Host specificity and genetics of host resistance in the *Daphnia-Pasteuria* host-parasite system.

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Cover: artistic impression of the *Daphnia-Pasteuria* system and the Red Queen Theory by artist and photographer Joep Luijckx

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Summary

Antagonistic coevolution plays an important role in a large number of evolutionary and ecological phenomena. For example, it may affect the strength and direction of change of host and parasite traits, promote reproductive isolation and enhance speciation. In addition, insights in coevolution are invaluable for combating diseases. Furthermore, according to the Red Queen Theory coevolution between hosts and their parasites may explain the maintenance of sexual reproduction, recombination and genetic variation, pertinent issues in evolutionary biology. For antagonistic coevolution to lead to negative frequency dependent selection, preserve genetic variation and select for sexual reproduction parasites need to have high host specificity and this specificity has to have a simple genetic basis. In this thesis I investigate these two criteria in the *Daphnia-Pasteuria* system. *Daphnia magna* and its bacterial pathogen *Pasteuria ramosa* have become one of the prime model systems for antagonistic coevolution between hosts and parasites and one of the few systems with empirical evidence consistent with antagonistic coevolution by frequency dependent selection.

In the first chapter I show that specificity in the *Daphnia-Pasteuria* system is much stronger than previously reported. By using a novel technique I obtain single genotypes (clones) from the unculturable *P. ramosa*. Infections with these single parasite genotypes either result in hosts that are fully resistant or in hosts that are fully susceptible. Previous reports of quantitative infection patterns in this system may have been caused by the presence of multiple parasite genotypes in the isolates that were used to infect the hosts in these studies. The finding of strong genotype-genotype interactions in the *Daphnia-Pasteuria* system are in support with antagonistic coevolution by negative frequency dependent selection and the maintenance of genetic variation and sexual recombination. Furthermore, the presence of multiple genotypes of *P. ramosa* in isolates suggests that multiple infections may occur frequently under natural conditions, which may play an important role in the evolution of parasite virulence.

High specificity for just some genotypes of *D. magna* as found in the first chapter contrast with reports from infections in natural populations which suggested that *P. ramosa* has a broad host range and is able to simultaneously infect highly diverged species of *Daphnia*. In the second chapter I address this apparent controversy. My findings of a controlled infection experiment with multiple host species and parasite lineages suggest that *P. ramosa* is a species complex consisting of multiple morphologically cryptic species each highly specialized for some genotypes within their host species. In addition I find that although infection does only occur in native host-parasite combinations, attachment of spores to the host esophagus, a necessary step in the infection process is conserved and polymorphic between highly diverged species of *Daphnia*. A potential ancient polymorphism for defence is consistent with long-term antagonistic coevolution by negative frequency dependent selection.

Chapters 3 & 4 investigate the inheritance of host resistance. Using a large array of crosses and two parasite genotypes I find that resistance is coded for by a single Mendelian inherited locus with three alleles with an allele hierarchy. An alternative, but more complex, explanation for our results is based on two closely linked diallelic loci with interlocus epistasis. Under both our genetic hypotheses the same host genotype is either resistant or susceptible depending on the genotype of the parasite and infection/resistance only occurs in specific combinations of host alleles and parasite genotypes, consistent with a matching allele model. Models of this family have played a central role in the theoretical development of antagonistic coevolution and where shown to readily lead to negative frequency dependent selection. My genetic results thus support the

notion that antagonistic coevolution between *D. magna* and *P. ramosa* can maintain genetic variation. Whether there is an advantage for sexual reproduction as envisioned under the Red Queen Theory remains an open question.

In conclusion both my findings on host specificity and the genetics of host resistance suggest that *Daphnia* and *Pasteuria* have the potential to undergo antagonistic coevolution by negative frequency dependent selection. Furthermore, the finding that genetics of resistance in *Daphnia* are consistent with a matching allele model will allow the *Daphnia-Pasteuria* system to become a powerful tool for empirical testing of population level predictions of this model. Indeed, the *Daphnia-Pasteuria* system could be used to experimentally test for negative frequency dependent selection, the maintenance of genetic variation and the notion that antagonistic coevolution may favor genetic mixing.

Introduction

Parasites harm their host and thereby select for host resistance, which in turn selects for parasites able to infect these hosts. This ongoing interaction between hosts and their parasites may result in antagonistic coevolution where a genetic change in one of the antagonists leads to a reciprocal response in the other. Antagonistic interactions may play an important role in a large number of evolutionary and ecological phenomena. For example, marine viruses are believed to affect global nutrient cycling. They are responsible for killing 20% of the marine microorganisms daily and thereby affect the retention of mineral elements in the photic zone and the export of carbon rich compounds to the deep ocean (Suttle 2007). Community structure and biodiversity may also be affected by host-parasite interactions (Hatcher *et al.* 2006). For example, the malaria parasite *Plasmodium azurophilum* is believed to enable the coexistence of *Anolis* lizards. In areas were the parasite is absent *Anolis gingivinius* outcompetes *Anolis wattsi*, while when it is present it reduces the competitive ability of *Anolis gingivinius* allowing *Anolis wattsi* to coexist (Schall 1992). Antagonistic coevolution may explain the presence of highly mutable loci in bacterial parasites (Moxon *et al.* 1994), and the maintenance of recombination and genetic variation (Peters & Lively 1999), e.g. human major histocompatibility loci A, B and C are known to be highly polymorphic (Frank 2002). Coevolution may also promote reproductive isolation and enhance speciation rates for both parasites and hosts (for review see Summers *et al.* 2003). In an experimental evolution experiment between bacteria and a phage it was shown that coevolved phage populations were much more diverged than populations that did not experience coevolution (Paterson *et al.* 2010). Furthermore, when parasites are locally adapted to their hosts, foreign parasites have a disadvantage and this may favor reproductive isolation between parasite populations (Summers *et al.* 2003). Insights in coevolution are also invaluable for combating diseases in livestock, crop-plants and humans (Woolhouse *et al.* 2002).

Two of the most discussed forms of antagonistic coevolution are; coevolution by selective sweeps (Woolhouse *et al.* 2002) and coevolution by negative frequency-dependent selection (Frank 1996). These are distinctly different with respect to their mechanism, time scale over which changes in gene frequencies can be observed and underlying genetics.

Coevolution by genetic sweeps describes the successive fixation of beneficial mutations in host and parasite populations. Novel beneficial mutations spread to fixation by directional selection (a genetic sweep) and lead to a continuous increase in host resistance and parasite infectivity (Buckling & Rainey 2002). Host specificity under this type of coevolution is thus expected to be low as is the level of genetic polymorphism for resistance and infectivity (Summers *et al.* 2003). In addition, as beneficial mutations are rare events and start at low initial frequencies genetic change under this type of coevolution is slow and may take hundreds of generations (Ebert 2008). Evidence for this form of coevolution has been found in for example bacteria and bacteriophage (Buckling & Rainey 2002) and plants and their pathogens (see for review Bergelson *et al.* 2001)

An alternative model is coevolution by negative frequency dependent selection, which leads to cycling of host and parasite genotypes. Natural selection will favour parasite genotypes that are able to infect common hosts, and, rare host genotypes, to which the parasite is not adapted, will thus have a competitive advantage and spread in the population till they become common. Changes in gene frequencies under this form of coevolution are expected to occur within few generations (Clarke 1976;Hamilton 1980). Furthermore, due to balancing selection genetic variation will be maintained for long periods of time leading to high levels of within population polymorphism. Evidence for

coevolution by frequency dependent selection is rare, but was suggested for soil bacteria and their phage (Gomez & Buckling 2011). A rare host advantage was demonstrated for snails and their trematode parasites (Dybdahl & Lively 1998) and long term balancing selection was found on the resistance gene rpm1 in *Arabidopsis* (Stahl *et al.* 1999). Besides maintaining genetic variation, coevolution of this form, may under some conditions also explain the widespread occurrence of sexual reproduction (Hamilton 1980;Jaenike 1978).

The paradox of sex and the Red Queen Theory

The majority of animals, plants and fungi reproduce sexually suggesting that sexual reproduction has advantages over asexual reproduction. Theory, however suggests that compared to asexual reproduction, sexual reproduction is believed to come with a substantial cost. In species where males provide little or no parental care, sexual reproduction has a two-fold disadvantage compared to asexual reproduction (Maynard Smith 1978). First, sexual females need to allocate resources to the production of males, whereas asexual females can produce daughters instead of sons resulting in a higher population growth rate. Second, a sexual female only transmits half of her genes to the next generation while an asexual female contributes all of her genes. Furthermore, searching for a suitable mate can be time consuming and costly. For example, male animals may sustain injuries in fights over access to females and female plants invest substantial resources in attracting pollinators. In addition, mating risks exposure to sexually transmitted diseases, parasitic genetic elements (Hurst & Werren 2001) and harmful seminal fluids (Chapman *et al.* 1995). Even in absence of these costs, it is unclear why there would be an advantage for sexual reproduction. Over time natural selection is expected to create favourable genetic associations, but shuffling of genetic material by recombination and segregation during sexual reproduction tends to break down these associations and transform them into unfavourable associations (Nei 1967;Turner 1967).

Breaking down genetic associations either between loci (recombination) or within loci (segregation) may be favoured when currently selected genetic associations are not favourable in the near future. For recombination to be maintained favourable associations between alleles on loci must fluctuate on the order of a few generations (Barton 1995;Charlesworth 1976). According to the Red Queen Theory antagonistic coevolution by frequency dependent selection will, under some circumstances, generate these conditions (Jaenike 1978;Salathe *et al.* 2008). Theory suggests that for recombination to be advantages there needs to be; 1) Strong selection on either the host or the parasite (Salathe *et al.* 2008). This condition may often be met as parasites that fail to infect their host have no fitness and infected host often have reduced fitness. 2) Hosts should be able to resist specific parasite genotypes and parasites should infect specific host genotypes. Infection outcome thus depends on the interaction between host and parasite genotypes. Examples for genotype-genotype interactions have been found in e.g. *Arabidopsis thaliana* and a fungal pathogen (Salvaudon *et al.* 2007), stickleback and trematode (Rauch *et al.* 2006) and *Caenorhabditis elegans* and the bacterium *Serratia marcescens* (Schulenburg & Ewbank 2004). 3) Genetics underlying the genotype-genotype interactions should have a specific genetic architecture. Theoretical modeling suggest that that for an advantage of recombination more than one but no more than around five loci should code for host resistance (Otto & Nuismer 2004). Furthermore, these loci need to be linked and their effect should be dependent on the combination of their genotypes (epistasis). Substantial evidence already exist for the genetic basis of host-parasite genotypic interactions in plants (Allen *et al.* 2004;Burdon & Jarosz 1991;Burdon 1994;Chaboudez &

Burdon 1995), but so far no studies have found evidence for epistasis between linked resistance loci (Wilfert & Schmid-Hempel 2008). Studies on the genetic basis of host-parasite genotypic interactions in invertebrates have also been unable to find the required genetic architecture but data is only available from few systems e.g. *C. bombi* and *B. terrestris* (Wilfert *et al.* 2007).

Genetic models

Several genetic models have been developed to capture the genetic mechanism underlying genotype-genotype interactions, the most used models are the gene-for-gene and the matching allele model. Under the gene-for-gene model resistance occurs when a gene in the host is able to recognize a virulence factor of the parasite. Susceptibility occurs when the host lacks the ability to recognize the parasite or if the parasite lacks the gene product that is recognized by the host. A key feature of this model is that there is a parasite that is able to infect all hosts. This genetic model is based on and well supported by empirical data from plants (Flor 1956; for a review see Thompson & Burdon 1992). As demonstrated by Parker (1994) this model does not lead to frequency dependent selection unless there are substantial costs associated with higher infectivity in which case host and parasite genotypes may cycle.

Under the matching allele model resistance occurs only in specific genotypic combinations between host and parasite. Each parasite genotype can only infect a specific set of host genotypes. Under this model universal virulence is absent and the model readily leads to frequency dependent selection. Therefore the matching allele model has been widely used in theoretical studies investigating the maintenance of sexual reproduction and genetic variation by antagonistic coevolution (Lively 2010;Otto & Nuismer 2004;Salathe *et al.* 2008). Direct empirical evidence for the matching allele model is however lacking.

Aim of this thesis

The *Daphnia-Pasteuria* host-parasite system has become one of the prime model systems for antagonistic coevolution between hosts and parasites. It is one of the few systems with empirical evidence for frequency dependent selection (Decaestecker *et al.* 2007) and it fulfils two of the three criteria required by the Red Queen Theory. The first criterion, strong selection on host and/or parasite (Salathe *et al.* 2008) was found for the *Daphnia-Pasteuria system* (Ebert *et al.* 2000;Little *et al.* 2006 and others) and will not be further discussed. The second criterion, the presence of strong host-parasite interactions was found (Carius *et al.* 2001). Ben-Ami *et al.*(2008) even suggested that the genotypic interactions may have been underestimated due to the use of isolates of *P. ramosa* which may have contained multiple parasite genotypes. Contrary to the finding of high specificity other studies have suggested that *P. ramosa* may have a very broad host range. Infections have been reported in several *Daphnia* species (Stirnadel & Ebert 1997) and even in other genera of cladocerans. An accurate estimate of *P. ramosa*'s host specificity is critical, besides implications for the Red Queen Theory it may, for example, play an important role in community structure, parasite mediated competition (Hatcher *et al.* 2006) and strength and direction of selection on host traits (Kirchner & Roy 2000). The first objective of this work is to investigate the genotype-genotype specificity in the *Daphnia-Pasteuria* system by using infections with cloned parasites, i.e. single parasite genotypes, thus negating potential confounding effects of parasite isolates (**chapter 1**). The second objective is to, for the first time, experimentally test the host range of *P. ramosa* using different *Daphnia* species (**chapter 2**). The third criterion required by the Red Queen Theory, the genetic architecture underlying the genotypic interactions, has been suggested to be simple (Little *et al.* 2006), but has not been tested. The third objective of this work is to determine the genetic architecture of

host resistance which underlies host specificity by performing a large array of crosses and assessing susceptibility of recombinant offspring against two genotypes of *P. ramosa* (**chapters 3 & 4**).

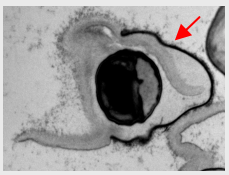
The host

Daphnia are cladocerans with a world wide distribution that occur in a variety of standing water bodies (e.g. rockpools, ponds, lakes and swamps). The majority of *Daphnia* reproduce by cyclic parthenogenesis, producing asexually for most of the season and sexually when conditions deteriorate (e.g. high densities, winter and desiccation). During sexual reproduction resting eggs, ephippia, are produced that are able to withstand harsh conditions. These characteristics make *Daphnia* especially suited for studying the inheritance of traits as genotypes can be crossed and offspring can be maintained as clonal lineages allowing multiple replicates to be tested for each recombinant genotype. In this work we will focus on the species *Daphnia magna* although I include some work with *D. pulex* and *D. longispina* as well. *D. magna* occurs in Eurasia, northern and western North America and some locations in Africa, and, in some parts of its range it co-occurs with *D. pulex* and *D. longispina* (Stirnadel & Ebert 1997;Ebert *et al.* 2001;Bengtsson 1986).

Box 1: The different steps in the infection process of *P. ramosa* (Duneau et al 2011)

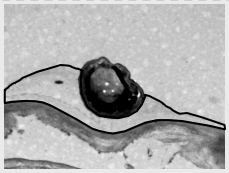
During filter feeding *Daphnia* encounter resting endospores of *P. ramosa*. Spores subsequently loose their outer coating, a process referred to as activation and upon ingestion by the host spores attach to the host esophagus (part of the foregut). The activation process is unspecific for host genotype but attachment only occurs in specific combinations of *Daphnia* and *P. ramosa*. Furthermore, attachment explains the great majority of variation in infection outcome and thus appears to be the key step in the infection process. Attachment can be visualized using fluorescent labelled spores and this technique can thus be used to assess if hosts are susceptible. After successful attachment *P. ramosa* is believed to penetrate the gutwall, enter the hosts bodycavity and subsequently proliferate in the hosts hemolymph and muscles. After host death several million spores are released from the decaying cadaver and transmission occurs.

Encounter: *Daphnia* ingests *P. ramosa* spores during filter feeding

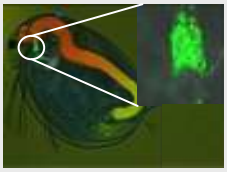


Spore activation: Spores loose their protective coating

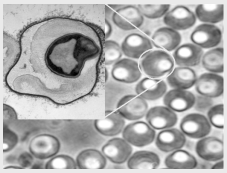
Activated spore



Attachment: Spores attach to the host esophagus



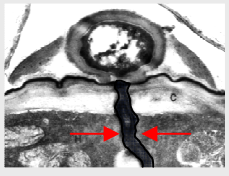
Attachment-test: Attachment can be visualized using fluorescent labeled spores



Waterborne spores

Host death: *P. ramosa* spores are released to the environment

Proliferation: *P. ramosa* grows within the host



Penetration: Spores have been proposed to penetrate host tissue (photo from related *P. penetrans*)

The parasite

The bacterial pathogen *Pasteuria ramosa* is a common parasite of *Daphnia* and infections have been reported from Europe and North America (Ebert 2005). Infection occurs when a susceptible host ingests waterborne spores that attach to the host esophagus, penetrate and subsequently proliferate within the host (Duneau *et al.* 2011) (see box 1). The step where spores attach to the host esophagus can be visualized using fluorescent labelled spores and this technique can be used to assess if hosts are susceptible (Duneau *et al.* 2011). Shortly after infection host are castrated and upon host dead several million spores are released. Infections are easily recognized as hosts are sterilized, show gigantism and a reddish body color. Infected host thus produce no more progeny, but still may live for several weeks, therefore infections with *P. ramosa* can have a strong impact on host populations (Ebert *et al.* 2000).

Outline

Chapter 1

In this chapter I present a novel technique to obtain single genotypes of the unculturable *P. ramosa*. I then use these clones to investigate if the strength of the genotypic interactions in the *Daphnia-Pasteuria* system was underestimated due to the use of isolates which may have contained multiple parasite genotypes. These experiments also test the binary infection hypothesis suggested by Ben-Ami *et al.* (2008) that states that infection of *D. magna* with *P. ramosa* either results in hosts that are fully resistant or in hosts that are fully susceptible (no partial resistance). By comparing infection patterns of single genotypes of *P. ramosa* with those of isolates I find support for this hypothesis. The genotype-genotype interactions in the *Daphnia-Pasteuria* system are thus much stronger as previously believed.

