

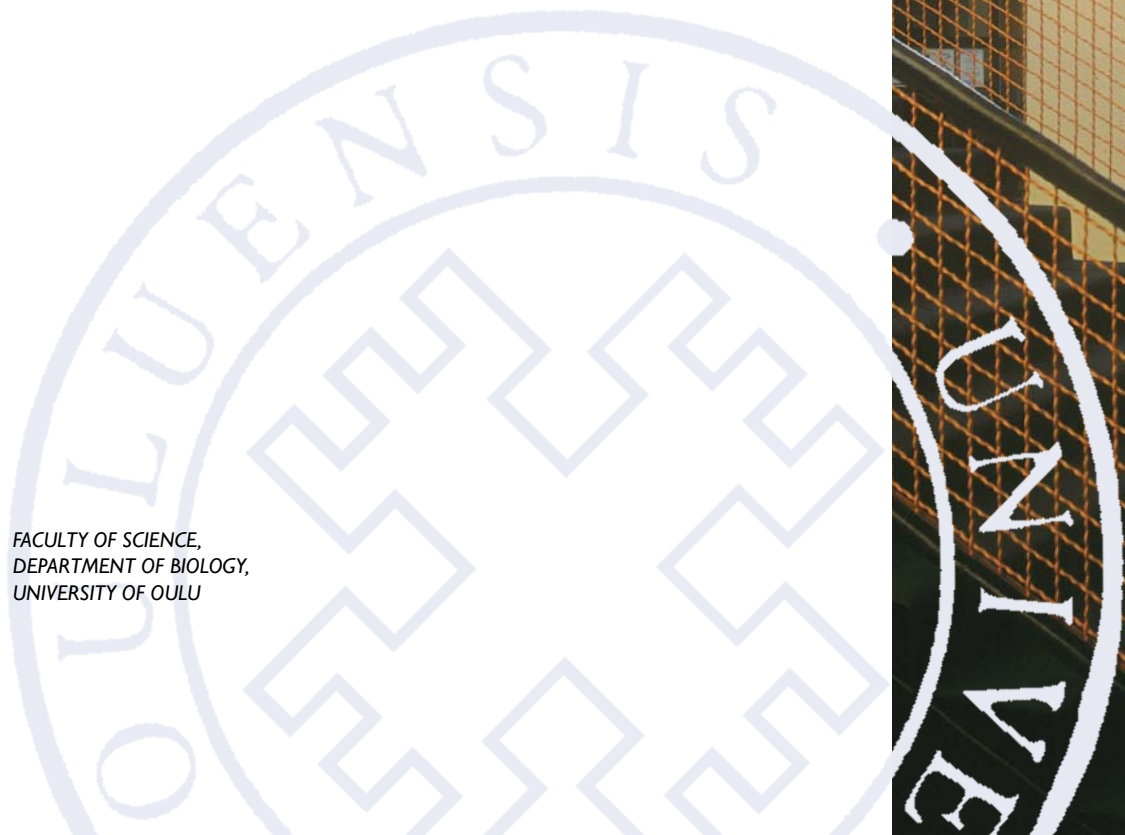
*Anu Sirviö*

THE ROLE OF FACTORS  
PROMOTING GENETIC  
DIVERSITY WITHIN SOCIAL  
INSECT COLONIES

FACULTY OF SCIENCE,  
DEPARTMENT OF BIOLOGY,  
UNIVERSITY OF OULU

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A Scientiae Rerum Naturalium 555

*ANU SIRVIÖ*

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## **Sirviö, Anu, The role of factors promoting genetic diversity within social insect colonies.**

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### ***Abstract***

The evolution of sociality is often associated with close relatedness and genetic similarity of interacting individuals. However, colonies of advanced social insects (e.g. ants, bees and wasps) characterized by large colony size and division of tasks, are also shaped by acquisition of genetic diversity by polyandry, polygyny, recombination and even by hybridization. The balance between forces selecting for high relatedness on one hand and for improved colony performance though increased genetic diversity on the other hand forms an intriguing area of research.

My study has produced the first genetic linkage maps for ants (*Acromyrmex echinator* and *Pogonomyrmex rugosus*) and social wasps (*Vespula vulgaris*). Together with the findings of earlier honeybee research, it is shown that advanced eusocial insects have higher recombination rates than any other insect (or animal) studied so far. The estimates obtained here were 14 cM/Mb for *P. rugosus*, 9.7 cM/Mb for *V. vulgaris* and 6.2 cM/Mb for *A. echinator*.

*Pogonomyrmex* harvester ants have a genetic caste determination system in which workers arise from mating between two hybridizing lineages whereas sexuals are produced by within-lineage mating. I evaluated the origin of the lineages and the caste determination system by using 751 variable nuclear genetic markers. Fertile hybrids would lead to introgression, particularly in genomic regions characterized by a high recombination rate and lack of strongly selected loci. The hybridizing lineages (lineage pairs J1/J2 and H1/H2) showed many fixed differences. Nineteen of them were in the constructed linkage map, scattered in different linkage groups. The results suggest that there has been no recent introgression. As the hybrids are viable (as workers), caste differentiation can be affected by many loci scattered throughout the ant genome or by a small number of very strongly selected loci.

Genetic diversity in colonies of the ant *Formica cinerea* is affected by varying levels of polygyny. I tested the hypotheses that the prevalence of endosymbiotic bacteria can vary in polygynous colonies but be either very low or very high in monogynous colonies. However, I found no association between the level of polygyny and endosymbiont prevalence. In addition to *Wolbachia*, I found two other endosymbiotic bacteria *Cardinium* and *Candidatus Serratia symbiotica* which have not been earlier reported from ants.

Genetic diversity in insect colonies is affected by polyandry and polygyny. My results indicate that high a recombination rate is also an important factor influencing diversity. Genotypically diverse progenies can enhance colony success, e.g. through effects on division of labour or defence against pathogens. Recombination differs from the other factors in its effects on genetic relatedness among colony members.

**Keywords:** AFLP, endosymbionts, recombination rate, sociality



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Oulu, March, 2010

Anu Sirviö



## **Abbreviations**

AFLP	amplified fragment length polymorphism
GCD	genetic caste determination
ECD	environmental caste determination
PCR	polymerase chain reaction
SSCP	single strand conformational polymorphism
NUMT	nuclear mitochondrial DNA



## List of original papers

- I Sirviö A, Gadau J, Rueppell O, Lamatsch D, Boomsma JJ, Pamilo P & Page RE Jr (2006) High recombination frequency creates genotypic diversity in colonies of the leaf-cutting ant *Acromyrmex echinator*. *J Evol Biol* 19: 1475–1485.
- II Sirviö A, Johnston S, Wenseleers T & Pamilo P (2010) A high recombination rate in the common wasp *Vespula vulgaris* adds independent support to the theory that advanced sociality selected for increased recombination rates. Manuscript.
- III Sirviö A, Pamilo P, Johnson RA, Page RE Jr & Gadau J (2010) Origin and evolution of the dependent lineages in the genetic caste determination system of *Pogonomyrmex* ants. Manuscript.
- IV Sirviö A & Pamilo P (2010) Multiple endosymbionts in populations of the ant *Formica cinerea*. Manuscript.



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# 1 Introduction

## 1.1 Evolution of sociality

Life on Earth has undergone several major transitions during its history (Szathmary & Maynard Smith 1995). Many of the stages include co-operation of units, whether they were first replicating molecules that joined together inside the first cells or the first multiple cell organisms that had several different co-operating cell types (e.g. muscle cells, nerve cells, blood cells of various kinds, germ cells) or the societies of individuals that co-operatively perform different tasks (e.g. amoeboid protozoan *Dictyostelium*, naked mole rats, social insect colonies).

Species can have different levels of sociality; usually the more individuals co-operate the more developed sociality is in the sense that the division of labour is more advanced. Group-living is observed in many species of mammals, birds, fish and spiders where communal nesting gives better protection against predators. Otherwise behaviour relating to feeding, breeding and nursing does not include strong cooperation between individuals and is not regarded as social. Contrary, the aggregation of closely related solitary individuals to form large colonies (of up to millions of individuals) where only a few individuals reproduce and other individuals co-operatively perform other tasks is considered eusocial (ants, bees, wasps, termites) and represent one of the latest major transitions in life. The evolutionary success of colony-forming species has been tremendous since it has been estimated that one-third of the biomass of the Amazon rain forests and other habitats consists of ants and termites (Wilson 1990). Sociality is presumed to have evolved for better offspring production and survival but the essential question is whether that benefits both the reproductive and non-reproductive colony members. Prerequisites for the formation of colonies or societies have been high relatedness among conspecifics and interactions that can be either co-operative or competitive (Hamilton 1964).

