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CROSS-SPECIES INFECTION TRIALS REVEAL CRYPTIC PARASITE VARIETIES AND A PUTATIVE POLYMORPHISM SHARED AMONG HOST SPECIES

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A parasite's host range can have important consequences for ecological and evolutionary processes but can be difficult to infer. Successful infection depends on the outcome of multiple steps and only some steps of the infection process may be critical in determining a parasites host range. To test this hypothesis, we investigated the host range of the bacterium *Pasteuria ramosa*, a *Daphnia* parasite, and determined the parasites success in different stages of the infection process. Multiple genotypes of *Daphnia pulex*, *Daphnia longispina* and *Daphnia magna* were tested with four *Pasteuria* genotypes using infection trials and an assay that determines the ability of the parasite to attach to the hosts esophagus. We find that attachment is not specific to host species but is specific to host genotype. This may suggest that alleles on the locus controlling attachment are shared among different host species that diverged 100 million year. However, in our trials, *Pasteuria* was never able to reproduce in nonnative host species, suggesting that *Pasteuria* infecting different host species are different varieties, each with a narrow host range. Our approach highlights the explanatory power of dissecting the steps of the infection process and resolves potentially conflicting reports on parasite host ranges.

KEY WORDS: Coevolution, *Daphnia magna*, host range and cryptic species, *Pasteuria ramosa*.

Understanding the host range of a parasite is critical, as it has important implications for numerous ecological and evolutionary phenomena. It can affect selection on both parasite and host traits (Woolhouse et al. 2001; Kirchner and Roy 2001; Regoes and Nowak 2000). Host range can also affect species interactions thereby altering community structure (e.g., parasite-mediated coexistence Janzen 1970; Connell 1971; for a review see Hatcher et al. 2006). Furthermore, it is an important predictor for the likelihood of host switches (Parker and Gilbert 2004) and therefore plays an important role in conservation and biocontrol.

Determining a parasite's host range is often difficult because apparent host ranges may overestimate actual host range. A common problem is that 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). A further problem in determining host ranges is the method used to test for host susceptibility. To incorporate a host into a parasite's host range, a parasite must be successful in all steps of the infection process. Failure to overcome a single step in the defence cascade results in failure of infection, regardless of the ease

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with which the parasite is able to overcome the other steps in the infection process (Solter and Maddox 1998; Schmid-hempel and Ebert 2003). Thus, assessments made based on only part of the infection process may not represent the outcome of the entire process. For example, infecting animals by injecting parasites into the body cavity circumvents possible resistance mechanisms related to the first steps of host–parasite encounter, such as attachment or penetration into the host. Here we test the hypothesis that a single step in the infection process is responsible for determining the host range of a parasite.

The bacterial parasite *Pasteuria ramosa* and its host *Daphnia magna* have become a model system for antagonistic coevolution (Ebert 2005; Decaestecker et al. 2007; Luijckx et al. 2013), and recent advances allow for the assessment of parasite's success at different steps of the infection process (Duneau et al. 2011). The infection process begins with ingestion by the host of the parasite's environmental transmission stage (endospore) followed by activation of the endospore (shedding of the outer spore coating). If host and parasite genotypes are compatible, the activated parasite spores may then attach to the host esophagus, penetrate the esophagus wall, and proliferate within the host (Duneau et al. 2011). Eventually, the parasite kills the host, and spores of the parasite are released into the environment from the decaying host cadaver. By separately testing the spore activation step, the attachment step, and the proliferation steps, one can assess which of these steps is critical in determining *Pasteuria's* host range.

The host range of *Pasteuria* is currently unresolved. However, *Pasteuria* has been reported from field samples of *D. magna*, *Daphnia longispina*, *Daphnia pulex* (Stirnadel and Ebert 1997), *Daphnia dentifera* (Duffy et al. 2010), *Daphnia curvirostris* (Goren and Ben-Ami 2012), and even cladocerans in other genera (Green 1974; Sayre and Wergin 1977; Goren and Ben-Ami 2012). These *Daphnia* species belong to different subgenera and are unlikely to have exchanged genes in the last 100 million years (Colbourne and Hebert 1996). However, within natural communities *Pasteuria* has been found to infect only a single *Daphnia* species, even if other sympatric *Daphnia* species are present (Ebert et al. 2001; J. Andras unpubl. data). Furthermore, individual genotypes of *Pasteuria* are known to be extremely specific, infecting only a few genotypes of *D. magna* (Luijckx et al. 2011).

