Genotypic variation in an ecologically important parasite is associated with host species, lake and spore size

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Abstract
Genetic variation in parasites has important consequences for host–parasite interactions. Prior studies of the ecologically important parasite Metschnikowia bicuspidata have suggested low genetic variation in the species. Here, we collected M. bicuspidata from two host species (Daphnia dentifera and Ceriodaphnia dubia) and two regions (Michigan and Indiana, USA). Within a lake, outbreaks tended to occur in one host species but not the other. Using microsatellite markers, we identified six parasite genotypes grouped within three distinct clades, one of which was rare. Of the two main clades, one was generally associated with D. dentifera, with lakes in both regions containing a single genotype. The other M. bicuspidata clade was mainly associated with C. dubia, with a different genotype dominating in each region. Despite these associations, both D. dentifera- and C. dubia-associated genotypes were found infecting both hosts in lakes. However, in lab experiments, the D. dentifera-associated genotype infected both D. dentifera and C. dubia, but the C. dubia-associated genotype, which had spores that were approximately 30% smaller, did not infect D. dentifera. We hypothesize that variation in spore size might help explain patterns of cross-species transmission. Future studies exploring the causes and consequences of variation in spore size may help explain patterns of infection and the maintenance of genotypic diversity in this ecologically important system.

Introduction
Most parasite species contain substantial diversity (Thompson and Lymbery, 1990), and one of the grand challenges in understanding the evolution of infectious diseases is to understand what promotes this genotype diversity (Metcalf et al., 2015). Genetic variation within parasites could lead to variation in infectivity (e.g. Luijckx et al., 2011; Thrall et al., 2012; Koskella, 2014), virulence (e.g. Morrison et al., 2010; Hawley et al., 2013; Audebert et al., 2020), and other important traits, such as the ability to survive and disperse in the environment (e.g. Tack et al., 2014; Mahmud et al., 2017; Rogalski and Duffy, 2020). Thus, not only is genetic variation within parasites common, it is also important to the ecology and evolution of host–parasite systems.

Although genetic variation is common and critical for predicting parasite evolution, it is not universal. Even just considering fungal parasites, some are highly diverse (e.g. the biocontrol agent Beauveria (Serna-Dominguez et al., 2019)), whereas others have extremely low diversity (e.g. Batrachochytrium dendrobatidis (James et al., 2009), Geomyces destructans (Ren et al., 2012), Raffaelea lauricola (Wuest et al., 2017)). Low genetic diversity equates to low effective population size, and may result from recent, rapid geographic spread or clonal reproduction (e.g. Leopardi et al., 2015; O’Hanlon et al., 2018). However, in other cases, diversity of a parasite is surprisingly low even in systems where the parasite is not thought to have recently invaded new hosts and habitats. One example of this is the ecologically important host–parasite system comprised of the fungus Metschnikowia bicuspidata and its zooplankton (daphnid) hosts, where infections can reach high prevalences (~60%) of the population with late stage infections at the peak of large outbreaks (Shaw et al., 2020). Intriguingly, research from the 1880s in Europe (Metschnikoff, 1884) and 1970s in the USA (Green, 1974) suggests that M. bicuspidata has likely had a world-wide distribution for centuries.

Given its widespread geographic distribution and high prevalence within populations, it is surprising that prior studies have failed to find significant intraspecific variation in M. bicuspidata. Parasites collected from different lakes and in different years did not differ in their infectivity or virulence (Duffy and Sivars-Becker, 2007; Searle et al., 2015); parasite populations did not respond to artificial selection on (a) infectivity or virulence (Duffy and Sivars-Becker, 2007), (b) within host growth rate (Auld et al., 2014), or (c) fungicide resistance (Cuco et al., 2020); and comparisons of the SSU ITS, and partial LSU regions found identical sequences for M. bicuspidata collected on different continents from different host species (Wolinska et al., 2009). However, these studies were not designed to characterize diversity across regions and hosts. First, the studies on phenotypes (Duffy and Sivars-Becker, 2007;
Auld et al. (2014; Searle et al., 2015; Cuco et al., 2020) used *M. bicuspidata* collected from a single region in a single host, but evolutionary forces could generate variation between regions and hosts. Second, the genetic study (Wolinska et al., 2009) used relatively conserved loci, which often cannot separate geographic populations or even species for certain fungal taxa. Thus, broader sampling with more sensitive markers might uncover diversity.