Advanced social breeding is based on high genetic relatedness among society members that has been crucial in the transition from solitary to social breeding. Social breeding by insects caused difficulties for Darwin, who stated “*with the working ant we have an insect differing greatly from its parents, yet absolutely sterile; so that it could never have transmitted successively acquired modifications of structure or instinct to its progeny*”. Sterile workers behave

altruistically and aid their mother queen to reproduce by caring for siblings and maintaining the colony. Darwin presented that possibly selection acts rather on the family level than on the individual level in social insects to ensure efficient production of offspring. The final solution was presented in the form of kin selection theory and inclusive fitness developed by Hamilton (1963, 1964). He stated that true fitness should be measured rather by the number of copies of a gene found in the progeny population than by measuring the number of offspring produced by an individual. Altruistic genes and behaviour can spread in the population if some individuals can pass more copies of their own genes to the next generation by helping to rear offspring of a close relative than by direct breeding (the inclusive fitness theory). This precludes that the cost (C), measured as the reduction of the number of an altruist's own offspring, is smaller than the benefit (B) to the recipient measured as the number of extra offspring weighted by the relatedness (r) between the altruist and recipient ( $rB > C$ ). The relatedness between individuals has played a fundamental role in the evolution of sociality but it is not considered as a crucial maintaining factor of a society because in social insect colonies individuals can be closely or remotely related. Yet, the organization of the colonies can be the same with efficient division of labour and communication independent of whether the relatedness was high or low. One potential factor maintaining societies can be family-level selection that was originally introduced by Darwin, where in between-group competition larger nests (and polyandry) are favoured which leads to division of labour in order to better organize the activities in large groups (see Wilson 1997, Korb & Heinze 2004). Indeed, the correlation between polyandry, colony size and the level of division of labour has been pointed out by many studies (e.g. Anderson & McShea 2001). Group-living brings advantages over a solitary mode of life and is selected to guarantee better competition against other colonies by enhanced offspring production and adaptation to changing environments. Mechanisms (such as sterility, lack of kin discrimination within a colony) have evolved to prevent or suppress the selfish interests of individuals that arise through variation in relatedness within colonies, thus stabilizing the group living by better tolerance of variation in relatedness (Korb & Heinze 2004).

In addition to the role kinship has had in the evolution of sociality, the genetic composition of animal societies is supposed to affect numerous social traits in societies such as resource allocation, partitioning of production and occurrence of conflict in the nest (Korb & Heinze 2004).



## 1.2 Social insects

High-level sociality (eusociality) is met among social insects that live and behave cooperatively in colonies. In addition to human societies they are the other major group of organisms that show highly structured societies and sociality between individuals. Social insects comprise at least 20 000 species from several orders, and most are found in the order Hymenoptera (ants, bees, wasps) where eusociality has evolved independently multiple times (Wilson 1971). In addition, all known termite species (~2300) in the order Isoptera (or family Termitidae according to Inward *et al.* 2007) are eusocial and their eusociality has evolved only once (Inward *et al.* 2007, Korb 2007). Some other truly social species are known among sphecid wasps, beetles and thrips (e.g. Crozier & Pamilo 1996). The haplodiploid sex determination system of Hymenopteran has been suggested to have influenced multiple evolution of eusociality in the families of the order Hymenopteran (Crozier & Pamilo 1996). Males produced parthenogenetically by a mother queen have a haploid genome whereas their sisters are produced by normal mating and are diploid. Female offspring obtain all genes of their haploid father and half of their mother's genes, thus differences in the ploidy level lead to different relatednesses among offspring. Monogamy produces high genetic relatedness within the progeny and has apparently been essential in the evolution of eusociality in such associations (Boomsma 2009). Worker individuals are female and they are more closely related to each other ( $r = 0.75$ ) than they would be to their own offspring ( $r = 0.5$ ), which can promote altruistic behaviour in the colony (Hamilton 1964, Wilson 1971). In addition to haplodiploidy other typical characters of social insect colonies are caste-based division of labour, overlapping generations, co-operative broodcare and good communication between individuals (behavioural and chemical). In many species sex is determined by a single locus where hemi- or rarely homozygous individuals are males and heterozygotes are females. This is known as the complementary sex determination system (e.g. Hasselmann *et al.* 2008)

Male haploidy produces close and asymmetric genetic relationships among colony members. Furthermore, female individuals can belong either to the queen or worker caste. The caste is a set of individuals that perform separate tasks in the colony. The queen caste monopolizes breeding whereas the worker caste takes care of all the other tasks in the colony according to their possible subcaste, including foraging, nursing the brood and the queen, nest defence, cleaning, ventilation and sustaining the stable temperature in the colony. Overlapping

generations are common as the mother queen outlives the workers, and also among the workers individuals of a previous generation may take care of a new generation.

The nesting sites can be open (army ants, Asian honeybees) or protected. Protected nests can have special constructed envelopes made of leaves, saliva, wood or excavated cavities in soil and trees. Nest structures can vary from multiple-layered underground chambers (Attini ants) to a single plane of nest cells (bumble bees). Typically queens found the nest alone after mating flights, and after nursing the first emerged workers they remain inside the nest to lay eggs whereas workers begin to take care of the functions of the nest by performing the other tasks of the colony. Individuals can discriminate nest members from intruders by sensing the nest and individual specific hydrocarbon profiles (e.g. El-Showk *et al.* 2010). Individual development in the Hymenoptera follows the holometabolous path where several larval instars precede the formation of an adult.

Sociality can be split into primitive and advanced levels of eusociality. Characteristically, primitively eusocial species have small colonies of a few hundred individuals (e.g. wasp *Dolichovespula sylvestris*, bumble bee *Bombus terrestris*) and weak division of labour between nestmates. Highly eusocial species have large colonies that can have up to millions of individuals (e.g. ants *Atta colombica* and *Anomma wilverthi*) and the division of labour is well-developed and multiple subcastes exist among the workers.

### **1.2.1 Genetics of sociality**

Social life has a biological basis and is therefore influenced by genes, even though it is difficult to specify genes directly causing sociality (Wilson 1971). Recent progress in the area of genetics of sociality includes the whole genome sequencing project of the honeybee (*Apis mellifera*) and discoveries of individual genes (e.g. *for*, *Gp-9*) that markedly affect social behaviour. Transcriptome studies have also brought valuable information about the rise of sociality by allowing better conception of how regulation of gene expression interacts with other genetic and environmental factors to promote the evolution of social characters and sociality in different species (e.g. Robinson *et al.* 2008).

The genes so far identified to be involved with social behaviour often have homologs in solitary species. The social dimension can be seen in the genome in the form of regulation of gene expression. Many findings indicate that genetic

regulatory pathways for reproduction in solitary insects have been used in the evolution of social insect societies (e.g. Ben-Shahar *et al.* 2002). Many of the genes known to be related to social traits are regulated by social behaviour itself. For example the foraging gene (*for*) is conserved across social insect species but is expressed differently among foragers and other workers in different species (Ben-Shahar *et al.* 2002, Ingram *et al.* 2005, Tobbach *et al.* 2008). In honey bees a foraging gene is involved in the onset age of foraging and it is based on the needs of the colony. If young bees sense a lack of foragers (transmitted by pheromones), precocious foraging occurs. Changes in gene regulation are seen in the mRNA levels and are associated with behavioural development (Ben-Shahar *et al.* 2002, Robinson & Ben-Shahar 2002). In addition the *for*-gene could be one of the QTLs that affect whether a honeybee collects pollen or nectar, another aspect of foraging influenced by social factors (e.g. Ruppell *et al.* 2004). Interestingly, foragers of the red harvester ant (*Pogonomyrmex barbatus*) have a lower level of the *for* gene expression in the brain than individuals that work inside the nest (Ingram *et al.* 2005). In general, the shift of worker behaviour from a within-hive worker to a pollen-and-nectar-collecting forager is associated with alteration of gene expression in hundreds of genes in the bee brain (see Robinson *et al.* 2008).

Vitellogenin lipoprotein is produced by worker honeybees and is used as a yolk protein in invertebrates and some vertebrates. Expression levels of the *Vg*-gene are highest among nurse honeybees that excrete it in the production of brood food (Amdam *et al.* 2006). Social behaviour in the form of maternal care can induce acetylation and methylation of the promoter region of a glucocorticoid receptor gene that leads to production of more stress tolerant individuals and those with good mothering skills in adulthood.

While the above examples concerned regulation of gene expression, specific genes are known with genotypic effects on sociality. For example sex determination loci (SDL) in the honeybee include *csd* and *fem* loci. The *csd* locus encodes a protein that regulates complementary sex determination so that heterozygous individuals develop into females whereas homozygous and hemizygous individuals become males. The *csd*-based sex determination system regulates sex-specific splicing of the transcript of its progenitor, *fem*-gene, and induces the switch of male and female pathways (Hasselmann *et al.* 2008). Even though the gene does not directly affect sociality, rather the sex, the system has direct consequences affecting social life by influencing the mating patterns which further influence genetic relatedness and genetic diversity within colonies.

Another gene *Gp9* that encodes for odorant-binding protein, is involved in the regulation of queen number by workers in colonies of the fire ant *Solenopsis invicta*. In general, only *B*-allelic variants are found in monogynous colonies whereas polygynous colonies harbour both *b*- and *B*-variants. Workers control the number of egg-laying queens so that the heterozygous workers that carry the *b*-allele favour queens with the same allele (polygynous colonies) whereas homozygous workers *BB* favour alike queens (monogynous colonies) (e.g. Krieger & Ross 2002, 2005, Gotzek & Ross 2007 but see Leal & Ishida 2008). Expression studies have shown that genotypically *BB* workers may adapt an expression profile similar to *Bb* workers when transferred into a nest characterized by multiple queens thus showing the effect of environment or behaviour on the expression level of the gene (Wang *et al.* 2008).

In insect societies the caste, the development of either queen or worker, is often environmentally determined in the form of nutritional or hormonal cues. RNA differential display and cDNA array analysis have revealed extensive differences between developing workers and queens in patterns of gene expression (e.g. Evans & Wheeler 2000). However, recent findings have revealed that caste can also be influenced by genotypic variation in some species of *Pogonomyrmex* and *Solenopsis* ants (see Parker 2004).

In addition to the distinction between the queen and worker castes, the worker caste can be divided into subcastes according to behavioural specialization to perform certain tasks that are often associated with age. Young workers often work inside the nests and older workers perform tasks in the nest entrances or outside the colony. Differential gene expression has been observed between subcastes such as nursers, foragers and soldiers (e.g. Whitfield *et al.* 2003). It has also been shown that worker subcastes associated with morphological differences may have a genotypic component as workers from different patriline were differently represented among morphological subcastes of the leaf-cutting ant *Acromyrmex echinator* (Hughes *et al.* 2003).

