecks because the distributions of quantitative variation are less sensitive to the loss of rare alleles than molecular markers (Lande 1980; Barton and Charlesworth 1984).

1.3 Ecological factors also contribute to the invasion success of ants

Ecological differences in the novel environment, or demographic and environmental stochasticity may be stronger influencers of population persistence than genetic factors (Lande 1988). While my thesis research does not focus on the many ecological mechanisms that might or might not affect the invasion success of *A. gracilipes*, the role of endogenous diversity (symbionts, parasites and pathogens) is of interest, as these endogenous organisms may affect variation in abundance, their effects may be affected by genetic diversity of the host (O'Brien and Evermann 1988; Shykoff and Schmid-Hempel 1991; Altermatt and Ebert 2008; Lefèvre *et al.* 2011), and they are often inherited through vertical transmission from parent to offspring (Zientz *et al.* 2005).

The relationship between ants, their parasites and symbionts is complex and not well understood compared to other ecological interactions. Although endogenous bacteria may be harmful to their insect hosts (Wenseleers *et al.* 2002; Schlüns and Crozier 2009), many are also beneficial (Dillon and Dillon 2003; Dale and Moran 2006; Feldhaar and Gross 2009). A number of bacteria play a role in nitrogen metabolism (Kikuchi *et al.* 2005; Stoll *et al.* 2007; Russell *et al.* 2009), and immune defense (de Souza *et al.* 2009), protecting insects from attack by pathogens, including viruses (Dillon and Charnley 2002; Hedges *et al.* 2008; Teixeira *et al.* 2008), and eukaryotic parasites (Currie *et al.* 1999; Santos *et al.* 2004; Moran *et al.* 2005). Parasites may even mediate kin recognition in ants (Martin *et al.* 2011), and their loss may be associated with invasion success (Cremer *et al.* 2008; Martin *et al.* 2011). Invasive ants may lose endosymbionts (e.g., *Wolbachia*) in the introduced range (Tsutsui *et al.* 2003a), but the function of common insect endosymbionts such as *Wolbachia* in ants is unclear and may vary (Zientz *et al.* 2005).

Mutualisms, including those with endosymbionts, are often associated with the ecological success of ants, and may contribute to invasion success. Carbohydrate resources obtained through extra-floral nectaries or tending scale insects are known to enhance ant abundance (Davidson 1997 and references therein), and the appropriation of these resources may often involve novel mutualistic interactions in the invaded range (Helms and Vinson 2002; Abbott 2004; Abbott and Green 2007; Wilder et al. 2011). Even ants that are not strictly herbivorous may use nitrogen-fixing endosymbionts to 'upgrade' their nutrition, gaining valuable amino acids that would be otherwise unavailable to them (Feldhaar et al. 2007). Ants may carry a suite of such mutualists, and if their function has a large effect on population dynamics, their loss could also result in population declines (Russell et al. 2009). In the invaded range symbiotic mutualists in particular may be easily lost as their persistence can depend on the host population size (Sachs and Simms 2006). Thus bottlenecks that affect the host may also affect the symbionts. Higher genetic diversity of the host can also enhance parasite resistance (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991; Keller 1995; Tarpy 2003; Ugelvig et al. 2010), so as well as losing mutualists a genetic bottleneck may result in reduced immune function. The changes in the dynamics of these endogenous populations may affect stress tolerance and parasite resistance, and ultimately invasion success (Feldhaar 2011).

Ecological and genetic factors are not necessarily mutually exclusive causes of invasion success, and different contexts may result in different interactions between these mechanisms (i.e., variation in ecological conditions in different parts of the invaded range, variation in propagule size, traits that assist introduction and establishment versus those that assist spread). For example in some contexts a loss of genetic diversity in *Spartina* sp. is associated with invasion success, while in other cases the opposite is the case (Petit 2004). In Argentine ants, for example, although unicoloniality may have enhanced the success of the ant throughout its invaded range (Suarez *et al.* 1999; Giraud *et al.* 2002), perhaps assisted by a loss of natural enemies (Suarez *et al.* 1999; Tsutsui *et al.* 2003a), abiotic factors also contribute to its success at the community scale (Menke and Holway 2006), predict its distribution and spread (Krushelnycky *et al.* 2005; Hartley *et al.* 2010), and likelihood of persistence (Cooling *et al.* 2011).

1.4 Focal study system: *Anoplolepis gracilipes* in Arnhem Land

The yellow crazy ant *Anoplolepis gracilipes* appears among a 'selection of 100 of the world's worst invasive species' (Lowe *et al.* 2000), and is considered one of the most widespread, abundant and damaging invasive ants (Holway *et al.* 2002). There is no doubt that *A. gracilipes* can be a serious pest. The ant can occur at very high densities, and its most extreme effects include ecosystem change on Christmas Island, owing to a classic case of 'ecological meltdown', promoted by a mutualism with a secondary introduction of a scale insect (O'Dowd *et al.* 1999; O'Dowd *et al.* 2003; Abbott 2006b). However, the abundance of the ant varies spatially and temporally throughout its range (Greenslade 1971; Baker 1976; Abbott 2006b; Abbott *et al.* 2007; Suwabe *et al.* 2009), and population declines have also been observed (Haines and Haines 1978b).

Anoplolepis gracilipes was first detected in Northeast Arnhem Land in Australia's Northern Territory around rehabilitated mine sites in 1984 (Majer 1984). By 1989 the ants distribution was estimated to patchily span an area of 2,500 km² in Arnhem Land (Young *et al.* 2001), which increased to around 16,000 km² by 2009 (B. Hoffmann, unpublished data). *Anoplolepis gracilipes* exhibits varying nest densities and abundance over this wide region. The abundance of *A. gracilipes* in Arnhem Land also appears relatively low compared to populations elsewhere. Carbohydrate resources obtained through novel mutualisms are not an obvious driver of the variation in abundance in Arnhem Land (B. Hoffmann, personal communication), unlike *A. gracilipes* invasions in Samoa (Savage *et al.* 2009), and on Christmas Island (O'Dowd *et al.* 2003). Nor are there obvious associations between some of the more commonly observed factors that contribute to the variation in abundance and success of invasive ants, such as abiotic factors, anthropogenic disturbance, or ecological release. Thus the invasion in Arnhem Land offered the opportunity for me to investigate whether genetic factors might contribute to the variation in abundance of *A. gracilipes*.

The potential contributions of genetic factors to the invasion success of A. gracilipes have not attracted much study compared to other invasive ants. Studies on this ant have overwhelmingly focussed on the potential ecological causes and effects of its invasion (e.g., Haines and Haines 1978b; Haines et al. 1994; Hill et al. 2003; Gerlach 2004; Green et al. 2004; Lester and Tavite 2004; Abbott 2005; Lach 2005; Ward and Beggs 2007; Bos et al. 2008; Davis et al. 2008; Neville et al. 2008; Pfeiffer et al. 2008; Lester et al. 2009; Matsui et al. 2009; Savage et al. 2009; Brühl and Eltz 2010; Davis et al. 2010; Hoffmann and Saul 2010; Drescher et al. 2011; Lach and Hoffmann 2011; Tanaka et al. 2011). Only recently have studies begun to investigate the genetic aspects of the ants' biology, and these studies have focussed primarily on population structure and behaviour, typically in relation to supercolony structure (Drescher et al. 2007; Drescher et al. 2010; Thomas et al. 2010). One notable exception is the finding that populations of the ant on the Tokelau atolls belonged to either of two common and behaviourally distinct mitochondrial haplotypes (A and D). Haplotype D was associated with more abundant populations and strong negative effects on the resident ant community (Abbott et al. 2007), indicating a potential association between genetic factors and invasion success.

As is often the case with invasive ants, density-dependent invasion success is likely to be generally true also of *A. gracilipes*. On Christmas Island management of the ant is only initiated once abundance has reached a threshold above which ecological effects are observed (Boland *et al.* 2011), and on Tokelau only more abundant populations have negatively affected resident ant species (Abbott *et al.* 2007; Lester *et al.* 2009). Some evidence suggests this is the case in Arnhem Land also (Hoffmann and Saul 2010). Many studies of *A. gracilipes* have focussed on the ant when it is at high abundance (e.g., Hill *et al.* 2003; Gerlach 2004; Lester *et al.* 2009; Savage *et al.* 2009; Brühl and Eltz 2010; Drescher *et al.* 2011). However, the abundance of the ant can vary temporally and spatially, and a few studies have documented this variation in a number of different places (Christmas Island: Abbott 2006b; Tokelau: Abbott *et al.* 2007; Japan: Suwabe *et al.* 2009). Reasons suggested for this variation included seasonal differences in ant activity (Abbott 2006b; Suwabe *et al.* 2009), or haplotype-specific abundance (Abbott *et al.* 2007). I hypothesised that the association Abbott *et al.* (2007) found between variation

in abundance and different haplotypes of the ant on Tokelau might also explain the variation in abundance in Arnhem Land.

Populations of A. gracilipes also appear to experience unexplained declines or local extinctions. Haines and Haines (1978) noted that A. gracilipes had inexplicably declined on Mahé in the Seychelles. Declines have also been anecdotally reported on Christmas Island (P. Green, personal communication). In both cases the reasons for the declines have not been empirically investigated. In Arnhem Land A. gracilipes has also experienced declines which have no obvious explanation (B. Hoffmann, personal communication). Sudden declines such as these occur occasionally in other invasive species also, and their causes are often unexplained (Simberloff and Gibbons 2004). Populations of A. gracilipes are known to harbour a number of endogenous bacteria, including sex-ratio manipulating Arsenophonus, Wolbachia, and herbivory-associated Rhizobiales (Sebastien et al. 2012), although other bacteria and endogenous eukaryotes have not been investigated in the species. The role of these endogenous bacteria in A. gracilipes is not known. However, it is possible that variation in microbial communities or bacterial associations with ants influence these population dynamics. Determining which, if any, bacteria are responsible for population declines may take considerable work and time. Here I undertook a study to gain preliminary insights into differences in the microbial communities of A. gracilipes populations.
1.5 Thesis aims

In this thesis I investigate the relationships between the variation in abundance of *A*. *gracilipes* populations and genetic factors that may affect invasion success. My overall objective is to determine if genetic factors are associated with the observed variation in abundance of *A*. *gracilipes*, primarily focussing on populations in Arnhem Land. Each chapter of my thesis addresses a single factor in relation to this objective.

Chapter 2 is concerned with how the reproductive mode might influence the variation in the abundance of *A. gracilipes*, and primarily investigates the possibility of lineageratio asymmetry within dependent lineage genetic caste determination, along with worker reproduction, and discussion regarding asexual reproduction.

In Chapter 3 I investigate the population structure and behaviour of *A. gracilipes*, to determine if introductions from multiple, different sources contribute to the variation in abundance of the ant in Arnhem Land (i.e., propagule pressure or potentially differential abundance of genetically distinct populations).

Chapter 4 investigates whether there is a positive or negative relationship between genetic diversity and invasion success of *A. gracilipes* via density-dependent effects on the invaded ant community, and whether selected ecological characteristics are also correlated with variation in abundance.

I revisit the study of Abbott *et al.* (2007) on Tokelau in Chapter 5 to determine if the *A. gracilipes* genetic haplotype identified earlier maintains its dominance, and whether the association between high abundance and this dominant haplotype persists.

In Chapter 6 I conduct a preliminary investigation of the diversity of endogenous parasites and symbionts of *A. gracilipes*, to identify candidates that may be associated with the variation in abundance of *A. gracilipes* populations.

Chapter 7 summarises and synthesises the main findings of my thesis, outlines the constraints faced, and opportunities for further research, including additional genetic factors that may affect abundance and invasion success.

Chapter 2. The conundrum of the *Anoplolepis gracilipes* reproductive mode: no evidence for dependent lineage genetic caste determination

2.1 Abstract

Asexual reproduction and hybridisation are often found among highly invasive plants and marine invertebrates. Recently, it has been suggested that clonality may enhance the success of invasive ants. By contrast, obligate hybridisation (dependent lineage genetic caste determination or DL GCD in ants) may decrease the chances of population persistence if one lineage is less prevalent than the other (asymmetry in lineage ratio). Genetic characteristics of the invasive yellow crazy ant (Anoplolepis gracilipes) suggest that it has an unconventional mode of reproduction that may involve asexual reproduction by workers or queens, or DL GCD. Anoplolepis gracilipes shows considerable spatial variation in abundance where it has invaded in Arnhem Land in Australia's Northern Territory. Here, we investigated whether A. gracilipes reproduction involved DL GCD, and if so, whether lower abundance could be owing to asymmetry in lineage ratio. The potential for worker reproduction was also assessed. We used microsatellite markers to assess the population structure of A. gracilipes workers, males, queens and sperm in queen spermathecae, from field collections in Arnhem Land. We found that a single queen lineage is present in Arnhem Land. The presence of a single lineage of queens discounts the possibility of DL GCD. Population structure separated queens and workers into different lineages, suggesting that these castes are determined genetically in A. gracilipes. Evidence for worker reproduction was weak. We conclude that the reproductive mode of A. gracilipes does not involve DL GCD, and thus the variation in abundance that we observe in the Arnhem Land population of A. gracilipes is not owing to asymmetry in lineage ratio. The resolution of the reproductive mode of A. gracilipes is complicated by a high prevalence of diploid males. The determination of the A. gracilipes reproductive mode remains a fascinating research question, and its resolution may improve our understanding of the contribution of the reproductive system to invasion success.

2.2 Introduction

Biological invasions are one of the chief threats to biodiversity, and affect ecosystem function, native organisms and economic well-being (Mack et al. 2000; Pimentel 2005). Although there is little consensus on the mechanisms and predictability of invasiveness, clonal reproduction and hybridisation are commonly observed traits in successful invaders of terrestrial plant and aquatic communities (Ren et al. 2005; Facon et al. 2006; Mergeay et al. 2006). It has become apparent that ant reproductive systems are more diverse than previously thought (Keller 2007; Heinze 2008; Schwander et al. 2010), and recently it has been suggested that the ecological success of some invasive ants may also be facilitated by clonal reproduction (Foucaud et al. 2009; Kellner and Heinze 2010; Pearcy et al. 2011). In clonal genetic caste determination (GCD) systems workers are produced sexually by mated queens, while queens and males are produced clonally. Variations on this system have been found in four species, Cataglyphis cursor (Pearcy et al. 2004), Wasmannia auropunctata (Fournier et al. 2005), Vollenhovia emeryi (Ohkawara et al. 2006; Kobayashi et al. 2008), and Paratrechina longicornis (Pearcy et al. 2011), although the exact mechanisms differ between species. In addition, unmated workers (gamergates) of many species reproduce clonally (reviewed in Heinze 2008).

A number of ant species are known to hybridise, often with negative fitness consequences for the population (Feldhaar *et al.* 2008). Some ant species avoid the fitness costs of hybridisation via dependent lineage genetic caste determination (DL GCD) (Anderson *et al.* 2006) The DL GCD system requires obligate hybridisation of two lineages (typically species) to produce workers, while matings within lineages result in queens (Helms Cahan *et al.* 2002; Volny and Gordon 2002; Helms Cahan and Vinson 2003). Queens must mate with multiple males to produce both workers and other reproductives. Thus the ratio of the two lineages present in the population has consequences for colony fitness. The chance of a queen acquiring sperm from only one lineage increases as the lineage ratio increases in skew, resulting in higher colony failure rates (Anderson *et al.* 2006; Schwander *et al.* 2006). Clearly, while clonal reproduction could enhance the chance of successful colonisation and persistence, DL GCD in invasive ants could result in a lower likelihood of invasion success if one lineage is underrepresented.

The yellow crazy ant (Anoplolepis gracilipes), considered to be one of the most damaging invasive ant species (Holway et al. 2002), has an unresolved reproductive mode. Although the reproductive mode remains elusive, and the ants native range is not known (Wetterer 2005), studies in the invaded range have consistently found that workers are typically heterozygous, queens are typically homozygous, and diploid males are common (Borneo: Drescher et al. 2007; Christmas Island: Thomas et al. 2010). High heterozygote frequencies in workers, and a prevalence of homozygous queens has been interpreted as possibly owing to DL GCD or clonal GCD (Drescher et al. 2007), or asexual reproduction by unmated workers (Heinze 2008). In addition, on Christmas Island at least two mitochondrial lineages of the ant are known to co-exist, although they appear to be reproductively isolated from each other (Thomas et al. 2010). In our study area, Arnhem Land in the Northern Territory of Australia, A. gracilipes worker populations vary spatially in abundance, and we wished to determine if the mode of reproduction contributed to this variation. For example, if A. gracilipes reproduction involves a DL GCD system, low abundance could be owing to a persistent skew in lineage ratio.

Here we use molecular markers to test the hypothesis that the reproductive mode of *A*. *gracilipes* involves DL GCD. If DL GCD was involved we would expect the population genetic structure to reflect queens and males resulting from pure lineages, and workers arising as a result of hybridisation between these pure lineages. It would have been desirable to also investigate the possibility of clonal production of queens and males, however field samples alone are insufficient to conduct the parentage analyses necessary to distinguish clonal versus sexual production of the different castes. We did, however, dissect workers and their ovaries to investigate the possibility of worker reproduction.

2.3 Methods

2.3.1 Study site and sampling

Anoplolepis gracilipes is patchily distributed throughout 16,000 km² in Arnhem Land (Fig 2.1). Workers were collected from ten sites throughout the ant's geographical range in October 2007, and queens, workers and brood were collected from an additional ten sites between August 2008 and March 2009 (Fig. 2.1). Samples were collected from a single nest at each site except site SC where we sampled seven nests, spaced 5-100 m apart, on different dates. This sampling regime allowed analysis at a broad (regional) scale, as well as the finer scale at which groups of ants are likely to interact. Nests contained between one and 16 queens (mean 4.1 ± 0.9 SE). Ants were stored in 95% ethanol at 4°C until DNA extraction. No adult males were found during the sampling, but four male brood were collected from a nest at site YI3 and one from a nest at site SC. In October 2009 (coinciding with the emergence of *A. gracilipes* sexuals), 24 males from a single nest (site SH) were sampled for genotyping. Workers from 13 nests at this site were also sampled for dissection to investigate the possibility of worker reproduction.

2.3.2 Genetic analyses

To determine the genotypes of males contributing to reproduction, we extracted sperm from the spermathecae of queens. Queen gasters were dissected, and the sperm contents extracted from the spermathecae and placed in 95% ethanol. Contamination by queen tissue from the spermatheca was considered unlikely as the sperm DNA content was likely to be greater than that of the spermathecal wall. Thus, sperm cell DNA would have preferentially amplified under PCR (Krieger and Keller 2000). This assumption was upheld by an earlier test of the method on four queens and their spermathecal contents from Christmas Island (unpublished data) for another project. Three of the four sperm samples had one allele (i.e., were likely to be haploid), which did not match an allele of the queen from which the spermatheca had been dissected. The fourth sperm sample had two alleles, one of which matched an allele of the queen from which it had been dissected.



Fig. 2.1: Sampling sites of *Anoplolepis gracilipes* in Arnhem Land. Grey circles (\bigcirc) indicate known locations of *A. gracilipes*. Open circles (\bigcirc) indicate sites where workers only were sampled in October 2007. Black circles (\bigcirc) indicate sites where queens, workers and male brood were sampled in August 2008 and March 2009. One nest was sampled at each site, with the exception of SC, where seven nests were sampled. The black square (\blacksquare) indicates the site (SH) where workers for dissection and males were collected in October 2009.

We extracted DNA using a modified Chelex protocol (Sepp *et al.* 1994). Spermathecal contents were placed in 1.7 mL microcentrifuge tubes with 5 μ l of 10 mg/mL Proteinase K and 60 μ l of 10% w/v Chelex-100 resin solution and heated for 2 hours at 60°C. Individual workers, brood, or heads of queens were ground using sterile plastic pestles in microcentrifuge tubes containing 150 μ l of a 10% w/v Chelex-100 resin solution. Tubes were centrifuged briefly, boiled for 15 minutes, chilled on ice for five minutes and then centrifuged for 15 minutes at 15,000 × g at 4°C. The supernatant containing DNA was stored at 4°C.

We used five microsatellite markers, Ano1, Ano3, Ano4, Ano7 and Ano8 (Feldhaar et al. 2006), to investigate population structure and identify multiple lineages. Each 15 µl PCR consisted of ~20 ng template DNA, 10× PCR Buffer, 0.4 mg/mL of bovine serum albumin (BSA), 1.5 - 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.1 mM of forward primer, 0.4 mM of reverse primer, 0.4 mM of universal fluorescent dye-labelled M13(-21) primer (Schuelke 2000) and 0.1 U of Taq DNA Polymerase (New England Biolabs). Thermal cycling was performed on an Eppendorf 2700 thermal cycler using conditions specified by Feldhaar et al. (2006), with modification for M13(-21) primers (Schuelke 2000). Amplified products were analysed on a 3730 Genetic Analyzer and genotypes scored using Genemapper v 3.7 (Applied Biosystems). Successful genotyping of 16 nests included three to 12 workers (mean \pm SE = 6.1 \pm 0.7, n = 96), one to 10 spermathecal contents (mean \pm SE = 3.6 \pm 0.6, n = 68), and one to 11 queens (mean \pm SE = 4.1 \pm 0.9, n = 73). Fifteen to 20 workers (mean \pm SE = 19.4 \pm 0.4, n = 214) were genotyped from 11 nests without queens. Four male brood from one nest and one from a second nest were also genotyped, along with the 24 adult males from a single nest. We used Micro-Checker v 2.2.3 (van Oosterhout et al. 2004) to validate genotyping quality. Scoring errors, presence of null alleles and large allele dropout were not apparent.

Sperm with homozygous multi-locus genotypes were considered to be haploid (hemizygous). Sperm with heterozygous genotypes at one or more loci were either diploid, and / or the queen was multiply mated, or the sperm sample was contaminated by queen tissue. We visually inspected the sperm genotypes to detect features that could indicate contamination. It was decided that sperm samples with relatively low volume that had a multi-locus genotype matching the genotype of the queen from which they

were dissected could be unreliable results and should be excluded from analysis. We excluded one sperm sample on this basis. In addition, sperm with genotype scoring peaks that were substantially lower for maternal alleles were also considered unreliable (i.e., we would expect maternal alleles to be non-preferentially amplified if the queen contribution of DNA from the spermatheca was much lower than the sperm contribution). No sperm samples were excluded on this basis.

2.3.3 Data analyses

2.3.3.1 Population genetic parameters

We tested for Hardy-Weinberg proportions (HW) and linkage disequilibrium (LD) using GENEPOP v 4.0.10 (Raymond and Rousset 1995; Rousset 2008). The close relatedness of workers within nests may result in non-independence of genotypes and thus pseudoreplication in HW and LD analyses (Drescher *et al.* 2007). We corrected for this by using a single randomly selected ant per nest.

We used Genclone v 2.0 (Arnaud-Haond and Belkhir 2007) to enumerate the multilocus genotypes (MLGs) of the ants sampled. To enable easier visualisation of MLGs we grouped and encoded alleles according to their observed distribution among queens, workers and sperm. Alleles that were not found in homozygous queens, but found in male brood, workers or sperm were designated as paternal alleles (i.e., putatively paternally inherited). The other of the pair of alleles found in heterozygous workers (i.e., putatively maternally inherited), which were found in homozygous queens, we designated as maternal alleles. We enumerated allele frequencies using GenAlEx v 6.3 (Peakall and Smouse 2006).

2.3.3.2 Population structure

We used AWclust (Gao and Starmer 2008) to estimate population structure. Unlike other methods (e.g., STRUCTURE: Pritchard *et al.* 2000), AWclust makes no assumptions about HW proportions or LD, and thus appears appropriate to use when an unusual reproductive mode is possible. In addition, the results enable easier identification of individuals on the resulting tree than STRUCTURE.

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$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		(0.000)	(0.500)	(0.481)	(0.338)	(0.341)					
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	8 4	0.234	0.997	0.674	0.414	0.496	NS	< 0.01 (E)	NS	NS	< 0.01 (E)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$		(0.207)	(0.550)	(0.447)	(0.400)	(0.311)					
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	4 2	0.000	0.997	0.711	0.276	0.609	NA	< 0.01 (E)	NS	NS	< 0.01 (E)
$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$		(0.000)	(0.500)	(0.458)	(0.285)	(0.435)					
Ano8 432 208-282 16 0.057 (0.628) (0.552) (0.438) 0.067 0.990 0.630 0.207 0.652) (0.815) (0.248) 0.6623 (0.815) (0.248) 0.6623 0.6623 0.6623 0.66663 0.66663 0.66663 0.66663 0.66663 0.66663 0.66663 0.66663 0.666663 0.666663 0.66666666666666666666666666666666666	9 9	0.026	0.984	0.911	0.517	0.474	0.03 (D)	< 0.01 (E)	NS	NS	NS
Ano8 432 208-282 16 0.067 0.990 0.630 0.207		(0.121)	(0.628)	(0.552)	(0.438)	(0.645)					
(0.242) (0.853) (0.816) (0.248)	2 16	0.067	066.0	0.630	0.207	0.580	< 0.01 (D)	< 0.01 (E)	< 0.01 (D)	NS	0.04 (D)
(0+7·0) (010·0) (CC0·0) (700·0)		(0.662)	(0.853)	(0.816)	(0.248)	(0.401)					

Table 2.1: Genetic diversity estimates for Anoplolepis gracilipes queens (Q), workers (W), sperm (S) and males (M) in Arnhem Land. Values given for heterozygote excess (E) or deficiency (D) represent P values. NA indicates values could not be determined as the locus showed no variation for the group.

2.4 Results

2.4.1 Population genetic parameters

Microsatellite diversity was consistent with the levels of diversity seen in single populations of *A. gracilipes* (Drescher *et al.* 2007; Thomas *et al.* 2010). Microsatellite genetic diversity among sites was low in two loci (*Ano1* and *Ano4*), which had the same 2 alleles in all heterozygotes, and one allele in all queens (Table 2.1). The remaining loci (*Ano3*, *Ano7* and *Ano8*) had four, six and 16 alleles respectively (Table 2.1).

Departures from Hardy-Weinberg (HW) proportions and linkage disequilibrium (LD) were consistent with an unusual reproductive mode. Departures from HW proportions were significant (Table 2.1). Heterozygote deficiency could not be determined for queens for *Ano1* and *Ano4* as these loci were fixed at two alleles, but was significant for *Ano7* and *Ano8*. Heterozygote excess was significant for workers for all loci (Table 2.1). Heterozygote deficiency was significant for sperm for *Ano8* (Table 2.1). Linkage disequilibrium could not be estimated for all 10 pairwise locus combinations, because of a lack of genetic variation in *Ano1* and *Ano4*. Only one comparison was possible for queens, and LD was not significant (P = 1.000). For sperm LD was significant in three of 10 pairwise comparisons: *Ano1* and *Ano4* (P = 0.002); *Ano4* and *Ano8* (P = 0.011); and *Ano1* and *Ano3* (P = 0.036). For workers LD could only be determined for three of 10 pairwise comparisons and was not significant for any of these comparisons.





Table 2.2: Multi-locus genotypic patterns among A. gracilipes queens, sperm, males (and male brood) and workers. Alleles have been encoded as letters for ease of interpretation. A,a = Ano1, B,b = Ano3, C,c = Ano4, D,d = Ano7, E,e = Ano8, -1 = failed to amplify. Lower case denotes alleles typically found in males, male brood, and workers and only occasionally in queens (putatively paternal). Upper case denotes alleles found in queens, workers and sperm (putatively maternal).

		N	Iulti	-locu	ıs ge	noty	pe			Queens	Sperm	Males	Workers
A	A	В	В	С	С	D	D	Ε	Ε	25 ^{1,2,3}	1 1,2,3	11 ^{1,2}	
A	A	В	В	С	С	D	D	Ε	е	2			
A	Α	В	В	С	С	d	D	Ε	Ε	1	5		
Α	Α	В	В	С	с	d	D	Ε	E		1		
Α	Α	В	b	С	С	D	D	Ε	е	1		1	
Α	Α	В	b	С	С	d	D	Ε	Ε		1		
Α	Α	В	b	С	С	D	D	Ε	Ε	10	1		1
а	а	b	b	С	С	d	d	Ε	е			1	
а	Α	В	В	С	С	d	D	Ε	е		5		
а	Α	В	b	С	С	d	D	Ε	Ε		3		
а	Α	В	b	С	С	d	d	Ε	е		1		4
а	Α	В	b	С	С	d	D	Ε	Ε			1	1
а	Α	В	b	С	С	D	D	Ε	Ε		1		
а	Α	В	В	С	С	d	D	Ε	Ε		2		1
а	Α	В	b	С	С	d	D	Ε	е		23^{4}	34 ⁴	303 ⁴
а	Α	В	В	С	С	d	D	Ε	Ε		1	1	
-1	-1	В	В	С	С	-1	-1	Ε	Ε	6			
-1	-1	В	В	С	С	-1	-1	-1	-1	2			
а	Α	В	b	-1	-1	-1	-1	Ε	E		1		
Α	Α	В	В	С	С	D	D	е	е			1	
Α	Α	В	В	С	С	d	D	Ε	е			1	
а	Α	В	В	С	С	D	D	Ε	е			4	
а	Α	В	В	С	С	D	D	Ε	Ε			2	
а	Α	В	b	С	С	D	D	Ε	е			1	
а	Α	В	b	С	С	D	D	Ε	Ε			1	
Α	Α	В	b	С	С	d	D	Ε	е			1	

 ¹ Homozygous multi-locus genotypes with maternal alleles at all loci
 ² Putative haploid male multi-locus genotypes. Heterozygous genotypes are assumed to be diploid

³ Putative haploid sperm multi-locus genotypes with alleles that differed from the genotype of the queen from which they were extracted

⁴ Heterozygous multi-locus genotypes with paternal and maternal alleles at all loci

2.4.2 Genotypic patterns

If the reproduction mode of *A. gracilipes* was a DL GCD system we would have expected to see genotypic patterns among workers that reflected a combination of queen and male genotypes, or complementary queen genotypes. Genotype and allele frequencies did differ considerably among queens, workers, sperm, and male brood. Queens were typically homozygous at most loci (Fig. 2.2; Table 2.2), and shared the same (putatively maternal) allele at each of the two loci (Fig. 2.2). Queens were also never heterozygous for different maternal alleles. Queen MLGs from a single nest were not always identical. In 10 of the 12 nests for which two or more queens were genotyped, queen genotypes differed (e.g., Table 2.3). No queens were homozygous for putatively paternal alleles at any locus, which indicates a single lineage of queens is present in Arnhem Land.

In almost all cases workers harboured both a paternal and maternal allele at all loci. Paternal alleles that were found in workers were rarely found in queens at loci *Ano3*, *Ano7* and *Ano8*. In all nests except BA8 and BY multiple worker MLGs were present, and often worker genotypes could not be assigned back to a queen and / or sperm from the same nest, which suggests that worker exchange among nests is common.

We found no evidence of males belonging to a lineage that corresponded with a putatively paternal lineage (i.e., no males had genotypes that would be expected if paternal MLGs were complementary to maternal MLGs [i.e., *aabbccddee* MLGs, Table 2.2]). Sperm samples displayed genotypic patterns that were similar to workers, typically being heterozygous at all loci, and possessing both maternal and paternal alleles (Fig. 2.2, Table 2.2). More than 60% of males and 90% of sperm genotypes were heterozygous. In addition, all heterozygote genotypes showed a bimodal distribution, with putatively paternal and maternal alleles clearly distinct from each other (Fig. 2.2). Eighteen of 46 sperm samples had alleles that did not match the maternal allele of the queen from which they had been dissected in at least one locus, which indicates the genotype of the mother of the males that produced the sperm differed from the queen genotype from which they had been dissected. The genotypic pattern of male brood and adult males was somewhat similar to that of sperm, although more male genotypes were similar to queen genotypes (Table 2.2).

Table 2.3: Examples of genotypes of males, queens, workers and sperm for three *A*. *gracilipes* nests sampled in Arnhem Land, indicative of the genotypic patterns found among nests.

Site	Туре	A	no1	A	no3	A	no4	A	no7	A	no8
SC3	Male	101	101	162	162	156	156	242	242	212	212
SC3	Queen	101	101	162	162	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Queen	101	101	162	162	156	156	242	242	216	216
SC3	Queen	101	101	162	176	156	156	242	242	216	216
SC3	Sperm	101	101	162	162	156	156	230	242	216	216
SC3	Sperm	101	101	162	176	156	156	230	242	216	216
SC3	Sperm	97	101	162	176	156	156	230	242	216	216
SC3	Sperm	97	101	162	176	156	156	242	242	226	226
SC3	Worker	97	101	162	176	156	174	230	242	216	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
SC3	Worker	97	101	162	176	156	174	230	242	216	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
SC3	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Male	97	101	162	176	156	174	230	242	216	276
YI2	Male	97	101	162	162	156	156	230	242	216	216
YI2	Male	97	101	162	176	156	174	230	244	216	216
YI2	Queen	101	101	162	162	156	156	242	242	214	214
YI2	Sperm	97	101	162	176	156	174	230	242	214	274
YI2	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Worker	97	101	162	176	156	174	230	242	212	274
YI2	Worker	97	101	162	176	156	174	230	242	214	274
YI2	Worker	97	101	162	176	156	174	230	242	216	274
MM3	Queen	101	101	162	162	156	156	242	242	212	212
MM3	Queen	101	101	162	162	156	156	244	244	212	212
MM3	Queen	101	101	162	162	156	156	242	242	212	212
MM3	Sperm	101	101	162	162	156	156	230	242	212	212
MM3	Sperm	97	101	162	176	156	174	230	244	212	272
MM3	Sperm	101	101	162	162	156	174	230	242	212	212
MM3	Worker	97	101	162	176	156	174	230	244	216	274
MM3	Worker	97	101	162	176	156	174	230	242	212	272
MM3	Worker	97	101	162	176	156	174	230	244	212	276

2.4.3 Population structure

The population structure among queens, workers, males and sperm was consistent with the genotypic patterns we observed. The population was divided into two main clusters: one consisting primarily of workers, and the other primarily of queens and males (Fig. 2.3). However, sperm and males were found in both clusters. Samples within sites did not cluster together (Fig. 2.4).



