



Daphnia parasite dynamics across multiple *Caullerya* epidemics indicate selection against common parasite genotypes[☆]

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ABSTRACT

Studies of parasite population dynamics in natural systems are crucial for our understanding of host–parasite coevolutionary processes. Some field studies have reported that host genotype frequencies in natural populations change over time according to parasite-driven negative frequency-dependent selection. However, the temporal patterns of parasite genotypes have rarely been investigated. Moreover, parasite-driven negative frequency-dependent selection is contingent on the existence of genetic specificity between hosts and parasites. In the present study, the population dynamics and host-genotype specificity of the ichthyosporean *Caullerya mesnili*, a common endoparasite of *Daphnia* water fleas, were analysed based on the observed sequence variation in the first internal transcribed spacer (ITS1) of the ribosomal DNA. The *Daphnia* population of lake Greifensee (Switzerland) was sampled and subjected to parasite screening and host genotyping during *C. mesnili* epidemics of four consecutive years. The ITS1 of wild-caught *C. mesnili*-infected *Daphnia* was sequenced using the 454 pyrosequencing platform. The relative frequencies of *C. mesnili* ITS1 sequences differed significantly among years: the most abundant *C. mesnili* ITS1 sequence decreased and rare sequences increased over the course of the study, a pattern consistent with negative frequency-dependent selection. However, only a weak signal of host-genotype specificity between *C. mesnili* and *Daphnia* genotypes was detected. Use of cutting edge genomic techniques will allow further investigation of the underlying micro-evolutionary relationships within the *Daphnia*–*C. mesnili* system.

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1. Introduction

One of the most important questions in evolutionary biology is why sexual reproduction persists when it usually has a 50% fit-

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ness disadvantage compared to asexual reproduction (Maynard Smith, 1978). One widely accepted explanation is that reciprocal selection arising from host–parasite interactions should accelerate evolutionary rates through the need for continual adaptation and counter-adaptation. Such rapid reciprocal adaptation can be driven by either directional selection or negative frequency-dependent selection (NFDS) (Gaba and Ebert, 2009; Brockhurst and Koskella, 2013; Papkou et al., 2016). In the former case, recurrent selective sweeps of novel host resistance and parasite infectivity alleles occur through time, leading to increases in the parasite’s host range and in host resistance traits. In the latter case, common genotypes of a given host have a higher probability of becoming infected by

coevolving parasites than do rare genotypes. Rare uninfected host genotypes thus tend to have a fitness advantage and eventually replace the previously common host genotypes. At this point, parasite genotypes that are able to infect surviving host genotypes will be favoured over those that are still adapted to previously common hosts. Parasite-driven NFDS could explain the long-term maintenance of sex in host and parasite populations (reviewed in Lively, 2010), since sex is efficient at promoting genetic diversity.

A prediction of NFDS is that common hosts (and thus, common parasites which are adapted to those common hosts) are at a disadvantage. Field studies of various systems have demonstrated that host frequencies are tracked by parasites, including the systems of *Daphnia* water fleas and microparasites (e.g., Decaestecker et al., 2007; Wolinska and Spaak, 2009), bryozoans and myxozoans (Vernon et al., 1996), plants and fungi (e.g., Burdon and Thompson, 1995; Siemens and Roy, 2005) and freshwater snails and trematodes (e.g., Jokela et al., 2009; King et al., 2009). However, the majority of field surveys investigated temporal changes in relative genotype frequencies only for hosts. This exclusion of parasites is striking because NFDS predicts changes to both host and parasite frequencies.

A prerequisite for NFDS is the existence of genetic specificity in host–parasite interactions, where the outcome of infection depends on the genotypic identity of both host and parasite (Dybdahl et al., 2014). Genotype-by-genotype interactions have been demonstrated in several host–parasite systems under experimental conditions (reviewed in Sadd and Schmid-Hempel, 2009). Thus, the pattern that might be expected under natural conditions is that parasite populations within hosts differ in their genetic composition depending on the host genotypes they infect (e.g., Lythgoe, 2002; Schmid-Hempel and Funk, 2004).

Water fleas of the genus *Daphnia* (Crustacea: Cladocera) and their microparasites were recently proposed as a model system to address coevolutionary questions (Ebert, 2008; Gaba and Ebert, 2009). One of the most common microparasites infecting *Daphnia* of large European lakes is the ichthyosporean *Caullerya mesnili* (Wolinska et al., 2007; Lohr et al., 2010). *C. mesnili* is a highly virulent parasite that reduces the survival and reproduction rate of its hosts (Wolinska et al., 2006). It has also been shown to be involved in driving frequencies of host species' abundance and genotypes (Wolinska et al., 2006). Moreover, *Daphnia* genotype dynamics have been associated with NFDS; the most common host genotypes decreased in frequency in infected but not in uninfected populations (Wolinska and Spaak, 2009). However, *C. mesnili* population dynamics over longer time scales have not been explored in detail. In the present study, we analyse the population dynamics of *C. mesnili* and its host-genotype specificity in a natural *Daphnia* population during parasite epidemics of four consecutive years, based on the observed variation in the first internal transcribed spacer ITS1 marker.