To summarize, the studies show that social behaviour and the separation of different castes or subcastes can be influenced both by genotypic differences (Hughes *et al.* 2003, Jones *et al.* 2004) and by social regulation of gene expression (e.g. Robinson *et al.* 2008, Smith *et al.* 2008).

### 1.3 Genetic diversity

The most important factors producing new genetic variation within species are mutation, recombination and hybridization whereas selection, genetic drift and migration are important in determining the destiny of the genetic polymorphisms. In the following recombination, hybridization and mutation are shortly discussed.

#### 1.3.1 Recombination

Recombination is an important factor in evolution because it generates genetic variation. Novel interlocus allelic and genotypic combinations are produced and the proper disjunction of chromosomes is ensured in meiosis. In addition to the individual-level effect on genetic variation, recombination is also important for population-level diversity through background selection or selective sweeps that result from selection within genomic regions that have linkage disequilibrium. Usually, a large physical distance between loci exposes them to a high level of recombination. Recombination can be measured as a recombination fraction which is the ratio of recombinant gametes to total gametes. Crossing over can occur either randomly within chromosomes or it can be genetically (e.g. Otto & Lenormand 2002) or environmentally regulated (Bridges 1915, Korol & Iliadi 1994). Many studies have pointed out the strong genetic control of recombination in plants (Säll 1991), animals (Charlesworth & Charlesworth 1985) and bacteria (Lanzov 1991) which is most probably polygenic in nature. Artificial selection experiments have altered recombination frequency, suggesting that it is amenable to manipulation (e.g. Otto & Lenormand 2002). High recombination frequency also benefits genetic studies by increasing the resolution of physical and linkage mapping. Recombination frequency can vary between species and within species among individuals of the same sex (e.g. Broman *et al.* 1998), between sexes (see Lynn *et al.* 2004) and different sections of the genome (Jensen-Seaman *et al.* 2004). Usually organisms with smaller genomes tend to have higher recombination frequencies. This is caused by a rather constant number of crossovers (one or two) between homologous chromosome pairs in meiosis. Usually organisms with a small genome also have small chromosomes which results in a greater frequency of recombination per nucleotide position (Lynch 2007). The distribution of recombination frequencies has some outliers; species that show exceptionally high recombination rates (Hunt & Page 1995, Solignac *et al.* 2004, Beye *et al.* 2006). *Apis mellifera*, the honeybee, has a 12- and 10-time

higher recombination rate than a human female and *Drosophila*, respectively. This cannot be explained only by genome size differences because *Drosophila* and the honeybee have genomes of almost the same size (180 and 238 Mb). The recombination rate of the honeybee is the highest of all the eukaryotes and has been associated with the characters of social life. On one hand, recombination reduces the variance of the number of alleles that are identical by descent between two offspring, thus making the multilocus (or genomic) kinship coefficient more uniform between the members of the colony. On the other hand, recombination increases the multilocus genotypic diversity underlying polygenic traits, also including traits which are beneficial for a social insect colony (e.g. pathogen resistance, division of labour and tolerance to environmental variation)

### **1.3.2 Hybridization**

Occasionally under some circumstances (e.g. lack of conspecifics, errors in mate recognition) individuals of different species may mate and produce offspring.

Often genetically incompatible hybrid progeny are either less viable or suffer from lowered levels of fertility due to annulment of the most advantageous and fittest allelic combinations of the parental species. However, sometimes hybridization can lead to offspring that are viable and fertile and may even have a fitness advantage over the parental species in certain ecological circumstances (Rieseberg *et al.* 2003). For example, hybrids with phenotypic traits that are intermediate of the parental species can inhabit new ecological niches, or can better tolerate different physical conditions (e.g. drought). Therefore, in some circumstances hybridization can produce new combinations of genetic material in a short period of time, and can be considered as one of the relevant evolutionary forces along with mutation and recombination (e.g. Dowling & Secor 1997, Mallet 2007). The increasing amount of empirical evidence points out that interspecies mating is rather common among plants and certain animal species where 25% and 10 % of the species hybridize (Mallet 2005).

Social insects form a unique system for hybridization because of the haplodiploid sex determination system and the caste-based division of labour. Diploid workers are normally sterile, thus infertile young resulting from hybridization do not necessarily harm the function of a nest. Queens can always produce haploid males parthenogenetically, thus allowing production of sexual non-hybrid offspring even if their diploid offspring might suffer from hybridization effects.

In social insects hybridization has been detected especially among ants and two well-documented cases come from the genera of *Solenopsis* fire ants and *Pogonomyrmex* seed-harvester ants. In these groups, hybridization is associated with the determination of caste so that between-species mating produces workers, whereas queens are generated from within-species mating (e.g. Helms Cahan & Vinson 2003, Helms Cahan & Keller 2003). This provides good possibilities to examine the genetic control of caste differentiation and the genetic differences associated with the formation and maintenance of new species.

### **1.3.3 Mutation and transposable elements**

The ultimate source of genetic variation is mutation that can operate on different levels. Point mutations focus on single nucleotides whereas insertions, deletions and transposons can affect larger sections of the genome. Most genes mutate at a low rate, the rate of nucleotide changes being in many eukaryotes on the order of  $10^{-8}$  per nucleotide per generation. The destiny of a mutation, whether it will spread or disappear, depends not only on the type of change but also on the genomic region (coding or non-coding) where the mutation arises. Selection for beneficial or against harmful mutations is most efficient in large populations. The effects of random genetic drift are prominent in small populations and can be more important than selection in determining the fate of a nearly neutral mutation (slightly deleterious or advantageous). In addition, migrants carry new mutations to adjacent populations where their fate depends on the local selection pressures.

Transposable elements are pieces of DNA sequences that can change their position in the genome. Often they carry a transposase gene that encodes a protein needed in the transposition to different parts of the genome via DNA or RNA intermediates. They can be inserted in any part of the genome whether it was coding or non-coding, and pairs of transposable elements can even carry out recombination and generate new chromosomal rearrangements. Thus transposable elements can affect functional genes, alter gene expression and cause genomic rearrangements in multiple ways and thus produce novel genetic variation. The infected genome can have multiple copies of transposable elements; when the old copy is left behind in the previous location a new copy is created elsewhere in the genome. One of the most widespread types of transposable elements in insects and other invertebrates is mariner (Robertson 1993). Several lines of evidence have been found for horizontal transmission between species and even distantly

related species can share transposable elements with a high sequence similarity percentage.

#### 1.4 Endosymbionts

Endosymbionts are intracellular bacteria that live exclusively within the host tissue and cannot be cultured in vitro. These obligate symbionts can have either beneficial (mutualistic) or harmful (parasitic) effects on their host. Mutualists live in a tight symbiosis-like relationship with their host and often provide supplements (vitamins, amino acids, nitrogen), thus extending the habitat diversity of their host species. In the long run, the host species can become dependent on their dietary endosymbionts and begin to generate special organ-like structures, bacteriocytes, where bacteria have a safe and stable microenvironment inside the gut- and ovary tissues. To assure the inheritance, mutualists are vertically transmitted to the next generation in the maternal line, from mother to offspring as with mitochondria. In fact, according to the endosymbiont theory, mitochondria have evolved from endosymbiotic bacteria belonging to  $\alpha$ -proteobacteria. Cospeciation of the host and bacterial species can be observed as a congruence of their respective phylogenetic trees (e.g. *Buchnera* in aphids, *Wigglesworthia* in tsetse flies, *Blochmannia* in *Camponotus* ants). Typically, beneficial bacteria have adapted to concurrent life with their host by reduction of the genome size (ranging 0.5–2Mb), and many genes relating to repair and metabolism have either degenerated or become integrated as a part of the host genome. Usually the gene content and order are conservative, and no gene uptake has occurred due to lack of horizontal transmission and neither bacteriophages nor transposable elements have been observed (review Bordenstein & Reznikoff 2005). The evolutionary rate is high, which can be explained by bottleneck-effect due to vertical transmission, which in turn leads to an accumulation of slightly deleterious mutations as purifying selection is weak in effectively small populations. The lack of selection can also be seen as a strong AT-nucleotide bias in the genome.

In contrast, harmful parasites can be regarded as selfish elements because they drive only for their own advantage by using hosts for proliferation and dispersal both vertically in the maternal line and horizontally between conspecifics and also between different host species. To ensure effective spreading some obligate parasitic endosymbionts have created mechanisms by which they can manipulate the reproduction of the host species (cytoplasmic



incompatibility, parthenogenesis, male killing, feminization of genetically determined males) by altering the sex bias in favour of the most beneficial sex which is usually female. These are also called reproductive parasites, and those most commonly met in invertebrates are *Wolbachia*, *Cardinium*, *Rickettsia* and *Spiroplasma* that infect multiple species of arthropods and nematodes. The genomes of parasites resemble those of free-living bacteria (e.g. *Escherichia coli*) by having large genomes (2–7Mb) that are under constant modification in the form of high recombination rate and have multiple phages and transposable elements (e.g. Cordaux *et al.* 2008). Therefore no strong coevolution has happened between endosymbiont and host, nor can strict congruence be detected in their phylogenies.

## **1.5 Study of sociality via genetic diversity**

Genetic diversity is essential for the evolution of organisms because it provides raw material on which forces of selection and drift can operate. Diversity can be measured at the level of individuals, colonies or populations depending on many factors like breeding system, effective population size, generation time, mutation, recombination and hybridization. In the following I present examples of how genomic studies have been used to explore the genetics and evolution of traits that are relevant for sociality (determination of caste, division of labour and the defence against pathogens).