Fig. 2.3: Population structure of *A. gracilipes* queens, workers, sperm and males in Arnhem Land. The hierarchical plot was based on Allele Sharing Distance calculated with Ward's minimum variance algorithm. Queens were found in cluster A, and workers were found in cluster B. Sperm and males were found in both clusters. Details of cluster membership are provided in Fig 2.4.



Fig. 2.4: Detailed population structure of *A. gracilipes* queens (Q), workers (W), sperm (S) and males (M) in Arnhem Land. The hierarchical plot was based on Allele Sharing Distance calculated with Ward's minimum variance algorithm, and shows details of cluster membership. Queens were found in cluster A, and workers were found in cluster B. Sperm and males were found in both clusters. The numbers in brackets summarise the number of ants on that branch for each caste within a site. Site codes refer to the locations in Fig. 2.1.

2.4.4 Worker reproduction

Dissections of workers indicated they were unlikely to be able to reproduce. Our dissections of 470 workers from 13 nests (mean = 36 ± 5 SE) found ovaries in 2% to 15% of workers from seven nests (Fig. 2.5). No spermathecae were found, which indicates the workers did not mate with males. Vitellogenesis, yolky oocytes, and yellow bodies that are characteristic of fertile eggs (Peeters 1987) were not apparent.



Fig. 2.5: Gaster dissection of *A. gracilipes* worker, showing ovarioles with no evidence of yolky oocyte development or yellow bodies.

2.5 Discussion

Recently it has been suggested that the ecological success of some invasive ants may be facilitated by clonal reproduction (Foucaud *et al.* 2009; Kellner and Heinze 2010; Pearcy *et al.* 2011). By contrast, populations of ant species with DL GCD could have reduced chances of invasion success if one lineage is under-represented. Although it has been suggested that *A. gracilipes* reproduction could involve DL GCD (Drescher *et al.* 2007), we found clear evidence to reject this hypothesis in Arnhem Land. We found evidence of only one lineage of queens, to which most male genotypes also belonged. Although we found no physiological or genetic evidence of asexual worker reproduction, we cannot entirely discount this possibility, as workers may reproduce seasonally during times we did not assess.

2.5.1 Dependent lineage genetic caste determination

We found no evidence of multiple lineages of reproductives to support DL GCD. Our sampling covered a very broad area, so that it was likely that we would have discovered at least a few examples of multiple lineages if they existed. If heterozygous workers were produced through DL GCD, we would have expected our data to show three overlapping populations, with homozygous queen genotypes matching each of the alleles found in the heterozygous worker genotypes. These features were not apparent in the population structure of *A. gracilipes* in Arnhem Land. The absence of any queens homozygous for the putatively paternal alleles in workers, despite extensive sampling, strongly suggests that the reproductive mode of *A. gracilipes* does not involve DL GCD. However, the divergent genotypic pattern between workers and queens does suggest that caste determination is genetic, and the microsatellite loci we have used might be strongly linked to a caste-determining locus. The disjoint distribution we found among microsatellite alleles are possible indicators of ancient hybridisation events, or clonal reproduction, but also may reflect the mode of microsatellite evolution.

2.5.2 Potential alternative reproductive modes

The pattern of heterozygous workers, predominantly homozygous queens and often heterozygous males and sperm found in this study is consistent with the results of other studies of this species (Borneo: Drescher *et al.* 2007; Christmas Island: Thomas *et al.* 2010), which suggests that the mode of reproduction of *A. gracilipes* may not vary throughout the invaded range. However, the actual reproductive mode remains unclear. The consistently observed patterns of fixed heterozygote genotypes among workers suggest a number of possible underlying genetic mechanisms, including the strong linkage of microsatellite loci to a caste-determining locus (Volny and Gordon 2002), or asexual reproduction by unmated workers (Balloux *et al.* 2003). Queen genotypes that are primarily homozygous may also indicate asexual reproduction among queens (Simon *et al.* 2003; Pearcy *et al.* 2006), albeit by a different mechanism to workers.

2.5.2.1 Asexual production of workers

Our dissections of workers provided little support for worker reproduction. We found ovaries in a minority of *A. gracilipes* workers, but no spermathecae, which indicates that *A. gracilipes* workers are not capable of sexual reproduction. The presence of ovaries in workers is not sufficient evidence for asexual reproduction because workers in many ant genera produce unfertilised trophic eggs that do not develop (Hölldobler and Wilson 1990), or eggs that develop into haploid males (Bourke and Franks 1995). Despite the presence of ovaries, there were no oocytes or evidence that viable eggs had been laid. However, workers may only reproduce when they are isolated for long periods from the queen (Bourke 1988). Thus, if the ovaries were functional, worker reproduction is either rare, or may occur seasonally differing to our collection time, or only when workers are isolated from queens.

Consistent with the morphological evidence, asexual worker reproduction was also not supported by the genotypic patterns observed. The two possible forms of asexual production of female castes in ants (thelytoky) are ameiotic (apomixis) and meiotic (automixis). Apomixis results in offspring genotypes that are identical to the parent (barring mutations and gene conversions), and overall levels of heterozygosity in the offspring are maintained (Suomalainen *et al.* 1987). By contrast, automixis results in a steep loss of heterozygosity over a few generations (Pearcy *et al.* 2006). Thus, if reproduction by *A. gracilipes* workers occurs by automixis, it must be very rare as we found only five workers with homozygous loci, and only one locus at most was homozygous in any single worker. The alternative mode of clonality, apomixis, would appear to be a more likely mode of parthenogenesis among *A. gracilipes* workers (if it occurs) because apomixis retains heterozygosity and offspring are identical to their mother. Low recombination rates, reflected in departures from HW expectations and significant LD, are typical indicators of asexual reproduction by apomixis (Balloux *et al.* 2003). Although we found departures from HW among workers, the degree of LD was not clearly resolved. In summary, there appear to be too many uncertainties to conclude that workers reproduce asexually. The disjoint bimodal allele distributions among heterozygous workers, together with the variability of loci *Ano3*, *Ano7* and *Ano8* could equally be interpreted as owing to sexual reproduction between queens and males with complementary alleles.

2.5.2.2 Asexual production of queens

Queen genotypes were almost exclusively homozygous with significant heterozygote deficiency, a pattern consistent with asexual reproduction via automixis (Simon *et al.* 2003; Pearcy *et al.* 2006). Automixis causes a rapid loss of heterozygosity over a few generations, but loci distant from the centromere retain heterozygosity (Suomalainen *et al.* 1987; Pearcy *et al.* 2006). We found heterozygote genotypes at the *Ano3*, *Ano7* or *Ano8* loci in 14 of 39 queens. The polymorphism in these loci, and occasional heterozygote genotypes, thus suggests that if automixis was occurring, these loci might undergo recombination. If workers reproduced asexually, the genotypic patterns suggest it would most likely be via apomixis. Thus, it is problematic to interpret how a system would evolve with different modes of asexuality between workers and queens. It seems simpler to conclude that if one of these castes is produced asexually, it is likely the other is not.

2.5.3 The enigma of diploid males

In haplo-diploid species, diploid males are diagnostic of complementary sex determination (CSD) (van Wilgenburg *et al.* 2006). Under CSD, individuals that are heterozygous at one or more sex loci develop into females, while individuals that are haploid or homozygous at one or more sex loci develop into males (Crozier 1971). Diploid males arise owing to a loss of variation at a CSD locus, are an indicator of inbreeding, are typically inviable or sterile, and are therefore rare (Hedrick and Parker 1997; Zayed and Packer 2005). However, a number of recent studies have found that diploid male Hymenopterans (including ants) can mate and produce viable offspring, although workers are triploid as a result (e.g., Liebert *et al.* 2005; Cournault and Aron 2009). If this was the case for *A. gracilipes*, we would perhaps expect to have found genotypes that indicated triploid workers. However, if matched matings occur, the maternal allele of the queen may mask the maternal allele of the diploid male and triploid worker genotypes would not be detectable.

The existence of heterozygous sperm genotypes in our results may not reflect diploid males. An alternative explanation for multiple sperm genotypes from a single queen is the gametes are haploid and that multiple matings occur. This pattern is similar to that seen in DL GCD, where queens mate once with a male of their own lineage and once with a male of the second lineage (Helms Cahan *et al.* 2002; Volny and Gordon 2002). While *A. gracilipes* may not require a second lineage, both paternal and maternal alleles are required to produce queens, workers and males, and caste fate may be controlled by a caste-determining locus. While sperm genotypes may be haploid, and queens may be mated to multiple males, this does not explain the presence of diploid male genotypes, or the origin of paternal alleles in workers and diploid males.

Another explanation for the presence of diploid male genotypes is that males may be mosaics of both maternal and paternal cells. Such sex mosaics (gynandromorphs) have been reported for more than 40 ant species among 22 genera (Jones and Phillips 1985), and matings involving mosaics can produce viable non-mosaic offspring (Yoshizawa *et al.* 2009). Importantly, sex mosaicism is known occur in species with clonal production of males and females (Dobata *et al.* 2012). *Anoplolepis gracilipes* males could thus be

haploid but with cells from both paternal and maternal sources. These haploid males could produce sperm with alleles inherited from either the maternal cells or from the paternal cells. The production of viable offspring, and their caste, could be determined by a combination of male and female alleles. The occurrence of sex mosaics in *A. gracilipes*, together with caste determination based on the allele combination inherited may provide an explanation for the presence of male and sperm genotypes in both worker and queen clusters.

2.6 Conclusion

The range of reproductive systems among Hymenopterans is likely to be far more varied than currently known (Keller 2007; Heinze 2008), and despite the reproductive mode of *A. gracilipes* remaining unresolved, it is clear that when it is determined it will be novel. Of the many uncertainties, the prevalence of diploid males is most problematic. The production of males, and the source and fate of paternal alleles remain enigmatic. However, the resolution of this enigma will also help determine the mode of production of queens and workers. While it is interesting to speculate on the reproductive system based on our data, experimental laboratory nests that allow the control of the parental relationships between individuals are clearly required to understand this unusual reproductive mode. The variation in abundance that we observe in the *A. gracilipes* population in Arnhem Land appears not to be owing to a skew in lineage-ratio in a DL GCD system, although it does appear that caste determination has a genetic basis. Whether the unusual reproductive mode affects the abundance, fitness and invasion success of this ant remains an intriguing question.



Chapter 3. Recent behavioural and population genetic divergence of an invasive ant in a novel environment

3.1 Abstract

Invasive species frequently exhibit high temporal and spatial variation in abundance. Although ecological aspects undoubtedly affect this variation, genetic factors may also play a part. The invasive unicolonial yellow crazy ant Anoplolepis gracilipes exhibits considerable variation in abundance throughout its extensive distribution in Arnhem Land in Australia's Northern Territory, where it was first detected in the 1980s. First, we aimed to determine if A. gracilipes variation in abundance was associated with behavioural and genetic differentiation of the population, and to determine whether one or more introductions occurred. Second, we investigated if the A. gracilipes population was genetically and behaviourally heterogeneous to determine if population divergence has occurred since introduction. Ant abundances were assessed at 13 sites throughout the study region. We used mitochondrial DNA sequences and microsatellite molecular markers to determine population genetic structure, which we correlated with abundance. Behavioural differentiation was assayed using aggression trials and analysed together with genetic data to investigate population divergence. Although we found considerable variation in abundance, we found no association between population structure and differences in abundance. These analyses suggest that A. gracilipes ants in Arnhem Land resulted from a single introduction. The population is not homogeneous, however, as aggression scores varied over both genetic and geographic distance. We also found a positive relationship between genetic and geographic distance. The variation in abundance in the Arnhem Land population of A. gracilipes is clearly not due to invasion by ants from different sources. The genetic and behavioural differentiation we observed is suggestive of incipient genetic and behavioral divergence, which may be expected over time when an invasive species enters in a new environment.

3.2 Introduction

The effects of invasive ant species on the recipient community are often densitydependent; therefore, elucidating the causes of variation in abundance is central to understanding invasive potential. The negative ecological effects of invasive ants on the invaded community typically increase in severity as the abundance of the invader increases (e.g., Macom and Porter 1996; Ross *et al.* 1996; O'Dowd *et al.* 2003; Le Breton *et al.* 2005; Abbott *et al.* 2007; Krushelnycky and Gillespie 2008; McNatty *et al.* 2009). Although ecological aspects undoubtedly affect variation in abundance, genetic factors may also contribute (e.g., Abbott *et al.* 2007). For example, genetic and behavioural differences among yellow crazy ant (*Anoplolepis gracilipes*) populations among islands of the Tokelau archipelago were associated with differences in population densities. More recently invading populations were genetically and behaviourally differentiated from populations that had been in Tokelau longer, and had substantially higher densities (Abbott *et al.* 2007).

The high population densities attained by invasive ants is often associated with unicoloniality. Although not all unicolonial ants are invasive, the ants listed among the 100 most damaging invasive species (Lowe *et al.* 2000) are unicolonial (Helanterä *et al.* 2009). Unicolonial ant species form supercolonies that exhibit behavioural and genetic differentiation between supercolonies, but no genetic or behavioural differentiation (i.e., aggression) within individual supercolonies (Pedersen *et al.* 2006). At the most extreme, individual supercolonies of the Argentine ant (*Linepithema humile*) that span different continents are entirely behaviourally, genetically and chemically undifferentiated, despite over 100 years of separation (van Wilgenburg *et al.* 2010). Not all Argentine ant supercolonies are undifferentiated, however. Ants from the Corsican supercolony are chemically and behaviourally different from their source supercolony on the European mainland, despite only 60 years of separation. The genetic relationships between the two colonies have not been investigated, however (Blight *et al.* 2010).

Populations of *A. gracilipes* present as mosaics of genetically and behaviourally differentiated supercolonies (Abbott *et al.* 2007; Drescher *et al.* 2007; Drescher *et al.* 2010). In fact, Drescher *et al.* (2010) suggested that the divergence between *A. gracilipes* supercolonies in Borneo may have resulted in incipient speciation. Although Drescher *et al.* (2010) focussed on divergence between, rather than within supercolonies, they extended their discussion to divergence within colonies that may occur in species where mating is intranidal (within the nest). This scenario entails fragmentation of the colony followed by spatial segregation. Isolation would result in population divergence through genetic drift, and the homogenising effects of gene flow would be eliminated as mating is locally restricted. As the origin of supercolonies may be a developmental process rather than an evolutionary one (Helanterä *et al.* 2009), the breakdown of individual supercolonies may occur in an observable timeframe, and be more common than has been reported to date.

Populations of *A. gracilipes* vary in size and density (Lewis *et al.* 1976; Hill *et al.* 2003; Abbott 2005; Abbott *et al.* 2007; Drescher *et al.* 2011). The native range of this ant is undetermined (Wetterer 2005), so its natural patterns of abundance, colony structure, and influence on the native ant community outside the invaded range are not known. It is clear, however, that in the invaded range its population densities, behaviour, colony structure, and ecological effects vary (Lewis *et al.* 1976; Hill *et al.* 2003; Abbott 2005; Abbott *et al.* 2007; Drescher *et al.* 2007; Drescher *et al.* 2011). Although habitat and ecological interactions fundamentally influence differences in abundance (O'Dowd *et al.* 2003), genetic differences may also be a contributing factor (Abbott *et al.* 2007). Understanding of the links between population density, behaviour and genetic population structure in *A. gracilipes* would improve our knowledge of its invasive potential, and the nature of unicoloniality in this species.

In Arnhem Land in Australia, *A. gracilipes* has a fragmented distribution over a wide region, and also exhibits varying nest and population densities. Unlike *A. gracilipes* invasions in Samoa (Savage *et al.* 2009) and on Christmas Island (O'Dowd *et al.* 2003), carbohydrate resources are not an obvious driver of differences in abundance (authors' unpublished data), nor are there clear associations with anthropogenic disturbance (Hoffmann and Saul 2010). Our study focuses on the relationships between abundance

and population differentiation of *A. gracilipes* in Arnhem Land. First, we test the hypothesis that spatially segregated populations with different densities are also behaviourally and genetically differentiated. Second, we investigate if Arnhem Land's *A. gracilipes* colony or colonies are genetically and behaviourally heterogeneous to test the hypothesis that divergence within colonies has occurred, and if divergence is related to patterns of abundance.

3.3 Methods

3.3.1 Study site

Arnhem Land is located in the Australia's Northern Territory. The regional climate is tropical monsoonal with temperatures ranging between 17 and 33°C throughout the year, and seasonal rainfall (December to July) of approximately 1200 mm. The vegetation is primarily savanna woodland. Although it is not known when *A. gracilipes* arrived in the region, it was first detected in 1982 (Majer 1984). Thorough surveys in 2009 found the ant to be patchily distributed throughout 16,000 km² in Arnhem Land (Fig. 3.1).

Sites throughout the geographical range of *A. gracilipes* in Arnhem Land were chosen in October 2007 based on our preliminary observations of differing population densities and abundance, and ecological similarity (a canopy dominated by *Eucalyptus tetrodonta*, an understorey primarily of *Acacia* spp., grasses and leaf litter, and similar drainage and topography). These preliminary observations were achieved through extensive surveys of the region, where all occurrences of the ant were recorded (Fig. 3.1).

3.3.2 Sampling

Approximately 30 workers from a single nest at each of seven sites were collected in October 2007 for genetic analyses (Fig. 3.1). Based on these earlier observations, between July 6 and 17 2009 we made additional collections of 100 - 200 workers from a single nest at 13 sites for genetic and behavioural analyses (Fig. 3.1). We recorded the location of each site using a GPS (Garmin), and measured the distance between sites using ArcMap v 9.3 (Esri). To test for genetic and behavioural differences relative to population densities, we measured the abundance of *A. gracilipes* at each site based on forager activity.

Counts of ant abundance were conducted before 10am when ambient temperatures do not prevent A. gracilipes from foraging. The site level abundance of A. gracilipes was assessed using card counts, which measure forager activity using a count of the number of ants crossing a white 10×10 cm laminated card (Green *et al.* 2004). At each site we measured A. gracilipes activity at 11 stations spaced 5 m apart on 50 m transects, with three replicate transects spaced 10 m apart. At each station the vegetation was scraped away to reveal bare ground. A 20×20 cm laminated card, divided into four quadrants, was placed on the ground and observed for 20 s. The first quadrant crossed by an A. gracilipes worker was used as the focal quadrant, and for the following 30 s all ants crossing that quadrant were counted. If no ants were observed on the card during the first 20 s, the first quadrant visited by an ant in the following 30 s was used. An individual walking across the quadrant multiple times was counted only once. Counts at all stations within a transect were summed, and the mean value of the three replicate transects was used as an index of relative abundance between sites. We also calculated the coefficient of variation (CV) of card counts as a measure of variability in abundance within and among sites.



Fig. 3.1: Anoplolepis gracilipes distribution and sample sites in Arnhem Land in the Northern Territory, Australia. Pale circles (\bigcirc) represent the known extent of the invasion at the time of sampling. Black circles (\bigcirc) are sites sampled in October 2007 for analysis of population structure. Black squares (\blacksquare) represent the 13 sites sampled in July 2009 for genetic analysis and behavioural assays. Numbers below site codes are the number of ants genotyped for the site.

3.3.3 Genetic analyses

Ants collected for genetic analysis were stored in 95% ethanol at 4°C. We extracted DNA using a modified Chelex protocol (Sepp *et al.* 1994). Individual workers were placed in microcentrifuge tubes, ground with sterile plastic pestles, and 150 μ l of a 10% w/v Chelex-100 resin solution was added. The tubes were centrifuged briefly, boiled for

15 minutes in a water bath, chilled on ice for five minutes, and centrifuged at $15,000 \times g$ for 15 minutes at 4°C. The supernatant containing DNA was stored at 4°C.

We used mitochondrial markers to determine if multiple haplotypes were present, targeting the *COI* (cytochrome oxidase I) region. We sequenced one ant per site with the exception of Y3 (one ant from each of five nests), G2, K4, M2, and K1 (one ant from each of two nests). PCR reagents and thermal cycling were the same as microsatellite analysis (see below), but with equal concentrations of only forward and reverse primers (CI-13 and CI-14, Quek *et al.* 2004), and with 40 cycles using a 40°C annealing temperature. Amplified products were purified using ExoSAP-IT (US Biochemicals) and sequenced on a 3730 Genetic Analyser (Applied Biosystems).

Microsatellite markers were used to assess the population genetic structure, genetic distance and relatedness between individuals within and among sites. Workers were genotyped using seven microsatellite loci: Ano1, Ano3, Ano4, Ano5, Ano7, Ano8 and Ano10 (Feldhaar et al. 2006), that are polymorphic in other populations (Drescher et al. 2007; Drescher et al. 2010; Thomas et al. 2010). Each 15 µl PCR consisted of ~20 ng template DNA, $10 \times$ PCR Buffer, 0.4 µg mL⁻¹ of bovine serum albumin (BSA), 1.5 - 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.1 mM forward primer (adapted with M13 tags, Schuelke 2000), 0.4 mM reverse primer, 0.4 mM universal fluorescent dye-labelled M13 primer (Schuelke 2000), and 0.3 U of Tag DNA Polymerase (either Bioline BIOTAQ, Invitrogen Platinum® or New England Biolabs). Fluorescent dyes used were FAM (Ano4, Ano5 and Ano8) and VIC (Ano1, Ano3, Ano7 and Ano10). Polymerase Chain Reactions were performed using the thermal cycling conditions specified by Feldhaar et al. (2006), with modification for M13 primers (Schuelke 2000). Amplified products were analyzed on a 3730 Genetic Analyzer using the LIZ size standard, and visualised and scored using Genemapper v 3.7 (both Applied Biosystems). Between 10 and 35 workers were genotyped per site (mean \pm SE = 23 \pm 1.58, n = 460). We used Micro-Checker v 2.2.3 (van Oosterhout et al. 2004) to validate genotyping quality (scoring errors, null alleles, and large allele dropout), and none of these features were observed in our dataset.

Hardy Weinberg (HW) proportions were tested using GENEPOP v 4.0.10 (Raymond and Rousset 1995). High relatedness among nestmates suggests non-independence of genotypes, and leads to pseudoreplication issues in HW analyses. Our relatedness estimates were relatively high (Table 3.1), so to be conservative we corrected for pseudoreplication by using a single randomly selected ant per site in our HW tests.

To estimate population structure we used STRUCTURE v 2.3.3 (Pritchard et al. 2000), together with a principal components analysis in PCAGEN (Goudet 2000). Unlike STRUCTURE, PCAGEN makes no assumptions about HW or linkage. We ran 15,000 permutations within PCAGEN to obtain significance values for the variation explained by each axis. STRUCTURE uses a Bayesian Markov Chain Monte Carlo (MCMC) method to assign individuals to one or several genetic clusters (K). We performed 10 STRUCTURE runs for values of K between one and 20, using a model that assumed admixture and correlated allele frequencies, with a burn-in of 100,000 simulations and 1,000,000 subsequent simulations. The best estimate of K was based on the run with the highest log likelihood, and confirmed using the ΔK method (Evanno *et al.* 2005). Using PCAGEN it is only possible to discriminate populations if samples clearly cluster together, and the ΔK method is able to discriminate two or more genetic clusters only. As we had 20 sites and also wished to determine if this was a single supercolony (i.e., K = 1), to determine the number of clusters we also used FLOCK v 2.0 (Duchesne and Turgeon 2009). We followed the FLOCK software guidelines and used 50 runs, increasing K until plateau lengths were zero for more than four consecutive values of K, or plateau lengths were ≥ 6 . In FLOCK analyses, if a plateau length of six is not reached, a single genetic cluster is assumed (i.e., K = 1).

To investigate genetic divergence among sites we tested for genetic isolation by distance (Slatkin 1993) using 10,000 permutations of the relationship between Nei's Distance and geographic distance with a Mantel test in the 'ade4' package (Dray and Dufour 2007) in R (R Development Core Team 2011). We also tested this relationship with Spearman's rank correlation, as the Mantel test has less power and is therefore prone to Type II errors (Legendre and Fortin 2010).

nd mean aggression scores (upper right diagonal, italic font) of within and between site	own in a single cell on the diagonal. Each cell refers to a single trial with the exception the	aggression score of trials is presented.
Table 3.1: Relatedness r values (lower left diagonal, plain font) and mean aggression scores (upper right di	behavioural trials of A. gracilipes workers. Within site values are shown in a single cell on the diagonal. Each c	cells marked * (two trials), and ** (three trials), for which the average aggression score of trials is presented.

	A1	A 2	B 1	B2	K 4	D2	D3	D4	G1	G2	M2	P1	P2
A1	0.81/1.10	ı	ı	ı	1.5	1.67	ı	0.97	,	ı	·	ı	ı
A 2	ı	0.92/1.23	I	I	I	ı	I	ı	ı	ı	ı	I	ı
B1	ı	ı	0.51/0.97	1.13	1.27	1.13	ı	ı	1.07	ı	2.03	ı	ı
B 2	ı	ı	0.51	0.53/1.07	ı	ı	1.27	,	·	ı	,	1.23	ı
K4	0.51	ı	0.54	ı	*0.61/0.92	0.83	0.90	*1.09	2.27	ı	0.97	ı	1.53
D2	0.85	ı	0.4	ı	0.52	**0.93/0.86	ı	ı	1.43	ı	1.10	ı	ı
D3	ı	ı	I	0.4	0.44	ı	0.49/0.80	ı	1.10	ı	ı	0.97	1.47
D4	0.61	I	I	ı	*0.56	ı	I	0.57/0.87	ı	ı	ı	ı	ı
G1	I	ı	0.49	ı	0.52	0.26	0.36	ı	0.69/0.80	1.13	1.10	1.20	ı
G2	ı	I	I	ı	I	ı	I	ı	0.61	0.66/1.23	ı	ı	ı
M2	ı	ı	0.53	ı	0.68	0.74	I	ı	0.43	ı	06.0/06.0	ı	ı
Ы	ı	·	·	0.34	ı	ı	0.60	·	0.31	ı	ı	0.84/0.80	1.13
2	ı	ı	ı	ı	0.51	ı	0.52	ı	ı	ı	·	0.72	0.90/0.83

To determine whether assignment of individuals to the genetic clusters identified by STRUCTURE had a geographical relationship, we used Spearman's rank correlation. We compared the relationship between proportional cluster assignments with distance along a north south axis. We used the northernmost site as a reference as this is the approximate location of a port, airport and only major human settlement, so could have been the initial invasion point. A Spearman's rank correlation was used to determine if the proportional cluster assignment of individuals was related to abundance. The standard error of mean abundance increased as abundance increased (Table 3.2). To test if there was a positive relationship between variability in abundance and genetic differentiation we used Spearman's rank correlation to compare the coefficient of variation and proportional cluster assignment.

Table 3.2: Abundance and relatedness estimates for *A. gracilipes* from the 13 sites included in behavioural trials. Abundance, mean \pm SE and co-efficient of variation (CV), measured using card counts. Values of zero reflect non-detection due to low density, rather than absence; N, number of individuals genotyped; Wang's *r*, mean within nest relatedness \pm SD (range).

Site	Abundan	ce	Ν	Wang's r
	Mean	CV		
A1	42.3 ± 9.0	2.8	31	$0.81 \pm 0.13 \; (0.30 - 1.00)$
A2	3.0 ± 0.3	3.4	18	$0.92 \pm 0.07 \; (0.85 - 1.00)$
B1	38.7 ± 3.8	1.3	32	$0.51 \pm 0.28 \; (0.24 - 1.00)$
B2	35.0 ± 3.1	1.2	29	$0.53 \pm 0.26 \; (0.26 - 1.00)$
K4	15.0 ± 1.8	1.2	28	$0.61 \pm 0.23 \; (0.19 - 1.00)$
D2	17.0 ± 1.6	1.2	24	$0.93 \pm 0.15 \; (0.74 - 1.00)$
D3	1.0 ± 0.2	3.2	27	$0.49 \pm 0.30 \; (0.30 - 1.00)$
D4	6.3 ± 1.0	2.2	25	$0.57 \pm 0.27 \; (0.30 - 1.00)$
G1	3.7 ± 0.3	2.3	35	$0.69 \pm 0.20 \; (0.30 - 1.00)$
G2	3.3 ± 0.2	1.9	22	$0.66 \pm 0.21 \; (0.30 - 1.00)$
M2	0.0 ± 0.0	0.0	25	$0.90 \pm 0.10 \; (0.79 - 1.00)$
P1	0.3 ± 0.1	5.7	19	$0.84 \pm 0.11 \; (0.30 - 1.00)$
P2	2.3 ± 0.4	2.8	30	$0.90 \pm 0.07 \; (0.30 - 1.00)$

Genetic diversity measures (observed and expected heterozygosities, allele frequencies) were calculated using GenAlEx v 6.3 (Peakall and Smouse 2006), and allelic richness was calculated using HP-RARE (Kalinowski 2005). We estimated relatedness using Wang's moment estimator of relatedness (r) implemented in MER v 3.0 (Wang 2002). Wang's r is comparable to other indices of relatedness but is a more reliable measure for biallelic loci, where allele frequencies are 0.5 or sample sizes are small, regardless of the allele frequency distribution, or number of alleles per locus (Wang 2002). Wang's r ranges from 0 – 1, although negative values are possible where individuals are unrelated. Relatedness values of 1 indicate identical multilocus genotypes. We conducted pairwise comparisons among all individuals, with 1000 bootstraps over loci, and then pooled individual results for each site. Pairwise genetic distances between sites were estimated using Nei's Distance (Nei and Roychoudhury 1974) calculated in GenAlEx v 6.3 (Peakall and Smouse 2006).

3.3.4 Behavioural assays

Behavioural assays were conducted to investigate the relationship between intraspecific aggression and geographic and genetic distance. Ants used in behavioural trials were collected from a single nest at each of 13 sites in July 2009 (Fig. 3.1; Table 3.1). Ants were kept at $\sim 23^{\circ}$ C, with a damp tissue for water but no food. Assays were completed within 72 hours of the ants being collected. We assessed aggression among pairs of sites (Table 3.1), using three replicates of five ants from each site for each trial, and controls of ants from the same site. The use of five ants ensures more consistent results across replicates than assays using a single ant (Roulston et al. 2003). Trials were conducted in a fluon-coated arena of 10 cm diameter with a removable barrier bisecting the arena. The arena floor was lined with a filter paper disc that was replaced after each replicate. Unmarked ants from two sites were placed into respective sides of the arena. Once ants had settled, after approximately 30 seconds, the divider was removed. A scan based approach was used to assess ant behaviour, checking the arena for 5 - 10 seconds every minute over a 10 minute period and recording the highest interaction score. We conducted 45 aggression trials of three replicates each (Table 3.1) over 22.5 hours of observation. As the trials were conducted over a period of two weeks, trials were repeated for some sites (Table 3.1).

Interactions were scored on a five point scale modified from Suarez *et al.* (1999): 0 = "Ignore" was a contact in which neither showed interest; 1 = "Contact" included antennation; 2 = "Avoidance" was a contact resulting in one or both ants retreating; 3 = "Aggression" consisted of biting, pulling, mouth gaping, twitching; 4 = "Fighting" included lengthy aggression, carrying, or grappling. Aggression scores between 0 and 2 were considered non-aggressive, and above 2 were considered aggressive. The mean score for each replicate was averaged to give a mean aggression score for each trial. Ants from each site were retained for genetic analyses and were processed as described earlier.

The relationships between aggression scores and geographic or genetic distance were analysed using Spearman's rank correlation implemented in R. We tested the relationships between mean aggression score and geographic distance (kilometres between sites), and genetic distance (Nei's Distance). To test if levels of aggression differed in trials within and between sites, we used Wilcoxon rank sum tests (with Monte-Carlo resampling to estimate P values), implemented in the 'coin' package in R (Hothorn *et al.* 2008). We applied a sequential Bonferroni correction (Rice 1989) across all statistical tests, and report Bonferroni corrected P values.
3.4 Results

3.4.1 Genetic differentiation

Our population genetic analyses indicated that A. gracilipes in Arnhem Land were derived from a single population, although the population structure was not homogeneous. Mitochondrial analyses of 28 samples found sequences of 533 base pairs of a single haplotype (GenBank Accession JN375978). The STRUCTURE analysis identified two genetic clusters using the ΔK method, which also had the highest mean log likelihood of -6267.04 (-6390.40 for K = 1 and -6533.50 for K = 3). STRUCTURE detects genetic clusters in the data and assigns individuals to these clusters based on genotypes. If there is strong differentiation, sites and clusters should correspond to each other. No differentiation is detectable if an individual is assigned equally to each cluster. Our results showed that although the clusters were not identically correlated with sites, individuals within a site typically assigned to one cluster more than the other (Fig. 3.2(a), (b)). The clusters identified by STRUCTURE followed an approximate north-south distribution (Fig. 3.2(b)). Individuals from the northern and eastern sites were assigned mostly to a single cluster (white; Fig. 3.2(b)). Individuals from the most southern sites were generally, but not always, assigned more often to the second cluster (grey; Fig. 3.2(b)). The presence of both clusters at all sites is consistent with a recent shared history or contemporary gene flow. A clinal pattern of genetic divergence was suggested by a decline in the proportion of individuals in a site assigning to the white cluster as distance from the northernmost site increased (Spearman's $r_s = 0.71$, S = 385.64, P = 0.001).



Fig. 3.2: Population genetic structure of *A. gracilipes* in Arnhem Land. (a) STRUCTURE identified two genetic clusters. Each individual ant is represented by a thin horizontal bar. The amount of each colour (grey or white) indicates the proportion of each inferred cluster to which the individual was assigned. Sites are presented in order of increasing abundance, followed by sites B3, D6, K1, K5, K6, T1, and Y3 for which abundance was not measured. Individuals within a site were typically assigned to both genetic clusters. (b) Geographical representation of the genetic clusters identified by STRUCTURE analysis. Pie graphs show the proportional assignment of individuals from each site to each cluster. The white cluster was more prevalent in the north, while the grey cluster was generally more prevalent in the west and south, a pattern that was significant (Spearman's $r_s = 0.71$, S = 385.64, P = 0.001). (c) Principal components analysis in PCAGEN revealed no strong clustering of sites. PCA axis 1 explains 38 % of the variance (P = 0.073), and axis 2 explains 26 % (P = 0.014).