We sought to uncover diversity in *M. bicuspidata* by genotyping parasites at microsatellite loci, which are generally more variable than the previously assayed SSU, ITS, and LSU loci (Chistiakov et al., 2006) and by collecting samples from two regions and from two host species. In particular, we hypothesized that *M. bicuspidata* genotypes might differ across host species because, while *M. bicuspidata* can infect multiple hosts (Auld et al., 2017), when two host species co-occur, it is common to see an outbreak in one host species but not the other. This is especially true in our studies of populations dominated by *Daphnia dentifera* and *Ceriodyphnia dubia* (data presented below). In prior studies, we have found that *C. dubia* is largely resistant to infections with *M. bicuspidata* isolated from *D. dentifera* (Strauss et al., 2015; Auld et al., 2017). These hosts vary substantially in adult body size (Dodson et al., 2010), and we had observed that *M. bicuspidata* spores in smaller-bodied hosts such as *C. dubia* were often notably smaller than those seen in *D. dentifera* in natural infections. Together, this led us to hypothesize that different host species harbour previously unseen variation in *M. bicuspidata*, and that this among-host variation might be associated with key parasite traits.

We conducted a study aimed at quantifying genetic variation in this ecologically important parasite. First, we monitored *M. bicuspidata* prevalence in two host species, *C. dubia* and *D. dentifera*. Second, we developed microsatellite markers and, with these, quantified intraspecific variation in *M. bicuspidata*. Second, we developed microsatellite markers and, with these, quantified intraspecific variation in *M. bicuspidata*.

**Materials and methods**

**Study system**

Zooplankton communities experience outbreaks of *M. bicuspidata* in late summer and autumn (Shaw et al., 2020). Grazing hosts consume infective, needle-shaped spores floating in the water; infection occurs if these spores pierce through the gut epithelium and are not successfully thwarted by the host immune response (Metschnikoff, 1884; Stewart Merrill and Cáceres, 2018; Stewart Merrill et al., 2020). The parasite replicates within the host body cavity (Stewart Merrill and Cáceres, 2018), and spores are released into the water after host death (Ebert, 2005) either as the cadaver decays or as a result of predation (Cáceres et al., 2009; Duffy, 2009).

Within the communities studied, *D. dentifera* and *C. dubia* are commonly infected hosts. However, these hosts are likely different selective environments for *M. bicuspidata* and potentially impact its diversity at the within-host or lake level. Importantly, the hosts differ in body size at maturity, with *C. dubia* adults being ~1 mm and *D. dentifera* adults being ~1.5–2.5 mm (Dodson et al., 2010), which could affect parasite infection or spore production (Auld et al., 2017). Indeed, within *D. dentifera*, *M. bicuspidata* produces more spores in larger hosts (Hall et al., 2009; Penczykowski et al., 2014; Civitello et al., 2015), likely due to space and/or resource constraints. Additional traits that affect infection such as spore capture during feeding, penetrability of the gut epithelium, or immune responses could also differ between the host species. In previous lab assays, infectivity and spore production was substantially lower in *C. dubia* than in *D. dentifera* (Strauss et al., 2015; Auld et al., 2017). However, in those studies, spores were sourced only from infected *D. dentifera* (i.e., collected by grinding up infected *D. dentifera* hosts) rather than *C. dubia* hosts. Although *D. dentifera* and *C. dubia* co-occur in many lakes (Tessier and Woodruff, 2002; Hall et al., 2010), their habitat preferences differ (Desmarais and Tessier, 1999; Strauss et al., 2016), so abundances of the two hosts vary. Parasite genetic diversity could thus be influenced by the distribution of hosts in lakes across a landscape.

**Field survey**

In order to quantify outbreak size in *D. dentifera* and *C. dubia*, we surveyed 15 lakes near Ann Arbor, Michigan and 35 lakes in Greene and Sullivan Counties, Indiana. Lakes were sampled approximately every 2 weeks from mid-July until mid-November 2015 by combining three vertical plankton tows from different locations in the deepest part of the lake. These live samples were subsampled within 36 h of collection until at least 200 *D. dentifera* and all *C. dubia* in those subsamples were counted and diagnosed visually (under a dissecting microscope) for infection with *M. bicuspidata*; hosts were diagnosed as infected if they contained asci, indicating they were fully infected (Stewart Merrill and Cáceres, 2018). To quantify outbreak size, we calculated area under the infection prevalence time series for each host and lake using the trapezoid rule (Penczykowski et al., 2014), thus units for this metric are prevalence × days. A linear model was used to test the association between outbreak sizes in the two host species.