### **1.5.1 The primary caste determination; queens and workers**

Often the caste (worker/queen) in social insects is determined environmentally, by quality of nutrition, by hormonal cues and by the intensity of care the larvae receive from nursing workers. The distinction between workers and queens is tremendous because queens can reproduce whereas workers perform other tasks relating to function and development of the colony (foraging, nursing, cleaning, defence), even though in many species the workers are capable of laying haploid eggs that develop into males. As explained in section 1.2.1, alternative expression of a set of specific genes leads to the development of either worker or queen trajectories and the caste differentiation thus depends on gene regulation differences. The presence of an allelic or genotypic factor underlying caste determination, like a selfish gene, has been speculated but empirical evidence has not been found or is weak. One case where the caste is genetically determined is

provided by a stingless *Melipona* bee in which the caste seems to be regulated by two loci (Kerr 1950) that have to be heterozygous for worker development. The first clear evidence for the presence of a genetic factor in caste determination was received recently when two sympatric species (*P. rugosus* and *P. barbatus*) of *Pogonomyrmex* seed harvester ants showed genotype differences between workers and queens at several marker loci (e.g. Helms Cahan & Keller 2003). Later studies revealed that some level of hybridization had happened between the parental species and genetic caste determination was detected only in the hybrid zone where dependent hybrid lines coexist. Mating between individuals belonging to two different lines are needed for the generation of heterozygous workers while within-lineage mating is necessary for the production of homozygous virgin queens. Whether the role of hybridization in the evolution of GCD was causal or just a secondary event that led to the introgression of GCD into new populations and species after the initial evolution of GCD within one population is unsettled (Helms Cahan & Keller 2003, Anderson *et al.* 2006).

Two *Solenopsis* fire ants (*S. invicta* and *S. xyloni*) unveiled a similar kind of hybrid-zone effect where workers were heterozygous and queens homozygous for studied markers (Helms-Cahan & Vinson 2003). Although hybridization can rapidly generate new gene combinations that can be advantageous in certain ecological circumstances, it usually leads to the production of unviable or sterile offspring. The evolution of the genetic caste determination system in these two ant genera has strongly relied on the fact that hybridization can be important in the production of sterile workers that are prerequisite for colony growth and function and can have certain advantages over pure-species workers in certain environmental conditions.

### **1.5.2 The secondary caste determination; worker castes and division of the labour**

The eusocial insect species have highly structured colonies where the organization of colony function is based on division of labour. The primary division of labour is based on the split between reproduction and colony maintenance according to main castes. The queens reproduce and worker individuals maintain the function of the colony by performing various tasks. The secondary division of labour is among workers where subcastes, a set of workers, can be specialized in certain occupations (e.g. nurses, cleaners, soldiers) (see section 1.2.1). To an extent the division of labour has a genetic component; the

function of the society can depend on the genetic diversity among its worker force (e.g. Smith *et al.* 2008).

The social structure of a colony can vary depending on the number of reproductive queens and the number of males the queens have mated with. In monogynous and monandrous colonies workers are highly related ‘super sisters’ and the level of genetic diversity is low. Such high relatedness agrees with the general principle of the kin selection theory, according to which cooperation is expected between related individuals. It is, however, well known that both polyandry and polygyny are common in social insects (e.g. Crozier & Pamilo 1996). They have been considered problematic from the point of view of the kin selection theory, as they reduce the level of relatedness within colonies. Possible benefits have therefore been sought from the increased genetic diversity they generate in the colonies (e.g. Crozier & Page 1985). Such benefits could relate to the division of labour among workers or to defence against parasites and pathogens.

The larger and more complex the colonies are, the more advanced division of labour can be seen, also in the form of physical characters of workers. For example in the colonies of leaf-cutter ant *Acromyrmex echinator* minor, medium- and major-sized workers accomplish tasks according to their size (Hughes *et al.* 2003). Minor workers occupy areas deep inside the nest nursing and feeding larvae and the queen whereas bigger workers occupy outer parts of the colony and outside the colony by foraging and defending the nest. Even though the tasks are also age-related, different patrilineages are differently represented in the task groups. This can reflect genetic differences in the response-threshold in the sense that different genotypes react differently to the external stimuli that influence the switch from one behavioural task to another. The response-threshold model has been used to explain the genotypic differences in honeybee foraging (Page & Mitchell 1991). Genotypic diversity within a honeybee colony has also been associated with a better ability to regulate the nest temperature and buffer it against environmental temperature fluctuations (Jones *et al.* 2004).

Genetic diversity within the colonies has also been associated with protection of the nest against pathogens. In a comparative analysis Schmid-Hempel & Crozier (1999) showed that genetic diversity resulting from polygyny was significantly correlated with the pathogen load and suggested that pathogen pressure has actually selected for polygyny.

In addition to polyandry and polygyny, recombination also affects the genotypic diversity within colonies. Polyandry and polygyny can increase the

number of alleles and thus the number of single-locus genotypes, whereas recombination produces a large number of multilocus genotypes resulting from novel allelic combinations in the maternal gametes. Studies on the honeybee genome have shown an exceptionally high recombination rate (Hunt & Page 1995, Solignac *et al.* 2004, Beye *et al.* 2006). This has been associated with an elevated level of genotypic diversity among workers and possible effects on colony function and resistance against pathogens.

### **1.5.3 Endosymbiotic bacteria and sociality**

Social insects interact with many other species, including insects, plants, fungi and bacteria because the nests are often constructed in the soil, hollows, caves, folded leaves or other sheltered places and they provide a suitable habitat for other species. Micro-organisms are common in these habitats and they can represent a serious threat to the existence of social insect colonies. Sociality is considered to be associated with a high rate of disease transmission (Alexander 1974). Typically the colonies are large (up to millions of individuals) and long-living, individuals are related and in close interaction with each other, and the physiological circumstances (humidity and temperature) are kept constant inside the colony, which forms an ideal platform for the transmission of microbial infections. Once entered into the system, micro-organisms can rapidly infect the whole colony especially if the group consist of closely related individuals. The rate of new infection is proposed to depend on the density of the individuals and parasites (Anderson & May 1981), known also as “mass-action” (McCallum *et al.* 2001). In long-living colonies, e.g. in polygynous ants with a turn-over of queen generations within a nest, infections are easily transmitted to new generations. However, group living can also be beneficial and improve disease resistance, e.g. in the form of antibiotic secretion (Hughes *et al.* 2002; Stow *et al.* 2007) and other forms of social defence (Cremer *et al.* 2007). The trade-off between parasite transmission and the beneficial effects of group living in defence against pathogens has been studied in many contexts (Loehle 1995). For example, in the leaf-cutting ant *Acromyrmex echinator*, group-living brings higher benefits than costs in defence against pathogens even though the number of individuals within its colonies is very high (Hughes *et al.* 2002).

Defence against pathogens has been one of the prerequisites for the evolution of sociality and social insects have evolved both external (antibiotic secretions and grooming) and internal defence mechanisms (immune defence) against

parasites. The shift from solitary to social bees seems to be associated with a major change in the efficiency of antimicrobial secretions, indicating the major role of pathogens in the evolution of sociality (Stow *et al.* 2007). The external or social defence constitutes the elimination of parasites from the surface of individuals by self-grooming or allogrooming by other workers and the secretion of antibiotic compounds from specialised mandibular and metapleural glands (e.g. Hughes *et al.* 2002). Internal defence is produced by the innate immune system where the recognition of a pathogen induces an immune response which eliminates the intruder. “The genetic diversity for disease resistance”- hypothesis predicts that a higher level of genetic diversity in the colony gives better resistance against microbes (Hamilton 1987, Sherman *et al.* 1988, Shykoff & Schmid-Hempel 1991). The genetic diversity in social insects can be measured at the levels of individuals and colonies. Individual-level diversity is determined by the allelic combinations given by parents. Within the colony, sister workers can have different genotypes and different multilocus combinations (depending on both Mendelian segregation and recombination) and these can interact in forming the functionality of the nest. In polygynous colonies the diversity is still larger. Studies conducted on eusocial Hymenoptera have shown that multiple mating by queens (polyandry) and the presence of several queens (polygyny) improve disease resistance (e.g. Hughes & Boomsma 2004, Seeley & Tarpay 2007, Reber *et al.* 2008). Parasites have been speculated to have a role in promoting the evolution of multiple mating by queens (Crozier & Fjerdingstad 2001) and polygyny (Schmid-Hempel & Crozier 1999), both affecting the social form of colonies in a major way.

Many species of ants and some social wasps (Stahlhut *et al.* 2006) carry parasitic *Wolbachia* endosymbionts even though the effect of the parasite remains largely unknown. Other parasitic endosymbionts have not been screened from social insects. Of the mutualistic symbionts, *Blochmannia* is commonly encountered in the species of carpenter ant genus (*Camponotus*) where it most probably provides some essential amino acids to its host, and the endosymbiont is essential for the individual development (Wolschin *et al.* 2004, de Souza *et al.* 2009). In non-endosymbiotic microbes, a tripartite mutualistic relationship is formed between a fungus (basidiomycete, family Lepiotaceae), a cuticle-bacteria (actinomycete, genus *Streptomyces*) and leaf-cutting ants in the tribe Attini. In this relationship the ants cultivate the fungus for food, while the bacteria on the ant surface produce antibiotics that aid in avoiding attacks by a parasitic fungus

(*Escovopsis*) against the beneficial fungus and provide vitamins or amino acids to promote the growth of the fungus garden.

The coevolutionary relationships between the carpenter ants and *Blochmannia* and the Attini ants and fungus Lepiotaceae have lasted for 150 and 50 million years, respectively (see review by Boursaux-Eude & Gross 2000). *Tetraponera* ants also have associated bacteria that provide nitrogen that enable the arboreal-life style of the ants (e.g. Stoll *et al.* 2007). In the case of mutualism and parasitism, bacteria can induce new colony-level phenotypes in the host and help to invade new ecological niches that would otherwise be elusive. In some cases co-adaptation with the endosymbionts has been so strong that the normal individual development has evolved to become bacteria-dependent even though the endosymbiont did not originally bring any beneficial effect to the host (e.g. Bordenstein & Reznikoff 2005).

