Development of molecular tools to study *Daphnia* - parasite dynamics

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Abstract

Evolutionary dynamics of hosts and their parasites are complex processes. In order to study these processes on genotype level, reliable molecular tools have to be developed. The goal of this thesis was to develop such tools for freshwater crustaceans - *Daphnia longispina* species complex and its parasites. On one hand, from the host side - an interspecific hybridization plays an important role. For tracing community dynamics and reticulate evolution in such a hybrid species complex, long-term comparative studies of natural populations are necessary. In order to conduct such a study, it is essential to access historical samples. These samples are usually suffering from low DNA quality due to the preservation chemical such as formaldehyde or denaturated ethanol, therefore traditional genotyping through length-based markers (such as microsatellites or allozymes) proved to be insufficient. For circumventing these issues, SNP- based markers were developed. Based on transcriptome data of one species belonging to the complex, it was possible to amplify and sequence several unlinked loci, which were then scanned for species-specific SNPs. Altogether 11 loci distinguishing all three species of the complex and their hybrids were developed and incorporated into PCR-RFLP assay. By comparing the taxon assignment from microsatellite and SNP data, there was found nearly perfect concordance. Finally, the genotyping method was successfully tested on samples dating back to the year 1960. On the other hand, parasite genetic studies are much more limited, in terms of availability of molecular markers. Only handful of parasites allows their cultivation under laboratory conditions inside the hosts. Even then, their disproportional amount of DNA compared to hosts and additional presence of other organisms present in media is forcing to rely on the traditional markers such as internal transcribed spacer (ITS). The traditional method of obtaining sequences to access the diversity (Sanger sequencing preceded by cloning – due to high intragenomic variation of this region) is becoming inefficient due to its high costs in terms of funds and time. Therefore, there was developed a molecular pipeline able to produce and process larger amount of sequence data with more accurate processing, specifically using a next-generation-sequencing platform (454). Afterwards a new bioinformatic pipeline QRS (quantification of representative sequences) was developed, inferring the representative sequences from the next generation sequencing (NGS) data sets (based on

neighbor joining or statistical parsimony) and calculating their frequencies. Verification of the method was done by comparing the dataset with the previous study of population structure of *Daphnia* parasite *Caullerya mesnili* based on cloning and Sanger sequencing. Pipelines were then used for accessing genetic diversity of the two parasite microsporidian species (*Berwaldia* and MIC1) commonly infecting *Daphnia longispina* complex in Central Europe. Specifically, the patterns of geographic population structure, intraspecific genetic variation, and the recombination events were examined, which are necessary for better characterization of the biology of these parasites. The limited geographical variation that was observed in *Berwaldia* and the different lake origin of recombinant and parental sequences supports usage of a mobile secondary host hypothesis during the life cycle of this species. Similarly MIC1 seems to have a secondary host, however the secondary hosts of both parasites likely differ and the *Berwaldia*`s one is assumed to have a higher mobility than the one transmitting MIC1.

Zusammenfassung

Die evolutionäre Dynamik von Wirtsarten und ihren Parasiten beinhaltet komplexe Prozesse. Zur Untersuchung dieser Prozesse auf der Ebene des Genotyps ist die Entwicklung zuverlässiger molekularer Werkzeuge notwendig. Das Ziel der vorliegenden Arbeit war die Entwicklung solcher Werkzeuge für den *Daphnia longispina*-Artenkomplex (Süßwasser-Crustaceen) und seine Parasiten. Einerseits spielt auf Seiten des Wirts interspezifische Hybridisierung eine große Rolle. Um die Dynamik der Artengemeinschaft sowie die verästelte Evolution innerhalb eines solchen Hybridartenkomplexes nachzuvollziehen, sind vergleichende Langzeitstudien natürlicher Populationen notwendig. Zur Durchführung einer solchen Studie ist die Einbeziehung historischer Proben essentiell. Diese Proben haben üblicherweise den Nachteil einer niedrigen DNA-Qualität durch Konservierungsmittel wie Formaldehyd oder vergällter Ethanol. Daher erwiesen sich traditionelle Methoden zur Genotypisierung mittels längenbasierter Marker wie Mikrosatelliten oder Allozyme als unzureichend. Aus diesem Grund wurden SNP-basierte Marker entwickelt. Basierend auf Transkriptomdaten einer Art aus dem *Daphnia longispina*-Artenkomplex konnten mehrere ungekoppelte Loci amplifiziert, sequenziert und nach artspezifischen SNPs durchsucht werden. Insgesamt wurden 11 SNP-Marker entwickelt, die alle drei Arten und ihre Hybriden unterscheiden und mit Hilfe eines PCR-RFLP-Tests untersucht wurden. Beim Vergleich der Taxonzuordnung von Mikrosatelliten- und SNP-Daten wurde annähernd perfekte Übereinstimmung gefunden. Schlussendlich konnte die Genotypisierungsmethode erfolgreich angewendet werden auf Proben, die aus dem Jahr 1960 stammen. Andererseits sind Studien zur Parasitengenetik viel beschränkter in Hinblick auf die Verfügbarkeit molekularer Marker. Nur wenige Parasiten können unter Laborbedingungen in den Wirtsorganismen kultiviert werden. Außerdem lässt die unverhältnismäßig kleine DNA-Menge der Parasiten im Vergleich zum Wirt und die Anwesenheit anderer Organismen im Kulturmedium wenig Alternativen zur Verwendung traditioneller Marker wie ITS (interne transkribierte Spacer). Die traditionelle Methode, Sequenzdaten zur Beurteilung der Diversität zu erhalten (Sanger-Sequenzierung gefolgt von Klonierung wegen der hohen intragenomischen Variation dieser Region) ist mittlerweile ineffizient durch den hohen finanziellen und zeitlichen Aufwand. Daher wurde eine molekulare Pipeline entwickelt, um größere Datenmengen zu produzieren und fehlerfreier zu verarbeiten unter

