Molecular evolution of the immune related gene transferrin in *Polyrhachis* ants and distantly related insect taxa

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Angela Jenny Shuetrim
(B.Sc.Hons)
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For the degree of Doctor of Philosophy
School of Marine and Tropical Biology
James Cook University
Townsville, Queensland
Australia

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Statement of contribution of others

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General Abstract

Host proteins involved in defence against parasites are expected to evolve rapidly and adaptively due to selective pressure from pathogens. Social insect taxa, which face an increased threat from pathogens due to demographic attributes such as high genetic similarities amongst nestmates and high population densities, differ in ways that allow us to examine the impact that variable pathogen loads have on the evolution of immune system genes. Social insects which nest in subterranean habitats come into contact with a wide range and large number of parasites. In contrast, many social insects nest in arboreal or lithocolous localities and so have less contact with the soil and the microbes within it. There is mounting evidence that social insect species which nest in subterranean habitats are associated with greater levels of parasitism. It was predicted that immunity genes will have evolved at a greater rate, as evidenced by positive selection, in social insect taxa that inhabit subterranean nests, as compared to their arboreal and lithocolous counterparts.

Transferrins are single polypeptide chains that play an important role in iron metabolism and resistance to infection in a variety of organisms. In order to limit the amount of iron microbial fauna receive, transferrin is up-regulated following infection in all insects studied to date. As the ability of a microbe to grow and develop within a host's body depends upon the availability of iron, conflict has arisen between host and parasite for procuring this essential element. For example, evidence of the battle to keep and acquire iron has been detected in salmon transferrins, which are evolving rapidly and adaptively. It has been proposed that transferrins in these fish are evolving in this manner because they occupy many different habitats during their

lifecycle, supporting the notion that the type and number of parasites encountered by an organism can affect the strength of selection on their immune system genes.

Polyrhachis is one of the most species rich ant genera in the world and is very well represented in Australia. Species within this genus exhibit extensive variation in nesting habit, making them a suitable and useful model for examining the evolution of immune genes under different selective regimes. Additionally, *Polyrhachis* ants lack metapleural glands, which secrete substances with antibiotic properties able to kill a wide range of pathogenic organisms. This lack enhances their use in an analysis of immune gene evolution, as without these secretions to help eliminate pathogenic microorganisms, other aspects of their immune system are likely to have experienced stronger selective pressure from pathogens than in the case of many other ants, due to a reduced armoury.

I used different models of evolution, based on the nonsynonymous to synonymous substitution ratio (dN/dS ratio), to compare the evolution of the immune related gene transferrin in 14 *Polyrhachis* species with different nesting habits. The type of selection in this protein across lineages ranged from strong purifying selection to positive selection, demonstrating that certain species of *Polyrhachis* have experienced different selective pressure to change the amino acid composition of their transferrin. Three lineages (with consensus between models) have a dN/dS ratio greater than one, indicative of positive selection. Of these three species, two nest in subterranean and one in arboreal localities. However, while predominantly arboreal, the third species is known to also nest in the ground. I suggest that the increased level of evolution in

certain lineages was brought about by variable loads and types of pathogens encountered by *Polyrhachis* ants as they radiated.

Transferrins of five species with subterranean nesting habits have a dN/dS ratio < 1, indicative of purifying selection. It may be that in these species other factors are more important than nesting habit in influencing parasite loads. Overall, predictions as to the parasite load a given species is likely to encounter are sound, however it seems that these predictions need to be inclusive of other factors, as many features are likely to influence parasite exposure.

As well as models designed to detect variable selective intensity across lineages, models that assign a dN/dS ratio to each site were used. Three individual sites were assigned a dN/dS ratio greater than one using consensus among models. These sites are located in regions that are likely to be bound by bacterial iron binding proteins, as they align with regions in vertebrate transferrins known to be so and that have also been subject to adaptive evolution. The occurrence of this type of evolution in equivalent sites and regions in such phylogenetically distant organisms suggests that these sites are important targets for microorganisms that seek to acquire iron from host transferrin.

Using transferrins of distantly related insect taxa, including three hymenopteran genera, I tested for positive selection across lineages and at individual sites. Positive selection was not detected at any site or in any lineage with consensus across models. Rather, the molecule appears to be under strong and significant purifying selection,

suggesting that the molecule is under selective pressure not to change its amino acid composition.

In this analysis of distantly related taxa I included the transferrin of a species of *Polyrhachis* that had a dN/dS ratio greater than one in the analysis *Polyrhachis* transferrins. I propose an explanation as to why positive selection was detected in transferrin of this species in one analysis and not another. Over time synonymous changes accumulate and can mask high rates of nonsynonymous change when it has occurred, as any detrimental nonsynonymous change is expected to be rapidly eliminated from the population. The insects used in this analysis are distantly related, therefore even if certain lineages have experienced selective pressure to change (and we know that at least one has), looking for evidence of this at this scale is likely to miss it, unless selection has been very strong and/or constant. As such, I suggest that where possible it is advisable to search for adaptive evolution in genes within shorter evolutionary time scales, such as in the analysis of *Polyrhachis* transferrins.

Most transferrins consist of two homologous lobes (the N and C lobes) and iron binding in each lobes involves six amino acid residues. With the exceptions of a termite and cockroach, insect transferrins studied to date are not generally conserved for binding motifs in their C termini, thus the capacity to bind iron in this region appears to have been lost. The degeneration of this region is thought to be due to antagonistic interactions between host transferrin and iron scavenging proteins of pathogenic bacteria. It is noteworthy that the positively selected sites in transferrin of *Polyrhachis* ants are located in the N -terminal, which is expected, if the C- terminal of transferrin in these insects is unable to bind iron. Based on alignments of

transferrins from distantly related animals (mammals to insects) doubt has been expressed as to as to whether most insect transferrins can bind iron at all {Lambert, 2005 #757}. My alignment differs to that presented by Lambert et al. {Lambert, 2005 #757}, and I conclude that there is no basis for suggesting that insect transferrins are unable to bind iron in the N-terminal.

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Chapter 1: General Introduction

Animals can be viewed as ideal vessels for the cultivation of the large number of pathogenic organisms they come into contact with every day. Preservation of host integrity requires the presence and maintenance of defences that exclude microbes or destroy them if they gain entry. All animals possess immune systems, integrated bodies of organs, tissues, cells and molecules, which swiftly and effectively differentiate self from non-self and seek to destroy foreign organisms. Immune responses are generated through two main arms of the immune system: the innate and the acquired. Invertebrates and plants generate innate responses, whereas vertebrates rely additionally on acquired responses to combat disease. This discussion focuses on invertebrate innate immune responses. However, the basic mechanisms of the acquired immune system shall also be discussed sufficiently to enable a brief comparison to be drawn between the two types of response, chiefly to highlight recent advances that demonstrate previously unsuspected complexities in invertebrate innate immune responses. Anti-viral defence is as yet poorly understood in insects, but involves a very different mechanism from those aimed at bacteria and fungi (Ip 2005), and I will not be considering it further.

Basic function of innate immune responses

Innate immune responses, which occur in all multicellular organisms, can be broadly divided into constitutive and inducible features (Beck and Habicht 1996; Janeway and Medzhitov 2002). Constitutive features (i.e. defence mechanisms which are always present regardless of physiological demand) include physical barriers such as hard cuticles, chitinous membranes which line the gut and trachea, low gut pH (hostile to

microbial colonisation) and destructive enzymes, such as lysozyme. When pathogens evade these chemical and physical barriers a great array of inducible defence responses are elicited (Figure 1). Inducible responses are classified as cellular (cell-mediated), or humoral (humorally occurring), based on the nature of the end product effector molecules produced. Cellular and humoral responses often act in concert, as both may occur due to the same infection (Janeway and Medzhitov 2002).

Cellular immune responses involve different classes of haemocytes (the invertebrate equivalent to white blood cells), known as plasmocytes, lamellocytes and crystal cells, which serve in processes such as phagocytosis, encapsulation and melanisation (Hultmark 2003; Schmid-Hempel 2005). Humoral inducible responses result in the production of a wide array of proteins which fight pathogens, such as antimicrobial peptides, stress response proteins and iron sequestration proteins (Janeway and Medzhitov 2002; Tzou, De Gregorio, and Lemaitre 2002).

Three main pathways operate in the innate immune system, namely the Toll, Imd and JAK/STAT pathways. The Toll signalling pathway mediates defence against gram-positive bacteria and fungi, whereas the Imd pathway acts in response to gram-negative bacteria (Lemaitre et al. 1995; Lemaitre et al. 1996). The JAK/STAT pathway is involved in both cellular and humoral responses (Agaisse and Perrimon 2004). Host pattern recognition molecules recognise surface determinants (pathogen-associated molecular patterns) present in microbes but absent in the host, such as peptidoglycans, lipopolysaccharides, and B-glucans (Kim et al. 2000). This message is passed to signalling molecules via signal transduction pathways and leads to the entry of transcription factors into the nucleus (Relish in the Imd pathway and Dorsal

and DIF in the Toll pathway) resulting in the synthesis of antimicrobial peptides (Figure 1).

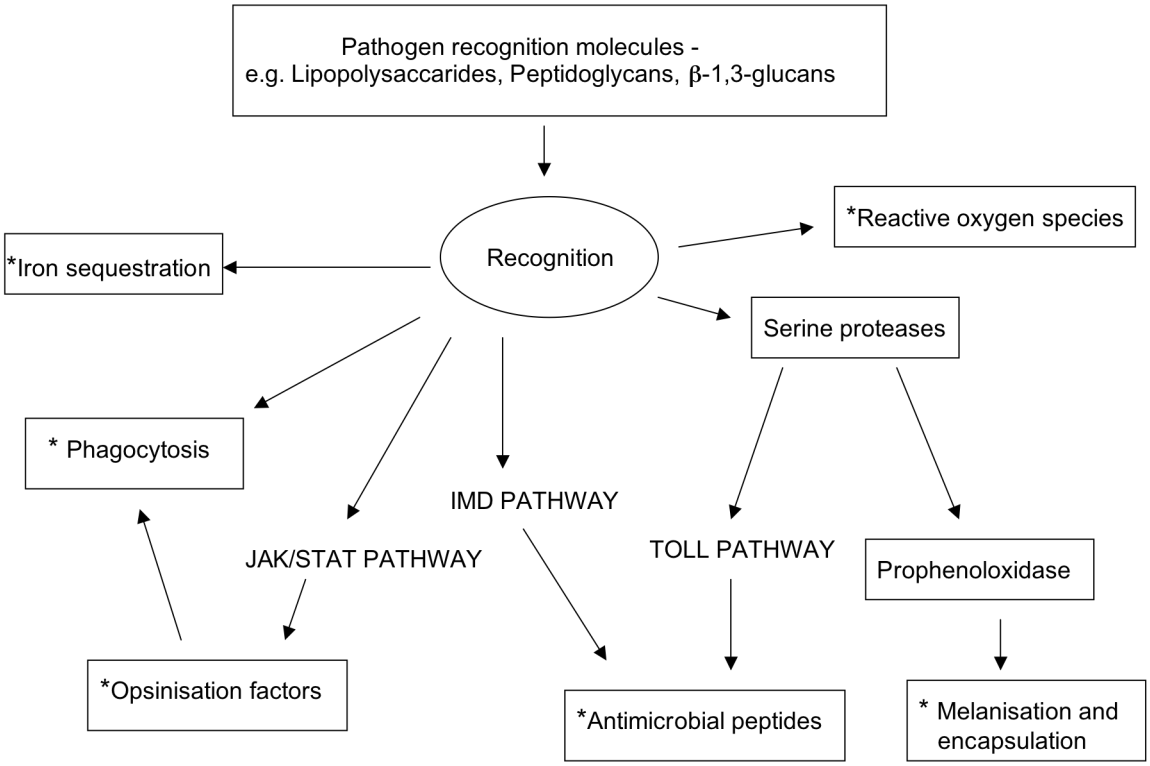

Figure 1. Overview of some known components of the innate immune system, based primarily on studies of *Drosophila*. Antigens are recognised by host pathogen recognition molecules and the signal is passed to signalling molecules, which results in the production of effector molecules involved in direct contact and attack (denoted by *). (Figure redrawn and modified from Tzou, De Gregorio and Lemaitre (2002))

Acquired immune responses

As acquired responses are not the focus of this research I shall just outline a few basic features characteristic of these types of responses. Acquired immune responses, based on immunoglobulin-type antigen receptors, are restricted to the jawed vertebrates i.e.,

all vertebrates except hagfish and lampreys. Acquired responses involve the generation of an extremely large number of recognition and defence molecules through somatic recombination of gene segments (Tonegawa 1988; Klein, Takahata, and Ayala 1993). When stimulated, the acquired system produces many types of T cell lymphocytes whose proteins bind to antigens on, or produced by, the pathogen, and complex cascades are set in motion to destroy the invader. The T cells remain in body fluids at high concentration for long periods of time, enabling the host to respond rapidly to previously encountered pathogens, and these responses are therefore said to have "immunological memory".

Memory and specificity of immune responses

Two key features are important in immune system responses. One is memory, the ability of a system to store and recall information about previously encountered antigens, allowing the host to respond more efficiently and strongly upon reinfection. Another key feature is specificity, whereby immune recognition molecules discriminate between antigens, enabling immune reactions to target specific pathogens (Kurtz 2005).

Memory and specificity have historically been the hallmarks of vertebrate acquired responses, as it was long thought that innate systems lack both memory and specificity. However, recent research has shown that this is not the case. There have been several demonstrations of immune priming in invertebrates, where initial contact with a parasite makes the host more resistant to the same, or a different, pathogen upon reinfection (Huang and Song 1999; Moret and Schmid-Hempel 2001; Moret and Siva-Jothy 2003). These responses can also be trans-generational, as offspring of

infected parents can display enhanced survival when faced with pathogens (Huang and Song 1999; Kurtz and Franz 2003; Sadd et al. 2005). Immune priming is not generally referred to as immunological memory, as the underlying mechanisms of priming in the innate system are unknown, and certainly likely to differ from mechanisms of the acquired system (Little and Kraaijeveld 2004; Kurtz 2005; Sadd and Schmid-Hempel 2006).

In the most thorough study of this kind to date, memory and specificity of the immune system in the bumble-bee *Bombus terrestris* was tested (Sadd and Schmid-Hempel 2006). Hosts survived better when reinfected with the same bacterium as in the initial challenge, as opposed to a reinfection with a different pathogen, and bacterial clearance was greater in groups reinfected with the same parasite as in the initial experiment. It does not appear that these responses were due to the lingering of antimicrobial peptides (AMPs) in the hemolymph following immune stimulation, as the transcription and activity of AMPs had ceased by the time effects were measured.

It may seem that immunological memory is likely to be of little use in invertebrate immune responses, as they are typically short-lived and therefore less likely to encounter the same type of parasite a second time. However, given that memory *has* been demonstrated in innate immune responses, there is reason to explore possible adaptive reasons for it, particularly in the case of social insects, which are the most abundant of all the insects (Wilson 1990; Keller and Genoud 1997). Ant queens can live for several decades and workers for several years (Hölldobler and Wilson 1990). Termite queens and kings can also live for many years (Thorne, Breisch, and Haverty 2002). Just as importantly, social insects engage in frequent contact with colony

members through processes that are exploited by parasites, such as allogrooming and trophallaxis (Bailey and Gibbs 1964; Bailey and Ball 1991). For these reasons I think the argument that insects, in particular social insects, are unlikely to benefit from "immunological memory" is unconvincing.