Chapter 2

The broad host range of *P. ramosa* as suggested by reports from infections in natural populations in highly diverged *Daphnia* species contrast sharply with the findings of strong specificity of *P. ramosa* for just some *D. magna* genotypes. This apparent contrast may be explained by the presence of cryptic parasite species with narrow host ranges or alternatively by a conservation of a genetic polymorphism for resistance across different host species. In this chapter I present the results of a large number of host genotypes from three *Daphnia* species that were tested for susceptibility against several *P. ramosa* collected from two *Daphnia* species. By using two different techniques, infection trials and attachment-tests, I am able to show that the attachment of *P. ramosa* spores to the host esophagus, a necessary step in the infection process, is conserved and polymorphic between the different *Daphnia* species. However, although attachment occurs infection is never observed in host species where the parasite did not originate from. *P. ramosa* thus consists of multiple cryptic species each highly specialized for some genotypes within their host species.

Chapter 3

In this chapter I describe how host resistance against one genotype of *P. ramosa* is inherited. I use a classical Mendelian approach to determine inheritance of resistance. I cross two parents to obtain an F1 which was selfed to obtain an F2. In addition, F1 was backcrossed and both parents where selfed. I test for susceptibility using two different methods, infection trials and the attachment-test. Both assays are highly consistent and results suggest resistance to be coded for by a single-locus with two alleles. Furthermore, a comparison with previous results suggests that host resistance is specific for the tested *P. ramosa* genotype. The genetics underlying host resistance may thus explain the strong genotypic interactions between *D. magna* and *P. ramosa*.

Chapter 4

In this chapter I present the results from a large set of genetic crosses that where designed to determine the inheritance of host resistance against two genotypes of *P. ramosa*. The results show that resistance is coded for by a single host locus for both *P. ramosa* genotypes. Resistance is determined by a specific match between host and parasite genotypes. A dominant allele provides resistance against one genotype of the parasite, but leads to susceptibility against the second. A second allele, recessive to the first, shows the reverse pattern. Double resistant hosts are never observed and parasites are unable to infect all hosts. This found genetic mechanism is consistent with the matching allele model that has been widely used in theoretical modeling pertaining negative frequency dependent selection and the Red Queen Theory.

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Chapter 1

Cloning of the unculturable parasite *Pasteuria ramosa* and its *Daphnia* host reveals extreme genotype-genotype interactions.

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Abstract: The degree of specificity in host-parasite interactions has important implications for ecology and evolution. Unfortunately, specificity can be difficult to determine when parasites cannot be cultured. In such cases, studies often use isolates of unknown genetic composition, which may lead to an underestimation of specificity. We obtained the first clones of the unculturable bacterium *Pasteuria ramosa,* a parasite of *Daphnia magna*. Clonal genotypes of the parasite exhibited much more specific interactions with host genotypes than previous studies using isolates. Clones of *P. ramosa* infected fewer *D. magna* genotypes than isolates and host clones were either fully susceptible or fully resistant to the parasite. Our finding enhances our understanding of the evolution of virulence and coevolutionary dynamics in this system. We recommend caution when using *P. ramosa* isolates since the presence of multiple genotypes may influence the outcome and interpretation of some experiments.

Keywords: Specificity, Host, Parasite, *Daphnia magna*, *Pasteuria ramosa,* Coevolution

Introduction

Parasites exhibit varying degrees of host specificity, ranging from generalists that are able to infect a wide range of host species, to specialists able to infect only one host or just a few genotypes within a host species. The degree of specificity has important implications for ecological and evolutionary phenomena related to host-parasite interactions (for review see Barrett *et al.* 2009). For example, host specificity is an important indicator of a parasite's ability to acquire a new host (Cleaveland *et al.* 2001) and may affect the likelihood of spread in biological invasions (Parker & Gilbert 2004). This is important because host switches or newly introduced parasites can drastically reduce biodiversity (e.g. Chestnut blight Anagnostakis & Hillman 1992). Host specificity can, in addition, influence community structure. For example, the Janzen-Connell hypothesis suggests that highly specific parasites decrease seedling survival close to the parent plant; thus, survival increases with distance from the parent plant, which promotes species coexistence and biodiversity (Connell 1971;Janzen 1970). Parasite specificity may also affect community structure by influencing species interactions (apparent competition, parasite mediated competition) (Hatcher *et al.* 2006). Specificity also plays an important role in coevolutionary interactions between host and parasites by influencing the strength and direction of selection on parasite (Kirchner & Roy 2002;Woolhouse *et al.* 2001) and host traits (Kirchner & Roy 2000). Extreme forms of specificity and host-parasite interactions may be important for maintaining genetic variation and sexual reproduction (Red Queen Theory, Jaenike 1978;Hamilton 1980)

Host-parasite specificity is largely considered to be under genetic control (Wilfert & Schmid-Hempel 2008). A desired test for specificity is thus to test for host genotype-parasite genotype interactions. Unfortunately, when parasites cannot be cultured, obtaining single genotypes is not possible. In such cases, studies often use isolates of unknown genetic composition to determine genetic interactions (e.g. Decaestecker *et al.* 2003;Solter *et al.*

2002). Isolates are defined here as parasite samples from infected hosts that may contain multiple genotypes, whereas clones are a single genotype. This distinction is important, as studies based on isolates composed of several clones, may infer incorrect patterns of specificity. The specificity of an isolate may in simple cases be lower than that of the clones it is composed of, but may be very complex if clones in the mixture interact with each other.

Bacteria of the genus *Pasteuria* are castrating parasites of nematodes and crustaceans with a nearly worldwide distribution (Sayre & Starr 2009). Interactions of *Daphnia magna* with *Pasteuria ramosa* have been shown to be highly specific (Carius *et al.* 2001;Decaestecker *et al.* 2003;Ebert 2008). However, as *P. ramosa* cannot be cultured outside its host, all previous studies of this parasite have used isolates, which may contain multiple genotypes. For example, microsatellite analysis revealed different alleles at the same locus within an isolate, suggesting the presence of multiple *P. ramosa* genotypes (Mouton & Ebert 2007). In addition, single isolates of *P. ramosa* were found to contain a slow and fast killing phenotype (Jensen *et al.* 2006;Little *et al.* 2008). Using a dose response curve, Ben-Ami *et al.* (2008b) found that an isolate of *P. ramosa* infected some host clones at low doses, while other host clones were only infected at very high doses. Propagation of high-dose infections resulted in a dose response similar to that of host clones that were infected with a low dose. This suggests that the infections seen at very high doses were caused by a second parasite genotype present within the isolate at a very low amount. Excluding infections caused by this second genotype, the observed infection patterns were binary: Some host-parasite combinations resulted in no infections while others resulted in a high proportion of infection. This led Ben-Ami *et al.* (2008b) to suggest that infection of *D. magna* clones by *P. ramosa* clones might be binary and that the previously observed patterns of quantitative variation in infectivity were due to the presence of multiple genotypes within isolates of *P. ramosa* (e.g. Carius *et al.* 2001;Ebert 2008;Little *et al.* 2006;Ebert *et al.* 1998). If the binary infection hypothesis holds, the host clone-parasite clone interactions will be much stronger than originally proposed for this system.

In this study, we describe the first clones of *P. ramosa* and test the binary infection hypothesis. We compare infection patterns of clones to those of the isolates from which they were obtained, and we perform infection trials on twelve host clones using five parasite clones to determine the specificity of the host clone-parasite clone interaction. Parasite clones showed higher specificity than natural isolates. They infected fewer *D. magna* genotypes and showed the strongest possible pattern of infectivity with hosts that are either fully susceptible or fully resistant.

Materials and Methods

Study system

Daphnia magna is a planktonic freshwater crustacean that acquires food by filter feeding and reproduces by cyclical parthenogenesis. *Pasteuria ramosa* is a gram-positive, endospore-forming bacterium that is an obligate parasite of *Daphnia* (Ebert 2005). Spores of *P. ramosa* are ingested during filter feeding and infect the *Daphnia* hemolymph and muscle. Successful infection by *P. ramosa* induces brownish coloration, gigantism and castration of the host (Ebert *et al.* 2004). Infections are thus easily recognizable. *P. ramosa* continues to grow until the host dies, whereupon several million endospores are released from the decaying cadaver. The severe fitness cost of infection by *P. ramosa*, in combination with generally high prevalence in natural populations (up to 100%) (Duncan *et al.* 2006), can exert substantial selection on its host (Little & Ebert 2000).

Table 1: Isolates of P .ramosa

Isolate	Origin (collected in the field)	Geographic region	Year of sampling
P1	One infected female	Germany, Gaarzerfeld	1997
P2	Single female infected from pond sediment	England, Kames	2002
P3	10 infected hosts	Finland, Tvärminne	2002
P4	8 infected hosts	Belgium, Heverlee	2003
P5	Single female infected from pond sediment	Russia, Moscow	1996

Host and parasite preparation

A total of 14 *D. magna* clones were isolated from several ponds in Europe: Belgium (B2, T10, M5 and M10), northern Germany (DG-1-106), Hungary (HO1, HO2 and HO3), southern Germany (Mu10, Mu11 and Mu12) and south-western Finland (SP1-2-3, X-clone and AL1-4-4). Four additional clones were the products of crosses performed in the lab: Inb1 is the once-selfed offspring of Mu11, Xinb3 and AL1-4-4 are the results of three generations selfing each, XFa6 is a cross between Xinb3 and AL1-4-4, and XI is a cross between Xinb3 and Iinb1. All clones were kept under standardized conditions for three generations prior to experiments (8 individuals per 400 ml jar filled with artificial medium (Ebert *et al.* 1998). Medium was replaced twice a week and each jar was fed 60 million cells of the chemostat-cultured unicellular algae *Scenedesmus obliquus* daily. Before and during experiments, *D. magna* were kept in an incubator on a 16h:8h light dark cycle at 20°C. Jars were kept in trays and randomly distributed across the shelves of the incubator, and their position was rearranged daily. Five isolates of *P. ramosa* were used to obtain clonal lineages (Table 1). All isolates were passaged at least twice in the laboratory through the same host clone before use. Clones of *P. ramosa* were derived from the 5 isolates using 2 methods: infection by limited dilution and single-spore infections.

Isolating clones of *P. ramosa* by limited dilution

Juvenile *D. magna* females (0 to 5 days old) of clones HO2 and AL1-4-4 were kept in groups of 10 in 400 ml jars filled with artificial culture medium. Each jar was fed a high ration of 100 million algae per day; medium was changed twice a week, and all newborn were removed. After two weeks, offspring born within a 5-day interval were collected and distributed across thirty-nine 400 ml jars at a density of approximately 80 animals per jar. Spore suspensions were prepared by homogenizing infected cadavers of *D. magna* in a 1.5 ml microcentrifuge tube with a plastic pestle. Spore concentrations were determined using a haemocytometer (Thoma ruling). For host clone HO2, one jar received an estimated 10,000 spores of *P. ramosa* isolate P5; two jars received 1,000 spores; six jars, 100 spores, and 30 jars, 10 spores. For host clone AL1-4-4, two jars received an estimated 1,000 spores, and 42 jars an estimated 100 spores. *D. magna* populations were fed 10^7 cells per jar/day for 20 days and 2 x 10^8 cells per jar/day thereafter. Females that produced clutches were removed because they were likely uninfected. With fewer females per jar, feeding regime was adjusted to represent good conditions. From day 40 to 50, all females that showed the typical symptoms of *P. ramosa* infection (castration, gigantism and brownish

Table 2: Clones of P. ramosa *obtained by limited dilution and single spore infections.*

Clone of *P.ramosa*	Cloning method	Cloned in host clone	Origin of material	Date of cloning	Single genotype
C1	limited dilution	HO2	P5	March 2006	likely
C2	limited dilution	HO2	P5	March 2006	likely
C3	limited dilution	HO2	P5	March 2006	no
C4	limited dilution	HO2	P5	March 2006	no
C14	limited dilution	AL1-4-4	P3	Aug. 2006	likely
C19	single spore	Xinb3	P1	Nov. 2007	yes
C20	single spore	HO2	P2	Feb. 2008	yes
C24	single spore	HO2	P4	June 2008	yes

colour) were frozen for later analysis. Using the same mothers, we repeated the limited dilution infections with two additional cohorts of juveniles. For isolate P5 in HO2, we found a total of seven infected *D. magna*: two individuals from two 100 spore jars (named C1, C2), two individuals from one 1000 spore jar (named C3, C4), and three individuals from the 10,000 spore jar (which were not used for further experiments). For isolate P3 in AL1-4-4, we found four infections in four different 100 spore jars, named C14 to C17 (Table 2).

Isolating clones of *P. ramosa* by single spore infection

Clones from *P. ramosa* isolates P1, P2, P3 and P4 were obtained by single spore infections. One three-day old *D. magna* of clone Xinb3, HO2 or AL1-4-4 was placed in each well of a 96-well plate (Falcon 354043). Wells contained about 100 µl of artificial medium. Spore suspensions of isolates were diluted to 0.1 million spores per ml., and 4 µl of this suspension was placed on a microscopic slide. The slide was placed under an inverse microscope at 400X magnification. Using a micropipette (1 mm O.D. 0/78 mm I.D. borosilicate micropipette elongated over a flame), single spores (about 5 µm in diameter) were drawn up from the microscope slide via capillary action and blown/transferred to a well of the 96-well plate. We added one spore per well. *D. magna* in well plates were fed between 10,000 and 20,000 algae cells daily. After three to four days, *D. magna* were transferred from the well plate to 100 ml glass jars containing 80 ml medium. Up to four individuals were kept in each jar. *D. magna* in jars were fed 4 to 20 million algae cells daily (depending on *D. magna* size and number) and transferred to new jars containing 80 ml of fresh medium weekly. *D. magna* individuals were screened by eye for infection daily, and infected individuals were placed in separate jars. Individuals that died 15 days or more after infection were immediately checked for *P. ramosa* spores or stored at -20°C in a 1.5 ml microcentrifuge tube containing minimal amount of medium for later analysis.

Nine out of 6,384 single spore infections were successful (0.14%): *D. magna* clone HO2 was infected by a spore of *P. ramosa* isolate P3 (named C18); Xinb3 was infected by a spore from isolate P1 (C19); four individuals of clone HO2 were infected by spores from isolate P2; (C20-C23), and three individuals of HO2 were infected by spores from isolate P4 (C24-C26). Infected animals were stored at -20°C until further use.

Spore sample preparation for experiments

To augment the cloned material, a second generation of each *P. ramosa* clone had to be produced. To produce infections, we added standardized concentrations of the appropriate spore suspension to 100 ml jars. These jars contained 20 ml of artificial culture medium and ≤15 three-day-old *D. magna* individuals from the *D. magna* clone that produced the *P. ramosa* clone. Spore doses were between 30,000 and 50,000 spores, depending on the amount of spores available. After five days, jars where filled to 80 ml of medium, and nine days after exposure, *D. magna* were transferred to 400 ml jars with up to 8 *D. magna* per jar. Infected individuals were kept under standard feeding conditions until natural death and were then stored at -20°C. Spores of a single infected *D. magna* individual from the second generation were used to create a third generation with a spore dose of 100,000 spores per jar.

Experiment 1: Comparing infection patterns of clones and an isolate of *P. ramosa*

In this experiment we compared the infection patterns of four putative clones of *P. ramosa* created by limited dilution to the infection patterns of the isolate from which they were cloned. Twelve *D. magna* clones (B2, T10, M5, M10, DG-1-106, HO1, HO2, HO3, Mu10, Mu11, Mu12 and SP1-2-3) were separately exposed to four putative *P. ramosa* clones (C1, C2, C3 and C4) and to the original isolate P5.

We used 14 replicates per treatment combination and 14 unexposed controls (a total of 12 x 6 x 14 = 1008 individuals). We placed four-day-old female juveniles from the third clutch of the standardized host clones singly into 100 ml jars containing 20 ml of artificial medium. The next day 50,000 *P. ramosa* spores of the second generation were added to each jar. A week after exposure, 80 ml of fresh medium was added to each jar, and medium was thereafter replaced on a weekly basis. Daily food levels were increased from 2 $x10^6$ cells per individual per day on day 10 to $10x10^6$ cells per individual per day on day 32 to accommodate for the increase in food demand of the growing animals. Dead individuals were recorded daily, but only those that died after day 14 were dissected and checked for *P. ramosa* spores. Individuals that died earlier could not be reliably checked for infection and were thus excluded from the analysis. On day 44, all remaining *D. magna* were scored phenotypically for infection. When in doubt, we dissected the animal and checked for infection under a phase contrast microscope (400x), but we found no discrepancies with our initial diagnosis.

Experiment 2: Genotype-genotype interactions

The infection specificity of five *P. ramosa* clones (C1 & C14 created by limited dilution, C19, C20 & C24 created by single spore infection), a mixture of these 5 clones, and an unexposed control was tested using a panel of 12 *D. magna* clones (HO1, HO2, HO3, Xinb3, AL1-4-4, M10, M5, Mu12, Dg106, Iinb1, XI and XFa6). We used 10 replicates for each host clone-parasite clone combination (a total of 12 x 7 x 10 = 840 individuals). In this experiment, *D. magna* were either exposed to 50,000 spores of the third generation of one of the five *P. ramosa* clones, exposed to a mixture containing 10,000 spores of each *P. ramosa* clone, or exposed to a negative control containing crushed, noninfected *Daphnia*. Experimental conditions were similar to Experiment 1 with the following exceptions: three-day-old females were exposed; fresh medium was added five days after exposure; and medium was changed every three days thereafter. Initially, 3 million algae cells were fed to each jar, but to accommodate the growing food demand of the animals, feeding levels were raised by 1 million algae on day 10 and again on day 20. The experiment was terminated 30 days after exposure. Animals that died 15 days after infection were taken into account in the analysis, and the infection status of all animals was verified with phase contrast microscopy (400x).

Results

Experiment 1: Comparing infection patterns of clones and an isolate of *P. ramosa*

In this experiment, the infection pattern of four putative *P. ramosa* clones created by limited dilution was compared to the original isolate. *P. ramosa* clones C1 and C2 infected *D. magna* clones HO2 and M10. Putative *P. ramosa* clones C3 and C4 also infected *D. magna* clone HO1. In contrast, the original isolate (P5) infected nine out of 12 *D. magna* clones (Table 3). Additionally, the rates of infection differed between clones and the isolate. While infection rates of the isolate and putative clones C3 and C4 were quite variable among the clones they infected (8-100%), clones C1 and C2 showed a strong binary pattern. Either they were able to infect close to 100% of exposed *D. magna,* or none at all. Finally, C1 and C2 were nearly identical in their infection pattern, while C3 and C4 showed strong differences. We speculate that C1 and C2 are indeed clones of *P. ramosa* while C3 and C4 are different mixtures of more than one clone.