Verwendung einer Next Generation Sequencing-Plattform (454). Anschließend wurde eine neuartige bioinformatische Pipeline ‚QRS‘ (Quantification of Representative Sequences, Quantifizierung Repräsentativer Sequenzen) entwickelt, die mithilfe von Neighbor Joining- und Parsimony-Ansätzen repräsentative Sequenzen aus Next Generation Sequencing (NGS)-Datensätzen ermittelt und deren Frequenzen berechnet. Die Methode wurde verifiziert durch den Vergleich des Datensatzes mit einer vorangegangenen Studie zur Populationsstruktur des *Daphnia*-Parasiten *Caullerya mesnili*, die auf Klonierung und Sanger-Sequenzierung basierte. Die Pipelines wurden anschließend zur Untersuchung der genetischen Diversität zweier parasitärer Mikrosporidienarten (*Berwaldia* und MIC1) verwendet, die häufig den *Daphnia longispina*-Komplex in Zentraleuropa infizieren. Besonders die Muster geografischer Populationsstruktur, intraspezifische genetische Variation und Rekombinationsereignisse wurden untersucht, da sie eine bessere Charakterisierung der Parasitenbiologie ermöglichen. Die bei *Berwaldia* beobachtete begrenzte geografische Variation und die Herkunft der rekombinanten und parentalen Sequenzen aus unterschiedlichen Seen unterstützt die Hypothese eines sekundären mobilen Wirts im Lebenszyklus dieser Art. Ebenso scheint MIC1 einen sekundären Wirt zu haben. Allerdings unterscheiden sich die sekundären Wirte der beiden Parasiten wahrscheinlich, wobei derjenige von Berwaldia vermutlich eine höhere Mobilität aufweist als der Wirt, der MIC1 überträgt.

1. Introduction

1. 1. Searching for a model system to enable the study of host-parasite interactions in the field and in the laboratory

Parasites are ubiquitous in nature (Lafferty *et al*., 2006, Poulin, 2011) and they seem to affect hosts from all taxa. They cause one of the strongest reciprocal selection pressure in nature leading to the co-evolution of hosts and themselves. Parasites reduce fitness of hosts and host defense systems are counteracting parasites or, in other words, co-evolution of host resistance and parasite virulence occurs. These relationships are very dynamic, many of which were discovered by studying *Escherichia coli* (e. g. Kashiwagi and Yomo, 2011, Meyer *et al*., 2012) or *Pseudomonas fluorescens* (e. g. Gómez and Buckling, 2011, Betts *et al*., 2014) with their respective bacteriophages, under laboratory settings. Laboratory conditions reduce the complexity of biological systems and simplify the environment under which the biological interactions occur. However, in order to fully understand host-parasite dynamics, both laboratory as well as field studies are needed. Therefore, a host-parasite model system which allows for both types of studies is required.

Several animal hosts with their parasites have been used so far as such model systems. The fruit fly *Drosophila melanogaster* is the most extensively studied animal model system in general, including host-parasite interactions (Starr and Cline, 2002, Veneti *et al*., 2004). For *Drosophila*, powerful genetic tools are available, including the sequenced genome (Adams *et al*., 2000), which is often lacking in other animal hosts. Moreover, it can be infected with a wide range of parasites such as bacteria, viruses, protozoans and fungi (reviewed in Keebaugh and Schlenke, 2014).
Nematode *Caenorhabditis elegans* offers similar advantages due to the extensive knowledge of its genetics (Brenner, 1974), resulting from its usage as a model system in other biological fields such as neural development (Schafer, 2005) and apoptosis (Peden *et al*., 2008). *Caenorhabditis elegans* together with its parasites: *Bacillus thuringiensis* and *Serratia marcescens*, makes a good model system to study the influence of antagonistic coevolution on parasite genetic diversity, gene

exchange, and selection for biparental sex (Morran *et al*., 2011, Gibson *et al*., 2015, Schulte *et al*., 2013).

Various snail species (e.g. *Potamopyrgus antipodarum, Littorina* sp.), and their parasitic castrating trematodes (e.g. *Microphallus* sp.) constitute another important model system because of the great variation in host reproductive strategies (i.e. coexistence of sexual and asexual lineages). Thus, these systems are used for studies of evolution and maintenance of sexual reproduction (Keogh *et al*., 2015, Vergara *et al*., 2014, King *et al*., 2009).

The red flour beetle, *Tribolium castaneum* – microsporidium *Parnosema whitei,* is another interesting system enabling to study the evolution of host resistance and trade-offs between virulence and transmission potential. The underlying theory is that an increase in transmission rate should come at a cost in terms of duration of infection (Massad, 1987). Unlike a majority of the other systems, this one allows for the transmissive stages of these spore-forming parasites to persist in the environment, which may have an important influence on the outcome of evolutionary dynamics (Rafaluk *et al*., 2015, Kerstes *et al*., 2012).

1. 1. 1. *Daphnia* as a model host

Daphnia and its parasites is a frequently used model particularly suitable to study host-parasite epidemiological, evolutionary, and genetic interactions. *Daphnia* are small planktonic freshwater crustaceans inhabiting freshwater bodies around the world. *Daphnia* reproduce mainly (under most conditions) asexually via apomictic parthenogenesis, producing offspring (daughters) genetically identical to their mother. Parthenogenetic reproduction system allows for individuals from the field to be maintained under laboratory conditions as clonal lineages. Consequently, by conducting experimental surveys (different treatments) with organisms that belong to the same clone, one could better control for the separation of genetic and non-genetic effects (e.g. environment). At the same time, *Daphnia* are also able to reproduce sexually, mainly during harsh conditions. Harsh conditions (e.g. overcrowding, scarcity of food, low temperatures) stimulate production of males and haploid eggs, which are being fertilized and formatted into resting eggs (ephippia). Ephippia are deposited in the lake sediment and they remain hatchable for decades (Frisch *et al*., 2014, Brendonck and De Meester, 2003). When different *Daphnia* species cross, this allows for

interspecific hybridization (e. g. Keller *et al*., 2008). Moreover, it is possible to collect resting eggs from dated sediment cores and hatch them under laboratory conditions (Orsini *et al*., 2013, Frisch *et al*., 2014), thus allowing for the evolutionary studies over time.