2. Materials and methods

2.1. Study site

Greifensee (N 47°20'41", E 8°40'21") is a eutrophic peri-alpine lake in Switzerland. The cladoceran community of this lake is dominated by hybridising members of the *Daphnia longispina* complex (Brede et al., 2009). Epidemics of *C. mesnili* in Greifensee have been documented in previous studies (Wolinska et al., 2004, 2006). Ongoing work since 2002 demonstrates that parasite prevalence in this lake follows an epidemic pattern, with infection of 20–30% of the *Daphnia* population during autumn, and parasite presence dropping to undetectable levels during the rest of the year (Fig. 1) (Wolinska et al., 2006).

2.2. *Daphnia* sampling

Daphnia specimens from Greifensee have been regularly sampled for *C. mesnili* screening (since 2002) and for microsatellite host genotyping (since 2007), biweekly or monthly (in winter). At the deepest point of the lake, zooplankton samples were taken using a 250 µm net. In the laboratory, ~80 adult *Daphnia* individuals were randomly chosen and visually screened for *C. mesnili* infection, using a stereomicroscope (Lohr et al., 2010). Then, during each *C. mesnili* epidemic, ~80 additional infected *Daphnia* were collected (i.e. "infected sample"). These infected *Daphnia* were then genotyped using microsatellites (see Section 2.3). Here, we focus our analysis on four *C. mesnili* epidemic peaks, occurring from 2010 to 2013 (Fig. 1). *C. mesnili* DNA was obtained from infected *Daphnia* from those years; one infected sample per epidemic year was analysed (2010-08-10, 2011-09-13, 2012-09-06 and 2013-08-29).

2.3. Selection of *Daphnia* hosts for characterisation of *C. mesnili* genetic structure

In order to assign each infected *Daphnia* individual to a particular multilocus genotype (MLG) (Yin et al., 2010), all *Daphnia* specimens from each "infected sample" were characterised by ten polymorphic microsatellite markers (Brede et al., 2006; for details see Data S1 in the supplementary online Appendix). The MLGs were used to assess host genotype abundance distributions in the "infected sample" of *Daphnia* collected during four consecutive *C. mesnili* epidemics (Fig. S1 in the supplementary online Appendix). "Common" *Daphnia* genotypes were defined as those making up more than 5% of the sample, and "rare" genotypes as those which were only detected once in a sample. For each sampling period, 15 randomly selected rare genotypes, as well as all of the common genotypes, were taken for *C. mesnili* genetic analyses (genotypes neither "common" nor "rare" were excluded). If a common genotype was represented by more than ten individuals, ten specimens were randomly selected for subsequent analysis; otherwise all individual *Daphnia* belonging to a given common genotype were analysed.

2.4. Molecular analyses of *C. mesnili* ITS1 region

Primers amplifying the ITS1 region of *C. mesnili* were constructed by fusing a specific core primer sequence (forward: ACAC-CGCCCCGCTACTACTAC and reverse: TGGATATACCACTCTCAAACAG) with a basal 25-mer for binding to the DNA capture beads (Lib-A) and a 10-base multiplex identifier (MID) chosen from the 454 Standard MID Set (Roche, Basel, Switzerland) targeting the approximately 425 bp long ITS1 region (González-Tortuero et al., 2015). DNA samples previously used for *Daphnia* microsatellite genotyping were purified by re-precipitation in 70% EtOH and resuspended in TE buffer. *C. mesnili* from infected *Daphnia* DNA samples was amplified using the following protocol: KAPA2 G Robust Ready Mix (Kappa Biosystems, Wilmington, MA, USA), 0.3 µM each of forward and reverse primer, 0.8 mM BSA, and 2 µl genomic DNA, for a total reaction volume of 14 µl. The PCR cycling parameters included initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, followed by a final elongation at 72 °C for 7 min. The success of amplification was verified by agarose gel electrophoresis.