Experiment 2: Genotype-genotype interactions

In this experiment, the infection patterns of five *P. ramosa* clones and a mixture of all five were tested against 12 *D. magna* clones (Table 4). Clones of *P. ramosa* showed a strong binary pattern, either infecting nearly all individuals of a given host clone or none at all. *P. ramosa* clones C1, C14, and C24 infected three of 12 *D. magna* clones. *P. ramosa* clones C19 & C20 infected five of 12 *D. magna* clones, and the mixture of all *P. ramosa* clones infected the combined set of *D. magna* clones. Based on their infection patterns, *P. ramosa* clones can be divided into two different infection phenotypes or infectotypes: Group one, containing clones C1, C14, and C24, originated from Russia, Finland and Belgium, respectively; and Group 2, containing clones C19 & C20, originated from Germany and England. Similarly, *D. magna* clones can be grouped into four resistance phenotypes or resistotypes: Four host clones were resistant to both P. ramosa infectotypes; two were susceptible to both infectotypes, and the others were susceptible to one and resistant to the other infectotype.

Table 3: Comparison of infections by putative clones obtained by limited dilution and the original isolate. C1 and C2 show a binary infection pattern, while C3 and C4 show low infectivity in D. magna clone HO1. Dilutions for the production of C1 and C2 were 10 times higher than those for C3 and C4. The original isolate, P5, infects many more D. magna *clones and shows a broader range of infection compared to C1 and C2. All numbers in % of exposed hosts. Each cell represents 14 replicates.*

	P. ramosa				
Clone of	Putative clones				Isolate
D. magna	C1	C2	C3	C4	P5
HO1	0	0	8	10	27
HO2	100	100	36	93	57
HO3	0	0	0	0	15
T10	0	0	0	0	0
M5	0	0	0	0	7
B2	0	0	0	0	0
M10	100	85	29	100	7
Mu10	0	0	0	0	8
Mu11	0	0	0	0	29
Mu12	0	0	0	0	9
SP1-2-3	0	0	0	0	21
DG-1-106	0	0	0	0	0

Discussion

Using two different techniques, we obtained the first clones of the obligate *Daphnia* parasite, *P. ramosa*. Clones of *P. ramosa* revealed much stronger patterns of specificity than previously reported. This suggests that isolates used in previous studies likely contained multiple genotypes, and that multiple infections in the *D. magna-P. ramosa* system may be common.

Clones of *P. ramosa*

Using single spore infections and infections produced by limited dilution, we obtained the first clones of *P. ramosa.* In cases where infections grew from a single spore, we knew for certain that the resulting *P. ramosa* infection consisted of only one genotype. When infections originated from limited dilution, we cannot rule out that infection with more than one spore occurred. To obtain *P. ramosa* C1 and C2, we used an estimated 100 spores per 80 *D. magna*, whereas for C3 and C4 we used 1000 spores per 80 *D. magna*, making infection with multiple genotypes more likely in the latter. Indeed, while C1 and C2 had infection patterns identical to C14 and C24, which were obtained by single spore infections, C3 and C4 showed a different infection pattern with low and intermediate infectivity in some host clones (Table 3 & 4). Low infectivity rates caused by mixtures of different genotypes may be explained by interference of *P. ramosa* genotypes during the infection process. We conclude that C1 and C2, in addition to the single spore infections, are infections with a single genotype, while C3 and C4 potentially contain multiple genotypes and for this reason were not further used. Furthermore, C1 and C2 may be the same genotype, while C3 and C4 may be mixtures with different composition.

Table 4: Outcome of infection trial with single genotype infections of P. ramosa*. C1 and C14 were obtained by limited dilution. C19, C24 and C20 were obtained by single spore infections. All numbers in % of exposed hosts. Each cell represents 10 replicates.*

		P. ramosa					
Clone of		C1	C14	C24	C19	C20	Mix
D. magna	Location	Russia	Finland	Belgium	Germany	England	-
HO1	Hungary	0	0	0	0	0	0
HO2	Hungary	100	86	90	100	100	88
HO3	Hungary	0	0	0	0	0	0
M5	Belgium	0	0	0	0	0	0
M10	Belgium	100	100	100	100	100	100
Xinb3	Finland (selfed)	0	0	0	100	100	100
AL1-4-4	Finland	100	100	100	0	0	100
Dg106	Germany	0	0	0	86	90	80
Mu12	Germany	0	0	0	0	0	0
Iinb1	Germany (selfed)	0	0	0	0	0	0
XI	Labcross	0	0	0	0	0	0
XFa6	Labcross	0	0	0	88	100	90

Clones reveal higher specificity

Our results on the specificity of *P. ramosa* clones differed markedly from previous studies that used isolates of *P. ramosa* (e.g. Carius *et al.* 2001;Decaestecker *et al.* 2003;Ebert 2008). These studies found that *P. ramosa* isolates were able to infect a wide range of *D. magna* genotypes with varying degree of infectivity. Here we report that *P. ramosa* clones show much higher specificity. They infect fewer *D. magna* genotypes and show the strongest possible pattern of infectivity: hosts are either fully susceptible or fully resistant (Table 4). Comparing *P. ramosa* clones C1 and C2 to the isolate they originated from (P5), it is clear that clones of *P. ramosa* are more specific, infecting fewer *D. magna* clones (Table 3). A similar pattern is observed when the five *P. ramosa* clones used in this study are compared to the infection rates of their respective isolates reported by Ebert (2008) (Table 5). While the isolates showed a range of infection rates, *P. ramosa* clones always showed binary infectivity.

An almost perfect binary pattern of resistance as found in our experiment is consistent with the binary infection hypothesis postulated by Ben-Ami *et al.* (2008b). This hypothesis posits that infection is binary in the *D. magna-P. ramosa* system and that the commonly observed pattern of quantitative infectivity is due to the presence of multiple genotypes within isolates of *P. ramosa*. Similar results and their implications have been discussed in plant pathogen interactions, where multiple (bulk) infections may produce a quantitative infection pattern while infections with a single genotype can show discontinuous variation for resistance (Burdon & Thrall 2001).

Table 5: Comparison of infection patterns of clones and the isolates from which these clones were obtained. Data for isolates taken from Ebert (2008). All numbers in % of exposed hosts. Note that M1 in Ebert 2008 is the same host clone as M10 in our study and that Xinb3 is the 3-times selfed X-clone whose infection pattern with P. ramosa *clones is identical.*

		P. ramosa									
		Russia		Finland		Belgium		Germany		England	
Clone of	Origin of	Clone	Isolate	Clone	Isolate	Clone	Isolate	Clone	Isolate	Clone	Isolate
D. magna	host clone	C1	P5	C14	P3	C24	P4	C19	P1	C20	P2
HO1	Hungary	0	50	0	0	0	0	0	13	0	0
HO2	Hungary	100	63	86	88	90	75	100	100	100	100
HO3	Hungary	0	88	0	13	0	13	0	0	0	13
Xinb3/X-clone	Finland	0	13	0	0	0	38	100	100	100	100
AL1-4-4	Finland	100	38	100	100	100	88	0	0	0	88
M10	Belgium	100	13	100	100	100	63	100	100	100	88
M5	Belgium	0	50	0	13	0	0	0	0	0	25
Mu12	Germany	0	25	0	0	0	0	0	0	0	50
Dg106	Germany	0	13	0	0	0	0	86	88	90	10

High specificity as found here has been found in plant pathogens (e.g. Zeigler *et al.* 1995;Thompson & Burdon 1992 and references therein) and in bacteria-bacteriophage interactions (Sullivan *et al.* 2003;Duplessis & Moineau 2001). To our knowledge, such high specificity has not been found in animal systems. The basis of strain specific resistance by innate immunity is well understood in plant-pathogen systems. Specific resistance follows the gene for gene principle "for each gene determining resistance in the host there is a corresponding gene for avirulence in the parasite with which it specifically interacts" (Kerr 1987). Numerous of the underlying genes have been identified in both the hosts and pathogens (see Nurnberger *et al.* 2004). Resistance in the *Daphnia-Pasteuria* system, as suggested by the binary pattern, is likely based on few loci, as a resistance mechanism based on many loci is likely to yield a continuum of infection rates. This is consistent with the proposed simple Mendelian inheritance of resistance for this system (Little *et al.* 2006) and similar to the mode of inheritance of resistance (R) genes in plants. In contrast to the majority of plant R-genes that act intracellularly (Jones & Dangl 2006), resistance in *Daphnia* is likely based on the failure of extracellular attachment of *P. ramosa* spores, as has been suggested for the related *Pasteuria penetrans* (Sayre & Starr 2009). This situation so far does not suggest any homology with any known mechanism in arthropods.

Multiple genotypes present in isolates

If our finding of binary infectivity is generally valid, it suggests that multiple genotypes are frequently present in isolates of *P. ramosa*. Looking at the distribution of infectivity of *P. ramosa* isolates in our study and Ebert (2008), we can speculate on the composition of some of the isolates (Table 3 & 5). The infection patterns of P1 and P3 suggest the presence of a second, low frequency *P. ramosa* genotype, different from the one revealed by cloning. P2, P4 and P5 show more complex patterns, indicating the presence of even more genotypes. A similar reasoning can be applied to the data from Carius *et al.* (2001), who used spores recovered from nine singly infected animals to infect nine host clones. One of the nine *P. ramosa* isolates shows a binary infection pattern indicative of an infection by a single genotype (*P. ramosa* number 15 in Carius *et al.* 2001), while all others show more complex patterns indicative of infections by more than one genotype. It thus appears that multiple infections were common in this study. This is consistent with earlier studies of *D. magna* and *P. ramosa* that found evidence for multiple genotypes within isolates (Jensen *et al.* 2006;Mouton & Ebert 2007). Infections with several strains of the same pathogen appear to be widespread among other pathogens (Balmer & Caccone 2008;Lopez-Villavicencio *et al.* 2007;Read & Taylor 2001). Our reasoning that *P. ramosa* cocktails are present in isolates also allows us to speculate that several other *P. ramosa* infectotypes might be present in natural populations, which we have not yet been able to clone. For example host clones HO1, HO3, M5, and Mu12 were never infected by our clones, but were infected by isolates. Although we continue to clone more *P. ramosa* genotypes, the low success rates (about 1 in 700) of single spore infections makes this a slow process.

Implications for coevolution and the evolution of virulence

Higher specificity and the presence of multiple genotypes within isolates of *P. ramosa* enhances our understanding of coevolution and the evolution of virulence in the *D. magna-P. ramosa* system. In addition, the presence of multiple genotypes within isolates may affect the interpretation of previously published studies.

Specificity is a strong determinant for antagonistic coevolution by negative frequency dependent selection (Agrawal & Lively 2002;Clarke 1976). Coevolutionary cycles may occur with specific genotypic interactions with

simple underlying genetics (Clarke 1976). Clones of *P. ramosa* revealed highly specific interactions. In addition, the binary infectivity of *P. ramosa* clones combined with the castration of the host leads to strong selection on both host and parasite. Strong discrepancy between the fitness of a successful and an unsuccessful host or parasite is favourable for coevolutionary cycles (Salathe *et al.* 2008). The binary infectivity pattern also suggests that the genetic control of resistance may be based on simple genetics (as discussed above). Thus, our findings support earlier evidence that coevolutionary cycles occur for infectivity in the *D. magna-P. ramosa* system (Decaestecker *et al.* 2007). As suggested by the Red Queen Theory, such cycles may be important for the maintenance of genetic variation and the evolution of recombination (Hamilton 1980;Jaenike 1978).

Evidence for the rapid evolution of infectivity has been previously reported in this system (Little *et al.* 2006). Two *P. ramosa* isolates were passaged five times on two *D. magna* clones. One *P. ramosa* isolate gained infectivity on the host clone it was grown on, but lost infectivity on the other *D. magna* clone, while the other *P. ramosa* isolate evolved higher infectivity on both *D. magna* clones. The presence of multiple genotypes within isolates may explain the rapid evolution of host infectivity. As suggested by Ben-Ami *et al.* (2008b), for Little *et al.* (2006), results may be interpreted as selection for infective and against noninfective parasite genotypes within a mixed-isolate infection.

The evolution of virulence may depend on host specificity (Woolhouse *et al.* 2001). Theory predicts that highly specialized parasites can evolve towards high levels of virulence (Regoes *et al.* 2000). Indeed, empirical data from the *D. magna-P. ramosa* system shows that the virulence of *P. ramosa* is high (Ebert *et al.* 2004) and evolved to optimize parasite reproductive success (Jensen *et al.* 2006). Frequent interactions between different genotypes of *P. ramosa*, as indicated by the presence of multiple genotypes within isolates, may, however, also influence the evolution of virulence. Models suggest that infections with multiple genotypes can either evolve higher (Bonhoeffer & Nowak 1994;Frank 1996) or lower virulence (Brown *et al.* 2002). For the *D. magna-P. ramosa* system, it is suggested that the most virulent competitor within a multiple infection produces the vast majority of transmission stages (Ben-Ami *et al.* 2008a). Based on this observation, one would expect that passaging *P. ramosa* isolates multiple times on the same host clone would lead to a loss of lower virulent genotypes within the isolate. However, isolates P1 & P5 had been passaged at least five times prior to the experiment of Ebert (2008) and still showed a highly quantitative pattern, indicating the presence of multiple genotypes (Table 5). Perhaps the *P. ramosa* genotypes within these isolates have similar virulence, or the interactions between *P. ramosa* genotypes are complex, perhaps depending on both the frequency and the identity of the interacting genotypes. Our data are consistent with this explanation. *P. ramosa* isolate P5 showed moderately high infectivity in host clone HO2 and very low infectivity in host clone M10, while clones obtained from this isolate showed equally high infectivity in both host clones (Table 3). Currently we have no explanation for how these interactions are produced. Other studies have reported that parasites may behave differently in single and multiple infections (Gower & Webster 2005). Interactions may also be caused by parasites that use the host immune system to harm competitors (Brown & Grenfell 2001). The outcome of multiple infections and the possible presence of a complex interaction between competing genotypes of *P. ramosa* remain to be investigated in more detail.

The presence of multiple genotypes within isolates may also have affected the outcome of an experiment performed by Little *et al.* (2008), in which the authors found that a low virulent isolate of *P. ramosa* had greater

infectivity and greater rate of replication compared to a highly virulent isolate of *P. ramosa*. This finding challenges the virulence trade-off hypothesis, which states that an increase in the production of transmission stages leads to an increase in virulence (Anderson & May 1982). In light of our findings, their explanation that the parasite isolates used in their study may have contained multiple genotypes is likely and can now be tested using *P. ramosa* clones.

Conclusion

Using natural isolates of parasites to determine specificity can greatly underestimate specificity in host-parasite interactions. Using the first clones of *P. ramosa,* we find much higher specificity than previously reported with isolates. Accurate estimates of specificity are important, as specificity plays a key role in understanding a number of ecological and evolutionary processes. In the *D. magna-P. ramosa* system, our findings have implications for coevolution between host and parasite, believed to be important for the maintenance of genetic variation and sexual recombination and for the evolution of virulence. We recommend caution when using isolates of *P. ramosa*, which may potentially contain multiple genotypes that can alter the outcome and interpretation of some experiments.

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Chapter 2

Cross species infection reveals the presence of cryptic parasite species and trans-species polymorphism for host defense.

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Abstract: Understanding the host range of parasites is critical as it can have important implications for ecological and evolutionary phenomena related to epidemiology and host-parasite interactions. The bacterial parasite *Pasteuria ramosa* has been reported to infect several different species of *Daphnia* and even other genera of Cladocera. In contrast to this apparent generalist life style, extreme specificity was reported for *P. ramosa* genotypes within the host species *Daphnia magna*. This apparent contrast may be explained by the presence of cryptic parasite species with narrow host ranges or alternatively by a conservation of a genetic polymorphism for resistance across different host species. To discern between these hypotheses we tested multiple genotypes of *D. pulex*, *D. longispina* and *D. magna* with four lines of *P. ramosa.* We use two methods to assess host range. Infection trials that show the outcome of the entire infection process and an assay that determines the ability of the parasite to attach to the hosts eosophagus, a necessary step in the infection process that was shown before to be responsible for host-parasite genotype-genotype specificity. Here we find attachment to the host esophagus in specific native, but also in non-native host-parasite combinations. Alleles allowing attachment to the esophagus thus seem to be conserved between the different host species that diverged more than 100 million years ago. However, despite successful attachment, *P. ramosa* were never able to infect non-native hosts, suggesting that *P. ramosa* reported from different host species are different host varieties each with a narrow range of host species.

Keywords: host range, cryptic species, ancient polymorphism, parasite, *Daphnia*, *Pasteuria ramosa*

Introduction

Understanding the host range of a parasite is critical as it can have important implications for numerous ecological and evolutionary phenomena. It can affect selection on both parasite (Kirchner & Roy 2002;Woolhouse *et al.* 2001) and host traits (Kirchner & Roy 2000) for example, parasites with a narrow host range may evolve toward higher levels of virulence (Regoes *et al.* 2000). A parasite's host range may also affect species interactions, and thereby alter community structure (Hatcher *et al.* 2006). Apparent competition may exclude one of two hosts in the presence of a parasite that would be able to coexist in the absence of the parasite (Bonsall & Hassell 1997). Parasites may also promote species coexistence. For example, tree seedling survival increases with distance from the parent plant by action of parasites with narrow host range (Janzen-Connell hypothesis: Connell 1971;Janzen 1970). Furthermore, host range is also an important predictor for the likelihood of host switches (Parker & Gilbert 2004) and therefore plays an important role in conservation and biocontrol.

Reports of a parasite's apparent host range may overestimate its actual host range. Parasites with an apparently broad host range may consist of multiple cryptic species each with a narrow host range (e.g. Bucheli *et al.* 2000;Mccoy *et al.* 2001;Steinauer *et al.* 2007). In addition, potential hosts and parasites may not come into contact under natural conditions due to non-overlapping ranges (Perlman & Jaenike 2003), or host behaviour (Hart 1990).

Even if parasites are able to overcome host defences they may still fail to transmit to the next host generation (Solter & Maddox 1998), or host abundance might be too low to sustain the infection (Anderson & May 1978). Thus, descriptions of parasite host ranges are difficult to interpret, as they don't differentiate between typical and accidental hosts and may fail to inform about possible host specialization (Tripet & Richner 1997).