Daphnia is the host of numerous parasites representing diverse taxonomic groups, especially microsporidia, bacteria, and fungi (but also nematodes or cestodes), mainly producing chronic infections (reviewed in Ebert, 2008). An impact of parasites on *Daphnia* population can be significant, especially through affecting host fecundity and survival (Stirnadel and Ebert, 1997), reaching prevalence peaks up to 100% (Lass and Ebert, 2006, Duncan and Little, 2007). Other traits influenced by parasites include reduced adult growth (Ebert, 1994, Lass and Bittner, 2002), parasite-induced host gigantism (Ebert *et al*., 2004), alternated life cycle (Lass and Ebert, 2006), altered host-predator interactions such as visibility to predators (Duffy *et al*., 2005), depth selection behavior (Fels *et al*., 2004) or inducible anti-predator defenses (Yin *et al*., 2011). Moreover, parasite-induced effects can be enhanced by environmental conditions such as food quality and quantity, temperature, host density, presence and density of competitors, predator cues, and toxins (e.g. Bittner, 2002, Lass and Bittner, 2002, Duffy *et al*., 2005, Mitchell *et al*., 2005, Jansen *et al*., 2011a, 2011b).

A handful of different parasite species with different transmission modes and the possibility of culturing some of them under laboratory conditions, together with the characteristics mentioned above, make *Daphnia*-parasite a convenient system to study host-parasite dynamics. This becomes even more of an importance since publishing the first *Daphnia* genome (Colbourne *et al*., 2005); *D. magna* and *D. galeata* in prep.) which allows a development of molecular markers and a wide range of research such as QTL (Routtu and Ebert, 2015), or the expression studies (Decaestecker *et al*., 2011) of *Daphnia* influenced by parasite pressure and environmental factors.

1. 1. 2. *Daphnia longispina* species complex as a model (host-parasite) system

Several hybrid species complexes have become model systems in evolutionary biology to study the role of interspecific hybridization in speciation and adaptation. Those are for example sunflowers (Rieseberg *et al*., 2007), irises (Arnold and Meyer, 2006), cichlids (Seehausen *et al*., 1997) and Darwin's finches (Grant and Grant, 1996). The specific reproductive system of *Daphnia* determines it to be one of them. Long-term maintenance of hybrid populations has been frequently

described in the *D. longispina* complex in Europe, involving a number of species such as *D. cucullata, D. galeata* and *D. longispina* (e. g. Schwenk and Spaak, 1997, Keller *et al.*, 2008, Petrusek *et al.*, 2008), producing several types of hybrids among themselves.

Daphnia longispina complex system is a host of numerous parasites. They significantly differ in the strength of selection pressure that they can exert (Lohr *et al.*, 2010), and the level of specialization on host species (Wolinska *et al.*, 2007) or temporal and spatial prevalence. Specifically, a variation in prevalence between seasons as well as a spatial variation were observed among, and within different *Daphnia* populations (Wolinska *et al.*, 2011a). The parasites of *D. longispina* complex belong to the whole range of taxa. Undefined bacteria grow in the body cavity of their host and strongly increase host opacity and reduce *Daphnia*`s fecundity (Bittner, 2001, Wolinska *et al.*, 2007). *Metschnikowia bicuspidata* (Metschnikoff, 1884) is an endoparasitic horizontally transmitted yeast Ascomycete (Endomycetales), producing needle-like ascospores penetrating *Daphnia*`s gut walls when ingesting with food. After germination in the hemolymph it grows inside the host until the entire cavity is filled with the spores, reducing host`s fecundity (Lohr *et al.*, 2010). Oomycete parasites appear to be generalist parasites infecting the brood pouch or the entire body cavity, and filling them with hypha-like structures (Wolinska *et al.*, 2008). Other important parasites of the *D. longispina* complex on which was this study focused are Microsporidia and Ichthyosporean *Caullerya mesnilli*. These parasites are described more in detail in the next two chapters.

1. 1. 2. 1. Microsporidia

Microsporidia are obligate intracellular parasites, forming the largest group of parasites infecting *Daphnia* (Ebert, 2005). As a group they are clearly distinguished from other eukaryotes, being the most divergent basal fungi clades (Capella-Gutiérrez *et al.*, 2012). Recently, microsporidia of *Daphnia* are becoming an important model system (Ebert, 2008, Stollewerk, 2010), also due to their complex life cycles (Refardt *et al.*, 2008, Weigl *et al.*, 2012). Microsporidia are usually tissue specific (ovaries, fat cells, hypodermis, gut) with spore size depending upon culture conditions (e.g., smaller spores were observed at lower temperatures; Friedrich *et al.*, 1996). The transmission

mode of this group of parasites is highly variable, also depending on the type of infection (e.g. gut infections are mainly transmitted horizontally - *Glugoides intestinalis* (Ebert, 1995), ovary infection vertically - *Flabelliforma magnivora* (Larsson *et al*., 1998)).

The most abundant microsporidia parasites infecting *Daphnia longispina* species complex are *Berwaldia schaefernai* (Jírovec, 1937) and an unidentified microsporidium MIC1 (Wolinska *et al*., 2009), frequently reported to occur in central Europe (Weigl *et al*., 2012). Their infections are specific for body cavity tissue of *Daphnia*. Taxonomically, they are closely related to other microsporidian parasites infecting other species of *Daphnia* such as *Gurleya daphnidae – D. pulex, Binucleata daphnidae – D. magna, Larssonia obtusa – D. pulex* (taxonomy reviewed in Weigl *et al*., 2012). There is no direct evidence about the transmission mode of *Berwaldia* and MIC1; however, the unsuccessful attempts to culture them in the laboratory and their close taxonomic relation with taxa infecting copepods transmitted by larval and adult mosquitoes (Vossbrinck *et al*., 2004) suggests that they also have indirect transmission mode with a mobile vector.