Another key element long thought to be absent in the innate system is the ability to generate a diverse range of pathogen recognition molecules. Recent work has shown this to be untrue (Watson et al. 2005), indicating an ability for enhanced specificity through the potential to produce thousands of isoforms of Dscam (Down Syndrome Cell Adhesion Molecule, a receptor in the immunoglobulin superfamily). In short, innate immune responses are not nearly as limited as once thought, and continuing research into this system is certainly going to reveal further complexities.

The cost of mounting an immune response

Immune responses are costly (and of course beneficial) to hosts, and the evolution of all immune responses, whether they involve short-term responses or long-term evolutionary moulding, can be boiled down to the dynamics of costs and benefits. Organisms have limited resources; therefore any immune response must be a trade off of some sort, as immune responses incur costs (Doums and Schmid-Hempel 2000; Bonneaud et al. 2003; Baer, Armitage, and Boomsma 2006).

The degree of melanisation of a foreign body is a useful tool for examining the ability of an animal to mount an immune response, and the intensity of that response. Using this approach, the immune response was found to be stronger in non-foraging bees than foraging bees, as measured by the degree of melanisation on small nylon

filaments (Doums and Schmid-Hempel 2000). Flying is an expensive activity, so presumably non-foraging workers (prevented from doing so by wing clipping) mounted a higher immune response than their foraging counterparts because they had more energy to devote to this process.

Promiscuity comes at a price. Primates with promiscuous females have a higher white blood cell count than those with less promiscuous females (Nunn, Gittleman, and Antonovics 2000), supporting the idea that behavioural traits which lead to differences in pathogen burden can lead to alterations in immune system function and affect the allocation of physiological resources. Parallel findings have been made for bumble bees, in which although higher levels of polyandry increase fitness, mating with one to two mates reduces it (Baer and Schmid-Hempel 2001).

Immune genes and adaptive evolution

Host immune genes and parasite genes which counter these defences are expected to evolve rapidly and adaptively in an arms race with eachother. In an evolutionary sense an arms race is when levels of defence and counter-defence continually escalate in order for each party to remain effective in combat with the other. Well known examples involve predation, when a predator evolves more efficient means of prey capture and the prey in turn evolves better means of evasion (Dawkins and Krebs 1979). Arms races can be detected at the molecular level, and indeed the evolution of many proteins involved in immune system processes appears to have been driven in this manner (Hughes and Yeager 1997; Ford, Thornton, and Park 1999; Hughes 1999; Begun and Whitley 2000; Ford 2000; Ford 2001; Boniotto et al. 2003; Morrison et al. 2003; Semple, Rolfe, and Dorin 2003; Bulmer and Crozier 2004; Lynn et al. 2004;

Bulmer and Crozier 2006). One of these proteins is transferrin, and as the evolution of this protein is of pivotal interest in this thesis, I shall outline the basic function and structure of this molecule and discuss work which demonstrates adaptive evolution in this molecule.

Transferrin

Transferrins are single polypeptide chains which play an important role in iron metabolism in multicellular organisms (Lambert, Perri, and Meehan 2005; Dunkov and Georgieva 2006). Within bodily fluids free iron can be deleterious as it can lead to the production of harmful reactive oxygen species (Nichol, Law, and Winzerling 2002; Dunkov and Georgieva 2006). Accordingly, specialised binding proteins, such as transferrins, have evolved to store iron and transport it to cells. The structure and function of transferrins has been studied extensively. Most transferrins contain two lobes (N and C), the result of a gene duplication estimated to have occurred between 850 and 670 mya (Lambert, Perri, and Meehan 2005). Vertebrate transferrins can bind a single iron molecule per lobe (Fe^{3+}) through the collective action of six amino acid residues (Chapter 3, Table 4). Vertebrate transferrins are typically around 80kDa in size, whereas insect transferrins studied to date range from around 66kDa to 80kDa (Yoshiga et al. 1997; Thompson, Crozier, and Crozier 2003) due to the reduction of the C lobe in many groups. Most insect transferrins studied to date are not conserved for iron binding residues in the C lobe, so the capacity to bind iron in this region appears to have been lost (Lambert et al. 2005). Notable exceptions are the cockroach *Blaberus discoidalis*, whose transferrin has been experimentally shown to bind iron in both lobes, and most likely the termite *Mastotermes darwiniensis* (Gasdaska et al. 1996; Thompson, Crozier, and Crozier 2003).

Transferrins and immunity

The ability of a microbe to grow and develop within a host's body is dependent upon the acquisition of many essential elements. Nutrients, such as organic carbon, nitrogen, phosphate and sulphate etc., are freely available to pathogens in tissue and body fluids (Ratledge and Dover 2000). However, the acquisition of iron poses an important problem for pathogens, as host iron is not maintained in solution. Accordingly, conflict has arisen between host and parasite for procuring this essential element. Transferrins are up-regulated following infection in many insects (Bartfeld and Law 1990; Jamroz et al. 1993; Yoshiga et al. 1997; Yoshiga et al. 1999; Thompson, Crozier, and Crozier 2003; Valles and Pereira 2005), and it is plausible that this has been selected for so as to limit the iron available to microbial fauna, reducing their ability to multiply.

In vertebrates, the related proteins lactoferrin and ovotransferrin also increase in plasma and other fluids in response to infection, providing strong protection against microbial proliferation (Lee, Mcknight, and Palmiter 1980; Baker, Rumball, and Anderson 1987; Aguila et al. 2001). In a "counter attack", microorganisms have evolved two principal systems which enable them to acquire iron from host transferrins.

One mechanism involves the production of siderophores, which are iron binding agents produced by pathogens in response to iron deficient conditions. Siderophores are secreted into the infected host and, due to their high affinity for iron, acquire iron from host transferrins. When bound to iron, the siderophores are taken back into to

the microbe through specific uptake carrier systems. The other major mechanism employed by pathogens to acquire iron involves the production of non-siderophore proteins which specifically bind to host transferrin and remove the iron (Ratledge and Dover 2000). Many of these microbial iron binding proteins act in the C-terminal of transferrin, and it has been suggested that the degeneration of the C-terminal in certain insects was selected for as a mechanism to reduce iron loss (Yoshiga et al. 1997).

Adaptive evolution in transferrins

The ratio of the substitution rate at nonsynonymous sites (dN) to that at synonymous sites (dS) provides a convenient indication of selection. A dN/dS ratio significantly greater than one is evidence of adaptive evolution (discussed below). Evidence of adaptive evolution has been found in salmon transferrins, where the dN/dS ratio between transferrins was found to be significantly greater than one (Ford, Thornton, and Park 1999). In an extension of this work, transferrin evolution was examined on a broader scale, using sequences from fish, amphibians and mammals (Ford 2001). Positive selection was restricted to salmon, and to outer areas of the molecule which interact with bacterial transferrin binding proteins, therefore supporting the notion that competition with bacteria led to this adaptive evolution. It is thought that salmon transferrins show positive selection because the fish travel long distances through varied environments (Ford, 2001). This suggests that the type and number of parasites encountered by an organism impact on the strength of selection on immune system genes.

Detecting positive selection

There has been much disagreement as to which force is the most dominant in molecular evolution and the history of these theoretical debates has been recently reviewed (Nei 2005). The neutral model of molecular evolution (Kimura, 1968) is used as the null model in tests for adaptive molecular evolution. Higher rates of evolution can be attributed to adaptive evolution and not an overall elevation of mutation by determining the relative rate of non-synonymous (dN) to synonymous (dS) changes. In the absence of adaptive evolution the rates of the two types of changes will be the same, whereas an elevated rate of nonsynonymous changes is evidence of adaptive evolution (Kreitman and Akashi 1995). In other words, a dN/dS ratio > 1 means that many nonsynonymous mutations offer a fitness advantage and are fixed in populations at a rate greater than that for synonymous changes, and is evidence of adaptive evolution (positive selection). A dN/dS ratio = 1 indicates neutral evolution and a dN/dS ratio < 1 is indicative of purifying selection.

In some tests designed to detect adaptive evolution, pairwise approaches are used. These tests estimate substitution rates by averaging over all sites which have changed between two proteins as they have diverged (Yang and Swanson 2002) (Yang, 2002). While this approach has in some cases been successful (Tanaka and Nei 1989; Riley 1993; Hughes 1999), it is likely that the simple determination of relative rates of nonsynonymous vs. synonymous sites averaged over all sites and lineages will be too conservative and not powerful enough to detect the footprints of adaptive evolution (Yang and Nielsen 2002; Yang and Swanson 2002).

The difficulty in attempting to detect natural selection at the molecular level is that generally only a few sites are under positive selection, as adaptive evolution most likely occurs at a few time points and affects only a few amino acids (Yang et al. 2000). Another shortcoming of pairwise methods is that they do not allow changes to be narrowed down to specific lineages in evolutionary history. The assumption that all sites and lineages are under the same selective pressure is unrealistic, and therefore by using this approach many attempts to detect adaptive evolution in proteins are likely to fail when it has indeed occurred. In addition, most nonsynonymous changes are expected to be removed from the population by purifying selection, because they are detrimental, so synonymous changes accumulate and are likely to surpass high rates of nonsynonymous change, unless selection is constant (Fay, Wyckoff, and Wu 2002).

Recent models (Yang and Nielsen 2002; Yang and Swanson 2002; Pond and Frost 2005b) allow for variable selection intensity across lineages and sites. Therefore, using a phylogenetic framework, positive selection can be detected when it occurs at only a few sites and/or in specific lineages, which is necessary for the research outlined in this thesis.

Social insects and parasites

A closed space with a colony of tightly packed, highly interactive and genetically similar individuals is surely an ideal place for the rapid proliferation of a parasite. As expected, social insect colonies have many parasites and diseases including many microparasites with extremely high rates of reproduction within the host's body and short generation times (Boomsma, Schmid-Hempel, and Hughes 2005). There is

much empirical evidence which shows that parasites have, and do, impose a considerable selective force upon social insects (Schmid-Hempel 1998).

Recognised methods to cope with this selective force are diverse and have been demonstrated in several studies. Social taxa have evolved behavioural, physiological and genetic mechanisms to cope with parasites. An example of a behavioural adaptation is found in the wood ants, *Formica paralugubris*, which collectively incorporate large amounts of microbial growth inhibiting conifer resin into their nest-material (Christe et al. 2003). Group living has also been shown to confer resistance to disease as ants and termites exploit behaviour such as trophallaxis to spread antibiotics through the colony and allogrooming to clean the cuticles of nestmates (Hughes, Eilenberg, and Boomsma 2002; Traniello, Rosengaus, and Savoie 2002).

There is evidence that parasites have influenced relatedness within social insect colonies. Hymenopteran colonies are often headed by one singly mated queen, which results in high relatedness and low genetic variability among colony members (Hamilton 1964b). Two common conditions alter this scenario by increasing genetic variance and lowering relatedness in a colony: polyandry, multiple mating by the queen, and polygyny, the presence of more than one queen in a colony. This realisation was pivotal in the understanding of hymenopteran evolution. Kin selection, the selection of genes favouring the survival and reproduction of relatives who possess genes identical by descent (Hamilton 1964a), is believed to have been the driving force behind the evolution of eusociality, but is undermined by low relatedness among nest mates. Additionally, extra matings consume time and energy and expose the queen to predation (Crozier 1996; Baer and Schmid-Hempel 1999).

Several hypotheses to explain the adaptive value of polyandry have been proposed

and reviewed (Crozier and Page 1985; Page, Robinson, and Fondrk 1989; Schmid-Hempel 1994; Keller and Reeve 1995; Crozier and Fjerdingstad 2001; Brown and Schmid-Hempel 2003). It is probably true that many factors contribute to the occurrence of polyandry across taxa, and that an explanation inclusive of many factors should be sought (Crozier and Fjerdingstad 2001). However, a hypothesis based on the notion that genetically variable colonies have an enhanced ability to fight disease has gained a lot of empirical support in recent years. This hypothesis was first was proposed by William Hamilton in 1987, and has since been called the variation versus parasitism hypothesis (Schmid-Hempel and Crozier 1999).

The essence of the variation vs parasites hypothesis is that polyandry and polygyny are adaptations to increase genetic diversity in order to combat epidemics (Hamilton 1987). This hypothesis assumes that genetically diverse colonies have a higher probability of survival and /or reproductive success when challenged by parasites due to the presence of diverse worker genotypes less likely to disastrously succumb to infection (Baer and Schmid-Hempel 1999). This hypothesis has been empirically tested and the superior ability of genetically diverse hymenopteran colonies to combat disease has been demonstrated on several occasions (Shykoff and Schmid-Hempel 1991; Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999). Furthermore, a phylogenetically based comparative approach reveals that high parasite loads correlate negatively with intra-colony genetic variability, i.e. species with lower relatedness (high genetic variability) have lower parasite loads (Schmid-Hempel and Crozier 1999).

The complete genome of the honey bee *Apis mellifera,* has recently been sequenced (Consortium 2006) and a genome wide analysis of immunity in these bees undertaken

(Evans et al. 2006). Far fewer immunity genes were identified in the honey bee genome than in *Drosophila* and *Anopheles*, which at first is unexpected given that social insects face such a great threat from parasites. It may be that, because honey bee DNA is so AT-biased, not all genes have been recognised. Alternatively, honey bees have evolved many advanced hygienic behaviours, possibly negating the need for a wide range of immunity molecules, and/or these bees may be primarily attacked by a restricted set of highly coevolved pathogens, making the possession of a vast range of immunity molecules unnecessary (Evans et al. 2006). Until the genomes of other Hymenoptera have been sequenced, we will not know if the small number of immune system genes in *Apis* reflects sociality or is a general characteristic of the order.

Life history characteristics of social insects likely to affect parasite load, with a focus on nesting habit

Several life history characteristics found within social insect taxa are expected to influence disease pressure. The four major social insect groups, bees, wasps, ants and termites, display non-uniform loads of parasites and diseases, and it is suggested that this can be explained by differences in ecology and life history (Boomsma, Schmid-Hempel, and Hughes 2005). For example, many bees and wasps build nests from freshly collected or manufactured substrates, which are likely to be associated with fewer pathogens than the soil and wood used by ants and termites to excavate their nests (Boomsma, Schmid-Hempel, and Hughes 2005).

Bacteria are ubiquitous in soils and many types of fungi are dispersed widely through passive processes. These fungi can infect a wide range of hosts, move through the soil

by growing hyphae and produce hard bodies composed of masses of tightly packed spores which are able to survive dormant for several years (Brock 1971; Soper, Whistler, and Davies 1976; Knox, Ladiges, and Evans 1994; Lynch et al. 2004; Boomsma, Schmid-Hempel, and Hughes 2005). Accordingly, social insects which nest in subterranean habitats are expected to come into contact with a wide range and a large number of pathogenic organisms. In contrast, many social insects nest arboreally and therefore have less contact with soil and the microbes within it. Differences in nesting behavior have been hypothesised (Michener 1985) to influence the parasite load encountered by a given species and evidence supports this (Wcislo 1996).