Pasteuria ramosa, a bacterial parasite of *Daphnia* has been reported to have a broad host range infecting *Daphnia magna, D. longispina*, *D. pulex* (Stirnadel & Ebert 1997), *D. dentifera* (Duffy *et al.* 2010), *D. dolichocephala* (Duneau *et al.* 2011) and other genera of cladocerans (Green 1974;Sayre *et al.* 1977). These assessments of host range are based on development and morphology of *P. ramosa* spores. Stirnadel and Ebert (1997), sampled three ponds in southern England over one year where *D. magna, D. pulex*, and *D. longispina* occur in sympatry and found *P. ramosa* infecting all species leading the authors to conclude that *P. ramosa* was able to simultaneously infect all three species. This suggests that *P. ramosa* has the ability to infect a large phylogenetic range of hosts, *D. magna,* (subgenus Ctenodaphnia) *D. longispina* and *D. pulex* (subgenus Hyaloaphnia) belong to different subgenera and are unlikely to have exchanged genes (by introgression) in the last 100 million year (Colbourne & Hebert 1996). This is even more true for reports of *P. ramosa* from different genera.

In contrast to its apparent ability to infect a broad phylogenetic range of species, *P. ramosa* is known to be extremely specific for some genotypes of *Daphnia magna* (Luijckx *et al.* 2011). The strong specificity of *P. ramosa* was suggested to be related to the way it enters the host. During filter feeding the host ingests spores of the parasite, which attach to the host esophagus, probably penetrate the esophagus wall and subsequently proliferate within the host in case of an infective parasite or fail to attach in case of an uninfective parasite (Duneau *et al.* 2011). For one parasite genotype it was suggested that the observed polymorphism for attachment depends on a single locus in *D. magna*. Inheritance patterns are consistent with a single Mendelian locus with two alleles, a dominant resistance allele leading to failure of parasite attachment and a recessive susceptibility allele that allows for attachment (**chapter 3**).

One possibility explaining the contrasting high specificity within species and apparent low specificity between species is that *P. ramosa* infecting the different host species are separate genetic entities each with a narrow host range (here defined as a variety) as suggested by Ebert (2005). Alternatively *P. ramosa* could be specific for some host genotypes within each host species but unspecific across species. Under this scenario different *Daphnia* species would share resistance alleles, as attachment depends on the alleles on the resistance locus/loci in the host, and thus have a trans-species polymorphism for defense against *P. ramosa.* Conservation of attachment was suggested by Duneau *et al.* (2011) who found attachment of one *P. ramosa* genotype in two related *Daphnia* species.

In this study, we test the host range of four *P. ramosa* lines using 1538 genotypes from the three host species, *D. magna*, *D. pulex* and *D. longispina*. We sampled a natural Finnish meta-population where all three host species occur in sympatry and test for resistance with natural co-occurring *P. ramosa* isolated from *D. magna* and *D. longispina*. In addition we determine resistance in *Daphnia* of all three host species from various locations in Europe with four different *P. ramosa* from both *D. magna* and *D. longispina*. We test for host range by both assessing spore attachment to the host esophagus (which can be visualized with fluorescent labeled spores Duneau *et al.* 2011) and by exposing animals to spores in infection trials. At high spore doses both methods are perfectly consistent when exposing *D. magna* to

P. ramosa sampled from *D. magna* (Duneau *et al.* 2011). The attachment test determines host-parasite compatibility in one step of the infection process, while infection trials give the outcome of the entire infection process (spore activation, attachment, penetration and proliferation). Spore activation was shown before to be non-specific with regard to host species (Duneau *et al.* 2011). Thus using both tests will allow us to determine if a failure of infection is due to failure of attachment or due to failure of post-attachment steps. We will use the term "native" here as a combination of *Pasteuria* with the *Daphnia* species it was sampled from (and thus naturally infects) and "non-native" as a combination of *Pasteuria* with a host species were it was not sampled from (a novel combination).

Materials and Methods

System description

P. ramosa is an obligate bacterial parasite of *Daphnia* and has a large geographic distribution e.g. infections have been reported from various places in Europe and North America (Ebert 2005). *P. ramosa* castrates its hosts (Ebert *et al.* 2004) and prevalence's are commonly high in natural populations (up to 100% Duncan *et al.* 2006), making it a potentially strong selective agent (Little & Ebert 2000). Its host *Daphnia* are cladocerans that occur in a variety of standing water bodies (e.g. rockpools, ponds, lakes and swamps). Multiple species of *Daphnia* can occupy the same location (De Bernardi & Giussani 1975) and the species *D. magna*, *D. longispina* and *D. pulex* have been found to occur in sympatry in several localities in England (Stirnadel & Ebert 1997), Sweden (Bengtsson 1986), Finland (Ebert *et al.* 2001) and Switzerland (J. Andras unpublished).

Attachment to host esophagus

We tested 1) if the polymorphism for spore attachment that was found for native combinations of *D. magna* and *P. ramosa* (Duneau *et al.* 2011) was also present in native combinations of *D. longispina* and *P. ramosa* and 2) if the polymorphism was conserved between non-native host species that diverged more than 100 million years ago (Colbourne & Hebert 1996).

The attachment-test is described in full detail by Duneau *et al.* (2011). In short, individual hosts were placed singly a 24-well plates in 1 ml of artificial medium (Klüttgen *et al.* 1994 ADaM modified by using only 5% of the recommended selenium dioxide concentration). Twenty thousand fluorescent labeled spores were added to each well and animals were incubated for one hour at room temperature. Attachment was determined by examining exposed *Daphnia* with a Leica fluorescent microscope and checking for the presence of fluorescently labeled spores attached to the esophagus of the transparent animal.

We performed attachment tests on *Daphnia* sampled from rockpools on islands along the Baltic coast of Finland where *P. ramosa* and the three *Daphnia* species, *D. longispina, D. magna* and *D. pulex*, naturally co-occur (Ebert *et al.* 2001). Twenty individuals of 6 *D. pulex*, 28 *D. magna* and 23 *D. longispina* populations were collected from 53 different rockpools located on 21 islands (4 rockpools contained more than one *Daphnia* species). To test consistency of the attachment test within clones, we obtained clonal lineages by collecting asexual offspring produced by a single mother from eight *D. magna*, seven *D. longispina*, eight sexual *D. pulex* (resting eggs are produced sexually) and eight asexual *D. pulex* (resting eggs are produced asexually) from the same metapopulation. Clonal lineages were propagated and maintained under standard laboratory conditions (fed 3x per week with $50 \cdot 10^6$ chemostat cultured *Scenedesmus obliques*, 16h:8h light dark cycle, 20°C, in artificial medium) and four individuals of each host clone were tested. Spore attachment to the esophagus was assessed using *P. ramosa* clone

C14 native to *D. magna* ($C14_{magna}$) and isolate P10 native to *D. longispina* ($P10_{longispina}$) (ten host clones per pond for each *P. ramosa* clone) that were previously collected from Tvärminne Finland where we sampled the majority of our *Daphnia*. $C14_{magna}$ is a single genotype of *P. ramosa* and is described in detail in Luijckx *et al.* (2011). $P10_{longispina}$ is a field isolate (containing possibly more than one genotype) of *P. ramosa* that was obtained by the propagation of spores from 3 infected *D. longispina* individuals (sampled in 2007 on Fyrgrundet island, pond FO-21 near Tvärminne, Finland) in *D. longispina* clone FS-30-1.

We extended our sampling to include *Daphnia* and *Pasteuria* from additional locations to verify the generality of our results. We sampled additional *Daphnia* from Finland (22 clones), England (6), Germany (29), Switzerland (12), France (3), Italy (6), Hungary (1), Iran (5) and Russia (3) (in some of these locations two or more *Daphnia* species co-occur) and obtained clonal lineages that were propagated and maintained under standard laboratory conditions. In addition to *P. ramosa* isolate $P10_{longispina}$ and clone $C14_{magna}$, we tested each host clone, with *P. ramosa* clone C1 from Russia and *P. ramosa* clone C20 from England using four replicates for each host-parasite combination, both are single genotypes of *P. ramosa* native to *D. magna* ($C1_{magna}$ and $C20_{magna}$) and are described in detail in Luijckx *et al.* (2011). In total we tested 612 native and 926 non-native host-parasite combinations with the attachment test.

Infection trials

Attachment of spores to the host esophagus in native combinations of *D. magna* and *P. ramosa* leads to successful infection (Duneau *et al.* 2011). We tested if 1) this holds in native combinations of *D. longispina* and *P. ramosa* and 2) if this holds in non-native combinations. Therefore we performed infection trials with the same or subsets of the same host clones used to determine attachment.

For infection trials, we took *Daphnia* from mass cultures that were kept under standard laboratory conditions. We placed groups of four one week old individuals of the appropriate host clones into separate 100-ml jars containing 20 ml of artificial medium. Jars received a first dose of 100,000 spores from the appropriate *P. ramosa* clone or isolate and a second dose of 100,000 spores the next day. This dose is known to cause 100% infection without lethal effect in *D. magna* (Regoes *et al.* 2003). Spore suspensions were produced by crushing dead infected hosts and assessing the spore density with a hemocytometer. Negative controls received placebo solution (crushed uninfected *Daphnia*). Replicates were randomized within an incubator. A week after exposure, 60 ml of fresh medium was added to each jar, and medium was replaced on a weekly basis thereafter. *Daphnia* were fed $2 \cdot 10^6$ cells per day at the start of the experiment and food levels were increased gradually to $4 \cdot 10^6$ cells per day for *D. pulex* and *D. longispina* and to $5 \cdot 10^6$ cells per day for *D. magna* to accommodate for the increase in food demand of the growing individuals. Dead individuals were recorded every other day, but only those that died 14 or more days after exposure were checked for presence of *P. ramosa* spores. Individuals that died earlier cannot be reliably checked for infection and were excluded from the analysis. Forty days after exposure, *Daphnia* were crushed and checked for presence of *P. ramosa* spores using a phase contrast microscope (400x).When one or more of the four individuals in a jar was infected we considered the replicate susceptible, when none were infected we considered it resistant.

We tested for infection by isolate $P10_{longispina}$ and clone $C14_{magna}$ in host clones of eight *D. magna*, seven *D. longispina*, eight sexual *D. pulex* and eight asexual *D. pulex* used above for determining the polymorphism of attachment (8 replicates per host clone). In addition, we performed infection trials with *P. ramosa* clones $C1_{magna}$, $C14_{magna}$ and $C20_{magna}$

and isolate $P10_{longispina}$ using all host clones from different locations across Europe and Iran where we had previously observed attachment in non-native combinations and a subset in which we had observed no attachment (using either 8 or 10 replicates). In total we tested 20 native and 107 non native host-parasite combinations with infection trials.

Results

Attachment-tests

We observed attachment to the esophagus of both native and non-native host genotypes for all four *P. ramosa* that were tested. We tested *P. ramosa* clone $C14_{magna}$ and isolate $P10_{longispina}$ from Finland in a Finnish meta-population where *D. longispina*, *D. magna* and *D. pulex* occur in sympatry. For both parasites there is substantial within and between population variation for attachment (Figure 1). Isolate $P10_{longispina}$ is able to attach to individuals from 6 of the 23 sampled populations of its native host *D. longispina* and to 3 of the 28 populations of its non-native host *D. magna.* Clone $C14_{magna}$ attached to 16 of the 28 populations of its native host *D. magna* and to 3 of the 6 populations of its non-native host *D. pulex.* We did not observe attachment of isolate $P10_{longispina}$ to non-native host *D. pulex* and clone $C14_{magna}$ to non-native host *D. longispina* (Figure 1).

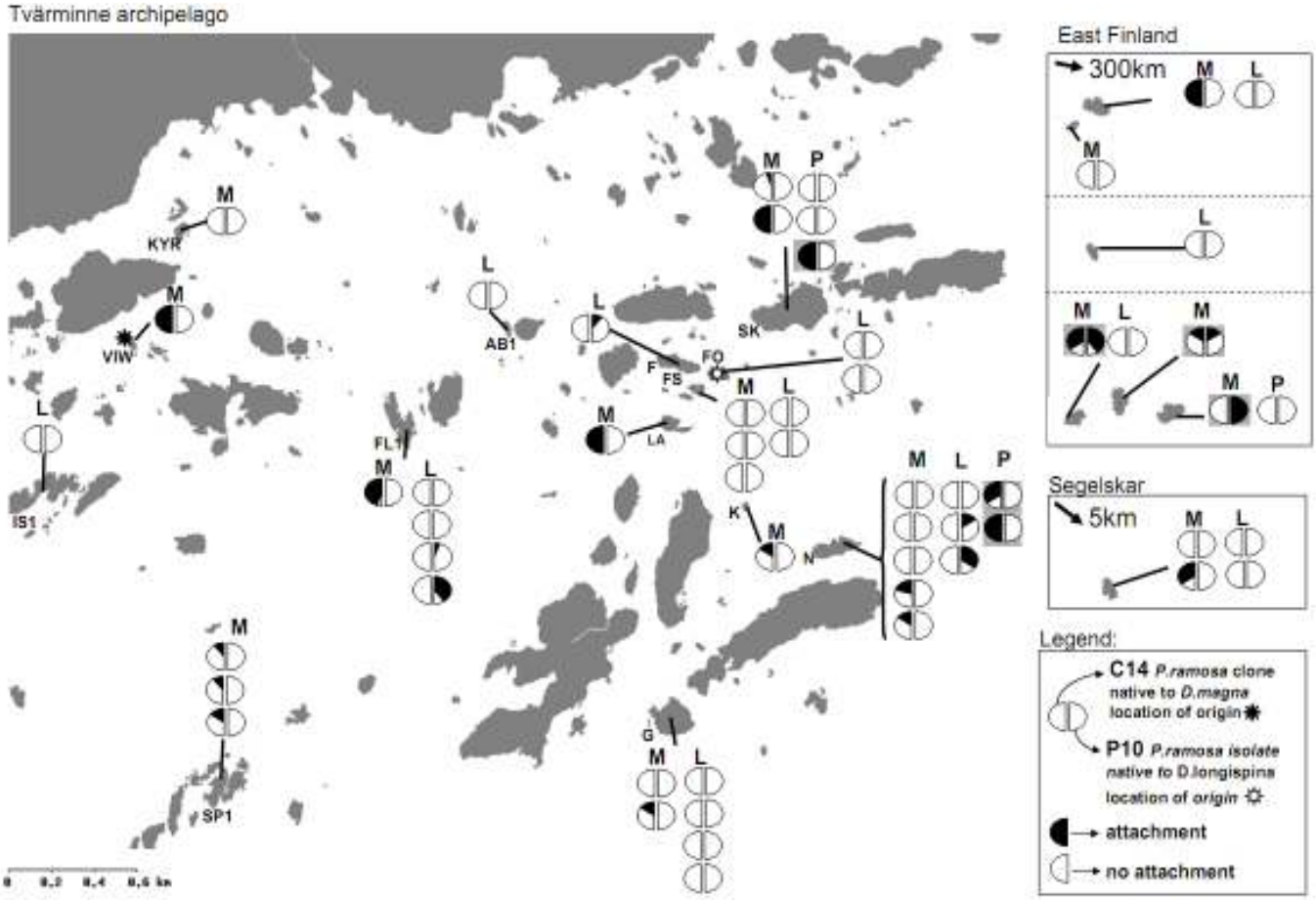

Figure 1: Daphnia *from rockpools of the islands of the Finnish Skerry archipelago, where the species* D. magna, D. longispina *and* D. pulex *co-occur, were tested for attachment of* P. ramosa *spores to the esophagus with two* P. ramosa *that were isolated from the same metapopulation.* P. ramosa *clone C14 native to* D. magna *and the isolate P10 native to* D .longispina. *Spores of C14 attached to individuals of* D. magna *and* D. pulex *but never in* D. longispina. *Spores of P10 attached to* D. longispina *and* D. magna *but never in* D. pulex *(attachment in non-native combinations with a grey box). From each population 10 individuals where tested with C14 and P10. Each population is represented by a circle diagram with on the left half attachment to C14 and on the right P10. Black indicates that attachment was observed, white indicates no attachment. Letters* ***M****,* ***L*** *and* ***P*** *indicate* D. magna, D. longispina *or* D. pulex.

However in our greatly extended sampling with *Daphnia* from several locations and additional *P. ramosa* we found that isolate $P10_{longispina}$ was also able to attach to non-native host *D. pulex* (from France) (Table 1). Clone $C14_{magna}$ never attached to non-native *D. longispina* but did attach to non-native *D. pulex* (from Iran). Clone $C1_{magna}$ showed a similar pattern as clone $C14_{magna}$, able to attach to its native host *D. magna* (from Finland, Italy, Hungary) and non native host *D. pulex* (from Finland and Iran). Attachment of clone $C20_{magna}$ was observed in its native host *D. magna* (from Germany, Italy and Hungary) and non-native hosts *D. longispina* (from Finland) and *D. pulex* (from Finland and Iran). In summary attachment of four lines of *P. ramosa* was observed in all native host species and almost all non-native host species. Attachment was in all cases highly specific for some host genotypes and showed within population variance.

Table 1: Attachment tests with four P. ramosa *on* D. magna D. pulex *and* D. longispina *from different locations. In most locations multiple* Daphnia *genotypes where sampled per pond and in some location multiple ponds where sampled. Numbers represent the number of genotypes where attachment was found / total number of genotypes tested.*

		P.ramosa			
		Native to *D.magna*			Native to *D.longispina*
Species	location (number of ponds)	C1	C14	C20	P10
D.magna	Germany (Stuttgart) (2)	0/6	0/6	**1/6**	0/6
	Germany (Gaarzerfeld)	0/2	0/2	**1/2**	0/2
	Finland (archipelago) (8)	0/1*	**3/8***	0/1*	**1/8***
	Italy (San Rossoro)	**3/3**	**3/3**	**2/3**	0/3
	Russia (Moscow)	0/2	0/2	0/2	0/2
	Switzerland (Winterthur)	0/3	0/3	0/3	0/3
	Hungary (Bogárzó Tó)	**1/1***	**1/1***	**1/1***	0/1*
D.longispina	Germany (Stuttgart)	0/2	0/2	0/2	0/2
	Germany (Gaarzerfeld)	0/4	0/4	0/4	0/4
	Germany (Rümmingen) (3)	0/11	0/11	0/11	0/11
	Germany (Märkt)	0/2	0/2	0/2	0/2
	Finland (archipelago)(17)	0/10*	0/7*	**4/10***	**3/7***
	Italy (San Rossoro)	0/3	0/3	0/3	0/3
	England (Oxford)	0/3	0/3	0/3	0/3
	Switzerland (Winterthur)	0/3	0/3	0/3	0/3
	Switzerland (Belenzona)	0/3	0/3	0/3	0/3
D.pulex	Germany (Stuttgart)	0/2	0/2	0/2	0/2
	Finland (archipelago) (28)	**4/12***	**5/16***	**4/12***	0/16*
	Russia (Moscow)	0/3	0/3	0/3	0/3
	England (Oxford)	0/3	0/3	0/3	0/3
	Switzerland (Winterthur)	0/3	0/3	0/3	0/3
	France (Connaux)	0/3	0/3	0/3	**3/3***
	Iran (Tabriz)	**3/3***	**3/3***	**3/3***	0/3*

*represent cases where infection trials were conducted (with either 8 or 10 replicates). The consistency between attachment and infection only held in native combinations of *Daphnia* and *Pasteuria* (in grey). In non-native host-parasite combinations no infections are observed.