To construct a 454 library, PCR products were purified independently for each MID-labelled sample (i.e., isolated from each infected *Daphnia*) with a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Taipei, Taiwan), their DNA concentration was measured on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and then they were pooled in equimolar concentrations. The final poolplex was further purified by separation

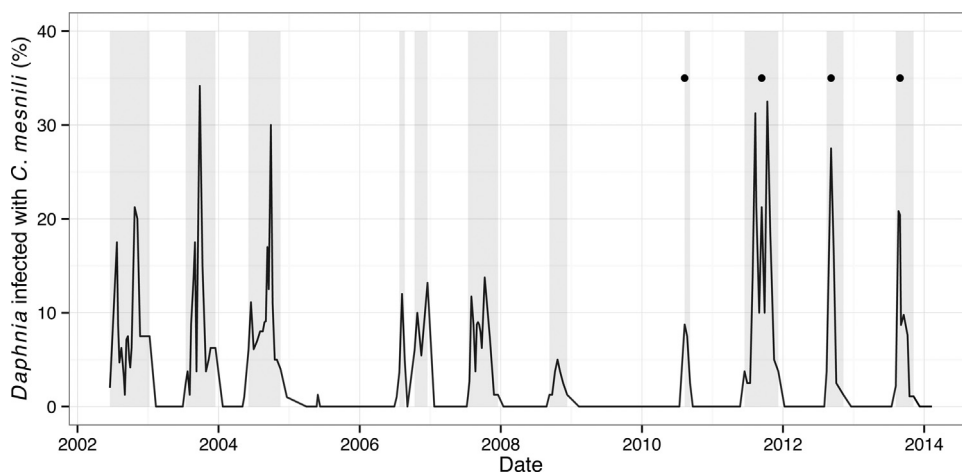


Fig. 1. *C. mesnili* prevalence in the *Daphnia* population of Greifensee, 2002 to 2014. Epidemic periods in which the prevalence of infection exceeded 2% are indicated in dark grey. Samples collected for genetic analyses are shown with a dot. X-axis labels indicate beginning of year.

on Pippin Prep (Pippin Prep Kit CSD2010; size-selection range 400–580 bp; Sage Science, Beverly, MA, USA) and purified with SPRI AMPure XP paramagnetic beads (Beckman Coulter Genomics, Danvers, MA, USA). Emulsion PCR and pyrosequencing were performed with the amplicon (Lib-A) kit, using GS Junior reagents and the manufacturer's protocols (454 Life Sciences, Branford, CT, USA), with the input ratio of DNA molecule-to-bead decreased to 0.4. DNA bead enrichment level was within the expected range (6%). The raw 454 dataset is available in the Sequence Read Archive (SRA) under accession number SRP065924.

2.5. Identifying representative ITS1 sequences of *C. mesnili*

The raw *C. mesnili* dataset was analysed using the Quantification of Representative Sequences (QRS) pipeline (González-Tortuero et al., 2015) to determine the number and frequency of representative sequence variants. A representative sequence is defined as the most abundant sequence per sequence cluster, and could be considered the presumed ancestral allelic reference (González-Tortuero et al., 2015). This reduction of variation is useful for population structure analysis when a multicopy marker (like the ITS1) is considered, as it helps mitigate potential overestimation of polymorphism (Giessler and Wolinska, 2013) as well as minor sequencing errors. These representative sequences were subsequently used instead of the raw data. The pipeline was run with default parameters (unless indicated otherwise; see Data S2 in the supplementary online Appendix). The most abundant ITS1 representative sequences are available in the GenBank sequence database under the accession numbers KU094678–KU094681.

2.6. Haplotype network of *C. mesnili*

A haplotype network was constructed for the abundant ITS1 representative sequences ("abundant" refers to representative sequences that reached overall frequencies higher than 0.5%). Connection distances between haplotypes were calculated using TCS (Clement et al., 2000) according to the statistical parsimony algorithm. The output was processed using a force-directed algorithm, implemented in Cytoscape 3.2.1 (Shannon et al., 2003). Additionally, to test if the ITS1 representative sequences detected here were present in previous studies, the abundant *C. mesnili* representative sequences obtained here were compared with those from a previous study (González-Tortuero et al., 2015). In that study, *C. mesnili*-infected *Daphnia* were sampled across seven reservoirs in

the Czech Republic (González-Tortuero et al., 2015). Here, all abundant representative sequences were re-aligned using the MUSCLE algorithm (Edgar, 2004) and manually corrected. Subsequently, a haplotype network was created as described above.

2.7. Temporal variation of *C. mesnili*

To investigate temporal variation in *C. mesnili*, two types of analyses were performed. First, the frequencies of *C. mesnili* representative sequences were compared among years, using a Chi-squared test. Second, to describe the temporal trend, a Mann–Kendall trend test (Mann, 1945; Kendall, 1948) was performed on the residuals from the locally weighted scatterplot smoothing (LOWESS) (Cleveland, 1979), with the frequency of *C. mesnili* representative sequences as the dependent variable and time (year) as the independent variable. These statistical tests were performed in R (R Core Team, 2015). The Kendall (Hipel and McLeod, 2005) package was used for the Mann–Kendall trend test. For these analyses, all *C. mesnili* sequences were pooled per year, regardless of their host genotype.

