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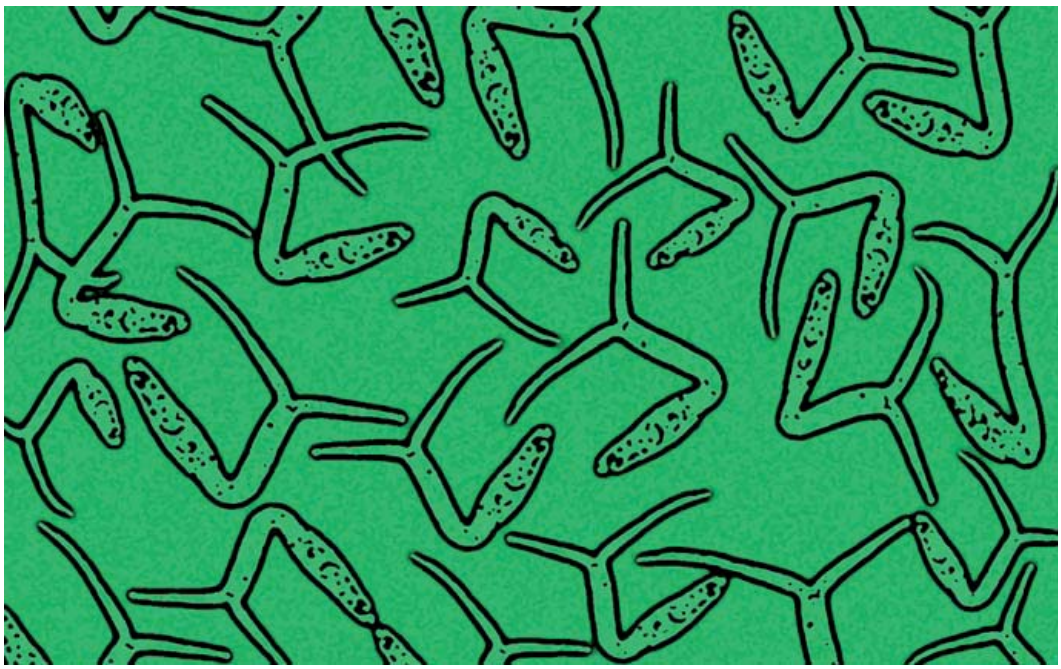
240

Katja-Riikka Louhi

Evolutionary Ecology of Complex Life Cycle Parasites

From Genotypes to Species Assemblages



JYVÄSKYLÄN YLIOPISTO

To my family

ABSTRACT

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Among the main aims in evolutionary ecology is to understand the organization of biological diversity in space and time as well as to explain why organisms vary in their life-history traits. Studying these aspects of biology in parasites furthers our understanding of between-species interactions and evolution of transmission strategies. In this thesis, I examined the genetic structure and community organization of *Diplostomum* (Trematoda: Digenea) parasites as well as variation in their transmission traits. These parasites have a complex life cycle (CLC) that includes snail, fish and bird hosts. I found that the species *D. pseudospathaceum* has no population genetic structure and exhibited high levels of neutral genetic diversity and gene flow among populations of its snail hosts, suggesting effective dissemination of parasite genotypes by the final bird hosts. Multiple genotype infections (MGI) of *D. pseudospathaceum* were common and aggregated in snail populations and their frequencies correlated with infection prevalence. Similar pattern of genotype distribution was true also in other aquatic snail-trematode systems that I reviewed from the literature. Multiple *Diplostomum*-species infections were common in the eye lenses of five freshwater fish species as revealed by pyrosequencing of pooled DNA samples. The fish species also harbored significantly different *Diplostomum*-species assemblages. Furthermore, I found genotypic and phenotypic variation in transmission traits of clonal stages of *D. pseudospathaceum* indicating either an adaptive 'bet hedging' –strategy or phenotypic plasticity in trait expression over time. These findings suggest that populations of CLC parasites maintain high levels of neutral genetic variation and probably also genetic variation in life-history traits, which may be a benefit in unpredictable environments. The novel analytical methods described in this thesis broaden our understanding of the causes and consequences of multiple genotype and multiple species infections for the evolutionary ecology of CLC parasites.

Keywords: Community structure; *Diplostomum pseudospathaceum*; multiple genotype infection; phenotypic variation; population genetics; Trematoda.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV. I participated in the planning and execution of all studies as well as collected the data for all studies together with Anssi Karvonen and Christian Rellstab. I conducted the data analysis in all studies except the building of the Bayesian analysis in study II and the statistical analyses in study III. I had the primary responsibility of preparing manuscripts I, II and IV and a significant role in the preparation of paper III.

I Louhi, K.-R., Karvonen, A., Rellstab, C. & Jokela, J. 2010. Is the population genetic structure of complex life cycle parasites determined by the geographic range of the most motile host? *Infection, Genetics and Evolution* 10: 1271-1277.

II Louhi, K.-R., Karvonen, A., Rellstab, C., Louhi, R. & Jokela, J. Prevalence of infection as a predictor of multiple genotype infection frequency in parasites with multiple-host life cycle. Submitted manuscript.

III Rellstab, C., Louhi, K.-R., Karvonen, A. & Jokela, J. 2011. Analysis of trematode parasite communities in fish eye lenses by pyrosequencing of naturally pooled DNA. *Infection, Genetics and Evolution* 11**:** 1276–1286.

IV Louhi, K.-R., Karvonen, A., Rellstab, C. & Jokela, J. Genotypic and phenotypic variation in transmission traits of a complex life cycle parasite. Manuscript.

1 INTRODUCTION

1.1 Parasitism

Parasitism has evolved several times independently in the history of life through adaptations that enabled the parasitic life style. It is argued that parasites now make up most of the species on our planet (e.g. Windsor 1998, Kuris et al. 2008). A parasite is defined as "an organism living in or on another living organism, obtaining from it part or all of its organic nutriment, commonly exhibiting some degree of adaptive structural modification, and causing some degree of real damage to its host" (Price 1977). Some of the parasite species that we see in nature today exploit only one host, whereas others, so-called complex life cycle (CLC) parasites, need to infect more than one consecutive host in order to successfully pass their genes to offspring. It is not agreed on whether CLCs evolved because they provide various benefits for parasites such as large, long-lived and motile definitive hosts, maintenance of genetic variability (Reusch et al. 2004, Rauch et al. 2005) and enhanced transmission (Choisy et al. 2003), or because of historical accidents, for example when the original host became frequently consumed by a new host species (Smith Trail 1980).

Questions in the evolutionary ecology of parasites have been facilitated recently by the rapid development of molecular methods. New methods for identification of parasite species lacking clear morphological characters, or to distinguish different parasite strains or genotypes enable evolutionary questions to be tackled at an appropriate level. Although the analysis of parasite populations with molecular methods has continued to increase, surprisingly little is known, for example, about the basic population genetic structure of many parasitic organisms. In what will follow, I will focus on two important aspects of evolutionary ecology of CLC parasites of the genus *Diplostomum* (Trematoda: Digenea), namely; on the spatial distribution of parasites among host individuals, populations and species as well as on the genotype-specific variation in parasite transmission-traits. *Diplostomum* parasites are especially suitable for such studies as the hosts that are required in the life cycle have fundamentally different geographical ranges and because

molecular markers already exist for identification of multiple genotype and multiple species infections. It is also an advantage that the CLC of *Diplostomum* includes production of clonal parasite stages that enables the characterization of within- and between-genotype variation in phenotypic traits. Studying these aspects of biology in CLC parasites is relevant for our understanding of host-parasite and parasite-parasite interactions as well as for understanding how parasite transmission evolves. As parasites with obligatory multihost life cycles are important for medical and veterinary science, studying their evolutionary ecology has practical applications in disease control and prevention.

1.2 Spatial distribution of parasites

1.2.1 Population genetic structure of parasites

One of the fundamental goals in evolutionary ecology is to understand organization of biological diversity in space and time as it determines what types of interactions may arise within and between species, and how these interactions shape biological systems and their evolution. In fact, spatial variation in the interactions between parasites and their hosts is considered as the major force shaping the coevolutionary process (Thompson 1994) and in maintaining genetic and phenotypic diversity within and among populations (Thompson 1999). It is therefore important to study the distribution of parasites at multiple scales in order to understand the different biological processes that operate at the different levels of biological organization.

The study of parasite populations is often complicated as parasites are distributed among metapopulations (i.e. local populations connected by dispersal) that may consist of several, clearly separate host populations (see Hess 1996, Lopez et al. 2005). Currently, the number of studies on parasite population genetic structure is still limited in comparison to the diversity of these systems (but see e.g. Anderson et al. 2000, McCoy et al. 2003, Archie & Ezenwa 2010, Blasco-Costa et al. 2012). The distribution of parasites within and between host populations has important implications for several biological processes such as speciation (reviewed by Huyse et al. 2005), transmission dynamics (e.g. Criscione et al. 2010) as well as for applied issues such as evolution of drug resistance (e.g. Cornell et al. 2003, Schwab et al. 2006). Moreover, the essential first step for understanding host-parasite coevolution is to determine the geographical scales of the interactions by analyzing the population genetic structure of parasites and their hosts (Thompson 2005a). Theoretical models predict that coevolution between host and parasite is affected by the relative rates of gene flow among the parasite and host populations (Lively 1999, Gandon & Michalakis 2002). According to these models, parasites are more likely to adapt to their local host population if the migration rate of the parasites is higher than the migration rate of their hosts.

This is because parasite migration can import beneficial alleles that may counteract host resistance alleles. However, extremely high levels of gene flow among parasite populations should attenuate the rate of local adaptation, or even make it unlikely (Gandon et al. 1996, Lively 1999, Gandon & Michalakis 2002) by swamping the beneficial alleles in the parasite population.

Early hypotheses of parasite population genetic structure have suggested that parasite populations are genetically differentiated, inbred and have a low level of genetic diversity (Price 1980). These predictions came from the idea that hosts represent transient and spatially isolated habitats for parasites and therefore parasite populations should be shaped by large population fluctuations, founder effects and genetic drift. Data now accumulating from diverse host-parasite systems (e.g. Anderson et al. 1998, Criscione & Blouin 2004) indicates that population genetic structure of parasites often does not follow these predictions.

In particular, specific features of parasite life histories will strongly influence their population genetic structure. Increasing complexity of the life cycle is thought to generate higher levels of genetic variation through increased demographic stability (Barrett et al. 2008) and through selection favoring different alleles in different obligatory hosts (Gower & Webster 2004). For example, it has been suggested that inclusion of a fish second intermediate host in the life cycle of trematodes would reduce the risk for inbreeding following the transmission to the avian definitive host (Rauch et al. 2005). According to this hypothesis the fish host accumulates different parasite genotypes over time and then transfers them into the final bird host as a cluster, which facilitates mating between different parasite genotypes. Thus, complexity of the life cycle should increase genetic variation in parasites by reducing probability for genetic bottlenecks and by decreasing genetic drift (Barrett et al. 2008).

Most parasites are dependent on their host for dispersal. Indeed, host mobility has been proposed as the main determinant of parasite gene flow (Blouin et al. 1995) because parasites often have free-living stages with restricted dispersal ability or lack free-living stages entirely. Gene flow in CLC parasites should therefore be determined mainly by the geographic range of the most motile host (Prugnolle et al. 2005).Those relatively few studies that have investigated the population genetic structure of CLC parasites have reported either strongly structured populations at small spatial scales (e.g. Prugnolle et al. 2005, Rudge et al. 2009) or no genetic structure at large spatial scales (e.g. Keeney et al. 2009). However, the geographical scale of the host stage in question is often neglected, making it difficult to estimate the level of parasite gene flow that local hosts of CLC parasites experience.

1.2.2 Distribution of parasite genotypes in snail hosts

In nature, parasites commonly infect and share hosts with other parasite strains or genotypes (Read & Taylor 2001, Woolhouse et al. 2002), suggesting that intraspecific parasite interactions are common within hosts. Interactions among co-infecting parasites can have significant effects both on host-parasite and

parasite-parasite dynamics. Examples of such interactions include selection for more virulent parasite strains (de Roode et al. 2005) and direct antagonism between conspecific parasite larvae (Hechinger et al. 2011).

Distribution of parasite genotypes within a host population is a key feature determining the likelihood of parasite-parasite interactions. If parasites are distributed randomly among the host individuals their distribution should follow a Poisson distribution (Shaw et al. 1998). Risk of infection (density of parasite infective stages in the environment) should affect the frequency of multiple genotype infections (hereafter MGIs), assuming that already infected hosts can be re-infected by another parasite genotype and that all parasite genotypes are present in similar proportions. Hence, under Poisson expectations, assuming parasite individuals do not directly interact and hosts do not differ in susceptibility, the more there are infective stages in the environment the higher should be the frequency of hosts that carry MGIs. However, in nature parasites show typically aggregated distribution patterns, which means that there are more hosts that are infected with multiple parasite individuals than expected by the Poisson null model (hereafter PNM) (Shaw & Dobson 1995). Aggregated distributions follow, for example when hosts differ in their risk of acquiring an infection due to ecological or demographic reasons or when they differ in susceptibility (Wilson et al. 2002).

Clonal parasite stages represent a challenge for the study of parasite distribution patterns, as genetic identification of individual clones is needed to distinguish MGIs. Understanding the distribution of clonal parasite stages within their hosts is especially interesting because competition is likely to be stronger in parasite stages that multiply rapidly and compete directly for host resources than in parasite stages that use the host mainly as a transmission vehicle (Karvonen et al. 2012). Such intensive competition should in turn have negative effects on parasite fitness through reduced production of larval stages due to shared resources (Karvonen et al. 2012) and/or increased host mortality (Davies et al. 2002).

Results from several studies suggest that MGIs of clonal trematodes are common in aquatic snails (e.g. Minchella et al. 1995, Dabo et al. 1997, Sire et al. 1999, Eppert et al. 2002, Rauch et al. 2005, Lagrue et al. 2007, Keeney et al. 2008). So far, however, only one study has analyzed the distribution of clonal parasite stages in snails. In this study schistosome (Trematoda: Digenea) populations showed both aggregated and non-aggregated distributions (Eppert et al. 2002). Moreover, it was recently proposed that there could be a positive relationship between parasite prevalence and frequency of MGIs in marine trematodes (Keeney et al. 2008). It is, however, not known if these results apply to snail-trematode systems in general. If the frequency of MGIs could be readily predicted from parasite prevalence, it would help in designing and performing MGI surveys, and facilitate the study of aggregation patterns and their ecological, epidemiological and evolutionary consequences.

1.2.3 Parasite communities

In addition to MGIs, infections where different parasite species share a host are also common in nature (e.g. Petney & Andrews 1998, Cox 2001, Fleming et al. 2006). The composition of such co-infecting parasite communities is the result of complex evolutionary and ecological host-parasite and parasite-parasite interactions (Janovy et al. 1992, Adamson & Caira 1994, Poulin 2001). These interactions can take many forms. For example, parasite species can interact through host mediated cross-immunity (Fenton & Perkins 2010), where host immune defenses directed against one parasite species are effective also against another closely related parasite species (Page et al. 2006). Sometimes co-infecting parasite species can facilitate each other (Graham 2008), because co-infection may compromise host immune defense (Seppälä et al. 2009). Some parasite species can also interact directly, for example, by attacking other co-infecting parasite species. This has been demonstrated for larval parasite stages of trematodes parasitizing their intermediate snail hosts (Sousa 1992, 1993, Hechinger et al. 2011). Additionally, infection patterns and community structure of the co-infecting parasites may be shaped by ecological features of the different hosts (Adamson & Caira 1994), like habitat preferences and seasonal population dynamics. These examples illustrate that the study of community-level processes within hosts is essential for our understanding of host-parasite interactions and disease dynamics.

One of the main difficulties in characterizing co-infecting parasite communities is that hosts are often infected with dozens or hundreds of parasite individuals belonging to closely related species that are morphologically identical in some or even in every developmental stage of their life cycle. Many parasite taxa also include cryptic species (Hung et al. 1999, Jousson et al. 2000, Leung et al. 2009, Locke et al. 2010a), which further complicates the analyses. Molecular methods can often be used to identify species at different developmental stages, which is a benefit when studying parasites with CLCs. Commonly, ribosomal DNA (rDNA) regions and mitochondrial DNA (mtDNA) genes are sequenced and analyzed for differences (e.g. Galazzo et al. 2002, Donald et al. 2004, Locke et al. 2010b, Locke et al. 2010a). The major drawback of these methods is that they are labor intensive, time-consuming and costly because individual parasites need to be analyzed separately. Therefore, a major breakthrough in the statistical power of community analyses could be achieved, if parasite communities could be analyzed as a whole from naturally pooled DNA samples. Such analyses, based on pyrosequencing technique, have been developed for example for the analysis of environmental samples (e.g. Ficetola et al. 2008) and for estimating the diets of predators (e.g. Deagle et al. 2009).

1.3 Variation in life-history traits of parasites

Life-history strategies are defined as combinations of evolved traits, including behavioral, physiological and anatomical traits, that influence survival and reproductive success of organisms (e.g. Ricklefs & Wikelski 2002). Numerous studies on life-history traits have demonstrated that many of the phenotypic differences observed among individuals have a genetic basis (Roff 1992, Stearns 1992). Since these traits are closely associated with fitness, selection should remove genetic variation in life-history traits (Roff 1997), but most traits seem to maintain additive genetic variation (reviewed by Merilä & Sheldon 1999) that is required for heritable response to selection. Thus, understanding how genetic variation in life-history traits is maintained within natural populations remains a central question in evolutionary ecology. Our current understanding of life-history strategies is based mainly on free-living organisms (but see review by Poulin 1996), however, understanding the processes that maintain variation in transmission traits of parasites is very important because they are directly related to evolution of transmission (e.g. Taylor & Read 1998) and virulence (e.g. Ebert & Mangin 1997).

Not surprisingly, parasites show also intraspecific variation in various transmission-related traits such as growth rate (e.g. Reilly et al. 2007), infection success (e.g. Seppälä et al. 2007) and virulence (e.g. Mackinnon & Read 1999). This variation may result from environmental effects such as differences in ecological condition of hosts (Keas & Esch 1997) or from genetic differences among the parasite individuals. For instance, developmental conditions within the hosts tend to vary substantially, and often unpredictably, among different host individuals (Poulin 1996, Reece et al. 2009). Conditions for parasite development, on the other hand, have important implications for within host infection dynamics and transmission success to the next host. Hosts in good condition might be hostile environments for parasites since they are effective in resisting infections, thus limiting opportunities for parasite development. On the other hand, hosts in bad condition might be easier targets for infection, but at the same time provide fewer resources for parasite growth and reproduction (Ebert 2000, Bedhomme et al. 2004, Ebert et al. 2004). Currently very little is known about the effect of host condition on parasite traits (but see Logan et al. 2005, Tschirren et al. 2007, Seppälä et al. 2008).