**Sample collection and genotyping**

We evaluated genetic structure of parasite populations using microsatellites. We genotyped *M. bicuspidata* from 51 infected hosts collected from five lakes in Livingston and Washtenaw counties, Michigan, and 11 lakes in Greene and Sullivan counties, Indiana, in July–November of 2015 (Tables S1 and S2). To create primers to amplify microsatellite regions, we located simple sequence repeats (with the MISA script; Thié, 2003) in the *M. bicuspidata* genome (Ahrendt et al., 2018) and then used Primer 3 software (Rozen and Skaltsky, 2000). Out of 24 candidate primer pairs, we selected nine that gave the most consistent amplification and variation between samples (Table S3). DNA extraction from infected *D. dentifera* and *C. dubia* and genotyping followed standard methods (see the Supplementary material).

Population genetic metrics were calculated using the R package poppr version 2.8.2 (Kamvar et al., 2014; see the Supplementary material). We calculated Preosti genetic distance between each parasite sample: the fraction of allelic differences between two parasite genotypes out of all loci (Wright, 1978). With these distances we constructed a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA). We generated support for each node using 1000 bootstrapped samples (Kamvar et al., 2014). The dendrogram allows for a visual inspection of how the diversity of *M. bicuspidata* genotypes are organized and if organization depends on host species, region (IN...
or MI), or lake. Then, to determine if host species, region, or lake was statistically associated with the structure of the parasite populations, we ran analyses of molecular variance (AMOVA) with the Prevosti distances among genotypes. In an AMOVA, genotypes are grouped into hierarchical categories (here: host species, region, and lake), and the significance of the similarity of genotypes in each category is tested (Excoffier et al., 1992). Since there was not an obvious hierarchy of categories in our study, we performed two AMOVAs. The first (AMOVA 1) designated host species as the highest level of hierarchy followed by region and lake. The second (AMOVA 2) designated region as the highest level of hierarchy followed by lake and host species.

Cross-infection experiment

Because M. bicuspidata infects both D. dentifera and C. dubia in nature, we tested if the parasite was equally successful infecting each host species with a cross-infection experiment. We quantified infectivity and spore production of parasites collected from D. dentifera and C. dubia in host clones of each species. For clarity we refer to animals exposed in the experiment as ‘exposed hosts’ and animals from which parasites were isolated for the experiment as ‘source hosts’. From our genotyping results, it seemed likely that cross infection patterns might differ for parasites collected from different lakes. Therefore, cross infection trials for parasites from different lakes were performed and analysed separately.

In September 2017, we used D. dentifera and C. dubia collected from plankton tows to establish unparasitized asexual isofemale lines (hereafter: ‘clones’) from Goose Lake, where an outbreak of M. bicuspidata was occurring in both host species. We used plankton tows collected from Benefiel Lake and Goose Lake in November 2017 to collect infected animals to be used as the source of M. bicuspidata spores from D. dentifera and C. dubia hosts for experimental infections. With these, we created spore slurries by homogenizing infected animals. For Benefiel Lake, we created one spore slurry by pooling infected D. dentifera and a second spore slurry by pooling infected C. dubia. Then, two to four groups of six 7-day old individuals of a given clone (5 D. dentifera clones and 5 C. dubia clones; Table 1) were exposed to 250 parasite spores/mL from the D. dentifera-sourced slurry or the C. dubia-sourced slurry. We performed the Goose Lake experiment in a similar fashion but in two blocks, with each block having different spore slurries composed of either infected D. dentifera or infected C. dubia. Due to difficulties growing up individuals of clones from both lakes, exposures were imbalanced, but this was especially the case for Goose Lake, since we had less time to grow up clones; we exposed zero to six group(s) of a given clone (5 D. dentifera clones and 5 C. dubia clones; Table 1) to the spore slurries. All exposures lasted 48 hours and took place in 80 mL of filtered (with A/E 1 μm filters, Pall) water from a lake near Ann Arbor, MI (North Lake). We routinely used filtered water from this lake for culturing Daphnia spp. and C. dubia and have never had animals become infected unintentionally (i.e. in a beaker to which we had not added Metschnikowia spores). On the day of exposure, we added algal food, 12 500 cells Ankistrodesmus falcatus/mL (‘AJT’ strain; Schomaker and Dudycha, 2021), to each beaker. On the second day of exposure, an additional 18 750 cells Ankistrodesmus falcatus/mL daily (at 20°C with a 16:8 light:dark cycle). Hosts were fed less food during exposure because this increases infection (Hall et al., 2007); afterwards, hosts were fed saturating food levels.