Infection trails

Although isolate $P10_{longispina}$ potentially may have contained multiple genotypes of *P. ramosa* highly specific infection and almost binary resistance, both signatures of infections with a *P. ramosa* clone (Luijckx *et al.* 2011), may suggest that this isolate is composed of a single *P. ramosa* genotype. All *P. ramosa* lines infected only certain genotypes of their native host and failed in all other native and non-native genotypes. The consistency between attachment and infection only holds in native combinations of *Daphnia* and *Pasteuria*. When non-native host-parasite combinations are used attachment is observed but no infection (Table 1&2).

Table 2: C14 from D. magna *and P10 from* D. longispina *were used to perform attachment tests and infection trials on 8 clones of* D. longispina*,* D. magna *and* D. pulex *(both asexual and sexual clones), all from one metapopulation in Finland. Attachment to the esophagus of some genotypes of* D. magna *and* D. pulex *(sexual) was observed for C14 and for P10 in* D. longispina*. Both methods, attachment-tests and infection trials, were perfectly consistent when the parasite was tested on the* Daphnia *species they were isolated from, however none of the non-native host-parasite combinations that showed attachment became infected. All numbers in percentages of exposed hosts. Each cell for the attachment test represent 4 replicates and each cell for the infection trial represents 8 replicates.*

		P.ramosa			
		C14 native to *D.magna*		P10 native to *D.longispina*	
Species	host clone	Attachment	Infection	Attachment	Infection
D.magna	AL1-4-4	**100**	**100**	0	0
	FS-13-c	**100**	**100**	0	0
	FS-26-b	0	0	0	0
	N-16-d	0	0	0	0
	N-44-c	**100**	**100**	0	0
	SP1-2-3	0	0	0	0
	Xinb3	0	0	0	0
	FHS2-11-3	0	0	**100**	0
D.longispina	F-7-a	0	0	0	0
	FS-13-c	0	0	0	0
	FS-30-1	0	0	**100**	**100**
	FS-6-14	0	0	**100**	**88**
	G-102-b	0	0	0	0
	G-122-1	0	0	**100**	**100**
	N-20-d	0	0	0	0
D.pulex (asex)	FU1-57-a	0	0	0	0
	FU2-83-c	0	0	0	0
	M-69-b	0	0	0	0
	SK-39-d	0	0	0	0
	SK-44-b	0	0	0	0
	SK-45-d	0	0	0	0
	SYN-3-d	0	0	0	0
	SYN-6-c	0	0	0	0
D.pulex (sex)	AB2-1-b	0	0	0	0
	KV1-1-d	**100**	0	0	0
	LH-3-c	**100**	0	0	0
	M-60-a	**100**	0	0	0
	N-69-b	**100**	0	0	0
	RO1-3-c	0	0	0	0
	SK-47-a	0	0	0	0
	VIN-4-b	**100**	0	0	0

Discussion

Attachment to the host esophagus, a necessary step in the infection process was found in specific native and non-native host-parasite combinations. Alleles allowing attachment to the esophagus thus seem to be conserved between the different host species that diverged 100 million years ago. However, despite successful attachment, *P. ramosa* lines were never able to infect non-native hosts indicating that *P. ramosa* reported from different host species are different varieties each with a narrow host range.

Attachment of *P. ramosa* is conserved between host species

Attachment to the host esophagus is believed to be responsible for the strong specificity of *Daphnia-Pasteuria* interactions, and is a necessary step for a successful infection (Duneau *et al.* 2011). It was suggested that attachment may be conserved between different host species (Duneau *et al.* 2011). Indeed, here we show that attachment to the host esophagus, although genotype specific within species, is unspecific between host species that diverged more than 100 million years ago (Colbourne & Hebert 1996) (Table 1, 2 & Figure 1). For *P. ramosa* clone $C1_{magna}$ it was demonstrated that the ability to attach to *D. magna* depends on a single locus in the host. The locus is Mendelian and has two alleles, a dominant resistance allele leading to failure of attachment and a recessive susceptibility allele that allows for attachment (**chapter 3**). The alleles that control specific attachment of *P. ramosa* may thus be shared between species of *Daphnia* as evident from attachment of clone $C1_{magna}$ to specific genotypes of *D. pulex* (Table 1) and specific attachment of *P. ramosa* to all three host species (Table 1&2 Figure 1). This suggests that *Daphnia* has an ancient polymorphism for defense against *P. ramosa* which predates the speciation of the subgenus Ctenodaphnia and the subgenus Hyalodaphnia (>100 millions years). Trans-species polymorphisms for defense against parasites have been found in other taxa e.g. vertebrate MHC (> 65 millions years) (Flajnik *et al.* 1999), TRIM5α in Old World monkeys (>8 million years) (Newman *et al.* 2006) and members of the interferon pathway in mice (3 million years) (Ferguson *et al.* 2008). If *Daphnia* indeed has a trans-species polymorphism for defense against *P. ramosa* it may be among the oldest examples ever recorded and the first in an invertebrate system. In addition, one may speculate that parasitism of *P. ramosa* on *Daphnia* is older than 100 million years.

Alternatively a pattern of specific attachment across species could be explained by convergent evolution. Similar to the finding of Ashfield *et al.* (2004), who found that Soybean and *Arabidopsis thaliana* evolved separate resistance genes recognizing the same bacterial avirulence protein (AvrB), the different species of *Daphnia* may have independently evolved mechanisms to prevent attachment of *P. ramosa.* Although we cannot discern between these hypotheses, the ancient polymorphism hypothesis seems more plausible. Balancing selection, the mechanism maintaining polymorphisms, can be generated by negative frequency-dependent selection, selection that varies in space and time and heterozygote advantage (Ferguson *et al.* 2008).

Coevolution by negative frequency dependent selection may occur with specific host parasite genotypic interactions with simple underlying genetics (Clarke 1976). Both strong genotype interactions (Carius *et al.* 2001;Luijckx *et al.* 2011) and simple genetics (**chapter 3**) have been shown for *D. magna* interacting with *P. ramosa* and empirical evidence for frequency dependent selection has been found (Decaestecker *et al.* 2007). If coevolution of *D. longispina* and *D. pulex* with their *P. ramosa* varieties follows similar dynamics, maintenance of ancient alleles in all three species would be possible.

Differences in selection for resistance against *P. ramosa* in time and space are also likely to occur. In the sampled Finnish meta-population that consists of large number of ponds scattered over thousands of islands, *P. ramosa* has been reported only from a few ponds (less than 1% of the *D. magna* population in one study, Ebert *et al.* 2001). Thus, selection for resistance occurs only in few ponds, while in the other ponds selection for resistance is neutral or disadvantageous if resistance is costly. This combined with frequent extinction and recolonization events reported for the host (Pajunen 1986;Altermatt *et al.* 2008) may create different selection regimes in time and space (Thompson 2005). In accordance with this, our data indicates that there can be large differences in resistance profiles of populations on a small geographic scale. In the sampled Finnish metapopulation, fully resistant and susceptible populations occur within small distance from each other (Figure 1).

The explanations above imply that host-parasite coevolution maintains the polymorphism in the host locus involved in attachment, however it is possible that *P. ramosa* interacts with an existing polymorphism that is maintained by another process, not related to host parasite coevolution. To distinguish these hypotheses and to identify the responsible mechanism, it would be powerful to identify the gene(s) responsible for attachment. A Quantitative Trait Locus panel (Routtu *et al.* 2010) has been developed and identification of the gene(s) is in progress.

Pasteuria consist of multiple host varieties

In contrast to a previous field study that suggested that *P. ramosa* was able to infect *D. magna*, *D. longispina* and *D. pulex* Stirnadel & Ebert (1997) we found that three *P. ramosa* clones native to *D. magna* and an isolate native to *D. longispina* showed specificity for certain genotypes of their native host species. Attachment is perfectly consistent with infection in native combinations, but not so in non-native combinations where infection never occurred (Table 1&2). Attachment in non-native hosts without infection indicates that the parasite fails in subsequent steps of the infection process (host penetration or proliferation). One possible explanation is that the Pasteuria is maladapted to the non-native host environment/innate immune system.

If our finding of a narrow host range is generally valid this would imply that *P. ramosa* consists of multiple co-occurring cryptic host varieties, which may explain the results of (Stirnadel & Ebert 1997) who were unable to distinguish between these varieties in their field study but observed clear differences in the epidemiology (Ebert unpublished). Co-occurrence of multiple host varieties is supported by our data from Finland where the three *Daphnia* species live in sympatry (Table 1&2), both *P. ramosa* only infect their native host. Consistent with the newly emerging picture is the finding of a field population with sympatric *D. magna*, *D. pulex* and *D. longispina* were only *D. magna* was infected with *P. ramosa* (J. Andras unpublished). Presence of multiple host varieties have been reported for other parasites e.g. anther smut fungus *Microbotryum violaceum* (Bucheli *et al.* 2000), seabird tick *Ixodes uriae* (Mccoy *et al.* 2001), spiny-headed worm *Leptorhynchoides thecatus* (Steinauer *et al.* 2007). The extreme specificity of *P. ramosa* for just some genotypes within each native host species was found previously for *P. ramosa* infecting *D. magna* (Luijckx *et al.* 2011) and may suggest that each of the different varieties of *P. ramosa* is restricted to some genotypes of a single host species. However cross infection to the South African species *D. dolichocephala*, a Daphnid closely related to *D. magna* (same subgenus), was reported for a *P. ramosa* clone isolated from *D. magna* (Duneau *et al.* 2011). Specificity of host varieties thus needs to be investigated in more detail using a finer phylogenetic scale as used in this study.

Finding of a narrow host range for *P. ramosa* may have implications for the expectations for demographic and evolutionary dynamics in this system as these can depend on the degree of host specialisation (Barrett *et al.* 2008). In comparison with generalists, parasites with a narrow host range are more likely to be locally adapted (Lajeunesse & Forbes 2002). In accordance with this, local adaptation for spore production was suggested for *P. ramosa* infecting *D. magna* (Ebert *et al.* 1998). Parasites restricted to a single host species, which may be the case for some varieties of *P. ramosa*, are expected to experience more frequent local extinction and recolonization which may influence genetic structure and effective population size (Barrett *et al.* 2008). Furthermore, highly specific infectivity of *P. ramosa* for genotypes within a single *Daphnia* species, such as suggested by our results and others (Luijckx *et al.* 2011), may lead to antagonistic coevolution by negative frequency dependent selection believed responsible for maintaining genetic variation and sexual reproduction (Red Queen Theory, Hamilton 1980;Jaenike 1978). Indeed support for frequency dependent selection was found for *D. magna* coevolving with *P. ramosa* (Decaestecker *et al.* 2007).

Finding of a narrow host range may also have implications for ecological phenomena. Varieties of *P. ramosa* may alter the potential for coexistence of *Daphnia* species by parasite mediated competition. For example, the malaria parasite *Plasmodium azurophilum* affects the coexistence of *Anolis* lizards. In areas were the parasite is absent *Anolis gingivinius* outcompetes *Anolis wattsi*, while when it is present it reduces the competitive ability of *Anolis gingivinius* allowing *Anolis wattsi* to coexist (Schall 1992).

Conclusion

Accurate estimates of host range are important, as it plays a critical role in understanding many ecological and evolutionary processes. Our suggestion of multiple host varieties with narrow host range for the *Daphnia* parasite *P. ramosa* may have implications for local adaptation, parasite genetic structure, parasite mediated competition and the outcome of coevolution in this system. In addition, by separating the steps in the infection process, we found that a part of the infection process, the attachment of spores to the host esophagus, seems conserved and polymorphic between host species that diverged 100 million years ago.

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Chapter 3

Resistance to a bacterial parasite in the Crustacean *Daphnia magna* shows Mendelian segregation with dominance.

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Abstract: The influence of host and parasite genetic background on infection outcome in invertebrates is a topic of great interest both because of its pertinence to theoretical issues in evolutionary biology and its relevance to combating devastating human parasites that use invertebrate vectors, such as malaria and schistosomiasis. In the present study we use a classical genetics approach to examine the mode of inheritance of infection outcome in the crustacean *Daphnia magna* when exposed to the bacterial parasite *Pasteuria ramosa*. In contrast to previous studies in this system we use a clone of *P. ramosa*, not field isolates, which allows for a more definitive interpretation of results. We test parental, F1, F2, backcross and selfed parental clones (total 284 genotypes) for susceptibility against a clone of *P. ramosa* using 2 different methods, infection trials and the recently developed attachment-test. We find that *D. magna* clones reliably exhibit either complete resistance or complete susceptibility to *P. ramosa* clone C1 and that resistance is dominant and inherited in a pattern consistent with Mendelian segregation of a single-locus with two alleles. Our results add to the growing body of evidence that resistance to parasites in invertebrates is mostly coded by one or few loci with dominance.

Keywords: Mendelian segregation, resistance, *Daphnia magna*, *Pasteuria ramosa, host, parasite*.

Introduction

The notion that both host and parasite genotypes are key determinants of infection outcome underlies much of the evolutionary theory pertaining to host-parasite interaction. Several models used to analyze the influences on and effect of parasitism explicitly rely on this premise. For example, the Red Queen Theory suggests that host-parasite genotype-genotype interactions with a simple genetic basis are important for the maintenance of genetic variation and genetic recombination in the host (Hamilton 1980; Little & Ebert 2000). Furthermore, host-parasite genotypic interactions have been implicated in other phenomena such as the evolution of virulence (Nowak & May 1994; Grigg & Suzuki 2003) and may be a significant complicating factor in dealing with infectious diseases in humans (Jelinek *et al.* 1999; Read & Taylor 2001; Lambrechts *et al.* 2005).

Substantial data and clear genetic models already exist on host-parasite genotypic interactions in plants (Burdon & Jarosz 1991; Burdon 1994; Chaboudez & Burdon 1995; Allen *et al.* 2004) and significant inroads have been made into unravelling host-parasite genotypic interactions in invertebrates in a number of systems including aphid (*Acyrthosiphon pisum)* and parasitic wasp (*Aphidus ervi*) (Henter & Via 1995), snail (*Bulinus globosus*) and schistosome (Morand *et al.* 1996; Webster & Woolhouse 1998), snail (*Potamopygrus antipodarum*) and trematode (*Microphallus sp.*) (Lively & Dybdahl 2000), bumble bee (*Bombus terrestris*) and trypanosome (*Crithidia bombi*) (Schmid-Hempel *et al.* 1999; Schmid-Hempel & Funk 2004), *Caenorhabditis elegans* and soil bacteria (Schulenburg & Ewbank 2004; Schulenburg &

Muller 2004) and mosquito (*Anopheles gambiae*) and malaria (*Plasmodium falciparum*) (Lambrechts *et al.* 2005). Information on the inheritance of the genotype-genotype interactions is available for very few invertebrate systems. For example, seven main effect QTLs where reported to affect infection intensity of *C. bombi* in *B. terrestris* (Wilfert *et al.* 2007) and the heritability of strain specific resistance has been demonstrated in the *B. globosus* / schistosome system (Webster & Woolhouse 1998). The complexity of the parasite life cycle in many invertebrate systems, which may involve multiple hosts, coupled with the difficulty of isolating either host or parasite clones has put significant hurdles in the way of discovering a clear pattern of inheritance of resistance and susceptibility amongst different combinations of host and parasite genotypes.

A recent study on water flea *Daphnia magna* infected with the castrating bacterial pathogen *Pasteuria ramosa* described extreme genotype-genotype interactions for infectivity (Luijckx *et al.* 2011). With evidence for genotypic interactions, fast acting selection (Little & Ebert 2000) and frequency-dependent selection in natural populations (Decaestecker *et al.* 2007) this host-parasite system has become one of the prime models for antagonistic coevolution. Genetics underlying the genotype-genotype interactions are however unknown. In the present study we use a classical genetic approach to examine the inheritance of resistance in the *D. magna*-*P. ramosa* system.

This research differs from previous work on host-parasite interactions with *D. magna* in several respects (e.g. Little & Ebert 2000; Carius *et al.* 2001; Decaestecker *et al.* 2003; Little *et al.* 2006). First, we employ a clone of *P. ramosa* (single genotype), not field isolates. Field isolates may contain more than one parasite clone (Jensen *et al.* 2006; Mouton *et al.* 2007; Ben-Ami *et al.* 2008; Luijckx *et al.* 2011). The use of *P. ramosa* clones negates the complicating factors intrinsic to mixed infections and allows for a more definitive interpretation of experimental results (Luijckx *et al.* 2011). Second, we use a recently developed assay that assesses host clones for susceptibility by visualizing attachment of the parasite to the host esophagus (part of the gut wall). This allows us to separate the step where the parasite attaches to the host, which is believed to be the key step in *P. ramosa*-*D. magna* coevolution, from the other steps in the infection process (encounter and proliferation within the host). In addition, it allows for higher sample sizes than classical infection trials (Duneau *et al.* 2011). Third, we employ a structured Mendelian approach in which *D. magna* inbred parental clones, a F1 clone, an array of F2 clones, an array of backcrossed clones and selfed parental clones are used to resolve the genetic pattern of inheritance underlying susceptibility. A further understanding of the genetics of this model system will greatly enhance its use in explaining the factors involved in and the evolutionary implications of host-parasite genotypic interactions.

Materials And Methods

Study system

Daphnia magna Straus is a cyclical parthenogenetic, freshwater cladoceran, found in rock pools, small ponds and medium sized lakes. *Pasteuria ramosa* is a bacterial endoparasite of *Daphnia magna* (Ebert *et al.* 1996; Ebert 2005). Transmission occurs when hosts ingest waterborne spores which attach to the esophagus, penetrate and subsequently cause infection (Duneau *et al.* 2011). During the infection millions of spores fill the host body cavity; upon death the spores are released from the decaying cadaver and the cycle begins anew (Ebert *et al.* 1996). Infection also results in castration, which often occurs before the production of any offspring and therefore entails severe fitness consequences for the host (Ebert *et al.* 2004).