One possible explanation for the high specificity of infection within species and apparent low specificity between host species is that *Pasteuria* infecting different host species are separate genetic entities with similar morphology, each with a narrow host range (here defined as a variety; Ebert 2005). Alternatively, *Pasteuria* could be specific for some host genotypes within each host species but unspecific across species (as suggested by Duneau et al. 2011). Under this scenario, *Daphnia* species might share the polymorphism coding for attachment that was identified in *D. magna*, which is believed to be responsible for the extreme specificity observed between *D. magna* and *Pasteuria* (Luijckx et al. 2013).

Here, we assess the host range of *Pasteuria* by assessing both spore attachment to the host esophagus (which can be visualized with fluorescent-labeled spores as described by Duneau et al. 2011) and by exposing animals to spores in infection trials. Spore activation has previously been described to be unspecific (spores are always activated upon contact with any *Daphnia*; Duneau et al. 2011). Spore attachment governs host–parasite compatibility in one step of the infection process, whereas infection trials quantify the outcome of the entire infection process (activation, attachment, penetration, and proliferation). Thus, using both tests allows us to determine if failure of infection is due to failure of spore attachment or due to failure during the post-attachment steps. We sampled a natural Finnish meta-population, where the three host species, *D. magna*, *D. pulex*, and *D. longispina* occur in sympatry and tested for attachment using two naturally co-occurring *Pasteuria* isolated from *D. magna* and *D. longispina*. In addition, we use four lines of *Pasteuria* to survey attachment and infection patterns in these three *Daphnia* species from various locations in Europe. We will use the term "native" here to refer to combinations of *Pasteuria* with the *Daphnia* species it was sampled from.

Materials and Methods

SYSTEM DESCRIPTION

Pasteuria is an obligate bacterial parasite of *Daphnia* and has a wide geographic distribution in the northern hemisphere (Ebert 2005). *Pasteuria* castrates its hosts (Ebert et al. 2004) and prevalence can be very high in natural populations (e.g., Duncan et al. 2006), making it a potentially strong selective agent (Little and Ebert 2000). *Daphnia* are cladocerans that occur in a variety of standing water bodies (e.g., rock pools, ponds, lakes and swamps). Multiple species of *Daphnia* can occupy the same body of water, and the species *D. magna*, *D. longispina*, and *D. pulex* have been found to occur in sympatry in several localities in England (Stirnadel and Ebert 1997), Sweden (Bengtsson 1986), Finland (Ebert et al. 2001), and Switzerland (J. Andras, unpubl. data).

ATTACHMENT TO HOST ESOPHAGUS

We tested if the polymorphism for spore attachment that was found for native combinations of *D. magna* and *Pasteuria* (Duneau et al. 2011) was also present in native combinations of *D. longispina* and *Pasteuria*. In total, we tested 623 native and 883 nonnative host–parasite combinations with the attachment test.

The attachment test is described in full detail by Duneau et al. (2011). In short, individual hosts were placed singly in 24-well plates in 1 mL of artificial medium (ADaM, modified by using only 5% of the recommended selenium dioxide concentration,

Klüttgen et al. 1994). Twenty thousand fluorescently labeled spores were added to each well, and animals were incubated for 1 hour at room temperature. Spore 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.

We sampled rock pools on islands along the Baltic coast of Finland, where *Pasteuria* and the three *Daphnia* species, *D. longispina*, *D. magna*, and *D. pulex*, naturally co-occur (Ebert et al. 2001). Twenty individuals were sampled from each of six *D. pulex*, 28 *D. magna*, and 23 *D. longispina* populations from 53 different rock pools located on 21 islands. Most samples were taken on islands close to Tvärminne Zoological station, but additional samples were collected on islands approximately 150, 225, and 250 km to the East (GPS coordinates of sampling locations can be found in Table S1). Sampled individuals were split into two groups of 10, and spore attachment to the esophagus was assessed using *Pasteuria* clone C14 native to *D. magna* ($C14_{magna}$) for the first group and isolate P10 native to *D. longispina* ($P10_{longispina}$) for the second group. Both parasites were originally collected close to Tvärminne Zoological station. $C14_{magna}$ is a single genotype of *Pasteuria* (see Luijckx et al. 2011). $P10_{longispina}$ is a field isolate (potentially containing more than one genotype) of *Pasteuria* that was obtained by the propagation of spores from 3 infected *D. longispina* individuals in *D. longispina* clone FS-30-1.