After 11 days, we diagnosed exposed hosts with a dissecting microscope; as with the field survey, animals were considered infected if they contained asci (Stewart Merrill and Cáceres, 2018). We ended the experiment before natural host death; death rates in natural populations indicate that hosts are likely to die from factors like predation prior to dying from virulent effects of parasites (Duffy and Hall, 2008), and spores remain infectious after infected hosts are killed by predators (Cáceres et al., 2009; Duffy, 2009).

Infected individuals from the experiment were frozen for later processing, which involved spore counts, measuring spore length, and genotyping. First, we counted spores: each infected individual was homogenized in 50 μL of water for 30 s with a battery-powered pestle. Three 10 μL aliquots of the homogenized solution were placed on a hemocytometer and spores within the grid were counted under 400× magnification. Average counts were used to quantify spore yields per infected individual. We then measured the length of a random sampling of spores from each infected individual: for each counted grid, one photograph was taken of spores at 400× magnification with a microscope camera (DP73, Olympus). The spores in view were measured with cellSens software (Olympus), and average spore length was computed across all three photographs. On average, 14.9 spores

| Experimental clone name | Host | Number of replicates exposed to M. bicuspidata from Daphnia Dentifera | Number of replicates exposed to M. bicuspidata from Ceriodaphnia dubia |
|--------------------------|------|-------------------------------------------------|-------------------------------------------------|
| BenefielDaphnia4          | D. dentifera | 3 | 3 |
| BenefielDaphnia6          | D. dentifera | 3 | 3 |
| BenefielDaphnia7          | D. dentifera | 2 | 2 |
| BenefielDaphnia14         | D. dentifera | 3 | 3 |
| BenefielDaphnia16         | D. dentifera | 3 | 3 |
| BenefielCerio13           | C. dubia | 3 | 4 |
| BenefielCerio6            | C. dubia | 4 | 4 |
| BenefielCerio10           | C. dubia | 2 | 2 |
| BenefielCerio1            | C. dubia | 4 | 4 |
| BenefielCerio15           | C. dubia | 4 | 4 |
| GooseCerioB               | C. dubia | 4/6 | 4/2 |
| GooseCerioA               | C. dubia | 4/3 | 3/3 |
| GooseCerioC               | C. dubia | 4/2 | 3/1 |
| GooseCerioi               | C. dubia | 3/6 | 3/2 |
| GooseCerioJ               | C. dubia | 3/2 | 3/1 |
| GooseDaphniaA             | D. dentifera | 1/1 | 1/1 |
| GooseDaphniaH             | D. dentifera | 1/5 | 1/4 |
| GooseDaphniaE             | D. dentifera | 1/0 | 1/1 |
| GooseDaphniaD             | D. dentifera | 0/0 | 0/1 |
| GooseDaphniaC             | D. dentifera | 0/0 | 0/1 |

The experiment with Goose Lake hosts and spores was completed in two blocks; the number before the slash indicates the numbers of beakers in the first block and the number after the slash indicates numbers of beakers in the second block.
were measured per infected animal although this ranged from 3 to 38 spores. Finally, we genotyped *M. bicuspidata* from a subset of the homogenized infected hosts (42 and 12 from the Benefiel and Goose cross-infection experiments respectively) in order to determine which parasite genotype was responsible for infection with similar methods to the genotyping study (see the Supplementary material).