The first two components of the PCAGEN analysis explained 64% (axis 1 = 38 % of variance [P = 0.073]; axis 2 = 26% [P = 0.014]) of the variability in the data and showed no definitive clustering of sampling sites (Fig. 3.2(c)). Consistent with this absence of defined population structure, the FLOCK analysis found plateau lengths of zero for values of 2 < k < 6, and plateau lengths of 3 and 2 for k = 2, indicating the best approximation of K is 1 (i.e., a single population). However, we found a significant pattern of genetic isolation by distance between sites included in the behavioural assays (Fig 3.3(a); Mantel test, r = 0.37, P = 0.002; Spearman's $r_s = 0.57$, S = 1725.96, P = 0.003), which indicates genetic divergence within the population. When all sites were included, the relationship was not significant using the Mantel test (Fig. 3.3(b); r = -0.04, P = 0.959), but was significant when tested using Spearman's rank correlation ($r_s = 0.23$, S = 875217.50, P = 0.003).

3.4.2 Genetic diversity



Fig. 3.3: Relationship between Nei's distance and geographical distance among *A. gracilipes* sites included in behavioural analyses only (a) and all sites (b), with Mantel correlation coefficients (r_s) and their respective significance values (P). The dashed lines indicate the line of best fit under a linear model.

Estimates of genetic diversity were consistent with a single supercolony. We found low genetic diversity relative to other *A. gracilipes* studies of multiple supercolonies (Drescher *et al.* 2007; Thomas *et al.* 2010) in six of the seven microsatellite loci used. Genetic diversity estimates found between two and 17 alleles per locus, and rarefied allelic richness of between 2.00 and 8.29 alleles per locus per site. All individuals had the same two alleles for the *Ano1*, *Ano4*, *Ano5* and *Ano10* loci. All loci except *Ano8* (the most diverse locus) exhibited significant heterozygote excess (Table 3.3), consistent with prior findings for this species (Drescher *et al.* 2007; Thomas *et al.* 2010).

Relatedness values within and between sites were consistent with a single supercolony. Mean relatedness within sites was 0.73 ± 0.04 SE (range: 0.49 - 0.93, Table 3.1). Mean relatedness between sites was 0.53 ± 0.02 SE (range: 0.26 - 0.93, Table 3.1). In three cases, relatedness within sites was lower than relatedness between sites (Table 3.1). Within individual sites, relatedness between individuals spanned a wide range, typically from 0.30 to 1.00 (Table 3.2).

Table 3.3: Microsatellite marker diversity estimates for *A. gracilipes* workers from 20 sites. N, number of individuals genotyped; Alleles, number of alleles detected per locus; AR₁₀, Allelic richness adjusted to the smallest sample size (n = 10); H_E , expected heterozygosity; H_O , observed heterozygosity; P_{HW} , significance values for tests of Hardy Weinberg proportions (heterozygote excess).

Locus	Ν	Size range (base pairs)	Alleles	AR ₁₀	H_0	H_{E}	P _{HW}
Anol	475	97-101	2	2.00	1.00	0.50	< 0.001
Ano3	475	162-178	5	2.64	1.00	0.54	< 0.001
Ano4	475	156-174	2	2.00	1.00	0.50	< 0.001
Ano5	475	116-118	2	2.00	1.00	0.50	< 0.001
Ano7	475	230-248	7	3.22	0.99	0.61	< 0.001
Ano8	475	208-284	17	8.29	0.93	0.87	0.083
Ano10	475	234-242	2	2.00	1.00	0.50	< 0.001

3.4.3 Behavioural differentiation in relation to population genetic structure

Overall aggression scores were relatively low (generally less than the aggression threshold of 2.00), although aggressive behaviours were observed. These aggressive behaviours typically included gaping of mandibles, twitching, and in one case, one ant subduing and carrying another. The mean aggression scores between ants from the same site did not exceed the aggression threshold, and ranged from 0.70 to 1.23 (Table 3.1; Fig. 3.4). The aggression scores in trials between ants from different sites ranged from 0.83 to 2.27, slightly exceeding the aggression threshold in three instances (Fig. 3.4). Aggression scores were significantly lower within sites than between sites (Fig. 3.4; Wilcoxon rank sum test, Z = -3.80, P < 0.001). Mean aggression scores were also positively correlated with genetic and geographic distance.



Fig. 3.4: Boxplot comparing aggression scores of *A. gracilipes* workers within and between sites. Aggression scores were significantly higher between than within sites (Wilcoxon rank sum test, Z = -3.80, P < 0.001). The rectangle shows the interquartile range ($25^{th} - 75^{th}$ percentile), the bold line is the median, the whiskers indicate the most extreme data points within 1.5 times the interquartile range from the median, and the open circles represent outliers (points greater than 1.5 times the interquartile range).

Bonferroni corrected Spearman tests were significant for Nei's Distance (Fig. 3.5(a); r_s = 0.55, S = 6896.92, P < 0.001), and geographic distance (Fig. 3.5(b); $r_s = 0.57$, S = 0.6467.50, P < 0.001). The most genetically or geographically distant sites were not the most behaviourally differentiated (Fig. 3.5). Although a few high scoring points drove the differences in aggression scores, the lowest scores were from within site comparisons.

3.4.4 Abundance in relation to population genetic structure

We observed considerable variation in abundance within and among sites (Table 3.2). The mean number of ants observed on card count transects ranged from zero at site M2 (i.e., ant numbers were too low for detection using card counts) to 42.3 at site A1 (Table 3.2). The co-efficient of variation of card counts within sites was 2.5 (range: 0 - 5.7, Table 3.2), and among all sites was 3.6.



Fig. 3.5: Relationships between aggression score and Nei's distance (a) and geographical distance (b) among nests of *A. gracilipes*. Spearman's correlation co-efficients (r_s) and significance values (*P*) of tests are shown for each plot. The dashed lines indicate the line of best fit under a linear model. Aggression scores higher than 2.0 were considered aggressive.

STRUCTURE, PCAGEN and FLOCK analyses indicated no strong genetic differentiation between sites of lower and higher abundance. The two genetic clusters identified by STRUCTURE were not associated with differences in abundance (Fig. 3.2(a)), and we found no relationship between cluster assignment and abundance (Spearman's $r_s = -0.34$, S = 490.17, P = 0.246). We also found no relationship between the variation in abundance within sites (CV) and genetic differentiation (Spearman's $r_s = -0.55$, S = 162, P = 0.079).

3.5 Discussion

3.5.1 The relationship between population structure and abundance

The invasion of A. gracilipes in Arnhem Land appears to consist of a single supercolony. We found no association between differences in abundance and population genetic structure. Relatedness within and between sites was high, which is also indicative of a single supercolony, as high relatedness is only observed within supercolonies of A. gracilipes (Drescher et al. 2007; Thomas et al. 2010). Relatedness within sites was similar to within colony relatedness found in other A. gracilipes studies (0.37 - 1.00, Drescher et al. 2007; 0.55 - 1.00, Thomas et al. 2010). In contrast, relatedness between sites was higher than other studies, in which between colony relatedness was effectively zero (Drescher et al. 2007; Thomas et al. 2010). Moreover, analysis of mitochondrial DNA shows that A. gracilipes in Arnhem Land share the same haplotype, which provides further corroboration that this is genetically a single population, as individual supercolonies of this species share a single or few haplotypes (Drescher et al. 2007; Thomas et al. 2010). The population genetic differentiation we observed was not correlated with the variation in abundance that we observed within or between sites. The factors responsible for variation in abundance in the Arnhem Land population of A. gracilipes remain unclear, but the variation is clearly not due to invasion by ants from different source populations.

3.5.2 Divergence of Anoplolepis gracilipes in Arnhem Land

Although we found that *A. gracilipes* in Arnhem Land has the genetic and behavioural characteristics of a single supercolony, and thus likely stemmed from a single source, the population is heterogeneous. In most tests we found higher genetic differentiation between distant sites, and higher aggression scores between sites than within sites. In contrast, many supercolonies of other invasive ants such as the *L. humile* show little or no intra-colonial genetic or behavioural differentiation (Giraud *et al.* 2002; Tsutsui *et al.* 2003b; Corin *et al.* 2007; Suhr *et al.* 2009), even in supercolonies that span multiple continents (van Wilgenburg *et al.* 2010). Similarly, workers of the big headed ant, *Pheidole megacephala* display little genetic or behavioural differentiation, and an absence of isolation by distance across much of the Australian continent (Fournier *et al.* 2009). A single apparent exception to this homogeneity is that of the Corsican colony of *L. humile*, which does appear to have differentiated from the main European colony (Blight *et al.* 2010).

Our results support the scenario proposed by Drescher *et al.* (2010) to explain divergence within *A. gracilipes* supercolonies. This scenario entails fragmentation of the colony followed by spatial segregation, which results in population differentiation over time. The mechanism assumes that intranidal mating prevents the homogenising effects of gene flow. Although the mode of reproduction of *A. gracilipes* is unconfirmed, relatively high within nest relatedness in the species suggests that mating is intranidal (Drescher *et al.* 2010). Flighted queens have not been observed in Arnhem Land (B. Hoffmann, personal communication), and as relatedness within sites is relatively high, it is likely that mating is intranidal in Arnhem Land. The population of *A. gracilipes* in Arnhem Land appears to be in the very early stages of divergence. Given sufficient time and spatial segregation we expect the differentiation of *A. gracilipes* in Arnhem Land to eventually resemble *A. gracilipes* in Borneo where divergence between populations has resulted in complete behavioural and genetic isolation (Drescher *et al.* 2010).

Recent studies suggest that the abandonment of aggression is a plastic trait (Foitzik *et al.* 2007; Steiner *et al.* 2007; Cremer *et al.* 2008; Helanterä *et al.* 2009; Martin *et al.* 2011). Although we do not know the behavioural characteristics of the source population, aggression scores above 1.00 have not been observed between ants from the same supercolony in *A. gracilipes* (Abbott 2005; Abbott *et al.* 2007), nor has within colony aggression been detected using other methods (Drescher *et al.* 2007; Drescher *et al.* 2010). Thus, the three instances of mild aggression we recorded suggest a partial recovery of nestmate discrimination in *A. gracilipes* in Arnhem Land. The results of Drescher *et al.* (2010) together with the genetic and behavioural divergence we observed suggests unicoloniality may be a plastic trait in this species.

3.5.3 Nestmate recognition and aggression

We have assumed that the variation in behaviour that we found is a result of genetic differentiation, however a number of different factors can affect behaviour among ants. Nestmate recognition and aggressive behaviour are dependent on cuticular hydrocarbon profiles that define recognition cues (Vander Meer and Morel 1998). Nestmate recognition is affected by cuticular hydrocarbon similarity (Suarez *et al.* 2002), which in turn can be affected by diet (Silverman and Liang 2001; Corin *et al.* 2007). Although we cannot entirely discount the effect of diet or other environmental aspects, we selected sites based on ecological similarity to minimise the effects of the environment on our results. In addition, genetic, chemical and behavioural differentiation are correlated in geographically diverse *A. gracilipes* populations in Borneo (Drescher *et al.* 2010). The correlations found by Drescher *et al.* (2010) indicate recognition in *A. gracilipes* has a genetic association. We do acknowledge, however, that environmental cues may have also contributed to the results of our behavioural assays.

A loss of the cues needed for nestmate recognition has been cited as causing the lack of aggression among invasive Argentine ants, and a potential mechanism for the evolution of unicoloniality in the species (Holway *et al.* 1998; Tsutsui *et al.* 2000; Giraud *et al.* 2002). Recently, it has also been suggested that abandoning aggression while retaining nestmate discrimination may be an early stage in the establishment of unicolonial populations, as it enables tolerance of worker exchange among nests (Steiner

et al. 2007), which is a cornerstone of unicoloniality (Helanterä *et al.* 2009). Although aggression scores in our study typically did not exceed the aggression threshold, scores were clearly higher between ants from different sites, indicating that *A. gracilipes* in Arnhem Land may discriminate non-nestmates from nestmates. A tolerance of worker exchange within the supercolony is implied by our finding that relatedness within sites was occasionally lower than between sites. Our genetic evidence for tolerance of worker exchange is also consistent with the worker interchange that is observed between *A. gracilipes* nests in the field (B. Hoffmann, unpublished data; Abbott 2005).

3.6 Conclusion

Our population structure and behavioural analyses suggest that the numerous and spatially discrete occurrences of *A. gracilipes* ants in Arnhem Land are genetically and behaviourally a single supercolony, and likely stemmed from a single introduction. The variation in abundance in this population is not associated with population genetic differentiation. Unlike the majority of supercolonies of other invasive ants, the *A. gracilipes* supercolony in Arnhem Land is neither genetically nor behaviourally homogeneous. The incipient population differentiation in Arnhem Land, together with the complete differentiation found between supercolonies of the ant in Borneo (Drescher *et al.* 2010) indicates that colony divergence in this species may be a common occurrence. We speculate that such divergence within ant species may be more common than has been observed to date, as the timescale of divergence may differ greatly among and within species.

Chapter 4. Higher genetic diversity is associated with finescale momentary abundance of an invasive ant

4.1 Abstract

Many introduced species become invasive despite genetic bottlenecks that reduce the genetic diversity of the population, and thus decrease the chances of persistence. By contrast, population genetic bottlenecks have been hypothesised to facilitate the ecological success of unicolonial ants by increasing the genetic similarity between descendent populations, and promoting co-operation between unrelated ants. We investigated these contrasting hypotheses in the yellow crazy ant Anoplolepis gracilipes, which has invaded Arnhem Land in Australia's Northern Territory, and exhibits variation in abundance in the region. We used abundance as a surrogate measure of short-term invasion success, and investigated the relationship between A. gracilipes genetic diversity and variation in abundance, and the density-dependent effects of the ant on native ant species diversity and community structure (i.e., ecological success) at a fine-scale. We also investigated whether selected habitat characteristics contributed to differences in A. gracilipes abundance. Our results revealed a significant positive association between measures of A. gracilipes genetic diversity and abundance. Ant communities invaded by A. gracilipes were less diverse and differed in structure from uninvaded communities, and these effects were positively density-dependent. Although higher genetic diversity may benefit individual nest clusters, the underlying mechanisms, the direction of the relationship between abundance and genetic diversity, and the implications for longer term invasion success on the wider population are unclear. Although the effects of A. gracilipes on the invaded ant community in Arnhem Land appear to be densitydependent, the variability in A. gracilipes abundance may be unlikely to have significant effects on regional native ant diversity over longer timescales. The relative importance of genetic diversity to A. gracilipes abundance may be context-dependent, and genetic diversity may be more important in a specific environment if novel ecological characteristics have less benefit.

4.2 Introduction

The success of invasive species is an apparent genetic paradox, because the loss of genetic diversity that invading populations typically experience should, in theory, limit the chances of invasion success (Allendorf and Lundquist 2003). The genetic bottlenecks that are typically experienced by small founding populations often result in reduced genetic variation relative to the parent population (Sakai *et al.* 2001). In the short term small introduced populations are thus susceptible to inbreeding and strong genetic variation may further erode genetic variation. In the longer term a lack of genetic variation may impede the potential for adaptive evolution. This apparent genetic paradox may be resolved if invading populations have higher genetic diversity than source populations owing to high propagule pressure (Kolbe *et al.* 2004; Roman and Darling 2007), the genetic diversity measured by commonly used molecular markers such as microsatellites does not reflect the total diversity in the genome (Väli *et al.* 2008), or if genetic variation is unimportant, as is observed in clonally reproducing species (Baker 1995; Dybdahl and Drown 2011).

In some cases colonisation success may be enhanced by a reduction in genetic diversity. Greater genetic similarity in the introduced population owing to a small number of founders could result in increased co-operation, thus enhancing ecological success (Suarez *et al.* 1999; Tsutsui *et al.* 2000). It has been suggested that population bottlenecks during the introduction event have promoted large-scale unicoloniality, and thus the invasion success, of the well-studied Argentine ant *Linepithema humile*. This is owing to a breakdown in kin recognition, so that individuals that are not related cooperate because they perceive each other as similar (Tsutsui *et al.* 2000). Although this bottleneck hypothesis has been disputed by others (Vogel *et al.* 2010), genetic bottlenecks may also aid in invasion success through the purging of deleterious alleles (Schmid-Hempel *et al.* 2007). As the bottleneck hypothesis specifically refers to differences between the native and introduced ranges (Suarez *et al.* 1999; Tsutsui *et al.* 2000), the putative benefits of a loss of genetic diversity may or may not persist subsequent to the initial bottleneck.

Invasion success can be difficult to define and measure. However, the effects of invasive ant species on the recipient community are typically density-dependent (e.g., Ross et al. 1996; O'Dowd et al. 2003; Le Breton et al. 2005; Krushelnycky and Gillespie 2008; Lester et al. 2009). The high abundance attained by invasive ants strengthens their competitive ability relative to native ants and furthers their ecological dominance through numerical monopolisation of resources (e.g., Holway 1999; Human and Gordon 1999; Morrison 2000; Rowles and O'Dowd 2007; Sagata and Lester 2009). In most organisms the census population size (Nc) and effective population size (Ne) are coupled, and genetic diversity typically increases with increasing population size. In ant species, however, worker abundance does not necessarily reflect effective population size, as reproduction involves relatively few individuals, and these are not workers (Wilson 1963). However, worker genotypic diversity is representative of the genetic diversity of the colony, as it reflects the contribution of reproductive individuals. As effective population size and worker abundance are independent in ants, worker abundance, along with the alteration of community structure in the invaded community (e.g., Sanders et al. 2003) are proxies by which short-term, or momentary, invasion success can be assessed. Here we define momentary invasion success as the ability for a species to reach sufficient abundance to cause a reduction in species diversity and a change in the structure of the community to which they have been introduced.

Anoplolepis gracilipes is one of the most widespread and damaging invasive ants (Holway *et al.* 2002). It can occur at very high densities (> 2000 ants m²) and can cause substantial ecosystem change (O'Dowd *et al.* 1999; O'Dowd *et al.* 2003; Abbott 2006b). In Arnhem Land in the Northern Territory of Australia, the abundance of this species exhibits considerable spatial variation. Unlike *A. gracilipes* invasions in Samoa (Savage *et al.* 2009) and on Christmas Island (O'Dowd *et al.* 2003), there appear to be no obvious single ecological mechanisms promoting differences in abundance, such as mutualisms with Homoptera (Young *et al.* 2001), nor are there clear associations between the abundance of this ant and anthropogenic disturbance (Hoffmann and Saul 2010). We therefore hypothesised that there could be an association between genetic diversity and the variation in abundance of *A. gracilipes* in Arnhem Land.

Although the genetic structure and behaviour of *A. gracilipes* in Arnhem Land is consistent with a single population, population genetic structure is not correlated with variation in abundance (Chapter 3). The population is somewhat heterogeneous and hypothesised to be in the process of divergence (Chapter 3). Thus, the variable but spatially discrete occurrences of *A. gracilipes* in Arnhem land resemble a mosaic of distinct nest clusters or 'meta-colony' (*sensu* Heller *et al.* 2008). In this study we use 'population' to refer to the entire distribution of *A. gracilipes* in Arnhem Land, and 'clusters' (or plots) to refer to individual localised occurrences of the ant.



Fig. 4.1: Our hypothesised relationships between the genetic diversity (e.g., allelic richness or genotypic richness) and abundance of the invading population, and relative influence on invaded communities under scenarios of: (a) a positive relationship between genetic diversity and abundance; and (b) a negative relationship between genetic diversity and abundance.

The aim of the present study is to explore the relationships between genetic diversity, abundance and ecological success. Chapter 3 investigated population genetic differentiation (i.e., beta diversity), while in this study we extend the analysis to investigate genetic diversity at the local scale at which ants are likely to interact (i.e., alpha diversity). If higher genetic diversity facilitates momentary invasion success, higher genetic diversity and greater *A. gracilipes* abundance would be co-observed, and would be associated with negative effects on the invaded community (Fig. 4.1(a)). Conversely, if reduced genetic diversity facilitates momentary success, lower genetic diversity would be

correlated with greater *A. gracilipes* abundance, and with negative effects on the invaded community (Fig. 4.1(b)). To test these competing hypotheses we address three specific questions. First, is there an association between *A. gracilipes* genetic diversity and abundance? Second, are there differences in native ant species diversity and community structure between invaded and uninvaded communities? Third, is there an association between *A. gracilipes* abundance and native ant species diversity in the invaded community? Further, to evaluate an effect of environment we investigate if habitat characteristics are associated with variation in *A. gracilipes* abundance.

4.3 Methods

4.3.1 Study area

Arnhem Land is located in the monsoonal tropics of Australia's Northern Territory (Fig. 4.2). The region experiences daytime high temperatures ranging between 17 and 33°C, and seasonal rainfall (December to July) of approximately 1200 mm. The vegetation is primarily fire-prone savanna woodland. Although it is not known when *A. gracilipes* arrived in the region, it was first detected in 1982 (Majer 1984). In 2009 the ant was patchily distributed throughout 16,000 km² in Arnhem Land (Fig. 4.2), mainly in anthropogenically undisturbed and ecologically intact sites. Genetic and behavioural analyses of population structure suggest this population stemmed from a single source (Chapter 3). However, the genetic structure and intra-specific behaviour of the population is heterogeneous, which suggests the population is in the process of divergence (Chapter 3). In this study we extend the analysis of Chapter 3 to investigate genetic diversity at the scale at which ants are likely to interact.

4.3.2 Ecological surveys

Nine study sites were selected for an ecological survey undertaken in July 2009 (Fig. 4.2), which intersect with the sites sampled in Chapter 3. The differences between these sampling sites and those of Chapter 3 are the additions of plots D1, M1, K6, K7, and K2. We selected sites with similar habitat characteristics in a stratified random fashion. All sites had a dominant canopy of *Eucalyptus tetrodonta*, an understorey primarily

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consisting of *Acacia* spp., grasses and leaf litter, and similar drainage and topography. At each of the nine sites we selected two visually similar plots in areas invaded by *A*. *gracilipes*. Plots were spaced at least 100 m apart to ensure their independence. The largest *A. gracilipes* foraging distance recorded in this region is 35 m, and within this distance ants freely move between nests (B. Hoffmann unpublished data). The presence of at least one *A. gracilipes* nest was confirmed in invaded plots, and we paired these invaded plots with nearby plots where the ant was absent. For the purposes of our study we inferred that *A. gracilipes* workers sampled in plots were representative of nest clusters within foraging range of the plot.



Fig. 4.2: *Anoplolepis gracilipes* distribution in Arnhem Land in the Northern Territory, Australia at the time of sampling in July 2009 (grey circles), and sampling plots (black squares with site codes).

At each plot we installed 16 pitfall traps (45 mm diameter) haphazardly placed approximately 1m apart in a 4×4 m grid around an *A. gracilipes* nest. Traps were 2/3 filled with propylene glycol and left for 48 hours. All ant species collected were counted and identified to species level. Species that could not be named were assigned to species groups according to Andersen (2000). Voucher specimens for all species were retained at the Tropical Ecosystems Research Centre in Darwin or Victoria University of Wellington.

In addition to *A. gracilipes* counts in pitfall traps, to assess population densities we also measured the abundance of *A. gracilipes* at the plot level based on forager activity, as described in Chapter 3. Briefly, this method uses a count of the number of ants crossing a laminated card in a 30 s period (Green *et al.* 2004). At each site we measured *A. gracilipes* activity at 11 stations spaced at 5 m intervals along three replicate 50 m transects spaced 10 m apart. Counts at all stations within a transect were summed, and the mean value of the three replicate transects was used as an index of relative abundance between plots. Our card counts ranged from 0 to 42, and were highly correlated with pitfall trap counts for each plot (Spearman's rank correlation $R_s = 0.80$, S = 195, P < 0.001, conducted in R v 2.13.1 (Ihaka and Gentleman 1996; R Development Core Team 2011)). We used the pitfall trap data in all analyses as these data were collected at the same spatial scale as the genetic diversity and habitat data.

Native ant species were also assigned to the functional groups used in studies of Australian ant communities (Andersen 1995): Dominant Dolichoderinae (DD); Subordinate Camponotini (SC); Climate Specialists [sub-divided into Hot (HCS) and Tropical (TCS)]; Generalised Myrmicinae (GM); Opportunists (OPP); Cryptic Species (CS); and Specialist Predators (SP). In addition to *A. gracilipes* we found three other non-native ant species: *Monomorium floricola* (one ant in one uninvaded plot), *Tetramorium simillimum* (62 ants in one invaded and five uninvaded plots) and *Paratrechina longicornis* (14 ants in two uninvaded plots). As these species are not known to have any effects on savanna ant communities (Hoffmann and Saul 2010), we included their data with the native species.

We recorded a number of habitat attributes at the plot level that we considered could contribute to differences in local ant community structure and abundances of A. gracilipes: availability of potential A. gracilipes nest sites (the proportion of the plot occupied by logs and tree basal area, as A. gracilipes often nest at the base of trees); canopy cover (measured with a spherical densioneter); the abundance of *Acacia* spp. (a count of the number of plants in the plot); and leaf litter depth (a mean of four estimates in the plot). Availability of nest sites has been considered to have a major influence on A. gracilipes population size (Haines and Haines 1978a). Denser canopy cover can facilitate extended foraging time for ants in extreme heat. Acacia spp. offer a potential novel carbohydrate resource, which can enhance ant abundance (Davidson 1997 and references therein). Novel carbohydrate resources are an important variable as they facilitate invasion of A. gracilipes on Christmas Island (O'Dowd et al. 2003) and Samoa (Savage et al. 2009). Finally, leaf litter depth is an approximate indicator of time since fire, with deeper leaf litter occurring in unburnt areas (Cook 2003; Russell-Smith et al. 2009). Fire is a major driver of vegetation structure and composition in savannas, which in turn are major drivers of ant community composition (Andersen and Hoffmann 2011). These habitat attributes are henceforth referred to as nest sites, canopy, Acacia and litter respectively.

4.3.3 Molecular analyses

For molecular analyses, we haphazardly selected *A. gracilipes* workers from all nests in our study plots in July 2009 (Fig. 4.2). We supplemented these with additional ants collected from pitfall traps at the same site when allele discovery curves did not flatten (Supplementary Fig. 4.1). Ants were stored in 95% ethanol at 4°C. We extracted genomic DNA using a modified Chelex protocol (Sepp *et al.* 1994). Individual workers were placed in microcentrifuge tubes, ground with sterile plastic pestles, and 150 µl of a 10% w/v Chelex-100 resin solution was added. The tubes were centrifuged briefly, boiled for 15 minutes, chilled on ice for five minutes, and centrifuged at 15,000 × *g* for 15 minutes at 4°C. The supernatant containing DNA was stored at 4°C. Microsatellite molecular markers were used to assess the genetic diversity of *A*. *gracilipes* at the 18 sampled plots. Workers were genotyped for seven microsatellite loci: *Ano1, Ano3, Ano4, Ano5, Ano7, Ano8* and *Ano10* (Feldhaar *et al.* 2006), using the methods outlined in Chapter 3. Fluorescent dyes used were FAM (*Ano4, Ano5* and *Ano8*) and VIC (*Ano1, Ano3, Ano7* and *Ano10*). Polymerase Chain Reactions were performed using the thermal cycling conditions specified by Feldhaar *et al.* (2006), with modification for M13 primers (Schuelke 2000). Amplified products were analyzed using the LIZ size standard on a 3730 Genetic Analyzer, and visualised and scored using Genemapper v 3.7 (both Applied Biosystems).

In order to determine whether sufficient worker ants were genotyped to represent allelic diversity, we generated allele discovery curves for the most polymorphic locus (*Ano8*) using the 'PopGenKit' package (Rioux Paquette 2011) in R, with jack-knifing using 1000 replicates. A flattening of allele discovery curves with increasing sample size indicates that the samples genotyped are a fair representation of the allelic diversity in the population. Where possible, we genotyped additional ants where discovery curves did not flatten (Supplementary Fig. 4.1), until there were no further ant samples remaining.

The majority of the 539 *A. gracilipes* workers genotyped were heterozygous at all loci, as is typical for this species (Drescher *et al.* 2007; Drescher *et al.* 2010; Thomas *et al.* 2010; Chapters 2 and 3). Forty ants were homozygous at the *Ano8* locus, and one was homozygous at the *Ano5* locus. The undetermined reproductive mode of *A. gracilipes* (Drescher *et al.* 2007; Chapter 2), and the existence of populations of *A. gracilipes* workers heterozygous at all loci (Thomas *et al.* 2010) indicates that homozygous loci could reflect allele dropout and thus possibly be biologically inaccurate. Therefore we conducted analyses with and without these worker genotypes as their presence may have inflated genotypic diversity estimates. Although we found no major differences between the two datasets we have chosen to report the analyses excluding these workers. These analyses were based on genotyping of 20 to 35 workers from each of the 18 plots (mean \pm SE = 27.67 \pm 1.07, n = 498). The removal of samples with homozygous genotypes resulted in a smaller sample size for some plots compared with Chapter 3, and the addition of genotypes as a result of allele discovery curves not flattening increased the sample sizes in other plots.

4.3.4 Statistical analyses

For all statistical tests a significance level of $\alpha = 0.05$ was used. For all analyses, effect sizes were interpreted as small ($R^2 \sim 0.01$, $R_s \sim 0.10$), medium ($R^2 \sim 0.09$, $R_s \sim 0.30$), or large ($R^2 \sim 0.25$, $R_s \sim 0.50$), according to Cohen (1988). We conducted all analyses at the plot level.

4.3.4.1 Is there an association between <u>Anopholepis gracilipes</u> genetic diversity and abundance?

Rarefied allelic richness was calculated in R using the 'PopGenKit' package (Rioux Paquette 2011), with jack-knifing of 1000 replicates. We estimated other genetic diversity parameters, including genotypic richness (G) and unbiased genotypic diversity (R_U), Shannon entropy (H'), and Simpson's index of diversity (D) using Genclone v 2.0 (Arnaud-Haond and Belkhir 2007). We then derived Hill's numbers from these measures using the same naming convention as species diversity (${}^{0}D$ = allelic richness (including only informative loci); ${}^{1}D$ = exp [Shannon's H']; and ${}^{2}D$ = 1/Simpson's D). We chose richness-based measures of diversity as these should be sensitive to the elimination of rare alleles by drift (Nei *et al.* 1975; Allendorf 1986). We chose genotypic diversity measures because the undetermined reproductive mode of *A. gracilipes* could involve clonality, and genotypic diversity would provide an estimate of the number of clones present. The relationships between diversity measures (${}^{0}D$, ${}^{1}D$ and ${}^{2}D$, and R_U) and differences in the abundance of *A. gracilipes* were analysed using Spearman's rank correlation implemented in R. We visualised the relationships between variables by fitting smoothed lines with the loess function in R using a span of 0.9.

4.3.4.2 Are there differences in native ant species diversity and community structure between invaded and uninvaded communities?

Species diversity measures (richness, Shannon's H' and dominance / Simpson's D) were estimated by individual-based rarefaction (Hurlbert 1971) using 1000 permutations implemented in EcoSim v 7 (Gotelli and Entsminger 2007). The lowest number of native ants sampled in a plot (52 individuals) was the rarefied sample size. We derived Hill's numbers from these measures following the terminology of Jost (2006): ${}^{q}D$ where ${}^{q} = 1, 2$ or 3 (i.e., ${}^{0}D$ = richness; ${}^{1}D$ = exp[Shannon's H']; and ${}^{2}D$ = 1/Simpson's D). These measures assign different weights to the relative abundance of species, with the importance of rare species decreasing as q increases, and together provide a biologically meaningful overview of the diversity of the community. To test if these measures of species diversity differed between invaded and uninvaded plots, we used Wilcoxon rank sum tests (with Monte-Carlo resampling to estimate P values), using the 'coin' R package (Hothorn *et al.* 2008). We used the Z score estimate to convert Z statistics to an R^2 effect size using the formula $R^2 = Z^2/N$ (Rosenthal 1991). Species accumulation curves (samplebased rarefaction curves) were generated for invaded and uninvaded sites using EstimateS v 8.2.0 (Colwell 2009). Pitfall traps were used as the sample and we generated curves for each site individually (Supplementary Fig. 4.2).

To assess if native ant community structure differed between invaded and uninvaded plots we used non-metric Multi-Dimensional Scaling (MDS) implemented in Primer v 6.1.11 (Clarke and Gorley 2006). We log-transformed data to even out the effects of rare and abundant species, and used the Bray-Curtis index as a similarity measure as recommended by Clarke and Warwick (2001). The MDS was run over 1000 iterations using Kruskal stress formula 1 and a minimum stress of 0.01. We tested for significant differences between invaded and uninvaded plots using PERMANOVA+ run over 99999 permutations, and checked for constant dispersion among groups using PERMDISP (Anderson *et al.* 2008). We used SIMPER analysis to assess how individual species contributed to differences between invaded and uninvaded plots (Clarke and Warwick 2001).

4.3.4.3 Is there an association between <u>Anopholepis gracilipes</u> abundance and native ant species diversity in the invaded community?

If higher abundance were associated with negative effects on the invaded community, we would have expected a decrease in species diversity as abundance increased. To determine if species diversity measures $({}^{0}D, {}^{1}D \text{ and }{}^{2}D)$ were correlated with variation in the abundance of *A. gracilipes* we used Spearman's rank correlation in R. We visualised the relationships between variables by fitting smoothed lines with the loess function in R using a span of 0.9.

4.3.4.4 Are habitat characteristics associated with variation in <u>Anoplolepis</u> <u>gracilipes</u> abundance?

We used non-parametric multiple regression with generalised additive models and automatic spline smoothing implemented in the 'mgcv' R package (Wood 2006) to test if the habitat attributes we measured contributed to differences in *A. gracilipes* abundance. We modelled *A. gracilipes* abundance as the dependent variable, site as a fixed factor, and litter, canopy, *Acacia*, nest sites as the independent variables. We ran models using all data, and data for invaded plots alone. As a single model could not be fit with all the model terms included, we used forward model selection, excluded terms that explained the least of the variation in the data, and selected the best models as those that explained most of the variation in the data. We visualised the relationships between environmental variables among plots using principal component analysis (PCA) in Primer v 6.1.11.