A study on termites showed that a ground-nesting termite had a significantly greater pathogen load than three arboreal-nesting termites (measured as colony-forming units), providing evidence that nesting habit influences pathogen load (Rosengaus et al, 2003). Importantly, it has been shown that three immunity genes, Relish and two GNBPs, were driven by positive selection in an ancestral lineage of ground nesting nasute termites (Bulmer and Crozier 2006). Along this branch it is inferred that there was a shift from a diet of grass to one of decayed wood, a change likely to expose the termites to a novel and elevated suite of fungal and bacterial pathogens (Bulmer and Crozier 2006), providing evidence of a long term evolutionary response to pathogen exposure.

Polyrhachis ants are a model for a study of immune gene evolution

The extraordinary ecological and social diversity of ants has been definitively reviewed by Hölldobler and Wilson (1990). Ants have evolved to fill a variety of

ecological niches, and are the most widely distributed eusocial group, found in six of the seven major biogeographic zones. They are the most numerically abundant of the eusocial insects (Wilson 1990). *Polyrhachis* (Formicinae) is one of the most species-rich genera in the world (Bolton 1995), containing approximately 500 described species belonging to twelve sub-genera (Kohout 1990b). *Polyrhachis* ants are particularly well represented in Australia where they inhabit all major faunal provinces (Dorow 1995; Shattuck 1999). This taxonomic diversity is accompanied by an extensive diversity in nest location (Table 1), making them a useful model group for examining the evolution of immune genes under different selective regimes. *Polyrhachis* nest in the soil (subterranean), in preformed cavities in trees (lignicolous), on rock faces (lithocolous), and some construct nests using larval and even spider silk (arboreal weavers) (Kohout 2000; Robson and Kohout 2005).

The use of *Polyrhachis* in an analysis of immune gene evolution is further enhanced by their lack of metapleural glands, which are an ancient and unique synapomorphy of ants (Hölldobler and Wilson 1990). These glands secrete substances with antibiotic properties able to kill a wide range of pathogenic organisms (Maschwitz 1974; Beattie et al. 1986; Poulsen, Hughes, and Boomsma 2006). *Polyrhachis* ants have secondarily lost metapleural glands (Hölldobler and Engel-Siegel 1994), and it is likely that without these secretions to help fight pathogenic microorganisms, other aspects of their immune system have been under stronger selection from pathogen pressure than in the case of other ants, due to a lack of armoury.

Table 1. Diversity of nest architecture in *Polyrhachis* ants

Subgenus	Nest locality	Larval silk ever present in nest	Source
Aulacomyrma	Arboreal	Yes	(Kohout pers. comm. to Robson)
Campomyrma	Arboreal	Yes	(Dorow and Kohout 1995)
	Subterranean	Yes	(Dorow and Kohout 1995)
Chariomyrma	Subterranean	No	(Kohout 2000)
Cryrtomyrma	Arboreal	Yes	(Robson and Kohout 2005)
Hagiomyrma	Lithocolous	No	(Robson and Kohout 2005)
	Subterranean	No	(Kohout 1988b)
Hedomyrma	Lithocolous	Yes	(Robson and Kohout 2005)
	Lignicolous	No	(Robson and Kohout 2005)
Hemioptica	Arboreal	Yes	(Dorow and Kohout 1995)
Myrma	Lignicolous	No	(Robson and Kohout 2005)
	Arboreal	Yes	(Robson and Kohout 2005)
	Subterranean	No	(Bolton 1973)
Myrmatopa	Arboreal	Yes	(Robson and Kohout 2005)
Myrmhopla	Arboreal	Yes	(Robson and Kohout 2005)
	Lignicolous	No	(Robson and Kohout 2005)
	Subterranean	No	(Kohout 1990a)
Myrmothrinax	Arboreal	Yes	(Robson and Kohout 2005)
Polyrhachis	Arboreal	Yes	(Robson and Kohout 2005)
	Lignicolous	Yes	(Kohout 1988a)
	Subterranean	Yes	Cited by Hung (1967)

The research outlined in this thesis was conducted in order to increase our understanding of the long term evolutionary response to altered pathogen loads, as measured by the rate of evolution in immune system genes, in social insects.

The two main questions addressed in this thesis are:

1. Has the evolution of the immune-related gene transferrin been more rapid in ground nesting than in arboreal species of *Polyrhachis* ants?

It is predicted that ground nesting social insects encounter a wider range of pathogens than their arboreal and lithocolous sisters, and that this will in turn lead to more rapid evolution of immune genes in the ground nesting species, as evidenced by positive selection in these lineages. An independently derived phylogeny based on the evolution of mitochondrial and nuclear genes of *Polyrhachis* ants is used in tests for heterogenous selective pressure in the gene as a whole, and for examination of differing evolutionary rates among lineages with varying parasite loads.

2. Has the evolution of the immune-related gene transferrin been more rapid in social insect species than in other phylogenetically distant, non social, insects?

It is predicted that social insects will have experienced greater selective pressure to change the amino acid composition of their transferrin, as evidenced by positive selection, than non-social insects. Widespread of evidence of adaptive evolution has not been found in insect transferrins (Thompson, Crozier, and Crozier 2003). However, more sequences are now available, including those of more social insects, so reassessment is warranted. Using a comparative phylogenetic approach, I look for evidence of adaptive evolution and examine the iron binding capacities of transferrins in a range of phylogenetically distant insect taxa.

My research will help to classify the evolutionary pressures which shape the functioning of specific regions in the innate immune system and increase our knowledge of this very important biological system. Future research can use this knowledge to develop methods to cripple the immune system in invasive species. Because the study subjects are the most numerically abundant of the social insects, which account for up to 75% of the entire insect biomass (Wilson 1990), the findings of this study will be of wide potential ecological interest and significance.

Chapter 2: Molecular evolution of the immune relate gene transferrin in Polyrhachis ants with variable nesting habits

Abstract

Host proteins involved in defence against parasites are expected to evolve rapidly and adaptively due to selective pressure from pathogens. Social insect taxa, which face an increased threat from pathogens, differ in ways that allow us to examine the impact that variable pathogen loads may have on the evolution of immune system genes. There is evidence that social insect species which nest in subterranean habitats are associated with greater levels of parasitism and it is predicted that immunity genes have evolved at a greater rate in social insect taxa that inhabit subterranean nests, as compared to their arboreal and lithocolous counterparts. The immune related gene transferrin, which plays an important role in iron metabolism and resistance to infection, was isolated from *Polyrhachis* ants. I used different models of evolution, based on the nonsynonymous to synonymous substitution ratio (dN/dS ratio), to compare the evolution of the immune related gene transferrin in 14 Polyrhachis species with different nesting habits. No strong association was found between nest type and rate of transferrin evolution, however, the type of selection in this protein across lineages ranged from strong purifying selection to positive selection, demonstrating that certain species of *Polyrhachis* have experienced different selective pressure to change the amino acid composition of their transferrin. I suggest that the increased level of evolution in certain lineages was brought about by variable loads and types of pathogens encountered by *Polyrhachis* ants as they radiated. As well as models designed to detect variable selective intensity across lineages, models that assign a dN/dS ratio to each site were used. Individual positively selected sites are located in regions that are likely to be bound by bacterial iron binding proteins, as

they align with regions in vertebrate transferrins known to be so and that have also been subject to adaptive evolution. The occurrence of this type of evolution in equivalent sites and regions in such phylogenetically distant organisms suggests that these sites are important targets for microorganisms that seek to acquire iron from host transferrin.

Introduction

Genes encoding host immune proteins are expected to evolve rapidly and adaptively due to selective pressure imposed by pathogenic microorganisms and indeed the evolution of many proteins involved in immune system processes indeed appears to have been driven in this manner (Hughes and Yeager 1997; Begun and Whitley 2000; Ford 2001; Boniotto et al. 2003; Lazzaro and Clark 2003; Morrison et al. 2003; Semple, Rolfe, and Dorin 2003; Bulmer and Crozier 2004; Lynn et al. 2004). Evolution of this kind has been documented in proteins involved in conflict for the acquisition of iron, which is an essential element required for the survival of a microorganism within a host's body.

Specialised proteins, such as transferrins, have arisen to store and transport iron within hosts' bodies (Nichol, Law, and Winzerling 2002; Dunkov and Georgieva 2006). Transferrins perform another essential role, as in order to prevent microbial fauna from receiving iron, reducing their ability to multiply, transferrin is up-regulated following infection in all insects examined to date (Bartfeld and Law 1990; Jamroz et al. 1993; Yoshiga et al. 1997; Yoshiga et al. 1999; Kucharski and Maleszka 2003; Thompson, Crozier, and Crozier 2003; Valles and Pereira 2005). Accordingly, the acquisition of iron poses an extremely important problem for pathogens within a

host, as without access to this element they will die (Ratledge and Dover 2000; Nichol, Law, and Winzerling 2002; Dunkov and Georgieva 2006). Microorganisms have evolved two known systems which enable them to acquire iron from host transferrin. One mechanism involves the production of siderophores, which are iron-binding agents with a very high affinity for iron which enable acquisition of iron from host transferrins. The other known mechanism involves non-siderophore proteins which specifically bind to, and remove, iron from host transferrin (Ratledge and Dover 2000). Evidence of the conflict to keep and acquire iron has been detected in salmon transferrins, which have, and most likely are, evolving rapidly and adaptively (Ford, Thornton, and Park 1999). It has been proposed that transferrins in these fish are evolving in this manner because they occupy many different habitats during their lifecycle (Ford 2001), suggesting that the type and number of parasites encountered by an organism can affect the strength of selection on their immune system genes.

Social insect colonies have many parasites and diseases, including many microparasites with extremely high rates of reproduction and short generation times (Schmid-Hempel and Crozier 1999; Boomsma, Schmid-Hempel, and Hughes 2005). There is empirical evidence that shows that parasites have, and do, impose a considerable selective force upon social insects (Shykoff and Schmid-Hempel 1991; Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999). This is expected, as a closed space with a colony of tightly packed, highly interactive and genetically similar individuals is an ideal place for the rapid proliferation of a parasite. The recent discovery of the low number of immunity genes in the honey bee (Consortium 2006) goes somewhat against these expectations. It is likely that the honey bee is exceptional in this regard, thus we await information on

the number of immunity genes in other social insect taxa, and in non-social Hymenoptera.

While costs and benefits incurred due to sociality are likely to vary across social insect taxa, so is the range of parasites to which they are exposed. Variable life history characteristics influence pathogen loads (Boomsma, Schmid-Hempel, and Hughes 2005), providing an opportunity to examine the effect that variable pathogen exposure has on the evolution of immune system genes. Bacteria are ubiquitous in soils, and many types of soil dwelling fungi able to infect a great range of hosts are dispersed widely through passive processes (Brock 1971; Soper, Whistler, and Davies 1976; Knox, Ladiges, and Evans 1994; Lynch et al. 2004; Boomsma, Schmid-Hempel, and Hughes 2005). Accordingly, social insects which nest in subterranean habitats come into contact with a wide range and large amount of parasites (Wcislo 1996; Boomsma, Schmid-Hempel, and Hughes 2005). In contrast, many social insects nest arboreally and so have less contact with the soil and the microbes within it. There is mounting evidence that the nesting habit adopted by a given social insect species influences the range of parasites to which they are exposed. For example, the evolution of three immunity genes, relish and two gram negative binding proteins (GNBPs), was driven by positive selection in an ancestral lineage of subterranean termites (Bulmer and Crozier 2006). Along this branch there was also a shift from a diet of grass to one of decayed wood, another transition likely to have exposed the termites to a novel and elevated suite of fungal and bacterial pathogens. Additionally, a study conducted with termites revealed that a ground nesting termite had a significantly greater parasite load than three arboreal nesting termites (Rosengaus et al. 2003). Ants are the most abundant and ecologically diverse of the social insects

(Wilson 1990), therefore in order to understand how widespread are these phenomena a study using ants as a model is timely.

Polyrhachis ants are taxonomically and ecologically diverse. These ants nest in the soil (subterranean), in preformed cavities in trees (lignicolous), on rock faces (lithocolous), and some construct nests using larval and even spider silk (arboreal weavers) (Kohout 2000; Robson and Kohout 2005). The use of *Polyrhachis* in an analysis of immune gene evolution is enhanced by their lack of metapleural glands, which are an ancient and unique synapomorphy of ants (Hölldobler and Wilson 1990). These glands, which have been secondarily lost in *Polyrhachis* ants, secrete substances with antibiotic properties able to kill a wide range of pathogenic organisms (Beattie et al. 1986; Hölldobler and Engel-Siegel 1994; Poulsen, Hughes, and Boomsma 2006). Without these secretions to help eliminate pathogenic microorganisms, other aspects of their immune system are likely to have experienced stronger selective pressure from pathogens than in the case of many other ants, due to a lack of armoury. For these reasons *Polyrhachis* is a useful model for examining the evolution of immune genes under different selective regimes.

Transferrin is predicted to have experienced relative high rates of positive selection in *Polyrhachis* ants that inhabit subterranean nests as compared to their arboreal and lithocolous sisters. A comparative phylogenetic approach, using methods that compare nonsynonymous substitution rates with synonymous substitution rates at individual codon sites and across lineages, is used to analyse the transferrins of *Polyrhachis* ants that nest in variable localities.

Materials and methods

Ants

Representatives of seven of the twelve recognised subgenera of *Polyrhachis* are used in this study (Table 1). The closely related formicine ant *Camponotus novaehollandiae* was used as the outgroup. Worker ants were collected from nests, snap frozen in liquid Nitrogen, and stored at –80°C until their mRNA was extracted. Information on taxonomy, nest sites, and collections is provided in Table 1.