To test for consistency of the attachment test within *Daphnia* clones, we produced 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 (see Table S2 for GPS coordinates). Clonal lineages were propagated and maintained under standard laboratory conditions (fed three times per week with 50×10^6 of the chemostat-cultured algae *Scenedesmus obliquus*, 16 h:8 h light dark cycle, 20°C, in artificial medium), and four individuals of each host clone were tested for spore attachment with *Pasteuria*. As found previously, attachment was a binary trait with spores either attaching in all individuals of one host clone or none (Duneau et al. 2011).

We extended our sampling to include *Daphnia* and *Pasteuria* from additional locations in Europe to verify the generality of our results. We sampled additional individuals from *D. magna*, *D. longispina*, and *D. pulex* from Finland (20 clones), England (6), Germany (29), Switzerland (12), France (3), Italy (6), Hungary (1), Iran (3), and Russia (5) and established clonal lineages that were propagated and maintained under standard laboratory conditions (for GPS locations, see Table S3). In addition to *Pasteuria* isolate $P10_{longispina}$ and clone $C14_{magna}$, we tested each of these host clones with *Pasteuria* clone C1 from a Russian *D. magna* population ($C1_{magna}$) and *Pasteuria* clone C20 from an English *D. magna* population ($C20_{magna}$). At least four individuals for each host–parasite combination were tested.

INFECTION TRIALS

Attachment of spores to the host esophagus in native combinations of *D. magna* and *Pasteuria* leads to successful infection (Duneau et al. 2011). We tested whether this also holds in native combinations of *D. longispina* and *Pasteuria* and if this holds in nonnative combinations. To do this, we performed infection trials with subsets of the same host clones used to determine spore attachment.

For infection trials, we took *Daphnia* from mass cultures that were kept under standard laboratory conditions. We placed groups of four 1-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 *Pasteuria* 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*). A week after exposure, jars were filled with 60 mL of fresh medium, and medium was replaced on a weekly basis thereafter. *Daphnia* were fed 2×10^6 cells per day at the start of the experiment, and food levels were increased gradually to 4×10^6 cells per day for *D. pulex* and *D. longispina* and to 5×10^6 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 *Pasteuria* spores. Individuals that died earlier cannot be reliably checked for infection and were excluded from the analysis. Forty days after exposure, remaining *Daphnia* were pooled per replicate, crushed, and checked for presence of *Pasteuria* spores using a phase contrast microscope (400×). 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 $P10_{longispina}$ and $C14_{magna}$ 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 (eight replicates per host clone). In addition, we performed infection trials with *Pasteuria* and host clones from other locations across Europe where we had previously observed attachment in nonnative combinations and a subset in which we had observed no attachment (eight to 10 replicates for each combination of host and parasite). In total, we tested 18 native and 83 nonnative host–parasite combinations with infection trials.

GENETIC IDENTIFICATION OF *PASTEURIA* CLONES AND ISOLATES

To verify that the clones and isolates of *Pasteuria* used in this study were genetically unique and to infer patterns of relatedness among them, we sequenced 1346 base pairs from the *Pasteuria* genome, spanning two genes (ribosomal protein RpsA—797 bp; predicted sporulation protein—374 bp) and 175 noncoding bases. Genomic DNA was extracted from resting spores of *Pasteuria* as described in McElroy et al. (2011). PCR reactions were performed in a volume of 50 μL containing 2 U of FastStart *Taq* Polymerase (Roche, Basel, Switzerland), 1× reaction buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer (h115F: AGCAGCTTCTTGGAAAGGAATTGGT; h115R: TGGGGTGAGAGGATTTGTACCTGC), 200 μM dNTP, and ∼100 ng of template DNA. The PCR profile consisted of an initial denaturation step at 95°C for 10 min, and 35 cycles of 95°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 60 sec, followed by a final extension at 72°C for 10 min. Amplicons were column purified (QIAquick PCR Purification Kit; Qiagen, Basel, Switzerland) and sequenced directly (Microsynth, Balgach, Switzerland).