We analysed experimental results (i.e. proportion infected and number and length of spores) for each lake separately with generalized linear mixed effects models or linear mixed effects models using the lme4 package version 1.1.21 (Bates et al., 2015). Proportion infected (binomial errors) and number and length of spores produced (Gaussian errors) were each modelled with an interaction between exposed host and source host (fixed effects) and with host clone included as a random effect. Beaker was included as an additional random effect for the latter two analyses where metrics were from infected individuals, to account for potential non-independence of individuals that were in the same beaker. Non-significant interactions were dropped. The experimental cross infections using spores from Goose Lake were completed in two temporal blocks (adding another random effect to the analysis for the Goose Lake experiment; Table 1). *Post hoc* in analyses where metrics were from infected individuals, to account for potential non-independence of individuals that were in the same beaker. Non-significant interactions were dropped. The experimental cross infections using spores from Goose Lake were completed in two temporal blocks (adding another random effect to the analysis for the Goose Lake experiment; Table 1). *Post hoc* in analyses where metrics were from infected individuals, to account for potential non-independence of individuals that were in the same beaker. Non-significant interactions were dropped. The experimental cross infections using spores from Goose Lake were completed in two temporal blocks (adding another random effect to the analysis for the Goose Lake experiment; Table 1). *Post hoc* in analyses where metrics were from infected individuals, to account for potential non-independence of individuals that were in the same beaker. Non-significant interactions were dropped. The experimental cross infections using spores from Goose Lake were completed in two temporal blocks (adding another random effect to the analysis for the Goose Lake experiment; Table 1). *Post hoc* in analyses where metrics were from infected individuals, to account for potential non-independence of individuals that were in the same beaker. Non-significant interactions were dropped. The experimental cross infections using spores from Goose Lake were completed in two temporal blocks (adding another random effect to the analysis for the Goose Lake experiment; Table 1). *Post hoc*

**Results**

**Field survey**

Outbreaks of *M. bicuspidata* tended to occur in either *D. dentifera* or *C. dubia*, but not in both in the same year (Fig. 1). Outbreak size in one host species was not correlated with outbreak size in the other host (*F*1,25 = 0.904, *P* = 0.351).

**Metschnikowia bicuspidata genotypes**

We found six parasite genotypes infecting *D. dentifera* and *C. dubia* hosts in our survey lakes, grouped within three distinct clades (Fig. 2). We found an average of 2.78 alleles per locus, and the six genotypes differed on average at 5.3 loci. Nei’s gene diversity (*H*<sub>G</sub>) measures the probability that two randomly drawn alleles from a given locus in a population will be different.

Over all parasites isolated from *D. dentifera* and *C. dubia*, *H*<sub>G</sub> was 0.409 (95% confidence interval (CI): (0.379, 0.422)), but for parasites infecting each host species, *H*<sub>G</sub> was lower [*D. dentifera* *H*<sub>G</sub> = 0.291, 95% CI: (0.222, 0.333); *C. dubia* *H*<sub>G</sub> = 0.290, 95% CI: (0.232, 0.324)], indicating lower diversity of genotypes infecting each individual host species.

We calculated the index of association, *I*<sub>Na</sub>, among alleles in clone corrected (data were filtered so that each multiplicity genotype was represented once) parasite genotypes to evaluate if parasites were outcrossing or clonal (Smith et al., 1993). The clone corrected index of association was 0.995 (*P* = 0.007) indicating that *M. bicuspidata* reproduces clonally.

Of the three most abundant *M. bicuspidata* genotypes, one genotype was present in both regions and found primarily infecting *D. dentifera* (Fig. 2; the single genotype in the *D. dentifera*-associated clade). The other two abundant *M. bicuspidata* genotypes were found primarily in *C. dubia* with one genotype common in Indiana lakes and the other genotype common in Michigan lakes (Fig. 2; the two most common genotypes in the *C. dubia*-associated clade). However, none of the three most prevalent *M. bicuspidata* genotypes was restricted to a single host species.