Host material

Two *D. magna* individuals were collected from separate rock pools near the Tvärminne Zoological Station in Southern Finland. These rock pools are part of a large *D. magna* metapopulation. The females were cloned (iso-female lines) under standard conditions, (20 ± 0.5°C, 16h:8h light:dark cycle and fed with chemostat-cultured algae *Scenedesmus obliquus*), and then were each selfed (sexual reproduction between clonal male and female offspring) over three generations to create the parental clones used in our study: Fainb3 and Xinb3. The parental clones were crossed and one F1 clone was selfed to create 71 F2 clones. In addition, the F1 clone was backcrossed to parental Fainb3 to create 164 backcrossed clones. Finally, both parent clones (Xinb3 and Fainb3) were selfed. We obtained 22 and 24 offspring clones for selfed Fainb3 and selfed Xinb3, respectively (Figure 1).

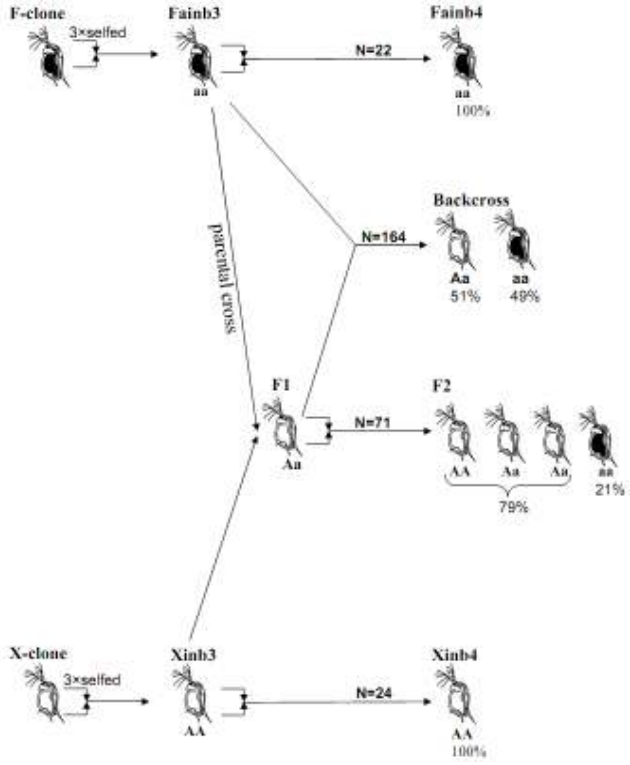
The cross to obtain F1 was performed by placing multiple individuals from both parent clones together in 400-ml beakers filled with artificial medium (ADaM, Klüttgen *et al.* 1994 modified as per Ebert *et al.* 1998). Beakers were filled to 90% of their maximum capacity unless otherwise stated. Epiphia containing the sexually produced eggs were removed as they appeared, stored in moist, dark conditions at 4°C for up to six months and then dried on filter paper for up to three weeks. Ephippia were then submerged in bleach (5% aqueous solution) for about 5 minutes to facilitate the hatching process, washed and placed in 400-ml beakers with medium under strong fluorescent artificial day light. After hatching of sexual eggs, microsatellite markers were used to distinguish hybrid clones from selfed clones (Colson *et al.* 2009). One hybrid was randomly picked to become the F1 and subsequently selfed to create the F2 clones.

A similar protocol was used to self the F1 and the two parent clones, except that genotyping in these crosses was not necessary. For the backcross, we used a slightly altered approach, as many microsatellites would have been needed to reliably distinguish selfed from outcrossed *Daphnia*. Fourteen day old virgin females of the F1 and males of Fainb3 were placed in 1000-ml beakers filled with medium, each three days parthenogenetic offspring were removed to prevent selfing of females with their sons, thereby ensuring that all offspring were outcrossed.

Parasite material

We used spores from *P. ramosa* clone C1 for our infection trials which, as other *P. ramosa* clones, shows highly specific infectivity (Luijckx *et al.* 2011). Spore suspensions were created by homogenizing *D. magna* with late stage infections in water. Spore concentration in each suspension was determined using a haemocytometer.

Figure 1: Pedigree showing the D. magna *crossing scheme and the resistance/ susceptibility profiles to* P. ramosa *for parental, F1, backcross and F2 host clones. Black* Daphnia *indicate susceptibility and unfilled* Daphnia *indicate resistance. In our proposed model of inheritance, resistance to* P. ramosa *clone C1 is conferred by a dominant allele, "A". Susceptibility by a recessive allele, "a".*



Tests for susceptibility

We employed two different techniques to determine host susceptibility: the attachment-test and infection trials. The attachment-test is a recently developed technique that employs fluorescent microscopy to assess the ability of *P. ramosa* spores to attach to the esophagus of *Daphnia*. Attachment perfectly correlates with susceptibility of the *Daphnia* host in infection trials (Duneau *et al.* 2011). Infection trials reflect the outcome of the entire infection process (encounter, attachment, penetration and proliferation within the host while the attachment-test only looks at one step in this process, the attachment step. Susceptibility of parental, F1 and F2 *Daphnia* clones was determined with both methods (numbers of tested clones differed between methods due to loss of some host clones before the end of all tests). Susceptibility in the backcross was determined with the attachment-test, with a representative subset of host clones tested with infection trials. Susceptibility of selfed parents was only tested using the attachment-test. Both assays agreed very well with each other, although variation between replicates in the infection trails was greater.

Infection trials

To remove maternal effects, mothers of the F1 and F2 were kept singly in 100-ml beakers filled with medium under standard conditions for three asexual generations prior to the start of the infection trials. For the backcross, this was reduced to one asexual generation. For infection trials, we used female *D. magna* of 1 to 4 days old at the time of parasite exposure and all were offspring from the third or later clutch. Juveniles were exposed to spores by placing them singly in 100-ml beakers filled with 20 ml of medium and adding spore suspensions containing 200,000 spores of *P. ramosa* clone C1. This dose is known to cause 100% infections without lethal effect in susceptible hosts (Regoes *et al.* 2003). Individual *D. magna* remained in 20 ml of medium for 4 days, at which point the beakers were filled up. For parental clones and the F1, we utilized a split-brood design with eight individuals from different mothers in separate 100-ml beakers with medium under standard conditions for both the treatment and the control. The medium was changed at day 7 of the experiment, and twice weekly for the following 23 days. *D. magna* were fed 3 x 10^5 cells/ml algae cells daily throughout the experiment. The F2 clones were tested using the same protocol with the following exceptions. We used four replicates per clone and to accommodate the increasing food demand of the growing animals, feeding was raised from 3 x 10^5 cells/ml to algae 6 x 10^5 cells/ml during the experiment. For the backcross, we tested 40 of the 164 clones using four randomly chosen juveniles (one to four days old) and fed 3 x 10^5 cells/ml algae daily at the start of the experiment and 5 x 10^5 cells/ml towards the end. All infection trials lasted 30 days. Mortality was monitored at least once every 48 hours. All animals dying after day 12 and those surviving till the end of the experiment were tested for infections by checking for parasite spores using phase-contrast microscopy (magnification 400x). Individuals dying before day 12 of the experiment were not assessed because detection of *P. ramosa* infection is unreliable during the early stage of infection.

Attachment-test

The attachment-test is described in full Duneau *et al.* (2011). In short, for assessment of susceptibility with the attachment-test four individuals of each clone were taken from stock cultures and placed singly in 24-well plates in 1 ml of medium. Twenty thousand fluorescent labelled spores and contrast dye were added to each well and plates were incubated for one hour at room temperature. Attachment was determined by examining exposed *Daphnia* with a fluorescent microscope and checking for the presence of fluorescently labelled spores on the *Daphnia* esophagus.

Results

We found a strong binary pattern of resistance, all or all but one replicate of susceptible clones became infected in infection trials while resistant clones never became infected. Three host clones from the backcross were an exception, in these only one of the replicates was infected during the infection trial. The attachment-test gave more consistent results, each host clone displayed either complete resistance or susceptibility.

Parental clones showed contrasting susceptibility, Fainb3 was susceptible while Xinb3 was resistant to *P. ramosa* C1. Parents were likely homozygous at the relevant loci due to three generations of selfing and indeed offspring of the selfed parents showed identical phenotypes as their parents and no segregation (Figure 1, Table 1). The F1 clone resembled Xinb3 and was resistant. The pattern for resistance in the F2 (56 resistant (79%) and 15 susceptible (21%), combined data from infection trial and attachment-test, Table 1), was not significantly different from the 3:1 pattern expected if resistance is determined by a dominant, single-locus trait exhibiting Mendelian segregation (Fisher's exact test p = 0.69). The single locus model with dominant inheritance of resistance was confirmed by the backcross in which 84 clones were resistant (51%) and 80 susceptible (49%) which was not significantly different from the expected 50:50 (Fisher's exact test p = 0.91, Figure 1, Table 1)

Discussion

By using two different methods to test for susceptibility, we show that the attachment to the host esophagus, an important step of the infection process, is controlled by single host locus. Alleles at this locus segregate in a Mendelian pattern and our crosses revealed the presence of two alleles, a dominant allele "A" preventing attachment of *P. ramosa* C1 and a recessive allele "a" allowing for attachment (Figure 1). Hosts thus show binary resistance patterns either being resistant (no attachment) or susceptible (attachment). Attachment to the host esophagus is responsible for the strong genotype-genotype interactions in the *D. magna-P. ramosa* system (Luijckx *et al.* 2011; Duneau *et al.* 2011). The finding of a single host locus controlling susceptibility to *P. ramosa* C1 thus suggests that these interactions might have a simple genetic basis. Genotypic interactions with a simple genetic basis may lead to coevolution cycles (Clarke 1976) suggested to be important for maintenance of genetic variation and recombination (Hamilton 1980;Jaenike 1978).

Binary resistance of *D. magna* clones to *P. ramosa* clones was found previously (Luijckx *et al.* 2011) and is related to the way the parasite enters the host (Duneau *et al.* 2011). During filter feeding spores attach to the esophagus of susceptible *Daphnia* and penetrate the gut wall. In resistant *Daphnia* no attachment is observed.

Table 1: Data from attachment-test and infection trials. The table shows the total number of clones tested per host clone or cross and the percentage of these clones that were either susceptible (susc.) or resistant (resist.) P-value, Fisher's exact test between number of observed and expected. - for not assessed, NA for not applicable.

Crosses or clones		total number	observed (%)						expected (%)		
			Attachment-test		infection trial		both methods		under one locus with dominance		
Name	Details		susc.	resist.	susc.	resist.	susc.	resist.	susc.	resist.	P-value
Xinb3	parental, 3×selfed X-clone	1	0	100	0	100	0	100	NA	NA	NA
Fainb3	parental, 3×selfed Fa-clone	1	100	0	100	0	100	0	NA	NA	NA
F1	Xinb3×Fainb3	1	0	100	0	100	0	100	0	100	p= 1
F2	selfed F1	71[a]	19	81	20	80	21	79	25	75	p= 0.69
Backcross	F1×Fainb3	164[b]	49	51	53	47	49	51	50	50	p= 0.91
Xinb4	selfed Xinb3	24	0	100	-	-	0	100	0	100	p= 1
Fainb4	selfed Fainb3	22	100	0	-	-	100	0	100	0	p= 1

a) Number of tested clones differed between attachment-test (57), infection trials (60) and the total amount of tested clones (71)
b) For infection trials a subset of 40 (38 survived the experiment) randomly selected clones was used.

The specificity of attachment is dependent on the genotype of both host and parasite and is not influenced by environmental effects (Duneau *et al.* 2011). Indeed, we found low variability within *Daphnia* genotypes in both infection trials and the attachment-test. Both assays agreed very well with each other, although variability between replicates within clones in infection trials was greater. The difference may be explained by outcome of the attachment test being only determined by the attachment step while the outcome of the infection trials is influenced by the entire infection process, including, encounter, attachment-step and proliferation within the host (Duneau *et al.* 2011). Contrary to the attachment-step the entire infection process has been shown to be influenced by environmental (Vale *et al.* 2008) and maternal effects (Ben-Ami *et al.* 2010), which may explain the greater variability found in our infection trials. It is likely that other loci than the one described here are involved in other steps of the infection process. For example encounter of the parasite spores, which reside in the sediment, may be depended on diel vertical migration which has been shown to have a strong genetic component (Decaestecker *et al.* 2002). It has also been suggested that genes affecting proliferation within the host might be different from those involved in attachment (Decaestecker *et al.* 2007; Duneau *et al.* 2011). Even though more loci might be involved in the entire infection process, the locus described here appears to be the major determinant of susceptibility to *P. ramosa* C1 and is involved in the attachment of the parasite to the host esophagus, which is a key step in *D. magna*-*P. ramosa* coevolution (Duneau *et al.* 2011).

Our interpretation that resistance is coded by a single dominant locus is partially consistent with previous studies on the genetic basis of resistance in *D. magna*. A pervious study speculated that resistance is due to one or a few loci (Little *et al.* 2006). However small sample size and use of *P. ramosa* isolates (not clones) in this earlier study makes comparison difficult. In other invertebrate-parasite systems the majority of resistance genes described today tend to be dominant and autosomal, examples include: *Drosophila*-parasitic Wasp (Carton *et al.* 1992); mosquito-malaria (Thathy *et al.* 1994); and snail-*Schistosoma* (Richards 1975; Knight *et al.* 1999; Lewis *et al.* 2003) with an exception being the sex-linked, recessive gene controlling mosquito resistance to filarial worms (Macdonald 1962; Macdonald & Ramachan 1965) (for review of invertebrate resistance see Carton *et al.* 2005).

P. ramosa clones not tested in this study also require attachment to the host esophagus for successful infection (Duneau *et al.* 2011) and all host genotypes tested with *P. ramosa* clones show binary resistance (Luijckx *et al.* 2011). Conservation of the infection mechanism among different parasite genotypes and similar phenotypic patterns of susceptibility of the host for other parasite genotypes suggests that our findings of a simple genetic mechanism conferring resistance to *P. ramosa* may also apply to untested *D. magna* and *P. ramosa* genotypes. Resistance to other *P. ramosa* genotypes may be conferred by additional alleles on the found locus or by additional loci possibly similar to the well described Gene-For-Gene resistance of plants (Keen 1990).

The finding of a simple genetic basis of host resistance has important implications for the outcome of host-parasite coevolution. Negative frequency-dependent selection may occur in presence of host-parasite genotype-genotype interactions with simple underlying genetics (Clarke 1976). Strong genotypic interactions were already described in the *Daphnia-Pasteuria* system by Luijckx et al. (2011) and their data shows that the locus described here only confers resistance to specific *P. ramosa* genotypes; the resistant parent (xinb3) was susceptible to three of the five tested *P. ramosa* genotypes while other host genotypes were resistant to these *P. ramosa*. A simple genetic basis for genotype-genotype interactions supports earlier findings of negative

frequency-dependent selection for infectivity in the *D. magna-P. ramosa* system (Decaestecker *et al.* 2007). Which may be important for the maintenance of genetic variation and the evolution of recombination as suggested by the Red Queen Theory (Hamilton 1980;Jaenike 1978).

Many theoretical models used to predict host-parasite coevolution assume simple genetics with binary resistance patterns (Salathe *et al.* 2008 and references therein) while data from empirical studies suggests that patterns are more quantitative (e.g. Schulenburg & Ewbank 2004). In addition, these models also (often) assume that parasites are highly specific to given host genotypes. We show that in one step of the infection process a single locus is responsible for binary resistance. Furthermore resistance is highly specific for the here tested *P. ramosa* genotype. Our results thus suggests that theoretical models for host-parasite coevolution may, in some cases, not be over-simplified and hold promise for understanding and interpreting empirical results.

Our results add to the experimental power of the *D. magna-P. ramosa* model system as a tool for understanding the evolution of host-parasite interactions. Furthermore, the isolation of more *P. ramosa* clones and additional crosses between host clones will allow for the creation of a *D. magna-P. ramosa* interaction matrix in which infectivity profiles can be determined by pair-wise matching of *D. magna* and *P. ramosa* genotypes. Through competition experiments, use of natural populations, and the development of a Quantitative Trait Locus panel (Routtu *et al.* 2010), this matrix has the possibility to serve as a powerful tool for testing evolutionary models.

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Chapter 4

Empirical evidence for specificity in host-parasite interactions consistent with a matching allele model

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The maintenance of genetic variation has long puzzled evolutionary biologists (Clarke 1976;Haldane 1949;Bergelson *et al.* 2001;Andrés 2011). A popular theory suggests that antagonistic coevolution between hosts and parasites may give an advantage to rare hosts, thus preserving genetic variation at disease loci (Berenos *et al.* 2011;Hamilton 1993). For this rare-host advantage to occur, parasites should infect specific host genotypes, and hosts should be able to resist specific parasite genotypes. The most prominently used genetic models capturing this specificity are matching allele models, which readily lead to selection in favour of rare genotypes (Frank 1993;Salathe *et al.* 2008;Dybdahl & Storfer 2003;Otto & Michalakis 1998;Hamilton 1993), but remain without empirical support from genetic studies. Here, we show that resistance of *Daphnia magna* against its bacterial parasite *Pasteuria ramosa* is determined by a highly specific match between the genotypes of the antagonists. Genetic crosses suggest that resistance against two parasite genotypes is coded for by a single locus with Mendelian segregation. A dominant allele provides resistance against one genotype of the parasite, but leads to susceptibility against another genotype. As envisioned by matching allele models, a second allele is recessive and shows the inverse effect. Double resistant hosts are never observed. In theory, genetic variation at this locus can be maintained endlessly. Antagonistic coevolution based on matching allele models have been widely used to explain the maintenance of genetic variation and sexual recombination, the so called Red Queen Theory (Salathe *et al.* 2008;Otto & Nuismer 2004;Lively 2010;Agrawal 2009;Otto & Michalakis 1998). Widespread genetic variation for resistance as well as rapid antagonistic coevolution had been reported earlier in the *Daphnia-Pasteuria* system, but without the knowledge of the underlying genetics (Decaestecker *et al.* 2007;Ebert *et al.* 1998;Carius *et al.* 2001).

Characteristic of matching allele models is that infection only occurs in a specific match between host and parasite genotypes. A parasite can only infect when its genotype matches those of its host and a host can only resist the parasite when its genotype matches those of the parasite. Although models have implemented forms of matching of hosts and parasite in different ways e.g. inverse matching and matching (Dybdahl & Storfer 2003;Frank 1993;Otto & Michalakis 1998), variable number of matching loci and variable number of alleles per locus (Lively & Howard 1994;Frank 1993;Hamilton *et al.* 1990;Gandon *et al.* 1996) and different ploidy levels of hosts and parasites (Agrawal 2009;Otto & Nuismer 2004;Hamilton 1993), all models have in common that there is no host that can resist all parasites and there is no parasite that can infect all hosts. Under such conditions parasites evolve to predominantly infect common hosts leading to a competitive advantage for rare host genotypes. The absence of both generally resistant host genotypes and generally virulent parasite genotypes prevents alleles to reach fixation and genetic variation in both antagonists is maintained.