1. 1. 2. 2. *Caullerya mesnili*

Caullerya mesnili is a protozoan parasite of *Daphnia*, recently classified into Ichthyosporea (Lohr *et al*., 2010). *Caullerya mesnilli* is a common parasite throughout communities of the *D. longispina* species complex in permanent European lakes, where they infect all of the paternal species and hybrids, reaching prevalence up to 40% (Wolinska *et al*., 2007). It forms clusters of spores in the gut epithelium of the host, causing severe harm. *Caullerya* infected hosts have shortened life-span, smaller body size and significantly reduced fecundity (e.g. number of eggs reduced by 95% compared to not infected hosts; Wolinska *et al*., 2006, 2007). Infection is transmitted horizontally – the spores released from one *Daphnia* are being up taken by another individual together with food - and then spread rapidly throughout the gut (Lohr *et al*., 2010).

1. 2. Tools to study dynamics in the Daphnia-parasite system

1. 2. 1. Host genotyping

1. 2. 1. 1. Available markers for genotyping of *D. longispina* species complex

Daphnia longispina species complex has already available several marker systems for identification parental and hybrid species. Historically the oldest was the genotyping via allozymes (e. g. Wolf and Mort, 1986, Haag *et al*., 2005, Seda *et al*., 2007). However, allozyme markers are resource-demanding in terms of required sample quantity and quality (Taylor *et al*., 1996, Gießler, 1997). When applied on old, low DNA quality samples, the allozyme markers are insufficient and additionally as only a limited number of loci can be genotyped they are unable to distinguish different hybrid classes. Most of the studies were able to score four loci (e. g. Spaak, 1996, Wolinska *et al*., 2006, Petrusek *et al*., 2013), from which only two were species specific (Wolf and Mort, 1986, Gießler, 1997). Two internal transcribed spacers (ITS) were the first nuclear markers (Billiones *et al*., 2004, Taylor *et al*., 2005, Petrusek *et al*., 2008) within *D. longispina* complex. This marker did not provide the possibility of distinguishing between all the species. The main problem though is that single, diploid marker can differentiate only F1 hybrids, whereas the detection of more complex hybrid classes have become impossible (Giessler and Englbrecht, 2009). Relatively recently developed microsatellites (Brede *et al*., 2006) are a more powerful genotyping system being used as a method of choice for parental and hybrid species identification based on information for up to 15 loci (e. g. Thielsch *et al*., 2009, Brede *et al*., 2009, Yin *et al*., 2010). However, as the taxon identification is based on the joint information from allele frequencies at all loci (Selkoe and Toonen, 2006, Dlouha *et al*., 2010) and the alleles are length-based, it makes them rather unsuitable when attempting to genotype DNA samples of limited quality.

1. 2. 1. 2. Molecular *exploration of host historical samples*

In order to study the population and community dynamics among hosts in *D. longispina* species complex, long-term surveys of natural populations are absolutely essential. One source of the historical samples are ephippia isolated from dated sediment cores (Duffy *et al*., 2000, Cousyn *et al*., 2001, Reid *et al*., 2002, Geerts *et al*., 2015). However, because ephippial eggs are produced sexually, such samples do not represent the actual lake population and there is a bias of sexual reproduction and hatching success rate towards pure (parental) species (Keller and Spaak, 2004). Reconstruction of actual populations is theoretically possible from sample archives. In the past, *Daphnia* samples (collected as net-tow material) were usually preserved in formaldehyde (or denaturated ethanol) which causes severe damages to the DNA, mainly fragmentation and cross-linking of the DNA molecules (thus blocking the accession for the enzymes). Consequently, DNA quality suffers and assays for genotyping are significantly limited. Therefore the development of new methods and tools, especially concerning improved DNA extraction from these samples and ways to genotype them, are essential for these kind of evolutionary studies.

1.2.2. Parasite genotyping

In contrast to host genotyping, parasite genetic studies are much more limited, especially in terms of availability of reliable molecular markers. Their development is being stunted by the impossibility of culturing parasites without the host. Therefore, when attempting to isolate parasite DNA, its amount is usually disproportionally low compared to host DNA, but also contaminated by DNA of other organisms found in cultivation media such as algae and bacteria. Commonly used markers are internal transcribed spacers (ITS), regions located between rRNA genes coding the subunits. Due to their multicopy nature (Hershkovitz and Lewis, 1996) it is possible to amplify them even in low DNA concentration samples. Regarding the parasites of the *Daphnia longispina* complex, these are the only available parasite markers to study intraspecific genetic diversity (Wolinska *et al*., 2011b, Giessler and Wolinska, 2013).

1. 2. 3. Bioinformatics tools to analyze next-generation-sequencing data

Traditional platforms for genomic studies (e.g. Sanger sequencing) are being replaced by faster and lower-cost Next Generation Sequencing (NGS) technologies producing large quantities of data enabling to understand links between observed diversity with ecological functions (Huber *et al*., 2007, Edgcomb *et al*., 2011, Kautz *et al*., 2013). Evolutionary studies examining changes of populations in time and space require additional reliable ways of genotype quantification, especially in the frame of bioinformatic analysis. That is challenging due to several types of artificial errors and artefacts produced by NGS methods (e.g. platform, homopolymer and indel misinterpretations (Margulies *et al*., 2005) by 454 pyrosequencing or systematic base-calling biases (Erlich *et al*., 2008, Rougemont *et al*., 2008, Renaud *et al*., 2013) by Illumina). Therefore the most demanding task in developing new bioinformatic tools performing such analyses is to identify the true alleles (natural variants present in a population or organism) and distinguish them from the variants resulting from methodological errors, when estimating their frequency.

1. 3. Outline of the thesis

The aim of this PhD project was to extend our understanding of the evolutionary dynamics firstly within the hosts, and secondly between the host and parasites, with particular focus on changes over time. Such ambitious studies suffer from the lack of reliable and precise tools, due to the nature of the examined samples (e.g. their quality), poor genomic knowledge of parasites (thus, limited availability of markers) and character of produced data (e.g. NGS output). This thesis aims to tackle these challenges and develop such molecular tools to address evolutionary questions.