2.8. Host-genotype specificity of *C. mesnili*

To assess differences in *C. mesnili* population structure between *Daphnia* genotypes, two types of statistical tests were performed. The tests were run by year (2010, 2011, 2012 and 2013) and only *C. mesnili* sequences detected on common *Daphnia* genotypes were considered. First, an analysis of molecular variance (AMOVA) was performed at three hierarchical levels: within a *Daphnia* individual, within a *Daphnia* genotype (i.e. among individuals that represented the same genotype) and among *Daphnia* genotypes. Second, to visualise the potential differences in the distribution of *C. mesnili* representative sequences sampled from different *Daphnia* genotypes, a non-metric multidimensional scaling (nMDS) plot was constructed, using the Bray–Curtis metric, and a permutational MANOVA was conducted (PERMANOVA) (Anderson, 2001). These statistical tests were performed in R using the *ade4* (Dray and Dufour, 2007) package for the AMOVA test and the *vegan* (Oksanen et al., 2015) and *MASS* (Venables and Ripley, 2003) packages for the nMDS and PERMANOVA.

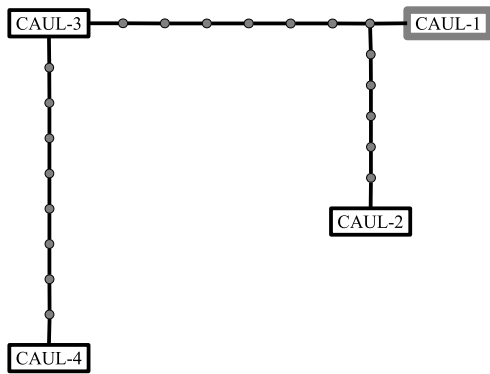


Fig. 2. Haplotype network of the abundant ITS1 representative sequences of *C. mesnili* in Greifensee. Each grey dot indicates a single connection step (i.e. a single mutation) between the ITS1 representative sequences. The grey-lined box indicates the most abundant ITS1 representative sequence.

3. Results

3.1. Selection of *Daphnia* hosts for characterisation of *C. mesnili* genetic structure

The genotype frequency distributions of infected *Daphnia* specimens fitted the expected pattern of many rare and few common genotypes (Fig. S1 in the supplementary online Appendix). There were 10 *Daphnia* genotypes that met the “common genotype” threshold: three in 2010 and 2013, and two in 2011 and 2012 (Table S1 in the supplementary online Appendix). An additional 15 *Daphnia* individuals per sampling year were randomly selected from the rare genotype pool. In total, *C. mesnili* ITS1 was separately sequenced from 145 infected *Daphnia* individuals (Table S1).

3.2. Description of the *C. mesnili* ITS1 dataset

Processing with the QRS pipeline yielded 20,645 *C. mesnili* ITS1 sequences (out of 76,719 available sequences). The majority of the sequences removed were singletons. Moreover, if less than 10 sequences remained per *Daphnia* host, these *Daphnia* hosts were excluded from further analyses, resulting in 138 analysed *Daphnia* (out of the 145 that were originally sequenced; Table S1). The average number of *C. mesnili* ITS1 sequences retained per *Daphnia* was 149.5, with a standard deviation of 82.9. The length of the alignment was 460 bp (see the Fasta file in the supplementary online Appendix).

3.3. Identifying representative ITS1 sequences of *C. mesnili*

1,062 unique representative sequences were detected in the *C. mesnili* dataset. Across the entire dataset (i.e. all *Daphnia* genotypes and years pooled), the most abundant *C. mesnili* ITS1 representative sequences reached a frequency of 77.45% (CAUL-1), 7.89% (CAUL-2), 1.83% (CAUL-3) and 0.86% (CAUL-4). The remaining 1,058 representative sequences were present at proportions lower than 0.5% and were classified as rare.

3.4. Haplotype network

The haplotype network had an almost linear structure, with the most abundant *C. mesnili* ITS1 representative sequence (CAUL-1) placed between the other abundant representative sequences (Fig. 2). The second most abundant *C. mesnili* representative sequence (CAUL-2) matched the C2.14-type from a previous study (González-Tortuero et al., 2015), which was the most abundant type in that study. In a joint haplotype network of representative

Table 1

Results of AMOVA tests of the distribution of *C. mesnili* ITS1 representative sequences in Greifensee, per year. Only *C. mesnili* sequences originating from “common” *Daphnia* genotypes were included in these analyses.