Table 1. Ant collection informatiom

Species	Subgenus	Nest locality	Site [1]	LatLong	Date	Collector [2]	DET [2]	Collection code [3]
P.sokolova	Chariomyrma	Subterranean	Three Mile Creek, Pallarenda, QLD, Australia	19º 10' " S 146º 50' " E	7.iii.2003	AJS	AJS	ADYS
P.senilis	Chariomyrma	Subterranean	Townsville, QLD, Australia	19º 19' " S 146º 45' " E	15.ix.2002	AJS	AJS	ADYR
P.schoopae	Chariomyrma	Subterranean	Townsville, QLD, Australia	19º 19' " S 146º 60' " E	3.v.2005	AJS	RJK	ADYO
P.pilosa	Cyrtomyrma	Arboreal	Bulimba, Brisbane, QLD, Australia	27º 27' " S 153º 03' " E	20.vii.2003	SKR	RJK	ADZB
P.thusnelda	Hagiomyrma	Lithocolous	Cape Hillsborough NP, QLD, Australia	20º 45' " S 149º 00' " E	17.iv.2003	SKAR, AJS, MJV	SKR	ADYV
P.trapezoidea	Hagiomyrma	Subterranean	Townsville, QLD, Australia	19º 19' " S 146º 45' " E	6.vi.2005	AJS	AJS	ADYQ
P. "hagio 10"	Hagiomyrma	Subterranean	Mt. Hartley, Cooktown, QLD, Australia	15º 45' " S 145º 18' " E	11.ix.2006	AJS, FSC	RJK	ADZE
P.turneri	Hedomyrma	Lithocolous	Townsville, QLD, Australia	19º 40' " S 146º 47' " E	7.iii.2003	AJS, MJV	SKAR	ADYT
P.andromache	Myrma	Arboreal	Iron Range NP, QLD, Australia	12º 43' " S 143º 17' " E	6.x.2000	SKAR	RJK	ADYX
P.foreli	Myrma	Subterranean	Mt. Hartley, Cooktown, QLD, Australia	15º 45' " S 145º 18' " E	11.ix.2006	AJS, FSC	RJK	ADZD
P.loweryi	Myrmhopla	Subterranean	Miles QLD, Australia	26º 36' " S 146º 10' " E	6.iii.2000	RJK	RJK	ADZA
P.mucronata	Myrmhopla	Arboreal	Mt. Hartley, Cooktown, QLD, Australia	15º 48' " S 145º 18' " E	9.xi.2003	AJS, MSB	AJS	ADYN
P.dives	Myrmhopla	Arboreal	Cardwell, QLD, Australia	20º 45' " S 149º 00' " E	1.v.2003	SKAR, AJS, MJV	SKAR	ADYU
P.bellicosa	Polyrhachis	Arboreal	Iron Range NP, QLD, Australia	12º 43' " S 143º 17' " E	4.x.2000	SKAR	RJK	ADYY
P.lamellidens	Polyrhachis	Subterranean	Yakushima Island, Kagoshima, Japan	30º 20' " N 130º 30' " E	23.i.2001	SY	RJK	ADYZ
C.novaehollandiae	Tanaemyrmex	Subterranean	Townsville, QLD, Australia	19º 19' " S 146º 60' " E	7.iii.2003	AJS	AJS	ADYW

1- NP = National Park

2 - Collectors and DET - AJS -Angela J Shuetrim, FSC -Sara Ceccarelli, MJV -Mathew J Vickers, MSB -Mark S Bulmer, RJK -RJ Kohout, SKAR-Simon K Robson, SY-Seiki Yamani

3- Collection codes in the Crozier accession database

Isolation and characterisation of transferrin

mRNA was isolated from five individuals of *Polyrhachis senilis.* Ants were ground in liquid nitrogen and mRNA isolated using a Quick Prep mRNA kit (Amersham Biosiences). Degenerate primers were designed using known transferrin sequences from *Mastotermes darwiniensis* (Thompson, Crozier, and Crozier 2003), *Blaberus discoidalis* (Jamroz et al. 1993), *Bombyx mori* (Yun et al. 1999) *Riptortus clavatus* (Hirai, Watanabe, and Chinzei 2000) *Aedes aegypti* (Harizanova et al. 2005) and *Drosophila melanogaster* (Yoshiga et al. 1999) using the program CODEHOP (Rose et. al, 1998). The forward and reverse degenerate primers CCT GGC TGT GGA CCC TGA RGA YAT GTA and CGT AAC CGG AGT ATT TGT CAG GRT ART YRCA respectively, were used for one-step reverse transcriptase PCR of mRNA (Invitrogen, San Diego, Calf.). RT-PCR conditions were: 55°C for 15mins, 94°C for 2 mins, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min with a final extension time of 72°C for 7 mins. An intense band of the expected size (~470bp's) was gel purified and cloned with the PGEM-T easy vector system (Promega, Annandale, Australia) and sequenced. The cloned product showed significant similarity to other known insect transferrins. From this sequence one specific primer was designed for 3'RACE (Trans For: GAT TCC ACT CAC GAA ACT AACC), and used with used with a poly-T primer (T25VN) for one step reverse transcriptase PCR (Invitrogen, San Diego, Calf.), resulting in a product of approximately 1,300 bps, which was cloned with the P-GEMT easy vector system and sequenced. Two specific primers were designed for 5'RACE, (Tranrev4; TGT TCA ACG ACT GTA TCC AGC TT and Tranrev5: CTG CAA GAT ACA TAT CCT CAG GGT CC). First strand cDNA was synthesised from *Polyrhachis* mRNA using the Clontech, SMART^{TM} PCR

cDNA synthesis kit. The primer Tranrev4 was used in place of the kits CDS primer. cDNA was cleaned with a Qiagen spin column and used for PCR with the primers Tranrev5 and the kits "PCR primer". A band of approximately 500 bps was gel purified, cloned and sequenced.

When the entire transferrin sequence was obtained, specific primers were designed for amplifying transferrin in other species of *Polyrhachis*. RT-PCR conditions varied only in annealing temperatures (Table 2), otherwise PCR conditions were; XX°C for 15mins, 94°C for 2 mins, followed by 35 cycles of 94°C for 15 sec, XX°C for 30 sec and 72°C for 1 min with a final extension time of 72°C for 7 mins. In this manner transferrin was isolated from fifteen species of *Polyrhachis* which vary in nesting habits and one species of *Camponotus* (Table 1). Transferrin mRNA transcripts were aligned and conceptually translated using the programs Sequencher, version 4.2, ClustalX, version 1.81 (Higgins, Thompson, and Gibson 1996)**,** and Se-Al version 2.a11 (Rambaut 2002).

Table 2. Primer sequences and annealing temperatures used for primer pairs PCR

Primer Pairs	Sequence (5'-3')	Annealing Temperature
5T4, T13	TAATCAAGATGCTGCACAGATTCA TGAACATTGTTAATGTCCAGATCCTTG	56°C
5T2, T13	ACCTTCGCTCCGCTTTACTC TGAACATTGTTAATGTCCAGATCCTTG	50°C
INTF2, T4	AGAGGGTTGGCAAGAAGGAAG CCCAATTGTGTTAGTTCTCTGAATAG	50°C
INTF3, T5	GACCGACATCAACAACAATCC CGATTACGTCCACATATTTTGC	50°C
T6, T11	GCTCAACGAAAAGACCTTGG AACGTCTCCCTTGCCATCTT	50°C
T14, T16	TCTCCCATAAATGGACTAGACGG CTAAAATGCCGTGTTTCAACTCTT	50°C
T9, T18	CAAGCGCCATCAATGAAT CTTGTTGAATATTTGCCCAAGTGT	52°C
T9, FTREV	CAAGCGCCATCAATGAAT AGAAGCAATATCACATAATACGACATTC	52°C

Tests for positive selection

Higher rates of evolution can be tested as due to positive selection and not an overall elevation of mutation by determining the relative rate of non-synonymous (dN) to synonymous (dS) changes. In the absence of adaptive evolution the rates of the two types of changes will be the same, whereas an elevated rate of nonsynonymous changes is evidence of adaptive evolution (Kreitman and Akashi 1995). A dN/dS ratio > 1 suggests that many nonsynonymous mutations offer a fitness advantage and are fixed in populations at a rate greater than synonymous changes, and is evidence of positive

selection. A dN/dS ratio = 1 indicates neutral evolution and a dN/dS ratio < 1, purifying selection. A phylogenetic comparative approach was adopted in this study, therefore a phylogeny of the included species was required. An independently derived phylogeny of *Polyrhachis* based on partial gene segments from three mitochondrial (Cox1, Cox2, Cob) and three nuclear genes (Ef-1-alpha, wingless and transferrin) was used as a framework in tests of positive selection in *Polyrhachis* transferrin (Figure 1).

Lineage specific selection

To determine whether different lineage have experienced different selective pressures PAML (Yang 1998) and HypHy (Pond and Frost 2005b) models that allow the dN/dS ratio to vary across lineages were implemented. The PAML free ratio model (Mb), which allows for different ratios across branches was compared to the fixed ratio model (MO), where values are restricted to one value and the HyPhy genetic algorithm (GA) model, which searches for the optimum model of lineage specific evolution by assigning unrestricted classes of dN/dS to lineages. Transferrins of different lineages were considered to be positively selected only if there was agreement between both models. For the nesting locality of each species see Table 1.

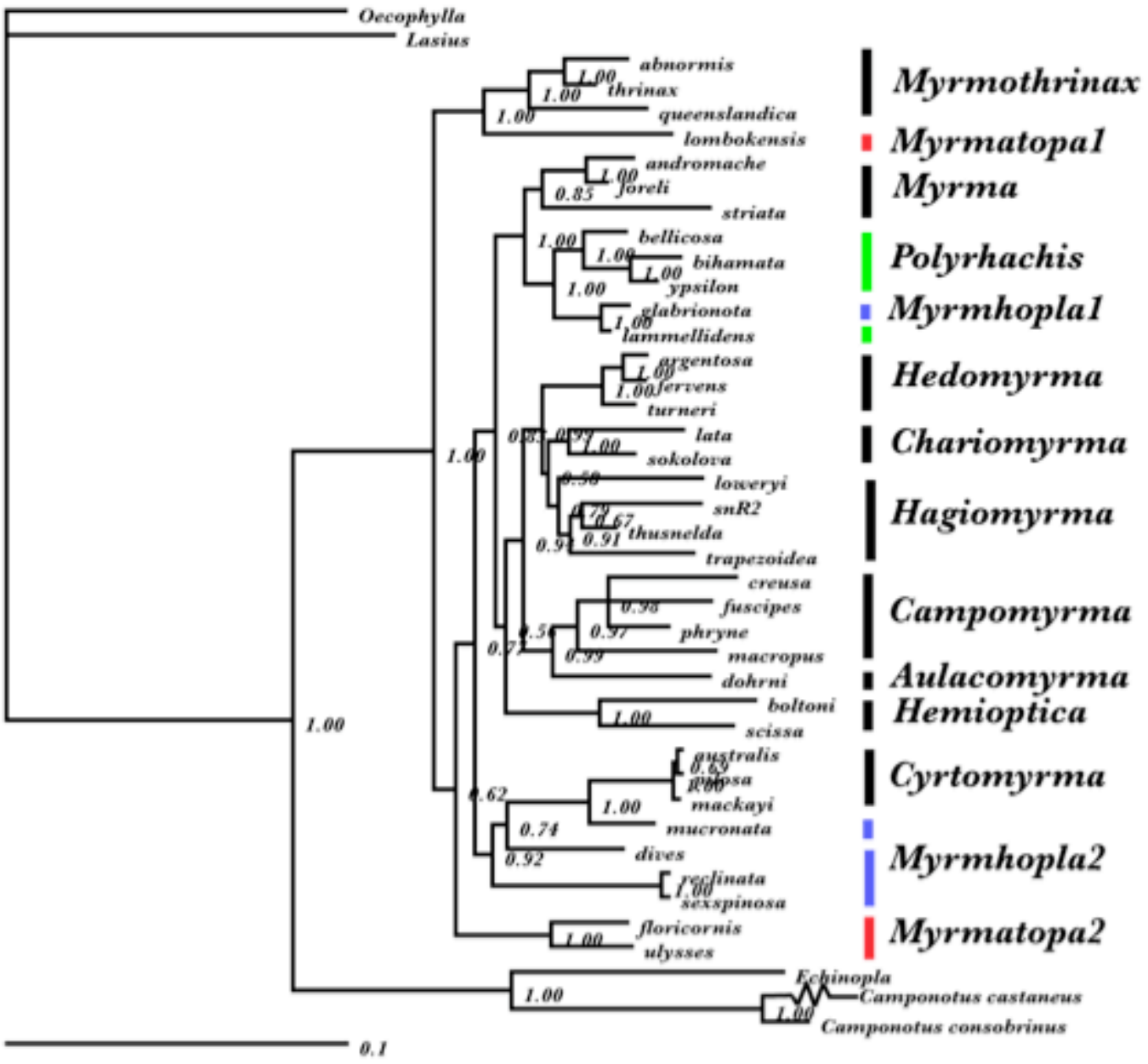

Figure 1. *Polyrhachis* phylogeny. Colored lines indicate apparently polyphyletic subgenera.

Site specific selection

To determine whether individual sites in transferrins have evolved in adaptive manner, different models of selection from the program package PAML were compared, namely M8, which allows for positive selection, and M8A, which allows only for neutral and/or purifying selection (Yang 1997; Yang and Nielsen 2002; Yang, Wong, and Nielsen 2005). The program HyPhy was also used to identify positively selected sites using the random effects (REL) and fixed effects (two rate FEL) models of evolution (Pond and Frost 2005a). An alpha level of 0.1 was used for FEL, 0.05 for REL and a posterior probability cut-off of 0.95 for M8. To reduce the chance of falsely accepting positively selected sites, sites were considered to be so only if there was consensus among all three models.

Results

Lineage specific selection

The PAML free ratio model (Mb), which allows different ratios across lineages is significantly better than the fixed ratio model (MO), where the dN/dS ratio is restricted to one value (Table 3). The free ratio model assigned a dN/dS ratio > 1 to three of the sixteen terminal lineages (Figure 2). Of these three species, two, "*Hagio 10"* and *P.senilis,* are subterranean nesters, and one, *P.andromache*, nests arboreally. The HyPhy genetic algorithm (GA) approach, which searches for the optimum model of lineage specific evolution by assigning unrestricted classes of dN/dS to lineages (Pond and Frost 2005b) assigned a dN/dS value greater than one to the same three lineages (Figure 3).

The models also assign a value greater than one to two internal branches with consensus (Figures 2 and 3).

Table 3. Results of likelihood ratio tests to detect positive selection

Models	2ΔL	χ^2 value	d.f.	P value for best model
M8A vs M8	2(-5068.99, -5048.38)	42	1	P <0.001
MO vs Mb	2(-5134.54, -5102.99)	63	26	P <0.001

Site specific selection

The PAML M8 model, which uses a beta distribution between the dN/dS interval from zero to one and an additional category that allows for positive selection, is significantly better than M8A where the extra category is restricted to one, indicating that transferrin has been subject to positive selection (Table 3). The sites assigned a dN/dS ratio greater than one by the different models are listed in Table 3. M8 identifies 11 sites, the HyPhy REL model 28 sites and the HyPhy FEL model ten sites (Table 4). The models are with consensus on the identity of three sites, 19, 55 and 312, so selection on transferrins appears to have been sufficiently strong to gain statistical support and consensus among the models in these cases.

Table 4. Positively selected sites identified with different models. The sites identified by all models are highlighted.