In Chapter 2, I tackled the challenges related to a need of following host dynamics over time. For conducting long-term evolutionary studies, it is essential to access historically archived samples suffering from low quality and damaged DNA. There were two main issues to be solved, which are addressed in Chapter 2. The first challenge was to develop and optimize DNA extraction protocol from such samples. Secondly, I needed to develop reliable molecular markers that are

able to distinguish between pure species and different classes of hybrids of the *D. longispina* complex. Since it was only possible to extract DNA of limited quality, previously developed methods of classification turned out to be insufficient. The method of choice was to develop the SNP (single nucleotide polymorphism) based markers which offer several advantages. The most important one is that each nucleotide base can be unambiguously identified, allowing addressing identical alleles in different studies. Moreover, as the detection of only one base at a given site is needed, short DNA fragments are sufficient to target the genomic region of interest, and at the same time even low quality DNA samples can be accessed. I compared transcripts of *D. galeata*, the only available sequence data of *D. longispina* species complex, with previously published genome of *D. pulex*, in order to identify putatively unlinked loci which were then screened for presence of species specific SNPs. Suitable SNPs were afterwards integrated into PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) assay and their concordance with other genotyping markers was tested on a dataset of field samples originating from across the Europe, previously identified by other classification methods. Finally, the markers were tested to access historical formaldehyde samples dating back to the year 1960.

Chapter 3 tackles the development of new tools for studying genetic diversity of parasites. An internal transcribed spacer is, in most cases, the only available nuclear marker for studies of non-model parasites or microorganisms (Nilsson *et al*., 2008). The traditional method of obtaining sequences to access the diversity (Sanger sequencing preceded by cloning – due to high intragenomic variation of this region) is becoming inefficient due to its high costs in terms of funds and time. I aimed to develop a molecular pipeline able to produce and process larger amount of sequence data with more accurate processing; i.e. using a next-generation-sequencing platform (454). To verify the method we used the same *C. mesnili* DNA samples analyzed in the previous study (Wolinska *et al*., 2014) which were genotyped by Sanger sequencing. Thus, NGS and Sanger sequencing generated data were compared in this chapter. First I designed primers that amplify shorter fragment of ITS1 region than the ones already optimized and published in a previous study, due to 454 pyrosequencing limitations (reliable sequence length read). Based on the alignment from the previous study (Wolinska *et al*., 2014), I targeted the most conserved regions, taking into account the machine capacity and a need of preserving of variable regions to be amplified by these primers. Then I optimized and verified the primer specificity, including their adapters (from the 454 Standard MID Set; Roche/454 Life Sciences). Each sample carried their unique combination

of adapters in order to separate them after sequencing. After generating the data, a co-author of Chapter 3, Enrique Gonzalez-Tortuero, developed a new bioinformatic pipeline (Quantification of Representative Sequences; QRS) that pools similar sequence variants and estimates their frequencies in NGS data sets from populations or communities. Pooling similar sequences was done in order to preserve the maximum of data, and to correct for the artificial errors at the same time. Then it was tested whether the estimated frequency of representative sequences generated by 454 amplicon sequencing differs significantly from that obtained by Sanger sequencing of cloned PCR products.

Once the molecular and bioinformatic pipelines were developed, we were able to use them for an actual study of intraspecific parasite sequence variation. In Chapter 4, we aimed to understand the transmission mode of the two most common microsporidians infecting *Daphnia* communities inhabiting large lakes and reservoirs in Central Europe, classified in the literature as *Berwaldia schaefernai* (Vávra and Larsson, 1994) and as the microsporidium MIC1 (Wolinska *et al*., 2009), by examining their population structure. For amplification of parasite ITS regions I used the core primer sequences form previous study (Wolinska *et al*., 2009), fused with the adapters (from the 454 Standard MID Set; Roche/454 Life Sciences). I optimized PCR reaction, taking into account additional nucleotides of adapters and diverse concentration of DNA samples. Then I carried on the amplification of 115 microsporidia-infected *Daphnia,* each labeled with unique combination of the adapters. We sequenced ITS region of both parasites originating from *D. longispina* samples from seven drinking water reservoirs in Czech Republic on 454 pyrosequencing platform and processed the data with QRS pipeline (as developed in Chapter 3). The patterns of geographic population structure, intraspecific genetic variation, and recombination events of the ITS1 sequence were compared between *Berwaldia* and MIC1, in order to better characterize the biology of these species.

The thesis concludes with Chapter 5, providing a general discussion and suggestions for future research.

2. New possibilities arise for studies of hybridization: SNP-based markers for the multi-species *Daphnia longispina* complex derived from transcriptome data

Jakub Rusek, Gokce B. Ayan, Patrick Turko, Christoph Tellenbach, Sabine Giessler, Piet Spaak, Justyna Wolinska

(2015)

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3. The Quantification of Representative Sequences pipeline for amplicon sequencing: case study on within-population ITS1 sequence variation in a microparasite infecting *Daphnia*

Enrique Gonzalez-Tortuero, Jakub Rusek, Adam Petrusek, Sabine Gießler, Dimitrios Lyras, Sonja Grath, F. Castro-Monzon, Justyna. Wolinska

(2015)

Molecular Ecology Resources (2015) 15 (6): 1385–1395

doi: 10.1111/1755-0998.12396

4. Genetic diversity of two *Daphnia*-infecting microsporidian parasites, based on sequence variation in the internal transcribed spacer region

Enrique González-Tortuero, Jakub Rusek, Inbar Maayan, Adam Petrusek, Lubomír Piálek, Stefan Laurent, Justyna Wolinska

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5. Discussion

5. 1. Summary of the thesis

This PhD project aimed at developing new molecular techniques and tools which would allow for better understanding of dynamics within model host-parasite system – *D. longispina* species complex together with two groups of parasites (microsporidia and ichthyosporea), and ideally, making it possible to track host-parasite dynamics over time.