From an evolutionary point of view, it is interesting to identify transmission traits that have a genetic component, since only such traits can change in response to selection. Parasites that have a clonal reproduction stage in their life cycle provide interesting opportunities to test to what extent variation in life-history traits has a genetic basis. This is possible as components of variance can be used to estimate broad–sense heritability (H^2), i.e. the degree to which a trait is determined by genotype. Both environmental and genetic factors are important sources of variation in life-history traits among clones, whereas variation in life-history traits of one clone should result from

environmental effects. Thus, variation among clonal parasite stages reflects the opportunity for natural selection to operate on life-history trait expression whereas a high variability within a clone may indicate phenotypic plasticity or classical bet-hedging strategy that is beneficial in stochastic environments (Fenton & Hudson 2002, Kussell & Leibler 2005, Donaldson-Matasci et al. 2008, Reece et al. 2009). Transmission-related traits of larval parasite stages (e.g. production rate, activity, survival, and infection success) are good candidates for characters that determine transmission success and subsequent parasite fitness as they affect the likelihood that the parasite reaches its final host. As the simultaneous maximization of all traits is unlikely due to physiological and ecological constraints (Stearns 1989), measuring infection success of clonal parasite stages together with other transmission traits might lead to a better understanding of the constraints underlying the evolution of parasite transmission.

1.4 Aims of the thesis

The main aim of this thesis was to gain understanding of the spatial structure and distribution of trematode (*Diplostomum* sp.) parasites within and among host individuals, populations and species, and to infer what the observed distribution patterns imply for the evolutionary ecology of host-parasite interactions in this system (I-III). The second aim was to determine genotype-specific variation in transmission traits of the parasite and to test if this variation is affected by host condition (IV).

More specifically, I first investigated the population genetic structure and the extent of gene flow in *Diplostomum pseudospathaceum* Niewiadomska, 1984 parasites within and among local freshwater snail (*L. stagnalis*) populations by using neutral genetic markers (I). Second, I asked how common MGIs are in snail populations, is the frequency of MGIs positively correlated with prevalence of infection, and whether MGIs are aggregating in snail hosts (II). To answer these questions I sampled *L. stagnalis* populations for *D. pseudospathaceum* infections over a broad geographical scale in Finland, as well as compiled literature data from various snail-trematode systems. Then I applied Bayesian analysis to describe the relationship between parasite prevalence and frequency of MGIs. Third, I developed parasite species-specific SNP markers to distinguish between 5 different *Diplostomum* species that co-occur in the eyes of several freshwater fish species (III). These markers were then used to analyze the frequency of multiple species infections and the composition of parasite species assemblages in five different fish species by pyrosequencing whole parasite communities in fish eye lenses. Finally, I measured transmission-related traits of parasite cercarial clones from naturally infected snails and tested experimentally if a short-term food deprivation of the snails affected the parasite traits (IV). I aimed to determine how variation in fitness traits of parasites was distributed within and among parasite genotypes

and whether manipulation of the external environment (host condition) could explain variability in these traits.

2 MATERIAL AND METHODS

2.1 *Diplostomum* (Trematoda: Digenea) parasites

Almost all trematodes (class Trematoda) are CLC parasites of vertebrates (few species mature in molluscs). Parasites in the subclass Digenea comprise several medically important parasite species such as blood flukes (schistosomes) infecting humans in the tropics. The parasite genus used in this study, *Diplostomum* (digenea), is a common cause of parasitic cataracts in wild and farmed fish. The taxonomy and nomenclature of this genus is not completely untangled as sequencing of COI and ITS1 regions suggest that cryptic species are present (Locke et al. 2010b, Locke et al. 2010a) and as some taxa (e.g. *D. spathaceum* and *D. parviventosum*) show identical ITS1 sequences, but also distinct morphological characters (Niewiadomska & Laskowski 2002).

Trematodes of the genus *Diplostomum* are common parasites in freshwater systems worldwide (Chappell 1995). They use aquatic snails as first intermediate hosts, fish as second intermediate hosts and fish-eating birds as final hosts. The life cycle of eye lens infecting *Diplostomum* spp. begins in the bird (e.g. gull) intestine, where adult parasites reproduce sexually (Dogiel et al. 1961)(Fig. 1). Parasite eggs are released with bird faeces and develop to miracidia larvae in water. Miracidia infect snails and form sporocysts in hepatopancreas and gonads of the snail. This typically leads to castration of the snail (Zbikowska 2011). The parasite multiplies asexually in the sporocysts producing cercarial clones. Thus, if the snail is infected with only one miracidium all cercariae released by the snail are genetically identical. Similarly, if the snail is infected with multiple miracidia, the snail may release parasites of different genotypes. A single snail can release up to tens of thousands of cercarial clones per day for several weeks (Karvonen et al. 2004b). Cercariae released from a snail infect their second intermediate hosts, freshwater fish, through skin and gills (Whyte et al. 1991, Höglund 1995) and migrate to the eye lenses. There they develop to metacercariae, but do not multiply. Most species of *Diplostomum* known so far infect the eye lens, but

some species (e.g. *D. baeri*) infect the vitreous humour of the eye or tissues under the retina. For successful establishment, the parasite needs to migrate to the fish eye lens within 24 hours from getting in contact with a fish (Whyte et al. 1991). Afterwards, the larva is either killed by the host immune system or it runs out of energy and dies. Heavily infected wild and farmed fish suffer from parasitic cataracts and blindness (Shariff et al. 1980, Karvonen et al. 2004a, Seppälä et al. 2010). The infection can be pathogenic to fish (Brassard et al. 1982, Chappell et al. 1994) and increase fish susceptibility to predation by birds (Seppälä et al. 2004). The life cycle is completed when an infected fish is predated by a suitable bird host.

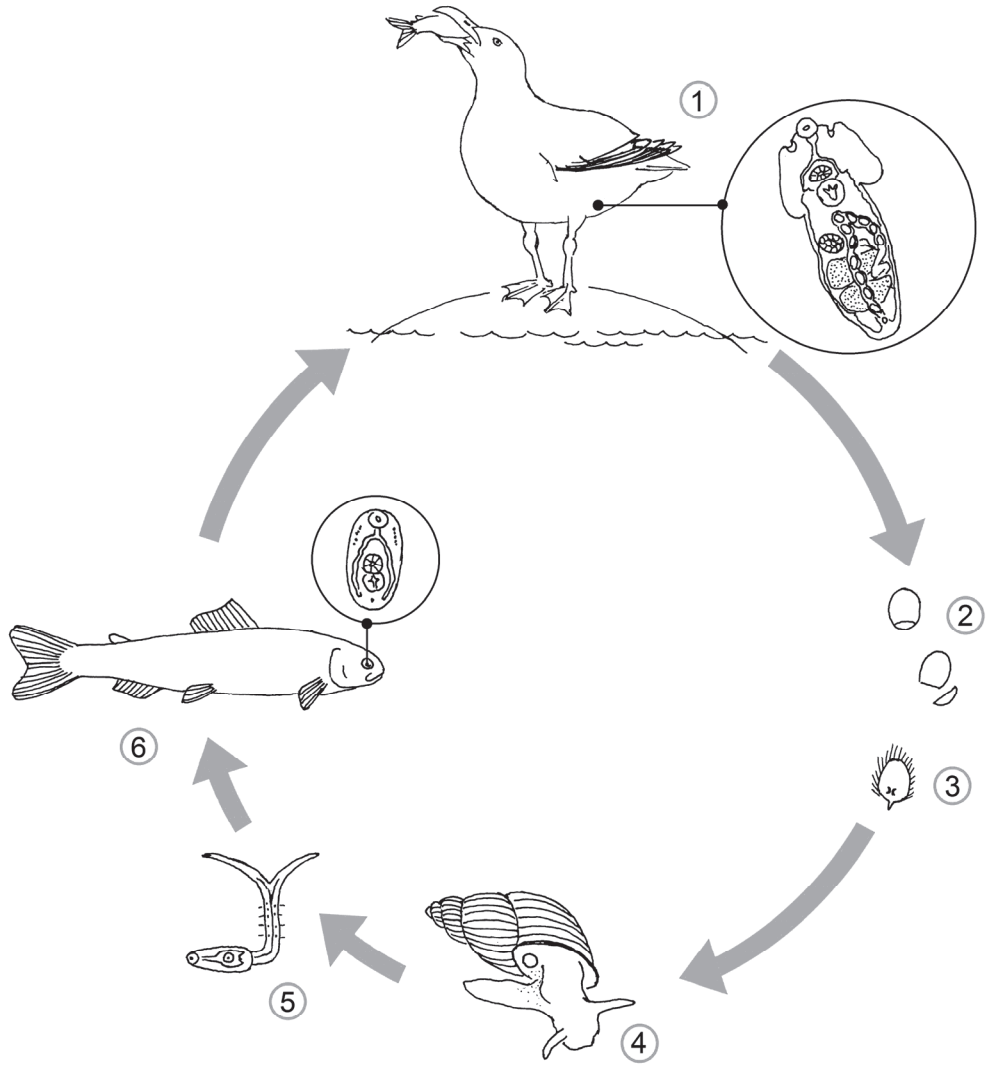

FIGURE 1 The life cycle of lens infecting *Diplostomum* parasites. 1) Adult worm in the intestine of a piscivorous bird, 2) eggs, 3) miracidium, 4) snail first intermediate host, 5) cercaria and 6) metacercaria in the lens of a fish second intermediate host. Modified from Valtonen, E. T., Hakalahti-Sirén, T., Karvonen, A. & Pulkkinen, K. (eds). 2012. Suomen kalojen loiset. Gaudeamus.

2.2 Host species and sampling locations

In studies I, II and VI naturally infected great pond snails, *Lymnaea stagnalis* were collected from the field. *L. stagnalis* is abundant in the littoral zone of lakes and ponds with lush vegetation. Snails were collected either from lake Vuojärvi (IV) or from several different lakes located in Central Finland (see I, II) during the peak period of cercarial release in July-August (Karvonen et al. 2006). To verify that the snails released *Diplostomum* cercariae and not larvae of other trematode species, cercariae were initially identified by observing their shape and movement under a light microscope.

In study III, various parasite individuals of different life stages and from different host species were sampled and sequenced for the development of the pyrosequencing assays. These host species included freshwater fish; bleak (*Alburnus alburnus*), hybrids of common bream (*Abramis brama*) and silver bream (*Blicca bjoerkna*), common dace (*Leuciscus leuciscus*), common whitefish (*Coregonus lavaretus*), perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*), ruffe (*Gymnocephalus cernuus*) and vendace (*Coregonus albula*) and pike-perch (*Sander lucioperca*) and freshwater snails; *L. stagnalis*, *Myxas glutinosa*, and *Lymnaea ovata* as well as gulls; (*Larus fuscus*). The exact sampling locations for the different host species are deposited together with the parasite sequences in GenBank under the accession numbers JF775679-JF775760.

2.3 Molecular methods

2.3.1 Genotyping

Genotyping of parasites using microsatellite markers was applied in three studies (I, II, IV). A detailed description of the protocol can be found in paper I. In these studies 7-15 cercariae per snail were genotyped at 3 loci (Diplo06, Diplo09 and Diplo23; Reusch et al. 2004). Parasite clones exhibiting the same snail host were removed before the statistical analysis to avoid artificially introduced population structure (I, II). Population genetic parameters were estimated using FSTAT 2.9.3.2 (Goudet 2001) and Genepop 3.4 or 4.0 (Raymond & Rousset 1995).

2.3.2 Sequencing

Parasite species determination was verified by sequencing the partial internal transcribed spacer 1 (ITS1) regions (I, III) as described in paper III.

2.3.3 Pyrosequencing

A novel pyrosequencing method was developed for the analysis of parasite species (*D. mergi*, *D. paracaudum*, *D. parviventosum/D. spathaceum* and *D. pseudospathaceum*) composition in fish eye lenses (III). The benefit of pyrosequencing is that it is quantitative and can thus be used to determine the allele frequency of species specific single nucleotide polymorphisms (SNPs) in naturally pooled DNA samples i.e. whole parasite communities in fish lenses. In this case SNPs in the ITS1 region of rDNA were utilized for this purpose. The SNPs were detected by sequencing and aligning 82 reference sequences of *Diplostomum* sp. originating from the host species described in section 2.2. Two pyrosequencing assays (Diplo197 and Diplo503), that included 4 interspecific SNPs, were developed to differentiate between the parasite species. The fifth species, *D. baeri,* was included in the analysis to detect and quantify possible contaminations on the surface of the lens by this species, but was excluded from the community analyses. The reliability of the pooled pyrosequencing approach was tested by cross-validating reference samples, examining repeatability and analysing artificial parasite-mixtures with known species ratios.

After development, the method was applied to analyze parasite communities in five different fish species; bleak, perch, roach, ruffe and vendace. For this purpose large numbers of fish were collected from the field with gill nets and the total number of metacercariae in the fish eye lenses was counted using a slit-lamp microscope (Karvonen et al. 2004a). Infected lenses were removed and stored at -80 ^{o}C. Prior to DNA extraction with DNeasy® Blood & Tissue Kit (Qiagen) the lenses were homogenized in a bead mill. The pooled DNA was then amplified using a nested PCR approach. In the first reaction, partial ITS1 region was amplified and in the second reaction biotinylated primers were incorporated in the PCR products. The biotinylated PCR products were extracted with streptavidin sepharose beads (Biotage) and released on a 96 well plate (Biotage) containing annealing buffer (Biotage) and pyrosequencing primer (TAG). The samples were analyzed on a PyroMark™ID (Biotage) pyrosequencer using Pyro Gold reagents (Biotage). The number of parasites belonging to each species was estimated by multiplying the total parasite number in a lens (obtained by the slit-lamp microscopy) with their relative frequencies (obtained by pyrosequencing). Finally, linear mixed models, principal component analysis, discriminant function analysis and specificity indices were applied to identify which factors explain the differences in infection patterns and parasite community structure among the fish species.

2.4 Analysis of parasite aggregation

The analysis of parasite aggregation (II) was done in three steps. First, Bayesian simulation was applied to describe the linear relationship between parasite prevalence and frequency of MGIs in our own field data as well as in a

combined data set consisting of our own data and literature data from 13 snail populations. The simulations were developed to fit simple linear relations to the observed data by combining information from the different snail populations. The strength of this method is that it takes into account the uncertainty that we have in the variables (infection prevalence and frequency of MGIs) about the true value in the whole population. This uncertainty arises from differences in sample sizes (number of collected snails and number of snails analyzed for MGIs). Second, the PNM, commonly used in parasitological literature to study parasite aggregation patterns (e.g. Shaw et al. 1998), was applied to calculate the expected frequency of MGIs at different levels of infection prevalence. In addition, an alternative null model (demographic PNM) was constructed to examine how inclusion of host age structure in the PNM would change the null expectations of parasite aggregation (II). Third, the results from the Bayesian simulations were compared with the expectations of the null models. This comparison was then used to determine if the observed MGI frequencies were significantly different from the expectations of the two null models.

2.5 Measurement of variation in parasite transmission traits

Variation in four different transmission traits (cercarial production rate, activity, survival and infection success) was measured from parasites that originated from food-deprived or normally fed *L. stagnalis* snails (IV). Before the beginning of the experiment, 32 field collected snails with single genotype infections (verified by microsatellite markers) were acclimated to laboratory conditions for 10 days and all snails were fed with lettuce *ad libitum.* During the experimental treatments half of the snails were fed with lettuce normally (referred to as '*ad libitum* snails') and the other half did not receive any food (referred to as 'starved snails'). Parasite performance was measured three times: before the food manipulation (week 0), after one week of food treatment (week 1) and after two weeks of food treatment (week 2).

Production of cercariae was followed by placing the snails individually in containers with 250 ml of water (20 ^{o}C). Production rate of cercariae was estimated by calculating the numbers of released cercariae from sub-samples (n = 5 per genotype) taken from the container after 3 hours. Cercarial activity was estimated by counting the number of cercarial swimming bursts executed in 2 minutes (n = 3 per genotype). Cercarial survival was estimated by determining the proportion of alive cercariae (n = 16 per genotype) after 24 hours.

Cercarial infection success to fish was assessed experimentally using rainbow trout. Fish were obtained from a fish farm where they were raised in ground water and therefore had no macroparasite infections. Fish were exposed individually in containers to 100 cercariae (10 fish per parasite genotype). All fish exposed to the same parasite genotype were placed in plastic cages that were distributed randomly into several 1500 l tanks supplied with lake water

for 48 h to allow parasites to reach the eye lenses. Fish were killed with an overdose of MS-222 (Sigma-Aldrich) and the number of successfully established parasites was determined by dissecting the eye lenses under a microscope. The total count of parasites in both eye lenses was used as an estimate of infection success.

Results of the week 0 were analyzed separately to test for differences between the treatment groups before the food treatment. Cercarial production rate, activity and infection success were analyzed with nested linear mixed models, whereas generalized linear mixed models were used for the analysis of cercarial survival. Components of variance were used to estimate broad–sense heritability (H^2) for cercarial activity and infection success, according to the model $V_G/(V_G+V_E)$, where V_G is the among-genotype variance component and V_E is the residual error variance component.

3 RESULTS AND DISCUSSION

3.1 The geographic range of the final host as determinant of parasite population genetic structure

Genetic differentiation among *D. pseudospathaceum* parasites over a geographically large scale (300 km) in Central Finland was very low. This was evident in the F_{ST} values that were all less than 0.05, indicating that most of the genetic variation was present within the sampling sites and that differentiation among the populations was insignificant. The observed pairwise F_{ST} values of the parasites were also much lower compared to the pairwise F_{ST} values of the snail populations (Puurtinen et al. 2004). There was also no indication of isolation by distance although the geographical distances among the sampled snail populations were considerable. Moreover, *Diplostomum* parasites had high levels of genetic diversity in terms of number of alleles (14-33 alleles per locus) and heterozygosity (up to 1). The estimated gene flow (N_m) between the sampling sites was also high (11.4). Although many trematode species can self-fertilize their offspring (e.g. Poulin & Cribb 2002), inbreeding did not seem to be common in *D. pseudospathaceum* as the populations showed low levels of homozygosity and no deviations from Hardy-Weinberg expectations.

These results suggest that *Diplostomum* parasites have a high effective population size and that they disperse effectively among the local snail populations along with their definitive bird host. It is nevertheless possible that population genetic structure and isolation by distance exist in this system in a much larger geographic scale than the one used in this study, or, for example, between the eastern and western migration routes of the bird host. Moreover, high gene flow does not make parasite local adaptation impossible (Gandon & Michalakis 1996, Lively 1999). For example, studies on other host–trematode systems have shown that trematode parasites may become locally adapted to their intermediate hosts (e.g. Ballabeni & Ward 1993, Lively et al. 2004). In New Zealand, *Microphallus* sp. parasites have been shown to adapt to their local freshwater snail (*Potamopyrgus antipodarum*) hosts (Lively et al. 2004) and this

parasite has somewhat similar population genetic structure compared to *D. pseudospathaceum*. However, in *Microphallus* sp., the population genetic structure is affected by specific spatial factors such as the arrangement of the lakes between the New Zealand Southern Alps (Dybdahl & Lively 1996). Lack of such geographic barriers in central Finland provides the likely explanation for the absence of population genetic structure in *D. pseudospahaceum* and makes the local cycling of the parasites within a lake unlikely. Moreover, *Microphallus* sp. has a two-host life cycle and a life span of only few months in the definitive host while *D. pseudospathaceum* has three obligatory hosts and probably lives much longer in the bird host. Short lifespan in the bird host significantly increases the opportunity for local cycling in the snail populations. Overall, it may be that the likelihood for local cycling of parasites in their intermediate host populations is lower in systems where migratory birds or birds with a wide foraging range are utilized as final hosts. Such highly motile hosts might be infected also at the overwintering areas and import parasites from distant areas to local snail populations. Currently, it is not known how seasonal migration patterns of birds affects the population genetic structure of CLC parasites migrating along these hosts. Future studies could look into this by sampling parasites from local snail populations covering regions where the migration patterns of the bird hosts differ.