Results

ATTACHMENT TESTS

Attachment of the four *Pasteuria* lines used in this study was observed in all native host species and almost all nonnative host species (Table 1). Attachment was in all cases specific for some host genotypes and showed within population variation. Within the Finnish meta-population where *Pasteuria* and the *Daphnia* species, *D. longispina, D. magna* and *D. pulex*, naturally co-occur, we found that isolate $P10_{longispina}$ is able to attach to its native host *D. longispina* as well as to *D. magna*. Clone $C14_{magna}$ attached to its native host *D. magna* as well as *D. pulex*. The other two nonnative combinations did not result in any attachment (Fig. 1). However, in our extended sampling we found that $P10_{longispina}$ was also able to attach to three of *D. pulex* (from France; Table 1). We also found that $C1_{magna}$ showed a similar pattern as $C14_{magna}$, able to attach to its native host *D. magna* (Finland, Italy, Hungary) as well as *D. pulex* (Finland and Iran). Attachment of $C20_{magna}$ was observed in its native host *D. magna* (Germany, Italy, and Hungary) and nonnative hosts *D. longispina* (Finland) and *D. pulex* (Finland and Iran).

INFECTION TRAILS

All *Pasteuria* lines infected only certain genotypes of their native host and failed in all other native and nonnative genotypes. The perfect correlation previously observed between attachment and infection (Duneau et al. 2011) thus only holds in native combinations of *Daphnia* and *Pasteuria*. All *Pasteuria* types that were able to attach to their nonnative hosts failed to produce infection (Tables 1, 2).

HAPLOTYPE NETWORK

A total of 19 single nucleotide polymorphisms (SNPs) were observed in the 1346 bases sequenced from the *Pasteuria* genome. These SNPs resolved all *Pasteuria* types used in this study, indicating that they were indeed distinct parasite genotypes. A median-joining network of haplotypes (Fig. 2) illustrates that the level of divergence between *Pasteuria* from different host species (P10 vs. C1, C14, C20) is greater than the divergence among *Pasteuria* isolated from a single host (C1, C14, C20).

Discussion

Attachment to the host esophagus, a necessary step in the infection process, was found in native and nonnative host–parasite combinations. This may indicate that the mechanism responsible for attachment to the esophagus is shared between host species that diverged more than 100 million years ago. Thus, with regard to the attachment step, *Pasteuria* has a wide host range. However, despite successful attachment, none of our *Pasteuria* was able to infect nonnative hosts. This finding indicates that the narrow host range of *Pasteuria* is determined by postattachment steps. Reports of *Pasteuria* infecting different host species thus suggest the existence of several parasite varieties, possibly different species, each with a narrow host range.

ATTACHMENT OF *PASTEURIA* IS CONSERVED BETWEEN HOST SPECIES

Attachment to the host esophagus is believed to be responsible for the strong specificity of *Daphnia–Pasteuria* interactions within *D. magna* (Duneau et al. 2011). Here we show that although attachment of the parasite spores is genotype-specific within species, spores can also attach to specific genotypes of nonnative host species (Table 1, 2 and Fig. 1). Furthermore, we show that the mechanism for attachment is polymorphic in all three *Daphnia* species studied, as is evident from specific attachment of *Pasteuria* to all three host species (Tables 1, 2 and Fig. 1). We offer three explanations for the observed patterns of attachment.

First, parasite spore attachment across different host species suggests that the same genetic mechanism operates in the different host species. It was recently shown that the genetics underlying specific attachment of *Pasteuria* in *D. magna* is based on few loci (one or two) that follow a matching allele model (Luijckx et al. 2013). A dominant allele provides resistance against *Pasteuria* C1, whereas homozygotes for the recessive allele were resistant against *Pasteuria* C19 (which has an infection pattern identical to the here used C20 Luijckx et al. 2011). The alleles that control specific attachment of *Pasteuria* may thus be shared between species of *Daphnia*. This would imply that *Daphnia* has an ancient polymorphism for attachment of *Pasteuria* predating the split of the subgenus *Ctenodaphnia* (with *D. magna*) and the

Table 1. Summary of attachment-tests with four *Pasteuria* on *Daphnia magna, Daphnia pulex,* and *Daphnia longispina* from different locations. In most locations, multiple *Daphnia* genotypes were sampled per pond and in some location multiple ponds were sampled. Numbers represent the number of host clones were *Pasteuria* attached to the esophagus over the total number of host clones tested per location. Each host clone was tested with at least four replicates and measurements within host clone were always consistent (all individuals either showing attachment or no attachment). A detailed representation of individual host clones can be found in Tables 2 and S4.