4.4 Results

4.4.1.1 Is there an association between <u>Anopholepis gracilipes</u> genetic diversity and abundance?

We found a significant correlation between most measures of genetic diversity and *A*. *gracilipes* abundance. *Anoplolepis gracilipes* was the most abundant species in all invaded plots, but no single species was universally the most abundant in uninvaded plots. *Anoplolepis gracilipes* abundances ranged from 62 to 5288 ants per invaded plot (mean \pm SE: 1134 \pm 328 ants), and from zero to 1016 ants per pitfall trap (mean \pm SE: 71 \pm 6 ants). Genetic diversity measures were positively correlated with each other, with the exception of ²D, which was negatively correlated with the other measures (Table 4.1; Fig. 4.3). The abundance of *A. gracilipes* was also positively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with all genetic diversity measures with the exception of ²D, which was negatively correlated with abundance (Fig. 4.3).

Although the positive relationship between *A. gracilipes* abundance and genetic diversity was not significant for ${}^{0}D$ (S = 674, P = 0.109, $R_s = 0.30$), this relationship nonetheless had a medium effect size. These relationships were positive and significant for ${}^{1}D$ (S = 431, P = 0.008, $R_s = 0.55$), and unbiased genotypic diversity (S = 540, P = 0.034, $R_s = 0.44$), and negative and significant for ${}^{2}D$ (S = 1514, P = 0.007, $R_s = 0.56$). All significant relationships also had large effect sizes. The relationships between abundance and genetic diversity were not linear, and instead were best fit by lines that tended toward a sigmoidal or inverse exponential function.



Fig. 4.3: The relationship between *A. gracilipes* abundance and genetic diversity: (a) ${}^{0}D$ (allelic richness); (b) ${}^{1}D$ (exp[Shannon's H']); (c) ${}^{2}D$ (1/Simpson's index); (d) genotypic diversity. Dashed lines indicate the smoothed spline line of best fit with a span of 0.9.

4.4.1.2 Are there differences in species diversity and community structure between invaded and uninvaded communities?

Seventy species in total were found in our survey (61 in uninvaded and 49 in invaded plots), and the number of species per plot ranged from 7 to 22 (mean \pm SE: 12 \pm 0.6 species in invaded plots and 15 \pm 0.8 species in uninvaded plots). Mean native ant abundances ranged from 52 to 918 ants per plot (mean \pm SE: 252 \pm 42.0 ants in invaded and 319 \pm 56.5 ants in uninvaded plots).

able 4.1: Abundance and genetic diversity parameters for Anoplolepis gracilipes in 18 invaded plots in Arnhem Land. Abundance = total number of
nts caught in pitfall traps in the plot, N = number of workers genotyped, Allelic richness = rarefied allelic richness for each locus based on the
mallest sample size (21), ^{0}D = rarefied allelic richness for the three informative loci, ^{1}D = exp[Shannon's H']), ^{2}D = (1/Simpson's index), G = multi-
cus genotypes, R_U = unbiased genotypic diversity.

				Alle	lic richnes	S	Gei	netic diver-	sity	Genotyp	oic richness
lot	Abundance	Z	Ano3	Ano7	Ano8	Other loci	$oldsymbol{G}_{ heta}$	\boldsymbol{Q}_{I}	D^2	6	R_U
A1	1353	31	2.00	2.00	6.43	2.00	10.43	4.16	1.48	7	0.20
A2	452	26	2.00	2.00	4.74	2.00	8.74	3.23	1.45	4	0.12
B1	5288	23	2.99	2.85	8.86	2.00	14.58	11.72	1.07	14	0.59
B2	868	25	2.87	4.60	10.01	2.00	16.61	13.59	1.05	16	0.63
D1	139	33	3.47	3.57	4.22	2.00	11.26	5.82	1.25	6	0.25
D2	1577	32	3.00	3.61	4.26	2.00	10.87	5.85	1.25	6	0.26
D3	1375	21	2.00	2.00	7.00	2.00	11.00	8.30	1.12	10	0.45
D4	3357	30	2.67	2.00	7.54	2.00	12.21	9.95	1.09	12	0.38
G	2320	35	2.00	2.79	6.77	2.00	11.56	7.80	1.14	10	0.27
G2	432	22	2.00	2.92	6.91	2.00	11.83	5.72	1.21	7	0.29
K2	566	20	2.00	2.50	9.00	2.00	13.00	6.68	1.18	6	0.42
K4	1183	25	2.79	3.00	8.54	2.00	14.33	9.98	1.09	12	0.46
K6	353	35	2.99	3.00	7.81	2.00	13.80	8.37	1.14	11	0.29
К7	256	27	2.00	2.93	69.9	2.00	11.62	5.82	1.24	6	0.31
P1	350	30	2.00	2.70	5.34	2.00	10.04	3.32	1.65	9	0.17
P2	383	29	2.00	2.69	4.60	2.00	9.29	2.42	2.18	5	0.14
M1	62	26	2.00	3.96	4.67	2.00	10.63	4.40	1.38	7	0.24
M2	76	28	2.00	3.92	5.35	2.00	11.27	4.70	1.43	8	0.26

Native ant species diversity differed between invaded and uninvaded plots, but only species richness (${}^{0}D$) was significantly higher in uninvaded plots than invaded plots (approximative Wilcoxon Mann-Whitney Rank Sum Test, Z = -1.96, $R^{2} = 0.11$, P = 0.047). Other species diversity measures did not significantly differ between invaded and uninvaded plots, but the observed effect sizes were progressively lower as q increased. (${}^{1}D$: Z = -1.76, $R^{2} = 0.09$, P = 0.077; ${}^{2}D$: Z = -1.24, $R^{2} = 0.04$, P = 0.227). As rare species are assigned a lower importance in the ${}^{1}D$ and ${}^{2}D$ measures, this decreasing effect size as q increases suggests that rare species are less likely to co-occur with A. gracilipes.

Three-dimensional MDS revealed clear differences in community structure between uninvaded and invaded plots (Fig. 4.4). The results of PERMANOVA+ analysis revealed significant differences between invaded and uninvaded plots (*Pseudo* $F = 4.43_{1,34}$, *P*[*perm*] < 0.001). PERMDISP analysis found that dispersions were homogeneous among groups ($F = 2.13_{1,34}$, *P*[*perm*] = 0.165), so the differences between groups were not owing to differences in dispersion within groups.



Fig. 4.4: Three-dimensional MDS plot of *A. gracilipes* invaded and uninvaded plots based on Bray-Curtis similarity of log (X+1) transformed abundance data. *Anoplolepis gracilipes* were excluded from the analysis. The accompanying PERMANOVA+ analysis revealed significant differences between invaded and uninvaded plots (*Pseudo F* = 4.43_{1,34}, *P[perm]* < 0.001).

SIMPER analysis found that invaded plots were less likely to include dominant species and more likely to contain insinuating species than uninvaded plots. 90% of the dissimilarity between invaded and uninvaded plots was attributable to 33 ant species, while 50% of the dissimilarity was attributable to 11 species. The abundances of a number of these species differed markedly between invaded and uninvaded plots (Table 4.2). The contribution of different functional groups also differed between invaded and uninvaded plots. Invaded plots had fewer Dominant Dolichoderinae (DD) and Tropical Climate Specialists (TCS) and more Generalised Myrmicinae (GM). Ecologically dominant species (DD and *Oecophylla smaragdina* (TCS)) were absent from many but not all invaded plots. In invaded plots. *Iridomyrmex pallidus* was found in plots B2 and G2, and *Iridomyrmex* sp. 1 *anceps* group was found in plots D3, D4, G2, K4, K7 and P2. *Iridomyrmex* sp. 3 *myobergi* group occurred at plot M2. When they occurred in invaded plots these species had lower abundance than when they were found in uninvaded plots (Table 4.2). *Iridomyrmex reburrus* was only found in uninvaded plots.

Species	Mean abu	ndance	0	% 0	Cumulative %
	uninvaded	invaded	difference	contribution	contribution
<i>heidole</i> sp. 3 <i>variabilis</i> group	0.61	2.12	247.5%	5.5	
<i>heidole</i> sp. 8 group F	1.24	2.05	65.3%	5.3	10.8
aratrechina sp. 4 vaga group	2.17	0.39	-82.0%	5.2	16.0
lonomorium sp. 8 carinatum group	2.25	1.15	-48.9%	5.2	21.2
lonomorium sp. A nigrius group	1.99	1.07	-46.2%	4.9	26.1
aratrechina sp. 13 vaga group	0.65	1.86	186.2%	4.8	30.9
'rematogaster queenslandica	0.92	1.47	59.8%	4.0	34.9
vecophylla smaragdina	1.67	0.25	-85.0%	4.0	38.9
lonomorium sp. 46 laeve group	1.14	1.18	3.5%	3.6	42.5
etramorium sp. 1 striolatum group	1.40	1.59	13.6%	3.6	46.1
lonomorium sp. 24 laeve group	3.18	3.46	8.8%	3.3	49.4
idomyrmex sp. 1 anceps group	1.09	0.60	-45.0%	3.3	52.7
dontomachus sp. near turneri	1.07	0.14	-86.9%	2.8	55.5
idomyrmex pallidus	1.11	0.39	-64.9%	2.8	58.3
amponotus sp. 11	1.14	0.61	-46.5%	2.7	61.0
<i>aratrechina</i> sp. 2 <i>minutula</i> group	0.88	0.78	-11.4%	2.7	63.7
heidole impressiceps	0.27	0.63	133.3%	2.2	62.9

Table 4.2: Differences in the abundances (log-transformed) of the ant species and functional groups that contributed to $\sim 90\%$ of the dissimilarity between A. gracilipes invaded plots compared with uninvaded plots in Arnhem Land, Australia. Anoplolepis

Species	Mean abu	ndance	0	%	Cumulative %
	uninvaded	invaded	difference	contribution	contribution
Pheidole sp. A variabilis group	0.37	0.55	48.6%	2.1	68.0
Monomorium sp. 13 nigrius group	0.88	0.15	-83.0%	2.1	70.1
Opisthopsis haddoni	0.73	0.53	-27.4%	2.0	72.1
Meranoplus sp. 8 group F	0.08	0.70	775.0%	2.0	74.1
Tetramorium lanuginosum	0.34	0.44	29.4%	1.8	75.9
Iridomyrmex reburrus	0.73	0.00	-100.0%	1.8	T.T.
Rhytidoponera sp. 9 tenuis group	0.44	0.49	11.4%	1.8	79.5
Meranoplus mjobergi	0.43	0.41	-4.7%	1.7	81.2
$Tetramorium\ simillimum$	0.10	0.58	480.0%	1.6	82.8
Crematogaster sp. 2 laeviceps group	0.56	0.06	-89.3%	1.4	84.2
Solenopsis sp. 1	0.66	0.04	-93.9%	1.4	85.6
Rhytidoponera sp. 3 turneri group	0.22	0.23	4.5%	1.1	86.7
Iridomyrmex sp. 3 mjobergi group	0.30	0.06	-80.0%	0.9	87.6
Rhytidoponera aurata	0.34	0.00	-100.0%	0.9	88.5
<i>Tapinoma</i> sp. 1	0.15	0.22	46.7%	0.8	89.3
Polyrhachis inconspicua	0.22	0.22	0.0%	0.8	90.1

Table 4.2 (continued):

Functional group	Mean abu	ndance	0	%	Cumulative %
	uninvaded	invaded	difference	contribution	contribution
Dominant Dolichoderinae (DD)	2.61	0.84	-67.8%	23.0	
Generalised Myrmicinae (GM)	3.12	4.09	31.1%	18.0	41.0
Tropical Climate Specialists (TCS)	1.67	0.28	-83.2%	16.7	57.7
Hot Climate Specialists (HCS)	4.45	4.02	-9.7%	11.9	69.6
Opportunists (OPP)	3.63	3.46	-4.7%	11.46	81.1
Subordinate Camponotini (SC)	1.82	1.32	-27.5%	10.77	91.9

Table 4.2 (continued):



4.4.1.3 Is there an association between <u>Anopholepis gracilipes</u> abundance and species diversity of the invaded community?

Fig. 4.5: Relationships between *A. gracilipes* abundance and native ant: (a) ${}^{0}D$ (species richness); (b) ${}^{1}D$ (exp [Shannon's H']); (c) ${}^{2}D$ (1/Simpson's index). Dashed lines indicate the smoothed spline line of best fit with a span of 0.9.

Greater *A. gracilipes* abundance was associated with lower native ant species diversity for all measures of species diversity (Fig. 4.5), but the effect size and statistical significance of this relationship was lower for the measures that placed less importance on rare species (${}^{0}D$: *S* = 11151, *R_s* = 0.44, *P* = 0.004; ${}^{1}D$: *S* = 10422, *R_s* = 0.34, *P* = 0.021; ${}^{2}D$: *S* = 9647, *R_s* = 0.24, *P* = 0.078). This result mirrored our finding that species diversity did not differ between invaded and uninvaded plots when rare species were assigned less importance, and supports our finding that rare species are less likely to co-occur with *A. gracilipes*. The relationships between abundance and ant species diversity were not linear, and instead were best fit by lines that tended toward a sigmoidal function. These relationships suggest a saturation point for these measures.

4.4.1.4 Are habitat characteristics associated with differences in <u>Anoplolepis</u> <u>gracilipes</u> abundance?

None of the habitat characteristics we measured were significantly correlated with differences in *A. gracilipes* abundance. For all plots combined the best model included site as a fixed factor and canopy, *Acacia* and litter as independent variables. The best model explained 48% of the variation in the data, but no variables were statistically significant ($R^2 = 0.20$, canopy: F = 1.02, P = 0.324, *Acacia*: F = 1.38, P = 0.273, litter: F = 3.06, P = 0.080). For invaded plots only the best statistical model included site and *Acacia*, and explained 65% of the variation in the data, but again, no variables were statistically significant ($R^2 = 0.18$, *Acacia*: F = 0.94, P = 0.44). The principal components analysis also did not reveal any differences between invaded and uninvaded plots for any of the habitat characteristics measured (Supplementary Fig. 4.3).

4.5 Discussion

Populations of invasive species often experience genetic bottlenecks that typically result in reduced genetic variation in the introduced population relative to the parent population (Sakai *et al.* 2001). Although introduced populations may persist in the short term, a lack of genetic variation may impede the potential for adaptive evolution in the longer term. We found that higher abundance of *A. gracilipes* was significantly positively correlated with most measures of genetic diversity, which is consistent with the

evolutionary theoretical expectations that invasive populations with higher genetic diversity should experience greater invasion success (Sakai *et al.* 2001). Our study was conducted at the scale at which ants are likely to interact (nest clusters), rather than at the population scale, and the abundance we measured reflects momentary invasion success. The longer term evolutionary benefits of higher genetic diversity to *A. gracilipes* populations are difficult to predict. The immediate functional benefits of higher genetic diversity, at both the scale of the nest cluster and the population as a whole, as well as the underlying causes of the relationship between genetic diversity and abundance, and the relationship between abundance and ecological effects on the invaded community merit further consideration.

4.5.1 The functional benefits of higher genetic diversity

We found that most, but not all, measures of genetic diversity were correlated with higher abundance. Although allelic richness (${}^{0}D$) was not significantly correlated with higher abundance of *A. gracilipes* in Arnhem Land, the relationship between the two was positive and moderately large. Genotypic diversity and ${}^{1}D$ genetic diversity were significantly positively correlated with higher *A. gracilipes* abundance, but ${}^{2}D$ was significantly negatively correlated with higher abundance. The interpretation of the biological relevance of the latter statistic is problematic. Thus, further discussion focuses on genotypic diversity, because: a) it relates directly to individuals; b) may indicate whether multiple queens and males contribute to reproduction within the nest cluster; and c) may be interpreted in light of asexual reproduction, which has been suggested possibly contributes to the reproductive mode of *A. gracilipes* (Drescher *et al.* 2007; Heinze 2008).

Nest clusters of *A. gracilipes* could obtain functional benefits from higher genetic diversity in a number of ways. The intra-colonial benefits of higher genetic diversity to social insects include increased resistance to infection (Sherman *et al.* 1988; Shykoff and Schmid-Hempel 1991; Keller 1995; Tarpy 2003; Reber *et al.* 2008; Ugelvig *et al.* 2010), genetically based task specialization (Oldroyd and Fewell 2007), enhanced colony growth (Cole and Wiernasz 1999; Tarpy 2003), and higher productivity and fitness (Mattila and Seeley 2007). However experimental tests have found no relationship between short term task efficiency and genetic diversity (Rosset *et al.* 2005). It remains to be seen whether

the fine scale, momentary correlation between genetic diversity and abundance that we observed translates into invasion success at larger spatial and temporal scales. However, given the findings of studies of other ants, it seems likely that higher genetic diversity of the worker population may have positive short term effects on local nest clusters.

If there are functional benefits of higher genetic diversity to some clusters, clearly other nest clusters do not receive these benefits. How then might variation in genetic diversity affect the dynamics of the *A. gracilipes* population in Arnhem Land as a whole? *Anoplolepis gracilipes* populations have been known to vary in abundance temporally, significantly decline or collapse entirely (Lewis *et al.* 1976; Haines and Haines 1978b; Hill *et al.* 2003; Abbott 2006b; B. Hoffmann, personal observation). If genetic diversity drives abundance, meta-population dynamics might result in 'sink' populations (Pulliam 1988) of lower genetic diversity that do not persist. The fluid nature of the population structure of *A. gracilipes* in Arnhem Land, and lack of significant aggression between geographically distant nests (Chapter 3) also suggests that 'sink' nest clusters may be able to receive additional propagules from 'source' nest clusters. If the relationship is reversed, and abundance drives higher genetic diversity, larger propagules may have better chances of persistence. Longer term study of the correlations between abundance and genetic diversity, and the spatio-temporal dynamics of abundance in *A. gracilipes* would reveal whether this was this case in Arnhem Land.

Although our findings appear to be in contrast to the hypothesis that genetic bottlenecks may promote unicoloniality, and thus invasion success, which has been suggested for *L. humile* (Suarez *et al.* 1999; Tsutsui *et al.* 2000), the potential positive effects of a bottleneck may only occur at the introduction event. Subsequent to the initial introduction event, relatively higher genetic diversity may offer benefits to local nest clusters as outlined earlier. Ideally, to determine the effects of potential bottlenecks that may have affected the success of *A. gracilipes* in the invaded range we would need a comparison with the native range. Unfortunately the native range of *A. gracilipes* is unknown. Although its congeners are African, the ant is suspected to have Asian origins (Wetterer 2005), which is supported by some evidence (Drescher 2011; Sebastien *et al.* 2012). For example, the closest relatives of some endosymbionts of *A. gracilipes* or the hosts of those endosymbionts appear to be from Asia (Sebastien *et al.* 2012), and lower

diversity at mitochondrial markers in Indo-Pacific populations suggests that these may have stemmed from populations in Asia (Drescher 2011). However, the entire range is not known and has not been sampled. Population divergence of *A. gracilipes* subsequent to introduction in Arnhem Land (Chapter 3) is also likely to have obscured evidence of a bottleneck that could have occurred on arrival. Thus, whether a bottleneck during the introduction event may have promoted unicoloniality in the Arnhem Land population is unknown.

4.5.2 Mechanisms underlying the relationship between genetic diversity and abundance

Variation in genetic diversity may be responsible for variation in the abundance of A. gracilipes in Arnhem Land, or it may be that more abundant populations are more genetically diverse because more individuals contribute to reproduction. In ant societies, census population size (Nc - workers together with reproductives) and effective population size (Ne – individuals contributing to reproduction) are potentially decoupled, as reproduction often involves relatively few individuals, and these are not workers (Wilson 1963). We assumed this might be the case for A. gracilipes. However, our results do show that genotypic diversity and abundance are to some degree coupled. In addition, there is the suggestion that A. gracilipes workers may contribute to reproduction (Heinze 2008), in which case worker abundance and genetic diversity could be more closely coupled if more worker clones result in higher abundance. Ant colonies can also be more genetically diverse if more queens and / or males contribute to reproduction (i.e., multiple queens reproduce [polygyny], or queens mate with multiple males [polyandry]: Pamilo 1991). Although the number of queens in A. gracilipes nests in Arnhem Land varies (1 – 16 queens per nest: Chapter 2) and is often much higher (up to 300 queens per nest: Haines and Haines 1978a; Rao and Veeresh 1991), polygyny has not been investigated in the species. If polygyny makes a significant contribution to A. gracilipes genetic diversity, a decline in queen abundance may result in lower genetic diversity within the nest (or nest cluster). Clearly, further exploration of the dynamics of colony structure and reproductive mode of A. gracilipes are required to answer these questions.

An alternative hypothesis is that the correlation between abundance and genetic diversity in A. gracilipes in Arnhem Land is owing to invasion history. In this scenario, genetic drift could result in an association between genetic diversity and abundance as a result of successive bottlenecks during dispersal throughout Arnhem Land. Indeed, the population appears to be in the process of divergence into multiple colonies (Chapter 3), a process hypothesised to be driven by colony fragmentation and genetic drift (Drescher et al. 2010). If this is the case, older populations would likely be more genetically diverse than younger populations, as the younger populations would be increasingly bottlenecked. Although possible, this hypothesis is not consistent with the invasion history and dynamics of abundance observed among a number of A. gracilipes populations. In Arnhem Land, for example, when the ant was first detected it was most abundant near sites K6 and K7, while in all other areas it was in low abundance (Young et al. 2001). In addition, nest clusters that have been highly abundant suddenly decline in Arnhem Land (B. Hoffmann, unpublished data), and in Tokelau, younger populations often appear to be more abundant than older populations (Lester and Tavite 2004; Abbott et al. 2007; Chapter 5), although the situation is thought to be reversed on Christmas Island (Abbott 2006b). Regardless, this hypothesis would be difficult to test without more detailed knowledge of the invasion history of nest clusters within the population, which is also complicated by the potential of lag times obscuring detection of younger nest clusters if they were at low abundance.

4.5.3 Density-dependent relationships between *Anoplolepis gracilipes* abundance and native ant community structure and diversity

Frequently the effects of invasive ants on the resident ant communities are densitydependent (e.g., Ross *et al.* 1996; Le Breton *et al.* 2005; Krushelnycky and Gillespie 2008). Although the negative ecological effects of *A. gracilipes* are clearly densitydependent on ant communities (Hill *et al.* 2003; Abbott *et al.* 2007; Lester *et al.* 2009; Hoffmann and Saul 2010; Drescher *et al.* 2011; this study) and other invertebrates (McNatty *et al.* 2009; Boland *et al.* 2011), in some instances *A. gracilipes* is not able to reach sufficient abundance for negative ecological effects to occur (Abbott *et al.* 2007; Hoffmann and Saul 2010; Boland *et al.* 2011; Drescher *et al.* 2011; this study; Chapter 5). The effect of invading ants on the recipient ant community does not always result in a
decline in species richness, as some species may increase in abundance while others decrease (Guénard and Dunn 2010). We used a combination of diversity measures, which revealed more than species richness alone regarding the nature of differences between communities. Species richness was not always lower in invaded plots than uninvaded plots. While rare species were less likely to occur with *A. gracilipes* in Arnhem Land, small, inconspicuous, insinuating species were commonly present. Other studies of *A. gracilipes* have found lower ant species richness in invaded sites (e.g., Sarty *et al.* 2007; Savage *et al.* 2009; Hoffmann and Saul 2010; Drescher *et al.* 2011), and that ecologically dominant species are less likely to co-occur with *A. gracilipes* (Hoffmann and Saul 2010). When *A. gracilipes* reaches high abundance it may cause more marked changes to ant community structure (Abbott *et al.* 2007; Lester *et al.* 2009). So while the absence of rare ant species when *A. gracilipes* is highly abundant may be of conservation concern, the patchy distribution and variable abundance of *A. gracilipes* in Arnhem Land may be unlikely to have significant effects on regional native ant diversity.

4.5.4 Habitat characteristics may be less important than genetic diversity

When viewed in a biogeographical context it is clear that many factors influence invasion success (Wilson *et al.* 2009a). Although many introduced populations experience a reduction in genetic diversity in the invaded range (e.g., Grapputo *et al.* 2005; Zayed *et al.* 2007; Dlugosch and Parker 2008), they may thrive if the new ecological conditions are more favourable than the native range (Sax and Brown 2000; Colautti *et al.* 2004; Moles *et al.* 2008). Alternatively, higher genetic diversity may be a more important contributor to invasion success where novel ecological conditions do not promote abundance. We suggest that this may be the case for *A. gracilipes* in Arnhem Land, where we found no association between the habitat characteristics we assessed and *A. gracilipes* abundance, yet we did find positive relationships between most measures of genetic diversity and abundance.

Of the habitat variables we measured, carbohydrate resources are perhaps the most important driver of *A. gracilipes* abundance elsewhere (O'Dowd *et al.* 1999; O'Dowd *et al.* 2003; Savage *et al.* 2009), and strongly influence ant abundance generally (Davidson 1997 and references therein). Thus, it was surprising that we found no effect of the

presence of *Acacia* on the abundance of *A. gracilipes*. The abundance of *A. gracilipes* in Arnhem Land appears very low compared to the ant on Christmas Island (our card counts ranged from 0 to 42, compared with a range of ~14 - 136 on Christmas Island, Abbott 2005), where *A. gracilipes* abundance is facilitated by honeydew-exuding scale insects (O'Dowd *et al.* 2003). The lesser abundance we observed may be owing to a lack of exploitation or availability of carbohydrate resources in Arnhem Land. Conversely, *A. gracilipes* may not have reached (or be able to reach) a minimum level of abundance required to monopolise exudate producing resources, as found in *Technomyrmex albipes* (Oliver *et al.* 2008). Regardless of the direction of the relationship between carbohydrate resources and abundance, our results suggest that in the absence of clear ecological drivers of abundance, genetic diversity may more likely to be associated with abundance. We do not deny that habitat characteristics, and particularly novel resources, often influence the abundance and ecological success of ants, but in this case the correlation with genetic diversity was much stronger than with the habitat variables we measured.

4.6 Conclusion

We found evidence of a positive association between measures of genetic diversity and the abundance of *A. gracilipes* in Arnhem Land. While higher genetic diversity may benefit individual nest clusters, the underlying mechanisms and the direction of the relationship between abundance and genetic diversity are unclear, and the implications for longer term invasion success on the wider population are difficult to predict. Although the effects of *A. gracilipes* on the invaded ant community in Arnhem Land are density-dependent, the variability in *A. gracilipes* abundance may be unlikely to have significant effects on regional native ant diversity over longer timescales. Finally, the relative importance of the relationship between genetic diversity and abundance may be context-dependent, may change over time, and be more obvious in the absence of highly favourable ecological characteristics.

Chapter 5. Population decline but increased distribution of an invasive ant genotype on a Pacific atoll

5.1 Abstract

Populations of invasive species are often studied when their effects are perceived as a problem. Yet much can be gained by observing the dynamics of populations over longer time periods, particularly if multiple cryptic populations are present that differ in their ecological effects. In this study we revisit an invasion of the yellow crazy ant (Anoplolepis gracilipes) in the Tokelau archipelago to determine if the distribution and abundance of the ant has changed \sim 7 years after surveys completed in 2004. We were particularly interested in whether populations of a previously identified invasive haplotype (D) had increased in distribution and abundance, as this haplotype was implicated in negative effects on the resident ant communities. Indeed, haplotype D populations have become more widespread since the initial survey, although this is more likely owing to new introductions or movement by humans, rather than intrinsic characteristics of the haplotype. We also found that although there has been no significant change in the abundance of A. gracilipes overall, haplotype D populations have significantly declined in abundance. Residents of the two Tokelau atolls where A. gracilipes has remained since 2004 no longer consider the ant to be a pest as they did seven years ago, when populations of this ant interfered with their food production and many other aspects of daily life. We observed no significant effects of A. gracilipes on the local ant community, which suggests that the ant is at a level of abundance below which significant negative ecological effects may occur. Population declines of invasive species are not infrequent, and understanding these population dynamics, particularly the underlying mechanisms promoting population declines or stabilisation, should be a high priority for invasion ecology.

5.2 Introduction

In invasion ecology, populations of invasive species are often sampled, and their effects described, when they are perceived as a problem (Strayer *et al.* 2006). These populations attract attention because of their high densities and negative effects on biodiversity and human populations. Invading populations are less often re-examined after long intervening periods (Strayer *et al.* 2006). It is often expected that invading populations that are already present in high numbers would persist and spread if left unchecked. Indeed, many studies have found that invading populations become more abundant and their impacts on the invaded community intensify over time (e.g., O'Dowd *et al.* 2003; Simberloff and Gibbons 2004; Hoffmann and Parr 2008). In other cases invading populations can expand or contract their range and abundance over time, and in some cases abundant populations can suffer local declines, or disappear entirely (e.g., Lewis *et al.* 1976; Morrison 2002; Hill *et al.* 2003; a number of studies reviewed by Simberloff and Gibbons 2004; Cooling *et al.* 2011). Thus, the predictive value of a single point-in-time study has limitations in relation to longer term invasion outcomes (Strayer *et al.* 2006).

In addition to changes occurring in population dynamics and ecological effects over time, the genetic nature of the population may affect invasion outcomes. For example, non-native populations of the reed grass *Phragmites australis* in North America have faster rates of spread than native populations. These cryptic non-native populations can only be differentiated from native populations by analysis of their genotypes (Saltonstall 2002). Similarly, the whitefly (*Bemisia tabaci* species complex) has a number of genetic biotypes, of which only two have attained pest status (Perring 2001). Identification of these genetic forms enables predictions regarding their spread and displacement of other genetic forms. Thus, genetic insights into the invading population can be an important aid to management if the abundance, spread and ecological effects of the invader vary according to the genotype.

In 2004, Abbott et al. (2007) conducted surveys of the invasive yellow crazy ant Anoplolepis gracilipes on the Tokelau atolls. Anoplolepis gracilipes is one of the most widespread invasive ants (Holway et al. 2002), and is listed among a selection of 100 of the world's worst invasive species (Lowe et al. 2000). Abbott et al. (2007) found that populations of the ant on the Tokelau atolls belonged to one of two common and behaviourally distinct mitochondrial haplotypes (A and D). Haplotype D was associated with more abundant populations and strong negative effects on the resident ant community. On Nukunonu atoll, these haplotype D populations were found on the western islets while haplotype A populations were found on the eastern islets. This distribution appeared to reflect the ages of the populations, with the western, more abundant haplotype D populations likely to be younger as they occurred on the inhabited islets (Fig. 5.1; Lester and Tavite 2004; Abbott *et al.* 2007). The less abundant haplotype A populations on the eastern islets likely represented an older invasion (Lester and Tavite 2004; Abbott et al. 2007). These eastern populations appear to be in densities low enough to allow resident ant species to co-exist (Lester et al. 2009). Haplotype D populations were also found on one islet of Fakaofo atoll at high abundance. At the time A. gracilipes was absent from the third atoll, Atafu. Clearly, if there is a persistent (i.e., causative) association between the haplotype, the abundance, and ecological effects of the ant, knowledge of the genetic identity of A. gracilipes populations would aid in the prediction of harmful effects.

In this study we revisited the *A. gracilipes* invasion on Tokelau, focussing in particular on differences in the distribution, abundance and effects of populations of the different haplotypes on resident ant communities. We addressed three specific questions: 1) Has the distribution of *A. gracilipes* among the three atolls changed? 2) Has the abundance of *A. gracilipes* changed on Nukunonu atoll? 3) Is there still an association between *A. gracilipes* abundance and effects on the invaded ant communities on Nukunonu atoll?

5.3 Methods

5.3.1 Study site

The atolls of the Tokelau archipelago lie in the humid tropics, ~500 km north of Samoa. The atolls experience a wet tropical rainforest climate with a mean annual temperature of 28°C, and mean annual rainfall of > 3000 mm (Mueller-Dombois and Fosberg 1998). Tokelau is made up of three atolls, Fakaofo, Nukunonu and Atafu, each with 31 - 58 low-lying coral islets encircling shallow lagoons. When Abbott et al. (2007) first studied A. gracilipes on Tokelau in 2004 the ant was observed on only two atolls: Nukunonu, where seven of about 42 islets were invaded, and Fakaofo, where two of about 51 islets were invaded. By 2006, the abundance of A. gracilipes appeared to be diminished, perhaps owing to the effects of Cyclone Percy in 2005 (K. Abbott, personal observation), but the ants remained in high densities in some areas. In 2006 poisoning was undertaken on the inhabited islets at Fakaofo and Nukunonu. These islets were Fenua Fala at Fakaofo atoll (at the time the only islet on which A. gracilipes was present at Fakaofo), and on Motuhaga and Nukunonu islets at Nukunonu atoll. Since then A. gracilipes has also been reported on Atafu atoll. Data for the current study were collected during a single visit to all three atolls in September - October 2011, which was seasonally comparable to the timeframe the Abbott et al. (2007) data were collected. The Tokelau atolls are serviced from Apia, Samoa by one of three vessels every 7 - 14 days. Apart from the occasional yacht these service vessels are the only apparent source of ants present on the atolls. Although distant from Samoa, the frequency of ship transport and biosecurity challenges mean that the likelihood of ant invasion is high.

5.3.2 Ecological surveys

To determine if *A. gracilipes* distribution has changed over the intervening years since the Abbott *et al.* (2007) study, we conducted hand searches on all islets at Nukunonu and Atafu atolls (approximately 42 and 40 islets respectively). Logistical constraints made it possible to survey Fakaofo only briefly (Fale islet). We conducted an ecological survey to assess the abundance of *A. gracilipes*, and ant community structure on nine islets on Nukunonu atoll (Fig. 5.1). Sites were selected based on the earlier presence of distinct genetic haplotypes (Abbott *et al.* 2007). These islets were Nukunonu, Motuhaga, Te Puka i Mua (all haplotype D), Te Fala, Pukapuka, Tokelau, Fenua Loa / Lalo (all haplotype A), Te Nonu and Te Palaoa (both previously uninvaded by *A. gracilipes*). Following the methods of earlier studies (Lester and Tavite 2004; Abbott *et al.* 2007), we selected two 15×15 m plots on each islet that were at least 100 m distant from each other. Pitfall traps were used to examine species richness and ant community composition, and assess the relative abundance of *A. gracilipes*. At each plot we installed five haphazardly placed pitfall traps (75 mm diameter). Traps were 1/3 filled with propylene glycol and left for 24 hours, after which all ants collected were counted, identified and named to species level where possible using the earlier collections of Abbott *et al.* (2007) and Lester *et al.* (2009) as a reference. Specimens of all species were retained at Victoria University of Wellington (VUW).