FEL	REL	PAML M8
	13	
	16	
19	**19**	**19**
	21	21
	22	
	24	
	54	
55	**55**	**55**
	92	92
	196	
	283	
	305	
306	306	
307	307	
312	**312**	**312**
	337	
	402	
	438	
		459
460	460	
	476	
		479
480	480	
		492
	503	
		513
514	514	
	531	
		543
544	544	
	549	
		668
670	670	
	672	

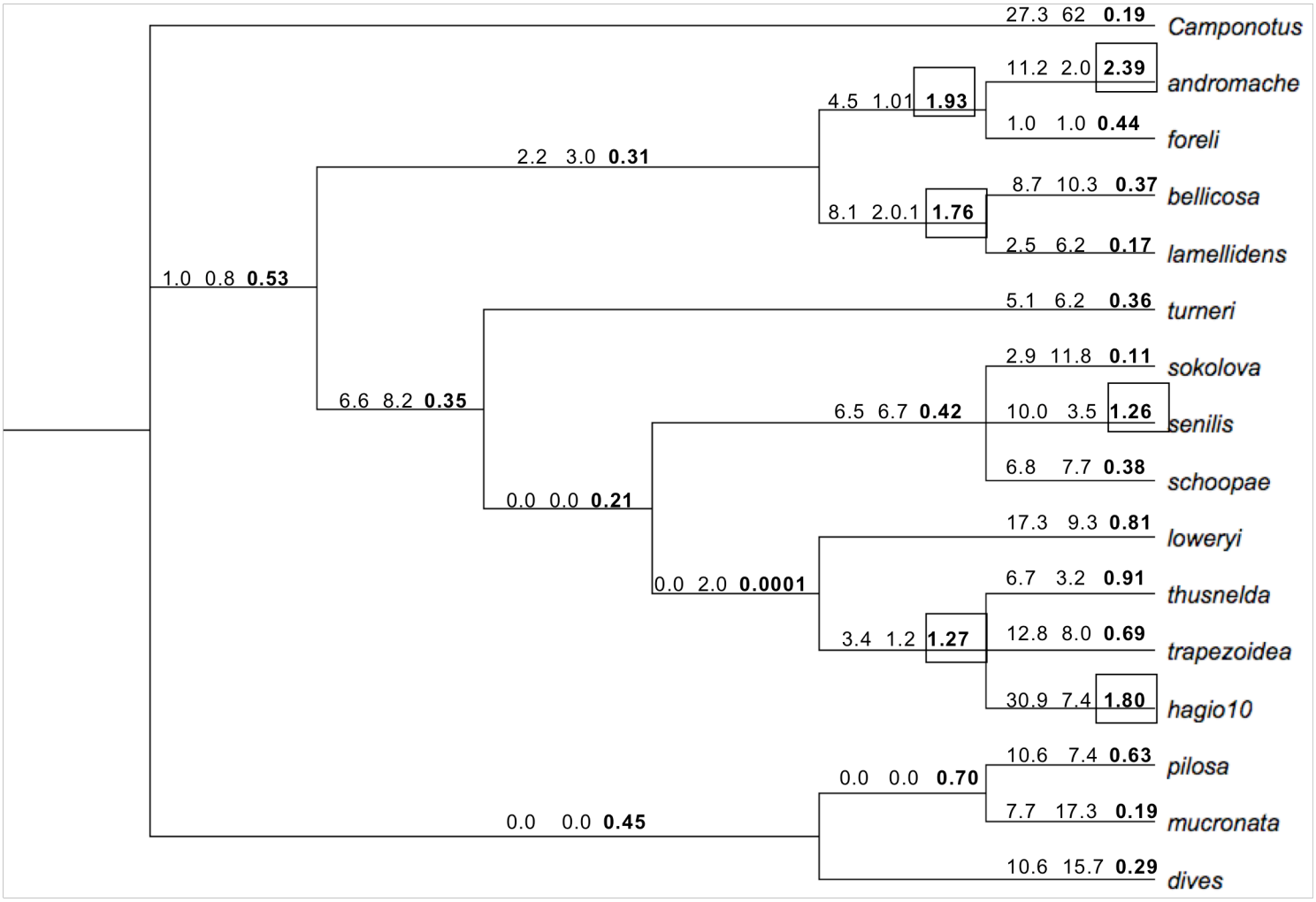

Figure 2. Estimates of nonsynonymous (N) to synonymous (S) substitutions and actual dN/dS ratios from the PAML free ratio model. Estimates of N are shown above branches on the left, estimates of S are the middle values and the dN/dS ratios are displayed in bold on the right. Values indicative of significant positive selection are boxed.

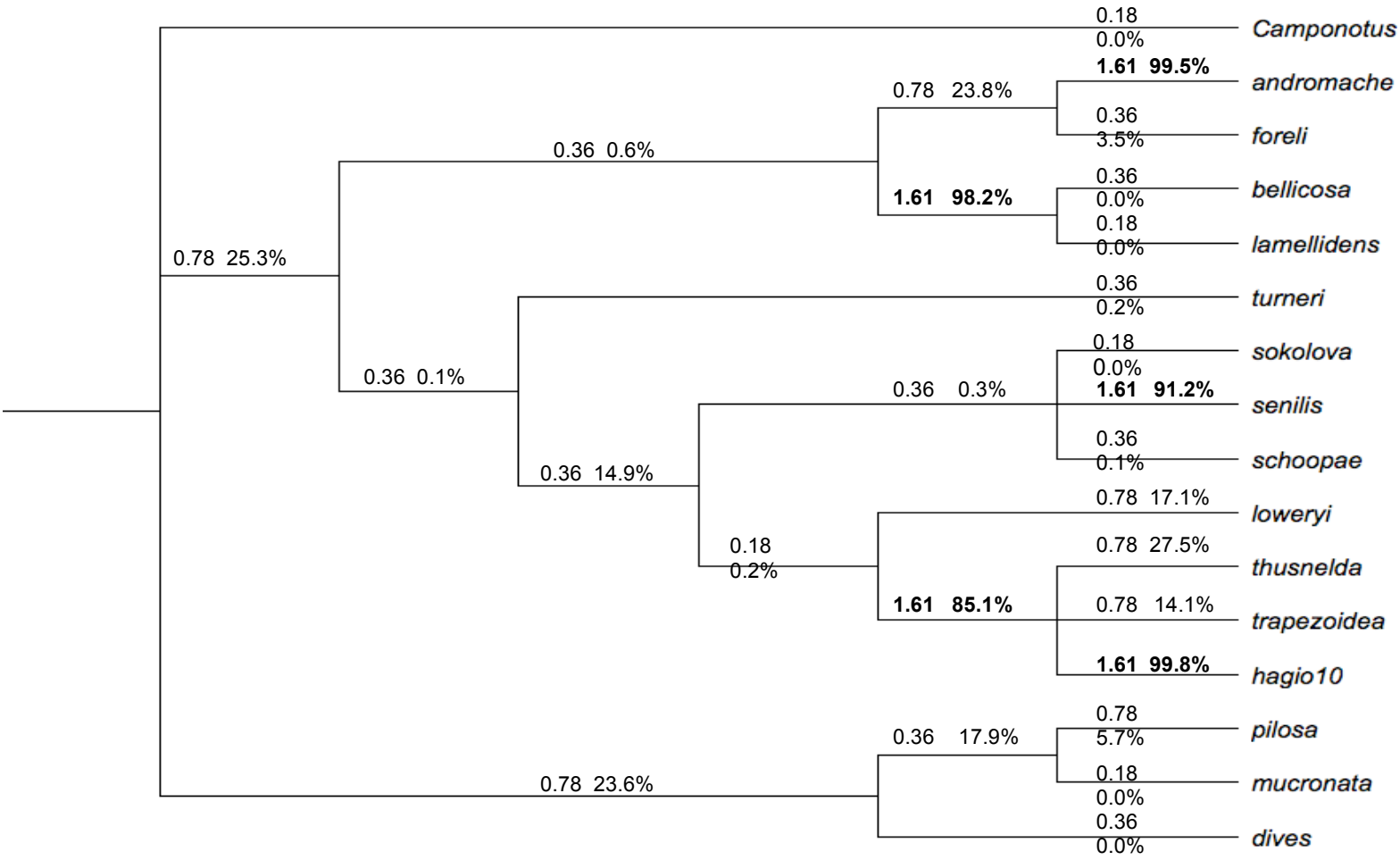

Figure 3. Results from the HyPhy genetic algorithm method of detecting lineage specific variation in selection. In the best model, four classes of dN/dS (0.18, 0.36, 0.78, 1.61) were assigned to branches. The class chosen for each branch by the best model is shown on the left hand side above branches. Percentages shown to the right of the dN/dS values represent model averaged probabilities that dN/dS > 1 along a lineage. Estimates where the dN/dS value is greater than one are highlighted in bold.

Location of positively selected sites

Bacterial iron binding proteins have been shown to attack specific stretches of host transferrin in humans (Retzer, Yu, and Schryvers 1999), and 29 of the 671 sites examined in salmon transferrins were found to be subject to positive selection (Ford 2001). *Polyrhachis* transferrin was aligned with human (NCBI accession NP001054) and salmon (BAA84102) transferrins in order to determine whether positively selected sites in *Polyrhachis* fall within the same regions. Site 19 in *Polyrhachis* transferrin does not fall into any of the regions attacked by bacterial binding proteins in humans, yet is only five positions away from a positively selected site in salmon transferrin (amino acid number 25). Site 55 in *Polyrhachis* transferrin aligns with site 53 in salmon transferrin, which is also positively selected in these fish. Site 312 in the *Polyrhachis* sequence aligns with site 280 in human transferrin and site 270 in salmon transferrin, which both lie in the centre of a region known to be targeted by bacterial transferrin-binding proteins. Transferrins are composed of two lobes known as the N and C lobes and in most insects studied to date the capacity to bind iron in the C lobe appears to have been lost (Lambert et al. 2005). All three positively selected sites in *Polyrhachis* transferrin are located in the N-terminal.

Discussion

Fifteen new transferrins from *Polyrhachis* ants have been characterised. We have not yet found a strong association between nest type and rate of transferrin evolution, however, the type of selection in this protein across lineages ranged from strong purifying selection to positive selection, demonstrating that certain species of *Polyrhachis* have experienced

different selective pressure to change the amino acid composition of their transferrin. The PAML free ratio model and HyPhy genetic algorithm assigned a dN/ds ratio greater than one to transferrin of three species. Two of these species were selected a priori as having a potentially high pathogen load due to their subterranean nesting habit. *P.senilis* is a widespread subterranean species in Queensland and The Northern Territory of Australia and the undescribed "hagio 10" nests in decomposing logs and soil in rainforests. One of the species assigned a dN/dS value greater than one with consensus between both models, *P.andromache*, nests in trees. While this appears to be the primary nesting habit of these ants, there are reports that this species sometimes nests in subterranean localities (SKAR, pers.comm.). Both models assigned a dN/dS value greater than one to two internal branches with consensus. The nesting habit, or any other characteristics, of these ancestral species is unknown, so I am unable to comment as to what specific characteristics may have driven the rapid evolution of transferrins along these branches. I suggest that the increased level of evolution in certain lineages is likely to have been brought about by high parasite loads encountered by *Polyrhachis* ants as they diverged.

Transferrins of five species with subterranean nesting habits have a dN/dS ratio significantly < 1, indicative of purifying selection. It may be that in these species other factors are more important than nesting habit in influencing parasite loads. For example, *P.sokolova* nests in mangrove mud, where nests are frequently submerged by salt water tides (Nielsen 1997). Salt concentrations limit the growth of many microorganisms (Brown 1976), therefore nesting in such a place may substantially decrease the parasite load of this species. Overall, predictions as to the parasite load a given species is likely to

encounter are sound, however, it seems that these predictions need to be inclusive of other factors. Many characteristics are expected to influence parasite loads in social insects, including colony size, colony density, feeding habits, macro-habitat and geographical range. A positive relationship has been found for group size and parasitism in taxa from birds (Brown and Brown 1986) to social spiders (Hieber and Uetz 1990). There are several studies (reviewed by Schmid-Hempel, 1998) that have shown that large social insect colonies are associated with more parasites. For example, large crowded honey bee colonies are especially susceptible to chronic bee paralysis (Bailey and Gibbs 1964). Increased transmission could be due to several factors such as higher thermal stability in large colonies (Seeley and Visscher 1985), providing a stable environment for the growth and development microorganisms and/or more grooming and greater activity levels (Schmid-Hempel 1998). Colony size is highly variable among social insect taxa. For example, colonies of army ants can have several million members, which is extreme when compared to colonies of the primitive group *Myrmecia,* where in some species there are only a few workers. There is also evidence that colony density influences exposure to parasites, as when population viscosity is high, infestation of the parasite *Acarapis woodi* is greater (Otis and Scottdupree 1992). Additionally, species that reproduce by budding or fissioning are generally poor dispersers and are usually characterized by a high population viscosity, which could explain why the bacterium *Wolbachia* is more common in dependently founding ant species (Wenseleers et al. 1998). As mentioned, the diet of social insects is expected to influence parasite loads. Trophallaxis (food exchange among colony members) is extremely common among social insect taxa and while such food exchange is of clear benefit to colony members,

the system is exploited by some parasites. For example, chronic bee paralysis is spread through colonies by trophallaxis, especially so in large crowded colonies (Bailey and Gibbs 1964), and the bacterium causing American foulbrood is passed from adult bee to larvae during food exchange (Bailey and Ball 1991). The feeding habits of social insects vary, ranging from highly specialised specific diets to broad generalist diets (Hölldobler and Wilson 1990). Considering that many parasites are contracted via ingestion, it seems reasonable to suggest that social insects that have a broad diet come into contact with a greater range of parasites as compared to species with specialised diets. Information regarding predictors of parasites loads discussed above is unknown and therefore unable to be incorporated into the current analysis of *Polyrhachis* transferrin.

The range of features that influence the parasite loads in social insects are of course going to be vast and naturally those discussed above are far from exhaustive. Even when sound predictions can be made, such as in this study, assessing the relative importance of different life history traits may be problematic, as one characteristic may negate the effect of another if it is sufficiently strong in the other direction. For example, if a species nests in subterranean habitats, but has small colonies, narrow geographic ranges and narrow diets etc., the net result may be that their parasite loads are not very high, negating the need for rapid evolution of immunity genes. By the same token, if an arboreal nester has large crowded colonies, a wide dietary range, and wide geographical range, for example, they may experience high parasite loads, and subsequently have rapidly evolving immunity genes. The nature of selection acting on the immune system is thus likely to be multifactorial (Dupas, Morand, and Eslin 2004). When predicting which species will

have experienced adaptive evolution in their immune system proteins due to increased pathogen exposure, it may be best to include another approach. By directly assessing the diversity of microbes on the bodies of the insects, in their nests and macro-environment, predictions could include direct evidence. Methods that would enable such a study to be undertaken have been recently developed.

The positively selected sites are located in the N-lobe of *Polyrhachis* transferrin. This is an important point as this is the lobe expected to under selection due to pressure from parasites for iron acquisition because the C-lobe of transferrin of *Polyrhachis* is unlikely to bind iron at all, as is the case in most other insects (Lambert et al. 2005). Importantly, these sites are located in areas that are likely to be attacked by bacterial iron binding proteins. Site 55 is of particular interest, as it aligns with the equivalent site in salmon transferrin, which has also been subject to positive selection. The other positively selected sites in *Polyrhachis* transferrin fall very close to positively selected sites in salmon, and one lies within a region that is known to be attacked by bacterial iron binding proteins in human transferrin. The occurrence of this type of evolution in equivalent sites and regions in such phylogenetically distant organisms suggests that these sites are important targets for microorganisms that seek to acquire iron from host transferrin.

Chapter 3: The evolution and iron binding capacity of transferrins in phylogenetically distant insect taxa

Abstract

Immunity proteins are predicted to be subject to selective pressure from pathogenic microorganisms and often show evidence of adaptive evolution. The acquisition of iron poses a very important problem for pathogens, as host iron is not maintained in solution. In order to limit the amount of iron microbial fauna receive, transferrin is up-regulated following infection in all insects studied to date. Widespread evidence of adaptive evolution has not been found in insect transferrins to date (Thompson, Crozier, and Crozier 2003). However, transferrin sequences from three hymenopteran genera are now available, so reassessment is warranted as social insect colonies face an increased threat from pathogens due to demographic attributes such as high genetic similarities amongst nestmates and high population densities, and are indeed associated with many parasites and diseases.