Sometimes a parasitic endosymbiont can also be regarded as a facultative mutualist to its carrier because the host sex ratio or fitness is manipulated in favour of the transmitting sex. The occurrence of parasitic endosymbionts has been studied in various ant species that represent different social types (monogyny/polygyny). According to one hypothesis, micro-organisms can be more common in monogynous species where the relatedness between nest members is high and the level of genetic diversity is low (Shoemaker *et al.* 2000). The other hypothesis presents that polygynous nests could be more infected because a larger number of breeding individuals can obtain the infection which is then effectively transmitted to the offspring (Wenseleers *et al.* 1998). Both hypotheses rest on the assumption that incompatible mating harms a monogynous colony but not necessarily a polygynous colony because the possible harmful effects are compensated for by other queens. The hypotheses differ regarding the reason for a lack of incompatible matings, that being either a lack (Wenseleers *et al.* 1998) or fixation (Shoemaker *et al.* 2000) of infections within the population. Cytoplasmic incompatibility is the most common effect of *Wolbachia*, leading to reproductive and sexual biases in colonies. Theoretically, a CI strain of *Wolbachia* can prevent gene flow in the host species and cause gradual speciation when individuals are differently infected. *Wolbachia* is also transmitted horizontally which helps its spreading and all the host individuals can be infected (e.g. in several *Formica* ants, Reuter & Keller 2003, Viljakainen *et al.* 2008.).

Several strains of parasitic fungi in leaf-cutting ants can infect the same host and there can be strict competition also between the parasites for survival (Hughes & Boomsma 2004). Recent studies have discovered several new

endosymbiotic bacteria in insects. Their coexistence and possible competition are still largely unstudied.

## 1.6 Marker linkage mapping

A genetic map is an abstract model of the linear arrangement of a group of genes/markers (either coding or non-coding pieces of DNA) that are linked or locate on the same chromosome. The first marker map was constructed for *Drosophila melanogaster* in 1913 by Sturtevant, and 6 linked genes were located on the sex chromosome. Since then improvements have been made in molecular techniques for providing appropriate markers and in statistical methods used in map construction. Recombination or crossing over between homologous chromosomes forms the basis of linkage map construction. The ratio between physical (number of DNA base pairs between the markers or genes) and map distances (multipoint corrected recombination fraction) is called the recombination frequency. For example in the honeybee genome, one centi-Morgan corresponds to ~53kb of DNA, indicating the physical distance between two crossing overs.

Genomic mapping analyses rely on the large number of genetic markers. For detecting and using the markers as reflectors of recombination events they must have alternative alleles, i.e. be variable, in the mapping populations. As indicators of the quality of the constructed map are the map density and the marker coverage that show a positive correlation if markers are randomly distributed in the genome as tags of recombination. The higher the marker density is (or the smaller the physical distance between adjacent markers is) the higher proportion of the genome is covered by markers in the map and the better the map quality is. In addition to the number of markers, also the size of the mapping population, the distribution of crossovers and the criteria of data analysis affect the marker density and the map coverage.

Linkage phase describes the chromatid association of the alleles at the linked loci. Two alleles that locate in the same chromosome are linked in a coupling phase, and alleles located on different chromosomes are linked in a repulsion phase. Depending on the mapping population, whether it is produced by controlled crosses or derived from natural matings, the linkage phase is either known or unknown. In many cases where the sample size is large enough and co-dominant markers are used, the phase can be determined by an algorithm built into linkage analysis computer software packages. Parental information would be

needed for determining the linkage phase, but it is not often available especially when natural populations are used.

Marker linkage mapping has many applications in medical studies, in animal and plant breeding but also in evolutionary studies. Examples include comparative mapping when mapping information can be transferred between organisms (e.g. human disease genes can be found with the aid of the mouse map), localization of genes of interest (e.g. individual disease genes or genes that control for important economic traits in plants or animals) and evolutionary studies of the genome structure. The synteny, the conservative order of the genes, is higher in vertebrates than invertebrates. For example between human and chicken 85 % of the genes show the same order but only 10% between *Drosophila* and *Apis mellifera* (Lynch 2007).

## **1.7 Goals of this work**

My aim has been to study some genetic diversity producing factors that can have an important influence on the evolution of social insects. So far, evolutionary genetic studies on social insects have either used neutral molecular markers for estimating kinship and social structures within colonies (Pamilo *et al.* 1997), or more recently tried to specify genes that might influence social behaviour or some aspects of social life. The aim of my work has been to focus on other genetic factors that are important in shaping genetic variability and that show specific features in social insects. The factors studied include recombination and hybridization that reorganize genetic diversity and create new combinations of alleles and genotypes. In addition to these factors, I have studied the connection between social organisation of colonies and the distribution of endosymbiotic bacteria.

The topics explored in the thesis concern factors that increase the genetic diversity within colonies. Hamilton's (1964) kin selection theory emphasizes the significance of close relatedness which implicitly implies low genetic diversity within a colony. However, it has been repeatedly suggested that social insect colonies can benefit from increased diversity even though such an increase simultaneously lowers the relatedness of nest mates. Therefore, studies of diversity-increasing mechanisms touch on some essential questions of social evolution.

Benefits arising from increased genetic diversity have been particularly associated with the following aspects that are central in social life.



1. Diversity can enable efficient division of labour among the worker subcastes and thus have an impact on the function of the nest.
2. More recently, the genetic variability has been associated not only with the division of labour among workers but with the determination of caste; whether diploid larvae will develop into a sterile worker or a fertile queen.
3. An adequate level of genetic diversity is also required for defence against pathogens which can form a serious threat to the existence of colonies comprising a high density of individuals.

My thesis focuses on diversity-increasing mechanisms and their role in social insects. The work has comparative aspects as the evolution of recombination rates (papers I-III) have been studied in phylogenetic lines in which eusociality has evolved separately, namely ants and wasps, and the results are also compared with a study of the honeybee (Beye *et al.* 2006). The subprojects of the thesis correspond to the points listed above in such a way that papers I and II focus on items 1 and 3, paper III concentrates on item 2 and the main focus of paper IV is on item 3.



## 2 Materials and methods

### 2.1 Ant samples and study areas

#### 2.1.1 *Pogonomyrmex* species complex

Seed-harvesters are the ants that collect and store seeds for later consumption and comprise at least 18 genera (Macmahon *et al.* 2000). They are common in deserts and grasslands in both warm temperate and tropical regions around the world (Johnson 2001). Half of the harvester ant species in the world belong to the genus *Pogonomyrmex* that has 60 species found in North, Central and South America (Macmahon *et al.* 2000). Because the individual density can be high (up to millions of individuals/hectare) they can harvest up to several millions seeds (primarily from annual plants) per hectare per year and thus have a significant impact on energy flow and on abundance of annual plants (Johnson 2001). Harvester ants are highly specialized granivores and occasionally also consume insects and termites. Group-foraging species have larger colonies (at least 5000–10 000 individuals) that can have high longevity (15–30 years) after they have reached the critical age of 2 years. After several years of rapid growth colonies produce several hundreds of alate males and females that participate in mating flights after summer rains. On a local scale mating flights are synchronous for most of the species and additional rains trigger more mating flights as long as alates are present in the nest (Johnson 2001).

*Pogonomyrmex rugosus* and *P. barbatus* (subfamily *Myrmicinae*) are closely related sister species that live in sympatry in hot and arid deserts of south-central and south-western parts of United States and show a partial overlap in their area of distribution. The queens of these species are highly polyandrous and mate with up to 12 males, the effective number of matings in *P. rugosus* being 4.7 (Gadau *et al.* 2003). Despite sympatry the species rarely coexist because they segregate in microhabitats based on differences in soil texture so that *P. rugosus* prefers more dry soils with lower clay content (Johnson 1998). Since the first morphological and molecular studies on sympatric populations of the two species, the signs of hybrid lineages have been detected in the form of exceptional colour morphs and deviations from Hardy-Weinberg equilibrium that were not observed in allopatric populations (Volny & Gordon 2002, Julian *et al.* 2002). Our mapping population male samples, originating from the colony number 3111 (RA Johnson collection),

are led by a single polyandrous pure *P. rugosus* queen from Pinal County, Arizona. Population samples were gathered from 44 colonies (1 worker and 1 virgin gyne/colony) in Arizona and New Mexico and include both J- and H- hybrid lineages

### **2.1.2 *Acromyrmex echinator***

Tribe *Attini* covers leaf-cutter ants of the world. Species of the tribe are farmer ants that cultivate fungi (mainly species from the family Lepiotaceae) for food in special growing chambers inside the underground nest by feeding them with the freshly cut leaves. One of the species *Acromyrmex echinator* (subfamily *Myrmicinae*, genus *Acromyrmex*) is commonly distributed in South America. Leaf-cutter ants have an ecological importance as they remould the soil.

After nuptial flights queens establish the nest by first excavating chambers and establishing a fungus farm with the piece of fungus carried from its natal nest. After the production of the first workers the queen focuses solely on egg-laying whereas workers maintain the colony. Colonies grow large and typically consist of tens of thousands of individuals. The nest can reach the underground size of a few cubic centimetres with multiple tunnels connecting hundreds of different kinds of chambers (e.g. fungus growing chamber, larvae growing chambers, queen chamber). These constructions have been compared to human- built skyscrapers in regard to their complexity, size and structure. The nest is headed by a single long-lived (10–20 years) queen that has mated with multiple males (up to 12) (Sumner *et al.* 2004). Division of labour is well developed and is associated with the physically polymorphic workers that differ in size. Minor workers take care of tasks inside the nest (brood care) whereas medium and major workers are specialized in guarding the nest entrances and foraging. The pathogen load is significant within large colonies.