In addition to *A. gracilipes* counts in pitfall traps, on Nukunonu we also measured the abundance of *A. gracilipes* at each site based on forager activity, as described in Chapter 3. Briefly, this method uses a count of the number of ants crossing a laminated card in a 30 s period (Green *et al.* 2004). At each site we measured *A. gracilipes* activity at 11 stations spaced at 5 m intervals along three replicate 50 m transects spaced 10 m apart. Counts at all stations within each transect were summed, and the mean value of the three replicate transects was used as an index of relative abundance between sites. We also conducted card counts of *A. gracilipes* on Atafu atoll, and mapped the extent of the large invasion we found there using a GPS (Garmin).

We also interviewed representatives of the local communities on each atoll regarding their perceptions of the *A. gracilipes* invasion. We asked whether the people had observed changes in *A. gracilipes* distribution or abundance over time, and if they perceived the ant to be a pest.



a white circle (\bigcirc) ; new detections in 2011 are indicated by a black circle (\bullet) ; and islets that were invaded in both 2004 and 2011 are indicated by a grey circle (). Islets without circles were uninvaded in 2004 and remain uninvaded in 2011. The dashed arcs indicate the islets with haplotypes A and D present in 2004. The pair of light solid lines around the islets indicates the encircling reef. The inhabited Nukunonu and Motuhaga islets had been treated with toxic bait in 2006 (Abbott 2006a).

5.3.3 Molecular analyses

To ascertain the prevalence of *A. gracilipes* haplotypes we sequenced one or two individuals from each collection (pitfall traps and hand collections) on Nukunonu atoll (19 samples). In addition, we sequenced *A. gracilipes* from Fakaofo (2 samples) and Atafu (5 samples) to determine the current distributions of populations with different haplotypes throughout Tokelau. We also amplified DNA extractions of ants from Tokelau (2 samples), Tonga (2 samples), and Samoa (4 samples) that had previously been sequenced for cytochrome b (*cyt b*) by Abbott *et al.* (2007) to confirm that our *COI* haplotypes were congruent with the haplotypes found by Abbott *et al.* (2007), and to enable comparison between studies.

Ants collected for genetic analysis were stored in ~95% ethanol at 4°C. We extracted DNA using a modified Chelex protocol (Sepp *et al.* 1994). Individual workers were placed in microcentrifuge tubes, ground with sterile plastic pestles, and 150 μ l of a ~10% w/v Chelex-100 resin solution was added. The tubes were centrifuged briefly, boiled for 15 minutes in a water bath, chilled on ice for five minutes, and centrifuged at 15,000 × *g* for 15 minutes at 4°C. The supernatant containing DNA was stored at 4°C.

We used mitochondrial markers to determine if multiple mitochondrial haplotypes were present, targeting the *COI* (cytochrome oxidase I) region. Each 15 μ l PCR consisted of ~20 ng template DNA, 10× PCR Buffer, 0.4 μ g mL⁻¹ of bovine serum albumin (BSA), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 mM forward and reverse primer (CI-13 and CI-14, Quek *et al.* 2004), and 0.3 U of *Taq* DNA Polymerase (either Bioline BIOTAQ, Invitrogen Platinum® or New England Biolabs). Thermal cycling consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified products were purified using ExoSAP-IT (US Biochemicals) and sequenced on a 3730 Genetic Analyser (Applied Biosystems). We checked and edited the sequences using ClustalW (Thompson *et al.* 1994) implemented in MEGA4 (Tamura *et al.* 2007), and translated our aligned *COI* sequences of 533 base pairs to detect any premature stop codons that could indicate a sequence of non-mitochondrial origin. A BLAST search in GenBank confirmed the authenticity of our sequences as *A. gracilipes*. We constructed a parsimony haplotype network using TCS v 1.21 (Clement *et al.* 2000) to determine the relationships among the sequences. We have deposited the *cyt b* haplotypes of Abbott *et al.* (2007) in GenBank together with our *COI* haplotypes (Accessions pending).

5.3.4 Statistical analyses

To determine if the abundance of *A. gracilipes* in 2011 differed from 2004, and if haplotype D populations differed in abundance from haplotype A populations, we fitted a generalised linear model in R v 2.14 (Ihaka and Gentleman 1996; R Development Core Team 2011). We included only those plots where *A. gracilipes* was detected in 2004 or 2011. To account for over-dispersion of the data, we used a negative binomial distribution with a log link function and theta = 1. *Anoplolepis gracilipes* abundance was the response variable, and haplotype nested within sampling year were the predictor variables.

We also used generalised linear models to determine if there still existed an association between the abundance of yellow crazy ants, the haplotype of the population, and the effects on species diversity in the invaded community. Again, we used a generalised linear model with a negative binomial distribution, log link function and theta = 1. The response variable was species richness, and haplotype identity and *A. gracilipes* abundance were predictor variables. We included data for 2011 only and ran models with the interaction term, main effects and single predictors. We used AIC scores (Akaike 1973; Burnham and Anderson 2002) to determine the model that best fit the data, using AICc scores generated by the 'AICcmodavg' package (Mazerolle 2011) in R.

To assess differences in ant community composition between sampling years, and in 2011 between invaded and uninvaded plots, and between different haplotypes we used non-metric Multi-Dimensional Scaling (MDS) and PERMANOVA+ implemented in PRIMER v 6.1.11 (Clarke and Gorley 2006). Data for *A. gracilipes* were excluded from

the analysis, along with two plots where no ants were recorded (Tokelau [2011] and Te Palaoa [2004]). We log-transformed data to even out the effects of rare and abundant species, and used the Bray-Curtis index as a similarity measure as recommended by Clarke and Warwick (2001). The MDS was run using 1000 iterations with a Kruskal stress formula 1 and a minimum stress of 0.01. The significance of differences between groups was tested with PERMANOVA+ run over 99999 permutations (Anderson *et al.* 2008).

5.4 Results

5.4.1 Changes in the distribution of *Anoplolepis gracilipes* in Tokelau

Since 2004 the distribution of A. gracilipes has increased on the Tokelau atolls, and the majority of new detections were of haplotype D. On Fakaofo atoll we were able to visit only one of the two inhabited islets. Along with the known presence of A. gracilipes on the first inhabited islet (Fenua Fala), the second inhabited islet (Fale) that was not invaded by A. gracilipes in 2004 was found to be invaded in 2011 with a haplotype D population. Of the 40 islets searched on Atafu atoll, the sole inhabited islet, which was not invaded by A. gracilipes in 2004, was found to be invaded in 2011, also with a haplotype D population. The major Atafu invasion covered an area of approximately 37 Ha. At Nukunonu atoll, where we searched 42 islets, we found a number of differences in the distribution of A. gracilipes compared to 2004 (Table 5.1). Although we were unable to find A. gracilipes on a number of islets where it was abundant in 2004, populations of the ant were found on new islets in 2011, and populations remained on other islets (both haplotypes; Fig. 5.1). The new invasions at Nukunonu atoll were primarily of haplotype D (three islets), along with one invasion of haplotype A (Table 5.1). The new invasion on Motu Akea at Nukunonu atoll also included a single ant with a new haplotype (e), which was closely related to haplotype D (Table 5.1, Fig. 5.2). The populations that had apparently disappeared since 2004 included the most abundant haplotype D population (Te Puka i Mua islet: Fig. 5.1, Table 5.1), and three haplotype A populations (Lalo, Pukapuka and Tokelau islets: Fig. 5.1, Table 5.1). Although we were unable to definitively conclude that A. gracilipes was locally extinct from these islets, their previously extremely high abundance had substantially decreased to undetectable levels.

All the populations that have remained on Nukunonu atoll since 2004 were of the same haplotype as detected in the Abbott *et al.* (2007) study. However, the definite pattern of haplotype D populations occurring on the western islets and haplotype A populations occurring on the eastern islets (Abbott *et al.* 2007) was no longer present, as the newly found eastern Te Nonu islet population was of haplotype D. New invasions were more often of haplotype D.

Table 5.1: Comparison of the genetic haplotypes and total pitfall trap abundance per site of *Anoplolepis gracilipes* on invaded islets on Nukunonu Atoll, Tokelau in 2004 (Abbott *et al.* 2007) and 2011. In addition, for 2011 we included card counts of ant activity. Dashes indicate the islet was not invaded. Zero values for abundance indicates non-detection in pitfall traps rather than absence, as the presence of the ant was confirmed at the site. The abundance of *A. gracilipes* on Motu Akea and Te Afua atolls was not assessed as we had insufficient time for pitfall trapping or card counts after the ants were detected. However, the ants were difficult to find on these two islets and thus abundance was assumed to be very low.

Islet	Haplo	otype	Abun	dance	Card
	2004	2011	2004	2011	Count 2011
Te Nonu	-	D	-	1937	63
Te Puka i Mua	D	-	6776	-	-
Nukunonu	D	D	108	0	0
Motuhaga	D	D	405	8	0
Te Fala	А	А	379	298	23
Pukapuka	А	-	9	-	-
Tokelau	А	-	71	-	-
Fenua Loa	-	А	-	76	18
Lalo	А	-	934	-	-
Motu Akea	-	D e	-	NA	NA
Te Afua	-	D	-	NA	NA



Fig. 5.2: Parsimony network describing the relationships between *A. gracilipes COI* haplotypes detected in Tokelau in 2004 and 2011. The number of samples included for each haplotype is given in brackets. Nucleotide substitution differences between haplotypes and their position in the sequence fragment are indicated. Table 5.1 details the islets on which the haplotypes were found. All substitutions were synonymous. Also included in the haplotype network were sequences using DNA extractions of 2004 samples from Tokelau, Samoa and Tonga for haplotype A (3 sequences), and Samoa and Tokelau for haplotype D (4 sequences), and 2011 samples from Atafu and Fakaofo (haplotype D, 7 sequences). The sizes of the circles reflect the number of sequences for the given haplotype.





Fig. 5.3: Interaction plot comparing differences in mean pitfall trap abundance (per plot \pm SE) of *A. gracilipes* haplotype A (open circles) and D (closed circles) in 2004 and 2011 on Nukunonu atoll, Tokelau. The differences in abundance were not significant between sampling years (GLM: estimate = -0.623, *t* = -0.681, *P* = 0.503), or between haplotypes in 2011 (GLM: estimate = 1.243, *t* = 1.294, *P* = 0.210) but haplotype D was significantly more abundant in 2004 (GLM: estimate = 1.943, *t* = 2.421, *P* = 0.025).

While the overall abundance of *A. gracilipes* at Nukunonu atoll appeared to be lower than in 2004, only the haplotype D populations had declined significantly (Fig. 5.3 & Fig. 5.4, Table 5.1). In 2011 the mean abundance of *A. gracilipes* per pitfall trap among haplotype D islets was 64.8 ± 23.2 SE (n = 30, 3 islets) compared to 2004, when haplotype D islands had 243.0 ± 112.6 SE ants per pitfall trap (n = 30, 3 islets: Abbott *et al.* 2007). Among haplotype A islets, mean pitfall trap abundance of *A. gracilipes* was 18.7 ± 4.7 SE in 2011 (n = 20, 2 islets), and 34.8 ± 8.3 SE ants per pitfall trap in 2004 (n = 40, 4 islets: Abbott *et al.* 2007). Pitfall trap abundances were highest for the new haplotype D invasion on Te Nonu islet (Table 5.1). Although the differences in pitfall trap abundance were not statistically significant between sampling years (GLM: estimate = -0.623, t = -0.681, P = 0.503), or between haplotypes in 2011 (Fig. 5.3; GLM: estimate = 1.243, t = 1.294, P = 0.210), haplotype D abundance had significantly declined since 2004 (Fig. 5.3; GLM: estimate = 1.943, t = 2.421, P = 0.025).



Fig. 5.4: The relationships between *A. gracilipes* abundance (total number of ants per invaded plot) and resident ant species richness in: (a) 2004; and (b) 2011. Dashed lines indicate the smoothed spline line of best fit with a span of 1, and solid lines indicate the line of best fit under the assumptions of a linear model for data from invaded plots only. Plots with haplotype D ants are indicated by black circles (\bullet), haplotype A by grey circles (\bullet), and uninvaded plots by open circles (\circ). In 2011 we found no relationship between the abundance of *A. gracilipes*, or haplotype D, and species richness of the invaded ant community (GLM: estimate = 0.2485, t = 0.608, P = 0.560).

Our card counts ranged from 0 to 63 at Nukunonu atoll, and were highly correlated with pitfall trap counts for each plot (Spearman's rank correlation $R_s = 0.83$, S = 27.9, P = 0.001). Both card counts and pitfall trap abundances were highest for the new haplotype D invasion on Te Nonu in Nukunonu (Table 5.1). The card counts for the 37 Ha invasion of haplotype D on Atafu atoll were 20, 52 and 63. The lowest card count at Atafu atoll was at a fuel storage area where local residents first noticed the ants.



Bray-Curtis similarity of log (X+1) transformed abundance data. Anoplolepis gracilipes were excluded from the analysis. On MDS plots stress values (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant community composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant composition in 2011 between invaded and uninvaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001), but no differences in ant composition in 2011 between invaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001, *P[perm]* < 0.001), but no differences in ant composition in 2011 between invaded sites (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001, *P[perm]* < 0.001, *P[perm]* < 0.001, *P[perm]* Fig. 5.5: Two-dimensional MDS plots of Nukunonu, Tokelau ant communities comparing differences between: (a) sites in 2004 and 2011; (b) Anoplolepis gracilipes invaded and uninvaded sites in 2011; and (c) Anoplolepis gracilipes haplotypes in invaded sites in 2011. The analysis used less than 0.2 indicate a good fit. The accompanying PERMANOVA+ revealed significant differences between ant communities in 2004 and 2011 $1.01_{1.15}$, P[perm] = 0.421) or between haplotypes (*Pseudo* $F = 1.30_{1.8}$, P[perm] = 0.218).

5.4.3 The association between *Anoplolepis gracilipes* abundance and effects on the resident ant communities

Excluding *A. gracilipes* we collected a total of 19 ant species in our pitfall traps (Table 5.2), compared to 16 species collected by Abbott *et al.* (2007). In 2011 species richness per plot ranged from 0 to 8 species (mean \pm SE: 3.8 ± 0.7 species in *A. gracilipes* invaded sites and 3.4 ± 0.7 species in uninvaded sites). Mean abundances of ants other than *A. gracilipes* ranged from 0 to 143 ants per plot (mean \pm SE: 30 ± 15.2 ants in *A. gracilipes* invaded sites and 18 ± 7.8 ants in uninvaded sites).

We found no significant relationship between haplotype or *A. gracilipes* abundance and species richness of the resident ant community. The generalised linear model with the lowest AICc score included haplotype as a main effect (AICc = 59.01; GLM: estimate = 0.2485, t = 0.608, P = 0.560). The next best fitting models included either *A. gracilipes* abundance (AICc = 59.10), or haplotype and *A. gracilipes* abundance as main effects (AICc = 64.97). In all the models no effects were statistically significant.

Two-dimensional MDS and PERMANOVA+ also revealed no significant differences in ant community composition in 2011 between *A. gracilipes* invaded and uninvaded sites (*Pseudo* $F = 1.01_{1,15}$, *P[perm]* = 0.421) or between haplotypes (*Pseudo* $F = 1.30_{1,8}$, *P[perm]* = 0.218). The PERMANOVA+ analysis did however, reveal significant differences between ant communities in 2004 and 2011 (*Pseudo* $F = 5.78_{1,32}$, *P[perm]* < 0.001; Fig. 5.5), which is consistent with a high rate of species turnover. PERMDISP analysis found that dispersions were homogeneous in 2011 between *A. gracilipes* invaded and uninvaded sites (*Pseudo* $F = 0.672_{1,15}$, *P[perm]* = 0.429) and between haplotypes (*Pseudo* $F = 2.44_{1,8}$, *P[perm]* = 0.214), but there were significant differences in dispersion between ant communities in 2004 and 2011 (*Pseudo* $F = 7.48_{1,32}$, *P[perm]* = 0.011), which indicates differences between years was specifically owing to differences in dispersion among sites.

Species	A. gracilipo isla	es invaded	Islets not in	waded by A.
	2004	2011	2004	2011
	(7 islets)	(7 islets)	(2 islets)	(4 islets)
Anochetus graeffei	1	3	2	2
Anoplolepis gracilipes	7	5		
Cardiocondyla nuda	2	2		
Iridomyrmex anceps		1		
Monomorium floricola	3	5		1
Monomorium minutum	1			
Paratrechina bourbonica		3		
Paratrechina longicornis		1		2
Paratrechina vaga	2		1	2
Pheidole fervens	6		2	
Pheidole oceanica	4	1	1	2
Pheidole sexspinosa	4	2		2
Pheidole umbonata	3		2	
Pheidole sp.		2		1
<i>Ponera</i> sp.				1
Pyramica membranifera	1			
Rogeria stigmatica	2		1	
Strumigenys godeffroyi	1			
Tapinoma melanocephalum		3		2
Tapinoma minutum		2		1
Tetramorium bicarinatum		2	1	2
Tetramorium lanuginosum	5	4	1	2
Tetramorium simillimum	2	2	1	
Tetramorium tonganum	1	1	1	
Tetramorium sp.				1

Table 5.2: Comparison of the ant species caught in pitfall traps on Nukunonu Atoll, Tokelau in 2004 (Abbott *et al.* 2007) and 2011, and the number of islets on which they found.

5.4.4 Tokelau residents perceptions of the Anoplolepis gracilipes invasion

The perceptions of the people of Tokelau were consistent with our ecological findings regarding the distribution and abundance of *A. gracilipes*. In 2003 and 2004 residents considered *A. gracilipes* to be a major pest. This ant had a devastating effect on crop production, irritated livestock, and interrupted the sleep of local residents by crawling over them during the

night. Residents attempted to halt the spread and limit the abundance of the ant by burning of trees (Lester and Tavite 2004). In 2011 the residents of Nukunonu atoll considered that the abundance of *A. gracilipes* had decreased markedly since 2004 and they no longer considered the ant a serious problem. Nor did they report variations in abundance throughout the year. Management via poisoning had taken place in 2006 on inhabited islets at Nukunonu and Fakaofo atolls (Abbott 2006a). However, a cyclone in late 2005 shortly before poisoning was also attributed by the residents to be responsible for a significant reduction in *A. gracilipes* abundance.

At Fakaofo atoll residents were similarly unconcerned with the abundance of the ant on the islet that had been initially invaded (Fenua Fale), as it had declined substantially. However, people were concerned with the new invasion on the islet of Fale, where they reported the ant was a nuisance around outdoor kitchens. It was uncertain when *A. gracilipes* was first detected on Fale, but it was some months rather than years prior to the current study.

Since the 2004 study, *A. gracilipes* has been observed on the inhabited islet at Atafu atoll. The ant first became apparent to residents in 2008 around a fuel storage area, had spread from there to cover approximately 37 Ha bordering on the village, and was also found at low abundance in a garden within the village. Many residents and the Atafu village council expressed serious concerns about the threat of the ant encroaching on the village, and were eager for management action.

There is no doubt that the supply vessels are a major source of ant species to the atolls. During our voyage from Samoa, we observed seven species of ants on the vessel These ant species were all later found to be resident on Tokelau, and included *Tetramorium bicarinatum*, *Paratrechina bourbonica*, *P. vaga*, *P longicornis*, *Tapinoma melanocephalum*, *Cardiocondyla nuda* and *Monomorium floricola*. No arrivals on the atolls during our visit were subjected to a quarantine process, and no biosecurity checks were in place in Apia prior to our departure for Tokelau.

5.5 Discussion

Although the effects of invasive species are often most severe when the invading population is in high numbers (Ehrlich 1986; Crawley 1987; Mack et al. 2000), their abundance and ecological effects clearly do vary temporally, and population declines or collapses occasionally occur (e.g., Lewis et al. 1976; Morrison 2002; Hill et al. 2003; a number of studies reviewed by Simberloff and Gibbons 2004; Cooling et al. 2011). The distribution of A. gracilipes and the abundance of haplotype D populations in Tokelau in 2011 differed substantially from 2004. Although haplotype D populations were responsible for most new invasions between 2004 and 2011, these populations were not significantly more abundant than those of haplotype A at Nukunonu atoll. Thus, the pattern of numerical dominance of haplotype D populations was no longer present, and there no longer existed an association between haplotype D populations and effects on the invaded ant communities. Indeed, the resident ant communities invaded by A. gracilipes did not differ from the uninvaded communities in structure or species richness, and other ants often suffer the earliest and most severe effects of invasive ants (Holway et al. 2002). The perceptions of the local Tokelauan people also indicated a substantial decline in A. gracilipes abundance and residents were no longer concerned about the ant when it was in low abundance.

5.5.1 Changes in Anoplolepis gracilipes distribution on Tokelau

The distribution of *A. gracilipes* among the atolls of Tokelau has increased to all three atolls, and although the abundance of haplotype D populations had declined, this haplotype featured in the majority of new detections. Although we were unable to sample ants from Samoa (the most likely origin of ants in Tokelau), haplotype D was present in Samoa and Tokelau in 2004 (Abbott *et al.* 2007). While we found a single occurrence of a new haplotype (e), this new haplotype may or may not be associated with a new invasion, as Abbott *et al.* (2007) also found rare haplotypes among the populations they sampled. However, we cannot be certain whether the new detections were due to arrivals from within Tokelau or from Samoa. Indeed, the ant communities at Nukunonu atoll are strikingly different to those of 2004, indicating high species turnover, and we found seven ant species in our brief survey of the ship to Tokelau. In 2002 three species (*Monomorium*)

minutum, *P. vaga*, and *T. bicarinatum*) were observed in only four small potted plants being moved onto the supply vessel to Nukunonu atoll (Lester and Tavite 2004). While all the species found on the supply vessels were all found in our pitfall traps, the risk of re-invasion (and invasion of new species) is clearly high as there were no biosecurity measures in place either before our departure from Samoa or on arrival in Tokelau.

Haplotype D populations seem more likely to feature in new detections because they were present on the inhabited islets in Tokelau, and are thus more likely to be transported. An alternative hypothesis, that haplotype D populations have displaced other ants due to enhanced competitive ability, seems less likely given the decline in abundance of these populations.

5.5.2 Density-dependent effects of Anoplolepis gracilipes invasion

Frequently the earliest and worst effects of invasive ants are on the resident ant community (Holway et al. 2002). Thus, these communities, which are straightforward to sample, can provide an indication of the potential wider ecological effects of the invasive ant. It is often the case that the effects of invasive ants on the resident ant communities are density-dependent (e.g., Ross et al. 1996; Le Breton et al. 2005; Krushelnycky and Gillespie 2008). The ecological effects of A. gracilipes are clearly density-dependent on ant communities (Hill et al. 2003; Abbott et al. 2007; Lester et al. 2009; Hoffmann and Saul 2010; Drescher et al. 2011; Chapter 4) and other invertebrates (McNatty et al. 2009; Boland *et al.* 2011). It is also clear that not all populations of *A. gracilipes* are sufficiently abundant for negative ecological effects to occur (Abbott et al. 2007; Hoffmann and Saul 2010; Boland et al. 2011; Drescher et al. 2011), and that abundance also varies naturally over time (Lewis et al. 1976; Haines and Haines 1978b; Hill et al. 2003; Abbott 2006b; this study). The overall A. gracilipes abundance we measured at Nukunonu atoll appeared to be below a threshold at which negative ecological effects may be significant on the resident ant community. While this threshold is difficult to ascertain quantitatively, card count abundances may provide an approximate indicator. On Christmas Island in the Indian Ocean for example, management of A. gracilipes is initiated if card count values exceed 37, the point beyond which negative effects on native land crabs become apparent (Boland *et al.* 2011). With the exception of the new haplotype D invasions on Te Nonu

islet (card count 63) and Atafu atoll (maximum card count 63), our card counts did not exceed this value. By contrast, card count abundance measured on Christmas Island ranged between ~14 and 136 in 2000 - 2002 (Abbott 2005), and card count abundance in the Northern Territory of Australia in 2009 ranged from 0 to 42 (Chapter 3). We advocate the use of card counts in *A. gracilipes* studies. Such an approach could enable a more accurate identification of the threshold at which density-dependent effects become apparent, enable comparison among studies, and highlight populations for which management options may be considered.

5.5.3 Anoplolepis gracilipes population dynamics

Although the differences in *A. gracilipes* abundance between 2004 and 2011 could be attributed to seasonal variation (Greenslade 1971; Baker 1976; Suwabe *et al.* 2009), we do not consider this to be the case. First, people on Tokelau were not concerned about the ant where it had persisted at low abundance since 2004. Residents were, however, concerned about the new *A. gracilipes* invasions, particularly on Atafu, where the ant had been noticeably abundant since 2008. Second, we found no differences in ant community structure or species richness between *A. gracilipes* invaded and uninvaded sites on Nukunonu atoll in 2011. As ant communities may take time to assemble (Badano *et al.* 2005), they have likely adjusted to lower densities of *A. gracilipes* over some time. Finally, overall we found no significant difference in overall abundance between years - the differences in abundance were primarily owing to the decline in haplotype D populations.

Explanations for the decline in abundance of *A. gracilipes* haplotype D populations are not immediately obvious, as is often the case for population collapses of invasive species (Simberloff and Gibbons 2004). Decline of *A. gracilipes* on Mahé in the Seychelles was attributed to a reduction in protein resources (Haines and Haines 1978b), although a decline in protein availability appears unlikely on Tokelau. The decline in *A. gracilipes* abundance on inhabited islets was attributed by Tokelauan people to Cyclone Percy in 2005, prior to poisoning. Although we cannot be certain of the relative effects of the cyclone or of poisoning, new invasions have certainly occurred since the cyclone, and many of the existing populations appear to have persisted. The eagerness of Atafu residents for *A. gracilipes* to be controlled there provides an opportunity to undertake management, and determine its efficacy, focussing on areas where *A. gracilipes* is highly abundant.

Cycles of population explosion and decline appear to be a feature of a number of A. gracilipes invasions. Documented declines have occurred on the Seychelles (Lewis et al. 1976; Hill et al. 2003), and temporal variation has been noted on Christmas Island (Abbott 2006b). Similar declines have occurred in the Northern Territory of Australia (B. Hoffmann, personal communication). The causes of such declines among invasive species are not well understood, although disease is sometimes invoked (Simberloff and Gibbons 2004). A number of endogenous parasites are known to affect ant population dynamics, and have been explored as methods for biological control. For example, the red imported fire ant Solenopsis invicta has populations infected with a number of parasites including a microsporidian and viruses (Oi et al. 2008; Briano 2009; Valles et al. 2010). Anoplolepis gracilipes is known to harbour multiple endogenous bacteria (Sebastien et al. 2012; Chapter 6), although it is not yet known if these bacteria affect population dynamics. If parasites or disease are involved, their effects need not be catastrophic by themselves. Rather, they may decrease the population size to a point where Allee effects may result in compromised fitness (Allee 1931). The exploitation of Allee effects could be employed to more effectively manage invasions (Taylor and Hastings 2005; Pachepsky and Levine 2010; Tobin et al. 2011).

Although it is tempting to assign a pathogenic cause to population declines, invertebrate population dynamics often involve strong cycles of alternating high and low abundance (Lester and Burns 2008). In addition, the abundance of invasive ants and their effects on the recipient ant community may be higher shortly after arrival, and then decline to an equilibrium (Morrison 2002). Lester and Tavite (2004) and Abbott *et al.* (2007) inferred that the abundant *A. gracilipes* populations on the inhabited islets were newer arrivals, and we also observed the same pattern. The association that Abbott *et al.* (2007) found between *A. gracilipes* haplotype D abundance and effects on the resident ant community may thus have been owing to their recent arrival, rather than to the haplotype itself. The abundance and potential effects of *A. gracilipes* on the Tokelau atolls may therefore be higher in the period shortly after the initial invasion, although how long this period may be is unknown, as is any preceding lag time when the ants are not observed. By contrast, on Christmas Island, high abundance has been inferred to occur at a later stage in the invasion process (Abbott 2006b).

5.6 Conclusion

In Tokelau the correlation that Abbott *et al.* (2007) identified between haplotype D and greater abundance of *A. gracilipes* was no longer detected in 2011. Although overall the distribution of the ant has increased in Tokelau, many *A. gracilipes* populations have declined or disappeared on some islets. Understanding the population dynamics of invasions, particularly the underlying mechanisms promoting population decline, should be a high priority for invasion ecology (Taylor and Hastings 2005; Pachepsky and Levine 2010; Cooling *et al.* 2011; Tobin *et al.* 2011). *Anoplolepis gracilipes* has significant effects not only on ant communities and invertebrates, but also on forest birds (Davis *et al.* 2008; Davis *et al.* 2010), and arboreal- and ground-nesting seabirds (Feare 1999), which are common on Tokelau. Thus, we do not suggest that occasional population declines may eliminate the need for invasive species management, as considerable ecological damage may occur in the interim. Rather, understanding and exploiting the underlying mechanisms of decline of these populations could provide a useful aid to management.

Chapter 6. A preliminary metagenomic survey of *Anoplolepis* gracilipes endogenous parasite and symbiont communities

6.1 Abstract

Invasive species often experience rapid increases in population size when introduced to a new range, which is followed by population stabilisation. However, occasionally declines of large populations of invasive species are observed, sometimes to the point of local extinction. Although the causes of these declines are difficult to ascertain, disease is often assumed. The invasive yellow crazy ant Anoplolepis gracilipes has been observed to experience unexplained population declines, and we hypothesised that these declines may be owing to endogenous bacterial or eukaryote parasite infection. We used Illumina GAIIx short-read sequencing of five samples, each of at least 50 ants that were pooled from A. gracilipes populations. Assemblies of the resulting reads were used to BLAST search existing public GenBank databases for potential eukaryote and prokaryote symbionts and parasites. Although our results suggested no evidence of eukaryotic parasites of insects, we found that bacterial community composition and diversity varied among our samples. Although the prevalence of many bacterial groups did not differ among A. gracilipes populations, the population with the lowest ant abundance had the lowest number of matches to bacterial sequences on the databases. Compared to other populations, this low abundance population also had a much higher prevalence of bacterial orders that have been identified as having beneficial relationships with ants, particularly involving antibiotic activity. The most prevalent of these orders was Burkholderiales, which has both pathogenic and beneficial members, but is only known to be beneficial in insects. This study has identified a range of bacteria that were previously unknown in A. gracilipes. Several of these are candidates for further research, which may shed light on the effects of endogenous parasites and symbionts on the population dynamics of the species.

6.2 Introduction

Invasive species often experience rapid increases in population size when introduced to a new range, which may be followed by population stabilisation to a lower level (Morrison 2002; Strayer *et al.* 2006). However, unexplained population declines of invasive species are occasionally observed, which sometimes lead to local extinction (e.g., Lewis *et al.* 1976; Morrison 2002; Hill *et al.* 2003; a number of studies reviewed by Simberloff and Gibbons 2004; Cooling *et al.* 2011). The causes of these declines are varied and difficult to determine, but may involve fluctuations in population dynamics, pathogens, or exhaustion of resources (Simberloff and Gibbons 2004). A number of parasites are known to affect insect population dynamics. For example, red imported fire ant (*Solenopsis invicta*) populations can be infected with a number of different parasites including a microsporidian and viruses (Oi *et al.* 2008; Briano 2009; Valles *et al.* 2010). Parasites not only affect the abundance of their hosts directly, but can also affect the host phenotype to ensure their own dispersal, to the detriment of the host (Yanoviak *et al.* 2008; Hoover *et al.* 2011).

The invasion success of pest species is sometimes attributed to a loss of natural enemies (herbivores, parasites or pathogens) in the invaded range (e.g., Mitchell and Power 2003; Torchin *et al.* 2003; Tsutsui *et al.* 2003a). However, when the host species is introduced to a new environment, a loss of parasites or symbionts from the native range may potentially be accompanied by exposure to a variety of new organisms that may be beneficial or harmful. The changes in the dynamics of these endogenous populations may cause changes in stress tolerance and parasite resistance, that could potentially affect invasion success (Feldhaar 2011).

The relationship between bacteria and their host insects is complex. Although endogenous bacteria may be harmful (Wenseleers *et al.* 2002), many are also beneficial (Dillon and Dillon 2003; Dale and Moran 2006; Feldhaar and Gross 2009), and if their function has a large effect on population dynamics, their loss could also result in population declines (Russell *et al.* 2009). For example, host sex-ratio manipulation in favour of females by bacteria such as *Wolbachia, Arsenonophonus*, and *Rickettsia* (Gherna *et al.* 1991; Engelstädter and Hurst 2009; Himler *et al.* 2011), may in some cases

have positive effects on the fitness of the host by increasing abundance and spread (Himler *et al.* 2011). However, *Wolbachia* may reduce fitness among ants (Wenseleers *et al.* 2002), and a number of invasive ant species have exhibited a loss of *Wolbachia* in the introduced range (Shoemaker *et al.* 2000; Tsutsui *et al.* 2003a), which may contribute to their invasion success (Zientz *et al.* 2005). However, the function of common insect endosymbionts such as *Wolbachia* in ants is unclear and may vary (Zientz *et al.* 2005). Thus, the relationships between bacteria and their hosts can be complex and context-dependent.

Metagenomic studies may be a useful tool to assist in understanding the role of symbionts in the population dynamics of insects (Gosalbes *et al.* 2010). Studies of this nature can enable characterisation of endogenous communities, to generate and test hypotheses concerning interactions among members of these communities (Kunin *et al.* 2008). The advent of short-read (next / second generation) sequencing technologies enables metagenomic categorisation of entire communities, and the comparison of these communities among populations. Typically, metagenomic studies focus on bacterial communities, and target the small-subunit rRNA (16S rRNA) gene as it has a large reference database (Kunin *et al.* 2008). The two common approaches are using 454 pyrosequencing (Ishak *et al.* 2011; Tamaki *et al.* 2011), or Illumina short-read sequencing (Bartram *et al.* 2011; Caporaso *et al.* 2011). Eurkaryote parasites such as nematodes and fungi can also be detected using a metagenomics approach (Kunin *et al.* 2008), although studies of this nature are rare (Petrosino *et al.* 2009).