Using transferrins of distantly related insect taxa I tested for positive selection across lineages and at individual sites. Positive selection was not detected at any site or in any lineage with consensus across models. Rather, the molecule appears to be under strong and significant purifying selection, suggesting that the molecule is under selective pressure not to change its amino acid composition. In this analysis I included the transferrin of a species of *Polyrhachis* (Hymenoptera) that had a dN/dS ratio greater than one in a previous analysis of *Polyrhachis* transferrins. There is a reasonable explanation as to why positive selection was detected in transferrin of this species in one analysis and

not another. Over time synonymous changes accumulate and can mask high rates of nonsynonymous change when it has occurred, as any detrimental nonsynonymous change is expected to be rapidly eliminated from the population. As such, I suggest that where possible it is advisable to search for adaptive evolution in genes within shorter evolutionary time scales, such as in the analysis of *Polyrhachis* transferrins.

The potential iron binding capacity of these tranferrins was also inferred. With the exceptions of a termite and cockroach, insect transferrins studied to date are not generally conserved for binding motifs in their C termini, thus the capacity to bind iron in this region appears to have been lost. Based on alignments of transferrins from distantly related animals (mammals to insects) doubt has been expressed as to as to whether the majority of insect transferrins can bind iron at all (Lambert et al. 2005). My alignment differs to that presented by Lambert et al. (2005), and I conclude that there is no basis for suggesting that insect transferrins are unable to bind iron in the N-terminal.

Introduction

Microorganisms have extremely short generation times and are therefore able to proliferate rapidly inside a host's body. The preservation of host integrity therefore relies on the presence of defences that detect and prevent the growth of these disease causing agents. When a parasite invades a host's body, pattern recognition molecules pass the message to intermediate molecules via signal transduction pathways, resulting in the synthesis of proteins able to directly attack and destroy the invader (Tzou, De Gregorio, and Lemaitre 2002). Genome wide analyses of the immune response in *Drosophila* and *Anopheles* reveals that when these insects are challenged, hundreds of genes are

upregulated (De Gregorio et al. 2001; Dimopoulos et al. 2002). Indeed this response has been shown to occur in immunity genes following infection in a wide range of insect taxa.

The ability of a microbe to grow and develop within a host's body requires the presence of many essential elements. The acquisition of iron poses a very important problem for pathogens, as host iron is not maintained in solution (Ratledge and Dover 2000). Iron is bound by such molecules as transferrins, which are single polypeptide chains that play an important role in iron metabolism and resistance to infection in animals (Yoshiga et al. 1997; Ford 2000). In order to limit the amount of iron microbial fauna receive, transferrin is up-regulated following infection in all insects studied to date (Bartfeld and Law 1990; Jamroz et al. 1993; Yoshiga et al. 1997; Yoshiga et al. 1999; Thompson, Crozier, and Crozier 2003; Valles and Pereira 2005). In vertebrates, the related proteins, lactoferrin and ovotransferrin, increase in plasma and other fluids in response to infection, providing strong protection against microbial proliferation (Lee, Mcknight, and Palmiter 1980; Baker, Rumball, and Anderson 1987; Aguila et al. 2001). Accordingly, conflict has arisen between host and parasite for procuring this essential element.

Immunity proteins are predicted to be subject to selective pressure from pathogenic microorganisms and often show evidence of adaptive evolution (Tanaka and Nei 1989; Riley 1993; Ford, Thornton, and Park 1999; Begun and Whitley 2000; Hughes 2002; Bulmer and Crozier 2004; Bulmer and Crozier 2006). Transferrins of *Polyrhachis* ants have evolved with episodes of positive selection (Chapter 2). Importantly, the positively

selected sites are located in areas likely to be directly targeted by iron binding proteins of pathogenic microorganisms, supporting the hypothesis that the evolution of these proteins is driven by the need for hosts and parasites to keep and acquire iron, respectively.

Most transferrins consist of two homologous lobes (or terminals), the N and C lobes, which are the result of an ancient gene duplication estimated to have occurred between 850 and 670 mya (Lambert, Perri, and Meehan 2005). Iron binding in human transferrin involves six amino acid residues in each lobe, two tyrosines, a histidine, an aspartic acid and two additional ligands, threonine and arginine. With the exceptions of a termite and cockroach (Gasdaska et al. 1996; Thompson, Crozier, and Crozier 2003) insect transferrins studied to date are not generally conserved for binding motifs in their C termini, thus the capacity to bind iron in this region appears to have been lost (Thompson, Crozier, and Crozier 2003; Lambert et al. 2005). The degeneration of this region is thought to be due to antagonistic interactions between host transferrin and iron scavenging proteins of pathogenic bacteria (Martinez, Delgado-Iribarren, and Baquero 1990; Hirst, Hastings, and Ellis 1991). Interestingly, the positively selected sites in transferrin of *Polyrhachis* ants are located in the N -terminal, as expected, if the C-terminal of transferrin in these insects is unable to bind iron.

Widespread evidence of adaptive evolution has not been found in insect transferrins to date (Thompson, Crozier, and Crozier 2003). However, transferrin sequences from three hymenopteran genera are now available, so reassessment is warranted, as social insect colonies face an increased threat from pathogens due to demographic attributes such as

high genetic similarities amongst nestmates and high population densities (Schmid-Hempel and Crozier 1999), and are indeed associated with many parasites and diseases (Hamilton 1964a; Shykoff and Schmid-Hempel 1991; Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999; Hughes, Eilenberg, and Boomsma 2002; Traniello, Rosengaus, and Savoie 2002; Boomsma, Schmid-Hempel, and Hughes 2005). But it should be noted that the honey bee *Apis mellifera* has fewer immunity genes than *Drosophila* and *Anopheles* (Consortium 2006). This at first appears odd considering that social insects face such a great threat from parasites, yet is not yet known if this is a common phenomenon across social insect taxa. If indeed a reduced number of immunity molecules is a widespread occurrence, or characteristic, of social insects, it could be a further reason to expect that immunity molecules in these animals are evolving at a rapid rate; as if they have limited tools with which to fight microorganisms, the tools they do have are likely to be under strong selective pressure to effectively perform their duties. Alternatively, the reduced number of immune system genes in *Apis* compared to *Drosophila* and *Anopheles* may reflect a general phenomenon within the Hymenoptera.

It is predicted that transferrins of many social insect taxa have are evolving in an adaptive manner, as in the case of *Polyrhachis* ants. A comparative phylogenetic approach, using methods that compare nonsynonymous substitution rates with synonymous substitution rates at individual codon sites and across lineages, is used to analyse the transferrins of phylogenetically distant insect taxa, and the potential iron binding capacity of these transferrins is examined.

Materials and Methods

A comparative phylogenetic approach was used, therefore a phylogeny of the insects used in this study (Table 1) was required. An order level phylogeny (Figure 1) of insects used in this study was obtained from two sources (Gullan and Cranston 2005; Consortium 2006). Relationships among species were narrowed down (Figure 2) using published phylogenies which have been amalgamated at the following web site http://tolweb.org/tree/phylogeny.html.

Table 1. Insects used in this study

Subclass	**Order**	**Species**	**Common name**	**NCBI Accession number**
Hemimetabola	Hemiptera	*Riptortus clavatus*	Bean Bug	AAD02419
	Isoptera	*Mastotermes darwiniensis*	Termite	AAN03488
	Orthoptera	*Romalea micoptera*	Grasshopper	AAQ62963
Holometabola	Coleptera	*Apriona germari*	Beetle	AAW70172
	Diptera	*Glossina morsitans*	Fly	AAM46784
	Diptera	*Sarcophaga peregrina*	Flesh Fly	D28940
	Diptera	*Drosophila melanogaster*	Fruit Fly	AAC67389
	Diptera	*Aedes aegypti*	Mosquito	AAL58079
	Diptera	*Anopheles gambiae*	Mosquito	XP310734
	Hymenoptera	*Apis mellifera*	Honeybee	AAO39761
	Hymenoptera	*Solenopsis invicta*	Ant	AAY21643
	Hymenoptera	*Polyrhachis "hagio 10"*	Ant	
	Lepidoptera	*Choristoneura fumiferana*	Butterfly	AAT08022
	Lepidoptera	*Galleria mellonella*	Wax Moth	AY364430
	Lepidoptera	*Manduca sexta*	Moth	P22297

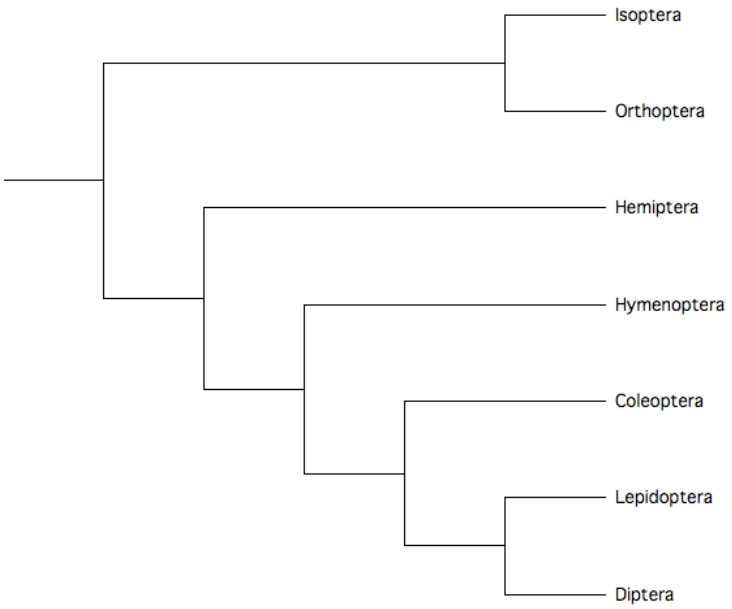

Figure 1. Order level phylogeny of insects included in this study

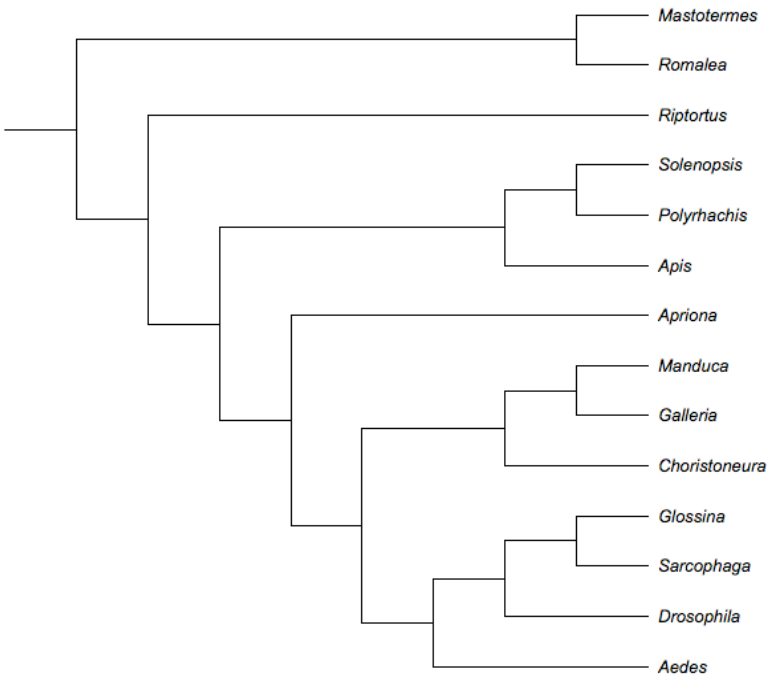

Figure 2. Topology of phylogeny used in analyses in this paper.

Lineage specific selection

The nonsynonymous (dN) to synonymous (dS) substitution ratio (dN/dS ratio) provides a measure by which selection can be detected at the molecular level. PAML and HyPhy models that allow dN/dS to vary across lineages were implemented in order to determine the dN/dS ratio of each lineage. The PAML free ratio model (Mb), which allows different ratios across branches was compared to the fixed ratio model (MO), where the dN/dS ratio is restricted to one value. The HYPHY genetic algorithm approach, searches for the optimum model of lineage specific evolution by assigning unrestricted classes of dN/dS to lineages (Pond and Frost 2005c).

Site specific selection

To obtain dN/dS ratios for individual sites, a model that allows for positive selection (M8) was compared to one that allows only for neutral (dN/dS=1) and/ or purifying selection (dN/dS<1) using the PAML program package (Yang 1997). The program package HyPhy (Pond and Frost 2005c) was also used to detect selection at individual sites using the random effects (REL) and fixed effects (FEL) models of evolution. A nominal alpha level of 0.05 was used for REL, 0.1 for FEL, and a posterior probability cutoff of 0.95 for M8. To reduce the chance of falsely accepting positively selected sites, sites were considered to be under positive selection only if there was consensus between all three models.

Inferred iron binding capacity of insect transferrins

Insect transferrin cDNA sequences were aligned and insepcted using ClustalX, version 1.81 (Higgins, Thompson, and Gibson 1996), and Se-Al, version 2.011 (Rambaut 2002). Specific residues in vertebrate transferrin collectively function to bind iron, and were used as guide to infer the potential iron binding capacity of insects (Bailey et al. 1988; Lambert et al. 2005).

Results

Lineage specific selection

To determine whether selective pressure varies across lineages, models that allow the dN/dS ratio to vary across branches were implemented in PAML and HYPHY. The PAML free ratio model (Mb), which allows different ratios across branches is significantly better than the fixed ratio model (MO), where the dN/dS ratio is restricted to one value (Table 2). Therefore there is variation in the rate of nonsynonymous to synonymous nucleotide substitutions; however, all terminal branches have a ratio less than one, indicating that the molecule is under strong and significant purifying selection (Figure 3). Four internal branches have high estimates of dN/dS under the free ratio model. The HYPHY genetic algorithm searches for the optimum model of lineage specific evolution by assigning unrestricted classes of dN/dS to lineages (Pond and Frost 2005c), and did not detect a dN/dS ratio above 1 for any lineage (Figure 4). In summary,

the PAML free ratio model and the HYPHY genetic algorithm identified no positively selected lineages with consensus.