5. 1. 1. SNP-based markers for genotyping *D. longispina* species complex

Despite the fact that *D. longispina* species complex features a rather wide range of classification methods (e.g. allozymes, microsatellites), it turned out that none of them could be used when accessing historical formaldehyde-preserved samples necessary for long-term evolutionary studies. Therefore, in Chapter 2, I aimed to resolve this issue by developing a set of completely new method based on SNPs which would allow distinguishing between pure (parental) species and different classes of their hybrids. As a detection of only one nucleotide is needed, the requirements for DNA quality (e.g. formaldehyde-preserved samples) is much lower. Because SNPs are biallelic markers, a single SNP site does not allow for direct discrimination among more than two species. Therefore I selected short fragments with two complementary informative SNP-sites. The set of eleven short fragments (SNP-based markers) developed in this study enabled reliable discrimination among three species of the *D. longispina* complex and their hybrids. This taxon assignment was concordant with other molecular markers; there was almost a perfect correspondence between microsatellite- and SNP-based marker assignments. The gene loci, with species-specific SNP-based markers developed here, are distributed over multiple chromosomes that I achieved through the mapping against the annotated genome of *D. pulex*, which suggests their putative linkage independence. Because of the high abundance of SNPs in a genome and their unambiguous nature, the statistical power to resolve hybrid and parental species is much higher compared to allozymes or microsatellites.

We have further demonstrated that old and poorly-preserved DNA samples spanning several decades can be successfully genotyped using short amplicons (and newly developed DNA extraction method from formaldehyde). This might allow for studies of hybridization over time. The field population samples preserved in formaldehyde provide better knowledge about the *Daphnia* community composition than the dormant egg banks from the sediment, which represent only the sexually active part of the population (e.g. Keller and Spaak, 2004). I showed that a relatively small set of biallelic SNPs provides sufficient information for obtaining concordant results in taxon assignment compared with microsatellites and other markers. This will make studies of long-term impact of various environmental factors (e. g. chemicals, parasite pressure, temperature) on biodiversity changes at community and population levels now possible.

5. 1. 2. Molecular and bioinformatic pipeline to study parasite genetic diversity

In Chapter 3, the aim was to test a new approach to access the genetic diversity of the parasites, replacing traditional, time and resources costly Sanger sequencing. The method of choice was the 454 pyrosequencing, due to the possibility of obtaining longer sequence reads at the time of method development. First, I designed and optimized a new set of fusion primers amplifying ITS1. According to alignments from the previous study (Wolinska *et al*., 2014), I aimed to develop primers amplifying a shorter region, and at the same time preserving the maximum of variable sites within the amplicon. Then, I amplified 16 samples (each representing one lake) of the ichthyosporean *Caullerya mesnili*, each one carrying a unique molecular identificator. Afterwards a new bioinformatic pipeline QRS (quantification of representative sequences) was developed, inferring the representative sequences from the next generation sequencing (NGS) data sets (based on neighbor joining or statistical parsimony) and calculating their frequencies (quantification of the sequence variants is important for tracing host-parasite dynamics). To verify the method, we compared data from 454 amplicon sequencing with data from Sanger sequencing of cloned ITS1 amplicons using QRS pipeline.

The frequencies of representative ITS1 sequences derived from both data sets were similar, with only one significant difference across the 16 studied cases. 454 pyrosequencing detected some

additional rare representative sequences what is caused by a higher sampling power of the method (Huse *et al*., 2007, Kröber *et al*., 2009, Liang *et al*., 2011). On the other hand, surprisingly, some rare sequences were detected also only by the Sanger sequencing, and missed by 454 pyrosequencing. This fact might be caused by poor sensitivity of the 454 pyrosequencing method for certain taxa, including the frequent ones (Tedersoo *et al*., 2010, Kauserud *et al*., 2012), or due to differences between primers used in both methods. Despite the maximum effort to place the primers in the most conserved regions, these were still partly polymorphic, and thus it is possible to miss certain variants due to this reason.

We showed that the Sanger sequencing can be replaced by the faster, and cheaper, 454 pyrosequencing. This was proved by re-analyzing the ITS1 region of *Caullerya mesnili* from the previous study and obtaining the same results. Specifically, same representative sequences were recovered at similar frequencies, despite the differences between the two sequencing platforms and associated potential methodological errors. The molecular and bioinformatic pipelines can be further used for studies requiring the insights into genetic diversity and the quantification of the sequence variants (e.g. various strains of parasites) when studying evolutionary dynamics of any non-model species. Hence, in Chapter 4 we used the developed here pipelines for such a study.

5. 1. 3. Genetic diversity of microsporidia

The molecular and bioinformatics methods developed in Chapter 3 (i.e. for high throughput analysis of the diversity and the quantification of the ITS sequences) were used in an ecological study addressed in Chapter 4. Thus, we wanted to access genetic diversity of the two microsporidian species commonly infecting cladocerans of the *Daphnia longispina* complex in Central Europe. More specifically, we aimed to have a closer look into the patterns of geographic population structure, intraspecific genetic variation, and the recombination events, all of which are necessary to better characterize the biology of these parasites. The available limited data on low among-population variation of *Berwaldia* supports the hypothesis of this parasite being spread by a highly mobile secondary vector (Wolinska *et al*., 2011b). This transmission mode might be able to effectively homogenize the parasite population, whereas generally high genetic diversity is found between populations of parasites infecting immobile or asocial hosts (reviewed in Huyse *et al*., 2005). For MIC1, no such information about a transmission mode was available.

Unlike the previous study (Chapter 3), newly developed protocols allowed to access genetic diversity on an individual level (whereas in Chapter 3, all ten *Caullerya* infected *Daphnia* per lake were pooled before DNA extraction). More specifically, ITS1 region of 87 *Daphnia* infected with *Berwaldia* (sampled across six water reservoirs), and 28 *Daphnia* infected with the microsporidium MIC1 (sampled from two reservoirs) was amplified with parasite-specific primers and sequenced. For each sample I provided a unique combination of molecular identificators, which allowed for sequencing of all samples in a single run, and their separation afterwards. Hence, the analyses could be done at an individual level, instead of per lake.

The limited geographical variation that was observed in *Berwaldia* and the different lake origin of recombinant and parental sequences supports usage of a mobile secondary host hypothesis during the life cycle of this species (Wolinska *et al*., 2011b). Close phylogenetical relatedness of MIC1 and genus *Gurleya* (Weigl *et al*., 2012) suggests the transmission through a secondary host (Voronin, 1996), also for MIC1 species. However, the observed differences in genetic structure between studied MIC1 and *Berwaldia* populations might imply a decrease in spreading efficiency through a secondary host for MIC1, compared to *Berwaldia*. In other words, the secondary hosts of both parasites likely differ and the *Berwaldia*`s one is assumed to have a higher mobility than

the one transmitting MIC1. Nevertheless, increased genetic variability of the parasite population might also be caused by other variables, especially effective population size (Huyse *et al*., 2005).