Year	Source of variation	df	Percent variation	P
2010	Among <i>Daphnia</i> genotypes	2	-0.05	0.003
	Within a <i>Daphnia</i> genotype	23	1.65	<0.001
	Within a <i>Daphnia</i> individual	5235	98.40	<0.001
2011	Among <i>Daphnia</i> genotypes	1	0.21	0.007
	Within a <i>Daphnia</i> genotype	13	0.69	<0.001
	Within a <i>Daphnia</i> individual	2137	99.10	<0.001
2012	Among <i>Daphnia</i> genotypes	1	-0.06	0.067
	Within a <i>Daphnia</i> genotype	14	1.70	<0.001
	Within a <i>Daphnia</i> individual	2550	98.36	<0.001
2013	Among <i>Daphnia</i> genotypes	2	0.22	0.050
	Within a <i>Daphnia</i> genotype	20	2.94	<0.001
	Within a <i>Daphnia</i> individual	1900	96.83	<0.001

sequences (i.e. those from the present study as well as from the previous study by González-Tortuero et al., 2015), the two most abundant representative sequences (CAUL-1 and C2.14-type) were present at the centre of the network (Fig. S2 in the supplementary online Appendix).

3.5. Temporal variation of *C. mesnili*

The relative frequencies of the *C. mesnili* ITS1 representative sequences differed among the years analysed (Chi-squared test: $\chi^2 = 1138.4$, $P < 2.2 \times 10^{-16}$; Fig. 3). A decrease in the most abundant *C. mesnili* ITS1 representative sequence (CAUL-1) and an increase in the rare representative sequences were the most pronounced trends. The significant changes in the relative abundance of representative frequencies over time were confirmed by a Mann–Kendall test (Fig. 4).

3.6. Host-genotype specificity of *C. mesnili*

Differences in the distribution of *C. mesnili* ITS1 representative sequences among different *Daphnia* genotypes were tested with an AMOVA (separately per epidemic year). The largest amount of variation was observed at the “within a *Daphnia* individual” level (up to 99.1% in 2011). Only a very small proportion of the variation was explained by differences in the frequencies of *C. mesnili* ITS1 representative sequences among *Daphnia* genotypes (this level of variation was significant in 2010 and 2011; Table 1). Differences in ITS1-based *C. mesnili* genetic structure between the *Daphnia* genotypes were visualised in nMDS plots, where the position of individual points is based on the frequencies of specific ITS1 representative sequences per *Daphnia* host (Fig. 5). The genotype centroids overlapped in 2010 (for two of the three analysed genotypes) and in 2011 (for both analysed genotypes). In 2012 and 2013, the group centroids were distinct. The results of the nMDS plots were supported by PERMANOVA tests, which indicated significant differences in the presence and abundance of representative *C. mesnili* ITS1 sequences among *Daphnia* genotypes in 2013 only (2010: $F = 0.782$, $P = 0.470$; 2011: $F = 0.590$, $P = 0.631$; 2012: $F = 2.220$, $P = 0.104$; 2013: $F = 2.342$, $P = 0.038$).

4. Discussion

In natural populations, tracking parasite dynamics over time remains difficult (reviewed in Penczykowski et al., 2015). Except for the *Trichostrongylus tenuis*–red grouse system (Hudson and Dobson, 1997) and the St. Kilda Soay Sheep Project (e.g., Wilson et al., 2004), the majority of studies about temporal changes in parasite populations to date have been performed in the laboratory under controlled conditions (e.g., Koskella and Lively, 2009; Schulte