There were also three less common *M. bicuspidata* genotypes. One was found in Sycamore Lake and Shake 1 Lake (both in Indiana). Sycamore Lake only had infections in *C. dubia*, and Shake 1 Lake had low infection levels in *D. dentifera* early in the season, but not when samples were collected. The other two less common *M. bicuspidata* genotypes were found infecting hosts in Michigan lakes, Woodland and Mill. In both of these lakes, it is possible that these infections spilled over from other host species. In Woodland Lake, two copepods collected in 2014 were infected by the same *M. bicuspidata* genotype as an infected *D. dentifera* that was collected in 2015 (Fig. 2). Marine copepods have previously been found to be infected with a different species of *Metschnikowia* (Seki and Fulton, 1969); this is the first published record of *M. bicuspidata* in copepods, although we have seen *M. bicuspidata* in copepods during other sampling of Indiana Lakes, as well (S.R. Hall, personal observation). In Mill Lake, only two infected *D. dentifera* were counted over the entire season. Although outbreaks didn’t take off in any species, one infected *C. dubia*, one infected *D. ambigua*, and two infected *D. retrocurva* were also documented in this lake during fall 2015, but parasites infecting these animals were not genotyped.

Overall, parasite genotypes from infected *D. dentifera* and *C. dubia* clustered by host species, although occasionally individuals of different host species in the same lake shared the same parasite genotype, showing that each parasite genotype can infect both hosts (Fig. 2). When host species was the highest level of hierarchy (AMOVA 1), host species groups explained 32.17% of the variation between samples (*P* = 0.001, Table 2), but when it was the lowest level (AMOVA 2) it only explained 6.29% of the variation between samples (*P* = 0.177, Table 2) with lake groups accounting for 73.37% of the variation (*P* = 0.011, Table 2). Together, the AMOVAs suggest genetic structure: *D. dentifera* and *C. dubia* tended to get infected by different *M. bicuspidata* genotypes when collected from different lakes. Within lakes, there was often transmission of a given *M. bicuspidata* genotype between the host species.

**Cross-infection experiment**

The results of our cross-infection experiment differed between the two lakes. In the cross-infection experiment using exposed and source hosts from Benefiel Lake, infection and spore production depended on both the source host species and the exposed host species. The proportion of infection in *C. dubia* was higher when exposed to *C. dubia*-sourced spores, as compared to *D. dentifera*-sourced spores (Fig. 3A; source × exposed host
Fig. 2. Microsatellite genotyping of M. bicuspidata from infected D. dentifera (blue font) and C. dubia (red font) collected in fall 2015 in Indiana (IN) and Michigan (MI) lakes (USA). Genotypes of M. bicuspidata infecting two copepods collected in fall 2014 are also included. We found three parasite clades. Of these, two were particularly common, with one primarily infecting D. dentifera and the other primarily infecting C. dubia. Within the C. dubia-associated clade, genotypes fall into different clades in IN and in MI. Tip labels follow the format LakeHostDate.Replicate(State). See supplemental Table S1 for a list of samples. Scale bar indicates Prevosti distance between individuals. Bootstrap support (>40%) is noted on nodes. Source hosts used in the lab experiments were collected in a subsequent year and pooled in spore slurries (see Materials and methods); thus, individual source hosts were not genotyped and are not on the dendrogram.

Table 2. Hierarchical analysis of variance suggests genotypic variance is partitioned by host and lake

| AMOVA 1 | Observed partition | Variance component | Variance | % total | ϕ-statistics | p* |
|---------|--------------------|-------------------|----------|---------|--------------|----|
|         |                    | Between hosts     | 1.51     | 32.17   | ϕ_{host-Total} = 0.32 (greater) 0.001 |
|         |                    | Between states    | 0.53     | 11.26   | ϕ_{state-host} = 0.17 (greater) 0.167 |
|         |                    | Between lakes     | 2.00     | 42.47   | ϕ_{lake-state} = 0.75 (greater) 0.001 |
|         |                    | Within lakes      | 0.66     | 14.10   | ϕ_{lake-total} = 0.86 (less) 0.001 |

| AMOVA 2 | Observed partition | Variance component | Variance | % total | ϕ-statistics | p* |
|---------|--------------------|-------------------|----------|---------|--------------|----|
|         |                    | Between states    | 0.16     | 3.98    | ϕ_{state-total} = 0.04 (greater) 0.301 |
|         |                    | Between lakes     | 2.98     | 73.37   | ϕ_{lake-state} = 0.76 (greater) 0.011 |
|         |                    | Between hosts     | 0.26     | 6.29    | ϕ_{host-lake} = 0.28 (greater) 0.177 |
|         |                    | Within hosts      | 0.66     | 16.36   | ϕ_{host-total} = 0.84 (less) 0.001 |

The two AMOVA analyses were designed to test if genetic variance was organized by host species, region (IN or MI), or lake using a hierarchical approach. AMOVA 1 designates host species as highest level of the hierarchical analysis followed by region and lake. AMOVA 2 designates region as the highest level followed by lake and host species.