The yellow crazy ant, *Anoplolepis gracilipes*, is considered one of the most widespread and damaging invasive ants (Holway *et al.* 2002). Although it can occur at very high densities (O'Dowd *et al.* 1999; O'Dowd *et al.* 2003; Abbott 2006b), some populations of the ant are found at very low abundance, and unexplained population declines and disappearances have also been reported (Haines and Haines 1978b; Abbott 2006b; B. Hoffmann, personal communication; P. Green, personal communication; Chapter 5). Populations of *A. gracilipes* are known to harbour a number of endogenous bacteria, including *Arsenophonus*, *Wolbachia*, and Rhizobiales (Sebastien *et al.* 2012), although other bacteria and endogenous eukaryotes have not been investigated in the species. We hypothesised that there could be associations between the prevalence of a

wider variety of parasites and the variation in abundance of *A. gracilipes*. In our focal study population in Arnhem Land in the Northern Territory of Australia, the ants show patterns of abundance that differ spatially, and abundance also appears relatively low compared to a number of populations elsewhere (Chapters 4 and 5). Here we use a short-read sequencing metagenomic approach to detect candidate symbionts and parasites from a number of *A. gracilipes* populations. The aim of the study is to: 1) explore the endogenous metagenomic diversity and community composition of *A. gracilipes*; 2) identify differences in diversity and community composition in the Arnhem Land population. Our study is a preliminary analysis for the purpose of generating hypotheses for further exploration of the effects that these candidate endogenous organisms (and their interactions) may have on the population dynamics of *A. gracilipes*.

6.3 Methods

6.3.1 Sample collection

Samples were collected from Arnhem Land in Australia's Northern Territory (March 2009), Upolu in Samoa (October 2009), Christmas Island in the Indian Ocean (November 2009), and Okinawa in Japan (April 2010). Samples were collected from a single location in Upolu, Samoa, at three locations in Okinawa, Japan, and at a number of locations on Christmas Island and in Arnhem Land, Australia (Fig. 6.1). Individual ants were collected by aspirator while foraging or during nest excavations. Ants were stored in 95% ethanol at 4°C, or in RNA*later* (AMBION, Inc., Austin, Texas) at -20°C and -80°C (from six months after collection) until DNA extraction. All ants from Okinawa were stored in 95% ethanol. Approximately half the samples from other sites were stored in ethanol, and the remainder in RNA*later*.

Estimates of abundance were not obtained in Okinawa or Samoa, however, on Christmas Island and in Arnhem Land abundance estimates were primarily based on card counts at the site (see Green *et al.* 2004 for a description of the method), or visual assessment in some cases when ants were at very low abundance. The abundance of samples was categorised as high when card count values exceeded 30. Typically card count values for low abundance sites were zero.

6.3.2 Illumina GAIIx sequencing

Ants from a number of collections were pooled into samples for sequencing in order to compare differences between populations (Fig 6.1). Ants were washed in ethanol to ensure no material remained on the integument. We extracted DNA from up to 10 ants at a time, using genomic kits (Bioline and Invitrogen), with further purification using a standard phenol and chloroform / isoamyl protocol, following which genomic DNA was precipitated in ethanol and dried. The DNA quantity was assessed using gel electrophoresis, and Nanodrop (Nanodrop Technologies Inc. 2005). The DNA extractions were of good quality, as indicated by gel bands at ~10,000 base pairs, minimal smearing and Nanodrop concentrations of ~200+ ng / μ l and A260/280 ratios of > 1.8, all of which met the standards expected for Illumina sequencing. The DNA from multiple extractions was pooled and was re-suspended in 20 μ l molecular grade H₂O and precipitated in 44 μ l 100% ethanol and 2 μ l 3M sodium acetate for transport to the sequencing facility (Macrogen Inc. Seoul, Korea). Quality tests following library preparation at Macrogen met the standards for Illumina sequencing, which confirmed the results of our quality assessments.

The original design intention was to sequence populations as separate samples, and also separate high and low abundance populations from Christmas Island and Arnhem Land. Unfortunately, a number of samples were inadvertently incorrectly pooled by the sequencing facility (Table 6.1). Fortunately, one sample of Arnhem Land ants only at low abundance was preserved (Arnhem Land 4). A second sample had a high prevalence of ants from low abundance Arnhem Land sites (Arnhem Land 3). Libraries were created for each of the five samples and each sample was run on a single lane of an Illumina Genome Analyzer IIx (GAIIx; five lanes in total) producing paired-end reads of 101 bases. Postprocessing at Macrogen included an analysis pipeline using RTA v 1.8 and CASAVA v 1.7.0 (Illumina). Sequencing resulted in an average of 7.7 Gbases of data per lane and a total of 34.5 Gbases over the five lanes.





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The For proportion of sample from low abundance sites refers to the proportion of ants in the sample that came from sites that were at low abundance. For Table 6.1: Sample name, locations sampled, number of sites per location, and total number of Anoplolepis gracilipes ants in the sample from which DNA was extracted for next generation Illumina GAIIx sequencing. Abundance at site (number of ants) indicates the abundance of ants at sites example, the Christmas Island 3 site included 45 / 55 ants from low abundance sites, whereas all ants in the Arnhem Land 4 sample came from sites at included in the sample. The number in brackets refers to the total number of ants in the sample that came from low and high abundance sites. example, the Christmas Island 3 sample included 10 ants sampled from sites at high abundance and 45 ants sampled from sites at low abundance. low abundance.

Sample name	Sampling	Number	Total number	Abundance at site	Proportion of sample from
	location	of sites	of ants	(number of ants)	low abundance sites
	Okinawa	3	80	Not known	VIV
OKIIIAWA / SAIIIUA	Samoa	1	76	Not known	W
Christmas Island 3	Christmas Island	8	55	High (10), Low (45)	0.81
Chan I and A / hadleI construction	Christmas Island	6	31	High (18), Low (13)	
Chilisthias Island / Annieni Land Z	Arnhem Land	6	72	High (24), Low (48)	60.0
Arnhem Land 3	Arnhem Land	6	51	High (10), Low (41)	0.80
Arnhem Land 4	Arnhem Land	11	57	Low (57)	1.00

6.3.3 Quality assessment of Illumina GAIIx sequencing

To validate the quality of the sequencing run we used the custom written java package SolexaStats (see Appendices). This function calculates the proportion of bases in the sequence files where the FASTQ Q value (quality score) exceeded 30, which is equivalent to a base call accuracy of 99.9 % (i.e., the probability of a base being incorrect is 0.001). SolexaStats revealed an average Q value > 30 of 0.91 (Table 6.2), which exceeded the minimum guidelines recommended by Illumina (Illumina 2011), which suggest that > 0.80 of Q values should be greater than 30.

To assess the quality of the run and detect systemic errors in the data we used SolexaQA.pl v 1.7 (Cox *et al.* 2010), using the software default of P = 0.05 (Q < 13, or 1) base call error every 20 nucleotides). SolexaQA analysis found occasional poor quality scores for sequences in the first two lanes of data. These had little effect on the overall quality of the run, and were not apparent in later lanes. We then used DynamicTrim.pl v 1.7 (Cox et al. 2010) to exclude bases with a more stringent Q < 30 or P = 0.001 (1-in-1000 probability of an incorrectly called base). We used this stringent quality score in order to minimise the inclusion of poor quality sequences. Trimming with DynamicTrim.pl to retain bases with scores of Q > 30 reduced file sizes to 80.4% of the original size, and average sequence length to 74 bases (Table 6.2). The LengthSort.pl v 1.7 (Cox et al. 2010) script was then used to remove sequences that were less than 25 base pairs in length. Once sequences shorter than 25 base pairs were removed, the average file size was 76.8% of the original size. In some cases, one of the paired-end reads (usually the second) was shorter than the 25 base pair threshold. This is typical of GAIIx data, where the second read of the pair is often less well resolved. LengthSort.pl separated these into single-end read file, which included 4.5% of the data on average, or approximately 3 million reads per file (Table 6.2).

Table 6.2: Results from Illumina GAIIx sequencing and quality filtering of A. gracilipes samples. GAIIx paired-end reads are the number of paired-
and sequences generated per sample. The SolexaStats score refers to the proportion of the sequences (reads) that had a quality (Q) score of > 30 (i.e.,
he probability of a base being in error was 0.0001 or less). The DynamicTrim results refer to the mean and median length of sequences once low
quality bases were trimmed (i.e., after trimming all bases remaining in the sequence had an error probability of 0.0001 or less). The reads input to
Velvet assembly refers to the number of reads longer than 25 bases after trimming that were included in the assembly input. For example, after quality
iltering of the 37,156,165 initial GAIIx reads for the Okinawa / Samoa sample a total of 35,048,267 reads > 25 base pairs long were used for
assembly. Of these reads 31,933,243 included both pairs of the initial paired-end reads. A further 3,115,024 reads were of only one of these pairs. In
hese cases all reads were the first of the pair, which is typical of GAIIx data, where the second read of the pair is often less well resolved.

Sample name	GAIIx paired- end reads	SolexaStats	Dynam	iicTrim	Reads in	put to Velvet a	ssembly
		•	Mean	Median	Paired-end	Single-end	Total
Okinawa / Samoa	37,156,165	06.0	72.3	81	31,933,243	3,115,024	35,048,267
Christmas Island 3	36,182,990	0.92	76.5	06	31,570,415	2,791,639	34,362,054
Christmas Island / Arnhem Land 2	38,837,785	0.19	74.4	87	33,282,102	3,295,769	36,577,871
Arnhem Land 3	38,711,456	0.91	74.0	86	32,948,217	3,363,427	36,311,644
Arnhem Land 4	39,665,532	0.91	73.4	86	33,289,077	3,657,152	36,946,229

We also used FastQC v 0.9.6 (Andrews 2011) to determine other indicators of poor quality including over-represented sequences, and inequality in base composition. FastQC analysis revealed that sequences in the second of the pair of reads were sometimes overrepresented by unresolved repeat sequences (NNNNn). These made up on average only 0.30% of all bases (compared to 0.16% of bases in the first of the pair of reads). These repeats are generally caused by sections which were joined by read-pairs but separated by a repeat pattern that could not be resolved (Andrews 2011). We trimmed these unresolved repeats using LengthSort.pl. FastQC identified a base inequality in all lanes, which consisted of an A+T bias (A+T = 0.68). All ant genomes sequenced have shown this feature (Bonasio et al. 2010; Smith et al. 2011a; Smith et al. 2011b; Suen et al. 2011; Wurm et al. 2011), thus we consider the bias was not owing to any failure in sequencing. FastQC identified a number of 5-mers that appeared in 3-5 fold greater number than expected. These consistently involved GC rich sequences such as CGCGC, GCGCT, CCGCG etc. As FastQC calculates the expected level at which a given 5-mer should have been observed in the sequence based on the base content of the library as a whole, the AT bias in our dataset may have caused an over-representation of GC / CG related patterns in this analysis.

6.3.4 Genomic sequence assembly

We assembled the genomic sequences using Velvet v 1.1.0.5 (Zerbino and Birney 2008; Zerbino 2010). Subsequent to quality checking and file trimming, paired-end sequence files were interleaved using the shuffleSequences_fastq.pl Perl script bundled with Velvet. Both the shuffled paired-end files and the single-end files output from LengthSort.pl were used in the assemblies. The VevetOptimiser.pl Perl script (Gladman 2011) was used to determine optimal assembly parameters. The initial parameter settings to determine the optimum parameters used k-mer lengths of between 43 and 57. We calculated the mean coverage for contigs > 100 base pairs in each lane in R v 2.14.0 (Ihaka and Gentleman 1996; R Development Core Team 2011). Our Velvet assemblies resulted in between 331k and 440k contigs per lane (Table 6.3). N50 values (50% of the assembly in contigs equal to or longer than this value) ranged from 1,656 to 4,052 bases (Table 6.3). Coverage, contig size, N50 and total bases in contigs were negatively related to k-mer length, which is typical of velvet assemblies. Longer k-mer length was also

associated with many more unused reads, with up to 55% of input reads not used in assemblies. However, longer k-mer lengths resulted in more contigs longer than 1,000 bases (Table 6.3).

6.3.5 BLAST sequence similarity searching and post-processing

To detect potential parasite and symbiont sequences in the assembled data, we used BLAST searches to query the assemblies against publicly available genome data. We used the BLASTn algorithm in BLAST+ to search the NCBI microbial 16S ribosomal sequence (16SMicrobial), non-model organism whole genome sequence (wgs), non-model organism genomic (other_genomic), and nucleotide (nt) databases. The databases downloaded from GenBank (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) were dated 5 December 2011. Searches were run locally on the Victoria University of Wellington (VUW) high performance computing facility on which the BLAST+ executables were installed (BLAST v 2.2.25; Zhang *et al.* 2000).We restricted searches to a threshold e-value of 0.0001 or lower, and used a word size of 11 base pairs, to enhance the specificity of the searches (Madden 2002). In practice the e-values in this study were orders of magnitude lower than the threshold, which indicated high-scoring, relatively specific results.

Once the BLAST searches had completed we used a custom Perl script taxon4blast.pl (see Appendices) to summarise the data. The taxon4blast.pl script accepts as input up to *n* files in BLAST+ output format 6, and has sub-routines to: 1) append taxonomic information to individual BLAST results from local copies of the NCBI taxonomy database (ftp://ftp.ncbi.nih.gov/pub/taxonomy); 2) summarise BLAST results by a user specified taxon level (genus, subfamily, family, order, class, phylum, kingdom or superkingdom,); 3) summarise the shared and unique results among the input files, also by user specified taxon level; 4) summarise the shared and unique results among the input files, where only exact sequence matches are included; 5) extract the original sequences input to BLAST for a specified taxon level (either to retain or discard those sequences from the original input file). We summarised our results by genus, family and order for community composition analyses, and report detailed results by genus and order.

of bases in ases in the c nger than th the averag	by Velvet corporated lataset (Ba his value. F e depth of	GAIIX re to constru into the as ses in cont figher N5(repeated c	ct the assem semblies. # igs > 1k). C) values are ontigs rather	bly. Total cor contigs > 1k a ontig N50 is t considered to : than genome	the control of the transmission of the transmission of the control (the control of the transmission of the coverage.	total numbe ber of asser assembled s er assembli	re assembled ar of assembled nbled sequences equence) length es (i.e., a greate	e k-mer leng sequences. To longer than where 50% o r number of l	th is the optimal otal bases in cont (,000 bases, which of the assembly is onger contigs). C	size of the tigs are the th were the in contigs toverage in
	Inused	%	k-mer	Total #	Contig	Longest	Total bases	# contigs	Bases in	Coverage
	reads	unused	length	contigs	N50	contig	in contigs	> 1k	contigs > 1k	D
19	,372,897	55%	51	331,534	1,656	38,250	230,064,414	68,875	154,890,044	$8 \times$
3	,429,317	27%	35	440,455	3,944	59,607	242,805,168	49,586	191,920,754	$13 \times$
12.	,585,350	34%	39	434,029	2,847	81,278	243,502,772	57,621	182,932,963	$14\times$
2										
6	,394,544	26%	33	429,989	4,052	55,541	241,889,871	48,688	191,288,850	$16 \times$
13.	,725,717	37%	41	377,116	3,672	41,517	241,249,423	52,057	190,473,749	$13 \times$
Similarity in BLAST search results is not a guarantee of identity, and results are dependent on the sequences of taxa lodged in the databases. In order to further exclude possible spurious matches, we used a number of criteria to prioritise the likely accuracy of the summarised BLAST matches. At the genus level, with the exception of the 16SMicrobial database we considered that matches on ant, Hymenopteran and other insect taxa were most likely to be homologues. Results of this nature were orders of magnitude more prevalent than matches for other taxa. Thus, we excluded all BLAST matches on eukaryote sequences that had low scores and were thus highly unlikely to have been biologically valid as potential parasites or symbionts. Results such as this we considered to be owing to the large number of eukaryote sequences present on the databases, and highly conserved sequence similarity, possibly along with undigested gut contents of the ants. The results for genera were thus limited to those with a relatively high numbers of matches, longer total lengths (these two measures were typically correlated), or longer average lengths than the potentially artefactual matches. This may have resulted in a subset result dataset that excluded valid results, but should have provided a conservative survey that did not inflate the prevalence of potential parasites.

6.3.6 Community composition and diversity

To determine whether metagenome community structure differed among our sampled populations, we used non-metric Multi-Dimensional Scaling (MDS), ANOSIM and SIMPER implemented in PRIMER v 6.1.11 (Clarke and Warwick 2001; Clarke and Gorley 2006). We used number of matches as the measure of abundance, log-transformed to even out the effects of rare and abundant taxonomic groups, and used the Bray-Curtis index of similarity measure as recommended by Clarke and Warwick (2001). The MDS was run using 1000 iterations with a Kruskal stress formula 1 and a minimum stress of 0.01. We used ANOSIM analyses run over 9999 permutations for significance testing of the differences between the Arnhem Land samples and other samples. In ANOSIM analyses the Global *R* statistic ranges from -1 to 1 (but typically from 0 to 1). Random grouping of samples is indicated by *R* values close to 0, and as *R* approaches 1 replicates within a group are increasingly more similar to each other than to replicates within other groups (Warwick *et al.* 1990). As the sample size was small we considered the ANOSIM analysis indicative only of the degree of difference between groups (i.e., the size of the

Global R statistic) rather than statistical significance of the result. Separate analyses were conducted by order, family, and genus for all databases. As potential eukaryote parasites did not figure strongly (see Results section 6.4.1) we limited these analyses to bacterial groups in all the databases.

6.4 Results

6.4.1 BLAST sequence similarity

At the genus level the highest number, and longest total length of matches (i.e., closest matches) were to other ant genera, other Hymenopterans, and insects in the wgs and other_genomic databases (e.g., *Camponotus, Linepithema, Solenopsis, Harpegnathos, Acromyrmex, Atta,* and *Pogonomyrmex* in the wgs database, in that order, followed by *Bombus* and *Apis*). The next most common matches were to other insects or to bacteria (often *Escherichia* and *Wolbachia*). Ants alone made up ~85 - 90% of matches in each sample in the wgs database. In the other_genomic database matches to ant sequences were lower than in the wgs database (~70 – 76% of each sample), because sequences for the ant genera *Camponotus, Harpegnathos* and *Pogonomyrmex* were not lodged in the other_genomic database. Matches to ant sequences within the nt database were very low, in contrast to the comparatively large amount of ant genomic data for these groups than other groups.

No sequences of fungi, nematode or other potential eukaryote parasites were found among the most common matches in the wgs, other_genomic and nt database results. Matches to bacterial sequences were at high prevalence in all databases. Thus, the remainder of our results focus on the bacterial sequence matches in all databases. The number and lengths of matches for each genus reflected the sizes of the databases (Table 6.4). Many more sequence matches were obtained from the larger wgs, nt and other_genomic databases than from the smallest 16SMicrobial database (Table 6.4). In all databases the search results for the Arnhem Land 4 sample differed substantially from the other samples. Compared to other samples, this sample had fewer matches to sequences in the wgs, other_genomic and nt databases, and the total length of all matches was shorter in all the databases (Table 6.4). The number of genera found did not differ substantially among the samples for any of the databases, although the Arnhem Land 4 sample had the highest number of matches to genera in the 16SMicrobial database (Table 6.4).

6.4.2 Community composition and diversity

The bacterial community composition of sequence matches among *A. gracilipes* samples showed a number of similar patterns. Across all the databases and levels of taxonomic hierarchy, the Arnhem Land 4 sample of ants from low abundance sites tended to be somewhat differentiated from other samples (Fig. 6.2). This was particularly evident in the nt database (Fig. 6.2, j - l), and in the 16SMicrobial database at the family level (Fig. 6.2, b). The Arnhem Land 3 sample, which included a mixture of ants from low and high abundance sites tended to group with the other samples (e.g., Fig. 6.2, k), or was intermediate between the Arnhem Land 4 sample and other samples (e.g., Fig 6.2, c).

In some cases the ANOSIM analyses found differences in community composition among the samples (Fig. 6.2). Analyses of the 16SMicrobial database results suggested the Arnhem Land samples were distinct from the other samples (ANOSIM, order: Global R = 0.63; family: Global R = 0.58; genus Global R = 0.67). The community composition of the Arnhem Land samples in the wgs database results were more distinct from the other samples at the genus level (Global R = 0.33), but less so at the order level (Global R= 0.17), and not at all at the family level (Global R = 0.00). In the other_genomic database results the community composition of the Arnhem Land samples was not distinct from the other samples (ANOSIM, order: Global R = -0.17; family: Global R = -0.08; genus Global R = 0.17). ANOSIM analyses in the nt database results identified the community composition of the Arnhem Land samples as distinct from the other samples at the order (Global R = 0.50) and genus levels (Global R = 0.58), but not at the family level (Global R = 0.08).



Fig. 6.2: Two-dimensional MDS plots of differences in *A. gracilipes* endogenous bacterial community composition based on BLAST search results. The MDS was based on Bray-Curtis similarity of log (X+1) transformed data, analysed by order, family and genus: (a - c) 16SMicrobial database; (d - f) other_genomic database; (g - i) wgs database; (j - l) nt database. Filled circles indicate the Arnhem Land samples (black = Arnhem Land 4; grey = Arnhem Land 3) and open circles indicate other samples. The numbers refer to the sampling sites: 1 = Okinawa / Samoa; 2 = Arnhem Land 4; 3 = Christmas Island / Arnhem Land 2; 4 = Arnhem Land 3; 5 = Christmas Island 3.

(a): BLAST results for bacterial genera sequences in the GenBank 16SMicrobial and wgs databases for five A. gracilipes samples,	number of matches, sum of the lengths of matches, and number of genera. Proportion of low abundance refers to the proportion of the	t included ants from sites of low ant abundance (refer to Table 6.1 for details).
Table 6.4 (a): BLAST	ncluding number of ma	ample that included an

	Proportion of	1	6SMicrobial			Ngs	
Sample name	low abundance	Matches	Length	Genera	Matches	Length	Genera
Okinawa / Samoa	NA	2,843	801,109	385	69,147	39,952,207	309
Christmas Island 3	0.81	2,000	766,957	278	57,090	39,024,428	294
Christmas Island / Arnhem Land 2	0.59	1,747	694,074	233	56,649	23,912,742	283
Arnhem Land 3	0.80	2,747	866,557	375	60,209	39,355,525	305
Arnhem Land 4	1.00	2,060	493,425	458	27,924	9,251,549	283

including number of matches, sum of the lengths of matches, and number of genera. Proportion of low abundance refers to the proportion of the sample that included ants from sites of low ant abundance (refer to Table 6.1 for details). Table 6.4 (b): BLAST results for bacterial genera sequences in the GenBank other_genomic and nt databases for five A. gracilipes samples,

	Proportion of	0	ther_genomic			nt	
Sample name	low abundance	Matches	Length	Genera	Matches	Length	Genera
Okinawa / Samoa	NA	83,020	37,757,317	475	73,401	35,241,892	466
Christmas Island 3	0.81	70,032	37,414,541	439	62,520	35,085,914	427
Christmas Island / Arnhem Land 2	0.59	65,949	26,317,356	431	63,968	26,730,790	418
Arnhem Land 3	0.80	75,781	39,404,290	472	64,478	35,925,461	491
Arnhem Land 4	1.00	34,569	10,769,405	436	30,112	9,264,107	388

6.4.2.1 GenBank 16SMicrobial database composition

At the order level in the 16SMicrobial database results, many bacterial groups were common to all samples, although there were some notable differences in the Arnhem Land 3 and 4 samples. The most common matches in all samples were to the orders Rhizobiales. Enterobacteriales, Rhodobacterales, Rickettsiales, Vibrionales, Pseudomonadales, Rhodospirillales, Rhodocyclales, Alteromonadales, and Sphingomonadales (Fig. 6.3) to varying degrees. Members of the Burkholderiales and Actinomycetales orders were relatively more common in the Arnhem Land samples, and rare in other samples. Matches to Enterobacteriales were less common in the Arnhem Land samples (Fig. 6.3), as were the Alteromonadales in the Arnhem Land 4 sample. Matches to Rhodospirillales featured highly in the Okinawa / Samoa sample.

Consistent with the results at the order level, at the genus level *Burkholderia* (order Burkholderiales) was the most commonly matched genus in the Arnhem Land samples, while *Vibrio* (order Vibrionales, Fig. 6.3), and a variety of genera in the Enterobacteriales order were common among all samples. *Acetobacter* (order Rhodospirillales, Fig. 6.3) was the most commonly matched genus in the Okinawa / Samoa sample, but was rare or absent in other samples.



Fig. 6.3: Summary of BLAST matches to bacterial orders in the 16SMicrobial database for five *A. gracilipes* samples. The figure in brackets below the sample name is the proportion of the sample that included ants from sites of low abundance (refer to Table 6.1 for details).

Genus	Order	Okinawa	a / Samoa	Christmas	s Island 3	Christm	as Island /	Arnhe	em Land 3	Arnhe	m Land 4
		Matches	Length	Matches	Length	Arnha Matches	em Land 2 Length	Matches	Length	Matches	Length
Burkholderia	Burkholderiales	0	0	0	0	0	0	143	55,139	107	12,571
Vibrio	Vibrionales	89	50,040	145	48,818	84	28,328	150	50,772	69	8,671
Rhizobium	Rhizobiales	52	11,053	68	32,216	68	29,725	65	17,171	58	28,695
Rickettsia	Rickettsiales	40	7,021	55	26,904	55	24,855	33	9,550	55	26,822
Xenorhabdus	Enterobacteriales	46	23,402	54	25,557	60	24,615	48	23,615	39	5,688
Pseudomonas	Pseudomonadales	123	14,003	73	4,822	42	5,317	80	5,835	38	4,141
Mesorhizobium	Rhizobiales	6	938	47	22,249	47	20,296	38	9,785	37	13,273
Ochrobactrum	Rhizobiales	45	9,991	39	18,332	39	16,939	34	8,402	33	16,793
Cupriavidus	Burkholderiales	5	341	0	0	0	0	31	11,446	29	3,244
Massilia	Burkholderiales	1	100	0	0	0	0	38	14,399	28	3,655
Brucella	Rhizobiales	18	4,374	27	12,558	27	11,487	25	5,801	27	12,378
Streptomyces	Actinomycetales	ŝ	733	1	55	0	0	5	124	26	764
Herbaspirillum	Burkholderiales	1	101	0	0	0	0	30	11,244	25	2,789
Acidovorax	Burkholderiales	0	0	C	0	0	0	L	3,003	23	2 346

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6.4.2.2 GenBank wgs database composition

The pattern of matches to orders in the wgs database was similar to the 16SMicrobial database. The most remarkable result in the wgs database was a very low number of matches to bacterial sequences in the Arnhem Land 4 sample compared to the other samples (Table 6.4), with the exception of *Wolbachia* (Table 6.6). Matches to *Escherichia* sequences were the most prevalent in all samples except Arnhem Land 4, where the number of matches was relatively low (Table 6.7). The genus *Burkholderia* (order Burkholderiales) was of interest as it was prevalent in the 16SMicrobial results for the Arnhem Land samples. Sequences of this genus received matches for all samples, and, although much less common than *Wolbachia* (order Rickettsiales), matches to it were the second most prevalent bacterial genus and order in the Arnhem Land samples (Table 6.6; Fig. 6.4). The related *Rickettsia* (order Rickettsiales) received matches in all samples, but with relatively low prevalence. Other bacterial genera (e.g., *Salmonella, Vibrio* and *Yersinia*) that received matches in all samples were at very low prevalence in the Arnhem Land 4 sample.



Fig. 6.4: Summary of BLAST matches to bacterial orders in the wgs database for five *A*. *gracilipes* samples. The figure in brackets below the sample name is the proportion of the sample that included ants from sites of low abundance (refer to Table 6.1 for details). The apparently high prevalence of matches to Rickettsiales (primarily sequences belonging to the genus *Wolbachia*) in the Arnhem Land 4 sample is open to misinterpretation. Matches to Rickettsiales sequences were no more prevalent than other groups in this sample (Table 6.7), and the distortion is owing to the markedly low number of matches to all other orders in the Arnhem Land 4 sample.

natched by BLAST searches in the GenBank wgs database for five A. gracilipes samples, the total number of	ch sample. The results are ordered by their prevalence in the Arnhem Land 4 sample, thus some genera that were	t appear (e.g., matches to Shigella were highly prevalent in non-Arnhem Land samples).
Table 6.6: The 14 most common genera matched by BLAST searche	matches, and total length of matches for each sample. The results are o	much more prevalent in other samples do not appear (e.g., matches to Sh

Genus	Order	Okinaw	'a / Samoa	Christm	is Island 3	Christn	as Island /	Arnhei	m Land 3	Arnhe	m Land 4
						Arnhe	m Land 2				
		Matches	Length	Matches	Length	Matches	Length	Matches	Length	Matches	Length
Wolbachia	Rickettsiales	14,908	6,353,101	13,612	6,761,837	14,389	6,912,741	12,263	6,909,780	14,653	6,889,007
Burkholderia	Burkholderiales	552	60,812	605	75,282	795	83,285	2,474	435,288	1,736	254,242
Escherichia	Enterobacteriales	21,824	17,348,374	18,398	16,260,109	19,343	7,968,235	18,807	16,439,075	1,307	173,087
Vibrio	Vibrionales	3,768	992,298	3,626	1,015,405	2,169	637,568	2,443	925,482	1,139	94,316
Neisseria	Neisseriales	237	24,755	286	34,724	236	35,702	1,182	311,337	982	186,733
Pseudomonas	Pseudomonadales	962	91,498	443	67,288	351	58,615	728	154,026	903	101,645
Salmonella	Enterobacteriales	5,839	3,820,786	3,971	3,738,665	3,480	1,586,180	4,299	3,412,557	565	76,975
Yersinia	Enterobacteriales	3,992	3,390,627	3,877	3,684,752	3,904	1,967,940	3,595	3,499,450	470	49,202
Acinetobacter	Pseudomonadales	695	252,125	513	224,482	578	253,577	694	210,544	300	101,441
Streptomyces	Actinomycetales	33	1,990	76	3,574	236	8,979	54	3,416	256	9,768
Streptococcus	Lactobacillales	419	44,856	547	86,063	332	43,457	521	63,471	249	29,302
Leptospira	Spirochaetales	180	28,785	50	1,641	187	7,161	16	582	225	7,994
Brucella	Rhizobiales	521	140,051	136	89,382	104	44,163	209	35,785	167	103,061
Clostridium	Clostridiales	93	23,097	102	15,706	96	15,543	101	13,424	153	16,104

6.4.2.3 GenBank other_genomic database composition

The results for the other_genomic database were similar to the wgs database, with high numbers of matches to *Wolbachia* and *Escherichia* (Table 6.7). Other bacterial groups also received a high number of matches, including *Vibrio* (order Vibrionales) and a number of Enterobacteriales. Once again, matches to these groups were less prevalent in the Arnhem Land 4 sample (Fig. 6.5). Matches to the genus *Burkholderia* were found for all samples, but again the number of matches was more than two times greater in the Arnhem Land 3 and 4 samples than in other samples (Table 6.7).



Fig. 6.5: Summary of BLAST matches to bacterial orders in the other_genomic database for five *A. gracilipes* samples. The figure in brackets below the sample name is the proportion of the sample that included ants from sites of low ant abundance (refer to Table 6.1 for details). As in Fig. 6.4 the distorted prevalence of Rickettsiales in the Arnhem Land 4 sample is owing to the low number of matches to most other orders.

|--|

Genus	Order	Okinaw	'a / Samoa	Christm	is Island 3	Christr	nas Island /	Arnhei	m Land 3	Arnhe	m Land 4
						Arnhe	m Land 2				
		Matches	Length	Matches	Length	Matches	Length	Matches	Length	Matches	Length
Wolbachia	Rickettsiales	11,177	4,981,368	11,410	5,457,377	11,946	5,587,767	9,913	5,583,231	12,033	5,544,979
Burkholderia	Burkholderiales	1,350	178,641	844	117,048	722	111,836	4,016	876,673	2,957	630,205
Escherichia	Enterobacteriales	17,740	10,352,241	16,397	10,052,329	15,685	5,924,934	15,793	10,055,720	2,669	457,699
Pseudomonas	Pseudomonadales	1,826	325,242	1,284	307,676	932	258,083	1,562	523,701	566	209,293
Salmonella	Enterobacteriales	5,150	2,688,722	4,714	2,766,758	4,601	1,862,286	4,268	2,552,246	815	130,251
Shewanella	Alteromonadales	3,548	1,323,978	2,716	1,042,186	2,377	945,813	3,396	1,754,614	507	123,572
Yersinia	Enterobacteriales	3,920	2,415,015	3,840	2,575,579	4,171	1,666,560	3,594	2,410,684	685	117,778
Brucella	Rhizobiales	640	162,739	184	93,855	137	47,905	213	35,301	205	112,569
Clostridium	Clostridiales	262	63,665	205	40,428	140	24,399	187	26,026	243	104, 386
Ehrlichia	Rickettsiales	154	54,123	177	101,459	165	98,116	171	97,335	168	101,501
Acinetobacter	Pseudomonadales	617	161, 177	405	199,328	382	172,599	559	243,904	311	100,603
Rickettsia	Rickettsiales	412	108,877	230	97,047	211	91,496	208	82,152	208	94,308
Cupriavidus	Burkholderiales	167	26,554	65	19,244	4	17,842	472	119,541	397	91,002
Neisseria	Neisseriales	224	28,701	134	13,146	102	11.419	504	143.244	436	77.676

6.4.2.4 GenBank nt database composition

The results for the nt database were similar to the wgs and other_genomic databases, with many matches to the Rickettsiales and Enterobacteriales orders (Fig. 6.6), and to the genera *Wolbachia* and *Escherichia* (Table 6.8). Matches to a number of bacterial groups were less prevalent in the Arnhem Land 4 sample than other samples (Table 6.8). Sequence matches to *Burkholderia* were higher in the Arnhem Land samples than in other samples, as were matches to *Arsenophonus* (order Enterobacteriales Table 6.8), which has previously been found in *A. gracilipes* (Sebastien *et al.* 2012).