		Pasteuria			
		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[1]	**3/8**[1]	0/1[1]	**1/8**[1]
	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]	**1/1**[1]	0/1[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[1]	0/7[1]	**4/10**[1]	**3/7**[1]
	Italy (San Rossoro)	0/3	0/3	0/3	0/3
	(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**[1]	**5/16**[1]	**4/12**[1]	**0/16**[1]
	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**[1]
	Iran (Tabriz)	**3/3**[1]	**3/3**[1]	**3/3**[1]	**0/3**[1]

[1]Represent cases were infection trials were conducted (with either eight or 10 replicates). The consistency between attachment and infection only held in native combinations of *Daphnia* and *Pasteuria* (in gray). In nonnative host–parasite combinations no infections are observed.

subgenus *Hyalodaphnia* (with *D. longispina*; >100 million years). Trans-species polymorphisms for defense against parasites have been found in other taxa, for example, vertebrate MHC (>65 million 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 spore attachment, it may be among the oldest trans-species polymorphisms for host defense ever recorded and the first outside the vertebrates. To maintain trans-species polymorphism, some form of balancing selection is needed. Both coevolution by negative frequency-dependent selection and selection that varies in space and time can generate balancing selection (Ferguson et al. 2008) and are likely to operate in the *Daphnia–Pasteuria* system. With high virulence (Ebert et al. 2004), highly specific infection (Luijckx et al. 2011) and host resistance congruent with a matching allele model (Luijckx et al. 2013), the prerequisites for coevolution by negative frequency-dependent selection are met, and indeed empirical evidence consistent with negative frequency-dependent selection has been found for *D. magna* interacting with *Pasteuria* (Decaestecker et al. 2007; J. Andras unpubl. data). Differences in selection for resistance against *Pasteuria* in time and space are also likely to occur. The Finnish meta-population in this study consists of a large number of ponds scattered over thousands of islands, yet *Pasteuria* has been reported from only a few ponds (Ebert et al. 2001), indicating that potential selection for resistance only occurs locally. Coupled with frequent extinction and recolonization (Pajunen 1986; Altermatt et al. 2008), this may create fluctuating selection regimes in time and space (Thompson 2005). In accordance with this, we find large