3.2 Multiple genotype infections and parasite aggregation in aquatic snails

MGIs were common in Finnish *L. stagnalis* snails as 20-55 % of the infected snails carried MGIs depending on the population (II).The data compilation showed that MGIs were common also in other snail-trematode systems where 5-90 % of the infected snails carried MGIs. The frequency of MGIs was strongly positively correlated with prevalence of infection in the Finnish snail populations (r_8 = 0.917, P < 0.01) and across the different snail-trematode systems (r_{11} = 0.937, P < 0.01). The Bayesian simulations revealed that parasite genotypes were significantly more aggregated than expected by the PNM, i.e. snail populations harbored a higher frequency of MGIs than expected by chance both in the Finnish data set (99.9 % of the simulated relations were above the neutral expectations) and when the Finnish and literature data were combined (all simulated relations were above the neutral expectations). The demographic PNM predicted higher frequency of MGIs than the PNM as it incorporated heterogeneous age distribution of the host population that contributes to the likelihood of MGIs. As a result, the proportion of relations that were above the null expectation decreased to 75 % for the Finnish field data and to 90 % for the combined data. However, all relations were above the expectations of the demographic PNM when prevalence of infection was < 30 %.

The observed aggregated genotype distributions could be caused by several biological processes. First, the exposure risk of the snails can vary as prevalence of infection often varies locally within snail populations (II). For example, a local concentration of the bird hosts, such as a colony, could result in high infection risk close to the colony and to low infection risk elsewhere (Jokela & Lively 1995, Byers et al. 2008). However, this explanation is unlikely in the present study as the snails were not sampled close to bird colonies. Moreover, miracidial dispersal through active swimming or with aid of water currents (Steinauer et al. 2009) as well as dispersal of snails through floating in surface water should lead to homogenization of the infection risk within a sampling site. Second, genetic factors can create differences in host susceptibility (see Shaw et al. 1998, Wilson et al. 2002) and such differences can lead to parasite aggregation if highly susceptible hosts attract multiple parasite genotypes. Results from microparasites support this hypothesis (van der Werf et al. 2011) and similar processes could also operate in our study system. For example, *L. stagnalis* snails show high among-family variation in immune defense traits (Seppälä & Jokela 2010), that could contribute to differences in susceptibility among individual snails.

Interestingly, recent infection experiments with fish indicate that the average infection success of *D. pseudospathaceum* genotypes from double-infected snails is higher than that of cercariae originating from singly-infected snails (Karvonen et al. 2012). Moreover, artificially mixed genotypes are also more successful in infecting fish than single genotypes suggesting that genetically diverse attack of the fish is advantageous for transmission between the parasite intermediate hosts. Infection of fishes by multiple parasite genotypes may be advantageous also because it decreases the probability of inbreeding once parasites are transmitted to the final host (Rauch et al. 2005). However, double-genotype infections produced fewer cercariae per parasite genotype indicating that the parasite genotypes compete in the snail (Karvonen et al. 2012). These results suggest that the higher infection success of mixed-genotype infections in fish may be constrained by resource competition in the snail (Karvonen et al. 2012). More experimental studies are needed to explore if the co-infecting parasite genotypes have higher infection success because of suppressed host resistance, and if co-infection comes with the cost of reduced cercarial output due to limiting host resources.

3.3 Multiple species infections and parasite communities in fish

Interspecific SNPs are especially useful for the development of pyrosequencing assays for pooled DNA samples as they enable analysis of whole parasite communities even with very small differences in allele frequencies. The initial testing phase confirmed that the pyrosequencing approach developed for the identification and quantification of different *Diplostomum* species in pooled samples gave reliable results as single specimens and species ratios in artificial

mixtures could be determined with high confidence (III). Also the repeatability of the results was high. The method was then applied to characterize the lens-infecting *Diplostomum*-communities in naturally infected fish (n = 315) belonging to 5 different fish species and corresponding to an estimated number of 1922 parasite individuals. Multiple species infections were common; 15-76 % of the fish carried multiple *Diplostomum* species depending on the fish species. Infection patterns differed significantly among the fish species and age classes. *Diplostomum mergi* and *D. parviventosum/spathaceum* were found commonly in bleak and roach, whereas *D. paracaudum* infected all fish species, but was most abundant in roach. *Diplostomum pseudospathaceum* was abundant in all studied fish species.

These results suggest that the different *Diplostomum* species are commonly transmitted to the final bird host as groups, indicating a potential for mating between closely related species. In fact, Locke et al. (2010a) detected signs, although rare, of possible hybridization between *Diplostomum* parasite species in Canadian populations by sequencing ITS and COI sequences of the parasites. It is possible that some of the species studied here represent previously unknown parasite species (e.g. the specimens 77-80 that did not fit well into the five *Diplostomum* species scheme; III) or hybrids of the existing species. However, future work is needed to verify these patterns.

There are many alternative explanations to the observed differences in the infection patterns in the studied fish species. For example, differences could emerge because of ecology of the hosts (Adamson & Caira 1994). In this system, such factors could be related to the extent of overlap between snail and fish habitats, which could influence the infection patterns as different snail species serve as hosts for different parasite species (Sousa 1992, Kuris & Lafferty 1994). Also seasonal or inter-annual variation in exposure to different *Diplostomum* species could induce variation in the infection pattern among the fish species. Moreover, some of the parasite species may show evolutionary host specificity. However, recent studies suggest that specificity of lens-infecting *Diplostomum*-species in fish is low, possibly because they do not interact intensively with their fish host after establishment since they are protected from host immune defenses in the lens (Locke et al. 2010b). My results are in agreement with this result; the overall host ranges of the parasite species were quite similar although the infection intensities in the different fish species varied among the parasite species. However, there are also other alternative explanations. For example, it is well known that an exposure to *Diplostomum* parasites induces immune responses in fish hosts (Chappell et al. 1994, Karvonen et al. 2005). Such responses of the host, possibly being specific to certain host-parasite species combinations, could explain the differences in infection patterns among the fish species. However, controlled experiments and field exposures are needed to determine which processes are shaping the species assemblages in these parasites.

Despite of the indisputable advantages in community analysis, pyrosequencing has also limitations. For example, the method relies on known SNP polymorphisms and it cannot detect new parasite species. Therefore, new

screening of the ITS1 sequence variation would be needed if the method was to be applied to parasite communities from other geographical areas. Moreover, the total number of parasites in a sample would need to be estimated so that the relative parasite frequencies could be converted to absolute numbers. In the present case, parasites were counted by using slit-lamp microscopy, but such counting is not possible for many other host-parasite systems or parasite stages that reside inside the host. Estimation of the total number of parasites could, however, be achieved e.g. by quantitative PCR or by estimating the total parasite numbers from a subsample.

3.4 Genotypic and phenotypic variation in transmission traits

Significant differences among the parasite genotypes were found for all measured life-history traits prior to the food-treatment (IV). For example, some genotypes had a relative low infection success whereas others were highly infective (infection success of individual genotypes ranged between 48% and 95 %). However, the subsequent starvation of the snails for two weeks did not explain variation in parasite traits to a significant degree. Instead, 51-95 % of the variance was attributable to parasite genotype alone, depending on the trait. In addition to parasite genotype, the week of the experiment and genotype by week interaction were the most important factors in the statistical tests. The significant genotype by week effect observed for cercarial production rate, activity and infection success indicates that the changes over time were significantly greater in some genotypes than in others. On the other hand, the significant week effect observed for cercarial production rate and infection success indicates a marked temporal variation in the traits. These results are in line with those from a marine trematode (*Maritrema novaezealandensis*), that show significant differences among cercarial clones in morphology, photoreactive behaviour and survivorship as well as significant effect of time and clone by time interaction on these traits (Koehler et al. 2011).

Broad-sense heritability estimates of cercarial activity and infection success were high (0.27-0.67) and relatively stable over time in both treatment groups. However, there was considerable variation in the traits over time within each genotype. The genotypes showed different mean trait values on different weeks for all measured traits, so that individual genotypes switched ranks during the experiment, and this happened both in the 'starvation group' and in the '*ad libitum* group'. Such phenotypic variation may be adaptive or non adaptive for the parasite. Variation within-genotypes could represent 'bet hedging –strategy' (reviewed by Hopper 1999) where variation among identical parasite clones would be a way to assure that at least some of the clones will survive and transmit successfully (Fenton & Hudson 2002, Kussell & Leibler 2005, Donaldson-Matasci et al. 2008). Another possibility is that the observed variation is mainly reflecting temporal host-parasite dynamics in the snail. Such temporal dynamics may be related to cercarial production in the snail e.g. if the

cercarial performance is determined by the level of synchrony between cercarial release and host energy levels. If this was the case, then the observed within-genotype variation can be considered as non-adaptive phenotypic plasticity in response to temporal dynamics in the snail. Similarly, Koehler et al. (2011) reported that morphological and behavioural traits of *M. novaezealandensis* cercariae may vary over time within a clone, indicating that phenotypic variation in life-history traits of trematodes is common. Unfortunately, these data do not allow separation of within-snail effects from genetic effect as it would require a system where several snail individuals could be infected with the same parasite genotype. This is not possible in the present study system as *Diplostomum* reproduce sexually in the bird host.

A negative correlation was found between cercarial production rate and activity (r_{30} = -0.451, P = 0.010) prior to the food treatment. After one week of treatment infection success was positively correlated with activity (r_{14} = 0.759, P = 0.001,) in the '*ad libitum*' group, but not in the 'no food' group (r_{14} = 0.268, P = 0.316). After two weeks, none of the traits showed significant correlations or trade-offs. Similarly, Koehler et al. (2011) found few correlations between the cercarial traits that they measured in *M. novaezealandensis* clones. The instability of the genetic correlations is probably related to the temporal within-genotype variation of the traits that could be regulated by environmental factors (see Gutteling et al. 2007) such as the level of snail resources.

4 CONCLUSIONS

I studied the spatial distribution of *Diplostomum* parasites on multiple hierarchical levels including population genetic structure of the parasites within and among snail populations (I), the distribution of parasite genotypes (individuals) among snails within a single snail population (II) as well as the distribution of *Diplostomum* species within and among different fish species. I also measured the level of variation in transmission-related life-history traits within and among *D. pseudospathaceum* genotypes originating from a single snail population (IV). Overall, I found consistent heterogeneity in the parasites across all levels, indicated by the high level of neutral genetic diversity (I) and significant variation in the transmission traits (IV), as well as by the prevalent occurrence of multiple genotype (II) and multiple species infections (III).

First, my results (I) were consistent with the expectation that population genetic structure of CLC parasites is determined by the geographic range of the most mobile host (Prugnolle et al. 2005). This highlights the need for determination of the spatial scale of host and parasite populations before formulating hypotheses on host-parasite coevolution. Coevolution needs continuity in the interspecific interactions to be able to operate (Thompson 2005b) and thus the high level of parasite gene flow is unlikely to promote strong reciprocal coevolution between *L. stagnalis* and *D. pseudospathaceum.* It is plausible that the population genetic structures of the other *Diplostomum* species identified in study III would be to some extent similar to that of *D. pseudospathaceum.* This depends, however, on the mobility of their avian final host species. Applied to aquaculture conditions, my results imply that *Diplostomum* parasites are unlikely to evolve highly virulent strains at fish farms through adaptation to high fish densities. This is because fish farms are often visited by migratory birds, such as gulls, that import parasites from natural populations and thus make the local cycling of the parasite within a farm unlikely.

I also observed that MGIs were common in *D. pseudospathaceum* as well as in other snail-trematode systems described in the literature (II). Moreover, MGIs were not randomly distributed among the snail hosts, but showed an

aggregated distribution pattern, which was consistent across different trematode taxa. This suggests that aggregation of trematode genotypes in aquatic snail hosts is a universal phenomenon and is in sharp contrast to the absence of multiple species infections in snails (Kuris & Lafferty 1994). Such distribution of MGIs may be promoted by variation in host exposure levels or in host susceptibility, which will be a fruitful field for future empirical work in snail-trematode systems. The Bayesian approach developed to predict MGIs from parasite prevalence might be useful in such studies.

I also found that coinfections of different *Diplostomum*-species were common in a range of fish species. Although the infection patterns were different among the fish, the overall host ranges of the parasite species were quite similar (III). High host specificity has been shown to be negatively correlated with geographic distribution in ectoparasites indicating that the same mechanisms that enable parasites to exploit either few or many host species can be the reason why they have either a small or a large geographic range (Krasnov et al. 2005). It is likely, that the geographic distribution of all *Diplostomum* species studied here is large. Such a distribution is unlikely to promote evolution of high host specificity for the fish second intermediate hosts as the same fish species is unlikely to be found in similar numbers across its distribution range. In such a situation, high parasite specificity would lead to low likelihood of successful transmission. The results from the population genetic study support the idea of low host specificity as high levels of within-population genetic variation is more likely to be maintained in life cycles where multiple alternative host species are utilized (Barrett et al. 2008). The next step in the analysis of *Diplostomum* communities would be to find out reasons underlying differences in the infection patterns of different parasite species. Analysis of SNP allele frequencies in pooled samples would be a useful tool in such studies as it shifts the sampling unit from individual parasites to whole parasite communities. This also increases statistical power in community analyses. The method could also be used to identify *Diplostomum* species in tissues or faeces of other hosts of the life cycle. Furthermore, pyrosequencing could be applied in the analysis of parasite species assemblages in controlled experiments or field exposures.

I found significant genotypic and phenotypic variation in parasite transmission traits. Assuming that the genetic variance also has a significant additive component, the observed genotype-specific differences in transmission traits suggest high evolutionary potential for CLC parasites living in stochastic environments. It may, however, be that 'snail maternal effects' explain at least some part of this variation. Separation of the effect of parasite genotype and 'snail maternal effects' would, however, require a system where replication of parasite genotypes in several different snail individuals is possible. Schistosomes parasitizing in *Biomphalaria glabrata* snails might provide one alternative for such studies.

Overall, the results of my thesis show that introduction of molecular methodology in host-parasite interactions opens new unexplored avenues in the study of evolutionary ecology of CLC parasites. Such approaches will be

important when investigating evolutionary phenomena such as parasite specificity, but also in research of medical and economical importance of parasite infections in natural and man-made systems. This will likely to gain more emphasis in the future as problems associated with parasitic diseases are probably increasing through phenomena such as evolution of drug resistance, introduction of invasive species, and progression of the climate change.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Imumatoloisten evolutiivinen ekologia – genotyypeistä lajiyhteisöihin

Monivaiheisten loisten elinkiertoon kuuluu useita eri isäntälajeja, joiden maantieteellinen levinneisyys ja kyky liikkua populaatioiden välillä voi olla hyvin erilainen. Tällä voi olla merkitystä mm. loisen paikallisen sopeutumisen tai loispopulaation geneettisen muuntelun ylläpitämisen kannalta. Loisten jakautuminen isäntäyksilöiden, -populaatioiden ja -lajien välillä taas määrää sen millaisia biologisia vuorovaikutussuhteita loisten ja niiden isäntien välille voi syntyä ja miten ne vaikuttavat esimerkiksi loisten haitallisuuden (virulenssi) evoluutioon. Loisgenotyyppien väliset erot isännästä toiseen siirtymisen (transmissio) kannalta olennaisten elinkiertopiirteitten suhteen vaikuttavat puolestaan siihen, miten luonnonvalinta voi muokata loisten transmissiota. Viimeaikainen DNA-menetelmien nopea kehittyminen on mahdollistanut näiden loisten evolutiivisen ekologian kannalta tärkeiden kysymysten tutkimisen yksittäisten genotyyppien tasolla.

Loisten maantieteellisen levinneisyyden ajatellaan yleisesti olevan riippuvainen loisten isäntien liikkeistä, koska loisilta usein puuttuvat vapaana elävät toukkavaiheet tai näiden liikuntakyky on merkityksetön maantieteellisessä mittakaavassa. Useita isäntiä elinkierrossaan käyttävien loisten populaatiogeneettinen rakenne riippuu siis todennäköisesti isäntälajista, jonka kyky liikkua populaatioiden välillä on kaikkein korkein. Empiiristä näyttöä tämän oletuksen tueksi on kuitenkin vielä varsin vähän.

Elinkiertonsa aikana loiset yleensä jakavat isäntänsä useiden saman loislajin yksilöiden tai toisen loislajin yksilöiden kanssa. Tällaiset yhteisinfektiot voivat vaikuttaa monella tapaa isäntien ja loisten vuorovaikutussuhteisiin. Loiset voivat esimerkiksi muuttua haitallisemmiksi isännilleen kilpaillessaan tilasta ja resursseista muiden loisyksilöiden kanssa. Ilmeisestä kilpailutilanteesta huolimatta loiset, lajista riippumatta, kertyvät säännönmukaisesti tiettyihin isäntäyksilöihin. Sen sijaan suvuttomasti lisääntyvien loisten kehitysvaiheiden jakautumista väli-isäntäyksilöiden kesken ei juuri ole tutkittu, koska se edellyttää loisgenotyyppien määrittämistä molekyylimenetelmin. Useiden loisgenotyyppien esiintymisellä samassa isännässä voi kuitenkin olla merkitystä loisten kelpoisuudelle (fitness) esimerkiksi voimakkaan resurssikilpailun kautta. Loisgenotyyppien välinen kilpailu laskee loisen kelpoisuutta esimerkiksi, jos se pienentää loisgenotyyppikohtaista jälkeläistuotantoa tai johtaa isännän aikaisempaan kuolemaan.

Lähisukuisten loislajien yhteisinfektioiden tutkimista hankaloittaa puolestaan se, että lajit ovat usein morfologisesti hyvin samannäköisiä. Lajit voidaan erottaa sekvenssianalyysillä, mutta menetelmän soveltaminen loisyhteisöjen analysoimiseen on kallista ja aikaa vievää. Ratkaisuna voivat olla menetelmät, jotka hyödyntävät lajispesifisiä markereita yhdistetyissä DNA-näytteissä (eng. pooled DNA samples).

Monilla loislajeilla on havaittu myös lajin sisäistä muuntelua elinkiertopiirteissä, mikä voi johtua yksilöiden välisten geneettisten erojen lisäksi ympäristötekijöistä. Loisten elinkiertojen evoluution kannalta on tärkeä ymmärtää näiden tekijöiden suhteellista merkitystä. Esimerkiksi elinkiertopiiteiden geneettisen muuntelun määrä vaikuttaa siihen, miten piirteet voivat muuttua luonnonvalinnan seurauksena. Imumatoloisten suvuton lisääntyminen kotiloisännissään mahdollistaa loisgenotyyppien sisäisen (elinkiertopiirteiden ajallinen vaihtelu) ja genotyyppien välisen vaihtelun vertailun. Jälkimmäinen antaa käsityksen loisten evoluutiopotentiaalista, kun taas merkittävä genotyypin sisäinen vaihtelu voi olla osoitus loisen fenotyyppisestä plastisuudesta tai ns. 'bet hedging' –strategiasta, jossa sama genotyyppi tuottaa erilaisia fenotyyppejä eri ajankohtina maksimoidakseen selviytymisen epävakaissa oloissa.