Fig. 6.6: Summary of BLAST matches to bacterial orders in the nt database for five *A*. *gracilipes* samples. The figure in brackets below the sample name is the proportion of the sample that included ants from sites of low ant abundance (refer to Table 6.1 for details). As in Fig 6.4 and 6.5, the distorted prevalence of Rickettsiales in the Arnhem Land 4 sample is owing to the low number of matches to most other orders.

e 14 most common genera matched by BLAST searches in the GenBank nt database for five A. gracilipes samples, the total number of matches,	th of matches for each sample. The results are ordered by their prevalence in the Arnhem Land 4 sample, thus some genera that were much more	other samples do not appear (e.g., Shigella, Vibrio, Shewanella).
Table 6.8: The 14 most comn	and total length of matches fc	prevalent in other samples do

Genus	Order	Okinaw	'a / Samoa	Christma	s Island 3	Christn	as Island /	Arnhe	m Land 3	Arnhen	a Land 4
						Arnhe	m Land 2				
		Matches	Length	Matches	Length	Matches	Length	Matches	Length	Matches	Length
Wolbachia	Rickettsiales	13,981	5,585,581	13,620	6,063,765	14,128	6,221,702	11,892	6,198,193	14,507	6,283,493
Burkholderia	Burkholderiales	593	33,440	509	40,822	421	37,874	2,131	449,797	2,043	349,921
Escherichia	Enterobacteriales	11,619	7,162,317	10,671	7,033,811	12,158	4,852,356	10,035	7,051,363	1,197	168,947
Arsenophonus	Enterobacteriales	1,316	2,892,549	1,057	2,953,607	2,567	2,589,427	1,120	2,989,214	721	120,349
Paracoccus	Rhodobacterales	295	17,766	270	14,352	434	19,431	137	10,747	641	29,672
Yersinia	Enterobacteriales	4,932	3,031,512	4,359	3,181,848	5,396	2,263,858	4,046	2,892,780	595	56,366
Neisseria	Neisseriales	183	14,598	194	17,853	147	16,107	671	186,173	536	71,513
Salmonella	Enterobacteriales	5,287	2,958,118	4,977	3,203,160	5,781	2,499,785	4,365	2,912,568	527	83,596
Vibrio	Vibrionales	2,360	590,647	2,188	616,408	1,348	308,704	1,932	540,931	452	54,506
Ralstonia	Burkholderiales	121	7,189	80	12,722	65	11,400	577	112,008	429	84,820
Cupriavidus	Burkholderiales	59	3,980	49	8,856	30	7,580	297	70,792	383	90,581
Xanthomonas	Xanthomonadales	82	5,923	LL	3,722	59	3,887	203	11,160	364	16,179
Pseudomonas	Pseudomonadales	1,088	104,240	936	132,562	616	157,449	1,145	245,528	292	143,302
Proteus	Enterobacteriales	1,050	483,672	1,148	541,223	609	287,171	1,240	539,375	273	24,886

6.5 Discussion

Animal populations introduced to new environments may bring with them a suite of beneficial or harmful endogenous symbionts and parasites. They may also encounter and assimilate new organisms. Population bottlenecks during the introduction may stochastically result in a loss of either or both beneficial and harmful symbionts, and endogenous symbiont and parasite composition may thus differ among introduced populations. In our study populations we found a low prevalence of eukaryote parasites of ants, such as fungi (Currie *et al.* 1999; Santos *et al.* 2004), microsporidia (Oi *et al.* 2008; Briano 2009; Valles *et al.* 2010), and nematodes (Yanoviak *et al.* 2008; Hoover *et al.* 2011), despite these groups being well represented in the databases. However, our study populations were apparent. The Arnhem Land samples exhibited results that were considerably different from the other samples among all four databases. The prevalence to groups common in other samples was much lower in the low abundance Arnhem Land 4 sample, with the exception of matches to sequences of the Burkholderiales order (and the genus *Burkholderia*).

6.5.1 Community composition differences between databases

We found differences in the results between the different databases. Genera that commonly received matches in some databases were absent from the results in others. For example, of the bacteria *A. gracilipes* is known to harbour (*Wolbachia, Arsenophonus* and Rhizobiales: Sebastien *et al.* 2012), *Wolbachia* sequences (order Rickettsiales) received no matches on the 16SMicrobial database, but had the most matches on the other databases. *Arsenophonus* (order Enterobacteriales) received matches for all samples but rarely in the 16SMicrobial database. This genus was absent from the wgs and other_genomic database results, but was one of the most prevalent results in the nt database. However, matches to Rhizobiales occurred at a much lower prevalence than other orders except in the 16SMicrobial database.

The reason for the differences in results among the databases is owing to differences in the data lodged in them. This potential bias may have resulted in the over-representation of some groups in the results. For example, organisms known to affect human health are likely to be over-represented. We excluded model-organism specific databases to minimise this effect. By chance, we also may have failed to detect some bacteria in the 16S database if our short-read sequences did not include the 16S region for any of these groups. However, the large amount of sequence data generated suggests this may be unlikely. Clearly the absence of matches to a particular group in a single database is not an indication that it is absent from the sample. We obtained more meaningful results from all the databases combined, rather than a single database. Our interpretation of the results attempts to take these issues into account.

6.5.2 The role of Burkholderiales and associated symbionts

Burkholderia species are among the most abundant bacteria in the environment, and are divided into two taxonomically distinct groups: one group whose members are pathogenic and another whose members are beneficial (Suárez-Moreno *et al.* 2012). Although some species are pathogenic in humans and other animals (Coenye and Vandamme 2003; Valvano *et al.* 2005), other species are plant-associated, both free-living in the soil among roots, and as endosymbionts or phytopathogens (Coenye and Vandamme 2003; Janssen 2006; Omarjee *et al.* 2008). Species of *Burkholderia* are not known to have pathogenic relationships with insects, although their ecological roles are often uncertain (Vandamme *et al.* 2007a). *Burkholderia* are common beneficial gut symbionts of insects (Compant *et al.* 2008). Beneficial relationships between species of *Burkholderia* and insects include assistance with nutrition (van Borm *et al.* 2002; Kikuchi *et al.* 2005), and antibiotic protection (Santos *et al.* 2004; Vandamme *et al.* 2007b).

Members of the Burkholderiales have been detected among a number of ant species, and are among a group of endogenous bacteria assumed to be symbionts that facilitate herbivory (Russell *et al.* 2009). Other bacterial groups suggested to be herbivory-associated among ants include members of the Rhizobiales, Pseudomonadales, Xanthomonadales and Verrucomicrobiales orders (Russell *et al.* 2009). Russell *et al.* (2009) screened a single *A. gracilipes* ant for these bacteria, with negative results. However, the prevalence of symbionts appears to vary among *A. gracilipes* populations (Sebastien *et al.* 2012; this study), so the Russell *et al.* (2009) study may have been unlikely to detect these organisms from a single sample, particularly if the symbionts are obtained facultatively. Of the other groups found to be associated with herbivory in ants (Russell *et al.* 2009), we found Rhizobiales, Pseudomonadales and Xanthomonadales to be common in many of our results. Verrucomicrobiales were also suggested by Russell *et al.* (2009) to be associated with herbivory. We found Verrucomicrobiales in the wgs, other_genomic and nt database results, but at such extremely low prevalence as to potentially be owing to sequence similarity, rather than a true indicator of prevalence.

The role of Burkholderia-related bacteria in A. gracilipes requires further attention before conclusion can be made regarding their function. Although A. gracilipes often dominates novel carbohydrate resources from plants directly (Lester and Tavite 2004; Savage et al. 2009), or indirectly (Abbott and Green 2007), it is also a generalist omnivore, that feeds on other insects, spiders, crabs and birds (Green et al. 1999). While it may seem unusual that an omnivorous ant such as A. gracilipes would require herbivory related symbionts, omnivorous ants may use symbionts to enhance their nutrition (Feldhaar et al. 2007). Moreover, as well as enhancing nitrogen recycling, Burkholderia species may function specifically to produce antibiotics as a protective function, as found in the leaf-cutting ant Atta sextens rubropilosa (Santos et al. 2004). Horizontal transmission of Burkholderia is common (Kikuchi et al. 2005), and the bacterium may be obtained from the soil (Santos et al. 2004). Additionally, Streptomyces are used by fungus-growing ants to protect against infection by fungal parasites (Currie et al. 1999). The Arnhem Land 4 sample had a much higher prevalence of matches to the order Actinomycetales, and to the Streptomyces genus within this group. We speculate that it is possible that the A. gracilipes population from which the Arnhem Land 4 sample was taken may have been infected by other organisms (perhaps fungi), and the facultative uptake of (potentially) antibiotic-producing bacteria from the soil may have been a response to infection. The lower prevalence of other bacteria in this sample that may be mutualistic suggests a hypothesis of fungal infection might be plausible. Although fungal and other eukaryote parasite sequences did not figure strongly in our results, when a population has declined due to disease, the extinction of the pathogen may precede that of the host (De Castro and Bolker 2005).

Alternatively, the species of Burkholderiales in *A. gracilipes* may be pathogenic members of the group. A number of species in the order Burkholderiales are pathogenic (Brogden 2009), and may consume antibiotics (Dantas *et al.* 2008) rather than produce them. However, this hypothesis is perhaps unlikely given the absence of documented pathogenic relationships between Burkholderiales and insects (Vandamme *et al.* 2007a).

6.5.3 The role of Rickettsiales

The order Rickettsiales includes the common insect symbiont *Wolbachia*, and its close relative *Rickettsia*. These bacteria are known to manipulate host sex-ratios in favour of females (Gherna *et al.* 1991; Engelstädter and Hurst 2009; Himler *et al.* 2011), which in some cases may have positive effects on the fitness of the host by increasing abundance and spread (Himler *et al.* 2011). However, *Wolbachia* may reduce fitness among ants (Wenseleers *et al.* 2002), and a number of invasive ant species have exhibited a loss of *Wolbachia* in the introduced range (Shoemaker *et al.* 2000; Tsutsui *et al.* 2003a). Although the loss of these bacteria may contribute to the invasion success of the host, the function of common insect endosymbionts such as *Wolbachia* in ants is unclear and may be context dependent (Zientz *et al.* 2005).

Both *Wolbachia* and *Rickettsia* featured in our results but were no more prevalent in any population. While it seems unlikely that either of these groups contributed to differences among our study populations, the role of *Wolbachia* and *Rickettsia* in *A*. *gracilipes* populations is of interest. *Wolbachia* has been detected previously in *A*. *gracilipes* at high prevalence (Sebastien *et al.* 2012). A high level of *Wolbachia* infection in the introduced range may be maintained through the genetic cleansing of incompatible strains (Wenseleers *et al.* 1998; Merçot and Poinsot 2009), which has been suggested for *A. gracilipes* (Sebastien *et al.* 2012). High prevalence of *Wolbachia* has been attributed to the virus protection it affords (Hedges *et al.* 2008; Teixeira *et al.* 2008), which may be the case for *A. gracilipes* (Sebastien *et al.* 2012). In addition, *Wolbachia* may spread more easily in species that experience repeated extinction and recolonisation because founder events may allow the frequencies of infection to exceed unstable levels (Turelli and Hoffmann 1995; Tsutsui *et al.* 2003a). This scenario may be applicable to *A. gracilipes*, which appears to experience dynamic population changes (Haines and Haines 1978b; Abbott 2006b; B. Hoffmann & P. Green, personal communication; Chapter 3; Chapter 5).

Wolbachia may also possibly affect the reproductive mode of A. *gracilipes*, as in some insects it can cause parthenogenetic production of females (Stouthamer *et al.* 1993). If *Wolbachia* has effects on reproduction in *A. gracilipes* it may contribute to the enigmatic reproductive mode of the species (Drescher *et al.* 2007; Chapter 2). Although to date no evidence has been found that *Wolbachia* affect reproduction among ant species (Wenseleers and Billen 2000; Keller *et al.* 2001), *Wolbachia* may be associated with a wider range of phenotypic effects on the host than previously assumed (Feldhaar 2011).

6.5.4 Enterobacteriales and other common bacterial groups

Enterobacteriales featured commonly in our results. However, our Arnhem Land population had a lower prevalence of sequence matches to this order than other populations. *Arsenophonus*, a member of the Enterobacteriales known to occur in *A. gracilipes* (Sebastien *et al.* 2012), was among the more prevalent sequences matched to in the nt database. Although the function of this bacterium is not known in *A. gracilipes* or other ants, *Arsenophonus* is known to modify the sex-ratio of host Hymenopterans (Gherna *et al.* 1991).

The Enterobacteriaceae family to which *Arsenophonus* belongs includes a number of members that are beneficial symbionts of ants and other insects, such as *Buchnera* (Buchner 1965), *Blochmannia* (Zientz *et al.* 2005; de Souza *et al.* 2009), *Wigglesworthia* (Heller 2011), *Regiella*, *Hamiltonella* and *Serratia* (Oliver *et al.* 2010; Tsuchida *et al.* 2010). *Arsenophonus*-like species are also common symbionts of aphids (Russell and Moran 2005), and whiteflies (Thao and Baumann 2004). Clearly, many bacteria, including *Arsenophonus* and *Wolbachia*, may have differing phenotypic effects in different host species, and these effects are beneficial in specific cases and harmful in others.

Among the other groups commonly encountered in our results, were the orders Rhodospirillales, Sphingomonadales, Vibrionales, Caulobacterales, Pasteurellales and Alteromonadales. These groups are large with diverse characteristics (Kersters *et al.* 2006). As the prevalence of matches to these groups did not vary a great deal among most populations, and may thus reflect a 'noisy' dataset, investigating their function further may provide little gain. However, if the majority of endogenous bacteria are not pathogenic, and are in fact beneficial, treatment with antibiotics may result in population declines (Mittler 1971; Feldhaar *et al.* 2007).

6.6 Conclusion

Our preliminary metagenomic study of *A. gracilipes* populations found no significant eukaryote parasites, but a diversity of bacteria among *A. gracilipes* populations. Many bacterial groups were detected that were previously unknown in the species. Several of these are candidates for further study, which may shed light on the effects of endogenous parasites and symbionts on the population dynamics of the species.



Chapter 7. General discussion

7.1 Summary

A key factor contributing to invasion success is the ability of an introduced population to reach sufficient abundance to persist, spread, and alter or dominate the recipient biological community (Ehrlich 1986; Crawley 1987; Mack *et al.* 2000). Genetic diversity is one factor that contributes to population dynamics, but has important ramifications for biological fitness, and thus invasion success in the long term. My dissertation investigated genetic factors associated with variation in abundance of the invasive yellow crazy ant *Anoplolepis gracilipes*. I investigated five aspects that I hypothesised could contribute to variation in the abundance of this ant: 1) dependent lineage genetic caste determination with lineage ratio asymmetry, which could be associated with low abundance; 2) multiple introductions which could increase abundance via propagule pressure, or different sources (i.e., genetically different colonies) with different levels of abundance; 3) differences in microsatellite genetic diversity between populations with different levels of abundance; 4) a specific haplotype associated with high abundance; 5) variation in endogenous parasite and symbiont diversity among populations which might be associated with variation in abundance.

My research suggests that genetic factors may contribute to the variation in abundance of *A. gracilipes*. I found no evidence for a dependent lineage genetic caste determination system in *A. gracilipes*, and thus abundance is not constrained by any lineage ratio asymmetry. Although ovaries were found in workers, no viable eggs were present. Based on this evidence, worker reproduction (either by production of males or clonal production of other workers) seems unlikely, or it may occur only seasonally. The reproductive mode of the species remains fascinating but enigmatic (Chapter 2). I also found no evidence that the variation in abundance of *A. gracilipes* in Arnhem Land was due to multiple introductions from different sources. By contrast, the population is a single heterogeneous supercolony that appears to be diverging, which is unusual in invasive ants (Chapter 3). While this divergence itself was not related to variation in abundance, genetic diversity was higher in more abundant nest clusters. These more abundant nest clusters were in

turn associated with lower native ant species diversity, and a difference in composition of the invaded ant community (Chapter 4). On Tokelau an earlier correlation identified between a specific haplotype and greater abundance was no longer detected, and although overall the distribution of the ant has increased, many *A. gracilipes* populations have declined or disappeared (Chapter 5). Variation in abundance among *A. gracilipes* populations in Arnhem Land was not obviously due to bacterial load on populations with low abundance, and I found no evidence of eukaryote parasites (Chapter 6). However, low abundance in *A. gracilipes* samples from Arnhem Land was correlated with lower microbial diversity overall, and higher prevalence of some bacterial groups, notably two that confer antibiotic properties (Chapter 6).

7.2 Synthesis

While dependent lineage GCD does not appear to be responsible for the variation in abundance of A. gracilipes in Arnhem Land (Chapter 2), the reproductive mode may contribute to the invasion success of the species if one or more castes or sexes are produced asexually. Recently it has been suggested that the ecological success of some invasive ants may be facilitated by clonal reproduction (Wasmannia auropunctata: Foucaud et al. 2009; Platythyrea punctata Cerapachys biroi, Pristomyrmex punctatus, Pyramica membranifera and Vollenhovia emeryi: Kellner and Heinze 2010, and Paratrechina longicornis: Pearcy et al. 2011). Asexual reproduction is a trait that evolved in the native range and is expressed more strongly, and selected for, in the invaded range of W. auropunctata (Foucaud et al. 2010). Although the native range of P. longicornis has not been studied it is suggested that the trait has 'pre-adapted' the ant to enable invasion success, by requiring few propagules for establishment (Pearcy et al. 2011). By contrast, if we assume that asexual reproduction does contribute to the variation in abundance of A. gracilipes, then it appears local abundance may be facilitated by a greater number of clonal lineages, as indicated by the higher genotypic diversity found in more abundant nest clusters in Arnhem Land (Chapter 4). This suggests that postestablishment, a larger propagule size (i.e., a greater diversity of reproductive individuals) might favour greater abundance than a small propagule size. Thus, even if the A. gracilipes reproduction mode involves asexuality, which could contribute to initial colonisation success, the reproductive mode may not contribute directly to the on-going persistence or spread of the ant.

The population divergence I detected (Chapter 3), together with the positive association between genotypic diversity and abundance (Chapter 4), may have implications for the long-term success of *A. gracilipes* in Arnhem Land. Population divergence may result in a loss of genetic diversity and decreased abundance over time. *Anoplolepis gracilipes* has been in Arnhem Land since at least the early 1980s. Nest clusters that were detected at that time which were at high abundance (Young *et al.* 2001) are now relatively less abundant than nest clusters at other sites (Chapter 4). Thus it appears that population divergence involving colony fragmentation (Chapter 3) may be associated with genetic bottlenecks that result in less abundant colonies with lower genetic diversity (Chapter 4).

Competition with native ant species may also further contribute to population fragmentation. The relationship between A. gracilipes and differences in the ecological community are density-dependent (Hill et al. 2003; Abbott 2005; Abbott et al. 2007; Lester et al. 2009; Hoffmann and Saul 2010; Drescher et al. 2011; Chapter 4). Anoplolepis gracilipes is unlikely to be able to compete with dominant ant species when the former are at low densities. In addition, A. gracilipes often occupies a dominant ecological position only in the absence of other highly aggressive and dominant species (Greenslade 1971; Bos et al. 2008; Kirschenbaum and Grace 2008; Brühl and Eltz 2010). This is consistent with my findings in Chapter 4, where A. gracilipes abundance was more negatively associated with dominant and rare species than with common and subdominant species. The ability of invasive ants to compete with native ants only when the invaders are in high abundance is well known (Oliver et al. 2008; Sagata and Lester 2009), which suggests that Allee effects (Allee 1931) may be mediated by competitive interactions among ant species. Thus, in areas with species-rich native ant communities such as those in Arnhem Land, A. gracilipes may not be an effective competitor at low abundance, which may further contribute to population fragmentation.

Although genetic bottlenecks generally, or specifically in nestmate recognition loci, may promote unicoloniality and thus the increased abundance of invasive ants (Suarez *et al.* 1999; Tsutsui *et al.* 2000; Giraud *et al.* 2002), post-introduction genetic bottlenecks

may also affect the dynamics of unicoloniality. Unicoloniality is thought to be an evolutionary dead-end, with the extinction of unicolonial species predicted to occur via kin-selected evolutionary mechanisms, including the development of finer-scale kin discrimination within the supercolony (Helanterä et al. 2009). This is consistent with the hypothesis of Drescher et al. (2010), together with my results (Chapters 3 and 4), which suggest that the loss of genetic diversity through serial bottlenecks may contribute to the breakdown of unicolonial populations by inducing fragmentation, and perhaps also decline at a local scale over ecological timeframes measured in decades rather than centuries. Such fragmentation potentially increases intra-specific aggression (Drescher et al. 2010), resulting in reduced colony sizes, and diminished competitive ability relative to native ants. Even if populations do not decline to the point of regional extinction, competition with native ant communities may result in alternative stable states of communities dominated by A. gracilipes in some cases, and native dominant ants in other cases (i.e., an ant mosaic: Leston 1973). This ant mosaic pattern evidently applies to the distribution of A. gracilipes in Borneo, for example (Pfeiffer et al. 2008), which is also consistent with the observation that colonies in Borneo (Drescher et al. 2010) are much smaller than the colony in Arnhem Land (Chapter 4).

Under the scenario described above, population bottlenecks may eventually result in local extinctions. A number of invasive ants show unpredictable, unexplained population declines (Greenslade 1971; Haines and Haines 1978b; Morrison 2002; Cooling *et al.* 2011). Serial population genetic bottlenecks may result in an extinction vortex, where the population declines to a size below which it can ecologically function (Gilpin and Soulé 1986). A hypothesis of population fragmentation, reduced genetic diversity and eventual decline is, however, based on the assumption that there is a persistent relationship between abundance and genetic diversity. Although higher genetic diversity may confer a number of short-term benefits (Cole and Wiernasz 1999; Tarpy 2003; Mattila and Seeley 2007; Oldroyd and Fewell 2007), particularly enhanced immune function (Sherman *et al.* 1988; Shykoff and Schmid-Hempel 1991; Keller 1995; Tarpy 2003; Ugelvig *et al.* 2010), the mechanisms that maintain genetic diversity, and how much higher genetic diversity needs to be to confer these benefits is not known in *A. gracilipes.* In addition, the long term adaptive benefits, and dynamics of the association between genetic diversity and abundance over time are unknown.

It is likely that colony fragmentation of *A. gracilipes* populations would not occur on small islands such as Tokelau, for example. On these islands dispersal is limited, ant colonies are likely to remain in sympatry, and the likelihood of re-invasion appears high (Chapter 5). In addition, competition with dominant native ants is unlikely to have an effect on islands such as Tokelau, as the ant fauna is cosmopolitan and comprises many introduced species (Abbott *et al.* 2006).

Based on what we know of the population dynamics of *A. gracilipes* on Tokelau (Lester and Tavite 2004; Abbott *et al.* 2007; Lester *et al.* 2009; Chapter 5), some aspects of the population dynamics of the ant there may be somewhat similar to that in Arnhem Land. On Tokelau the more recent haplotype D populations declined, while the older haplotype A populations appeared to remain relatively constant at lower abundance (Chapter 5). This mirrors the finding that the earliest detections in Arnhem Land, which were highly abundant (Young *et al.* 2001) are no longer abundant, while the abundance of the ant in more recently invaded sites appears higher (Chapter 4). The density-dependent ecological effects of other invasive ants can also be higher shortly after arrival, and then decline (Morrison 2002). Invasive species generally may experience an acute phase during which their abundance and effects are most severe, followed by a long chronic phase of stability (Strayer *et al.* 2006). Clearly, understanding these dynamics requires further work.

Although the metagenomic investigation in Chapter 6 was preliminary, and was qualitative rather than quantitative, it does raise interesting questions regarding the potential role of endosymbionts in the variation in abundance and dynamics of *A*. *gracilipes* populations. I had initially hypothesised that parasites or pathogens could be responsible for the low abundance (presumably through population decline) of *A*. *gracilipes* in Arnhem Land. If bacterial pathogens were responsible for variation in abundance, bacterial load would be much higher in the groups with lower abundance. In addition, if pathogenic bacteria were responsible for population declines bacterial diversity would differ in *A. gracilipes* populations of low abundance, and that pathogenic organisms would be more prevalent in these populations. My results suggested that the opposite was the case for both hypotheses. The most common bacterial species in samples from Arnhem Land with low abundance were *Burkholderia*. *Burkholderia* species fall

into two groups: some that are widely known as pathogenic to humans and animals, and some are beneficial to plants (Suárez-Moreno *et al.* 2012), and insects including ants (van Borm *et al.* 2002; Santos *et al.* 2004; Kikuchi *et al.* 2005; Vandamme *et al.* 2007b; Compant *et al.* 2008). There are no reports of pathogenic *Burkholderia* in insects. Thus, the function of *Burkholderia*-like species in *A. gracilipes* is clearly worthy of further consideration.

Given the potential importance of beneficial endosymbionts such as Burkholderiales to enhance nutrition among ant species (Currie *et al.* 1999; Feldhaar *et al.* 2007; Russell *et al.* 2009; Feldhaar 2011), the potential use of novel carbohydrates deserves closer attention, in light of the findings of Chapter 6. Although the presence of *Acacia* spp. was investigated (Chapter 4), no estimates were made of possible differences in usage of *Acacia* extra floral nectaries by *A. gracilipes*. One of the roles of candidate endosymbionts such as Burkholderiales (Chapter 6) is the provision of nutritional upgrading in non-herbivorous ant species (Feldhaar *et al.* 2007). Predatory invasive ants are known to shift their diet from animals to plants in the introduced range as protein resources are exhausted (Tillberg *et al.* 2007), and depletion of protein resources had been suggested as a possible, but untested, reason for the decline of *A. gracilipes* on Mahé in the Seychelles (Haines and Haines 1978b). Thus, endosymbionts such as Burkholderiales may possibly be used facultatively by *A. gracilipes* to facilitate uptake of plant-based nutrients when protein resources are locally depleted.

In ants, a positive relationship exists between genetic diversity and immunity (Sherman *et al.* 1988; Shykoff and Schmid-Hempel 1991; Keller 1995; Tarpy 2003; Ugelvig *et al.* 2010). Social insects, including ants, have fewer immune genes than other insects, and thus their populations typically have naturally high pathogen loads (Schmid-Hempel 1998). Pathogen spread is facilitated by the social organisation of these insects, owing to aggregations of close relatives (Sherman *et al.* 1988). Consequently, social insects employ a number of behavioural and physiological mechanisms to enhance disease resistance (Cremer and Sixt 2009). Enhanced disease resistance through improved immune defence has been hypothesised to drive increased genetic diversity within social insect colonies, favouring colonies with a higher number of reproductives (i.e., multiple queens reproduce [polygyny], and / or queens mate with multiple males [polyandry]), and

consequently lower parasite loads (Liersch and Schmid-Hempel 1998; Schmid-Hempel and Crozier 1999; Tarpy 2003). While both low genotypic diversity (Chapter 4) and low bacterial diversity (Chapter 6) were associated with low abundance of *A. gracilipes* in Arnhem Land, it is not possible to conclude that there is a direct or interactive relationship between genetic diversity and bacterial diversity. However, given the apparent benefits of genetic diversity, and possible effects of endogenous parasites, the combination of these factors would benefit from further study.

Without knowledge of the native range of A. gracilipes it is not possible to discover which differences may enhance or hinder establishment success in the introduced range. Although other members of the genus are found only in Africa, Madagascar, the Arabian peninsula and Réunion (Wetterer 2005), there have been suggestions that Asia may be within the native range of the species (Wetterer 2005; Drescher 2011; Sebastien et al. 2012; H. Feldhaar, personal communication). India has not been sampled for genetic analysis, although it has been suggested that this may ultimately prove to be the native range (Drescher 2011; H. Feldhaar, personal communication). Hypothetically, if the closest relatives, and potentially ancestors, of A. gracilipes were in Madagascar and Africa, the origin of A. gracilipes could be vicariant owing to the break-up of Gondwana (i.e., the out-of-India hypothesis: McKenna 1973). If this were the case, India would have been a natural point of origin for dispersal of the ants throughout Asia. The origin of ants pre-dates the break-up of Pangaea (Crozier et al. 1997; Moreau 2009). Formicinae (the sub-family to which A. gracilipes belongs) appear to have evolved ~100 Ma (Moreau et al. 2006), not long before the approximate separation of India and Madagascar from Gondwana ~90 Ma (Scortese 1997), so a vicariant origin is possible.

Differences in colony structure among *A. gracilipes* populations may enable inferences to be made regarding the native range. Invasive unicolonial ants often exhibit differences in colony structure in the introduced range compared to the native range, and a striking difference is colony size. For example, the diameter of Argentine ant colonies in their native range has been estimated to not exceed 2 km, and colonies are frequently much smaller (Heller 2004). In the introduced ranges colony sizes are reported to be many orders of magnitude larger (Giraud *et al.* 2002; Corin *et al.* 2007; Suhr *et al.* 2009; van Wilgenburg *et al.* 2010). The extent of supercolonies in these ants are therefore much

greater in the introduced range (Suarez *et al.* 2008). Most invasions of *A. gracilipes* have been studied on relatively small islands, where the size that supercolonies can extend to is spatially limited (Haines and Haines 1978b; Feare 1999; Abbott 2006b; Abbott *et al.* 2007; Suwabe *et al.* 2009). These spatial limits clearly do not exist in Arnhem Land (Chapter 4). By contrast, other than my work the only study of *A. gracilipes* on a large scale has been in Borneo, where despite the absence of geographical constraints, genetically and behaviourally differentiated colonies span only a few hundred metres (Drescher *et al.* 2010). Colonies of this size are more akin to small polydomous colonies (Bourke and Franks 1995; Suarez *et al.* 2008), as observed in the native range of the Argentine ant (Tsutsui *et al.* 2000; Pedersen *et al.* 2006).

If a phenomenon of colony 'gigantism' in the invaded range, due to a loss of intraspecific aggression, is common among unicolonial invasive ants, this may be an indicator that Borneo is within the native range of A. gracilipes. Even on Christmas Island, the social structure appears very different to that in Borneo, whereby two supercolonies share an overlapping distribution throughout the entire island (Thomas et al. 2010). The allelic richness of individual A. gracilipes colonies in Borneo has not been reported (Drescher et al. 2007; Drescher et al. 2010; Drescher 2011), so we do not know if colonies in Borneo are more genetically diverse that those on Christmas Island (Thomas et al. 2010), or than those in Arnhem Land (Chapter 4). Based on these features it appears possible that Borneo is within the native range of A. gracilipes, as others have suggested (Wetterer 2005; Drescher 2011; Sebastien et al. 2012; H. Feldhaar, personal communication). Given the clear differences in colony structure between Arnhem Land (Chapter 4) and Borneo (Drescher et al. 2010), that appear to mirror differences between the native and introduced ranges in Argentine ants (Tsutsui et al. 2000; Giraud et al. 2002; Pedersen et al. 2006; Corin et al. 2007; Suhr et al. 2009; van Wilgenburg et al. 2010), more direct comparisons between A. gracilipes in Borneo (and India) and the introduced range may shed more light on the potential causes of the differences between the ranges.

7.3 Constraints and further research

A number of further interesting avenues of research are possible in relation to the genetic factors contributing to the abundance and population dynamics of *A. gracilipes*. Many of these research directions require longer term research on the changes in population dynamics over time. During the course of my thesis I faced a number of constraints which also highlighted significant unanswered questions regarding the biology and ecology of this under-studied species.

As part of my work in Chapter 2, I originally attempted to determine whether clonal reproduction occurred in *A. gracilipes*. As lab facilities were not available in either in New Zealand or in Australia, I (somewhat naïvely in hindsight) attempted to determine statistically if the genotypes of workers and queens were consistent with clonal or sexual reproduction (primarily using the GenClone functions of Arnaud-Haond and Belkhir 2007). The results of these tests were mixed and asexual reproduction by queens or workers appeared no more likely than sexual reproduction by mated queens. I did not include these analyses in Chapter 2, however, as when a manuscript including these analyses was submitted, reviewers consistently pointed out that these questions could only be answered through experimental laboratory nests that allow the control of the parental relationships between individuals. I agreed with this assessment, and thus I modified Chapter 2 to focus only on DL GCD, a hypothesis I consider can be rejected based upon the evidence of a single queen lineage in Arnhem Land.

The social systems of ants have traditionally been viewed as families of sterile workers, all offspring of a single diploid mother queen mated to a single haploid male (Pamilo 1991; Heinze 2008), resulting in relatively low genetic diversity compared to species that are not haplo-diploid (Hedrick and Parker 1997). However, the diversity of reproductive systems that have been discovered among ants in recent decades means that very little can be assumed about the reproductive mode of a given species (Keller 2007; Heinze 2008). We lack knowledge of a number of aspects of *A. gracilipes* biology, and reproduction in particular, that relate directly to the genetic diversity of ant populations (Pamilo 1991). How many queens and males contribute to reproduction? Does the species mate only within the nest or do nuptial flights occur, and if they do, are they common or

rare events? Is there sex-biased dispersal? How does high heterozygote frequency among workers and homozygote frequency among queens contribute to fitness? How is worker exchange among nests mediated? What is the structure of nest clusters within the colony and how does this vary? How do these aspects vary in different parts of the invaded range, and how does this compare with the (unknown) native range? Some of these questions can perhaps be answered through field assessments. However, answering many of these fundamental questions requires experimental manipulation of colonies.

The genomic short-read-sequence dataset generated for this project could not be fully exploited due to a number of timing constraints regarding sequencing, and provision of computing resources. The results reflect the short amount of time that was available for the study once computing resources were in place. In addition to exploring metagenomic diversity, my original intent was to determine if genetic diversity differed between functional genes and neutral markers (i.e., bottlenecks may affect the diversity of functional genes less than neutral markers (Väli *et al.* 2008)), and to identify markers that may provide greater resolution of the genome (i.e., single nucleotide polymorphisms or SNP markers (Morin *et al.* 2004)). These types of markers might be particularly useful given the small number of existing polymorphic microsatellite markers for *A. gracilipes*, which as a result have limited use in determining fine-scale genetic structure (Koskinen *et al.* 2004). Illumina data may also be used to generate new microsatellite markers (Castoe *et al.* 2012).

Another use of the short-read-sequence dataset is to search for potential genes that may be related to variation in abundance (e.g., genes associated with functions such as insulin pathways, recognition or immunity). This latter aim may be more feasible now than when I started my thesis, as six ant genomes have been sequenced and annotated over the last year or so (Bonasio *et al.* 2010; Smith *et al.* 2011a; Smith *et al.* 2011b; Suen *et al.* 2011; Wurm *et al.* 2011), together with a number of other Hymenopteran genomes that have been more extensively annotated (Munoz-Torres *et al.* 2011). A number of candidate genes have also been identified for pest control of ants (Rodovalho *et al.* 2011). These include genes that are involved development and longevity, immunity, resistance to pathogens, pheromone function, behaviour and polysaccharide metabolism (Rodovalho *et al.* 2011). In addition, genes coding for defensin antimicrobial proteins, which are under positive selection in ants (Viljakainen and Pamilo 2008), and vitellogenin, which is involved in stress resilience and social organisation in honeybees (Amdam *et al.* 2012) may be among candidates that contribute to variation in abundance.