Our samples came from a laboratory colony that was originally dug up in 1996 in Gamboa, Panama as a young colony with a single queen and a single bell-shaped fungus garden with a radius of ca. 5 cm. The colony was kept in the laboratory where it grew to an appreciable size so that males were produced and could be collected in the summer of 1998 for marker mapping purposes.

### **2.1.3 *Formica cinerea***

*Formica cinerea* (subfamily *Formicidae*, genus *Formica*) known also as the silky or velvet ant (due to its silk-like shining abdomen) has a distribution that ranges from the Pyrenees to Altay mountains and is common in continental Europe and southern Sweden. Instead in northern Europe it occurs as scattered populations in sandy habitat patches, e.g. at beaches where it excavates nest chambers and tunnels underground; the only visible part of the nest can be seen on the ground as ~10 cm in diameter circular sandy patches that have hole in the centre. The nest size can reach thousands of individuals and they can be headed by either a single queen or multiple queens (Goropashnaya *et al.* 2001). The species feed on insects and plants.

Our samples were collected from 10 populations around the Oulu region in Northern Finland in September and October 2001. From each population 3 to 22 colonies were sampled including 5 workers from each colony. The distances between colonies were at least 5 metres within populations and the distance between different populations varied from 2 to 50 km.

## **2.2 Wasp samples and study area**

### **2.2.1 *Vespula vulgaris***

*Vespula vulgaris* (family *Vespidae*), known also as the common wasp, construct elaborate paper-like nests that are made from the wood-saliva paste that individuals excrete. The queen can mate with up to 4 males, and the effective mating number is 1.9 (Foster & Ratnieks 2001). Nests are composed of a series of round combs made of paper-like material that are attached below each other horizontally by pillars and are surrounded by multi-layered envelopes. Large colonies can locate either above or below ground and can contain up to 30 000 combs thus having diameter of half a metre and the nest may consist of 20 000 individuals (Spradbery 1973). Wasps feed on both plant and animal material and they are regarded as pests than can ravage fruit crops and honey from beekeepers colonies.

My male, worker and queen samples were collected from one colony from Sint-Truiden, Belgium, in September 2005.

## **2.3 Molecular methods**

Molecular methods used in this study are presented here only briefly because some of them are rather common in molecular biology and they are described more explicitly in the original papers.

### **2.3.1 AFLP**

Amplified Fragment Length Polymorphism, also called as AFLP is used to visualize hundreds of amplified DNA restriction fragments simultaneously. The AFLP technology combines restriction fragment length polymorphism (RFLP) and adaptor-sequence-based PCR where primer-recognition sequences or adaptors are ligated in the ends of produced restriction fragments and are used as templates for PCR primers (Vos *et al.* 1995). Advantages of AFLP are that only a small amount of DNA is needed, results are reproducible due to use of adaptor sequence-based primers, numerous markers can be produced simultaneously, the resolution is good due to stringent PCR conditions and no prior knowledge of the genomic sequence is required. AFLP can be used, e.g. in producing markers for linkage mapping purposes and in estimating the relatedness between individuals.

### **2.3.2 Cloning and DNA sequencing**

The Topo TA cloning kit by Invitrogen was used in all steps involving cloning. The advantage of the method is that only a small amount of DNA is needed as an insert that is efficiently amplified by bacteria to increase the volume of the desired PCR product. The outline of the method is the direct insertion of Taq-polymerase amplified PCR products into *Echerichi coli* derived plasmid vector pCR 2.1-TOPO that is cultured overnight to enrich the amount of insert along the bacterial DNA. After the extraction of cloned DNA there are massive numbers of original PCR product to be used as a template, for example in sequencing. All the sequencing, either standard or cloned insert-based, was done according to the protocol of the manufacturer of the Big Dye Sequencing Kits by Applied Biosystems.

### **2.3.3 *Microsatellites***

Microsatellites are short tandem repeat sequences in the genome that consist of a varying number of repeats of short sequences (1–6 bp). Microsatellite primers target the flanking regions of the repeat sequence. Markers can be used as neutral markers because often the repeat sequences locate in non-coding parts of the genome and thus also the mutation rate is rather high. Markers are also useful because they amplify repeat sequences in closely related species. Because of the neutral nature of the markers and their universality, they can be used in different kinds of population genetic, forensic and marker map studies and in addition they can be used to estimate relatedness between individuals in populations.

### **2.3.4 *Heminested PCR***

In heminested PCR two sets of primer pairs are used to enrich the volume of the desired region in the amplification and to increase the fidelity of the PCR. The method is especially useful when DNA quantity in the original sample is low and it also enables quick screening of samples for the required DNA-region.

## **2.4 Statistical methods**

### **2.4.1 *Map generation by using MapMaker v. 3.0***

Multiple software programs are available for constructing the linkage map and part of these are commercial. In this work the program MapMaker v. 3.0 (Lander *et al.* 1987, Lincoln *et al.* 1992) was used because it is freely available, it is suitable for many kinds of mapping populations and earlier results from honeybee studies were mostly done using MapMaker. The first steps include the determination of the phase of the markers, determination of the parameters (maximum marker distance in cM and LOD value) used in marker grouping, generation of primary marker groups based on two point linkage, the ordering of markers within a group based on three point linkage, joining of additional unlinked markers and confirmation of the marker order by three point linkage.

#### **2.4.2 Genetic structure of populations; Arlequin, Fstat, Genepop**

Population genetic parameters were estimated by using various methods and programs (papers III and IV, *Pogonomyrmex* and endosymbionts). The microsatellite data were reformatted for different population genetic software packages by the program Convert (Glaubitz 2004). Population differentiation was analyzed using the GENEPOP package version on the web (Raymond & Rousset 1995), and Arlequin version 3.11 (Excoffier *et al.* 2005). Principal co-ordinate analysis was calculated by the FORTRAN computer program (Anderson 2003).

#### **2.4.3 Social structure of populations**

The genetic relatedness and inbreeding coefficients for ant nests and populations were calculated in paper IV by using the algorithms of Queller and Goodnight (1989) and Pamilo (1984) included in the program Genrel.

#### **2.4.4 DNA polymorphism**

Sequences were edited and aligned with Sequencher version 4.1 (Gene Codes Corporation) and BioEdit version 7.0.0. Genetic diversity values were analysed using the programs DnaSP version 4.0 (Rozas *et al.* 2003), Mega version 3.1 (Kumar *et al.* 2004) and the GENEPOP package version on the web (Raymond & Rousset 1995). Estimated values are presented in papers III and IV (*Pogonomyrmex* and endosymbionts).

#### **2.4.5 Phylogenetic trees**

The genetic distances and phylogenetic analyses were conducted using Mega version 3.1 (Kumar *et al.* 2004) and BioEdit version 7.0.0.



### 3 Results and Discussion

It has been often stated that social insect colonies need genetic diversity to cope with varying environmental pressures (e.g. with pathogens) and with the needs arising from genetically influenced division of labour among the colony members. In addition to factors maintaining genetic diversity (heterozygosity) within individual genotypes, the diversity of genotypes within a social insect colony can be affected by several factors. Some aspects of these factors have been studied in this thesis. Polygyny increases the number of maternal lineages, and the hypothesised association between polygyny and the spreading of bacterial infections was studied in the ant *Formica cinerea* in paper IV. Polyandry produces separate patrilineages within the colony and polyandry connected to mating of dependent hybrid lines forms the basis of the caste system in the *Pogonomyrmex* ants studied in paper III. While polygyny and polyandry increase the number of genotypes within a single locus in a colony, recombination can increase the number of multilocus combinations among the offspring. Recombination was the target of my study in papers I, II and to some extent in III.

#### 3.1 Marker maps of social insects

During this thesis work I produced the first genetic maps for ants and social wasps, thus completing the picture of the Hymenopteran social insect maps that were earlier based on information from bees (honeybee and bumble bee). In addition, by using the map size estimates and the physical genome size I compared the recombination rates among the social insects and between different insect groups. This is discussed further in section 3.2 “The level of sociality and recombination rates”.

The maps generated in this work (papers I–III) were based on dominant AFLP-markers. Dominant markers can be used without any problem in ants as haploid males directly represent the maternal gametes and were used in the map construction with MapMaker v. 3.0. Maps were generated with the male haploidy-adjusted phase unknown method (see Gadau *et al.* 2001) because the linkage phase of the grandparents of males was not known. None of the generated maps were saturated and the number of linkage groups exceeded the haploid chromosome numbers. This is rather common a phenomenon, depending on the number of markers used. By increasing the number of markers (density of the map) and the size of the mapping population (enough crossing overs that can be

detected) a saturated map could be achieved. The maps constructed here included 145 markers in *Acromyrmex echinator*, 215 in *Pogonomyrmex rugosus* and 197 in *Vespula vulgaris*. The average density of markers was 14 cM (*A. echinator*), 16.5 cM (*P. rugosus*) and 10.8 cM (*Vespula vulgaris*). The estimates of the recombination frequency were 161 kb/cM (or 6.2 cM/Mb, *A. echinator*), 72 kb/cM (14 cM/Mb, *P. rugosus*) and 103 kb/cM (9.7 cM/Mb *V. vulgaris*). In general, the estimates of the recombination rate were of the same level than observed for the honeybee 52 kb/cM (19 cM/Mb) (Beye *et al.* 2006).