Väitöskirjatyössäni käytin mallilajina *Diplostomum*-suvun imumatoloisia (Trematoda: Digenea), jotka ovat yleisiä makean veden kaloissa ympäri maailmaa. Loiset hyödyntävät elinkierrossaan kotilo-, kala- sekä lintuisäntiä. Työssäni selvitin loisten populaatiogeneettistä rakennetta sekä loisgenotyyppien yhteisinfektioiden yleisyyttä ja jakaumaa kotilopopulaatioissa. Lisäksi selvitin, miten eri *Diplostomum*-lajit esiintyvät kalayhteisöissä. Selvitin myös loisen siirtymisen kannalta tärkeiden elinkiertopiirteiden muuntelun määrää genotyyppitasolla sekä tutkin kokeellisesti miten kotiloisännän kunto vaikuttaa näihin elinkiertopiirteisiin.

Tulokset osoittivat, että loisilla oli paljon geneettistä muuntelua, ja että niillä ei ollut populaatiogeneettistä rakennetta tai sisäsiittoisuutta. Havaitsin myös merkkejä voimakkaasta loisten geenivirrasta yli 300 km päässä toisistaan olevien isäntäpopulaatioiden välillä. Nämä tulokset viittaavat siihen, että loisen lintuisännät (esim. lokki) levittävät loisia tehokkaasti kotilopopulaatioiden välillä. Geneettisen rakenteen puuttuminen ei tue ajatusta loisten paikallisesta sopeutumisesta tai yhteisevoluutiosta isäntäpopulaatioiden kanssa. Tällä voi olla merkitystä mm. näiden loisten kalanviljelylle aiheuttamien ongelmien kannalta; voimakas geenivirta estänee haitallisten loiskantojen kehittymisen kalanviljelylaitoksilla.

Havaitsin myös, että loisgenotyyppien yhteisinfektiot olivat erittäin yleisiä *L. stagnalis* -kotilopopulaatioissa sekä julkaistuun kirjallisuuteen perustuneen tarkastelun mukaan myös muissa kotilo-imumato -vuorovaikutussuhteissa. Yhteisinfektioiden esiintyminen korreloi vahvasti kotilopopulaation loisintaasteen (prevalenssi) kanssa ja yhteisinfektiot olivat keskittyneinä tiettyihin kotiloyksilöihin verrattuna satunnaisjakaumaan. Jälkimmäinen tulos saattaa johtua siitä, että osa kotiloista altistuu suuremmalla todennäköisyydellä loisille tai niillä on suurempi alttius saada useita tartuntoja esimerkiksi heikomman immuunipuolustuksen takia.

Kehitin väitöskirjatyöni yhteydessä myös SNP-markereihin (eng. single nucleotide polymorphism) perustuvan menetelmän, jolla kalan silmän linssin *Diplostomum*-loisyhteisöjä voidaan analysoida käyttämällä yhdistettyjä DNA-näytteitä. Sovelsin menetelmää luonnonkalojen loisyhteisöjen analysointiin ja havaitsin, että eri lajien yhteisinfektiot olivat yleisiä kaikissa tutkituissa kalala-

jeissa. Loisyhteisöjen rakenne kuitenkin erosi merkittävästi eri kalalajien välillä ja myös saman kalalajin erikokoisten yksilöiden välillä. Tämä voi johtua useista eri tekijöistä. Esimerkiksi eri kala- ja kotilolajien suosimien elinympäristöjen eriasteinen päällekkäisyys voi johtaa eroihin loisyhteisöjen rakenteessa, koska eri kotilolajit toimivat isäntinä eri loislajeille. Myös ajallinen vaihtelu kalojen altistumisessa eri loislajeille voi osaltaan selittää havaitut erot. Altistuminen yhdelle *Diplostomum*-lajille voi myös suojata kaloja muilta *Diplostomum*-lajeilta (eng. cross-immunity). Näiden tekijöiden merkityksen selvittäminen vaatii kuitenkin kokeellisia lisätutkimuksia.

Havaitsin myös, että loisgenotyyppien välillä oli merkittäviä eroja loisten transmission kannalta tärkeissä elinkiertopiirteissä. Elinkiertopiirteet erosivat kuitenkin myös genotyypin sisällä eri mittausajankohtina. Sen sijaan lyhytkestoinen kotiloisäntien ravintovaje ei vaikuttanut loisten elinkiertopiirteisiin. Genotyypin sisäinen fenotyyppinen variaatio voi olla loisen strategia selviytyä vaihtelevissa olosuhteissa (ns. 'bet hedging –strategia') tai osoitus fenotyyppisestä plastisuudesta. Jälkimmäiseen voi vaikuttaa esimerkiksi se, onko loistuotanto synkroniassa kotilon energiaresurssien kanssa.

Kaiken kaikkiaan tulokseni osoittavat, että *Diplostomum*-loisten monivaiheinen elinkierto ylläpitää suurta neutraalia geneettistä vaihtelua sekä mahdollisesti myös geneettistä vaihtelua elinkiertopiirteissä, mikä tekee näistä loisista hyvin sopeutuneita elämään ennalta arvaamattomissa elinympäristöissä. Yhteisinfektioiden yleisyyden syyt sekä niiden evolutiivis-ekologiset seuraukset vaativat kuitenkin lisätutkimuksia, joissa väitöskirjani yhteydessä kehitetyt menetelmät voivat olla hyödyllisiä.

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ORIGINAL PAPERS

I

IS THE POPULATION GENETIC STRUCTURE OF COMPLEX LIFE CYCLE PARASITES DETERMINED BY THE GEOGRAPHIC RANGE OF THE MOST MOTILE HOST?

by

Katja-Riikka Louhi, Anssi Karvonen, Christian Rellstab & Jukka Jokela 2010

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Is the population genetic structure of complex life cycle parasites determined by the geographic range of the most motile host?

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ABSTRACT

Due to their particular way of life, dispersal of parasites is often mediated by their host's biology. Dispersal distance is relevant for parasites because high degree of dispersal leads to high gene flow, which counters the rate of parasite local adaptation in the host populations. Parasites with complex life cycles need to exploit sequentially more than one host species to complete their life cycle. Most trematode parasites have such complex life cycles involving invertebrate and vertebrate hosts. The spatial scales of invertebrate and vertebrate host populations are often different, which may decrease the probability that the parasite cycles locally in the intermediate host population. We used neutral microsatellite markers to determine genetic structure in *Diplostomum pseudospathaceum* parasites collected from local populations of freshwater snails (*Lymnaea stagnalis*). *D. pseudospathaceum* is a trematode that has two intermediate hosts (snail and fish) and a highly motile definitive host (bird). We found that the parasite population infecting the local snail populations showed no genetic structure over a large geographic range (over 300 km). We also did not detect evidence for isolation by distance in the parasite. We conclude that dispersal in the motile definitive host is likely to prevent emergence of local population genetic structure in the parasite. Our results suggest that parasite dispersal in the definitive host may limit local cycling of the parasites in the intermediate host populations.

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1. Introduction

Theory predicts that the spatial population structure of parasites and hosts, their dispersal rates, and differences in demography drive host–parasite coevolution leading to a geographic mosaic of coevolutionary hot spots and cold spots (Thompson, 1994; Lively, 1999; King et al., 2009). One of the predictions is, that parasites are generally more likely to adapt to their local host population if the migration rate of the parasites is higher than the migration rate of their hosts (Gandon et al., 1996). This is because migration brings into the parasite population locally novel alleles that promote adaptation to new host resistance alleles. However, very high gene flow among parasite populations should slow the rate of local adaptation, or even make it unlikely (Lively, 1999). This is because high gene flow leads to swamping of the advantageous local alleles making response to selection slower. In other words, differences in dispersal rates of the host and the parasite may largely determine the rate of change in the host and parasite genes that are under selection in interacting populations (Gandon et al., 1996; Ebert, 1998; Kaltz and Shykoff, 1998; Mutikainen and Koskela, 2002) and significantly contribute to the spatial coevolutionary mosaic (Thompson, 1994).

Host mobility has been proposed to be the main determinant of gene flow in parasites since they are commonly dependent on their host for dispersal (Blouin et al., 1995). This seems highly plausible, particularly in parasites that lack free-living stages, or have free-living stages with limited mobility (Nadler, 1995). In complex parasite life cycles, including multiple host species, gene flow is expected to be determined by the host with the highest dispersal rate (Prugnolle et al., 2005).

In their review, Lajeunesse and Forbes (2002) asked how the strength of local adaptation is associated with host range and concluded that parasites with a wide host range tend to be less locally adapted, most likely because exposure to several hosts may weaken species-specific selection in an interbreeding parasite population. In other words, the gene flow between parasite lineages

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going through different host species is expected to be high. Similarly, local adaptation of parasites to their intermediate hosts may be constrained by high gene flow caused by highly motile definitive hosts. Such life cycles are common among trematodes where the intermediate hosts (snails) have genetically structured local populations that experience little gene flow, but the definitive hosts are often highly motile vertebrates that move over a large geographic area covering many intermediate host populations (Dybdahl and Lively, 1996). Interestingly, some of the best evidence for local adaptation comes from trematodes adapting to intermediate host populations (Lively et al., 2004), which shows that dispersal in the motile definitive hosts does not always lead to gene flow that is strong enough to counter local adaptation.

However, we still know relatively little of parasite population genetic structure in comparison to that of their hosts (but see Dybdahl and Lively, 1996; Criscione and Blouin, 2004; Stohler et al., 2004; Rauch et al., 2005; Keeney et al., 2007). In one of the few examples, Criscione and Blouin (2004) studied population genetic structure in trematode species infecting out-migrating smolts in four Pacific Northwest Rivers. They found that parasites that cycled only through freshwater hosts had much more structured populations and less gene flow among subpopulations than parasite species which had both freshwater and terrestrial hosts in their life cycle. Trematode populations can also be genetically structured over relatively small spatial scales, as shown by studies conducted on schistosomes (Prugnolle et al., 2005; Rudge et al., 2009).

Taken the diversity of parasites, their ecology and life cycles, more studies are needed to understand the factors that shape their population genetic structure. It would be especially interesting to know if movement of the definitive host, which often is the most motile of the host species, commonly leads to a high gene flow among parasite populations within a given geographic area. Analyzing genetic data can also shed light on the reproductive strategies of parasites. For example, the population genetic structure can be very different in outcrossing parasite species compared to those using self-fertilization (see Lagrue et al., 2007a,b for an example of self-fertilization in trematodes).

In the present paper we studied a trematode parasite, *Diplostomum pseudospathaceum* Niewiadomska 1984, which has a complex life cycle. The parasite reproduces asexually in the first intermediate host (a freshwater snail) and uses the second intermediate host (fish) for transmission to the definitive host (bird) where sexual reproduction takes place. The first intermediate hosts belong to the family Lymnaeidae, which are used as intermediate hosts by a range of trematode, nematode and cestode species (Bargues et al., 2001). These snails are known to have local and genetically strongly structured populations (Puurtinen et al., 2004), while the second intermediate host populations (fish) are regionally restricted to specific lakes, and the definitive hosts are highly motile fish-eating birds, such as gulls and terns.

In this study we asked whether these parasites show local population structure in their first intermediate host or whether the final bird host imposes strong gene flow between parasites from distinct lakes thus eroding parasite genetic structure. To address this question we studied microsatellite polymorphism in parasites collected from various populations of the first intermediate host, freshwater snail (*Lymnaea stagnalis*), covering a large geographic area, and examined the population genetic structure of the parasites within and between the snail populations. In this paper we present evidence for high gene flow and a lack of local population genetic structure in these parasites. Together with a study in a marine trematode species (*Maritrema novaezealandensis*; Keeney et al., 2009), this study is among the first to describe the genetic structure of a trematode species that has a three-host life cycle in populations of local intermediate hosts sampled over a large geographic range.

2. Materials and methods

2.1. Study system and sampling

Diplostomum (Trematoda) parasites are widespread and abundant in brackish water and freshwater systems worldwide. *D. pseudospathaceum* reproduces sexually in the intestine of fish-eating birds. When the parasite eggs are released to the water they hatch into miracidia, free-swimming larvae which infect the first intermediate host, freshwater snail (*L. stagnalis*). In the snail the parasites produce asexually thousands of cercariae, short-lived larvae that leave the snail and infect the eye lenses of the fish second intermediate host. Heavy *Diplostomum* infections are known to cause parasitic cataracts in several species of wild and farmed fish (Karvonen et al., 2004). The life cycle is completed when the infected fish is eaten by a bird. Most commonly used definitive hosts are gulls (Laridae) and terns (Sternidae).

In summer 2007 (July 23 to August 14), we sampled five *L. stagnalis* populations for parasite infections (Table 1). Two of the sampling sites (Pynnölänniemi and Ämmänlahti) were located in the same water system (Lake Konnevesi and Lake Liesvesi), but the other three populations were not connected. Distance between the sampling sites varied from 1.5 km to 300 km (Fig. 1). This distance is sufficient to lead to strong genetic structure in these mixed mating snails as earlier studies on population genetic structure of *L. stagnalis* report average pairwise F_{ST} values of 0.12–0.86 for similar snail populations in Finland (Puurtinen et al., 2004).

In the laboratory the snails were placed individually in 2 dl cups filled with water. After a few hours the cups were checked under a microscope for cercariae to determine the prevalence of *D. pseudospathaceum* in the snails. From every infected snail, individual *D. pseudospathaceum* cercariae were transferred into Eppendorf tubes and stored in −20 °C. Species determination was verified by sequencing the partial internal transcribed spacer 1 (ITS-1) region from a subset of cercariae from each sampling site (Niewiadomska and Laskowski, 2002).

2.2. Microsatellite analysis

We extracted DNA from frozen parasites according to Criscione and Blouin (2004) and genotyped haphazardly chosen cercariae

Table 1
Overview of the sampling sites.

Sampling site (lake)	Sampling date	Coordinates (WGS84)	No. of snails	No. of parasites	Prevalence
Lake Huumojärvi	14-08-2007	65°06′20″N, 26°07′50″E	283	136	36.8%
Lake Kuivasjärvi	14-08-2007	65°03′58″N, 25°28′51″E	90	42	6.7%
Pynnölänniemi (L. Konnevesi)	23-07-2007, 25-07-2007	62°36′58″N, 26°22′16″E	108	137	27.8%
Lake Vuojärvi	06-08-2007, 08-08-2007	62°24′50″N, 25°56′31″E	264	139	16.3%
Ämmänlahti (L. Konnevesi)	31-07-2007, 01-08-2007, 02-08-2007	62°36′46″N, 26°20′38″E	68	139	38.2%

Abbreviations: No. of snails, total number of snails collected per sampling site; No. of parasites, total number of parasites included in the analysis; prevalence, percentage of snails infected with *Diplostomum pseudospathaceum*.

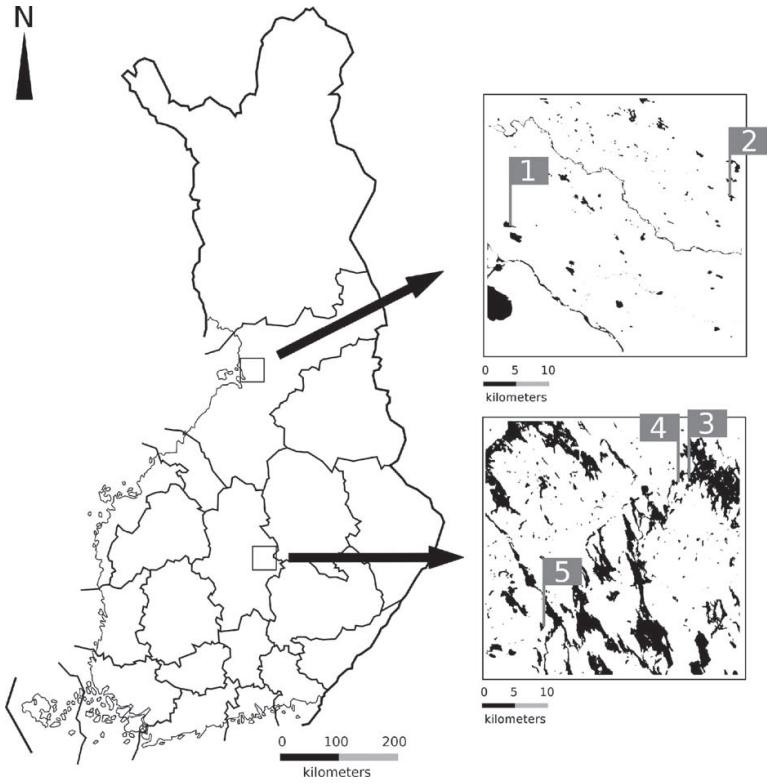

Fig. 1. Map of Finland. Sampling sites are marked with numbers: (1) Kuivasjärvi, (2) Huumojärvi, (3) Pynnölänniemi, (4) Ämmänlahti and (5) Vuojärvi.

(n = 7) from each snail (n = 20, except for Kuivasjärvi n = 6) at three microsatellite loci Diplo06, Diplo09 and Diplo23 (Genbank accession numbers: AJ629250, AJ629252 and AJ629253; Reusch et al., 2004). The microsatellites were amplified using a multiplex PCR kit (Qiagen, Basel, Switzerland) in a 6 μl total volume. Each reaction included 3 μl multiplex PCR master mix, 0.5 μM of each primer (Applied Biosystems, Cheshire, UK), 0.64 μl RNase-free water and 2 μl extracted DNA. Thermocycling profile started with 15 min at 95 °C, followed by 30 cycles of 94 °C for 0.5 min, 56 °C for 1.5 min and 72 °C for 1 min. A further extension step of 30 min at 60 °C ended the program. 1 μl of each PCR product was mixed with 15 μl Hi-Di™ formamide (Applied Biosystems, CA, USA) and 0.5 μl GeneScan™-500 LIZ® size standard (Applied Biosystems, CA, USA). Samples were analyzed on an ABI Prism® 3130xl Genetic Analyzer and alleles were identified using the software Peak Scanner 1.0 (Applied Biosystems Inc.).

2.3. Statistical analyses

We used Micro-Checker 2.2.3 to check the data for null alleles and scoring errors due to stuttering and large allele drop-out (Van Oosterhout et al., 2004). We excluded identical cercariae genotypes from the same snail host to avoid artificially introduced population structure (due to the asexual reproduction that takes place in the snails) in the statistical analysis. We estimated population genetic parameters using FSTAT 2.9.3.2 (Goudet, 2001) and Genepop 3.4 (Raymond and Rousset, 1995). The latter program was also used to calculate and test deviations from HWE for all loci and sampling sites, to determine linkage disequilibrium between all pairs of loci and to estimate the number of migrants by using the private alleles method (Barton and Slatkin, 1986). We tested the significance of F_{IS} values by applying 1500 randomizations with FSTAT 2.9.3.2 using a value of 0.003 as the adjusted 5% nominal level for multiple comparisons. We additionally used RMES to determine selfing rates from the genetic data (David et al., 2007) and tested the isolation by distance [$F_{ST}/(1 - F_{ST})$ versus ln-transformed distances; Rousset, 1997] with Mantel test applying 5000 permutations (Mantel, 1967) using Genepop 4.0. Finally we performed a factorial analysis of correspondence with Genetix 4.05 (Belkhir et al., 1996–2004) and examined if population membership of a genotype can be predicted using Structure 2.2 (Pritchard et al., 2000). We used default settings in Structure, prior information of population membership and the following parameters: K = 5, length of burn-in = 10 000 repetitions and run length 100 000 repetitions.