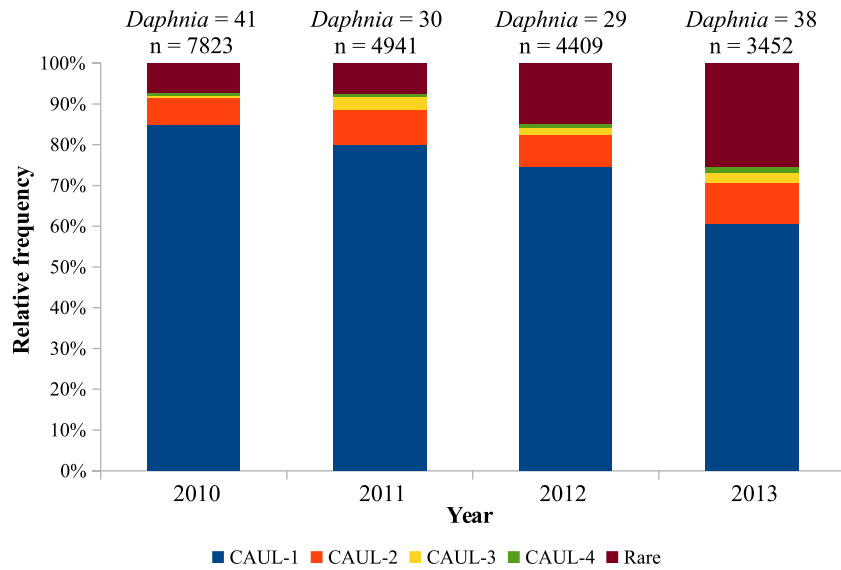


Fig. 3. Comparison of relative frequencies of the ITS1 representative sequences of *C. mesnili* among epidemic years in Greifensee, 2010 to 2013. The number of infected *Daphnia* individuals that were used for *C. mesnili* ITS1 sequencing and the number of analysed ITS1 sequences are shown above each stacked bar graph.

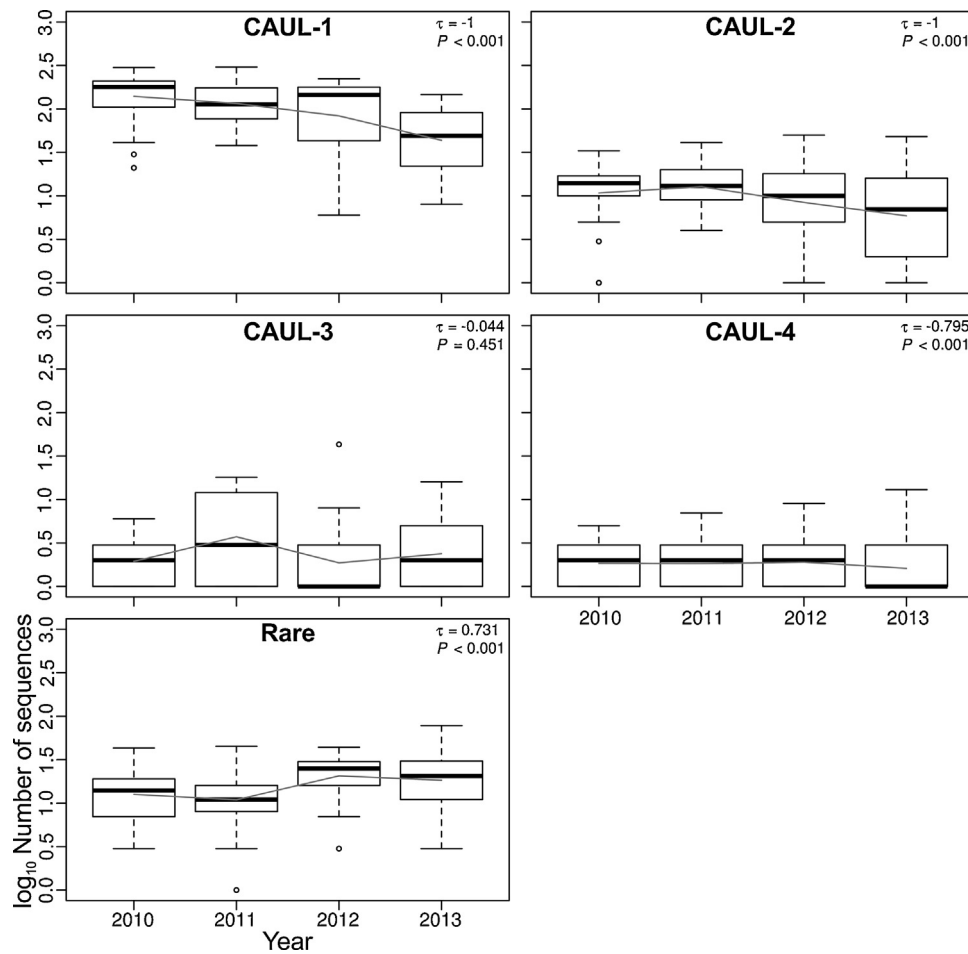


Fig. 4. Change over time in the number of *C. mesnili* representative sequences (i.e. CAUL-1, CAUL-2, CAUL-3, CAUL-4, and rare group) in Greifensee, 2010 to 2013. The grey line connecting the boxes describes the temporal trend of the representative sequences' abundance based on the LOWESS smoothing graph. Results from a Mann-Kendall trend test (i.e. Kendall's τ and P value) are shown in the top right corner.

et al., 2010). Although such experiments are necessary to reduce environmental noise which can otherwise conceal important factors and processes, they tend to oversimplify natural conditions.