The metagenomic aspect of my study could be refined further. The variation in data publicly available on GenBank is likely to be a constraint, and there are other microbial database options including those that are publicly available (e.g., the RDP database used by Anderson et al. 2012) or privately held (e.g., the Microbial Diversity Analysis Service used by Ishak et al. 2011). Identifying, and correcting for, the bias in the GenBank databases (i.e., whether high numbers of matches are due to prevalence of particular organisms in the database) will also be useful. Further work on this aspect includes investigating ways of extracting more data from the many unused reads in the dataset by refining the assemblies (initially by varying the assembly parameters, including combining assemblies with different k-mer lengths [B. Schoenfeld personal communication]), and metagenome analyses using different bioinformatic approaches (e.g., different assemblers such as MEGAN (Huson et al. 2011), ABySS (Simpson et al. 2009), SOAP (Li et al. 2009) or PE-Assembler (Nuwantha Ariyaratne and Sung 2011)). Unused or unmapped reads are sometimes biologically meaningful, however, and can be due to viral sequences (Li et al. 2011), which may also prove a useful avenue to explore in relation to causes of population declines. Further analysis of the short-read-sequence dataset may be complicated, however, by the challenge of heterozygote genotypes that were, by necessity, used for sequencing. This 'phasing' problem perhaps contributes to the number of unused reads in the assemblies, and is a further challenge for which to investigate a solution.

The actual function of bacteria (and potential parasites) in *A. gracilipes* populations can only be assessed via controlled experiments. Experiments would also enable the direct assessment of any interaction between the genetic diversity of the host and its susceptibility to infection. If this were possible, a good sampling design would include multiple replicates from populations that are known to have declined together with those that have not, and comparisons with the native range (or at least colonies from possible candidate native ranges). Now that we have some idea of the types of bacteria that may be present in *A. gracilipes*, assays could be developed with specific primers to test for the

prevalence of these bacteria. Treatment with antibiotics and infection challenges would also be informative, and protocols are available for culturing *Burkholderia* (Vandamme *et al.* 2007a). However isolating and culturing other more general bacterial strains may prove more difficult.

7.4 Conclusion

A key factor in invasion success is the ability for a population to reach sufficient abundance to persist, spread, and alter or dominate the recipient biological community (Ehrlich 1986; Crawley 1987; Mack *et al.* 2000). Genetic diversity has important ramifications for biological fitness, and thus invasion success in the long term. The vast majority of studies of *A. gracilipes* to date have focussed on the ecological causes of the success of this invasive ant. Genetic factors play an important role in the success of other invasive ants, and this is the first study to directly investigate the possible role of multiple genetic factors in the variation in abundance, and thus differential invasion success of *A. gracilipes*. In addition, this is to my knowledge the first study to use Illumina short-read-sequencing to investigate metagenomic diversity in an ant species. While my metagenomic work is only preliminary, and although all my thesis findings are essentially correlational rather than mechanistic, the study highlights a number of avenues of research into the causes of variation in *A. gracilipes* abundance, which may also be applicable more generally to other invasive ant species.

Together, my findings suggest that propagule pressure, reproductive mode and haplotype-specific effects do not appear to be associated with variation in *A. gracilipes* abundance. Even if clonal reproduction occurs in the species, it may assist establishment success rather than long term persistence. Other genetic factors I investigated do appear to be associated with variation in *A. gracilipes* abundance. Genotypic diversity was positively related to the abundance of *A. gracilipes* in Arnhem Land, and this relationship may be affected by population divergence through population bottlenecks. In addition, differences in bacterial diversity may also be associated with variation in abundance among populations. Although genetic factors are often implicated in the successful establishment of invasive species, my thesis demonstrates that genetic factors may also be associated with post-establishment population dynamics.

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Appendices

Supplementary figures



Supplementary Fig. 4.1: Allele discovery curves for the most polymorphic locus (*Ano8*) for all nine *Anoplolepis gracilipes* invaded sites (18 plots) in the study, based on the complete dataset of 539 ants. Where curves have not flattened completely there were no further samples for genotyping.



Supplementary Fig. 4.2: Species accumulation curves based on pitfall trap catches for *Anoplolepis gracilipes*: (a) invaded; and (b) uninvaded sites in Arnhem Land, Australia.



Supplementary Fig. 4.3: Principal component analysis of *Anoplolepis gracilipes* invaded and uninvaded plots based on Euclidean distance of standardised values for the availability of nest sites, canopy cover, presence of *Acacia* spp. and leaf litter depth.

Supplementary tables

Supplementary Table 2.1: Allele frequencies and sample sizes for Chapter 2.

Locus	Allele	Queens	Sperm	Males	Workers
Anol	Ν	39	46	29	310
	97	0.000	0.402	0.259	0.498
	101	1.000	0.598	0.741	0.502
Ano3	Ν	47	46	29	310
	162	0.883	0.663	0.724	0.502
	174	0.000	0.000	0.000	0.011
	176	0.117	0.337	0.276	0.444
	178	0.000	0.000	0.000	0.044
Ano4	Ν	47	45	29	310
	156	1.000	0.644	0.828	0.502
	174	0.000	0.356	0.172	0.498
Ano7	Ν	39	45	29	310
	228	0.013	0.000	0.000	0.000
	230	0.000	0.478	0.293	0.505
	240	0.000	0.033	0.000	0.005
	242	0.936	0.467	0.690	0.263
	244	0.051	0.022	0.017	0.219
	246	0.000	0.000	0.000	0.008
Ano8	Ν	45	46	29	310
	208	0.000	0.043	0.000	0.000
	210	0.000	0.011	0.000	0.015
	212	0.444	0.293	0.034	0.156
	214	0.300	0.185	0.000	0.108
	216	0.222	0.130	0.862	0.210
	218	0.000	0.022	0.000	0.016
	262	0.000	0.000	0.000	0.024
	264	0.000	0.000	0.000	0.019
	266	0.000	0.000	0.000	0.045
	268	0.000	0.033	0.000	0.042
	270	0.000	0.000	0.000	0.006
	272	0.022	0.196	0.000	0.090
	274	0.011	0.065	0.086	0.232
	276	0.000	0.000	0.017	0.026
	278	0.000	0.000	0.000	0.002
	282	0.000	0.022	0.000	0.008

		Sample						
Locus	Allele	A1	A2	B1	B2	D1	D2	
Ano3	Ν	31	26	23	25	33	32	
	162	0.500	0.500	0.478	0.500	0.500	0.500	
	164	0.000	0.000	0.022	0.000	0.000	0.000	
	174	0.000	0.000	0.000	0.000	0.030	0.000	
	176	0.500	0.500	0.500	0.500	0.455	0.297	
	178	0.000	0.000	0.000	0.000	0.015	0.203	
Ano7	Ν	31	26	23	25	33	32	
	230	0.500	0.500	0.500	0.480	0.500	0.500	
	232	0.000	0.000	0.000	0.020	0.000	0.000	
	240	0.000	0.000	0.000	0.000	0.015	0.016	
	242	0.000	0.000	0.000	0.080	0.273	0.078	
	244	0.500	0.500	0.478	0.400	0.212	0.406	
	246	0.000	0.000	0.022	0.020	0.000	0.000	
	248	0.000	0.000	0.000	0.000	0.000	0.000	
Ano8	Ν	31	26	23	25	33	32	
	208	0.000	0.000	0.000	0.020	0.000	0.000	
	210	0.000	0.000	0.087	0.020	0.000	0.000	
	212	0.000	0.000	0.239	0.240	0.000	0.000	
	214	0.048	0.000	0.130	0.200	0.015	0.000	
	216	0.403	0.500	0.043	0.020	0.470	0.500	
	218	0.048	0.000	0.000	0.000	0.015	0.000	
	254	0.000	0.000	0.000	0.000	0.000	0.000	
	256	0.000	0.000	0.000	0.000	0.000	0.000	
	264	0.000	0.000	0.000	0.000	0.000	0.000	
	266	0.000	0.000	0.000	0.000	0.000	0.000	
	268	0.000	0.000	0.022	0.040	0.000	0.000	
	270	0.032	0.019	0.043	0.160	0.000	0.000	
	272	0.097	0.096	0.130	0.100	0.000	0.016	
	274	0.355	0.173	0.130	0.160	0.394	0.391	
	276	0.016	0.212	0.174	0.000	0.106	0.078	
	278	0.000	0.000	0.000	0.020	0.000	0.000	
	282	0.000	0.000	0.000	0.000	0.000	0.016	
	284	0.000	0.000	0.000	0.020	0.000	0.000	

Supplementary Table 3.1 (a): Allele frequencies and sample sizes for Chapter 3, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples A1 – D2.

		Sample								
Locus	Allele	D3	D4	G1	G2	K2	K4			
Ano3	Ν	21	30	35	22	20	25			
	162	0.500	0.500	0.500	0.500	0.500	0.500			
	164	0.000	0.000	0.000	0.000	0.000	0.000			
	174	0.000	0.000	0.000	0.000	0.000	0.000			
	176	0.500	0.483	0.500	0.500	0.500	0.480			
	178	0.000	0.017	0.000	0.000	0.000	0.020			
Ano7	Ν	21	30	35	22	20	25			
	230	0.500	0.500	0.500	0.500	0.500	0.500			
	232	0.000	0.000	0.000	0.000	0.000	0.000			
	240	0.000	0.000	0.000	0.000	0.000	0.000			
	242	0.000	0.000	0.029	0.023	0.000	0.100			
	244	0.500	0.500	0.471	0.477	0.500	0.400			
	246	0.000	0.000	0.000	0.000	0.000	0.000			
	248	0.000	0.000	0.000	0.000	0.000	0.000			
Ano8	Ν	21	30	35	22	20	25			
	208	0.000	0.000	0.000	0.000	0.025	0.000			
	210	0.000	0.000	0.114	0.182	0.000	0.040			
	212	0.048	0.233	0.314	0.273	0.325	0.260			
	214	0.143	0.100	0.071	0.045	0.150	0.180			
	216	0.143	0.167	0.000	0.000	0.000	0.020			
	218	0.167	0.000	0.000	0.000	0.000	0.000			
	254	0.000	0.000	0.000	0.000	0.000	0.000			
	256	0.000	0.017	0.000	0.000	0.000	0.000			
	264	0.000	0.000	0.271	0.023	0.000	0.020			
	266	0.000	0.000	0.143	0.273	0.050	0.000			
	268	0.000	0.000	0.057	0.159	0.000	0.060			
	270	0.000	0.000	0.000	0.045	0.050	0.000			
	272	0.095	0.100	0.029	0.000	0.100	0.060			
	274	0.119	0.217	0.000	0.000	0.225	0.280			
	276	0.286	0.133	0.000	0.000	0.050	0.080			
	278	0.000	0.033	0.000	0.000	0.025	0.000			
	282	0.000	0.000	0.000	0.000	0.000	0.000			
	284	0.000	0.000	0.000	0.000	0.000	0.000			

Supplementary Table 3.1 (b): Allele frequencies and sample sizes for Chapter 3, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples D3 – K4.

		Sample						
Locus	Allele	K6	K7	M1	M2	P1	P2	
Ano3	Ν	35	27	26	28	30	29	
	162	0.500	0.500	0.500	0.500	0.500	0.500	
	164	0.000	0.000	0.000	0.000	0.000	0.000	
	174	0.071	0.000	0.000	0.000	0.000	0.000	
	176	0.429	0.500	0.500	0.500	0.500	0.500	
	178	0.000	0.000	0.000	0.000	0.000	0.000	
Ano7	Ν	35	27	26	28	30	29	
	230	0.500	0.500	0.500	0.500	0.500	0.500	
	232	0.000	0.000	0.000	0.000	0.000	0.000	
	240	0.000	0.000	0.000	0.000	0.000	0.000	
	242	0.300	0.037	0.000	0.000	0.017	0.017	
	244	0.200	0.463	0.308	0.393	0.483	0.483	
	246	0.000	0.000	0.154	0.071	0.000	0.000	
	248	0.000	0.000	0.038	0.036	0.000	0.000	
Ano8	Ν	35	27	26	28	30	29	
	208	0.000	0.000	0.000	0.000	0.000	0.000	
	210	0.000	0.000	0.000	0.000	0.000	0.000	
	212	0.243	0.296	0.019	0.107	0.000	0.000	
	214	0.229	0.185	0.442	0.375	0.050	0.000	
	216	0.029	0.019	0.038	0.018	0.433	0.483	
	218	0.000	0.000	0.000	0.000	0.017	0.017	
	254	0.014	0.000	0.000	0.000	0.000	0.000	
	256	0.000	0.000	0.000	0.000	0.000	0.000	
	264	0.000	0.000	0.000	0.000	0.000	0.000	
	266	0.286	0.000	0.000	0.000	0.000	0.000	
	268	0.029	0.037	0.000	0.000	0.000	0.000	
	270	0.000	0.167	0.000	0.000	0.000	0.000	
	272	0.057	0.111	0.000	0.036	0.017	0.086	
	274	0.100	0.185	0.462	0.446	0.100	0.379	
	276	0.014	0.000	0.038	0.018	0.383	0.034	
	278	0.000	0.000	0.000	0.000	0.000	0.000	
	282	0.000	0.000	0.000	0.000	0.000	0.000	
	284	0.000	0.000	0.000	0.000	0.000	0.000	

Supplementary Table 3.1 (c): Allele frequencies and sample sizes for Chapter 3, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples K6 – P2.

				Sample		
Locus	Allele	A1	A2	<u>B1</u>	B2	B3
Ano3	Ν	31	18	32	29	10
	162	0.500	0.500	0.484	0.500	0.500
	164	0.000	0.000	0.016	0.000	0.000
	174	0.000	0.000	0.000	0.000	0.000
	176	0.500	0.500	0.500	0.500	0.500
	178	0.000	0.000	0.000	0.000	0.000
Ano7	Ν	31	18	32	29	10
	230	0.500	0.500	0.500	0.483	0.500
	232	0.000	0.000	0.000	0.017	0.000
	240	0.000	0.000	0.000	0.000	0.000
	242	0.000	0.000	0.016	0.103	0.000
	244	0.500	0.500	0.453	0.362	0.500
	246	0.000	0.000	0.031	0.034	0.000
	248	0.000	0.000	0.000	0.000	0.000
Ano8	Ν	31	18	32	29	10
	208	0.000	0.000	0.000	0.017	0.000
	210	0.000	0.000	0.063	0.017	0.000
	212	0.000	0.000	0.328	0.310	0.000
	214	0.048	0.000	0.219	0.207	0.000
	216	0.419	0.500	0.031	0.017	0.500
	218	0.048	0.000	0.000	0.000	0.000
	256	0.000	0.000	0.000	0.000	0.000
	262	0.000	0.000	0.000	0.000	0.000
	264	0.000	0.000	0.000	0.000	0.000
	266	0.000	0.000	0.000	0.000	0.000
	268	0.000	0.000	0.016	0.034	0.500
	270	0.032	0.000	0.031	0.138	0.000
	272	0.097	0.028	0.094	0.086	0.000
	274	0.339	0.167	0.094	0.138	0.000
	276	0.016	0.306	0.125	0.000	0.000
	278	0.000	0.000	0.000	0.017	0.000
	284	0.000	0.000	0.000	0.017	0.000

Supplementary Table 4.1 (a): Allele frequencies and sample sizes for Chapter 4, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples A1– B3.

				Sample		
Locus	Allele	D2	D3	D4	D6	G1
Ano3	Ν	24	27	25	20	35
	162	0.500	0.500	0.500	0.500	0.500
	164	0.000	0.000	0.000	0.000	0.000
	174	0.000	0.000	0.000	0.050	0.000
	176	0.250	0.500	0.500	0.450	0.500
	178	0.250	0.000	0.000	0.000	0.000
Ano7	Ν	24	27	25	20	35
	230	0.500	0.500	0.500	0.500	0.500
	232	0.000	0.000	0.000	0.000	0.000
	240	0.021	0.000	0.000	0.025	0.000
	242	0.083	0.000	0.000	0.450	0.029
	244	0.396	0.500	0.500	0.025	0.471
	246	0.000	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000
Ano8	Ν	24	27	25	20	35
	208	0.000	0.000	0.000	0.000	0.000
	210	0.000	0.000	0.000	0.000	0.114
	212	0.000	0.130	0.220	0.000	0.314
	214	0.000	0.148	0.120	0.025	0.071
	216	0.500	0.278	0.200	0.450	0.000
	218	0.000	0.130	0.000	0.025	0.000
	256	0.000	0.000	0.020	0.000	0.000
	262	0.000	0.000	0.000	0.000	0.000
	264	0.000	0.000	0.000	0.000	0.271
	266	0.000	0.000	0.000	0.000	0.143
	268	0.000	0.000	0.000	0.000	0.057
	270	0.000	0.000	0.000	0.000	0.000
	272	0.000	0.056	0.060	0.000	0.029
	274	0.458	0.074	0.200	0.450	0.000
	276	0.042	0.185	0.140	0.050	0.000
	278	0.000	0.000	0.040	0.000	0.000
	284	0.000	0.000	0.000	0.000	0.000

Supplementary Table 4.1 (b): Allele frequencies and sample sizes for Chapter 4, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples D2 – G1.

				Sample	!	
Locus	Allele	G2	K1	K4	K5	K6
Ano3	Ν	22	19	28	20	21
	162	0.500	0.500	0.500	0.500	0.500
	164	0.000	0.000	0.000	0.000	0.000
	174	0.000	0.000	0.000	0.000	0.119
	176	0.500	0.500	0.482	0.150	0.381
	178	0.000	0.000	0.018	0.350	0.000
Ano7	Ν	22	19	28	20	21
	230	0.500	0.500	0.500	0.500	0.500
	232	0.000	0.000	0.000	0.000	0.000
	240	0.000	0.000	0.000	0.000	0.000
	242	0.000	0.474	0.089	0.500	0.500
	244	0.500	0.000	0.411	0.000	0.000
	246	0.000	0.026	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000
Ano8	Ν	22	19	28	20	21
	208	0.000	0.000	0.000	0.000	0.000
	210	0.205	0.000	0.036	0.000	0.000
	212	0.273	0.132	0.339	0.500	0.310
	214	0.045	0.342	0.161	0.000	0.143
	216	0.000	0.026	0.018	0.000	0.048
	218	0.000	0.000	0.000	0.000	0.000
	256	0.000	0.000	0.000	0.000	0.000
	262	0.000	0.000	0.000	0.375	0.000
	264	0.000	0.000	0.018	0.025	0.000
	266	0.273	0.000	0.000	0.100	0.429
	268	0.159	0.000	0.054	0.000	0.048
	270	0.045	0.000	0.000	0.000	0.000
	272	0.000	0.079	0.054	0.000	0.024
	274	0.000	0.263	0.250	0.000	0.000
	276	0.000	0.132	0.071	0.000	0.000
	278	0.000	0.026	0.000	0.000	0.000
	284	0.000	0.000	0.000	0.000	0.000

Supplementary Table 4.1 (c): Allele frequencies and sample sizes for Chapter 4, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples G2 –K6.

				Sample		
Locus	Allele	M2	P2	P1	T1	Y3
Ano3	Ν	25	30	19	10	15
	162	0.500	0.500	0.500	0.500	0.500
	164	0.000	0.000	0.000	0.000	0.000
	174	0.000	0.000	0.000	0.000	0.033
	176	0.500	0.500	0.500	0.500	0.467
	178	0.000	0.000	0.000	0.000	0.000
Ano7	Ν	25	30	19	10	15
	230	0.500	0.500	0.500	0.500	0.600
	232	0.000	0.000	0.000	0.000	0.000
	240	0.000	0.000	0.000	0.000	0.000
	242	0.000	0.017	0.000	0.300	0.400
	244	0.380	0.483	0.500	0.200	0.000
	246	0.080	0.000	0.000	0.000	0.000
	248	0.040	0.000	0.000	0.000	0.000
Ano8	Ν	25	30	19	10	15
	208	0.000	0.000	0.000	0.000	0.000
	210	0.000	0.000	0.000	0.000	0.000
	212	0.120	0.000	0.000	0.050	0.000
	214	0.380	0.000	0.079	0.150	0.233
	216	0.000	0.500	0.447	0.300	0.267
	218	0.000	0.017	0.026	0.000	0.000
	256	0.000	0.000	0.000	0.000	0.000
	262	0.000	0.000	0.000	0.000	0.000
	264	0.000	0.000	0.000	0.000	0.000
	266	0.000	0.000	0.000	0.000	0.000
	268	0.000	0.000	0.000	0.450	0.000
	270	0.000	0.000	0.000	0.000	0.100
	272	0.000	0.083	0.026	0.050	0.167
	274	0.480	0.367	0.000	0.000	0.233
	276	0.020	0.033	0.421	0.000	0.000
	278	0.000	0.000	0.000	0.000	0.000
	284	0.000	0.000	0.000	0.000	0.000

Supplementary Table 4.1 (d): Allele frequencies and sample sizes for Chapter 4, for loci with > 2 alleles only (loci with two alleles had 0.500:0.500 allele frequencies). Samples M2 – Y3.

Computer code

The taxon4blast.pl Perl script is publicly available at Github: https://github.com/AnnaFriedlander/taxon4blast

It is planned to tmake the SolexaStats java function available in a public repository in the future.

Taxon4blast.pl README

```
#
# Copyright 2012 Anna Friedlander and Monica Gruber.
                                                                      #
# (anna.fr@gmail.com and monica.gruber@vuw.ac.nz)
                                                                      #
#
                                                                      #
# This program is free software; you can redistribute it and/or modify it
                                                                      #
# under the same terms as Perl itself (either: the GNU General Public License #
# as published by the Free Software Foundation; or the Artistic License).
                                                                      #
# See http://dev.perl.org/licenses/ for more information.
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# Comments and queries are welcome to either or both authors, however we are
                                                                      #
# unable to provide dedicated technical support.
                                                                      #
                                                                      #
# Please acknowledge the authors if you use the programme in any work
                                                                      #
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# resulting in publication. Please cite it as:
                                                                      #
#
#
   Friedlander, A.M. and Gruber, M.A.M 2012. taxon4blast.pl v 1.0.
                                                                      #
   available from anna.fr@gmail.com or monica.gruber@vuw.ac.nz
#
                                                                      #
#
                                                                      #
*****
-----PROGRAM DESCRIPTION-----
taxon4blast.pl is a program to parse BLAST+ output (output format 6 with
default fields) and append taxonomic information.
It contains functions to:
   * summarise BLAST+ output by taxon and calculate summary statistics
     (number of hits; total, average, and median sequence length; average
     e-value; average bit-score);
   * compare BLAST+ output files by number of hits and sequence length at
     a particular taxon level (or taxon level and GI)
   * create FASTA files and split data based on whether an input sequence
     hit a particular taxon.
More detailed information can be found in the help menu by using the command:
     % perl taxon4blast.pl -help
-----RUNNING REQUIREMENTS------
taxon4blast.pl parses files of the following format:
   BLAST+ output in output format 6 (tab-delimited, no comment lines) with
   default fields (qseqid sseqid pident length mismatch gapopen qstart qend
   sstart send evalue bitscore)
To run taxon4blast.pl, Perl 5 (or later) and the following Perl modules must be
installed:
   *Bioperl
```
*Bio::LITE::Taxonomy::NCBI::Gi2taxid *Sort::Naturally As well as the default modules Getopt::Long and List::Util The appropriate (nucl or prot) NCBI Taxonomy flatfiles are required: *nodes.dmp *names.dmp *gi taxid nucl.dmp or gi taxid prot.dmp which can be obtained from ftp://ftp.ncbi.nih.gov/pub/taxonomy (the first two files will be in a taxdump archive) NOTE: the first time the program is run, use the gi taxid <nucl/prot>.dmp file; a gi taxid <nucl/prot>.bin file will be created, which can be used subsequently To use -taxon summary, input files to the BLAST+ search (in FASTA format) are required. -----FUNCTION SUMMARY------The following functions are available to the user: taxon info fetches taxid and taxonomic hierarchy given GI, appends to BLAST+ output and prints to file (output used in the other four main functions) taxon_summary sorts taxon_info output according to taxon specified by the user, calculates summary stats (number of hits; total, average, median sequence length; average e-value; average bit-score) and prints to file taxon overlap sorts taxon info output according to taxon specified by user compares n files by number of hits and total length of hits for each taxon taxon sequence overlap sorts taxon info output according to taxon specified by user compares n files by number of hits and total length of hits for each taxon+GI using -nonunique prints only records found in >1 file sequence extract given taxon info output and the original input files to BLAST (which were used to create the BLAST output used as input to taxon_info), and a taxon level and taxon name (e.g. -level superkingdom -name bacteria) this function creates two fasta files. One file contains all sequences that hit the taxon name specified, the other contains all sequences that hit other taxa. Note that there will likely be overlap in the output files (eg an input sequence may hit both bacterial sequences and nonbacterial sequences). Using the subseq option prints out only the sequence that was matched (using qstart and qend) -----SUGGESTED IMPROVEMENTS------The following are improvements that the user may wish to make to the program: *allow input of any BLAST output type, by requiring the user to specify what column the qseqid, sseqid, evalue etc are in and how fields are delimited (at the moment BLAST+ output format 6 with default fields is hardcoded in). *graphical output *improve efficiency (e.g. use three hashes in taxon_info (line->gi; gi->taxid; taxid->taxon_info) - at present there is duplication in this function *utilise parallel processing (at present processing is sequential)

SolexaStats java package

```
#
                                                                          #
# Copyright 2010 Cameron Jack and Monica Gruber.
                                                                          #
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                                                                          #
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#
   Jack, C.A. and Gruber, M.A.M 2010. SolexaStats v 0.1.
                                                                          #
#
   available from cam.jack.nz@gmail.com or monica.gruber@vuw.ac.nz
                                                                          #
#
                                                                          #
**************
/*
 * SolexaStats v0.1
 * Cameron Jack and Monica Gruber, 2010. Victoria University of Wellington.
 *
 * SolexaStats implements Illumina's suggest quality baseline of the total
 * FASTQ file
 * having a minimum of 0.80 of bases of Phred score 30 or better. This
 * corresponds to * p < 0.001 or the ^_`abcdefgh symbols.</pre>
 * We can also get some other basic quality measures at the same time to
 * compare with other QA software such as SolexaQA.
 */
package solexastats;
import java.io.BufferedReader;
import java.io.File;
import java.io.FileReader;
import java.io.IOException;
import java.util.Iterator;
import java.util.Vector;
class Stats {
   public long numBasesConsidered_ = 0;
   public long numBasesOverS30_ = 0;
   public final int MAXLINES_ = 1000000;
   public Vector<String> allQualityLines_;
   public void doWork() {
       Iterator dataItr_ = allQualityLines_.iterator();
       String dataLine_; char c_;
       while (dataItr_.hasNext()) {
```

```
dataLine_ = (String) dataItr_.next();
        for (int i = 0; i < dataLine_.length(); i++) {</pre>
             numBasesConsidered_++;
             c_ = dataLine_.charAt(i);
             switch(c_) {
    //case ';
                          ;':
                                //-5
                                        0.7597
                 //case '<':
                                //-4
                                        0.7153
                 //case '=':
                                //-3
                                        0.6661
                 //case '>':
                                //-2
                                        0.6131
                 //case '?':
                                        0.5573
                                //-1
                 //case '@':
                                        0.5000
                                //0
                 //case 'A':
//case 'B':
                                //1
                                        0.4427
                                //2
                                        0.3869
                 //case 'C':
                                //3
                                        0.3339
                 //case 'D':
                                        0.2847
                                //4
                 //case 'E':
                                //5
                                        0.2403
                 //case 'F':
                                //6
                                        0.2008
                 //case 'G':
                                //7
                                        0.1663
                 //case 'H':
                                        0.1368
                                //8
                 //case 'I':
//case 'J':
//case 'K':
                                //9
                                        0.1118
                                //10
                                        0.0909
                                //11
                                        0.0736
                 //case 'L':
                                //12
                                        0.0594
                 //case 'M':
                                //13
                                        0.0477
                 //case 'N':
                                //14
                                        0.0383
                 //case '0':
                                //15
                                        0.0307
                 //case 'P':
                                        0.0245
                                //16
                 //case 'Q':
//case 'R':
                                        0.0196
                                //17
                                        0.0156
                                //18
                 //case 'S':
                                //19
                                        0.0124
                 //case 'T': //20
                                        0.0099
                 //case 'U':
                               //21
                                        0.0079
                 //case 'V':
                                        0.0063
                                //22
                 //case 'W':
                                //23
                                        0.0050
                 //case 'X':
                                        0.0040
                                //24
                 //case 'Y':
                                //25
                                        0.0032
                 //case 'Z':
//case '[':
                                //26
                                        0.0025
                                //27
                                        0.0020
                 //case '\\': //28
                                        0.0016
                 //case ']': //29
                                        0.0013
                 case '^': numBasesOverS30 ++; break;//
                                                                30
                                                                        0.0010
                 case ' ': numBasesOverS30 ++; break;//
                                                                31
                                                                        0.0008
                 case '`': numBasesOverS30_++; break;//
                                                                32
                                                                        0.0006
                 case 'a': numBasesOverS30_++; break;//
                                                                33
                                                                        0.0005
                 case 'b': numBasesOverS30_++; break;//
                                                                34
                                                                        0.0004
                 case 'c': numBasesOverS30 ++; break;//
                                                                35
                                                                        0.0003
                 case 'd': numBasesOverS30 ++; break;//
                                                                36
                                                                        0.0003
                 case 'e': numBasesOverS30_++; break;//
                                                                37
                                                                        0.0002
                 case 'f': numBasesOverS30_++; break;//
                                                                38
                                                                        0.0002
                 case 'g': numBasesOverS30_++; break;//
                                                                39
                                                                        0.0001
                 case 'h': numBasesOverS30 ++; break;//
                                                                 40
                                                                        0.0001
             }
       }
    }
}
```

}

```
public class SolexaStats{
       public static void main(String[] args) {
           if (args.length != 1) {
               System.out.println("Usage: solexastats filename.ext");
               System.exit(0);
           }
           Stats stats_ = new Stats();
           stats_.allQualityLines_ = new Vector<String>();
       try {
               File inputDataFile_ = new File(args[0]);
               // The next line checks to if the output file has already been created
and exits if it has
               //if (outputFile.exists()) { System.out.println("Output file already
exists!"); System.exit(-1);}
               // create file readers and their buffers
               FileReader inputDataFileReader_ = new FileReader (inputDataFile_);
               BufferedReader
                                                                          BufferedReader
                                    inDataFileBR_
                                                                new
                                                       =
(inputDataFileReader_);
               try { // open data file
                   String dataLine_ = null;
                   int lineCounter_ = 0;
                   Iterator dataItr_;
                   char c_;
                   while (inDataFileBR_.ready()) { // until the end of the file
                       if (lineCounter_ < stats_.MAXLINES_) {</pre>
                           dataLine_ = inDataFileBR_.readLine();
                           dataLine_ = dataLine_.trim();
                           lineCounter_++;
                           if (lineCounter_%4 == 0) { // the 4th line is the quality
scores
                               stats_.allQualityLines_.add(dataLine_);
                           }
                       } else {
                           stats_.doWork();
                           lineCounter_ = 0;
                           stats .allQualityLines .clear();
                       }
                   } // end read file
                   stats .doWork();
               } catch (IOException e) {
                   System.out.println("File cannot be read from " + e);
               }
               try {
                   inDataFileBR_.close();
               } catch (IOException e) {
                   System.out.println("File close error " + e);
               }
           } catch (IOException e) {
               System.out.println("Problem opening input data file " + e);
           }
```

```
float megaBases_ = (float) stats_.numBasesConsidered_ / 1000000;
           float fractionPassed_
                                      = (float) stats_.numBasesOverS30_/
                                                                                     (float)
stats_.numBasesConsidered_;
           System.out.println(megaBases_ + " Megabases evaluated in " + args[0]);
           System.out.println("The proportion of bases of Sanger quality score 30 or
greater is " + fractionPassed_);
       }
   }
      FASTQ symbol, ASCII number, Sanger score, p value
   /*
       59
               -5
                      0.7597
   ;
       60
               -4
                      0.7153
   <
                      0.6661
       61
               -3
   =
       62
               -2
                       0.6131
   ?
               -1
                      0.5573
       63
               0
                      0.5000
  @
       64
  А
       65
               1
                      0.4427
  В
       66
               2
                      0.3869
               3
  С
       67
                      0.3339
       68
               4
                      0.2847
  D
               5
                      0.2403
  Е
       69
  F
       70
               6
                       0.2008
   G
       71
               7
                      0.1663
               8
  Н
       72
                      0.1368
               9
   Ι
       73
                       0.1118
   J
       74
               10
                       0.0909
       75
   Κ
               11
                      0.0736
  L
       76
               12
                      0.0594
       77
  Μ
               13
                      0.0477
  Ν
       78
               14
                       0.0383
  0
       79
               15
                      0.0307
  Ρ
                      0.0245
       80
               16
  Q
       81
               17
                      0.0196
  R
       82
               18
                       0.0156
  S
       83
               19
                      0.0124
  Т
       84
               20
                      0.0099
                      0.0079
  U
       85
               21
  ٧
       86
               22
                       0.0063
  W
       87
               23
                       0.0050
               24
  Х
       88
                      0.0040
   Y
               25
       89
                      0.0032
   Ζ
       90
               26
                       0.0025
               27
   [
       91
                       0.0020
       92
               28
                      0.0016
   ١
       93
               29
                      0.0013
   ]
   Ā
       94
               30
                       0.0010
                       0.0008
       95
               31
  ~
                      0.0006
       96
               32
                      0.0005
       97
               33
  а
       98
               34
                       0.0004
  b
       99
               35
                      0.0003
   с
       100
               36
                      0.0003
   d
               37
                      0.0002
       101
   e
   f
       102
               38
                       0.0002
       103
               39
                       0.0001
   g
               40
                      0.0001
   h
       104
```

```
*/
```