3. Results

3.1. Genetic diversity

Parasites were identified based on morphology and sequencing of the partial ITS-1 region. Obtained sequences (n = 12) were 100% identical with the ITS-1 sequence from *D. pseudospathaceum* (BLASTN search, NCBI Genbank). Prevalence of *D. pseudospathaceum* infection varied between 6.7% and 38.2%. Note that the snails were not dissected to detect possible developing infections (sporocysts not releasing cercariae), which is why the prevalence values should be taken as minimum estimates. Genotyping using the three chosen microsatellite loci was highly reliable (only ~1% of the alleles could not be determined). All loci were variable with

Table 2
Summary of the microsatellite data.

Sampling site	Parameter	Microsatellite loci			
		Diplo06	Diplo09	Diplo23	
		n_{tot} = 32	n_{tot} = 33	n_{tot} = 14	All loci
HUUMOJÄRVI	n	16	22	10	48
N_{mlg} = 32	H_e	0.907	0.940	0.841	0.896
	H_o	0.818	0.970	0.844	0.877
	F_{IS}	0.098	−0.032	−0.003	0.021
KUIVASJÄRVI	n	9	11	5	25
N_{mlg} = 7	H_e	0.905	0.964	0.774	0.881
	H_o	1.000	1.000	0.714	0.905
	F_{IS}	−0.105*	−0.037*	0.077	−0.022
PYNNÖLÄNNIEMI	n	21	26	10	57
N_{mlg} = 33	H_e	0.936	0.960	0.798	0.898
	H_o	0.909	1.000	0.667	0.859
	F_{IS}	0.028	−0.042*	0.165	0.050
VUOJÄRVI	n	16	22	10	48
N_{mlg} = 25	H_e	0.897	0.955	0.798	0.883
	H_o	0.840	1.000	0.880	0.907
	F_{IS}	0.063	−0.047*	−0.102	−0.029
ÄMMÄNLAHTI	n	18	27	10	55
N_{mlg} = 38	H_e	0.914	0.960	0.847	0.907
	H_o	1.000	0.921	0.816	0.912
	F_{IS}	−0.094*	0.041	0.037	−0.005

Abbreviations: n_{tot}, total number of alleles per locus; n, number of alleles per sampling site; N_{mlg}, number of complete multilocus genotypes included in the analysis; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{IS}, fixation index ($1 - [H_o/H_e]$) values.
* $P < 0.05$, level of significance for F_{IS} values (determined after 1500 randomizations).

14–33 alleles per locus, the total amount of alleles being 79 with an average of 26.3 (Table 2). The number of alleles ranged from 25 to 57 between the host populations, with an average of 47. Allele numbers per locus were similar in all host populations (except for Kuivasjärvi, which had fewer samples). All locus pairs were in linkage equilibrium ($P = 1$). All snail populations had some snails with multiple genotype parasite infections and thus the number of multilocus genotypes included in the analysis exceeded the number of sampled infected snails (Table 2). One parasite genotype from Huumojärvi could not be distinguished from another parasite genotype from Ämmänlahti since only two loci could be scored from the former, in other cases the same genotype was never found in different snails.

The observed and expected heterozygosities (H_o and H_e) varied from 0.667 to 1 and from 0.774 to 0.964, with means of 0.892 and 0.893, respectively (Table 2). Overall F_{IS} values for parasites collected from the five different host populations ranged from −0.027 to 0.044 (Table 2). Some F_{IS} values were significantly different from zero and all significant F_{IS} values were negative. Selfing rates were not significantly different from zero ($g2 = s = 0$) in any of the sampling sites.

3.2. Absence of genetic structure and the estimated number of migrants

The overall F_{ST} of the parasites from different host populations was −0.001. The pairwise F_{ST} values ranged from −0.0058 to 0.0048 (Fig. 2) and 50% of the pairwise comparisons even resulted in a negative F_{ST} value (estimates can be negative close to zero). The highest F_{ST} value, 0.0048, was found between Kuivasjärvi and Ämmänlahti populations. All non-adjusted P-values for each population pair were non-significant (P for all comparisons was > 0.05). None of the pairwise estimates of F_{ST} were significantly larger than zero after Bonferroni correction. Allele frequencies were in Hardy–Weinberg equilibrium (HWE) for all loci and populations (x^2-test, $P = 0.516$). Furthermore, analysis with the software Structure suggested that every parasite individual from the five host populations could belong to any other population with 20% probability (data not shown). A factorial analysis of correspondence supported the finding that there is no population structure in the data set (data not shown).

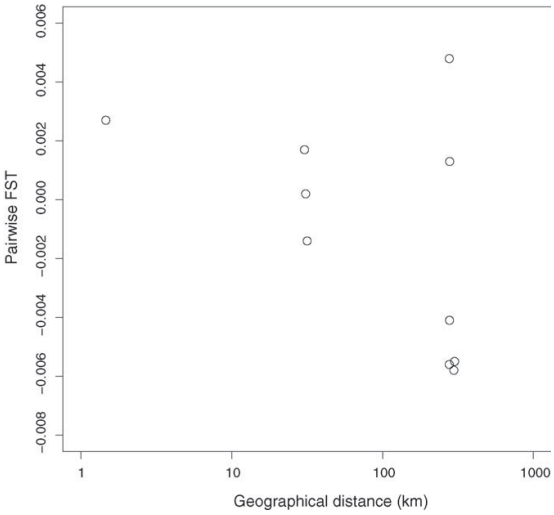

Fig. 2. Pairwise F_{ST} values plotted against geographic distance. None of the F_{ST} values is significant after Bonferroni correction.

The estimate for the number of migrants in every generation (N_m) was 11.4 after correction for sample size (mean sample size was 27.13 and mean frequency of private alleles was 0.0182). There was no significant correlation between geographic distance and the genetic estimates (Mantel test with 5000 permutations, $P = 0.96$) indicating the absence of any spatial genetic differentiation between the sampling locations (Fig. 2).

4. Discussion

Gene flow among natural populations of parasites is of fundamental importance for coevolutionary dynamics of parasites

and their hosts. Gene flow should be high enough to provide genetic resources for the parasite population to better adapt to the local host, but it should be low enough not to attenuate the adaptive response to selection (Gandon et al., 1996; Lively, 1999). In addition to genetic factors, a range of ecological factors such as host mobility, reproductive mode of the parasite, complexity of the parasite life cycle and the size of the parasite population within a host may influence the genetic structure of parasite populations (reviewed by Barrett et al., 2008). Factors that are predicted to decrease the genetic differentiation among local parasite populations include mobile hosts, long generation time and low host specificity (Nadler, 1995)

4.1. Few loci with many alleles versus many loci with few alleles

Our results are based on three microsatellite loci, which is a low number for many other systems for which microsatellites are available. There are good reasons to expect that for our purposes the number of loci is sufficient. First, the three microsatellite loci analyzed in this study were highly polymorphic (14–33 alleles/locus). Kalinowski (2002) used computer simulations to investigate how the number of alleles at loci affects the estimates of genetic distances, such as F_{ST} values. He showed that equivalent results could be achieved by examining either few loci with many alleles or many loci with few alleles. For example, 16 loci each having two alleles had about the same coefficient of variation for the genetic distances as two loci having 16 alleles each. Therefore it is very unlikely that a different population genetic structure would have been observed in our study system even if more microsatellite loci had been analyzed. Second, we use these loci as markers of neutral genetic structure, i.e. regions of the genome that are not under selection. All three loci give a similar signal and behave similarly in the analysis. Therefore, we expect that these loci reflect the genetic structure of the regions in the genome that are not directly involved in the host–parasite interaction. Third, the polymorphism in our loci was still high enough for all but two parasite individuals to return a unique genotype. This indicates that the resolution of our markers was at the level expected for a sexual species.

4.2. Panmixia

Multi-host parasite populations are expected to maintain higher levels of genetic polymorphism than parasites with a narrow host range because they are unlikely to experience extreme size fluctuations (Barrett et al., 2008). Our results are in agreement with this prediction as the polymorphism we detected was high at all three microsatellite loci. Many trematodes are facultative hermaphrodites, which potentially could self-fertilize their offspring. Self-fertilization is more common in trematodes that have abbreviated their life cycle and use two instead of three hosts (Poulin and Cribb, 2002). Facultative self-fertilization may lead to significant heterozygozity defiency, as reported for *Coitocaecum parvum* (Lagrue et al., 2007a,b), which can omit the definitive fish host and produce eggs by selfing in its amphipod host. However, selfing does not seem to be common in our study population as we found low homozygosity and no deviation from HWE. The RMES estimated selfing rates were not significantly different from zero. The negative F_{IS} values indicate an excess of heterozygotes. Most of the F_{IS} values, even if statistically significant, were very close to zero, suggesting that mating was close to panmixia. In the present system selfing simply may be rare because wild common gulls and herring gulls are typically infected with tens or even hundreds of adult parasites (Karvonen et al., 2006).

In general, our results show that the genetic differentiation among *D. pseudospathaceum* parasites in a geographically large area in Central Finland is very low (Fig. 2 and Table 2). Our results suggest that these parasites have a high effective population size and high level of gene flow among the sampled local host populations. The observed pairwise F_{ST} values of the parasites are much lower than pairwise F_{ST} values obtained for the snail host (*L. stagnalis*) populations in a previous study conducted in Finland. For example, the *L. stagnalis* populations obtained from lakes that were 2–30 km apart had pairwise F_{ST} values between 0.12 and 0.86 (Puurtinen et al., 2004).

4.3. Mobility of the final bird host enables parasite dispersal

We did not find evidence for isolation by distance in the parasite population, which is striking because the distances among the sampled host populations were considerable. The most likely explanation for this is a high level of non-gradual gene flow between the sampling sites. In our study system the mobility of the final bird host can potentially be very high. For instance, common gulls (*L. canus*) overwinter in Denmark, at the North Sea coast or in Britain (Kilpi and Saurola, 1985), whereas herring gulls (*L. argentatus*) overwinter within the Baltic (Kilpi and Saurola, 1984). Adult common and herring gulls return each spring to their natal breeding sites in Finland. Young common gulls stay 1–2 years in the overwintering areas before returning to the breeding areas in the North (Kilpi and Saurola, 1985), whereas juvenile herring gulls mostly return to the breeding areas every spring (Kilpi and Saurola, 1984). It is likely that birds are infected with *D. pseudospathaceum* throughout the year, also at the overwintering areas, and that these parasites are imported to Finland when the gulls return. During summer 75% of adult common gulls stay within 50 km of the natal site, but some individuals are recruited into more distant areas (up to 300 km away from the natal site) (Kilpi and Saurola, 1985). Around 15% of adult herring gulls are found more than 100 km away from the natal site during the breeding season (Kilpi and Saurola, 1983). It is likely that high mobility in the two main definitive host species is sufficient to cause high levels of gene flow between spatially isolated *Diplostomum* parasites in Finland when taking into account that each gull can harbour dozens or hundreds of adult parasites. However, it is possible that population genetic structure and isolation by distance could be detected in this system if parasites would be sampled from lakes separated by a much larger geographic distance than 300 km. Especially interesting would be to sample a transect from east to west, for example, covering regions of Siberia where the overwintering areas of birds differ.

4.4. Factors affecting local cycling of parasites

Studies on various host–trematode systems have shown that trematode parasites may be locally adapted to their intermediate host populations (Ballabeni and Ward, 1993; Lively et al., 2004). For example, in the well-studied New Zealand *Potamopyrgus–Microphallus* system, parasites have been shown to adapt to their local snail populations (Lively et al., 2004) and even specifically to some common asexual snail genotypes (Lively et al., 2004; Jokela et al., 2009). Our results are mainly concordant with the population genetic structure of *Microphallus* sp. in New Zealand (Dybdahl and Lively, 1996). *Microphallus* sp. populations were in HWE, gene flow seems high (tens of migrants per generation) and differentiation of allozyme markers relatively low (0.012–0.028). In contrast to our results, the spatial arrangement of the lakes between the east and west side of the New Zealand Southern Alps had an effect on *Microphallus* sp. population structure. The lack of major geographic barriers (mountains) in central Finland might provide one

explanation for the absence of population structure in *D. pseudospathaceum*. The other important difference between *Microphallus* sp. and *D. pseudospathaceum* is that *Microphallus* sp. has a two host life cycle, while *D. pseudospathaceum* has three obligatory hosts. Moreover, *Microphallus* sp. is a small and short lived (few months, Lively, unpublished) parasite in the definitive host while *D. pseudospathaceum* is medium sized parasite which probably lives much longer. Short lifespan in the definitive host significantly increases the opportunity for local cycling in the intermediate host populations, which might be one reason why strong local adaptation by parasites on snail populations has frequently been observed in the *Microphallus–Potamopyrgus* system (Lively et al., 2004).

5. Conclusions

In summary, we analyzed the population genetic structure of the trematode parasite *D. pseudospathaceum* in its first intermediate host, a freshwater snail (*L. stagnalis*) at five different locations in Finland. We could not find evidence for strong genetic differentiation, inbreeding or isolation by distance in parasites collected from local host populations. The estimate of gene flow (N_m) between the sampling sites was high, suggesting that dispersal, with the aid of the definitive bird host, acts as the homogenizing force limiting genetic differentiation in the parasites. High gene flow does not make parasite local adaptation impossible (Gandon and Michalakis, 1996; Lively, 1999), but in the case of *Diplostomum* the likelihood of local cycling of the parasites may be reduced because of the wide foraging range of bird hosts, the longevity of the parasite in this final host and possible temporal stochasticity of the snail populations.

Conflict of interest

The authors declare no conflict of interest.

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II

PREVALENCE OF INFECTION AS A PREDICTOR OF MULTIPLE GENOTYPE INFECTION FREQUENCY IN PARASITES WITH MULTIPLE-HOST LIFE CYCLE

by

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Submitted manuscript

III

ANALYSIS OF TREMATODE PARASITE COMMUNITIES IN FISH EYE LENSES BY PYROSEQUENCING OF NATURALLY POOLED DNA

by

Christian Rellstab, Katja-Riikka Louhi, Anssi Karvonen & Jukka Jokela 2011

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Analysis of trematode parasite communities in fish eye lenses by pyrosequencing of naturally pooled DNA☆

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ABSTRACT

Infections by multiple parasite species are common in nature and have important consequences for between-species interactions and coevolutionary dynamics with the host populations. For example, ecological and evolutionary factors underlying the structure of parasite communities determine the range of hosts a parasite can infect and set the basis for both evolution of host defences and parasite virulence, as well as management of diseases. Studies investigating these factors have been facilitated in the recent past by genetic methods, which surmount difficulties of traditional morphological taxonomy in identifying individual parasite species. Here we take a step further and present a novel methodological approach to analyze parasite communities as a whole. We determined the relative frequencies of interspecific SNP alleles by pyrosequencing naturally pooled samples of closely related *Diplostomum* spp. trematodes infecting eye lenses of freshwater fish. Pyrosequencing allowed us to use naturally pooled community samples (lenses) to increase the sample size and statistical power, without sequencing single parasite specimens. In a case study, we applied this method to analyze the community structure of the eye flukes across fish host species of a freshwater system in Finland. We found that the fish species harboured significantly different parasite communities and that multiple species infections were common. Our study provides an example of how quantitative pyrosequencing can be used to answer evolutionary and ecological questions in natural communities of parasites.

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1. Introduction

Multiple infections, where host individuals are simultaneously infected with several different parasite species (or different genotypes of the same species), are common and diverse in nature (Cox, 2001; Fleming et al., 2006; Petney and Andrews, 1998). Communities of the co-infecting parasites in individual hosts and host populations show structure in various degrees (Poulin, 2007), and this structure is strongly influenced by ecologically and evolutionary driven parasite–parasite and host–parasite interactions (Adamson and Caira, 1994; Janovy et al., 1992; Mideo, 2009; Poulin, 2001). Community processes may have significant consequences for the evolution of host–parasite relationships and the management of medically important parasite species as well as of those infecting our food chain. For example, competition among co-infecting parasite strains can select for higher parasite virulence, i.e. harm caused to the host (de Roode et al., 2005; Van Baalen and Sabelis, 1995). In malaria, a prior or co-infection with one species can decrease (Maitland et al., 1997) or increase (May et al., 2000) the effect of another species, but these interactions also depend on other factors like seasonality and infection intensity (Bruce et al., 2008).

The above examples illustrate that community level approaches are necessary for understanding the evolutionary ecology of host–parasite relationships. Such approaches, however, may be associated with practical difficulties in many parasite groups. One of the major hurdles in characterizing parasite communities is that same host individuals (e.g., Valtonen and Gibson, 1997), same organs (e.g., Lello et al., 2004) and particular tissues (e.g., Karvonen et al., 2009) can be infected by dozens or hundreds of parasite individuals belonging to different but closely related species. The species are often morphologically similar, without a proper resolution of morphological taxonomy, or accompanied by cryptic species (Donald et al., 2004; Jousson et al., 2000; Leung et al., 2009; Locke et al., 2010b). As a consequence, traditional community level analyses are time consuming and require very high level of parasitological expertise.

☆ *GenBank:* Sequences of the 82 reference samples have been deposited in GenBank under accession numbers JF775679–JF775760.

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DNA based methods provide an alternative method of discriminating among taxa (McManus and Bowles, 1996) and analyzing the structure of communities of co-infecting parasite species. Species identification by sequencing regions (Sanger et al., 1977) of ribosomal and mitochondrial DNA has therefore become more common (e.g., Donald et al., 2004; Locke et al., 2010b) and various other mutation scanning methods exist (for examples see, e.g., Gasser, 2006). However, the fact that only one parasite specimen can be analyzed at a time limits the sample size and power of community analyses, and results in high processing times and costs.

Pyrosequencing (Ronaghi et al., 1998) represents an ideal tool in this context. It can be used for a wide range of applications such as sequencing of PCR amplified gene fragments (Kolak et al., 2003), methylation analyses (Tost et al., 2003) and analyses of single nucleotide polymorphisms (SNPs, Ahmadian et al., 2000). Pyrosequencing is also the method underlying one of the high throughput sequencing systems, which are frequently used for, e.g., transcriptome characterization, gene expression profiling, and whole genome (re-)sequencing (Ekblom and Galindo, 2010; Hudson, 2008). So far, however, the method has not been extensively applied in studies of parasite/pathogen taxa or genotypes (but see Sreekumar et al., 2005; Troell et al., 2003). In particular, pyrosequencing allows reliable and accurate allele quantification of SNPs in pooled samples, even with very small differences in allele frequencies (Gruber et al., 2002). Given that knowledge about the interspecific sequence variation is available, pyrosequencing can be used to analyze parasite communities in naturally pooled samples including morphologically similar, but genetically different species. This allows communities to be characterized as a whole using pooled DNA samples, thus representing a major breakthrough in the capacity to examine parasite community structure.