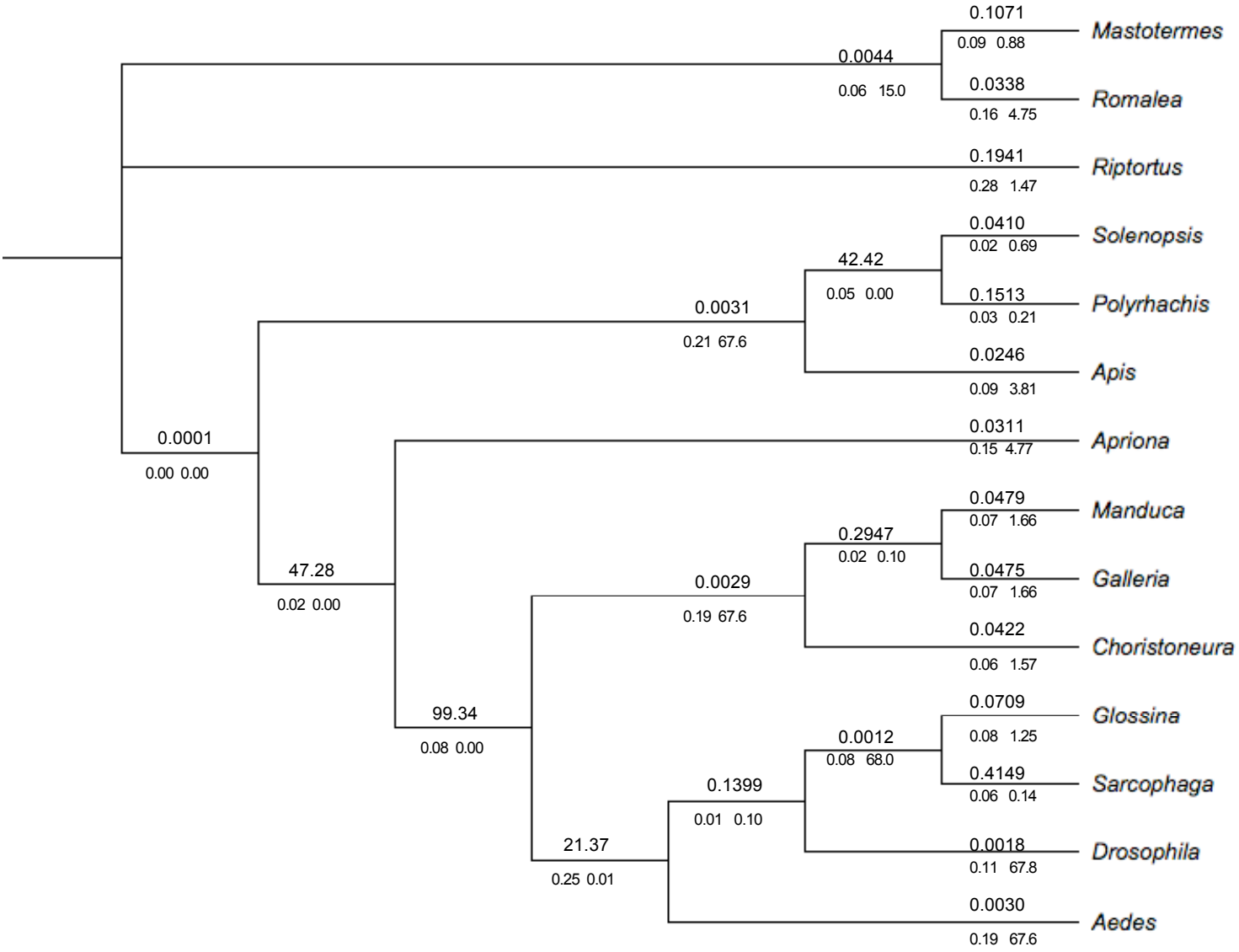

Figure 3. Nonsynonymous (N) and synonymous (S) substitutions and actual dN/dS ratios from the PAML free ratio model. dN/dS ratios are displayed above branches, estimates of N below branches on the left and estimates of S below branches on the right.

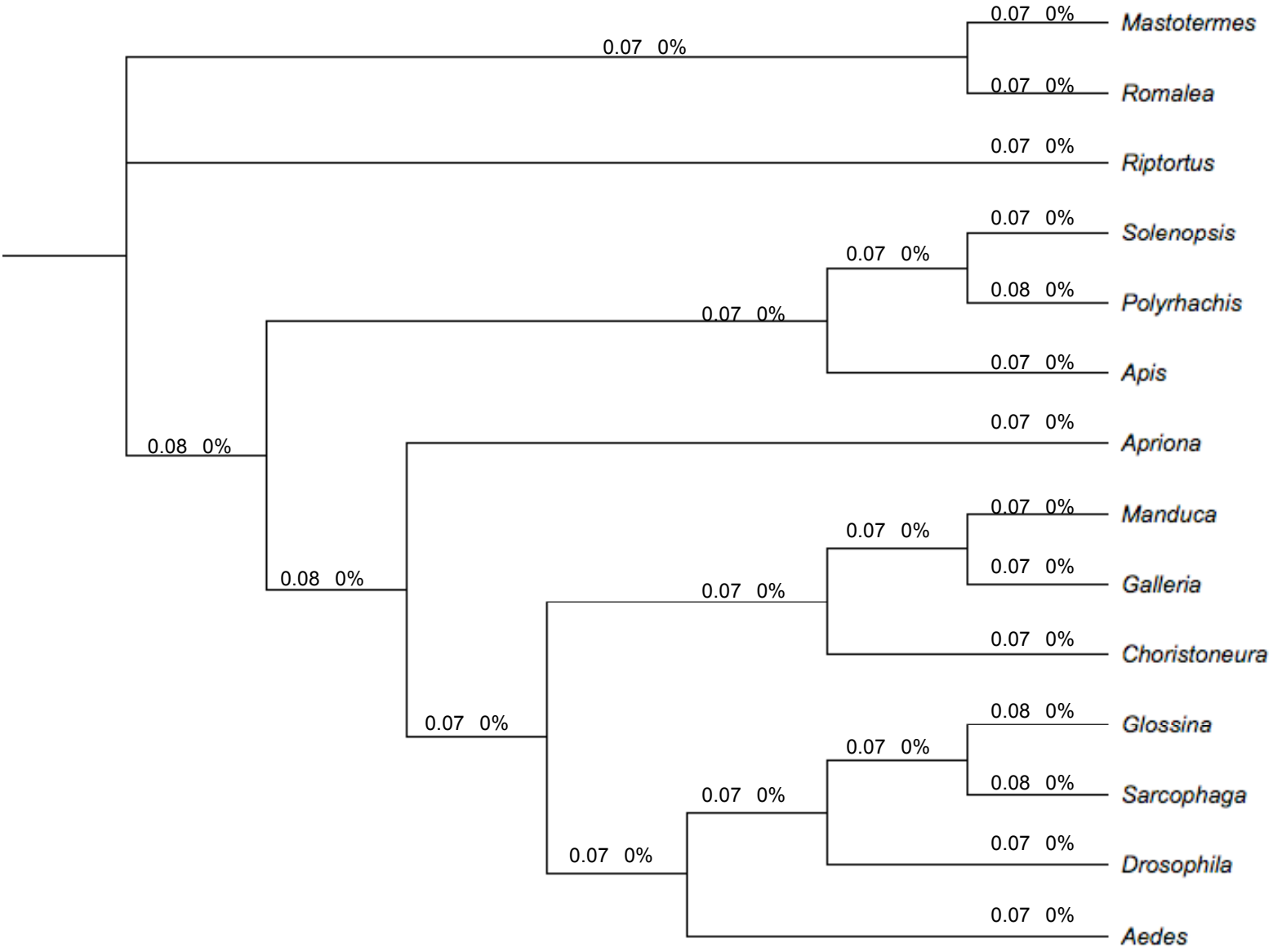

Figure 4. Results from the HyPhy genetic algorithm method of detecting lineage specific variation in selection. In the best model, 2 classes of dN/dS (0.07 and 0.08) were assigned to branches. The class chosen for each branch by the best model is shown on the left hand side above branches. Percentages shown to the right of the dN/dS values represent model averaged probabilities that dN/dS > 1 along a lineage.

Site specific selection

A comparison of the M8 and M8A models in PAML indicates that the molecule is under purifying selection, as M8, which uses a beta distribution between the dN/dS interval from zero to one with an extra category that allows for positive selection is not significantly better than M8A, where the extra category is restricted to one (Table 2). The global dN/dS value from the M8A model is 0.0594, indicative of strong purifying selection. The FEL model in HyPhy identifies two positively selected sites, 532 and 592, with an alpha value of 0.1 (Table 3); however the REL HyPhy model does not identify any sites as positively selected. No positive selection with consensus among the models has been detected, all sites have a dN/dS value equal to, or less than one.

Table 2. Likelihood Ratio Tests (LRT) to detect positive selection for insect analyses

Models	2ΔL	X^2 Value	df	*P*
M8 vs M8A	2(-20175.061, -20175.024)	0.74	1	0.389
M0vsMb	2(-20620.365, -20573.115)	94.5	24	< 0.05

The global dN/dS value was 0.0594

Table 3. Positively selected sites identified with different models for insect analyses

Model	Positively selected sites
CODEML, M8	None
HYPHY, FEL	532, 592
HYPHY, REL	None

Iron binding

The amino acids present in transferrin of each species which correspond to iron binding residues in human transferrin are shown in Table 4. Transferrin of the cockroach *B. discoidalis,* which has been experimentally shown to bind one iron molecule per terminal (Gasdaska et al. 1996), shares all except one of the iron binding residues with mammalian transferrins. At the equivalent of position 249 in mammalian transferrin, cockroach transferrin codes for the amino acid glutamine (Q), whereas mammalian transferrins have a histidine (H). All of the insect transferrins aligned here share the same iron binding residues in the N-terminal as the cockroach *B. discoidalis*, except for *R. clavatus, G. mortisans, S. peregrina, D. melanogaster* and *A. aegypti.* With the exceptions of *M. darwiniensis, B. discoiladlis* and *R. micoptera,* the insects' transferrins here are not generally conserved for iron binding motifs in the C-terminal.

Table 4. Conservation of iron and anion binding residues in insect transferrins. Asterisks represent conserved amino acids. The number of each residue corresponds to human transferrin.

				N-lobe						C-lobe					
				Fe-binding				Anion		Fe-binding				Anion	
Subclass	**Order**	**Species**	**Residue**	**Asp**	**Tyr**	**Tyr**	**His**	**Thr**	**Arg**	**Asp**	**Tyr**	**Tyr**	**His**	**Thr**	**Arg**
			Position	**63**	**95**	**188**	**249**	**120**	**124**	**392**	**426**	**517**	**585**	**452**	**456**
Hemimetabola	Blattodea	*Blaberbus discoidalis*		*	*	*	Q	*	*	*	*	*	*	*	*
	Hemiptera	*Riptortus clavatus*		E	*	*	P	*	*	*	Q	DEL	K	L	D
	Isoptera	*Mastotermes darwiniensis*		*	*	*	Q	*	*	*	*	*	*	*	*
	Orthoptera	*Romalea micoptera*		*	*	*	Q	*	*	*	*	*	*	*	*
Holometabola	Coleptera	*Apriona germari*		*	*	*	Q	*	*	*	*	*	*	P	DEL
	Diptera	*Glossina morsitans*		E	*	*	T	*	*	H	D	DEL	V	D	Q
	Diptera	*Sarcophaga peregrina*		E	*	*	T	*	*	P	N	DEL	V	D	K
	Diptera	*Drosophila melanogaster*		E	*	*	S	*	*	R	D	DEL	R	C	A
	Diptera	*Aedes aegypti*		*	*	*	Q	*	*	K	D	DEL	T	D	S
	Hymenoptera	*Apis mellifera*		*	*	*	Q	*	*	S	R	G	R	S	S
	Hymenoptera	*Solenopsis invicta*		*	*	*	Q	*	*	*	R	G	R	S	D
	Hymenoptera	*Polyrhachis hagio 10*		*	*	*	Q	*	*	N	T	G	K	S	D
	Lepidoptera	*Choristoneura fumiferana*		*	*	*	Q	*	*	*	R	D	R	D	S
	Lepidoptera	*Manduca sexta*		*	*	*	Q	*	*	*	N	D	R	S	T
	Lepidoptera	*Galleria mellonella*		*	*	*	Q	*	*	*	N	DEL	R	D	S

Discussion

Models designed to detect variable selective intensity across lineages did not assign a dN/dS ratio greater than one to any lineage with consensus. All terminal branches had a dN/dS ratio much less than one, indicative of strong purifying selection. Likewise, models that assign a dN/dS ratio to each individual site did not assign a value greater than one to any site with consensus. The global dN/dS value from the M8A model, which the selection model M8 is not significantly better than, is very low (0.0594), indicative of strong purifying selection, suggesting that the molecule is under selective pressure not to change its amino acid composition.

The *Polyrhachis* transferrin used in this analysis was included in a previous analysis of transferrins and was assigned a dN/dS ratio significantly greater than one by the PAML free-ratio model and the HyPhy genetic algorithm (Chapter 2). There is a resonable explanation for why positive selection was detected in transferrin of this species in one study and not another. Nonsynonymous changes result in an actual change in the amino acid composition of a protein, therefore any change of this kind that is detrimental is expected to be rapidly eliminated from a population (Fay, Wyckoff, and Wu 2002). Synonymous changes on the other hand, are much more likely to remain, as no functional change occurs in the molecule, hence such substitutions are referred to as "silent". Therefore, over time synonymous changes accumulate and can mask high rates of nonsynonymous change when it has occurred. The insects used in this analysis are distantly related, therefore even if certain lineages have experienced selective pressure to change (and we know that at least one has), looking for evidence of this at this scale is likely to miss it, unless selection has been very strong and/or constant. As such, where possible it seems important to search for

evidence of positive selection within the shorter evolutionary time scales, as a comparison of closely related species seems more likely to detect adaptive evolution, such as in the analysis of *Polyrhachis* transferrins (Chapter 2). In this regard, Ford's (2001) finding of positive selection only in salmonid transferrins may have arisen because he included closely related salmonid fishes but all the other organisms were relatively distant relatives.

Evidence suggests that the C-terminals of transferrin in the majority of insects, with the exception of a cockroach (Gasdaska et al. 1996) and a termite (Thompson, Crozier, and Crozier 2003), have lost the capacity to bind iron; as the amino acid residues involved in iron binding in this region are not conserved (Table 2). The loss of the ability to bind iron in the C-terminal of transferrin seems at first to be detrimental to a host. However the "iron scavenging" bacteria mentioned above have been shown to act largely in the C-terminal (Martinez, Delgado-Iribarren, and Baquero 1990; Hirst, Hastings, and Ellis 1991). Therefore this loss may be an adaptive trait which increases a hosts ability to limit the growth of pathogenic organisms inside their bodies.

Doubt has been expressed as to as to whether the majority of insect transferrins can bind iron in the N-terminal either, the implication therefore being that transferrins in these insects do not serve a role in iron metabolism (Lambert et al. 2005). In this work, transferrins of distantly related organisms (mammals to insects) were aligned and the conserved residues in each species examined to infer iron binding potential. The argument is as follows. As transferrin of the cockroach *B. discoidalis*, which can bind iron in both lobes (Gasdaska et al. 1996), is the only insect transferrin that has the same iron binding residues as mammalian transferrins in the N-terminal, other

insect transferrins may have lost the ability to bind in this region. However, my alignment differs to that presented by Lambert et al. (2005) The cockroach transferrin actually does differ from mammalian transferrin at position 249. In this position mammalian transferrins code for histidine (H), whereas cockroach transferrin codes for glutamine (Q, Table 4). This difference is noted by the authors of the cockroach transferrin paper (Jamroz et al. 1993), and my alignment agrees with that of Thompson, Crozier and Crozier, (2003). Of the 14 insect tranferrins aligned in this study, ten share iron binding residues identical to cockroach transferrin in the N-terminal, therefore is no basis for suggesting that these insect transferrins are unable to bind iron in the N-terminal.

Chapter 4: General discussion

All animals possess immune systems, which defend them against the large number of pathogenic organisms they come into contact with every day. The molecules involved in these defensive activities are expected to evolve rapidly and adaptively due to selective pressure from pathogenic microorganisms. As social insect colonies face an increased threat from pathogens due to demographic attributes such as high genetic similarities amongst nestmates and high population densities, immunity genes in these animals are expected to be under particularly strong selective pressure. Additionally closely related social insect taxa vary in ways that allow us to examine the impact that different pathogen loads may have on the evolution of immune system genes. In order to increase our understanding of the long term evolutionary response to altered pathogen loads, as measured by the rate of evolution of immune system genes, this research consisted of two main components which are outlined below.

