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LETTER

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 as the presence of multiple genotypes may influence the outcome and interpretation of some experiments.

Keywords

Coevolution, *Daphnia magna*, host, parasite, *Pasteuria ramosa*, specificity.

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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 (Janzen 1970; Connell 1971). 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 (Woolhouse *et al.* 2001; Kirchner & Roy 2002) 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. Solter *et al.* 2002; Decaestecker *et al.* 2003). 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 *et al.* 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, whereas other host clones were infected only 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, whereas 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. Ebert *et al.* 1998; Carius *et al.* 2001; Little *et al.* 2006; Ebert 2008). 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 12 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.

MATERIAL 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 colouration, gigantism and castration of the host (Ebert *et al.* 2004). Infections are thus easily recognizable. *Pasteuria 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).

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 laboratory: Inb1 is the once-selfed offspring of Mu11, Xinb3 and Fainb3 are the results of three generations selfing X-clone and AL1-4-4, XFa6 is a cross between Xinb3 and Fainb3 and XI is a cross between Xinb3 and Iinb1. All clones were kept under standardized conditions for three generations prior to experiments [eight 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 16 : 8 h 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 five isolates using two methods: infection by limited dilution and single-spore infections.

Isolating clones of *P. ramosa* by limited dilution

Juvenile *D. magna* females (0–5 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 2 weeks, offspring born within a 5-day interval were collected and distributed across thirty-nine 400 mL jars at a density of *c.* 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, Hawksley, Sussex, UK). For host clone HO2, one jar received an estimated 10 000 spores of *P. ramosa* isolate P5; two jars received 1000 spores; six jars, 100 spores and thirty jars, 10 spores. For host clone AL1-4-4, 2 jars received an estimated 1000 spores and 42 jars an estimated 100 spores. *Daphnia magna* populations were fed 10^7 cells per jar day^{-1} for 20 days and 2×10^8 cells per jar day^{-1} 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 days 40 to 50, all females that showed the typical symptoms of *P. ramosa* infection (castration, gigantism and brownish 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–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 3-day-old *D. magna* of clone Xinb3, HO2 or AL1-4-4 was placed in each well of a 96-well plate (Falcon 354043, Becton Dickinson AG, Allschwil, Switzerland). Wells contained *c.* 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 400× magnification. Using a micropipette (1 mm O.D. 0/78 mm I.D. borosilicate micropipette elongated over a flame), single spores (*c.* 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

Table 1 Isolates of *Pasteuria 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, Kains	2002
P3	Ten infected hosts	Finland, Tvärminne	2002
P4	Eight infected hosts	Belgium, Heverlee	2003
P5	Single female infected from pond sediment	Russia, Moscow	1996

Table 2 Clones of *Pasteuria 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	August 2006	Likely
C19	Single spore	Xinb3	P1	November 2007	Yes
C20	Single spore	HO2	P2	February 2008	Yes
C24	Single spore	HO2	P4	June 2008	Yes

per well. *Daphnia magna* in well plates were fed between 10 000 and 20 000 algae cells daily. After 3–4 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. *Daphnia magna* in jars were fed 4–20 million algae cells daily (depending on *D. magna* size and number) and transferred to new jars containing 80 mL of fresh medium weekly. *Daphnia 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 6384 single-spore infections were successful (0.14%): *D. magna* clone HO2 was infected by a spore of *P. ramosa* from 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 3-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 5 days, jars were filled with 80 mL of medium, and 9 days after exposure, *D. magna* were transferred to 400 mL jars with up to eight *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 $12 \times 6 \times 14 = 1008$ individuals). We placed 4-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 2×10^6 cells per individual per day on day 10 to 10×10^6 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 (400×), but we found no discrepancies with our initial diagnosis.

Experiment 2: Genotype–genotype interactions

The infection specificity of five *P. ramosa* clones (C1 and C14 created by limited dilution; C19, C20 and C24 created by single-spore infection), a mixture of these five 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 $12 \times 7 \times 10 = 840$ 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, non-infected *Daphnia*. Experimental conditions were similar to Experiment 1 with the following exceptions: 3-day-old females were exposed, fresh medium was added 5 days after exposure and medium was changed every 3 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 (400×).

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.

Table 3 Comparison of infections by putative clones obtained by limited dilution and the original isolate

Clone of *Daphnia magna*	*Pasteuria ramosa*				
	Putative clones				Isolate
	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

C1 and C2 show a binary infection pattern, whereas 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.

Table 4 Outcome of infection trial with single genotype infections of *Pasteuria ramosa*

Clone of *Daphnia magna*	Location	*P. ramosa*					
		C1	C14	C24	C19	C20	Mix
		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

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.

Pasteuria 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 9 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, whereas C3 and C4 showed strong differences. We speculate that C1 and C2 are indeed clones of *P. ramosa*, whereas 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. *Pasteuria ramosa* clones C1, C14 and C24 infected 3 of 12 *D. magna* clones. *Pasteuria ramosa* clones C19 and C20 infected 5 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 1, containing clones C1, C14 and C24, originated from Russia, Finland and Belgium, respectively; and Group 2, containing clones C19 and C20, originated from Germany and England, respectively. 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.

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 (Tables 3 and 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, whereas C3 and C4 potentially contain multiple genotypes and for this reason were not further used. Furthermore, C1 and C2 may be the same genotype, whereas C3 and C4 may be mixtures with different composition.

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

Table 5 Comparison of infection patterns of clones and the isolates from which these clones were obtained

		Pasteuria ramosa									
		Russia		Finland		Belgium		Germany		England	
Clone of *Daphnia magna*	Origin of host clone	Clone C1	Isolate P5	Clone C14	Isolate P3	Clone C24	Isolate P4	Clone C19	Isolate P1	Clone C20	Isolate 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

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 three times selfed X-clone whose infection pattern with *P. ramosa* clones is identical.

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 to 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).

High specificity as found here has been found in plant pathogens (e.g. Thompson & Burdon 1992; Zeigler *et al.* 1995 and references therein) and in bacteria–bacteriophage interactions (Duplessis & Moineau 2001; Sullivan *et al.* 2003). 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 (Tables 3 and 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), whereas 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 *et al.* 2007). Infections with several strains of the same pathogen appear to be widespread among other pathogens (Read & Taylor 2001; Lopez-Villavicencio *et al.* 2007; Balmer & Caccone 2008). 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 (*c.* 1 in 700) of single-spore infections make 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 (Clarke 1976; Agrawal & Lively 2002). 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 (Jaenike 1978; Hamilton 1980).

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, whereas 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 non-infective 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 show 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 and 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. *Pasteuria ramosa* isolate P5 showed moderately high infectivity in host clone HO2 and very low infectivity in host clone M10, whereas 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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