In the present study, we demonstrate how pyrosequencing can be used to analyze parasite communities within individual hosts and across host populations. More precisely, we perform allele quantification of interspecific SNPs in naturally pooled samples (i.e. whole parasite communities of closely related and previously described species) in order to determine the species composition in the hosts. Our model system is a trematode species complex (*Diplostomum* spp.) infecting the eye lenses of several co-occurring freshwater fish species. In a case study, we sampled the lenses of different fish species and compared the species composition and frequency of co-infection among host species. We propose that pyrosequencing of naturally pooled parasite samples represents an efficient and powerful method to characterize parasite communities.

2. Materials and methods

2.1. Study organism

Trematodes of the genus *Diplostomum* parasitize snail, fish and bird hosts during their life cycle (Chappell et al., 1994). Adult worms mature in the intestine of fish eating birds, where they reproduce sexually. Eggs are released with the bird faeces into the water, where they hatch to free-swimming miracidia larvae and infect the first intermediate host, a freshwater snail. In the snail, asexual reproduction takes place and the parasite larvae leave the snail as free-living cercariae which penetrate the second intermediate host, a freshwater fish. Cercariae migrate to the eye and develop to metacercariae in the lens (some species infect the vitreous humour of the eye or tissues under the retina). *Diplostomum* spp. are common in many freshwater and brackish water fish species (Valtonen and Gibson, 1997). The life cycle is completed when the fish is eaten by a predator bird.

2.2. Reference samples

We sampled various parasite individuals at different life stages and from different host species in order to characterize parasite species-specific (interspecific) SNP alleles and develop the pyrosequencing assays used in this study. In total 82 specimens were sampled, including 21 cercariae from 21 snails representing three different snail species, 57 metacercariae from lenses of ten different fish species, and four adult parasites isolated from gulls (*Larus fuscus*). The samples originated from five different lakes in Finland up to 300 km apart (Lake Huumojärvi, L. Konnevesi, L. Kuivasjärvi, L. Liesvesi, and L. Vuojärvi, for locations see Louhi et al., 2010) and from one fish farm located in central Finland (rainbow trout and gull samples). We froze the samples at −20 °C or −80 °C until further analysis, except for the adult parasites that were preserved in 95% ethanol at room temperature.

DNA was either isolated in 90 μl of 5% chelex (Bio-Rad Laboratories) containing 0.2 mg ml^{-1} proteinase K (Roche), incubated at 56 °C for 2 h and heated at 100 °C for 8 min, or by using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. We sequenced the partial internal transcribed spacer 1 (ITS1) region of the ribosomal DNA (rDNA) to determine the species of the individual parasites. PCR amplification was carried out in a final volume of 20 μl containing 5 μl genomic DNA, 0.5 μM of forward and reverse primers (TAG, named here SPA-F and SPA-R, primers from Niewiadomska and Laskowski, 2002, see Appendix A), 0.25 mM of each dNTP (Fermentas), 2 μl 10× $MgCl_2$ free buffer (BioTools), 1.5 mM $MgCl_2$ (BioTools), and 0.5 U DNA polymerase (BioTools). The thermocycling profile started with 2 min at 95 °C, followed by 25 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. A further 5 min extension at 72 °C ended the program. 15 μl of the PCR product was treated with Exonuclease (Exo I, Fermentas) and Shrimp Alkaline Phosphatase (SAP, Fermentas). 3 μl of each Exo I-SAP treated PCR amplification was used in direct cycle sequencing with BigDye® v.3.1 chemistry (Applied Biosystems) according to the manufacturer's instructions. To obtain a complete sequence of both strands of the PCR product, two internal sequencing primers, KRP-F and KRP-R (TAG, Appendix A), were designed and used together with the PCR primers. Sequencing reactions were purified with DyeEx 2.0 spin kit (Qiagen) and resolved on an ABI PRISM® 3130XL genetic analyzer (Applied Biosystems). The sequence chromatograms were analyzed with Sequence Scanner™ v1.0 (Applied Biosystems).

We did multiple alignments and consensus sequences with BioEdit Sequence Alignment Editor v7.0.9.0 (Hall, 1999) using ClustalW multiple alignment. We compared our sequences to already existing datasets in the NCBI GenBank. All sequences could be assigned to five different lens-infecting species or species groups (*D. mergi*, GenBank accession number AF419279.1, *D. paracaudum* AF419272.1, *D. parviventosum* AF419277.1/*D. spathaceum* AF419275.1, and *D. pseudospathaceum* AF419273.1) and one humour-infecting species (*D. baeri* AF419274.1) described in Niewiadomska and Laskowski (2002). According to these authors, *D. parviventosum* and *D. spathaceum* share the same sequence, but can be distinguished morphologically. However, as our study does not include any morphological analyses, we will refer to the species *D. parviventosum/spathaceum* throughout the manuscript, acknowledging that this taxon may consist of one or two species. Genetic distances within and between species (Kimura-2-parameter distance with pairwise deletion of gaps, Kimura, 1980) were calculated with MEGA 4.0 (Tamura et al., 2007). The same software was also used to draw a bootstrapped neighbour-joining tree to illustrate genetic distance and taxonomic groups. The five species were genetically distinct from each other (Table 1 and Fig. 1), *D. paracaudum* and *D. pseudospathaceum* being the most closely related. Most of the 82 individuals fitted well into this five species

Table 1
Pairwise genetic distances (Kimura-2-parameter model, pairwise deletion of gaps) between and within (grey area) the five different *Diplostomum* species, based on 82 reference samples used for development of the pyrosequencing assays. Distances are based on partial ITS1 sequences with a length up to 630 bp. Errors represent standard errors, calculated by computing 500 bootstrap iterations.

Species	*D. baeri*	*D. mergi*	*D. paracaudum*	*D. parviventosum/spathaceum*	*D. pseudospathaceum*
No. of samples	7	5	33	4	33
D. baeri	0.003 ± 0.002				
D. mergi	0.059 ± 0.010	0.001 ± 0.001			
D. paracaudum	0.032 ± 0.007	0.051 ± 0.009	0.001 ± 0.000		
D. parviventosum/spathaceum	0.034 ± 0.008	0.035 ± 0.008	0.040 ± 0.008	0.000 ± 0.000	
D. pseudospathaceum	0.025 ± 0.006	0.045 ± 0.008	0.014 ± 0.005	0.032 ± 0.008	0.003 ± 0.001

scheme, except for four individuals classified as *D. pseudospathaceum* (specimens 77–80, Fig. 1). Seven parasites were *D. baeri*, a synonym for *D. gasterostei* (used, for example, in Karvonen et al., 2006a; Valtonen and Gibson, 1997). Since we were only interested in the parasite community in the lenses, *D. baeri* samples were included in the pyrosequencing analysis to detect and quantify possible contaminations on the surface of the lens by this species.

2.3. Development of pyrosequencing assays

The sequences amplified by the ITS1 primers were up to 630 base pairs (bp) and the alignment 634 bp in length (Appendix A). All 82 sequences have been deposited in GenBank under accession numbers JF775679–JF775760. Over a common range of 556 bp that was analyzed for pyrosequencing assay development, the individuals shared 495 nucleotides (89.0%). From the 61 detected SNPs, 19 had at least one interspecific allele. The remaining SNPs had either intraspecific variation or alleles common to several parasite species. Additionally, in 25 sequences and in a total of 36 positions (0.08%), potential intra-individual variation was detected, which is common for the ITS1 region that exists in multiple copies in the cell (Hillis and Dixon, 1991). However, when developing the pyrosequencing assays these positions were excluded. Using the Pyrosequencing® Assay Design Software (Biotage) we developed two pyrosequencing assays (Diplo197 and Diplo503) to analyze two regions in the ITS1 region that included four interspecific SNPs to differentiate between the five species (Table 2, Appendix A). The sequences presented in Niewiadomska and Laskowski (2002) have the same interspecific SNPs.

We used a nested PCR approach. In the first step, a PCR targeting the ITS1 region was done (as described in Section 2.2) followed by a PCR with a total volume of 40 μl for each pyrosequencing assay. In the second PCRs, each reaction contained 3 μl diluted (1:50 to 1:150 depending on the number of parasites in the sample) PCR product from the first step, 0.2 mM of each dNTP (Fermentas), 0.2 μM of each primer (TAG, Appendix A), 2.0 or 2.5 mM $MgCl_2$ (BioTools, Appendix A), 1.2 U of DNA Polymerase (BioTools) and the associated buffer at 1× concentration. An initial denaturation step of 5 min at 95 °C was followed by 50 cycles of 15 s at 95 °C, 30 s at the primer-specific annealing temperature (Appendix A) and 15 s at 72 °C, and a final step of 4 min at 72 °C. The biotinylated PCR products were extracted with streptavidin sepharose beads (Biotage) according to the manufacturer's instructions and released into a PSQ 96 Plate Low (Biotage) containing 39 μl annealing buffer (Biotage) and 1 μl of 10 μM pyrosequencing primer (Appendix A). The plate was incubated at 80 °C for 2 min and analyzed on a PyroMark™ ID instrument (Biotage) using Pyro Gold reagents (Biotage) as specified by the manufacturer. The dispensation order of the four nucleotides and allele quantification was determined automatically by the software using default settings.

Primers were chosen and optimized according to their ability to deliver reliable and repeatable results (more than the four SNP loci presented here were originally tested). First, we analyzed 34 of the 82 reference samples of known species identity based on dideoxy sequencing (Sanger et al., 1977). Samples from all species (including *D. baeri*) were included in this analysis. Second, repeatability tests were performed on the same DNA template, i.e. certain lenses were analyzed up to five times. Finally, measurements on artificial mixtures of known lens-infecting species were performed, measuring nine different DNA ratios of four parasite pairs (from the same lens) belonging to two of the four different parasite species (for details, see Appendix A).

2.4. Determination of relative taxon frequencies

Relative taxon frequencies within a sample were determined directly or stepwise. The frequency of *D. mergi* was determined by the frequency of allele T in locus a, *D. paracaudum* by the frequency of the allele T in locus c, and *D. parviventosum/spathaceum* by the frequency of allele A in locus d (Table 2 and Appendix A). The frequency of *D. baeri* was determined by the frequency of allele G in locus b after subtracting the frequency of allele T in locus c. If this resulted in negative frequencies for *D. baeri*, these erroneous (but rare) estimates were set to zero. The remaining frequency represented *D. pseudospathaceum*. In samples including only a single specimen, original frequencies were multiplied by a factor so they summed up to 100%. In pooled samples, the frequency of the humour-infecting *D. baeri* was ignored and the remaining frequencies were multiplied by a factor to sum up to 100%. Pooled samples with a frequency of *D. baeri* exceeding 50% were excluded from the analyses.

2.5. Case study – parasite communities in the lenses of five freshwater fish species

2.5.1. Fish and parasite sampling

We sampled different fish species using gillnets between 11th of July and 26th of August 2008, at three different locations (within a range of 1 km) and dates in the shallows of Lake Konnevesi (62°37′N, 26°21′E), an oligotrophic lake in Central Finland. Two different mesh sizes were used (16 and 30 mm). Additionally, we sampled juvenile fish using a fine mesh size seine net and received vendace (*Coregonus albula*) from a local fisherman (16 mm mesh size). Throughout this manuscript we will refer to the different size classes of fish by using "small" (fine mesh size), "medium" (16 mm mesh size) and "large" (30 mm). The length of the fish was determined and the number of metacercariae in the lenses was counted using a slit-lamp microscope (KOWA SL-14, Karvonen et al., 2004). Finally, analyzed lenses were removed, stored in Eppendorf tubes and frozen at −80 °C until further analyses.

We pyrosequenced one randomly chosen infected lens from each fish. However, to test lens-specific variation within fish individuals, we also analyzed both lenses from 20 randomly chosen fish. To homogenize the lens and the parasite material, and to improve DNA extraction, we added a 5 mm stainless steel bead (Qiagen) in each tube and shook the tubes in a bead mill (MM2000, Retsch) at 80 Hz for 2 min. The extraction was performed with the DNeasy® Blood &

Fig. 1. Neighbour-joining tree of *Diplostomum* reference specimens. The sequences of these 82 specimens were used to develop the pyrosequencing assays. Based on the partial ITS1 region of the rDNA and Kimura-2-parameter distances with pairwise deletion of gaps. Bootstrap values (in %) are based on 500 iterations; values below 50% are not shown. Species names (italic) of the different taxa (to which the specimens were assigned, larger font) according to reference sequences of Niewiadomska and Laskowski (2002) which are also included in the tree. Sequences are deposited in GenBank under accession numbers JF775679–JF775760.

Table 2
Species specific loci at the target regions of the two pyrosequencing assays. Bold letters with grey background indicate species specific SNP alleles. For more information, see Appendix A.

Assay/position:	Diplo197			Diplo503
Locus:	a	b	c	d
Species				
D. baeri	CACTTGTG**G**AGGGTTC			GCATGCC
D. mergi	**T**ACTTGTGAAGGGTTC			GCATGCC
D. paracaudum	CACTTGTG**G**AGGGTT**T**			GCATGCC
D. parviventosum/spathaceum	CACTTGTGAAGGGTTC			**A**CATGCC
D. pseudospathaceum	CACTTGTGAAGGGTTC			GCATGCC

Tissue Kit (Qiagen) according to the manufacturer's instructions, but with a double concentration of proteinase K, a lysis time of at least 14 h and two elution steps each with 50 μl buffer at the end of the protocol. The extracted DNA was then amplified using the nested PCR described in Section 2.3.

2.5.2. Statistical analysis

The effect of fish species and size class on parasite richness (ln-transformed) and diversity (Simpson's Index of Diversity 1-D) was analyzed using a MANOVA, followed by Tukey's post hoc tests to distinguish among significant treatments. Since richness was based on presence–absence data, we used a 5% detection limit in the pyrosequencing analysis for each species, thus excluding possible confusion between low allele frequency and background noise. This limit was based on previous literature (Keller et al., 2008) and confidence intervals found in single specimen samples (see Section 3.1).

We used linear mixed models (LMMs) to analyze species-specific infection patterns, representing an ideal tool for analyzing data with both fixed and random (including nested) factors. The number of metacercariae ($\ln(x+0.1)$-transformed) per parasite species was set as a dependent variable, and calculated by multiplying the total parasite number in a lens with the relative frequencies of the corresponding species. To exclude random noise associated with variation among individual hosts, lens was defined as a random factor. Host species, parasite species, fish size class (roughly representing age classes), sampling location, and sampling date were used as fixed factors in different combinations. Fish length was used as a covariate in cases where the fish size class was not used as fixed factor (in order not to violate the assumption of a normally distributed covariate). Essentially, a significant main effect of host species indicates that fish species differ in infection of any of the *Diplostomum* species, while the main effect of parasite species shows that some parasite species are more common than others, independent of the fish species in which they are found. We were mainly interested in the interaction between these two factors, which would indicate species-specific infection patterns. Due to logistic reasons, the locations could not be sampled at all time points, which is why spatial and temporal effects of sampling were analyzed separately.

We also performed a principal component analysis (PCA) followed by a discriminant function analysis (DFA) on the parasite numbers. $\ln(x+0.1)$-transformed numbers of each of the four parasite species per lens were used to compute two principle components that best explained the community structure. The same numbers were used in the DFA to tests if the parasite numbers discriminate between the fish species (used as a grouping variable). Squared Mahalanobis distances and their significances were also computed with the DFA to test if and to what extent parasite communities differed among the different fish species. Finally, we calculated two host specificity indices, S_i (based on parasite intensity) and S_p (based on parasite prevalence), that calculate how a parasite species is distributed among the host species (Rohde, 1994). Both indices range from 1 (infecting only one host species) to 0 (equally distributed). As S can only approach zero when the number of host species is very high, it was corrected for the minimum possible S (Rohde and Rohde, 2005). Note that these indices were not used to analyze parasite specificity in a coevolutionary sense, but to analyze differences in infection patterns. All analyses were carried out using SPSS 16.0 and Statistica 6.0.

3. Results

3.1. SNP analysis

Accuracy and repeatability of the SNP analysis was high. Pyrosequencing produced correct species identification in 31 cases of 34 single specimen samples, while the remaining three samples belonged to the *D. pseudospathaceum* group that had a nucleotide G in locus b (specimens 77, 79, and 80 in Fig. 1, see also Section 2.2). These samples were therefore misclassified as *D. baeri*. Within the 31 correctly classified specimens the average frequency of the interspecific SNP allele was 96.6% ± 1.0 confidence interval (95% level). The confidence intervals of repeatedly measured species or SNP allele frequencies were also low, normally ranging between 0 and 4.7%, and 9.7% in one case. Analyses of artificial mixtures of DNA of different species showed that the pooling method was reliable; linear regressions on pipetted vs. measured species proportions were highly significant ($p < 0.001$) with R^2 values of 0.96–0.99 (Appendix A).

3.2. Case study

3.2.1. Parasite and host populations

In total, we caught 492 fish including five species (Table 3): the cyprinids bleak (*Alburnus alburnus*) and roach (*Rutilus rutilus*), the

Table 3
Details of *Diplostomum* infections in the lenses of fish. Parasite intensity: average number of metacercariae in the lenses of infected fish. Number of lenses: lenses included in the analyses. Parasite richness: average number of species in infected lenses. Parasite diversity: average Simpson's Index of Diversity in infected lenses. Multiple species infection reports the proportion of infected fish that were carrying more than one species in the lens. Errors represent standard error.

Fish species	Bleak (*Alburnus alburnus*)	Roach (*Rutilus rutilus*)		Vendace (*Coregonus albula*)	Ruffe (*Gymnocephalus cernuus*)	Perch (*Perca fluviatilis*)		
Size class	Medium	Medium	Large	Medium	Medium	Small	Medium	Large
Number of fish	66	62	70	30	59	60	113	32
Length (cm)	12.9 ± 1.1	11.4 ± 0.7	19.8 ± 1.6	12.0 ± 0.8	9.5 ± 1.1	5.0 ± 0.5	10.5 ± 1.0	17.7 ± 1.9
Prevalence of infection	0.86	0.98	1.00	1.00	1.00	0.53	0.54	0.38
Parasite intensity	2.7 ± 0.3	4.9 ± 0.4	30.7 ± 5.0	10.8 ± 0.8	10.8 ± 0.6	1.4 ± 0.1	2.0 ± 0.2	3.2 ± 0.4
Number of lenses	43	58	67	29	54	13	43	10
Parasite richness	1.53 ± 0.10	2.02 ± 0.10	2.12 ± 0.10	1.45 ± 0.12	1.48 ± 0.07	1.15 ± 0.10	1.46 ± 0.09	1.35 ± 0.13
Parasite diversity	0.19 ± 0.03	0.34 ± 0.03	0.32 ± 0.02	0.16 ± 0.04	0.19 ± 0.03	0.06 ± 0.03	0.12 ± 0.02	0.12 ± 0.04
Multiple species infection	0.46	0.74	0.76	0.38	0.46	0.15	0.30	0.20

salmonid vendace (*C. albula*), and the percids ruffe (*Gymnocephalus cernuus*) and perch (*Perca fluviatilis*). These fish species represent most of the common fish species found in Lake Konnevesi (Toivonen et al., 1982). Samples of roach included two size classes (medium and large), while those of perch included all three. Except for perch, the overall prevalence of infection (all parasite species combined) was close to 100% (Table 3). Parasite intensity differed substantially among the fish species with perch and bleak carrying on average 1–3 parasites per fish, while in large roach the average was 31 (Table 3). The most heavily infected roach had an estimated total number of 300 metacercariae.