Since parasite population dynamics are highly sensitive to environmental changes (reviewed in Wolinska and King, 2009), studies

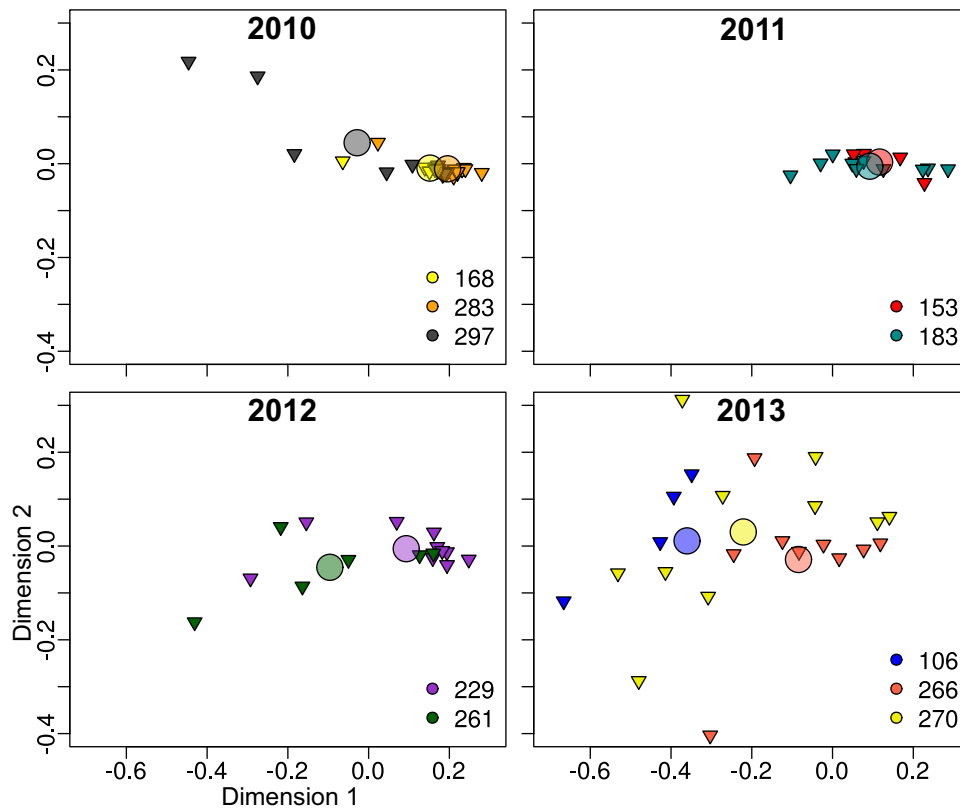


Fig. 5. Non-metric MDS plots of the structure of the *C. mesnili* population infecting *Daphnia* genotypes in Greifensee, 2010 to 2013. The analysis was based on the frequencies of ITS1 representative sequences of *C. mesnili* calculated per individual *Daphnia* host. Triangles represent individual *Daphnia* hosts while circles indicate the centroids per *Daphnia* genotype (different *Daphnia* genotypes are marked by different colours). “Stress” (the rank dissimilarities between the distance matrix and the plotted distances) was 0.079 for all plots.

under natural conditions are essential to understand host–parasite coevolution.

In the present study, we detected a decrease of the most abundant *C. mesnili* ITS1 representative sequence (CAUL-1) and an increase of the rare representative sequences in a natural *Daphnia* population over the four study years. This observation is in line with NFDS, as common parasites which presumably are adapted to the most common host genotypes are at a disadvantage and must then decrease in abundance. In a similar study examining seven drinking water reservoirs in the Czech Republic, the frequencies of *C. mesnili* ITS1 representative sequences changed across time as well (Wolinska et al., 2014). However, here we were able to analyse a much larger sequence dataset; the number of *C. mesnili* ITS1 representative sequences analysed per infected *Daphnia* was 25–33 in the study by Wolinska et al. (2014) in contrast to 3,452–7,823 sequences in the present study (due to the use of Sanger sequencing and 454 pyrosequencing platforms, respectively).

One alternative explanation that could have led to the trend of parasite genetic change over time observed here could be that different parasite genotypes are being favoured over time due to changes in the external environment (reviewed in Wolinska and King, 2009). In fact, interaction between parasites and temperature was significant in the *C. mesnili*–*Daphnia* system when assessed experimentally (Schoebel et al., 2011). However, there is no indication that conditions in Greifensee have changed in any particular direction within the four years examined. Another alternative explanation is that a yearly expansion following a bottleneck might explain the trend seen in the data. It is still unknown how *C. mesnili* survives between the epidemics; it may persist either at very low and undetectable densities in the *Daphnia* host, in as yet unknown alternative hosts (like fish) or in the sediment as spores.