The genetic basis of specific resistance is well understood for a number of plant-pathogen systems, but their genotypic interactions do not fit with a matching allele model due to presence of universally infective parasites (Parker 1994). For animal-parasite systems, interactions between arbitrarily chosen host and parasite genotypes have been reported in a number of studies (Henter & Via 1995;Schulenburg & Ewbank 2004;Rauch *et al.* 2006) but little is known about the underlying genetics and it is unclear if these are congruent with a matching allele model. The same is true for the here studied *Daphnia magna* and its parasite *Pasteuria ramosa*. This system shows substantial genetic variation for parasite infectivity and host resistance in natural populations, with only specific parasite genotypes being able to infect specific host genotypes (Carius *et al.* 2001;Luijckx *et al.* 2011). Resistance shows a binary pattern, with *Daphnia* genotypes being either fully resistant or susceptible (Luijckx *et al.* 2011), which is predominantly determined by the success or failure of attachment of the parasite spores to the host esophagus. Attachment is believed to be based on a recognition process between host and parasite (Duneau *et al.* 2011). The genetic basis of the genotypic interactions has been suggested to be simple (**Chapter 3**;Little *et al.* 2006). Here we present an in depth breeding study aimed to uncover the genetic basis of host resistance to multiple parasite genotypes.

Daphnia are cyclic parthenogens, allowing for genetic crosses to be performed and recombinant *Daphnia* to be maintained as clonal lineages. Two *Daphnia* genotypes (Fa and X) from a rockpool metapopulation in Southwestern-Finland were used to create a set of crosses (Figure 1) that were tested for resistance against two genotypes of *P. ramosa* (C1 & C19). The Fa genotype is resistant to *Pasteuria* clone C19 and susceptible to C1, while the opposite was the case for the X genotype (Table 1). Both parental *Daphnia* were selfed for three generations (resulting in genotypes Fainb3 & Xinb3) after which they were crossed to produce a F1 offspring that was selfed to produce 68 F2 offspring. The F1 offspring was backcrossed to one of its parents (Fainb3) to produce 164 BackCross offspring. Ten of these cloned BackCross offspring and both parents were selfed and two selected pairs of BackCross hosts were crossed (heterozygote crossed with recessive homozygote). In total 820 recombinant genotypes were cloned (isofemale lines), maintained in the laboratory and tested for resistance using two methods, infection trials and spore attachment-tests. The latter test assesses the attachment of *P. ramosa* spores to the host esophagus with the use of fluorescently labeled spores (Duneau *et al.* 2011). Both assays have been shown to give congruent results (Duneau *et al.* 2011).

Our results allowed us to build a genetic model with one resistance/susceptibility locus with 3 alleles (Table 1 & Figure 1). The parents of our crosses were differentially susceptible to *P. ramosa* genotypes C1 and C19, and we observed Mendelian segregation of resistance against C1 and C19 in two separate crosses (F2 & Fainb4). In both cases resistance was dominant and susceptibility recessive. A backcross revealed that individuals where either susceptible to both *P. ramosa* clones (26%), C1 susceptible and C19 resistant (23%) or C1 resistant and C19 susceptible (51%). The absence of individuals resistant to both *P. ramosa* genotypes excluded the possibility that resistance was coded for by two independent Mendelian segregating loci. Segregation patterns of selfed individuals from the backcross confirmed resistance to both *P. ramosa* genotypes to be coded for by the same locus. Results indicated that the allele (**x**) which provides resistance against C1 but susceptibility to C19, was dominant over the allele (**y**) that provides resistance against C19 and susceptibility to C1. Furthermore, both resistance alleles (**x** & **y**) were dominant over a third allele, which did not confer resistance to the either *P. ramosa* C1 or C19.

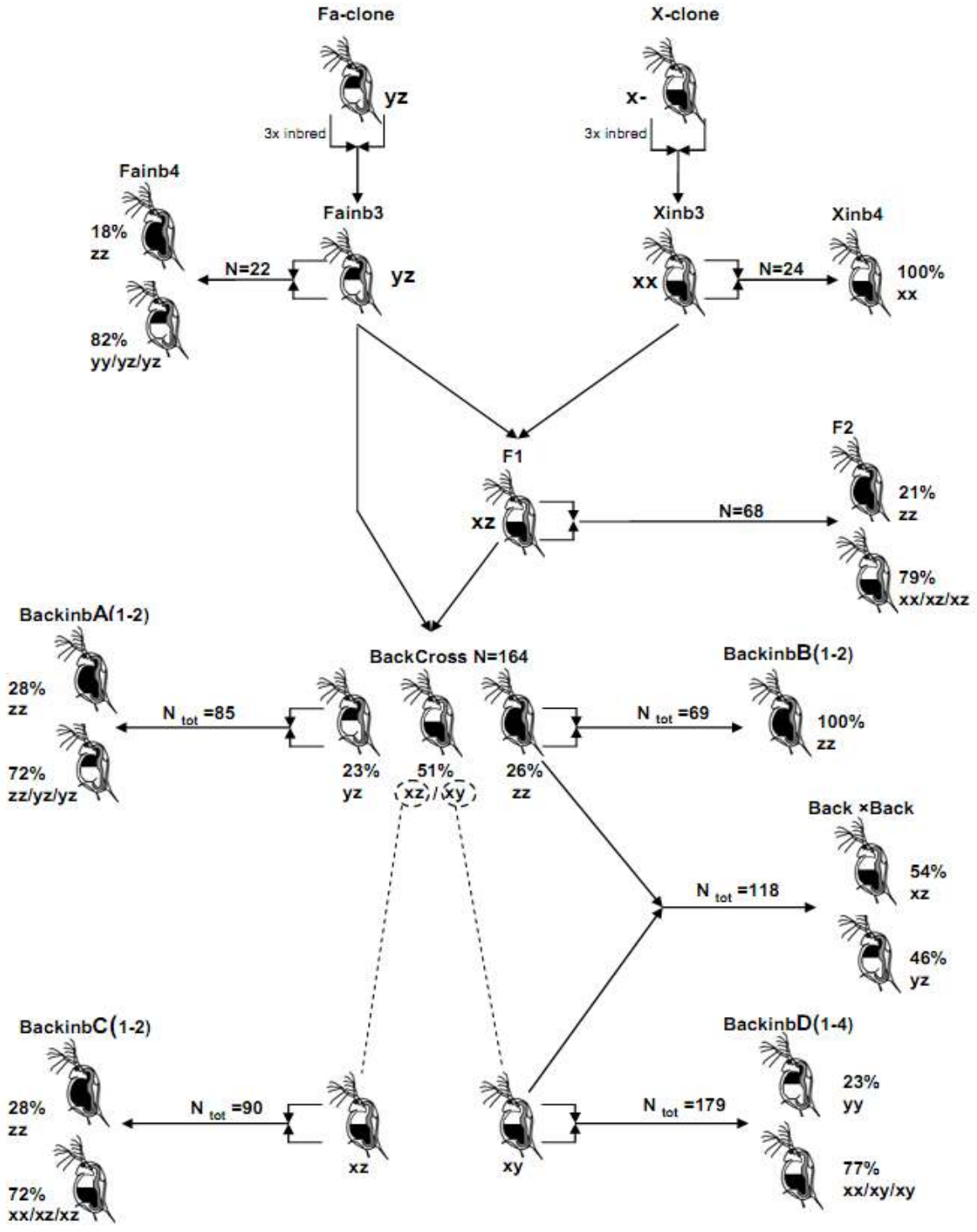

Figure 1: *Pedigree showing the crossing schedule and resistance profiles of* Daphnia magna *genotypes for two* Pasteuria ramosa *genotypes (C1 & C19).* **N** *represents the total number of recombinants tested and percentages reflect segregation patterns. Letters e.g.* **xx**, ***xy*** *represent the genotypes under the hypothesis of one locus with three alleles and an allele hierarchy* ***x***>***y***>***z***.

Phenotypes: *C1 & C19 susceptible*

C1 susceptible and C19 resistant

C1 resistant and C19 susceptible

Our interpretation that resistance is coded by a single locus with an allele hierarchy (**x**>**y**>**z**) is consistent with the results from a previous study which speculated that resistance of *D. magna* against two isolates of *P. ramosa* was dominant for one and recessive for the other isolate (Little *et al.* 2006). Dominance hierarchies have been found in other systems such as self incompatibility in plants (Schierup *et al.* 1997) and color morphs in insects (Cordero 1990). An alternative, but more complex, explanation for our results is based on two closely linked diallelic loci with interlocus epistasis (see Appendix 1).

Under the 1 and the 2-locus genetic models the same host genotype is either resistant or susceptible depending on the genotype of the parasite and infection/resistance only occurs in specific combinations of host alleles and parasite genotypes (table 2), which are trademarks of a matching allele model. Although we cannot exclude that we did not sample parasites that are universally infective or hosts that are universally resistant as envisioned by (inverse) gene-for-gene models (Fenton *et al.* 2009;Thompson & Burdon 1992), previous data from the *Daphnia-Pasteuria* system suggest that these either do not occur in this system or are rare. Studies testing large numbers of host and parasite genotypes found no host clone being resistant to all parasites and no parasite isolate being most infective in all host clones (Carius *et al.* 2001;Decaestecker *et al.* 2003;Ebert 2008). Furthermore, time series using archived *D. magna* and *P. ramosa* resting stages from sediment cores, did not observe any sweeping events associated with universally infective parasites or universally resistant host genotypes (Decaestecker *et al.* 2007).

Table 2: The Daphnia-Pasteuria *genetics matrix. Our results are congruent with a 1-locus matching genotype model with dominance. As envisioned under such models the same host genotype is either resistant or susceptible depending on the genotype of the parasite and infection/resistance only occurs in specific combinations of host alleles and parasite genotypes. (The z allele does not confer resistance and thus will likely be selected against and lost, unless it codes for resistance against an untested* P. ramosa *genotype.)*

	Parasite genotype	
Host genotype	C1	C19
xx	**resistant**	susceptible
xy	**resistant**	susceptible
xz	**resistant**	susceptible
yy	susceptible	**resistant**
yz	susceptible	**resistant**
zz	susceptible	susceptible

*Table 1: Crossing details. The table shows the total number of host clones tested per cross (N) and the percentage of these clones that where either susceptible (sus.) or resistant (res). P-value represents Fisher's exact test between number of observed and expected under the hypothesis that resistance is coded for by one locus with three alleles with an allele hierarchy **x**>**y**>**z**.*

				Observed/Expected (in %)				
Cross	**Suggested genotype**	**Name**	**N**	C1: / C19:	sus. / sus.	sus. / res.	res. / sus.	res. / res.	**P-value**
3 × selfed X	xx	Xinb3	1		0/-	0/-	100/-	0/-	-
3 × selfed Fa	yz	Fainb3	1		0/-	100/-	0/-	0/-	-
selfed Xinb3	xx • xx	Xinb4	24		0/0	0/0	100/100	0/0	P=1.00
selfed Fainb3	yz • yz	Fainb4	22		18/25	82/75	0/0	0/0	P=0.72
Xinb3×Fainb3	xx • yz	F1	1		0/0	0/0	100/100	0/0	-
Selfed F1	xz • xz	F2	68		21/25	0/0	79/75	0/0	P=0.69
F1×Fainb3	xz • yz	BackCross	164		23/25	26/25	51/50	0/0	P=0.94
selfed BackCross**A**	yz • yz	Backinb**A**-1	38		21/25	79/75	0/0	0/0	P=0.79
selfed BackCross**A**	yz • yz	Backinb**A**-2	47		17/25	83/75	0/0	0/0	P=0.45
selfed BackCross**B**	zz • zz	Backinb**B**-1	30		100/100	0/0	0/0	0/0	P=1.00
selfed BackCross**B**	zz • zz	Backinb**B**-2	39		100/100	0/0	0/0	0/0	P=1.00
selfed BackCross**C**	xz • xz	Backinb**C**-1	26		19/25	0/0	81/75	0/0	P=0.74
selfed BackCross**C**	xz • xz	Backinb**C**-2	64		31/25	0/0	69/75	0/0	P=0.56
selfed BackCross**D**	xy • xy	Backinb**D**-1	35		0/0	17/25	83/75	0/0	P=0.56
selfed BackCross**D**	xy • xy	Backinb**D**-2	68		0/0	25/25	75/75	0/0	P=1.00
selfed BackCross**D**	xy • xy	Backinb**D**-3	49		0/0	22/25	78/75	0/0	P=1.00
selfed BackCross**D**	xy • xy	Backinb**D**-4	27		0/0	30/25	70/75	0/0	P=1.00
BackCross×BackCross	zz • xy	Back×Back-1	62		0/0	45/50	55/50	0/0	P=0.85
BackCross×BackCross	zz • xy	Back×Back-2	56		0/0	46/50	54/50	0/0	P=0.72
			822						

Under a matching allele model infection is based on a recognition process between host and parasites. In the *Daphnia-Pasteuria* system recognition is believed to occur when ingested spores attach to the esophagus of susceptible hosts. Upon successful attachment *P. ramosa* enters the host and proliferates in the hemolymph and muscle (Duneau *et al.* 2011). A putative explanation consistent with our results and others (Duneau *et al.* 2011) is that geneproducts of the loci/locus described here prevent attachment of *P. ramosa* to the host esophagus either by actively disrupting adherence of *P. ramosa* spores or by blocking target receptors. Such a mechanism would fit with the family of matching allele models which are based on the ability of the host to recognize and resist attack by parasites (e.g. Frank 1993;Gandon *et al.* 1996).

This study on host resistance genetics in the *Daphnia-Pasteuria* system provides the first empirical support for a matching allele model. Models of this family have played a central role in the theoretical development of antagonistic coevolution and where shown to readily lead to negative frequency dependent selection and thus to the maintenance of genetic variation (Sardanyes & Sole 2008;Frank 1993;Hamilton 1993;Clarke 1976), a pertinent issue in evolutionary biology. Indeed, the *Daphnia-Pasteuria* system is one of the few systems with empirical evidence for rapid antagonistic coevolution, and a parameterized simulation model with a matching allele assumption supported the interpretation that coevolution works by negative frequency dependent selection (Decaestecker *et al.* 2007). Further support for frequency dependent selection comes from the observation of high within population variation for resistance (Carius *et al.* 2001), but low among population variance (Ebert *et al.* 1998) which is consistent with expectations for traits experiencing such a selection regime (Schierup *et al.* 2000). In addition, dominance hierarchies, as found in this study have been shown to be associated with maintenance of polymorphism in traits such as self incompatibility in plants (Schierup *et al.* 1997) and color morphs in insects (Cordero 1992;Cordero 1990). Besides theoretical work pertaining the maintenance of genetic variation matching allele models have been used extensively in simulations to test if host-parasite coevolution can explain the widespread occurrence of sex and recombination (Salathe *et al.* 2008;Otto & Nuismer 2004;Lively 2010;Agrawal 2009;Otto & Michalakis 1998). Invariably, matching allele models were able to explain the maintenance of sex better than alternative host-parasite genotype matrices were able to do.

Our results suggest that findings from theoretical models, which use a matching allele assumption hold promise for understanding and interpreting empirical results in the *Daphnia-Pasteuria* system. These organisms can now be used to empirically test predictions of matching allele models; most prominently aspects of the Red Queen Theory such as negative frequency dependent selection, the maintenance of genetic variation and the notion that antagonistic coevolution may favor genetic mixing.

Method summary

Details for crosses, infection trials and attachment-tests can be found in (**Chapter 3**), where assays with *P. ramosa* genotypes C19 were similar to C1; protocols for the selfed backcross were identical to that of the selfed parents; and protocols for crosses between two pairs of the backcross were identical to that of the backcross. In short, selfed lineages were created by collecting sexual resting eggs from laboratory populations of their respective parent clone. For the creation of the F1 offspring we collected sexual resting eggs from a mixed laboratory population of both parents and used microsatellites to distinguish between selfed and outcrossed offspring. For the other crosses we used a slightly different approach, as many microsatellites would have been needed to distinguish between selfed and outcrossed

offspring. We placed virgin females of one parent together with males of the other and removed all parthenogenetic offspring to prevent selfing. All sexual resting eggs were removed as they appeared, dried, hatched and maintained as clonal lineages in the laboratory. Four individuals of each recombinant lineage were tested with the attachment test. This test visualizes the attachment of the spores to the host esophagus by using fluorescent labeled spores and correlates perfectly with host susceptibility (Duneau *et al.* 2011). For a subset of the crosses, we also performed infection trials by exposing up to eight *D. magna* females per recombinant genotype individually to 200.000 spores of *P. ramosa* and scoring infection status 30 days after exposure. All statistics were performed with Fisher's exact test.

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Appendix 1

An alternative genetic model to the one locus – three allele model is a model with 2 diallelic loci and epistatic interaction (Figure S1 & Table S1). Alleles at the two loci (A locus and B locus, with alleles A and a and B and b respectively) segregate in a Mendelian pattern and resistance is dominant for both (C1 resistance allele "A" and susceptibility allele "a", C19 resistance allele "B" and susceptibility allele "b"). Furthermore, there is an epistatic interaction between alleles "A" and "B" as evident from the absence of double resistant host clones and overrepresentation of single resistance host clones in the backcross. Individuals carrying the "A" allele are susceptible to *P. ramosa* C19 regardless of the presence of the "B" allele, which, in absence of "A", normally confers resistance to C19. Epistasis is consistent with the results of selfing of backcrossed host clones, which revealed that two genotypes (Aabb & AaBb) expressed the same phenotype. In addition, absence of expected double susceptible host clones (for AaBb) suggested that both loci may be linked. Two independent test crosses for linkage (aabb * AaBb) did not detect any recombinants (N=118). Under the assumption that the next offspring would have resulted in a recombinant genotype, the maximum distance between both loci would be 0.8 cM. Using the mapping distance of the related *Daphnia pulex* (Cristescu *et al.* 2006) this corresponds to a distance of 110 kb.

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Table S1: Crossing details. The table shows the total number of host clones tested per cross (N) and the percentage of these clones that where either susceptible (sus.) or resistant (res). P-value, Fisher's exact test between number of observed and expected under the hypothesis that resistance is coded for by two linked loci with 2 alleles and inter locus epistasis.