From a total of 362 lenses, 315 were included in the analyses. Nine samples did not amplify in the nested PCR and 38 samples had to be discarded due to high frequency of *D. baeri*. Thirty-seven of these came from perch, which are typically heavily infected with *D. baeri* (Karvonen et al., 2006b). In total, we pyrosequenced an estimated number of 1922 parasite individuals when analyzing the communities. The most abundant species were *D. paracaudum* ($n = 1086$) and *D. pseudospathaceum* ($n = 722$), whereas *D. mergi* ($n = 98$) and *D. parviventosum/spathaceum* ($n = 16$) were rare. Uninfected lenses did not amplify in the nested PCR and did not result in peaks in the pyrosequencer.

3.2.2. Parasite communities

Multiple parasite species infections were common (Table 3). A total of 76% of roach carried multiple species infections and more than 20% were infected with three or four species. In the other fish species, multiple infections were found in 15–46% of the individuals. The MANOVA revealed significant effects of host species (Wilk's $\lambda = 0.878$, $F_{8,612} = 5.149$, $p < 0.001$), but not of fish size class ($\lambda = 0.995$, $F_{4,612} = 0.349$, $p = 0.845$) or their interaction ($\lambda = 0.985$, $F_{2,306} = 5.149$, $p = 0.103$), on parasite richness and diversity. Tukey's post hoc tests showed that roach exhibited significantly higher parasite richness and diversity compared to the other four fish species that did not differ from each other.

Our LMM analyses showed that the fish species were infected with different *Diplostomum* communities. When pooling data over different sampling locations and sampling dates, the effects of host species, parasite species, fish size classes and their interactions were highly significant (Table 4, model 1). Vendace, ruffe and perch were mainly infected with *D. pseudospathaceum* (Fig. 2). The majority of parasites in the lenses of bleak were *D. mergi*, but also *D. pseudospathaceum* was present in high frequencies. Finally, younger individuals of roach carried both *D. pseudospathaceum* and *D. paracaudum* (rarely also *D. parviventosum/spathaceum*), but adult individuals were mainly infected by *D. paracaudum*. Sampling location or date did not affect the result; only the interaction of sampling date with host species was statistically significant (Table 4, models 2 and 3). These two models also showed that fish length (within the medium size class) significantly affected the number of parasites in the lens. There were also significant differences in parasite infection patterns among the age classes of roach (significant effect of fish size class; Table 4, model 4) and perch (significant interaction between parasite species and fish size class; Table 4, model 5). An additional LMM showed no significant effect of lens (left or right, $F_{1,133} = 2.330$, $p = 0.129$) or its interaction ($F_{3,133} = 0.597$, $p = 0.618$) with parasite species ($F_{3,133} = 31.911$, $p < 0.001$), on the species specific number of parasites per lens in the same fish (defined as random factor). In other words, the species composition did not differ between the two lenses of an individual fish.

In the PCA, the first two axes explained 32.92% and 27.88% of the variation in the data (Fig. 3). Parasite communities of bleak and roach were substantially different, while those in vendace, ruffe and perch were overlapping. The DFA showed that parasite numbers significantly discriminated between the fish species (Wilk's $\lambda = 0.302$, $F_{16,398} = 28.142$, $p < 0.001$) and revealed significant effects of three variables used in the model (number of *D. mergi*, *D. paracaudum* and *D. pseudospathaceum*, $p < 0.001$), while the fourth variable (number of *D. parviventosum/spathaceum*) was marginally significant ($p = 0.06$). Squared Mahalanobis distances were highly significant ($p < 0.001$) among the parasite communities of all fish species, except between ruffe and vendace. Bleak was the species most distant from the others, followed by roach and lastly perch.

3.2.3. Host range and parasite distribution

The overall host range of the parasite species was quite similar. *D. mergi* was never present in ruffe and *D. parviventosum/spathaceum* was never found in vendace, but the remaining two parasite species were found in all fish species. However, the parasite species differed greatly in their infection intensities in the different fish species. Individuals of *D. mergi* were mostly found in bleak and roach, while those of *D. paracaudum* and *D. parviventosum/spathaceum* were mainly in roach. *D. pseudospathaceum* was abundant in all fish species. The specificity indices also supported this pattern of infection. *D. parviventosum/spathaceum* showed the highest tendency to infect only certain host species ($S_i = 0.86$, $S_p = 0.60$), while *D. mergi* showed intermediate values ($S_i = 0.53$, $S_p = 0.54$) and *D. pseudospathaceum* ($S_i = 0.38$, $S_p = 0.17$) was most equally distributed among the host species. In the case of *D. paracaudum* ($S_i = 0.79$, $S_p = 0.34$) the two indices differed markedly. While the prevalence index suggested equal distribution among the host species, the intensity index showed rather the opposite; this is mainly due to the very high infection intensity of this species in roach.

4. Discussion

Individual hosts are typically infected by a community of parasites. The community structure is shaped by complex ecological and evolutionary interactions among the co-infecting parasite species, and those between parasite and host species (Adamson and Caira, 1994; Janovy et al., 1992; Mideo, 2009; Poulin, 2007). However, analyzing parasite community structure is often challenging due to taxonomical difficulties and low sample sizes. Here we present a novel methodological approach to analyze parasite communities by allele quantification of interspecific SNPs using pyrosequencing. The method enables analyzing the communities as a whole and therefore increases the sample size and power of statistical analyses. To show the usefulness of the method, we performed a case study where we investigated multiple infections and community composition of *Diplostomum* eye flukes in several fish species of one freshwater system. We found a high frequency of multiple species infections and significantly different parasite communities among the fish species.

4.1. Pyrosequencing of natural parasite communities

Our results demonstrate that pyrosequencing gives a reliable and repeatable description of the parasite community. Instead of Sanger sequencing (Sanger et al., 1977) individual parasites infecting the same host tissue (estimated total $n = 1922$), we analyzed the tissue as a whole using pyrosequencing (Ronaghi et al., 1998) of pooled parasite DNA in 315 fish lenses. We demonstrated that the method is reliable; a large proportion of single specimen samples were identified correctly, repeatability was very high, and it was possible to accurately determine the species ratios in artificial mixtures. Thus, analysis of SNP allele frequencies in pooled samples enables fast and accurate charac-

Table 4
Results of the five linear mixed models analyzing the parasite communities in the lenses of different fish species. Dependent variable is the number of metacercariae (of the same parasite species) in one lens. Lens is used as a random factor. Significant p-values ($p < 0.05$) in bold.

Source	Numerator df	Denominator df	F	p
Model 1: Overall effects				
Intercept	1	1228	767.284	**<0.001**
Parasite species	3	1228	220.124	**<0.001**
Host species	4	1228	27.420	**<0.001**
Fish size class	2	1228	18.518	**<0.001**
Parasite species × host species	12	1228	38.078	**<0.001**
Parasite species × fish size class	6	1228	3.538	**0.002**
Host species × fish size class	1	1228	19.671	**<0.001**
Parasite species × host species × fish size class	3	1228	23.581	**<0.001**
Model 2: Sampling location effects (medium fish size class only)				
Intercept	1	735	36.207	**<0.001**
Parasite species	3	735	99.572	**<0.001**
Host species	3	735	9.832	**<0.001**
Sampling location	2	735	0.364	0.695
Parasite species × host species	9	735	12.370	**<0.001**
Parasite species × sampling location	6	735	0.538	0.780
Host species × sampling location	5	735	1.155	0.330
Parasite species × host species × sampling location	15	735	0.961	0.495
Fish length (covariate)	1	735	7.364	**0.007**
Model 3: Sampling date effects (medium fish size class only)				
Intercept	1	727	31.617	**<0.001**
Parasite species	3	727	158.058	**<0.001**
Host species	3	727	10.258	**<0.001**
Sampling date	3	727	1.068	0.362
Parasite species × host species	9	727	13.417	**<0.001**
Parasite species × sampling date	9	727	0.808	0.609
Host species × sampling date	6	727	2.338	**0.030**
Parasite species × host species × sampling date	18	727	0.862	0.626
Fish length (covariate)	1	727	5.502	**0.019**
Model 4: Size class effects in perch				
Intercept	1	244	661.500	**<0.001**
Parasite species	3	244	95.334	**<0.001**
Fish size class	2	244	1.494	0.227
Parasite species × fish size class	6	244	2.799	**0.012**
Model 5: Size class effects in roach				
Intercept	1	123	188.649	**<0.001**
Parasite species	3	369	213.622	**<0.001**
Fish size class	1	123	85.651	**<0.001**
Parasite species × fish size class	3	369	50.253	**<0.001**

terization of a parasite community and shifts the sampling unit from individual parasites to whole parasite communities. This significantly increases the statistical power of community analyses. To our knowledge, no similar studies exist, except for detecting multiple infections of a pathogen in medical science (Sreekumar et al., 2005). Our approach resembles approaches used in environmental sampling (e.g., Ficetola et al., 2008) and faecal analyses (e.g., Deagle et al., 2009).

However, there are important methodological limitations that need to be taken into account. Most importantly, the use of pyrosequencing requires known SNP polymorphisms in the targeted region. In other words, the analysis cannot detect new species that have been possibly missed when acquiring the genetic data for assay development. This would result in misclassification of these novel species. The requirement of known SNP polymorphisms also means that the same markers cannot necessarily be used in a new geographic region without a new screening for species composition using Sanger sequencing. Second, the method assumes that all parasite individuals in a community contain the same amount of rDNA. In reality, differences between individuals and species most likely exist, and the ITS1 region in the rDNA is known to exist in multiple copies within a cell (Hillis and Dixon, 1991). In the present system, results from the artificial species mixtures suggest variation in rDNA content, but it seems to be rather small. Due to the multi-copy nature of the rDNA, even intra-individual variation is possible, as shown for other well-studied trematodes (Blair, 2006). In *Diplostomum* this was reported by Locke et al. (2010a) who found rare double peaks in some sequences, which they interpret as signature of hybridization. We also found signs of intra-individual variation, but suspicious loci were excluded when developing the pyrosequencing assays. Third, there is a risk of co-amplification of host tissue during PCR, which should be carefully tested for. In our setup, there is a three- to four-fold control incorporated, namely two rounds of PCR, one pyrosequencing reaction (in assay Diplo503), as well as the order of nucleotide insertion during pyrosequencing. Lastly, the method requires that the total parasite intensity in the community can be evaluated. We solved this by using non-invasive slit-lamp microscopy for the estimation of the total number of parasites in the lens and then converting the relative species frequencies into absolute numbers. In other systems, where such an approach is not possible, quantitative PCR or counting the parasite intensity from a subsample could represent an alternative.

We found four species of lens-infecting *Diplostomum* in the present study, but it is possible that this represents an underestimation. For example, we do not know if the *D. parviventosum/spathaceum* group actually includes one species, or two species having the same ITS1 sequence (Niewiadomska

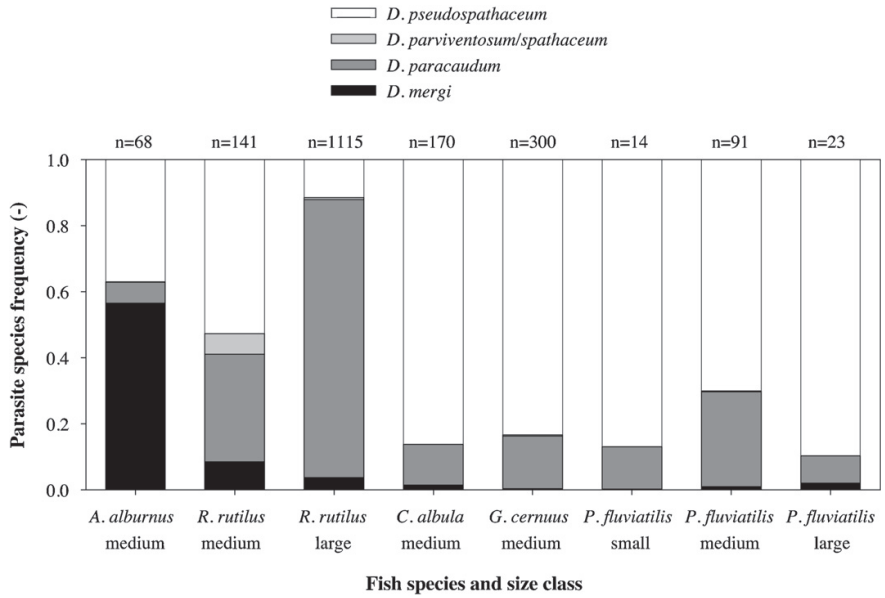

Fig. 2. Distribution of *Diplostomum* parasite species in the lenses of different fish species. Five fish species were analyzed: *Alburnus alburnus, Rutilus rutilus*, *Coregonus albula, Gymnocephalus cernuus, Perca fluviatilis.* Some fish species include different size classes. Sample size (*n*) denotes the estimated number of metacercariae per fish group included in the graph. For sample sizes of fish lenses see Table 3.

and Laskowski, 2002). According to the latter authors, the species can be separated morphologically, but this is very difficult, if not impossible, from metacercarial stages of the parasites. Moreover, specimens 77–80 (classified as *D. pseudospathaceum*) might also represent a fifth lens-infecting species that was misclassified as humour-infecting *D. baeri* with the pyrosequencing method. Nevertheless, it had very low prevalence. Only four of the 75 lens-infecting specimens collected for assay development belonged to this potential fifth species. In the field samples, it may have been present in the 21 of 315 samples, where the proportion of *D. baeri* was higher than 5%

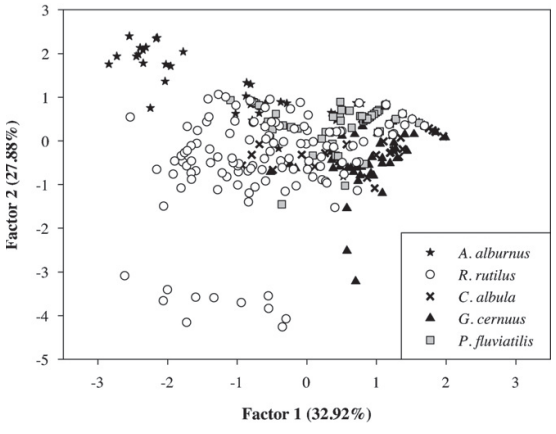

Fig. 3. PCA showing *Diplostomum* parasite communities in the lenses of different fish species. Five fish species were analyzed: *Alburnus alburnus, Rutilus rutilus*, *Coregonus albula, Gymnocephalus cernuus*, and *Perca fluviatilis.* Axes are composed of the number of the four different parasite species in each host. Each dot represents the parasite community of one lens.

(detection level) but lower than 50% (samples above this level were discarded). However, it should be noted that these *D. baeri* frequencies were excluded from the statistical analysis and do not affect the results on the presence of the four species. Finally, as already discussed, some species might have been completely missed when collecting the reference samples. We are also aware that the numbers of reference samples for *D. mergi* and *D. parviventosum/spathaceum* are small, which is mainly because they seem to be rare species as corroborated by our field data. However, we nevertheless consider the markers to show high reliability. Both parasite species showed very low intraspecific variation in ITS1 sequences (Table 1); the reference specimens of *D. mergi* exhibited differences in two positions of the ITS1 sequence and those of *D. parviventosum/spathaceum* only in one. Moreover, Niewiadomska and Laskowski (2002) found exactly the same interspecific SNPs in a geographically distinct study system.

We used the term "species" to differentiate among the different *Diplostomum* taxa. It is important to note that the taxonomic identity and nomenclature of *Diplostomum* species has not been resolved. As mentioned above, Niewiadomska and Laskowski (2002) showed that *D. spathaceum* and *D. parviventosum* are clearly distinguishable morphologically, but have identical ITS1 sequences. Furthermore, North-American specimens of *D. baeri* were hardly distinguishable morphologically from their European counterparts, but differed in 23 of 604 bp in the ITS1 region (Galazzo et al., 2002). In the same freshwater system, three new (or cryptic) lens-infecting species have been found using CO1 and ITS sequences in addition to two species already known (Locke et al., 2010a,b). As Niewiadomska and Laskowski (2002) is the only study so far presenting both detailed morphological and molecular characters in European *Diplostomum*, we chose to use their nomenclature. The fact that we find differences in infection patterns, e.g., between the genetically similar *D. paracaudum* and *D. pseudospathaceum*, supports that we are actually looking at different species. Earlier studies on *Diplostomum* have commonly

used the name *D. spathaceum* (or *Diplostomum* sp.) for the parasites infecting the lenses of freshwater fishes around the world. For example, in Finland (e.g., Karvonen et al., 2006b, 2009; Valtonen and Gibson, 1997) it has been used for metacercariae in field studies with wild fishes and for cercariae produced in the snail *Lymnaea stagnalis* in experimental infections, while clearly acknowledging the fact that problems with species identification and nomenclature exist. The present results (and those of Louhi et al., 2010) indicated that wild fish species in Finland may carry several eye fluke species in their lenses, and that *L. stagnalis* is typically infected by *D. pseudospathaceum* according the nomenclature of Niewiadomska and Laskowski (2002). However, the goal of the present study was not to disentangle the taxonomic twists in the present species complex, but to show how pyrosequencing can be used to describe parasite communities, and how these communities differ between host species.

4.2. Ecological and evolutionary factors influencing parasite communities

The principal findings of our case study indicated that multiple species infections of *Diplostomum* were common in the fish community (Table 3) and infection patterns significantly differed among the different fish species and age classes (Figs. 2 and 3). These results were corroborated by different statistical approaches (MANOVA, LMM, DFA, and specificity indices). For example, roach was infected with all four parasite species in considerable numbers, showing the highest parasite diversity and richness. The parasite community of bleak with the high representation of *D. mergi* differed markedly from the other fish species. Moreover, *D. pseudospathaceum* was found in all five fish species in relatively high intensities, whereas *D. mergi* and *D. parviventosum/spathaceum* were found almost exclusively in the two cyprinids, bleak and roach.

There are several possible ecological and evolutionary mechanisms that could underlie the observed patterns of infection. First, the patterns could be determined by the ecology of the different hosts (Adamson and Caira, 1994). This is particularly likely in complex life cycle parasites such as *Diplostomum*, where the risk of infection is spatially structured according to the habitat of the first intermediate hosts. For example, given that these parasites are typically specialists regarding their first intermediate hosts, freshwater snails (Adamson and Caira, 1994; Gibson and Bray, 1994; Nunez and De Jong-Brink, 1997), and snails occupy certain habitats in a lake, extensive habitat coupling between consecutive snail and fish hosts could strongly influence the infection patterns. In our system, infection patterns in the first intermediate hosts are not known in detail. Some of the freshwater snail species known to carry *Diplostomum* infections (*L. stagnalis*, *Myxas glutinosa*, and *Radix balthica*) co-occur in the same habitats (Karvonen et al., 2006b), but, for example, the snail host of the most abundant species *D. paracaudum* is unknown. Moreover, the fish species investigated here show differences in their habitat preferences, but all spend also time in the littoral zone of lakes (Muus and Dahlström, 1993) where infected snails typically occur. This is the case even for vendace, which move to shallow areas during spawning. In summary, possible differences in habitat overlap between the first and the second intermediate hosts may result in different parasite communities in different fish species.