To be able to discard this potential explanation, population genetic tests are needed.

NFDS is associated with the parasite-driven evolution of sex (reviewed in Brockhurst et al., 2014). In fact, the existence of sexual cycles could potentially explain the origin of the rare *C. mesnili* ITS1 representative sequences. However, cryptic sexuality has only been described in the ichthyosporean shellfish symbiont *Sphaeroforma tapetis* so far (Marshall and Berbee, 2010). Alternative mechanisms might also account for the origin of rare variants. For instance, ribosomal DNA is present in multiple copies throughout the genome and each copy is a potential target for mutations leading to intragenomic variation. It has long been thought that such regions (including the ITS1) evolve under concerted evolution, i.e., become homogenised and evolve as a unit (reviewed in Liao, 1999). Although ITS1 polymorphism is not well studied in the class Ichthyosporea, high intragenomic ITS1 variability has been described in other protozoa such as the human intestinal parasite *Dientamoeba fragilis* (Bart et al., 2008) and the foraminiferan *Elphidium macellum* (Pillet et al., 2012). This high ITS1 variability argues against the concerted evolution of the ribosomal genes (at least in those particular taxa) and suggests a birth-and-death process, where new genes originate from gene duplication and some are maintained in the genomes while others are eliminated or become non-functional (Nei et al., 1997). To elucidate ribosomal gene evolution according to the above hypotheses (i.e. concerted evolution or birth-and-death processes), cytogenetic and genomic techniques should be implemented.

The weak signals of host-genotype specificity we found between *C. mesnili* and *Daphnia* genotypes support the lack of host-genotype specificity in this system, as indicated in a previous study in which we compared the distribution of *C. mesnili* ITS1 sequences between

Daphnia hybrids and their parental species (Wolinska et al., 2014). Two main arguments are commonly used to explain host specificity and its related factors: the specialisation of parasites and the relationship between host specificity and parasite transmission mode. Parasites tend to infect specific hosts that are phylogenetically and/or eco(physio)logically related (Adamson and Caira, 1994; Pedersen et al., 2005). In invertebrates, parasite specificity is associated with differential expression of the innate immune system, the genetic diversity of receptors or effectors, dosage effects and the host's microbiota (Schulenburg et al., 2007; Riddell et al., 2009; Koch and Schmid-Hempel, 2012). Host ecology is an important factor that affects specificity when the parasites interact minimally with the host's physiology and/or evade the host immune system (Adamson and Caira, 1994; Schmid-Hempel, 2009). Ecological parameters like composition of the community (e.g., Poulin, 1997; Marcogliese, 2002), host foraging strategies (Salathé and Schmid-Hempel, 2011) and the presence of vectors (reviewed in Hoberg and Brooks, 2008) have been associated with a lack of parasite specificity. In contrast, factors including similarity between habitats (Šimková et al., 2006) and host geographical distribution (reviewed in Poulin et al., 2011) could reinforce host-genotype specificity. Additionally, non-structured parasite populations could be the result of weak genetic interactions or of frequent genetic exchange (Schmid-Hempel and Funk, 2004; Bruyndonckx et al., 2009). Host specificity might also relate to parasite transmission mode; specific forms of transmission might facilitate encounters with multiple types of host (Pedersen et al., 2005). For instance, *Daphnia* become infected when they ingest *C. mesnili* spores during grazing (Lohr et al., 2010). This method of transmission, common in gut parasites, is expected to be a non-specific mode of parasite transmission (Marcogliese, 2002).

Finally, despite its many benefits, ITS1 may not be the optimal marker for host-genotype specificity tests. However, ITS1 sequences are the only known polymorphic marker in *C. mesnili* which could allow for the identification of different parasite strains or variants (Giessler and Wolinska, 2013). In a similar way, the use of ITS1 to discriminate strains or variants was successful in amoebzoa (Köhler et al., 2006) and trichomonads (Ibáñez-Escribano et al., 2014). Nevertheless, the resolution of this marker is not fine enough to discriminate between strains or variants in oomycetes (Robideau et al., 2011), dinoflagellates (Stern et al., 2012) or in other protozoa (Homan et al., 1997; Lollis et al., 2011). For this reason, strains or variants identified with the ITS1 marker should be confirmed with other neutral markers which identify strains at a higher resolution.

In conclusion, we detected a decrease in the most abundant *C. mesnili* ITS1 representative sequence and an increase in the rare representative sequences over four consecutive epidemics. These findings are consistent with the assumptions of NFDS. However, only weak host-genotype specificity between *C. mesnili* and *Daphnia* was detected in our survey. In future studies, NFDS should be further confirmed by performing simultaneous genetic screening of host and parasite populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.zool.2016.04.003>.

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