Some segregation distortion markers were detected in the maps of *A. echinator* (17%), in *P. rugosus* (20%) and in *V. vulgaris* (3.6%). Biased segregation can occur because of premeiotic (meiotic drive) or postmeiotic (viability/fertility) selection. Also other reasons, like methodological artefacts in the production of markers, small sample size (Hackett & Broadfoot 2003) and breakdown of long-chain DNA during genome extraction stage can generate segregation distortion among markers. In addition, deleterious alleles (Solignac *et al.* 2004) and strong gender-specific segregation distortion (Pamilo 1993) can be associated with biased marker allele frequencies.

### **3.2 The level of sociality and recombination rates**

My aim was to explore whether the high frequency of genome reorganization is associated with the evolution of advanced sociality. This was done by determining the recombination frequency for highly eusocial species representing different phylogenetic lineages: ants (*Acromyrmex echinator*, *Pogonomyrmex rugosus*) and wasps (*Vespula vulgaris*). Earlier results from solitary wasps (*Bracon hebetor*, *Nasonia vitripennis*), the primitively eusocial bumble bee (*Bombus terrestris*) and highly eusocial honeybee (*A. mellifera*), had pointed out the extremely high recombination rate of honeybees (Hunt & Page 1995, Solignac *et al.* 2004, Beye *et al.* 2006), the highest known for animals. Our results confirmed that the recombination frequency is high in other highly eusocial species, thus emphasizing the possible relevance of genome-reorganizing factors as diversity-producing mechanisms in the evolution of complex insect societies. Frequent recombination can benefit the societies in various ways. In addition to providing genotypic variation for polygenic traits such as division of labour and defence against pathogens, recombination also reduces the variance of multilocus genetic relatedness among nest-mates. The latter aspect underlies the genetic nest-mate recognition system.

In advanced eusocial species recombination frequency varied from 72 kb/cM to 161 kb/cM, indicating that recombination is much more frequent than in other insects (e.g. *Drosophila melanogaster* 643 kb/cM, *Nasonia* 410 kb/cM, *Tribolium castaneum* 350kb/cM) (Adams *et al.* 2000, Gadau *et al.* 1999, Zhong *et al.* 2004). High recombination rate produces genotypic diversity at the colony level. Division of labour, like other traits of sociality, has both environmental (including social interactions) and genotypic components. The phenotype of a trait is encoded with interaction between genes and environment and the phenotype can be altered by differential gene expression caused by the other genes and by the social behaviour itself (e.g. Robinson *et al.* 2008). As mentioned in sections 1.2.1 and 1.5.2, worker subcastes can have differences in the expression levels of many genes, for example in the foraging gene (*for*). Considerable differences in the expression levels may occur depending on the encoding allele, variation in the regulatory regions of the genome and by social behaviour.

In addition to expression differences, there is good empirical support from honeybee, ants and wasps for genotypic differences associated with the division of labour among worker subcastes (O'Donnell 1996, Hughes *et al.* 2003, Jones *et al.* 2004, Robinson *et al.* 2008). The response threshold model provides a theoretical framework for such differences in social behaviour (Page & Mitchell 1991). This has been confirmed by various recent studies where the interaction network of genetic variability, development of brain cells (the organ that is largely responsible for sociality), expression and social behaviour are indicated (e.g. Robinson *et al.* 2008)

Social insects also have extensive pathogen loads that may have led to the evolution of high recombination rate for providing enough genetic diversity in the nest established by single queen to better resist the microbes (Schmid-Hempel 2000). Indeed genetic variation has been associated in disease resistance in the *A. echinator* (Hughes & Boomsma 2004). The precise effect of recombination on genetic diversity at different hierarchical levels depends on the genetic architecture of the trait in question. Schmid-Hempel (2000) made some simple calculations comparing the effects of polyandry and recombination but a proper treatment of the topic requires much further work.

In addition, many advanced social insect colonies have a single queen in the nest that leads to a small effective population size. Because also the generation times are long (ant queens can live up to 20 years or even longer) high rates of recombination may have been selected to compensate for small effective

population size and long generation time to generate enough genetic diversity into the colonies.

The common characters of the species studied in this work are monogyny and a polyandrous mating system that is similar to that in the honeybee, *Apis mellifera*. The effective number of matings has been estimated to be 5.3 in *Acromyrmex* (Sumner *et al.* 2004), 4.7 in *Pogonomyrmex* (Gadau *et al.* 2003) and 1.9 in *Vespula* (Foster & Ratnieks 2001). Polyandry also increases the level of genetic diversity but with different consequences than recombination. Recombination influences polygenic traits and the variance of genetic relatedness whereas polyandry also affects single-gene traits and decreases the overall level of genetic relatedness. Because polyandry results in allelic differences between workers and thus alters the levels of genetic relatedness among offspring, it also influences the genetic conflicts among queens and workers. This is not the case with recombination that tends to reduce the genetic variance among offspring. It can therefore be hypothesized that polyandry and recombination could be under different selection pressures even though high recombination rate and a high level of polyandry characterize all the studied species (*A. mellifera*, *A. echinator*, *P. rugosus* and *V. vulgaris*). One possibility to distinguish between the effects of recombination and polyandry could be to study social insects that have complex societies but are characterized by a monandrous mating system. Other relevant comparisons could include species with less developed social structures (e.g. small ant colonies with poorly developed division of labour) and socially parasitic species that do not have their own worker force and in which the hypotheses based on the diversity of workers do not apply. Whether recombination is essential in producing a genotypically variable worker force for the development of advanced division of labour in large and complex colonies of highly eusocial insects or for better defence against pathogens remains to be studied in further projects.

Together with the earlier studies the present results clearly point out the high rate of genome reorganization in social insects. By having a well-developed division of labour, defence against pathogens and an efficient nest-mate recognition system, the nest can be more competitive against other colonies and species and have better tolerance to environmental variation. Even though the kin selection theory emphasizes the importance of high relatedness and thus low levels of diversity for the origin and maintenance of social insect colonies, recent theoretical work has expanded the view by including multiple levels and especially group-level selection as an important promoter for the evolution of

colonies (review Korb & Heinze 2004) thus emphasizing the need for a high level of genetic diversity within colonies to increase the productivity (e.g. Mattila & Seeley 2007).

### **3.3 Caste determination and hybridization**

Recent studies based on molecular markers have revealed a genetic basis for caste determination in a complex of North American seed harvester ant species of the genus *Pogonomyrmex* (e.g. Helms Cahan & Keller 2003). Genetic caste determination (GCD) in this species group involves genetically differentiated lineages, where mating within lineages normally produces queens and mating between lineages results in workers. Hence the lineages depend on each other to successfully reproduce. The proximate genetic architecture (number and genomic distribution of the involved genes) underlying the caste determination in the *Pogonomyrmex* species complex with hybridizing lines is unknown. The goals of my work were to clarify the genetic architecture and origin of the dependent lineages that inhabit the hybrid zone between the parental species *P. rugosus* and *P. barbatus* and to evaluate if there has been introgression between the lineages. Introgression is expected to be restricted in those areas of the genome that have low recombination rates and that include strongly selected genes. As GCD selects against hybrids, one expects that introgression is restricted in regions linked with caste-determining genes but can occur in other parts of the genome. Three alternative models have been proposed for the genetic basis of the caste determination system in this species group: 1. a single nuclear gene, 2. two interacting nuclear genes or 3. cyto-nuclear incompatibilities.

Since there is very little gene flow between the lineages it is also inherently difficult to distinguish between markers causally linked with GCD and markers that co-segregate with GCD and show fixed differences between the dependent lineages. To overcome the problem we combined population genomics and linkage mapping using nuclear markers and a partial DNA sequence of 600 bp of the mitochondrial gene *coxI*. We genotyped 94 individuals (a virgin queen and a worker from 44 colonies) derived from the previously studied GCD populations that were known to include four hybrid lineages with morphological resemblance of one of the parental species, either *P. barbatus* or *P. rugosus*. Individuals were screened with 17 AFLP primer pairs that resulted in 1147 polymorphic markers. By using principal coordinate analysis for the nuclear markers we could detect the admixture of genomic DNA of the dependent lineages. The individuals clustered

into seven main groups and the first two axes explained 80 % of the variation. Large nuclear distances between the pairs of the dependent lineages clearly point to the lack of regular gene flow. The existence of four main lineages was confirmed and the lineages corresponded well with the branches of the mitochondrial neighbour joining tree constructed on the basis of ~600 bp of *coxI* gene for all the samples. Statistically significant differences between workers and queens in the AFLP genotypes identified 165 candidate genetic caste determination markers (workers band present = heterozygote, queens band absent = recessive homozygote). Of these 19 were in the linkage map constructed for 92 haploid non-GCD *P. rugosus* males that had been produced by a single queen. The 19 markers were distributed throughout the genome, suggesting that either the genome has many caste-determining factors or that there are fixed differences and none (or only very little introgression) between the lines and the number of differences does not reflect the number of caste-determining genes.

We re-estimated the history of the lineages on the basis of the 1147 AFLP markers. This was done with the help of evolutionary simulations because the present-day allele frequencies do not represent those at the time of lineage formation and do not reveal old hybridization events. The results gave support to the conclusion that the dependent lines are some type of hybrids, but they did not give strong (or any) support to the hypothesis that the genomes of dependent lines J1 and J2 would be equal mixtures of the parental genomes. The mitochondrial haplotypes suggest that there has been later introgression between some of the lines. Evidence for that is provided by the sequence similarity of the mitochondrial haplotypes in *P. rugosus* and J1, and between J2 and the H-lineages. Furthermore, the females of both H1 and H2 are morphologically of the *P. rugosus* type and those of J1 and J2 of the *P. barbatus* type, even though such similarities are not evident in the nuclear AFLP distances. It thus seems clear that the dependent lineages themselves have some hybrid origin, and the workers resulting from a mating between two dependent lineages are hybrid offspring of hybrid parents. The AFLP results indicate that the level of introgression in the dependent lines is relatively weak, 85–100% of the nuclear genes coming from one of the parental species. The comparison of our simulation model with the obtained AFLP data supports a model where new lines would have been formed by hybridization of the initial parental species *P. barbatus* and *P. rugosus*. Even though we had a very large nuclear data set covering the ant genome, the precise history of the lineages may be difficult to infer because of repeated, even though rare, hybridization events.