*p values are calculated by 999 random permutations of the distance matrix (composed of Prevosti distances) between genotyped parasites. Significance is attained if the observed ϕ-statistic (and variance component) is greater or less (noted in parentheses) than it would be by chance (Excoffier et al., 1992).
The combination of exposed and source hosts from Benefiel (IN) mattered for infection and spore production. (A) The proportion of infected animals depended on an exposed × source host interaction: C. dubia were most infected by C. dubia-sourced spores. Points represent beakers, and shapes represent different experimental host clones. (B) More spores were produced in exposed D. dentifera hosts; in exposed C. dubia, more spores were produced when infected with C. dubia-sourced M. bicuspidata. (C) Spores in C. dubia exposed hosts were smaller when sourced from C. dubia. The smaller spores belonged to the C. dubia-associated M. bicuspidata genotype (red fill) found in Benefiel in 2015, while the larger spores belonged to the D. dentifera-associated M. bicuspidata genotype (blue fill). (D) When infected with the C. dubia-associated genotype, exposed C. dubia hosts produced a relatively large number of small spores (red border-red fill symbols); in contrast, when infected with the D. dentifera-associated genotype, exposed C. dubia hosts produced fewer and larger spores (red border-blue fill symbols). Exposed D. dentifera hosts (blue border) only produced relatively large spores. Within exposed D. dentifera, animals that had larger spores also produced more spores. In (B)-(D) points represent individual infected hosts with shapes designating different experimental host clones. Beaker was also included as a random effect in statistical models.

Results from the cross infection with hosts and parasites from Goose Lake showed different patterns. Overall infection levels were low, and therefore no influence of source or exposed host on infection rates could be detected (Fig. 4A). More spores were produced in D. dentifera hosts (LRT = 3.84, P = 0.05; Fig. 4B), although there was no difference in spore quantities produced by M. bicuspidata from the two source host species (LRT = 0.00, P = 0.46; source species: LRT = 7.77, P = 0.003; post-hoc: D. dentifera vs C. dubia-sourced spores: z = 3.18, P = 0.008). In contrast, prevalence of infection in D. dentifera was consistent when they were exposed to C. dubia-sourced spores and D. dentifera-sourced spores (Fig. 3A).

Spore production at 11 days post infection also depended on source and exposed hosts (source host: LRT = 8.86, P = 0.003; exposed host: LRT = 6.77, P = 0.009; Fig. 3B). In exposed C. dubia, C. dubia-sourced M. bicuspidata produced more spores than D. dentifera-sourced M. bicuspidata (post-hoc: t-ratio = 2.81, P = 0.04; Fig. 3B). In exposed D. dentifera, spore production at 11 days did not differ significantly between animals infected by C. dubia-sourced and D. dentifera-sourced spores (Fig. 3B).

The size of spores produced in infections depended on source and exposed host (Fig. 3C; LRT = 25.46, P < 0.001): C. dubia-sourced M. bicuspidata produced smaller spores in exposed C. dubia hosts as compared to spores produced in exposed D. dentifera hosts sourced from either host species (post-hoc: from D. dentifera: t-ratio = −8.94, P < 0.001; from C. dubia: t-ratio = −7.77, P < 0.001) and to spores produced in C. dubia when sourced from D. dentifera (post-hoc: t-ratio = −8.69, P < 0.001). These smaller spores belonged to the most prevalent Indiana (IN) C. dubia-associated genotype (i.e. in the C. dubia-associated clade) in the 2015 survey (Fig. 2). In contrast, the larger spores belonged to the main D. dentifera-associated genotype. Ceriodaphnia dubia exposed to M. bicuspidata sourced from C. dubia became infected by both genotypes, whereas the D. dentifera exposed to spores sourced from C. dubia only became infected by the main D. dentifera-associated genotype (Fig. 3C).

Furthermore, C. dubia produced more spores when infected by the smaller-spored genotype, as compared to when they were infected by the larger-spored genotype (Fig. 3D; spore size × host species: LRT = 15.97, P < 0.001). In summary, C. dubia source hosts from Benefiel must have been infected by both genotypes when they were collected from the field. Then, in the experiment, exposed C. dubia hosts became infected by both genotypes; in contrast, D. dentifera only became infected by the larger, D. dentifera-associated genotype.