Also other ecological factors may explain the observed patterns of infection. For example, seasonal variation in infection among the different *Diplostomum* species could play an important role. However, in high latitude regions like Finland, the temporal window for infection in summer is short, and previous evidence suggests similarity rather than differences in seasonal patterns of infection in two species of *Diplostomum* (Karvonen et al., 2006b). Moreover, as these parasites accumulate in fish over time and as there is variation in age structure among and within fish species sampled in our case study, inter-annual variation in exposure may also explain the infection patterns.

In addition to ecological factors, evolutionary processes may also play a role in structuring the parasite communities. One such mechanism is host specificity, which means that a parasite species has a narrow host range and/or infects certain host species in higher prevalence or intensity than others (Poulin, 2007). In *Diplostomum*, variation in specificity can be explained, for example, by the degree of interaction with the host. Recent studies on metacercariae in fish showed that lens-infecting species show a wider host range than species found in other tissues (Locke et al., 2010a,b). This is most likely because metacercariae of the lens-infecting species have less interaction with their hosts, as they are protected from the immune system once they have reached the lens. Another possible factor explaining the differences in co-infections and community species composition in this system is host immunity (Cox, 2001). It is well known that exposure to *Diplostomum* parasites leads to immune responses in fish hosts (Chappell et al., 1994; Karvonen et al., 2005). These responses could differ among the host species because of variation in levels of exposure and of coevolutionary history with the parasites. In this system, it is unknown if there is cross-immunity in hosts against closely related parasite species. Hosts could also become infected at different times resulting in sequential immunization to different parasite species, which may have significant consequences for the community structure (Karvonen et al., 2009). The fact that we found significantly different parasite communities in different size classes of the fish species suggests that sequential immunization might exist at the species level. However these differences could also result from parasite-induced mortality or temporal variation in exposure.

It has been shown that the predictability of parasite community structure in space and time can be poor (Poulin and Valtonen, 2002) and that differences in sampling effort among the different host species can account for variation found in infection patterns (Poulin, 2007). Therefore, given that our case study represents a snapshot of the community structure in one location and at one time point, and the fact that the five fish species exhibit different ecological characteristics and age structures, general conclusions on factors underlying the observed infection patterns should be made carefully. Now that we found that non-random infection patterns exist, we can make specific hypotheses to test if our findings represent general patterns and what explains the underlying mechanisms. Therefore, further research including controlled experiments is needed, and the pyrosequencing approach described in this study represents an ideal tool to perform such studies.

5. Conclusions

Despite the few limitations, we propose that pyrosequencing of naturally pooled parasite samples represents a more efficient method to characterize parasite community structure compared to traditional Sanger sequencing of individual parasites. This approach is likely to facilitate studies on infection pathways and community structure of parasites, and helps to answer classical ecological and evolutionary questions related to host specificity and resistance. In the case of complex life cycle parasites, the method also provides a possibility for studying parasite taxa in tissues or faeces of other hosts of the life cycle, and could be used as a tool in controlled experiments or field exposures. Moreover, the approach can also be extended to artificially pooled samples, and

those from individual parasites, presenting a flexible and efficient method for species identification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.04.018.

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Appendix 1: PCR, sequencing, and pyrosequencing primers used for allele quantification of the SNPs.

Given are also PCR product length, $MgCl_2$ concentration and annealing temperature (T_A). SPA-F and SPA-R are the forward and reverse primers from Niewiadomska & Laskowski (2002).

Primer name	Primer type	Primer sequence 5'-3'	PCR product size	$MgCl_2$	T_A
SPA-F	PCR/sequencing ITS1 forward	ACAAGGTTTCCGTAGGT	~650 bp	2.0 mM	54°C
SPA-R	PCR/sequencing ITS1 reverse	AGTGATCCACCGCTCAGAGTT			
KRP-F	Sequencing ITS1 forward	GACTACTATGTCCAGCCTCCGC			
KRP-R	Sequencing ITS1 reverse	GAAGCCACGAGCCAACCATCAGGG			
197-F	PCR Diplo197 forward	GGGATTGACGGACGAACTCT	64/65 bp	2.0 mM	57°C
197-R	PCR Diplo197 reverse	*bio*-CGTCTCAGGTATGGCCAATAG			
197-S	Pyrosequencing Diplo197	TGACGGACGAACTCTT			
503-F	PCR Diplo503 forward	TCCGCCCCATCTTGTTGT	128 bp	2.5 mM	54°C
503-R	PCR Diplo503 reverse	*bio*-CTGTACATGCGGACCAGTCATA			
503-S	Pyrosequencing Diplo503	TAGCTAGCTGCCCATA			

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Niewiadomska, K., Laskowski, Z., 2002. Systematic relationships among six species of *Diplostomum* Nordmann, 1832 (Digenea) based on morphological and molecular data. Acta Parasitolog. 47, 20-28.

Appendix 2: Partial ITS1 sequence alignment of typical representatives of the five *Diplostomum* species.

Gaps are indicated by "-". Species names according to Niewiadomska & Laskowski (2002). Green regions indicate binding sites of the PCR primers and red regions binding sites of the sequencing primers. Interspecific SNP loci (a-d) used in this study are shown in yellow and bold. Shown are specimens 05, 08, 18, 47, and 51 of Figure 1. Sequences of all specimens have been deposited in GenBank under accession numbers JF775679-JF775760.

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                        ....|....| ....|....| ....|....| ....|....| ....|....|
                            5          15         25         35         45
D. baeri                GAACCTGCGG AAGGATCATT ACAAGTCCCC TATCTGAAAC TTATCGAACT
D. mergi                 AACCTGCGG AAGGATCATT ACAAGTCCC- TATCTGAAAC TTATCGAACT
D. paracaudum                CGG AAGGATCATT ACAAGTCCC- TATCTGAAAC TTATCGAACT
D. parviv./spathac.
D. pseudospathaceum     GAACCTGCGG AAGGATCATT ACAAGTCCC- TATCTGAAAC TTATCGAACT

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                            55         65         75         85         95
D. baeri                CGGTCTCGGC CGGGTTTGGA AATAATTGGC GCGTTGGGTT AGCAATTGAG
D. mergi                CGGTTTCGAC CGGGTTCGGA TTTAATTGGC GCGTTGGGTT GGCAATTGAG
D. paracaudum           CGGTTTCGGC CGGGTTCGGA TTTAATTGGC GCGTTGGGTT GGCAATTGAG
D. parviv./spathac.
D. pseudospathaceum     CGGTCTCGGC CGGGTTCGGA TTTAATTGGC GCGTTGGGTT GGCAATTGAG

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                           105        115        125        135        145
D. baeri                TTAACCTAGC GTGTCAAGGA ATTGACGGAT GGGCTTCCCG CAAGGGACCC
D. mergi                TTAACCTGGC GTGTCAAGGA ATTGACGGAT GGGCTTCCCG CAAGGGACCC
D. paracaudum           TTAACCTAGC GTGTCAAGGA ATTGACGGAT GGGCTTCCCG TAAGGGACCC
D. parviv./spathac.                   GGA ATTGACGGAT GGGATTCCCG TAAGGGACCC
D. pseudospathaceum     TTAACCTAGC GTGTCAAGGA ATTGACGGAT GGGCTTCCCG TAAGGGACCC

                        ....|....| ....|....| ....|....| ...Primer 197S.|.a..|
                        ....|....| ....|....| ....|....| Primer 197F....|.a..|
                            155        165        175        185        195
D. baeri                GCGAATTACA GTGCAT---A TAAACGGGAT TGACGGACGA ACTCTTCACT
D. mergi                GCACATAACA GTGCAT-ATA CAAACGGGAT TGACGGACGA ACTCTTTACT
D. paracaudum           GCGAATTACA GTGCAA---A CAAACGGGAT TGACGGACGA ACTCTTCACT
D. parviv./spathac.     GCGAATAACA GTGCATCATA TAAACGGGAT TGACGGACGA ACTCTTCACT
D. pseudospathaceum     GCGAATTACA GTGCAG---A CAAACGGGAT TGACGGACGA ACTCTTCACT

                        ....b....| .c..|....| ....|Primer 197R....| ....|....|
                            205        215        225        235        245
D. baeri                TGTGGAGGGT TCG-CGATAC TATTGGCCAT ACCTGAGACG TGGTTCTGCT
D. mergi                TGTGAAGGGT TCG-CGATTC TATTGGCCAT ACCTGAGACG TGGTTCTACT
D. paracaudum           TGTGGAGGGT TTGGCAATAC TATTGGCCAT ACCTGAGACG TAGTTCTGCT
D. parviv./spathac.     TGTGAAGGGT TCG-CGATTC TATTGGCCAT ACCTGAGACG TGGTTCTACT
D. pseudospathaceum     TGTGAAGGGT TCG-CGATAC TATTGGCCAT ACCTGAGACG TAGTTCTGCT

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                            255        265        275        285        295
D. baeri                GCGTTTCACA AGTACGGTCC GTATTGTGGT GGGGTGCCTA TCCTGTCTGA
D. mergi                GCGTTTCATA AGTACGGTCC GTATTTTGGT GGGGTGCCTA TCCTGTCTGA
D. paracaudum           GCGTTTCACA AGTACGGCCC GAATTTTGGT GGGGTGCCTA TCCTGTCTGA
D. parviv./spathac.     GCGTTTCATA AGTACGGTCC GTATTCTGGT GGGGTGCCTA TCCTGTCTGA
D. pseudospathaceum     GCGTTTCACA AGTACGGCCC GTATTTTGGT GGGGTGCCTA TCCTGTCTGA
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       305        315        325        335        345
D. baeri            TACCCTGATG GTTGGCTCGT GGCTTCGGCT GCCTTGTCAT GCCAAGGGTG
D. mergi            TACCCTGATG GTTGGCTCGT GGCTTCGGCT GCCTTGTCAT GCCAAGGGTG
D. paracaudum       TACCCTGATG GTTGGCTCGT GGCTTCGGCT GCCTTGTCAT GCCAAGGGTG
D. parviv./spathac. TACCCTGATG GTTGGCTCGT GGCTTCGGCT GCCTTGTCAT GCCAAGGGTG
D. pseudospathaceum TACCCTGATG GTTGGCTCGT GGCTTCGGCT GCCTTGTCAT GCCAAGGGTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       355        365        375        385        395
D. baeri            ATGGGAAAGT ACTGTATCTA TCTCAGTGCA AGGCTCAAAG AGGGTCTCGG
D. mergi            ATGGGAAAGT ACTGTATCTA TCTCAGTGCA AGGCTCAAAG AGAGTGCTGG
D. paracaudum       ATGGGAAAGT ACTGTATCTA TCTCAGTGCA AGGCTCAAAG AGGGTCTCGG
D. parviv./spathac. ATGGGAAAGT ACTGTATCTA TCTCAGTGCA AGGCTCAAAG AGGGTCTCGG
D. pseudospathaceum ATGGGAAAGT ACTGTATCTA TCTCAGTGCA AGGCTCAAAG AGGGTCTCGG

                    ....|....| ....|...| .Primer 503F...|....| ....|....|
                       405        415        425        435        445
D. baeri            ACTAATA-GT CCAGCCTCCG CCCCATCTTG TTGTTTCTAC TACCATTTTT
D. mergi            ACTATTA-GT TCAGCCTCCG CCCCATCTTG TTGTTTCTAC TACCATTTTT
D. paracaudum       ACTACTATGT CCAGCCTCCG CCCCATCTTG TTGTTTCTAC TACCATTCTT
D. parviv./spathac. ACTAATA-GT CCAGCCTCCG CCCCATCTTG TTGTTTCTAC TACCATTTTT
D. pseudospathaceum ACTACTATGT CCAGCCTCCG CCCCATCTTG TTGTTTCTAC TACCATTTTT

                    ....|....| ....|....| ....|....| ....|....|Primer 503S
                       455        465        475        485        495
D. baeri            ACACTGTTTA AGTTGGTTAG GTCGGCTTG- CCGGTCTAGC TAGCTGCCCA
D. mergi            ACACTGTTTA AGTTGGTTAG TTCGGCTTGT CCGATCTAGC TAGCTGCCCA
D. paracaudum       ACACTGTTTA AGTTGGTTAG GTCGGCTTGT CCGGTCTAGC TAGCTGCCCA
D. parviv./spathac. ACACTGTTTA AGTTAGTTAG GTCGGCTTGT CCGGTCTAGC TAGCTGCCCA
D. pseudospathaceum ACACTGTTTA AGTTGGTTAG GTCGGCTTGT CCGGTCTAGC TAGCTGCCCA

                    ..d.|....| ....|....| ..|..|..Primer 503R..| ....|....|
                       505        515        525        535        545
D. baeri            TAGCATGCCT CCAGACATCT TGTATGACTG GTCCGCATGT ACAGTCGCCT
D. mergi            TAGCATGCCT CCAGACATCT TGTATGACTG GTCCGCATGT ACAGTCGCCT
D. paracaudum       TAGCATGCCT CCAGACATCT TGTATGACTG GTCCGCATGT ACAGTCGCCT
D. parviv./spathac. TAACATGCCT CCAGACATCT TGTATGACTG GTCCGCATGT ACAGTCGCCT
D. pseudospathaceum TAGCATGCCT CCAGACATCT TGTATGACTG GTCCGCATGT ACAGTCGCCT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       555        565        575        585        595
D. baeri            GGCGGTGCCT CATCCTGGGC TAGACTGCAA ACCTATATGT TCTCATTGAC
D. mergi            GGCGGTGCCT CATCCTGGGC TAGACTGCAA ACCTATATGT TCTCATTGGC
D. paracaudum       GGCGGTGCCT CATCCTGGGC TAGACTGCAA ACCTATATGT TCTCATTGGC
D. parviv./spathac. GGCGGTGCCT CATCCTGGGC TAGACTGCAA ACCTATATGT TCTCATTGGC
D. pseudospathaceum GGCGGTGCCT CATCCTGGGC TAGACTGCAA ACCTATATGT TCTCATTGGC

                    ....|....| ....|....| ....|....| ....
                       605        615        625
D. baeri            TCGGTTTACC GGGTCGGTGG GTGCATTACA GTAC
D. mergi            TCGGTTTACC GGGTCGGTGG GTGCATTACA GTAC
D. paracaudum       TCGGTTTACC GGGTTGGTGG GTGCATTACA GTAC
D. parviv./spathac. TCG
D. pseudospathaceum TCGGTTTACC GGGTTGGTGG GTGCATTACA GTAC
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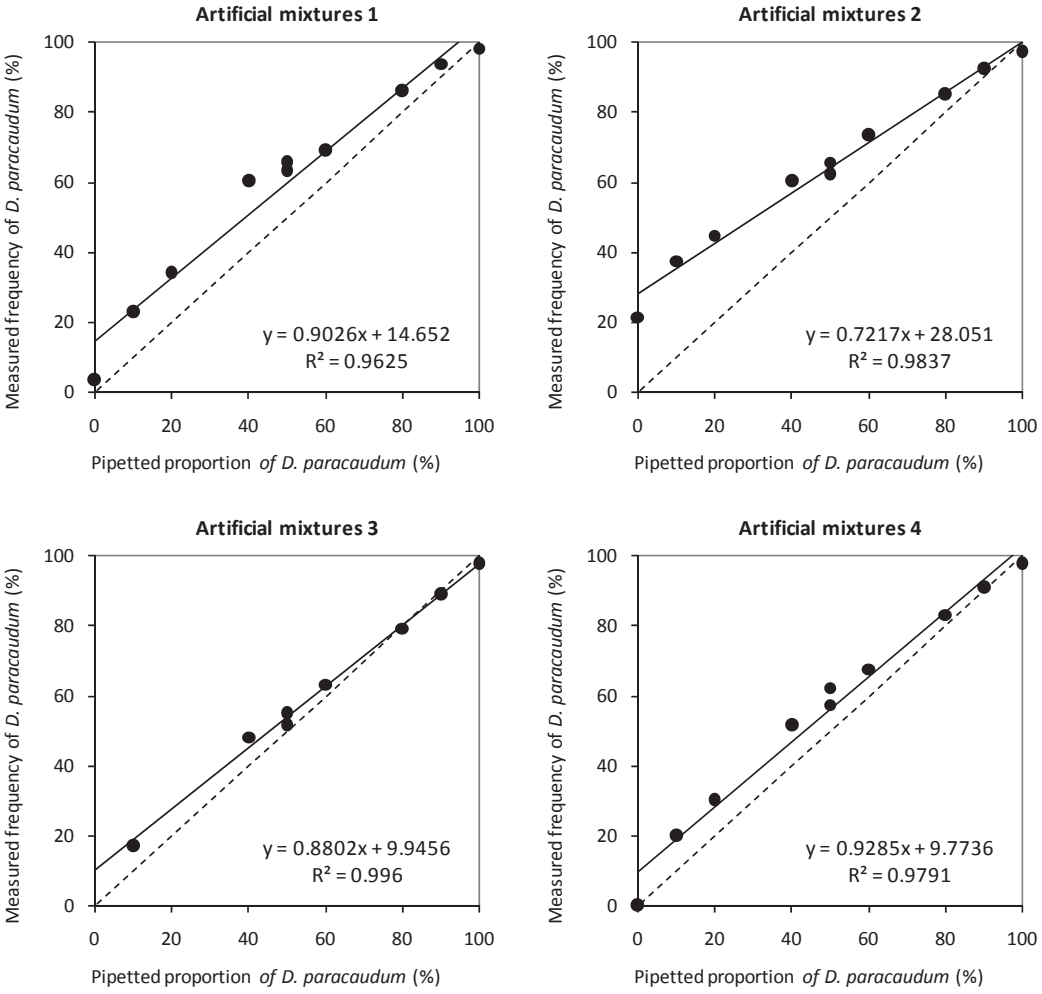
Appendix 3: Artificial mixtures of two species to test the accuracy of the SNP analysis.

Species determination of single parasite larvae originating from the same lens of adult roach (*Rutilus rutilus*) was conducted using pyrosequencing. Subsequently, four *D. paracaudum* and four *D. pseudospathaceum* samples were randomly grouped in pairs and artificial mixtures in nine different concentrations were prepared. These samples were then pyrosequenced as if they were naturally pooled samples. Results of the four pairs are shown below. All linear regressions (straight lines) are highly significant ($p<0.001$). The accuracy of DNA pooling, indicated by the R^2 value, was very high. Deviation of the data points from the ideal (dashed) line is most likely caused by differences in rDNA content of the specimens/samples or contamination (in the case of mixture 2). The relative frequency of *D. paracaudum* was higher in all four mixtures. Two data points of mixture 3 are missing due to PCR/pyrosequencing failure.



IV

GENOTYPIC AND PHENOTYPIC VARIATION IN TRANSMISSION TRAITS OF A COMPLEX LIFE CYCLE PARASITE

by

Katja-Riikka Louhi, Anssi Karvonen, Christian Rellstab & Jukka Jokela

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