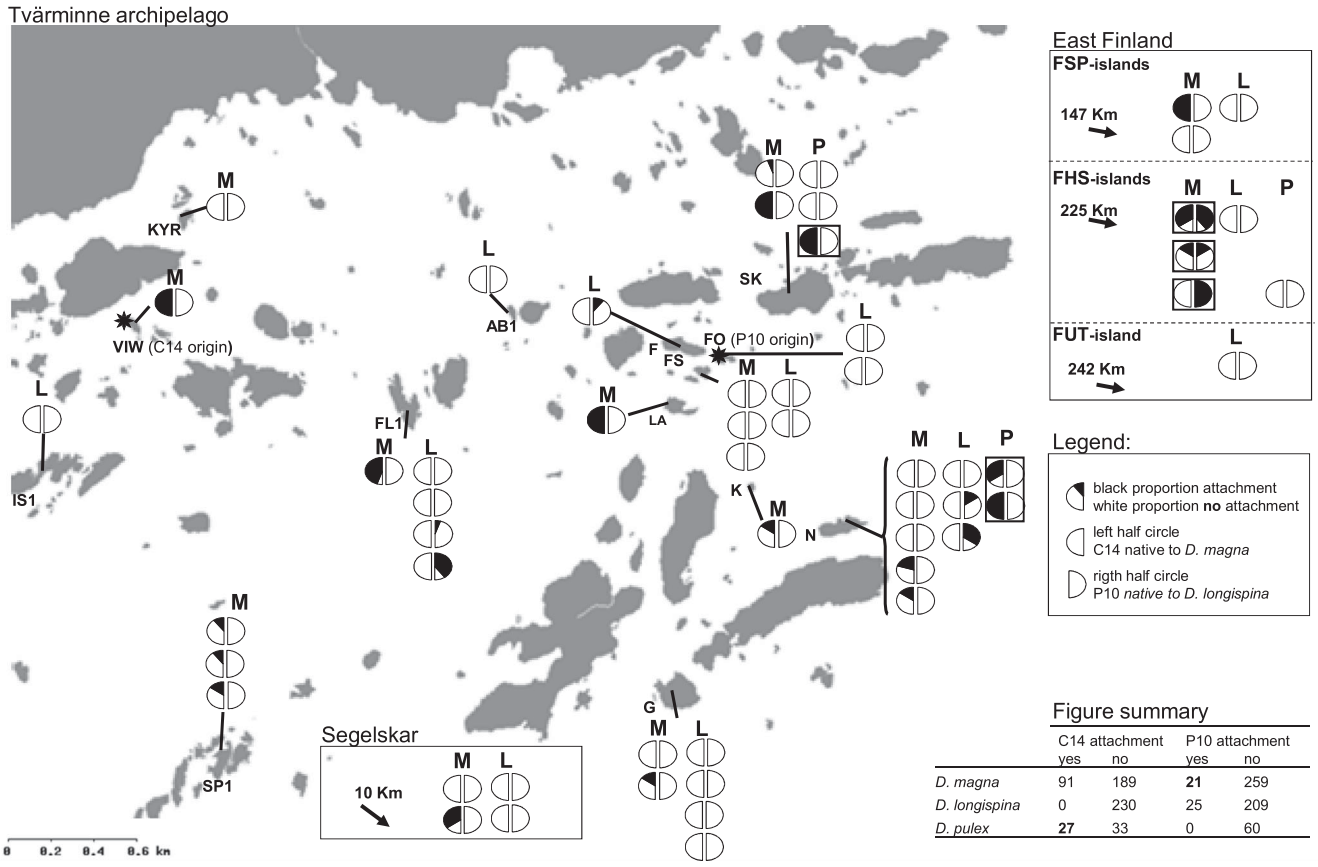

Figure 1. ***Daphnia*** **from rockpools of the islands of the Finnish Skerry archipelago, where the species *Daphnia magna*, *Daphnia longispina*, and *Daphnia pulex* co-occur, were tested for attachment of *Pasteuria* spores to the esophagus with two *Pasteuria* that were isolated from the same metapopulation. *Pasteuria* 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 nonnative combinations represented by black box). Each population is represented by a circle diagram with on the left half proportion of attachment of C14 out of 10 host clones 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*. Total number of observations per host species can be found in the summary table (bottom right, nonnative combinations in bold).**

differences in resistance profiles of populations on a small geographic scale (Fig. 1).

The second explanation for the specific attachment across species is based on convergent evolution. Similar to the results 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 *Pasteuria*.

The third explanation posits that *Pasteuria* interacts with a genetic polymorphism that is maintained by another process, not related to host parasite coevolution. We are not aware of examples for such a scenario. Given the apparent strong selection of *Pasteuria* on the host population (Little and Ebert 2000), it seems unlikely that the polymorphism is not influenced by *Pasteuria–Daphnia* interactions.

PASTEURIA CONSIST OF MULTIPLE HOST VARIETIES

One of the aims of this study was to test whether *Pasteuria* is composed of different varieties, which are specific to different host species. The presence of multiple host varieties within parasites "species" has been reported for other parasites, and the difficulties in distinguishing cryptic species is usually attributed to the highly derived and reduced morphology of parasites. For example, anther smut fungus *Microbotryum violaceum* (Bucheli et al. 2000), the seabird tick *Ixodes uriae* (Mccoy et al. 2001), and the spiny-headed worm *Leptorhynchoides thecatus* (Steinauer et al. 2007) were shown to be composed of cryptic host-specific species. *Pasteuria* seems to be another example. We found that three *Pasteuria* clones native to *D. magna* and an isolate native to *D. longispina* showed specificity for infection in their native host species. Attachment is perfectly consistent with infection within native combinations, but not in nonnative combinations where

Table 2. Attachment-tests and infection trials with C14 from *Daphnia magna* and P10 from *Daphnia longispina* on clones of *D. longispina*, *D. magna*, and *Daphnia 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 (gray "cases"), however none of the nonnative host–parasite combinations that showed attachment became infected. Each cell for the attachment-test represents the percentage of observed attachment in four individuals and each cell for the infection trial represents the percentage out of eight replicates were infection was observed in infection trials. Attachment and infection data from other locations in Europe (and Iran) showed similar patterns (Table S4) and the total number of host clones per location where attachment was observed/the total number of tested host clones per location can be found in Table 1.