### 3.4 The sociality, genetic diversity and endosymbionts

In social insects, high rate of recombination, polyandry and polygyny sustain the high level of genetic diversity in a colony, putatively helping to create better defence against pathogens (e.g. Hunt & Page 1995, Hughes *et al.* 2003). In this work I studied bacterial endosymbionts in the ant *Formica cinerea* that is known to have both monogynous and polygynous colonies that differ in relatedness values. In the colonies led by single queens, individuals are genetically more similar and the level of genetic diversity is lower than in colonies with multiple queens. This can have relevant consequences on the infection level of colonies by various microbes. Two hypotheses have been presented concerning the association of polygyny and the infection frequency of *Wolbachia* endosymbionts within populations (Wenseleers *et al.* 1998, Shoemaker *et al.* 2000; see section 1.5.3). Both hypotheses rely on the prediction that polygynous colonies can tolerate varying infection rates when the endosymbionts cause cytoplasmic incompatibility. Even though some queens would suffer from an incompatible mating type, they may not harm the total success of a polygynous colony. For a species with monogynous colonies, an incompatible mating is fatal. For this reason, Wenseleers *et al.* (1998) first proposed that *Wolbachia* infections can spread more easily in species with polygynous colonies. Later, Shoemaker *et al.* (2000) predicted that it would be advantageous for a monogynous species to avoid incompatibilities by always being infected. The hypotheses thus make opposing predictions concerning the association between the infection frequency and polygyny.

The present work (paper IV) found that the ant populations had a lot of variation in the levels of polygyny; the populations showed clear genetic differences and they were also infected by multiple endosymbiotic bacteria that can interfere with host reproduction. In total 19%, of the nests were infected by *Wolbachia*, 3.8% *Cardinium* and 33% with *Serratia symbiotica*. The first two are known to be reproductive manipulators of the host species and the third is commonly encountered in aphids. *Wolbachia* infection percentages (on average 5.6% infected individuals) was lower than is reported in the other *Formica* species (e.g. Viljakainen *et al.* 2008) that may suggest either a new *Wolbachia* strain in ants or a clearance of infection either by workers or by a demographic effect (bottle-neck effect). *Wolbachia* infections were associated with the degree of genetic differentiation of the host populations but the direction of effects remains speculative; whether *Wolbachia* infections lead to genetic separation of

populations (that could refer to a CI effect) or whether separation of the ant populations allow different dynamics of the *Wolbachia* infections within the populations.

*Cardinium* was found at a low frequency (1.1 % infected individuals) in *F. cinerea*, this is of the same level reported in some other studies (Zchori-Fein & Perlman 2004, but see Matalon *et al.* 2007). *Cardinium* is found in the insect orders Hymenoptera, Hemiptera and in some ticks and spiders as a manipulator of host reproduction in the form of cytoplasmic incompatibility, parthenogenesis and feminization (Duron *et al.* 2008) and it can thus influence the host sex ratio. It can also enhance the fecundity of some species (Weeks *et al.* 2003). *Cardinium* found in *F. cinerea* had high levels of nucleotide diversity which suggests either a long-term infection or frequent horizontal transmission from diverse sources. *Cardinium* has not been earlier reported from ants, and my work is the first to confirm *Cardinium* infection in social insects. Its effects on hosts remain to be clarified in further studies.

Our study showed a second new bacteria, *Serratia symbiotica*, in ants. The role it has on host biology is not clear, although in one *F. cinerea* population nest relatedness was positively correlated with the *Serratia* infection. *Serratia symbiotica* occurred in nine out of ten populations (13% of individuals were infected) and was the most common of the bacteria found in *F. cinerea*. *Serratia* is commonly found among aphids as a secondary symbiont (*Buchnera* is the primary symbiont) and it can bring beneficial effects to the host in the form of heat resistance and defence against parasitoids. Many of the ant species and aphids have a mutualistic relationship which can offer a potential transmission route for *Serratia* to *F. cinerea* similarly to that reported for many gut symbionts in ant species (Russell *et al.* 2010).

Overall, the infection frequencies for the separate bacteria species and the pooled set of endosymbionts varied significantly among the populations but we did not find clear correlations between the level of relatedness and infection frequency. Polygynous colonies were not more, or less, infected than monogynous colonies, and the results lend no support to either of the hypotheses connecting polygyny and the level of endosymbiont infections.

Resistance against pathogens creates selection pressure on a social insect colony to have enough genetic diversity, in addition microbes can directly interfere with the evolution of the host genome. Endosymbionts, whether mutualistic or facultative, may transfer genes to the host genome and can cause reorganization of the genome. Although the transfer of bacterial genes to the host



nucleus has not been ruled out in the lack of experimental evidence among obligate endosymbionts (Dale & Moran 2006) it is known that facultative endosymbionts equipped with multiple transposons and bacteriophages have a potential to also affect the organization of the host genome. Recent genome sequencing projects have shown that *Wolbachia*-derived lateral gene transfer has occurred in the genomes of some nematodes and insects (Dunning-Hotopp *et al.* 2007, Klasson *et al.* 2009). The honeybee genome is also characterized by a high density of NUMTs, nuclear copies of mitochondrial genes. Mitochondria are originally ancient bacteria belonging to the class of alpha-proteobacteria together with *Wolbachia* and *Rickettsia* that were captured inside the eukaryote cell to form a symbiosis (Margulis & Sagan 2001). Mitochondrial gene copies are more frequent in the honeybee genome (NUMTs) than in the genomes of other animals showing a ten-times higher density than in humans (Pamilo *et al.* 2007, Behura 2007). We found that NUMTs are associated with transposon sequences in the honeybee genome, indicating that they may have spread along with transposons. Recombination and insertion of NUMTs can thus be connected in two ways. First, both rely on a similar process requiring a double strand breakage of DNA. Second, NUMTs (as well as other inserted sequences such as transposons, possible transfers from endosymbiont genomes) establish new homologous sites which can promote recombination.

The genome of no other advanced social insect has been sequenced yet. If recombination and the spreading of NUMTs are causally linked, we can predict a high density of NUMTs in other advanced eusocial insects.



## 4 Concluding remarks

In multicellular eukaryotes the generation of novel allele combinations are created by sexual reproduction between individuals belonging to the same or different populations (migration), or between individuals that represent different species (hybridization). At the gametic level alleles of different loci are further recombined to produce additional diversity.

In Hymenopteran social insects (ants, bees, wasps) allelic richness can be increased by multiple mating of the mother queen or by the presence of multiple reproducing queens in the colony. The size of marker linkage maps constructed in this work for two new phylogenetic lineages (ants and wasps in addition to previously studied honeybees) showed that the eusocial insects have a high rate of genome reorganization by recombination. Recombination increases genotype diversity efficiently in large social insect colonies (with thousands or tens of thousands of individuals) produced by a single polyandrous queen. Such a genotypically diverse work force can be beneficial in regard to division of labour, resistance against pathogens and survival in changing environmental conditions. Recombination also decreases the variance in pair-wise relatedness between nestmates thus reducing potential intracolony genetic conflicts.

Genetic variability has been associated with the determination of primary (queen-worker) and secondary castes (worker-worker). In this work the determination of the primary caste in *Pogonomyrmex* ant lineages was studied in hybrid zone populations where the split between the queen and worker caste is determined genetically. The hybridizing lineages showed many fixed nuclear differences dispersed across the constructed linkage map. This reflects the long separation of hybrid lineages and a lack of recent introgression in most parts of the genome. Alternatively, the observed scatter of fixed differences could indicate that the primary caste is determined by many genes in different linkage groups.

Social insects form an ideal platform for the spread of microbes by having a high density of closely related individuals that maintain colony temperature constant and often have stored food resources in the colony. We studied host reproduction manipulator endosymbionts in the species of *Formica cinerea* ant that establish both monogynous and polygynous colonies. In addition to *Wolbachia* two new endosymbionts of social insects were found, *Cardinium* and *Serratia symbiotica*, that showed different infection frequencies between ant populations. Multiple- and single-queen colonies were similarly infected, thus we

could not associate the infection rate and the social structure (single queen/multiple queen) of the *F. cinerea* ant populations.

Evolution of sociality is often associated with close relatedness which means genetic similarity of interacting individuals. However, the evolution of social insects characterized by large colonies and complex social behaviour, has also been shaped by acquisition of genetic diversity by polyandry, polygyny and recombination. The balance between forces selecting on one hand for high relatedness and genetic homogeneity and on the other hand for improved colony performance through increased genetic diversity is an intriguing area of research. My study has particularly emphasized the potential importance of recombination, a factor which has previously been largely neglected in this context.

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## Original papers

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- II Sirviö A, Johnston S, Wenseleers T & Pamilo P (2010) A high recombination rate in the common wasp *Vespula vulgaris* adds independent support to the theory that advanced sociality selected for increased recombination rates. Manuscript.
- III Sirviö A, Pamilo P, Johnson RA, Page RE Jr & Gadau J (2010) Origin and evolution of the dependent lineages in the genetic caste determination system of *Pogonomyrmex* ants. Manuscript.
- IV Sirviö A & Pamilo P (2010) Multiple endosymbionts in populations of the ant *Formica cinerea*. Manuscript.

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