Additionally, in this study, phylogenetic analysis was conducted and the results were compared with the published trees (Vossbrinck *et al*., 2004, Wolinska *et al*., 2009, Weigl *et al*., 2012) which were based on the small subunit ribosomal DNA. This was done in order to examine whether the recombination events in the ITS region of *Berwaldia* have an effect on tree topology (Posada and Crandall, 2002), as it has been seen in other microsporidian species like *Vairimorpha*/*Nosema* (Choi *et al*., 2011). Phylogenetic trees based on both markers were consistent with each other, and the positioning of both *Berwaldia* and MIC1 supports the hypothesis of using more than one host during their life cycle.

5.2. Future directions

5.2.1 Access to historical samples and the possibility to perform long-term studies

In order to study the significance of hybridization events over time, reliable methods for detecting species, hybrids, and backcrosses are needed. I addressed this issue in Chapter 2 where I developed a completely new classification method based on SNP markers (eleven SNP-based markers in total). I showed that this method enables reliable discrimination among three species of the *D. longispina* complex and their hybrids. SNP-based genotyping has been concordant with other molecular markers. Moreover, I achieved a higher statistical power in resolving hybrid and parental species, hence this method is much more precise compared to allozymes or microsatellites. Further, Chapter 2 also describes the newly developed protocol for DNA extraction from formaldehyde preserved samples spanning several decades. These two innovations combined together might provide an essential tool for hybridization studies over time. *Daphnia* seems to be an excellent system for such studies, due to the natural archives of resting stages in lake sediments, and also the availability of old plankton samples stored in limnological collections worldwide; being preserved in the DNA-damaging chemical formaldehyde. The latter samples provide better knowledge about the *Daphnia* community composition than dormant egg banks from sediment, which represent only the sexually active part of the population (e.g. Keller and Spaak, 2004). I have already demonstrated in Chapter 2 that old and poorly-preserved DNA samples spanning over several decades can be successfully genotyped using short amplicons. Classification based on the eleven SNP-based markers (or their subset) can be facilitated by their multiplexing, both at PCR and at genotyping level. As a method of detection, an ideal method appears to be SNaPshot multiplex based on minisequencing (Huang *et al*., 2011, Kitpipit *et al*., 2012), for which the application on *Daphnia* samples is currently being developed (Turko *et al*., in prep.). This will make studies of long-term impact of various environmental factors (e. g. chemicals, parasite pressure, temperature) on biodiversity changes at *Daphnia* community and population levels now possible.

In Chapter 2, PCR-RFLP was chosen as a method of SNP detection for community screenings. PCR-RFLP is a simple method, which enables relatively quick and cost-efficient detection of SNPs using equipment common in most laboratories (e.g. Laguerre *et al*., 1994, Ota *et al*., 2007, Rusek

et al., 2013). This opens a possibility to study community dynamics for research groups unprivileged by the grade of funding or accessibility to technical facilities, thus fill in the gap of knowledge about the *D. longispina* species complex system from diverse geographical locations.

Additionally, the alignments of sequences from eleven loci revealed numerous intraspecific SNPs, which could be used in future population genetic studies of the *D. longispina* species complex, including low quality samples by providing more accurate estimations compared to microsatellites. It is possible to develop more of such SNP-based markers by a simple application of the here developed pipeline – resequencing of transcriptome. Lastly, a similar strategy could be applied for other non-model species with prior knowledge of transcriptome data only (reviewed in Ekblom and Galindo, 2011).

5.2.2. Host-parasite dynamics

In Chapter 3, we have developed new molecular and bioinformatic pipelines to analyze sequence variation within populations, as obtained from NGS platforms. Both neighbor joining and statistical parsimony (Templeton *et al.*, 1992) were used as a clustering algorithm and a network approach, respectively, making it useful to identify and quantify representative sequences for a variety of purposes (with possible usage of a big variety of alignment algorithms). In order to verify the pipeline, we reanalyzed DNA samples used in the Sanger sequencing-based study of the *Daphnia* parasite *Caullerya mesnilli* ITS1 region (Wolinska *et al.*, 2014). With QRS, it was possible to identify and characterize variation among closely related populations from the same samples, while obtaining comparable patterns with Sanger sequencing. Specifically, the same representative sequences were recovered at similar frequencies, despite the differences between the two sequencing approaches and associated potential methodological errors.

Several kinds of biological questions can be answered with the tools that have been developed. In case of *Daphnia*, the most interesting seems to be the tracking of changes in the frequencies in natural populations over time, in accordance to parasite-driven negative frequency-dependent selection (NFDS; Brockhurst and Koskella, 2013, Gaba and Ebert, 2009). The concept of the NFDS is that common genotypes of a host will be more likely to become infected by coevolving parasites, in respect to the rare genotypes. Rare host genotypes have then a higher fitness, and

eventually will replace the previously common host genotypes, because they are uninfected. The parasite population genotype frequencies will change accordingly. Specifically, parasite genotypes able to infect previously rare host genotypes will be favored. Because sex is efficient in promoting genetic diversity, NFDS can additionally explain the long-term maintenance of sex in host and parasite populations (reviewed in Lively, 2010). Under the experimental conditions, genotype-by-genotype interactions of both actors have been demonstrated in multiple host-parasite systems (Forsman, 2014, reviewed in Sadd and Schmid-Hempel, 2009). On the other hand, the field studies also show the increased susceptibility of the most common host genotypes to the parasites. That was shown for example in *Daphnia* and its parasites (Decaestecker *et al*., 2007, Little and Ebert, 1999, Wolinska and Spaak, 2009) and snail *Potamopyrgus* – trematode *Microphallus* sp. systems (e.g. Jokela *et al*., 2009, King *et al*., 2009). As implied, the previous studies were host-focused, while from the parasite side, measuring such changes in genotype interactions seems to be rather exceptional. With the new available tools (molecular and bioinformatic pipelines) we can examine parasite population genetic structure, which is a crucial component in the analysis of host-parasite coevolution. The pipelines were already successfully applied for such a survey (González-Tortuero *et al*., 2016). In this study, the population dynamics and host-genotype specificity of the *C mesnili* were analysed based on the observed sequence variation in the first internal transcribed spacer (ITS1) of the ribosomal DNA.