		Pasteuria			
		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

attachment sometimes, but infection never occurs (Tables 1, 2). Attachment in nonnative hosts without infection indicates that the parasite fails in subsequent steps of the infection process (host penetration or proliferation). Thus, although spore attachment and activation seem to be conserved, later steps in the infection process appear to have diverged among the different host species. This is consistent with the notion of Decaestecker et al. (2007) and Duneau et al. (2011) who suggested that genes underlying the different stages in the infection process follow different evolutionary dynamics. Yet, most theoretical studies pertaining to evolutionary dynamics of infection consider infection to be a single-step process coded by a single set of genes. Studies that have considered a multistep process have found that the outcome of the evolutionary process can be dominated by a single step in the infection

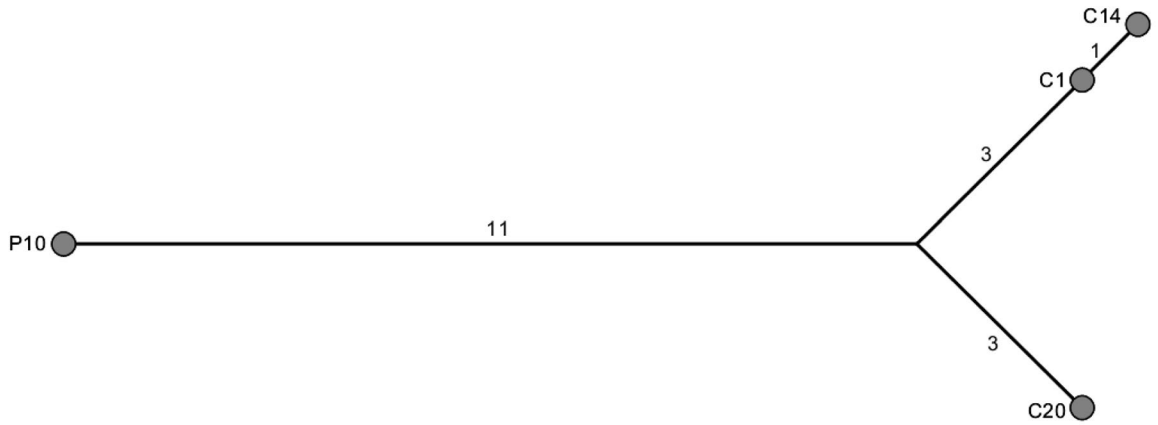

Figure 2. Haplotype network of clones/isolates of *Pasteuria*. A median-joining maximum parsimony network based on 19 SNPs across 1346 base pairs of sequence illustrates the relatively high divergence of P10 originating from *Daphnia longispina* relative to *Pasteuria* types originating from *Daphnia magna*. Each node is labeled with the clone/isolate name and branch lengths are scaled to the number of mutational steps (reported as numbers above the branches).

process (Agrawal and Lively 2003), although another study using an alternative genetic basis of resistance found many parameter settings where evolution happened across multiple steps in the infection process (Fenton et al. 2012). Our study and others (Duneau et al. 2011; Luijckx et al. 2013) strongly suggest that, within native combinations of *Daphnia* and *Pasteuria*, the attachment step dominates the outcome of the infection process. However, it is also clear that subsequent steps of the infection process limit the host range.