Molecular evolution of the immune relate gene transferrin in *Polyrhachis* ants with variable nesting habits

No strong association was found between nest type and rate of transferrin evolution, however, the type of selection in this protein across lineages ranged from strong purifying selection to positive selection, demonstrating that certain species of *Polyrhachis* have experienced different selective pressure to change the amino acid composition of their transferrin. Three species were assigned a dN/dS ratio significantly greater than one with consensus with different models. Two of these species were selected a priori as having a potentially high pathogen load due to their subterranean nesting habit. *P. senilis* is a widespread subterranean species in

Queensland and The Northern Territory of Australia and the undescribed *P. hagio 10* nests in decomposing logs and soil in rainforests. One of the species with dN/dS value greater than one with consensus between both models, *P. andromache*, nests in trees. While this appears to be the primary nesting habit of these ants, there are reports that this species sometimes nests in subterranean localities (SKAR, pers.comm.) I suggest that the increased rate of evolution in certain lineages was brought about by variable loads and types of pathogens encountered by *Polyrhachis* ants as they diverged.

Transferrins of six species of the eight species examined in this study with subterranean nesting habits have a dN/dS ratio < 1, indicative of purifying selection. It may be that in these species other factors are more important than nesting habit in influencing parasite loads. Overall, predictions as to the parasite load a given species is likely to encounter are sound, however it seems that these predictions need to be inclusive of several factors. Many characteristics are expected to influence parasite loads in social insects, including colony size, colony density, feeding habits, macro-habitat and geographical range. However, assessing the relative importance of different life history traits may be problematic, as one characteristic may negate the effect of another if it is sufficiently strong in the other direction. For example, if a species nests in subterranean habitats, but has small colonies, narrow geographic ranges and narrow diets etc., the net result may be that the parasite loads are not very high, negating the need for rapid evolution of immunity genes. By the same token, if an arboreal nester has large crowded colonies, a wide dietary range, and wide geographical range, for example, they may experience high parasite loads, and subsequently have rapidly evolving immunity genes. When predicting which species

will have experienced adaptive evolution in their immune system proteins due to increased pathogen exposure, it may be best to include another approach (discussed in future directions).

The positively selected sites are located in the N-lobe of *Polyrhachis* transferrin. This is an important point as this is the lobe expected to be under selection due to pressure from parasites for iron acquisition because the C-lobe of transferrin of *Polyrhachis* is unlikely to bind iron at all, as appears to be the case for holometabolous insects in general (Lambert et al. 2005). Importantly, these sites are located in areas that are likely to be attacked by bacterial iron binding proteins. The occurrence of this type of evolution in equivalent sites and regions in such phylogenetically distant organisms suggests that these sites are important targets for microorganisms that seek to acquire iron from host transferrin.

The evolution and iron binding capacity of transferrins in phylogenetically distant insect taxa

There was no agreement across models that there was positive selection at any site or in any lineage. Rather the molecule appears to be under strong and significant purifying selection. I suggest that due to the accumulation of synonymous substitutions over time, which can mask elevated rates of nonsynonymous change, where possible a comparison of closely related species seems more likely to detect adaptive evolution, such as in the analysis of *Polyrhachis* transferrins (Chapter 2).

Doubt has been expressed as to as to whether the majority of insect transferrins can bind iron in either the N-lobe or the C-lobe, the implication therefore being that transferrins in these insects do not serve a role in iron metabolism (Lambert et al. 2005). Of the 14 insect tranferrins aligned in this study, ten have iron binding residues identical to cockroach transferrin in the N-lobe, therefore is no basis for suggesting that these insect transferrins are unable to bind iron in the N-terminal, because cockroach transferrin has been shown to bind iron (Gasdaska et al. 1996).

Future Work

Other immunity genes

Different classes of immunity genes are likely to have responded to selection from pathogens in different ways. Three main pathways operate in the innate immune system, namely the Toll, Imd and JAK/STAT pathways. The Toll signalling pathway mediates defence against gram-positive bacteria and fungi, whereas the Imd pathway acts in response to gram-negative bacteria (Lemaitre et al. 1995; Lemaitre et al. 1996). Dorsal is an important signal transduction protein in the Toll pathway. When host pattern recognition molecules, such as GNBPs and peptidoglycans detect the presence of a microbe, the message is passed to signalling molecules via signal transduction pathways and leads to the translocation of transcription factors Dorsal, Dif (Toll pathway) and Relish (Imd pathway) into the nucleus. When in the nucleus these proteins initiate the synthesis of antimicrobial peptides, which are effector molecules directly involved in eliminating pathogens.

Evidence of adaptive evolution in Relish has been found in *Drosophila simulans*, but not in *D. melanogaster* (Begun and Whitley 2000) and nasute termites (Bulmer and

Crozier 2006). Given that Relish and Dorsal play very similar roles in the different pathways, Dorsal is also an ideal candidate for a study of adaptive evolution.

To date I have sequenced ~ 1000 base pairs of Dorsal from one species of *Polyrhachis* and ~ 300bp's from two other species of *Polyrhachis* and *Mastotermes*. I aligned the 1000bp's of Dorsal of *Polyrhachis* with that of other insect species. This molecule displays variability at the amino acid level across species, where similarity ranges from 71 – 85% (Table 1). When the gene is sequenced it in its entirety in several *Polyrhachis* ants, it will again be possible to perform analyses of positive selection to determine whether selection is variable among species with variable nesting habits. It will be of interest to see if this immunity gene provides us with a different story to transferrin.

Table 1. Percent amino acid identities of Dorsal. Identities shown are among insect Dorsal from GAP-generated pair-wise alignments employing the Blosum 62 amino acid substitution matrix.

	Apis	*Drosophila*	*Aedes*
Polyrhachis	85%	71%	73%
Aedes	75%	75%	
Drosophila	73%		

Direct assessment of microbial diversity

The relationship between parasite pressure and the rate of immunity genes could be better understood by directly measuring the microbial diversity encountered by given taxa and testing this against the rate of evolution of immune system genes. The microbial diversity (a surrogate for pathogen pressure) to which different social insect taxa are exposed, could be estimated by sampling microbes from the insects bodies, their nests and nest surroundings. Using such an approach in combination with predictors of pathogen load, such as nesting habit, is likely to provide great insight into the relationships between parasitism and immunity gene evolution.

Measuring the diversity, structure and composition of microbial communities has long posed a problem, as many traditional methods are well known to have many shortcomings, however, a powerful tool to differentiate between and compare microbial communities from environmental samples has been developed. Terminal restriction fragment length polymorphism (TRFLP) is a tool that can be used to compare the diversity of microbial DNA sequences from environmental samples (Dunbar, Ticknor, and Kuske 2001). Its operation is based on variation in the position of restriction sites in sequences and diversity is estimated by the length of fluorescently labeled TRFs, which are measured by high resolution gel-electrophoresis. This method has been successfully used to differentiate between bacterial communities from many different types of environmental samples, using the 16S rRNA region)see Dunbar, Ticknor and Kuske (2001)).

Primers used in TRFLP should flank conserved regions in the target gene of choice (Clement et al. 1998), so that they successfully amplify the gene from a wide range of microbes, such as in the case of the 16S primers for bacterial samples. Primers

designed to amplify SSU rDNA from 12 microbial groups known to contain parasites and symbionts of insects have recently been developed (Van Borm and Boomsma 2002). These primers could be used in TRFLP analysis enabling a comparison of a wide range microbial types in environmental samples. Naturally time and money will dictate how many microbial types are examined. One set of these primers was designed to amplify fungi within the division Eumycota (true fungi). As a first step approach, I suggest that the 16S primers be used to measure bacterial diversity and the Eumycota primers (Van Borm and Boomsma 2002) used to measure fungal diversity from samples taken from a range of closely related social insects, such as *Polyrhachis*. Knowledge of the diversity of these two microbial types would give a good indication of the microbial diversity encountered by a given species, and provide a sound basis for predictions as to which species are likely candidates for high rates of immune gene evolution.

Duplication

If the amino acid composition, and therefore function, of a host immunity protein changes in response to an evolving parasite, the host may gain the ability to successfully ward off that parasite, but in turn become vulnerable to other parasites which they were previously able to defend themselves against. The presence of multiple copies of immunity proteins would be beneficial under these conditions. As such duplications, along with adaptive evolution, are likely to be a common occurrences in such proteins. Indeed multiple copies of defensive proteins have been found in genome wide and gene specific studies in a variety of insects (De Gregorio et al. 2001; Christophides et al. 2002; Bulmer and Crozier 2004; Bulmer and Crozier 2006; Consortium 2006).

The complete genome of the honey bee *Apis mellifera,* has recently been sequenced (Consortium 2006) and a genome wide analysis of immunity in these bees undertaken (Evans et al. 2006). Far fewer immunity genes were identified in the honey bee genome than in those of *Drosophila* and *Anopheles*. This comparative reduction in immunity gene number poses a potential problem to the hypothesis that social insects face a greater threat from pathogens than their non-social counterparts, as under this hypothesis social insects would be expected to possess more, or at least as many, copies of proteins involved in defence than solitary insects. It is likely that honey bees are exceptional is this regard as they are very well protected against disease due to the evolution of many advanced hygienic behaviours, and/or these bees may be primarily attacked by a restricted set of highly coevolved pathogens, possibly negating the need for a wide range of immunity molecules (Evans et al. 2006). Alternatively, the reduced number of immune system genes in *Apis* compared to *Drosophila* and *Anopheles* may reflect a general phenomenon within the Hymenoptera.

The genome of the solitary jewel wasp, *Nasonia*, is currently being sequenced (http://www.hgsc.bcm.tmc.edu/projects/nasonia/). This will be of importance for determining how wide spread low numbers of immunity genes are in the hymenoptera. However, it will be a many years until the genome of several social insect species is sequenced, and as such a long while until we can compare the number of immunity molecules in a range of social and non social insects at the genome level. I propose that in order to get a decent, albeit preliminary, understanding of how widespread the reduction in number of immunity proteins is we do not have to wait for the genome of many social insect taxa to be sequenced. An alternative approach is outlined below.

Basic approach

The basic premise is to isolate certain immunity genes from a range of insect taxa, including an ant, a termite, a social bee, a social wasp, a solitary bee and a solitary wasp. By cloning products obtained from PCR with degenerate primers and screening several clones of each, the copy number present in each species can be determined and compared across species. A given number of immunity gene families could be randomly chosen in order to determine whether the number of immune genes in new species being examined is closer to that found in *Apis* or in the dipterans.

Candidate species

As we know, social insect taxa vary in ways that are likely to influence the range of pathogens to which they are exposed. It seems desirable to choose social insects that have life history traits predictive of a high pathogen load, because if duplication of immunity genes has occurred at a wide scale, examination of such species is likely to reveal it. For example, ideal candidates would nest in the ground, have a large colonies, broad diets and low genetic variability within colonies.

Behaviours which alter pathogen loads should also be taken into account. This is especially so considering one of the hypotheses to explain the reduction of immunity genes in the honey bee is the presence of advanced hygienic behaviours. The rationale for choosing species on this basis could go both ways. Firstly, it would be of interest to see if species that also have elaborate hygienic behaviours also have low numbers of immune genes. Although the honey bee is exceptional in this regard, a useful comparison is likely to be difficult to draw. However considering this approach is

designed to determine whether some social species have high numbers of immune genes, species that do not exhibit high levels of hygienic behaviour (at least that we know of) may more likely to reveal high immunity gene copy number, and therefore should be chosen on this basis.

Proteins involved in metapleural gland function - discovery of novel ant immunity genes

There are proteins involved in immune defense in ants that are yet to be recognised and characterised. Such genes are those involved in the production and regulation of metapleural gland secretions, which are able to kill a wide range of pathogenic organisms (Beattie et al. 1986; Hölldobler and Engel-Siegel 1984; Poulsen, Hughes, and Boomsma 2006). The products secreted from these glands have been well characterised in certain species however nothing is known of the genes behind their production and regulation.

The production of metapleural gland secretions stops quickly in when the glands are blocked with nail polish, i.e. when the ants are incapable of secreting them (Poulsen, Hughes, and Boomsma 2006). Assuming the same occurs in other ants, and that when the secretions are terminated, the genes involved in their production are turned off, we are able to perform experiments to find some of these genes.

An ideal species for such work is the recently described ant, *Camponotus thadeus* (Shattuck 2005), which lives at high elevation in rainforests of North Queensland, Australia. These ants are one only of two species of *Camponotus* known to possess

metapleural glands, a trait which they are most likely to have evolved secondarily. The benefit of using this species is that two approaches could be combined in order to increase the chance of identifying such genes. The approaches are based on subtractive hybridisation, which is a method used to identify transcripts present in one sample but not in another.

Outline of approach

Subtraction 1

Infected *C.thadeus* ants with metapleural glands blocked *vs* infected *C.thadeus* ants with metapleural glands unblocked

- Experimentally block the metapleural glands of a group of *C.thadeus* ants.
- Infect these ants with a known immune response elictor, along with ants without their glands blocked
- Perform subtractive hybridisation on these samples.
- Determine which genes are expressed in the "unblocked" group that are not expressed in the "blocked group"

Subtraction 2 :

Infected *C.thadeus* ants vs infected ants of the *Camponotus aureopilus* Species Group

C.thadeus is the only member of the *Camponotus aureopilus* species group, which contains eight species, that has metapleural glands (Shattuck 2005). Following

infection, *C.thadeus* is likely to express genes that these other closely related species do not, due to the production and secretion of metapleural gland compounds.

- Experimentally infect *C.thadeus* ants and closely related ants from the *Camponotus aureopilus* Species Group that does not have metapleural glands with a known immune response elictor.
- Perform subtractive hybridisation on these samples.
- Determine which genes are expressed in the *C.thadeus* sample which are not present in other species.

The benefit of using both approaches is that if a gene is upregulated in the infected *C. thadeus* ants without their glands blocked, and in infected *C. thadeus* ants that were infected (compared to species without glands), we can be more confident of its involvement in metapleural gland function. Many of these genes are unlikely to match with published gene sequences; more likely genes will be of unknown function, and further approaches be required to confirm their role in metapleural gland function. In order to determine how important candidate genes (as determined from subtractions) are in metapleural gland function this approach could be extended.

RNA interference (RNAi) is an important tool which can be used to selectively reduce the expression of individual genes. The hypothesis in this case is that if candidate metapleural gland genes are "knocked out", or silenced, metapleural gland secretions will be eliminated, or impaired. Thus the roles of such genes could be confirmed. RNAi is an expensive and time consuming approach, particularly since several genes are likely to be chosen for further analysis. However considering the

importance of understanding how innate immune responses operate, and the effort being expended by many researchers to do so, this is type of research is worthwhile. The novel study outlined above is particularly relevant as it will increase our understanding of an extremely important immune function within the formicidae, enabling further comparisons to be drawn between social and non-social insect immune responses.

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Appendix 1: CLUSTAL W (1.83) *Polyrhachis* transferrin nucleotide sequence alignment

DATA APPENDICES HAVE BEEN REMOVED

Appendix 2: CLUSTAL W (1.83) *Polyrhachis* transferrin protein sequence alignment

DATA APPENDICES HAVE BEEN REMOVED

Appendix 3: CLUSTAL W (1.83) multiple amino acid sequence alignment of transferrin from distantly related insect taxa

DATA APPENDICES HAVE BEEN REMOVED

Appendix 4: CLUSTAL W (1.83) amino acid sequence alignment of Dorsal

DATA APPENDICES HAVE BEEN REMOVED