5.2.3. Role of variable environments on hosts and parasite dynamics

By applying the here developed tools, possible areas of research can expand one step further. The focus of research can involve not only within and between host or parasite dynamics per se, but also to examine the role of additional (environmental) factors influencing these dynamics. This can be done by looking at the changes in genotype frequencies of the players involved over the changing environmental factors.

Species can rapidly expand their ecological tolerance and geographical range. Moreover, hybridization between species can provide the genetic variation necessary for natural selection to produce a new adaptive norm (Lewontin and Birch, 1966). The regulation of hybrid zones seems to be greatly influenced by environmental (exogenous) selection which might be changing in short or long-term ecological time frames (Arnold, 1997, Fitzpatrick and Shaffer, 2004, Culumber *et al*.,

2011). It is quite likely that temporal changes are only detectable over longer periods than covered by typical studies (Carson *et al*., 2012). Therefore it is crucial to conduct long-term evolutionary studies, for which it will be necessary to access the archived samples. Several studies already suggest that consideration of spatio-temporal dynamics in hybrid zones can provide better understanding of the dynamics of hybridization (Grant and Grant, 1993, Grant *et al*., 2004, Gee, 2004) and predicting the long-term impact of various environmental factors on community changes.

Similarly, the environmental factors can have a significant influence on the parasite itself, as well as on the co-evolutionary dynamics (reviewed in Wolinska and King, 2009). Temperature, for example, can strongly influence the parasite and host physiology (Murdock *et al*., 2012). Moreover, ambient temperature does have an influence also on host resistance and parasite virulence as shown in *D. magna* (Mitchell *et al*., 2005). Host nutrition can push these dynamics both ways. Lower nutrient availability can cause both the elevated costs of resistance (a direct effect of nutrient availability), and reduced benefits of resistance when population sizes of hosts and parasites are lower; i.e. indicating an indirect effect (Lopez Pascua *et al*., 2014).

Better knowledge about the environmental factors influencing dynamics within hosts and parasites may also aid the development of means for improved protection against epizootics and epidemics (Grenfell and Dobson, 1995). Finally, in the light of global change, environmental factors have been shown to have consequences on changes in pathogen diversity and patterns of exposure for people and animals (Kutz *et al*., 2005, Burek *et al*., 2008, Hoberg and Brooks, 2013). For example, melted sea-ice area due to warming influences the abundance, distribution, seasonality, and interactions of marine and terrestrial species. One of the consequences is crowding during hauling out, higher disease transmission and finally increased mortality of hosts such as walruses (Post *et al*., 2013). Thus, long-term evolutionary studies about host-parasite dynamics by using here developed tools will significantly contribute to future predictions of ecological scenarios.

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Authors Contribution

Chapter 2:

J. Wolinska and P. Spaak supervised the project. J. Rusek and J. Wolinska designed the study, S. Giessler and J. Wolinska selected reference clones. J. Rusek designed the workflow of SNP discovery. P. Turko performed the mapping of the transcriptome and optimized historical samples DNA extraction protocol from formaldehyde. J. Rusek, G. Ayan and C. Tellenbach developed and analysed the SNP markers. J. Rusek and G. Ayan developed the PCR-RFLP assay, and tested historical samples. G. Ayan performed the "Large-scale" analysis. S. Giessler constructed FCA plots. J.Rusek drafted the manuscript.

Chapter 3:

J. Wolinska and A. Petrusek designed the study. J.Rusek designed sequencing primers and established the PCR protocol and amplified the samples for the study. A. Petrusek prepared and performed the pyrosequencing reactions. E. Gonzalez-Tortuero developed the QRS pipeline. D. Lyras, S. Grath and F. Castro -Monzon contributed to the development of QRS pipeline in earlier stages. E. Gonzalez-Tortuero, with the help of S. Giessler performed statistical analyses and, together with J. Wolinska, S. Giessler and A. Petrusek wrote the manuscript. D. Lyras and S. Grath helped with writing the Supporting Information.

Chapter 4:

J. Rusek and J. Wolinska designed the study. A. Petrusek contributed to sampling. J. Rusek designed sequencing primers, and J. Rusek and I. Maayan established and carried out the PCR protocol. L. Pialek and A. Petrusek designed, and L. Pialek performed the pyrosequencing reactions. E. Gonzalez-Tortuero performed all bioinformatics and statistical analyses. S. Laurent contributed to the interpretation of the genetic tests' results. E. Gonzalez-Tortuero and J. Wolinska wrote the manuscript, with comments and editing by A. Petrusek and I. Maayan.

Statutory Declaration and Statement

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 1.11.16 .... Jakub Rusek ....

(Unterschrift)

Erklärung

Hiermit erkläre ich, *

[X] dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

[X] dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

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und in den Nebenfächern ....
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(Hochschule/Universität)
unterzogen habe.

[ ] dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, den 1.11.16 .... Jakub Rusek ....

(Unterschrift)

*) Nichtzutreffendes streichen

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Curriculum Vitae

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Publications

Peer-reviewed publications:

Imhof H., Rusek J., Thiel M., Wolinska J., Laforsch C (2016) Do microplastic particles affect *Daphnia magna* at the morphology, life history and molecular level*?*. PloS one. submitted.

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Rusek J, Tellenbach C, Ayan G, Turko P, Theodosiu L, Giessler S, Spaak P, Wolinska J (2013) "Unlocking poor quality Daphnia samples by SNP genotyping“ DZG 2013, September 13-16, Munich, Germany

Rusek J, Tellenbach C, Ayan G, Turko P, Theodosiu L, Giessler S, Spaak P, Wolinska J (2013) „Unlocking poor quality Daphnia samples by SNP genotyping“ ESEB 2013, August 19-24, Lisbon, Portugal

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