Results from the cross infection with hosts and parasites from Goose Lake showed different patterns. Overall infection levels were low, and therefore no influence of source or exposed host on infection rates could be detected (Fig. 4A). More spores were produced in D. dentifera hosts (LRT = 3.84, P = 0.05; Fig. 4B), although there was no difference in spore quantities produced by M. bicuspidata from the two source host species (LRT = 0.00, P = 0.46; source species: LRT = 7.77, P = 0.003; post-hoc: D. dentifera vs C. dubia-sourced spores: z = 3.18, P = 0.008). In contrast, prevalence of infection in D. dentifera was consistent when they were exposed to C. dubia-sourced spores and D. dentifera-sourced spores (Fig. 3A).
with the lack of a source host effect on infection rate, spore yield, and spore size on exposed host species in this lake.

Discussion

*Metchnikowia bicuspidata* is a widespread parasite of *Daphnia* (Green, 1974; Ebert, 2005) with substantial impacts on the ecology (Duffy, 2007; Duffy and Hall, 2008; Penczykowski et al., 2020) and evolution (Duffy and Sivars-Becker, 2007; Duffy et al., 2008, 2012) of its hosts. As reviewed in the introduction, prior studies failed to detect phenotypic or genetic variation in *Daphnia* hosts, even though outbreaks are large, common, and occur in multiple hosts on multiple continents. Here, using more sensitive techniques, we found significant intraspecific variation in *M. bicuspidata*. We found six parasite genotypes grouped within three distinct clades. One of these clades was rare (but included a *M. bicuspidata* genotype that infected copepods and *D. dentifera* – notable given that the most recent common ancestor of these taxa lived ~550 MYA during the Cambrian Era (Schwentner et al., 2017)). Of the two main parasite clades, one was primarily associated with *D. dentifera* and the other was primarily associated with *C. dubia*. In lake populations, outbreaks tended to occur in one species or the other. However, each of these genotypes could be found in both hosts within a lake, indicating that parasite genotypes were not completely restricted to the host species with which they were most commonly associated. In laboratory cross-infection experiments, infection outcomes depended on the lake from which parasite spores were collected, likely because only one of the two lakes contained the *C. dubia*-associated genotype. In the experiment where spores were collected from this lake (Benefiel), the *D. dentifera*-associated genotype was able to infect both host species, but produced fewer spores at 11 days post infection in *C. dubia* hosts than the *C. dubia*-associated genotype did. In contrast, the *C. dubia*-associated genotype did not infect *D. dentifera*.

The *C. dubia*-associated genotype produced smaller spores, as compared to the *D. dentifera* genotype, even when they both infected the same host species, *C. dubia*. In the experiment where spores were collected from the other lake (Goose), there was a lack of effect of source host on infection rate, spore yield, and spore size, which is consistent with only the *D. dentifera*-associated genotype causing infection in this lake.

We hypothesize that spore size might influence the ability of *M. bicuspidata* to infect different hosts, by influencing the likelihood of encountering a spore and/or the probability of infection given encounter. First, the likelihood of encountering a spore will vary based on both filtering rate (Burns, 1969) and feeding appendage structure (Geller and Müller, 1981), both of which correlate with body size. Second, once a spore is encountered, infection is a mechanical process in which spores penetrate the host’s gut wall (Stewart Merrill and Cáceres, 2018; Stewart Merrill et al., 2020); one possibility is that size could impact the probability of piercing through this barrier. Infection usually begins at the anterior or posterior bends in the gut where long, needle-like spores may ram straight into the gut wall instead of making the ‘turn’ with the

![Fig. 4.](https://doi.org/10.1017/S0031182021000949) Published online by Cambridge University Press
Fig. 5. Although both D. dentifera and C. dubia grow continuously, adult D. dentifera are larger than adult C. dubia, potentially influencing competence for the parasite. The photograph shows two adult female D. dubia (on top) and one adult female D. dentifera (below). Arrows show bends where spores most likely pierce the gut wall (Stewart Merrill and Cáceres, 2018). Photo credit: Meghan A. Duffy.
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