				Observed/Expected (in %)				
Cross	**Suggested genotype**	**Name**	**N**	C1: C19:	sus. sus.	sus. res.	res. sus.	res. res.	**P-value**
3 × selfed X	AAbb	Xinb3	1		0/-	0/-	100/-	0/-	-
3 × selfed Fa	aaBb	Fainb3	1		0/-	100/-	0/-	0/-	-
selfed Xinb3	Aabb • AAbb	Xinb4	24		0/0	0/0	100/100	0/0	P=1.00
selfed Fainb3	aaBb • aaBb	Fainb4	22		18/25	82/75	0/0	0/0	P=0.72
Xinb3×Fainb3	Aabb • aaBb	F1	1		0/0	0/0	100/100	0/0	-
Selfed F1	Aabb • Aabb	F2	68		21/25	0/0	79/75	0/0	P=0.69
F1×Fainb3	Aabb • aaBb	BackCross	164		23/25	26/25	51/50	0/0	P=0.94
selfed BackCross**A**	aabb • aabb	Backinb**A**-1	38		21/25	79/75	0/0	0/0	P=0.79
selfed BackCross**A**	aabb • aabb	Backinb**A**-2	47		17/25	83/75	0/0	0/0	P=0.45
selfed BackCross**B**	aaBb • aaBb	Backinb**B**-1	30		100/100	0/0	0/0	0/0	P=1.00
selfed BackCross**B**	aaBb • aaBb	Backinb**B**-2	39		100/100	0/0	0/0	0/0	P=1.00
selfed BackCross**C**	Aabb • Aabb	Backinb**C**-1	26		19/25	0/0	81/75	0/0	P=0.74
selfed BackCross**C**	Aabb • Aabb	Backinb**C**-2	64		31/25	0/0	69/75	0/0	P=0.56
selfed BackCross**D**	AaBb • AaBb	Backinb**D**-1	35		0/0	17/25	83/75	0/0	P=0.56
selfed BackCross**D**	AaBb • AaBb	Backinb**D**-2	68		0/0	25/25	75/75	0/0	P=1.00
selfed BackCross**D**	AaBb • AaBb	Backinb**D**-3	49		0/0	22/25	78/75	0/0	P=1.00
selfed BackCross**D**	AaBb • AaBb	Backinb**D**-4	27		0/0	30/25	70/75	0/0	P=1.00
BackCross×BackCross	aabb • AaBb*	Back×Back-1	62		0/0	45/50	55/50	0/0	P=0.85
BackCross×BackCross	aabb • AaBb*	Back×Back-2	56		0/0	46/50	54/50	0/0	P=0.72
			822						

*as expected under perfect linkage

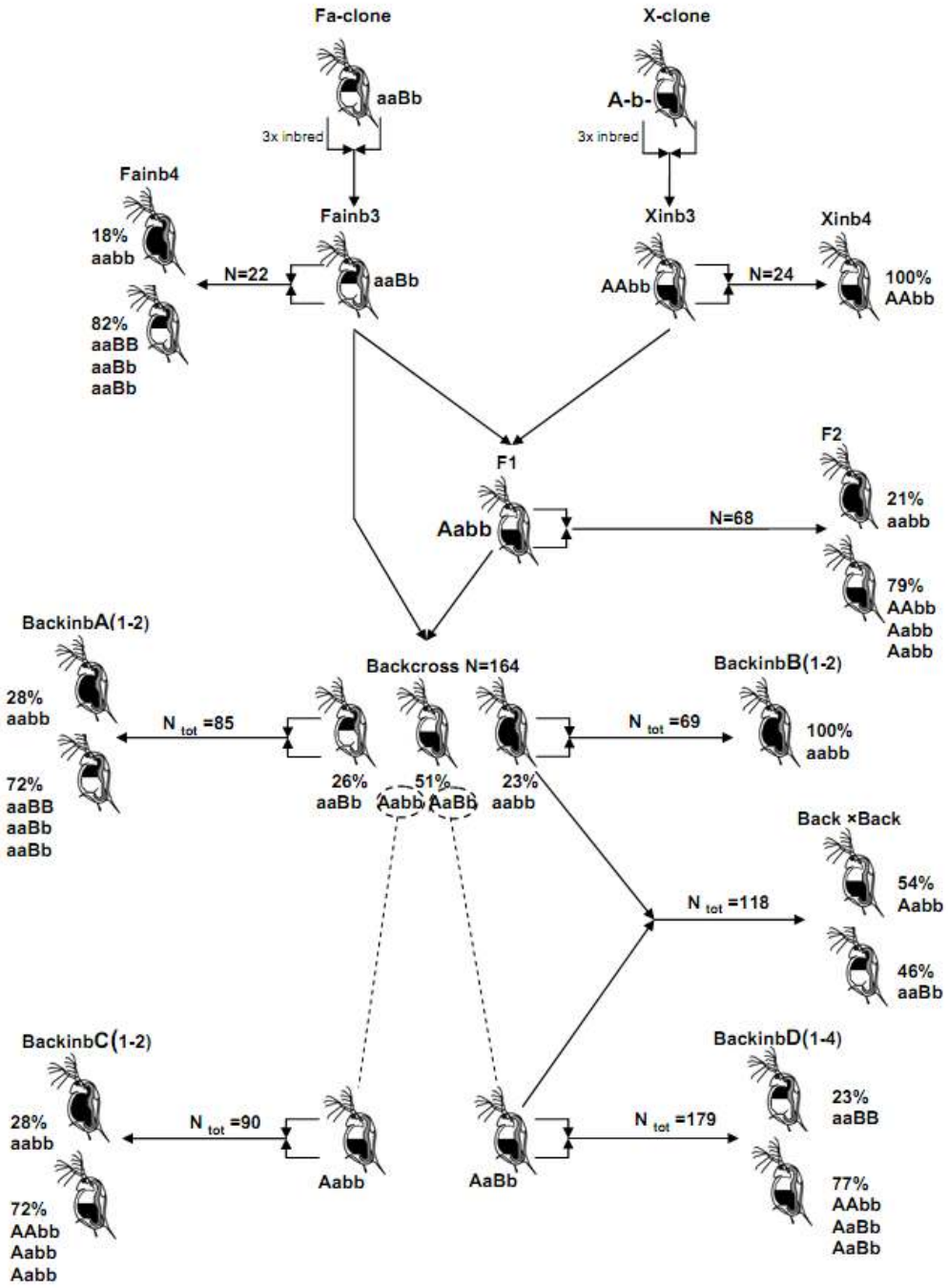

Figure S1: *Pedigree showing the crossing schedule and resistance profiles of* Daphnia magna *genotypes for two* Pasteuria ramosa *genotypes (C1 & C19).* **N** *represents the total number of recombinants tested and percentages reflect segregation patterns. Letters e.g. Aabb, aaBb represent the genotypes under the hypothesis of two linked loci with 2 alleles and inter locus epistasis.*

Phenotypes: *C1 & C19 susceptible*

C1 susceptible and C19 resistant

C1 resistant and C19 susceptible

Chapter 5

Concluding remarks

Antagonistic coevolution between host and parasites can lead to negative frequency dependent selection and thereby maintain genetic variation, a pertinent issue in evolutionary biology (Clarke 1976). Furthermore, according to the Red Queen Theory such a form of coevolution may also explain why sexual reproduction is favored over asexual reproduction (Hamilton 1980;Jaenike 1978). In my thesis I tested the *Daphnia-Pasteuria* model system for two assumptions required by the Red Queen Theory, host-parasite specificity and a specific genetic architecture of host resistance.

First, I investigated host specificity, which is a strong determinant for antagonistic coevolution by negative frequency dependent selection (Agrawal & Lively 2002;Clarke 1976). I show that specificity in the *Daphnia-Pasteuria* system has been underestimated for two reasons. First, *Pasteuria* consists of multiple species that are morphologically identical. Each of these varieties of *Pasteuria* has a narrow host range infecting only one of the three tested species of *Daphnia*. Second, specificity has been underestimated due to the presence of multiple genotypes of *P. ramosa* in isolates used to perform infection trials in previous studies. Infections with single genotypes of *P. ramosa* showed much higher specificity. Hosts were either fully susceptible or fully resistant and infection outcome depended on both host and parasite genotype. The finding of very high specificity has implications for a number of ecological and evolutionary phenomena in the *Daphnia-Pasteuria* system e.g. local adaptation (Lajeunesse & Forbes 2002), evolution of virulence (Woolhouse *et al.* 2001) and parasite mediated competition. Furthermore theory suggests that strong genotype-genotype interactions such as found here are favourable for antagonistic coevolution by negative frequency dependent selection and the Red Queen Theory (Salathe *et al.* 2008).

For antagonistic coevolution to select for recombination, as suggested by the Red Queen Theory, host resistance should have a specific genetic architecture (Otto & Nuismer 2004;Parker 1994). In my thesis I show that host resistance is coded for by a single locus with three alleles and a dominance hierarchy. A dominant allele provides resistance against one genotype of *P. ramosa*, but leads to susceptibility against the second. A second allele that is recessive to the first shows the reverse pattern, providing resistance to the second and susceptibility to the first *P. ramosa* genotype. A third allele recessive to both other alleles leads to *Daphnia* that are susceptible to both parasite genotypes. An alternative, but more complex explanation is that resistance is coded for by two very closely linked loci with both two alleles and strong interlocus epistasis. Both hypotheses on the genetic architecture of host resistance in *D. magna* are congruent with a matching allele model, thereby providing the first empirical evidence for such models. This class of models has been widely used in the modeling of antagonistic coevolution and readily show frequency dependent selection and maintenance of genetic variation and sexual reproduction (Frank 1993;Salathe *et al.* 2008;Otto & Michalakis 1998). Antagonistic coevolution in the *Daphnia-Pasteuria* system had been reported earlier (Decaestecker *et al.* 2007), but without the knowledge of the underlying genetics. Our genetic results support the notion that antagonistic coevolution between *D.magna* and *P. ramosa* can maintain genetic variation. Indeed the finding of a cross host species polymorphism for attachment of *Pasteuria* spores to the *Daphnia* esophagus is consistent

with long term preservation of genetic variation on the resistance locus. It however, remains an open question if the inferred genetic architecture can explain the maintenance of sexual reproduction as suggested by the Red Queen Theory. Under the most parsimonious hypothesis "one locus with three alleles" there can be no advantage for recombination as this would require more than one locus to code for resistance (Otto & Nuismer 2004). Under the second hypothesis "interlocus epistasis between two closely linked loci" there might be an advantage for recombination. For recombination to be advantageous selection on favourable allele combinations must fluctuate on the order of a few generations (Barton 1995;Charlesworth 1976). The interlocus epistasis between the resistance alleles under this hypothesis may generate such patterns under negative frequency depended selection but only when genetic variation is maintained on both loci. With our 2-linked loci model, the recessive susceptibility allele on the second locus would be lost as there is no advantage for maintaining it. However, if epistasis between both loci occurs for more allele combinations then found so far, variation on the second locus could be maintained. Alternatively variation on this locus could be maintained if there is a cost for carrying the resistance allele. Further research is needed to determine if antagonistic coevolution between *Daphnia* and *Pasteuria* could lead to an advantage for sexual reproduction. Nevertheless, the *Daphnia-Pasteuria* system may fall into the parameter space where a model found an advantage for sexual reproduction by segregation (Agrawal 2009). The most important parameter pertaining to the evolution of sex in this model, describes dominance relationships in heterozygote individuals and under a matching allele model this study suggest that the found dominance relationships in the *Daphnia- Pasteuria* system are favorable for the evolution of segregation.

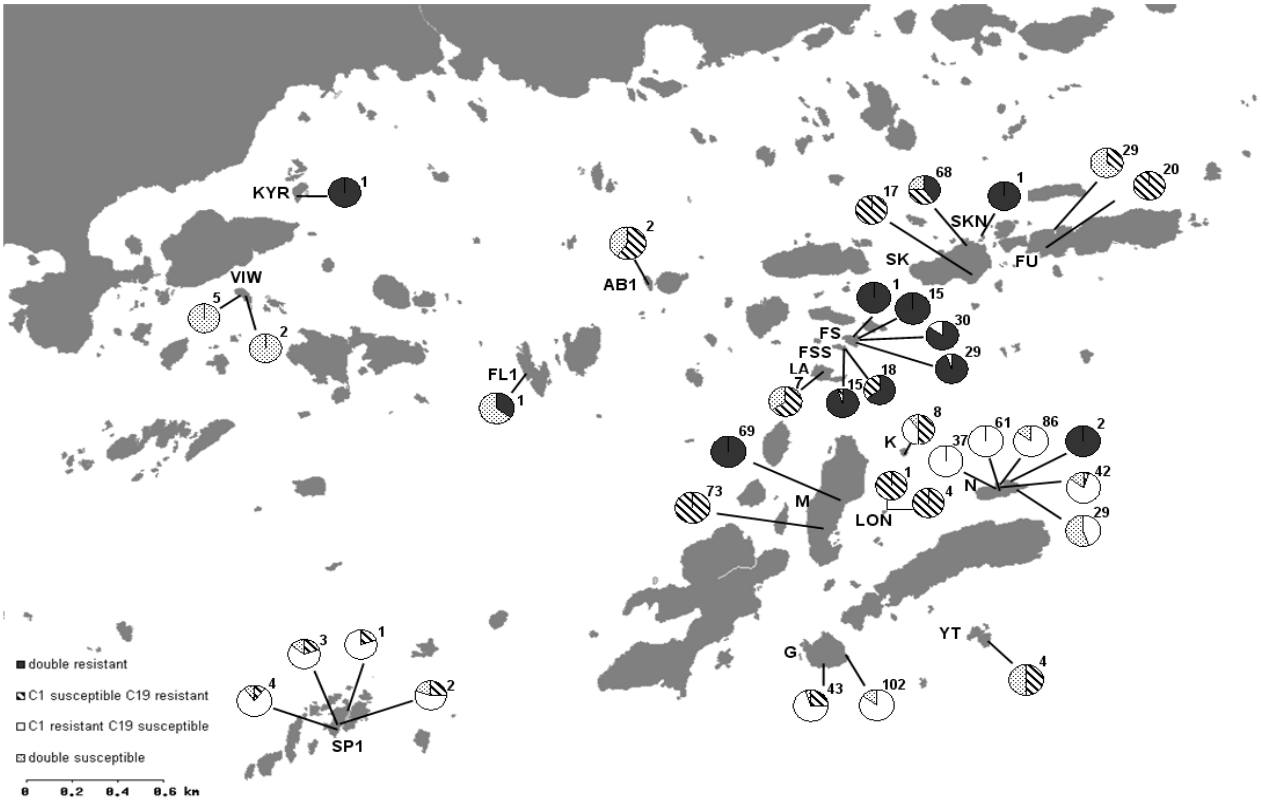

Figure 1: Resistance profiles of D. magna *from rockpools of the island archipelago near Tvärminne, Finland. I sampled 20 individuals per pond and tested simultaneously for attachment with C1 and C19 using red and green fluorescently labeled spores. I observed 33% double resistant individuals, a phenotype we cannot explain with our current understanding of the genetics. The white area is the Baltic Sea, the grey area are the mainland and islands. The 2-3 letters close to islands are codes for island names. The numbers beside the pie charts are the numbers for rock pools on the islands.*

This thesis represents a significant advancement in the *Daphnia-Pasteuria* system which has been widely used for testing of theories pertaining to the antagonistic coevolution such as the evolution of virulence (Ben-Ami *et al.* 2008a;Jensen *et al.* 2006), local adaptation (Ebert *et al.* 1998), genotype by environment interactions (Mitchell *et al.* 2005;Vale *et al.* 2008;Vale & Little 2009), mass-action principle (Ben-Ami *et al.* 2008b;Regoes *et al.* 2003), maternal effects (Ben-Ami *et al.* 2010) and frequency dependent selection (Decaestecker *et al.* 2007). The knowledge that *P. ramosa* consists of multiple cryptic host varieties, together with the availability of *P. ramosa* clones, that negate the complicating factors intrinsic to mixed infections, allows for a more definitive interpretation of experimental results. Furthermore, the finding that genetics of resistance in *Daphnia* are consistent with a matching allele model will allow the *Daphnia-Pasteuria* system to become a powerful tool for empirical testing of population level predictions of this model. Indeed, the *Daphnia-Pasteuria* system could be used to experimentally test for negative frequency dependent selection, the maintenance of genetic variation and the notion that antagonistic coevolution may favor genetic mixing.

Further directions for research

It is likely that the genetics for host resistance against *P. ramosa* is more complex involving more alleles or loci. First, in this work we tested only two parental genotypes of *P. ramosa*, and data (unpublished) indicates that there are at least three, but likely, many more *P. ramosa* with different infection profiles. One of these (isolate inb1P5) was tested on the parents and a subset of the backcrossed hosts used in this work and all *Daphnia* where found to be susceptible. Unfortunately these *P. ramosa* are not yet cloned and thus these isolates may contain multiple genotypes, which complicates the determination of inheritance of resistance. Further evidence suggesting that host resistance is more complex comes from an unpublished study where I tested resistance in *Daphnia* from 41 ponds on 19 islands of the coast of Finland. Although I could explain the majority of field observations with the current genetic model, 33% of the Daphnia taken directly from the field were resistant to both *P. ramosa* C1 and C19 (Figure 1). Such double resistant genotypes should be absent according to the current understanding of the genetics. Selfing these *Daphnia* revealed that this trait is Mendelian inherited and resistance is dominant. It is however unclear if resistance to both *P. ramosa* is coded for by an additional allele on the known locus or on a further locus. To determine how this trait is inherited I suggest to create three new F2 panels by crossing homozygous *Daphnia* resistant to both C1 and C19 to both parents used in this thesis and a *Daphnia* susceptible to both *P. ramosa* genotypes and self the resulting F1 clones to produce many F2 offspring. In addition, these panels could be used to distinguish between the two genetic hypotheses. Under the allele hierarchy hypothesis only two alleles can segregate at a time while under the two linked loci with interlocus epistasis hypothesis four alleles at two loci can segregate. By testing these new F2 panels with additional *P. ramosa* clones with different infection profiles we could determine the number of simultaneous segregating resistance alleles and thus determine the correct hypotheses. Identification of the gene or genes for host resistance by QTL mapping (Routtu *et al.* 2010), followed by a verification by reverse genetics (e.g. RNAi knockdown (Kato *et al.* 2011) may also help distinguish between both hypothesis. Furthermore, identification of the gene will give an estimate on the number of resistance alleles and allow for the tracking of these in natural populations. In addition, it will allow for the testing of the proposed ancient polymorphism for attachment to the host esophagus on the genetic level.

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