If our finding of a narrow host range is generally valid, it would imply that *Pasteuria* consists of a complex of multiple co-occurring cryptic host varieties. The findings of Stirnadel and Ebert (1997) that *Pasteuria* infected three *Daphnia* species in populations in South England, could then be explained by the presence of multiple *Pasteuria* varieties within a multihost community. This hypothesis is consistent with the clear differences in the epidemiological dynamics of *Pasteuria* infections in the three host species observed in these populations (H. A. Stirnadel and D. Ebert, unpubl. data). The haplotype network of *Pasteuria* lines used in this study gives further support to the hypothesis of cryptic varieties. The four lines of *Pasteuria* used in our study were morphologically indistinguishable, but showed substantial genetic differentiation (Fig. 2). Co-occurrence of multiple host varieties is supported by our data from Finland where the three *Daphnia* species live in sympatry (Tables 1, 2), yet *Pasteuria* lines infect only their native host species. Consistent with the newly emerging picture, are findings from ongoing field studies in Swiss and Finish ponds with sympatric *D. magna*, *D. pulex*, and *D. longispina* where over periods of several years only one *Daphnia* species was found to be infected with *Pasteuria* (J. Andras, unpubl. data; D. Ebert, unpubl. data). Cross-infection of *Pasteuria* native to *D. magna* to the South African species *D. dolichocephala*, which is in the same subgenus as *D. magna* was previously reported (Duneau et al. 2011). However, the finding that parasites are often host-specific, but occasionally are able to infect so-called nonhosts, is well known for other systems (see Antonovics et al. 2013, for a conceptual review).

The high specificity of *Pasteuria* may have implications for host and parasite demographic and evolutionary dynamics, which can depend on the degree of host specialization (Barrett et al. 2008). In contrast to generalists, parasites with a narrow host range are more likely to be locally adapted (Lajeunesse and Forbes 2002). In accordance with this, local adaptation for spore production was suggested for *Pasteuria* infecting *D. magna* (Ebert et al. 1998). In addition, parasites restricted to a single host species are expected to experience more frequent local extinction and recolonization events, which may influence genetic structure and effective population size (Barrett et al. 2008). The host range of a parasite may also have consequences for the evolution of virulence (Woolhouse et al. 2001). According to theory, parasites with a narrow host range can evolve to high levels of virulence whereas generalist parasites need to trade-off virulence across different host species (Regoes and Nowak 2000). High virulence is indeed observed in the *D. magna–Pasteuria* system (Ebert et al. 2004). Finally, our finding of narrow host range supports previous evidence for antagonistic coevolution by negative frequency-dependent selection in the *D. magna* and *Pasteuria* system (Decaestecker et al. 2007; Ebert 2008) and may suggest that coevolution among the different combinations of *Daphnia* and *Pasteuria* can maintain genetic variation and sexual reproduction (Jaenike 1978; Red Queen Theory, Hamilton 1980).

The narrow host range of *Pasteuria* may also have implications for community dynamics. The occurrence of *Pasteuria* may alter the potential for coexistence of *Daphnia* species by

parasite-mediated competition similar to the *Anolis* lizard parasite *Plasmodium azurophilum*. In areas where this parasite is absent *Anolis gingivinius* outcompetes *Anolis wattsi*, whereas the presence of *P. azurophilum* reduces the competitive ability of *A. gingivinius* allowing *A. wattsi* to coexist (Schall 1992). Similar evidence is missing for *Daphnia*, but *Daphnia* are known to differentially alter phytoplankton composition (Kasprzak and Lathrop 1997) and a change in *Daphnia* composition could thus have consequences for the entire community.

Conclusion

Our study highlights the explanatory power of separating the different steps in the infection process for understanding the evolution of host–parasite interactions. The three easily distinguishable steps in the infection of *Daphnia* by *Pasteuria* (activation, attachment, and proliferation) show contrasting patterns. The activation step showed neither within nor between host species variation (Duneau et al. 2011), the attachment step shows polymorphism both within and between host species, and the postattachment step(s) reveals host species specificity. Thus, it is likely that different forms of selection act on the genes underlying these steps. Using a step-wise approach, our study resolved the conflicting reports on host range in the *Daphnia–Pasteuria* system. This has consequences for our understanding of local adaptation, evolution of virulence, parasite-mediated competition, and coevolution in this model system. Similar approaches may help to gain explanatory power in other host–parasite systems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. GPS coordinates of sampled rockpools of the islands of the Finnish Skerry archipelago (corresponds to locations shown in Fig. 1 in the main text).

Table S2. GPS coordinates of sampling locations of *Daphnia* used to test for consistency of the attachment test within host clone (corresponds to Tables 1 and 2 in the main text).

Table S3. GPS coordinates of the additional sampling locations from Europe and Iran used to verify the generality of our results (locations correspond to Tables S4 and 1 in the main text).

Table S4. Full data table of the attachment-tests showing the results of four *Pasteuria* lines tested on *Daphnia magna*, *Daphnia pulex*, and *Daphnia longispina* from different locations.